Establishment of an ultra scale-down model of a lignocellulosic ethanol process

A thesis submitted to University College London for the degree of Doctor of Engineering

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Abstract

Lignocellulosic bioethanol processes have significant potential to reduce global greenhouse gas emissions. Such processes involve several steps, all of which require experimental optimisation. A significant aid to this research would be a validated Ultra Scale-Down (USD) platform that could be used to perform rapid and wide ranging screening and optimisation experiments on key operations in the process; namely enzymatic saccharification and microbial fermentation. Such experimental systems are widely used in the pharmaceutical industry but their applicability to lignocellulosic ethanol processes has been limited by the engineering complexity presented by biomass substrates, through, for example, the high viscosity and high levels of suspended solids involved.

As a basis for development of the USD methods, initial studies focused on the engineering characterisation of the current standard laboratory-scale, 4L, Stirred Tank Reactors (STRs) used for lignocellulosic bioprocess development. These yielded quantitative values of key engineering parameters that needed to be matched in the small scale models such as solid-liquid suspension, liquid mixing and oxygen mass transfer. Along with practical considerations related to the volumes of sample required for analysis, orbitally shaken, conical bottomed tubes (30 mL) were identified as a potential USD bioreactor geometry. The suitability of the USD tubes as a model system for enzyme hydrolysis and fermentation processes was evaluated using Distillers' Dried Grains with Solubles (DDGS).

Detailed studies on enzyme hydrolysis of DDGS (steam pre-treated, solids loadings from 5 – 25 % (w/w)) in the USD tubes closely match those obtained from STRs, in terms of the rate, composition and concentrations of sugars released. This represents an eighty fold scale reduction. The utility of the USD approach was further illustrated by investigating factors that may be limiting hydrolysis yields at high solids loadings. Washing the residual solids periodically during hydrolysis allowed 100% of the available sugar to be hydrolysed using commercially available enzymes.

The biomass hydrolysate is next used for fermentation by the thermophilic organism *Geobacillus thermoglucosidasius*. Here the presence of the suspended solid complicates the use of existing engineering correlations used as a basis for scale transfer in conventional STRs. Consequently, specific

correlations were developed for prediction of k_La as a function of gassed power input and superficial air velocity at the USD and laboratory scales, accounting for the effect of suspended solids. This led to successful demonstration of comparability of fermentation performance between the USD tubes and STRs in terms of product titres, conversion yields and substrate utilisation rates. This again represented an 80-fold reduction in scale of operation and material requirements.

The final studies aimed at validating the results from the USD saccharification and fermentation steps by using the USD data to predict operational expenditure, process step timings and feedstock conversion yields for a planned DDGS to ethanol production facility. These predictions are based on a validated process model from ReBio and can be validated against existing large scale data. Several model outputs were examined for scenarios using USD platform and demonstration scale data, for example the volume of ethanol produced from 100,000 tons of DDGS and the predicted rate of return on investment. There was good agreement between the outputs of the two models. Likewise a breakdown of expenditure by plant area showed a close agreement between the two models. The work in this thesis has therefore established the shaken USD tubes as a small scale alternative to STRs for early stage bioprocess development of lignocellulosic bioethanol processes. The USD tubes have been used successfully within ReBio in order to carry out strain screening experiments under conditions that closely reflect target process operating conditions.

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Nomenclature

Symbols

AFEX	Ammonia Freeze Explosion	-
C^*	Saturated dissolved oxygen concentration	%
C_{L1}	Dissolved oxygen concentration at t_0	%
C_{L2}	Dissolved oxygen concentration at time t	%
C_s	Concentration of sugars when feedstock completely hydrolysed	$g\ mL^{-1}$
C_{si}	Concentration of sugar i when feedstock completely hydrolysed	$g\ mL^{-1}$
DDGS	Distillers Dried Grains with Solubles	-
f	Shaking frequency	RPM
HPLC	High performance liquid chromatography	
k_L	Film mass transfer coefficient	
k_La	Volumetric oxygen transfer coefficient	h^{-1}
k_La_{exp}	Experimentally derived vale of k_La	h^{-1}
k_La_{the}	Value of k_La predicted by a correlation	h^{-1}
M_H	Mass of water of hydrolysis	Kg
M_p	Mass of polymeric sugar on a dry matter basis	Kg
MSW	Municipal solid waste	-
M_W	Mass of water	Kg
NREL	National Renewable Energy Laboratory (USA)	-
OD_{600}	Optical Density at a wavelength of 600 nm	
P_g	Gassed power input to STR	W
RPM	Revolutions per minute	-
STR	Stirred tank reactor	
t	Time	s
t_0	Time zero (start of experiment)	s
U_s	Superficial gas velocity	$m\ s^{-1}$
USD	Ultra scale-down	-
V	Volume of liquid	L
vvm	Volume of air (per minute) per volume of culture	$L\ min^{-1}\ L^{-1}$
X+G+M+F	Combined concentration of xylose, galactose, mannose and fructose	-
Y_i	Proportion of sugar i on a dry matter basis	$kg\ kg^{-1}$

Greek Letters

ρ_s	Density of sugar solution	$g\ cm^{-3}$

1. Introduction

1.1 Background to biofuels

1.1.1 Introduction

According to the Oxford English Dictionary, a biofuel is any fuel that is produced immediately from organic matter, separating it from fossil fuels such as oil, which, although they have their origins in organic matter, take millions of years to form. In its simplest form this encompasses the use of wood fires for cooking and industrial purposes - practices which date back to the earliest times. Indeed this remained the primary source of energy for most of the world until, with the beginning of the industrial revolution in the 18th Century, there began an inexorable rise in the use of fossil fuels which has continued to this day. The term is often defined more narrowly to signify liquid based transportation fuels (Demirbas, 2007) and are often offered up as alternatives to the current fossil fuel derived transportation fuels such as petrol, diesel and kerosene.

Over the last few decades a variety of factors have led to renewed interest in biofuels, most importantly concerns over global warming, declining oil supplies and energy security. The link between global warming and the burning of fossil fuels was first proposed in the 1970s (Broecker, 1975). It was not however until the following decade that the issue gained mainstream attention and this is often now cited as a primary reason for the expanded use of biofuels (Farrell et al., 2006). There are however several other reasons for the current global interest in biofuels, both economic and political. The oil crisis of the 1970s prompted many countries, especially Brazil, to look for alternative sources of fuel to reduce their dependence on imported oil (Goldemberg, 2006). Although such interest waned when the crisis ended and global oil prices fell, it is now a focus again due to the declining reserves of easily accessible oil (Hirsch et al., 2006).

There are a wide variety of potential transportation biofuels and production methods (Naik et al., 2010), however the two most common are biodiesel and bioethanol. In the year 2010 biodiesel was estimated to have a 3.7% share of the total diesel market where it is available, whilst bioethanol, an alternative to petrol, which has a segment share of 9% over the same period (OECD, 2013). The total worldwide production of bioethanol was just over 100 billion litres, around three quarters of which was US corn ethanol and Brazilian production from sugarcane (Renewable Fuels Association, 2013). Both bioethanol and biodiesel have been demonstrated to offer substantial reductions in greenhouse gas (GHG) emissions (Hill et al., 2006).

Ethanol has become the most common biofuel largely due to its ability to be used blended with regular petrol in proportions of up to 15% (v/v) with no modifications to engines or infrastructure needed. It can also be used neat or in blends of up to 85% (v/v) ethanol in 'flex fuel' engines which have been extensively developed in the Brazilian market (Joseph, 2007). The use of up to 10% (v/v) ethanol in petrol blends is permitted by the US Environmental Protection Agency (EPA) and indeed several states have mandated the use of so called E10 fuel (Solomon et al., 2007). This along with a tax credits system that has operated since the 1970's have caused a rapid expansion in the industrial production of ethanol in the USA, and in 2013 US production was over 50 billion litres (Renewable Fuels Association, 2013).

1.1.2 Current production and utilisation of ethanol

The vast majority of current ethanol production comes from first generation plants utilising either sugarcane or corn grain, although other sources such as wheat, sugar beet, sorghum or cassava are used where localised environmental factors favour them (Thomas and Ingledew, 1992, Worley et al., 1992, Atthasampunna et al., 1987, Halleux et al., 2008).

All crops generally require a similar set of pre-processing steps prior to conversion. For the sugar based crops, the feed is washed and chopped then pressed to extract the juice. The juice is then concentrated to an appropriate strength for fermentation. Industrial strains of *Saccharomyces cerevisiae* are then used to convert the sugars into beer which passes through a series of distillation columns and molecular sieves to produce anhydrous ethanol as the final product. This process is sometimes combined with a crystal sugar production process in Brazilian plants (Ensinas et al., 2007).

Corn (or other grain) ethanol plants are generally divided into ‘wet mill’ and ‘dry mill’ processes depending on how the grains are disrupted at the beginning of the process. Wet mill plants soak the grains in an acid solution for 24-48 hours to separate out the different portions of the grain; endosperm (starch rich), germ (protein rich) and husk (fibre rich). The starch is then separated from the gluten and used in a fermentation process very similar to that of sugar cane plants. Wet mill plants possess the advantage of producing valuable corn oil and gluten meal as by products (Naik et al., 2010).

The basic unit operations of a dry mill plant are shown in Figure 1.1. The dry grain entering the process is ground into flour which is heated with water and cooked to liberate the starch. Amylase based enzyme mixtures are then added in a liquefaction step that converts the starch into glucose. This glucose stream is then fermented by *S. cerevisiae* to produce a beer. The beer is then fed through a series of distillation columns with the ethanol passing through a set of molecular sieves to remove remaining water. The residue from the distillation columns is then centrifuged to separate the solids, known as Wet Distillers Grains (WDG) or wet cake, from the thin stillage. The thin stillage is then evaporated to form a syrup which is subsequently mixed back in with the solids and dried to produce a product called Distillers Dried Grains with Solubles (DDGS) which is sold as an animal feed component (Kwiatkowski et al., 2006). Dry mill plants have become the standard for ethanol production in the US largely due to their lower construction costs and lower energy requirements (Wu, 2008, Renewable Fuels Association, 2013).

The ethanol industry in the US has now become a major economic and political influence, directly and indirectly employing an estimated 400,000 people in 2009, and recent policy, such as the Energy Independence and Security Act (2007) , which mandated that 36 billion US gallons of renewable fuel should be used by 2022 is expected to increase production still further (Solomon et al., 2007, Urbanchuk, 2009, Energy Independence and Security act of 2007). In addition to the environmental and energy security arguments, politics can now be considered to be one of the driving influences behind governmental support for ethanol in the United States where the so called ‘ethanol lobby’ is a powerful influence in several mid-western states (Hahn, 2008).

The main reason for the heavy government incentives that led to the highly developed Brazilian ethanol industry was a desire by the government of the 1970’s to reduce its reliance on imported oil and replace it with a locally produced alternative (Goldemberg, 2006). This is also often cited as a reason for other countries to invest in biofuels. Ongoing concerns about diminishing fossil fuel reserves and instability in the major oil producing regions have led many western countries to become increasing concerned about these issues in recent years, and many authors have suggested that increased use of biofuels such as ethanol could form part of the solution to reducing demand for imported oil (Bielecki, 2002, Field et al., 2008, Liska et al., 2009, Demain, 2009).

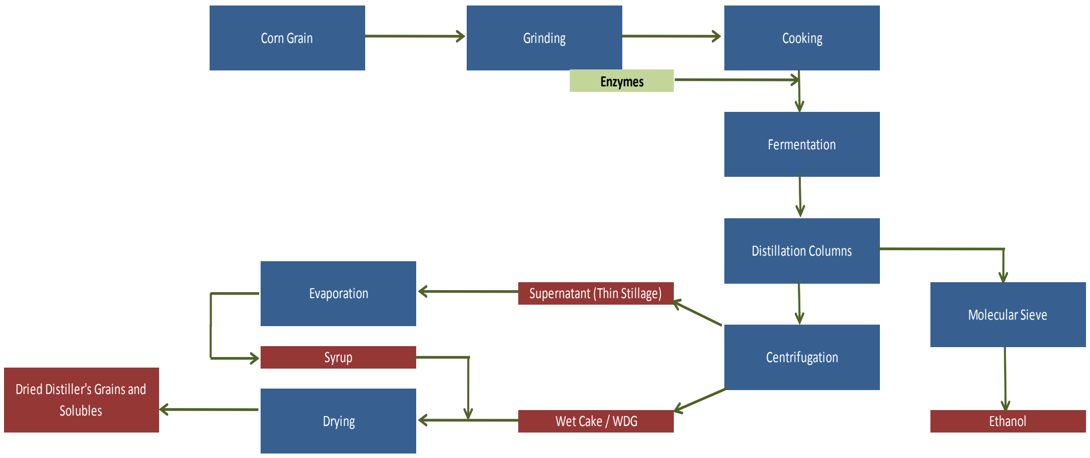


Figure 1.1. Schematic outline of a typical dry grind, first generation corn ethanol plant. Adapted from RFA(2011).

1.1.3 Environmental concerns

The scientific community has by no means been consistently behind these government support programmes, with doubts being cast over both bioethanol’s environmental impact and its ability to improve energy security. A brief overview of these issues is provided here, however more detail can be found in a dedicated review of assessments that have been published on the environmental and economic impacts of bioethanol (von Blottnitz and Curran, 2007).

Concerns about the environmental impact of bioethanol have generally been focussed on two interconnected areas: the levels of reduction in GHG emissions and fossil fuel use associated with different production processes, and the impact that production has on food supplies. Some authors have estimated that US ethanol production has a net negative energy balance of almost 25% and that US ethanol production also has a significant detrimental effect on food production (Pimentel, 2003). Other studies however have concluded that ethanol production has a positive energy balance of the same or even greater magnitude, up to a 50% positive energy balance for a modern plant (Shapouri et al., 1995, Liska et al., 2009). One problem of such studies is that they are heavily dependent on the boundaries set and assumptions made; a 2006 study recalculated the results from several different papers using common system boundaries and estimated that ethanol plants today had net energy balances ranging from approximately neutral through to 45% positive (Farrell et al., 2006).

Likewise, estimates of the effect of bioethanol on GHG emissions vary widely in the literature. A 2006 comparison of US bioethanol and biodiesel concluded that bioethanol reduced GHG emissions by 12% relative to the petrol it displaces and the figure for biodiesel was 41% (Hill et al., 2006). A study that modelled several different types of ethanol plants gave figures that range from a very small increase in GHG emissions for older coal powered plants through to a 39% reduction for the most modern natural gas powered plants (Wang et al., 2007). A separate study modelling the more modern generation of plants suggested a GHG emission reduction of 50% that could be improved further with the new technologies that are coming on line, whilst the same 2006 comparison mentioned above suggested an average reduction of 13% in GHG emissions (Farrell et al., 2006, Liska et al., 2009). It is generally accepted that the Brazilian sugarcane ethanol process offers substantially larger reductions in GHG emissions and net energy consumption than US corn ethanol processes (Goldemberg and Guardabassi, 2010).

After moving towards apparent consensus, this debate was reignited by two articles written in 2008, both asserting that when land use change was taken into account (non-agricultural land that is converted to farming use to replace the food crops being used to produce ethanol) corn ethanol actually leads to the emission of 93% more GHG than petrol (Searchinger et al., 2008, Fargione et al., 2008). The models and assumptions used in these works have come in for criticism from other authors, and there seems to be no consensus on the issue, which is not surprising given the difficulties of life cycle analyses such as these which rely on making predictions about future trends (Wang and Haq, 2008, Mathews and Tan, 2009, Tyner et al., 2010).

Whilst there has been considerable debate over the relative effect of current methods of bioethanol production on GHG emissions and net energy requirements, perhaps of more notice to the wider public has been the effect of bioethanol production on food markets – the so called ‘food versus fuel' debate. In essence some authors are concerned that large scale diversion of food crops for ethanol production will lead to rising food prices, which will likely have a disproportionate impact on poorer countries (Runge and Senauer, 2007, Tenenbaum, 2008). This has led to debate in the media about the merits of biofuel production (Harrabin, 2008). Most of the above concerns about the environmental debate have however been focussed on first generation bioethanol produced from the starch and sugar content of crops. There is a broad agreement in the literature, even from authors who are critical of the current generation of biofuel technologies, that the solution to these problems lie in second generation, or cellulosic, biofuels which are produced from either non-food crops grown on marginal land, from by-products of the agricultural, forestry or paper industries or from the organic components of municipal waste (Fargione et al., 2008, Tilman et al., 2009).

Cellulosic ethanol will form the focus of this project, and therefore this review will now focus on this subject.

1.2 Lignocellulosic ethanol

The ultimate goal of a 2nd generation cellulosic ethanol process is the same as for the 1st generation process discussed above: to release sugars from recently grown organic material and convert them into ethanol. The process involved is however considerably more complex with the first commercial scale plants only becoming operational from 2014 onwards (European Biofuels Technology Platform, 2015).

The reason for the added complexity of a cellulosic ethanol process is due to the form in which the target sugars are found in the biomass. In a first generation process the target sugar is glucose stored in the plant as either sucrose (sugar crops) or starch (grains). Both of these can be easily liberated from the plant material and can be readily hydrolysed to easily fermented sugars with readily available, cheap enzymes.

When considering cellulosic ethanol processes, the sugars that are targeted are generally found in the biomass as lignocellulose, a complex structural polymer consisting of three main components, cellulose, hemicelluloses and lignin, closely associated together. Cellulose is composed of glucose, with the individual units being linked together by β 1-4 glycosidic bonds (Shen and Gnanakaran, 2009). Hemicellulose is significantly more varied in composition consisting of varying proportions of several different sugars, including the C6 sugars glucose, mannose and galactose and the C5 sugars xylose and arabinose, with several possible branched side groups including acetate and uronic acids. The composition of hemicelluloses varies between different biomass types (for example hemicellulose from softwoods is typically of a different type to that found in grasses) and between different parts of the same plant (Schädel et al., 2010). Lignin is a complex structural polymer that is found in the cell walls of plants where it both provides structural support (through its cross-linkage with hemicellulose) and helps to prevent microbial attack of the plant. There are three different forms of lignin *p*-hydroxypehnyl (H), guaiacyl (G) and syringal (S) each based around a different monomer, and again the relative proportions of each vary according to the type of plant and the particular tissue within a plant (Boerjan et al., 2003).

In contrast to the first generation starch processes, simply opening up the plant structure in a crude way and then adding enzymes is not sufficient to deliver efficient hydrolysis of lignocelluloses as it naturally has low susceptibility to enzymatic conversion. Thus it is necessary for a cellulosic ethanol process to have a pretreatment step before enzyme hydrolysis that will in some way alter the structure of the lignocellulose and render it more susceptible to attack by enzymes. This would then be followed by an enzymatic hydrolysis step which requires a more complex mixture of enzymes that a 1st generation process due to the more complex sugar containing structures found in lignocellulose. As in the 1st generation process these sugars will then be fermented, however due to the mixed sugar composition of the post hydrolysis stream, it will be necessary to have an organism that is capable of utilising a wider range of sugars than currently used industrial strains of *S. cerevisiae* which has a relatively narrow substrate range.

Whilst this biological process towards cellulosic ethanol production is generally considered more likely to lead to economic production, an alternative process is via synthetic gas, commonly referred to as syngas. In this process biomass is heated by steam in the absence of oxygen, with thermal decomposition leading to the production of a gas that consists mainly of carbon monoxide and hydrogen, with smaller amounts of carbon dioxide and nitrogen. The syngas can then be converted to ethanol either catalytically or fermentatively (Younesi et al., 2005, Subramani, 2008) . The main proposed advantage of the syngas method is that it eliminates the potentially costly and problematic enzyme hydrolysis step and allows a broad range of substrates to be converted to an essentially similar feed stream for further processing; however, the capital costs for the syngas process are likely to be higher due to the high temperatures and pressures involved. A recent comparison of the gasification-fermentation process with conventional enzyme hydrolysis and fermentation suggested that the enzyme hydrolysis based process offered a better energetic return on investment (Piccolo and Bezzo, 2009).

As this thesis is focussing on a second generation cellulosic ethanol process based on the pretreatment – enzyme hydrolysis - fermentation route, this review will now look at the potential forms such a process could take in more detail.

1.2.1 Feedstocks

Before considering what form any potential process may take it is worth considering the various sources of lignocellulosic biomass that may be available for ethanol production. Recent literature encompasses a steady stream of papers reporting the production of ethanol from a novel source of biomass, however, one thing that is apparent is the scale of the potential resource is immense.

The two most commonly mentioned biomass resources are the waste streams generated by the agricultural and forestry industries. A 2005 US Department of Energy report set out how an annual production of 1.3 billion tons of biomass from the agricultural and forestry industries could be sustainably achieved (Perlack et al., 2005). In terms of agricultural waste, corn stover, the husks, stalk and leaves left behind after harvesting of the grain, is commonly referred to as a potential source of biomass in the US where it has the advantage of being produced close to existing grain fed ethanol plants. It can easily be seen how this supply could change according to which agricultural crops are present in a particular area e.g. wheat, rice or cassava. A 2004 paper estimated that globally rice straw, wheat straw, corn stover and sugarcane bagasse could be used to produce 418 billion litres of ethanol, over two thirds of it in Asia. It should be noted that this study looked only at the availability of the feedstock, not the feasibility of using them (Kim, 2004). Forestry residue comes in a variety of forms, from small branches and bark that are currently left behind in forests to sawdust and other wastes from lumber mills. It is especially attractive, from an ecological point of view, when it comes from well managed forests of fast growing trees (Duff and Murray, 1996).

There are also other potential sources of biomass, for example a recent report estimated that the cellulosic component of the US's Municipal Solid Waste (MSW) that is currently sent to landfill could be used to produce 13.7 billion litres of ethanol, approximately as much as is currently produced by first generation corn ethanol processes (Kalogo et al., 2007).

Numerous authors have speculated that crops dedicated to providing biomass for ethanol production could be grown on marginal agricultural land that is not currently used for crop production. The two most commonly cited dedicated ethanol crops are switchgrass (*Panicum virgatum*) and hybrid Miscanthus (e.g. *Miscanthus x giganteus).* Compared with the agricultural or forest residues they have several advantages. They require less intensive agricultural practices meaning that less fossil fuel is needed to grow them improving the net energy balance. They are perennial plants and, therefore, there is no need for an annual growing period thus increasing their productivity (although normally they need to grow for at least two years before they can be harvested). In terms of future productivity, they may also present more possibilities for the use of genetic modification to enhance and tailor their use in fuel ethanol production (Yuan et al., 2008). There would be more opportunity to modify these energy crops as there would be no risk of interfering with their use in the food chain, either through interfering with food quality or due to public concerns.

These different sources of biomass all possess different properties. The wood derived material will have higher levels of lignin than grasses and cereals but will also have higher total levels of carbohydrates. Compared to the woody material the grasses/cereals also have a lower proportion of glucose to C5 sugars. Hardwoods tend to have much higher levels of xylan but much lower levels of mannan when compared to softwoods. Typical compositions for a range of feedstocks are given in Table 1.1. The generation of enough bioethanol to replace a significant portion of petrol usage will require the utilisation of several different sources of biomass, and the types of biomass used will vary according to what is available locally.

Table 1.1. Typical compositions of a range of feedstocks potentially useful for bioethanol production.

Feedstock	**Glucan**	**Xylan**	**Arabinan**	**Lignin**	**Reference.**
DDGS	22.0	8.0	6.3	<5.0	(Banerjee et al., 2010a)
Wet Cake	18.5	14.9	5.5	n.d	(Kim et al., 2008b)
Sugar Cane Bagasse	38.8	23.5	2.5	n.d	(da Silva et al., 2010)
Corn Stover	35.6	18.9	2.9	12.3	(Jørgensen et al., 2007a)
Wheat Straw	38.2	21.2	2.5	23.4	
Spruce	43.4	4.9	1.1	28.1	
Pine	46.4	8.8	2.4	29.4	
Willow	43.0	24.9	1.2	24.2	

1.2.1.2 DDGS Composition

As outlined in Section 1.2.2 DDGS is a by-product of existing ethanol production process, containing the non-starch portion of the grain that is not utilised for product formation. Ethanol is a commodity product, produced in large volumes, and therefore production in a given country will tend to utilise the predominantly available local feedstock. As discussed in Section 1.2.1 in the USA this is corn (known as maize in the USA) from which large quantities of DDGS are produced as a by-product of ethanol production. It is also possible to use other grains to produce ethanol where local conditions are favourable in a process essentially identical to that for the production of ethanol from corn. In Canada and Europe climate conditions favour the production of wheat over corn and thus in these locations the significant quantities of wheat are used for the production of ethanol.

Naturally due to the differences in the composition of corn and wheat, there are also differences to be found in the DDGS co-products of the two grains. Wheat DDGS is typically reported to be higher in protein content that corn DDGS with typical compositions for the two showing wheat DDGS has around 35 to 37% (w/w) protein whereas corn DDGS is around 28 to 30% (w/w) protein. The value of DDGS as an animal feed is, generally speaking, related to its crude protein content and therefore wheat DDGS would be expected to be a more valuable commodity. When looking at the oil content of DDGS the picture is reversed with corn DDGS containing around 5% (w/w) and wheat DDGS around 10% (w/w). As much of the analysis of DDGS composition has been focused on its use as an animal feed there is a relative lack of detailed compositional information on the sugars in DDGS. Instead it is typically analysed in terms of acid detergent fibre (ADF) and neutral detergent fibre (NDF). NDF contains all of the 'structural' components of the plant cell and thus includes cellulose and hemicelluloses as well as lignin. ADF contains only the lignin and so called 'recalcitrant' cellulose. As there is only a small amount of lignin present in corn or wheat, the NDF figure should correspond to the approximate amount of sugar available in DDGS for conversion into second generation bioethanol. In this regard it would appear that corn DDGS is better suited for further processing into bioethanol with typical NDF values of around 40 % (w/w) compared to around 30% for wheat DDGS (Chatzifragkou et al., 2015, FOBI, 2015, US Grains Council, 2012). This fits in with what reported sugar composition is available for corn DDGS, with typical samples found to contain for example 18.5 % (w/w) glucose sugars, 15% (w/w) xylose and 5.5% (w/w) arabinose (Kim et al., 2008b).

It should also be noted that despite the extensive use of wheat for ethanol production in some locations, the quantities used are small compared to bioethanol production from corn, and indeed even in locations favourable to wheat production such as the EU, more bioethanol is produced from corn that wheat (ePURE, 2015). For example, in 2010, Canada, the world's largest wheat producer, produced 0.26 million tons of wheat DDGS whereas over 40 million tons of corn DDGS were produced in the USA. Thus while wheat DDGS may in some local situations be a suitable feedstock for second generation bioethanol production, the greater sugar content and much greater availability of corn DDGS makes it a more widely applicable feedstock and thus corn DDGS is to be used in this work.

The following sections describe in detail the main process stages envisioned for a biologically based lignocellulosic ethanol production process; namely pretreatment, enzyme hydrolysis and fermentation.

1.2.2 Pretreatment

The purpose of pretreatment is to open up the structure of biomass and improve the subsequent hydrolysis of the cellulose and hemicellulose by enzymes (although concentrated acid hydrolysis will directly solubilise large proportions of the sugar). A 2009 review set out the goals of pretreatment as in Table 1.2

Pretreatments can be broadly split into three main categories, physical, chemical and others, although very often more than one approach is combined, for example physio-chemical pretreatment. Much effort has been focussed on improving pre-treatment methods for biomass, both due to its importance to subsequent steps of the process and due to the fact that it is one of the most expensive steps in cellulosic ethanol processes, leading Charles Wyman to state that “The only step more expensive than pretreatment is no pretreatment” (Wyman, 2007). Given the importance of this step it is of no surprise that several comprehensive reviews of the topic have appeared in the literature (Hendriks and Zeeman, 2009, Alvira et al., 2010, Haghighi Mood et al., 2013).

This review will provide a brief outline of the major pretreatment technologies reported on the literature, but will focus especially on those which are of most relevance to this project.

Table 1.2. The Goals of a Pretreatment Step. Adapted from (Kumar et al., 2009)

Pretreatment should ...	**Pretreatment should not ...**
Improve the formation of sugars or their formation by subsequent hydrolysis steps	Lead to the formation of compounds which will inhibit subsequent hydrolysis or fermentation steps
Be cost effective	Degrade sugars or cause loss of carbohydrate

1.2.2.1 Physical pretreatment

This class of pretreatment methods involve the use of cutting or shearing forces to reduce particle size and increase surface area of biomass. Such milling techniques have been reported to enhance sugar release following enzyme hydrolysis (da Silva et al., 2010). Data has been published that seems to suggest that the benefits of milling type pretreatments are actually largely derived from their effect in reducing the degree of crystallinity of biomass (Yu and Wu, 2011). Although these pretreatment methods are often easily applicable to the large scale, thanks for example to their use in the food and grain industries, the very high energy requirements make milling pretreatments uneconomic for use in cellulosic ethanol processes (Schell and Harwood, 1994). Milling is however, commonly used as a precursor to laboratory scale trials of a wide range of pretreatment technologies as there is a need to reduce what are often large pieces of biomass, for example straw, into a feedstock that will flow through small litre scale trial vessels. What impact, if any, this may have on results when these technologies are scaled up is currently unclear.

1.2.2.2 Chemical pretreatment

Chemical pretreatments use a catalyst to break down the physical and chemical structure of the biomass, liberating sugar and/or opening up the structure for further degradation by enzymes. The effects are highly dependent on the specific chemical and biomass used.

The first category of chemical pretreatment is acid pretreatment. In a concentrated acid catalytic process, a strong acid is added to the biomass slurry in relatively high concentrations. This results in the hydrolysis of a large portion of the lignin and almost all of the hemicellulose along with a small amount of cellulose and leaves behind a solid fraction that is heavily enriched in cellulose. This makes the subsequent enzyme hydrolysis step easier, but results in the sugar being split between two process streams. Acid hydrolysis also tends to cause degradation of some of the sugars present, especially those in the more easily hydrolysed hemicellulose. Thus, pretreatment liquors from acid hydrolysis can have high concentrations of monomeric lignin and compounds such as furfural or hydroxymethylfurfural and are very inhibitory to subsequent biological steps (Larsson, 1999). Although this process found some utility during the World War II, the costs of the acid and materials needed for this process has resulted in it falling out of favour (Gnansounou and Dauriat, 2010).

More typically used is a dilute acid process. Here the concentrations of acid used are lower, around 0.5% to 2% (w/w), but the temperature of the reaction is higher, from roughly 130°C to 200°C. Typically the acid used is sulphuric, although nitric, peracetic and hydrochloric are sometimes used and the process has been shown to be effective on a wide range of biomass sources including hardwoods, softwoods and herbaceous crops (Mosier, 2005). It has similar advantages to acid hydrolysis in that it solubilises the majority of the hemicellulose leaving a solid residue that can be easily hydrolysed with enzymes. For example, Jeong et al. working with rape seed straw achieved solubilisation of 80% (w/w) of the hemicellulose during dilute sulphuric acid pretreatment and were then able to achieve a 95% (w/w) yield during subsequent enzymatic hydrolysis of the cellulose enriched solids (Jeong et al., 2010). Similar results have been seen with softwood biomass sources with reported examples of the solubilisation of over 90% of the hemicellulose and 20% of the cellulose after pretreatment with greater than 90% of the remaining cellulose being solubilised by subsequent enzymatic hydrolysis (Nguyen et al., 1998). However, dilute acid pretreatment is not without its problems. The resulting sugar degradation is an important issue and the costs associated with the acid and its use (e.g. expensive alloys for the reactor due to corrosion) can be significant. Dilute acid is one of the pretreatment methods that has been scaled up to pilot scale, with NREL operating a continuous dilute acid pretreatment reactor fed with around 32kg/hr dry corn stover. Following enzyme hydrolysis, they were able to achieve over 80% cellulose conversion and xylose yields of over 70% (Schell et al., 2003).

Alternative chemical pretreatment methods involve the use of bases to break down the lignocellulose structure. One of the simplest pretreatment methods involves simply mixing lime in with the feedstock and leaving the mixture to react for a period of several weeks in a warm room (Wyman et al., 2005). Although this pretreatment method would involve little capital expenditure, its protracted nature may make it difficult to implement in reality. It is also possible to use lime as a catalyst in pretreatments of much shorter duration at higher temperatures, and this has been found to give comparable results to dilute acid pretreatment (Saha and Cotta, 2010).

An alternative alkaline chemical commonly used for pretreatment is ammonia, generally used in dilute form in Ammonia Recycled Percolation (ARP) processes or in concentrated form for Ammonia Fibre Explosion (AFEX); due to the elevated pressures used in AFEX it is often considered a physio-chemical method and will be discussed later. In ARP, the feedstock is immobilised inside a reactor through which a dilute aqueous ammonia solution is recirculated. Significant amounts of lignin and hemicellulose can be removed by the process leaving behind a residue with increased enzyme digestibility that is enriched in cellulose (Yoon et al., 1995). An alternative approach to using ammonia in pretreatment has been reported where the authors used ammonia as a catalyst for the pretreatment of sorghum at elevated temperatures and pressures, resulting in significant improvements in feedstock digestibility (Salvi et al., 2010). The range of dilute ammonia pretreatments has recently been reviewed (Kim et al., 2009b).

Oxidative pretreatment involves the use of an oxidising agent to disrupt the chemical bonds within biomass, reducing the lignin content of the feedstock, and actually has its origins as a pretreatment in the similar processes carried out to bleach pulp in the paper industries. Originally hydrogen peroxide under alkaline conditions was employed, although this has the drawback of also degrading some of the sugars present in the biomass (Gould, 1984). More recently peracetic acid has been trialled for use as an oxidising agent as it is reported to have a greater specificity for lignin and therefore leaves the sugar content of the feedstock largely intact. Enzyme hydrolysis yields of up to 95% were reported when a combined peracetic acid and sodium hydroxide pretreatment was tested on hybrid poplar (Teixeira et al., 1999).

Finally amongst the common chemical pretreatment methods is the organosolv process, which again developed out of processes used in the paper industries. These processes use organic solvents, often with a mineral acid catalyst, to dissolve the lignin and hemicellulose portions of the biomass, leaving the cellulose behind. These processes, which have been reviewed elsewhere (Zhao et al., 2009), produce three separate streams; solid lignin, which can be burnt or used as a chemical precursor, hemicellulose and cellulose, which could potentially simplify further processing. However there are significant costs associated with the use of such solvents and their recovery, and organosolv processes need to be tightly controlled. As such the authors felt that whilst not cost effective at the moment, they could be useful in future scenarios where each of the product streams can be utilised individually to maximise revenues.

1.2.2.3 Physiochemical pretreatment

Physiochemical pretreatments are those where chemical catalytic effects are combined with destructive physical forces, often high pressure, in a single pretreatment step. The two most common pretreatments in this category are steam explosion and ammonia freeze explosion (AFEX).

The notion of using ammonia at high pressure to increase the digestibility of lignocellulosic biomass dates back to the early 1980's and has been significantly developed since then (Abdullah et al., 1985). In this process aqueous ammonia is mixed with the biomass (generally with a ratio around 1g ammonia/1g biomass) and heated in a pressurised reactor to approximately 100°C for up to 15 minutes. At the end of the reaction time the material is rapidly discharged from the reactor and some of the ammonia can be recovered from the vapour phase (Bals et al., 2011). AFEX is an unusual pretreatment in that little or no material is solubilised by the pretreatment itself, but sugar yields in excess of 90% of the maximum can be achieved. This effect was seen clearly in a comparison of several different pretreatment technologies on corn stover where AFEX was the only pretreatment that did not even solubilise some of the hemicellulose during pretreatment (Wyman et al., 2005). AFEX pretreatment has also been tested on DDGS, the feedstock of interest for this project, resulting in a glucose yield of 100% at low solids loadings; C5 sugar solubilisation was significantly lower at these conditions but was un-optimised (Kim et al., 2008a). Although AFEX has been shown to be a highly effective pretreatment for grass or cereal based feedstocks, it has been reported that it is far less effective as a pretreatment for feedstocks with higher lignin content such as newspaper or hardwood, although it does seem that significantly harsher AFEX conditions can improve yields somewhat on these recalcitrant feedstocks (Holtzapple et al., 1991, Balan et al., 2009)′. Traditionally this increased recalcitrance has been attributed to a higher lignin content, although recent work on hardwood Aspen led the authors to suggest it may also be related to the degree of arabinoxylan cross linkage (Sun and Cheng, 2002, Balan et al., 2009).

Steam explosion is a relatively simple, widely used feedstock pretreatment process, and was the subject of a previous extensive review (Ramos, 2003). Feedstock slurries are heated by direct steam injection to temperatures in excess of 100°C and sometimes in excess of 200°C. After a specified residence period in the reactor, the pressure in the reactor is suddenly released in a process often referred to as a 'flash'. The technology is referred to as physiochemical rather than simply physical due to the fact that the solubilisation of hemicellulose that occurs at the temperatures involved releases organic acids that were part of the side chains on the hemicellulose. These acids lower the pH of the slurry and promote further solubilisation of the biomass in a process known as autohydrolysis (Lamptey et al., 1985). In a modified form (including the addition of exogenous sulphuric acid as a catalyst) it has been used at near commercial scale, and thus seems to be a leading contender for application in commercial cellulosic ethanol plants (Tolan, 2002).

Steam explosion has been used successfully on a range of feedstocks including softwood, hardwood, sugar cane bagasse, corn stover and wheat straw (Foody, 1980, Schultz et al., 1984, Zabihi et al., 2010, Yang et al., 2011). It has also been used, with and without an acid catalyst with DDGS, however this work was focussed on directly fermenting the sugars released after pretreatment without a subsequent enzyme hydrolysis step, with the intent of creating a higher value feed with ethanol as a by-product (Tucker et al., 2004). This meant that most focus was placed on the acid catalysed conditions which directly released far more sugars. It is possible that had enzyme hydrolysis been included the non-catalysed steam explosion only condition could have achieved good levels of sugar solubilisation and ethanol production. It should be noted however that this use of steam explosion combined with an acid catalyst is very common with examples dating from the earliest days of the technology through to the present day (Ramos, 2003).

In the literature it is common to see steam explosion pretreatments described by a 'severity factor' ($\mathrm{LogR_o}$), which combines temperature, residence time and sometimes the addition of an acid catalyst into one numerical, unit free, value for ease of comparison.

$$Severity\ Factor = \log\left(t \times exp\left(\frac{T-100}{14.75}\right)\right) \qquad [1.1]$$

In their evaluation of the use of severity factors Pederson and Meyer found that whilst having one value that could encompass all of the physical and pH effects on pretreatments would be particularly helpful, none of the severity factor calculations derived to date accurately predicted monomeric sugar yields following hydrolysis (Pedersen and Meyer, 2010).

1.3 Enzyme hydrolysis

1.3.1 General and industrial background

The pretreatment stage described in Section 1.2 opens up the biomass and, depending upon the pretreatment method used, may liberate some monomeric or short chain oligomeric sugars. However it is likely that large amounts of soluble long chain oligomers and complex insoluble sugars will remain in the solid or liquid phase of the pretreated slurry. In order for these sugars to be effectively converted to ethanol they need to be broken down into monomers or perhaps short chain oligomers. Historically this was done using acid hydrolysis; however enzymatic hydrolysis has been favoured recently due in large part to lower production of sugar degradation products, such hydroxymethylformaldehyde or furfuraldehyde, which are inhibitory to fermentative microorganisms (Wilson et al., 1989).

The process of enzyme hydrolysis is a complex one. It involves both the solubilisation of sugars from the solid phase into the liquid phase and the subsequent cleavage of these oligomers into short chain or monomeric forms. The solids are a mixture of varying proportions of cellulose, hemicelluloses of varying structures and chemistries, lignin, protein, ash and other components. This composition will vary according to the feedstock used, as discussed in Section 1.2.1, and it will also be heavily influenced by the pretreatment method used. Some such as dilute acid, or to a lesser extent steam explosion, will solubilise significant amounts of hemicellulose and lignin, whilst others such as AFEX do not seem to significantly alter the chemical composition of the feedstock. The differing proportions of the varying components will necessitate different ratios of the various enzyme activities that are required to break down lignocellulose. This is a key step in the bioethanol process, and has been the subject of several review articles (Jørgensen et al., 2007a, Kumar et al., 2008, Karimi and Taherzadeh, 2007, Sun and Cheng, 2002).

The enzymes used in the hydrolysis of lignocellulosic biomass are generally divided into three main categories; cellulases, hemicellulases and accessory enzymes. Cellulases and hemicellulases are responsible for the hydrolysis of cellulose and hemicellulose respectively. Accessory enzymes tend to have a role in removing side groups from the hemicellulose although hemicellulases are still occasionally referred to as accessory enzymes due to the traditional focus on cellulose hydrolysis. Due to its more complex structure a greater number of activities are required for the hydrolysis of hemicellulose (Saha, 2003).

Table 1.3. List of enzymes commonly reported to be involved in the hydrolysis of lignocellulose. Additional details of enzymes can be found in the KEGG database (Kanehisa and Goto, 2000). The following list is by no means exhaustive for every enzyme that may play a role in hydrolysis.

General Grouping	**Name**	**Classification**	**Activity**	**Comments**
Cellulases Sources: (Nidetzky et al., 1994) (Bélaich et al., 1997)	Endoglucanases	EC 3.2.1.4	Endohydrolysis of β 1-4 D-glycosidic linkages in the cellulose chain	
	Exoglucanases	EC 3.2.1.91	Hydrolysis of β 1-4 linkages at non-reducing end releasing cellobiose	Also referred to as cellobiohydrolase, CBH
	β-glucosidases	EC 3.2.1.21	Hydrolysis of terminal bonds at non reducing end releasing D-glucose	
Hemicellulases Sources: (Bachmann and McCarthy, 1991) (Alvira et al., 2011)	Endoxylanase	EC 3.2.1.8	Endohydrolysis of β 1-4 xylosidic linkages in xylan	
	Exoxylanases	EC 3.2.1.156	Hydrolysis of β 1-4 bonds at reducing end of xylan releasing xylose	
	β-xylosidase	EC 3.2.1.37	Hydrolysis of β 1-4 xylan bonds, releasing xylose from non-reducing end	
	α-L-arabinofuranosidase	EC 3.2.1.55	Hydrolysis of non-reducing terminal arabinofuranoside residues	
Accessory Enzymes Sources: (Akin and Rigsby, 2007) (Jeffries, 1990) (Moore et al., 1996) (Saha and Cotta, 2010)	Ferulic acid esterase	EC 3.1.1.73	Hydrolysis of feruloyl residues releasing ferulic acid	Found to be absent in a study of commercial enzyme cocktails
	Acetyl xylan esterase	EC 3.1.1.72	Deacetylation of xylo-oligomers	

It is important to note that although the general classifications of activity found above are useful when discussing the hydrolysis of lignocellulose they understate the system's complexity. It is believed that different enzymes with the same activity classification can still display a synergistic activity, suggesting that many of these enzymes also possess side activities (Nidetzky et al., 1994).

There are two main sources of enzymes for the degradation of lignocellulose – fungi and bacteria. Earlier work tended to be centred around the cellulolytic fungi which have been under investigation since the 1940's (Waksman, 1940). Of particular interest were *Trichoderma reesei, Trichoderma viride and Aspergillus niger* (Montenecourt, 1983, Toyama, 1976, King and Smibert, 1963). More recently researchers have begun looking for novel lignocellulose degrading enzymes in various prokaryotes, for example in the genera *Clostridium, Bacillus* and *Thermomonospora;* the topic of bacterial enzymes for lignocellulose degradation was reviewed recently with the authors concluding that the diversity of inhospitable environments bacteria are found in suggests the interesting possibility of discovering enzymes with far broader physical tolerances than fungal ones (Maki et al., 2009)(Maki et al., 2009)(Maki et al., 2009)(Maki et al., 2009)(Maki et al., 2009)(Maki et al., 2009)(Maki et al., 2009)(Maki et al., 2009)(Maki et al., 2009)(Maki et al., 2009)(Maki et al., 2009)(Maki et al., 2009)(Maki et al., 2009)(Maki et al., 2009)(Maki et al., 2009).

There are some areas of ongoing work which appear to offer significant potential for future development. White rot fungi, which naturally grow on and degrade lignocellulosic biomass, are known to produce several lignin degrading enzymes. It has been speculated that these could be of use in the enzymatic degradation of biomass for bioethanol production, although their use is still at an experimental stage (Hatakka, 1994, Banerjee et al., 2010a). Another topic that is currently the focus of much research is the fact that the cellulases of some species, notably *Clostridium thermocellum,* form complexes that are attached to the cell surface (Bayer et al., 1985, Bayer et al., 2007). It is hoped that this concentration of enzyme activities in a small area may improve the efficiency of biomass hydrolysis.

Despite the interest that has been shown in bacterial enzymes, most current commercially available enzyme cocktails are almost all fungally derived, particularly from strains of *Trichoderma.* The current situation in the commercial lignocellulosic enzyme market has recently been reviewed, with a focus on research and development funding. Whilst the authors were pleased with the desire shown by a recent US DOE report to fully develop the potential of novel bacterial enzymes, they were concerned by the fact that almost all DOE enzyme research funding was concentrated in four large commercial producers with a focus on one feedstock, dilute acid pretreated corn stover, which could lead to under development of hemicellulose degrading enzymes (Banerjee et al., 2010b, US Department of Energy, 2006). Another concern with the current situation is that the focus of fungal enzymes is a potential disadvantage to some novel fermentative organisms; fungal enzymes tend to work best a slightly acidic pH values and moderate temperatures whereas some promising ethanologenic bacteria are better suited to neutral pHs and higher temperatures (Section 1.4.3). Development of enzyme technology has been rapid in recent years aided by the development of high throughput screening technologies and a greater understanding of the role of non-catalytic proteins in biomass degradation. It is still felt however that there is significant scope for further improvement in coming years (Harris et al., 2014)

1.3.2 Optimisation of enzyme cocktails

Several efforts have been made to design a base set of enzymes that can hydrolyse any source of available biomass. Generally this work has taken the form of defining a core set of enzymes that can be supplemented with various accessory enzymes. Attempts have been made to define an optimal set of enzymes for both cellulose and hemicellulose. It was discovered that by varying the proportions of the various cellulases produced by *Trichoderma viride*, glucose release from steam exploded corn stover could be increased 2.1-fold over a crude enzyme preparation (Zhou et al., 2009). Work supported by Novozymes, one of the main commercial enzyme producers, found that when enzyme activities were tailored to the composition of different wheat aribinoxylans, good hydrolysis could be achieved with significantly lower enzyme dosages than with commercially available enzyme mixtures (Sørensen et al., 2007). Whilst this shows the potential for optimising the hydrolysis of hemicellulose, the authors put extensive effort into tailoring the enzyme cocktails to particular relatively pure substrates and it is unlikely that this could be carried out for all real-world feedstocks of interest. Attempts have also been made to design optimal cocktails for the hydrolysis of whole feedstocks; for example an optimised mixture of four cellulases and two hemicellulases was developed for the hydrolysis of AFEX pretreated corn stover (Gao et al., 2010).

Banerjee et al. carried out an extensive set of experiments looking to optimise the enzyme cocktails for five feedstocks, including DDGS, subjected to three alkaline pretreatments (Banerjee et al., 2010a). Working with a similar base set of four cellulases and two hemicellulases (both xylanases), differences were seen that related to both feedstock and pretreatment. In terms of DDGS, the optimal cocktail contained relatively more of the xylanases although release of xylose was still very poor, a fact that was attributed by the authors to the high levels of arabinose in DDGS. The authors continued the work by adding an additional 10 enzymes alongside the core six (all glycosyl hydrolases). Further optimisation of this mixture of up to sixteen enzymes produced enzyme mixtures whose performance was superior to two commercial cellulase mixtures. In terms of DDGS the most important of these extra activities were arabinofuranosidase, amyloglucosidase, β-mannanase and α-glucuronidase. The authors were of the opinion that current knowledge of the lignocellulosic structure of DDGS was insufficient to explain the activities required in the optimal mixture. It was also observed that a mixture of four commercial enzyme complexes gave significantly improved xylose yields when compared to the most efficient mix of individual enzymes prepared by the authors. It seems that the authors 16 enzyme mix was lacking esterase activities, possibly explaining its inferiority compared to the commercial mixtures.

This last fact highlights one of the main limitations of developing optimised mixtures of purified enzyme activities; commercial preparations are crude extracts from microbiological cultures and thus may contain low levels of a very wide range of activities, different ones of which may be important under different circumstances. Given that over 80 different proteins were detected in a commercially produced cellulose preparation, it would take significant effort to isolate and optimise the ratios of all the activities found in commercial preparations (Nagendran et al., 2009). A further complication is that whilst many of these proteins will be enzymes whose catalytic activity can in theory be determined, recent discoveries suggest that there are some proteins secreted by cellulase degrading organisms, known as swollenins and expansins, that whilst not catalytically active themselves, still serve to enhance the hydrolysis of lignocellulose (Saloheimo et al., 2002, Kim et al., 2009a).

Thus whilst such optimisations of individual enzyme ratios may be useful in terms of suggesting activities to target for up-regulation in commercial mixtures, of more commercial relevance is the approach taken above and by others (Berlin et al., 2007), wherein optimised ratios of different commercially available enzyme mixtures are investigated.

1.3.3 Kinetics of lignocellulose hydrolysis

The kinetics of lignocellulosic hydrolysis are relatively complex, in part caused by the range of different enzymes involved and the two physical phase (solid and liquid) interactions. A general schematic diagram of the mechanism of lignocellulose hydrolysis down to the dimer sugar stage is shown in Figure 1.2. Where the product is a dimer, this will be further cleaved to monomeric sugars by β-glycosidases in a simpler step that is believed to follow Michaelis-Menten kinetics with competitive product inhibition (Chauve et al., 2010).

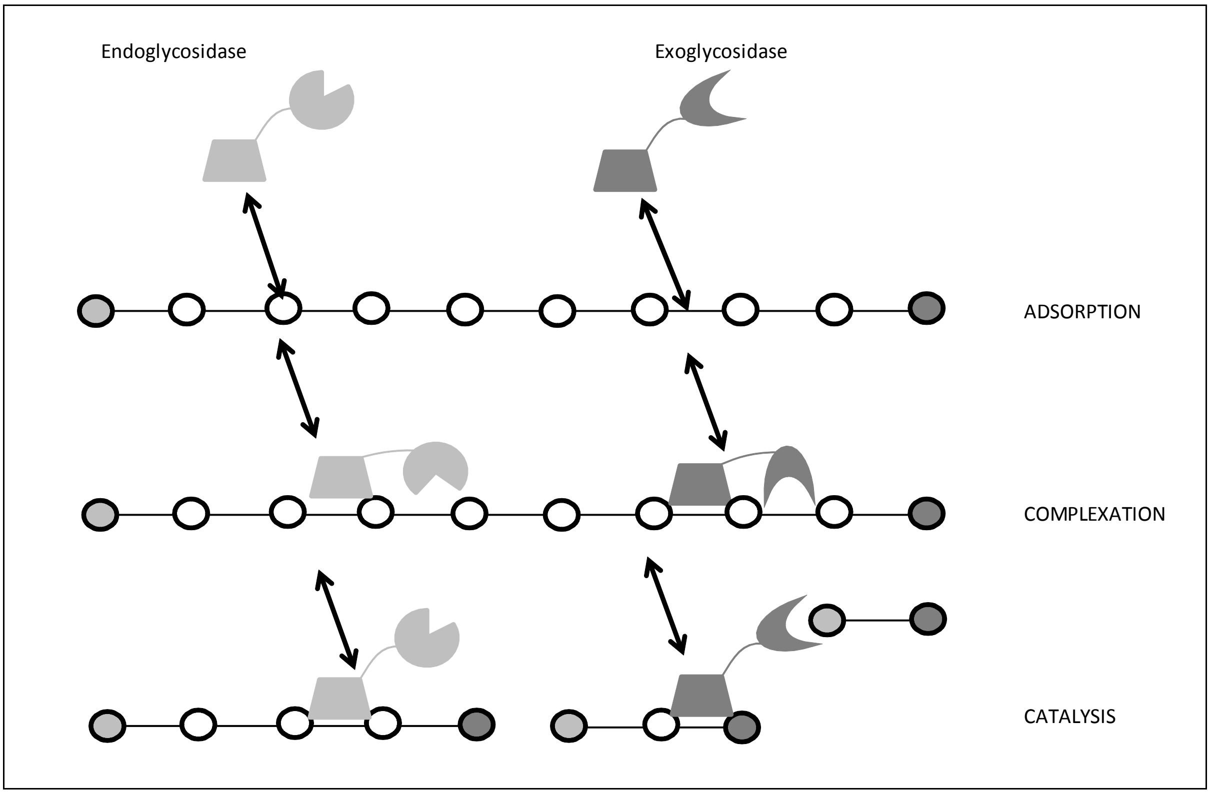


Figure 1.2. General schematic mechanism for the solubilisation of lignocellulosic polysaccharides by endo and exo glycosidases. Adapted from Levine et al. (2010).

There are believed to be three stages to the process with the enzyme first adsorbing to the surface of the substrate, followed by the active site complexing with the substrate. Catalytic cleavage then occurs and products are released. The mechanism for the solubilisation and cleavage of the longer chain polysaccharides is heavily dependent on an unusual feature found in the structure of many lignocellulose degrading enzymes, a Carbohydrate Binding Module (CBM) (sometimes referred to as Carbohydrate Binding Domain (CBD)). These features have been reported in a wide variety of cellulases and hemicellulases, and seem to be responsible for the adhesion of the enzyme to the substrate, but are entirely separate from the catalytic domain (Selvaraj et al., 2010, Ljungdahl, 2008, Ferreira et al., 1990, Boraston et al., 2004). It has been reported in many cases that there is a progressive action of cellulases along the surface of the substrate meaning that dissociation and adsorption do not need to occur fully every time there is a catalytic event (Li et al., 2007).

Trying to develop full computational models of the enzyme hydrolysis of real world feedstocks is immensely challenging due to the numerous linked reactions which are occurring, and therefore most work on the subject takes the approach of simply quantifying sugar concentrations generated following a period of hydrolysis. There is a very wide range of reported sugar yields in the literature due to the vast number of permutations of feedstock, pretreatment method and enzyme cocktail; thus it is impossible to present a comprehensive review of reported hydrolysis yields. Instead, this review will focus on what has been reported on DDGS and closely related feedstocks as these are the focus of this project.

It is evident that arabinose, despite representing approximately 17% of the sugar available from DDGS, is rarely reported in the results of hydrolysis experiments. This is likely due to the fact that the yeasts often used in fermentation steps have only recently been engineered to ferment arabinose successfully, meaning that it has often been ignored in terms of hydrolysis. Table 1.4 also shows that generally yields of xylose are lower than those of glucose, and the same reasons likely apply. The highly variable results for xylose, with yields ranging from less than 20% to over 80% is likely to be in part due to what is considered the 'standard' protocol for the hydrolysis of lignocellulose. Published in 1996 by the National Renewable Energy Laboratory (NREL), this aimed to provide a standard method that could provide comparable data across different groups working with different feedstocks and pretreatments in the emerging bioethanol field. It calls for the hydrolysis of biomass at low solids loadings with cellulase and β-glucosidase at certain activity loadings (Brown and Torget, 1996). Whilst this was of value at the time, its focus on cellulose hydrolysis, and therefore glucose, which represents only around half of the available sugar in DDGS is dated given the bioethanol industry's focus on using all of the fermentable sugars available in order to maximise the economic returns of the process. Whilst some published work included all three of the main sugars in the hydrolysis data, in much of the literature there is still a focus just on one or two sugars.

Table 1.4. Summary of published hydrolysis results with DDGS or WDG as a feedstock. a) Performed in microwells with very low loading of substrate b) Calculated from data published in paper c) Hydrolyses performed without hemicellulase mixtures so results expected to be low. SOURCES: 1:(Kim et al., 2008a), 2: (Kim et al., 2010b), 3:(Banerjee et al., 2010a), 4: (Dien et al., 2008)

Material	PT	Hydrolysis Solids Loading	Overall Hydrolysis Yield	Glucose Yield	Xylose Yield	Arabinose Yield	Ref.
DDGS	LHW	5% DS		98%	<45%[c]		1
DDGS	AFEX	5% DS		103%	<20%[c]		1
DDGS	LHW	6% DS	85%[b]	91%	82%	70%	4
DDGS	AFEX	6% DS	95%[b]	101%	81%	98%	4
DDGS	LHW	20% DS	63%[b]	70%	54%	59%	4
DDGS	AFEX	20% DS	69%[b]	75%	55%	70%	4
DDGS	AFEX	0.5% glucan[a]		57%	29%		3
WDG	LHW	5% DS		75%	N/A[c]		2
WDG	AFEX	5% DS		75%	N/A[c]		2
WDG	LHW	15% DS		71%	41%		1
WDG	AFEX	15% DS		72%	45%		1
WDG	LHW	15% DS		85%	50%		2
WDG	AFEX	15% DS		75%	40%		2
WDG	LHW	20% DS		83%	50%		1
WDG		30%DS		67%	38%		1

Several attempts have been made to develop mathematical models that adequately describe the complexities found in the hydrolysis of lignocellulose. Schell and co-workers used empirical data to model the effect of enzyme loading and solids content on the hydrolysis of Douglas fir and developed a model that had a reasonably good fit to the experimental data; however no attempt was made to test predictions from the model in the laboratory (Schell et al., 1999). More recently, Levine et al. published a model based around theoretical spheres and two cellulase enzymes (an endoglucanase and an exoglucanase). The model was developed to give a good fit to experimental data during the early stages of hydrolysis, but was unable to predict accurately experimental sugar release (from a simple model substrate) during the latter stages of hydrolysis without making unrealistic assumptions (Levine et al., 2010). Further development of the model suggested that complexation of an enzyme with a glucan chain was the rate limiting step for degradation. The authors used accessory enzymes which had been reported to speed up this step of the enzyme hydrolysis process and did indeed see an increase in the overall rate of hydrolysis (Fox et al., 2012). Although clearly suggesting potential for the use of mechanistic models to identify rate limiting steps in the enzyme hydrolysis process it should be noted that the experimental portion of this work used a relatively simplistic model feedstock which would eliminate many factors found in a commercial process.

Given the difficulties encountered in a comparatively recent work using just two enzymes and a model substrate, it is perhaps not unexpected that other authors looking to model lignocellulose hydrolysis have often focussed on just one aspect rather than the whole system. Thus models have been developed to explain decreased specific performance at high enzyme to substrate ratios, the sharp decrease in hydrolysis rates seen after the initial period of hydrolysis and the effect that certain chemical additives can apparently have on enzyme hydrolysis of lignocellulose (Bommarius et al., 2008, Jalak and Väljamäe, 2010, Wang et al., 2007).

Another approach has been to model the effect of mixing on enzyme hydrolysis with the authors concluding that the best results would be achieved with good macromixing but limited micromixing (Chakraborty and Gaikwad, 2010). These predictions appear to have support from experimental data, where it was shown that continuous high speed shaking gave superior hydrolysis performance to intermittent mixing (Ingesson et al., 2001, Mais et al., 2002). Both of these experiments however compared high speed shaking with low and intermittent speeds. There was no investigation of whether there was a critical shaking speed above which further increases were ineffectual, as has been reported elsewhere. When the hydrolysis of high solids content cellulose fibre suspensions over the initial few minutes of hydrolysis was investigated, no benefit to conversion or particle size reduction was seen with a doubling of agitation rate once a critical point was reached (Samaniuk et al., 2011).

1.3.4 Effects of solids loading on enzyme hydrolysis performance

From the work described above there are two trends that stand out as being consistent across different feedstock and enzyme combinations; higher levels of dry solids content hydrolysis with the same enzymes under the same conditions will give a lower hydrolysis yield, and that secondly that regardless of the feedstock or enzymes used there is an initial, very rapid burst of enzymatic conversion before the rate slows markedly after the first few hours.

The effect of solids loading on hydrolysis has been observed on many occasions although it has seldom been the focus of investigations (Sims, 2013, Kim et al., 2008a, Roche et al., 2009b, Yang et al., 2011). Although working at low solids loading may prove useful for initial investigations into optimisation of various process parameters, if bioethanol processes are to be viable then it is vital that studies are carried out at commercially viable solids levels (Humbird et al., 2010). The relatively limited body of work that has been focussed on identifying the reasons for the relatively poorer performance at higher solids loadings is therefore somewhat surprising. One often mooted cause of the drop in hydrolysis performance seen at high solids loadings is end product inhibition by the sugars that are released (Cara et al., 2007). However, Pristavka and co-workers by using the hydrolysate liquor from previous high solids hydrolyses of steam exploded willow as a diluent for fresh hydrolyses of the same material reached a different conclusion. A halving of the initial rate of hydrolysis was seen in the presence of the hydrolysate liquor; however the authors concluded that due to the high concentrations of sugar that were being added in (>150g L^{-1} sugar) if end product inhibition were the main issue at high solids loadings then a greater effect would be seen (Pristavka et al., 2000).

A mixed mode of inhibition has been suggested from experiments using washed pretreated corn stover. The authors concluded that below approximately 20% solids loading inhibition was primarily due to soluble inhibitors in the pretreatment liquor, with sugars being the main inhibitors, but that at above this solids loading the well-known high viscosities of high solids biomass slurries caused mass transfer limitation which became the primary limit on the extent of hydrolysis (Hodge, 2008, Ehrhardt et al., 2010). This however fails to explain the fact that performance at high solids remains worse even after the initial high viscosity has been reduced.

Perhaps the most comprehensive work to date on the limitations to enzymatic hydrolysis of biomass imposed by high initial solids loadings was carried out by Kristensen et al. (2009) where the authors investigated various proposed causes for the solids effect. The authors summarised previous examples of the solids effect and found they covered a wide range of feedstocks and solids compositions and that there was nearly always a linear decrease in hydrolysis yield as solids loading increased. On this basis they proposed that a single factor is likely to be the cause in contrast to the mixed model outlined above. The authors found that enzyme adsorption to the filter paper they were using as a substrate decreased linearly as solids loading increased, and that there was a statistically significant link between the decreased in enzyme adsorption and hydrolysis yield (Kristensen et al., 2009). They suggested that decreased enzyme adsorption was the cause of the solids effect and suggested that it may be mediated by products such as sugars. This would not be the same as classical product inhibition which affects the active site, however they were unable to produce experimental data to back this assertion, and it seems not to fit with other data in the paper which led the authors to conclude that direct product inhibition, whilst undoubtedly occurring, was not the cause of the solids effect. It is also worthwhile noting that in a previously mentioned study on shaking regimes, degree of enzyme adsorption under the different mixing regimes did not correlate well with their respective hydrolysis conversion yields (Mais et al., 2002).

The idea of product inhibition being an important factor at high solids levels is however supported by recent work in which a 3 stage hydrolysis (3 separate hydrolyses with the solids after each stage being washed before passing to the next hydrolysis step) produced significantly superior yields when compared to batch hydrolysis of 25% or 30% solids pretreated corn stover (Yang et al., 2011).

Thus it seems that whilst enzyme adsorption may have been identified as the proximate cause of the solids effect, the ultimate cause remains to be uncovered. This problem needs to be an important focus of research due to the key effect solids loading will have on process economics.

Due to the problems identified with mixing at high solids loadings of lignocellulosic biomass, several authors have attempted to devise systems which improve the hydrolysis of biomass at high solids loadings. One such system is the ‘Roller Bottle Reactor’ where hydrolysis is carried out inside sealed bottles which are then rotated horizontally, providing superior solids distribution and hydrolysis performance compared to regular shake flasks and giving consistent results at scales from 125mL to 2L (Roche et al., 2009b, Roche et al., 2009a). Horizontal agitation systems have also been described in a more scalable form, with paddles on a central rotating shaft running through a horizontal reactor allowing successful hydrolysis of corn stover at up to 40% solids loading (Sims, 2013). An alternative, chemical, approach has also been taken whereby rheology modifiers such as cetyl trimethylammonium bromide (CTAB) were found to significantly reduce the viscosity of corn stover slurries at very low addition levels. This work focussed on the physical and economic effects of the chemicals and thus whilst they were found to be an economical means of reducing viscosity levels, no work was undertaken on what effect, if any, they would have on sugar release from the slurries (Knutsen and Liberatore, 2010). Finally it has also been proposed that a fed batch mode of hydrolysis, whereby the insoluble solids content in the reactor would be maintained constant, whilst the overall solids level would increase as soluble solids are converted to soluble sugars. Whilst this appeared effective with washed feedstocks, it was not tested with whole biomass slurries which may have a significant effect on its utility (Hodge et al., 2009).

1.3.5 Rates of enzyme hydrolysis

Another feature common to the enzymatic hydrolysis of various lignocellulosic feedstocks is that rates of enzymatic degradation are not constant; there tends to be a short period of very rapid hydrolysis early in the time course, followed by continuing hydrolysis at a much reduced rate for the remaining time. This phenomenon has been seen with a wide variety of feedstocks, including model substrates, and enzymes (Zabihi et al., 2010, Kim et al., 2008a, Saha and Cotta, 2010, Lee and Fan, 1982). The mechanism of this reduction in rate and possible measures to reduce its impact is the focus of much work. If the initial very rapid rates of hydrolysis could be maintained for a greater portion of the whole enzymatic hydrolysis, overall process times could be considerably reduced and the economics of the whole process improved.

Using calorimetry as a method for continually monitoring the progress of enzymatic hydrolysis, it was recently proposed that in the initial stages of hydrolysis, when conversion is below approximately 10%, available attack points on the biomass are the limiting factor, but that at higher conversions, the rate of hydrolysis is proportional to enzyme loading, with the slowdown being governed by the degree of conversion and possibly build-up of products (Olsen et al., 2010).

Jalak and Väljamäe (2010) attempted to estimate the observed catalytic rate for CBH enzymes from two fungi indirectly by noting the reduction in the rate of hydrolysis of a reporter molecule over the first 60 minutes of hydrolysis. They discounted product inhibition due to the excess of CBH and found no link between the degree of conversion of a feedstock and the decline in the catalytic rate. They also found that the drop in observed catalytic rate was similar for different enzyme: substrate ratios and so discounted the possibility of steric hindrance on the substrate surface. In the end the authors concluded that their data best fit with the hypothesis that hydrolysis continues with the enzymes progressing along the substrate using the carbohydrate binding domain until reaching an obstacle such as a branched side group, at which point hydrolysis is slowed dramatically due to a low dissociation constant for the enzyme. However the authors note that this hypothesis does not wholly fit with the data in that lignocellulose, which would be expected to have more obstacles than amorphous cellulose, in fact had a similar rate of decline in catalytic rate. Also the same rate of decline was seen regardless of whether hemicellulases and accessory enzymes were present, even though these enzymes would be expected to remove obstructions (Jalak and Väljamäe, 2010).

Similar conclusions were reached in a separate study working with Avicel, a model substrate that contains in excess of 95% cellulose. It was shown that whilst the hydrolysis rate (i.e. solubilisation per hour) decreased sharply over the first 5 hours of standard hydrolysis, washing the remaining solids with a protease and then restarting hydrolysis recovered the initial hourly rate of hydrolysis repeatedly upon the restart of hydrolysis. Whilst the rate of a regular hydrolysis dropped to only 10% of its initial value by the time half of the original substrate had been converted in unwashed samples, washing and restarting the hydrolysis maintained the hourly rate at least at the initial level up 80% overall conversion. The effect was only observed when a protease was included in the washing, leading the authors to conclude that enzymes were in some way getting ‘stuck’ to the substrate (Yang et al., 2006).

The reduction in rate could also be related to the phenomenon of unproductive enzyme binding to lignin, whereby it is proposed that cellulases become adsorbed to lignin within the biomass, but are unable to detach due to a low dissociation constant and thus become stuck (Karlsson et al., 1999). It is widely believed that this unproductive binding can to some extent be overcome by the addition of surfactants or Bovine Serum Albumin (BSA) into the hydrolysis slurry in order to increase the dissociation rate of adsorbed enzymes (Börjesson et al., 2007, Kaar and Holtzapple, 1998, Eriksson, 2002, Yang and Wyman, 2006).

In addition to the above considerations, there are also several pieces of work that support the idea that the slowdown in the rate of hydrolysis is due in large part to the accumulation of products in the liquid fraction of the hydrolysate. It is well established that glucose and cellobiose are strong inhibitors of β-glucosidases and cellulases respectively (Mandels and Reese, 1965), but more recently inhibitory effects have also been attributed to other sugars, xylose, mannose and galactose, on endo and exo-glucanases but interestingly not β-glucosidase (Xiao et al., 2004). Qing et al. have shown that xylooligomers have an even stronger negative effect than monomers on both the extent and the initial rate of hydrolysis of Avicel, with initial rates reduced by up to 85% and final yields reduced by approximately 50% (Qing et al., 2010). As Avicel contains virtually no hemicellulose, it seems unlikely that end product inhibition of hemicellulase enzymes is the cause suggesting that the effect really is on the cellulases. Because of the inhibitory effect that sugars are known to have on the enzymatic hydrolysis of biomass it has been suggested that membrane technology could be used to continuously remove glucose (and by extension other sugars) in order reduce inhibitory effects (Andrić et al., 2010).

It is also believed that soluble chemicals other than sugars, especially phenolic compounds, have an inhibitory effect on biomass enzymatic hydrolysis. Tests have shown that such compounds can cause up to 50% inhibition of cellulases and almost 90% inhibition of β-glucosidase activity during the hydrolysis of wet cake (Vohra et al., 1980, Ximenes et al., 2010). In this case the compounds were added exogenously at concentrations somewhat in excess of those found in the actual feedstock; however the same effect has been observed with real pretreatment liquors through the selective removal of phenolic compounds (Kim et al., 2011). Both this paper and another published by the same authors note that as well as having an inhibitory effect on cellulases, phenolic compounds can also cause the precipitation of proteins and hence reduce the effective quantities of enzyme present over time (Ximenes et al., 2011).

Thus, as with the effects of solids on enzyme hydrolysis performance (Section 1.3.4), there is consensus that there are factors limiting the rate of enzyme hydrolysis, but it is far from certain exactly what they are. It should be noted that especially in the case of soluble inhibitors there may well be a link between the two phenomena. If a build-up of compounds solubilised during the enzyme hydrolysis process can limit the rate of sugar solubilisation, this problem would be exacerbated at higher biomass concentrations.

Historically the enzymatic conversion step has been seen as one of the more complex and costly steps in the lignocellulosic ethanol process, and continues to be considered by leading industry figures as one of the key technological areas into which government research funds should be targeted (Lynd et al., 1991, Wyman, 2007). Intensive research efforts have already resulted in substantial reduction in the cost of commercial cellulase and hemicellulase enzymes (Bryant, 2009). Yet the above discussion makes it clear that there are substantial areas of uncertainty regarding the enzymatic hydrolysis of lignocellulosic biomass and thus there is hope that far greater cost savings for this step will come along in the future years as understanding increases.

1.4 Fermentation

1.4.1 Background

The next step in the bioethanol process is the conversion of the sugars released during enzyme hydrolysis into ethanol via fermentation in a process that dates back many thousands of years. The original wild yeasts responsible have been replaced with cultivated strains of brewer's yeast, *S. cerevisiae,* and it is this organism that is still responsible for fermentation in 1st generation starch and sugar processes today.

Because of its ubiquity *S. cerevisiae* was also used in many of the initial investigations into lignocellulosic ethanol processes. However, its limitations for such processes were quickly evident, due to the yeast's inability to ferment the hemicellulose derived sugars (mainly xylose and arabinose) and its susceptibility to the inhibitors found in lignocellulosic hydrolysates (Duff and Murray, 1996).

Early attempts to circumvent this problem often involved the use of yeasts of the genus *Pichia* which were naturally able to ferment many of the sugars which *S. cerevisiae* was not (Wilson et al., 1989). Attempts at fully utilising the sugars in lignocellulosic hydrolysate streams were thus made based around the idea of a co-fermentation involving both *Pichia* and *S. cerevisiae* or in some cases sequential fermentation of glucose followed by xylose (Grootjen et al., 1990, Slininger and Bothast, 1988). Additionally, some studies continue to show that having separate xylose and glucose fermentation streams is a more attractive process economically, largely due to the lower efficiencies seen when bioprocessing combined streams (Dutta et al., 2010). Theoretically however a process that had a combined fermentation should be economically more attractive, and the simultaneous fermentation of the full post hydrolysis sugar stream continues to be seen as the ideal for a cellulosic ethanol process (Kim et al., 2010a). Table 1.5 shows a list of suggested requirements for what may be considered an ideal ethanologenic organism for a 2nd generation bioethanol process adapted from an extensive review of potential fermentative organisms for a lignocellulosic ethanol process.

Table 1.5. Requirements for an ideal fermentative organism for a 2nd generation bioethanol process. Adapted from(Dien et al., 2003).

Substrate Usage	Able to utilise all significant sugars in feedstock
Ethanol Yield	> 90% of theoretical
Ethanol Titre	$>40gl^{-1}$
Ethanol Productivity	$>1gl^{-1}h^{-1}$
Hydrolysate Tolerant	Should be resistant to major lignocellulosic inhibitors
Robust Growth	Simple, inexpensive nutrition requirements
Contamination Resistance	Growth at acidic pH or high temperature

1.4.2 Common second generation bioethanol organisms

A wild type organism that fulfils all of these requirements has yet to be discovered, leading to many attempts to introduce the desired characteristics into various strains. Approaches to the problem have been attempted from two different directions. One is to take an organism which has an efficient fermentative metabolism for glucose, and introduce into it the metabolic pathways necessary to utilise the other major sugars found in biomass, the other is to start from a strain that can already utilise a broad range of sugars and introduce into it a fermentative metabolism.

It is simpler from the point of view of this review to begin with the former approach since generally only two organisms are involved, S. *cerevisiae* and *Zymomonas mobilis*, a gram negative bacterium which efficiently ferments glucose, fructose and sucrose (Rogers et al., 1982). *Z. mobilis* was for a period considered by some as a serious alternative to *S. cerevisiae* in first generation ethanol processes although the yeast's greater hardiness meant it continued to be preferred even though the bacteria has a higher yield of ethanol per mole of glucose and produces ethanol at a higher rate (Lin and Tanaka, 2006, Doelle et al., 1993). Strains of both of these organisms have now been engineered to utilise a much broader range of sugars.

Z. mobilis was altered in the mid-1990s to ferment both xylose and arabinose, the two main pentose sugars found in biomass, in the case of the former by introducing the *xylA, xylB* and pentose phosphate pathway from *Escherichia coli*, the latter by introducing *araA, araB* and the pentose phosphate pathway genes, again from *E. coli* (Zhang et al., 1995, Deanda et al., 1996). More recently a stably integrated strain of *Z .mobilis* named AX101 that was capable of co-fermenting mixtures of glucose, xylose and arabinose (albeit large proportions of the sugar were used sequentially rather than simultaneously) was created and is now considered one of the leading 2nd generation bioethanol microbes (Mohagheghi et al., 2002). A modified strain of *Z. mobilis* is to be used in the $250 million production plant currently under construction by DuPont and Danisco (Winters, 2011).

Genetic and metabolic engineering of *S*. cerevisiae with the goal of improving its use of pentose sugars has been a focus of interest for many years and continues to be to this day. Whilst performance initially was poor, significant advances in our understanding of xylose metabolism in yeasts have led to strains with significantly improved fermentation performance on xylose (Sarthy et al., 1987, Kuyper et al., 2005, Jeffries, 2006, Liu and Hu, 2010). The first yeast strain engineered for xylose fermentation with acceptable fermentation performance was reported in 1998 and was created by introducing the xylose reductase, xylitol dehydrogenase and xylulokinase genes of *Pichia stipitis,* a naturally xylose utilising yeast (Ho et al., 1998). Earlier work introducing only the first two of these genes resulted in xylose fermentation, but with poor kinetics (Walfridsson et al., 1997). The strain reported by Ho et al. in 1998 has undergone significant further development, and the group recently reported that they had engineered the strain to successfully ferment arabinose, meaning that the strain was now capable of fermenting all of the main biomass sugars (Bera et al., 2010). Again, this strain is considered a leading candidate as a successful fermentative organism (Lau and Dale, 2009). Two other commercial scale facilities (one under construction, one recently completed) are intending to use modified yeast strains as a fermentative organism (Evans, 2014, Voegele, 2013).

Taking the opposite approach and looking to improve the fermentative metabolism of an organism with an already wide sugar utilisation profile was likely to involve *E. coli* due to its wide substrate range, easy growth and well established genetic manipulation techniques. As far back as 1987 the pyruvate decarboxylase (PDC) and alcohol dehydrogenase II (ADH2) genes from *Z. mobilis* were introduced into *E. coli* via the so called *pet* operon*,* significantly improving its fermentative metabolism (Ingram et al., 1987, Ohta et al., 1991). Further work to improve the ethanol tolerance of this strain followed, and it is now widely used for research purposes in the bioethanol field (Yomano et al., 1998, Lau et al., 2008).

These three strains *Z. mobilis* AX101, *E. coli* KO11 and *S. cerevisiae* 424A(LNH-ST), considered by many to be the leading strains available for academic bioethanol researchers (other strains exist within companies conducting privately funded research into bioethanol, but these are commercially sensitive and not easily available for published research), have been compared side by side in a single publication. All three achieved target yields of ethanol (>40g L^{-1}) on mixed sugars in a media background, albeit with the rate of the two bacteria be being significantly higher than the yeast. However, when the strains were grown in corn stover hydrolysate (from an AFEX pretreatment) it was 424A which was the best performer, producing approximately one third more ethanol than the two bacterial strains, apparently due to a much higher tolerance to the inhibitors present in the hydrolysate stream (Lau et al., 2010). This work shows clearly that caution needs to be attached to performance figures reported for new organisms when based on growth in synthetic media unless verified on hydrolysate. Given the wide variability that will be expected in the composition of hydrolysate streams from different biomass, pretreatment and enzyme combinations, it also suggests that the organism which performs best in one process may not necessarily be the best solution for all processes.

1.4.3 Thermophilic ethanologens

All of the organisms cited above are mesophilic, meaning they grow best at moderate temperatures, and both of the bacteria also have neutral pH optima. Processes utilising these strains are therefore likely to be at risk from contamination from competing microorganisms, often Lactobacilli, in much the same way as current first generation processes are. This problem would be exasperated if 2nd generation bioethanol fermentations last significantly longer then first generation glucose only fermentations. This would be expected from the fermentation profiles in Lau et. Al (2010), where peak ethanol often did not occur until 100h hours into the fermentation (Lau et al., 2010, Skinner and Leathers, 2004, Schell et al., 2007).

These two factors, risk of contamination and process time, are significant drivers in the search for thermophilic bacteria as ethanologens. These, along with reduced process cooling requirements and possibly the opportunity for continuous product stripping, could in theory give a thermophilic fermentation process significant economic advantages (Sonnleitner and Fiechter, 1983). In addition, thermophiles are commonly found in environmental situations where the ability to utilise lignocellulosic biomass could be of an evolutionary advantage such as such as compost heaps and waste streams in facilities such as breweries or paper mills. Screening tests of isolates from such locations identified several microorganisms which were capable of growing on the hydrolysate portion of pretreated material without it being enzymatically hydrolysed, suggesting an ability to utilise oligosaccharides, something which could significantly reduce enzyme uses in a 2nd generation ethanol process (Sommer et al., 2004, Payton, 1984, Maki et al., 2009).

Despite their potential advantages, the development of the production of biofuels by Thermophilic organisms has traditionally been held back by the fact that they have a mixed acid metabolism (as opposed to the systems in *S. cerevisiae* or *Z. mobilis* which produce only ethanol as a product under fermentative conditions) and relatively poor tolerance to products such as ethanol, combined with a lack of genetic manipulation tools to help improve these characteristics. However, as covered in a recent review, substantial progress has been made in recent years in developing such genetic techniques and applying them to deliver substantial improvements in performance in several of the organisms identified as having potential for the production of ethanol by Payton as far back as 1984 (Payton, 1984, Taylor et al., 2009).

Given its unusual cellusome based hydrolytic system that gives it the ability to grow directly on cellulosic substrates, there has been long standing interest in the development of *Clostridia* species, particularly *C. thermocellum,* for bioethanol production (Weimer and Zeikus, 1977, Ng et al., 1981). In terms on enhancing suitability as an ethanol producer, traditional UV mutagenesis and selection for mutants with impaired lactate production led to the isolation of strain LD1 which was capable of faster growth in 38 g L^{-1} ethanol than the wild type was in 10g L^{-1}, albeit the doubling time of 0.2 h^{-1} was only a fraction of what was achieved at lower ethanol concentrations. The strain was capable of utilising over 60 gL^{-1} cellulose, from which it produced 14.5 gL^{-1} ethanol (Tailliez et al., 1989). Early work also demonstrated that growth temperature and ethanol tolerance have significant interaction, and suggested that this was related to the fatty acid composition of the cell membrane with ethanol tolerance favouring a more rigid membrane and higher growth temperatures a more fluid one (Herrero and Gomez, 1980). This subject was reviewed in detail elsewhere, and many of the issues and mechanisms may be expected to be relatives common across different species and even genera (Ramos et al., 2002, Taylor et al., 2008a). Since those early studies were published, genetic manipulation techniques have been developed for a closely related organism *Thermoanaerobacterium thermosaccharolyticum* suggesting that such manipulations should also be possible for *C. thermocellum* (Tyurin et al., 2006). Despite this, and probably due to the fact that compared to other thermophiles reports of very high ethanol tolerances are few, the focus for *C. thermocellum* has shifted away from the production of ethanol and towards fermentative production of hydrogen or butanol to which they are perhaps better suited (Magnusson et al., 2009, Levin et al., 2006).

The next group of potential thermophilic ethanologens are from the genus *Thermoanaerobacterium* or the closely related *Thermoanaerobacter*, both of which used to be classified under the genus *Clostridia* (Collins et al., 1994). *Thermoanaerobacter mathranii*, isolated from a thermal spring in Iceland, was found to be capable of the fermentation of the hemicellulase portion of pretreated wet oxidised wheat straw (Larsen et al., 1997). Subsequent work in collaboration with the Danish company BioGasol has developed the recombinant BG1G1 strain which has the *ldh* gene deleted to eliminate lactate production and an NAD^+ dependant glycerol dehydrogenase *gldA* from *Thermotoga maritima* inserted in order to aid fermentative redox balance (Mikkelsen and Ahring, 2007, Yao and Mikkelsen, 2010). The resultant strain showed increased ethanol production and decreased acetate compared to the wild type when grown in batch culture. This was especially so with xylose as the sugar source where acetate production was reduced by 65% and ethanol production increased by over 40%. The strain has been reported to utilise over 40% of the xylose available in continuous culture in a background of 8.3% v/v (approximately 6.5% w/v) ethanol at 70°C (Georgieva et al., 2007). In a continuous immobilised bed reactor, the stain was able to effectively utilise non detoxified dilute acid pretreated corn stover at solids loadings of up to 15% (w/w). At 10% (w/w) solids around 90% of both the glucose and xylose in the feed was consumed with a yield of 0.4g/g (grams of ethanol per gram of sugar consumed); increasing the solids loading to 15% reduced the consumption figures to 72% and 67%, but the ethanol production yield remained high at 0.39g/g (Georgieva and Ahring, 2007)(Georgieva and Ahring, 2007).

Thermoanaerobacterium saccharolyticum is another organism isolated from hot springs, reported to be capable of utilising xylan and starch, but not cellulose, with a mixed acid metabolism including some ethanol (Lee et al., 1993). Genetic manipulation techniques have been developed for this organism, and this allowed the *ldh* gene to be successfully and stably deleted, eliminating the production of lactate in strain TD1, although the effect this deletion had on ethanol production was only marginal (Mai and Wiegel, 2000, Desai et al., 2004). Further work, supported by the Mascoma Corporation, deleted the acetate kinase and phosphate acetyltransferase genes which led to the elimination of acetate production during fermentation; subsequent continuous culture with increasing xylose concentrations led to strain ALK2. This strain produced 37gl^{-1} ethanol in a mixed sugar (glucose, xylose mannose and galactose) in a fed batch which was claimed to be the highest titre at that time reported for a thermophilic anaerobe (Shaw et al., 2008).

The other main thermophiles considered as viable candidates for bioethanol production are species of the genus *Geobacillus*, composed of a variety of organisms originally classified as *Bacilli (Nazina et al., 2001)*. In particular *G. stearothermophilus* and *G. thermoglucosidasius* have been identified as producing ethanol with a strain of *G. stearothermophilus* being reported to use almost 90% of the feed sugar in continuous culture using wheat straw hydrolysate (Atkinson et al., 1975, San Martin et al., 1992, Amarteyl and Leungl, 2000, Fong et al., 2006). In particular, one of the strains isolated by Fong et al. *G. thermoglucosidasius* M10EXG, with an optimum growth temperature of 60°C, is of interest due to the wide range of sugars it utilised (including glucose, xylose, arabinose and mannose) with a mixed acid metabolism, and its reported tolerance to 10%v/v ethanol. Further work carried out on M10EXG provided a detailed metabolic flux analysis which confirmed its mixed acid metabolism and could be used as the basis for future metabolic engineering of the strain to enhance ethanol production (Tang et al., 2009). The M10EXG genome has now reportedly been sequenced and modified to enhance its ethanol production (Pawate et al., 2009, Kozina et al., 2009).

Another strain of *G. thermoglucosidasius* has also been developed, again in partnership with a commercial organisation, ReBio Technologies Ltd. This work has involved the creation of a shuttle vector allowing manipulation of the strain, which was initially used to insert a copy of the *pdc* gene from *Zymomonas palmae* in an *ldh*$^{-}$ negative *G. thermoglucosidasius* mutant. The resultant enzyme was found to have a good level of activity at 45°C, but essentially no activity at 50°C (Taylor et al., 2008b). Subsequent work on a wild type strain generated a recombinant strain that had the lactate dehydrogenase (*ldhA*) and pyruvate formate lyase (*pfl*) genes deleted and expression of pyruvate dehydrogenase (*pdh*) upregulated (the latter modification being necessary due to an accumulation of pyruvate in *ldhA*$^{-}$ strains). The resulting strain, TM242, could ferment glucose in batch culture with an ethanol yield greater than 90% of the theoretical, and mixture of three sugars (glucose, xylose and arabinose) was utilised simultaneously with an ethanol yield over 80% of the theoretical, albeit with the rate of utilisation of pentoses being significantly slower than for hexoses. The resulting strain is also claimed to be capable of fermenting xylo-oligomers (Atkinson et al., 2007).

Thus there appears to be significant promise in the development of thermophiles for use as fermentative organisms for 2nd generation lignocellulosic ethanol processes. Whilst there continues to be much less published work for these thermophiles than for the mesophilic organisms outlined previously, this may in part be due to the fact that several of the promising thermophilic organisms are being developed with commercial partners, restricting what can be published.

1.4.4 Modes of operation

Whichever organism is chosen to be used in a bioethanol process, there are two basic modes of operation available: Separate Hydrolysis and Fermentation (SHF) and Simultaneous Saccharification and Fermentation (SSF). During SHF the hydrolysis and fermentation steps are separated, either physically in different vessels or temporally in the same vessel, whilst in SSF the two steps occur at the same time in the same vessel, although sometimes a short pre-hydrolysis step is included. Both have advantages and disadvantages. SHF allows each step to be run at its optimal conditions, SSF however reduces process times and costs, and in particular the consumption of sugars significantly reduces product inhibition of enzymes, allowing greater conversion of biomass. This is believed to be the reason why SSF is generally found to outperform SHF (Schell et al., 1999, Wingren et al., 2003, Tomás-Pejó et al., 2008, Marques et al., 2008, Drissen et al., 2009). In each of these cases however the fermentative organism used was a species of yeast. As most commercially available lignocellulose degrading enzymes are derived from fungi, the temperature and pH optima of the enzymes and fermentative organism will be expected to overlap and both can operate at close to their optimum conditions. It is clear that this will generally not be the case if the fermentative organism was a recombinant *E.coli* or *Z.mobilis* (mismatch of pH) or a thermophilic bacterium (mismatch of temperature and pH). Thus processes employing such organisms will not have SSF as a viable option until such time as enzymes with much broader operating conditions are available. In this regard the work of Dyadic, a US enzyme manufacturer selling products based around preparations of broth from *Myceliophthora thermophila*, is of particular note. Their enzyme preparations are reported to have wider optimal pH and temperature ranges than those from other producers (Gusakov, 2013). A recent review of progress towards developing SSF 2nd generation bioethanol processes covers reports of performance with a wide variety of process combinations (Geddes et al., 2011).

The other main distinction in terms of process operation is between batch fermentation and continuous fermentation. In batch fermentation the fermenter is filled with hydrolysate and then inoculum is added. The fermentation is then allowed to proceed through to the desired end point at which time the vessel is emptied and the contents harvested. In continuous culture, once the vessel is inoculated, fresh hydrolysate is fed into the vessel whilst broth is removed from the fermenter and sent to downstream processing at the same rate. It has long been known that there is an economic advantage associated with continuous culture largely due to increased specific rates of ethanol production and the significantly reduced equipment sizes that are required to achieve the same level of annual productivity (Ghose and Tyagi, 1979, Maiorella et al., 1984). However application of this technology has tended to be limited in practice by instability in cultures over extended growth periods and due to the increased risk of contamination that continuous processes face (Lacis and Lawford, 1988, Terrell et al., 1984, Roukas, 1996, Bayrock and Ingledew, 2001). It remains to be seen whether any of the novel strains proposed for 2nd generation bioethanol production are more amenable to continuous culture than yeasts, with the greatest chance probably lying with one of the thermophilic bacteria due to the increased contamination resistance such high growth temperatures would provide. In this regard the instability seen with *Thermoanaerobacter ethanolicus* by Lacis and Lawford (1988) is concerning, however our knowledge of the biology of thermophiles has improved dramatically in the years since their work was published and it is to be hoped that this may allow successful continuous fermentation as seems to be suggested by some of the recent published (Georgieva and Ahring, 2007, Shaw et al., 2008, Lacis and Lawford, 1988).

1.4.5 Inhibition of fermentation by compounds in hydrolysate streams

One thing that is clear from the literature is that regardless of the fermentative organism used in a process, inhibition by compounds in the hydrolysate stream is likely to have a significant negative effect on performance. Numerous examples can be found in the literature, including in several of the papers previously cited, in which the effect was seen with a variety of feedstocks and fermentative organisms (Lau et al., 2009, Lau and Dale, 2010, Larsson, 1999, Palmqvist et al., 1996, Lau et al., 2010). There have also been detailed reviews of the topic which can be consulted for further information on the mechanisms of action (Klinke et al., 2004, Palmqvist and Hahn-Hägerdal, 2000a, Palmqvist and Hahn-Hägerdal, 2000b). Due to its importance in the commercialisation of 2nd generation bioethanol, a brief overview of the recent work found to be effective in reducing inhibition will be provided here.

Two of the simplest methods that have been reported to be successful strategies are adaptation of the culture to the inhibitor or overliming i.e. the use of calcium hydroxide to precipitate out inhibitory compounds. The former largely restored fermentative performance when selected ‘model’ inhibitors were added into synthetic media fermentations at representative level. Overliming meanwhile was shown to restore full glucose consumption in a situation where a complex mixture of inhibitors derived from willow was added at varying concentrations to synthetic media. Overliming was successful in restoring performance at moderate inhibitor levels, but was not effective when the inhibitors were present at higher levels (Lau and Dale, 2010, Palmqvist et al., 1996). Furthermore overliming also been shown to be effective with whole hydrolysate, allowing fermentation of steam exploded spruce which was unfermentable when not detoxified (Cantarella et al., 2004).

Another relatively crude method of detoxification was recently used to improve the fermentative performance of the thermophile *T. saccharolyticum* (Section 1.4.3). When grown on mixed hardwood hydrolysate supplemented with defined medium, no ethanol was produced on non-detoxified hydrolysate; however treatment with 2.5% (w/w) activated charcoal allowed nearly full sugar consumption and ethanol production at near theoretical yields. The trade-off for this increased performance is the removal of just under 10% of the fermentable sugar along with the inhibitors (Lee et al., 2011). This loss of sugars along with the associated cost of the activated charcoal may well render this process uneconomical for commercial use, but it could be a relatively simple and cost effective approach for R&D purposes. When the hydrolysate was supplemented instead with complex medium (containing yeast extract), fermentative performance was at least as good in the controls as it was when the hydrolysate was detoxified. This would suggest that the inhibitory effect of hydrolysates can be modified to some extent either through enhancement of core metabolic activities or the enabling of secondary detoxifying metabolisms. A similar effect had earlier been seen on work with rice straw hydrolysate looking at the production of xylitol, which has commercial value in the food and pharmaceutical industries (Mussatto and Roberto, 2005).

Another approach is to use reducing chemicals which, in comparison to alkaline detoxification, would have the advantage of not altering the pH of the slurry and not leading to the formation of a precipitate. Sodium dithionite and sodium sulphite were compared with alkaline detoxification with ammonium hydroxide (similar to overliming) for the detoxification of spruce and sugar cane bagasse hydrolysates. 10mM sodium dithionite was found to give similar performance to alkaline detoxification in both of the hydrolysates. Ethanol production yields were around 0.4g/g with both detoxification methods in the spruce hydrolysate and 0.35g/g in the bagasse hydrolysate. When no detoxification was carried out prior to fermentation the ethanol production yield was only 0.2g/g and sugar consumption was substantially reduced. Interestingly detoxified hydrolysate outperformed a reference media fermentation – something which is frequently observed in experiments with *S. cerevisiae* and has been attributed to the presence of small amounts of weak acids in the hydrolysates (Alriksson et al., 2011).

There has also been much interest in biological detoxification, whereby various species of fungi with the ability to utilise common biomass inhibitors as carbon sources for growth, are used to detoxifying the hydrolysate stream. An example is the use of the fungus *Amorphotheca resinae* following dilute acid pretreatment of corn stover at high solids (Zhang et al., 2010). Corn stover was inoculated with the fungus prior to enzyme hydrolysis and left as a solid state fermentation (the fungus was used at this stage as it is also capable of rapidly using free sugars, so performing the detoxification at this point minimises sugar loss). Over a period of several days inhibitors were gradually removed and following this enzyme hydrolysis and fermentation was carried out in a normal manner. Fermentation of the non-detoxified material produced less than 10g L^{-1} ethanol, whilst detoxified material produced over 40g L^{-1}. With steam explosion pretreatment instead of dilute acid there was no benefit to the overall titre from detoxification, however it did improve the rate of fermentation. Another example is the use of *Aspergillus nidulans*, again with corn stover as the feedstock. This fungus was found to have a much lower glucose consumption rate than *S. cerevisiae* and so was tested for both detoxification after pretreatment as above, but also for detoxification during SSF of the corn stover. In a simple batch fermentation of hydrolysed corn stover, supplemented with addition glucose, the ethanol titre in detoxified medium was improved by 80% relative to a control, whilst in SSF, following 96hours of cultivation, the co-culture of *S. cerevisiae* and *A. nidulans* produced over 200% more ethanol than SSF using *S. cerevisiae* alone (Yu et al., 2011). Therefore there appears to be significant potential for biological detoxification of hydrolysates, although the slow rate of growth of these fungi may limit their utility in industrial processes.

1.5 Recovery and purification of ethanol

Once the ethanol has been produced during fermentation, it needs to be recovered and purified. This operation is outside the scope of this project, so only a brief review of the most salient points will be provided.

In the existing 1st generation ethanol industry the use of distillation and molecular sieves in order to recover and purify the ethanol produced in the fermentation beer is well established (Kwiatkowski et al., 2006). In principle there is no reason that this should not be applied to lignocellulosic ethanol processes. The issue that arises is that as the concentration of ethanol in the fermentation beer decreases, the energy requirements for distillation increases markedly, hence the generally stated requirement for lignocellulosic ethanol processes to produce at least 4%w/w ethanol in the fermentation step (Zacchi and Axelsson, 1989). This has led to much interest potential alternative technologies that may be more cost effective for both 1st and 2nd generation processes, and these technologies, of which continuous ethanol stripping may be of most interest to this project, have been reviewed in detail elsewhere (Vane, 2008).

However, compared with the other steps in the lignocellulosic ethanol process, the cost of distillation is low and as such this area is not regarded as being a priority for research that will help in the commercialisation of lignocellulosic ethanol (McAloon et al., 2000, Wyman, 2007).

1.6 Scale transfer in lignocellulosic ethanol processes

1.6.1 Scale transfer methodologies

Scale-up and scale-down techniques for the transfer of processes between different scales of stirred tank rector are well known and have been the subject of several excellent reviews (Junker, 2004, Schmidt, 2005, Hewitt and Nienow, 2007, Marques et al., 2010). Traditionally the scale-up of biological processes involved optimising the process at gradually larger scales until the desired production capacity had been reached. Such methods are however time consuming and both labour and capital intensive with large numbers of experiments to be run and multiple pieces of equipment to purchase. Because of this, there is now a far greater emphasis on transferring processes from R&D scales to production scales with the minimum of intermediate steps. The development of models and systems that can be used to accurately predict performance at multiple scales is vitally important to this. Small scale model systems can either be geometrically similar to the larger systems they are designed to mimic (and are then known as scale-down models) or they can be geometrically dissimilar in which case they are known as ultra scale-down (USD) models. Traditionally geometrically similar models were preferred as it was felt that systems possessing similar engineering characteristics would be more likely to lead to successful process scale-up. Examples of miniature STRs can be found can be found with volumes of around 10mL, although such systems tend to have limitations in terms of operating ranges and motor torque that would render them more suited to pharmaceutical applications (Gill et al., 2008a). In general as scale-down systems become increasing small it becomes difficult to continue to maintain geometric similarity to process scale equipment and therefore the use of USD models have become more common.

Researchers have for a long time tried to characterise the best approach to scaling up processes involving biocatalysts (Oldshue, 1966). In their review of the literature, Ju and Chase found that there were six commonly used scale-up factors (Ju and Chase, 1992):

- Vessel geometry
- Volumetric oxygen mass transfer coefficient, k_La
- Maximum shear
- Volumetric power input, $P_g V^{-1}$
- Volumetric gas flow rate, Q/V or VVM
- Superficial gas velocity, U_s

Of these, vessel geometry is clearly only of utility for scale-down systems and has no relevance to the increasingly common USD systems. Some of the other methodologies, for example scaling the aeration rate by matching superficial gas velocity and the stirrer speed by matching volumetric power input or the shear rate in the vessel, are also unlikely to be well suited to USD models. The utility of these techniques will mainly be found in scale-down systems with geometric and operational similarity where such factors may be expected to behave in similar ways. The utility of such metrics is of far less importance when USD models are involved as the same result will often be achieved by different means in the different systems, for example aeration by sparging in an STR compared to the use of surface aeration in an orbitally shaken system. They are also useful in specific instances where a physical parameter is of particular importance to the process, for instance when scaling up mammalian cell cultures it is important not to exceed the tolerance of the cells to shear stress (Kretzmer and Schügerl, 1991). This situation is however not believed to be the case in the process under investigation and so these parameters are likely to be of limited utility in the current project. More recently, for scale translation between orbitally shaken and small scale stirred bioreactors, mixing time has also been demonstrated to be a suitable basis for predictive scale translation (Tissot et al., 2010). Nevertheless it is felt that maintaining mixing time as a scale-up parameter leads to an overestimation of the power requirement of large stirred tanks and that this factor is not widely used industrially (Ju and Chase, 1992).

The remaining scale transfer methodology, the volumetric mass transfer coefficient (k_La), is the only parameter identified by Ju and Chase (1992) which is readily applicable to both geometrically similar and USD models (Ju and Chase, 1992). k_La is made up of two components, K_L, the volumetric mass transfer coefficient for oxygen and a, the gas transfer area available per unit volume. In essence the mass transfer coefficient provides the resisting force for diffusion of oxygen from the gas phase into the liquid phase whilst the volumetric area is the surface area available for oxygen diffusion to take place. It should be noted that the actual oxygen transfer rate into a vessel is given by the product of k_La and the difference in oxygen concentration between the bulk liquid and the gas-liquid interface. In most operations it is impractical at larger scales to use anything other than atmospheric air for vessel aeration and so the concentration gradient will remain unchanged at different operating conditions.

K_L can be influenced by reducing the thickness of the boundary layer or though improving the diffusion coefficient in the film whilst a can be increased by having more numerous, smaller bubbles in an STR or a larger free liquid surface area in orbitally shaken systems. The higher the oxygen demand of a culture, the higher the k_La of a system will need to be in order to maintain a given dissolved oxygen tension

Although there are two separate components to the volumetric oxygen mass transfer coefficient they are usually considered as a combined term as it is difficult to separate them experimentally without specialised equipment. The use of k_La as a scale-up factor does not require it to be separated out, rather the combined term is used. There are several published correlations which aim to relate k_La to the agitation and aeration inputs to an STR for use when experimentally derived values are not available and to predict k_La at untested conditions. This will be discussed further in Section 3.3.

There are many examples in the literature where k_La has successfully been used to transfer processes between conventional STRs of different scales (Flores et al., 1997, Herbst et al., 1992, Bandaiphet and Prasertsan, 2006). The examples cited cover processes producing a range of different products using different organisms and as such it can be regarded as a robust method for the transfer of processes between STRs of different scales. There are also reports of the use of k_La as a metric for scaling processes between conventional laboratory scale STRs and miniature STR bioreactors (Gill et al., 2008b). More recently the volumetric oxygen mass transfer coefficient has also been used to transfer processes between conventional STRs and geometrically dissimilar USD bioreactors. Islam et al. reported the scale-up of a process using a recombinant strain of *E. coli* from microwell plates to 7.5L and 75L STRs using k_La as a basis for scale-up (Islam et al., 2008). Conditions for culture growth and protein expression were optimised in the microwell format before transfer to the larger scale where the process kinetics were well matched to those in the microwell format. In another example of the concept the production of docosahexaenoic acid was successfully scaled up from shake flasks to STRs with volumes of up to 1.5m^3 (Qu et al., 2013). An attempt to transfer the process to a 7m^3 STR was also made however the product titre in the larger tank was around 40% higher than was achieved in shake flasks or several smaller STRs. Thus k_La has been shown to be useful not only in traditional applications where processes are scaled up between geometrically similar STRs but also in for use transferring processes between USD systems and STRs.

1.6.2 Scale-down of lignocellulosic ethanol processes

There is no reason why the techniques described in Section 1.6.1 would not be applicable to the scale-up/down of lignocellulosic ethanol fermentations provided the effects of the solids in the hydrolysate on issues such as fluid flow, mixing and gas-liquid mass transfer can be addressed. Numerous studies have been published examining the effects of solids loading on fluid flow and gas hold-up in stirred tanks. However, problems with non-uniform mixing and nutrient dispersion that have reportedly been problematic in process scale-up could well be significant issues with complex solids containing substrates (Dohi et al., 1999, Humphrey, 1998).

Whilst scale-up techniques for processes optimised in stirred tanks are undoubtedly useful, even the smallest stirred tanks are relatively large and labour intensive to set up and operate, limiting throughput and therefore the number of experimental conditions that can be tested. There has therefore been a trend in recent years to try and match the performance of microtiter plates, which have the additional advantage of being highly amenable to automation, with large scale stirred tank reactors. There have been several reports of the successful scale-up of culture profiles over several orders of magnitude to STRs of up to 10m^3 (Kensy et al., 2009, Islam et al., 2008). This work has been greatly aided by the successful characterisation of gas mass transfer coefficients in individual microwells (Zhang et al., 2008). Further information on the background and implementation of such techniques is found in several detailed reviews (Micheletti and Lye, 2006, Duetz, 2007).

It is of note that in terms of bioethanol production, whilst most of the processes scaled up from microtiter plates to large vessels have been aerobic with an emphasis on high oxygen transfer, to which microtiter plates are well suited thanks to the large surface area relative to the volume of liquids in them, work has recently been published examining their suitability for scale-down of the fermentation of grape juice. Growth of brewer’s yeast in microtiter plates was found to be very similar to that in oxygen limiting shake flasks, which have traditionally been used in vinification research, as was glucose consumption. Ethanol production was however significantly lower, although the authors attributed this to evaporative losses (Liccioli et al., 2010). Despite the mismatch in ethanol production the results could generally be considered promising for what was an un-optimised process, suggesting that microtiter plate fermentation may be of interest for low oxygen as well as aerobic processes.

Currently most initial development work for lignocellulosic ethanol processes is carried out in shake flasks before transfer to laboratory scale STRs for further development and optimisation. These methods, although effective, lack the published characterisations that are available for other sectors of the biotechnology industry. Most of the recent advances in scale-down technology, particularly in terms of USD methods, have been developed with the pharmaceutical industry in mind and have limitation with regards to their use in lignocellulosic ethanol processes.

There have been several instances where microplates have been used for both enzymatic hydrolysis and fermentation stages of a lignocellulosic bioethanol process. One approach involved forming pretreated corn stover into solid sheets that were cut up into very small disks and then hydrolysed in 96 well microplates with different enzyme mixtures; the equivalent solids loading was approximately 0.6% (w/w) (Berlin et al., 2007). Chundawat et al. used very finely milled (< 100µm) AFEX pretreated corn stover as a substrate for the development of an automated hydrolysis screening assay using an Eppendorf epMotion Liquid Handling Workstation (Chundawat et al., 2008). Solids loadings up to approximately 3% (w/w) were hydrolysed in 96 deep well microtiter plates with integrated HPLC analysis of the sugars released (although the authors found that slurries of up to 10% solids in the feed reservoir could be successfully pipetted). Glucan conversion yields that took 96 hours to achieve in shake flasks were achieved in around 6 hours in the microwell plates, opening up the possibility for much more rapid screening of new enzyme cocktails (Chundawat et al., 2008). The methodology has been further developed and used for the optimisation of loadings for core enzyme activities (Gao et al., 2010). Such a difference between microwell plates and shake flask models emphasises the importance of not modifying the biomass solids to be hydrolysed to make work at smaller scales more manageable

In terms of fermentation in microwell plates, the comparison three recombinant mesophiles discussed in Section 1.4.2 also included a microwell scale fermentation of a hydrolysate wash stream with mixing aided by the addition of a glass bead. The results from these trials played a significant role in forming the conclusions of the experiment; however there was no attempt to quantify how scalable the microwell fermentations were (Lau et al., 2010).

In all of the above, the lignocellulosic materials were either devoid of solids or contained very low levels of solids that had been substantially processed. This is long way from the 'real world' situation which will involve the processing of slurries at high solids loadings with a minimal number of steps. Given the issues associated with un-hydrolysed slurries of pretreated biomass (high viscosities, very difficult to pipette accurately due to particulates), it seems that it is unlikely that the microwell format can be used to operate trials at levels of solids close to those that will be found in commercial processes and, as was discussed earlier, the solids loading at which hydrolysis is carried out has a substantial impact on performance. It seems therefore that the role for the microwell plate format in research on lignocellulosic materials will likely to be restricted to initial screening studies, as was the case in all of the examples above. It may be expected for example that an enzyme which substantially outperforms a control in one of the above formats will also boost performance at the large scale, but it will not be possible to accurately predict the overall process performance and kinetics accurately.

1.7 Aims and objectives

As described in Section 1.6.2, shake flasks of various sizes have traditionally been used as small scale models for early stage bioprocess development. Their advantage when applied to hydrolysis and fermentation studies with lignocellulosic feedstocks is that they can mix relatively high solids loadings effectively. The disadvantage of shake flasks, and one of the prime drivers for the move to microwell plates in the pharmaceutical industry, is their relatively large footprint and material requirements. This limits the number of conditions and replicates that could potentially be tested. Microwells, while smaller and offering highly parallel experimentation, have the additional advantage of easy automation (Section 1.6.2). The challenge, however, is to be able to suspend solids at industrially relevant loadings without extensive modification. It appears therefore, that there is a need for an intermediate scale experimental system that would allow for increased throughput and decreased use of reagents whilst maintaining the ability to work with higher loadings of unmodified solids.

One option is the use of 50mL conical bottom polystyrene tubes as ultra scale-down mimics (Section 1.6.1) of conventional STRs. These are inexpensive, disposable and widely available in laboratories and have a much smaller footprint than shake flasks. For instance, sixteen 50mL tubes (with holders) occupy the same shaker platform surface area as four or five small (250 mL) flasks. Only a limited amount of work has been done on such systems to date. One group has developed 'TubeSpin' technology for early stage mammalian cell culture, however this is a process which occurs in dilute media with low viscosity and where the only suspended solids are the cells themselves. It is thus significantly different to a lignocellulosic ethanol process which presents a far more challenging environment from a scale-up point of view (De Jesus et al., 2004, Xie et al., 2011b).

ReBio Technologies, the industrial sponsor for this project, has an interest in developing a full scale lignocellulosic ethanol process (Section 1.2) that can utilise a wide variety of biomass feedstocks (Section 1.2.1) and so be used in a variety of situations globally. Development studies being undertaken by the company are focused around a three stage process with pretreatment, enzyme hydrolysis and fermentation stages. The pretreatment technology chosen for this work is steam explosion (Section 1.2.2.3). ReBio has steam explosion pretreatment reactors at both a $1m^3$ scale and at a pretreatment volume of 100L. As described in Section 1.2.2.3, studies of the scale-down of steam explosion reactors have proven difficult and hence the material produced inconsistent and not representative of large scale performance. Consequently in this work the pre-treatment stage will be fixed in order to provide reproducible batches of material for subsequent stages.

Enzyme hydrolysis is carried out at ReBio at scales from 50mL to $12m^3$ in both shaken vessels and stirred tanks; the company utilises commercially available enzymes from a variety of manufacturers for this process step. Fermentation is carried out at scales of up to $6m^3$ using a strain of *G. thermoglucosidasius* that is capable of utilising the major sugars found in biomass to produce ethanol at a high yield (Cripps et al., 2009). Substantial preparatory work on each of these stages has been undertaken prior to the start of this project, and such work will be referred to where relevant later in this thesis. Due to the limited number of STRs available, most initial development work is carried out in orbital shakers using shake flasks. Large numbers of trials can be run using these methods, and such work is used to define conditions that will be scaled up to progressively larger stirred tanks. There is clear value in creating a USD model of the STRs to drive process development.

As outlined in Section 1.3, there are several important performance limitations to enzyme hydrolysis, centred around kinetics and the effect of solids loading on hydrolysis performance. These are highly relevant to ReBio since the company is seeking to optimise and commercialise a lignocellulosic ethanol process. It would be of significant commercial utility for higher throughput USD systems to be available for screening and optimisation work. For the reasons outlined in Section 1.6.2 the pretreated biomass used must not be subject to any modifications that would not be used at commercial scales

There is also significant interest in further scaling down the fermentation stage of the process. Due to the place of fermentation as the last stage of the process there will be an even larger number of trials that will need to be performed. Each combination of pretreatment and enzyme hydrolysis conditions would ideally be checked for fermentability in order to try and find the optimum overall process with regard to enzyme hydrolysis yield and inhibitor levels (Section 1.4.5). In addition, there are various inherent properties of the fermentation that will need to be optimised to maximise process performance once a suitable pretreatment and enzyme hydrolysis combination is selected. Here factors such as oxygen transfer and pH control are likely to have a significant effect (Section 1.4.3), and these will require investigation if direct transfer of processes from USD systems to currently used stirred tanks is to be possible.

The **aim** of this thesis is thus to develop an experimental ultra scale-down model of the enzymatic hydrolysis and fermentation stages involved in the conversion of lignocellulosic feedstocks to bioethanol. This fills a gap identified in the published literature for small scale systems capable of dealing with high solids loadings without modification (Section 1.6.2) and will also deliver a low-cost, high throughput technology platform of immediate industrial relevance. Fundamental to this approach will be the need to understand and demonstrate how results obtained in the USD model are predictive of processes performed in currently used stirred tank scale-down models (4 L scale). The project will focus on DDGS (Section 1.2.1) as a commercially relevant lignocellulosic feedstock of particular significance to the UK. Commercially available enzymes will be used for the hydrolysis stage while *G. thermoglucosidasius* will be used for bioethanol production because of its ability to utilise a wide substrate range and the inherent advantages that a thermophilic ethanologen would bring to a process (Section 1.4.3). The specific **objectives** necessary to achieve the aim of this thesis are outlined below.

- Engineering characterisation of the current stirred tank scale-down reactor used at ReBio for both enzyme hydrolysis and fermentation and identification and engineering characterisation of the chosen USD experimental model. This will be described in Chapter 3.

- Establishment of the selected USD model for enzymatic hydrolysis of pre-treated DDGS with regard to the rate and extent of sugar release relative to the established STR process. This work will be presented in Chapter 4.

- Establishment of an optimised fermentation protocol for cultivation of *G. thermoglucosidasiusG. thermoglucosidasius* on hydrolysed DDGS (from Chapter 3) in the USD tubes. Studies will again focus on characterisation of fermentation performance at USD and standard STR scales in order to identify suitable conditions for scale translation. This work will be described in Chapter 5.

In addition to the above, Chapter 2 provides a detailed description of the Materials and Methods used, Chapter 6 a consideration of the industrial implementation of the USD approach (as required in an EngD thesis) while Chapter 7 provides overall conclusions and suggestions for future work.

2. Materials and Methods

2.1 Materials

DDGS was purchased from Trident Feeds (Cambridgeshire, UK). The moisture content was analysed using standard National Renewable Energy Laboratory (NREL) techniques and was determined to be 10.2% (w/w). All chemicals used in this work were of analytical grade unless otherwise stated and were sourced from Sigma Aldrich (Poole, Dorset, UK) or Fisher Scientific (Loughborough, UK). The supply of the enzymes used is covered by non-disclosure agreements with commercial suppliers and so they will simply be referred to as A, B and C. Enzymes A and B are liquid mixtures containing mainly cellulases supplemented with hemicellulases. Enzyme C is a food processing aid that contains mainly hemicellulases and was supplied in the form of a dry powder and so was prepared as a 20% (w/w) stock solution prior to use. Stock solutions were stored for up to one week and then discarded. Enzymes A and B were used at a loading of 3.33 mL Kg^{-1} dry DDGS and enzyme C was added at 5 g Kg^{-1} dry DDGS. This loading was determined to be the optimal commercial loading based on confidential price information from suppliers and ReBio process-economic models and was consequently used for all experimental work. The 15 mL and 50 mL conical bottom polypropylene tubes used in this study were purchased from Starlab (Milton Keynes, UK).

Table 2.1. Enzymes used in this work and their standard loading

Enzyme Identifier	Description	Standard Loading '1x'
Enzyme A	Mixture of beta glucosidase, cellulase and hemicellulase activities	3.33 mL per Kg dry DDGS
Enzyme B	Mixed cellulase, hemicellulase and beta glucosidase activities	3.33 mL per Kg dry DDGS
Enzyme C	Mainly hemicellulase activities	5 g per Kg dry DDGS

2.2 DDGS pretreatment

Slurries of DDGS were pretreated in a custom built, pilot scale steam explosion reactor with a total volume of 68 L and multiple steam injection nozzles. Appropriate amounts of DDGS were weighed out (correcting for the moisture content of the DDGS) and then diluted with hot water until the desired level of dry solids, 30% (w/w), was reached. All dry solids loadings, abbreviated to %DS, in this work are given as the percentage by mass of bone dry solids in a slurry. The slurry was then mixed thoroughly before being transferred into the pretreatment reactor. Dry saturated steam was added to the reactor to reach a pressure of 6.5 bar and maintain the contents at this pressure for 5 minutes. After the hold time was completed the steam valve was opened again, and the pressure was increased to 20 bar. At this point, the steam valve was closed and immediately afterwards a discharge valve located at the bottom of the reactor was opened and the material was discharged into a second vessel at atmospheric pressure. This pretreated material was then well-mixed and discharged into suitable 5 L containers to be stored at -20 °C. Pretreated DDGS was stored for a maximum of one month, after which time fresh material was generated.

2.3 Enzymatic hydrolysis in STRs

Stirred tank hydrolyses were carried out in 4 L working volume (7.5 L total volume) sterilise in place stainless steel vessels (Biostat CT-DCU-5-2, Sartorius UK). The stirrer shaft was fitted with two Rushton turbine impellors (d_i = 65 mm) spaced equidistantly up to 100 mm below the fill level with a stirring speed of 200 rpm. The tanks were fitted with four equally spaced rectangular baffles and a sparger located centrally below the bottom impellor. Slurries were prepared from DDGS that had been pretreated as described in Section 2.2, diluted with an appropriate volume of tap water to reach the desired dry solids content. Slurries were then adjusted to a pH of 5.0 ± 0.1 with 32% (w/v) NaOH (Brentag, Leeds, UK). 0.04% (w/v) Sodium Azide was then added in order to prevent contamination by microorganisms. Enzyme hydrolysis was initiated by adding Enzymes A, B and C at the ‘1x’ loading set out in Table 2.1.

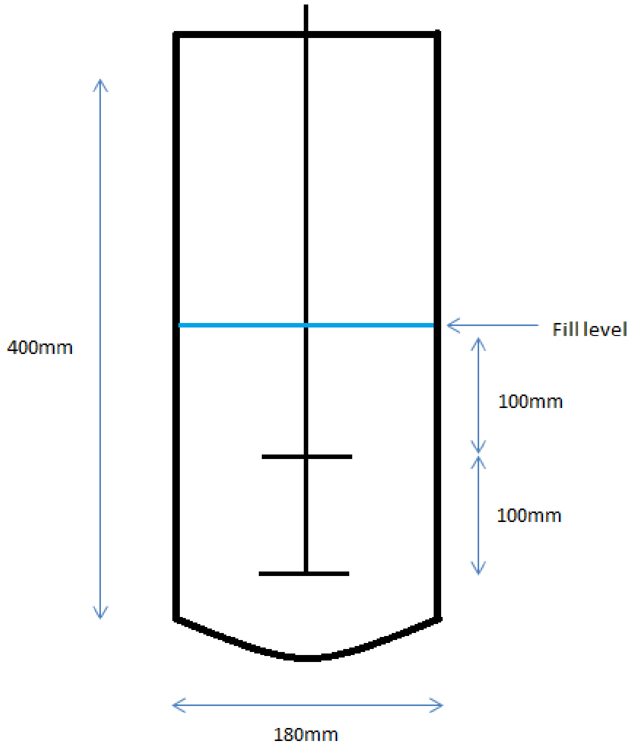


Figure 2.1. Schematic representation of the STR used for hydrolysis experiments.

2.4 Enzymatic hydrolysis in USD tubes

50 mL conical bottomed tubes used for scaled down enzyme hydrolysis reactions are referred to hereafter as USD tubes. Slurries of pretreated DDGS were prepared by diluting the pretreated material from Section 2.2 with an appropriate quantity of tap water. The pH of the slurry was adjusted to 5.0 ± 0.1 with 32% (w/v) NaOH (Brentag, Leeds, UK). 0.04% (w/v) Sodium Azide was then added in order to

prevent contamination by microorganisms. Hydrolysis was carried out in 50 mL conical bottom polypropylene tubes filled with 30 ± 0.1 g of slurry. These were warmed in an oven set to 50 °C for 1 hour prior to the addition of enzymes. Following addition of the enzymes, the tubes were mixed vigorously by hand and then placed in racks into an incubator shaker (Sartorius Certomat BS-1, Sartorius UK) with a 50 mm orbital diameter that was set to 50 °C and a shaking frequency of 250 rpm for the desired hydrolysis time. In order to generate time course data for tube hydrolysis, multiple tubes were started at the same time, with sacrificial tubes being removed at desired intervals. For all tube experiments, each condition was run either in duplicate or triplicate. Unless otherwise noted, all hydrolyses were run with the '1x' enzyme loading defined in Table 2.1 added in one dose at the beginning of the hydrolysis period.

2.4.1 Enzyme hydrolysis in USD tubes with additional inversion

USD tubes were set up as described in Section 2.4. Every four hours during the course of hydrolysis the tubes were briefly removed from the shaker and vigorously mixed by hand, for a period of approximately 30 seconds, before being returned to the shaking incubator.

2.4.2 Enzyme hydrolysis in USD tubes with multi-stage enzyme addition

For experiments with multi-stage enzyme addition, USD tubes were set up as described in Section 2.4 but a portion of the enzyme dose was held back and added at a later period in the hydrolysis as summarised Table 2.2. In all cases the total enzyme dose added was equivalent to the '1x' loading used for the control tubes. All conditions were run in duplicate with sacrificial USD tubes used to determine the progression of the hydrolysis.

Table 2.2. Enzyme dosing schedule for split enzyme addition experiment.

Tubes	0h	4h	8h	Enzyme
Control	20 uL 120 uL			A, B C
0h, 4h	10 uL 60 uL	10 uL 60 uL		A, B C
0h, 8h (50:50)	10 uL 60 uL		10 uL 60 uL	A, B C
0h, 8h (67:33)	13.5 uL 80.5 uL		6.5 uL 39.5 uL	A, B C

2.4.3 Enzyme hydrolysis in USD tubes with washed solids

For these experiments, hydrolysis was started in USD tubes as described in Section2.4 and at certain time points, USD tubes were removed from the shaker and centrifuged to separate the solids (3820 g, 5 minutes). The supernatant was poured off, weighed and then stored for further analysis. The solids were re-suspended in 30 mL of water, mixed well and then centrifuged; again the supernatant was weighed and stored. The pellet was re-suspended in 0.5 M citrate buffer (pH 5.0) to a total of 30 g and then fresh enzyme was added and the tubes returned to the shaker. The study is summarised in Table 2.3.

Table 2.3. Frequency and interval of washing for interrupted hydrolysis experiments

	Control	**1 Wash**	**2 Washes**	**3 Washes**
Initial Enzyme Loading	2x	1x	1x	1x
Wash 1 Time	---	8 hours	4 hours	2 hours
Restart 1 Enzyme Loading	---	1x	0.5x	0.25x
Wash 2 Time	---	---	8 hours	4 hours
Restart 2 Enzyme Loading	---	---	0.5x	0.25x
Wash 3 Time	---	---	---	8 hours
Restart 3 Enzyme Loading	---	---	---	0.5x
Total Hydrolysis Time	24 hours	24 hours	24 hours	24 hours

2.4.4 Enzyme hydrolysis in USD tubes held at various angles to the shaker platform

Hydrolysis was carried out as described in Section 2.4 using racks where the angle at which the tubes are held relative to the shaker platform could be varied. With the exception of the angle of the rack, all other details were unchanged.

2.4.5 Enzyme hydrolysis in USD tubes with addition of exogenous glucose

In order to investigate the effect of glucose as an inhibitor of the hydrolysis of DDGS investigations were carried out using washed pretreated DDGS as a substrate. In order to prepare the washed feedstock, DDGS was pretreated as set out in Section 2.2. The solids were separated by centrifuging at 4000 g for 60 minutes. The supernatant was poured off and the solids were made back up to the original volume with water and resuspended. This slurry was again centrifuged at 4000 g for 60 minutes and the supernatant poured off. The solids were collected and stored frozen for later use. This process removed all other inhibitors allowing the effect of glucose to be examined in isolation.

A sample of the washed DDGS solids was analysed (Section 2.6.1) and found to contain 64.8% (w/w) moisture. The solids were found to contain, on a dry matter basis, 18.9% (w/w) glucose, 11.8% (w/w) xylose, galactose mannose and fructose (X+G+M+F) and 3.4% (w/w) arabinose. The washed DDGS solids were added to the USD tubes and diluted with an appropriate quantity of water to produce a final dry solids content of 20% (w/w) at the standard 30 g fill. The contents were then adjusted to pH 5.0 using NaOH and Sodium Azide was added as in Section 2.4. In the tubes that were to have extra glucose added an appropriate quantity of water was replaced with a corresponding mass of a 530 g L^{-1} glucose stock solution. The concentrations of glucose added were 4 g L^{-1}, 8 g L^{-1}, 12 g L^{-1}, 16 g L^{-1}, and 22 g L^{-1}.

All hydrolyses were carried out with the 1x enzyme loading in Table 2.1 and run for a total of 24 hours. All other conditions were as set out in section 2.4.

2.5 Imaging of solid suspension and mixing in USD tubes

Images were recorded using a Fastcam DVR high speed video recorder (Photron Europe Limited, West Wycombe, UK). The USD tube was held on an orbital shaking platform using a combination of clamps that allowed the angle at which it was held to be varied without impeding the field of vision of the camera. Images were recorded at a rate of 500 frames per second using at various shaking frequencies. The USD tubes were filled with hydrolysed DDGS produced as described in Section 2.4. Hydrolysis was not carried out in real time due to the inability to control temperature using the camera set up available. The images produced from the recordings were stored for later use and analysis.

2.6 Determination of oxygen mass transfer coefficients (k_La)

2.6.1 Determination of k_La in STRs

Determination of k_La in the stirred tank reactors, Section 2.3, was carried out using the static gassing out method (Stanbury and Whitaker, 1984). The measurements were carried out in the same vessels and with the same impellor arrangement as described in Section 2.3 and filled to a volume of 4L. The dissolved oxygen concentration in the tanks was measured using a VisiFerm DO optical dissolved oxygen probe (Hamilton Bonaduz AG, Bonaduz, GR, Switzerland). The response time of the probe, defined as the time taken to record a stepwise increase in dissolved oxygen from 0% to 63%, was determined. In all cases this was found to be less than $1/K_La$ and so was determined to be unlikely to significantly impact on the results generated (Van't Riet, 1979, Tribe et al., 1995).

The contents of the reactor were degassed by sparging with Nitrogen following which the agitation and aeration set points were adjusted to the test conditions. The air supply was then immediately switched from N_2 to air and the change in dissolved oxygen recorded. The data was exported to Microsoft Excel in order to calculate the oxygen transfer rate according to the Equation 2.1

$$\ln\left(\frac{C^{*}-C_{L1}}{C^{*}-C_{L2}}\right) = k_L a \qquad (2.1)$$

where C* is the saturation oxygen concentration of the system, C_{L1} is the initial concentration of dissolved oxygen in the tank and C_{L2} is the concentration of dissolved oxygen in the tank at a given time, t, after oxygen was introduced to the tank. Plotting the logarithm of the change in oxygen concentration against time gives a straight line of slope K_La. Each measurement was carried out in triplicate and the mean slope calculated.

k_La determination was carried out using lab media, as described in Table 2.5, and the same media to which the residual solids from an enzymatic hydrolysis of DDGS at 25% DS (w/w) had been added. The solids were produced by carrying out enzymatic hydrolysis as described in Section 2.3 then recovering the residual solids by centrifuging at 3500 g for 15 minutes. The recovered solids were then washed with RO water and separated again by centrifugation. The solids were then autoclaved and stored at -20 °C until needed.

2.6.2 Determination of k_La in USD tubes

Measurement of K_La in USD tubes was carried out using the same principles as in Section 2.6.1. A USD tube was modified so that it contained an OX-Eddy micro dissolved oxygen probe (Unisense A/S, Aarhus, Denmark) and a needle through which nitrogen could be sparged. Prior to making k_La measurements the tube was vented to fill the headspace with air and replicate standard operating conditions. Dissolved oxygen data was exported to Microsoft Excel and processed as in Section 2.6.1. Each measurement was carried out in duplicate and the mean slope calculated.

2.7 Fermentation Media Formulation

All of the fermentation work was carried out using 50mM urea sulphate media (USM) with an additional 20 g L^{-1} yeast extract as a background. This formulation was developed and optimised through significant development work at ReBio. This background was supplemented with various carbon sources as outlined in Tables 2.4 to 2.6. Glycerol was used as a carbon source during seed culture growth to minimise pH drift caused by acidic metabolic products.

Table 2.4. Formulation of 50mM USM / 20 g L^{-1} yeast extract / 30 g L^{-1} glycerol

Component	**Concentration (g L^{-1})**
NaH_2PO_4	3
Urea	3
K_2SO_4	4.4
Citric Acid	0.96
$MgSO_4$	0.38
$CaCl_2$	0.006
Na_2MoO_4	0.0005
Biotin	7.5×10^{-2}
H_2SO_4	0.12
$ZnSO_4$	0.02
$FeSO_4$	0.07
$MnSO_4$	0.02
$CuSO_4$	0.003
$CoSO_4$	0.007
H_3BO_3	0.00075
$NiSO_4$	0.01
Yeast Extract	20
Glycerol	30

Table 2.5. Formulation of 50mM USM / 20g L^{-1} yeast extract / 40 g L^{-1} glucose

Component	**Concentration (g L-1)**
NaH_2PO_4	3
Urea	3
K_2SO_4	4.4
Citric Acid	0.96
$MgSO_4$	0.38
$CaCl_2$	0.006
Na_2MoO_4	0.0005
Biotin	7.5x10-2
H_2SO_4	0.12
$ZnSO_4$	0.02
$FeSO_4$	0.07
$MnSO_4$	0.02
$CuSO_4$	0.003
$CoSO_4$	0.007
H_3BO_3	0.00075
$NiSO_4$	0.01
Yeast Extract	20
Glucose	40

Table 2.6. Formulation of 50mM USM / 20 g L^{-1} yeast extract / 40 g L^{-1} mixed sugar

Component	**Concentration (g L^{-1})**
NaH_2PO_4	3
Urea	3
K_2SO_4	4.4
Citric Acid	0.96
$MgSO_4$	0.38
$CaCl_2$	0.006
Na_2MoO_4	0.0005
Biotin	$7.5x10^{-2}$
H_2SO_4	0.12
$ZnSO_4$	0.02
$FeSO_4$	0.07
$MnSO_4$	0.02
$CuSO_4$	0.003
$CoSO_4$	0.007
H_3BO_3	0.00075
$NiSO_4$	0.01
Yeast Extract	20
Glucose	30
Xylose	10

Table 2.7. Formulation of buffered 50mM USM / 20 g L^{-1} yeast extract / 40 g L^{-1} glucose

Component	**Concentration (g L-1)**
NaH_2PO_4	3
Urea	3
K_2SO_4	4.4
Citric Acid	0.96
$MgSO_4$	0.38
$CaCl_2$	0.006
Na_2MoO_4	0.0005
Biotin	7.5x10-2
H_2SO_4	0.12
$ZnSO_4$	0.02
$FeSO_4$	0.07
$MnSO_4$	0.02
$CuSO_4$	0.003
$CoSO_4$	0.007
H_3BO_3	0.00075
$NiSO_4$	0.01
Yeast Extract	20
Glucose	40
1M MOPES (pH 7)	0.4
1M HEPES (pH 7)	0.4
1M Bis-tris (pH 7)	0.4

Table 2.8. Formulation of bis-tris buffered 50mM USM / 20 g L^{-1} yeast extract / 40 g L^{-1} glucose

Component	**Concentration (g L-1)**
NaH_2PO_4	3
Urea	3
K_2SO_4	4.4
Citric Acid	0.96
$MgSO_4$	0.38
$CaCl_2$	0.006
Na_2MoO_4	0.0005
Biotin	7.5x10-2
H_2SO_4	0.12
$ZnSO_4$	0.02
$FeSO_4$	0.07
$MnSO_4$	0.02
$CuSO_4$	0.003
$CoSO_4$	0.007
H_3BO_3	0.00075
$NiSO_4$	0.01
Yeast Extract	20
Glucose	30
Xylose	10
1M Bis-tris (pH 7)	1.2

2.8 Fermentation in STRs

2.8.1 Stirred Tank Reactor Fermentation with soluble media

Fermentations were carried out in the same tanks and with the same impellor arrangement as for the hydrolysis experiments described in Section 2.3. The vessels were equipped with online pH and redox sensors (Broadley-James, Bedford, UK) with analysis of the fermentation off gas carried out using a Prima dB process mass spectrometer (Thermo Fisher, Winsford, Cheshire, UK). Measurements of biomass (where applicable) were made by taking an offline sample and recording the optical density at 600nm using a Jenway 6300 spectrophotometer (Bibby Scientific, Stone, Staffs. UK).

Inoculum for the fermentations was developed as follows. A vial of *G. thermoglucosidasius* strain TM242 (Cripps et al., 2009) from a working cell bank stored at -80°C was defrosted at room temperature for 30 minutes. A 1:1000 dilution was performed by aliquoting 50 µL into 50 mL of USM / 20 g L^{-1} yeast extract / 30 g L^{-1} glycerol at pH 7.0 (Table 2.4) that had been sterilised at 121 °C for 15 minutes and then pre-warmed at 60 °C for 3 hours prior to use. 5 mL of this dilution was then used to inoculate 1 L baffled shake flasks containing 200 mL of the same media, again pre-warmed for 3 hours prior to use. The culture was then placed in a shaking incubator set at 60 °C and 200rpm shaking frequency for 14 to 16 hours overnight.

For fermentation, the vessels were filled with 4L of the media in Table 2.6 which was then sterilised by heating in situ to 121°C for 15 minutes. The tanks were then cooled to 8°C and left overnight prior to the fermentation. Prior to inoculation the media in the vessel was heated to 60°C and the probes in the vessel were checked and re-calibrated where necessary. The optical density of the inoculum flasks which had been growing overnight was checked to ensure that the culture was still growing (increasing biomass) and that an OD_{600} of at least 15 au had been reached. At this point 400 mL of inoculum, 9% (v/v) of the final volume, was aseptically transferred to each vessel and data logging of the fermentation parameters begun. Samples were removed at regular intervals to check the optical density and for analysis of sugars and metabolites in the fermentation broth as described in Section 2.10.2.

Agitation and aeration conditions were determined using the correlations developed in Section 3.3.1.

2.8.2 Stirred Tank Rector Fermentation with DDGS solids in soluble media

DDGS was hydrolysed at 20% (w/w) solids loading and 4 L working volume as set out in Section 2.3. Following 48 hours of hydrolysis the hydrolysate was removed from the tank and centrifuged at 4000 g for 30 minutes to separate the solids. The solids were collected and then re-suspended in clean water to a final volume 4 L in order to wash them. The solids were again then separated by centrifugation and stored at -20°C prior to use. Typically around 8% (w/w) insoluble solids was recovered following enzyme hydrolysis.

Prior to use in a fermentation, the solids were defrosted and autoclaved at 121 °C for 15 minutes. They were then aseptically transferred to the vessel prior to inoculation. The effect of adding the solids to the vessel increased the volume in the tank by approximately 1 litre to 5 litres. However, the concentration of sugars etc. was unaffected since the volume of water present in the system was unchanged, and thus the volume of inoculum was not varied for these experiments. It was determined that this had a minimal impact on the measured K_La of the system.

Fermentations were otherwise carried out in the same way as described in Section 2.8.1 with the exception that it was not possible to measure the OD in the vessel due to the presence of the solids.

2.9 USD tube fermentations

2.9.1 Comparison of 15mL and 50mL conical bottom tubes

15mL and 50mL conical bottom polypropylene tubes were filled with various volumes of the media in Table 2.7. The tubes additionally contained a mix of buffers designed to limit pH drift during the experiment. This comprised 11% of the final volume of the tube, and was made up of equal parts of 1M MOPS, 1M HEPES and 1M Bis-Tris, all adjusted to pH 7.0.

Inoculum for the tubes was prepared in the same was as for the STRs in Section 2.8. The inoculum comprised 9% of the final volume in each tube. Following inoculation the tubes were placed in a shaking incubator set at 250 rpm and 60 °C for 24 hours. The tubes were pierced twice with a 12 gauge needle in order to prevent build-up of carbon dioxide during fermentation. Following fermentation, the tubes were removed from the incubator, their pH and OD_{600} recorded, and the concentration of sugars and metabolites determined as in Section 2.10.2. All conditions were tested in triplicate.

2.9.2 USD fermentations at various shaking frequencies, fill volumes and initial glucose concentrations

For these experiments the USD tubes were set up in the same way as described in Section 2.9.1, with the exception that the shaking frequency and glucose concentration in the media was adjusted to 50 rpm, 150 rpm or 250 rpm and 20 g L^{-1}, 40 g L^{-1} or 60 g L^{-1} according to the condition being tested. Again the fermentations lasted for 24 hours and the post-fermentation analysis remained unchanged.

2.9.3 Investigating alternative buffers in USD tube fermentation

USD tubes were filled with the USM media formulation in Table 2.7 and run as described in Section 2.9.1, but containing 60g L^{-1} glucose rather than 40g L^{-1} where required and with the pH buffers used altered according to Table 2.9. All tubes in this experiment were filled to a final volume of 20mL. The total proportion of buffers contained in the tubes was unchanged at 11% of the final volume, but four different compositions for the buffer mix were investigated as set out in Table 2.9. Again the experiment was run for 24 hours, and analysed as described in section 2.10.2.

Table 2.9. pH buffer mixtures tested in SDD fermentations. All volumes given are based on a 20 mL final volume

Buffer formulation	1M MOPES (mL)	1M Bis-Tris (mL)	1M HEPES (mL)
A	1.1	1.1	0
B	0.7	1.5	0
C	0	2.2	0
D	0.7	0.7	0.7

2.9.4 USD tube fermentations at specific K_La values

USD tubes were filled with 20mL of the medium in set out in Table 2.8 using buffer formulation C from Table 2.9. All experiments were carried out at 60 °C whilst the shaking frequency was determined according the correlations described in Sections 3.6.1 and 3.6.2. All other details were as described in 2.9.1.

2.9.5 USD tube fermentation with DDGS solids in soluble media

DDGS was hydrolysed at 20% (w/w) solids loading and 20 mL working volume as set out in Section 2.3. Following 48 hours of hydrolysis the hydrolysate was removed from the tank and centrifuged at 4000 g for 30 minutes to separate the solids. The solids were collected and then re-suspended in clean water to a final volume 20 mL in order to wash them. The solids were again then separated by centrifugation and stored at -20°C prior to use. Typically around 8% (w/w) insoluble solids was recovered following enzyme hydrolysis.

Prior to use in a fermentation, the solids were defrosted and autoclaved at 121°C for 15 minutes. They were then aseptically transferred to the vessel prior to inoculation. The effect of adding the solids to the vessel increased the volume in the tank by approximately 20%. However, the concentration of sugars etc. was unaffected since the volume of water present in the system was unchanged, and thus the volume of inoculum was not varied for these experiments. It was determined that this had a minimal impact on the measured K_La of the system.

Fermentations were otherwise carried out in the same way as described in Section 2.9.4 with the exception that it was not possible to measure the OD in the vessel due to the presence of the solids.

2.10 Analytical techniques

2.10.1 Dry solids analysis

The moisture content of feedstocks and slurries was determined using a MA150 moisture balance (Sartorius, Epsom, UK) in accordance with the standard NREL procedure for moisture determination (Sluiter et al., 2008). All measurements were carried out in triplicate.

2.10.2 Monosaccharide, ethanol and organic acid analysis

All samples were centrifuged in order to remove solids, and the supernatant passed through a 0.2 µm nylon membrane filter (PEHNEX NY, Phenomenex, Macclesfield, UK) to remove any particulates. Sugars were determined by HPLC (Alliance 2695, Waters, Elstree, UK) using a Rezex RHM column (7.8 x 300 mm, 8 µm packing) (Phenomenex, UK) with a 4mM H2SO4 mobile phase at a flow rate of 0.6 mL min^{-1}. Detection of compounds was by UV adsorption (Waters 2996, Waters, UK) and differential Refractive Index (RI) (Waters 2414, Waters, UK) detectors operated in series. Concentrations of glucose, xylose, galactose, mannose, fructose, cellobiose, arabinose, ethanol and acetic acid were determined using calibration curves prepared with standards of known concentration. The RI and UV traces for the standard curve are shown in Appendices 1 and 2 and for a typical fermentation sample in Appendices 3 and 4. Using this method, xylose, mannose, galactose and fructose co-elute and so cannot be separated.

The concentrations of these four sugars are therefore reported combined as X+G+M+F. In DDGS this peak will mainly represent xylose as only small amounts of the other sugars are present (Kim et al., 2008b).

2.10.3 Oligosaccharide analysis

Sample preparation is as described in Section 2.10.2 and after the sample is filtered 72% (w/w) H_2SO_4 is added to the sample to a final concentration of 4% (w/w) in a sealed glass bijou bottle and the sample autoclaved at 121 °C for 60 minutes. The sample is cooled on the bench and then neutralised to pH 5-7 using $CaCO_3$. The sample is then filtered through a 0.2 µm filter as before and analysed as in Section 2.10.2. Concentrations are determined using a calibration curve prepared with standards of oligomeric and monomeric sugars at known concentrations. The concentration of oligomers is determined by subtracting the concentration measured in a non-acid hydrolysed sample from that achieved following acid hydrolysis.

2.10.4 Determination of feedstock composition

The composition of sugars in the DDGS was determined by acid hydrolysis of the solids followed by determination according to the methods described in Section 2.10.2. For the feedstock used in all the experiments here it was determined to be, on a dry matter basis, 19.5% (w/w) glucose, 9.5% (w/w) X+G+M+F, 6.4% (w/w) arabinose. This is in line with the values reported by others (Kim et al., 2008b)

2.10.5 Calculation of hydrolysis yields

When calculating hydrolysis yields, there are a variety of factors that can complicate the interpretation of sugar concentrations following hydrolysis. These include the fact that sugars solubilised during hydrolysis have a greater density than the water present, the fact that some water is consumed to hydrolyse the polymeric sugars into monomers, and the fact that a proportion of the total hydrolysate volume is occupied by unhydrolysed solids. Methods have been proposed to mitigate these effects by diluting samples following hydrolysis (Hodge, 2008).

The work in this project instead uses a formula to calculate a corrected maximum possible observable sugar concentration. Sugar concentration values from hydrolysis samples are then compared to this corrected sugar concentration, taking the corrected value as the 100% value in order to calculate hydrolysis yields. This corrected sugar concentration, C_s, is calculated as follows:

$$C_S = \frac{M_p + M_H}{(M_W - M_H) + \left(\frac{M_p + M_H}{\rho_S}\right)} \tag{2.2}$$

where C_s = Concentration of sugars at 100% hydrolysis (g mL^{-1}), M_P = Mass of polymeric sugar on a dry matter basis (Kg), M_H = Mass of water of hydrolysis = 0.1 x M_P (Kg), M_W = Mass of water (Kg), ρ_s = Density of sugar in solution = 1.5 (g cm^{-3})

The above value, C_s, is used for the calculation of overall hydrolysis yields. The maximum possible concentration of any individual sugar is calculated according to the following:

$$C_{si} = C_s \times Y_i \qquad (2.3)$$

where C_{si} = Concentration of an individual sugar, i, at 100% hydrolysis, Y_i = The proportion of sugar in the feedstock on a dry matter basis.

2.10.6 Determination of cell dry weight

Cell dry weight was determined by centrifuging (13,000 g for 5 minutes) 1 mL of culture in a pre-weighed centrifuge tube. The cell pellet was re-suspended in RO and centrifuged again to wash off residual media. The pellet was then dried in the centrifuge tube overnight in an oven set to 105°C. The wry weight of biomass is determined by subtracting the mass of the tube from the mass of the tube and pellet. Multiplying this figure by 1000 gives the mass of biomass in one litre of culture.

2.11 Statistical treatment of data

Where possible all experiments were carried out with at least three replicates, with means and standard deviations then calculated. The statistical tests used to analyse the data were carried out using the functions built into Microsoft Excel unless otherwise noted.

Direct comparisons of two experimental conditions, such as comparing the overall yield achieved at two different solids loadings in the same system, were analysed using a two tailed unpaired t-test. Results were deemed to be significant if the p value of the test was <0.05. Examination of larger sets of data at multiple conditions used analysis of variance (ANOVA). This test is a measure of whether the entire data set can be said to be one population around a central mean. A p value of <0.05 indicates that the data set does not form one population. The degree of correlation between two variables was examined using the regression analysis tool in Excel. This gives both an r value that measures how good the fit between two variables is and a p value that measure the significance of the fit using ANOVA. A p value of <0.05 indicates that there is a significant fit to the correlation.

3. Engineering characterisation of laboratory and ultra scale-down bioreactors

3.1 Introduction

The first stage in the development of a validated, useable ultra scale-down model is to understand how the engineering environment within a particular unit operation affects the product and/or process under investigation (Li et al., 2006, Titchener-Hooker et al., 2008). This is necessary for both the ultra scale-down system and for the larger scale system to which it is being compared; in the case of this work ReBio's existing laboratory scale STRs.

The 4L STRs described in Section 2.3 represent an established scale-down model for both the enzymatic hydrolysis and fermentation stages of ReBio's lignocellulosic ethanol processes. As described in Section 1.7, the overall aim of this thesis is to establish a USD system as a small scale alternative to these STR reactors, particularly for application in the early stages of process development. In order to fully validate the USD system as a small scale experimental model of the STRs it is necessary to better characterise the engineering environment at both scales. It is also necessary to consider the differing process requirements for the enzyme hydrolysis and fermentation stages. In the enzyme hydrolysis stage of the process, the primary requirement is for there to be sufficient mixing in the system to adequately mix the enzyme mixture and DDGS together and then prevent any suspended solids from being so poorly mixed that enzymatic hydrolysis rate is impaired. It is established from the literature that above a certain critical level, necessary to suspend the solids present, higher levels of mixing have no effect on enzyme hydrolysis performance (Mais et al., 2002). Thus of the methodologies discussed in Section 1.6 mixing time would appear to be most applicable to DDGS. Mixing time has been used to successfully, in a modified form accounting for geometry, to scale up a mammalian cell process in similar vessels to those identified as a USD system in this work (Tissot et al., 2010). This scale up used a colorimetric method to determine mixing time, and such methods are commonly used to determine mixing time. Regrettably however they are not suitable for the current application as the colour of the DDGS would mask any colour change. Similarly mixing time can be determined using CFD methods; however CFD simulations are not well suited to systems containing large quantities of suspended solids and are therefore again unsuitable for the problem presented by DDGS (Panneerselvam et al., 2008). It was therefore decided that within the timelines and scope of the project it was appropriate a more empirical route. In the first instance high speed imagining will be used to visualise the solid-liquid dispersion and the pattern of fluid dispersion in the USD tubes and qualitatively assess their suitability for use in this work. Secondly, following the visualisation of mixing, initial experimental trials will assess actual enzyme hydrolysis performance in the USD tubes. For the fermentation process stage, mixing is still important in order to maintain a homogenous environment within the culture. However it is also known from the literature that the organism being used in fermentation is affected by other factors and specifically the fact that the concentrations of dissolved oxygen during culture have a significant effect on: (i) the activity of gene promoters and (ii) on the metabolic flux to ethanol in *G. thermoglucosidasius* (Lin et al., 2014, Van Zyl et al., 2014). It was therefore considered that a scaling methodology based around k_La in the culture medium was most likely to lead to establishment of successful scale-up and scale-down approaches. k_La is a commonly used scaling parameter, with many examples in the literature of scaling different fermentations at matched k_La. Because of its widespread success with a range of fermentations it is also considered a low risk scale-up strategy (Marques et al., 2010). It should be noted however that it is not unusual for differences to be observed when processes are scaled up from laboratory to production scales, even when using constant k_La as a scale up metric (Garcia-Ochoa and Gomez, 2009). Factors such as local variation in mixing time can become important at very large scales but are difficult to predict from small scale models.

As set out in Section 1.7 the aim of this chapter is to characterise the current laboratory scale STR system in use at ReBio, consider potential USD systems that could be used as an alternative and then to characterise the chosen USD system to assess its suitability for further study. The specific objectives are outlined below:

- To characterise the STR system currently in use, quantifying k_La as a function of stirrer speed and aeration conditions.
- To consider and select an appropriate USD system for evaluation based on process characteristics.
- To visualise solids suspension and fluid flow in the chosen USD system to ensure it can handle the solids level and fluid viscosities found in industrial processes.
- To characterise the USD system in a way that allows comparison with the STR system, e.g. by the establishment of engineering correlations for prediction of kLa.

3.2 Measurement of oxygen mass transfer coefficient (k_La) in STRs

3.2.1 Measurement of k_La in STRs

The overall volumetric oxygen mass transfer coefficient, k_La, was determined experimentally using the static gassing out method as described in Section 2.6. Examples of the dissolved oxygen concentration versus time profiles, from which k_La values were calculated, are shown in Figure 3.1. The raw data shows the expected rapid initial increase over time followed by a slower increase with saturation being reached in around 150 seconds. The steeper rise in the system without suspended solids suggests that the presence of solids reduces the rate of oxygen transfer. This is reflected in the calculated k_La values of 167 h^{-1} and 197 h^{-1} respectively.

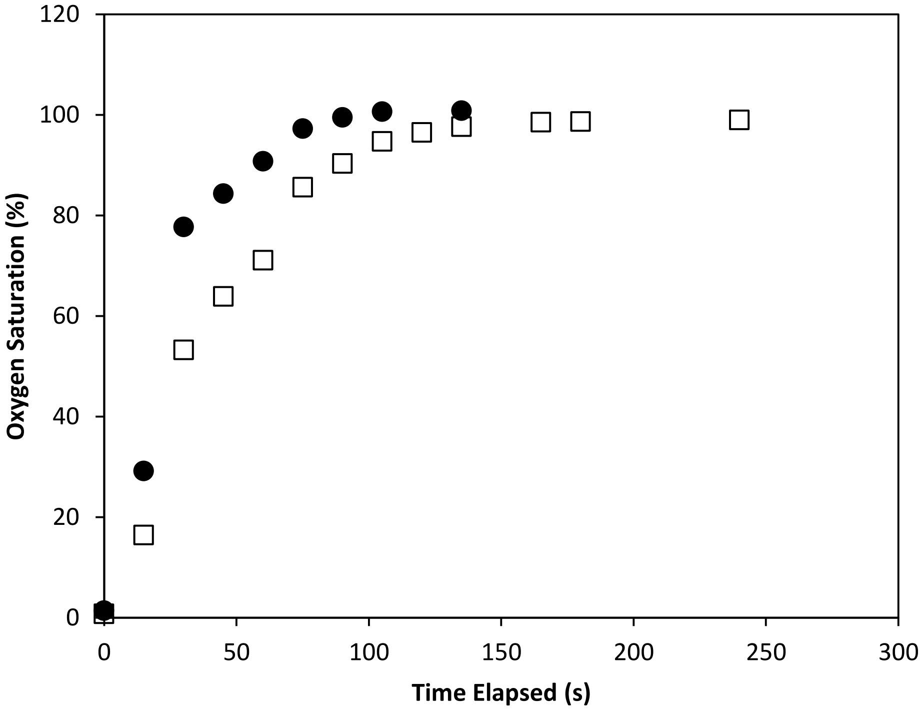


Figure 3.1. Typical examples of dissolved oxygen versus time profiles obtained using the static gassing out method. Examples shown were obtained at a stirrer speed of 800rpm and an aeration rate of 1.0 vvm at a temperature of 60°C.:● denotes data collected in solids free 50mM USM media (Table 2.6) ; ☐ denotes data collected with 8% (w/w) DDGS solids added to the same media. Experiments performed as described in Section 2.6.1.

Figure 3.2 shows the measured k_La values determined over a wider range of stirrer speeds and aeration conditions in the STRs again in either solids free media or media to which solids that had been separated from DDGS hydrolysate were added. The suspended solids used were those insoluble solids that were recovered following enzyme hydrolysis (Section 2.9.5) and were added at 8% (w/w), the concentration at which they were recovered from the hydrolysate. It is clear from this data that the presence or absence of solids in the hydrolysate has a substantial effect on the k_La of the system; the values calculated in the presence of solids being significantly lower at comparable conditions. The one exception to this observation is in the case of a low stirrer speed but relatively high aeration where the oxygen mass transfer coefficient was slightly higher in the media containing DDGS solids. The magnitude of the K_La measured under these conditions was however low and so a small error in measurement could make a significant difference to the calculated result. Additionally, visual observation suggested that dispersion of the gas bubbles by the impellors was sub-optimal under these conditions with ‘flooding’ occurring.

Regardless of the presence of solids in the STR the expected general trend of increasing K_La as the stirrer speed and aeration are raised is observed. There is, however, a significant difference in the degree to which they respond to changing stirrer speeds and aeration rates. Using a fixed aeration rate of 0.2vvm as a basis for comparison, Table 3.1 shows the measured K_La increase at different stirrer speeds in both simple media and media that contained suspended solids. At the lowest stirrer speed the

K_La values of the two systems are similar, albeit low, with a difference of just 8%. Identical increases in stirrer speed led to greater increases in K_La in the solids free system and at a stirrer speed of 800rpm, the highest investigated, there was a 30% difference in K_La values between the two systems. Although the K_La achieved with the highest settings for stirrer speed and aeration in the solids free media was higher than what was achievable in the media with suspended solids there was a significant window where K_La values could be replicated in both systems.

Table 3.1. Measured K_La values at different stirrer speeds with 0.2vvm airflow in 50mM USM media (Table 2.6) with and without 8% (w/w) DDGS solids at 60°C. k_La values determined as described in Section 2.6.1.

Stirrer Speed (rpm)	K_La values in media containing DDGS solids (h^{-1})	K_La values in in solids free media (h^{-1})
200	11.9	13.1
500	46.3	69.3
800	87.6	124.9

Table 3.2. Measured K_La values at different aeration rates with 800rpm stirrer speed in 50mM USM media (Table 2.6) with and without 8% (w/w) DDGS solids at 60°C. k_La values determined as described in Section 2.6.1.

Aeration Rate (vvm)	K_La values in media containing DDGS solids (h^{-1})	K_La values in in solids free media (h^{-1})
0.2	87.6	124.9
0.5	135.9	166.0
1.0	166.7	197.2

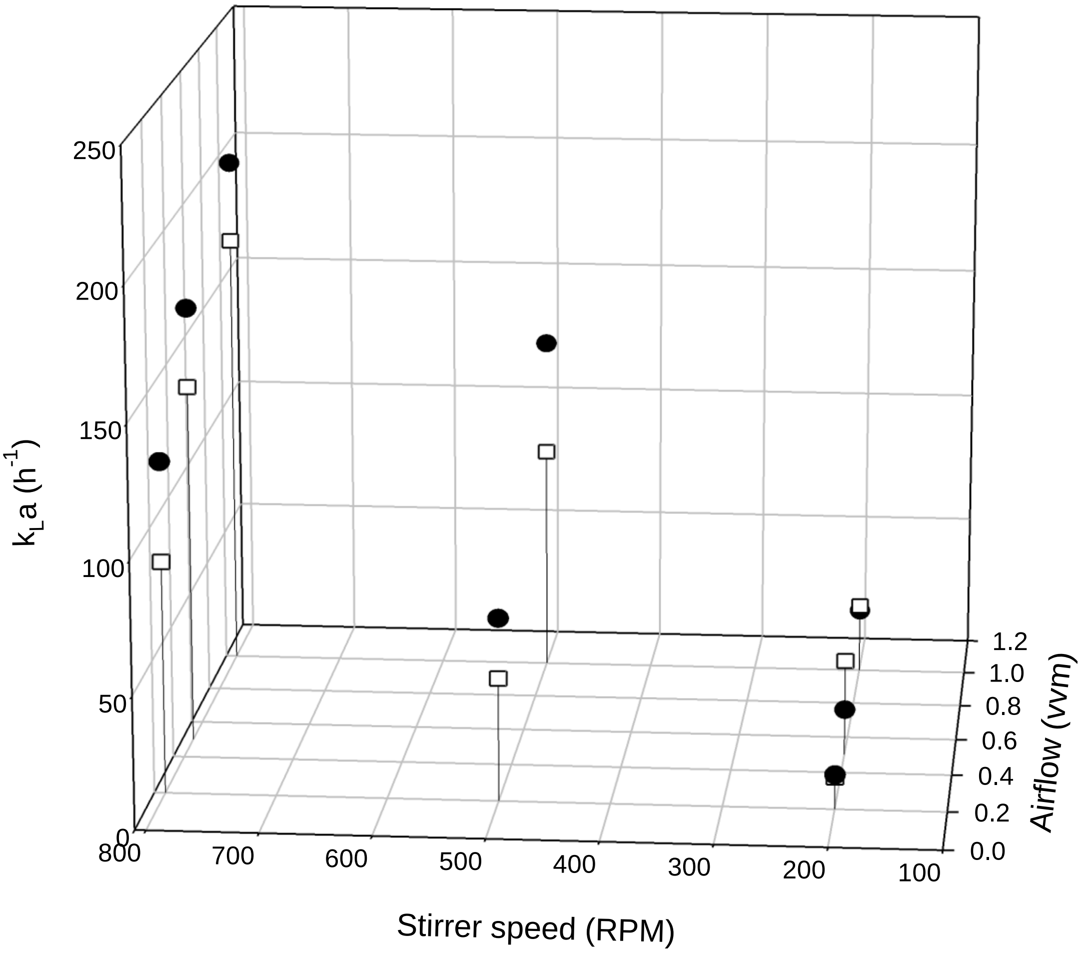


Figure 3.2. K_La measured in 50mM USM / 20 g L^{-1} yeast extract / 40 g L^{-1} glucose media with and without 8% (w/w) hydrolysed DDGS solids added in a 4L STR at 60°C. ●: denotes data collected in solids free 50mM USM media (Table 2.6) ; ☐ denotes data collected with 8% (w/w) DDGS solids added to the same media. Droplines show the agitation and airflow conditions at which measurements were made. Experiments performed as described in Section 2.6.1.
. Data points represent the average of n=3 data points.

A thorough review of the literature did not identify any work where K_La values had been determined using a wholly comparable experimental set up. One notable aspect of this work is that the determination of K_La values was carried out at 60 °C as this is the optimal operating condition for the fermentative organism used in Chapter 5. The vast majority of published K_La values are concerned with mesophilic processes and thus the known effect of temperature on recorded K_La values limits the degree to which the values of K_La measured in STRs in this work can be compared to those in the literature (Vogelaar et al., 2000). No published K_La vales were found that had been determined in the exact specification and model of STR used here, however there were several reports of K_La values that had been determined in comparable laboratory scale STRs with a similar volume and geometry.

The K_La values obtained in this work in the absence of solids ranged from 13h^{-1} to 197h^{-1} and these values are broadly similar to the values found in the literature using similar STRs but at much lower temperatures. In a study examining different experimental approaches to the measurement of K_La, values of 8 h^{-1} and 23 h^{-1} were determined at 200 rpm and 400 rpm (the airflow rate used is unclear) using the static gassing-out method as used here (Poughon et al., 2003). Other published work includes K_La values of around 10 h^{-1} measured at 200 rpm and 0.2 vvm which is in very good agreement with the data in Table 3.1 (Littlejohns and Daugulis, 2007). This same study included a maximum value of around 85 h^{-1} measured at 400 rpm and 0.3 vvm. Corroboration of the k_La values at the higher end of the measured range in Figure 3.2 can also be found with reports of K_La values in excess of 200 h^{-1} being measured in comparable experimental set ups (Mills et al., 1987, Dreher et al., 2013).

3.2.2 Effect of suspended solids on k_La values in STRs

The published literature on the effects of dissolved and suspended solids on oxygen transfer into slurries is limited and in many cases is apparently contradictory. It has been reported that at very low levels of just a few grams of solids per litre of slurry, solids can increase K_La values, i.e. enhance the oxygen transfer rate. This has been attributed to the solids increasing the interfacial area between the gas and liquid thus increasing the surface area for gas transfer. Equally it has been suggested that for suspensions of very fine solids the effect may be attributed to effects on the film mass transfer coefficient, K_L, rather than the available surface area per unit volume (Casey and Karmo, 1974, Ozkan et al., 2000, Linek et al., 2008). The published concentrations of solids reported to increase K_La values are at least an order of magnitude below those used in this work.

As the level of solids in a slurry is increased, the reported effect of the solids becomes more variable, with reports of both increased and decreased K_La values. The published data appears to be heavily influenced by the nature of the solids and liquid involved as well as the configuration of the bioreactor being studied. In addition to the above mentioned increase in K_La with a small amount of solids, the same study also found that larger concentrations of flocculent solids led to a decrease in K_La, whilst concentrations of up to 12.5g L^{-1} of fine suspended solids had no effect on K_La (Casey and Karmo, 1974). These results were obtained with a surface aeration system that may behave differently to the sparged system in this work. In a paper primarily focussed on wastewater treatment in a tank aerated by a fine bubble membrane, kaolin and diatomaceous earth were used to represent suspended and dissolved solids respectively (Leu et al., 1998). At all concentrations tested, up to 200mg L^{-1} for kaolin and 2000mg L^{-1} for diatomaceous earth, there was a decrease in K_La. This decrease was proportional to the increase in solids loading for the dissolved solids, but somewhat more complicated for the suspended solids with the largest decrease in K_La coming in the middle of the concentration range in this case.

Looking at the literature available for the effects of higher concentrations of suspended solids (> 10% v/v) in slurries the results available are again contradictory. In an investigation into the effects of 10% to 40% (v/v) of 66µm glass beads in a mechanically agitated sparged tank it was found that as the concentration of beads increased, the recorded k_La decreased (Mills et al., 1987). This decrease was approximately proportional to the solids loading and at the highest levels of solids the K_La recorded was only 40% of that from the base media of 0.1M NaCl. At 20% (v/v) solids loading, approximately correlating to the level of solids used in this work, the K_La was around 75% of the value recorded in the salt water with no solids. The authors established a correlation for K_La in the presence of solids based upon the volume concentration of solids, total power input and superficial gas velocity but its applicability to systems containing solids different to the glass beads used is likely to be limited. The authors attributed the decrease in k_La seen in the presence of glass beads to a reduction in interfacial area caused by decreased gas hold-up when the beads are present.

Investigations have also been carried into the effects of different types of solids loaded at 17% (w/v) in an agitated bioreactor. Spheres of two inert substances, glass and nylon 6,6, and two with some affinity for oxygen, silicone rubber and a styrene-butadiene copolymer, were used for these investigations. It was found that the two inert substances increased the K_La of the system whilst the two reactive compounds decreased K_La (Littlejohns and Daugulis, 2007). There is other literature supporting the observation that K_La can be increased by the presence of high concentrations of solids (Guo et al., 1997), however the majority of the published literature suggests that anything above a very low level of solids in a system is associated with a decrease in the oxygen mass transfer coefficient. Comparing directly the study cited which found that glass beads increased K_La with that by Mills et al. which found a decrease in K_La, the most obvious substantive difference is that the glass beads used in the study which found a decrease of K_La were at least an order of magnitude smaller than those which were found to increase K_La. Differences in the size of suspended particles having such a significant effect on measured K_La values would be of relevance to this work as the size of particles present will not be homogenous. A review of the literature available did not find any works looking at the effects of multiple sizes of particles of the same material and so any such effect remains speculative.

The data in Figure 3.2 clearly demonstrates that in the STR bioreactor system used by ReBio the presence of suspended solids reduces significantly the observed K_La under almost all conditions. Indeed the effect of the solids seems to be broadly similar in magnitude to that observed by Mills et al. (1987) in their work. Thus the observations reported here can be said to fit within part of a complicated picture that emerges from the literature.

Potentially of particular relevance to this study are the observations made with solids which are able to absorb oxygen. The literature data shows that the time taken to reach oxygen saturation when oxygen absorbing materials were present in the bioreactor is influenced by the sometimes substantial concentrations of oxygen absorbing to the solids. This naturally led to reduced K_La values being recorded. However, the original authors also calculated that due to this absorption substantially more oxygen in total was being transferred to the system when the oxygen absorbing solids were present. Accounting for the extra oxygen being transferred to the solids, the overall rate of oxygen transfer into the system was actually higher in the presence of these solids, aided by the solids creating a greater oxygen transfer gradient through their absorption of oxygen (which had the effect of reducing the concentration dissolved in the liquid phase). The amount of oxygen absorbed by the solids was greatest at the lowest airflow rate which the authors speculated was due to the K_La being so high at higher airflow rates that the solids did not have time to absorb significant amounts of oxygen. The authors have speculated that this effect could be used to alleviate oxygen limitation in systems with transient, high oxygen demand (Boudreau and Daugulis, 2006, Littlejohns and Daugulis, 2007).

During the determination of the k_La values reported here it was observed that if the DDGS solids were added to air saturated water the measured dissolved oxygen concentration decreased by 20%. This observation could support the possibility that the solids from DDGS have some capacity to absorb oxygen and therefore may be behaving in a manner similar to the oxygen absorbing solids mentioned above.

3.3 Correlation of STR k_La values with Pg and vs

3.3.1 Development of correlations to fit experimental data

The published literature contains several correlations relating measured K_La values in different bioreactor configurations with the stirrer speed and aeration rates (Gill et al., 2008b, Van't Riet, 1979, Vilaça et al., 2000, Linek et al., 2004, Smith et al., 1977, Shin et al., 1996, Zhu et al., 2001). Such correlations are useful as they allow the control of a parameter which is difficult to quantify in real time by the manipulation of factors which are readily quantified and controlled, i.e. stirrer speed and airflow rate. Correlations have been published using a wide variety of reactor designs containing a range of media formulations, some highly similar to that reported here, but no data has been reported to date for identical experimental set-ups. Nevertheless it was decided to compare the experimental results obtained with those from published correlations. Most correlations take the form:

$$K_La = a.(\frac{P_g}{V})^b.U_s{}^c \tag{3.1}$$

Where, P_g is the power input to the system under gassed conditions (W), V is the volume of liquid in the bioreactor (m^3) and U_s is the superficial gas velocity in the bioreactor ($m\ s^{-1}$). The differences between correlations are thus not in terms of the factors affecting K_La but rather the values of the constant *a* and the exponents *b* and *c*. These values are influenced by characteristics such as vessel geometry, stirrer design and fluid characteristics.

All of the published correlations require a measurement of P_g to the vessel in order for an estimation of the K_La to be made. Such information is not always available, as was the case here, and as such correlations for the estimation of gassed power input have also been published. One widely cited example is that derived by Cui et al. (1996) which calculates the gassed power input to a fermenter from the airflow, stirrer speed and the ungassed power input (Cui et al., 1996). This particular correlation was selected based on the similarities to the physical system used in this work. It was used to calculate the gassed power input for the parameter settings tested in this work, as well as to estimate P_g at other conditions to give a desired value of K_La.

The values of P_g calculated in this work show good agreement with those in the literature for a bioreactor of this size. As would be expected from the literature the calculated gassed power consumption increased as stirrer speed increases and decrease as the aeration rate increases. Volumetric gassed power consumption figures calculated ranged from 0.05 kW m^3 to 4.2 kW m3 at stirrer speeds between 200 – 800 rpm and aeration rates, in terms of superficial gas velocity, between 0.07 s^{-1} - 0.33 s^{-1}. Hassan and Robinson using a tank with similar diameter to those in this work but a smaller working volume measured volumetric gassed power inputs ranging from 0.03 kW m^3 to 16.2 kW m^3 (Hassan and Robinson, 1977). The operating conditions used in this paper spanned a wider range than those in this work and so a greater spread of gassed power inputs would be expected. Mills et al., using a slightly larger tank than in this work, measured gassed power inputs ranging from 1.5kW m^3 to 4kw m^3 over a relatively narrow operating range that corresponds to the higher end of the values used here. (Mills et al., 1987). This work is also notable as the gassed power measurements were taken in the presence and absence of suspended solids. The solids did not have a substantial effect on the measured power consumption. Thus the gassed power figures calculated for this work fit well with comparable measured data from the literature and thus their use is realistic for the purposes of developing correlations of k_La.

When predictions from several published K_La correlations were compared to the experimental data collected here it was apparent that there was not a close relationship between the predicted and actual values. This was true regardless of the presence or absence of solids in the STR. It was therefore necessary to develop a correlation specific to the STRs and experimental systems used in this work. Information from this gassed power correlation and the known values of U_s were used to establish correlation models in Excel of the K_La correlations published by several authors. The values of *a*, *b* and *c* from Eq. (3.1) for each of the correlations considered are given in Table 3.3. Two different correlations were developed from the experimental data collected, based on the data derived with and without solids in the medium. Equation (3.2) shows the correlation developed for solids free media and Eq. (3.3.) shows the correlation developed with DDGS solids added to the media.

$$K_L a = 0.019.(\frac{P_g}{V})^{0.45}.{U_s}^{0.45} \tag{3.2}$$

$$K_L a = 0.025.(\frac{P_g}{V})^{0.44}.{U_s}^{0.48} \tag{3.3}$$

Table 3.3. Values of constants and exponents reported for Eq 3.1 for various published K_La correlations.

Correlation	***a***	***b***	***c***
This work (without solids) This work (with solids)	0.0249 0.0194	0.443 0.447	0.479 0.447
(Gill et al., 2008b)	0.224	0.35	0.52
(Shin et al., 1996)	0.0195	0.55	0.64
(Van't Riet, 1979)	0.002	0.7	0.2
(Vilaça et al., 2000)	0.00676	0.94	0.65
(Linek et al., 2004)	0.01	0.699	0.65
(Smith et al., 1977)	0.01	0.475	0.4
(Zhu et al., 2001)	0.031	0.4	0.5

3.3.2 Comparison of developed correlations with those from the literature

One common method of determining which of several correlations provides the best fit to a set of experimental data is to look for the one which gives the lowest sum of residual squares according to the formula.

$$\Sigma(K_L a_{the} - K_L a_{exp})^2 \qquad (3.4)$$

Where, K_La_{the} is the value of K_La predicted by a given correlation at a particular condition and K_La_{exp} is the experimentally derived value of K_La at the same condition

An alternative method used by some authors to assess the best fit amongst several correlations of K_La is to solve equation (3.3) for each. The best correlation will have a value close to zero for c and values of close to one for m and R^2.

$$K_L a_{the} = c + m.K_L a_{exp} \qquad (3.5)$$

Table 3.4. Comparison of the goodness of fit of various correlations for predicting values of K_La (using Equation 3.1) with experimentally derived data (from Figure 3.2). m and C as defined in Eq. 3.5. Squared error calculated using Eq. 3.4.

Correlation	**m**	**c**	**R^2**	**Squared error**
This work	0.96	0.00092	0.98	0.00018
(Gill et al., 2008b)	0.27	0.0017	0.93	0.010
(Shin et al., 1996)	0.70	-0.0009	0.94	0.0024
(Van't Riet, 1979)	3.19	8.00E-05	0.76	0.12
(Vilaça et al., 2000)	5.29	0.0291	0.90	0.29
(Linek et al., 2004)	1.14	0.0041	0.93	0.00088
(Smith et al., 1977)	0.77	0.0022	0.93	0.00099
(Zhu et al., 2001)	0.71	0.003	0.95	0.0012

Table 3.4 shows both the sum of residual squares (from Eq. 3.4) as well as the equation fitting parameters from Eq. 3.5. For both measures the correlations developed in this work are substantially better at predicting the experimental K_La values than any of the published correlations.

Figure 3.3 shows the actual K_La values obtained along with predictions based on several published correlations and the correlation developed in this work (Eq. 3.2) for tanks containing solids free medium at low and high airflow rates. It can be seen that the correlation of Smith et al. (1977) provides a reasonable estimation of the measured K_La at several data points, although the fit is not as good as for the specific correlation developed here. The exception to this is at very low values of K_La where Eq. (3.4) overestimates K_La. The correlation produced by Linek et al. provides an excellent fit under low aeration and high stirrer speed conditions but tends to underestimate the K_La at lower stirrer speeds. The improved fit of Eq. (3.4) relative to the other correlations is clear. Figure 3.5 A shows the values predicted by the correlation developed in this work against the experimentally derived values for solids-free media. It can be seen that there is a good fit to the experimental data points at all values of K_La.

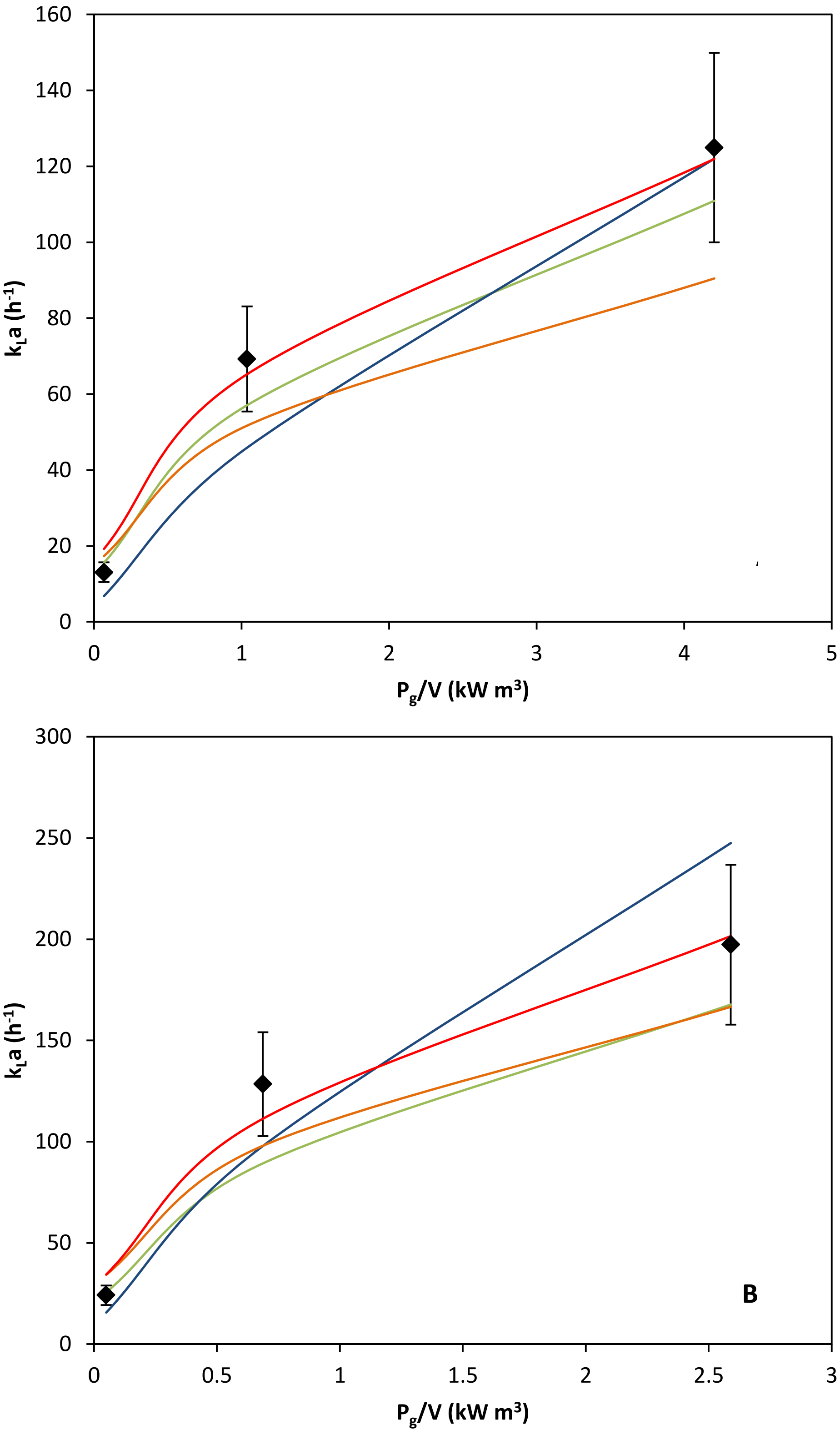
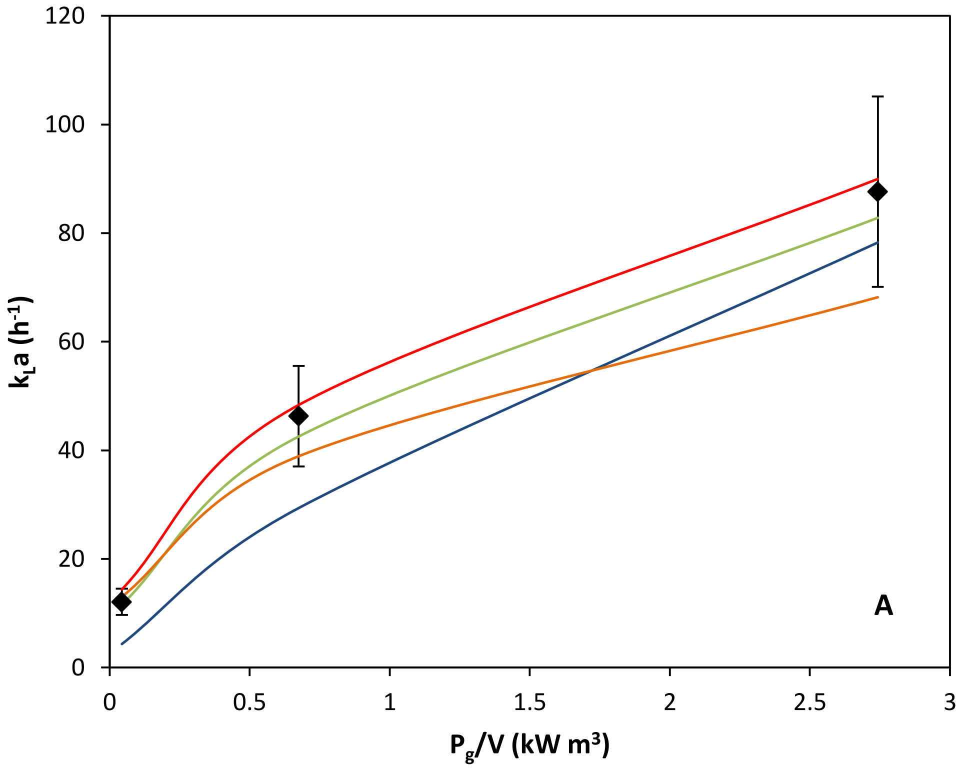


Figure 3.3. Comparison of measured (symbol) and predicted k_La values (lines) determined in solids-free 50mM USM media (Table 2.6) at 60°C and (A) 0.2 vvm airflow rate and (B) 1 vvm airflow rate. – This work; — (Linek et al., 2004); — (Smith et al., 1977); — (Zhu et al., 2001). Experimental data points plotted are the average of N=3 replicates and the error bars show one standard deviation around the mean. Experiments carried out as described in Section 2.7.1.

When solids are introduced into the system the pattern is somewhat different as seen in Figure 3.4. Here the published correlations tend to be a better fit to the experimental data. The correlation of Smith et al. (1997) provides a good fit to the experimental data at a 0.2 vvm aeration rate and a reasonable estimation at 1.0 vvm with all but one of the predicted values falling within one standard deviation of the experimental values. The Linek et al. (2004) correlation again tends to underestimate the experimental value at low stirrer speeds before moving closer to or even overestimating the measured value at the highest stirrer speed. The correlation derived from the data in Section 3.2 (Eq. 3.3) has the best fit to the experimental data and, in contrast to the solids free situation, does not overestimate the K_La at low values. Figure 3.5 B shows that, as was the case in solids free media, the correlation developed for solids-containing media has an excellent fit to the experiential data. In this case there is one point where the developed correlation significantly underestimates the experimental value and could be considered an outlier. This data point was measured at an intermediate airflow rate not shown in Figure 3.4.



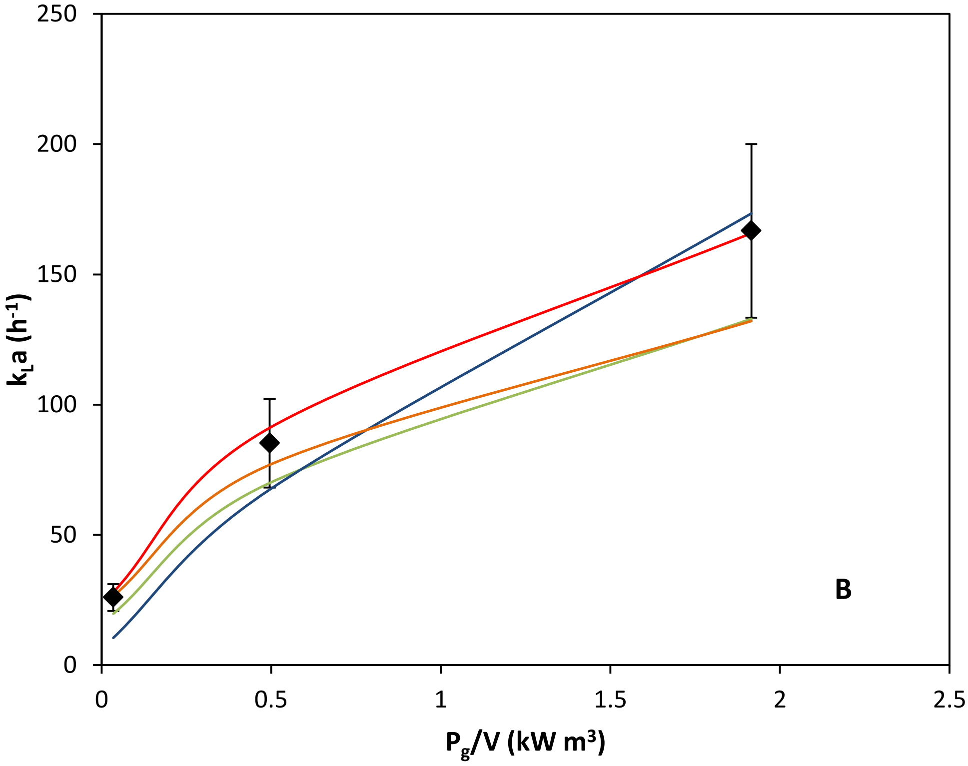


Figure 3.4. Comparison of measured (symbol) and predicted kLa values (lines) determined in media containing 8% (w/w) DDGS solids in 50mM USM media (Table 2.6) at 60°C and (A) 0.2 vvm airflow rate and (B) 1 vvm airflow rate. – This work; — (Linek et al., 2004); — (Smith et al., 1977); — (Zhu et al., 2001). Experimental data points plotted are the average of N=3 replicates and the error bars show one standard deviation around the mean. Experiments carried out as described in Section 2.6.1.

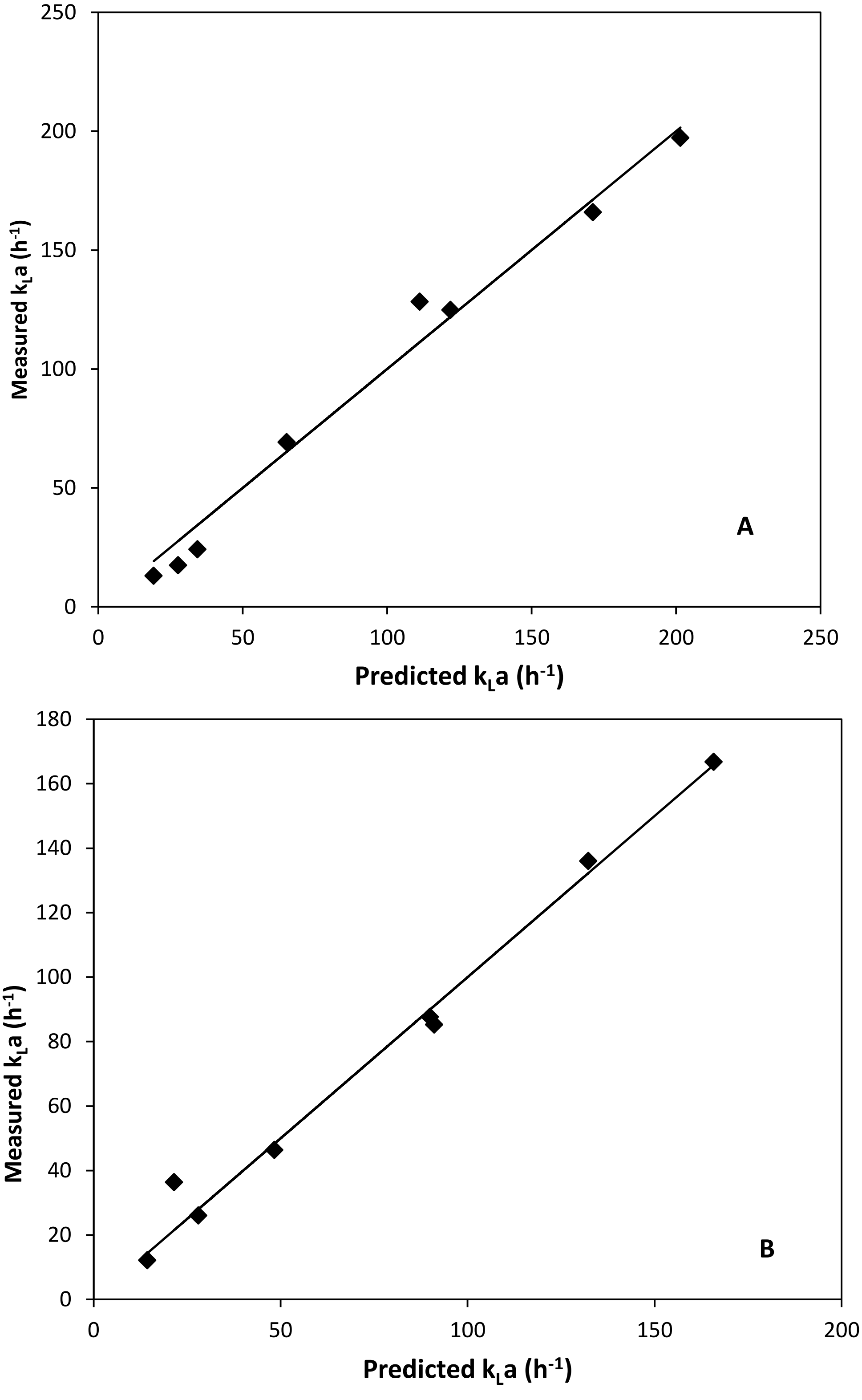


Figure 3.5. Parity plots of measured and predicted k_La values using the correlations developed in this work for (A) solids free 50mM USM media (Equation 3.2) and (B) 50mM USM media containing 8% (w/w) solids derived from DDGS (Equation 3.3). Data points show the average of N=3 replicates. Experiments carried out as described in Section 2.6.1.

Although published correlations were as far as possible chosen based on similarities to this work, all were based on measurements carried out at room temperature whereas the measurements for this work were carried out at the process condition of 60°C in the vessel. In a study of the effect of temperature on oxygen transfer in bubble column reactors containing tap water it was found that as the temperature increased so did the K_La (Vogelaar et al., 2000). An increase in the temperature in the column from 20°C to 55°C was sufficient to raise the K_La of the system from 0.006 s^{-1} to 0.01 s^{-1}. In terms of the overall transfer of oxygen into the system however the authors calculated that the increase in K_La would be offset by a decrease in the saturation concentration of oxygen and that the actual rate of oxygen transfer in terms of the mass of oxygen transferred per litre per hour would remain largely unchanged. Similar observations were reported in a study using a conventional stirred tank reactor (Krahe et al., 1996) and thus the observed increase in K_La at increased temperatures could be used to explain the fact that in the solids free hydrolysate the measured values for K_La were generally higher that was predicted by most of the published correlations. The addition of solids into the experimental set up had a clear negative effect on the values of K_La recorded and this drop when combined with the effect of the elevated temperature would explain why the published correlation are a better fit to the results in the presence of solids even though all of those correlations were determined in solids free media.

The validity of the correlations determined was confirmed by using them to predict the stirrer speed and aeration set points required to achieve particular K_La values. These K_La measurements showed that the correlations produced a close estimate of the actual K_La recorded (data not shown).

3.4 Rationale for the selection of conical bottom tubes as the USD model

In the establishment of USD approaches to industrial lignocellulosic ethanol process development there are several key requirements that need to be met. Briefly, any system must be of a small scale (ideally less than 100mL in volume) and with a small footprint to minimise material use and maximise the number of experiments carried out simultaneously. In order to enhance their utility they should use pretreated lignocellulose slurries without modification. Finally, the results obtained from the USD system need to be reproducible across experiments and be capable of accurately replicating results from larger scales.

A review of the current literature in Section 1.6 suggested that the selection of 50 mL conical bottom tubes for use as a USD model is a suitable compromise between the various options available. Microwell plates would reduce material use further and offer greater opportunities for automation but would likely require particle size reduction in the slurries which is considered undesirable. Methods have been patented that allow for the use of microwell plates without particle size reduction but there is a lack of published data using this methodology examining kinetics or comparability to STRs (Quinlan et al., 2014). Shake flasks have often been used for lignocellulosic hydrolysis experiments and for fermentation and the larger quantities of material used should improve reproducibility since a major source of error when working with slurries is their non-homogenous nature. However, shake flasks do not offer particularly large reductions in material or space requirements relative to STRs. Conical tubes therefore offer the best compromise between these two options. A rack for sixteen conical tubes occupies the same space on a shaker platform as four 500mL shake flasks and thus provides a four-fold increase in experimental throughput. The quantities of biomass, enzymes and other reagents required will be likewise reduced.

While such systems have previously been used for suspension culture of mammalian cells the dry solids fraction in cell culture applications is negligible and the cells were small, 10-20 µm, with a density close to that of water (De Jesus et al., 2004, Strnad et al., 2010). The current application is considerably more challenging with the requirement to work at dry solids fractions up to 25% (w/w) and with unmilled DDGS with particle sizes in excess of 5 mm. A similar system has been used to validate theoretical calculations of hydrolysis yield but the published work contains no kinetic data or comparison with STRs (Zhu et al., 2011). Thus there is a lack of data to determine the suitability of such systems for widespread industrial use.

Two different sizes of conical bottom tubes are available with 15 mL and 50 mL nominal volumes. The dimensions of the tubes are shown in Figure 3.6. It can be seen that the aspect ratios of the different sizes are significantly different, with the smaller tube being slightly taller but significantly narrower. There is also a longer, more gradual taper to the cone on the 15mL tube. In terms of their suitability for use as a scale-down system of a lignocellulosic ethanol process their applicability to the enzyme hydrolysis and fermentation stages should be considered separately due to the different requirements of each operation.

The greater diameter of the 50 mL tube makes loading them with highly viscous pretreated DDGS much easier and so only this size was used for experimental studies of enzyme hydrolysis. Once the DDGS has been through the enzyme hydrolysis stage its viscosity is reduced substantially. The concerns around loading the tubes are therefore not relevant to the fermentation stage and the lower requirements for material and space make the 15 mL tubes a potentially more suitable option. Both tube sizes were therefore taken forward for investigation for USD studies on the fermentation stage. For reference, images of DDGS prior to pretreatment, following pretreatment and following enzyme hydrolysis are shown in Figure 3.7.

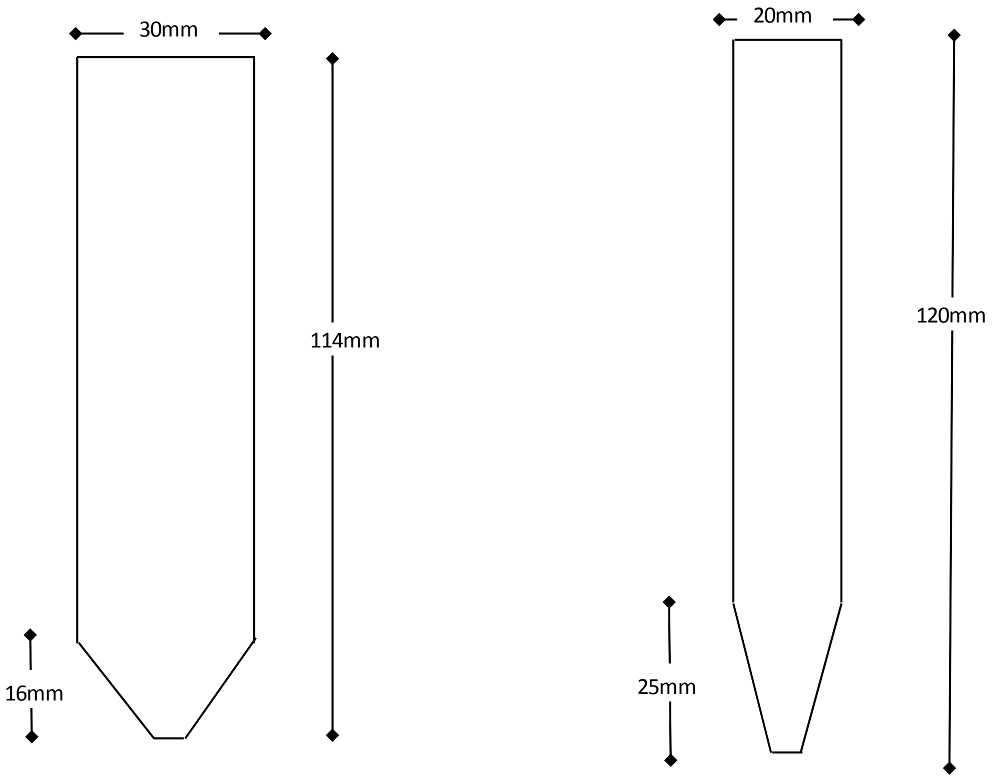


Figure 3.6. Dimensions of the 50 mL (H:D 3.8:1) and 15 mL (H:D 6:1) conical bottom tubes investigated as USD models of enzymatic hydrolysis and fermentation operations.

Figure 3.7. Images of DDGS prior to pretreatment, following pretreatment at 30% (w/w) solids content at 6.5 bar pressure for 5 minutes and following enzyme hydrolysis at 20% (w/w) DS for 24 hours with standard enzyme loading. Pretreatment carried out as described in Section 2.2. Enzyme hydrolysis carried out as described in Section 2.4.

3.5 Solid-Liquid Dispersion and Fluid Flow in USD tubes

As mentioned in Section 3.4 the ability to fully suspend and disperse the DDGS in the shaken tubes will be critical to their successful use as USD models of larger scale enzymatic hydrolysis (Chapter 4) and bioethanol fermentation (Chapter 5) processes. In order to assess the solid-liquid dispersion capabilities of the USD tubes, they were filled with previously hydrolysed DDGS and orbitally shaken at various frequencies (50 – 250 rpm) and angles (0 – 90 degrees) relative to a shaker platform. This work was not designed to be a full characterisation of the mixing in the system as others have performed for microwell systems (Barrett et al., 2010, Zhang et al., 2008). Rather it was designed as an initial investigation of whether the conditions under which the USD tubes were shaken were capable of suspending and dispersing the DDGS solids as these factors could impact on the kinetics of the enzyme hydrolysis stage.

The images in Figures 3.8, 3.9 and 3.10 are representative of maximum height of the free liquid surface as it passes around the orbitally shaken USD tube. Figure 3.8 shows images taken with the USD tube at 90° to the shaker platform, that is to say vertically, and shaken at frequencies of 50 – 250 rpm. The extent of solid-liquid dispersion and fluid motion in the tubes was judged by observation suspended solids distribution and through measurement of the free liquid surface height. As expected, as the shaking frequency increased so did the height of the free liquid surface (Barrett et al., 2010). With the shaking frequency at 50 rpm the height of the free liquid surface was 18 mm. This increased to 42 mm with a shaking frequency of 150 rpm and 68 mm with 250 rpm. Thus over the shaking frequency range the free liquid surface height increased by over 270%. As this surface is the primary means of gas exchange in shaken tubes it is expected that this will have a significant effect on the k_La values that can be achieved (Section 3.6). Visually there was dispersion and movement of the solids in a portion of material extending approximately 15 mm below the base of the free liquid surface, however there was a portion of poorly mixed material in the cone of the tube where there appeared to be some settling of solids occurring. At all shaking frequencies the fluid flow observed appeared to be laminar.

Figure 3.9 shows fluid flow and solids dispersion in USD tubes at an angle of 45°. With a shaking frequency of 50rpm or 150rpm the fluid flow and solids dispersion observed was similar to that when the tubes were held at 90° with the height of the free liquid surface reaching down to approximately the same position in the tubes. When the shaking frequency was increased to 250rpm however the angle at which the tube was held has a significant impact on the fluid flow observed. Comparing Figures 3.8 C and 3.9 C it is clear that the height of the free liquid surface is similar however the liquid surface itself is no longer uniform when the USD tube is held at 45°. Thus the fluid flow under this condition appears to be closer to a turbulent flow regime, at least at the surface. There are still some apparently poorly dispersed solids in the base of the cone however this shaking regime visibly reduced the settling out of solids. Holding the USD tube at an angle of 45° to the shaker platform appears to substantially improve fluid flow and solids dispersion at higher shaking frequencies.

Figure 3.10 shows that with the USD tube at 0° (horizontally) to the shaker platform there is substantially increased fluid flow and solids dispersion even at the lowest shaking frequency of 50rpm.

The pattern of fluid flow seen, with the wave passing fully from cone to the cap of the tube regardless of shaking frequency, was visibly more energetic than when the USD tubes were held at 0° or at 45°. The change in the direction of fluid movement makes it impossible to directly compare the height of the free liquid surface in the USD tubes however the images recorded show evidence of turbulent fluid flow at all frequencies investigated. This is likely due to the asymmetrical dimensions of the tube when held at this angle. This had the effect of achieving a far more even dispersion of the suspended solids within the liquid. As was the case at the other tube angles, the degree of fluid flow and solids dispersion visibly increased as the shaking frequency increased. This was especially so at 250rpm where the fluid flow, rather than passing along the USD tube with one free liquid surface, had multiple interacting free liquid surfaces that resulted in noticeable more turbulent fluid flow and dispersion of solids.

The data from this initial investigation indicates that the degree of solid – liquid dispersion and the pattern of fluid flow in USD tubes containing slurries of DDGS can be influenced by the shaking frequency of the shaker and even more so by the angle at which they are held relative to the shaker bed. The role of mixing in enzyme hydrolysis processes is currently not fully established in the literature, as discussed in Section 1.3.3, and so is examined directly in Chapter 4 of this thesis.

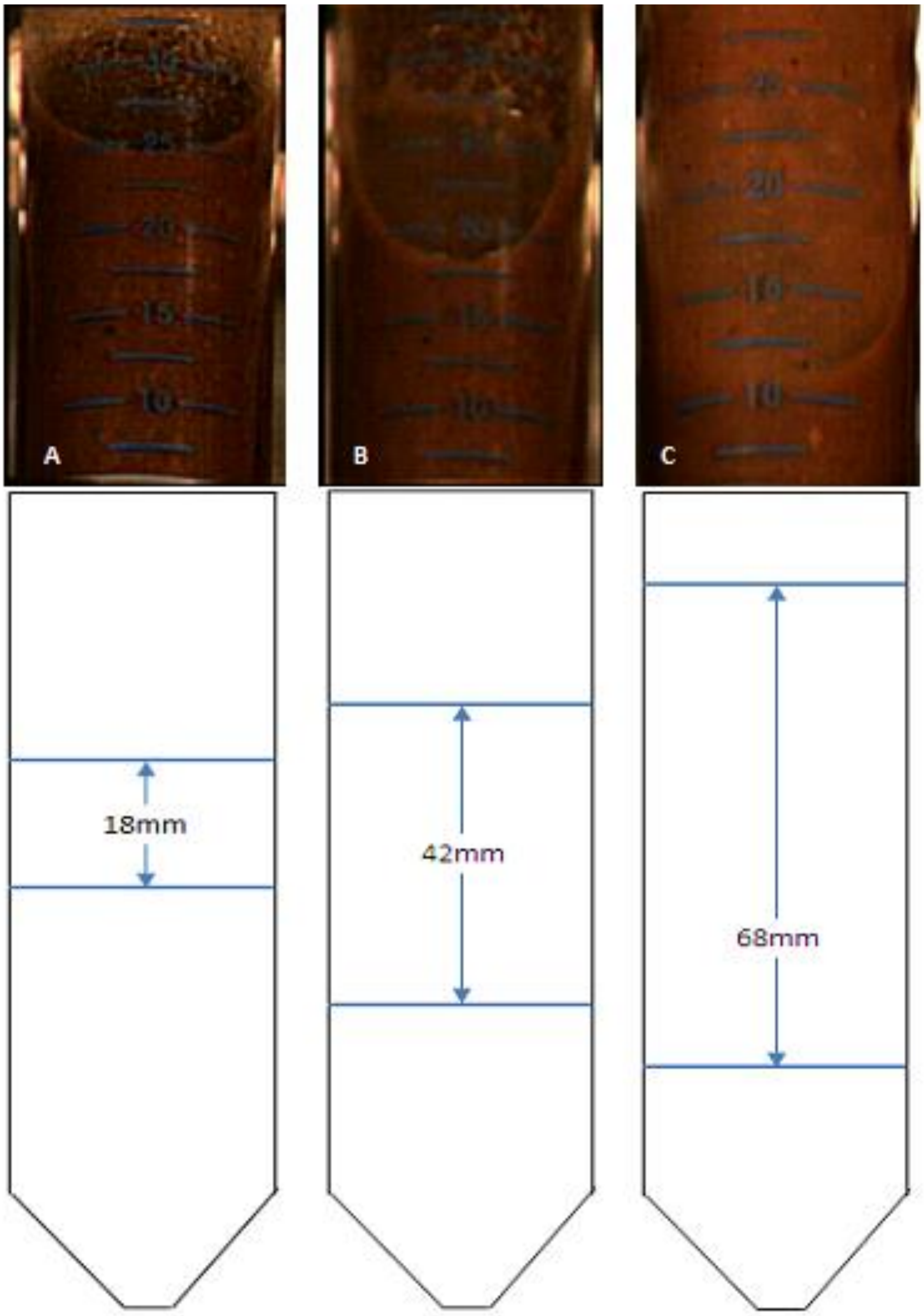


Figure 3.8. Fluid flow patterns and solid–liquid dispersion in USD tubes filled with 30 mL of DDGS hydrolysate, held at 90° to the shaker bed at shaking frequencies of (A) 50rpm, (B) 150rpm, (C) 250rpm. Also shown are schematic representations of the height of the free liquid surface. Experiments carried out as described in Section 2.5.

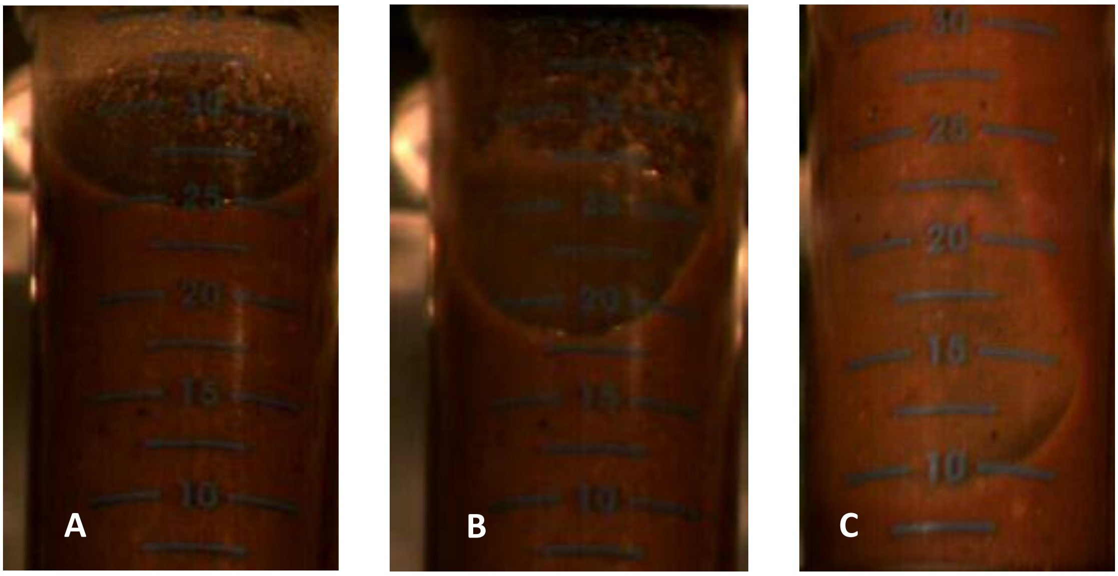


Figure 3.9. Fluid flow patterns and solid–liquid dispersion in USD tubes filled with 30 mL of DDGS hydrolysate, held at 45° to the shaker bed at shaking frequencies of (A) 50rpm, (B) 150rpm, (C) 250rpm. Experiments carried out as described in Section 2.5.

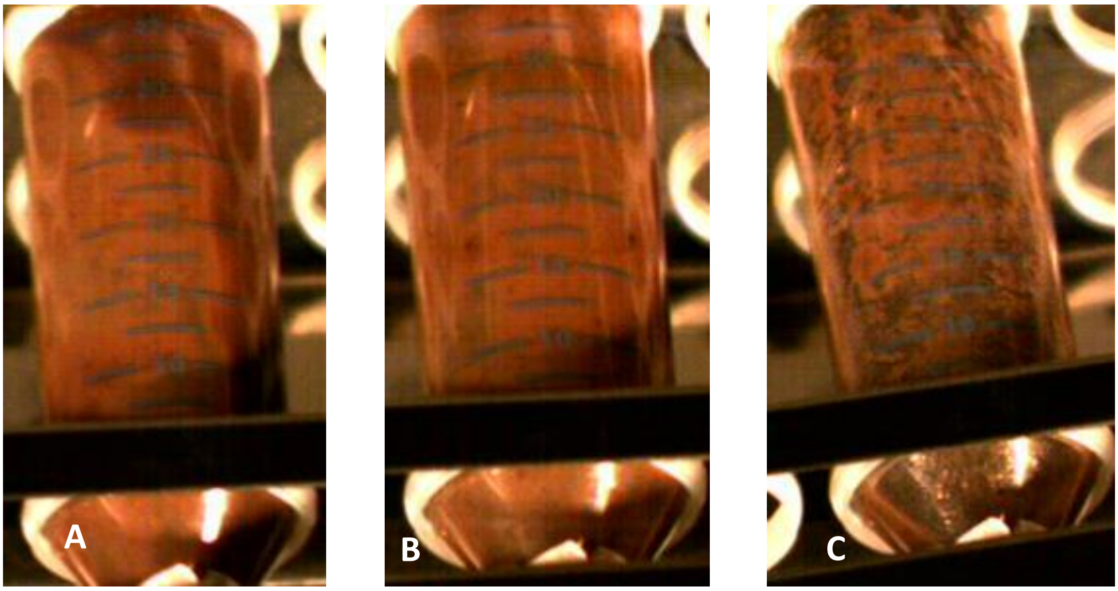


Figure 3.10. Fluid flow patterns and solid–liquid dispersion in USD tubes filled with 30 mL of DDGS hydrolysate, held at 0° to the shaker bed at shaking frequencies of (A) 50rpm, (B) 150rpm, (C) 250rpm. Experiments carried out as described in Section 2.5.

3.6 Measurement of k_La values in USD tubes

In Section 3.5 it was shown that DDGS solids could be adequately suspended and dispersed in the USD tubes and that the height of the liquid surface increased with shaking frequency. This is likely to impact on oxygen transfer rate and hence k_La values in the tubes. Initial fermentation studies in the tubes (data not shown) indicated that the best fermentation performance was obtained at a fill volume of 20 mL, as described in Chapter 5, hence all k_La values were measured at this fill volume

The data in Figures 3.8, 3.9 and 3.10 shows very clearly that the angle at which the USD tubes are held on the shaker platform has a significant impact on fluid flow and solid - liquid dispersion. Tubes held at 0° relative to the shaker had more turbulent fluid flow characteristics and better solid – liquid dispersion. Tubes held at 45° to the shaker bed showed intermediate levels of solid-liquid dispersion, and so any effect of adjusting the angle at which the USD tubes were held would only be increased if they were held at 0° rather than at 45°. Therefore the determination of $K_L a$ was carried out with the tubes held at 0° and 90° to the shaker platform. As with the STR vessels, data was obtained in the presence and absence of suspended solids in media.

3.6.1 Measurement of $K_L a$ in USD tubes in the absence of suspended solids

Typical examples of the dissolved oxygen – time profiles obtained from static gassing out experiments are shown in Figure 3.11. The experimentally derived $K_L a$ values are shown in Figure 3.12. From this data it is clear that shaking frequency, inclusion of suspended solids and the angle at which the USD tubes are held all have a significant effect on the oxygen mass transfer coefficient. There is a strong, statistically significant, correlation (coefficients > 0.97, $p < 0.05$) between increasing shaking frequency and increasing $K_L a$ value regardless of the specific system set up. This is consistent with the observations from Section 3.5 where area of the free liquid surface increased as the shaking frequency was increased across the same range. The relationship between shaking frequency and $k_L a$ is essentially linear for all conditions. Looking first at tubes held at 90° to the shaker platform, at the three shaking frequencies examined the $K_L a$ values recorded were 4 h^{-1}, 50 h^{-1} and 97 h^{-1}. These values are in line with published values in a similar system (Xie et al., 2011a). When the USD tubes were instead held at 0° to the shaker platform $K_L a$ values were significantly enhanced with the increases ranging from 36% at 50 rpm to 158% at 150 rpm. The maximum $K_L a$ recorded during these tests was when the USD tube was held horizontally and shaken at a frequency of 250 pm with a value of 210 h^{-1}. This is not unexpected given the clear difference that the angle at which the USD tubes were held was observed to make to the fluid flow (Section 3.5).

Correlations were developed to predict $K_L a$ in with USD tubes held at 0° to the shaker platform (Eq. 3.6) and 90° to the shaker platform (Eq. 3.7):

$$k_L a = 1.06f - 41.42 \qquad (3.6)$$

$$k_L a = 0.46f - 19.15 \qquad (3.7)$$

where f is the shaking frequency in RPM.

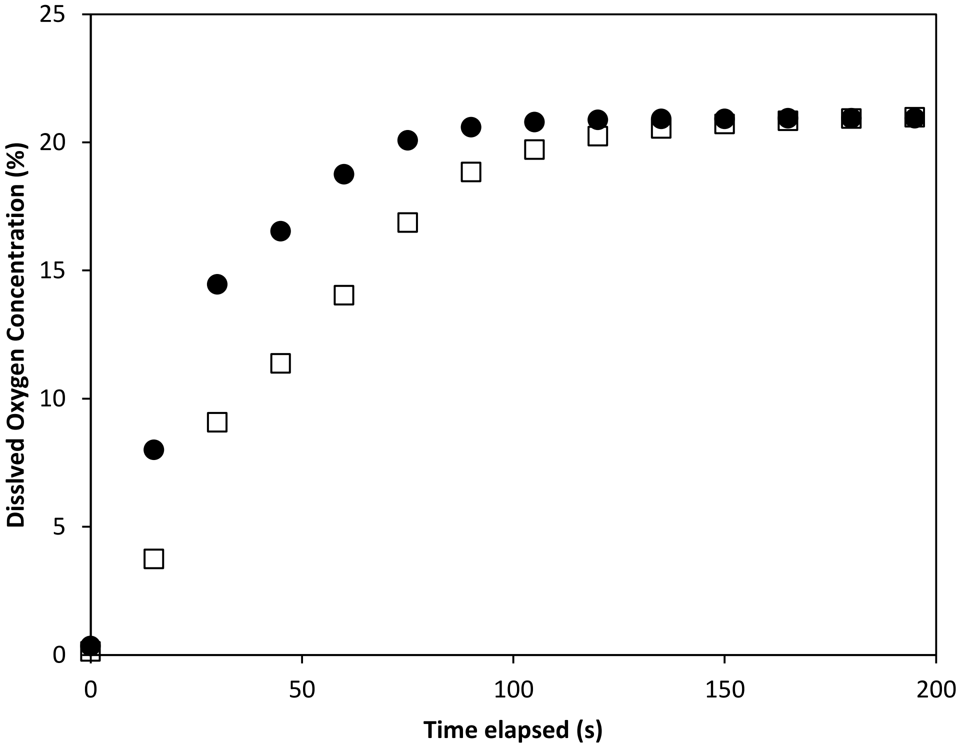


Figure 3.11. Typical examples of dissolved oxygen concentration measurements obtained in USD tubes. Examples shown were obtained at a shaking frequency of 150rpm with USD tubes held at 90° to shaker platform in a shaking incubator at 60°C. ●: data collected in solids free 50mM USM media (Table 2.8); ☐: data collected with 8% (w/w) solids added to the same media. Experiments carried out as described in Section 2.6.2

3.6.2 Measurement of K_La in USD tubes in the presence of suspended solids

When solids were introduced into the system with the USD tubes held at 90°, measured K_La values were reduced significantly as can be seen in Figure 3.12. In the extreme case at 50 rpm no increase in dissolved oxygen concentration was recorded in the USD tube over a period of an hour. At 150 rpm and 250 rpm the K_La values determined were reduced by 87% and 77% respectively when compared to USD tubes without solids in them. There was a similar observation when suspended solids were included in the system with the USD tubes held at 0° with decreases of 17%, 86% and 36% in K_La at shaking frequencies of 50 rpm, 150 rpm and 250 rpm. As in the solids free set up, there is a strong, statistically significant, correlation between increased shaking frequency and increased k_La (correlation > 0.90, $p < 0.05$)

Thus, as was the case in STRs, increased fluid flow in the USD tubes led to increases in k_La whilst the presence of suspended solids in the media leads to a clear reduction in the oxygen mass transfer coefficient, although this can be significantly mitigated by holding the tubes at 0° on the shaker bed rather than at 90°.

Correlations were developed to predict K_La in with USD tubes held at 0° to the shaker platform (Eq. 3.8) and 90° to the shaker platform (Eq. 3.9):

$$k_La = 0.68f - 46.94 \qquad (3.8)$$

$$k_La = 0.11f - 7.08 \qquad (3.9)$$

where *f* is the shaking frequency in RPM.

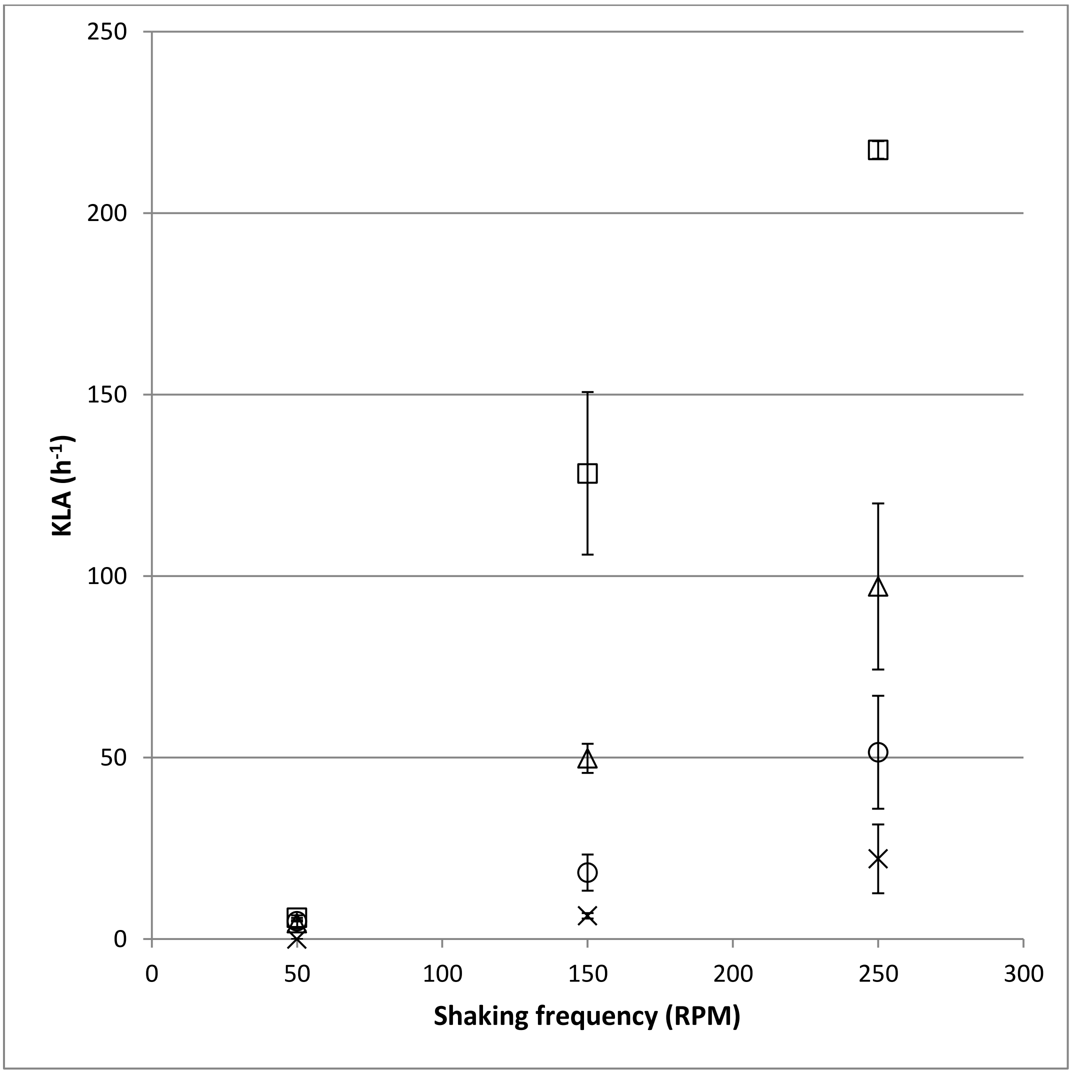


Figure 3.12. The effect of suspended solids, tube orientation and shaking frequency on K_La values in USD tubes. In all cases the tubes were held in a shaking incubator set to 60°C in a background of 50mM USM media (Table 2.8). △: 90° USD, no solids; ×: 90° USD, with 8% (w/w) suspended solids from DDGS; ☐: 0° USD, no solids; ○: 0° USD, with 8% (w/w) suspended solids from DDGS. Data points are an average of 2 replicates. Error bars show one standard deviation around the mean. Experiments carried out as described in Section 2.6.2.

3.6.3 Comparison of k_La values in STRs and USD tubes

The K_La values recorded in the USD tubes span a comparable range to those recorded in the STRs when the USD tubes are held at 0° to the shaker platform. In this orientation, the K_La values in the USD tubes ranged from 6h^{-1} to 217h^{-1} in the absence of solids and 5h^{-1} to 140h^{-1} with solids present. In the

STRs K_La values ranging from 13h^{-1} to 197h^{-1} were recorded in the absence of solids whilst the values recorded in the presence of solids ranged from 12h^{-1} to 165h^{-1}. Thus, at least in terms of the volumetric oxygen transfer rate, this makes the USD tubes a potentially suitable mimic for STRs. Importantly this is true for both a clean media and also when solids are introduced to the system.

3.7 Summary

The aim of this chapter, as described in Section 3.1 was to characterise the current laboratory scale STR system in use at ReBio, select an appropriate USD model for testing and then to compare the engineering characteristics of the two systems in terms of fluid flow, solid-liquid dispersion and oxygen mass transfer.

The first objective was to characterise the STR system at a range of operating parameters. The oxygen mass transfer coefficient, k_La, was chosen for this characterisation based on analysis of the options available and the known importance of oxygen levels in the fermentation process. This characterisation was described in Section 3.2. Values for k_La were determined over a range of operating conditions ranging from aerobic through to micro-aerobic in both solids-free media and in media containing solids that had been separated from DDGS after enzyme hydrolysis. The values determined in solids-free media were found to be comparable to those for similar systems in the literature.

The presence of suspended solids in a second generation ethanol process is one of its notable properties, and the effect of solids on k_La is something about which there are varying reports in the literature. In this work it was found that they had a significant negative effect on k_La, with the values in the presence of the suspended solids being approximately 75% of that in the solids free media (Figure 3.2). It is suggested that this may be due to oxygen absorption by the DDGS solids.

In Section 3.3 the k_La data was used to develop correlations predicting k_La based on the gassed power input and superficial gas velocity. The correlations developed were found to better predict the measured k_La values compared to several published correlations (Figures 3.3 and 3.4). The correlations developed will be used later in Chapter 5 to define the optimal process conditions and to compare fermentation performance at different scales.

The second objective was to select an appropriate system to act as a scale-down model for the characterised STRs. The rationale for selection of conical bottomed tubes with a nominal volume of 50mL was described in Section 3.4. This was based on the degree of scale reduction offered as well as their suitability for use with the viscous slurries found in a cellulosic ethanol process. Similar tubes were found to be in use with mammalian cell culture but their detailed characterisation and use here would be novel for an industrial biotechnology application.

Subsequently the solids dispersion and fluid flow that could be achieved in the USD tubes was visualised using a high speed video system. The work in Section 3.5 demonstrated that both the shaking frequency and the angle at which the USD tubes were held on the shaker platform affected fluid flow and solid - liquid dispersion. When the tubes were held at 90° the degree of solids dispersion increased with shaking frequency but even at the highest shaking frequency there appeared to be a portion of poorly dispersed solids in the base of the tube. Holding the tube at 0° substantially improved solids dispersion, and at the highest shaking frequency led to fluid flow that was increasingly turbulent in appearance. The implications of these observations are discussed further in terms of enzyme hydrolysis performance in Chapter 4.

The final objective was to characterise the USD tubes in terms of k_La to allow a comparison with the STR characterisation in Section 3.2. This was addressed in Section 3.6, and the broad trends are the same as were observed in STRs. As the shaking frequency increased, so did the magnitude of the k_La values recorded. The observation of apparently more turbulent fluid flow when the USD tubes were held at 0° was confirmed by the substantial increases in k_La seen when the tubes were held in this orientation. Finally as was the case in the STRs the introduction of suspended solids led to a substantial decrease in the determined k_La vales. It was further observed that there was a large range where the k_La values measured at the two scales overlapped. It should be noted however that the k_La in the USD tubes was measured in a state where the tube headspace was filled with air prior to measurement but the air was not replenished during use except by diffusion through vent holes. It may therefore be that in practice the oxygen transfer rate in the USD tubes would decline over time as oxygen is depleted from the headspace. This would not be an issue with the continuously sparged STRs.

Having now selected and characterised the USD tubes, relative to the current STRs, their application to the enzymatic hydrolysis stage of the lignocellulosic bioethanol process is described in the next chapter.

4. Establishment of a USD model for the enzymatic hydrolysis of DDGS[1]

4.1 Introduction

The enzymatic hydrolysis stage of a lignocellulosic ethanol process is, as noted in Section1.3, one of the key areas in which process improvements are required for profitable production of lignocellulosic ethanol. The 4L STRs described in Section 2.3 represent an established scale-down model for DDGS hydrolysis that is in use at ReBio, with some additional scouting experiments performed in shake flasks.

As noted in Section 1.6, several papers have been published in which microwell plates have been used for enzymatic hydrolysis processes as part of a screening study. These have, however, been used primarily to identify the best performing conditions with the belief that the optimum condition from the small scale trials is also likely to be the optimum if the trials were to be replicated at larger scales. What is not, however, claimed is that the actual quantitative performance seen at the small scale will be replicated at the larger scale. That is to say that if condition A gave a maximum yield in small scale trials of say 85% hydrolysis in X hours, it may be hoped that condition A would also give the same rate and yield at larger scale. The review of the literature also found that the substrate used in the microwells had generally been altered to make it more amenable to use, i.e. feed material is ground down into fine particles (< 0.5mm) to facilitate adequate solid suspension. Recent work has to some extent alleviated the issue with particle size but the lack of comparability to larger scales remains (Quinlan et al., 2014). Finally, enzyme hydrolysis can be carried out in shake flasks without any need for the substrate used to be modified. These have a relatively large footprint compared to their volume however, and require moderately large volumes of material for experiments.

From the critical review of the literature in Section 3.4 it appeared that conical bottomed tubes offer an alternative system that would offer a good compromise between microwell plates and shake flasks. In order to offer a commercially relevant improvement over existing microwell or shake flask methods the USD tubes must use non-modified slurries of pretreated biomass. They should also allow the direct replication of results from the USD system in the larger scale stirred tanks currently in use at

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[1] The majority of the results in this chapter have been published as: Conroy, N., Tebble, I. and Lye, G. J. (2015), Creation of an ultra scale-down bioreactor mimic for rapid development of lignocellulosic enzymatic hydrolysis processes. J. Chem. Technol. Biotechnol., 90: 1983–1990

ReBio. If such a system can be established then it would be of significant benefit to researchers in both academic and industrial setting.

In terms of enzyme hydrolysis there are a several metrics that can be used to characterise the success of a scale down strategy. Most important is that the concentration of sugar released from the feedstock by the end of the reaction is the same at both scales. The composition of that sugar in terms of the concentration of the main sugars present as well as the split between monomers and oligomers should ideally also be similar. A second metric is that the kinetics of hydrolysis, that is to say the rate of release of the different sugars from the DDGS, should also ideally be similar at the different scales in order that the USD system is able to be used to examine the kinetics of hydrolysis. The comparison of STR and USD tube performance in the following sections will be considered in terms of these criteria in Section 4.8.

As described in Section 1.7 the aim of this chapter is to establish the USD model selected in Section 3.4 for use with an enzymatic hydrolysis process using DDGS as an industrially relevant model feedstock. This can be broken down into the following objectives

- Understand and control the degree of variability introduced into USD hydrolysis arising from the DDGS pretreatment stage.
- Quantify the performance of the current STR system as a benchmark against which to evaluate USD system performance.
- Investigate how the solid-liquid dispersion and fluid flow patterns identified in Section 3.5 affect enzyme hydrolysis kinetics.
- Establish comparability of performance in USD and STR systems at industrially relevant solids loadings.
- Use the validated USD model to investigate factors that are limiting to enzyme hydrolysis performance.

4.2 Variability resulting from pretreatment

4.2.1 Hydrolysis of DDGS in USD tubes

Prior to detailed investigation of enzyme hydrolysis of DDGS in USD tubes or STRs, initial investigations were carried out to assess the general suitability of the USD tubes for enzyme hydrolysis. These experiments also provide data on how enzyme hydrolysis affects the sugars found in pretreated DDGS.

Figure 4.1 shows the concentrations of the various sugars determined in DDGS following pretreatment and enzymatic hydrolysis of a single batch at two different solids levels. Slightly under 45g L^{-1} sugar is solubilised during the pretreatment stage, comprising of 19 g L^{-1} glucose sugars, 18 g L^{-1} of sugars that elute as X+G+M+F sugars (Section 2.10.2) and 8 g L^{-1} of arabinose sugars. In the case of the glucose this is almost totally present as oligomers whilst the others are mainly present as oligomers, but with a significant proportion of monomers. That there is a higher proportion of monomeric arabinose and X+G+M+F present is as expected given the lower thermal stability of the hemicellulose from which they derive compared to the cellulose which contains the majority of the glucose (Section 1.2).

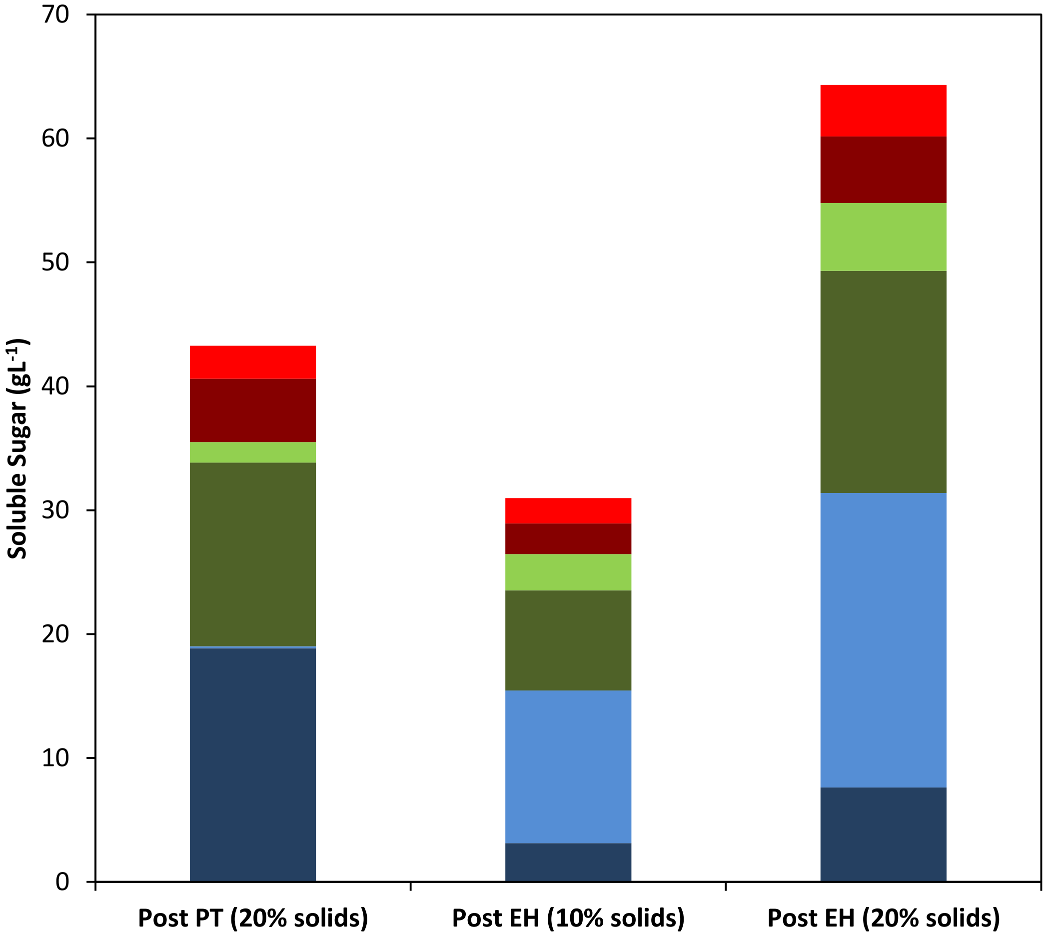

Figure 4.1. Concentrations of oligomeric and monomeric soluble sugars in slurries of DDGS following pretreatment at 30% (w/w) solids and enzyme hydrolysis at 10% (w/w) and 20% (w/w) solids loadings for 48 hours at 50°C. Data shown is the mean of n=2 points. 🟦: Glucose oligomers; 🟦: glucose monomer; 🟩: X+G+M+F oligomers; 🟩: X+G+M+F monomers; 🟥: arabinose oligomers; 🟥: arabinose monomer. Experiments carried out as in Section 2.4. Sugar concentrations determined as described in Section 2.10 and data analysed as in Section 2.11.

To understand the effect of enzyme hydrolysis on the pretreated material it is informative to look first at the data from the hydrolysis that was carried out at 20% (w/w) solids. Due to the issues noted in Section 2.10.5, it is difficult to directly compare sugar concentrations from hydrolyses carried out at different solids loadings without turning them into hydrolysis yields. Following the enzyme hydrolysis stage the total concentration of soluble sugars is substantially increased, as is the proportion of monomeric sugars present. The total soluble sugar concentration following enzyme hydrolysis at 20% (w/w) solids increased to 64 g L^{-1}. The proportional differences for the three different groups of sugars were a 65% increase in the glucose sugars, a 42% increase in the X+G+M+F sugars and a 22% increase in the level of arabinose sugars. The differences in the degree to which the concentrations of the different sugars increased may be due to the extent to which they were solubilised in pretreatment, with a higher proportion of the glucose remaining unsolubilised.

The increase in the degree to which the sugars present are monomerised is far more pronounced when looking at the glucose data. From representing only 1% of the glucose sugars present following pretreatment the monomer form accounts for 76% of the glucose sugar following enzyme hydrolysis. For the X+G+M+F and arabinose, although the proportion of monomers is increased compared to the post pretreatment sample they still represents less than 50% of those sugars following enzyme hydrolysis. Thus the enzymes used in this study are substantially more effective at reducing the glucose sugars to monomers than they are the other sugars. This likely reflects the historical focus on enzyme producers on the hydrolysis of cellulose (Banerjee et al., 2010b).

These experiments demonstrated the practical suitability of the USD tubes for the enzymatic hydrolysis of DDGS, with hydrolysis successfully occurring at both high and low solids loadings.

4.2.2 Variability occurring in the pretreatment stage

Although DDGS pretreatment technology is not a focus of this project, any variability occurring at this initial stage of the process could potentially lead to differences being observed during subsequent enzymatic hydrolysis and fermentation experiments. By necessity, material produced from several different pretreatment batches will be used during the course of this project and so it is important to know how much, if any, variability in hydrolysis performance could be attributed to differences carried forward from the pretreatment stage. To this end an initial investigation was carried out into the reproducibility of pretreatment batches. Six pretreatment batches were produced as set out in Section 2.2 and for each batch enzyme hydrolysis reactions were performed in USD tubes at 10% (w/w) and 20% (w/w) dry solids loadings. All of the hydrolysis reactions were run for 48 hours.

Figure 4.2 shows the overall hydrolysis yield for each of the 6 pre-treatment batches (PT 1-6) at 10% (w/w) solids loading along with the yields of monomeric sugar for glucose and other monomers. The overall yields (Section 2.10.5) seen varied between 83% for PT1 and 69% for PT6 with a mean overall yield of 75.3%. The standard deviation around the mean in terms of the overall yield was 6.1, thus for five of the six PT batches the overall yield fell within one standard deviation of the mean. The glucose monomer yield varied between 65% in pre-treatment batches PT1 and PT2 to 58% in batch PT6 with an average of 60%. The standard deviation across the whole data set was 4.8. All of the PT batches in this case giving glucose monomer yields that were within one standard deviation of the mean. The yield of other monomeric sugars was substantially lower, ranging from 22% to 26% with an average of 22.7%. All of the pre-treatment batches were within one standard deviation of the mean in terms of the yield of other monomers. Analysis of variance (ANOVA) carried out on this data (Section 2.11) showed that there was no statistically significant variation within the data set ($p > 0.1$ for the overall, glucose, and other monomer yields).

In Figure 4.3 it can be seen that hydrolysis yields are lower when the hydrolysis was carried out at 20% (w/w) solids. Overall yields at this solids level ranged from 69% in batch PT1 to 77% from batch PT5 with an average of 72% and a standard deviation of 4.2. Glucose monomer yields ranged from 49% (PT3) to 57% (PT5) whilst the yield of other monomers ranged from 19% (PT3 and PT6) to 22% (PT5). Again, the vast majority of yields fell within one standard deviation of the mean, the one exception being the overall yield of batch PT5 which was marginally above the upper limit. ANOVA analysis suggested that there was a statistically significant difference within the data set for all yields (overall yield $p < 0.01$, glucose and other monomer yields $p < 0.05$).

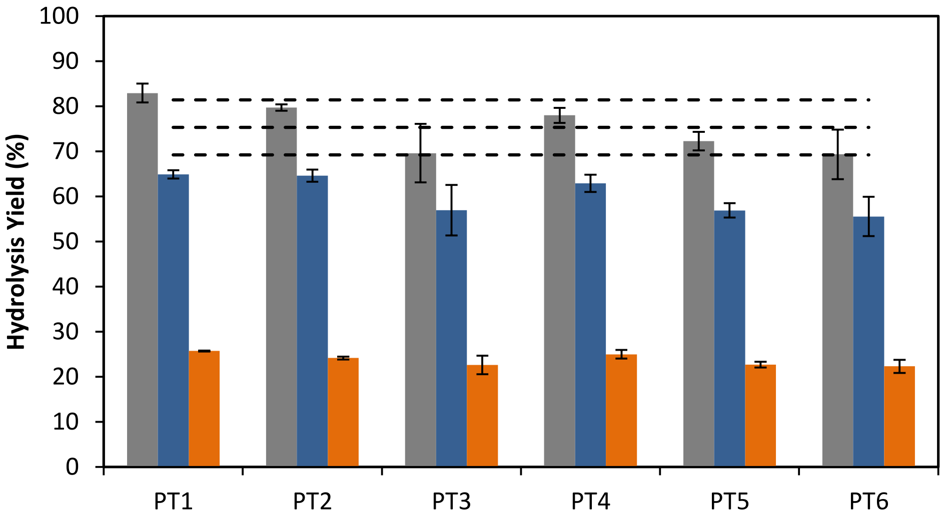

Figure 4.2. Average overall sugar solubilisation yield from the enzymatic hydrolysis of six batches of pretreated DDGS (PT1-6) and the yields of glucose monomer and other monomers at 10% (w/w) dry solids loading. Enzyme hydrolysis was carried out in a shaking incubator set to 50°C and 250 rpm for 48 hours. ⬛ = overall yield; 🟦 glucose yield; 🟧 other monomers yield. Error bars show one standard deviation about the mean (n=3). Dashed lines represent one standard deviation around the mean of the whole sample for the overall yield. Experiments performed as described in Section 2.4. Sugar concentrations determined as in Section 2.10 and data analysed as in Section 2.11.

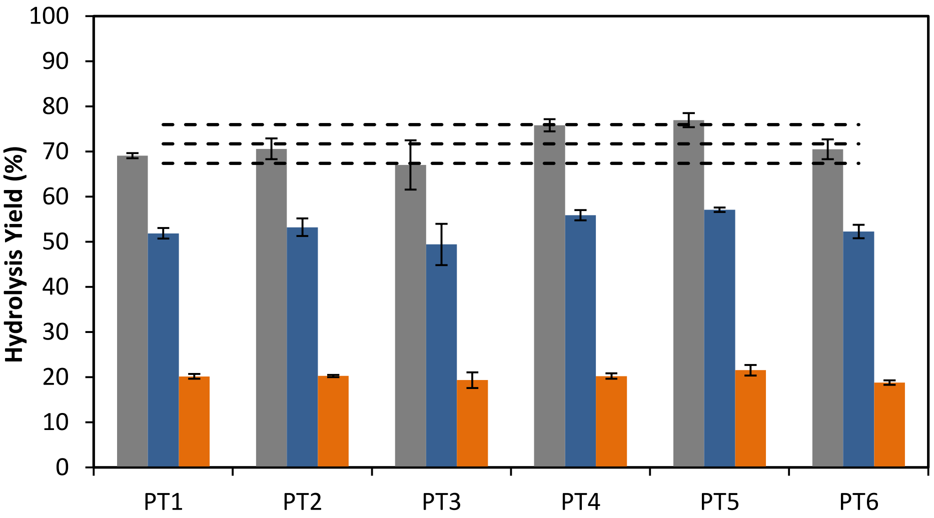

Figure 4.3. Sugar solubilisation yield from the enzymatic hydrolysis of six batches of pretreated DDGS (PT1-6) and the yields of glucose monomer and other monomers at 20% (w/w) dry solids loading. Enzyme hydrolysis was carried out in a shaking incubator set to 50°C and 250 rpm for 48 hours. ▮. ▮ = overall yield; 🟦 glucose yield; 🟧 other monomers yield. Error bars show one standard deviation about the mean (n=3). Dashed lines represent one standard deviation around the mean of the whole sample for the overall yield. Experiments performed as described in Section 2.4. Sugar concentrations determined as in Section 2.10 and data analysed as in Section 2.11.

If the six pretreatment batches are ranked according to the overall yield achieved as in Table 4.1, there is little correlation between a pretreatment's performance at the two solids loadings (correlation = 0.085). There was little change in the rankings if the glucose or other monomer yields were used for the comparison instead (data not shown). The fact that rankings were not correlated between the two solids levels suggests that the differences seen at 20% solids, although statistically significant, were more likely due to the inherent variability of the methods used rather than variability in pretreatment performance.

Nevertheless, as a statistically significant difference had been observed, the performance of each pretreatment was compared to key metrics recorded from the pretreatment stage to look for a correlation. Table 4.2 shows these metrics for each of the pretreatments. From Table 4.2, it seems that only the solids content of the material immediately after pretreatment shows any substantial degree of variability, with solids contents ranging from 21.9% to 28.1% due to variability in the condensed water that remains within the slurry after pressure release in the pretreatment vessel. Figure 4.4 however makes it clear that there is no correlation between the solids content after the pretreatment step and subsequent hydrolysis performance.

Table 4.1. Ranking of six individual pretreatment batches (PT1-6) according to the overall hydrolysis yield achieved following hydrolysis of pretreated DDGS at 10% (w/w) and 20% (w/w) solids. Enzyme hydrolysis was carried out in a shaking incubator set to 50°C and 250 rpm for 48 hours. Ranking based on data in Figures 4.2 and 4.3 respectively.

Rank	10% (w/w)Solids Loading Rank	20% (w/w) Solids Loading Rank
PT1	1	5
PT2	2	3
PT3	5	6
PT4	3	2
PT5	4	1
PT6	6	4

Table 4.2. Key process metrics recorded for each of six pretreatment batches (PT 1-6) of DDGS (pH was recorded at a standard solids content and temperature to remove any influence from these variables). Pretreatment was carried out at 30% (w/w) solids loading at 6.5bar for 5 minutes as described in Section 2.2.

PT Batch	Dry solids content following pretreatment (% w/w)	pH at 20% (w/w) DS and 50°C	Temperature in Reactor (°C)
1	21.9	3.89	165
2	22.9	3.92	162
3	23.4	3.87	170
4	26.3	3.89	163
5	26.7	3.85	162
6	28.1	3.84	160

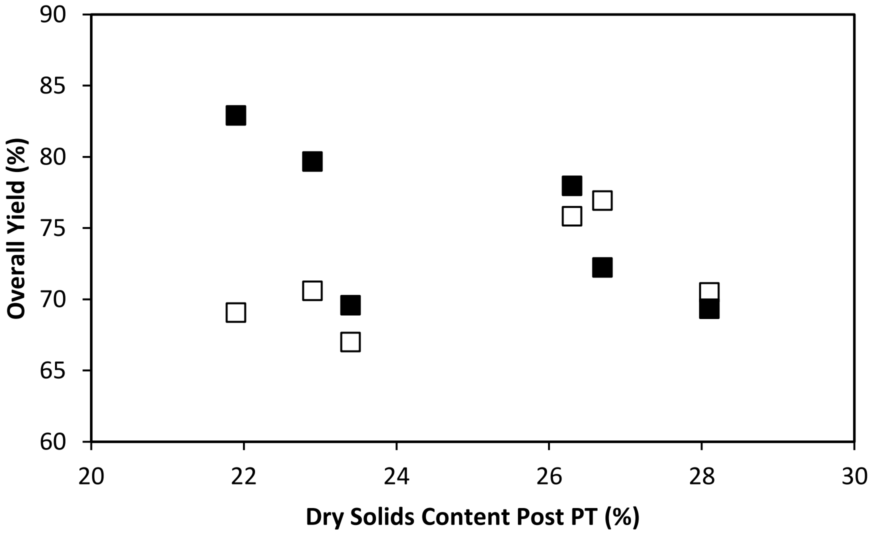

Figure 4.4. Correlation of dry solids content post pretreatment with overall hydrolysis yield for the hydrolysis of pretreated DDGS. ■ = 10% (w/w) solids hydrolysis; □ = 20% (w/w) solids hydrolysis. Mean values for each set of hydrolysis USD tubes are shown (n=3). Enzyme hydrolysis was carried out in a shaking incubator set to 50°C and 250 rpm for 48 hours. Experiments carried out as in Section 2.4. Hydrolysis yields and dry solids content determined as described in Section 2.10. Data analysed as in Section 2.11.

As the focus of this project is the enzyme hydrolysis stage of a second generation bioethanol process, it was decided not to devote further effort into reducing the variability in the pretreatment stage at this time This is based on the fact that no metric could be identified that correlated with the hydrolysis yields achieved from a given batch of pretreated slurry. Variability will instead be accounted for by including suitable control hydrolysis tubes in all subsequent experiments and comparing results only within experiments on a single pre-treatment batch. The performance of such control tubes can be monitored over time to look for long term drift in performance.

The utilisation of the USD tubes to examine pretreatment variability, as shown here, is a good example of the commercial utility of having a validated small scale model of the STRs. Hydrolysing six separate pretreatments in triplicate would require eighteen STRs to be set up, meaning that what can easily be achieved in one week using the USD tubes would have taken around a month to achieve in ReBio's STRs.

4.3 Effect of varying solids content on enzyme hydrolysis efficiency

As noted in Section 1.3.4, many authors have reported that increasing the dry solids content of a hydrolysis treatment leads to reduced hydrolysis yields. It was decided to confirm whether this was also the case using DDGS as a feedstock as a search of the literature did not find any studies where this had been definitively investigated. Pretreatment was carried out as specified in Section 2.2 before enzymatic hydrolyses were set up at a range of solids loadings in USD tubes and 4L STRs according to Sections 2.4 and 2.3 respectively.

Figures 4.5 and 4.6 show the overall hydrolysis yield and the yield for glucose monomer respectively. In both cases there is a clear trend for the yield obtained to decrease as the dry solids content of the hydrolysis is increased. The decreases in yield observed as the solids loading of the hydrolysis increased over a range 15% were in the range of 15-20% of the value at the lowest solids loading. With the exception of the glucose monomer data from the USD tubes, regression analysis showed the trends to be statistically significant ($p < 0.05$). The yields seen in the STRs are somewhat higher than in the USD tubes with a difference of around 5% for the overall yield and 5-10% for the glucose monomer yield. Importantly however, the trends observed in the STRs and the USD tubes are very similar with the increase in solids content causing yields to decrease by the same amount in both systems.

The decrease seen in hydrolysis yield as the solids content of the process increases is linear. The fact that a linear trend with good correlation (coefficient > 0.88 in both USD tubes and STRs) was observed for the effect of solids content on hydrolysis yield is helpful when trying to obtain as much data as possible from a minimum number of experiments. Using the observed relationship it is possible to extrapolate experimental data obtained at one solids loading to predict the yields that would be achieved at a range of solids loadings. This is likely to be particularly useful in early screening experiments where a very broad range of conditions need to be investigated whilst using as little resource as possible.

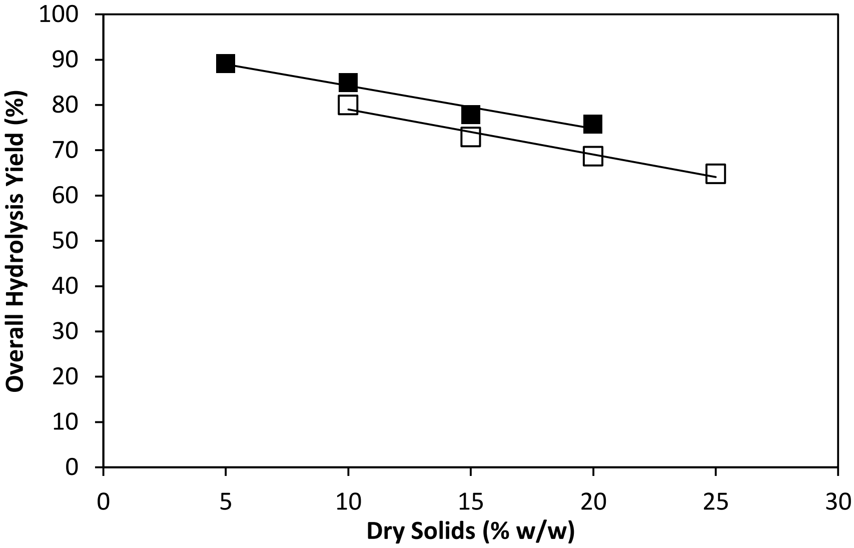

Figure 4.5. Effect of initial solids content during enzymatic hydrolysis on the overall sugar solubilisation achieved following 48 hours of enzymatic hydrolysis of pretreated DDGS in STRs and USD tubes. Enzyme hydrolysis was carried out at 50°C for 48 hours in either a shaking incubator set to 250 rpm or a 4L STR agitated at 200rpm. For USD tubes the mean for each set of replicates is shown (n=3). ■ =STR data; □ USD tube data. Solid lines obtained by linear regression ($R^2 > 0.98$). Experiments carried out as in Section 2.3 and 2.4.Sugar concentrations determined as described in Section 2.10 and data analysed as in Section 2.10.

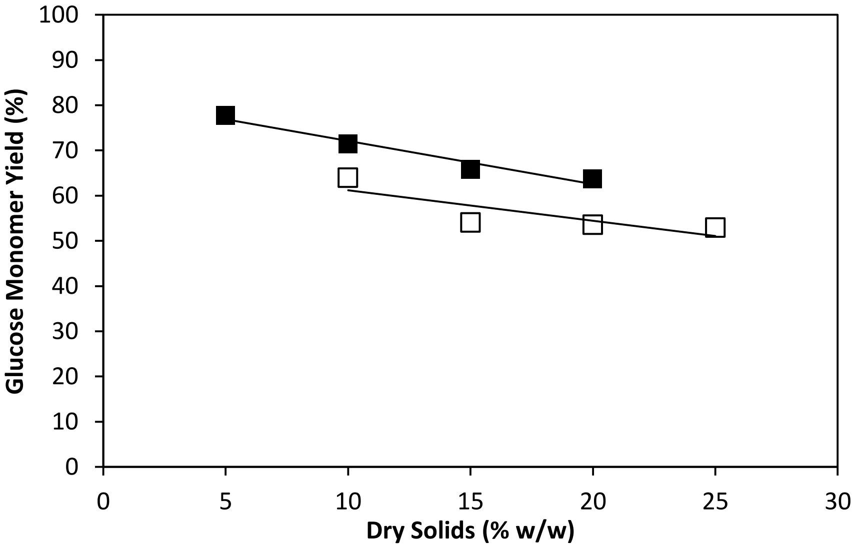

Figure 4.6. Effect of initial solids content of a hydrolysis on the yield of glucose monomer achieved following 48 hours of enzymatic hydrolysis of pretreated DDGS in STRs and USD tubes. Enzyme hydrolysis was carried out at 50°C for 48 hours in either a shaking incubator set to 250 rpm or a 4L STR agitated at 200rpm.. For USD tubes the mean for each set of replicates is shown (n=3). ■ =STR data; ☐ USD tube data. Solid lines obtained by linear regression (R^2 = 0.68 for USD tubes, 0.89 for STRs). Experiments carried out as in Section 2.3 and 2.4 Sugar concentrations determined as described in Section 2.10 and data analysed as in Section 2.10.

This data is in broad agreement with other published work. Using filter paper as a model substrate a decrease of 33% in the hydrolysis yield was seen as the solids content increased from 5 to 20%DS (w/w) (Kristensen et al., 2009). Jorgensen et al. (2007) working with pretreated wheat straw observed a decrease of 25% in the cellulose conversion as the solids content was increased from 5% (w/w) to 25% (w/w) (Jørgensen et al., 2007b). In data published using olive wood subjected to different pretreatments, the apparent solids effect was much lower. As solids content was increased from 2% (w/w) to 20% (w/w) the observed decrease in hydrolysis yield was as low at 11% when steam explosion pretreatment was utilised (Cara et al., 2007). This may in part be due to the fact that the hydrolysis yields seen in that work were generally lower than seen by Jorgensen et al. (2007b) and in this work. This view would be supported by the fact that when the olive wood was treated by liquid hot water the hydrolysis yields were generally higher and so was the decrease in yield as solids content increased.

It is not possible from the data seen here to draw any conclusions about what lies behind the decrease in hydrolysis yield that is observed as solids loading is increased. These results do however reinforce the message of Humbird and others that it is it is important to work at a solids concentration that is high enough to deliver commercially viable concentrations of sugar (Humbird et al., 2010). It is of little value to develop a process that delivers improved hydrolysis yields at low solids contents if those improvements are not maintained as solids content is increased.

Further experiments will therefore focus on delivering process improvements at solids concentrations capable of generating the nominal target of 40 g L^{-1} ethanol, a metric often cited in the literature (Section 1.4.1). In the case of the feedstock used in this work, containing approximately 40% by mass sugar, 40 g L^{-1} ethanol could be produced from the complete fermentation of the sugars found in 20% (w/w) solids content slurry. All of the pretreatments generated for the variability study had a post pre-treatment solids content of greater than 20% (w/w) (Table 4.2) suggesting that there should be no issue with the generation of slurries at a suitable solids content.

4.4 Comparison of USD tube and STR Hydrolyses

In order to compare the performance of USD tubes and STRs, hydrolysis reactions were performed using a single pretreatment batch at 10% and 20% solids (w/w). It is important that the results achieved in USD tubes should be indicative of the actual performance of a particular condition at larger scale rather than just the relative performance of a range of conditions. Consequently the results shown in Figures 4.7 and 4.8 are encouraging. These show that for both the overall and monomeric sugar yields there was generally little difference between the STR and USD tube data.

Looking first at the total sugar solubilisation at 10% (w/w) solids loading, shown in Figure 4.7, the final yields were 80% in the USD tubes and 79% in the STRs. Just over 40% of the available sugar was solubilised during the pretreatment step. Hydrolysis then progressed rapidly in the first four hours such that around 65% of the available sugar had been solubilised at this point. Thus more sugar is solubilised in the first four hours of hydrolysis then in the remaining 44 hours, fitting in with the general trend reported in the literature and discussed in Section 1.3.3. At both the 4 hour and 48 hour sample points the overall hydrolysis yield was not statistically different in the two different systems (t-test, $p>0.05$). In between these points however the yields in the USD tubes were slightly higher than in the STR.

Figure 4.8 shows the hydrolysis experiments with a solids loading of 20% (w/w) and the general trend was similar, with a rapid period of sugar solubilisation in the first four hours followed by solubilisation continuing at a decreasing rate for the remainder of the reaction. Indeed in both STRs and USD tubes there was essentially no solubilisation of sugars during the final 24 hours of hydrolysis. As would be expected from the results in Figure 4.5, the overall hydrolysis yield was lower at the higher solids level by slightly over 10%. Unlike at the 10% (w/w) solids level there was a clear difference between the overall yields in USD tubes and STRs at 48 hours and through most of the hydrolysis period with the yield being around 7% higher in the USD tubes. The difference between the two systems at 48 hours was statistically significant (t-test, $p<0.05$), however it should be noted that in Figure 4.5 the yield in STRs under similar operational set up showed the opposite trend with the overall yield in USD tubes being lower than in STRs.

Figure 4.7 shows that the amount of monomeric sugar present at 10% (w/w) solids increases rapidly from less than 2 g L^{-1} after only the pretreatment stage to around 11.5 g L^{-1} after four hours of hydrolysis, representing around 25% of the sugar available. The monomer yield then increases through to the end of the hydrolysis, but at a decreasing rate. Comparing the two scales, there was little difference between USD tubes and STRs. The yield in USD tubes was slightly higher towards the end of the hydrolysis, but at 48 hours the yields were 61% in the STRs to 63% in USD tubes, within the margins of error expected from this sort of process and not statistically significant (t-test, $p>0.05$). Comparing

Figures 4.7 and 4.8, after 4 hours of hydrolysis the difference between the two solids levels was small at both scales. However by 8 hours the monomer yield at 20% (w/w) solids loading was around 8% lower and this difference was maintained for the remainder of the hydrolysis. Again there was no significant difference between the STR and USD tube data. The trends described hold true when the concentrations of individual monomers are examined although as would be expected from the data in Figure 4.1 the concentrations of glucose were higher than the other sugars (data not shown).

As expected from the results in Figures 4.5 and 4.6 the solids content of a hydrolysis has a significant effect on the maximum sugar solubilisation achieved. However for the most part the rates of sugar solubilisation and monomerisation seen in Figures 4.7 and 4.8 show similar trends regardless of the solids level and whether the hydrolysis was carried out in USD tubes or STRs. The differences that were observed between the two solids levels were apparent by 8 hours into the hydrolysis and did not significantly alter over the remaining 40 hours of the experiment. Thus the total solids content of a hydrolysis seems to have a clear negative effect on the level sugar solubilised but the kinetics that lead up to that final yield have the same trends regardless of the solids level. It is therefore not an unreasonable expectation that the same issues will affect the kinetics at all solids level and that improvements to the process kinetics would be equally applicable to all solids levels.

The rapid initial period of solubilisation followed by a much longer slower phase, with around 80% of the final soluble sugar total being present after 4 hours of hydrolysis, is in agreement with the literature (Section1.3.3). Kim et al, working with wet cake, a similar feedstock to DDGS, saw 80-90% of the total solubilisation occurring in the first six hours of hydrolysis regardless of the pretreatment method (Kim et al., 2010b). Earlier work had found a similar profile to the hydrolysis of DDGS although in this case the proportion of hydrolysis occurring during the initial period varied somewhat with the type of pretreatment, from around 70% to over 90% of the final yield (Kim et al., 2008a). In neither of these papers was this aspect a focus of investigation.

If the very rapid initial rate of hydrolysis could be maintained for longer, the enzymatic hydrolysis stage of the process could be much reduced in length, improving process economics. It may also be hoped that higher overall yields may be achieved, especially at higher solids levels. Thus there is logic in focusing on what is limiting the rate of hydrolysis after the initial period of rapid solubilisation. In discussing the fact that the vast majority of sugar is solubilised in the first few hours of hydrolysis it is important to note that as was discussed in Section 1.4 most fermentative organisms are only capable of utilising monomeric sugars and that therefore the kinetics of sugar monomerisation are as important as those of solubilisation.

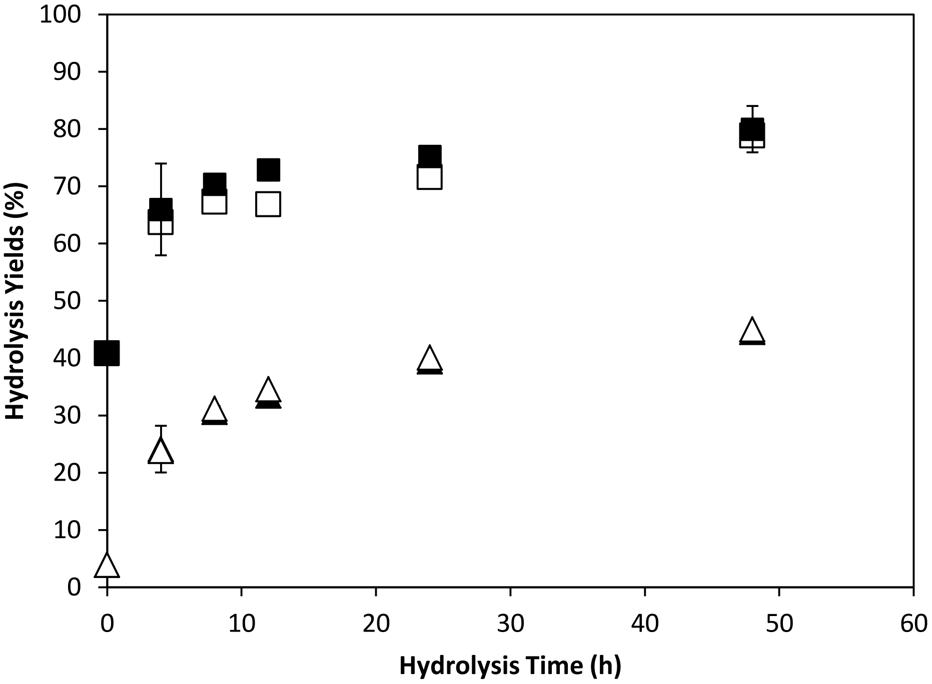

Figure 4.7. Overall hydrolysis yield and monomeric sugar yield from the enzymatic hydrolysis of pretreated DDGS in USD tubes and STRs at a solids loading of 10% (w/w). Enzyme hydrolysis was carried out at 50°C for 48 hours in either a shaking incubator set to 250 rpm or a 4L STR agitated at 200rpm. Data is the average of n=3 points for USD tubes and n=2 points for STRs. ■ = STR overall yield; ☐ USD tube overall yield; ▲ STR monomeric sugar yield; △ USD tube monomeric sugar yield. Experiments carried out as described in Section 2.3 and 2.4 and sugar concentrations determined as in Section 2.10. Data analysed as described in Section 2.11. Error bars show one standard deviation around the mean for USD tubes.

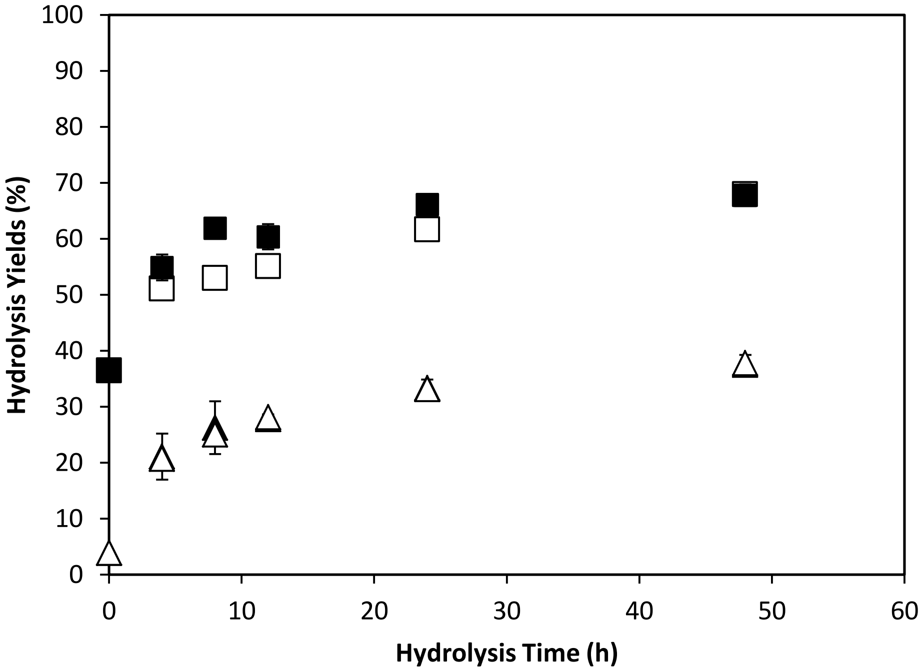

Figure 4.8. Overall hydrolysis yield and monomeric sugar yield from the hydrolysis of pretreated DDGS in USD tubes and STRs at a solids loading of 20% (w/w). Enzyme hydrolysis was carried out at 50°C for 48 hours in either a shaking incubator set to 250 rpm or a 4L STR agitated at 200rpm. Data is the average of n=3 points for USD tubes and n=2 points for STRs. ■ = STR overall yield; ☐ USD tube overall yield; ▲ STR monomeric sugar yield; △ USD tube monomeric sugar yield. Experiments carried out as described in Section 2.3 and 2.4 and sugar concentrations determined as in Section 2.10. Data analysed as described in Section 2.11. Error bars show one standard deviation around the mean for USD tubes.

4.5 Investigation of Enzyme Degradation

One possible explanation for the decrease in the rate of solubilisation with time seen in Figures 4.7 and 4.8 is that the enzymes are being damaged or degraded during the first few hours of hydrolysis, limiting performance later on in the process. This would fit with the fact that the phenomenon seems to be reported with virtually all combinations of feedstock and pretreatment (Section 1.3.4). According to this hypothesis the reduction in rate is due to a reduction in the remaining level of enzyme activity through denaturation of the protein. The idea of denaturation of enzymes being a factor limiting the rates of hydrolysis is not new, with several references to it being found in the literature (Jørgensen et al., 2007a, Kaar and Holtzapple, 1998, Ximenes et al., 2011).

A simple way to investigate enzyme denaturation is to split the addition of enzymes such that a portion is retained to be added after the initial rapid hydrolysis period had passed. Hydrolyses were therefore carried out with the enzyme dose being split into two or more additions at different times as described in Section 2.4.2.

In Figure 4.9 it is clear that splitting the enzyme addition has had very little effect on the progress of the hydrolysis. As well as no improvement from splitting the enzyme addition, neither is there a large decrease in yield caused by having only a portion of the standard enzyme dose added at the beginning of the hydrolysis. There is a slight decrease in overall yield observable at 8 hours caused by adding either two thirds or half of the normal dose of enzyme at the beginning of hydrolysis. Following the addition of the remaining enzyme after 8 hours of hydrolysis the yields recover and by the time of the next data point are identical to those from USD tubes where the entire enzyme dose was added at the start of hydrolysis. The fact that reducing the initial enzyme dose by half has such a small effect on the progress of sugar solubilisation in the first 8 hours of hydrolysis suggests that there is likely to be an excess of enzyme present during the initial period of hydrolysis. At the end of the experiment there was no statistically significant difference detected between any of the conditions tested in terms of the overall hydrolysis yield achieved (ANOVA, $p > 0.05$).

Splitting the enzyme addition up into several doses had somewhat more of an effect on the glucose yields, as seen in Figure 4.10. Here there is a general trend for the tubes with split enzyme addition to have lower glucose yields early on in the process. By 24 hours however the yields recover to approximately the same levels as the control. The fact that the lower yields seen are attributable to the lower levels of enzyme present in the split addition USD tubes is highlighted by the data from the two sets of USD tubes with enzyme added at 0 hours and 8 hours. Here there were two different enzyme split ratios used, a 50:50 split and a 67:33 split. The USD tubes with 50% of the enzyme added at the start show a relative reduction in yield of 30% at 8 hours, whilst the USD tubes with 67% of the enzyme added at the start show a reduction of only 16%. However, once the second dose of enzyme is added, yields rapidly begin to recover, as can be seen from the data at 12 hours and 24 hours. By the end of the hydrolysis yield, there was again no statistically significant difference observed (ANOVA, $p > 0.05$). Similar patterns were seen with the other monomeric sugars measured (data not shown).

The data in Figures 4.9 and 4.10 thus provides no evidence of any deactivation of enzymes during the initial period of hydrolysis. In terms of the overall hydrolysis yield it appears there is an excess of enzyme activity present as removing a portion of the enzyme during the early part of the hydrolysis had very little effect on levels of solubilisation. Although removing a portion of the enzyme load certainly had a negative effect on the glucose yield in the early stage of hydrolysis once the remaining dose of enzymes was added in, yields recovered to the same levels as in the control tubes. In no case was any benefit to splitting the enzyme dose observed.

An alternative approach to overcoming potential issues with enzyme degradation is to investigate the addition of a greater concentration of enzyme such that there would be sufficient present to overcome any degradation occurring. This approach was not used primarily because it would also be expected to be effective if the limitation to hydrolysis were to be caused by product inhibition, complicating interpretation of results. Nevertheless as a proof of principle USD tubes were set up as before with ten times the normal enzyme loading. Figure 4.11 shows the concentrations of sugar of following 24 hours of hydrolysis with either the standard enzyme dose or 10 times the normal enzyme dose. As would be expected substantially more sugar was solubilised with the high enzyme loading, however the concentration reached only represents 80% of the sugar available in the feedstock. The proportion of the different sugars that was monomerised was also increased with the higher enzyme loading. The fact that an enzyme loading far in excess of what could be commercially considered increased the overall yield only from 63% to 81% shows that this is not a viable approach to improving enzyme hydrolysis yields industrially.

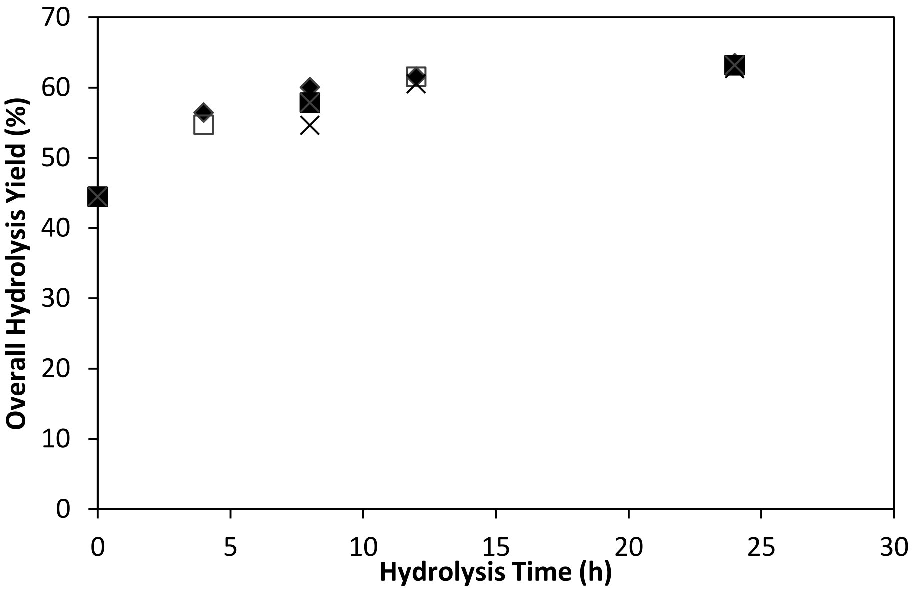

Figure 4.9. Effect of splitting enzyme addition into two doses on overall hydrolysis yields achieved during the enzymatic hydrolysis of pretreated DDGS. Enzyme hydrolysis was carried out at 50°C for 48 hours in either a shaking incubator set to 250 rpm or a 4L STR agitated at 200rpm . Total enzyme loading was the same for all conditions. Data is the mean of n=2 points. ♦= control; ☐ 0h, 4h split; × 0h, 8h (50:50 split); ■ 0h, 8h (67:33 split). Experiments were performed as described in Section 2.4.2. Sugars determined as described in Section 2.10 and data analysed as described in Section 2.11.

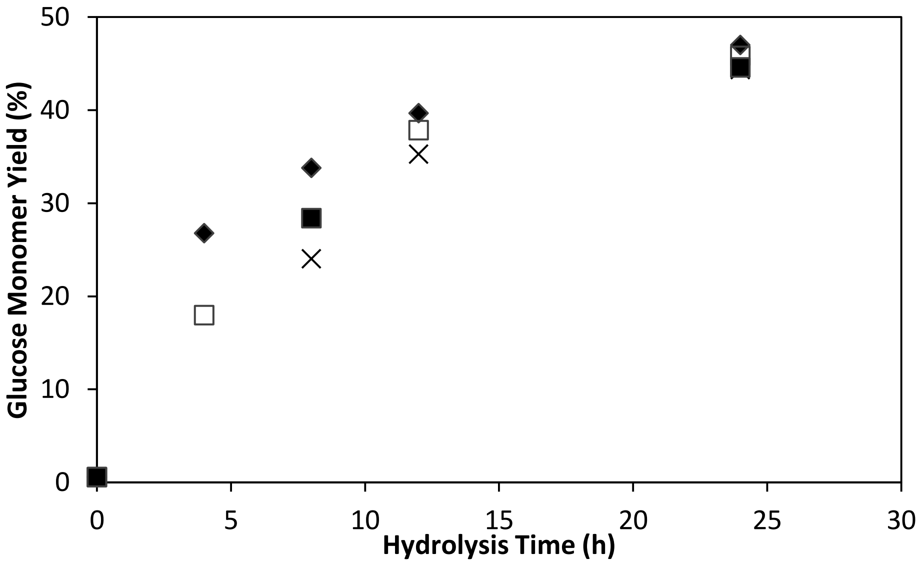

Figure 4.10. Effect of splitting enzyme addition into two doses on glucose monomer yields achieved during the enzyme hydrolysis of pretreated DDGS. Enzyme hydrolysis was carried out at 50°C for 48 hours in either a shaking incubator set to 250 rpm or a 4L STR agitated at 200rpm. Total enzyme loading was the same for all conditions. Data is the mean of n=2 points. ♦ = control; ☐ 0h, 4h split; × 0h, 8h (50:50 split); ■ 0h, 8h (67:33 split). Experiments were performed as described in Section 2.4.2. Sugars determined as described in Section 2.10 and data analysed as described in Section 2.11.

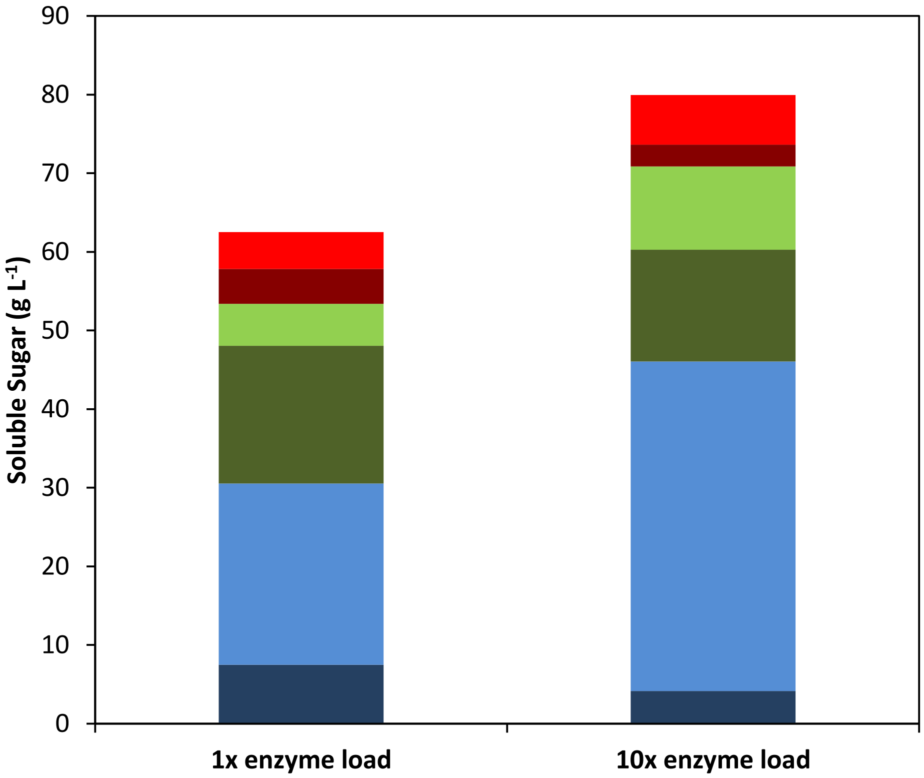

Figure 4.11. Sugars solubilised from the enzymatic hydrolysis of pretreated DDGS at 20% (w/w) DS with either standard or '10x standard' enzyme loading. Enzyme hydrolysis was carried out at 50°C for 48 hours in a shaking incubator set to 250 rpm. Data shown is the mean of n=2 points. 🟦: Glucose oligomers; 🟦: glucose monomer; 🟩: X+G+M+F oligomers; 🟩: X+G+M+F monomers; 🟥: arabinose oligomers; 🟥: arabinose monomer. Experiments carried out as in section 2.4. Sugars determined as described in Section 2.10 and data analysed as in Section 2.11.

4.6 Inhibition of enzymes by soluble compounds

4.6.1 Effect of removing inhibitors on enzyme hydrolysis

Another potential cause for the decrease in solubilisation rate occurring after the first few hours is that the enzymes are being inhibited by compounds present in the hydrolysate (Horváth et al., 2004, Palmqvist et al., 1996). If this was the case, stopping the hydrolysis process, washing the solids and then restarting the hydrolysis with fresh enzyme should improve both the rates of solubilisation and the titres achieved. In order to test this hypothesis hydrolyses were interrupted and the solids washed on up to three occasions as outlined in Section 2.4.3. The procedure for washing was the same on each occasion, but the quantity of enzymes added to restart the hydrolysis was varied according to the number of washes. This was done in order to ensure that by the end of the hydrolysis the same quantity of enzymes had been added to each tube, albeit that some enzyme would be lost during the washing stages. Any enzyme carry over is likely to be restricted to that which was adsorbed to the solids but it was not possible to quantify this.

The results of this approach on overall hydrolysis yields are shown in Figure 4.12. Interrupting the hydrolysis and washing the solids clearly has a significant effect on the overall yield. This effect also increases with the number of washing steps. In the case of the tubes with three washing steps all of the available sugar was solubilised, increasing the overall yield by 40% relative to the controls. With each interruption step added to the process there was a clear increase in the proportion of the potentially available sugar which was solubilised. Even with just one washing step there was a significant increase in the overall yield at the 24 hour time point when compared to the control (t-test, $p < 0.05$).

The benefit in yield was not confined to the final time point with, for example, a statistically significant improvement in the overall yield at the 4 hour time point of the three interruptions USD tubes, even though they had only been interrupted and washed once at this point (t-test, $p < 0.05$). Interestingly at the time points when the interrupted sets of USD tubes were washed for the first time, their yields were not decreased relative to the controls despite having a lower enzyme loading. This agrees with the suggestion from Figure 4.10 that there is an excess of enzyme activity in terms of the overall solubilisation of sugars.

In addition to the increase in final yield achieved, it can also be seen from Figure 4.12 that the rate of hydrolysis is also improved by the washing of the solids, although the rate of sugar solubilisation does drop as the hydrolysis progresses regardless of whether the solids are washed during the process. For example when the solids were washed three times the rate of sugar solubilisation between 4 and 8 hours was 54% of the rate between 2 and 4 hours whereas in the control tube the rate was only 41% of the 2 to 4 hour rate. This is perhaps to be expected as the lignocellulose in a feedstock like DDGS is unlikely to be homogenous. As discussed in Section 1.2.1 there are a variety of side groups and branching arrangements found in feedstocks, some of which are more rapidly digested by enzymes than others. It could be reasonably expected that the more readily digestible portion of the feedstock will be degraded early in the enzyme hydrolysis process, leading to a natural decline in the rate of sugar solubilisation as the proportion of recalcitrant feedstock increases. The higher rates of hydrolysis and the smaller decline in those rates seen in the interrupted hydrolysis experiments show however that the change in feedstock composition is not the sole cause of the decline in sugar solubilisation.

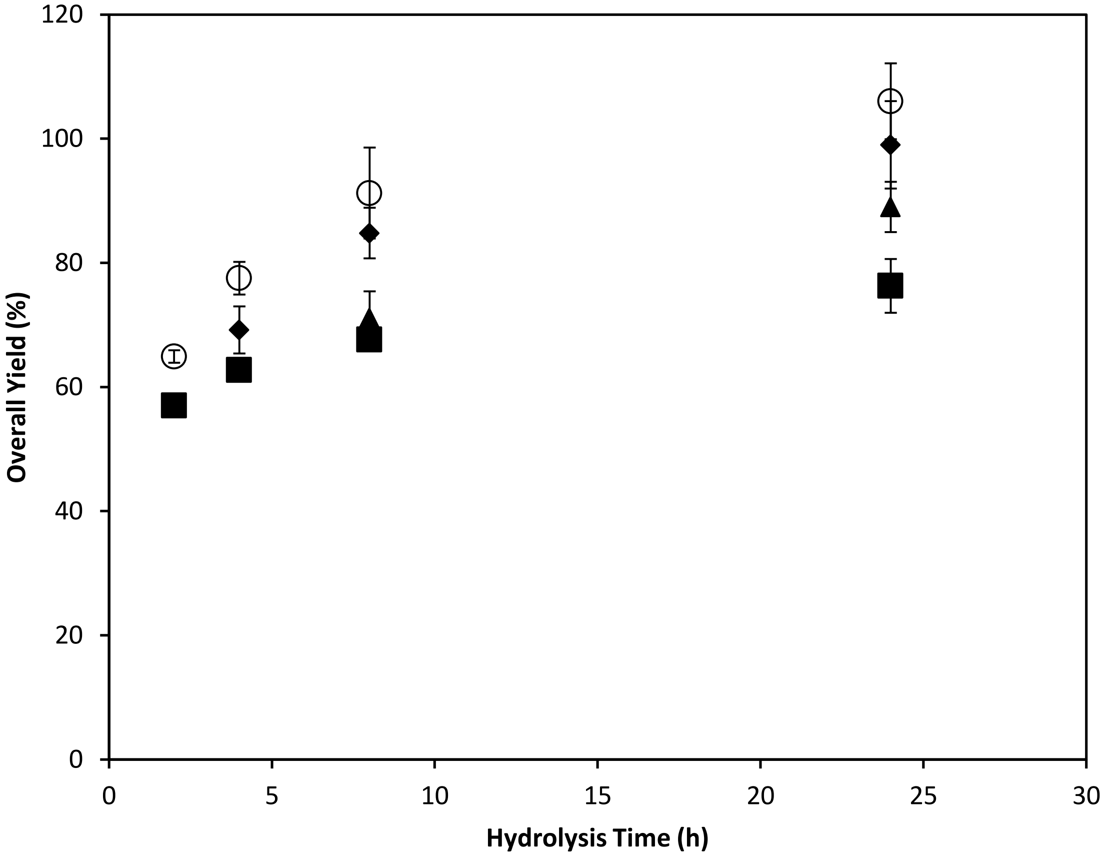

Figure 4.12. Effect of interrupting hydrolysis and washing residual solids on overall hydrolysis yields. Data is the mean of n=3 samples for the interrupted samples and n=2 for the control. Enzyme hydrolysis was carried out at with pretreated DDGS at an initial solids loading of 20% (w/w) at 50°C for 24 hours in a shaking incubator set to 250 rpm. Total enzyme addition was the same for all conditions. ■ =control; ▲1 interruption & wash; ♦ 2 interruptions & washes; ○3 interruptions & washes. Experiments carried out as described in Section 2.4.3. Sugar concentrations determined as described in Section 2.10 and data analysed as in Section 2.11. Error bars show one standard deviation around the mean.

Perhaps surprisingly given the large effect seen on the overall yield, interrupting the hydrolysis did not lead to any increases in the yields of monomeric sugars at the 24 hour time point as seen in Figure 4.13. Upon closer examination however, it does seem as though there may have been some benefit attributable to washing the residual solids at intermediate time points in the experiment. For example at the 8 hour time point both the 2 wash and 3 wash USD tubes had significantly higher glucose and other monomer yields than the control USD tubes (t-test, $p < 0.05$). By the end of the experiment however there was no significant difference in those yields (t-test, $p > 0.05$).

One possibility is simply that there are different factors limiting the two main stages of hydrolysis: hydrolysing the insoluble solids to release soluble oligomers and further hydrolysing these oligomers to monomers. This would not however explain the increases in monomer yield seen during the early stages of the experiment. Another is that the enzymes responsible for releasing monomeric sugars, primarily β-glucosidases, have a lower K_i for the inhibitors in question meaning that the interruption steps were not enough to prevent the inhibitors reaching an effective level. The fact that yields at 8 hours were improved in the USD tubes containing washed solids would argue against this hypothesis, showing that interrupting the hydrolysis in such a way can improve glucose monomerisation in certain circumstances. The explanation that seems most appropriate at this time is that the lack of improvement in the monomer yields is an artefact of the methods used. When the interruptions occurred any oligomers present would have been removed from the process and thus not become subject to further hydrolysis into monomers because they were not exposed to the enzyme cocktail for long enough. Although monomerisation proceeded at a slower rate in the control tubes, ultimately the longer exposure of all of the substrate to the enzymes allowed the same concentration of glucose monomer to be achieved even if less sugar was solubilised overall. Further work could help to determine which, if any, of these hypotheses is correct.

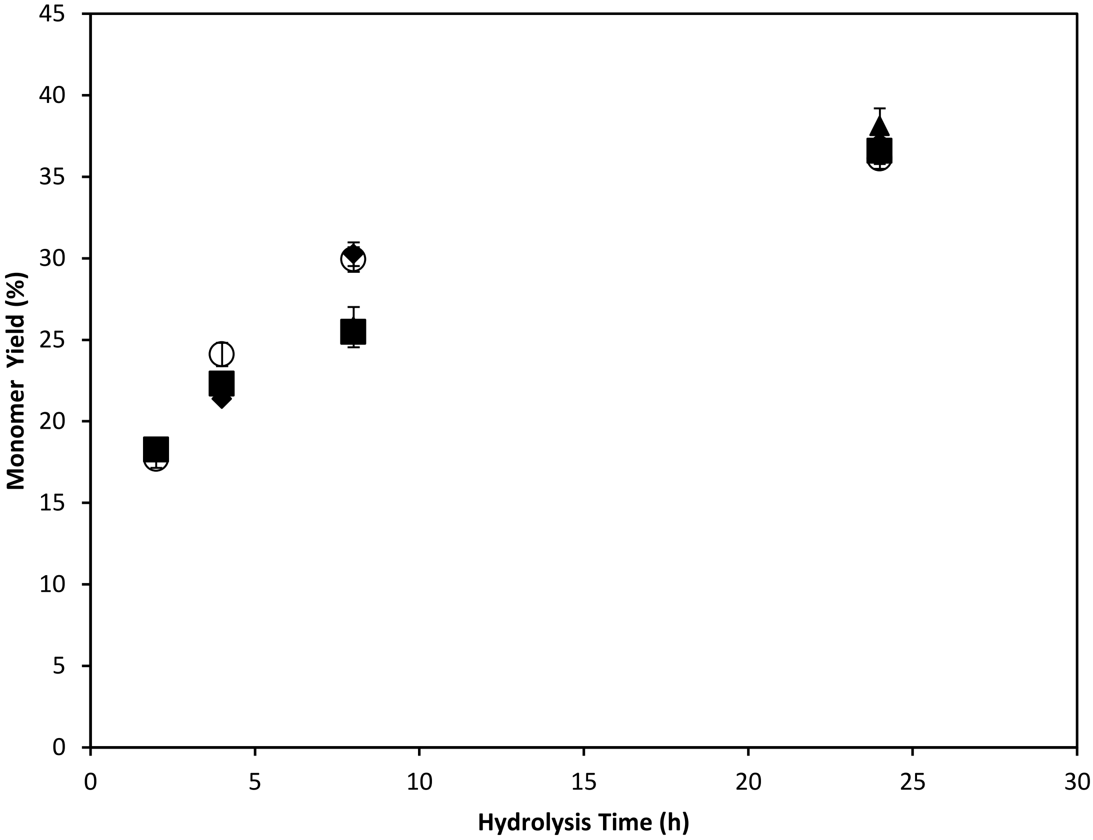

Figure 4.13. Effect of interrupting hydrolysis and washing residual solids on the monomeric sugar yield. Data is the mean of n=3 samples for the interrupted samples and n=2 for the control. Enzyme hydrolysis was carried out at with pretreated DDGS at an initial solids loading of 20% (w/w) at 50°C for 24 hours in a shaking incubator set to 250 rpm. Total enzyme addition was the same for all conditions. ■ =control; ▲1 interruption & wash; ♦ 2 interruptions & washes; ○3 interruptions & washes. Experiments carried out as described in Section 2.4.3. Sugar concentrations determined as described in Section 2.10 and data analysed as in Section 2.11. Error bars show one standard deviation around the mean.

The data in Figure 4.11 clearly demonstrates that the solubilisation of the sugars in DDGS is limited by soluble inhibitors that are present in the hydrolysate. Removing these inhibitors by periodically interrupting the hydrolysis and washing the remaining solids allows for significant increase to both the overall hydrolysis yield and the rate at which this yield increases. On this basis there seems to be considerable scope for further work to establish which individual compounds within the hydrolysate are responsible for the inhibition and there are several potential candidates in the literature. These include glucose and cellobiose, monomeric pentose sugars, xylo oligomers, soluble phenolic compounds and insoluble lignin (Xiao et al., 2004, Qing et al., 2010, Kim et al., 2011, Berlin et al., 2006). It remains to be explained however why the same improvement in performance was less apparent in terms of the monomer yields.

In Section 4.2 it was also noted that the enzymes used in this study are substantially more effective on the cellulose portion of the feedstock than on the hemicellulose portion both in terms of solubilising the feedstock to produce oligomeric sugar and when further degrading those oligomers to monomers. There are two likely factors that could be the cause of this discrepancy in performance. Firstly, the molecular structure of hemicellulose is more complex and varied and will therefore require more enzyme activities in order to digest it. This will in turn make enhancing the catalytic rate and minimising sensitivity to inhibitors more complicated as there will be more targets to focus on. The data from the interrupted hydrolysis experiments provide support to this theory as the removal of inhibitors by washing led to full solubilisation of the hemicellulose. The second linked factor is that most potential lignocellulosic ethanol organisms perform far better fermenting glucose than other sugars and there is therefore less commercial drive for enzyme manufacturers to improve the performance of their enzymes in relation to these other sugars. Nevertheless the evolution of enzyme cocktails has been rapid with new products emerging every 18-24 months (Lane, 2012). The enzymes used in this project, whist representing the best commercially available products at the beginning of this project, have been superseded by newer enzyme products that are claimed to have much improved performance in terms of all sugars.

4.6.2 Effect of glucose on enzyme hydrolysis

At the end of a typical DDGS enzyme hydrolysis process, the largest single component present in the soluble phase of the hydrolysate is monomeric glucose. In screening for potential inhibitors present in the hydrolysate it is therefore to be regarded as a potential candidate.

It has been known for many years that glucose has the potential to be a significant inhibitor of both β-glucosidase and endo and exo-glucosidases (Holtzapple et al., 1990). More recent studies however found that glucose was only of limited influence as an inhibitor of commercial cellulase preparations, and mainly acted by inhibiting cellulases rather than β-glucosidase (Kim et al., 2011). This apparent discrepancy can be explained by the fact that extensive research carried out on lignocellulose hydrolysis over the 20 years separating the two papers has produced enzymes that are far less susceptible to end product inhibition. Nevertheless a comparatively recent study found that glucose was a more potent inhibitor of both cellulases and β-glucosidase than xylose, galactose or mannose (Xiao et al., 2004). The authors reported that 20 g L^{-1} glucose caused a 30% loss of activity for cellulases and a 15% loss of activity for β-glucosidase.

A wide range of inhibitory concentrations for glucose can be found in the literature however two factors limit their comparability to the data reported here. Firstly it has been noted that the source of a particular enzyme can have a substantial effect on its susceptibility to inhibitors with, for example, β-glucosidases derived from *A. niger* and *T. reesei* showing markedly different sensitivities to glucose (Holtzapple et al., 1990). Secondly, the degree of inhibition by glucose is often reported to be related to the ratio of enzyme to inhibitor. The precise origin, quantity of enzyme and specific activity levels of the enzyme cocktail used in this work are proprietary commercial information that is not disclosed by the manufacturer and so precise ratios of enzyme to inhibitor cannot be worked out using a metric that provides comparability to published data.

Consequently an experiment was performed to examine the effect on hydrolysis yields of a range of glucose concentrations. The results of this experiment are shown in Figure 4.14. The total concentration of sugar contained in the hydrolysate increased significantly due to the exogenous sugar added, and so corrected sugar concentrations were calculated which excluded the added glucose. It is clear that additional glucose had very little effect on overall yields. Analysis confirmed that there was no significant difference in the total amount of sugar that was actually solubilised from the feedstock regardless of the amount of exogenous glucose added (ANOVA, $p>0.1$).

Although the addition of glucose did not appear to affect the total solubilisation of sugars it did have some impact on the hydrolysis, specifically on the glucose yield. In Figure 4.14 it can be seen that there is a tendency for the concentration of monomeric glucose produced during the hydrolysis to decrease as the concentration of exogenous glucose increased. There is a corresponding increase in the concentration of glucose oligomers. Regression analysis confirmed that this was a statistically significant trend ($R^2 = 0.83$, $p < 0.05$).

Thus glucose does have an inhibitory effect on the monomerisation of glucose from the feedstock. It seems that this effect is not due to product inhibition of the β-glucosidase as no cellobiose was detected even at the highest levels of glucose addition. This suggests instead that the inhibitory effect of glucose is mainly on the endo and exoglucanases, and possibly more specifically the exoglucanases since these enzymes release cellobiose as a product. This highlights the complicated nature of the enzymatic hydrolysis of lignocellulose. The data clearly shows that exogenous glucose has no effect on the solubilisation of sugars from the insoluble feedstock present. It does have a negative effect on the monomerisation of glucose, but not on the final enzyme in this process β-glucosidase. It must therefore be assumed that its inhibitory effect occurs at some point in between, presumably on the stages where the chain length of soluble oligomers is being reduced prior to the final reduction to glucose. This is in agreement with other recent studies on potential inhibitors of enzyme hydrolysis processes (Kim et al., 2011).

Whilst effects in terms of glucose monomerisation have been seen in response to the addition of exogenous glucose into the hydrolysis liquid they are relatively small in relation to the concentrations of sugar added; the addition of 22 g L^{-1} glucose led to a 10% decrease in the level of glucose monomer produced. Additionally the pattern of inhibition seen in Figure 4.14 is markedly different from that in

Figures 4.12 and 4.13 suggesting that glucose was not a primary factor in the inhibition which was relieved by washing the residual DDGS solids.

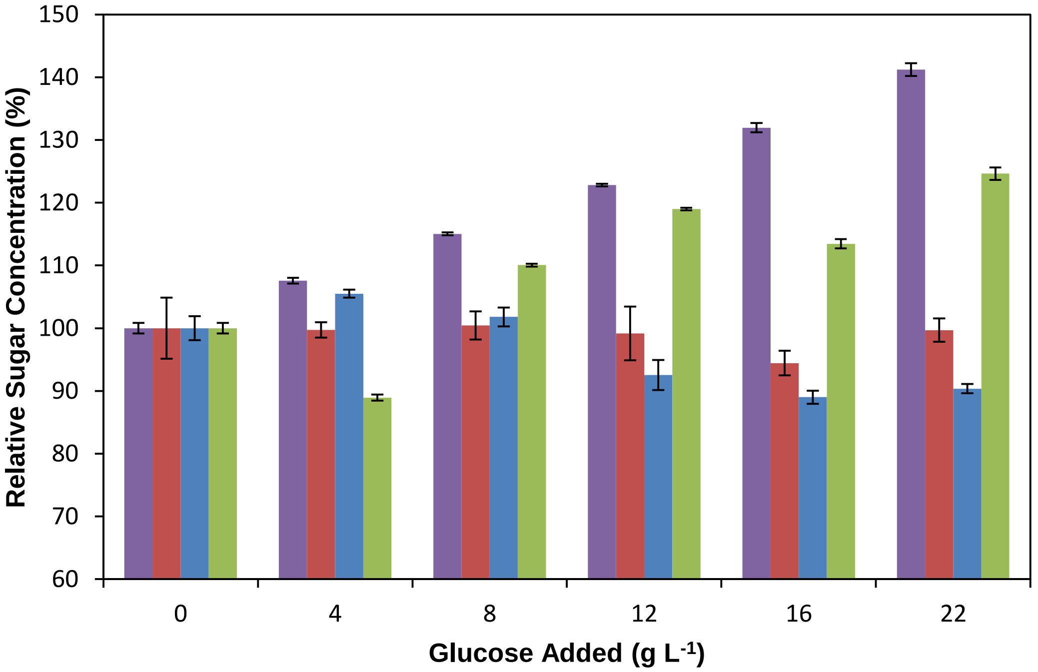

Figure 4.14. Effect of addition of exogenous glucose on the hydrolysis of pretreated DDGS that had been washed to remove inhibitors formed during pretreatment. Enzyme hydrolysis was carried out at with pretreated DDGS at an initial solids loading of 20% (w/w) at 50°C for 24 hours in a shaking incubator set to 250 rpm. Total enzyme addition was the same for all conditions. Data is the mean of n=3 points and each concentration was normalised against data from hydrolyses with no additional glucose. 🟪: Non adjusted total sugar concentration; 🟥: Total sugar concentration adjusted for additional glucose; 🟦: Adjusted glucose monomer concentration; 🟩: Glucose oligomer concentration. Error bars show ± 1 standard deviation. Experiments performed as described in Section 2.5. Sugar concentrations determined as described in Section 2.10 and data analysed as in Section 2.11.

Effect of USD tube angle on enzyme hydrolysis in USD tubes

From Section 3.5 it was clear that when the USD tubes were held at 90° on an orbital shaker, i.e. vertically, solids were not evenly dispersed throughout the USD tubes, rather the solids were prone to some settling at the base of the tube. Holding the tubes at either 45° to the vertical or especially at 0° (horizontal) was found to substantially improve the degree of dispersion of the solids in the system. Experiments were therefore set up to investigate whether the angle the USD tubes were held at in the shaker had any effect on the kinetics of enzyme hydrolysis.

The concentrations of all sugars and glucose specifically are shown for three USD tube angles in Figures 4.15 and 4.16 respectively. From both figures it is apparent that altering the angle at which the USD tubes are held relative to the shaker platform does not have a substantial effect on either the solubilisation of sugars during the hydrolysis or the digestion of oligomeric sugar into monomeric. This is the case even early in the hydrolysis period when it may have been expected that the additional fluid flow caused by altering the angle the USD tubes were held at would have had the greatest effect due to the initially high viscosity of the hydrolysate. Figure 4.17 shows a more detailed breakdown of the sugars present at the end of the experiment following 24 hours of hydrolysis.

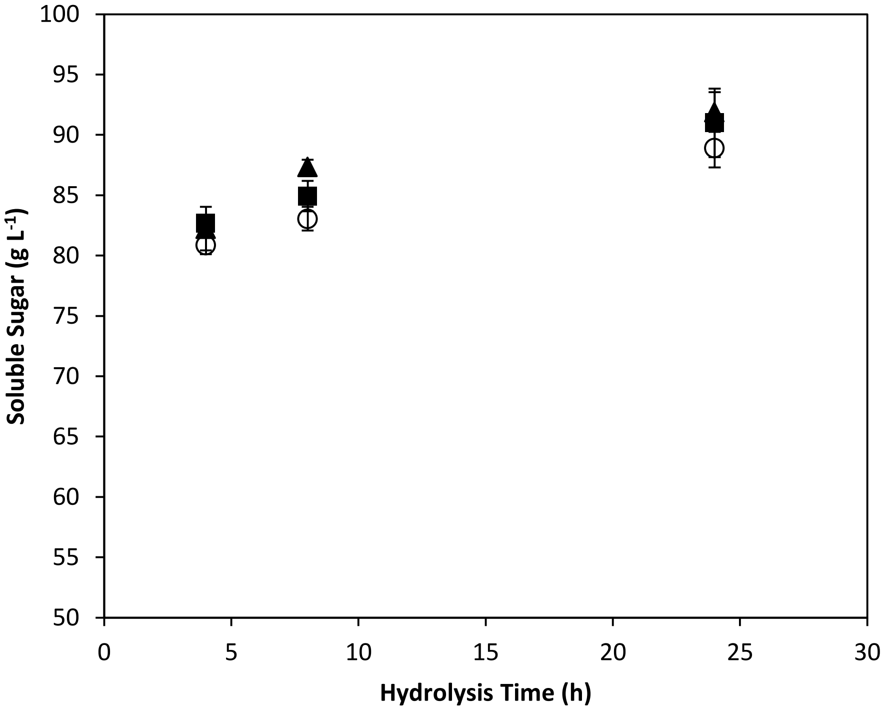

Figure 4.15. Solubilisation of sugars from pretreated DDGS during enzymatic hydrolysis in USD tubes held at various angles relative to the shaking platform. Enzyme hydrolysis was carried out at with pretreated DDGS at an initial solids loading of 20% (w/w) at 50°C for 24 hours in a shaking incubator set to 250 rpm. Data is the average of n=3 data points. ■: 90° (vertical); ▲: 45°; ○: 0° (horizontal).Error bars show one standard deviation around mean. Experiments carried out as in Section 2.4.4. Sugars determined as described in Section 2.10 and data analysed as in Section 2.11.

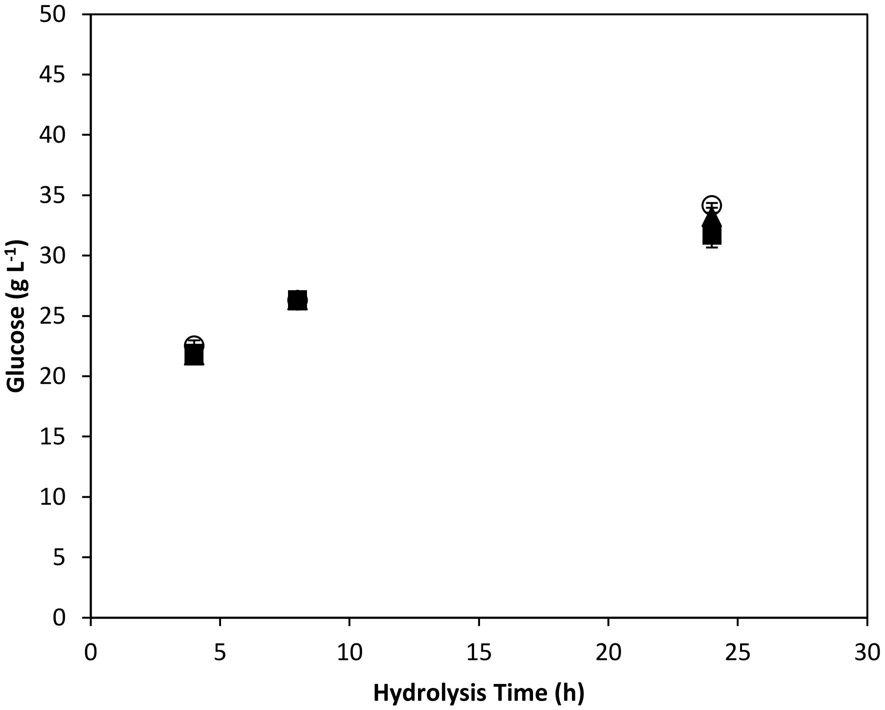

Figure 4.16. Solubilisation of glucose from pretreated DDGS during enzymatic hydrolysis in USD tubes held at various angles relative to the shaking platform. Enzyme hydrolysis was carried out at with pretreated DDGS at an initial solids loading of 20% (w/w) at 50°C for 24 hours in a shaking incubator set to 250 rpm.. Data is the average of n=3 data points. ■: 90° (vertical); ▲: 45°; ○: 0° (horizontal). Error bars show one standard deviation around mean. Experiments carried out as in Section 2.4.4. Sugars determined as described in Section 2.10 and data analysed as in Section 2.11.

From this data it is again apparent that the shaking angle of the USD tubes did not substantially affect the composition of the released sugars. There was only a small difference in the concentration of sugar solubilised, ranging from 89 g L^{-1} to 92 g L^{-1}. Paired t-tests confirmed that the differences seen in the total levels of sugar solubilised in the control and angled tubes were not statistically significant ($p > 0.1$). From Figure 4.17 it does appear however that the proportion of sugar that is present in a monomeric form was slightly higher in the tubes held at an angle. For this data paired t-tests revealed that the concentrations of glucose and of all monomeric sugars taken together were increased to a statistically significant degree ($p < 0.05$) when the USD tubes were held at angle of 90° to the shaker platform rather than at 0°. These differences, although statistically significant, are not large. The concentration of glucose was 31.7 g L^{-1} in the control tubes and 34.1 g L^{-1} in the 90° tubes. Likewise the total concentration of monomers only increased from 42.9 g L^{-1} to 46.0 g L^{-1}.

Interpreting the observation that shaking angle has no significant effect on overall hydrolysis yield in light of published work suggests that there is adequate solid-liquid dispersion when the tubes are held at 0°. In line with the earlier observations by other authors, discussed in Section 1.3.3, increased fluid flow does not lead to improved performance of the enzyme hydrolysis process once a critical level is reached. The remainder of the work in this chapter was carried out with the USD tubes held at 0° on the shaker platform as loading the USD tubes at 90° on the shaker platform takes up more space and therefore reduced the number of experiments that can be performed in parallel. Based on the data here it appears that this decision may have had a small negative impact on the yields of monomeric sugars seen in the other experiments, but not on the total level of sugar solubilised. The level of difference in the monomeric sugar concentrations is small and additionally it would be expected that the difference seen would likely to have applied to all conditions in the other experiments equally. It is therefore unlikely that the interpretation of the results from the other sections would have been substantially altered had the experiments been run with the USD tubes held at 90° to the shaker platform rather than at 0°.

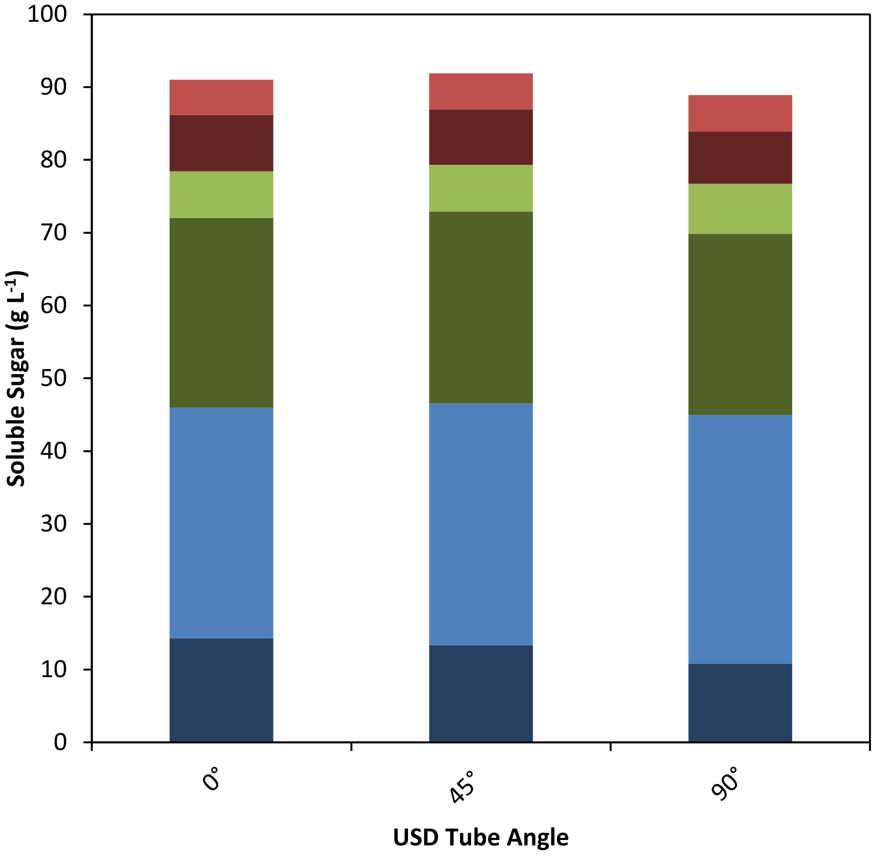

Figure 4.17. Solubilisation of sugars from pretreated DDGS during enzymatic hydrolysis in USD tubes held at various angles relative to the shaking platform. Enzyme hydrolysis was carried out at with pretreated DDGS at an initial solids loading of 20% (w/w) at 50°C for 24 hours in a shaking incubator set to 250 rpm.. Data is the average of n=3 data points. 🟦: Glucose oligomers; 🟦: glucose monomer; 🟩: X+G+M+F oligomers; 🟩: X+G+M+F monomers; 🟥: arabinose oligomers; 🟥: arabinose monomer. Experiments carried out as in Section 2.4.4. Sugars determined as described in Section 2.10 and data analysed as in Section 2.11.

4.8 Summary

The main aim of the work in this chapter, as described in Section 4.1, was to establish whether the USD tube geometry selected in Section 3.4 is suitable for use as a smaller scale alternative to the STRs currently used for investigating enzymatic hydrolysis processes at ReBio. The results demonstrate that the USD system reported successfully mimics the performance of conventional stirred tanks under industrially relevant conditions for carrying out the enzyme hydrolysis stage of a lignocellulosic ethanol process. The utility of the USD tubes was confirmed through its use to investigate performance limitation using a commercially relevant feedstock.

Work was carried out to investigate the practical effects of the observations on the dispersion of solids in shaken USD tubes from Section 3.5. This was carried out by performing otherwise identical enzyme hydrolyses with the USD tubes held at different angles relative to the shaker platform. Importantly Figure 4.15 shows that altering the angle at which the USD tube was held had no effect on the overall hydrolysis yield despite the substantially improved fluid flow and solids dispersion that was observed when tubes were shaken horizontally. These experiments found there was a small increase in sugar monomerisation when the USD tubes were held at 0° on the shaking platform (Figure 4.16). This difference, although statistically significant, was small and considered unlikely to have altered interpretation of results and was not felt to justify the increase in footprint caused.

In Section 4.1 criteria were set out for successful USD performance against which the results of this chapter could be judged. The first of these was that that the concentration of sugar released at the end of hydrolysis should be the same at both scales.. Figure 4.5 showed the effect of varying the solids content of an enzymatic hydrolysis on final yields in both USD tubes and STRs. It was shown clearly that as the dry solids content of a hydrolysis was increased, the final yield achieved in the hydrolysis decreased. Importantly the trends for decreasing final yields were similar in both USD tubes and STRs. As noted earlier (Section 1.3.4) this phenomenon is well established in the literature for a variety of feedstocks so it’s observation in this work fits well with the established literature. The data in Figure 4.5 thus shows clearly that the amount of sugar released was the same in STRs and USD tubes across a range of solids loadings. Data from Section 4.3 also demonstrated that the composition of sugar released at the two scales was similar and thus that one of the criteria for good scale down replication has been met for the USD tube system.

Not only was the effect of solids content similar in USD tubes and STRs, the hydrolysis time course profiles were extremely similar as demonstrated by the data in Figures 4.7 and 4.8. At two different solids levels the hydrolysis profiles from both scales were highly comparable; the relative difference in overall yield between scales was less than 10%. This data demonstrates that the kinetics of hydrolysis are highly similar at the two scales and thus satisfies the second criteria for successful scale down that was identified in Section 4.1.

The experiments described in Sections 4.3 and 4.4 using USD tubes and STRs generated some data in which the performance of the STRs was slightly superior to the USD tubes and some where the opposite was true. Taken together, the results of from these experiments suggest that the degree of variability within the experimental set ups used is greater than any difference in performance between the two systems. In practical terms therefore the USD tubes and 4L STRs were extremely similar, satisfying the criteria identified in Section 4 and showing that the USD tubes are an effective scale-down model for the enzymatic hydrolysis of DDGS, satisfying the main objective of the chapter. The USD tube system lacks the ability for on-line pH control found in STRs, however the lack of pH control is demonstrably not important for the enzyme hydrolysis stage and thus this difference is of little relevance. If further pH control was found be necessary in further work then there is the option to add buffers into the USD tube system in order to reduce pH drift.

One notable aspect of the time course profiles was that hydrolysis rates quickly declined after an initial rapid period of sugar solubilisation. Typically around 80% of all the sugar solubilised during the enzymatic hydrolysis of DDGS was present after the first 4 hours of the hydrolysis. Therefore the USD tubes were used in order to investigate potential causes of the slowdown. Section 4.6 provided clear evidence that soluble inhibitors are a significant limiting factor to the enzymatic hydrolysis of DDGS. When the hydrolysis reactions were periodically interrupted, the residual solids washed and then the hydrolysis process started again with fresh enzyme significant increases in solubilisation were observed. Figure 4.12 shows that the degree of improvement was related to the number of washes. Three interruption - washing steps led to a 40% improvement in sugar solubilisation relative to the control and the solubilisation of all available sugars. Thus all of the objectives stated at the outset of this chapter have been met with the comparability of performance in USD tubes and STRs being demonstrated before the tubes were used to investigate a commercially relevant process bottleneck. The successful use of 30mL conical tubes as USD tubes is of both academic and commercial relevance. The USD tube system has advantages over both shake flasks and microwell plates having a smaller footprint than the former and improved scalability compared to the latter. Thus there is potential for the use of the USD tubes to enhance research timelines. The observation that repeated washing steps lead to large increases in solubilisation is not directly commercially relevant. Although a much larger quantity of sugar was solubilised, the end result was several dilute process streams that would need to be combined and concentrated in order to achieve target volumetric sugar concentrations. The associated costs would be likely to negate a significant proportion of the increase achieved (Maiorella et al., 1984). There is however significant commercial value in identifying what factors are limiting to the process in order that research efforts can be focused on problem areas. As demonstrated here the USD system would also be of significant utility in any such experiments due to the much larger number of conditions that can be tested compared to using STRs.

The next stage in a lignocellulosic ethanol process is fermentation of the released sugars. The use of the USD tubes as small scale mimics of ReBio’s stirred-tank fermenters is addressed in the next chapter.

5. Creation of a USD Model for the Fermentation of Hydrolysed DDGS

5.1 Introduction

Fermentation is the final biological stage in a lignocellulosic ethanol process in which the sugars released from biomass by the pretreatment and enzyme hydrolysis steps (Chapter 4) is converted into ethanol by a fermentative organism. It is a more complex process step than enzyme hydrolysis, with more variables to consider. For example, dissolved oxygen can be of critical importance to a fermentation process (Tang et al., 2009) but has little influence on the enzyme hydrolysis stage. Likewise, pH tends to drift in both enzyme hydrolysis and fermentation processes but the changes that occur during fermentation are both larger and more rapid making control of pH much more importance during fermentation (Babuska et al., 2002). Whilst there is a large amount of published literature available for both process stages, as described in Section 1.3 and 1.4 respectively, the information available for fermentation is far less universally applicable, since it is tied to a particular organism and fermentation system. Therefore substantially more development work needs to be carried out when establishing a novel fermentation system and, hence, this is the stage at which USD technologies could have the greatest commercial impact. A significant portion of this chapter is therefore devoted to initial experiments to better define appropriate operating parameters for the shaken tubes when applied as an ultra scale-down fermentation system.

The organism used for the fermentation studies in this work is *G. thermoglucosidasius* TM242 as described in Section 1.4.3 (Atkinson et al., 2007, Cripps et al., 2009). Some prior characterisation of this strain, for example determination of the optimal pH and growth temperature, has taken place at ReBio and this knowledge will be utilised here as appropriate to avoid repetition. In comparison to more established organisms such as *S. cerevisiae* or *E. coli* there is comparatively little published work to use as a basis for developing and improving processes involving thermophilic organisms (Section 1.4.3). There would therefore be considerable scientific benefit in establishing a higher throughput, smaller volume system that could speed up investigation of thermophilic fermentation processes, especially when using complex, partly insoluble substrates such as hydrolysed DDGS.

The literature reviewed in Section 1.6 highlighted the need to fully characterise all of the process scales in use in order for a particular unit operation to be successfully scaled up or down. The physical characterisation of both the current STR system and the USD tube system that is being established was described in Chapter 3. The aim of this chapter is therefore to evaluate the USD tube system for use as a small scale fermentation model of the current 4L STRs used at ReBio for *G. thermoglucosidasius* TM242 fermentation. Ethanol production by *Geobacillus* is a relatively straightforward fermentative process where the product is produced as a primary metabolite. They naturally use a mixed acid fermentative process producing a mixture of acetic acid, lactic acid and ethanol under oxygen limited conditions. Strain TM242 retains the ability to produce acetic acid and ethanol. Of critical importance to process economics is the amount of ethanol produced and therefore the key metrics against which the scale down system will be judged are the ethanol titre and the ethanol production yield relative to substrate consumed. Small differences in the rate of fermentation are less likely to have a substantial effect on process economics and so will be considered as secondary metrics. The specific objectives are as follows:

- To characterise the effect of the volumetric oxygen mass transfer coefficient, k_La, on fermentation performance in the current 4L scale STR system.
- To develop protocols for use of the USD tubes for thermophilic fermentation and to evaluate fermentation performance at this scale.
- To establish k_La as a basis for scale translation of fermentations carried out at the USD and STR scales under comparable conditions.

5.2. Effect of oxygen transfer on fermentation in STR

An important objective of this project was to establish whether the USD tubes could be used a scale-down system to successfully predict fermentation performance at larger scales in conventional STRs. This requires a method of transferring a process from one scale to another with an expectation of achieving comparable process performance. This is most commonly considered in terms of scaling up a novel process developed at laboratory scale to pilot or production scale equipment. However there is also a converse approach whereby a process already operating at larger scales is transferred down to smaller scale apparatus (Betts et al., 2006). This can be useful for investigating issues that are occurring at production scales or characterising established production processes with techniques that were not available when initial process development occurred (Li et al., 2006).

The fermentative production of ethanol using *G. thermoglucosidasius* as carried out by ReBio Technologies was largely developed using lab scale STRs. The goal now is to transfer this process into the USD tubes to provide a high throughput approach to support future fermentation process development. As noted in Section 1.6 the volumetric oxygen mass transfer coefficient, k_La, is commonly reported to achieve good outcomes when used as a basis for scale transfer (Marques et al., 2010). Consequently it was considered that, due to the known importance of oxygenation to the strain of *G. thermoglucosidasius* being used, k_La was an appropriate scale transfer criteria for use in this work (Lin et al., 2014, Van Zyl et al., 2014). This section describes a series of experiments designed to achieve a scalable process for the fermentative production of ethanol based on the specific oxygen transfer rate in the STRs.

5.2.1 Fermentation performance at a range of k_La values

Fermentations were carried out using a mixture of glucose and xylose, the two most abundant sugars in DDGS, at the C5:C6 (w/w) ratio they would be found in biomass hydrolysate (Figure 4.1) (Kim et al., 2008b). In general terms it was expected that there would be an optimum rate of oxygen transfer for the culture below which growth would be sub optimal and above which products of aerobic metabolism, primarily acetic acid, would begin to be produced at the expense of ethanol. Using the data from Section 3.3 a series of experiments were therefore carried out at fixed k_La values ranging from 20 h^{-1} to 61 h^{-1} aiming to find this optimal point. This range was based on prior work at ReBio, and the stirrer speeds and airflow rates tested are listed in Table 6.1. Most examples of the use of k_La for process scaling focus on aerobic processes and thus the investigation of micro aerobic processes at low k_La values is novel in this work (Islam et al., 2008, Flores et al., 1997).

The experiments used lab media, 50mM USM, supplemented with 20 g L^{-1} yeast extract and a mixture of glucose and xylose (in a ratio of 3:1) in order to assess the utilisation of both C6 and C5 sugars (Table 2.6). This is important as it mimics the requirement for efficient fermentation of both the sugars derived from cellulose and those derived from hemicellulose into ethanol as would be required in a final process (Section 1.4.1). The use of laboratory media rather than DDGS hydrolysate for these experiments allows for the elimination of several compounding factors such as the efficiency of pretreatment and enzyme hydrolysis and the influence of any inhibitors and solids in the hydrolysate. This baseline data can then be used to analyse these compounding factors in a well-defined system.

Figures 5.1 and 5.2 show the consumption of glucose and xylose respectively in the different fermentations. Full utilisation of both sugars was achieved within the 24 hour fermentation with the exception of the culture grown at the lowest k_La value of 20 h^{-1} where only around 20% (w/w) of the glucose and 40% (w/w) of the xylose was consumed. In this culture the rate of consumption of xylose and especially glucose was largely constant throughout the fermentation, suggesting the fermentation was limited by the oxygen mass transfer rate. When the oxygen mass transfer coefficient was increased to 36 h^{-1} full utilisation of both sugars occurred but at a noticeably slower rate. This is particularly clear in Figure 5.1 where, with the exception of the 20 h^{-1} and 36 h^{-1} cultures, all glucose utilisation profiles are closely grouped together with little to distinguish between them. The 36 h^{-1} culture, in common with all others except the 20 h^{-1} culture, showed an initially slower rate of glucose utilisation which then increased markedly later in the fermentation. Relative to the cultures run at higher k_La values the inflection point in the rate of utilisation was later and the rate post inflection lower in the 36 h^{-1} culture. The time of glucose exhaustion in the other cultures, ranging in k_La value from 40 h^{-1} to 61 h^{-1} only varied by around two hours and the peak rates of utilisation were very similar. In all cultures with the exception of the 20 h^{-1} culture the rate of glucose utilisation in the first 3 hours was around 2 g L^{-1} h^{-1}. At this point there is a clear inflection in glucose concentration in most of the cultures and the rate of utilisation increases to around 6 g L^{-1} h^{-1} (Figure 5.6). This was observed in the cultures grown at k_La values ranging 40 h^{-1} to 61 h^{-1}. When the culture grown was grown at 36 h^{-1} the initial lower rate of glucose utilisation was maintained for a longer period, for the first 6 hours, before the rate of utilisation increased. The higher rate of utilisation, 3.5 g L^{-1} h^{-1}, was significantly lower than in the cultures grown at higher values of k_La.

From analysing the xylose utilisation in Figure 5.2 it is clear that there is a greater spread of performance associated with the different oxygenation regimes. As was the case with glucose, all of the conditions tested with the exception of 20 h^{-1} allowed complete utilisation of the available xylose within 24 hours. In all of the other cultures full utilisation of xylose occurred in the overnight sampling gap between 10 hours and 24 hours. The pattern of xylose utilisation was similar to glucose with the rate of utilisation being slow for the first 4-6 hours then increasing significantly. Rates of utilisation of xylose were substantially lower than those of glucose with rates in the initial period typically around 0.3 g L^{-1} h^{-1}, increasing to values around 0.8 g L^{-1} h^{-1}in the later period of rapid utilisation. There is though clear evidence for the co-utilisation of glucose and xylose under all of the conditions investigated. Looking at the amount of xylose that had been consumed by the time of the 10 hour sample reveals a potentially interesting pattern. Xylose utilisation increases with increasing k_La values over the range 20 h^{-1} to 50 h^{-1} where the lowest residual xylose of 1.7 g L^{-1} was recorded. The amount of xylose remaining after 10 hours of fermentation then increased at 56 h^{-1} and again at 61 h^{-1}. Thus there appears to be an optimum rate of oxygen transfer for the utilisation of xylose lying towards the middle of the range tested as can be seen in Figure 5.6 where the peak rates of utilisation of glucose and xylose are plotted against the culture k_La values.

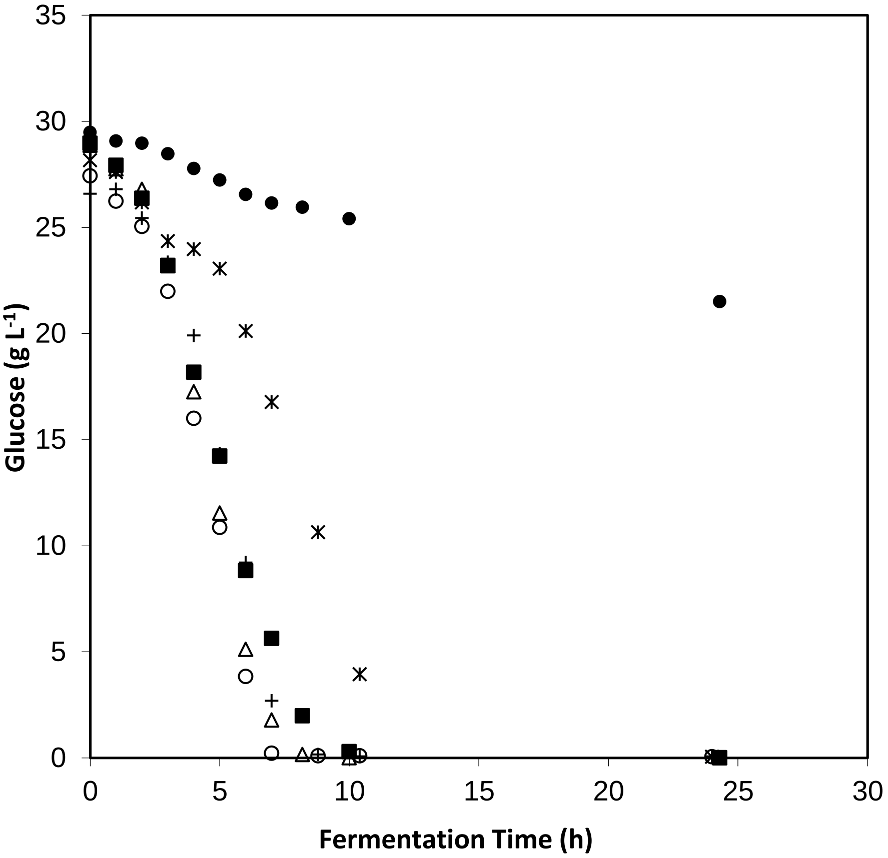

Figure 5.1. Glucose consumption during fermentation of 40 g L^{-1} mixed sugar in 50mM USM / 20 g L^{-1} yeast extract (Table 2.6) by *G. thermoglucosidasius* TM242 in 4L STRs at different k_La values. Fermentations were carried out at 60°C and pH 6.7. ●: 20 h^{-1}; ×: 36 h^{-1}; ■: 40 h^{-1}; ○: 50 h^{-1}; +: 56 h^{-1}; △: 61 h^{-1}. Experiments carried out as described in Section 2.9 and glucose concentration determined as described in Section 2.10. Data analysed as described in Section 2.11.

Table 5.1. Stirrer speeds and airflow rates used to achieve STR k_La values of 20 h^{-1} – 60 h^{-1}. Values were calculated using Eq. 3.2.

STR k_La value (h^{-1})	Stirrer speed (rpm)	Airflow rate (L min^{-1})
20	256	1.0
36	290	2.0
40	409	1.0
50	365	2.0
56	395	2.0
61	413	2.0

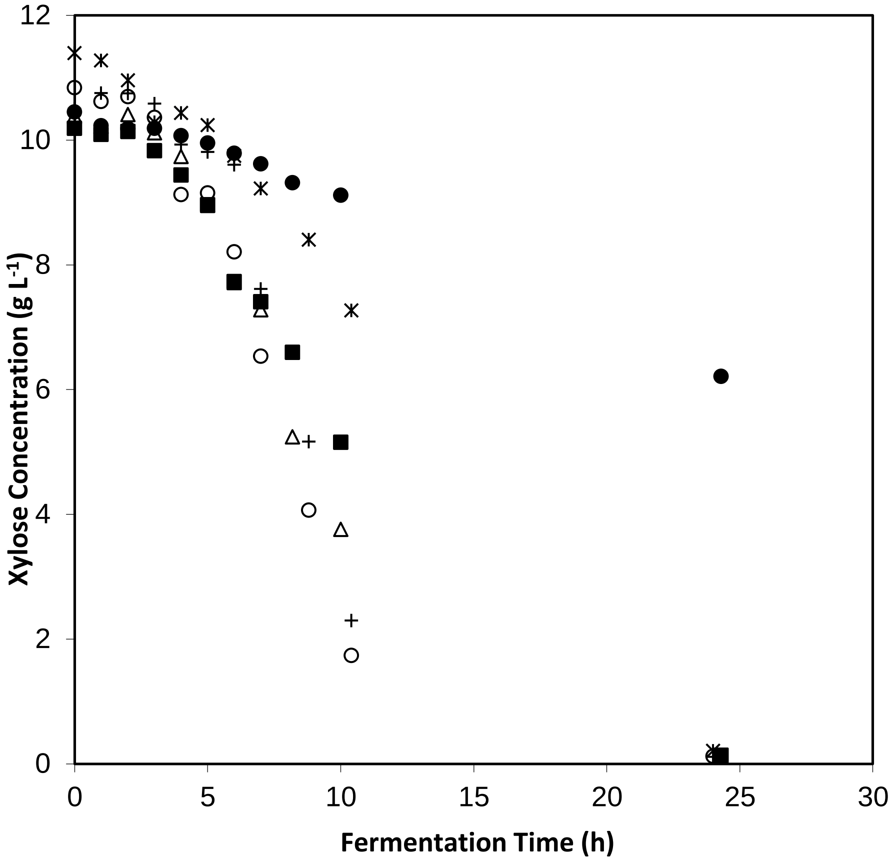

Figure 5.2. Xylose consumption during fermentation of 40 g L^{-1} mixed sugar in 50mM USM / 20 g L^{-1} yeast extract (Table 2.6) by *G. thermoglucosidasius* TM242 in 4L STRs at different k_La values. Fermentations were carried out at 60°C and pH 6.7.*G. thermoglucosidasius* ●: 20 h^{-1}; ×: 36 h^{-1}; ■: 40 h^{-1}; ○: 50 h^{-1}; +: 56 h^{-1}; △: 61 h^{-1}. Experiments carried out as described in Section 2.9 and xylose concentration determined as described in Section 2.10. Data analysed as described in Section 2.11.

The two main metabolic products produced during the course of the fermentation were ethanol and acetate, the profiles for which are shown in Figures 5.3 and 5.4 respectively. In Figure 5.3 it can be seen that as with the glucose utilisation in Figure 5.1, the cultures grown at k_La values of 36 h^{-1} and especially 20 h^{-1} are noticeable outliers. Comparison of Figures 5.1, 5.2 and 5.3 show that ethanol production is closely linked to sugar consumption, as would be expected in a strain that produces ethanol as its primary metabolite under conditions of low oxygenation. Thus the ethanol production in the 20 h^{-1} and 36 h^{-1} cultures reflects the lower rates of glucose consumption. In the four cultures grown at higher oxygen transfer rates both the rates of ethanol production and the maximum titres achieved are largely similar although small differences can be observed. The cultures grown at 40 h^{-1}, 50 h^{-1} and 56 h^{-1} all produced around 11.5 g L^{-1} ethanol, albeit with slight differences in timing; the culture at 50 h^{-1} achieved complete glucose utilisation marginally sooner than the others and this is reflected in this culture reaching peak ethanol slightly sooner. The peak ethanol titre in the 61 h^{-1} culture was slightly lower than in those three cultures at 10.8 g L^{-1}, although the difference was not statistically significant. As would be expected the rates of ethanol production show a similar pattern to the rates of glucose consumption; rates are low in the first few hours of the culture but then increase sharply after around 3 hours of growth and peak at around 2.5 g L^{-1} h^{-1}in the period leading up to glucose exhaustion. The peak ethanol titre and ethanol production yield of each of the cultures is plotted against the corresponding k_La value in Figure 5.7. It is observed that optimal ethanol production, in terms of titre is seen at a k_La of 56 h^{-1} whilst the optimal ethanol production yield occurs in the 50 h^{-1} culture.

Figure 5.4 shows that during the first few hours of culture growth acetate production was minimal for all aeration regimes. No extracellular acetate was detected during the first four hours with detectable concentrations being produced after 4 to 6 hours of culture growth in most conditions. As with all other metrics, the two outliers were the cultures grown at 20 h^{-1} and 36 h^{-1} where acetate production began later in the culture and reached much lower final concentrations. In the STRs run at the other four aeration conditions the final acetate concentration is between 6.1 g L^{-1} in the culture grown at 40 h^{-1} and 7.3 g L^{-1} in the culture grown at 61 h^{-1}. The majority of the acetate was produced from the metabolism of xylose as the concentrations of acetate after 10 hours of culture growth, by which time all glucose had been consumed, were significantly lower. The concentrations produced by the 40 h^{-1} and 61 h^{-1} cultures were 1.7 g L^{-1} and 2.6 g L^{-1} respectively. On this basis it seems to be the case that little ethanol was produced from the consumption of xylose once glucose exhaustion had been reached.

The levels of biomass produced in the tanks, recorded as optical density, are shown in Figure 5.5. As in all other aspects of fermentation performance the most noticeable outlier is the low biomass production in the culture grown at 20 h^{-1}. In terms of both the maximal level of biomass production and the highest rate of production the best performing condition was 50 h^{-1} where a peak value of 16 au was reached at 7 hours. The increase in biomass in this culture was essentially linear up to the peak value, before declining somewhat after all of the glucose in the culture had been consumed. In three of the remaining cultures peak biomass readings were recorded from the samples taken after 10 to 11 hours of growth, the exception being the 61 h^{-1} culture where the peak OD value was recorded after 24 hours of fermentation but the gap in sampling makes interpreting this complicated. Maximal readings of around

14 au were achieved in the 40 h^{-1} and 56 h^{-1} cultures and of around 11 au in the 36 h^{-1} and 61 h^{-1} cultures.

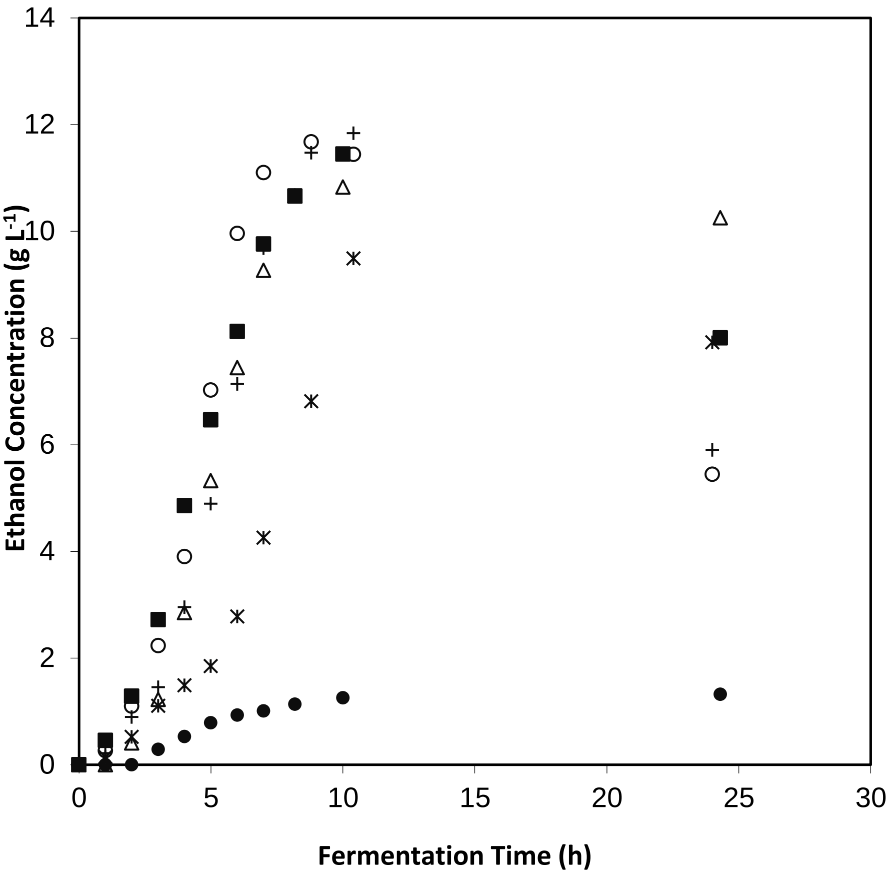

Figure 5.3. Ethanol production during fermentation of 40 g L^{-1} mixed sugar in 50mM USM / 20 g L^{-1} yeast extract (Table 2.6) by *G. thermoglucosidasius* TM242 in 4L STRs at different k_La values. Fermentations were carried out at 60°C and pH 6.7*G. thermoglucosidasius*. ●: 20 h^{-1}; ×: 36 h^{-1}; ■: 40 h^{-1}; ○: 50 h^{-1}; +: 56 h^{-1}; △: 61 h^{-1}. Experiments carried out as described in Section 2.9 and ethanol concentration determined as in Section 2.10. Data analysed as described in Section 2.11.

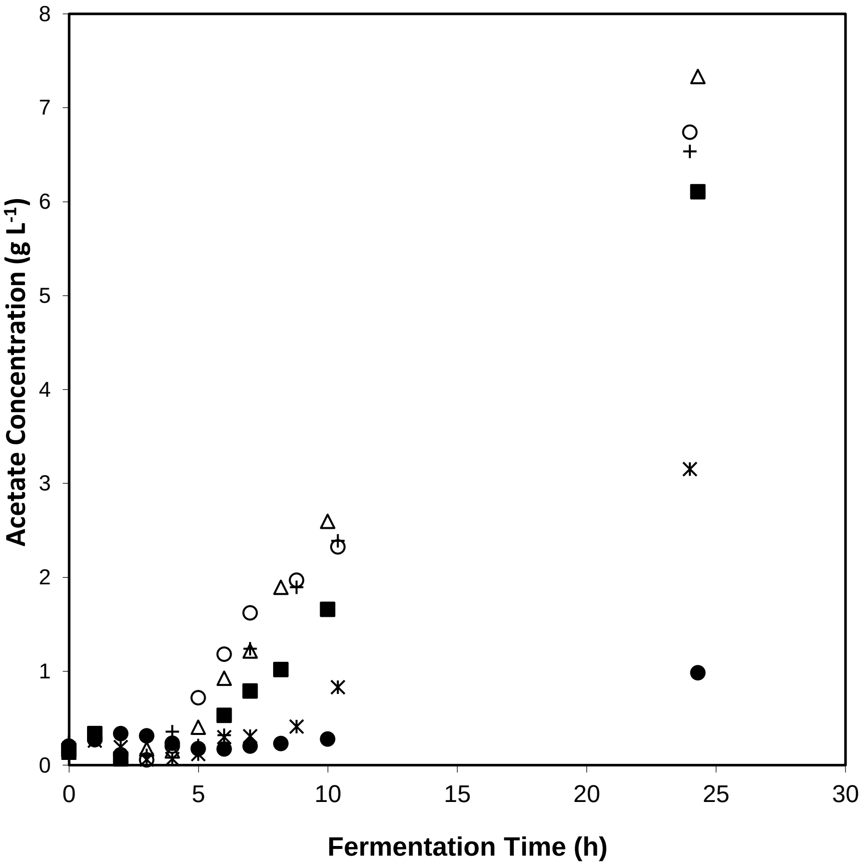

Figure 5.4. Acetate production during fermentation of 40 g L^{-1} mixed sugar in 50mM USM / 20 g L^{-1} yeast extract (Table 2.6) by *G. thermoglucosidasius* TM242 in 4L STRs at different k_La values. Fermentations were carried out at 60°C and pH 6.7*G. thermoglucosidasius*. ●: 20 h^{-1}; ×: 36 h^{-1}; ■: 40 h^{-1}; ○: 50 h^{-1}; +: 56 h^{-1}; △: 61 h^{-1}. Experiments carried out as described in Section 2.9 and acetate concentration determined as in Section 2.10. Data analysed as described in Section 2.11.

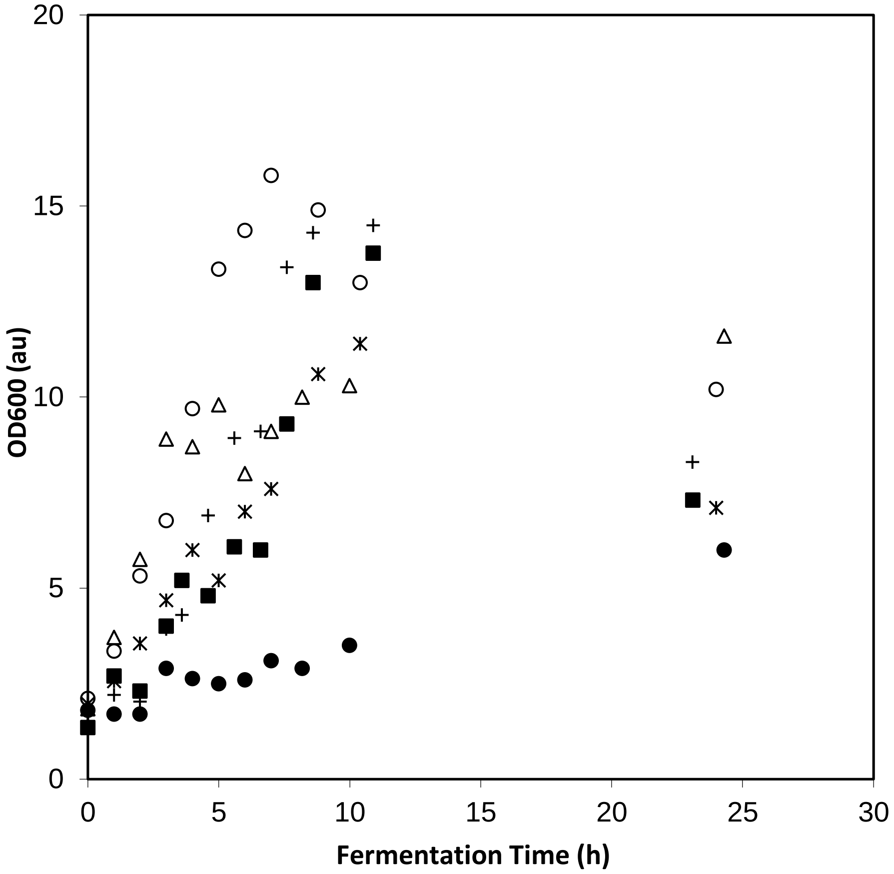

Figure 5.5. Culture optical density (600 nm) values during fermentation of 40 g L^{-1} mixed sugar in 50mM USM / 20 g L^{-1} yeast extract (Table 2.6) by *G. thermoglucosidasius* TM242 in 4L STRs at different k_La values. Fermentations were carried out at 60°C and pH 6.7*G. thermoglucosidasius*. ●: 20 h^{-1}; ×: 36 h^{-1}; ■: 40 h^{-1}; ○: 50 h^{-1}; +: 56 h^{-1}; △: 61 h^{-1}. Experiments carried out as described in Section 2.9. Data analysed as described in Section 2.11.

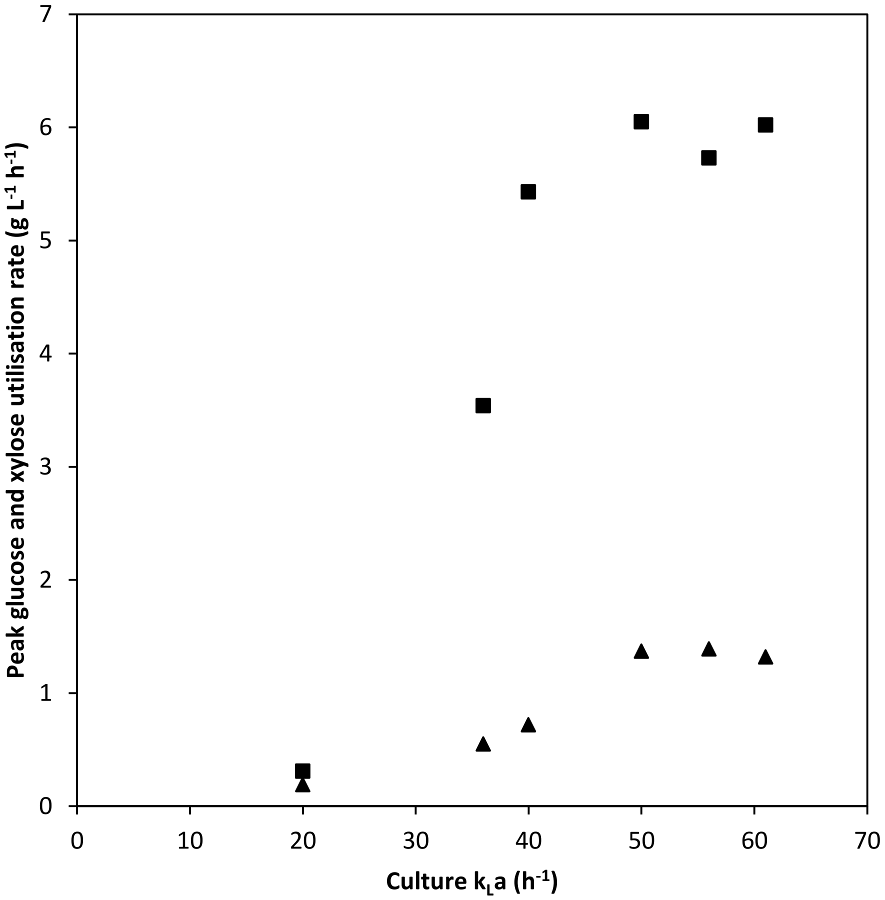

Figure 5.6. Peak rates of consumption of glucose and xylose during fermentation of 40 g L^{-1} mixed sugar in 50mM USM / 20 g L^{-1} yeast extract (Table 2.6) by *G. thermoglucosidasius* TM242 in 4L STRs at different k_La values. Fermentations were carried out at 60°C and pH 6.7*G. thermoglucosidasius*. ■: Glucose consumption rate; ▲: xylose consumption rate. Experiments carried out as described in Section 2.9. Data analysed as described in Section 2.11.

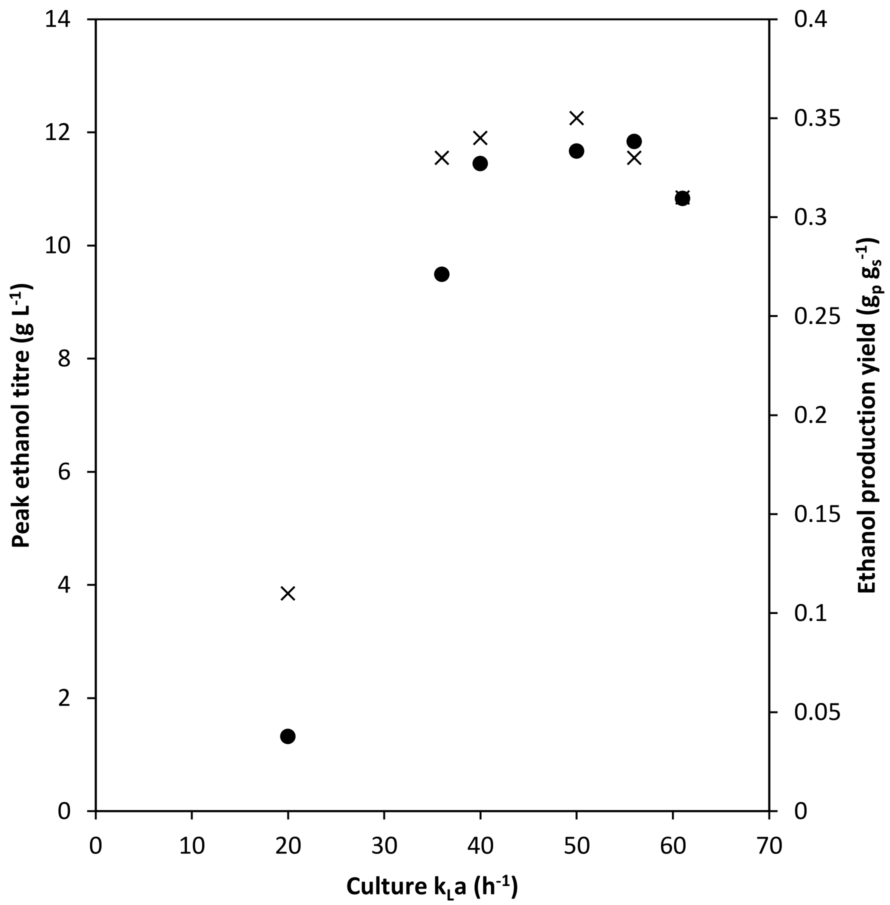

Figure 5.7. Peak ethanol titre and ethanol production yield (gp gs^{-1}) during fermentation of 40 g L^{-1} mixed sugar in 50mM USM / 20 g L^{-1} yeast extract (Table 2.6) by *G. thermoglucosidasius* TM242 in 4L STRs at different k_La values. Fermentations were carried out at 60°C and pH 6.7*G. thermoglucosidasius*. ●: peak ethanol titre; ×: ethanol production yield. Experiments carried out as described in Section 2.9. Data analysed as described in Section 2.11.

The fermentation profiles in Figures 5.1 to 5.5, when examined alongside the plots of k_La against peak substrate consumption, ethanol titre and ethanol production yield in Figures 5.6 and 5.7, suggest that an optimal oxygen mass transfer coefficient for the growth of *G. thermoglucosidasius* strain TM242 lies around a value of 50 h^{-1}. When grown under these conditions TM242 was seen to have the highest rates of sugar utilisation and ethanol production, whilst the ethanol titre achieved was in the group of peak titres. At k_La values slightly above or below this sugar utilisation and ethanol production rates were slightly lower, although the differences in timing were small and the final ethanol titres achieved were unchanged. At k_La values further below the optimum rates of sugar utilisation were much lower as ultimately was the ethanol titre achieved. At the highest k_La value tested there seemed to be an increase in the production of the by-product acetate which is suggestive of the level of oxygen in the system being higher than optimal for ethanol production, although the difference was not large enough to be statistically significant on the data available. In all culture conditions co-utilisation of glucose and xylose was seen to occur, although utilisation of glucose occurred at a much higher rate and thus was exhausted before the xylose. Xylose consumed after the exhaustion of the glucose seemed to be primarily metabolised to acetate rather than ethanol.

5.2.2 Reproducibility of fermentations in STRs

In order to assess the reproducibility of fermentation in STRs, three separate experiments were carried out at the same conditions using a k_La value of 40 h^{-1} for comparative purposes. The sugar utilisation and product formation achieved is shown Figures 5.8 and 5.9 respectively. It can be seen that the profiles of two of the three replicates are closely matched, with the third lying somewhat away from the other two. The differences are mainly observed in the glucose and ethanol profiles and are largely issues of rates. In Figure 5.8 it is observed that the initial slower period of glucose utilisation continues for longer in run two than in runs one and three, continuing for around five hours rather than around 2 hours. The increase in the rate of utilisation that occurs in run two is however greater than in the other runs with the net effect that glucose exhaustion occurs at approximately the same time in all three cultures despite their somewhat different utilisation profiles. As would be expected from the close relationship between glucose utilisation and ethanol production, ethanol production is somewhat delayed during the majority of the fermentation in run two. There is, however, no reduction in the peak glucose titre achieved; indeed the peak titre achieved is somewhat higher in run two at around 13 g L^{-1} rather than 11.5 g L^{-1}. The xylose utilisation and acetate production profiles of run two are not significantly different from the other runs. There were no other differences observed that could explain the profile of run two. All three runs were carried out with identical experimental protocols and in all cases seed stage performance was within ReBio's internal acceptance criteria.

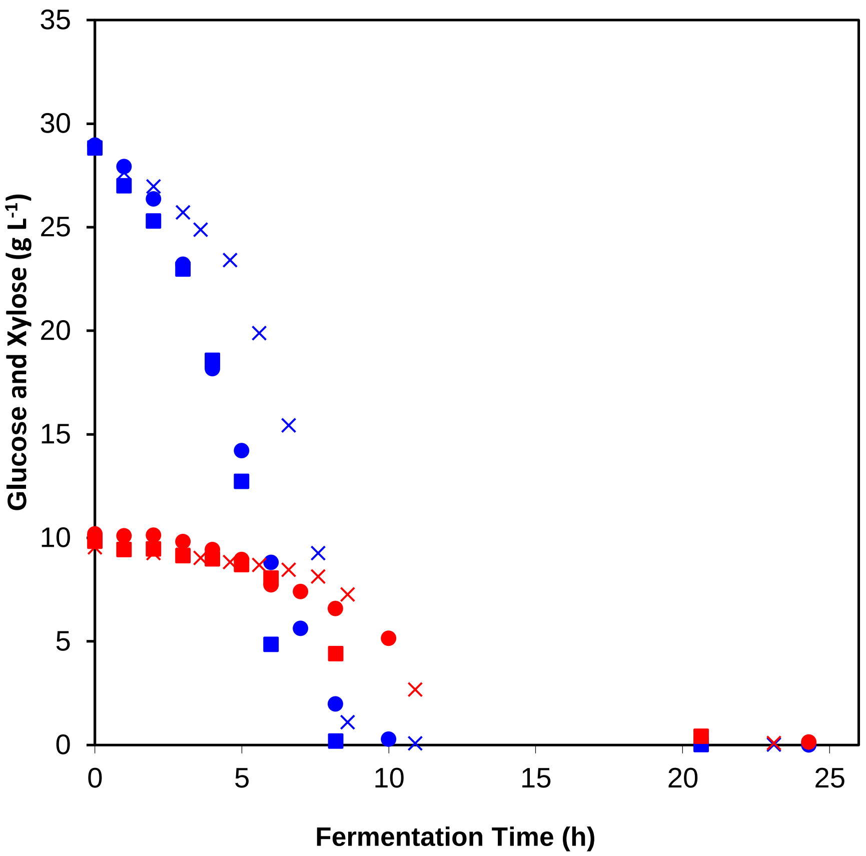

Figure 5.8. Utilisation of 40 g L^{-1} mixed sugar during fermentation in 50mM USM / 20 g L^{-1} yeast extract (Table 2.6) by *G. thermoglucosidasius* TM242 in 4L STRs at a k_La value of 40 h^{-1} and identical agitation and aeration set points. Fermentations were carried out at 60°C and pH 6.7*G. thermoglucosidasius* ●: Glucose (1); ×: Glucose (2); ■: Glucose (3); ●: Xylose (1); ×: Xylose (2); ■: Xylose (3). Experiments carried out as described in Section 2.9 and concentrations determined as in Section 2.10. Data analysed as described in Section 2.11.

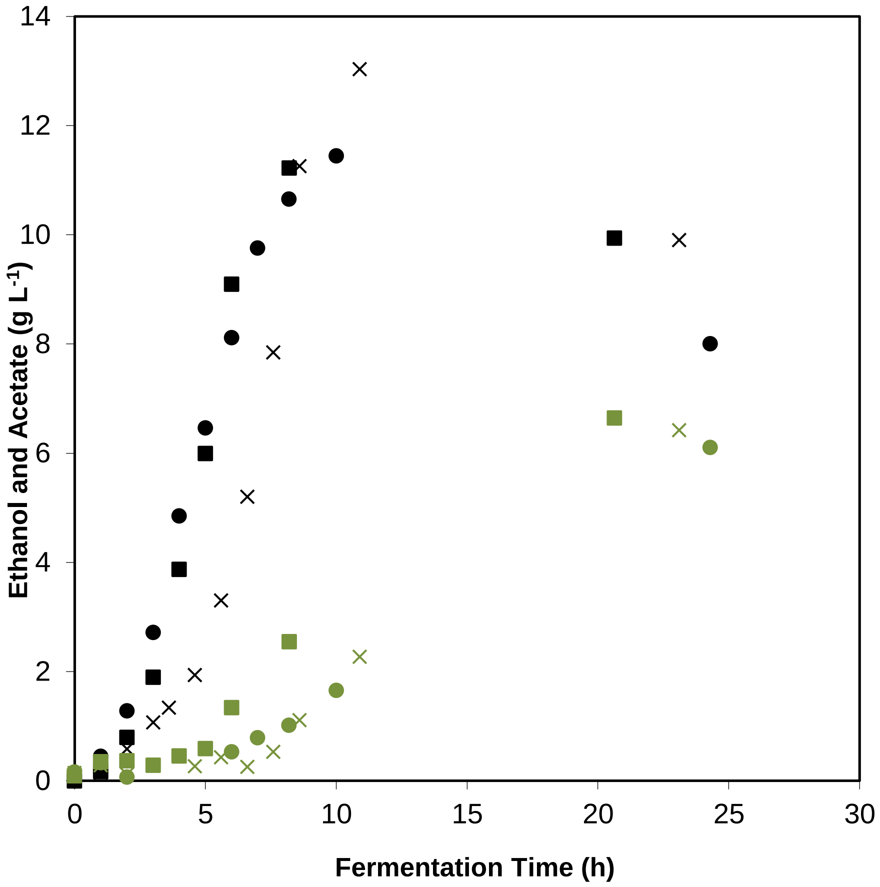

Figure 5.9. Product formation during fermentation of 40 g L^{-1} mixed sugar in 50mM USM / 20 g L^{-1} yeast extract (Table 2.6) by *G. thermoglucosidasius* TM242 in 4L STRs at a k_La value of 40 h^{-1} and identical agitation and aeration set points. Fermentations were carried out at 60°C and pH 6.7*G. thermoglucosidasius*. ●: Ethanol (1); ×: Ethanol (2); ■: Ethanol (3); ●: Acetate (1); ×: Acetate (2); ■: Acetate (3). Experiments carried out as described in Section 2.9 and concentrations determined as in Section 2.10. Data analysed as described in Section 2.11.

5.2.3 Reproducibility of fermentations performed at matched k_La under different agitation and aeration conditions

In order to better validate the k_La correlation developed in Section 3.3 two fermentations were performed at different agitation speeds and aeration rates that were predicted to give the same absolute value of k_La (Table 5.2). Sugar utilisation and product formation under both conditions is shown in Figures 5.10 and 5.11 respectively and there is good similarity between the two runs. The data for run two represents a single run, whilst the data for run one is the average of runs one and three described in Figures 5.8 and 5.9. Due to the fact that its performance was noticeably different to the other runs, run two from those experiments was discarded for these purposes.

Looking first at the glucose utilisation in Figure 5.10 it is noticeable that run two had a slightly lower initial glucose concentration due to a formulation error. Glucose utilisation occurs at a slightly higher rate in run two, such that glucose exhaustion occurs following around 6 hours of fermentation compared to 8 hours in run one. At least some of this difference is attributable to the difference in starting glucose concentration between the two cultures. For the first six hours of fermentation there was no difference between the two trends in terms of xylose utilisation. Following this however xylose utilisation increases noticeably in run two, but not in run one. In Figures 5.1 and 5.2 it was observed that xylose utilisation tended to increase considerably once all of the glucose consumed had been exhausted. Given this observation and the timing of the increase in xylose utilisation in run one it is reasonable to attribute this observation the differences in glucose utilisation that occurred.

From the product formation in Figure 5.11, it can be seen that there is a difference in the rate of ethanol production that corresponds with the difference in glucose utilisation rate. The peak ethanol titres achieved are very similar however at around 12 g L^{-1}. Both acetate profiles are very similar for approximately 10 hours of fermentation during which time 2 g L^{-1} was produced. Following a gap in sampling overnight the concentration had increased under both conditions, with around 1.2 g L^{-1} less being produced in run two. Given that much of this acetate was likely produced after the peak ethanol titre the difference may be considered to have limited value.

Figures 5.8 and 5.9 demonstrate the variability that is seen when three fermentations are carried out at the same conditions whilst Figures 5.10 and 5.11 compare an average of those runs with a separate fermentation carried out at a second set of conditions that were predicted to produce the same k_La value. The variability that is seen in the latter two figures is no greater than that seen in the former two suggesting that by matching k_La, fermentation performance can be replicated even if the fermentation is carried out at different stirrer and aeration conditions. This helps to validate the k_La models produced by showing that matched k_La leads to similar performance regardless of the absolute stirring and aeration rates, providing these are set at values that provide homogeneous mixing and good gas-liquid dispersion within the tanks. It also lends credence to the selection of k_La as a metric for the transfer of processes between STRs and USDs by showing that process performance can be matched by maintaining a constant k_La.

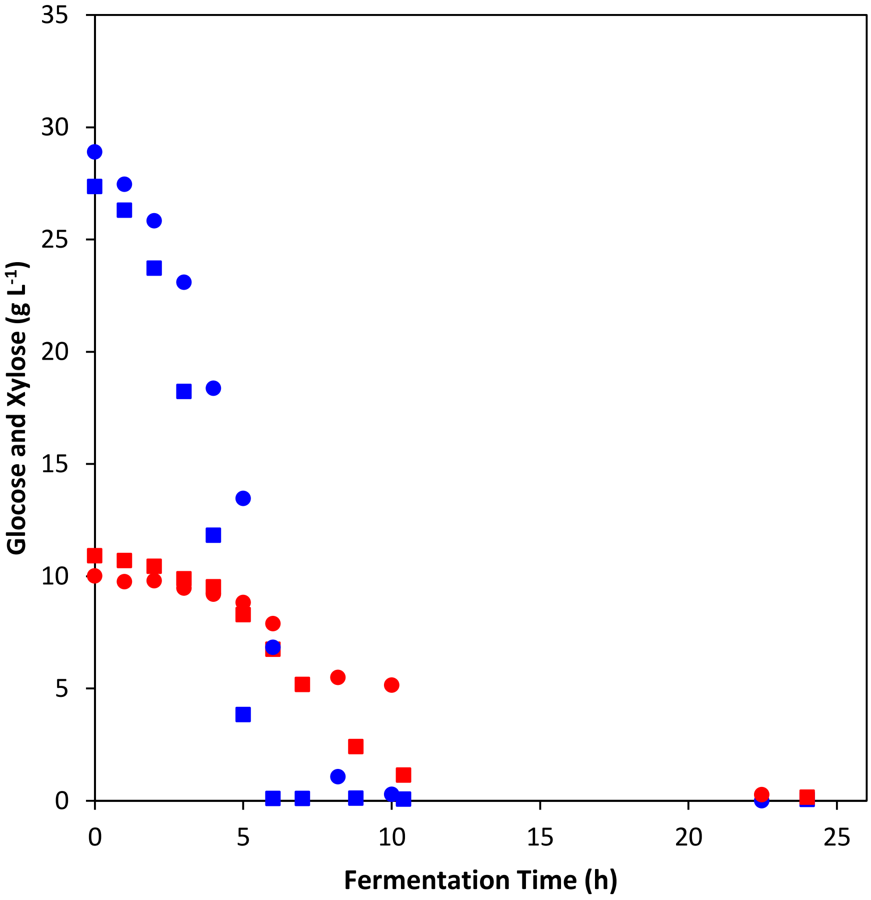

Figure 5.10. Sugar utilisation during fermentation of 40 g L^{-1} mixed sugar in 50mM USM / 20 g L^{-1} yeast extract (Table 2.6) by *G. thermoglucosidasius* TM242 in 4L STRs at a k_La value of 40 h^{-1}. Fermentations were carried out at 60°C, pH 6.7 and the same k_La value but different agitation and aeration set points (Table 5.2) *G. thermoglucosidasius*. ●: Glucose (1); ■: Glucose (2); ●: Xylose (1); ■: Xylose (2). Experiments carried out as described in Section 2.9 and concentrations determined as in Section 2.10. Data analysed as described in Section 2.11.

Table 5.2. Stirrer speeds and airflow rates used to achieve k_La values of 40 h^{-1} (Figures 5.10 and 5.11). Agitation and aeration conditions determined by fitting to Eq. 3.2.

K_La 40 h^{-1} Condition	**Stirrer speed (rpm)**	**Airflow rate (L min^{-1})**
Condition 1	409	1.0
Condition 2	314	2.0

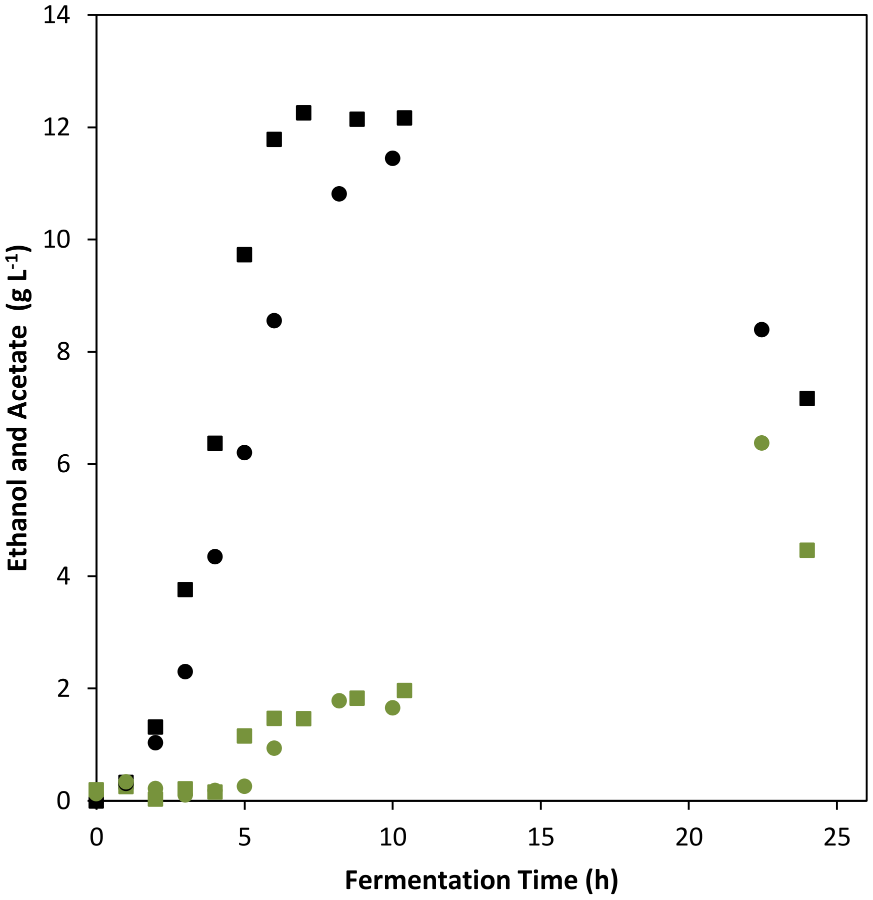

Figure 5.11. Product accumulation during fermentation of 40 g L^{-1} mixed sugar in 50mM USM / 20 g L^{-1} yeast extract (Table 2.6) by *G. thermoglucosidasius* TM242 in 4L STRs at a k_La value of 40 h^{-1}. Fermentations were carried out at 60°C, pH 6.7 and the same k_La value but different agitation and aeration set points (Table 5.2)*G. thermoglucosidasius*. ●: Ethanol (1); ■: Ethanol (2); 🟢: Acetate (1); 🟩: Acetate (2). Experiments carried out as described in Section 2.9. Data analysed as described in Section 2.11.

5.3 Effect of culture and tube volume of USD tube fermentations

Conical bottomed polypropylene tubes of the sort being used in this work come in two standard sizes with working volumes of 15 mL and 50 mL. As discussed in Section 3.4, the two sizes have substantially different geometries with the 15 mL tubes having a much larger height to width ratio than the 50 mL tubes. For the hydrolysis stage, the high initial viscosity of the hydrolysate dictated that the work be carried out in the larger, wider 50 mL tubes (Section 3.4). By the end of hydrolysis the viscosity of hydrolysates is very substantially reduced meaning that the former restriction no longer applies and therefore both tube sizes would be equally valid choices. Indeed the reduced material requirements and footprint of the 15 mL tubes would make them a more attractive choice for scale-down studies and could mean that multiple USD fermentations could be done from a single USD hydrolysis tube.

An experiment was therefore set up looking to investigate the influence of culture volume and tube geometry on the performance of fermentations carried out in them. The media composition (Table 2.5) and inoculum loading (% v/v) were identical in all cases, however tubes were filled to several different final volumes in order to examine whether this had any effect on fermentation performance.

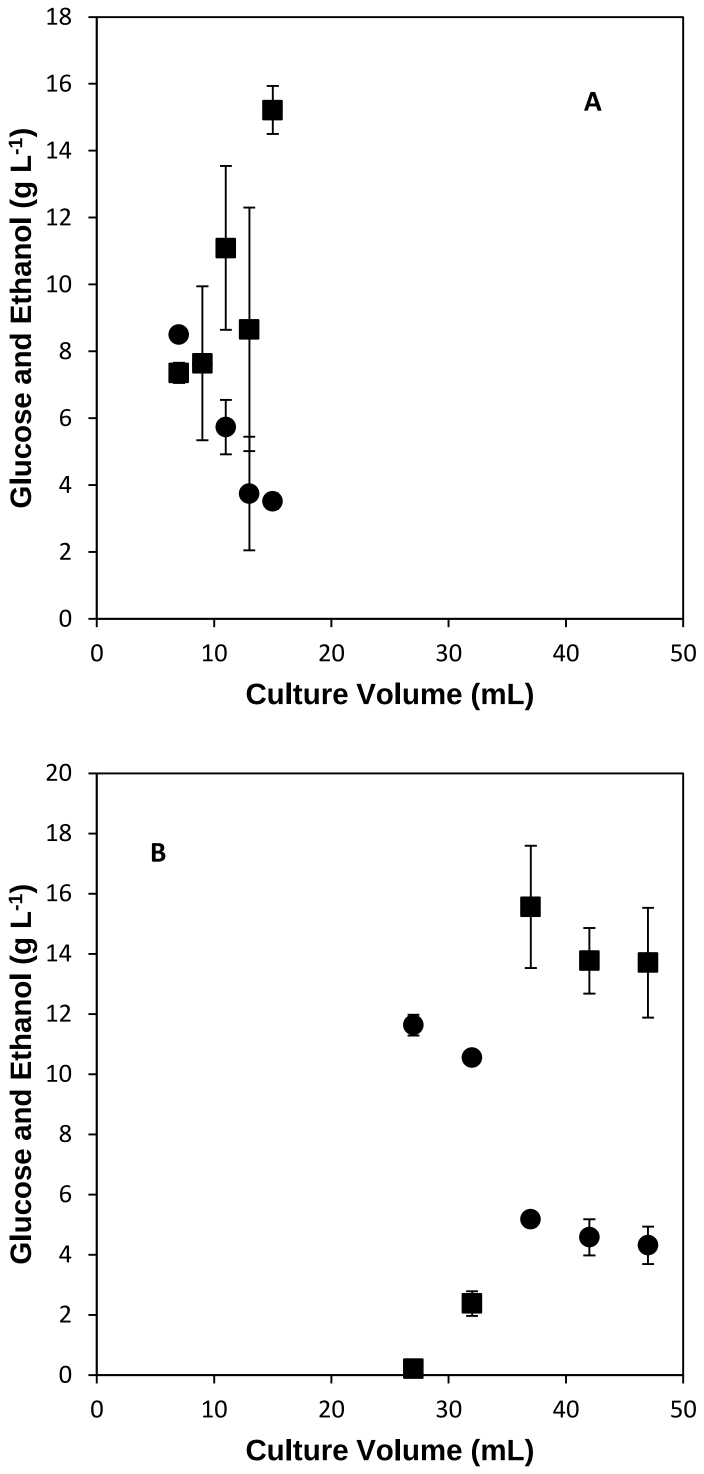

Figure 5.12. Effect of working volume on ethanol production and residual glucose during fermentation of 40 g L^{-1} glucose in 50mM USM / 20 g L^{-1} yeast extract (Table 2.7) by *G. thermoglucosidasius* strain TM242 in conical bottomed tubes with working volumes of A) 15 mL and B) 50 mL. Fermentations were carried out at an initial pH of 7.0 in a shaking incubator set to 60°C and 250 rpm and were run for 24 hours. ■: Residual glucose; ●: Ethanol produced. Data shown is the average of n=3 samples. Error bars show 1 standard deviation around the mean. Experiments were carried out as described in Section 2.9 and glucose and ethanol concentration determined as in Section 2.10. Data analysed as described in Section 2.11.

Figure 5.12 shows the concentrations of ethanol produced and glucose remaining at the end of the fermentation period. Both the tube size and the level to which the tubes were filled had a substantial effect on the fermentation performance of the cultures they contained. The maximum ethanol concentration recorded in the 15 mL tubes was 8.5 g L^{-1} whilst in the 50 mL tubes concentrations of up to 11.6 g L^{-1} were achieved. In terms of residual glucose the lowest concentration in the 15 mL tubes was 7.4 g L^{-1} whilst in the 50 mL tubes it was just 0.2 g L^{-1}. In both tube sizes there was a good correlation between glucose consumption and ethanol production and the highest figures for ethanol production and glucose consumption were seen at the lowest working volumes.

This suggested that there is link between fermentation performance and ratio of headspace to culture volume in the tubes. Figure 5.13 shows this ratio plotted against ethanol production as an indicator of fermentation performance.

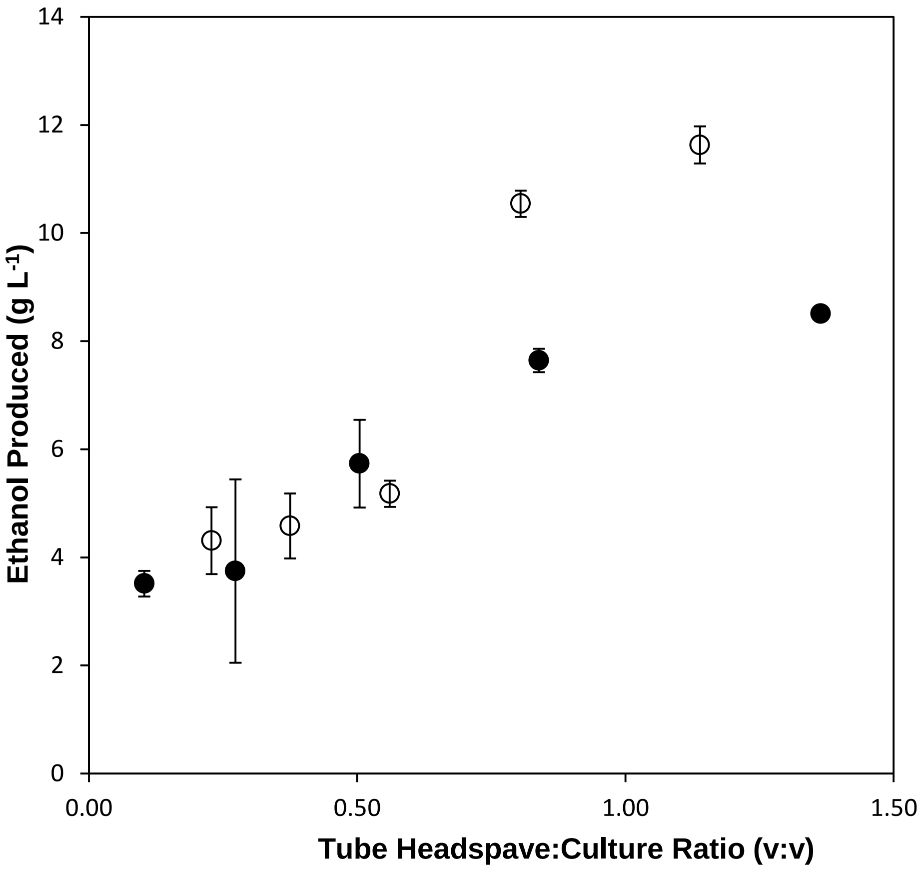

Figure 5.13. The relationship between ethanol production and headspace volume during fermentation of 40 g L^{-1} glucose in 50mM USM / 20 g L^{-1} (Table 2.7) by *G. thermoglucosidasius* strain TM242 in 15 mL and 50 mL conical bottom tubes filled to various volumes with culture. Fermentations were carried out at an initial pH of 7.0 in a shaking incubator set to 60°C and 250 rpm and were run for 24 hours. ●: Ethanol produced, 15 mL tubes; ○: Ethanol produced, 50 mL tubes. Data shown is the average of n=3 replicates. Error bars show one standard deviation around the mean. Experiments performed as described in Section 2.9 and ethanol concentration determined as in Section 2.10. Data analysed as described in Section 2.11

It can be seen that there is a clear link between a larger headspace volume and increased ethanol production. Linear relationship plots returned R^2 values of 0.92 for the 15 mL tube data and 0.89 for the 50 mL tube data. However if this were the only factor involved then the trends from both tube sizes would overlay. This is clearly not the case at the higher ratios of headspace to culture with ethanol production in the 50 mL tubes being around 25% higher than in 15 mL tubes with comparable ratios. The working volume is known to affect the degree of mixing and the k_La values in a tube and it is likely that these factors also have an effect on the data in Figure 5.12 (Backoff et al., 2012).

The data from this experiment clearly demonstrates that the volume of culture in a tube has filled has a substantial effect on the fermentation in the tubes. It has also provided clear evidence that fermentation performance is substantially better in 50 mL tubes than 15 mL tubes regardless of culture volumes. Therefore 50 mL tubes were chosen as the USD tube for the fermentation process and will be referred to as such from this point forwards.

5.4 Establishment of a robust USD tube fermentation protocol

Following the selection of 50 mL tubes as USD tubes for fermentation, a series of experiments were performed to examine the effect of initial sugar concentration, culture volume and shaker agitation rate on fermentation performance. Glucose concentrations of 20 g L^{-1} to 60 g L^{-1}, working volumes of 20 mL to 40 mL and shaking frequencies of 50 rpm to 250 rpm were evaluated.

5.4.1 The effect of culture volume and shaking frequency on glucose consumption during fermentation in USD tubes

From Figure 5.14 it is clear that a shaking frequency of 50 rpm leads to only very limited utilisation of glucose, regardless of the initial concentration present. From the k_La studies carried out in the USD tubes (Figure 3.12) it is known that the k_La values achieved at 50 rpm are very low, around 5 h^{-1}. Given the decline in fermentation performance in STR cultures grown at low k_La values (Section 5.2.1) it is perhaps not surprising that the performance seen in the tubes at 50 rpm was very poor, and confirms that the volumetric oxygen transfer coefficient is in important factor affecting fermentation performance in USD tubes as well as STRs. Given the poor utilisation of glucose at 50 rpm, the results of tubes grown at this shaking frequency will not be discussed at length in this section.

With 20 g L^{-1} glucose present in the culture, full utilisation of the sugar was achieved at both 150 rpm and 250 rpm regardless of the culture volume in the tubes. This concentration of glucose is clearly well within the capacity of the USD tubes and as such it is difficult to gain any information about the optimal culture shaking frequency or working volume from the 20 g L^{-1} USD tube cultures. Again this data will therefore not be discussed at length in this section.

With the initial glucose concentration increased to 40 g L^{-1} a clear difference between shaking frequencies of 150 rpm and 250 rpm can be seen. At 150 rpm sugar utilisation is incomplete, with only around 50% of the glucose being consumed by the end of the fermentation with the volume of culture making little difference to the amount of glucose consumed. At 250 rpm with an initial glucose concentration of 40 g L^{-1} there was very little residual glucose with any of the working volumes. Utilisation was complete with a working volume of 20mL or 40mL with a very small residual glucose concentration with a fill of 30mL. This is likely just due to experimental variability with the working volume having little impact on sugar consumption with an initial glucose concentration of 40 g L^{-1}.

The proportion of glucose consumed when the initial glucose concentration was 60 g L^{-1} was substantially reduced relative to the lower glucose concentrations. As at the lower glucose concentrations, consumption of glucose improved at higher shaking frequencies, although even at 250 rpm a maximum of around 50% of the glucose was consumed - thus less glucose was consumed than with a 40 g L^{-1} starting concentration.

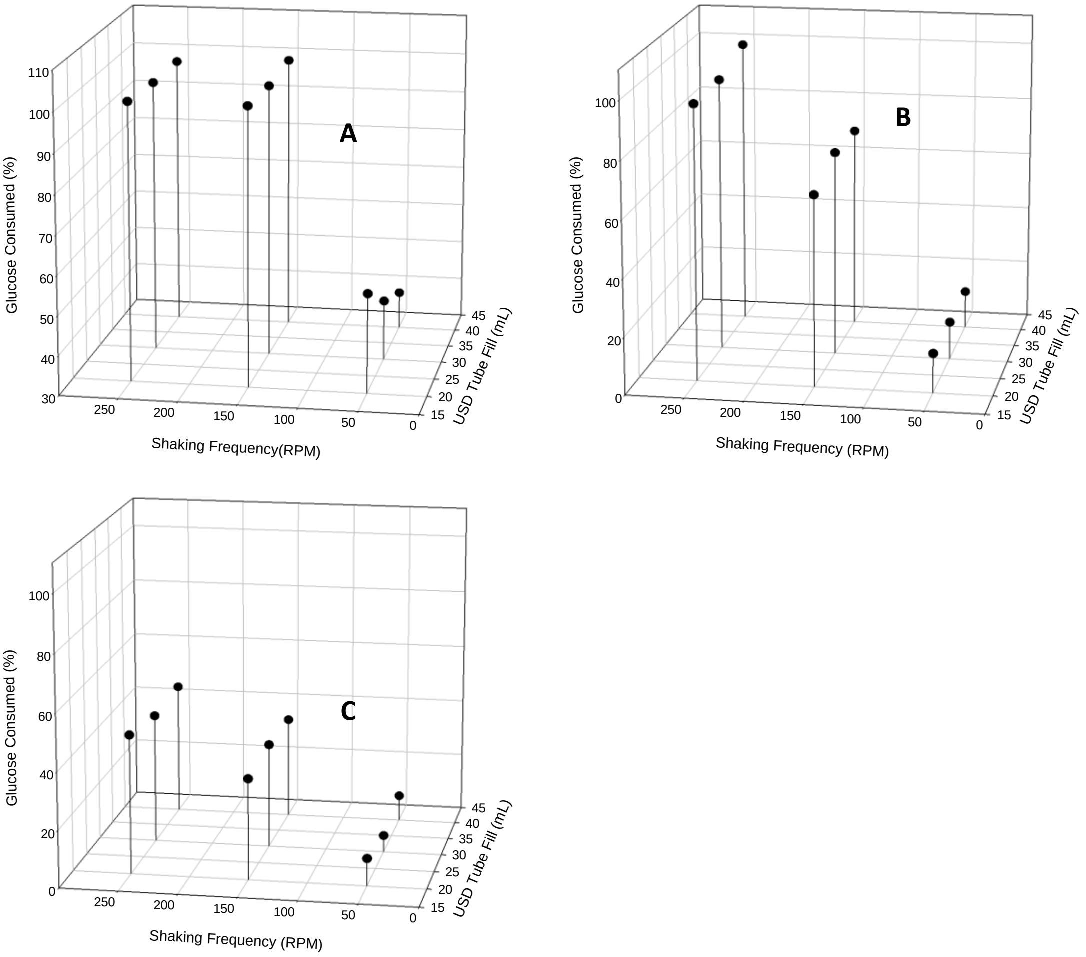

Figure 5.14. Glucose consumed following 24 hours of fermentation by *G. thermoglucosidasius* strain TM242 in USD tubes at working volumes of 20 mL, 30 mL and 40 mL and shaking frequencies of 50 rpm, 150 rpm and 250 rpm in 50mM USM / 20 g L^{-1} yeast extract media and initial glucose concentrations of A) 20 g L^{-1}; B) 40 g L^{-1} C) 60 g L^{-1} Droplines show the fill level and shaking frequency used. Fermentations were carried out at an initial pH of 7.0 in a shaking incubator set to 60°C. Data shown is the mean of n =2 replicates. Experiments carried out as described in Section 2.9 and glucose determined as in Section 2.10. Data analysed as described in Section 2.11.

5.4.2 Effect of working volume and shaking frequency on ethanol production during fermentation in USD tubes

In general terms the pattern of ethanol production is similar to that for the consumption of glucose seen in the previous section. This is to be expected given that ethanol is the primary product of the strain of *G. thermoglucosidasius* used in this work (Cripps et al., 2009). With a 20 g L^{-1} initial glucose concentration, peak ethanol titres slightly over 8 g L^{-1} were achieved as shown in Figure 5.15. This was achieved with a shaking frequency of 150 rpm and lower titres were seen at 50 rpm in line with the glucose utilisation. Interestingly ethanol production was also reduced at 250 rpm accompanied by an increase in acetate production. The ethanol production yields, defined as the mass of ethanol produced divided by the mass of glucose consumed, show a similar pattern with the highest yield, 0.4 g g^{-1}, being achieved with a fill of 40mL and a shaking frequency of 150 rpm.

With a glucose concentration of 40 g L^{-1} the highest ethanol titre was 14.3 g L^{-1}. This was achieved with a working volume of 40mL and a shaking frequency of 250 rpm. At lower working volumes the ethanol titres achieved were somewhat lower, at around 12-13 g L^{-1}, however the effect of working volume on ethanol titre was not statistically significant (ANOVA, $p > 0.05$). The accompanying ethanol yields, shown in Figure 5.16, reveal an interesting pattern with yields highest at the 40mL working volume, somewhat lower with the 20mL working volume and lower still at the intermediate working volume of 30mL. The peak yield of around 0.37 g g^{-1} was slightly lower than with a 20 g L^{-1} initial glucose concentration but the higher glucose consumption led to higher ethanol titres overall. As would be expected from the glucose utilisation figures, ethanol production at lower agitation rates was significantly lower regardless of working volume. The ethanol yields seen at 150 rpm were generally slightly lower than those seen at 250 rpm. Ethanol yields at 50 rpm were lower again, although this is of little relevance due to the very low levels of glucose utilisation.

As would be expected from the amount of glucose consumed with a 60 g L^{-1} starting concentration less ethanol was produced in the USD tubes with the highest initial concentration of glucose. As with the other initial glucose concentrations, ethanol production increased with shaking frequency. The peak ethanol titre achieved was just under 10 g L^{-1} with a 20mL working volume and shaking frequency of 250 rpm. Figure 5.16 shows that the ethanol yields for the 60 gl^{-1} USD tubes were highest at a shaking frequency of 50 rpm, although very little sugar was used at this agitation speed and so ethanol titres were still lower than at the higher shaking frequencies. Ethanol yields at the higher agitation rates were lower than seen with the smaller initial glucose concentrations.

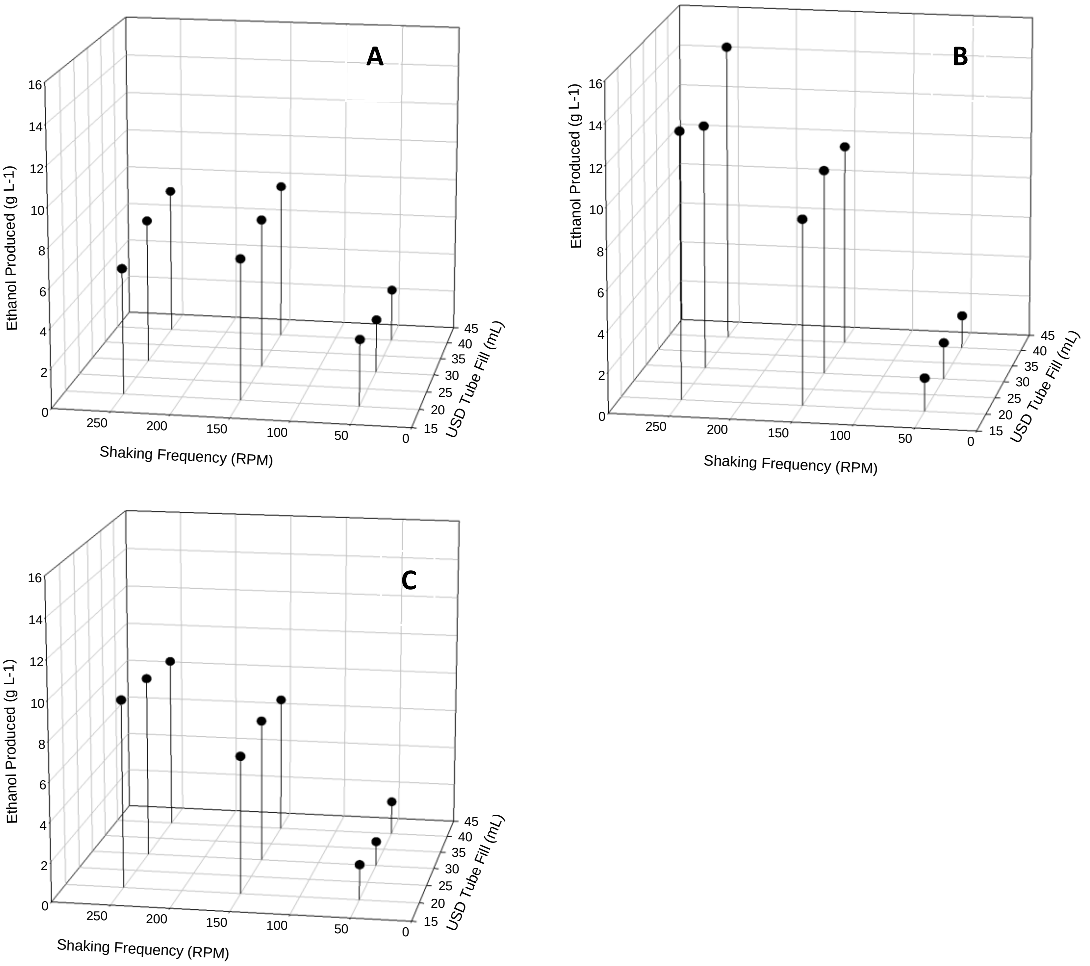

Figure 5.15. Ethanol produced following 24 hours of fermentation by *G. thermoglucosidasius* strain TM242 in USD tubes at working volumes of 20 mL, 30 mL and 40 mL and shaking frequencies of 50 rpm, 150 rpm and 250 rpm in 50mM USM / 20 g L^{-1} yeast extract media and initial glucose concentrations of A) 20 g L^{-1}; B) 40 g L^{-1} C) 60 g L^{-1}. Droplines show the fill level and shaking frequency used. Fermentations were carried out at an initial pH of 7.0 in a shaking incubator set to 60°C. Data shown is the mean of n =2 replicates. Experiments carried out as described in Section 2.9 and ethanol concentration determined as in Section 2.10. Data analysed as described in Section 2.11.

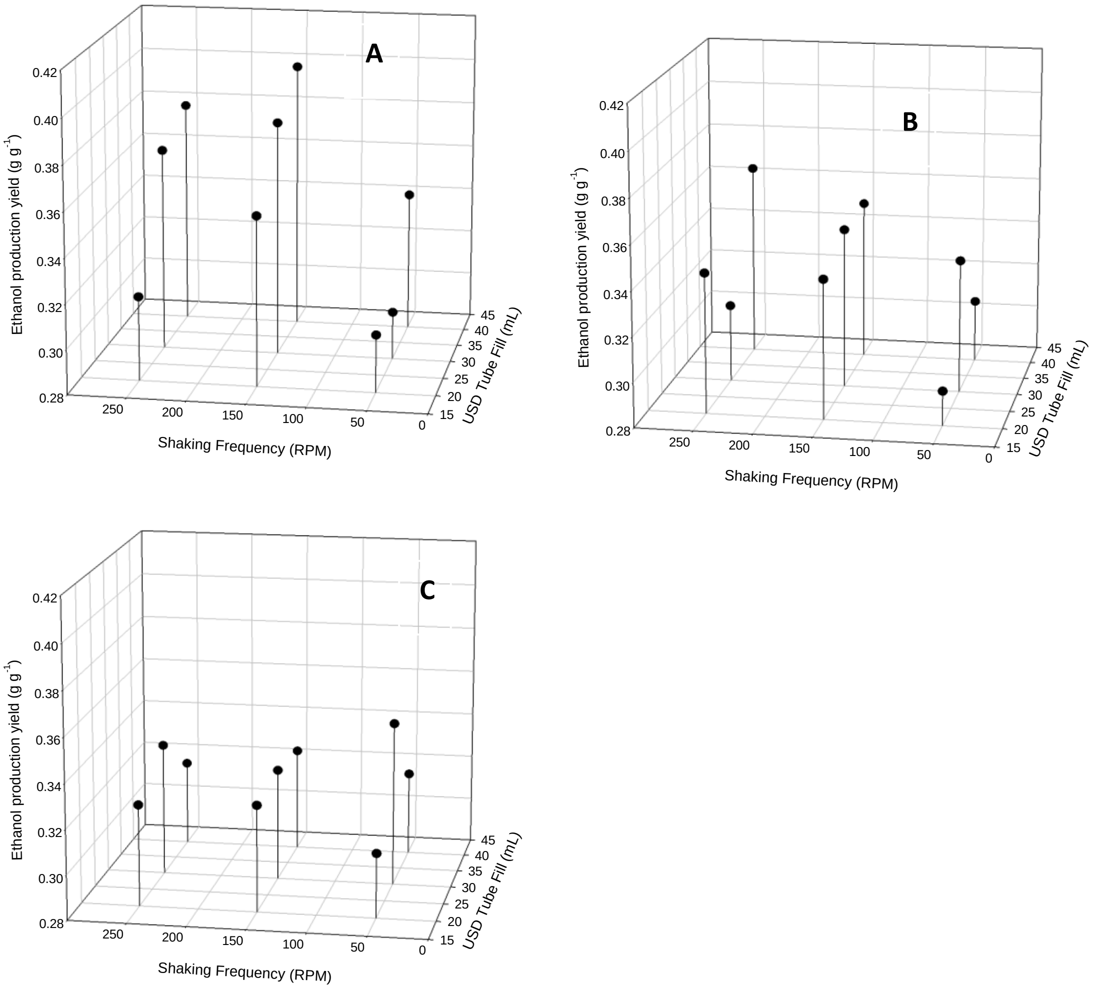

Figure 5.16. Ethanol production yield ($g_p\ g_s^{-1}$) following 24 hours of fermentation by *G. thermoglucosidasius* strain TM242 in USD tubes at working volumes of 20 mL, 30 mL and 40 mL and shaking frequencies of 50 rpm, 150 rpm and 250 rpm in 50mM USM / 20 g L^{-1} yeast extract media and initial glucose concentrations of A) 20 g L^{-1}; B) 40 g L^{-1} C) 60 g L^{-1} . Fermentations were carried out at an initial pH of 7.0 in a shaking incubator set to 60°C.y. Droplines show the shaking frequency and fill level used. Data shown is the mean of n =2 replicates. Experiments carried out as described in Section 2.9 and ethanol and glucose concentrations determined as in Section 2.10. Data analysed as described in Section 2.11.

5.4.3 Final pH in Tube fermentations at different agitation speeds, working volumes and initial sugar concentrations

In the USD tube cultures, control of pH was achieved solely by media buffering meaning that under circumstances where the buffering capacity is exceeded the pH of the USD tube cultures will be prone to variability. This is not an issue in the STR fermentations where automatic addition of acid or base is used to control pH within a narrow band.

Figure 5.17 shown the pH of the USD tube cultures measured at the end of the fermentation period and comparing it with the sugar utilisation data in Figure 5.15 there is a general trend for the final pH in the USD tubes to drop as the amount of glucose consumed increases particularly in the USD tubes with an initial glucose concentration of 40 g L^{-1} or 60 g L^{-1}. With the USD tubes shaken at a frequency of 50 rpm and little glucose consumed, the pH values at the end of the fermentation are close to the initial pH of 7.0. When the USD tubes were shaken at 150 rpm the final pH in both the 40 g L^{-1} glucose and 60 g L^{-1} glucose runs was around 6.2. At the highest shaking frequency tested the final pH was slightly lower again, generally 6.1 or lower. With both concentrations of sugar and USD tubes shaken at 250 rpm there was a trend for the final pH to decrease as the working volume of the tubes decreases. This is particularly noticeable in Figure 5.17 C where at the highest shaking frequency and lowest working volume the final pH dropped as low as 5.7.

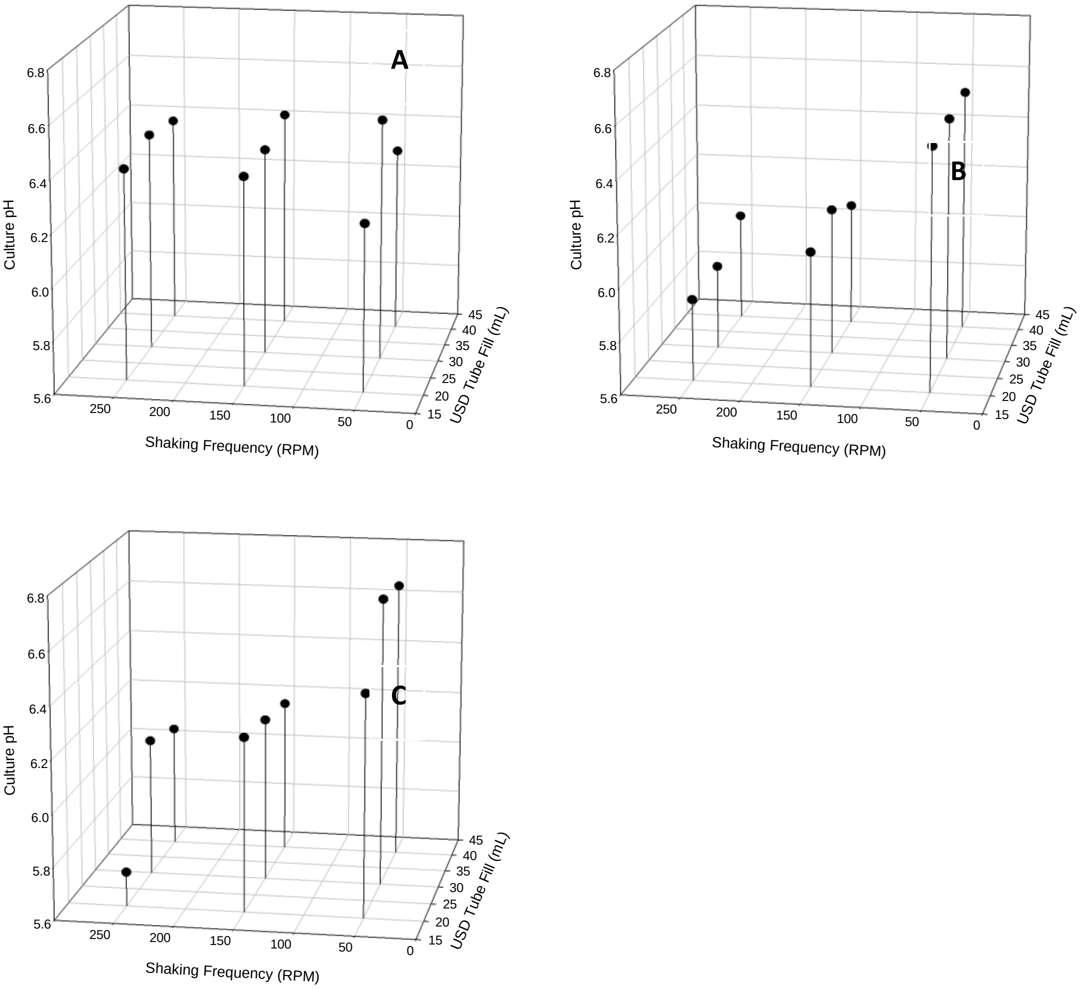

Figure 5.17. Final culture pH following 24 hours of fermentation by *G. thermoglucosidasius* strain TM242 in USD tubes at working volumes of 20 mL, 30 mL and 40 mL and shaking frequencies of 50 rpm, 150 rpm and 250 rpm in 50mM USM / 20 g L^{-1} yeast extract media and initial glucose concentrations of A) 20 g L^{-1}; B) 40 g L^{-1} C) 60 g L^{-1} Fermentations were carried out at an initial pH of 7.0 in a shaking incubator set to 60°C. Droplines show the shaking frequency and fill level used. Data shown is the mean of n =2 replicates. Experiments carried out as described in Section 2.9. Data analysed as described in Section 2.11.

The most obvious pattern discernible from these initial studies is that the best fermentation results, in terms of both glucose consumption and ethanol production, were achieved when using a shaking frequency of 250 rpm. The USD tubes are capable of achieving full utilisation of an initial concentration of 20 g L^{-1} or 40 g L^{-1} glucose, but not an initial concentration of 60 g L^{-1}. Indeed with this higher initial concentration, less glucose was consumed than with an initial 40 g L^{-1} concentration. One possible explanation for poor performance at a higher initial sugar concentration would be limitation of other nutrients within the media formulation, with the fixed quantities of nutrients available being insufficient to allow consumption of more than a certain concentration of sugar. However, this does not fit with the observation that less sugar was used when 60 g L^{-1} was present initially than when 40 g L^{-1} was. With nutrient limitation consumption of at least 40 g L^{-1} would be expected in both cases. Additionally, the media formulation used in these experiments, USM with 2% (w/w) yeast extract is a 'rich' media and should contain sufficient nutrients to allow utilisation of 60 g L^{-1} glucose. Similar reasoning would rule out any limitation in the total supply of oxygen available to the USD tubes.

From the evidence available it therefore seems more likely that the substantial drop in pH seen at the end of fermentation with higher glucose concentrations could in fact be causing fermentation to be inhibited. The pH values seen at the end of the fermentation are significantly outside of the optimum pH of the strain of *G. thermoglucosidasius* used and therefore poor fermentation performance would be expected. The pH drop appears to be more acute when the initial concentration of glucose was 60 g L^{-1} which suggests that there may also be some toxicity to the level of sugar used, leading to a stress response that alters the metabolism of the organism and causes greater production of acidic metabolites. This is supported by the data in Figure 5.16 which show generally lower fermentation yields in the USD tubes that were started with 60 g L^{-1} glucose. The lower production of ethanol was accompanied by a corresponding increase in the concentration of acetate, the anion of acetic acid, formed.

From an industrial perspective the results obtained with the highest initial sugar concentration are of most relevance. Although better ethanol production was seen at higher working volumes at the lower glucose concentrations, the best performance with a 60 g L^{-1} initial concentration was seen with a 20 mL working volume. This working volume was therefore used for future work.

5.5 Control of pH in USD tube fermentations

As there is no on-line pH control facility available for use with the USD tubes control of pH can be achieved through the use of buffer solutions. The experiments in Section 5.4 were carried out using a mixture of buffers solutions that had been developed at ReBio Technologies during previous work. As the data in Figure 5.17 suggested that inadequate pH control was limiting fermentation performance in the USD tubes an experiment was set up to investigate whether alternative mixtures of buffer solutions could improve control of pH and therefore fermentation performance. Examination of the pK_a values of the buffers used in the current mixture suggested that better buffering against acidic pH drift would be achieved by increasing the proportion of bis-tris in the medium (Table 2.9). The ability to overcome a pH drift towards acidic values will be enhanced at the expense of the ability to compensate for a drift towards basic pH values, however, given that Figure 5.17 showed a very clear trend for the cultures to drift towards acidic pH values this is unlikely to be of practical relevance. Several alternative buffer formulations, set out in Table 5.3, were therefore examined for their effect on the fermentation performance of TM242 in USD tube cultures.

Figure 5.18 shows the effect of the different buffer formulations on both consumption of glucose and production of ethanol. These experiments were carried out with two initial sugar concentrations, 40 g L^{-1} and 60 g L^{-1}. With an initial glucose concentration of 40 g L^{-1} there was essentially no difference between any of the three alternative formulations and the control tubes. Full utilisation of the glucose was achieved, and approximately 15.5 g L^{-1} of ethanol was produced. When the initial concentration of glucose was increased to 60 g L^{-1} there was a clear difference between the performance of the control tubes and that of the new buffer formulations. There was not however any significant difference between the three alternative buffer mixtures tested. In the control tubes, labelled as D, there was approximately 18 g L^{-1} glucose remaining and 15.6 g L^{-1} of ethanol was produced. With the altered buffer formulations, the residual glucose concentration fell to between 13 g L^{-1} and 14 g L^{-1} whilst ethanol production increased to just under 18 g L^{-1}. The difference in performance between the control and formulations B and C were statistically significant (*Student's t-test, $p<0.05$*). The difference between formulation A and the control was not statistically significant due to the high variability seen with formulation A. This high variability could also be seen with a 40 g L^{-1} glucose concentration suggesting performance with this formulation may be inherently more variable than with the other mixtures. As it offers no significant benefits over formulations B or C there would be no reason to progress with formulation A.

The improved fermentation performance seen with two alternative buffer formulations, that offer better buffering capacity when a culture is drifting towards acidic pH values, corroborates the hypothesis that pH drift in the cultures limits performance in USD tubes. However the final pH values, recorded in Table 5.4, with formulations B and C were only very slightly higher than in the control USD tubes. The final values of 6.22 and 6.23 are still well below the optimum for the organism used and are in the region where inhibition of growth will occur. It seems reasonable to assume therefore that the alternative buffer formulations somewhat slowed the acidic drift in pH in the USD tubes but still lacked sufficient buffering capacity to stop the drift completely. As was the case in the earlier experiments the main secondary product produced alongside ethanol was acetic acid (data not shown). This was responsible for the acidic drift in pH that is occurring and limiting the fermentation potential of the *G. thermoglucosidasius* strain used.

Although the benefit obtained through altering the buffer formulation in use was small, the statistically significant improvement in performance seen meant that all further work was carried out using formulation C from Table 5.3 and thus media buffering was henceforth achieved with bis-tris only (Table 2.8).

Table 5.3. Buffer formulations tested for effect on pH drop in tube fermentations. Volumes shown are for addition to a USD tube with a working volume of 20 mL. Buffers were included in 50mM USM media with 20 gL^{-1} yeast extract and 40 gL^{-1} glucose (Table 2.7).

Formulation	**MOPS (mL)**	**Bis-Tris (mL)**	**HEPES (mL)**
A	1.1	1.1	0
B	0.7	1.5	0
C	0	2.2	0
D (standard)	0.7	0.7	0.7

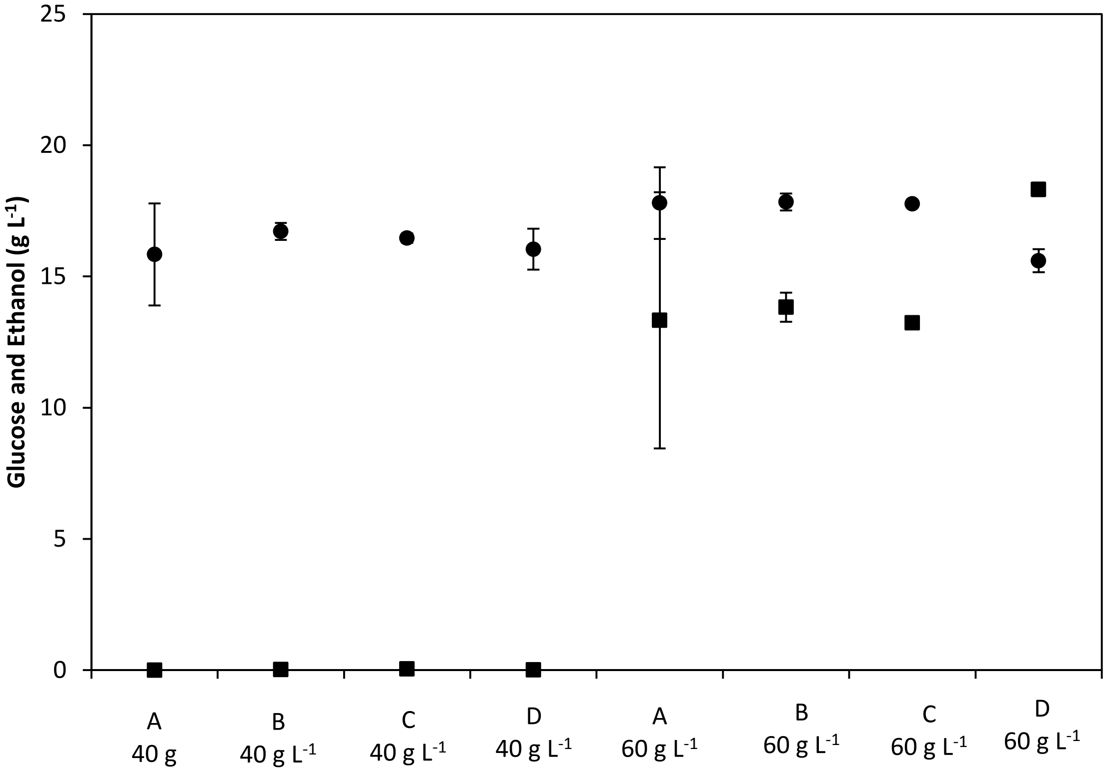

Figure 5.18. Residual glucose and ethanol produced following 24 hours of fermentation by *G. thermoglucosidasius* strain TM242 in USD tubes with four buffer formulations, as outlined in Table 5.3, and initial glucose concentrations of 40 g L^{-1} and 60 g L^{-1}. Fermentations were carried out in a shaking incubator set to 60°C and 250 rpm in a background of 50mM USM / 20 g L^{-1} yeast extract (Table 2.7). ■: Residual glucose; ●: Ethanol produced. Data shown is the average of n=3 replicates and error bars show one standard deviation around the mean. Experiments carried out as described in Section 2.9 and concentrations determined as in Section 2.10. Data analysed as described in Section 2.11.

Table 5.4. Key fermentation performance metrics for with various pH buffer mixes, as in Table 5.3, in USD tubes with 60 g L^{-1} starting glucose in a background of 50mM USM / 20 g L^{-1} yeast extract. Data is taken from the experiment shown in Figure 5.18.

Buffer Formulation	**A**	**B**	**C**	**D**
Glucose used (gl^{-1})	46.7	46.2	46.8	41.7
Ethanol produced (gl^{-1})	17.8	17.8	17.8	15.6
Product yield (g g^{-1})	0.38	0.39	0.38	0.37
Final pH	6.16	6.22	6.23	6.16

5.6 Fermentation in STRs and USD tubes at matched k_La values

5.6.1 Comparability of fermentations in soluble (solid-free) media

A review of the literature available suggested that k_La was the most suitable basis for scale translation between STRs and the USD tubes (Section 3.1). Characterisations of k_La in both STRs and the USD tubes were therefore carried out and correlations were developed that allowed prediction of the conditions required to achieve a given value of k_La (Sections 3.3 and 3.6). The experiments described in Section 5.2.1 suggested that optimal conversion of 40 g L^{-1} of mixed sugars to ethanol by TM242 occurred at a k_La value of 50 h^{-1}. Comparable experiments were therefore run using the USD tubes at the same k_La value. The utilisation of glucose and xylose and the production of ethanol in the USD fermentations and a comparable experiment in STRs is shown in Figure 5.19.

A generally good comparison between the two scales is observed with broadly similar rates of glucose utilisation and ethanol production being achieved. Looking at the glucose utilisation rate in detail it is observed that utilisation is initially slightly slower in the USD tubes before increasing to a level above that of the STRs. Over the first four hours of fermentation glucose utilisation in the STR is at an average rate of 2.9 g L^{-1} h^{-1} whilst in the USDs the rate was 2.2 g L^{-1} h^{-1}. Over the following two hours the peak rates of glucose utilisation were seen in both systems although the rate in the USD tubes (9.1 g L h^{-1}) was around 50% higher than in the STRs (6.3 g L^{-1} h^{-1}). It should be noted however that the rate in USD tubes is calculated from only two data points and should be treated with caution. Full utilisation of the available glucose appears to have occurred at approximately the same point in both systems.

Ethanol production is similar in the two systems in terms of both rate and peak titre. The maximum titre achieved in the STR was 11.7 g L^{-1} compared to 12 g L^{-1} in the USD tube and taking into account variations in the amount of sugar used both systems gave ethanol production yields of around 0.34 grams of ethanol produced per gram of glucose consumed. The peak ethanol titre was observed following around 9 hours of fermentation in the STR and after 12 hours fermentation in the USD tubes. Thus the time difference between complete utilisation of glucose occurring and peak ethanol being observed is greater in the USD tube fermentations, although given the very similar titres and production yields this is unlikely to be of significant importance.

The most noticeable difference between the two systems in Figure 5.19 is in the utilisation of xylose. Utilisation is essentially identical in the two systems first six hours of fermentation when little xylose is consumed in either. Following this point the rate of utilisation in the STR increases and the xylose is fully consumed over the following six hours. In the USD tube fermentations by contrast, xylose utilisation remains incomplete at the end of the fermentation.

The levels of biomass produced in the fermentations are shown in Figure 5.20. It can be seen that at all three sample points the level of biomass measured was higher in the STR than in the USD tubes. After six hours of fermentation the difference was around 3 g L^{-1}, increasing to 5 g L^{-1} by the 12 hour sample. Peak biomass was recorded in the final sample in both systems (24 hours in the STRs, 25 hours in the USDs) with concentrations of 34.5 g L^{-1} in the STR and 30.5 g L^{-1} in the USD tubes, a difference of 13%.

This difference was not calculated to be statistically significant (*p>0.05*). The differences in biomass concentration in the six and 12 hour samples were likewise not statistically significant.

Taken together the results in Figures 5.19 and 5.20 show good comparability between the STR and USD tube fermentations using soluble media suggesting that the USD tubes are a suitable scale-down model as an alternative to the STR system currently in use. The differences in fermentation profiles seen between USD tubes and STRs in Figure 5.19 are no greater than the differences seen between STR replicates in Figures 5.8 and 5.9. Other differences observed, such as with biomass levels, were not found to be statistically significant. The results also confirm the suitability of k_La as a basis for scale translation between the two systems.

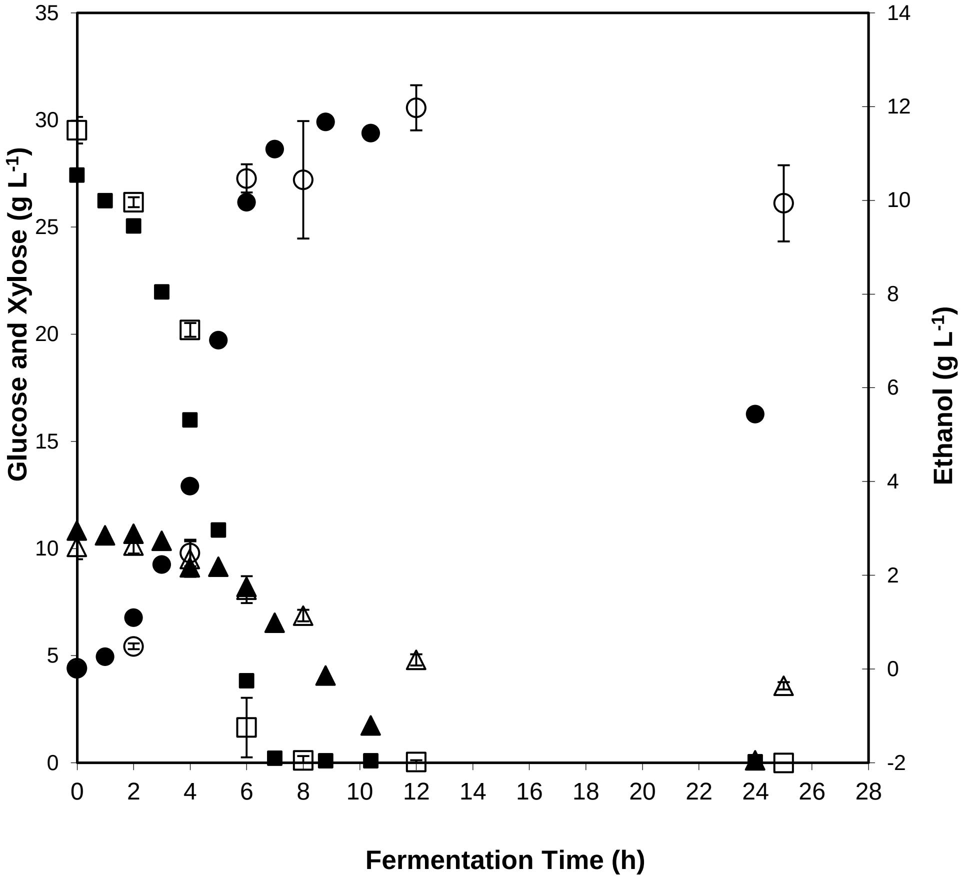

Figure 5.19. Comparison of sugar utilisation and ethanol production during fermentation of 40 g L^{-1} mixed sugars by *G. thermoglucosidasius* strain TM242 in STRs and USD tubes at a matched k_La of 50 h^{-1}. STR fermentations were run at 60 °C and a working volume of 4L with on-line pH control in a background of 50mM USM / 20 g L^{-1} yeast extract (Table 2.6). USD fermentations were carried out at a working volume of 20 mL in a shaking incubator set to 60 °C in a background of buffered 50mM USM / 20 g L^{-1} yeast extract (Table 2.8). ■: Glucose in STRs; □: Glucose in USD tubes; ▲: Xylose in STRs; △: Xylose in USD tubes; ●: Ethanol in STRs; ○: Ethanol in USD tubes. Data shown is the average of n=3 replicates for the USD tubes and error bars show one standard deviation around the mean. Experiments carried out as described in Sections 2.8 and 2.9 and concentrations determined as in Section 2.10. Data analysed as described in Section 2.11.

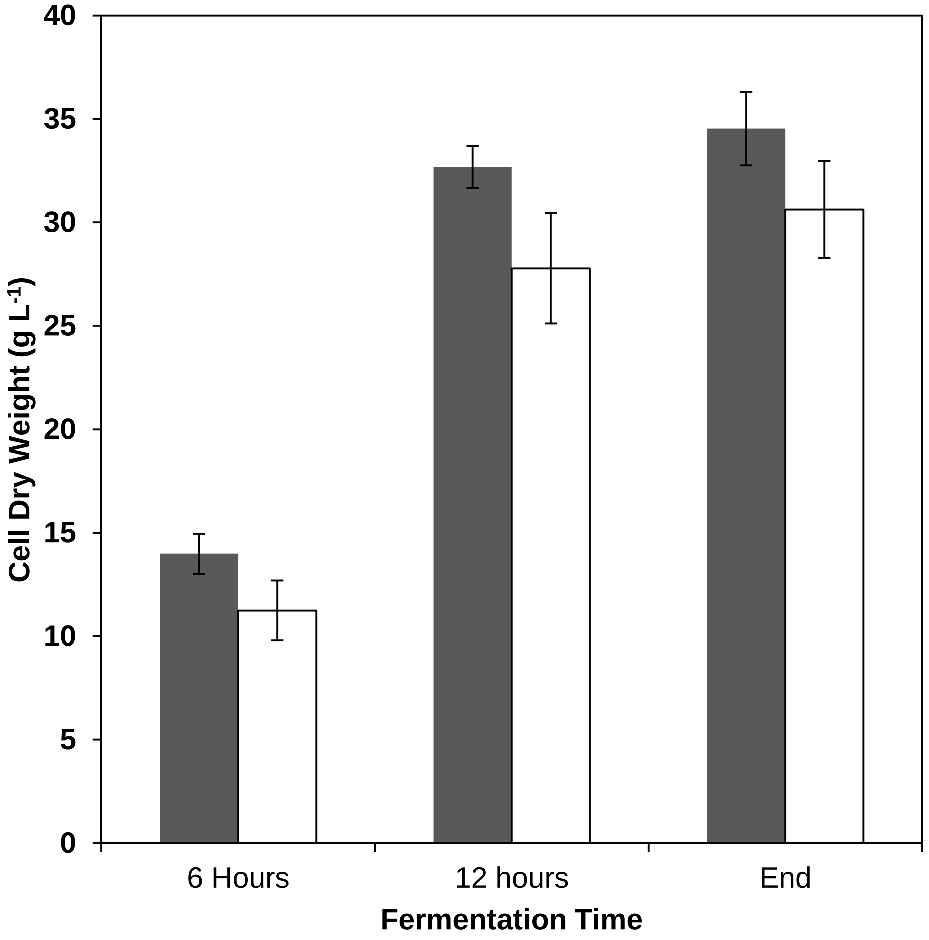

Figure 5.20. Biomass (Cell Dry Weight) produced during the fermentation of 40 g L^{-1} mixed sugars by *G. thermoglucosidasius* strain TM242 in STRs and USD tubes at a matched k_La of 50 h^{-1}. STR fermentations were run at 60 °C and a working volume of 4L with on-line pH control in a background of 50mM USM / 20 g L^{-1} yeast extract (Table 2.6). USD fermentations were carried out at a working volume of 20 mL in a shaking incubator set to 60 °C in a background of buffered 50mM USM / 20 g L^{-1} yeast extract (Table 2.8). Filled bars show data from STRs; hollow bars show data from USD tubes. Data shown is the average of n=3 replicates for the USD tubes and error bars show one standard deviation around the mean. Experiments carried out as described in Sections 2.8 and 2.9. Cell dry weight determined as described in Section 2.10.6. Data analysed as described in Section 2.11.

5.6.2 Effect of suspended solids on fermentation

It has been shown that the suspended solids in DDGS have a substantial effect on the volumetric oxygen transfer rate in STRs and USD tubes in Sections 3.2 and 3.6 respectively. The presence of suspended solids leads to a reduction in k_La values in both STRs and USD tubes. Because of this observation it was decided to examine what effect, if any, the presence of suspended solids has on fermentation in USD tubes. The suspended solids used were those insoluble solids that were recovered following enzyme hydrolysis (Section 2.9.5) and were added at 8% (w/w), the concentration at which they were recovered from the hydrolysate. Figure 5.21 shows that, when USD tubes are used to ferment media with and without suspended solids at identical shaking frequencies, the presence of suspended solids in the media has a substantial effect on the profile of the fermentation. In the absence of solids, utilisation of glucose is detected from the first sample whereas the presence of solids causes a lag period of approximately 4 hours before substantial glucose utilisation occurs. The rate of glucose utilisation is also reduced by the addition of the suspended solids. The rate of glucose utilisation during the peak period of growth was reduced by around 50% to 3.3 g L^{-1} h^{-1} with solids present. By the end of the fermentation full glucose utilisation occurred in both systems, although full utilisation occurred in an overnight sampling gap for the tubes with solids added. The utilisation of xylose by TM242 was similarly delayed and reduced in rate when solids were added to the USD tubes. As was the case with glucose the residual concentration of xylose at the end of the fermentation was similarly unaffected by the presence of solids.

Analysing the ethanol production trend, it is clear that adding solids into the USD tubes has an effect on both the ethanol titre and yield of ethanol relative to sugar consumed. In the absence of solids a peak titre of 12 g L^{-1} was recorded after 12 hours of fermentation; with solids a peak of 8 g L^{-1} was recorded at 24 hours. Given the amount of sugar consumed was not significantly affected by the presence of the solids ($p > 0.05$) it is the yield of ethanol produced per gram of sugar consumed that has been reduced. The yield figures are 0.34 g g^{-1} and 0.21 g g^{-1} for the USD tubes without and with suspended solids respectively.

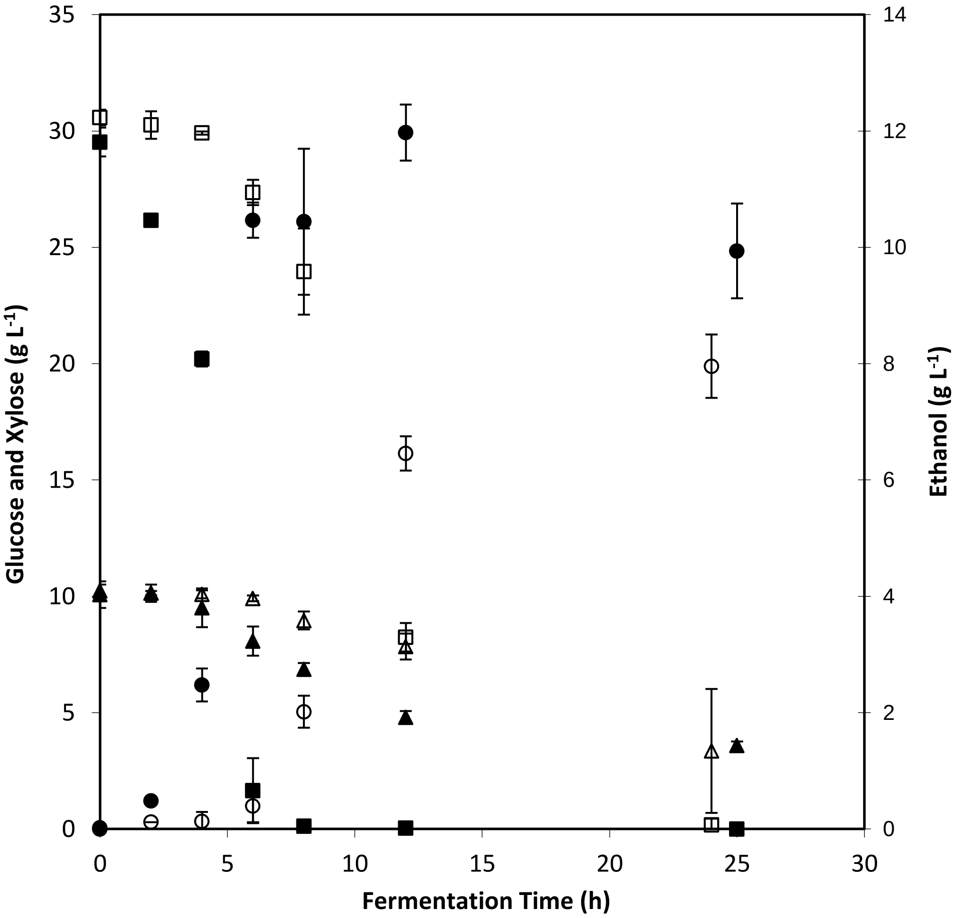

Figure 5.21. Effect of 8% (w/w) suspended hydrolysed DDGS solids on the fermentation of 40 g L^{-1} mixed sugars in a buffered 50mM USM / 20 g L^{-1} yeast extract (Table 2.8) background by *G. thermoglucosidasius* strain TM242 in USD tubes run at matched shaking frequencies. Fermentations were carried out at a working volume of 20 mL and initial pH of 7.0 in a shaking incubator set to 60°C for

24 hours. ■: Glucose without solids; ▲: xylose without solids; ●: ethanol without solids; □ : glucose with solids; △: xylose with solids; ○: ethanol with solids. Experiments carried out as described in Section 2.9 and concentrations determined as in Section 2.10. Data analysed as described in Section 2.11.

The observations from Section 3.6 mean that when USD tubes are shaken at the same frequency with and without suspended solids substantially lower rates of oxygen transfer will occur in the tubes with suspended solids added. It is therefore a reasonable expectation that if the shaking frequency used were to be increased in order to restore the system k_La to that seen in the absence of solids, fermentation performance in USD tubes containing suspended solids should match that seen in solids free media. Eq. 3.8 was therefore used to calculate the shaking frequency needed to match k_La values in the presence and absence of solids. Figure 5.22 demonstrates that that the increased oxygen transfer in the USD tubes run at matched k_La does indeed lead to an improvement in the fermentation profile. The rate of glucose consumption during the period of peak growth increased to 4.2 g L h^{-1} and by 12 hours into the fermentation only 1.6 g L^{-1} glucose remained. Ethanol production was likewise improved with a titre of 9.1 g L^{-1} being achieved at a yield of 0.24 grams of ethanol produced for each gram of sugar consumed. As would be expected given the longer lag period, the utilisation of xylose began later when suspended solids were added to the media in the USD tubes. Once utilisation was underway the rates of consumption were similar regardless of the presence of solids and indeed by the end of the fermentation residual xylose was 1.5 g L^{-1} lower in the system containing suspended solids.

The improvements in performance in the solids containing USD tubes run at matched k_La rather than matched shaking conditions are more clearly seen in Figure 5.23 where they are directly compared. The lag period before glucose utilisation begins is similar with both matched shaking frequency and matched k_La, the rate of glucose utilisation following this is, as noted, higher when the fermentations occur under conditions of matched k_La. In the USD tubes run at matched shaking frequency 73% of the glucose had been consumed within the first 12 hours of fermentation; when the tubes were instead run at matched k_La this increased to 95%. The differences seen in glucose utilisation did not correspond to any changes in xylose utilisation as there were no statistically significant differences in any of the experimental samples. The increase in ethanol production can be clearly seen and, as discussed above, is related both to the rate at which glucose is consumed in the USD tubes and the conversion yield of sugars to ethanol. The difference in peak ethanol tires seen was found to be statistically significant (paired t-test, $p < 0.05$).

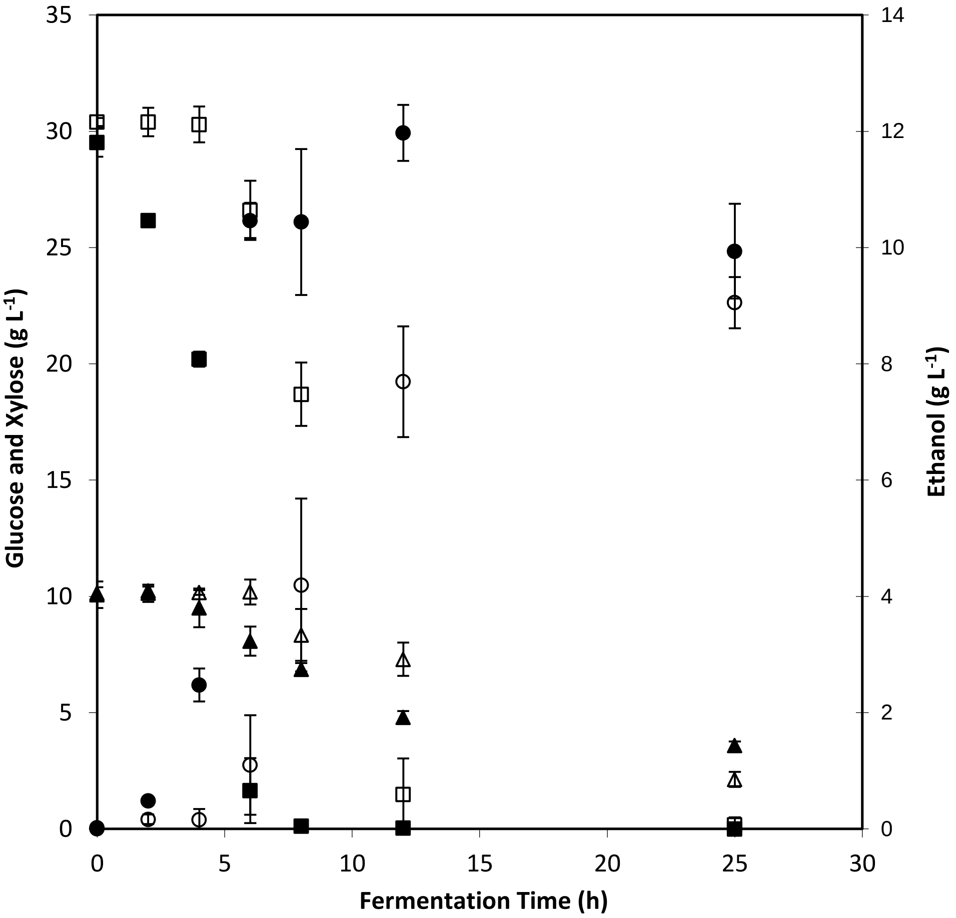

Figure 5.22. Effect of 8% (w/w) suspended hydrolysed DDGS solids on the fermentation of 40 g L^{-1} mixed sugars in a buffered 50mM USM / 20 g L^{-1} yeast extract (Table 2.8) background by *G. thermoglucosidasius* strain TM242 in USD tubes run at matched k_La values of 50 h^{-1}, correcting for the effect of the suspended solids on k_La. Fermentations were carried out at a working volume of 20 mL in a shaking incubator set to 60°C for 24 hours. *G. thermoglucosidasius*. ■: Glucose without solids; ▲: xylose without solids; ●: ethanol without solids; □ : glucose with solids; △: xylose with solids; ○: ethanol with solids. Experiments carried out as described in Section 2.9 and concentrations determined as in Section 2.10. Data analysed as described in Section 2.11.

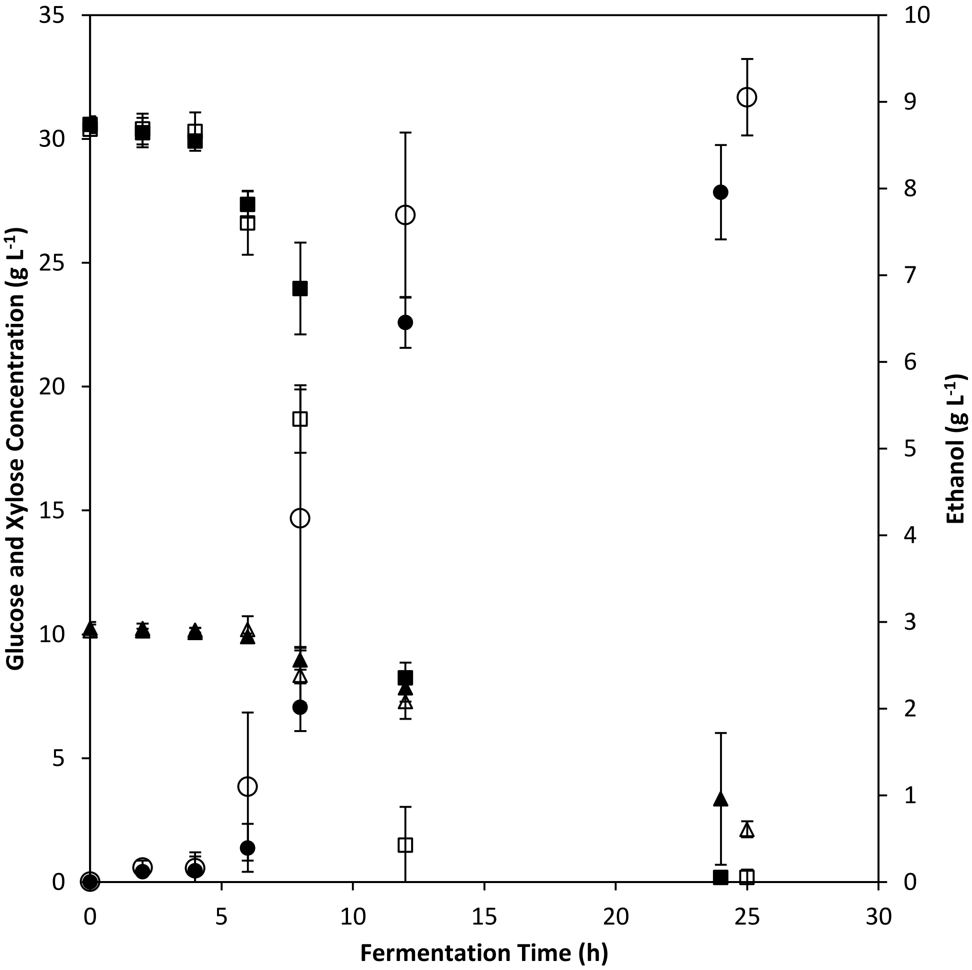

Figure 5.23. Fermentation of 40 g L^{-1} mixed sugars in the presence of 8% (w/w) suspended DDGS solids by *G. thermoglucosidasius* strain TM242 in USD tubes run at either matched shaking frequency or matched k_La values. Fermentations were carried out in a background of 50mM USM / 20 g L^{-1} yeast extract (Table 2.8) at a working volume of 20 mL. Data is taken from Figures 5.21 and 5.22 ■: Glucose, matched shaking frequency; ▲: xylose, matched shaking frequency; ●: ethanol, matched shaking frequency; □ : glucose, matched k_La; △: xylose, matched k_La; ○: ethanol, matched k_La. Experiments carried out as described in Section 2.9 and concentrations determined as in Section 2.10. Data analysed as described in Section 2.11.

5.6.3 Comparability of fermentations in the presence of suspended solids

The data in the previous section demonstrated that the addition of suspended solids derived from DDGS clearly has an effect on the fermentation of mixed sugars in USD tubes. Some of this can be explained by the effect of suspended solids on k_La that was observed in Section 3.6 but Figure 5.22 demonstrates that there is an additional effect above and beyond that caused by differences in k_La. In order for the USD tubes to be an effective scale-down mimic of STRs for use in lignocellulosic ethanol projects it is important that they are effective mimics of performance when suspended solids are a part of the system as well as in solids-free media.

Therefore fermentations were carried out in an STR using the same suspended solids containing media as was used in the USD tubes. The fermentations were run at a matched k_La in both systems. Figure 5.24 shows that the profile obtained in the STR was similar to that obtained using USD tubes.

Looking at the glucose concentration it can be seen that there is a longer lag period in the USD tubes at around 4 four hours rather than two in the STRs. Once utilisation of glucose begins the utilisation profiles in the two systems are very similar. Over the first two hours of growth the rate of glucose utilisation was 1.8 g L^{-1} h^{-1} in the USD tubes and 1.4 g L^{-1} h^{-1} in the STRs. As seen in the absence of any solids the rate of glucose utilisation increases noticeably after the first few hours of growth until glucose exhaustion occurs. In the USD tubes this higher rate of utilisation was 4.2 g L^{-1} h^{-1} whilst in the STRs it was 4.5 g L^{-1} h^{-1}. Thus the differences in glucose utilisation seen in Figure 5.24 are attributable to variability in the lag period at the start of the fermentation.

As seen in the absence of suspended solids xylose utilisation begins after glucose utilisation and proceeds at a significantly lower rate leaving some residual xylose at both scales. The initial xylose concentration in the USD tubes was around 2 g L^{-1} higher than in the STRs and this differential remained throughout much of the fermentation. At the end of the fermentation residual xylose was around 1 g L^{-1} in the STR and 2 g L^{-1} in the USD tubes.

Ethanol production was similar at both scales in terms of peak titres and production yields. In the STR 9.7 g L^{-1} was produced at a yield of 0.26 g g^{-1} whilst in the USD tubes 9.1 g L^{-1} was produced at a yield of 0.24 g g^{-1} (paired t-test, $p > 0.05$ for ethanol titre and production yield). Ethanol production yields at intermediate time points ranged from 0.26 g g^{-1} to 0.36 g g^{-1} in the STR and 0.24 g g^{-1} to 0.31 g g^{-1} in the USD tubes. These ethanol yields are significantly lower than seen in the solids free fermentations and this is explained by the production of acetate. In the solids free fermentations production of acetate was minimal whilst in Figure 5.24 it can be seen that acetate concentrations at the end of the fermentation are of similar levels to those of ethanol.

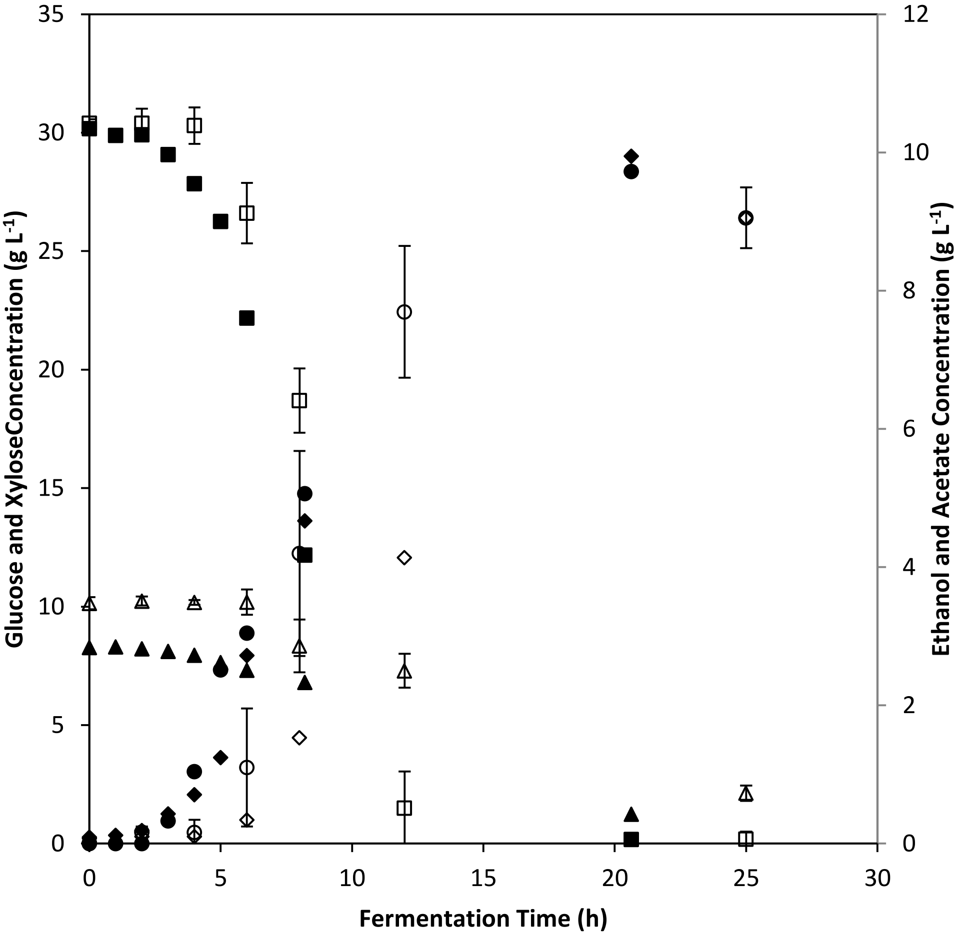

Figure 5.24. Utilisation of sugars and production of ethanol during fermentation of 40 g L^{-1} mixed sugars by *G. thermoglucosidasius* strain TM242 in STRs and USD tubes containing DDGS derived suspended solids at a matched k_La value of 50 h^{-1}. STR fermentations were carried out at in a background of 50mM USM / 20 g L^{-1} yeast extract (Table 2.6) at a working volume of 4 L at 60°C with on-line pH control. USD fermentations were carried out in a background of buffered 50mM USM / 20 g L^{-1} yeast extract (Table 2.8) at a working volume of 20 mL in a shaking incubator set to 60°C. ■: Glucose in STRs; □: Glucose in USD tubes; ▲: Xylose in STRs; △: Xylose in USD tubes; ●: Ethanol in STRs; ○: Ethanol in USD tubes; ♦:Acetate in STRs; ◊: Acetate in USD tubes. USD tube data shown is the average of n=3 replicates and error bars show one standard deviation around the mean. Experiments carried out as described in Sections 2.8 and 2.9 and concentrations determined as in Section 2.10. Data analysed as described in Section 2.11.

5.7 Summary

Section 5.1 set out the aims for this chapter which were to determine the optimal fermentation conditions at STR scale, to develop methods allowing fermentation to be carried out in USD tubes and then to compare fermentation performance in the USD tubes and STRs to assess the suitability of the USD tubes as a scale-down model of the fermentation stage of a lignocellulosic ethanol process. It also set out the criteria which would be used to evaluate the relative performance of the two systems.

In Section 3.3 correlations predicting k_La were developed and these were used to carry out fermentations at a range of volumetric oxygen transfer rates with the aim of finding the value which delivered optimal formation performance. Several performance metrics, covering substrate utilisation and product formation, were examined in Figures 5.1 to 5.5. This led to the conclusion that peak fermentation performance was achieved with a k_La value of 50 h^{-1}, with sugar consumption being reduced at values below this and increased by-product formation occurring at higher values. Additional work, shown in Figures 5.8 and 5.11, determined that a limited amount of variability was seen in replicate fermentations at the same k_La value.

Considerations in Section 3.4 had determined that 15 mL and 50 mL conical bottom tubes would be equally suitable for use as USD tubes for the fermentation stage of the process. Scouting experiments determined that significantly improved fermentation performance was seen using 50 mL tubes (Figure 5.12) and that there was a correlation between tube headspace volume and fermentation performance (Figure 5.13). On the basis of these experiments 50 mL USD tubes were selected for all further work.

Following on from the observation that the tube working volume has a significant effect on fermentation performance, a series of experiments were run to define robust obverting conditions through examination of the effect of the USD working volume, initial sugar concentration and shaking frequency. These experiments, shown in Figure 5.14 to 5.17, demonstrated clearly that at high sugar concentrations the best fermentation performance, in terms of sugar utilisation and ethanol production, was seen with the lowest working volume of 20 mL. Fermentation performance was best at an intermediate sugar concentration of 40 g L^{-1} with performance declining at a higher sugar concentration. The data in Figure 5.17 suggested that this may be due to a pH drop occurring at higher sugar concentration that was in excess of the buffering capacity of the media. Experiments carried out using alternative media buffers confirmed that increasing the buffering capacity around pH 7 allowed for improved fermentation performance at higher sugar concentrations (Table 5.4). These observations may be related to the fact that the USD tubes are not continuously replenished with fresh oxygen during fermentation as STRs are. Equally the limitation in performance may be related to the lack of the on-line pH control in the USD tube system, with the buffering capacity of the media being exceeded. Obviously there is also a possibility for an interaction between the two limitations; for example the lack of oxygen could cause a switch in metabolism that leads to increased production of acidic by-products. Thus performance was best with the lowest fill level as these tubes had the most oxygen available initially and fermentation of 60 g L^{-1} glucose was incomplete as there was insufficient oxygen available. A concentration of 40 g L^{-1} sugar was therefore used for the comparison of STR and USD tube performance.

Having established a protocol that allowed successful fermentations to be performed in USD tubes, a comparison of USD tubes and STRs in terms of fermentation performance was performed. Using the correlations developed in Section 3.6 USD tube fermentations were run at the optimal k_La values determined in Section5.2. Figure 5.19 shows that the ethanol titres differed by only 0.3 g L^{-1} whilst in both systems the ethanol production yield was 0.35 g g^{-1}. This in terms of the main criteria identified in Section 5.1 performance was identical in the two systems. The fermentation rates, secondary criteria for comparison, were also highly similar. The most significant differences occurred following the peak ethanol titre, when the process would be ended at scale, and so are of limited importance.

The USD tubes were used to investigate how the observed effect of suspended solids on k_La (Sections 3.2 and 3.6) impacted on fermentation performance. Figure 5.21 showed that when the difference in k_La caused by suspended solids was not corrected for there was a substantial impact on fermentation performance. Figure 5.23 showed that altering the shaking frequency to correct for the decrease in k_La led to some restoration of fermentation performance but not to the level seen in solids free media. Following this observation in USD tubes, a comparable STR fermentation was performed to compare performance at the two scales when solids were included with the media. Figure 5.24 showed that as was the case with solids free media there was good comparability the in the sugar utilisation and product production profiles from USD tubes and STRs. For the primary ethanol production criteria, differences of 0.6 g L^{-1} in ethanol titre and 0.02 g g^{-1} in ethanol yield do not represent significant differences in process performance. The ethanol production rates showed some differences between the two scales but in light of the highly similar titres and generally poor performance seen these would not affect interpretation of the results. Thus although the inclusion of solids recovered from DDGS hydrolysate has led to a substantial decline in terms of fermentation performance, especially in terms of ethanol production, the USD tubes represent a good scale down model of the STRs used when judged against the criteria from Section 5.1.

The following chapter considers the wider industrial implications of the work presented throughout this thesis in terms of the practical utilisation of the USD tubes as a scale-down mimic of the enzymatic hydrolysis and fermentation stages of bioethanol production.

6. Industrial Relevance of the USD Platform[2]

6.1 Relationship between the USD platform and commercial process modelling

Commercial scale lignocellulosic ethanol facilities are extremely large and complicated plants, often costing in excess of £100 million to design and construct (DuPont, 2015).Any such processes therefore go through several rounds of design that model the process in increasing detail and confirm at each stage that the project is commercially viable. A key stage in this design process is production of a high level techno-economic model of a process (Schell et al., 2016). This requires a detailed mass balance on the proposal identifying all of the key inputs, outputs and conversion yields. At this stage in the design the size of most key process vessels will be identified and an initial estimate of project capital expenditure (CAPEX) can be made. The detailed mass balance listing all inputs and outputs of the process also allows estimates to be made for the operational expenditure (OPEX). This early estimate of the running costs and likely profitability of a project is key to determining whether a project will deliver a sufficient return to proceed towards further stages of development.

In use at ReBio such models are used to help to discriminate between various possible options for potential processes, for example between different pretreatment conditions or commercial enzyme mixtures. It is import that a techno-economic model such as this is used to as part of the decision making process as it may be observed that the most profitable process will result from conditions that give slightly suboptimal yields in individual steps once OPEX differences are considered. Data from laboratory scale STRs are normally used to fill in several key process parameters that are easily determined at this scale whilst standard values are used for other parameters. The most promising process options are then taken forward for development at pilot and ultimately demonstration scale at which point the remaining assumptions in the model can be replaced with plant data. The close relationship established between the USD tubes and STRs in Chapters 4 and 5 means that data from the USD tubes scale down model can be used to populate such techno-economic process models. This allows process economics to be used as a guide even at early stages of process development to focus research on areas that will be most beneficial to the overall process.

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[2] This chapter is included as part of the formal requirement for the award of the UCL EngD in Bioprocess Engineeering Leadership.

6.2 Overview of model structure

The model used for this validation was developed for a plant processing approximately 100,000 tons of DDGS into ethanol per year. It was designed to be sited in the Mid-West USA adjacent to an existing 1st generation corn ethanol plant with which it would share some common facilities. Electrical power and steam for distillation and pretreatment was produced by a biomass boiler using corn stover as a fuel. The organism used in Chapter 5 is classified as a non-GM organism and so the residue after extraction of the ethanol produced was destined for use as animal feed and priced as such. A block flow diagram of the process is shown in Figure 6.1. Greater detail is unable to be provided due to the commercially sensitive nature of some of the information contained.

The model was constructed using a mixture of data sources including standard engineering factors, laboratory scale experiments and trials in a demonstration scale facility capable of producing 100,000 litres of ethanol per year. This allowed an accurate estimation of factors such as the steam requirement for pretreatment and the electrical consumption of pumps and agitators. A summary of the key inputs and outputs of the model of the model are given in Table 6.1.

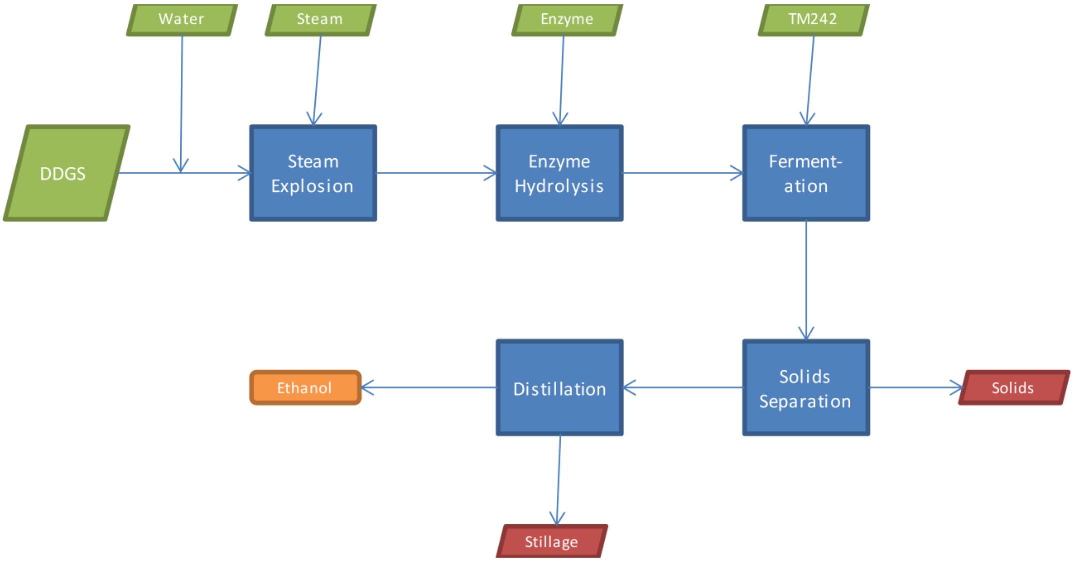

Figure 6.1. Block flow diagram of commercial scale process in ReBio’s DDGS techno-economic plant model.

In order to illustrate the utility of the USD tubes key process metrics from Chapters 4 and 5 were entered into the process techno-economic model described above. For comparative purposes the model was also used to calculate what values would need to be achieved for these metrics in order for the process to meet standard internal viability requirements based on the internal rate of return and project payback time. Table 6.1 gives the key input metrics for the model used as well as the values determined from the data in this thesis. Table 6.2 lists the main outputs of the model that are used to determine the potential economic viability of the process.

Table 6.1. Key inputs of ReBio's 100,000 tons per annum DDGS to ethanol techno-economic plant model. Values provided from data generated in either the USD platform or from a previously evaluated demonstration scale process.

Inputs	**Value (USD Tube Results)**
DDGS cost ($ per ton)	Fixed
Enzyme cost ($ per tonne)	Fixed
Enzyme loading (% w/w)	Fixed
Steam production cost ($/t)	Fixed
Distillation costs ($ per tonne broth)	Variable
Pretreatment pressure (bar)	6.5
Pretreatment Time (minutes)	5
Electricity requirement (kW per year)	Variable
Electricity cost ($ per kWh)	0.21
DDGS sugar composition	As per Section 2.10
Enzyme hydrolysis yield (C6 sugars)	65%
Enzyme hydrolysis yield (C5 Sugars)	60%
Enzyme hydrolysis length (hours)	12
Fermentation sugar consumption	70%
Fermentation length (hours)	12
Ethanol production yield (g ethanol per g sugar consumed)	0.24
Ethanol price ($ per litre)	Market Price
Co-product price ($ per kg)	0.175

Table 6.2. Key outputs of ReBio's 100,000 tons per annum DDGS to etnaol techno-economic plant model

Output	Metric
Ethanol production	Millions of litres per year
Co-product production	Tons per year
Internal Rate of Return (IRR)	Rate of return (%)
Project payback time	Years taken to payback CAPEX

6.3 Analysis of model outputs

Figure 6.2 shows that process outputs from the model when it is populated with data from the USD tubes are some way off those that would be considered ideal for a viable process. In the scenario being modelled the quantity of DDGS available for ethanol production is fixed and thus changes in process yields primarily affect the volume of ethanol produced rather than the operational expenditure (which would vary in a scenario where the quantity of DDGS used was adjusted to maintain a constant ethanol output). As the quantity of DDGS processed in both the USD tube data and ideal data models is the same the CAPEX for the two processes is also the same. The increase in ethanol revenues in the ideal process means that the IRR is greatly increased and thus the payback period for the capital invested is much reduced. In addition to the major effect of increased revenue from the extra ethanol production there is also a smaller secondary benefit in the form of reduced product recovery OPEX through the greater efficiency of distillation when the beer ethanol concentration is increased.

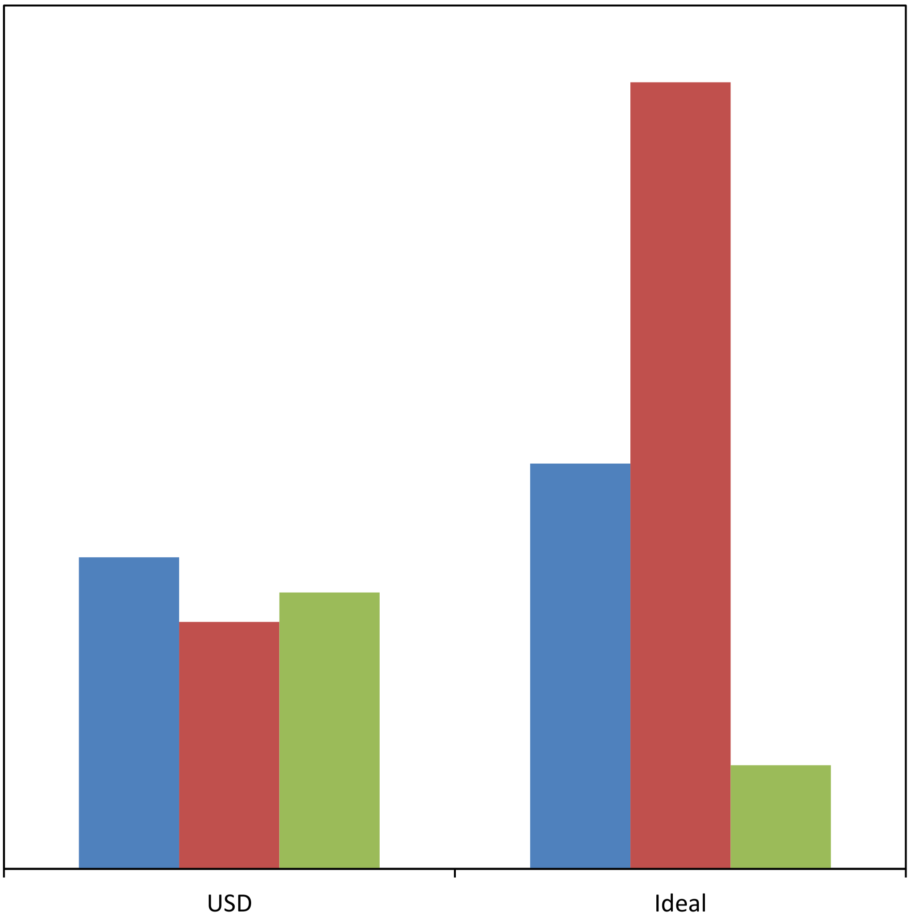

Figure 6.2. Key outputs from a process techno-economic model for the production of ethanol from DDGS constructed using data the USD platform (USD) and the same metrics from an ideal model that would be considered viable. 🟦 : Annual ethanol production (millions of litres); 🟥: Project IRR (%); 🟩: Project payback (years)

The data in Figure 6.2 clearly identifies a need to significantly improve the performance of the DDGS process investigated in USD tubes but does not identify the areas of the process in which such improvements need to be made. There are two complimentary approaches for identifying areas in which R&D activities should be focussed. Figure 6.3 shows the breakdown of the OPEX within the main process areas for the above plant. General expenditure accounts for approximately one third of OPEX in both cases. This category includes expenditure that cannot be attributed to a particular process stage such as the cost of the feedstock and labour and maintenance costs. These items are unaffected by any of the variables investigated in the USD platform and so the figures used are identical in both cases. The pretreatment stage only makes up a small proportion, 4%, of the overall OPEX, the majority of this coming from the cost of the steam required. Although it is outside the scope of this project it is easy to envision the utility of the USD platform system in evaluating the effectiveness of various pretreatment conditions on a feedstock. Nevertheless the small proportion of OPEX allocated for pretreatment means that any development in this area should be carefully targeted as research in this area will only have a substantial impact on process economics through effects on subsequent enzyme hydrolysis performance. Research into reducing steam usage in pretreatment would only have a very slight effect on process viability.

Enzyme hydrolysis accounted for just over a third of OPEX in the USD tube data model whilst 19% of OPEX was allocated to the fermentation stage. Thus there is scope for examining these two stages in more detail and identifying the major differences in process metrics between the USD tube data from this thesis and the ideal model which may be commercially attractive. Figure 6.4 shows relative improvement required in the main metrics for enzyme hydrolysis and fermentation in order achieve a viable process. In Chapter 4 it was noted that the enzyme cocktail used in this thesis was more effective at hydrolysing the C6 sugars in cellulose than the C5 sugars in hemicellulose. This is reflected in the fact that greater improvement is required in the C5 sugar yield than the C6 sugar yield, albeit both yields need to be significantly increased from the levels seen in Chapter 4 at higher solids loadings.

In terms of fermentation performance it can be seen that along with a need for increased solubilisation of sugars during hydrolysis there is also a need for a greater proportion of those sugars to be consumed during fermentation. It should be noted sugar consumption for these purposes was determined at the point at which the maximum ethanol titre was recorded as this marks the point at which a fermentation would be terminated industrially. The data in Chapter 5 shows that more sugar was consumed but that consumption was not productive in terms of ethanol. Of even greater importance according to Figure 6.4 is the yield at which sugar is converted into ethanol, which requires by far the largest increase in performance in order to deliver a viable process. This emphasises the importance in understanding the effect of DDGS solids on TM242 as seen in Section 5.6.2 . The drop in ethanol production yield observed is clearly something that will have a highly detrimental on process economics if not resolved.

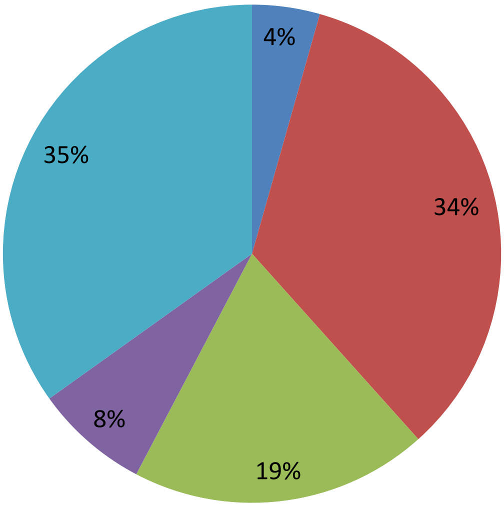

Figure 6.3. Breakdown of OPEX between main process areas in a techno-economic model of a 100,000 tons per year DDGS to ethanol plant based on USD platform data. 🟦: General expenditure; 🟦:Pretreatment; 🟥: Enzyme hydrolysis; 🟩: Fermentation; 🟪: Downstream Processing.

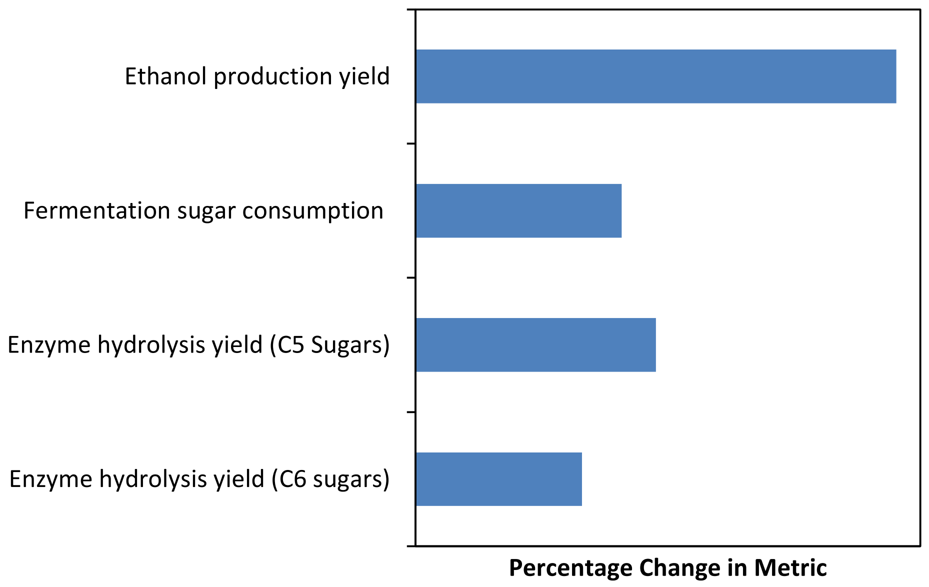

Figure 6.4. Relative improvement required in key enzyme hydrolysis and fermentation metrics in order to increase process performance to economically viable levels.

6.4 Impact of the USD platform on development timescales

That USD tube data can be used to supplement data generated in a demonstration scale facility in this way greatly adds to the commercial utility of the work described in Chapters 3, 4 and 5. The demonstrated ability of the USD tubes to accurately mimic fermentation and enzyme hydrolysis performance providing useful data for modelling purposes presents opportunities for a smaller company such as ReBio. Previously laboratory STRs represented the smallest scale at which reliable data could be generated for process models. This represented a limitation in terms of throughput that meant that initial process trials had to be carried out without consideration of economic factors. Carrying out a typical screening trial on a new feedstock with 8 pretreatment conditions, 4 enzyme hydrolysis and 4 fermentation conditions represents 128 experiments without any replicates. Even using design of experiments (DOE) to reduce the number of experiments somewhat this represents around three months' worth of work on an initial screening, a prohibitive use of resource for a small company such as ReBio. By carrying out these trials in USD tubes instead of STRs the same experiments could be completed in approximately 3 weeks with substantially reduced labour requirements compared to carrying out the same trials in laboratory STRs. Using internal cost information from ReBio including data such as salaries, overheads and consumables, the costs to the company of carrying out the limited screening trial described above would be reduced by approximately 95%. This combined with the 75% reduction in the time taken to generate all the data would allow all trials data to be examined in with regard to the economic as well as the technical outputs. This would have clear advantages for a company such as ReBio, allowing it to focus its limited resources on the projects with the most promising prospects.

6.5 Summary

This Chapter has shown the utility of having validated scale down models of the enzyme hydrolysis and fermentation process stages. The validated USD model data can be fed into process economic models in place of STR data allowing modelling of process options at a much earlier stage of development. This can then be used to assess whether a process should be developed further and if so identify the areas of the process in greatest need of development, allowing the correct focusing of resources. For example in the case of the data from this thesis the need to improve the conversion yield of sugar to ethanol is highlighted as a key limitation in the current process. As shown in Section 6.4 utilisation of the USD platform also leads to significant reductions in development time and cost.

7. Conclusions and Future Work

7.1 Overall Summary

Currently much of the world is dependent on transportation fuels that are produced from crude oil, reserves of which are finite. Additionally the burning of fossil fuels releases into the atmosphere carbon, in the form of carbon dioxide (CO_2), that had been sequestered underground for millions of years. This release of CO_2 has been identified as one of the main factors behind global warming; the rapid rise in global temperatures that has occurred over recent decades (Broecker, 1975, Puppan, 2001). Demand for transportation fuels will increase markedly in coming years as developing countries industrialise and become more dependent on motor vehicles in the way Western countries already are. Some progress towards a renewable, carbon efficient, source of transportation fuel has been made through the large scale production of 1st generation bioethanol. The use of 1st generation bioethanol is not without controversy however due to the direct and indirect competition between these fuels and food production (Section 1.1.3). The production of 2nd generation bioethanol from lignocellulose rich waste streams is considered to have much potential for reducing global greenhouse gas emissions without the concerns associated with existing processes (Tilman et al., 2009). Such processes are complicated with several distinct stages of operations (Sections 1.2, 1.3 and 1.4). Firstly a pretreatment step is used to partially deconstruct the biomass source being used. Complex enzyme mixtures are then used to solubilise monomeric and oligomeric sugars from the biomass in an enzyme hydrolysis step. The sugars produced are then converted into ethanol using a fermentative organism before the ethanol is recovered, generally via distillation.

Lignocellulosic ethanol processes are, however, still immature and will require significant further development to reach their full potential (Kim and Kim, 2014). In order to ensure that experimental performance has a strong chance of being replicated at larger scales most process development intended for scale-up to production is carried out in laboratory scale stirred tank reactors which limits the rate of process development (Section 1.6). In the pharmaceutical industry this rate limitation has been addressed through the development of many small, often microliter scale, systems that are capable of accurately mimicking process performance at volumes that are orders of magnitude larger (Micheletti and Lye, 2006). For the purposes of scaling down performance from stirred tank reactors, (STRs) microwell plates are commonly and successfully used as a well characterised small scale mimic (Islam et al., 2008, Zhang et al., 2008). Microwell plates are however of limited utility in development of a 2nd generation bioethanol ethanol processes due to the viscous, highly particulate nature of the slurries which are to be processed (Figure 3.7).This work has therefore sought to develop an alternative system that would offer a compromise between the high throughput and low cost offered by microwell plates and the excellent scalability and solids handling of STRs. DDGS, a co-product of corn based first generation ethanol plants with the potential to be valorised, was chosen as the feedstock for this project.

As with other industrial biotechnology companies, STRs form the predominant system in use at ReBio for process development purposes and these tanks formed the starting point for the development of a smaller, higher throughput system. A review of the available options for system characterisation of the enzyme hydrolysis and fermentation stages identified options for process characterisation to guide scale down strategies (Section 3.1) A review of the available options for a USD system suggested that 30mL conical bottom tubes offered the best compromise between the competing priorities of throughput and industrial relevance (Section 3.4). The characterisation work carried out in Chapter 3 demonstrated that the USD tubes were capable of adequately dispersing suspended solids and thus suitable for use in the enzyme hydrolysis stage. It was further shown that k_La values determined in the USD tubes were similar to those determined over a range of operating conditions in STRs and thus the USD tubes were suitable for evaluation in the fermentation stage of the process. The general consensus in the literature is that it is necessary to fully suspended solids during enzyme hydrolysis in order to achieve effective performance. It was noted in Chapter 3 that under some circumstances there appeared to be some non-suspended solids present in the USD tubes. When enzyme hydrolysis reactions were carried out under these circumstances however no significant effect on performance was seen suggesting that the relationship may be more complicated than the literature suggests.

. The pattern of solid-liquid dispersion in a two-phase slurry and how this was affected by shaking frequency and the angle at which the USD tubes were held was therefore investigated visually using a high speed camera system in order to assess the suitability of the tubes for work with lignocellulosic feedstocks (Figures 3.8, 3.9 and 3.10). It was observed that the height of the free liquid surface, through which gas exchange occurs, increased with shaking frequency regardless of the angle of the USD tube relative to the shaker bed. The angle of the USD tubes had an impact on solid-liquid dispersion with dispersion and the gas exchange area increasing as the angle of the tubes moved away from 90°.

Having established in Chapter 3 that the USD tubes were, based on an examination of their engineering characteristics, suitable for use as an ultra scale-down system their practical application to the enzyme hydrolysis stage of the process was examined in Chapter 4. Enzyme hydrolysis reactions carried out with a range of DDGS concentrations, ranging from 5% (w/w) to 25% (w/w) showed that, in line with literature data from other feedstocks, there was a linear decrease in enzyme hydrolysis yield over the solids range investigated, the overall yield at the highest solids loading being 78% of that seen at the lowest (Section 4.3). A detailed comparison of the USD tube and STR processes showed high similarity for all of the key enzyme hydrolysis metrics (Section 4.4). Thus the USD tube system has been validated against an STR system for the hydrolysis of a lignocellulosic feedstock, corn DDGS, at solids loadings of up to 25% DS (w/w). This represents a substantial improvement over scale down systems described in the literature which were either larger and more resource intensive or not validated against standard STRs.

The utility of the USD tube system was exemplified by using the USD tubes to examine what factors were limiting the rate of enzyme hydrolysis following the first eight hours of the reaction. By washing away soluble inhibitors present in the hydrolysate it was demonstrated that the sugars in DDGS were readily, and rapidly, completely hydrolysed by the enzyme mixture used in this work (Figure 4.12). This was in line with published observations from other feedstocks but represents the first time that such evidence has been reported using DDGS as a substrate. Having demonstrated the suitability of the USD tubes for the enzyme hydrolysis stage their use in the more complex fermentation step using a modified strain of *G. thermoglucosidasius* was examined in Chapter 5. Firstly, an optimal k_La of 50 h^{-1} was identified for cultures growing in 4L STRs. At k_La values below this, the rate of sugar consumption was reduced, as was the amount of glucose consumed at the lowest k_La values. When fermentations were run at higher k_La values there was a reduction in the production of the target product, ethanol, with increased production of acetate seen in its place. With this performance established as a baseline the next requirement was to develop protocols to allow the USD tubes to be used for fermentation processes.

Initial fermentation experiments in the USD tube showed that there was a limit to the concentration of sugar that could be consumed during fermentation, with concentrations above 40 g L^{-1} being incompletely utilised. Increasing the buffering capacity of the culture media to acidic pH drift improved the utilisation of 60 g L^{-1} sugar in the USD tubes, albeit not to the point of complete utilisation. It is suggested that this limitation may be related to depletion of oxygen in the USD tubes as they, unlike conventional STRs, are not continuously supplied with air during fermentation.

At this stage the methodology for USD tube fermentations had been developed sufficiently for experiments to be performed comparing the fermentation profiles of cultures in USD tubes and STRs (Section 5.6). In the first instance this was performed in laboratory media with no suspended solids at the optimal k_La value of 50 h^{-1}. There was good agreement between the two different systems in terms of the key metrics and secondary metrics that were identified for successful scale down. For example the peak ethanol titre in the STR culture was 11.7 g L^{-1} whilst in the USD tube it was 12 g L^{-1}. In order for the USD tubes to be useful for lignocellulosic ethanol processes it was also necessary to examine the performance of the USD tubes in the presence of the suspended solids found in real-world processes.

The negative effect that DDGS solids were shown to have on culture k_La in the USD tubes (Section 3.6) was confirmed to have a negative effect on culture performance as would have been expected from the data from Section 5.1. Correcting for the effect of the solids on culture k_La only partially restored fermentation performance, suggesting that the DDGS solids being used were in some way inhibitory to the organism being used. A comparison of fermentation performance in DDGS solids containing USM media in tubes and STRs showed that this issue was not related to the fermentation system. Cultures run at matched k_La values in STRs and USD tubes again showed good agreement for the main fermentation performance metrics, with only minor discrepancies for secondary metrics. There is a strong possibility that this inhibitory effect could be an artefact of the processing methods used to producer them and so it is important to establish whether this inhibition would occur with unprocessed hydrolysate,

Thus the main objectives of this thesis, developing a validated USD model for a lignocellulosic ethanol enzyme hydrolysis and fermentation process has been developed. There remain limitations to the system, notably the incomplete utilisation of high sugar concentrations during fermentation, however further work may reduce this limitation and the system as it stands is still a novel addition to the state of the art. Small scale alternatives to STRs have been reported for enzyme hydrolysis in the literature previously but this is the first time that a USD system has been described in which the detailed kinetics of enzyme hydrolysis have been shown to match a conventional STR. There are no reports of validated scale down systems for lignocellulosic fermentation systems in the literature and thus the demonstration of a simple, cheap, disposable USD system capable of use in fermentations with significant suspended solids content is of significant utility in the field. The fact that the same conical bottom tubes have been demonstrated as a USD system for both the enzyme hydrolysis and fermentation stages of a lignocellulosic ethanol process enhances the simplicity and utility of the developments in this thesis. The USD tubes used in this work are cheap, readily available and disposable and require little upfront investment meaning that there are no significant barriers to the uptake and use of this technology for similar applications.

The excellent comparability between USD tubes and STRs seen in this thesis allows the data generated to be used to populate techno-economic models. This highlighted that several areas examined in this thesis would need to be further developed in order to deliver a profitable process. The combination of USD systems and techno-economic models has the potential to be a powerful tool for the correct focussing of R&D efforts.

7.2 Future work

This work has developed a practically applicable small scale mimic of two of the main process stages of a lignocellulosic ethanol process. The results in previous chapters have shown good comparability between the USD tubes and STRs using a commercially relevant lignocellulosic feedstock and have provided some understanding of the reasons why the USD tubes are an effective mimic. There is however further work which could be carried out, both to increase understanding of the fundamental operation of the USD tubes and to increase their practical utility.

The fluid flow and solid-liquid dispersion studies carried out in Section 3.5 were useful in demonstrating the potential utility of the USD tubes. However a more complete understanding of the mixing in the USD tubes, as well as the STRs, could be obtained using computational fluid dynamics (CFD) techniques (Murthy et al., 2007). Such techniques have been shown to be helpful in developing robust scale-down models, although the level of suspended solids present may represent an issue to such an approach. (Barrett et al., 2010, Panneerselvam et al., 2008) These models could be used to better understand the results of Section 4.7 where the angle of the USD tube was found not to significantly affect the overall hydrolysis yield despite the differences which were observed in solid-liquid dispersion. Likewise CFD modelling could be a useful companion to the determination of k_La values in the USD tubes which was carried out over a relatively limited range of conditions.

There are several topics worthy of further investigation related to the determination of k_La in Sections 3.2 and 3.6. The first of these was the observation that under some circumstances oxygen appeared to be adsorbing to the DDGS solids, reducing the concentration in the liquid. This has implications for the k_La measurements made as well as potentially practical implications for cultures containing these solids which may not be receiving the anticipated levels of oxygen. It would therefore be valuable to study this phenomenon in isolation under controlled circumstances. Additionally, whilst reviewing the literature reports were found where particles of the same material but of different sizes had opposing effects on k_La. This has obvious implications for processes using DDGS, or any other lignocellulosic feedstock, where the particles present will be of a range of sizes. Whilst it did not impact on the direct measurements of k_La made in this work, in order to gain a fuller understanding of how the solids present in a feedstock affect k_La it would be advisable to sort the particles into various size fractions and examine their effect on k_La in isolation and in differing combinations. Lastly in terms of k_La, when developing the correlations in Section 3.3, direct measurement of the gassed power input into the STR was not possible. Whilst a widely cited correlation was used to estimate these values, it would be preferable if direct measurements of the power input were made.

In Section 4.6 initial attempts to identify the nature of the soluble inhibitors limiting enzyme hydrolysis performance were made, eliminating glucose as a possibility. It was decided however that further investigation of such inhibitors, while of significant potential utility, lay outside the scope of the project. There are a number of compounds identified as inhibitors of the enzyme hydrolysis of biomass in the literature (Kim et al., 2011, Qing et al., 2010, Ximenes et al., 2011). Determining which particular inhibitors are of most importance when using DDGS as a substrate could significantly aid those looking to valorise DDGS in order to improve the efficiency of existing corn ethanol plants. Additionally all of the enzyme hydrolysis reactions in this work used commercially available enzyme cocktails that, whilst representing the state of the art at the start of this project, have been superseded by improved formulations. It would therefore be of value to repeat certain observations using enzyme products that represent the current state of the art.

In Chapter 5 the protocols for fermentation in USD tubes were developed sufficiently to allow comparison to STR cultures. There remains room to improve the methods however. The determination of the effect of sugar concentrations, shaking frequency and fill volume would ideally be repeated using a mixture of C5 and C6 sugars in order to make it more relevant to the situation in feedstocks. The optimal culture parameters could also be more satisfactorily identified by incorporating design of experiments (DOE) techniques into the experimental design and using them to identify the sweet spot for optimal operation.

In order to maximise the utility of the USD tubes, they should be capable of being used with starting sugar concentrations greater than was found possible in Section 5.4. The experiments in Section 5.5 suggested that in order to do this the pH buffering system in the tubes needed to be modified to give it a higher capacity. Initial work in this direction produced some promising results but in order to make best use of the USD tubes further improvements in this regard would be required.

The DDGS solids used in Section 5.6 to investigate the effect of suspended solids on fermentation had a very substantial impact on process performance. It would be useful for future work to understand what about the solids was so inhibitory. They had been washed during their recovery and so any carryover of soluble inhibitors from the hydrolysate should have been minimal. The isolation of the suspended solids from a lignocellulosic feedstock could be useful in a variety of circumstances where it is wished to examine their effect in isolation and so thorough examination of their isolation and processing may be useful in identifying and then removing any steps which are generating inhibitors that limit what can be done with the solids.

Finally this work has focussed exclusively on the production of ethanol from DDGS. There is significant opportunity to expand both the range of feedstocks validated in the system, for example to include wheat straw, corn stover, MSW or cane bagasse, as well as the range of products produced. There is significant interest currently in using industrial biotechnology to produce a wide range of products from lignocellulosic feedstocks, with examples such as lactic acid for the production of biodegradable polymers or succinic acid as a platform chemical. Expanding the use of the USD tubes in either of these directions would be of both academic and industrial interest.

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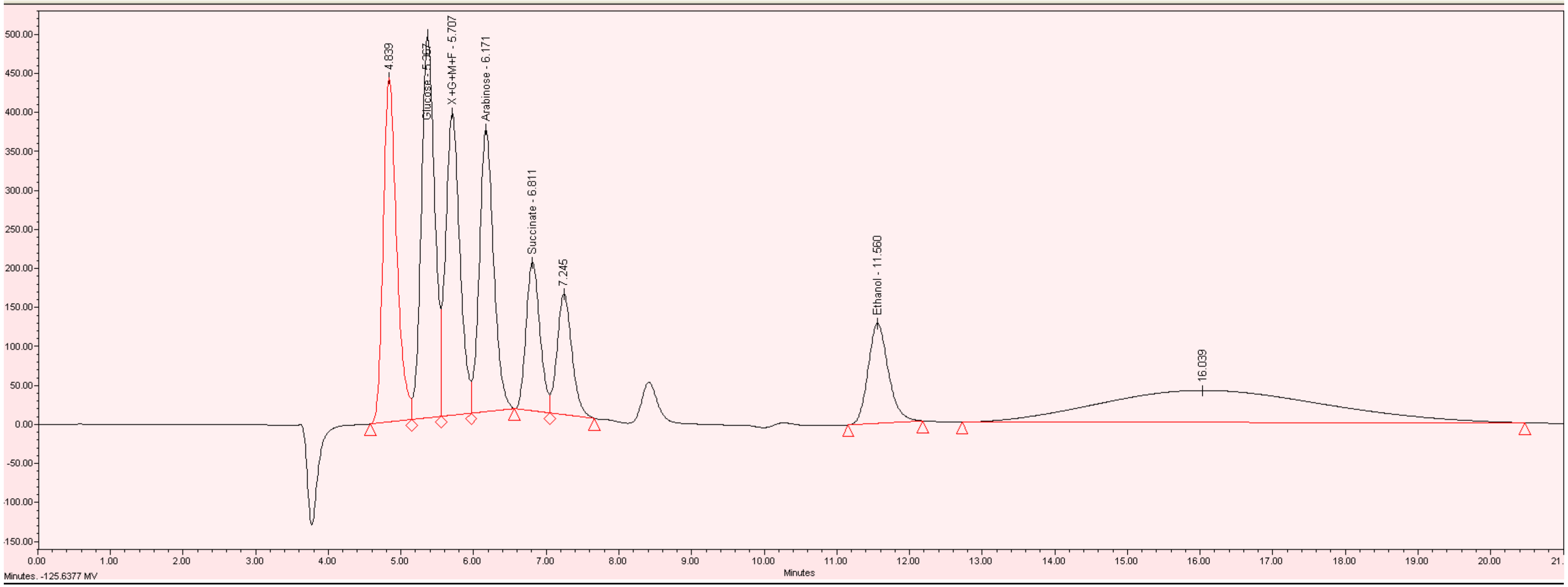
Appendix

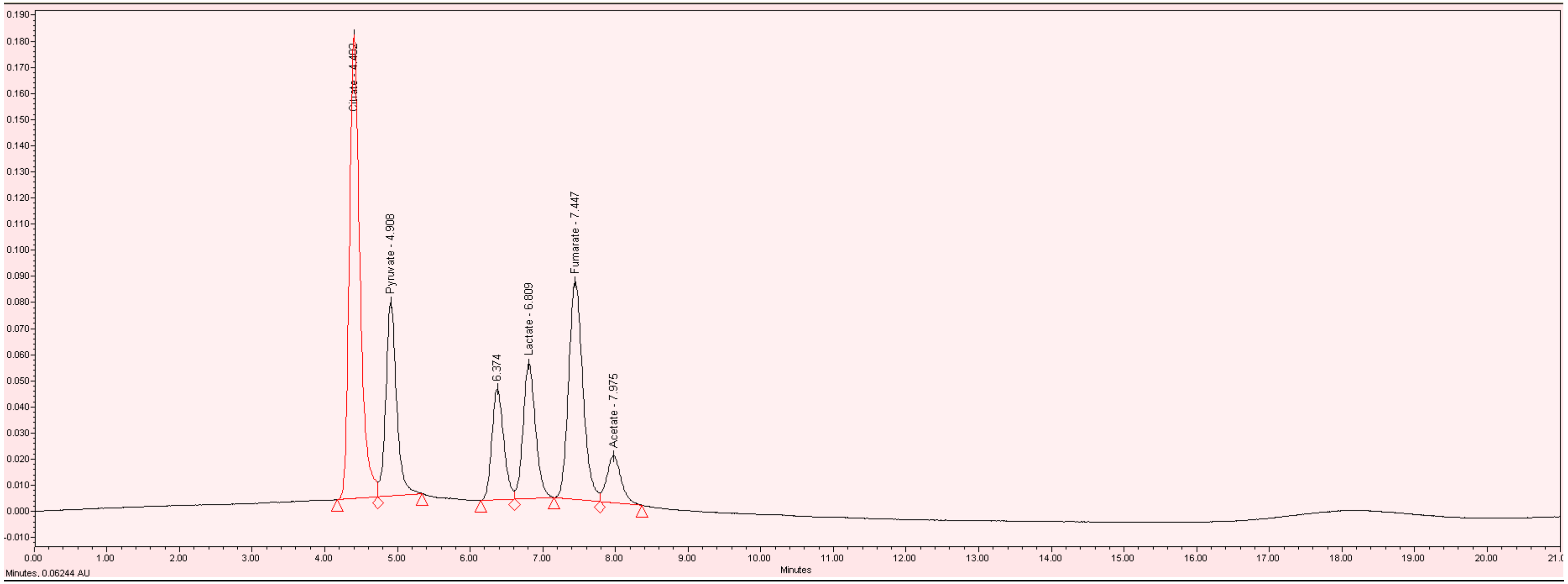
Appendix 1

RI trace of HPLC standard calibration



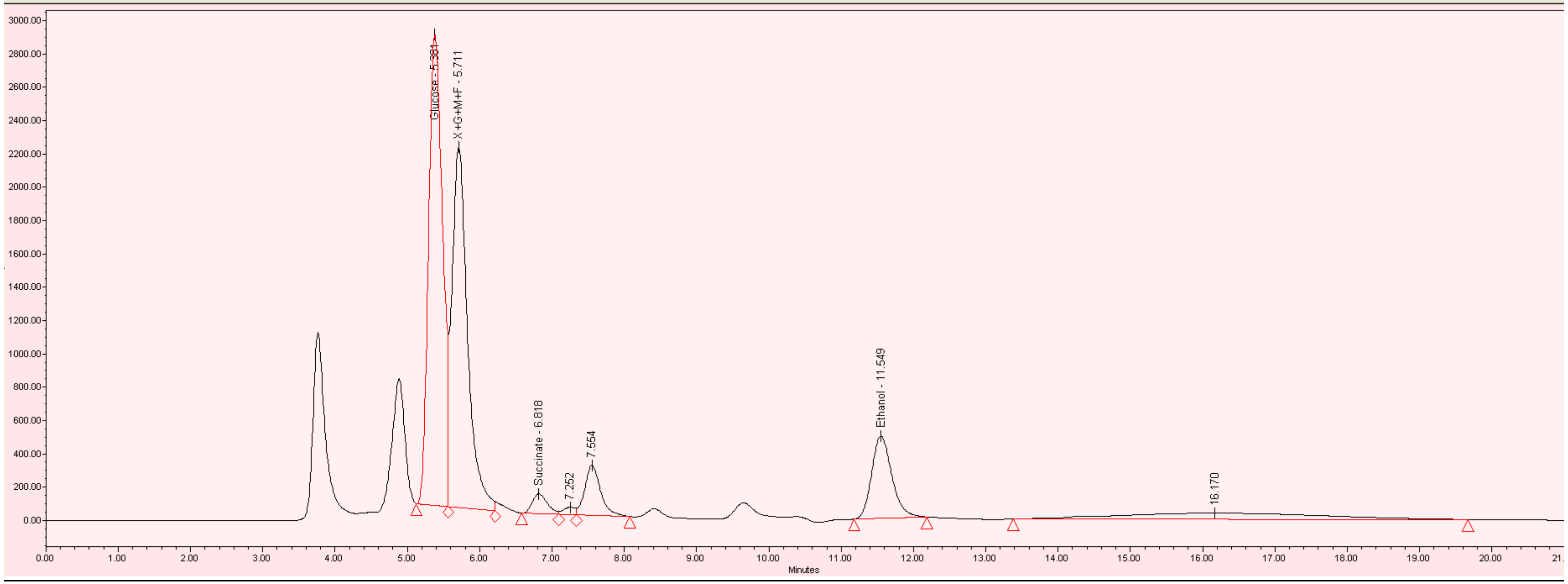
Appendix 2

UV trace of HPLC standard calibration



Appendix 3

RI trace of typical HPLC sample (data taken from experiment shown in Figures 5.1-5.4)



Appendix 4

UV trace of typical HPLC sample (data taken from experiment shown in Figures 5.1-5.4)