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Development of physio-chemical pre-treatments and mixed microbial cultures for the conversion of lignocellulosic biomass to useful products

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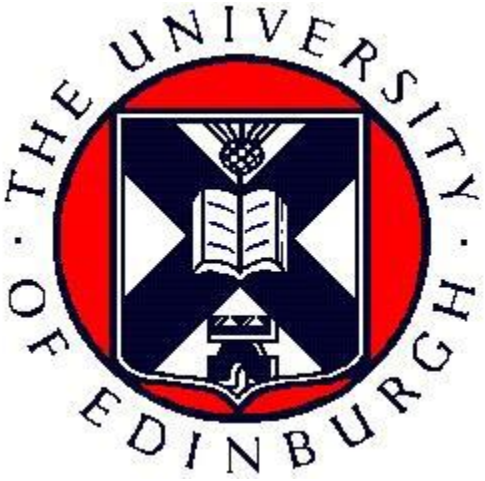

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Declaration

I hereby declare that the research reported in this thesis is original and was carried out by me and supervised by Prof. Christopher E. French. Work from other authors where cited have been duly acknowledged. This work has not been submitted at any institution wholly or partially for the award of any degree.

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Craig Christopher Robert Munns

Lay Summary

There is an increasing need for biofuels and other useful products to be produced from non-food plant matter by micro-organisms. The problem in using plant matter as a feed stock is its natural resistance to bacterial degradation. A method of overcoming this hurdle is to physically and chemically process the biomass to make the sugars present in the plant cell walls more accessible for microbial use and conversion to useful products such as ethanol. This study concentrated on developing pre-treatments that would allow cellulose degrading micro-organisms to release sugars from the biomass. These sugars could then be used by a secondary micro-organism in co-culture to ferment these sugars into useful products such as ethanol or lactic acid. These pre-treatments and biological degradation processes were effective at releasing sugars into the medium, which could then be used for biofuel production, but they also released compounds that were inhibitory to microbial growth. Several promising combinations of micro-organisms were found and further development of these may lead to novel processes for manufacturing useful products from non-food biomass.

Abstract

There is increasing interest in producing biofuels; biofuels are preferable to fossil fuels as the biomass from which they are derived is seen as a renewable source, as opposed to fossil fuels which are a finite resource. “First Generation” biofuels are derived from food crops such as grains and sugar cane. The use of food crops is not sustainable in this age of increasing food insecurity. A promising alternative appears to be what is termed “Second Generation” feedstocks, such as energy crops like *Miscanthus spp.*, and agricultural by-products. The problem with the use of second generation feedstocks is firstly that the sugars are locked up in the cell wall polymers (CWP), which need to be released by physio-chemical pre-treatments, that are costly and time consuming. The second problem is that not all the sugars that are released from CWP are able to be utilised by wild type product-forming organisms. However, model chassis organisms can be genetically modified to utilise these sugars and /or produce enzymes to degrade biomass which reduces the time and costs involved in the process. While engineering these organisms to utilise a range of monosaccharides has already been successful, engineering them to produce degradation enzymes is proving to be problematic. A potentially more effective system is to use co-cultures of both cellulose-degrading and product-forming organisms. Since this is a novel approach it is not known whether the two organisms are able to live together without any adverse effects.

The aims of this study were firstly to determine whether mixed cultures of both cellulose-degrading and potential product-forming organisms could survive in the presence of one another, secondly whether the cellulose-degrading organisms could degrade potential feedstock down into their monosaccharide building blocks and thirdly whether the potential product-forming organisms could survive and utilise these monosaccharides for growth and potential fermentation. It was discovered that *C. hutchinsonii* can degrade both paper and *Triticum aestivum* straw polymers into their monosaccharide components and that *B. subtilis* can survive on the sugars released by *C. hutchinsonii*. It was also discovered that *C. hutchinsonii* and *B. subtilis* 168 can only tolerate an ethanol concentration of up to 2% (v/v) and that this is below the baseline for a biofuel system to be economically viable. Likewise, *C. hutchinsonii* and *B. subtilis* 168 have an even poorer tolerance for butanol; growth is inhibited by < 1% butanol in its growth media.

A series of physio-chemical pre-treatments were developed in order to make the monosaccharides present in the cell wall polymers more accessible to microbial saccharification. Sequential pre-treatments, both physical milling and chemical hydrolysis in tandem, had the greatest effect on the bio chemistry of the biomass, but that these physio-chemical pre-treatments produced inhibitory compounds in the medium that retarded microbial growth.

Attempts were made to genetically modified *Bacillus subtilis 168* to produce lactic acid and ethanol by over expressing the native *ldh* gene under the highly-expressed promoter of the *cspD* gene and by integrating the fused *pdc:adh* gene from *Z. mobilis* under the same promoter. Transformation of *B. subtilis* to over express LDH was successful, with PCR confirmation of the correct insertion and enzyme activity for the *ldh* both in vitro and in vivo, with the latter producing more lactic acid aerobically than the wild type. Transformation of *B. subtilis* to express *pdc:adh* and subsequent production of ethanol was not successful.

Dedicated to the memory of Dr Robert Owen MacRae

“There’s never a chemist around when you need one”.

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And finally, because he has thanked me on the sleeve of every single, EP and album he has ever released my deepest thanks to my oldest and dearest friend, Mr Liam Brett.

… oh, and Skaz!

Contents

1 Introduction

This review will summarise the background information needed to fully utilise a range of feed stocks for the production of liquid fuels and the current knowledge of this area.

1.1 Current biofuels

There has been an interest in biofuels for a number of years, particularly in Brazil and the United States of America. This bioethanol, however, is mainly derived from sugarcane and grain, which has raised concerns about food security. Brazil produces 3.4 billion gallons of bioethanol from sugarcane annually (Borrion et al., 2012a, Borrion et al., 2012b) There has been interest in second generation biofuels, fuels derived from non-food sources, for a number of years and several pilot production plants have been set up, particularly in Europe and Scandinavia. While the production methods vary slightly across the range due to biomass used etc., a general overview is given below.

1.1.1 Ethanol

Bioethanol is currently the main focus of research into the use of lignocellulose degrading biomass (French *et al*., 2013) and is one of the first generation biofuels, currently produced from sugar cane waste, maize grains and starch in the Americas (Visser, 2013). The reason bioethanol is one of the most used biofuel is because it is readily produced by wild type micro-organisms, such as *Saccharomyces cerevisiae*, *Escherichia coli, Zymomonas mobilis, Pichia stipitis, Clostridium thermocellum* and Clostridium *phytofermentans* (Alper, 2009, Fletcher, 2014, French, 2015). Bioethanol is seen as cleaner fuel than petrol and can be used in the place of petrol in modified spark ignition engines (Coombes, 2004) but due to bioethanol being a lower energy density fuel than petrol, more ethanol is needed than petrol to do the same amount of work. To counter this most use a mix of up to 15% ethanol to petrol (v/v) which also helps the fuel to burn cleaner and more efficiently by oxygenating the fuel mix (Fernando, 2004, Sebayang et al., 2016). Once a system for pre-treatment and fermentation is developed, the micro-organisms can be either swapped out for ones that make higher energy fuels such as butanol from *Clostridium acetobutylicum* (Alper, 2009) or are engineered to make them (Fletcher, 2014), as higher energy density fuels should prove to be more economical.

1.1.2 Butanol

Butanol is a next generation biofuel as it has an energy density comparable to petrol (32 MJ/l : 27 MJ/l respectively) (Alper, 2009, Krutsakorn et al., 2013, Antoni et al., 2007) so does not face the same issues with lower energy density that are present when bioethanol is used (see above), and can be used in unmodified petrol engines so is preferable to ethanol. Butanol is naturally produced by *Clostridium acetobutylicum* via the ABE fermentation (Alper, 2009, Nakayama et al., 2011, Krutsakorn et al., 2013). Due to butanol production by *Clostridium acetobutylicum* being anaerobic, it makes this system difficult to work with. However, several organisms have been engineered to produce it, particularly *S. cerevisiae* and *E. coli* (Steen et al., 2008, Alper, 2009, Fletcher, 2014, French, 2015). The engineering of *S. cerevisiae* produced yields of only butanol at 2.5 mg/l but this system used cellulose as the carbon source, which is cheaper than glucose and which would go some way to offset the low amount produced as E. coli has been modified to produce 30g/l butanol when the butanol is continually removed to prevent toxic levels (see chapter four) of the solvent building up (Steen et al., 2008, Bialkowska et al., 2016). There has been some success in using cell free systems to produce butanol *in vitro* from glucose (Krutsakorn et al., 2013), which would circumvent the issues encountered in the microbial production systems.

1.1.3 Biodiesel

Biodiesel is seen as a renewable source of transport fuel as it is easily made from oils via transesterification and so biodiesel is produced from either specifically grown oil crops, such as oil seed rape or by recycling used cooking oil (Ma, 1999, Meher et al., 2006). Biodiesel can be used in modified compression ignition engines (Coombes, 2004, Fernando, 2004) There have also advances in the modification of organisms to produce biodiesel, both microbial (Gomez, 2008, Alper, 2009, Schirmer et al., 2010, Wahlen et al., 2013, Fletcher, 2014, French, 2015) and algal (Mata et al., 2010, Wargacki et al., 2012, Wahlen et al., 2013, Zhou et al., 2013, Roy, 2015). But since biodiesel is easily made from oils and recycled cooking oils, there seems to be little need to produce it microbially. However, if its use becomes much more wide spread in which case microbial and/or algal production will be needed to meet demand. However, both traditionally produced diesel and biodiesel produce nitrous oxide which I both a greenhouse gas and contributes heavily to air pollution, especially in metropolitan areas, so biodiesel is not quite as “green” as it appears on first glance.

1.1.4 Biohydrogen

Hydrogen has the possibility of being the greenest alternative fuel as its combustion in oxygen produces only heat and water (Antoni et al., 2007). Hydrogen production is possible via passive acid electrolysis but if hydrogen fuel is to meet the ever-growing energy demands a more productive method of production is needed. One that makes uses of second generation lignocellulose degrading biomass would seem ideal. Hydrogen is released through anaerobic butanol fermentation by *Clostridium* (Nandi and Sengupta, 1998, Nath and Das, 2004, Mathews and Wang, 2009, Krupp and Widmann, 2009, Christos Nitsos and Ulrika Rova, 2016) and co-cultures of *Clostridium thermocellum* and *Clostridium saccharoperbutylacetonicum* have been shown to be able to utilise cellulose as a carbon source for butanol and hydrogen production (Nakayama et al., 2011).

1.1.5 Lactic acid

Lactic acid is naturally produced by many bacteria, such as those found in natural yogurt and silage. One potential use of lactic acid is (poly)lactic acid, touted as a biodegradable plastic (MacRae, 2010). However when it contaminates regular polyethylene plastic recycling, it ruins the whole batch so none can be recycled and it is also only biodegradable in anaerobic digesters, after it has been shredded (Agarwal, 1998) and not in compost heaps as often stated.

1.1.6 Sources of biomass feedstock

There are a wide range of sources for feedstock that can be utilised for the production of liquid biofuels. These are often seen as waste and simply sent to landfill or occasionally utilised as livestock feed. However, there is growing interest in using these as the raw materials for biofuel production.

1.1.7 Food waste and by-products

The food industry produces abundant waste, from vegetable and fruit peelings, to whole vegetables that do not meet the supermarkets' rigorous aesthetic standards, to food by-products such as brewers' grains and sugar beet pulp. The traditional destination for these is utilisation as livestock feed in the agricultural industry. In the UK ≈576,000 tonnes of sugar beet pulp were produced in the 2010/2011 growing season (CEFS, 2011). This is a rich source of sugars that can easily be utilised for biofuel production (Van Dyk et al., 2013). The UK also produces over 700,000 tonnes of carrots a year (GBC, 2016) with some sources estimating 30% of carrots produced being rejected by supermarkets (GBC, 2016) and thousands of tonnes of carrot pulp are produced by the carrot juice industry annually (Hsu, 2006). There is an estimated 70 million tonnes of apple waste produced globally every year (Dhillon et al., 2013). The citrus fruit industry (juice and marmalade etc.)

produces approx. 80 million tonnes of citrus fruit waste globally each year (Kalaugher, 2013, Van Dyk et al., 2013); it is possible to recover roughly 250g/l of sugars, mainly glucose, from this waste alone (Scordino et al., 2007, Pourbafrani et al., 2010), and this is an excellent source of potential sugars for fermentation, yielding up ≈40 litres of ethanol from one tonne of citrus dry matter waste alone (Pourbafrani et al., 2010). There is the potential and increased interest in utilising these by-products and particularly the cell wall polymers for bio-ethanol production. Perhaps it is time to eliminate the concept of "waste" and instead look upon it as not a problem but an opportunity.

1.1.8 Agricultural and horticultural by-products

The straw of *B. napus* (oil seed rape; OSR) is an agricultural by-product with very few uses. While the straw of other agricultural crops can either be fed to live-stock (*H. vulgaris* straw) or used as livestock bedding (*T. aestivum* straw), OSR straw is inedible and impermeable to liquids due to the high oil content of the straw, so can neither be eaten or used as bedding. Due to the high oil content of the crop it cannot be safely burned in standard straw and/or wood burners often utilised on farms, because, again, due to the high oil content, it simply burns too hot (Caslin, 2016).

The current method of disposing of horticultural waste (grass cuttings, hedge trimmings etc.) is mainly through composting. While this is an economic and environmentally friendly practice, which helps to enrich soils with humus and nutrients, a more economically attractive method could be to utilise the sugars locked up in this material for the production of biofuels or another renewable chemical feedstock. Since several hundred thousand tonnes of horticultural waste are produced each year in the UK, this is a sustainable and renewable source of biomass. A major downside to this is the loss of, or reduction in, soil-improving carbon (Aspinall, 1954 , Carpita, 1996, Kerr and Fry, 2003). Soil erosion, nutrient depletion and reduction in the carbon content of soil are all major factors that lead to a lack of soil fertility. When crop residues are removed from the field, rather than being ploughed back, there is no recycling of nutrients and these nutrients must be added as artificial fertilisers, a process which is not sustainable. This process not only depletes the soil of nutrients and carbon but also of soil born micro-organisms, essential in the carbon and nitrogen cycles. Soil can become a close to sterile medium, whose main function is to provide a medium for plant roots to hold onto and not as a living ecosystem that actually cycles nutrients to feed the crops.

1.1.9 Energy crops

The main energy crops grown in the UK are *Miscanthus* spp., mainly *Miscanthus giganteus*, and short rotation coppice willow (*Salix* spp.). The current method of using energy crops is to harvest them, once they have reached the appropriate dry matter percentage, and then to burn them in the place of coal in power stations, such as Drax in North Yorkshire (Stephenson et al., 2010). This is seen as a carbon neutral method of energy production, as the carbon released during their combustion is believed to be the same amount that has been sequestered during the crops' growth. However this does not take into account the amount of carbon that is still locked up in the root system of the crop, especially willow, nor does it take into account the amount of carbon released during the production, cultivation and transportation of these crops (Stephenson et al., 2010). This use of biofuel crops in the place of fossil fuels, when factors such as the energy needed to grow, cut, transport and process is actually carbon producing, but while biofuel crops have a lower energy density than coal, they are renewable and coal is not.

To grow these crops, land needs to be taken out of food production for anywhere between one year (*Miscanthus* production) to 20 years (willow production). This is also not sustainable. There is also the concern about the negative effect these monocultures have on the biodiversity of the site on which they are grown. While all modern agriculture consists of fields of monoculture crops, at least in the monoculture of grain crops there is some scope for other plants to grow, particularly the growth of poppies in crops of oil seed rape and there are some small communities of insects and small mammals and birds in the field. However, when coppice willow is grown, it is quite literally the definition of a monoculture as all other species of plant are shaded out and due to the dense sward of tall woody plants, most animal species will avoid it. A potential alternative is to grow *Buddleja davidii* which is extremely attractive to pollinator insect species and there is increasing interest in using *Buddleja davidii* as a source of biomass for biofuel production (Hallac, 2009, Hallac et al., 2010).

1.1.10 Algal sources

Algae are among the fastest growing plants on the planet (Demirbas, 2009, Demirbas, 2010, Demirbas, 2011) and thus are a source of abundant biomass which is quickly renewed. There is an increasing interest in using algal species as a source of biofuels, particularly biodiesel (Demirbas, 2009, Demirbas, 2010, Demirbas, 2011). However, the structure and biochemistry of algae differs significantly from that of land plants, and varies greatly between species of algae themselves. There are three distinct lineages of multicellular macroalgae: *Chlorophyta* (green algae), *Rhodophyta* (red

algae) and *Phaeophyta* (brown algae) (Popper et al., 2011, Enquist-Newman et al., 2014). While each lineage contains cellulose, they each have distinct non-cellulose cell wall polysaccharides (see figure 1).

Taxa / Polysaccharide	Chloroplastida			Rhodophyta	Phaeophyceae
	Embryophyceae	Charophyceae	Chlorophyta		
Crystalline polysaccharides	Cellulose	Cellulose	Cellulose	Cellulose (1→4)-β-D-mannan (1→4)-β-D-xylan (1→3)-β-D-xylan	Cellulose
Hemicelluloses	Xyloglucan Mannans Xylans MLG (1→3)-β-glucan	Xyloglucan Mannans Xylans (1→3)-β-glucan	Xyloglucan Mannans Glucuronan (1→3)-β-glucan	Glucomannan Sulfated MLG (1→3),(1→4)-β-D-xylan	Sulfated xylofucoglucan Sulfated xylofucoglucuronan (1→3)-β-glucan
Matrix carboxylic polysaccharides	Pectins	Pectins	Ulvans	—	Alginates
Matrix sulfated polysaccharides	—	—	Ulvans	Agars Carrageenans Porphyran	Homofucans

Figure 1 - Cell wall polysaccharides of algae lineages. *Embryophyceae* = land plants, *Charophyceae* = fresh water green algae, *Chlorophyta* = marine green algae, *Rhodophyta* = marine red algae, *Phaeophyceae* = marine brown algae. Adapted from (Popper et al., 2011).

Due to the difference in cell wall polymers between algae and land plants (see Fig. 1), the monosaccharide composition will also differ from the monosaccharides that can be liberated from land plant cell wall polymers. To this end micro-organisms will need to be engineered to fully utilise the sugars released. There has already been some advancement in this area with engineered *E. coli* to utilise the monosaccharides released from algal glycans, mannan and alginates (Wargacki et al., 2012).

1.1.11 Other micro-organisms as a source of biomass

Cyanobacteria, particularly *Nostoc* spp., are quick, cheap and easy to grow and produce large volumes of biomass, which contain abundant extra- and intra- cellular polysaccharides (Deakin, 2012), particularly polymers of glucose, mannose and xylose (Mehta, 1978, Parikh and Madamwar, 2006) and are thus a potential source of biomass for biofuel production. Species of the Cyanobacteria *Synechocystis* can be easily engineered for direct ethanol production from photosynthesis, by expressing bacterial pyruvate decarboxylase and alcohol dehydrogenase, rather than fermenting the sugars produced through photosynthesis to ethanol(Weber et al., 2010).

1.2 Biomass sources

The main and upcoming biomass sources for second generation biofuels, and the ones investigated in this study are;

- Agricultural straws – As cereals are monocot plants the main cell wall polymers are cellulose and arabinoxylan, meaning the majority of the monosaccharides that could be released would be; glucose (hexose), arabinose and xylose (pentose) (Lee, 1997, Wang, 2008, Carroll and Somerville, 2009, Geddes et al., 2011, Albersheim, 2011, Borrion et al., 2012b, Youngs and Somerville, 2012, Moreno et al., 2013c).
- Switchgrass and *Miscanthus* spp. - the main cell wall polymers in these sources of biomass will also be cellulose and arabinoxylans, as they are also monocot plants, so the monosaccharides that would be released would also be; glucose, arabinose and xylose (Carroll and Somerville, 2009, Sipos et al., 2010, Geddes et al., 2011, Albersheim, 2011).
- *Buddleja* spp., willow and pine wood – in wood the main cell wall polymers are cellulose, mannan and xylan, which would yield glucose, mannose and xylose. There will also be a high amount of lignin present in the cell walls, as that is a major component, up to 25%, of wood (Lee, 1997, Hallac, 2009, Carroll and Somerville, 2009, Stephenson et al., 2010, Geddes et al., 2011, Albersheim, 2011, Youngs and Somerville, 2012).
- Algae – depending on the phylum of the algae (see section 1.1.10) the main cell wall polymers in algae include; cellulose (in all cases), xyloglucan, mannan, xylan, glucans, xylofucoglucan, pectins, ulvans, alginates and fucans. So, major sugars yielded may include common monosaccharides such as glucose, mannose, xylose as well as glucuronic acid, fucose and aldobiouronic acid (Lahaye, 2007, John et al., 2011, Albersheim, 2011, Popper et al., 2011, Wargacki et al., 2012).
- Food waste, including waste from the citrus industry; the major cell wall polymer in citrus is pectin, meaning that majority of monosaccharides released would be galacturonic acid, galactose, arabinose and some rhamnose (Pourbafrani et al., 2010, Edwards and Doran-Peterson, 2012, Van Dyk et al., 2013).
- Wastepaper – paper is made of cellulose, so the main sugar released will be glucose, however depending on the intensity of the production methods, there may also be mannose and xylose present (Geddes et al., 2011, Albersheim, 2011, Borrion et al., 2012b, Wang et al., 2013, Prasetyo and Park, 2013, Jameson, 2015).

These sources of biomass cover a range of taxa and consequently have a range of different cell wall polymers and therefore a different range of monosaccharide building blocks. For this reason, it is unlikely that there will be a "one size fits all" method of biofuel production from these feedstocks. A range of different saccharification and fermentation systems will need to be employed. For instance; waste paper and algal sources will have no lignin present so will require minimal pre-treatments compared to *Miscanthus*, *Buddleja* and straw, however these latter sources will offer a wider and more commonly utilisable profile of monosaccharides, compared to fucans and sulphated glycans from algal sources.

1.3 Cell wall polymers (CWP)

Plant cell walls are not the simple "wooden box" they were once perceived to be. They are in fact dynamic and adaptive matrices of ever changing polymers. These polymers are made up of monosaccharide building blocks. It is these monosaccharides that form the basis of the energy that can be derived from biomass, either as fuel for respiration in animals or through utilisation for the production of ethanol and other liquid fuels.

1.3.1 Cellulose

Cellulose is the most abundant polymer on earth and consists of a β (1→4) linked D-glucose chain, single glucose molecules linked by their 1 and 4 carbons. So because cellulose is made up purely of glucose molecules its breakdown products are: glucose, the dimer cellobiose, the trimer cellotriose and so on (Fry, 1988, Albersheim, 2011). These breakdown products are readily utilised by most micro-organisms (French, 2013, Kane, 2014). Each glucan chain is synthesised in the plasma membrane and is linked via hydrogen bonds to 16 other glucan chains to form a nanofibril, which in turn is linked to others to form microfibrils which link to form a fibril (Albersheim, 2011) and (figure 1). It is because of cellulose's relative strength and abundance that is also one of the most important polymers on the earth, and is utilised for everything from paper to buildings, to biofuel production to animal feed. This is also the reason why attempts are made to utilise lignocellulose degrading biomass for second generation biofuel production; if the glucose locked up in the cellulose can be liberated it will provide an abundant, renewable source of glucose for microbial growth and chemical conversation.

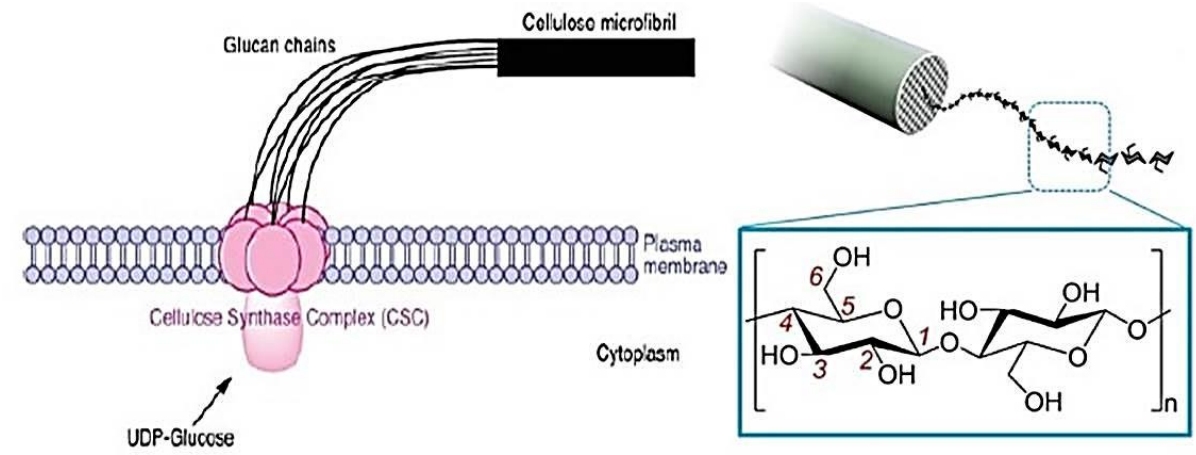


Figure 2 - Schematic of the synthesis and structure of cellulose microfibrils. Adapted from (Zhang et al., 2010, Wightman and Turner, 2010)

1.3.2 Lignin

Lignin is the second most abundant polymer on earth, after cellulose, and is synthesised in the middle lamella and cell wall, through oxidative polymerisation of phenolic compounds (Hallac et al., 2010, Albersheim, 2011). The lignin and cellulose are often referred to as "lignocellulose" particularly when discussing biomass. Lignin is a complex non- linear polymer (Fig. 2), consisting of aromatic repeating structures known as monolignols (Fig.3). Indeed, it is lignin that gives different woods such as sandalwood etc. their distinctive aromas. The composition of lignin differs across cell wall domains and cell types. Lignin is covalently bonded to hemicellulose and cross links other cell wall polymers (CWP), "filling in" the gaps between then, to convey strength to the cell wall (Albersheim, 2011). In the case of grasses, lignin

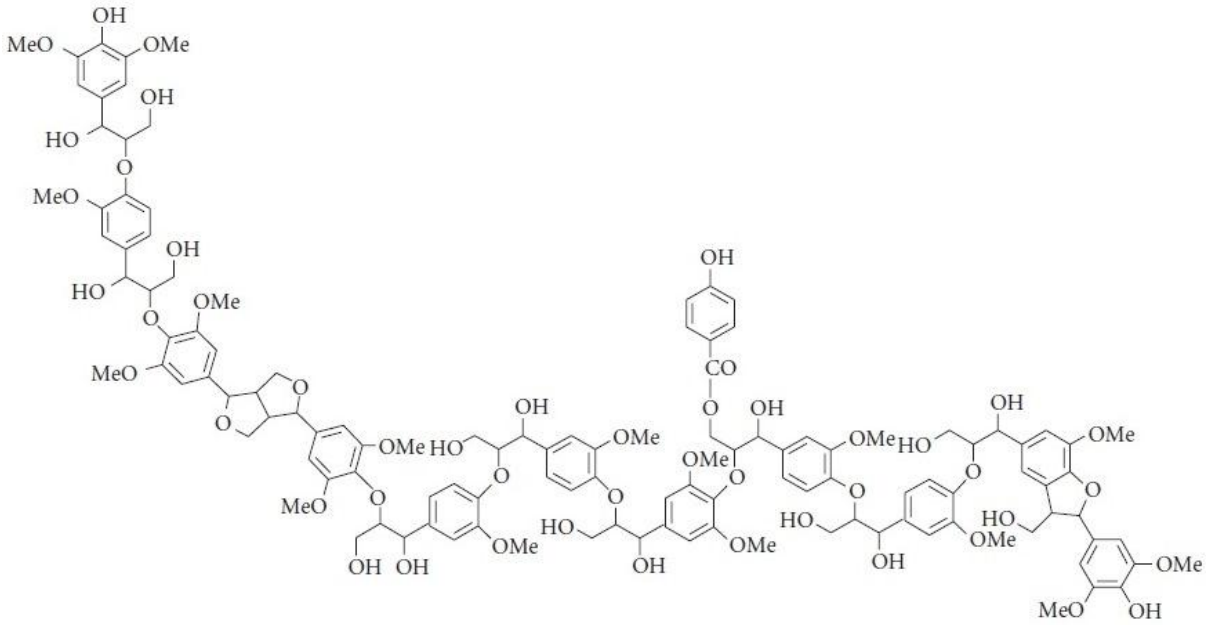


Figure 3 - A model structure of lignin, adapted from (Brosse et al., 2011)

A major stumbling block in the use of lignocellulose biomass for biofuel production is the presence of lignin. As lignin was evolved by plants to counter the pressures involved in terrestrial colonisation it is also able to resist microbial enzymatic degradation. It is highly resistant to this form of degradation due to the three-dimensional non-repeating pattern in its structure, multiple enzymes are needed, each with a different active site, to break down its structure, there is not a "one size fits all" enzyme for lignin, as there is for other cell wall polymers. All of this means that the lignin network needs to be disrupted and/or removed before the sugars locked up in the other cell wall can be released for microbial utilisation. The first step is often to physically disrupt the lignin and increase the surface area by mechanical grinding. Once lignocellulosic biomass has been milled the lignin can be removed by oxidation by potassium permanganate or sodium hydroxide/urea (Shi et al., 2014), by enzymatic digestion (Bugg et al., 2011, Furukawa et al., 2014) or through fungal peroxidase (Lundell et al., 2010).

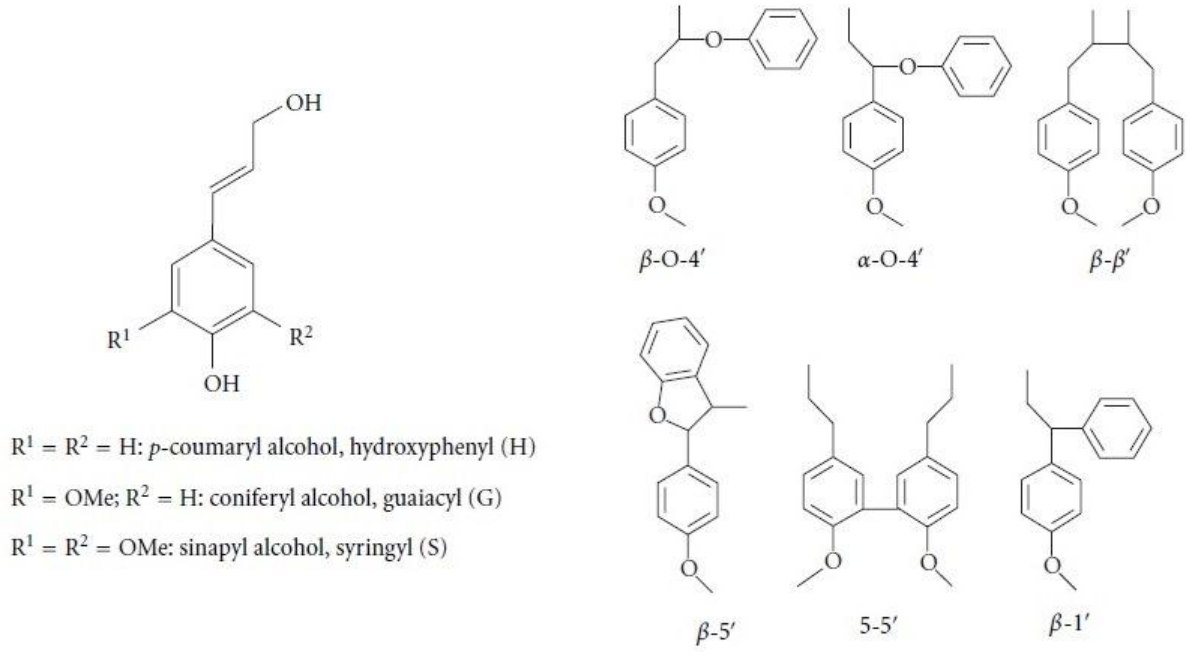


Figure 4 - The building blocks and common cross links of lignin, adapted from (Brosse et al., 2011, Hallac et al., 2010)

When physio-chemical pre-treatments are utilised to increase the digestibility of the lignocellulosic biomass, unless the lignin has been removed, these pre-treatments can release inhibitor compounds from the component parts of the lignin (see figure 1) particularly if hot water and/or dilute acids are used. These compounds inhibit the growth of microbes in the resultant hydrolysate, particularly the growth of yeasts, and so need to be removed before the hydrolysate can be used for microbial growth, although there has been some work into producing lignin deficient plants (Dauwe, 2007, Novaes et al., 2010) which may lead to greater ease in using these as a biomass source. However, the concern is that plants deficient in lignin would suffer retarded growth and increased damage due to this absence. Studies have shown that lignin deficient plants suffer greater insect herbivory (Johnson et al., 2010), increased microbial degradation (Tilson et al., 2013) and structural deformity and collapse (Jones et al., 2001, Novaes et al., 2010, Nguyen et al., 2016), issues that will need to be addressed before lignin deficient plants can be grown in a real-world sense.

1.3.3 Hemicellulose

Hemicelluloses are cell wall polymers consisting of repeating units of different monosaccharides (figures 4 and 5) synthesised in the Golgi apparatus. They are bonded to cellulose microfibrils via hydrogen bonds. Within angiosperms, primary cell walls can be split into two distinct architectural types; type I and type II. Type I walls occur in dicots and non-commelinid monocots and they contain roughly equal amounts of cellulose and xyloglucan, whereas type II walls occur in commelinid monocots and also contain cellulose but instead of xyloglucan being the predominant hemicellulose, in this case it is xylans and glucuronoarabinoxylan (GAX) although small amounts of xyloglucan are still present and are tightly bound to the cellulose (Carpita, 1996, Carpita, 2015). Xyloglucan and GAX are linked to cellulose and to themselves via hydrogen bonds. Type II walls generally contain low amounts of pectin (Albersheim, 2011, Carpita, 2015). In the secondary cell walls of non- Poaceae angiosperms predominant hemicelluloses are xylans and in gymnosperms, such as conifers, glucomannan is the predominate hemicellulose (Albersheim, 2011, Simmons et al., 2016 Busse-Wicher et al., 2016). When these polymers are broken down by enzymes, their constituent monosaccharide building blocks are released as monomers or as dimers, depending on the enzyme in question. The monomers released from hemicellulose, i.e. glucose and xylose, are either readily taken up by some wild type organisms see chapter four; in other cases there are already modified microorganisms, such as *S. cerevisiae* and *E. coli* strains, that can utilise these arabinose and xylose (Karhumaa et al., 2006, Bettiga et al., 2009, Garcia Sanchez, 2010, Vinuselvi and Lee, 2011).

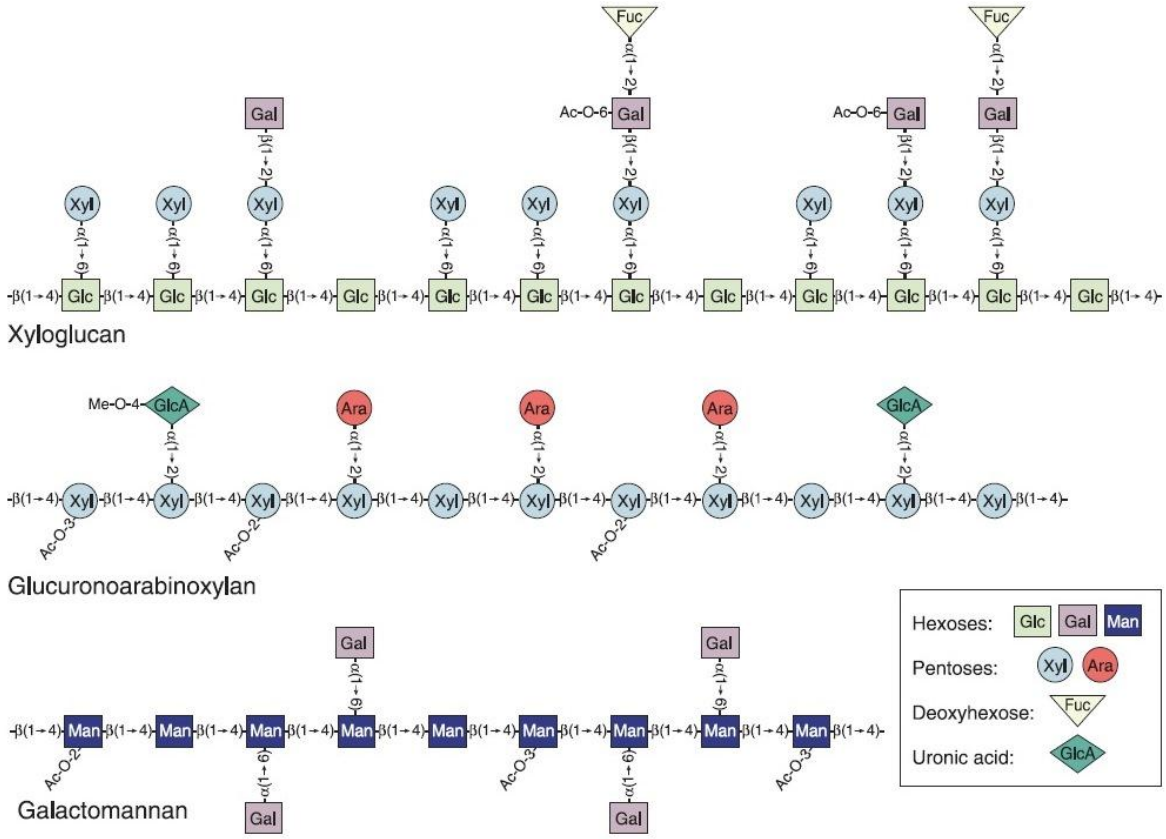


Figure 5 – Biochemical structure of the hemicellulose xyloglucan, (glucurono)arabinoxylan and (galacto)mannan - Adapted from (Pauly and Keegstra, 2008)

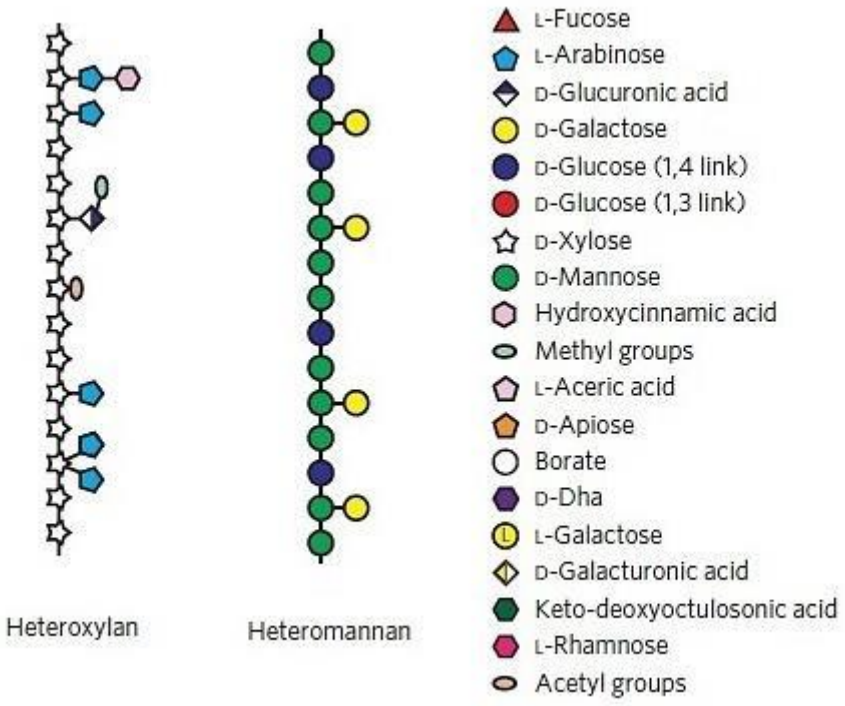


Figure 6 -Biochemical structure of the hemicellulose (hetero)xylan and (hetero)mannan - Adapted from (Burton et al., 2010)

1.3.4 Pectin

Pectin is also a polymer made up of repeating units of monosaccharides (figure 6). Both cellulose and its linked hemicelluloses are embedded in a matrix of pectin which is also synthesised in the Golgi apparatus. Pectin can be split into “pectic domains” (figure 6) These are: Rhamnogalacturonan I (RGI), Rhamnogalacturonan II (RGII), Homogalacturonan (HG) and Xylogalacturonan (XGA) (Scheller et al., 2006). Pectic homogalacturonan is cross-linked via calcium bonds to adjacent nonesterified homogalacturonan regions; this is what forms the pectic matrix in which cellulose and hemicellulose is embedded (Albersheim, 2011). Depending on the species of plant in question the amount of pectin varies; there is more pectin present in the cell walls of dicots than in the cell walls of monocots. When pectin is broken down into its monomers, dimers and trimers etc., the sugars released are mainly galacturonic acid, galactose and arabinose. So any biofuel system utilising predominantly pectin rich plant species would need to engineer and utilise specific modified organisms to utilise the sugars released from pectin for the production of biofuels; such as *S. cerevisiae* and *E. coli* strains that utilise xylose, arabinose (Karhumaa et al., 2006, Bettiga et al., 2009, Vinuselvi and Lee, 2011), and galactose (Timson, 2007).

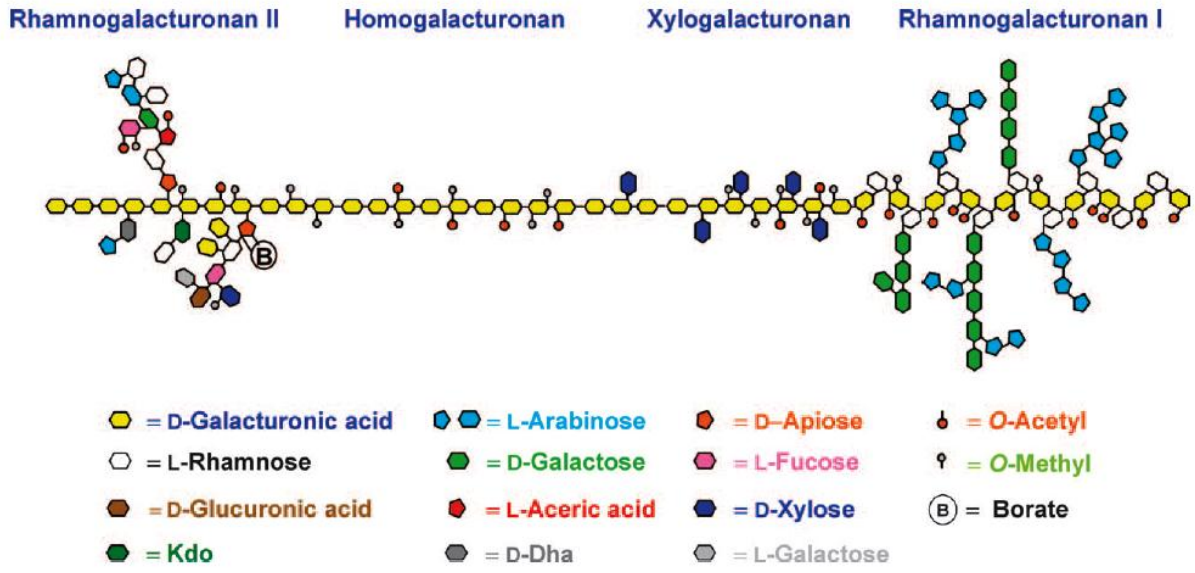


Figure 7 - Schematic of the pectic domains - Adapted from (Scheller et al., 2006)

1.3.5 Mixed linkage glucans

In grass species and in *Equisetum* spp. a cell wall component is mixed linkage glucans, often touted as a super food in oats. Mixed linkage glucans, as the name suggests, are glucans chains, with mixed β-1→3/1→4 linkages between the glucose molecules (figure 8). Due to the mixed nature of the linkages, these bonds are easily hydrolysed compared to those in cellulose (Albersheim, 2011). The role of mixed linkage glucans in plant cells walls has been suggested as being involved in cellulose microfibril tethering (Fry, 1989) but has been shown to also play a role in cellulose/xyloglucan tethering (Fry et al., 2008) and in cell wall remodelling where they are utilised as a donor substrate for xyloglucan synthesis (Fry et al., 2008 and Simmons et al., 2015).

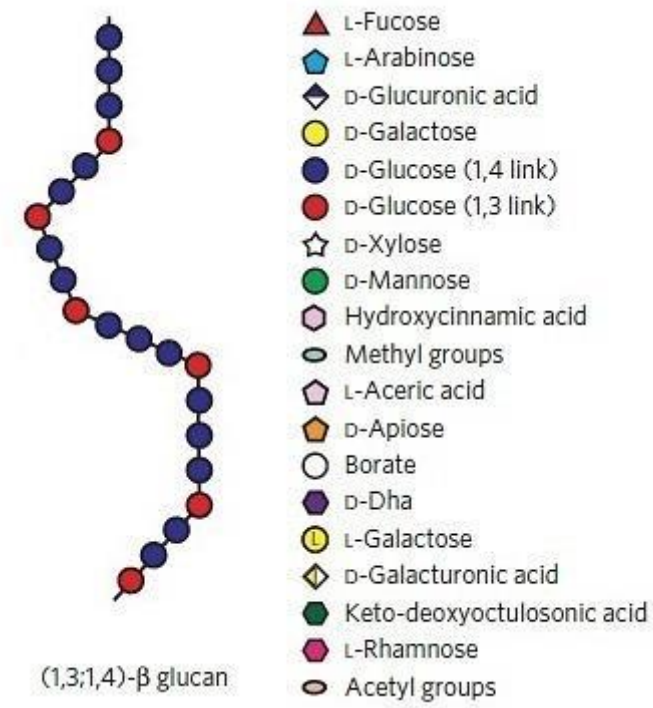


Figure 8 - Biochemical structure of mixed linkage glucans - Adapted from (Burton et al., 2010)

1.3.6 Other cell wall components and starch

Different plant cell walls also contain many hundreds of different proteins, glycoproteins and proteoproteins, as well as cutins, lipids, waxes and numerous enzymes, each having a unique and important role in the complex bioactive biochemistry of the plant cell wall, such as initiating and stopping cell wall expansion. Starch, like cellulose and mixed linkage glucans, is composed of glucose but the with an α-1→4 linkage between the glucose molecules (figure 8), which prevents the close packing seen in cellulose, and so is easily hydrolysed or digested by the enzyme α- amylase into glucose, maltose and isomaltose (Albersheim, 2011). Plants form starch in the chloroplasts to use for respiration at night when the plant is not photosynthesising and as a method of storing energy for future growth, such as in seeds for germination and in biennial plants, such as carrots, over-wintering ready to flower in spring.

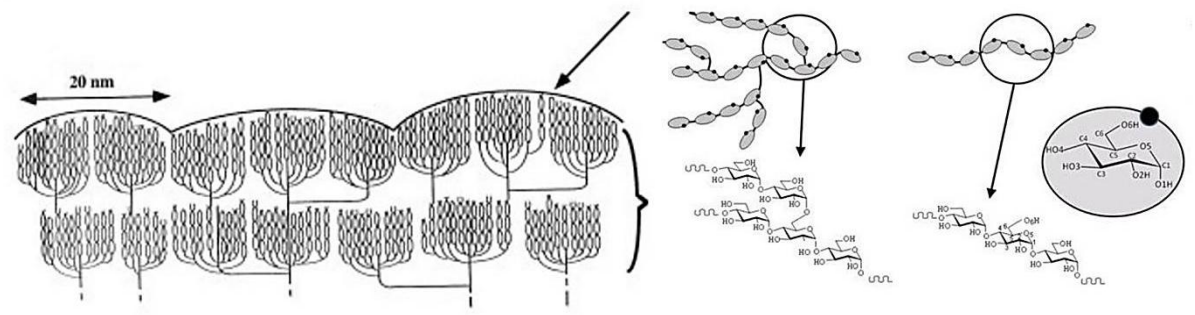


Figure 9 - Schematic of the structure of starch adapted from (Pérez and Bertoft, 2010, White, 2014)

1.4 Biomass pre-treatments

Due to the nature of the polysaccharides present in the plant cell walls, such as crystalline cellulose and the presence of lignin bound to cell wall polymers (CWP), pre-treatment of the biomass is needed before saccharification and/or fermentation can take place. This pre-treatment can either be physical, such as grinding the biomass to small particulate sizes, or chemical such as acid hydrolysis. These pre-treatments are designed to hydrolyse cell wall polymers into monosaccharides, expose and remove crystalline cellulose and lignin. The problems with these pre-treatments are that they are either expensive, produce fermentation inhibiting compounds such as furfurals from the breakdown of xylose (Kumar et al., 2009, Carroll and Somerville, 2009). However, the most common methods of biomass pre-treatment are given below with a brief explanation:

- Mechanical pre-treatment – this is the most basic of pre-treatments and is normally included with most other pre-treatment methods mentioned below. The biomass is chipped, milled and/or ground to a power, or sawdust, with a fine particulate size, to increase the surface area

of the biomass and also reduce the crystallinity of the cellulose, which increases its susceptible to enzymatic digestion (Kumar et al., 2008, Kumar et al., 2009, Moreno et al., 2013b).

- Partial acid hydrolysis – in this process the glyosidic bonds between the monosaccharide building blocks of cell wall polymers are hydrolysed, releasing them into the liquid hydrolysate, which can be neutralised and fermented. Once certain cell wall polymers have been hydrolysed, others will be revealed for further saccharification. However, this process can be expensive if the acids are not recovered and/or specialist equipment is needed to resist corrosion caused by the acids used. Hot acid hydrolysis can also lead to the breakdown of the monosaccharides, to produce fermentation inhibiting compounds such as acetic acid and furfural from the breakdown of hemicellulose and xylose respectively (Harmsen, 2010). It does remain the best way to degrade all polymers into monosaccharides (Kumar et al., 2008, Wang, 2008, Kumar et al., 2009, Brodeur et al., 2011, Harmsen, 2010).
- Ethanol-organosolv pre-treatment – biomass is pulped using a solvent, normally ethanol or methanol along with a catalyst (normally an acid) and heated to approximately 200^{o}C. This removes lignin and partially hydrolyses hemicellulose. The solvent can be recovered to reduce costs of this method, and should be routinely removed as the solvent can be a fermentation inhibitor. Evaporation of the solvent should leave any monosaccharides hydrolysed from the hemicelluloses behind for fermentation (Kumar et al., 2009, Hallac et al., 2010, Harmsen, 2010, Tong, 2013).
- *In vitro* enzyme and/or biological pre-treatment – this method utilises enzymes produced by saprophytic micro-organisms, either by adding the extracted and purified enzymes to the biomass *in vitro* or by inoculating the biomass with the micro-organisms themselves. Most methods of biomass conversation utilise enzymes at some stage, as they are an excellent method of reducing the cell wall polymers to fermentable monosaccharides, without the risk of producing fermentation inhibiting compounds and they can be used in relatively mild conditions, unlike hot acid hydrolysis (Lee, 1997, Kumar et al., 2008, Wang, 2008, Kumar et al., 2009, Harmsen, 2010, Moreno et al., 2013a, Moreno et al., 2013b, Moreno et al., 2013c).
- SO_2 impregnation – In this process a vessel containing the biomass is filled with sulphur dioxide and pressurised. This disrupts the biochemical structure of the biomass and has been shown to result in a 30% increase in fermentable sugars and a 65% reduction in fermentation inhibiting compounds compared to acid hydrolysis pre-treatment (Sipos et al., 2010, Geddes et al., 2011, Tong, 2013).
- Ammonia fibre expansion (AFEX) / alkaline hydrolysis - these processes are employed to remove lignin, reduce the crystallinity of cellulose, increasing the surface area and remove the

acetyl groups from the hemicellulose, which can form fermentation inhibiting acetic acid. With AFEX, the biomass is exposed to liquid ammonia at between 60°C-100°C (Tong, 2013). This breaks the ester and glyosidic bonds in the polymers and increases the surface area but does not remove any hemicellulose, leaving the sugars present in them available for degradation and fermentation. In the case of alkaline hydrolysis biomass is packed into a reactor tube and liquid ammonia passed over it at high temperatures under slight pressure (Harmsen, 2010). A further benefit of these processes is the ability to recover the ammonia, so reducing costs. It is possible to utilise other alkalis such as lime, sodium, calcium and/or potassium hydroxides; these can be used at low temperatures and pressures and result in far less sugar degradation than ammonia (Kumar et al., 2009, Harmsen, 2010, Brodeur et al., 2011, Geddes et al., 2011, Tong, 2013).

- Steam explosion – This process is the most commonly used method as it does not employ any dangerous and expensive chemicals. Biomass is heated up to between 160-260°C, under pressure, for up to twenty minutes, the pressure is then released and the biomass undergoes explosive decompression (Moreno et al., 2013a, Moreno et al., 2013b, Moreno et al., 2013c). The water acts as an acid at high temperatures breaking glycosidic bonds. This process can be hastened by adding CO_2 or SO_2 to the water used for steam. This process increases the surface area of the biomass and exposes cellulose microfibrils. However, these processes can hydrolyse the hemicellulose and result in acetic acid, an inhibitory compound. Fortunately, it also removes lignin (Wang, 2008, Kumar et al., 2009, Sipos et al., 2010, Brodeur et al., 2011, Moreno et al., 2013a, Moreno et al., 2013b, Moreno et al., 2013c).
- Hot water pre-treatment - Biomass is mixed with water in a metal reactor tube and heated to 190°C – 240°C at a pressure of 12.5 atmospheres for approximately 30-60 minutes and then is rapidly cooled (Kim et al., 2009, Harmsen, 2010). This dissolves up to 60% of the biomass (Brodeur et al., 2011), which hydrolyses the cellulose and hemicellulose to oligosaccharides and monosaccharides, which are then fermented, without producing any inhibitor compounds (Kim et al., 2009, Harmsen, 2010, Brodeur et al., 2011, Pedersen et al., 2011, Meyer et al., 2013, Li, 2013).
- Oxidation – This process utilises oxygen to solubilise hemicellulose and remove lignin by converting it to carbon dioxide, water and carbolic acids. Biomass is added to water, aerated and pressurised to approximately 12 atmospheres, and heated to approximately 200°C. This can also be achieved heating biomass to 30°C, for eight hours in hydrogen peroxide (Kumar et al., 2008, Kumar et al., 2009, Harmsen, 2010, Brodeur et al., 2011). However, this also process

forms fermentation inhibiting compounds which would need to be removed before fermentation, which would increase production costs.

1.5 Inhibitor compounds

Several of the pre-treatments mentioned above are essential steps to avoid the production of compounds that inhibit microbial growth, which would reduce the effectiveness of these systems. If a liquid hot water treatment and/or the removal of hemicellulose are not undertaken then the acetyl groups in hemicellulose will form acetic acid (Scheller and Ulvskov, 2010, Eiteman, 2014). Furfural is also produced by the hot acid hydrolysis of hemicellulose. Furfural is formed when arabinose and xylose are dehydrated (Ibraheem and Ndimba, 2013, Baral and Shah, 2014), and high concentrations of the compound inhibit respiratory microbial growth (Favaro et al., 2013, Cuevas et al., 2014, Kumar, 2014, Monlau et al., 2014). Lignin can be removed by oxidation in potassium permanganate or sodium hydroxide/urea (Shi et al., 2014). If not removed, the lignin is degraded into phenolic compounds which inhibit microbial growth by compromising the cell membrane (Borneman, 1986, Ibraheem and Ndimba, 2013, Baral and Shah, 2014, Monlau et al., 2014).

Furfural can be removed by heating and vaporization to alleviate the toxic effect (Li, 2013) or by filtering the hydrolysate through activated carbon (Cuevas et al., 2014).

However furfural and acetic acid are not necessarily inhibitory in small amounts; *E. coli* and some yeast can convert furfural into $NAD(P)H^+$ (Liu et al., 2009) and acetate is used to produce Acetyl Co-A, (Garrett, 2016), a vital intermediary in many cell functions (see section 1.9 and 1.10) .

1.6 Biofuel fermentation, harvest and yield

After the chemical or biological hydrolysis of the biomass, the resulting product is a mixture of different oligo – and mono- saccharides from the polymers present in the biomass cell walls. Once this solution has been neutralised (if need be), it is then fermented with the appropriate micro-organisms, depending on the monosaccharides present, which is dependent on the biomass used. For instance, if the biomass was waste paper, the resulting liquor could easily be fermented with wild type *Saccharomyces cerevisiae*. In most cases the resulting ethanol is recovered by distillation and/or molecular sieves (Kumar and Murthy, 2011, Monot et al., 2013, Meyer et al., 2013).

1.7 Potential wild type degrader, fermenter and transformable micro-organisms

The aim of this study is to develop mixed microbial cultures to convert lignocellulose degrading biomass into biofuels. Therefore, background about the abilities of commonly used microorganisms needs to be understood.

There are several possible organisms that could potentially be used in this study, either as biomass degrading organisms (*Cytophaga hutchinsonii, Cellulomonas fimi* and/or *Trichoderma reesei*), as ethanol producing organisms (*Saccharomyces cerevisiae* or *Pichia* spp.) or as organisms to be transformed (*Bacillus subtilis, Escherichia coli, Citrobacter freundii* and *Saccharomyces cerevisiae*), that can be genetically modified to either produce enzymes to degrade biomass and/or have foreign pathways inserted to utilise the different monosaccharides released from the biomass, for the production of ethanol and other important liquid biofuels such as butanol (Antoni et al., 2007, French, 2013).

1.7.1 Cytophaga hutchinsonii

The complete genome sequence of *C. hutchinsonii* has been published (Xie et al., 2007) *C. hutchinsonii* is a gliding gram negative soil bacterium (Xie et al., 2007, Liu, 2012, Duedu, 2015). *C. hutchinsonii* is capable of degrading crystalline cellulose and xyloglucan (Brenner, 2007, Xie et al., 2007, Rubin, 2008, Wilson, 2009, Duedu, 2015). The presence of these enzymes, however, is more than sufficient to make *C. hutchinsonii* an ideal candidate for use in biomass degradation, but for degradation of these polysaccharides direct contact between the bacteria and the substrate is needed (Liu, 2012), achieved by the *Cytophaga* characteristic yellow mucilage, which consists of polysaccharides composed of glucose, mannose, arabinose, xylose and glucuronic acid residues (Liu, 2012).

1.7.2 Cellulomonas fimi

Cellulomonas fimi is also a soil living, gram positive bacterium capable of degrading cellulose and xyloglucan (Kane, 2014, Diaz, 2015). There are 12 known extracellular enzymes involved in cellulose and hemicellulose degradation produced by *Cellulomonas fimi* including exo- cellulases, β-glucosidases, β-mannanase and endo- xylanases (Brenner, 2007, Chen et al., 2012, Srivastava, 2012, Kane, 2014, Duedu, 2015). This micro-organism is also a candidate for biomass degradation, as it is able to grow on wood, agricultural wastes and crystalline cellulose (Poulsen, 1989). It is also a good candidate for mixed culture systems as mixed cultures of *Cellulomonas* and *Ruminococcus* spp. have been shown to produce competitive yields of hydrogen utilising cellulose as the substrate (Nandi and Sengupta, 1998).

1.7.3 Trichoderma reesei

Trichoderma reesei is a filamentous fungus capable of producing exo- and endo- cellulases, β-glucosidase and hemicellulases (Nidetzky, 1994, Martinez et al., 2008, Kumar et al., 2008, Seiboth,

2011). This micro-organism is said to be the "work horse" of industrial cellulases production, producing over 100g of cellulases enzymes per litre of medium (Seiboth, 2011). For this reason, it is an excellent candidate for biomass degradation for use in this study.

1.7.4 Bacillus subtilis

Bacillus subtilis 168 is derived from a soil dwelling, gram positive, bacterium (Zeigler et al., 2008). *B. subtilis* 168 is itself a tryptophan auxotroph, meaning it need an *ex vivo* source of tryptophan normally as yeast extract, which grows well on monosaccharides commonly derived from biomass, such as galactose, glucose, xylose and arabinose (Jacob, 1991, Lindner, 1994, Schmiedel, 1996, Krispin, 1998a, Krispin, 1998b, Brenner, 2007, French, 2013), although *B. subtilis* 168 use of monosaccharides is induced and/or repressed by the presence of other monosaccharides (Jacob, 1991). *B. subtilis* 168 naturally secretes cell wall polymer degrading enzymes, such as (glucurono)xylanases (Welker, 1967, St John et al., 2006, Brenner, 2007, Oyeleke, 2012). However, it does not naturally ferment these monosaccharides to ethanol and modifying it to produce ethanol as proved difficult (Romero *et al*., 2007 and French *et al*., 2013), but in any case *B. subtilis* has been shown to tolerate up to 13% ethanol (v/v) (Sivagurunathan, 2013, Fletcher, 2014). *B. subtilis* also naturally produces lactic acid, another potentially important chemical, via the homolactic fermentation pathway (Abdel-Rahman, 2011, Abdel-Rahman et al., 2013, Poudel et al., 2015). Regardless, it may be possible to modify this organism to produce other liquid biofuels of interest and/or be modified to over express its biomass degrading enzymes as it is easily engineered and rapidly grows in minimal media (French, 2013). Several strains have been produced that are deficient in certain cell membrane proteases (Lee, 2000, Westers et al., 2004) which may be useful for over-expression of useful proteins and enzymes. *B. subtilis* is "*Generally Regarded as Safe*", unlike *E. coli*, so can be used for food grade applications and is indeed actually consumed in south East Asia as *Natto. Bacillus licheniformis* has been shown to produce hydrogen (Nandi and Sengupta, 1998). That *B. subtilis* 168 is a tryptophan auxotroph also adds the safe-guard that it should not be able to survive outside the lab, although it does produce long lived endospores which are highly resistant to heat and desiccation, so can survive for many years (Nicholson, 2008, French, 2015).

B. subtilis' natural competence

Bacillus subtilis has an interesting ability; it becomes naturally competent under starvation conditions (Claverys et al., 2006) and this has been suggested to be a survival mechanism, either to utilise any uptake of DNA as a carbon source (Claverys and Havarstein, 2007, Overballe-Petersena, 2010) or to incorporate the DNA into the *Bacillus* genome as a survival mechanism (Claverys et al.,

2006). This ability can be utilised to genetically modify *B. subtilis* 168, without the need for chemically or electro- competent cells, required for the transformation of *E. coli*.

1.7.5 Lactobacillus spp.

Lactobacillus are gram positive bacteria and can either be hetro- or homo- fermentive, able to produce lactic acid (homo) or lactic acid and ethanol (hetero) from a range of hexose sugars (Thomas, 1979, Brenner, 2007). *Lactobacilli are* found in a variety of environments and have no adverse effects on human health. They are one of the bacteria utilised in natural yogurt and so, like *B. subtilis*, are "Generally Regarded as Safe" so are not subject to the same stringent regulation as *E. coli*.

1.7.6 Escherichia coli

Escherichia coli is a gram-negative bacterium, found in the intestines of humans. It is a fast-growing model organism and has been used in laboratories for genetic manipulation for many years, giving rise to many different strains and modification techniques (Lederberg, 1946, Russo, 2003, French, 2013). It is easily transformed through chemically induced competence and heat shock or mutation through the use of UV radiation (Yun, 2008). For these reasons, it is a favoured organism for genetic modification and is often utilised in synthetic biology. *E. coli* is a well characterised micro-organism and is naturally capable of fermenting a wide range of sugars normally obtained from biomass, both pentoses and hexoses, such as glucose, arabinose and xylose into ethanol (Weber et al., 2010). However, this is achieved through the mixed acid pathway, which means *E. coli* produce equal amounts of acetic acid and ethanol. While this may seem a negative aspect, acetic acid is a valuable chemical and should be seen as an added value product rather than waste. If desired, acetate production can easily be overcome by expressing pyruvate decarboxalase (*pdc*) and alcohol dehydrogenase (*adh*) genes (French, 2013). *E. coli* has been modified to produce other liquid biofuels such as butanol and propanol (Lee et al., 2008) and to secrete a range of biomass degrading enzymes, such as pectate lyase, endo- and exo- glucanases and β-glucosidase (French, 2013, French, 2009); however, it does not seem to secrete these enzymes to a sufficient level to be used in current lignocellulose degrading conversion systems.

1.7.7 Citrobacter freundii

Citrobacter freundii is a soil borne, gram negative, bacterium, involved in the reduction of nitrate to nitrite in the nitrogen cycle and is a symbiotic bacterium in the digestive tract of termites (Rosswall, 1981, Deakin, 2012) and humans (Pereira et al., 2010). It can readily be used as a chassis organism as it is similar to *E. coli* and so can utilise *E. coli* vectors etc. *C freundii* naturally contains the pathways to utilise a range of sugar sources including cellobiose (Lewicka, 2014, Duedu, 2015), a major breakdown product of the biomass used in this study, and therefore should be relatively easy to modify for the production of liquid biofuels.

1.7.8 Saccharomyces cerevisiae

Saccharomyces cerevisiae, also known as Brewer's yeast, is naturally able to ferment glucose and sucrose into ethanol, but cannot naturally ferment the monosaccharides released from the hydrolysis of hemicellulose (French, 2013). This has limited its use in the conversation of cellulosic biomass to ethanol, since it is only able to utilise the glucose released through the hydrolysis of cellulose and xyloglucan, so failing to utilise a large amount of the monosaccharides released (see figures 5 and 6). *Saccharomyces cerevisiae* is, unsurprisingly, "Generally Regarded As Safe", so can be used for food grade applications. However, since this is an industrially important micro-organism and one that is easily genetically modified there has been a great deal interest in engineering this organism to be able to fully utilise the commonly released monosaccharides from biomass. Strains that are able to utilise xylose (Ho, 1998, Sedlak, 2004, Matsushika et al., 2009, Bera et al., 2011) arabinose (Sedlak, 2001, Richard et al., 2003, Becker and Boles, 2003) and both xylose and arabinose (Karhumaa et al., 2006, Bettiga et al., 2009, Garcia Sanchez, 2010, Bera et al., 2010) have now been developed. These strains are able to utilise xylose and arabinose by converting the sugars to xylitol and arabitol, respectively (Garcia Sanchez, 2010, French, 2013). These engineered strains are able to utilise glucose, xylose and arabinose to such an extent, that they can produce between 60% (arabinose) 83% (xylose) and 100% (glucose) of the theoretical yield of ethanol from the sugars present over 72 hours, depending on which sugars and strains were studied (Sedlak, 2004, Karhumaa et al., 2006, Matsushika et al., 2009).

1.7.9 Pichia spp.

Pichia spp. are a genus of yeasts that are naturally able to ferment xylose, arabinose and more interestingly galactose, as well as glucose and cellobiose (Knoshaug et al., 2009, Weber et al., 2010). This ability makes it a very attractive fermentation organism for the use in biomass conversation, as it would be able to utilise the galactose released from the RG1 domain of pectin, if used in biofuel

systems that utilise pectin rich species. However, its uptake of monosaccharides is lower than that of *S. cerevisiae*, with its preferred monosaccharide being glucose, as is its yield of ethanol, approximately 40% of the potential yield from sugars available (Weber et al., 2010). However, if it were to be grown in a mixed culture with other fermenter organisms, these shortcomings may be negated by its ability to utilise the monosaccharide (galactose) other organisms do not, once the glucose supply had been exhausted. This versatility is demonstrated in a strain of *Pichia anomala* that is able to produce ethanol from a range of biomass sources, both aerobically and anaerobically, utilising both pentose and hexose sugars (Zhaab, 2013).

1.7.10 Clostridium spp.

Clostridium is a genus of soil dwelling gram-positive rod-shaped bacteria. They are obligate anaerobes capable of producing endospores, and some species are pathogenic. *Clostridium thermocellum* is capable of producing ethanol and hydrogen, from cellulose containing biomass due to its range of cellulases and is capable of using glucose, xylose, cellobiose and xylobiose for growth (Lee, 1975, Freier, 1988, Nakayama et al., 2011, French, 2013). Co-cultures of different species of *Clostridium* (*Clostridium acetobutylicum* and *Clostridium cellulolyticum*, *Clostridium thermocellum* and *Clostridium saccharoperbutylacetonicum*) have been shown to be synergetic, yielding higher quantities of hydrogen, ethanol and butanol than single cultures when grown on cellulose biomass, (Nakayama et al., 2011, Salimi, 2013). Co-cultures of *Clostridium butyricum* and *Rhodobacter sphaeroides* have also been shown to be possible and that this co-culture also yielded higher amounts of biohydrogen than a mono-culture (Chen et al., 2005, Fang et al., 2006). It may be possible to utilise *Clostridium* as a partner with either a cellulose degrading bacterial partner or a chassis organism, engineered to complement the product produced by its *Clostridial* partner. Failing that it would be possible to grow *Clostridium* spp. on cellulose degrading biomass in mono-culture and still generate reasonable yield of hydrogen, ethanol and butanol from this cheap and abundant source of biomass.

1.7.11 Isolated cell wall degrading enzymes

While the micro-organisms *C. hutchinsonii*, *C. fimi* and *T. reesei* have a range of *endogenous* enzymes (Alper, 2009), a more efficient method of utilising enzymes is to add them *in vitro* in a higher concentration that may be possible by the microbes, especially if those microbes are growing in a minimal medium. There are a range of commercial enzymes available, such as cellulases, xylanases, etc. but a handy one is Driselase as it yields specific disaccharides depending on the source of the biomass, isopromeverose from xyloglucan from dicots and xylobiose from xylans (Kerr and Fry,

2003). Driselase is a cocktail of enzymes from the white rot fungus *Irpex lacteus* and contains fucosidases, glucanases, β- mannosidase α-cellobiohydrolase, β-galactosidases, β-xylanases and cellobiohydrolase cellulases β-glucanases β-galactanase α-arabinanase pectinases β-mannanase and β-xylanases so can completely digest plant cell walls (Fry, 1988). These enzymes allow for a more robust analysis of the biomass used in this study.

1.8 The use of mixed cultures of cellulose-degrading and product-producing organisms for production of cellulose degrading biofuels

There have been several papers that utilise the use of mixed saccharophytic and fermentive micro-organisms for synergetic growth. Mixed cultures of *Cellulomonas* and *Xanthomonas* were demonstrated (Ponce-Noyola, 1993), showing that thanks to both micro-organisms having coinciding growth requirements, they were able to grow using sugarcane waste, as a lignocellulosic carbon source. A study using a mixed culture of *C. fimi* and *Z. mobilis*, producing ethanol from straw, produced yields of 18% ethanol (v/v) by seven days (Srivastava, 2012). A similar system was developed by (Haruta et al., 2002), although the exact micro-organisms were not specifically selected, wild microbial communities found in sugarcane waste, rice straw and chicken, pig and cattle faeces were used as the starter cultures for this system. While the microbes in these environments undoubtedly would have the ability to degrade lignocellulosic biomass because they either live on rice straw and/or live in the digestive systems of animals that subsist on lignocellulosic biomass, using 16S ribosomal RNA PCR identification seems inefficient due to the fact that along with cellulose degrading micro-organism that are present there will also be microbes that are of no concern to any work interested in developing microbial conversion of lignocellulosic biomass to useful presents. A better system would have been to isolate, as far as possible, the individual microbe colonies and then sequence the “pure” culture. Of course, there is no guarantee that the microbes of interest would be able to be cultured in the lab on agar plates. Some isolation did take place after ten days’ growth of the mixed cultures with samples being isolated for growth on filter paper, but this only shows that cellulose degrading micro-organisms are present. By the very nature of the systems described, non-cellulose degrading micro-organisms may have been present and simply living off the glucose released from the filter paper (Haruta et al., 2002). A similar study, using microbes isolated from straw biomass and using 16S ribosomal RNA PCR identification showed that the species present included *Sporocytophaga* sp., *Xanthomonas* sp, *Pseudomonas* sp. *Alcaligenes* sp., *Ochrobactrum* sp., *Achromobacter* sp. and *Pseudomonas* sp., (Dumova and Kruglov, 2009). Studies investigating the use of mixed cultures including *Lactobacillus acidophilus* and *Debaryomyces hansenii* (Portilla et al., 2008) to produce lactic acid from grape vine waste, *Trichoderma reesei* and *Saccharomyces cerevisiae* (Brethauer and Studer, 2014) to produce ethanol from cellulose,

Clostridium butyricum and *Rhodobacter sphaeroides* to produce hydrogen from glucose (Fang et al., 2006) and *S. cerevisiae* and yeasts *Candida shehatae* and *Pichia stipites* to utilise glucose and xylose from lignocellulosic biomass (Monlau et al., 2014) have also been attempted.

1.9 The biochemistry of fermentation

Below is outlined some basic biochemistry essential to understand the experiments set out in chapters 2-6.

1.10 Metabolic pathways and fermentation

The three main metabolic pathways in this study are; glycolysis/pentose phosphate pathway (figure 10); pentose sugar utilisation (figures 11 and 12); ethanolic fermentation (figure 13) homolactic fermentation (figure 13 and 14) and butanol production (figure 15).

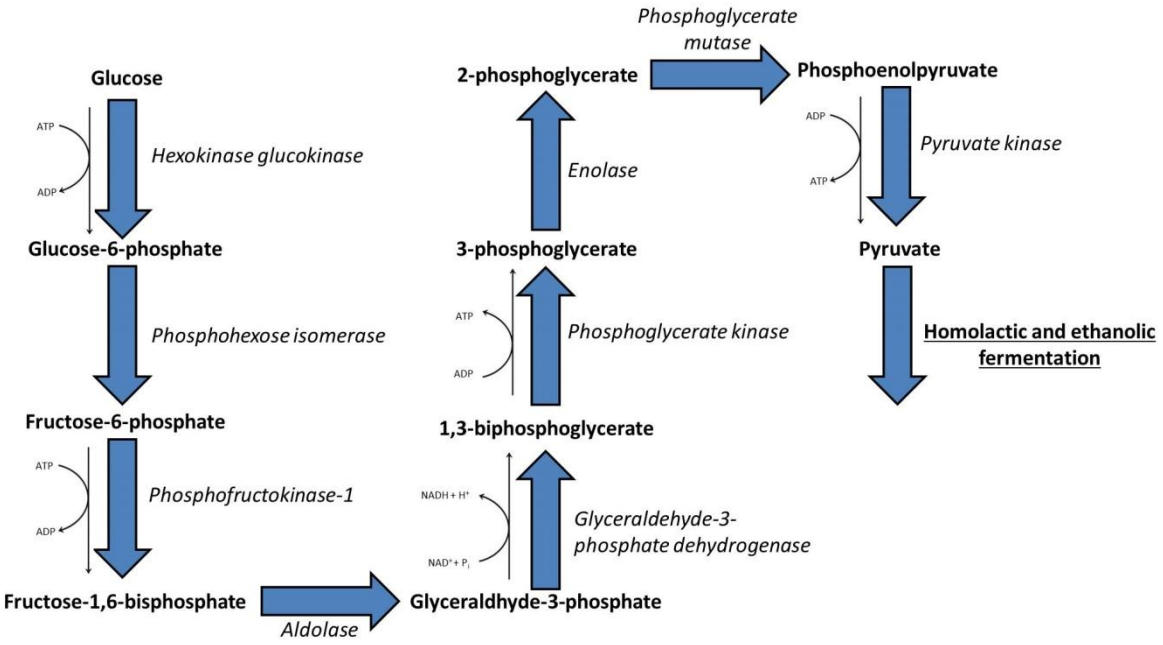


Figure 10 – The glycolysis pathway for glucose adapted from (Lehninger, 1975)

Glucose is the sugar of choice for most organisms. It is normally utilised via the glycolysis/ Embden–Meyerhof–Parnas (EMP) pathway, as are other hexose sugars, such as galactose or mannose. These are converted to pyruvate, with the energy released by this reaction used to form ATP. In the case of bacteria and yeasts, pyruvate is then converted to the waste products lactic acid/ethanol by anaerobic fermentation (figs 13 and 14). If glucose is not available organisms are able to utilise pentose, 5-carbon sugars. These cannot be directly used via glycolysis; they must first be transformed to D-xylulose-5-phosphate before entering the pentose phosphate pathway (fig 11).

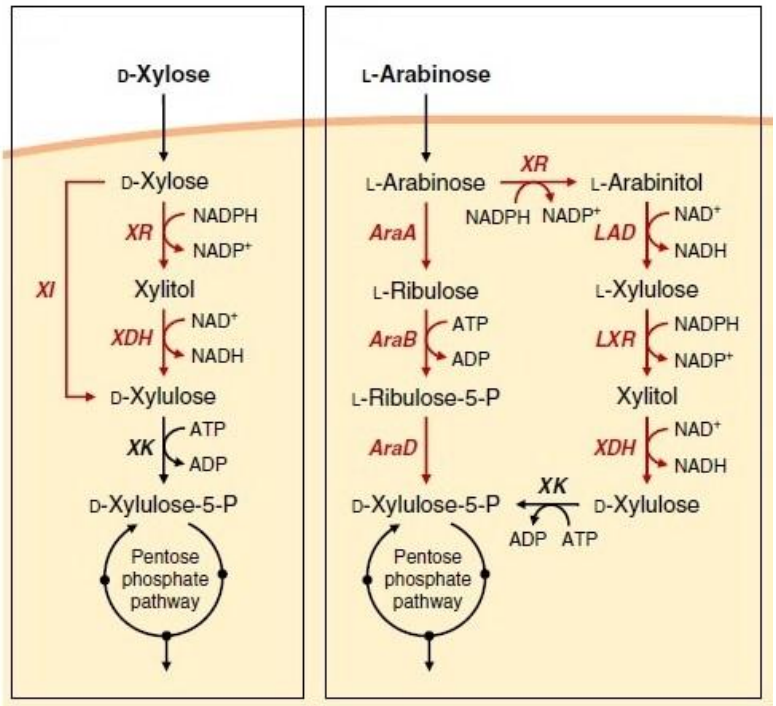


Figure 11 – Over view of metabolism of non-glucose sugars adapted from (Bettiga et al., 2009) and (Zhang et al., 2015). XI=xylose isomerase, XR=xylose reductase, XDH=xylitol dehydrogenase, XK=xylokinase, LAD=L-arabitol dehydrogenase, LXR=L-xylulose reductase.

Pentoses, five-carbon sugars, are converted to D-xylulose-5-phosphate before they enter the pentose phosphate pathway (figures 11 and 12). This is achieved either via xylose reductase, to xylitol, then xylose dehydrogenase, to D-xylulose or xylose isomerase, D-xylulose is then phosphorylated to D-xyulose-5-phosphate (fig 12).

Arabinose is first reduced to L-arabitol, by xylose reductase, then to L-xylulose via L-arabitol dehydrogenase then to xylitol by L-xylulose reductase then to D-xylulose by xylitol dehydrogenase, D-xylulose is then phosphorylated to D-xyulose-5-phosphate.

Once both sugars are at the D-xylulose-5-phosphate stage they enter the non-oxidative phase of the pentose phosphate pathway, which facilitates their complete degradation into hexose sugars to be utilised via glycolysis (Lehninger, 1975).

Once the pentose sugar is converted to D-xylulose-5-phosphate it enters the non-oxidative phase of the pentose phosphate pathway and is converted to D-ribose-5-phosphate; D-sedheptulose-7-phosphate; D-Glyceraldehyde-3-phosphate; via the transketolase reaction, then to D-Fructose-6-phosphate, via the transaldolase reaction. The D-Fructose-6-phosphate then enters the glycolysis pathway (fig 10). After glycolysis, the product has been converted to pyruvate, which is then reduced to either lactic acid or ethanol anaerobically (fig 13).

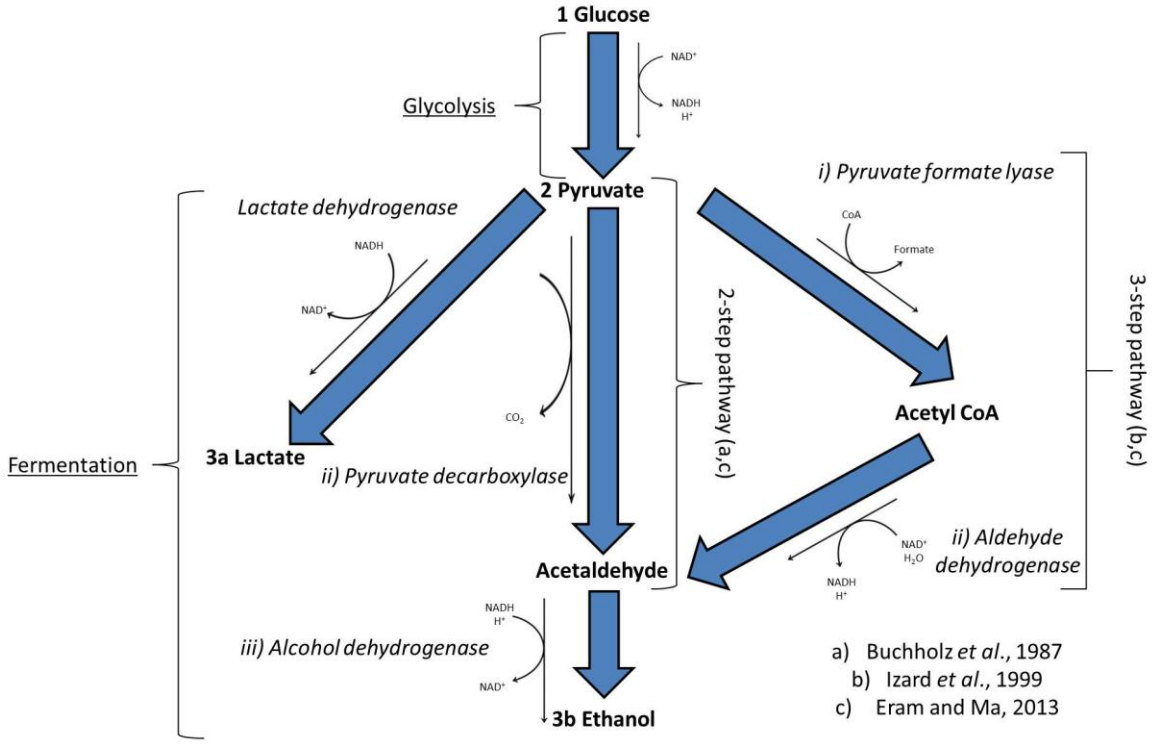


Figure 12 – Homolactic and ethanolic fermentation pathways. The 2-way pathway is typical of *Z. mobilis*; the 3-way pathway is typical of *E. coli et al*. Adapted from (Buchholz, 1987, Izard, 1999, Eram and Ma, 2013)

To utilise monosaccharides for growth and the production of ATP micro-organisms convert these sugars to pyruvate. This pyruvate is then reduced to either lactic acid or ethanol as waste products. These waste products are of interest to humanity for a variety of uses (section 1.1).

Depending on the organism, pyruvate can be reduced to lactate via lactate dehydrogenase via homolactic fermentation, giving pure L-lactic acid (figure 13 and 14 and Poudel *et al*, 2015). This pathway is present in *Lactobacillus* species, of the type found in natural yogurt. In the case of heterofermentative lactic acid bacteria, pyruvate can also be reduced to the mixed products of D/L-lactate acetic acid and ethanol via heterolactic fermentation (Figure 14) and (Busse, 1961, Thomas, 1979, Poudel et al., 2015).

In the case of ethanol fermentation, this is achieved as either a two-stage pathway, or a three-stage pathway. In the two-stage pathway, pyruvate is converted to acetaldehyde via the action of pyruvate decarboxylase, and then the acetaldehyde is converted to ethanol by alcohol dehydrogenase. This pathway is typical of *Zymomonas mobilis* and *Saccharomyces cerevisiae* (Buchholz, 1987, Eram and Ma, 2013). Ethanol can also be produced through the three-stage fermentation, typical of *E. coli*. In this pathway pyruvate is first converted to acetyl Co-A by the action of pyruvate formate lyase. The acetyl Co-A is then reduced to acetaldehyde by the action of

CoA-dependent aldehyde dehydrogenase. The acetaldehyde is then converted to ethanol by the action of alcohol dehydrogenase (Izard, 1999, Eram and Ma, 2013).

Both of these bacterial waste products can then be harvested for uses such as biodegradable plastic production (MacRae, 2010) or for fuel (Sebayang et al., 2016).

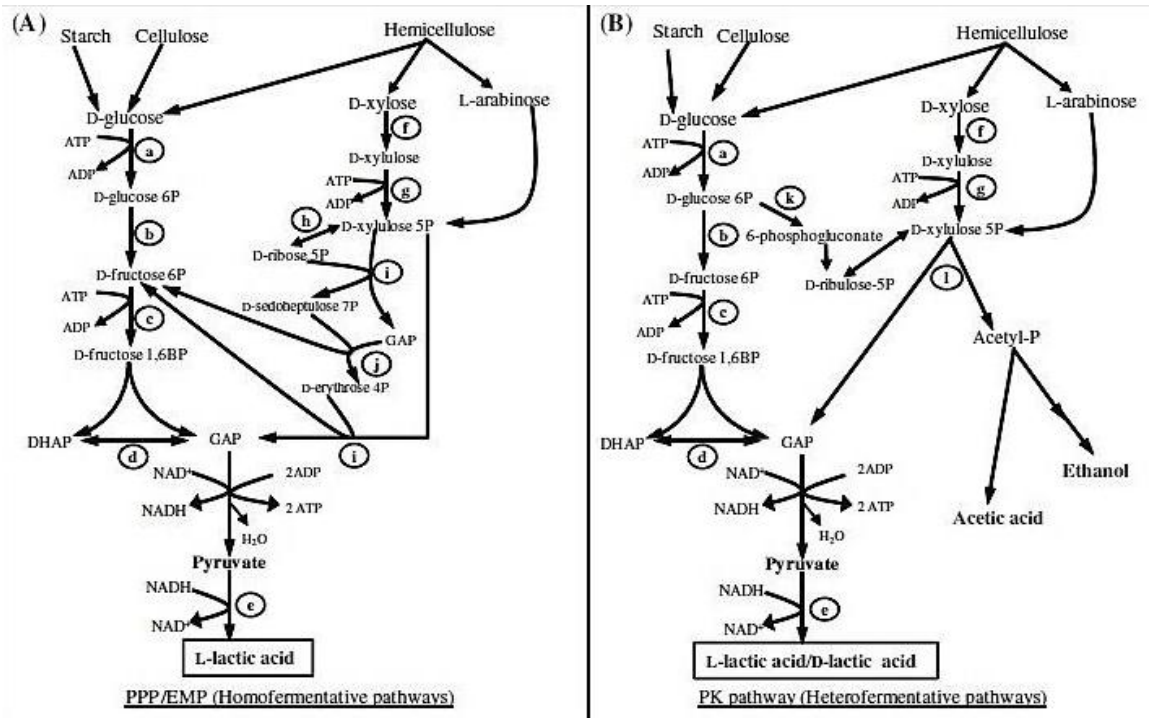


Figure 13 – Over view of the metabolic pathways for homo- and hetero- lactic fermentation. A = homofermentative; B = heterofermentative. Enzyme; (a) hexokinase; (b) glucose-6-phosphate isomerase; (c) 6-phosphatefructo kinase; (d) triose phosphate isomerase; (e) lactate dehydrogenase; (f) xylose isomerase; (g)xylulose kinase; (h) epimerase; (i) transaldolase; (j) transketolase; (k) glucose -6-phosphate dehydrogenase; (l) phosphoketolase. adapted from (Poudel et al., 2015)

Micro-organisms can also produce other useful products other than lactic acid and ethanol. *Clostridium* spp. can produce hydrogen gas and butanol (see section 1.1.2, 1.1.4) and (Nakayama et al., 2011). As figure 15 shows the butanol pathway starts far beyond the glycolysis pathway, at the acetyl Co-A stage of possibly the ethanol fermentation pathway, although the end product will be butanol not ethanol.

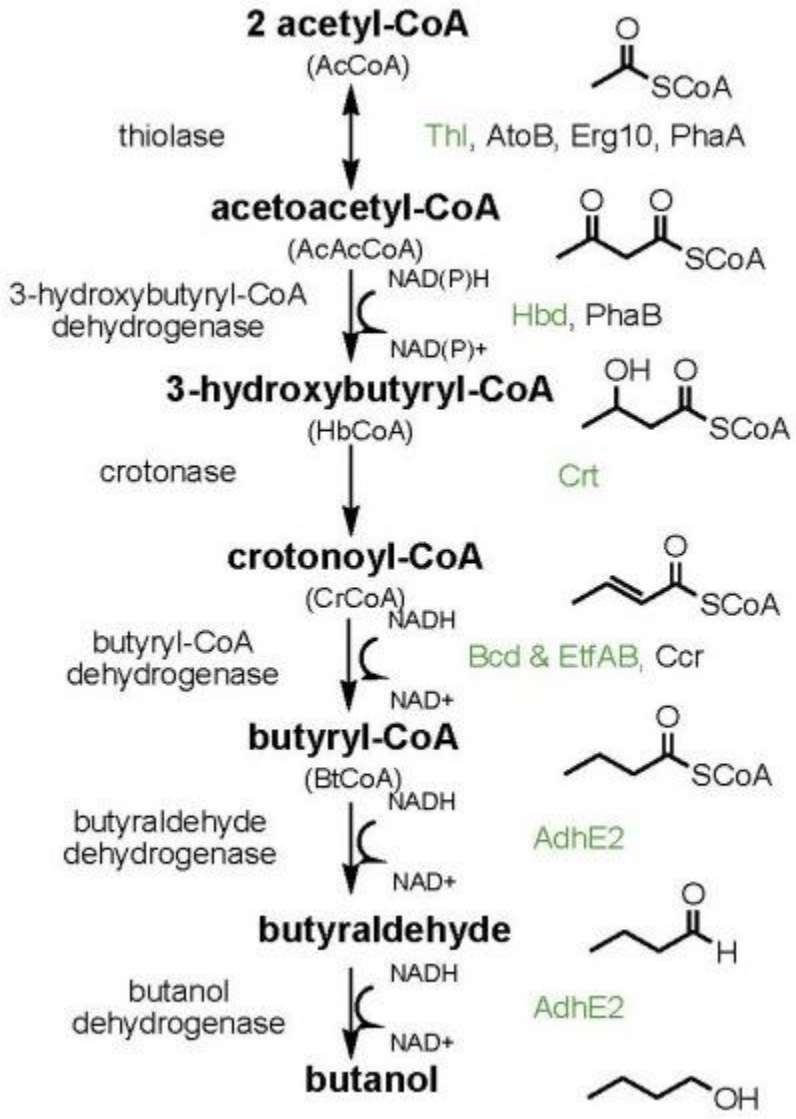


Figure 14 – The metabolic pathway for butanol production from (Steen et al., 2008)

As figure 13 and 15 shows, it is in theory possible to hijack the pyruvate and/or Acetyl Co-A to produce butanol using either live cells or isolated enzymes. But since *Clostridium* spp. naturally produce butanol and can do so from cellulose (Nakayama et al., 2011) this *in vitro* conversion would be a purely academic exercise, but is possibly feasible (Krutsakorn et al., 2013).

1.10.1.1 Colourimetric enzyme activity detection

It is possible to detect the presence of a substrate and/ or the activity of an enzyme using a colourimetric biochemical assay. As figure 16 shows, the cycling of NADH and/or NAD^+ driven by the actions of either ldh or adh, causes a chain reaction that ultimately leads to photochromic changes to **i**odo**n**itrote**t**razolium **v**iolet (INTV) that are detectable visually.

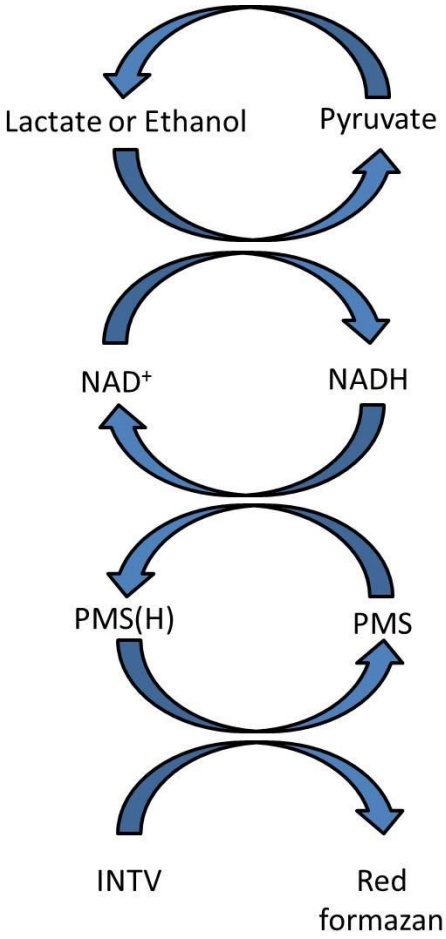


Figure 15 - Biochemical reaction of colourimetric forward and reverse reactions for activity assays, native gels and in vitro conversion. PMS(H) = the reduced form of PMS.

This assay is able to detect the activity of ldh and adh both in liquid reaction mix and in native poly acrylamide (PAGE) gels and /or the presence of lactic acid or ethanol, depending whether the enzyme or the substrate is the catalyst in the reaction. The oxidation of NADH to NAD is facilitated by either LDH or ADH, depending on the substrate used. **p**henazine **m**etho**s**ulphate (PMS) acts as an electron carrier for the NADH/NAD^+ cycle which causes the reduction of the INTV to formazan dyes (Greymore, 1969, Worsfold, 1977).

1.11 Synthetic biology, artificial DNA synthesis and assembly

Synthetic biology an interdisciplinary area of biology encompassing engineering, genetics, biophysics and biotechnology, which allows for, amongst other things, complete genomes to be synthesised, which has led to synthetic organisms being created (Gibson, 2010, Annaluru, 2014, Hutchison, 2016). It enables us to produce human serum albumin in plants (Sijmons, 1990) and produce spider silk from goats milk (Vollrath, 2001, Chung et al., 2012). Synthetic biology allows for the synthesis of unnatural base pairs, expanding the genetic alphabet past C-G A-T base pairs (Malyshev et al., 2012, Malyshev, 2014b) and artificial gametes (Nagy and Chang, 2007), allowing for the potential for a child to be born containing genetic material from two fathers or two mothers (Testa, 2005 , Master, 2006, Zhou, 2016). It is also ideally placed to fill in the gaps found in current lignocellulosic second generation biofuel production systems.

Shortly after Watson, Crick, Wilkins (and Franklin *posthumously*) published their findings on the double helix structure of DNA in 1953 (Watson, 1953), Michelson and Todd published their work on the first oligonucleotide synthesis (Michelson, 1955) and by the 1970s the process of synthesising oligonucleotides was highly successful (Beaucage, 1992) but it was not until the invention of polymerase chain reaction (PCR) by Kary Mullis in 1985 (Saiki, 1989) that the age of genetic modification could dawn. While cloning requires extracted genomic or plasmid DNA to be replicated by PCR, the synthesis of oligonucleotides, allows for truly unique and novel genomes to be constructed, only loosely based on those occurring in nature. The insertion of foreign DNA into an organism genome is nothing new; transgenic *E. coli* was first produced in the 1970's (Cohen, 1972). The method of genetically modifying bacteria is well known and understood and can be surmised as involving making bacterial cell membranes "porous" to facilitate the uptake of the foreign DNA, normally coding for antibiotic resistance, in the form of a plasmid. Transgenic plants were first produced in the 1980's (Fraley, 1983, Rani, 2013) the main use being to produce crop plants that are either resistant to certain herbicides or to produce toxins to kill pest insects (Herbers, 1999, Rani, 2013). The method of transforming plants requires either cell cultures where the foreign DNA is inserted into the single plant cell via the use of the *Agrobacterium tumefaciens* derived Ti plasmid, which has the desired gene inserted and then the whole plant recovered from this single transgenic cell, since some plant cells are totipotent (Rani, 2013) or by the "floral dip" method involving dipping the floral meristem in an inoculum of *Agrobacterium tumefaciens* containing the modified Ti plasmid. The plants are then allowed to grow and isolated so only self-fertilisation occurs. The seed are then collected and transgenic seedlings selected for by specific resistance (Clough, 1998). The first transgenic mice were produced in the late 1970's, early 1980's (Costantini, 1981, Kiermer, 2007). The most common methods for the production of mammalian transgenic animals are either

via embryonic stem cell modification (Stice, 1997, Lin, 2011) or pronucleus method (Costantini, 1981, Ittner and Gotz, 2007). In the case of embryonic stem cell modifications, embryonic stem cells with the desired gene are cultured in vitro, these cells are then injected into the developing blastocyst and then implanted into a surrogate and then the offspring are tested to see which contain the target gene (Stice, 1997, Lin, 2011). For the pronuclear method, just fertilised ova are taken before the nucleus of the sperm and ova fuse, the target DNA, in a vector, are injected into the male pronucleus, once the pronucleus' fuse, the zygote is implanted in the surrogate and then the offspring are tested for the desired gene (Costantini, 1981, Ittner and Gotz, 2007).

1.11.1 Self-replicating synthetic micro-organism

While transgenic bacteria have been around for many years, the transgenes used have always been copied / cloned from other organisms. With the advent of cheaper, more efficient synthetic nucleotide techniques it became possible to completely synthesise an entire genome from scratch. This completely synthetic genome could then be inserted into the cell and via homologous recombination, the synthetic genome was swapped with the native one, piece by piece, allowing the now synthetic cells to continue through the cell cycle and reproduce viable daughter cells (Gibson, 2010, Annaluru, 2014, Hutchison, 2016).

1.11.2 Synthetic nucleotides, artificial base pairs and the expanded genetic alphabet

Throughout all known life, there are four nucleotides (guanine-cytosine and adenine-thymine; G-C, A-T) present in an organism's DNA. These nucleotides make up genes which code for protein synthesis and are in turn transcribed into specific proteins causing the specialisation of cells. However, even as far back as 1962, an artificial third base pair; the possibility of an artificial, third base pair between isoguanine (isoG, 6-amino-2-ketopurine) and isocytosine (isoC, 2-amino-4-ketopyrimidine) was suggested (Rich, 1962), with these nucleotides being synthesised in 1989 (Switzer, 1989). Since then the genetic alphabet has been expanded to include several un-natural base pairs such as *s-y* (Hirao, 2002, Hirao and Kimoto, 2012, Malyshev et al., 2012, Malyshev, 2014b, Malyshev, 2014a) and recently the first organism to propagate stably an expanded genetic alphabet was reported (Yamashige et al., 2012, Malyshev, 2014b, Malyshev, 2014a). With the transcription of these un-natural genes the number of amino acids is increasing (Hirao, 2002, Hirao and Kimoto, 2012) and so increases the variety of proteins produced, which fundamentally changes the raw materials of life.

1.11.3 Recent genetic modification techniques

The field of genetic modification and synthetic biology is an ever expanding, fast paced sector. Below are some of the more recent advances in the field.

1.11.3.1 CRISPR/Cas9

This is currently the cutting edge in genome editing and relies on a system based on the "immune system" of the bacteria; *Streptococcus pyogenes* which identifies and removes foreign viral DNA. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) are elements of viral DNA incorporated into the bacterial genome so that the bacterium has a defence against the same infection at a later stage, in a similar way as antibodies in our immune system work. These elements of the viral DNA are used to produce crRNA and tracerRNA which bind to CRISPR associated protein 9 (Cas9), an endonuclease that uses these RNA molecules to identify foreign DNA which it then cuts at a site specified by the crRNA, and at a site specified by the tracer RNA. These restriction sites are either upstream or downstream of each other so that the cut is staggered and the cut site cannot simply join back together and so that gene is disabled.

In the CRISPR/Cas9 system, the Cas9 nucleases uses guide RNA, analogous to fused tracerRNA-crRNA, specifically designed to deliver the enzyme to a specific locus on the genome, Cas9 then cuts out the DNA at that site, allowing either homologous recombination integration of a cassette with homology to the DNA on either side of the cut or for deletion of a gene when the DNA repair mechanism of the cell ligates the cut ends back together. This system also allows several cuts to the same DNA, allowing for multiple deletions and/or insertions at the same time (Hovath, 2010, Jinek, 2012, Le Cong, 2013, Sander and Joung, 2014).

1.11.3.2 Zinc finger nucleases and TALEN

Both zinc finger nucleases and Transcription Activator Like Effector Nucleases (TALENs) are artificial restriction enzymes. Both the zinc finger nuclease and TALEN can be designed to bind to any sequences of DNA and when fused with a nuclease the adjacent region is cut (Pabo, 2001, Boch, 2011).

1.11.3.3 Other recombinases

Synthetic biology is also leading the fight against HIV, with the development of artificial recombinases that targets and removes HIV DNA present in the nucleus of infected cells in a similar manner to Cas9 (Karpinski et al., 2016).

1.11.3.4 Restriction *enzyme* ligase procedures

For many years, the use of restriction enzymes, which cut at specific nucleotide sequences in DNA, creating “sticky ends” were the standard method of DNA assembly. The sequence of interest was designed/scrutinised to determine which, if any, restriction sites were percent, the appropriate restriction enzyme was then used to digest the DNA at that specific site. Meanwhile the insertion cassette was digested with a complementary restriction enzyme so that the restriction sites had complementary i.e. A-T/G-C over hangs or “sticky ends”. These digested fragments were then ligated back together in the desired order (Lee, 1996, Pagett, 1996 , Knight, 2003, Canton et al., 2008, Shetty et al., 2008, Engler et al., 2008). There are several issues with this method of DNA assembly, such as the presence of forbidden sites, sequences of DNA that have a restriction site in them, but that the site is in the wrong place and does not want to be cut, which limits which restriction enzyme could be used, this is also a long process compared to other methods, with multiple purifications and PCR reactions and long digestion and ligation steps. However, they are very effective methods of DNA assembly with solid success rates and simple enough that the most novice member of a lab can utilise them successfully.

1.11.3.5 Gibson assembly

Unlike the use of restriction enzymes Gibson assembly uses a T5 exonuclease to chew back DNA from the 5' end of DNA cassettes which can have specific regions designed for the enzyme to digest created through PCR. The resulting single-stranded regions on adjacent DNA fragments “sticky ends” anneal together. A thermostable DNA Taq ligase is used to covalently join the DNA of adjacent segments and a DNA polymerase Phusion, to incorporate nucleotides to fill in any gaps. (Because the Taq ligase is thermostable it is able to work in the same conditions of the polymerase). It has been shown that up to 10x1kb fragments can be assembled with this method (Barnes, 1994, Gibson et al., 2009, Gibson, 2010).

1.11.3.6 Paperclip

The Paperclip method uses homology of “full clips” parts to build a genetic cassette. These full clips have homology to the different parts of the genetic cassette and serve as primers to amplify up the different parts (fig 17).

These parts can then be assembled alongside a plasmid backbone, using another PCR reaction to anneal the parts together and so amplify the full plasmid or they can be assembled by inserting them into competent *E. coli* cells and the native DNA repair mechanism will assemble the pieces due to their homology (see chapter 2).

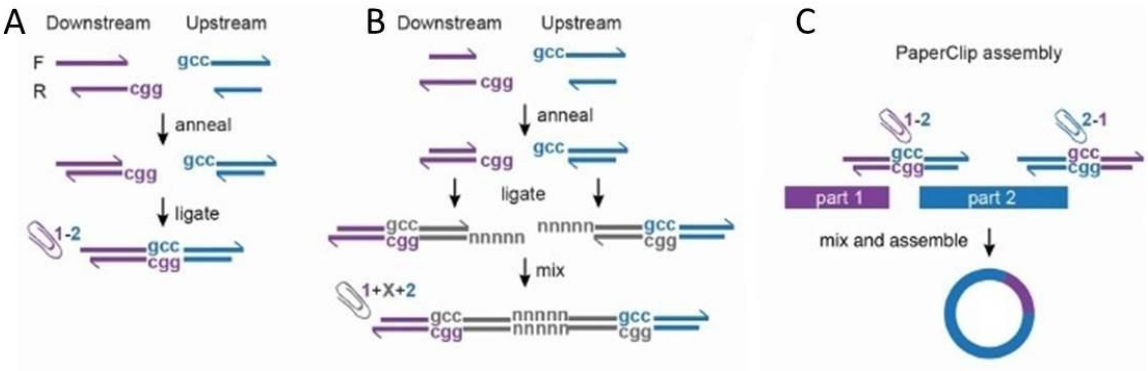


Figure 16 - Schematic of the Paperclip DNA assembly method. A) shows the half clip – full clip ligation assembly with GCC-CCG homology; B) follows on from A and shows how the homology of the full clips serves as primers to amplify up the different DNA parts; C) shows how the full clips are used to ligate the different parts of the cassette together. Adapted from (Trubitsyna et al., 2014).

This plasmid can then be miniprepped out of the recovered cells and either used to directly transform other competent cells or be digested with restriction enzymes to form a linear DNA cassette to use for homologous recombination (Trubitsyna et al., 2014, Storch et al., 2015). Ligase chain reaction is a similar method of DNA assembly (de Kok et al., 2014).

1.11.3.7 Splice and overlap extension

This method of DNA assembly is similar to that of the paperclip method, as it relies on homology, created by the primers, between the parts of the DNA cassette as they are amplified as opposed to producing them by using restriction enzymes, so there are no forbidden sites and the whole construction of the DNA cassette is achieved via two PCR cycles, where the homology between two parts anneal after the denaturation step of the PCR process (Horton, 1990, Wurch, 1998, Vallejo et al., 2008, Luo, 2013). However, the homology of the parts means that they can only annealed in a certain order, the 3' of one pieces has homology to the 5' of the sequential pieces and so on (see chapter 2).

1.12 Aim and Objectives

The project aims are to develop mixed microbial cultures to investigate ways of improving microbial degradation of cellulose degrading biomass and to use synthetic biology tools to engineer chassis organisms for use in the mixed microbial cultures to produce useful products.

1.12.1 Specific Objectives:

1. Determine which combinations of microbial partners show synergetic growth on cellulosic biomass in a minimal medium.

2. Develop an efficient system of physio-chemical pre-treatments to enable the most productive use of cell wall polymer derived monosaccharides.

3. Engineer the chassis organism *Bacillus subtilis* 168 to produce useful substance from cellulose degrading biomass, using modern synthetic biology tools.

2 Materials and Methods

2.1 Chemicals, strains and reagents

All chemicals and reagents were purchased from Sigma-Aldrich unless otherwise stated.

Strains of *Cytophaga hutchinsonii* ATCC 33406, *Cellulomonas fimi* ATCC 484 and *Escherichia coli* JM109 are from stocks maintained by the French lab, University of Edinburgh, *Saccharomyces cerevisiae* BY4741 from stocks maintained by the Cai lab, University of Edinburgh, *Bacillus subtilis* 168, *Bacillus subtilis* Eco4Red, *Bacillus subtilis* WB700 are from strains developed by Cobra Bio PLC and maintained by Dr D. Radford, *Bacillus natto* 21A1 is from Ajioka lab University of Cambridge and maintained by Dr D. Radford. The *Lactobacillus acidophilus* was isolated from Sainsbury's value natural yogurt by the author.

Table 1 - List of bacterial strains

Strain	Genotype	Reference/Description
Cytophaga hutchinsonii ATCC 33406		Strain ATCC 33406 Obtained from DSMZ, (strain No. 1761)
Cellulomonas fimi ATCC 484		NCIB 9469
Escherichia coli JM109	*endA1, glnV44, thi-1, relA1, gyrA96, recA1, mcrB$^+$, Δ(lac-proAB), e14-, [F' traD36 proAB$^+$ lacI^q lacZΔM15] hsdR17(rK$^-$ mK$^+$)*	Yanish-Perron et al., 1985 Obtained from Promega. (Catalog No. P9751)
Saccharomyces cerevisiae BY4741	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0*	Brachmann et al., 1998 Obtained from Cai lab, University of Edinburgh ATCC 4040005
Bacillus subtilis 168	*trpC2*	Kunst et al., 1997 Obtained from Cobra Bio PLC
Bacillus subtilis WB700	*nprE aprE epr bpr mpr::ble nprB::bsr Δ vpr*	Wu et al., 2002 Obtained from Cobra Bio PLC
Bacillus subtilis Eco4Red		Obtained from Cobra Bio PLC
Bacillus natto 21A1		Obtained from Ajioka lab University of Cambridge
Lactobacillus acidophilus		Isolated from Sainsbury's value natural yogurt

2.2 Pre-treatments

2.2.1 Selection of biomass

Due to the wide range of potential sources of biomass suitable for this study and with them the wide range of cell wall biochemistry, it was necessary to determine which cell wall polymers and sugars were present in which biomass.

2.2.1.1 Production of alcohol insoluble residue (AIR)

Miscanthus sinensis and *Triticum aestivum* straw were selected as the lignocellulosic biomass used in this study. *M. sinensis* was grown at outdoors at Edinburgh Botanical Gardens and the T*. aestivum* was grown on Askham Bryan College Estates in the Vale of York. They were dried after harvest and stored at room temperature until use.

The biomass was weighed out and homogenised in a food processor in 50% ethanol (v/v), and then washed in 50% ethanol (v/v) for 24h. The solid fractions were separated from the ethanol by filtration through "Miracloth" rayon-polyester fibre sheets (EMD Millipore PN: 475855, USA), as using filter paper may contaminate the samples with non-native cellulose. The tissue was then washed in 90% ethanol (1:10 w/v) at room temperature with agitation for seven days, with ethanol changes every 24h. After seven days, once there was no more change to the colour of the tissue and the ethanol remained clear and colourless, it was assumed that all the low molecular weight compounds had been removed and the residue consisted of purely crude cell wall material. This was then washed in acetone and allowed to air dry before being stored at room temperature until use.

2.2.1.2 TFA hydrolysis and thin layer chromatography

For each type of biomass 10 mg was weighed out and 1 ml of 2 M trifluoroacetic acid (TFA) was added in a screw top 1 ml Microcentrifuge tube. The biomass was then acid hydrolysed at 110^{O}C for 1 hr. Once cool, the tubes were spun down at 11000 g for 10 min to separate the residue (which should only contain crystalline cellulose and lignin) and the hydrolysate. The hydrolysate was decanted off and dried down to approx. 10 µl, with a consistency of syrup in a speed vac set at 38^{O}C overnight. This syrup was then redisolved in 100 µl of 0.5% chlorobutanol, a volatile antimicrobial solvent. Of this mix, which is a X10 concentrate of the original hydrolysate, 3 µl was loaded onto a silica thin layer chromatography plate (Sigma-Aldrich, USA; PN Z185310). Once all samples had been loaded and allowed to dry, the chromatogram was run in a solvent mix of Ethyl acetate: pyridine: propanol: acetic acid: water (10:2:2:1:1) for 3 h and allowed to air dry. Once dry the plate was

stained with thymol stain (10g thymol: 95 ml ethanol: 5 ml conc sulphuric acid) and allowed to dry. Once dry the chromatogram was developed at 105^{O}C for 10 min and immediately scanned.

2.2.2 Physio-chemical pre-treatments

To improve saccharification of cell wall polymers, the biomass was subjected to several different physio-chemical pre-treatments, shown in figure 1, to reduce cellulose crystallinity, increase surface area and to hydrolysis and/or digests the cell wall polymers.

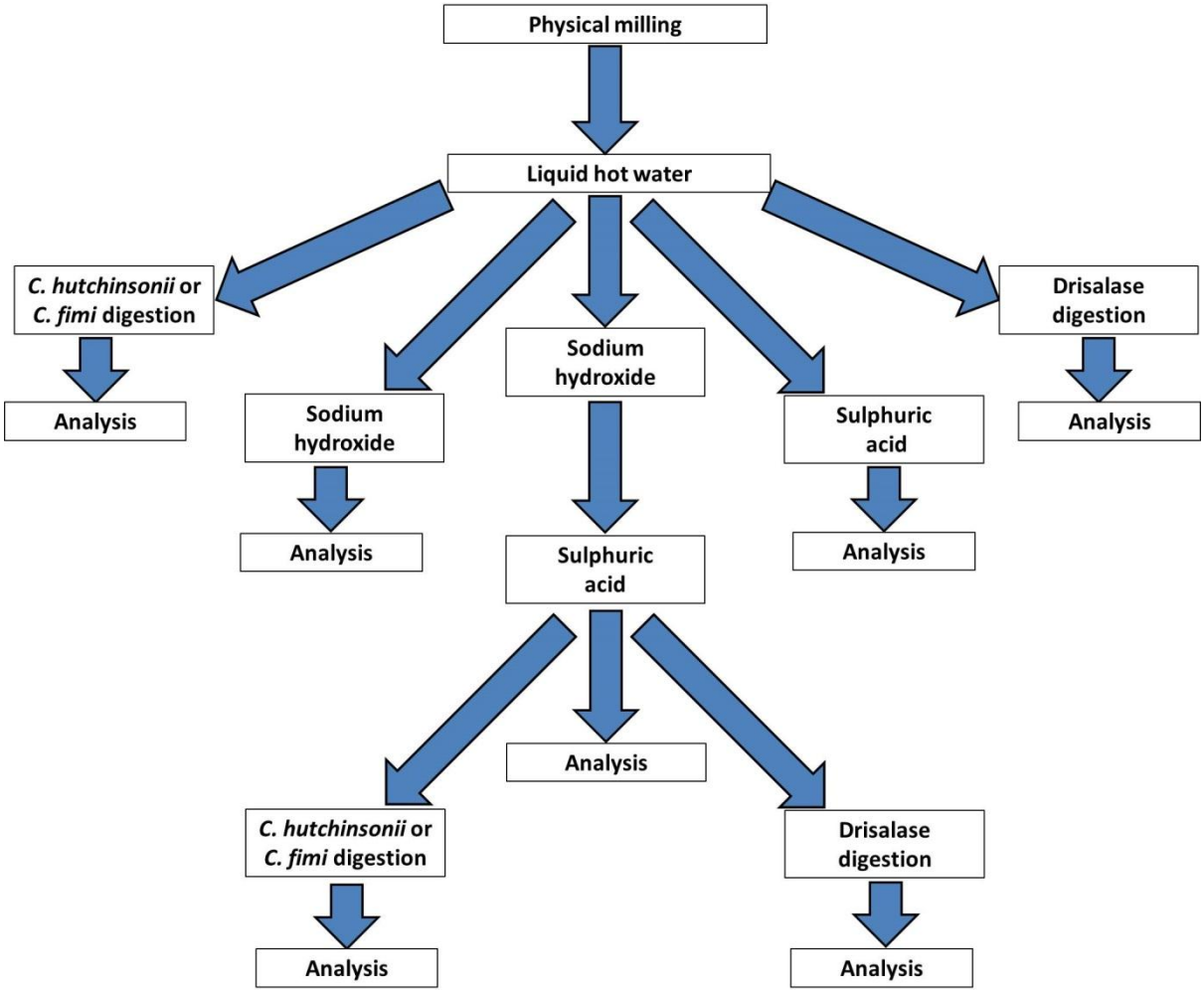


Figure 17 - Flow diagram of single and sequential physio-chemical pre-treatments of lignocellulosic biomass used in this study

2.2.2.1 Mechanical milling

To increase the surface area of the biomass, wheat straw was milled in a commercial food blender (Kenwood; PN FPP225) for approx. 10-20 minutes, until the particle size was less than 1 cm.

2.2.2.2 Liquid hot water (LHW)

Ten grams of the pre-milled wheat straw was added to 2 litres of dH_20 and then boiled for two hours over a Bunsen flame. This left approx. 100 ml of hydrolysate along with the biomass. These were separated by filtration and the biomass was dried ready for the chemical pre-treatments (section 2.2.2) and the hydrolysate sterilised by autoclaving for future assays (section 2.3.3)

2.2.2.3 Sodium hydroxide (NaOH)

Three grams of milled and LHW pre-treated wheat straw was weighed out and hydrolysed in 300 ml of 1 M NaOH for 2 hr at 50^{O}C. The biomass was separated from the hydrolysate by filtering through Miracloth. The solid fraction was washed in 2 l water for ten minutes and then resuspended in X1 PBS and neutralised with 1M sulphuric acid and then separated from the PBS by filtration through miracloth and air dried at room temperature overnight for use in further assays. The NaOH hydrolysate was also neutralised with 1M sulphuric acid and pooled with the neutralised PBS wash.

2.2.2.4 Dilute sulphuric acid (H_2SO_4)

Three grams of milled and LHW pre-treated wheat straw was weighed out and hydrolysed in 300 ml of 1 M H_2SO_4 for 2 hr at 150^{O}C. The biomass was separated from the hydrolysate by filtering through miracloth. The solid fraction was washed in 2 l water for ten minutes and then resuspended in X1 PBS and neutralised with 1M NaOH and then separated from the PBS by filtration through miracloth and dried for use in further assays. The H_2SO_4 hydrolysate was also neutralised with 1M NaOH and pooled with the neutralised PBS wash.

2.2.2.5 Driselase digestion

One gram of pre-treated wheat straw was added to 10 ml of P:A:W (pyridine: acetic acid: water) (1:1:98 v/v) containing 0.5% Driselase (Sigma-Aldrich, USA; PN D9515) and incubated for 3 days at 37^{o}C with rotary shaking. The digestion was stopped by adding 50 ml of 90% ethanol and heating to 80^{o}C for 10 minutes. The reaction was spun down, at *11000 g* for 10 min to separate the residue with the solid fraction fixed in 2.5% glutaraldehyde/PEM (v/v) and stored at room temperature for in situ antibody labelling. The digestate was dried in a speed vac overnight and re-suspended in 100 µl 0.5% chlorobutanol/water (v/v) for TLC analysis.

2.2.2.6 Bacterial digestion

500mg biomass was added to 50 ml DSM3+YE in 250 ml conical flasks and inoculated with 1 ml of either *C. fimi* or *C. hutchinsonii* liquid culture, grown in DMS3+YE at 30^{O}C, 100 rpm for 48 hours (OD < 1.000). This inoculated culture was then incubated for 10 days at 30^{o}C, 100 rpm. After 10 days, the culture was spun at *11000 g* for 10 min to separate the solid and liquid fractions as above. The solid fraction was fixed in 2.5% glutaraldehyde and stored for in situ antibody labelling. The liquid fraction was dried in a speed vac overnight and re-suspended in 100 µl 0.5% chlorobutanol for TLC analysis.

2.2.2.7 Sequential pre-treatments

Once the neutralised pre-treated biomass was dried it was sterilised by autoclaving for storage. Before being added to flasks containing appropriate growth media the biomass was milled to reduce the particle size and break up any clumps and then sterilised again by autoclaving (section 2.2.2). This biomass was then subjected to the same pre-treatments as before but in varying combinations and sequences i.e. NaOH + Driselase, H_2SO_4 + *C. fimi* etc. (see figure 18 above).

2.2.3 Fixing and resin infiltration of biomass

Plant material was fixed by immersion in 2.5% glutaraldehyde and washed twice for 10 min in PBS. It was then dehydrated in an ethanol series at 4^{o}C, in 10%, 20%, 30%, 50%, 70% then 90% ethanol then in 100% ethanol twice for 30 min each concentration. The material was resin infiltrated in 10% (v/v) LR White resin (a hard grade acrylic resin) in 100% ethanol, then in 20% (v/v), 30% (v/v), 50% (v/v), 70% (v/v), and 90% (v/v) for 1 h per step. The material was left in 100% resin overnight and then in a fresh amount of 100% resin for 8 h. The material was placed in gelatine capsules in 100% resin for five days at 37^{o}C allowing the resin to polymerise. The capsules of resin were sectioned to a thickness of 1-µm using a diamond knife on an ultra-microtome and fixed onto Vectabond-coated slides. These sections were either stained with Toluidine Blue O to stain cell or labelled with CWP monoclonal antibodies (section 2.2.4).

2.2.4 In situ monoclonal antibody labelling of cell wall polymers in acrylic resin sections

Each slide was incubated with 5% milk/PBS emulsion for 30 min. The slides were rinsed with PBS and then incubated for 90 min with primary antibodies raised to identify cell wall polymer epitopes (see figure 2). These antibodies were diluted 1:5 in 5% milk/PBS. The slides were washed three times in PBS for 5 min per wash. They were incubated in the dark, for 1 h with the secondary anti-rat FITC antibody, which was diluted to 1:100 concentration in 5% milk/PBS. The slides were washed three times, for five min in PBS. The slides were then stained with either Calcofluor white (if also labelled with monoclonal antibodies) at 0.25 mg/ml or Toluidine Blue O (0.1 % w/v) (if not). Both the

Calcofluor and Toluidine Blue were left for 5 min, and the slides were then washed three times in PBS for 5 min each wash and then rinsed with distilled water to remove any salt crystals. The slides were covered with a cover slip mounted with anti-fade glycerol/PBS 10:90 (v/v). The slides were stored in a cool dark place until viewed.

Once resin sections of plant material had been prepared as above, the slides were examined using an epifluorescence microscope (Elipse E600 NIKON) excited at 475 nm (FITC filter) and 600 nm (auto-fluorescence filter) respectively. Images were captured using a Leica DFC365 FX digital camera.

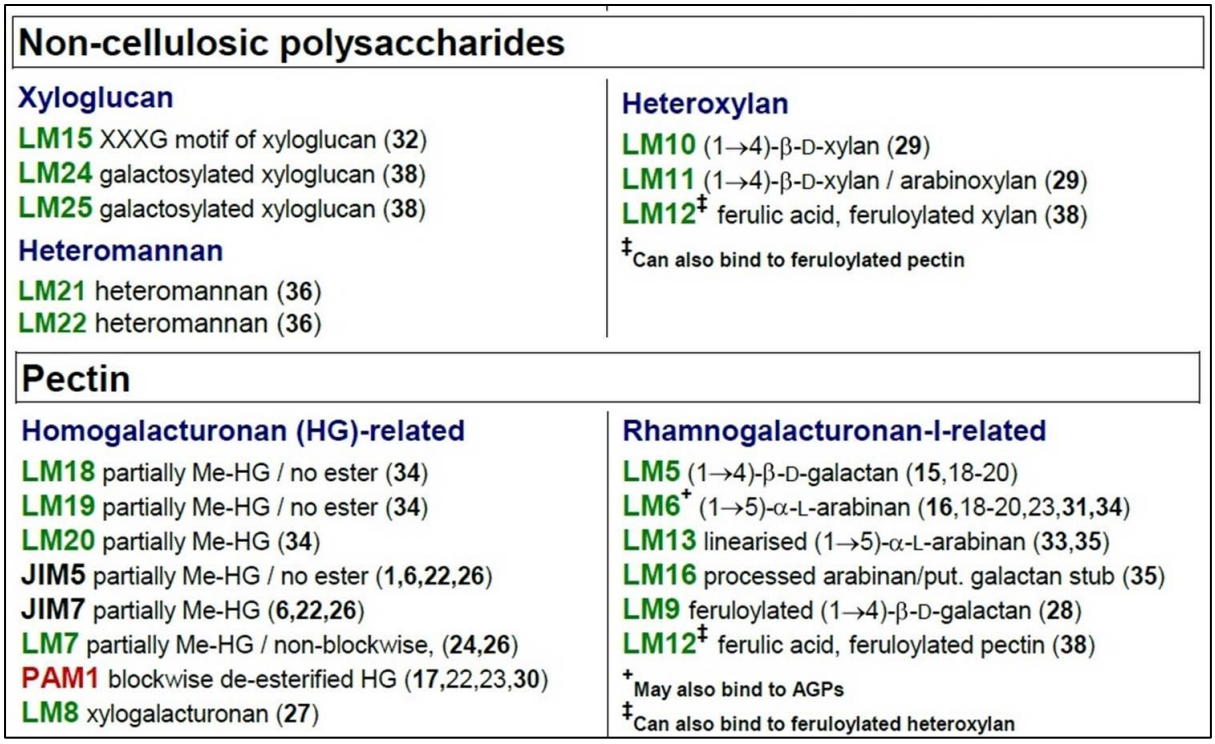


Figure 18 - List of cell wall polymer antibodies and their epitope of recognition raised in the Paul Knox lab, University of Leeds. Adapted from (Knox, 2016)

2.3 Co -culture and liquid growth experiments

The main medium used in these experiments was DSM3+YE (see table 1).

Table 2 – Growth media used. 1% agar was added to all media for plates except for Rogasa media. *see appendix. All media sterilised by autoclave apart from YNB which was filter sterilised. Vitamins added by sterile filtration after autoclaving † The volume was corrected to 1000 ml and heated until boiling with agitation until dissolved. The pH at this point is pH7. The mix was boiled for one minute, and 1.32 ml of acetic acid added, bringing the pH down to pH 5/5.5, and then mixed. The mixture was boiled for a further two minutes. then the mix was allowed to cool to 45°C before being decanted into sterile glassware and stored in the cold room until needed. ± Adjusted pH to 7.0 with 1M hydrochloric acid or sodium hydroxide. ≠ Filter sterilise. ± add after autoclaving

Media/Reagent	Components	Quantities
Dubos Salts Medium (DSM3) (DSMZ.de)	K_2HPO_4	8 g
	KH_2PO_4	2 g
	$MgSO_4$	5 g
	KCL	5 g
	$NaNO_3$	20 mg
	Distilled water	1000 ml
	(Yeast extract)	(400 mg)
PBS	KH_2PO_4	0.21 g
	NaCl	9 g
	Na_2HPO_4	0.726 g
	Distilled water	Up to 1000 ml
M9 Minimal Medium	4x M9 Salts consisting of: -	25 ml
	64g Na_2HPO_4-$7H_20$	Per 1000 ml
	15g KH_2PO_4	
	2.5g NaCl	10 µl
	5.0g NH_4Cl	200 µl
	1M $CaCl2^{\pm\neq}$	Up to 300ml
	1M $MgSO4^{\pm\neq}$	0.04%
	Distilled water	Either 0.5% monosaccharide or
	Yeast $extract^{\pm\neq}$	filter paper
	Carbon $source^{\pm\neq}$	
Luria Broth (LB)	Bacto Tryptone	10 g
	Oxoid Yeast Extract	5 g
	NaCl	10 g
	NaOH	0.2 g
	Distilled Water	1 L

Media/Reagent	Components	Quantities
Fortified yeast nitrogen base (x10 conc stock solution)	yeast nitrogen base	10 g
	Na_2HPO_4	0.15 M
	yeast extract	400 mg
	B-vitamin complex B-100*	100 mg
	multi vitamin complex*	100 mg
	selenium	260 µg
	Distilled water	1 L
MMM3 (0.1 M working solution)	NH_4Cl	5.34 g
	$NaSO_4$,	14.2 g
	MgCl	20.36 g
	CaCl,	14.7 g
	$CaHPO_4$	13.6 g
	H_3BO_3	1.48 g
	$MnSO_4$	4.46 g
	Na_2MoO_4	360 mg
	$CuSO_4$	2.49 g
	$CoCl_2$	280 mg
	$ZnSO_4$	28.78 g
	KI	298 mg
	$FeCl_3$	16.22g
	$CaCl_2$	12 g
	B-vitamin complex B-100	1g
	Distilled water	Up to 1000 ml
DSM3a	K_2HPO_4	800 mg
	H_3BO_3	1.48 g
	$MnSO_4$	4.46 g
	$Na2MoO_4$	360 mg
	$CuSO_4$	2.49 g
	$CoCl_2$	280 mg
	$ZnSO_4$	2.878 g
	KI	298 mg
	$FeCl_3$	16.22 g
	$CaCl_2$	12 g
	Distilled water	Up to 1000 ml

Media/Reagent	Components	Quantities
DSM3b	K2HPO4 NH4SO4 H3BO3 MnSO4 Na2MoO4 CuSO4 CoCl2 ZnSO4 KI FeCl3 CaCl2 Distilled water	800 mg 500 mg 1.48 g 4.46 g 360 mg 2.49 g 280 mg 2.878 g 298 mg 16.22 g 12 g Up to 1000 ml
DSM3c	K_2HPO_4 NH_4Cl H_3BO_3 $MnSO_4$ Na_2MoO_4 $CuSO_4$ $CoCl_2$ $ZnSO_4$ KI $FeCl_3$ $CaCl_2$ Distilled water	800 mg 500 mg 1.48 g 4.46 g 360 mg 2.49 g 280 mg 2.878 g 298 mg 16.22 g 12 g Up to 1000 ml
Wickerhams minimal media (Wickerham 1964)	KH_2PO_4 K_2HPO_4 $MgSO_4$ NaCl $CaCl_2$ glucose NH_4SO_4 H_3BO_3 $CuSO_4$ KI $FeCl_3$ $ZnSO_4$ B-vitamin complex B-100* Distilled water	875 mg 125 mg 500 mg 100 mg 100 mg 10 g 100mg 10 mg 10 mg 100 mg 50 mg 70 mg 1 g Up to 1000 ml

Media/Reagent	Components	Quantities
Neranderts minimal media (x10 stock solution) (Narendranath, 2001)	$(NH_4)_2SO_4$	49.9 g
	K_2HPO_4	1.49 g
	KH_2PO_4	9.28 g
	$MgSO_4$	5 g
	NaCl	1.2 g
	H_3BO_3	1.48 g
	$MnSO_4$	4.46 g
	Na_2MoO_4	360 mg
	$CuSO_4$	2.49
	$CoCl_2$	280 mg
	$ZnSO_4$	28.78 g
	KI	298 mg
	$FeCl_3$	16.22 g
	$CaCl_2$	12 g
	B-vitamin complex B-100*	20 mg
	Distilled water	90 ml
Rogasa media† (see appendix)	$C_2H_3NaO_2$	15 g
	Tryptone	10 g
	Dextrose	10 g
	KH_2PO	6 g
	yeast extract	5 g
	Sucrose	5 g
	Arabinose	5 g
	$Na_3C_6H_5O_7$ (sodium citrate)	2 g
	polysorbate 80	1 ml
	$MgSO_4$	570 µg
	$MnSO_4$	120 µg
	$FeSO_4$	30 µg
	bacteriological agar	15 g
	Distilled water	1000 ml

Media/Reagent	Components	Quantities
Minimal Bacillus salt solution (X5 conc)	$NH_4{}_2SO_4$	2 g
	K_2HPO_4	14.8 g
	KH_2PO_4	5.4 g
	$Na_3C_6H_5O_7$ (sodium citrate)	1.9 g
	$MgSO_4$	0.2 g
	Distilled water±	Up to 200 ml
Minimal *Bacillus* media (To be prepared fresh each time) ≠ (Bron 1997)	Minimal salt solution (above)	10 ml
	Glucose (50% (w/v)	0.5 ml
	Casamino acids (2% (w/v)	0.5 ml
	Tryptophan (10 mg/ml)	0.1 ml
	$C_6H_8FeNO_7$ (Ammonium ferric citrate) (2.2 mg/ml)	0.05 ml
	Distilled water	Up to 50 ml
Starvation *Bacillus* media ≠ (Bron 1997)	Minimal salt solution (above)	10 ml
	Glucose (50% (w/v))	0.5 ml
	Distilled water	Up to 50 ml
Super Optimal broth with Catabolite repression (SOC) (Bron 1997)	Tryptone	2%
	yeast extract	0.5%
	NaCl	10 mM
	KCl	2.5 mM
	$MgCl_2$	10 mM
	$MgSO_4$	10 mM
	Glucose	20 mM
Yeast extract peptone dextrose media (YPD)	Yeast extract	10g
	Peptone	10g
	Dextrose	20g
	Distilled water	Up to 1000 ml
Nutrient Broth	Peptone	5 g
	Beef extract	3 g
	Distilled water	Up to 1000 ml
	Adjust pH to 7 with NaOH or HCL	

Table 3 - Antibiotic resistance cassettes

Antibiotic and amount	Plasmid used or cloned from	Organism
Kanamycin	pSB1K3	*B. subtilis* 168 ΔcspD::ldh+^{R}cat
Chloramphenicol	pSB1C3	*B. subtilis* 168 $^{\Delta}$cspD::pdc:adhB^{+R}kan

2.3.1 Co-culture in liquid media with filter paper as the carbon source

100 ml of DSM3+YE (or other appropriate media, see above) was measured out into six 250 ml conical flasks, four of which contained 15 1cm^2 (approx. 500 mg) squares of cellulose filter paper (Ford 428), of the remaining two, one contained 500 mg glucose (positive control) and the final flask only contained DSM3 (negative control). To four of these flasks, 1 ml of overnight *B. subtilis* or *S. cerevisiae* in LB / YPD respectively (OD_{600} >1.000) was added. To one of these flasks 1 cm^2 of filter papers colonised by *C. hutchinsonii* (as described in Liu, 2012) or 1 ml of *C. fimi* culture, grown in DMS3+YE as above (OD>1.000) was added (mixed culture flask) and was also added to the one flask containing only paper. The six-flask containing only paper was not inoculated, to serve as background for the filter paper degradation. The final set up was:

DSM3 + YE + filter paper only
DSM3 + YE + filter paper + *C. hutchinsonii* or *C. fimi*
DSM3 + YE + filter paper + *B. subtilis or S. cerevisiae*
DSM3 + YE + filter paper + *C. hutchinsonii* or *C. fimi* + *B. subtilis* or *S. cerevisiae*
DSM3 + YE + glucose + *B. subtilis* or *S. cerevisiae*
DSM3 + YE + *B. subtilis* or *S. cerevisiae* only

The flasks were incubated at 30^{O}C at 100 RPM for 10 days. Samples were analysed every 24 hours.

2.3.2 Co-culture in liquid media with pre-treated wheat straw as the carbon source

100 ml of DSM3+YE or other appropriate media, see above) was measured out into six 250 ml conical flasks, four of which contained approx. 500 mg of pre-treated wheat straw, of the remaining two, one contained 500 mg glucose (positive control) and the final flask only contained DSM3+YE (negative control). To four of these flasks, 1 ml of overnight *B. subtilis* in LB (OD_{600} >1.000) or 1 ml S*. cerevisiae* grown in YPD over night was added. To one of these flasks 1 cm^2 of filter papers colonised by *C. hutchinsonii* (as described in Liu, 2012) or 1 ml of *C. fimi* culture, grown in DMS3+YE as above (OD>1.000) was added (mixed culture flask) and *C. hutchinsonii* / *C. fimi* was also added to one flask containing only wheat straw. The sixth flask contained only pre-treated straw and was not inoculated; this was so it could serve as background for the OD changes caused by the straw degradation. The final set up was:

DSM3 + YE + pre-treated wheat straw only
DSM3 + YE + wheat straw + *C. hutchinsonii* or *C. fimi*
DSM3 + YE + wheat straw + *B. subtilis* or *S. cerevisiae*
DSM3 + YE + wheat straw + *C. hutchinsonii* or *C. fimi* + *B. subtilis* or *S. cerevisiae*
DSM3 + YE + glucose + *B. subtilis* or *S. cerevisiae*
DSM3 + YE + *B. subtilis* or *S. cerevisiae* only

The flasks were incubated at 30^{O}C at 100 RPM for 10 days. Samples were analysed every 24 hours.

2.3.3 Hydrolysate growth assays

The liquid fraction from the wheat pre-treatment steps (see section …) were neutralised to pH 7, pooled and sterilised in an autoclave. Before sterilisation 1 ml of the hydrolysate was removed for TLC analysis (see section …). 100 ml of this hydrolysate was inoculated with 1ml of overnight *B. subtilis* / *E. coli* culture in LB (OD_{600} >1.000) or with 1ml of overnight *S. cerevisiae* in YPDA (OD_{600} >1.000). Measurements for optical density and plate counts were taken every 24 hours.

2.3.4 Optical density measurements (OD)

Every 24 hours for ten days (including a 0 hour measurements) 1 ml of liquid culture was pipetted aseptically into a spectrometer curvet and t optical density of the sample was measured at OD_{600}, with a water blank.

2.3.5 Serial dilution for colony forming units (CFU)

From the aliquot used to measure OD 100 µl of liquid culture was removed and used in serial dilution for plate counts of CFU as a measure of viable bacterial cells. 100 µl was added to 900 µl of

sterile dH_20, ranging from 10^{-3} dilution at 0h and up to 10^{-7} by day ten. At the final dilution 100 µl was pipetted and spread onto LB agar plates and incubated at 37^OC overnight. The number of colonies was then counted and multiplied by the next dilution up x 10^, i.e. if the dilution plated out was 10^6, then the number of colonies was multiplied by 10^7 to give the number of CFU per ml.

2.3.6 Total protein assay (Bradford assay)

From the aliquot used to measure OD 100 µl of liquid culture was removed added to 900 µl of Coomassie reagent (Thermo Scientific Inc., PN. 23236) in a spectrometer curvet. These curvets we then incubated at 70^OC for 1 hour to lyse the cells. The curvets were then allowed to cool for at least 30 minutes and then the OD was measured at A_{595}, with a Coomassie only blank. The absorbance was then compared to a standard curve of known protein content prepared in the same way and the total protein per ml of culture calculated.

2.3.7 Solvent tolerance assay

Flasks containing 100 ml of a DMS3/ethanol/butanol/ D/L lactic acid mix (between 0% solvent – 100% solvent, rising in 1% increments) and also containing 500 mg of glucose or filter paper were prepared under aseptic conditions and inoculated with 1ml of overnight *B. subtilis* in LB (OD_{600} >1.000) or a 1 cm^2 piece of filter paper hosting *C. hutchinsonii* cells or 1 ml of *C. fimi* culture was used to measure optical density of the sample and at 10 days 100 µl was used to assess whether there were still viable cells in the media through plate counts for *B. subtilis* or used to inoculate 1 cm^2 filter paper for *C. hutchinsonii*.

2.3.8 Monosaccharide utilisation assay

2.3.8.1 Liquid culture

100 ml of DSM3+YE was measured out into nine 250 ml flasks and 500 mg of individual monosaccharides that make up the main seven plant cell wall polymers added. To the eighth flask approx. 72 mg of each monosaccharide was added (approx. 500 mg in total) and none added to the ninth flask (negative control). To each flask 1 ml of overnight *B. subtilis* / *S. cerevisiae* / *E. coli* (OD 1.000) was added. The final set up was:

DSM3 + YE + microorganism + galacturonic acid (neutralised to pH 7 with 20 µl 1M sodium hydroxide)
DSM3 + YE + microorganism + galactose
DSM3 + YE + microorganism + glucose
DSM3 + YE + microorganism + mannose
DSM3 + YE + microorganism + arabinose

DSM3 + YE + microorganism + xylose
DSM3 + YE + microorganism + rhamnose
DSM3 + YE + microorganism + all monosaccharides
DSM3 + YE + microorganism only

Flasks were incubated at 30^{O}C at 100 RPM for 10 days. Samples were analysed every 24 hours for OD, CFU, total protein and a further 1 ml taken for thin layer chromatography.

2.3.8.2 Plate cultures

Agar plates of an appropriate medium (see above) for each microorganism had one of the above monosaccharides added, with one plate containing all the monosaccharides and one containing none, as above. These plates were then inoculated with 100 µl of overnight microbial culture (OD >1.000). These plates were incubated at 37^{O}C overnight and assayed visually to determine whether that specific micro-organism was able to utilise the monosaccharide present as a carbon source for growth.

2.3.9 Thin layer chromatography (TLC)

One ml of liquid culture was taken and spun down to separate out any bacterial cells. The resultant supernatant was then dried in a speed vac until almost dry, until it had the consistence of thick syrup. This was then rehydrated in 100 µl of 0.5% chlorobutanol. Once the syrup was completely re-dissolved and of uniform consistence 3 µl was pipetted onto a 20 x 20 cm^2 silica thin layer chromatography plate, dispensed as 5 drops. Once all samples and a monosaccharide marker mix had been loaded and dried, the plate was run in the solvent ethyl acetate: pyridine: propanol: acetic acid: water (4:2:2:1:1) and run in a vertical tank for three hours. The plate was then dried in a fume hood over night until completely dry and all traces of the solvent removed. The plate was then stained in thymol, allowed to air dry before being developed at 105^{O}C for two minutes. It was immediately photographed.

2.4 Molecular and biochemical techniques and transformations

2.4.1 Chemicals and Reagents

All PCR was performed using Phusion polymerase or Phusion master mix. All primers were purchased from Sigma. Growth medium was Luria Broth or Luria agar. For transforment selection Luria agar plates were supplemented with either 50 µg/ml kanamycin or 35 µg/ml chloramphenicol for *E. coli* or 5 µg/ml kanamycin or 5 µg/ml chloramphenicol for *B. subtilis*. PCR solution purification, Gel-extraction and mini-prep kits were purchased from Qiagen. Restriction enzymes were purchased

from New England Biolabs (NEB). T4 DNA ligase was purchased from Promega. A complete list of primers and their sequences can be found in the appendix.

2.4.2 Inducing chemical competence of E. coli *JM109 based on (Chung, 1989)*

Insertion of the genetic cassette using the *Paperclip* method requires the transformation of *E. coli* cells, based on Chung *et al.,* 1989.

Transformation and Storage Solution (TSS); was prepared by adding 5 ml of sterile 40% (w/v) Polyethylene Glycol (PEG) 3350, 1 ml of sterile 1 M MgCl2 and 1 ml (filter) sterile 90% (v/v) DSMO to 17 ml of sterile LB broth, under aseptic conditions. This was mixed and stored at 4^{o}C.

From an overnight plate of *E. coli* on LB agar, 50 ml LB in a 250-ml conical flask was inoculated and incubated at 37^{o}C, with shaking for 3-4 hours, until an OD of 0.200/0.500 was reached. The flask was chilled in ice/water slurry to rapidly cool it along with a microcentrifuge tube. One ml of the cooled culture was pipetted into pre-chilled microcentrifuge tube and spun down, in a chilled centrifuge, at 5500 *g* for 5 mins. The supernatant was decanted off and the pellet was re-suspended in 100 µl of TSS and placed on ice for 30 mins, then snap frozen and stored at -80^{o}C.

2.4.3 Transformation of chemically competent E. coli *JM109 cells based on (Chung, 1989)*

An appropriate number of frozen competent cells was removed from storage (section 2.4.2) and thawed in an ice/water slurry then transferred to ice. Cells needed to be kept cold from this point on. To each aliquot 1 µl of plasmid DNA (conc 73.19 pMol) or 5µl ligation / PCR product (conc 25.25 pMol) was added and mixed by inversion then incubated on ice for 40 mins. After 40 mins, the cells were heat shocked at 42^{o}C for 90 seconds and then placed back on ice for 90 seconds. To this 900 µl of SOC was added and mixed by inversion. This transformation mix was incubated at 37^{o}C for 2 hours on a shaker at 200 rpm to ensure proper aeration. 100 µl of recovered cells were plated out onto LB plates with the appropriate antibiotic selector for transformation efficiency calculations. The remaining 900 µl of the transformation mix was spun down at 5500 *g* for one minute and re suspend in 100 µl LB, then plated out to increase the chances of successful propagation of transformed cells.

2.4.4 Inducing natural competence in B. subtilis 168

B. subtilis 168 becomes naturally competent under stress starvation conditions.

One colony from stock plates was sub cultured onto a fresh LB agar plate and grown overnight at 37^{o}C. One colony was then taken (stab culture method) from this fresh plate and used to inoculate 10 ml of *Bacillus* minimal medium (see table one, above) in 250 ml conical flask and was incubated at

37°C 200 rpm for 18 hours. 1.4 ml of this culture was used to inoculate 10 ml, pre-warmed, minimal medium in a 250-ml conical flask and was then incubated for a further 3 hours, as above. After three hours 11 ml of *Bacillus* starvation media (table one, above) was added and the culture incubated for a further 2.5 hours. The cells were then competent and were transformed as in (section 2.4.5). N.B. For larger batches, glycerol stocks of competent cells can be made by adding 250 µl of competent cells to 1.70 ml of sterile 50% glycerol and snap freezing in either dry ice or liquid nitrogen. These aliquots can be stored at -80°C indefinitely.

2.4.5 Transformation of B. subtilis by homologous recombination

An appropriate number of frozen competent cell aliquots (section 2.4.2) were thawed in an ice/water slurry (approx. 10 minutes), and immediately placed tubes on ice, cells must be kept cold from here on out.

To each 2-ml aliquot 60 µl of linear PCR product or linear Pet plasmid was added and the whole volume was decanted into a 15-ml falcon tube.

The falcon tube was placed on a shaker at a 45° angle, for maximum aeration, and incubated at 37°C, 200 rpm for 1 hour. After 1 hour, 2 ml SOC was added and then incubated for a further 2 hours at 37°C (to ensure the resistance protein are being expressed). 100 µl was plated out onto an LB medium with 5 µg/ml chloramphenicol or kanamycin. This plate was used to calculate the transformation efficiency. To increase the chances of getting a successfully transformed colony, the remainder of the culture was spun down at 5500 *g* for 10 minutes, the supernatant decanted off, and the pellet resuspended in 100 µl LB and then the total volume plated onto a LB plate with appropriate antibiotic. Plates were incubated overnight at 37°C. Any colonies were then sub cultured onto individual LB plates with appropriate antibiotic as above to ensure the transformation was successful and not a false positive.

The successfully transformed *B. subtilis* 168 should be described as *B. subtilis* ΔcspD::ldh+^{R}cat (ldh) and *B. subtilis* ΔcspD::pdc:adhB^{R}kan (pdcadh).

2.4.6 Cloning of B. subtilis genes, iGem constructs and construction of cspD *locus inserts*

This section describes the construction (table 3) and insertion of cloned *ldh* and *pdcadh* at the *cspD* locus under the native promoter, by homologous recombination, to produce the economically interesting products; lactic acid and ethanol. The template for *ldh* was native *ldh* and was amplified out of *B. subtilis* 168 genomic DNA, the fused pdcadh template was amplified out of plasmid BBa_K173003, see (Lewicka, 2014).

Table 4 - Primer sequences used in the amplification and construction of ldh and pdcadh producing cassette. Full cassette sequence in appendix

Primer / Part	Sequence
cspD upstream forward primer	GATCGGCACTCATCCAAGCAATGTAAAAATCACAGGAATTCCAATCAGG
cspD upstream reverse primer	GATTAAAGCTACTTTATTTACATGTTTGTTCATCATATTGCTTAATTCCTCCTAGTA
cspD downstream forward primer	TTTTCTAATGTCACTAACCTGCCCCGTTAGTTGAAGATTTCAATCGCCAAAATGTTT
cspD downstream reverse primer	AAATTAGACCGTAACGGAGATGACGATATTGGATAAGAAAATGATCTT
lactate dehydrogenase forward primer	TACTAGGAGGAATTAAGCAATATGATGAACAAACATGTAAATAAAGTTAGCTTTAAT
lactate dehydrogenase reverse primer	GTTTGTTGAACTAATGGGTGCTTTAGTTGAAGAATAGTTGACTTTTTGTTCTGCAAAATGAGGTTTTAAAAT
chloramphenicol resistance forward primer	ATTTTAAAACCTCATTTTGCAGAACAAAAAGTCAACTATTCTTCAACTAAAGCACCCATTAGTTCAACAAAC
chloramphenicol resistance reverse primer	AAACATTTTGGCGATTGAAATCTTCAACTAACGGGGCAGGTTAGTGACATTAGAAAA

fused pyruvate decarboxylase-alcohol dehydrogenase forward primer	CATACCAACACTTCAACGCACCGGCTCTAGTATTATCAAAAGGCACTC
fused pyruvate decarboxylase-alcohol dehydrogenase reverse primer	GAGTGCCTTTTGATAATACTAGAGCCGGTGCGTTGAAGTGTTGGTATG
neomycin (kanamycin) resistance forward primer	CTCATCATCATGTATTGAGTTTAGCGCAGATCAATTCTGACAGCCATG
neomycin (kanamycin) resistance reverse primer	CATGGCTGTCAGAATTGATCTGCGCTAAACTCAATACATGATGATGAG

2.4.6.1 Parts amplification by polymerase chain reaction

A PCR reaction mix for each part was assembled as follows (table 4);

Table 5 - PCR reaction mix recipe

Component	50 µl Reaction	Final Concentration
5X Phusion Reaction Buffer	1 µl	1×
10 mM dNTP mixture	2.5 µl	200 µM
10 µM Forward Primer	2.5 µl	0.5 µM
10 µM Reverse Primer	2.5 µl	0.5 µM
Template DNA	1 µl	
Phusion DNA Polymerase	0.5 µl	0.02 U/µl
Nuclease-Free Water	to 50 µl	

Standard PCR reactions settings were used (table 5);

Table 6 - PCR reaction settings used

Step	Temperature	Time
Initial Denaturation	98^{O}C	10 minutes
35 cycles	98^{O}C	10 sec
	55^{O}C-68^{O}C	30 sec
	72^{O}C	30 sec per kb DNA
Final Extension	72^{O}C	10 minutes
Hold	10°C	∞

Once these parts A; upstream homology, B; ldh or pdcadh, C; kanamycin or chloramphenicol resistance gene and D; downstream homology were cloned and amplified (tables three and four), they were used as template DNA for construction of inserts using either splicing overlap extension PCR (Wurch, 1998) or the paperclip method (Trubitsyna et al., 2014).

2.4.6.2 Splicing overlap extension

The sequences for all parts were entered "A plasmid Editor v2.0.47" (ApE) and primers (other than the extreme 5' and 3' primers) were designed with 25 bp homology to the previous part. The extreme 5' primer was designed to anneal to the first 25 bp and 3' primer designed as a reverse complement to the last 25 bp of the final construct. Parts were amplified in a standard PCR reaction with these primers so each amplified part had 25 bp homology to the previous and consecutive parts. These parts were either gel or column purified (see section 2.8.4, below) and then equimolar amounts of all four parts (four-way fusion) or parts A-B, C-D and then AB-CD (2x2 way fusions) were used in a primer less PCR reaction (as above), with the annealing temperature set at one degree lower than the melting temperature of the homologous regions. This enabled these complementary regions to anneal to each other over 35 cycles. Once these cycles were over the outermost primers were added and a standard PCR reaction was run to amplify the fused parts, with the extension time adjusted depending on the size of the resultant fused constructs. This reaction mix was visualised on a 0.8% agarose gel, run at 80 V for 50 minutes and stained with GelGreen ^{TM} Nucleic Acid Gel Stain (BIOTIUM Inc.) per the manufacturer's instructions. The DNA bands were visualized on a Bio-Rad Gel Doc system and then purified by either gel or column purification and frozen for later use for the transformation of *B. subtilis* 168 competent cells (section 2.4.5)

2.4.6.3 Paperclip DNA Assembly

The sequences for all parts were entered "A plasmid Editor v2.0.47" (ApE) and primers were designed based on (Trubitsyna et al., 2014).

For each part of the DNA cassette, four oligosaccharides (primers) are needed, upstream forward (UF), upstream reverse (UR), downstream forward (DF) and downstream reverse (DR).

To create the upstream forward clip/primer the first 40 bases of the sequence were selected and GCC added to the 5' end. For UR the first 37 bases were selected and using the option in the software a reverse complementary sequence created, this sequence anneals to UF with a GCC overhang at 5' and a three base over hang at 3'.

To create DF clip/primer, the last 40 bases were selected and provided that the first three bases were not GCC or GGC. If the first three bases were GGC or GCC then the first base was removed to prevent incorrect ligation at later stages. For DR, the last 37 bases were selected and a reverse complementary sequence created that annealed to DF and GGC added to the 5' end.

These single stranded oligonucleotides were dissolved in distilled, sterile dH_20 to a stock concentration of 100 µM. 20 µl (equivalent of 50 µM final concentration) of the forward and reverse oligonucleotides (UF + UR, DF + DR) were mixed and the reaction slowly cooled from 95^{O}C to 4^{O}C (≈ 0.1^{o}C/s) to allow the half clips to anneal.

Full clips, to link parts in a specific order, were prepared by phosphorylating and ligating half clips in a certain order. This reaction was carried out in 20 µl final volume; containing 7 µl of both half clips (final concentration 14 µM) along with 2 µl T4 DNA ligase buffer, 2 µl of PNK buffer 1 µl of T4 polynucleotide kinase and 1 µl of T4 DNA ligase (Thermo Scientific). Phosphorylation and ligation were carried out at 16^{o}C overnight and the ligation stopped by heat inactivation at 65^{o}C for 20 minutes.

To check if the ligation was successful 0.5 µl of the reaction mix, along with 4.5 µl dH_20 and 1 µl x6 loading dye (NEB), was run on a precast Bio-Rad 12% PAGE gradient gel, alongside a 50 bp standard ladder (NEB), in 0.5% TBE (stock X5 concentration) at 50 V for 10 minutes and then 100 V for 1 h at room temperature. The gel was stained with GelGreen^{TM} Nucleic Acid Gel Stain (BIOTIUM Inc.) 66 µl in 100 ml dH_20. The DNA bands were visualized on a Bio-Rad Gel Doc system.

Once the formation of full clips was ascertained they were frozen for further use.

To generate parts with homology to the clips, the UF and DR oligonucleotides for each part and the plasmid backbone were used in place of traditional primers and used in a two-step PCR reaction

using Phusion master mix for 35 cycles as described in table 4. The parts were then purified using Qiagen PCR purification kit (QIAGEN GmbH, Germany) and eluted in 50 µl of EB buffer. This was then concentrated by evaporation to a concentration of 100 ng / µl. One microliter of each part was added along with 0.21 µl of appropriate clips (final concentration 60nM) and run in a PCR reaction as above but for 20 cycles. Once the construct had been assembled, 5 µl of the reaction mix was used to serve as template DNA for a standard PCR reaction using KOD Hotstart (2.4.6.1) with appropriate primers for 35 cycles. After this PCR reaction, the reaction mix (50 µl) was added to 100 µl of competent JM109 cells (see section 2.4.2) and recovered with SOC by incubating for 2 hours at 37^{o}C and plated out of agar plates containing 30 µg of appropriate antibiotic and 40 µl of 0.1M IPTG and grow at 37^{o}C overnight. Once colonies had developed, red colonies were disregarded while white colonies (containing the plasmid with the Pet cassette in the place of the RFP) were grow up overnight in LB with appropriate antibiotic and 40 µl 0.1M IPTG and then extracted using a Qiagen Miniprep kit (QIAGEN GmbH, Germany).

Once the PET plasmid had been isolated it was digested with Spe1 and Antarctic phosphatase enzyme to linearize it and prevent self-ligation, 10 µl of this was analysed by agarose gel electrophoresis and gave a 8 kb band. The rest was used to transform competent *B. subtilis* 168 cells (section 2.4.5) The plasmid overhang (approx. 2kb) should have been digested by the native exonuclease in the *Bacillus* cell.

2.4.7 Transformation efficiency

To calculate the efficiency of the transformation; the number of colonies was divided by the concentration of DNA added to competent cells divided by the dilution spread on the plate (NEB C3019)

TE = No colonies / µg DNA / dilution x 10^

2.4.8 Extraction of chromosomal DNA and PCR to determine locus of insertion

To show that the construct had integrated in the correct position in the *B. subtilis* 168 genome, the *cspD* locus was amplified by PCR for the two transformed *B. subtilis* cultures and in WT *B. subtilis* 168. This was achieved using a modified protocol for a Qiagen DNeasy Blood and Tissue kit.

From overnight plates of LB, containing the appropriate antibiotic, an overnight culture of wild type *B. subtilis* 168 (WT) and the transformed *B. subtilis* 168 $^{\Delta}$cspD::ldh^{+R}cat (ldh) and *B. subtilis* 168 $^{\Delta}$cspD::pdc:adhB^{R}kan (pdcadh) were prepared in 50 ml LB in 250 ml conical flasks, for maximum aeration at 37^{o}C, 200 RPM overnight. One ml of culture was spun down for 10 minutes at 6000 *g* and

the supernatant discarded. The pellet was resuspended in 180 µl of enzymatic lysis solution and was incubated at 37°C for 30 minutes. To the resuspension 200 µl buffer AL containing 25 µl proteinase K was added and mixed by vortexing then incubated at 57°C for 30 minutes. After 30 minutes 200 µl 95% ethanol was added then mixed by vortexing. The mixture was pipetted into a Qiagen miniprep column (QIAGEN GmbH, Germany) placed in a 2 ml microcentrifuge tube and spun at 6000 *g* for one minute and the flow through discarded. The miniprep column was placed in a fresh 2 ml Microcentrifuge tube, and 500 µl of buffer AW1 added then spun at 6000 *g* for one minute and the flow through and microcentrifuge tube both discarded. The miniprep column was placed in a fresh 2 ml microcentrifuge tube, and 500 µl of buffer AW2 added then spun at 6000 *g* for one minute and the flow through and microcentrifuge tube discarded again. The miniprep column was then placed in a clean and autoclaved 1.5 ml Microcentrifuge tube and 200 µl of buffer AE added directly to the membrane. This was incubated at room temperature for five minutes, then spun for one minute at 6000 *g*. This step was then repeated. The extract was stored at -20°C or used as the template for PCR to amplify out the inserted DNA cassette, this PCR product was then run on 0.8% agarose gel, at 80 V for 50 minutes and stained with GelGreen™ Nucleic Acid Gel Stain (BIOTIUM Inc.) (66 µl in 100 ml dH_20). The gel was visualised the on a Bio-Rad Gel Doc system.

2.4.9 Preparation of crude extracts and total protein assay

To determine whether the genes for *ldh* and/or *pdcadh* had been transcribed to produce those enzymes, it is necessary to prepare and examine crude proteins from the cells extracts.

An overnight culture of wild type *B. subtilis* 168 (WT) and the transformed *B. subtilis* 168 $^{\Delta}$cspD::ldh^{+R}cat (ldh) and *B. subtilis* 168 $^{\Delta}$cspD::pdc:adhB^{R}kan (pdcadh) were grown in 50 ml LB in 250 ml conical flasks, for maximum aeration at 37°C, 200 RPM overnight. These cultures were then spun down at 7500 *g* for 20 minutes, the supernatant decanted off and the pellet resuspended in 5 ml of 15% glycerol in x1 PBS and then frozen overnight. Once thawed this was sonicated for three minutes, on ice, in one minute bursts at 10 µm amplitude. It was then spun down at 7500 *g* again and the supernatant decanted off. This supernatant (the crude extract) was then concentrated using Millipore protein concentrators (MWCO 10kD) down to a final volume of 500 µl of crude extract (final concentration x100). This was then used for total protein, SDS PAGE and native PAGE assays and enzyme activity assays.

2.4.10 Total protein assay

To get optimal results from SDS and activity assays, the total protein content (including enzymes) of crude extracts is needed.

To a volume of 990 µl of Pierce™ Coomassie Plus (Bradford) Assay Kit (Thermo Scientific, P/N 23236), 1 µl of concentrated crude extract, normalised in 9 µl dH_20, to a final volume of 10 µl, was added at room temperature. The reaction was allowed to develop for 10 minutes before the A_{595} was measured in a spectrophotometer and compared to a standard curve. The standard curve was produced as described above.

2.4.11 *SDS PAGE of crude extract to detect* ldh/pdcadh

The MW of LDH and PDCADH are known, so it should be possible to visualise their presence in crude extracts.

1 µl of x100 concentrated crude extract at a concentration of 1.25-2.5 µg protein per ml and 19 µl of SDS loading buffer were heated to 95^oC for 10 minutes to denature the proteins before running on a precast Bio-Rad 4-15% pre-cast gradient gels (Bio-Rad). The SDS loading buffer consisted of 250 µl of stacking buffer, which itself consisted of 7.87g Tris HCL and 8 mg of Tris Base in 100 ml dH_20, 600 µl of 50% glycerol, 100 µl of 10% SDS and 50 µl 1M DTT (added fresh to the SDS loading buffer just prior to use). The gel was run at 200 V for 1 hour and stained overnight in 50 ml Coomassie Brilliant Blue R250 stain in acetic acid: methanol: dH_20 (10:50:40) (50mg / 100 ml w/v) with gentle agitation. The gels were destained using 50 ml of the same solvent ($AcOH:CH_4O:dH_20$) minus the Coomassie Brilliant Blue, for 3 hours, with the destain changed every 30 minutes. Once the destain was complete, the gel was rehydrated in 50 ml dH_20 for 30 minutes and then scanned.

2.4.12 Native PAGE to detect ldh/pdcadh *activity*

20 µl of crude extract at a concentration of 1.25-2.5 µg protein per ml was loaded onto a 12% non-denaturing PAGE gel and run at 200 volts for 1 hour. The gels were removed and stained overnight in the activity reagents shown in Table 6.

Table 7 - Composition of native gel activity stains

Stain	Regents	Amount
Lactate dehydrogenase activity	50 mM Tris-HCL pH 9 0.5% D/L lactic acid (v/v) 1mM INTV 0.25 mM NAD+ 0.05 mM PMS	30 ml 150 µl 600 µl of 50 mM stock 5 µl of 50 mM stock 1 µl of 50 mM stock
Pyruvate decarboxylase activity	50 mM Tris-HCL pH 7.5 0.05 mM sodium pyruvate 25 mg/ml sodium bisulphate 0.025% basic fuchsin	30 ml 0.1 µl of 1 M stock 750 mg 7.5 µl of 2% fuchsin: ethanol (v/v) stock
Alcohol dehydrogenase activity	50 mM Tris-HCL pH 7.5 0.5% ethanol (v/v) 1mM INTV 0.25 mM NAD+ 0.05 mM PMS	30 ml 150 µl 600 µl of 50 mM stock 5 µl of 50 mM stock 1 µl of 50 mM stock

The gels were removed from the stains and immediately scanned.

2.4.13 LDH PDCADH *activity assay*

To determine whether there was enzymatic activity in the crude extract, a decrease in the OD of samples, caused by the oxidation of NADH to NAD^+, were measured. This is the reduction of pyruvate to either ethanol or lactate (see chapter 1).

2.4.13.1 LDH

To 1 ml of Tris buffer pH 9; 0.3 mM NADH (2 µl of 15 mM stock) and 1 µl of crude extract from *B. subtilis* $^{\Delta}$cspD::ldh^{+R}cat (ldh) was used. The background decrease in OD was measured at A_{340}, every

10 seconds for one minute. One µl of 1 mM sodium pyruvate (1 M stock) was then added and the decrease in OD 340_A measured every 10 seconds for three minutes.

2.4.13.2 PDCADH

To 1 ml of Tris buffer pH 7.5; 0.15 mM NADH (1 µl of 15 mM stock) and 1 µl of crude extract from $^{\Delta}$cspD::pdc:adhB^{R}kan (pdcadh) was added. The background decrease in OD at A_{340}, every 10 seconds for one minute was measured. One µl of 1 mM sodium pyruvate (1 M stock) was then added and measure the decrease in OD A_{340} measured every 10 seconds for three minutes.

This reaction was also carried out for WT *B. subtilis* 168, as above and all measurements were compared to a standard curve of known NAD^+ concentrations as described above.

2.4.14 Total lactic acid and ethanol assay

To determine if the conversation has been successful the levels of lactic acid and ethanol needed to be assayed. Ten microliters of sample were assayed in 1 ml of the appropriate reaction mix (table 7) below.

Table 8 - Reaction mix for total lactic acid and total ethanol assays

Substance	Reagents	Amount
Ethanol	50 mM Tris-HCL pH 7.5	1 ml
	YADH	5 µl (10 mg/ml stock)
	NAD+	5 µl (0.25 mM stock)
	PMS	0.5 µl (0.05 mM stock)
	INTV	200 µl INTV (1 mM stock)
Lactic acid	50 mM Tris-HCL pH 9	1 ml
	LDH	5 µl (10 mg/ml stock)
	NAD+	5 µl (0.25 mM stock)
	PMS	0.5 µl (0.05 mM stock)
	INTV	200 µl INTV (1 mM stock)

The colour was allowed to develop for 15 minutes exactly (this is not an endpoint assay) and the absorbance of each sample at 500_A measured with a one minute gap between each sample, so all samples had developed for 15 minutes only. The results were compared to a standard curve of known ethanol or lactic acid concentrations produced as above.

3 Biomass selection and physio-chemical pre-treatments

3.1 Background:

There is abundant literature regarding the different methods of pre-treating a wide range of biomass for utilisation in the production of biofuels, likewise there have been many studies with regards to which sugars different micro-organisms can utilise. The most common methods of pre-treating lignocellulosic biomass are to grind or mill to increase the surface area and partially disrupt the crystalline regions of cellulose. This biomass is then partially hydrolysed by hydrochloric or sulphuric acid. Other methods include alkaline pre-treatments, such as ammonia or sodium hydroxide, to reduce the crystallinity of the cellulose or by steam infiltration under a vacuum and then sudden depressurisation to explode the biomass.

The most common sugar utilised by the majority of micro-organisms is unsurprisingly glucose, which is utilised for growth through glycolysis. Pentose monosaccharides are utilised via the pentose phosphate pathway prior to glycolysis.

Since paper is mainly cellulose and cellulose is chains of glucose, the use of waste paper as the main carbon source is an appealing system. Lignocellulosic biomass, however, is made up of not only cellulose, but hemicellulose and pectin, as well as lignin. Hemicellulose and pectin are made up of different monosaccharides in different amounts and lignin limits enzymatic degradation of cell walls, which is why pre-treatments are necessary and why knowing which monosaccharides are released by which pre-treatments from which biomass and which microorganisms can use those monosaccharides is vital if this is to be developed as an economically feasible system.

3.1.1 Physio-chemical pre-treatments

There are many arrays of different types of physical and chemical pre-treatments available to treat lignocellulosic biomass to enable microbes to utilise it as a carbon source. Pre-treatment is essential as plants have evolved to prevent degradation by microbes. The main pre-treatments can be surmised as;

Physical grinding or milling to increase surface area and reduce the crystallinity of cellulose.

Acid hydrolysis to hydrolyse amorphous cellulose and hemicellulose to expose more cellulose to chemical degradation (Kumar et al., 2008, Kumar et al., 2009, Wang, 2008, Harmsen, 2010, Brodeur et al., 2011).

Alkaline treatments to swell crystalline areas of cellulose by breaking hydrogen bonds between the microfibrils and remove cross links between cell wall polymers (Kumar et al., 2008, Harmsen, 2010, Brodeur et al., 2011, Geddes et al., 2011, Tong, 2013).

Biological degradation; this is mainly done after previous physio-chemical pre-treatments and usually involves cell free enzymes derived from cellulosic organisms such as *T. reesei* (Lee, 1997, Kumar et al., 2008, Kumar et al., 2009, Wang, 2008, Harmsen, 2010, Meyer et al., 2013).

3.2 Methods:

3.2.1 Thin layer chromatography for biomass monosaccharide analysis

To determine which monosaccharides were present at any stage of the experiment thin layer chromatography was used to give a clear profile. This analyse was done using either;

Alcohol insoluble residue (AIR) was produced for each biomass by rehydration in distilled water, then washed in 50% ethanol (50:50 H_2O v/v) for 24 hours and then in absolute ethanol, with fresh ethanol added every 24 hours, until no more colour change of the liquid was seen. The AIR was then washed in neat acetone to dry it. Once dry and all acetone evaporated, 10 mg of each biomass was hydrolysed in 2M trifluoroacetic acid (TFA) at 100^{O}C for 1 hour. Once cool the reaction was spun down and the liquid hydrolysate decanted off.

One ml of growth media from each flask was removed.

This was then dried almost completely, leaving syrup. This syrup was then re suspended in 100 µl 0.5% chlorobutanol and 3 µl loaded onto a silica thin layer chromatography plate and then run in the solvent mix ethyl acetate; pyridine; propanol; acetic acid; water (4:2:2:1:1) for three hours. The plate was then allowed to air dry overnight. Once all solvent had evaporated it was stained in thymol stain and then developed at 110^{O}C for 10 minutes and immediately scanned.

3.2.2 In situ labelling of cell wall polymer epitopes on pre-treated biomass

Plant material was first fixed in glutaraldehyde and then subjected to ethanol dehydration, immersion in increasing concentrations of ethanol: water until saturation by 100% ethanol was achieved. The plant material was then resin infiltrated by immersion in increasing concentrations of ethanol:acrylic resin until 100% resin infiltration was achieved, all at 5^{o}C. The plant material was then encapsulated and the resin polymerised at 37^{o}C. Once hard the plant material was sectioned on a diamond microtome and fixed onto vectabond slides. The sections were blocked with 10% milk:PBS (w/v) mix, then each section was labelled with the appropriate antibody by incubating the antibody in milk for one hour. The slides were washed with PBS and then incubated with a secondary antibody linked with FITC which attached to the primary antibody. The slides were washed again in PBS and a coverslip mounted with glycerol to prevent fading. The slides were then viewed on a fluorescence microscope with appropriate excitation of the FITC as selected on the pre-set microscope settings, literally “FITC” option.

3.2.3 Physiochemical pre-treatments of biomass

If untreated biomass was to be used, then 5000 mg of the untreated biomass was added to 100 ml of appropriate minimal growth media. If pre-treated biomass was to be utilised, then the biomass was first ground to a practical size of less than one centimetre in a food blender. It was then boiled in tap water for two hours and the solid and liquid fractions separated and stored. The solid fraction from the previous step was then immersed in 1M NaOH at 50^{o}C for two hours, again the solid and liquid fractions separated and stored. The solid fraction from the previous step was then immersed in 1M sulphuric acid at 150^{o}C for two hours. The solid and liquid fractions were separated and stored as before. The solid fraction was then submersed in distilled water and neutralised to pH7, it was then dried for use as the main carbon source in growth assays. The liquid fractions from these pre-treatments were pooled and neutralised to pH 7 and then either supplemented with appropriate mineral salts to convert it to a suitable growth medium dependant on which micro-organism is to be used, or filtered through activated carbon before the mineral salts being added.

3.3 Results

3.3.1 Monosaccharide profile of common biomass

To determine which potential monosaccharides would be available for microbial growth, the monosaccharide profile of the biomass used in this study was investigated, through incomplete TFA acid hydrolysis and thin layer chromatography. The biomasses selected for investigation were; Filter paper, office paper, newspaper, wheat straw and *Miscanthus sinensis*.

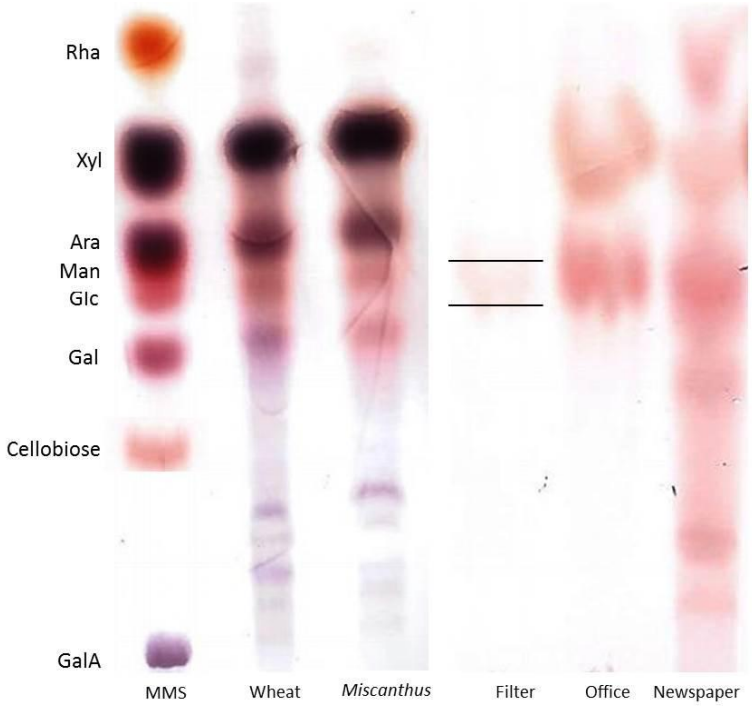


Figure 19 – Composite thin layer chromatograph of the sugar profiles of biomass utilised in this study. Biomass was subjected to incomplete trifluoro acetic acid (TFA) acid hydrolysis. Wheat and *Miscanthus* lanes were run on one silica plate and the filter paper, office paper and newspaper ran on a second plate. MMS = monosaccharide marker standard. Representative of three biological replicates.

As figure 19 shows;

Filter paper contains glucose. The glucose is presumed to be mainly be derived from cellulose. Since filter paper is utilised in research a high level of quality is needed, hence the low amounts of xylose, filter paper would be mainly cellulose.

Office paper contains mainly glucose, again derived mainly from cellulose, but also contains xylose probably derived from xylans, due to the lack of arabinose, although this may have been present in an altered form, as arabinose is particularly sensitive to acid hydrolysis. The higher amount of xylose present is probably due to the purity of office papers not needing to be as high quality as lab filter paper, so fewer alkaline treatments in the Kraft process were possibly used to reduce production costs. Alkaline pre-treatments break the hydrogen bonds linking hemicelluloses (xylan) to cellulose fibrils (Fry, 1988).

The hydrolysed newspaper shows bands for glucose, again mainly derived from cellulose, some galactose and (possibly) galacturonic acid; mannose, some xylose (although surprisingly seemingly less than is present in the office paper) and a relatively large amount of rhamnose. The mannose is probably derived from hetro- and/or gluco- mannans, and are again probably due to fewer/less intense alkaline treatments as newspaper is a very low quality paper so needs to be cheap to produce. The galacturonic acid, galactose and rhamnose are surprising but are possibly because newspaper is

commonly made from “groundwood” (Victoria and Albert Museum, 2016, Glatfelter, 2016) which is produced from grinding whole wood to pulp, without the extensive chemical treatments used in Kraft paper production, and as such still contains acids from the wood (Victoria and Albert Museum, 2016, Glatfelter, 2016).

When wheat straw was analysed there appears to be a surprising lack of glucose in the hydrolysate when compared to arabinose and xylose, since glucose will have been present in the cellulose and hemicelluloses, so a greater amount of glucose in total was expected compared to the other monosaccharides, in this case glucose derived from cellulose, mixed linkage glucans and xyloglucans. However, TFA hydrolysis would not have affected crystalline cellulose, which would leave a large amount of potential glucose in the residue. To determine which cell wall polymers are present it is possible to digest plant material with the enzyme cocktail “Driselase” which yield the dimer xylobiose from arabinoxylans and the dimer isoprimeverose from xyloglucan (Fry, 1988). However, figure 19 is only for TFA hydrolysis. There is also a large amount of mannose which will have come from mannans in the secondary cell walls, as wheat at harvest has senesced, therefore is at the end of its growing season and the majority of secondary cell walls are constructed after a plant has stopped actively growing (Fry, 1988).

In the profile of *Miscanthus sinensis*, the monosaccharide present in largest amounts is xylose with lesser amounts of arabinose, these will have come from arabinoxylans which are a primary cell wall polymer in grass species, while the rest of the xylose will probably have been derived from xylans. There are also bands present for galactose and glucose. It is also surprising that there appears to be very little mannose present in the *Miscanthus* as the plant material was quite mature when harvested, so we would expect a larger amount of secondary cell walls, of which mannans are a major component, than in newer growth (Fry, 1988).

3.3.2 Development of simple pre-treatments

The next step was to determine whether *Cytophaga hutchinsonii* and *Cellulomonas fimi* could utilise this biomass for growth. The results presented in chapter four, demonstrate that both micro-organisms can use paper as a carbon source for growth. Preliminary experiments detailed here to determine if *C. hutchinsonii* and *C. fimi* could grow in DSM3+ 0.5% yeast extract (w/v) on untreated *Miscanthus* biomass over 28 days. As figures 21 and 22 demonstrate, there was no visually obvious physical breakdown of the biomass, by either micro-organism, but thin layer chromatography analysis show the presence of different monosaccharides in the growth medium, presumed to have come from the cell wall polymers present in the *Miscanthus* biomass.

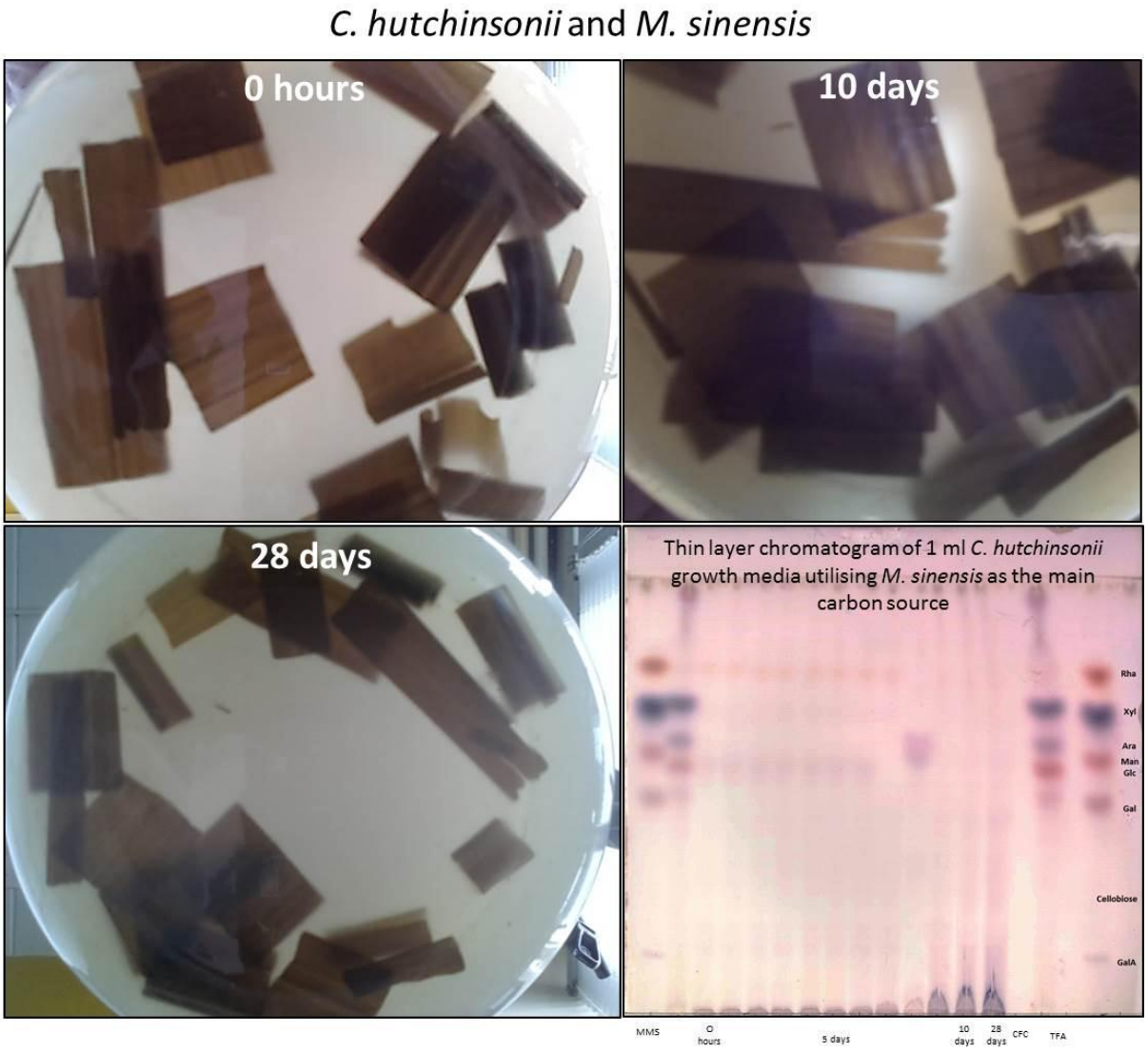


Figure 20 – Attempted growth of *C. hutchinsonii* on untreated *Miscanthus sinensis* biomass over 28 days and monosaccharides released into media by the enzymatic actions of *C. hutchinsonii*. 100g of biomass was added to 100 ml of minimal DSM3 media and each flask was inoculated with 1 ml of liquid bacterial culture with an OD > 1.000 and cultured at 100 RPM at 30°C for 28 days. The lane CFC = cell free control, sampled after 28 days. Representative of three technical replicates of open biological replicate.

As figure 20 shows, there is no discernible breakdown of the *Miscanthus* biomass from the start of the growth trial to the end at 28 days, although the TLC analysis does shows evidence of glucose, xylose and rhamnose being released into the growth medium up to 7 days, these sugars are presumed to be being released from the plant cell wall polymers. There are no spots in the cell free control lane (CFC), taken after 28 days, which suggests no passive chemical degradation of the cell wall polymers by this media. These spots disappearing after seven days, which is taken to mean that these monosaccharides are no longer present in detectable quantities after this points. There is a large spot for arabinose at day nine, but the cause of this is a mystery.

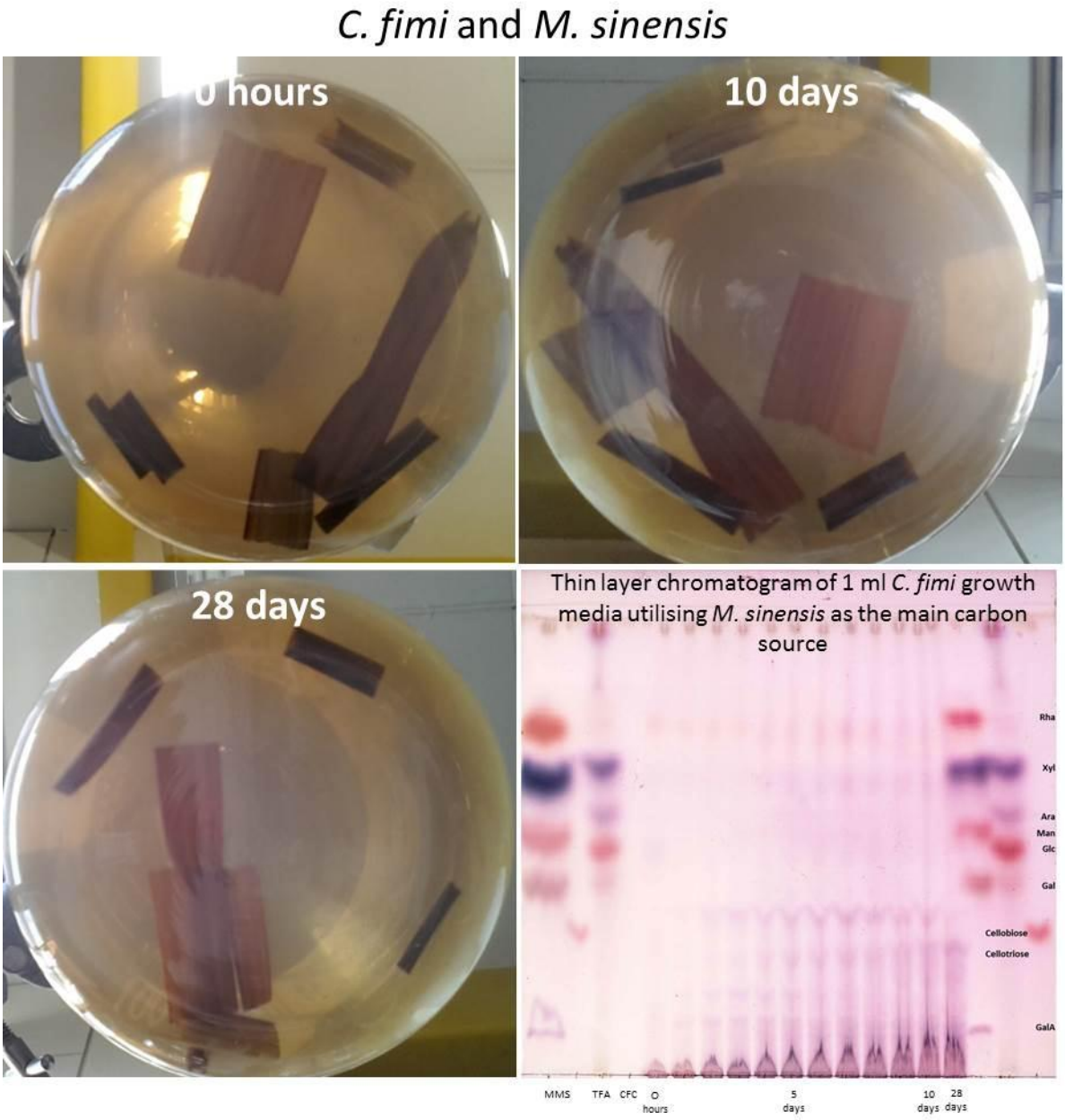


Figure 21 - Attempted growth of *C. fimi* on untreated *Miscanthus sinensis* biomass over 28 days and monosaccharides released into media by the enzymatic actions of *C. fimi*. 100g of biomass was added to 100 ml of minimal DSM3 media and each flask was inoculated with 1 ml of liquid bacterial culture with an OD > 1.000 and cultured at 100 RPM at 30°C for 28 days. The lane CFC = cell free control sampled at 28 days. Representative of three technical replicates of open biological replicate.

Figure 21 again shows little if any physical breakdown of the *Miscanthus* biomass across the 28-day growth period. However, there is some evidence of micro-scale degradation with the presence of cello biose and triose being detected by TLC from 48 hours to 20 days' xylose being detected from 4 days until 20 days and rhamnose being present from 0 hours to 20 days. There were no spots in the cell free control (CFC) lane, which was taken at the 28-day time point, suggesting no passive degradation by the medium. Since there was no visually obvious physical degradation or increase in

optical density for either the *C. hutchinsonii* or *C. fimi* growth trials, it was presumed that there was no real microbial growth taking place.

3.3.3 Physio-chemical pre-treatments

Since the experiments detailed above show little degradation of the biomass and no real microbial growth it was determined that some form of physio-chemical pre-treatment of the biomass would be required before the micro-organism could utilise the biomass for growth. The pre-treatments devised were based on techniques outlined in the literature (see chapter one). These pre-treatments are outlined in chapter two and simply laid out in figure 22 below.

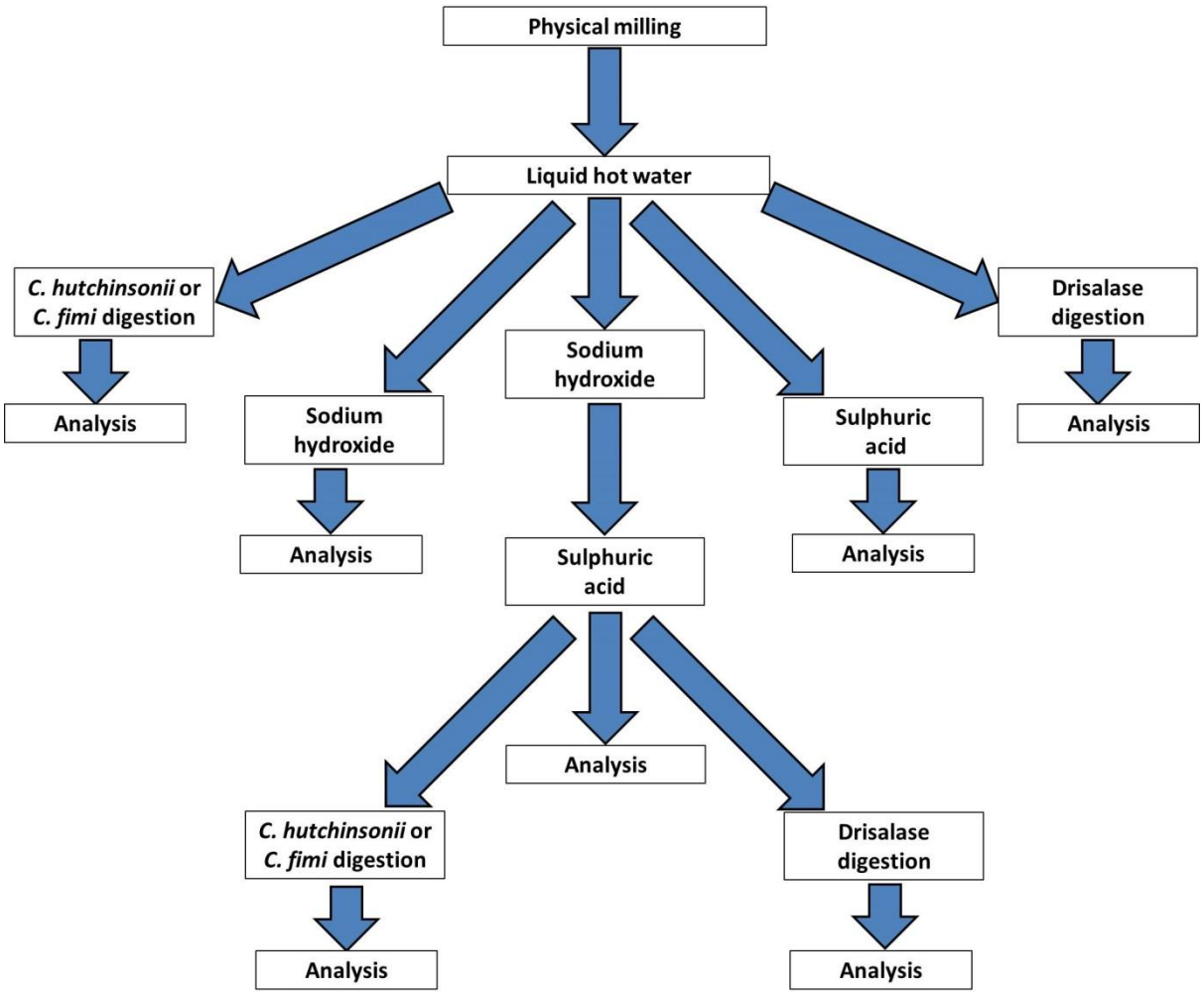


Figure 22 - Simplified flow diagram of the physio-chemical pre-treatments used on wheat straw and *Miscanthus* biomass.

The liquid fractions / hydrolysates from these pre-treatments were collected, neutralised and then analysed using thin layer chromatography, to visualise the monosaccharides present in them and so removed from the cell wall polymers by the pre-treatments, shown in figure 24.

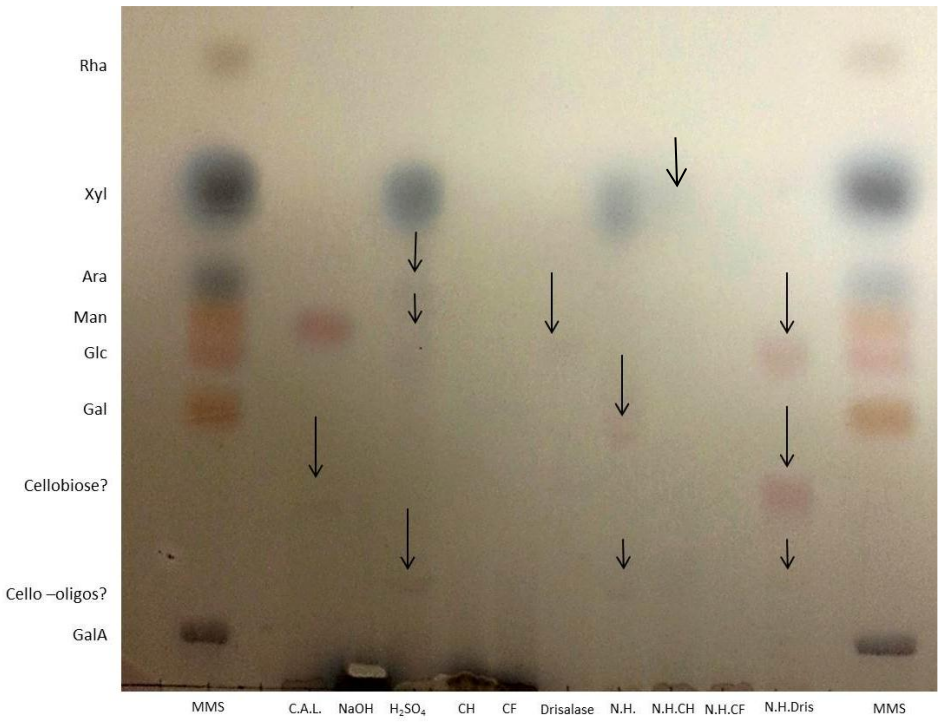


Figure 23 – Qualitive TLC of the pre-treatment hydrolysates from wheat straw. After each pre-treatment, the liquid hydrolysate was collected and neutralised, 1 ml was then concentrated and run on the thin layer chromatography silica plate and then stained with thymol. C.A.L. = Chopped + Autoclaved + Liquid hot water treatment; NaOH= sodium hydroxide treatment; H_2SO_4 = sulphuric acid treatment; CH= *C. hutchinsonii* inoculation; CF = *C. fimi* inoculation; Driselase = Driselase digestion; N.H.= sodium hydroxide + sulphuric acid treatments; N.H.CH = Sodium hydroxide = sulphuric acid treatments followed *by C. hutchinsonii* inoculation; N.H.CF = Sodium hydroxide + sulphuric acid treatments followed by *C. fimi* inoculation; N.H.Dris = Sodium hydroxide + sulphuric acid treatments followed by Driselase digestion (see chapter two and figure 4). Representative of three biological replicates.

As figure 23 shows milling and liquid hot water pre-treatments (CAL) resulted in the release of glucose and possibly cellobiose. These sugars were probably derived from the hydrolysis of amorphous cellulose, since milling was used to increase the surface area of the biomass and reduce the crystallinity of the cellulose. No monosaccharides were detected in the sodium hydroxide liquid fraction, probably due to sodium hydroxide being used to reduce the crystallinity of the cellulose and to break the hydrogen bonds between cellulose and hemicellulose. Hydrolysis by dilute sulphuric acid results in the presence of small amounts of cellulose derived oligosaccharides, small amounts of glucose or mannose, small amounts of arabinose and large amounts of xylose. There were no monosaccharides detected from the *C. hutchinsonii* or *C. fimi* fractions, although as figures 20 and 21 above show these organisms are capable of releasing monosaccharides from the plant cell walls. Driselase digestion resulted in a small amount of glucose.

Sequential pre-treatments of sodium hydroxide and dilute sulphuric acid (NH) resulted in cellulose derived oligosaccharides, galactose and a smaller amount of xylose than was released by dilute sulphuric acid alone. A trace of xylose was released by sequential sodium hydroxide/sulphuric acid/ *C. hutchinsonii* but no monosaccharides were detected in the sodium hydroxide/sulphuric acid/ *C.*

fimi treatments. This may be due to the monosaccharides being utilised by the micro-organism as *C. fimi* can utilise all the potential monosaccharides released, as can *C. hutchinsonii* with the exception of xylose (see chapter four) and (Liu, 2012, Kane, 2014). Sequential pre-treatments of sodium hydroxide/sulphuric acid/ Driselase resulted in some cellulose derived oligosaccharides and relatively large amounts of cellobiose and glucose. This method of analysis is qualitive only, not quantitive. To determine the amount on sugars present in each pre-treatment hydrolysate, analysis by HPLC or semi-quantitive paper chromatography would be needed.

3.3.4 Effect of pre-treatments on biomass biochemistry

As figure 23 shows, different physio-chemical pre-treatments result in different monosaccharides being released as different cell wall polymers are deconstructed. Since certain cell wall polymers can mask others until the former are removed (Xue et al., 2013), it may be advantageous to know in what order the cell wall polymers are degraded and which polymers mask each other, so enabling us to know in what order and amount certain monosaccharides are released making the process more efficient.

This pattern of masking and unmasking of cell wall polymers and can be visualised using in situ antibody labelling of the side chains of certain cell wall polymers, antibodies that are raised to recognise epitopes derived from cell wall polymers. The antibodies used for the labelling of wheat straw cell walls are;

LM11 recognises the 1→4 β linkage of the xylose backbone of xylan/arabinoxylan, figure 18 and (McCartney et al., 2005)

LM19 recognises partially methyl esterified homogalacturonan in pectin, figure 89 and (Verhertbruggen et al., 2009)

LM21 recognises heteromannan, figure 18 and (Marcus et al., 2010, Donaldson and Knox, 2012)

LM25 recognises galactosylated xyloglucan, figure 18 and (Pedersen et al., 2012)

However, there proved to be no signal at all or no conclusive signal for the LM19, LM21 and LM25 epitopes so those micrographs are not shown.

It is possible to use FITC linked secondary antibodies to visualise the position of the primary antibody in the plant cell walls. FITC is green when excited, so on the following micrographs, the green areas are where the epitopes are located and it is the strength of the green signal that illustrates the abundance and location of these epitopes.

The biomass was subjected to different physio-chemical pre-treatments (fig 24 -27) and then the cell walls were labelled with CWP antibodies above to demonstrate what, if any, biochemical changes occurred in the biomass once it had been pre-treated.

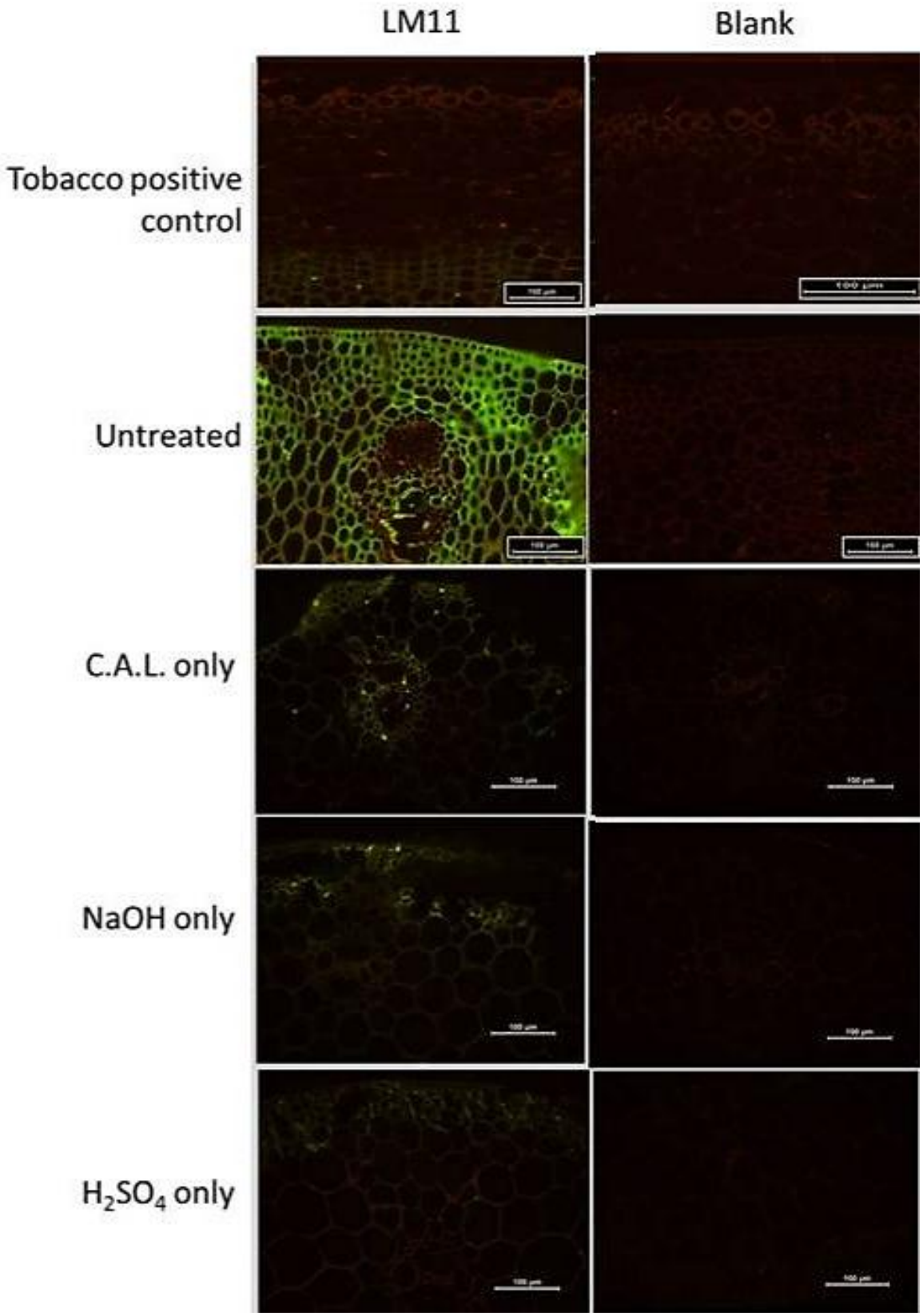


Figure 24 - *in situ* labelling of wheat straw cell walls of single pre-treatment treated wheat straw biomass. Resin embedded biomass was labelled with a primary antibody to bind to the LM11 epitope and then with a secondary FITC linked antibody to bind to the primary antibody. Micrographs where the scale bar is enclosed were added post capture; all other scale bars were present at exposure, scale bar = 100 µm. Images shown are representative of two technical replicates of three biological replicates in all cases.

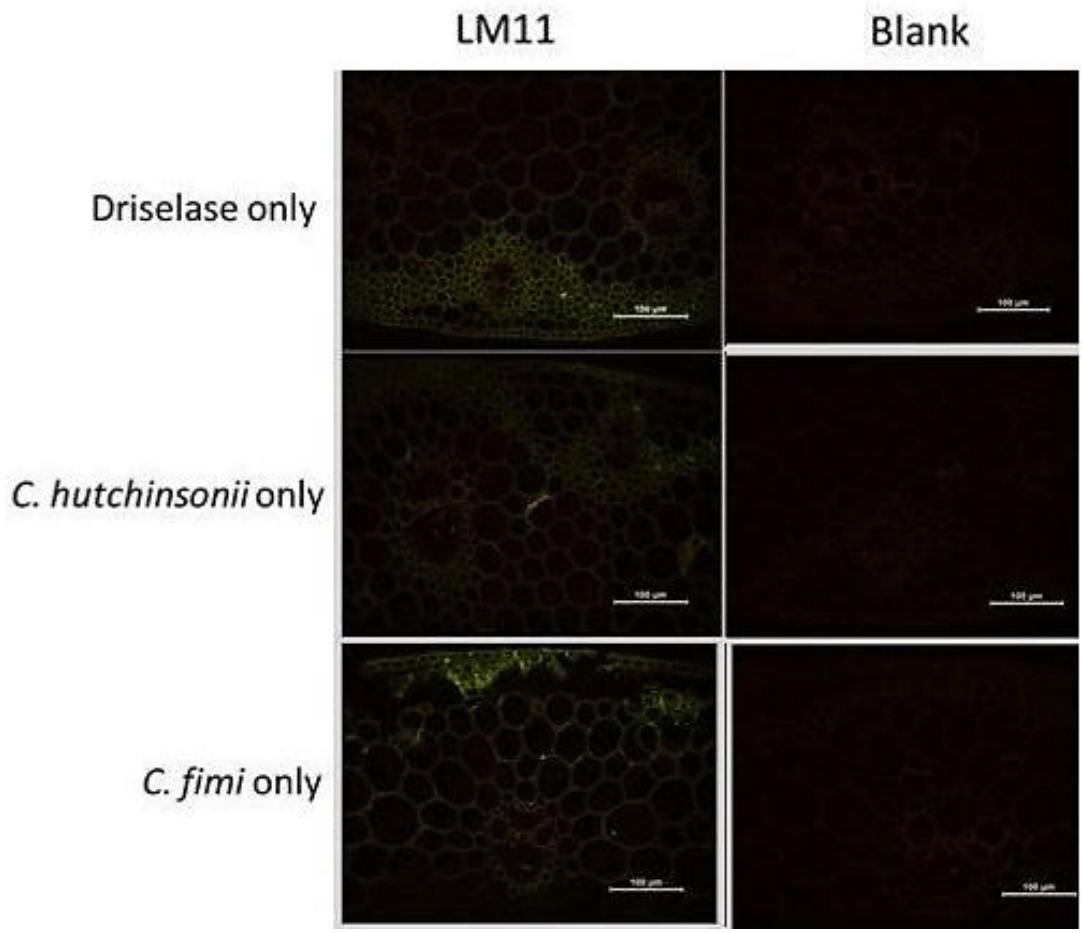


Figure 25 - *in situ* labelling of wheat straw cell walls of single pre-treatment treated wheat straw biomass. Resin embedded biomass was labelled with a primary antibody to bind to the LM11 epitope and then with a secondary FITC linked antibody to bind to the primary antibody. Micrographs where the scale bar is enclosed were added post capture; all other scale bars were present at exposure, scale bar = 100 µm. Images shown are representative of two technical replicates of three biological replicates in all cases

The micrographs of wheat straw subjected to single pre-treatments are shown in figure 24 and 25. The tobacco positive control is to show that the antibodies do label cell walls, as the antibodies were raised against epitopes derived from cell wall polymers extracted from tobacco plants. The "untreated" control is to show what epitopes are present in the cell wall polymers before any pre-treatments and the blank control is to show that the signal is from the secondary antibodies that attaches to the primary antibody only and not to the epitope on its own, to show that the antibodies are specific and don't just bind to anything.

We would expect to see a signal for LM11 and LM21 as wheat straw cell walls contain xylan, arabinoxylan and mannan. Since wheat is a grass, we would not expect to see a signal for LM19 as grass cell walls are poor in pectin nor see much of a signal for LM25 as xyloglucan is the predominant hemicellulos in dicots not in monocots (Albersheim, 2011, Carpita, 1996).

In the tobacco, positive control the LM11 epitope is present in the epidermis and medulla (pith). This controls shows that the antibodies recognise specific epitopes from different polymers in plant cell walls, as they were raised against epitopes derived from tobacco cell wall polymers.

In the untreated wheat sample; there is a strong signal for LM11 throughout all tissues, apart from the xylem and phloem.

In the C.A.L treated sample the LM11 epitope is present throughout the tissue, apart from the xylem and phloem.

In the sodium hydroxide treated sample the LM11 epitope is present in the strongest concentrations in the epidermis and the signal is reduced in the medulla, indicating that the sodium hydroxide has had some effect on the abundance of the LM11 epitope, which is present in the backbone of xylan and/or arabinoxylan. The epitope is completely absent from the xylem and phloem. All other epitopes are absent.

In the sulphuric acid treated sample the LM11 epitope is present in all tissues but there is only a weak signal compared to the untreated control, indicating that sulphuric acid has had a similar effect on the abundance of this epitope to the effect of the sodium hydroxide. All other epitopes are absent.

In the Driselase treated sample the LM11 epitope is present in all tissues, apart from the phloem and xylem as expected.

In samples that have been inoculated with 1 ml of liquid culture of *C. hutchinsonii* and so were subjected to the enzymatic digestion by *C. hutchinsonii* the LM11 epitope is present throughout the

tissues, but giving a weaker signal compared to the untreated sample, indicating that the enzymes produced by *C. hutchinsonii* are influencing the xylan/arabinoxylan present in the cell walls, which is to be expected (see section 1.4.1). The LM 11 epitope is absent from the xylem and phloem.

In samples exposed to *C. fimi* the LM11 epitope is present throughout but is strongest in the epidermis and absent from the xylem and phloem indicating that the enzymes of *C. fimi* are having a specific effect on specific cell wall polymers, as would be expected as that is what they are designed to do.

Figures 26 and 27 shows the micrographs of wheat straw subjected to sequential pre-treatments. These should illustrate the exposure of different cell wall polymers as bonds are broken by different pre-treatments and so unmask different cell wall polymers.

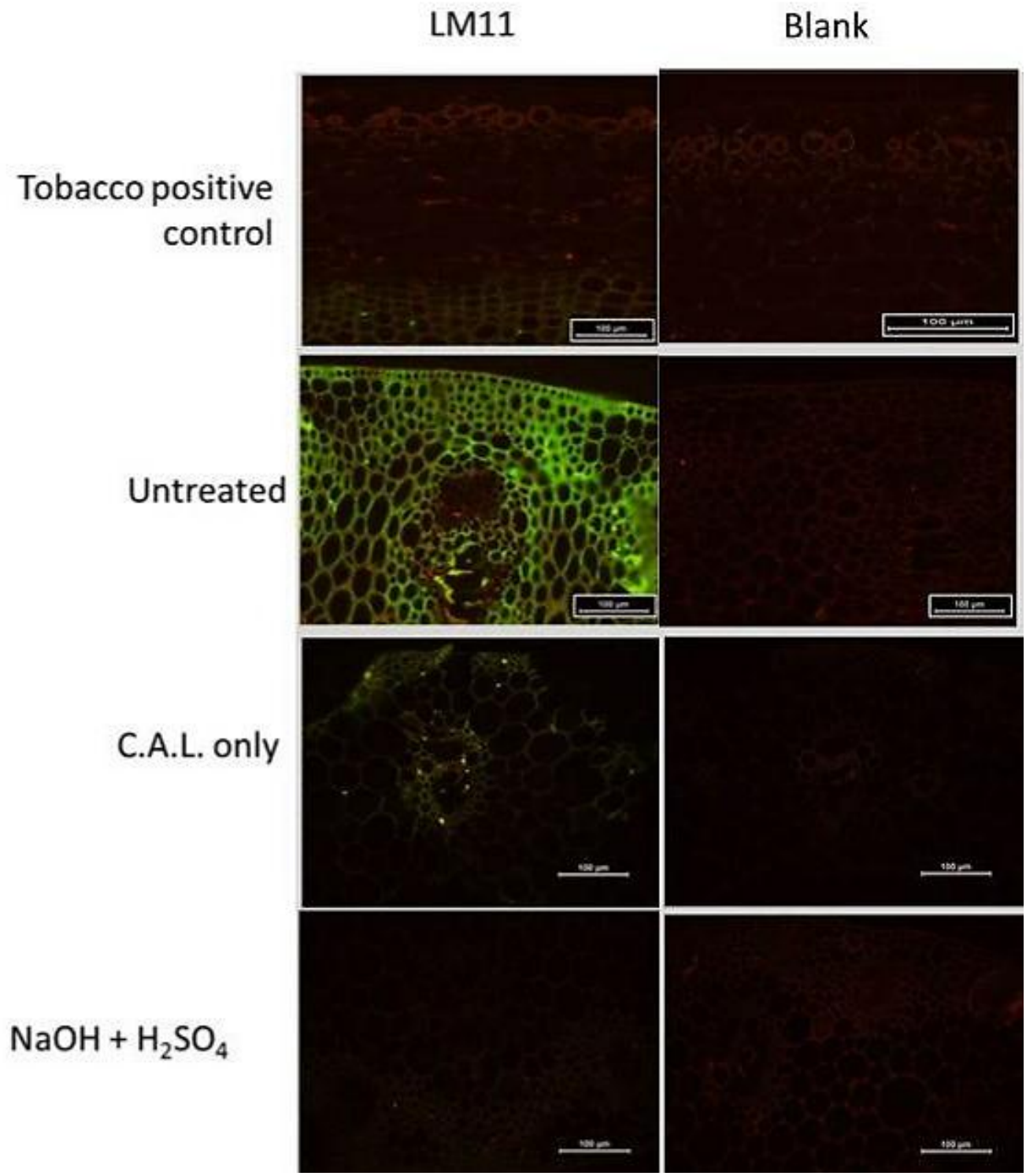


Figure 26 – *in situ* labelling of wheat straw cell walls of sequentially pre-treated biomass. Resin embedded wheat straw biomass was labelled with a primary antibody to bind to the LM11 epitope and then with a secondary FITC linked antibody to bind to the primary antibody. Micrographs where the scale bar is enclosed were added post capture; all other scale bars were present at exposure, scale bar = 100 µm. Controls as above. Images shown are representative of two technical replicates of three biological replicates in all cases.

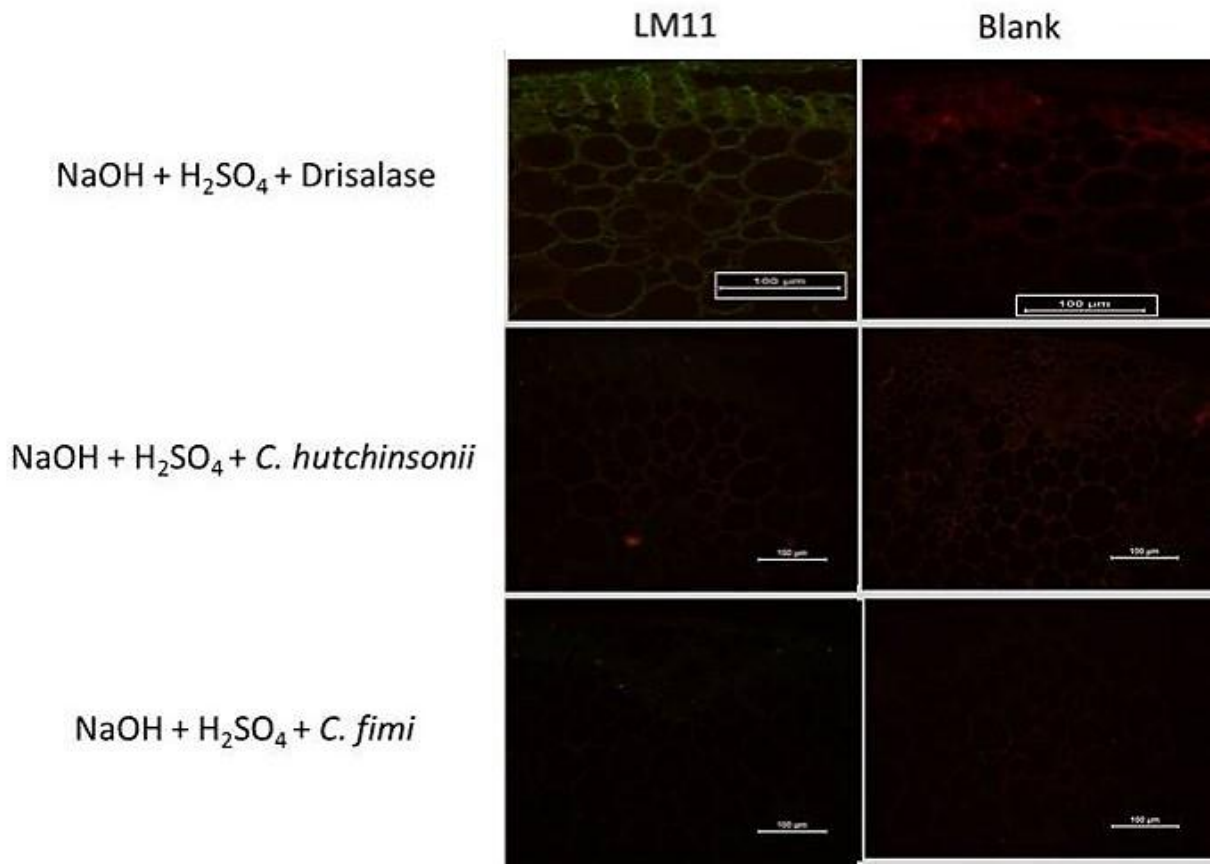


Figure 27 - *in situ* labelling of wheat straw cell walls of sequentially pre-treated biomass. Resin embedded wheat straw biomass was labelled with a primary antibody to bind to the LM11 epitope and then with a secondary FITC linked antibody to bind to the primary antibody. Micrographs where the scale bar is enclosed were added post capture; all other scale bars were present at exposure, scale bar = 100 µm. Controls as above. Images shown are representative of two technical replicates of three biological replicates in all cases.

In samples subjected to sodium hydroxide and then sulphuric acid pre-treatments the LM11 epitope is faintly present in the epidermis and cortex, but is absent from the medulla. This pattern is different to that of the untreated samples, the sodium hydroxide and sulphuric acid pre-treatments when used on their own. This indicates that the sodium hydroxide is altering the cell wall in such a way as to expose more of the cell wall polymers to chemical degradation. All other epitopes are absent.

In samples pre-treated with sodium hydroxide then sulphuric acid and then Driselase the LM11 epitope is present throughout all tissues, which is different to the pattern caused by just sodium hydroxide and sulphuric acid and suggests the Driselase is unmasking cell wall polymers in a specific way. There is a signal after Driselase digestion but not after sodium hydroxide and sulphuric acid pre-treatments. It also shows that chemical pre-treatments enable greater biological digestion when compared to samples that are just exposed to biological attack without the chemical pre-treatments.

In samples subjected to alkali, acid treatments and then exposed to *C. hutchinsonii*, there is no signal the LM11 epitope, compared to the untreated control. The growth of *C. hutchinsonii* on the

sequentially pre-treated wheat straw was better than the growth on untreated wheat straw (see chapter five).

For samples subjected to these chemical pre-treatments and then *C. fimi* the LM11 epitope is absent, while it is present in the untreated control, showing that that different bacteria, with different enzymes degrade the same cell wall polymers in different ways, suggesting that mixed cultures of one chassis organism and one cellulosic organism may be enhanced by using more than one cellulosic organism for greater biological saccharification.

While there is a range of monosaccharides present in the various pre-treatment hydrolysates (fig 23), the purpose of the pre-treatments is not to hydrolyse the biomass, but to make it more amenable to bacterial and enzymatic digestion.

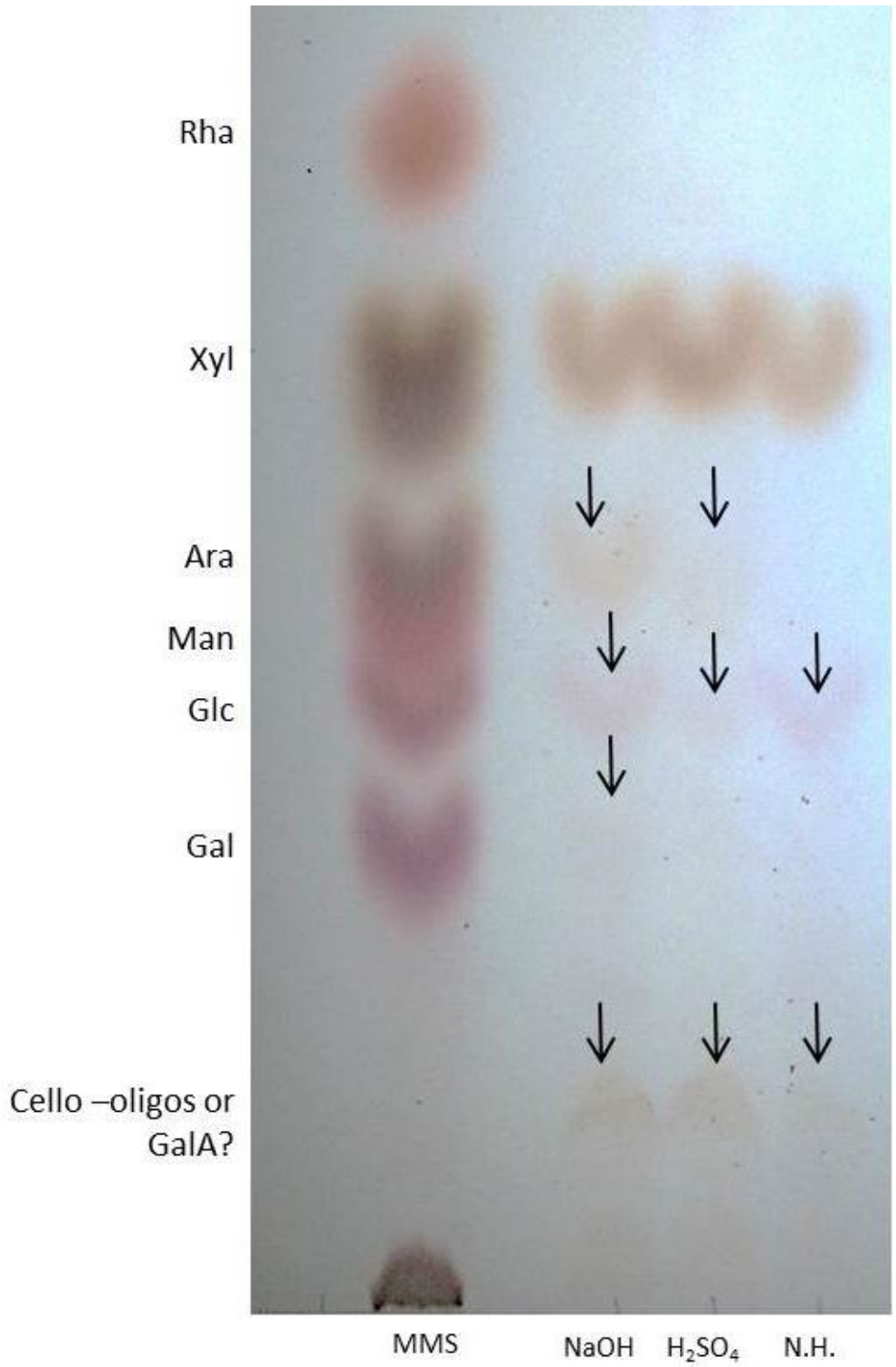


Figure 28 - TLC of TFA hydrolysed pre-treated wheat residue, which was subjected to incomplete TFA acid hydrolysis, before the hydrolysate was run on a silica TLC plate and stained with thymol. This shows which monosaccharides are present in the residue before the biological saccharification (CH, CF and Driselase) begins. Representative of two biological replicates.

To determine what polysaccharides and therefore what potential monosaccharides were still present in the pre-treated biomass, after pre-treatments, a sample of the residue from each pre-treatment was subjected to acid hydrolysis by TFA and analysed by TLC. The results are shown in

figure 28 and show that in the residue left after sodium hydroxide pre-treatments, there remained cell wall polymers that yielded galactose, glucose, arabinose and xylose. In the residue left after dilute sulphuric acid treatment there were polymers that yield a small amount of glucose and arabinose and a large amount of xylose. In the residue left after sequential sodium hydroxide and sulphuric acid treatments there were polymers that yielded glucose in slightly higher amounts than that left in the sulphuric acid residue but a smaller amount of xylose than was present in the first two residues.

3.3.5 Utilisation of hydrolysates from pre-treatments as growth media

We know that specific pre-treatments lead to changes in the biochemistry of lignocellulosic biomass, hydrolysis and removal of mono and oligosaccharides from cell wall polymers into the hydrolysate (figures 23 -28). Therefore, there is a potential carbon source in the liquid fractions of the pre-treatments as well as in the solid fraction. The liquid fraction contains monosaccharides as well as inhibitor compounds such as furfurals, acetic acid and phenolic compounds from the hydrolysation of hemicellulose and the lignin in the cell walls. We decided to investigate whether the neutralised, filtered and unfiltered liquid fractions could be used as a growth medium for the micro-organisms used in this study. As figure 29 shows, only *B. subtilis* 168 had any viable cells after ten days when the hydrolysate was unfiltered, although there was no change in the optical density.

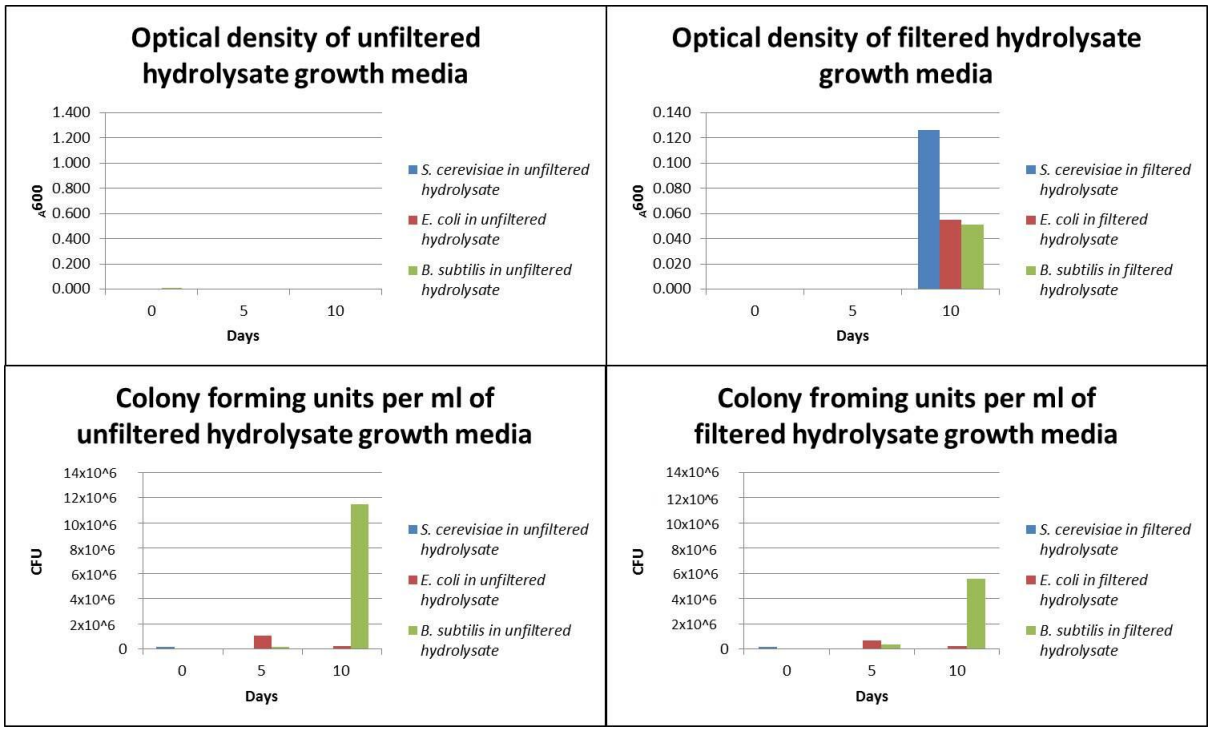


Figure 29 - Optical density and CFU counts for select micro-organisms as a measure of growth in neutralised pre-treatment hydrolysate when supplemented with necessary mineral salts to constitute a growth medium. Representative of one biological replicate.

The lack of growth in the former is possibly due to the presence of inhibitor compounds such as furfurals and acetic acid (Ibraheem and Ndimba, 2013, Eiteman, 2014, Zhang and Wu, 2014, Fletcher, 2014). To try and counter this we filtered the hydrolysate through activated carbon, based on the literature (Cuevas et al., 2014). In the filtered hydrolysate, there was an increase in the optical density for all three micro-organisms after ten days, with the largest increase being in the *S. cerevisiae* culture and the lowest being in *B. subtilis*, which was unexpected compared to the unfiltered results. In the unfiltered hydrolysate, the only organisms with any quantifiable viable cells were *S. cerevisiae* and even then, the numbers where quite low (12x10^6). This result was surprising so it was decided to analyse the filtered hydrolysate with thin layer chromatography. As figure 30 shows the filtration through activated carbon not only presumably removed the inhibitor compounds, as the filtrate was clear, but also the monosaccharides present, as illustrated in figure 30 by the lack of spots on the TLC plate.

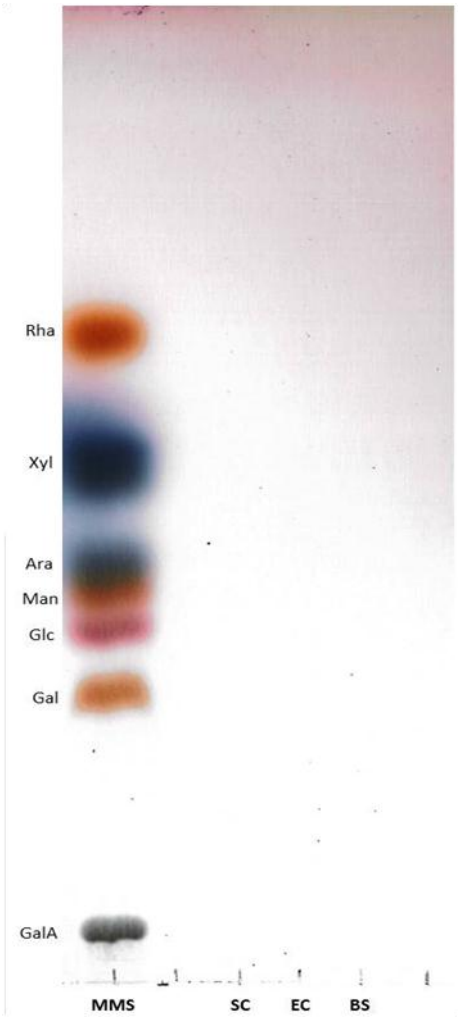


Figure 30 – Monosaccharides present in filtered neutralised pre-treatment hydrolysate after filtration through activated charcoal. The filtered samples were used as a growth media (fig 29) after ten days 1 ml was collected, condensed and run on a TLC silica plate and stained with thymol. SC = S. *cerevisiae*, EC = *E. coli* and BS = *B. subtilis* cultures. Representative of one biological replicate.

Since there is no growth in neutralised unfiltered hydrolysates, probably due to phenolic compounds and acetic acid and no growth in filtered hydrolysates, probably due to the lack of monosaccharides, all the monosaccharides in the hydrolysates are essentially wasted.

3.4 Short discussion and summary

The results presented here show that different pre-treatments have different effects on the biochemistry of wheat straw. These treatments result in the breakdown of cell wall polymers into their constituent monosaccharide building blocks and these monosaccharides are released into the resultant hydrolysates, with only certain monosaccharide yielding polymers being left in any residue, however the monosaccharides in the hydrolysates are unable to be utilised for growth, possibly due to inhibitor compounds being present created by the pre-treatments and the loss of these monosaccharides when these impurities are removed. Although these pre-treatments should make the biomass more amenable to microbial utilisation, the limited range of potential monosaccharides in the residue negate any perceived benefits to this increase in digestibility as the remaining sugars are not readily utilised by the organisms investigated in this study.

4 Microbial monosaccharide utilisation

4.1 Background:

As chapter three demonstrated, the different cell wall polymers present in different sources of biomass yield different monosaccharides. In an effort to make the conversion of lignocellulosic biomass to useful products via the actions of micro-organisms, it would be helpful to determine which monosaccharides can be utilised by which microorganisms, under the conditions used in this study. Looking at microbial tolerance to lactic acid, butanol and ethanol under the same growth conditions will also be investigated.

4.2 Wild type monosaccharide utilisation

Lignocellulosic biomass is made up of cell wall polymers constituted from a range of monosaccharides. Through the actions of enzymes produced by some micro-organisms and/or via physiochemical pre-treatments, these polymers yield their monosaccharide building blocks. The ability to utilise most, if not all, of these sugars is essential to increase the economic viability of using lignocellulosic biomass for the production of economically relevant substances.

According to Mayer et al., 2006 *C. fimi* is able to degrade cellulose, xylan, and chitin and can utilise galactose, glucose, mannose, arabinose, xylose, fructose, rhamnose and lactose all of which are fully metabolised and assimilated.

Xie et al., 2007 and Lui, 2012 state that *C. hutchinsonii* can only utilise glucose and cellobiose, however it does contain genes that code for xylanases and pectic lyases but lacks genes that code for the transporters of xylose, arabinose and galacturonic acid, which are components of xylans and pectin respectively The presumption that xylanases are present, without the ability to use the components, is that the enzymatic digestion of hemicellulose such as xylans, is to unmask cellulose for digestion (Xie et al., 2007).

Bacillus subtilis can utilise galactose, glucose, mannose, arabinose, xylose, fructose, ribose, cellobiose, sucrose and maltose. *Saccharomyces cerevisiae* preferentially uses hexose sugars such as glucose and is unable to utilise arabinose and xylose (Barnett, 1976, Hsiao, 1981, Kotter, 1992, Sedlak, 2004) but can use galactose via the Leloir pathway and glucose, mannose and fructose via Embden–Meyerhof pathway / glycolysis (van Maris et al., 2006). *Saccharomyces* cerevisiae contains the genes that code for xylose reductase (which converts xylose to xylitol) and xylitol dehydrogenase (which converts xylitol to xylulose), so it can be utilised in the pentose phosphate pathway. In *S. cerevisiae*, xylitol dehydrogenase is active in the presence of xylose but xylose reductase is not active under any conditions regardless of carbon source (Toivari et al., 2004, Toivari et al., 2007). This means that *S. cerevisiae* can use xylitol for growth but cannot convert xylose into xylitol. Nor can it

utilise arabinose, even though xylose reductase would convert arabinose to arabitol were it active (Karhumaa et al., 2006). However, since several papers have reported they have modified *S. cerevisiae* to utilise arabitol (Toivari et al., 2007, Kordowska-Wiater, 2012) we can assume that wild type *S. cerevisiae* cannot utilise arabitol for growth regardless of whether xylose reductase is active or not. Both these factors point to *S. cerevisiae* being unable to utilise arabinose or xylose under any circumstances.

Escherichia coli can utilise glucose (Clark, 1989) mannose, arabinose, xylose and fructose (Dien, 1998, Luo et al., 2014) and rhamnose (Boronat, 1981, Baldoma, 1988).

4.3 Solvent tolerances

Certain micro-organisms naturally produce economically relevant substances, be they butanol (*Clostridium* spp.), ethanol (*S. cerevisiae* or *Z. mobilis*) or lactic acid (*Lactobacillus* spp.) so obviously, these micro-organisms have a natural tolerance to these chemicals (Inoue, 1989, Katahira et al., 2006). However, if other micro-organisms are to be modified to produce these chemicals then they will need to be able to tolerate an economically viable amount in their media (French, 2009), in the case of ethanol; this is 4% (Lau and Dale, 2009). *Bacillus subtilis* has been shown to tolerate up to 13% ethanol in its media (Sivagurunathan, 2013) and *S. cerevisiae* has similar tolerances, as evidenced by the percentage ethanol in most wines. *E. coli* has also shown tolerance to hexane and chlorohexane (Horikoshi, 2011) but *E. coli* JM109 has been shown to only tolerate between 2-4% ethanol and between 0.5 -1% butanol; there were no viable cells present. The growth of *E. coli* above 2% ethanol and 0.1% butanol was retarded (Fletcher, 2014). The tolerance of cellulose degrading micro-organisms is relatively unexplored but this knowledge is vital if this study is ever to be used in an industrial setting.

4.4 Methods:

4.4.1 Thin layer chromatography for micro-organism monosaccharide utilisation

To determine which monosaccharides were present at any stage of the experiment thin layer chromatography was used to give a clear profile. This analysis was done using one ml of growth medium from each flask that was removed and dried overnight in a rotary evaporator at 37^{o}C almost completely, leaving a syrup. This syrup was then resuspended in 100 µl 0.5% chlorobutanol and 3 µl loaded onto a silica thin layer chromatography plate and then run in the solvent mix ethyl acetate; pyridine; propanol; acetic acid; water (4:2:2:1:1) for three hours. The plate was then allowed to air dry overnight. Once all solvent had evaporated it was stained in thymol stain and then developed at 110^{o}C for 10 minutes and immediately scanned.

4.4.2 Plate screening for monosaccharide utilisation

100 µl of overnight liquid culture (OD>1.000) was spread onto minimal medium plates, giving an on-plate dilution of -1, (DSM3 *B. subtilis,* M9 *E. coli* and *C. fimi*, yeast nitrogen base *S. cerevisiae*) containing 0.5% galacturonic acid; galactose; glucose; mannose; arabinose; xylose and rhamnose. These plates were then incubated at 37^{o}C for 72 hrs and then photographed. Since *C. hutchinsonii* can only grow on plates if it is on filter paper, which it will use as the main carbon source, liquid cultures of *C. hutchinsonii* were grown in DSM3 containing 0.5% of each monosaccharide and the optical density of 1 ml used as a measure of whether *C. hutchinsonii* can use each monosaccharide as the main carbon source.

4.4.3 Microbial tolerance of chemicals of interest

Flasks containing up to 100 ml (total volume) of either LB, nutrient broth or YPD were inoculated with one colony of the appropriate micro-organisms (LB; *Bacillus* and *E. coli*; nutrient broth; *C. fimi*; YPD *S. cerevisiae*) and up to 10 ml (10%) of either lactic acid, ethanol or butanol, up to a total volume of 100ml. Optical density of 1 ml was measured every 24 hours for 10 days and on the 10th day, 100 µl of the culture was pipetted onto an appropriate medium plate to check that any cells present were still viable.

4.5 Results

4.5.1 Monosaccharide utilisation profiles of select micro-organisms

Since there is a range of potential monosaccharides to be released from the biomasses above, we need to know which of these sugars can be utilised by which micro-organism under the growth conditions utilised in this system.

4.5.2 Cytophaga hutchinsonii

A ten-day liquid growth trail was conducted for *C. hutchinsonii* in DSM3+YE with the seven main cell wall monosaccharides added as the main carbon source shown in figure 31.

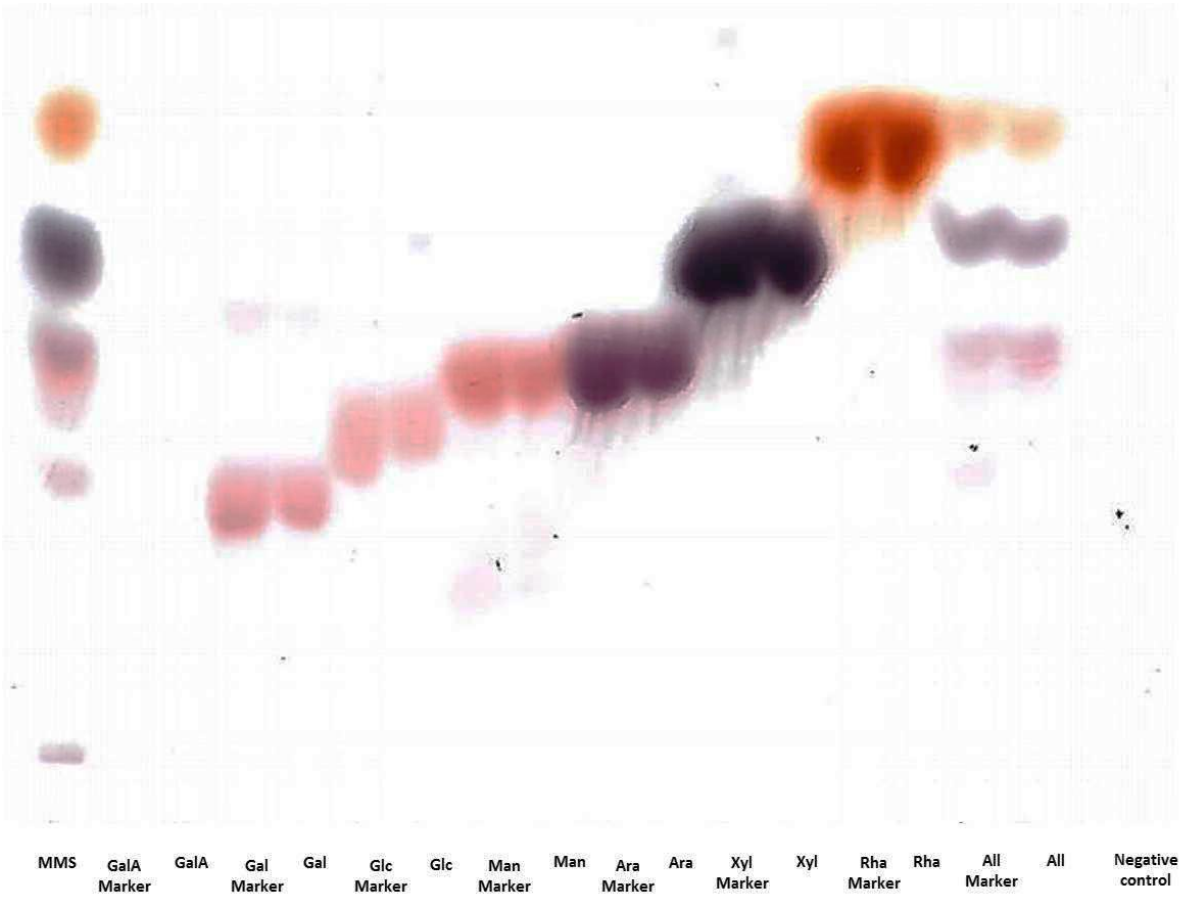


Figure 31 - TLC of liquid cultures of *C. hutchinsonii*, where the main carbon source was one of the seven main cell wall monosaccharides, 500 mg of each sugar added to 100 ml DSM3 media. Where all seven monosaccharides are present 72 mg of each sugar was added to 100 ml media. The monosaccharide marker lane is a 0-hour sample, taken before the flasks were inoculated. In this and following figures 1 ml of culture was taken after 10 days growth, condensed and run on a silica TLC plate and stained with thymol. The galacturonic acid (GalA) samples are missing, due to operator error. MMS = monosaccharide marker standard. Representative of one biological replicate.

Since *C. hutchinsonii* does not form colonies on agar, it is necessary to grow cultures on filter paper. These inoculated squares of filter paper are used to inoculate flasks with the bacteria; however it would appear that *C. hutchinsonii* will preferentially use this filter paper for growth rather than the monosaccharides present in the media. A better system to use in this case would be to grow up cultures of *C. hutchinsonii* to a high OD and then use the liquid culture to inoculate the flasks, thus reducing the amount of paper for growth, forcing the bacteria to use the sugars in solution. Time constraints prevented this approach. This could explain why there is no reduction in the intensity of spots after ten days growth in figure 31, when compared to the day zero amount (GalA marker, Gal marker etc.), but *C. hutchinsonii* can use glucose for growth (Liu, 2012).

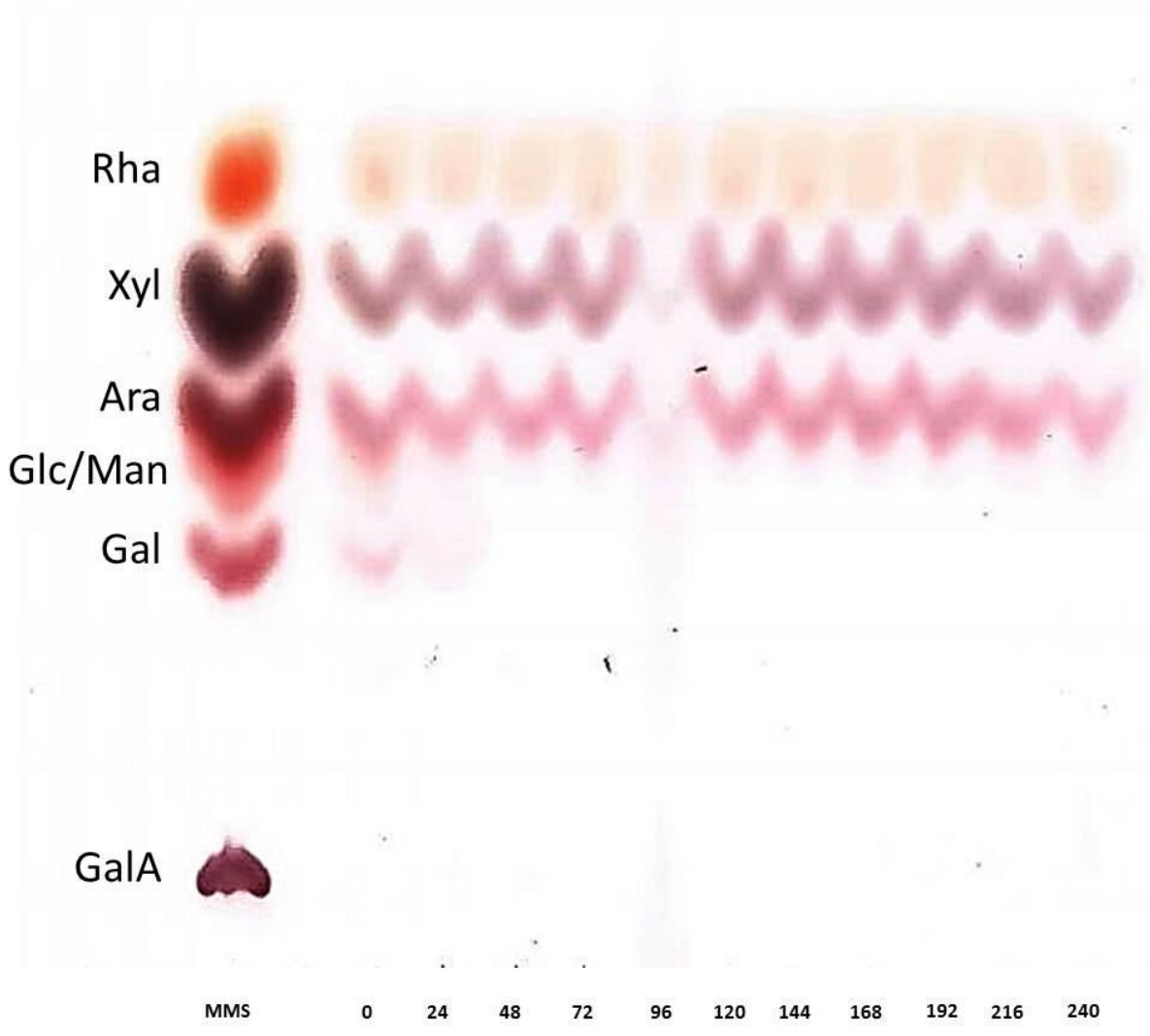


Figure 32 - TLC of liquid culture of *C. hutchinsonii*, where the seven cell wall monosaccharides were present in equal amounts (72 mg in 100 ml media). The 96-hour sample was burned during drying so is unreliable. MMS = monosaccharide marker standard. Representative of one biological replicates.

Interestingly when *C. hutchinsonii* is in media containing all seven cell wall monosaccharides, it appears to use under these growth conditions the galactose and glucose in solution. As figure 32 shows It appears that all (≈ 78 mg /4mM equivalent or 0.07%) of glucose is used up within 24 hours, followed by ≈ 78 mg (4mM/0.07%) galactose being used up by 48 hours. No other monosaccharides appear to be used due as there was no decrease in the intensity of the spots.

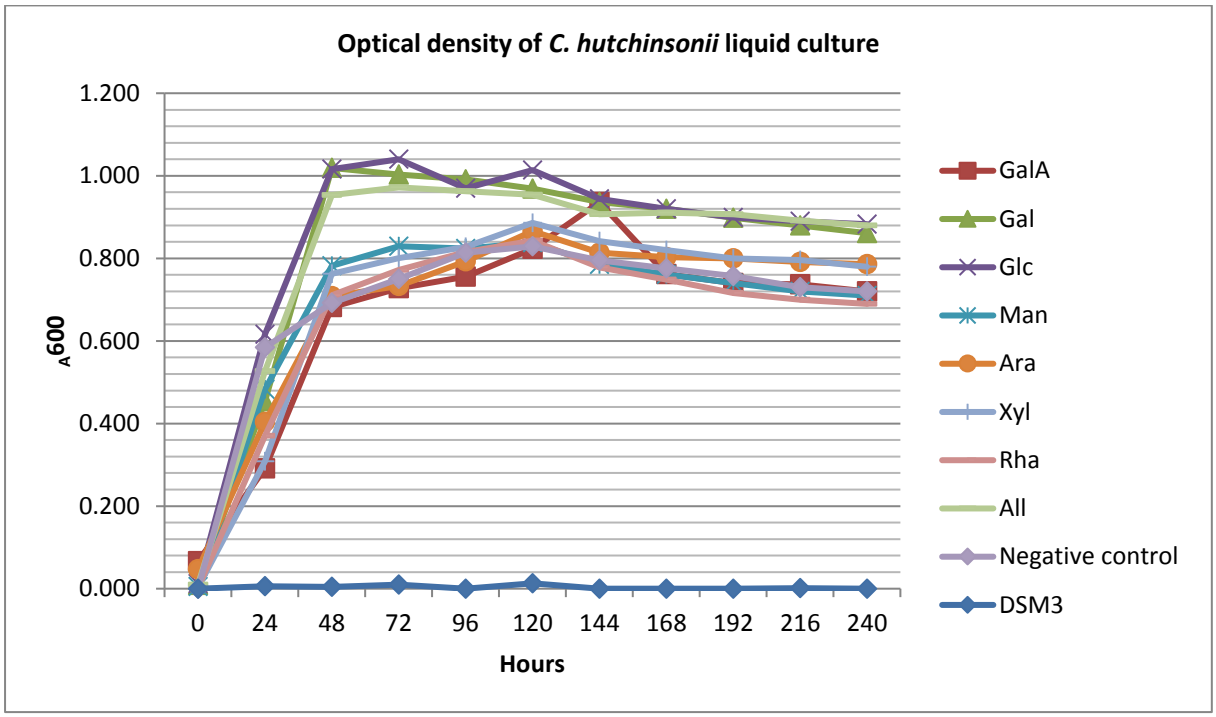


Figure 33 - Optical density of *C. hutchinsonii* liquid culture for growth utilising cell wall monosaccharides as the main carbon source 500 mg in 100 ml media and/or 72 mg of each sugar in 100 ml media. In this and following figures the negative control consists of DSM3+YE with no other carbon source and DSM3 is a cell free control. Representative of one biological replicate.

The only evidence of the *C. hutchinsonii* utilising the monosaccharides for growth come from cultures where the main carbon source was galactose, glucose or all the monosaccharides. As expected, figure 33 shows that *C. hutchinsonii* can readily use glucose for growth; this data also suggests it can use galactose for growth both as the single sugar and in mixture. The relatively high ODs come from the fact that the culture was inoculated with *C. hutchinsonii* on a square of filter paper, so it was most likely utilising that paper as the main carbon source which resulted in high background growth. The results for galactose utilisation are also verified by fig 32, as the galactose is taken up by 48 hours. The ability of *C. hutchinsonii* to use galactose as the main carbon source warrants further investigation, as it contradicts previous reports that *C. hutchinsonii* can only utilise glucose, cellobiose and cellulose (Xie et al., 2007, Liu, 2012).

4.5.3 Cellulomonas fimi

A ten-day liquid growth trial was conducted for *C. fimi* in DSM3+YE with the seven main cell wall monosaccharides added as the main carbon source.

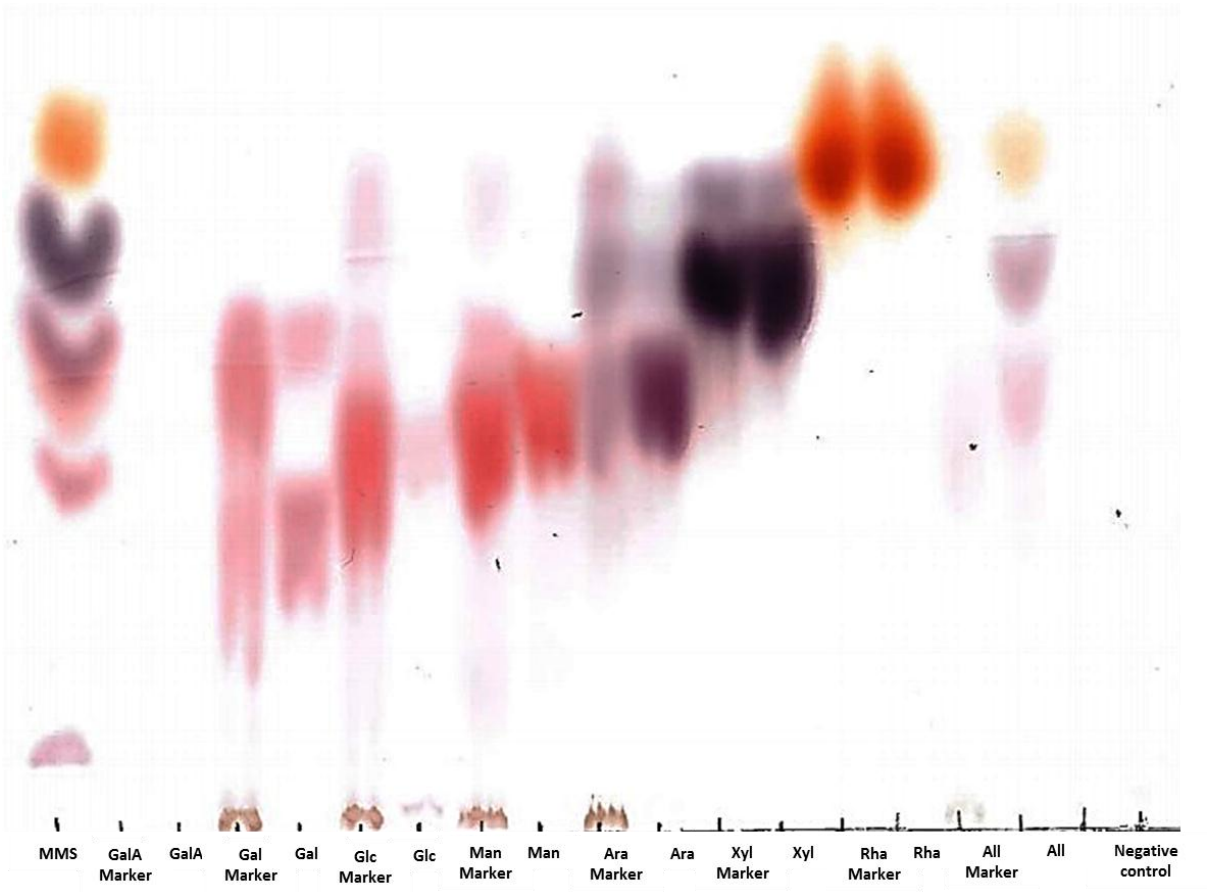


Figure 34 – Thin layer chromatogram of the growth medium of liquid culture of *C. fimi* with individual monosaccharides as the main carbon source. The monosaccharide marker lane is a 0-hour sample, taken before the flasks were inoculated. Where single cell wall monosaccharides as the main carbon source 500 mg in 100 ml media and/or 72 mg of each sugar in 100 ml media when all sugar are present. There appears to have been an error when loading the GalA lanes on to the TLC plate, as both spots are missing. MMS = monosaccharide marker standard. Representative of one biological replicate.

In figure 34, there is a decrease in the intensity of the glucose spot when compared to its control. This indicates that *C. fimi* is able to utilise glucose as the main carbon source, but after ten days has used less than 500 mg (28mM equivalent or 0.5%). There is no decrease in any other monosaccharides indicating that they were not taken up by *C. fimi*.

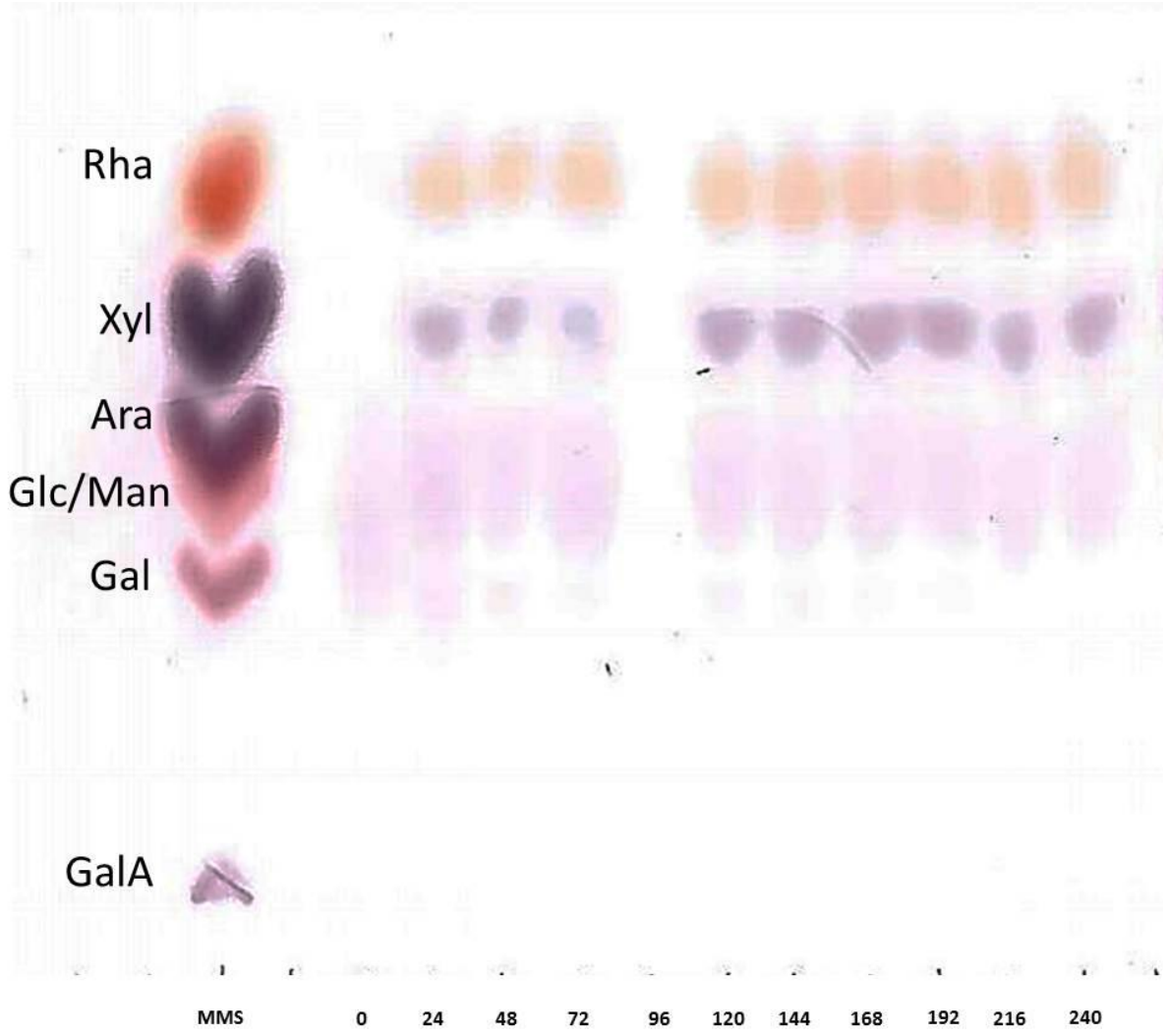


Figure 35 - TLC of *C. fimi* liquid culture where monosaccharides were all present as 72 mg of each sugar in 100 ml media. The 96-hour sample is missing because the sample burned when drying. MMS = monosaccharide marker standard. Representative of one biological replicate.

As figures 34 and 35 show *C. fimi* is only able to utilise glucose for growth as only the glucose is missing from the TLC after ten days, both as the main carbon source and when a mixture of all seven common cell wall monosaccharides are present in equal amounts (4mM equivalent of each monosaccharide).

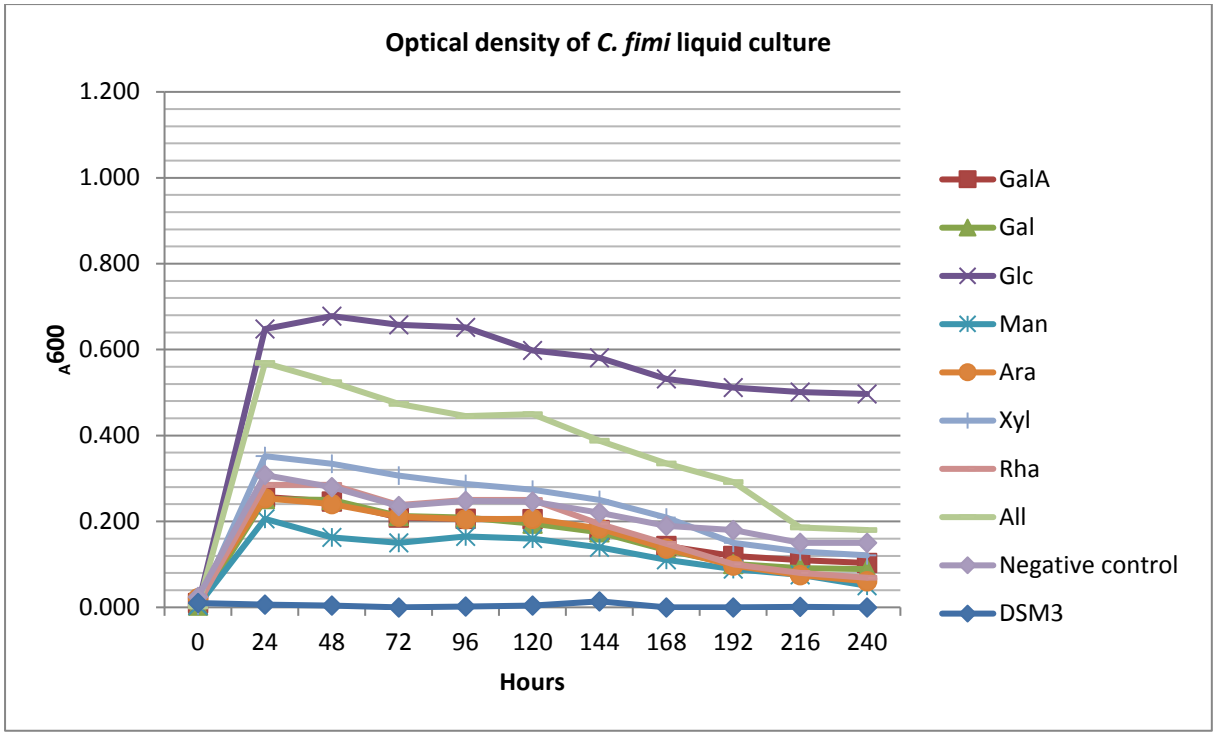


Figure 36 - Optical density of *C. fimi* liquid culture liquid culture for growth utilising cell wall monosaccharides as the main carbon source 500 mg in 100 ml media and/or 72 mg of each sugar in 100 ml media. In this and following figures the negative control consists of DSM3+YE with no other carbon source and DSM3 is a cell free control. Representative of one biological replicate.

Figure 36 suggests that under these growth conditions, *C. fimi* can only use glucose for growth as evidenced by the higher OD. Growth in cultures with all monosaccharides is attributed to the presence of glucose in the mix. However, it is known that *C. fimi* can utilise mannose, arabinose, xylose, rhamnose, fructose and lactose for growth under different growth conditions (Kane, 2014). This warrants further investigation to determine why *C. fimi* failed these sugars under the conditions used in this study, since effective assimilation of all these sugars will be required for efficient utilisation of biomass hydrolysates.

4.5.4 Bacillus subtilis 168

A ten-day liquid growth trail was conducted for *B. subtilis* 168 in DSM3+YE with the seven main cell wall monosaccharides added as the main carbon source.

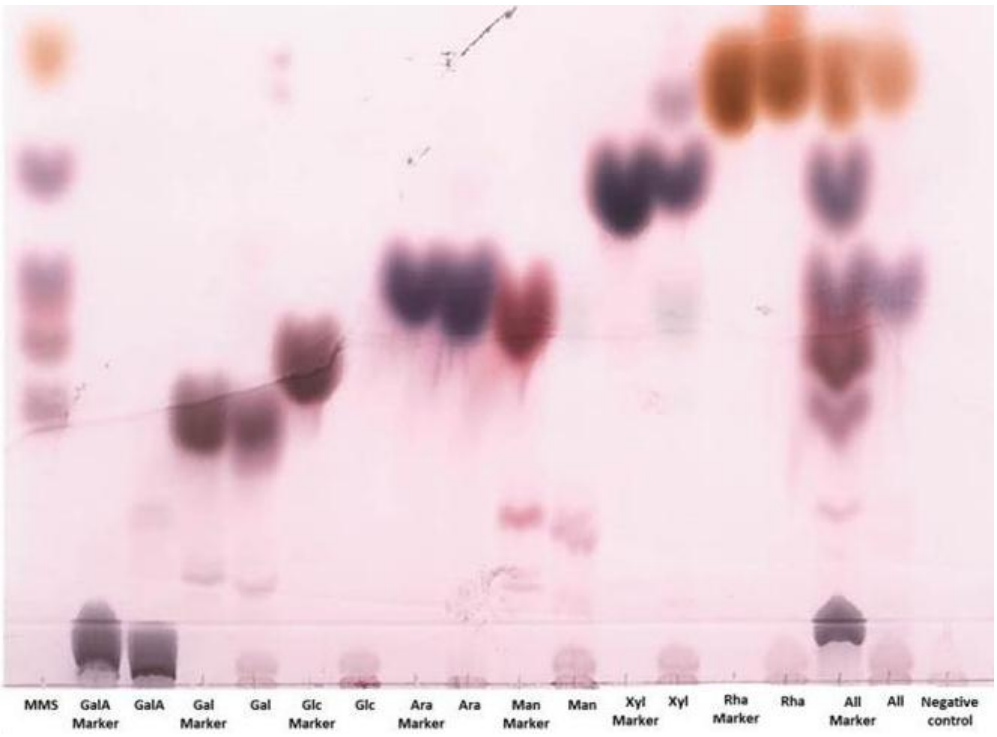


Figure 37 - *Bacillus subtilis* 168 monosaccharide utilisation of single monosaccharides in liquid culture, when *B. subtilis* is grown in DSM3+YE with one of the seven main cell wall polymer constituent monosaccharides 500mg per 100 ml media. The monosaccharide marker lane is a 0-hour sample, taken before the flasks were inoculated. Representative of two biological replicates. MMS = monosaccharide marker standard.

Figure 37 shows that *Bacillus* subtilis 168 is able to use the totality of 0.5% (5 mg per ml / 28mM equivalent) of glucose and mannose for growth over a ten day period as evidenced by the lack of spot after ten days, indicating *B. subtilis* has completely consumed the glucose and mannose when they are the sole carbon source, as predicted in the literature (Sulke, 2000) and supported by the optical density for the mannose containing culture in figure 37. *B. subtilis* 168 is also able to take up some (<5 mg/ ml) of galactose and xylose, as evidenced by the reduction in the intensity of the spot compared to the marker spots, which equate to the same concentration of sugar. The galacturonic acid, arabinose and rhamnose have not been utilised and are present in the same concentrations as at the start.

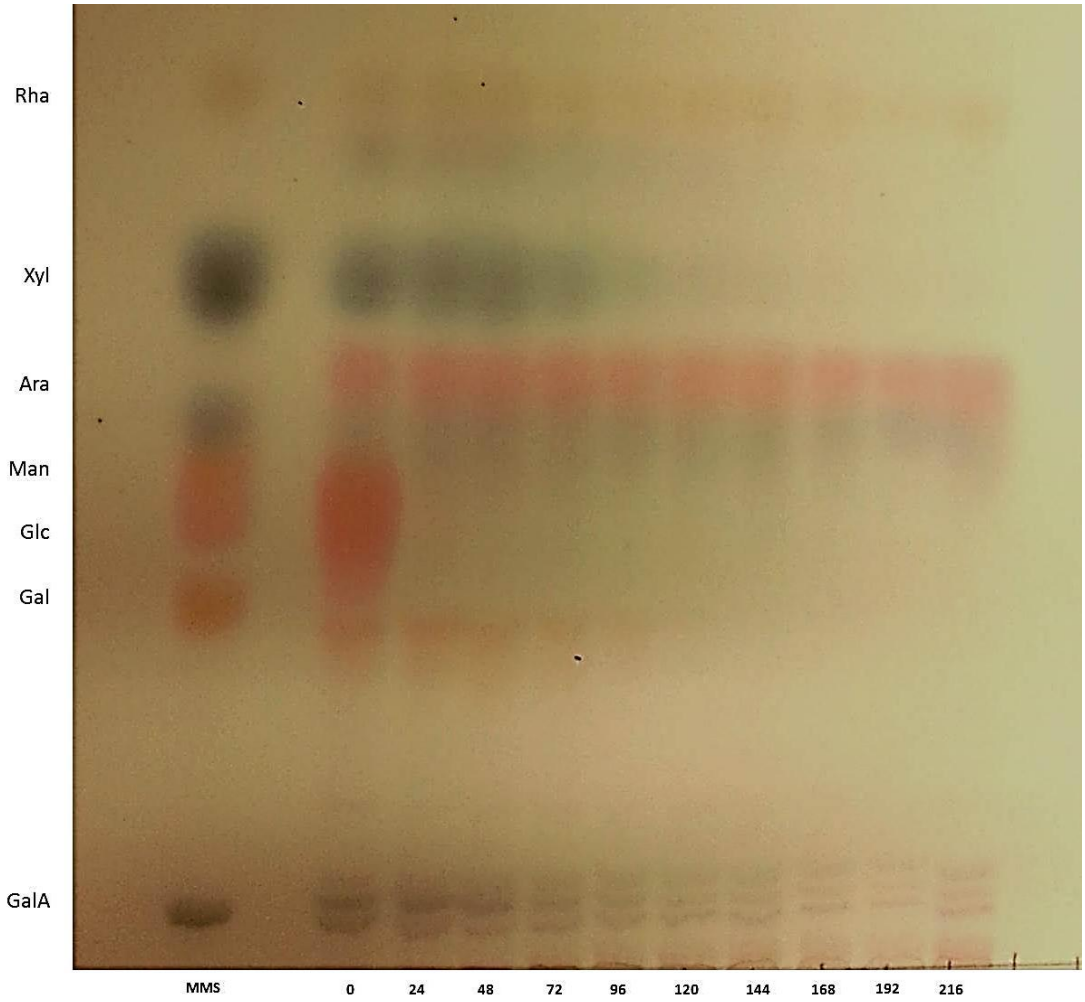


Figure 38 - *B. subtilis* 168 monosaccharide utilisation of mixed monosaccharides in liquid culture with 72 mg of each sugar per 100 ml media. MMS = monosaccharide marker standard. Representative of two biological replicates (see appendix).

As figure 38 shows, when all seven monosaccharides are present in the growth medium in equal amounts, equating to 500 mg in total, *B. subtilis* is able to utilise all of them apart from arabinose and rhamnose. Glucose is used by 24 h, mannose is used up by 48 h, xylose is used up by 96 h and galactose is used up by 120 h. When these monosaccharides are present together in liquid culture at a concentration of 0.07% (0.7 mg per ml / 4mM) in DSM3+YE the pattern of monosaccharide usage differs from that when a single monosaccharide is the main carbon source. This range of monosaccharides is closer to what we would expect in the growth media when lignocellulosic biomass is saccharified (25 and 28). The first monosaccharide to be utilised is glucose which is used up in the first 24 h, then mannose is used up by 48 h, xylose is used up by 96 h and galactose is used up by 120 h. There also appears to be a reduction in the intensity of the galacturonic acid spot after 96 h but there appears to be no reduction in the arabinose or rhamnose spots.

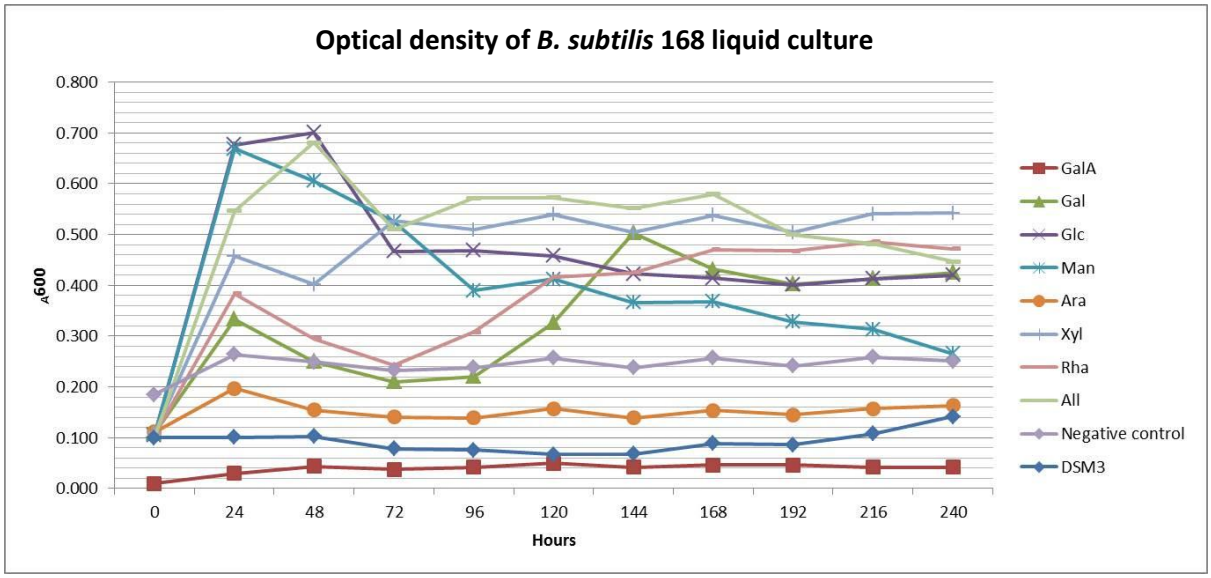


Figure 39 - Optical density of *B. subtilis* 168 liquid culture utilising one or all the common cell wall polymer monosaccharides as the main carbon source, error bars are too small to be seen but show standard error. DSM3 is the cell free control. Representative of two biological replicates.

The optical density of *B. subtilis* cultures are shown in figure 39, Xylose and rhamnose give growth better than the glucose "positive" control from 72 hours and galactose give a comparable optical density after 144 hours. Culture containing mannose gives an optical density lower than the positive control but higher than the negative control and interestingly the cultures containing arabinose and galacturonic acid has an optical density lower than the negative control.

4.5.5 *Escherichia coli*

Due to time constraints, a different approach was needed for *E. coli*. A simple growth trial to determine whether *E. coli* could utilise the seven main cell wall monosaccharides was conducted by plating out a $1x10^1$ sample of *E. coli* was plated onto M9 agar plates to see which plates colonies could grow on (data not shown). *E. coli* JM 109 can utilise all monosaccharides apart from galacturonic acid. This is not due to the acidity on the sugar as the *E. coli* was grown on neutralised M9 plates. It seems that *E. coli* can use all the monosaccharides as stated in the literature (Boronat, 1981, Baldoma, 1988, Clark, 1989, Dien, 1998, Luo et al., 2014).

4.5.6 *Saccharomyces cerevisiae*

A simple growth trial to determine whether *S. cerevisiae* could utilise the seven main cell wall monosaccharides was conducted by a ten-day liquid growth trial in 1% yeast nitrogen base with the seven main cell wall monosaccharides added as the main carbons source.

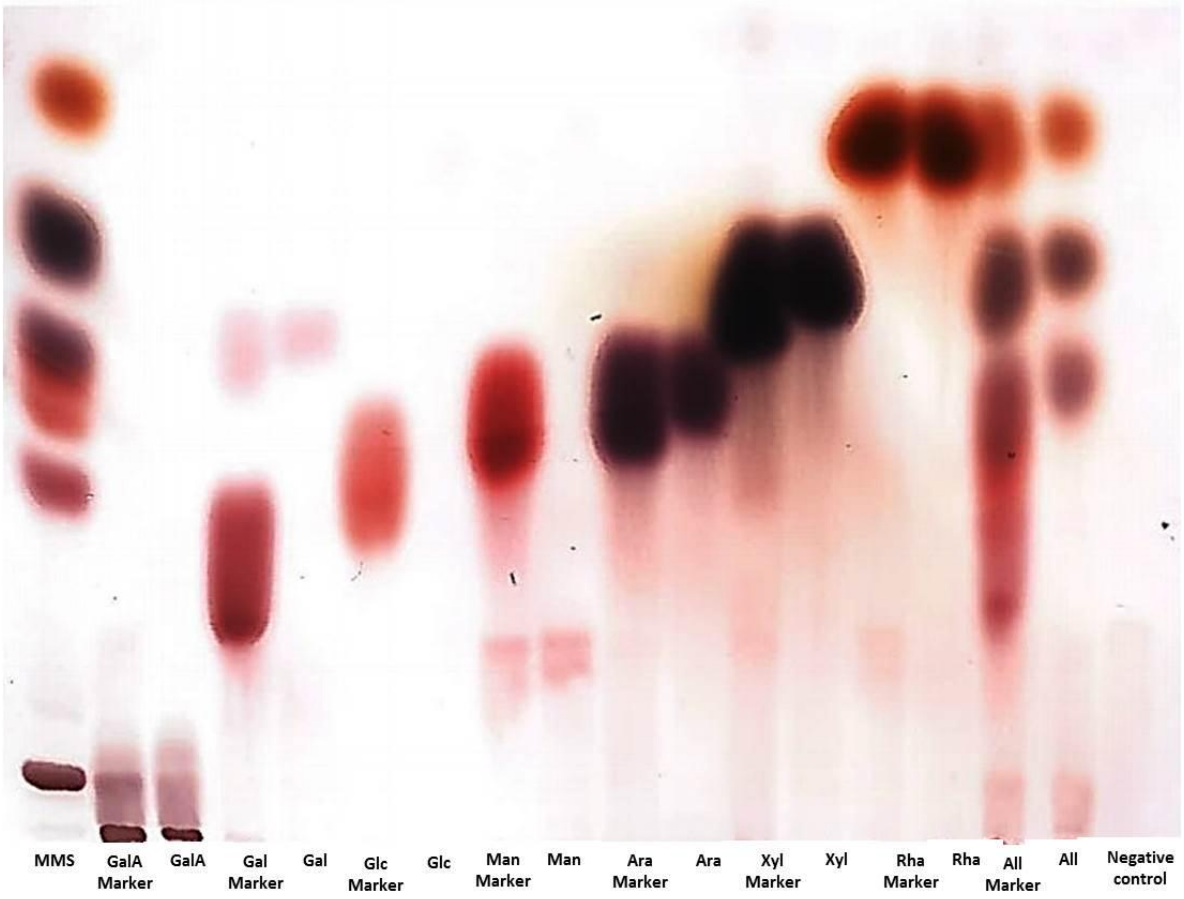


Figure 40 - *S. cerevisiae* monosaccharide use in ten-day liquid growth trial. Where single cell wall monosaccharides as the main carbon source 500 mg in 100 ml media and/or 72 mg of each sugar in 100 ml media when all sugar are present.

The monosaccharide marker lane is a 0-hour sample, taken before the flasks were inoculated. MMS = monosaccharide marker standard. Representative of one biological replicate.

As figure 40 shows *S. cerevisiae* is able to take up galactose, glucose and mannose. This is demonstrated by the lack of these monosaccharide spots compared to their controls after ten days' growth. *S. cerevisiae* has taken up 500 mg (0.5%/28mM) of these monosaccharides for growth over ten days. There seems to be a slight reduction in the arabinose spot when compared to its zero-hour control spot. There is no reduction in the xylose spot.

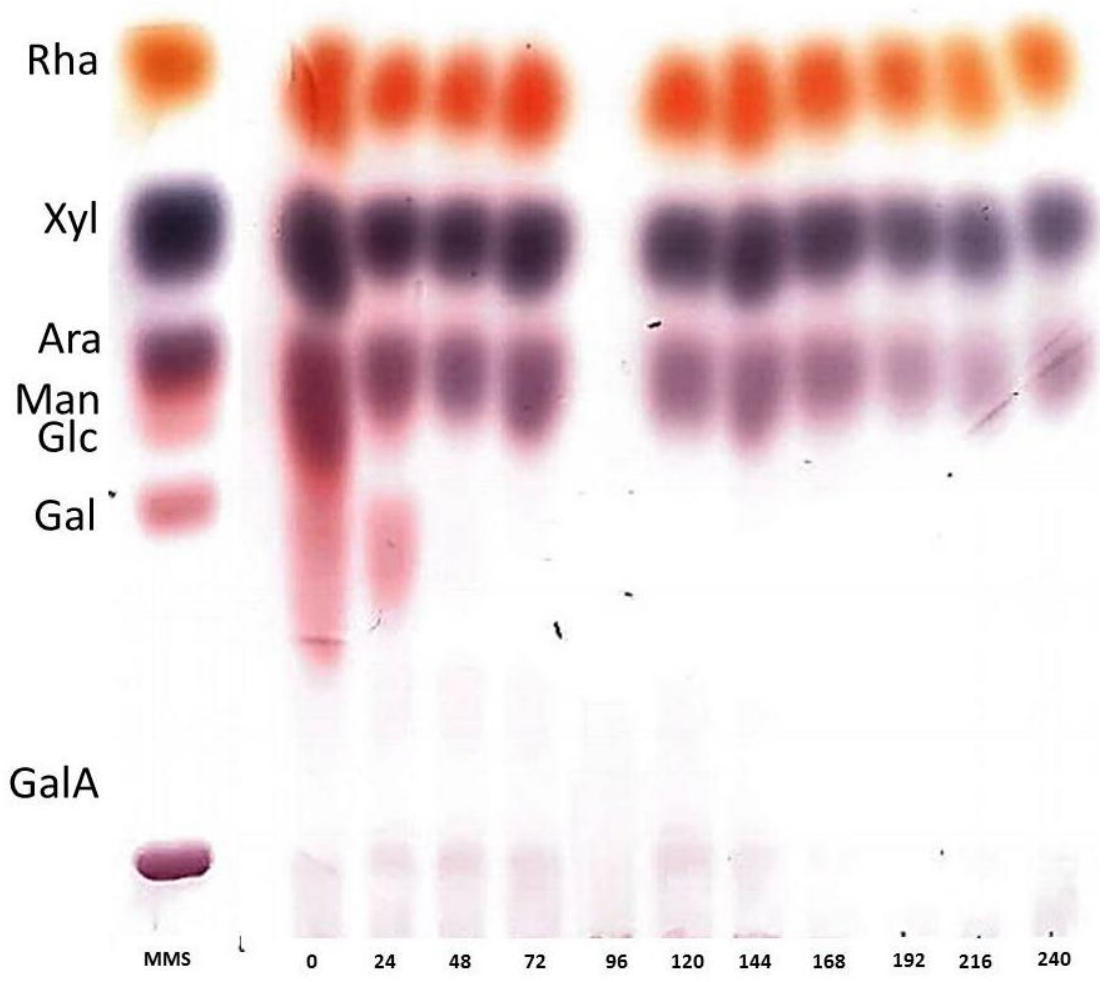


Figure 41 - *S. cerevisiae* ten-day liquid cultures when all cell wall monosaccharides are present with 72 mg of each sugar per 100 ml media. The 96-hour sample are missing because the sample burned when drying. MMS = monosaccharide marker standard. Representative of one biological replicate.

Figure 41 shows that *S. cerevisiae* utilises glucose preferentially over other monosaccharides in a mixed carbon source and then utilises galactose and then mannose, as the glucose is used up by 24 hours, the galactose by 48 hours and the mannose by 120 hours. There is also a reduction in the intensity of the arabinose spot after 196 hours. The reduction in the amount of arabinose shown on

the TLCs indicated that the arabinose and xylose were taken into the cell, as *S. cerevisiae* has the metabolism to do so.

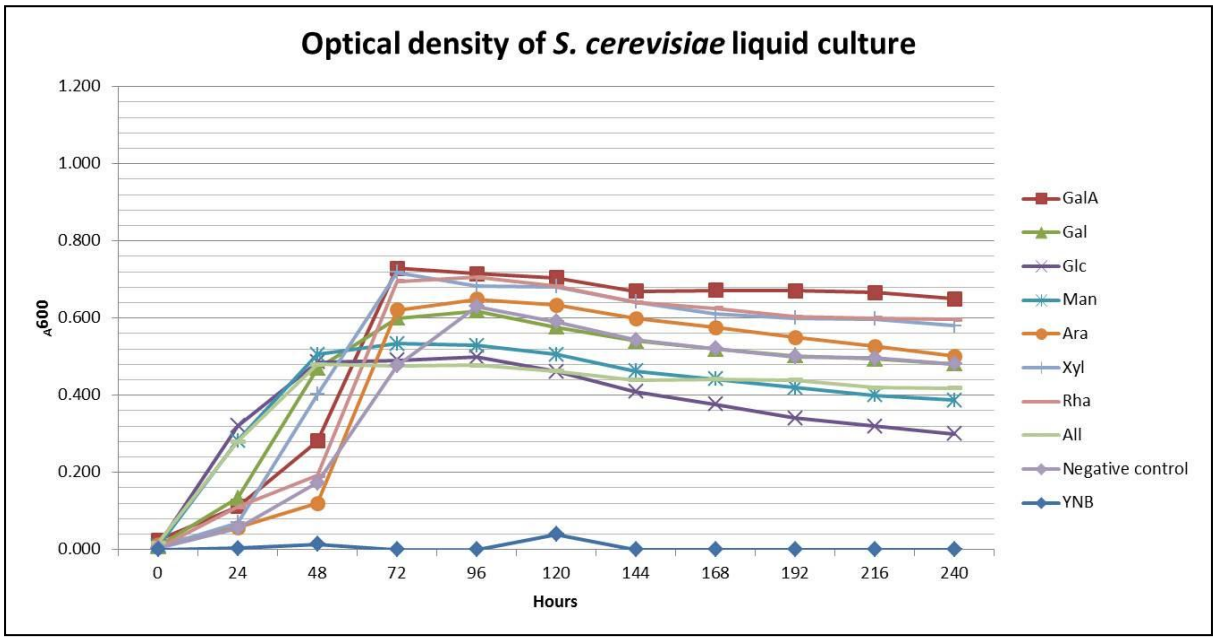
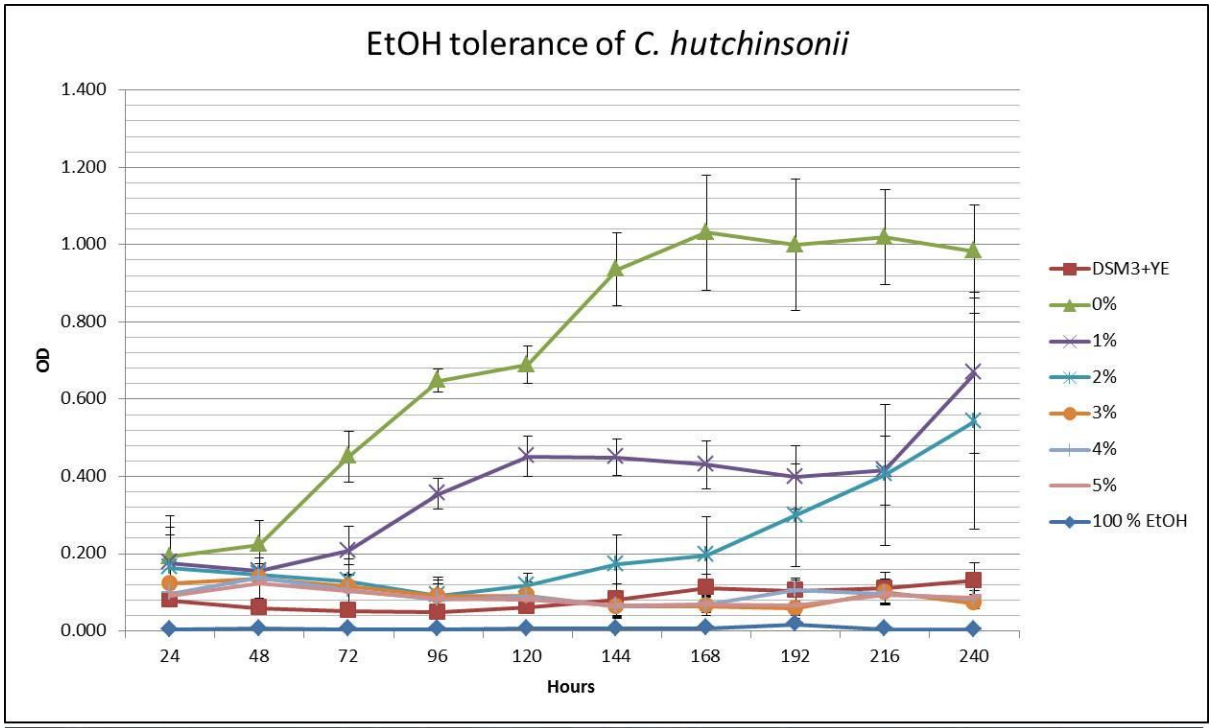


Figure 42 - Optical density of *S. cerevisiae* liquid culture utilising one or all the common cell wall polymer monosaccharides as the main carbon source 500 mg in 100 ml media for single sugar cultures and/or 72 mg of each sugar in 100 ml media. YNB is a cell free control. Representative of one biological replicate.

Figure 42 shows the optical density of S. cerevisiae liquid cultures where one of the seven main monosaccharides is the carbon source. This result would indicate that cultures where galacturonic acid had the highest rates of growth and that cultures where glucose is the carbon source had the lowest growth. This seems very unlikely and these results are probably not valid. Replicates are needed to verify these results.

4.5.7 Microbial tolerance of economically interesting substances within a liquid growth media and their effects on growth

It is important to note that certain chemicals, particularly economically important ones are also fatal to micro-organisms at a certain level. Therefore, to determine which substances were feasible to produce, the tolerance of the micro-organisms in co-culture to these substances needed to be determined and whether that tolerance was to economically viable amounts.



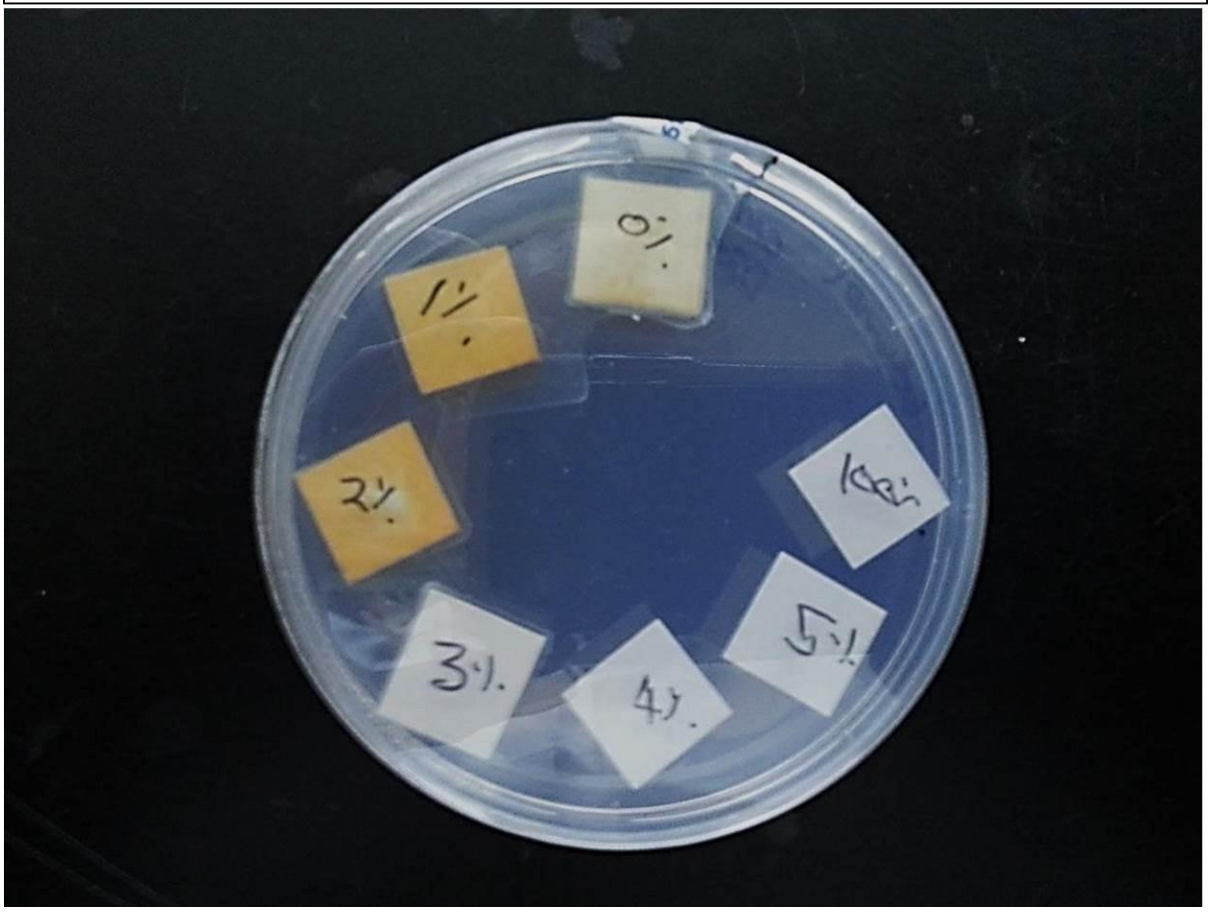


Figure 43 - Tolerance of *C. hutchinsonii* to specific percentages of ethanol in their liquid growth media. The specified percentages of ethanol were added to 100 ml DSM3 before being inoculated. After ten days 100 µl of culture was added to squares of filter paper on DMS3 agar and allowed to grow to determine the presence of viable cells. Representative of three biological replicates.

Figure 43 shows that *C. hutchinsonii* can survive with up to 2% (v/v) of ethanol in its growth medium as demonstrated by the optical density and live cells being able to colonise filter paper on DSM3+YE agar plates. Above 2% there was no increase in optical density nor were the aliquots from the media of this percentage and above able to colonise filter paper on agar plates (fig 42).

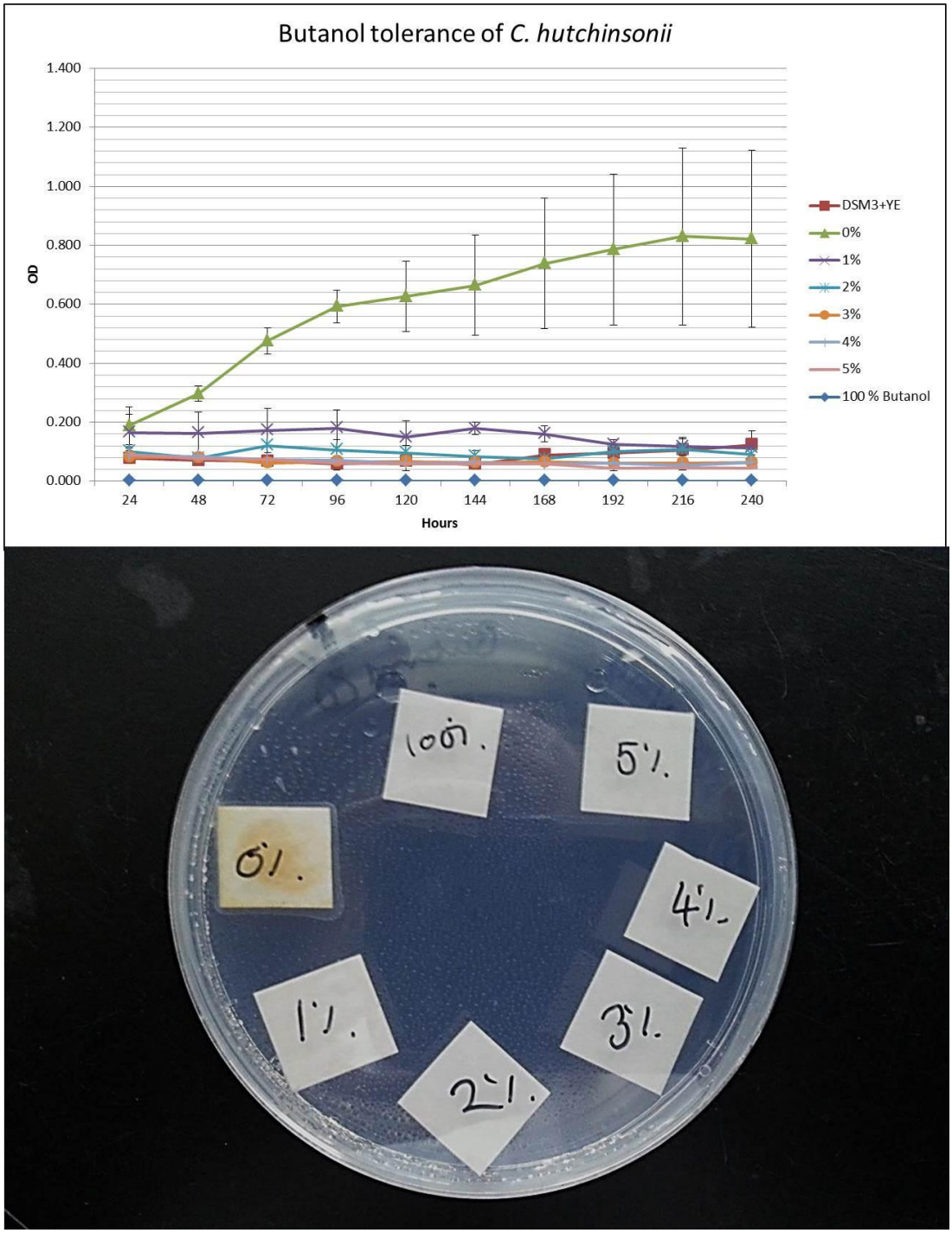


Figure 44 - Tolerance of *C. hutchinsonii* to specific percentages of butanol in their liquid growth media. The specified percentages of butanol were added to 100 ml DSM3 before being inoculated. After ten days 100 µl of culture was added to squares of filter paper on DMS3 agar and allowed to grow to determine the presence of viable cells. Representative of three biological replicates.

Figure 44 demonstrates that *C. hutchinsonii* is unable to tolerate any butanol (>= 1% v/v) in its growth medium, shown by no increase of the optical density of cultures containing >0% butanol (v/v) and only samples from the culture containing no butanol being able to colonise filter paper on agar plates. A wider range of butanol concentrations i.e. 0.1%, 0.2% etc., should be examined.

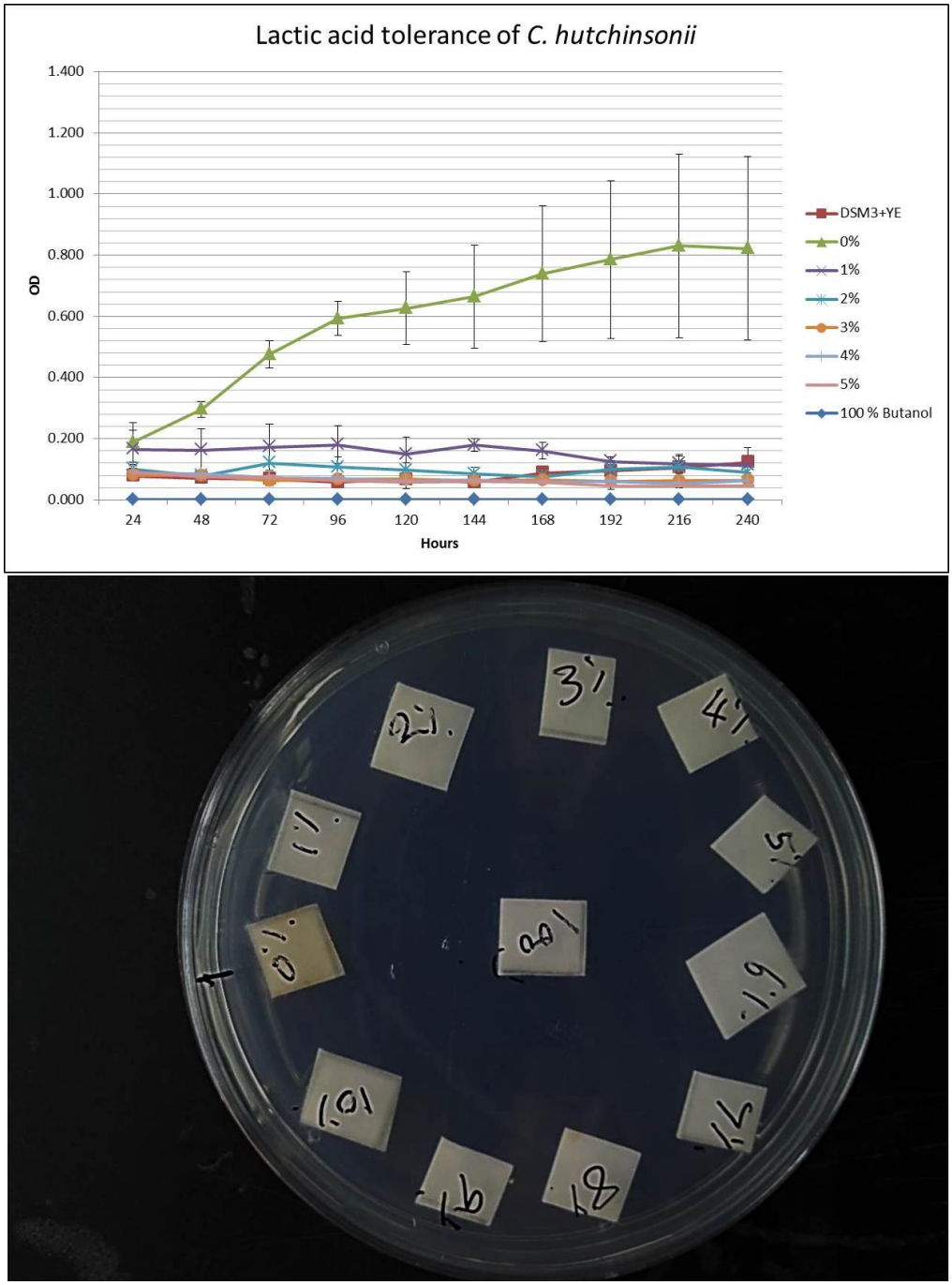


Figure 45 - Tolerance of *C. hutchinsonii* to specific percentages of lactic acid in their liquid growth media. The specified percentages of lactic acid were added to 100 ml DSM3 before being inoculated. After ten days 100 µl of culture was added to squares of filter paper on DMS3 agar and allowed to grow to determine the presence of viable cells. Representative of three biological replicates.

Figure 45 shows that *C. hutchinsonii* is unable to grow with the presence of lactic acid in its growth media demonstrated by the optical density of the cultures over ten days and the inability for samples of these cultures to colonise filter paper on agar plates. This is most likely due to the lactic acid lowering the pH to approximately pH 2-3; as DSM3 is a poorly buffered medium and we were unable to find a better buffered medium that *C. hutchinsonii* could grow in, during this study.

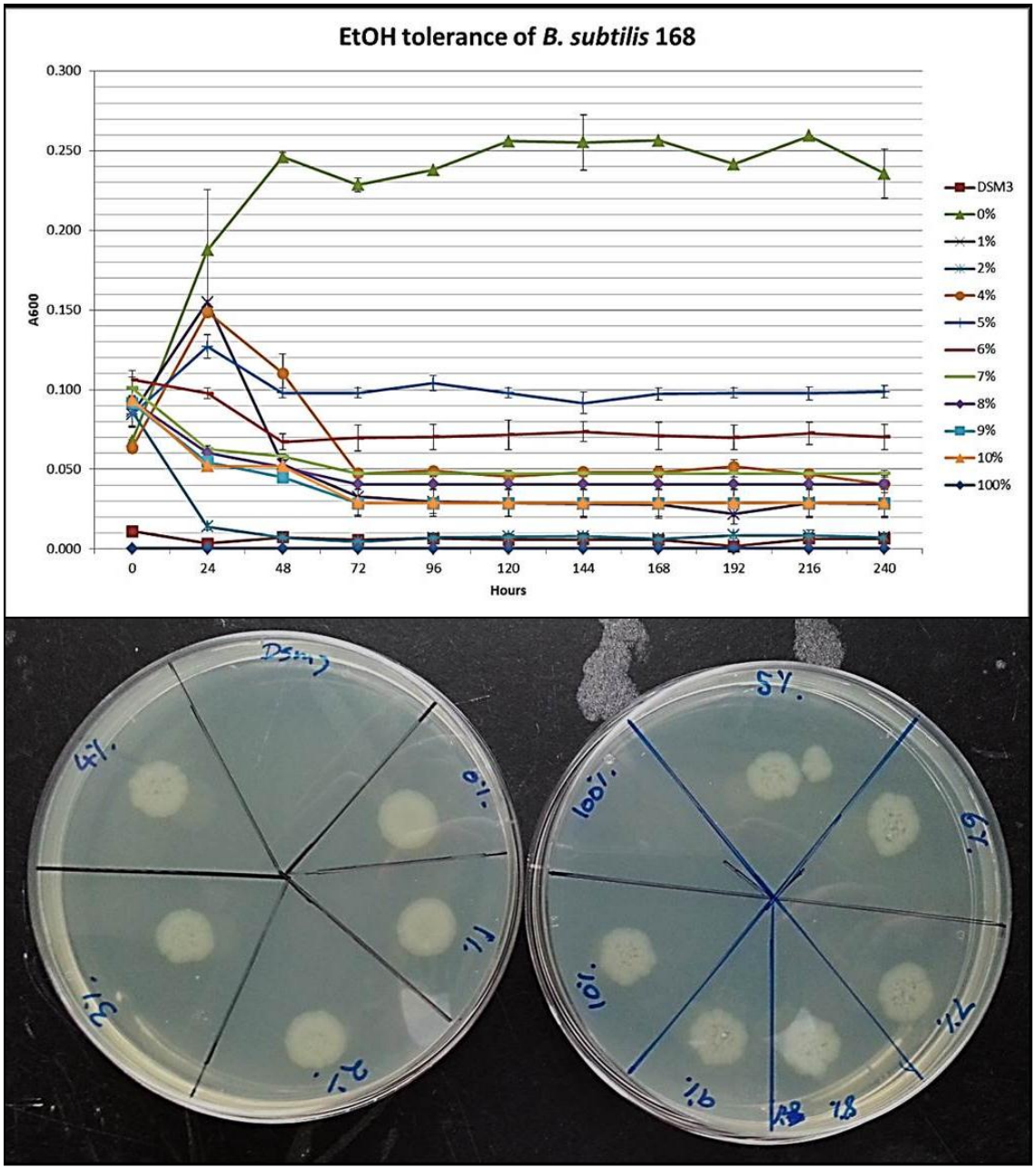


Figure 46 - Tolerance of *B. subtilis* 168 to specific percentages of ethanol in their liquid growth media. The specified percentages of ethanol were added to 100 ml DSM3 before being inoculated. After ten days 10 µl of culture was added to LB agar and allowed to grow to determine the presence of viable cells. Representative of three biological replicates.

Figure 46 shows that *B. subtilis 168* can survive with up to 10% ethanol (v/v) in DSM3+YE growth medium, as aliquots of these cultures could form colonies on agar plates; however actual growth of *B. subtilis 168* is impeded by levels >=1%(v/v), as demonstrated by the optical density readings (fig 45) which suggests that the *B. subtilis* was surviving as spores.

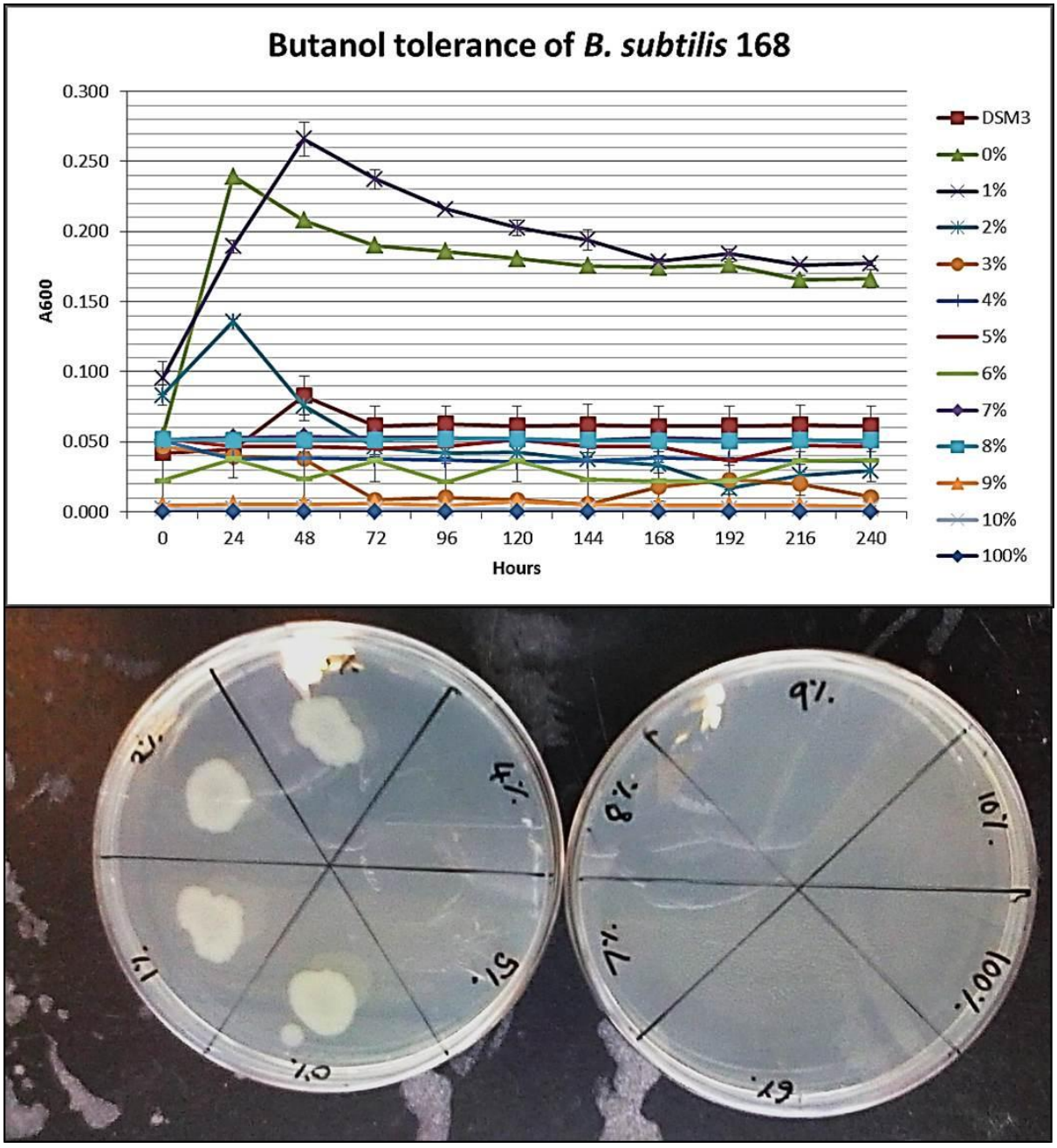


Figure 47 - Tolerance of *B. subtilis* 168 to specific percentages of butanol in their liquid growth media. The specified percentages of butanol were added to 100 ml DSM3 before being inoculated. After ten days 10 µl of culture was added to LB agar and allowed to grow to determine the presence of viable cells. Representative of three biological replicates.

Figure 47 shows that *B. subtilis 168* can survive with up to 3% butanol (v/v) in its growth media, again probably surviving as spores as opposed to vegetative cells as growth, denoted by optical densities, is impeded at 2%.

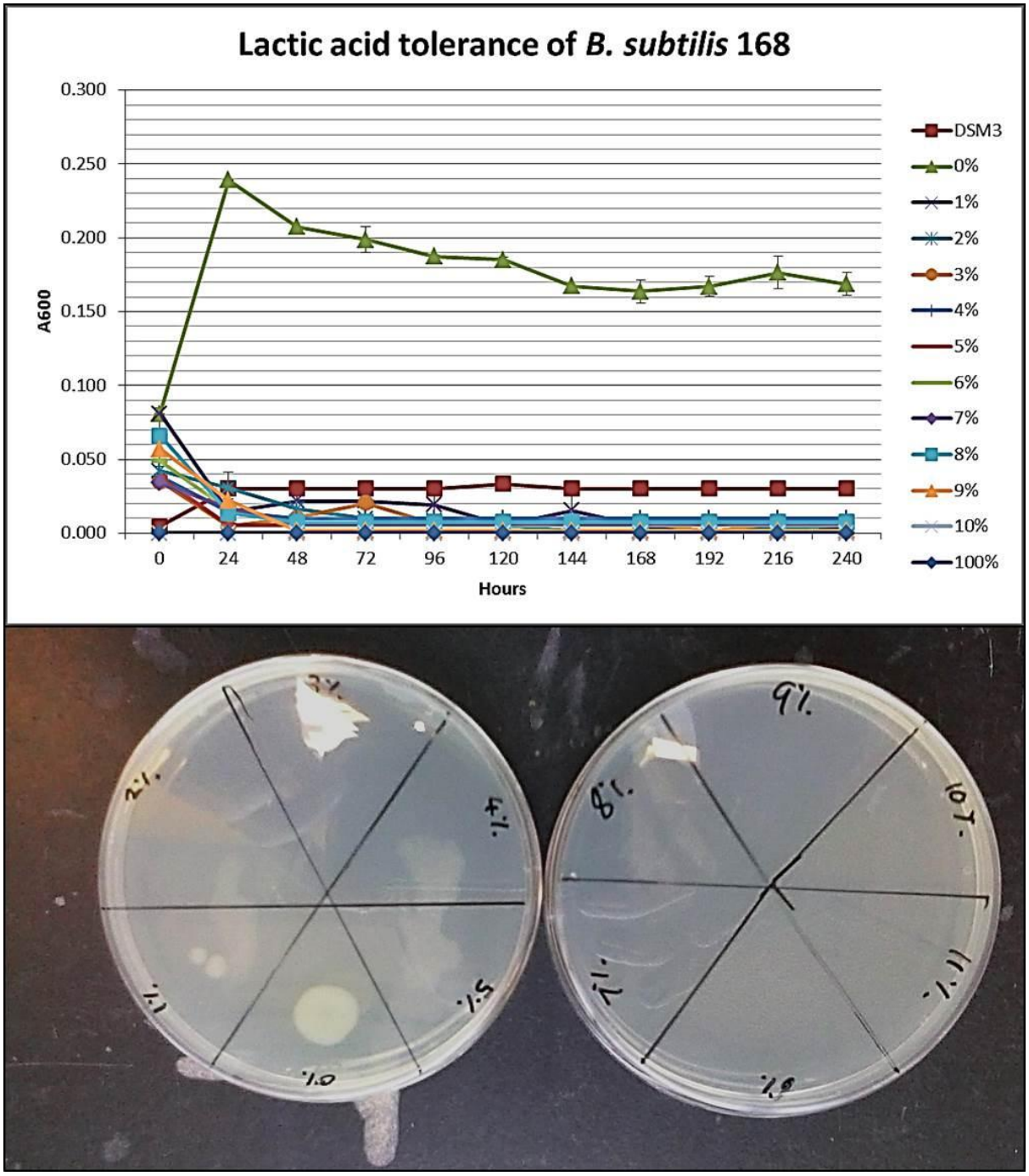


Figure 48 - Tolerance of *B. subtilis* 168 to specific percentages of lactic acid in their liquid growth media. The specified percentages of lactic acid were added to 100 ml DSM3 before being inoculated. After ten days 10 µl of culture was added to LB agar and allowed to grow to determine the presence of viable cells. Representative of three biological replicates.

Figure 48 *B. subtilis 168* grown in DSM3+YE cannot grow in the presence of lactic acid in the media, but this is due to drop in pH (from pH7 to pH 3.2) caused by the lactic acid as DSM3+YE is a poorly buffered medium. *B. subtilis 168* can grow in the presence of lactic acid in a better buffered medium M9 (data not shown), as the pH remains at pH 7. However, since this study is looking at the co-culture *of B. subtilis* 168 and *C. hutchinsonii*, this avenue was not pursued as *C. hutchinsonii* cannot grow in M9. Since there is no actual growth above certain levels of the chemicals listed above as demonstrated by the optical densities of the cultures (fig 47), it is likely that the *Bacillus* was surviving as spores rather than vegetative cells.

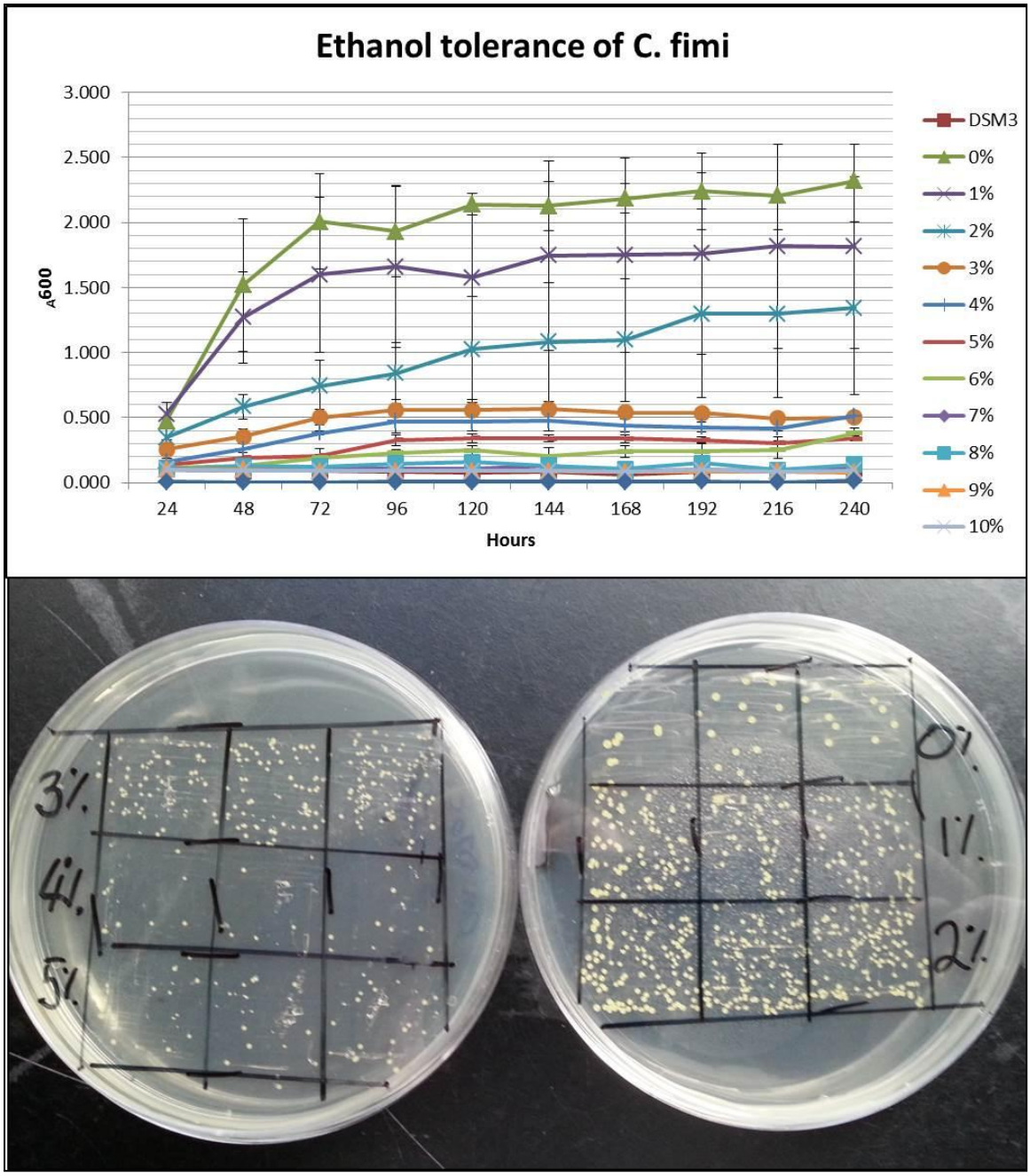


Figure 49 - Tolerance of *C. fimi* to specific percentages of ethanol in their liquid growth media. The specified percentages of ethanol were added to 100 ml DSM3 before being inoculated. After ten days 10 µl of culture was added to LB agar and allowed to grow to determine the presence of viable cells. Representative of three biological replicates.

Figure 49 shows that *C. fimi* can survive with up to 5% (v/v) ethanol in its growth medium, as shown as colonies on agar plates (figure 49) however actual growth is retarded after 2%, when optical density of the cultures is used as a measure of growth. Since only mixed cultures of *C. fimi* and *S. cerevisiae* showed any promise of success, unlike *C. hutchinsonii* and *B. subtilis,* tolerance of butanol and lactic acid by *C. fimi* was not explored, due to time constraints and the fact that *S. cerevisiae* naturally produces ethanol.

4.6 Short discussion and summary

4.6.1 Monosaccharide utilisation

Under the growth conditions used in this study, it was determined that *C. hutchinsonii* could utilise galactose and glucose for growth and that *C. fimi* was only able to utilise glucose for growth, which runs counter to the literature. A more robust method of determining which monosaccharides *C. hutchinsonii* can use is needed, possibly by inoculating flasks with an aliquot of liquid culture rather than an inoculated piece of filter paper, as the *C. hutchinsonii* probably utilised the cellulose in the filter paper for growth rather than the monosaccharides present in its growth medium. That *C. fimi* only utilised glucose for growth is very surprising, but since both monosaccharide utilisation growth trials were only done once, replicates are needed before any real conclusion can be drawn.

Replicates were possible in the time frame for the *B. subtilis* 168 monosaccharide growth trials and it was established that *B. subtilis* 168 can utilise ≈ 28mM glucose and mannose when they are the sole carbon source. *B. subtilis* 168 is also able to take up some (< 28mM) of galactose and xylose when a mixture of all seven monosaccharides were present in equal amounts in its growth medium.

Under these growth conditions, *E. coli* was able to utilise six of the seven monosaccharides, galacturonic acid was not utilised, which is what was predicted in the literature. Replicates are needed to verify these results and to generate robust and useable data.

Saccharomyces cerevisiae was able to utilise ≈28mM of galactose, glucose and mannose both as individual monosaccharides and when all seven monosaccharides were present in equal amounts in the growth medium. There was also a reduction in the amount of arabinose and xylose on the TLC plates, which indicates that some amount of these monosaccharides were taken into the cell, but not utilised for growth. The optical densities for the *S. cerevisiae* growth trials are extremely unusual and replicates are needed to verify them.

4.6.2 Solvents

Utilising the growth medium set out in this study, it was found that *C. hutchinsonii* can tolerate 2% ethanol (v/v) in its growth medium but no butanol or lactic acid, the latter most likely to the change in pH. *C. fimi* is able to tolerate up to 5% ethanol (v/v) in its growth medium, but active growth is suppressed at concentrations above 2% demonstrated by optical density.

In DSM3+YE *B. subtilis* 168 is able to survive as spores up to 10% (v/v) ethanol but vegetative growth is reduced above 2%. *B. subtilis* 168 can also survive as spore when 3% butanol (v/v) is present in its growth medium, but actual growth is retarded above 2% (v/v). *B. subtilis* 168 is unable to tolerate

any lactic acid in its growth medium under these conditions, which would seem to indicate that this system, utilising the media as described, under the growth conditions described, would not be suitable for the practical production of biofuels.

5 Verification of viable co-cultures of cellulose degrading and chassis micro-organisms in liquid minimal medium

5.1 Background:

5.1.1 Mixed cultures

There have been several studies conducted into the use of mixed cultures of micro-organisms for the production of high value chemicals, however, in most of these studies, the carbon source is either added as sugar rich liquor or in the case of utilising biomass, in vitro enzyme cocktails are used (Chu and Feng, 2013, Bornscheuer et al., 2014). Some studies have used wild type or modified micro-organisms that both saccharified the biomass and fermented the resultant sugars (Mbaneme-Smith, 2015), and some studies have used mixed cultures using micro-organisms of the same species mainly *Clostridium spp.* (Salimi, 2013) or same family i.e. *Pichia stipitis* with *Saccharomyces cerevisiae* (Chen, 2011) but there are few studies that have attempted to use mixed cultures of a cellulose degrading micro-organism and a fermentive micro-organism (Tran et al., 2013) and even fewer that have attempted cross kingdom co-cultures.

Previous work in the French lab (Kane, 2014) established that M9 minimal medium was suitable for the growth of *Cellulomonas fimi* and *E. coli* when supplemented with a carbon source. Previous work also showed that DSM3 when supplemented with yeast extract and cellulose based carbon source, was a suitable minimal medium for the growth of *Cytophaga hutchinsonii* (Liu, 2012).

5.2 Methods:

5.2.1 Co-cultures

Initial growth trials of 72 hours in specific medium (see table 8) with 28mM glucose as the carbon source were preformed to determine compatible co-cultures of the micro-organisms in this study. Ten-day growth trials consisted of 100 ml of an appropriate minimal medium (see chapter four) added to 250 ml conical flasks containing 500 mg of biomass (either cellulose filter paper or pre-treated wheat straw, with 500 mg of glucose as a positive control; 28mM equivalent). These flasks were then sterilised in an autoclave. Once cool each was inoculated with 1 ml of overnight liquid culture (OD>1.000) of appropriate combination of micro-organisms (see below). Optical density, total protein and CFU counts were taken every 24 hours as measures of growth over a ten-day period.

5.3 Results

5.3.1 Development of mixed microbial cultures to utilise cellulose filter paper as the main carbon source in liquid cultures.

Several different combinations of a range on organisms were tested, with some combinations proving to be fatal to one or both of the organisms, some that were non-fatal but did not result in measurable growth of one or both organisms and two that proved to provide synergetic growth of both organisms, to a level equal to or greater than a positive control.

5.3.2 *Overview of fatal, non-fatal and complementary co-cultures*

Table 8 shows which organisms are able to live in co-culture, firstly, in a liquid medium that supports growth of both organisms and then whether this combination is fatal or non-fatal to one or more of the microorganisms therein.

Table 9 - Showing an overview of fatal, non-fatal and synergetic combinations of microorganisms in minimal medium. Representative of at least 2 biological replicates It is important to note that *C. hutchinsonii* can only grow in DSM3+YE, no other medium investigated in this study allowed for its growth. Likewise, it should be noted that *E. coli* cannot use $NaNO_3$ as a nitrogen source so cannot grow in DSM3. (See chapter two for medium recipes)

Organism 1	Organism 2	Medium	Result
B. subtilis 168	*C. hutchinsonii*	DSM3+YE with 28mM glucose	Viable cells for both micro-organism present after 72 hours.
B. subtilis 168	*C. fimi*	DSM3+YE with 28mM glucose	Viable *C. fimi after 72 hours*, no viable *B. subtilis* 168 cells after 72 hours.
B. subtilis WB700	*C. hutchinsonii*	DSM3+YE with 28mM glucose	Viable *C. hutchinsonii* cells after 72 hours, no viable *B. subtilis* WB700 cells after 72 hours.
B. subtilis WB700	*C. fimi*	DSM3+YE with 28mM glucose	Viable *C. fimi after 72 hours*, no viable *B. subtilis* WB700 cells after 72 hours.
B. subtilis natto 21A1	*C. hutchinsonii*	DSM3+YE with 28mM glucose	Viable cells for both micro-organism present after 72 hours.
B. subtilis natto 21A1	*C. fimi*	DSM3+YE with 28mM glucose	Viable *C. fimi* cells after 72 hours, no viable *B. subtilis* natto 21A1 cells after 72 hours.
B. subtilis Efor-Red	*C. hutchinsonii*	DSM3+YE with 28mM glucose	Viable *C. hutchinsonii* cells after 72 hours, no viable *B. subtilis* Efor-Red cells after 72 hours.
B. subtilis Efor-Red	*C. fimi*	DSM3+YE with 28mM glucose	Viable *C. fimi* cells after 72 hours, no viable *B. subtilis* Efor-Red cells after 72 hours.
S. cerevisiae BY4742	*C. hutchinsonii*	DSM3+YE with 28mM glucose	*C. hutchinsonii* did not grow but *S. cerevisiae* did although poorly
S. cerevisiae BY4742	*C. fimi*	1% yeast nitrogen base + YE with 28mM glucose	Viable cells for both micro-organism present after 72 hours.
E. coli JM109	*C. hutchinsonii*	DSM3+YE and M9+YE with 28mM glucose	Incompatible medium; *C. hutchinsonii* cannot grow in M9 and *E. coli* cannot grow in DSM3
E. coli JM109	*C. fimi*	M9+YE	Viable cells for both micro-organism present after 72 hours.
L. acidophilus	*C. hutchinsonii*	DSM3+YE with 28mM glucose and Rogasa medium	Both organisms grew in DSM3+YE but only *L. acidophilus* grew in Rogasa medium
L. acidophilus	*C. fimi*	DSM3+YE with 28mM glucose and Rogasa medium	Both organisms grew in DSM3+YE but only *L. acidophilus* grew in Rogasa medium

Co-culture of *B. subtilis* 168 and *C. hutchinsonii* resulted in complementary growth (figure 50), as did a co-culture of *Bacillus natto 21A1* and *C. hutchinsonii*, whereas a co-culture of *B. subtilis* 168 and *C. fimi* proved to be fatal to *B. subtilis* 168. The co-culture of *B. subtilis* WB700 and *B. subtilis Efor-Red* with both *C. hutchinsonii* and *C. fimi* proved to be fatal to the *B. subtilis* spp. (table 9).

Growth of *S. cerevisiae* and *C. hutchinsonii* in DSM3+YE proved to be fatal to the *C. hutchinsonii* and resulted in poor growth of the *S. cerevisiae*, indicating that DSM3+YE is not a compatible medium for *S. cerevisiae* growth. However, co-culture of *S. cerevisiae* and *C. fimi* resulted in growth of both the *C. fimi* and *S. cerevisiae* in a fortified yeast nitrogen base minimal medium (table 9).

The co-culture *of E. coli* and *C. hutchinsonii* is not possible due to *E. coli* being unable to grow in DSM3+YE and we were unable to find another medium that *C. hutchinsonii* could to grow in. However, preliminary results show that *E. coli* in co-culture with *C. fimi* was non-fatal to either micro-organism (table 9).

The preliminary 72-hour growth trials also showed that *B. subtilis* natto 21A1 and *C. hutchinsonii* were non-fatal. *Lactobacillus acidophilus* (isolated from yogurt) in co-culture with *C. hutchinsonii* and *C. fimi* in DSM3+YE also proved to be non-fatal (table 8), as viable cells were still present in the culture, although the number was low ($5x10^5$) (data not shown).

The most effective co-culture proved to be *B. subtilis* 168 in co-culture with *C. hutchinsonii* in DSM3 + YE. This resulted in growth of *B. subtilis* greater than or equal to the positive control (see figures 50-52 and table 9) across all three measures of growth, across all replicates (n=7).

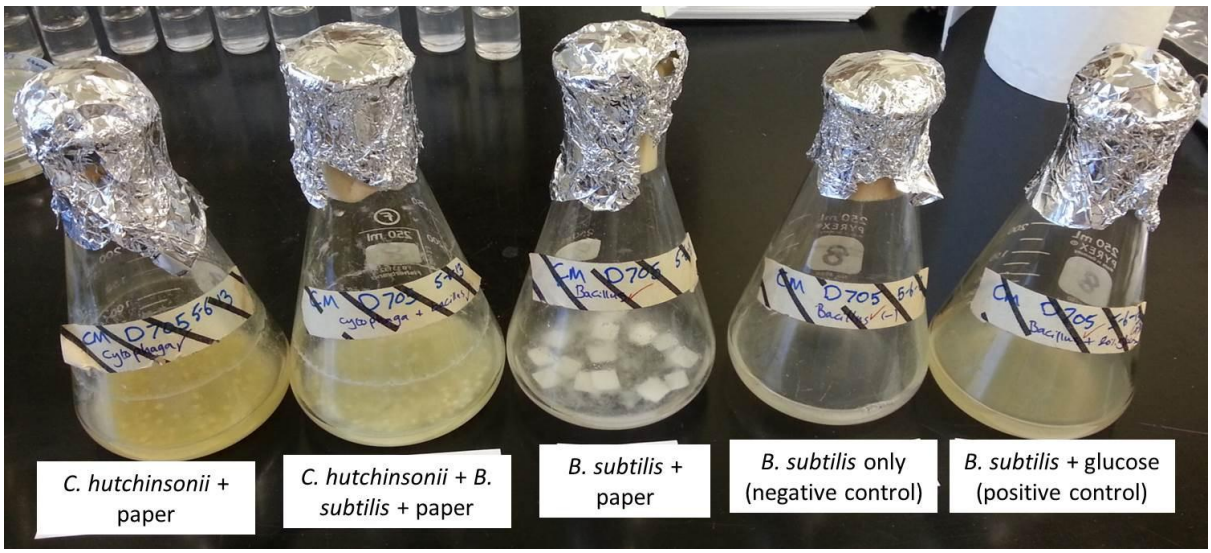


Figure 50 - Flasks showing single and co-cultured *C. hutchinsonii* and/or *B. subtilis* 168 after 10 days' growth at 30°C in DSM3 + YE with 500 mg of filter paper or glucose per 100 ml media. Negative control = DSM3+YE with no additional carbon source, positive control = DSM3+YE + 0.5% (28mM) of glucose. Representative of seven biological replicates.

5.3.3 *Investigation into the growth of* C. hutchinsonii *and* B. subtilis *168 in liquid co-culture utilising a range of carbon sources*

To determine whether the microbial partners were actually growing, i.e. increasing in number, rather than just surviving in the medium, three different measures of growth were used; measuring changes in the optical density (OD) of the culture, monitoring the total protein concentration of the culture and plate counts of colony forming units (CFU), to give an approximation of the number of viable cells in one millilitre of culture. Note; *C. hutchinsonii* does not form colonies on agar.

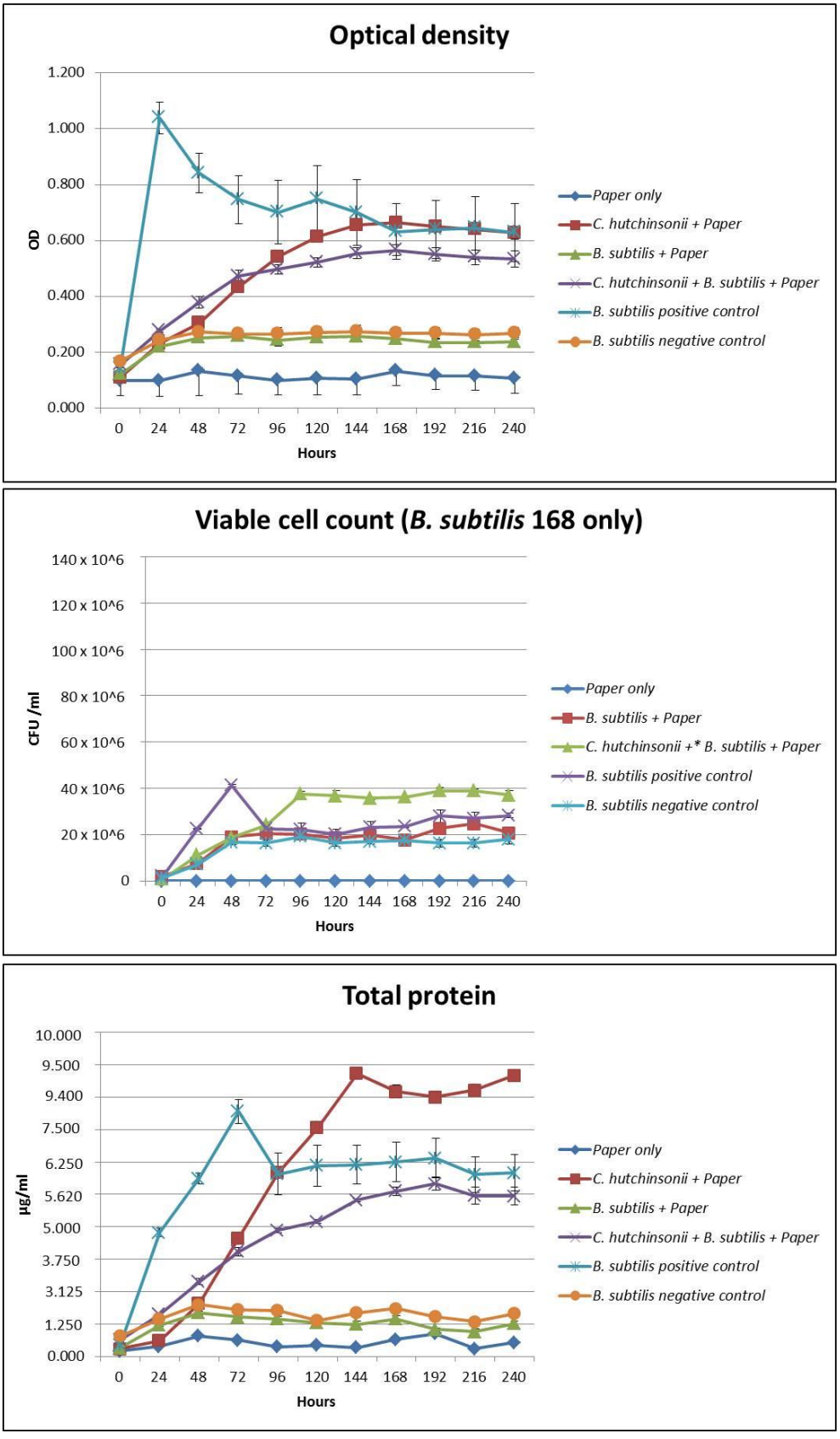


Figure 51 - Measures of growth of *B. subtilis* 168 in co-culture with *C. hutchinsonii* in DSM3+YE with 500 mg cellulose filter paper per 100 ml media as the main carbon source, inoculated with one colonised square of filter paper per flask (fig 50). Representative of seven biological replicates. Error bars show standard error.

As figure 51 shows, co-culture of *C. hutchinsonii* and *B. subtilis* 168 liquid cultures where the main carbon source is filter paper, the culture with the highest optical density is the *B. subtilis* 168 positive control, containing 0.05% w/v glucose (28mM), with the optical density peaking at 24 hours, then falling, until it plateaus at OD 0.600 at seven days (168 hours). The next highest optical densities are in the *C. hutchinsonii* monoculture and the *C. hutchinsonii* and *B. subtilis* 168 co-culture, both achieving similar levels to each other and the *B. subtilis* 168 positive control (within standard error), suggesting that filter paper can be utilised to achieve growth similar to when glucose is the main carbon source. The cultures with the lowest optical density and therefore growth are the *B. subtilis* 168 negative control where there is no carbon source and the *B. subtilis* 168 culture where filter paper is the main carbon source. This shows that *B. subtilis* 168 cannot grow in DSM3+YE without a carbon source and that *B. subtilis* 168, on its own, cannot utilise filter paper as a carbon source. When colony forming, units are used as the measure of growth, figure three also shows that *the B. subtilis* 168 positive control reaches the highest number of colonies at 24 hours before falling away and plateaus at 72 hours at approximately $20x10^6$ cfu/ml. The co-culture of *C. hutchinsonii* and *B. subtilis* 168 colony numbers rise more slowly than the *B. subtilis* 168 positive control, peaking at 96 hours and then maintaining a colony count of approximately $40x10^6$ cfu/ml from 96-240 hours. This gives the co-culture a higher colony count than the *B. subtilis* 168 monoculture, positive and negative controls, all of which colony counts of approximately 20-$30x10^6$ cfu/ml from 72 hours to 240 hours. When total protein is used as a measure of growth, in figure 51, we see that the culture with the fastest growth is the *B. subtilis* 168 positive control, peaking at approximately 9.000 µg of protein/ml at 72 hours before dropping and plateauing at approximately 6.250 µg/ml. The *C. hutchinsonii* monoculture has a slower rate of growth but reaches a higher amount of protein at 9.500 µg /ml by 144 hours. The mixed culture of *C. hutchinsonii* and *B. subtilis* 168 also have a slower rate of growth similar to the trend seen in optical density and colony numbers for this culture, the amount of protein for the mixed culture peaks at around 5.620 µg of protein / ml by 168 hours (seven days) where it plateaus, giving it a level similar to the *B. subtilis* 168 positive control, again mirroring the trends seen for these cultures for optical density and colony counts. The *B. subtilis* monoculture and negative control again have the lowest growth rates as indicated by total protein, showing that *B. subtilis* cannot grow in DSM3+YE without a carbon source and that it cannot utilise filter paper as that carbon source.

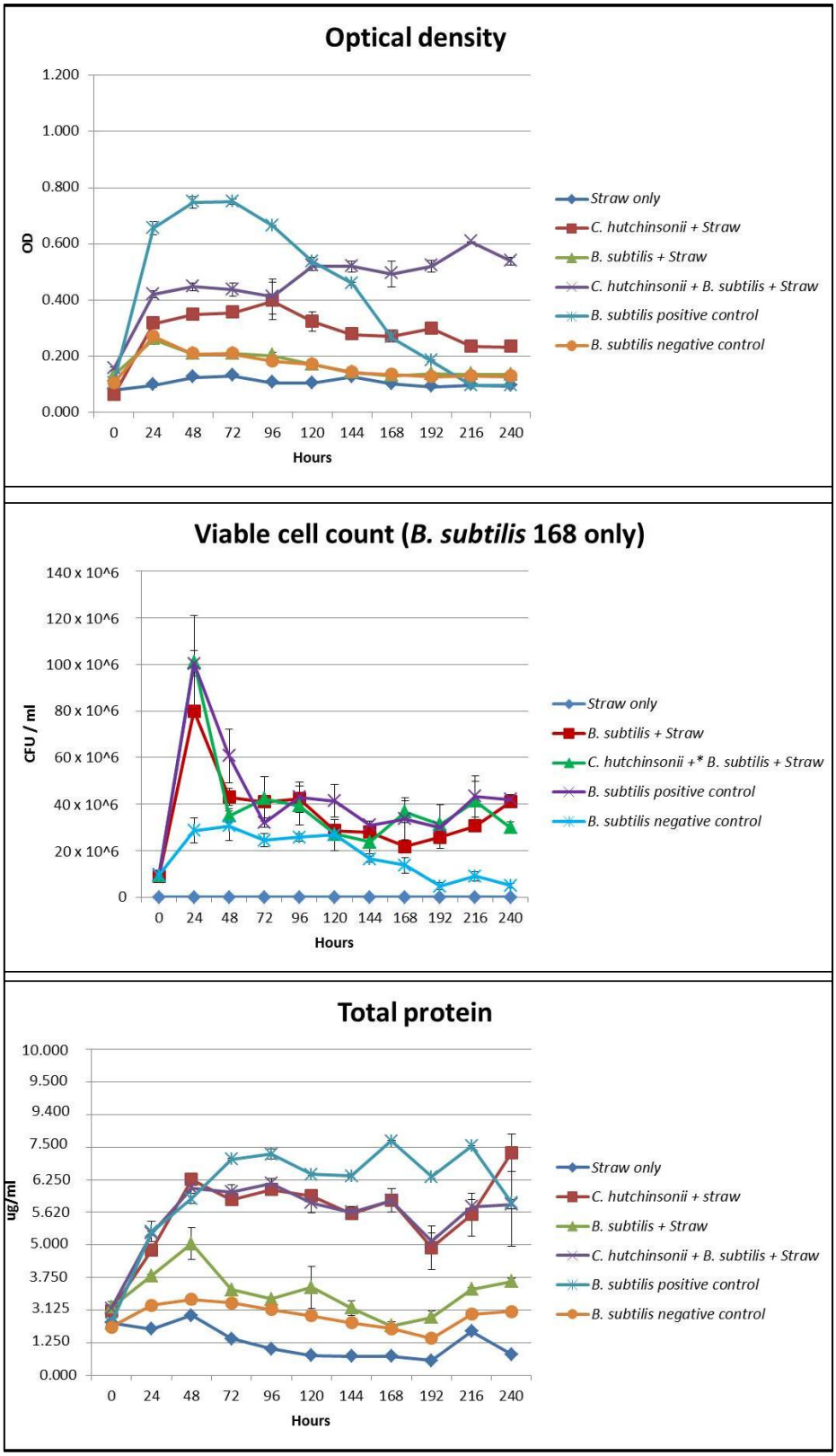


Figure 52 - Measures of growth of *B. subtilis* 168 in co-culture with *C. hutchinsonii* in DSM3+YE with 500 mg pre-treated wheat straw per 100 ml media as the main carbon source inoculated with one colonised square of filter paper per flask. Representative of seven biological replicates. Error bars show standard error.

When pre-treated wheat straw (see chapter three) is used as the main carbon source for co-cultures of *C. hutchinsonii* and *B. subtilis* 168 growth measures, shown in figure 52, indicate that the culture with the highest optical density is the *B. subtilis* 168 positive control, containing 0.05% W/V glucose (28mM), with the optical density peaking at 48-72 hours before falling away. The optical density of the mixed culture of *C. hutchinsonii* and *B. subtilis* 168 again raises more slowly but more consistently than the positive control over ten days, peaking at OD 0.600 before falling slightly. The monoculture of *C. hutchinsonii* on pre-treated wheat straw has a much lower optical density compared to when filter paper is used, with its optical density peaking at 96 hours at OD 0.400 before falling away over ten days to approximately OD 0.200, suggesting a lower ability to utilise lignocellulosic biomass compared to cellulose filter paper, which is to be expected as a wider range of enzymes are needed to break down lignocellulosic biomass, than are required for filter paper. The optical density of the *B. subtilis* 168 negative control and the *B. subtilis* 168 monoculture have the lowest optical density, suggesting again that *B. subtilis* 168 cannot grown in DSM3+YE without a carbon source and that *B. subtilis* 168 cannot utilise pre-treated wheat straw as a carbon source. The latter is surprising as *B. subtilis* 168 have native hemicellulases (St John et al., 2006), which should allow it to utilise polymers such as xylans. When viable cell counts are used as the measure of growth, the results are less clear (figure 52) with only the low number for the *B. subtilis* 168 negative control being clear. The picture becomes somewhat clearer when the total protein is the measure of growth, again the *B. subtilis* 168 positive control has the highest amount, followed by the mixed culture of *C. hutchinsonii* and *B. subtilis* 168, suggesting synergetic growth and the monoculture of *C. hutchinsonii* having extremely similar amounts of protein until ten days when the amount in the monoculture of *C. hutchinsonii* pulls away. Again the *B. subtilis* 168 negative control and monoculture have the lowest amount of growth, as described above. These results are the most robust and convincing but to clear differences in the measures of growth and the number of replicates (seven biological replicates).

5.3.4 *Development of a minimal medium that supports the growth of* Cellulomonas fimi *and* Saccharomyces cerevisiae

One of the aims of this study is to produce economically interesting chemicals and while it is possible to genetically modify *B. subtilis* and/or *E. coli*, another model organism already naturally produces one of these chemicals, *Saccharomyces cerevisiae*.

Table 8 shows, while co-cultures of *S. cerevisiae* and *C. hutchinsonii* proved to be fatal for the *C. hutchinsonii*, a co-culture of *C. fimi* and *S. cerevisiae* showed co-operative growth, once a suitable medium was found. A great deal of effort was made into finding a suitable medium for both organisms as *C. fimi* is unable to grow in the yeast minimal medium, 1% yeast nitrogen base (YNB) and *S. cerevisiae* cannot grow in DSM3+YE. After screening several types of unsuitable medium (see appendix), it was determined that both organisms could grow in 1% YNB, if it was supplemented with µM amounts of trace elements, amino acids and vitamin complex (see chapter 2 and appendix).

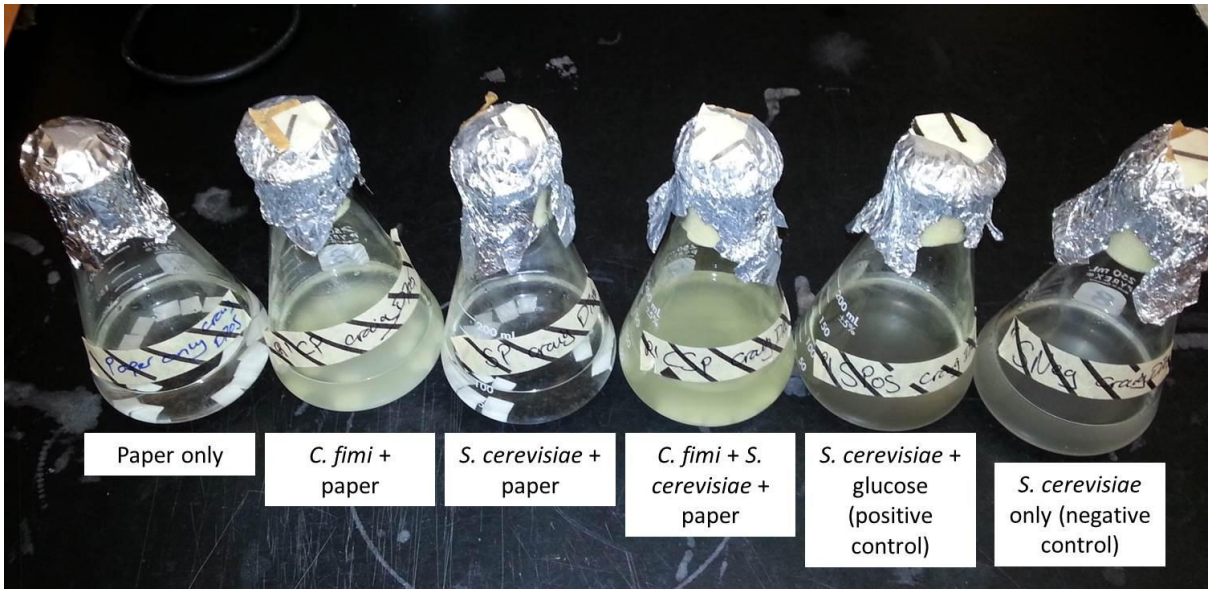


Figure 53 – Flasks of single and co-cultured *C. fimi* and/or *S. cerevisiae* after 5 days' growth at 30°C in 1% fortified YNB, with 500 mg filter paper / glucose per 100 ml media inoculated with one millilitre of culture (OD >1.000) per flask. Paper only = cell free control, positive control = 1% YNB + 0.5% (28mM) of glucose, negative control = 1% YNB with no additional carbon source. Representative of one biological replicate.

From a quick visualisation shown in figure 53*, C. fimi* can grow in fortified 1% YNB, as evidenced by the yellowing of the filter paper and that there is growth of potentially both organisms in the mixed culture flask. *S. cerevisiae* is seemingly unable to use filter paper as a carbon source and cannot grow in fortified YNB without a carbon source. Once growth was confirmed a 5-day growth assay was set up to measure a range of growth indicators (figure 54).

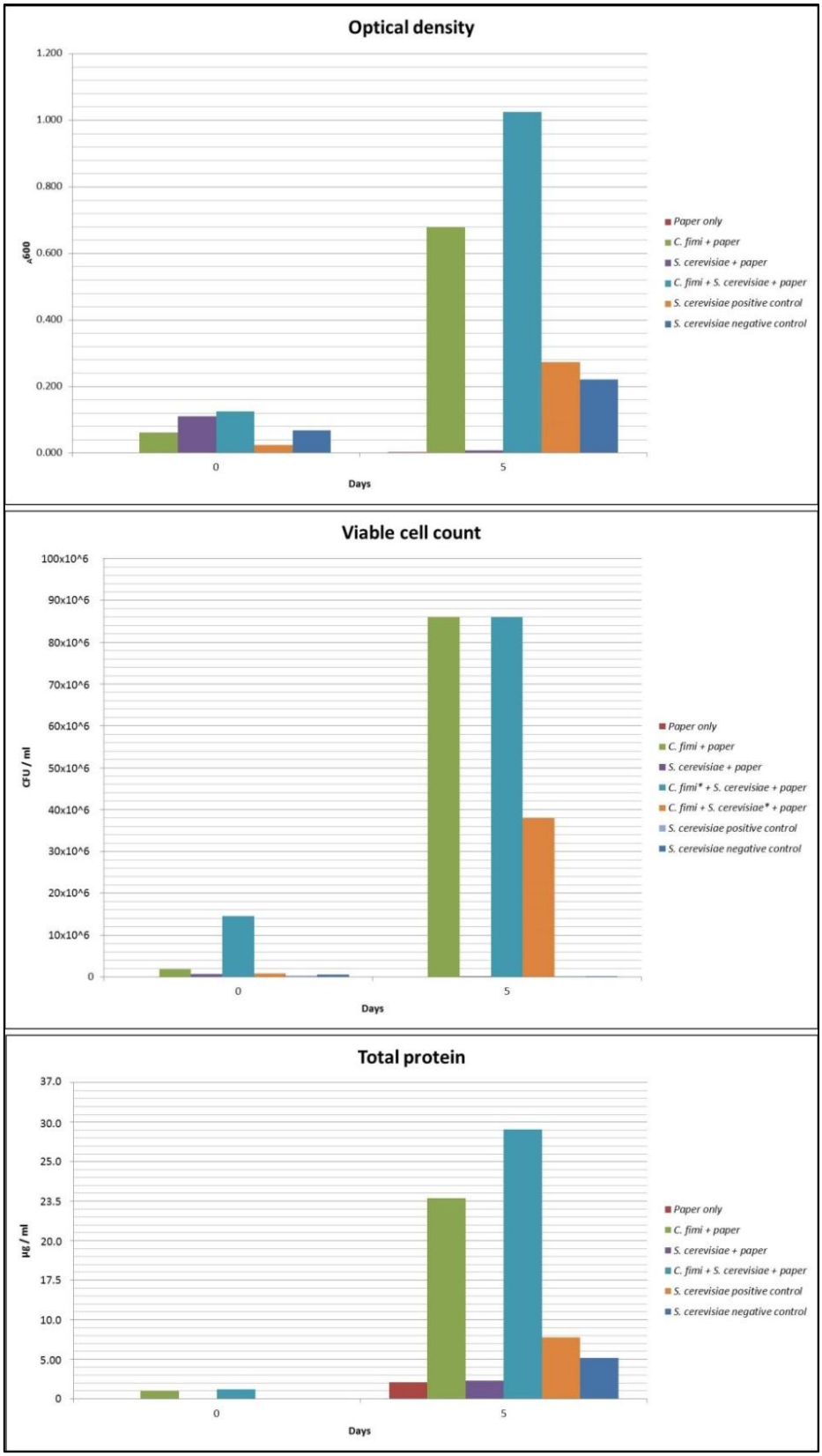


Figure 54 - Measures of growth of *S. cerevisiae* in co-culture with *C. fimi* in fortified yeast nitrogen base (YNB) after five days at 30°C with 500 mg cellulose filter paper or glucose the main carbon source (fig 53). Paper only = cell free control, positive control = 1%YNB + 0.5% (28mM) of glucose, negative control = 1% YNB with no additional carbon source. Representative of one biological replicate.

Figure 54 shows that after five days co-culture the mixed culture of *C. fimi* and *S. cerevisiae* had a higher optical density (at approximately OD 1.000) than the *S. cerevisiae* positive control (approximately OD 0.300). After five days, the monoculture of *C. fimi* had an OD of approximately 0.620 which was higher than the *S. cerevisiae* negative control and *S. cerevisiae* monoculture with paper as the carbon source. The number of viable cells of *C. fimi* in the co-culture after five days was equal to that of the *C. fimi* monoculture and the number of viable cells of *S. cerevisiae* in the co-culture was again higher than the *S. cerevisiae* positive control, negative control and monoculture. Figure 54 also shows that the total amount of protein in the co-culture was higher than both the *C. fimi* monoculture and the *S. cerevisiae* positive control, indicating that; overall, the co-culture of *C. fimi* and *S. cerevisiae* had better growth than their controls. Further replicates showed that heat lysis does not work on *S. cerevisiae* cells. Alkali, sonication and freeze-thaw lysis were also tried but failed to give reproducible results across replicates. For this reason, the total protein results are not as robust as the OD and cfu/ml results.

5.3.5 *Co-culture of* C. fimi *and* E. coli *JM109 in M9 minimal medium*

Since *E. coli* is the most widely studied bacterium, is easily genetically engineered to produce useful products and has the widest range of products shown in the literature the possibility of using this microorganism in co-culture was investigated.

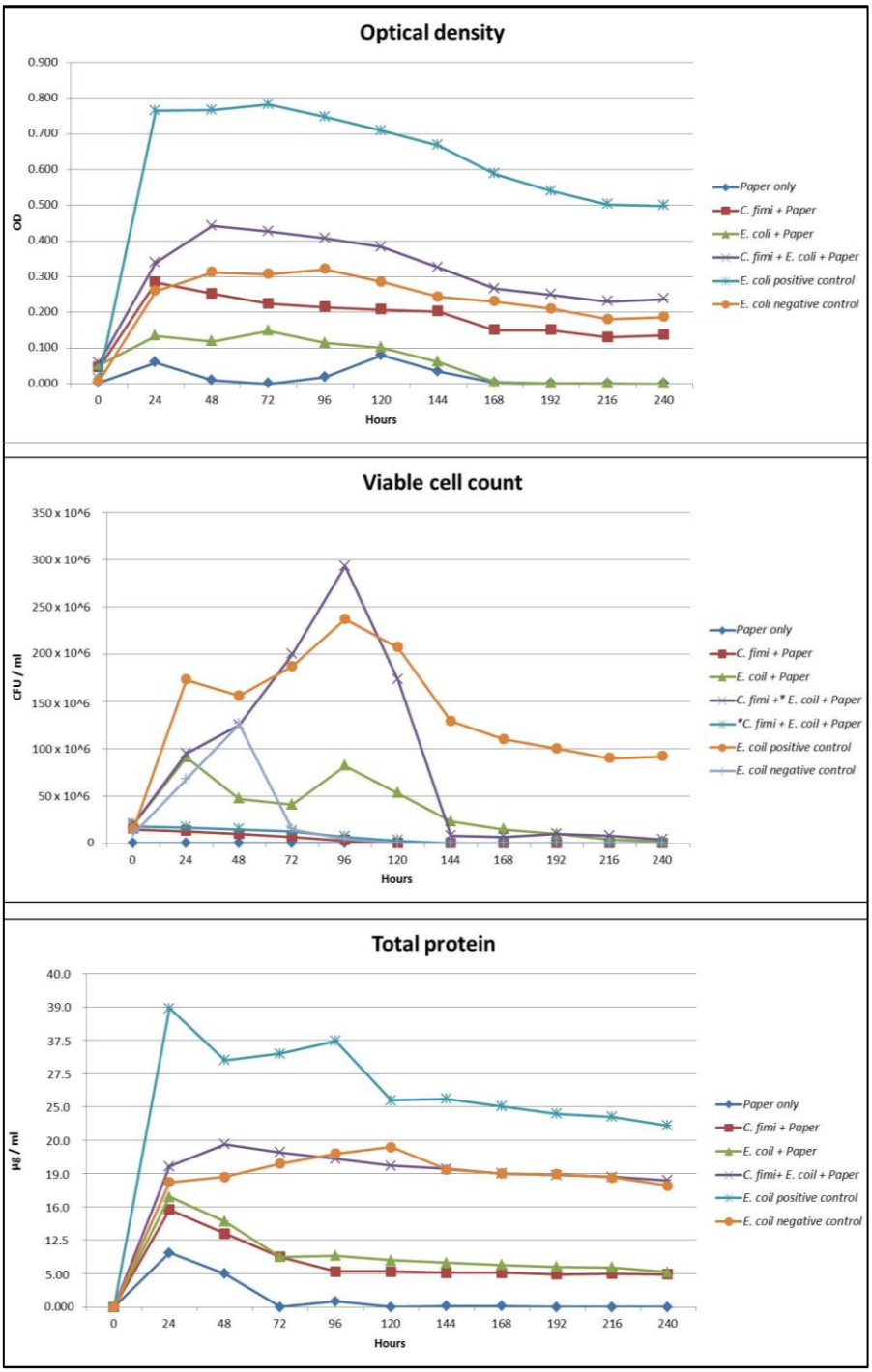


Figure 55 - Measures of growth of *E. coli* in co-culture with *C. fimi* in M9 with 500 mg filter paper per 100 ml media as the main carbon source. Paper only = cell free control, positive control M9 + 0.5% (28mM) of glucose, negative control = M9 with no additional carbon source. Representative of one biological replicate.

As fig 55 shows the optical density of the co-culture of *C. fimi* and *E. coli* was higher than the negative control and the *E. coli* and paper flask, but far below the glucose positive control (0.05% w/v glucose/28mM). The viable cell counts for the mixed culture were higher than the positive control by 96h after a lag, since it takes time for the filter paper to be broken down to usable sugars. However, the *E. coli* colony numbers drop dramatically after 96 h; this may be due to a technical fault with the incubator being turned off, but should be examined with further replicates. The total protein of the mixed is well below the glucose positive control but of similar levels to the negative control and well above the *E. coli* plus paper flask. These results do not show complementary growth of *C. fimi* and *E. coli*, but it is worth repeating to make sure these results are accurate, specifically the relatively high growth of the *E. coli* negative controls.

5.4 Short summary and discussion

Initial tests show that there were a range of different microbial co-culture combinations if a compatible medium, one that supported the growth of both micro-organisms, could be found. Co-cultures of *C. hutchinsonii* and *B. subtilis* 168; *C. fimi* and *S. cerevisiae* and *C. fimi* and *E. coli* are possible. The most promising of these co-cultures was the co-culture of *C. hutchinsonii* and *B. subtilis* 168 utilising either filter paper or pre-treated wheat straw as the main carbon source. The co-cultures of *B. subtilis* 168 and *C. hutchinsonii* over ten days, demonstrated that when the biomass was broken down by the enzymatic actions of *C. hutchinsonii*, *B. subtilis* is able to use the oligo – and mono- saccharides released for growth, which is comparable to growth achieved when the main carbon source is glucose. This is an important factor to consider, as (waste) paper is cheaper than glucose. It also shows that, while *B. subtilis* does naturally produces polymer digesting enzymes, such as α-amylase and xylanases, it does not naturally produce cellulases and so cannot use paper or, surprisingly, pre-treated wheat straw as the main carbon source for growth in isolation; a mixed culture, with compatible cellulose degrading micro-organism is needed, if these forms of biomass are to be utilised.

6 Modification of *Bacillus subtilis* 168 for the production of lactic acid and ethanol

6.1 Background:

It is widely known that under anaerobic conditions *B. subtilis* 168 will produce lactic acid via the homolactic fermentation pathway (Romero-Garcia et al., 2009, BsubCyc, 2016) however growth of *B. subtilis* 168 under anaerobic conditions is massively reduced compared to aerobic growth and so, due to this low growth, low amounts of lactic acid are produced. Some work has been done to engineer *B. subtilis* 168 to produce larger amounts of lactic acid, by reducing metabolic competition for pyruvate (Romero-Garcia et al., 2009) or using UV driven mutagenesis, to produce levels of lactic acid, aerobically, that compete with levels produced by commercial lactic acid bacteria based systems (Gao et al., 2012, Gao and Ho, 2013). Attempts have also already been made to engineer *B. subtilis* 168 to produce ethanol by disrupting native *ldh* placing *pdc* and *adh* under the *ldh* promoter (Romero et al., 2007). There has also been some success in engineering *B. subtilis* to produce butanol by expressing the acetolactate synthase *alsS* gene (Romero et al., 2007) by incorporating into plasmids under the P_{43} promoter so the *alsS* gene would be transcribed during exponential and lag phases (Li et al., 2011, Li, 2012).

In this chapter, we aimed to produce lactic acid and ethanol by inserting genetic cassettes under the *cspD* locus. *B. subtilis* 168 has three cold shock proteins analogous to cold shock protein A found in *E. coli* (Schindler, 1999) of these cold shock protein D CspD is the most highly expressed non-essential protein produced by *B. subtilis* 168 (Nicolas et al., 2012) suggesting that this locus could be utilised for the production of useful proteins in high amounts, without any major detrimental effect to the growth of *B. subtilis* 168 and would provide a non-chemically induced promoter. Since CspD is the most highly expressed non-essential protein (Graumann, 1997, Marahiel, 1999, Schindler, 1999, Nicolas et al., 2012) in *B. subtilis* using this locus for over production of lactate dehydrogenase and fused pyruvate decarboxylase: alcohol dehydrogenase aerobically should be possible. Since *Cytophaga hutchinsonii* cannot grow anaerobically, this should lead to a co-culture system for these products (chapter four). The use of the *cspD* promoter may also lead to the utilisation of *B. subtilis* as a platform for protein production such as already found in literature (Lee, 2000, Simiqueli, 2009, Oyeleke, 2012, Barros et al., 2013, Liu et al., 2013, Pant et al., 2015).

6.2 Methods:

6.2.1 Cassette construction

Construction of the lactate dehydrogenase (*ldh*) cassette was completed using splicing overlap extension PCR (Vallejo et al., 2008, Luo, 2013), using Phusion master mix (NEB).

6.2.2 B. subtilis competence and transformations

Natural competence of *B. subtilis* 168 was induced as per lab protocols. To 1 ml of *B. subtilis* 168 competent cells glycerol stock, 60 µl (0.0038 pMol [*ldh*] and 0.0018 pMol [Pet]) of column purified linear PCR product was added and the reaction incubated in a 15 ml Falcon tube at 37^{o}C for 1 hour. The cells were recovered in 2 ml of SOC for two hours at 37^{o}C before being plated out onto LB plates containing the appropriate antibiotic.

6.2.3 Crude extract total protein

Isolates of *Bacillus subtilis* were grown up overnight in 50 ml LB, which was then spun down and resuspended in 15% glycerol/x1 PBS and sonicated for three minutes in one minute bursts. This was then spun down and the supernatant concentrated with Millipore protein concentrators to x100 conc. This crude extract was then used for total protein SDS, native PAGE gels and enzymatic activity assays.

6.2.4 Total protein

The total protein of the crude extracts was determined using Bradford reagent. One microliter of crude extract was diluted in 9µl of distilled water which was then added to 990 µl of Pierce™ Coomassie Plus (Bradford) Assay Kit (Thermo Scientific, P/N. 23236), the reaction was allowed to develop for 10 minutes before the absorbance was measured at 595nm.

6.2.5 Native and SDS gels

For SDS PAGE 1 µl of crude extract was added to 19 µl of SDS buffer and heated at 95^{o}C for 10 minutes. The gel was run at 200 V for 1 hour and stained overnight in 50 ml Coomassie brilliant blue stain with gentle agitation. The gel was destained using 50 ml of the same solvent for 3 hours. Once the distaining was complete, the gel was rehydrated in 50 ml dH_20 for 30 minutes and then scanned.

For native gels 20 µl of crude extract was loaded onto a 12% non- denaturing PAGE gel and run at 200 volts for 1 hour. The gels were removed and stained overnight for *ldh*, *pdc* and *adh* activity (see chapter two).

6.2.6 Enzyme activity assay

To 1 ml of Tris buffer pH 7.5 (pdc:adh) pH 9 (ldh); 0.3 mM NADH (2 µl of 15 mM stock) and 1 µl of crude extract from either the wild type, ldh or Pet strain were added. The background decrease in OD at 340nm was measured every 10 seconds for one minute. Then 1µl of 1 mM sodium pyruvate (1 M stock) was added and the decrease in OD 340nm every 10 seconds for three minutes was measured.

6.2.7 Lactic acid production

One millilitre of overnight culture of wild type and ldh producing *B. subtilis* was used to inoculate either 15 ml universals (anaerobic culture) or 25 ml conical flasks (aerobic culture) containing 10 ml of both standard LB and LB supplemented with 0.5% glucose. These were then grown up overnight at 37^{o}C with half receiving 1 hour cold shock at 5^{o}C as described in (Lottering, 1995), at the start of exponential stage, to induce the cold shock protein D (*cspD*) promoter. The control flasks were maintained at 37^{o}C. After 30 minutes the cold shocked flasks were returned to 37^{o}C and, along with the controls, grown overnight to stationary phase. These cultures were then assayed for lactic acid content using colourimetric assay detailed above (section 6.2.5).

6.3 Results:

6.3.1 Construction of cassettes

To transform *B. subtilis* 168 to produce ethanol and over produce lactic acid, DNA cassettes were constructed using; splicing overlap extension PCR (lactic acid production) and paperclip assembly (ethanol production) (see section 2.4.6). Each cassette had a region of homology to the genomic DNA flanking the cold shock protein D (*cspD*) locus and contained genes that code for lactate dehydrogenase (lactic acid production) cloned from native *B. subtilis* 168, pyruvate decarboxylase :alcohol dehydrogenase (ethanol production), originally cloned from *Z. mobilis* (Lewicka, 2014) along with genes that confer either kanamycin or chloramphenicol resistance, cloned from BioBrick plasmids pSB1K3 and pSB1C3 respectively. These areas of flanking homology corresponded to the loci UDP-glucose diacylglyceroltransferase (*ugtP*) and regulatory protein DegR (*degR*) in *B. subtilis* genomic DNA which flank the cold shock protein D (*cspD*) gene. When this linear DNA was taken up by the competent *B. subtilis* 168 cells, it was inserted into the *cspD* locus by homologous recombination.

cspD upstream primer	GATCGGCACTCATCCAAGCAATG
CspD upstream reverse primer	GTACTAGGAGGAATTAAGCAAT
Lactate dehydrogenase forward primer	TACTAGGAGGAATTAAGCAATATGATGAACAAACATGTAAATAAAGTTAGCTTTAAT
Lactate dehydrogenase reverse primer	ATTTTAAAACCTCATTTTGCAGAACAAAAAGTCAACT
Chloramphenicol resistance cassette forward primer	ATTCTTCAACTAAAGCACCCATTAGTTCAACAAAC
Chloramphenicol resistance cassette reverse primer	AAACATTTTGGCGATTGAAATCTTCAACTAACGGGGCAGGTTAGTGACATTAGAAAA
fused pyruvate decarboxylase-alcohol dehydrogenase forward primer	CATACCAACACTTCAACGCACCGGCTCTAGTATTATCAAAAGGCACTC
fused pyruvate decarboxylase-alcohol dehydrogenase reverse primer	GAGTGCCTTTTGATAATACTAGAGCCGGTGCGTTGAAGTGTTGGTATG
neomycin (kanamycin) resistance forward primer	CTCATCATCATGTATTGAGTTTAGCGCAGATCAATTCTGACAGCCATG
neomycin (kanamycin) resistance reverse primer	CATGGCTGTCAGAATTGATCTGCGCTAAACTCAATACATGATGATGAG
cspD downstream forward primer	CTAAACTCAATACATGATGATGAG
cspD downstream reverse primer	ATCGTGCAGTTCTGTCAGTTCTTTAAAG

Table 10 – Sequence of primers used (see appendix for full insertion cassette sequences)

Table ten shows the sequence of primers used to amplify constituent DNA parts for the construction of lactic acid and ethanol producing cassettes under the native *cspD* promoter. These primers were also used for analytical PCR amplification to verify the correct insertion sites of these constructs. A southern blot could also be used.

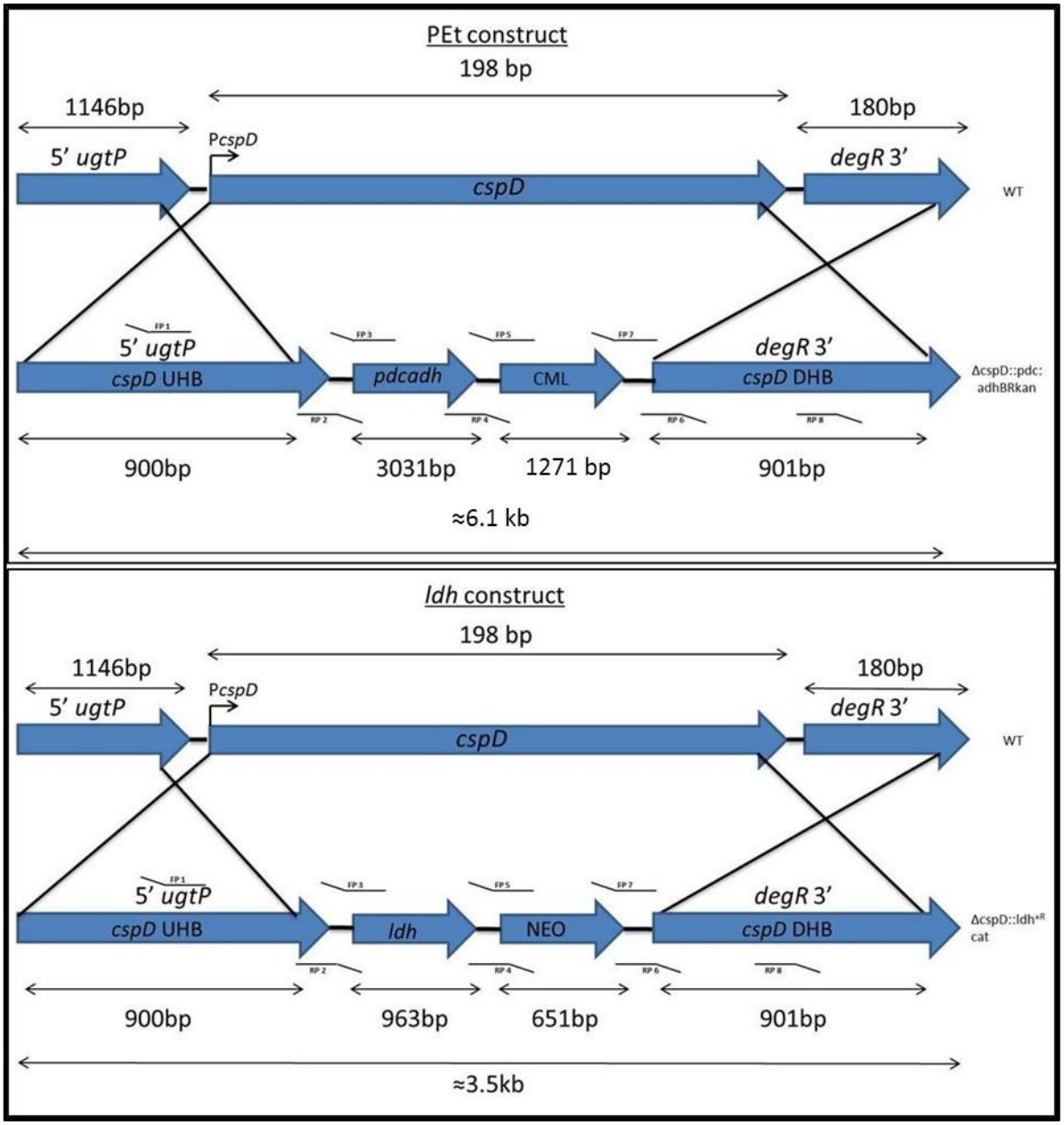


Figure 56 - Schematic of genetic cassettes, including parts; order and sizes. In paperclip assembly, the full clips join the upstream homology to the *ldh*/*pdc*:*adh* genes; the *ldh*/*pdc*:*adh* genes to the antibiotic resistance genes and the antibiotic resistance genes to the downstream homology. In splicing overlap extension, these regions are joined by their overlapping homology (see section 2.4.6)

Figure 56 shows how the parts of the cassettes were amplified with primers that had an overlapping homology to the sequential DNA piece; these parts would then be combined and fused together in a secondary PCR reaction. This linear DNA was then used to transform competent *B. subtilis* 168 cells. This method proved highly effective when constructing the lactate dehydrogenase (*ldh*) cassette, but repeatedly failed to amplify the different DNA parts for PEt, particularly the *pdc*:*adh* sequence and completely and repeatedly failed to construct the full cassette. In the end this method was abandoned in favour of the "paperclip" assembly method (Trubitsyna et al., 2014). In this method, rather than amplifying the cassette parts with overlapping homology to each other, the homology

between parts is present in “clips”, short oligos that join the parts together as a paperclip (or staple) would. The construction of these parts in this method, is not done via PCR, rather the parts, and a plasmid backbone, are taken up by competent *E. coli* cells where the in-cell mechanisms construct a plasmid (Trubitsyna et al., 2014), which can be recovered and used to transform *B. subtilis* 168 competent cells.

To determine whether the PCR amplification of parts and construction of both the full and intermediary parts of the cassette was successful 10 µl of the PCR reaction were visualised on 0.8% agarose gels.

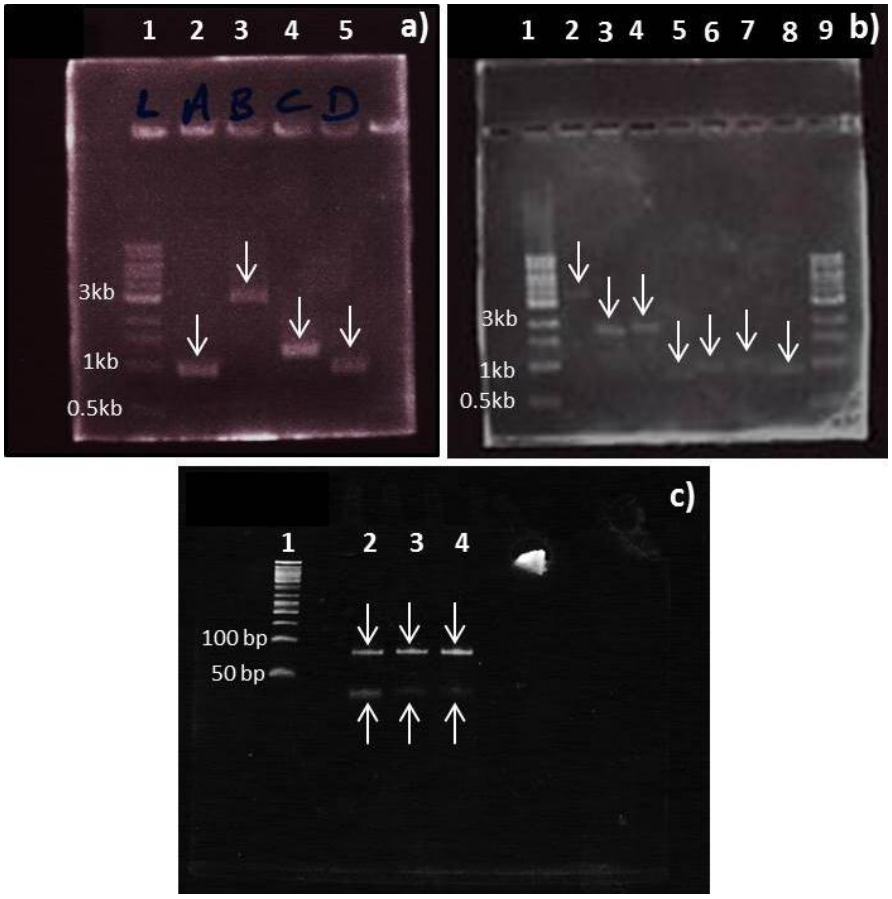


Figure 57 – Genetic cassette parts visualised on electrophoresis gels and stained with GelGreen (see section 2.4.8) Bands indicated with arrows for clarity.

a) *pdcadh* parts lane 1 = DNA ladder, (NEB); lane 2 = Upstream homology, 900 bp; lane 3 = *pdcadh*, 3031 bp; lane 4 = chloramphenicol resistance, 1271 bp; lane 5= downstream homology, 901bp.

b) *ldh* parts lane 1 = DNA ladder; lane 2 = full 3.5 kb assembled cassette; lane 3 = assembled upstream homology: *ldh* cassette, 1863bp; lane 4 = fused neomycin/kanamycin resistance cassette: downstream homology, 1552bp; lane 5 = Upstream homology, 900 bp; lane 6 = *ldh*, 963bp; lane 7 = neomycin/kanamycin resistance cassette, 651bp; lane 8 = downstream homology, 901bp; lane 9= DNA ladder (NEB).

Bridging oligos for paperclip assembly c) Clips and half clips for paperclip assembly; half-clips = 40bp; full-clips, 80 bp; visualised on 10% native PAGE gel and stained with GelGreen (see section 2.4.8) Lane 1 is a DNA marker ladder (NEB), lane 2 is the upstream homology: *pdcadh* clip, lane 3 is the pdcadh: chloramphenicol resistance clips and lane 4 is the chloramphenicol cassette: downstream homology clip.

As figure 57 shows the parts, clips and constructs are of the expected size, when the gels are compared to the sequence sizes. The PCR reactions for these parts were column purified and the amount of DNA per µl quantified on a nanodrop spectrophotometer. After construction of the full cassette (and digestion in the case of the paperclip assembled cassette) and their column purification, the amount of DNA present was very low at: 15 µg/ µl for the *ldh* cassette and 11 µg/ µl for the Pet cassette.

6.3.2 Transformation of B. subtilis 168

The purified DNA cassettes were used to transform 1 ml of competent *B. subtilis* 168 cells.

6.3.2.1 Strain names of transformed B. subtilis 168

Once the PEt (**P**roduces **Et**hanol) or the *ldh* (**l**actate **d**e**h**ydrogenase) cassettes have been inserted into the genomic DNA of *B. subtilis* 168, any transformed cells become:

PEt construct *B. subtilis* 168 $^{\Delta}$*cspD*::*pdc*:*adh*B^{+}Rneo

Ldh construct *B. subtilis* 168 $^{\Delta}$*cspD*::*ldh*$^{+}$Rcml

6.3.2.2 Transformation efficiency TE

The transformation efficiency of competent cells transformed to become; *B. subtilis* 168 $^{\Delta}$*cspD*::*pdc*:*adh*B^{+}Rkan, where 7.14 cfu/µg and the transformation efficiency of competent cells transformed to become *B. subtilis* 168 $^{\Delta}$*cspD*::*ldh*$^{+}$Rcat, where 28.33 cfu/µg.

6.4 Evidence of presence and activity of cassettes and associated proteins

Once the cells were transformed the next step was to test for the presence and activity of transcribed lactate dehydrogenase and/or pyruvate decarboxylase: alcohol dehydrogenase. To this end crude protein extracts were produced via sonication from cell cultures for use in activity assays and PAGE gels.

6.4.1 PCR identification of transformed loci in B. subtilis 168 $^{\Delta}$cspD::pdc:adhB^{+}Rkan and B. subtilis 168 $^{\Delta}$cspD::ldh^{+}Rcat

To determine whether the genetic cassette has inserted in the correct position within the genomic DNA of the transformed *B. subtilis*, the genomic DNA from all strains was extracted and amplified using primers designed to anneal outside of, and half way down (figure 59), the cassette. These were then PCR amplified. These were then visualised on a 0.8% agarose gel (figure 59).

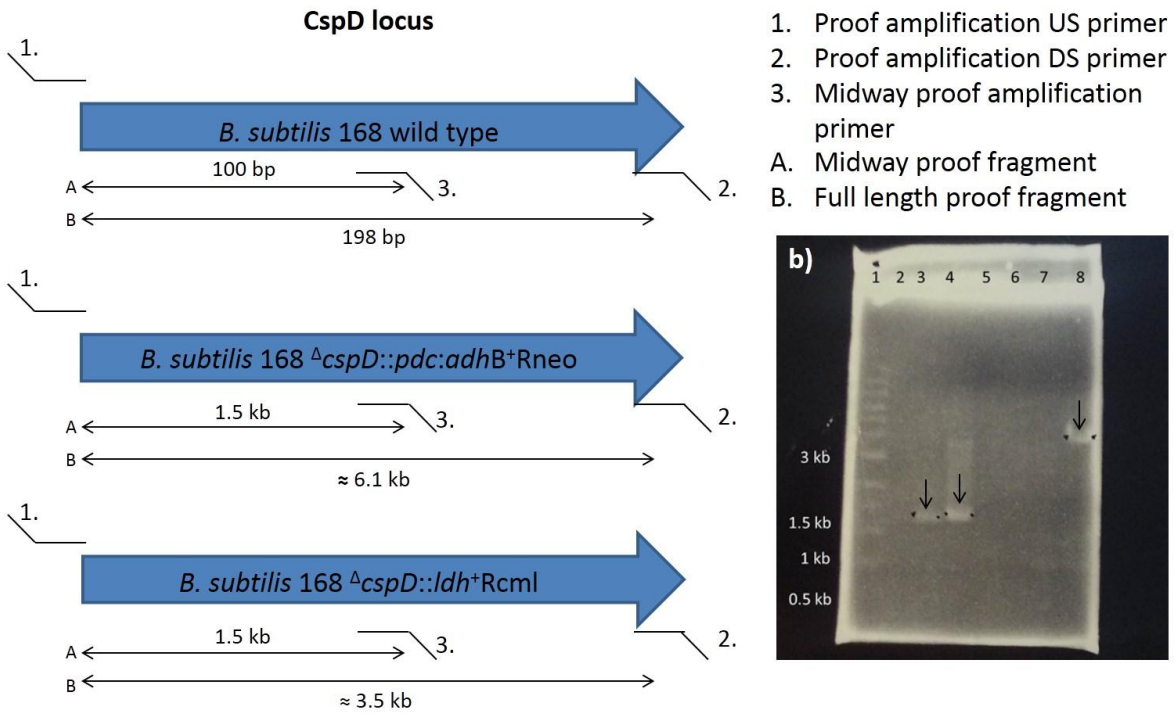


Figure 58 – Proof PCR schematic shown in a) and agarose gel for loci confirmation shown in b).

a) Schematic shows the approximate binding sites for primers designed to amplify the whole and mid-way down the insertion sequence and the approximate size of the bands expected on the gel. Excise primers 1 and 2 were designed to anneal outside the inserted DNA cassette just past the upstream and downstream homology, in the *ugtP* and *degR* genes respectively. Primer 3 was designed to be used with primer one and should have annealed mid-way down the inserted DNA cassette, giving fragments of 100 bp for the wild type DNA and at 1.5 kb for both *B. subtilis* 168 Δ*cspD*::*pdc*:*adh*B⁺Rneo and *B. subtilis* 168 Δ*cspD*::*ldh*⁺Rcml.

b) Agarose gel 1) NEB DNA ladder, 2) WT midway fragment ≈ 100 bp, not shown due to operator error, 3) PEt midway fragment ≈ 1.5 kb, 4) *ldh* midway fragment ≈ 1.5 kb, 5) empty, 6) WT excise 198 bp, not shown due to operator error 7) Pet excise ≈ 6 kb, 8) *ldh* excise ≈ 3.5 kb. Bands indicated with arrows for clarity. The bands expected for the wild type DNA are too small to appear on this gel at 100bp and 198 bp respectively. One biological replicate.

Figure 58 shows the agarose gel for the confirmation PCR for the transformed and wild type genomic DNA. Primer 1 was designed to anneal outside the construct locus, upstream of the *CspD* promoter in the *ugtP* gene (figures 57 and 59). Primer 2 was designed to anneal outside the construct locus, after the downstream homology, in the *degR* gene (figures 57 and 59). Primer 3 was designed to anneal part way down the *cspD* locus for wild type, at 100 bp, and partway down the PEt and *ldh* cassettes, at 1.5 kb, which should give PCR products at 1.5 kb (see figures 57 and 59). Primers 1 and 2 annealed outside the *cspD* locus were used for proof PCR, amplifying the whole region in that locus i.e. 198 bp for wild type, ≈ 3.5 kb for *ldh* and ≈ 6kb for Pet.

No bands for the wild type DNA were present, as they were two small to be visualised on this gel. There are midway confirmation bands in both the Pet (lane 3) and *ldh* (lane 4) transformations at approx. 1.5 kb as expected (figure 58).

There are no bands indicating the region for Pet ≈ 6 kb (fig 59 b lane 7), however there is a band at ≈ 3.5 kb for the *ldh* (lane 8), as expected (figure 58), as the primers amplifying out the whole construct. We can be confident that the *ldh* cassette has successfully inserted in the *cspD* locus, with both midway fragments and full region bands of the correct size, but the Pet cassette has not.

Since the $^{\Delta}$*cspD*::*pdc*:*adh*B^{+}Rkan cells were resistant to chloramphenicol (see section …) it is possible the resistance cassette integrated somewhere else with in the genomic DNA, which may explain why there is a midway fragment for this strain (figure 59b) or that the polymerase was unable to amply the full 6kb (figures 57 and 58).

6.4.2 Sequencing

The insertion locus from B. subtilis 168 ΔcspD::ldh+Rcml was amplified out of extracted genomic DNA of the transformed B. subtilis using primers that anneal upstream and downstream of the cspD locus, as above. These were sent for sequencing with appropriate primers. DNA sequencing was performed by the GenePool services of the University of Edinburgh with ABI 3730 Sanger technology platform. The attempt to sequence the excised DNA failed.

6.4.3 Production of crude cell extracts

Once crude extracts of the wild type and transformed *B. subtilis* strains had been produced, the total protein concentration of each extract was tested. As figure 59 shows the concentrations present in original samples was very low. To increase efficiency of activity and PAGE assays, the crude extracts were concentrated down to a theoretical x100 concentration (5000 µl concentrated to 50 µl). A sample of this was then assayed for total protein again and as figure 59 shows the increase in concentration was not x 100 but approx. 2-3 times the concentration, possibly due to operator error or equipment failure.

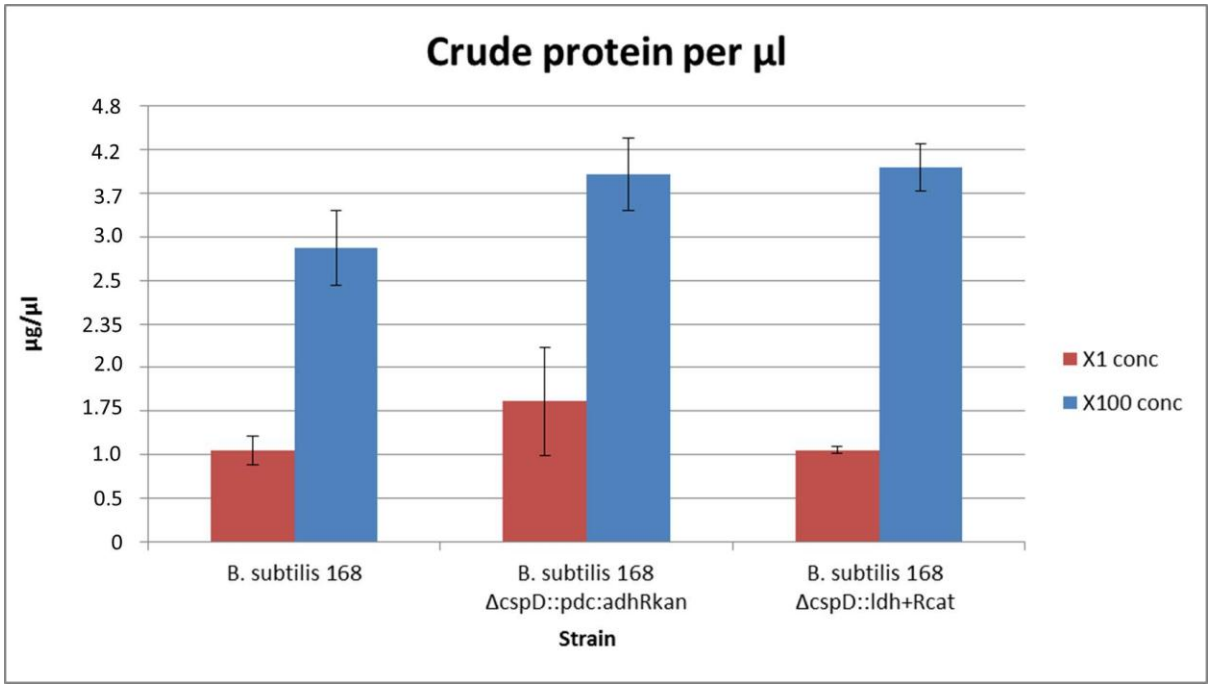


Figure 59 - Amount of crude protein (µg/µl) in sonicated cell extracts for wild type and transformed *B. subtilis.* The concentrated crude protein was used for enzyme activity assay, SDS and native gels. Representative of three biological replicates, error bars show standard error.

These concentrated crude extracts were used for PAGE analysis to detect both enzyme activity and protein size, to confirm that the enzymes coded for in the genetic cassette were transcribed and active.

6.4.4 Native and SDS PAGE gel empirical analysis

Since we know the action of these enzymes and their size we are able to use native gel activity stains and SDS to determine the presence of enzyme at the right size and stain for their activity.

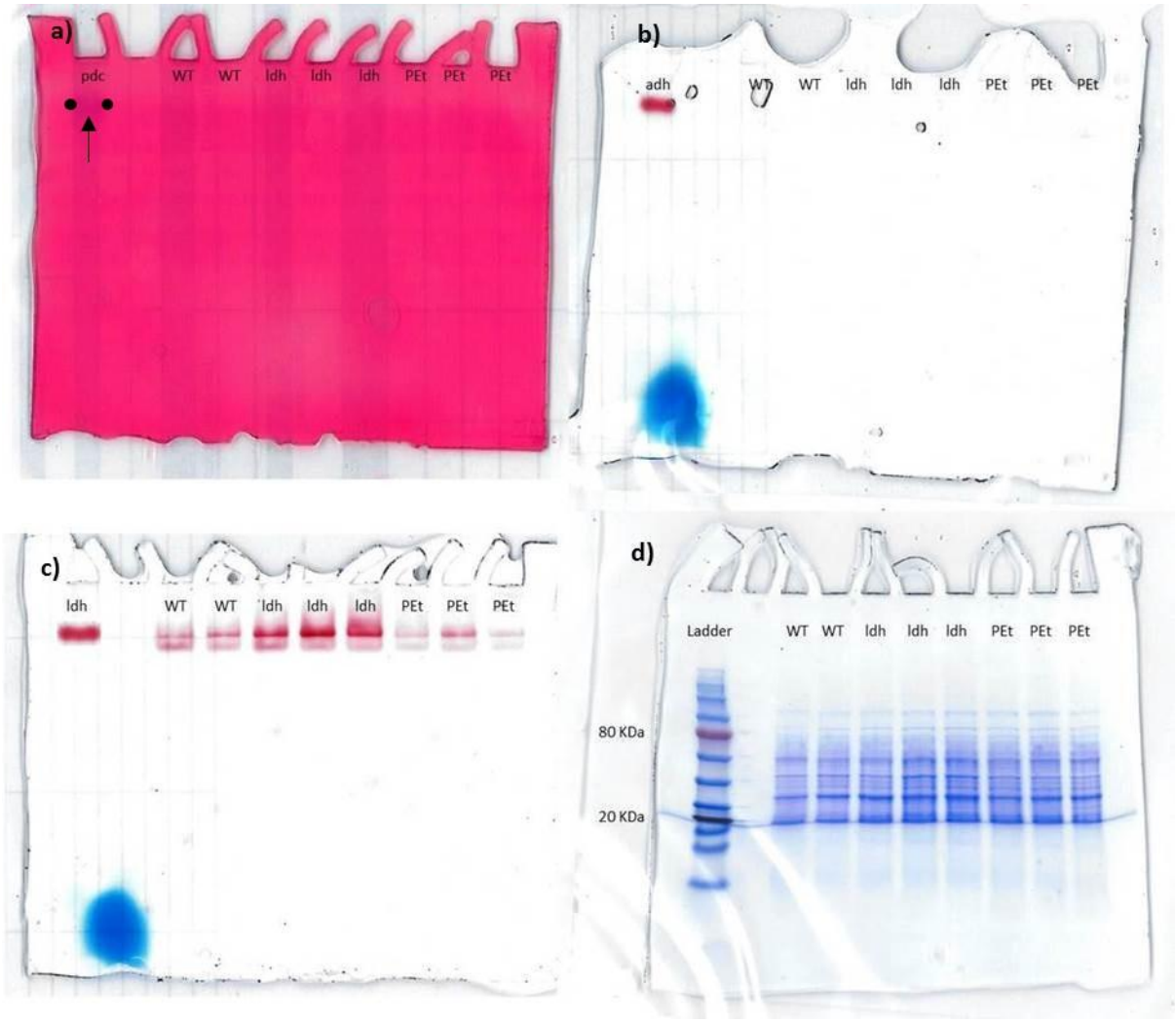


Figure 60 - Native and SDS PAGE gels. The size of fused Pdc:Adh is 98kDa (Pdc 58 kDa and Adh 40 kDa) and native L-Ldh is 35 kDa. Native gels were stained with appropriate stain (see chapter two) to detect expressed enzymes (pdc, adh and ldh). SDS PAGE gel was run to detect expressed enzymes by size. Representative of four technical replicates of two biological replicates for wild type and three biological replicates for both *B. subtilis* 168 $^{\Delta}$*cspD*::*pdc*:*adh*B^{+}Rneo (PEt) and *B. subtilis* 168 $^{\Delta}$*cspD*::*ldh*$^{+}$Rcml (ldh).

Figure 60 shows that there was no activity stain for the actions of Pdc or Adh, other than the control markers in the first lane (fig 60a and 60b), but there is clearly heavy staining in the over expressed Ldh gel (fig 60c). Ldh is a native enzyme which is why there is some staining in the WT and Pet lanes. There is no band for fused Pdc:Adh in the SDS gel (fig 60d). Ldh is a native protein and there seems to be a slightly more intense band at around 34KDa in all three Ldh lanes (fig 60d).

To stain native gels for pdc activity, the stain used is Schiff's reagent, which shows pdc activity by staining the aldehyde in acetaldehyde that is formed as pyruvate is oxidised to acetaldehyde (Vogel, 1989, Zhou et al., 2009). Other than the marker in the Pdc lane, no pdc activity is detected in this gel (fig 60a). The activity stain for Adh and Ldh is of a similar composition, other than inclusion of ethanol and lactic acid respectively. The enzyme activity drives the oxidation of NADH, which in turn drives PMS, then INTV to produce a red stain (see section 1.9, 1.10 and 2.4.11-2.4.13).

6.4.5 *Enzyme activity assay*

To determine whether the crude extracts contained active *ldh* and/or fused *pdc*:*adh* proteins a sample of the crude extract was used as the catalyst in a colorimetric assay.

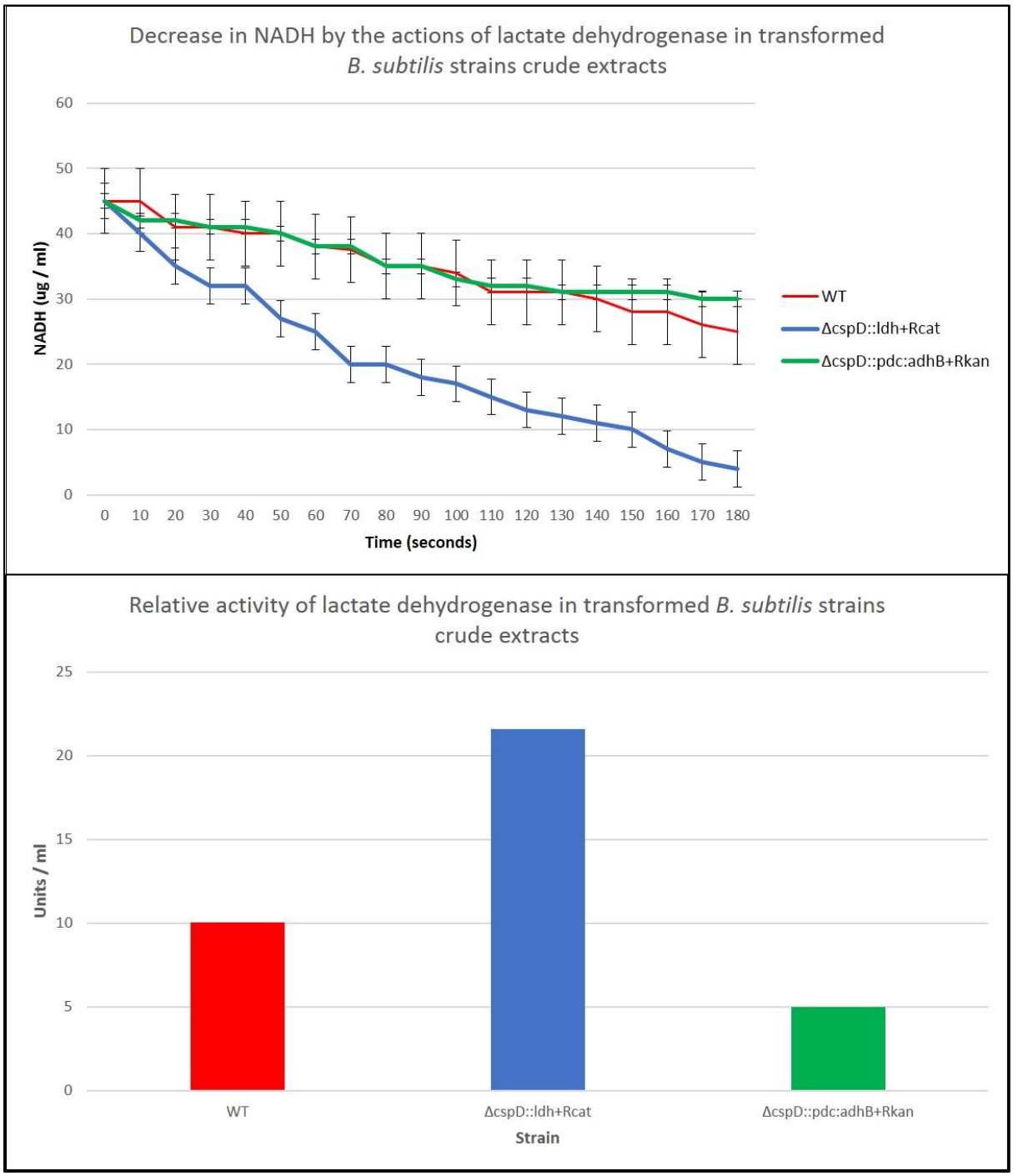


Figure 61 - Activity of over expressed lactate dehydrogenase in transformed *B. subtilis* compared to WT. Assay measures the oxidation of NADH to NAD^+ by *ldh* in one millilitre reaction mix. The rate of oxidation of NADH to NAD^+ was used to calculate the specific activity of *ldh* units per millilitre of reaction mix. Representative of three biological and three technical replicates. Error bars represent standard error.

Figure 61 shows that the decrease in the optical density of the reaction mix containing cell extracts from $^{\Delta}$*cspD*::*ldh*$^{+}$Rcat as the catalyst is markedly greater than either $^{\Delta}$*cspD*::*pdc*:*adh*B^{+}Rkan, the wild type *B. subtilis* 168 and well above the background decreases. This indicates that the reaction; converting pyruvate to lactic acid, is occurring and that this cell extract contains active *ldh* and that this *ldh* is either in greater quantities or more active than the *ldh* in wild type *B. subtilis* 168 and $^{\Delta}$*cspD*::*pdc*:*adh*B^{+}Rkan.

6.4.6 *Presence of lactic acid in the supernatant of wild type and transformed B. subtilis*

Wild type *B. subtilis 168* and *B. subtilis* Δ*cspD*::*ldh*$^{+}$Rcat cells were grown in LB overnight, either with or without 0.5% glucose, and/or aerobically or anaerobically and either subjected to cold shock treatment or not and the supernatant of the culture assayed to determine whether any lactic acid was present.

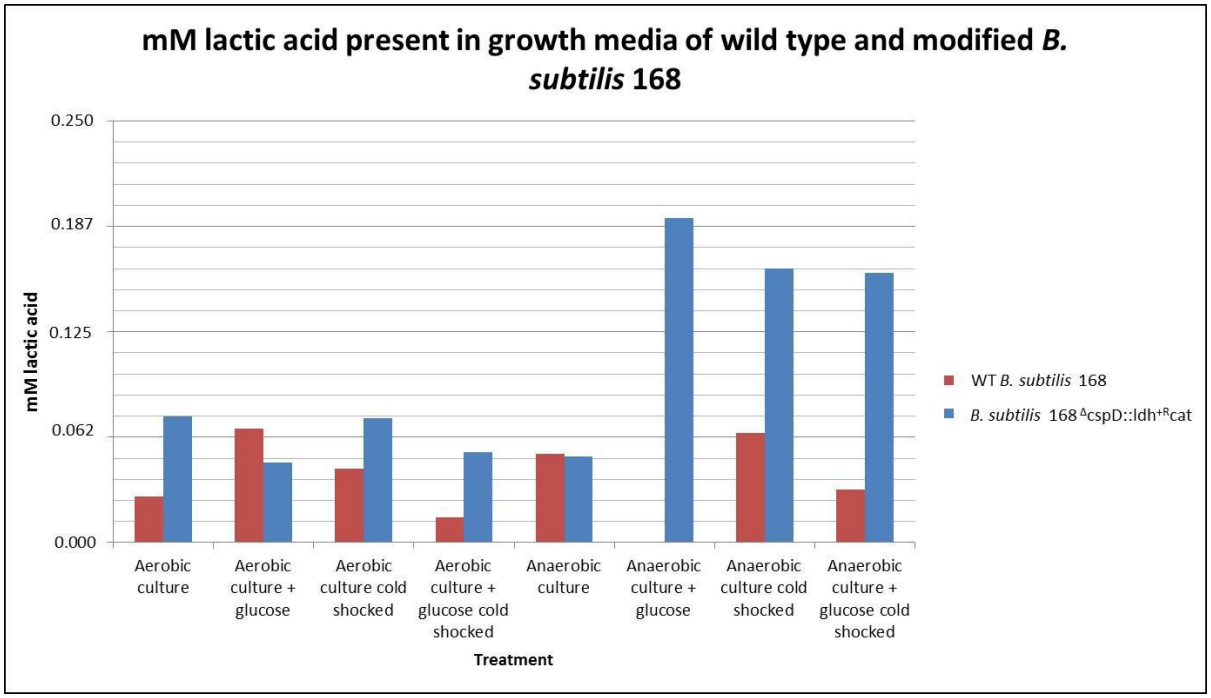


Figure 62 - Lactic acid (mM) present in the growth media of wild type *B. subtilis* 168 and *B. subtilis ΔcspD::ldh*$^{+}$*Rcat* engineered to over express lactate dehydrogenase after overnight growth at 30°C, grown both aerobically and anaerobically and with and without the presence of glucose. Representative of one biological replicate.

When grown aerobically, the modified *B. subtilis* produced more lactic acid than the wild type, without glucose and more lactic acid than the wild type when subjected to cold shock treatment, regardless of the presence of glucose (figure 62). This may mean it is possible to use this modified strain to produce lactic acid in co- culture with *Cytophaga hutchinsonii* as *C. hutchinsonii* can only grow aerobically.

When the modified *B. subtilis* is grown anaerobically the modified strain produces more lactic acid than the wild type. The modified strain produces larger amounts of lactic acid when glucose was present in the LB media compared to levels of lactic acid levels produced by it in plain LB.

No lactic acid was detected in the wild type cultures grown anaerobically with glucose (figure 62). Replicates are needed to verify these results.

6.5 Short summary and discussion

We attempted to transform *Bacillus subtilis* 168 to over express native **l**actate **de**h**y**drogenase (Ldh) and express the fused protein Pyruvate decarboxalase:alcohol dehydrogenase (pdc:adh, denoted as Pet, **P**roduces **Et**hanol) (Lewicka, 2014) under the native cold shock protein D (*cspD*) promoter. The Ldh cassette was constructed using Splice and Overlap Extension PCR and the Pet cassette was constructed using the Paperclip method (Trubitsyna et al., 2014). *B. subtilis* competent cells transformed with the Pet cassette showed resistance to chloramphenicol, but did not show any activity for the Pdc:Adh cassette in *in vitro* enzyme activity assays, native gels or SDS PAGE gel nor was its presence detected through proof amplification PCR or sequencing. It is likely only the resistance cassette integrated into the *B. subtilis* genome.

However, *B. subtilis* cell transformed with the Ldh cassette, showed activity for the cassette *in vitro* enzymatic action assays, native gels and proof amplification PCR. We did not definitively detect the over expressed protein on a SDS PAGE gel as Ldh is a native protein so would be present in WT *B. subtilis*, i.e. there were bands at 35KDa in every lane of the gel, nor were we able to get a reliable sequence extracted genomic DNA from the transformed *B. subtilis*. This could be overcome by using southern blot and His tag protein purification assays.

When *B. subtilis* was modified to over express Ldh, it produced more lactic acid than the wild type, both anaerobically and crucially aerobically, suggesting that this strain could be grown in aerobic co-culture with *C. hutchinsonii*, to produce lactic acid.

7 Discussion, conclusion and further work

7.1 Biomass selection and physio-chemical pre-treatments

7.1.1 Monosaccharide profiles of common biomass

To determine the suitability of different sources of biomass for microbial conversion to useful products the potential available sugar from that biomass needs to be established. To that end a selection of biomass was subjected to incomplete TFA acid hydrolysis and the resultant hydrolysate screened by thin layer chromatography to show the monosaccharide profiles.

Different sources of biomass present different ranges of potential monosaccharides that can be released. In order to make the system outlined here more efficient it is necessary to know which monosaccharides will be released from which biomass stream and subsequently which monosaccharides the micro-organisms in this study are able to utilise.

Using incomplete TFA hydrolysis, filter paper yields mainly glucose which is derived from cellulose, which can yield cellobiose –triose. The filter paper also yields a small amount of xylose, most probably derived from xylans or possibly from xyloglucans (Donaldson and Knox, 2012). If other monosaccharides were detected i.e. arabinose this would suggests the presence of arabinoxylans etc. The presence of xylose presents an interesting opportunity if other, cheaper, sources of paper such as office paper or newspaper yield mainly glucose, this would provide a cheaper carbon source than pure glucose (filter paper is £330 / kg whereas pure glucose powder is £5 / kg (Sigma) office paper and newspaper, however, are cheaper).

Shredded office paper yields large amounts of xylose as well as glucose. The larger amount of xylose is again probably derived from xylans and/or xyloglucans. Xylose is present in higher amounts as the alkali / hemicellulose removal step for office paper is not as intensive as it is for filter paper, as there is less need for the former to be as pure as filter paper. It would be possible to determine which cell wall polymer the xylose came from by digesting the office paper in Driselase, which would yield isoprimeverose (xyl-glc) if the source was xyloglucans or xylobiose (xyl-xyl) if the source were xylans (Fry, 1988, Kerr and Fry, 2003, Popper and Fry, 2005). The presence of xylose in office paper may present another opportunity if one or both of the microbial partners in a co-culture can grow using either glucose or xylose or both. A rough visual estimate of fig 20 suggests that there is an equal amount of glucose and xylose released by the TFA (trifluoroacetic acid) hydrolysis of 10 mg of shredded office paper (exact amounts would need to be determined using high pressure liquid chromatography [HPLC]). Extrapolating up, if one microbial partner only uses glucose for growth and the second partner only uses xylose an efficient system for utilising shredded office paper as the main carbon source for microbial co-cultures presents itself.

Newspaper yields a broader range of monosaccharides, probably due to less intensive chemical pre-treatment as there is no need of purity for newspaper print and/or due to the incorporation of cardboard and other recycled materials. When TFA hydrolysed, 10 mg of newspaper yields galactose, glucose, mannose, arabinose, xylose and rhamnose. The range of monosaccharides yielded by this cheap carbon source, gives it an advantage over filter paper and office paper as carbon sources, provided that the micro-organisms used in this study can utilise the sugars released as described above. Paper also has the advantage of not needing any expensive chemical or physical pre-treatment before the monosaccharides are made available by the cellulose degrading partner, which makes any process using them as the main carbon source cheaper, more so than the low price of the biomass alone.

Wheat straw is a natural by-product of food production so unlike *Miscanthus* does not compete with food production for land, a fact that needs to be taken into consideration in a world of seven billion, facing the uncertainty of climate change, although utilising wheat straw in this way does stop it from being incorporated back into the soil, a process that returns nutrients to the soil and improves soil structure. Wheat straw yields, galactose, glucose, mannose and larger amounts of arabinose and xylose (fig 19). The relatively small amount of glucose in comparison to arabinose is due to the fact that TFA cannot hydrolyse crystalline cellulose, which accounts for up to 90% of cellulose in plant cell walls (Albersheim, 2011) as the wheat straw has not been subjected to any chemical pre-treatments at this point (see sections 2.2.2., 3.3.2 and 3.3.3) and as such will remain crystalline until the sodium hydroxide pre-treatment breaks the hydrogen bonds in the microfibrils, turning the cellulose amorphous (Fry, 1988). This fact may also account for the lack of mannose, as mannose is found in the secondary cell walls as heteromannan which is also crystalline. Although the biomass did contain starch (identified by iodine staining) this starch was probably removed during the alcohol insoluble residue (A.I.R.) production, in the initial water: ethanol steps. We would expect to see mannans/mannose, as the wheat straw is senesced plant matter and therefore should have a higher amount of secondary cell walls, compared to actively growing plant material (Albersheim, 2011). The large amount of arabinose and xylose are probably derived from arabinoxylans and xylans, as arabinoxylans are the predominate hemicellulose on monocot plants (Albersheim, 2011) and so will yield arabinose and xylose when hydrolysed. Xylans will only yield xylose when hydrolysed, which accounts for a larger amount of xylose compared to arabinose, since both cell wall polymers are present in the wheat straw. Unlike the papers wheat straw also contains up to 30% lignin (Scheller and Ulvskov, 2010, Albersheim, 2011) and so requires physiochemical pre-treatments before the cell wall polymers are exposed for saccharification and for the micro-organisms to utilise. This lignin could be removed and sold to improve the economics of this system, as lignin and its derivatives

have a wide range of uses. However, wheat straw would be a suitable source of biomass if the microbial partners especially favoured pentose sugars for growth.

Unlike wheat straw, *Miscanthus sinensis* does compete for growing space with food crops and has higher lignin content than wheat straw, so needs more pre-treatment before it is suitable for use as a microbial carbon source (figs 22 and 23). When TFA hydrolysed there was a small amount of glucose released, again this is due to the crystallinity of the untreated cellulose, and again the lack of mannan from a biomass rich in secondary cell walls, is probably also due to the crystalline structure of the mannan, which is resistant to TFA hydrolysis. Figure 20 visually shows an apparently lower amount of arabinose and a greater amount of xylose compared to wheat straw, showing that biomass with similar monosaccharide profiles yield different amounts of those monosaccharides, due to the different amounts of cell wall polymers they contain. Again, this is a rough visual estimate and needs to be verified with HPLC. *Miscanthus sinensis* would also be a suitable biomass for a co-culture that could utilise arabinose and xylose, both of which were derived from arabinoxylans, as arabinoxylans are also the predominant hemicellulose in *Miscanthus* spp. (Albersheim, 2011).

The biomasses used here provide a range of monosaccharide profile and pre-treatment requirements. Whilst filter paper and office paper require no pre-treatments, they only yield a limited range of monosaccharides, being limited to glucose and xylose, the latter being in greater amounts in office paper, whilst wheat straw and *Miscanthus sinensis* biomass have a wider range of monosaccharides with arabinose and xylose being predominant in untreated samples, but they do require pre-treatments, particularly an alkali pre-treatment to reduce the crystallinity of cellulose and mannans, for the full range of cell wall polymers to become accessible for biological saccharification. Fortunately newspaper has the widest range of monosaccharides from the biomass studied here and requires no pre-treatments, other than a solvent wash to remove inks, for its sugars to be accessed and so could provide the carbon source for a range of co-cultures depending on the monosaccharide utilisation profiles of their microbial partners.

7.1.2 Development of simple pre-treatments

Simple visual assays (figs 20 and 21) showed that *C. hutchinsonii* and *C. fimi* do not seem to grow in the longer term (28+ days) on untreated *Miscanthus sinensis* biomass, from the lack of obvious physical degradation (fig 20 and 21) probably due to the presence of undisrupted cutin and lignin (Albersheim, 2011). As figure 21 and 22 show, after 28 days in liquid culture with either *C. hutchinsonii* or *C. fimi*, there was no obvious degradation of the non-pre-treated biomass, by either micro-organism, however, monosaccharides were released as visualised on the TLC plates (fig 20 and

21) suggesting bacterial cells survived long enough to produce lignocellulosic degrading enzymes, which may have still been present in the media *post mortem*.

As figure 21 shows, between zero hours and 240 hours the enzymatic actions of *C. hutchinsonii* resulted in the release of: glucose, a small amount of xylose and rhamnose. The glucose is used up by six days and the xylose is used up after 7 days. The amount of rhamnose does not appear to change, as it is not used up, although we would expect there to be an increase in the amount of rhamnose as more should be released over time by the enzymatic actions of *C. hutchinsonii*, when none is used. This shows that enzymatic actions of *C. hutchinsonii* cause biochemical changes in the biomass (Brenner, 2007, Xie et al., 2007, Rubin, 2008, Wilson, 2009, Wilson, 2011, Liu, 2012, Duedu, 2015). The glucose released will have been utilised by the *C. hutchinsonii* for growth, the absence of xylose was not expected as figures 30-32 demonstrate that *C. hutchinsonii* cannot use xylose for growth either as single carbon source or in a mixed sugar culture.

Figure 22 shows that the enzymatic actions of *C. fimi* resulted in the release of rhamnose from zero hours, none of which was used by the *C. fimi*; cellobiose was released after 48 hours and used up after ten days, being absent at 28 days as expected (Kane, 2014); xylose was released after 72 hours but was not used. It is assumed that any glucose released was immediately used by the *C. fimi*, hence the absence of the spot, but we know that *C. fimi* uses glucose and we also know it has enzymes which would result in the released of glucose amongst other monosaccharides (Chen et al., 2012, Srivastava, 2012, Kane, 2014, Duedu, 2015) and that enzymes are being produced hence the presence of other monosaccharides. Since *C. hutchinsonii* and *C. fimi* cannot survive long term on untreated biomass, physio-chemical pretreatments are needed.

Wheat straw was processed in a normal food blender until pieces were less than 1 cm. This served to increase the surface area of the biomass, disrupt cutin and lignin. Increasing the surface area of the biomass allows for more efficient chemical pre-treatments as the chemicals are able to reach more of the biomass and exposed cell wall polymers at different levels in the biomass. This milled biomass was then subjected to liquid hot water treatments which potentially removes acetyl- groups from the side chains of hemicellulose (Scheller and Ulvskov, 2010, Li, 2013, Eiteman, 2014, Cuevas et al., 2014) a process that reduces the amount of acetic acid, an inhibitory compound (see chapter one, section 1.3.8) produced by later pre-treatments. The liquid hot water treatment also hydrolyses some hemicellulose, releasing monosaccharides into the liquid fraction for later recovery (see section 3.3.5 and figure 24) and exposes or unmasks more of the cellulose to enzymatic/chemical degradation, increasing the efficiency of the biological saccharification process (Li, 2013, Xue et al., 2013). As figure 25 shows, the liquid hot water (C.A.L.) pre-treatment of wheat straw, results in the

release of cellobiose and glucose (as opposed to mannose as the spot is red, not orange/brown). However, figure 25 shows that this pre-treatment resulted in a reduction in the abundance of the LM11 epitope, which recognises the 1→4 β linkage (xyl-xyl) in xylan/arabinoxylan (McCartney et al., 2005), throughout the cortex of the biomass, apart from the vascular bundle, so we would expect there to be xylose or xylose and arabinose (dependent on cell wall polymer affected) in the C.A.L. liquid fraction. This glucose is probably not from cellulose as the majority of the cellulose (≈ 90%) is still crystalline and hot water is not sufficient to hydrolyse the 1→4 linkage and the inter-fibril hydrogen bonds (Fry, 1988). The glucose may be from the hydrolysis of the 1→3 linkage of mixed linkage glucans, which are not crystalline, or from the hydrolysis of starch present in the biomass (as indicated by iodine staining, data not shown). The C.A.L. solid fraction yielded the same monosaccharide profile as wheat in figure 24. C.A.L. pre-treated biomass was used for all subsequent pre-treatments.

The C.A.L. biomass was then subjected to sodium hydroxide pre-treatment to reduce the crystallinity of the cellulose by breaking the hydrogen bonds between microfibrils, exposing the 1→4 β linkage to chemical hydrolysis and also to break the hydrogen bonds linking cellulose to hemicellulose (Fry, 1988, Wang, 2008, Albersheim, 2011). Sodium hydroxide has also been shown to remove lignin (Shi et al., 2014) which would go some way to explaining the results in Chapter five, section 5.3.3, figure 51, with the absence of lignin making the degradation of wheat straw easier. No monosaccharides were detected in the neutralised hydrolysate (fig 24), which is due to the sodium hydroxide only breaking the hydrogen bonds within and between the cell wall polymers, but not hydrolysing those cell wall polymers (Fry, 1988). This also explains the reduction in the signal for the LM11 epitope (fig 25), which recognises the 1→4 β linkage (xyl:xyl) backbone of xylans/arabinoxylans (McCartney et al., 2005) (see chapter three, section 3.3.3, figure 25), in the vascular bundle of tissue samples (see fig 25). The hydrogen bonds between the xylan/arabinoxylans have been broken by the actions of the sodium hydroxide and the hemicellulose was extracted from the tissue but not hydrolysed (Fry, 1988). When TFA hydrolysed the residue/ solid fraction from this pre-treatment yielded galactose, glucose, arabinose and xylose (see fig 27) indicating that these monosaccharides will be available to the microbial partners in a co-culture after enzymatic/bacterial degradation and that this residue would be amenable to such digestion after this pre-treatment.

The acid hydrolysis pre-treatment consisted of immersion of the biomass in 1M hot 50^{o}C sulphuric acid for two hours. The solid and liquid fractions were separated and neutralised. Figure 24 shows that the sugars present in the hydrolysate were: cellulose derived oligosaccharides, a small amount of glucose and arabinose and a large amount of xylose. The glucose was possibly derived from starch

present in the biomass, detected by iodine staining, or from mixed linkage glucans, but we would expect more glucose than this to be yielded from these polymers, so it is likely that the glucose came from the hydrolysation of amorphous cellulose as 1M sulphuric acid at 50^{o}C is not sufficient to hydrolyse crystalline cellulose. The hydrolysis of only amorphous cellulose which makes up only ≈10% of cellulose, unless alkali pre-treated, explains the low amounts of glucose released. The arabinose and some of the xylose is derived from arabinoxylans, but the majority of the xylose will be derived from xylans. This is because arabinoxylans have an approximate ratio of 3:1 (xyl:ara) (Albersheim, 2011) so if the xylose were solely derived from arabinoxylans, we would expect much more arabinose. We would also see other monosaccharides if the xylose were derived from any other xylose containing cell wall polymers such as pectin (Scheller et al., 2006). This assertion is further corroborated by the reduction in the LM11 epitope, which recognises the 1→4 β linkage (xyl:xyl) backbone of xylans/arabinoxylans (see chapter two, section 2.2.4, figure 19). The signal for the LM11 epitope in H_2SO_4 pre-treated biomass, is lower than in the samples subjected to C.A.L. and sodium hydroxide pre-treatments (fig 25) which is to be expected since the sulphuric acid pre-treatment is a harsher pre-treatment than hot water, however, unlike with sodium hydroxide, the hemicelluloses (arabinoxylan and/or xylans) are being hydrolysed to their monosaccharides rather than simply being removed intact (Fry, 1988). This monosaccharide poor residue was expected as sulphuric acid is a very effective pre-treatment designed to hydrolyse a large amount of cell wall polymers to monosaccharides, so the liquid fraction can be used as carbon source for biofuel production (Blakeney, 1983, Cuevas et al., 2014, Kharina et al., 2016).

The enzymatic actions of *C. hutchinsonii* and *C. fimi* did not result in the release of any monosaccharides from the untreated biomass (fig 24) but any released may have been utilised by the bacteria straight away. They also appear to have had a lesser effect on the abundance of the LM11 epitope than did the sodium hydroxide and sulphuric acid pre-treatments. This was expected as previous work showed that *C. hutchinsonii* and *C. fimi* were unable to utilise untreated biomass for growth (see above). While figures 22 and 23 show that the enzymatic actions of *C. hutchinsonii* and *C. fimi* resulted in the release of some monosaccharides from the untreated biomass over one month, the time frame used in this assay was much shorter for ease of replication and comparison. When wheat straw was digested with 1% Driselase in P:A:W buffer some glucose was released (fig 24) and Driselase digestion resulted in a reduction of the LM11 epitope in the cortex, compared to the untreated sample (fig 25) but not in the vascular bundle, which suggests a masking of cell wall polymers.

7.1.3 Sequential pre-treatments

The dual pre-treatment of sodium hydroxide and then sulphuric acid resulted in the release of small amounts of cellulose derived oligosaccharides and xylose although the latter was in far smaller amounts than were released by the sulphuric acid treatment only (fig 24), which was expected as the hemicellulose (arabinoxylan/ xylan) had already been removed by the sodium hydroxide stage (Fry, 1988). Figure 26 shows that there is almost a complete lack of the LM11 epitope after the sequential use of sodium hydroxide and sulphuric acid. It can be argued that this indicates a total lack of arabinoxylan/xylan in the tissue, particularly when you consider the effects these pre-treatments have on the cell wall polymers; the sodium hydroxide facilitates the removal of arabinoxylan/xylan by breaking the hydrogen bonds between cellulose and the arabinoxylan/xylan and then the sulphuric acid hydrolysed any remaining arabinoxylan/xylan or any arabinoxylan/xylan exposed by the removal of the previous layer (Xue et al., 2013). With the arabinoxylan/xylan removed there was nothing for LM11 antibody to bind to, hence little or no signal. The xylose present in the pooled hydrolysate (fig 24) is from the hydrolysed xylan after these pre-treatments. These pre-treatments resulted in a residue that contained a small amount of glucose and xylose, from hydrolysed amorphous cellulose and any remaining xylans (fig 27). The absence of arabinose from this residue again suggests that any arabinoxylans were removed by the sodium hydroxide treatment.

The chemical sodium hydroxide and sulphuric acid pre-treatments followed by the biological Driselase pre-treatment of wheat straw resulted in the release of a comparatively large amount of glucose and xylobiose (fig 24) (the latter identified by colour and position as we lacked the appropriate marker) (Fry, 1988, Kerr and Fry, 2003) but a miniscule amount of xylose. This verifies that at least one of the xylose containing cell wall polymers was xylan, as Driselase digestion resulting in the release of xylobiose is a diagnostic tool for identifying the presence of xylans (Kerr and Fry, 2003, Scheller and Ulvskov, 2010). Driselase also contains a range of endo- and exo-glucanases and cellulases (Fry, 1988) so it is able to digest both crystalline and amorphous cellulose, although the majority of the remaining cellulose would now be amorphous due to the actions of the sodium hydroxide in this treatment (Fry, 1988). *In situ* labelling shows an increase in the LM11 epitope (fig 27) after this three-way pre-treatment compared to sodium hydroxide and sulphuric acid treatments, which suggests that Driselase is unmasking more arabinoxylan/xylan. If you compare the results of this three-way treatment with the effects of sodium hydroxide, sulphuric acid and Driselase on their own, this three-way treatment seems to act by the chemical treatments removing some unidentified cell wall polymer, this then allows the Driselase to digest whatever is

masking the lower arabinoxylan/xylan. The chemicals remove the (hypothetical) first layer and the Driselase removes the (hypothetical) second layer, revealing the arabinoxylan/xylan present on the (hypothetical) lower third layer. Interestingly this result is very different to that when sodium hydroxide and sulphuric acid pre-treated wheat straw is digested by the enzymatic actions of *C. hutchinsonii* and *C. fimi*. Chemically pre-treated wheat straw biomass exposed to the enzymatic actions of *C. hutchinsonii* released only a very small amount of xylose into the liquid fraction with even less (if any) xylose released by the enzymes of *C. fimi* (fig 24), which implies that a much wider enzyme profile maybe needed to efficiently degrade lignocellulosic biomass, than is offered by *C. hutchinsonii* and *C. fimi*, a fungal degrader partner may be needed, similar to the one Driselase is isolated from. This seeming lack of saccharification is due to the time scale used in this assay, 72 hours, which was used for ease of replication and comparison because as figures 21 and 22 shows, both of these organisms can biochemically pre-treated wheat straw and as fig 51 shows, *C. hutchinsonii* is able to utilise pre-treated wheat straw as the main carbon source for growth, even in co-culture, where the demands on available sugar are higher. Any monosaccharides released in this 72-hour window will probably have been used by the bacteria for growth, other than the xylose present in fig 24, which neither micro-organism can use (sections 4.5.2, 4.5.3 and figures 30-35).

The difference in the biochemical changes caused by *C. hutchinsonii* and *C. fimi* and by those caused by Driselase are interesting in that, as figure 28 shows, the *in situ* labelling of the cell wall polymers exposed to *C. hutchinsonii* and *C. fimi* after the sodium hydroxide and sulphuric acid, show a total lack of the LM11 epitope, just as they do when sodium hydroxide and sulphuric acid alone are used as outlined above. But, after the sodium hydroxide and H_2SO_4 pre-treatments, Driselase seems to unmask arabinoxylan/xylan whereas *C. hutchinsonii* and *C. fimi* do not. This implies that there is an enzyme present in the profile of Driselase that is not present on the enzyme profile of *C. hutchinsonii* or *C. fimi*. Driselase is isolated from the brown rot fungus *IPEX lacteus* (Fry, 1988) which may account for any differences but more time should be allocated to determining which enzyme in Driselase is responsible for the unmasking and compare it to *C. hutchinsonii* and *C. fimi*, since the complete enzymatic profiles of all three are known (Fry, 1988, Kane, 2014) to make the system more efficient.

7.1.4 Other antibodies

Other than very weak signals for LM25 at the cell junctures and for LM19 and LM25 in the vascular bundle there is no other signal for LM19, LM21 and LM25 when compared to the blank negative control so it is impossible to say whether the pre-treatments had an effect on these epitopes and any perceived changes may in fact be false positives. We know that these antibodies are active as they all show signal in the tobacco tissue positive controls, as the antibodies were raised against

tobacco (McCartney et al., 2005, Verhertbruggen et al., 2009, Marcus et al., 2010, Pedersen et al., 2012). There are antibodies available that have been specifically raised against epitopes derived from grass cell walls, which may give better results. The lack of signal for the LM21 epitope, which is associated with heteromannan, is unexpected, as mannans are found in secondary cell walls and the tissue used in this study had senesced when it was harvested, so should have been rich in secondary cell walls (Fry, 1988, Albersheim, 2011). There are antibodies available that have been raised specifically against epitopes from grass cell walls, however the ones used in this study were provided *gratis*, and time constraints prevented further use of antibody labelling, as lignin specific antibodies were also envisaged being used.

7.2 Hydrolysate growth

As these pre-treatments resulted in a monosaccharide containing hydrolysate, monosaccharides that are released will be wasted unless utilised by the micro-organisms in this study. To this end, 100 ml of pooled hydrolysate was neutralised and was either used as was or filtered through activated charcoal to remove any inhibitory compounds, as per the literature (Cuevas et al., 2014). The hydrolysate was then supplemented with appropriate salts to make a growth medium dependent on the micro-organism screened. Chapter four, figure 28 shows that there was no change to the optical density of the unfiltered hydrolysate after ten days' incubation and the only growth detected was that of B. subtilis 168, with a CFU count of 11x106 (fig 28). When compared to growth of B. subtilis 168 in DMS3 alone, this cannot be said to be growth (see chapter four, section 4.5.4). With the filtered hydrolysate, there was only an increase in the optical density after ten days' incubation from 0.000 to 0.060-0.140 and in the B. subtilis 168 culture, which gave a CFU count of ≈ 6x106 (fig 28). When compared to growth in DSM3 alone, this is not growth. It is clear that unfiltered hydrolysate contains compounds which inhibit microbial growth, most likely furfurals and acetic acid and that filtering through activated charcoal will remove these compounds (Cuevas et al., 2014); however, this filtering also removes any monosaccharides in the hydrolysate (fig 29), which was unexpected and contrary to the literature (Cuevas et al., 2014). It was suggested that the removal of monosaccharides was due to the filtration system failing to take into account the polarity of the compunds present in the hydrolysate, due to operator error. Under the system described here, it is not possible to utilise the monosaccharides present in the pre-treatments hydrolysate and as such, they are wasted. Another method of removing inhibitor compounds without also removing the monosaccharides needs to be devised or the micro-organisms need to be engineered to tolerate the inhibitory compounds (Liu et al., 2009, Eiteman, 2014, Xiros and Olsson, 2014).

7.3 Conclusions

Common sources of lignocellulosic biomass have a wide range of cell wall polymers, depending on the biochemistry of the plant, which are made from different combinations of the seven cell wall polymer monosaccharides. These monosaccharides are released from cell wall polymers in differing quantities and in different sequences depending on the physio-chemical pre-treatment. These pre-treatments can unmask cell wall polymers for sequential degradation and increase surface area, reduce cellulose crystallinity, break hydrogen and covalent bonds and hydrolyse the cell wall polymers to their constituent monosaccharide building blocks, leaving behind a polysaccharide containing substrate that is more amenable to enzymatic digestion, while the hydrolysate provides a monosaccharide containing liquid. However, this may also contains inhibitory compounds which adversely affect microbial growth (section 3.3.5). The compounds present in the hydrolysate inhibit bacterial growth and while filtering through activated carbon removes them, it also removes many of the monosaccharides as well, so developing organisms with a tolerance to these compounds, as opposed to simply removing them, is an important step in making the utilisation of lignocellulosic biomass an economic reality.

7.3.1 Limitations and further experiments

Optimisation of the pre-treatments for wheat straw and *Miscanthus sinensis* is needed to ensure that the maximum amount of sugars are released and utilised. To that end, a system that also incorporates the removal of lignin as part of the pre-treatments needs to be developed and a method of removing inhibitor compounds present in the liquid fractions of the pre-treatments is needed, one that will leave the sugars present for microbial utilisation. The *in situ* labelling assay should be repeated and should include antibodies more likely to bind to monocot plant tissue, as the tissue is pectin (LM19) and xyloglucan (LM25) poor (Albersheim, 2011).

7.4 Microbial monosaccharide utilisation and solvent tolerance

7.4.1 Monosaccharide utilisation profiles of micro-organisms used in this study

7.4.1.1 Cytophaga hutchinsonii

Aside from being able to utilise glucose, figure 31 and 32 shows that *C. hutchinsonii* can use galactose for growth when in a medium with mixed monosaccharides. This may mean that *C. hutchinsonii* would be a the suitable biomass degrading partner in a system that utilises pectin rich biomass, such as fruit pulps, as the RG1 region of pectin is galactose rich (Scheller et al., 2006) leaving the glucose for the fermentive/chassis partner. The optical density of liquid cultures of *C. hutchinsonii* utilising one of the seven main monosaccharides as the main carbon source do not give

any reliable results as the *C. hutchinsonii* is using the filter paper used to inoculate the flasks for growth, which resulted in high background growth. However, the optical density of cultures containing galactose and glucose are slightly higher than the rest (fig 32) and this coupled with the results of the TLCs (figs 30 and 31) support the assertion that *C. hutchinsonii* can use galactose and glucose for growth. The apparent use of galactose contradicts to the literature (Xie et al., 2007, Liu, 2012, Diaz, 2015) so this should be investigated further.

7.4.1.2 Cellulomonas fimi

As figure 33 shows, *C. fimi* can only use glucose, as shown by the lack of only the glucose spot in fig 34 and by the optical density of the liquid cultures in fig 35. This this is unexpected, as literature indicates that *C. fimi* should be able to assimilate a wide variety of sugars, and it is not clear why it failed to do so in these experiments. That *C. fimi* can only use glucose in this system presents an interesting scenario, whilst only being able to utilise glucose for growth limits the efficiency of a process where a monosaccharide rich biomass is used when *C. fimi* is the cellulosic partner, it does mean that the sugars that are not used by *C. fimi* are available for the fermentive/chassis partner.

7.4.1.3 Bacillus subtilis 168

When *B. subtilis* 168 is grown in liquid culture it uses > 0.5% (28mM) of glucose and mannose over ten days (figs 36-38). In liquid culture *B. subtilis* 168 is seemingly able to utilise some galactose (fig 36) when it is the sole carbon source, as there appears to be a slight reduction in the intensity of the galactose spot after ten days, similar to xylose, this would need to be verified by HPLC. However, *B. subtilis* 168 can utilise galactose when arabinose is also present (see section 1.10) and arabinose also regulates the uptake of xylose (Lindner, 1994, Krispin, 1998a, Krispin, 1998b, Sulke, 2000, Nicolas et al., 2012). However, figure 37 shows some (< 0.5% 28mM) of xylose is used by *B. subtilis* 168, when it is the sole carbon source, independent of any other sugar, like galactose. This result is not as clear cut as that of the use of glucose and mannose, so the levels of xylose still present in the media after ten days would need to be quantified by HPLC before any definite conclusions could be drawn, but if *B. subtilis* 168 is able to utilise galactose and xylose as the sole carbon sources, without the presence of arabinose, this is a novel result and should be investigated further. In any case the fact that *B. subtilis* can use xylose in the presence of arabinose lends itself to a system where arabinose/xylose rich biomass, such as wheat straw or *Miscanthus sinensis*, are being utilised (see chapter 3). This is particularly true as *B. subtilis* 168 naturally produces xylanases (St John et al., 2006). With single monosaccharide cultures, the optical density when the main carbon source was galactose, glucose, mannose, xylose and rhamnose were higher than the negative control and gave comparable growth to the glucose "positive control" culture. The high optical density for cultures containing rhamnose was unexpected. From the TLC results, there does not seem to be any reduction in the amount of

rhamnose over ten days; this needs to be verified with HPLC. If *B. subtilis* can utilise rhamnose for growth, then like its ability to use galactose, in the presence of arabinose, *B. subtilis* 168 would be a good choice if the co-culture were to be utilising pectin rich biomass as the RG1 and RG2 regions of pectin are rhamnose rich.

When *B. subtilis* 168 is grown in liquid culture with all seven-common cell wall polymer monosaccharides in equal amounts (≈ 71 mg 0.07%), the pattern of monosaccharide utilisations is different to that seen when the sugars are present as the sole carbon source. *B. subtilis* 168 is able to use at least >71 mg of galactose, glucose, mannose and xylose, but is unable to utilise galacturonic acid, arabinose and, seemingly, rhamnose (fig 38). This total use of galactose and xylose is possibly due to the presence of arabinose (Krispin, 1998a, Krispin, 1998b, Sulke, 2000). In mixed sugar culture (fig 37), glucose is used up first, the whole ≈ 71 mg is consumed by 24 hours and mannose is used up by 48 hours. Galactose, also a hexose, is however not taken up in this manner as its uptake in *B. subtilis* 168 is reportedly mediated by a nonspecific pentose transporter encoded by the *araE* gene, which is regulated by the presence or absence of arabinose (Krispin, 1998a, Krispin, 1998b, Sulke, 2000). Once the glucose and mannose have been consumed, the presence of arabinose allows the uptake of galactose and xylose via the nonspecific pentose transporter mentioned above. This allows for the xylose to be totally used up by 96 hours and all the galactose to be consumed by 120 hours (fig 37). Once galactose and xylose are taken up by *B. subtilis* 168 via the nonspecific pentose transporter and they enter the pentose phosphate pathway (see chapter 1, section 1.9 and 1.10), which is analogous to glycolysis. Since in mixed sugar cultures, *B. subtilis* is able to use xylose as well as glucose, it would again be a better partner in co-cultures that utilise wheat straw or *Miscanthus sinensis*, compared to a co-culture where *Z. mobilis*, which can only use glucose, is the fermentive/chassis organism.

7.4.1.4 Escherichia coli JM109

A quick visual monosaccharide utilisation assay was conducted, rather than a full 10-day liquid culture assay, as above, *E. coli* JM109 was grown in LB overnight and 100 µl was plated out onto M9 minimal media, containing one of the seven main cell walls monosaccharides. *Escherichia coli* was found to be able to grow on M9 minimal plates containing, galactose, glucose, mannose, arabinose, xylose and rhamnose. *E. coli* failed to grow on the negative control (plain) plate and the plate containing galacturonic acid, even though this was at pH 7. As *E. coli,* can utilise six of the seven common monosaccharides and is one of the most well studied and readily engineered micro-organisms, a system utilising this micro-organism would seem ideal. As an example, a co-culture where *E. coli* is in partnership with *C. fimi* on lignocellulosic biomass, or newspaper, the *C. fimi* could utilise the glucose released by its enzymes and the *E. coli* could utilise the remaining

monosaccharides for In vivo natural ethanol production or once engineered to make some other chemically relevant product.

7.4.1.5 Saccharomyces cerevisiae BY4741

The optical density measurements for the monosaccharide utilisation profile of are unreliable, in so much that the optical density of *S. cerevisiae* cultures containing monosaccharides was higher than the glucose positive control (figure 41). Due to this, this assay needs to be repeated to verify the following results. Since the optical density of *S. cerevisiae* cultures is unreliable the reduction in the amount of monosaccharide present may demonstrate a measure of growth. *Saccharomyces cerevisiae* was able to grow in 1% yeast nitrogen base liquid minimal medium containing galactose, glucose and mannose. This is proposed by the lack of these spots after ten days when analysed using TLC (fig 39), compared to the 0 hours' control which suggests that the full ≈ 500 mg (0.5% 28 mM) of these sugars was used up, however when optical density of the galactose, glucose and mannose are among the lowest, alongside the negative control (fig 41). This pattern of monosaccharide usage is the same when *S. cerevisiae* is grown in liquid culture with equal amounts ≈ 71 mg (0.07%) of each of the seven monosaccharides (fig 40) with glucose being used up by 24 hours, galactose by 48 hours and mannose by 120 hours. The amount of the other sugars remains approximately the same as the amount at zero hours in both single and mixed monosaccharide cultures, other than a slight reduction in the arabinose spot in the mixed sugar culture after 168 hours (fig 40).

The results of the liquid culture where arabinose and xylose as the main carbon source show there is no real decrease in the amount of arabinose and xylose after ten days than from the zero-hour control in the single sugar cultures (fig 39), suggesting that they are not being utilised for growth, as described in the literature.

In the mixed sugar culture arabinose is starting to disappear after 168 hours (fig 40), something that does not happen when arabinose is the sole sugar present (fig 39) which may suggest that the uptake of arabinose may be affected by the presence of other monosaccharides. *Saccharomyces cerevisiae* is seemingly able to take small amounts of arabinose and xylose into the cell (fig 40), but cannot convert them past the arabitol/xylitol stage (Karhumaa et al., 2006, Garcia Sanchez, 2010).These results need to be verified with replicates, and the exact amount of sugars present verified with HPLC. The optical densities for the galacturonic acid and rhamnose cultures are the highest (fig 41) but seemingly these sugars are not taken into the cell, as demonstrated by TLC (Figs 39 and 40).

The results of the optical density do not seem to match the results visualised on the TLCs and therefore replicates should be carried out before any worthwhile conclusion can be drawn.

Fortunately, *S. cerevisiae* may prove compatible in terms of monosaccharide utilisation with *C. fimi*. Again, the *C. fimi* could use the glucose and the *S. cerevisiae* could use the galactose and mannose if the biomass utilised was newspaper (3.2.1) as *S. cerevisiae* is the only micro-organism in this study that has been proven to partake in co-culture with *C. fimi* (although *E. coli* shows promise, see section 5.3.5). The main conclusion is that *S. cerevisiae* has produced some very strange results and needs far more investigation before a co-culture including this micro-organism is investigated further on anything other than filter paper as the main carbon source, but since S. cerevisiae naturally produces high quantities of ethanol and has been engineered to produce other biofuels, this potential co-culture is worth pursuing.

We have demonstrated that the micro-organisms described here are able to utilise a range of monosaccharides for growth and that the monosaccharide utilisation profile of each micro-organism is different, which can complement the different monosaccharide profile of the biomass types used (fig 19). We now need to determine whether these micro-organisms are compatible with the range of economically relevant products envisioned to be produced from lignocellulosic biomass by mixed cultures of micro-organisms.

7.4.2 Solvent tolerances

The co-culture of *C. hutchinsonii* and *B. subtilis* 168 affords complementary growth (see section 5.3.3) and *B. subtilis* is readily engineered, including with the ability to produce economically relevant chemicals but if this system is to work the tolerance of *B. subtilis* and any potential cellulose degrading partners to these chemicals needs to be established.

7.4.2.1 Cytophaga hutchinsonii

Cytophaga hutchinsonii is able to tolerate up to 2% (v/v) ethanol in its growth media concentrations above this proved fatal to *C. hutchinsonii* (fig 42). Since the lower economically viable threshold for ethanol production is 4%, an ethanol production system that employs *C. hutchinsonii* as the cellulose degrading partner would not be economically viable. In regards to butanol tolerance, *C. hutchinsonii* is only able to tolerate <1% (v/v) butanol in its growth media. It was also determined that *C. hutchinsonii* was unable to tolerate any lactic acid in its growth media. This is due to DMS3 being a very poorly buffered medium. *C. hutchinsonii* is an unsuitable partner for any system hoping to produce lactic acid, as well as any system to produce butanol. While the tolerance of *C. hutchinsonii* to other economically relevant chemicals has not been tested, a system using *C. hutchinsonii* as the cellulosic partner does not look promising in the context of economically relevant chemical production. However, if the product produced by *B. subtilis* in a co-culture with *C. hutchinsonii* (see

chapter five and six, sections 5.3.3 and 6.4.4) were proteins, it is likely that this system would be economically viable as *B. subtilis* is regularly used for this sort of protein production (Lee, 2000, Simiqueli, 2009, Oyeleke, 2012, Barros et al., 2013, Liu et al., 2013, Pant et al., 2015) and waste paper is significantly cheaper than glucose as a carbon source for *Bacillus* growth (see sections 3.3.1 and 5.3.3).

7.4.2.2 Bacillus subtilis 168

When *B. subtilis* 168 is grown in DSM3+YE minimal media, it is able to survive > 10% (v/v) ethanol, but only as spores, vegetative growth was retarded above 1% (v/v) as demonstrated by the optical density of the cultures (fig 45). Interestingly *B. subtilis* 168 is able to tolerate up to 2% (v/v) butanol in a vegetative state and is able to survive as spores up to 3% (v/v) when grown in DSM3 (fig 46), *B. subtilis* 168 has been engineered to produce butanol (Li et al., 2011, Li, 2012) so these results suggest promise in that area. *B. subtilis* is only able to tolerate <1% (v/v) lactic acid in its growth media (fig 47), but this is again attributed to the low pH as DSM3 is poorly buffered. This is a factor, as in the context of this study, *B. subtilis* is grown in co-culture with *C. hutchinsonii* (see section 5.3.3) and DSM3 is the only minimal media for *C. hutchinsonii* investigated to use in this study. So even though *B. subtilis* has complementary growth in co-culture with *C. hutchinsonii* (see chapter four) if we were to modify *B. subtilis* 168 to produce any of the chemicals examined here, it would produce an environment toxic to itself and probably toxic to *C. hutchinsonii* before an economically viable threshold/yield of the chemical were reached (chapter 5). However, *B. subtilis* can be engineered to produce things other than chemicals (see above).

7.4.2.3 Cellulomonas fimi

Co-cultures of *C. fimi* and *B. subtilis* 168 proved fatal to the *Bacillus* and co-cultures of *S. cerevisiae* and *C. hutchinsonii* proved fatal to the *C. hutchinsonii*, however co-cultures of *C. fimi* and *S. cerevisiae* appear complementary (see section 5.3.4). So, with that in mind and knowing that *S. cerevisiae* is able to naturally produce and tolerate high (>10% v/v) levels of ethanol, we concentrated on establishing the ethanol tolerance of *C. fimi*. The ethanol tolerance of *C. fimi* is not greatly understood, when grown in a minimal media (1% YNB), *C. fimi* is able to tolerate up to 2% (v/v) before growth is retarded. This is still far short of the 4% (v/v) economically viable threshold, but as fig 48 shows, *C. fimi* is still alive at 5% (v/v) ethanol, so it may be possible to select for a strain that is still actively growing at 5% ethanol or higher, without the need to genetically modify the *C. fimi*, which has proved difficult in the past (C. French, 2014 Pers. Comm.). As figure 48 shows, the colony counts of *C. fimi* from media containing 1% and 2% ethanol are actually higher than the CFU in the medium containing no ethanol. This may suggest that *C. fimi* can utilise ethanol as a carbon source.

So, while the tolerance of micro-organisms in this study, to economically relevant chemicals, is, at present, a hindrance, there are still options open to us, such as developing a better buffered minimal growth medium that *C. hutchinsonii* can grow in; selecting/developing a strain of *C. fimi* that can actively grow at higher levels of ethanol or to engineer *B. subtilis* to produce non-toxic products such as enzymes or other proteins.

7.5 Conclusion

The range of monosaccharides outlined here, representing those possibly released from ligno-cellulosic biomass is utilised by a range of model organisms used in this study, which either produce economically interesting chemicals or can be engineered to do so.

Bacillus subtilis can tolerate economically viable levels of ethanol, butanol and lactic acid in its growth medium, provided the medium is well buffered to keep pH stable, however growth is retarded at levels above 1%. *C. hutchinsonii* is able to tolerate 2% ethanol in its growth media, half the level needed to be economically viable. It cannot tolerate any butanol or lactic acid in its growth media, although the latter may only be due to the changes in pH, since DSM3 is poorly buffered. The fact *C. fimi* can only tolerate up to 5% ethanol in its growth media and that its growth is inhibited by more than 2% ethanol is troubling as a) *S. cerevisiae* is able to produce much higher levels of ethanol as a matter of course and b) since the growth of *C. fimi* is restricted this means that it will take longer for the biomass to be broken down into simple sugars.

It may be possible to improve the ethanol tolerance of *C. hutchinsonii* and *C. fimi* through directed evolution; by raising multiple generations of these organisms in the presence of ethanol to select for more tolerant isolates. It would also be possible to genetically modify *C. hutchinsonii* and/or *C. fimi* with genes from organisms such as *S. cerevisiae* or *Z. mobilis* to increase its ethanol tolerance but it has proven difficult to modifying these organisms in the past (C. French *Pers. Comm*).

7.5.1 Limitations and further experiments

Results for *S. cerevisiae* should be repeated to determine whether the optical density measurements are an actual measure of growth since they seem to be at odds with the utilisation of the monosaccharides from the liquid culture visualised on TLCs. It may also be worth while attempting to select for a strain of *C. fimi* that can tolerate higher levels of ethanol as co-cultures of *C. fimi* and *S. cerevisiae* show promise (see below).

7.6 Verification of viable co-cultures of cellulose degrading and chassis micro-organisms in liquid minimal medium

A range of co-cultures were envisaged and we initially attempted to use micro-organisms that are *Generally Regarded As Safe*; such as *Bacillus subtilis* natto, used to ferment soya beans in Japan, *Lactobacillus acidophilus*, used in live yoghurts, *Saccharomyces cerevisiae*, used in bread and alcoholic beverages. While this was not possible with the *Cytophaga hutchinsonii* and *Cellulomonas fimi* both have level one biosafety rating (American Type Culture Collection, 2016, American Type Culture Collection, 2016). Initial co-culture viability assays consisted of co-culture in an appropriate minimal medium (see chapter four) with 500 mg (0.5%) of cellulose filter paper (see chapter three, section 5.3.2) as the main carbon source. Further to this we attempted to use *B. subtilis* Efor-Red so we could determine growth rates using fluorescence as a measure; this method of measuring growth worked well in *E. coli* (Duedu, 2015). We initially attempted to use WB700 because of its lack of proteases, with the view that there would be less chance of the foreign proteins *pdc* and *adh* and *pdc*:*adh* being destroyed but its lack of proteases would also make it a good candidate for a system that produces proteins and enzymes as the final product (Lee, 2000, Westers et al., 2004). As shown in table 8 not all co-cultures were successful. Co-cultures containing *C. fimi* proved to be fatal to *B. subtilis* 168, WB700, Efor-Red and natto 21A1. When *B. subtilis* WB700 and Efor-Red were grown with *C. hutchinsonii*, this combination proved fatal for both *Bacillus* species. This may be due to *B. subtilis* WB700 being deficient in a range of proteases (Westers et al., 2004), so is not a very competitive strain. However the only modifications of Efor-Red is mCherry chromoprotein in the α-amylase locus (Ranford, 2014, Yorkoni, 2016) so should not have had WB700 handicap. Unfortunately, neither stain was compatible with *C. hutchinsonii* or *C. fimi*. Co-cultures with *C. hutchinsonii* were possible with *B. subtilis* 168 and *B. natto* 21A1.

When a co-culture of *C. hutchinsonii* and *S. cerevisiae* was attempted this proved fatal to the *C. hutchinsonii*, whereas there were still some viable *S. cerevisiae* cells at the end of the trial. Co-cultures of *C. fimi* and *S. cerevisiae* were viable, with both micro-organisms having viable cells at the end of the trial. It also transpired that *C. fimi* and *E. coli* were a potential synergetic co-culture but a co-culture using *E. coli* and *C. hutchinsonii* was not possible as we were unable to find a compatible medium that both *E. coli* and *C. hutchinsonii* could grow in.

There is also a potential for co-cultures of *L. acidophilus* and *C. hutchinsonii* and/or *C. fimi* as all three are able to grow in DSM3+YE when supplemented with a carbon source. With these preliminary results, it was decided that co-cultures of: *C. hutchinsonii* and *B. subtilis* 168, *C. fimi* and *S. cerevisiae* and *C. fimi* and *E. coli* would be investigated further.

The most successful co-culture was that of *C. hutchinsonii* and *B. subtilis* 168. Over seven replicates the growth of *B. subtilis* 168, in all three measures of growth (optical density, CFU count and total protein), had values equal to, or greater than the positive control (DSM3+YE+0.5% / 28mM glucose), and well above the negative control (DSM3+YE only). This growth was presumably based on sugars, mainly glucose but also cellobiose and cellotriose, released from the filter paper by the enzymatic actions of *C. hutchinsonii* and this apparently led to better growth than when the carbon source consisted of only glucose. *B. subtilis* 168 was unable to use filter paper as the carbon source when grown in isolation. This lack of growth is likely due to *B. subtilis* 168 not naturally producing cellulases, although it does naturally produce α-amylase (Welker, 1967) and xylanases (St John et al., 2006). So, for *B. subtilis* to utilise filter paper as the main carbon source in a minimal media, co-culture with *C. hutchinsonii*, is necessary. As figures 50 and 551 show, after ten days' growth, *C. hutchinsonii*, both in isolation and in co-culture with *B. subtilis* 168, is able to completely degrade filter paper, *B. subtilis* on its own, is not and has an approximate optical density to that of the negative (DSM3+YE only). Due to these basic visual measures of growth (fig 50), the growth of *B. subtilis* 168 under this system was examined with more robust measures, consisting of optical density, colony forming units and total protein per ml of culture. Optical density does not take into account whether the cells causing the change in optical density are alive or dead. To determine this, serial dilutions of culture were plated out to determine the approximate number of live cells per ml of culture. This method is also not as precise as we would like, as the dilutions add error to the calculations and while in principle each colony is representative of one bacterial cell, if two cells are spread on the plate in close proximity to one another, they will appear as a single colony. It may be possible to address this potential for error but using a lower number of dilutions and spreading the culture over a large agar plate. Growth was also measured by calculating the total protein per ml of culture, using Bradford reagent, but again this does not differentiate between protein from live cells and protein from dead cells. While none of these measures of growth are perfect, taken together they give a relatively robust picture of growth and a clear trend starts to emerge, as each measure complements the others, showing that together, they are good measures of total growth.

As fig 50 shows by ten days growth the optical density and total protein of the mixed *C. hutchinsonii* and *B. subtilis* 168 culture with filter paper as the main carbon source had comparable growth (within standard error) to the glucose positive control and by ten days a higher *B. subtilis* 168 CFU count (*C. hutchinsonii* does not form colonies) than the positive control, ≈ $40x10^6$ compared to ≈ $30x10^6$ and the growth rates for the mixed culture is much greater than the negative control and the *B. subtilis* 168 + paper culture, again showing that co-culture provides the necessary sugars for the growth of *B. subtilis* 168.

A co-culture of *C. hutchinsonii* and *B. subtilis* 168 was set up with non-pre-treated *Miscanthus sinensis* biomass as the carbon source (not shown), however no growth of the *B. subtilis* 168 was detected, and this was probably due to the *C. hutchinsonii* not releasing enough sugars from the biomass to support both microbial partners. However, some growth (in so much as there were still viable *B. subtilis* 168 cells after ten days) was detected when the carbon source was wheat straw (not shown). Due to the results in figures 21 and 22 it was decided to concentrate on developing a system utilising wheat straw and to attempt to increase growth rates by developing a system of pre-treatments for the wheat straw, based on techniques found in the literature (see chapter one, section 1.3.7).

Figure 51 shows that when pre-treated wheat straw is the main carbon source, the mixed culture of *C. hutchinsonii* and *B. subtilis* 168 has an optical density equal to that of the positive glucose control by 120 hours and that by 240 hours the optical density of the mixed culture is nearly three times that of all other cultures. The total protein of the mixed culture is equal to that of the positive control by ten days' growth and is well above the negative control and the *B. subtilis* 168 + pre-treated wheat straw culture, which was unexpected (see below).

The colony forming units of the mixed culture, however are below the glucose positive control and, interestingly, lower than the *B. subtilis* 168 + pre-treated wheat straw culture, but the colony number is comparable to the number of colonies when filter paper is the main carbon source after ten days' growth (see figures 50 and 51). The fact that the CFU count for the *B. subtilis* 168 + wheat straw is so comparatively high is likely due to the native α-amylase digesting any remaining starch in the biomass and the native xylanases (St John et al., 2006) digesting the xylans present in the biomass (see chapters two and four). Both sets of results do show that *B. subtilis* 168 is capable of complementary growth in a mixed culture with *C. hutchinsonii* on both filter paper and pre-treated wheat straw as the main carbon source.

To determine which monosaccharides were present in the media of these cultures, one ml of the culture was analysed with thin layer chromatography. Figure 52 shows the three TLC analysed; *C. hutchinsonii* + pre-treated wheat straw, *B. subtilis* 168 + pre-treated wheat straw and the mixed culture of *C. hutchinsonii* and *B. subtilis* 168 on pre-treated wheat straw. The TLC of the media from the culture of *C. hutchinsonii* and wheat straw appears not to show any trace of monosaccharides, which is odd since we know that *C. hutchinsonii* has the enzymes to digest the cell wall polymers present in wheat straw, and that *C. hutchinsonii* cannot use all of the monosaccharides it releases (chapter four), so other monosaccharides, such as arabinose should still be present on the TLC. In the *B. subtilis* 168 with pre-treated wheat straw we would expect glucose from starch and xylose and

possibly xylobiose, from xylans due to its native enzymes (see chapter four). There are faint spots for xylose and oligosaccharides one of which appears to be cellobiose or possibly xylobiose (see chapter three). Unfortunately, we did not have the marker for xylobiose, but there does definitely seem to be some digestion of cell wall polymers by *B. subtilis* 168 visualised on this plate.

Bacillus subtilis 168 can be engineered to produce a range of biofuels (Romero et al., 2007, Li, 2012) or their potential precursors (Romero-Garcia et al., 2009, Gao et al., 2012, Gao and Ho, 2013)(see chapter six) or for enzyme or medicinal protein production (Simiqueli, 2009, Barros et al., 2013, Liu et al., 2013, Pant et al., 2015) and a co-culture system that utilised waste paper, a cheap carbon source needing few if any pre-treatments, to produce these can only be a good thing, both financially and ethically.

Initial visual growth assays suggested that a co-culture of *C. fimi* and *S. cerevisiae* may be possible as neither micro-organism proved fatal to its partner (table 8 and figures 53 and 54). These growth trials only showed that both organisms survived the presence of each other and that there were viable cells still present at the end of the trial but there was nothing that suggested there was any actual growth, they were merely alive. The problem was finding a minimal growth medium that could support both of these micro-organisms. This proved to be extremely difficult (see appendix for the full list of growth media trialled, along with grow measurements and discussion).

It was determined that 1% YNB + YE worked as a minimal media for both these micro-organisms. Figures 53 and 54 show that after five days' growth the optical density, colony count and total protein of the *C. fimi* with filter paper as the main carbon source, were much higher than the values for growth rates for *C. fimi* in other media where glucose, not filter paper, was the main carbon source, possibly because the *C. fimi* was using the cellulose derived oligosaccharides as well as the glucose released for growth. Figures 53 and 54 also show that where *C. fimi* and *S. cerevisiae* are grown in co-culture with filter paper as the main carbon source, the optical density, colony counts (for both micro-organisms) and total protein were higher than the glucose positive control after five days growth and much higher than the negative control and the culture where *S. cerevisiae* is grown with filter paper as a pure culture, showing that *S. cerevisiae* cannot utilise filter paper as a carbon source for growth without a cellulose degrading partner. These results need to be verified with replicates and be run over the full ten-day growth trial as the *C. hutchinsonii* and *B. subtilis* 168 co-cultures are, but this demonstrates that a variety of co-cultures are possible, with *C. fimi* and *S. cerevisiae* co-culture being especially expedient as it potentially means that wild type micro-organisms can produce an economically relevant substance from paper, a cheap carbon source,

without the need for genetic modification. However a much cheaper growth media would need to be devised first, as using YNB as the growth media is not economically viable.

There are already many strains of modified *S. cerevisiae* able to utilise pentose sugars (Becker and Boles, 2003, Hughes et al., 2015) and with the advent of synthetic biology; it may be possible to synthesise *S. cerevisiae* genome (Annaluru, 2014) that codes for the metabolism of the full range of common cell wall polymer monosaccharides and that will also produce a wide range of end products, enabling lignocellulosic biomass to be utilised under this so-culture system.

In the interest of completeness, a co-culture of *C. fimi* and *E. coli* was set up. Previous work in the lab has shown that both organisms can live in M9 minimal medium (Kane, 2014) and that *E. coli* can be engineered to produce ethanol using fused pdc:adh (Lewicka, 2014, Yang et al., 2014) although ethanol producing strains are already known (Leite, 2000). Unlike the *C. fimi* and *S. cerevisiae* trial, the initial media trials were not needed thanks to the previous work in the lab, so a full ten-day co-culture of *C. fimi* and *E. coli* was run with filter paper as the main carbon source.

As figure 55 shows, the optical density of the *C. fimi* and *E. coli* co-culture is slightly higher than the *E. coli* negative control but far below the optical density of the positive control. The OD of the culture where *E. coli* is cultured alone with filter paper is far lower than the negative control. The colony count of viable *E. coli* cells in the co-culture is initially higher than the number of viable *E. coli* cells in the positive control. The fact that the colonies of viable *E. coli* cells, along with the CFU for other cultures, drops so dramatically at 96 hours, is because the temperature on the incubator was lowered at that time point and the cultures were unable to survive or recover from this cold shock, and this also may have had a slight effect on the total protein for the glucose positive control, so it is not possible to draw any conclusions from this data set. The total protein of the mixed culture is equal to that of the negative control, but again above that of the *E. coli* + filter paper culture. The total protein for the glucose positive control is far above all the other cultures. Although the results that we do have warrant replicates of this co-culture being undertaken, replicates will tell us whether the poor growth was a result of the incubator malfunction or whether this co-culture is not viable. There is potentially a better system for utilising *E.coli* using a mixed culture of *E. coli* that produce endo- exo- and glucosidases (Salinas, 2016) and *E. coli* that produce ethanol using the fused pdcadh protein (Lewicka, 2014, Yang et al., 2014) (see chapter six).

7.7 Conclusions

The results presented here show that certain combinations of microbial partners can give rates of growth equal to or greater than growth found in positive glucose controls but from far cheaper carbon sources. This growth can be translated into economically important substances if one microbial partner produces them naturally or is engineered to do so, provided both the micro-organisms can tolerate an economically viable amount of these chemicals in the growth media (Lau and Dale, 2009). While there is still work to be done in optimising growth conditions, further screening of suitable co-cultures and testing their tolerance of, and their modification to produce, economically interesting substances, this system could go some way towards making the production of biofuels etc. from lignocellulosic biomass more economically viable.

7.7.1 Limitations and further experiments

While the *B. subtilis* results have been verified with replicates, the assays for the other micro-organisms have only been trialled once, so cannot be said to be robust and reproducible results, but they do give us the impetus to investigate these mixed cultures further.

Optimisation of the growth media should be attempted as the *C. fimi* / *S. cerevisiae* and *C. fimi* / *E. coli* work is far from perfect but does demonstrate this combination is viable.

Another area to be investigated is into the establishment of new co-cultures, such as ones utilising lactic acid bacteria, *Z. mobilis* and other fungi such as *T. reesii* and utilising other sources of biomass such as pectin rich waste and algal sources.

7.8 Modification of *Bacillus subtilis* 168 for the production of lactic acid and ethanol

It was decided to attempt engineer *Bacillus subtilis* 168 to aerobically produce lactic acid and ethanol (see section 6.3.2). This was done by producing a genetic cassette containing the genes to code for native lactate dehydrogenase and to code for the fused protein pyruvate decarboxylase: alcohol dehydrogenase (Lewicka, 2014). Each cassette contained upstream and downstream homology to the *cspD* locus, which should enable the cassettes to be integrated by homologous recombination at this region and to be expressed under the *cspD* promoter (figure 56 and 58). The *cspD* gene locus was selected as *cspD* is the most highly expressed non-essential *B. subtilis* gene (Nicolas et al., 2012), so we believed that replacing this gene would have no detrimental effect on the *B. subtilis* and would result in the production of large amounts of the desired enzyme.

In theory and in the case of lactate dehydrogenase, this experiment has been designed in such a way as to override the propensity of *B. subtilis* to only produce lactic acid anaerobically via the homolactic fermentation pathway, since over expressing *ldh* under the *cspD* promoter, would result in large amounts of lactate dehydrogenase being produced aerobically and pyruvate being converted to lactic acid aerobically.

This is a similar method to the one we hoped to employ for the aerobic production of ethanol. We again wanted to utilise the *cspD* promoter to produce the fused protein pdc:adh, but in this case and since neither pyruvate decarboxylase nor alcohol dehydrogenase are native enzymes in *B. subtilis* 168 (Romero et al., 2007) they needed to be introduced. The presence of large amounts of this fused enzyme (pdc:adh) would convert pyruvate to ethanol. The use of the fused protein means that the two enzymes are next to each other and so should be more effective (Lewicka, 2014, Trubitsyna et al., 2014). This should enable both lactic acid and ethanol would be produced aerobically and so enable a system that could be used with *C. hutchinsonii* (see chapters four and five).

The use of splicing overlap extension (SOE) (Horton, 1990) and Paperclip (Trubitsyna et al., 2014) PCR for the construction of the insertion cassettes was due to these methods being used, and in the case of Paperclip, being developed, in the French lab. But these methods of DNA assembly have advantages over other methods. There are no forbidden sites as neither method uses restriction enzymes, so designing the cassettes is much easier and quicker. In the case of overlap splicing extension, the method simply consists of two PCR cycles, where the homologous regions of the sequential parts anneal to one another (figures 56 and 58a) (Horton, 1990, Luo, 2013). This linear DNA can then be purified to sequence or used to directly transform competent cells via homologous recombination.

In the case of this study, we were easily able to produce the ldh cassette by SOE and use this linear PCR product to transform competent *B. subtilis* 168 cells. We were unable to use SOE to produce the PEt cassette. Construction of the Pet cassette was then attempted using Paperclip, to produce a linear product to transform *B. subtilis* 168 as we did with the ldh cassette; however, as fig 57 and 58 show either the PCR reaction failed due to the size of the cassette (6kb) being constructed or that the full Pet cassette did not integrate into the *B. subtilis* genome. It seems that the full 6 kb cassette was not assembled. Had we attempted to use *E. coli* to produce a plasmid, rather than linear PCR product, we could have digested this and used it as a linear product to transform the *B. subtilis* 168 competent cells, with the plasmid over hang being digested by the *B. subtilis* 168 native exonucleases. This is the method that should be adopted in any repeat attempts of this work.

Once the DNA cassette construction was completed, as determined by gel electrophoresis the PCR reaction was column purified which resulted in a reduction the amount of DNA, as determined by A260 measurements using the nanodrop, which left us with; ldh construct = 15 µg/µl (0.0038 pMol) and the PEt cassette = 11 µg/µl (0.0018 pMol), which gave a transformation efficiency of 28.33 colonies per µg of DNA for the ldh construct and 7.14 colonies / µg of DNA for the PEt construct. These relatively low transformation efficiencies are attributed to the loss of DNA during purification, but they did result in colonies that showed resistance to the appropriate antibiotic after two subsequent subcultures onto fresh antibiotic plates. These colonies were then grown overnight in LB to extract genomic DNA for sequencing and analytical PCR and to produce crude extract for PAGE gel and enzyme activity assays.

Crude extract was produced via sonication and once concentrated to a high protein level (see figure 59) was used for SDS PAGE gel analysis. As figure 60 shows, there is no band in the SDS gel for the proposed fused pdc:adh at 98 KDa. This coupled with a lack of staining for the activity of pdc and adh in the native gels (fig 60) indicates that the fused protein is not being produced by *B. subtilis* 168 $^{\Delta}$*cspD*::*pdc*:*adh*B^{+}Rneo. When we consider the lack of a PCR product for the PEt cassette (fig 58b) in the genomic DNA of the transformed *Bacillus* and the complete lack of enzyme activity for pdc:adh in the enzymatic activity assay (data not shown) we can draw the conclusion that the PEt cassette did not integrate into the transformed *Bacillus* genome, and the resistant colonies were due to either a random mutation, or that only the antibiotic resistance part integrated into the genomic DNA, which may explain why there is a 1.5 kb band for the PEt construct genomic DNA (chapter 6, figure 3). Sequencing of this genomic DNA failed.

Since Ldh is a native enzyme to *B. subtilis* 168, we would expect bands at 34 KDa for all isolates, which we do see, although there seems to be slightly more intense bands at around 34 KDa for *B.*

subtilis 168 $^{\Delta}$*cspD*::*ldh*$^{+}$Rcml (fig 60). Figures 60 and 61 also shows that there is staining/activity for ldh activity in all isolates, but there is a much higher intensity of the staining in the Ldh isolates, as the native Ldh is being over expressed under a different promoter to the native Ldh promoter. Figure 58b shows that there is a 1.5 kb band for the ldh construct, indicating that the cassette has inserted into the genomic DNA, as primers were designed to anneal half way down (at 1.5 kb) and flanking the beginning of the construct. A PCR product of 4 kb is also generated for this isolate, as expected, when primers amplifying the entire *cspD* locus are used. An attempt to sequence this PCR product failed.

This demonstrates that the production and integration of a genetic cassette that results in the over expression of native ldh was successful but the analogous PEt cassette construction and integration was not. With the ldh cassette integrated under the *cspD* locus there is an over production of ldh compared to wild type and that the transcription of the ldh genetic cassette and its overproduction of ldh resulted higher levels of ldh activity compared to wild type.

Bacillus subtilis 168 $^{\Delta}$*cspD*::*ldh*$^{+}$Rcml, was tested under different growth conditions to determine whether it produced more lactic acid than the wild type. The amount of lactic acid produced was greater than the amount produced by the wild type *B. subtilis* 168, under both aerobic and anaerobic conditions, with and without the presence of glucose. *Bacillus subtilis* 168 naturally produces lactic acid in anaerobic conditions through homolactic fermentation. However, as figure 62 shows when *B. subtilis* 168 that has been engineered to overexpress lactate dehydrogenase is grown in aerobic conditions and subjected to cold shock treatment, the amount of lactic acid produced is greater than that produced by the wild type *B. subtilis* 168 under the same conditions.

This system shows that expression of a desired protein under the *cspD* locus results in higher enzyme amounts compared to wild type and more enzymatic activity than wild type. While it is probably not economical using this system to produce lactic acid or indeed ethanol, since extremely efficient systems for the productions of these chemicals are already in place, this system of utilising the *cspD* locus in *B. subtilis* to produce enzymes/proteins of economic or medical value would probably prove advantageous. This, coupled with the fact that *B. subtilis* can grow on the relatively cheap carbon source of paper when in co-culture with *C. hutchinsonii* (see chapter four) provided an extremely economic method of protein production.

7.9 Conclusion

The transformation of WT *B. subtilis* 168 to *B. subtilis* 168 $^{\Delta}$*cspD*::pdc:adhB^{+}Rkan failed, because even though we have a confirmation band for the Pet cassette (fig 59) and these cells were resistant to chloramphenicol, amplification of the *cpsD* locus did not give a PCR product of the expected size (Chapter 6, Figure 3), and no activity staining was seen, a southern blot test may have yielded results however. There were no proteins present at 98 kDa in the SDS PAGE gel, nor any pdc:adh activity in the enzyme activity assay. The chloramphenicol resistance observed could be due to just the antibiotic resistance genes being integrated somewhere within the genome, as cells grew on subsequent agar plates containing chloramphenicol but the fused protein pdc:adh is not present in these cells.

Conversely the transformation of *B. subtilis* 168 to $^{\Delta}$*cspD*::ldh^{+}Rcat was successful, both the proof primers, that amplify the insertion locus showed bands of the correct size after PCR amplification, there was increased intensity of the extracts of the overexpressed ldh strain when compared to the wild type and the Pet transformed cells, and also, possibly, increased amounts of a protein at 35 kDa in the SDS PAGE gel. There was enzyme activity in the enzyme activity assay, showing that pyruvate was being converted to lactic acid and a greater amount of lactic acid was detected in the ldh transformed culture.

7.9.1 Limitations and further experiments

Time constraints limited the number of replicates for some results so it is imperative that these replicates are carried out before any actual conclusions about the results presented here can be drawn. Another attempt to transform *B. subtilis* 168 to produce ethanol by inserting the pdc:adh cassette should be attempted. It would also be interesting to place the fused pdc:adh under the ldh promoter, in a similar way as described in Romero *et al*., 2007 and investigate how that would affect levels of lactic acid and possible ethanol produced by *B. subtilis* 168. Once any transformations are successful, sequencing of *B. subtilis* 168 $^{\Delta}$*cspD*::ldh^{+R}cat and *B. subtilis* 168 $^{\Delta}$*cspD*::pdc:adhB^{+R}kan genomic DNA should be attempted again (section 6.4.2). It would also be interesting to investigate the practicality of using the *cspD* locus in *B. subtilis* 168 for the production of enzymes and other proteins for extraction and purification and possibly modifying *B. subtilis* 168 to produce cellulases, possibly under the α-amylase promoter/locus.

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9 Appendix

9.1 Chapter 2, materials and methods

9.1.1 Minimal media

There follows addition information to supplement chapter two.

9.1.1.1 Vitamin complex

Figure 63 shows the composition of the B vitamin supplement added to the media listed in table 1

Two caplets contain:	%EC RDA*
Thiamin (Vitamin B1)	10mg 909%
Riboflavin (Vitamin B2)	15mg 1,071%
Niacin	25mg NE 156%
Pantothenic Acid	100mg 1,667%
Vitamin B6	10mg 714%
Vitamin B12	25µg 1,000%
Folic Acid	400µg 200%
Biotin	100µg 200%
Choline Bitartrate	250mg **
Inositol	250mg **
PABA	50mg **
*RDA = Recommended Daily Allowance **No RDA established	

Other Ingredients:
Stabiliser (Calcium Carbonate), Bulking Agents (Microcrystaline Cellulose, Dicalcium Phosphate)< Choline Bitartrate, Inositol, Pantothenic Acid (as d-Calcium Pantothenate), Anti-Caking Agents (stearic Acid, Silicon Dioxide, Magnesium Stearate), Maltodextrin, PABA (ParaAminobenzoic Acid) Niacin (as Nicotinamide), Riboflavin (Vitamin B2), Vitamin B6 (as Pyridoxine Hydrochloride), Firming Agent (Povidone), Thiamin Hydrochloride (Vitamin B1), Glazing Agents (Hydroxypropyl Methylcellulose, Glycerine, Camauba Wax), Folic Acid, Biotin (as d-Biotin), Vitamin B12 (as Cyanocobalamin).

Figure 63 - Overview of vitamin complex mentioned in chapter two, table 1

9.1.1.2 Rogasa medium

Figure 64 shows the complete composition and methods for Rogasa medium listed in table 1.

Fluka SIGMA www.sigma-aldrich.com

R1148 Rogosa SL Agar

Rogosa SL Agar is used as a selective solid medium for cultivation of oral and faecal *Lactobacilli*. Tryptose and yeast extract provide nitrogenous compounds, sulphur and vitamin B complex which are essential for the growth of *Lactobacilli*.

Composition:

Ingredients	Grams/Litre
Tryptose	10.0
Yeast Extract	5.0
Dextrose	10.0
Arabinose	5.0
Saccharose	5.0
Sodium Acetate	15.0
Ammonium Citrate	2.0
Monopotassium Phosphate	6.0
Magnesium Sulfate	0.57
Manganese Sulfate	0.12
Ferrous Sulfate	0.03
Polysorbate 80	1.0
Agar	15.0

Final pH 5.4 +/- 0.2 at 25°C

Store prepared media below 8°C, protected from direct light. Store dehydrated powder in a dry place in tightly-sealed containers at 4°C.

Appearance:	Light yellow colored, homogeneous powder containing soft lumps.
Gelling:	Firm
Color and Clarity:	Light yellow colored, slightly opalescent gel forms in petri plates.

Directions:
Suspend 75 g of Rogosa SL Agar in 1000 ml of distilled water. Boil to dissolve the medium completely. Add 1.32 ml of glacial acetic acid (Fluka 45726). Mix thoroughly, and distribute into culture tubes or flasks. Heat to 90-100°C for 2-3 minutes. Cool to 45°C for direct inoculation. DO NOT AUTOCLAVE.

Principle and Interpretation:
Dextrose, arabinose, saccharose are the fermentable carbohydrates. Polysorbate 80 is the source of fatty acids. Ammonium citrate and sodium acetate inhibit molds and Streptococci. The low pH of the agar and the addition of acetic acid makes the medium selective for Lactobacilli while inhibiting other bacteria. It is recommended the inoculated plates should be incubated for 3 days at 37°C in 5% CO_2 and 95% H_2. If this is not possible overlay the inoculated plates with a second layer of agar before the incubation.

Figure 64 - Additional information on Rogasa medium mentioned in chapter 2, table 1

9.2 Chapter four - *B. subtilis* 168 mixed monosaccharide utilisation plate replicate

Below is the replicate TLC of B. subtilis utilising mixed monosaccharides as the main carbon source. It lacks a galactose marker, galacturonic acid was left out of the mix and it is of poor visual quality so it was not presented in the main body of work for these reasons but the results are verified by replicates.

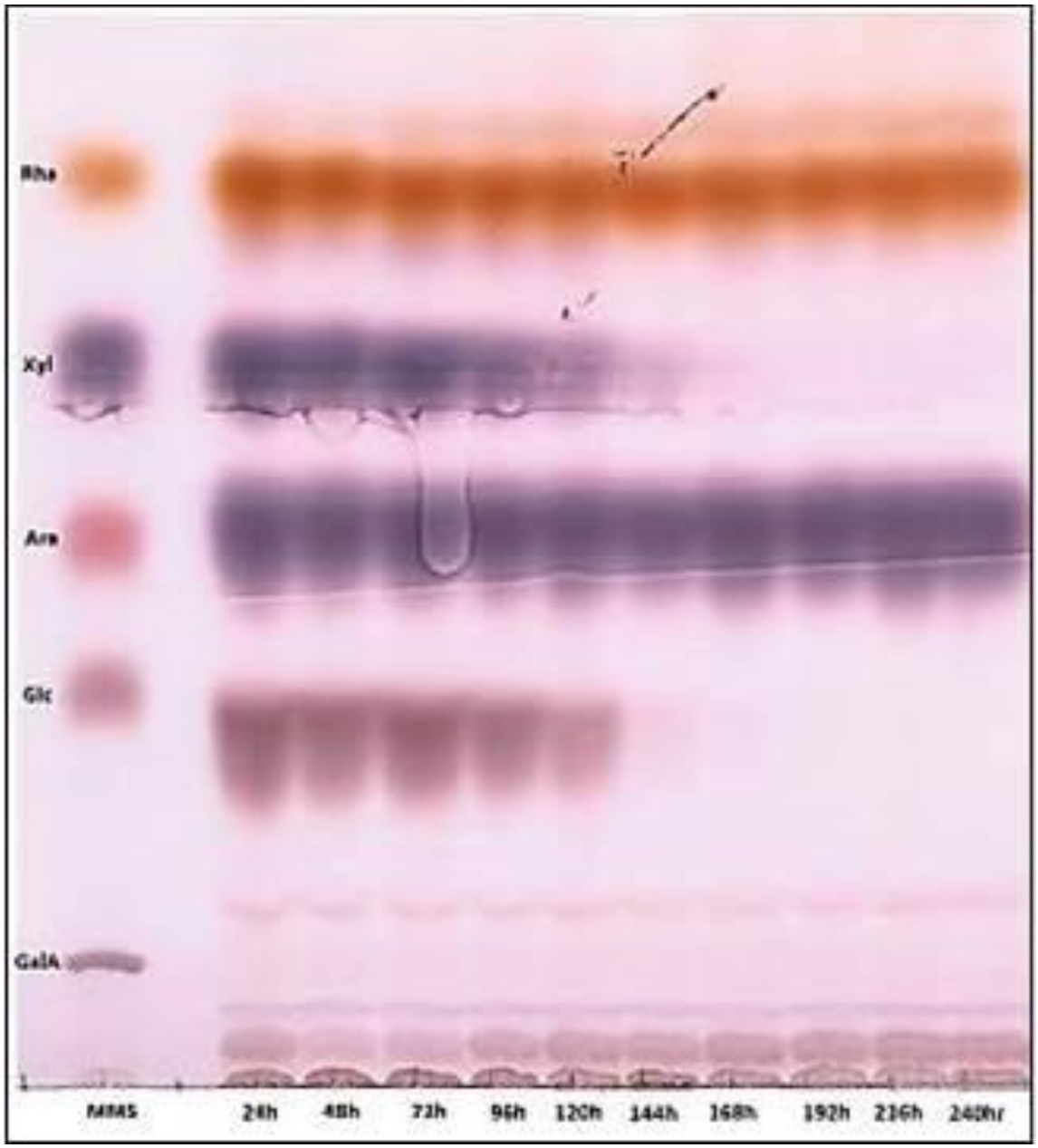

Figure 65 - *B. subtilis* 168 mixed monosaccharide utilisation TLC plate replicate

9.3 Chapter 5 – co-cultures

9.3.1 Screened minimal media

There follows a complete list and rational of screened minimal media for co-culture of *C. fimi* and *S. cerevisiae*. Figure 63 shows eleven different recipes for a minimal medium that were trialled, for co-operative growth of both *C. fimi* and *S. cerevisiae*, with various amounts of the component salts in each recipe. As figure 63 shows, the use of Narendranat (Narendranath and Power, 2005), MM3, 0.5% solution, and *T. reesii* media (see chapter two and appendix) results in no real change in the optical density and a decrease in the number of colonies over a 72 hour period. There is also no change in the optical density for the Wickerhams (Wickerham, 1946) media but there is a sharp increase in the number of colonies at 48 hours before dropping away. We then attempted to alter DSM3 to bring it more in line with a yeast minimal medium but the only growth from these media was the *C. fimi* positive control (fig 63). The main difference in media we noticed was that the yeast media all had trace elements, amino acids and vitamin complexes present, whereas the DSM3 did not. We decided to test how both organisms would grow in 1% yeast nitrogen base minimal media, if supplemented with trace elements, amino acids and vitamins. This lead to growth of both micro-organism, with the *C. fimi* growth lagging behind the *S. cerevisiae*, when we supplemented the 1% yeast nitrogen base with sodium phosphate, this resulted in for better *C. fimi* growth (figs 63 and 64), suggesting that the low growth of the *C. fimi* was due to the lack of sodium and/or phosphate (SIGMA).

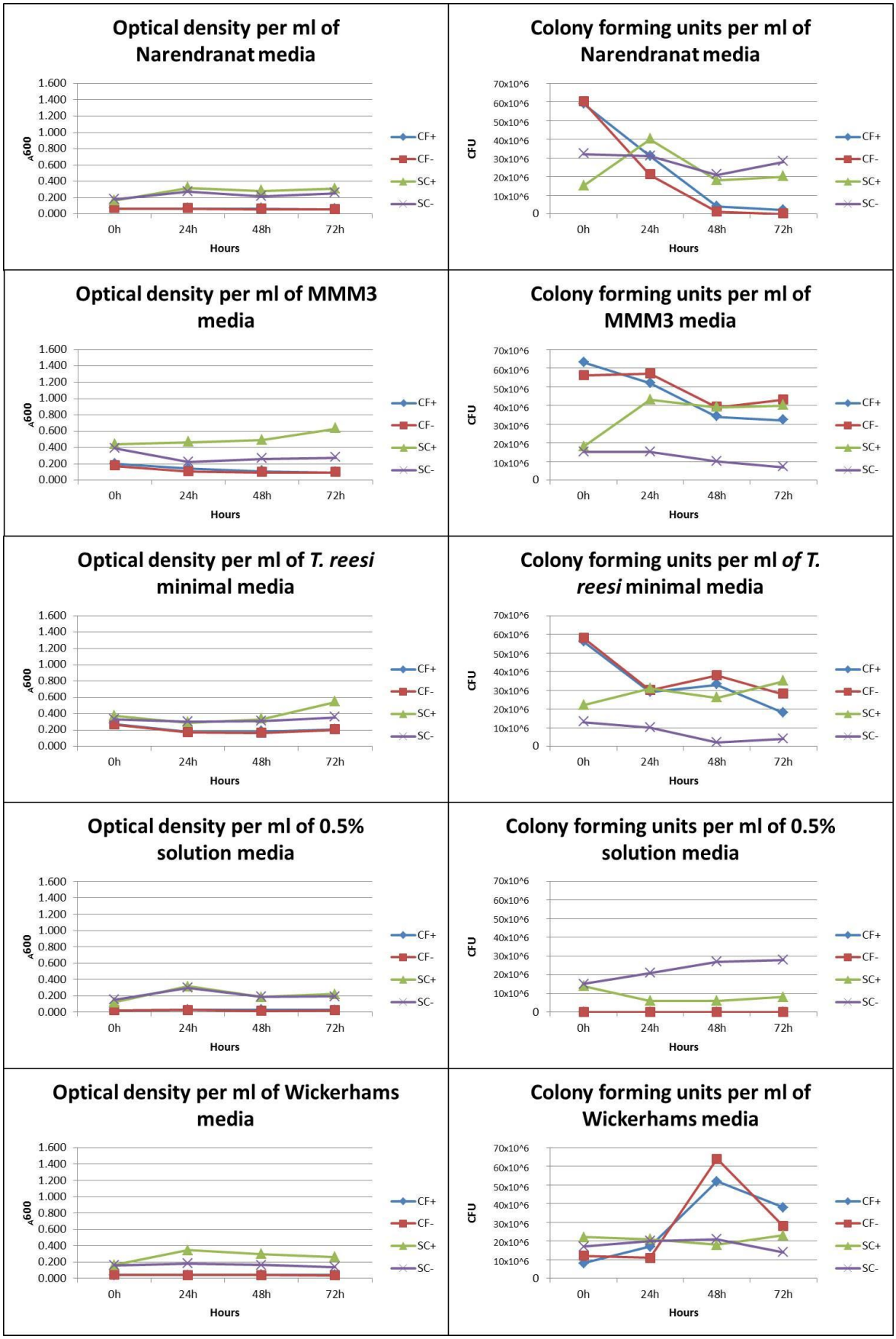

Figure 66 - Failed minimal medium for both *C. fimi* and *S. cerevisiae* micro-organisms. Optical density and CFU count were used as measures of growth.

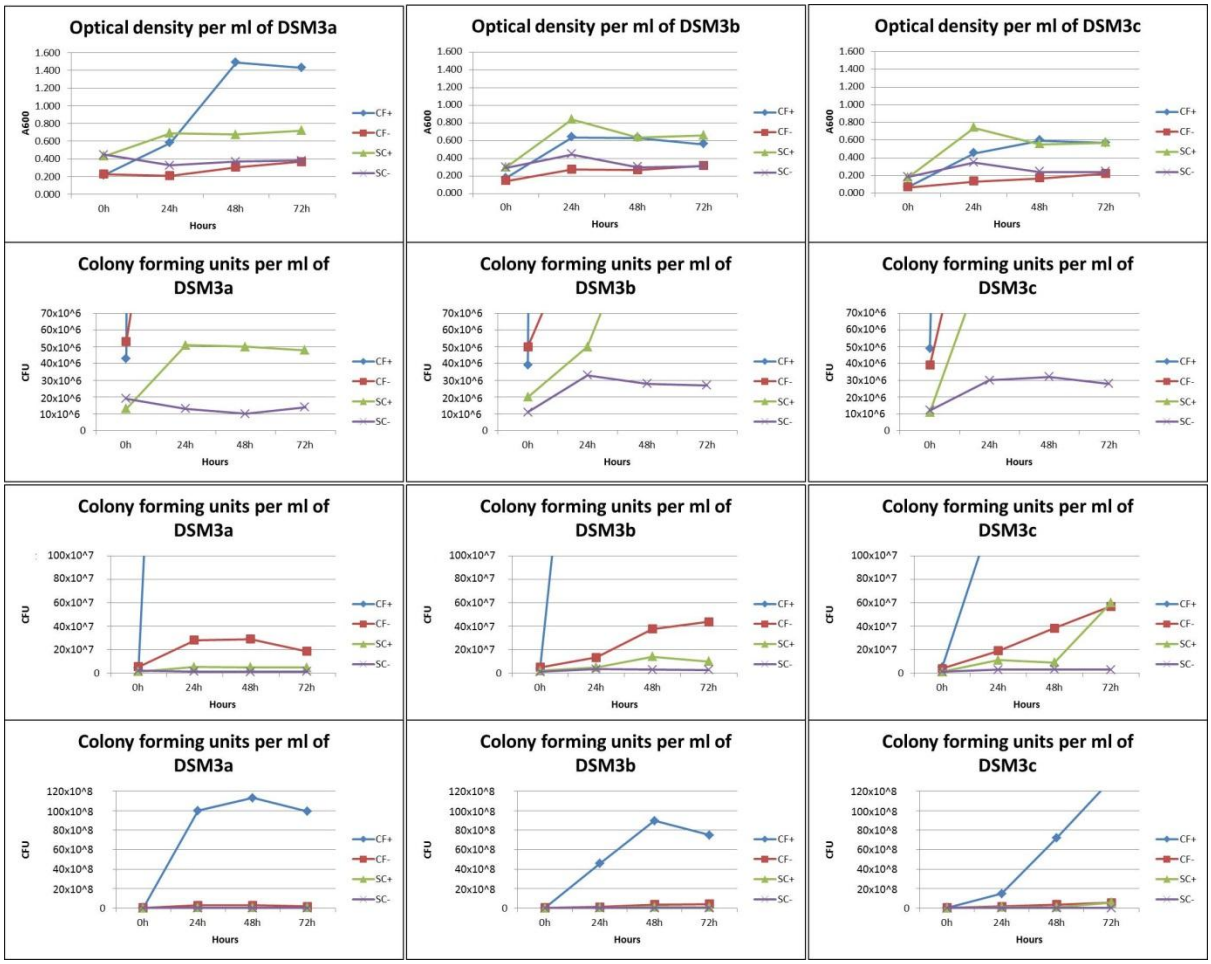

Figure 67 - Variants of DSM3 that failed to provide for growth of *C. fimi* and/or *S. cerevisiae.* Optical density and CFU count were used as measures of growth.

9.4 Chapter 6 - Transformations

There follows the full genetic cassette sequence of the transformed B. subtilis 168 mentioned in chapter six, table 9.

9.4.1 Cassette sequences

Table 11 - Complete genetic sequence of ldh and PEt cassettes used to transform *B. subtilis* 168

lactic acid producing cassette sequence	GATCGGCACTCATCCAAGCAATGTAAAAATCACAGGAATTCCAATCAGGCCGCAATT TGAAGAATCCATGCCTGTTGGCCCGATATATAAAAAGTACAATCTTTCACCAAACAAA AAAGTGCTTCTGATCATGGCAGGTGCTCACGGTGTATTAAAGAACGTAAAAGAGCTG TGCGAAAACCTTGTCAAGGATGACCAAGTGCAAGTAGTTGTCGTGTGCGGGAAAAA TACGGCTTTAAAAGAATCTTTGAGTGCGCTTGAAGCGGAAAATGGTGACAAATTAAA AGTTCTGGGCTATGTGGAGCGCATTGATGAGCTATTTCGGATCACAGATTGCATGAT TACCAAGCCCGGCGGCATTACTTTGACAGAAGCCACAGCCATTGGAGTGCCTGTCAT TCTGTACAAACCCGTGCCTGGCCAGGAAAAAGAAAATGCAAACTTCTTTGAAGACCG

	CGGAGCTGCCATCGTTGTGAACCGTCATGAAGAGATTCTCGAGTCAGTCACTTCCCTT CTTGCAGATGAAGATACCTTGCATCGCATGAAGAAAAACATTAAGGACCTTCATTTA GCAAACTCCTCTGAAGTGATTTTAGAGGATATCCTGAAGGAATCAGAAATGATGACC GCCAAACAAAAAGCCAAAGTGCTATCGTAATGGCGTACTTGAGAGCATACGAAAATC GTGTGCTCTTTTTATTTATATTCAGCCATCAATAAAAGCGGTTACATTTTTTTATGGAA CTTGCCCTTCTTTTGAAAAATAAGCCGTTTCGCAACTTGACGGGTGCTCCCAGATGGT GTATAGTTGAACCATCATTTAACAATGAATCAAAGTTAGATGATGACAAAATTTTTTT TAGCACATGGGTGCTACAGTACTAGGAGGAATTAAGCAATATGATGAACAAACATG TAAATAAAGTAGCTTTAATCGGAGCGGGTTTTGTTGGAAGCAGTTATGCATTTGCGT TAATTAACCAAGGGATCACAGATGAGCTTGTGGTCATTGATGTAAATAAAGAAAAAG CAATGGGCGATGTGATGGATTTACCCCACGGAAAGGCGTTTGGGCTACAACCGGTC AAAACATCTTACGGAACATATGAAGACTGCAAGGATGCTGATATTGTCTGCATTTGC GCCGGAGCAAACCAAAAACCTGGTGAGACACGCCTTGAATTAGTAGAAAAGAACTT GAAGATTTTCAAAGGCATCGTTAGTGAAGTCATGGCGAGCGGATTTGACGGCATTTT CTTAGTCGCGACAAATCCGGTTGATATCCTGACTTACGCAACATGGAAATTCAGCGG CCTGCCAAAAGAGCGGGTGATTGGAAGCGGCACAACACTTGATTCTGCGAGATTCC GTTTCATGCTGAGCGAATACTTTGGCGCAGCGCCTCAAAACGTACACGCGCATATTA TCGGAGAGCACGGCGACACAGAGCTTCCTGTTTGGAGCCACGCGAATGTCGGCGGT GTGCCGGTCAGTGAACTCGTTGAGAAAAACGATGCGTACAAACAAGAGGAGCTGGA CCAAATTGTAGATGATGTGAAAAACGCAGCTTACCATATCATTGAGAAAAAAGGCGC GACTTATTATGGGGTTGCGATGAGTCTTGCTCGCATTACAAAAGCCATTCTTCATAAT GAAAACAGCATATTAACTGTCAGCACATATTTGGACGGGCAATACGGTGCAGATGAC GTGTACATCGGTGTGCCGGCTGTCGTGAATCGCGGAGGGATCGCAGGTATCACTGA GCTGAACTTAAATGAGAAAGAAAAAGAACAGTTCCTTCACAGCGCCGGCGTCCTTAA AAACATTTTAAAACCTCATTTTGCAGAACAAAAAGTCAACATGAACTTTAATAAAATT GATTTAGACAATTGGAAGAGAAAAGAGATATTTAATCATTATTTGAACCAACAAACG ACTTTTAGTATAACCACAGAAATTGATATTAGTGTTTTATACCGAAACATAAAACAAG AAGGATATAAATTTTACCCTGCATTTATTTTCTTAGTGACAAGGGTGATAAACTCAAA TACAGCTTTTAGAACTGGTTACAATAGCGACGGAGAGTTAGGTTATTGGGATAAGTT AGAGCCACTTTATACAATTTTTGATGGTGTATCTAAAACATTCTCTGGTATTTGGACTC CTGTAAAGAATGACTTCAAAGAGTTTTATGATTTATACCTTTCTGATGTAGAGAAATA TAATGGTTCGGGGAAATTGTTTCCCAAAACACCTATACCTGAAAATGCTTTTTCTCTTT CTATTATTCCATGGACTTCATTTACTGGGTTTAACTTAAATATCAATAATAATAGTAAT TACCTTCTACCCATTATTACAGCAGGAAAATTCATTAATAAAGGTAATTCAATATATTT

	ACCGCTATCTTTACAGGTACATCATTCTGTTTGTGATGGTTATCATGCAGGATTGTTTA TGAACTCTATTCAGGAATTGTCAGATAGGCCTAATGACTGGCTTTTATAACTAAACTC AATACATGATGATGAGATGACAAATAGAGGAGAGGCATTCGCCTCTCCTATTTGATT TTCATCTGGATTTCAATCGCCAAAATGTTTTGTTTACACCTCTGTTCAATCTCTTCAACA TACTTTTCCATTGATGGTTTTCCTTTTTTCGCGATATCGGCCAGTTCTTCTAAATCACTG TATATTTCTATAAATGTTTTGTGAAGGATCAACTTCAAGTCTTTATCATCCATAGCTGC GTTCCCCTTCTCTATCAAGATACCTCAAATAGTAAAACGGTCCTATTTATTCTGGTTCA ATAGTTATATCGGCTTATTTTTATTTTCTTTTCTATTTTGGTACATAAATTTCAAAAAAA CTCTGCAAAATAATGGCGGAGGTGTTTTTTGTGACTTCAGAATTTCATAATGAGGATC AGACCGGCTTTACGGATAAGCGGCAGCTGGAACTAGCGGTGGAAACAGCGCAGAA AACAACAGGAGCCGCGACGAGAGGCCAAAGCAAAACATTAGTCGACTCTGCATACC AAGCCATTGAGGATGCTAGAGAACTGTCACAATCTGAAGAGCTGGCAGCTCTCGAT GATCCTGAATTTGTAAAGCAGCAACAGCAGCTGCTAGATGACAGCGAGCATCAGCT GGATGAATTCAAAGAATAAAAACCGCAGCTTCTGCGGTTTTTATTTTTAGTGATTGGT TAAGACATCTCCGTATTGTTTCAACTTTTCACCAACCGTACATTTATTGATACAAAATG AATGCGCATAAGTTTTGCCAAATTCTTTGCGAAACTGCTTCTTAATAAAACAGTCCTT GCAATATTCATCGTGCAGTTCTGTCAGTTCTTTAAAGATCATTTTCTTATCCAATATCG TCATCTCCGTTACGGTCTAATTT
ethanol producing cassette sequence	GATCGGCACTCATCCAAGCAATGTAAAAATCACAGGAATTCCAATCAGGCCGCAATT TGAAGAATCCATGCCTGTTGGCCCGATATATAAAAAGTACAATCTTTCACCAAACAAA AAAGTGCTTCTGATCATGGCAGGTGCTCACGGTGTATTAAAGAACGTAAAAGAGCTG TGCGAAAACCTTGTCAAGGATGACCAAGTGCAAGTAGTTGTCGTGTGCGGGAAAAA TACGGCTTTAAAAGAATCTTTGAGTGCGCTTGAAGCGGAAAATGGTGACAAATTAAA AGTTCTGGGCTATGTGGAGCGCATTGATGAGCTATTTCGGATCACAGATTGCATGAT TACCAAGCCCGGCGGCATTACTTTGACAGAAGCCACAGCCATTGGAGTGCCTGTCAT TCTGTACAAACCCGTGCCTGGCCAGGAAAAAGAAAATGCAAACTTCTTTGAAGACCG CGGAGCTGCCATCGTTGTGAACCGTCATGAAGAGATTCTCGAGTCAGTCACTTCCCTT CTTGCAGATGAAGATACCTTGCATCGCATGAAGAAAAACATTAAGGACCTTCATTTA GCAAACTCCTCTGAAGTGATTTTAGAGGATATCCTGAAGGAATCAGAAATGATGACC GCCAAACAAAAAGCCAAAGTGCTATCGTAATGGCGTACTTGAGAGCATACGAAAATC GTGTGCTCTTTTTATTTATATTCAGCCATCAATAAAAGCGGTTACATTTTTTTATGGAA CTTGCCCTTCTTTTGAAAAATAAGCCGTTTCGCAACTTGACGGGTGCTCCCAGATGGT GTATAGTTGAACCATCATTTAACAATGAATCAAAGTTAGATGATGACAAAATTTTTTT TAGCACATGGGTGCTACAGTACTAGGAGGAATTAAGCAATATGTCTTATACCGTGGG

	GACCTATCTGGCAGAACGCCTGGTTCAAATTGGGCTGAAACACCACTTCGCTGTTGC CGGTGATTATAACCTGGTGCTGCTGGACAATCTGCTGCTGAATAAAAACATGGAGCA AGTCTATTGCTGTAACGAACTGAACTGTGGCTTTAGCGCTGAAGGCTATGCCCGTGC CAAAGGTGCCGCCGCTGCTGTTGTAACCTATAGTGTGGGTGCCCTGAGTGCTTTCGA TGCTATTGGAGGGGCTTATGCTGAAAACCTGCCGGTAATCCTGATTTCTGGTGCCCC GAACAATAACGATCATGCCGCCGGTCATGTGCTGCATCATGCTCTGGGTAAAACCGA CTATCATTATCAGCTGGAAATGGCCAAAAACATTACTGCCGCTGCCGAGGCGATTTA TACTCCGGAAGAGGCTCCGGCCAAAATTGATCATGTCATCAAAACCGCACTGCGTGA GAAAAAACCGGTCTATCTGGAAATTGCTTGTAACATTGCCTCAATGCCGTGTGCCGCT CCTGGTCCGGCTAGTGCCCTGTTTAACGATGAAGCTTCGGATGAAGCAAGCCTGAAT GCTGCCGTTGAAGAAACGCTGAAATTCATTGCCAACCGTGACAAAGTCGCTGTACTG GTGGGTTCTAAACTGCGCGCTGCTGGTGCTGAGGAAGCCGCTGTAAAATTCGCTGAC GCCCTGGGTGGAGCTGTAGCAACCATGGCTGCCGCCAAATCCTTCTTTCCTGAGGAG AATCCTCATTATATCGGCACGAGCTGGGGCGAGGTTTCATATCCAGGGGTGGAGAA AACCATGAAAGAGGCAGACGCCGTTATCGCTCTGGCTCCTGTGTTCAATGACTATTCC ACCACCGGGTGGACTGATATTCCTGACCCGAAAAAACTGGTGCTGGCTGAACCTCGT TCTGTTGTGGTGAATGGCATCCGTTTTCCGAGCGTTCACCTGAAAGACTATCTGACTC GTCTGGCCCAAAAAGTGTCCAAAAAAACTGGCGCTCTGGACTTCTTTAAAAGTCTGA ATGCCGGGGAACTGAAAAAAGCTGCTCCGGCCGATCCTTCTGCTCCTCTGGTTAATG CCGAGATTGCTCGTCAAGTTGAAGCTCTGCTGACTCCGAATACCACCGTTATCGCCGA AACTGGTGATTCCTGGTTCAATGCCCAGCGTATGAAACTGCCGAATGGTGCTCGTGT TGAGTATGAGATGCAATGGGGCCATATTGGTTGGTCTGTGCCTGCTGCTTTTGGTTA TGCCGTTGGTGCTCCTGAACGTCGTAACATCCTGATGGTAGGGGATGGATCATTTCA ACTGACCGCCCAGGAAGTTGCTCAAATGGTTCGTCTGAAACTGCCTGTGATCATCTTT CTGATTAACAACTATGGCTATACGATCGAGGTCATGATTCACGATGGTCCGTATAAC AACATTAAAAACTGGGACTATGCTGGTCTGATGGAGGTGTTCAATGGTAATGGCGG GTATGATTCTGGAGCCGGGAAAGGCCTGAAAGCGAAAACCGGGGGTGAGCTGGCT GAGGCTATTAAAGTTGCCCTGGCCAATACAGATGGGCCTACCCTGATCGAATGCTTT ATTGGGCGTGAGGACTGTACAGAAGAACTGGTAAAATGGGGGAAACGTGTTGCCGC TGCTAACAGCCGTAAACCGGTGAATAAACTGCTGTAATAATACTAGAGATTAAAGAG GAGAAATACTAGATGGCATCAAGCACCTTTTATATCCCGTTTGTTAATGAAATGGGC GAGGGTTCTCTGGAAAAAGCGATCAAAGACCTGAATGGTAGTGGGTTCAAAAATGC CCTGATTGTGTCAGACGCCTTCATGAATAAAAGTGGCGTGGTGAAACAGGTTGCTGA TCTGCTGAAAGCACAGGGTATCAATAGCGCCGTGTATGACGGTGTTATGCCGAATCC

	GACCGTTACAGCCGTACTGGAGGGACTGAAAATCCTGAAAGACAACAACAGCGACT TCGTGATTTCTCTGGGTGGTGGTTCTCCACATGATTGTGCCAAAGCCATTGCCCTGGT GGCTACAAATGGAGGTGAAGTGAAAGATTATGAGGGGATCGACAAAAGCAAAAAA CCGGCACTGCCTCTGATGAGCATCAATACCACAGCGGGTACAGCATCTGAGATGACT CGTTTCTGTATTATCACCGACGAGGTTCGCCATGTGAAAATGGCCATCGTGGATCGTC ATGTTACACCGATGGTTAGCGTGAATGATCCTCTGCTGATGGTGGGTATGCCTAAAG GGCTGACTGCCGCTACAGGAATGGACGCCCTGACTCATGCCTTCGAAGCCTATTCTA GCACCGCCGCTACTCCTATTACCGACGCTTGTGCCCTGAAAGCTGCCTCTATGATTGC CAAAAATCTGAAAACCGCTTGTGACAACGGCAAAGACATGCCTGCTCGTGAGGCCAT GGCCTATGCCCAATTTCTGGCCGGTATGGCGTTTAATAACGCCTCTCTGGGCTATGTT CATGCTATGGCTCACCAACTGGGAGGCTATTATAACCTGCCACACGGCGTCTGTAAT GCTGTGCTGCTGCCTCATGTTCTGGCCTATAATGCCAGCGTGGTTGCTGGTCGCCTGA AAGATGTTGGAGTGGCTATGGGTCTGGATATTGCCAATCTGGGGGACAAAGAGGGT GCTGAAGCGACAATTCAAGCCGTCCGTGACCTGGCTGCTTCTATCGGAATCCCGGCA AACCTGACAGAACTGGGCGCCAAAAAAGAAGATGTGCCTCTGCTGGCTGATCATGCT CTGAAAGACGCTTGTGCTCTGACTAATCCTCGTCAGGGAGATCAGAAAGAAGTCGA GGAGCTGTTTCTGAGTGCCTTTTGATAATACTAGAGCCAGGCATCAAATAAAACGAA AGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCT CTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATAGGTGCGTT GAAGTGTTGGTATGTATGTGTTTTAAAGTATTGAAAACCCTTAAAATTGGTTGCACAG AAAAACCCCATCTGTTAAAGTTATAAGTGACTAAACAAATAACTAAATAGATGGGGG TTTCTTTTAATATTATGTGTCCTAATAGTAGCATTTATTCAGATGAAAAATCAAGGGTT TTAGTGGACAAGACAAAAAGTGGAAAAGTGAGACCATGATGCTTAGGAAGACGAGT TATTAATAGCTGAATAAGAACGGTGCTCTCCAAATATTCTTATTTAGAAAAGCAAATC TAAAATTATCTGAAAAGGGAATGAGAATAGTGAATGGACCAATAATAATGACTAGA GAAGAAAGAATGAAGATTGTTCATGAAATTAAGGAACGAATATTGGATAAATATGG GGATGATGTTAAGGCTATTGGTGTTTATGGCTCTCTTGGTCGTCAGACTGATGGGCC CTATTCGGATATTGAGATGATGTGTGTCATGTCAACAGAGGAAGCAGAGTTCAGCCA TGAATGGACAACCGGTGAGTGGAAGGTGGAAGTGAATTTTGATAGCGAAGAGATTC TACTAGATTATGCATCTCAGGTGGAATCAGATTGGCCGCTTACACATGGTCAATTTTT CTCTATTTTGCCGATTTATGATTCAGGTGGATACTTAGAGAAAGTGTATCAAACTGCT AAATCGGTAGAAGCCCAAACGTTCCACGATGCGATTTGTGCCCTTATCGTAGAAGAG CTGTTTGAATATGCAGGCAAATGGCGTAATATTCGTGTGCAAGGACCGACAACATTT CTACCATCCTTGACTGTACAGGTAGCAATGGCAGGTGCCATGTTGATTGGTCTGCATC

	ATCGCATCTGTTATACGACGAGCGCTTCGGTCTTAACTGAAGCAGTTAAGCAATCAG ATCTTCCTTCAGGTTATGACCATCTGTGCCAGTTCGTAATGTCTGGTCAACTTTCCGAC TCTGAGAAACTTCTGGAATCGCTAGAGAATTTCTGGAATGGGATTCAGGAGTGGACA GAACGACACGGATATATAGTGGATGTGTCAAAACGCATACCATTTTGAACGATGACC TCTAATAATTGTTAATCATGTTGGTTACGTATTTATTAACTTCTCCTAGTATTAGTAATT ATCATGGCTGTCAGAATTGATCTGCGCTAAACTCAATACATGATGATGAGATGACAA ATAGAGGAGAGGCATTCGCCTCTCCTATTTGATTTTCATCTGGATTTCAATCGCCAAA ATGTTTTGTTTACACCTCTGTTCAATCTCTTCAACATACTTTTCCATTGATGGTTTTCCT TTTTTCGCGATATCGGCCAGTTCTTCTAAATCACTGTATATTTCTATAAATGTTTTGTG AAGGATCAACTTCAAGTCTTTATCATCCATAGCTGCGTTCCCCTTCTCTATCAAGATAC CTCAAATAGTAAAACGGTCCTATTTATTCTGGTTCAATAGTTATATCGGCTTATTTTTA TTTTCTTTTCTATTTTGGTACATAAATTTCAAAAAAACTCTGCAAAATAATGGCGGAG GTGTTTTTTGTGACTTCAGAATTTCATAATGAGGATCAGACCGGCTTTACGGATAAGC GGCAGCTGGAACTAGCGGTGGAAACAGCGCAGAAAACAACAGGAGCCGCGACGAG AGGCCAAAGCAAAACATTAGTCGACTCTGCATACCAAGCCATTGAGGATGCTAGAGA ACTGTCACAATCTGAAGAGCTGGCAGCTCTCGATGATCCTGAATTTGTAAAGCAGCA ACAGCAGCTGCTAGATGACAGCGAGCATCAGCTGGATGAATTCAAAGAATAAAAAC CGCAGCTTCTGCGGTTTTTATTTTTAGTGATTGGTTAAGACATCTCCGTATTGTTTCAA CTTTTCACCAACCGTACATTTATTGATACAAAATGAATGCGCATAAGTTTTGCCAAATT CTTTGCGAAACTGCTTCTTAATAAAACAGTCCTTGCAATATTCATCGTGCAGTTCTGTC AGTTCTTTAAAGATCATTTTCTTATCCAATATCGTCATCTCCGTTACGGTCTAATTT