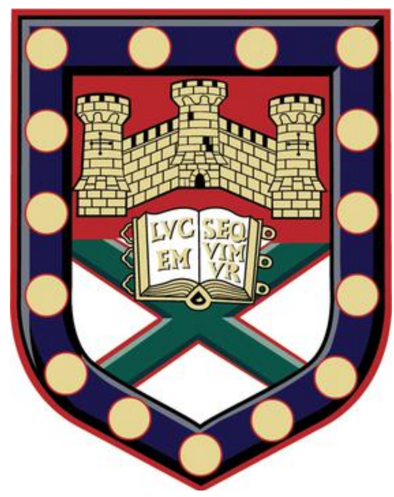


Developing synthetic consortia for industrial biofuel production from lignocellulose

Submitted by Tessa Reid to the University of Exeter as a thesis for the degree of

Master of Science by Research in Biological Sciences

in July 2018

This thesis is available for Library use on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature:…………………………………………………………………………

Abstract

This report describes work initiated at the Shell Biodomain Houston to evaluate candidate species for use as consolidated bioprocessing (CBP) hosts for conversion of lignocellulose (LC) to fuel. A systematic literature review regarding desirable characteristics for LC CBP hosts identified eight potential species: *Bacillus subtilis 168, Cellulomonas fimi, Clostridium cellulolyticum, Clostridium thermocellum, Escherichia coli, Lactobacillus plantarum, Thermoanaerobacterium saccharolyticum* and *Thermobifida fusca*. Commercially available API CH 50 kits were shown to be a rapid convenient method of testing the ability of all the species to ferment a wide range of carbohydrates. Analysis of batches cultured in the most important LC-derived sugars (D-glucose, cellobiose, D-xylose, L-arabinose) for biomass, ethanol, acetate and lactate production prompted *T. fusca* to be discarded as a potential host. This highlighted the high efficiency to which *L. plantarum* could achieve high product (lactic acid) yields. Physiological characterisation by imaging flow cytometry (IFC) established that it is possible to separate populations of different bacteria for the purpose of future sorting of complex consortia. Based on all these results a two-member microbial consortium was designed in which the biomass-to-model fuel product (lactate) pathway was shared among two species in co-culture with each species being specialised for specific processes *i.e. C. fimi* as the cellulolytic, and *L. plantarum* as the biofuel-producing specialist. For population monitoring of consortia IFC technology was utilised to establish a microbial demographic of *C. fimi* and *L. plantarum* separately and co-cultured together. Subsequently, a novel method to determine species abundance in synthetic consortia was developed allowing high-throughput testing. This could be used in industry for rapid process optimisation of synthetic consortia, and as an online monitoring and management system to detect consortium population-balance in industrial fermentations.

Acknowledgements

Firstly, I would like to thank my supervisor Prof. John Love, to whom I am extremely grateful for providing this unique opportunity; he continually and convincingly conveys a spirit of adventure towards research and scholarship, and excitement in regard to teaching. His enthusiasm, creativity and love of science is infectious and inspires the high standard of work to which his lab operates.

Secondly, to Dr Chloe Singleton, for her unwavering support in my academic development; thank you for consistently offering kind advice and guidance, providing stringent thesis corrections and investing valuable hours listening to me rehearse presentations. Chloe’s commitment to teaching is endless and I feel privileged to have learnt from such a teacher.

My gratitude also to the members of the Shell Biodomain Lab (Houston) and Exeter Microbial Biofuels Group, for providing a fun and inspiring workplace. I thank you all but in particular Professor David Parker, Kelly Showalter, Dr. James Winkler, Dr. Kun Zhang, Dr Ann Power and Professor Rob Lee. I thank Shell for their financial support.

Finally, I wish to express my gratitude and appreciation to Dr Sara Burton, to whom I am gratefully indebted; without her guidance and persistence this thesis would not have been possible. It is my secret belief that Sara should have a career as a motivational speaker and I have no doubt she will help many other students ‘cross the finish line’!

Thank you all for teaching me how to be a scientist.

Contents

Abstract 2

Acknowledgements 3

Contents 4

List of Figures 6

List of Tables 7

List of Abbreviations 8

1. Introduction 9
   1.1. Energy and Fuel 9
   1.2. Biofuel Production from Lignocellulose 10
      1.2.1. Lignocellulosic biomass 10
      1.2.2. Lignocellulosic biofuels 10
      1.2.3. Consolidated Bioprocessing 12
   1.3. Microbial Consortia for Biofuel Production by Consolidated Bioprocessing 17
      1.3.1. Minimising complexity in microbial consortia 17
      1.3.2. Target microorganism selection for optimal consortia 18

2. Project aims 20

3. Materials and methods 21
   3.1. Selection of industrially-relevant, CBP candidate hosts 21
   3.2. Revival of lyophilized species 21
   3.3. Media composition 21
   3.4. Growth of species on complex fermentation media 24
   3.5. Cryo-storage of species 24
   3.6. Carbon utilization of the species 24
   3.7. Carbohydrate fermentation profiles using API strips 25
   3.8. Growth Curves to determine time-points for imaging 25
   3.9. Imaging flow cytometry 25
   3.10. Growth of bacteria in different media 26
   3.11. *C. fimi/L. plantarum* co-culture experiment 27

4. Results 29

4.1. Selection of industrially-relevant hosts for CBP 29
4.1.1. Literature-based study of potential chassis for CBP 29
4.2. Metabolic characterisation of selected hosts 34
4.2.1. Rationale 34
4.2.2. Carbohydrate fermentation profiles for candidate industrially-relevant species 35
4.2.3. Investigation of utilisation of hexose and pentose sugars by candidate industrially-relevant species 37
4.2.4. In silico analysis of sugar utilisation pathways for hexose and pentose sugars by selected industrially-relevant species 40
4.3. Physiological characterisation of selected host species 43
4.3.1. Assessment of monoculture bacterial morphologies during candidate growth phases 43
4.3.1.1. Establishment of lag, log and stationary phase of growth for selected hosts 43
4.3.1.2. Characterisation of single cell monoculture bacterial morphology using imaging flow cytometry 45
4.3.2. Using Imaging flow cytometry to separate populations of different bacteria during co-culture 47
4.4. Co-culture of *C. fimi* and *L. plantarum* 49
4.4.1. Design of co-culture model 49
4.4.2. Exploring the use of 96-well plates for co-culture 50
4.4.3. Validation of growth in selected media in larger culture volume 52
4.4.4. Separating populations of *C. fimi* and *L. plantarum* by autofluorescence of the cells from imaging flow cytometry 54
4.4.5. Co-culture of *C. fimi* and *L. plantarum* on a hexose and pentose monosaccharide 56
5. Discussion 59
5.1. Selection of a chassis for consolidated bioprocessing 59
5.2. Combined metabolic and physiological characterisation of candidate species 60
5.3. Microbial consortia for CBP of LC 61
5.4. Imaging flow cytometry for study of microbial consortia 62
5.5. Progress and further work 63
6. Conclusion 66
7. References 67

List of Figures

Figure 1. Microbial Route to Lignocellulose Degradation
Figure 2. Consolidation of biomass processing technology
Figure 3. Carbohydrate fermentation profiles for selected industrially-relevant hosts
Figure 4. Dry cell weight, lactic acid, acetic acid and ethanol production on four main LC-derived sugars for industrially-relevant hosts
Figure 5. Reconstructed sugar utilisation pathways for glucose, cellobiose, arabinose and xylose in the selected host species
Figure 6. Growth curves for selected bacterial hosts in order to determine lag, log, and stationary phase of growth to identify time points for ImageStream sampling
Figure 7. Representative brightfield images of bacterial hosts during lag phase (t_{lag}), log phase (t_{log}) and stationary phase (t_{sta}) of growth
Figure 8. Imaging flow cytometry (ImageStream) plots comparing area of cell populations for the selected hosts
Figure 9. Assessment of growth of *C. fimi and L. plantarum* in Dubos salt medium (DBS), Luria Bertani broth (LB) and MRS medium (MRS)
Figure 10. Confirmation of growth of *C. fimi, E. coli* and *L. plantarum* in DBS media in 50 ml culture volume
Figure 11. Separating cell populations of *C. fimi* and *L. plantarum* in a 'model' co-culture from imaging flow cytometry
Figure 12. Percentage abundance and cells per ml of *C. fimi* and *L. plantarum* co-culture grown on glucose (A) and xylose (B)
Figure 13. Media management of consortia based on species abundance as determined by imaging flow cytometry (IFC)

List of Tables

Table 1. Common CBP hosts in the literature

Table 2. List of traits by which each microorganism was assessed by in the literature in order to test its suitability as a CBP host

Table 3. Overview of host species ordered from corresponding culture collection detailing recommended culture conditions.

Table 4. Inoculation ratio of *C. fimi* and *L. plantarum* for co-culture

Table 5a. Industrially-utilised species for use as hosts for biofuel-production from lignocellulose

Table 5b. Aerobic species with native advantageous traits for use as hosts for biofuel-production from lignocellulose

Table 5c. Anaerobic species with native advantageous traits for use as hosts for biofuel-production from lignocellulose

Table 6. Absolute (Abslt.) and relative (Rel;%) change in dry cell weight (DCW), lactic acid, acetic acid and ethanol production on four main LC-derived sugars for industrially-relevant hosts

Table 7. Inoculation ratio of *C. fimi* and *L. plantarum* co-culture for sampling by imaging flow cytometry

List of Abbreviations

μl	Microlitre
μm	Micrometre
ATCC	American Type Culture Collection
CBP	Consolidated bioprocessing
DBS	Dubos Salt Media
DSMZ	Deutsche Sammlung von Mikroorganism und Zellkulturen
EC	Enzyme commission
h	Hours
HPLC	High Performance Liquid Chromatography
IFC	Imaging Flow Cytometry
KEGG	Kyoto Encyclopedia of Genes and Genomes
LB	Luria Broth
LC	Lignocellulose
min	Minutes
ml	Millilitre
M	Mole
nm	Nanometre
OD_{600}	Optical Density at 600 nm
rpm	Revolutions Per Minute
PTS	Phosphotransferase
SHF	Separate Hydrolysis and Fermentation
SSCF	Simultaneous Saccharification and Co-fermentation
SSF	Simultaneous Saccharification and Fermentation

1. Introduction

1.1. *Energy and Fuel*

Transport fuel currently represents 65.6 % of oil consumption (1) and is predicted to rise from 95 million barrels per day in 2017 to 106 million barrels per day in 2040; transportation accounting for 60.6 % of this (2). Moreover, transport is the second largest producer of global greenhouse gases, with attendant environmental pollution and putative global warming (2). World population is projected to increase to nearly 8.8 billion people by 2035, with increasing global prosperity leading to demand for energy (3). CO_2 emission mandates are currently in place for transportation: several governments are set to ban new fossil fuel-only cars by 2040 and set lower, increasing, limits on biofuel content (4) with the USA specifying lignocellulosic sources (5).

Transport is dominated by non-renewable fossil fuels, primarily oil (3). Fossil fuels are energy dense and extremely versatile, however, for a number of practical, political and environmental reasons the combustion of fossil fuels is not sustainable (6). Biofuels (biologically-derived combustible fuels) are the current practical commercial solution for “carbon-neutralisation” of the transport sector; combustion of plant-derived biofuel releases CO_2, previously removed by plant growth, back into the atmosphere with no net increase in the atmospheric carbon stock (7,8). This in theory makes biofuels carbon neutral, however, in practice, differing factors contributing to biofuel production means the process is unlikely to be entirely carbon neutral. Although the CO_2 released from biofuels is comparatively less than fossil fuels. Fossil “carbon positive” fuel combustion releases carbon dioxide sequestered for millions of years (9). The widespread use of biofuels in the place of fossil fuels would help reduce CO_2 emissions in the transport sector.

Today we can at least begin the transition from oil to future more sustainable alternatives. Utilising the most abundant, renewable biopolymer on the planet – lignocellulose – as some form of energy carrier is an attractive place to start.

1.2. Biofuel Production from Lignocellulose

Biofuel production from LC biomass (second generation biofuels) is a superior alternative to biofuel production from food crops (first generation biofuels, predominantly ethanol) as it diminishes the 'food *vs* fuel' debate currently surrounding first generation biofuel production. Although, it is important to consider that this debate is not entirely negated for LC biomass if agricultural land/fresh water is required for biomass production. Additionally, low-cost technology for overcoming recalcitrance of LC biomass is still lacking (10).

1.2.1. Lignocellulosic biomass

LC biomass can be obtained from a number of plant sources including dedicated bioenergy crops (*e.g. Sorghum, Miscanthus,* Corn stover, switchgrass), agricultural and forestry residues (*e.g.* rice straw, crop residue, sugarcane bagasse) and some types of industrial or municipal wastes (rice straw, crop residue, sugarcane bagasse (10,11). This reduces the 'food *vs* fuel' controversy as land/resources required for energy production do not compromise food production. These sources of LC can subsequently be converted into various liquid fuels, such as ethanol, isobutanol, biodiesel, and others (12).

LC comprises lignin, hemicellulose, and cellulose in a complex, strong nanoscale composite (Figure 1). Lignin is an insoluble, branched phenolic polymer strengthening plant cell walls by cross-linking phenylpropane units via carbon-carbon or ether linkages. Hemicellulose is a network of cross-linked heteropolymeric fibres (xylan, xyloglucan, arabinoxylan and mannan) enclosing cellulose fibrils in a matrix. Cellulose comprises $\beta(1\rightarrow4)$ linked D-glucose units that assemble into larger units known as protofibrils or elementary fibrils packed into microfibrils and subsequently into cellulose fibres.

1.2.2. Lignocellulosic biofuels

Lignocellulose is intrinsically recalcitrant requiring expensive pre-treatment processes for conversion to biofuel. Pretreatment alters the biomass structure as well as its overall chemical composition to facilitate rapid and efficient enzyme access and hydrolysis of carbohydrates to fermentable sugars (13,14).

There are two main routes for biofuel production from LC biomass:

1) Thermochemical conversion: commonly referred to as biomass to liquid conversion process whereby biomass is pyrolyzed or gasified to produce "syngas" (synthesis gas, a mixture of carbon monoxide and hydrogen), subsequently reformed to fuels using catalytic or biological conversion.
2) Biochemical conversion: transforms sugar polymers in biomass (cellulose and hemicellulose) to monomeric sugars, which are fermented to fuels or chemicals using natural or genetically modified microorganisms.

A hybrid of these two routes can be used *e.g.* a chemical intermediate, produced by a biochemical process is then transformed into higher value products using a thermochemical route. This report will focus on biofuel production by the biochemical route, broadly categorisable into four biologically mediated events (Figure 2):

1) Pretreatment of LC biomass: (i) physical pretreatment (size reduction by grinding, milling (15), extrusion at elevated temperature (16), etc.); (ii) chemical pre-treatment under alkaline conditions (AFEX (17), ammonia percolation process (18), soaking aqueous ammonia lime (19), alkaline wet oxidation (20), steam explosion under alkaline condition, etc.), neutral conditions (ionic liquid (21), liquid hot water (22), ozonolysis (23), super critical water), or acidic conditions (dilute sulfuric acid (24), organic acid (25,26), concentrated acid (27), organosolv under acidic condition (28), etc.); (iii) physiochemical pretreatment (steam explosion under acidic conditions (29)) and (iv) biological pretreatment (30,31).
2) Enzyme production: involves numerous microorganisms for the production of cellulases and hemicellulases, most commonly the cellulolytic fungus *Trichoderma reesei* (32,33). Research has focused on isolating optimised enzyme cocktails for cellulose and hemicellulose depolymerization, and genetic-engineering-based strain improvement (34) for producing significantly more cellulases than the original QM6a isolate, up to 100g of cellulase per litre of fungus (35).
3) Hydrolysis of cellulose and hemicellulose: deconstructs cellulose and other carbohydrate polymers into sugar monomers and/or oligomers convertible into valuable products through biological or chemical

processes (36). This is carried out by native or engineered microorganisms (37).

4) Fermentation of soluble cellulose/hemicellulose hydrolysis products: performed by microorganisms, usually genetically engineered. For example, the industrial bioethanol producer *Saccharomyces cerevisiae* has been engineered for pentose as well as hexose fermentation (38–41).

Product recovery completes the conversion process from LC to biofuel.

1.2.3. *Consolidated Bioprocessing*

Recent estimates of biofuel production costs show that second generation biofuels are two to three times more expensive than petroleum fuels on an energy equivalent basis (42). Pretreatment and saccharification dominate operational costs and so determine the economic feasibility of biofuel production from LC biomass (43); efficient technologies to accomplish these stages are crucial to square the economic circle.

An example of such a technology is consolidated bioprocessing (CBP) in which enzyme production and fermentation of cellulosic and hemicellulosic products occur in a single process step (Figure 2; 44,45). Biomass processing technology has shifted towards increasing consolidation in which cellulase production, hydrolysis, and fermentation of all products are accomplished in a single process step. Reducing multiple unit operations to this single CBP operation is modelled to reduce ethanol production cost over fourfold (44). Therefore, the incentive for developing such processes is obvious.

The typical CBP strategy entails engineering all functionalities into a single microbial biocatalyst (46–51). The anaerobic cellulolytic bacterium *Clostridium thermocellum* is the most widely studied host for CBP followed by industrial *Saccharomyces cerevisiae* (Table 1). However, engineering and optimising multiple heterologous functionalities in a single microbe is inherently challenging, and there are few reports of commercially viable CBP yields and titres (45). The highest reported CBP yield is from a *Thermoanaerobacterium saccharolyticum-C. thermocellum* co-culture combining efficient cellulose hydrolysis by *C. thermocellum* with broad sugar utilisation and high ethanol yield of *T. saccharolyticum* (52) which led to 40% conversion efficiency. CBP successes in fuel ethanol production (52)

have not yet been replicated for advanced biofuels, such as higher molecular- weight alcohols and hydrocarbons.

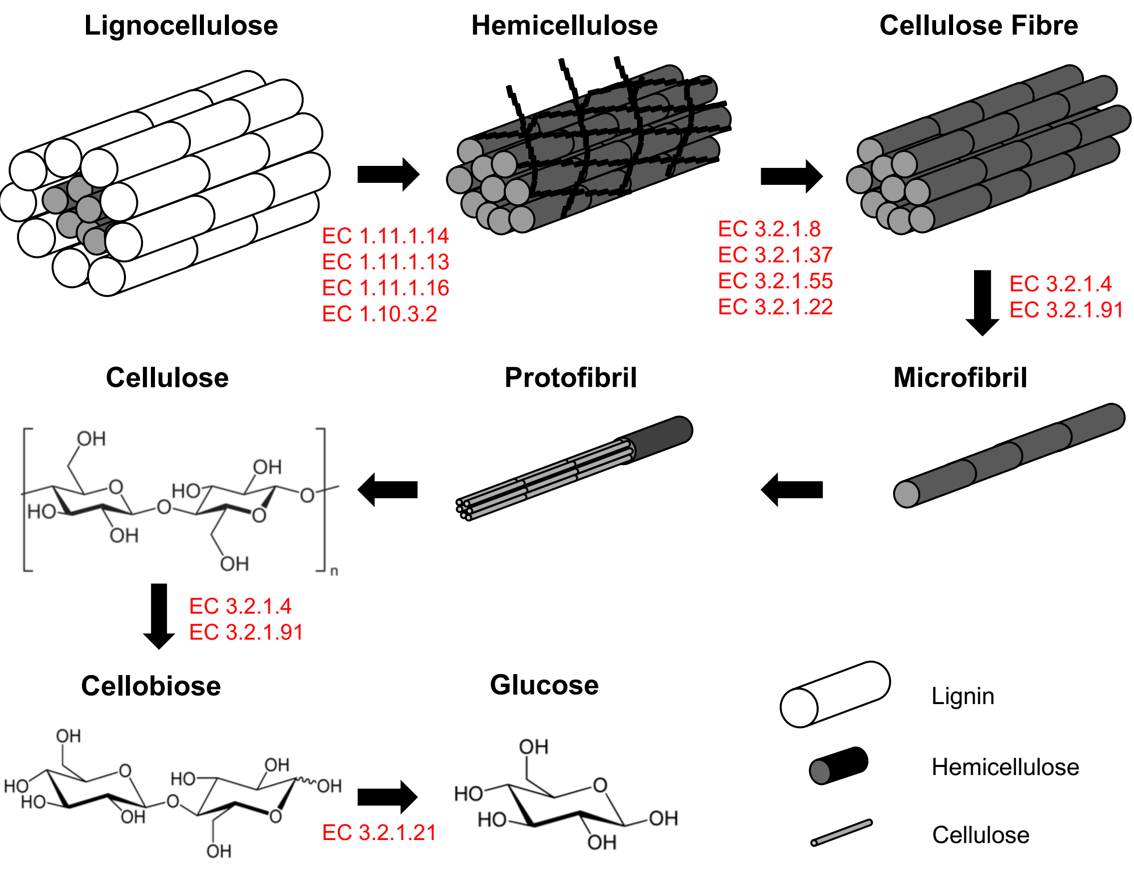


Figure 1. Microbial Route to Lignocellulose Degradation

Simplified schematic diagram of lignocellulose degradation detailing each key step and the enzymes responsible, highlighted in red (Enzyme commission (EC) number shown). Approximately 30 individual cellulose molecules are assembled into larger units known as protofibrils, which are packed into larger units known as microfibrils and these in turn assemble into cellulose fibres. Hemicellulose forms a matrix surrounding the cellulose fibre consisting of structures such as xylan, xyloglucan, galactomannan, galactoglucomannan. Finally, lignin surrounds this structure forming a protective layer. Firstly, the lignin layer is degraded by lignin peroxidases (EC 1.11.1.14), manganese peroxidases (EC 1.11.1.13), versatile peroxidases (EC 1.11.1.16) and laccases (EC 1.10.3.2). Hemicellulose structures are degraded by xylanases (EC 3.2.1.8), endo-1,4-β-xylanase/β-D-xylosidase (EC 3.2.1.37), arabinosidase (EC 3.2.1.55), α-galactosidase (EC 3.2.1.22). Endoglucanases (EC 3.2.1.4) hydrolyse cellulose bonds internally, cellobiohydrolases/exoglucanases (EC 3.2.1.91) cleave cellobiose units from the ends of the polysaccharide chains and β-glucosidases (EC 3.2.1.21) hydrolyse cellobiose units, releasing glucose, the main carbon source readily metabolised by bacteria.

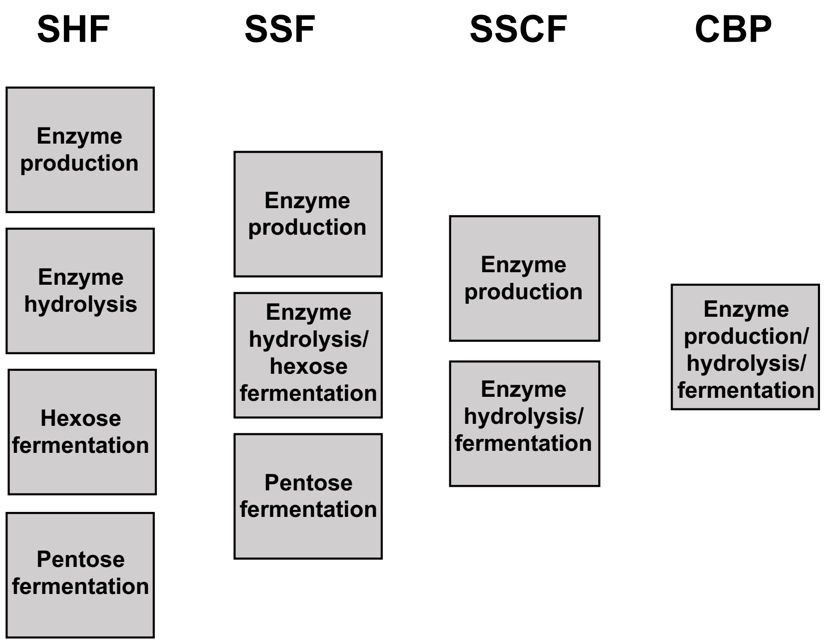


Figure 2. Consolidation of biomass processing technology

Separate hydrolysis and fermentation (SHF) involves four discrete process steps and up to four different biocatalysts. Simultaneous saccharification and fermentation (SSF) consolidates hydrolysis and fermentation of cellulose hydrolysis products into one step, with cellulase production and fermentation of hemicellulosic products occurring in two additional discrete process steps. Simultaneous saccharification and cofermentation (SSCF) involves two process steps: cellulase production, and cellulose hydrolysis and fermentation of both cellulose and hemicellulose hydrolysis products. In CBP, cellulase production, hydrolysis, and fermentation of all products, are accomplished in a single process step. Each grey box is representative of a separate unit operation required for that processing step i.e. in SHF, enzymes are first produced in one reaction, such as cultivation of a cellulase-producing bacterium and isolation of resulting enzymes, followed by another reaction step where the isolated enzymes are added to biomass for hydrolysis.

	Number of hits with organism name AND CBP on Web of Science (as searched in February, 2019)
Saccharomyces cerevisiae	160
Escherichia coli	143
Clostridium thermocellum	76
Trichoderma reesei	35
Clostridium acetobutylicum	17
Bacillus subtilis	16
Fusarium oxysporum	10
Clostridium cellulolyticum	7
Caldicellulosiruptor bescii	6
Corynebacterium glutamicum	5
Clostridium phytofermentans	4
Thermoanaerobacterium saccharolyticum	3
Yarrowia lipolytica	3
Lactobacillus plantarum	2
Thermobifida fusca	2
Geobacillus thermodenitrificans	1
Rhodosporidium toruloides	0
Thermoanaerobacterium ethanolicus	0
Cellulomonas fimi	0

Table 1. Common CBP hosts in the literature

Common species used for CBP development were identified in the literature then searched in the paper archive Web of Science against the term ‘CBP’’. The number of ‘hits’ was recorded, and species are listed in order of decreasing popularity.

1.3. *Microbial Consortia for Biofuel Production by Consolidated Bioprocessing*

In contrast to the CBP "superbug" paradigm, microbes live in synergistic communities in most natural environments in which individual species with specialised roles cooperate to survive and thrive together (53). Natural microbial consortia hold many appealing properties - stability, functional robustness, and the ability to perform complex tasks (54). Thus there is growing interest in engineering synthetic consortia for biotechnology applications (55,56).

Consortia can be defined as groupings of microorganisms undergoing interactions via modes such as commensalism, mutualism, competition, and predation, with synthetic consortia artificially designed for a specific purpose. These are often fragile and unstable, unlike their natural counterparts, currently limiting their use in industrial bioprocessing.

1.3.1. *Minimising complexity in microbial consortia*

To apply synthetic consortia to industrial processes such as CBP, variables contributing to complexity should be reduced, such as: 1) number of populations, 2) degree of physical separation between populations, 3) difference between population local environments, 4) volume of cultures and 5) time-scale of co-culture (54). These variables can mostly be characterised for respective microorganisms in single culture (55). So far, most studies of synthetic microbial consortia focus on a 'build a consortium to understand it' principle. For synthetic microbial consortia, more design principles and models are required to reveal the interactions and dynamic changes of high-performance synthetic systems (56).

Mathematical modelling and computational biology are integral to understanding consortia dynamics (57). Efficient computational modelling can address experimentally inaccessible aspects of microbial communities, such as inter-species interactions (57). For example, Shou *et al.* (58) built a mathematical model to analyse the requirements and constraints of their engineered yeast strain co-culture; stable population equilibrium was achieved through mutualistic cooperation for metabolite exchange, modelled using initial growth, survival, and metabolite production, rates.

In silico metabolic modelling can also elucidate interactions within microbial communities (59). For instance, an analysis of the syntrophic bacteria *Desulfovibrio vulgaris* and *Methanococcus maripaludis* generated a dual-species stoichiometric model, including 170 reactions and 147 metabolites comprising the central metabolism of both species (60). Hanly *et al.* (61) combined genome-scale metabolic models for two *E. coli* strains ALS1008 and ZSC113, engineered for glucose and xylose uptake respectively as a way to optimise batch co-cultures through computer simulations. However, despite design of complex computer and mathematical models of the co-culture by flux balance analysis (mathematical approach for analysing the flow of metabolites through a metabolic network), the model was not validated experimentally – a key step in model development, refinement and application. In addition, metabolic models are highly complex to curate, disadvantageous to minimising complexity in microbial consortia.

Successful co-cultures for biofuel production have previously been reported (52,61–63). Here, co-cultures are often tested in large volumes using bioreactors, which involves time and resources to set up. This can be time-consuming if numerous conditions are required for testing co-culture compatibility. High throughput experiments with predictable output is a defining aspiration of synthetic biology. Increasingly, protocols are being designed to produce higher outputs than their standard counterparts by, for example, reducing culture volume allowing an increase in sample size (*i.e.* 96-well plate transformations).

Therefore, it seems an optimum microbial consortia design could be achieved by use of a mathematical model followed by testing using a high throughput method.

1.3.2. *Target microorganism selection for optimal consortia*

A key challenge and one of the least studied aspects in microbial biofuel production is host selection. A discussion of the ‘ideal microorganism for biofuel production’ frames two alternatives to host selection: ground-up genetic engineering in an ‘industry-friendly’ model host (e.g. *Escherichia coli* or *Saccharomyces cerevisiae*) or enhancing a novel host which already possesses some of the required functional elements for substrate degradation or fuel production (64). These can also be called the recombinant strategy and the native strategy respectively. Given the wealth of potential hosts in the literature, it is difficult to select the final host species merely

from a literature search; also with complexities of consortia it is best not to be constrained by the species initially chosen. Subsequent strain characterisation of the most promising CBP species will allow experimental validation of their potential and assessment of between-laboratory discrepancies.

2. Project aims

This project aims to:

- Define desirable criteria for host selection to discern potential candidates for industrial CBP of lignocellulose
- Systematically review species in-literature using developed criteria to select microbial species with highest potential as an industrial CBP host
- Characterise selected candidate species metabolically and physiologically in order to design a co-culture model for the conversion of cellulose to fuel
- Development of a novel method to separate different bacterial populations and determine species abundance in co-culture to allow high-throughput testing.

3. Materials and methods

3.1. *Selection of industrially-relevant, CBP candidate hosts*

Novel host selection began by initial broad scoping of potential microorganisms suited for CBP and genetic engineering by searching for organisms commonly associated with CBP research (Table 1), followed by an extensive literature review of the final chosen candidates. Candidates were assessed on a list of traits (Table 2).

3.2. *Revival of lyophilized species*

Eight bacterial species were investigated: *Bacillus subtilis* 168 (DSM 402), *Cellulomonas fimi* (DSM 20113), *Clostridium cellulolyticum* (DSM 5812), *Clostridium thermocellum* (DSM 1237), *Escherichia coli, Lactobacillus plantarum* (DSM 20174), *Thermoanaerobacterium saccharolyticum* (DSM 7060), and *Thermobifida fusca* (ATCC BAA629) (Table 3). Species were obtained from two separate culture collections in freeze dried form - the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) and the ATCC (American Type Culture Collection), and stored at 4 °C until rehydration according to the culture collection's instructions. For aerobic species, 100 µl of culture was plated onto the recommended agar and 250 µl of culture was inoculated into 25 ml of the recommended media, in a 125 ml vented, baffled flask. For anaerobic species, all inoculation and handling of active cultures was carried out in an anaerobic hood (Whitley A35 Anaerobic Chamber, Yorkshire, UK). Subsequently, 250 µl of culture was plated onto recommended agar and 250 µl cells were inoculated into 5 ml of the recommended media in a 50 ml centrifuge tube. All species were incubated under the conditions recommended by the culture collection (Table 3).

3.3. *Media composition*

All chemicals were reagent grade and obtained from Sigma-Aldrich (Poole, UK) and Fisher Scientific (Loughborough, UK) unless indicated otherwise. All solutions were made with water purified using a MilliQ system and either autoclaved at 121 °C for 20 min or filter sterilised (0.22 µm filter, ThermoFisher) prior to use.

Trait	Notes
Growth conditions	O_2 requirements e.g. aerobic or anaerobic, optimal growth temperature and temperature range, pH tolerance.
Publically available genome sequence	Allows genomic analysis of host without re-sequencing.
Hazard class	Only the use of hazard class 1 organisms will be considered as these are generally regarded as safe (GRAS) e.g. non-pathogenic.
Previous industrial utilisation	Assumes microorganism has well-established genetic tools and a track record of industrial applications.
Genetic tractability	Ease of genetic manipulation either by genetic or metabolic engineering is advantageous as this will likely be necessary for strain improvement.
Cellulolytic capabilities	Shows whether organisms have LC-degrading capabilities
Metabolism	Five- and six-carbon sugars that are commonly found in lignocellulosic material include xylose, arabinose, glucose, mannose, galactose and the disaccharide cellobiose. The ability to metabolise all of these sugars increases efficiency of the biofuel production process
Product	A microorganism that naturally produces a biofuel molecule avoids the need to engineer a novel biofuel-producing pathway, which is energetically costly to the cell

Table 2. List of traits by which each microorganism was assessed by in the literature in order to test suitability as a CBP host

Host	Culture collection number[a]	Growth conditions[b]			Recommended Media[d]
		Temperature (ºC)	pH[c]	O_2 requirements	
Bacillus subtilis	DSM 402	30	7.0-7.2	Aerobic	Typtone soya broth (DSM-545)
Cellulomonas fimi	DSM 20113	30	7.0-7.2	Aerobic	Dubos salt medium (DSM-1161)
Clostridium cellulolyticum	DSM 5812	35	7.0-7.2	Anaerobic	Clostridium cellulolyticum (CM3) medium (DSM-520)
Clostridium thermocellum	DSM 1237	60	7.0-7.2	Anaerobic	Clostridium thermocellum medium (DSM-122)
Escherichia coli		37	7.0-7.2	Aerobic	Luria Bertani (LB) Broth
Lactobacillus plantarum	DSM 20174	37	6.2-6.5	Aerobic	MRS medium (DSM-11)
Thermoanaerobacterium saccharolyticum	DSM 7060	60	7.2-7.4	Anaerobic	Thermoanaerobacter SP. medium (DSM-144)
Thermobifida fusca	ATCC BAA629	50	7.2-7.4	Aerobic	Hagerdahl medium (ATCC 2382)

Table 3. Overview of host species ordered from corresponding culture collection detailing recommended culture conditions.

[a] The specific strain ordered for each species. All species ordered were the type strain other than *Bacillus subtilis* (strain 168) and *Thermobifida fusca* (strain YX).

[b] The optimal growth conditions for each host species as specified on the strain product sheet. Product sheets are available online at: www.dsmz.de. or www.atcc.org. Liquid cultures for all bacteria with aerobic O2 requirements were incubated in a shaking incubator at 200rpm.

[c] The pH ranges of the media in which the bacteria were cultured.

[d] The media the culture collection recommends reviving each species in plus the corresponding media code i.e. DSM-53. Composition can be found online.

[e] Anaerobic cultures incubated in either a Whitley a35 anaerobic cabinet or in a BD GasPak 100 using GasPak™ EZ anaerobe container system sachets to achieve anaerobic conditions.

3.4. *Growth of species on complex fermentation media*

If a given strain showed single colony formation upon revival on agar, a single colony was used to inoculate liquid media. All aerobic species were inoculated into 25 ml of medium in a 125 ml vented, baffled flask using a single colony and incubated at identical conditions as described previously. All anaerobic species were inoculated identically as aerobic strains but in a 50 ml falcon flask. All handling of active cultures was performed inside an anaerobic hood. Growth was confirmed by media turbidity as measured by absorbance (at 600 nm).

3.5. *Cryo-storage of species*

For long-term microbial storage, 500 μl stationary phase culture was added to 500 μl 50 % w/v sterile glycerol and thoroughly mixed by inversion. Samples were snap frozen in liquid nitrogen and stored at -80 °C. The viability of the cryo-stocked species was tested by streaking onto recommended agar and incubating under conditions as described above.

3.6. *Carbon utilization of the species*

Carbon source versatility was tested for all aerobic species by culturing in 125 ml vented, baffled flasks in standard media supplemented with 10 g l^{-1} D-(+)-Glucose, D-(+)-Cellobiose, L-(+)-Arabinose, or D-(+)-Xylose (conditions as in Table 3). Growth was monitored after 24 h by dry cell weight in the absence of added carbon source. Additionally, 800 µl samples were taken at the beginning and end of culture and centrifuged at 15,000 rpm, 3 min, room temperature (in Eppendorf 5424 R; Corning® Costar® Spin-X® centrifuge tubes containing 0.22 µm nylon membrane filter) in order to remove solid particulates and cells; 200 µl of resultant filtrate was analysed by high performance liquid chromatography (HPLC). Sugars and organic acids were separated using an HPX-87H (Biorad) column with Refractive index (RI) detection (512.00 µ RIU), mobile phase 0.005 % H_2SO_4, flow rate 0.500 ml min^{-1}, 50 °C, 20 µl injections. Cellobiose, glucose, xylose, arabinose, glycerol, lactic acid, acetic acid and ethanol were quantified

by comparison with standards. Anaerobic species were assessed identically except that cultivation was performed in 50 ml centrifuge tubes.

3.7. *Carbohydrate fermentation profiles using API strips*

API 50 CH (bioMérieux, Basingstoke, UK) strip tests for carbohydrate fermentation were used to study fermentation of 49 carbohydrate and related substrates (heterosides, polyalcohols, uronic acids). The species recommended medium was dyed with 0.1 g l^{-1} of bromothymol blue pH indicator. For all species, 10 ml of media was inoculated with a loopful of bacteria which was used to inoculate the API strip wells. A drop of mineral oil was used to seal each well to prevent medium evaporation and allow anaerobic fermentation. Strips were incubated at species growth temperature (Table 3) for at least 48 h or until a colour change (blue to yellow) was visually detected indicating acidification by fermentation. The first well contained no substrate so acts as a negative control.

3.8. *Growth Curves to determine time-points for imaging*

Growth curves were performed for each species to identify suitable time points at which to sample for lag, log and stationary phases. Single colonies of each species were used to inoculate 5 ml of species-specific media with 5 biological replicates. Cultures were grown overnight under species-specific conditions (Table 3). Overnight cultures were used to inoculate 10 ml of the relevant media to an optical density of 0.05 measured at 600 nm (OD_{600nm} = 0.05) in a 50 ml centrifuge tube. Identical incubation conditions were kept for sub-cultures as per overnight cultures. OD measurements were taken with a Tecan Sunrise (Tecan, Switzerland) until stationary phase was reached.

3.9. *Imaging flow cytometry*

An ImageStream (Amnis Corporation, WA, USA) flow cytometer was used to determine morphological features for each species at lag, log and stationary phases. To prepare samples for analysis, 150 μl of culture was transferred to a 2 ml centrifuge tube, and filtered through a 100 μM pore filter. All samples were acquired at 60x magnification (pixel size 0.3 μM^2), with the low flow rate/high sensitivity setting using INSPIRE software. Each

sample was imaged at six wavelengths: 435-405 nm, 505-560 nm, 560-595 nm, 595-642 nm, 622-745 nm and 745-780 nm. 10,000 events were collected for each sample. Raw ImageStream data resulting from analysis of culture was analysed using IDEAS software (Amnis Corporation, Seattle, WA) was used.

3.10. *Growth of bacteria in different media*

To test whether *C. fimi* and *L. plantarum* could grow in the same media so that the species can be co-cultured, each bacterium was grown separately in Dubos salt medium (DBS: 1 g l^{-1} $NaNO_3$, 2.0 g l^{-1} K_2HPO_4, 1 g l^{-1} $MgSO_4.7H_2O$, 1 g l^{-1} KCl, 0.002 g l^{-1} $FeSO_4.7H_2O$), Lysogeny broth (LB: 10 g l^{-1} NaCl; 10 g l^{-1} Tryptone; 5g l^{-1} yeast extract [BD, United States of America]) and MRS broth (MRS: 10 g l^{-1} peptone, 10 g l^{-1} meat extract, 5 g l^{-1} yeast extract, 20 g l^{-1} glucose, 1 g l^{-1} Tween 80, 2 g l^{-1} K_2HPO_4, 5 g l^{-1} Na-acetate, 2 g l^{-1} $(NH_4)_3$ citrate, 0.20 g l^{-1} $MgSO_4.7H_2O$ and 0.05 g l-1 $MnSO_4.H_2O$). For each species, single colonies were used to inoculate 5 ml of species-recommended media and grown overnight at 30 °C, with shaking at 200 rpm. For each of the resulting cultures, 1.6 ml was transferred to 3x tubes and centrifuged at 13, 000 rpm for 3 min to pellet cells. 1x cells were then re-suspended in either 1.6 ml of DBS, LB or MRS. Cultures were subsequently diluted to an OD_{600} = 0.05 with relevant media; 200 μl sub-culture was pipetted into 5x wells on a 96-well plate. This included 4 biological replicates for each medium for each species (1x plate per species). The outermost wells contained sterile LB media in order to reduce edge effects. Plates were then incubated at 30 °C, 800 rpm. Absorbance measurements were taken as previously described.

Following growth of all species in DBS, growth in larger DBS culture volumes was tested. A single colony chosen from an agar plate was inoculated into 5 ml of species recommended media and grown overnight at 30 °C, 200 rpm in a shaking incubator. The resulting culture was centrifuged at 5,000 rpm for 10 minutes to pellet cells and remove supernatant. Cells were re-suspended in 5 ml DBS. Resulting culture was diluted to an absorbance of 0.05 with DBS media with 5 biological replicates for each species. Cultures were incubated at 30°C, 200 rpm, and absorbance measured as previously.

3.11. *C. fimi/L. plantarum co-culture experiment*

A 96-well plate co-culture experiment was performed to determine whether the ImageStream could be used to determine percentage abundance of species. Co-cultures were tested with growth on either glucose or xylose. Overnight cultures of *C. fimi* and *L. plantarum* were prepared as described previously (Section 3.10). 2 ml of each of the resulting cultures were transferred to 4x tubes and centrifuged at 13, 000 rpm for 3 min to pellet cells. Cells were then re-suspended in 2 ml DBS (plus 10 g l^{-1} glucose or 10 g l^{-1} xylose). Cultures were subsequently diluted (Table 4). 200 μl sub-culture was pipetted into 3x wells on a 96-well plate. This included 4 biological replicates for each consortium (1x plate for glucose and 1x plate for xylose). The outermost wells contained sterile DBS media to reduce edge effects. Two identical plates were prepared, one of which was immediately sampled using the ImageStream 96-well plate auto-sampler, while the other was incubated at 30 °C, 800 rpm for 24 h then sampled identically as above.

	Inoculation Ratio (OD_{600nm})	
Consortium Name	*C. fimi*	*L. plantarum*
CF100	0.1	-
LP100	-	0.1
CF50LP50	0.05	0.05
CF25LP75	0.25	0.75
CF75LP25	0.75	0.25

Table 4. Inoculation ratio of *C. fimi* and *L. plantarum* for co-culture

Numbers indicate optical density at 600nm (OD_{600nm}) of both microbial species inoculated on 96-well plates. 4x biological replicates were included with 3x technical replicates for each co-culture. 2x plates were prepared for destructive sampling at 0 h and 24 h. Cultures were inoculated from a 5 ml overnight bacterial culture to an OD_{600nm} = 0.1. Positive controls of *C. fimi* and *L. plantarum* were included to check for accurate growth of both species. CF = *C. fimi,* LP = *L. plantarum*.

4. Results

4.1. Selection of industrially-relevant hosts for CBP

4.1.1. Literature-based study of potential chassis for CBP

Sixteen bacterial and fungal species were reviewed by considering a broad-spectrum of primary literature using the search engine Web of Science. Species were appraised according to desirable traits for biofuel production from lignocellulose. Only species which are considered non-pathogenic to humans, plants and animals were considered. Phenotype, oxygen requirement and growth conditions were informative for adaption of optimal culture conditions *in vitro*. Some species commonly used in industry have well-established genetic tools and a long track record of successful industrial applications so are considered likely to perform reliably as industrial biofuel hosts (37). An innate ability of a host species to naturally degrade lignocellulose substrates, and/or possession of biochemical pathways for biomass conversion into biofuel-like products indicates the potential as biofuel production hosts. In addition, genetic competence and high transformation efficiency are important traits as metabolic engineering approaches will be necessary to optimise biofuel-processing competency in selected species.

Using the results of the literature review (Table 5), eight bacterial species were identified by the industrial sponsor Shell Research Ltd. for investigation as potential industrial CBP hosts, comprising five microorganisms with natural characteristics advantageous to biofuel production, and three species already widely used in industry.

Cellulomonas fimi, Thermobifida fusca, Clostridium cellulolyticum and *Clostridium thermocellum* were chosen for their ability to hydrolyse pure cellulose and natural lignocellulosic materials (65–68). *C. fimi* and *T. fusca* are aerobes from the phylum *Actinobacteria; C. fimi* is mesophilic and *T. fusca* is thermophilic. *C. cellulolyticum* and *C. thermocellum* are obligate anaerobes from the phylum *Clostridia; C. cellulolyticum* is mesophilic and *C. thermocellum* is thermophilic. *Thermoanaerobacterium saccharolyticum* is also a member of the class '*Clostridia'* and was chosen for its CBP

capabilities as it is xylanolytic and may ferment a wide array of carbohydrates with ethanol as the main product (69).

Following the reported strategy of host selection, three industrially-utilised species were chosen: *Bacillus subtilis, Escherichia coli* and *Lactobacillus plantarum*. *B. subtilis* and *L. plantarum* are both widely studied industrial hosts and model organisms in their field (70,71). *E. coli* remains one of the most frequently used hosts for demonstrating production of biofuel candidates including alcohol-, fatty acid- and terpenoid-based biofuels (72).

Species	Phenotype	O_2 Requirements	Growth Conditions (literature)	Used in Industry	Genetic Competence	Lignocellulose degrading	Biofuel production (native or engineered strains)
*Bacillus subtilis**	Bacteria, Gram +ve,	Facultative aerobe	25-35 °C, pH 6.0-9.0	Yes	10^3 – 10^4 cfu; several transformation protocols available (70,73)	No	• Engineered for production of few higher alcohols; 24 mg l^{-1} Butanol (74) and 6.1 g l^{-1} isobutanol (75) • Native isoprene producer
*Escherichia coli**	Bacteria, Gram -ve	Facultative anaerobe	Opt 37 °C	Yes (extensively)	10^9 cfu; Most quick and efficient transformation system known; extensive genetic toolkit	No	• Engineered for production of many higher alcohols: 143 g l^{-1} isopropanol (76), 50.9 g/l isobutanol (77), 30 g l^{-1} 1-butanol (78), etc. • Engineered for production of many fatty acid derivatives: 1.95 g l^{-1} fatty alcohols (79), 0.6 g/l fatty alkanes (80), 1.1 g l^{-1} fatty ester (81) etc. • Engineered for production of many terpenoids via MEP or MVA pathway: 2.2 g/l isopentenol (82), 0.53 g l^{-1} farnesol (83), 1.1 g l^{-1} bisabolene (84), >60 g l^{-1} isoprene (85) etc.
*Lactobacillus plantarum**	Bacteria, Gram +ve	Facultative anaerobe	15-45 °C, pH>3.2	Yes	Very good; 10^9 efficiencies reported (86); established genetic toolbox	No (however engineered to show cellulolytic and xylanitic activity (87,88)	• Engineered to produce ethanol however at very low titer: 90-130 mM ethanol (89)
Yarrowia lipolytica	Fungi	Micro-aerobic	20-40 °C (opt 15-24), pH 2.5-8.0	Yes	10^4 – 10^6 cfu; Established electroporation protocol (90); developed genetic toolbox	No (however engineered to show cellulolytic activity (91) and xylanase activity (92))	• Natively produces lipids >20% of its dry biomass • Engineered for production of fatty acid derivatives: 0.5 g/l decanol (93), 4.98 mg/l pentane (94), and 0.64 g/l hexadecanol (95) • Engineered for α-farnesene production via MVA pathway: 0.26 g/l α-farnesene (96)

Table 5a. Industrially-utilised species for use as hosts for biofuel-production from lignocellulose

Species	Phenotype	O_2 Requirements	Growth Conditions (literature)	Used in Industry	Genetic Competence	Lignocellulose degrading	Biofuel production (native or engineered strains)
Acidithiobacillus ferooxidans/Acetobacter aceti	Bacteria, Gram –ve, acidophilic	Aerobic	Opt 30 °C, pH<1-2 (opt 2)	Yes (bioleaching)	Few attempts (97)	No	• Natively CO_2 and N_2 fixing
Bacillus pseudofirmus	Bacteria, Gram +ve, alkaliphilic	Facultative anaerobe	37 °C, pH 7.5-11.4 (opt>9.5)	No	Few attempts (98,99)	No	
Burkholderia thailandensis	Bacteria, Gram -ve	Aerobic	25-42 °C, pH 6.0-7.0	No	Naturally transformable (100); lambda red	No	• Native rhamnolipid producer on glycerol and canola oil (101)
*Cellulomonas fimi**	Bacteria, Gram +ve	Facultative anaerobe	30 °C, pH 7.0-7.2	No	Few attempts	Yes (secreted cellulase system)	• Developed as a microbial fuel cell to generate electricity (38.7 mW/m over a 7 day period) from cellulose (102)
Halobacterium salinarum	Archaea, Gram –ve, extreme halophile	Obligate aerobe	35-50 °C (opt 37), pH 7.2-7.4	No	10^2 cfu; by spheroblast transformation; Established protocol (103); development of genetic tools (104); cannot electroporate	No	• Photosynthetic • Natively ferments arginine
Rhodotorula toruloides	Fungi, yeast,	Micro-aerobic	30-37 °C, pH 5	No	Few transformation attempts; laborious protocol (105–107)	No	• Natively produces lipid <70% of its dry biomass
Shewanella oneidensis	Bacteria, Gram -ve	Facultative anaerobe	0-35 °C, pH 6.0-9.0	No	10^3 cfu; Established electroporation protocol (108)	No	• Developed to be a microbial fuel cell for electricity production from pentose and hexose sugars (109)
Sulfolobus islandicus	Archaea, hyper-thermophile	Aerobic	65-85 °C, pH 2.0-4.0	No	Established electroporation protocol (110); CRISPR-Cas (111)	No	
*Thermobifida fusca**	Bacteria	Aerobic	45-55 °C (opt 55), pH 4.0-10.0	No	10^3 cfu (112)	Yes (reported cellulase and xylanase activity; identified ligninase)	

Table 5b. Aerobic species with native advantageous traits for use as hosts for biofuel-production from lignocellulose

Species	Phenotype	O_2 Requirements	Growth Conditions (literature)	Used in Industry	Genetic Competence	Lignocellulose degrading	Biofuel production (native or engineered strains)
*Clostridium/Ruminiclostridium cellulolyticum***	Bacteria, Gram +ve	Obligate anaerobe	25-45 °C (opt 32-35), pH>5.0	No	10^5 – 10^7 cfu; Established conjugation and electro-transformation protocol (113,114); CRISPR/Cas9 (115)	Yes (by multi-enzymatic structure – the cellulosome)	• Native producer of ethanol on pentose and hexose sugar but at very low titres • Engineered for production of butanol; titre: 40 mg l^{-1} and 120 mg l^{-1} n-butanol from cellobiose and cellulose respectively (116)
Clostridium thermocellum	Bacteria, Gram +ve	Obligate anaerobe	37-69 °C (opt 60°C), pH 6.0-7.7	No	10^3 – 10^4 cfu; Established electroporation protocol (117); genetic tool development	Yes (by multi-enzymatic structure – the cellulosome)	• Native producer of ethanol on hexose sugars at very low titre; engineered for enhanced ethanol production: 3.0 g l^{-1} (53 % theoretical yield) from cellulose (118) • Natively CO_2 fixing by novel pathway (119)
*Thermoanaerobacterium saccharolyticum**	Bacteria, Gram +ve	Obligate anaerobe	45-70 °C, pH 4.5-7.0	In developmen t	Established electroporation protocol (120,121)		• Natively ethanol producing from pentose and hexose sugars; engineered strain produces highest titre of ethanol thus reported for a thermophilic anaerobe: 37 g l^{-1} (122) • Engineered for production of higher alcohols: 1.05 g l^{-1} n-butanol (26 % theoretical yield; (123))

Table 5c. Anaerobic species with native advantageous traits for use as hosts for biofuel-production from lignocellulose

Selection based on following criteria: 1) Organism description, oxygen requirement and growth conditions determine the feasibility of culturing microorganism in a laboratory environment 2) Previous use of microorganism in industry assumes there is already a large prior knowledge base for this species 3) High transformation efficiency and a well characterized genetic system are desirable for selected host species 4) Inherent cellulolytic capability gives species competitive advantage to release fermentable sugars from lignocellulose for conversion to biofuel 5) Natively-producing biofuel is advantageous as it avoids the energetic constraints of genetic engineering pathway. The symbol ‘*’ after a species name denotes that it was one of the selected host species for further study.

4.2. Metabolic characterisation of selected hosts

4.2.1. *Rationale*

Lignocellulose (LC) is the most abundant and readily available raw material on Earth for the production of biofuels (12). LC consists mainly of the polysaccharides cellulose and hemicellulose and the phenolic polymer lignin. Enzymatic hydrolysis of lignocellulosic biomass deconstructs cellulose and hemicellulose into fermentable sugars that can be converted into valuable products through biological or chemical processes (14). The prevalent fermentable sugars released from cellulose is the monosaccharide glucose and the disaccharide cellobiose (124). The main fermentable sugar released from hemicellulose is the monosaccharide D-xylose; D-galactose, L-arabinose, D-mannose, L-fucose and L-rhamnose are also released (124). The ability of a biofuel production host to ferment these sugars (in addition to glucose) significantly increases production efficiency (125). This is therefore a highly desired characteristic for a biofuel production host.

The aim of this study is to test the potential of the eight bacterial species chosen by Shell Research Ltd. to ferment a variety of LC derived sugars, using the API 50 CH biochemical strip test, and by batch fermentations of the four predominant LC sugars. The range of utilisable, fermentable sugars will indicate the microbial candidate strains potential and may highlight sugars that can be used across all species, thus determining a novel potential management strategy for optimisation of a defined LC/CBP co-culture.

4.2.2. *Carbohydrate fermentation profiles for candidate industrially-relevant species*

API 50 CH is a standardised system composed of 50 biochemical tests for the determination of sole carbohydrate utilisation of microorganisms. API 50 CH strip experiments established which of the seven species selected by Shell were capable of fermenting carbohydrates commonly derived from LC biomass (Figure 3). *T. fusca* was discounted from this study as it showed inconsistent growth and was difficult to work with due to culture heterogeneity caused by its filamentous growth. The fermentation tests were inoculated with bacterial culture with a pH indicator. During incubation, fermentation was revealed by a colour change in the tube, caused by the anaerobic production of acid and detected by the pH indicator colour change. There was an absence of sugar in first tube to act as a negative control.

All seven bacterial species tested fermented L-arabinose and D-glucose whereas none fermented L-fucose, D-arabitol, L-arabitol, esculin or methyl-βD-xylopyranoside. The aerobic species - *B. subtilis, C. fimi, E. coli,* and *L. plantarum* – fermented 53 %, 57 %, 41 % and 45 % of the test carbohydrates, respectively (Figure 3). The anaerobic species – *C. cellulolyticum, C. thermocellum* and *T. saccharolyticum* – fermented 12 %, 33 % and 41 % of the carbohydrates, respectively (Figure 3).

The carbohydrates of most relevance *i.e.* LC-derived were the monosaccharides, disaccharides, trisaccharides and polysaccharides (Figure 3). When considering these carbohydrates alone, *C. fimi* fermented 75 % of total carbohydrates, *B. subtilis* and *T. saccharolyticum* fermented 57 % of total carbohydrates, *C. thermocellum* fermented 50 % of total carbohydrates, and *L. plantarum, E. coli* and *C. cellulolyticum* fermented 46 %, 39 % and 11 % of total carbohydrates, respectively.

Carbohydrate fermentation results for *L. plantarum* were inputted into the API web service for Lactobacillus and related genera identification. This was to act as a test quality control. The input of *L. plantarum* API CH 50 test result into the API web service returned a ‘very good identification’ result with 99.9 % ID for *Lactobacillus plantarum*.

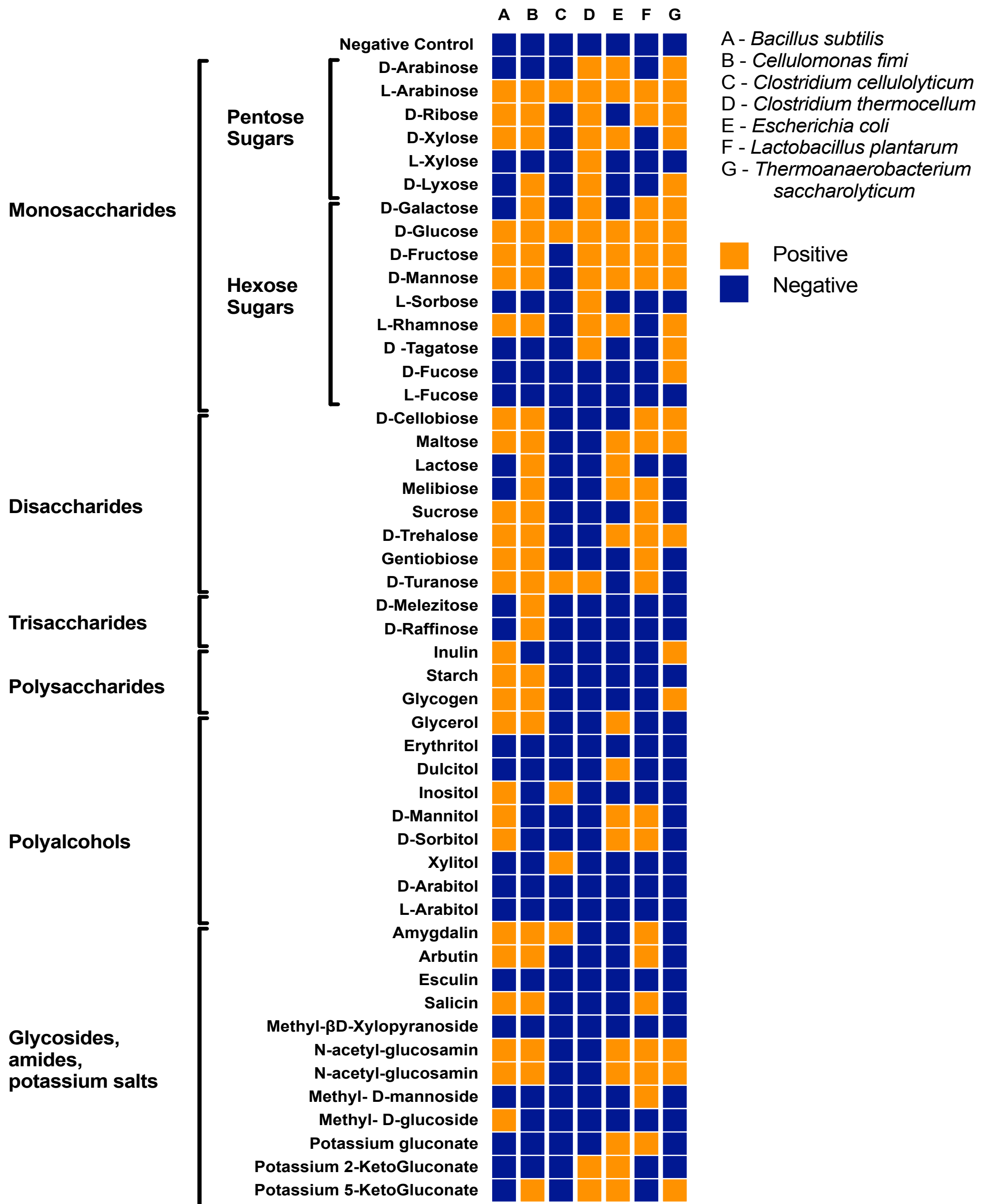


Figure 3. Carbohydrate fermentation profiles for selected industrially-relevant hosts

Carbohydrate fermentation was determined using API CH 50 strips (BioMérieux). Bacterial media were dyed with a pH indicator (bromothymol blue) before inoculating with bacteria cultured on an agar plate. 1 ml of culture was then pipetted into each well on the API strip (total 50: 49 carbohydrates and one control well with no carbohydrate). Strips were incubated at the species optimal growth temperature (Table 3) for 48 h or until colour change could be seen. A positive result was indicated by a colour change from blue to orange.

4.2.3. *Investigation of utilisation of hexose and pentose sugars by candidate industrially-relevant species*

It was important to understand which LC hydrolysis derived sugars could be utilised by the eight species selected by Shell *i.e.* glucose, cellobiose, arabinose and xylose. By measuring dry cell weight and lactic acid, acetic acid and ethanol production before and after growth on the species recommended media containing glucose, cellobiose, arabinose or xylose, the ability of the eight species to utilise products of LC degradation was assessed (Figure 4). This was compared to growth in a no sugar control. To determine if there was a significant difference between growth on glucose, cellobiose, arabinose and xylose with the no sugar control an ordinary one-way ANOVA with a *post-hoc* Tukey multiple comparison test was performed (Table 6).

From the results it can be concluded that in these conditions *C. cellulolyticum, C. fimi* and *T. fusca* may utilise glucose, cellobiose, arabinose and xylose. *E. coli* can utilise glucose, arabinose and xylose but not cellobiose. *L. plantarum* can utilise glucose, cellobiose and arabinose but not xylose. *T. fusca* can utilise glucose, cellobiose and xylose but not arabinose. *C. thermocellum* can utilise cellobiose but not glucose, arabinose and xylose. Results for *B. subtilis* were inconclusive as *B. subtilis* recommended media was a rich medium containing additional sugars that could have supported growth in addition to the added glucose, cellobiose, arabinose or xylose. *L. plantarum* produces the highest concentration of lactic acid as its main product compared to the other seven species whereas *T. saccharolyticum* produces the highest concentration of ethanol on glucose, cellobiose, arabinose and xylose.

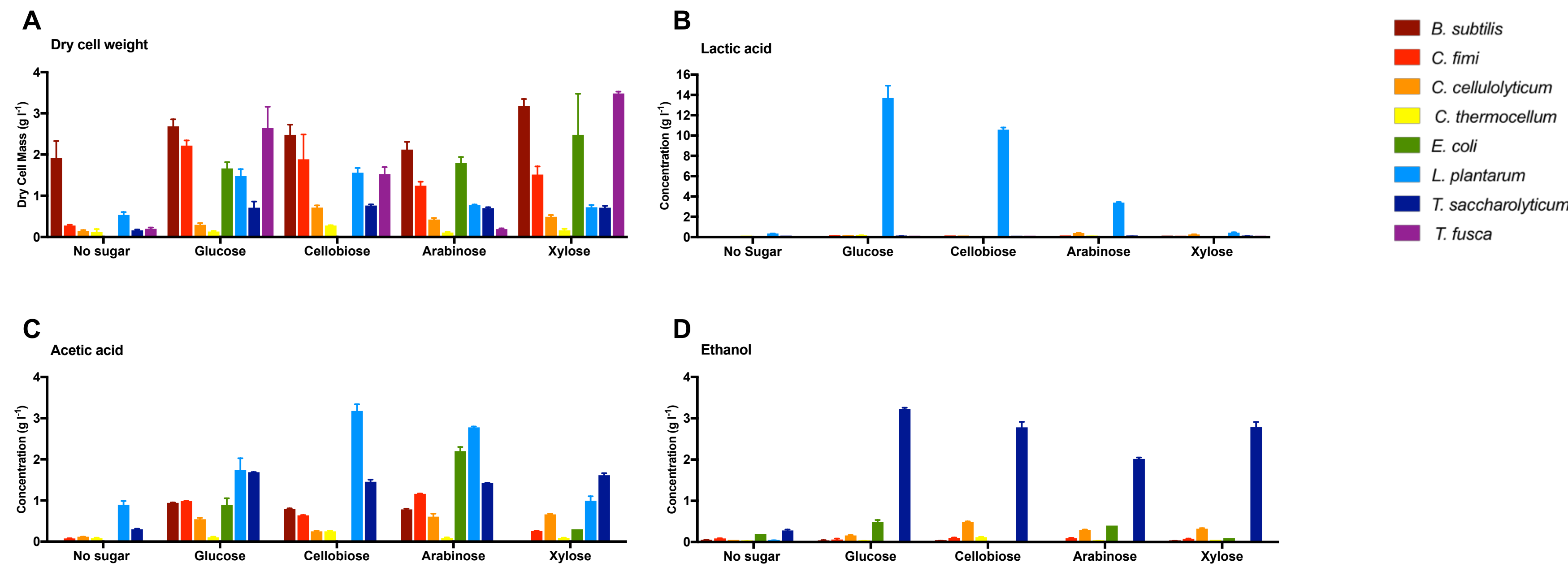


Figure 4. Dry cell weight, lactic acid, acetic acid and ethanol production on four main LC-derived sugars for industrially-relevant hosts

Growth was determined by dry cell weight measurements in gram per litre ($g\ l^{-1}$) after 24 h. Product concentration ($g\ l^{-1}$) was determined by HPLC analysis of culture broth after 24 hour fermentations and for each time point. Experiments were performed in triplicate. The initial concentration of each sugar in the growth media was 10 $g\ l^{-1}$. All species were grown in their recommended media and culture conditions. Growth in sugar-enriched media was compared to growth in identical media with no sugar. Standard deviation bars shown. (Graphs rendered in Prism).

Organism	Change	Glucose				Cellobiose				Arabinose				Xylose			
		DCW	Lactic acid	Acetic acid	Ethanol	DCW	Lactic acid	Acetic acid	Ethanol	DCW	Lactic acid	Acetic acid	Ethanol	DCW	Lactic acid	Acetic acid	Ethanol
B. subtilis	Abslt. (g l-1)	2.69*	0	0.94*	0	2.48*	0	0.79*	0	2.12	0	0.78*	0	3.18*	0	0	0
	Rel. (%)	40	0	94	0	29	0	79	0	10	0	78	0	66	0	0	0
C. fimi	Abslt. (g l-1)	2.22*	0	0.99*	0	1.89*	0	0.64*	0	1.25*	0	1.16*	0	1.52*	0	0.26*	0
	Rel. (%)	696	0	1142	0	578	0	703	0	348	0	1364	0	445	0	226	0
C. cellulolyticum	Abslt. (g l-1)	0.29*	0.1*	0.54*	0.16*	0.72*	0.4*	0.63*	0.48*	0.42*	0.4*	0.61*	0.29*	0.49*	0.26*	0.66*	0.33*
	Rel. (%)	106	196	361	278	400	432	436	103	196	432	414	570	242	376	460	655
C. thermocellum	Abslt. (g l-1)	0.13	0	0.11	0	0.28*	0	0.25*	0	0.108	0	0	0	0.16	0	0	0
	Rel. (%)	6	0	32	0	106	0	211	0	-61	0	0	0	48	0	0	0
E. coli	Abslt. (g l-1)	1.66*	0	0.89*	0.49	0	0	0	0	1.79*	0	2.2*	0.4	2.48*	0	0.3*	0.4
	Rel. (%)	166	0	89	143	0	0	0	0	179	0	220	100	248	0	30	100
L. plantarum	Abslt. (g l-1)	1.48*	13.7*	5.43*	0	1.56*	10.6*	6.85*	0	0.77*	3.41*	6.52*	0	0.72*	0.45*	4.69*	0
	Rel. (%)	173	3749	17.6	0	188	2868	48.2	0	43	855	41	0	34	26	2	0
T. saccharolyticum	Abslt. (g l-1)	0.71*	0	1.69*	3.23*	0.76*	0	1.46*	2.78*	0.7*	0	1.42*	2.01*	0.71*	0	1.61*	2.78*
	Rel. (%)	341	0	465	1043	373	0	387	886	335	0	375	613	341	0	440	886
T. fusca	Abslt. (g l-1)	2.64*	0	0	0	1.53*	0	0	0	0	0	0	0	3.48*	0	0	0
	Rel. (%)	1234	0	0	0	672	0	0	0	0	0	0	0	1658	0	0	0

Table 6. Absolute (Abslt.) and relative (Rel) change in dry cell weight (DCW), lactic acid, acetic acid and ethanol production on four main LC-derived sugars for industrially-relevant hosts

Absolute change represents the value for DCW, lactic acid, acetic acid and ethanol in grams per litre (g l^{-1}) for each species after 24 h fermentation with either glucose, cellobiose, arabinose or xylose. Relative change represents the percentage increase or decrease in DCW, lactic acid, acetic acid and ethanol for each sugar as compared to the no sugar control at 24 h. '*' symbol next to number means there is a significant difference ($P<0.05$) between growth on sugar compared to the no sugar control. This was determined by an ordinary one-way ANOVA with a *post-hoc* Tukey multiple comparison test. Analysis was performed using the statistics software Prism.

__4.2.4.__ In silico analysis of sugar utilisation pathways for hexose and pentose sugars by selected industrially-relevant species

The Kyoto Encyclopedia of Genes and Genomes (KEGG; 126) was used to investigate whether the eight industrially-relevant species selected by Shell had the corresponding genes coding for enzymes in utilisation pathways for glucose, cellobiose, arabinose and xylose (Figure 5). This was used to verify and support the results obtained from batch fermentation analysis for the eight species with glucose, cellobiose, arabinose and xylose (Figure 4).

D-glucose is transported into the cell by the phosphotransferase (PTS) system where it is converted to pyruvate by glycolysis which can then be anaerobically broken down to lactate, ethanol or acetate, which requires the enzymes: lactate dehydrogenase, alcohol dehydrogenase and aldehyde dehydrogenase respectively. These products are excreted from the cell and hence can be detected in the culture broth. The disaccharide cellobiose can be cleaved into glucose monomers by beta-glucosidase or can be transported into the cell by a PTS system and then cleaved by phospho-beta-glucosidase. Arabinose and xylose require the transporter L-arabinose isomerase and xylose isomerase in order to be transported into the cell. Arabinose is then converted to L-ribulose, phosphorylated, converted to D-xylulose and subsequently to D-ribulose which enters the pentose phosphate pathway. The pentose phosphate pathway converts D-ribulose to pyruvate which is metabolised by glycolysis. Xylose follows the same pathway however, once transported into the cell it is converted to D-xylulose and then phosphorylated.

C. thermocellum only utilised cellobiose and not glucose, arabinose or xylose; this corresponds to *C. thermocellum* encoding for only a beta-glucosidase enzyme for cellobiose utilisation (Figure 5). *L. plantarum* could not utilise xylose which corresponds to a reported lack of the enzyme xylose isomerase for xylose utilisation. *T. fusca* was not observed to utilise arabinose which corresponds to a reported absence of the enzyme arabinose isomerase for arabinose utilisation. *C. fimi* and *C. cellulolyticum* were observed to utilise all sugars tested which corresponds to the reported presence of

beta-glucosidase, arabinose isomerase and xylose isomerase enzymes. Both genomes are not reported to encode for a glucose PTS system however code for a hexose kinase enzyme for the utilisation of glucose. *E. coli* could not utilise cellobiose which corresponds to a lack of beta-glucosidase enzyme. *B. subtilis* has all the required enzymes for the utilisation of glucose, cellobiose, arabinose and xylose (Figure 5). Statistical significance was inconclusive for *B. subtilis,* most likely because this bacterium was grown in a rich medium therefore there was less difference between the no-sugar control and the sugar supplemented media. Therefore, further work would be required to confirm sugar utilisation in *B. subtilis*. *T. saccharolyticum* showed statistically significant growth and ethanol production on all four sugars which corresponds to the reported presence of necessary enzymes for fermentation of all sugars (Figure 5). This shows the successful prediction of the performance of the organisms on the different sugars.

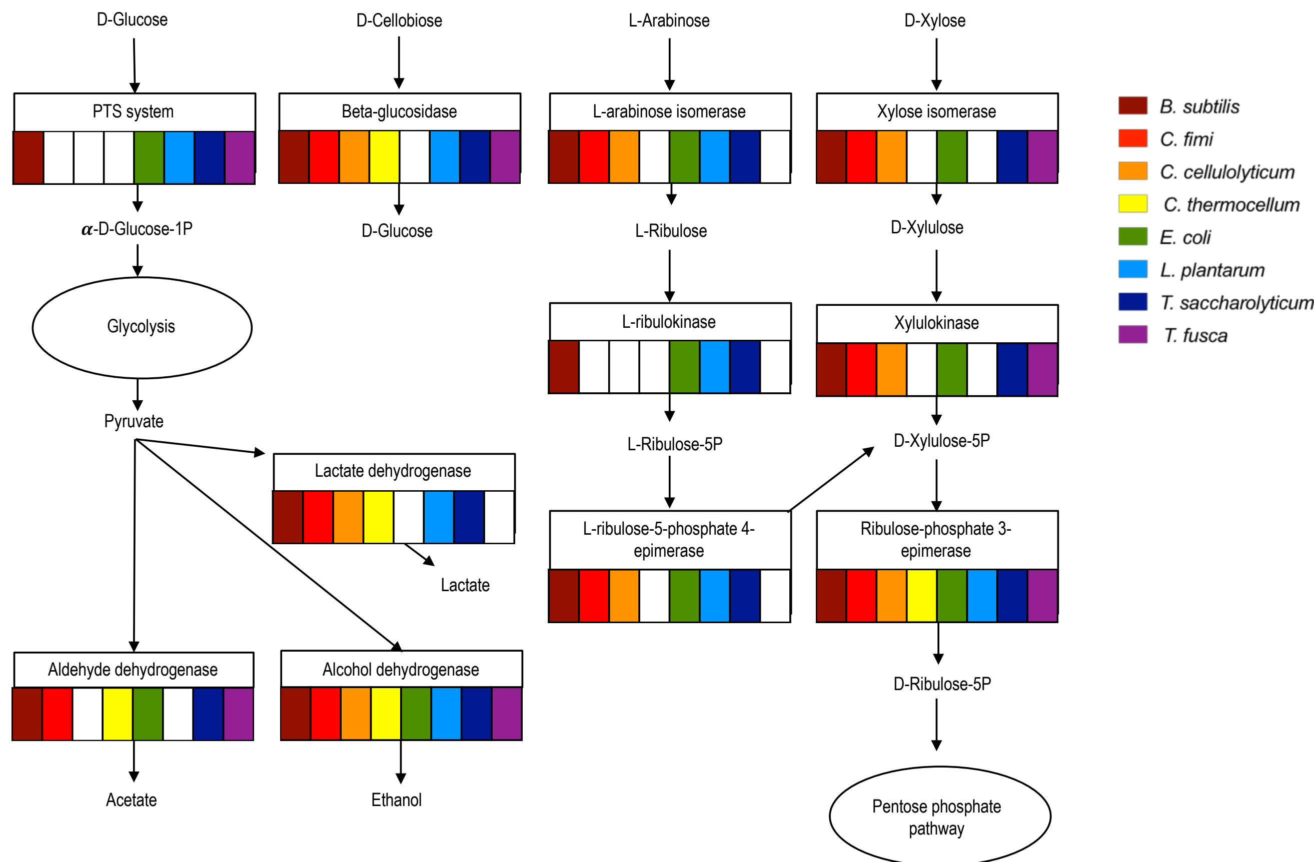


Figure 5. Reconstructed sugar utilisation pathways for glucose, cellobiose, arabinose and xylose in the selected host species

Reproduced from KEGG Pathways. Boxes represent the key enzymes involved in sugar utilisation; ovals represent pathways involved in sugar utilisation. Boxes highlighted in species colour (see key) represent the presence of this enzyme.

4.3. Physiological characterisation of selected host species

4.3.1. *Assessment of monoculture bacterial morphologies during candidate growth phases*

4.3.1.1. *Establishment of lag, log and stationary phase of growth for selected hosts*

Mono-species morphology was assessed using an imaging flow cytometer (ImageStream) to identify distinct morphological features that could distinguish between bacterial species in a consortium. This was assessed at three key stages of bacterial growth: 1) Lag phase, 2) Log phase, 3) Stationary phase, to discriminate if significant changes occurred in cell morphology.

Before ImageStream samples could be taken, the lag, log and stationary phases of growth for each bacterial host had to be established to identify at which time points sampling was most appropriate. Each species was grown in 10 ml culture volume in 50 ml centrifuge tubes. OD_{600nm} measurements were taken to monitor growth over time (Figure 5). The natural log of these values were then plotted against time to establish each phase of growth – where the points lie in a straight line determine when cells are growing exponentially compared to lag/stationary phase. This is represented by the red lines in Figure 5 establishing t_{lag}, t_{log} and t_{sta} for each species.

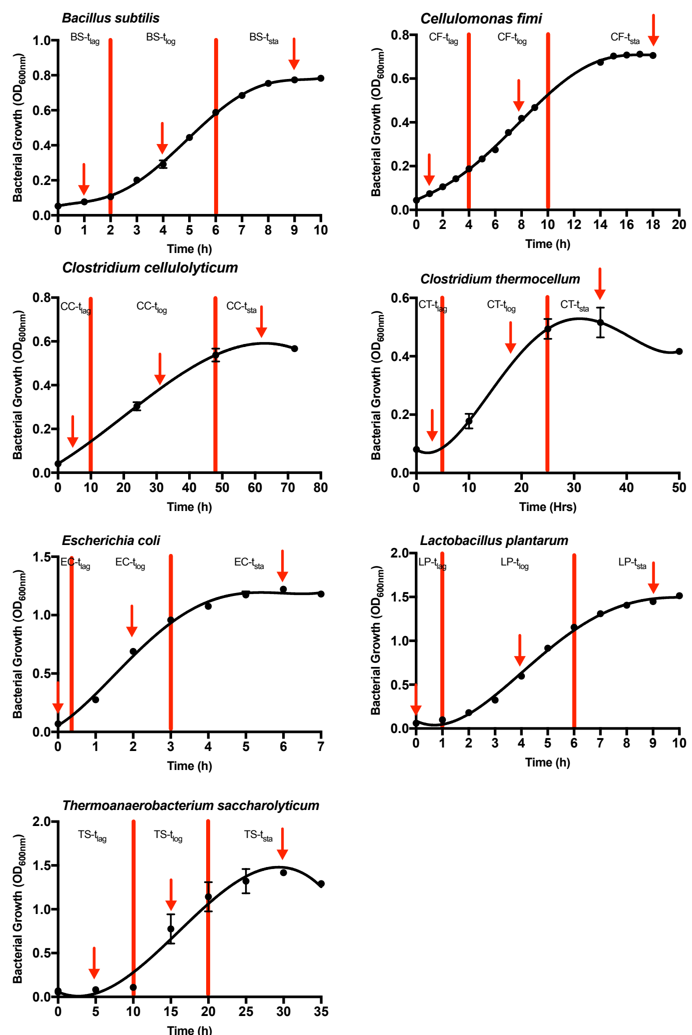


Figure 6. Growth curves for selected bacterial hosts in order to determine lag, log, and stationary phase of growth to identify time points for ImageStream sampling

All species were grown in 10ml culture volume in the species recommended media in 50 ml centrifuge tubes. Incubation was at the species optimal temperature; aerobic species were incubated with shaking at 220 rpm, anaerobic species were incubated in a static incubator. Cultures were inoculated from a 5 ml overnight bacterial culture to an OD_{600} = 0.05. Points represent the mean from 5 biological replicates, with standard deviation bars shown unless hidden by the symbol. Curves represent the best fit of the data using a third, fourth or fifth order polynomial equation, rendered using Prism software. Red lines separate lag phase, log phase and stationary phase; ‘BS-t_{lag}’ means *Bacillus subtilis* at time = lag phase. Red arrows indicate where samples will be taken to image using the ImageStream in order to identify distinct morphological features at each phase of growth.

4.3.1.2. *Characterisation of single cell monoculture bacterial morphology using imaging flow cytometry*

To identify distinct morphological features between the species and to ascertain whether this could be used to distinguish between species in a co-culture, single cell morphology was studied using imaging flow cytometry. The ImageStream filters cells through a channel and takes an image of each cell at six different wavelengths. Corresponding feature values can be extracted for each image *e.g.* cell length, width, area etc. In this study, brightfield microscopy was used to discern the morphology of cells as this channel gave the clearest morphological images.

At the three key stages of growth (as established from growth curves), flow cytometry images were taken of cells (Figure 6). From the images it can be seen that all the species studied are rod shaped and similar in size; images were chosen that had the median cell length from the sample population.

From stationary phase images (as this assumes that cells have grown to their full length), *C. fimi* has the smallest mean in length (5.7 μm (±0.2 μm)) followed by *C. thermocellum* (7.7 μm (±0.4 μm)) and *E. coli* (8 μm (±0.2 μm)); *C. cellulolyticum* and *T. saccharolyticum* have the longest cell length (13.3 μm (±0.9 μm) and 12.7 μm (±0.9 μm) respectively) and *B. subtilis* and *L. plantarum* both have a cell length 10.7 μm (±0.4 μm) and 11.3 μm (±0.6 μm) respectively. Mean values are an average of 5 biological replicates plus or minus the standard deviation. Therefore, cell length could potentially be useful as a parameter for separating species in a co-culture by, for example, plotting all events on a histogram of cell length to discern if there are two distinguishable peaks at the expected lengths for the individual species.

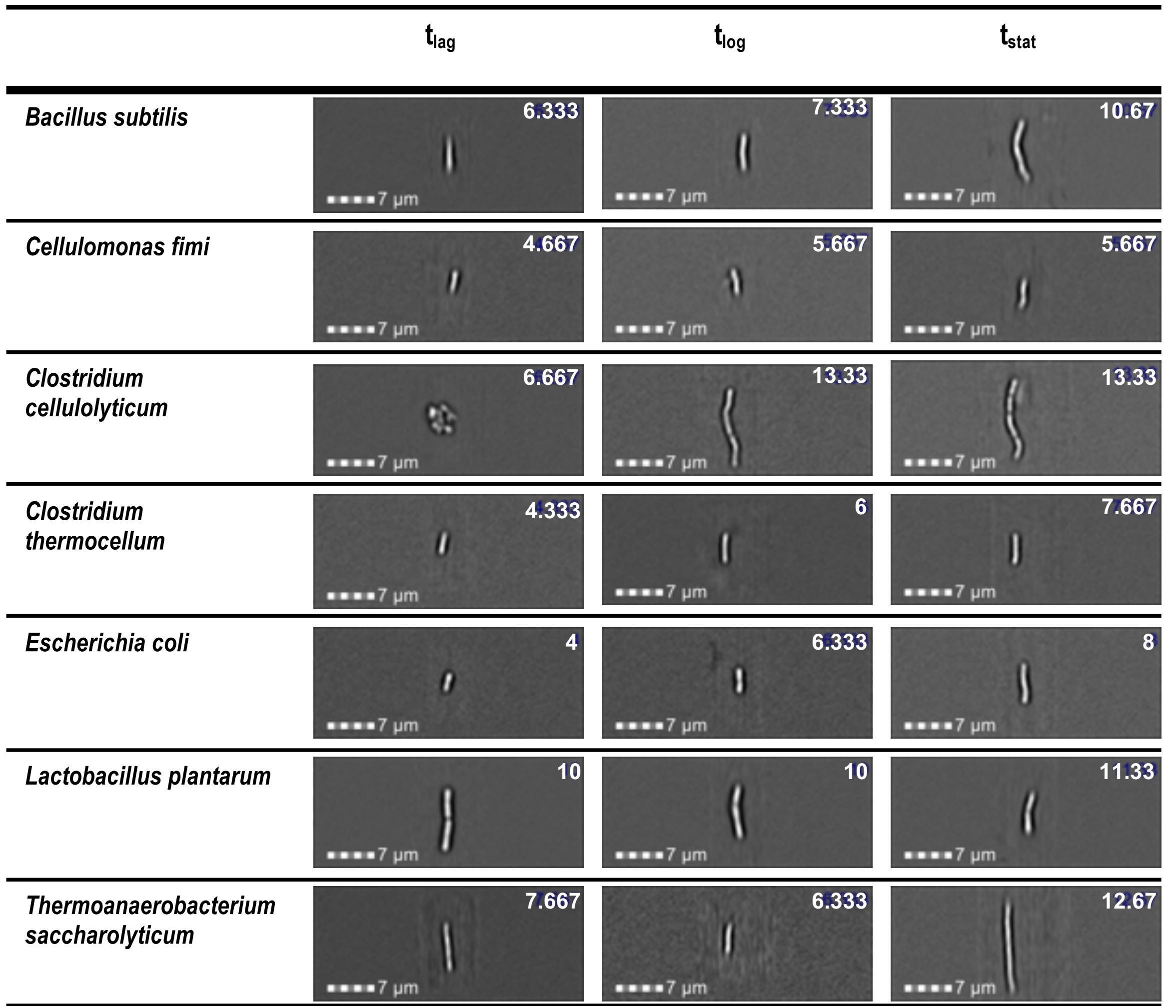


Figure 7. Representative brightfield images of bacterial hosts during lag phase (t_{lag}), log phase (t_{log}) and stationary phase (t_{sta}) of growth

Images acquired from ImageStream^{x} imaging cytometer and processed using IDEAS software. Scale bars shown in bottom left hand corner of each image. White numbers in top right hand corner of each image represents length of cell in µm. Length represents median cell length of sample (10,000 cells in one sample). 150 µl of bacterial culture was sampled at relevant time point, as identified in Figure 5. Each species grown in the same conditions as in Figure 5.

4.3.2. *Using Imaging flow cytometry to separate populations of different bacteria during co-culture*

In addition to bacterial cell morphology, it is possible to separate populations of different bacteria by comparative cell area plots (Figure 8). These data can also be acquired on the ImageStream. To assess this, the data acquired from stationary phase samples (as described above) were used to combine the 10,000 events analysed for each species into one dot plot.

The three bacteria chosen by recombinant selection were compared against the four bacteria chosen by native selection. This is based on the principle that the recombinant bacteria will be co-cultured with a native bacterium.

Figure 8 showed clear differences in cell area in bacterial populations between some species. For example, cell populations for *C. cellulolyticum*/*E. coli* and *C. cellulolyticum/L. plantarum* could be more clearly distinguished compared to *T. saccharolyticum/B. subtilis* or *T. saccharolyticum/E.coli*. This approach and these data could be useful when gating the cell populations in a co-culture in order to distinguish the ratio of each species.

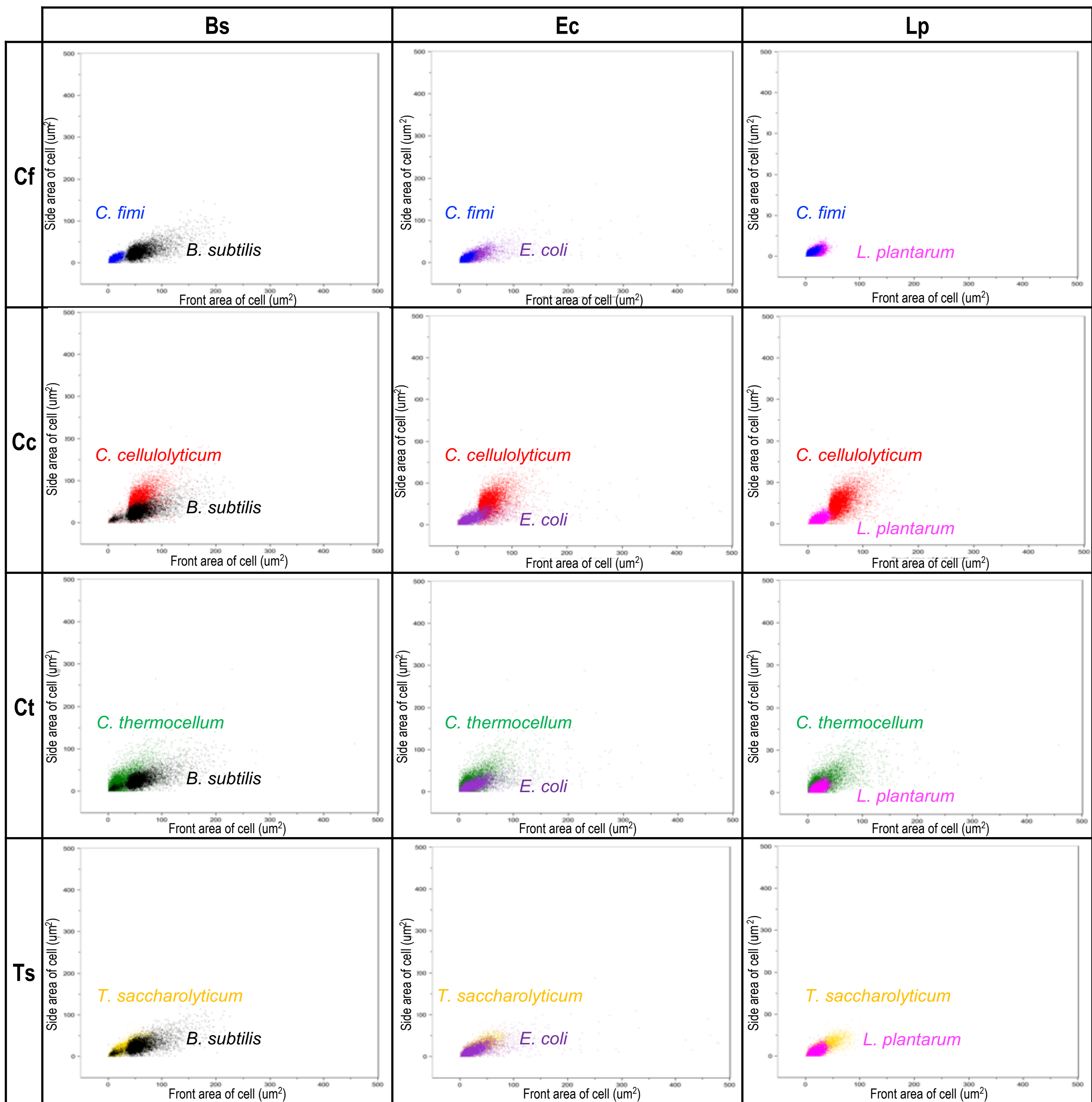


Figure 8. Imaging flow cytometry (ImageStream) plots comparing area of cell populations for the selected hosts

Plots of area in μm^2 of front cell face (determined by bright field microscopy on the ImageStream) vs. area in μm^2 of side cell face (determined by laser 745-780 nm). Data acquired from ImageStream^x imaging cytometer and processed using IDEAS software. 10,000 events shown for each population. X-axis scaled from 0 μm^2 to 500 μm^2. Y-axis scaled from 0 μm^2 to 500 μm^2.

4.4. Co-culture of *C. fimi* and *L. plantarum*

4.4.1. *Design of co-culture model*

Following physiological and morphological characterisation of the eight bacterial species, a co-culture model was designed. This was with the ultimate goal of converting LC biomass into a fuel molecule, for example alkanes. Here, the consolidated process will be split across the two bacteria in the co-culture, with a ‘cellulolytic specialist’ performing the enzyme production and hydrolysis stages of CBP and a ‘production specialist’ performing the hexose/pentose fermentation stage of CBP.

Of the cellulolytic species, *T. fusca* showed inconsistent growth and *C. cellulolyticum* and *C. thermocellum* were slow to grow and grew to a low culture density compared to the other hosts. Therefore, *C. fimi* shows most potential as the cellulolytic specialist in co-culture.

For the production specialist, *B. subtilis, E. coli* and *L. plantarum* all show promise; growth rates are similar (0.43 h^{-1}, 0.8 h^{-1}, 0.58 h^{-1} respectively) and each species can grow at the same growth temperature as *C. fimi*. Furthermore, from an industrial fermentation perspective, *L. plantarum* is the most relevant host given the higher product yield in keeping with previous data.

4.4.2. *Exploring the use of 96-well plates for co-culture*

In this study, the use of 96-well plates for co-cultures will be assessed. This will allow high-throughput testing of different co-culture conditions *e.g.* different starting population ratios of bacteria, different substrates (glucose, xylose etc.), different species of bacteria. Evolution of the co-culture populations can be tested using the ImageStream which has a 96-well plate sampler allowing time-course experiments.

To initially test the feasibility of a 96-well plate co-culture format, *C. fimi* and *L. plantarum* were grown separately in a 96-well plate, in each of their corresponding media and LB (Figure 9). One 96-well plate was used for *C. fimi* and one 96-well plate was used for *L. plantarum.* Each plate was set up as follows: 20 wells were used for DBS medium, 20 wells were used for LB medium and 20 wells were used for MRS medium – each medium had 5x technical replicates with 4x biological replicates; peripheral wells contained sterile LB medium to reduce edge effects. 96-well plate benchtop shaking incubators were used at 30 °C with shaking at 800 rpm for all bacteria.

C. fimi and *L. plantarum* grew in DBS medium and LB medium (Figure 9). *C. fimi* was unable to grow in MRS broth. Given that both species grew to a similar OD_{600nm} (0.9 – 1.1 at 600 nm) in DBS medium, this shows promise to be used for future co-culture experiments. DBS is a minimal medium supplemented with 1 g l^{-1} of yeast extract and 10 g l^{-1} glucose therefore reduced growth compared to LB and MRS media (rich media) is to be expected.

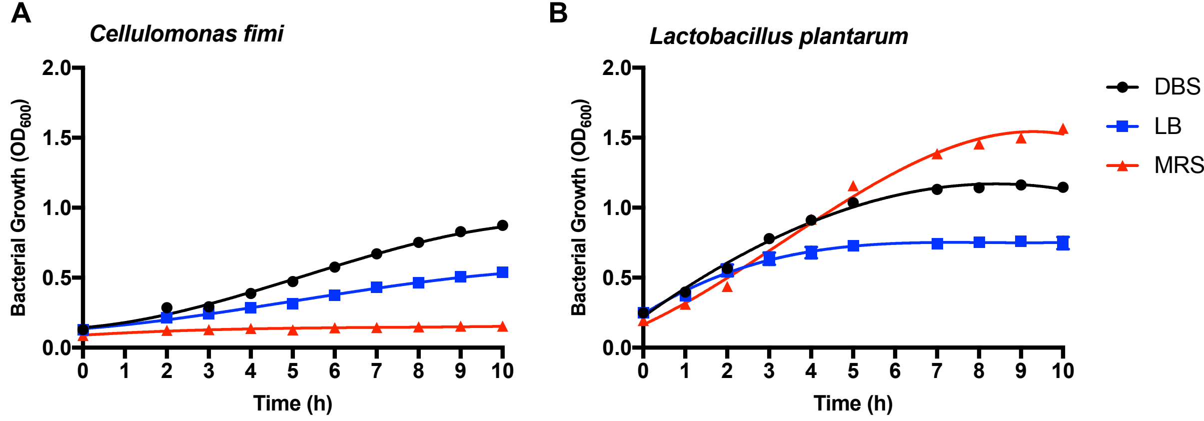


Figure 9. Assessment of growth of *C. fimi and L. plantarum* in Dubos salt medium (DBS), Luria Bertani broth (LB) and MRS medium (MRS)

C. fimi and *L. plantarum* were cultured separately in DBS media, LB media and MRS broth. Both species were cultured in a 96-well plate; peripheral wells on the plates were filled will blank LB medium to reduce edge effect. Benchtop shaking incubators were used to incubate bacteria at 30 °C, 800 rpm. Cultures were inoculated from a 5 ml overnight bacterial culture to an OD_{600nm} = 0.05. Points represent the mean of four biological replicates from five technical replicates, with standard deviation bars shown, unless hidden by the symbol. Curves represent the best fit of the data using a third order polynomial (cubic) equation, rendered using Prism software.

4.4.3. *Validation of growth in selected media in larger culture volume*

Before continuing with the 96-well plate co-culture experiments, confirmation of growth of *C. fimi* and *L. plantarum* in DBS media in a larger culture volume was tested. This was to validate that the 96-well plate method is representative if the experiment is to be scaled-up. For growth of cultures at larger volumes, *C. fimi* and *L. plantarum* were grown in 50 ml DBS media in 250 ml flasks, at 30 °C with shaking at 200 rpm (Figure 10).

DBS media supports growth of *C. fimi* and *L. plantarum* in a larger culture volume as shown by an increase in OD_{600nm} (Figure 10). *C. fimi* and *L. plantarum* grow at similar rates in DBS medium (0.17 h^{-1} and 0.13 h^{-1} respectively) suggesting that this may be a suitable growth medium for a co-culture such that one species will not out-compete the other.

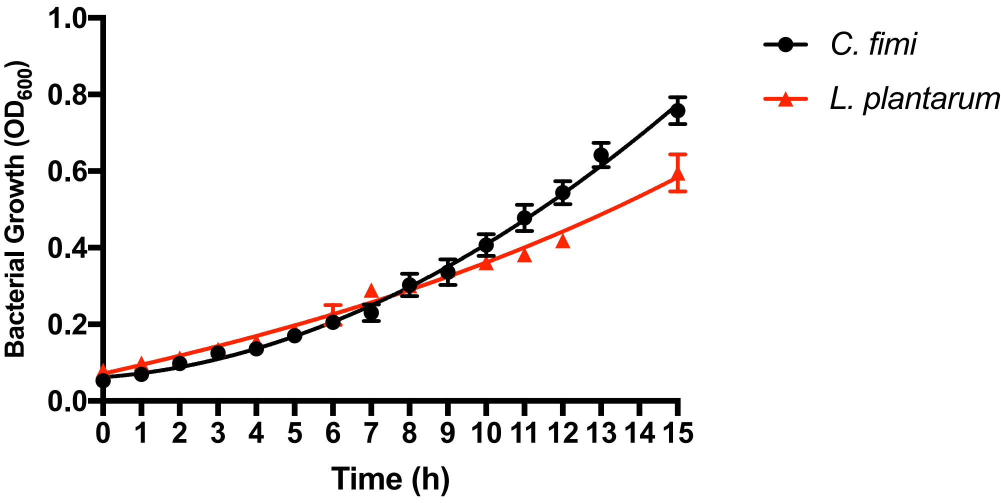


Figure 10. Confirmation of growth of *C. fimi* and *L. plantarum* in DBS media in 50 ml culture volume

150 ml flasks were used to culture *C. fimi* and *L. plantarum* in DBS media with 10 g l^{-1} glucose at 30 °C, 200 rpm. Cultures were inoculated from a 5 ml overnight bacterial culture to an OD_{600nm} = 0.05. Points represent the mean of 5 biological replicates, with standard deviation bars shown, unless hidden by the symbol. Curves represent the best fit of the data using a third order polynomial (cubic) equation, rendered using Prism software.

4.4.4. *Separating populations of C. fimi and L. plantarum by autofluorescence of the cells from imaging flow cytometry*

Based on data from ImageStream studies, it was determined that different populations of bacteria could be separated using imaging flow cytometry (Figure 7). This was established by using forward scatter vs. side scatter of the cells. For the two species of interest to co-culture, *C. fimi* and *L. plantarum,* forward scatter vs. side scatter of the cells was not an appropriate parameter to use to separate these two particular populations of bacteria. Therefore, a different parameter to separate populations of *C. fimi* and *L. plantarum* was explored.

C. fimi and *L. plantarum* were grown separately in 96-well plate format from a starting OD_{600nm} = 0.05 and sampled using the ImageStream 96-well plate sampler after 24 h. This was to replicate the exact conditions of the co-culture experiment. To assess whether populations of *C. fimi* and *L. plantarum* could be separated using imaging flow cytometry, the data acquired from ImageStream samples were used to combine the 10,000 events analysed for each species into one dot plot.

Figure 11 shows that *L. plantarum* cells auto-fluoresced at 505-560 nm wavelength in channel two of the ImageStream whereas *C. fimi* cells did not. Therefore, *C. fimi* and *L. plantarum* populations could be separated using the intensity parameter at 505-560 nm vs. 745-780 nm (side-scatter of cells) on the ImageStream. Also present in both samples were round particles which were discovered to be a medium component due to presence in media controls. These particles could also be separated using the intensity feature. Percentage abundance can be determined on the ImageStream: *C. fimi* accounted for 48 % of the population; *L. plantarum* accounted for 48 % of the population and the round particles accounted for 4 % of the population (Figure 12).

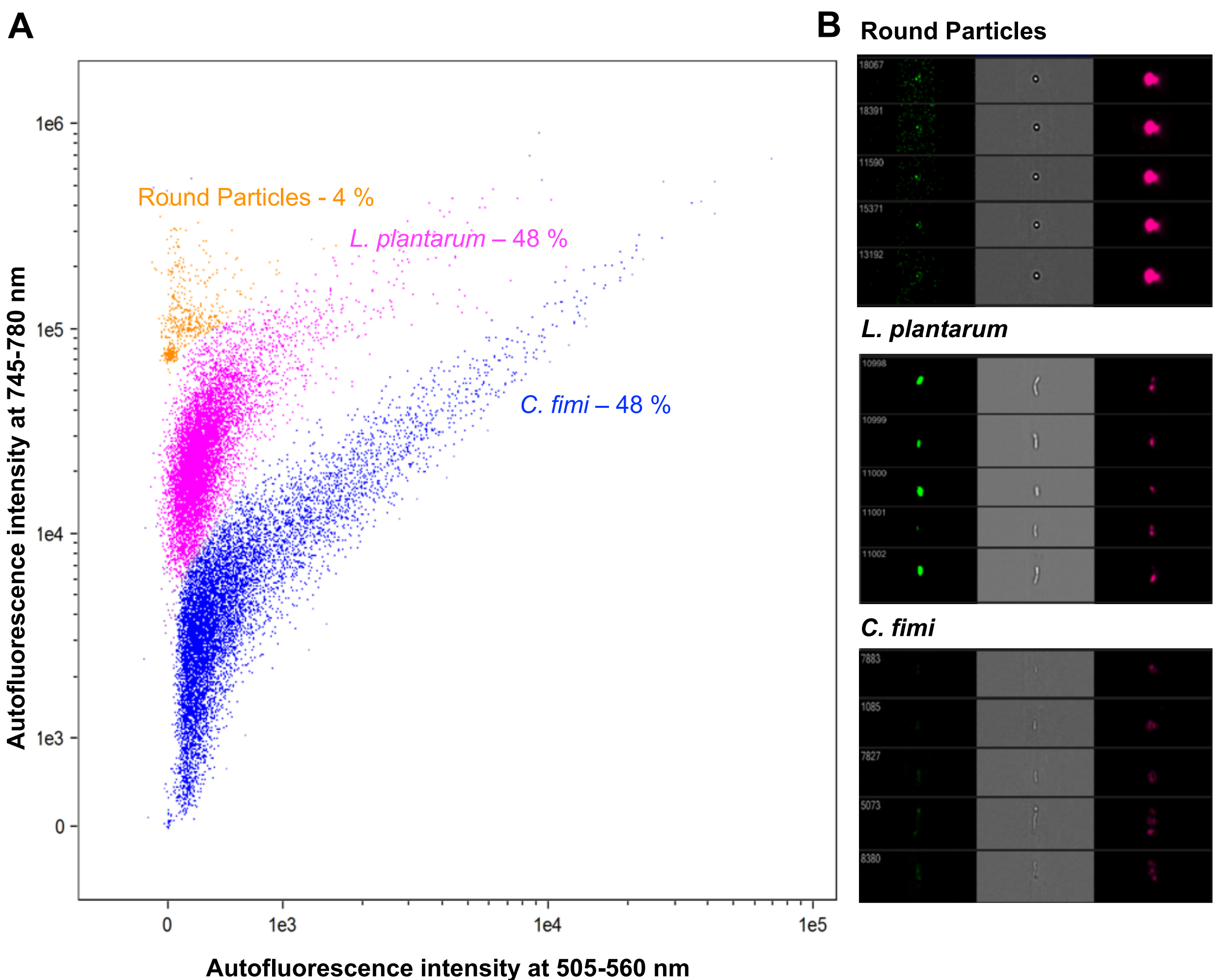


Figure 11. Separating cell populations of *C. fimi* and *L. plantarum* in a 'model' co-culture from imaging flow cytometry

Imaging flow cytometry (ImageStream) plot of autofluorescence intensity of cells at 505-560 nm vs. 745-780 nm wavelength (A) with corresponding images of gated populations from three channels (505-560 nm; Brightfield; 745-780 nm). Data acquired from ImageStream^x imaging cytometer and processed using IDEAS software. 10,000 events shown for each population. Percentage abundance determined on IDEAS software by gating populations.

4.4.5. *Co-culture of C. fimi and L. plantarum on a hexose and pentose monosaccharide*

To assess whether the cellulolytic specialist, *C. fimi* and the biofuel-production specialist, *L. plantarum* could be co-cultured, an experiment was performed in which *C. fimi* and *L. plantarum* were co-cultured on a 96-well plate and sampled at 0 h and 24 h using the ImageStream. This was to determine percentage abundance and cell density of each species at the beginning and end of the fermentation (as shown from Figure 11). Different starting volume inoculation ratios of each species were tested (Table 6). Previous studies showed that *C. fimi* and *L. plantarum* both grew in DBS medium supplemented with glucose (Figure 10) therefore co-culture experiments were performed in this medium. DBS medium supplemented with xylose was also tested as it was previously observed that *L. plantarum* was unable to utilise xylose whereas *C. fimi* could (Figure 4).

Figure 12 shows that *L. plantarum* is the dominant species compared to *C. fimi* in the co-culture when grown on glucose and *C. fimi* is the dominant species in the co-culture compared to *L. plantarum* when grown on xylose.

The set template for separation of *C. fimi* and *L. plantarum* populations (Figure 11) showed some inconsistencies with expectation. There was a ~10 % error for the *C. fimi* positive control (10 % of the population was identified as *L. plantarum*) and a ~3 % error for the *L. plantarum* positive control (3 % of the population was identified at *C. fimi*) (Figure 12A). Additionally, there was a ~3 % error for the *C. fimi* positive control (3 % of the population was identified as *L. plantarum*) and a ~20 % error for the *L. plantarum* positive control (20 % of the population was identified at *C. fimi*) (Figure 12B). The difference in error of population overlap for glucose and xylose highlights the variability between experiments, or the error inherent in the measurement methodology, or both.

	Inoculation Ratio (%)	
Consortium Name	*C. fimi*	*L. plantarum*
CF100	100	-
LP100	-	100
CF50LP50	50	50
CF25LP75	25	75
CF75LP25	75	25

Table 7. Inoculation ratio of *C. fimi* and *L. plantarum* co-culture for sampling by imaging flow cytometry

Numbers indicate percentage inoculation ratio of both microbial species inoculated on 96-well plates. 4x biological replicates were included with 3x technical replicates for each co-culture.2x plates were prepared for destructive sampling at 0 h and 24 h. Cultures were inoculated from a 5 ml overnight bacterial culture to an OD_{600nm} = 0.1. Positive controls of *C. fimi* and *L. plantarum* were included to check for accurate growth of both species. CF = *C. fimi,* LP = *L. plantarum*.

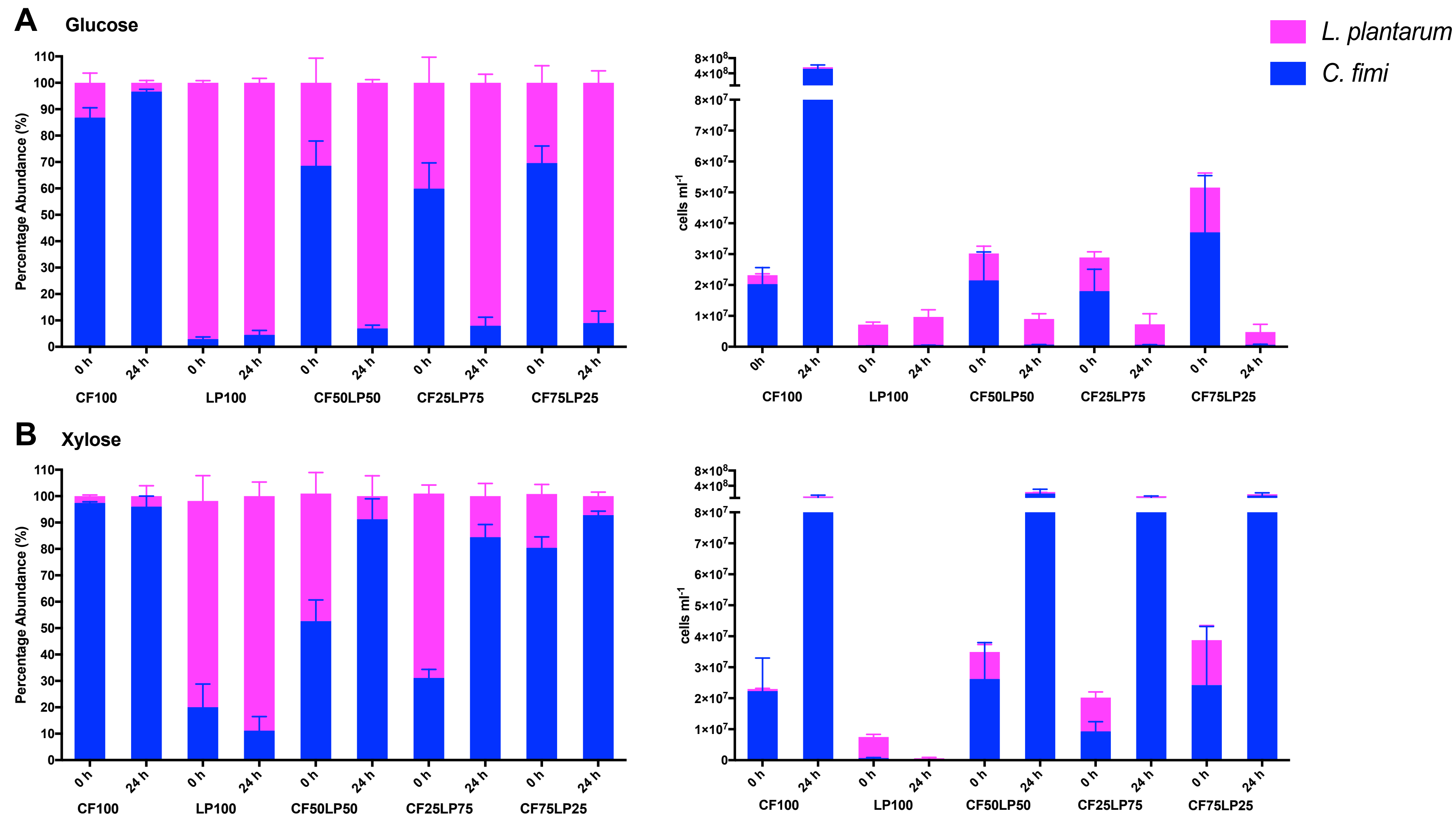


Figure 12. Percentage abundance and cells per ml of *C. fimi* and *L. plantarum* co-culture grown on glucose (A) and xylose (B)

Percentage abundance and cells per ml determined on the ImageStream. Cultures grown in 96-well plates. Bars represent the mean from 4 biological replicates and 3 technical replicates, with standard deviation bars shown.

5. Discussion

A review of the long-range Biodomain research-bioengineering project objectives at Shell made clear that as a priority the program should re-visit potential microbial CBP host selection and progress in the wider scientific/industrial community. This was after extensive research was performed on the then-relevant host *Geobacillus*. Despite promising outcomes with *Geobacillus* strains i.e. successful expression of endo- and exo-glucanases in *Geobacillus thermoglucosidasius* DSM 2542 and positive activity of two natural cellulase enzymes (127), the ongoing challenges of transformation within this species and the lack of intrinsic desirable characteristics of a consolidated bioprocessing host prompted an executive decision to stop work on this micro-organism. Therefore, the search for an alternative novel host began by initial broad scoping of potential micro-organisms suited for Consolidated Bioprocessing (CPB) and genetic engineering, followed by an extensive literature review of the chosen candidates.

The aim of this project was to explore and identify (from the literature) a suite of microorganisms suitable for CBP of cellulosic biomass into useful products. The host selection yielded eight species with potential as CBP hosts. These species were subsequently ordered from a culture collection (ATCC, DSMZ) and characterised metabolically and physiologically for use in a CBP microbial co-culture for bioprocessing of LC.

5.1. *Selection of a chassis for consolidated bioprocessing*

Although the need to use cheap feedstock for bioprocessing of commodity products such as biofuels has long been well appreciated (128), remarkably little research has been carried out to evaluate the suitability of industrial production host strains to utilise these substrates. One study focused on directly comparing six widely used industrial species by evaluating performance in growth conditions commonly encountered in second-generation production processes (129). Despite the development of a unified synthetic minimal medium to support growth of all species, no simple, clear conclusion regarding the hosts suitability due to differences in growth conditions between species was reached.

This study aimed to define desirable criteria for host selection in order to assess species impartially from the literature (Table 2). These criteria were based on previously identified desirable characteristics identified for CBP production hosts either in the literature (51) or in-house (127). Other reviews on CBP hosts have focused on a review of the literature rather than a systematic approach to identify the best candidate species for the process (51,128). Here, 16 species were systematically reviewed, resulting in identification of eight candidate species that show the most potential for use as CBP hosts. However, before deciding on the final species, it was important to locally characterise each species *in vitro* in order to confirm compatibility.

5.2. *Combined metabolic and physiological characterisation of candidate species*

The combined studies of sugar metabolism, growth and morphology allowed for candidate host species to be assessed based on adaptability to LC-derived sugars in biomass and cell size and shape parameters (Figure 3-7). This study reports the use of API 50 CH strips as a suitable, rapid and simple method for testing of candidate host species to ferment a wide range of LC-relevant sugars; utilisation and product generation of the four predominant sugars released from LC degradation (glucose, cellobiose, arabinose, xylose); confirmation of sugar pathway presence with KEGG and cell morphology using imaging flow cytometry.

The API 50 CH fermentation strips manufacturers ascribed use is the identification of *Lactobacillus* and related genera. In this study, API 50 CH strips were applied to study carbohydrate metabolism of seven different species of bacteria in order to determine adaptability to LC-derived sugars. They proved a rapid and reliable method to initially screen candidate species for carbohydrate fermentation (Figure 3). Batch-culture fermentations highlighted *L. plantarum* as the species with greatest biofuel-producing potential with the highest product yield (Figure 4). Despite the anaerobic species, particularly *C. thermocellum* and *T. saccharolyticum,* proving prevalent in literature (Table 1), in-house results with these species proved them to be slow-growing, unreliable and

laborious to work with (Figure 5). *T. saccharolyticum* was noted to have the greatest potential as a biofuel-producing specialist as it produces the highest ethanol yield on glucose, cellobiose, arabinose and xylose (Figure 3) and has a faster growth rate compared to the other anaerobes (Figure 5). However, due to the assay method for the co-culture, aerobic growth is preferred therefore *L. plantarum* was favoured as the biofuel-producing specialist and *C. fimi* as the cellulolytic specialist.

5.3. *Microbial consortia for CBP of LC*

LC biomass is an attractive renewable carbon source for bioenergy applications because of its large-scale availability, low cost, and environmentally benign production. The primary barrier for bioconversion of lignocellulosic bio-mass to liquid fuels is the absence of the low-cost technologies to overcome the structural recalcitrance of these feedstocks to enzymatic degradation. Consolidated bioprocessing (CBP), a process that consolidates lignocellulolytic enzyme production, LC biomass deconstruction, and sugar fermentation into a single step, is a promising approach to overcome this barrier as it has the potential to significantly reduce costs in a biorefinery (45).

The industrial and scientific community are moving toward the use of microbial consortia to perform complex industrial processes (54). The complexity of processes that consortia can perform exceed that of monocultures and are less susceptible to contamination and elimination by viruses. In this study, the cellulolytic bacterium *Cellulomonas fimi* and the industrially-utilised bacterium *Lactobacillus plantarum* were used to act as a proof of principle co-culture for CBP of LC.

C. fimi is considered an “un-domesticated” species in a laboratory context, as there is no reported use in industry or transformation protocol. However, the naturally ability to degrade cellulose and hemicellulose provide an incentive to characterise this species for use as an industrial host.

L. plantarum has recognised potential as a biofuel-producing specialist due to natural tolerance to fermentation conditions *i.e.* acidophile (130) and prevalence in silage systems (131). Further engineering of this species would be required if lactic acid were

not the desired product of the bioprocess, which *L. plantarum* is capable of producing in high yields (Figure 4). In fact, an engineered strain of *L. plantarum is c*apable of degrading cellulose and hemicellulose through use of a cellulosome complex (87). Thus the potential of *L. plantarum* as a CBP host has previously been recognised and the development of this species into a CBP host either as part of a consortium or single host is promising. Most notably, *L. plantarum* has been usefully engineered.

5.4. *Imaging flow cytometry for study of microbial consortia*

To effectively monitor, understand and optimise microbial consortia for industrial use, their accurate characterisation and quantification must be robust and reliable (132,133). Historically, a variety of microbiological, biochemical and molecular biology based approaches have been used to assess cellular abundance, metabolic characteristics and taxonomic diversity profiles (134). Microbiological approaches include community-level physiological profiles, and cell-counting microscopic and colony-forming unit measurements, and biochemical approaches include metabolic assays, cell lipid-composition studies, and DNA association studies to measure sample complexity (134). However, these methods provide characteristics of the consortium community as a whole, but cannot infer presence or function of particular species (134).

For process optimisation of industrial consortia the assessment of the relative cellular abundance of the different species is important to determine whether bacterial populations are balanced or out-competing (48). However, current methods to study bacterial populations within consortia are labour-intensive or expensive (134).

This study applied the technique of imaging flow cytometry (IFC) to synthetic CBP consortia for biofuel production (Figure 8,12,13) to answer the question: is a microbial demographic sufficiently accessible using IFC? IFC combines features of flow cytometry with advanced microscopy to analyse thousands of cellular events with real images (135). Previous use has focused primarily on the analysis of eukaryotic cells in medical research. Here however, IFC was applied to better understand microbial consortia

dynamics. To the best of our knowledge, it is the first study to utilise IFC to evaluate consortia dynamics to evaluate percentage abundance of species in synthetic consortia.

To better understand consortia dynamics during fermentation, a high throughput method for quantifying percentage abundance of species present was developed. This 96-well plate assay for bacterial fermentation as previously tested (Figure 10) and sampled using the 96-well plate sampler on the ImageStream with the applied template (from Figure 12) to determine percentage abundance and cells per ml of the two species (Figure 13). This method for study of consortia proved effective as a high-throughput method as minimal set up was required; one limitation is the overlap of the template for the two species. Further work would require optimising this parameter.

Applying IFC to the study of microbial consortia is most effective when monitoring population changes in synthetic consortia; it requires a pure culture of each species so that a species-specific template can be determined. Once species profiles have been characterised on the IFC software, then this method is robust and reliable in that it can be routinely applied to test the population dynamics during fermentation of the synthetic consortia. The accessibility of this method would depend on the laboratory's availability to an imaging flow cytometer; after the preliminary cost of the machine the running cost is minimal. The application of this technique to complex microbial communities *e.g.* environmental samples, would be less effective at determining the species present however could give an idea of different populations present.

5.5. *Progress and further work*

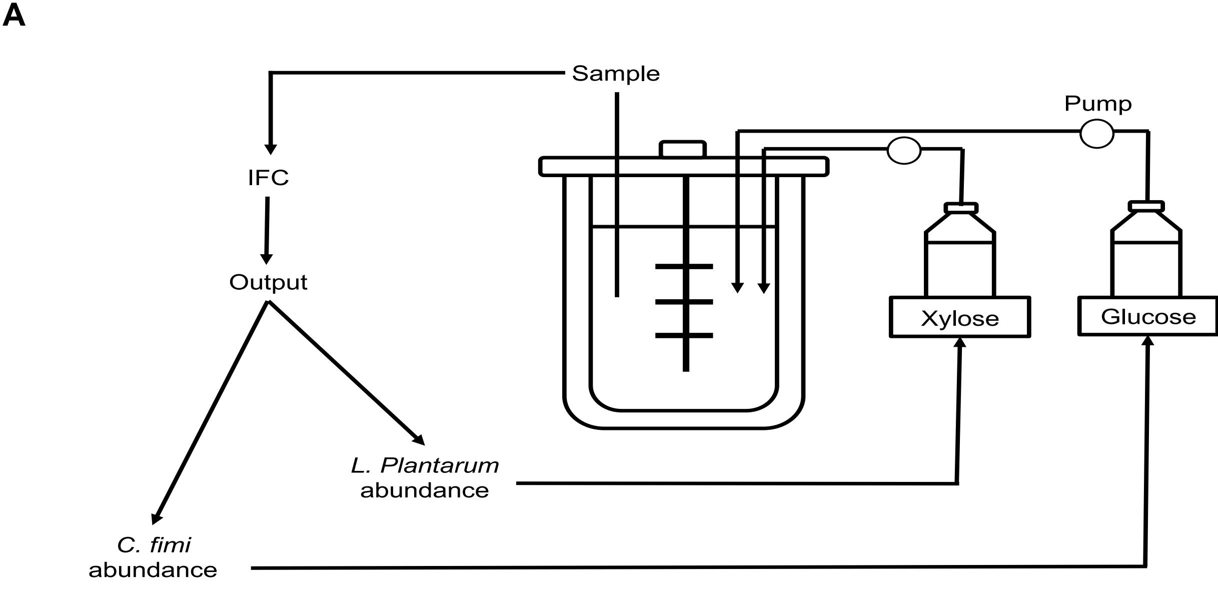
With regards to further experimental work, a number of approaches can be taken to clarify findings or to continue experiments that could not be completed during the course of this project. It is interesting that under certain experimental conditions *L. plantarum* is noted to be outcompeting *C. fimi* (on glucose) whereas *C. fimi* appears to outcompete *L. plantarum* on xylose (Figure 13). It would therefore be interesting to investigate the effect

different substrates have on consortia dynamics for example on cellulose, filter paper and a LC substrate (*Sorghum*).

Similarly, Figure 13 illustrates that inter-species competition is a problem for synthetic co-cultures. General strategies have been suggested to control species populations in a 'division of labour' consortium (*e.g.* saccharolytic and fermentation specialists) (62,63). For example, one study showed that equilibrium population composition can be tuned by modulating relative co-operator-cheater benefits and possibly other ecological parameters (62). More broadly, the co-operator-cheater concept could serve as a general tool for designing and regulating consortia, and represents a complementary approach to previous efforts to stabilise/tune consortia via mutualistic interactions (62,63). Co-operator-cheater tuning could be accomplished via manipulation of culture conditions or genetically programmed cellular behaviour. The IFC method described in this study allows high throughput of consortia and therefore allows different culture conditions to be tested quickly allowing for tuning of consortia dynamics to be achieved rapidly.

This study offers a novel approach to the use of IFC for the optimisation of industrial fermentation systems (Figure 14). For example, in a continuous bioreactor, percentage abundance of *C. fimi* and *L. plantarum* by IFC would allow population balance to be determined so that the respective sugar substrate could be added to increase/decrease the respective population (*e.g.* glucose addition to increase *L. plantarum* abundance; xylose to increase *C. fimi* abundance). This would allow a rapid assessment of synthetic consortium dynamics and culture progression during industrial fermentation with minimal sample sacrificed (maximum IFC sample = 200 μl).

A



B

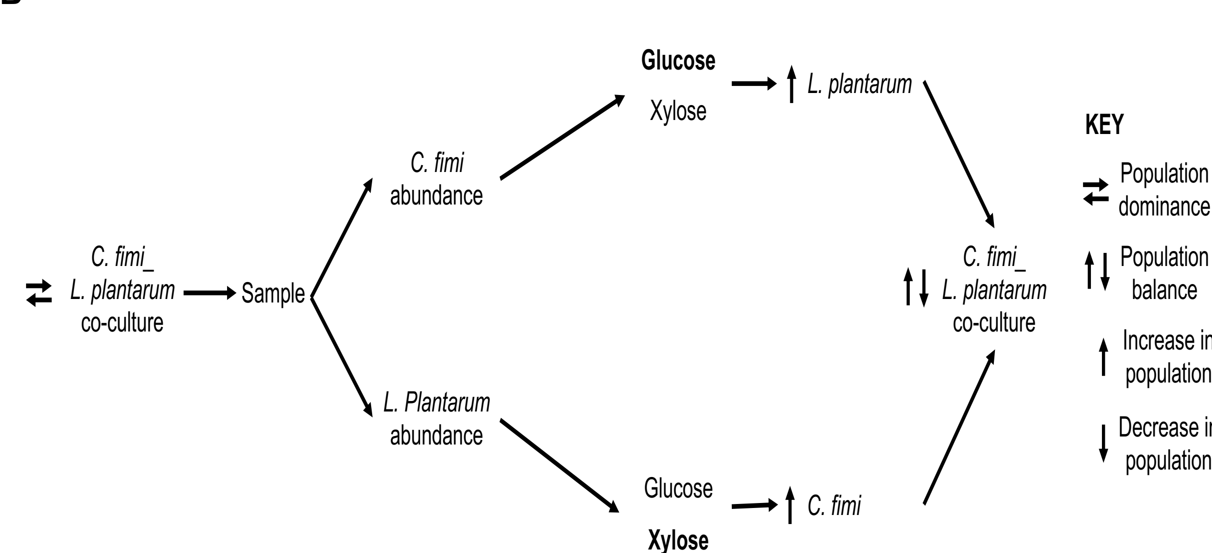


Figure 13. Media management of consortia based on species abundance as determined by imaging flow cytometry (IFC)

Schematic diagram to show bioreactor set-up for industrial fermentation of synthetic co-culture (A) and sequence of events to allow population balance by the increase of either glucose or xylose concentration in media (as depicted by bold lettering).

6. Conclusion

At the beginning of the study the following criteria for CBP hosts were identified as important features to determine a species potential to perform effectively and co-operatively as an industrial CBP chassis. : O_2 requirement, growth conditions, previous and established industrial use, genetic competence, ability to degrade lignocellulose, and inherent biofuel-producing pathways. This analysis highlighted eight microbial hosts with industrial relevance for selection to be tested for CBP of LC. Physiological and metabolic characterisation highlighted *C. fimi* and *L. plantarum* as suitable species to co-culture in exploring the design of a model biomass-to-fuel consortium.

IFC was shown to be an effective method to monitor bacterial populations in synthetic consortia, and suitable to be effectively used as a high throughput method for monitoring population dynamics in future process optimisation. To the best of our knowledge, it is the first study to utilise IFC to evaluate consortium dynamics.

Clear avenues for future study include high throughput testing of *C. fimi* and *L. plantarum* in order to find optimal conditions to balance populations, and testing consortium performance on a wider range of substrates, including standard cellulose and LC biomass. Our novel IFC based method will facilitate the construction of reliable and robust synthetic consortia for efficient LC biomass conversion into commercial products.

7. References

1. IEA. Key World Energy Statistics 2018. *Statistics*. 2018;40.
2. IEA. World Energy Outlook 2018. *Int Energy Agency Paris, Fr.* 2018;28.
3. BP. BP Energy outlook 2017. *BP Stat Rev World Energy.* 2017;52.
4. European Parliament. Directive 2009/28/EC of the European Parliament and of the Council of 23 April 2009. *Off J Eur Union.* 2009;140(16):16–62.
5. U.S. Government Printing Office. Energy Independence and Security Act of 2007. *Security.* 2007;Public Law:1–311.
6. IEA. Energy Technology Perspectives 2012: Pathways to a Clean Energy System. 2012;1–12.
7. Sims REH, Mabee W, Saddler JN, Taylor M. An overview of second generation biofuel technologies. Vol. 101, *Bioresource Technology.* 2010. p. 1570–80.
8. Naik SN, Goud V V., Rout PK, Dalai AK. Production of first and second generation biofuels: A comprehensive review. Vol. 14, *Renewable and Sustainable Energy Reviews.* 2010. p. 578–97.
9. Fulton LM, Lynd LR, Körner A, Greene N, Tonachel LR. The need for biofuels as part of a low carbon energy future. *Biofuels, Bioprod Biorefining.* 2015;9(5):476–83.
10. Balan V. Current Challenges in Commercially Producing Biofuels from Lignocellulosic Biomass. *ISRN Biotechnol.* 2014;2014:1–31.
11. Tao G, Lestander TA, Geladi P, Xiong S. Biomass properties in association with plant species and assortments I: A synthesis based on literature data of energy properties. Vol. 16, *Renewable and Sustainable Energy Reviews.* 2012. p. 3481–506.
12. Wu X, McLaren J, Madl R, Wang D. Biofuels from lignocellulosic biomass. *Sustainable Biotechnology: Sources of Renewable Energy.* 2010. p. 19–41.
13. Sun S, Sun S, Cao X, Sun R. The role of pretreatment in improving the enzymatic hydrolysis of lignocellulosic materials. Vol. 199, *Bioresource Technology.* 2016. p. 49–58.
14. Yang B, Dai Z, Ding S-Y, Wyman CE. Enzymatic hydrolysis of cellulosic biomass.

Biofuels. 2011;2(4):421–49.

15. Hideno A, Inoue H, Tsukahara K, Fujimoto S, Minowa T, Inoue S, et al. Wet disk milling pretreatment without sulfuric acid for enzymatic hydrolysis of rice straw. *Bioresour Technol.* 2009;100(10):2706–11.
16. Yoo J, Alavi S, Vadlani P, Amanor-Boadu V. Thermo-mechanical extrusion pretreatment for conversion of soybean hulls to fermentable sugars. *Bioresour Technol.* 2011;102(16):7583–90.
17. Teymouri F, Laureano-Perez L, Alizadeh H, Dale BE. Optimization of the ammonia fiber explosion (AFEX) treatment parameters for enzymatic hydrolysis of corn stover. *Bioresour Technol.* 2005;96(18 SPEC. ISS.):2014–8.
18. Tae HK, Lee YY. Pretreatment and fractionation of corn stover by ammonia recycle percolation process. *Bioresour Technol.* 2005;96(18 SPEC. ISS.):2007–13.
19. Li X, Kim TH, Nghiem NP. Bioethanol production from corn stover using aqueous ammonia pretreatment and two-phase simultaneous saccharification and fermentation (TPSSF). *Bioresour Technol.* 2010;101(15):5910–6.
20. Banerjee G, Car S, Liu T, Williams DL, Meza SL, Walton JD, et al. Scale-up and integration of alkaline hydrogen peroxide pretreatment, enzymatic hydrolysis, and ethanolic fermentation. *Biotechnol Bioeng.* 2012;109(4):922–31.
21. Dadi AP, Schall CA, Varanasi S. Mitigation of cellulose recalcitrance to enzymatic hydrolysis by ionic liquid pretreatment. *Applied Biochemistry and Biotechnology.* 2007. p. 407–21.
22. Zhuang X, Wang W, Yu Q, Qi W, Wang Q, Tan X, et al. Liquid hot water pretreatment of lignocellulosic biomass for bioethanol production accompanying with high valuable products. *Bioresource Technology.* 2016; 199:68–75.
23. García-Cubero MT, González-Benito G, Indacoechea I, Coca M, Bolado S. Effect of ozonolysis pretreatment on enzymatic digestibility of wheat and rye straw. *Bioresour Technol.* 2009;100(4):1608–13.
24. Lloyd TA, Wyman CE. Combined sugar yields for dilute sulfuric acid pretreatment of corn stover followed by enzymatic hydrolysis of the remaining solids. *Bioresour Technol.* 2005;96:1967–77.

25. Qin L, Liu ZH, Li BZ, Dale BE, Yuan YJ. Mass balance and transformation of corn stover by pretreatment with different dilute organic acids. *Bioresour Technol.* 2012;112:319–26.
26. Gírio FM, Fonseca C, Carvalheiro F, Duarte LC, Marques S, Bogel-Łukasik R. Hemicelluloses for fuel ethanol: A review. *Bioresource Technology.* 2010;101:4775–800.
27. Galbe M, Zacchi G. A review of the production of ethanol from softwood. *Applied Microbiology and Biotechnology.* 2002;59:618–28.
28. Sun F, Chen H. Organosolv pretreatment by crude glycerol from oleochemicals industry for enzymatic hydrolysis of wheat straw. *Bioresour Technol.* 2008;99(13):5474–9.
29. Söderström J, Pilcher L, Galbe M, Zacchi G. Combined use of H2SO4 and SO2 impregnation for steam pretreatment of spruce in ethanol production. *Applied Biochemistry and Biotechnology - Part A Enzyme Engineering and Biotechnology.* 2003:127–40.
30. Patel SJ. Comparative Study of Ethanol Production from Microbial Pretreated Agricultural Residues. *J Appl Sci Environ Manag.* 2007;11(4):137–41.
31. Wan C, Li Y. Fungal pretreatment of lignocellulosic biomass. *Biotechnology Advances.* 2012;30:1447–57.
32. Reese ET. History of the Cellulase Program at the U.S. Army Natick Development Center. *Biotechnol Bioeng Symp.* 1976;6(6):9–20.
33. Gupta VK, Schmoll M, Herrera-Estrella A, Upadhyay RS, Druzhinina I, Tuohy MG. Biotechnology and Biology of Trichoderma. *Biotechnology and Biology of Trichoderma.* 2014:1-549
34. Bischof RH, Ramoni J, Seiboth B. Cellulases and beyond: The first 70 years of the enzyme producer Trichoderma reesei. *Microbial Cell Factories.* 2016;15.
35. Seiboth B, Ivanova C, Seidl-Seiboth V. Trichoderma reesei: A Fungal Enzyme Producer for Cellulosic Biofuels. *Biofuel Production-Recent Developments and Prospects.* 2011.
36. Binod P, Janu KU, Sindhu R, Pandey A. Hydrolysis of lignocellulosic biomass for bioethanol production. *Biofuels.* 2011:229–50.

37. Alper H, Stephanopoulos G. Engineering for biofuels: Exploiting innate microbial capacity or importing biosynthetic potential? *Nature Reviews Microbiology.* 2009;7:715–23.

38. Den Haan R, Kroukamp H, Mert M, Bloom M, Görgens JF, Van Zyl WH. Engineering Saccharomyces cerevisiae for next generation ethanol production. Vol. 88, *Journal of Chemical Technology and Biotechnology.* 2013;88:983–91.

39. Matsushika A, Inoue H, Kodaki T, Sawayama S. Ethanol production from xylose in engineered Saccharomyces cerevisiae strains: Current state and perspectives. *Applied Microbiology and Biotechnology.* 2009;84:37–53.

40. Van Vleet JH, Jeffries TW. Yeast metabolic engineering for hemicellulosic ethanol production. *Current Opinion in Biotechnology*. 2009;20:300–6.

41. Träff KL, Cordero RRO, Van Zyl WH, Hahn-Hägerdal B. Deletion of the GRE3 Aldose Reductase Gene and Its Influence on Xylose Metabolism in Recombinant Strains of Saccharomyces cerevisiae Expressing the xylA and XKS1 Genes. *Appl Environ Microbiol.* 2001;67(12):5668–74.

42. Carriquiry MA, Du X, Timilsina GR. Second generation biofuels: Economics and policies. *Energy Policy*. 2011;39(7):4222–34.

43. Hamelinck CN, Van Hooijdonk G, Faaij APC. Ethanol from lignocellulosic biomass: Techno-economic performance in short-, middle- and long-term. *Biomass and Bioenergy.* 2005;28:384–410.

44. Lynd LR, Van Zyl WH, McBride JE, Laser M. Consolidated bioprocessing of cellulosic biomass: An update. *Current Opinion in Biotechnology.* 2005;16:577–83.

45. Olson DG, McBride JE, Joe Shaw A, Lynd LR. Recent progress in consolidated bioprocessing. *Current Opinion in Biotechnology.* 2012;23:396–405.

46. Salehi Jouzani G, Taherzadeh MJ. Advances in consolidated bioprocessing systems for bioethanol and butanol production from biomass: a comprehensive review. *Biofuel Res J.* 2015;152–95.

47. Kök MS. An integrated approach: advances in the use of *Clostridium* for biofuel. *Biotechnol Genet Eng Rev.* 2015;31(1–2):69–81.

48. Brethauer S, Studer MH. Consolidated bioprocessing of lignocellulose by a

microbial consortium. *Energy Environ Sci.* 2014;7(4):1446.

49. Parisutham V, Kim TH, Lee SK. Feasibilities of consolidated bioprocessing microbes: From pretreatment to biofuel production. Vol. 161, *Bioresource Technology.* 2014;161:431–40.
50. Zuroff TR, Curtis WR. Developing symbiotic consortia for lignocellulosic biofuel production. *Appl Microbiol Biotechnol.* 2012;93(4):1423–35.
51. van Zyl WH, den Haan R, la Grange DC. Developing organisms for consolidated bioprocessing of biomass to ethanol. *Biofuel Prod Dev Prospect.* 2011;137–62.
52. Argyros DA, Tripathi SA, Barrett TF, Rogers SR, Feinberg LF, Olson DG, et al. High ethanol Titers from cellulose by using metabolically engineered thermophilic, anaerobic microbes. *Appl Environ Microbiol.* 2011;77(23):8288–94.
53. Klitgord N, Segrè D. Environments that induce synthetic microbial ecosystems. *PLoS Comput Biol.* 2010;6(11).
54. Goers L, Freemont P, Polizzi KM. Co-culture systems and technologies: taking synthetic biology to the next level. *J R Soc Interface.* 2014;11(96):20140065–20140065.
55. Shong J, Jimenez Diaz MR, Collins CH. Towards synthetic microbial consortia for bioprocessing. *Current Opinion in Biotechnology.* 2012;23:798–802.
56. Brenner K, You L, Arnold FH. Engineering microbial consortia: a new frontier in synthetic biology. *Trends in Biotechnology.* 2008;26:483–9.
57. Zomorrodi AR, Segrè D. Synthetic Ecology of Microbes: Mathematical Models and Applications. *Journal of Molecular Biology.* 2016;428:837–61.
58. Shou W, Ram S, Vilar JMG. Synthetic cooperation in engineered yeast populations. *Proc Natl Acad Sci.* 2007;104(6):1877–82.
59. Bosi E, Bacci G, Mengoni A, Fondi M. Perspectives and challenges in microbial communities metabolic modeling. *Front Genet.* 2017;8:88.
60. Stolyar S, Van Dien S, Hillesland KL, Pinel N, Lie TJ, Leigh JA, et al. Metabolic modeling of a mutualistic microbial community. *Mol Syst Biol.* 2007;3:1–14.
61. Hanly TJ, Henson MA. Dynamic flux balance modeling of microbial co-cultures for efficient batch fermentation of glucose and xylose mixtures. *Biotechnol Bioeng.* 2011;108(2):376–85.

62. Minty JJ, Singer ME, Scholz SA, Bae C-H, Ahn J-H, Foster CE, et al. Design and characterization of synthetic fungal-bacterial consortia for direct production of isobutanol from cellulosic biomass. *Proc Natl Acad Sci.* 2013;110(36):14592–7.
63. Zuroff TR, Xiques SB, Curtis WR. Consortia-mediated bioprocessing of cellulose to ethanol with a symbiotic Clostridium phytofermentans/yeast co-culture. *Biotechnol Biofuels.* 2013;6(1).
64. Fischer CR, Klein-Marcuschamer D, Stephanopoulos G. Selection and optimization of microbial hosts for biofuels production. *Metab Eng.* 2008;10(6):295–304.
65. Wakarchuk WW, Brochu D, Foote S, Robotham A, Saxena H, Erak T, et al. Proteomic analysis of the secretome of cellulomonas fimi ATCC 484 and cellulomonas flavigena ATCC 482. *PLoS One.* 2016;11(3).
66. Wilson DB. Studies of Thermobifida fusca plant cell wall degrading enzymes. *Chem Rec.* 2004;4(2):72–82.
67. Feinberg L, Foden J, Barrett T, Davenport KW, Bruce D, Detter C, et al. Complete genome sequence of the cellulolytic thermophile *Clostridium thermocellum* DSM1313. *J Bacteriol.* 2011;193(11):2906–7.
68. Desvaux M. Clostridium cellulolyticum: Model organism of mesophilic cellulolytic clostridia. *FEMS Microbiol Rev.* 2005;29(4):741–64.
69. Lee YE, Jain MK, Lee C, Zeikus JG. Taxonomic Distinction of Saccharolytic Thermophilic Anaerobes. *Int J Syst Bacteriol.* 1993;43(2):41–51.
70. Harwood CR. Bacillus subtilis and its relatives: molecular biological and industrial workhorses. *Trends in Biotechnology.* 1992;10:247–56.
71. Siezen RJ, van Hylckama Vlieg JE. Genomic diversity and versatility of Lactobacillus plantarum, a natural metabolic engineer. *Microb Cell Fact.* 2011;10(Suppl 1):S3.
72. Wang C, Pfleger BF, Kim SW. Reassessing Escherichia coli as a cell factory for biofuel production. Vol. 45, *Current Opinion in Biotechnology.* 2017. p. 92–103.
73. Smith MCM. Molecular biological methods for bacillus. *FEBS Lett.* 1991;287(1–2):227.
74. Nielsen DR, Leonard E, Yoon SH, Tseng HC, Yuan C, Prather KLJ. Engineering

alternative butanol production platforms in heterologous bacteria. *Metab Eng.* 2009;11(4–5):262–73.

75. Qi H, Li S, Zhao S, Huang D, Xia M, Wen J. Model-driven redox pathway manipulation for improved isobutanol production in Bacillus subtilis complemented with experimental validation and metabolic profiling analysis. *PLoS One.* 2014;9(4).
76. Baez A, Cho KM, Liao JC. High-flux isobutanol production using engineered Escherichia coli: A bioreactor study with in situ product removal. *Appl Microbiol Biotechnol*. 2011;90(5):1681–90.
77. Inokuma K, Liao JC, Okamoto M, Hanai T. Improvement of isopropanol production by metabolically engineered Escherichia coli using gas stripping. *J Biosci Bioeng.* 2010;110(6):696–701.
78. Shen CR, Lan EI, Dekishima Y, Baez A, Cho KM, Liao JC. Driving forces enable high-titer anaerobic 1-butanol synthesis in Escherichia coli. *Appl Environ Microbiol.* 2011;77(9):2905–15.
79. Cao YX, Xiao WH, Liu D, Zhang JL, Ding MZ, Yuan YJ. Biosynthesis of odd-chain fatty alcohols in escherichia coli. *Metab Eng.* 2015;29:113–23.
80. Choi YJ, Lee SY. Microbial production of short-chain alkanes. *Nature.* 2013;502(7472):571–4.
81. Zhang F, Carothers JM, Keasling JD. Design of a dynamic sensor-regulator system for production of chemicals and fuels derived from fatty acids. *Nat Biotechnol.* 2012;30(4):354–9.
82. George KW, Thompson MG, Kang A, Baidoo E, Wang G, Chan LJG, et al. Metabolic engineering for the high-yield production of isoprenoid-based C5 alcohols in E. coli. *Sci Rep*. 2015;5(June):11128.
83. Wang C, Park JE, Choi ES, Kim SW. Farnesol production in Escherichia coli through the construction of a farnesol biosynthesis pathway ??? application of PgpB and YbjG phosphatases. *Biotechnol J*. 2016;11(10):1291–7.
84. Alonso-Gutierrez J, Kim EM, Batth TS, Cho N, Hu Q, Chan LJG, et al. Principal component analysis of proteomics (PCAP) as a tool to direct metabolic engineering. *Metab Eng*. 2015;28:123–33.

85. Alonso-Gutierrez J, Chan R, Batth TS, Adams PD, Keasling JD, Petzold CJ, et al. Metabolic engineering of Escherichia coli for limonene and perillyl alcohol production. *Metab Eng.* 2013;19:33–41.

86. Spath K, Heinl S, Grabherr R. "Direct cloning in Lactobacillus plantarum: electroporation with non-methylated plasmid DNA enhances transformation efficiency and makes shuttle vectors obsolete". *Microb Cell Fact.* 2012;11(1):141.

87. Moraïs S, Shterzer N, Grinberg IR, Mathiesen G, Eijsink VGH, Axelsson L, et al. Establishment of a simple lactobacillus plantarum cell consortium for cellulase-xylanase synergistic interactions. *Appl Environ Microbiol*. 2013;79(17):5242–9.

88. Moraïs S, Shterzer N, Lamed R, Bayer EA, Mizrahi I. A combined cell-consortium approach for lignocellulose degradation by specialized Lactobacillus plantarum cells. *Biotechnol Biofuels.* 2014;7(1):112.

89. Liu S, Nichols NN, Dien BS, Cotta MA. Metabolic engineering of a Lactobacillus plantarum double ldh knockout strain for enhanced ethanol production. *J Ind Microbiol Biotechnol*. 2006;33(1):1–7.

90. Wang JH, Hung W, Tsai SH. High efficiency transformation by electroporation of *Yarrowia lipolytica. J Microbiol.* 2011;49(3):469–72.

91. Guo Z, Duquesne S, Bozonnet S, Cioci G, Nicaud J-M, Marty A, et al. Conferring cellulose-degrading ability to Yarrowia lipolytica to facilitate a consolidated bioprocessing approach. *Biotechnol Biofuels*. 2017;10(1):132.

92. Wang W, Wei H, Alahuhta M, Chen X, Hyman D, Johnson DK, et al. Heterologous expression of xylanase enzymes in lipogenic yeast *Yarrowia lipolytica. PLoS One*. 2014;9(12).

93. Rutter CD, Rao C V. Production of 1-decanol by metabolically engineered *Yarrowia lipolytica. Metab Eng*. 2016;38:139–47.

94. Blazeck J, Liu L, Knight R, Alper HS. Heterologous production of pentane in the oleaginous yeast *Yarrowia lipolytica. J Biotechnol*. 2013;165(3–4):184–94.

95. Xu P, Qiao K, Ahn WS, Stephanopoulos G. Engineering *Yarrowia lipolytica* as a platform for synthesis of drop-in transportation fuels and oleochemicals. *Proc Natl Acad Sci*. 2016;113(39):10848–53.

96. Yang X, Nambou K, Wei L, Hua Q. Heterologous production of α-farnesene in

metabolically engineered strains of *Yarrowia lipolytica. Bioresour Technol.* 2016;216:1040–8.

97. Peng JB, Yan WM, Bao XZ. Plasmid and transposon transfer to *Thiobacillus ferrooxidans. J Bacteriol.* 1994;176(10):2892–7.

98. Ito M, Nagane M. Improvement of the Electro-transformation Efficiency of Facultatively Alkaliphilic *Bacillus pseudofirmus* OF4 by High Osmolarity and Glycine Treatment. *Biosci Biotechnol Biochem.* 2001;65(12):2773–5.

99. Gao C, Xue Y, Ma Y. Protoplast transformation of recalcitrant alkaliphilic bacillus sp. with methylated plasmid DNA and a developed hard agar regeneration medium. *PLoS One*. 2011;6(11).

100. Kang Y, Norris MH, Wilcox BA, Tuanyok A, Keim PS, Hoang TT. Knockout and pullout recombineering for naturally transformable *Burkholderia thailandensis* and *Burkholderia pseudomallei*. *Nat Protoc.* 2011;6(8):1085–104.

101. Funston SJ, Tsaousi K, Rudden M, Smyth TJ, Stevenson PS, Marchant R, et al. Characterising rhamnolipid production in *Burkholderia thailandensis* E264, a non-pathogenic producer. *Appl Microbiol Biotechnol.* 2016;100(18):7945–56.

102. Takeuchi Y, Khawdas W, Aso Y, Ohara H. Microbial fuel cells using *Cellulomonas* spp. with cellulose as fuel. *J Biosci Bioeng*. 2017;123(3):358–63.

103. Zibat A. Efficient Transformation of *Halobacterium salinarum* by a “Freeze and Thaw” Technique. *Biotechniques*. 2001;31(5):1010–2.

104. Silva-Rocha R, Pontelli MC, Furtado GP, Zaramela LS, Koide T. Development of new modular genetic tools for engineering the halophilic archaeon *Halobacterium salinarum. PLoS One*. 2015;10(6).

105. Liu Y, Koh CMJ, Sun L, Hlaing MM, Du M, Peng N, et al. Characterization of glyceraldehyde-3-phosphate dehydrogenase gene RtGPD1 and development of genetic transformation method by dominant selection in oleaginous yeast *Rhodosporidium toruloides. Appl Microbiol Biotechnol*. 2013;97(2):719–29.

106. Lin X, Wang Y, Zhang S, Zhu Z, Zhou YJ, Yang F, et al. Functional integration of multiple genes into the genome of the oleaginous yeast *Rhodosporidium toruloides. FEMS Yeast Res*. 2014;14(4):547–55.

107. Johns AMB, Love J, Aves SJ. Four inducible promoters for controlled gene

expression in the oleaginous yeast *Rhodotorula toruloides. Front Microbiol.* 2016;7(OCT).

108. Rachkevych N, Sybirna K, Boyko S, Boretsky Y, Sibirny A. Improving the efficiency of plasmid transformation in *Shewanella oneidensis* MR-1 by removing ClaI restriction site. *J Microbiol Methods*. 2014;99(1):35–7.
109. Li F, Li Y, Sun L, Li X, Yin C, An X, et al. Engineering *Shewanella oneidensis* enables xylose-fed microbial fuel cell. *Biotechnol Biofuels.* 2017;10(1):196.
110. Schleper C, Kubo K, Zillig W. The particle SSV1 from the extremely thermophilic archaeon *Sulfolobus* is a virus: Demonstration of infectivity and of transfection with viral DNA (site-specific ntegration/lysogeny/electroporation). *Microbiology.* 1992;89:7645–9.
111. Li Y, Pan S, Zhang Y, Ren M, Feng M, Peng N, et al. Harnessing Type i and Type III CRISPR-Cas systems for genome editing. *Nucleic Acids Res*. 2015;44(4).
112. Deng Y, Fong SS. Development and application of a PCR-targeted gene disruption method for studying CelR function in *Thermobifida fusca. Appl Environ Microbiol.* 2010;76(7):2098–106.
113. Jennert KCB, Tardif C, Young DI, Young M. Gene transfer to *Clostridium cellulolyticum* ATCC 35319. *Microbiology.* 2000;146(12):3071–80.
114. Tardif C, Maamar H, Balfin M, Belaich JP. Electrotransformation studies in *Clostridium cellulolyticum. J Ind Microbiol Biotechnol.* 2001;27(5):271–4.
115. Xu T, Li Y, Shi Z, Hemme CL, Li Y, Zhu Y, et al. Efficient genome editing in *Clostridium cellulolyticum* via CRISPR-Cas9 nickase. *Appl Environ Microbiol.* 2015;81(13):4423–31.
116. Higashide W, Li Y, Yang Y, Liao JC. Metabolic Engineering of *Clostridium cellulolyticum* for Isobutanol Production From Cellulose. *Appl Environ Microbiol*. 2011;77(8):AEM.02454-10.
117. Olson DG, Lynd LR. Transformation of *Clostridium thermocellum* by electroporation. *Methods Enzymol.* 2012;510:317–30.
118. Kannuchamy S, Mukund N, Saleena LM. Genetic engineering of *Clostridium thermocellum* DSM1313 for enhanced ethanol production. *BMC Biotechnol.* 2016;16(S1):34.

119. Xiong W, Lin PP, Magnusson L, Warner L, Liao JC, Maness P-C, et al. CO2-fixing one-carbon metabolism in a cellulose-degrading bacterium *Clostridium thermocellum. Proc Natl Acad Sci U S A.* 2016;113(46):13180–5.
120. Mai V, Lorenz WW, Wiegel J. Transformation of *Thermoanaerobacterium* sp. strain JW/SL-YS485 with plasmid pIKM1 conferring kanamycin resistance. *Fems Microbiol Lett.* 1997;148(2):163–7.
121. Tyurin M V., Sullivan CR, Lynd LR. Role of spontaneous current oscillations during high-efficiency electrotransformation of thermophilic anaerobes. *Appl Environ Microbiol.* 2005;71(12):8069–76.
122. Shaw AJ, Podkaminer KK, Desai SG, Bardsley JS, Rogers SR, Thorne PG, et al. Metabolic engineering of a thermophilic bacterium to produce ethanol at high yield. *Proc Natl Acad Sci U S A.* 2008;105(37):13769–74.
123. Bhandiwad A, Shaw AJ, Guss A, Guseva A, Bahl H, Lynd LR. Metabolic engineering of *Thermoanaerobacterium saccharolyticum* for n-butanol production. *Metab Eng*. 2014;21:17–25.
124. de Souza WR. Microbial Degradation of Lignocellulosic Biomass. In: Sustainable Degradation of Lignocellulosic Biomass - Techniques, Applications and Commercialization. 2013.
125. Chandel AK, Chandrasekhar G, Radhika K, Ravinder R, Ravindra P. Bioconversion of pentose sugars into ethanol: A review and future directions. *Biotechnol Mol Biol Rev.* 2011;6(1):8–20.
126. Ogata H, Goto S, Sato K, Fujibuchi W, Bono H, Kanehisa M. KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Research.* 1999;27: 29–34.
127. Kent RW. Screening and Engineering of *Geobacillus* spp. for Consolidated Bioprocessing of Lignocellulosic Biomass. Masters by Res Thesis Univ Exet. 2015;
128. Jouzani GS, Taherzadeh MJ. Advances in consolidated bioprocessing systems for bioethanol and butanol production from biomass: a comprehensive review. *Biofuel Res J.* 2015;5(MARCH):152–95.
129. Rumbold K, van Buijsen HJJ, Overkamp KM, van Groenestijn JW, Punt PJ, Werf MJVD. Microbial production host selection for converting second-generation

feedstocks into bioproducts. *Microb Cell Fact.* 2009;8.

130. Siezen RJ, van Hylckama Vlieg JET. Genomic diversity and versatility of *Lactobacillus plantarum*, a natural metabolic engineer. *Microb Cell Fact.* 2011;10(SUPPL. 1).
131. Gallagher D, Parker D, Allen DJ, Tsesmetzis N. Dynamic bacterial and fungal microbiomes during sweet sorghum ensiling impact bioethanol production. *Bioresour Technol*. 2018;264:163–73.
132. VerBerkmoes NC, Denef VJ, Hettich RL, Banfield JF. Functional analysis of natural microbial consortia using community proteomics. *Nat Rev Microbiol.* 2009;7(3):196–205.
133. Lindemann SR, Bernstein HC, Song HS, Fredrickson JK, Fields MW, Shou W, et al. Engineering microbial consortia for controllable outputs. *ISME Journal.* 2016;10:2077–84.
134. Spiegelman D, Whissell G, Greer CW. A survey of the methods for the characterization of microbial consortia and communities. *Can J Microbiol.* 2005;51(5):355–86.
135. Basiji D, O’Gorman MRG. Imaging flow cytometry. *J Immunol Methods.* 2015;423:1–2.