Common and novel approaches to convert cellulose to platform chemicals

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Abstract

Renewable sources of cellulose, such as crop residues and food industry by-products, can be a valuable resource for energy production and the generation of platform chemicals. In order to enable this, simple sugars need firstly to be liberated from the cellulose via enzymatic or chemical hydrolysis (saccharification) before being fermented; generating the desired end products. The hydrolysis process, however, is hindered by the structure and composition of the lignocellulosic material, and its associated physicochemical properties. Pretreatment methods are used to overcome some of these issues, with many different options available. In this study, the development and application of a novel continuous pretreatment method was investigated: extrusion cooking. Application of the extrusion approach to cellulose and sugar beet fibres (fibrex) was performed in a lab scale extruder, and issues associated with the operation of the extruder overcome by the addition of a plastifying agent. In order to optimise the effectiveness of the extrusion pretreatment, a range of different parameters were assessed. Enzymatic hydrolysis of the resulting extruded material demonstrated that extrusion did improve the conversion of cellulosic biomass to glucose, with a maximum conversion rate of 53% obtained. Furthermore, a novel fermentation method was also explored in this study utilising anaerobic fungi (*Neocallimastigomycota*). Anaerobic fungi are found in the intestinal tract of herbivores, and have become of increasing biotechnical interest in the recent years due to their highly active cellulolytic enzyme system. A standardised rumen-fluid free medium for the growth of anaerobic fungi was developed, and then the cellulolytic ability of anaerobic fungal strains from various genera (*Anaeromyces, Caecomyces, Neocallimastix, Orpinomyces and Piromyces)* investigated in isolation or in co-culture with *Saccharomyces cerevisiae*. *Neocallimastix* was found to exhibit the best overall culture activity, and co-culturing with *Saccharomyces cerevisiae* had a stimulating effect on fungal fermentation results depending on the culturepartner.

Keywords: Cellulose, Extrusion, Anaerobic Fungi, Pretreatment, Saccharification

Kurzfassung

Nachwachsende Rohstoffe, wie z.B. Ernterückstände und Nebenprodukte der Lebensmittelindustrie, können eine wertvolle Basis für die Erzeugung von Plattformchemikalien und Energie darstellen. Um dies zu erreichen, müssen zunächst die Glucosemoleküle, aus denen die Zellulosemolekül in der Biomasse aufgebaut sind, durch enzymatisch oder chemische Hydrolyse freigesetzt werden, um in einer darauf folgenden Fermentation die gewünschten Endprodukte zu generieren. Jedoch wird eine effektive Hydrolyse durch die Struktur und Zusammensetzung des zellulosegehalten Materials sowie die physikochemischen Eigenschaften der Cellulose behindert. Durch eine Vorbehandlung des Materials kann allerdings die Effektivität der Hydrolyse gesteigert werden. In dieser Arbeit werden aktuelle Vorbehandlungsmethoden vorgestellt und diskutiert sowie zwei neue Ansätze, Kochextrusion und Fermentation mit anaeroben Pilzen, auf ihre Anwendbarkeit zur Zellulose-Saccharifikation hin untersucht. Hierzu wurden Zuckerrübenschnitzel (Fibrex) und Zellulose in einem Laborextruder mit Hilfe von Stärke als Prozesshilfsmittel extrudiert. Um den Prozess zu optimieren, wurden verschiedene Prozessparameter untersucht. An die Extrusion anschließend wurden die vorbehandelten Materialien einer enzymatischen Hydrolisation unterzogen. Die Ergebnisse der Umsetzungsraten zeigten, daß die Extrusion die Effektivität der Hydrolyse steigern konnte, bei einer maximalen Umsetzungsrate von 53%. Eine neue Fermentationsmethode unter Verwendung von anaeroben Pilzen (*Neocallimastigomycota*) wurde ebenfalls untersucht. Anaerobe Pilze kommen im Verdauungstrackt von Pflanzenfressern vor und stellen dort eine der Hauptgruppen der funktionalen Mikroorganismen dar. In den letzten Jahren sind anaerobe Pilze auf Grund ihrer hoch aktiven Enzymsysteme in den Focus biotechnologischer Forschung gerückt. Ein standardisiertes pansenflüssigkeitfreies Medium für die Kultivierung von anaeroben Pilzen wurde entwickelt, anschließend wurde die zellulolytische Fähigkeit von verschiedenen Gattungen (*Anaeromyces, Caecomyces, Neocallimastix, Orpinomyces* und *Piromyces*) in Einzel- oder Co-Kultur sowie in Co-Kultur mit *Saccharomyces cerevisiae* getestet. *Neocallimastix* zeigte die beste gesamt Kulturaktivität. Co-Kultivierung mit *Saccharomyces cerevisiae* zeigte einen positiven Effekt auf die Fermentationsergebnisse abhängig vom Kultivierungspartner.

Schlagwörter: Cellulose, Extrusion, Anaerobe Pilze, Vorbehandlung, Saccharifikation

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Preface

Due to the continuously growing demand for energy and the ever growing scarceness of fossil fuels, the world's scientific interest has laid it´s eye on one of the most abundant molecules and resources on earth, cellulose. The development of human civilisation has been underpinned by the utilization of cellulose containing materials; whether as a fuel for fires in early settlements, use as a construction material (shelter, housing and roads) or food for livestock. Cellulose is also historically important in our current widespread use of fossil fuels; which were formed from polysaccharide derived organic materials that were transformed over time by intense pressure and heat. The utilization of cellulose has also received much attention due to the ability to convert it to glucose (which is contained within its structure), and then to other platform chemicals such as ethanol.

To address future energy security challenges, it is not possible to rely on the currently used starch containing renewable resources such as maize and wheat to generate ethanol as a fuel. Production of these resources, often referred to as first generation platform chemicals, results in competition for resources that could also be used for agricultural production of food and animal feed (the food versus fuel debate). This is already causing problems in some areas of the world as costs for crops are on a continuous rise, and people in poorer regions of the world are increasingly unable to afford the cost of basic foods. Second generation platform chemicals derived from renewable resources of cellulose (i.e. wood, crop residues and grasses) however do not have this same fundamental issue as these resources cannot be directly utilised by man as a source of food. This therefore makes them a more feasible and ethical renewable resource for the generation of energy and platform chemicals. In this work, commonly used and up to date approaches to cellulose conversion are discussed. A novel pre-treatment, extrusion cooking of cellulosic containing materials as well as the applicability of a before not used microorganism group, the anaerobic fungi are investigated for their respective perspectives.

1 State of the art

1.1 Cellulosic materials

The chemical structure of cellulose underpins the many diverse features of plants, for example the stability of wood and structural flexibility of grass. Cellulose, chemically described as $(C_6H_{10}O_5)_n$, is an unbranched polysaccharide that consists of (1→4) *β*-glycosidic linked glucose-molecules (also named cellobiose units) that interlink to form fibrillate structures. There can be anywhere from hundreds to thousands of glucose molecules in a cellulose molecule. The stability of cellulose molecules results from this strong covalent linkage, with intramolecular hydrogen bridges between single cellulose molecules adding a further level of stability to cellulose fibres. A cellulose fibre is formed when many cellulose fibrils are aligned, and micro tubes regulate the alignment.

Up to 30 individual cellulose molecules form a larger unit, the elementary fibrils, which are in turn packed into a bigger unit the microfribrils that make up the macro cellulose fibres. Intra- and inter-chain hydrogen bonds solidify the cellulose fibres and mediate the forming of cellulose sheets. The singular sheets in turn are kept together by van der Waals forces acting between the sheets. In the crystalline region of the cellulose fibres the atoms are fixed in discrete positions. This positioning is one of the main reasons for the difficulties in cellulose saccharification.

The close packing of molecules from the different fibrils leads to a tight structure, which cannot be permeated, even by small molecules such as water. Amorphous parts of the fibres lack this close proximity of the molecules. In nature the cellulose fibers consist of various degrees of amorphous and crystalline sections of the cellulose fibre and also show micropores and capillaries.

This structural heterogeneity allows for a partial hydration of the fibres in aqueous media and allow for a certain amount of swelling enabling larger molecules, like cellulases to insert themselves into the cellulose fibre interior.

Cellulose is formed in the plasma membrane of plant cells, along with another molecule that is also found in plant fibre: hemicellulose.

Hemicellulose is a heteropolymer consisting of pentoses, hexoses and sugar acids: with the exact components influencing the biological properties of the plant fibre. For example mannose is the main sugar found in softwoods (e.g. oak, walnut) and xylose in hardwoods (e.g. pine). Hemicelluloses are similar to the cellulose molecules in structure, but diverge by having branched, random and amorphous sections, which incorporate mono sugars (i.e. fructose, galactose, arabinose and xylose). The branches differ in their chemical structure, and consist of four to six carbon-atoms. Plant fibres also incorporate another component: lignin. (Marchessault 1994; Fengel & Wegener 1984)

Lignin is a three-dimensional and amorphous network of aromatic basic modules that are linked in various ways, as well as carbon to carbon single and double-bonds. These consist of monomethoxylated, dimethoxylated and non-methoxylated phenylpropanoid units. Lignin also contains phenolic groups. It can be found in the cell wall and between plant cell walls. Together with hemicelluloses, it forms the matrix in which the cellulose fibers are embedded and bound together. This matrix not only supports the stable structure of plants, but also prevents degradation of the fibres (Martínez et al. 2005; Fengel & Wegener 1984).

In wood, the combination and interlinkage of the three main plant fibre components (lignin, hemicellulose and cellulose) are the basis for its stable structure. As a result, wood is very difficult to break down. It has taken bacteria and fungi many millennia to evolve ways to degrade it. The stability of lignocellulosic materials is also the key challenge in the utilisation of cellulose for energy or platform chemical generation (Ewanick & Bura 2010; South et al. 1995; Aden et al. 2002).

Due to the complex structure and stability of lignocellulosic plant material, pretreatment methods are needed to enable the cellulose within the material to be accessed/utilised by enzymes and/or micro-organisms during the hydrolysis of the cellulose (saccharification). This is similar irrespective of whether ethanol or other platform chemicals are to be produced.

1.2 Overview of methods used in pretreatment of cellulosic materials

Prior to any cellulose saccharification process a mechanical pretreatment (milling, grinding and cutting) and then a physical, chemical or biological pretreatment is normally performed.

The aim of mechanical pretreatment is to generate a bigger surface area as well as to break up the fibres to allow for easier decomposition and higher accessibility in subsequent pretreatment steps. This is also partly necessary due to the fact that the penetration depth of reaction solvents during a hydrothermal fractionation is generally not more than 1-2mm into the fibre. Following mechanical pretreatment, the next steps normally involve thermal or chemical treatment processes: for example treatment with hot water overcritical steam (steam explosion), acids (hydrochloric and phosphoric) or basics (Ammoniac). Biological treatments methods (brown rot fungi) are less commonly used, but will also be covered within this section (Bobleter & Binder 1980; Dumitriu 2004).

Depending on the source material and the pretreatment methods used, unwanted side reactions can occur, for example the formation of furfurals. These can be hindersome for the subsequent fermentation of the treated cellulosic material. A partly or complete fractionation of the base material into its components (cellulose, hemicellulose and lignin), or to a partial subtraction of components (lignine) can now occur (Donaldson 2007).

The target components, cellulose and hemicellulose are now separated or are still in a mixed variation, a complete separation is not yet possible as well as the complete subtraction of lignin is not yet possible. Using the organosolve method the subtraction of lignin is very effective but at the same time quite energy-consuming.

The next fundamental step is the saccharification or hydrolysation of the cellulose, using for example acid catalysis or enzyme reactions.

Acid catalysis is not used commonly any more due to the high cost and energy required to manage the process and the strong acids.

Enzymatic reactions are therefore now much more common, and are mediated by a group of enzymes commonly referred to as cellulases. These enzymes decompose cellulose to cellobiose and further to glucose. Mainly fungi are used in the industrial production of these enzymes.

After the saccharification step, glucose is available and can be fermented with microorganisms to platform chemicals, such as ethanol.

Due to the complex and very stable structure of cellulosic materials, pretreatment is mainly beneficial for the subsequent saccharification as it increases the accessible surface area for enzymatic and/or microbial attack. It can also remove hindersome elements such as lignin and hemicellulose, therefore increasing the amount of amorphous regions. This results in increased sugar yield relative to decreased processing costs (Sun & Cheng 2002; Hahn-Hägerdal et al. 2006; Alriksson et al. 2009; Dashtban et al. 2009; Otieno 2014).

The effectiveness of a pretreatment process can be judged on the following key points:

- Forming of reactive fibre parts for the saccharification.
- No production of hinder some or inhibiting components for enzymes and microorganisms used in the fermentation.
- Low energy demand, low-cost for maintenance and process as well as cheap construction costs.
- Low chemical needs and minimal or no environmental costs.

In addition to the above, removal of lignin from lignocellulosic biomass is also important as it is one of the main obstacles for an effective saccharification. It is suggested that the disruption of the plant cell wall structures and increase of accessible surface are alone sufficient for an effective enzyme attack on the polymers in spite of the crystallinity also negating the effect that delignified cellulosic biomass can exhibit a higher crystallinity of the cellulose (Yoshida et al. 2008; Lee et al. 2009; Ouajai & Shanks 2006).

This is due to fact that the matrix in which the cellulose fibers are embedded mainly comprises lignin. The recalcitrant structure of the polymer hinders effective access to the cellulose fibre and is therefore hindersome during saccharification. In order to effectively access cellulose fibre, the matrix in which they are embedded is reasonable to be removed. In addition, increasing the surface area with a lowering of crystalinity due to mechanical pretreatments such as milling has also been shown to lead to an increase in saccharification yields (Rowell 2005).

The different features and benefits of physical, chemical and biological pretreatments, which are currently available, have been systematically reviewed in the following.

1.2.1 Physical treatments

Many types of pretreatment methods mainly utilise a physical mode of action. In order to decrease crystallinity regions and increase surface area, the source material are commonly processed using mills and/or cutting tools: milling, chipping or grinding the material into a smaller particle size prior to most pretreatments (Schubert 2012).

1.2.1.1 Pyrolysis

Pyrolysis, is a thermal treatment of the source material with temperatures higher than 300°C in the absence of oxygen. In this thermolysis process, cellulose decomposes rapidly to gaseous products and residual char: simultaneously changing the physical phase and chemical composition. Due to the high temperatures involved in this process, the links in the bigger molecules are broken and smaller molecules form. Pyrolysis can also be performed at lower temperatures in combination with a mild acid hydrolysis. Under these conditions, 85% of the cellulose content of the material is converted to reducing sugars, of which the glucose content is more than 50% (Fan et al. 1987).

Additives (oxygen, zinc chloride or sodium carbonate) can also act as catalysts, allowing for cellulose decomposition at much lower temperatures. Heat and pressure can also be used in combination to disrupt cellulosic material (Bridgwater 2012; Narobe et al. 2014; Sukiran et al. 2009; Pollex et al. 2011).

1.2.1.2 Steam explosion

Steam Explosion is a physical process where heated cellulosic material is disrupted due to a rapid decrease in pressure. Material is heated under high pressure in a pressure vessel, with water or steam until the desired pressure is reached. The pressure is then rapidly decreased, causing a spontaneous evaporation of the water contained in the material. This disrupts the material due to forced expansion and associated explosive decompression. The temperature range that is generally used is 160°C-270°C, with pressures ranging from 0.7 to 4.8 Mpa. Treatment times vary from several seconds to a few minutes before the pressure is released. Apart from the process parameters pressure, treatment time and temperature the moisture content and particle size also influence the effect of the treatment. According to Holzapple et al. 1989, this process is roughly 70% less energy consuming than mechanical methods and also has the advantage that it has hardly any recycling or environmental costs.

However, the harsh conditions in the process can lead to the formation of condensation substances between polymers of wheat straw that can form a recalcitrant residue that can be sometimes hinder the enzymatic digestibility of the cellulose material (Vignon et al. 1996; Ballesteros et al. 2006; Sun et al. 2005).

1.2.1.3 Ammonia fibre explosion (afex)

A physico-chemical process where the source material is treated with liquid ammonia at high temperatures and high pressures in a pressure chamber, where after the desired treatment time the pressure is rapidly reduced. The process is influenced by the following key parameters: amount of liquid ammo-

nia (1-2kg ammonia / kg material), water content, temperature (90-100°C), treatment time, pressure reduction, number of treatments and composition of the lignocellulosic source material. Although some types of inhibitory components are not formed during treatment, some phenolic fragments of lignin and various other components can still remain on the cellulose surface. An additional washing step is necessary, producing wastewater in addition to ammonia waste. This wastewater is recycled back into the process to reduce costs and environmental pollution (Teymouri et al. 2005; J. M. Lee et al. 2010; Holtzapple et al. 1992).

1.2.1.4 CO_2 explosion

Like steam explosion and Afex, this carbon dioxide explosion uses spontaneous release of pressure to disrupt the cellulose fibrils and increase the surface area available for enzymatic hydrolysation. Carbon dioxide (CO_2) is used in supercritical condition as extraction agent without extractive purposes. In the normal state, this aggregation state CO_2 shows different properties compared to the same density as a fluid while retaining the viscosity of a gas. It also shows a liquid like solvating power as well as a gas-like mass transfer capacity.

CO_2 enters the supercritical state when the temperature is over 304.13k and pressure is above 73.75 bar, also known as the critical point for carbon dioxide. The solvating power is mainly adjacent to the density, therefore a higher density also increases the solvating power. Higher pressure in the treatment is therefore beneficial for higher glucose yield in the following enzyme-saccharification.

Advantages are low cost due to ample availability, non-toxicity, non- flammability, good recovery and environmental safe. Zheng et al. showed that CO_2 explosion compared to an ammonia explosion pretreatment of bagasse and recycled paper was more cost-efficient. Compared to other explosion pretreatments, it also had a second advantage in that no inhibitory compounds were formed(Kim & Hong 2001; Zheng et al. 1995; Santos et al. 2011; Phan & Tan 2014).

1.2.1.5 Liquid hot water pretreatment (LHW)

Liquid hot water pretreatment (LHW) is a hydrothermal process where cellulosic material is cooked under high pressure in water. The water is able to penetrate the fibres of the base material under high pressure conditions: hydrating the fibres, partly removing hemicelluose and lignin and enlarging the accessible surface area. LHW does not require additional chemicals and expensive corrosive resistant materials. LHW also produces less waste products compared to acid pretreatments whilst being as effective as a dilute acid pretreatment. It is hypothesized that a prior size reduction is not needed for LHW due to the advance penetration capabilities of the water under pressure. Numerous process parameters (pH, temperature, solid concentration and time) can be adjusted to optimise the process (Yu et al. 2010; Pérez et al. 2008; Laser et al. 2002; Mosier et al. 2005).

1.2.1.6 Irradiation

Irradiation is a process where microwaves, gamma rays or electron beams are used to alter the structure of cellulose fibres for a better saccharification.

Kumakura et al found that saccharification of irradiated bagasse produce twice the glucose yield than that of untreated bagasse. Irradiation also leads to degradation of the cellulose-material and to splitting of low molecular weight oligosaccarides (Bak et al. 2009; Yang et al. 2008; Khan et al. 1986; Kumakura & Kaetsu 1983).

1.2.2 Chemical pretreatments

Chemicals can be combined with physical treatments, as in the case of afex etc. However there are numerous pretreatment methods that primarily use chemicals to disrupt cellulosic plant material: alkaline hydrolysis, acid hydrolysis, organosolv amd wet oxidation. The mechanisms and features of these different methods will be reviewed within this section.

1.2.2.1 Alkaline hydrolysis

During alkaline hydrolysis, the source material is treated in alkaline solutions (i.e. Calcium hydroxide ($Ca(OH)_2$), sodium hydroxide (NaOH) or ammonia) to remove parts of the lignin and hemicellulose. This increases the enzyme accessible surface area due to the saponification of intermolecular ester bonds cross linking the hemicelluloses and other components. Dilute NaOH treatment can increase accessible surface area due to a decrease in crystallinity and swelling of the internal surface area. Alkali treatments also seem to be more effective than acid or oxidative methods due to a greater the ester bond breaking capability (Gaspar et al).

Effective treatment parameters were reported to be 1-2% NaOH, temperatures around 120°C and 90-120 minutes of treatment time. If lower temperatures are desired, for example room temperature, the concentration of the alkaline solution has to be adjusted as well as the treatment time, for example the alkaline solution has to be raised by a tenfold and the treatment time increase up to 24 hours.

The process can also be carried out with the addition of hydrogen peroxide (H_2O_2) also known as alkaline peroxide treatment. The ph of the solution is then adjusted to 11-12 pH 7.5%-10% h2o2 35°C and 24h treatment time. For the treatment of rice hulls, for example, the formation of furfural and hydroxymethylfurfural couldn´t be detected indicating that the process is fairly free of the formation of fermentation inhibiting components. This makes the process com-

pared to for example dilute acid pretreatment more desirable as the fermentation rate is not hindered by reaction products (Gáspár et al. 2007; Bjerre et al. 1996; Zhao et al. 2008; Mishima et al. 2006; Curreli et al. 1997).

1.2.2.2 Organosolv process

In order to obtain reactive cellulose in the organsolv process, the chosen source material is treated with an organic or aqueous organic solvent with the option of additional of catalysts. A wide variety of chemicals such as ketones, phenols, ethers, methanol, ethanol, acetone, ethylene, tetrahydrofurfuryl alcohol can be applied as a solvent. Catalysts such as oxalic, acetylsalicylic and salicylic acid can also be added to the treatment liquid, which can also partly consist of water. Treatment temperatures range from 150 to 200°C, depending on the solvents and their concentration. When considering which solvent is to be applied, the factors of cost and recovery are of interest, as solvent has to be removed from the treated material due to an inhibiting effect on the following enzyme reaction and fermentation. When lignocellulosic source material is used as source material, relatively pure lignin can be gained as a byproduct of the recovery process, a feature that differs compared to other chemical pretreatments. Treatment times vary depending on the used chemicals (Pan et al. 2008; Ruiz et al. 2011; Araque et al. 2008; Park et al. 2010; Sun & Chen 2008).

1.2.2.3 Wet oxidation

With the wet oxidation process, temperatures above 120°C are applied to the source material and water mixture under a high oxygen pressure, using air or pure oxygen gas. Due to the exothermic nature of the process it can be self-sustaining once initiated and it actively solubiles as well as degrades the material. Oxidative reactions and formation of acids from hydrolytic processes are the main functions in the formation of reactive cellulose fibres. However, due to the exothermic nature of this pretreatment process, it has to be controlled as the reaction rates and the heat generation are fast and when pure oxygen is

used can get critical. Martin et al reported a treatment scheme where the best results were: fractionization of 94% of hemicelluloses 50% of lignin and formation of reactive cellulose for enzymatic degradation. This was achieved with a treatment time of 15 min and 195°C process temperature. An increase of 35-70 % of methane production from various bio wastes have been reported by Lissens et al. following a wet oxidation pretreatment scheme. In this case, operation parameters were: temperatures ranging from 185-220 °C, oxygen pressure from 1-12 bar and 15 minutes treatment time. Due to the cleavage and oxidation of the lignin byproduct that is formed, the byproduct has limited use and consequently has a lower value (Klinke et al. 2002; Klinke et al. 2003; Schmidt & Thomsen 1998; Schultz et al. 1984).

1.2.2.4 Acid hydrolysis

Acid hydrolysis is a process which uses acids such as sulphuric, nitric and hydrochloric acid to pretreat cellulosic material, and like most other pretreatment methods also implements high temperatures in the process parameters. The acids can be used in combination of either high acid concentration and lower process temperatures or lower acid concentrations and higher temperatures respectively. Lower temperatures are favourable, on the one hand, for lower energy costs, but on the other hand the higher concentrated acids require specialised expensive non-corrosive, non-metal or alloy constructions and a capital and energy expensive acid recovery in order to decrease process cost and environmental pollution. This essentially negates the cost saved by the lower operating temperatures. Using a process with higher temperatures and lower acid concentration is therefore generally more favourable. Apart from the pretreatment for the enzymatic hydrolysis, this method can also be used for direct hydrolysation of cellulose material to fermentable sugars. Acid hydrolysis is one of the most commonly used pretreatment methods and different types of reactors can be used: plug flow percolation, batch, counter current and shrink bead.

Pretreatment methods with a low pH value such as the acid hydrolysis can exhibit a negative side effect in the formation of inhibitor compounds such as furans, phenolic compounds and carboxylic acids. These compounds can inhibit

microbial growth and the fermentation effectiveness leading to lower yields and make a washing or removing process necessary adding to the process costs (Luo et al. 2007; Chen & Dixon 2007; Xiao et al. 2001; Xiao & Clarkson n.d.; Lloyd & Wyman 2005; Chen et al. 2012; Taherzadeh & Karimi 2007a).

1.2.3 Biological pretreatments

For the generation of reactive cellulose fibres from cellulosic biomass, microorganisms can also be used. Brown, white and soft-rot fungi (*Phanerochaete chrysosporium, Trametes versicolor , Ceripororopsis subvermispora , pleurotus*) *Aspergillus sportrichikum* ,or bacteria (*Sphiningomonas paucimonilis and Bacillus curcilans*) have all been effectively used. Microorganisms generally act by degrading the outer structure of the fibres: opening these up and degrading lignin and hemicellulose, while only slightly degrading the cellulose. In addition to the structural and composition changes, other compounds may also be removed from the fermentation material as a result of biological pretreatment. The main key advantages of this type of approach are: low energy consumption, mild environmental conditions, no toxic chemical requirements and easy application and availability. The long treatment times required in the biological pretreatment and relatively low conversion rates can be a disadvantage however when compared to other pretreatment methods (Zhong et al. 2011; Abdelhafidh Dhouib et al. 2006; A Dhouib et al. 2006; Kurakake et al. 2007; Taniguchi et al. 2005).

1.2.4 Current challenges and future opportunities.

All of the above mentioned pretreatment methods have a common aim: to generate cellulosic biomass with improved access for enzymes and/or microbes for saccharification of the cellulose polymer. Reactive cellulose fibres are generated using different techniques, change of the superstructure by removing lignin and hemicelluloses, hydrolysation of the fibres, enlarging the accessible surface area by swelling or tearing/sheering and decreasing crystallinity. Although the methods have the same goal, they vary significantly in their properties and ad-

vantages. Technical factors such as corrosion, steam generation, pressure endurance of the material, solvent and treatment phase recycling, waste water treatment (AFEX, steam explosion, dilute acid) and process specific factors such as cost for chemical and other material, special equipment, pollution control as well as treatment time (Biological pretreatment) influence the cost effectiveness of the process. This can also be optimised by the sale of byproducts such as hemicellulose and lignin. Therefore, a decision regarding what is needed / crucial for the pretreatment process is very flexible, and requires profound knowledge of the biomass material, its production and the pretreatment aim.

1.2.5 Extrusion

The core action of extrusion can be described as forcing a viscous material through a die. Extruders are basically screw compressors that force viscose or high viscose masses under high pressure and temperature through a die with one or more specific openings. Pressure can range from 2 – 300 bar and temperatures can be realised from below zero and up to 300°C. In specialised processes, even higher pressures and temperatures can be realised. Dosable materials such as starch or plastic pellets are continuously fed into the extruder reaction chamber and are mixed, transformed into a plasticisable mass under temperature. The shear force and pressure the forces the mass through a die to from a desired shape. The conveying of the material and the pressure generation are mediated by the friction of the material on the barrel wall and the screw itself.

The extruder simultaneously kneads, mixes, conveys and forms. It can also be seen as a biochemical reactor with feed batch properties. Various plastic items and foods such as pasta, ice cream, snacks, cereals and pet food are produced using this technique. Extrusion is energy efficient, can process both wet and dry materials and consolidated processing and continuous treatment with a high throughput is also possible.

Extruders in theory are compartmented into four sections. Catchment or entrance sections, mixing sections, cooking or melting sections and the forming section (die). The desired material is dosed into the entrance of the extruder

barrel and is conveyed to the mixing and kneading section, the screw ideally shows a high flight depth in this section, water or other chemicals can be added in this section as well. In the mixing and kneading section, the granulated products are kneaded by screw elements and transferred into an amorphous state. The material is then further conveyed into the cooking section where mechanical and thermically energy heat the product to the desired temperatures; pressure is thus built which is needed to force the material through the die, forming the product according to the geometry of the die openings and possible expansion. This occurs when the melt pressure in front of the die is higher than the partial melt pressure outside of the die at the according temperature, resulting in pressure equalisation, that for example, transforms contained water into flashed steam that rapidly expands, and with it the extruded material. Pressure is generated mainly by the screw geometry. Other unit operations such as mixing, shearing, kneeing and conveying are also accomplished based on the section of the screw and the screw configuration.

Extruders can have one, two or more worm shafts, but are generally divided into single- or double screw extruders. Each category displays specific characteristics and has to be chosen according to the desired product properties. Twin-screw extruders have an advantage over single extruders as the screws gear into each other, allowing for a self-cleaning and the higher flight depth allows for a better mixing and conveying. Dosage of water or other chemicals and degassing is possible in almost any section of the extruder barrel.

Other previously discussed pretreatment methods aim at altering of the structure of the cellulosic substrates. The main action is cell wall disruption, thus increasing surface area and porosity as well as opening up the fibres for enzymatic saccharification. Twin screw extruders can mediate good heat and mass transfer capabilities in a closed thermo-chemical reactor with highly controllable treatment times and mixing, dispersing, homogenizing and shearing possibilities. In contrast to the most dominant pretreatment methods, no liquid side fraction is produced that has to be disposed of and no solid loss is therefore associated. The whole substrate can be used for subsequent fermentation. There have been reports of some components being hindered during extrusion treat-

ment. As the effectiveness of the extrusion process relies on a good combination of different parameters, the combinations of high screw speed and high temperature (short treatment time) or a combination of low temperature and low screw speed (long treatment time) have been reported to show the best saccharification results (Lamsal et al. 2010; Lee et al. 2009; Otieno 2014).

High pressures, temperatures and shear generated by the rotating screw lead to shortening of cellulose fibres, generating optimal sites for enzymatic hydrolysation of the fibres. As the substrate moves through the extruder barrel, the high shear stress and constant mixing expose unaffected parts of the interior of the cellulosic substrate to the chemical and thermal treatment (Lamsal et al. 2010; Yoo et al. 2009).

Other effects have been reported to decrease in cellulose crystallinity, inductions of micro/nano fibrillation, and increase in pore quantity and size (Zhao et al. 2009; Karunanithy & Muthukumarappan 2011a; Ayadi et al. 2011; Senturk-Ozer et al. 2011; DEVRIJE 2002).

Karunanithy and Muthukumarappan (2010a) used a single screw extruder in their pretreatment study of corn cob varying screw speeds and extruder barrel temperatures ranging from 25 up to 125 rpm and 25 up to 125°C respectively. The best results of 75% glucose recovery, 2.0 times higher than the control samples, were obtained at 75 rpm and 125°C using a mixture of cellulase and β-glucosidase enzymes. In a second study by Karunanithy and Muthukumarappan (2010b), the effect of extruder barrel temperature, different moisture content and screw speed on the enzymatic saccharification of switchgrass and prairie cordgrass was investigated. Screw speeds of 50, 100 and 150 rpm, barrel temperatures of 50, 100, and 150°C and moisture contents ob 15, 25, 35 and 45% were alternated. After enzymatic saccharification of switchgrass, the highest glucose content 45,2% was obtained for pretreatment with a barrel temperature of 150°C, a moisture content of 15% and a screw speed of 50 rpm. Pretreatment of prairie cordgrass with a barrel temperature of 50°C, a moisture content of 25% and a screw speed of 50 rpm yielded the highest glucose recovery of 61,4%. In addition, the effect of single screw extrusion pretreatment on the enzymatic saccharification of soy hulls was investigated in another study. Extruder barrel temperatures of 100, 110, 120 and 130ºC, screw speeds of 50,

60, 70 and 80 rpm and three moisture contents 10, 12,5 and 15% were varied. The best results of 1.7 higher glucose yield of 62,5% compared to the untreated samples were generated with an extruder barrel temperature of 130ºC, a screw speed of 60 rpm and a moisture content of 12.5%. In a study by Zhang (2011) corn stover was used as the cellulosic material. The pretreatment was conducted with a twin-screw extruder with applied screw speeds ranging from 40-140 rpm and moisture contents of 22.5, 25 and 27.5%. At pretreatment conditions of 27.5% moisture content and a screw speed of 80 rpm the best results of 2.2 times higher glucose recovery compared to that of untreated corn stover could be obtained (Karunanithy & Muthukumarappan 2010; C. Karunanithy & Muthukumarappan 2011; Zhang 2011).

The influence of screw speed on cellulose saccharification was investigated by Lamsal (2010) and Zhang (2012). Corn stover as well as wheat bran were pretreated using twin-screw extruder with barrel temperature of 140°C and 150°C. The results showed an increase of saccharification with increasing screw speed. It was proposed that a higher sheering of the cellulosic biomass was realized by the higher screw speeds increasing specific surface areas for an optimal enzymatic attack. The relatively shortened residence times were also of interest as this allows for more material to be treated in shorter time as well as the prevention of thermal degradation at the higher processing temperatures (Lamsal 2010; Zhang 2012)

1.3 Cellulosic biomass processing

Cellulosic biomass processing via enzymatic saccharification of the pretreated cellulose fibres consists of different separated biological units of operation, namely: cellulase production, enzymatic hydrolysis of the material and the fermentation of hydrolysis products (pentose as well as hexose).

- Different approaches to the combination of the single treatment steps have been developed over the years: Separate hydrolysis and fermentation (SHF),
- Simultaneous saccharification and fermentation (SSF),
- Simultaneous saccharification and Co-fermentation (SSCF) and
- Consolidated Bioprocessing (CBP).

SHF consists of completely separate steps where cellulases are produced outside of the process that are used for the saccharification of cellulose containing material. The generated sugar yields are then fermented in another separate process step to the desired chemicals.

SSF also uses pre-produced cellulases, but combines enzymatic saccharification and fermentation of cellulose hydrolysation products in a single step with an additional fermentation step of hemicellulose hydrolysation products.

SSCF uses pre produced cellulases as well but combines the enzymatic saccharification and fermentation of all saccharification products in a single step

CBP combines enzyme production, saccharification and fermentation of generated sugars in a single process.

CBP seems to be the most cost efficient cellulosic biomass processing via enzymatic saccharification, as the cost for enzyme production is omitted and only a single process step is needed. In order for this process to be applied successfully, the applied microorganisms have to remain metabolically active during the processing time and fairly resistant to possible inhibitory compounds originating from potential pretreatments (M. Jin et al. 2011; Bayer et al. 2007; Geddes et al.

2011; Taherzadeh & Karimi 2007b; Kadam & McMillan 2003; Wyman & Davison 1996).

1.4 Cellulose degrading enzymes and cellulosome

In order to utilize insoluble cellulose as a carbon source which is accessible after pretreatment, various types of microorganisms (mainly fungi and bacteria) have evolved and/or acquired enzymes to effectively hydrolyse and metabolize polysaccharides. These extracellular enzymes are commonly referred to as cellulases.

Cellulase enzymes have recently been categorised based on their structural properties {260} into three different groups: (i) endoglucanases or 1,4-ß-D-glucan-4-glucanohydrolases (EC 3.2.1.4); (ii) exoglucanases, including 1,4-ß-D-glucan glucanohydrolases (also known as cellodextrinases) (EC 3.2.1.74) and1,4-ß-D glucan cellobiohydrolases (cellobiohydrolases) (EC 3.2.1.91); (iii) ß-glucosidases or ß-glucoside glucohydrolases (EC 3.2.1.21) (Tipton & Boyce 2000; Babbitt 2003).

Endoglucancase enzymes cut randomly in amorphous regions of the intertwined polymer complex, liberating singular oligosaccharide chains of varying lengths and generating new chain ends. Exoglucanase enzymes attack liberated cellulose polysaccharides, either from the reducing or non-reducing end. Thereby cellobiose (cellobiohydrolases) or glucose (glucanohydrolases) molecules are split off.

The liberated cellodextrins or cellobiose molecules are then hydrolyzed by the ß-glucosidase to glucose molecules by an acid hydrolysis mechanism, using a nucleophile and a proton donor. Hemicellulases hydrolyse the variety of glycoside linkages present in the structure of the hemicellulose and are secreted by many cellulolytic microorganisms.

All the different types of cellulase enzymes can be simply secreted out of the microbial cell: penetrating openings or exposed inner surface areas of plant materialin in order to target specific cell wall components and saccharides. Cellulases exhibit a modular structure with both catalytic and carbohydrate binding modules as well as catalytic centres. The binding modules mediate the attachment to the cellulose and bring the active centres into close proximity of the corresponding carbohydrates. The different types of cellulases exhibit a synergism when endo-, exo-, and ß-glucanases are present: showing a higher enzyme activity than the sum of the singular enzyme activities. This is due to the fact that reaction products like cellobiose are removed via degradation, and therefore no product inhibition occurs limiting the enzyme activity.

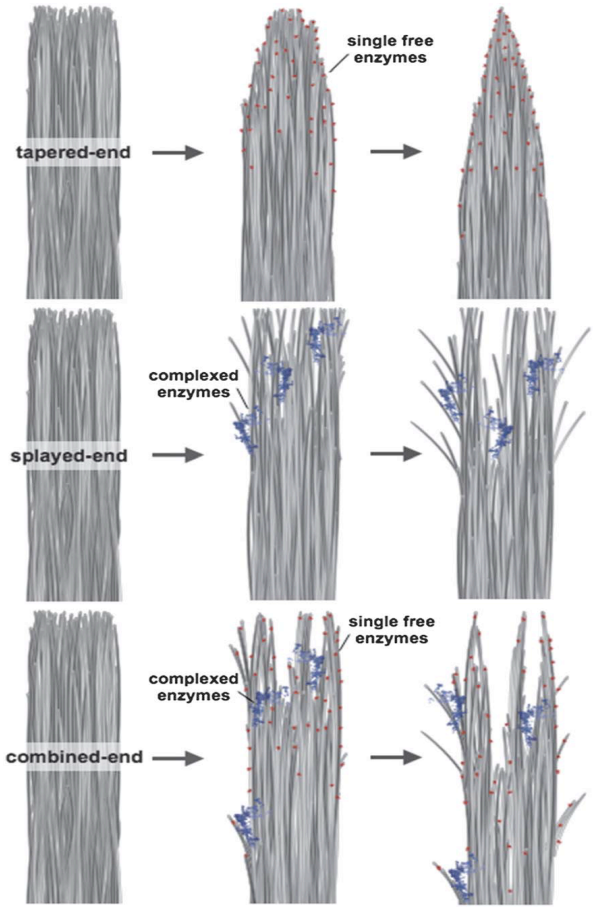


Figure 1: Illustration of the mechanisms by which free enzymes (top) and cellulosomes (middle) differ in their action on cellulose microfibril bundles and act synergistically to degrade cellulose (bottom).(Resch, Donohoe, Baker, Decker, E. a. Bayer, et al. 2013)

In contrast to the free enzymes that are secreted into the area around the microbial cell, various bacteria and fungi have developed a self-assembling macro multi-enzyme complex of synergistically acting enzymes. This complex is attached to the exterior of the microbial cell via a protein scaffold, and is called a cellulosome.

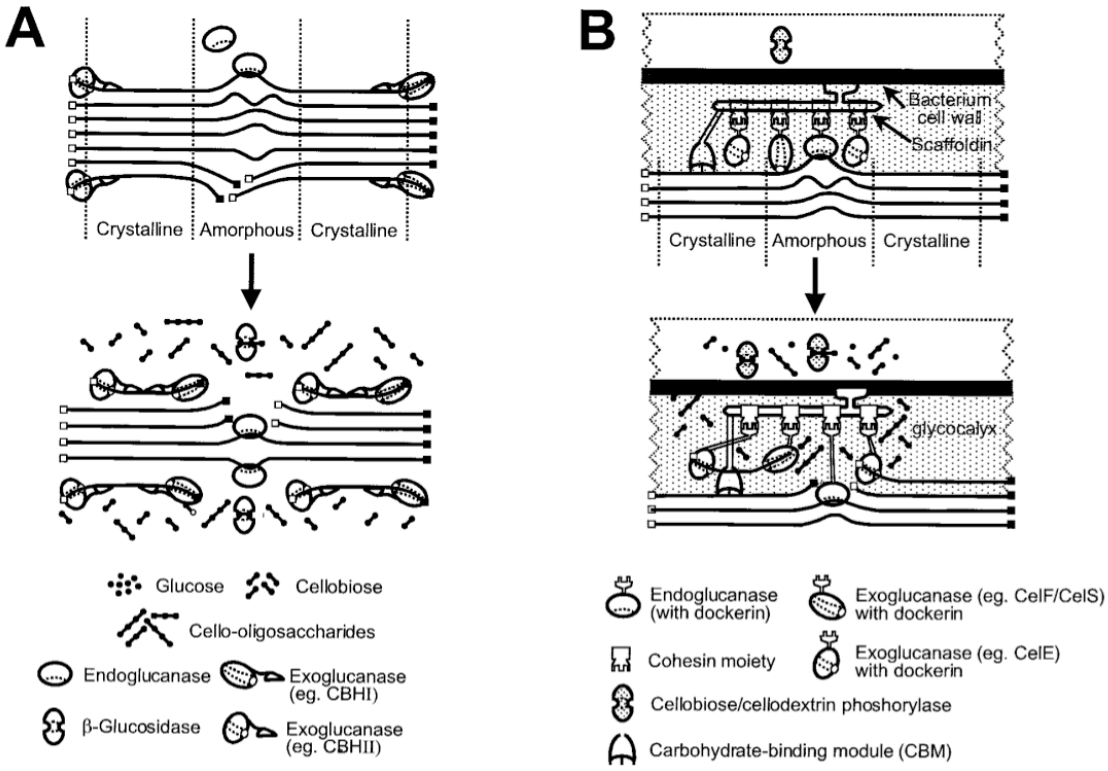


Figure 2: Schematic representation of the hydrolysis of amorphous and microcrystalline cellulose by non complexed (A) and complexed (B) cellulase systems. The solid squares represent reducing ends, and the open squares represent nonreducing ends. Amorphous and crystalline regions are indicated. Cellulose, enzymes, and hydrolytic products are not shown to scale. (Lynd et al. 2002)

Cellulosomes consist of processive and non-processive carbohydrate active enzymes, various cellulases and hemicellulases fixated on protein scaffoldings with multiple catalytic domains. They exhibit a good ratio between catalytic domains and the individual components are at optimal distances from each other. This enables cellulose and other polysaccharides to be effectively and efficiently removed.

Native cellulosomes are curled closed, and open when attaching to cellulose fibre. A synergistic effect can be observed when the cellulosome is paired with free enzymes. This is due to a faster conversion rate, as the free enzymes seem to liberate cellulose ends and cellulosomes mediate surface area expansion and splaying of the fibrils. Adhesion of the cell via cellulosomes to the fiber can also have some other beneficial effects such as concentration of enzymes on the cell surface, protection against protozoal and bacteriophage attack and protection of hydrolytic enzymes from the action of proteases. Cellulosomes also enable the direct utilization of the liberated cellulose fibrils and glucose, which is very important in complex microbial ecosystems that utilise lignocellulosic plant material, such as the rumen (Harris et al. 2014; Garvey et al. 2013; Payne et al. 2013; Resch, Donohoe, Baker, Decker, E. A. Bayer, et al. 2013).

1.5 Anaerobic fungi and their role in the utilisation of lignocellulosic biomass

Anaerobe fungi are commonly found in the intestinal tracts of ruminant and non-ruminant herbivorous mammals such as cows, sheep, horses and deer. The importance of bacteria and protozoa for the digestion of lignocellulose in the forestomach compartments of ruminants has been widely acknowledged since the middle of the 20th century. However the existence of anaerobic fungi was originally thought unfathomable. The scientific community was of the opinion that anaerobic fungi were different from bacteria and protozoa in that they could never exist anaerobically as they needed oxygen to grow. It was not until the late 1960's that it was discovered that the flagellated zoospores of anaerobic fungi had been incorrectly identified as flagellated protozoa.

Anaerobe fungi exhibit proteolytic activity, which allows them to penetrate the outer protein containing layer quite easy and allow for a fast access to the second layer of plant matter. This ability allows for a faster colonization and utilization of the cellulosic substrate compared to cellulytical bacteria. Colonisation of plant material in the rumen has been reported to be successful after only 3 hours ingestion and occurs primary on damaged plant material and stomata. Anaerobic fungi seem to be the first group of microorganisms in the rumen to render the in the plant material accessible and also lower the resistance of the plant material for biological degradation by the action of bacteria and protozoa (Klieve & Bauchop 1988; T Bauchop 1989; Bauchop 1979; Akin 1980; Akin & Rigsby 1987).

1.5.1 Life cycle

The lifecycle of the anaerobic fungi consist of a mobile phase (zoospore) and a stable vegetative phase (thallus), which results in one or more fruiting body (sporangia). The vegetative phase is found on plant particles and living saprophytic. The rhizoid of the thalli is penetrating the plant particle and develop spo-

rangia on the outer surface of the plant particle. After development and maturation of the sporangia, numerous flagellated zoospores are released in the zoosporulation process 8-32 hours after the encystment of the original zoospore (Trinci et al. 1988; Theodorou et al. 1990a; Kingston-Smith et al. 2003; Fen et al. 2009; Theodorou et al. 1990b).

Zoospores have one or more flagella, protein fibres that are attached to the cell body and can be moved exhibiting a lashing motion that can propel the cell amöbiacly into a direction mediated by chemotaxis. Form and shape of the sporangia can vary and sporulation can be induced by newly available plant material in the media and when animals are fed shortly after the ingestion of the plant material.

Newly available material is colonized, or the surrounding surface is recolonised by the zoospores that can be mobile in the liquid phase for many hours before encystment. Prior to encystment, the flagella are discarded and the spore develops a thallus. Orpin reported a maximum in spore count 30 minutes after fresh plant material was ingested by ruminants for Caecomyces and piromyces this time was roughly 60 minutes. It is also stated that the zoosporgeneses is mediated by soluble components in the media or rumen originating from fresh plant material. For Neocallimastix spores, a chemotaxic reaction on soluble carbohydrates and haem have been reported (Orpin and Greenwood 1986). Chemorceptors for Manose, Manitol, Fructose and Glucose have been discovered, and the chemotaxic reaction is sensitive and has been reported to be affective at a concentration of 1µl sucrose (Orpin and Joblin 1988). It is hypothesized that zoospores follow the carbohydrate gradient originating from damaged plant material to find a suitable spot for encystment. (Orpin & Bountiff 1978; Orpin 1977a; Orpin 1975; Orpin 1977c; Orpin 1984; Akin & Borneman 1990; Grenet et al. 1989).

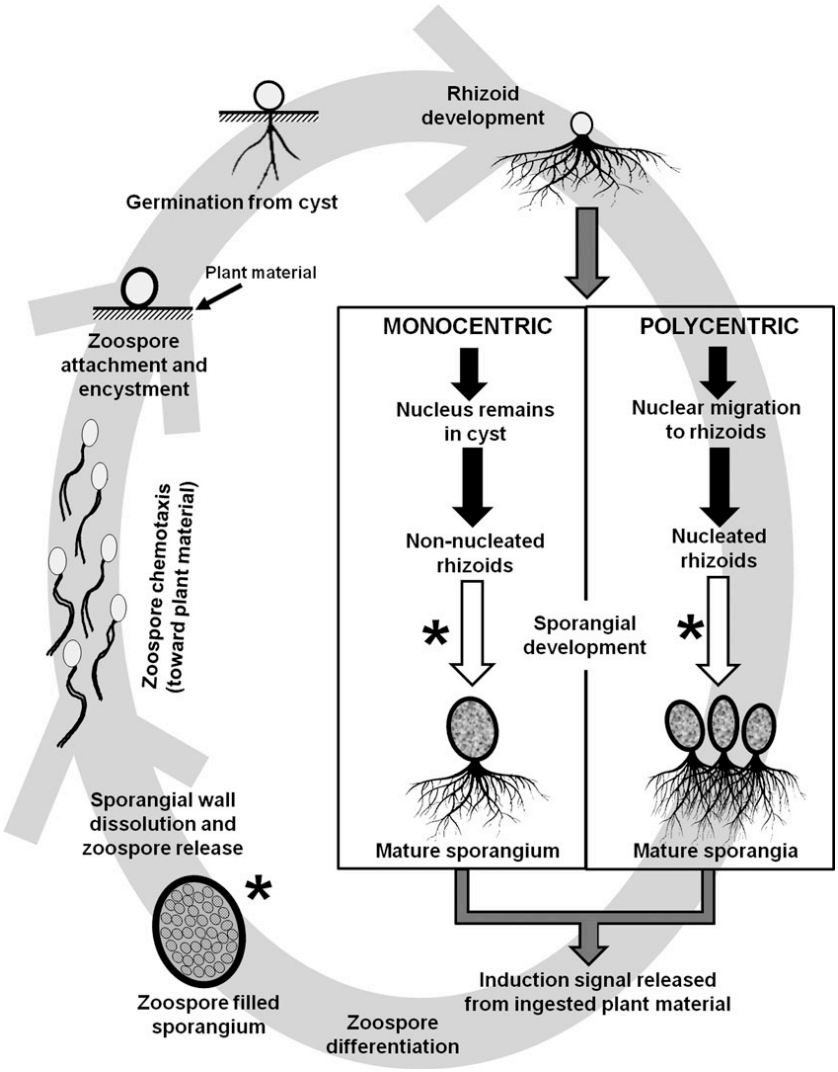


Figure 3: Summary of the anaerobic fungal life cycle. The stages in the life cycle where 'resistant' structures (that have been reported to date) may be formed are also indicated (*) (Gruninger et al. 2014).

Theodorou as well as Orpin and Joblin suggested that the life cycle of the anaerobic chytridomycetes can be viewed as a 2 stage cycle with an additional survival or resistant stage. These being the motile zoospore, the vegetative thallus, and an aero tolerant survival stage. The latter stage allows them to be transferred from one animal to another, be dormant in dried fees and also be probably disseminated into herbage. This depicts an interesting anomaly of a strict anaerobic microorganism, and is similar to the lifecycle of some aerobic chytridomycetes(K. N. Joblin 1981; S E Lowe, Theodorou & A. P. Trinci 1987a; S. E. Lowe et al. 1987; S E Lowe, Theodorou & A. P. Trinci 1987b; Edwards, Kingston-Smith, Jimenez, S. A. Huws, et al. 2008)

1.5.2 Morphological based classification of anaerobic fungal genera

The general assignment of a neocallimastigaceae is based on various morphological features that occur during and in their life cycle.

1.5.2.1 Monocentric anaerobic fungi

Monocentric anaerobe chytridomycetes are reported to either have an endogen or exogen monocentric development. In endogen development, the nucleus remains in the encysted zoospore undergoes mitosis and forms a single zoosporangium. In exogen development, the zoospore nucleus migrates into a germ tube and undergoes mitosis and a single zoosporangium is formed. Thus only one sporangium is formed per thallus. The nucleus multiplies during the development of the sporangium and nucleii are not present in the rhizoidal system, nuclei free vegetative thalli are developed that can´t develop further and dissolve after sporulation (Barr et al. 2011; Kudo et al. 1990; S. E. Lowe et al. 1987).

1.5.2.1.1 Neocallimastix

The vegetative state shows single sporangia and an interlaced filamentous rhizoid. The zoospores are ovoid to ellipsoid and polyflagellated with diverging numbers of flagella ranging from 7 up to 17. Flagella are protruding from two parallel lines and exhibit a central nucleus(Munn et al. 1988; Munn et al. 1981; Orpin and Joblin 1988)

1.5.2.1.2 Piromyces

Exhibits one or two filamentous rhizoids that can be branched and partly protruding single sporangia. The zoospores are round to ovoid and monoflagelated, sometimes showing 2-4 flagella. (Orpin 1977a; Barr 1989; Kudo et al. 1990)

1.5.2.1.3 Caecomyces

The vegetative stage is characterized by an unbranched coralline rhizoid with ovoid structures partly with fibrillar roots and also vesicular structures. The zoospores are round to ellipsoid or amoeboid, monoflagelated. Hydrogenosomes are scattered between the flagella roots. Ribosomes can be found in helixes and cluster. (Theodorou et al. 1996a; Gordon & Phillips 1998a)

1.5.2.2 Polycentric anaerobic fungi

Polycentric anaerobe chytridomycetes show an exogenous polycentric development where the nucleus migrates into the germ tube and undergoes mitosis and is spreading to form numerous zoosporangia. A highly branched and intertwined rhizoid ripens. Sporangia are formed at the end of the rhizoid but also various other places of the rhizoid. These can be either singular or in a group of up to six, which differs from monocentric genera in the way that no central core of sporangia is formed. As vegetative reproduction is realized through the fragmentation of the rhizoid, a continuous stable lifecycle that isn´t as dependent on zoospores as that of the monocentric anaerobe chytridomycetes is possible (Trinci et al. 1994; Barr 1989; Grenet et al. 1989; Theodorou et al. 1996a).

1.5.2.2.1 Orpinomyces

The rhizoid is polycentric, extensive , filamentous and has multiple nucleii. Sporangiophores are produced at terminal or middle position on the rhizoid. The zoospores are ovoid to ellipsoid and polyflagellated with diverging numbers of flagella ranging. Flagella protrude from two parallel lines and exhibit a central nucleus (Barr et al. 2011; Barr 1989; Hodrovd & Kdg 1998; Kittelmann et al. 2012).

1.5.2.2.2 Anaeromyces

The rhizoid is polycentric, extensive , filamentous and has multiple nucleii and can exhibit branched hyphae that can be fine and root like. The sporangia can be elliptical and fusiform with a pointy tip. The zoospores can be round to polymorph and are monoflagelated. (Fliegerová et al. 2002; Fliegerová et al. 2010; Denman et al. 2008; Barr 2008; Theodorou et al. 1996b)

1.5.3 Hexosematabolism

Yarlett 1986 as well as o´Fallon 1991 describe the electron and carbohydrate flow for the anaerobe chytridiomycetes as follows, shown exemplarily for *Neocallimastix frontalis*. During glycolysis, pyruvate is generated which is converted into oxaloacetate by the enzyme pyruvate-carboxylase. The oxaloacetate is then subsequently reduced by the malate-dehydrogenase to malate. Malate can then be transported into the hydrogenosome where hydrogenosomal enzymes generate energy through its oxidation via a NADH:ferridoxin oxidoreductase. The proton reduction is mediated through the ferridoxin and hydrogenases simultaneously generate hydrogen. Additional pyruvate is produced after the oxidation of malate, which can be transported back into the cytoplasm.

Cytoplasmic pyruvate can be transformed into oxalacetate and subsequently into lactate by the cytosolic lactate- dehydrogenase. The pyruvate-decarboxylase then can change to acetate, or it can be decarboxylated to acetaldehyde. Subsequently the acetaldehyde can be transformed into ethanol by an alcohol-dehydrogenase. The hydrogenosomal production of acetate and the cytosyolic production of the ethanol can be inhibited by carbon dioxide. This is due to end product inhibition of the pyruvate:ferridoxin-oxidoreductrase and pyruvic-decarboxylase-reaction.

Glucose can also be transformed into phosphoenolpyruvate, and subsequently carboxylated to oxaloacetate and reduced to malate. The hydrogenosomally

generated pyruvate can then be converted with a pyruvate kinase to phosphoenolpyruvate or other fermentation products respectively. The generation of formate requires a pyruvate-fomate-lyase, which splits pyruvate into formate and acetyl-CoA. The acetyl-CoA can then be further metabolized to ethanol and acetate. (O'Fallon et al. 1991; Yarlett et al. 1986)

Interestingly, lowe 1987 reported that *N. huleyensis* produces the same fermentation end products when grown with glucose or xylose as carbon source, but the relative abundance of the fermentation end products differed. Formate is the main fermentation product when glucose is provided as a substrate, whereas acetate dominates when xylose is the sole carbon source (S E Lowe et al. 1987; S E Lowe, Theodorou & A. P. Trinci 1987b; Susan E Lowe et al. 1987; Marvin-Sikkema et al. 1990a).

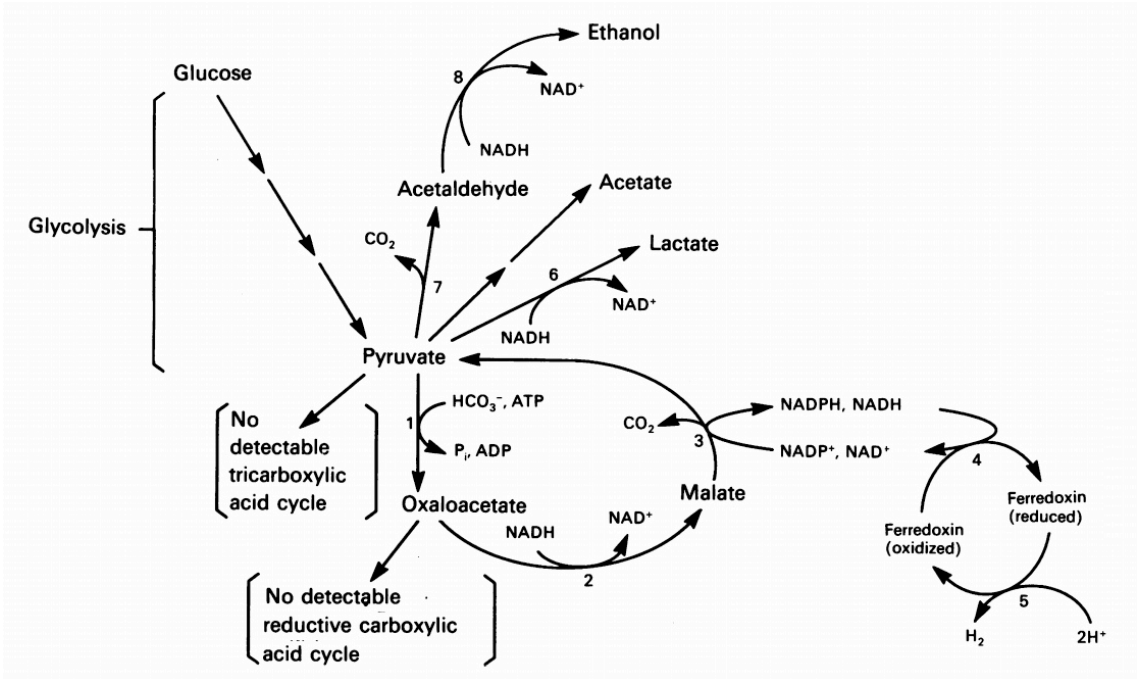


Figure 4: Primary metabolic conversion of glucose into acetate, ethanol and lactate by N. frontalis EB188 Numbers designate the following enzymes: 1, pyruvate carboxylase; 2, malate dehydrogenase; 3, 'malic' enzyme; 4, NADH (NADPH): ferredoxin oxidoreductases; 5, hydrogenase; 6, lactate dehydrogenase; 7, pyruvate decarboxylase; 8, alcohol dehydrogenase. (O'Fallon et al. 1991)

1.5.4 Anaerobic fungal enzymes and their catabolic regulation

The vegetative stage and the zoospores of the anaerobic chytridiomycetes produce a wide array of effective plant degrading enzymes, enabling utilization of the main structure carbohydrates of plant material. Known groups of anaerobic fungal enzymes include: cellulases, hemicellulases, glucosidases, xylosidase, disaccharidases, pectinases, amylases, proteases, beta glucosidase and xylanases. Anaerobic fungal enzymes can attack a wide variety of cellulosic substrates, with some enzymes exhibiting several active centres with multiple substrate affinities (O'Fallon et al. 1991; Li & Calza 1991; S. E. Lowe et al. 1987; Teunissen & Op Den Camp 1993; Borneman et al. 1991; Chen et al. 1994).

Anaerobic fungal enzymes can be found cell bound as well as secreted into the media. There is also evidence that anaerobic fungi can exhibit a cellulosome on the cell surface, similar to that in *Clostridum thermocellum* (which appears to be smaller in comparison). (Wilson & Wood 1992; Li & Calza 1991; Chen et al. 1994).

It now appears that anaerobe fungi have acquired the ability to construct cellulosomes from bacteria. The close proximity of microorganisms in the rumen and research by Garcia Vallvé who performed sequence homology analysis between anaerobic fungi and *F. succinogenes* as well as the G+C content and codon bias of glycoside hydrolase genes as well as a comparison of the phylogenetic trees of multi-alignment of orthologous sequences indicate a that in fact horizontal gene transfer had occurred. Other horizontal gene transfers have also been reported in the natural environment of the fungi, the rumen (Garcia-Vallve et al. 2000).

The degradation of highly stable cellulose has to be mediated by complex and sequential processes by close interacting enzymes. Other microorganisms also produce cellulases that can degrade amorphous, crystalline and highly ordered cellulose: for example *Trichoderma* spp, *Fusarium solani* and *Clostridium thermocellum*. A wide variety of polysaccharide hydrolases can similarly be found in these microbes. Research has shown that various genes are involved in producing the enzymes, and production of specific enzymes can be triggered by the corresponding carbohydrates (Li & Calza 1991; Barichievich & Calza 1990).

Many enzyme activities of the anaerobe chytridomycetes are governed by catabolic regulations mechanisms, and are suppressed or stimulated by various mono- and di-saccharides like cellobiose, glucose, fructose and xylose as well as cellulose and xylan. The cellulytical systems can be stimulated by cellulose, cello-oligosaccharids as well as cellobiose and exhibit an constitutive stimulation as well.

It is of interest to note that the cellulases of anaerobe chytridomycetes seem to be highly active, and even exceed the most active cellulases in *Trichoderma ressei* (Wood 1986). Cellulases from *Neocallimastix frontalis* have shown a several fold higher solubilisation rate of cotton fiber per unit of endo-1,4-β-glucanase than those found in the culture filtrate of *Trichoderma reseii* mutant c-30 (Wood 1986). The pH optimum of anaerobic fungal cellulases has been found to be in a range 5.1 to 6.4, and a temperature optimum from 45°C to 55°C. The cellulases also possess endoglucanase, beta glucosidase and cellobiose activity (Puls & Wood 1991; Bhat & Bhat 1997; Wilson et al. 1994; Morrison et al. 1990; Borneman et al. 1990).

1.5.5 Interactions of anaerobe chytridomycetes with other microorganisms

Spatial juxtaposition is also needed for effective cellulose fermentation. Cellulose activity can also be enhanced by a co-culture of anaerobic fungi with methanogenic archaea, which are also common in the rumen. Fermentation products then shift from lactate and ethanol to acetate and methane.

In their natural gut environment, anaerobe Chytridiomycetes are also found in close proximity to bacteria and protozoa. Various interaction between the different kinds have been studied they can exhibit an antagonistic and synergistic effect. Bacteria and protozoa can support or hinder anaerobe fungi in growth and cellulytical activity which can vary based on the genera of anaerobic fungi as well as bacteria and protozoa genera present in the co culture. Anaerobe fungi, on the other hand, provide beneficial actions for the other kinds, for example the lowering of the structural integrity of plant fiber mass allowing for a

cellulytical vantage point that otherwise wouldn´t be accessible for bacteria. (Akin et al. 1983; Joblin et al. 1990; Theodorou et al. 1994; Orpin 1977a; Bauchop 1977; Tom Bauchop 1989; Mountfort et al. 1982; Bauchop & Mountfort 1981)

The ATP production of anaerobic fungi can be enhanced when methanogen bacteria are present in a coculture and hydrogen transfer to methanogens (Ruminococcus flavefaciens, Methanobrevibacter ruminatium for example) and acetogene bacteria (Eubacterium limosum, Acetitomaculum ruminis for example) is reported to take place. The formation of fermentation products is shifted to the production of more acetate and methane and fungal growth, whereas as extracellular enzyme production is also elevated. A catabolic suppression of methanogens and acetogenes can also be observed in later fermentation states. Joblin 1990 Degradation of celluosic biomass can be observed to be accelerated. Co cultures with acetogenes have a less enhancing effect than methanogense (Joblin et al. 1990; Hodrová et al. 1995; Kopecný et al. 1996; Marvin-Sikkema et al. 1990a).

Fibrolytic bacteria such as Butyrivibrio fibrisovens, Fibrobacter succinogenes Ruminococcus albus, Ruminocucus flavefaciens seem to exhibit no synergetic interactions with the anaerobe chytridomycetes but can be of support in the degradation of plant fiber or instead compete for available cellulosic biomass. R. albus and R. flavefaciens seem to have a rather inhibiting effect on the cellolyse of anaerobe fungi but are also reported to have no effect or a slight enhancing effect. The different effects may be attributed to the different characteristics of the single species be it bacteria or fungi (Joblin & Orpin 1993; Roger et al. 1992; Bernalier et al. 1992; Gaillard et al. 1989; Marvin-Sikkema et al. 1990b).

Protozoa seem to have a synergistic as well as an antagonistic effect, depending on the type of co culture and species used. In the natural environment of fungi, the protozoa are part of the plant fiber degraders and some protozoa prey on the fungi taking them up via phagocytosis and following digestion, As observed for Eudioldinium maggii Entodinomorphe, Polypastron multivesiculatum and Entodimium spp. Hemicellulytical activity is reported to be enhanced with an inhibiting effect on cellulytical activity by the protozoa. Fluctuations in zoo-

spore count are reported in the vicinity as well but to date there has been no concrete evidence whether a lowering or stabilizing or enhancing effect takes place. An explanation for this divergence can be the natural fluctuation of the zoospore count depending of the culture age or the fact that the different species exhibit different properties in coexistence or are due to the errors and difference of the applied counting method (Morgavi et al. 1994; Of et al. 1981; K N Joblin 1981; Joblin et al. 1990; Fonty et al. 1995; Joblin & Orpin 1993).

As the main focus research on anaerobic fungi lies on co-culture, research with microorganisms that are present in the natural habitat. Very little research has been conducted on co-cultures with non-gut microorganisms. The interaction with yeast as a functional microorganism for simultaneous bioprocessing could be of interest. (Flint 1997; Krause et al. 2013)

Chaucheyras studied the effects of live yeast, saccharomyces cerevisiae as promotive additive to cultures of Neocallimastix frontalis in media with and without vitamin deficiency. Various additions of different amounts of *S. cerevisiae* cells, both viable and autoclaved, were added and both types stimulated fungal zoosporulation. Addition also enhanced cellulase production and production of fermentation products such as hydrogen, format, lactate and acetate. Higher responses could be observed for viable cells added. It seems that yeast propagates fungal activity in two ways, supplementing the fungi with supportive vitamins such as Thiamine, essential for fungal growth and scavenging inhibiting fermentation products from the medium and supplying additional micro nutrition. Making yeast an interesting co fermenter also for a utilization for ethanol production in a consolidated bioprocessing approach. (Chaucheyras et al. 1995; Dawson et al. 1990)

1.5.6 Current challenges and future potential for the use of anaerobic fungi in the industrial utilisation of cellulose.

Anaerobic fungi have been of interest for some time as part of the digestion of ruminants, as the nutrition of farm animals is of industrial interest in terms of both their health and productivity. Anaerobic fungal cellulytical activity has exceptional potential to provide a cheap and efficient way of transforming cellulosic biomass into platform chemicals and energy. Their complex enzyme system allows for the fast and effective rhizomycelial penetration of plant material, facilitating their ability to effectively degradation and utilise cellulosic biomass (Gruninger et al. 2014).

The generation time is slower than that of bacteria and protozoa but that is made up by the faster and more effective utilization of the plant fiber. Their ability to work synergistically with other microorganisms is another possible application and their cellulytical and fibrolytical activity that is comparable to or even exciding that of aerobe fibrolyticall microorganisms has recently become of great interest in the search for more effective cellulytical enzymes. Lowe 1986 Anaerobe enzyme production still poses a difficult solution for the production of cellulytical active enzymes but could be improved by the modification of specific microorganisms via biotechnological techniques. Further research into this field has already begun, but is yet to be proven to be effectively implemented in an industrial scale process (Newbold et al. 1996; Newbold et al. 1998; Fen et al. 2009; Gruninger et al. 2014; Edwards et al. 2008; S E Lowe, Theodorou & A. P. Trinci 1987b).

2 General Introduction and Objectives

Whilst striving for clean and affordable energy and platform chemicals, the generation of cellulose derived chemicals has become of more interest for academic and industrial research. America for example is aiming for a continuous increase in produced bio-ethanol derived from corn in their renewable fuel standard 2 RFS service 2010 (Schnepf & Yacobucci 2013). Brazil is widely using fuel generated from sugarcane. Other countries are similarly interested in the enhancement of production of renewable energy and platform chemicals.

A problem with this first generation platform chemical process is that it is in competition with an increasing human population and their need for affordable food. Food prices have been increasing all over the world due to environmental conditions as well as the utilization of starch containing foods for the production of bio ethanol. A solution for this food rivalry can be found in the use in the ever abundant cellulosic biomass, be it wood, grass, sugarcane bagasse, switch grass, wheat straw, wheat bran, soybean hulls or other agricultural crop residues, which also shows beneficial environmental properties is not in use as food and is reducing greenhouse gas emissions.

Most crop residues and food industry byproducts are used in ruminant animal feed due to the low value or low value for ethanol distillation and other application. When industrialby-products are used, an extensive pretreatment via grinding and other pretreatment methods to generate reactive cellulose fibres is not as extensively needed as with lignocellulosic materials.

The generation of glucose from cellulose via enzymes has proven to be quite difficult due to the recalcitrant condition of the source material deriving from the chemical structure of the cellulose and the resistance to enzymatic hydrolysation in the native state due to the lignin matrix that shields the cellulose fibres (Galbe et al. 2007; Galbe & Zacchi 2007; Wu et al. 2007).

Other limiting factors in research are for example, the currently high production costs for enzymes, transportation logistics of materials that exhibit only a limited energy content or concentration, uncertainties in the sponsorship and support of

policymakers as well as politic in general and oversupply of first generation biochemicals. New approaches are therefore needed to overcome the current gaps in technology and encourage further research and funding of said research. New and/orenzyme systems as well as more efficient pretreatment methods with a high throughput and a continuous operation schematic as well as more efficient microorganism or application of yet unused potentially promising microorganisms are therefore in focus. The thus discussed pretreatment methods offer different advantages and disadvantages.

Energy consumption, low solids loading, small capacity, non-continuously nature of batch processes and difficulties for a scale up are the key issues that have to be addressed. A pretreatment method that is cost effective on an industrial scale has yet to be implemented, as the various disadvantages are mostly too expensive with respect to energy costs, materials and chemicals which mainly means that the generated value from the cellulosic source material is negated by the process costs. (Saha & Cotta 2007; Galbe & Zacchi 2007; Galbe et al. 2007; Corredor 2008; Corredor et al. 2008)

The challenge at hand is the optimization of the cellulose conversion process with respect to cost and energy efficieny. The extrusion process can be an interesting addition to the known cellulose conversion processes. It can provide a novel and unique continuous process that combines mechanical shear within a stirred thermo-chemical reactor and a pressure treatment. The high-throughput rate that could be realized is of special interest. Shear forces as well allow for an effective lowering of the structural integrity of cellulosic biomass with a simultaneous thermal chemical and pressure treatment of the opened up fibres (Lamsal et al. 2010).

In this work, pure cellulose and sugar beet fibres (Fibrex), are used to evaluate the extrusion process with the development of a protocol for a combined pretreatment process and downstream enzymatic hydrolysation to glucose.

As mentioned above, naturally accruing cellulosic substrates have to be pretreated prior to an enzymatic saccharification with an industrially produced enzyme cocktail. Due to the fact that the cellulose in the cellulosic base materials occurs naturally in heterogenous intertwined polysaccharied chains. In addition, incorporating Hemicellulose polysaccharides and various other sugars and

commonly embedded in lignin. The polysaccharides exhibit varying degrees of crystallinity that can be hindersome for an enzymatic attack, as the enzymes simply cannot reach the cellulose chains. The enzyme cocktails can also show activity to degrade hemicelluloses as the microorganisms used to produce the enzymes also commonly coproduce enzymes that are active on hemicelluloses as well and act that way depending on the purity of the enzyme cocktail. A novel approach to this task has also been investigated in this work using anaerobic fungi.

The novel aproach of the utilization of anaerobic fungi for the task of cellulose saccharification is also an interesting step towards a more efficent use of renewable energy containing material. Anaerobic fungi are tested for their potential as a novel microorganism group that simultaneously break up the cellulose containing materials and starts to hydrolyse the freed cellulose fibres. Thus providing glucose for a fermenation to platform chemicals.

3 Materials and Methods

3.1 Extrusion

Thermo-mechanical extrusion, also known as extrusion cooking as a pretreatment of cellulosic biomass, was investigated using pure cellulose and fibrex, fibre of sugar beet. The effect of different extrusion conditions of enzymatic digestibility of the base material can be determined by glucose yield obtained after enzymatic hydrolisation with cellulases. (S.-H. Lee et al. 2010)

3.1.1 Cellulosic materials and their preparation for extrusion

Cellulose (VWR GmbH) and Fibrex (Welding GmbH & Co) were extruded with starch (KMC GmbH) to aid the process, as the cellulosic materials were found not to be plastificating when extruded alone. Substrate preparations were mixed prior to extrusion in a rolling barrel mixer to provide a good mixing of the cellulose material and the starch that mediated product flow. The in-barrel-moisture of the mixture was adjusted in line in the process chamber of the extruder via an electrical governed pump (Dräger) to 20-30% (w/v) moisture content.

3.1.2 Technical specifications and monitoring of the double screw extruder

The thermo-mechanical-pressure pretreatment of the cellulose containing materials was performed using a laboratory scale twin-screw-extruder (Berstorff ZE 25) with a 2 diameter head plate, a screw diameter of 20 mm and a 2.4 mm circular opening.

The drive power at maximal screw speed was 8.6 kW and the drive torque per axel was 82Nm: , a maximal screw speed of 500 U/min could therefore be realised.

The process part of the extruder was composed of 5 body casings and the head plate, which also functioned as a die . Each body casing comprised an independently adjustable air cooling systematic and electric heat circuit, which were connected in series. Each body casing module could be heated and cooled independently.

The solids (matrix mixtures of different compositions) were added using a dosing scale (Brabender). The dosing accuracy was gravimetrically determined and showed a deviation of around 0.3-0.5% from the target value. Water dosage was performed by a digitally controlled pump (Dräger).

The product pressure in the screw chamber was measured inline using a pressure dynometer in the head-plate module (MDA 420-1/2-2C-15, Dynisco, USA) to monitor the extrudate flow.

The product (extrudant) temperature was measured with a Fe/Co-thermoelement (Coperion Werner & Pfleiderer, Stuttgart). Measurements of the product temperature were acquired inline before the die with a resistance thermometer (PT 100, Coperion Werner & Pfleiderer, Stuttgart).

The casing temperature was adjusted and displayed independently on the control panels of the extruder.

These measured extruder parameters and those for temperature and product pressure in the screw chamber were recorded manually.

The following extrusionparameter sets have been used in the experiments.

Extrusionconditions	Temperature	Moisture	RPM
EX1	100°C	20,00%	250
EX2	100°C	20,00%	350
EX3	100°C	20,00%	450
EX4	100°C	30,00%	250
EX5	100°C	30,00%	350
EX6	100°C	30,00%	450
Ex7	120°C	20,00%	250
Ex8	120°C	20,00%	350
Ex9	120°C	20,00%	450
Ex10	120°C	30,00%	250
Ex11	120°C	30,00%	350
Ex12	120°C	30,00%	450
Ex13	140°C	20,00%	250
Ex14	140°C	20,00%	350
Ex15	140°C	20,00%	450
Ex16	140°C	30,00%	250
Ex17	140°C	30,00%	350
Ex18	140°C	30,00%	450

Table 1: Extrusion parameter combinations

3.1.3 Operation parameters of the double screw extruder, and extrudant storage

All extrudants were produced with a constant mass flow of 8.5 kg/h. Changes of the substrate moisture content, when required, were adjusted by changing the dosing of the solids (cellulosic material ans starch) and the water online as changes in the respective dosing. Different screw speeds (250, 350 and 450 rpm) were tested with substrates prepared under different parameters (varying extruder barrel temperatures and in moisture combinations). Specific mechanical energy increased with screw speed, as expected. Temperature profiles with discharge or die temperatures of 100^{o}C, 120 ^{o}C and 140^{o}C, respectively, were used, and the feed rate was set to 8.8 kg/h using a Brabender automated feeder.

The extruded cellulose and fibrex mixtures were dried after extrusion in a convection oven at 70°C for 24h to reduce the water content. The dried extrudats

were ground and were stored at room temperature prior to enzymatic saccharification. Low amounts of added starch as process aid resulted in burning of the substrate or complete blocking of the extruder die or the extruder barrel as a whole.

3.2.4. Enzymatic saccharification

A combination of different cellulases (obtained from Sternenzyme) from a culture of *Penicillium janthinellum* and a culture of *Pyrococuus furiosus* in solid and as liquid state were used for enzymatic saccharification of the treated (extruded) and untreated cellulosic materials. The enzyme activity of the cellulase mix was 24 FPU and 27 FPU (filter paper unit / ml) for the solid and liquid states respectively. The cellulase mix had an optimal pH range of 4.6-6.0, and an optimal temperature range of 50-55°C.

The calculated optimum enzyme load was used in the subsequent enzymatic hydrolisation experiments. Treated (extruded) and untreated cellulose and fibrex mixtures were hydrolysed with different amounts of the enzyme cocktail in a 0.05M acetate buffer (pH 6,5). Incubation conditions were 50°C for 72 hours. Untreated substrate mixtures (including the equivalent amount of process aid starch) were used as a control, and were subjected to exactly the same enzymatic hydrolysis conditions.

3.1.4 Sugar analysis of the treated and untreated cellulosic materials

Glucose concentrations in the supernatant was analysed by high performance liquid chromatography HPLC (ERC GmbH, Riemerling, Germany) using a Nucleogel Ipn 300 OA colomn (Macherey-Nagel GmbH & co. KG, Düren, Germany). For the mobile phase sterile 5 mM H_2SO_4 was used at a flowrate of 0.4 ml/minat room temperature.

Glucose yields were expressed in terms of g/g substrate mixture. Degree of saccharification (C res %) was calculated based on the total cellulose content and added starch in the substrate mixtures as follows:

$$C\ res\ \% = Yrs/\ (c)*1.1\ *100$$

where ‘c’ was the amount of cellulose in 1 g of substrate mixture, 1.1 the conversion factor for polymer to monomer sugar and Yrs was the glucose yield.

Glucose is the main saccharid in cellulose, mannose and xylose on the other hand are the main saccharids in hemicellulose which makes it possible to distinguish between cellulose degradation and hemicellulose degradation.

As described before, cellulosefibers are composed of microfibrils that are sheathed with hemicellulose and cross linked with lignine along the cell walls. Pectin can also be found in plant material and can consist of arabinose, galactose, rhamnose fructose and uronic acid. The main origin of the released glucose are cellulose polymers, but released glucose originating from the process aid starch also needs to be accounted for as starch is a carbohydrate consisting of a large number of glucose units joined by glycosidic bonds (which will also be acted upon by the enzyme cocktail).

The yields were then calculated as 00,69 g /g material mixture for both pure cellulose and fibrex. The multiplication factor 1.1 accounts for water originating from the hydrolysis. Cellulases act directly on the cellulose in the material mixtures degrading it to glucose-molecules. Typical used enzyme loading have been reported to be applied in the range of 7 to 33 FPU / g Substrate, Sun and cheng 2002, and in this work the optimal enzyme load was assessed to be 31 FPU / g Substrate. Some inconsistency of the enzymatic saccharification could also be derived from the fact that the enzyme cocktail is also degrading hemicelluloses and glucose could also be derived from the starch used and hydrolysed from coenzymes from the culture supernatant of the supplied cellulases.

The correct concentration or amount of cellulases is of importance as an overdosage can lead to a reduced glucose yield. This is hypothesized due to the fact that a high initial glucose generation leads to product inhibition of the hydrolysis.

The prevention of overdosage of cellulases is also an important factor in cost reduction or control of the overall saccharification process as enzymes are one of biggest cost factors and overdosage would not only increase costs, but also inhibit cellulose saccharification. (Xiros et al. 2009; Bansal et al. 2009; Begum & Alimon n.d.; Anon 1982; Begum & Alimon n.d.; Corredor 2008)

3.2 Anaerobic Fungi

3.2.1 Isolation and culture methods of anaerobic fungi

The cultivation and isolation of anaerobic fungi can be a challenging task and requires specialised anaerobic methods. Most of the methods used today have been established by Hungate (Hungate 1969), and have been optimised over the years by different researchers (Miller & Wolin 1974; LOWE et al. 1985).

All anaerobic fungal cultivation techniques are carried out under anaerobic conditions, either with a carbon dioxide atmosphere or using a combination of hydrogen, nitrogen and carbon dioxide. In order to isolate anaerobic fungi from their natural environment, as well as faeces, a continuous set of subculturing and antibioti treatments with agar isolation techniques are needed. Various isolation techniques have been implemented to date. (France et al. 1990; Orpin 1977b; Milne et al. 1989; Bryant 1972a; LOWE et al. 1985; Finegold et al. 1969)

Orpin used agar media containing antibiotics to cultivate anaerobic fungi from rumen particles. The particles were immersed in the agar medium and after incubation, the top layer was removed allowing access to the subsequently developed vegetative fungal growth that originated from zoospores that had migrated into the medium. Single colonies were picked and transferred into a new agar medium containing antibiotics to further purify the culture. This step was repeated until a bacterial free pure culture was obtained.

The isolation process steps can also be performed using a previous enrichment of plant particles from the source sample (i.e. rumen content or faeces) in anaerobic media in order to enhance the chances of recovering anaerobic fungal populations. These enriched fungal cultures can then be transferred to agar medium containing cellulose. Alternatively, the enrichments can be used to prepare a series of dilutions, which are then used to inoculate roll tubes (an agar medium supplemented with antibiotics, with the agar solidified on the wall of the glass tubes). After incubation of the roll tubes, single colonies can be trans-

ferred to new medium containing antibiotics and cultivated. As a control step, selected colonies can be transferred into antibiotic free glucose containing media and incubated in order to test for remaining contaminating bacteria. In liquid media free swimming zoospores can be observed under a microscope after 48 hours. (Lowe et al. 1985; Finegold et al. 1969; Holdeman & Hungate 1972; Bryant 1972b)

3.3.2 Culture conditions

The gut ecosystems normally inhabited by anaerobic chytridiomycetes can be described as an open steady-state system with continuous input (feed) and output (digesta outflow). Recreating the properties of this anaerobic steady-state system on a lab scale is difficult and cost-intensive, which leads to the general practice of cultivating anaerobic fungi in batch culture. Culture media used varies depending on the cultivation purpose and the specific requirements of a desired species. In general however, a carbohydrate limited anaerobic media is used with plant biomass (like wheat straw or oat bran), cellulose or soluble carbohydrates (i.e. xylose or cellobiose) as a substrate.

Generally zoospore suspensions are used for inoculation, but it can be necessary to use rhizoid and colonised plant particles due to the fact that the polycentric anaerobe chytridomycetes are producing less zoospores then the monocentric counterparts. Batch fermentations (up to 20 L in volume), continuous flow fermenter and continuous or chemostatic fermentations have all been reported to be suitable for the propagation of the anaerobe chytridiomycetes (Jouany 1994; Anon 1991; Welch et al. 1996; Tsai & Calza 1993; Hungate 1969).

3.2.2 Cultures and culture conditions

Anaerobic Fungi were all acquired from the culture collection of the Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, Wales, UK. *Saccharomyces cerevisie* used was a commercially available dried baking yeast.

In this study, the cultivation of anaerobic fungi as mono and co-cultures (with *Saccharomyces cerevisiae*) was conducted following the established techniques of Hungate, Orpin and Callaghan (Callaghan 2014). Anaerobic medium was either dispensed into CO_2 flushed Hungate tubes (16 ml) or 250ml serum bottles and sealed with a butyl rubber septum held in place by a screw lid or metal crimp respectively and then autoclaved.

Prior to inoculation, sampling or suspension extraction the surface of the septum was sterilised. Media in Hungate tubes (10 ml) were inoculated with 0.5 ml zoospore suspension (or rhizoid suspension) or for serum bottles (100 ml) 2 ml of suspensions. All transfers and sampling was performed using medical grade sterile needles and syringes. The suspensions were generated from 72 h old cultures.

Saccharomyces cerevisiae cultures were grown over night (approx. 16 h) using the same media and conditions as the anaerobic fungal cultures. *S. cerevisiae* cells were counted microscopically using a counting chamber, and cellconcentrations adjusted to a concentration of 10^6 and 10^7 cells/ml. Co-cultures of anaerobic fungi and *S. cereivisiae* were simultaneously inoculated. To do this co-cultures were either started with a 24 hour incubated anaerobe chytridomycetes culture or a 24 hour incubated *Saccharomyces cerevisiae* culture. All incubations were conducted anaerobically at 39°- 40°C, and performed in triplicate.

3.2.3 Media composition

3.2.3.1 Full medium

Orpin as one of the pioneer researchers in the field of the anaerobe chytridiomycetes set the standard for the composition of the culture media. Most of the media used and described are incorporating clarified rumen fluid (CRF) and are labeled non-defined complex, full or environment simulating media. Trypton, Yeast extract, a carbon source (cellulose, cellobiose, glucose, xylan), L-cysteinhydrochlorid, natrium sulfite as a reducing agent, a pH-buffer for a pH

range from 6.5 to 6.9(carbondioxid-bicarbonat or phophatbuffer), haem, resazurin as oxygen indicator and vitamins as well as some trace elements are the other most defining materials. In order to prevent contamination with bacteria or when the media is used to isolate anaerobic chytridiomycetes, antibiotica such as penicillin, ampicillin, streptomycin and chloramphenicol (which inhibits methanogen bacteria) can be added as the shorter generation interval of the bacteria would lead to an overgrow of the fungi. The choice of carbohydrate source is based on the intended use of the medium. Cellobiose is commonly used for general purposes as it allows for a good visible and optical measurable liquid phase as well as stable cultures, insoluble cellulose is used in the isolation of fungi as the dissolving cellulose fraction indicates the activity of cellulytical microorganism, glucose allows for a more accurate quantification of a culture than cellobiose and is reported to yield a higher biomass yield but fungal cultures have to be subcultured 24- 72 hours to be kept alive and cellulosic biomass / plant material such as wheat straw and oat bran propagate and conserve in vitro the cellulytical activity of anaerobic chytridomycetes and allow batch cultures to be viable for 7-10 days (Dehority & Orpin 1997; Dehority 1997; Li et al. 1997; Orpin 1975; Trinci et al. 1994; Teunissen et al. 1991; Yarlett et al. 1986).

The full medium used in this study is based on the medium by Orpin (1977) and Davies (1993) (Davies et al. 1993). The following components were used per litre: 150 ml salts solution 1 (3 $g.L^{-1}$ K_2HP0_4 in distilled water), 150 ml salts solution 2 (3 g KH_2PO_4, 6 g $(NH_4)_2SO_4$, 6 g NaCl, 0.6 g $MgSO_4.7H_20$ and 0.6 g $CaCl_2.H_2O$ dissolved in 1 L of distilled water (in the order listed), yeast extract (3 g; Oxoid), tryptone (10 g; Fisher) and 0.1% (w/v) resazurin solution (1 ml) were then added and the solution made up to 850 ml with distilled water. After boiling (until light red in colour) and cooling the following was added: 150 ml clarified rumen fluid, 6 g $NaHCO_3$, 1 g L-cysteine-HCl and 5 g of a carbon source. The solution was then simultaneously mixed and deoxygenated by gassing with CO_2 for 1 hr at room temperature (Callaghan 2014).

Carbon sources used in medium preparation included: cellobiose D (+98% purity, Acros Organics); dry wheat straw; fibrous cellulose powder (Whatman); glucose (LabM); soluble xylan. Dry wheat straw was prepared by milling through a 2 mm sieve followed by further sieving to remove excess wheat dust. Soluble

xylan (~4g) was prepared as follows: 10 g of xylan (xylan from beechwood, Apollo Scientific) was dissolved in 200 ml dH_20, adjusted to pH 10 using 1 M NaOH, incubated at room temperature for 1 hr and centrifuged for 10 min at 10,000x g. The supernatant (containing soluble xylan) was decanted and pH neutralised using glacial acetic acid, and a representative portion was freeze dried for quantification of mg/ml soluble xylan. Liquid soluble xylan was then added to the media in the appropriate volume before heating.

Standard media contained 0.5 % (w/v) of a stated individual carbon source, and an adapted media termed 'wheat straw enrichment media' was also used containing 0.5% (w/v)dry wheat straw, 0.2% (w/v) cellobiose and 0.2% (w/v) soluble xylan. For roll tubes, wheat straw could not be used an 'enrichment substrate', therefore 0.3% (w/v) cellobiose and 0.3% (w/v) soluble xylan was used instead.

Rumen fluid was collected under PPL 40/3579 (Dairy Cow Licence) from the rumen of cannulated cows at Trawscoed farm (Aberystwyth University, Wales, U.K.). Clarified rumen fluid was prepared as follows: rumen solids were removed, the liquid squeezed into a bucket and then filtered through a muslin cloth. Filtered rumen fluid was then centrifuged in 150 ml bottles at 12,000 g for 10 min (with the brake off) using a Sorvall RC-5B Refrigerated Superspeed Centrifuge (Du Pont Instruments). The clarified rumen fluid was then decanted (leaving the pellet behind), and was autoclaved (121ºC/15 min) before being stored at -20°C for later use in media preparation (Callaghan 2014).

Prior to dispensing the media, the pH was checked using a Digital pH meter (Hach H170), and adjusted to pH 6.9 using 5 M NaOH). The medium was dispensed either in 10 ml aliquots into Hungate tubes under anaerobic conditions or in larger volumes into serum bottles using a Perimatic™ GP Pump Dispenser (Jencons). Tubes and bottles were then capped with a polypropylene bung held in place by a screw cap or an aluminium crimp and autoclaved (121ºC/15 min).

3.2.3.2 Rumen fluid free medium

Lowe (1985) developed a defined medium (Medium B) that does not require clarified rumen fluid as a component. Medium B essentially uses many of the

same key ingredients as the complex medium (see 3.2.3.1) but with a higher concentration of minerals, salts and organic and non-organic sources of nitrogen. Cultures grown in Medium B were shown to exhibit the same growth characteristics as cultures cultivated in complex media (Lowe (1985)). In this study, a modified form of Medium B was devised in order to allow for a standardised assessment of the growth and cellulose utilisation of anaerobic fungi.

The rumen fluid free standardised (RFFS) medium was prepared in the following manner. The following components were used per L of medium: 150 ml of salts solution 1 (3 gL^{-1} K_2HP0_4 in distilled water), 150 ml salts solution 2 (3 g KH_2PO_4, 6 g $(NH_4)_2SO_4$, 6 g NaCl, 0.6 g $MgSO_4.7H_20$ and 0.6 g $CaCl_2.H_2O$ dissolved in 1 L distilled water (in the order listed)), yeast extract (3 g; Oxoid), tryptone (10 g; Fisher) and 0.1% (w/v) resazurin solution (1 ml) were mixed and made up to 850 ml with distilled water. After boiling (until light red in colour) and cooling, 10 ml of trace element solution, 6 g $NaHCO_3$,, 1 g L-cysteine-HCl and a carbon source were added and deoxygenated by gassing with CO_2 for 1 h. The trace element solution was prepared in 0.2 M-HCl and contained (g L-1): MnCI.4H2O, 0.25; NiCl.6H20, 0.25; NaMo0, 2H,O, 0.25; H3B03, 0.25; FeSO, 7H,O, 0.20; CoCl, .6Hz0, 0-05; SeO, 0.05; NaVO 4H20, 0.05; ZnCI, 0.025; CuCl. 2H20, 0.025)

3.2.3.3 Cryomedium

Fungi can be conserved cryogenetically by using a medium containing a cryoprotectant (such as dimethyl sulfoxide) and using controlled freezing and storage in liquid nitrogen. For cryopreservation of cultures, a method based on the work of Sakurada *et al*. (1995) and Yarlett *et al*. (1986) was used.

A 5 times concentrated cryopreservation solution was prepared by mixing 49.7 g ethylene glycol with 155 ml clarified rumen fluid, 200 µl of 0.1% (w.v) resazurin, 0.2 g L-cysteine and 1.2 g $NaHCO_3$ under anaerobic conditions (total volume 200ml; 3.2 M ethylene glycol). This solution was rendered anaerobic using deoxygenated CO_2, prior to dispensing in 10 ml aliquots in Hungate tubes and storage at -20°C. The 5 times cryopreservation solution was added to 3-5 d old wheat straw cultures (2.5 ml per 10 ml culture) under anaerobic conditions in

Hungate tubes. After mixing, tubes were chilled in ice water for 15 min and dispensed in 2 ml aliquots into sterile 2 ml cryovials under anaerobic conditions. Cryovials were placed at -80ºC overnight and then transferred to liquid nitrogen. Storage of cryopreserved cultures at -80ºC is possible for up to a few months but for prolonged storage, liquid nitrogen was found to be more reliable.

Resuscitation of vials was carried out as follows. Media was prewarmed with antibiotics (section 3.3.3.4) added to prevent bacterial contamination. The vial was then removed from the liquid nitrogen and placed in a water bath at 39°C. Once thawed the vial was quickly opened and poured into the pre-warmed media under CO_2. The media was then recapped and incubated at 39°C (Callaghan 2014).

3.2.3.4 Antibiotics and other preparations

Anaerobic fungi are resistant to the following antibiotics: Avoparcin, Bacitracin, Penicillin, Rifampicin, Streptomycin, Tylosin and Virginiamycin. These compounds can therefore be used for anaerobic fungal selective media or to inhibit bacterial contamination of axenic cultures. Fungicides like Nystatin, Amphotericin B and sterol synthase inhibitors have no effect on anaerobic fungi. In contrast anaerobic fungi are highly sensitive to eukaryiotic biocides like cycloheximide, and fungicides like Nikkomycin. Ionophores like Monensin can also have an inhibiting effect but it has been reported that a resistance can be developed by anaerobic fungi. (Marounek & Hodrova 1989; Gordon & Phillips 1998b; Hobson & Stewart 1997; Orpin 1975)

An antibiotic cocktail was used to inhibit bacterial growth in anaerobic fungal cultures, and to reduce the risk of contamination associated with antibiotic resistance. A stock solution containing 10 mg/ml each of penicillin G, ampicillin and streptomycin sulphate was prepared in 50% (v/v) ethanol. This was filter-sterilized using a syringe attached to a 0.22µm filter unit (Millex GP). Antibiotics were used at 1% (v/v) in the final media (X ug/ml final concentration) and were stored at -20°C with repeated freeze thawing avoided.

A 0.05% hemin solution was prepared 1:1 in Ethanol: 0.05M NaOH and a resazurin solution was prepared 0.1%w/v in distilled water both were filter sterilized as above .

3.2.4 Control parameters during culture growth and assessment of culture condition.

Enumeration and general assessmenta of anaerobic fungal cultures is challenging as standard counting and assessment techniques cannot easily be applied and/or adapted. This is due to the mobile and static stages of the life cycle, and growth characteristics of the vegetative phase. The number of motile zoospores in a batch culture can fluctuate, and the vegetative thalli grow on and within plant particles. In addition, biomass quantity and form can vary depending on the age of the culture, making enumeration difficult and inaccurate. Inaccuracy in counting can also originate from zoospores that are bound to plant particles or detached sporangia, as well as the difficulty in distinguishing zoospores from thalli when using thallus forming units to enumerate. (Marano et al. 2012; Akin et al. 1983; S E Lowe, Theodorou & A. P. Trinci 1987b; Susan E Lowe et al. 1987; Lowe et al. 1987).

To assess the growth kinetics of cultures grown with insoluble substrate (like cellulose) or wheat bran, a quantitative measuring of gaseous or liquid fermentations products can be of interest, however this approach involves alteration or destruction of the culture via the sampling method. It is also possible, however, to assess the growth or fermentation kinetics via the transducer method developed by Theodorou et al (1994 1996). Using a pressure transducer, the gas pressure in the batch culture is measured and released (returning it to ambient pressure) to allow measurements to be made at different time intervals. The correlation between gas production and fungal biomass growth has been shown to be a useful indicator for the growth kinetics of anaerobic fungal cultures. *N. hurleyensis* showed a biomass doubling time of 12.4 hours and the culture entered a stationary phase of gas production 84 hours after inoculation. An accurate and direct biomass enumeration method is still to be implemented however, and current techniques show a trend rather than an actual stable result (Hobson & Stewart 1997; Akin 1986; Theodorou et al. 1995; Coleman 1978; Theodorou 1990; Hobson & Summers 1967; Hobson & Stewart 1997)

In order to assess the feasibility of the rumen free medium as well as the growth effectiveness of the different co-cultures and media, two different approaches were used: the non-destructive gas production method and fermentation end-point sampling of the culture medium for volatile fatty acids production. The gas pressure transducer (Datatrack, Process Instruments. Tracker 200) had a pre-sterilised 23G needle attached and the rubber septum on the culture bottles was flame sterilised prior to inserting the needle. Gas pressure readings were displayed (in PSI) on the digital display.

3.3 Monitoring of anaerobic fungal growth

In order to assess the feasibility of the rumen free medium as well as the growth effectiveness of the different co-cultures and media, two different approaches were used to monitor anaerobic fungal growth: the non-destructive gas production method and fermentation end-point sampling of the culture medium for volatile fatty acids production.

3.3.1 Gas pressure

Generally, the utilization of cellulosic substrates by anaerobic fungi in batch culture has been assessed by the quantification of soluble and gaseous end products of fermentation and/or quantification of dry matter loss (Akin 1983; Lowe 1987; Mountfort 1982; Mountfort and Asher 1983).

These approaches involve the discontinuation of the culture due to destructive sampling and require the use of many replicate cultures. The quantification of dry matter can be inaccurate at the beginning of the fermentation stages as weight changes are small. Overall, the build-up of adherent and intrusive fungal biomass on the degradable substrates also complicates accurate measuring as it requires a washing step of the substrate which can also lead to a wash out of substrate, therefore impeding a reliable and accurate assessment of amount of substrate utilised.

As an alternative a non-destructive sampling method, the measurement of the gas accumulation and production of anaerobic cultures using pressure and vol-

volumetric sensing instruments has been proposed. The gas accumulation can be seen as an indicator for carbon metabolism and cellulosic substrate degradation in the proposed method. In this work, this approach was applied using a pressure transducer with an attached syringe for gas volume measurement. The transducer was used to assess the culture headspace gas pressures and the associated volumes of gas produced during the batch fermentations. (Michael K Theodorou et al. 1995; Davies et al. 2000; Mountfort 1987; Kongmun et al. 2010)

Cultures were incubated without agitation under 100% CO_2 atmosphere and measured every day. Cultures were sealed with rubber septa held tightly in places with aluminium crimp seals. A pressure transducer with a digital readout and an attached syringe for volumetric measurement and gas release was used in this work. The syringe was connected with to a Luer-lock three-way stopcock that was fitted on the culture sampling end with a disposable hypodermic syringe needle. For sampling, a small number of cultures were taken out of the incubator to assure ambient temperature conditions throughout sampling and prevention of temperature drops as well as having a unaltered volume of gas in the headspace. The top of the cultures were sterilised prior to triplicate sampling. The hypodermic syringe needle was inserted through the septum and head space gas pressure was read from the LED display and gas volume was assessed via volumetric measurement of the headspace gas displaced in the syringe barrel until ambient head space gas pressure was displayed on the LED display. After measurement, the transducer was removed from the culture and measured head space gas discarded. Variations in samplings can be attributed to differences in media dispensation and small variations of manufactured tubes and bottles.

As culture gas production is closely related to cellulosic substrate utilization, this factor was assumed to be the main source of the gases produced by the anaerobic fungi. Other factors can contribute to differences in the amount of gas production however: the concentration and type of growth inhibiting media components used, type of microorganism used (single or co-culture in the case of this study) and the rate of growth (Beuvink & Spoelstra 1992; Theodorou et al. 1994). If these factors are taken into account, the assessment of the gas production can be used as a rapid and non-destructive primer for culture growth

and substrate utilization (Mountfort & Asher 1983). Another factor or positive side effect to for this method of measurement is that the culture are vented periodically preventing a build up gases in the headspace of gases in the media liquid that can have an inhibiting effect on growth. Hydrogen for example is removed from the batch culture, which has been shown to have a positive effect of growth rate of the culture. A lower hydrogen partial pressure allows for a re-oxidation of cofactors like NADH and production of electron-sink products, ATP production for example is increased which in turn provides more energy for biomass growth and metabolic activity. (Bauchop & Douglas O Mountfort 1981; Nielsen 2002; Davies et al. 2000; Theodorou et al. 1994; Theodorou et al. 1996b)

A gas pressure transducer (Datatrack, Process Instruments. Tracker200) with a pre-sterilised 23G needle attached was used to monitor the pressure of the accumulated gas in the culture bottle headspace over a 96 h incubation perios. The rubber septum on the culture bottles was flame sterilised prior to inserting the needle. Gas pressure readings were displayed (in PSI) on the digital display.The gas pressure of the cultures was measured 12, 24, 36, 48, 72 hours after inoculation, and the excess gas pressure released (returning the headspace pressure to ambient conditions).

3.3.2 Analysis of fermentation end products

The fermentation end products acetic acid, propionic acid, butyric acid, valeric acid, caproic acid were analysed via gas chromatography following the method originally devised by Jouany (Cardozo et al. 2004; Ahring et al. 1995; Playne 1985; Fussell & McCalley 1987).

4 Results

4.1 Extrusion

4.1.1 Cellulose and Fibrex mixtures

As previously discussed (section 1.2.5), extrusion is a widely understood and well-researched process, which is applied mainly in the plastic and food industries. In these industries, extrusion process parameters are constantly optimized and improved. In contrast, extrusion of cellulosic material has not been widely applied or researched as a pretreatment method. The main reason for this is the recalcitrant nature of the cellulosic material presents a challenging and complex task. This could also be observed in this study (Table 2).

Process aid %	Cellulose	Fibrex
0	-	-
10	-	-
20	+	-
30	+	+

Table 2: Summary of the ability to extrude different cellulosic material. Cellulose and fibrex were tested alone or mixed with different amounts of process aid starch, and the ability to extrude the mixture assessed as either being successful (+) or unsuccessful (-).

Pure cellulose and fibrex could not be extruded. This was due to the poor flow properties of the mixture in the barrel and the die, which lead to frequent blockages. This also increased the danger of machine damage as well as expensive repair and replacement costs. As a consequence process aid starch was used, which melts and forms a viscous matrix, which incorporates the cellulosic materials, and facilitates the conveying as well as incorporation of the extruder specific unit operations (namely shearing, mixing and pressure treatment). The use

of 10 % process aid starch did not improve the flow properties but higher amounts did (Table 2), particularly for the 30% amount which gave consistent success for both types of cellulosic material tested (Table 2). This was not the case for the 20% amount, which was only successful for the cellulose. Due to these findings only the following mixtures could be further assessed: 80% cellulose and 20% starch (C8S2); 70% cellulose and 30% starch (C7S3); 70% fibrex and 30% starch (F7S3).

4.1.2 Optimization of extrusion parameters

The effect of different combinations of extrusion parameters on the following three cellulosic mixtures was assessed: C8S2C, C7S3 and F7S3. In order to quantify the effectiveness of the different parameters, conversion efficiency of cellulose to glucose (based on glucose yields obtained after enzymatic hydrolysation of the extruded cellulosic mixtures) were compared.

All extrusion pretreatments led to increased glucose yield and conversion efficiency relative to the non-extruded cellulosic mixtures. After subtracting glucose yields originating from the process aid starch, and calculating back to 100% of the cellulosic material, conversion efficiency of the cellulosic material to glucose ranged from 24% - 53% (Figure 5). With respect to the comparison of the different cellulosic mixtures, many of the extrusion treatments had the same effect on the conversion efficiency of the different mixtures (Figure 5). This was also true for the optimal combination of extrusion parameters: 120°C / 30% / 350 rpm (Ex11). In contrast, large differences between one or more of the mixtures was always seen with the 140^{o}C barrel temperature and 30 % moisture content, irrespective of the screw speed.

In general the 450 rpm screw speed setting resulted in lower conversion efficiency relative to the other two slower speeds (250 & 350 rpm) tested (Figure 5). For example comparison of samples treated with 250 rpm and 450 rpm showed on average a decrease of conversion by 20-21% for all the cellulosic mixtures. The higher screw speed effectively shortened the retention time of the mixture in the barrel, resulting in the material also having a shorter heat

treatment. The effect of the barrel temperature itself on conversion efficiency however appeared to be complex, and influenced to some extent by the amount of moisture used. For example while 120^{o}C always gave the highest conversion within each screw speed setting tested, the optimal value was only obtained in combination with 30% moisture.

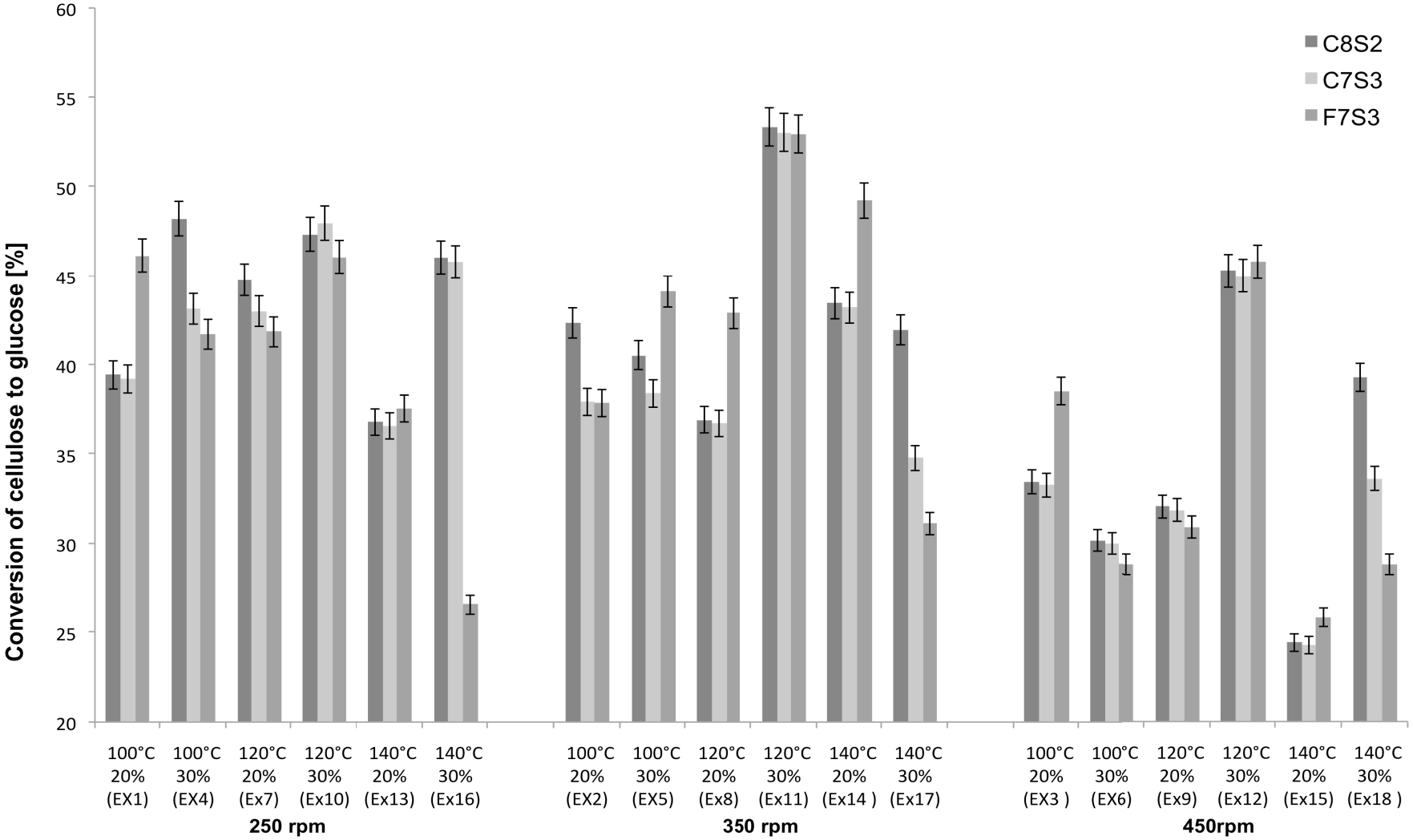


Figure 5: Effect of different extrusion treatments on the conversion efficiency of the cellulosic mixtures to glucose after enzymatic saccharification of the pretreated mixture. Three different cellulosic mixtures were assessed: C8S2 (cellulose 80% and starch 20%), C7S3 (cellulose 70% and starch 30%) and F7S3 (Fibrex 70% and starch 30%). Extrusion treatments were conducted using different combinations of the following parameters: extruder barrel temperature (100°C, 120°C, 140°C), screw speed (250rpm, 350rpm, 450rpm) and moisture content (20%, 30%). The extrusion treatments shown are grouped by screw speed. Corresponding treatment variables are shown in front of the experiment classification (Ex1-Ex18). Data presented are mean values (n=3), and error bars repesent the standard error of the mean.

4.2 Anaerobic Fungi

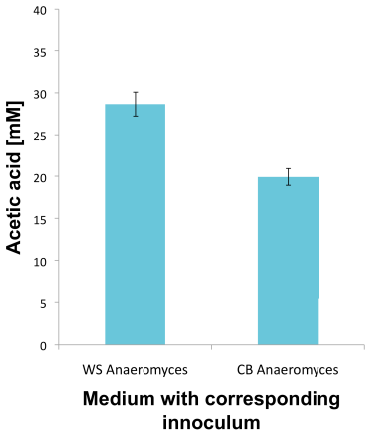
Anaerobic fungal fermentation of pretreated substrates can be an interesting alternative for the production of platform chemicals compared to enzymatic hydrolysis and subsequent fermentation. In order to develop baseline knowledge about the fermentation properties of a morphologically diverse range of anaerobic fungi for this study, the ability of five representative cultures to ferment wheat straw, filter paper and cellobiose in a widely used cultivation medium as well as a new medium without rumen fluid was assessed. Co-culture behavior with *Saccharomyces cerevisiae* and other anaerobic fungi co-cultures was then asessed in order to optimise culture growth and performance for potential usage in a consolidated bioprocess.

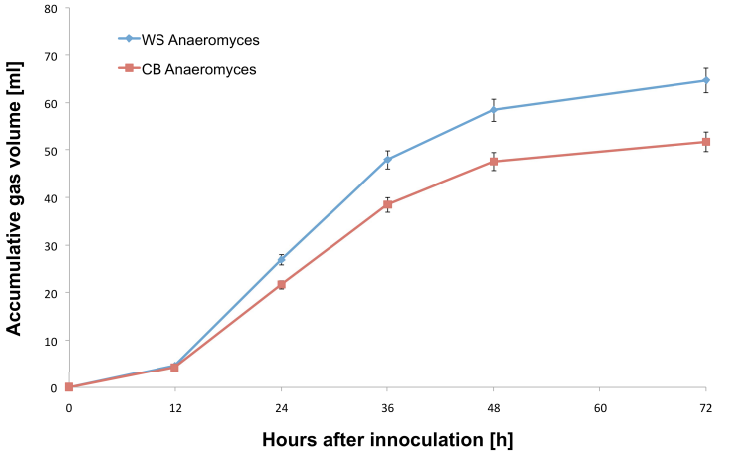
All fungal cultures selected for this study were known to be able to utilize cellulose containing biomass or pure cellulose. Growth dynamics and fermentation of the mono- and co-cultures was monitored using a gas production technique. Gas production has been correlated with fiber degradation (Theodorou et al. 1995), and is used in this study to assess if the cellulolytic activity of the anaerobic fungi could be improved by the presence of other anaerobic fungi and/or *Saccharomyces cerevisiae*. (Edwards, Kingston-Smith, Jimenez, S. a Huws, et al. 2008; Mauricio et al. 1999). In addition to monitoring gas production, the volatile fatty acids (VFA) at the end of the fermentation were also assessed. Although total VFA was measured, the anaerobic fungal cultures primarily produced acetate as also previously reported. Therefore only acetate is reported, in this study, relative to the corresponding gas production data.

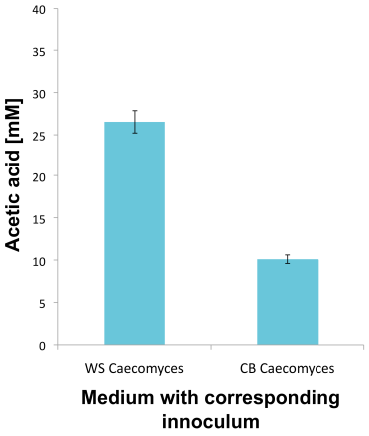
4.2.1 Comparison of Anaerobic Fungal monocultures grown in complex media

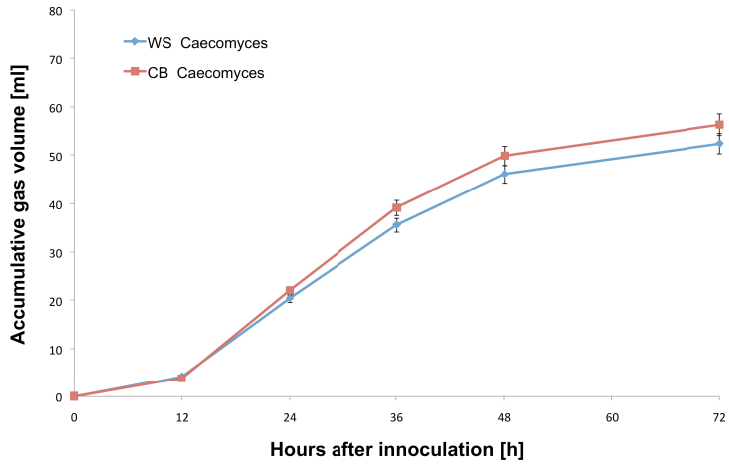
The fermentation properties of five representative anaerobic fungal monocultures in terms of their ability to utilise wheat straw and cellobiose in a widely used cultivation medium were assessed. Cultures exhibited similar accumulative gas production dynamics in complex medium with added wheat straw (WS) and added cellobiose (CB) (Figure 6). Fermentations with *Neocallimastix* showed elevated total gas production compared to the total gas production of *Anaeromyces* and *Orpinomyces* (by 16% and 18% respectively on average) as well as Caecomyces and Piromyces (by 25% and 29% respectively on average). Fermentations with *Caecomyces* and *Piromyces* showed the lowest gas production. *Anaeromyces* and *Neocallimastix* exhibited higher accumulative gas volumes (by 25% and 8% respectively) on WS than on CB. *Caecomyces* and *Orpinomyces* on the other hand showed higher accumulative gas volumes (by 7%) on CB than on WS. *Pirmoyces* exhibited similar gas production on CB and WS. Of note is that *Anaeromyces* produced a lot less gas with cellobiose relative to wheat straw - unlike all the other cultures.

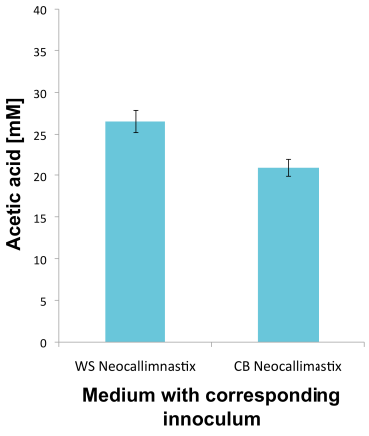
A similar trend could not be observed for acetate fermentation end product (Figure 6). Cultures of *Anaeromyces, Caecomyces, Neocallimastix, Orpinomyces* and *Piromyces* consistently exhibited higher acetate concentration in complex medium WS than in CB of the respective culture. *Caecomyces* showed the biggest difference in acetate concentration in complex medium WS compared to CB. Acetate concentrations in CB were proportionally much lower than the WS, compared to the corresponding total gas production.

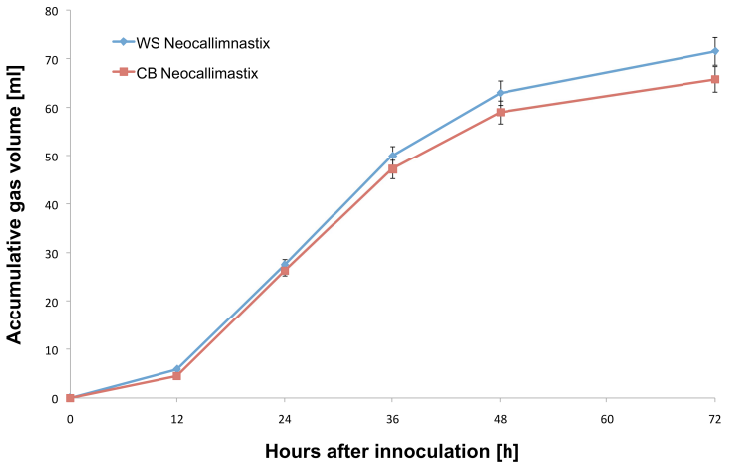

Acetic acid [mM]
40
35
30
25
20
15
10
5
0
WS Anaeromyces
CB Anaeromyces
Medium with corresponding innoculum


Accumulative gas volume [ml]
80
70
60
50
40
30
20
10
0
WS Anaeromyces
CB Anaeromyces
0
12
24
36
48
60
72
Hours after innoculation [h]


Acetic acid [mM]
40
35
30
25
20
15
10
5
0
WS Caecomyces
CB Caecomyces
Medium with corresponding innoculum


Accumulative gas volume [ml]
80
70
60
50
40
30
20
10
0
WS Caecomyces
CB Caecomyces
0
12
24
36
48
60
72
Hours after innoculation [h]


Acetic acid [mM]
40
35
30
25
20
15
10
5
0
WS Neocallimnastix
CB Neocallimastix
Medium with corresponding innoculum


Accumulative gas volume [ml]
80
70
60
50
40
30
20
10
0
WS Neocallimnastix
CB Neocallimastix
0
12
24
36
48
60
72
Hours after innoculation [h]

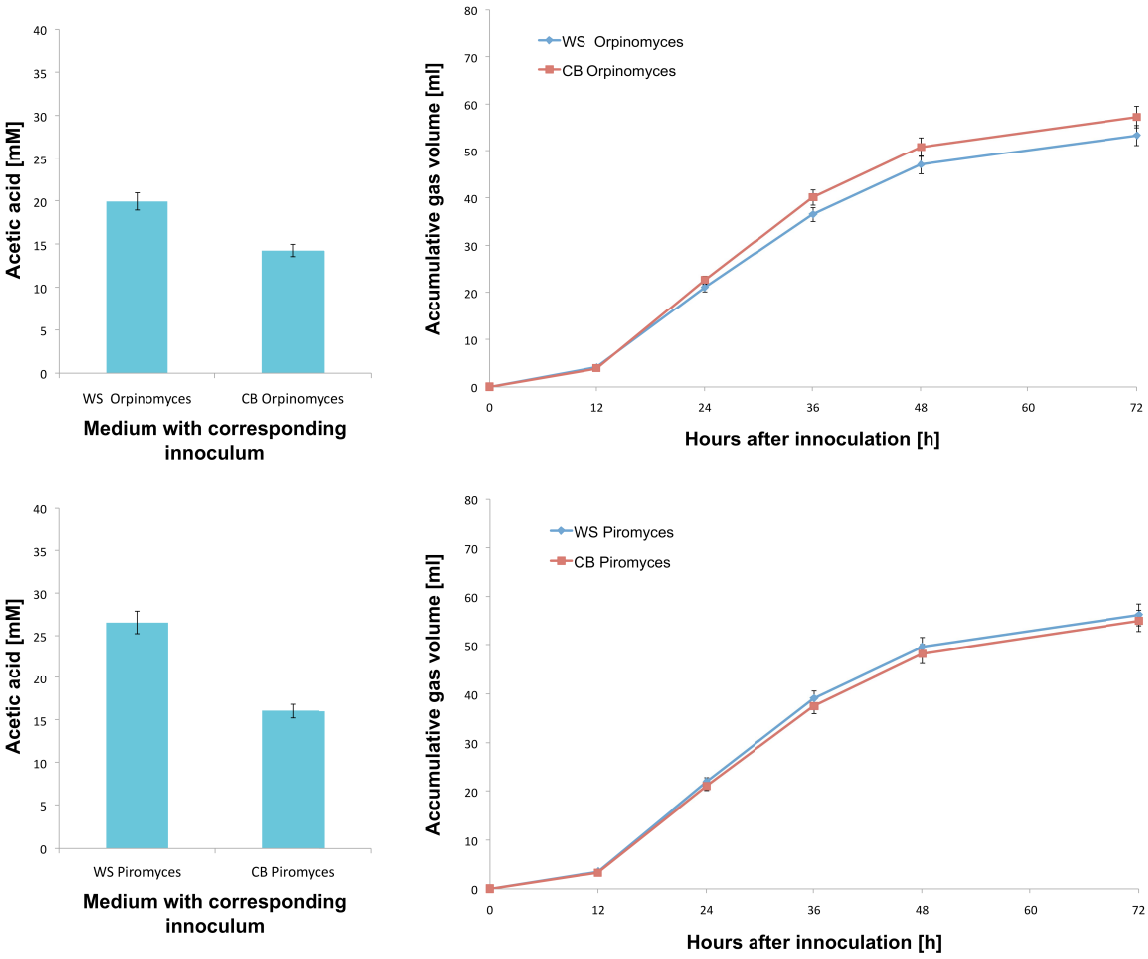


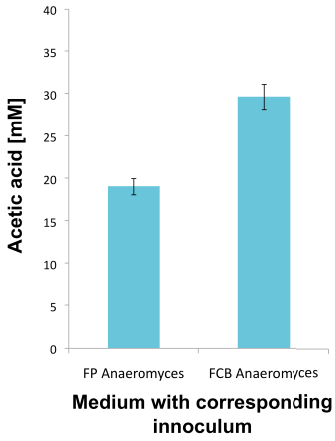
Figure 6: Comparison of anaerobic fungal monocultures grown in complex medium with either wheat straw (WS) or cellobiose (CB) as a substrate. Left column of figure showing acetic acid content at end of fermentation. Right column showing the accumulative gas production of corresponding cultures. From top to bottom Anaerobic fungi *Anaeromyces, Caecomyces, Neocallimastix, Orpinomyces* and *Piromyces* are the innoculi in the fermentations respectively. Values shown are mean values (n=3).

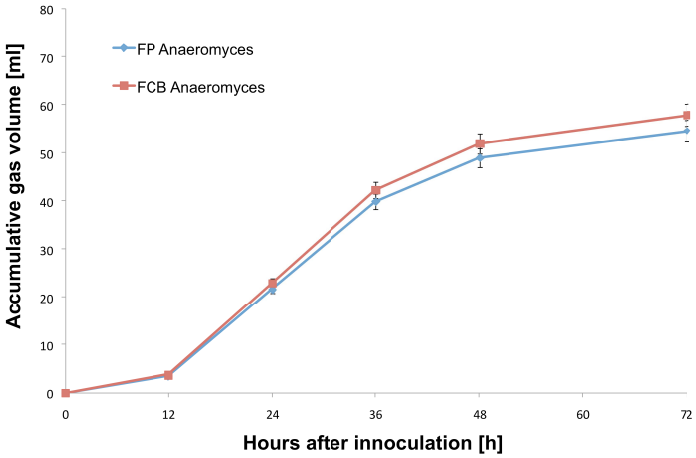
4.2.2 Comparison of Anaerobic Fungal monocultures grown in standardized rumen-fluid free media with different substrates

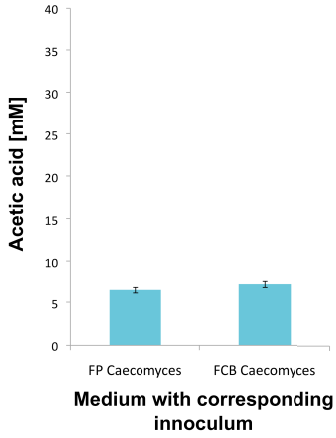
The fermentation properties of five representative anaerobic fungal monocultures to utilise filter paper and cellobiose in a new ucultivation medium without the addition of rumen fluid were assessed.Cultures exhibited comparable accumulative gas production dynamics in rumen fluid free standardised medium with added filter paper (FP) and added cellobiose (FCB) (Figure 7). Fermentations with *Neocallimastix* showed elevated accumulative gas production compared to the accumulative gas production of *Anaeromyces, Caecomyces, Orpinomyces and Piromyces* (by 10%, 20%, 17% and 19% respectively on average) in FP as well as in FCB. Fermentations with *Caecomyces* showed the lowest gas production.

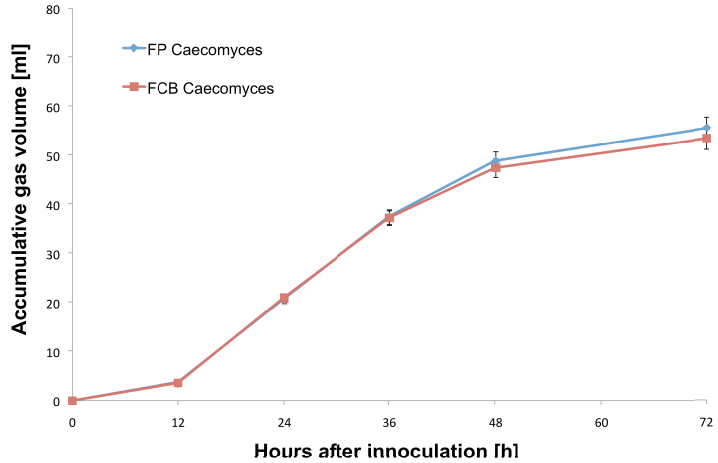
Cultures of *Anaeromyces* and *Piromyces* exhibited similar acetate concentration in rumen fluid free standardised medium with added filter paper (FP) and compared to the low alike amounts in *Caecomyces, Neocallimastix and Orpinomyces* substantial higher amounts of acetic acid. On the contrary only *Anaeromyces* showed a significant higher concentration of acetate in FCB compared to acetate concentration of other fungal cultures.

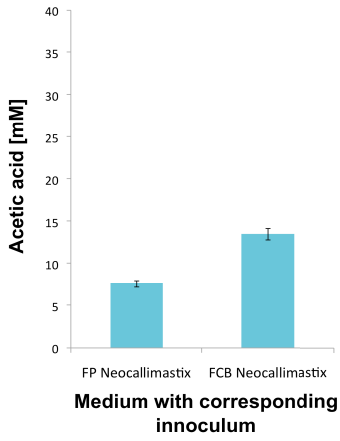
Aneromyces, Neocallimastix and *Orpinomyces* produced more acetate in FCB relative to FP, in contrast to *Piromyces*, which produced more acetate in FP relative to the FCB. Unlike with the gas production, only *Caecomyces* showed similar amounts of acetate in both FP and FCB.

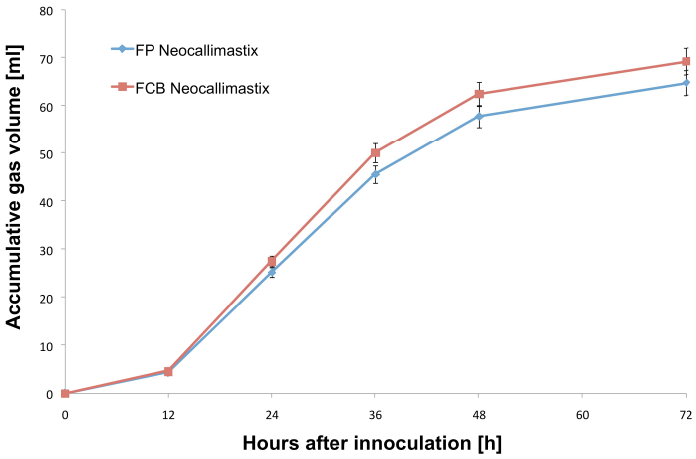

Acetic acid [mM]
40
35
30
25
20
15
10
5
0
FP Anaeromyces
FCB Anaeromyces
Medium with corresponding innoculum


Accumulative gas volume [ml]
80
70
60
50
40
30
20
10
0
FP Anaeromyces
FCB Anaeromyces
0
12
24
36
48
60
72
Hours after innoculation [h]


Acetic acid [mM]
40
35
30
25
20
15
10
5
0
FP Caecomyces
FCB Caecomyces
Medium with corresponding innoculum


Accumulative gas volume [ml]
80
70
60
50
40
30
20
10
0
FP Caecomyces
FCB Caecomyces
0
12
24
36
48
60
72
Hours after innoculation [h]


Acetic acid [mM]
40
35
30
25
20
15
10
5
0
FP Neocallimastix
FCB Neocallimastix
Medium with corresponding innoculum


Accumulative gas volume [ml]
80
70
60
50
40
30
20
10
0
FP Neocallimastix
FCB Neocallimastix
0
12
24
36
48
60
72
Hours after innoculation [h]

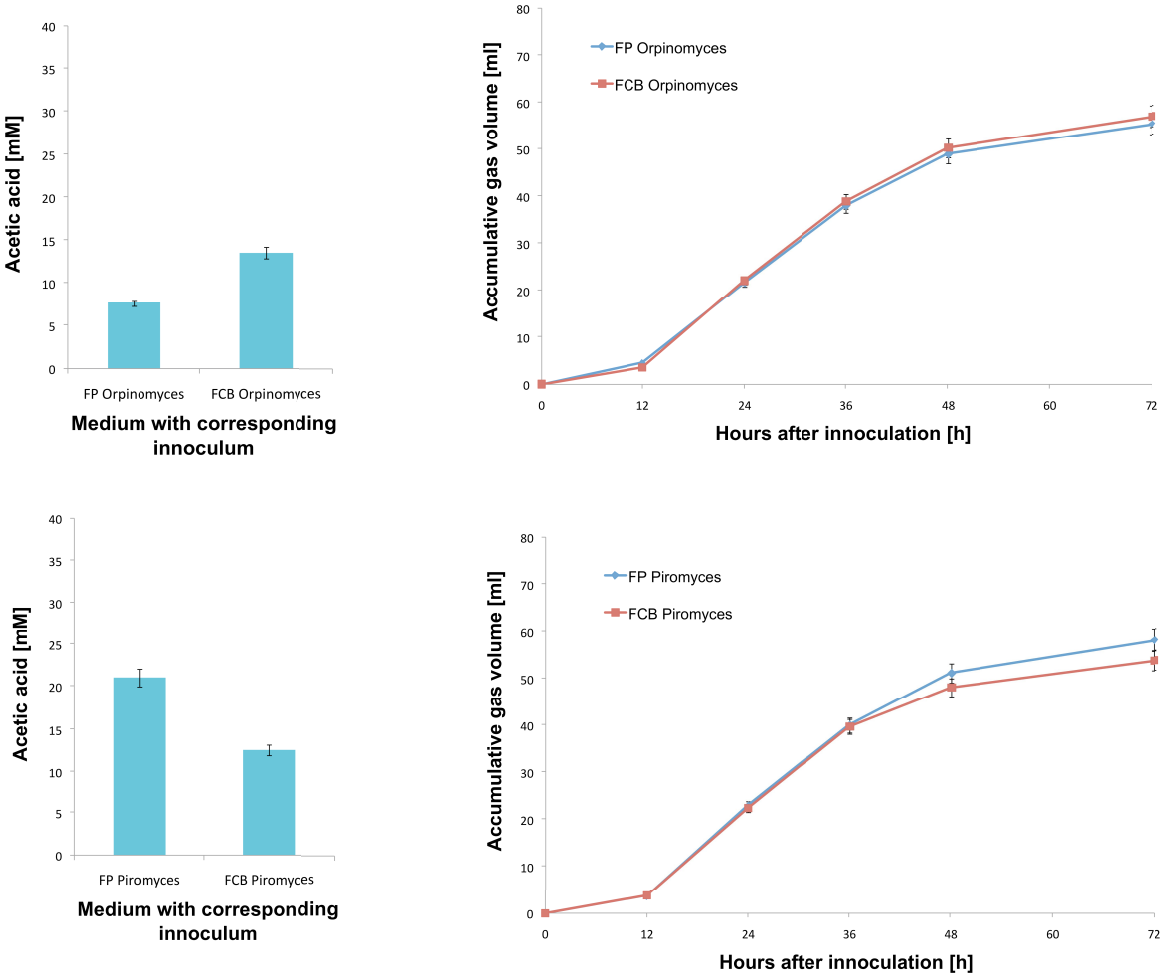


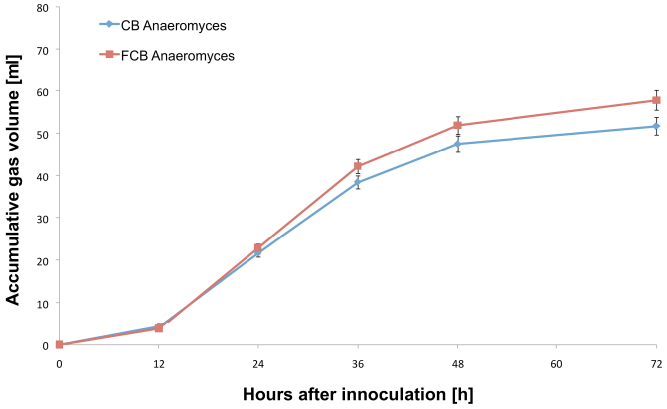
Figure 7: Comparison of anaerobic fungal monocultures grown in rumen fluid free standardized medium with either filter paper (FP) or cellobiose (FCB) as a substrate. Left colum of figure showing acetic acid content at end of fermentation. Right column showing the accumulative gas production of the corresponding cultures. From top to bottom Anaerobic fungi *Anaeromyces, Caecomyces, Neocallimastix, Orpinomyces* and *Piromyces* are the innoculi in the fermentations respectively. Values shown are mean values (n=3).

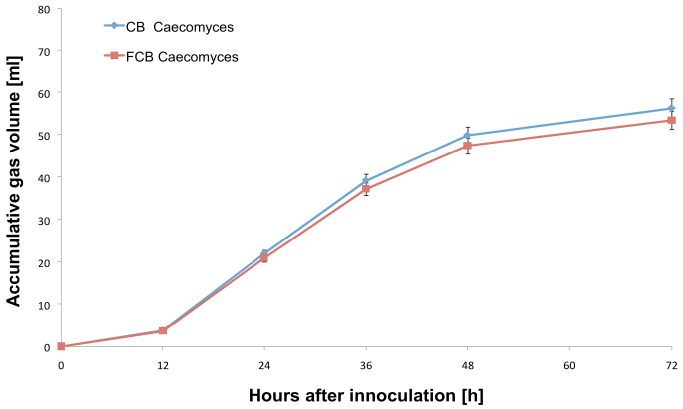
4.2.3 Comparison of complex media and rumen fluid free standardized media

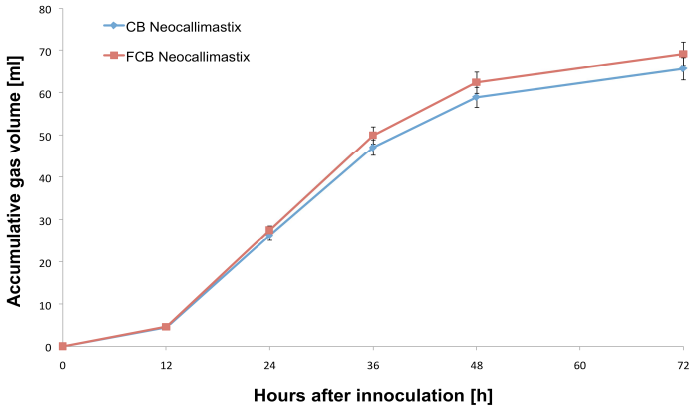
In order to assess if the anaerobic fungal fermentations properties were comparable in a standardised environment, the complex medium with rumen fluid was compared to the rumen fluid free standardised medium. Cellobiose was used as a substrate for both types of media.
Cultures exhibited similar dynamics and total amounts of gas productions for complex medium (CB) and rumen fluid free standardised medium FCB, with the exception of *Anaeromyces* (Figure 8 and Figure 9). *Anaeromyces* exhibited a 10% increase of gas production in FCB compared to CB. Overall, *Neocallimastix* showed the highest total gas production of all the anaerobic fungal cultures, irrespective of the type of medium used. Fermentations with *Caecomyces* and *Orpinomyces* showed the lowest total gas production.

Unlike the gas production data, the observed acetic acid fermentation end concentrations in the different media varied greatly (Figure 8 and Figure 9). Cultures of *Neocallimastix, Caecomyces*, and *Piromyces* exhibited a decrease of acetate production in FCB compared to CB. *Orpinomyces* exhibited similar acetate production in complex medium CB compared to standardised medium FCB. *Anaeromyces*, like with the gas production, showed an elevation of acetate production in FCB compared to CB.

These results indicate that the standardised rumen fluid free medium can be used as a growth medium for anaerobic fungi, particularly when gas production is used to monitor culture growth in real-time. This medium allows for a more reproducible assessment of culture fermentation as all the medium ingredients are ‘defined’. Ideal medium composition for acetate production however appears to depend on the anaerobic fungal culture used.


CB Anaeromyces
FCB Anaeromyces
Accumulative gas volume [ml]
Hours after innoculation [h]
0
10
20
30
40
50
60
70
80
0
12
24
36
48
60
72


CB Caecomyces
FCB Caecomyces
Accumulative gas volume [ml]
Hours after innoculation [h]
0
10
20
30
40
50
60
70
80
0
12
24
36
48
60
72


CB Neocallimastix
FCB Neocallimastix
Accumulative gas volume [ml]
Hours after innoculation [h]
0
10
20
30
40
50
60
70
80
0
12
24
36
48
60
72

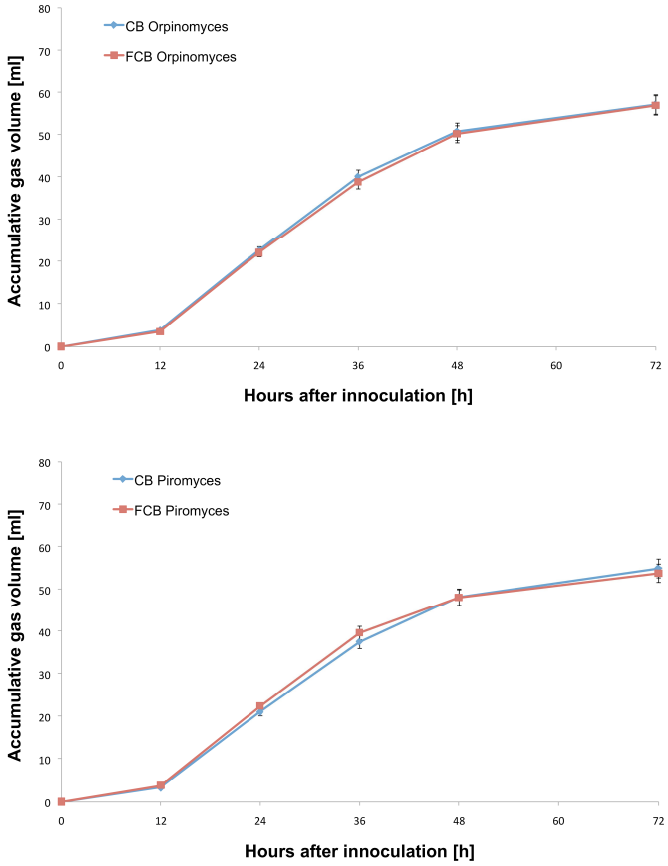


Figure 8: Gas production dynamics of Anaerobic Fungal monocultures grown in complex (CB) and rumen fluid free standardized medium with cellobiose (FCB) as a substrate. Graphs show the accumulative gas production of the anaerobic fungal cultures (*Anaeromyces, Caecomyces, Neocallimastix, Orpinomyces* and *Piromyces*) which were used in the respective fermentations. Values shown are mean values (n=3).

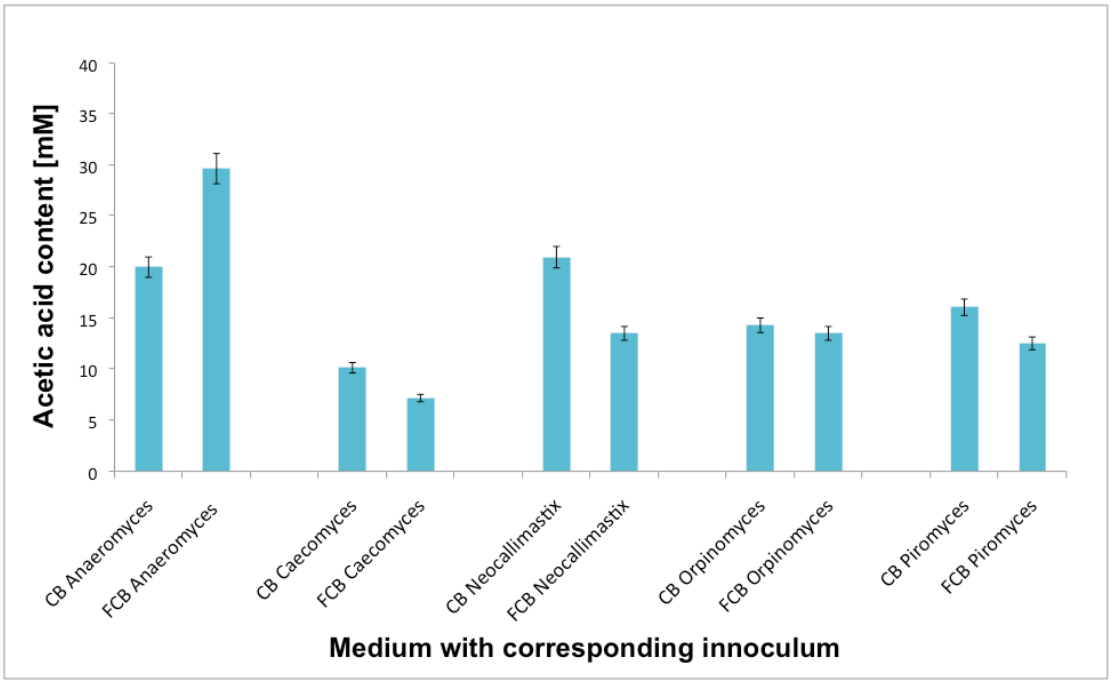


Figure 9: Acetate production of Anaerobic Fungal monocultures grown in complex (CB) and rumen fluid free standardized medium with cellobiose (FCB) as a substrate. Anaerobic fungi (*Anaeromyces, Caecomyces, Neocallimastix, Orpinomyces* and *Piromyces*) were the innoculi in the fermentations respectively. Values shown are mean values (n=3).

4.2.4 Effect of *Saccharomyces cerevisiae* addition on Anaerobic Fungal monocultures grown in standardized rumen fluid free medium.

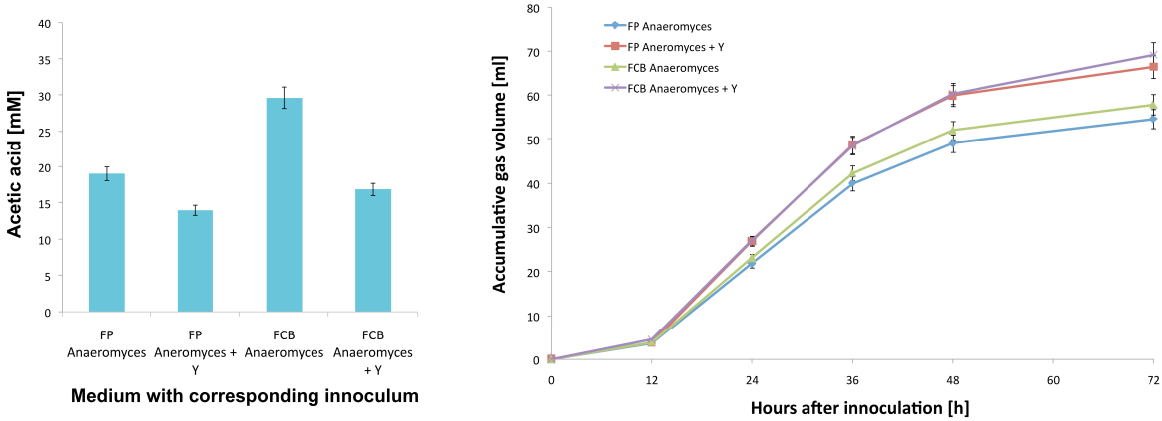
The effect of *Saccharomyces cerevisiae* addition on the behavior of anaerobic fungal monocultures was asessed using standardized rumen fluid free medium and two different susbtrates: filter paper and cellbiose. Cultures of *Anaeromyces, Caecomyces*, *Neocallimastix, Orpinomyces,* and *Piromyces* exhibited different fermentation responses to the addition of *Saccharomyces cerevisiae*, both when fermentations in media containing filter paper (FP) and cellobiose (FCB) as substrates were compared for accumulative gas development and concentration of acetic acid end concentrations.

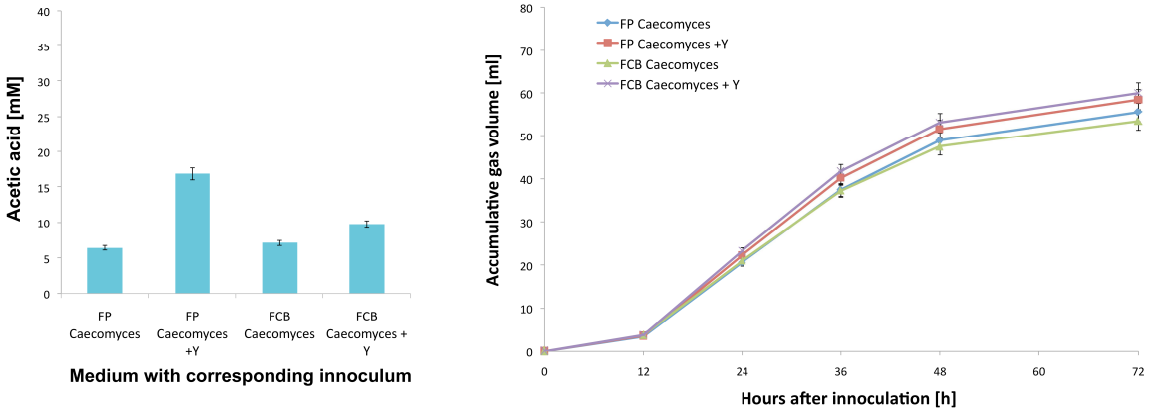
Of all the anaerobic fungal cultures, Anaeromyces showed the highest increase with Saccharomyces cerevisiae addition in both FP (21%) and FCB (19%) medium compared to the corresponding monocultures. Both Caecomyces and Piromyces also had elevated gas production in both media due to Saccharomyces cerevisiae addition compared to the corresponding monocultures. The increase was minimal however relative to that observed with the Anaeromyces cultures (Figure 10). The Neocallimastix and Saccharomyces cerevisiae coculture showed elevated gas production only in the FP medium, whereas with Piromyces an increase with Saccharomyces cerevisiae was only observed in the FCB medium.

Acetate production in co-cultures was elevated for almost all the fermentations with *Saccharomyces cerevisiae* in FP and in FCB, except for *Anaeromyces* where a decrease in acetate production was noted in both FP and FCB. Cocultures in medium FCB showed overall lower improvements in acetate production compared to fermentations in FP medium. Co-cultures in medium FP for *Piromyces* with added *Saccharomyces cerevisiae* showed the highest production of acetate compared to other co-cultures. *Caecomyces* showed the highest increase with *Saccharomyces cerevisiae* addition, resulting in 10.4 mM of acetate compared to the monoculture. *Neocallimastix* and *Orpinomyces* showed an increase of 6.4 mM and 6.3 mM comparable to the increase in *Piromyces*

by 6.7 mM. *Anaeromyces,* as mentioned earlier, exhibited a decrease by 5.1 mM acetate.

Co-cultures in medium FCB for *Piromyces* with added *Saccharomyces cerevisiae* showed again the highest production of acetate compared to other co-cultures. Acetate production of *Caecomyces, Orpinomyces* and Piromyces increase by 6,4 mM, 6,3 mM and 6,53 mM acetate respectively compared to the monoculture. *Neocallimastix* exhibited a lower production of 5,5 mM acetate compared to the monoculture. *Anaeromyces* cocultures exhibited a decrease, by 12,7 mM acetate compared to the monoculture, in contrast to the increased acetate production in medium FP.





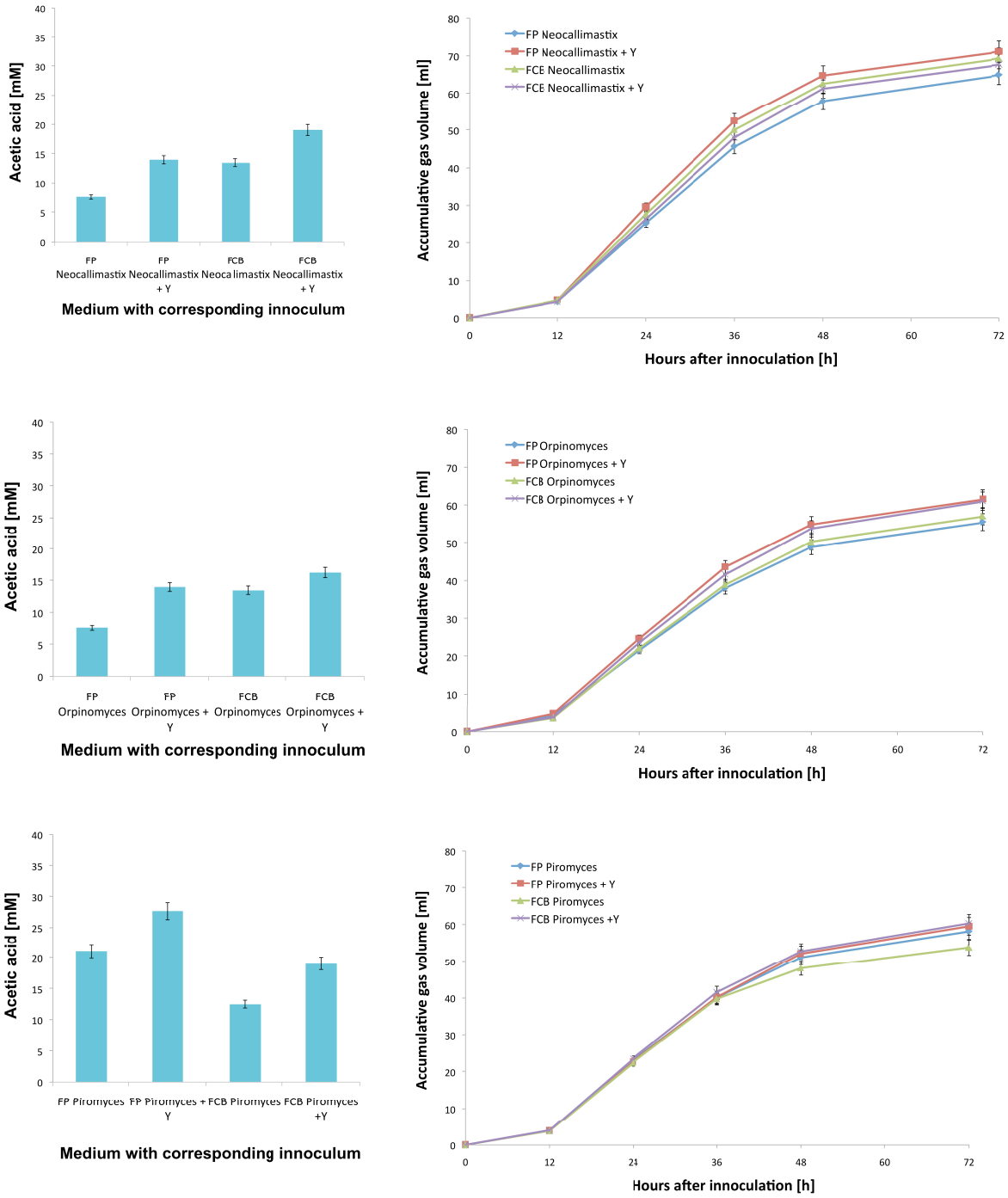


Figure 10: Comparison of the effect of *Saccharomyces cerevisiae* addition on the anaerobic fungal monocultures: *Anaeromyces, Caecomyces, Neocallimastix, Orpinomyces* and *Piromyces*. The *Saccharomyces cerevisiae* inoculum was added to the growing anaerobic fungal monoculture in standardized rumen fluid free medium, containing either filter paper (FP) or cellobiose (FCB) as a substrate. Left column of figure shows acetic acid concentration at the end of the fermentation. Right column showing accumulative gas production dynamics of the same cultures. Values shown are mean values (n=3).

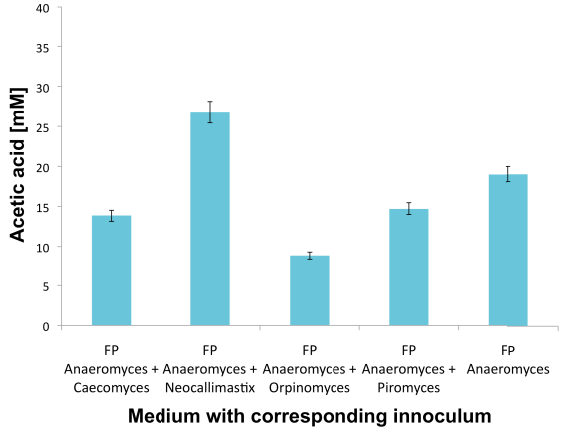
4.2.5 Comparison of Anaerobic Fungi cocultures in rumenfluid free media FP and FCB

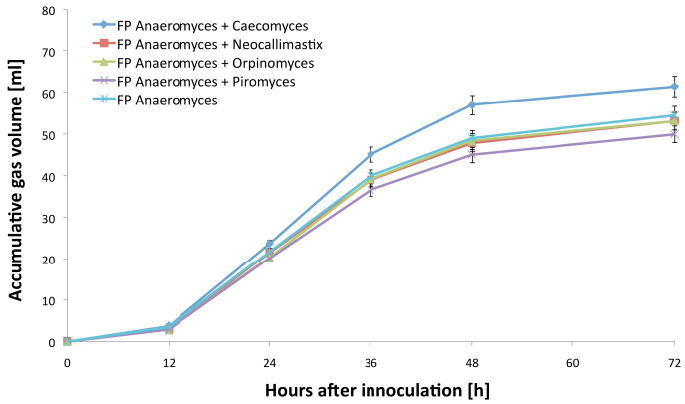
The fermentation of anaerobic fungal cocultures with another anaerobic fungus were compared using FP and FCB medium. *Anaeromyces, Caecomyces*, *Neocallimastix, Orpinomyces* (Figure 11) with an anaerobic fungal culture partner showed minor increases of gas productions on average in FP compared to the respective single fungal culture. *Piromyces* actually showed a slight decrease when compared with other anaerobic fungi.

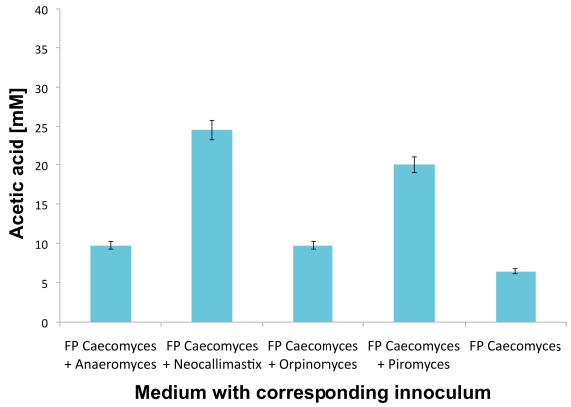
With respect to culture partner, no significant best partner was observed. Co-cultures with *Neocallimastix* and *Orpinomyces* tended to show lower gas production compared to other co-culturing partners. This is contrary to the fact that *Neocallimastix* showed the biggest gas production compared to the other fungal cultures.

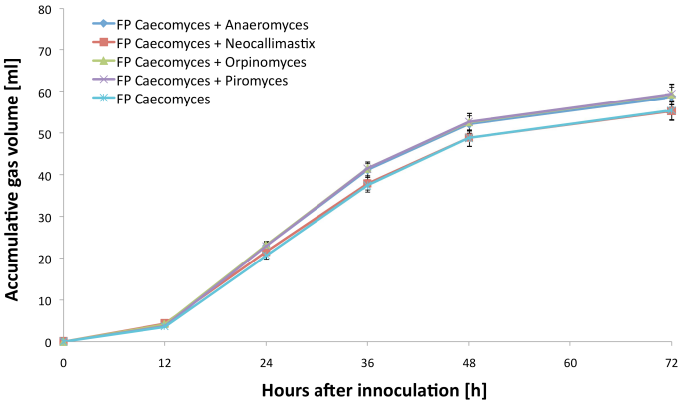
Co-cultures of *Caecomyces*, *Neocallimastix, Orpinomyces* with anaerobic fungi as a culture partner showed substantial elevations of acetate production in standardised medium FP on average. In comparison *Piromyces* showed a moderate increase when paired with other anaerobic fungi, and *Anaeromyces* showed a decrease.

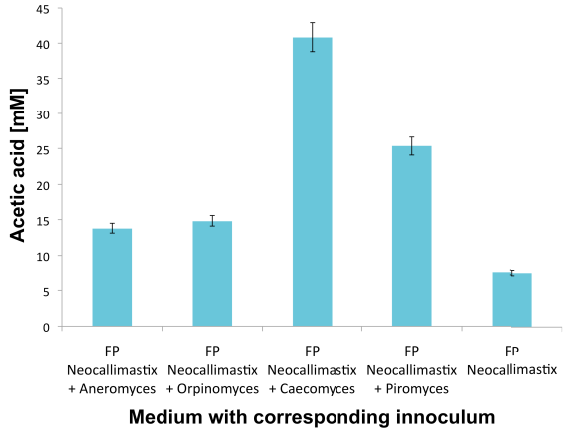
With respect to culture partner, *Neocallimastix, Piromyces* and *Caecomyces* exhibited the best improvement in acetate production in the respective cultures. *Anaeromyces* and *Orpinomyces* showed the least improvement when added as a culture partner.

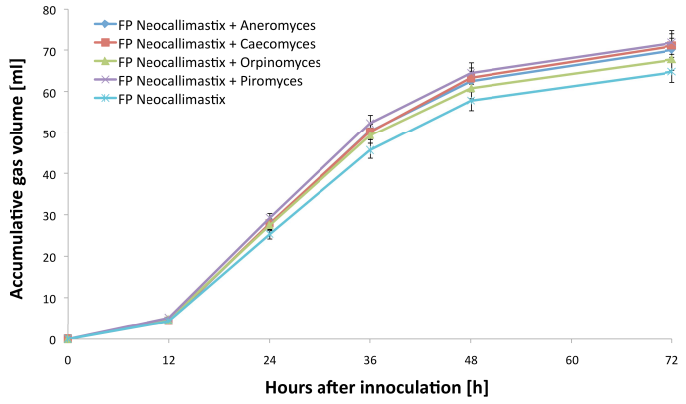

Acetic acid [mM]
40
35
30
25
20
15
10
5
0
FP Anaeromyces + Caecomyces
FP Anaeromyces + Neocallimastix
FP Anaeromyces + Orpinomyces
FP Anaeromyces + Piromyces
FP Anaeromyces
Medium with corresponding innoculum


Accumulative gas volume [ml]
80
70
60
50
40
30
20
10
0
FP Anaeromyces + Caecomyces
FP Anaeromyces + Neocallimastix
FP Anaeromyces + Orpinomyces
FP Anaeromyces + Piromyces
FP Anaeromyces
0
12
24
36
48
60
72
Hours after innoculation [h]


Acetic acid [mM]
40
35
30
25
20
15
10
5
0
FP Caecomyces + Anaeromyces
FP Caecomyces + Neocallimastix
FP Caecomyces + Orpinomyces
FP Caecomyces + Piromyces
FP Caecomyces
Medium with corresponding innoculum


Accumulative gas volume [ml]
80
70
60
50
40
30
20
10
0
FP Caecomyces + Anaeromyces
FP Caecomyces + Neocallimastix
FP Caecomyces + Orpinomyces
FP Caecomyces + Piromyces
FP Caecomyces
0
12
24
36
48
60
72
Hours after innoculation [h]


Acetic acid [mM]
45
40
35
30
25
20
15
10
5
0
FP Neocallimastix + Aneromyces
FP Neocallimastix + Orpinomyces
FP Neocallimastix + Caecomyces
FP Neocallimastix + Piromyces
FP Neocallimastix
Medium with corresponding innoculum


Accumulative gas volume [ml]
80
70
60
50
40
30
20
10
0
FP Neocallimastix + Aneromyces
FP Neocallimastix + Caecomyces
FP Neocallimastix + Orpinomyces
FP Neocallimastix + Piromyces
FP Neocallimastix
0
12
24
36
48
60
72
Hours after innoculation [h]

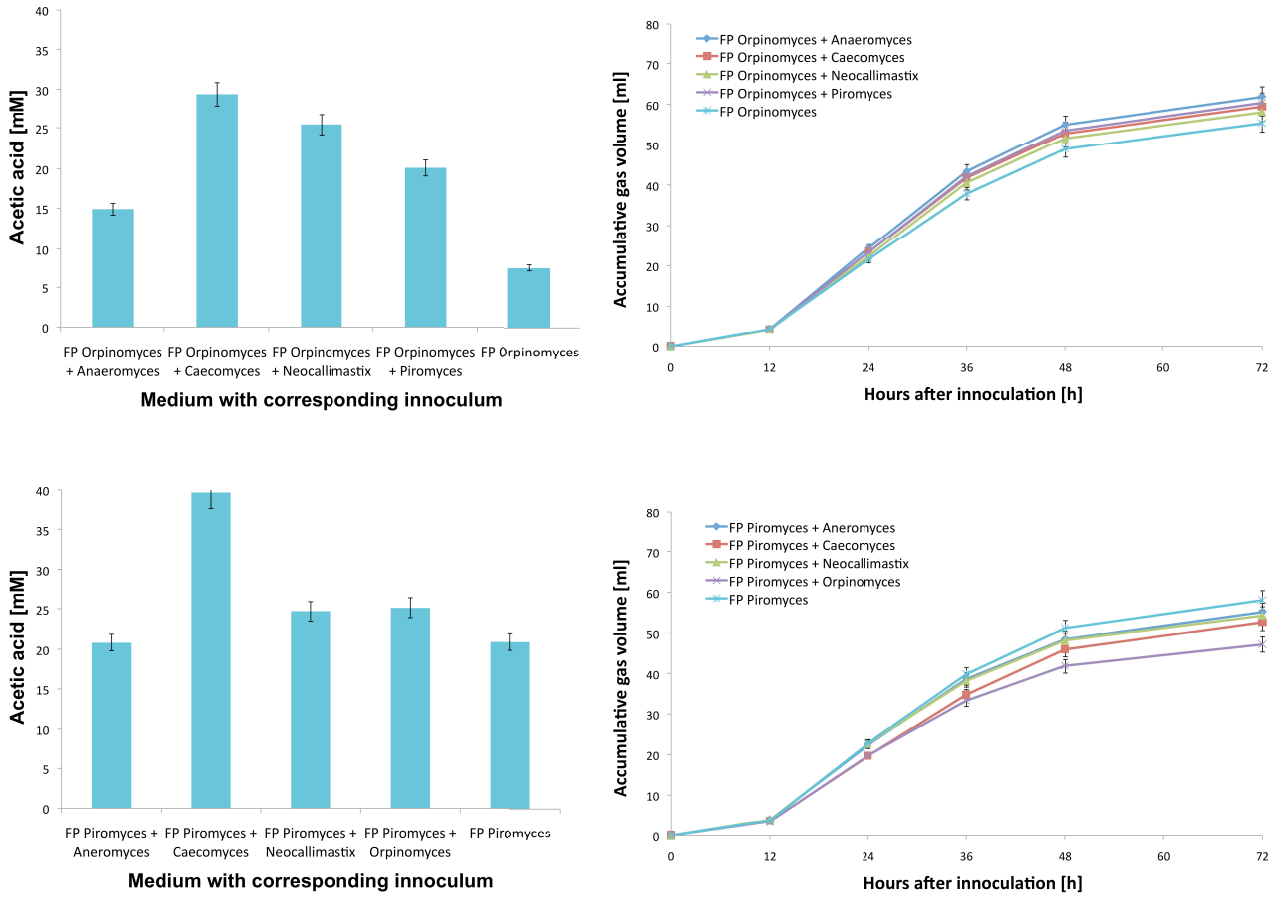


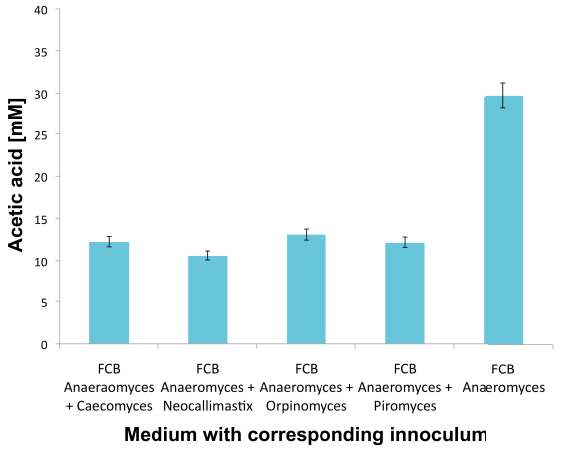
Figure 11: Comparison of the effect of anaerobic fungal co-cultures grown in standardized rumen fluid free medium with filter paper as a substrate (FP). Respective coculture innoculi have been added to a growing culture of single anaerobic fungus. Left column of figure showing acetic acid content at end of fermentation. Right column showing accumulative gas production of the corresponding cultures. Values shown are mean values (n=3).

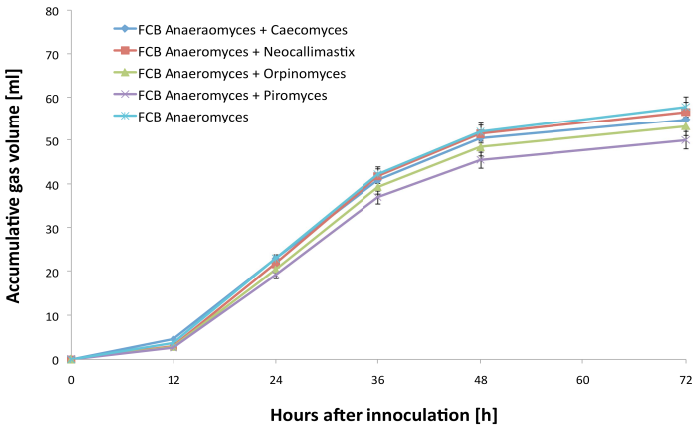
Co-cultures of *Anaeromyces, Orpinomyces and Piromyces (Figure 12)* with anaerobic fungi as a culture partner showed minor increases of gas productions on average in FCB compared to the respective single fungal culture. In contrast *Neocallimastix* showed an average decrease by 10 % and *Caecomyces* an increase by 11%. This is could be attributable in part to the medium composition, as *Caecomyces* is not exhibiting a filamentous rhizoid and is specialised on the utilisation of cellobiose. *Neocallimastix* on the other hand is exhibiting a filamentous rhizoid and is more specialised for fibrous carbon sources. With respect to

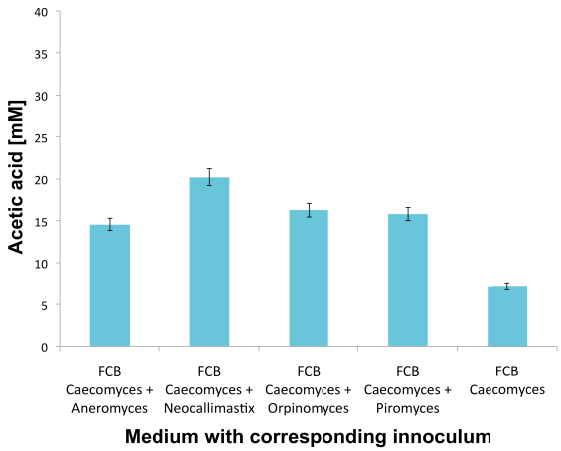
culture partner, *Neocallimastix* and *Anaeromyces* exhibited the best improvement in gas production in the respective cultures. *Piromyces* and *Orpinomyces* showed the least improvement.

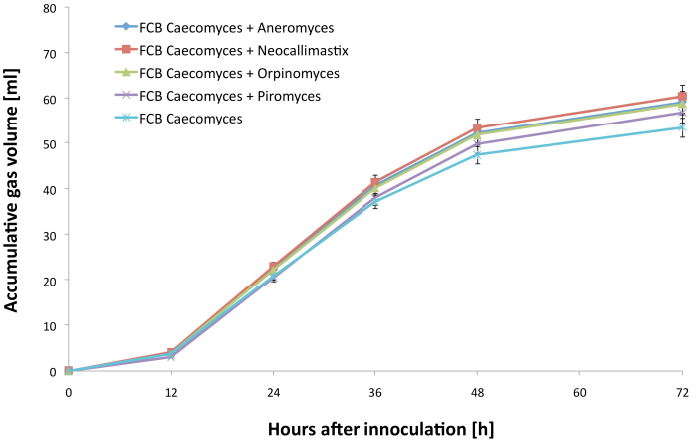
Co-cultures of *Caecomyces*, *Neocallimastix, and Orpinomyces* with anaerobic fungi as a culture partner showed substantial elevations of acetate concentration in standardised medium FCB on average. In comparison the single fungal culture *Piromyces* showed a moderate increase when paired with other anaerobic fungi, and *Anaeromyces* showed a decrease.

With respect to culture partner, no significant best partner could be determined. Co-cultures with *Anaeromyces* and *Piromyces* showed lower gas productions compared to other co-culturing partners.









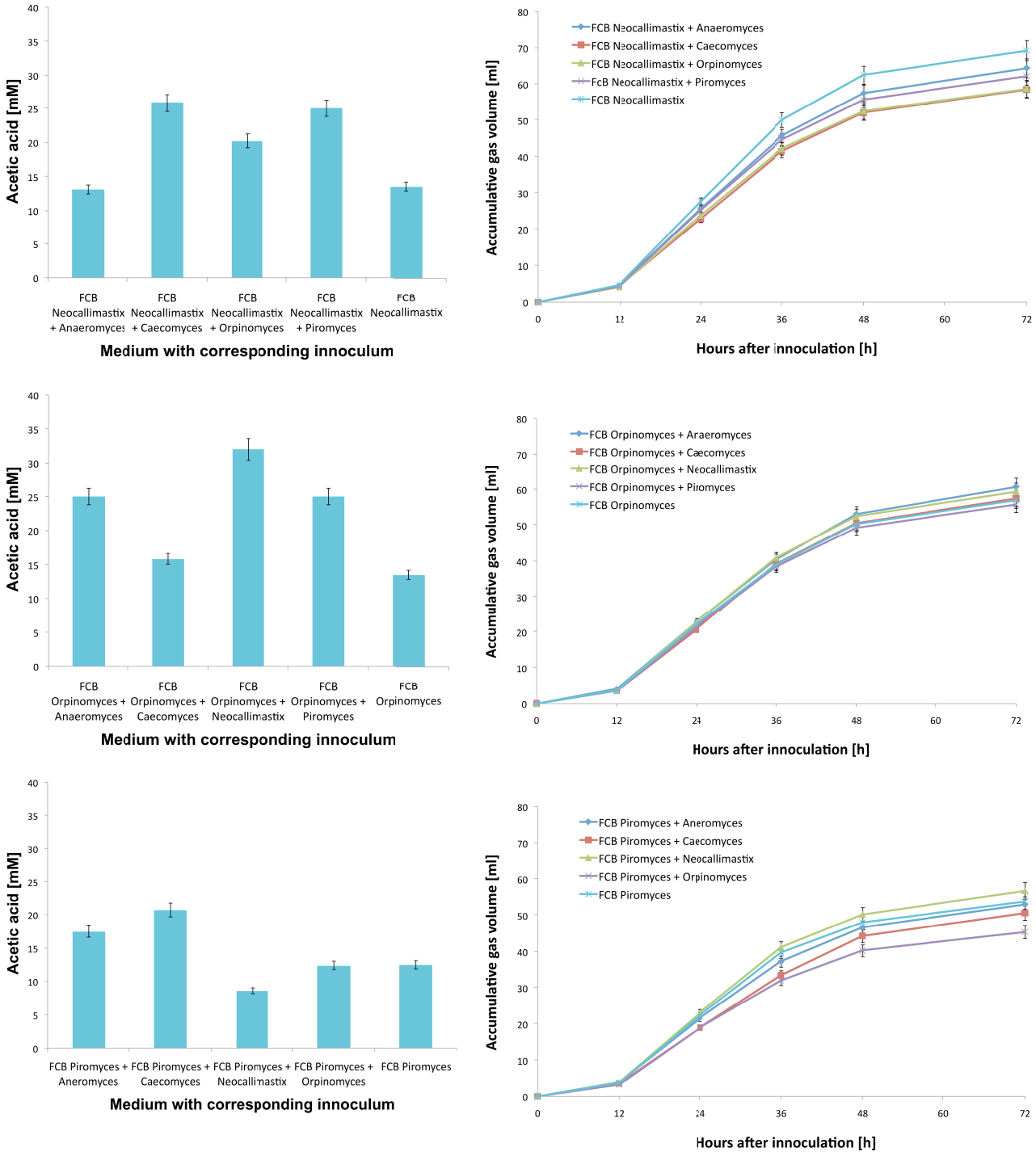


Figure 12: Comparison of Effect of Anaerobic Fungal co-cultures grown in standardized rumen fluid free medium with cellobiose as a substrate. Respective co-culture innoculi have been added to a growing culture of single anaerobic fungus. Left coloum of figure showing acetic acid content at end of fermentation. Right column showing accumulative gas production of the corresponding culture. Values shown are mean values (n=3).

5 Discussion and future opportunities

5.1 Extrusion

5.1.1 Extrusion process and conversion

The extrusion process is an interesting pretreatment method which causes disruption of the physical structure of the material, allowing effective enzymatic saccharification of the generated reactive fibres In recent years, the extrusion process with its unique combination of different pretreatment methods and realizable high shear stress has received increasing attention. It has also been shown to be capable of improving enzymatic saccharification of cellulosic substrates as discussed earlier in this work.

The first step in conversion of biomass is, as discussed previously, the pretreatment to allow for an effective saccharification. This is generally one of the most capital consuming process steps. The research in this area is focused on three main goals: (i) lowering the amount of enzymes needed for the bioconversion, (ii) a reduction of treatment times and (iii) the increase in platform chemical yields. Various pretreatment methods such as wet oxidation, dilute acid, alkali, ammonia fiber explosion and steam explosion have been investigated. To date, however, very few commercially effective pretreatment and saccharification methods have been able to be established on an industrial scale (Ewanick & Bura 2010).

As discussed previously (section 1.2.5), extrusion pretreatment utilizes rapid heat transfer, high pressure, high shear and effective mixing in a continuous process with short treatment times. The extrusion process therefore offers some key advantages over other pretreatment methods, namely: better control of variables, lower process costs, no sugar degradation products, a high continuous throughput and good adaptability to different biomass sources and requirements such as addition of chemical solutions. This shows the high potential of extrusion as a pretreatment for cellulosic biomass for enzymatic saccharification

and subsequent platform chemical production (Kohlgrüber 2008; C Karunanithy & Muthukumarappan 2011; Devrije 2002; Ahmed 2009).

Several studies have focused on assessing the effect of extrusion parameters on the subsequent enzymatic saccharification process, as the effectiveness of the pretreatment has been shown to be heavily influenced by moisture content, barrel temperature and screw speed (Table 3).

Conversion of extruded cellulosic material has been reported to range from 41.1% up to 90% (Table 3). The optimal conversion of 53% achieved in this work is higher than some of the lower conversions achieved for Switchgrass,but lower than other reported conversions. Conversion seems not to be heavily influenced by source of material as the highest and lowest conversion is reported for switchgrass, although various variables of the pretreatment influence conversion (Table 3). In the case of switchgrass a pretreatment with alkali extrusion lead to the highest conversion.

A more critical analysis of the literature looking at the effect of different extrusion treatment parameters on substrates proved to be difficult, as many different factors influenced the effect of the treatment. These factors include the different types of extruders, the high flexibility of the screw configuration, applied additional chemicals and process aids, enzymes and enzyme combinations and saccharification conditions used, and post extrusion treatments. However some studies have been performed to specifically assess individual process parameters, particularly barrel temperature.

Barrel temperature has been reported to have an impact on enzymatic cellulose saccharification, depending on the substrates and the temperature range used (Akdogan 1996; Ayadi et al. 2011; Li et al. 2011). Higher temperatures tend to have a negative effect on sugar conversion, although this also depends on the substrate used. Higher temperatures can lead to higher moisture evaporation, which leads to more friction and a decreased residence time due to lowered viscosity. A temperature change from 80°C to 120°C in the extrusion treatment of rice straw for example lead to an increase in saccharification rate, whereas a further increase to 160°C had an negative effect on the rate (Cheng et al. 2010).

Pretreatment	Cellulosic material	Pretreatment settings	Results	Literature
Single screw extrusion	Corn cob	125°C, 75 rpm	75% of the original polysaccharides were converted	Karunanithy and Muthukumarappan (2010a)
Single screw extrusion	Switchgrass	150°C, 50 rpm, moisture content 15%	45.2% of the original polysaccharides were converted	Karunanithy and Muthukumarappan (2010b)
Single screw extrusion	Pine wood	180°C, 150 rpm, 25% moisture content	65.8% of the original polysaccharides were converted	Karunanithy et al. (2013)
Single screw extrusion	Switchgrass	176°C, 155 rpm, moisture content 20%	41.1 % of the original polysaccharides were converted	Karunanithy & Muthukumarappan (2011)
Twin screw extrusion	Corn stover	140°C, 80 rpm, moisture content 27.5%	48,79% glucose recovery, 2.6 times higher than for untreated corn stover	Zhang (2012)
Twin screw extrusion	Wheat bran	7 Hz/150 °C and 3.7 Hz/110 °C	total reducing sugar yields of 60–73%	Lamsal (2010)
Alkali soaking followed by single screw extrusion	Prairie cord	114°C, 122 rpm, 1.7% NaOH concentration, ratio of 7 liquid/1 biomass	86.8% of the original polysaccharides were converted	Karunanithy and Muthukumarappan (2011)Karunan
Specialy designed alkaline twin-screw extrusion	Corn stover	99°C, 325 rpm, biomass/liquid ratio of 1/2 (w/w)	78% of the original polysaccharides were converted	Liu (2013)
Alkali soaking followed by single screw extrusion	Switchgrass	180°C, 118 rpm, 2% NaOH concentration, ratio of 7 liquid/ 1 biomass	90.5% of the original polysaccharides were converted	Karunanithy and Muthukumarappan (2011)

Table 3: Comparison of optimised extrusion conditions obtained with varying extrusion, pretreatment settings and cellulosic material.

This is consistent with the findings of this study, where temperatures above 120^{o}C had a negative impact on the saccharifaction yield. The higher barrel temperature also resulted in a darker extrudate in this study, indicating that the mixtures were being 'burnt'.

Of note is the fact that the highest conversion observed at each screw speed were all in combination with 120°C and 30% moisture. In comparison, lower conversions were always obtained with 120°C and 20% moisture, highlighting a consistent interaction between these parameters regardless of substrate and amount of process aid starch. The effects of screw speed in contrast appeared to be more independent of these two parameters.

As the residence time of the substrate in the extruder is inversely proportional to screw speed, a change in residence time results in a complex range of effects on hydration, thermal degradation and mechanical energy input (Lamsal 2010). This is perhaps why both negative as well positive effects have been reported when looking at different combinations of screw speed and barrel temperature settings, for example an increase in screw speed has been reported to lead to an increased shear rate that in turn lead to a higher fracture of the cellulose fibrils (Lamsal et al. 2010; Bak et al. 2009; Lee et al. 2009).

In this study a moderate screw speed (350 rpm) was found to be optimal, with a higher screw speed (450 rpm) decreasing conversion efficiency by 14,5 %. This is consistent with previous studies (Table 3) that reported optimal cellulose saccharification with moderate screw speeds.

In terms of optimizing extrusion conditions, it is clear that assessment of the parameters discussed above is key. Of even more importance however was the use of an appropriate amount of process aid starch, without which extrusion of cellulosic feedstocks was not feasible due to frequent blockage and cessation of screw rotation. Increased amounts of the process aid starch led to a more stable process, however this is economically an unfavorable modification as the increased cost of the starch substantially affects the production costs of the end products generated via enzymatic saccharification.

5.1.2 Conclusion

Corredor (2009) reported a conversation rate of 73% for soybean hulls pretreated with sulfuric acid 2% at 140°C, following steam explosion and enzymatic saccharification in 36 hours. Martin (2007) reported a cellulose conversion rate of 61% for pretreated rice hulls. Saha (2005) reported a conversion to glucose of 60% of rice hulls subjected to acid hydrolysis although the sugar yield varied depending on the pretreatment conditions and Wang (2010) reported that conversation to glucose of alkali hydrolysis subjected Bermuda grass was around 90%. Karuppuchamy and Muthukumarappan (2009) extruded corn stover, switchgrass and prairiecord grass and subjected the pretreated materials to enzymatic saccharification, resulting in an increased conversion rate of 45 to 66% converted cellulose. The extrusion pretreatment parameters investigated in this work led to an increase in conversion from 24 % up to 53%, with the highest results obtained for all the cellulosic mixtures with the extrusion parameters 120°C extruder barrel temperature and 30% in process moisture at 350 rpm respectively. Other potential influencing factors which can also be of interest to further assess include: interaction of starch with the cellulosic material, distribution of water (between starch and cellulosic material) and residence time in the extruder (Lee et al. 2009; S.-H. Lee et al. 2010; Lamsal et al. 2010; Yoo et al. 2012).

Conversion rates could be increased compared to untreated mixtures, but the issue of the poor flow properties of cellulosic materials had to be addressed. The use of an in-process aid as well as a high in barrel moisture alleviated the issues associated with unstable flow. This optimization however does have associated drawbacks. Less cellulose can be treated in one run, therefore lowering the effectiveness of the pretreatment relative to operational costs. High moisture results in a suboptimal treatment of the cellulosic substrate and the use of process aids (such as starch) increases production costs. The use of starch as a process aid is also questionable in sustainability terms, as it can also be directly utilized in food and platform chemical production. Further work needs to be conducted therefore to find a more sustainable (and ideally cost-effective) substance as a process aid or a process without need for a process aid.

It is of paramount importance to optimize the stability of the extrusion process before scale up is undertaken. A constant and reproducible process is needed for a continuous process: which is a potential key advantage of this system. It is also central in order to enable the pretreatment to be performed with minimal equipment maintenance and repair costs.

In addition to maintenance costs, start-up costs with industrial scale extruders need a large capital investment, as well as high costs in terms of energy demand (for heating and motor) and operating personnel. Also, the requirement for off-line produced enzymes in the saccharification as well as the consecutive fermentation of the generated sugars requires more funds and energy, and increases process costs. The transport of the cellulosic materials results in increased energy demand and costs as well.

In summary, extrusion pretreatment can potentially be an important addition to the already used and approved pretreatment processes. However, high energy and capital demands make it challenging at the moment to be a feasible alternative to other pretreatment methods currently used. Despite the difficulties deriving from the recalcitrant nature of the substrate, as well as the costs associated with a new extrusion plant, more research in this field seems promising. Development of a continuous pretreatment process that could be be carried out in a decentralized manner, allows for the cellulosic material to be efficiently utilized for production of platform chemicals and renewable energy generation.

5.2 Anaerobic fungi

5.2.1 Anaerobic fungi

Anaerobic fungi have been of interest for some time as part of the natural microbiota of the ruminant gut, as the nutrition of farm animals is of industrial interest in order to maximise milk and meat production and improve animal health. Their potent cellulolytic activity has brought them to the interest of the wider scientific community, particularly now that new efficient and cost effective approaches transforming cellulosic biomass into platform chemicals and energy is high on the global research agenda. (Sehgal et al. 2008; Wallace 1994; Lee et al. 2000)

The presence of anaerobic fungi in the digestive tract of mammalian herbivores enables them to effectively colonise, utilise and degrade ingested lignocellulosic plant biomass. Generation time in the rumen may be slower than that of other major taxa, but their invasive colonisation of plant material provides them with a key ecological niche, as well as an effective means to deliver their vast array of plant degrading enzymes. Their ability to grow in synergy with other microorganisms, like methanogens, is another applied aspect of their biology that is also of commercial interest, in addition to their potential to generate effective cellulolytic enzymes for a range of biotechnological applications (Gruninger et al. 2014).

Anaerobic enzyme production still poses a difficult solution for the production of cellulytical active enzymes, but could be improved by the modification of specific microorganisms via biotechnological techniques. Further research into this field has already started but is yet to be proven to be able to be effectively implemented at an industrial scale. (Fuller 1992; Cabe & Clem Kuek n.d.; Theodorou et al. 1996a)

5.2.2 Culture gas production in relation to cellulosic substrate utilization

According to Theodorou (1995), cellulosic substrate utilization is closely related to gas production by anaerobic fungal cultures. On this basis, the monitoring of the gas production of anaerobic fungal cultures in real-time has been widely used to assess their fermentation of cellulosic substrates *in vitro*. As culture gas production is closely related to cellulosic substrate utilization this factor was assumed as the main source of the gases produced by the anaerobic fungi, but other factors can influence/contribute to the gas production. These factors include: concentration and type of growth inhibiting medium components, produced components, type of microorganism used as single or co-culture and rate of growth. (Theodorou 1996; Beuvink and Spolestra 1992) If these factors are taken into account, the assessment of the gas production can be used as a rapid and non-destructive method to monitor culture growth and substrate utilization.

Another factor, or positive side effect, of this method of measurement is that the cultures are vented periodically preventing a build up gases in the culture bottle headspace, which can have an inhibiting effect on growth. Hydrogen, for example, is removed from the batch culture, and this has been reported to have a positive effect on the culture growth rate (Theodorou et al. 1996a; Davies et al. 2000; Nielsen 2002; Trinci et al. 1994). Lower hydrogen partial pressure allows for a re-oxidation of cofactors like NADH and production of electron-sink products. ATP production, for example, is increased which in turn provides more energy for biomass growth and metabolic activity. The noted gas production showed similarities with the typical growth patterns of anaerobic cultures. (Nielsen 2002; Trinci et al. 1994; Davies et al. 2000; Miller & Wolin 1974; Bauchop 1981)

For a more accurate representation of growth rate, an automated continuous measurement of gas production in combination with a computerised data capture system could be used, but the applied technique in this work is widely acknowledged to be feasible for the basic interpretation of co-culture responses of the anaerobic fungi to different treatments and/or parameters.

Gas production measurements in this study showed similarities with the typical growth patterns reported for anaerobic fungi (Phillips & Gordon 1989; Teunissen, Op den Camp, et al. 1991). Anaerobic fungal cultures of *Caecomyces* showed the least active gas production, and *Neocallimastix* the most. This is consistent with the previous observations of Lowe (1988) and Gordon (1989). Combinations of anaerobic fungal cultures could be characterised as giving low, moderate or good improvements with regard to increasing gas production. In summary, the majority of cultures investigated in this study showed varying degradation ability and optimization potential, with *Neocallimastix* displaying the best overall results. *Orpinomyces* and *Caecomyces* were found to be less effective culture partners or general degraders (Davies et al. 2000; Anon n.d.; Theodorou et al. 1988; Phillips & Gordon 1989; Zhu 1996; Cheng et al. 2009; Sun et al. 2006; W. Jin et al. 2011).

In order to assess the produced volatile fatty acids in the batch fermentations, volatile fatty acid concentrations in uninoculated media were used as a calibration base, i.e. mean values were subtracted from the concentrations of volatile fatty acids measured in inoculated media. Concentrations of the following volatile fatty acids were measured as indicators for the evaluation of the fermentation: acetic, propionic, iso-butyric, N-butyric, iso-valeric N-valeric, iso-caprioic and N-caprioc. Propionic, iso-butyric, N-butyric, iso-valeric, N-valeric, iso-caprioic and N-caprioc acid were low or undetactable in all single and co-culture supernatants. In the case of the media, which contained clarified rumen fluid as an ingredient, these values were comparable to the concentrations of volatile fatty acids in uninoculated culture medium. By contrast, concentrations of acetate in all the single and co-cultures were higher than in the uninoculated culture medium. This is consistent with previous reports that acetate is the major VFA formed by anaerobic fungal metabolism and fibre degradation in both mono- and co-cultures. (Mountfort et al. 1982; Wood et al. 1986; Teunissen et al. 1993; Teunissen, Smits, et al. 1991)

5.2.3 Growth of anaerobic fungal monocultures in different media

Single cultures of *Anaeromyces*, *Caecomyces*, *Neocallimastix*, *Orpinmyces* and *Piromyces* have been compared in WS, CB, FP and FCB media within this study. With all the different media used, and compared to the other anaerobic fungi, *Neocallimastix* generally showed the best activity based on gas and acetate production in the various media and culture combinations used in this study. *Caecomyces* on the other hand generally exhibited the lowest culture activity, but had higher activity in FCB relative to the other media used for this culture. This could in part be attributed to diverging structures of the fungi.

The vegetative state of *Neocallimastix* shows an interlaced filamentous rhizoid. Caecomyces exhibits an unbranched coralline rhizoid with ovoid structures partly with fibrilar roots and also vesicular structures and is of bulbous character. An interlaced and highly branched rhizoid seems to be better equipped for cellulosic substrate utilisation. This is also supported by the fact the *Caecomyces* exhibited better growth in media with cellobiose, seemingly being better able to utilize the smaller disaccharide as opposed to the polysaccharide cellulose.

5.2.4 Comparison of complex media and rumen fluid free standardised media

Rumen fluid is a non-standardisable addition to the complex medium, and requires invasive sampling of animals. The complex medium containing rumen fluid is designed to simulate the natural habitat of most anaerobic fungi. As described in the previous chapters, the complex medium was and is mainly used to culture new detected fungal strains and for culture collections, both in growing state and in cryopreservation.

In order to assess anaerobic fungal cultures for biotechnical applications, a standardised medium is needed. One of the advantages of the standardised medium is that volatile fatty acids can be measured more accurately as no uncertainty deriving from rumen fluid has to be accounted for. Other advantages

are easier access to media components, a reduction of cost and no ethical implications with animal sampling.

All fungal cultures were able to grow equally well in both complex and standardised medium in terms of gas production. *Anaeromyces* also exhibited higher acetate production in the standardised medium. Results presented in 4.2.1 confirmed that the standardised medium can be used as a substitution for a complex medium, as all tested fungi were able to grow and most cultures exhibited similar growth to the complex medium. This allows a standardised basis for further research studies.

5.2.5 Anaerobic Fungal cocultures in rumenfluid free media FP and FCB

Effects or partnering different anaerobic fungi in coculture varied in the media FP and FCB. *Neocallimastix* monocultures with added anaerobic fungal culture partners showed again more activity, based on gas and acetate produced, than other fungal monocultures. In contrast, an adverse effect could be observed when *Neocallimastix* was used as culture partner in FP. A decrease in gas production for *Neocallimastix* cocultures in FCB could be observed as well. Of note is that the addition of *Neocallimastix* was supportive for acetate production in FCB. *Caecomyces* on the other hand showed an increase of culture activity based on gas and acetate produced as a culture partner in FCB. The amount of acetate produced also changed with the different coculturing combinations. *Caecomyces*, *Neocallimastix* and *Orpinomyces* generally benefited from cocultures with other anaerobic fungi, as more acetate was produced. *Neocallimastix* and *Caecomyces* also are improving acetate production when used as a coculture partner when added to an already growing monoculture. Coculturing of anaerobic fungal cultures is thus possible, but culture partners have to be chosen according to media/substrate preferences. Also only a minor enhancement of the culture activity was observed.

5.2.6 Effect of *Saccharomyces cerevisiae* addition on anaerobic fungal monocultures grown in standardized rumen fluid free medium.

In this study, the effect of *Sacharomyces cerevisiae* on different monocultures of anaerobic fungi was investigated. The results indicated that fungal metabolism can be influenced through the careful selection of the partner organism. Anaerobe fungal partners showed small positive effects (section 1.5), whilst the addition of live *Saccharomyces cerevisiae* enhanced more the fermentation of *Anaeromyces* and *Orpinomyces*.

This stimulating effect could be attributed to the fact that liberated soluble sugar-molecules, that can have an inhibiting effect on cellulases through product inhibition, are scavenged by the yeast cells. Oxygen scavenging is also a possible mode of action of added yeast and seems to be beneficial for the anaerobic fungal co-culture. In addition *Saccharomyces cerevisiae* can provide the anaerobe fungi with vitamins, thiamine especially, as shown by Chaucheyras (1995).

This stimulating effect of *Saccharomyces cerevisiae* on anaerobic fungal activity as well as the fact that cultures showed generally no negative effect in co-culture allows for a use of *Saccharomyces cerevisiae* as a culture partner.

5.2.7 Conclusion and future prospects

As mentioned earlier, the majority of cultures investigated showed similar degradation ability and optimization potential with *Neocallimastix* being the best overall combination partner, and *Orpinomyces* and *Caecomyces* being the lesser effective culture partners or general degraders. This is also consistent with the findings of other studies (Joblin 1993; Theodorou et al. 1988; Zhu 1996; Theodorou et al. 1996a; Lowe 1987).

The successful application and optimization of a standardised media for the growth of anaerobic fungi in this work supports future research on anaerobic fungal utilization of lignocellulosic biomass. It was shown to be feasible to support growth and fermentation of anaerobic fungi. Its lack of rumen fluid allows

for a more standardised and easier work with anaerobic fungi, as no clarified rumen fluid is needed. Rumen fluid can be difficult to acquire, as special cows are needed and it fluctuates in its properties. Further research into the utilization of anaerobic fungi for the conversion of cellulosic biomass seems highly promising.

The addition of *Saccharomyces cerevisiae* could be a promising step in the optimization of conversion of cellulolytic biomass to platform chemicals in a consolidated bioprocess. Further research into the direct utilization of anaerobe fungi for the conversion of cellulosic biomass therefore seems very encouraging. Of interest could also be the fermentation of cellulose producing algael biomass (macro algae, seaweed for example) as a substrate alternative to cellulosic land crop and food waste biomass (John et al. 2011). Marine algae were tested as a substrate in this study (data not shown), and the anaerobic fungi exhibited growth on the algal fibres and appeared to degrade them. Micro or single cell algae with cell walls containing cellulose may also be of interest. Wastewater could be treated with these algae, the algae functioning as a nitrogen scavenger and can later be degraded by the fungi and utilized in a fermentation process. Proteolytic activity of the fungal rhizoid could also be of interest, hereby being utilized for the opening of algae cells to exploit intracellular components, mediated by the rhizoidal attack mode of the fungi (Lissens et al. 2004).

The inimitable mode of action of anaerobic fungi in degrading recalcitrant cellulose-containing material, by enzymatic hydrolysation mediated from cell-bound and free enzymes as well as invasive rhizoidal growth into the plant fibre, makes them highly desirable as a novel microorganism group. The mechanical and enzymatic action increases reactive surface area, thus allowing for faster saccharification after the initial colonialisation of the substrate (Ho et al., 1988a,b; Li et al. 1997; Dehority & Orpin 1997).

One future task at hand is the development of a continuous culturing method for anaerobic fungi. At present, repeated batch cultures are needed that require frequent transfers, which are costly and time consuming. A continuous process could be supported by identifying a strain that is more suitable for continuous fermentation or/and a better understanding of the forming and nature of resist-

ant or dormant states of anaerobic fungi. Another possibility is the immobilisation of anaerobic fungal cultures and zoospores, allowing for various industrial applications as well as potentially large scale enzyme production (Nagpal et al. 2009; Sridhar & Kumar 2010).

Anaerobic fungi are already improving biogas yields in bioreactors functioning as an anaerobic pretreatment step that could replace an aerobic pretreatment. Further biotechnological applications of anaerobic fungi are already being investigated (Leis et al. 2014; Haitjema et al. 2014; Procházka et al. 2012). Improved culturing methods and the complex cellulase system make the anaerobic fungi a very promising candidate for the generation of platform chemicals from cellulosic materials.

Anaerobic fungi could be used in a unique way to convert cellulose containing materials in a consolidated bioprocess, pretreating and hydrolysing the base material in the same process step. This omits a pretreatment step, as needed with the enzymatic saccharification method. This could result in improving the common practice to generate glucose by enzymatic hydrolysation of pre-treated materials, and subsequent fermentation to platform chemicals. Furthermore, it could even be realised in a continuous fermentation system, as the anaerobic fungi are already adapted to a continuous system, the rumen.

In conclusion, more research is needed in order to make the implementation of anaerobic fungi as a novel group of microorganism for cellulose saccharification feasible. The results of this study indicate that anaerobic fungi, with their effective cellulose hydrolysation enzymes, are a highly promising addition to the energy solutions of the future.

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6.1 Publications to which contributions were made

Kubo Yuji, Saito Katsuichi, Hohlweck Daniel, Nakagawa Rikio, Kimura Keitarou. Black Soybean Fermentation using a rpoB Mutant Strain of Bacillus subtilis (natto). 581, 577–581 (2013). **菌体外分解酵素がされた豆菌**rpoB **株による 大豆 豆の 造**

Callaghan, T. M., S.M. Podmirseg, D. Hohlweck, J.E. Edwards, A.K. Puniya, S.S. Dagar & G.W. Griffith. Buwchfawromyces eastonii gen . nov ., sp . nov : a new anaerobic fungus (Neocallimastigomycota) isolated from buffalo faeces. Accepted for publishing by MycoKeys (2015)