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Exploring Anaerobic Bacteria for Industrial Biotechnology - Diversity Studies, Screening and Biorefinery Applications

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Exploring Anaerobic Bacteria for Industrial Biotechnology

Diversity Studies, Screening and Biorefinery Applications

Rosa Aragão Börner

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Doctoral Dissertation
December 2013

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The Faculty opponent is Associate Professor Peter Stougaard, from the Department of Plant and Environmental Sciences, University of Copenhagen, Denmark.

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Abstract

Depletion of easily accessible fossil energy resources, threat of climate change and political priority to achieve energy self-sufficiency and sustainable solutions prioritise a conscious and smart use of renewable resources to generate a bio-based economy. Bio-based compounds can replace chemicals and fuels that are now mainly produced from crude oil. Efficient processes for the conversion of plant biomass into compounds of interest to the biorefinery industry occur naturally in anaerobic environments such as in the forestomach of herbivores. Exploration of anaerobic microorganisms for industrial biotechnological applications creates the possibility to convey efficient and flexible processes, with lower implementation and running costs, making it also applicable to developing and emerging economies.

Despite the growing interest in anaerobic microorganisms for applications in industrial biotechnology, there is less information available concerning their diversity and function compared to what is known for their aerobic counterparts. To counter this, microbial diversity studies on an unexplored environment for microbial applications, was investigated by molecular and traditional cultivation techniques. The bacterial diversity of the forestomach of the llama, showed differences in the prokaryotes populations according to the complexity of the material type digested. Bacterial isolates were selected by their ability to produce compounds such as organic acids and alcohols and hydrolytic enzymes.

Also, a new strategy for cultivation of anaerobic microorganisms with the potential for an improved isolation rate and screening has been developed in this thesis work. The technique is based on single cell entrapment in alginate microbeads. A method was optimized for simple preparation under anaerobic conditions and successful cultivation of single cells was observed.

Further applications of anaerobic bacteria towards the biorefinery were also studied. The production of 1,3-propanediol, a compound used as building block for polymer materials, was investigated from selected llama isolates. The use of wheat straw as co-substrate and/or support material improved the concentration of 1,3-propanediol by 29% for *C. butyricum* BSL59 and 65% for *C. butyricum* BSL61 in comparison to using sole glycerol in the medium. The use of wheat straw was also superior in comparison to addition of pure sugars. Moreover, the solid residue from sequence batch fermentation using wheat straw as co-substrate showed to have high methane potential yield. Demonstrating that agriculture residue can be used in an integrated process for the production of valuable chemical compounds and energy carriers. Finally, a new method for cell immobilization forming a macroporous material was evaluated for butanol production which reaches high yields and allows repeated use of the cell-based material.

Key words

Anaerobic bacteria, industrial biotechnology, biorefinery, microbial diversity, single cell entrapment, llama rumen

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Cover photo: Llamas in the Bolivian Altiplano by Rodrigo W. Soria Auza and scanning electron microscopy images of bacteria studied in this thesis

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"Seja quem você for, seja qual for a posição social
que você tenha na vida, tenha sempre como meta
muita força, muita determinação e sempre
faça tudo com muito amor e com muita
fé em Deus, que um dia você chega lá.
De alguma maneira você chega lá"
Ayrton Senna

Popular Summary

Look around and notice how many items have been manufactured or transported using petroleum-based products. In modern life, it is almost an impossible task to exclude them from our daily routine. They are present in plastics, cosmetics, cleaning products, medicaments, food, etc. To diminish our dependence on the demanded black liquid, a lot of research efforts have been focused on replacing oil-derived chemicals by renewable sources.

Many microorganisms can naturally produce compounds that are considered as building blocks, comparable to "Lego® pieces", for the industrial production of several items. These compounds can replace oil-based products. Moreover, microorganisms can also produce fuels, for example ethanol. Among the vast diversity of microorganisms, anaerobic bacteria (which thrive in environments without oxygen) have been pioneers in industrial production of chemicals and fuels. With the urge to replace oil products, they gain once more interest due to their ability to naturally transform what can be considered as waste (such as crop parts which are not used for food) into valuable products. This is achieved thanks to a complex metabolism which allows them to consume different types of substrates. However, their sensitivity to oxygen and their peculiar metabolism has restricted the ability to cultivate these bacteria in the laboratory, and potentially limit their examination in comparison to their aerobic counterpart.

This thesis concerns about studies on anaerobic bacteria and their applications in industrial biotechnology. In particular, targeting the production of compounds relevant to a biorefinery, which is the counterpart of the petroleum refinery based on renewable feedstock. For that, the microorganisms from an unexplored environment, the stomach of the llama, were investigated for their potential use as producers of chemical compounds. The stomach of herbivores such as llamas is considered one of the most efficient natural processes for transformation of plants and is colonised by anaerobic microorganisms. These are likely to produce valuable industrially relevant compounds. However, it is not possible to cultivate all bacteria under laboratory conditions and therefore, a new method for cultivating single bacterial cells inside capsules was developed. This method can potentially allow the growth of "slow" microorganisms, be used to isolate bacteria and at the same time, speed up the detection of the industrially important ones.

Finally, the bacteria were used for the production of valuable compounds for the biorefinery sector, such as 1,3-propanediol (1,3-PD) and butanol, which can be used as solvents, building blocks and fuel. Bacteria were cultivated via different

strategies, which can be used to improve industrial production, aiming to take a little step forward towards a bio-based economy.

Abstract

Depletion of easily accessible fossil energy resources, threat of climate change and political priority to achieve energy self-sufficiency and sustainable solutions prioritize a conscious and smart use of renewable resources to generate a bio-based economy. Bio-based compounds can replace chemicals and fuels that are now mainly produced from crude oil. Efficient processes for the conversion of plant biomass into compounds of interest to the biorefinery industry occur naturally in anaerobic environments such as in the forestomach of herbivores. Exploration of anaerobic microorganisms for industrial biotechnological applications creates the possibility to convey efficient and flexible processes, with lower implementation and running costs, making it also applicable to developing and emerging economies.

Despite the growing interest in anaerobic microorganisms for applications in industrial biotechnology, there is less information available concerning their diversity and function compared to what is known for their aerobic counterparts. To counter this, microbial diversity studies on an unexplored environment for microbial applications, was investigated by molecular and traditional cultivation techniques. The bacterial diversity of the forestomach of the llama, showed differences in the prokaryotes populations according to the complexity of the material type digested. Bacterial isolates were selected by their ability to produce compounds such as organic acids and alcohols and hydrolytic enzymes.

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and energy carriers. Finally, a new method for cell immobilization forming a macroporous material was evaluated for butanol production which reaches high yields and allows repeated use of the cell-based material.

List of Publications

The thesis is based on the following papers, referred to in the text by their Roman numerals. Papers II and IV are reproduced by permission of the publishers. The papers are attached as appendices at the end of the thesis.

I. Bacteria from the forestomach of the llama (*Lama glama*) and their biorefinery potential.

Aragão Börner R, Pereyra L, Scaccia N, Pilgaard B, Alvarez Aliaga MT, Lange L, Mattiasson B. *Manuscript*

II. Microcultivation of anaerobic bacteria single cells entrapped in alginate microbeads.

Aragão Börner R, Alvarez Aliaga MT, Mattiasson B. *Biotechnology Letters*, 2013, 35:397-405.

III. Production of 1,3-propanediol from glycerol and wheat straw using isolates from llama rumen.

Aragão Börner R, Scaccia N, Viloria-Cols M, Badshah M, Alvarez Aliaga MT, Mattiasson B. *Manuscript*

IV. Immobilization of *Clostridium acetobutylicum* DSM 792 as macroporous aggregates through cryogelation for butanol production.

Aragão Börner R, Zaushytsina O, Berillo D, Scacia N, Mattiasson B, Kirsebom H. *Process Biochemistry, in press*

My Contribution to the Publications

I. The project was initiated by me and I was involved in the experimental design. I performed the experimental work together with Nazareno Scaccia, Luciana Pereyra and Bo Pilgaard. Data analysis was performed together with L. Pereyra. I wrote the first draft of the manuscript.

II. The project idea was initiated by Bo Mattiasson and I came up with the internal gelation system to make the microbeads. I was involved in the experimental design and performed all the experimental work. Data analysis was performed together with Maria Teresa Alvarez Aliaga. I wrote the first draft of the manuscript and finalised it in collaboration with the co-authors.

III. The project was initiated by me and I was involved in the experimental design. I performed the experimental work with N. Scaccia, Maria Viloria-Cols and Malik Badshah and analysed the data. I wrote the first draft of the manuscript.

IV. I was involved in the experimental design for the fermentation studies. I performed the cell cultivations and batch tests together with Oksana Zaushytsina and analytical quantification with N. Scaccia. Also, I wrote the first draft of the manuscript and finalised it in collaboration with the co-authors.

Abbreviations

1,3-PD	1,3-Propanediol
ABE	Acetone-butnaol-ethanol
ATP	Adenosine -5'-triphosphate
BS	Brave straw
CMC	Carboxylmethylcellulose
DGGE	Denaturing gradient gel electrophoresis
FP	Filter paper
FF	Fiber rumen fraction
LF	Liquid rumen fraction
NAD^+	Oxidized nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
PBS	Phosphate buffer solution
PCR	Polymerase chain reaction
PEI-GTA	Polyethyleneimine-glutaraldehyde
PVA-GTA	Poly(vinyl alcohol)-glutaraldehyde
RMS	Rapeseed methyl esther
rrs	16S rRNA gene
WS	Wheat straw
Xyl	xylan

Contents

1. Introduction

The depletion of easily accessible fossil resources, oil price fluctuation and the threat of climate change, has set the need for replacing the current oil-based economy. A political priority to achieve self-sufficiency and a sustainable industrial society, urge a conscious and smart use of renewable resources to generate a bio-based economy. With this focus, an upgraded use of bioresources to both feed the growing population and to use as a substitute for fossil-based products is needed. Microorganisms can play a crucial role in the development of a sustainable economy replacing oil derivatives by transforming agro-related and industrial waste streams into chemicals, materials and energy carriers (Souer *et al.*, 2008; Rittman, 2008; Rogers *et al.*, 2013). These production processes can be integrated within a biorefinery, which is the manufacturing facility that uses biomass as feedstock to produce the traditional oil-based products (Cherubini, 2010; Yang & Yu, 2013). The presented thesis contributes to the search for new microorganisms and processes to advance their application in the industrial biotechnology.

In this context, anaerobic bacteria have had a long history of industrial application for the production of fuels and chemicals (Goldstein, 1995; Wolfe, 1999). The pioneer process in the fermentation industry was the use of *Clostridium acetobutylicum* to produce alcohols and solvents since 1920 (Kumar & Gaven, 2011). Nowadays, anaerobic bacteria have regained attention for their unique biosynthetic capabilities, and advantages regarding substrate flexibility and toxicity tolerance for their industrial application (Tracy *et al.*, 2012). However due to their complex metabolism and often special cultivation requirements, they have been less explored than their aerobic counterparts.

In natural environments, anaerobic bacteria thrive where oxygen has been depleted and play a crucial role in carbon recirculation. They usually form consortia where organic matter is degraded in sequential steps in a synergetic action (Khanal, 2008). Examples of such environments are sediments from lakes and rivers and the gastrointestinal tract of ruminants, where a highly specialized microbial system has evolved to convert biomass. Microorganisms of industrial relevance for the biorefinery sector are likely to be found in such environments. To reach these potentialities, novel and smart cultivation techniques that facilitate microbial screening would be an advantage.

Moreover, the complex and interesting metabolism of anaerobic microorganisms, which result in various products with potential in many fields, brings advantages for their industrial application. Anaerobic processes for the

production of fuels and chemicals are advantageous over aerobic processes, considering broad substrate utilization, yields and productivities, flexibility, and also the investment in equipment and energy supply (Yazdani & Gonzales, 2007; Weusthuis *et al.*, 2011). A lower effort required for implementation and running costs (e.g., reduced energy required for tank aeration and cooling) is an advantage for the development of the biorefineries (Davis *et al.*, 2013) also in developing countries, where this venture could have an even higher social impact. Describing new microorganisms and products, and the further development of anaerobic processes can help a broader and global implementation, stepping closer towards a sustainable society.

Scope of the thesis

The aim of this thesis is to study anaerobic bacteria and their applications in industrial biotechnology. This is done by exploring the microbiota of new sources, isolating, screening and cultivating microorganisms of relevance for the biorefinery industry.

The thesis is divided into two sections. The first section provides scientific background information about the topics covered in the thesis and summarizes the results and potential of the presented research. The second section consists of the publications upon which this thesis is based on.

For the search for microorganisms with potential utilization in the biorefinery sector, a specialized system for biomass degradation, the llama rumen, was studied. Molecular techniques and traditional cultivation methods were applied, to assess its microbial diversity and microorganisms able to produce compounds of interest for the biorefinery industry (e.g., organic acids, alcohols, enzymes) were selected. Considering the limitations of the cultivability of anaerobic bacteria, a novel cultivation strategy based on single cell entrapment was developed and optimized for its application on strict anaerobes.

Regarding the applications of anaerobic bacteria, llama isolates able to convert glycerol into 1,3-propanediol (1,3-PD) were further investigated using wheat straw as a co-substrate to evaluate the production. Finally, the production of another alcohol of interest for the biroefinery industry, butanol, was studied. A new strategy for cell immobilization was evaluated using *Clostridium acetobutylicum* which aimed to improve the production of butanol and favor cell recycling.

2. Anaerobic microorganisms

Microorganisms that thrive in the absence of oxygen are called anaerobes. These are distinguished from their counterparts, the aerobes, for their ability to use alternative electron acceptors than oxygen to complete their energy/carbon cycle (anaerobic respiration / fermentation). In the case of anaerobic respiration, ultimate electron acceptors are reduced compounds. Such examples are nitrate (NO_3^-), ferric (Fe_3^+), sulfate (SO_4^{2-}), carbonate (CO_3^{2-}) and occasionally certain organic compounds (Madigan & Martinko, 2006). In the case of fermentative energy generation, organic compounds serve as electron donors and acceptors. These compounds are usually two different metabolites derived from sugars by cleavage. Fermentations are accompanied by the production of more or fewer reduced compounds, such as alcohols, organic acids, ammonia, hydrogen (depending on the regeneration of the cofactor) and carbon dioxide as the oxidized product (Schlegel and Jannasch, 2006). However, via this strategy, anaerobes obtain less net energy gain (Figure 1). This fact has implications for their metabolism, with the result that they generally are considered as "slow growers" than their aerobic counterparts.

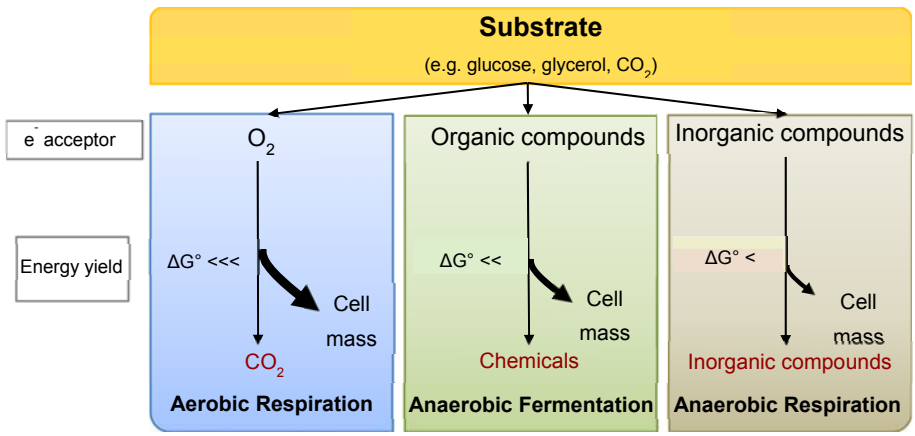


Figure 1: Schematic representation of differences between aerobic and anaerobic metabolism regarding the electron acceptor type, energy yield and cell mass formation. The free-energy change (ΔG°) depends on the type of the electron (e-) acceptor. The more oxidized the e- acceptor, the higher the energy yield available for the cell.

Anaerobes can be differentiated into aerotolerant anaerobes, which are microorganisms unable to respire oxygen (O_2) but whose growth is not affected by its presence and obligate (or strict) anaerobes. The alternative energy strategy of anaerobes has allowed them to colonize diverse environments on earth where oxygen is not present or has been depleted by aerobic organisms.

Obligate anaerobes are found in all three domains of life. The eukaryotes are represented by anaerobic fungi, ciliates and flagellates, and the archaea by the methanogens. Most genera of obligate anaerobes belong to the bacteria which were the main targets of investigations in this thesis.

2.1 Biological and metabolic considerations of advantages for industrial biotechnology

As mentioned above, the energy generation system of anaerobic bacteria is not as efficient as in their aerobic counterparts. For example, anaerobic fermentation of 1 mole glucose results in 2 molecules of ATP for the cell in comparison to 36-38 ATP molecules formed by aerobic respiration (Madigan & Martinko, 2006). However, in terms of biotechnological process application, this provides the advantage of less carbon being used for cell biomass generation and maintenance and more carbon directed towards product formation, which leads to a high product yield (Weusthius *et al.*, 2010).

Another point of interest for biotechnology is the wide range of products formed and substrates fermented (Zeikus, 1980). The formation of diverse products during fermentation can have negative implications on the downstream steps. However, when the metabolic routes are known for the microorganism, shifting the production towards one (or more) specific products can be achieved by process engineering (Dishisha, 2013) or by metabolic engineering (Jeng *et al.*, 2012). Opting for process engineering adaptations brings the advantage of maintaining all the routes still active in the microorganism. It also offers the benefit of using the same strain to produce different products, while obtaining high productivities by shifting the process operational conditions according to the market demand.

Most anaerobic bacteria have a substrate versatility and flexibility that is lacking in their aerobic counterparts (Tracy *et al.*, 2012), e.g. adaptability for the uptake of hexose and pentose sugars. Moreover, they do not only ferment sugars, but also have the machinery to degrade complex structural biomass (e.g., cellulose,

hemicellulose) (Zeiku *et al.*, 1980; Tracy *et al.*, 2012). This offers the possibility to potentially hydrolyse and ferment plant biomass into an efficient and integrated process, termed Consolidated BioProcessing (Lynd *et al.*, 2005). The simplification of several processing steps could provide many operational and economic advantages on an industrial scale (Tamaru *et al.*, 2010; Svetlitchnyi *et al.*, 2013).

Cellulases and hemicellulases have also been described from anaerobes (Leschine, 1995). The multifunctional enzyme systems from *Caldicellulosiruptor* thermophilic bacteria (Willquist *et al.*, 2010), and the cellulosomal system in e.g. *Clostridia* strains are examples of such unique systems (Bayer *et al.*, 2006). These enzyme complexes have a high biotechnological potential due to the optimized system to access the cellulose/hemicellulose fractions in plant biomass.

The provision of a renewable, consistent, affordable and regular supply of feedstock is an important stage in biorefinery systems (Cherubini, 2010; Hughes *et al.*, 2013). The broad range of substrate utilisation (from polymeric carbohydrates to sugars) by anaerobic microorganisms (especially bacteria), facilitates a nonrestrictive process to a single type of biomass feedstock (Lowe *et al.*, 1993). The process would then be more flexible in the possibility to use any available raw material and also by adapting the same microorganism to different geographical settings. In this way, technology transfer to developing countries would also be simplified.

2.2 Applications in industrial biotechnology

Anaerobic microorganisms, and especially bacteria, have played an outstanding role in the rise and expansion of industrial biotechnology. The first industrial fermentation process for the production of chemicals and fuels was the acetone-butanol-ethanol process (ABE) by *Clostridium acetobutylicum* in 1920's (Wolfe, 1999). The process was discovered and patented by Chaim Weizmann in the United Kingdom and was soon upgraded to an industrial scale, initially to supply acetone to the British artillery during World War I (Goldstein, 1995). Subsequently, the solvents were used in the chemical industry and as fuel for the expanding automobile industry (Kumar & Gayen, 2011) and resulting in the construction of industrial plants *(*Rogers *et al.,* 2006*)*.

With the expansion of the petrochemical industry, the production of chemicals and fuels by fermentation was mostly replaced by oil refining. However, in certain places (e.g. South Africa), the ABE process continued to be in operation until end of the 1980's (Rogers *et al.*, 2006). After oil crises at the beginning of the 21st century, the concern for replacing oil-derived products by renewable resources has

once again focused attention on biological processes and is now one of the main targets of the chemical industry. Genetically modified strains with anaerobic microbial metabolic routes are the core for the production of such compounds by industry nowadays. One example is the butanol production by Butamax™ (Donaldson *et al.*, 2011). Another successful example of a chemicals in large-scale production that can be produced by an anaerobic bacteria, but is commercialized from a genetically modified strain is 1,3-propanediol (Bio-PDO™), by DuPont (Erickson *et al.*, 2012).

Anaerobic microorganisms have been used in fermentation processes for food production for thousends of years (Goldstein, 1995). Food items (such as beer, cheese and vinager) that are produced by anaerobic fermentation by yeast or bacteria, obtain their characteristics by the formation of compounds such as propionic acid, lactic acid, ethanol, acetic acid and carbon dioxide. These compounds are also of interest for the chemical industry nowadays. To use traditional food-fermenting microorganisms for the chemical industry is an approach in the study of chemicals and fuels produced from renewable resources (Dishisha *et al.*, 2012). A commercially exploited example is lactic acid from *Lactobacillus bulgaricus* for the use in formulations for the cosmetic and pharmaceutical industry and polymer production (Datta *et al.*, 1995). However, wild anaerobic microorganisms encountered in natural selective processes (such as cellulose/hemicellulose rich environments) are a potential source of microorganisms or genes for reaching next generation processes for the conversion of biomass into value added products.

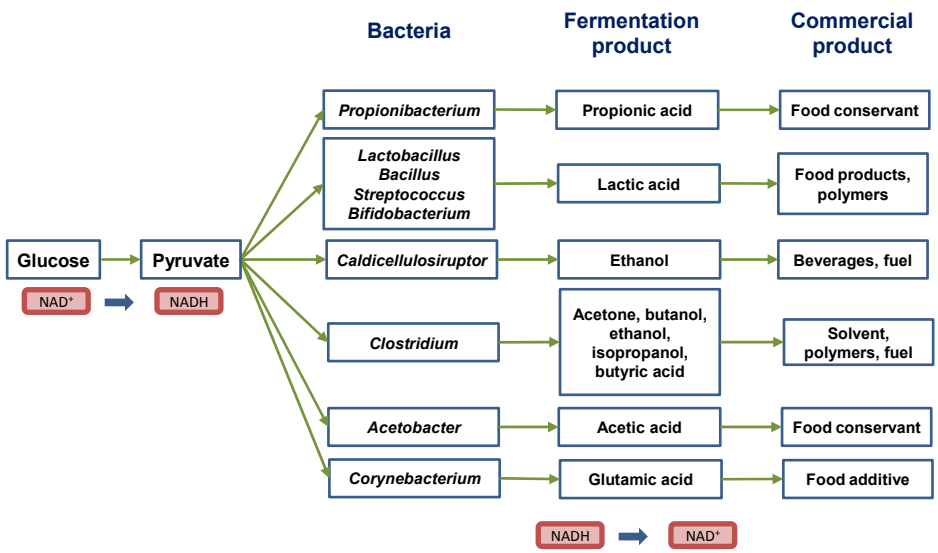


Figure 2: Examples of fermentation processes by anaerobic bacteria applied in industry.

2.3 Natural habitats

Anaerobic bacteria are widespread in almost all environments on Earth. Natural systems such as the sediment of rivers, lakes and oceans or the gastrointestinal tract (GI) of animals are habitats for these microorganisms. They can also be found in micro-environments where oxygen has been depleted by other aerobic organisms, such as in soil and decaying plant material (Zehnder & Stumm, 1988). Some microorganisms are enriched by human activities, such as in sewage plants, compost piles and anaerobic digesters for the production of biogas (Tamaru *et al.*, 2013).

These microorganisms play an important role in the carbon cycle by contributing to organic biomass degradation and by converting insoluble organic material to soluble compounds and gases that can circulate back to aerobic environments (Shmitz *et al.*, 2006). Considering their natural capability, it is a promising venture to search for interesting microorganisms in systems that have evolved under high organic load, with views for biotechnological applications.

2.3.1 Rumen (or foregut of herbivore animals)

The gastrointestinal (GI) tract of animals is colonized by microorganisms soon after birth or hatching (Ley *et al.*, 2008). The functional and metabolic characteristics of these microorganisms are relevant to the animal nutrition and health of the host. In herbivores, microorganisms play a key role, as they are responsible for processing plant material (Tamura *et al.*, 2013). The rumen of herbivores contains a vast collection of microorganisms that specialize in the rapid conversion of lignocellulosic plant biomass imposed by the continuous transit of feed through their gut (Morrison *et al.*, 2009). Ruminant and non-ruminant herbivores are distinguished by their ability to regurgitate swallowed feed material for remastication. This provides a greater feed surface area for rumen microbes and allows a greater extent of biomass degradation (Van Saun, 2006). The long evolution of this system under high biomass pressure makes the rumen of herbivores one of the most efficient natural processes (Flint *et al.*, 2012).

The microbiota of the rumen is comprised of representatives of all kingdoms of life: bacteria, ciliate and flagellate protozoa, anaerobic phycomycete fungi, methanogenic archaea and bacteriophage (Mc Sweeney & Mackie, 2012). The most predominant group is obligate anaerobic bacteria (Kim *et al.*, 2010). A large fraction of bacteria is attached to plant fiber (or undigested feed), as the cellulolytic

machinery of anaerobic bacteria it is commonly present on the surface of the cell (Weimar *et al.*, 2009).

In the rumen, a fine interaction between cellulose/hemicellulose- degrading microorganisms and non-cellulolytic bacteria occurs (Flint *et al.*, 2008). Many non-cellulolytic bacteria depend of the soluble polysaccharides released by the primary degraders. These bacteria ferment sugar and polysaccharides into products such as acetate, propionate, butyrate, carbon dioxide and hydrogen which are then used as nutrient by the animal (Weimar *et al.*, 2009). Moreover, the forage of herbivores contains other compounds such as proteins and lipids, which are also metabolized by the rumen bacteria (Mackie *et al.*, 1991). The focus of the research in **Paper I** was to explore the natural process of the rumen to find microorganisms with potential use for industrial biotechnological applications.

Although the cow rumen has been extensively studied since the 1960's with both traditional and later also with biomolecular methods (Bryant, 1996), the advancement of DNA sequencing techniques has demonstrated findings such as unique enzyme functions from isolated strains (Suen *et al.*, 2011) and an extremely rich biodiversity which still remains to a large extent "unknown" (Kim *et al.*, 2011). These reports demonstrate that there remains much more to be discovered and used from this system (Brulc *et al.*, 2009; Mc Sweeney & Mackie, 2012). Other animals with different feed type (e.g. fiber-rich) have the potential to be the source of even more useful metabolic systems for the conversion of biomass into value-added products (Weimer *et al.*, 2009; Qi *et al.*, 2011; Bhatt *et al.*, 2013).

The rumen of llama

Llamas (*Llama glama*) are a domesticated species of South American camelids (SAC) from the Andean Altiplano (3,500 to 4,500 m altitude) (Figure 2). They have traditionally been used for labor, food, leather and wool (del Valle *et al.*, 2008). Their dung is used for fuel (Israel, 2002) and has been studied for energy generation in the form of biogas (Alvarez *et al.*, 2006). These animals are the most abundant herds in the Altiplano and are of economic importance for the local population (Genin *et al.*, 1994; Tichit & Genin, 1997). Furthermore, llama farming and breeding is a growing industry in North America, Europe and Australia (Van Saun, 2006).

The vegetation on the Altiplano is dominated by coarse bunchgrasses, primarly formed of brave straw (*Festuca* and *Stipa* genera*)* due to the climate conditions (Genin *et al.*, 1994). As a consequence, the native pasture of llamas is characterized as being high in fiber and poor in nitrogen forage (Genin & Tichit, 1997). Despite their low nutritive diet (e.g., compared to cows), llamas are tall, robust and the

largest of the SAC species (San Martin & Bryant, 1989), reaching 110-140 kg. This indicates a highly efficient system for the conversion of coarse biomass for animal nutrition.

Llamas have three stomach compartments, instead of four as in cows (Alzola *et al.*, 2004), however they chew their cud and have an expanded rumen to facilitate the microbial fermentation of ingested feedstuff, a similar digestive strategy as in the "true ruminants" (Van Saun *et al.*, 2009). As their forestomach has the same function as a rumen, that is how it will be designated from now in this thesis. Based on their metabolism and feeding characteristics, the study of the microorganisms from the llama's rumen represents an opportunity to improve our understanding of biomass degradation and synergistic systems for the conversion of biomass into added-value compounds.

Figure 3: A) Llama (*Llama glama*), b) Rumen content, fiber fraction, c) Rumen content, liquid fraction, d) Brave straw (Photos by R. Aragão Börner).

The llama rumen is a very interesting system and little is known about its microbiota of llamas or native SACs and their function. The only report on the microbial characterization of llamas is related to protozoa diversity (del Valle *et al.*, 2008). The concentration and diversity of protozoa in llamas were lower than those of alpacas and other camelids and even lower compared to "true ruminants" such as cows and sheeps. This indicates that llamas have a strong selection pressure

on the microorganisms able to colonize their foregut, due to the fact that they feed on coarse pastures.

Selective medium containing structurally complex polysaccharides was used for the enrichment of specialized microorganisms from a sample collected from the rumen of a llama (**Paper I**). Initial results showed that the microorganisms had high potential for hemicellulose/cellulose degradation. A cellulosic substrate (filter paper) was completely degraded by the microorganisms 15 days after inoculation (Figure 4). Enzyme screening studies of the liquid and fiber fraction of the rumen, confirmed the presence of different enzymes involved in plant biomass decomposition.

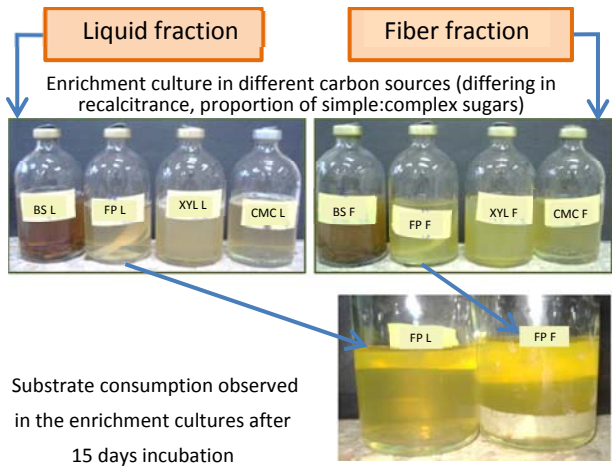


Figure 4: Enrichment of liquid (L) and fiber (F) sample from the llama forestomach on A) selective medium with complex polysaccharides. BS: brave straw, FP: filter paper, XYL: xylan, CMC: carboxylmethylcellulose B) Enrichments in FP from liquid and fiber fraction after 15 days of incubation.

To the best of the author's knowledge, the first description of bacterial diversity in the llama rumen is presented in **Paper I** and will be further discussed in Chapter 3 (Microbial Diversity). Knowledge about the microbial diversity of llamas can help the understanding of the synergetic interactions of microorganisms in this highly specialized system. Moreover, the production of compounds with potential for biorefinery applications such as 1,3-PD, lactic acid and succinic acid were detected in the isolates (**Paper I** and **Paper III**). The initial results obtained in this these findings encourage further studies into this system.

3. Microbial diversity

Bacteria are an essential component of the Earth's biota and account for the vast majority of its biomass (Whitman *et al.*, 1998). Prokaryotes also represent a large portion of life's genetic diversity, which also implies metabolic diversification and the formation of numerous chemical compounds. The characterization of microbial diversity has furthered the understanding of the metabolic potential in microorganisms occupying various ecological niches and has resulted in the discovery of many novel biotechnological products (Keller & Ramos, 2008; Prakash *et al.*, 2013). Although microbial diversity can be defined using several criteria (eg. phylogeny, physiology, metabolism and genomics), in this thesis, it is referred to as the different species in a given environment.

The interest in microbial diversity from a biotechnological perspective is not only to describe what inhabits in a particular environment, but to explore it towards a specific application. However, our understanding and ability to exploit microbial diversity in any system is limited to the technology available with which to assess and manipulate these microorganisms (Egan *et al.*, 2008).

The isolation and cultivation of new microorganisms, using traditional cultivation methods has resulted in innumerable discoveries for the biotechnology-related industry (Keller & Zengler, 2004). The cultivation of microorganisms is still a relevant approach, as it provides access to the whole microorganism including all its metabolites, moreover allowing potential in-depth physiological studies and genetic modifications (Prakash *et al.*, 2013; Zengler, 2013). Cultivable representatives are also required for the description of new species (Kämpfer *et al.*, 2003).

However, the advancement of molecular techniques and the information from the sequencing of the 16S rRNA gene or its hypervariable regions, helped to confirm that > 99% of the prokaryotes on Earth cannot be successfully cultivated via classical methods (Staley & Konopka, 1985; Pace *et al.*, 1997; Rappé & Giovannoni, 2003; Kim *et al.*, 2011b). Some of the currently uncultivable bacteria might have escaped discovery because they grow too slowly to be easily cultivable by traditional laboratory techniques or because they lack compounds or conditions necessary to support their growth (Zengler *et al.*, 2000). This is especially relevant to anaerobic bacteria, considering their particular metabolic characteristics and requirements such as a gas phase and the availability of electron acceptors, among other conditions, for their cultivation (Schmitz *et al.*, 2006; Speers *et al.*, 2009).

Therefore, new cultivation methods are needed to access anaerobic diversity and function.

3.1 Molecular techniques

The use of molecular techniques based on DNA sequencing has caused a revolution in microbiology based on the use of molecular phylogeny to characterize microbial diversity and to classify organisms based on their evolutionary relationships (Hugenholtz, *et al.*, 1998).

Molecular-based methods have given further insight into natural and man-made anaerobic environments, such as the rumen of cows (McSweeney & Mackie, 2012) and anaerobic digestors (Sundberg *et al.*, 2013) to cite a few. For an initial exploration of the bacteria present in the llama rumen (**Paper I**), DNA fingerprinting techniques, which allow a simpler differentiation among the communities studied were used.

3.1.1 16S rDNA

The corner-stone of current molecular microbial diversity is the use of DNA sequence analysis of the ribosomal small subunit, the 16S rRNA gene (*rrs*) (approximately 1540 bp) (Forney *et al.*, 2004; Kim *et al.*, 2011b). The *rrs* gene contains both highly conserved regions, which allow the positioning of DNA sequence alignments, and highly variable regions (V), which allow evolutionary comparisons between the microorganisms (Pontes *et al.*, 2007). This makes the *rrs* gene a suitable marker to examine microbial diversity and to classify microbes (Kim *et al.*, 2011b). Partial *rrs* information based on the sequence of one or more V regions (200-600 bp), has also been used as a phylogenetic marker when it is not possible to access the full length of the 16S rRNA gene due to technology or cost limitations (Kim *et al.*, 2011b).

Phylogenetic identification of the llama isolates (**Paper I**), was performed using the information from the partial sequence of the *rrs* gene (~1200 bp). In this study, only selected microorganisms that produced industrially relevant compounds (such as 1,3-propanediol, succinic acid or lactic acid) were sequenced. All sequenced isolates were closely related (>97%) to C*lostridium butyricum,* a known solvent-producing and biotechnologically relevant bacteria (Weigel *et al.*, 2006). This species is known to produce butyric acid from sugars, which in salt form is

incorporated into a large number of commercial products such as food additives and flavors, plastics, drug formulations and fragrances (Zidwick *et al.*, 2013).

The genus *Clostridium* contains by far the most described species of anaerobic bacteria, followed by *Bacteroidetes* and *Bifidobacterium* (Schmitz *et al.*, 2013). The high abundance of *Clostridium* isolates from environmental samples might be related to the high cultivability of the members of this genus under conventional laboratory conditions. However, besides *C. butyricum,* this genus includes other relevant biotechnological species such as *C. acetobutylicum*, known as a solvent-producing bacteria and also used in this thesis and *C. thermocellum*, a known highly cellulolytic strain (Weigel *et al.*, 2006).

Methods based on the 16S rRNA fragment such as cloning, metagenomics and direct sequencing using next-generation-sequencing techniques have been applied to environmental samples for biodiversity studies (Keller & Zengler, 2004). The use of such of next-generation-sequencing techniques can provide greater in-depth access to microbial diversity and should be used in further studies. However, other molecular-based techniques can be used to initially access the structure of an environmental sample, such as DNA fingerprinting.

3.1.2 DGGE

DNA fingerprinting techniques exploit the fact that each microorganism has a different DNA gene variant, depending on the targeted region. The difference can be visualized using electrophoresis. The use of 16S rRNA gene markers is the most common approach to assess community structure, although the method can also be used with metabolic genes (Hiibel *et al.*, 2008; Vasileiadis *et al.*, 2012).

Fingerprinting methods have been extensively applied to study microbial diversity from environmental samples. Among these, denaturing gradient gel electrophoresis (DGGE) has been the most widely used molecular fingerprinting technique to provide information about changes in the numerically dominant bacterial populations from anaerobic environments (Torsvik *et al.*, 1998; Yu & Morrison, 2004; Dhamwichukorn, 2008).

In PCR-DGGE, either a single hypervariable region (V) or a combination of two or three V regions in *rrs* genes is amplified (Muyzer & Smalla, 1998; Yu & Morrison, 2004). The amplification is performed using primers attached to a GC-clamp to one side of the DNA fragment and the resulting amplicons of the same length are distinguished by their sequences. A different DNA base composition in the V region gives PCR products with different melting points (Torsvik *et al.*, 1998). The DNA fragments are then separated on a polyacrylamide gel containing

a linear gradient of denaturant (a mixture of urea and formamide). Separation within the gel is based on the decrease of electrophoretical mobility of the partially melted double-stranded DNA molecule (Muyzer & Smalla, 1998). The molecules with different sequences will cease migrating at different positions in the gel, depending on their melting point. Finally, the GC-rich sequence acts as a high melting domain preventing the two DNA strands from complete dissociation into single strands.

With community fingerprinting, an overall understanding of a community structure based on the most abundant bacterial groups can be achieved with the advantage that it can be performed relatively quickly and at low-cost (Muyzer & Smalla, 1998). The technique is, however, limited by low sensitivity as it restraints the detection to populations that are present below 1% (Muyzer *et al.*, 1993). Nevertheless, many samples can be analyzed simultaneously and changes in the microbial populations will be reflected in fingerprint differences induced by specific changes in the environment which can be followed.

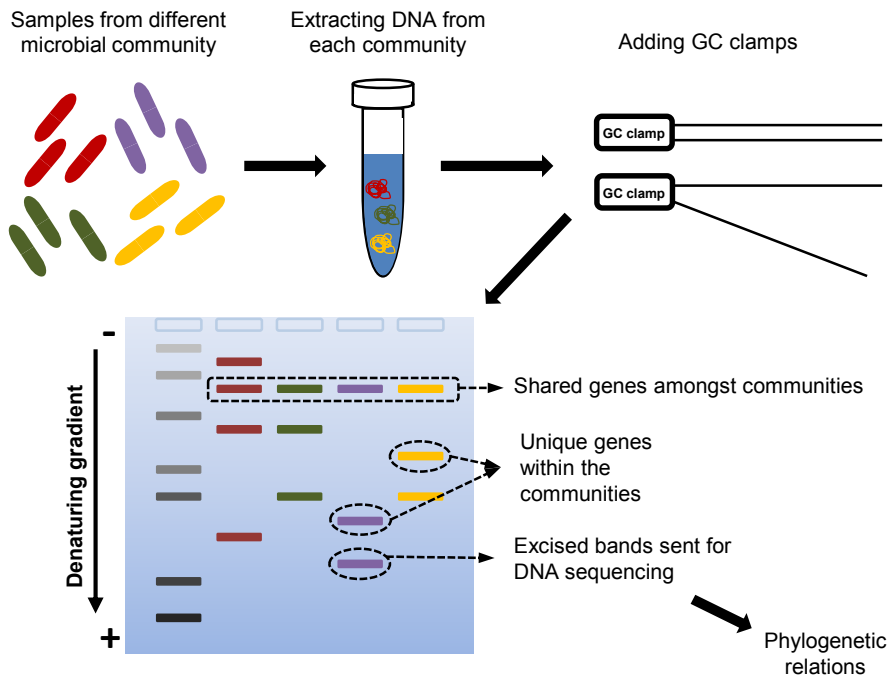


Figure 5: Schematic representation of the PCR-DGGE technique to assess microbial community structure.

This approach has been used in **Paper I** to assess the community structure in the fiber and liquid fractions of the llama rumen, as well as in the communities following enrichment of these two samples on different polymeric carbon sources (CMC: catboxymethylcellulose, BS: brave straw, Xyl: xylan, and FP: filter paper).

The sample richness (number of bands present) in the sample and the clustering of the banding patterns showed fewer bands in the fiber rumen fraction, indicating that the there is a difference in community composition in the liquid or fiber fraction. The clustering of the band patterns indicates changes in the original community after enrichment on the selective carbon sources (Figure 6). A remarkable change was observed in the fiber fraction enrichment in brave straw. Its separation from the other samples into a unique cluster indicates that the complexity of this material used for the enrichment, selects for the formation of a more specialized community of microorganisms. This selection process was also reflected in the presence of exclusive banding patterns in the liquid (five bands) or fiber fractions (four bands).

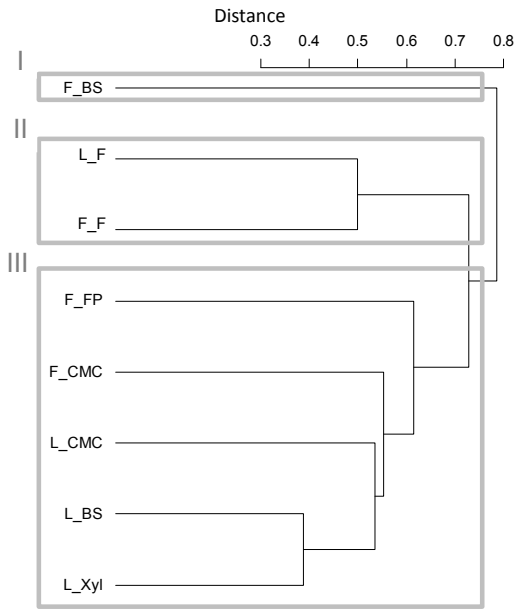


Figure 6: Clustering results of 16S rRNA fingerprinting of rumen fractions and enrichment cultures using UPGMA and the Jaccard coefficient based on banding patterns. L_F: liquid rumen fraction; F_F: fiber rumen fraction; L: liquid enrichment; F: fiber enrichment.

Identification of community members of the llama rumen

Sequencing of the obtained DNA fingerprints from the PCR-DGGE can be used to access the phylogenetic information of the community, bypassing cultivability limitations. It was possible to identify members of the phyla Firmicutes and Bacteroidetes from the llama samples by applying this approach. Several band sequences were closely related to microorganisms that do not have a cultivable representative in both phyla. This is observed in the phylogenetic tree constructed from the phylogenetic relationships between the DNA sequences (Figure 7). Some of the uncultured-related sequences have also been previously described to be present in the rumen (Fernando *et al.*, 2010). However, their cultivation remains a challenge that requires new approaches.

The sequences of most bands were related to the Firmicutes phylum and belonged to different families of the Order Clostridiales, mainly represented by the genus *Clostridium*. Some sequences detected in the llama samples were closely related to other *Clostridium* representatives known for their cellulolytic/hemicellulolytic activity such as *Clostridium cellulolyticum* and *C. cellulosolvens* (Scmitz *et al.*, 2013) and were associated with the liquid enrichment cultures (Fig 7). Among the Bacteroidetes, another banding pattern present in the liquid sample and enrichments was closely related to *Provetella ruminicula*, a frequently described species in the rumen of cows and sheep (Kocherginskaya etl., 2011; Stevensson & Weimar, 2007).

The molecular-based analysis of the microbial diversity structure in the llama rumen indicated that especially the fiber fraction and the enrichments in brave straw (BS) create a selective environment. The distribution of the microbial populations is probably related to the ability of the microorganisms to degrade complex material (Brulc *et al.*, 2009). However, hemicellulolytic and cellulolytic bacteria appeared were also present in the liquid fraction. More in-depth diversity studies using high-throughput sequencing might shed more light on the patterns of substrate colonization and free-swiming prokaryotes in the llama rumen.

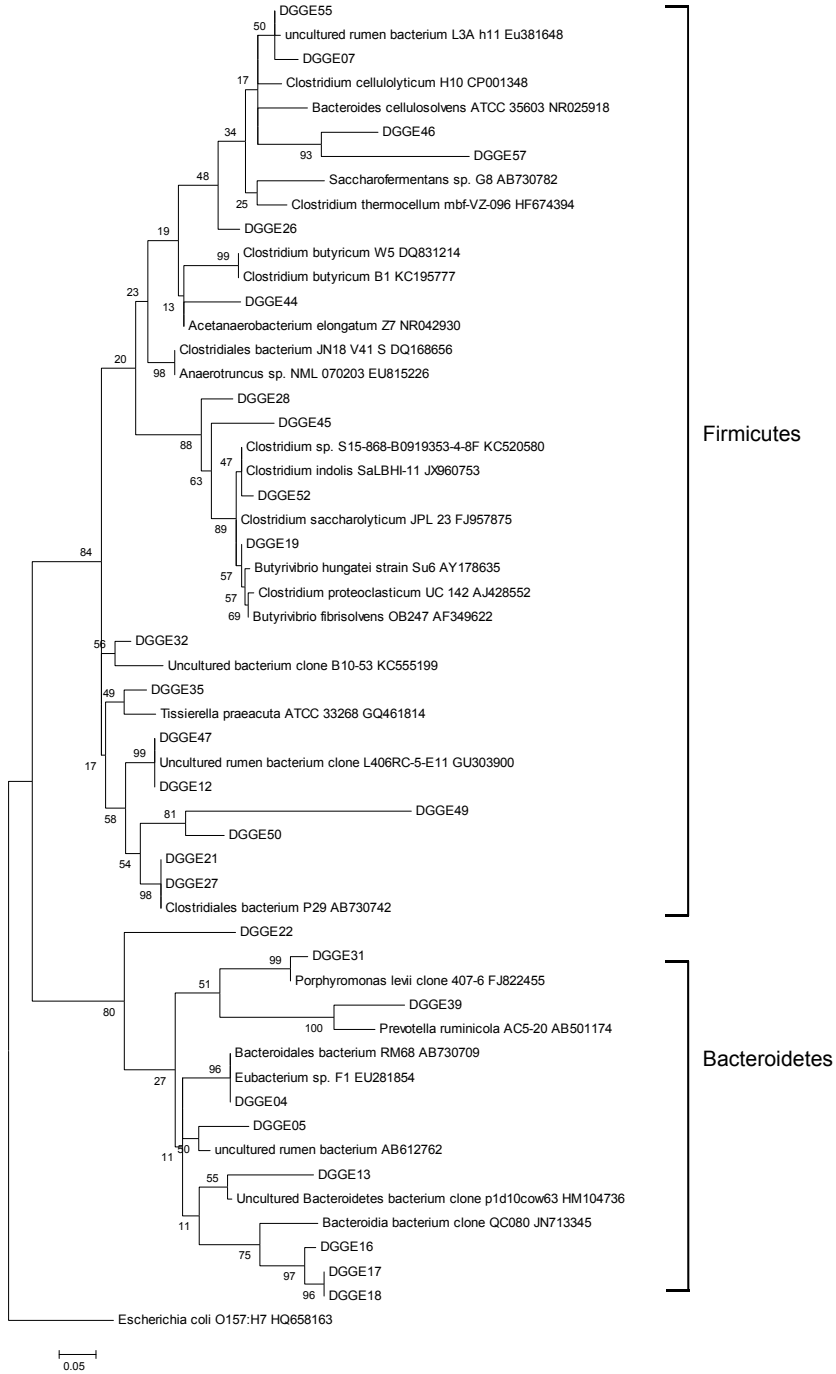


Figure 7: Phylogenetic tree from the DNA sequences of DGGE bands from the llama rumen content.

3.2 Cultivation techniques

The cultivation of microorganisms is still relevant to advance knowledge of microbial diversity for the following reasons: it provides reference strains for in-depth physiological studies, broadens our view in the area of basic microbial research and provides access to new organisms for novel metabolites of commercial interest.

An analysis of the large number of novel cultivation methods described over the last 15 years, indicates that at least some so-called "unculturable" microorganisms are not fastidious, but they might be too rare to be captured (Buerger *et al.*, 2012). One of the strategies to overcome this is simply to increase the conventional cultivation effort and geography of sampling (Epstein, 2013), as many environments on Earth have not been explored, such as the llama rumen. Considering that the access to new isolates can potentially bring new metabolites and applications, the isolation effort is justified.

In this thesis, conventional anaerobic cultivation techniques were used to isolate bacteria from the rumen of llama (**Paper I**) based on the Hungate and roller agar technique. However, a new method for the cultivation of cells was also developed, based on single-cell entrapment, with a potential application in bacteria isolation (**Paper II**).

3.2.1 Conventional cultivation techniques

Strict anaerobes require an oxygen-free environment and a low redox potential for growth. The anaerobic technique developed by Robert Hungate (Hungate, 1969) was later modified and is now commonly used in all major laboratories that deal with strict anaerobic cultures. It is based on the formation of anoxic gases by the passage of an inert gas such as N_2 and CO_2 through a heated copper column (previously prereduced with H_2) (Speers *et al.*, 2009). The anoxic gas produced is then used to replace air in glass vials containing culture medium, which are sealed with rubber stoppers and closed with aluminum caps. To generate a low redox potential in the medium, reducing agents such as cysteine hydrochloride are commonly used. Other components of the medium that can be limiting for certain anaerobic groups are the absence of metals as trace elements and vitamins (especially vitamin B) (Mc Sweeney *et al.*, 2005). To isolate anaerobic bacteria, solid media are usually prepared according to the roller-tube technique, although

classical microbiological techniques on Petri dishes can also be used if handled inside an anaerobic chamber.

In general, the steps involved in obtaining a microbial culture can be challenging. Many factors can increase the isolation rate and allow cultivability of bacteria from an environmental sample. It has been suggested, that the best approach given a limited time and budget to obtain cultures of interest, is to maximize the cultivation success by simulating conditions of the natural environment,as this is considered the most critical step (Zengler, 2013).

Therefore, for the enrichment and isolation of llama rumen bacteria, clarified rumen liquid was used as a medium supplement (**Paper I**). In this way, metabolites and other compounds existing in the rumen were also present to some extent in the cultivation medium, with the aim of mimicking as closely as possible the conditions the original environment. The cultivation temperature and pH in the medium were also adjusted according to the *in situ* measurements from the llama rumen.

Enrichment of the rumen samples with selective polysaccharide substrates was used to limit the isolation based on their ability to grow on the specific cellulosic and hemicellulosic materials. Using this procedure, 156 bacterial isolates were obtained from the llama rumen and were further studied for detecting the production of biorefinery-relevant metabolites. The results obtained in this llama rumen study, related to complex substrate utilization provide important data for the functional biodiversity within the llama rumen.

3.2.2 New cultivation techniques

Although it is difficult to affirm that all microorganisms are culturable in certain way, it is possible to suggest that most microorganisms that are so-called "unculturable" should actually be considered "not yet-cultured", reflecting the fact that insufficient knowledge exists concerning how to cultivate these microorganisms under laboratory conditions.

Progress has been made in developing novel cultivation strategies that allow the improved growth of bacteria. In the particular case of anaerobes, strategies using different media compositions, including alternative gelling agents and reducing media (Carbonero *et al.*, 2010; Nyonyo *et al.*, 2013) were efficient in the cultivation of bacteria oreviously considered "unculturable". Other methods have focused on simulating the natural environment using *in vivo* incubation and special chambers (Aoi *et al.*, 2009). However, very promising methods for obtaining anaerobic cultures and coupling these with a high-throughput screening method

are based on the use of single cells. This can be performed with free (Hamilton-Brehm *et al.*, 2012) or cells entrapped into a polymer matrix (Zengler *et al.*, 2002; Zengler *et al.*, 2005; **Paper II**).

Single cell entrapment

Single cells entrapped into a polymer matrix (agarose or alginate) can be further cultivated in liquid medium to allow microcolony formation. This strategy allows a 3D structure for cell cultivation, offering support and mechanical stability for easier handling. When single cells of two or more bacterial species entrapped in the microbeads are cultivated together in liquid medium, community conditions can be simulated. In this case, the polymer beads provide protection from competitors and toxic compounds in the environment. Nevertheless, due to the porosity of the material, nutrient exchange with the medium and other metabolites and compounds necessary for cell-to-cell communication in cultivated communities can occur.

This strategy has been proven to be successful with slow-growing bacteria such as *Mycobacterium* strains (Weaver *et al.* 1988, Manome *et al.* 2001, Akselband *et al.* 2005). However, the most interesting results were obtained by Zengler *et al.* (2000), who developed a high-throughput method for single cell entrapment and cultivation of bacterial cells from sea and soil samples. This allowed the detection of strains that were previously considered uncultured. Recently, it has also been shown that gel microbeads can be used to enrich single cells from an environmental sample for whole-genome sequencing (Fitzsimons *et al.*, 2013).

Most published studies on the cultivation of single cells in microdroplets, have used polymer microbeads produced consisting of agarose as matrix and prepared with a special equipment (Once Cell Systems Inc., Cambridge, MA, USA). However, an alternative method of microbead preparation was desired, which could easily be performed inside an anaerobic chamber, to maintain anaerobic conditions during the whole entrapment process. Optimally, no special equipment would be required so that the method could be easily performed under different laboratory conditions.

For this purpose, a method using alginate as an entrapment material was optimized in the work presented in **Paper II**. The alginate polymer is inexpensive and widely available as a food or medical-grade material (Reis *et al* 2006). It has several unique properties that have enabled its use as a matrix for the entrapment of different biological materials such as biocompatibility and mild process for polymerization. Using an emulsion formation with vegetable oil, alginate microbeads with less than 100 µm diameter can be produced (Poncelet, *et al.* 2001). This method has the advantage of being safe, simple, low-cost and

adaptable to any laboratory conditions, as it has also been tested in Bolivia and India.

Conditions were optimized to obtain microbeads in the range of 15-80 µm with the emulsion method (**Paper II**).This size-range was desired, to ensure that most beads contained single cell during the entrapment procedure, whilst allowing space for microcolony formation within the beads. The type of oil used to form the emulsion was shown to be the main factor that determines the formation of smaller beads. Rapeseed Methyl Ester (RME, or biodiesel) is ten-fold less viscous than rapeseed oil, offering less resistance for the emulsion, forming smaller aqueous droplets, which will then generate smaller alginate beads. Finally, the conditions selected to form the alginate beads consisted of RME as hydrophobic phase, presence of baffles in the beaker and rotation impeller of 100 rpm.

Alginate microbeads containing cells of the anaerobic strain *Clostridium sulfatirreducens* CCUG 50825 were cultivated in minimal medium with cellobiose. After 24 h cultivation, microcolonies were observed inside the beads (Figure 8), showing that it is possible to cultivate strict anaerobes using this technique. Although the technique has only been tested with bacteria, it has the potential to be used for other anaerobic microorganisms, such as archea.

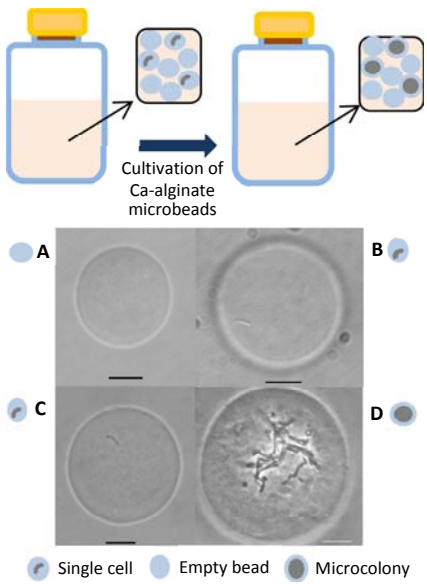


Figure 8: Microcultivation of single cells of *Clostridium sulfatirreducens* CCUG 508225.

Detection by flow cytometery

Flow cytometry and sorting techniques have been applied to the isolation and cultivation of uncharacterized or slow-growing microorganisms from the environment, including anaerobic systems (Lundin, 2013; Müller *et al.*, 2012; Hamilton-Brehm *et al.*, 2012).

In an initial study, it was possible to distinguish alginate microbeads with entrapped cells from free cells, empty beads and alginate debris in a suspension using flow cytometry (Figure 9) (Aragão Börner *et al.*, 2011). Cells were stained with Syto 9, which only marks viable cells. The results showed that this technique is also suitable for selecting and sorting alginate microbeads containing microcolonies. The sorted microbeads can be further cultivated in separate containers, such as the wells of a microtiter plate. However, in the particular case of anaerobic bacteria entrapped in microbeads, modifications are needed in the equipment to maintain the sheet fluid free of oxygen and avoid cell damage during the detection and sorting procedure (unpublished data).

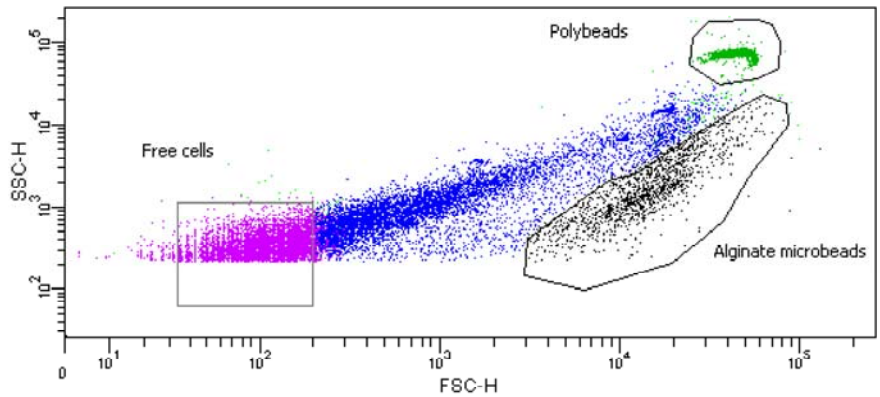


Figure 9: Dot-plot representation of side versus forward scatter data of suspended particles in a flow cytometer Aria II. Dots visualized in purple are free cells in the suspension (not entrapped); the polybeads for size reference are in green and, the alginate beads with entrapped cells are in black. All blue particles visible are alginate debris (Aragão Börner *et al.*, 2011).

This experiment also showed that it is possible to read fluorescent signals from entrapped cells in the alginate beads. It demonstrates that screening techniques that include fluorescent markers can be used for further selection of the targeted microorganisms.

3.3 Screening strategies

The search for novel chemicals and catalysts for application in industrial biotechnology is dependent on adequate methods for the detection of such compounds. These might originate from a collection of microbial strains or for direct detection of functional genes from environmental samples, such as in the case of metagenomics.

3.3.1 Chemicals

For the detection of many metabolites, in special catalysts, there are different techniques for the rapid detection of their production, using for example, color indicators or color substrates (Percival Zhang *et al.*, 2006). However, for the detection of chemicals such as 1,3-PD or succinic and lactic acid in wild strains, there is no optimized process for rapid screening (Petidemage *et al.*, 1995; Zhang *et al.*, 2007; Szymanowska-Powalowska *et al.*, 2013). For this, compounds in the supernatant of cell cultures from llama isolates were detected using HPLC (**Paper I**). These compounds were alcohols (1,3-PD, ethanol) and organic acids (succinic, lactic). From 148 isolates studied, 52 were able to produce one of the targeted compounds and from those positive producers, 10 isolates were selected by their ability to produce more than one of the compounds of interest. Furthermore, sugars were released into the medium from the hydrolysis of the complex carbon substrate.

3.3.2 Enzymes

The presence of enzymes able to hydrolyze different components of plant biomass, such as starch, cellulose and hemicellulose, was evaluated in the 10 selected isolates. For this, a rapid screening method was used, which is based on insoluble chromogenic substrates; if cleavage of the substrate occurs, a blue coloration will appear (Figure 10). This technique can be used to test whole cells by colony transfer onto agar plates (Bastien *et al.*, 2013) or using cell extracts (Pedersen *et al.*, 2009; Schmidt *et al.*, 2012).

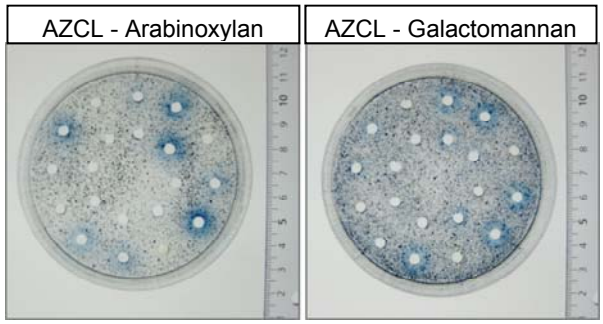


Figure 10: Enzyme screening assay using insoluble chromogenic substrates.

For the selected llama isolates, different cell fractions were screened, considering the soluble enzyme (in the supernatant), membrane-bound and intracellular systems. This was performed bearing in mind that the presence of insoluble cellulase systems (cellulosomes), which are associated to the cell wall are common in anaerobes (Bayer *et al.*, 2006). All isolates tested demonstrated activity against at least one substrate; malt-β-glucanase was the most common enzyme activity detected. In addition, the isolates grown in xylan showed hemicellulolytic activity against galactomannan. Besides the isolates, the rumen fractions were also tested and showed broad enzyme activities against starch, cellulose and hemicellulose.

Substrate use and bacterial chemical production profiles provide important data for studies on functional biodiversity (Pontes *et al.*, 2007). The llama rumen appears to contain microorganisms with potential for biotechnological applications, and some compounds of interest for biorefinery were detected from the isolates. Such a system offers a great potential for further study on the enzymes. Other methods such as sequence-based metagenomics could be used to explore the system even more towards the detection of efficient catalysts.

3.3.3 Screening strategy using microbeads

It is possible to combine high-throughput cultivation with screening tests using the strategy of single-cell entrapment in polymer microbeads. Selected compounds added to the liquid medium can be used to target specific metabolic activities, such as the use of sugars as cellobiose. However, to select microorganisms able to degrade complex polymers, it might be possible to entrap the substrate together with the cell within the alginate microbead.

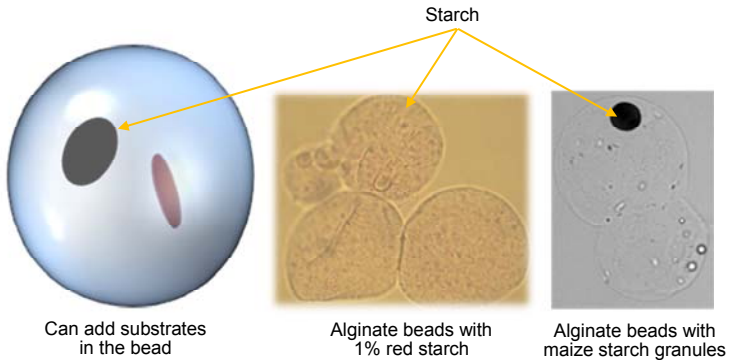


Figure 11: Addition of the substrate in the polymer alginate microbead.

This possibility was tested in initial studies using maize-based and chromogenic starch (Fig. 11). The cultivation of cells in alginate beads has been previously described (Sultana *et al.*, 2000). In this way, microorganisms able to grow using starch as a substrate would potentially form microcolonies in the bead which could be sorted out by flow cytometery or by another sorting technique. Another possibility would be to add fluorochrome-coupled substrates to the bead or to modify the polymer structure by the addition of fluorochromes that are activated when a certain metabolic activity in the cell is present. In this way, the polymer bead would fluoresce with different intensities, indicating different activity levels. This system has a high potential for applications in industrial biotechnology, however it requires more research.

4. Industrial biotechnology for the development of the biorefinery

The use of microorganisms as fermentation agents producers of biocatalysts is extremely relevant for the conversion of biomass and the development of biorefineries. It provides a cleaner, energy-efficient and less toxic process compared to conventional chemical processes (Dishisha, 2013). Industrial biotechnology processes have a great impact on the economy, with over US$ 89 billion dollars revenue reported in the year 2012 (Ernst & Young, 2013).

The use of the multiple components in biomass, intermediates, and derived products is intended in a biorefinery industry setting. The goal is therefore, to maximize the value derived from the feedstock while minimizing the waste (Cherubini, 2010; Yang & Yu, 2013). For this, a biorefinery might produce one or several low-volume but high-value products (e.g. pharmaceuticals) but also a low-value, but high-volume liquid transportation fuels (e.g. butanol), while generating process heat and electricity (e.g. methane).

New microbial sources or processes for the production of valuable compounds that can help to achieve this goal are needed, to improve commercialization of biorefinery products.

4.1 The need to valorize waste – as raw material for the biorefinery

Sugars (mainly glucose) derived from maize and sugar cane, and oils from soybean have been the main initial resources for the establishment of the bio-production of industrial compounds (Yang & Yu, 2013). However, it is not sustainable to use food and feed sources for the industry, considering the growing global population. This can be avoided by using the agricultural residues from different crops as raw materials. The same scenario applies to industrial residues from the processing of organic raw materials, such as by-products from milk and fat processing (Stabnikova *et al.*, 2010). The use of agricultural and agro-industrial by-products (or waste streams) would maximize the resource use, reduce waste management, and diversify the economy by creation of added-value products. It also affects positively affects the environment by reducing greenhouse gas emissions and

diminishing potential eutrophication of water bodies near the production plants in comparison to the exclusive use of carbohydrates for the conversion process (Cherubini & Ulgiati, 2010).

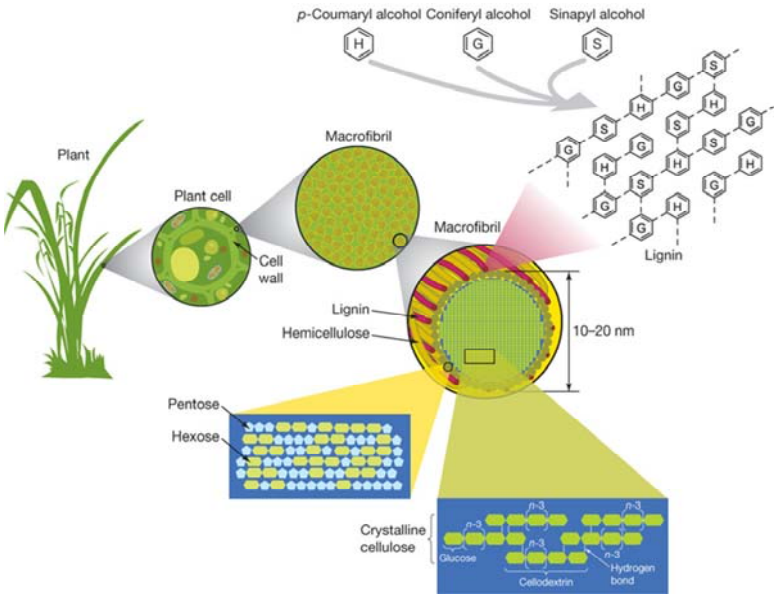


Figure 12: Structure of lignocellulose. Reprinted by permission from Macmillan Publishers Ltd: Nature (EM Rubin (2008) 454, 841-845 doi: 10.1038/nature07190), copyright 2008.

Agricultural residues are mainly formed by lignocellulosic materials, which consist of a complex structure of interwoven complexes of biopolymers such as cellulose, hemicellulose and lignin (Figure 12) (Taherzadeh & Karimi, 2008). Although lignocellulose is the main component, proteins and lipids are also part of plant composition in different concentrations (Sluiter & Sluiter, 2010). To access the polymeric sugars in cellulose and hemicellulose fractions, a pretreatment step for the biomass is required (Figure 11). Such pretreatment is a costly and energy-demanding process for the industry and includes mechanical, chemical and enzymatic steps (Takara & Kumar Khanal, 2012). The high cost of biomass conversion is recognized as the major obstacle for its commercialization (Himmel *et al.*, 2010). The use of microorganisms that are able to degrade complex carbohydrates and simultaneously ferment the released sugars and produce compounds of interest for the development of a biorefinery would reduce the burden of the pretreatment processes. Moreover, enzymes produced from such

microorganisms can be purified and used as catalysts for the final hydrolytic step of a pretreatment to release sugars.

4.1.1 Wheat straw as an agricultural by-product

Wheat is the most widely grown crop worldwide, with availability in 115 countries and grown under different environmental conditions (Talebina *et al.*, 2010). About 21% of the world's food depends on wheat and its global production needs to be increased to satisfy the growing demand for human consumption (Ortiz *et al.*, 2008). The large-scale production of wheat generates a large quantity of straw (1 kg of wheat straw per kg of harvested wheat), which can be left in the field, plowed back into the soil for nutrient recirculation, burned or removed from the land (Jiménez *et al.*, 2002; Talebina *et al.*, 2010). The production of wheat straw doubles of what is needed to plow back in the soil to avoid soil impoverishment. Moreover burning straw results in air pollution and is a risk for human health (Li *et al.*, 2008). Thus, there is a need to find an alternative way to treat this residue material.

From a biorefinery perspective, wheat straw is an abundant and low-cost lignocellulosic agricultural residue which does not compete with the food/feed chain and can represent a good feedstock for bioconversion processes. Consequently, several research groups have focused on the valorization of wheat straw for the production of chemicals and fuels such as succinic acid (Li *et al.*, 2010), butanol (Qureshi *et al.*, 2013), ethanol (Erdei *et al.*, 2010) and biogas (Nkemka & Murto, 2012).

In **Paper I**, wheat straw was used as the sole carbon source for cultivation of llama isolates that were enriched and isolated from brave straw. This was performed with the aim of detecting microorganisms able to produce organic acids or alcohols using this straw as substrate. Brave straw (BS) is the natural feed of llamas and has a similar composition to wheat straw (Carrasco *et al.*, 2011). To have access to microorganisms able to produce value-added products from wheat straw opens the possibility to have a more cost-efficient process, considering the abundance and low-cost of the raw material. Furthermore, isolates able to grow on wheat straw and glycerol as the sole carbon source were further studied for production of 1,3 propanediol using both substrates in **Paper III.**

4.1.2 Glycerol as an agro-industrial by-product

Glycerol is a polyhydric alcohol that can be produced from renewable feedstock either by microbial fermentation or trans-esterification of vegetable and animal fat, and from petrochemical feedstock by chemical synthesis (da Silva *et al.*, 2009). Currently, it is mainly formed as a by-product of the production of biofuels (mainly biodiesel), representing 10% of total biodiesel produced (Mattam *et al.*, 2013). As the production of biofuels increases every year, so does the production of glycerol (Almeida *et al.*, 2012), with disposable costs attached to it (Yazdani & Gonzalez, 2007). Moreover, the surplus glycerol produced from the biodiesel industry has resulted in a 10-fold decrease in its market price during the past decade (Yazdani & Gonzalez, 2007) and opportunities for its industrial conversion into valuable products have developed.

Glycerol can be used as a carbon source for microbial fermentation. In recent years, the availability of the substrate has led to significant research efforts into the microbial conversion of glycerol into value added chemicals and fuels (Mattam *et al.*, 2013). One example is the conversion of glycerol into 1,3-PD, which was studied in **Papers I** and **III**.

4.2 Bio-based chemicals as products

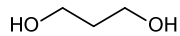
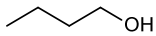
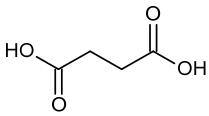
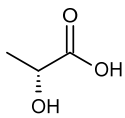
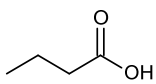
Nowadays most chemicals are synthesized from non-renewable feedstock; however, the microbial production of organic acids and alcohols is a promising approach to obtain such industrially important building blocks from renewable resources. Building-blocks or "platform chemicals" are molecules with one or multiple functional groups that can be transformed into new families of usable molecules (Sauer *et al.*, 2008).

According to the estimation of McKinsey and Company, the consulting firm, 2% of today's chemical market, $1.25 trillion total value, is produced using biotechnology processes. The fraction of the market is expected to grow, as biotechnological conversion processes have demonstrated such benefits that investment in this area has increased (Stabnikova *et al.*, 2010). This is also due to a recent slowdown in the market of biofuels, based on sugars as feedstock (Clean Energy Trends, 2012). Although the tendency is to move towards lignocellulose-based biofuels, many producers are looking for alternative end-uses for their

products and a number of companies have changed their focus from producing fuels, to producing chemicals of high-value (Aylott & Higson, 2013).

Finding innovative fermentation processes for the cost-effective production of chemicals from renewable resources is a necessity (Weusthius *et al.*, 2011; Lin *et al.*, 2011) and new strategies for the production of alcohols and solvents such as 1,3-PD and butanol were evaluated in **Paper III** and **IV**. Microorganisms with more efficient conversion and a broader substrate uptake, including complex polymeric carbohydrates, would be an advantage for the improvement of bioprocessing in the production of such compounds. For this reason, isolates from the llama rumen that are able to grow on lignocellulose or its derivatives were tested for the production of organic acids, such as succinic acid, lactic acid and alcohols such as 1,3-PD and ethanol in **Paper I**.

Table 1: Different chemicals for potential bio-based production that were studied in the present thesis

Chemical	Structure	Annual production (MT)	Main current production process	Applications
1,3-PD		120,000	Petrochemical	Monomer for terephtalic acid (building block)
n-Butanol		2,800,000	Petrochemical	Solvent (pharmacy); monomer for acrylates, ethers and butyl acetate (building block); transport fuel,
Acetone		5,700,000	Petrochemical	Solvent, chemical intermediate
Ethanol		67,200,000	Microbial fermentation	Beverages (food); transport fuel, solvent, chemical intermediate
Succinic acid		30,000	Petrochemical	Conserving agent and surfactant (food, pharmacy, cosmetics); building block for formation of solvents, fiber and polymer production
Lactic acid		450,000	Microbial fermentation	Formulae (pharmacy and cosmetic) solvent; building block for polylactic acid (PLA)
Butyric acid		80,000	Petrochemical	Pharmaceuticals, aroma, fragrance; building block for cellulose acetate butyrate
Acetic Acid		~10,000,000	Petrochemical	ethylacetate as solvent, vinylacetate for polymers

MT, metric tons; *Sources*: Sauer *et al.* (2008), Almeida *et al.*, (2012); Yang & Yu (2013).

4.2.1 Alcohols

For years, alcohols produced from biomass such as ethanol and butanol have been mainly considered as a renewable alternative fuel for transportation. However, these compounds are also relevant as renewable solvents and building blocks for the chemical industry.

1,3-propanediol

1,3-propanediol (1,3-PD) is an emerging commodity chemical with an estimated market price of $1.76 per kg (Kraus, 2008) and can be produced naturally by bacteria under anaerobic conditions, via the fermentation of glycerol. Its main commercial interest is related to the production of the modern polymer, polytrimethylene terephthalate (PTT) (Straathof, 2013). PTT is used in the manufacture of carpets and textiles and is related to polyethylene terephthalate (PET), a widely used polymer in packaging material (Erickson *et al.*, 2012) (Figure 13). The industrial production of 1,3-PD is mainly based on chemical synthesis using oil-based ethylene and propylene (Biebl *et al.*, 1999). This process has a high energy consumption, uses expensive catalysts and results in highly toxic wastes, which has serious environmental and economic implications for the chemical industry (Wendisch *et al.*, 2011). However, a highly competitive industrial process via the fermentation of glucose was recently introduced by DuPont and Tate & Lyle using recombinant yeast strains. Fermentation plants based on glycerol are under construction in France (Straathof, 2013).

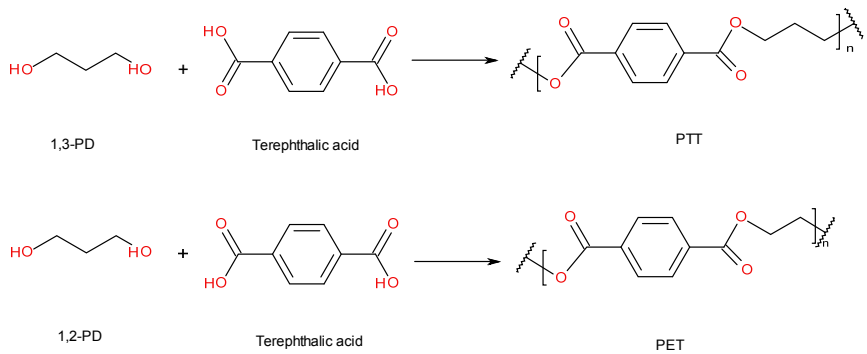


Figure 13: 1,3-propanediol as a building block for the formation of PTT, a polymer with similar characteristics to PET.

Among the different microorganisms that can produce 1,3-PD, *Clostridium butyricum* and *Klebsiella pneumonia* have been paid special attention due to their substrate tolerance, yield and productivity (Saxena *et al.*, 2009). However, *K. pneumonia* is classified as an opportunistic pathogen and industrial scale-up would demand special safety precautions. In this context, *C. butyricum,* a non-pathogenic and GRAS (Generally Regarded As Safe) microorganism, has an advantage for industrialization (Johnson & Taconi, 2007; Willke *et al.*, 2008).

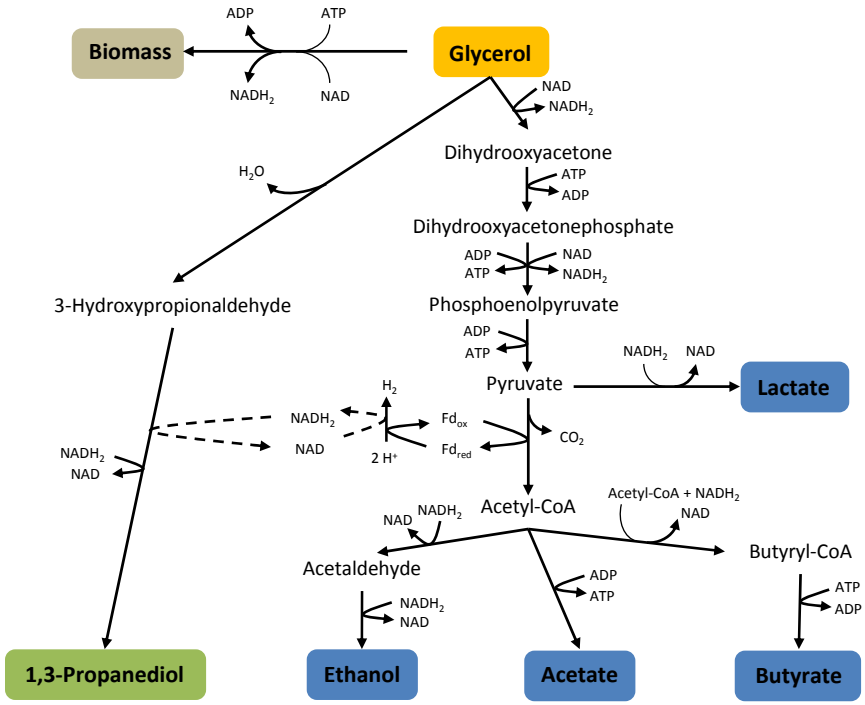


Figure 14: Metabolic pathways of anaerobic glycerol fermentation by *C. butyricum*. (Modified from Zeng, 1996).

The metabolism of glycerol in *Clostridium spp.* occurs via two different concurrent pathways. One part of the glycerol is reduced via 3-hydroxypropionaldehyde (3-HPA), with 1,3-PD as an intermediate (Figure 14). Another part is oxidized to pyruvate via dihydroxyacetone (DHA) which is further converted into the central metabolite, pyruvate, via the glycolytic pathway. This

pathway leads to the formation of different by-products such as lactate, acetate, butyrate and ethanol. This latter pathway is also associated with the generation of reducing equivalents that forces the utilization of glycerol as an electron acceptor for achieving the redox balance with the formation of 1,3-PD (Biebl *et al.*, 1999; Johnson & Taconi, 2007; Saxena *et al.*, 2009). The production of acetate and butyrate is necessary to generate the ATP needed for biomass synthesis. A small amount of up to 5% of glycerol is used for biomass and energy production if no other carbon source is present (Willke *et al.*, 2008).

From the screening of llama rumen isolates in **Paper I**, 1,3-PD was primarily detected in nine isolates in medium containing 1.5 g/L glycerol and complex polysaccharides. All isolates from the enrichment in BS were then tested for growth on 5 g/L glycerol as the sole carbon source and four isolates identified as *C. butyricum* produced 1,3-PD ranging between 1.4-1.8 g/l. Further sequence-batch production studies and the evaluation of the addition of co-substrate were continued in **Paper III**.

Butanol

Butanol (n-butanol or 1-butanol) is produced by ABE fermentation using *Clostridium* bacteria. Fermentation via ABE was one of the pioneer processes in industrial fermentation for the production of chemicals. Butanol is the main product of the fermentation and is considered as a good replacement for gasoline, due to its high octane content and less corrosion risk for the current fuel pipeline and automobile motors (Lee *et al.*, 2008). Besides butanol, acetone and ethanol are also important as solvents and building blocks for the chemical industry. Due to the interest in its use as a fuel and as a solvent and building block (Table 1), commercial fermentation processes have gained renewed interest and production plants are functioning in the USA and China (Donaldson *et al.*, 2011, Straathof, 2013).

The ABE fermentation from sugars typically includes two phases mediated by the formation of Acetyl-CoA from pyruvate (Figure 15) (Lee *et al.*, 2008; Straathof, 2013). It includes the formation of acids (acidogenic phase) and their re-assimilation for further solvent formation during the solventogenic phase. The acidogenic phase usually occurs during the exponential growth phase, whereas the solventogenic is associated witho the stationary phase (Kumar & Gayen, 2011).

Butanol is mainly fermented produced by sugar fermentation, although it can also be obtained from glycerol by *Clostridium pasteurianum* (Almeida *et al.*, 2012). Nevertheless the most-studied strain for ABE fermentation is *C. acetobutylicum*. Due to the importance of butanol production and because there is extensive

information for this bacteria, *C. acetobutylicum* was chosen for study in a novel immobilization process through cryogelation (**Paper IV**). This was performed with the intention of improving the production of butanol based on cell immobilization. Concentrations of up to 18.2 g/l with high yields of 0.41 g/g were achieved.

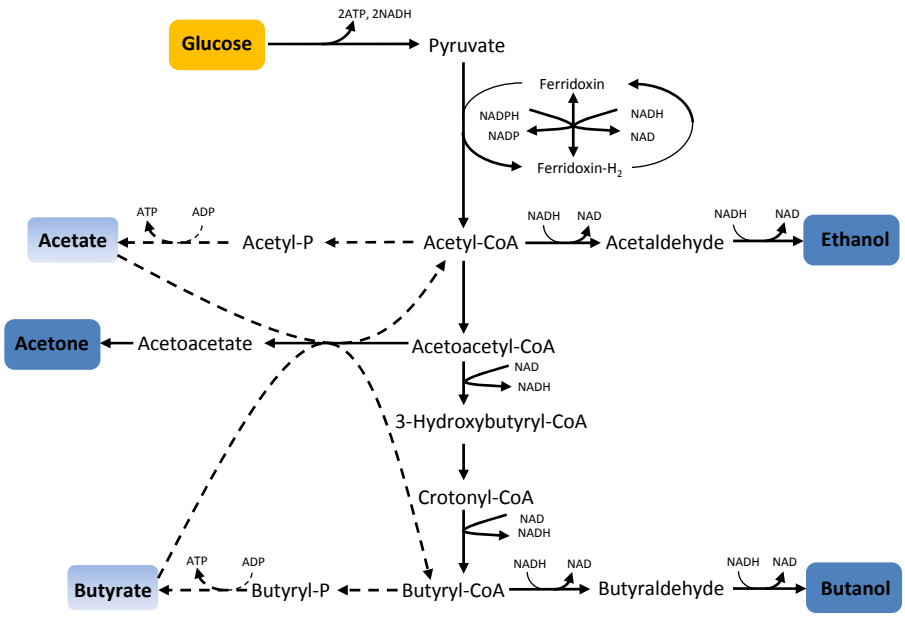


Figure 15: The acetone-butanol-ethanol fermentation pathway, modified from Lee *et al.*, 2008.

Ethanol

Ethanol fermentation has been known since ancient times for the production of beverages. Nowadays it has great industrial impact as a renewable biofuel. Ethanol can also be used as a precursor for ethylene, a highly important building block in the petrochemical industry (Yang & Yu, 2013). It is produced industrially mainly by the fermentation of sugars using *Saccharomyces cereviseae* (baker's yeast). Prokaryotes such as *Zymomonas mobilis* and the anaerobic thermophilic genera *Caldicellulosiruptor*, *Thermoanaerobacter* and *Caloramator* have a great potential to produce ethanol from lignocellulosic-derived material (Svetlitchnyi *et al.*, 2013; Tomás *et al.*, 2013; Crespo *et al.*, 2012).

Ethanol was formed during ABE fermentation by *C. acetobutylicum* (**Paper IV**). It was also formed as by-product by *C. butyricum* llama isolates (**Paper III**) when grown in glycerol as sole carbon source.

4.2.2 Organic acids

Organic acids are used as preservatives in the food and pharmaceutical industries, as chemical intermediates for the production of fine chemicals and as building blocks in the polymer industry (Straathof, 2013). Examples are succinic, lactic, propionic, gluconic, citric, malic, itaconic, maleic, pyruvic, fumaric acids (Stabnikova *et al.*, 2010).

Succinic acid

Succinic acid is a dicarboxylic acid that can be found in almost all plant and animal tissues, and in microorganisms as an intermediate of the citric cycle (Rogers *et al.*, 2013). It can also be excreted as a major fermentation end-product by certain anaerobic bacteria such as *Anaerobiospirillum succiniciproducens,* and the bovine rumen isolates *Actinobacillus succinogenes*, and *Manheimia succiniciproducens* (Song & Lee, 2006). The succinic acid producers have the advantage of fixing the greenhouse gas CO_2, thus providing an additional environmental advantage (Lin *et al.*, 2011).

Commercial production of bio-based succinic acid has been announced by several companies using different microorganisms. Genetically modified yeast is used by Myriant and DSM with Roquete. *Basfia succiniproducens*, a bovine rumen isolate, is used by Bioamber with Mitsui and BASF with Purac (Straathof, 2013; Jansen *et al.*, 2013).

Succinic acid was detected in 34 isolates from the llama rumen (**Paper I**). In **Paper III**, it appeared as a by-product of glycerol fermentation by the llama isolates *C. butyricum* BSL59 and BSL 61, when carbohydrates were used as a co-substrate. It reached 0.1 g/l and 0.2 g/l respectively when adding 15 g/l glucose to the medium but was 30% and 65% lower when having 5 g/l WS as a co-substrate. It is also a by-product formed during the co-fermentation of glycerol and sugars and wheat straw by *C. butyricum* BSL59 and BSL61, reaching concentrations of 0.2 g/l.

Lactic acid

Lactic acid is a traditional product from food fermentation by lactic acid bacteria. Its industrial production is based on the fermentation of sugars by *Lactobacillus strains*, although glycerol can also be used for its production by other naturally occurring microorganisms including *Klebsiella*, *E. coli* and the strict anaerobe *Clostridia* (Almeida *et al*., 2012; Rogers *et al*., 2013).

Besides conventional food processing, it has several industrial applications (Table 1); however the main current use is as a building block for polylactic acid (PLA). PLA is one of the most promising biodegradable polymers that can be used in textiles, packing materials, and films (Straathof, 2013). It has different properties depending on the enantiomeric form, however, both optical forms (L and D) have good processability, biocompatibility and biodegradability (Rahman *et al*., 2013).

Similar to succinic acid, lactic acid production was detected in the cultivation broth of llama rumen isolates, with 20 representatives (**Paper I**). Lactic acid was formed as by-product of glycerol fermentation in selected isolates, reaching 0.2 g/l in *C. butyricum* BSL59 and 1.4 g/L in *C. butyricum* BSL61. It was also formed as by-product in the co-fermentation of glycerol and carbohydrates; with a maximum concentration of 2.2 g/L in *C. butyricum* BSL59 and *C. butyricum* BSL61when cells were grown on 15 g/L glucose.

Table 2: Overall concentration and yield of organic acids produced as by-products in three sequence batch fermentations.

	C. butyricum BSL61 from glycerol (**Paper III**)		*C. acetobutylicum* DSM 792 from glucose (**Paper IV**)	
	Concentration (g/l)	Yield (mol/mol)	Concentration (g/l)	Yield (mol/mol)
Lactic acid	1.3	0.06	0	0
Acetic acid	0.6	0.01	2.9	0.08
Butyric acid	3.4	0.15	4.8	0.09

Other products of industrial interest that were also formed in the studied fermentations include acetic acid and butyric acid, which are common metabolites produced under anaerobic conditions by several bacteria. Both acids were typically formed as by-products from the solventogenic fermentation of *Clostridium*

acetobutylicum (**Paper IV**). They were also formed during the fermentation of glycerol by *C. butyricum* strains isolated from the llama rumen (**Paper III**). Butyric acid was the main product from *C. butyricum* BSL59 and 61 when growing on 20 g/L wheat straw, reaching 0.7 g/l and 0.6 g/l respectively.

In general, these different by-products were detected in low concentrations (Table 2), a typical trait of anaerobic bacteria. It provides single microorganisms with the potential for the production of a wide range of valuable compounds; however it can be troublesome for downstream processing. Some methods for separation of these acids have been reported, including crystallization, ion exchange and membrane separation (Sauer *et al.*, 2008; Lin *et al.*, 2012). The effort for the separation of such chemicals from the broth would be profitable if the production process itself is of low-cost. The type of feedstock used is an example of the significant parameters to be considered (Saeuer *et al.*, 2008).

4.3 Biotechnological processes

Innovative fermentation processes are necessary for the cost-effective production of bio-based chemicals. Indicators of bioprocess efficiency are production titer, yield and productivity (Yang & Yu, 2013). Process efficiency affects the production cost and process economics (Whesthuis *et al.*, 2011). For an initial evaluation of a new producer strain (**Paper III**) and a new fermentation process (**Paper IV**), batch fermentations were used.

The batch system is adequate for initial evaluation; however, it is restricted to inhibition of the accumulation of fermentation by-products, as observed in **Papers III** and **IV**. To overcome this, simultaneous process removal has been successfully reported to improve butanol production (Qureshi *et al.*, 2013; Zhao *et al.*, 2013). To change the fermentation set-up to continuous mode is also an option for the production of both alcohols (Yang & Yu, 2013), but in this case, cells immobilized to a support to avoid cell loss, is required.

4.3.1 Cell immobilization

Immobilization of microorganisms can improve fermentation and allow the easy recovery and reuse of bacterial cells (Willke *et al.*, 2008). For immobilization, the conventional approach is to entrap cells in a polymeric material such as alginate, (**Paper II**) or to attach them to an inert support material. However, in **Paper IV**, a

new method based on the crosslinking of bacterial cells, without the need of an extra support material, was developed.

Table 3: Comparison between butanol production by crosslinked cells in a cryogel plug or by free cells.

	Immobilized cells	**Free cells**
Average butanol (g/l)	8.3	4.4
Average total acids (g/l)	2.8	3.3
Overall butanol yield (g/g)	0.25	0.26
Overall butanol productivity (g/l/h)	0.07	0.04

Immobilized cells have the advantage that cell recycling is simplified than when counting with free cells. Culture broth is easily separated from the immobilized cells without the need for centrifugation or special separation membranes (Zhao *et al.*, 2013). This was tested in five consecutive batches in **Paper IV,** to compare cross-linked cells in cryogel plugs and free cells for the production of butanol (Table 3). In both cases, the same cell mass was used at the start of fermentation. Although no significant difference was observed between the yield values, the butanol titer and concentration was two-fold higher when cells were immobilized. It demonstrates that cells in the cryogel plug are more efficient in the uptake and recycling of acids, detoxifying the environment and contributing to additional carbon for an improved solventogenesis (Formanek *et al.*, 1997).

Sequential batch for cell recycling was also used to evaluate the production of 1,3-PD using glycerol and WS as co-substrate (**Paper III**). It is possible that WS can also be considered as a support material as cell growth on the material was observed in extended fermentation times.

Crosslinked cells in cryogel for butanol production

The use of polymeric crosslinkers to create viable cell aggregates has been previously used in yeast (Parascandola *et al.*, 1990, Bečka *et al.*, 2003). However, a new mixture of crosslinkers was optimized for its use to immobilize viable cells of *C. acetobutylicum* DSM 729 forming cryogel plugs in **Paper IV**. The crosslinker mixture used, PEI-GTA/PVA-GTA, is mild and cells maintain viability throughout cultivation. Due to the type of cryogel material, the cell-based plug formed is highly porous, with thin walls formed of densely packed cells (Figure

15). This gives the advantage of having an immobilized system that offers a high density of active cells with a reduced mass transfer limitation.

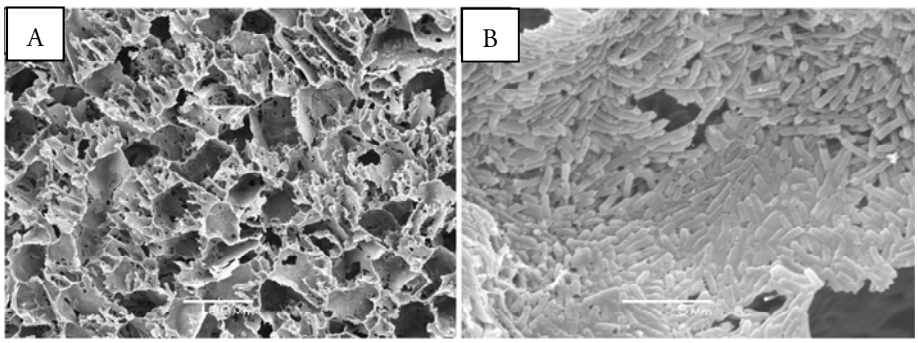


Figure 16: Scanning electron microscopy images of cryogel from *C. acetobutylicum* DSM729 prepared using PEI-GTA and PVA-GTA as crosslinkers. A) cross-section of cryogel plug and B) detail of the walls of the cryogel.

In repeated batch cultivation cycles, immobilized cells remained active in cell proliferation and butanol production. Improved efficiency in acid assimilation and recycling by the cell was one of the remarkable characteristics of the immobilized systems during the fermentation. High concentrations of butanol were obtained with the crosslinked cells in cryogel (Table 4). Variations between the cycles are mainly due to inhibition by the products formed and accumulated in the batch cultivation, which reached toxic levels for the cell. The maximum butanol titer reached in this study is superior in comparison to that of other immobilization methods tested in batch mode (Tripathi *et al.*, 2010; Efremenko *et al.*, 2012; Chen *et al.*, 2013). This indicates that immobilized cells with direct contact with the cultivation medium can be a good alternative for improving butanol production.

Table 4: Sequential butanol production by immobilized cells of *C. acetobutylicum* DSM 729 with 60 g/l glucose. Cryogel plugs were washed with PBS before the start of a new cycle. Results are the mean of three repetitions.

Cycle number	Glucose consumed (%)	Butanol (g/l)	Total acids (g/l)	*Y* butanol (g/g)	*Q* butanol (g/l/h)
1	62.7	4.9	2.8	0.13	0.04
2	74.0	18.2	0.8	0.41	0.14
3	51.5	2.4	1.5	0.08	0.02

Y: yield; *Q*: productivity

Although glucose was used as a substrate in this study, biomass hydrolysates, such as WS hydrolysate could also be tested. It has been shown that WS acid pretreated and enzymatically hydrolyzed could be a profitable substrate for the production of butanol in conventional batch process, with adjustment in the recovery process (Qureshi *et al.*, 2013).

4.3.2 Use of a co-substrate for improved production of 1,3-propanediol

The intracellular redox balance is important for a continuous 1,3-PD production by the cell. As more reducing equivalent is generated by the pyruvate pathway, more metabolic flux is directed towards 1,3-PD biosynthesis (Zeng, 1996). Alteration of the redox balance (intracellular $NADH_2/NAD^+$ level) by genetic manipulation had deleterious effects on glycerol uptake efficiency, inhibited the growth of the producer strain or had a limited effect on conversion (Jin *et al.*, 2011a). The addition of a carbohydrate substrate, such as glucose or xylose to the glycerol fermentation, can be a simpler solution to improve the production of 1,3-PD by regulating the internal redox balance of the cell (Biebl & Marten, 1995; Abbad-Andaloussi *et al.*, 1998; Jin *et al.*, 2011a).

Addition of a carbohydrate substrate, such as glucose or xylose, would improve the $NADH_2$ generation, as this molecule is formed in the hexose and pentose pathways (Zeikus, 1980). The addition of sugars would also improve cell biomass formation, leaving glycerol to be used mainly in the conversion step (Malaoui & Marczak, 2001). Moreover, current fluctuations in the market price of glycerol, due to the increasing demand for other applications of interest and its dependence on the biodiesel market, makes the upscale process unstable for 1,3-PD production based only on glycerol (Willke & Vorlop, 2008). The addition of a low-cost and abundantly available substrate such as wheat straw, would balance the process economy.

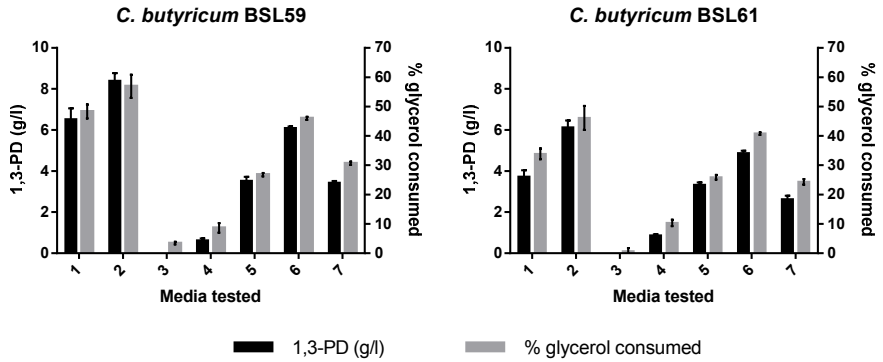


Figure 17: Production of 1,3-PD by *C. butyricum* strains isolated from the llama rumen from 30 g/l glycerol using different co-substrates. Media *1*: 30 g/l glycerol, *2*: 30 g/l glycerol + 5 g/l WS, *3*: 30 g/l glycerol + 15 g/l glucose, *4*: 30 g/l glycerol + 15 g/l xylose, *5*: 30 g/l glycerol + 2 g/l glucose, *6*: 30 g/l glycerol + 1.3 g/l xylose, 7: 30 g/l glycerol + 2 g/l glucose + 1.3 g/l xylose.

Llama isolates that were able to produce 1,3-PD from glycerol were tested for fermentation using WS as a co-substrate (**Paper I**). The two strains that produced higher concentration of 1,3-PD were selected for further tests using glucose, xylose and WS as a co-substrate (**Paper III**). It was observed that the addition of WS in the medium significantly improved the consumption of glycerol and boosted the production of 1,3-PD in *C. butyricum* BSL59 (8.4 g/l) and BSL61 (6.1 g/l) in comparison to the absence of the co-substrate (29% and 65% more, respectively) (Figure 17). Addition of WS was also superior in 1,3-PD titer in comparison to the addition of pure sugars in different concentrations. This might be due to the presence of carbohydrates in WS which cells could access by hydrolytic enzymes (**Paper I**). As sugars in WS are not freely available, its slow release by the cells avoids suppression of the glycerol utilization pathway (Mitchel *et al.*, 1995; Tracy *et al.*, 2012). Despite no high cell density was observed in the medium, cell attachment to WS was observed under the microscope. Cell attachment to WS might be due to the action of cellulases associated in cellulosomes (Bayer *et al.*, 2004), which is commonly found in *Clostridia.* Another possible reason is that cells are associated to WS as a growth support material (Survase *et al.*, 2012).

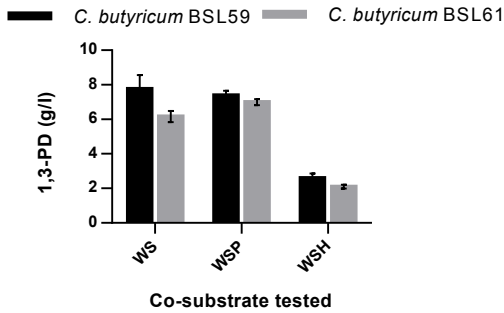


Figure 18: The production of 1,3-PD by *C. butyricum* strains isolated from the llama rumen from 30 g/l glycerol and different fractions of pretreated wheat straw. WS: wheat straw; WSP: steam exploded wheat straw, fiber fraction; WSH: steam exploded wheat straw, liquid fraction enzymatically hydrolysed.

Pretreated WS by steam explosion was also studied as a co-substrate. The pretreatment was intended to improve the substrate biodegradability by the dissolution of hemicellulose and lignin, releasing the cellulose fibrils (Hendriks & Zeeman, 2009). Steam explosion is commonly applied for improving the conversion of the sugars from the biomass for bioethanol conversion (Gnansounou, 2010). Two different fractions obtained from pretreated WS were evaluated in both *Clostridia* strains in comparison to untreated WS. Enzymatically treated hydrolysate, which contains free sugars from WS, had a negative effect on 1,3PD production in comparison to the WS-exploded fiber or the untreated material (Figure 18). Exploded fiber did significantly improve the 1,3-PD production on C. *butyricum* BSL61 by 13% in comparison to untreated WS. However, it did not improve the 1,3-PD concentration in *C. butyricum* BSL59. The low performance in the pretreated material, can also be associated to low tolerance by the strain to inhibitor compounds formed during the pretreatment, such as furfurals and hydroxymethylfurfural (HMF) (Erdei *et al.*, 2010).

The use of WS as a co-substrate is more significant as carbon source than solely as support material in *C. butyricum* BSL61. Hence, the production in sequential batch mode under controlled pH was studied only in this strain. The use of sole glycerol as carbon source and the addition of 15 g/l of wheat straw were compared. Cyclic variations were observed in the cycles and in general, no significant difference was observed in between the two substrates strategy used (Table 5). The yields achieved in the fermentation (0.64 mol/mol) were comparable to other studies using sole glycerol as substrate in *C. butyricum* (0.65 mol/mol) (Colin *et al.*, 2000) and hemicellulosic hydrolysate in *Klebsiella pneumonia* (0.65 mol/mol) (Jin

et al., 2011b). However, more studies are needed to understand the fermentation process using a complex co-substrate such as wheat straw.

Table 5: Sequence batch production of 1,3-PD by *C. butyricum* BSL61

	Glycerol	**Glycerol + WS**
Overall yield (mol/mol)	0.64	0.64
Overall productivity (g/l/h)	0.13	0.14

Nevertheless, the solid residue from the sequential fermentation has a potential to be converted into biogas (Figure 19). A methane yield of 0.81 (l CH_4/ g VS added) was achieved from the cells and wheat straw left as residues from the glycerol fermentation. This value is 3-fold the methane yield obtained from chopped wheat straw (0.27 L CH_4/g VS added). The elevated methane yield, however, is not only due to the WS, which would be considered as "pretreated" during the fermentation. Cell biomass and other compounds such as volatile fatty acids, are part of the degradable organic mass and were not accounted in the total and volatile solids calculations (VS), contributing to the increase in methane yield in comparison to unfermented WS (Kreuger *et al.*, 2011). Nevertheless, the possibility to generate energy from the fermentation residues represents an advantage over synthetic support material. In this way, the use of agriculture and agro-industrial residue is maximized by the use of anaerobic fermentation and anaerobic digestion forming products of interest for the biorefinery.

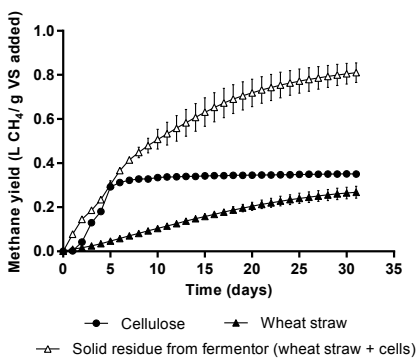


Figure 19: Methane yield after 31 days digestion of solid residues from the sequential fermentation, wheat straw and cellulose

Overall, the use of WS as co-substrate improved the production of 1,3-PD in the *C. butyricum* strains tested. It demonstrates that an agricultural residue combined with an agro-industrial by-product can be used to generate an added-value compound which is demanded by the chemical industry. Moreover, WS might be useful as a carbohydrate source as well as a support material. This provides the opportunity to convert the fermentation residue into energy by anaerobic digestion, fulfilling the biorefinery concept by maximizing the use of the biomass.

5. Conclusions & future perspectives

In this thesis, several industrial biotechnological applications of anaerobic microorganisms, especially bacteria, were demonstrated. For this, different approaches were assessed for the ability to develop biotechnological applications from anaerobic bacteria, with a focus on industrial biotechnology and biorefinery. The ability of the studied bacteria to degrade structurally complex material and transform it into value-added products has been exploited.

Microbial diversity studies from unexplored environments, the screening of novel isolates and development of new technologies to facilitate access to the "unknowns" or slow growing bacteria with the potential for simultaneous high-throughput screening were the first steps. This approach was evaluated for bacteria but can also be extended to other anaerobic microorganisms.

The study of a previously unexplored environment, the llama foregut, demonstrated the high potential of this efficient natural system for finding microorganisms that produced relevant compounds. Microbial diversity studies based on molecular tools, showed changes in the prokaryote populations according to the complexity of the composition of the material being digested. It was also possible to identify several bacteria (known to degrade biomass) that have a potential use in biotechnological processes.

Isolated strains from the llama foregut were screened for the production of organic acids, and alcohols combined with their ability to produce enzymes for converting the plant biomass. These products are of high interest for the industry to reduce the dependence on oil in the manufacture of goods. From these products, 1,3-propanediol production by selected strains was then evaluated. The use of a combination of wheat straw and glycerol improved the production in batch tests. Much attention has been given to using wheat straw as a feedstock for biofuel production. Using it initially as a co-substrate in glycerol fermentation by *Clostridium*, it brings potential economic benefits to the 1,3-PD process before being converted into biogas, fulfilling the biorefinery concept.

A new immobilization technique was developed for bioprocess improvement and optimized for the production of butanol. Cells were crosslinked to form a cryogel and were evaluated in consecutive batches. This approach resulted in a higher butanol concentration and yield. The results suggest that the system maintained stability and could be used for sequential batch production with simultaneous product removal in biorefinery systems.

Future perspectives

To learn more about the efficiency of biomass conversion by the llama rumen microbiota, in-depth studies on the microbial community and their function should be carried out. For this, the use of high-throughput sequencing and sequenced-based metagenomics can aid in the "exploration" of this system.

Potential for production of other compounds should be explored from the collection of isolates obtained from the llama rumen. Special attention should be given to the enzymatic potential of these microorganisms, which can lead to novel applications.

The alginate microbeads technology is a promising tool for cultivating the "not-yet-cultivable" microbial diversity. Further investigation based on this system can lead to solutions for one of the biggest challenges in microbiology, which is to access not just the genes, but the whole microorganisms of the large "not-yet-cultivable" majority. Optimization of parameters for the sorting of the microbeads under anaerobic conditions via flow cytometery and studies exploring this technique as a screening method, would have a great impact on industrial biotechnology discoveries.

Studies on cell biofilm formation and the internal cell redox balance would be useful to understand more about the positive effect of wheat straw on the glycerol production by *Clostridium butyricum*.

The technique on crosslinking cells into cryogels for cell immobilization has a high potential for increasing biomass in fermentations using "slow grower" microorganisms. It would be recommended to apply this immobilization system on different bacterial species.

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