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PREFACE

This thesis comprises the research done during a PhD project carried out at the Department of Environmental Engineering, Technical University of Denmark, from November 1, 2008 to March 31, 2013. Professor Irini Angelidaki was the main supervisor; Senior Researcher Dimitar Karakashev was the co-supervisor. It was funded by the Portuguese Foundation for Science and Technology, through the Human Potential Thematic Operational Programme, funded by the Portuguese Government and the European Social Fund, under grant SFRH/BD/43863/2008.

The thesis is organized in two parts: the first part puts into context the findings of the PhD in an introductive review; the second part consists of the papers listed below. These will be referred to in the text by their paper number written with the Roman numerals I-IV.

I Tomás AF, Karakashev D, Angelidaki I. 2012. *Thermoanaerobacter pentosaceus* sp. nov., an anaerobic, extreme thermophilic, high ethanol-yielding bacterium isolated from household waste. Int J Syst Evol Microbiol (in press) doi:10.1099/ijs.0.045211-0.

II Tomás AF, Karakashev D, Angelidaki I. 2011. Effect of xylose and nutrients concentration on ethanol production by a newly isolated extreme thermophilic bacterium. Water Science and Technology 64 (2) 341-347.

III Tomás AF, Karagöz P, Karakashev D, Angelidaki I. 2013. Extreme thermophilic ethanol production from rapeseed straw: using the newly isolated *Thermoanaerobacter pentosaceus* and combining it with *Saccharomyces cerevisiae* in a two-step process. Biotechnol Bioeng (in press) doi:10.1002/bit.24813.

IV Sittijunda S, Tomás AF, Reungsang A, O-thong S, Angelidaki I. 2013. Ethanol production from glucose and xylose by immobilized *Thermoanaerobacter pentosaceus* at 70 °C in an up-flow anaerobic sludge blanket (UASB) reactor. Submitted.

In addition, the following publications, not included in this thesis, were also concluded during this PhD study:

Angelidaki I, Tomás AF, Karakashev D. 2012. DSMZ 24726 for second generation bioethanol production. Patent number WO/2012/059105.

In this online version of the thesis, the papers are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from:

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ABSTRACT

Second-generation bioethanol is produced from residual biomass such as industrial and municipal waste or agricultural and forestry residues. However, *Saccharomyces cerevisiae*, the microorganism currently used in industrial first-generation bioethanol production, is not capable of converting all of the carbohydrates present in these complex substrates into ethanol. This is in particular true for pentose sugars such as xylose, generally the second major sugar present in lignocellulosic biomass. The transition of second-generation bioethanol production from pilot to industrial scale is hindered by the recalcitrance of the lignocellulosic biomass, and by the lack of a microorganism capable of converting this feedstock to bioethanol with high yield, efficiency and productivity.

In this study, a new extreme thermophilic ethanologenic bacterium was isolated from household waste. When assessed for ethanol production from xylose, an ethanol yield of 1.39 mol mol^{-1} xylose was obtained. This represents 83 % of the theoretical ethanol yield from xylose and is to date the highest reported value for a native, not genetically modified microorganism.

The bacterium was identified as a new member of the genus *Thermoanaerobacter*, named *Thermoanaerobacter pentosaceus* and was subsequently used to investigate some of the factors that influence second-generation bioethanol production, such as initial substrate concentration and sensitivity to inhibitors. Furthermore, *T. pentosaceus* was used to develop and optimize bioethanol production from lignocellulosic biomass using a range of different approaches, including combination with other microorganisms and immobilization of the cells.

T. pentosaceus could produce ethanol from a wide range of substrates without the addition of nutrients such as yeast extract and vitamins to the medium. It was initially sensitive to concentrations of 10 g l^{-1} of xylose and 1 % (v/v) ethanol. However, long term repeated batch cultivation showed that the strain was capable of adaptation to higher substrate concentrations, at least up to 20 g l^{-1} xylose.

T. pentosaceus was able to metabolize two typical inhibitors present in lignocellulosic hydrolysate, 5-hydroxymethylfurfural (HMF) and 2-furfural, up to concentrations of 1 and 0.5 g l^{-1}, respectively. Above these levels, xylose consumption was inhibited up to 75 % (at 3.4 g l^{-1} 5-HMF) and 70 % (at

3.4 g l^{-1} furfural). *T. pentosaceus* could grow and produce ethanol directly from the liquid fraction of pretreated rapeseed straw, without any dilution or need for additives.

When *T. pentosaceus* was used in combination with *S. cerevisiae* in a sequential fermentation of pretreated rapeseed straw, it achieved 85 % of the theoretical ethanol yield based on the sugar composition of the rapeseed straw. This was 50 % and 14 % higher than the yield obtained with the bacteria or the yeast alone, respectively.

When *T. pentosaceus* was immobilized in rapeseed straw, an improvement of 11 % in ethanol production was observed in batch mode. In continuous mode, it was shown that hydraulic retention time (HRT) affected ethanol yield, and a dramatic shift from ethanol to acetate and lactate production occurred at an HRT of 6 h. The maximum ethanol yield and concentration, 1.50 mol mol^{-1} consumed sugars and 12.4 g l^{-1}, were obtained with an HRT of 12 h. The latter represented an improvement of 60 % in relation to previously obtained results.

The results obtained confirm that the extreme thermophile *T. pentosaceus* is a promising candidate for bioethanol production from lignocellulosic biomass, and that improvement and optimization of existing processes are possible using different approaches. Further insight into the metabolism of the strain, as well as its improvement by genetic engineering can bring second-generation ethanol production one step closer to its industrial application.

DANSK SAMMENFATNING

Restbiomasse fra industri og kommunalt affald eller restmaterialer fra land- og skovbrug bliver brugt til produktion af andengenerations-bioethanol. Mikroorganismen *Saccharomyces cerevisiae*, der i dag industrielt bruges til fremstilling af førestegenerationsbioethanol, er ikke i stand til at omdanne alle de kulhydrater, der findes i de nævnte komplekse biomasser, til ethanol. Det gælder især for pentoser såsom xylose, der generelt udgør det næststørste indhold af lignocellulosebiomasse. Overgangen fra produktionen af andengenerations-bioethanol i pilotanlæg til industriskala er besværliggjort af det forhold, at lignocellulosebiomassen ikke er nedbrydelig og af manglen på mikroorganismer, der er i stand til at omdanne dette råmateriale til bioethanol met et højt udbytte, med stor effektivitet og produktivitet.

I nærværende PhD studium blev en ekstrem thermofil ethanolproducerende bakteriestamme isoleret fra husholdningsaffald. Vurderet ud fra ethanolproduktionen fra xylose, blev der dannet 1.39 mol mol^{-1} xylose. Det svarer til et udbytte på 83 % af det teoretisk mulige og er den højeste rapporterede værdi for naturlige, ikke genetisk modificerede mikroorganismer.

Bakterien blev identificeret som en ny stamme indenfor slægten *Thermoanaerobacter* og blev således navngivet *Thermoanaerobacter pentosaceus* og blev efterfølgende brugt til at undersøge forskellige faktorers indflydelse på produktionen af andengenerations-bioethanol, såsom råvarematerialets begyndelseskoncentration og følsomheden over for kendte proceshæmmere. Derudover blev *T. pentosaceus* ved forskellige fremgangsmåder brugt til at optimere bioethanolproduktionen fra lignocelluloseholdig biomasse, herunder i kombination med andre mikroorganismer og immobilisering af cellerne.

T. pentosaceus kan producere ethanol fra en bred vifte af substrater uden tilsætning af næringsstoffer som gærekstrakt og vitaminer til vækstmediet. I begyndelsen var denne bakterie følsom overfor koncentrationer af xylose på 10 g l^{-1} og ethanol på 1 % (v/v). Men, gentagne langtidsbatchkultiveringer viste, at denne stamme var i stand til at tilpasse sig høje substratkoncentrationer, i det mindste op til 20 g l^{-1}.

T. pentosaceus var i stand til at nedbryde to velkendte proceshæmmere, der er tilstedet i lignocellulosehydrolysater; 5-hydroxymethylfurfural (HMF) og 2-furfural i koncentrationer op til henholdsvis 1 og 0.5 g l^{-1}. Over disse niveauer blev xyloseforbruget hæmmet med op til henholdsvis 75 % og 70 % ved 5-HMF-

og 2-furfuralkoncentrationer på 3.4 g l^{-1}. *T. pentosaceus* kunne vokse og producere ethanol direkte fra væskefraktionen af forbehandlet rapsstrå, uden nogle former for fortynding eller behov for tilsætningsstoffer.

Da *T. pentosaceus* blev anvendt i kombination med *S. cerevisiae* i en sekventiel gæringsproces af forbehandlet rapsstrå var det opnåede udbytte 85 % af det teoretiske ethanoludbytte bestemt ud fra sukkersammensætningen af rapsstrå. Dette var henholdsvis 50 % og 14 % højere end det opnåede udbytte når enten bakteriestammen eller gærstammen blev brugt alene.

Da *T. pentosaceus* blev immobiliseret i rapsstrå, blev en forbedring af ethanolproduktionen på 11 % observeret i batchtest. I kontinuerte test i en "up-flow anaerobic sludge blanket" (UASB) reaktor og ved brug af en blanding af glucose og xylose i en koncentration på 20 g l^{-1}, blev der opnået en ethanolkoncentration på 12.4 g l^{-1}, svarende til et ethanol udbytte på 1.50 mol mol^{-1} forbrugt sukker.

De opnåede resultater bekræfter at den ekstremt termofile *T. pentosaceus* er en lovende kandidat til bioethanolproduktion ud fra lignocellulosebiomasse, og at forbedringer og optimeringer af eksisterende processer er mulige ved brug af forskellige fremgangsmåder. Yderligere viden om denne stammes metabolisme, og ligeså forbedringer gennem genmodifikation, kan bringe andengenerationsethanolproduktion et skridt tættere på industriel anvendelse.

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ABBREVIATIONS

ADH	Alcohol dehydrogenase
AFEX	Ammonia fibre explosion
CoA	Coenzyme A
COD	Chemical oxygen demand
HMF	Hydroxymethylfurfural
LDH	Lactate dehydrogenase
PDC	Pyruvate decarboxylase
PFL	Pyruvate formate lyase
UASB	Up-flow anaerobic sludge blanket

1 INTRODUCTION

1.1 Background

In 2011, 87 % of the world energy consumption came from fossil fuels: oil, natural gas and coal (33 %, 24 % and 30 %, respectively) (BP, 2012). The dependence of modern societies on these primary energy sources is known to be on the basis of increasing rates of natural resource depletion and climate change stresses (IPCC, 2007). The extensive use of fossil fuels is also one of the causes of several other concerns, such as high levels of pollution, destruction of natural landscapes and habitats, and environmental catastrophes (National Research Council, 1999).

In light of these facts, the interest in developing viable, clean, and sustainable energy sources has risen considerably. The remaining 13 % of the world's energy sources in 2011 were nuclear power, hydroelectricity and other renewable energies, which included biofuels, such as biodiesel, biohydrogen and bioethanol. The latter is currently the most produced biofuel in the world, with almost 110 billion litres in 2011 (RFA, 2012).

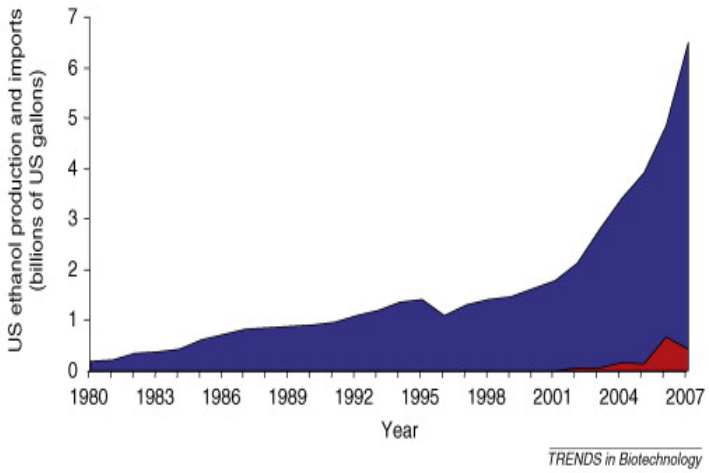

Figure 1. The production of fuel ethanol in the USA between 2002 and 2007. The area shown in blue is the bioethanol production by the US, whereas the area in red indicates recent imports. Data from http://www.ethanolrfa.org/industry/statistics/. Reprinted from Trends in Biotechnology, vol. 27, Taylor *et al.*, Thermophilic ethanologenesis: future prospects for second-generation bioethanol production, 398-405, Copyright (2009) with permission from Elsevier.

Ethanol produced by microbial fermentation of biomass is used primarily as a substitute for gasoline, usually blended, although it can also be used on its own. Minor applications commonly include industrial use or rocket fuel. In developing countries it is also commonly used to replace kerosene for cooking and illumination. Besides being a renewable energy source, bioethanol has other advantages over fossil fuels: it emits less CO_2 when burned (Brown *et al.*, 1998), which in addition is compensated by CO_2 uptake from the biomass; it does not emit toxic gases (such as CO and nitrate oxides) or particles when burned; less energy is required to produce ethanol than the equivalent amount of gasoline; it is rapidly biodegraded in the environment; it has a higher octane rating than gasoline (Bailey, 1996), allowing for increased thermal efficiency (Ecklund, 1978).

Currently, commercial bioethanol is produced mainly from starch extracted from corn (USA) and from sucrose extracted from sugar cane (Brazil) (Mielenz, 2001; Rosillo-Calle & Cortez, 1998). Other starch-rich types of biomass include potatoes, wheat, cassava, rye and barley; sucrose can also be directly obtained from sugar beet. Starch is easily hydrolysed into glucose; sucrose, a disaccharide composed by the hexoses fructose and glucose, is readily fermented by the yeast *Saccharomyces cerevisiae*, the most employed microorganism in industrial bioethanol production.

Bioethanol produced from energy crops – commonly referred to as first-generation bioethanol – has, however, not been exempt from criticism. In spite of the benefits listed above, the reduction of greenhouse gas emissions is not as high as expected (Farrell *et al.*, 2006); raw material can cost up to 40 % of the ethanol production process (Von Sivers *et al.*, 1994), and its use for this purpose competes directly with production of food and animal feed. Furthermore, the extensive cultivation of crops that would be needed if ethanol was to completely replace gasoline would have several negative impacts on land use (soil fertility, water availability, use of fertilizers, etc.).

These concerns can be addressed when ethanol is produced from residual biomass. Agricultural, forestry, industrial, household and municipal waste contain carbohydrates which can also be converted into bioethanol. In particular, they can contain lignocellulosic biomass (Wiselogel *et al.*, 1996), the most abundant type of biomass in the planet (Claassen *et al.*, 1999), and therefore a potential, affordable raw material for second-generation bioethanol production.

The efficient conversion of the carbohydrates present in the different residual resources into biofuels is one important part of the road towards a sustainable fuel economy, away from the current one on relying fossil fuels. However, before commercialization of bioethanol produced from non-food feedstock can become a reality; several challenges have to be addressed. The major one is the characteristic recalcitrance of lignocellulosic biomass, which renders the fermentable sugars inaccessible to most microorganisms. The current pre-treatment technologies used to extract the carbohydrates generate inhibiting compounds that can affect the fermentation process. Furthermore, *S. cerevisiae* and other microorganisms typically used in first-generation bioethanol processes are not able to convert all the sugars which constitute lignocellulosic biomass (Bothast *et al.*, 1999), therefore compromising the efficiency of the process.

The lack of microorganisms capable of such conversion while still achieving the high yield and productivity required for a cost-effective process served as motivation for the investigation that resulted in this PhD thesis.

1.2 Objectives and structure of the thesis

The trigger of this project was the establishment of an enrichment culture able to produce ethanol with a yield up to 1.6 mol mol^{-1} xylose (Zhao *et al.*, 2010). The main goal was to select, from this culture, a microbe capable of yielding comparable amounts of ethanol, and ultimately to use it as the main driver in a second-generation ethanol production process. In particular to:

- Isolate at least one microorganism from this enriched culture capable of producing high yields of ethanol from pentoses.
- Identify and characterize this microorganism in terms of its physiology, phylogeny, chemotaxonomic and metabolic properties.
- Test the influence of different factors in the microorganism's ethanol production capacity: substrate concentration, nutrient addition, tolerance to exogenously added ethanol, tolerance to typical inhibitors derived from biomass pre-treatment.
- Test the ability of the microorganism to convert a lignocellulosic substrate to ethanol.

- Improve the microorganism's ethanol production potential using different process configurations, such as immobilization and combined use with other microorganisms.
- Assess the microorganism's amenability to strain improvement by metabolic engineering.
- Contextualize the main findings into the broader picture of the current state of second-generation bioethanol production.

In Chapter 2 the fundamentals of second-generation ethanol production are presented, with focus on the thermophilic case. Advantages and limitations of this approach are highlighted. Some of the findings from Papers I, II and III are included in this section.

In Chapter 3, the different strategies that can be used to bring second-generation ethanol production a step closer to industrial ethanol production are presented. The main results obtained during this research are included in the respective sub-chapters and described in more detail in Papers I, II, III and IV.

2 SECOND-GENERATION BIOETHANOL PRODUCTION

By definition, second-generation bioethanol refers to ethanol produced by biological fermentation of residual biomass (Taylor *et al.*, 2009). Its production typically comprises the following steps:

- Pre-treatment: process where the structural carbohydrates that compose the biomass are made more accessible for the subsequent steps;
- Enzymatic hydrolysis: break down of the polymeric carbohydrates into simple sugars that can be fermented by the microorganisms into ethanol;
- Fermentation: conversion of the carbohydrates into ethanol by the selected microorganism or culture;
- Downstream processing: recovery of the ethanol from the fermentation broth (typically by distillation) and management of the remaining streams.

The need for a pre-treatment step is the major distinction between a first- and a second-generation process. Despite this, and although some of the other stages have correspondence in the two generations of bioethanol production processes, there are many differences. In this section some of these differences will be highlighted.

2.1 Substrate considerations: lignocellulosic biomass

Residual biomass comprehends a vast diversity of organic materials. While in principle all types of carbohydrate containing waste can be used, vegetable-sourced biomass is usually preferred to other types of waste, such as industrial wastewaters or household waste (Van Wyk, 2001). The latter have complex and heterogeneous compositions that typically include proteins, fats, and different organic and inorganic substances that can be toxic to microorganisms that produce ethanol.

Agricultural and forestry waste contain almost exclusively polymeric carbohydrates, such as cellulose and xylan. Once broken down to its smaller constitutional units, the mono and disaccharides can be easily converted into ethanol by fermentative microorganisms. Industrial waste from the sugar, cereal and paper industries is also rich in carbohydrates and therefore also used often in

second-generation bioethanol production. Table 1 lists the composition of a few types of biomass that are being considered for ethanol production.

Table 1. Composition of different types of lignocellulosic biomass.

Biomass	Glucan	Xylan	Galactan	Arabinan	Mannan	Lignin
Agricultural waste						
Corn stover[1]	36.4	18.0	1.0	3.0	0.6	16.6
Wheat straw[1]	38.2	21.2	0.7	2.5	0.3	23.4
Switchgrass[1]	31.0	20.4	0.9	2.8	0.3	17.6
Bagasse[1]	40.2	21.1	0.5	1.9	0.3	25.2
Rapeseed straw[3]	34.7	19.4		8.3		28.1
Rice straw[1]	34.2	24.5	-	-	-	11.9
Softwoods						
Spruce[6]	43.4	4.9	-	1.1	12.0	28.1
Pine[1]	46.4	8.8	-	2.4	11.7	29.4
Hardwoods						
Sycamore[1]	53.1	17.1	-	1.7	2.7	23.2
Willow[5]	43.0	24.9	-	1.2	3.2	24.2
Paper waste						
From MSW[1]	56.0	8.3	-	-	5.6	30.1
From newsprint[2]	64.4	4.6	-	0.5	16.6	21.0
From chemical pulps[4]	60-70			10-20		5-10

MSW, municipal solid waste; References: [1]Wiselogel *et al.*, 1996, [2]Lee, 1997, [3]Jeong *et al.*, 2010, [4]Sun & Cheng, 2002, [5] Sassner *et al.*, 2006, [6]Tengborg *et al.*, 1998

Despite some differences in the chemical composition of plant biomass, in all cases they are embedded in the same kind of matrix: lignocellulose. This complex arrangement of structural carbohydrates (Figure 2) imposes serious limitations to the efficient conversion of residual plant biomass to ethanol.

The main component of lignocellulose is the polysaccharide cellulose, in which glucose molecules are connected by β(1-4) links. The chains are then linked by hydrogen bonds, forming a crystalline or amorphous structure (Zaldivar *et al.*, 2001). Hemicellulose, a heterogeneous family of polysaccharides, contains a mixture of hexoses and pentoses, such as arabinose, mannose, glucose, xylose, galactose, etc., and it is the second most abundant component of lignocellulose (Van Wyk, 2001). Lignin, an intricate macromolecule consisting of cross-linked phenylpropanoid monomers, is covalently linked with hemicellulose, creating a complex mesh in which the cellulose is embedded. This structure provides the biomass with mechanical strength and resistance to degradation, but also poses

the biggest obstacle to the commercialization of second-generation bioethanol (Himmel *et al.*, 2007). There are no microorganisms that can convert lignin into ethanol (Zaldivar *et al.*, 2001). Natural degradation of lignocellulosic materials is typically mediated by fungi (Namhyun *et al.*, 2000). Therefore, before the carbohydrates in lignocellulosic biomass can be converted into ethanol by microorganisms, a pre-treatment step is required.

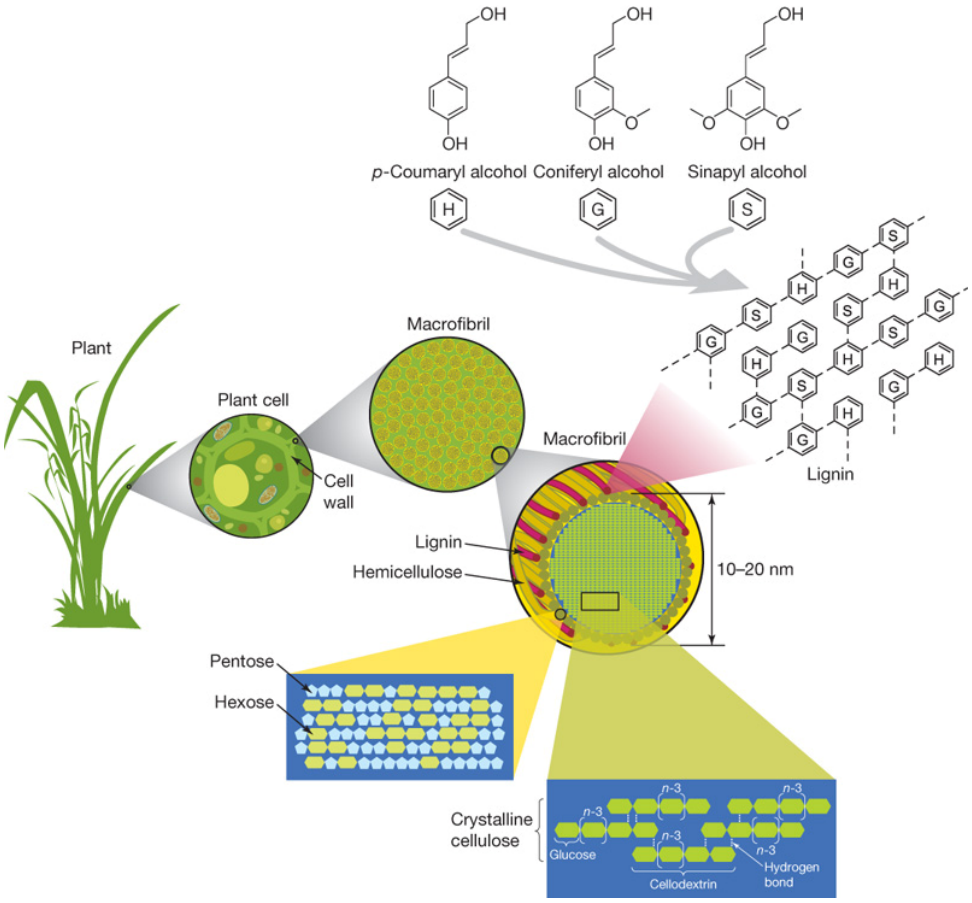

Figure 2. Structure of lignocellulose. Reprinted by permission from Macmillan Publishers Ltd: Nature (EM Rubin Nature 454, 841-845 (2008) doi:10.1038/nature07190), copyright 2008.

2.2 Pre-treatment strategies

An effective pre-treatment method should both be able to break down the structure of lignin, and to disrupt the links between hemicellulose, lignin and cellulose, as well as the crystalline structure of cellulose, in order to make the sugar polymers accessible for the subsequent hydrolytic steps. Additionally, the ideal pre-treatment method avoids the need for reducing the particle size, preserves the hemicellulose fraction, limits the sugar degradation and formation of inhibiting compounds, and has a minimal cost and energy consumption,

among other factors (Alvira *et al.*, 2010; Mosier *et al.*, 2005; Taherzadeh & Karimi, 2008). Pre-treatment techniques are typically categorized according to their mode of action and the reagents used (if any) in physical, chemical, physicochemical or biological techniques. The most commonly used physicochemical method is steam explosion (Alvira *et al.*, 2010), while H_2SO_4 and NaOH are the most widely reported chemical pre-treatment agents (Taherzadeh & Karimi, 2008).

Each method has different advantages and limitations. Therefore the most adequate one will depend not only on the type and composition of lignocellulosic material to be pretreated, but also on the nature of the subsequent steps. For instance, if the fermentative microorganism is significantly inhibited by sugar degradation products, then methods such as alkaline peroxide pre-treatment or wet oxidation, which generate low or no amounts of inhibitors such as 2-furfural or 5-hydroxymethylfurfural (Paper III; Saha and Cotta, 2006; Saha and Cotta, 2007) can be used. If the intention is to use a cellulolytic microorganism and skip the enzymatic hydrolysis step, special emphasis should be put on using a method that favours cellulose accessibility and structural disruption, such as ammonia fibre explosion (AFEX) (Laureano-Perez *et al.*, 2005) or ammonia recycle percolation (Yang & Wyman, 2008).

Another important factor that does not receive much attention is the distribution of fermentative sugars in the different fractions resulting of a pre-treatment. Most methods yield two different phases, and the slurry phase is often discarded (Erdei *et al.*, 2012; Georgieva & Ahring, 2007; Nigam, 2001). This phase can contain a considerable amount of sugars, in most cases derived from hemicellulose (Alvira *et al.*, 2010). Maximizing the use of all the fermentable carbohydrates is a relevant goal in order to decrease the cost of bioethanol production; therefore, all the pre-treatment fractions should be used, especially if a pentose-fermenting microorganism is to be used (Paper III). This could however have the drawbacks of salt inhibition and excessive dilution of the substrate (Paper III, Banerjee *et al.*, 2012). An alternative would be to use AFEX, which, due to the evaporation of ammonia, generates only one (solid) fraction (Alvira *et al.*, 2010).

Despite these positive efforts, pre-treatment remains one of the most expensive steps in the process (Lynd, 1996), and the one drawing the most attention in recent research reports.

2.3 Metabolic aspects of ethanol fermentation

Theoretically, 2 moles of ethanol can be obtained from fermentation of 1 mole of hexose (Eq. 1). The yield from 1 mole of pentose is lower (Eq. 2).

$$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2 \quad \text{Eq. 1}$$

$$C_5H_{10}O_5 \rightarrow 1.67\ C_2H_5OH + 1.67\ CO_2 \quad \text{Eq. 2}$$

However, part of the sugars is used for growth and biomass production, and therefore the experimentally obtained yields can never correspond to the theoretical ones. Furthermore, depending on the metabolic pathway that the microorganism uses, other metabolites can be produced alongside ethanol, therefore decreasing the final yield. For *S. cerevisiae*, ethanol is formed via pyruvate conversion to acetaldehyde and carbon dioxide, by action of the enzyme pyruvate decarboxylase (PDC) (Figure 3). Acetaldehyde is then converted into ethanol by the action of an alcohol dehydrogenase (ADH), while one molecule of NAD(P)H is consumed. Since during glycolysis one molecule of NAD(P)H is formed per molecule of pyruvate, there is no redox imbalance and the microorganism can afford a homoethanolic fermentation (Dellomonaco *et al.*, 2010). However, few other microorganisms besides *S. cerevisiae* ferment ethanol using PDC, the bacteria *Zymomonas mobilis* being one of them (Wiegel, 1980). Most bacteria lacking this enzyme convert pyruvate to ethanol with an additional NAD(P)H consuming step, via acetyl-coenzyme A (CoA) (Figure 3). If no other product was formed, a redox imbalance would occur; therefore, most bacteria that produce ethanol in this way also produce acetate, a more oxidized product. In addition to acetate, mixed acid fermentation can also include lactate formation from pyruvate, and formate if acetyl-CoA formation is catalysed by a pyruvate formate lyase (PFL) (Figure 3).

Variations in the fermentation product ratios occur due to differences in the properties of the enzymes that direct the electron flow, such as specific activities and regulatory mechanisms (Lowe *et al.*, 1993). Changes in the culture conditions (pH, temperature) can also have an effect on product formation (Paper II). However, it is thermodynamically implausible that homoethanologenesis, the formation of ethanol as the single metabolic product, from pyruvate, can happen via acetyl-CoA (Wiegel, 1980).

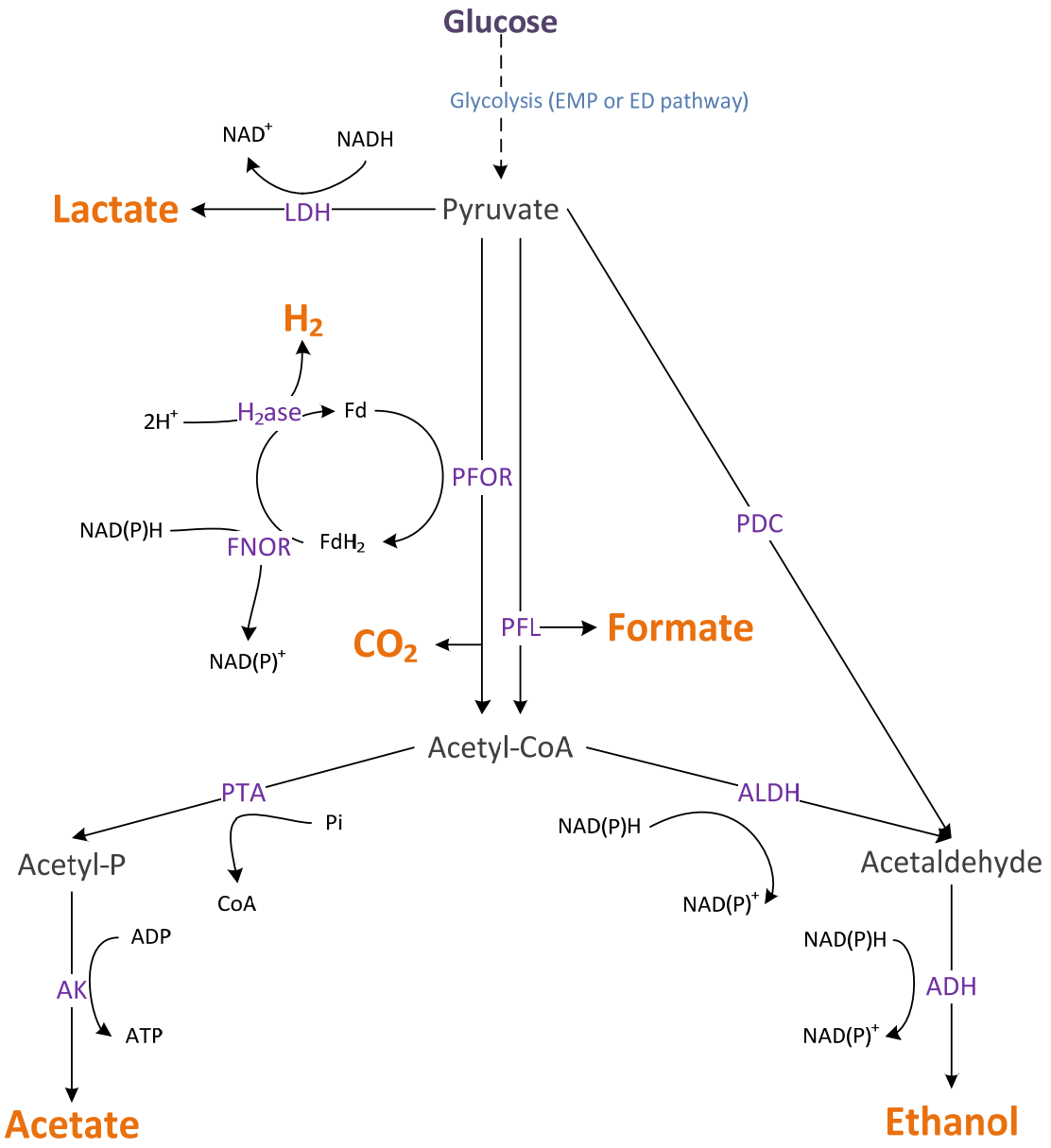

Figure 3. Different metabolic routes for ethanol formation from pyruvate, and other products of mixed acid fermentation. EMP, Embden-Meyerhof-Parnas pathway; ED, Entner-Doudoroff pathway; LDH, lactate dehydrogenase; PDC, pyruvate decarboxylase; PFOR, pyruvate ferredoxin oxidoreductase; H_2ase, hydrogenase; FNOR, ferredoxin/NAD(P)H oxidoreductase; PFL, pyruvate formate lyase; PTA, phosphate acetyltransferase; AK, acetate kinase; ALDH, acetaldehyde dehydrogenase; ADH, alcohol dehydrogenase.

2.4 Extreme thermophilic ethanologens

An efficient, economically viable conversion of second-generation substrates into bioethanol production requires the use of microorganisms with specific properties. A broad substrate range is desirable, given the composition of lignocellulosic biomass (Table 1); the microorganism should at least be able to

ferment both glucose and xylose, which represent the majority of the sugars in these materials. The ability to hydrolyse cellulose, xylan and other carbohydrate polymers would be a significant advantage. The ethanol yield should ideally be in the range of 90-100 % of the theoretical yield (Eq. 1, Eq. 2); such an ethanol yield is intrinsically linked to minimal by-product formation. The ethanol productivity should be at least 1 g l^{-1} h^{-1}; for downstream processing facilitation, the ethanol titer should not be lower than 4-5 % (v/v), and the microorganism should also be able to grow in the presence that amount of ethanol. Finally, an ideal microorganism should be robust in several other aspects: tolerant to variation in environmental conditions (pH, temperature), ability to grow without special nutrients or supplements, and to tolerate pre-treatment derived inhibitors (Dien *et al.*, 2003; Zaldivar *et al.*, 2001).

A microorganism with all these traits is, however, yet to be found. The yeast *S. cerevisiae* and the bacterium *Z. mobilis* both can achieve yields in the range of 90-97 % of the theoretical one, and productivities of 1.3-3.5 g g^{-1} cell mass h^{-1} (Rogers *et al.*, 1982; Zaldivar *et al.*, 2001). However, they lack the ability to ferment pentoses (Olofsson *et al.*, 2008; Weber *et al.*, 2010). The only yeast that can efficiently convert xylose to ethanol is *Pichia stipitis* (Parekh & Wayman, 1986). However, this can only be achieved under very strict microaerophilic conditions and with the addition of determined amounts of nitrogen sources (Slininger *et al.*, 2006).

Recently, much of the search for new ethanologenic strains has been focusing in a particular category of microorganisms: extreme thermophiles, which have an optimal growth temperature in the range of 65-75 °C (Chang & Yao, 2011; Taylor *et al.*, 2009). The advantages of a thermophilic ethanol production process were first mentioned by Wiegel in 1980: higher yields due to high catabolic activity (in contrast with biomass production), which results in shorter fermentation times; a wide range of fermentable substrates; minor risk of process contamination by other microorganisms; no requirement for aeration, and minimal need for mixing, cooling or heating of vessels in-between process steps (energy savings); ease of direct ethanol recovery from the fermentation broth by vacuum distillation.

Indeed, many of these extreme thermophiles have ethanol as their major fermentation product (Table 2). Wiegel (1980) has attributed this to the fact that ethanol is a neutral and volatile compound, therefore causing no accumulation problems to the thermophiles that produce it. In addition to the generally high yields, many

thermophiles have the capacity to hydrolyse lignocellulosic polymers, like cellulose and xylan. This characteristic could allow for the development of a consolidated bioprocess (Lynd *et al.*, 2005) by skipping the step of enzymatic hydrolysis after pre-treatment of lignocellulosic biomass. The most relevant extreme thermophilic microorganisms that are currently being studied for bioethanol production are listed in Table 2.

The wild-type microorganism with the highest ethanol yield from xylose reported so far, *Thermoanaerobacter pentosaceus*, was recently isolated and described (Paper I). It is the most recent member of the *Thermoanaerobacter* genus, which contains several other species of interest for bioethanol production. Besides the typically high ethanol yields (Table 2), most *Thermoanaerobacter* strains do not have significant nutrient requirements, because of their ability to synthetize cofactors, such as vitamin B_{12} (Hemme *et al.*, 2011); furthermore, they are known to contain special ADH enzymes that enhance ethanol fermentation (Burdette & Zeikus, 1994; Burdette *et al.*, 1996; Peng *et al.*, 2008). *Thermoanaerobacter ethanolicus*, the first member to be assigned to the genus, is one of the most studied thermophilic anaerobes regarding bioethanol production (He *et al.*, 2009; Hild *et al.*, 2003; Lacis & Lawford, 1991; Lee *et al.*, 1993; Ljungdahl & Carriera, 1983; Wiegel & Ljungdahl, 1981).

Despite their potential, and unlike *S. cerevisiae* and *Z. mobilis*, thermophiles produce ethanol via acetyl-CoA (Figure 3) and therefore are not true homoethanologens. Due to this, many of them are already being the subject of metabolic engineering in order to improve their performance.

Table 2. Overview of extreme thermophilic, anaerobic microorganisms with relevant properties for second-generation bioethanol production.

Microorganism	Growth temperature (°C)	Cellulolytic ability	Inhibitor tolerance	Ethanol tolerance	Fermentation products	Maximum ethanol yield (g g^{-1})	Reference
Caldicellulosiruptor bescii	78-80	C	n.r.	n.r.	n.r.	n.r.	Chung *et al.*, 2012; Dam *et al.*, 2011; Yang *et al.*, 2010
Caldicellulosiruptor obsidiansis	78	C, X, S, Pc	n.r.	1 %	E, A, L, H	n.r.	Hamilton-Brehm *et al.*, 2010
Caloramator boliviensis	60	X	n.r.	n.r.	E, A, L, P, H	0.39 (xylose)	Crespo *et al.*, 2012a, b
Clostridium thermocellum	60	C	n.r.	4-5 %	E, A, L, F, H	0.37 (glucose)	Balusu *et al.*, 2004; Rani & Seenayya, 1999; Roberts *et al.*, 2010; Tyurin *et al.*, 2004
Thermoanaerobacter ethanolicus	70	X, S	n.r.	0.5-5 %	E, A, L, H	0.49 (glucose) 0.42 (xylose)	Hild *et al.*, 2003; Lacis & Lawford, 1988; Wiegel & Ljungdahl, 1981
Thermoanaerobacter mathranii	70	I, Pc, S, X	2 mM aromatic compounds	5 %	E, A, L, H	0.35 (xylose)	Larsen & Nielsen, 1997; Yao & Mikkelsen, 2010a
Thermoanaerobacter pentosaceus	70	I, Pc, S, X	3.4 g l^{-1} 2-furfural and 5-HMF	0.5 %	E, A, L, H	0.43 (xylose)	Paper I, Paper III
Thermoanaerobacter pseudethanolicus	67-69	X, Pc, S	n.r.	4 %	E, A, L, H	0.48 (glucose)	Lovitt *et al.*, 1988; Zeikus, 1980
Thermoanaerobacter AK_5	65	S	n.r.	n.r.	E, A, L, H	0.43 (glucose) 0.41 (xylose)	Brynjarsdottir *et al.*, 2012
Thermoanaerobacter J1	65	S	n.r.	n.r.	E, A, L, H	0.43 (glucose) 0.38 (xylose)	Jessen & Orlygsson, 2012
Thermoanaerobacterium saccharolyticum	60	X, S	n.r.	n.r.	E, A, L, H	0.30 (xylose)	Lee *et al.*, 1993; Shaw *et al.*, 2008
Thermoanaerobacterium AK_{17}	60	C, Pc	4 g-furfural l^{-1} 6 g-HMF l^{-1}	n.r.	E, A, H	0.38 (glucose) 0.34 (xylose)	Almarsdóttir *et al.*, 2012; Orlygsson & Baldursson, 2007; Sveinsdottir *et al.*, 2009

C, cellulose; I, inulin; S, starch; Pc, pectin; X, xylan; A, acetate; E, ethanol; F, formate; H, hydrogen; L, lactate; P, propionate; n.r., not reported;

2.5 Factors affecting extreme thermophilic ethanol production

2.5.1 Substrate

The concentration of substrate is known to have a significant effect on a microorganism's growth and fermentation capacity (Edwards, 1970). Growth inhibition due to high carbon concentration is typically associated with ineffective regulation of the carbon flow and accumulation of reducing power (Guedon *et al.*, 1999). The initial concentration of substrate can also have an effect on the soluble metabolites profile, and consequently on the ethanol yield. It has been reported that thermophiles show a lower tolerance to high sugar concentrations, when compared to mesophiles. This was attributed to the increased solubility of inorganic salts and other organic compounds at higher temperatures, resulting in higher osmotic pressure (Lynd, 1989).

For *T. ethanolicus*, it was found that at substrate concentrations above 10 g l^{-1}, a shift from ethanol production to other products would occur (Ljungdahl & Carriera, 1983). For *T. pentosaceus*, at initial concentrations of xylose of 10 g l^{-1}, only 60 % of the xylose was consumed, and at 20 g l^{-1}, 70 % of the initial xylose remained in the fermentation broth. Furthermore, higher concentrations of xylose redirected the metabolism from high ethanol production to a more even carbon distribution between ethanol, acetate and lactate (Paper II). A similar effect was reported for high concentrations of glucose on *Thermoanaerobacter brockii*, where more lactate was produced. This has been attributed to the accumulation of intermediate compounds such as fructose diphosphate, which stimulates the enzyme lactate dehydrogenase (LDH) (Germain *et al.*, 1986; Lamed & Zeikus, 1980).

In the case of extreme thermophiles, inhibition at high substrate concentrations could also be caused by the formation of inhibitory compounds through the Maillard reaction. At high initial sugar concentrations, and when these are not immediately utilized, their reaction with the amino acids present in the medium can be catalysed by the exposure to high temperatures (Maillard, 1912). This can be confirmed by observation of browning in the culture medium (Paper II), HPLC detection of inhibitory compounds (2-furfural, 5-HMF) in the medium (Paper III), and an incomplete chemical oxygen demand (COD) recovery if no detection of the inhibitors is performed (Paper II).

However, for second-generation ethanol production, a microorganism that can tolerate high substrate concentrations is not necessarily a requirement, depending on the concentration of sugars obtained after pre-treatment and enzymatic hydrolysis. Furthermore, a higher tolerance can be obtained by immobilization of the microorganism, evolutionary adaptation, or genetic engineering (Shao *et al.*, 2011; Xue *et al.*, 2008; Paper IV).

The type of substrate used can also have an effect on ethanol yield. Depending on the oxidation state of the substrate, different quantities of reducing equivalents are generated and need to be recycled during fermentation. This will affect the fermentation metabolites and consequently the ethanol yield. A higher ethanol yield has been reported for strains *Thermoanaerobacter mathranii* and *Caloramator boliviensis* when mannitol is used as the single carbon source (Crespo, 2012; Yao & Mikkelsen, 2010a). Mannitol is a more reduced substrate when compared to glucose or xylose (Yao & Mikkelsen, 2010a), therefore generating more reducing power during its conversion. Ethanol formation, which regenerates more $NAD(P)^+$, is therefore the preferred metabolite when a more reduced substrate is used (Yao & Mikkelsen, 2010a).

For second-generation bioethanol however, the type of substrate available is restricted by the composition of the locally available biomass, the pre-treatment method, and the enzymatic hydrolysis step. Reduced substrates such as mannitol are not major components of lignocellulosic biomass (Table 1). However, mannitol is a significant component of some species of seaweed (Holdt & Kraan, 2011), which are being considered for ethanol production (Kim *et al.*, 2011).

2.5.2 Pre-treatment derived compounds

One of the main consequences of the use of some pre-treatment methods in lignocellulosic biomass is the formation of compounds that are toxic to the fermentative microorganisms. These include weak acids such as acetic or formic acid, phenolic compounds such as vanillin and syringaldehyde and furaldehydes such as 2-furfural and 5-hydroxymethylfurfural (HMF) (Palmqvist & Hahn-Hägerdal, 2000b). The latter are dehydration products of hexoses and pentoses, and are found among most types of lignocellulosic biomass (Hahn-Hägerdal, 1996); they are also the best studied. Their mechanisms of inhibition are thought to be related to their level of hydrophobicity (Hahn-Hägerdal, 1996), their chemical reactivity with cellular macromolecules (Zaldivar *et al.*, 1999), and interference with enzyme and RNA synthesis. Most of this knowledge was however generated from studies on *S. cerevisiae*, which do not necessarily

translate to other microorganisms. For example, although in *S. cerevisiae* the mode of action of aldehyde inhibition is thought to be related to cell membrane damage (Hahn-Hägerdal, 1996), this was not observed in a strain of *Escherichia coli* (Zaldivar *et al.*, 1999). In thermophilic, anaerobic bacteria, little is known about the mode of action of pre-treatment derived inhibitors. Klinke *et al*. (2001) have reported complete inhibition of growth and ethanol production by several phenolic compounds at 2.7 g l^{-1} for *T. mathranii*. More recently, *Thermoanaerobacterium* AK_{17} was reported to be the most tolerant thermophilic anaerobe to date: total inhibition of end-product formation from glucose was only detected for 4 and 6 g l^{-1} of 2-furfural and 5-HMF (Almarsdóttir *et al.*, 2012). On the other hand, *T. pentosaceus* was shown to have its ethanol production completely inhibited at 1.2 and 2.8 g l^{-1} of 2-furfural and 5-HMF, respectively (Paper III).

These values indicate a lower tolerance to inhibitors for anaerobic thermophiles when compared to *S. cerevisiae* (Klinke *et al.*, 2004). However, at low inhibitor concentrations, which typically correspond to low hydrolysate loadings (less than 10 % dry biomass), there are several reports of thermophilic bacteria being able to grow and produce ethanol without restrictions (Ahring *et al.*, 1996; Crespo *et al.*, 2012b; Georgieva & Ahring, 2007; Georgieva *et al.*, 2008; Ng *et al.*, 1981). In some cases, this capacity is attributed to adaptability to the cultivation conditions (Thomasser *et al.*, 2002).

In Paper III, it was reported that *T. pentosaceus* was able to remove 5-HMF and 2-furfural from the cultivation medium up to 1 g l^{-1} and 0.5 g l^{-1}, respectively. It was also observed that at these concentrations, the yields of some fermentation products (ethanol, acetate and lactate for 5-HMF, and acetate for 2-furfural) had been enhanced, rather than decreased. Comparable beneficial effects had been previously observed in other related anaerobic bacteria (Almarsdóttir *et al.*, 2012; Ezeji *et al.*, 2007; Zhang *et al.*, 2012). Although to date there are no known ethanologenic strains that contain the gene cluster responsible for 5-HMF and 2-furfural metabolism (Wierckx *et al.*, 2011), these findings indicate that there is ground for strain improvement in this field, either with evolutionary or metabolic engineering approaches.

Sensitivity to pre-treatment derived inhibitors remains a limitation for second-generation bioethanol production for most of the ethanologenic microorganisms. Depending on the concentration of inhibitors in the pretreated biomass, the addition of a detoxification step before the fermentation could prove beneficial

for the ethanol yield; however these additional costs could potentially increase the price of the final product (Palmqvist & Hahn-Hägerdal, 2000a). A better understanding of the microbiology of the inhibition mechanisms, combined with the development of the existing and possibly new pre-treatment strategies is definitely the best approach to overcome this limitation.

2.5.3 Ethanol tolerance

One of the features usually required for a good ethanol producing microorganism is the ability to grow and produce ethanol in the presence of at least 4 % (v/v) ethanol. This threshold also corresponds to the minimal ethanol titer required for an economic ethanol recovery using the classical downstream approaches (Hahn-Hägerdal *et al.*, 2006).

Low tolerance to ethanol concentration in the cultivation medium (i.e. below 4 % (w/v)) is widely claimed to be a major limitation for the use of thermophilic, anaerobic bacteria for industrial ethanol production (Herrero, 1983; Olsson & Hahn-Hägerdal, 1996; Taylor *et al.*, 2009). However, there are very few reports in which such low tolerance values are reported: Carlier *et al*. (2006) reported that strains of *T. mathranii* subsp. *alimentarius* presents only weak growth in the presence of 4 % (v/v) ethanol; Lovitt *et al*. (1984) claimed that the native strain of *Thermoanaerobacter pseudethanolicus* 39E does not grow above 1 % (w/v) ethanol; Wang *et al*., 1983 showed that the native strain of *Clostridium thermocellum* has its growth reduced to 40 % at 1 % (v/v) ethanol; the recent isolate *T. pentosaceus* was shown to have its growth reduced by 50 % and to stop producing ethanol at a concentration of (exogenously added) ethanol of only 0.5 % (v/v) (Figure 4).

The data pool on ethanol-adapted strains that can grow above 4 % (w/v) ethanol is, on the other hand, larger, and there are reports of tolerance as high as 10 % for *T. ethanolicus*, 8 % for *T. mathranii*, and 6 % for *C. thermocellum* (Georgieva *et al*., 2007; Rani & Seenayya, 1999; Shao *et al*., 2011; reviewed in Lynd *et al*., 2002 and Lynd *et al*., 1991). Despite these achievements, the highest ethanol titres achieved by thermophilic, anaerobic bacterial cultures are still in the range of 30-40 g l^{-1} (Argyros *et al.*, 2011; Shaw *et al.*, 2008). The reason for this gap between tolerance to exogenously added ethanol and the maximum titer achieved by thermophilic anaerobes is still the cause of much debate (Lynd *et al.*, 2002).

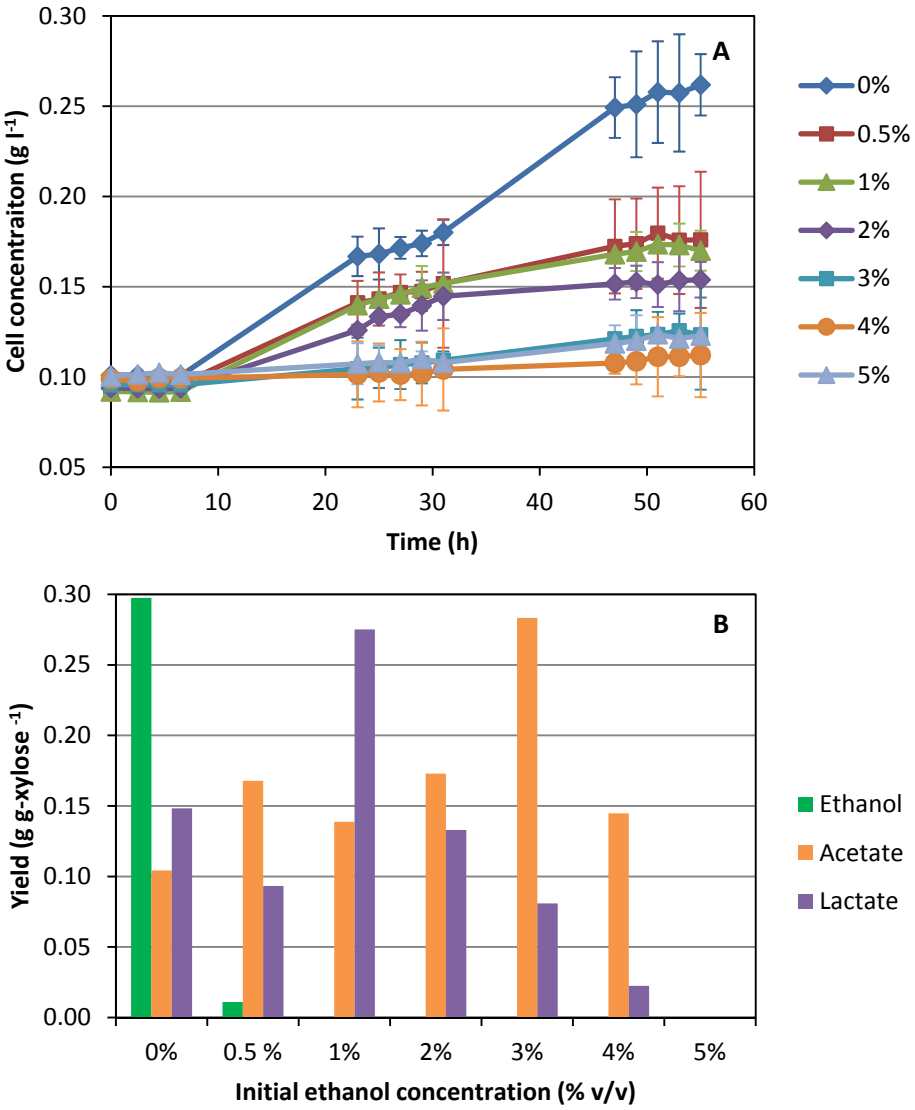

Figure 4. Biomass evolution (A) and soluble metabolic profile (B) of *T. pentosaceus* at different concentrations of exogeneously added ethanol to the cultivation medium.

Only very recently a culture of a genetically modified strain *T. saccharolyticum* in medium supplemented with urea as a nitrogen source was able to produce 54.3 g l^{-1} (Shaw *et al.*, 2012). This outcome was attributed to the lower osmolality in the cultivation medium, since there was no need to add ammonium salt as nitrogen source, nor base to control the pH of the medium. This explanation supports the hypothesis that the discrepancy between ethanol tolerance and achieved ethanol titres is a result of the high salt concentration added for the purpose of pH control in high feed continuous fermentations (Lynd *et al.*, 2001).

From the perspective of process design, second-generation ethanol fermentations are unlikely to involve ethanol concentrations above 5 %, due to limitations in high solid content handling (Zambare *et al.*, 2011). In addition to this, in thermophilic fermentations ethanol can be more easily removed from the fermentation broth by vacuum distillation (Cysewski & Wilke, 1977), when compared to the first-generation ethanol production process for which the threshold of 4-5 % minimum titer was required for.

In light of these facts, the current accomplishments in adapting thermophilic ethanologens to high ethanol concentrations seem more significant than expected in the path leading to a good alternative to *S. cerevisiae*.

A better understanding on the mechanisms of ethanol tolerance in anaerobic thermophiles - cell membrane alterations, redox potential imbalance and enzymatic regulation (Herrero *et al.*, 1982; Lovitt *et al.*, 1988; Pei *et al.*, 2010; Timmons *et al.*, 2009) - will undoubtedly accelerate the complete overcome of this limitation.

2.5.4 Nutrients and other supplements

Although most thermophilic ethanologens are able to grow and produce ethanol without the addition of complex supplemental nutrients to the cultivation medium (peptone, yeast extract, vitamins), the latter can have a visible effect in the fermentation outcome (Lynd *et al.*, 2002; Zhao *et al.*, 2010).

For *T. pentosaceus* (Paper II), it was reported that adding 2 g l^{-1} of yeast extract or 5 g l^{-1} peptone has a negative effect on the yield. This was likely due to increased concentration of amino acids in the medium, which in turn increase the likelihood of the Maillard reaction occurring (Maillard, 1912). Therefore, a high amount of amino acids present in the medium is not desirable. However, a low amount of yeast extract (0.5 g l^{-1}) had a beneficial effect on ethanol yield and xylose consumption rate. This effect was also observed in a strain of *T. ethanolicus* (Hild *et al.*, 2003) and *T. pseudethanolicus* (He *et al.*, 2009). Some thermophiles have the ability to synthetize vitamins (Hemme *et al.*, 2011; Sato *et al.*, 1992), which is a major advantage.

However, complex nutrients are a costly addition in an industrial ethanol production process, and therefore identifying exactly which nutrients are limiting for a particular strain is important. Another solution could be the use of alternative, inexpensive sources of nutrients, such as corn steep liquor (Amartley & Leungl, 2000) or urea (Shaw *et al.*, 2012).

Sulfur-related compounds have also been shown to affect cell growth and metabolic product distribution in some thermophilic, anaerobic strains. The addition of thiosulfate, an electron scavenger, to cultures of several *Thermoanaerobacter* strains has been shown to have a positive effect on cell growth rate and yield, while oxidizing H_2 and decreasing its inhibitory effect. (Fardeau *et al.*, 1994, 1996; Faudon *et al.*, 1995). However, in most strains the metabolic profile shifted dramatically from ethanol to acetate production (Brynjarsdottir *et al.*, 2012; Fardeau *et al.*, 1996). It was speculated that the use of thiosulfate caused a shift in the flow of electrons, channelling them away from ethanol production. This resulted in an increased flow of carbon to acetate production, which does not consume electrons (Figure 3). Another electron acceptor, sulfite, was shown to have the opposite effect: redirecting the metabolism of *T. pentosaceus* to ethanol production, drastically increasing the ethanol to acetate ratio from 4.8 to 8.2, while maintaining cell yield, as well as lactate and hydrogen production. This resulted in an ethanol yield of 1.39 mol mol^{-1} xylose (Paper I, Figure 5). The mechanism and effects of sulfite reduction have not been studied in detail in thermophilic anaerobes and it would be of great interest to explore this path.

Although the addition of supplements and nutrients to the fermentation process might have a beneficial effect on a microorganism's growth and ethanol yield, it is not essential. Therefore, in order to reduce the costs of second-generation ethanol production, these should only be considered when the benefits clearly exceed the costs.

3 IMPROVEMENT STRATEGIES

Bioethanol produced from residual biomass is still far from being implemented at the industrial scale. Although lignocellulose remains widely available, and despite the advances in pre-treatment techniques, its recalcitrance is still the main cause for the high production costs of second-generation bioethanol. Typically large volumes of chemicals are required, and the price of the enzymes required for the breakdown of the different polymers is high. There are very few examples of successful pre-treatment scale-up, since handling industrial quantities of slurry is also an issue.

Furthermore, extreme thermophilic microorganisms, a promising alternative to *S. cerevisiae* due to their native abilities of achieving high ethanol yields from a wide range of carbohydrates, are still behind in terms of productivity and ethanol concentration. Moreover, most of them produce other metabolites besides ethanol, so there is still room for yield improvement.

In this chapter, some of the possible approaches that can be used to tackle the aforementioned limitations in the fermentation step are discussed.

3.1 Isolation of new microorganisms

A microorganism with all the ideal characteristics of a second-generation ethanol producer is yet to be found. Attempts at finding interesting isolates have led scientists to search for them in environments where desirable properties for the conversion of residual substrates can act as a selective pressure. Considerable efforts have been dedicated to natural thermophilic environments in geothermal areas, but anthropogenic habitats should not be excluded. Galactose-fermenting strains of *S. cerevisiae* have been found in a spent sulfite liquor fermentation plant (Lindén *et al.*, 1992), and cellulolytic and xylanolytic bacteria have been isolated from compost and landfills (Sizova *et al.*, 2011; Westlake *et al.*, 1995).

In Paper I the isolation and characterization of a new member of the genus *Thermoanaerobacter* that produces ethanol from a wide range of sugars is described. *T. pentosaceus* was isolated from an enrichment culture capable of producing 0.49 g g^{-1} xylose, which is 95 % of the theoretical ethanol yield from pentose sugars. This culture had been originally enriched from a household waste inoculum (Liu *et al.*, 2008). *T. pentosaceus* was shown to be able to convert xylose to ethanol with a yield of 1.28 mol mol^{-1} xylose, which is 77 % of the theoretical ethanol yield (Paper II). To date, that was the highest ethanol yield achieved from

pentose by a wild-type thermophilic anaerobe. Upon its characterization, it was found that the ethanol yield could be enhanced by adding 2.5 mM sodium sulfite to the cultivation medium. In these conditions, the ethanol yield increased to 1.39 mol mol^{-1} xylose (Paper I, Figure 5).

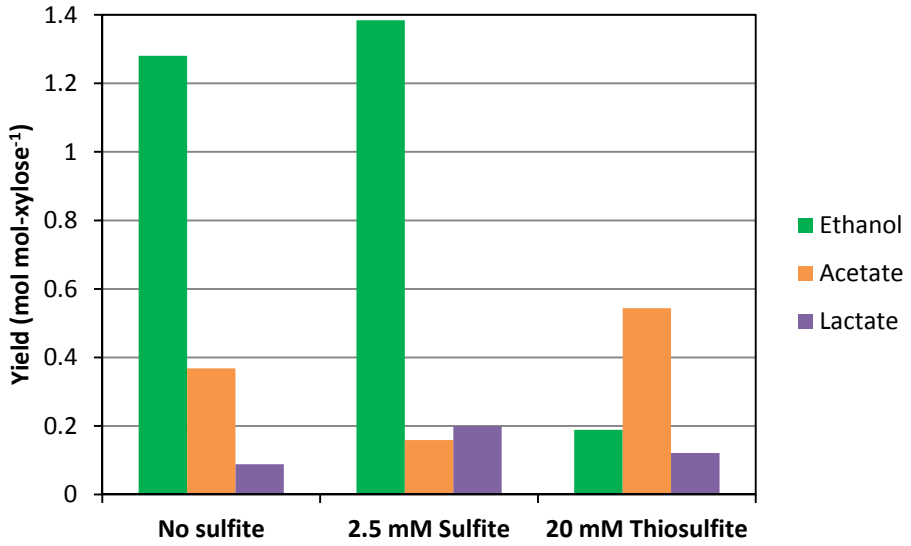

Figure 5. Metabolic product distribution with different electron acceptors added to the cultivation medium of *T. pentosaceus*, using xylose as the carbon source.

Besides this characteristic, *T. pentosaceus* has the same advantages and features as other thermophilic, anaerobic ethanologens. Therefore it is a promising candidate as the fermenting microorganism in a second-generation ethanol production process. However, like the other potential microorganisms (

), it can be the subject of further improvements. This will be discussed in the following sections.

The possibility of unearthing microorganisms with interesting abilities in all kinds of anthropogenic or natural environments remains. However, in spite of the advances in cultivation and isolation techniques (Börner *et al.*, 2012; Sizova *et al.*, 2011), the gap between cultivable and detected microbes in a determined environment remains wide. This is especially true in the case of anaerobic microorganisms in extreme environments (Staley & Gosink, 1999).

3.2 Combined used of microorganisms

Defined co-cultures of microorganisms have been used in the context of bioethanol production with several different purposes, such as accommodating carbohydrate hydrolysis and sugar fermentation in the same vessel (Coughlan &

Kierstan, 1988), or improving the efficiency of sugar consumption in complex substrates (reviewed by Chen, 2011). One of the approaches that can be used to achieve the latter is to complement the efficient glucose fermentation of *S. cerevisiae* with a microorganism that can ferment the remaining sugars, i.e. pentoses. This strategy has been widely used with various mesophilic hexose and pentose fermenting yeasts and bacteria. Close-to-theoretical yields were achieved, such as in the case reported by Taniguchi *et al.* (1997), where a co-culture of *P. stipitis* and a mutant strain of *S. cerevisiae* were able to ferment a mixture of 75 g l^{-1} glucose and xylose to an ethanol yield of 0.50 g g^{-1}.

Thermophilic anaerobes have also been the target of this strategy, an early example being the co-cultivation of *C. thermocellum* and *T. pseudethanolicus* (Ng *et al.*, 1981). More recently, Argyros and co-workers (2011) were able to improve fermentation of 92 g l^{-1} Avicel by a mutant strain of *C. thermocellum* by co-cultivating it with *T. saccharolyticum*. Although the reasons for improvement were not clear, it was hypothesized that *T. saccharolyticum* could minimize the possible effect of substrate inhibition by metabolic overflow in *C. thermocellum* by consuming excess sugar.

However, in order to achieve a stable co-culture, the two selected strains must have similar growth requirements in terms of pH, temperature and oxygen supply, which greatly limits the range of choice. Furthermore, if the consumption of the different sugars is not simultaneous, the pentose consuming strain must be able to do so in the presence of the ethanol produced by the glucose consuming strain.

Another approach is the use of sequential cultivation. This option allows for the use of microorganisms with very different growth requirements; however it is often overlooked as cumbersome due to the number of steps involved. Despite this, inactivation of the bacterial cells in-between steps (Fu *et al.*, 2009) is not necessarily a requirement, and taking advantage of the existence of a well-established bioethanol production process and adding a second step can be a simple way of increasing process efficiency.

In Paper III, *T. pentosaceus* was used with *S. cerevisiae* to ferment pre-treated rapeseed straw in a two-step process. In the first step, *S. cerevisiae* was expected to efficiently convert the hexose sugars to ethanol, and in the second step *T. pentosaceus* would ferment the remaining pentose sugars to ethanol. It was found that the combined use of the two microorganisms resulted in an ethanol yield of 0.45 g g^{-1} initial total sugars. This represented an increase of 14 % and

33 % when compared to a fermentation of the same substrate by only *S. cerevisiae* and *T. pentosaceus*, respectively, and 88 % of the theoretical yield. An even better result could have been obtained by decreasing the length of the first fermentation step to 24 h or less, in order to avoid xylose conversion to xylitol by *S. cerevisiae*.

This is the first example of the combined use of an extreme thermophile with yeast to produce bioethanol. This approach could be extended to other microorganism combinations, possibly using a microorganism in the second step that is highly tolerant to ethanol in order to skip the distillation step. Alternatively, if the second step is thermophilic, simultaneous vacuum ethanol removal can be performed.

3.3 Immobilization of ethanol producing bacteria

Immobilization of bacterial cells is an attractive strategy to increase ethanol productivity and yield, as well as to facilitate downstream processing, since it provides a means to increase cell density and retain the cells inside a continuous reactor (Olsson & Hahn-Hägerdal, 1996). Studies on cell immobilization for second-generation ethanol production focus both on: the immobilization of cellulose producing microorganisms, for improving the enzymatic hydrolysis step (Tamada *et al.*, 1987); and on the fermentation step. In the latter case, most reports concern the immobilization of strains of *S. cerevisiae* and *Z. mobilis*. Ethanol productivities as high as 73 g l^{-1} h^{-1} have been achieved for immobilized *Z. mobilis* in a continuous reactor fed with acid hydrolysate of aspen cellulose (Parekh *et al.*, 1989), while *S. cerevisiae* immobilized in corn stalk could produce up to 86 g l^{-1} ethanol (Yan *et al.*, 2012). Despite these achievements, the problem of the residual pentose sugars remains.

There are however surprisingly few reports pertaining immobilization of thermophilic bacteria for ethanol production. The genetically modified strain *Thermoanaerobacter* BG1L1, derived from *T. mathranii*, has been successfully immobilized in mesophilic granules from a wastewater treatment up-flow anaerobic sludge blanket (UASB) reactor. When fed with un-detoxified wet-exploded wheat straw hydrolysate in a fluidized bed reactor, the immobilized microorganism was able to convert the sugars to ethanol with an efficiency of 68 to 78 % and a yield of 0.39 to 0.42 g g^{-1}. Immobilization and high cell density protected the bacteria from the toxicity of the hydrolysate (up to 10 g l^{-1} acetate in the effluent).

In Paper IV, *T. pentosaceus* was immobilized in different supports (UASB granules, rapeseed straw and activated carbon) and tested for ethanol production from a mixture of xylose and glucose (20 g l^{-1}). The best ethanol producing results were obtained with UASB granules, where a yield of 0.44 g g^{-1} was achieved at an HRT of 24 h. The other immobilization supports tested, rapeseed straw and activated carbon, yielded a maximum ethanol yield of 0.42 and 0.41 g g^{-1} in the same conditions. Furthermore, a maximum ethanol titer of 12.4 g l^{-1} was obtained in these conditions. This is the maximum titer of ethanol reported for *T. pentosaceus*, which was shown to have its ethanol production inhibited by of 5 g l^{-1} of exogenously added ethanol to the growth medium. This result further supports the protective effect that cell immobilization can confer during fermentation (Cassidy *et al.*, 1996) by allowing for higher cell density (Olsson & Hahn-Hägerdal, 1996).

Besides the characteristics typically required for a good carrier (inexpensive, stable, re-usable, nontoxic and that minimizes internal mass transfer limitations (Yan *et al.*, 2012)), stability at extreme thermophilic temperatures (60-80 °C) is an additional requirement for immobilization of thermophilic bacteria. Due to the lack of this property, the typical gel matrices used for cell entrapment, such as calcium alginate or k-carrageenan, are not appropriate (Kanasawud *et al.*, 1989; Norton & Lacroix, 1990).

A recent approach is the utilization of naturally occurring supporting materials to which the cells can adhere, such as sugarcane bagasse, rice husk, loofah sponge and other natural structural fibrous networks (Chandel *et al*., 2009; Iqbal and Saeed, 2005; Yan *et al*., 2012; Yu *et al*., 2007, Paper IV). These are typically nontoxic, reusable, strong and porous enough to allow for cellular growth without rupture and diffusion problems (Iqbal & Saeed, 2005).

Immobilization of thermophilic ethanologens is therefore a promising strategy to improve their characteristic low productivities and sensibility to ethanol. However, more studies on inexpensive and adequate carriers are required before advancing to the industrial stage.

3.4 Metabolic engineering

Until very recently, metabolic engineering of microorganisms was almost exclusive to well-studied organisms, such as *S. cerevisiae* and *E. coli*. Besides having their genome sequenced and despite the vast knowledge on their metabolism, the available genetic tools, such as plasmids and promoters,

pertained almost only to these microorganisms. Using these tools, hexose-only fermenting strains of *S. cerevisiae* and *Z. mobilis* have been engineered to be able to convert pentoses to ethanol (Wisselink *et al.*, 2009; Yanase *et al.*, 2012). However, it is often the case that these strains prefer one sugar over the other; furthermore, formation of undesired side products, such as arabitol and xylitol has been observed in *S. cerevisiae*, possibly as a result of limited enzyme activity in the pentose metabolism pathway (Jeffries & Jin, 2004; Karhumaa *et al.*, 2006). *E. coli* and *Klebsiella oxytoca* strains have also been the subject of genetic modifications with the purpose of redirecting the carbon flow to ethanol production (Dien *et al.*, 2003), but often at the cost of productivity loss (Trinh *et al.*, 2008; Woodruff *et al.*, 2013).

As the genome sequencing technologies become cheaper and more accessible to researchers and novel techniques and tools become available, the development of engineered thermophilic ethanologens also becomes a reality. The first successful transfection of an extreme thermophile with a plasmid for ethanol production purposes was achieved by Mai, Lorenz and Wiegel (1997) with *Thermoanaerobacterium saccharolyticum* JW/SL-YS485. Since then, other ethanologenic thermophiles (Table 3) have been the subject of genetic modifications that have led to an improved ethanol production. Amongst the different approaches used, the most common is the deletion of a gene involved in the formation of an undesirable fermentation by-product, such as lactate dehydrogenase (*ldh*). This has been the first step in most of the strain improvement trials, as reported for *T. saccharolyticum* TD1 (Desai *et al.*, 2004), *T. mathranii* BG1L1 (Georgieva *et al.*, 2007), *C. thermocellum* (Argyros *et al.*, 2011) and *Geobacillus thermoglucosidasius* (Cripps *et al.*, 2009). *C. thermocellum* was subject to yet another deletion mutation, of a phosphotransacetylase, in order to block the acetate production pathway. The resulting strain did produce ethanol as the only fermentation product; however the intermediary compound pyruvate was detected in the fermentation medium and the ethanol yield was not significantly improved: 0.27 g g^{-1} Avicel (Argyros *et al.*, 2011). This was attributed to a redox imbalance.

Another strategy is the insertion of genes that attribute new properties to a strain, for example the ability to convert additional substrates into ethanol. Such is the case of *T. mathranii* BG1G1, which contains a NAD^+ dependent glycerol dehydrogenase and has been shown to successfully convert glycerol into ethanol (Yao & Mikkelsen, 2010a). Other mutant strain of *T. mathranii* has been further

developed using a distinct strategy: strain BG1E1 contains a xylose induced promoter that allows for overexpression of an ADH, resulting in an ethanol yield of 0.46 g g^{-1} when grown on 5 g l^{-1} xylose (Yao & Mikkelsen, 2010b).

However, most of these strategies rely on the selection of mutants using antibiotic markers. Besides the constant need of using antibiotics to cultivate the mutant strains, this limits the number of gene modifications that can be made in a microorganism. With this in mind, Shaw and coworkers (2011) have developed a marker-less genetic system of plasmids that has allowed for more complex mutants to be developed. Such is the case of *T. saccharolyticum* M2907, which encompasses a mutation for upregulating oxidative stress response that increases oxygen tolerance, and a modified histidine-containing protein that relieves carbon catabolite repression and consequently enables simultaneous sugar consumption (Tsakraklides *et al.*, 2012). Another example is *T. saccharolyticum* M1051, which contains a urease gene cluster from *C. thermocellum*, preceded by a cellobiose phosphorylate promoter, allowing to use urea as a nitrogen source, which can significantly decrease the fermentation cost (Shaw *et al.*, 2012). Both these strains are derived from strain M0355 (Shaw *et al.*, 2011), which already contains deletions that hinder the production of lactate and acetate, making ethanol the only fermentation product.

Despite these efforts, not all attempts at metabolic engineering of promising microorganisms for second-generation bioethanol production have been successful. Strains of the genus *Caldicellulosiruptor*, while possessing promising hydrolytic abilities (Blumer-Schuette *et al.*, 2012; Yang *et al.*, 2009), are not amenable to transformation because they contain very specific DNA restriction systems that destroy any foreign DNA that might enter the cells (Chung *et al.*, 2011). Only very recently did Chung and co-workers (2012) identify, in *Caldicellulosiruptor bescii*, a novel methyltransferase capable of methylation of DNA in such a way that it is not targeted by the restriction enzyme CbeI of *C. bescii*. This allowed for the first successful transformation of *C. bescii* with a non-replicative plasmid, thus paving the way for metabolic engineering of this promising strain.

Table 3. Genetically modified thermophilic, anaerobic bacteria for ethanol production.

Microorganism	Genotype	Substrate	Fermentation mode	Ethanol yield (g g^{-1})	Reference
C. thermocellum M0971	*ΔpyrF, Δpta::gapDHp-cat*	5 g l^{-1} Avicel	Batch	0.19	(Tripathi *et al.*, 2010)
C. thermocellum M1570	*Δldh, Δhpt, Δpta,*	18.4 g l^{-1} Avicel	Batch	0.27	(Argyros *et al.*, 2011)
T. mathranii BG1L1	*Δldh*	3-12 % WSH	Continuous	0.39-0.42	(Georgieva *et al.*, 2008)
T. mathranii BG1E1	*Δldh, adhE*$_{upregulated}$	5 g l^{-1} xylose	Batch	0.49	(Yao & Mikkelsen, 2010b)
T. mathranii BG1G1	*Δldh, P*$_{xyl}$ *gldA*	12.8 g l^{-1} xylose 7.2 g l^{-1} glycerol	Continuous	0.47	(Yao & Mikkelsen, 2010a)
T. saccharolyticum TD1	*Δldh*	5 g l^{-1} xylose	Batch	0.25	(Desai *et al.*, 2004)
T. saccharolyticum ALK2	*Δldh, Δack, Δpta*	70 g l^{-1} xylose	Continuous	0.46	(Shaw *et al.*, 2008)
T. saccharolyticum HKO7	*Δldh, Δhfs*	1.8 g l^{-1} cellobiose	Batch	0.22	(Shaw *et al.*, 2009)
T. saccharolyticum M0355	*Δldh, Δack, Δpta*	50 g l^{-1} cellobiose	Batch	0.44	(Shaw *et al.*, 2011)
T. saccharolyticum M1051	*Δldh, Δack, Δpta, ureABCDEFG*	27.5 g l^{-1} cellobiose	Batch	0.44	(Shaw *et al.*, 2012)
G. thermoclucosidasius TM180	*Δldh, pdh*$_{upregulated}$	34 g l^{-1} glucose	Batch	0.39	(Cripps *et al.*, 2009)
G. thermoclucosidasius TM242	*Δldh, pdh*$_{upregulated}$, *Δpfl*	34 g l^{-1} glucose	Batch	0.42	(Cripps *et al.*, 2009)

WSH, Wheat straw hydrolysate;

Given the promising ethanol producing capacity of *T. pentosaceus* and its relatedness to *T. mathranii* (Paper I), which has been a successful target for several genetic changes, attempts at creating a *Δldh* mutant strain of *T. pentosaceus* were made. Based on sequence similarity with *T. mathranii*, the *ldh* gene of *T. pentosaceus* and its flanking sequences were successfully amplified and sequenced (Genbank accession number KC769509).

With this information, a knock-out plasmid for *T. pentosaceus* was designed (plasmid pDTU001, Figure 6), with the objective of deleting the *ldh* gene. The plasmid contained a thermostable kanamycin resistance encoding gene (*htk*) (Hoseki *et al.*, 1999), flanked by two regions homologous to the flanking regions of the *ldh* gene in the genome of *T. pentosaceus*, and it was based on the commercial plasmid pUC57 (Genscript, USA).

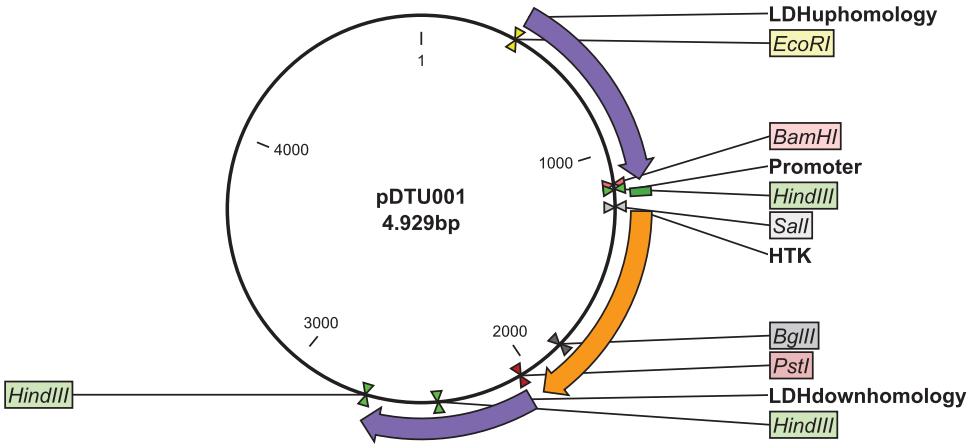

Figure 6. Knock-out plasmid construct pDTU001 was designed based on commercial plasmid pUC57; it contains at the multiple cloning site an insert consisting of a thermostable kanamycin resistance gene (*htk*) preceded by a promoter and flanked by two regions homologous to the flanking regions to the *ldh* gene in the genome of *T. pentosaceus* (LDHuphomology and LDHdownhomology).

Despite several trials to transform the knockout plasmid into *T. pentosaceus,* the deletion was unsuccessful. Additional attempts of transformation of *T. pentosaceus* with the replicative plasmids conferring kanamycin resistance pIKM1 (Mai *et al.*, 1997) and pMU131 (Caiazza *et al.*, 2009) were also unsuccessful. In order to isolate the cause of this result, cell extracts of *T. pentosaceus* were incubated with plasmid pIKM1 under several conditions, and the results were analysed in an agarose gel (Figure 7).

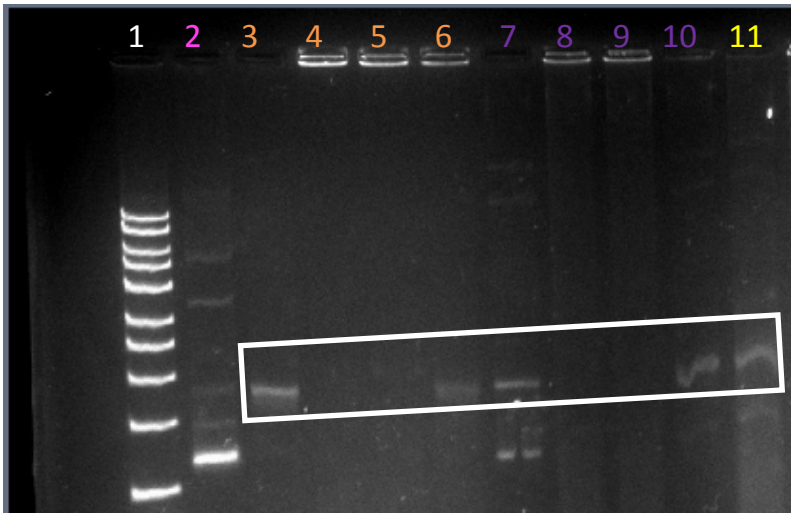

Figure 7. Agarose gel of plasmid pIKM1 incubated for 2 h with cell extracts of *T. pentosaceus* under several different conditions. Lanes: 1, 1 kb ladder; 2, original plasmid; 3, plasmid incubated at 70 °C; 4 and 5, plasmid incubated at 70 °C with cell extract of *T. pentosaceus*; 6, plasmid incubated at 70 °C with inactivated cell extract of *T. pentosaceus*; 7, 8, 9 and 10 correspond to 3, 4, 5 and 6 but at 60 °C; 11, plasmid incubated at 37 °C with cell extract of *T. pentosaceus.*

When incubated at 60 °C and 70 °C for 2 h, there was no visible plasmid band (lanes 4, 5, 8 and 9). Incubation at 37 °C (lane 11) seems however to have no effect on the plasmid, as well as incubation at 60 °C and 70 °C without cell extract or with inactivated cell extract (by treatment at 90 °C for 30 min; lanes 3, 6, 7 and 10). These results indicate that a possible cause of the difficulties in transforming *T. pentosaceus* with foreign plasmid DNA is a native DNA restriction system that, at least *in vitro*, can degrade DNA in less than 2 h.

This shows that *T. pentosaceus* is not as amenable to metabolic engineering as some of the other extreme thermophilic ethanologens. In order to overcome this obstacle, future work should focus on the characterization of the restriction system of *T. pentosaceus*, in a similar fashion to what was discovered for *C. bescii*.

With the aid of metabolic engineering, extreme thermophilic bacteria are now closer to become the ideal ethanologens. However, one has to keep in mind that a strain thus obtained and tested in laboratory conditions might behave differently in industrial conditions; robustness is still a key trait that should not be lost during the strain engineering process.

3.5 Evolutionary adaptation

Despite the constant advances in the knowledge of the metabolic aspects of second-generation bioethanol production, there are many gaps that hinder the use of metabolic engineering strategies to their full potential.

The use of the classical approach of evolutionary adaptation remains therefore a valid strategy to improve a microorganism's performance, and it has been widely use in the second-generation bioethanol production context. An early example is described in a patent by Ljungdahl and Carriera (1983), where three new strains of *T. ethanolicus* were obtained by adaptation of the parent strain by selection on pyruvate and iron deprivation. These new strains were able to tolerate up to 10 % (v/v) ethanol and to produce high yields at substrate concentrations above 1 % (w/v) substrate. Klapatch *et al*. (1994) were able to adapt a strain of *Clostridium thermosaccharolyticum* to stable growth and substrate consumption in exogenous ethanol concentrations up to 36 g l^{-1}. More recently, Shao and co-workers (2011) were able to increase ethanol tolerance of *C. thermocellum* up to 50 g l^{-1} by continuously transferring cultures to media containing increasing concentrations of ethanol.

Besides adaptation to higher concentrations of ethanol, there are also successful examples of adaptation to higher substrate concentrations and to compounds typically present in pre-treated substrates. Parekh *et al*. (1986) have increased the ethanol yield from wood hydrolysate of the yeast strains *Candida shehatae* and *P. stipitis* from 0.39 and 0.41 to 0.45 and 0.47 g l^{-1} ethanol. This was achieved by repeated batch cultivation with a constant concentration of fresh wood hydrolysate. In another study (Tran & Chambers, 1986) *P. stipitis* CBS 5776 was able to ferment un-detoxified red oak acid hydrolysate only after inoculum concentration post-acclimatization to this same substrate.

Although a direct attempt at promoting evolutionary adaptation in *T. pentosaceus* was not performed in the scope of this work, the concentration of substrate used for routine cultivations was increased over time. The microorganism was isolated using 2 g l^{-1} xylose; however, during more than 3 years it was routinely cultivated using 5 g l^{-1} xylose. In the last months, the routine cultivation concentration was increased to 10 g l^{-1} xylose. The changes in xylose consumption, ethanol titer, ethanol yield and ethanol to acetate plus lactate ratio are represented in respectively graphs A, B, C and D.

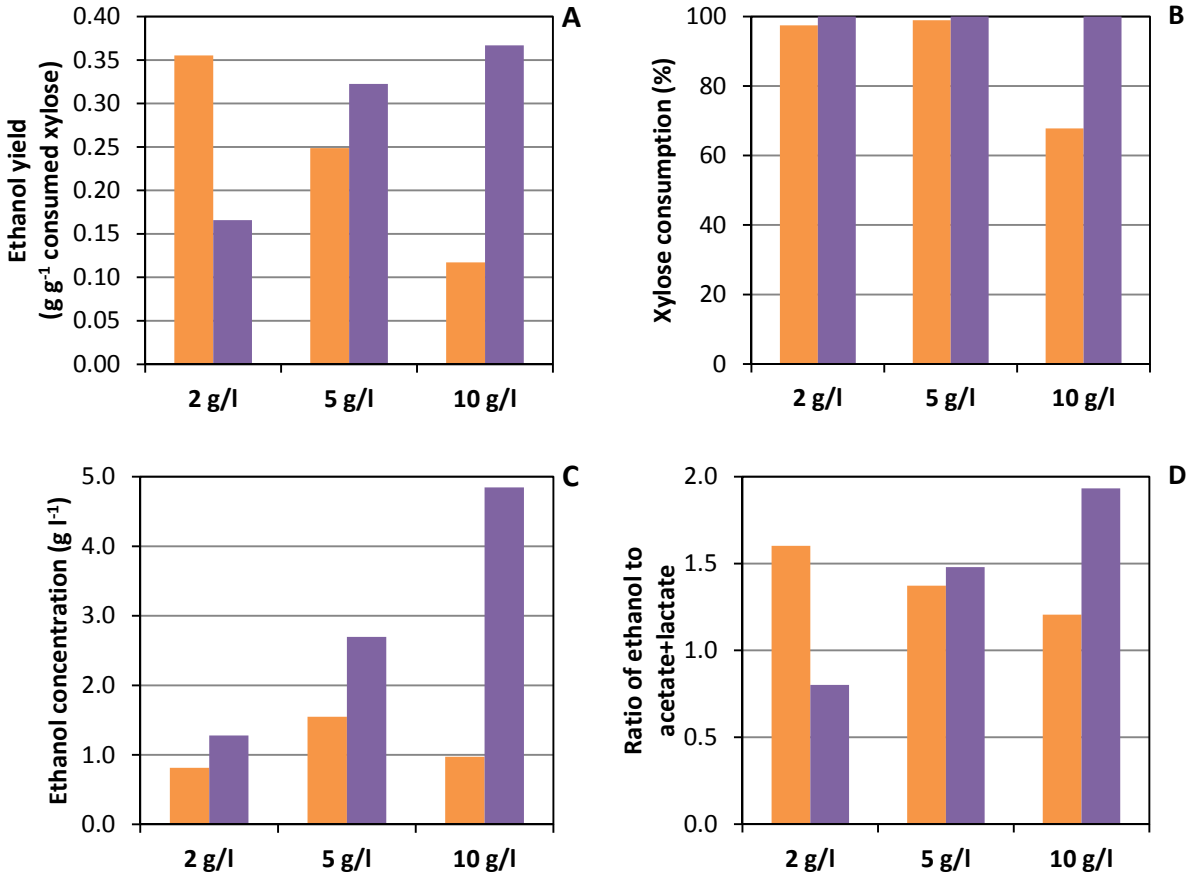

Figure 8. Ethanol yield (A), percentage of xylose consumption (B), ethanol titer (C) and ethanol to acetate plus lactate ratio (D) of *T. pentosaceus* cultivated in BA medium supplied with different concentrations of xylose just after isolation (orange bars, left), and after 3.5 years of repeated batch cultivation in 5 g l^{-1} xylose (purple bars, right).

It is clear that *T. pentosaceus* was adapted to higher substrate concentrations. Soon after its isolation, at 10 g l^{-1} xylose only 68 % of the xylose was consumed (Figure 8B) (and an incomplete COD balance and browning of the medium indicated that it was not completely converted into ethanol (Paper II)). After 3.5 years of routine cultivations in 5 g l^{-1} xylose, *T. pentosaceus* was able to completely consume 10 g l^{-1} xylose in the medium while also increasing the ethanol yield (Figure 8A).

These findings show that evolutionary adaptation, while not causing dramatic changes in the behaviour of a microorganism, seems to leave marks on its genome. Genome sequencing of ethanol-tolerant adapted strains of *C. thermocellum* revealed mutations in ADH genes and in genes related to

carbamoyl-P metabolism, a compound whose derivatives react with ethanol (Shao *et al.*, 2011).

Therefore, evolutionary adaptation can be considered as a valid strategy to improve second-generation ethanol production, especially in situations where the required metabolic tools for a particular organism are not yet available or do not work.

4 CONCLUSIONS

This thesis focused on bioethanol production from residual biomass, by means of isolating a new ethanol-producing microorganism, characterizing it, and analysing its behaviour under different cultivation conditions. Furthermore, the potential of this new *Thermoanaerobacter* species was explored by applying its properties in different approaches for overcoming some of the limitations of thermophilic ethanol production. The major contributions resulting from these studies are summarized below.

- The use of locally sourced waste as a possible source of new, industrially relevant microorganisms was demonstrated.
- A new member of the *Thermoanaerobacter* genus, *T. pentosaceus*, was identified. It grows optimally at 70 °C, it can ferment a wide range of carbohydrates, and it has the highest ethanol yield from xylose reported for a wild-type strain.
- A previously unreported metabolic shift to increased ethanol production in the presence of low concentrations of sulfite was detected in batch cultures of *T. pentosaceus*. Exploring the metabolism behind this shift could lead to new insights in the yet relatively unknown mechanisms of ethanol formation in the *Thermoanaerobacter* genus.
- *T. pentosaceus* was able to produce ethanol directly from alkaline-peroxide pretreated rapeseed straw hydrolysate, and to metabolize low concentrations of the inhibitors 5-HMF and 2-furfural.
- A two-step process for ethanol production from residual wastes was developed, where the yeast *S. cerevisiae* converts hexoses in the first step and *T. pentosaceus* converts pentoses in the second step. The yield was improved when compared to the individual performance by each microorganism. This concept could be adapted to existing ethanol producing facilities by adding an extra thermophilic fermentation step, which would use the pentose-rich heated distillate as a substrate.
- It was demonstrated that all the fractions of pretreated rapeseed straw (solid and liquid, pre and post-enzymatic hydrolysis) can be combined and successfully converted to ethanol in the same process.

- *T. pentosaceus* was immobilized in a variety of supports and continuous ethanol production was observed, adding to the few reports on immobilization of thermophilic microorganisms. Rapeseed straw, a cheap and accessible support material was successfully used. The maximum ethanol titer for this strain, 12 g l^{-1}, was observed at an HRT of 12 h for immobilization in UASB granules.

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The Department of Environmental Engineering (DTU Environment) conducts science-based engineering research within four sections:
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The department dates back to 1865, when Ludvig August Colding, the founder of the department, gave the first lecture on sanitary engineering as response to the cholera epidemics in Copenhagen in the late 1800s.

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