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Department of Systems Biology
Technical University of Denmark

Lactic Acid Bacteria as a new platform for sustainable production of fuels and chemicals

PhD thesis

Anna Monika Boguta

Supervisors:

Associate Professor Jan Martinussen
Professor Peter Ruhdal Jensen

Submitted: March 2016

Preface and acknowledgements

The work described in this PhD thesis has been performed at the Department of Systems Biology, Technical University of Denmark, from January 2012 to March 2016. The project was supervised by Associate Professor Jan Martinussen and Professor Peter Ruhdal Jensen. The research was initially carried out at the Center for Systems Microbiology, which has then split into two smaller groups: Center for Systems Biotechnology with Peter Ruhdal Jensen as a group leader and Metabolic Signaling and Regulation Group, formed by Jan Martinussen and Mogens Kilstrup.

First and foremost, I would like to thank my supervisors, Jan Martinussen and Peter Ruhdal Jensen, for their support, guidance, and valuable suggestions throughout my entire PhD project. Thank you for being inspiring and encouraging, and for always having the time for scientific discussions.

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Finally, I would like to thank my husband, Tomasz, and my lovely daughter, Zuzanna, for their love, patience and unconditional support. I couldn't have done it without you.

Anna Monika Boguta

Kgs. Lyngby, March 2016

Summary

The diminishing natural resources and environmental issues lead us to consider other ways of producing materials, chemicals and energy to satisfy the ever-increasing needs of our society. Lignocellulosic biomass is the most abundant type of substrate in the world; it is also cheap and renewable which makes it a perfect candidate substrate for production of value added products. The second generation biorefineries, employing microorganisms for conversion of lignocellulosic feedstocks into value added products, are not yet employed commercially in a large scale. To increase the economic feasibility of the process, robust microbial catalysts are necessary, both having a broad substrate utilization range and being tolerant to the common inhibitors generated during the lignocellulose pretreatment. Even though many microorganisms are already well characterized and commercially employed in 1st generation biorefineries, the conversion of lignocellulose is a more complex process; thus, the pursue for a suitable microbe continues.

In this PhD study, a wide collection of Lactic Acid Bacteria was systematically screened for the strains' tolerance levels towards various inhibitors coming from the pretreatment of lignocellulosic biomass, as well as for their capabilities to utilize various sugar substrates, including both pentoses and hexoses. Almost 300 strains were tested, including 141 different isolates of *Lactobacillus plantarum*, *L. paraplantarum*, *L. pentosus*, *L. brevis*, *L. buchneri* and *L. paracasei*, and all available *Lactobacillus* and *Pediococcus* type strains. Five most promising strains were subjected to further studies; these included *L. pentosus* LMG 17672, *L. pentosus* LMG 17673, *L. pentosus* 10-16, *P. pentosaceous* ATCC 25745 and *P. acidilactici* DSM 20284. The strains were tested in growth experiments with increased concentrations of the key inhibitors, such as furfural and HMF, as well as with the presence of the most common combinations of inhibitors, mimicking real-life lignocellulosic feedstocks: sugarcane bagasse, wheat straw and soft wood. The two most promising strains were selected; these were *L. pentosus* LMG 17673 and *P. acidilactici* DSM 20284. They were not only found highly resistant to the key inhibitors, but they were also demonstrated to utilize pentoses, xylose and arabinose.

For one of the selected most promising strains, *P. acidilactici* DSM 20284, a chemically defined medium was developed and optimized. The resulting *Pediococcus* Defined Medium (PDM) proved to support the growth of a variety of other species as well, including all *Pediococcus* species and several fastidious Lactobacilli. Thus, the PDM medium appears to be superior to the previously published defined media, and can therefore be suitable for physiological, biochemical or nutritional investigations in other LAB species.

An efficient transformation procedure is necessary for strain's rational genetic engineering. To ease strategies for further strain improvement, a transformation procedure was developed and optimized for *P. acidilactici* DSM 20284, increasing the transformation efficiency by 2 log units. An optimized method allows for the transformation with an efficiency of $2.8 \cdot 10^3$ transformants per µg DNA, permitting the genetic modification of this strain.

In order to even further enhance the *P. acidilactici* DSM 20284 tolerance to furfural, an adaptation experiment was performed by continuous serial-transfer method. After 408 generations, an adapted strain A28 was isolated and showed an increased growth rate on the rich MRS medium with addition of furfural; yet, it also demonstrated a 27% better growth in MRS medium alone. A whole genome resequencing analysis revealed 62 mutations in the genome of the adapted strain compared to the wild-type. The mutations were mainly single nucleotide polymorphisms, but there were also 12 single insertions

identified. More than half of the mutations were non-synonymous substitutions, leading to an amino acid change. Two transcriptional regulators, HrcA and CtsR, were affected by non-synonymous substitutions within the protein or the Shine-Dalgarno sequence, respectively. Several membrane proteins as well as proteins involved in the cell redox homeostasis were also mutated. Purine biosynthesis, salvage and transport related genes were also affected by mutations, likely having an influence on the intracellular nucleotide pool sizes, thereby allowing for an increased growth rate.

The analysis of the transcriptomic profiles of the wild-type *P. acidilactici* DSM 20284 and the adapted strain A28 revealed that the applied furfural concentration did not induce the stress response neither in the wild-type nor in the adapted strain. This finding indicates that both strains are already well adapted to furfural; thereby during the adaptive laboratory evolution experiment the strain adapted towards a faster and more efficient growth on the medium rather than towards furfural resistance. However, several genes related to exopolysaccharide biosynthesis or encoding membrane proteins were induced in the adapted strain, indicating that the cell wall structure might be important for the cell's protection against furfural. The higher growth rate on the other hand, occurred to be enabled by an optimization of the purine and pyrimidine biosynthesis and salvage pathways, up-regulation of the folic acid biosynthesis as well as several enzymes involved in glycolysis.

Finally, the study confirmed the remarkable potential of LAB for their use as microbial cell factories for conversion of lignocellulosic substrates into value-added products.

Dansk Resumé

De faldende ressourcer kombineret med et stadigt stigende globalt forbrug, gør det tvingende nødvendigt at overveje andre måder at producere blandt andet kemikalier på. Biomasse der indeholder lignocellulose er det der er mest af i verden; det er derfor tvingende nødvendigt at udnytte det til produktion af forædlede produkter fremadrettet. Anden generations bioraffinaderier der bruger mikroorganismer til at omdanne lignocellulose-holdige råmaterialer til forædlede produkter, findes endnu ikke i kommercielt stor skala. For at øge de økonomiske muligheder i denne type af anlæg, er det nødvendigt med robuste, mikrobielle katalysatorer, der kombinerer bred substratspecificitet med tolerance overfor de inhibitorer der genereres under forbehandling af lignocellulose. Selvom mange mikroorganismer allerede er velkarakteriserede og brugt rutinemæssigt i første generation bioraffinaderier, er omdannelsen af lignocellulose en mere kompleks proces, der har andre krav til mikroorganismerne. Derfor er jagten på egnede mikrober stadigvæk højaktuel.

I dette studium, blev en stor samling af mælkesyrebakterier systematisk screenet for stammernes tolerancetærskler mod forskellige inhibitorer kendt fra forbehandlingen af lignocellulose, samt for deres evner til at udnytte forskellige pentoser og hexoser. Næsten 300 stammer blev testet, herunder forskellige isolater af *Lactobacillus plantarum*, *L. paraplantarum*, *L. pentosus*, *L. brevis*, *L. buchneri* og *L. paracasei*, samt alle tilgængelige *Lactobacillus* og *Pediococcus* typestammer. De fem mest lovende stammer (*L. pentosus* LMG 17672, *L. pentosus* LMG 17673, *L. pentosus* 10-16, *P. pentosaceous* ATCC 25.745 og *P. acidilactici* DSM 20284) blev underkastet yderligere undersøgelser. Stammerne blev testet i vækstforsøg med høje koncentrationer af mest almindelige hæmmere - furfural og HMF, både alene og i kombinationer, der bedst efterligner virkelige lignocellulose-holdige råmaterialer, som fx bagasse (restproduktet fra sukkerrør), halm og blødt træ. To stammer var særlig lovende *L. pentosus* LMG 17673 og *P. acidilactici* DSM 20284. De blev ikke kun fundet meget modstandsdygtigt over for de centrale inhibitorer, men de kunne også vokse på pentoserne xylose og arabinose.

For en af de udvalgte stammer, *P. acidilactici* DSM 20284, blev et kemisk defineret medium udviklet og sammenlignet med andre publicerede medier. Det resulterende *Pediococcus* Defined Medium (PDM) viste sig at understøtte væksten af en række andre arter, herunder alle Pediococci og flere af de mest krævende Lactobacilli. PDM mediet udmærkede sig ved at være signifikant bedre end de tidligere offentliggjorte definerede medier.

En effektiv transformation procedure er forudsætningen for at man kan udvikle effektive stammer ved genteknologi. En forbedret transformationsprocedure blev udviklet til *P. acidilactici* DSM 20284, og det lykkedes at øge transformationeffektivitet 100 gange i forhold til tidligere publicerede procedurer. Der blev opnået en transformationseffektivitet på $2{,}8 \cdot 10^3$ transformanter per µg DNA, hvilket tillader en udnyttelse af genetiske metoder til optimering af denne stamme.

For yderligere at forbedre *P. acidilactici* DSM 20284 tolerance over for furfural, blev et "Adaptive evolution" eksperiment udført. Efter 408 generationers vækst i det rige MRS medium med tilsætning af furfural, blev en stamme isoleret og den viste sig at have en øget vækstrate på 27 % i mediet. Stammen viste sig både at have en forøget vækst rate i MRS alene, samt en øget tolerance overfor fufural. En hel genom sekventering af den tilpassede stamme afslørede 62 mutationer i genomet. Mutationerne var hovedsagelig enkelt basepar udskiftninger, men der blev også identificeret 12 enkelt basepar insertioner. Mere end halvdelen af

mutationerne var ikke-synonyme substitutioner, der medførte aminosyreændringen. To transkriptionelle regulatorer, HrcA og CtsR, var påvirket af henholdsvis ikke-synonyme substitutioner i proteinet eller i Shine-Dalgarno sekvensen. Der blev identificeret aminosyreændringer i adskillige membranproteiner og enzymer involveret i cellens redox homeostase. Særlig udtalt var tilstedeværelsen af mutationer i gener involveret i biosyntese, salvage og transport og puriner. Disse mutationer står sandsynligvis bag den forøgede vækstrate i MRS.

Analysen af transkriptionsprofilerne for vildtype *P. acidilactici* DSM 20284 og den tilpassede stamme *P. acidilactici* A28 afslørede, at en furfural koncentration der betyder en nedsættelse af vækstraten på 10 % ikke havde nogen effekt på transkriptionen. Denne observation tyder på, at *P. acidilactici* DSM 20284 allerede er godt tilpasset til furfural. Dette forklarer også hvorfor det adaptive evolutions forsøg primært selekterede for mere effektiv vækst på MRS fremfor en øget resistens overfor furfural. Imidlertid blev flere gener relateret til exopolysaccharid biosyntese eller syntesen af membranproteiner induceret i den tilpassede stamme A28. Dette kunne indikere, at cellenvæggen er vigtig for cellens beskyttelse mod furfural. På den anden side, kan den højere vækstrate forklares ved en optimering af purin og pyrimidin biosyntese og salvage pathways, opregulering af folinsyre biosyntesen samt flere enzymer involveret i glycolysen.

Dette studium har klart dokumenteret mælkesyrebakteriers særlige potentiale som cellefabrikker i omdannelsen af biomasse indeholdende lignocellulose til værdifulde produkter.

Outline of the thesis

The thesis consists of 7 chapters. **Chapter 1** contains a general introduction to the concept of biorefinery, the structure of lignocellulose and the challenges associated with the use of lignocellulosic biomass for microbial conversion into value-added products. It also introduces lactic acid bacteria, their phylogeny and mechanisms of stress responses. **Chapter 2** includes an article published in Microbial Cell Factories; the article covers the screening of numerous lactic acid bacteria for their potential as microbial cell factories. The study identified several most promising strains, which were able to ferment both hexoses and pentoses as well as tolerate the key inhibitors derived from the pretreated lignocellulosic substrates. **Chapter 3** presents the development and optimization of the chemically defined medium for one of the selected most promising strains, *Pediococcus acidilactici* DSM 20284. One of the parameters hampering both the industrial use and the research on many lactic acid bacteria species is the lack of efficient methods for their transformation; thus **Chapter 4** includes the development and optimization of the transformation procedure for *P. acidilactici*. Since the utilization of lignocellulosic feedstocks is inevitably associated with the presence of growth inhibitors, **Chapter 5** aims at developing a superior strain by adaptive laboratory evolution; the adapted strain is able to withstand and grow in the presence of even higher concentrations of the key inhibitors, furfural and acetic acid. Last but not least, **Chapter 6** focuses on the transcriptomic analysis of the stress response to furfural; the findings might help to better understand the metabolism and stress response mechanisms of both the wild-type and the adapted strains. Finally, **Chapter 7** presents the study conclusions and future perspectives.

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Chapter 1

Introduction

1.1. Biorefinery concept

The diminishing natural resources, soon to be unable to satisfy the needs of our ever-growing population as well as the environmental issues have recently drawn considerable attention towards using renewable resources. Such renewable substrates can range from agricultural products and energy crops to various wastes and residues of forestry, agricultural or municipal origin. In one of the definitions, a biorefinery was described as "*the sustainable processing of biomass into a spectrum of bio-based products (food, feed, chemicals, and/or materials) and bioenergy (biofuels, power and/or heat)*" (IEA Bioenergy, 2007). The overall aim of a biorefinery is thus biosustainable production of a variety of value-added products from such renewable feedstocks. The biorefineries can be classified as 1st or 2nd generation based on the substrates they use. The 1st generation biorefinery production utilizes substrates such as sugar crops or oil seeds; it thus competes for substrates with food and animal feed which rises numerous ethical, political and environmental concerns (Cherubini, 2010). In contrast, the 2nd generation biorefinery uses lignocellulosic biomass as substrates. Lignocellulose is non-edible for either humans or animals, so its use poses no competition to the food or animal feed supplies (Sun and Cheng, 2002). Lignocellulose is therefore gaining considerable interest as the most promising alternative to fossil fuels or conventional biofuels produced in a 1st generation biorefinery (Cherubini, 2010). The lignocellulosic biomass is the most abundant feedstock in the world (Zhou et al., 2011); it is also cheap and renewable. It thus manifests tremendous potential for production of both fuels and chemicals. Lignocellulosic substrates include various forestry and agricultural residues, municipal solid wastes (e.g. wood products), wastes from pulp and paper industry, and energy crops (Himmel et al., 2007; Jönsson et al., 2013; Saha, 2005). In this context, conversion of lignocellulosic feedstocks into value-added products is not only economical and sustainable but it also resolves the waste disposal, processing and storage issues (Taherzadeh and Karimi, 2008).

1.2. Lignocellulose structure

Lignocellulosic biomass is a complex matrix of polymers, built of three main components: cellulose, hemicellulose and lignin (Figure 1.1). Cellulose is a linear polymer of glucose, made of cellobiose units, tightly bound by both intra- and inter-molecular hydrogen bonding networks, forming a crystalline structure. Cellulose constitutes the highest fraction of lignocellulose (Isikgor and Becer, 2015). Hemicellulose is a branched polymer of different sugars, including both C5 sugars (such as xylose and arabinose) and C6 sugars (such as glucose, mannose, galactose and rhamnose). Hemicellulose has a random, amorphous structure which makes it easier to hydrolyze when compared to cellulose. In most

types of biomasses, the predominant sugar monomer derived from hemicellulose is xylose. Last but not least, lignin is a complex polymer of aromatic compounds, made of phenylpropane units, covalently bound to the hemicellulose fraction. Lignin provides the plant cell wall with rigidity, and it makes the lignocellulose naturally resistant to microbial or enzymatic decomposition (Himmel et al., 2007).

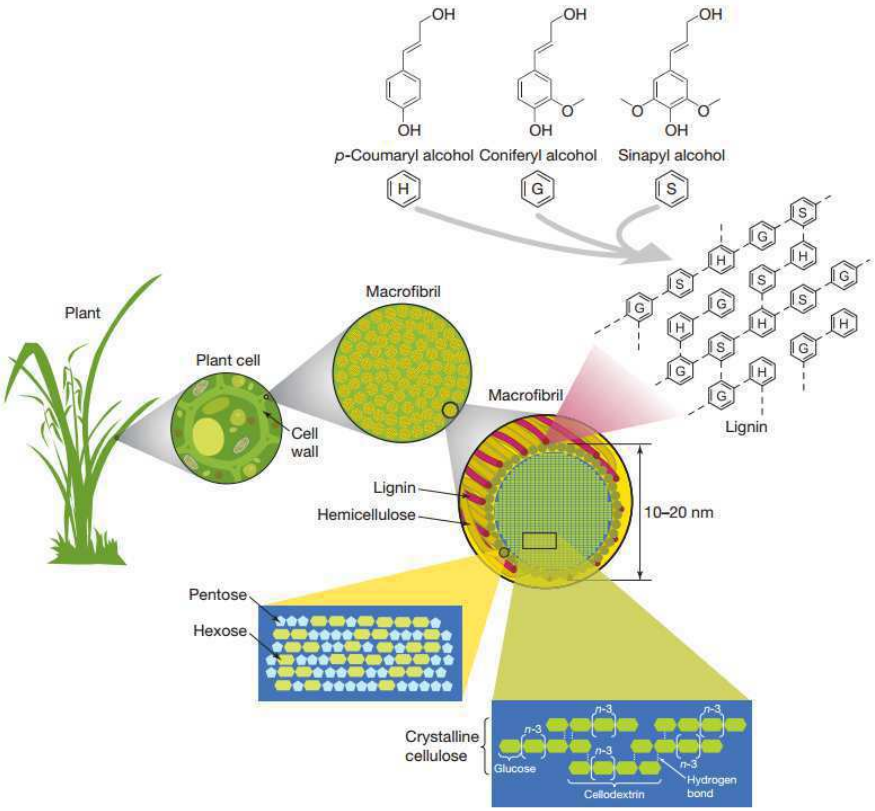

Figure 1.1. The structure of lignocellulosic biomass (Rubin, 2008).

The exact proportions of the three components in the lignocellulosic material depend on the source of biomass (Almeida et al., 2007). For example, softwoods tend to have higher percentages of lignin compared to the hardwoods; likewise, hardwoods usually contain higher amounts of xylose in the hemicellulose fraction (Table 1.1).

Table 1.1. Percentage composition of various common agricultural wastes or energy crops, based on Mood et al. (2013).

Lignocellulosic feedstock	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Reference
Sugarcane bagasse	43.1	31.1	11.4	Martin et al., 2007
Wheat straw	30.2	22.3	17	Ballesteros et al., 2006
Rice straw	31.1	22.3	13.3	Chen et al., 2011b
Sweet sorghum	45	27	21	Kim and Day, 2011
Corn stover	38.3	25.8	17.4	Li et al., 2010a
Switchgrass	39.5	25	21.8	Li et al., 2010b
Oak (hardwood)	45.2	24.5	24.3	Shafiei et al., 2010
Pine (softwood)	46.4	8.8	29.4	Wiselogel, 1996
Spruce (softwood)	43.8	20.8	28.8	Shafiei et al., 2010

1.3. Lignocellulose pretreatment methods

The natural recalcitrance and the compact structure of lignocellulose make it impossible for the microorganisms to reach the cellulose fraction inside. Therefore, the lignocellulosic biomass has to be pretreated before it can be fermented by microorganisms. An ideal pretreatment method would separate lignin from cellulose and hemicellulose, hydrolyze cellulose and hemicellulose into fermentable sugars without generation of any inhibitory substances, and would have a low energy demand, minimizing the cost of the pretreatment process. Various pretreatment technologies have been developed with the common purpose to break open the physical structure of the lignocellulose, making it more accessible for bacteria; the desired effect of pretreatment is illustrated in Figure 1.2. The technologies have been categorized into physical (e.g. milling and grinding), chemical (e.g. acid, alkaline), physicochemical (e.g. steam explosion, wet oxidation) and biological methods. Often, a combination of different technologies is used in order to overcome the limitations of individual pretreatment methods, adding up to the overall cost and energy expenses (Isikgor and Becer, 2015).

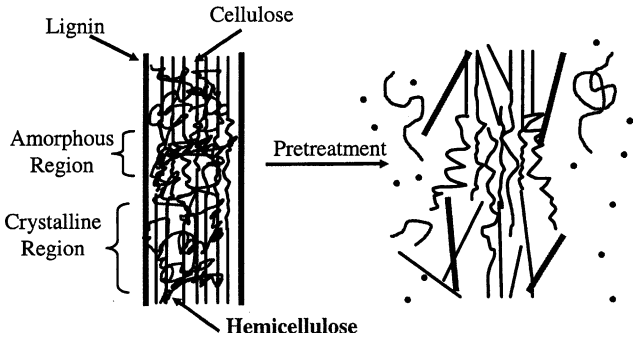

Figure 1.2. The effect of lignocellulose pretreatment (Mosier, 2005).

1.4. Inhibitors in the lignocellulosic biomass hydrolysates

The pretreatment, apart from the fermentable sugars, results in the release of numerous undesirable compounds as well, and those may have inhibitory effects on the growth and performance of microorganisms. There are three major groups of compounds that inhibit the following fermentation step. These include furan derivatives, weak acids and phenolic compounds. As depicted in Figure 1.3, the inhibitors may be derived from decomposition of lignin, but they can also be degradation products of sugars and other inhibitors.

Furan derivatives include mainly 2-furaldehyde (furfural) and 5-hydroxymethyl-2-furaldehyde (HMF). These two compounds come from dehydration of pentoses and hexoses, respectively (Dunlop, 1948; Ulbricht et al., 1984). Both furfural and HMF were demonstrated to inhibit the growth of several industrially relevant microorganisms, including *Escherichia coli*, *Saccharomyces cerevisiae*, *Pichia stipitis*, and *Zymomonas mobilis* (Almeida et al., 2007; Delgenes and Moletta, 1996; Palmqvist et al., 1999; Parawira and Tekere, 2011; Ranatunga et al., 1997; Zaldivar et al., 1999). They were also demonstrated to decrease the productivity and yield of ethanol in *S. cerevisiae* (Jönsson et al., 2013; Larsson et al., 1999a; Liu et al., 2004). Furfural was also widely recognized to induce DNA damage, inhibit protein and RNA synthesis, and induce increased levels of reactive oxygen species (ROS) (Allen et al., 2010; Almeida et al., 2009; Parawira and Tekere, 2011). Both furfural and ROS can then cause protein misfolding and fragmentation as well as

damage to the cell membrane (Almeida et al., 2009). Under anaerobic conditions, both *E. coli* and *S. cerevisiae* were shown to cope with furfural and HMF by converting them into their less toxic alcohol derivatives (Lewis Liu and Blaschek, 2010; Liu et al., 2008; Wang et al., 2011).

Weak acids include mainly acetic, formic and levulinic acids. Acetic acid comes from deacetylation of lignin, whereas formic and levulinic acids are products of further HMF decomposition. Formic acid can also be generated from furfural in specific set of conditions (acidic pH, elevated temperature) (Almeida et al., 2007).

Phenolic compounds are derived from lignin. These can include vanillin, vanillic acid, syringaldehyde, catechol, methylcatechol, guaiacol, and many others, depending on the source of biomass used. The effect of phenolics and other aromatic compounds on the growth of microorganisms varies, depending on the specific functional groups (Jönsson et al., 2013). Phenolic compounds might cause loss of integrity of cell membranes and were shown to be toxic to yeasts, although the exact inhibition mechanism has not been elucidated (Palmqvist and Hahn-Hägerdal, 2000).

Furthermore, the inhibitors may exhibit synergistic or antagonistic effects in combination with other compounds, which means that the total influence on the microorganism may be enhanced or reduced when compared to what would be expected by the sum the individual impacts of the inhibitors (Klinke et al., 2004). Indeed, furfural was demonstrated to act synergistically in combinations with other inhibitors, increasing the toxicity of the hydrolysates (Zaldivar et al., 1999). For instance, the effect of combined treatment of *S. cerevisiae* with furfural and acetic acid was found to be greater than the additive effect of both inhibitors (Palmqvist et al., 1999). Similarly, furfural was also found to significantly enhance the

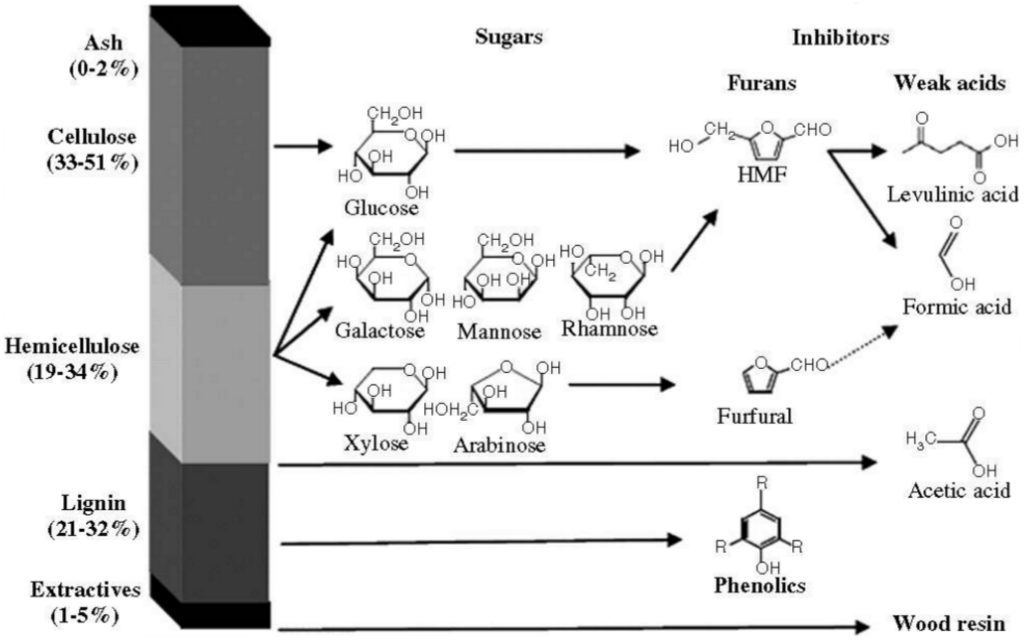

Figure 1.3. Composition of lignocellulosic biomass and its main degradation products (Almeida et al., 2007).

inhibition of *E. coli* fermentation when present in combination with phenolic compounds (Zaldivar et al., 2000, 1999).

The inhibitors profile and the amounts of the different inhibitors depend on the source of biomass used as well as on the severity of the pretreatment method and conditions. For instance, the pretreatment of spruce generates up to 5.9 g/L of HMF, whereas no HMF is released during the pretreatment of wheat straw (Almeida et al., 2007; Klinke et al., 2003; Nilvebrant et al., 2003). Also, the pretreatment of vine-trimming wastes can yield from 0 to 8.6 g/L furfural and from 0.8 to 6.3 g/L of acetic acid, depending on the time of pretreatment and the concentration of sulfuric acid used (Bustos et al., 2004). One should keep in mind, however, that the pretreatment conditions also influence the amounts of sugars released and available for the subsequent fermentation step. Thus, it is important to balance the pretreatment severity in order to release majority of sugars with as low concentrations of generated inhibitors as possible. Table 1.2 presents several examples of the inhibitors generated during lignocellulose pretreatment; it can be easily noticed that both the profile and the amounts of inhibitors differ in the various types of biomass.

Table 1.2. The profile of inhibitors in various pretreated lignocellulosic feedstocks, adapted from Chandel et al. (2011).

Biomass type	Profile of inhibitors (g/L)	Reference
Sugarcane bagasse	Furfural, 0.3 HMF, 0.04 Acetic acid, 2.7	Canilha et al., 2010
Wheat straw	Furfural, 0.15 ± 0.02 Acetic acid, 2.7 ± 0.33	Nigam, 2001
Softwood	Furfural, 2.2 Acetic, acid 5.3	Qian et al., 2006

A detoxification process may be employed in order to reduce the amounts of inhibitors in the lignocellulosic hydrolysates. Indeed, the treatments with $Ca(OH)_2$ (referred to as "*overliming*") or ion-exchange treatment proved to significantly enhance the fermentation step (Larsson et al., 1999b; Martinez et al., 2000; Nilvebrant et al., 2003). However, this is done at the expense of fermentable sugars available for microorganisms, as well as the increase in the overall process costs.

1.5. Challenges in lignocellulose-based biorefineries

There are several challenges that need to be addressed before the lignocellulosic biomass-based biorefineries become economically feasible and applied on a large scale. Firstly, the pretreatment of lignocellulosic feedstocks yields a hydrolysate where a mixture of sugars, including both hexoses and pentoses is present. In order to fully utilize the potential of lignocellulosic biomass, both C5 and C6 sugars should be converted into value-added products. It is therefore of crucial importance that the biocatalyst is capable of utilizing both hexoses and pentoses, preferably simultaneously. Secondly, as described earlier, the hydrolysates contain numerous compounds that have been widely shown to inhibit the growth and productivity of the most common industrial microorganisms. Therefore, a very robust organism is needed, that demonstrates high tolerance levels to these inhibitors. In addition, the microorganism should be easy to manipulate, able to deliver the value-added products with a high yield and productivity, and with

minimal by-product formation. If the lignocellulosic feedstocks are going to be employed in the food industry, it is a prerequisite that the organism is certified as Generally Regarded As Safe (GRAS). At present, no organism meets all the above criteria; the search for a robust and tolerant organism continues both by screening of new promising candidates, and by random as well as rational engineering of already known industrial microorganisms. The two main challenges for the feasibility of the lignocellulosic feedstocks-based biorefinery, are the co-fermentation of pentoses and hexoses and the resistance to inhibitors, described in more details below.

1.5.1. Co-fermentation of pentoses and hexoses

The pretreated lignocellulosic biomass can contain more than 30% of the pentoses xylose and arabinose (Weber et al., 2010). As mentioned earlier, xylose is the most abundant pentose sugar in the lignocellulose; thus the research has focused on development of xylose-fermenting microorganisms (Hahn-Hägerdal et al., 2006). Although many microorganisms, both bacteria, yeasts and fungi, were found to be naturally capable of utilizing xylose and/or arabinose, none of them were suitable for direct application as microbial cell factories converting lignocellulosic feedstocks (Hahn-Hägerdal et al., 2006). *E. coli*, which can use a wide spectrum of sugars, including pentoses and is highly amenable for genetic manipulations, not only possesses a carbon catabolite repression system, interfering with simultaneous use of C5 and C6 sugars, but it is also very sensitive towards inhibitors and ethanol as an end-product (Limayem and Ricke, 2012). *S. cerevisiae*, on the other hand, is naturally adapted to high ethanol concentrations and other inhibitors, but it cannot utilize pentoses. Yet, another yeast, *Pichia stipitis*, was demonstrated a good performance on xylose; however, similarly to *E. coli*, it shows low tolerance to inhibitors derived from the pretreatment of lignocellulosic biomass (Hahn-Hägerdal et al., 1994; Jeffries et al., 2007; Limayem and Ricke, 2012). *Zymomonas mobilis* was recognized as a promising bio-ethanol producer due to its high ethanol tolerance, productivity and close-to-theoretical ethanol yield (Limayem and Ricke, 2012). It does not, however, meet any of the two main requirements for a biocatalyst of lignocellulosic feedstocks conversion; it is sensitive to the inhibitors and it is not capable of utilizing pentoses. Last but not least, filamentous fungi, which are both resistant to the fermentation inhibitors and are capable of using both hexoses and pentoses, cannot compete with bacteria or yeast in an industrial setting due to their slow growth (Hahn-Hägerdal et al., 2006). Thus, a lot of research efforts were focused on engineering recombinant strains of yeast and bacteria that would overcome their individual drawbacks; either the inability to utilize pentoses or the intolerance to lignocellulose-derived inhibitors.

For instance, the inability of *S. cerevisiae* to utilize xylose was complemented by inserting the xylose-metabolizing genes from both bacterium, *Thermus thermophiles*, yeast, *P. stipitis*, and fungus, *Piromyces* spp. (Kuyper et al., 2003; Kötter and Ciriacy, 1993; Walfridsson et al., 1996). The arabinose fermentation pathway was also introduced into *S. cerevisiae* from both bacteria and fungi (Becker and Boles, 2003; Richard et al., 2003); however, the fungal pathway did not result in ethanolic fermentation (Richard et al., 2003). Yet, the rate of pentose fermentation was found to be one to two orders of magnitude lower than that of hexose fermentation; thus, further metabolic engineering strategies were necessary to increase both the xylose flux and the ethanol yield (Hahn-Hägerdal et al., 2007). Indeed, different strategies have been applied in laboratory yeast strains, including engineering of xylose transport, reducing the formation of xylitol by-product, relieving carbon catabolite repression or improving the general metabolism efficiency (Hahn-Hägerdal et al., 2007; Karhumaa et al., 2007, 2005; Kuyper et al., 2005; Leandro et al., 2006; Ohgren

et al., 2006; Roca et al., 2004). As the industrial yeast strains are much more tolerant to inhibitors and generally more robust compared to the laboratory strains, many efforts were also put into improving those (Sonderegger et al., 2004; Wahlbom et al., 2003). Indeed, some *S. cerevisiae* strains were used in simultaneous saccharification and fermentation of nondetoxified corn stover hydrolysates, producing relatively high ethanol yields (Ohgren et al., 2006). Furthermore, Karhumaa et al. (2006) demonstrated that both a laboratory and an industrial strain of *S. cerevisiae* was able to co-utilize xylose and arabinose; the xylose and arabinose fermentation was then further improved by evolutionary engineering strategies (Sanchez et al., 2010).

Also, *Z. mobilis* was successfully engineered to utilize xylose and arabinose by inserting the four and five *E. coli* genes, required for the fermentation of xylose and arabinose, respectively (Deanda et al., 1996; Min Zhang et al., 1995). The recombinant xylose-fermenting strain showed 86% ethanol yield when grown on xylose (Min Zhang et al., 1995). In a later attempt, the seven genes from the pentose assimilation and pentose phosphate pathways were introduced into *Z. mobilis* genomic DNA, and the recombinant strain was demonstrated to co-ferment glucose, xylose and arabinose (Mohagheghi et al., 2002). However, *Z. mobilis* cannot ferment mannose and galactose, which also constitute considerable parts of lignocellulosic substrates; thus, further metabolic engineering is necessary (Hahn-Hägerdal et al., 2007).

1.5.2. Tolerance to pretreatment-derived fermentation inhibitors

The fermentation of lignocellulosic hydrolysates requires the strains to be tolerant to various inhibitors generated during the biomass pretreatment. The development of highly tolerant strains capable of converting nondetoxified lignocellulosic hydrolysates has recently gained considerable attention. Numerous studies report recombinant strains of both *S. cerevisiae* and *E. coli* that were engineered for a higher resistance to the common inhibitors.

For example, the tolerance against furfural in *S. cerevisiae* was demonstrated to be conveyed by overexpression of specific enzymes, such as glucose 6-phosphate dehydrogenase, the first and rate-controlling enzyme in the pentose phosphate pathway (Gorsich et al., 2006). The deletion mutants could not reduce furfural to furfuryl alcohol, which was suggested to be due to lower levels of reducing equivalents or NADPH available for detoxification (Gorsich et al., 2006). Also, the overexpression of laccase, an extracellular enzyme produced by a range of fungi and plants, was reported to enhance the *S. cerevisiae* resistance to phenolic compounds (Larsson et al., 2001). The enzyme catalyzes the reduction of oxygen to water, oxidizing phenolic compounds to unstable radicals, which then associate to form polymers; the net effect is the removal of low-molecular-weight phenolic compounds from the hydrolysate (Jönsson et al., 1998; Larsson et al., 2001). The resistance to phenolic compounds was also demonstrated to be increased by overexpression of phenylacrylic acid decarboxylase; the transformants showed both higher conversion rates of ferulic and cinnamic acids, but also higher ethanol productivities (Larsson et al., 2001). The overexpression of alcohol dehydrogenase in *S. cerevisiae* was shown to increase the tolerance towards HMF by at least 4-fold (Petersson et al., 2006). Another method commonly employed for increasing the tolerance of strains towards the lignocellulose-derived inhibitors is evolutionary engineering. For instance, the resistance of *S. cerevisiae* towards furfural was enhanced by continuous transfer of the strains to higher concentrations of the inhibitors (Liu et al., 2005). The resulting adapted strains showed higher tolerance levels towards both furfural and HMF (Almeida et al., 2007; Liu et al., 2005).

Furfural was suggested to inhibit the growth of *E. coli* due to competition between the furfural metabolism and the biosynthesis of NADPH. Indeed, the down-regulation of two NADPH-dependent oxidoreductases was found to increase the strain's tolerance to both furfural and HMF (Miller et al., 2010, 2009). Moreover, the overexpression of an NADH-dependent oxidoreductase was demonstrated to increase furfural tolerance by 50% (Wang et al., 2011). Furfural tolerance was also improved in *E. coli* by expressing thymidylate synthase ThyA, a key enzyme in the *de novo* biosynthesis of dTMP for DNA biosynthesis and repair (Zheng et al., 2012). Furthermore, it was found that supplementation with thymine or thymidine, or serine and tetrahydrofolate resulted in the same benefit in furfural tolerance; this finding proved that furfural resistance can be enhanced by supplying pyrimidine deoxyribonucleotides to the medium (Zheng et al., 2012). Moreover, a global regulator engineering strategy was also applied, using *irrE*, an exogenous global regulator isolated from *Deinococcus radiodurans.* The IrrE was previously shown to increase *E. coli* tolerances towards radiation as well as various types of stresses, including osmotic, heat, oxidative, alcohol and acid stresses (Chen et al., 2011a; Gao et al., 2003; Ma et al., 2011; Pan et al., 2009). The obtained *irrE* mutants were demonstrated a significantly improved tolerance towards furfural, as well as HMF, vanillin, and real lignocellulosic hydrolysates (Wang et al., 2012). More recently, polyamines and polyamine transporters were suggested to play a role in *E. coli* resistance to furfural, enhancing both the cells' growth and ethanol production (Geddes et al., 2014). The mechanism behind the enhanced tolerance was proposed to be the binding of polyamines to the negatively charged nucleic acids and phospholipid membranes, thus protecting them from the damage exerted by furfural (Geddes et al., 2014). In addition, Glebes et al. (2014) identified three additional genes related to enhanced tolerance towards furfural: *lpcA*, encoding the first enzyme in the lipopolysaccharide biosynthesis, *groES* and *groEL*, encoding a chaperonin complex.

1.6. Cell factories utilizing lignocellulosic biomass

There is many microorganisms employed in the industrial fermentations; the most promising biocatalysts for conversion of lignocellulosic biomass are *E. coli*, *S. cerevisiae* and *Z. mobilis* (Almeida et al., 2007). A range of value-added products can be manufactured, including various commodity chemicals or biofuels. In 2004, the US Department of Energy has identified top 12 building block chemicals that can be produced from lignocellulose-derived sugars. The compounds can subsequently be converted into high-value chemicals or materials that can replace the petroleum-derived products. The list include, among others, 1,4-diacids (malic, formic and succinic acids), aspartic, glutamic and glutaric acids, as well as glycerol, sorbitol, and xylitol (Pacific Northwest National Laboratory, 2004). So far, however, most of the efforts were put into development of microbial catalysts converting lignocellulose biomass into bioethanol.

A traditionally used organism for ethanol production is *S. cerevisiae*; however, at present ethanol is mainly produced from sucrose, molasses or corn starch (Adsul et al., 2011). Other organisms with high potential for industrial ethanol production are *E. coli* and *Z. mobilis*. *E. coli* have been engineered for homoethanolic fermentation; many strains have also been manipulated for production of more advanced biofuels, including n-butanol and n-propanol (Atsumi et al., 2008; Shen and Liao, 2008). However, one of the major drawbacks of *E. coli* is the possession of the carbon catabolite repression system, which makes *E. coli* have preference for glucose, delaying the utilization of pentoses (Nichols et al., 2001). It was found, however, that the deletion of a *ptsG*, encoding glucose phosphotransferase system allowed for simultaneous fermentation of glucose, xylose and arabinose (Nichols et al., 2001). The PTS system plays a role in

regulation of the carbon catabolite repression by transporting and phosphorylating glucose and repressing the other carbon sources until glucose is depleted. Such *ptsG* deletion mutants were successfully employed for both ethanol and lactate production (Dien et al., 2001; Nichols et al., 2001).

Numerous efforts were put into metabolic engineering of *Z. mobilis* as it is highly tolerant to ethanol and demonstrates 2.5 fold higher ethanol productivity than *S. cerevisiae* (Weber et al., 2010). Thus, it was manipulated with the objective of broadening its substrate spectrum to include, apart from glucose, fructose and sucrose, also xylose, arabinose and cellobiose (Deanda et al., 1996; Yanase et al., 2005; Zhang et al., 1995). *Z. mobilis* performance was evaluated on various lignocellulosic feedstocks, including cassava and sugarcane bagasse (da Silveira dos Santos et al., 2010; Patle and Lal, 2007).

Furthermore, *Lactobacillus buchneri* was also demonstrated to utilize mixed sugars, including both pentoses and hexoses, and convert them into ethanol with reasonable yields. In addition, the strain was shown to be tolerant to various inhibitors present in corn stover and wheat straw hydrolysates (Liu et al., 2009).

1.6.1. Production of lactic acid from lignocellulose

The conversion of lignocellulosic biomass to lactic acid has recently gained a lot of attention as well. Lactic acid is a versatile chemical widely used in food and pharmaceutical industries (Singhvi et al., 2010). Due to the fact that it contains both hydroxyl and carboxylic groups, it is an excellent building block that can be converted to a range of value-added products, including methyl lactate, lactide, and polylactic acid (PLA) (FitzPatrick et al., 2010). PLA is a biodegradable polymer, employed for production of packaging and containers, as an environmentally friendly replacement for the commonly used polyethylene terephtalates (PETs) (FitzPatrick et al., 2010; Singhvi et al., 2014).

Lactic acid has traditionally been produced by lactic acid bacteria, and they are still the prevailing candidates for the industrial lactic acid production. Moreover, lactic acid bacteria have already been demonstrated to perform very well in lignocellulosic biomass hydrolysates. For instance, *Lactobacillus brevis* was demonstrated to utilize 57 g/L mixed sugars from corncob hydrolysate to produce 39 g/L of lactic acid. At the same time, it was also shown to ferment both hexoses and pentoses simultaneously, and grow in the presence of furfural and ferulic acid without any growth inhibition or reduction in lactic acid formation (Guo et al., 2010). More recently, a mutated strain of *Lactobacillus paracasei* was found to represent tremendous potential for the industrial use: it could utilize non-detoxified wood or rice straw hydrolysates to produce optically pure L-lactic acid with remarkable efficiency (Kuo et al., 2015). Other *Lactobacillus* strains were also employed for lactic acid production from various lignocellulose-derived materials. Wet-oxidized wheat straw was used for fermentation by *Lactobacillus pentosus* and *Lactobacillus brevis* with up to 88% of the theoretical maximum yield (Garde et al., 2002). *Lactobacillus delbrueckii* and *Lactobacillus lactis* were shown to efficiently ferment sugarcane bagasse-derived cellulose and cellobiose to produce L-lactic acid and D-lactic acid, respectively (Adsul et al., 2007; Singhvi et al., 2010). *Lactobacillus rhamnosus* was demonstrated to readily convert recycled paper sludge into lactic acid with high yield and productivity (Marques et al., 2008). Similarly, *Lactobacillus coryniformis* subsp. *torquens* was shown to metabolize filter paper and pretreated cardboard waste (Yanez et al., 2005, 2003). Other studies reported the production of lactic acid by LAB from other biomass materials, among others soft wood, cassava bagasse, apple pomace, beet molasses, alfalfa fibers, corn stover and vine-trimming wastes (Bustos et al.,

2004; Cui et al., 2011; Gullon et al., 2008; Iyer et al., 2000; John, 2006; Kotzamanidis et al., 2002; Sreenath et al., 2001).

1.7. Lactic Acid Bacteria (LAB)

Lactic Acid Bacteria (LAB) constitute a highly heterogeneous group of Gram-positive, microaerophilic, non-motile, non-spore forming organisms which readily utilize glucose to produce lactic acid as a main product (Franz and Holzapfel, 2011). Taxonomically, LAB belong to the phylum *Firmicutes*, class *Bacilli*, and order *Lactobacillales* (Wright and Axelsson, 2011). The genera of LAB include *Lactococcus, Lactobacillus*, *Pediococcus*, *Streptococcus*, *Leuconostoc, Enterococcus*, *Oenococcus*, *Carnobacterium, Tetragenococcus, Vagococcus, Aerococcus* and *Weisella* (Figure 1.4).

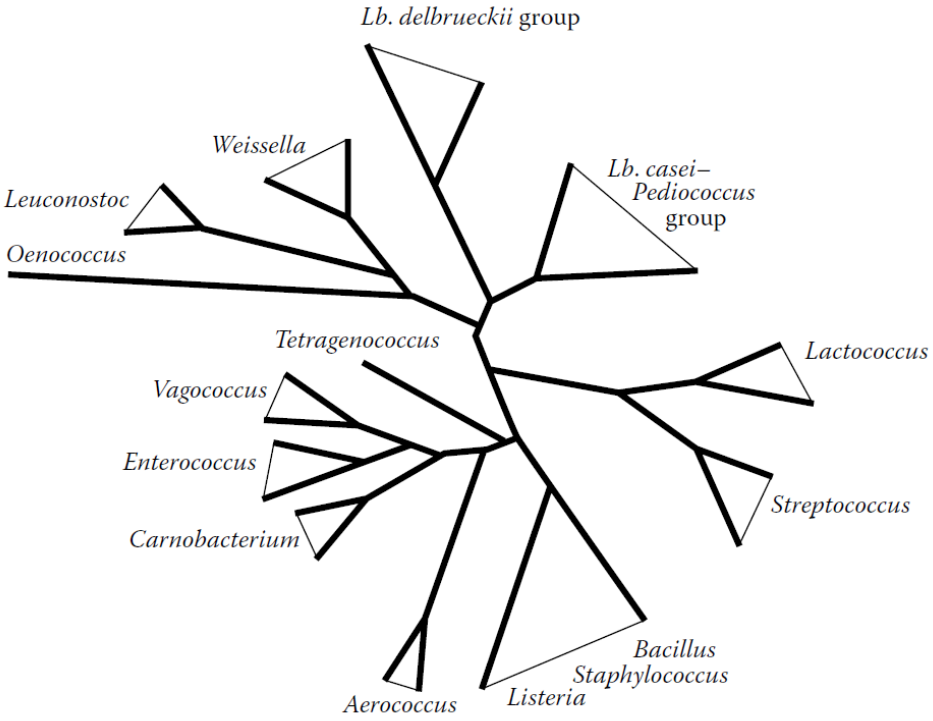

Figure 1.4. Phylogenetic tree of LAB (Wright and Axelsson, 2011).

The LAB genera include many highly important industrial species, widely used in dairy and other food and beverages-related fermentations. The different species are commonly involved in the production of yogurts, cheese, pickles, sauerkraut, olives, fermented sausages and fish products; they are also employed for fermentation of alcoholic beverages. In dairy products, LAB are known to contribute to the taste, aroma, flavour and texture. LAB are also recognized for their production of natural antimicrobials such as bacteriocins which inhibit the growth of foodborne pathogens including *Staphylococcus aureus*, *Clostridium botulinum* and *Listeria monocytogenes* (Cesselin et al., 2011; Leroy and De Vuyst, 2004). Their production of organic acids (primarily lactic acid, but also acetic, formic, phenyllactic and caproic acids) results in rapid acidification of raw material and also plays a role in prolonging the product's shelf life and increasing its microbial safety (Leroy and De Vuyst, 2004). Consequently, various LAB species are often employed as food preservatives (Corr et al., 2007; Gálvez et al., 2007; Lücke, 2000).

Due to their long history and use, LAB are generally regarded as safe and beneficial microorganisms. Some of them are even considered to have health-promoting features and are therefore employed in probiotic functional foods. Examples include *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus fermentum* and *Bifidobacterium lactis*, which are used by numerous companies around the world (Upadrasta et al., 2011). On the other hand, however, there are also some LAB species which are human or animal pathogens (Wright and Axelsson, 2011). For instance, *Streptococcus pyogenes* and *Streptococcus pneumonia* are both recognized to be opportunistic pathogens causing infections such as strep throat, impetigo, pneumonia, scarlet fever, and meningitis (Henningham et al., 2012).

LAB can be found in a variety of ecological niches, from human gastrointestinal tract and cavities, through dairy products, fermented meat, fish and vegetables, to silage, soil and decaying plant material (König and Fröhlich, 2009). In general, LAB are isolated from nutritionally rich habitats; being evolved in environments rich in sugars, amino acids, vitamins and nucleotides, they often demonstrate a limited scope of biosynthetic capabilities which is reflected by their complex nutritional requirements. Such a wide spectrum of natural habitats makes the LAB group highly heterogeneous; yet, it also reflects the scope of their catabolic potential.

1.8. Stress responses of lactic acid bacteria

Microorganisms have evolved mechanisms that allow them to quickly respond to sudden changes in the environment and survive harsh conditions (van de Guchte et al., 2002). By altering their gene expression, which means by inducing and/or repressing specific set of genes, the bacteria can very fast re-program their metabolism and adapt to the new environment. These mechanisms and systems allow microorganisms to survive in an industrial setting as well, where they are exposed to a variety of substances and conditions that are likely to induce stress. The different stresses are likely to trigger similar stress responses, thereby conferring resistance to several different environmental stresses (so-called cross-protection)(Desmond et al., 2001). For instance, the classical chaperones DnaK and GroEL were found to be induced both during heat, acid, osmotic and UV-irradiation stresses in *L. lactis* (Hartke et al., 1997; Kilstrup et al., 1997). *Enterococcus faecalis*, under starvation, became tolerant towards heat, acid, osmotic and UV-irradation stresses (Hartke et al., 1998). Likewise, *L. acidophilus* and *L. paracasei* exposed to osmotic stress were found to be less sensitive to bile and heat stresses as well (Desmond et al., 2001; Kim et al., 2001).

1.8.1. Heat stress

Rising temperature to sublethal levels primarily causes protein denaturation, but it was also shown to affect nucleic acids, ribosomes and cell membranes (De Angelis and Gobbetti, 2004; Teixeira et al., 1997). The cells respond to the heat shock by induction of the biosynthesis of certain set of proteins, referred to as heat-shock proteins. In *Bacillus subtilis*, a Gram-positive model organism, the heat shock proteins were classified into 4 classes based on their regulation. Class I heat shock proteins consists of genes controlled by a negative regulator HrcA that binds to a so called CIRCE operator sequence; the HrcA regulator is known to control the universal chaperones DnaK and GroEL in many Lactobacilli (Desmond et al., 2004; van de Guchte et al., 2002; Walker et al., 1999; Woodbury and Haldenwang, 2003). The DnaK family consists of DnaK, DnaJ and GrpE, and GroEL family consists of GroEL and GroES. Both work as molecular chaperones to promote correct folding of nascent proteins as well as denatured proteins' refolding. Indeed, the DnaK and GroEL were found to be induced by heat shock in *L. acidophilus*, *L. casei* and *L. helveticus* (Broadbent et al.,

1997). Class II heat shock proteins are regulated by the *B. subtilis* alternate sigma factor σ^B, which regulates the expression of numerous general stress responsive genes, induced by a variety of stress conditions (Petersohn et al., 2001). The activity of the σ^B increases during stress conditions; however, such factor has not been identified in other LAB (Girgis et al., 2003). Class III heat shock proteins contains genes under the control of a negative regulator CtsR. This includes the genes encoding Clp family proteins, consisting of Clp proteases and Clp ATPases. The Clp ATPases can have chaperone functions, and when they associate with a Clp protease to form a complex, they can also play a role in degrading proteins that cannot be correctly refolded by chaperones. Indeed, heat stress was demonstrated to result in induction of the Clp family proteins in *L. rhamnosus* and *L. gasseri* (Suokko et al., 2005, 2008). In *L. lactis*, mutations in the ClpP protease resulted in an increased sensitivity to heat stress (Frees et al., 2001). Last but not least, the class IV consists of heat shock proteins which mechanism of regulation is unknown.

In addition, a widely conserved gene, *htrA*, which encodes a serine protease, was also suggested to be involved in the heat shock response (Pallen and Wren, 1997). In *L. lactis,* the *htrA* deletion mutant showed increased sensitivity towards the heat stress; the gene was also found to be induced during sublethal heat stress in *L. helveticus* (Poquet et al., 2000; Smeds et al., 1998). HtrA works as a chaperone, but it also possesses proteolytic activity (Varmanen and Savijoki, 2011). Furthermore, RecA, which is widely spread among bacteria and functions in homologous recombination, DNA repair and SOS response (Varmanen and Savijoki, 2011) has also been found to be implicated in the heat stress response in *L. lactis*, where a *recA* deletion mutant was found to be less heat tolerant than the wild-type strain (Duwat et al., 1995).

1.8.2. Acid stress

One of the methods LAB cope with acid stress is by employing proton-translocating enzymes, such as F_0F_1-ATPase. This proton pump plays a role in a regulation of the cell's intracellular pH homeostasis by pumping protons out of the cell with the expense of ATP. Arginine deiminase pathway (ADI) is another mechanism allowing the cells to cope with acid-related stress. ADI pathway is composed of three enzymes: arginine deiminase, ornithine carbamoyltransferase and carbamate kinase, and they catalyze the conversion of arginine, through a citrulline intermediate, to ornithine, ammonia, carbon dioxide and energy in the form of ATP. By producing ammonia, the ADI pathway helps to restore the optimal pH (De Angelis and Gobbetti, 2011). Moreover, the ATP formed via the ADI pathway can be utilized by the F_0F_1-ATPase (Sanders et al., 1999).

A phenomenon known as Acid-Tolerance Response (ATR) also positively influences strain's tolerance to acid-induced stress. It's an adaptation mechanism, in which a previous slight or moderate treatment with acid increases further strain's resistance to stress conditions (Wilmes-Riesenberg et al., 1996). It was demonstrated in *L. delbrueckii* subsp. *bulgaricus* that the cells subjected to an acid pre-incubation at a pH between 4.6 and 5.2 for 40 min were 250 to 700 times more tolerant to acid challenge at pH 3.7 when compared to the non-adapted cells (Zhai et al., 2014). The mechanisms behind these findings were studied in various species by proteomics; the strains after acid adaptation demonstrated induction of numerous acid-shock proteins, among others 15 proteins in *L. sanfraciscensis* and 30 proteins in *L. delbrueckii* subs. *bulgaricus* (De Angelis et al., 2001; Lim et al., 2000). The same studies revealed the induction of heat shock proteins during acid adaptation: the molecular chaperones GroES, GroEL, DnaK and DnaJ were induced in *L. delbrueckii* subs. *bulgaricus*; however, they were not affected in *L. sanfraciscensis* where only GrpE was found to be induced. Similarly, the above mentioned chaperones together with ClpE and several other

proteins involved also in oxidative stress response were up-regulated in *L. lactis* at low pH (Frees et al., 2003). Proteases ClpC and ClpP were also found to be induced in response to acid stress in *S. mutans* (Lemos and Burne, 2002).

Moreover, acid stress was also found to induce changes in the cell membrane. The low pH was shown to induce modifications in the composition of fatty acids in the cell membrane of *L. delbrueckii* subsp. *bulgaricus* (Streit et al., 2008). Similar observations were made in *L. casei*, where the proportions of monounsaturated fatty acids as well as the mean chain lengths increased after the exposure to acidic conditions (Wu et al., 2012). What is more, several genes responsible for the biosynthesis of exopolysaccharides (EPS) were found to be up-regulated in some Lactobacilli under acid-related stress (Gaenzle and Schwab, 2009). The EPS were suggested to have a protective function against several adverse environmental conditions, including acid-induced and osmotic stress (Ruas-Madiedo et al., 2002).

1.8.3. Oxidative stress

LAB are facultative anaerobic microorganisms, meaning that they do not need oxygen for growth. As a matter of fact, oxygen has generally been demonstrated to exert negative effects on their growth (van de Guchte et al., 2002). Oxygen is a small molecule that can easily pass thorugh cell membrane and access the catalytic site of the proteins. It has been found that enzymes that contain the glycyl radical are especially sensitive to oxygen; an example of such an enzyme is pyruvate formate lyase (PFL), which catalyzes the conversion of pyruvate into acetyl Co-A. PFL has been demonstrated to be highly sensitive to oxidative stress in *L. lactis*, getting irreversibly inactivated in the presence of oxygen (Cesselin et al., 2011; Zhang et al., 2001). However, the toxicity of oxygen is not only attributed to the presence of oxygen itself; in fact, oxygen derivatives are much more toxic to the cells. These include the reactive oxygen species (ROS) such as superoxide anion radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\cdot}$). Moreover, other molecules, such as cysteine, pyruvate and ppGpp were also found to play a role in protection against ROS (Cesselin et al., 2011; Vido et al., 2005). In *L. lactis*, elevated ppGpp levels were correlated with increased strain's resistance to multiple stress factors (Rallu et al., 2000).

In Lactobacilli, the oxidative stress is dealt with by means of enzymes such as NADH oxidase, NADH peroxidase, superoxide dismutase or catalases, or by means of other compounds, including glutathione or Mn^{2+} ions (De Angelis and Gobbetti, 2011; van de Guchte et al., 2002). NADH oxidase reduces O_2 to H_2O_2, while NADH peroxidase reduces it further to H_2O. Manganese ions either work to scavenge the oxygen radicals, or they work as cofactors in manganese-dependent catalases (De Angelis and Gobbetti, 2011; Groot et al., 2005; Rochat et al., 2006). Superoxide dismutase eliminates $O_2^{\cdot-}$ by converting it to oxygen and H_2O_2. The hydrogen peroxide can be further converted to H_2O by the action of a catalase. In fact, it was demonstrated that the coexpression of superoxide dismutase and a catalase significantly increases the resistance to oxidative stress in *L. rhamnosus* (An et al., 2011). However, the expression of a catalase alone was also shown to have a positive impact on the oxidative stress resistance in various hosts, including *L. casei*, *L. plantarum* and *L. lactis* (Noonpakdee et al., 2004; Rochat et al., 2006, 2005). Moreover, oxidative stress was shown to induce changes in the fatty acid composition of the cell wall in *L. helveticus* (Guerzoni et al., 2001). A thioredoxin reductase, identified in genomes of several LAB, including *L. lactis*, *L. johnsonii* and *L. delbruecki* subsp. *bulgaricus*, was also suggested to be involved in the oxidative stress response and work to remove the ROS before they inactivate the proteins (Cesselin et al., 2011). Indeed, it was demonstrated that in *L. plantarum*, the overexpression of *trxB* gene resulted in increased resistance to

oxygen (Serrano et al., 2007). In *L. sanfranciscensis*, oxidative stress caused increase in the activity of another enzyme, glutathione reductase, which was shown to be involved in the stress resistance by maintaining the balance between the oxidized and reduced forms of glutathione, a key cell antioxidant molecule (De Angelis and Gobbetti, 2011; Jänsch et al., 2007). Apart from oxidative stress, glutathione was also shown to be involved in the resistance to acid and osmotic stress (Kajfasz and Quivey, 2011; Lee et al., 2010; Zhang et al., 2007).

1.8.4. Osmotic stress

Salts, such as NaCl or KCl, or sugars, such as sucrose or lactose, present in the immediate surroundings of a microorganism, impose osmotic stress on the cell. To equilibrate the external osmotic pressure, water is transported to the external environment, resulting in a loss of cell's turgor pressure. Studies done in *L. plantarum* have shown that the presence of salts in the medium is more detrimental to the cells than the equimolar amounts of sugars. This observation can be explained by the fact that the osmotic stress caused by sugars is only transient as the sugar concentrations inside and outside of the cell can be quickly balanced (Glaasker et al., 1998).

As a defense mechanism against osmotic stress, LAB accumulate compatible solutes, which primarily serve as osmoprotectants, but were also shown to provide thermostability and increase the resistance to drying (Kets et al., 1996). During hypo-osmotic conditions, the bacteria rapidly release them into the external environment which helps them to maintain cell's turgor pressure. It was shown that *L. plantarum* preferred organic compatible solutes, such as glycine betaine and carnithine; however, other compounds such as glutamate or proline can also provide osmotolerance (De Angelis and Gobbetti, 2004). Indeed, Glaasker et al. (1996a) demonstrated that when *L. plantarum* was grown in the presence of 0.8M KCl, glutamate and proline were accumulated in the cell. If glycine betaine was provided additionally in the medium, it became the preferred solute. During hypo-osmotic shock, *L. plantarum* was demonstrated to release glycine betaine, glutamate and proline, while the concentrations of other amino acids remained unchanged (De Angelis and Gobbetti, 2004; Glaasker et al., 1996b). Furthermore, glycine betaine was also shown to provide osmotolerance in *L. lactis*, *L. casei* and *L. acidophilus* (Hutkins et al., 1987; Piuri et al., 2003; Van der Heide and Poolman, 2000).

1.9. Aim of the thesis

The following PhD thesis aimed at investigating the potential of a variety of LAB with regard to their use as microbial cell factories for conversion of lignocellulosic biomass into value-added products. LAB, such as Lactococci and Lactobacilli are distinguished by a substantial range of useful properties that make them promising candidates to be employed in 2nd generation microbial cell factories. They can grow anaerobically with high growth rates, and were shown to have high glycolytic capacity for conversion of sugars. LAB model organisms, such as *Lactococcus lactis* or *Lactobacillus plantarum*, were studied for decades to establish molecular and genetic tools as well as metabolic models (Rud et al., 2006; Solem, n.d.; Teusink et al., 2006). LAB are also naturally highly tolerant to organic acids and alcohols; indeed, high yield ethanol production was recently demonstrated in *L. lactis* (Solem et al., 2013). Another *L. lactis* strain was also earlier engineered to produce butanol, a more advanced biofuel (Liu et al., 2010). Last but not least, some of the LAB species have already been demonstrated to be promising utilizers of the lignocellulosic biomass to produce value-added products (Berezina et al., 2010; Aarnikunnas et al., 2003).

The study aimed at screening of a range of LAB species for their sugar utilization profiles and tolerance levels towards several most important inhibitors derived from the pretreatment of lignocellulosic biomass. The objective was to select a best-performing LAB strain and develop a chemically defined medium supporting good growth of the strain as well as an efficient transformation protocol allowing for the introduction of exogenous DNA into the strain. Another goal of the study was to further improve the selected strain for a better growth in the presence of lignocellulose-derived inhibitors.

1.10. References

Adsul, M., Khire, J., Bastawde, K., Gokhale, D., 2007. Production of Lactic Acid from Cellobiose and Cellotriose by *Lactobacillus delbrueckii* Mutant Uc-3. Appl. Environ. Microbiol. 73, 5055–5057.

Adsul, M.G., Singhvi, M.S., Gaikaiwari, S.A., Gokhale, D. V, 2011. Development of biocatalysts for production of commodity chemicals from lignocellulosic biomass. Bioresour. Technol. 102, 4304–4312.

Allen, S.A., Clark, W., McCaffery, J.M., Cai, Z., Lanctot, A., Slininger, P.J., Liu, Z.L., Gorsich, S.W., 2010. Furfural induces reactive oxygen species accumulation and cellular damage in *Saccharomyces cerevisiae*. Biotechnol. Biofuels 3, 1–10.

Almeida, J.R.M., Bertilsson, M., Gorwa-Grauslund, M.F., Gorsich, S., Lidén, G., 2009. Metabolic effects of furaldehydes and impacts on biotechnological processes. Appl. Microbiol. Biotechnol. 82, 625–638.

Almeida, J.R.M., Modig, T., Petersson, A., Hahn-Hagerdal, B., Liden, G., Gorwa-Grauslund, M.F., 2007. Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. J. Chem. Technol. Biotechnol. 82, 340–349.

An, H., Zhai, Z., Yin, S., Luo, Y., Han, B., Hao, Y., 2011. Coexpression of the Superoxide Dismutase and the Catalase Provides Remarkable Oxidative Stress Resistance in *Lactobacillus rhamnosus*. J. Agric. Food Chem. 59, 3851–3856.

Atsumi, S., Cann, A.F., Connor, M.R., Shen, C.R., Smith, K.M., Brynildsen, M.P., Chou, K.J.Y., Hanai, T., Liao, J.C., 2008. Metabolic engineering of *Escherichia coli* for 1-butanol production. Metab. Eng. 10, 305–311.

Ballesteros, I., Negro, J.M., Oliva, J.M., Cabanas, A., Manzanares, P., Ballesteros, M., 2006. Ethanol Production From Steam-Explosion Pretreated Wheat Straw. Appl. Biochem. Biotechnol. 129-132, 496–508.

Becker, J., Boles, E., 2003. A Modified *Saccharomyces cerevisiae* Strain That Consumes L -Arabinose and Produces Ethanol. Appl. Environ. Microbiol. 69, 4144–4150.

Berezina, O. V, Zakharova, N. V, Brandt, A., Yarotsky, S. V, Schwarz, W.H., Zverlov, V. V, 2010. Reconstructing the clostridial n-butanol metabolic pathway in *Lactobacillus brevis*. Appl. Microbiol. Biotechnol. 87, 635–646.

Broadbent, J.R., Oberg, C.J., Wang, H.U.A., Wei, L., 1997. Attributes of the Heat Shock Response in Three Species of Dairy Lactobacillus. Syst. Appl. Microbiol. 20, 12–19.

Bustos, G., Moldes, A.B., Cruz, J.M., Dominguez, J.M., 2004. Production of fermentable media from vine-trimming wastes and bioconversion into lactic acid by *Lactobacillus pentosus*. J. Sci. Food Agric. 84, 2105–2112.

Canilha, L., Carvalho, W., Felipe, M.D.G.D.A., Silva, J.B.D.A.E., Giulietti, M., 2010. Ethanol production from sugarcane bagasse hydrolysate using *Pichia stipitis*. Appl. Biochem. Biotechnol. 161, 84-92.

Cesselin, B., Derré-bobillot, A., Fernandez, A., Lamberet, G., Lechardeur, D., Yamamoto, Y., Bastian, M., Garrigues, C., Gruss, A., Gaudu, P., 2011. Responses of Lactic Acid Bacteria to Oxidative Stress, in: Stress Responses of Lactic Acid Bacteria. pp. 111–127.

Chandel, A.K., da Silva, S.S., Singh, O. V, 2011. Detoxification of Lignocellulosic Hydrolysates for Improved Bioethanol Production, in: Biofuel Production – Recent Developments and Prospects. pp. 225–246.

Chen, T., Wang, J., Yang, R., Li, J., Lin, M., Lin, Z., 2011a. Laboratory-Evolved Mutants of an Exogenous Global Regulator, IrrE from *Deinococcus radiodurans*, Enhance Stress Tolerances of *Escherichia coli*.

PLoS One 6.

Chen, W.-H., Pen, B.-L., Yu, C.-T., Hwang, W.-S., 2011b. Pretreatment efficiency and structural characterization of rice straw by an integrated process of dilute-acid and steam explosion for bioethanol production. Bioresour. Technol. 102, 2916–2924.

Cherubini, F., 2010. The biorefinery concept: Using biomass instead of oil for producing energy and chemicals. Energy Convers. Manag. 51, 1412–1421.

Corr, C., Li, Y., Riedel, C.U., O´Toole, P.W., Hill, C., Gahan, C.G.M., 2007. Bacteriocin production as a mechanism for the antiinfective activity of *Lactobacillus salivarius* UCC118. Proc. Natl. Acad. Sci. 104, 7617–7621.

Cui, F., Li, Y., Wan, C., 2011. Lactic acid production from corn stover using mixed cultures of *Lactobacillus rhamnosus* and *Lactobacillus brevis*. Bioresour. Technol. 102, 1831–1836.

da Silveira dos Santos, D., Camelo, A.C., Cristina, K., Rodrigues, P., Carlos, L.C., Pereira, N.J., 2010. Ethanol Production from Sugarcane Bagasse by *Zymomonas mobilis* Using Simultaneous Saccharification and Fermentation (SSF) Process. Appl. Biochem. Biotechnol. 161, 93–105.

De Angelis, M., Bini, L., Pallini, V., Cocconcelli, P.S., Gobbetti, M., 2001. The acid-stress response in *Lactobacillus sanfranciscensis* CB1. Microbiology 147, 1863–1873.

De Angelis, M., Gobbetti, M., 2011. Stress Responses of Lactobacilli, in: Stress Responses of Lactic Acid Bacteria. pp. 219–249.

De Angelis, M., Gobbetti, M., 2004. Environmental stress responses in Lactobacillus: a review. Proteomics 4, 106–122.

Deanda, K., Zhang, M., Eddy, C., Picataggio, S., 1996. Development of an Arabinose-Fermenting *Zymomonas mobilis* Strain by Metabolic Pathway Engineering. Appl. Environ. Microbiol. 62, 4465–4470.

Delgenes, J.P., Moletta, R., 1996. Effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by *Saccharomyces cerevisiae*, *Zymomonas mobilis*, *Pichia stipitis*, and *Candida shehatae*. Enzyme Microb. Technol. 19, 220–225.

Desmond, C., Fitzgerald, G.F., Stanton, C., Ross, R.P., 2004. Improved Stress Tolerance of GroESL-Overproducing *Lactococcus lactis* and Probiotic *Lactobacillus paracasei* NFBC 338. Appl. Environ. Microbiol. 70, 5929–5936.

Desmond, C., Stanton, C., Fitzgerald, G.F., Collins, K., Ross, R.P., 2001. Environmental adaptation of probiotic lactobacilli towards improvement of performance during spray drying. Int. Dairy J. 11, 801–808.

Dien, B.S., Nichols, N.N., Bothast, R.J., 2001. Recombinant *Escherichia coli* engineered for production of L-lactic acid from hexose and pentose sugars. J. Ind. Microbiol. Biotechnol. 27, 259–264.

Dunlop, A.P., 1948. Furfural formation and behavior. Ind. Eng. Chem. 40, 204–209.

Duwat, P., Ehrlich, S.D., Gruss, A., 1995. The *recA* gene of *Lactococcus lactis*: characterization and involvement in oxidative and thermal stress. Mol. Microbiol. 17, 1121–1131.

FitzPatrick, M., Champagne, P., Cunningham, M.F., Whitney, R.A., 2010. A biorefinery processing perspective: Treatment of lignocellulosic materials for the production of value-added products. Bioresour. Technol. 101, 8915–8922.

Franz, C.M.A.P., Holzapfel, W.H., 2011. The Importance of Understanding the Stress Physiology of Lactic Acid Bacteria, in: Stress Responses of Lactic Acid Bacteria. pp. 3–20.

Frees, D., Varmanen, P., Ingmer, H., 2001. Inactivation of a gene that is highly conserved in Gram-positive bacteria stimulates degradation of non-native proteins and concomitantly increases stress tolerance in *Lactococcus lactis*. Mol. Microbiol. 41, 93–103.

Frees, D., Vogensen, F.K., Ingmer, H., 2003. Identification of proteins induced at low pH in *Lactococcus lactis*. Int. J. Food Microbiol. 87, 293–300.

Gaenzle, M., Schwab, C., 2009. Ecology of Exopolysaccharide Formation by Lactic Acid Bacteria: Sucrose Utilization, Stress Tolerance, and Biofilm Formation, in: Bacterial Polysaccharides. Current Innovations and Future Trends. pp. 263–278.

Gálvez, A., Abriouel, H., López, R.L., Omar, N. Ben, 2007. Bacteriocin-based strategies for food biopreservation. Int. J. Food Microbiol. 120, 51–70.

Gao, G., Tian, B., Liu, L., Sheng, D., Shen, B., Hua, Y., 2003. Expression of *Deinococcus radiodurans* PprI enhances the radioresistance of *Escherichia coli*. DNA Repair (Amst). 2, 1419–1427.

Garde, A., Jonsson, G., Schmidt, A.S., Ahring, B.K., 2002. Lactic acid production from wheat straw hemicellulose hydrolysate by *Lactobacillus pentosus* and *Lactobacillus brevis*. Bioresour. Technol. 81, 217–223.

Geddes, R.D., Wang, X., Yomano, L.P., Miller, E.N., Zheng, H., Shanmugam, K.T., Ingram, L.O., 2014. Polyamine Transporters and Polyamines Increase Furfural Tolerance during Xylose Fermentation with Ethanologenic *Escherichia coli* Strain LY180. Appl. Environ. Microbiol. 80, 5955–5964.

Girgis, H.S., Smith, J., Luchansky, J.B., Klaenhammer, T.R., 2003. Stress Adaptations of Lactic Acid Bacteria, in: Microbial Stress Adaptation and Food Safety.

Glebes, T.Y., Sandoval, N.R., Reeder, P.J., Schilling, K.D., Zhang, M., Gill, R.T., 2014. Genome-Wide Mapping of Furfural Tolerance Genes in *Escherichia coli*. PLoS One 9.

Glaasker, E., Konings, W.N., Poolman, B., 1996a. Osmotic Regulation of Intracellular Solute Pools in *Lactobacillus plantarum*. J. Bacteriol. 178, 575–582.

Glaasker, E., Konings, W.N., Poolman, B., 1996b. Glycine Betaine Fluxes in *Lactobacillus plantarum* during Osmostasis and Hyper- and Hypo-osmotic Shock. J. Biol. Chem. 271, 10060–10065.

Glaasker, E., Tjan, F.S.B., Steeg, P.F. Ter, Konings, W.N., Poolman, B., 1998. Physiological Response of *Lactobacillus plantarum* to Salt and Nonelectrolyte Stress. J. Bacteriol. 180, 4718–4723.

Gorsich, S.W., Dien, B.S., Nichols, N.N., Slininger, P.J., Liu, Z.L., Skory, C.D., 2006. Tolerance to furfural-induced stress is associated with pentose phosphate pathway genes ZWF1, GND1, RPE1, and TKL1 in *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. 71, 339–349.

Groot, M.N.N., Klaassens, E., de Vos, W.M., Delcour, J., Hols, P., Kleerebezem, M., 2005. Genome-based in silico detection of putative manganese transport systems in *Lactobacillus plantarum* and their genetic analysis. Microbiology 151, 1229–1238.

Guerzoni, M.E., Lanciotti, R., Cocconcelli, P.S., 2001. Alteration in cellular fatty acid composition as a response to salt, acid, oxidative and thermal stresses in *Lactobacillus helveticus*. Microbiology 147, 2255–2264.

Gullon, B., Yanez, R., Alonso, L.J., Parajo, J.C., 2008. L-Lactic acid production from apple pomace by sequential hydrolysis and fermentation. Bioresour. Technol. 99, 308–319.

Guo, W., Jia, W., Li, Y., Chen, S., 2010. Performances of *Lactobacillus brevis* for Producing Lactic Acid from Hydrolysate of Lignocellulosics. Appl. Biochem. Biotechnol. 161, 124–36.

Hahn-Hägerdal, B., Galbe, M., Gorwa-Grauslund, M.-F., Liden, G., Zacchi, G., 2006. Bio-ethanol – the fuel of tomorrow from the residues of today. TRENDS Biotechnol. 24, 549–556.

Hahn-Hägerdal, B., Jeppsson, H., Olsson, L., Mohagheghi, A., 1994. An interlaboratory comparison of the performance of ethanol-producing micro-organisms in a xylose-rich acid hydrolysate. Appl. Microbiol. Biotechnol. 41, 62–72.

Hahn-Hägerdal, B., Karhumaa, K., Fonseca, C., Spencer-Martins, I., Gorwa-Grauslund, M.F., 2007. Towards industrial pentose-fermenting yeast strains. Appl. Microbiol. Biotechnol. 74, 937–953.

Hartke, A., Frere, J., Boutibonnes, P., Auffray, Y., 1997. Differential Induction of the Chaperonin GroEL and the Co-Chaperonin GroES by Heat, Acid, and UV-Irradiation in *Lactococcus lactis* subsp. *lactis*. Curr. Microbiol. 34, 23–26.

Hartke, A., Giard, J.-C., Laplace, J.-M., Auffray, Y., 1998. Survival of *Enterococcus faecalis* in an Oligotrophic Microcosm: Changes in Morphology, Development of General Stress Resistance, and Analysis of Protein Synthesis. Appl. Environ. Microbiol. 64, 4238–4245.

Henningham, A., Barnett, T.I.C., Maamary, P.G., Walker, M.J., 2012. Pathogenesis of Group A Streptococcal Infections. Discov. Med. 13, 329–342.

Himmel, M.E., Ding, S.-Y., Johnson, D.K., Adney, W.S., Nimlos, M.R., Brady, J.W., Foust, T.D., 2007. Biomass Recalcitrance: Engineering Plants and Enzymes for Biofuels Production. Science (80-.). 315, 804–808.

Hutkins, R.W., Ellefson, W.L., Kashket, E.R., 1987. Betaine Transport Imparts Osmotolerance on a Strain of *Lactobacillus acidophilus*. Appl. Environ. Microbiol. 53, 2275–2281.

IEA Bioenergy, 2007. Biorefining in a future BioEconomy [WWW Document]. Task 42 Biorefining. URL www.iea-bioenergy.task42-biorefineries.com (accessed 2.19.16).

Isikgor, F.H., Becer, C.R., 2015. Lignocellulosic biomass: a sustainable platform for the production of bio-based chemicals and polymers. Polym. Chem. 6, 4497–4559.

Iyer, P. V, Thomas, S., Lee, Y.Y., 2000. High-Yield Fermentation of Pentoses into Lactic Acid. Appl. Biochem. Biotechnol. 84-86, 665–677.

Jeffries, T.W., Grigoriev, I. V, Grimwood, J., Laplaza, M., Aerts, A., Salamov, A., Schmutz, J., Lindquist, E., Dehal, P., Shapiro, H., Jin, Y., Passoth, V., Richardson, P.M., 2007. Genome sequence of the lignocellulose-bioconverting and xylose-fermenting yeast *Pichia stipitis*. Nat. Biotechnol. 25, 319–326.

John, R.P., 2006. Simultaneous Saccharification and Fermentation of Cassava Bagasse for L-(+)-Lactic Acid Production. Appl. Biochem. Biotechnol. 134, 263–272.

Jänsch, A., Korakli, M., Vogel, R., Gänzle, M., 2007. Glutathione Reductase *from Lactobacillus sanfranciscensis* DSM 20451T: Contribution to Oxygen Tolerance and Thiol Exchange Reactions in Wheat Sourdoughs. Appl. Environ. Microbiol. 73, 4469–4476.

Jönsson, L.J., Alriksson, B., Nilvebrant, N.O., 2013. Bioconversion of lignocellulose: inhibitors and detoxification. Biotechnol. Biofuels 6.

Jönsson, L.J., Palmqvist, E., Nilvebrant, N.-O., Hahn-Hägerdal, B., 1998. Detoxification of wood hydrolysates with laccase and peroxidase from the white-rot fungus Trametes versicolor. Appl. Biochem. Biotechnol. 49, 691–697.

Kajfasz, J.K., Quivey, R.G.J., 2011. Responses of Lactic Acid Bacteria to Acid Stress, in: Stress Responses of Lactic Acid Bacteria. pp. 23–53.

Karhumaa, K., Fromanger, R., Hahn-Hägerdal, B., Gorwa, 2007. High activity of xylose reductase and xylitol

dehydrogenase improves xylose fermentation by recombinant *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. 73, 1039–1046.

Karhumaa, K., Hahn-Hägerdal, B., Gorwa-Grauslund, M.F., 2005. Investigation of limiting metabolic steps in the utilization of xylose by recombinant *Saccharomyces cerevisiae* using metabolic engineering. Yeast 22, 359–368.

Karhumaa, K., Wiedemann, B., Hahn-Hägerdal, B., Boles, E., Gorwa-Grauslund, M.F., 2006. Co-utilization of L-arabinose and D-xylose by laboratory and industrial *Saccharomyces cerevisiae* strains. Microb. Cell Fact. 5, 18.

Kets, E.P.W., Teunissen, P.J.M., de Bont, J.A.M., 1996. Effect of Compatible Solutes on Survival of Lactic Acid Bacteria Subjected to Drying. Appl. Environ. Microbiol. 62, 259–261.

Kilstrup, M., Jacobsen, S., Hammer, K., 1997. Induction of Heat Shock Proteins DnaK , GroEL , and GroES by Salt Stress in *Lactococcus lactis* 63, 1826–1837.

Kim, M., Day, D.F., 2011. Composition of sugar cane, energy cane, and sweet sorghum suitable for ethanol production at Louisiana sugar mills. J. Ind. Microbiol. Biotechnol. 38, 803–807.

Kim, W.S., Perl, L., Park, J.H., Tandianus, J.E., Dunn, N.W., 2001. Assessment of Stress Response of the Probiotic *Lactobacillus acidophilus*. Curr. Microbiol. 43, 346–350.

Klinke, H.B., Olsson, L., Thomsen, A.B., Ahring, B.K., 2003. Potential Inhibitors From Wet Oxidation of Wheat Straw and Their Effect on Ethanol Production of *Saccharomyces cerevisiae*: Wet Oxidation and Fermentation by Yeast. Biotechnol. Bioeng. 81, 738–747.

Klinke, H.B., Thomsen, a B., Ahring, B.K., 2004. Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. Appl. Microbiol. Biotechnol. 66, 10–26.

Kotzamanidis, C., Roukas, T., Skaracis, G., 2002. Optimization of lactic acid production from beet molasses by *Lactobacillus delbrueckii* NCIMB 8130. World J. Microbiol. Biotechnol. 18, 441–448.

Kuo, Y.-C., Yuan, S.-F., Wang, C.-A., Huang, Y.-J., Guo, G.-L., Hwang, W.-S., 2015. Production of optically pure L-lactic acid from lignocellulosic hydrolysate by using a newly isolated and D-lactate dehydrogenase gene-deficient *Lactobacillus paracasei* strain. Bioresour. Technol. 198, 651–657.

Kuyper, M., Harhangi, H.R., Stave, A.K., Winkler, A.A., Jetten, M.S.M., de Laat, W.T.A.M., den Ridder, J.J.J., den Camp, H.J.M.O., van Dijken, J.P., Pronk, J.T., 2003. High-level functional expression of a fungal xylose isomerase: the key to efficient ethanolic fermentation of xylose by *Saccharomyces cerevisiae*? FEMS Yeast Res. 4, 69–78.

Kuyper, M., Hartog, M.M.P., Toirkens, M.J., Almering, M.J.H., Winkler, A.A., van Dijken, J.P., Pronk, J.T., 2005. Metabolic engineering of a xylose-isomerase-expressing *Saccharomyces cerevisiae* strain for rapid anaerobic xylose fermentation. FEMS Yeast Res. 5, 399–409.

König, H., Fröhlich, J., 2009. Lactic Acid Bacteria, in: Biology of Microorganisms on Grapes, in Must and in Wine. pp. 3–29.

Kötter, P., Ciriacy, M., 1993. Xylose fermentation by *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. 38, 776–783.

Larsson, S., Cassland, P., Jönsson, L.J., 2001. Development of a *Saccharomyces cerevisiae* Strain with Enhanced Resistance to Phenolic Fermentation Inhibitors in Lignocellulose Hydrolysates by Heterologous Expression of Laccase. Appl. Environ. Microbiol. 67, 1163–1170.

Larsson, S., Nilvebrant, N.-O., Jönsson, L.J., 2001. Effect of overexpression of *Saccharomyces cerevisiae* Pad1p on the resistance to phenylacrylic acids and lignocellulose hydrolysates under aerobic and oxygen-limited conditions. Appl. Microbiol. Biotechnol. 57, 167–174.

Larsson, S., Palmqvist, E., Hahn-Hägerdal, B., Tengborg, C., Stenberg, K., Zacchi, G., Nilvebrant, N.-O., 1999a. The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. Enzyme Microb. Technol. 24, 151–159.

Larsson, S., Reimann, A., Nilvebrant, N.-O., Jönsson, L.J., 1999b. Comparison of Different Methods for the Detoxification of Lignocellulose Hydrolyzates of Spruce. Appl. Biochem. Biotechnol. 77-79, 91–103.

Leandro, M.J., Goncalves, P., Spencer-Martins, I., 2006. Two glucose/xylose transporter genes from the yeast *Candida intermedia*: first molecular characterization of a yeast xylose –H+ symporter. Biochem. J. 395, 543–549.

Lee, K., Pi, K., Kim, E.B., Rho, B.-S., Kang, S.-K., Lee, H.G., Choi, Y.-J., 2010. Glutathione-mediated response to acid stress in the probiotic bacterium, *Lactobacillus salivarius*. Biotechnol. Lett. 32, 969–972.

Lemos, A.C., Burne, R.A., 2002. Regulation and Physiological Significance of ClpC and ClpP in *Streptococcus mutans*. J. Bacteriol. 184, 6357–6366.

Leroy, F., De Vuyst, L., 2004. Lactic acid bacteria as functional starter cultures for the food fermentation industry. Trends Food Sci. Technol. 15, 67–78.

Lewis Liu, Z., Blaschek, H.P., 2010. Biomass conversion inhibitors and in situ detoxification, in: Biomass to Biofuels: Strategies for Global Industries. pp. 233–259.

Li, X., Kim, T.H., Nghiem, N.P., 2010a. Bioethanol production from corn stover using aqueous ammonia pretreatment and two-phase simultaneous saccharification and fermentation (TPSSF). Bioresour. Technol. 101, 5910–5916.

Li, C., Knierim, B., Manisseri, C., Arora, R., Scheller, H. V, Auer, M., Vogel, K.P., Simmons, B.A., Singh, S., 2010b. Comparison of dilute acid and ionic liquid pretreatment of switchgrass: Biomass recalcitrance, delignification and enzymatic saccharification. Bioresour. Technol. 101, 4900–4906.

Lim, E.M., Ehrlich, S.D., Maguin, E., 2000. Identification of stress-inducible proteins in *Lactobacillus delbrueckii* subsp. *bulgaricus*. Electrophoresis 21, 2557–2561.

Limayem, A., Ricke, S.C., 2012. Lignocellulosic biomass for bioethanol production: Current perspectives, potential issues and future prospects. Prog. Energy Combust. Sci. 38, 449–467.

Liu, S., Bischoff, K.M., Hughes, S.R., Leathers, T.D., Price, N.P., Qureshi, N., Rich, J.O., 2009. Conversion of biomass hydrolysates and other substrates to ethanol and other chemicals by *Lactobacillus buchneri*. Lett. Appl. Microbiol. 48, 337–342.

Liu, S., Bischoff, K.M., Qureshi, N., Hughes, S.R., Rich, J.O., 2010. Functional expression of the thiolase gene thl from *Clostridium beijerinckii* P260 in *Lactococcus lactis* and *Lactobacillus buchneri*. N. Biotechnol. 27, 283–288. doi:10.1016/j.nbt.2010.03.007

Liu, Z.L., Moon, J., Andersh, B.J., Slininger, P.J., Weber, S., 2008. Multiple gene-mediated NAD(P)H-dependent aldehyde reduction is a mechanism of in situ detoxification of furfural and 5-hydroxymethylfurfural by *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. 81, 743–753.

Liu, Z.L., Slininger, P.J., Dien, B.S., Berhow, M. a, Kurtzman, C.P., Gorsich, S.W., 2004. Adaptive response of yeasts to furfural and 5-hydroxymethylfurfural and new chemical evidence for HMF conversion to 2,5-bis-hydroxymethylfuran. J. Ind. Microbiol. Biotechnol. 31, 345–352.

Liu, Z.L., Slininger, P.J., Gorsich, S.W., 2005. Enhanced Biotransformation of Furfural and Hydroxymethylfurfural by Newly Developed Ethanologenic Yeast Strains. Appl. Biochem. Biotechnol. 121-124, 451–460.

Lücke, F., 2000. Utilization of microbes to process and preserve meat. Meat Sci. 56, 105–115.

Ma, R., Zhang, Y., Hong, H., Lu, W., Lin, M., Chen, M., Zhang, W., 2011. Improved Osmotic Tolerance and Ethanol Production of Ethanologenic *Escherichia coli* by IrrE, a Global Regulator of Radiation-Resistance of *Deinococcus radiodurans*. Curr. Microbiol. 62, 659–664.

Marques, S., Santos, A.L., Girio, F.M., Roseiro, J.C., 2008. Lactic acid production from recycled paper sludge by simultaneous saccharification and fermentation. Biochem. Eng. J. 41, 210–216.

Martin, C., Klinke, H.B., Thomsen, A.B., 2007. Wet oxidation as a pretreatment method for enhancing the enzymatic convertibility of sugarcane bagasse. Enzyme Microb. Technol. 40, 426–432.

Martinez, A., Rodriguez, M.E., York, S.W., Preston, J.F., Ingram, L.O., 2000. Effects of Ca(OH)2 Treatments ("overliming") on the Composition and Toxicity of Bagasse Hemicellulose Hydrolysates. Biotechnol. Bioeng. 69, 526–36.

Miller, E.N., Jarboe, L.R., Yomano, L.P., York, S.W., Shanmugam, K.T., Ingram, L.O., 2009. Silencing of NADPH-dependent oxidoreductase genes (yqhD and dkgA) in furfural-resistant ethanologenic *Escherichia coli*. Appl. Environ. Microbiol. 75, 4315–23.

Miller, E.N., Turner, P.C., Jarboe, L.R., Ingram, L.O., 2010. Genetic changes that increase 5-hydroxymethyl furfural resistance in ethanol-producing *Escherichia coli* LY180. Biotechnol. Lett. 32, 661–667.

Mohagheghi, A., Evans, K., Chou, Y.-C., Zhang, M., 2002. Cofermentation of Glucose, Xylose, and Arabinose by Genomic DNA – Integrated Xylose/Arabinose Fermenting Strain of *Zymomonas mobilis* AX101. Appl. Biochem. Biotechnol. 98-100, 885–898.

Mood, S.H., Golfeshan, A.H., Tabatabaei, M., Jouzani, G.S., Najafi, G.H., Gholami, M., Ardjmand, M., 2013. Lignocellulosic biomass to bioethanol, a comprehensive review with a focus on pretreatment. Renew. Sustain. Rev. 27, 77–93.

Nichols, N.N., Dien, B.S., Bothast, R.J., 2001. Use of catabolite repression mutants for fermentation of sugar mixtures to ethanol. Appl. Microbiol. Biotechnol. 56, 120–125.

Nigam, J.N., 2001. Ethanol production from wheat straw hemicellulose hydrolysate by *Pichia stipitis*. J. Biotechnol 87, 17-27.

Nilvebrant, N.-O., Persson, P., Reimann, A., de Sousa, F., Gorton, L., Jönsson, L.J., 2003. Limits for Alkaline Detoxification of Dilute-Acid Lignocellulose Hydrolysates. Appl. Biochem. Biotechnol. 105-108, 615–628.

Noonpakdee, W., Sitthimonchai, S., Panyim, S., Lertsiri, S., 2004. Expression of the catalase gene *katA* in starter culture *Lactobacillus plantarum* TISTR850 tolerates oxidative stress and reduces lipid oxidation in fermented meat product. Int. J. Food Microbiol. 95, 127–135.

Ohgren, K., Bengtsson, O., Gorwa-Grauslund, M.F., Galbe, M., Hahn-Hagerdal, B., Zacchi, G., 2006. Simultaneous saccharification and co-fermentation of glucose and xylose in steam-pretreated corn stover at high fiber content with *Saccharomyces cerevisiae* TMB3400. J. Biotechnol. 126, 488–498.

Pacific Northwest National Laboratory, N.R.E.L., 2004. Top Value Added Chemicals from Biomass: Results of Screening for Potential Candidates from Sugars and Synthesis Gas. US Dep. Energy, Oak Ridge, TN.

Pallen, M.J., Wren, B.W., 1997. The HtrA family of serine proteases. Mol. Microbiol. 26, 209–221.

Palmqvist, E., Grage, H., Meinander, N.Q., Hahn-Hägerdal, B., 1999. Main and interaction effects of acetic acid, furfural, and p-hydroxybenzoic acid on growth and ethanol productivity of yeasts. Biotechnol. Bioeng. 63, 46–55.

Palmqvist, E., Hahn-Hägerdal, B., 2000. Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibitions. Bioresour. Technol. 74, 25–33.

Pan, J., Wang, J., Zhou, Z., Yan, Y., Zhang, W., Lu, W., Ping, S., Dai, Q., Yuan, M., Feng, B., Hou, X., Zhang, Y., Ma, R., Liu, T., Feng, L., Wang, L., Chen, M., Lin, M., 2009. IrrE, a Global Regulator of Extreme Radiation Resistance in *Deinococcus radiodurans*, Enhances Salt Tolerance in *Escherichia coli* and *Brassica napus*. PLoS One 4, 1–9.

Parawira, W., Tekere, M., 2011. Biotechnological strategies to overcome inhibitors in lignocellulose hydrolysates for ethanol production: review. Crit. Rev. Biotechnol. 31, 20–31.

Patle, S., Lal, B., 2007. Ethanol production from hydrolysed agricultural wastes using mixed culture of *Zymomonas mobilis* and *Candida tropicalis*. Biotechnol. Lett. 29, 1839–1843.

Petersohn, A., Brigulla, M., Haas, S., Hoheisel, J.D., Völker, U., Hecker, M., 2001. Global Analysis of the General Stress Response of *Bacillus subtilis*. J. Bacteriol. 183, 5617–5631.

Petersson, A., Almeida, J.R.M., Modig, T., Karhumaa, K., Hahn-Hägerdal, B., Gorwa-Grauslund, M.F., Lidén, G., 2006. A 5-hydroxymethyl furfural reducing enzyme encoded by the *Saccharomyces cerevisiae* ADH6 gene conveys HMF tolerance. Yeast 23, 455–464.

Piuri, M., Sanchez-Rivas, C., Ruzal, S.M., 2003. Adaptation to high salt in Lactobacillus: role of peptides and proteolytic enzymes. J. Appl. Microbiol. 95, 372–379.

Poquet, I., Saint, V., Seznec, E., Simoes, N., Bolotin, A., Gruss, A., 2000. HtrA is the unique surface housekeeping protease in *Lactococcus lactis* and is required for natural protein processing. Mol. Microbiol. 35, 1042–1051.

Qian, M., Tian, S., Li, X., Zhang, J., Pan, Y., Yang, X., 2006. Ethanol production from dilute-acid softwood hydrolysate by co-culture. Appl. Biochem. Biotechnol. 134, 273-284.

Rallu, F., Gruss, A., Ehrlich, S.D., Maguin, E., 2000. Acid- and multistress-resistant mutants of *Lactococcus lactis*: identification of intracellular stress signals. Mol. Microbiol. 35, 517–528.

Ranatunga, T.D., Jervis, I.J., Helm, I.R.F., McMillan, J.D., Hatzis, C., 1997. Identification of Inhibitory Components Toxic Toward *Zymomonas mobilis* CP4 (pZB5) Xylose Fermentation. Appl. Biochem. Biotechnol. 67, 185–198.

Richard, P., Verho, R., Putkonen, M., Londesborough, J., Penttilä, M., 2003. Production of ethanol from L-arabinose by *Saccharomyces cerevisiae* containing a fungal L-arabinose pathway. FEMS Yeast Res. 3, 185–189.

Roca, C., Haack, M.B., Olsson, L., 2004. Engineering of carbon catabolite repression in recombinant xylose fermenting *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. 63, 578–583.

Rochat, T., Gratadoux, J.-J., Gruss, A., Corthier, G., Maguin, E., Langella, P., van de Guchte, M., 2006. Production of a Heterologous Nonheme Catalase by *Lactobacillus casei*: an Efficient Tool for Removal of H2O2 and Protection of *Lactobacillus bulgaricus* from Oxidative Stress in Milk. Appl. Environ. Microbiol. 72, 5143–5149.

Rochat, T., Miyoshi, A., Gratadoux, J.J., Duwat, P., Sourice, S., Azevedo, V., Langella, P., 2005. High-level resistance to oxidative stress in *Lactococcus lactis* conferred by *Bacillus subtilis* catalase KatE.

Microbiology 151, 3011–3018.

Ruas-Madiedo, P., Hugenholtz, J., Zoon, P., 2002. An overview of the functionality of exopolysaccharides produced by lactic acid bacteria. Int. Dairy J. 12, 163–171.

Rubin, E.M., 2008. Genomics of cellulosic biofuels. Nature 454, 841–845.

Rud, I., Jensen, P.R., Naterstad, K., Axelsson, L., 2006. A synthetic promoter library for constitutive gene expression in *Lactobacillus plantarum*. Microbiology. doi:10.1099/mic.0.28599-0

Saha, B.C., 2005. Enzymes as Biocatalysts for Conversion of Lignocellulosic Biomass to Fermentable Sugars, in: Handbook of Industrial Biocatalysis. pp. 1–12.

Sanchez, R.G., Karhumaa, K., Fonseca, C., Nogué, V.S., Almeida, J.R.M., Larsson, C.U., Bengtsson, O., Bettiga, M., Hahn-Hägerdal, B., Gorwa-Grauslund, M.F., 2010. Improved xylose and arabinose utilization by an industrial recombinant *Saccharomyces cerevisiae* strain using evolutionary engineering. Biotechnol. Biofuels 3, 1–11.

Sanders, J.W., Venema, G., Kok, J., 1999. Environmental stress responses in *Lactococcus lactis*. FEMS Microbiol. Rev. 23, 483–501.

Serrano, L.M., Molenaar, D., Wels, M., Teusink, B., Bron, P. a, de Vos, W.M., Smid, E.J., 2007. Thioredoxin reductase is a key factor in the oxidative stress response of *Lactobacillus plantarum* WCFS1. Microb. Cell Fact. 6, 1–14.

Shafiei, M., Karimi, K., Taherzadeh, M.J., 2010. Pretreatment of spruce and oak by N-methylmorpholine-N-oxide (NMMO) for efficient conversion of their cellulose to ethanol. Bioresour. Technol. 101, 4914–4918.

Shen, C.R., Liao, J.C., 2008. Metabolic engineering of *Escherichia coli* for 1-butanol and 1-propanol production via the keto-acid pathways. Metab. Eng. 10, 312–320.

Singhvi, M., Joshi, D., Adsul, M., Varma, A., Gokhale, D., 2010. D-(-)-Lactic acid production from cellobiose and cellulose by *Lactobacillus lactis* mutant RM2-2†4. Green Chem. 12, 1106–1109.

Singhvi, M.S., Chaudhari, S., Gokhale, D. V, 2014. Lignocellulose processing: a current challenge. R. Soc. Chem. Adv. 4, 8271–8277.

Smeds, A.S., Varmanen, P., Palva, A., 1998. Molecular Characterization of a Stress-Inducible Gene from *Lactobacillus helveticus*. J. Bacteriol. 180, 6148–6153.

Solem, C., Defoor, E., Jensen, P.R., Martinussen, J., 2008. Plasmid pCS1966, a New Selection/Counterselection Tool for Lactic Acid Bacterium Strain Construction Based on the *oroP* Gene, Encoding an Orotate Transporter from *Lactococcus lactis*. Appl. Environ. Microbiol. 74, 4772–4775.

Solem, C., Dehli, T., Ruhdal Jensen, P., 2013. Rewiring *Lactococcus lactis* for Ethanol Production. Appl. Environ. Microbiol. 79, 2512–2518.

Sonderegger, M., Jeppsson, M., Larsson, C., Gorwa-Grauslund, M.F., Boles, E., Olsson, L., Spencer-Martins, I., Hahn-Hägerdal, B., Sauer, U., 2004. Fermentation Performance of Engineered and Evolved Xylose-Fermenting *Saccharomyces cerevisiae* Strains. Biotechnol. Bioeng. 87, 90–98.

Sreenath, H.K., Moldes, A.B., Koegel, R.G., Straub, R.J., 2001. Lactic Acid Production by Simultaneous Saccharification and Fermentation of Alfalfa Fiber. J. Biosci. Bioeng. 92, 518–523.

Streit, F., Delettre, J., Corrieu, G., Beal, C., 2008. Acid adaptation of *Lactobacillus delbrueckii* subsp .

bulgaricus induces physiological responses at membrane and cytosolic levels that improves cryotolerance. J. Appl. Microbiol. 105, 1071–1080.

Sun, Y., Cheng, J., 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour. Technol. 83, 1–11.

Suokko, A., Poutanen, M., Savijoki, K., Kalkkinen, N., Varmanen, P., 2008. ClpL is essential for induction of thermotolerance and is potentially part of the HrcA regulon in *Lactobacillus gasseri*. Proteomics 8, 1029–1041.

Suokko, A., Savijoki, K., Malinen, E., Palva, A., Varmanen, P., 2005. Characterization of a Mobile *clpL* Gene from *Lactobacillus rhamnosus* 71, 2061–2069.

Taherzadeh, M.J., Karimi, K., 2008. Pretreatment of Lignocellulosic Wastes to Improve Ethanol and Biogas Production: A Review. Int. J. Mol. Sci. 9, 1621–1651.

Teixeira, P., Castro, H., Mohacsi-Farkas, C., Kirby, R., 1997. Identification of sites of injury in *Lactobacillus bulgaricus* during heat stress. J. Appl. Microbiol. 83, 219–226.

Teusink, B., Wiersma, A., Molenaar, D., Francke, C., de Vos, W.M., Siezen, R.J., Smid, E.J., 2006. Analysis of growth of *Lactobacillus plantarum* WCFS1 on a complex medium using a genome-scale metabolic model. J. Biol. Chem.

Ulbricht, R.J., Northup, S.J., Thomas, J.A., 1984. A review of 5-Hydroxymethylfurfural (HMF) in Parenteral Solutions. Fundam. Appl. Toxicol. 4, 843–853.

Upadrasta, A., Stanton, C., Hill, C., Fitzgerald, G.F., Ross, R.P., 2011. Improving the Stress Tolerance of Probiotic Cultures: Recent Trends and Future Directions, in: Stress Responses of Lactic Acid Bacteria. pp. 395–438.

van de Guchte, M., Serror, P., Chervaux, C., Smokvina, T., Ehrlich, S.D., Maguin, E., 2002. Stress responses in lactic acid bacteria. Antonie Van Leeuwenhoek, Int. J. Gen. Mol. Microbiol. 82, 187–216.

Van der Heide, T., Poolman, B., 2000. Glycine Betaine Transport in *Lactococcus lactis* Is Osmotically Regulated at the Level of Expression and Translocation Activity. J. Bacteriol. 182, 203–206.

Varmanen, P., Savijoki, K., 2011. Responses of Lactic Acid Bacteria to Heat Stress, in: Stress Responses of Lactic Acid Bacteria. pp. 55–66.

Vido, K., Diemer, H., Dorsselaer, A. Van, Leize, E., Juillard, V., Gruss, A., Gaudu, P., 2005. Roles of Thioredoxin Reductase during the Aerobic Life of *Lactococcus lactis*. J. Bacteriol. 187, 601–610.

Wahlbom, C.F., van Zyl, W.H., Jönsson, L.J., Hahn-Hägerdal, B., Cordero Otero, R.R., 2003. Generation of the improved recombinant xylose-utilizing *Saccharomyces cerevisiae* TMB 3400 by random mutagenesis and physiological comparison with *Pichia stipitis* CBS 6054. FEMS Yeast Res. 3, 319–326.

Walfridsson, M., Bao, X., Anderlund, M., Lilius, G., Bülow, L., Hahn-Hägerdal, B., 1996. Ethanolic Fermentation of Xylose with *Saccharomyces cerevisiae* Harboring the Thermus thermophilus *xylA* Gene, Which Expresses an Active Xylose (Glucose) Isomerase. Appl. Environ. Microbiol. 62, 4648–4651.

Walker, D.C., Girgis, H.S., Klaenhammer, T.R., 1999. The groESL Chaperone Operon of *Lactobacillus johnsonii*. Appl. Environ. Microbiol. 65, 3033–3041.

Wang, J., Zhang, Y., Chen, Y., Lin, M., Lin, Z., 2012. Global Regulator Engineering Significantly Improved *Escherichia coli* Tolerances Toward Inhibitors of Lignocellulosic Hydrolysates. Biotechnol. Bioeng. 109, 3133–3142.

Wang, X., Miller, E.N., Yomano, L.P., Zhang, X., Shanmugam, K.T., Ingram, L.O., 2011. Increased Furfural Tolerance Due To Overexpression of NADH-Dependent Oxidoreductase FucO in *Escherichia coli* Strains Engineered for the Production of Ethanol and Lactate. Appl. Environ. Microbiol. 77, 5132–5140.

Weber, C., Farwick, A., Benisch, F., Brat, D., Dietz, H., Subtil, T., Boles, E., 2010. Trends and challenges in the microbial production of lignocellulosic bioalcohol fuels. Appl. Microbiol. Biotechnol. 87, 1303–1315.

Wilmes-Riesenberg, M.R., Bearson, B., Foster, J.W., Curtis, R., 1996. Role of the Acid Tolerance Response in Virulence of *Salmonella typhimurium*. Infect. Immun. 64, 1085–1092.

Wiselogel, 1996. Biomass feedstock resources and composition, in: Handbook on Bioethanol: Production and Utilization.

Woodbury, R., Haldenwang, W.., 2003. HrcA is a negative regulator of the dnaK and groESL operons of *Streptococcus pyogenes*. Biochem. Biophys. Res. Commun. 302, 722–727.

Wright, A. Von, Axelsson, L., 2011. Lactic Acid Bacteria: An Introduction, in: Lactic Acid Bacteria: Microbiological and Functional Aspects. pp. 1–16.

Wu, C., Zhang, J., Wang, M., Du, G., Chen, J., 2012. *Lactobacillus casei* combats acid stress by maintaining cell membrane functionality. J. Ind. Microbiol. Biotechnol. 39, 1031–1039.

Yanase, H., Nozaki, K., Okamoto, K., 2005. Ethanol production from cellulosic materials by genetically engineered *Zymomonas mobilis*. Biotechnol. Lett. 27, 259–263.

Yanez, R., Alonso, L., Parajo, J.C., 2005. D-Lactic acid production from waste cardbord. J. Chem. Technol. Biotechnol. 80, 76–84.

Yanez, R., Moldes, A.B., Alonso, J.L., Parajo, J.C., 2003. Production of D(-)-lactic acid from cellulose by simultaneous saccharification and fermentation using *Lactobacillus coryniformis* subsp. *torquens*. Biotechnol. Lett. 25, 1161–1164.

Zaldivar, J., Martinez, A., Ingram, L.O., 2000. Effect of Alcohol Compounds Found in Hemicellulose Hydrolysate on the Growth and Fermentation of Ethanologenic *Escherichia coli*. Biotechnol. Bioeng. 68, 524–30.

Zaldivar, J., Martinez, A., Ingram, L.O., 1999. Effect of Selected Aldehydes on the Growth and Fermentation of Ethanologenic *Escherichia coli*. Biotechnol. Bioeng. 65, 24–33.

Zhai, Z., Douillard, F.P., An, H., Wang, G., Guo, X., Luo, Y., Hao, Y., 2014. Proteomic characterization of the acid tolerance response in *Lactobacillus delbrueckii* subsp. *bulgaricus* CAUH1 and functional identification of a novel acid stress-related transcriptional regulator Ldb0677. Environ. Microbiol. 16, 1524–1537.

Zhang, J., Fu, R.-Y., Hugenholtz, J., Li, Y., Chen, J., 2007. Glutathione Protects *Lactococcus lactis* against Acid Stress. Appl. Environ. Microbiol. 73, 5268–5275.

Zhang, M., Eddy, C., Deanda, K., Finkelstein, M., Picataggio, S., 1995. Metabolic Engineering of a Pentose Metabolism Pathway in Ethanologenic *Zymomonas mobilis*. Science. 267, 240–243.

Zhang, W., Wong, K.K., Magliozzo, R.S., Kozarich, J.W., 2001. Inactivation of Pyruvate Formate-Lyase by Dioxygen: Defining the Mechanistic Interplay of Glycine 734 and Cysteine 419 by Rapid Freeze-Quench EPR. Biochemistry 40, 4123–4130.

Zheng, H., Wang, X., Yomano, L.P., Shanmugam, K.T., Ingram, L.O., 2012. Increase in Furfural Tolerance in Ethanologenic *Escherichia coli* LY180 by Plasmid-Based Expression of *thyA*. Appl. Environ. Microbiol. 78, 4346–4352.

Zhou, C.-H., Xia, X., Lin, C.-X., Tong, D.-S., Beltramini, J., 2011. Catalytic conversion of lignocellulosic biomass to fine chemicals and fuels. Chem. Soc. Rev. 40, 5588–5617.

Aarnikunnas, J., Von Weymarn, N., Rönnholm, K., Leisola, M., Palva, A., 2003. Metabolic Engineering of *Lactobacillus fermentum* for Production of Mannitol and Pure L-Lactic Acid or Pyruvate. Biotechnol. Bioeng. 82, 653–63.

Chapter 2

Screening of lactic acid bacteria for their potential as microbial cell factories for bioconversion of lignocellulosic feedstocks

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MICROBIAL CELL FACTORIES

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Screening of lactic acid bacteria for their potential as microbial cell factories for bioconversion of lignocellulosic feedstocks

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Abstract

Background: The use of fossil carbon sources for fuels and petrochemicals has serious impacts on our environment and is unable to meet the demand in the future. A promising and sustainable alternative is to substitute fossil carbon sources with microbial cell factories converting lignocellulosic biomass into desirable value added products. However, such bioprocesses require availability of suitable and efficient microbial biocatalysts, capable of utilizing C5 sugars and tolerant to inhibitory compounds generated during pretreatment of biomass. In this study, the performance of a collection of lactic acid bacteria was evaluated regarding their properties with respect to the conversion of lignocellulosic feedstocks. The strains were examined for their ability to utilize xylose and arabinose as well as their resistance towards common inhibitors from pretreated lignocellulosic biomass (furan derivatives, phenolic compounds, weak acids).

Results: Among 296 tested Lactobacillus and Pediococcus strains, 3 *L. pentosus*, 1 *P. acidilactici* and 1 *P. pentosaceus* isolates were found to be both capable of utilizing xylose and arabinose and highly resistant to the key inhibitors from chemically pretreated lignocellulosic biomass. When tested in broth with commonly found combinations of inhibitors, the selected strains showed merely 4%, 1% and 37% drop in growth rates for sugarcane bagasse, wheat straw and soft wood representatives, respectively, as compared to *Escherichia coli* MG1655 showing decreased growth rates by 36%, 21% and 90%, respectively, under the same conditions.

Conclusion: The study showed that some strains of Lactobacilli and Pediococci have the potential to be used as production platforms for value-added products from pretreated lignocellulosic biomass. Selected Lactobacilli and Pediococci strains were able to tolerate the key inhibitors in higher concentrations compared to *E.coli*; in addition, as these isolates were also capable of fermenting xylose and arabinose, they constitute good candidates for efficient lignocellulosic feedstock bioconversions.

Keywords: Lactic acid bacteria, Fermentation inhibitors, Furfural, HMF, Lignocellulosic biomass, C5 sugars

Background

The 21st century brought us to the point where increasing needs for food and energy can no longer be satisfied by the diminishing natural resources. Both the limiting oil and coal supplies and the environmental issues including greenhouse gas emissions into the atmosphere make it crucial to explore microbial bioconversion from renewable feedstocks. One source of renewable raw material with a high potential is lignocellulosic biomass. This substrate is highly abundant worldwide and therefore much cheaper than the first generation biomass used at present. Additionally, the lignocellulose, in contrast to the first generation feedstocks, poses no competition to the food or animal feed supplies. However, this environmentally friendly solution has not been yet implemented commercially on a large scale with one of the obstacles being the lack of an efficient organism to allow an economically feasible conversion process.

Lignocellulose consists of three main component fractions: cellulose, hemicellulose and lignin. The fermentable sugars, which include both hexoses and pentoses,

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are protected from microbial or chemical attack inside the lignin fraction. Thus lignocellulose needs to be pretreated before the microorganisms can ferment the sugars from the cellulose and hemicellulose inside [1]. Unfortunately, the different pretreatment methods will not only release fermentable sugars, but also substances with inhibitory effects towards microorganisms in the subsequent fermentation step. These toxic substances can be categorized into three major groups: furan derivatives, phenolic compounds and weak organic acids. Furan aldehydes, furfural and HMF, are of sugar origin and are produced from pentoses and hexoses, respectively, while phenolic compounds are generated during degradation of lignin [2,3]. Acetic acid, formed in high concentrations (up to 12 g/L) [4], comes from deacetylation of hemicellulose, while other organic acids (formic and levulinic acids) are released when sugars are further degraded [3]. The concentrations of inhibitors and their composition highly depend on the chosen method of pretreatment, the process conditions and the type of substrate used.

Lactic acid bacteria are characterized by their ability to grow anaerobically with high growth rates at low pH values caused by the presence of organic acids. Within the lactic acid bacteria, *Lactobacillus* is a highly diversified genus with over 150 different species displaying a large panel of catabolic activities. Lactobacilli have been isolated from varied environments, from human gastrointestinal tract to soil and decaying plant material. These features suggest that *Lactobacilli* could be interesting candidates for becoming efficient utilizers of the second-generation lignocellulosic feedstocks, perhaps even superior to the strains traditionally used (e.g. *Escherichia coli* or *Saccharomyces cerevisiae*). Some of the *Lactobacillus* strains have already been reported to be suitable for conversion of biomass to value added products [5-7] but no systematic studies have been performed on this group of organisms.

In this study, we screened several hundred species of the *Lactobacillus* genus along with a closely related *Pediococcus* genus with regard to several important properties for becoming potential workhorses for microbial bioconversion of lignocellulosic biomass into value-added products. We evaluated a collection of strains with respect to their ability to utilize xylose and arabinose, their resistance towards common inhibitors from pretreated lignocellulosic biomass, and their performance at high concentrations of acidic products potentially formed during the fermentation process.

Results and discussion

A commonly adopted approach when selecting a production host is a product-oriented strategy, which investigates the organism's ability to produce a specific product and further uses genetic engineering to make the organism utilize the required substrate. Another approach could be a substrate-oriented strategy focusing on the capacity of an organism to utilize a certain feedstock in order to select best fitted strain and then add the required metabolic steps by genetic engineering. To provide an initial screening, 296 strains were tested, including 155 type strains of different lactic acid bacteria species and a collection of 141 isolates of *L. plantarum*, *L. paraplantarum*, *L. pentosus*, *L. brevis*, *L. buchneri* and *L. paracasei*. This covered all available species of Lactobacilli and Pediococci. Of those, 23 strains were obligate anaerobes or displayed poor growth on MRS medium, and were discarded as being less interesting as potential future workhorses. Additionally, strains of the model organism *Lactococcus lactis* MG1363 and *Escherichia coli* MG1655 were included in the tests for comparison.

Growth media test

Strains were tested for their ability to grow on three media: MRS, GSA and DLA. MRS is a complex rich undefined medium supporting the growth of Lactobacilli. GSA and DLA are defined media for growth of Lactococci and *Lactobacillus plantarum,* respectively. The results of the growth tests on MRS, DLA and GSA media are presented in (Additional file 1: Table S2). All but 23 strains grew on MRS plates and these strains were excluded from further tests. Out of the 125 tested *L. plantarum* isolates, 115 (92%) strains showed good or moderate growth on DLA plates. Out of the remaining 171 tested strains, including the various type strains, only a small fraction showed good (26 strains) or moderate (8 strains) growth on DLA, including a close relative *L. pentosus* (all tested isolates) and 2 isolates of *L. buchneri*. The other defined medium, GSA, supported good or moderate growth of 159 strains, including different *L. plantarum* (111) and *L. pentosus* (7) isolates, 38 other Lactobacilli species, 2 Pediococci strains and a strain of *L. lactis*, for which the medium was originally developed. Due to the inability of many of the strains to grow on DLA medium, only GSA and MRS media were used for the subsequent screening.

Test of sensitivity towards key inhibitors from lignocellulose

The inhibitory compounds used for the screening tests were selected based on a literature study and their concentrations were chosen to be the highest reported to be present in pretreated lignocellulosic biomass (Table 1). The susceptibility of a collection of lactic acid bacteria strains to a number of common inhibitors from pretreated lignocellulosic biomass was evaluated. Out of 274 strains, which showed good or moderate growth on MRS, 256 and 141 were able to grow on high concentrations of furfural (3.5 g/L) and HMF (5.9 g/L), respectively, which are the two key inhibitors found in lignocellulosic biomass.

Table 1 Inhibitors and their concentrations used in this study

	Compounds found in hemicellulose hydrolysates	Max. concentration in biomass (g/L)	Tested concentration (g/L)	References
Aldehydes	Furfural	3.75	3.75	[2]
	HMF (5-hydroxymethyl-furfural)	5.9; 7.3	5.9; 7.3	[8,9]
	4-Hydroxybenzaldehyde	0.01	0.01	[10]
	Syringaldehyde (3,5-Dimethoxy-4-hydroxybenzaldehyde)	0.213	0.213	[4]
	Vanillin (4-Hydroxy-3-methoxybenzaldehyde)	0.43	0.43	[10]
Alcohols	Pyrocatechol	0.44	0.44	[10]
	Furfuryl alcohol		20	[11]
	Guaiacol (2-Methoxyphenol)	0.615	0.615	[10]
	Methylcatechol		0.15	
	Vanillin alcohol (4-Hydroxy-3-methoxybenzyl alcohol)		9	
	Ethanol		55	
	Syringyl alcohol (2,6-dimethoxyphenol, syringol)	0.156	0.156	[12]
Acids	Formic acid	7.7	7.7	[13]
	Levulinic acid	23.3	23.3	[2]
	Acetic acid	12.14	12.14	[4]
	Syringic acid	0.092	0.092	[4]
	Vanillic acid (4-Hydroxy-3-methoxybenzoic acid)	0.122	0.122	[13]
	Ferulic acid	0.018	0.018	[13]

The results of inhibitor screening on MRS plates for the best performing strains are presented in Table 2; the results obtained for all tested species can be found in (Additional file 1: Table S3). A subset of sixteen strains with the best tolerance for the inhibitors were subjected to an additional test where the concentration of furfural was raised to 7 g/L, 10 g/L and 15 g/L (Table 3). All tested strains could grow well on plates containing 7 g/L furfural; the higher concentrations were not tolerated by most of the strains, and only 2 strains showed a moderate growth on 10 g/L and 15 g/L furfural. Similarly, HMF concentration was raised from 5.9 g/L to 7.3 g/L and 10 g/L. None of the strains were able to grow well on 7.3 g/L HMF, a concentration reported to be present in dilute sulfuric acid hydrolyzed spruce [8], but a few strains showed moderate growth on that concentration of HMF. HMF concentration of 10 g/L completely inhibited strains' growth.

When tested with a panel of other inhibitors, the best performing strains showed high resistance towards furfuryl alcohol, vanillin and vanillin alcohol. When testing higher concentrations of vanillin (0.86 g/L, 1.72 g/L and 3.44 g/L), it was found that 11 out of 16 tested strains could grow at a concentration of 1.72 g/L which is a 4 times higher concentration than the maximum concentration reported to be found in pretreated lignocellulosic biomass. Doubling the concentration of vanillin alcohol from 9 g/L to 18 g/L revealed that all tested strains that were able to grow well on 9 g/L, also exhibited good or moderate growth on 18 g/L.

In the case of furfuryl alcohol, no literature data has been reported regarding the concentrations found in the lignocellulosic biomass and therefore a minimal inhibitory concentration of *Escherichia coli* strain LY01 [11] was used for the screening (20 g/L). 40.5% of the screened lactic acid bacteria strain collection was found to be resistant to that concentration of furfuryl alcohol. Out of 16 best performing strains, only 3 showed no growth. In addition, most of the strains that were able to grow at a concentration of 20 g/L, showed also a moderate growth at a higher concentration of 25 g/L.

When tested for the tolerance to ethanol, a potential value-added product that could be made out of lignocellulosic biomass, 88% of the tested strains were tolerant to 55 g/L ethanol, including all isolates of *L. plantarum*, *L. pentosus*, and *L. brevis*. After raising the concentration of ethanol on plates to 70 g/L and 85 g/L, all sixteen tested strains produced colonies of similar sizes when compared to the colonies they produced on MRS control plates.

Many of the strains, especially *L. plantarum* and *L. pentosus* isolates could easily grow at high concentrations of acetate, levulinate and formate (79%, 82.5% and 74% of all strains, respectively). When grown on plates containing syringic, vanillic and ferulic acids, most strains (>90%) were hardly affected; however, the tested concentrations

Table 2 Results of the screening on MRS for the 15 best-performing strains

Species	Strain	MRS	Furfural	HMF	4-hydroxybenzaldehyde	Syringaldehyde	Vanillin	Catechol	Furfuryl alcohol	Guaiacol	Methylcatechol
Lactobacillus brevis	LMG 19215	+	+	+	+	+	+	+	±	±	-
Lactobacillus brevis	LMG 19191	+	+	+	+	+	+	+	±	±	-
Lactobacillus brevis	LMG 19188	+	+	+	+	-	+	+	-	+	-
Lactobacillus brevis	LMG 19216	+	+	+	+	-	±	±	±	±	-
Lactobacillus brevis	LMG 19186	+	+	+	+	-	±	+	-	+	-
Lactobacillus hammesii	DSM 16381	+	+	+	+	-	+	±	-	+	-
Lactobacillus pentosus	DSMZ 20314 T	+	+	+	+	±	+	-	+	+	-
Lactobacillus pentosus	B148	+	+	+	+	-	+	+	-	+	-
Lactobacillus pentosus	LMG 17678	+	+	+	+	-	+	+	-	+	-
Lactobacillus pentosus	LMG 17682	+	+	+	+	+	+	+	+	+	-
Lactobacillus rossiae	DSM 15814	+	+	+	+	-	±	±	-	+	-
Lactobacillus spicheri	DSM 15429	+	+	+	+	-	+	±	±	+	-
Lactobacillus suebicus	DSM 5007	+	+	+	+	±	±	-	-	±	-
Pediococcus acidilactici	DSM 20284	+	+	+	+	±	-	+	+	+	-
Pediococcus pentosaceus	ATCC 25745	+	+	+	+	+	+	+	±	+	-

Table 2 Results of the screening on MRS for the 15 best-performing strains *(Continued)*

Species	Vanillin alcohol	Ethanol	Syringyl alcohol	Formic acid	Levulinic acid	Acetic acid	Syringic acid	Vanillic acid	Ferulic acid
Lactobacillus brevis	±	+	+	±	+	+	+	+	+
Lactobacillus brevis	±	+	+	±	+	+	+	+	+
Lactobacillus brevis	±	+	+	±	+	+	+	+	+
Lactobacillus brevis	±	+	±	±	+	+	±	+	±
Lactobacillus brevis	±	+	+	±	+	+	+	+	+
Lactobacillus hammesii	-	+	+	+	+	+	+	+	+
Lactobacillus pentosus	+	+	+	+	+	+	+	+	+
Lactobacillus pentosus	+	+	+	±	+	+	+	+	+
Lactobacillus pentosus	+	+	+	+	±	±	+	+	+
Lactobacillus pentosus	±	+	+	+	+	+	+	+	+
Lactobacillus rossiae	-	+	+	+	+	+	+	+	+
Lactobacillus spicheri	-	+	+	+	+	±	+	+	+
Lactobacillus suebicus	-	±	+	-	-	-	±	±	±
Pediococcus acidilactici	±	+	+	±	+	+	+	+	+
Pediococcus pentosaceus	+	+	+	±	+	+	+	+	+

+, good growth; ±, moderate growth; −, no or poor growth; nd, not determined.

Table 3 Results of the screening of 16 best-performing strains for growth on higher concentrations of selected inhibitors

Species	Strain	MRS	Furfural 3.5 g/L	Furfural 7 g/L	Furfural 10 g/L	Furfural 15 g/L	HMF 5.9 g/L	HMF 7.3 g/L	HMF 10 g/L	Vanillin 0.43 g/L	Vanillin 0.86 g/L	Vanillin 1.72 g/L
Lactobacillus brevis	LMG 19215	+	+	+	±	±	+	-	-	+	+	-
Lactobacillus brevis	LMG 19217	+	+	+	±	±	+	±	-	+	+	-
Lactobacillus hammesii	DSM 16381	+	+	+	-	-	+	-	-	+	+	-
Lactobacillus pentosus	LMG 17673	+	+	+	-	-	+	±	-	+	+	+
Lactobacillus pentosus	LMG 17672	+	+	+	-	-	+	-	-	+	+	+
Lactobacillus pentosus	10-16	+	+	+	-	-	+	-	-	+	+	+
Lactobacillus pentosus	B148	+	+	+	-	-	+	-	-	+	+	±
Lactobacillus plantarum	JCL1279	+	+	+	-	-	+	-	-	+	+	±
Lactobacillus plantarum	A7	+	+	+	-	-	+	-	-	+	+	±
Lactobacillus plantarum	R4698	+	+	+	-	-	+	-	-	+	+	+
Lactobacillus plantarum	KOG8	+	+	+	-	-	+	±	-	+	+	±
Lactobacillus plantarum	NCFB1206	+	+	+	-	-	±	-	-	+	+	+
Lactobacillus spicheri	DSM 15429	+	+	+	-	-	+	-	-	+	+	±
Lactobacillus suebicus	DSM 5007	+	+	+	-	-	+	-	-	±	±	-
Pediococcus acidilactici	DSM 20284	+	+	+	-	-	+	-	-	-	-	-
Pediococcus pentosaceus	ATCC 25745	+	+	+	-	-	+	-	-	+	+	+

Table 3 Results of the screening of 16 best-performing strains for growth on higher concentrations of selected inhibitors *(Continued)*

Species	Vanillin 3.44 g/L	Furfuryl alcohol 20 g/L	Furfuryl alcohol 25 g/L	Vanillin alcohol 4.5 g/L	Vanillin alcohol 9 g/L	Vanillin alcohol 13.5 g/L	Vanillin alcohol 18 g/L	Ethanol 55 g/L	Ethanol 70 g/L	Ethanol 85 g/L
Lactobacillus brevis	-	±	-	+	±	±	±	+	+	+
Lactobacillus brevis	-	±	-	+	±	±	±	+	+	+
Lactobacillus hammesii	-	-	-	+	-	-	-	+	±	±
Lactobacillus pentosus	±	+	±	+	+	+	+	+	+	+
Lactobacillus pentosus	±	+	+	+	+	+	+	+	+	+
Lactobacillus pentosus	-	+	±	+	+	+	+	+	+	+
Lactobacillus pentosus	-	±	±	+	+	+	±	+	+	+
Lactobacillus plantarum	-	-	-	+	+	+	±	+	+	+
Lactobacillus plantarum	-	+	±	+	+	±	±	+	+	+
Lactobacillus plantarum	-	+	±	+	+	+	±	+	+	+
Lactobacillus plantarum	-	+	±	+	+	+	±	+	+	+
Lactobacillus plantarum	-	+	±	+	+	+	+	+	+	+
Lactobacillus spicheri	-	±	±	+	±	±	±	+	+	+
Lactobacillus suebicus	-	-	-	+	-	-	-	±	±	±
Pediococcus acidilactici	-	+	±	+	+	+	±	+	+	+
Pediococcus pentosaceus	-	+	±	+	+	+	±	+	+	+

+, good growth; ±, moderate growth; −, no or poor growth; nd, not determined.

were very low, similarly to the ones found in the pretreated lignocellulosic biomass [4,13].

Methylcatechol was the compound found to impair growth of the majority of microorganisms the most compared to the other tested compounds and already at concentration as low as 0.15 g/L. No literature data was available on methylcatechol concentrations in lignocellulose, and therefore the minimal inhibitory concentration of *E. coli* LY01 [11] was tested (1.5 g/L). However, since none of the tested strains showed any growth on that concentration, it was decreased to 0.15 g/L. Nevertheless, none of the strains could grow well even on the 10 times decreased concentration and only 18% of the strains showed moderate growth. This finding can result from methylcatechol's mode of action as it causes partition or loss of integrity of biological membranes [14]. Thus, the outer membrane of Gram negative organisms makes them much less vulnerable for the action of methylcatechol. However, despite the lack of data on its concentrations in lignocellulose, methylcatechol is one of the products that can be generated during degradation of lignin [15] and is therefore relevant to consider.

A known mechanism for detoxification used by cells is a modification of the inhibitors into less toxic derivatives, e.g. reduction of aldehydes to alcohols or oxidation to acids [16,17]. Accordingly, furfural would be reduced to furfuryl alcohol or oxidized to feroic acid; vanillin would be converted into vanillin alcohol or vanillic acid, and syringaldehyde - either to syringyl alcohol or syringic acid. Indeed, the negative impact of tested derivatives on the growth of the strains was slightly lower and in general the organisms tolerated higher concentrations of these compounds.

The screening was repeated on GSA plates for 159 strains, which showed good or moderate growth on this defined medium. On GSA plates containing furfural, 42.8% of the strains could grow well but none of the strains could tolerate HMF well at the tested concentration of 5.9 g/L; 32% of the strains showed only moderate growth. The vast majority of the strains grew well with vanillin, whereas only 30.2% could well tolerate the presence of vanillin alcohol. The strains were generally sensitive to furfuryl alcohol, as only 17% of the strains showed good growth when it was present in the medium. The most toxic compounds were found to be methylcatechol and pyrocatechol, which completely inhibited the growth of 88% and 75.5% of the strains, respectively. All results are presented in (Additional file 1: Table S4).

The strains exhibiting the best performance on GSA were *L. lactis* MG1363 and various *L. plantarum* isolates (FOEB9106, NCFB1193, LMG 17678, KOG10, NICMB8826, KOG21, KOG2, DK32, Lactolabo). These results indicate that on GSA medium *L. lactis* performs equally well as the other lactic acid bacteria with regard to inhibitor tolerance. However, GSA medium was originally developed specifically for *L. lactis,* and it does not support the growth of Lactobacilli to a similar extent. On MRS medium there were several strains found which showed an even better resistance profile than *L. lactis*. Moreover, the best performing strains of Lactobacilli and Pediococci have two significant advantages over *L. lactis*: they can utilize the C5 sugars and they can grow at higher temperatures (37°C-42°C vs 30°C for *L. lactis*).

Stirred flask fermentation experiments

The 10 best performing strains identified during the initial screening on solid media were chosen to quantify the effects of the inhibitors on growth rates in MRS broth. Controls were performed by cultivating the strains in the same conditions but with no inhibitors added. The growth rates with and without inhibitors were compared for each strain, and *L. pentosus* LMG 17672, LMG 17673 and 10–16 were found to be the most resistant strains (Table 4). All of them performed well in presence of 3.5 g/L furfural or 5.9 g/L HMF, showing decreased growth rates by up to 32%. They all tolerated the presence of 20 g/L furfuryl alcohol which caused a 24 to 46% decrease in their growth rates. Last but not least, they performed remarkably well when grown in the presence of 0.43 g/L vanillin showing a similar or better growth compared to growth in MRS with no inhibitors. However, one of these strains, LMG 17673, was found to be susceptible to acetate and showed no growth during fermentation in the presence of 30 g/L acetate.

Two strains, *L. spicheri* DSM 15429 and *L. brevis* LMG 19215, demonstrated very good performance in the presence of furfural and HMF. Two other strains (*L. suebicus* DSM 5007 and *L. hammesii* DSM 16381) showed very slow growth when compared to other tested strains (about 6 times lower when compared to the growth rate of the fastest-growing strain *P. pentosaceus* ATCC 25745), and were therefore not considered for further investigation as they are probably less promising as potential workhorses.

The concentrations of furfural and HMF used in this study which do not severely inhibit the growth of the tested strains are very high when compared to inhibitory concentrations for *E. coli* or *S. cerevisiae* strains reported in the literature. Furfural was shown to cause a 50% inhibition of growth of *E. coli* strains already at concentrations of 1 – 2.4 g/L [18,19]; *S. cerevisiae* strains were inhibited at 1 g/L furfural; with HMF, the growth was shown to be inhibited by 2 g/L for *E. coli* and 1 g/L for *S. cerevisiae* [18]. Moreover, Zaldivar et al. (1999) showed that furfural and HMF completely inhibited the growth of *E. coli* strains at a concentration of 3.5 g/L and 4.0 g/L, respectively; *S. cerevisiae* was completely inhibited by 5.09 g/L furfural [20]. The best performing strains selected in this study were able to grow in presence of 3.5 g/L furfural

Table 4 Growth of the best performing strains in MRS medium with inhibitors

		Mean growth rates [1/h]													
		MRS		Furfural (3.5 g/L)		HMF (5.9 g/L)		Ethanol (55 g/L)		Acetic acid (30 g/L)		Furfuryl alcohol (20 g/L)		Vanillin (0.43 g/L)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Lactobacillus brevis	LMG 19215	0.528	0.093	0.402	0.008	0.290	0.030	0.356	0.031	0.522	0.034	nd	nd	nd	nd
Lactobacillus hammesii	DSM 16381	0.167	0.020	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Lactobacillus pentosus	10-16	0.794	0.047	0.538	0.036	0.554	0.027	0.550	0.066	0.484	0.015	0.511	0.073	0.765	0.038
Lactobacillus pentosus	LMG 17672	0.712	0.062	0.530	0.024	0.540	0.000	0.504	0.061	0.642	0.083	0.383	0.108	0.729	0.013
Lactobacillus pentosus	LMG 17673	0.826	0.022	0.558	0.042	0.636	0.067	0.586	0.018	ng	ng	0.627	0.013	0.825	0.021
Lactobacillus plantarum	JCL1279	0.675	0.004	0.399	0.013	0.332	0.009	0.316	0.048	0.561	0.030	nd	nd	nd	nd
Lactobacillus spicheri	DSM 15429	0.531	0.036	0.420	0.034	0.338	0.087	0.376	0.018	0.408	0.027	nd	nd	nd	nd
Lactobacillus suebicus	DSM 5007	0.147	0.038	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
*Lactobacillus vaccinostercus**	DSM 20634	0.690	0.017	0.435	0.021	0.258	0.085	0.444	0.017	0.711	0.030	0.351	0.013	0.660	0.034
Pediococcus acidilactici	DSM 20284	0.886	0.061	0.405	0.025	0.372	0.026	0.315	0.038	0.450	0.090	nd	nd	nd	nd
Pediococcus pentosaceus	ATCC 25745	0.995	0.042	0.684	0.076	0.606	0.079	0.566	0.091	0.404	0.034	0.552	0.085	0.960	0.008

nd, not determined.
ng, no growth.
*grown in MRS medium with glucose replaced with xylose.

with 26-54% inhibition of growth, whereas the growth with 5.9 g/L HMF was inhibited by 24-58%.

When looking at the ethanol tolerance of the strains, the growth of *E. coli* was inhibited completely by a concentration of 55 g/L [19], while the same concentration caused only 29% to 64% decrease in growth rates of strains selected in this study. Yet, ethanol is only one of the potential value-added products that could be made out of lignocellulose and the selected strains may be further tested for their tolerance to other products as well; however this is beyond the scope of this study.

Moreover, the tested inhibitor concentrations are the highest measured and reported in the pretreated lignocellulosic biomass, usually coming from soft or hard wood which is a specific type of biomass that needs longer pretreatment time and harsher conditions, therefore containing higher quantities of inhibitors. The average amounts of inhibitors present in different types of biomass are frequently much lower [21].

Pentose utilization test

Since the lignocellulose contains significant amounts of C5 sugars xylose and arabinose, all of the strains from the collection were also screened on plates for their abilities to utilize xylose and arabinose. Since MRS medium supports significant growth of the strains even with no sugar added, a modified MRS medium (10% MRS) was used, containing 90% lowered quantities of casein peptone, yeast extract and meat extract, and having all other ingredients in the original amount. 37 strains could utilize both xylose and arabinose very well (producing colonies of the same size as on glucose) and 9 strains could utilize both of them well or moderately (producing smaller colonies when compared to glucose plate) (Additional file 1: Table S5). Some strains were able to utilize only one of the tested pentoses: 10 strains were found to utilize xylose well or moderately; 40 and 34 strains could utilize arabinose well or moderately, respectively (Additional file 1: Table S5).

The strains that showed good growth on plates with xylose and arabinose were further tested in 10% MRS broth with xylose or arabinose, and their growth rates on C5 sugars were compared to their growth rates on glucose (Table 5). The best performing strains were *P. acidilactici* DSM 20284 and *P. pentosaceus* ATCC

Table 5 Growth of the best performing strains in 10% MRS medium with glucose, xylose or arabinose

Mean growth rates [1/h]							
		2% glucose		2% xylose		2% arabinose	
		Mean	SD	Mean	SD	Mean	SD
Lactobacillus brevis	LMG 19215	0.108	0.017	0.099	0.038	0.099	0.021
Lactobacillus pentosus	10-16	0.610	0.051	0.304	0.043	0.374	0.025
Lactobacillus pentosus	LMG 17672	0.594	0.055	0.199	0.038	0.460	0.018
Lactobacillus pentosus	LMG 17673	0.577	0.119	0.259	0.035	0.372	0.031
Lactobacillus spicheri	DSM 15429	0.111	0.072	ng	ng	0.066	0,000
Pediococcus acidilactici	DSM 20284	0.482	0.063	0.415	0.034	0.494	0.057
Pediococcus pentosaceus	ATCC 25745	0.391	0.026	0.202	0.014	0.390	0.016

nd, not determined.
ng, no growth.

25745 with 14% and 48% lower growth rates on xylose compared to glucose. On arabinose, both *P. acidilactici* DSM 20284 and *P. pentosaceus* ATCC 25745 showed similar growth rates when compared to glucose. Some of the other tested strains showed up to 66% and 41% lower growth rates on xylose and arabinose, respectively.

Performance in combination of inhibitors

As mentioned above, the concentrations of inhibitors in the pretreated lignocellulose depend both on the initial plant material (e.g. sugarcane bagasse, wheat straw, rice straw) and the method of pretreatment (the temperature, chemicals and their concentrations, time of pretreatment). The method can be chosen so that it is optimal for a given plant substrate; however, it is always a matter of a compromise between the inhibitors and the amount of released sugars available for the microorganisms. Usually, a mixture of different inhibitors is formed during pretreatment, and since some of them were previously shown to have additive or synergistic effects [19,22,23], we chose to investigate strain performance on a mixture of different inhibitors. MRS medium with combination of inhibitors was used as representatives of three different types of lignocellulosic biomass and was used to test for any additive or synergistic effects between different inhibitors and to simulate the strains' performance on real-life feedstocks. Five of the best performing strains were selected for the test: *L. pentosus* LMG 17672, *L. pentosus* LMG 17673, *L. pentosus* 10–16, *P. pentosaceus* ATCC 25745, and *P. acidilactici* DSM 20284. The effects of inhibitors on the growth rates were investigated both separately for each inhibitor and in combinations to reveal any additive or synergistic effects.

No apparent differences were found between the individual strains with regard to their resistance to the inhibitors (Table 6). The combined treatment with furfural, HMF and acetate representing sugarcane bagasse (0.3 g/L, 0.04 g/L and 2.7 g/L, respectively) and furfural and acetate representing wheat straw (0.15 g/L and 2.7 g/L, respectively) were found not to affect the growth rates of the tested strains significantly. The combined effect of furfural and acetate found in soft wood affected the growth rates of microorganisms by up to 37%. The most severe effects were due to the presence of furfural, since acetate, with one exception, did not influence the growth of the strains when added as a single inhibitor. Neither 2.7 g/L nor 5.3 g/L acetate exerted negative effects in 4 of the tested strains; the growth rates were even slightly enhanced in the presence of acetate. Thus, no synergistic effects were found between furfural and acetate for these strains at the tested concentrations. For *P. acidilactici*, however, 5.3 g/L acetate caused a 4% growth inhibition and showed a synergistic effect when the strain was grown with both acetate and furfural.

To evaluate if the strains perform equally well when they grow on xylose instead of glucose, the tests with combination of inhibitors were repeated for four of the best strains but in 10% MRS containing glucose or xylose (Table 7). In all but one cases, the growth rates on xylose were decreased 2–3 times when compared to glucose, as shown before; only *P. acidilactici* showed similar growth rates on both glucose and xylose. However, when the strains were grown on xylose, the inhibition effect caused by the presence of inhibitors was lessened when compared to when the strains were grown on glucose; only a strain of *P. pentosaceus* showed higher drops in growth rates on xylose than on glucose when grown with the combination of inhibitors.

The performance of the best strains of Lactobacilli and Pediococci was compared with the performance of *E. coli* MG1655 which was tested in LB with either glucose or xylose and with combinations of inhibitors representing sugarcane bagasse, wheat straw and soft wood (Additional file 1: Table S6). The growth rates were high both on glucose and xylose, however, the strain showed much worse performance in the presence of inhibitors than Lactobacilli and Pediococci. *E. coli* was inhibited by all tested combinations of inhibitors; in particular it was severely inhibited by furfural and acetate from soft wood (87-90% drop in growth rates on xylose and glucose, respectively); same conditions caused up to 37% lower growth rates in the selected strains of Lactobacilli and Pediococci. The presence of inhibitors found in sugarcane bagasse and wheat straw caused an inhibition of *E. coli* growth by 36% and 21%, respectively, whereas the same conditions caused up to 4% inhibition of growth of *L. pentosus* LMG 17672, and had no impact on the growth of the other four tested strains.

Conclusions

Lactic Acid Bacteria were systematically screened for tolerance towards inhibitors from pretreated lignocellulosic biomass. The results show that some of the identified isolates of *L. pentosus*, *P. pentosaceus* and *P. acidilactici* are not only highly resistant to the different inhibitors, also at higher concentrations than are usually present in the biomass, but they can also utilize xylose and arabinose. These findings stress that some LAB has the potential to become platforms for second generation bioconversion processes. The investigation of the transformability of selected strains is currently underway to ease metabolic and genetic engineering strategies to further improve their performance as production organisms.

Materials and methods

Strains and media

All strains used in this study including their origin are listed in (Additional file 1: Table S1). Some of the strains were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) or

Table 6 Performance of the best-performing strains in representatives of three feedstock hydrolysate types

		Conditions			Growth rate (1/h)		Gen. time (min)	% difference vs control
					Mean	SD		
L. pentosus LMG 17672	*Sugarcane bagasse*	Control (MRS)			0.712	0.062	60	0
		Furfural 0.3 g/L			0.666	0.043	62	6
		HMF 0.04 g/L			0.692	0.023	60	3
		Acetic acid 2.7 g/L			0.699	0.004	59	2
		Furfural 0.3 g/L	HMF 0.04 g/L		0.663	0.021	63	7
		Furfural 0.3 g/L	Acetic acid 2.7 g/L		0.723	0.013	58	−2
		HMF 0.04 g/L	Acetic acid 2.7 g/L		0.720	0.008	58	−1
		Furfural 0.3 g/L	HMF 0.04 g/L	Acetic acid 2.7 g/L	0.684	0.000	61	4
	Wheat straw	Furfural 0.15 g/L			0.696	0.000	60	2
		Furfural 0.15 g/L	Acetic acid 2.7 g/L		0.714	0.039	58	0
	Soft wood	Furfural 2.2 g/L			0.480	0.027	87	33
		Acetic Acid 5.3 g/L			0.750	0.008	55	−5
		Furfural 2.2 g/L	Acetic Acid 5.3 g/L		0.532	0.023	78	25
L. pentosus LMG 17673	*Sugarcane bagasse*	Control (MRS)			0.826	0.022	50	0
		Furfural 0.3 g/L			0.794	0.048	52	4
		HMF 0.04 g/L			0.849	0.021	49	−3
		Acetic acid 2.7 g/L			0.873	0.047	48	−6
		Furfural 0.3 g/L	HMF 0.04 g/L		0.795	0.013	52	4
		Furfural 0.3 g/L	Acetic acid 2.7 g/L		0.825	0.013	50	0
		HMF 0.04 g/L	Acetic acid 2.7 g/L		0.852	0.000	49	−3
		Furfural 0.3 g/L	HMF 0.04 g/L	Acetic acid 2.7 g/L	0.834	0.000	50	−1
	Wheat straw	Furfural 0.15 g/L			0.819	0.038	51	1
		Furfural 0.15 g/L	Acetic acid 2.7 g/L		0.831	0.021	50	−1
	Soft wood	Furfural 2.2 g/L			0.621	0.013	67	25
		Acetic Acid 5.3 g/L			0.864	0.000	48	−5
		Furfural 2.2 g/L	Acetic Acid 5.3 g/L		0.651	0.038	64	21
L. pentosus 10-16	*Sugarcane bagasse*	Control (MRS)			0.794	0.047	55	0
		Furfural 0.3 g/L			0.768	0.008	54	3
		HMF 0.04 g/L			0.765	0.013	54	4
		Acetic acid 2.7 g/L			0.753	0.013	55	5
		Furfural 0.3 g/L	HMF 0.04 g/L		0.747	0.013	56	6
		Furfural 0.3 g/L	Acetic acid 2.7 g/L		0.765	0.004	54	4

Table 6 Performance of the best-performing strains in representatives of three feedstock hydrolysate types *(Continued)*

		HMF 0.04 g/L	Acetic acid 2.7 g/L		0.762	0.000	55	4
		Furfural 0.3 g/L	HMF 0.04 g/L	Acetic acid 2.7 g/L	0.783	0.004	53	1
	Wheat straw	Furfural 0.15 g/L			0.780	0.025	53	2
		Furfural 0.15 g/L	Acetic acid 2.7 g/L		0.789	0.004	53	1
	Soft wood	Furfural 2.2 g/L			0.570	0.017	73	28
		Acetic Acid 5.3 g/L			0.807	0.004	52	−2
		Furfural 2.2 g/L	Acetic Acid 5.3 g/L		0.594	0.000	70	25
P. pentosaceus ATCC 25745	*Sugarcane bagasse*	Control (MRS)			0.995	0.042	41	0
		Furfural 0.3 g/L			0.981	0.004	42	1
		HMF 0.04 g/L			1.026	0.025	41	−3
		Acetic acid 2.7 g/L			0.951	0.004	44	4
		Furfural 0.3 g/L	HMF 0.04 g/L		1.011	0.047	41	−2
		Furfural 0.3 g/L	Acetic acid 2.7 g/L		1.002	0.034	42	−1
		HMF 0.04 g/L	Acetic acid 2.7 g/L		1.038	0.025	40	−4
		Furfural 0.3 g/L	HMF 0.04 g/L	Acetic acid 2.7 g/L	1.008	0.059	41	−1
	Wheat straw	Furfural 0.15 g/L			1.011	0.013	41	−2
		Furfural 0.15 g/L	Acetic acid 2.7 g/L		1.020	0.008	41	−3
	Soft wood	Furfural 2.2 g/L			0.708	0.036	59	29
		Acetic Acid 5.3 g/L			1.026	0.017	41	−3
		Furfural 2.2 g/L	Acetic Acid 5.3 g/L		0.753	0.030	55	24
P. acidilactici DSM 20284	*Sugarcane bagasse*	Control (MRS)			0.886	0.061	44	0
		Furfural 0.3 g/L			0.936	0.010	44	−6
		HMF 0.04 g/L			0.934	0.019	45	−5
		Acetic acid 2.7 g/L			0.882	0.006	47	0
		Furfural 0.3 g/L	HMF 0.04 g/L		0.936	0.006	44	−6
		Furfural 0.3 g/L	Acetic acid 2.7 g/L		0.978	0.027	43	−10
		HMF 0.04 g/L	Acetic acid 2.7 g/L		0.920	0.021	45	−4
		Furfural 0.3 g/L	HMF 0.04 g/L	Acetic acid 2.7 g/L	0.872	0.074	48	2
	Wheat straw	Furfural 0.15 g/L			0.872	0.009	48	2
		Furfural 0.15 g/L	Acetic acid 2.7 g/L		0.938	0.051	44	−6
	Soft wood	Furfural 2.2 g/L			0.676	0.012	62	24
		Acetic Acid 5.3 g/L			0.850	0.033	49	4
		Furfural 2.2 g/L	Acetic Acid 5.3 g/L		0.561	0.013	74	37

Table 7 Performance of the best strains in 10% MRS with glucose or xylose and combinations of inhibitors representing three feedstock hydrolysate types

	Conditions		Growth rate (1/h)		Gen. time (min)	% difference vs control
			Mean	SD		
L. pentosus LMG 17672	*10% MRS with glucose*	Control (10% MRS)	0.594	0.055	70	0
		Sugarcane bagasse (Furfural 0,3 g/L; HMF 0,04 g/L; Acetic acid 2,7 g/L)	0.552	0.017	75	7.1
		Wheat straw (Furfural 0.15 g/L; Acetic acid 2.7 g/L)	0.627	0.021	66	−5.6
		Soft wood (Furfural 2.2 g/L; Acetic Acid 5.3 g/L)	0.357	0.013	116	39.9
	10% MRS with xylose	Control (10% MRS)	0.199	0.038	209	0
		Sugarcane bagasse (Furfural 0.3 g/L; HMF 0.04 g/L; Acetic acid 2.7 g/L)	0.213	0.013	195	−6.9
		Wheat straw (Furfural 0.15 g/L; Acetic acid 2.7 g/L)	0.195	0.004	213	2.1
		Soft wood (Furfural 2.2 g/L; Acetic Acid 5.3 g/L)	0.180	0.000	231	9.6
L. pentosus LMG 17673	*10% MRS with glucose*	Control (10% MRS)	0.577	0.119	72	0
		Sugarcane bagasse (Furfural 0.3 g/L; HMF 0.04 g/L; Acetic acid 2.7 g/L)	0.612	0.025	68	−6.0
		Wheat straw (Furfural 0.15 g/L; Acetic acid 2.7 g/L)	0.624	0.017	67	−8.1
		Soft wood (Furfural 2.2 g/L; Acetic Acid 5.3 g/L)	0.435	0.013	96	24.6
	10% MRS with xylose	Control (10% MRS)	0.259	0.035	160	0
		Sugarcane bagasse (Furfural 0.3 g/L; HMF 0.04 g/L; Acetic acid 2.7 g/L)	0.315	0.030	132	−21.5
		Wheat straw (Furfural 0.15 g/L; Acetic acid 2.7 g/L)	0.243	0.064	171	6.3
		Soft wood (Furfural 2.2 g/L; Acetic Acid 5.3 g/L)	0.249	0.021	167	3.9
P. acidilactici DSM 20284	*10% MRS with glucose*	Control (10% MRS)	0.482	0.063	86	0
		Sugarcane bagasse (Furfural 0.3 g/L; HMF 0.04 g/L; Acetic acid 2.7 g/L)	0.387	0.013	107	19.8
		Wheat straw (Furfural 0.15 g/L; Acetic acid 2.7 g/L)	0.414	0.000	100	14.2
		Soft wood (Furfural 2.2 g/L; Acetic Acid 5.3 g/L)	0.324	0.034	128	32.8
	10% MRS with xylose	Control (10% MRS)	0.415	0.034	100	0
		Sugarcane bagasse (Furfural 0.3 g/L; HMF 0.04 g/L; Acetic acid 2.7 g/L)	0.378	0.000	110	9.0
		Wheat straw (Furfural 0.15 g/L; Acetic acid 2.7 g/L)	0.390	0.017	107	6.1
		Soft wood (Furfural 2.2 g/L; Acetic Acid 5.3 g/L)	0.300	0.034	139	27.7
P. pentosaceus ATCC 25745	*10% MRS with glucose*	Control (10% MRS)	0.391	0.026	106	0
		Sugarcane bagasse (Furfural 0.3 g/L; HMF 0.04 g/L; Acetic acid 2.7 g/L)	0.405	0.013	103	−3.5
		Wheat straw (Furfural 0.15 g/L; Acetic acid 2.7 g/L)	0.414	0.017	100	−5.8
		Soft wood (Furfural 2.2 g/L; Acetic Acid 5.3 g/L)	0.267	0.030	156	31.7
	10% MRS with xylose	Control (10% MRS)	0.202	0.014	206	0
		Sugarcane bagasse (Furfural 0.3 g/L; HMF 0.04 g/L; Acetic acid 2.7 g/L)	0.135	0.013	308	33.0
		Wheat straw (Furfural 0.15 g/L; Acetic acid 2.7 g/L)	0.171	0.004	243	15.2
		Soft wood (Furfural 2.2 g/L; Acetic Acid 5.3 g/L)	0.129	0.004	322	36.0

kindly obtained Jørgen Leisner, Copenhagen University (Copenhagen, Denmark). All strains except for *Lactococcus lactis* and *Escherichia coli* that were propagated in M17 (Oxoid) supplied with glucose to 1% at 30°C and Lysogeny Broth (LB) at 37°C, respectively, were grown on MRS agar plates (Oxoid) containing, per liter: 10 g casein peptone (tryptic digest), 10 g meat extract, 5 g yeast extract, 20 g glucose, 1 g Tween 80, 2 g K_2HPO_4, 5 g sodium acetate, 2 g diammonium citrate, 0.2 g $MgSO_4 \cdot 7H_2O$ and 0.05 g $MnSO_4 \cdot H_2O$ at optimal temperature (25°C, 28°C, 30°C, 37°C or 40°C) for 24-48 h. For storage cultures in stationary phase were harvested by centrifugation, resuspended in fresh medium supplied with 25% glycerol and frozen at −80°C.

For the screening purpose two media formulations were used: complex MRS medium and defined SA medium with 2% glucose (GSA) [24] supplemented with 25 mg/L uracil and 50 mg/L hypoxanthine. In case of media containing organic acids, the pH was adjusted to 6.5 ± 0.1 with 2 M NaOH or 10 M KOH. The strains were also tested for growth on defined DLA medium. The medium was prepared as described by Bringel et al. (1997) [25]; the following solutions were used: 100 ml of autoclaved solution 1 (50 g glucose, 50 g sodium acetate, 0.05 g oleic acid, 5 g Tween 40, 2.5 g ascorbic acid, 0.04 g $MnSO_4 \cdot H_2O$, 1 g $MgSO_4 \cdot 7H_2O$, and H_2O to 500 ml), 200 ml of filter-sterilized salt solution (8.75 g $Na_2HPO_4 \cdot 2H_2O$, 15 g KCl, and H_2O to 1 liter), 200 ml of a filter-sterilized solution of L-amino acids (0.2 g Pro; 0.25 g Lys and Thr; 1.25 g Asn; 1 g Gly, Trp, Ser, Ala, Phe, Leu, and Tyr; 2.5 g His, Iso, Met, and Val; 5 g Glu; 10 g Asp; and H_2O to 1 liter), 10 ml of filter-sterilized riboflavin solution (0.01 g dissolved in 100 ml of 0.02 M acetic acid and stored in the dark), 250 ml of filter-sterilized purine solution (0.2 g hypoxanthine, 0.3 g deoxyguanosine and guanine HCl, 0.5 g adenine, and H_2O to 1.5 liters), 0.1 ml of filter-sterilized solution 3 (0.05 g biotin in 50 ml of 50% ethanol, 0.025 g vitamin B_{12}, 0.08 g pyridoxamine · 2HCl, and H_2O to 500 ml), 10 ml of filter-sterilized solution 4 (0.025 g pyridoxal HCl in 100 ml of 20% ethanol, 0.02 g p-aminobenzoic acid, 0.085 g of nicotinic acid, 0.016 g of folic acid in 100 ml of 20% ethanol, 0.05 g of calcium pantothenate, 0.05 g spermine HCl, and H_2O to 500 ml), 50 ml of filter-sterilized solution 5 (2 g L-cysteine, 1.5 g L-glutamine, and H_2O to 250 ml), 1 ml of filter-sterilized 0.1% thiamine HCl solution, and 100 ml of autoclaved 0.1% L-cystine solution. The solution was adjusted to pH 6.5 with KOH or HCl and brought to 1 L with H_2O.

The ability of the strains to utilize xylose and arabinose was tested in 10% MRS medium containing per liter: 1 g casein peptone (tryptic digest) 0.8 g meat extract, 0.4 g yeast extract, 1 mL Tween 80, 2 g K_2HPO_4, 5 g sodium acetate, 2 g diammonium citrate, 0.2 g $MgSO_4 \cdot 7H_2O$ and 0.05 g $MnSO_4 \cdot H_2O$, and 20 g of carbon source (glucose, xylose, or arabinose). The pH of the medium was adjusted to 6.5 ± 0.1 with 2 M NaOH or 10 M KOH. For preparation of plates, 10 g/L Bacto agar was added.

Reagents

The following chemicals were purchased from Sigma Aldrich: HMF (5-hydroxymethylfurfural), 4-hydroxybenzaldehyde, syringaldehyde, vanillin, pyrocatechol, methylcatechol, guaiacol, furfuryl alcohol, vanillin alcohol, syringyl alcohol, levulinic acid, syringic acid, vanillic acid and ferulic acid. All other chemicals were obtained from Kemetyl (ethanol), Bie&Berntsen A-S (acetic acid), or Merck (furfural, formic acid).

Preliminary inhibitor screening

The resistance of strains towards the inhibitors from lignocellulosic biomass was investigated on MRS agar plates with a single inhibitor added at a specified concentration. The colonies were transferred onto plates from a dilution series made in a 96-well microtiter plates (TPP). The growth of strains was examined after 48 hour incubation at optimal temperature (25°C, 28°C, 30°C, 37°C or 40°C) by comparing the colony sizes on plates containing an inhibitor and control MRS plates. For several best performing strains a similar screening was repeated with higher concentrations of selected inhibitors: furfural (7.5 g/L, 10 g/L, 15 g/L), HMF (10 g/L, 15 g/L), vanillin (0.86 g/L, 1.72 g/L, 3.44 g/L), vanillin alcohol (4.5 g/L, 13.5 g/L, 18 g/L), furfuryl alcohol (5 g/L, 10 g/L, 20 g/L, 25 g/L) and ethanol (70 g/L, 85 g/L).

Screening in broth

The experiments were performed by inoculating 100 mL flasks containing 50 mL MRS broth and an inhibitor with an overnight culture to a starting OD_{600} of 0.04. The cells were cultivated under aerobic conditions at 30°C with 220 rpm magnetic stirring (2mag MIXdrive 15). To monitor the growth, 1 mL samples were taken every 30 min and the optical density at 600 nm was investigated by Genesys 10 spectrophotometer (Thermo Spectronic). At least 2 replicates were made for each strain and media type. For determination of specific growth rates, more than 5 experimental data points in the exponential growth phase were used.

Pentose utilization tests

The strains were streaked on 10% MRS agar plates containing glucose, xylose, arabinose, or no carbon source added and incubated at 30°C for 48 hours. The growth of the strains was evaluated as good growth (+), when the colonies produced on xylose and arabinose plates were of similar size as the ones on glucose plate; moderate (±)

when they were smaller, and no growth (–) when there were no colonies or they were small and comparable to the control plate with no sugar added.

Combination effect of inhibitors

The strains' performance in the presence of combination of inhibitors was evaluated by inoculating 100 mL flasks containing 50 mL medium and the inhibitors with overnight cultures to a starting OD_{600} of 0.04 and incubating at 30°C with 220 rpm magnetic stirring. The OD_{600} measurements were performed at 30 min intervals by Genesys 10 spectrophotometer (Thermo Spectronic). The medium was MRS containing 20 g/L glucose or 10% MRS containing 20 g/L glucose or xylose. The analyzed combinations of inhibitors were representative of sugarcane bagasse (0.3 g/L furfural, 0.04 g/L HMF, and 2.7 g/L acetate) [26], wheat straw (0.15 g/L furfural and 2.7 g/L acetate) [27] and soft wood (2.2 g/L furfural and 5.3 g/L acetate) [28]. For comparison, *E. coli* MG1655 was tested; 250 mL Erlenmeyer flasks with 50 mL LB with 20 g/L glucose or xylose and combination of inhibitors were inoculated with overnight cultures to an OD_{450} of 0.04 and incubated at 37°C with 180 rpm shaking. The OD_{450} measurements were done at 20 min intervals.

Additional file

Additional file 1: Table S1. Bacterial strains used in this study. **Table S2.** Growth of the tested strains on MRS, DLA and GSA media. **Table S3.** Results of the screening on MRS plates. **Table S4.** Results of the screening on GSA plates. **Table S5.** Results of the pentose utilization tests on 10% MRS plates with glucose, xylose or arabinose as sole carbon sources. **Table S6.** Performance of *E.coli* MG1655 in LB with glucose or xylose and combinations of inhibitors representing three feedstock hydolysate types.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AMB carried out experiments and drafted the manuscript. FB supplied with 127 *L. plantarum* isolates. JM and PRJ participated in the design of the study, supervised the experiments and helped to draft the manuscript. All authors read and approved the final manuscript.

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References

1. Himmel ME, Ding SY, Johnson DK, Adney WS, Nimlos MR, Brady JW, Foust TD: **Biomass recalcitrance: engineering plants and enzymes for biofuels production.** *Science* 2007, **315:**804–807.
2. Larsson S, Palmqvist E, Hahn-Hägerdal B, Tengborg C, Stenberg K, Zacchi G, Nilvebrant NO: **The generation of fermentation inhibitors during dilute acid hydrolysis of softwood.** *Enzyme Microb Technol* 1999, **24:**151–159.
3. Almeida JRM, Modig T, Petersson A, Hähn-Hägerdal B, Lidén G, Gorwa-Grauslund MF: **Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *S. cerevisiae*.** *J Chem Technol Biotechnol* 2007, **82:**340–349.
4. Tran AV, Chambers RP: **Ethanol fermentation of red oak acid prehydrolysate by the yeast *Pichia stipitis* CBS 5776.** *Enzyme Microb Technol* 1986, **8:**439–444.
5. Aarnikunnas J, von Weymarn N, Ronnholm K, Leisola M, Palva A: **Metabolic engineering of *Lactobacillus fermentum* for production of mannitol and pure L-lactic acid or pyruvate.** *Biotechnol Bioeng* 2003, **82:**653–663.
6. Berezina OV, Zakharova NV, Brandt A, Yarotsky SV, Schwarz WH, Zverlov VV: **Reconstructing the clostridial n-butanol metabolic pathway in *Lactobacillus brevis*.** *Appl Microbiol Biotechnol* 2010, **87:**635–646.
7. John RP, Nampoothiri KM, Pandey A: **Fermentative production of lactic acid from biomass: an overview on process developments and future perspectives.** *Appl Microbiol Biotechnol* 2007, **74:**524–534.
8. Taherzadeh MJ, Niklasson C, Lidén G: **Conversion of dilute-acid hydrolyzates of spruce and birch to ethanol by fed-batch fermentation.** *Biores Technol* 1999, **69:**59–66.
9. Klinke HB, Thomsen AB, Ahring BK: **Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass.** *Appl Microbiol Biotechnol* 2004, **66:**10–26.
10. Jönsson LJ, Palmqvist E, Nilvebrant NO, Hahn-Hägerdal B: **Detoxification of wood hydrolysates with laccase and peroxidase from the white-rot fungus *Trametes versicolor*.** *Appl Microbiol Biotechnol* 1998, **49:**691–697.
11. Zaldivar J, Martinez A, Ingram LO: **Effect of alcohol compounds found in hemicellulose hydrolysate on the growth and fermentation of ethanologenic *Escherichia coli*.** *Biotechnol Bioeng* 2000, **68:**524–530.
12. Fenske JJ, Griffin DA, Penner MH: **Comparison of aromatic monomers in lignocellulosic biomass prehydrolysates.** *J Ind Microbiol Biotechnol* 1998, **20:**364–368.
13. Klinke HB, Ahring BK, Schmidt AS, Thomsen AB: **Characterization of degradation products from alkaline wet oxidation of wheat straw.** *Bioresoure Technol* 2002, **82:**15–26.
14. Palmqvist E, Hahn-Hägerdal B: **Fermentation of lignocellulosic hydrolysates: II - inhibitors and mechanisms of inhibition.** *Biores Technol* 2000, **74:**25–33.
15. Schweigert N, Zehnder AJB, Eggen RIL: **Chemical properties of catechols and their molecular modes of toxic action in cells, from microorganisms to mammals.** *Environ Microbiol* 2001, **3**(2):81–91.
16. Boopathy R, Bokang H, Daniels L: **Biotransformation of furfural and 5-hydroxymethyl furfural by enteric bacteria.** *J Ind Microbiol* 1993, **11:**147–150.
17. Mills TY, Sandoval NR, Gill RT: **Cellulosic hydrolysate toxicity and tolerance mechanisms in *Escherichia coli*.** *Biotechnol Biofuels* 2009, **2:**26.
18. Rumbold K, van Buijsen HJJ, Overkamp KM, van Groenestijn JW, Punt PJ, van der Werf MJ: **Microbial production host selection for converting second-generation feedstocks into bioproducts.** *Microb Cell Fact* 2009, **8:**64.
19. Zaldivar J, Martinez A, Ingram LO: **Effect of selected aldehydes on the growth and fermentation of ethanologenic *Escherichia coli*.** *Biotechnol Bioeng* 1999, **65:**24–33.
20. Palmqvist E, Almeida JS, Hahn-Hägerdal B: **Influence of furfural on anaerobic glycolytic kinetics of *Saccharomyces cerevisiae* in batch culture.** *Biotechnol Bioeng* 1999, **62:**447–454.
21. Chandel AK, Silva S, Om VS: **Detoxification of lignocellulose hydrolysates: biochemical and metabolic engineering toward white biotechnology.** *Bioenerg Res* 2013, **6:**388–401.
22. Zaldivar J, Ingram LO: **Effect of organic acids on the growth and fermentation of ethanologenic *Escherichia coli* LY01.** *Biotechnol Bioeng* 1999, **66:**203–210.

23. Palmqvist E, Grage H, Meinander NQ, Hahn-Hägerdal B: **Main and interaction effects of acetic acid, furfural, and p-hydroxybenzoic acid on growth and ethanol productivity of yeasts.** *Biotechnol Bioeng* 1999, **63:**46–55.
24. Jensen PR, Hammer K: **Minimal requirements for exponential growth of *Lactococcus lactis*.** *Appl Environ Microbiol* 1993, **59:**4363–4366.
25. Bringel F, Frey L, Boivin S, Hubert JC: **Arginine biosynthesis and regulation in *Lactobacillus plantarum*: the carA gene and the argCJBDF cluster are divergently transcribed.** *J Bacteriol* 1997, **79:**2697–2706.
26. Canilha L, Carvalho W, Felipe M, Silva JB, Giulietti M: **Ethanol production from sugarcane bagasse hydrolysate using *Pichia stipitis*.** *Appl Biochem Biotechnol* 2010, **161:**84–92.
27. Nigam JN: **Ethanol production from wheat straw hemicellulose hydrolysate by *Pichia stipitis*.** *J Biotechnol* 2001, **87:**17–27.
28. Qian M, Tian S, Li X, Zhang J, Pan Y, Yang X: **Ethanol production from dilute-acid softwood hydrolysate by co-culture.** *Appl Biochem Biotechnol* 2006, **134:**273–284.

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Chapter 3

Media optimization for *Pediococcus acidilactici* DSM 20284

3.1. Introduction

Lactic Acid Bacteria (LAB) are industrially relevant microorganisms widely used in starter cultures for food fermentations, especially for dairy products, where they play a role in acidification of the product, prolonging its shelf life and influencing its texture and flavor (Leroy and De Vuyst, 2004). In addition, LAB are increasingly used for the production of flavor compounds, vitamins or exopolysaccharides (Teusink et al., 2005). LAB are also known to be fastidious organisms with complex nutritional requirements. They are usually grown in complex media that support rapid growth to high cell densities, such as M17 for Lactococci (Terzaghi and Sandine, 1975) or MRS for Lactobacilli and Pediococci (De Man et al., 1960). MRS is a rich medium consisting of several complex ingredients (yeast extract, meat extract and casein peptone) and therefore its exact chemical composition might differ slightly from batch to batch, resulting in irreproducible growth conditions. In contrast, a chemically defined medium offers reproducibility of chemical composition (Hayek and Ibrahim, 2013), and avoids unnecessary excess of nutrients (Møretrø et al., 1998), being therefore suitable for use in physiological, metabolic and nutritional studies (Hayek and Ibrahim, 2013; Zhang et al., 2009). Chemically defined media usually do not support as high-cell-density growth as the complex media (Zhang et al., 2009); they should, however, support the microorganism's exponential growth at a reasonably high rate (Foucaud et al., 1997).

The formulation of a chemically defined medium for LAB is challenging due to several reasons. Firstly, LAB are fastidious microorganisms and have numerous nutritional requirements, including multiple auxotrophies with respect to amino acids and vitamins (Møretrø et al., 1998; O'Sullivan et al., 2009). Secondly, the nutritional requirements can vary remarkably between the individual species and even strains (Hayek and Ibrahim, 2013; Morishita et al., 1981; Saguir and De Nadra, 2007). This can be attributed to the fact that LAB are isolated from and naturally adapted to a variety of different niches, from wine, milk, silages, soil, plants to human and animal gastrointestinal tracts or oral cavities. Many of LAB natural habitats are rich in nutrients which explains the abundance of amino acid and vitamin auxotrophies among LAB.

Many chemically defined media has been developed for different LAB species, among others for *L. plantarum* (Bringel et al., 1997; Saguir and De Nadra, 2007; Teusink et al., 2005; Wegkamp et al., 2010), *L. casei* (Morishita et al., 1974), *L. delbrueckii* subsp. *bulgaricus* (Chervaux et al., 2000), *L. helveticus* (Torino et

al., 2005), *L. fermentum* (Fukuda et al., 2010), *Lactococcus lactis* (Jensen and Hammer, 1993) and *Leuconostoc mesenteroides* (Foucaud et al., 1997).

Earlier investigations (Chapter 2) showed that the growth of *P. acidilactici* was not supported by defined media developed for other LAB: either by SA medium, developed for *Lactococcus lactis* (Jensen and Hammer, 1993), or by DLA medium, developed for *Lactobacillus plantarum* (Bringel et al., 1997). There is very limited data on the nutritional requirements or defined media development for *Pediococcus* species. This study aimed at developing a chemically defined medium supporting reasonable growth of *P. acidilactici* DSM 20284.

3.2. Materials and Methods

3.2.1. Strains employed in this study

For the development of a chemically defined medium, the strain *Pediococcus acidilactici* DSM 20284 was used. The strain was routinely cultivated in MRS medium at 30°C. Several other LAB strains were also employed in this study in order to test if the developed *Pediococcus* Defined Medium (PDM) supports their growth. These were several strains of *Lactobacillus* species: *L. acidophilus* DSM 20079, *L. alimentarius* DSM 20249, *L. brevis* LMG 19186, *L. brevis* DSM 20054, *L. buchneri* Ketchup-1, *L. casei* DSM 20011, *L. delbrueckii* subsp. *bulgaricus* DSM 20081, *L. equi* DSM 15833, *L. fermentum* DSM 20052, *L. helveticus* DSM 20075, *L. plantarum* KOG5, *L. plantarum* FOEB9106, *L. plantarum* JCL1280, *L. plantarum* JCL1285, *L. pentosus* DSMZ 20314T, *L. pentosus* 10-16, *L. reuteri* DSM 20016, *L. sakei* subsp. *sakei* DSM 20017, *L. salivarius* subsp. *salivarius* DSM 20555, *L. spicheri* DSM 15429 and all available *Pediococcus* species: *P. argentinicus* DSM 23026, *P. cellicola* DSM 17757, *P. claussenii* DSM 14800, *P. damnosus* DSM 20331, *P. ethanolidurans* DSM 22301, *P. inopinatus* DSM 20285, *P. lolii* DSM 19927, *P. parvulus* DSM 20332, *P. pentosaceus* ATCC 25745 and *P. stilesii* DSM 18001. The strains were grown at their optimal growth temperature (26°C, 28°C, 30°C or 37°C).

3.2.2. Defined medium

The defined medium was prepared from concentrated stock solutions of individual components. The stock solutions were filter-sterilized and stored at either 5°C or -20°C until use. After all ingredients were mixed, the pH of the medium was adjusted to 6.5±0.1 with 2M NaOH or 18.5% HCl, and the medium was sterilized by filtration (0.22 μm).

3.2.3. Growth tests

For the growth test and single omission experiments, the *P. acidilactici* strain was inoculated from an overnight culture into 10 mL volume of defined medium. When necessary, the cells from MRS overnight culture were harvested by centrifugation, washed three times and resuspended in sterile distilled water; this cell suspension was further used for inoculation. The growth of the strain was evaluated after 24 h of incubation at appropriate temperature. For the growth test of the other tested strains, single colonies were inoculated into the developed defined medium (PDM) from MRS plates.

For growth experiment, the strain was inoculated into 100 mL bottles containing 50 mL of PDM medium and grown at 30°C with 220 rpm stirring. The growth of the strain was followed by regular optical density (OD_{600}) measurements. The experiment was done in triplicates.

3.3. Results and Discussion

As a basis for development of a chemically defined medium for *P. acidilactici* DSM 20284, a CDM medium supporting the growth of another *P. acidilactici* strain (TISTR 425) was used. The CDM medium as described by Sriphochanart et al. (2011) did not support the growth of the strain used in this study. This finding indicates that the nutritional requirements of *Pediococci* are highly variable even between different strains of the same species. It's worth mentioning that the two *P. acidilactici* strains were isolated from different niches: TISTR 425 was isolated from fermented pork, while DSM 20284 was isolated from barley. Their various nutritional requirements might thus result from adaptation to the specific environments they lived in.

As mentioned before, neither SA nor DLA media could support the growth of the tested strain DSM 20284. Another medium formulation, found over the World Wide Web as a commercially offered medium[1] supporting the growth of *Lactobacilli* and proposed to be a defined equivalent of MRS medium, was also tested. Instead of the complex MRS ingredients (such as the meat extract, yeast extract and casein peptone), defined supplements and vitamins were used. The medium was slightly modified, and three of the ingredients were omitted during the test (DL-aminobutyric acid, cobalt sulphate and calcium lactate). No growth of the tested strain was observed in this media formulation. For a control, a strain of *L. pentosus* (LMG 17673) was also inoculated and showed very good growth in the defined MRS broth. Comparing the two media compositions (Table 3.1), it is evident that while the CDM contains extra nucleotides, the defined MRS broth contains glutamic acid instead of glutamine, and additionally aspartic acid as well as several vitamins. Moreover, several ingredients (Mn^{2+}, Mg^{2+}, amino acids and vitamins) were used in higher concentrations in the defined MRS broth. The next step was to supplement the basic CDM medium with all additional components used in the defined MRS broth formulation, as well as to increase the concentrations of several components. Adenine, guanine and uracil, present in the CDM medium in extremely low concentrations, were omitted as the new medium was additionally supplemented with 2'-deoxyuridine, cytidine and hypoxanthine which can be interconverted to the other purines. Adding all the missing ingredients (cytidine, 2'-deoxyadenosine, 2'-deoxyuridine, myo-inositol, cyanocobalamin, biotin, ascorbic acid, ammonium citrate, aspartic acid, glutamic acid, and additionally NaH_2PO_4 and hypoxanthine) resulted in a good growth of *P. acidilactici*. To further examine which of these nutrients are essential for growth, the single omission technique was applied where one of the ingredients of the chemically defined medium was omitted at a time. To avoid carry-over of essential nutrients, the cells were washed before inoculation. The conditions tested as well as the test results are summarized in Table 3.2.

Table 3.1. The composition of the basal CDM medium and the modified *Pediococcus* Defined Medium (PDM).

Compound	CDM (g/L)	Defined MRS broth (g/L)	PDM (g/L)
Glucose	10	10	10

[1] http://www.usbio.net/technicalSheet.php?item=L1021-04, accessed at 19.11.2015

Sodium acetate	2		10
Tween 80	1	1	1
Na_2HPO_4 x H20	1,75		1,75
KCl	0,75		0,75
$MnSO_4$ x H2O	0,0077	0,02	0,0200
$MgSO_4$ x 7H2O	0,2	0,5	0,5
Sodium gluconate	10		10
4-aminobenzoic acid	0,0004	0,0002	0,0004
Pyridoxal	0,000502	0,01	0,01
Folic acid	0,0002	0,0002	0,0002
Nicotinic acid	0,001	0,01	0,01
Calcium panthotenate	0,001	0,01	0,01
Riboflavin	0,001	0,01	0,01
Thiamine	0,001		0,001
Adenine	0,000005		
Guanine	0,00001	0,1	
Uracil	0,00001		
Glycine	0,05	0,2	0,05
Asparagine	0,05	0,1	0,05
Tryptophan	0,05	0,1	0,05
Serine	0,05	0,1	0,05
Alanine	0,05	0,2	0,05
Phenylalanine	0,05	0,1	0,05
Histidine	0,05	0,2	0,05
Isoleucine	0,05	0,1	0,05
Leucine	0,05	0,2	0,05
Methionine	0,05	0,1	0,05
Lysine	0,05	0,2	0,05
Proline	0,05	0,2	0,05
Threonine	0,05	0,1	0,05
Valine	0,05	0,1	0,05
Arginine	0,05	0,1	0,05
Tyrosine	0,05	0,1	0,05
Cysteine	0,05	0,1	0,05
Glutamine	0,05		0,05
Aspartic acid		0,3	0,05
Glutamic acid		0,3	0,05
Biotin		0,001	0,001
Cyanocobalamin (vit. B_{12})		0,00002	0,00002
Myo-Inositol		0,01	0,01
Ascorbic acid		0,5	0,5
2'-Deoxyadenosine		0,1	
2'-Deoxyuridine		0,1	0,1
Cytidine		0,1	0,1
Ammonium citrate		2	2
KH_2PO_4		1,5	
K_2HPO_4		3,1	
Potassium acetate		10	
NaCl		0,02	
NaH_2PO_4			0,85
Hypoxanthine			0,1

The deletion of individual ingredients from the medium revealed the requirement of *P. acidilactici* for thiamine and 16 amino acids, which were found to be essential for growth. Mn^{2+}, nicotinic acid, calcium panthotenate, riboflavin and glutamine were found to be stimulatory nutrients; they were not essential for growth, but their presence in the medium significantly improved it. Other vitamins and amino acids were demonstrated to be nonessential. These results are in agreement with previous reports, which demonstrated that riboflavin, panthotenic and nicotinic acids are important for growth of most LAB strains (Foucaud et al., 1997; Hayek and Ibrahim, 2013; Letort et al., 2001; Wegkamp et al., 2010). Mn^{2+} was shown to be essential or stimulatory for most organisms, including LAB (Foucaud et al., 1997; Hayek and Ibrahim, 2013). As mentioned earlier, data on *P. acidilactici* nutritional requirements is scarce; only single studies were performed. A previous report by Sakagughi (1960) suggested that *P. acidilactici* required riboflavin, pyridoxine, pantothenic acid, nicotinic acid and biotin as well as all amino acids apart from methionine. The findings of this study revealed thiamine to be essential for growth instead of biotin and pyridoxal, while the other mentioned vitamins were stimulatory for growth. Moreover, the DSM 20284 strain needed methionine, yet asparagine, alanine and glutamic acid were found nonessential. The differences between the findings of this and the earlier study emphasize the discrepancy of nutritional requirements of various *P. acidilactici* strains.

Table 3.2. Results of leave-one-out experiment.

Omitted nutrient	Concentration (g/L)	Growth	Omitted nutrient	Concentration (g/L)	Growth
Sodium acetate	10	+	2'-deoxyadenosine	0,1	+
Tween 80	1	+	2'-deoxyuridine	0,1	+
Na_2HPO_4 x H_20	1,75	+	Glycine	0,05	-
NaH_2PO_4	0,85	+	Asparagine	0,05	+
KCl	0,75	+	Tryptophan	0,05	-
$MnSO_4$ x H_2O	0,0200	±	Serine	0,05	-
$MgSO_4$ x $7H_2O$	0,5	+	Alanine	0,05	+
Sodium gluconate	10	+	Phenylalanine	0,05	-
Ammonium citrate dibasic	2	+	Histidine	0,05	-
4-aminobenzoic acid	0,0004	+	Isoleucine	0,05	-
Pyridoxal	0,01	+	Leucine	0,05	-
Folic acid	0,0002	+	Methionine	0,05	-
Nicotinic acid	0,01	±	Lysine	0,05	-
Calcium panthotenate	0,01	±	Proline	0,05	-
Riboflavin	0,01	±	Threonine	0,05	-
Thiamine	0,001	-	Valine	0,05	-
Myo-inositol	0,01	+	Arginine	0,05	-
Cyanocobalamin	0,00002	+	Tyrosine	0,05	-
Biotin	0,001	+	Cysteine	0,05	-
Ascorbic acid	0,5	+	Glutamine	0,05	±
Hypoxanthine	0,1	+	Aspartic acid	0,05	-
Guanine	0,1	+	Glutamic acid	0,05	+
Cytidine	0,1	+	All ingredients (control)		+

+, good growth; ±, weak growth; -, no growth

The *P. acidilactici* was then also tested for growth in a minimal medium, which contained only the essential ingredients; however, no growth was observed. The experiment was repeated with a medium containing all essential as well as stimulatory nutrients; still no growth was detected. That implies that there are more ingredients essential for the growth of *P. acidilactici* that were not uncovered during the single omission experiment. This can be due to the fact that e.g. the different purines can be interconverted inside the cell, and therefore an experiment omitting all the purines at once rather than single-omission experiment would be more suitable for determining the strain's requirements for purines. Likewise, some vitamins can replace each other, which was not taken into account in this study. Furthermore, the cultures might require being subcultivated into the new medium a few times, which is especially important for investigation of the vitamin requirements; this was, however, not done in this study. Yet, it is important to keep in mind, that serial subcultivation can result in adaptation to the new medium deficient of an essential nutrient, which can lead to a wrong conclusion (Hayek and Ibrahim, 2013).

The medium containing all 44 ingredients could support good growth of the tested strain and was designated as *Pediococcus* Defined Medium (PDM). A growth experiment was performed in order to investigate the strain growth rate and final growth yield in the defined PDM medium. The experiment was done in triplicates; the growth rate was found to be equal to 0.416±0.003 h^{-1} which corresponds to a generation time of 100 min. The medium supported exponential growth until OD_{600} of around 1.1. The final optical density was also observed and was found to be equal to 1.4. For comparison, in MRS medium, the strain demonstrated a growth rate of 0.654±0.00 h^{-1} and a final OD_{600} of 6.9.

Since the PDM medium supported good growth of the tested *P. acidilactici* strain, several other LAB strains were also tested for their growth in this defined medium. Several species of Lactobacilli and all available Pediococci species were tested. The strains selected for the test were shown earlier (Chapter 2) to exhibit no growth on GSA or DLA media, or both. The growth tests were done in 10mL volume of PDM medium. As it can be seen in Table 3.3, the newly developed PDM medium is able to support the growth of all Pediococci species and several fastidious Lactobacilli that showed no growth in earlier developed defined media. The PDM medium is therefore superior to the other media tested and can perhaps be used as a general defined medium for the growth of various LAB. It is worth mentioning that many of the Lactobacilli that were tested are industrially relevant organisms used as probiotics or employed in food fermentations (Giraffa et al., 2010; Hammes and Hertel, 1998; Kim et al., 2014; Ramos et al., 2013).

Table 3.3. The growth of the tested strains in PDM medium. For comparison, the growth in MRS, DLA and GSA media is also reported.

Species	Strain	MRS	DLA	GSA	PDM
Lactobacillus acidophilus	DSM 20079	+	-	-	±
Lactobacillus alimentarius	DSM 20249	+	-	-	+
Lactobacillus brevis	DSM 20054	+	-	+	+
Lactobacillus brevis	LMG 19186	+	-	-	+
Lactobacillus buchneri	Ketchup-1	+	+	-	+
Lactobacillus casei	DSM 20011	+	-	+	+
Lactobacillus delbrueckii subsp. bulgaricus	DSM 20081	+	-	-	-
Lactobacillus equi	DSM 15833	+	-	±	+
Lactobacillus fermentum	DSM 20052	+	-	+	±

Lactobacillus helveticus	DSM 20075	+	-	-	-
Lactobacillus pentosus	10-16	+	+	+	+
Lactobacillus pentosus	DSMZ 20314T	+	+	+	+
Lactobacillus pentosus	LMG 17673	+	+	+	+
Lactobacillus plantarum	FOEB9106	+	-	+	+
Lactobacillus plantarum	JCL1280	+	-	-	+
Lactobacillus plantarum	JCL1285	+	-	-	+
Lactobacillus plantarum	KOG5	+	-	-	+
Lactobacillus reuteri	DSM 20016	+	-	-	+
Lactobacillus sakei subsp. sakei	DSM 20017	+	-	+	+
Lactobacillus salivarius subsp. salivarius	DSM 20555	+	-	-	+
Lactobacillus spicheri	DSM 15429	+	-	-	+
Pediococcus argentinicus	DSM 23026	+	-	-	+
Pediococcus cellicola	DSM 17757	+	-	-	+
Pediococcus claussenii	DSM 14800	+	-	+	+
Pediococcus damnosus	DSM 20331	±	-	-	±
Pediococcus ethanolidurans	DSM 22301	+	-	-	+
Pediococcus inopinatus	DSM 20285	+	-	-	+
Pediococcus lolii	DSM 19927	+	-	-	+
Pediococcus parvulus	DSM 20332	+	-	-	+
Pediococcus pentosaceus	ATCC 25745	+	-	-	+
Pediococcus stilesii	DSM 18001	+	-	+	+

+, good growth; ±, weak growth; -, no growth

3.4. Conclusions

The medium developed in this study was shown to support growth of *P. acidilactici* DSM 20284 with a reasonable growth rate (0.416±0.003 h^{-1}). The medium allowed exponential growth of the strain until OD_{600} of around 1.1. The growth experiments gave reproducible results. Single omission experiments revealed that thiamine and 16 amino acids were essential nutrients while Mn^{2+}, nicotinic acid, calcium panthotenate, riboflavin and glutamine were stimulatory for growth of the *P. acidilactici* strain. However, to investigate the vitamin requirements in more details, the strain should be several times subcultivated into the new medium; similarly for purines, an additional multiple-omission experiment should be performed in order to determine which of the purines are essential for growth.

The chemically defined medium PDM (*Pediococcus* defined medium) containing 44 ingredients was shown to support the growth of all other Pediococci species, as well as several fastidious Lactobacilli. Therefore, the PDM medium proved to be suitable for a variety of LAB species and can therefore be very useful in performing physiological, biochemical or nutritional investigations in Pediococci and other LAB species.

3.5. References

Bringel, F., Frey, L., Boivin, S., Hubert, J.C., 1997. Arginine biosynthesis and regulation in *Lactobacillus plantarum*: the *carA* gene and the *argCJBDF* cluster are divergently transcribed. J. Bacteriol. 179, 2697–2706.

Chervaux, C., Ehrlich, S.D., Maguin, E., 2000. Physiological Study of *Lactobacillus delbrueckii* subsp. *bulgaricus* Strains in a Novel Chemically Defined Medium. Appl. Environ. Microbiol. 66, 5306–5311.

De Man, J.C., Rogosa, M., Sharpe, M.E., 1960. A medium for the cultivation of Lactobacilli. J. Appl. Bacteriol. 23, 130–135.

Foucaud, C., Francois, A., Richard, J., 1997. Development of a Chemically Defined Medium for the Growth of *Leuconostoc mesenteroides*. Appl. Environ. Microbiol. 63, 301–304.

Fukuda, K., Shi, T., Nagami, K., Leo, F., Nakamura, T., Yasuda, K., Senda, A., Motoshima, H., Urashima, T., 2010. Effects of carbohydrate source on physicochemical properties of the exopolysaccharide produced by *Lactobacillus fermentum* TDS030603 in a chemically defined medium. Carbohydr. Polym. 79, 1040–1045.

Giraffa, G., Chanishvili, N., Widyastuti, Y., 2010. Importance of lactobacilli in food and feed biotechnology. Res. Microbiol. 161, 480–487.

Hammes, W.P., Hertel, C., 1998. New developments in meat starter cultures. Meat Sci. 49, 125–138.

Hayek, S.A., Ibrahim, S.A., 2013. Current Limitations and Challenges with Lactic Acid Bacteria: A Review. Food Nutr. Sci. 4, 73–87.

Jensen, P.R., Hammer, K., 1993. Minimal Requirements for Exponential Growth of *Lactococcus lactis*. Appl. Environ. Microbiol. 59, 4363–4366.

Kim, Y.-W., Jeong, Y.-J., Kim, A.-Y., Son, H.-H., Lee, J.-A., Jung, C.-H., Kim, C.-H., Kim, J., 2014. *Lactobacillus brevis* Strains from Fermented Aloe vera Survive Gastroduodenal Environment and Suppress Common Food Borne Enteropathogens. PLoS One 9, e90866.

Leroy, F., De Vuyst, L., 2004. Lactic acid bacteria as functional starter cultures for the food fermentation industry. Trends Food Sci. Technol. 15, 67–78.

Letort, C., Letort, C., Juillard, V., Juillard, V., 2001. Development of a minimal chemically-defined medium for the exponential growth of *Streptococcus thermophilus*. J. Appl. Microbiol. 91, 1023–1029.

Morishita, T., Deguchi, Y., Yajima, M., Sakurai, T., Yura, T., 1981. Multiple Nutritional Requirements of Lactobacilli: Genetic Lesions Affecting Amino Acid Biosynthetic Pathways. J. Bacteriol. 148, 64–71.

Morishita, T., Fukada, T., Shirota, M., Yuira, T., 1974. Genetic Basis of Nutritional Requirements in *Lactobacillus casei*. J. Bacteriol. 120, 1078–1084.

Møretrø, T., Hagen, B.F., Axelsson, L., 1998. A new, completely defined medium for meat Lactobacilli. J. Appl. Microbiol. 85, 715–722.

O'Sullivan, O., O'Callaghan, J., Sangrador-Vegas, A., McAuliffe, O., Slattery, L., Kaleta, P., Callanan, M., Fitzgerald, G.F., Ross, R.P., Beresford, T., 2009. Comparative genomics of lactic acid bacteria reveals a niche-specific gene set. BMC Microbiol. 9, 50.

Ramos, C.L., Thorsen, L., Schwan, R.F., Jespersen, L., 2013. Strain-specific probiotics properties of *Lactobacillus fermentum*, *Lactobacillus plantarum* and *Lactobacillus brevis* isolates from Brazilian food products. Food Microbiol. 36, 22–29.

Saguir, F.M., De Nadra, M.C.M., 2007. Improvement of a Chemically Defined Medium for the Sustained Growth of *Lactobacillus plantarum*: Nutritional requirements. Curr. Microbiol. 54, 414–418.

Sakagughi, K., 1960. Vitamin and Amino Acid Requirements of *Pediococcus soyae* and *Pediococcus acidilactici* Kitahara's Strain. Bull. Agric. Chem. Soc. Japan 24, 638–643.

Sriphochanart, W., Skolpap, W., Scharer, J.M., Moo-young, M., Douglas, P.L., 2011. Effect of amino acid requirements on the growth and lactic acid production of *Pediococcus acidilactici* culture. African J. Microbiol. Res. 5, 3815–3822.

Terzaghi, B.E., Sandine, W.E., 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. 29, 807–813.

Teusink, B., van Enckevort, F.H.J., Francke, C., Wiersma, A., Wegkamp, A., Smid, E.J., Siezen, R.J., 2005. In Silico Reconstruction of the Metabolic Pathways of *Lactobacillus plantarum*: Comparing Predictions of Nutrient Requirements with Those from Growth Experiments. Appl. Environ. Microbiol. 71, 7253–7262.

Torino, M.I., Mozzi, F., Font De Valdez, G., 2005. Exopolysaccharide biosynthesis by *Lactobacillus helveticus* ATCC 15807. Appl. Microbiol. Biotechnol. 68, 259–265.

Wegkamp, A., Teusink, B., de Vos, W.M., Smid, E.J., 2010. Development of a minimal growth medium for *Lactobacillus plantarum*. Lett. Appl. Microbiol. 50, 57–64.

Zhang, G., Mills, D. a, Block, D.E., 2009. Development of Chemically Defined Media Supporting High-Cell-Density Growth of Lactococci, Enterococci, and Streptococci. Appl. Environ. Microbiol.

Chapter 4

Optimization of the transformation method for *Pediococcus acidilactici* DSM 20284

4.1. Introduction

It is highly desirable that industrial strains can be genetically manipulated, either to introduce desirable metabolic characteristics or novel phenotypes or to remove unwanted traits (Shareck et al., 2004). As the lactic acid bacteria are industrially important group of microorganisms, there is substantial interest to improve their properties for biotechnological applications. The lack of efficient methods for the introduction of heterologous DNA into Lactobacilli and Pediococci, however, hampered both the industrial use and the research on many species.

The Gram positive bacteria possess a thick cell wall made of several layers of peptidoglycan which works as a barrier to transforming DNA, and they are therefore more difficult to transform when compared to the Gram negatives (Powell et al., 1988; Trevors et al., 1992). The main challenge in the transformation procedure is to make the cell wall penetrable for the plasmid DNA without losing the cell viability. Glycine and DL-threonine are the most commonly used cell-weakening agents that are shown to enhance the transformability of a range of different species, including *Lactobacillus*, *Pediococcus* and other LAB strains (Buckley et al., 1999; Caldwell et al., 1996; David et al., 1989; Helmark et al., 2004; Holo and Nes, 1989; Kim et al., 1992; Mason et al., 2005; Thompson and Collins, 1996). However, cell-wall degrading enzymes or β-lactam antibiotics, such as ampicillin were also employed as cell-weakening agents (Aune and Aachmann, 2010; Bonnassie et al., 1990). The various strains can differ in their tolerance levels to these additives, and thus optimal concentrations of these agents are strain- dependent and have to be found separately for each strain. Often, to increase the viability of the cells, there are osmoprotectants added to the medium along with the cell-weakening agents that can help to increase the transformation efficiency (Heravi et al., 2012; Mason et al., 2005).

Due to a high degree of heterogeneity within the *Lactobacillus* and *Pediococcus* genera, the protocols need to be optimized for each microorganism, as the different species and even strains can vary considerably in the cell wall morphology, endonuclease restriction-modification systems or the amount of extracellular nucleases (Alegre et al., 2004; Aune and Aachmann, 2010; Rixon and Warner, 2003). That means that an optimized method resulting in high transformation efficiencies for particular strain, might not work effectively for another one. Nonetheless, the successful transformation depends both on the strain and plasmid vector employed (Kim et al., 2005; Rixon and Warner, 2003; Serror et al., 2002).

Over the years a lot of effort was put into developing techniques that could allow efficient transformation; numerous methods have been published for both Lactobacilli and Pediococci (Alegre et al., 2004; Berthier et al., 1996; Bringel and Hubert, 1990; Kim et al., 1992, 2005; Palomino et al., 2010; Rodríguez et al., 2007; Serror et al., 2002). Yet, the efficiencies obtained for Pediococci were low, namely 10^2-10^3 transformants per µg DNA used (Caldwell et al., 1996; Kim et al., 1992). The optimized method proposed by Rodríguez et al. (2007) managed to increase these values by 1-log unit; the method, however, did not yield the same efficiencies for the strain used in this study. Recently, Landete et al. (2014) reported an improved method allowing for the electrotransformation of several LAB species, including *Pediococcus acidilactici*, for which the achieved efficiencies were as high as $1.6 \cdot 10^5$. Nonetheless, it has been published after the work described in this chapter was performed.

This chapter describes an attempt to optimize the transformation protocol for *P. acidilactici* DSM 20284 strain. A number of parameters have been reported to influence the transformation efficiency. Among others: the growth conditions, composition of the washing and electroporation buffers, the growth phase at which the cells are harvested, and electroporation conditions. To determine the optimal conditions, the effects of modifications of the above parameters were evaluated.

4.2. Materials and Methods

4.2.1. Bacterial strains, plasmids and growth conditions

E.coli MC1061 was grown in LB medium at 37°C with shaking. For selection purposes, LB supplemented with 150 µg/mL erythromycin was used. *Lactococcus lactis* MG1363 was grown in GM17 at 28°C. *Pediococcus acidilactici* was grown in MRS (Oxoid) at 30°C or 37°C depending on the protocol, without shaking. For solid medium, 10 g/L of Bacto agar was added to MRS broth. For selection of transformants, MRS plates were supplemented with 10 µg/mL erythromycin.

The plasmid used in this study was pG$^+$host4, a thermosensitive derivative of pGK12 with erythromycin resistance gene (Maguin et al., 1992). The thermosensitivity mutations of pG$^+$host4 were reversed in this study and a temperature-resistant version of the plasmid was obtained. The WT mutations were re-introduced by PCR reaction with pGH4ts-fwd and –rev primers. PCR amplification was performed using Phusion polymerase (Thermo Fisher Scientific). The PCR product was purified with the GFX PCR DNA and gel band purification kit (GE Healthcare). The PCR product was then phosphorylated by a T4 polynucleotide kinase (Thermo Fisher Scientific) according to the manufacturer's protocol. T4 DNA Ligase (Thermo Fisher Scientific) was used for self-circularization of linear DNA. The ligation mix was then electroporated into *E.coli* MC1061. The thermo-resistant derivative of the pG$^+$host4 plasmid was called pAMB1 and was then isolated from *E.coli* and its sequence was verified by sequencing (Macrogen). The plasmid pAMB1 was then used for electroporation into *L.lactis* MG1363 as described by Holo and Nes (1995), with the difference in plates used for selection of transformants (GM17 supplemented with 1% NaCl and 5µg/mL erythromycin). The plates were incubated at both 28°C and 37°C.

Primers used in the study are shown in Table 4.1.

Table 4.1. Primers used in the study. Forward primer covers all mutated region (4 bases replaced with WT sequence – shown in red); reverse primer starts exactly at the same place as the forward primer, but goes in the opposite direction).

Primer name	Sequence (5'-3')
pGH4ts-fwd	GGAATAGTAGTGATGTTATACGAAATGGAAAGCACTAT
pGH4ts-rev	ATGTATCTTTATCTTTTTTTTCGTCCATATCGTGTAA

4.2.2. Extraction of plasmid DNA

Plasmids were purified from *E.coli* by Zyppy Plasmid Miniprep Kit (Zymo Research) according to the protocol supplied by the manufacturer. For isolation of plasmids from *L. lactis* or *P. acidilactici*, the cells were harvested by centrifugation, resuspended in 500 µL TNEX buffer containing 20 mg/mL lysozyme and incubated for 20 min at 37°C. Before proceeding with Zyppy Plasmid Miniprep Kit, the cells were harvested by centrifugation and resuspended in 600 µL TE buffer.

4.2.3. Initial electroporation protocols

Six electrotransformation protocols from literature were used for initial transformability tests (Table 4.2). The methods were published either for Lactobacilli (Heravi et al., 2012; Leer et al., 1992; Posno et al., 1991; Ye et al., 2013) or Pediococci (Caldwell et al., 1996; Rodríguez et al., 2007).

Table 4.2. Summary of initial transformability test methods and buffers.

Method	Reference	Competent medium	Washing buffer	Electroporation buffer	Recovery medium	Harvest OD_{600}
1	Rodríguez et al., 2007[1]	MRS with 40mM DL-threonine	0.6M sucrose, 7mM K_2HPO_4 1mM $MgCl_2$ pH 7.5	0.6M sucrose, 7mM K_2HPO_4 1mM $MgCl_2$ pH 7.5	MRS with 0.5M sucrose	1.0-1.2
2	Caldwell et al., 1996[2]	MRS with 0.5M sorbitol, 3% glycine and 40mM DL-threonine	0.5M sorbitol 10% glycerol	0.5M sorbitol 1mM K_2HPO_4 1mM $MgCl_2$ pH 7.0	MRS with 0.5M sorbitol, 20mM $MgCl_2$, 2mM $CaCl_2$	0.4-0.6
3	Heravi et al., 2012	MRS with 2% glycine and 0.5M sucrose	ice-cold H_2O ice-cold EDTA ice-cold H_2O	0.3M sucrose	MRS	0.6
4	Leer et al., 1992	MRS with 20mM DL-threonine	RT H_2O	30% PEG	MRS	0.5-1.0
5	Posno et al., 1991	MRS with 1% glycine	15mM NaH_2PO_4, 3mM $MgCl_2$ (ice-cold)	ice-cold 0.9M sucrose, 15mM NaH_2PO_4, 3mM $MgCl_2$	MRS	0.6
6	Ye et al., 2013	MRS with 0.7M NaCl	ice-cold H_2O	ice-cold H_2O	MRS	2.0

[1]The protocol contains an additional step: lysozyme treatment with 2000U/mL lysozyme, incubation time 20 min at 37°C

[2]Inoculated from an overnight culture in MRS supplemented with 0.5M sorbitol

Competent cells were prepared by growing the strain in an appropriate competent medium until OD_{600} of 0.4-2.0, depending on the method applied. The cells were grown at 30°C (Rodríguez et al., 2007) or at 37°C

(Caldwell et al., 1996; Heravi et al., 2012; Leer et al., 1992; Posno et al., 1991; Rodríguez et al., 2007; Ye et al., 2013). After harvesting by centrifugation, the cells were washed three times by appropriate washing buffers and then resuspended in an electroporation buffer. 50-100 µl of resuspended cells was mixed with 0.3-5 µg of plasmid DNA, and then transferred into pre-chilled electroporation cuvettes (2-mm inter-electrode gap). Electrotransformation was carried out by applying a single pulse at 1.4-2.5 kV in a MicroPulser^{TM} apparatus (Bio-Rad Laboratories). Immediately after the pulse, the cells were recovered in an appropriate recovery medium and then incubated at either 30°C or at 37°C for 1.5-3 h. The cells were afterwards plated on MRS plates supplemented with 10 µg/mL erythromycin for 2-5 days. The transformation efficiency is expressed as the number of transformants per µg of DNA. The experiments were done in one replicate.

4.2.4. An improved protocol for *P. acidilactici* DSM 20284 transformation

For the preparation of competent cells, an overnight culture of *P. acidilactici* was used to inoculate 50 mL MRS containing 0.7M NaCl (1:50 dilution). The cells were grown at 37°C until they reached an OD_{600} of around 1.1 when they were placed on ice for 10 min. Subsequently, the cells were harvested by 10 min centrifugation at 4000 rpm at 4°C, washed three times with ice-cold de-ionized (DI) water and concentrated in 1 mL DI water. 50 µL aliquots of resuspended cells were made and either used directly for electroporation or stored at -80°C until use. For electroporation, the competent cells were mixed with 1.1 µg plasmid DNA, transferred to a pre-chilled 2-mm gap electroporation cuvette, and electroporated at a voltage of 3 kV. Afterwards the cells were allowed to recover in 1 mL MRS medium for 2 hours at 37°C. The cells were then plated on MRS plates with 10 µg/mL erythromycin for 2-3 days.

4.3. Results and Discussion

4.3.1. Reversing thermosensitivity mutations

The broad-range plasmid pG^{+}host4, a thermosensitive derivative of pGK12, containing a thermosensitive replicon was used in this study (Maguin et al., 1992). The plasmid can be transformed and maintained at the permissive temperature (28°C), however, after increasing the temperature to 37°C, its replication is inhibited and it is forced to integrate into the chromosome. Despite of the obvious advantages that the thermosensitivity gives (e.g. allowing low-frequency events such as integration in poorly transformable bacteria)(Biswas et al., 1993), however, the plasmid does not work efficiently in lactobacilli and pediococci as the permissive temperature is not optimal for growth of these strains (Kullen and Klaenhammer, 2000). Therefore, by reversing the thermosensitivity mutations in the replicon, a temperature-resistant variant was made that could replicate also at 37°C. The thermosensitivity was caused by four point mutations in the *repA* gene; all being transitions from G to A. Every base alteration caused an amino acid change (Maguin et al., 1992); as it is shown in Figure 5. The WT mutations were re-introduced by a PCR reaction; a T4 polynucleotide kinase was then employed for phosphorylation, and T4 DNA ligase was used for self-circulization. The modified plasmid was called pAMB1. It was electroporated into *E. coli* and propagated at 37°C. The plasmid was also transformed successfully into *L. lactis* at both 28°C and 37°C.

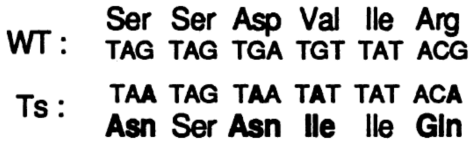


Figure 4.1. The WT and TS regions on the pG$^+$host4 replicon (Maguin et al., 1992).

4.3.2. Initial transformability tests

Initially, *P. acidilactici* strain was tested for its ability to undergo genetic transformation using 6 different methods (Table 4.2). The methods were diverse both with regard to the protocols for preparation of competent cells, but also with regard to the electroporation parameters. Glycine or DL-threonine or a combination of both, were used as cell-weakening agents either with or without the help of sucrose or sorbitol as osmotic stabilizers. Different washing and electroporation buffers were employed, containing sucrose, sorbitol, salts, polyethylene glycol (PEG) or ice-cold H_2O. Regarding the electroporation parameters, in most methods 0.5 µg plasmid DNA was used, however the amounts ranged from 0.3 to 5 µg DNA. The voltages applied were between 1.4 to 2.5 kV and time for expression varied from 1.5 to 3 hours.

The transformation worked very poorly and the efficiencies were extremely low for all of the methods employed; though the highest efficiency was observed for the method of Ye et al. (2013) (Table 4.3). This method was therefore selected to serve as a basic protocol for further enhancement. Surprisingly, the method of Ye et al. (2013) was developed for *Lactobacillus plantarum*, not for *Pediococcus* spp. A method optimized for *P. acidilactici* but another strain, was evaluated as well, but did not show better results. This indicates that there is a considerable variation among different strains, and a method allowing for an efficient introduction of DNA for one strain might not work for another.

Table 4.3. Transformation efficiencies obtained by different protocols.

Method	Method used for	Transformation efficiency [transformants per µg DNA]
Rodríguez et al., 2007	*P. acidilactici*	10
Caldwell et al., 1996	*P. pentosaceus* *L. salivarius*	0
Heravi et al., 2012	*L. crispatus* *L.rhamnosus* *L. casei*	2
Leer et al., 1992	*L. pentosus* *L. plantarum*	3
Posno et al., 1991	*L. plantarum* *L. pentosus* *L. acidophilus* *L. fermentum* *L. brevis*	7
Ye et al., 2013	*L. pentosus*	34

4.3.3. Optimization of electroporation parameters

This study was aimed at developing an optimized protocol for transformation of *P. acidilactici* DSM 20284. As the success of transformation depends on a number of different parameters, all individual steps of the basic electroporation protocol were modified in order to identify conditions that would improve the transformation efficiency. A variety of modifications were attempted: variations in salt concentration in competent media, time of harvest, washing buffer, voltages applied, DNA amount used for electroporation, expression time, erythromycin concentration used for selection as well as the cuvette type. These conditions were evaluated for their effect on transformation efficiency, while the strain was electroporated with the same plasmid DNA (pAMB1). A summary of the conditions tested can be found in Table 4.4.

Table 4.4. Summary of the tested transformation parameters.

Transformation parameters	Range of parameters
Competent media	0.7M NaCl, 0.9M NaCl
Harvesting OD_{600}	1.1, 2.0, 3.4
Washing buffer	Ice-cold H_2O, ES[1]
Voltage [kV]	1.5, 2, 2.5, 3
Plasmid DNA amount [µg]	0.57, 0.76, 1.14, 1.70, 2.27
Expression time [h]	0, 1, 2, 3, 4, o/n
Erythromycin concentration [µg/mL]	5, 10, 15, 20, 30
Cuvette type	1-mm, 2-mm

[1]ES – electroporation solution (0.6M sucrose, 7mM K_2HPO_4, 1mM $MgCl_2$, pH 7.5)

4.3.4. The effect of variations in electroporation parameters

4.3.4.1. The effect of DNA amount

Five different amounts of plasmid DNA were used: 0.57 µg, 0.76 µg, 1.14 µg, 1.70 µg and 2.27 µg. In general, the DNA amount did not seem to have a significant impact on the transformation efficiencies. However, a slight enhancement of the protocol could be observed while using 1.14 µg plasmid DNA. Using more than 1.14 µg of transforming DNA lowered the transformation efficiency, as illustrated in Figure 4.2A.

4.3.4.2. The effect of voltage

The electrical conditions used for electroporation are an important factor. It was shown that higher field strengths can yield more transformants and increase the transformation efficiency (Rixon and Warner, 2003). Four different voltages were tested for their impact on the transformation efficiency: 1.5 kV, 2.0 kV, 2.5 kV and 3 kV. Lowering the voltage used for electroporation to 1.5 kV yielded only 1 transformant. Setting the voltage at 2 kV had a similar effect as the initial condition 2.5 kV. Yet, increasing the voltage to 3 kV showed a 2-fold increase in efficiency when compared to 2 kV or 2.5 kV (Figure 4.2B).

4.3.4.3. The effect of expression time

After electroporation, the *Pediococcus* strain was either directly plated on MRS plates supplemented with 10 µg/mL erythromycin, or allowed to recover during incubation at 37°C for 1 hour, 2 hours, 3 hours, 4 hours, or o/n. First of all, when the cells were directly plated on selection plates, no transformants were obtained. The efficiency was lowered also when the strains were allowed an o/n incubation time. The optimal expression time was found to be 2 hours and it caused an increase in the efficiency of transformation of more than 4-fold (Figure 4.2C).

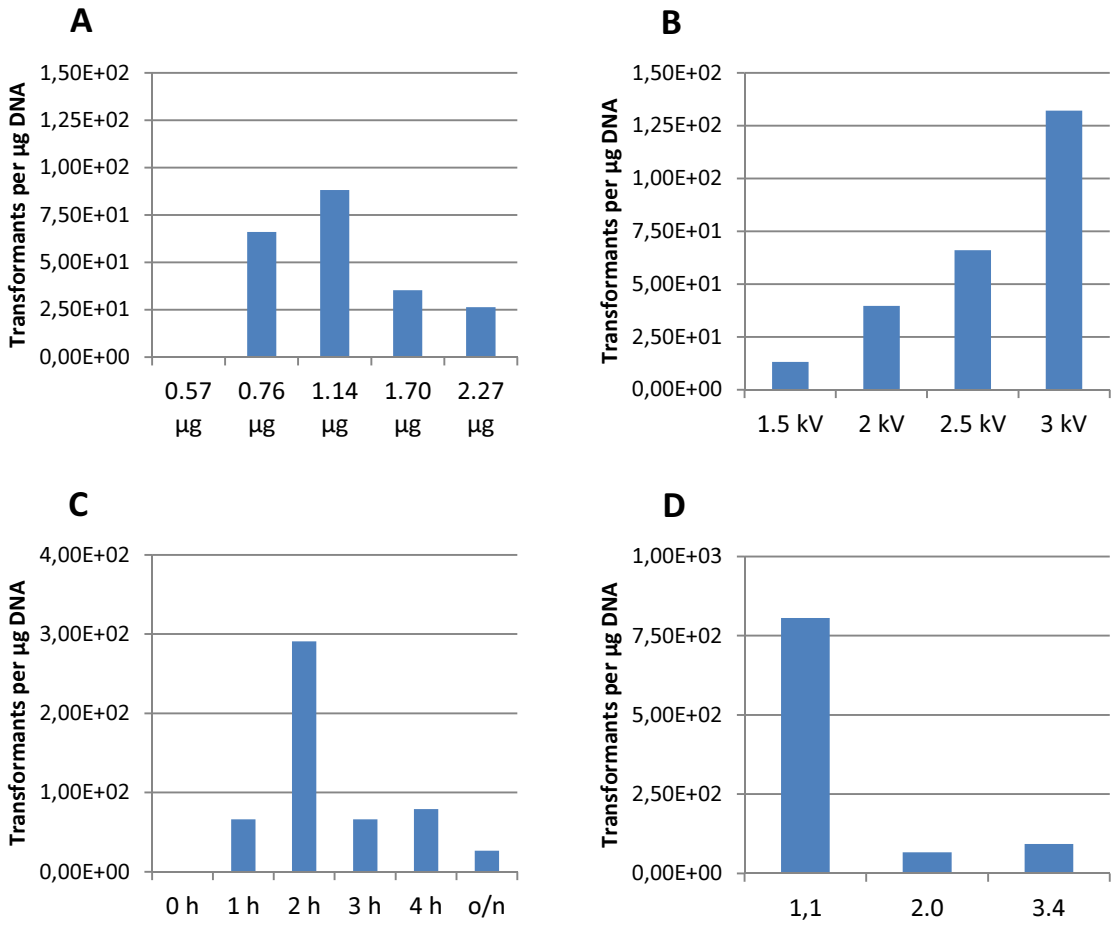


Figure 4.2. The effects of different parameters on the transformation efficiencies. Basic transformation protocol was used as described by Ye et al. (2013), apart from point D, were the harvesting OD_{600} varied. A) The effect of DNA amount. B) The effect of voltage used for electroporation. C) The effect of expression time. D) The effect of harvesting OD_{600}.

4.3.5. The effect of variations in competent media preparation protocol

4.3.5.1. The effect of salt concentration in competent media

In the transformation methods developed for Pediococci, the strains were grown in MRS with DL-threonine (method 1), MRS with sorbitol, glycine and DL-threonine (method 2) or MRS with glycine and sucrose (Landete et al., 2014). None of these, however, worked for the strain used in this study, as shown earlier in the initial transformability tests. The DL-threonine or glycine used as single MRS additives (methods 4, 5)

did not show better results either. It was found that a high salt concentration (method 6) can be used for a transformation that gives single transformants. An even higher salt concentration (Palomino et al., 2010) was then tested in order to examine its effect on the transformation efficiency. The cells were grown in MRS with 0.7M NaCl or 0.9M NaCl, and all the other conditions remained unchanged according to the protocol given by Ye et al. (2013). The salt concentration, however, has shown no effect on the transformation efficiency, and only a slightly negative effect on the growth rate. Therefore, it was decided to use 0.7M NaCl for further experiments.

4.3.5.2. The effect of harvesting OD_{600}

It was shown that the growth phase at which the cells are harvested, can also have a remarkable impact on the transformation efficiency. Moreover, the level of cell competency at the different growth phases was reported to be species- or even strain-dependent (Berthier et al., 1996). Certain species can be more susceptible to undergo transformation at a lower OD, but have higher survival rates during the electroporation at a higher OD; thus an optimum needs to be found (Aune and Aachmann, 2010).

The cells were harvested at OD_{600} of 1.1, 2.0 and 3.4. It was found that the transformation efficiency increased 10-fold when the cells were harvested at an early stationary phase (OD_{600} around 1.1). Harvesting the cells at an OD_{600} of 3.4 did not have a significant influence on the transformation efficiency. With the higher OD_{600} there are more cells, so in order to make the efficiencies comparable, the efficiency needs to be recalculated taking into account the cells' OD_{600}. Bearing this in mind, the transformation efficiencies appeared to be the same for both 2.0 and 3.4 OD_{600}. The results are illustrated in Figure 4.2D.

4.3.5.3. The effect of the washing buffer

The basic protocol employed ice-cold de-ionized water as a washing buffer. The effect of using another washing buffer was examined; the buffer to be tested was selected from the second-best transformation method from the initial transformability tests (method 1). Notably, the method was designed for another strain of *P. acidilactici*. The buffer contained 0.6M sucrose, 7 mM K_2HPO_4 and 1 mM $MgCl_2$ (pH 7.5). The transformation of competent cells washed in the electroporation solution, however, yielded no transformants.

4.3.6. The effect of other parameters

4.3.6.1. The effect of erythromycin concentration used for selection of transformants

The strains were plated on MRS supplemented with 5, 10, 15, 20, or 30 μg/mL erythromycin. The efficiency of transformation was the highest for selection at 10 or 15 μg/mL erythromycin (Figure 4.3A). It is worth noting that the transformation efficiencies in this experiment are higher when compared to the ones obtained with basic protocol, and this is because the competent cells used for this experiment were harvested at an OD_{600} of 1.1 and electroporated with previously found optimal parameters (1.14 μg plasmid DNA, 3 kV electric pulse and 2 hours expression time).

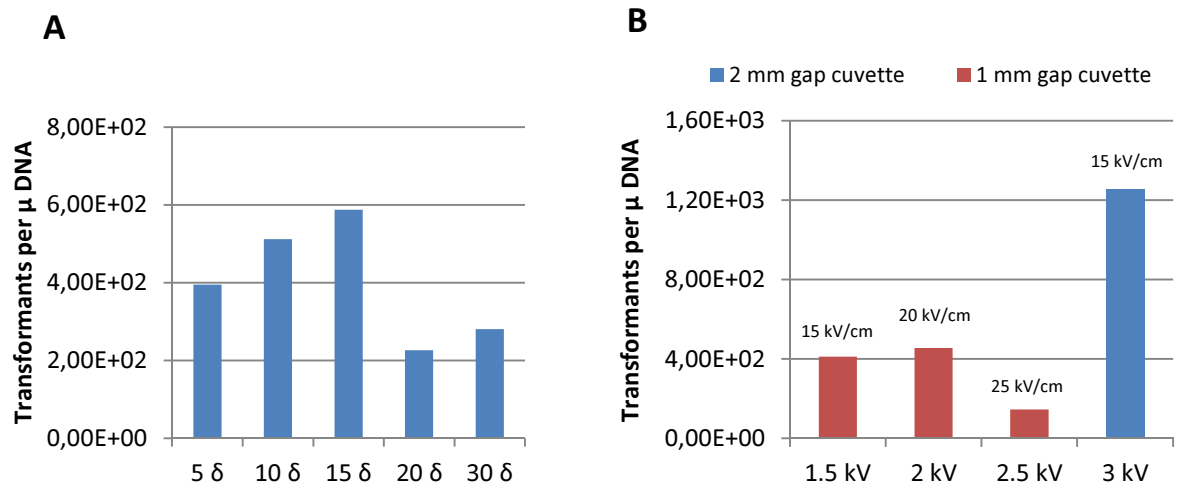


Figure 4.3. The effects of different parameters on the transformation efficiencies. Optimized transformation protocol was used for preparation of competent cells. A) The effect of erythromycin concentration used for selection of transformants. B) The effect of the type of cuvette used for electroporation at different voltages. Corresponding field strengths are given above the individual columns.

4.3.6.2. The effect of cuvette type

Either 1- or 2-mm gap cuvettes were used for electroporation at different voltages: 1.5 kV, 2kV and 2.5 kV. For 1 mm-gap cuvettes the respective field strengths are 15, 20 and 25 kV/cm; for 2-mm gap cuvettes – 7.5, 10, 12.5 kV/cm, respectively. The earlier found optimal voltage (3 kV with the use of a 2 mm gap cuvette) corresponed to a field strength of 15 kV/cm (Figure 4.3B). While using 1-mm gap cuvettes, the transformation efficiencies were lower when compared to the use of 2-mm gap cuvettes; therefore it was decided to continue using 2-mm gap cuvettes.

4.3.7. The optimal conditions

Based on the results obtained in this study, an improved electrotransformation protocol for the DSM 20284 strain was developed. Shortly, the competent cells were grown at 37°C in 50 mL MRS with 0.7M NaCl, harvested at OD_{600} of around 1.1, washed three times with ice-cold DI water and concentrated in 1 mL DI water. The cells were then mixed with 1.14 µg plasmid DNA and electroporated at 3 kV voltage. After the pulse, the cells were immediately resuspended in 1 mL MRS and incubated at 37°C for 2 hours. This setting increased the transformation efficiency from $6.6 \cdot 10^1$ to $1.3 \cdot 10^3$, as depicted in Figure 4.4. The variations in the single parameters caused increasing transformation efficiencies; the parameter with the highest influence on the efficiency was found to be the harvesting OD_{600}. Combining optimized values for all relevant parameters induced the transformation efficiency to raise by 1-log unit. Electroporation of three independently prepared competent cells batches harvested with OD_{600} around 1.0 gave average transformation efficiency equal to $1.59 \cdot 10^3 \pm 2.85 \cdot 10^2$. The presence of plasmids in transformants was confirmed by agarose gel electrophoresis, and by successful isolation of plasmid DNA from the transformants.

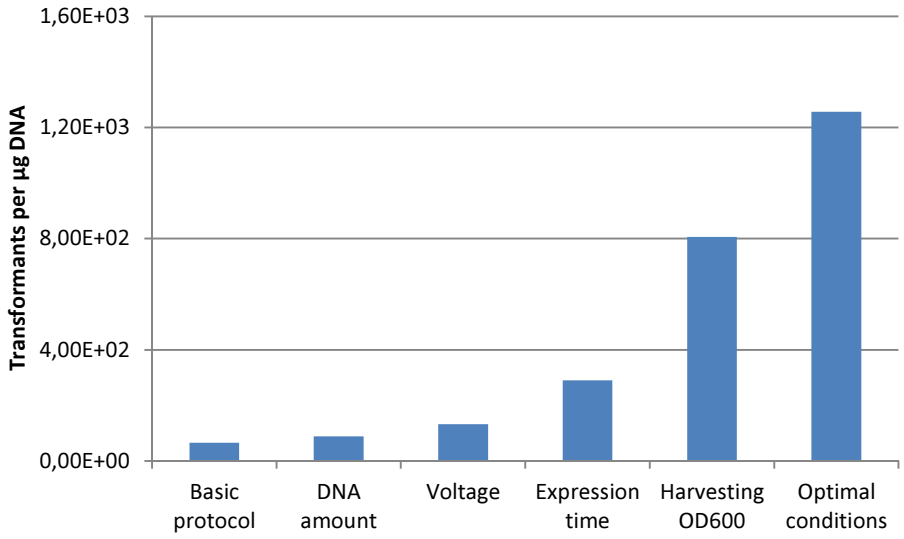


Figure 4.4. Effects of different parameter variations on the transformation efficiency. The selection of optimal values for each of the parameters resulted in an increased transformation efficiency.

4.3.8. The effect of number of generations

Finally, the effect of the harvesting OD_{600} in relation to the number of generations in the competent media was examined. The competent cells were inoculated from o/n culture to an OD_{600} of 0.04 or 0.08. The cells were then grown in MRS with 0.7M NaCl, according to the optimized protocol. The cells were harvested after approximately 1, 2, 3, 4, or 5 generations (Table 4.5). The transformation efficiencies can be found in Table 4.5; the results are also illustrated in Figure 4.5.

Table 4.5. Transformation efficiencies vs harvesting OD_{600} and number of generations.

$OD_{starting}$	$OD_{harvesting}$	Number of generations	Transformation efficiency	Transformation efficiency (takes OD_{600} into account)
0.04	0.10	1	$2.80 \cdot 10^2$	$2.80 \cdot 10^3$
0.04	0.16	2	$3.93 \cdot 10^1$	$2.46 \cdot 10^2$
0.04	0.37	3	$6.47 \cdot 10^2$	$1.75 \cdot 10^3$
0.04	0.66	4	$8.91 \cdot 10^2$	$1.35 \cdot 10^3$
0.04	1.58	5	$1.74 \cdot 10^3$	$1.10 \cdot 10^3$
0.08	0.17	1	$6.29 \cdot 10^1$	$3.70 \cdot 10^2$
0.08	0.36	2	$5.32 \cdot 10^2$	$1.48 \cdot 10^3$
0.08	0.81	3	$1.76 \cdot 10^3$	$2.17 \cdot 10^3$
0.08	1.26	4	$5.86 \cdot 10^2$	$4.65 \cdot 10^2$

The transformation efficiency appeared to be the highest for the cells harvested after 5 and 3 generations, for the starting OD_{600} of 0.04 and 0.08, respectively. The corresponding harvesting OD_{600} were 1.58 and 0.81. It thus seems that it is not the OD_{600} that solely influences the transformability of the cells; the number of generations and the starting OD_{600} appear to be of importance as well. However, taking into account the differences in cell counts due to the variations in OD_{600} values, a significant increase in the transformation efficiency was observed for the cells harvested after 3 generations, irrespective of their

starting or harvesting OD_{600}. Therefore, the highest transformability of the cells occurred when the cells were harvested after 3 generations; on the other hand, using older cultures with higher OD_{600} could yield a higher number of transformants.

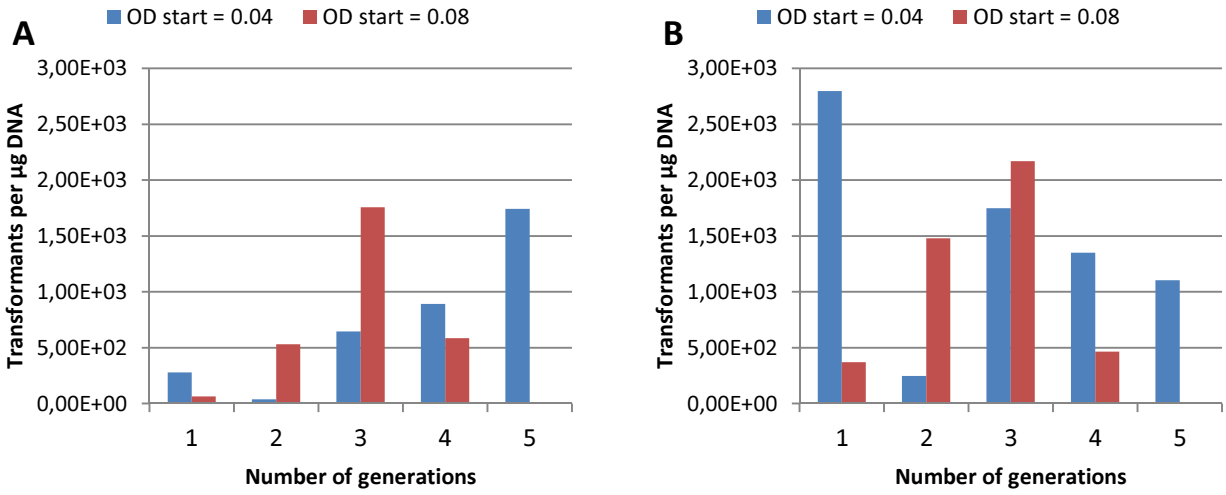


Figure 4.5. The effect of number of generations grown in competent medium after which the cells are harvested. A) Transformation efficiency vs number of generations; B) Transformation efficiency, taking into account the different OD_{600} of harvested cells, vs number of generations.

4.3.9. Electroporation with the use of pAMB1 plasmids isolated from *L. lactis* or *P. acidilactici*

The optimal conditions were further tested by attempting to transform the strain with plasmids purified from *L. lactis* or from the same strain of *P. acidilactici*. It has been reported that many species can be transformed with higher efficiencies when the plasmid DNA isolated from the host strain is used, or when an *E. coli* purified plasmid is further modified in vitro by cell-free extracts of the host strain (Alegre et al., 2004; Rodríguez et al., 2007). The increased transformation efficiencies possibly result from in vivo modification of the plasmid DNA, and thus using the host-isolated or in vitro modified DNA can help to circumvent host restriction mechanisms (Alegre et al., 2004). However, in this experiment the use of plasmids from both *L. lactis* and *P. acidilactici* resulted in lowered efficiencies (Table 4.6); the latter can be, however, attributable to poor plasmid preparation used for the electroporation. The concentrations of plasmids purified from this strain were low (<100 ng/µL), and therefore a substantial amount of plasmid had to be added to the cells in order to reach the appropriate, optimal amount of transforming DNA, hence diluting the cells.

Table 4.6. Transformation efficiencies for competent cells transformed with plasmids amplified in LAB.

Plasmid amplified in:	Transformation efficiency
P. acidilactici DSM 20284	$3.83 \cdot 10^2$
L. lactis MG 1363	$9.06 \cdot 10^1$

4.3.10. Other protocols

As mentioned earlier, several protocols for transformation of different LAB species have been developed over the years. One recent study done by Landete et al. (2014) developed a robust protocol allowing for an efficient electrotransformation of several species, including *P. acidilactici*. The reported transformation efficiencies were as high as $1.6 \cdot 10^5$. However, this work has been published after the protocol optimization described in this chapter was performed. It is also worth mentioning that the study employed a different strain of *P. acidilactici* and another plasmid, which both can have an influence on the transformation efficiency. As for the method, to increase the membrane permeability, Landete et al. (2014) used MRS supplemented with 1% glycine and 0.5M sucrose. The cells were harvested at an OD_{600} of 0.6 and washed in a solution containing 10% glycerol and 0.3M sucrose; it was also shown that the transformation efficiency can be raised by supplementing the washing buffer with 5 mM KH_2PO_4 and 2 mM $MgCl_2$. The electroporation was performed in 2-mm gap cuvettes at 1.7 kV; immediately after the pulse the cells were mixed with a recovery medium, which was MRS with 0.3M sucrose, 20 mM $MgCl_2$ and 2 mM $CaCl_2$. After incubation for 2.5 h at 37°C, the cells were plated on MRS plates supplemented with 0.3M sucrose and 5µg/mL erythromycin. Such conditions were not tested in this study; however, two competent media containing MRS with 1% glycine or MRS with 2% glycine and 0.5M sucrose did not yield more than 7 transformants. A similar washing buffer, containing sucrose, K_2HPO_4 and $MgCl_2$, was also tested in nearly optimized conditions and resulted in a zero transformation efficiency. However, as the method proposed by (Landete et al., 2014) was demonstrated to work for several LAB and was suggested to be applicable for other species as well, it is likely to work for the strain employed in this study; yet, the transformation efficiency should be investigated.

4.4. Conclusions

The most critical parameters evaluated in this study that enhanced the transformation efficiency proved to be the competent cells harvesting OD_{600}, voltage used for electroporation and time allowed for expression after the transformation. The number of generation times was also evaluated as a very relevant parameter. With these parameters modified, an optimized protocol could be developed that increased the efficiency of transformation by almost 20 fold; a maximum of $2.8 \cdot 10^3$ transformants per µg DNA was obtained in this study using the optimized method.

Although the obtained transformation efficiency of 10^3 is low when compared to other species being transformed with efficiencies in the range 10^4-10^7, it still allows genetic modification of this strain, which is of crucial importance for its possible industrial applications.

4.5. References

Alegre, T.M., Rodríguez, C.M., Mesas, J.M., 2004. Transformation of *Lactobacillus plantarum* by electroporation with in vitro modified plasmid DNA. FEMS Microbiol. Lett. 241, 73–77.

Aune, T.E.V., Aachmann, F.L., 2010. Methodologies to increase the transformation efficiencies and the range of bacteria that can be transformed. Appl. Microbiol. Biotechnol. 85, 1301–1313.

Berthier, F., Zagorec, M., Champomier-Verges, M., Ehrlich, S.D., Morel-Deville, F., 1996. Efficient transformation of *Lactobacillus sake* by electroporation. Microbiology 142, 1273–1279.

Biswas, I., Gruss, A., Ehrlich, S.D., Maguin, E., 1993. High-Efficiency Gene Inactivation and Replacement System for Gram-Positive Bacteria. J. Bacteriol. 175, 3628–3635.

Bonnassie, S., Burini, J.F., Oreglia, J., Trautwetter, A., Patte, J.C., Sicard, a M., 1990. Transfer of plasmid DNA to *Brevibacterium lactofermentum* by electrotransformation. J. Gen. Microbiol. 136, 2107–2112.

Bringel, F., Hubert, J., 1990. Optimized transformation by electroporation of *Lactobacillus plantarum* strains with plasmid vectors. Appl. Microbiol. Biotechnol. 33, 664–670.

Buckley, N.D., Vadeboncoeur, C., LeBlanc, D.J., Lee, L.N., Frenette, M., 1999. An Effective Strategy, Applicable to *Streptococcus salivarius* and Related Bacteria, To Enhance or Confer Electroporation Competence. Appl. Environ. Microbiol. 65, 3800–3804.

Caldwell, S.L., Mahon, D.J.M.C., Oberg, C.J., Broadbent, J.R., 1996. Development and Characterization of Lactose-Positive Pediococcus Species for Milk Fermentation. Appl. Environ. Microbiol. 62, 936–941.

David, S., Simons, G., de Vos, W.M., 1989. Plasmid Transformation by Electroporation of *Leuconostoc paramesenteroides* and Its Use in Molecular Cloning. Appl. Environ. Microbiol. 55, 1483–1489.

Helmark, S., Hansen, M.E., Jelle, B., Sørensen, K.I., Jensen, P.R., 2004. Transformation of *Leuconostoc carnosum* 4010 and Evidence for Natural Competence of the Organism. Appl. Environ. Microbiol. 70, 3695–3699.

Heravi, R.M., Nasiraii, L.R., Sankian, M., Kermanshah, H., Varasteh, A.R., 2012. Optimization and Comparison of Two Electrotansformation Methods for Lactobacilli. Biotechnology 11, 50–54.

Holo, H., Nes, I., 1995. Transformation of Lactococcus by Electroporation, in: Methods in Molecular Biology. pp. 195–199.

Holo, H., Nes, I.F., 1989. High-Frequency Transformation, by Electroporation, of *Lactococcus lactis* subsp. *cremoris* Grown with Glycine in Osmotically Stabilized Media. Appl. Environ. Microbiol. 55, 3119–3123.

Kim, W., Ray, B., Johnson, M., 1992. Plasmid transfer by conjugation and electroporation in *Pediococcus acidilactici*. J. Appl. Bacteriol. 72, 201–207.

Kim, Y.H., Han, K.S., Oh, S., You, S., Kim, S.H., 2005. Optimization of technical conditions for the transformation of *Lactobacillus acidophilus* strains by electroporation. J. Appl. Microbiol. 99, 167–74.

Kullen, M.J., Klaenhammer, T.R., 2000. Genetic Modification of Intestinal Lactobacilli and Bifidobacteria. Curr. Issues Mol. Biol. 2, 41–50.

Landete, J.M., Arqués, J.L., Peirotén, Á., Langa, S., Medina, M., 2014. An improved method for the electrotransformation of lactic acid bacteria: A comparative survey. J. Microbiol. Methods 105, 130–133.

Leer, R.J., van Luijk, N., Posno, M., Pouwels, P.H., 1992. Structural and functional analysis of two cryptic

plasmids from *Lactobacillus pentosus* MD353 and *Lactobacillus plantarum* ATCC 8014. Mol. Gen. Genet. 234, 265–74.

Maguin, E., Duwat, P., Hege, T., Ehrlich, D., Gruss, A., 1992. New thermosensitive plasmid for gram-positive bacteria. J. Bacteriol. 174, 5633–5638.

Mason, C.K., Collins, M.A., Thompson, K., 2005. Modified electroporation protocol for Lactobacilli isolated from the chicken crop facilitates transformation and the use of a genetic tool. J. Microbiol. Methods 60, 353–363.

Palomino, M.M., Allievi, M.C., Prado-Acosta, M., Sanchez-Rivas, C., Ruzal, S.M., 2010. New method for electroporation of Lactobacillus species grown in high salt. J. Microbiol. Methods 83, 164–167.

Posno, M., Leer, R.J., van Luijk, N., van Giezen, M.J.F., Lokman, B.C., Pouwels, P.H., 1991. Incompatibility of Lactobacillus Vectors with Replicons Derived from Small Cryptic Lactobacillus Plasmids and Segregational Instability of the Introduced Vectors. Appl. Environ. Microbiol. 57, 1822–1828.

Powell, I.B., Achen, M.G., Hillier, A.J., Davidson, B.E., 1988. A Simple and Rapid Method for Genetic Transformation of Lactic Streptococci by Electroporation. Appl. Environ. Microbiol. 54, 655–660.

Rixon, J., Warner, P.J., 2003. Introduction: background, relevant genetic techniques and terms, in: Genetics of Lactic Acid Bacteria. pp. 1–24.

Rodríguez, M.C., Alegre, M.T., Mesas, J.M., 2007. Optimization of technical conditions for the transformation of *Pediococcus acidilactici* P60 by electroporation. Plasmid 58, 44–50.

Serror, P., Sasaki, T., Ehrlich, S.D., Maguin, E., 2002. Electrotransformation of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus delbrueckii* subsp. *lactis* with Various Plasmids. Appl. Environ. Microbiol. 68, 46–52.

Shareck, J., Choi, Y., Lee, B., Miguez, C.B., 2004. Cloning Vectors Based on Cryptic Plasmids Isolated from Lactic Acid Bacteria: Their Characteristics and Potential Applications in Biotechnology. Crit. Rev. Biotechnol. 24, 155–208.

Thompson, K., Collins, M.A., 1996. Improvement in electroporation efficiency for *Lactobacillus plantarum* by the inclusion of high concentrations of glycine in the growth medium. J. Microbiol. Methods 26, 73–79.

Trevors, J.T., Chassy, B.M., Dower, W.J., Blaschek, H.P., 1992. Electrotransformation of Bacteria by Plasmid DNA, in: Guide to Electroporation and Electrofusion. pp. 265–290.

Ye, L., Zhao, H., Li, Z., Wu, J.C., 2013. Improved acid tolerance of *Lactobacillus pentosus* by error-prone whole genome amplification. Bioresour. Technol. 135, 459–463.

Chapter 5

Adaptive evolution of *Pediococcus acidilactici* DSM 20284 for enhanced furfural resistance

5.1. Introduction

Adaptive laboratory evolution is one of the approaches for strain development and optimization and is considered to be a powerful tool for the use in metabolic engineering. It allows nonintuitive engineering of industrial strains for important properties (e.g. substrate utilization, product formation, growth temperature, tolerance to inhibitors) without the need to understand the underlying genetic mechanisms (Shui et al., 2015). Developing a strain with a higher environmental robustness is a challenging task both because the underlying stress response mechanisms are largely uncharacterized, but also due to the physiological complexity of microbial tolerance to stress conditions which is very likely to require simultaneous manipulation of several genes and pathways (Jia et al., 2010; Shui et al., 2015). Therefore, the adaptive evolution strategy can be a much more effective approach for enhancing inhibitor tolerance in microorganisms compared to rational metabolic engineering (Cakar et al., 2005).

During adaptive evolution, microorganisms are cultivated under defined conditions either as batch cultivations with sequential serial-transfer to fresh medium, or as continuous cultures in chemostats. While chemostat cultivations offer tight control of many growth conditions and parameters, including nutrient supply, pH, dissolved oxygen, growth rates and population densities, the batch cultivation is an easier and cheaper method, widely used for microorganisms with short generation times. Such microbial cultivation for extended periods of time allows for accumulation of beneficial mutations and thus for the selection of desirable phenotype (Dragosits and Mattanovich, 2013). The recent advances in the next-generation sequencing as well as transcriptomics methods make it possible to obtain phenotype-genotype correlations, in this way allowing to gain insight into genetic bases of microbial evolution (Dragosits and Mattanovich, 2013).

Many microorganisms have been successfully adapted to various stress conditions, including high temperatures (Wallace-Salinas and Gorwa-Grauslund, 2013), saline stress (Dhar et al., 2011), and tolerance to ethanol (Brown and Oliver, 1982; Stanley et al., 2010; Yomano et al., 1998), butanol (Liu et al., 2012), and acetate (Steiner and Sauer, 2003; Wright et al., 2011; Aarnio et al., 1991). Much effort has also been put to generate microorganisms tolerant to inhibitors coming from the pretreatment of lignocellulosic biomass to improve their industrial performance. Although there are methods allowing for detoxification of lignocellulosic hydrolysates, including chemical, enzymatic and microbial detoxifications (Mussatto and Roberto, 2004), it is desirable that the fermenting microorganism requires no or minimum detoxification

treatment. This is important both because it prevents the loss of fermentable sugars, but also the detoxification processes tend to be complex and result in additional costs (Parawira and Tekere, 2011). Among others, adaptation to furfural and HMF has been performed in *Zymomonas mobilis* (Shui et al., 2015) and *Saccharomyces cerevisiae* (Almario et al., 2013; Demeke et al., 2013; Hawkins and Doran-Peterson, 2011; Heer and Sauer, 2008; Liu et al., 2005; Martín et al., 2007; Smith et al., 2014).

This chapter describes an adaptive evolution experiment of *P. acidilactici* DSM 20284 in order to enhance its growth in the presence of furfural and acetic acid inhibitors (the most prevalent inhibitors in softwood hydrolysates). An adapted strain A28 was isolated that showed increased growth rates both in the presence of inhibitors and in the medium alone. The genome of the adapted strain has been sequenced in order to find mutations responsible for the changed phenotype.

5.2. Materials and Methods

5.2.1. Strains and growth conditions

Pediococcus acidilactici DSM 20284 was grown in 10 mL filtered MRS medium (Oxoid) supplemented with 2.2 g/L furfural and 5.3 g/L acetic acid. The pH was set at 6.5±0.1. The strain was grown at 30°C. Serial transfers into fresh medium were performed on a daily basis in 100-fold dilution. Samples of the cultures were taken at regular intervals and plated on MRS plates to check for possible contamination and to isolate adapted mutants; freeze cultures were made weekly. To screen for adapted strains growth experiments were performed in 1 to 7 replicates in 50 mL of the MRS with and without the inhibitors with 220 rpm stirring. The growth of strains was followed by regular optical density (OD_{600}) measurements.

5.2.2. Whole genome resequencing

For the purification of genomic DNA, *P. acidilactici* adapted strain was cultivated in MRS medium overnight at 30°C. The genomic DNA was isolated from the strain as described previously (Grimberg et al., 1989) with several modifications; briefly: 1 mL of the overnight culture was harvested by centrifugation, washed with 1 mL TNE (10 mM Tris pH 8, 10 mM NaCl, 10 mM EDTA) and then resuspended in 270 µL TNEX (TNE with 1% Triton X-100). Then 30 µL of freshly prepared lysozyme solution (5 mg/mL) was added and the suspension was incubated at 37°C for 2 hours. Afterwards, proteinase K was added to 1 mg/mL, followed by 2-hour incubation at 37°C and 2-hour incubation at 65°C. The DNA was precipitated by adding 1/20 volume of 5M NaCl and 2-3 volumes of 96% ethanol. After all ethanol was removed, the DNA was resuspended in 100-200 µL TER (TE with RNase) and stored at -20°C. The quality of DNA was verified by gel electrophoresis and Tecan's plate reader equipped with the NanoQuant plate.

Whole genome sequencing was performed with Illumina HighSeq 2000 by BGI (Hong Kong). The genome was assembled and analyzed by CLC Genomics Workbench. The reference genome of *P. acidilactici* DSM 20284 with NCBI accession number NZ_GL397067.1 was used for reads mapping. For detection of single nucleotide polymorphisms (SNPs) and insertions or deletions, the probabilistic variant detector was used.

5.3. Results and Discussion

Based on the results from Chapter 2, *Pediococcus acidilactici* was selected as a promising candidate for converting lignocellulosic biomass; as described previously, it possesses the ability of utilizing pentoses,

xylose and arabinose, with almost the same growth rate as it utilizes glucose. This is an important property as the lignocellulosic biomass contains significant amounts of C5 sugars and an efficient utilizer of the 2nd generation biomass is required to be able to use both C-5 and C-6 sugars at similar levels. Moreover, *P. acidilactici* was also selected among almost 300 strains as one of the five best performing strains in the presence of common inhibitors coming from the pretreatment of the lignocellulose. When tested in 10% MRS medium, with either glucose or xylose, *P. acidilactici* DSM 20284 performed the best of all tested strains (Table 5.1). It was not markedly inhibited either by utilization of xylose instead of glucose, or by the presence of inhibitors in the medium, while other tested strains showed reduced growth rates when xylose instead of glucose was used as a carbon source. On glucose, all the strains showed similar or better results when grown together with the inhibitors. However, when looking at the cumulative % drops in growth rates caused both by the presence of inhibitors in the medium and by using xylose instead of glucose as a carbon source, the strain of *P. acidilactici* demonstrates significantly better performance than all other tested strains. This is the reason why it was *P. acidilactici* that was selected for the study; the adaptive evolution experiment was performed in order to further improve its robustness towards the common inhibitors from pretreated lignocellulosic biomass. The combination of furfural and acetic acid representing inhibitors from pretreated softwood was selected for the adaptive laboratory evolution. Softwood is a specific type of biomass as it requires a more severe pretreatment which thus generates higher concentrations of inhibitors. It was assumed that the mutant adapted to harsher conditions would perform better than the wild-type strain also at lower inhibitor concentrations.

Table 5.1. Performance of the best strains in 10% MRS with glucose or xylose and combination of inhibitors from softwood (furfural and acetic acid).

Strain	Conditions	Growth rate (1/h)		% drop on softwood inhibitors[1]	% drop on xylose[2]	Cummulative % drop[3]
		Glucose	Xylose			
L. pentosus LMG 17672	Control	0.594	0.199	39.9	66.5	69.7
	+ softwood inhibitors	0.357	0.180			
L. pentosus LMG 17673	Control	0.577	0.259	24.6	55.1	56.8
	+ softwood inhibitors	0.435	0.249			
P. acidilactici DSM 20284	Control	0.482	0.415	32.8	13.9	37.8
	+ softwood inhibitors	0.324	0.300			
P. pentosaceus ATCC 25745	Control	0.391	0.202	31.7	48.3	67.0
	+ softwood inhibitors	0.267	0.129			

[1]Calculated as (growth rate$_{glucose}$ – growth rate$_{glucose + softwood}$) * 100 / growth rate$_{glucose}$
[2]Calculated as (growth rate$_{glucose}$ – growth rate$_{xylose}$) * 100 / growth rate$_{glucose}$
[3]Calculated as (growth rate$_{glucose}$ – growth rate$_{xylose + softwood}$) * 100 / growth rate$_{glucose}$

5.3.1. Screening for adapted strains

The adaptive evolution experiment was conducted over 93 days, giving a total of 454 generations. During the course of evolution, 34 colonies were isolated and tested in growth experiments in MRS medium with furfural and acetic acid with 1 to 7 replicates (Figure 5.1). The different growth experiments were performed at different days and using different batches of the MRS medium, which resulted in variations in the growth rates of the wild-type strain obtained from the different media batches. Therefore, for comparisons between the different adaptive evolution strains, relative growth rates were used, calculated as $\mu_{adaptive\ evolution\ strain}/\mu_{wildtype\ strain}$. As illustrated in Figure 5.1, the best performance in MRS with inhibitors was shown by A28, an adapted strain isolated after approximately 408 generations; this strain was selected

for further characterization. Interestingly, the screened adaptive evolution strains also showed increased growth rates on MRS medium alone, without the addition of any inhibitors (Figure 5.2). This finding indicates that the strains, apart from the tolerance to the tested inhibitors, became adapted to the medium itself as well.

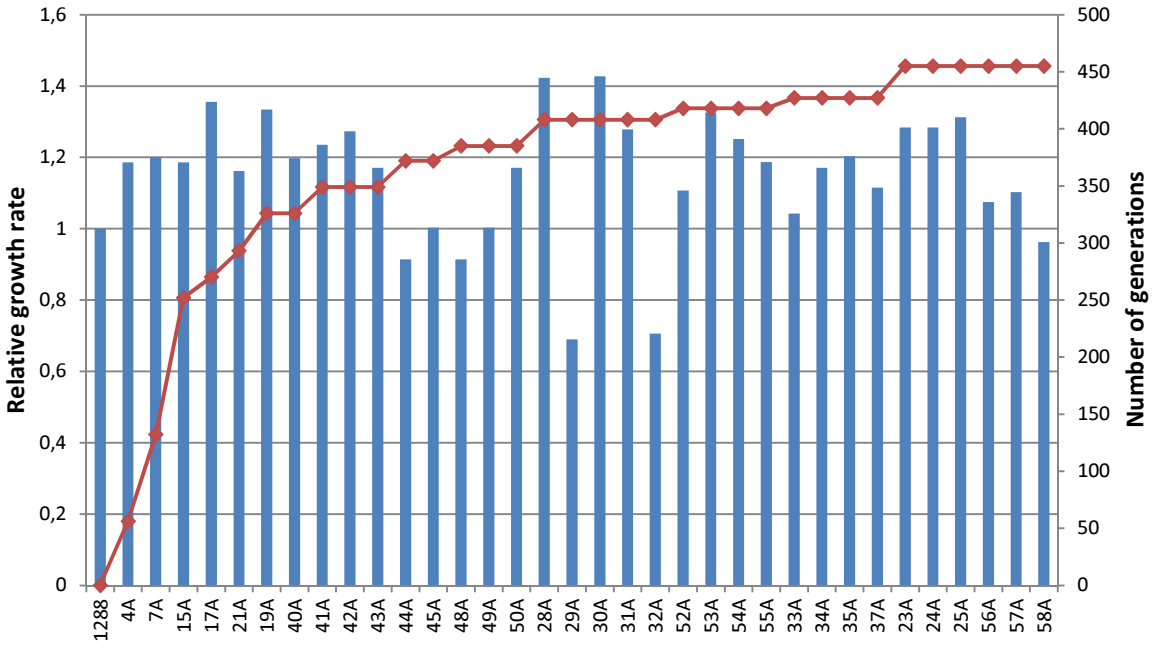


Figure 5.1. Screening of adaptive evolution strains for improved growth on MRS with the inhibitors representing pretreated softwood. Relative growth rate was calculated as $\mu_{\text{adaptive evolution strain}}/\mu_{\text{wildtype strain}}$.

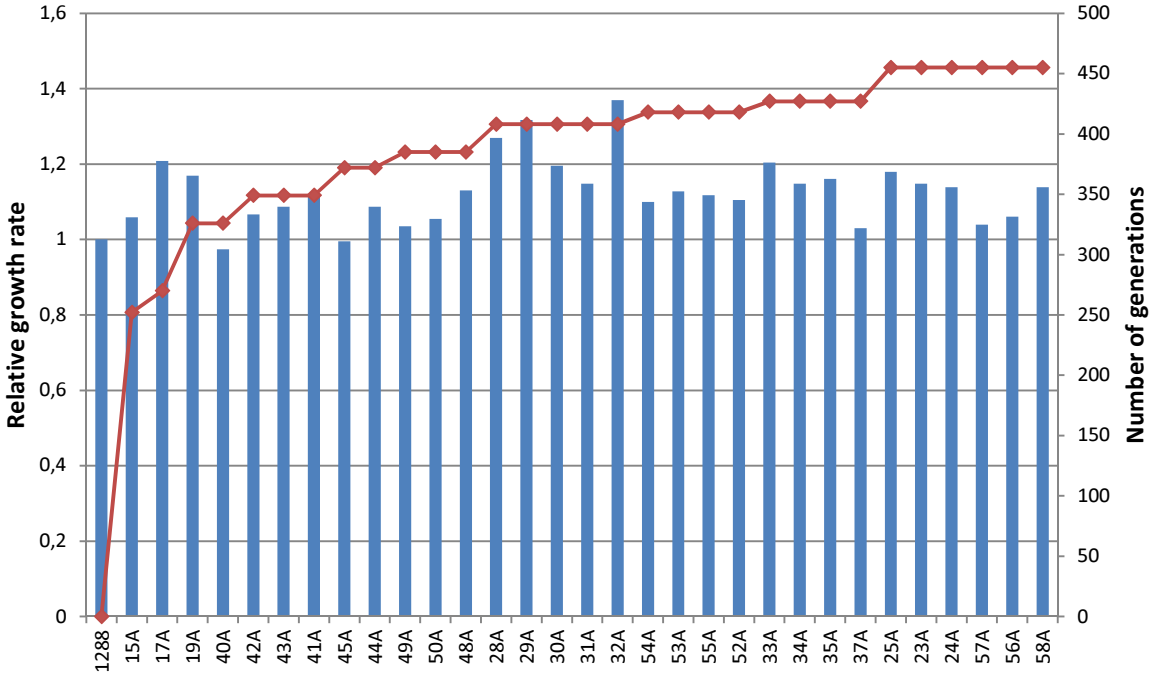


Figure 5.2. Screening of adaptive evolution strains for their improvement of growth on MRS medium. Relative growth rate was calculated as $\mu_{\text{adaptive evolution strain}}/\mu_{\text{wildtype strain}}$.

5.3.2. Characterisation of adapted strain A28

The adapted strain A28 was tested in growth experiments in duplicates to compare its growth rate to the wild-type strain in higher concentrations of furfural (0-8 g/L). It was confirmed that the adapted strain exhibits a higher growth rate on MRS medium without addition of furfural when compared to the wild-type strain; however, with the increasing concentration of furfural, the difference in growth rates of the two strains was smaller and smaller (Figure 5.3A). The differences in final optical densities were also observed (Figure 5.3B). The adapted strain was shown to yield a higher final OD_{600} than the wild-type strain, until furfural concentration reached 6 g/L; with higher concentrations, the OD_{600} of both the wild-type and the adapted strain were at similar levels, showing very limited growth. The growth rates of both the wildtype and the adapted strain were lowering with increasing furfural concentrations, while the final optical densities remained unchanged until the concentration of furfural of 2.0 g/L for the wildtype strain and 3.5 g/L for the adapted strain. The wild-type strain's final optical density was significantly lowered already at 4.5 g/L furfural, while the adapted strain was still able to grow to an OD_{600} of 5.4 at that furfural concentration; it went down to an OD_{600} of 0.2 with the addition of 6.0 g/L furfural. It is evident that the adapted strain was improved with regard to the growth yield; at a concentration of furfural equal to 4.0 g/L, the wild-type strain showed a very low yield, while the same furfural concentration allowed the adapted strain to achieve final OD_{600} of 6.9.

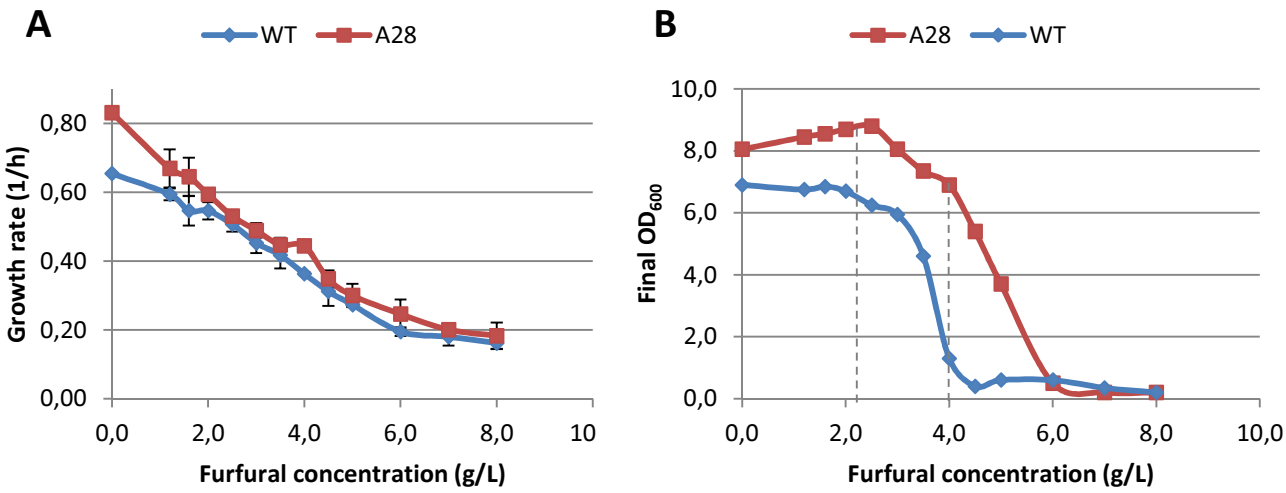


Figure 5.3. Growth rates (A) and final OD (B) of the wild-type and adapted strains versus furfural concentration. The dashed lines correspond to a furfural concentration of 2.2 g/L (at which the adaptive evolution experiment was performed) and 4 g/L, where the biggest effect on the growth yield could be observed.

As the growth rate of the adapted strain A28 is higher in the medium both with and without furfural, it seems that the strain adapted to the growth in the rich MRS medium. To verify whether it also adapted to the presence of furfural, a growth experiment in a different medium was performed. The medium used was a *Pediococcus* Defined Medium (PDM); the adapted strain showed higher growth rates in the medium both with and without furfural present (0.398 h^{-1} ±0.015 and 0.572 h^{-1} ±0.003, respectively). These results confirmed that the strain adapted both to the presence of the inhibitor, and to the faster growth in MRS medium. It is worth to mention that the growth rate of A28 in PDM medium in the presence of furfural was almost the same as the growth rate of the wild-type without the addition of furfural (0.398 h^{-1} ±0.003 vs

0.416 h^{-1} ±0.003)(Figure 5.4). The final OD_{600} was also investigated for both the wildtype and adapted strain in PDM with and without inhibitors. As it can be seen on the graph, the adapted strain A28 reaches higher ODs than the wildtype both with and without the presence of the inhibitors.

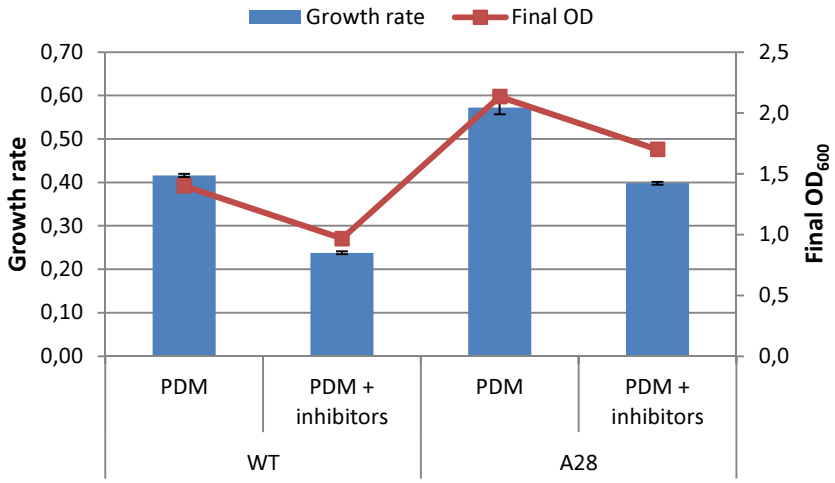


Figure 5.4. Growth rates of the wild-type (WT) and adapted strain (A28) in PDM media with and without the inhibitors; final optical densities has been shown as well.

5.3.3. Characterisation of mutations

A complete set of mutations responsible for an evolved phenotype can be studied by whole genome resequencing and comparing the genomes of the starting reference strain with the adapted strain (Conrad et al., 2011). The mutations found in the adapted strain A28 can be both associated with the adaptation of the strain to the faster growth in MRS medium, or with the higher tolerance to furfural and acetic acid. Furfural can influence microorganisms in a number of ways. It was shown to inhibit protein and RNA synthesis (Liu et al., 2004; Parawira and Tekere, 2011), and it is known to cause DNA damage in *Escherichia coli* and *Salmonella enterica* (Almeida et al., 2009; Khan and Hadi, 1993; Zdzienicka et al., 1978). In yeasts, it inhibits key glycolytic enzymes (hexokinase and glyceraldehyde-3-phosphate dehydrogenase)(Banerjee et al., 1981; Parawira and Tekere, 2011) and induces the accumulation of reactive oxygen species (ROS) (Allen et al., 2010; Glebes et al., 2014). Apart from the oxidative damage, it can also cause protein misfolding and fragmentation and membrane damage (Almeida et al., 2009).

There were 62 mutations identified within the genome, consisting of 50 single nucleotide polymorphisms and 12 single insertions (Table 5.2). Most of the mutations were non-synonymous substitutions that lead to amino acid changes, influencing the composition of the proteins. 7 out of 36 non-synonymous mutations resulted in frameshifts, likely rendering the proteins inactive. 15 mutations were silent, and 11 mutations were found to be located in the intergenic regions.

One of the non-synonymous mutations was found within a gene encoding heat-inducible transcription repressor HrcA (mut. 4). This is a negative regulator of the class I heat shock genes, regulating the expression of *dnaK* and *groEL* operons in Gram-positive bacteria (Kilstrup et al., 1997; Woodbury and Haldenwang, 2003). HrcA protein binds to a promoter region of the *dnaK* and *groEL* operons (CIRCE sequence) disallowing their expression. During a heat shock stress, the HrcA protein dissociates from the

CIRCE element, derepressing the expression of the *dnaK* and *groEL* stress genes (Woodbury and Haldenwang, 2003). The HrcA regulon was also shown to be induced in response to acid, cold, ethanol and salt stress (De Angelis and Gobbetti, 2004; Lemos et al., 2001; Van Bokhorst-van de Veen et al., 2013). An amino acid change from glutamine into arginine within the HrcA protein could potentially change its conformation and prevent it from interacting with the CIRCE sequence, resulting in an overexpression of DnaK and GroEL chaperones. Changes in the expression of these chaperones were also observed during acid adaptation in *L. delbruekii* subsp. *bulgaricus* (Lim et al., 2000; Silva et al., 2005).

One of the intergenic mutations was also interesting to look at (mut. 56). The mutation occurred 15 nucleotides away from a gene encoding a hypothetical protein, and was located close to a ribosomal binding site (RBS), possibly having an influence on the protein translation process. The protein sequence was blasted at NCBI and it showed high identity (81% with 100% coverage) to a transcriptional regulator CtsR from *Pediococcus clausenii* ATCC BAA-344. The CtsR family consists of several transcriptional repressor of class III stress genes proteins, mainly from Firmicute species. In *Lactococcus lactis*, CtsR is a key regulator found to negatively regulate the heat shock response (Varmanen et al., 2000). In other Gram-positive bacteria, the inactivation of the *ctsR* gene caused enhanced stress tolerance under adverse conditions (Hufner et al., 2007; Nair et al., 2000; Zotta et al., 2009). The CstR protein was shown to derepress the *clp* operon (encoding Clp ATPases, facilitating proper folding of proteins) not only during the heat shock, but also a range of other environmental stresses, including osmotic and oxidative stresses (Elsholz et al., 2011; Zotta et al., 2009).

Two of the non-synonymous mutations were found to be located in genes annotated to encode proteins involved in DNA repair processes. One of the mutations was in *ruvB* gene (mut. 10) inducing a conservative substitution of isoleucine into valine. RuvB protein forms a complex with RuvA and catalyzes the resolution of Holliday junctions that appear during homologous recombination and double strand break repair. Thus, it can be involved in repairing damaged microbial DNA (Shinagawa et al., 1991).

The second mutation was within *mutT* gene (mut. 17) encoding the DNA-mismatch repair protein. In *E. coli,* MutT protein was shown to hydrolyze analogs of ribonucleotides, 8-oxo-dGTP and 8-oxo-GTP (produced by oxidation of dGTP by reactive oxygen species), in this way preventing them from being incorporated into DNA and RNA. Thus, the MutT protein decreases the number of errors during transcription and DNA replication (Fowler and Schaaper, 1997; Taddei et al., 1997) and plays a role in cells' protection against oxidative damage.

Two of the single nucleotide polymorphisms (SNPs) were found to be localized within genes encoding proteins involved in the cell redox homeostasis. One of the mutated genes was found to encode a pyridine nucleotide-disulfide oxidoreductase (mut. 3); the other encoded thioredoxin (mut. 21), which serves as a disulfide oxidoreductase and is a part of a thioredoxin system, consisting of an NADPH, thioredoxin and thioredoxin reductase. This system is recognized as one of the key antioxidant systems in the defence against oxidative stress both in *E. coli* and lactic acid bacteria (Guzzo et al., 2000; Lu and Holmgren, 2014; Serata et al., 2012). Another non-synonymous mutation was found in a gene encoding for dithiol-disulfide isomerase (mut. 33) which facilitates the formation of correct disulfide cross-linking between thiols (Gilbert, 2011), thus playing a role in the interconversion between thiols and disulfides. Since furfural can cause oxidative damage to the cells, it is very likely that these can confer some of the resistance by helping to maintain cell redox homeostasis.

Table 5.2. Mutations identified in the adapted strain A28 when compared to the wild-type *P. acidilactici* DSM 20284.

No.	Region	Type	Nucleotide change	Amino acid change			CDS	Product
1	51842	SNP	G → A	Glu	503	Lys	HMPREF0623_0042	hypothetical protein
2	53288^53289	SNI	- → T	Val	49	fs	HMPREF0623_0044	hypothetical protein
3	97264^97265	SNI	- → A	Gln	515	fs	HMPREF0623_RS00495	pyridine nucleotide-disulfide oxidoreductase
4	335530	SNP	A → G	Gln	192	Arg	hrcA	heat-inducible transcription repressor
5	340740^340741	SNI	- → A	Lys	249	fs	HMPREF0623_0344	hypothetical protein
6	388825	SNP	C → T	Glu	550	Lys	FbpA	hypothetical protein
7	458125	SNP	C → T	Gly	7	Glu	purF	amidophosphoribosyltransferase
8	578070	SNP	C → T	Gly	141	Arg	apt	adenine phosphoribosyltransferase
9	640842	SNP	C → T	Val	679	Met	pbpC	penicillin-binding protein 2B
10	710848	SNP	T → C	Ile	297	Val	ruvB	Holliday junction DNA helicase
11	794231	SNP	T → C	Lys	41	Arg	HMPREF0623_0793	DNA-binding response regulator
12	1269904	SNP	G → A	Pro	258	Leu	HMPREF0623_1254	membrane protein / Acyltransferase
13	1282332	SNP	C → T	Arg	109	Gln	HMPREF0623_1266	hypothetical protein
14	1600973^1600974	SNI	- → T	*	81	fs	cpsY	CpsY protein / Putative exopolysaccharide phosphotransferase
15	1646886	SNP	T → C	Val	65	Ala	dapE2	succinyl-diaminopimelate desuccinylase
16	1656925	SNP	C → T	Arg	460	Gln	nrdD	Anaerobic ribonucleoside triphosphate reductase
17	1820451	SNP	T → C	Val	196	Ala	HMPREF0623_1771	DNA mismatch repair protein MutT
18	544466	SNP	T → C	Gln	394	Arg	hisS	histidine--tRNA ligase
19	1097908	SNP	C → T	Gly	540	Arg	HMPREF0623_1085	alpha-glucosidase
20	1719727	SNP	C → T	Gly	58	Arg	galU	UTP--glucose-1-phosphate uridylyltransferase
21	134693	SNP	G → A	Gly	124	Ser	HMPREF0623_0111	thioredoxin
22	74411^74412	SNI	- → A	Gln	412	fs	HMPREF0623_0061	MFS sugar transporter
23	429662	SNP	C → T	Gly	77	Ser	hslV	ATP-dependent protease
24	266162	SNP	A → G	His	250	Arg	fabI	enoyl-ACP reductase
25	1787847	SNP	T → C	Val	274	Ala	add	adenosine deaminase
26	904250	SNP	C → T	Met	367	Ile	pbuX	Uric acid permease PucJ / xanthine permease
27	1356079	SNP	G → A	Val	274	Ile	HMPREF0623_1340	PTS lactose transporter subunit IIC
28	1266563^1266564	SNI	- → A	Leu	173	fs	HMPREF0623_1250	hypothetical protein
29	1716776	SNP	T → C	Gln	296	Arg	pgm2	phosphoglucomutase
30	1169842	SNP	G → A	Asp	253	Asn	cfa	cyclopropane-fatty-acyl-phospholipid synthase
31	1650098	SNP	T → C	Ile	86	Thr	HMPREF0623_1612	membrane protein
32	234463	SNP	T → C	Leu	141	Pro	HMPREF0623_0218	protein kinase
33	656088	SNP	A → G	Tyr	170	Cys	HMPREF0623_0654	dithiol-disulfide isomerase
34	437872	SNP	T → C	Leu	58	Ser	HMPREF0623_RS09255	hypothetical protein
35	1246415	SNP	T → C	Ser	666	Gly	HMPREF0623_1235	membrane protein

No.	Region	Type	Nucleotide change	Amino acid change			CDS	Product
36	373283^373284	SNI	- → A	Lys	58	fs	HMPREF0623_0375	transposase
37	244349	SNP	C → T	None			rnc	ribonuclease III
38	576830	SNP	C → T	None			HMPREF0623_0577	ABC transporter
39	606947	SNP	C → T	None			HMPREF0623_0606 / typA	GTP-binding protein
40	943036	SNP	T → C	None			HMPREF0623_0951	twitching motility protein PilT
41	945329	SNP	T → C	None			HMPREF0623_0953 / dut	dUTP diphosphatase
42	1159850	SNP	C → T	None			HMPREF0623_1151	ABC transporter permease
43	1378092	SNP	G → A	None			HMPREF0623_RS06695	hypothetical protein
44	1728987	SNP	C → T	None			HMPREF0623_1690	two-component system sensor histidine kinase
45	1510906	SNP	A → G	None			HMPREF0623_1478	hypothetical protein
46	1650895	SNP	C → T	None			HMPREF0623_1613 / sufB	Fe-S cluster assembly protein SufB
47	176159	SNP	A → G	None			HMPREF0623_0152	RNA-binding protein
48	441852	SNP	C → T	None			HMPREF0623_RS02220	hypothetical protein
49	413112	SNP	C → T	None			HMPREF0623_0414	gluconate:proton symporter
50	1317947	SNP	T → C	None			HMPREF0623_1304 / cls2	cardiolipin synthase
51	106996	SNP	C → T	None			HMPREF0623_0089	alpha-mannosidase
52	174297	SNP	A → G	Intergenic				
53	253085^253086	SNI	- → T	Intergenic				
54	306105^306106	SNI	- → T	Intergenic				
55	408741	SNP	A → G	Intergenic				
56	885034	SNP	C → T	Intergenic				Transcriptional repressor of class III stress genes (CtsR)
57	1116508	SNP	C → T	Intergenic				
58	1184747^1184748	SNI	- → T	Intergenic				
59	1557134^1557135	SNI	- → A	Intergenic				
60	1625424^1625425	SNI	- → A	Intergenic				
61	1681753	SNP	C → T	Intergenic				
62	1765346	SNP	T → C	Intergenic				

SNP, single nucleotide polymorphism; SNI, single nucleotide insertion; fs, frameshift; *, stop codon; color code of amino acids: blue, basic; red, acidic; green, neutral-polar; orange, neutral-nonpolar.

Apart from the regulators and DNA repair proteins, four non-synonymous mutations occurred in the genes annotated to encode membrane proteins. The first mutation resulting in amino acid change from valine to methionine was located in the *pbpC* gene encoding penicillin-binding protein 2B (mut. 9). The penicillin-binding proteins are involved in the biosynthesis and crosslinking of peptidoglycan, a constituent of bacterial cell wall. Disruption of the genes encoding for these proteins results in an altered cell morphology; both in *E. coli* and in *B. subtilis* the deletion of penicillin-binding protein 2 caused cells to grow as spheres (Popham and Young, 2003). The finding suggests that the cell wall density or thickness might play a role in the resistance of the bacteria to furfural and/or acetic acid.

Other mutations in genes encoding membrane proteins were also investigated. Protein BLAST searches revealed that the first one (mut. 12; HMPREF0623_1254) possesses an acyltransferase domain, however showing low similarity to acyltransferases from other organisms; the second one (mut. 31; HMPREF0623_1612) has a domain with homology to a small neutral amino acid transporter SnatA. It also showed high identity (99%) to a multidrug ABC transporter from *P. lolii*, which is a heterotypic synonym for *P. acidilactici* (Wieme et al., 2012). The third and last gene encoding a membrane protein (mut. 35; HMPREF0623_1235) was also BLASTed but it demonstrated no homology to any membrane protein with a known function. Yet, according to the UniProt database, the gene encodes a methyl-accepting chemotaxis protein which might play a role in the signal transduction. The protein spans the membrane multiple times and might work in response to changes in the concentrations of attractants and repellants in the environment, mediating chemotaxis.

A non-synonymous mutation in a gene encoding for a protein kinase was also found (mut. 32). A protein BLAST revealed 99% identity (100% coverage) to a protein from *P. acidilactici* strain D3, functioning as a TOMM system kinase/cyclase fusion protein. The protein has a transmembrane domain and plays a role in intracellular signal transduction and cyclic nucleotide biosynthetic process. The mutation induced an amino acid change from leucine to proline in the active site of the protein, which is likely to impair the protein's function.

Four genes associated with purine biosynthesis, salvage and transport were also affected by non-synonymous mutations. Among these, there were:

- Amidophosphoribosyltransferase (mut. 7), which catalyzes the formation of phosphoribosylamine from phosphoribosylpyrophosphate (PRPP) and glutamine, constituting the first step of de novo purine nucleotide biosynthesis (purF)
- adenine phosphoribosyltransferase (mut. 8), which catalyzes the formation of AMP from adenine and PRPP
- adenine deaminase (mut. 25), involved in the deamination of adenine to hypoxanthine
- uric acid permease (mut. 26); a protein BLAST revealed that it also possesses a xanthine permease domain; indeed, the gene has been annotated in the UniProt database to be a xanthine permease *pbuX*. Nevetherless, the protein encoded by this gene likely plays a role in the transport of purines, xanthine and/or its degradation product, uric acid.

The mutations in the above described genes are likely to influence the intracellular nucleotide pool sizes, which in turn can affect many biochemical pathways, altering the cell physiology (Martinussen et al., 2003). Purines are important for the synthesis of DNA, RNA and several coenzymes; yet, various LAB are known to

have purine partial requirements (Jan Martinussen, personal communication). The rich MRS medium contains large amounts of purine nucleobases and nucleosides, so adding additional purines likely might not have an influence on the growth rate; however, optimization of the purine transport into the cell as well as pathways for purine biosynthesis and salvage, resulting in an optimal turnover of the nucleotides might allow the organism for a faster growth.

A mutation in *purF* gene was a change from glycine to glutamic acid at amino acid position no.7; a more detailed investigation of this mutation revealed that in many microorganisms this amino acid position is taken either by a hydrophilic serine or by aspartic acid. As mentioned above, PurF catalyzes the first step of de novo purine biosynthesis; it is also feedback inhibited, rate-limiting step. Its enhancement can potentially increase the flux through the whole pathway. The mutation in *purF* might also relieve the inhibition of the biosynthetic pathway, which would in turn mean that both the biosynthesis and salvage pathways could be active at the same time, supplying the strain with more purines and thereby allowing for a higher growth rate.

Among the various non-synonymous mutations there were also several SNPs within the genes coding for proteins involved in sugar transport (MFS sugar transporter and PTS lactose transporter, mut. 22 and 27, respectively) and metabolism (UTP--glucose-1-phosphate uridylyltransferase and phosphoglucomutase). These mutations are likely to be related to a faster growth in rich MRS medium; even though glucose was added as the primary carbon source, there can be other sugars including lactose present in the medium as well, coming from the non-defined medium' components. Indeed, it was shown earlier that different *Lactobacillus* and *Pediococcus* species can grow on MRS without glucose added (data not shown), implying the presence of additional carbon source(s) in MRS. Nonetheless, an improvement in sugar uptake is likely to positively correlate with the microorganism's growth rate.

Two of the mutations within the sugar metabolism are enzymes involved in the production of exopolysaccharides (EPSs). Phosphoglucomutase (mut. 29) converts the glucose-6-phosphate into glucose-1-phosphate, while UTP--glucose-1-phosphate uridylyltransferase (mut. 20), also known as UDP-glucose phosphorylase, catalyzes the conversion of glucose-1-phosphate into UDP-glucose, which is a precursor for EPS synthesis. These two reactions are considered to be control points in the EPS production (Papagianni, 2012). EPSs are long-chain polysaccharides secreted by bacteria into the extracellular environment and were suggested to play a role in growth and survival of microorganisms' under adverse environmental conditions. EPSs are believed to have a protective function against dehydration, attacks by bacteriophages, osmotic stress, antibiotics and toxic compounds (Looijesteijn et al., 2001; Ozturk and Aslim, 2010; Patel et al., 2012). Therefore, a mutation within these two genes is likely to have an effect on the cell's tolerance to furfural or acetic acid present in the medium.

5.4. Conclusions

An adapted strain A28 was isolated after 408 generations and showed higher growth rates both on the MRS medium alone, and with addition of furfural and acetic acid, the most prevalent inhibitors found in pretreated softwood. The results were confirmed on a defined medium PDM, where the adapted strain also exhibited elevated growth rates both with and without the inhibitors present. When grown with furfural and acetic acid, the adapted strain A28 reached a growth rate similar to the one obtained by the wildtype strain without the inhibitors added. By whole genome resequencing, 62 mutations were found

within the genome of adapted strain A28 when compared to the reference wild-type strain. Over half of the mutations were non-synonymous single nucleotide substitutions, imposing changes in the proteins; either an amino acid change or a frameshift. Many mutations were found likely to be associated with the changed phenotype. The mutations could be divided into several functional groups, including mutations in the DNA repair proteins, stress response transcriptional regulators, membrane proteins and proteins involved in cell redox homeostasis, which all could be linked to the elevated tolerance of the adapted strain to a combination of furfural and acetic acid. The mutations in the purine biosynthesis and salvage related proteins, as well as in proteins associated with sugar transport and metabolism, are most probably related to the adapted strain's improved growth on MRS medium. In conclusion, *P. acidilactici* DSM 20284 strain was found to be very well adapted to furfural in advance; that is a possible reason for the strain's adaptation for a faster growth on the MRS medium. It was demonstrated that the growth yield rather than the growth rate was considerably enhanced, possibly due to a better utilization of energy by the adapted strain.

Additional experiments are necessary to verify and confirm the effect of the individual mutations on the changed phenotype. However, due to the limited time, these were not within the scope of this PhD project.

5.5. References

Allen, S.A., Clark, W., McCaffery, J.M., Cai, Z., Lanctot, A., Slininger, P.J., Liu, Z.L., Gorsich, S.W., 2010. Furfural induces reactive oxygen species accumulation and cellular damage in *Saccharomyces cerevisiae*. Biotechnol. Biofuels 3, 1–10.

Almario, M.P., Reyes, L.H., Kao, K.C., 2013. Evolutionary Engineering of *Saccharomyces cerevisiae* for Enhanced Tolerance to Hydrolysates of Lignocellulosic Biomass. Biotechnol. Bioeng. 110, 2616–2623.

Almeida, J.R.M., Bertilsson, M., Gorwa-Grauslund, M.F., Gorsich, S., Lidén, G., 2009. Metabolic effects of furaldehydes and impacts on biotechnological processes. Appl. Microbiol. Biotechnol. 82, 625–638.

Banerjee, N., Bhatnagar, R., Viswanathan, L., 1981. Inhibition of Glycolysis by Furfural in *Saccharomyces cerevisiae*. Eur. J. Appl. Microbiol. Biotechnol. 11, 226–228.

Brown, S.W., Oliver, S.G., 1982. Isolation of Ethanol-Tolerant Mutants of Yeast by Continuous Selection. Eur. J. Appl. Microbiol. Biotechnol. 16, 119–122. doi:10.1007/BF00500738

Cakar, Z., Seker, U., Tamerler, C., Sonderegger, M., Sauer, U., 2005. Evolutionary engineering of multiple-stress resistant. FEMS Yeast Res. 5, 569–578. doi:10.1016/j.femsyr.2004.10.010

Conrad, T.M., Lewis, N.E., Palsson, B.Ø., 2011. Microbial laboratory evolution in the era of genome-scale science. Mol. Syst. Biol. 7, 1–11.

De Angelis, M., Gobbetti, M., 2004. Environmental stress responses in Lactobacillus: a review. Proteomics 4, 106–122.

Demeke, M.M., Dietz, H., Li, Y., Foulquié-Moreno, M.R., Mutturi, S., Deprez, S., Den Abt, T., Bonini, B.M., Liden, G., Dumortier, F., Verplaetse, A., Boles, E., Thevelein, J.M., 2013. Development of a D-xylose fermenting and inhibitor tolerant industrial *Saccharomyces cerevisiae* strain with high performance in lignocellulose hydrolysates using metabolic and evolutionary engineering. Biotechnol. Biofuels 6, 1–24.

Dhar, R., Sägesser, R., Weikert, C., Yuan, J., Wagner, A., 2011. Adaptation of *Saccharomyces cerevisiae* to saline stress through laboratory evolution. J. Evol. Biol. 24, 1135–1153.

Dragosits, M., Mattanovich, D., 2013. Adaptive laboratory evolution - principles and applications for biotechnology. Microb. Cell Fact. 12, 1–17.

Elsholz, A.K.W., Hempel, K., Pöther, D.-C., Becher, D., Hecker, M., Gerth, U., 2011. CtsR inactivation during thiol-specific stress in low GC, Gram+ bacteria. Mol. Microbiol. 79, 772–785.

Fowler, R.G., Schaaper, R.M., 1997. The role of the *mutT* gene of *Escherichia coli* in maintaining replication fidelity. FEMS Microbiol. Rev. 21, 43–54.

Gilbert, H.F., 2011. Protein Disulfide Isomerases. Encycl. Life Sci.

Glebes, T.Y., Sandoval, N.R., Reeder, P.J., Schilling, K.D., Zhang, M., Gill, R.T., 2014. Genome-Wide Mapping of Furfural Tolerance Genes in *Escherichia coli*. PLoS One 9.

Grimberg, J., Maguire, S., Belluscio, L., 1989. A simple method for the preparation of plasmid and chromosomal *E. coli* DNA. Nucleic Acids Res. 17, 8893.

Guzzo, J., Jobin, M.P., Delmas, F., Fortier, L.C., Garmyn, D., Tourdot-Marechal, R., Lee, B., Divies, C., 2000. Regulation of stress response in *Oenococcus oeni* as a function of environmental changes and growth phase. Int. J. Food Microbiol. 55, 27–31.

Hawkins, G.M., Doran-Peterson, J., 2011. A strain of *Saccharomyces cerevisiae* evolved for fermentation of

lignocellulosic biomass displays improved growth and fermentative ability in high solids concentrations and in the presence of inhibitory compounds. Biotechnol. Biofuels 4, 1–14.

Heer, D., Sauer, U., 2008. Identification of furfural as a key toxin in lignocellulosic hydrolysates and evolution of a tolerant yeast strain. Microb. Biotechnol. 1, 497–506.

Hufner, E., Markieton, T., Chaillou, S., Crutz-Le Coq, A.-M., Zagorec, M., Hertel, C., 2007. Identification of *Lactobacillus sakei* Genes Induced during Meat Fermentation and Their Role in Survival and Growth. Appl. Environ. Microbiol. 73, 2522–2531.

Jia, K., Zhang, Y., Li, Y., 2010. Systematic engineering of microorganisms to improve alcohol tolerance. Eng. Life Sci. 10, 422–429.

Khan, Q.A., Hadi, S.M., 1993. Effect of furfural on plasmid DNA. Biochem. Mol. Biol. Int. 29, 1153–1160.

Kilstrup, M., Jacobsen, S., Hammer, K., Vogensen, F.K., 1997. Induction of Heat Shock Proteins DnaK, GroEL, and GroES by Salt Stress in *Lactococcus lactis*. Appl. Environ. Microbiol. 63, 1826–1837.

Lemos, J. a, Chen, Y.Y., Burne, R. a, 2001. Genetic and Physiologic Analysis of the groE Operon and Role of the HrcA Repressor in Stress Gene Regulation and Acid Tolerance in *Streptococcus mutans*. J. Bacteriol. 183, 6074–6084.

Lim, E.M., Ehrlich, S.D., Maguin, E., 2000. Identification of stress-inducible proteins in *Lactobacillus delbrueckii* subsp. *bulgaricus*. Electrophoresis 21, 2557–2561.

Liu, S., Bischoff, K.M., Leathers, T.D., Qureshi, N., Rich, J.O., Hughes, S.R., 2012. Adaptation of lactic acid bacteria to butanol. Biocatal. Agric. Biotechnol. 1, 57–61.

Liu, Z.L., Slininger, P.J., Dien, B.S., Berhow, M. a, Kurtzman, C.P., Gorsich, S.W., 2004. Adaptive response of yeasts to furfural and 5-hydroxymethylfurfural and new chemical evidence for HMF conversion to 2,5-bis-hydroxymethylfuran. J. Ind. Microbiol. Biotechnol. 31, 345–352.

Liu, Z.L., Slininger, P.J., Gorsich, S.W., 2005. Enhanced Biotransformation of Furfural and Hydroxymethylfurfural by Newly Developed Ethanologenic Yeast Strains. Appl. Biochem. Biotechnol. 121-124, 451–460.

Looijesteijn, P.J., Trapet, L., De Vries, E., Abee, T., Hugenholtz, J., 2001. Physiological function of exopolysaccharides produced by *Lactococcus lactis*. Int. J. Food Microbiol. 64, 71–80.

Lu, J., Holmgren, A., 2014. The thioredoxin antioxidant system. Free Radic. Biol. Med. 66, 75–87.

Martín, C., Marcet, M., Almazán, O., Jönsson, L.J., 2007. Adaptation of a recombinant xylose-utilizing *Saccharomyces cerevisiae* strain to a sugarcane bagasse hydrolysate with high content of fermentation inhibitors. Bioresour. Technol.

Martinussen, J., Wadskov-Hansen, S.L.L., Hammer, K., 2003. Two Nucleoside Uptake Systems in *Lactococcus lactis*: Competition between Purine Nucleosides and Cytidine Allows for Modulation of intracellular nucleotide pools. Microbiology 185, 1503–1508.

Mussatto, S.I., Roberto, I.C., 2004. Alternatives for detoxification of diluted-acid lignocellulosic hydrolyzates for use in fermentative processes: a review. Bioresour. Technol. 93, 1-10.

Nair, S., Derré, I., Msadek, T., Gaillot, O., Berche, P., 2000. CtsR controls class III heat shock gene expression in the human pathogen *Listeria monocytogenes*. Mol. Microbiol. 35, 800–811.

Ozturk, S., Aslim, B., 2010. Modification of exopolysaccharide composition and production by three cyanobacterial isolates under salt stress. Environ. Sci. Pollut. Res. Int. 17, 595–602.

Papagianni, M., 2012. Metabolic engineering of lactic acid bacteria for the production of industrially important compounds. Comput. Struct. Biotechnol. J. 3, 1–8.

Parawira, W., Tekere, M., 2011. Biotechnological strategies to overcome inhibitors in lignocellulose hydrolysates for ethanol production: review. Crit. Rev. Biotechnol. 31, 20–31.

Patel, S., Majumder, A., Goyal, A., 2012. Potentials of Exopolysaccharides from Lactic Acid Bacteria. Indian J. Microbiol. 52, 3–12.

Popham, D.L., Young, K.D., 2003. Role of penicillin-binding proteins in bacterial cell morphogenesis. Curr. Opin. Microbiol. 6, 594–599.

Serata, M., Iino, T., Yasuda, E., Sako, T., 2012. Roles of thioredoxin and thioredoxin reductase in the resistance to oxidative stress in *Lactobacillus casei*. Microbiology 158, 953–962.

Shinagawa, H., Shiba, T., Iwasaki, H., Makino, K., Takahagi, M., Nakata, a., 1991. Properties of the *Escherichia coli* RuvA and RuvB proteins involved in DNA repair, recombination and mutagenesis. Biochimie 73, 505–507.

Shui, Z.-X., Qin, H., Wu, B., Ruan, Z., Wang, L., Tan, F.-R., Wang, J.-L., Tang, X.-Y., Dai, L.-C., Hu, G.-Q., He, M.-X., 2015. Adaptive laboratory evolution of ethanologenic *Zymomonas mobilis* strain tolerant to furfural and acetic acid inhibitors. Appl. Microbiol. Biotechnol. 99, 5739–5748.

Silva, J., Carvalho, a. S., Ferreira, R., Vitorino, R., Amado, F., Domingues, P., Teixeira, P., Gibbs, P. A., 2005. Effect of the pH of growth on the survival of *Lactobacillus delbrueckii* subsp. *bulgaricus* to stress conditions during spray-drying. J. Appl. Microbiol. 98, 775–782.

Smith, J., van Rensburg, E., Görgens, J.F., 2014. Simultaneously improving xylose fermentation and tolerance to lignocellulosic inhibitors through evolutionary engineering of recombinant *Saccharomyces cerevisiae* harbouring xylose isomerase. BMC Biotechnol. 14, 1–17.

Stanley, D., Fraser, S., Chambers, P.J., Rogers, P., Stanley, G.A., 2010. Generation and characterisation of stable ethanol-tolerant mutants of *Saccharomyces cerevisiae*. J. Ind. Microbiol. Biotechnol. 37, 139–149.

Steiner, P., Sauer, U., 2003. Long-term Continuous Evolution of Acetate Resistant *Acetobacter aceti*. Biotechnol. Bioeng. 84, 40–44.

Taddei, F., Hayakawa, H., Bouton, M., Cirinesi, A., Matic, I., Sekiguchi, M., Radman, M., 1997. Counteraction by MutT Protein of Transcriptional Errors Caused by Oxidative Damage. Science. 278, 128–130.

Van Bokhorst-van de Veen, H., Bongers, R.S., Wels, M., Bron, P. a, Kleerebezem, M., 2013. Transcriptome signatures of class I and III stress response deregulation in *Lactobacillus plantarum* reveal pleiotropic adaptation. Microb. Cell Fact. 12, 1–15.

Varmanen, P., Ingmer, H., Vogensen, F.K., 2000. *ctsR* of *Lactococcus lactis* encodes a negative regulator of *clp* gene expression. Microbiology 146, 1447–1455.

Wallace-Salinas, V., Gorwa-Grauslund, M.F., 2013. Adaptive evolution of an industrial strain of *Saccharomyces cerevisiae* for combined tolerance to inhibitors and temperature. Biotechnol. Biofuels. 6, 1-9.

Wieme, A., Cleenwerck, I., Van Landschoot, A., Vandamme, P., 2012. *Pediococcus lolii* DSM 19927T and JCM 15055T are strains of *Pediococcus acidilactici*. Int. J. Syst. Evol. Microbiol. 62, 3105–3108.

Woodbury, R., Haldenwang, W.., 2003. HrcA is a negative regulator of the *dnaK* and *groESL* operons of *Streptococcus pyogenes*. Biochem. Biophys. Res. Commun. 302, 722–727.

Wright, J., Bellissimi, E., de Hulster, E., Wagner, A., Pronk, J.T., Van Maris, A.J. a, 2011. Batch and continuous culture-based selection strategies for acetic acid tolerance in xylose-fermenting *Saccharomyces cerevisiae*. FEMS Yeast Res. 11, 299–306.

Yomano, L.P., York, S.W., Ingram, L.O., 1998. Isolation and characterization of ethanol-tolerant mutants of *Escherichia coli* KO11 for fuel ethanol production. J. Ind. Microbiol. Biotechnol. 20, 132–138.

Zdzienicka, M., Tudek, B., Zielenska, M., Szymczyk, T., 1978. Mutagenic Activity of Furfural in *Salmonella typhimurium* TA100. Mutat. Res. 58, 205–209.

Zotta, T., Asterinou, K., Rossano, R., Ricciardi, A., Varcamonti, M., Parente, E., 2009. Effect of inactivation of stress response regulators on the growth and survival of *Streptococcus thermophilus* Sfi39. Int. J. Food Microbiol. 129, 211–220.

Aarnio, T.H., Suihko, M.L., Kauppinen, V.S., 1991. Isolation of Acetic Acid-Tolerant Baker's Yeast Variants in a Turbidostat. Appl. Biochem. Biotechnol. 27, 55–63.

Chapter 6

Transcriptomics analysis of furfural stress response in *Pediococcus acidilactici* DSM 20284

6.1. Introduction

Lignocellulosic biomass is a cheap, abundant and renewable resource that can be used for the production of fuels and valuable chemicals, and it does not compete with land used for the production of food (Winkler and Kao, 2011). Lignocellulosic feedstocks, however, need to be processed before being available for fermentation by microorganisms. The processing usually consists of a pretreatment step during which the lignocellulosic structures are broken down to sugars: hexoses and pentoses. During this step, however, substances often being growth inhibitors for fermenting microorganisms are released as well. One of the main inhibitors, arising as a dehydration product of xylose, is furfural. Furfural was shown to cause DNA damage, inhibit or inactivate glycolytic enzymes, disrupt transcriptional regulation and induce oxidative stress and damage to cell membranes (Allen et al., 2010; Almeida et al., 2009; Glebes et al., 2014; Parawira and Tekere, 2011; Park et al., 2015).

To survive harsh environmental conditions such as extreme pH or temperature, scarce nutrient sources, oxygen levels, or the presence of toxic or inhibitory substances, bacteria have developed complex mechanisms helping them to cope with the stresses. By altering their gene expression, organisms can adjust their metabolism in response to changes in environmental conditions (Ferenci and Spira, 2007). For instance, in *S. cerevisiae,* the presence of furfural in the growth media caused upregulation of the stress-responsive genes (including genes associated with anti-oxidant stress, DNA repair and redox metabolism) and downregulated transcriptional and translational control genes as well as genes involved in metabolism of essential chemicals (Li and Yuan, 2010; Ma and Liu, 2010). In *E. coli*, furfural was found to limit the assimilation of sulfur, and its presence upregulated genes and regulators associated with cysteine and methionine biosynthesis, while many other biosynthetic genes were downregulated (Miller et al., 2009). In *Zymomonas mobilis*, over 400 genes were found to have altered expression levels in response to furfural; the differentially expressed genes were mainly associated with cell wall and membrane biosynthesis, DNA repair system, metabolism and transcription (He et al., 2012).

High-throughput technologies developed for biotechnology and systems biology such as microarrays or RNA sequencing (RNA-seq) offer efficient approaches for understanding complex biological processes. By measuring the transcriptome (all mRNA in a cell at any given time) one can uncover all actively expressed genes in a microorganism. By comparing the genes differentially expressed at various environmental

conditions, it is possible to unravel biological functions of some genes and get an insight into gene regulatory networks (Horgan and Kenny, 2011).

P. acidilactici was demonstrated to be highly tolerant to various inhibitors originating from the pretreatment of lignocellulosic biomass, and also to be capable of utilizing both hexoses and pentoses with similar growth rates. For this reason, *P. acidilactici* is a promising microorganism suitable for future use as a cell factory converting 2nd generation biomass into value-added products.

To provide insight into the mechanisms involved in furfural tolerance in *P. acidilactici*, this study presents transcriptional analysis of *P. acidilactici* DSM 20284 (WT) and the adapted strain A28 in response to furfural stress. The study also aimed at investigating the differences in gene expression between the wild-type and the adapted strain growing in MRS without furfural for an improved understanding of mechanisms behind the adapted strain's faster growth on rich MRS medium.

6.2. Materials and Methods

6.2.1. Strains and growth conditions

The wild-type *Pediococcus acidilactici* DSM 20284 strain and mutant strain A28 adapted to furfural and acetic acid were grown in MRS medium at 30°C. Growth experiments were performed in 3 biological replicates in 100 mL of MRS medium with magnetic stirring at 220 rpm. The growth of strains was followed by regular optical density (OD_{600}) measurements. At an OD_{600} of ~0.3, furfural was added to the cultures to a final concentration of 0.88 g/L. This concentration was selected as it was earlier found (data not shown) to give ~10% change in growth rate for both the wild-type and the adapted strain. During growth experiments, cell samples were taken: first samples were taken 10 min before adding furfural; second samples were taken 10 min after furfural addition; for the wild-type strain, third samples were collected 60 min after furfural addition.

6.2.2. Cell harvest

For the isolation of RNA, 25 mL samples of exponentially growing cells were transferred to pre-chilled flasks. The cells were centrifuged at 4000 rpm for 15 min at 4°C. The cells' pellets were resuspended in 2 mL of ice-cold 0.9% NaCl and then collected by centrifugation at 6000 g for 5 min at 4°C. The pellets were kept at -80°C.

6.2.3. RNA purification

For extraction of RNA, the following protocol was followed. 400 µL of phenol/acetate (phenol saturated with 50mM sodium acetate, pH 4.8) was added to 0.5 mL glass beads (106 microns, acid washed; Sigma), mixed and preheated to 65°C. The cell pellets were resuspended in 200 uL ice cold RNA solution I (0.3M sucrose, 0.01M sodium acetate, pH 4.8). Then 200 uL of preheated RNA solution II (2% SDS, 0.01M sodium acetate, pH 4.8) was added, the cell suspensions were transferred to preheated glass beads/phenol, and vortexed 3 times for 45 s at speed 4 in FastPrep instrument with 1 min intervals. Following 5 min incubation on ice, the suspensions were kept at 65°C for 3 min, vortexed and cooled on dry ice for 3 min. After centrifuging at 13 000 g for 5 min at room temperature, the appearing upper phases were transferred to new Eppendorf tubes. The following procedure was repeated two more times: 400 uL of phenol/acetate

solution was added, vortexed and incubated for 3 min at 65°C; vortexed again, cooled on dry ice for 3 min, and centrifuged at 13 000 g for 5 min at room temperature. The upper phases were then again transferred to new Eppendorf tubes. Then 400 µL of phenol/acetate:chloroform (1:1) was added, vortexed, centrifuged at 13 000 g for 5 min at room temperature, and the upper phases were transferred to new Eppendorf tubes. Finally, 40 µL of 3M sodium acetate and 900 µL of 96% ethanol were added, the samples were vortexed and placed for 5 min at -20°C. The samples were centrifuged at 13 000 g for 15 min at 4°C. The pellets were washed carefully with 500 µL 70% ethanol and centrifuged again at 13 000 g for 5 min. The pellets were dried briefly (10 min) in a vacuum centrifuge, the RNA was dissolved in 25 µL RNAse-free water and frozen at -80°C.

The RNA quantity and quality were assessed using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). For the examination of RNA integrity 2100 BioAnalyzer (Agilent) was used.

6.2.4. Microarray design

The custom DNA microarray covering all known and putative genes from *Pediococcus acidilactici* DSM 20284 was designed using an online Agilent tool, eArray (http://earray.chem.agilent.com). Each gene was covered by two different 60-mer probes designed using the best distribution methodology; each probe was replicated on the slide 4 times, except for 28 probes which were replicated 3 times on the slide. The Cy3 labelling and cDNA hybridization was performed by DMAC (DTU, Kgs. Lyngby, Denmark).

6.2.5. Data processing and analysis

Data preprocessing was done using the statistical software R (R Core Team, 2015) and Bioconductor (Gentleman et al., 2004). Linear models and empirical Bayesian methods from the Limma package (Smyth, 2005) were used for testing for differentially expressed genes and to adjust for multiple-testing. The density plots before and after normalization are shown in 8.2.1 in the Appendix. Genes were considered to be significantly differentially expressed when the fold change was at least 1.5 and the adjusted p values were less than 0.05.

6.3. Results and Discussion

P. acidilactici cells were grown exponentially in MRS medium, and at an OD_{600} of around 0.3 the strains were stressed by the addition of 0.88 g/L furfural. The concentration of furfural to be added was selected carefully to influence the growth rate only slightly (by around 10%), since higher perturbations in the growth rate might induce additional metabolic alterations caused by slower growth rather than by furfural stress. Samples were taken 10 minutes before the addition of furfural, 10 minutes after the addition of furfural, and for the wildtype also 60 minutes after the stress (Figure 6.1).

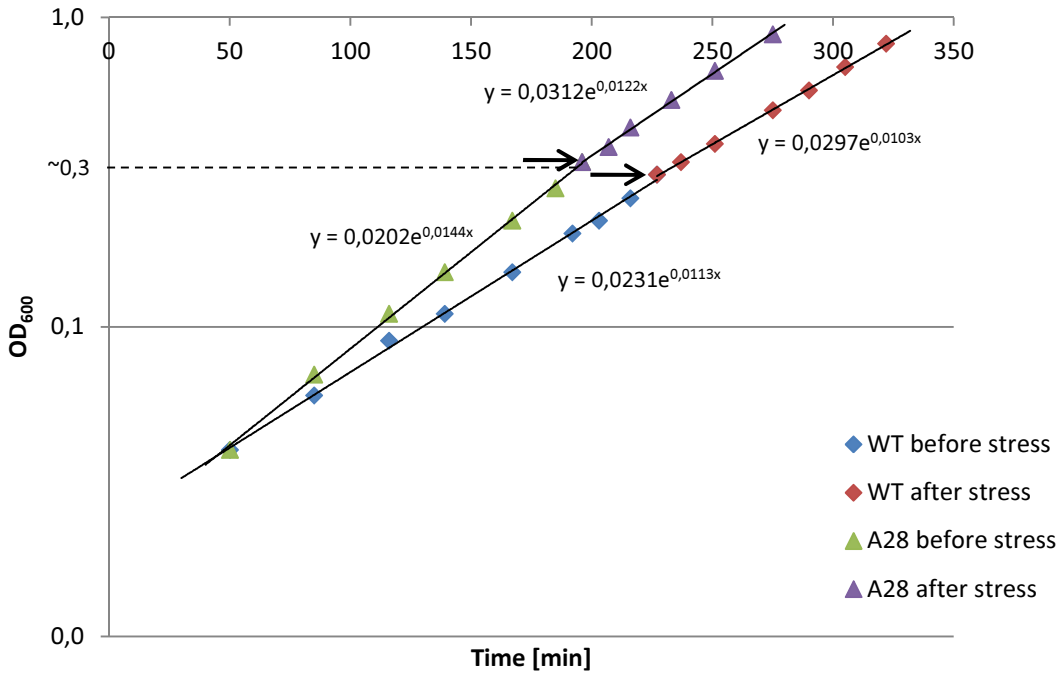


Figure 6.1. Growth curves of the wild-type strain (WT) and adapted strain (A28). Black arrows indicate time points, were furfural was added to the cultures to the final concentration of 0.88 g/L.

6.3.1. Transcriptomic profile of the wild-type *P. acidilactici* during furfural-induced stress

Global gene expression profiles were compared between the controls and the stressed strains. The cutoff values were set at a fold change of the gene expression of at least 1.5 and the Benjamini adjusted p value was set to be lower than 0.05. Under these conditions, 7 and 23 genes were found to be differentially expressed in the wild-type strain DSM 20284 after 10 min and 60 min of exposure to furfural, respectively. Most of the genes were found to be down-regulated in stress conditions; only 4 genes were up-regulated when furfural was present in the growth medium. For the adapted strain A28, there were 14 statistically significant differentially expressed genes identified; 8 of them were down-regulated whereas 6 of them were up-regulated when furfural was present in the medium. The list of all differentially regulated genes in the wild-type strain 10 and 60 minutes after the addition of furfural is presented in Table 6.1.

The data analysis revealed a down-regulation of all the components of the arginine deiminase (ADI) pathway in the furfural stressed wild-type strain. The arginine deiminase pathway consists of three enzymes: arginine deiminase, ornithine carbamoyltransferase and carbamate kinase (Figure 6.2). In several species, including *Lactococcus lactis* and *Lactobacillus sakei*, the operon includes also a fourth component which catalyzes the arginine-ornithine exchange (Girgis et al., 2003). This membrane-bound antiporter was found to be down-regulated along with the rest of the genes from the ADI operon in the wild-type strain of *P. acidilactici*. The ADI pathway metabolizes arginine to produce ornithine, ammonia and carbon dioxide with the formation of ATP. Arginine is utilized via ADI pathway mainly for the production of energy; however, by producing ammonia, the pathway was also shown to work against acidification of the environment (Rimaux et al., 2012). Indeed, the pathway was demonstrated to function at a very low pH (Casiano-Colon and Marquis, 1988). The ADI pathway has been shown to be active also under other types

of stresses, including temperature and salt stress in *Lactobacillus fermentum* (Vrancken et al., 2009). The pathway was triggered in response to various factors, among others the availability of arginine, depletion of energy, catabolite repression, oxygenation or low pH (van de Guchte et al., 2002). Consistent down-regulation of the full operon encoding the arginine deiminase pathway might indicate a role of this pathway in organism's response to furfural-induced stress. Another explanation could be that the strain prefers to utilize the arginine for biosynthetic processes rather than to convert it into ornithine, ammonia and ATP, which would allow the organism for a faster growth.

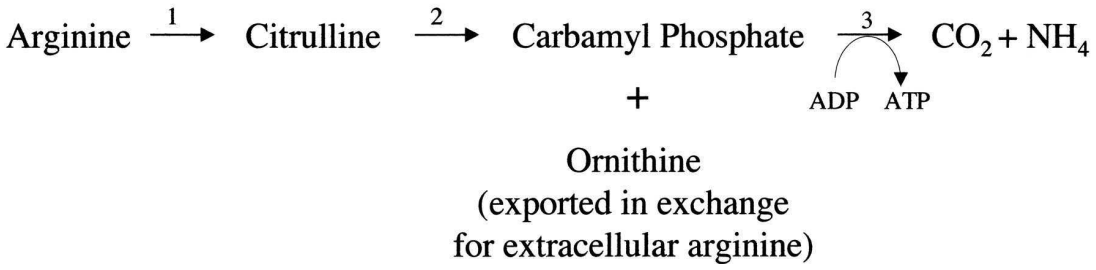


Figure 6.2. Arginine deiminase (ADI) pathway. (1) arginine deiminase; (2) ornithine carbamoyltransferase; (3) carbamate kinase (Rodney, 2001).

Two alkaline-shock proteins were found to be repressed after addition of furfural. The alkaline-shock proteins, as their name implies, are involved in stress response to alkaline pH. Similar proteins were identified in *Lactobacillus plantarum* and are expected to be involved in the strain's pH tolerance (Liu et al., 2015). Interestingly, 4 other genes located in a close proximity to the two repressed alkaline-shock proteins were also down-regulated when furfural was added. The genes encoded hypothetical proteins, all predicted to be integral membrane components. The genes could be co-regulated in an operon and could possibly be involved in the strain's response to furfural.

Also, a PTS mannose transporter, which is a major glucose transporter in many LAB, was found to be down-regulated during furfural-related stress conditions. This finding might indicate that the cell's metabolism slows down during furfural stress. However, the gene was only repressed 10 minutes after the addition of furfural, and was not differentially expressed 60 min after the onset of stress conditions.

Among other significantly down-regulated genes there was a hypothetical protein HMPREF0623_1577. A protein BLAST search, however, revealed 50% identity to various CsbD proteins in other Gram positive species, including *Lactobacillus kimchicus*, *Lactobacillus brevis*, *Brevibacterium mcbrellneri* and *Leuconostoc mesenteroides*. CsbD protein is a general stress response protein in bacteria; yet, it's exact function stays unknown (Akbar et al., 1999).

Another down-regulated gene was found to be a DNA recombination and repair protein RecF. This protein is involved in single-strand DNA repair and is required for the induction of the SOS response (Mckenzie et al., 2000). As furfural was shown to cause DNA damage, RecF could be expected to be up-regulated during stress conditions. However, it was found to be repressed rather than induced during furfural-induced stress. Interestingly, it was not differentially regulated 10 min after the addition of furfural; it became down-regulated 60 min after the stress.

Glutathione reductase was also found among the genes repressed during furfural stress. Glutathione reductase catalyzes reduction of glutathione disulfide to glutathione, which is believed to be the cell's key antioxidant molecule. By conversion between the oxidized and reduced forms of glutathione, it acts to maintain intracellular redox homeostasis and is involved in the cell's response to oxidative stress (Jänsch et al., 2007; Li et al., 2003). Indeed, the activity of glutathione reductase increased during oxidative stress in *Lactobacillus sanfranciscensis* (Jänsch et al., 2007); it was also shown to be affected by osmotic stress in *E.coli* (Smirnova et al., 2001). However, this study showed an opposite trend. The glutathione reductase was found to be down-regulated after addition of furfural. Interestingly, similarly to the RecF protein, the gene was not differentially expressed 10 min after the addition of furfural, but became down-regulated with time.

6.3.2. Transcriptomic profile of the adapted strain A28 in response to furfural-induced stress

When the gene expression profiles of the adapted strain were compared between the stress and non-stress conditions, it was easy to notice that the nucleotide metabolism-related genes were down-regulated during furfural-induced stress (Table 6.2). However, when the adapted strain was analyzed relative to the wild-type strain, the nucleotide metabolism genes were found to be highly up-regulated. Since the genes were slightly less induced during stress conditions compared to the non-stress conditions, they appear down-regulated during stress when the two conditions for the adapted strain are compared; however, that only results from the selected reference.

Furthermore, similarly to what was demonstrated for the wild-type strain in stress conditions, the genes encoding the PTS mannose transporters were slightly down-regulated. Among other genes differentially expressed during stress conditions, there were ribosome-binding factor A, which was down-regulated during furfural stress, an NADPH-dependent oxidoreductase and a camphor resistance protein, which were both induced in the presence of furfural. The ribosome-binding factor A was suggested to be involved in ribosomal maturation or the initiation of a translation process; it was also demonstrated to be a cold-shock protein that, if absent, would induce a cold-shock response in *E. coli* (Jones and Inouye, 1996). An up-regulation of an oxidoreductase might indicate that the cell tries to cope with oxidative stress. The function of the camphor resistance protein in uncharacterized; however, its overexpression in *E. coli* was linked to camphor resistance and chromosome condensation (Hu et al., 1996).

Both the fact that only a handful of genes were found to be differentially expressed during furfural-induced stress conditions, as well as the low level of their induction or repression suggest that neither the wild-type nor the adapted strain were highly affected by the presence of furfural in the growth medium. This finding indicates that the selected concentration of furfural did not activate the stress response system in any of the two strains. As shown in Chapter 2, *P. acidilactici* strain was found to be among the most robust strains, being able to resist as high furfural concentrations as 7 g/L; whereas furfural concentrations up to 4 g/L caused less than 50% drop in its growth rate. In addition, as the strain was initially isolated from barley, it is possible that it was pre-adapted to deteriorating plant material and thus showed intrinsic high resistance to furfural. Accordingly, low furfural concentrations would not affect the strain to a high extent. It was observed, however, that the addition of 0.88 g/L furfural, resulted in a reduction in the strain's growth rate. The reason behind the slower growth might be the direct effect of furfural on the bacterial cell wall as a certain amount of energy needed to be employed for the protection of cell wall instead of for growth. The furfural effect on the cell wall, however, might have occurred to be too mild to induce the stress response.

Table 6.1. Differentially expressed genes in the wild-type strain DSM 20284 10 minutes and 60 minutes after the addition of furfural.

			10 min		60 min	
Systematic Name	**Locus Tag**	**Annotation**	**logFC**	**Adj. p.value**	**logFC**	**Adj. p.value**
EFL95962	HMPREF0623_0013	cell wall anchor protein	-0,60	1,70E-02		
EFL95966	HMPREF0623_0017	deoxyadenosine kinase			0,73	1,72E-03
EFL96296	HMPREF0623_0347	DUF378 domain-containing protein			-0,78	1,73E-03
EFL96352	HMPREF0623_0403	hypothetical protein			-0,71	1,19E-04
EFL96391	HMPREF0623_0442	NAD(FAD)-dependent dehydrogenase			-0,66	2,55E-02
EFL96513	HMPREF0623_0564	glucose starvation-inducible protein B			-0,80	2,33E-03
EFL95651	HMPREF0623_0687	aquaporin			0,71	3,32E-04
EFL95936	HMPREF0623_0972	PTS mannose transporter subunit EIIAB	-0,67	1,19E-03		
EFL95383	HMPREF0623_1120	alkaline-shock protein			-1,02	1,78E-04
EFL95384	HMPREF0623_1121	alkaline-shock protein	-0,61	2,72E-02	-1,04	2,45E-06
EFL95384	HMPREF0623_1121	alkaline-shock protein	-0,61	2,72E-02		
EFL95385	HMPREF0623_1122	hypothetical protein			-1,04	1,90E-05
EFL95386	HMPREF0623_1123	hypothetical protein			-0,77	1,61E-03
EFL95387	HMPREF0623_1124	hypothetical protein			-0,94	4,52E-03
EFL95388	HMPREF0623_1125	membrane protein	-0,54	5,43E-04	-0,90	2,00E-09
EFL95423	HMPREF0623_1160	lipoprotein			-0,72	1,36E-03
EFL95442	HMPREF0623_1179	nucleoside transporter			0,92	1,56E-02
EFL95584	HMPREF0623_1321	DNA recombination protein RecF			-0,69	1,87E-03
EFL95611	HMPREF0623_1348	ornithine carbamoyltransferase	-0,72	1,47E-02		
EFL95612	HMPREF0623_1349	carbamate kinase	-0,68	2,18E-02		
EFL95613	HMPREF0623_1350	arginine deiminase	-0,75	1,03E-04	-0,65	4,05E-04
EFL95614	HMPREF0623_1351	amino acid permease	-0,74	7,36E-04		
EFL95094	HMPREF0623_1406	manganese catalase			-0,65	3,40E-02
EFL95189	HMPREF0623_1501	peptidase			-1,13	4,08E-05
EFL95190	HMPREF0623_1502	hypothetical protein			-0,75	1,14E-02
EFL95071	HMPREF0623_1577	hypothetical protein			-0,89	2,98E-03
EFL95087	HMPREF0623_1593	glutathione reductase			-0,70	3,73E-04
EFL95090	HMPREF0623_1596	integrase			0,61	4,14E-02
EFL94911	HMPREF0623_1779	hypothetical protein			-1,37	2,14E-12

Table 6.2. Differentially expressed genes in the adapted strain A28 10 minutes after the addition of furfural.

Systematic Name	Locus Tag	Annotation	logFC	Adj. p.value
EFL94776	HMPREF0623_1644	glutamine--fructose-6-phosphate aminotransferase	0,81	1,09E-02
EFL94868	HMPREF0623_1736	amino acid permease	0,71	5,93E-03
EFL94869	HMPREF0623_1737	adenine deaminase	-0,77	1,40E-04
EFL94870	HMPREF0623_1738	guanine permease	-0,60	8,06E-04
EFL94871	HMPREF0623_1739	adenosine deaminase	-1,04	2,22E-03
EFL94968	HMPREF0623_1836	ABC transporter	-0,81	5,94E-04
EFL95337	HMPREF0623_1074	hypothetical protein	0,61	1,64E-03
EFL95934	HMPREF0623_0970	PTS mannose transporter subunit IID	-0,63	1,10E-05
EFL95935	HMPREF0623_0971	PTS mannose/fructose/sorbose transporter subunit IIC	-0,62	5,90E-04
EFL96139	HMPREF0623_0190	dUTPase	-0,59	4,11E-02
EFL96285	HMPREF0623_0336	ribosome-binding factor A	-0,87	1,66E-05
EFL96342	HMPREF0623_0393	camphor resistance protein CrcB	0,74	3,09E-03
EFL96512	HMPREF0623_0563	NADPH-dependent oxidoreductase	0,63	3,79E-03
EFL96609	HMPREF0623_0660	hypothetical protein	0,81	9,24E-03

6.3.3. Transcriptomic analysis of the adapted strain

Additional comparison was done between the unstressed wild-type strain and the unstressed adapted strain to elucidate any changes in the transcriptome induced by an earlier performed adaptation experiment, which allowed the adapted strain both a higher growth rate in MRS and a better tolerance to furfural. 314 genes were identified to be differentially regulated in the wild-type strain DSM 20284 compared to the adapted strain A28 when grown in MRS medium without the addition of furfural. 167 genes were found to be down-regulated, while 147 genes were up-regulated in the adapted strain. A selection of several differentially expressed genes selected as the most critical to the adapted strain's higher growth rate and enhanced furfural tolerance are shown in Table 6.3. A complete list of the genes found to be differentially regulated in the adapted strain both in non-stress conditions and during furfural stress, are listed in Table S7 in the Appendix; the lists with 50 most up- and down-regulated genes are presented in Appendix in Table S8 and Table S9, respectively.

For functional analysis, the differently expressed genes were categorized using Clusters of Orthologous Groups (COG). The majority of genes was not assigned to any category, or was assigned to the COG category S (function unknown) (Figure 6.3). The next most represented COG category was category G (carbohydrate metabolism and transport). This implies that the faster growth of the adapted strain may result from a more efficient metabolism and transport of sugars. Most of the genes belonging to this COG category were repressed in the adapted strain; these were, however, mainly PTS system sugar transporters. Among the few up-regulated genes in the adapted strain, there were glucokinase, fructose-bisphosphate aldolase and pyruvate kinase, which are all involved in glycolysis. Glucokinase is a hexokinase enzyme highly specific for glucose, and it catalyzes the first step of glycolysis (glucose + ATP -> glucose-6-phosphate + ADP). Fructose bisphosphate aldolase catalyzes the fourth glycolysis step, converting fructose-1,6-bisphosphate into dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. Finally, pyruvate kinase catalyzes the last glycolysis step; conversion of phosphoenolpyruvate into pyruvate with the formation of ATP. It thus seems that the glycolysis is up-regulated in the adapted strain, indicating an increased flux through glycolysis.

Among other COG categories, most highly represented were those related to nucleotide, amino acid and inorganic ion metabolism and transport as well as cell wall/membrane/envelope biogenesis (COG categories F, E, P and M). The down-regulated genes were mostly found to be associated with carbohydrate and amino acid metabolism and transport (representing COG categories G and E), while the up-regulated genes were related to carbohydrate, nucleotide and inorganic ion metabolism and transport as well as signal transduction (categories G, F, P and T).

All the genes differentially expressed that were categorized to be involved in the nucleotide metabolism and transport were induced in the adapted strain compared to the wild-type strain. Adenine deaminase and guanosine monophosphate reductase were both more than 17 fold up-regulated in the adapted strain and were found to be the most highly up-regulated genes in the whole transcriptome; also adenosine deaminase showed more than 10 times up-regulation compared to the wild-type strain. Adenine and adenosine deaminases catalyze deamination of adenine to hypoxanthine and adenosine to inosine, respectively; guanosine monophosphate (GMP) reductase is involved in conversion of GMP into inosine monophosphate (IMP). All three of these enzymes play important roles in purine metabolism and their overexpression following the adaptive laboratory evolution in rich MRS medium demonstrates that

optimization of purine metabolism can significantly improve the *P. acidilactici* rate of growth. Several other enzymes, involved in purine synthesis and salvage, were also up-regulated in the adapted strain. These include, among others:

- adenylosuccinate synthase (*purA*) and adenylosuccinate lyase (*purB*) which catalyze the conversion of IMP into adenosine monophosphate (AMP) through adenyl succinate (sAMP) as an intermediate
- deoxyadenosine kinase, which catalyzes phosphorylation of deoxyadenosine into deoxyadenosine monophosphate (dAMP)
- guanine deaminase and permeases
- xanthine and uracil permeases
- xanthine and uracil phosphoribosyltransferases

The genes encoding for pyrimidine biosynthesis and salvage were also found to be induced in the adapted strain; however, they were less induced (1.5 to 2 fold up-regulation) when compared to genes encoding for purine metabolism; still, their up-regulation can be a consequence of an increased need for NTPs.

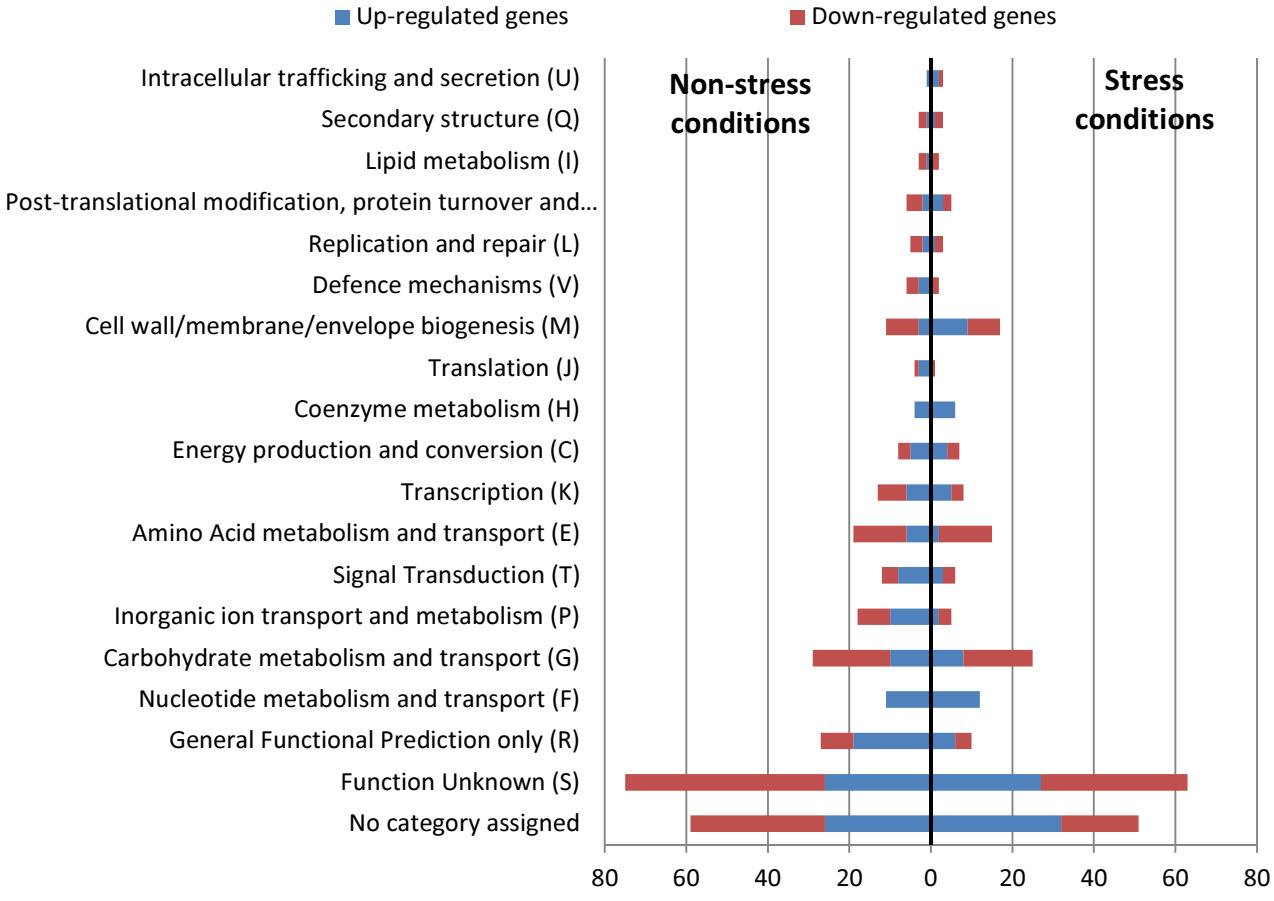


Figure 6.3. Comparison of the COG categories of genes differentially expressed in the adapted strain relative to the wild-type strain in both non-stress and stress conditions.

Out of 16 genes differentially regulated in the adapted strain categorized to the COG category E, 13 (81%) were down-regulated; most of them were annotated as amino acid transporters or permeases. Only 3

genes (19%) were found to be up-regulated in the adapted strain. These were a transporter, an amino acid permease and cysteine desulfurase SufS, which is, among others, involved in the Fe-S cluster biosynthesis (Mihara and Esaki, 2002). Indeed, the cluster of five genes encoding the SUF (sulfur assimilation) system were found to be up-regulated in the adapted strain. The operon constists of SufC (an iron-sulfur assembly ATPase), SufD (an iron-sulfur cluster assembly protein), SufS (a cysteine desulfurase), and SufB (an iron-sulfur cluster assembly protein). The cluster of genes contains also a putative iron-sulfur cluster assembly scaffold protein, SufE2. The SUF system was shown to be involved in iron-sulfur cluster assembly under stress conditions, including oxidative stress and iron starvation in *E.coli* and a plant pathogen bacteria, *Erwinia chrysanthemi* (Loiseau et al., 2003; Nachin et al., 2003; Outten et al., 2004; Zheng et al., 2001). They are considered to be sensory proteins, allowing bacteria to adapt to the changes in the environment (Aguado-Urda et al., 2013).

The ADI pathway was found to be significantly repressed in the adapted strain. Carbamate kinase, ornithine carbamoyltransferase and arginine deiminase were all 9 to 11 fold down-regulated; the arginine/ornithine antiporter *arcD* also showed 6 fold down-regulation in the adapted strain. Interestingly, the genome of *P. acidilactici* is predicted to have another gene annotated to be an arginine/ornithine antiporter and it was also down-regulated in the adapted strain. What is more, arginine repressor *argR*, which controls the expression of the arginine deiminase pathway, was found to be almost 2 fold up-regulated. The repressor usually binds to an operator site close to the *arc* promoter, activating the ADI pathway in the presence of arginine (Fulde et al., 2011). LAB often possess two transcriptional regulators of the ArgR family (Larsen et al., 2008); it was demonstrated that in some species, such as *L. plantarum* and *L. lactis*, both are required for the regulation of arginine metabolism (Larsen et al., 2004; Nicoloff et al., 2004). Indeed, the *P. acidilactici* DSM 20284 is predicted to possess two repressors; yet, the other one was not found to be differentially regulated in the adapted strain or during furfural stress. It is worth mentioning that after the addition of furfural, the genes involved in the ADI pathway remained highly repressed. Similar, but not as pronounced results of ADI pathway down-regulation, were seen in the wild-type strain under conditions of furfural stress. This finding suggests a role of the ADI pathway in furfural stress response of *P. acidilactici*; however, the exact mechanisms remain to be elucidated.

Within the COG category M, a gene related to exopolysaccharide biosynthesis was slightly up-regulated in the adapted strain relative to the wild-type. The exopolysaccharides (EPSs) were suggested to be involved in the protection of bacteria against adverse environmental conditions, allowing their survival during stress. In particular, they were proposed to play a role in protection against toxic compounds (Looijesteijn et al., 2001; Patel et al., 2012). EPSs are secreted to the extracellular environment where they accumulate stabilizing the structure of cell membrane (Mishra and Jha, 2013). Indeed, it was shown that the cell membrane might be involved in the cell's response to furfural, and that changing the membrane composition might help to minimize the detrimental effects of furfural on the cell (He et al., 2012).

Among the most highly up-regulated genes in the adapted strain, there were genes involved in folic acid biosynthesis. All the genes were more than 3 fold induced in the adapted strain relative to the wild-type. Folate is used as a cofactor in a wide variety of biosynthetic reactions (Bermingham and Derrick, 2002; Wegkamp et al., 2004). As presented in Figure 6.4, it is synthetized from three building blocks: a GTP, p-aminobenzoate and glutamate (Sybesma et al., 2003b). First five genes within the folic acid biosynthesis pathway were induced in the adapted strain. These were:

- GTP cyclohydrolase I, folE
- dihydroneopterin aldolase, folB
- 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase, folK
- Dihydropteroate synthase, folP
- tetrahydrofolate synthase, folC

The genes formed a putative operon with a hypothetical protein and a non-canonical purine NTP pyrophosphatase. In *Lactococcus lactis*, the folic acid biosynthesis operon consists of six genes; apart from the ones mentioned above, it contains an additional folA gene, encoding dihydrofolate reductase, which catalyzes the conversion of dihydrofolate into tetrahydrofolate (Sybesma et al., 2003a). *P. acidilactici* is predicted to possess a dihydrofolate reductase; however, it was not found to be differentially regulated in the adapted strain compared to the wild-type in this study.

It was demonstrated in *L. lactis* that an overexpression of GTP cyclohydrolase, the first enzyme in the folic acid biosynthesis pathway, led to an increase in folate production (Sybesma et al., 2003a). Upregulation of all the enzymes involved in folic acid biosynthesis possibly results in formation of higher levels of folate. Folate serves as a cofactor for C1 interconversion in numerous biosynthetic processes, including the biosynthesis of purines (Kilstrup et al., 2005). As described above, several enzymes involved in purine biosynthesis and salvage were significantly up-regulated in the adapted strain; thus increased amounts of folate might be necessary to support an optimized purine metabolism in the adapted strain. Moreover, in many lactic acid bacteria, several genes involved in folate metabolism are co-regulated with the genes in the purine biosynthesis pathway (Kilstrup et al., 2005).

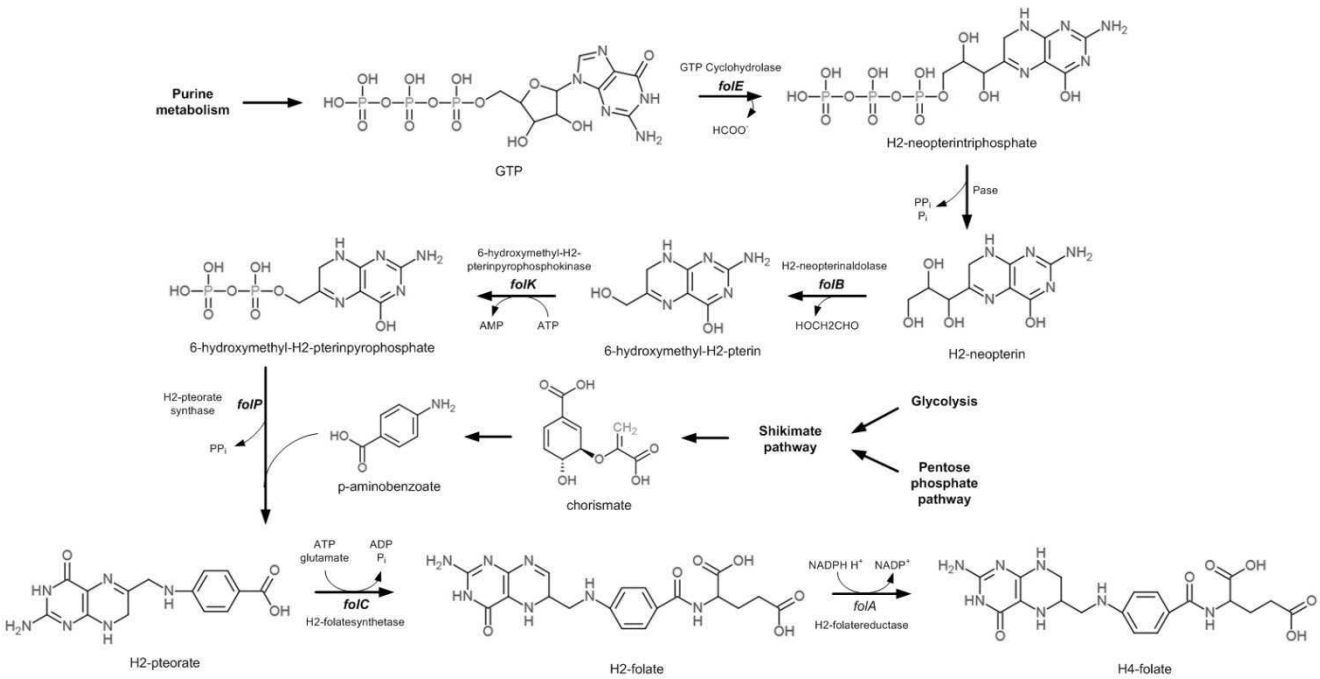


Figure 6.4. Folic acid biosynthesis pathway. Up-regulated genes are shown in bold. Figure adapted from Sybesma et al. (2003a).

Table 6.3. Selected differentially expressed genes in DSM 20284 and A28 grown in MRS with and without furfural.

			Without furfural		With furfural		
Systematic Name	**Locus Tag**	**Annotation**	**logFC**	**Adj. p.value**	**logFC**	**Adj. p.value**	**COG group**
EFL96129	HMPREF0623_0180	glucokinase	0,80	1,43E-03			G
EFL95145	HMPREF0623_1457	fructose-bisphosphate aldolase	-0,69	4,67E-02			G
EFL96469	HMPREF0623_0520	pyruvate kinase	0,81	1,74E-03			G
EFL94869	HMPREF0623_1737	adenine deaminase	4,22	7,61E-39	3,47	6,76E-32	F
EFL95022	HMPREF0623_1528	guanosine monophosphate reductase, guaC	4,13	4,58E-27	3,92	2,61E-24	F
EFL94871	HMPREF0623_1739	adenosine deaminase, add	3,35	1,15E-21	2,36	2,22E-13	F
EFL95646	HMPREF0623_0682	adenylosuccinate synthase, purA	1,46	2,29E-13	1,54	4,51E-13	F
EFL95645	HMPREF0623_0681	adenylosuccinate lyase, purB	1,69	1,21E-12	1,36	4,35E-09	F
EFL95966	HMPREF0623_0017	deoxyadenosine kinase	1,25	1,34E-09	0,76	3,16E-04	F
EFL95363	HMPREF0623_1100	xanthine/uracil permease	1,74	5,43E-14	1,67	2,80E-12	F
EFL95873	HMPREF0623_0909	Uric acid/xanthine permease	1,09	1,35E-10	0,60	1,34E-04	F
EFL95733	HMPREF0623_0769	uracil phosphoribosyltransferase, upp	0,87	4,19E-05			F
EFL95795	HMPREF0623_0831	xanthine phosphoribosyltransferase	0,62	7,20E-04			F
EFL95278	HMPREF0623_1015	CTP synthetase, pyrG	0,89	1,76E-03	0,64	4,41E-02	F
EFL96408	HMPREF0623_0459	N5-carboxyaminoimidazole ribonucleotide mutase, purE	0,68	1,04E-03			F
EFL95182	HMPREF0623_1494	pyrimidine-nucleoside phosphorylase, pdp	0,73	1,56E-04			F
EFL94751	HMPREF0623_1619	ribonucleoside-triphosphate reductase	0,63	1,22E-02			F
EFL96451	HMPREF0623_0502	nucleoside deoxyribosyltransferase	0,68	1,93E-03			F
EFL95856	HMPREF0623_0892	deoxyadenosine kinase	0,79	5,17E-05			F
EFL95441	HMPREF0623_1178	ribonucleoside hydrolase	1,13	1,57E-08	1,01	1,15E-06	F
EFL95442	HMPREF0623_1179	nucleoside transporter	1,21	3,94E-05	1,07	8,72E-04	F
EFL95364	HMPREF0623_1101	chlorohydrolase	1,66	4,51E-14	1,64	1,32E-12	F
EFL94747	HMPREF0623_1615	cysteine desulfurase, SufS	1,02	4,21E-06	0,84	5,67E-04	E
EFL94856	HMPREF0623_1724	amino acid permease	0,96	4,82E-09	0,96	8,24E-08	E
EFL96055	HMPREF0623_0106	transporter	0,84	1,33E-04			E
EFL94974	HMPREF0623_1842	Arginine/ornithine antiporter, ArcD	-0,78	1,38E-04	-0,80	9,77E-04	E

			Without furfural		With furfural		
Systematic Name	**Locus Tag**	**Annotation**	**logFC**	**Adj. p.value**	**logFC**	**Adj. p.value**	**COG group**
EFL95611	HMPREF0623_1348	ornithine carbamoyltransferase	-3,19	1,58E-24	-2,98	2,04E-21	E
EFL95612	HMPREF0623_1349	carbamate kinase	-3,46	8,23E-28	-3,13	1,26E-23	E
EFL95613	HMPREF0623_1350	arginine deiminase	-3,22	2,82E-32	-2,75	1,20E-26	E
EFL95614	HMPREF0623_1351	amino acid permease	-2,68	5,51E-24	-2,36	8,49E-20	E
EFL96005	HMPREF0623_0056	amino acid permease	-0,82	1,31E-04	-0,93	8,70E-05	E
EFL96309	HMPREF0623_0360	amino acid permease	-0,77	2,18E-05			E
EFL96523	HMPREF0623_0574	succinyl-diaminopimelate desuccinylase			-0,62	2,89E-02	E
EFL95225	HMPREF0623_1402	histidinol-phosphatase	-0,88	1,43E-03	-1,11	4,08E-04	E
EFL95160	HMPREF0623_1472	amino acid:proton antiporter			-0,66	3,66E-04	E
EFL94859	HMPREF0623_1727	glutamine ABC transporter permease	-1,40	3,77E-15	-1,19	6,09E-11	E
EFL94860	HMPREF0623_1728	glutamine ABC transporter substrate-binding protein	-1,62	1,98E-13	-1,57	1,24E-11	E
EFL94861	HMPREF0623_1729	arginine ABC transporter ATP-binding protein	-1,68	6,02E-17	-1,57	1,84E-14	E
EFL94868	HMPREF0623_1736	amino acid permease	-1,37	2,50E-11	-0,93	2,75E-06	E
EFL95010	HMPREF0623_1878	ABC transporter ATP-binding protein	-0,81	3,26E-05			E
EFL94749	HMPREF0623_1617	ABC transporter ATP-binding protein, SufC	0,84	5,89E-06	0,74	1,34E-04	O
EFL94748	HMPREF0623_1616	Fe-S cluster assembly protein, SufD	0,77	1,03E-04			O
EFL94745	HMPREF0623_1613	Fe-S cluster assembly protein, SufB	0,94	4,88E-08	0,86	3,75E-06	O
EFL94746	HMPREF0623_1614	iron-sulfur cluster assembly scaffold protein, SufE2	0,81	3,21E-07	0,61	2,16E-04	C
EFL96067	HMPREF0623_0118	transcriptional regulator, ArgR family	0,95	4,84E-05	0,86	8,94E-04	K
EFL94731	HMPREF0623_1599	exopolysaccharide biosynthesis protein	0,87	2,81E-03	0,79	1,27E-02	M
EFL96352	HMPREF0623_0403	hypothetical protein	1,79	2,01E-19	2,01	3,52E-20	
EFL96353	HMPREF0623_0404	dihydropteroate synthase, folP	1,92	3,27E-13	1,84	1,15E-11	H
EFL96354	HMPREF0623_0405	non-canonical purine NTP pyrophosphatase	1,42	1,96E-08	1,71	1,12E-09	
EFL96355	HMPREF0623_0406	tetrahydrofolate synthase, folC	1,68	4,24E-12	1,66	2,14E-11	H
EFL96356	HMPREF0623_0407	GTP cyclohydrolase I, folE	1,64	3,76E-17	1,73	6,96E-17	H
EFL96357	HMPREF0623_0408	2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase, folK	1,80	3,73E-11	1,64	3,05E-09	H
EFL96358	HMPREF0623_0409	dihydroneopterin aldolase, folB	1,79	1,61E-12	1,71	3,17E-11	H

			Without furfural		With furfural		
Systematic Name	**Locus Tag**	**Annotation**	**logFC**	**Adj. p.value**	**logFC**	**Adj. p.value**	**COG group**
EFL95768	HMPREF0623_0804	hypothetical protein	1,03	1,48E-06	1,06	4,81E-06	S
EFL95769	HMPREF0623_0805	glycosyl transferase	0,94	1,64E-07	0,95	4,42E-07	M
EFL95770	HMPREF0623_0806	accessory Sec system protein Asp3	1,25	2,24E-07	1,37	1,48E-07	S
EFL95771	HMPREF0623_0807	accessory Sec system protein Asp2	0,98	7,16E-05	1,05	1,03E-04	S
EFL95772	HMPREF0623_0808	accessory Sec system protein Asp1	1,41	1,46E-07	1,39	1,63E-06	S
EFL95773	HMPREF0623_0809	preprotein translocase subunit SecY	0,77	3,75E-06	0,92	1,19E-06	U
EFL95774	HMPREF0623_0810	accessory Sec system translocase SecA2	1,35	8,20E-14	1,31	4,19E-12	U
EFL95775	HMPREF0623_0811	hypothetical protein	1,51	4,80E-06	1,47	3,73E-05	
EFL95776	HMPREF0623_0812	hypothetical protein	0,79	3,66E-04	1,07	4,89E-06	S
EFL95383	HMPREF0623_1120	alkaline-shock protein	-3,76	4,28E-26	-3,02	5,59E-20	S
EFL95384	HMPREF0623_1121	alkaline-shock protein	-3,76	9,47E-32	-3,14	8,31E-26	S
EFL95385	HMPREF0623_1122	hypothetical protein	-3,47	1,62E-27	-2,80	3,09E-21	
EFL95386	HMPREF0623_1123	hypothetical protein	-3,11	8,20E-25	-2,86	2,39E-21	
EFL95387	HMPREF0623_1124	hypothetical protein	-3,54	3,67E-22	-2,99	2,41E-17	S
EFL95388	HMPREF0623_1125	membrane protein	-3,07	8,10E-36	-2,58	6,19E-30	S
EFL95087	HMPREF0623_1593	glutathione reductase	-1,71	5,73E-17	-1,33	1,58E-11	C
EFL96394	HMPREF0623_0445	ATP-dependent Clp protease ATP-binding subunit, ClpL	-1,31	9,89E-08	-1,01	1,09E-04	O
EFL95852	HMPREF0623_0888	ATP-dependent Clp protease ATP-binding subunit, ClpC	-0,76	9,32E-04			O
EFL95021	HMPREF0623_1527	Clp protease, ClpX	-0,75	1,14E-03			O
EFL95853	HMPREF0623_0889	transcriptional regulator, CtsR	-0,65	2,78E-05			K
EFL94832	HMPREF0623_1700	molecular chaperone GroEL	0,63	9,58E-05			O
EFL95705	HMPREF0623_0741	universal stress protein, UspA	-0,73	1,01E-02	-0,76	2,10E-02	T
EFL96264	HMPREF0623_0315	SOS response repressor LexA	-0,69	2,38E-04	-0,70	8,76E-04	K
EFL95964	HMPREF0623_0015	transcriptional regulator, GntR family			0,74	1,35E-04	K
EFL95136	HMPREF0623_1448	transcriptional regulator			0,68	8,15E-04	K
EFL96210	HMPREF0623_0261	transcriptional regulator			0,69	8,96E-04	K
EFL96512	HMPREF0623_0563	NADPH-dependent oxidoreductase			1,00	1,56E-07	C

			Without furfural		With furfural		
Systematic Name	**Locus Tag**	**Annotation**	**logFC**	**Adj. p.value**	**logFC**	**Adj. p.value**	**COG group**
EFL95081	HMPREF0623_1587	recombinase, RecD			0,65	9,90E-04	
EFL95660	HMPREF0623_0696	thiol reductase thioredoxin			-0,59	1,94E-02	O

Intriguingly, nine consecutive genes, presumably encoding an operon encoding accessory Sec system proteins, were found to be significantly up-regulated in the adapted strain. The putative operon was formed by three accessory Sec system proteins (Asp1, Asp2 and Asp3), two translocases (SecY and SecA2), a glycosyl transferase and three hypothetical proteins. The accessory Sec system plays a role in exporting proteins to the external environment or to the cell surface, being involved in establishing the structure of the cell wall (Braunstein et al., 2003; Rigel et al., 2009). The Sec system is essential for bacteria, however some of Gram-positive bacteria were found to possess an extra Sec system; this accessory Sec systems are believed to be specialized for certain substrates, and were recognized to be linked to virulence in some pathogenic strains (Braunstein et al., 2003; Rigel et al., 2009).

Furthermore, two genes, both predicted to be alkaline-shock proteins, were found to be significantly repressed in the adapted strain when comparing differentially regulated genes between the adapted and the wild-type strain in non-stress conditions. The two repressed alkaline-shock proteins were down-regulated together with four other neighboring genes, presumably forming an operon of co-regulated genes. All six genes demonstrated 8 to 11 fold repression in the adapted strain, and therefore were among 10 most highly down-regulated genes in the adapted strain. It is worth noticing that the same operon was found to be down-regulated in the wild-type strain in response to furfural stress. The genes were shown to stay down-regulated in the adapted strain also during furfural stress. It thus seems that they are constitutively repressed in the adapted strain even in non-stress conditions.

Glutathione reductase was also found to be repressed in the adapted strain. Interestingly, it was down-regulated both during furfural-associated stress and in normal non-stress conditions. As mentioned earlier, glutathione reductase is involved in maintaining cell redox homeostasis, and by keeping appropriate ratios of reduced glutathione and its oxidized disulfide form, it can be implicated in the cell defense against oxidative stress (Jänsch et al., 2007; Li et al., 2003). As furfural is known to cause oxidative stress in different organisms (Allen et al., 2010; Almeida et al., 2009), it was unexpected to see decreased expression of glutathione reductase during furfural-related stress.

Three heat shock proteins, ClpL, ClpC and ClpX were found to be down-regulated in the adapted strain. The Clp ATPases act as molecular chaperones to facilitate the process of correct protein folding; when they form a complex with a Clp protease, they also possess protease activity and thus play a role in degradation of misfolded proteins. The Clp protease subunit, ClpP, however, was not found to be differentially regulated in the adapted strain. The Clp proteins are implicated in stress responses, including acid, bile, heat and osmotic stresses (Frees et al., 2007; Wall et al., 2007). The transcription of Clp proteins was demonstrated to be negatively regulated by CtsR repressor (Kru et al., 2001). The CtsR homologues were identified in numerous species of Gram-positive bacteria, including species of *Lactococcus*, *Lactobacillus*, *Leuconostoc* and *Streptococcus* and they were suggested to be highly conserved (Derré et al., 1999). The transcriptional regulator CtsR was found to be down-regulated in the adapted strain. The decreased levels of the CtsR repressor would in turn lead to an up-regulation of the Clp family proteins; however, the microarray data analysis revealed that the Clp proteins were actually repressed rather than induced in the adapted strain. The reason for the repression of both the Clp proteins and their negative regulator might be a more efficient translation process of the CtsR protein. Recalling Chapter 5, one of the mutations in the adapted strain was found to be in a close proximity of the ribosomal binding site of the CtsR repressor. It was postulated there that the single nucleotide substitution within this region could possibly have an

impact on the translation process. Indeed, such a change might influence the transcript's affinity towards the ribosome and increase the efficiency of translation, yielding higher amounts of the protein. Thereby, a down-regulation of the CtsR might still result in the same or even higher amount of the CtsR regulator. Yet, further experiments could be performed in order to verify this finding.

One of the genes found to be differently regulated in the adapted strain compared to the wild-type, was the molecular chaperone groEL. The chaperone works together with GroES and the complex participates in the correct folding of proteins. It was demonstrated to be highly induced under stress conditions in many different organisms, among others, in *L. paracasei*, *L. rhamnosus* and *L. gasseri* under heat and/or osmotic stresses (Desmond et al., 2004; Prasad et al., 2003; Suokko et al., 2008). In this study, the chaperone was only induced in non-stress conditions, and did not show any up- or down-regulation either in the adapted or the wild-type strain cultures after the addition of furfural. The GroEL chaperone is controlled by a negative regulator HrcA, which was found to be mutated in the adapted strain. Interestingly, neither GroES, nor DnaK, DnaJ, or GrpE, which are also a part of HrcA regulon, were not differentially expressed in the adapted strain, indicating that the HrcA activity was not affected by the mutation.

Interestingly, a gene encoding a universal stress protein UspA was also found to be down-regulated in the adapted strain, both during furfural stress and during non-stress conditions. The exact function of the universal stress proteins is unknown; however, the production of the UspA protein was found to be induced in *E. coli* by a wide range of conditions, including starvation, heat and oxidative stress, and exposure to numerous stimulants (Gustavsson et al., 2002; Kvint et al., 2003; Nachin et al., 2005). The protein Usp1 was also suggested to be involved in *L. plantarum* response to acid-related stress (Gury, 2009). During stress conditions, the universal stress proteins become up-regulated with a few exceptions; a cold shock as well as a tetracycline treatment were both demonstrated to cause a reduction in the *usp* expression levels (Gustavsson et al., 2002). This can be explained by the fact that both mentioned stress conditions cause reduction in guanosine tetraphosphate (ppGpp) levels, which was suggested to be a positive regulator of the *usp* genes (Gustavsson et al., 2002; Kvint et al., 2003). In this study, the UspA protein was repressed in the adapted strain both with and without a stress factor present.

One of the genes encoding a transcriptional repressor of SOS response, LexA, was also found to be significantly down-regulated in the adapted strain both during non-stress conditions and during furfural-induced stress. SOS response is the cells' DNA repair system which is induced by accumulation of single stranded DNA within the cell; this can be due to UV radiation or chemicals, which cause damage to DNA or interrupt DNA synthesis (Janion, 2008). LexA is a transcriptional regulator and it is of major importance in the regulation of the SOS response. LexA is normally bound to the so called ´SOS box´ located upstream to the DNA repair-related genes, repressing their expression if no DNA repair is necessary. When DNA is damaged, RecA protein becomes activated by the single-stranded DNA and it facilitates LexA dissociation from the operator region, thereby inducing the expression of the DNA repair genes (Janion, 2008). By regulating mutagenesis, the SOS response system can also be involved in bacterial adaptation to the environment (Mckenzie et al., 2000; Schons-Fonseca et al., 2016). As furfural is known to cause DNA damage, the down-regulation of LexA expression in the adapted strain would induce the expression of DNA-repair genes, and therefore help the cells to cope with the furfural-damaged DNA. However, none of the genes regulated by LexA were found to be differentially expressed in the adapted strain.

As described above, several stress proteins were found to be differentially regulated in the adapted strain when compared to the wild-type strain. Among these there were alkaline-shock proteins, Clp proteases, molecular chaperone GroEL, universal stress protein UspA and a transcriptional regulator of SOS response, LexA. Surprisingly, however, most of the above mentioned stress-related proteins were down-regulated in the adapted strain. The rationale for the repression rather than induction of the commonly known stress proteins in the adapted strain is not straightforward. First of all, it can result from the fact that the general perturbation of the strains was not that pronounced; the experiment was designed to minimize the possible pleiotropic effects on the growth rate. Therefore, the amount of furfural added to the cultures was designed not to decrease the growth rate more than 10%. Perhaps the perturbation was too small to see the effects exerted by furfural stress. What is more, since it was the first study of furfural stress response in *P. acidilactici*, it is possible that furfural affects the strain's metabolism in a different way than it was seen in other microorganisms. However, the exact reasons behind the down-regulation of the stress genes remain to be revealed.

6.3.4. Genes differentially expressed in the adapted strain solely during furfural-induced stress

When looking at the gene expression data for the wild-type strain versus the adapted strain during growth in MRS with the furfural added, there were 244 genes identified to be differentially expressed in the adapted strain; 121 genes were repressed, while 123 genes were induced in response to furfural stress. A close inspection of the data revealed that 196 genes were differently expressed in the adapted strain compared to the wild-type strain at both conditions: both with and without the furfural present in the growth medium. Apart from that, 118 genes were differently regulated in the absence of stress factor, while 48 other genes were differently regulated during the furfural stress.

Having a closer look at genes specifically differentially regulated only with furfural present, it is easily noticeable that three transcriptional regulators were up-regulated in the adapted strain. Moreover, an NADPH-dependent oxidoreductase was found to be 2-fold up-regulated. This finding might indicate an oxidative stress and an enhanced need for maintaining cell's redox homeostasis under furfural stress; as opposed to it, however, a thioredoxin, which also plays a role in the redox homeostasis, was found to be down-regulated. A recombinase protein, RecD, which is involved in the DNA recombination and repair, was found to be up-regulated in the stressed adapted strain; this finding might point to the DNA-damaging effect possibly exerted by furfural.

6.4. Conclusions

This study aimed at investigating the stress response of *P. acidilactici* DSM 20284 wild-type and adapted strain A28 to furfural. To my knowledge, this was the first report on transcriptomics analysis and furfural effect on the metabolism and stress response done in *P. acidilactici*.

The influence of furfural on both the wild-type and adapted strains was very subtle. It can therefore be concluded that the concentration of furfural selected for the experiment was too low to enough perturb the strains in order to see the induction of the stress response pathway. It indicates that both the wild-type strain and the adapted strain are already well adapted to furfural. Such competitive advantage, however, costs energy, and that is possibly the reason for why the cells became adapted towards a faster and more efficient growth on the medium rather than towards furfural resistance.

As the study also aimed at exploring the mechanisms behind the adapted strain's faster growth on rich MRS medium compared to the wild-type strain, the transcriptomes of the wild-type and adapted strains in non-stress conditions were compared. The data analysis revealed that the faster growth of the adapted strain A28 is achieved by optimization of the nucleotide metabolism and the up-regulation of the folic acid biosynthetic pathway as well as several enzymes involved in glycolysis. It has also been found that the genes involved in exopolysaccharides biosynthesis were induced in the adapted strain. This finding might indicate that the structure of the cell wall might be important for the cell's protection against furfural. Furthermore, the accessory Sec system, as it is also involved in establishing the cell wall and was found to be highly up-regulated in the adapted strain relative to the wild-type, might also play a role in the strain's adaptation to the growth in the presence of furfural.

To further study the mechanisms of how furfural influences the cell's metabolism, new experiments with higher perturbation levels could be performed. Otherwise, the results of this transcriptomic study could be validated by knockouts of appropriate genes to confirm both their predicted functions and their role in *P. acidilactici* stress response.

6.5. References

Aguado-Urda, M., Gibello, A., Blanco, M. del M., Fernandez-Garayzabal, J.F., Lopez-Alonso, V., Lopez-Campos, G.H., 2013. Global Transcriptome Analysis of *Lactococcus garvieae* Strains in Response to Temperature. PLoS One 8.

Akbar, S., Lee, S.Y., Boylan, S.A., Price, C.W., 1999. Two genes from *Bacillus subtilis* under the sole control of the general stress transcription factor σ(B). Microbiology 145, 1069–1078.

Allen, S.A., Clark, W., McCaffery, J.M., Cai, Z., Lanctot, A., Slininger, P.J., Liu, Z.L., Gorsich, S.W., 2010. Furfural induces reactive oxygen species accumulation and cellular damage in *Saccharomyces cerevisiae*. Biotechnol. Biofuels 3, 1–10.

Almeida, J.R.M., Bertilsson, M., Gorwa-Grauslund, M.F., Gorsich, S., Lidén, G., 2009. Metabolic effects of furaldehydes and impacts on biotechnological processes. Appl. Microbiol. Biotechnol. 82, 625–638.

Bermingham, A., Derrick, J.P., 2002. The folic acid biosynthesis pathway in bacteria: evaluation of potential for antibacterial drug discovery. BioEssays 24, 637–648.

Braunstein, M., Espinosa, B.J., Belisle, J.T., Jacobs, W.R., 2003. SecA2 functions in the secretion of superoxide dismutase A and in the virulence of *Mycobacterium tuberculosis*. Mol. Microbiol. 48, 453–464.

Casiano-Colon, A., Marquis, R.E., 1988. Role of the Arginine Deiminase System in Protecting Oral Bacteria and an Enzymatic Basis for Acid Tolerance. Appl. Environ. Microbiol. 54, 1318–1324.

Derré, I., Rapoport, G., Msadek, T., 1999. CtsR, a novel regulator of stress and heat shock response, controls *clp* and molecular chaperone gene expression in Gram-positive bacteria. Mol. Microbiol. 31, 117–131.

Desmond, C., Fitzgerald, G.F., Stanton, C., Ross, R.P., 2004. Improved Stress Tolerance of GroESL-Overproducing *Lactococcus lactis* and Probiotic *Lactobacillus paracasei* NFBC 338. Appl. Environ. Microbiol. 70, 5929–5936.

Ferenci, T., Spira, B., 2007. Variation in Stress Responses within a Bacterial Species and the Indirect Costs of Stress Resistance. Ann. N. Y. Acad. Sci. 1113, 105–113.

Frees, D., Savijoki, K., Varmanen, P., Ingmer, H., 2007. Clp ATPases and ClpP proteolytic complexes regulate vital biological processes in low GC, Gram-positive bacteria. Mol. Microbiol. 63, 1285–1295.

Fulde, M., Willenborg, J., Greeff, A. De, Benga, L., Smith, H.E., Valentin-weigand, P., Goethe, R., 2011. ArgR is an essential local transcriptional regulator of the arcABC operon in *Streptococcus suis* and is crucial for biological fitness in an acidic environment. Microbiology 157, 572–582.

Gentleman, R.C., Carey, V.J., Bates, D.M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J., Hornik, K., Hothorn, T., Huber, W., Iacus, S., Irizarry, R., Leisch, F., Li, C., Maechler, M., Rossini, A.J., Sawitzki, G., Smith, C., Smyth, G., Tierney, L., Yang, J.Y., Zhang, J., 2004. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. 5, R80.

Girgis, H.S., Smith, J., Luchansky, J.B., Klaenhammer, T.R., 2003. Stress Adaptations of Lactic Acid Bacteria, in: Microbial Stress Adaptation and Food Safety. pp. 159–212.

Glebes, T.Y., Sandoval, N.R., Reeder, P.J., Schilling, K.D., Zhang, M., Gill, R.T., 2014. Genome-Wide Mapping of Furfural Tolerance Genes in *Escherichia coli*. PLoS One 9.

Gury, J., 2009. Inactivation of PadR, the Repressor of the Phenolic Acid Stress Response, by Molecular Interaction with Usp1, a Universal Stress Protein from *Lactobacillus plantarum*, in *Escherichia coli*. Appl. Environ. Microbiol. 75, 5273–5283.

Gustavsson, N., Diez, A.A., Nyström, T., 2002. The universal stress protein paralogues of *Escherichia coli* are co-ordinately regulated and co-operate in the defence against DNA damage. Mol. Microbiol. 43, 107–117.

He, M., Wu, B., Shui, Z., Hu, Q., Wang, W., Tan, F., Tang, X., Zhu, Q., Pan, K., Li, Q., Su, X., 2012. Transcriptome profiling of *Zymomonas mobilis* under furfural stress. Biotechnol. Biofuels 95, 189–199.

Horgan, R.P., Kenny, L.C., 2011. "Omic" technologies: genomics, transcriptomics, proteomics and metabolomics. Obstet. Gynaecol. 13, 189–195.

Hu, K.H., Liu, E., Dean, K., Gingas, M., Degrafft, W., Trun, N.J., 1996. Overproduction of Three Genes Leads to Camphor Resistance and Chromosome Condensation in *Escherichia coli*. Genetics 143, 1521–1532.

Janion, C., 2008. Inducible SOS Response System of DNA Repair and Mutagenesis in *Escherichia coli*. Int. J. Biol. Sci. 4, 338–344.

Jones, P.G., Inouye, M., 1996. RbfA, a 30S ribosomal binding factor, is a cold-shock protein whose absence triggers the cold-shock response. Mol. Microbiol. 21, 1207–1218.

Jänsch, A., Korakli, M., Vogel, R., Gänzle, M., 2007. Glutathione Reductase from *Lactobacillus sanfranciscensis* DSM 20451T: Contribution to Oxygen Tolerance and Thiol Exchange Reactions in Wheat Sourdoughs. Appl. Environ. Microbiol. 73, 4469–4476.

Kilstrup, M., Hammer, K., Ruhdal Jensen, P., Martinussen, J., 2005. Nucleotide metabolism and its control in lactic acid bacteria. FEMS Microbiol. Rev. 29, 555–590.

Kru, E., Zu, D., Witt, E., Ludwig, H., Hecker, M., 2001. Clp-mediated proteolysis in Gram-positive bacteria is autoregulated by the stability of a repressor. EMBO J. 20, 852–863.

Kvint, K., Nachin, L., Diez, A., Nyström, T., 2003. The bacterial universal stress protein: function and regulation. Curr. Opin. Microbiol. 6, 140–145.

Larsen, R., Buist, G., Kuipers, O.P., Kok, J., 2004. ArgR and AhrC Are Both Required for Regulation of Arginine Metabolism in *Lactococcus lactis*. J. Bacteriol. 186, 1147–1157.

Larsen, R., van Hijum, S.A.F.T., Martinussen, J., Kuipers, O.P., Kok, J., 2008. Transcriptome Analysis of the *Lactococcus lactis* ArgR and AhrC Regulons. Appl. Environ. Microbiol. 74, 4768–4771.

Li, B.Z., Yuan, Y.J., 2010. Transcriptome shifts in response to furfural and acetic acid in *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. 86, 1915–1924.

Li, Y., Hugenholtz, J., Abee, T., Molenaar, D., 2003. Glutathione Protects *Lactococcus lactis* against Oxidative Stress. Appl. Environ. Microbiol. 69, 5739–5745.

Liu, C., Wang, R., Gong, F., Liu, X., Zheng, H., Luo, Y., Li, X., 2015. Complete genome sequences and comparative genome analysis of *Lactobacillus plantarum* strain 5-2 isolated from fermented soybean. Genomics 106, 404–411.

Loiseau, L., Ollagnier-de-Choudens, S., Nachin, L., Fontecave, M., 2003. Biogenesis of Fe-S Cluster by the Bacterial Suf System. J. Biol. Chem. 278, 38352–38359.

Looijesteijn, P.J., Trapet, L., De Vries, E., Abee, T., Hugenholtz, J., 2001. Physiological function of exopolysaccharides produced by *Lactococcus lactis*. Int. J. Food Microbiol. 64, 71–80.

Ma, M., Liu, Z.L., 2010. Comparative transcriptome profiling analyses during the lag phase uncover YAP1, PDR1, PDR3, RPN4, and HSF1 as key regulatory genes in genomic adaptation to the lignocellulose derived inhibitor HMF for *Saccharomyces cerevisiae*. BMC Genomics 11, 660.

Mckenzie, G.J., Harris, R.S., Lee, P.L., Rosenberg, S.M., 2000. The SOS response regulates adaptive mutation. Proc. Natl. Acad. Sci. U. S. A. 97, 6646–6651.

Mihara, H., Esaki, N., 2002. Bacterial cysteine desulfurases: their function and mechanisms. Appl. Microbiol. Biotechnol. 60, 12–23.

Miller, E.N., Jarboe, L.R., Turner, P.C., Pharkya, P., Yomano, L.P., York, S.W., Nunn, D., Shanmugam, K.T., Ingram, L.O., 2009. Furfural Inhibits Growth by Limiting Sulfur Assimilation in Ethanologenic *Escherichia coli* Strain LY180. Appl. Environ. Microbiol. 75, 6132–6141.

Mishra, A., Jha, B., 2013. Microbial Exopolysaccharides, in: The Prokaryotes - Applied Bacteriology and Biotechnology. pp. 179–192.

Nachin, L., Loiseau, L., Expert, D., Barras, F., 2003. SufC: an unorthodox cytoplasmic ABC/ATPase required for [Fe-S] biogenesis under oxidative stress. EMBO J. 22, 427–437.

Nachin, L., Nannmark, U., Nyström, T., 2005. Differential Roles of the Universal Stress Proteins of *Escherichia coli* in Oxidative Stress Resistance, Adhesion, and Motility. J. Bacteriol. 187, 6265–6272.

Nicoloff, H., Arsene-Ploetze, F., Malandain, C., Kleerebezem, M., Bringel, F., 2004. Two Arginine Repressors Regulate Arginine Biosynthesis in *Lactobacillus plantarum*. J. Bacteriol. 186, 6059–6069.

Outten, F.W., Djaman, O., Storz, G., 2004. A suf operon requirement for Fe-S cluster assembly during iron starvation in *Escherichia coli*. Mol. Microbiol. 52, 861–872.

Parawira, W., Tekere, M., 2011. Biotechnological strategies to overcome inhibitors in lignocellulose hydrolysates for ethanol production: review. Crit. Rev. Biotechnol. 31, 20–31.

Park, H.-S., Um, Y., Sim, S.J., Lee, S.Y., Woo, H.M., 2015. Transcriptomic analysis of *Corynebacterium glutamicum* in the response to the toxicity of furfural present in lignocellulosic hydrolysates. Process Biochem. 50, 347–356.

Patel, S., Majumder, A., Goyal, A., 2012. Potentials of Exopolysaccharides from Lactic Acid Bacteria. Indian J. Microbiol. 52, 3–12.

Prasad, J., McJarrow, P., Gopal, P., 2003. Heat and Osmotic Stress Responses of Probiotic *Lactobacillus rhamnosus* HN001 (DR20) in Relation to Viability after Drying. Appl. Environ. Microbiol. 69, 917–925.

R Core Team, 2015. R: A language and environment for statistical computing. R Found. Stat. Comput. Vienna, Austria.

Rigel, N.W., Gibbons, H.S., McCann, J.R., McDonough, J.A., Kurtz, S., Braunstein, M., 2009. The Accessory SecA2 System of Mycobacteria Requires ATP Binding and the Canonical SecA1. J. Biol. Chem. 284, 9927–9936.

Rimaux, T., Rivière, A., Illeghems, K., Weckx, S., Vuyst, L. De, Leroy, F., 2012. Expression of the Arginine Deiminase Pathway Genes in *Lactobacillus sakei* Is Strain Dependent and Is Affected by the Environmental pH. Appl. Environ. Microbiol. 78, 4874–4883.

Rodney, A.D., 2001. Biology of *Giardia lamblia*. Clin. Microbiol. Rev. 14, 447–469.

Schons-Fonseca, L., da Silva, J.B., Milanez, J.S., Domingos, H., Smith, J.L., Nakaya, H.I., Grossman, A.D., Ho, P.L., da Costa, R., 2016. Analysis of LexA binding sites and transcriptomics in response to genotoxic stress in *Leptospira interrogans*. Nucleic Acids Res. 1–13.

Smirnova, G. V, Krasnykh, T.A., Oktyabrsky, O.N., 2001. Role of Glutathione in the Response of *Escherichia coli* to Osmotic Stress. Biokhimiya 66, 1195–1201.

Smyth, G.K., 2005. Limma: Linear Models for Microarray Data, in: Bioinformatics and Computational Biology Solutions Using R and Bioconductor. pp. 397–420.

Suokko, A., Poutanen, M., Savijoki, K., Kalkkinen, N., Varmanen, P., 2008. ClpL is essential for induction of thermotolerance and is potentially part of the HrcA regulon in *Lactobacillus gasseri*. Proteomics 8, 1029–1041.

Sybesma, W., Starrenburg, M., Kleerebezem, M., Mierau, I., Vos, W.M. De, Hugenholtz, J., 2003a. Increased Production of Folate by Metabolic Engineering of *Lactococcus lactis*. Appl. Environ. Microbiol. 69, 3069–3076.

Sybesma, W., Starrenburg, M., Tijsseling, L., Hoefnagel, M.H.N., Hugenholtz, J., 2003b. Effects of Cultivation Conditions on Folate Production by Lactic Acid Bacteria. Appl. Environ. Microbiol. 69, 4542–4548.

van de Guchte, M., Serror, P., Chervaux, C., Smokvina, T., Ehrlich, S.D., Maguin, E., 2002. Stress responses in lactic acid bacteria. Antonie Van Leeuwenhoek, Int. J. Gen. Mol. Microbiol. 82, 187–216.

Vrancken, G., Rimaux, T., Wouters, D., Leroy, F., De Vuyst, L., 2009. The arginine deiminase pathway of *Lactobacillus fermentum* IMDO 130101 responds to growth under stress conditions of both temperature and salt. Food Microbiol. 26, 720–727.

Wall, T., Båth, K., Britton, R.A., Jonsson, H., Versalovic, J., Roos, S., 2007. The Early Response to Acid Shock in *Lactobacillus reuteri* Involves the ClpL Chaperone and a Putative Cell Wall-Altering Esterase. Appl. Environ. Microbiol. 73, 3924–3935.

Wegkamp, A., Starrenburg, M., de Vos, W.M., Hugenholtz, J., Sybesma, W., 2004. Transformation of Folate-Consuming *Lactobacillus gasseri* into a Folate Producer. Appl. Environ. Microbiol. 70, 3146–3148.

Winkler, J., Kao, K.C., 2011. Transcriptional Analysis of *Lactobacillus brevis* to N-Butanol and Ferulic Acid Stress Responses. PLoS One 6.

Zheng, M., Wang, X., Templeton, L.J., Smulski, D.R., Larossa, R.A., Storz, G., 2001. DNA Microarray-Mediated Transcriptional Profiling of the *Escherichia coli* Response to Hydrogen Peroxide. J. Bacteriol. 183, 4562–4570.

Chapter 7

Conclusions and Future Perspectives

This PhD study was set out to explore the potential of Lactic Acid Bacteria (LAB) for becoming microbial catalysts for conversion of lignocellulosic feedstocks into value-added products. There are several requirements that need to be fulfilled by a microorganism in order to be considered a promising candidate. Firstly, it should have a broad substrate utilization range, being able to utilize both pentoses and hexoses. Secondly, it should be tolerant to various inhibitors generated during the pretreatment of lignocellulosic biomass. Thirdly, it should be easy to engineer, with a range of developed tools for its genetic manipulation.

In this PhD study, almost 300 different LAB strains, including all type strains, were systematically screened for their tolerance levels towards the most common inhibitors coming from the pretreatment of lignocellulosic biomass as well as for the strains' abilities to ferment a variety of carbon sources, including both pentoses and hexoses. There were many LAB strains identified that were both tolerant to high concentrations of the key inhibitors and capable of utilizing pentoses and hexoses. Therefore, the findings confirmed the remarkable potential of LAB to become future microbial cell factories converting lignocellulosic substrates into chemicals, materials and energy. Moreover, the study has provided new valuable insight on the general robustness of various LAB species as well as new information concerning their growth on a variety of media and carbon sources.

As mentioned earlier, one of the important requirements for an efficient microbial catalyst is the ease of its genetic engineering. Genetic manipulation allows to engineer the strains for production of desired value-added products; it allows to increase the specific product yield and productivity, minimize the formation of by-products, further increase their substrate utilization range or enhance their tolerance levels towards inhibitors or newly-introduced fermentation products. That is why efficient transformation methods are necessary and one protocol was successfully developed and optimized in this study, allowing for the transformation of *Pediococcus acidilactici* DSM 20284, one of the selected best-performing strains, with efficiencies up to $2.8 \cdot 10^3$. The method permits the introduction of exogenous DNA into this strain, fulfilling the last requirement for the strain's industrial applications.

An adaptive laboratory evolution was performed to further enhance the tolerance of *P. acidilactici* towards the combination of furfural and acetic acid, the inhibitors generated during the pretreatment of softwood. An isolated adapted strain A28 was found to grow with a higher growth rate both in the presence and absence of inhibitors. The phenotype was amenable to 62 mutations identified by whole genome

resequencing; the mutations were mostly in the genes coding for DNA repair proteins, stress response transcriptional regulators, membrane proteins and proteins involved in the cell redox homeostasis. These mutations are likely to play a role in the enhanced furfural tolerance of the adapted strain. A second group of mutations, mainly in the purine biosynthesis and salvage pathways, as well as in genes related to carbohydrate metabolism and transport, are likely associated with the strain's adaptation to the MRS medium. The faster growth of the adapted strain was confirmed in *Pediococcus* Defined Medium (PDM), developed specifically for *P. acidilactici*; that points to a general improvement of the adapted strain metabolism.

The transcriptomic study of *P. acidilactici* wild-type and adapted strain showed that neither the wild-type nor the adapted strain induced the stress-related genes in response to furfural, signifying that *P. acidilactici* is adapted to furfural. This finding is not unexpected as the *P. acidilactici* strain has been identified as one of the most inhibitor-tolerant strains from almost 300 screened strains. Moreover, the DSM 20284 strain was isolated from barley; thus, it might already be pre-adapted to deteriorating plant material. However, several membrane proteins and genes related to exopolysaccharide biosynthesis were induced in the adapted strain, indicating that the structure of the cell wall might be involved in cell's protection against furfural.

The transcriptome analysis also helped to increase our understanding of the reasons for which the adapted strain was able to grow at a higher growth rate: it was most likely achieved by optimization of the nucleotide metabolism and the up-regulation of the folic acid biosynthetic pathway along with several enzymes involved in glycolysis. LAB are known to have purine partial requirements; it is thus not surprising that the optimization of the metabolism of purines and pyrimidines, especially relieving the PurF feedback inhibition, would result in an enhanced growth.

Based on the findings above, it can be concluded that the *P. acidilactici* DSM 20284 is pre-adapted to furfural. Previous investigations proved it to be tolerant to a variety of other inhibitors as well; moreover, the strain was demonstrated to utilize both xylose and arabinose with similar growth rates as it utilized glucose. Since the strain was also shown to be transformable with reasonable transformation efficiencies, it therefore fulfills all the criteria for an efficient biocatalyst for conversion of lignocellulosic biomass into value-added products, which confirms its remarkable potential to be used in future industrial applications.

Based on the results of this PhD study, the following issues could potentially be addressed in a further study:

1. The transformation method for *P. acidilactici* was optimized in this study and the efficiency was increased to reasonable levels; yet, the protocol could be further optimized.
2. In order to study the furfural stress response in *P. acidilactici*, a higher perturbation level is needed. A transcriptomic study employing an increased concentration of furfural would allow identifying the genes related to the strain's resistance to furfural; the findings could aid in rational strain engineering for an even higher inhibitor tolerance.
3. The findings of the transcriptomic study should be further verified either by qPCR or by enzyme assays of the relevant proteins. Especially, the down-regulation of the stress response-related genes was unexpected and could be investigated in more details.

4. Tests in commercial pretreated lignocellulosic biomass could prove the concept of using *P. acidilactici* as a 2nd generation cell factory.
5. The strain should be further investigated in order to determine the effect of furfural and other inhibitors from the pretreated lignocellulosic biomass on the product formation; it should also be tested for the presence of a carbon catabolite repression system that would not allow for simultaneous consumption of various sugars.

Appendix

Supplementary materials for Chapter 2 consist of Tables S1 – S6, published in Microbial Cell Factories.

Table S1. Bacterial strains used in this study

Table S2. Growth of the tested strains on MRS, DLA and GSA media

Table S3. Results of the screening on MRS plates

Table S4. Results of the screening on GSA plates

Table S5. Results of the pentose utilization tests on 10% MRS plates with glucose, xylose or arabinose as sole carbon sources

Table S6. Performance of *E.coli* MG1655 in LB with glucose or xylose and combinations of inhibitors representing three feedstock hydolysate types

Supplementary materials for Chapter 6 consist of the following:

Figure S1. Microarray density plots before and after normalization

Table S7. Differentially expressed genes in DSM 20284 and A28 grown in MRS with and without furfural

Table S8. Top 50 most up-regulated genes in DSM 20284 and A28 grown in MRS with and without furfural

Table S9. Top 50 most down-regulated genes in DSM 20284 and A28 grown in MRS with and without furfural

Table S1. Bacterial strains used in this study.

Species	Strain	Source	Optimal temperature
Lactobacillus acetotolerans	DSM 20749	fermented vinegar broth	30°C
Lactobacillus acidifarinae	DSM 19394	artisanal wheat sourdough (Belgium)	30°C
Lactobacillus acidipiscis	DSM 15836	fermented fish (Thailand)	30°C
Lactobacillus acidophilus	DSM 20079	human	37°C
Lactobacillus agilis	DSM 20509	municipal sewage	37°C
Lactobacillus algidus	DSM 15638	vacuum-packaged beef	20°C
Lactobacillus alimentarius	DSM 20249	marinated fish product	30°C
Lactobacillus amylolyticus	DSM 11664	acidified beer wort	45°C
Lactobacillus amylophilus	DSM 20533	swine waste-corn fermentation	28°C
Lactobacillus amylotrophicus	DSM 20534	swine waste-corn fermentation	28°C
Lactobacillus amylovorus	DSM 20531	cattle waste-corn fermentation	37°C
Lactobacillus animalis	DSM 20602	dental plaque of baboon	37°C
Lactobacillus antri	DSM 16041	gastric biopsies, human stomach mucosa (Sweden)	37°C
Lactobacillus apodemi	DSM 16634	faeces, wild Japanese wood mouse	37°C
Lactobacillus aquaticus	DSM 21051	surface of a eutrophic freshwater pond (Korea)	37°C
Lactobacillus aviarius subsp. araffinosus	DSM 20653	intestine of chicken	37°C
Lactobacillus aviarius subsp. aviarius	DSM 20655	faeces of chicken	37°C
Lactobacillus bifermentans	DSM 20003	blown cheese	30°C
Lactobacillus bobalius	DSM 19674	Spanish Bobal grape must (Spain)	28°C
Lactobacillus brevis	LMG 19186		30°C
Lactobacillus brevis	LMG 19188		30°C
Lactobacillus brevis	LMG 19191		30°C
Lactobacillus brevis	LMG 19217		30°C
Lactobacillus brevis	LMG 19215		30°C
Lactobacillus brevis	LMG 19216		30°C
Lactobacillus brevis	DSM 20054	faeces	30°C
Lactobacillus buchneri	Ketchup-3		30°C
Lactobacillus buchneri	DSM 20057	tomato pulp	37°C
Lactobacillus buchneri	Ketchup-1		30°C
Lactobacillus cacaonum	DSM 21116	cocoa bean heap fermentation (Ghana) [1]	30°C
Lactobacillus camelliae	DSM 22697	fermented tea leaves (miang) (Thailand)	37°C
Lactobacillus capillatus	DSM 19910	isolated from fermented brine used for stinky tofu production (Taiwan) [2]	30°C

Species	Strain	Source	Optimal temperature
Lactobacillus casei	DSM 20011	cheese	30°C
Lactobacillus ceti	DSM 22408	lungs of a beaked whale (*Ziphius cavirostris*) (Spain)	37°C
Lactobacillus coleohominis	DSM 14060	human vagina (Sweden)	37°C
Lactobacillus collinoides	DSM 20515	fermenting apple juice	26°C
Lactobacillus composti	DSM 18527	composting material of distilled shochu residue (Japan)	30°C
Lactobacillus concavus	DSM 17758	distilled pirit-fermenting cellar (China)	37°C
Lactobacillus coryniformis subsp. coryniformis	DSM 20001	silage	30°C
Lactobacillus coryniformis subsp. torquens	DSM 20004	air of cow shed	30°C
Lactobacillus crispatus	DSM 20584	eye	37°C
Lactobacillus curvatus	DSM 20019	milk	30°C
Lactobacillus delbrueckii subsp. bulgaricus	DSM 20081	bulgarian yoghourt	37°C
Lactobacillus delbrueckii subsp. delbrueckii	DSM 20074	sour grain mash	37°C
Lactobacillus delbrueckii subsp. indicus	DSM 15996	traditional dairy fermented product (Dahi type) [3]	37°C
Lactobacillus delbrueckii subsp. lactis	DSM 20072	emmental cheese	37°C
Lactobacillus dextrinicus	DSM 20335	silage	30°C
Lactobacillus diolivorans	DSM 14421	maize silage (Netherlands)	30°C
Lactobacillus equi	DSM 15833	faeces of horses (Japan)	37°C
Lactobacillus equicursoris	DSM 19284	healthy thoroughbred racehorse (Japan)	37°C
Lactobacillus equigenerosi	DSM 18793	thoroughbred horses (Japan)	37°C
Lactobacillus fabifermentans	DSM 21115	cocoa bean heap fermentation (Ghana) [1]	30°C
Lactobacillus farciminis	DSM 20184	sausage	30°C
Lactobacillus farraginis	DSM 18382	composting material of distilled shochu residue (Japan)	30°C
Lactobacillus fermentum	DSM 20052	fermented beets	37°C
Lactobacillus floricola	DSM 23037	flower of *Caltha palustris* (Japan)	30°C
Lactobacillus florum	DSM 22689	peony (*Paeonia suffruticosa*) (South Africa)	30°C
Lactobacillus fructivorans	DSM 20203		30°C
Lactobacillus frumenti	DSM 13145	rye-bran sourdough (Germany)	40°C
Lactobacillus fuchuensis	DSM 14340	vacuum-packaged beef (Japan)	20°C
Lactobacillus gallinarum	DSM 10532	chicken crop	37°C
Lactobacillus gasseri	DSM 20243	human	37°C
Lactobacillus gastricus	DSM 16045	gastric biopsies, human stomach mucosa (Sweden)	37°C
Lactobacillus ghanensis	DSM 18630	cocoa fermentation (Ghana)	30°C
Lactobacillus graminis	DSM 20719	grass silage	30°C
Lactobacillus hammesii	DSM 16381	wheat sourdough (France)	30°C

Species	Strain	Source	Optimal temperature
Lactobacillus hamsteri	DSM 5661	faeces of hamster	37°C
Lactobacillus harbinensis	DSM 16991	chinese traditional fermented vegetable Suan cai (China)	37°C
Lactobacillus hayakitensis	DSM 18933	faeces of thoroughbred (horse) (Japan)	30°C
Lactobacillus helveticus	DSM 20075	emmental cheese	37°C
Lactobacillus hilgardii	DSM 20176	wine	30°C
Lactobacillus homohiochii	DSM 20571	spoilt sake	26°C
Lactobacillus hordei	DSM 19519	malted barley (Belgium)	30°C
Lactobacillus iners	DSM 13335	human urine	37°C
Lactobacillus ingluviei	DSM 15946	pigeon, crop (Belgium)	37°C
Lactobacillus intestinalis	DSM 6629	intestine of rat	37°C
Lactobacillus jensenii	DSM 20557	human vaginal discharge	37°C
Lactobacillus johnsonii	DSM 10533	human blood	37°C
Lactobacillus kalixensis	DSM 16043	gastric biopsies, human stomach mucosa (Sweden)	37°C
Lactobacillus kefiranofaciens subsp. kefiranofaciens	DSM 5016	kefir grains	28°C
Lactobacillus kefiranofaciens subsp. kefirgranum	DSM 10550	kefir grains	30°C
Lactobacillus kefiri	DSM 20587	kefir grains	30°C
Lactobacillus kimchii	DSM 13961	fermented vegetable (kimchi) (South Korea)	30°C
Lactobacillus kisonensis	DSM 19906	non-salted pickle solution used in production of sunki (Japan) [2]	30°C
Lactobacillus kitasatonis	DSM 16761	chicken, intestine (Japan)	37°C
Lactobacillus kunkeei	DSM 12361	commercial grape wine undergoing a sluggish/stuck alcoholic fermentation (USA)	30°C
Lactobacillus lindneri	DSM 20690	spoilt beer	28°C
Lactobacillus malefermentans	DSM 5705	beer	28°C
Lactobacillus mali	DSM 20444	apple juice from cider press	30°C
Lactobacillus manihotivorans	DSM 13343	cassava sour starch fermentation (Colombia)	30°C
Lactobacillus mindensis	DSM 14500	sourdough (Germany)	30°C
Lactobacillus mucosae	DSM 13345	pig small intestine (Sweden)	37°C
Lactobacillus murinus	DSM 20452	intestine of rat	37°C
Lactobacillus nagelii	DSM 13675	partially fermented wine	30°C
Lactobacillus namurensis	DSM 19117	sourdough, manufactured with wheat, rye and spelt flour (Belgium)	30°C
Lactobacillus nantensis	DSM 16982	wheat sourdough (France)	30°C
Lactobacillus nodensis	DSM 19682	Japanese pickles (Japan)	30°C
Lactobacillus odoratitofui	DSM 19909	fermented brine used for stinky tofu production (Taiwan) [2]	30°C
Lactobacillus oeni	DSM 19972	Bobal wine (Spain)	30°C
Lactobacillus oligofermentans	DSM 15707	broiler leg (Finland)	25°C

Species	Strain	Source	Optimal temperature
Lactobacillus oris	DSM 4864	human saliva	37°C
Lactobacillus otakiensis	DSM 19908	non-salted pickle solution used in production of sunki (Japan) [2]	30°C
Lactobacillus ozensis	DSM 23829	*Inula ciliaris* var. *glandulosa*, a chrysanthemum (Japan)	30°C
Lactobacillus panis	DSM 6035	sourdough (Germany)	37°C
Lactobacillus pantheris	DSM 15945	jaguar, faeces (China)	37°C
Lactobacillus parabuchneri	DSM 5707	human saliva	28°C
Lactobacillus paracasei	LMG 19719		30°C
Lactobacillus paracasei subsp. *paracasei*	DSM 5622		30°C
Lactobacillus paracollinoides	DSM 15502	brewery environment (Japan)	25°C
Lactobacillus parafarraginis	DSM 18390	composting material of distilled shochun residue (Japan)	30°C
Lactobacillus parakefiri	DSM 10551	kefir grains	30°C
Lactobacillus paralimentarius	DSM 13238	sourdough (Japan)	30°C
Lactobacillus paraplantarum	DSM 10667T	beer contaminant (France) [4]	30°C
Lactobacillus paucivorans	DSM 22467	yeast storage tank containing lager beer (Germany)	28°C
Lactobacillus pentosus	DSM 20314T	sawdust fermentation	30°C
Lactobacillus pentosus	LMG 17672		30°C
Lactobacillus pentosus	LMG 17673		30°C
Lactobacillus pentosus	LMG 17678		30°C
Lactobacillus pentosus	LMG 17682		30°C
Lactobacillus pentosus	B148		30°C
Lactobacillus pentosus	10-16		30°C
Lactobacillus perolens	DSM 12744	orange lemonade (Germany) [5]	28°C
Lactobacillus plantarum subsp. *plantarum*	NCIMB 6461	unknown, [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	NCIMB 8102	unknown, *	30°C
Lactobacillus plantarum subsp. *argentoratensis*	NCIMB 12120	fermented cereal ogi (Nigeria) [7]	30°C
Lactobacillus plantarum subsp. *plantarum*	CST 10928	recycled beer bottle (France) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	CST 12007	dairy products (France) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	CST 12008	dairy products (France) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	NCIMB 8016	unknown, *	30°C
Lactobacillus plantarum subsp. *plantarum*	CST 11019	beer (France) [6]	30°C
Lactobacillus *plantarum* subsp. *plantarum*	DSM 2648	silage, [6]	30°C
Lactobacillus *plantarum* subsp. *plantarum*	CST 10967	beer (France) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	CST 11023	beer (France) [7]	30°C
Lactobacillus plantarum subsp. *plantarum*	CIP 71.39	pickled cabbage (United Kingdom) [6]	30°C

Species	Strain	Source	Optimal temperature
Lactobacillus plantarum subsp. *plantarum*	CST 12009	Fresh dairy product. [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	CST 10952		30°C
Lactobacillus plantarum subsp. *plantarum*	CST 11031	beer (France) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	NCFB 2171	cheese (New Zealand) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	FB 115	Hawaiian fermented taro (USA) [6]	30°C
Lactobacillus plantarum subsp. *argentoratensis*	DK 28J	fermented millet (Nigeria) *	30°C
Lactobacillus plantarum subsp. *plantarum*	FOEB 8402	Bordeaux grapes (France) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	NCIMB 5914	unknown, [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	FOEB 9106	Porto grapes (Portugal) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	FOEB 9113	white wine (France) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	FOEB 9532	Pineau wine (France) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	NCIMB 6105	silage, *	30°C
Lactobacillus plantarum subsp. *plantarum*	CNRZ 1220	cheese (Egypt) [7]	30°C
Lactobacillus plantarum subsp. *plantarum*	CNRZ 1838	unknown, *	30°C
Lactobacillus plantarum subsp. *plantarum*	CNRZ 1849	unknown, [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	CNRZ 1850	unknown, *	30°C
Lactobacillus plantarum subsp. *argentoratensis*	SF2A35B	sour cassava starch fermentation (South America) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	SF2B37-1	sour cassava starch fermentation (South America) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	SF2B41-1	sour cassava starch fermentation (South America) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	SF2A33	sour cassava starch fermentation (South America) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	SF2A31B	sour cassava starch fermentation (South America) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	SF2A39	sour cassava starch fermentation (South America) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	ALAB20	sour cassava starch fermentation (South America) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	CIP 102021	unknown, [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	NCFB 1088	cheese, [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	JCL1275	unknown, *	30°C
Lactobacillus plantarum subsp. *plantarum*	JCL1278	unknown, *	30°C
Lactobacillus plantarum subsp. *plantarum*	JCL1279	fermented cucumber (Spain) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	JCL1280	unknown, *	30°C
Lactobacillus plantarum subsp. *plantarum*	JCL1267	unknown, *	30°C
Lactobacillus plantarum subsp. *plantarum*	JCL1283	fermented cucumber (Spain) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	JCL1284	unknown, *	30°C
Lactobacillus plantarum subsp. *plantarum*	JCL1285	unknown, *	30°C
Lactobacillus plantarum subsp. *plantarum*	JCL1268	unknown, *	30°C

Species	Strain	Source	Optimal temperature
Lactobacillus plantarum subsp. *plantarum*	NCFB 772 (NCIMB 700772)	cheese (Sweden) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	NCFB 773 (NCIMB 700773)	cheese (Sweden) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	NCFB 963 (NCIMB 700963)	cheese (Sweden) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	NCFB 965 (NCIMB 700965)	cheese (Sweden) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	NCFB 1042 (NCIMB 701042)	hard cheese (England) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	NCFB 1193 (NCIMB 8299)	silage, [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	JCL1269	(Spain) *	30°C
Lactobacillus plantarum subsp. *plantarum*	NCFB 1204 (NCIMB 701204)	cheese starter (United Kingdom) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	NCFB 1206 (NCIMB 701206)	starter cheese (United Kingdom) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	JCL1271	(Spain) *	30°C
Lactobacillus plantarum subsp. *plantarum*	CNRZ 738	silage (France) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	CNRZ 1229	Domiatri cheese (Egypt) *	30°C
Lactobacillus plantarum subsp. *plantarum*	DSM 9296	Munster cheese (France) *	30°C
Lactobacillus plantarum subsp. *plantarum*	NCIMB 8826	human saliva, [7]	30°C
Lactobacillus plantarum subsp. *argentoratensis*	LP85-2	silage (France) [7]	30°C
Lactobacillus plantarum subsp. *plantarum*	CNRZ 184	dairy products (France) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	NCIMB 7220	pickled cabbage, [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	CNRZ 424	sourdough (France) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	Agrano 15b	sourdough (France) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	CNRZ 432	sourdough (France) *	30°C
Lactobacillus plantarum subsp. *plantarum*	CNRZ 764	dairy products (France) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	CNRZ 1228	Domiatri cheese (Egypt) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	CNRZ 1246	Domiatri cheese (Egypt) [7]	30°C
Lactobacillus plantarum subsp. *plantarum*	NCIMB 11974T	pickled cabbage, [7]	30°C
Lactobacillus plantarum subsp. *plantarum*	LMAB1	pickled cabbage (France) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	LMAB2	cheese (France) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	CCM 3626	Pecorino romano cheese (Italy) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	CCM 4279	hard cheese, [6]	30°C
Lactobacillus plantarum subsp. *argentoratensis*	A1	cassava (Colombia) [7]	30°C
Lactobacillus plantarum subsp. *plantarum*	A2	cassava (Colombia) *	30°C
Lactobacillus plantarum subsp. *argentoratensis*	A4	cassava (Colombia) [7]	30°C
Lactobacillus plantarum subsp. *plantarum*	CIP 104453	pickled cabbage, [6]	30°C
Lactobacillus plantarum subsp. *argentoratensis*	A7	cassava (Colombia) [7]	30°C
Lactobacillus plantarum subsp. *plantarum*	A9	cassava (Colombia) [6]	30°C

Species	Strain	Source	Optimal temperature
Lactobacillus plantarum subsp. *plantarum*	A12	cassava (Colombia) [7]	30°C
Lactobacillus plantarum subsp. *plantarum*	38AA	fermented cassava (Columbia) [7]	30°C
Lactobacillus plantarum subsp. *plantarum*	R4698	unknown, *	30°C
Lactobacillus plantarum subsp. *plantarum*	R4700	unknown, *	30°C
Lactobacillus plantarum subsp. *plantarum*	CIP 104454	Cantal cheese (France) [6]	30°C
Lactobacillus plantarum subsp. *argentoratensis*	DK 9	fermented cucumber (Nigeria) [7]	30°C
Lactobacillus plantarum subsp. *plantarum*	DK 15	fermented millet (Nigeria) [6]	30°C
Lactobacillus plantarum subsp. *argentoratensis*	DK 19	White maize kenkey (Nigeria) [7]	30°C
Lactobacillus plantarum subsp. *plantarum*	DK 21	fermented oil bean (Nigeria) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	DK 30	fermented cereals (Nigeria) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	DK0 12	fermented cereals (Nigeria) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	DK0 18	cucumber (Nigeria) [6]	30°C
Lactobacillus plantarum subsp. *argentoratensis*	DK0 22T (DSM 16265)	fermented cassava (Nigeria) [7]	30°C
Lactobacillus plantarum subsp. *plantarum*	DK 32	fermented cow milk (Nigeria) [6]	30°C
Lactobacillus plantarum subsp. *argentoratensis*	DK 36	tapioca (Nigeria) [7]	30°C
Lactobacillus plantarum subsp. *plantarum*	DK 38	fermented cassava (Nigeria) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	DK0 2A	tapioca (Nigeria)	30°C
Lactobacillus plantarum subsp. *plantarum*	DK0 7	fermented cereals (Nigeria) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	DK0 8	fermented cereals (Nigeria) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	ATCC 10012	unknown, [7]	30°C
Lactobacillus plantarum subsp. *plantarum*	B41	silage (Italy) [7]	30°C
Lactobacillus plantarum subsp. *plantarum*	NCFB 340	silage (United Kingdom) [7]	30°C
Lactobacillus plantarum subsp. *plantarum*	KOG 8	cabbage kimchi (Korea) [8]	30°C
Lactobacillus plantarum subsp. *plantarum*	KOG 10	pickled eggplant (Japan) [8]	30°C
Lactobacillus plantarum subsp. *plantarum*	KOG 11	pickled eggplant (Japan) [8]	30°C
Lactobacillus plantarum subsp. *plantarum*	KOG 12	pickled radish (Japan) [8]	30°C
Lactobacillus plantarum subsp. *plantarum*	KOG 4	pickled curcumber (Japan) [6, 8]	30°C
Lactobacillus plantarum subsp. *plantarum*	KOG 13	radish kimchi (Korea) [8]	30°C
Lactobacillus plantarum subsp. *plantarum*	KOG 14	pickled eggplant (Japan) [8]	30°C
Lactobacillus plantarum subsp. *plantarum*	KOG 18	turnips (Japan) [8]	30°C
Lactobacillus plantarum subsp. *plantarum*	KOG 19	pickled vegetables (Japan) [8]	30°C
Lactobacillus plantarum subsp. *plantarum*	KOG 21	pickled vegetables (Japan) [8]	30°C
Lactobacillus plantarum subsp. *plantarum*	KOG 22	pickled vegetables (Japan) [8]	30°C
Lactobacillus plantarum subsp. *plantarum*	KOG 5	pickled vegetables (Japan) [8]	30°C

Species	Strain	Source	Optimal temperature
Lactobacillus plantarum subsp. *plantarum*	KOG 23	radish kimchi (Korea) [8]	30°C
Lactobacillus plantarum subsp. *plantarum*	KOG 2	pickled turnips (Japan) [8]	30°C
Lactobacillus plantarum subsp. *plantarum*	LMG 12167	homede soft cheese (Yugoslavia)*	30°C
Lactobacillus plantarum subsp. *plantarum*	LMG 18021	milk (Senegal) *	30°C
Lactobacillus plantarum subsp. *plantarum*	FB101	crashed corn (Guatemala) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	Lactolabo	commercial starter culture, *	30°C
Lactobacillus plantarum subsp. *plantarum*	Hd4	unknown, [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	CCM 1904	corn silage. [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	DKO 20A	fermented cassava (Nigeria) [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	Hd17	unknown, [6]	30°C
Lactobacillus plantarum subsp. *plantarum*	LP80	unknown, [6]	30°C
Lactobacillus pontis	DSM 8475	rye sourdough	30°C
Lactobacillus psittaci	DSM 15354	lung of parrot (Sweden)	37°C
Lactobacillus rapi	DSM 19907	non-salted pickle solution used in production of sunki (Japan) [2]	30°C
Lactobacillus rennini	DSM 20253	rennin	30°C
Lactobacillus reuteri	DSM 20016	intestine of adult	37°C
Lactobacillus rhamnosus	DSM 20021		37°C
Lactobacillus rossiae	DSM 15814	wheat sourdough (Italy)	30°C
Lactobacillus ruminis	DSM 20403	bovine rumen	37°C
Lactobacillus saerimneri	DSM 16049	pig faeces (Sweden)	37°C
Lactobacillus sakei subsp. carnosus	DSM 15831	fermented meat produkt (Germany)	37°C
Lactobacillus sakei subsp. sakei	DSM 20017	"Moto" starter of sake	30°C
Lactobacillus salivarius subsp. salicinius	DSM 20554	saliva	37°C
Lactobacillus salivarius subsp. salivarius	DSM 20555	saliva	37°C
Lactobacillus sanfranciscensis	DSM 20451	San Francisco sourdough	30°C
Lactobacillus saniviri	DSM 24301	feces of a Japanese healthy adult male [9]	37°C
Lactobacillus satsumensis	DSM 16230	shochu mash (Japan)	30°C
Lactobacillus secaliphilus	DSM 17896	sourdough (Germany)	37°C
Lactobacillus selangorensis	DSM 13344	chili bo (Malaysia)	30°C
Lactobacillus senioris	DSM 24302	feces of a healthy 100-year-old Japanese female (Japan, Okinawa) [9]	37°C
Lactobacillus senmaizukei	DSM 21775	pickles (Japan)	30°C
Lactobacillus sharpeae	DSM 20505	municipal sewage	30°C
Lactobacillus siliginis	DSM 22696	wheat sourdough (Republic of Korea, Daejeon)	37°C
Lactobacillus similis	DSM 23365	fermented cane molasses at alcohol plants (Thailand)	35°C

Species	Strain	Source	Optimal temperature
Lactobacillus spicheri	DSM 15429	rice sourdough (Germany)	30°C
Lactobacillus sucicola	DSM 21376	sap of Quercus sp (Japan)	30°C
Lactobacillus suebicus	DSM 5007	apple mash	30°C
Lactobacillus sunkii	DSM 19904	non-salted pickle solution used in production of sunki (Japan) [2]	30°C
Lactobacillus taiwanensis	DSM 21401	silage cattle feed (Taiwan)	37°C
Lactobacillus thailandensis	DSM 22698	fermented tea leaves (miang) (Thailand)	37°C
Lactobacillus tucceti	DSM 20183	sausage	30°C
Lactobacillus ultunensis	DSM 16047	gastric biopsies, human stomach mucosa (Sweden)	37°C
Lactobacillus uvarum	DSM 19971	must of Bobal grape variety (Spain)	30°C
Lactobacillus vaccinostercus	DSM 20634	cow dung	30°C
Lactobacillus vaginalis	DSM 5837	vaginal swab from patient with trichomoniasis	37°C
Lactobacillus versmoldensis	DSM 14857	poultry salami (Germany)	30°C
Lactobacillus vini	DSM 20605	grape must, fermenting at high temperature	37°C
Lactobacillus zeae	DSM 20178	corn steep liquor	37°C
Lactobacillus zymae	DSM 19395	artisanal wheat sourdough (Belgium)	30°C
Lactococcus lactis	MG 1363		30°C
Pediococcus acidilactici	DSM 20284	barley	30°C
Pediococcus argentinicus	DSM 23026	fermented wheat flour (Argentina)	30°C
Pediococcus cellicola	DSM 17757	distilled pirit-fermenting cellar (China)	30°C
Pediococcus claussenii	DSM 14800	spoiled beer (Canada)	28°C
Pediococcus damnosus	DSM 20331	lager beer yeast	26°C
Pediococcus ethanolidurans	DSM 22301	walls of a distilled-spirit-fermenting cellar (China)	37°C
Pediococcus inopinatus	DSM 20285	brewery yeast	30°C
Pediococcus lolii	DSM 19927	Ryegrass silage (Japan)	30°C
Pediococcus parvulus	DSM 20332	silage	30°C
Pediococcus pentosaceus	ATCC 25745		30°C
Pediococcus stilesii	DSM 18001	white maize grains (Nigeria)	30°C

*, personal communication, unpublished

1. Prof. Luc de Vuyst, Vrije Universiteit Brussel, IMDO (Brussel, Belgium).

2. Dr. Koichi Watanabe, Yakult Central Institute for Microbial Research (Tokyo, Japan).

3. Dr. J.E. Germond, Nestle Research Center (Lausanne 26, Switzerland).

4. Curk MC, Hubert JC, Bringel F: **Lactobacillus paraplantarum sp. now., a new species related to *Lactobacillus plantarum*.** *Int J Syst Bacteriol* 1996, **46**(2)**:**595–598.

5. Dr. Ingrid Bohak, TU München, Technologie der Brauerei I (Freising-Weihenstephan, Germany).

6. Bringel F, Quénée P, Tailliez P: **Polyphasic investigation of the diversity within *Lactobacillus plantarum* related strains revealed two *L. plantarum* subgroups.** *Syst Appl Microbiol* 2001, **24:**561–571.

7. Bringel F, Castioni A, Olukoya DK, Felis GE, Torriani S, Dellaglio F: ***Lactobacillus plantarum* subsp. *argentoratensis* subsp. nov., isolated from vegetable matrices.** *Int J Syst Evol Microbiol* 2005, **55:**1629–1634.

8. Osawa R, Kuroiso K, Goto S, Shimizu A: **Isolation of tannin-degrading lactobacilli from humans and fermented foods.** *Appl Environ Microbiol* 2000, **66:**3093–3097.

9. Dr. Kaihei Oki, Culture Collection and Microbial Systematics Laboratory, Bioresource Department, Yakult Central Institute for Microbial Research (Tokyo, Japan).

Table S2. Growth of the tested strains on MRS, DLA and GSA media.

Species	Strain	MRS	DLA	GSA
Lactobacillus acetotolerans	DSM 20749	+	-	-
Lactobacillus acidifarinae	DSM 19394	+	-	-
Lactobacillus acidipiscis	DSM 15836	-	nd	nd
Lactobacillus acidophilus	DSM 20079	+	-	-
Lactobacillus agilis	DSM 20509	+	-	-
Lactobacillus algidus	DSM 15638	-	nd	nd
Lactobacillus alimentarius	DSM 20249	+	-	-
Lactobacillus amylolyticus	DSM 11664	-	nd	nd
Lactobacillus amylophilus	DSM 20533	+	-	-
Lactobacillus amylotrophicus	DSM 20534	+	-	-
Lactobacillus amylovorus	DSM 20531	+	-	-
Lactobacillus animalis	DSM 20602	+	-	-
Lactobacillus antri	DSM 16041	±	-	+
Lactobacillus apodemi	DSM 16634	+	-	±
Lactobacillus aquaticus	DSM 21051	+	+	+
Lactobacillus aviarius subsp. araffinosus	DSM 20653	-	nd	nd
Lactobacillus aviarius subsp. aviarius	DSM 20655	-	nd	nd
Lactobacillus bifermentans	DSM 20003	+	-	-
Lactobacillus bobalius	DSM 19674	+	-	-
Lactobacillus brevis	LMG 19186	+	-	-
Lactobacillus brevis	LMG 19188	+	-	-
Lactobacillus brevis	LMG 19191	+	-	±
Lactobacillus brevis	LMG 19217	+	-	-
Lactobacillus brevis	LMG 19215	+	-	+
Lactobacillus brevis	LMG 19216	+	-	-
Lactobacillus brevis	DSM 20054	+	-	+
Lactobacillus buchneri	Ketchup-1	+	+	-
Lactobacillus buchneri	DSM 20057	+	-	-
Lactobacillus buchneri	Ketchup-3	+	+	-
Lactobacillus cacaonum	DSM 21116	+	-	-
Lactobacillus camelliae	DSM 22697	+	-	-
Lactobacillus capillatus	DSM 19910	+	+	+
Lactobacillus casei	DSM 20011	+	-	+
Lactobacillus ceti	DSM 22408	-	nd	nd
Lactobacillus coleohominis	DSM 14060	+	-	-
Lactobacillus collinoides	DSM 20515	-	nd	nd
Lactobacillus composti	DSM 18527	+	+	±
Lactobacillus concavus	DSM 17758	+	-	±
Lactobacillus coryniformis subsp. coryniformis	DSM 20001	+	-	+
Lactobacillus coryniformis subsp. torquens	DSM 20004	+	-	+
Lactobacillus crispatus	DSM 20584	+	-	-
Lactobacillus curvatus	DSM 20019	+	-	±

Species	Strain	MRS	DLA	GSA
Lactobacillus delbrueckii subsp. bulgaricus	DSM 20081	+	-	-
Lactobacillus delbrueckii subsp. delbrueckii	DSM 20074	±	-	-
Lactobacillus delbrueckii subsp. indicus	DSM 15996	+	-	-
Lactobacillus delbrueckii subsp. lactis	DSM 20072	±	-	-
Lactobacillus dextrinicus	DSM 20335	+	-	-
Lactobacillus diolivorans	DSM 14421	+	±	-
Lactobacillus equi	DSM 15833	+	-	±
Lactobacillus equicursoris	DSM 19284	-	nd	nd
Lactobacillus equigenerosi	DSM 18793	+	-	-
Lactobacillus fabifermentans	DSM 21115	+	+	+
Lactobacillus farciminis	DSM 20184	+	±	-
Lactobacillus farraginis	DSM 18382	+	-	-
Lactobacillus fermentum	DSM 20052	+	-	+
Lactobacillus floricola	DSM 23037	+	-	-
Lactobacillus florum	DSM 22689	+	-	-
Lactobacillus fructivorans	DSM 20203	+	-	-
Lactobacillus frumenti	DSM 13145	+	-	-
Lactobacillus fuchuensis	DSM 14340	+	+	-
Lactobacillus gallinarum	DSM 10532	+	-	-
Lactobacillus gasseri	DSM 20243	+	-	-
Lactobacillus gastricus	DSM 16045	+	-	-
Lactobacillus ghanensis	DSM 18630	±	-	-
Lactobacillus graminis	DSM 20719	+	-	+
Lactobacillus hammesii	DSM 16381	+	-	-
Lactobacillus hamsteri	DSM 5661	-	nd	nd
Lactobacillus harbinensis	DSM 16991	+	-	+
Lactobacillus hayakitensis	DSM 18933	+	-	-
Lactobacillus helveticus	DSM 20075	+	-	-
Lactobacillus hilgardii	DSM 20176	+	-	+
Lactobacillus homohiochii	DSM 20571	-	nd	nd
Lactobacillus hordei	DSM 19519	+	+	+
Lactobacillus iners	DSM 13335	-	nd	nd
Lactobacillus ingluviei	DSM 15946	+	-	±
Lactobacillus intestinalis	DSM 6629	+	-	-
Lactobacillus jensenii	DSM 20557	+	-	-
Lactobacillus johnsonii	DSM 10533	+	-	-
Lactobacillus kalixensis	DSM 16043	+	-	-
Lactobacillus kefiranofaciens subsp. kefiranofaciens	DSM 5016	-	nd	nd
Lactobacillus kefiranofaciens subsp. kefirgranum	DSM 10550	-	nd	nd
Lactobacillus kefiri	DSM 20587	+	-	-
Lactobacillus kimchii	DSM 13961	+	-	+
Lactobacillus kisonensis	DSM 19906	+	-	-
Lactobacillus kitasatonis	DSM 16761	+	-	-

Species	Strain	MRS	DLA	GSA
Lactobacillus kunkeei	DSM 12361	+	+	-
Lactobacillus lindneri	DSM 20690	-	nd	nd
Lactobacillus malefermentans	DSM 5705	+	-	-
Lactobacillus mali	DSM 20444	+	+	+
Lactobacillus manihotivorans	DSM 13343	+	+	-
Lactobacillus mindensis	DSM 14500	+	-	-
Lactobacillus mucosae	DSM 13345	+	-	-
Lactobacillus murinus	DSM 20452	+	-	±
Lactobacillus nagelii	DSM 13675	+	-	+
Lactobacillus namurensis	DSM 19117	+	-	-
Lactobacillus nantensis	DSM 16982	+	-	-
Lactobacillus nodensis	DSM 19682	+	-	-
Lactobacillus odoratitofui	DSM 19909	+	±	-
Lactobacillus oeni	DSM 19972	+	-	+
Lactobacillus oligofermentans	DSM 15707	+	+	-
Lactobacillus oris	DSM 4864	+	-	-
Lactobacillus otakiensis	DSM 19908	+	-	-
Lactobacillus ozensis	DSM 23829	±	±	-
Lactobacillus panis	DSM 6035	-	nd	nd
Lactobacillus pantheris	DSM 15945	+	-	-
Lactobacillus parabuchneri	DSM 5707	+	-	-
Lactobacillus paracasei	LMG 19719	+	-	+
Lactobacillus paracasei subsp. paracasei	DSM 5622	+	-	+
Lactobacillus paracollinoides	DSM 15502	-	nd	nd
Lactobacillus parafarraginis	DSM 18390	+	±	-
Lactobacillus parakefiri	DSM 10551	±	-	-
Lactobacillus paralimentarius	DSM 13238	+	-	±
Lactobacillus paraplantarum	DSM 10667T	+	+	+
Lactobacillus paucivorans	DSM 22467	-	nd	nd
Lactobacillus pentosus	DSM 20314T	+	+	+
Lactobacillus pentosus	LMG 17672	+	+	+
Lactobacillus pentosus	LMG 17673	+	+	+
Lactobacillus pentosus	LMG 17678	+	+	+
Lactobacillus pentosus	LMG 17682	+	+	+
Lactobacillus pentosus	B148	+	+	+
Lactobacillus pentosus	10-16	+	+	+
Lactobacillus perolens	DSM 12744	+	-	-
Lactobacillus plantarum subsp. *plantarum*	NCIMB 6461	+	+	-
Lactobacillus plantarum subsp. *plantarum*	NCIMB 8102	+	+	+
Lactobacillus plantarum subsp. *argentoratensis*	NCIMB 12120	+	+	+
Lactobacillus plantarum subsp. *plantarum*	CST 10928	+	+	+
Lactobacillus plantarum subsp. *plantarum*	CST 12007	+	+	+
Lactobacillus plantarum subsp. *plantarum*	CST 12008	+	+	+

Species	Strain	MRS	DLA	GSA
Lactobacillus *plantarum* subsp. *plantarum*	NCIMB 8016	+	+	-
Lactobacillus plantarum subsp. *plantarum*	CST 11019	+	±	+
Lactobacillus *plantarum* subsp. *plantarum*	DSM 2648	+	+	+
Lactobacillus *plantarum* subsp. *plantarum*	CST 10967	+	+	+
Lactobacillus plantarum subsp. *plantarum*	CST 11023	+	+	+
Lactobacillus plantarum subsp. *plantarum*	CIP 71.39	+	+	+
Lactobacillus plantarum subsp. *plantarum*	CST 12009	+	+	+
Lactobacillus plantarum subsp. *plantarum*	CST 10952	+	+	+
Lactobacillus plantarum subsp. *plantarum*	CST 11031	+	+	+
Lactobacillus plantarum subsp. *plantarum*	NCFB 2171	+	-	+
Lactobacillus plantarum subsp. *plantarum*	FB115	+	+	+
Lactobacillus plantarum subsp. *argentoratensis*	DK 28J	+	+	+
Lactobacillus plantarum subsp. *plantarum*	FOEB 8402	+	+	+
Lactobacillus plantarum subsp. *plantarum*	NCIMB 5914	+	+	+
Lactobacillus plantarum subsp. *plantarum*	FOEB 9106	+	-	+
Lactobacillus plantarum subsp. *plantarum*	FOEB 9113	+	+	+
Lactobacillus plantarum subsp. *plantarum*	FOEB 9532	+	+	+
Lactobacillus plantarum subsp. *plantarum*	NCIMB 6105	+	+	+
Lactobacillus plantarum subsp. *plantarum*	CNRZ 1220	+	+	±
Lactobacillus plantarum subsp. *plantarum*	CNRZ 1838	+	+	+
Lactobacillus plantarum subsp. *plantarum*	CNRZ 1849	+	+	+
Lactobacillus plantarum subsp. *plantarum*	CNRZ 1850	+	+	+
Lactobacillus plantarum subsp. *argentoratensis*	SF2A35B	+	+	+
Lactobacillus plantarum subsp. *plantarum*	SF2B37-1	+	+	+
Lactobacillus plantarum subsp. *plantarum*	SF2B41-1	+	+	+
Lactobacillus plantarum subsp. *plantarum*	SF2A33	+	+	+
Lactobacillus plantarum subsp. *plantarum*	SF2A31B	+	+	+
Lactobacillus plantarum subsp. *plantarum*	SF2A39	+	+	+
Lactobacillus plantarum subsp. *plantarum*	ALAB20	+	+	+
Lactobacillus plantarum subsp. *plantarum*	CIP 102021	+	+	+
Lactobacillus plantarum subsp. *plantarum*	NCFB 1088	+	+	+
Lactobacillus plantarum subsp. *plantarum*	JCL1275	+	+	+
Lactobacillus plantarum subsp. *plantarum*	JCL1278	+	+	+
Lactobacillus plantarum subsp. *plantarum*	JCL1279	+	+	+
Lactobacillus plantarum subsp. *plantarum*	JCL1280	+	-	-
Lactobacillus plantarum subsp. *plantarum*	JCL1267	+	+	+
Lactobacillus plantarum subsp. *plantarum*	JCL1283	+	+	+
Lactobacillus plantarum subsp. *plantarum*	JCL1284	+	+	-
Lactobacillus plantarum subsp. *plantarum*	JCL1285	+	-	-
Lactobacillus plantarum subsp. *plantarum*	JCL1268	+	±	+
Lactobacillus plantarum subsp. *plantarum*	NCFB 772 (NCIMB 700772)	+	-	+
Lactobacillus plantarum subsp. *plantarum*	NCFB 773 (NCIMB 700773)	+	+	+
Lactobacillus plantarum subsp. *plantarum*	NCFB 963 (NCIMB 700963)	+	+	+

Species	Strain	MRS	DLA	GSA
Lactobacillus plantarum subsp. *plantarum*	NCFB 965 (NCIMB 700965)	+	-	+
Lactobacillus plantarum subsp. *plantarum*	NCFB 1042 (NCIMB 701042)	+	+	+
Lactobacillus plantarum subsp. *plantarum*	NCFB 1193 (NCIMB 8299)	+	+	+
Lactobacillus plantarum subsp. *plantarum*	JCL1269	+	+	+
Lactobacillus plantarum subsp. *plantarum*	NCFB 1204 (NCIMB 701204)	+	+	+
Lactobacillus plantarum subsp. *plantarum*	NCFB 1206 (NCIMB 701206)	+	+	+
Lactobacillus plantarum subsp. *plantarum*	JCL1271	+	+	+
Lactobacillus plantarum subsp. *plantarum*	CNRZ 738J	+	+	+
Lactobacillus plantarum subsp. *plantarum*	CNRZ 1229	+	+	+
Lactobacillus plantarum subsp. *plantarum*	DSM9296	+	+	+
Lactobacillus plantarum subsp. *plantarum*	NCIMB 8826	+	-	+
Lactobacillus plantarum subsp. *argentoratensis*	LP85-2	+	-	+
Lactobacillus plantarum subsp. *plantarum*	CNRZ 184	+	+	+
Lactobacillus plantarum subsp. *plantarum*	NCIMB 7220	+	+	+
Lactobacillus plantarum subsp. *plantarum*	CNRZ 424	+	+	+
Lactobacillus plantarum subsp. *plantarum*	Agrano 15b	+	+	+
Lactobacillus plantarum subsp. *plantarum*	CNRZ 432	+	+	+
Lactobacillus plantarum subsp. *plantarum*	CNRZ 764	+	±	+
Lactobacillus plantarum subsp. *plantarum*	CNRZ 1228	+	+	+
Lactobacillus plantarum subsp. *plantarum*	CNRZ 1246	+	+	+
Lactobacillus plantarum subsp. *plantarum*	NCIMB 11974T	+	+	+
Lactobacillus plantarum subsp. *plantarum*	LMAB1	+	+	+
Lactobacillus plantarum subsp. *plantarum*	LMAB2	+	+	+
Lactobacillus plantarum subsp. *plantarum*	CCM 3626	+	-	+
Lactobacillus plantarum subsp. *plantarum*	CCM4279	+	+	+
Lactobacillus plantarum subsp. *argentoratensis*	A1	+	+	+
Lactobacillus plantarum subsp. *plantarum*	A2	+	±	+
Lactobacillus plantarum subsp. *argentoratensis*	A4	+	+	+
Lactobacillus plantarum subsp. *plantarum*	CIP104453	+	±	+
Lactobacillus plantarum subsp. *argentoratensis*	A7	+	+	+
Lactobacillus plantarum subsp. *plantarum*	A9	+	+	+
Lactobacillus plantarum subsp. *plantarum*	A12	+	+	+
Lactobacillus plantarum subsp. *plantarum*	38AA	+	+	+
Lactobacillus plantarum subsp. *plantarum*	R4698	+	+	+
Lactobacillus plantarum subsp. *plantarum*	R4700	+	+	+
Lactobacillus plantarum subsp. *plantarum*	CIP104454	+	+	+
Lactobacillus plantarum subsp. *argentoratensis*	DK 9	+	+	+
Lactobacillus plantarum subsp. *plantarum*	DK 15	+	+	+
Lactobacillus plantarum subsp. *argentoratensis*	DK 19	+	+	+
Lactobacillus plantarum subsp. *plantarum*	DK 21	+	+	+
Lactobacillus plantarum subsp. *plantarum*	DK 30	+	+	+
Lactobacillus plantarum subsp. *plantarum*	DK0 12	+	+	+
Lactobacillus plantarum subsp. *plantarum*	DK0 18	+	+	+

Species	Strain	MRS	DLA	GSA
Lactobacillus plantarum subsp. *argentoratensis*	DK0 22T (DSM 16265)	+	+	+
Lactobacillus plantarum subsp. *plantarum*	DK 32	+	+	+
Lactobacillus plantarum subsp. *argentoratensis*	DK 36	+	+	+
Lactobacillus plantarum subsp. *plantarum*	DK 38	+	+	+
Lactobacillus plantarum subsp. *plantarum*	DK0 2A	+	+	+
Lactobacillus plantarum subsp. *plantarum*	DK0 7	+	+	+
Lactobacillus plantarum subsp. *plantarum*	DK0 8	+	+	+
Lactobacillus plantarum subsp. *plantarum*	ATCC 10012	+	+	+
Lactobacillus plantarum subsp. *plantarum*	B41	+	+	+
Lactobacillus plantarum subsp. *plantarum*	NCFB 340	+	+	+
Lactobacillus plantarum subsp. *plantarum*	KOG 8	+	+	+
Lactobacillus plantarum subsp. *plantarum*	KOG 10	+	+	+
Lactobacillus plantarum subsp. *plantarum*	KOG 11	+	+	-
Lactobacillus plantarum subsp. *plantarum*	KOG 12	+	+	-
Lactobacillus plantarum subsp. *plantarum*	KOG 4	+	+	-
Lactobacillus plantarum subsp. *plantarum*	KOG 13	+	+	-
Lactobacillus plantarum subsp. *plantarum*	KOG 14	+	+	+
Lactobacillus plantarum subsp. *plantarum*	KOG 18	+	+	-
Lactobacillus plantarum subsp. *plantarum*	KOG 19	+	+	-
Lactobacillus plantarum subsp. *plantarum*	KOG 21	+	+	+
Lactobacillus plantarum subsp. *plantarum*	KOG 22	+	+	-
Lactobacillus plantarum subsp. *plantarum*	KOG 5	+	-	-
Lactobacillus plantarum subsp. *plantarum*	KOG 23	+	+	-
Lactobacillus plantarum subsp. *plantarum*	KOG 2	+	+	+
Lactobacillus plantarum subsp. *plantarum*	LMG 12167	+	+	+
Lactobacillus plantarum subsp. *plantarum*	LMG 18021	+	+	+
Lactobacillus plantarum subsp. *plantarum*	FB101	+	+	+
Lactobacillus plantarum subsp. *plantarum*	Lactolabo	+	+	+
Lactobacillus plantarum subsp. *plantarum*	Hd4	+	+	+
Lactobacillus plantarum subsp. *plantarum*	CCM 1904	+	+	+
Lactobacillus plantarum subsp. *plantarum*	DKO 20A	+	+	+
Lactobacillus plantarum subsp. *plantarum*	Hd17	+	+	+
Lactobacillus plantarum subsp. *plantarum*	LP80	+	+	+
Lactobacillus pontis	DSM 8475	-	nd	nd
Lactobacillus psittaci	DSM 15354	+	+	+
Lactobacillus rapi	DSM 19907	+	-	-
Lactobacillus rennini	DSM 20253	+	+	-
Lactobacillus reuteri	DSM 20016	+	-	-
Lactobacillus rhamnosus	DSM 20021	+	±	+
Lactobacillus rossiae	DSM 15814	+	-	-
Lactobacillus ruminis	DSM 20403	+	-	-
Lactobacillus saerimneri	DSM 16049	+	-	-
Lactobacillus sakei subsp. carnosus	DSM 15831	+	-	±

Species	Strain	MRS	DLA	GSA
Lactobacillus sakei subsp. sakei	DSM 20017	+	-	+
Lactobacillus salivarius subsp. salicinius	DSM 20554	+	-	-
Lactobacillus salivarius subsp. salivarius	DSM 20555	+	-	-
Lactobacillus sanfranciscensis	DSM 20451	-	nd	nd
Lactobacillus saniviri	DSM 24301	+	+	-
Lactobacillus satsumensis	DSM 16230	+	+	-
Lactobacillus secaliphilus	DSM 17896	-	nd	nd
Lactobacillus selangorensis	DSM 13344	+	-	±
Lactobacillus senioris	DSM 24302	+	-	-
Lactobacillus senmaizukei	DSM 21775	+	-	-
Lactobacillus sharpeae	DSM 20505	+	-	-
Lactobacillus siliginis	DSM 22696	-	nd	nd
Lactobacillus similis	DSM 23365	-	nd	nd
Lactobacillus spicheri	DSM 15429	+	-	-
Lactobacillus sucicola	DSM 21376	+	+	+
Lactobacillus suebicus	DSM 5007	+	±	-
Lactobacillus sunkii	DSM 19904	+	-	-
Lactobacillus taiwanensis	DSM 21401	+	-	-
Lactobacillus thailandensis	DSM 22698	+	-	-
Lactobacillus tucceti	DSM 20183	+	-	±
Lactobacillus ultunensis	DSM 16047	-	nd	nd
Lactobacillus uvarum	DSM 19971	+	+	+
Lactobacillus vaccinostercus	DSM 20634	±	-	-
Lactobacillus vaginalis	DSM 5837	+	-	-
Lactobacillus versmoldensis	DSM 14857	+	-	-
Lactobacillus vini	DSM 20605	+	-	-
Lactobacillus zeae	DSM 20178	+	-	-
Lactobacillus zymae	DSM 19395	+	±	-
Pediococcus acidilactici	DSM 20284	+	-	-
Pediococcus argentinicus	DSM 23026	+	-	-
Pediococcus cellicola	DSM 17757	+	-	-
Pediococcus claussenii	DSM 14800	+	-	+
Pediococcus damnosus	DSM 20331	±	-	-
Pediococcus ethanolidurans	DSM 22301	+	-	-
Pediococcus inopinatus	DSM 20285	+	-	-
Pediococcus lolii	DSM 19927	+	-	-
Pediococcus parvulus	DSM 20332	+	-	-
Pediococcus pentosaceus	ATCC 25745	+	-	-
Pediococcus stilesii	DSM 18001	+	-	+

+, good growth; ±, moderate growth; -, no or poor growth; nd, not determined

Table S3. Results of the screening on MRS plates.

Strain	MRS	Furfural	HMF	4-hydroxybenzaldehyde	Syringaldehyde	Vanillin	Catechol	Furfuryl alcohol	Guaiacol	Methylcatechol	Vanillin alcohol	Ethanol	Syringyl alcohol	Formic acid	Levulinic acid	Acetic acid	Syringic acid	Vanillic acid	Ferulic acid
10-16	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
38AA	+	+	-	-	-	+	-	-	+	-	±	+	±	+	+	+	+	+	+
A1	+	+	±	+	-	+	±	±	+	-	+	+	+	+	+	+	+	+	+
A12	+	+	-	+	-	+	-	-	+	-	+	+	+	+	+	+	+	+	+
A2	+	+	±	+	-	+	±	±	+	-	+	+	+	±	+	+	+	+	+
A4	+	+	-	+	-	+	±	±	+	-	-	+	+	+	+	+	+	+	+
A7	+	+	+	+	-	+	+	+	+	-	+	+	±	+	+	+	+	+	+
A9	+	+	-	+	-	+	-	±	+	-	±	+	+	±	+	+	+	+	+
Agrano 15b	+	+	±	+	-	+	-	±	+	-	+	+	+	+	+	+	+	+	+
ALAB20	+	±	-	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+
ATCC 10012	+	+	±	+	-	+	+	±	+	-	+	+	+	+	+	+	+	+	+
ATCC 25745	+	+	+	+	+	+	+	+	+	-	+	+	+	±	+	+	+	+	+
B148	+	+	+	+	-	+	+	±	+	-	+	+	+	+	±	±	+	+	+
B41	+	+	±	+	-	+	+	±	+	-	+	+	+	+	+	+	+	+	+
CCM 1904	+	+	±	+	±	+	+	±	+	-	+	+	+	±	+	+	±	+	+
CCM 3626	+	+	-	+	-	+	±	-	+	-	±	+	+	+	+	+	±	+	+
CCM4279	+	+	±	+	-	+	+	±	+	-	+	+	+	+	+	+	+	+	+
CIP 102021	+	+	-	+	-	+	+	-	+	-	+	±	+	+	+	+	+	+	+
CIP104453	+	+	-	+	-	+	±	±	+	-	±	+	+	-	-	-	+	+	+
CIP104454	+	+	+	+	-	-	+	-	+	-	±	+	+	±	+	+	+	+	+
CIP71.39	+	+	-	+	-	±	+	-	+	+	+	±	±	±	±	+	+	+	+
CNRZ 1220	+	+	±	+	-	+	+	-	+	+	+	+	+	-	+	+	±	+	±
CNRZ 1228	+	+	±	+	±	+	+	±	+	-	±	+	+	±	+	+	+	+	+
CNRZ 1229	+	±	±	+	+	+	+	±	+	+	+	+	+	±	+	+	+	+	+
CNRZ 1246	+	+	±	+	-	+	+	-	+	-	±	+	+	±	+	+	+	+	+
CNRZ 1838	+	+	±	+	-	+	+	-	+	±	+	+	+	±	+	+	+	+	+
CNRZ 184	+	+	±	+	-	+	+	±	+	+	±	+	+	±	+	+	+	+	+
CNRZ 1849	+	±	±	+	-	±	+	-	+	+	+	±	+	+	+	+	±	+	±
CNRZ 1850	+	+	±	+	+	+	+	+	+	±	+	+	+	±	+	+	+	+	+
CNRZ 424	+	+	-	+	-	+	±	±	+	+	+	+	+	±	+	+	+	+	+
CNRZ 432	+	+	-	+	-	+	±	±	+	-	±	+	+	+	+	+	+	+	+
CNRZ 738J	+	+	±	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CNRZ 764	+	+	±	+	±	+	+	±	+	-	±	+	+	+	+	+	+	+	+
CST 10928	+	+	±	+	±	+	+	±	+	±	±	±	+	+	+	+	+	+	+
CST 12009	+	+	±	+	+	+	+	±	+	+	+	+	+	±	+	+	+	+	+
CST 10952	+	+	+	+	±	+	+	±	+	+	+	+	+	+	+	+	+	+	+

Strain	MRS	Furfural	HMF	4-hydroxybenzaldehyde	Syringaldehyde	Vanillin	Catechol	Furfuryl alcohol	Guaiacol	Methylcatechol	Vanillin alcohol	Ethanol	Syringyl alcohol	Formic acid	Levulinic acid	Acetic acid	Syringic acid	Vanillic acid	Ferulic acid
CST 10967	+	+	±	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+
CST 11019	+	+	-	+	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+
CST 11023	+	+	±	+	-	+	+	±	+	+	+	+	+	±	+	+	+	+	+
CST 11031	+	+	±	+	+	+	+	±	+	+	+	+	+	+	+	+	+	+	+
CST 12007	+	+	+	+	-	-	+	-	+	-	+	+	+	+	+	+	+	+	+
CST 12008	+	+	-	+	+	+	+	-	+	+	+	+	+	±	+	+	+	+	+
DK0 12	+	+	±	+	±	±	±	±	+	-	±	+	+	+	+	+	+	+	+
DK0 18	+	+	±	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
DK0 22T (DSM 16265)	+	+	+	+	±	+	-	-	+	-	+	+	+	±	+	+	+	+	+
DK0 2A	+	+	+	+	±	+	-	-	+	-	+	+	+	+	±	+	+	+	+
DK0 7	+	+	±	+	-	+	+	±	+	-	+	+	+	+	+	+	+	+	+
DK0 8	+	+	±	+	-	+	+	±	+	-	+	+	+	+	+	+	+	+	+
DK 15	+	+	-	+	-	-	+	±	+	-	±	+	+	+	+	+	+	+	+
DK 19	+	+	+	+	±	+	-	-	+	-	+	+	+	+	+	+	+	+	+
DK 21	+	+	±	+	±	+	+	±	+	±	+	+	+	+	+	+	+	+	+
DK 28J	+	+	+	+	±	±	±	±	+	-	+	+	±	+	+	+	±	+	+
DK 30	+	+	-	+	+	±	±	±	+	-	±	+	+	+	+	+	+	+	+
DK 32	+	+	±	+	±	+	+	+	+	-	+	+	+	+	+	+	+	+	+
DK 36	+	+	±	+	+	+	+	±	+	-	+	+	+	+	+	+	+	+	+
DK 38	+	+	±	+	-	±	±	±	+	-	±	+	±	+	+	+	±	+	±
DK 9	+	+	+	+	-	-	+	-	+	-	-	+	+	+	+	+	+	+	+
DKO 20A	+	+	±	+	+	+	+	±	+	-	+	+	+	+	+	+	+	+	+
DSM 10532	+	-	-	±	-	-	-	-	+	-	+	+	+	±	-	±	+	+	+
DSM 10533	+	±	±	+	-	±	±	±	+	-	±	+	+	+	+	+	+	+	+
DSM 10551	±	-	-	±	-	±	-	-	±	-	-	±	±	-	-	-	-	±	±
DSM 10667T	+	+	-	+	-	+	+	±	+	-	+	+	+	±	±	±	+	+	+
DSM 12361	+	+	+	+	-	+	+	-	+	±	+	+	+	±	+	+	+	+	+
DSM 12744	+	±	-	-	-	-	-	-	-	-	-	±	-	±	+	+	-	-	-
DSM 13145	+	-	-	±	-	-	-	-	±	-	-	±	±	-	-	-	±	+	±
DSM 13238	+	+	±	+	+	+	+	±	+	-	+	+	+	+	+	+	+	+	+
DSM 13343	+	+	-	+	-	-	-	-	+	-	-	-	-	-	+	+	+	±	±
DSM 13344	+	+	-	+	-	±	±	±	+	-	-	-	+	-	-	-	+	+	-
DSM 13345	+	-	±	+	-	±	-	+	+	-	±	+	+	-	-	-	±	+	+
DSM 13675	+	±	-	+	-	±	-	-	±	-	-	-	±	-	-	-	±	±	-
DSM 13961	+	+	±	+	±	+	±	±	+	-	±	+	+	+	+	+	+	+	+
DSM 14060	+	+	-	nd	-	±	-	-	+	-	±	+	+	+	+	+	-	+	+
DSM 14340	+	-	-	+	-	-	-	-	+	-	-	+	+	±	+	+	+	+	+
DSM 14421	+	+	-	+	-	+	±	-	+	-	-	+	+	-	±	±	+	+	+

Strain	MRS	Furfural	HMF	4-hydroxybenzaldehyde	Syringaldehyde	Vanillin	Catechol	Furfuryl alcohol	Guaiacol	Methylcatechol	Vanillin alcohol	Ethanol	Syringyl alcohol	Formic acid	Levulinic acid	Acetic acid	Syringic acid	Vanillic acid	Ferulic acid
DSM 14500	+	-	±	±	-	-	-	-	+	-	-	-	-	-	-	-	±	±	±
DSM 14800	+	-	-	+	-	+	±	-	+	-	+	+	+	±	±	±	+	+	+
DSM 14857	+	-	-	±	-	-	-	-	±	-	-	-	-	-	-	-	±	±	-
DSM 15354	+	+	±	+	-	+	+	-	+	-	+	+	+	+	+	+	+	+	+
DSM 15429	+	+	+	+	-	+	±	±	+	-	±	+	+	+	+	±	+	+	+
DSM 15707	+	+	nd	nd	-	+	-	±	+	-	+	+	+	±	+	+	+	+	+
DSM 15814	+	+	+	+	-	±	±	-	+	-	-	+	+	+	+	+	+	+	+
DSM 15831	+	±	-	+	-	±	+	-	+	-	-	+	+	-	-	-	+	+	+
DSM 15833	+	-	-	+	-	±	±	-	+	-	-	+	±	±	±	-	+	+	+
DSM 15945	+	-	-	+	-	±	-	-	+	-	±	+	+	-	±	±	+	+	+
DSM 15946	+	+	+	-	-	-	-	-	-	-	-	-	-	±	+	+	±	±	±
DSM 15996	+	-	-	±	-	-	-	-	±	-	-	±	±	±	-	-	±	±	±
DSM 16041	±	-	-	-	-	-	-	-	-	-	-	-	-	-	±	±	±	-	±
DSM 16043	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	±	±	±
DSM 16045	+	±	-	-	-	-	-	-	-	-	-	-	-	-	-	-	±	±	-
DSM 16049	+	+	±	+	-	+	+	-	+	-	±	+	+	+	+	+	+	+	+
DSM 16230	+	±	-	+	-	-	-	-	+	-	-	±	+	±	+	+	+	+	+
DSM 16381	+	+	+	+	-	+	±	-	+	-	-	+	+	+	+	+	+	+	+
DSM 16634	+	-	-	±	-	-	-	-	±	-	-	-	±	-	-	-	±	±	±
DSM 16761	+	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+	±	-	-
DSM 16982	+	+	+	+	-	+	-	-	-	-	-	+	+	+	+	+	+	+	+
DSM 16991	+	-	-	±	-	±	±	-	+	-	-	+	±	±	±	-	±	+	+
DSM 17757	+	±	-	+	-	±	-	-	+	-	-	±	±	-	-	-	±	+	±
DSM 17758	+	-	-	±	-	-	-	-	±	-	-	-	-	±	-	-	-	±	±
DSM 18001	+	+	±	+	-	±	-	±	±	-	±	±	±	+	+	+	+	+	+
DSM 18382	+	±	+	+	-	+	+	-	+	-	+	+	+	-	±	±	+	+	+
DSM 18390	+	±	+	+	-	-	-	-	+	-	-	±	±	-	-	-	+	+	+
DSM 18527	+	+	±	+	-	±	±	-	+	-	±	+	+	-	+	±	+	+	+
DSM 18630	±	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DSM 18793	+	-	-	-	-	-	-	-	+	-	±	-	+	-	±	-	-	+	+
DSM 18933	+	-	-	-	-	-	-	-	±	-	-	-	-	-	-	-	±	-	-
DSM 19117	+	+	+	+	-	-	-	-	+	-	-	±	±	±	+	±	+	+	+
DSM 19394	+	+	±	+	±	+	-	±	+	-	-	+	+	±	+	+	+	+	+
DSM 19395	+	±	±	±	±	+	-	±	+	-	+	+	+	±	+	+	±	+	+
DSM 19519	+	±	-	+	-	+	+	-	+	-	-	+	+	-	+	+	+	+	+
DSM 19674	+	+	±	+	±	+	+	-	+	-	+	+	+	+	+	+	+	+	+
DSM 19682	+	+	-	+	-	+	-	-	+	-	-	±	+	+	+	+	+	+	+
DSM 19904	+	+	-	±	-	-	-	-	-	-	-	-	-	-	-	-	-	±	-

Strain	MRS	Furfural	HMF	4-hydroxybenzaldehyde	Syringaldehyde	Vanillin	Catechol	Furfuryl alcohol	Guaiacol	Methylcatechol	Vanillin alcohol	Ethanol	Syringyl alcohol	Formic acid	Levulinic acid	Acetic acid	Syringic acid	Vanillic acid	Ferulic acid
DSM 19906	±	-	-	±	-	±	-	-	±	-	-	±	±	±	-	±	±	±	±
DSM 19907	+	±	-	+	-	-	-	-	±	-	-	+	±	-	-	-	+	+	+
DSM 19908	+	+	-	+	-	±	-	-	±	-	-	±	±	-	-	-	±	±	±
DSM 19909	+	±	±	+	±	-	-	±	+	-	-	+	+	+	+	+	+	+	+
DSM 19910	+	-	-	+	-	-	-	-	-	-	-	-	±	-	-	-	±	±	-
DSM 19927	+	+	±	+	+	±	-	±	+	-	-	±	+	±	+	+	±	+	±
DSM 19971	+	-	-	±	-	+	-	-	+	-	+	+	+	+	±	±	+	+	+
DSM 19972	+	±	-	+	-	±	-	-	+	-	-	±	±	±	+	+	±	±	+
DSM 20001	+	+	-	-	-	-	±	-	-	-	-	-	-	±	+	+	-	-	-
DSM 20003	+	-	-	+	-	+	±	-	±	-	±	±	±	-	-	-	-	+	+
DSM 20004	+	+	-	+	-	+	±	-	+	-	±	+	+	-	-	-	-	+	+
DSM 20011	+	+	-	+	-	±	±	-	±	-	±	+	±	-	±	-	-	+	+
DSM 20016	+	-	-	-	-	-	-	-	-	-	-	-	-	±	±	-	-	-	-
DSM 20017	+	±	±	+	±	+	±	-	+	-	+	+	+	+	±	+	+	+	+
DSM 20019	+	±	-	+	-	+	+	-	+	-	+	+	+	±	+	+	-	+	+
DSM 20021	+	-	±	+	-	+	+	±	+	-	+	+	+	±	+	+	+	+	+
DSM 20052	+	+	±	+	-	+	±	±	+	-	+	+	+	+	+	+	+	+	+
DSM 20057	+	-	±	+	-	+	±	-	+	-	-	+	+	-	-	-	+	+	+
DSM 20072	±	-	-	±	-	±	-	-	±	-	-	-	±	-	-	-	±	±	±
DSM 20074	±	-	-	±	-	±	±	-	±	-	-	±	±	-	-	-	±	±	±
DSM 20075	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DSM 20079	+	-	-	±	-	±	-	-	±	-	-	±	±	-	-	-	±	±	-
DSM 20081	+	+	-	-	-	-	-	-	-	-	-	-	-	±	+	+	±	-	±
DSM 20176	+	±	-	+	-	-	-	-	+	-	-	±	±	-	-	-	+	+	±
DSM 20178	+	±	-	+	-	+	±	±	+	-	±	+	+	±	+	+	+	+	+
DSM 20183	+	+	-	+	-	±	±	-	+	-	-	±	+	±	±	±	+	+	±
DSM 20184	+	+	±	+	-	+	+	±	+	-	±	+	+	+	+	+	+	+	+
DSM 20203	+	±	-	+	-	+	±	-	+	-	-	+	+	±	±	±	+	+	+
DSM 20243	+	-	±	±	-	-	-	-	±	-	±	±	±	-	±	±	±	+	±
DSM 20249	+	+	-	+	-	+	+	±	+	-	±	+	+	±	±	-	-	+	+
DSM 20253	+	+	-	±	-	+	-	±	+	-	+	+	+	+	+	+	-	+	+
DSM 20284	+	+	+	+	±	-	+	+	+	-	+	+	+	±	+	+	+	+	+
DSM 20285	+	±	±	+	-	±	±	-	+	-	-	+	+	-	-	-	+	+	+
DSM 20331	±	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DSM 20332	+	+	±	+	-	±	±	-	+	-	-	±	+	+	+	+	+	+	+
DSM 20335	+	±	±	-	-	-	-	-	-	-	-	-	-	±	+	+	+	-	-
DSM 20403	+	-	-	-	-	-	-	±	+	-	±	±	+	-	-	-	-	+	+
DSM 20444	+	±	-	±	+	+	-	-	+	-	±	+	+	±	±	-	-	+	+

Strain	MRS	Furfural	HMF	4-hydroxybenzaldehyde	Syringaldehyde	Vanillin	Catechol	Furfuryl alcohol	Guaiacol	Methylcatechol	Vanillin alcohol	Ethanol	Syringyl alcohol	Formic acid	Levulinic acid	Acetic acid	Syringic acid	Vanillic acid	Ferulic acid
DSM 20452	+	-	±	+	-	+	-	-	+	-	±	+	+	±	+	±	+	+	+
DSM 20505	+	+	+	+	-	±	-	-	-	-	-	±	±	±	±	±	±	+	+
DSM 20509	+	+	-	+	-	+	+	±	+	-	+	+	+	+	+	+	+	+	+
DSM 20531	+	+	±	+	-	±	±	±	+	-	-	+	+	±	±	-	+	+	+
DSM 20533	+	+	-	+	-	+	+	-	+	-	+	+	+	+	+	+	+	+	+
DSM 20534	+	+	-	+	±	+	±	-	+	-	±	+	+	±	+	+	+	+	+
DSM 20554	+	+	-	±	-	±	±	-	+	-	±	±	±	+	±	±	±	+	±
DSM 20555	+	-	-	+	-	+	±	-	+	-	+	+	+	+	+	+	+	+	+
DSM 20557	+	-	-	±	-	-	-	-	-	-	-	-	-	-	-	±	±	±	±
DSM 20584	+	±	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DSM 20587	+	±	-	+	-	-	-	-	+	-	-	+	+	+	+	+	+	+	+
DSM 20602	+	+	-	+	-	+	±	-	+	-	±	+	+	±	+	+	+	+	+
DSM 20605	+	-	-	+	-	±	-	-	+	-	-	+	+	-	-	-	+	+	+
DSM 20634	±	±	±	±	-	±	-	-	-	-	-	±	±	±	±	±	±	±	±
DSM 20719	+	±	-	+	-	±	-	-	+	-	-	+	+	-	±	±	+	+	+
DSM 20749	+	±	nd	-	-	-	-	-	±	-	-	±	+	±	+	+	±	±	±
DSM 21051	+	-	-	+	-	±	-	-	+	-	-	+	+	-	±	±	+	+	+
DSM 21115	+	+	±	+	-	+	±	-	+	-	+	+	+	+	+	+	+	+	+
DSM 21116	+	±	-	+	-	-	±	-	+	-	-	-	+	-	-	-	+	+	+
DSM 21376	+	±	-	+	-	-	-	-	-	-	-	-	-	-	-	-	±	±	±
DSM 21401	+	+	±	+	±	+	+	±	+	-	±	+	+	±	+	+	+	+	+
DSM 21775	+	-	-	±	-	-	-	-	-	-	-	-	-	-	±	-	±	±	-
DSM 22301	+	-	-	+	-	±	±	±	+	-	±	±	+	-	±	±	+	+	+
DSM 22689	+	-	-	+	±	±	±	-	+	-	-	+	+	-	-	-	+	+	+
DSM 22697	+	-	-	±	-	-	-	-	±	-	-	±	±	-	-	-	±	±	±
DSM 22698	+	-	-	±	-	-	-	-	±	-	-	-	-	-	±	±	+	+	+
DSM 23026	+	+	±	+	+	±	-	-	±	-	±	±	+	+	+	+	+	+	±
DSM 23037	+	-	-	±	-	-	-	-	±	-	-	-	-	-	-	-	-	±	-
DSM 23829	±	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	±	±	±
DSM 24301	+	±	-	nd	-	±	-	-	+	-	-	+	+	±	+	+	-	+	+
DSM 24302	+	+	-	+	±	±	±	+	+	-	+	+	+	±	+	+	+	+	+
DSM 4864	+	+	-	±	-	±	-	-	+	-	-	±	±	+	±	+	±	±	±
DSM 5007	+	+	+	+	±	±	-	-	±	-	-	±	+	-	-	-	±	±	±
DSM 5622	+	+	±	+	-	+	+	±	+	-	-	+	+	±	+	+	+	+	+
DSM 5705	+	-	-	+	±	+	±	-	+	-	±	±	±	±	+	+	+	+	+
DSM 5707	+	+	-	+	-	+	±	-	+	-	+	±	+	-	+	±	+	+	+
DSM 5837	+	-	-	+	-	+	±	-	+	-	-	+	±	-	±	-	+	+	±
DSM 6629	+	±	-	±	-	+	-	-	+	-	-	±	+	±	±	+	+	+	+

Strain	MRS	Furfural	HMF	4-hydroxybenzaldehyde	Syringaldehyde	Vanillin	Catechol	Furfuryl alcohol	Guaiacol	Methylcatechol	Vanillin alcohol	Ethanol	Syringyl alcohol	Formic acid	Levulinic acid	Acetic acid	Syringic acid	Vanillic acid	Ferulic acid
DSM2648	+	±	±	+	+	+	+	±	+	±	+	+	+	+	+	+	+	+	+
DSM9296	+	+	±	+	-	+	-	±	+	-	+	+	+	+	+	+	+	+	+
DSM 20314T	+	+	-	+	-	+	+	-	+	-	±	+	+	±	+	+	+	+	+
FB101	+	±	-	+	-	+	+	-	+	±	±	+	+	-	+	+	+	+	+
FB115	+	+	±	+	±	+	+	±	±	±	+	+	+	-	+	+	±	+	+
FOEB 8402	+	+	±	+	-	+	+	-	±	+	+	+	+	+	+	+	+	+	+
FOEB 9106	+	+	+	+	+	+	+	±	+	+	+	+	+	+	+	+	+	+	+
FOEB 9113	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FOEB 9532	+	+	±	+	-	+	+	-	+	+	±	+	+	±	+	+	+	+	+
Hd17	+	+	±	+	+	+	+	±	+	-	+	+	+	+	+	+	+	+	+
Hd4	+	+	±	+	-	+	+	±	+	-	+	+	+	-	±	-	±	+	+
JCL1267	+	+	±	+	-	+	+	±	+	±	+	+	+	±	+	+	+	+	+
JCL1268	+	+	±	+	-	+	+	+	+	±	+	+	+	±	+	+	+	+	+
JCL1269	+	±	-	+	-	+	+	-	+	+	±	+	+	±	+	+	+	+	+
JCL1271	+	+	±	+	-	-	+	-	+	-	±	+	+	+	+	+	+	+	+
JCL1275	+	+	-	+	-	+	+	-	+	-	+	+	+	+	+	+	+	+	+
JCL1278	+	+	+	+	-	-	+	-	+	-	+	+	+	+	+	+	+	+	+
JCL1279	+	+	+	+	-	+	+	-	+	-	+	+	+	+	+	+	+	+	+
JCL1280	+	+	-	±	-	+	±	-	+	-	-	+	±	-	+	+	±	+	±
JCL1283	+	+	-	+	-	±	+	-	+	-	±	+	+	+	+	+	+	+	+
JCL1284	+	+	±	+	-	+	+	±	+	±	+	+	+	-	-	-	+	+	+
JCL1285	+	+	+	+	-	+	+	±	+	+	+	+	+	±	+	+	+	+	+
Ketchup-1	+	+	±	+	-	+	+	-	±	-	-	+	+	-	-	-	±	+	±
Ketchup-3	+	+	±	+	-	-	-	-	+	-	-	-	-	±	+	+	+	+	+
KOG 10	+	+	+	+	-	+	+	±	+	-	±	±	+	+	+	+	+	+	+
KOG 11	+	+	+	+	-	+	+	-	+	-	+	+	+	+	+	+	+	+	+
KOG 12	+	+	±	+	-	+	+	-	+	-	+	+	+	+	+	+	+	+	+
KOG 13	+	+	-	+	-	+	+	±	+	-	+	+	+	+	+	+	+	+	+
KOG 14	+	+	-	+	-	+	±	±	+	-	±	+	+	+	+	+	+	+	+
KOG 18	+	+	-	+	-	+	-	-	+	-	±	+	+	+	+	+	+	+	+
KOG 19	+	-	-	±	-	+	±	-	±	-	-	+	+	-	-	-	+	+	+
KOG 2	+	+	±	+	-	±	+	±	+	-	+	+	+	+	+	+	+	+	+
KOG 21	+	+	-	+	-	+	+	-	±	-	-	+	+	+	+	+	±	+	+
KOG 22	+	+	-	±	-	±	-	-	+	-	±	+	+	+	±	+	+	+	+
KOG 23	+	±	-	+	-	-	-	-	+	-	+	+	+	-	±	-	+	+	+
KOG 4	+	+	±	+	-	+	+	-	+	-	+	+	+	+	+	+	+	+	+
KOG 5	+	+	-	+	-	+	+	±	+	-	±	+	+	+	±	+	+	+	+
KOG 8	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+

Strain	MRS	Furfural	HMF	4-hydroxybenzaldehyde	Syringaldehyde	Vanillin	Catechol	Furfuryl alcohol	Guaiacol	Methylcatechol	Vanillin alcohol	Ethanol	Syringyl alcohol	Formic acid	Levulinic acid	Acetic acid	Syringic acid	Vanillic acid	Ferulic acid
Lactolabo	+	+	-	+	-	+	+	-	+	-	±	+	+	+	+	+	±	+	±
LMAB1	+	+	-	+	-	+	±	-	+	-	±	+	±	±	+	+	+	+	±
LMAB2	+	+	±	+	-	+	+	±	+	±	±	+	+	±	+	+	+	+	+
LMG 17672	+	+	+	+	-	+	+	+	+	-	+	+	+	±	+	+	+	+	+
LMG 17673	+	+	+	+	±	+	-	+	+	-	+	+	+	+	+	+	+	+	+
LMG 17678	+	±	±	±	-	-	-	±	+	-	+	+	+	±	±	+	+	+	+
LMG 17682	+	+	±	+	-	+	-	±	+	-	+	+	+	±	+	+	+	+	+
LMG 19186	+	+	±	+	-	±	+	-	+	-	±	+	+	±	+	+	+	+	+
LMG 19188	+	+	+	+	-	±	+	-	+	-	±	+	+	±	+	+	+	+	+
LMG 19191	+	+	+	+	-	+	+	-	+	-	±	+	+	±	+	+	+	+	+
LMG 19215	+	+	+	+	+	+	+	±	±	-	±	+	+	±	+	+	+	+	+
LMG 19216	+	+	+	+	-	±	±	±	±	-	±	+	±	±	+	+	±	+	±
LMG 19217	+	+	+	+	+	+	+	±	±	-	±	+	+	±	+	+	+	+	+
LMG 19217	+	+	-	+	-	+	+	±	+	-	+	+	+	±	+	+	-	+	+
LMG 19719	+	+	-	±	-	-	-	-	-	-	-	±	±	-	+	+	-	±	-
LMG 12167	+	±	-	+	+	+	+	±	+	-	±	+	+	+	+	+	+	+	+
LMG 18021	+	+	-	+	+	+	+	±	+	-	±	+	+	±	+	+	+	+	+
LP80	+	+	±	+	-	+	+	±	+	-	±	+	+	+	+	+	+	+	+
LP85-2	+	+	±	+	-	+	-	±	+	+	+	+	+	-	+	+	+	+	+
MG1363	+	-	-	+	±	+	+	-	+	-	-	+	+	+	+	+	+	+	+
NCFB 1042 (NCIMB 701042)	+	+	±	+	-	+	+	-	+	-	+	+	+	+	+	+	+	+	+
NCFB 1088	+	+	-	+	-	+	+	±	+	+	+	±	+	±	+	+	+	+	+
NCFB 1193(NCIMB 8299)	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+
NCFB 1204 (NCIMB 701204)	+	+	+	+	-	+	+	-	+	±	+	+	+	±	±	+	+	+	+
NCFB 1206 (NCIMB 701206)	+	+	±	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
NCFB 2171	+	+	-	+	-	+	+	-	+	±	+	+	+	-	±	±	+	+	+
NCFB 340	+	+	-	+	-	+	+	±	+	-	+	+	+	±	+	+	+	+	+
NCFB 772 (NCIMB 700772)	+	+	±	+	-	+	+	+	+	+	+	+	+	±	+	+	+	+	+
NCFB 773 (NCIMB 700773)	+	+	±	+	-	-	+	-	+	-	±	+	+	+	+	+	+	+	+
NCFB 963 (NCIMB 700963)	+	+	-	+	-	+	+	-	+	-	+	+	+	+	+	+	+	+	+
NCFB 965 (NCIMB 700965)	+	+	-	+	-	+	+	-	+	-	+	+	+	±	±	+	+	+	+
NCIMB 11974T	+	+	±	+	±	+	+	±	+	-	±	+	+	±	+	+	+	+	+
NCIMB 12120	+	±	±	+	-	+	+	-	+	±	±	±	+	-	±	±	+	+	+
NCIMB 5914	+	+	±	+	+	+	+	+	+	±	+	+	+	+	+	+	+	±	+
NCIMB 6105	+	+	±	+	±	+	+	±	+	+	+	+	+	+	+	+	+	+	+
NCIMB 6461	+	+	±	+	+	+	±	-	+	-	±	+	+	+	+	+	+	+	+
NCIMB 7220	+	+	±	+	±	+	+	±	+	+	-	+	+	+	+	+	+	+	+
NCIMB 8016	+	+	±	+	+	+	+	±	+	+	+	+	+	+	+	+	+	+	+

Strain	MRS	Furfural	HMF	4-hydroxybenzaldehyde	Syringaldehyde	Vanillin	Catechol	Furfuryl alcohol	Guaiacol	Methylcatechol	Vanillin alcohol	Ethanol	Syringyl alcohol	Formic acid	Levulinic acid	Acetic acid	Syringic acid	Vanillic acid	Ferulic acid
NCIMB 8102	+	+	±	+	+	+	+	±	+	-	±	+	+	±	±	+	+	+	+
NCIMB 8826	+	+	±	+	±	+	+	±	+	+	±	+	+	±	+	+	+	+	+
R4698	+	+	+	+	-	+	+	+	+	-	+	+	±	+	+	+	+	+	+
R4700	+	+	-	+	-	+	+	±	+	-	+	+	+	±	+	+	+	+	+
SF2A31B	+	+	-	+	-	+	+	-	+	-	+	+	+	+	+	+	+	+	+
SF2A33	+	+	-	+	-	+	+	-	+	+	+	+	+	±	+	+	+	+	+
SF2A35B	+	+	+	+	+	+	+	±	+	+	+	+	+	±	+	+	+	+	+
SF2A39	+	+	±	+	-	+	+	-	+	-	+	+	+	+	+	+	±	+	+
SF2B37-1	+	+	+	+	+	+	+	±	+	±	+	±	+	-	+	+	+	+	+
SF2B41-1	+	±	-	+	-	-	+	-	+	-	±	+	+	±	+	+	+	+	+

+, good growth; ±, moderate growth; -, no or poor growth; nd, not determined

Table S4. Results of the screening on GSA plates.

Strain	GSA	Furfural	HMF	4-Hydroxybenzaldehyde	Syringaldehyde	Vanillin	Catechol	Furfuryl alcohol	Guaiacol	Methylcatechol	Vanillin alcohol	Ethanol	Syringyl alcohol	Acetic acid	Syringic acid	Vanillic acid	Ferulic acid
10-16	+	-	-	+	+	+	±	±	+	±	±	+	+	+	+	+	+
38AA	+	+	-	+	±	+	-	-	+	-	-	+	+	+	+	+	+
A1	+	+	±	+	-	+	-	±	+	-	+	+	+	+	±	±	±
A12	+	-	-	+	-	+	-	-	+	-	-	±	-	+	+	+	+
A2	+	+	±	+	+	+	±	±	+	-	±	±	+	+	+	+	+
A4	+	±	-	+	+	+	-	-	+	-	±	±	+	+	+	+	+
A7	+	±	-	+	+	+	-	-	+	-	±	±	+	+	+	+	+
A9	+	±	-	+	+	+	±	-	+	-	-	±	+	+	+	+	+
Agrano 15b	+	±	±	+	-	+	-	-	+	-	-	+	+	+	+	+	+
ALAB20	+	+	-	+	±	+	±	-	+	-	±	±	+	+	+	+	+
ATCC 10012	+	+	±	+	+	+	-	±	+	-	+	+	+	+	±	±	±
B148	+	±	±	+	+	+	-	-	+	-	-	+	+	+	+	+	+
B41	+	+	±	+	+	+	-	±	+	-	+	+	+	+	±	±	±
CCM 1904	+	+	-	+	+	+	-	±	+	±	+	+	+	+	+	+	+
CCM 3626	+	-	-	+	±	+	±	-	+	-	-	-	±	+	±	±	±
CCM4279	+	+	-	+	+	+	-	±	+	-	+	+	+	+	+	+	+
CIP 102021	+	+	-	+	+	+	-	±	+	-	-	+	+	+	+	+	+
CIP104453	+	±	-	+	+	+	±	-	+	-	-	±	+	+	+	+	+
CIP104454	+	-	-	+	+	+	±	-	+	-	-	-	+	+	+	+	+
CIP71.39	+	±	-	+	±	+	-	-	+	-	-	±	+	+	±	±	+
CNRZ 1220	±	-	-	±	±	±	-	-	±	-	-	-	±	±	±	±	±
CNRZ 1228	+	+	±	+	+	+	±	+	+	-	+	+	+	+	+	+	+
CNRZ 1229	+	+	±	+	+	+	-	+	+	-	+	+	+	+	+	+	+
CNRZ 1246	+	-	-	+	-	+	±	-	+	-	-	-	±	+	+	+	+
CNRZ 1838	+	±	-	+	+	+	-	±	+	-	+	+	+	+	+	+	+
CNRZ 184	+	+	±	+	+	+	±	±	+	-	+	+	+	+	+	+	+
CNRZ 1849	+	+	-	+	±	+	-	±	+	-	±	+	+	+	+	+	+
CNRZ 1850	+	+	±	+	+	+	-	+	+	-	+	+	+	+	+	±	+
CNRZ 424	+	+	±	+	+	+	±	±	+	-	±	+	+	+	+	+	+
CNRZ 432	+	+	±	+	+	+	-	+	+	-	+	+	+	+	+	+	+
CNRZ 738J	+	+	±	+	+	+	-	+	+	-	+	+	+	+	±	±	±
CNRZ 764	+	+	±	+	+	+	-	+	+	-	+	+	+	+	+	+	+
CST 10928	+	+	-	+	±	+	-	-	+	±	-	+	+	+	+	+	+
CST 12009	+	±	-	+	+	+	±	-	+	-	±	±	+	+	+	+	+
CST 10952	+	+	-	+	±	+	-	+	+	-	-	+	+	+	+	+	+
CST 10967	+	+	-	+	±	+	-	-	+	-	±	+	+	+	±	+	+

Strain	GSA	Furfural	HMF	4-Hydroxybenzaldehyde	Syringaldehyde	Vanillin	Catechol	Furfuryl alcohol	Guaiacol	Methylcatechol	Vanillin alcohol	Ethanol	Syringyl alcohol	Acetic acid	Syringic acid	Vanillic acid	Ferulic acid
CST 11019	+	-	-	±	-	-	-	-	-	-	-	-	±	±	+	+	+
CST 11023	+	±	-	+	±	+	-	-	+	-	-	±	+	+	±	+	±
CST 11031	+	±	-	+	+	+	-	-	+	-	±	±	+	+	+	+	+
CST 12007	+	-	-	+	±	+	-	-	+	-	-	±	+	+	+	+	+
CST 12008	+	+	-	+	+	+	±	-	+	-	+	±	+	+	+	+	+
DK0 12	+	+	±	+	+	+	-	+	+	-	+	+	+	+	+	+	+
DK0 18	+	+	±	+	+	+	-	+	+	-	+	+	+	+	+	+	+
DK0 22T (DSM 16265)	+	+	-	+	+	+	-	±	+	-	-	+	+	+	+	+	+
DK0 2A	+	±	±	+	-	+	±	±	+	±	-	+	±	+	+	+	+
DK0 7	+	+	±	+	+	+	-	±	+	-	+	+	+	+	+	+	+
DK0 8	+	+	±	+	+	+	-	±	+	-	+	+	+	+	±	±	±
DK 15	+	+	-	+	+	+	-	±	+	-	±	+	+	+	+	+	+
DK 19	+	±	-	+	±	+	-	±	+	-	-	+	±	+	+	+	+
DK 21	+	+	±	+	+	+	-	+	+	-	+	+	+	+	+	+	+
DK 28J	+	+	±	+	+	+	±	+	+	-	±	+	+	+	+	+	+
DK 30	+	+	±	+	+	+	-	+	+	-	+	+	+	+	+	+	+
DK 32	+	+	±	+	+	+	-	+	+	±	+	+	+	+	+	+	+
DK 36	+	+	±	+	+	+	-	±	+	-	±	+	+	+	+	+	+
DK 38	+	+	±	+	+	+	-	±	+	-	±	+	+	+	+	+	+
DK 9	+	+	-	+	+	+	-	-	+	-	-	-	+	+	+	+	+
DKO 20A	+	+	-	+	+	+	-	±	+	±	+	+	+	+	+	+	+
DSM 10667T	+	-	-	+	-	+	-	-	+	-	-	-	±	±	+	+	+
DSM 13238	±	-	-	±	-	±	-	-	±	-	-	-	-	±	±	-	±
DSM 13344	±	-	-	-	-	-	-	-	-	-	-	-	-	-	±	±	±
DSM 13675	+	-	-	+	±	+	-	-	±	-	-	-	+	+	+	+	+
DSM 13961	+	±	±	+	-	+	-	±	+	-	±	+	±	+	+	+	+
DSM 14800	+	-	-	+	-	+	-	-	±	-	-	±	±	±	-	±	-
DSM 15354	+	±	-	+	+	+	-	-	+	±	-	+	+	+	+	+	+
DSM 15831	±	-	-	±	-	±	-	-	-	-	-	-	±	-	±	±	±
DSM 15833	±	-	-	±	-	±	-	-	±	-	-	-	±	±	±	±	±
DSM 15946	±	-	-	-	-	±	-	-	±	-	-	-	-	-	±	±	±
DSM 16041	+	±	-	+	+	+	-	-	+	-	±	+	+	+	-	-	-
DSM 16634	±	-	-	±	-	±	-	-	±	-	-	-	±	±	-	±	±
DSM 16991	+	±	-	+	-	+	-	-	±	-	-	±	+	±	+	+	+
DSM 17758	±	±	-	±	-	±	-	-	±	-	-	±	±	-	±	±	±
DSM 18001	+	+	±	+	+	+	-	±	±	-	+	+	+	+	+	+	+
DSM 18527	±	-	-	-	-	±	-	-	±	-	-	±	±	-	±	±	±

Strain	GSA	Furfural	HMF	4-Hydroxybenzaldehyde	Syringaldehyde	Vanillin	Catechol	Furfuryl alcohol	Guaiacol	Methylcatechol	Vanillin alcohol	Ethanol	Syringyl alcohol	Acetic acid	Syringic acid	Vanillic acid	Ferulic acid
DSM 19519	+	-	-	+	±	+	-	-	±	-	-	±	+	+	+	+	+
DSM 19910	+	±	-	+	±	+	-	-	±	-	±	+	±	+	+	+	+
DSM 19971	+	-	-	+	±	+	-	-	±	-	-	+	+	±	+	+	+
DSM 19972	+	-	-	+	-	+	-	-	±	-	-	±	+	+	+	+	+
DSM 20001	+	-	±	+	±	+	-	+	+	±	+	+	+	±	+	+	+
DSM 20004	+	-	-	+	±	+	-	-	+	-	-	-	-	-	+	+	±
DSM 20011	+	-	-	+	+	+	-	-	+	-	-	±	±	+	+	+	+
DSM 20017	+	-	-	±	±	+	-	-	±	-	-	±	+	+	+	+	+
DSM 20019	±	±	-	±	±	±	-	-	±	-	±	±	±	-	±	±	±
DSM 20021	+	±	-	+	+	+	-	±	+	-	±	+	+	+	+	+	+
DSM 20052	+	+	±	+	+	+	-	-	+	-	±	+	+	+	+	+	+
DSM 20054	+	±	-	+	+	+	-	-	+	-	+	-	+	+	+	+	+
DSM 20176	+	-	-	-	-	-	-	-	±	-	-	±	±	-	+	±	+
DSM 20183	±	-	-	±	-	±	-	-	-	-	-	±	-	-	±	±	±
DSM 20444	+	-	-	+	-	+	-	-	±	-	-	±	+	+	+	+	+
DSM 20452	±	-	-	±	±	±	-	-	±	-	-	-	±	-	-	-	-
DSM 20719	+	-	-	±	-	±	-	-	+	-	-	+	±	-	+	+	+
DSM 21051	+	+	-	+	±	+	-	-	+	-	-	+	+	+	+	+	+
DSM 21115	+	+	-	+	+	+	-	-	±	-	±	+	+	+	+	+	+
DSM 21376	+	±	-	+	+	+	-	-	±	-	-	+	+	+	+	+	+
DSM 5622	+	-	-	+	±	+	-	-	+	-	-	±	+	+	+	+	+
DSM2648	+	-	-	+	+	+	±	-	+	-	+	±	+	+	+	+	+
DSM9296	+	±	-	+	+	+	-	±	+	-	-	+	+	+	+	+	+
DSM 20314T	+	±	-	+	+	+	-	±	+	-	+	+	+	+	+	+	+
FB101	+	-	-	+	±	+	±	-	+	-	-	-	±	±	+	+	+
FB115	+	±	-	+	+	+	-	±	+	-	±	±	+	+	+	+	+
FOEB 8402	+	±	-	+	±	+	±	-	+	±	±	±	+	±	±	±	±
FOEB 9106	+	+	±	+	+	+	+	±	+	±	+	+	+	+	+	+	+
FOEB 9113	+	+	-	+	±	+	-	±	+	-	+	+	+	+	+	+	+
FOEB 9532	+	±	-	+	±	+	±	-	+	-	±	+	+	+	±	±	±
Hd17	+	+	-	+	+	+	-	±	+	±	+	+	+	+	+	+	+
Hd4	+	-	-	+	+	+	-	-	+	-	±	-	+	+	±	±	±
JCL1267	+	+	±	+	+	+	±	±	+	-	±	±	+	+	+	+	+
JCL1268	+	+	±	+	+	+	±	±	+	-	+	±	+	+	+	+	+
JCL1269	+	-	-	+	-	+	-	-	+	-	-	-	±	±	+	+	+
JCL1271	+	+	-	+	±	+	-	±	+	-	±	+	±	+	+	+	+
JCL1275	+	-	-	+	-	+	-	-	+	-	-	-	+	-	+	+	+

Strain	GSA	Furfural	HMF	4-Hydroxybenzaldehyde	Syringaldehyde	Vanillin	Catechol	Furfuryl alcohol	Guaiacol	Methylcatechol	Vanillin alcohol	Ethanol	Syringyl alcohol	Acetic acid	Syringic acid	Vanillic acid	Ferulic acid
JCL1278	+	+	±	+	+	+	-	+	+	-	+	+	+	+	+	+	+
JCL1279	+	±	-	+	±	+	-	-	+	-	-	+	+	+	+	+	+
JCL1283	+	±	-	+	±	+	-	-	+	-	-	+	±	+	+	+	+
KOG 10	+	+	±	+	+	+	±	+	+	±	+	+	+	+	+	+	+
KOG 14	+	±	-	+	±	+	-	-	+	-	-	+	+	+	±	±	±
KOG 2	+	+	±	+	+	+	±	±	+	±	+	+	+	+	+	+	+
KOG 21	+	+	±	+	+	+	±	±	+	±	+	+	+	+	±	+	+
KOG 8	+	+	±	+	+	+	-	+	+	-	+	+	+	+	+	+	+
Lactolabo	+	+	±	+	+	+	-	+	+	±	+	+	+	+	+	+	+
LMAB1	+	±	-	+	+	+	±	-	+	-	-	+	+	+	+	+	+
LMAB2	+	±	-	+	±	+	-	-	+	-	-	+	+	+	+	+	+
LMG 17672	+	-	-	+	+	+	±	±	+	-	±	+	+	+	+	+	+
LMG 17673	+	-	-	+	±	+	-	-	+	-	-	±	+	+	+	±	+
LMG 17678	+	+	±	+	+	+	±	+	+	±	+	+	+	+	±	±	±
LMG 17682	+	±	±	+	+	+	-	+	+	-	±	+	+	+	±	±	±
LMG 19191	±	-	-	-	±	-	-	-	-	-	-	-	±	-	-	-	-
LMG 19215	+	-	-	+	±	+	-	-	+	-	-	±	+	-	+	+	+
LMG 19719	+	-	-	+	-	+	-	-	+	-	-	+	+	+	±	±	+
LMG 12167	+	-	-	+	±	+	-	-	+	-	-	-	+	+	+	+	±
LMG 18021	+	±	-	+	+	+	-	±	+	-	+	+	+	+	±	±	±
LP80	+	+	±	+	+	+	-	±	+	±	±	+	+	+	+	+	+
LP85-2	+	+	±	+	+	+	±	+	+	-	-	+	+	+	+	+	+
MG1363	+	+	±	+	+	+	-	-	+	-	+	+	+	+	+	+	+
NCFB 1042 (NCIMB 701042)	+	+	-	+	+	+	-	-	+	-	-	+	+	+	+	+	+
NCFB 1088	+	±	-	+	+	+	+	-	+	±	-	±	+	+	+	+	+
NCFB 1193 (NCIMB 8299)	+	+	±	+	+	+	-	±	+	-	+	+	+	+	+	+	+
NCFB 1204 (NCIMB 701204)	+	-	-	+	-	+	±	-	+	-	±	±	±	+	+	+	+
NCFB 1206 (NCIMB 701206)	+	+	±	+	+	+	±	±	+	-	±	+	+	+	+	+	+
NCFB 2171	+	±	-	+	±	+	-	-	+	-	-	+	+	+	±	+	+
NCFB 340	+	+	±	+	+	+	-	+	+	-	+	+	+	+	+	+	+
NCFB 772 (NCIMB 700772)	+	-	-	+	±	+	-	-	+	-	-	-	+	±	+	+	+
NCFB 773 (NCIMB 700773)	+	±	-	+	+	+	-	-	+	-	-	+	+	+	±	±	+
NCFB 963 (NCIMB 700963)	+	-	-	+	+	+	-	-	+	-	±	-	+	+	+	+	+
NCFB 965 (NCIMB 700965)	+	-	-	+	+	+	-	-	+	-	-	+	±	+	+	+	+
NCIMB 11974T	+	+	±	+	+	+	-	+	+	-	+	+	±	+	+	+	+
NCIMB 12120	+	±	-	+	+	+	±	-	+	-	-	±	+	+	+	+	+
NCIMB 5914	+	+	-	+	+	+	-	±	+	-	+	+	+	+	-	+	+

Strain	GSA	Furfural	HMF	4-Hydroxybenzaldehyde	Syringaldehyde	Vanillin	Catechol	Furfuryl alcohol	Guaiacol	Methylcatechol	Vanillin alcohol	Ethanol	Syringyl alcohol	Acetic acid	Syringic acid	Vanillic acid	Ferulic acid
NCIMB 6105	+	+	±	+	+	+	-	+	+	-	+	+	+	+	±	±	-
NCIMB 7220	+	+	±	+	+	+	-	+	+	-	+	+	+	+	+	+	+
NCIMB 8102	+	+	-	+	+	+	-	-	+	-	+	±	+	+	+	+	+
NCIMB 8826	+	+	±	+	+	+	±	±	+	±	+	+	+	+	+	+	+
R4698	+	±	-	+	±	+	±	±	+	-	-	±	+	+	+	+	+
R4700	+	-	-	+	+	+	±	-	+	-	±	-	+	+	+	+	+
SF2A31B	+	-	-	+	-	+	±	-	+	-	-	±	+	+	+	±	+
SF2A33	+	+	-	+	±	+	-	+	+	-	±	+	+	+	+	+	+
SF2A35B	+	±	±	+	+	+	±	±	+	-	±	±	+	+	+	+	+
SF2A39	+	+	-	+	+	+	-	+	+	-	±	+	+	+	+	+	+
SF2B37-1	+	±	-	+	+	+	±	±	+	-	±	±	+	+	±	±	±
SF2B41-1	+	±	-	+	-	+	-	-	+	-	-	+	±	+	+	+	+

+, good growth; ±, moderate growth; -, no or poor growth; nd, not determined

Table S5. Results of the pentose utilization tests on 10% MRS plates with glucose, xylose or arabinose as sole carbon sources.

Strain	MRS	Glucose	Xylose	Arabinose
10-16	+	+	+	+
38AA	+	+	-	±
A1	+	+	-	-
A12	+	+	-	-
A2	+	+	-	-
A4	+	+	-	-
A7	+	+	-	-
A9	+	+	-	-
Agrano 15b	+	+	-	+
ALAB20	+	+	-	±
ATCC 10012	+	+	-	+
ATCC 25745	+	+	+	+
B148	+	+	±	±
B41	+	+	-	+
CCM 1904	+	+	-	+
CCM 3626	+	+	-	-
CCM4279	+	+	-	±
CIP 102021	+	+	-	-
CIP104453	+	+	-	-
CIP104454	+	+	-	-
CIP71.39	+	+	-	-
CNRZ 1220	+	+	-	-
CNRZ 1228	+	+	-	±
CNRZ 1229	+	+	-	±
CNRZ 1246	+	+	-	-
CNRZ 1838	+	+	-	-
CNRZ 184	+	+	-	±
CNRZ 1849	+	+	-	±
CNRZ 1850	+	+	-	+
CNRZ 424	+	+	-	-
CNRZ 432	+	+	-	+
CNRZ 738J	+	+	-	±
CNRZ 764	+	+	-	-
CST 10928	+	+	-	±
CST 12009	+	+	-	±
CST 10952	+	+	-	+
CST 10967	+	+	-	-
CST 11019	+	+	-	-
CST 11023	+	+	-	-
CST 11031	+	+	-	+

Strain	MRS	Glucose	Xylose	Arabinose
CST 12007	+	+	-	-
CST 12008	+	+	-	±
DK0 12	+	+	-	+
DK0 18	+	+	-	+
DK0 22T (DSM 16265)	+	+	-	-
DK0 2A	+	+	-	-
DK0 7	+	+	-	+
DK0 8	+	+	-	+
DK 15	+	+	-	+
DK 19	+	+	-	-
DK 21	+	+	-	+
DK 28J	+	+	-	±
DK 30	+	+	-	+
DK 32	+	+	±	+
DK 36	+	+	-	-
DK 38	+	+	-	+
DK 9	+	+	-	-
DKO 20A	+	+	-	+
DSM 10532	+	+	-	-
DSM 10533	+	-	-	-
DSM 10551	±	±	-	-
DSM 10667T	+	+	-	-
DSM 12361	+	+	-	-
DSM 12744	+	+	-	-
DSM 13145	+	+	+	+
DSM 13238	+	+	-	-
DSM 13343	+	+	-	-
DSM 13344	+	+	-	-
DSM 13345	+	-	-	-
DSM 13675	+	±	-	-
DSM 13961	+	+	-	-
DSM 14060	+	+	+	+
DSM 14340	+	+	+	-
DSM 14421	+	+	+	+
DSM 14500	+	+	-	-
DSM 14800	+	+	-	-
DSM 14857	+	-	-	-
DSM 15354	+	+	+	±
DSM 15429	+	+	+	+
DSM 15707	+	+	+	+
DSM 15814	+	+	+	+
DSM 15831	+	+	-	-
DSM 15833	+	+	-	+

Strain	MRS	Glucose	Xylose	Arabinose
DSM 15945	+	+	+	+
DSM 15946	+	+	-	-
DSM 15996	+	-	-	-
DSM 16041	+	±	-	±
DSM 16043	+	±	-	-
DSM 16045	+	+	-	-
DSM 16049	+	+	-	-
DSM 16230	+	+	-	-
DSM 16381	+	+	+	-
DSM 16634	+	+	-	-
DSM 16761	+	+	-	-
DSM 16982	+	+	-	-
DSM 16991	+	+	-	+
DSM 17757	+	+	-	-
DSM 17758	+	+	-	-
DSM 18001	+	+	-	-
DSM 18382	+	+	-	+
DSM 18390	+	+	+	+
DSM 18527	+	+	+	+
DSM 18630	+	+	-	-
DSM 18793	+	-	-	-
DSM 18933	+	+	-	-
DSM 19117	+	+	-	+
DSM 19394	+	+	+	+
DSM 19395	+	+	±	±
DSM 19519	+	+	-	-
DSM 19674	+	+	-	-
DSM 19682	+	+	-	-
DSM 19904	+	+	-	+
DSM 19906	+	-	+	+
DSM 19907	+	±	+	+
DSM 19908	+	+	-	+
DSM 19909	+	+	+	±
DSM 19910	+	+	-	-
DSM 19927	+	+	+	+
DSM 19971	+	+	-	-
DSM 19972	+	+	-	-
DSM 20001	+	+	-	-
DSM 20003	+	+	-	-
DSM 20004	+	+	-	-
DSM 20011	+	+	-	-
DSM 20016	+	+	-	±
DSM 20017	+	+	-	-

Strain	MRS	Glucose	Xylose	Arabinose
DSM 20019	+	+	-	-
DSM 20021	+	+	-	-
DSM 20052	+	+	-	-
DSM 20054	+	+	±	-
DSM 20057	+	+	+	+
DSM 20072	+	±	-	-
DSM 20074	+	±	-	-
DSM 20075	+	+	-	-
DSM 20079	+	+	-	-
DSM 20081	+	+	-	-
DSM 20176	+	+	+	-
DSM 20178	+	+	-	-
DSM 20183	+	+	-	-
DSM 20184	+	+	-	-
DSM 20203	+	+	-	-
DSM 20243	+	±	±	-
DSM 20249	+	+	-	-
DSM 20253	+	+	+	+
DSM 20284	+	+	+	+
DSM 20285	+	+	-	-
DSM 20331	±	±	-	-
DSM 20332	+	±	-	-
DSM 20335	+	±	-	-
DSM 20403	+	+	+	+
DSM 20444	+	+	+	+
DSM 20452	+	±	±	±
DSM 20505	+	+	-	-
DSM 20509	+	+	-	-
DSM 20531	+	+	-	-
DSM 20533	+	±	+	-
DSM 20534	+	±	-	-
DSM 20554	+	+	+	+
DSM 20555	+	+	-	-
DSM 20557	+	+	-	-
DSM 20584	+	+	+	+
DSM 20587	+	+	-	+
DSM 20602	+	+	-	-
DSM 20605	+	-	-	-
DSM 20634	±	±	+	+
DSM 20719	+	+	±	-
DSM 20749	+	+	+	+
DSM 21051	+	+	-	-
DSM 21115	+	+	±	+

Strain	MRS	Glucose	Xylose	Arabinose
DSM 21116	+	+	-	-
DSM 21376	+	+	-	-
DSM 21401	+	+	-	-
DSM 21775	+	±	±	-
DSM 22301	+	+	-	-
DSM 22689	+	±	-	-
DSM 22697	+	+	-	-
DSM 22698	+	+	-	-
DSM 23026	+	+	-	-
DSM 23037	+	+	+	+
DSM 23829	+	+	-	-
DSM 24301	+	+	+	-
DSM 24302	+	+	+	+
DSM 4864	+	+	-	-
DSM 5007	+	+	+	+
DSM 5622	+	+	-	-
DSM 5705	+	+	-	-
DSM 5707	+	+	-	+
DSM 5837	+	±	-	-
DSM 6629	+	±	-	-
DSM2648	+	+	-	±
DSM9296	+	+	-	-
DSM 20314T	+	+	±	±
FB101	+	+	-	-
FB115	+	+	-	±
FOEB 8402	+	+	-	±
FOEB 9106	+	+	-	±
FOEB 9113	+	+	-	+
FOEB 9532	+	+	-	±
Hd17	+	+	-	±
Hd4	+	+	-	±
JCL1267	+	+	-	+
JCL1268	+	+	-	+
JCL1269	+	+	-	-
JCL1271	+	+	-	+
JCL1275	+	+	-	-
JCL1278	+	+	-	±
JCL1279	+	+	±	-
JCL1280	+	+	±	±
JCL1283	+	+	-	-
JCL1284	+	+	-	±
JCL1285	+	+	-	+
Ketchup-1	+	+	-	+

Strain	MRS	Glucose	Xylose	Arabinose
Ketchup-3	+	+	-	+
KOG 10	+	+	-	+
KOG 11	+	+	-	-
KOG 12	+	+	-	-
KOG 13	+	+	-	±
KOG 14	+	+	-	±
KOG 18	+	+	-	±
KOG 19	+	+	-	-
KOG 2	+	+	-	-
KOG 21	+	+	-	-
KOG 22	+	+	-	-
KOG 23	+	+	-	-
KOG 4	+	+	-	-
KOG 5	+	±	-	-
KOG 8	+	+	-	+
Lactolabo	+	+	-	+
LMAB1	+	+	-	-
LMAB2	+	+	-	-
LMG 17672	+	+	+	+
LMG 17673	+	+	+	+
LMG 17678	+	+	+	+
LMG 17682	+	+	+	+
LMG 19186	+	+	+	+
LMG 19188	+	+	+	+
LMG 19191	+	+	+	+
LMG 19215	+	+	+	+
LMG 19216	+	+	+	+
LMG 19217	+	+	+	+
LMG 19719	+	+	-	-
LMG 12167	+	+	-	-
LMG 18021	+	+	-	-
LP80	+	+	-	+
LP85-2	+	+	-	-
NCFB 1042 (NCIMB 701042)	+	+	-	-
NCFB 1088	+	+	-	-
NCFB 1193 (NCIMB 8299)	+	+	-	+
NCFB 1204 (NCIMB 701204)	+	+	-	-
NCFB 1206 (NCIMB 701206)	+	+	-	+
NCFB 2171	+	+	-	±
NCFB 340	+	+	-	+
NCFB 772 (NCIMB 700772)	+	+	-	-
NCFB 773 (NCIMB 700773)	+	+	-	-
NCFB 963 (NCIMB 700963)	+	+	-	-

Strain	MRS	Glucose	Xylose	Arabinose
NCFB 965 (NCIMB 700965)	+	+	-	-
NCIMB 11974T	+	+	-	±
NCIMB 12120	+	+	-	±
NCIMB 5914	+	+	-	±
NCIMB 6105	+	+	-	+
NCIMB 6461	+	+	-	-
NCIMB 7220	+	+	-	-
NCIMB 8016	+	+	-	±
NCIMB 8102	+	+	-	±
NCIMB 8826	+	+	-	-
R4698	+	+	-	±
R4700	+	+	-	-
SF2A31B	+	+	-	-
SF2A33	+	+	-	-
SF2A35B	+	+	-	-
SF2A39	+	+	-	±
SF2B37-1	+	+	-	-
SF2B41-1	+	+	-	-

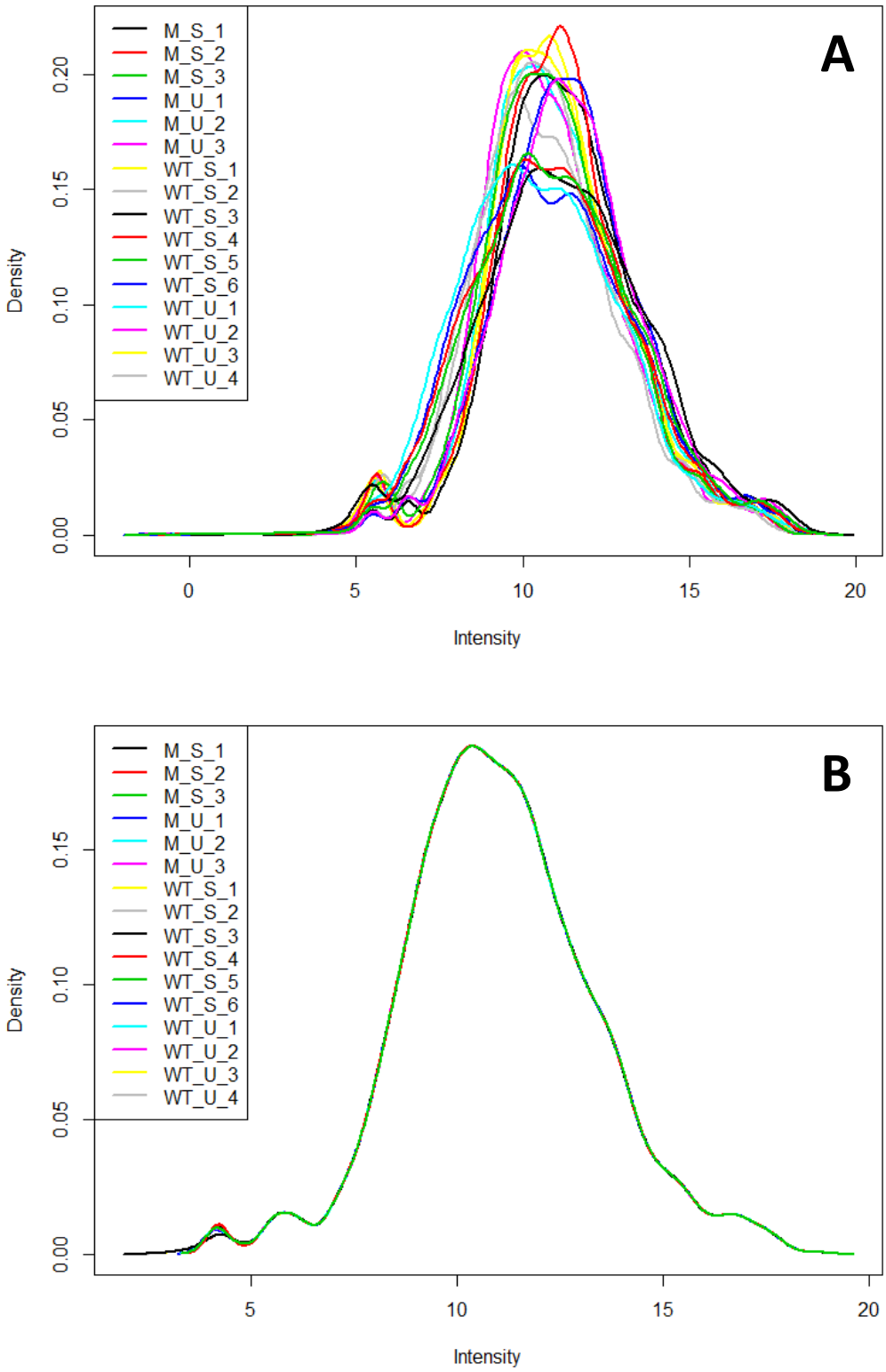
+, good growth; ±, moderate growth; -, no or poor growth

Table S6. Performance of *E.coli* MG1655 in LB with glucose or xylose and combinations of inhibitors representing three feedstock hydolysate types.

Conditions		Growth rate (1/h)		Gen. time (min)	% difference vs control
		Mean	SD		
LB with glucose	Control	1,875	0,072	22	0
	Sugarcane bagasse (Furfural 0,3 g/L; HMF 0,04 g/L; Acetic acid 2,7 g/L)	1,194	0,127	35	36
	Wheat straw (Furfural 0,15 g/L; Acetic acid 2,7 g/L)	1,473	0,004	28	21
	Soft wood (Furfural 2,2 g/L; Acetic Acid 5,3 g/L)	0,183	0,038	232	90
LB with xylose	Control	1,665	0,115	25	0
	Sugarcane bagasse (Furfural 0,3 g/L; HMF 0,04 g/L; Acetic acid 2,7 g/L)	1,014	0,068	41	39
	Wheat straw (Furfural 0,15 g/L; Acetic acid 2,7 g/L)	1,221	0,030	34	27
	Soft wood (Furfural 2,2 g/L; Acetic Acid 5,3 g/L)	0,213	0,055	202	87

8.2 Supplementary materials for Chapter 6

8.2.1 Figure S1. Microarray density plots before (A) and after (B) normalization.



8.2.2 Table S7. Differentially expressed genes in DSM 20284 and A28 grown in MRS with and without furfural, sorted by COG category.

			Without furfural		With furfural		
Systematic Name	**Locus Tag**	**Annotation**	**logFC**	**Adj. p.value**	**logFC**	**Adj. p.value**	**COG group**
EFL95087	HMPREF0623_1593	glutathione reductase	-1,71	5,73E-17	-1,33	1,58E-11	C
EFL96114	HMPREF0623_0165	acylphosphatase	-1,01	1,98E-09	-0,61	4,46E-04	C
EFL95325	HMPREF0623_1062	oxidoreductase ion channel protein, IolS	-0,70	6,66E-07	-0,59	9,67E-05	C
EFL96367	HMPREF0623_0418	manganese-dependent inorganic pyrophosphatase	0,59	3,80E-02			C
EFL95323	HMPREF0623_1060	NAD-dependent malic enzyme	0,69	1,74E-03			C
EFL95723	HMPREF0623_0759	F0F1 ATP synthase subunit epsilon	0,70	1,33E-03			C
EFL94746	HMPREF0623_1614	iron-sulfur cluster assembly scaffold protein	0,81	3,21E-07	0,61	2,16E-04	C
EFL95233	HMPREF0623_1369	glutamate:protein symporter	1,49	1,09E-15	1,27	1,48E-12	C
EFL96512	HMPREF0623_0563	NADPH-dependent oxidoreductase			1,00	1,56E-07	C
EFL95322	HMPREF0623_1059	class II fumarate hydratase			0,61	1,46E-04	C
EFL95612	HMPREF0623_1349	carbamate kinase	-3,46	8,23E-28	-3,13	1,26E-23	E
EFL95613	HMPREF0623_1350	arginine deiminase	-3,22	2,82E-32	-2,75	1,20E-26	E
EFL95611	HMPREF0623_1348	ornithine carbamoyltransferase	-3,19	1,58E-24	-2,98	2,04E-21	E
EFL95614	HMPREF0623_1351	amino acid permease	-2,68	5,51E-24	-2,36	8,49E-20	E
EFL94861	HMPREF0623_1729	arginine ABC transporter ATP-binding protein	-1,68	6,02E-17	-1,57	1,84E-14	E
EFL94860	HMPREF0623_1728	glutamine ABC transporter substrate-binding protein	-1,62	1,98E-13	-1,57	1,24E-11	E
EFL94859	HMPREF0623_1727	glutamine ABC transporter permease	-1,40	3,77E-15	-1,19	6,09E-11	E
EFL94868	HMPREF0623_1736	amino acid permease	-1,37	2,50E-11	-0,93	2,75E-06	E
EFL95225	HMPREF0623_1402	histidinol-phosphatase	-0,88	1,43E-03	-1,11	4,08E-04	E
EFL96005	HMPREF0623_0056	amino acid permease	-0,82	1,31E-04	-0,93	8,70E-05	E
EFL95010	HMPREF0623_1878	ABC transporter ATP-binding protein	-0,81	3,26E-05			E
EFL94974	HMPREF0623_1842	Arginine/ornithine antiporter, ArcD	-0,78	1,38E-04	-0,80	9,77E-04	E
EFL96309	HMPREF0623_0360	amino acid permease	-0,77	2,18E-05			E
EFL96055	HMPREF0623_0106	transporter	0,84	1,33E-04			E
EFL94856	HMPREF0623_1724	amino acid permease	0,96	4,82E-09	0,96	8,24E-08	E

			Without furfural		With furfural		
Systematic Name	**Locus Tag**	**Annotation**	**logFC**	**Adj. p.value**	**logFC**	**Adj. p.value**	**COG group**
EFL94747	HMPREF0623_1615	cysteine desulfurase	1,02	4,21E-06	0,84	5,67E-04	E
EFL96523	HMPREF0623_0574	succinyl-diaminopimelate desuccinylase			-0,62	2,89E-02	E
EFL95160	HMPREF0623_1472	amino acid:proton antiporter			-0,66	3,66E-04	E
EFL95795	HMPREF0623_0831	xanthine phosphoribosyltransferase	0,62	7,20E-04			F
EFL94751	HMPREF0623_1619	ribonucleoside-triphosphate reductase	0,63	1,22E-02			F
EFL96451	HMPREF0623_0502	nucleoside deoxyribosyltransferase	0,68	1,93E-03			F
EFL96408	HMPREF0623_0459	N5-carboxyaminoimidazole ribonucleotide mutase, purE	0,68	1,04E-03			F
EFL95182	HMPREF0623_1494	pyrimidine-nucleoside phosphorylase, pdp	0,73	1,56E-04			F
EFL95856	HMPREF0623_0892	deoxyadenosine kinase	0,79	5,17E-05			F
EFL95733	HMPREF0623_0769	uracil phosphoribosyltransferase, upp	0,87	4,19E-05			F
EFL95278	HMPREF0623_1015	CTP synthetase, pyrG	0,89	1,76E-03	0,64	4,41E-02	F
EFL95873	HMPREF0623_0909	Uric acid/xanthine permease	1,09	1,35E-10	0,60	1,34E-04	F
EFL95441	HMPREF0623_1178	ribonucleoside hydrolase	1,13	1,57E-08	1,01	1,15E-06	F
EFL95442	HMPREF0623_1179	nucleoside transporter	1,21	3,94E-05	1,07	8,72E-04	F
EFL95966	HMPREF0623_0017	deoxyadenosine kinase	1,25	1,34E-09	0,76	3,16E-04	F
EFL95646	HMPREF0623_0682	adenylosuccinate synthase, purA	1,46	2,29E-13	1,54	4,51E-13	F
EFL95364	HMPREF0623_1101	chlorohydrolase	1,66	4,51E-14	1,64	1,32E-12	F
EFL95645	HMPREF0623_0681	adenylosuccinate lyase, purB	1,69	1,21E-12	1,36	4,35E-09	F
EFL95363	HMPREF0623_1100	xanthine/uracil permease	1,74	5,43E-14	1,67	2,80E-12	F
EFL94871	HMPREF0623_1739	adenosine deaminase, add	3,35	1,15E-21	2,36	2,22E-13	F
EFL95022	HMPREF0623_1528	guanosine monophosphate reductase, guaC	4,13	4,58E-27	3,92	2,61E-24	F
EFL94869	HMPREF0623_1737	adenine deaminase	4,22	7,61E-39	3,47	6,76E-32	F
EFL96040	HMPREF0623_0091	PTS mannitol transporter subunit IIA	-1,31	6,21E-03	-1,40	3,11E-03	G
EFL95466	HMPREF0623_1203	GPH family xyloside:cation symporter	-1,04	2,84E-06	-1,06	1,35E-05	G
EFL95042	HMPREF0623_1548	protein-N(pi)-phosphohistidine--sugar phosphotransferase	-1,00	9,92E-06	-0,91	1,03E-04	G
EFL96041	HMPREF0623_0092	PTS lactose transporter subunit IIB	-0,99	1,01E-02	-1,17	4,59E-03	G
EFL95153	HMPREF0623_1465	alpha-glucosidase	-0,96	2,10E-06	-0,83	1,70E-04	G

			Without furfural		With furfural		
Systematic Name	**Locus Tag**	**Annotation**	**logFC**	**Adj. p.value**	**logFC**	**Adj. p.value**	**COG group**
EFL96049	HMPREF0623_0100	beta-galactosidase	-0,95	3,67E-06			G
EFL95465	HMPREF0623_1202	beta-xylosidase	-0,83	4,93E-06	-0,94	1,49E-06	G
EFL95172	HMPREF0623_1484	PTS mannose transporter subunit IID	-0,82	6,57E-04	-0,87	6,52E-04	G
EFL96037	HMPREF0623_0088	PTS cellobiose transporter subunit IIC	-0,78	7,91E-04	-1,02	5,35E-05	G
EFL95541	HMPREF0623_1278	alpha-L-fucosidase	-0,77	1,09E-03	-0,95	2,83E-04	G
EFL96053	HMPREF0623_0104	peptidase S24	-0,76	1,39E-04			G
EFL96031	HMPREF0623_0082	D-galactonate transporter	-0,76	7,39E-03			G
EFL95171	HMPREF0623_1483	PTS sugar transporter	-0,70	2,05E-05	-0,93	9,52E-07	G
EFL95145	HMPREF0623_1457	fructose-bisphosphate aldolase	-0,69	4,67E-02			G
EFL96010	HMPREF0623_0061	sugar transporter	-0,68	2,91E-03	-0,70	7,79E-03	G
EFL95041	HMPREF0623_1547	PTS lactose transporter subunit IIA	-0,67	1,39E-02			G
EFL95999	HMPREF0623_0050	sugar:proton symporter	-0,66	6,42E-04	-0,77	1,06E-03	G
EFL96002	HMPREF0623_0053	arabinose isomerase	-0,61	5,79E-03	-0,59	1,68E-02	G
EFL95987	HMPREF0623_0038	PTS glucose transporter subunit IIABC	-0,59	1,20E-04			G
EFL94855	HMPREF0623_1723	fructose 2,6-bisphosphatase	0,60	7,63E-04			G
EFL94732	HMPREF0623_1600	hypothetical protein	0,60	9,83E-04	0,79	4,44E-04	G
EFL95755	HMPREF0623_0791	fructose-bisphosphate aldolase	0,62	2,90E-03			G
EFL95503	HMPREF0623_1240	6-phosphogluconate dehydrogenase	0,62	6,47E-03			G
EFL96034	HMPREF0623_0085	PTS N-acetylgalactosamine transporter subunit IID	0,64	2,82E-04	0,67	5,43E-04	G
EFL95404	HMPREF0623_1141	ribose transporter RbsU	0,77	4,97E-04	0,62	1,19E-02	G
EFL96129	HMPREF0623_0180	glucokinase	0,80	1,43E-03			G
EFL96469	HMPREF0623_0520	pyruvate kinase	0,81	1,74E-03			G
EFL96035	HMPREF0623_0086	PTS sugar transporter	0,83	4,00E-03	0,69	3,67E-02	G
EFL94733	HMPREF0623_1601	hypothetical protein	1,04	9,39E-12	1,19	1,09E-12	G
EFL94857	HMPREF0623_1725	fructose 2,6-bisphosphatase	1,10	9,14E-06	0,76	5,62E-04	G
EFL95544	HMPREF0623_1281	PTS cellobiose transporter subunit IIC			-0,72	5,25E-04	G
EFL96024	HMPREF0623_0075	alpha-mannosidase			-0,65	4,68E-03	G

			Without furfural		With furfural		
Systematic Name	**Locus Tag**	**Annotation**	**logFC**	**Adj. p.value**	**logFC**	**Adj. p.value**	**COG group**
EFL95040	HMPREF0623_1546	6-phospho-beta-glucosidase			-0,64	4,09E-02	G
EFL95468	HMPREF0623_1205	xylulokinase, xylB			-0,59	2,63E-02	G
EFL96033	HMPREF0623_0084	PTS sucrose transporter subunit IIBC			0,64	3,78E-03	G
EFL95963	HMPREF0623_0014	alpha-amylase			0,67	4,25E-05	G
EFL95070	HMPREF0623_1576	hydroxyethylthiazole kinase	0,75	6,92E-04	0,70	1,17E-03	H
EFL96356	HMPREF0623_0407	GTP cyclohydrolase I, folE	1,64	3,76E-17	1,73	6,96E-17	H
EFL96355	HMPREF0623_0406	tetrahydrofolate synthase, folC	1,68	4,24E-12	1,66	2,14E-11	H
EFL96358	HMPREF0623_0409	dihydroneopterin aldolase, folB	1,79	1,61E-12	1,71	3,17E-11	H
EFL96357	HMPREF0623_0408	2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase, folK	1,80	3,73E-11	1,64	3,05E-09	H
EFL96353	HMPREF0623_0404	dihydropteroate synthase, folP	1,92	3,27E-13	1,84	1,15E-11	H
EFL95688	HMPREF0623_0724	cardiolipin synthase	-0,89	3,82E-07	-0,77	6,50E-05	I
EFL95294	HMPREF0623_1031	D-alanine--poly(phosphoribitol) ligase subunit 2	-0,81	8,05E-06	-0,61	2,98E-03	I
EFL95567	HMPREF0623_1304	cardiolipin synthase	0,59	5,88E-03			I
EFL94826	HMPREF0623_1694	sigma-54 modulation protein	-1,30	5,81E-05	-0,99	5,14E-03	J
EFL95903	HMPREF0623_0939	50S ribosomal protein L7/L12	0,62	6,77E-04			J
EFL95809	HMPREF0623_0845	30S ribosomal protein S9	0,67	3,08E-03			J
EFL96133	HMPREF0623_0184	tRNA dimethylallyltransferase	0,80	4,59E-03			J
EFL95808	HMPREF0623_0844	hypothetical protein	-1,08	4,15E-05	-0,70	1,48E-02	K
EFL96025	HMPREF0623_0076	hypothetical protein	-0,82	2,37E-05	-0,94	2,87E-05	K
EFL94759	HMPREF0623_1627	transcriptional regulator, Xre family	-0,80	1,22E-06			K
EFL96016	HMPREF0623_0067	transcriptional regulator, AraC family	-0,71	2,60E-03			K
EFL96264	HMPREF0623_0315	SOS response repressor LexA	-0,69	2,38E-04	-0,70	8,76E-04	K
EFL96345	HMPREF0623_0396	transcriptional regulator, LacI family	-0,65	1,64E-04			K
EFL95853	HMPREF0623_0889	transcriptional regulator CtsR	-0,65	2,78E-05			K
EFL96301	HMPREF0623_0352	transcriptional regulator, LysR family	-0,62	1,07E-02			K
EFL94891	HMPREF0623_1759	transcriptional regulator, Xre family	0,60	3,46E-02			K
EFL96067	HMPREF0623_0118	transcriptional regulator, ArgR family	0,95	4,84E-05	0,86	8,94E-04	K

			Without furfural		With furfural		
Systematic Name	**Locus Tag**	**Annotation**	**logFC**	**Adj. p.value**	**logFC**	**Adj. p.value**	**COG group**
EFL95438	HMPREF0623_1175	chromosome partitioning protein, ParB			0,67	9,91E-04	K
EFL95136	HMPREF0623_1448	transcriptional regulator			0,68	8,15E-04	K
EFL95964	HMPREF0623_0015	transcriptional regulator, GntR family			0,74	1,35E-04	K
EFL95049	HMPREF0623_1555	hypothetical protein			0,80	6,73E-03	K
EFL96375	HMPREF0623_0426	tyrosine recombinase XerC	-1,13	5,35E-05			L
EFL94734	HMPREF0623_1602	hypothetical protein	-0,65	2,69E-03			L
EFL95668	HMPREF0623_0704	DEAD box RNA helicase	-0,63	1,77E-05			L
EFL94867	HMPREF0623_1735	NUDIX hydrolase	0,60	4,71E-04			L
EFL95460	HMPREF0623_1197	DNA-3-methyladenine glycosylase	0,74	7,20E-03	0,88	4,08E-03	L
EFL95090	HMPREF0623_1596	integrase			-0,61	2,05E-02	L
EFL96550	HMPREF0623_0601	transporter			-0,60	6,49E-03	L
EFL95189	HMPREF0623_1501	peptidase	-3,30	1,56E-23	-2,80	1,39E-18	M
EFL94727	HMPREF0623_1882	hypothetical protein	-1,78	9,84E-12	-1,59	3,81E-09	M
EFL95761	HMPREF0623_0797	peptidoglycan-binding protein, LysM	-1,22	8,49E-06	-1,21	5,14E-05	M
EFL95295	HMPREF0623_1032	D-alanyl-lipoteichoic acid biosynthesis protein, DltB	-0,78	6,03E-04	-0,70	6,06E-03	M
EFL96429	HMPREF0623_0480	cell surface protein	-0,77	1,44E-04	-0,61	6,33E-03	M
EFL96585	HMPREF0623_0636	UDP-N-acetylglucosamine--N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase	-0,74	4,90E-05			M
EFL95293	HMPREF0623_1030	D-alanyl-lipoteichoic acid biosynthesis protein, DltD	-0,72	1,82E-06			M
EFL95173	HMPREF0623_1485	sugar isomerase	-0,70	1,40E-03			M
EFL95412	HMPREF0623_1149	glycosyl transferase	0,61	2,84E-03	0,61	8,97E-03	M
EFL95062	HMPREF0623_1568	polysaccharide biosynthesis protein	0,62	5,86E-05			M
EFL94776	HMPREF0623_1644	glutamine--fructose-6-phosphate aminotransferase	0,67	2,22E-03	0,91	1,59E-04	M
EFL96082	HMPREF0623_0133	UDP-N-acetylmuramate--alanine ligase	0,68	2,67E-02			M
EFL95439	HMPREF0623_1176	16S rRNA methyltransferase	0,70	5,85E-04	0,61	6,37E-03	M
EFL94949	HMPREF0623_1817	glycosyltransferase family 2	0,83	1,28E-06	0,81	1,00E-05	M
EFL94731	HMPREF0623_1599	exopolysaccharide biosynthesis protein	0,87	2,81E-03	0,79	1,27E-02	M
EFL95053	HMPREF0623_1559	hypothetical protein	0,88	9,46E-04	0,88	1,93E-03	M

			Without furfural		With furfural		
Systematic Name	**Locus Tag**	**Annotation**	**logFC**	**Adj. p.value**	**logFC**	**Adj. p.value**	**COG group**
EFL95052	HMPREF0623_1558	UDP-galactopyranose mutase	0,92	5,79E-06	0,95	1,60E-05	M
EFL95769	HMPREF0623_0805	glycosyl transferase	0,94	1,64E-07	0,95	4,42E-07	M
EFL96094	HMPREF0623_0145	antiholin			-0,78	5,60E-05	M
EFL95521	HMPREF0623_1258	UDP-N-acetyl-D-mannosamine transferase			-0,62	2,88E-02	M
EFL95059	HMPREF0623_1565	multidrug MFS transporter			0,75	8,53E-06	M
EFL96245	HMPREF0623_0296	phage tail tape measure protein			-0,73	6,80E-04	M
EFL96394	HMPREF0623_0445	ATP-dependent Clp protease ATP-binding subunit, clpL	-1,31	9,89E-08	-1,01	1,09E-04	O
EFL95852	HMPREF0623_0888	ATP-dependent Clp protease ATP-binding subunit, ClpC	-0,76	9,32E-04			O
EFL95021	HMPREF0623_1527	Clp protease, ClpX	-0,75	1,14E-03			O
EFL96360	HMPREF0623_0411	peptide-methionine (S)-S-oxide reductase	-0,70	1,29E-03			O
EFL94832	HMPREF0623_1700	molecular chaperone GroEL	0,63	9,58E-05			O
EFL95175	HMPREF0623_1487	peptidyl-prolyl cis-trans isomerase	0,67	2,53E-04			O
EFL94748	HMPREF0623_1616	Fe-S cluster assembly protein, SufD	0,77	1,03E-04			O
EFL94749	HMPREF0623_1617	ABC transporter ATP-binding protein, sufC	0,84	5,89E-06	0,74	1,34E-04	O
EFL94750	HMPREF0623_1618	anaerobic ribonucleoside-triphosphate reductase activating protein	0,91	2,33E-04	0,65	1,32E-02	O
EFL94745	HMPREF0623_1613	Fe-S cluster assembly protein, SufB	0,94	4,88E-08	0,86	3,75E-06	O
EFL95660	HMPREF0623_0696	thiol reductase thioredoxin			-0,59	1,94E-02	O
EFL95094	HMPREF0623_1406	manganese catalase	-2,03	1,50E-14	-1,98	7,85E-13	P
EFL95366	HMPREF0623_1103	magnesium-transporting ATPase	-1,46	4,57E-10	-1,35	3,21E-08	P
EFL96524	HMPREF0623_0575	catalase	-1,37	3,14E-07	-1,42	1,01E-06	P
EFL94889	HMPREF0623_1757	sodium:proton antiporter	-0,80	3,54E-06			P
EFL95207	HMPREF0623_1519	MFS transporter	-0,77	6,57E-05			P
EFL96349	HMPREF0623_0400	magnesium-translocating P-type ATPase	-0,75	3,99E-06			P
EFL95084	HMPREF0623_1590	metal transporter CorA	-0,66	4,59E-03			P
EFL94821	HMPREF0623_1689	phosphate ABC transporter substrate-binding protein	-0,59	1,38E-03			P
EFL95814	HMPREF0623_0850	energy-coupling factor transporter ATPase	0,60	5,14E-03			P
EFL95717	HMPREF0623_0753	methionine ABC transporter ATP-binding protein	0,61	2,95E-03			P

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Systematic Name	**Locus Tag**	**Annotation**	**logFC**	**Adj. p.value**	**logFC**	**Adj. p.value**	**COG group**
EFL96130	HMPREF0623_0181	sulfurtransferase	0,61	2,73E-04			P
EFL96134	HMPREF0623_0185	aluminum resistance protein	0,61	2,13E-03			P
EFL95715	HMPREF0623_0751	methionine ABC transporter substrate-binding protein	0,61	1,45E-04	0,70	7,72E-05	P
EFL94968	HMPREF0623_1836	ABC transporter	0,62	3,37E-04			P
EFL94864	HMPREF0623_1732	transcriptional repressor	0,70	1,17E-03			P
EFL96606	HMPREF0623_0657	transcriptional regulator	0,90	7,11E-05			P
EFL95716	HMPREF0623_0752	ABC transporter permease			0,67	1,23E-04	P
EFL96603	HMPREF0623_0654	dithiol-disulfide isomerase	-1,00	4,61E-04	-1,17	1,87E-04	Q
EFL95296	HMPREF0623_1033	D-alanine--poly(phosphoribitol) ligase	-0,94	7,02E-06			Q
EFL95644	HMPREF0623_0680	isochorismatase	1,01	3,29E-08	0,97	3,44E-08	Q
EFL94762	HMPREF0623_1630	phenolic acid decarboxylase, padC			-0,75	2,09E-03	Q
EFL96513	HMPREF0623_0564	glucose starvation-inducible protein B	-1,51	1,22E-10	-1,72	3,41E-10	R
EFL96391	HMPREF0623_0442	NAD(FAD)-dependent dehydrogenase	-1,46	4,05E-09	-1,07	5,14E-05	R
EFL95655	HMPREF0623_0691	chemotaxis protein	-1,22	6,32E-08	-1,16	1,44E-06	R
EFL94807	HMPREF0623_1675	hydrolase	-1,00	7,27E-07			R
EFL95174	HMPREF0623_1486	haloacid dehalogenase	-0,73	1,80E-04	-0,62	4,11E-03	R
EFL96544	HMPREF0623_0595	RNase J family beta-CASP ribonuclease	-0,69	4,86E-03			R
EFL94849	HMPREF0623_1717	sodium:dicarboxylate symporter	-0,64	1,70E-02			R
EFL96116	HMPREF0623_0167	hydrolase	-0,63	4,68E-06			R
EFL95595	HMPREF0623_1332	permease	0,62	5,91E-06			R
EFL95246	HMPREF0623_1382	2,5-diketo-D-gluconic acid reductase	0,67	1,14E-04	0,61	5,00E-04	R
EFL94804	HMPREF0623_1672	ATPase P	0,67	1,24E-02			R
EFL96607	HMPREF0623_0658	hypothetical protein	0,70	2,49E-03			R
EFL95362	HMPREF0623_1099	heme ABC transporter ATP-binding protein	0,90	3,46E-07	0,81	9,82E-06	R
EFL95051	HMPREF0623_1557	polysaccharide biosynthesis protein	0,91	7,20E-04	0,85	4,02E-03	R
EFL96307	HMPREF0623_0358	TIGR00730 family Rossman fold protein	0,92	5,61E-03	1,18	1,06E-03	R
EFL96314	HMPREF0623_0365	hypothetical protein	0,94	6,99E-05			R

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EFL94954	HMPREF0623_1822	guanine permease	2,58	1,02E-16	2,53	3,29E-15	R
EFL94870	HMPREF0623_1738	guanine permease	3,01	6,95E-31	2,41	2,41E-24	R
EFL95384	HMPREF0623_1121	alkaline-shock protein	-3,76	9,47E-32	-3,14	8,31E-26	S
EFL95383	HMPREF0623_1120	alkaline-shock protein	-3,76	4,28E-26	-3,02	5,59E-20	S
EFL95387	HMPREF0623_1124	hypothetical protein	-3,54	3,67E-22	-2,99	2,41E-17	S
EFL95388	HMPREF0623_1125	membrane protein	-3,07	8,10E-36	-2,58	6,19E-30	S
EFL96296	HMPREF0623_0347	DUF378 domain-containing protein	-2,91	1,21E-23	-2,53	3,83E-19	S
EFL95071	HMPREF0623_1577	hypothetical protein	-2,53	2,18E-17	-2,13	5,00E-13	S
EFL95511	HMPREF0623_1248	hypothetical protein	-2,18	1,07E-15	-2,21	2,05E-14	S
EFL94911	HMPREF0623_1779	hypothetical protein	-2,07	5,94E-22	-1,94	3,25E-19	S
EFL95123	HMPREF0623_1435	hypothetical protein	-2,06	1,74E-18	-1,85	5,72E-15	S
EFL95122	HMPREF0623_1434	oxidoreductase	-1,99	1,00E-16	-1,75	1,65E-13	S
EFL94928	HMPREF0623_1796	hypothetical protein	-1,63	4,88E-09	-1,01	5,37E-04	S
EFL95871	HMPREF0623_0907	hypothetical protein	-1,63	7,53E-10	-1,74	7,38E-10	S
EFL94866	HMPREF0623_1734	hypothetical protein	-1,39	2,49E-11	-0,98	1,68E-06	S
EFL96029	HMPREF0623_0080	hypothetical protein	-1,38	2,65E-08	-1,42	8,92E-08	S
EFL94760	HMPREF0623_1628	glucose-1-dehydrogenase	-1,20	1,13E-12	-0,86	3,52E-07	S
EFL96131	HMPREF0623_0182	hypothetical protein	-1,17	1,06E-10	-0,85	3,06E-06	S
EFL95759	HMPREF0623_0795	hypothetical protein	-1,05	1,93E-03	-0,81	4,27E-02	S
EFL95435	HMPREF0623_1172	ABC transporter permease	-1,03	3,22E-10	-0,92	7,96E-07	S
EFL96233	HMPREF0623_0284	phage portal protein	-1,03	6,96E-03	-0,85	4,42E-02	S
EFL96251	HMPREF0623_0302	phage capsid protein	-1,02	1,82E-02			S
EFL95289	HMPREF0623_1026	hypothetical protein	-1,02	3,11E-07	-0,68	1,48E-03	S
EFL96246	HMPREF0623_0297	hypothetical protein	-1,00	9,59E-03	-1,23	2,73E-03	S
EFL96519	HMPREF0623_0570	hypothetical protein	-0,95	4,48E-06	-0,93	6,48E-05	S
EFL95288	HMPREF0623_1025	peptidase	-0,92	1,44E-08	-0,66	1,22E-04	S
EFL96234	HMPREF0623_0285	gp4 family protein	-0,92	1,24E-06	-1,03	6,56E-07	S

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Systematic Name	**Locus Tag**	**Annotation**	**logFC**	**Adj. p.value**	**logFC**	**Adj. p.value**	**COG group**
EFL95420	HMPREF0623_1157	hypothetical protein	-0,89	1,74E-04	-0,89	8,39E-04	S
EFL96241	HMPREF0623_0292	hypothetical protein	-0,88	4,03E-05	-1,00	1,82E-05	S
EFL95760	HMPREF0623_0796	peptidoglycan-binding protein LysM	-0,86	5,31E-07	-1,10	2,87E-09	S
EFL95452	HMPREF0623_1189	alkaline phosphatase	-0,86	3,35E-09			S
EFL96236	HMPREF0623_0287	phage capsid protein	-0,84	1,99E-04	-1,06	3,97E-05	S
EFL96515	HMPREF0623_0566	membrane protein	-0,83	1,63E-06			S
EFL96472	HMPREF0623_0523	hypothetical protein	-0,81	1,05E-06			S
EFL95556	HMPREF0623_1293	hypothetical protein	-0,80	7,66E-04	-0,64	1,68E-02	S
EFL94931	HMPREF0623_1799	hypothetical protein	-0,79	7,86E-08			S
EFL95880	HMPREF0623_0916	hypothetical protein	-0,79	1,69E-03			S
EFL96232	HMPREF0623_0283	terminase	-0,78	3,75E-04	-1,04	1,66E-04	S
EFL95453	HMPREF0623_1190	hypothetical protein	-0,77	1,43E-02			S
EFL95473	HMPREF0623_1210	hypothetical protein	-0,74	4,30E-04	-0,63	1,60E-02	S
EFL95093	HMPREF0623_1405	RNA polymerase subunit sigma	-0,71	1,75E-03			S
EFL95028	HMPREF0623_1534	hypothetical protein	-0,70	5,75E-05			S
EFL96432	HMPREF0623_0483	hypothetical protein	-0,69	2,69E-04			S
EFL96235	HMPREF0623_0286	phage minor structural protein GP20	-0,69	7,57E-04	-1,06	4,06E-06	S
EFL95089	HMPREF0623_1595	plasmid replication initiation protein	-0,68	2,20E-04			S
EFL96300	HMPREF0623_0351	ABC transporter permease	-0,67	6,16E-04			S
EFL95454	HMPREF0623_1191	membrane protein	-0,66	1,21E-03			S
EFL94972	HMPREF0623_1840	hypothetical protein	-0,65	4,50E-04	-0,59	4,63E-03	S
EFL94814	HMPREF0623_1682	membrane protein	-0,62	2,67E-03			S
EFL95523	HMPREF0623_1260	hypothetical protein	-0,61	1,02E-02	-0,69	1,04E-02	S
EFL95440	HMPREF0623_1177	acetyltransferase	-0,59	3,27E-04			S
EFL94775	HMPREF0623_1643	hypothetical protein	0,61	1,11E-02			S
EFL94950	HMPREF0623_1818	hypothetical protein	0,62	1,70E-03	0,63	2,66E-03	S
EFL94838	HMPREF0623_1706	type II CRISPR RNA-guided endonuclease Cas9	0,62	1,14E-03	0,59	4,98E-03	S

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Systematic Name	**Locus Tag**	**Annotation**	**logFC**	**Adj. p.value**	**logFC**	**Adj. p.value**	**COG group**
EFL94952	HMPREF0623_1820	hypothetical protein	0,63	3,51E-03	0,72	2,46E-03	S
EFL95058	HMPREF0623_1564	multidrug MFS transporter	0,63	1,13E-07	0,67	3,50E-06	S
EFL95498	HMPREF0623_1235	membrane protein	0,68	3,47E-04			S
EFL94948	HMPREF0623_1816	cellulose synthase	0,68	5,30E-04			S
EFL96393	HMPREF0623_0444	hypothetical protein	0,71	7,48E-04	0,62	9,27E-03	S
EFL95057	HMPREF0623_1563	glycosyl transferase	0,76	4,64E-03	0,87	5,88E-04	S
EFL95712	HMPREF0623_0748	ABC transporter	0,76	7,45E-03			S
EFL95339	HMPREF0623_1076	hypothetical protein	0,78	2,38E-04	0,97	3,76E-05	S
EFL95776	HMPREF0623_0812	hypothetical protein	0,79	3,66E-04	1,07	4,89E-06	S
EFL95054	HMPREF0623_1560	ABC superfamily ATP binding cassette transporter permease subunit	0,83	1,64E-04	0,89	1,08E-04	S
EFL94802	HMPREF0623_1670	sporulation regulator WhiA	0,85	2,87E-05			S
EFL95037	HMPREF0623_1543	2-hydroxyacid dehydrogenase	0,88	1,60E-04	1,22	1,45E-06	S
EFL96206	HMPREF0623_0257	hypothetical protein	0,92	8,98E-03	0,84	2,67E-02	S
EFL95342	HMPREF0623_1079	hypothetical protein	0,96	1,08E-05	1,18	8,70E-07	S
EFL95771	HMPREF0623_0807	accessory Sec system protein Asp2	0,98	7,16E-05	1,05	1,03E-04	S
EFL95768	HMPREF0623_0804	hypothetical protein	1,03	1,48E-06	1,06	4,81E-06	S
EFL95335	HMPREF0623_1072	hypothetical protein	1,12	1,37E-08	1,26	2,90E-09	S
EFL95038	HMPREF0623_1544	hypothetical protein	1,24	3,35E-08	1,58	2,12E-10	S
EFL95770	HMPREF0623_0806	accessory Sec system protein Asp3	1,25	2,24E-07	1,37	1,48E-07	S
EFL95332	HMPREF0623_1069	hypothetical protein	1,27	5,32E-12	1,40	4,99E-13	S
EFL95050	HMPREF0623_1556	hypothetical protein	1,36	9,09E-07	0,91	1,49E-03	S
EFL95772	HMPREF0623_0808	accessory Sec system protein Asp1	1,41	1,46E-07	1,39	1,63E-06	S
EFL95502	HMPREF0623_1239	hypothetical protein	1,92	5,96E-13	1,70	3,99E-10	S
EFL94729	HMPREF0623_1597	hypothetical protein			0,60	6,55E-04	S
EFL95299	HMPREF0623_1036	hypothetical protein			0,71	9,09E-03	S
EFL96247	HMPREF0623_0298	hypothetical protein			-0,98	8,49E-05	S
EFL95985	HMPREF0623_0036	hypothetical protein			-0,81	1,66E-03	S

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Systematic Name	**Locus Tag**	**Annotation**	**logFC**	**Adj. p.value**	**logFC**	**Adj. p.value**	**COG group**
EFL94736	HMPREF0623_1604	hydrolase			0,60	1,96E-04	S
EFL96208	HMPREF0623_0259	hypothetical protein			0,66	1,30E-03	S
EFL95036	HMPREF0623_1542	hypothetical protein			0,74	6,59E-05	S
EFL95232	HMPREF0623_1368	N-acetyltransferase GCN5			0,84	5,01E-03	S
EFL96295	HMPREF0623_0346	tryptophan-rich sensory protein	-1,53	2,44E-11	-1,31	2,90E-08	T
EFL95705	HMPREF0623_0741	universal stress protein, UspA	-0,73	1,01E-02	-0,76	2,10E-02	T
EFL95429	HMPREF0623_1166	two-component sensor histidine kinase	-0,73	1,75E-05			T
EFL94813	HMPREF0623_1681	HPr kinase/phosphorylase	-0,63	3,05E-04			T
EFL94947	HMPREF0623_1815	diguanylate cyclase	0,60	5,48E-04			T
EFL95462	HMPREF0623_1199	EAL domain-containing protein	0,97	1,15E-08	0,78	7,06E-06	T
EFL96066	HMPREF0623_0117	cAMP-binding protein	1,10	6,41E-03	1,16	9,93E-03	T
EFL95463	HMPREF0623_1200	diguanylate cyclase	1,24	1,76E-06	0,91	1,12E-03	T
EFL94973	HMPREF0623_1841	arginine deiminase			-0,68	1,48E-02	T
EFL95569	HMPREF0623_1306	signal peptidase	0,73	1,31E-05			U
EFL95773	HMPREF0623_0809	preprotein translocase subunit SecY	0,77	3,75E-06	0,92	1,19E-06	U
EFL95774	HMPREF0623_0810	accessory Sec system translocase SecA2	1,35	8,20E-14	1,31	4,19E-12	U
EFL95636	HMPREF0623_0672	competence protein ComGC			-0,68	2,86E-04	U
EFL95365	HMPREF0623_1102	ABC transporter ATP-binding protein	-0,98	7,72E-11	-0,79	4,36E-07	V
EFL95287	HMPREF0623_1024	MATE family efflux transporter	-0,79	2,40E-04	-0,78	8,90E-04	V
EFL95215	HMPREF0623_1392	GntR family transcriptional regulator	-0,71	3,68E-03			V
EFL95063	HMPREF0623_1569	serine hydrolase	0,62	4,28E-04			V
EFL95385	HMPREF0623_1122	hypothetical protein	-3,47	1,62E-27	-2,80	3,09E-21	
EFL95386	HMPREF0623_1123	hypothetical protein	-3,11	8,20E-25	-2,86	2,39E-21	
EFL95190	HMPREF0623_1502	hypothetical protein	-1,94	2,27E-13	-1,56	3,58E-09	
EFL96537	HMPREF0623_0588	hypothetical protein	-1,74	3,10E-09	-0,97	1,49E-03	
EFL96242	HMPREF0623_0293	phosphoenolpyruvate synthase	-1,35	1,29E-07	-1,20	1,04E-05	
EFL95983	HMPREF0623_0034	hypothetical protein	-1,16	6,18E-03			

			Without furfural		With furfural		
Systematic Name	**Locus Tag**	**Annotation**	**logFC**	**Adj. p.value**	**logFC**	**Adj. p.value**	**COG group**
EFL95075	HMPREF0623_1581	hypothetical protein	-1,15	3,46E-06	-1,17	1,60E-05	
EFL96243	HMPREF0623_0294	hypothetical protein	-1,15	1,85E-05	-1,16	1,35E-04	
EFL96428	HMPREF0623_0479	hypothetical protein	-1,14	4,80E-05	-0,72	1,10E-02	
EFL95554	HMPREF0623_1291	hypothetical protein	-1,00	7,49E-05	-0,80	5,74E-03	
EFL95557	HMPREF0623_1294	hypothetical protein	-0,94	8,58E-08			
EFL96438	HMPREF0623_0489	fimbrial chaperone	-0,92	5,35E-03			
EFL95879	HMPREF0623_0915	hypothetical protein	-0,90	9,26E-03	-0,71	4,82E-02	
EFL95436	HMPREF0623_1173	hypothetical protein	-0,87	2,98E-05			
EFL95553	HMPREF0623_1290	hypothetical protein	-0,87	1,25E-04			
EFL95654	HMPREF0623_0690	hypothetical protein	-0,83	2,54E-03	-0,73	1,95E-02	
EFL95229	HMPREF0623_1365	hypothetical protein	-0,81	7,49E-03	-0,80	1,90E-02	
EFL96057	HMPREF0623_0108	hypothetical protein	-0,79	3,32E-04	-0,83	1,07E-03	
EFL95922	HMPREF0623_0958	hypothetical protein	-0,75	1,34E-04			
EFL96237	HMPREF0623_0288	hypothetical protein	-0,75	8,23E-04	-0,89	2,53E-04	
EFL96238	HMPREF0623_0289	hypothetical protein	-0,74	2,68E-02	-0,94	7,94E-03	
EFL95297	HMPREF0623_1034	hypothetical protein	-0,73	9,35E-04	-0,62	1,39E-02	
EFL95208	HMPREF0623_1385	hypothetical protein	-0,70	9,79E-04			
EFL95188	HMPREF0623_1500	hypothetical protein	-0,68	4,87E-03			
EFL94773	HMPREF0623_1641	oleate hydratase	-0,67	5,22E-05			
EFL96244	HMPREF0623_0295	bacteriophage Gp15 protein	-0,66	4,75E-02			
EFL96608	HMPREF0623_0659	monooxygenase	-0,66	2,82E-04			
EFL95457	HMPREF0623_1194	hypothetical protein	-0,65	5,41E-03	-0,69	7,59E-03	
EFL95121	HMPREF0623_1433	magnesium transporter	-0,65	1,93E-03			
EFL95847	HMPREF0623_0883	hypothetical protein	-0,63	2,37E-04			
EFL95357	HMPREF0623_1094	hypothetical protein	-0,63	4,27E-02			
EFL95894	HMPREF0623_0930	hypothetical protein	-0,59	2,86E-02			
EFL95749	HMPREF0623_0785	hypothetical protein	0,59	1,47E-04			

			Without furfural		With furfural		
Systematic Name	**Locus Tag**	**Annotation**	**logFC**	**Adj. p.value**	**logFC**	**Adj. p.value**	**COG group**
EFL95680	HMPREF0623_0716	hypothetical protein	0,62	3,07E-02			
EFL95343	HMPREF0623_1080	hypothetical protein	0,63	7,22E-06	0,84	9,78E-08	
EFL96286	HMPREF0623_0337	hypothetical protein	0,63	1,65E-03	0,59	6,76E-03	
EFL95077	HMPREF0623_1583	hypothetical protein	0,65	1,96E-05	0,81	3,13E-06	
EFL94962	HMPREF0623_1830	hypothetical protein	0,67	1,94E-03			
EFL95337	HMPREF0623_1074	hypothetical protein	0,68	5,26E-05	1,37	2,31E-11	
EFL96344	HMPREF0623_0395	hypothetical protein	0,71	1,27E-03			
EFL96431	HMPREF0623_0482	M protein trans-acting positive regulator	0,75	1,22E-03			
EFL95596	HMPREF0623_1333	#N/A	0,76	6,58E-05	0,59	4,88E-03	
EFL96527	HMPREF0623_0578	hypothetical protein	0,76	4,51E-05	0,70	6,06E-04	
EFL96260	HMPREF0623_0311	hypothetical protein	0,78	1,31E-05	1,01	4,86E-06	
EFL95055	HMPREF0623_1561	capsular polysaccharide phosphotransferase WcwK	0,82	2,63E-05	0,84	2,99E-05	
EFL96261	HMPREF0623_0312	hypothetical protein	0,83	1,44E-04	1,11	6,02E-06	
EFL96384	HMPREF0623_0435	hypothetical protein	0,87	5,60E-06			
EFL95338	HMPREF0623_1075	hypothetical protein	0,96	1,38E-07	1,19	1,51E-09	
EFL96409	HMPREF0623_0460	hypothetical protein	0,96	1,62E-08			
EFL96255	HMPREF0623_0306	hypothetical protein	0,99	9,28E-04	0,88	1,09E-02	
EFL96207	HMPREF0623_0258	hypothetical protein	0,99	6,66E-07	0,95	8,56E-06	
EFL95333	HMPREF0623_1070	hypothetical protein	1,00	4,85E-06	1,09	4,85E-06	
EFL95334	HMPREF0623_1071	hypothetical protein	1,02	1,70E-09	1,28	1,10E-11	
EFL95340	HMPREF0623_1077	hypothetical protein	1,19	8,80E-06	1,19	4,99E-05	
EFL95336	HMPREF0623_1073	hypothetical protein	1,30	2,33E-13	1,50	1,02E-14	
EFL96354	HMPREF0623_0405	non-canonical purine NTP pyrophosphatase	1,42	1,96E-08	1,71	1,12E-09	
EFL95775	HMPREF0623_0811	hypothetical protein	1,51	4,80E-06	1,47	3,73E-05	
EFL96352	HMPREF0623_0403	hypothetical protein	1,79	2,01E-19	2,01	3,52E-20	
EFL96250	HMPREF0623_0301	hypothetical protein			-1,44	1,30E-02	
EFL96239	HMPREF0623_0290	hypothetical protein			-0,72	3,56E-02	

			Without furfural		With furfural		
Systematic Name	**Locus Tag**	**Annotation**	**logFC**	**Adj. p.value**	**logFC**	**Adj. p.value**	**COG group**
EFL95085	HMPREF0623_1591	hypothetical protein			0,60	3,90E-03	
EFL95081	HMPREF0623_1587	recombinase RecD			0,65	9,90E-04	
EFL94850	HMPREF0623_1718	hypothetical protein			0,66	1,31E-04	
EFL96211	HMPREF0623_0262	hypothetical protein			0,68	1,54E-03	
EFL96210	HMPREF0623_0261	transcriptional regulator			0,69	8,96E-04	
EFL96382	HMPREF0623_0433	hypothetical protein			0,73	1,60E-02	
EFL95056	HMPREF0623_1562	CpsY protein			0,77	1,07E-05	
EFL95080	HMPREF0623_1586	hypothetical protein			0,79	2,55E-04	
EFL96383	HMPREF0623_0434	hypothetical protein			0,84	4,21E-02	
EFL96297	HMPREF0623_0348	hypothetical protein			0,90	7,09E-04	
EFL95971	HMPREF0623_0022	hypothetical protein			0,91	2,89E-04	
EFL96609	HMPREF0623_0660	hypothetical protein			0,93	1,05E-04	
EFL96204	HMPREF0623_0255	hypothetical protein			1,08	7,17E-04	

8.2.3 Table S8. Top 50 most up-regulated genes in DSM 20284 and A28 grown in MRS with and without furfural, sorted by logFC.

			Without furfural		With furfural		
Systematic Name	**Locus Tag**	**Annotation**	**logFC**	**Adj. p.value**	**logFC**	**Adj. p.value**	**COG group**
EFL94869	HMPREF0623_1737	adenine deaminase	4,22	7,61E-39	3,47	6,76E-32	F
EFL95022	HMPREF0623_1528	guanosine monophosphate reductase, guaC	4,13	4,58E-27	3,92	2,61E-24	F
EFL94871	HMPREF0623_1739	adenosine deaminase, add	3,35	1,15E-21	2,36	2,22E-13	F
EFL94870	HMPREF0623_1738	guanine permease	3,01	6,95E-31	2,41	2,41E-24	R
EFL94954	HMPREF0623_1822	guanine permease	2,58	1,02E-16	2,53	3,29E-15	R
EFL96353	HMPREF0623_0404	dihydropteroate synthase, folP	1,92	3,27E-13	1,84	1,15E-11	H
EFL95502	HMPREF0623_1239	hypothetical protein	1,92	5,96E-13	1,70	3,99E-10	S
EFL96357	HMPREF0623_0408	2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase, folK	1,80	3,73E-11	1,64	3,05E-09	H
EFL96352	HMPREF0623_0403	hypothetical protein	1,79	2,01E-19	2,01	3,52E-20	
EFL96358	HMPREF0623_0409	dihydroneopterin aldolase, folB	1,79	1,61E-12	1,71	3,17E-11	H
EFL95363	HMPREF0623_1100	xanthine/uracil permease	1,74	5,43E-14	1,67	2,80E-12	F
EFL95645	HMPREF0623_0681	adenylosuccinate lyase, purB	1,69	1,21E-12	1,36	4,35E-09	F
EFL96355	HMPREF0623_0406	tetrahydrofolate synthase, folC	1,68	4,24E-12	1,66	2,14E-11	H
EFL95364	HMPREF0623_1101	chlorohydrolase	1,66	4,51E-14	1,64	1,32E-12	F
EFL96356	HMPREF0623_0407	GTP cyclohydrolase I, folE	1,64	3,76E-17	1,73	6,96E-17	H
EFL95775	HMPREF0623_0811	hypothetical protein	1,51	4,80E-06	1,47	3,73E-05	
EFL95233	HMPREF0623_1369	glutamate:protein symporter	1,49	1,09E-15	1,27	1,48E-12	C
EFL95646	HMPREF0623_0682	adenylosuccinate synthase, purA	1,46	2,29E-13	1,54	4,51E-13	F
EFL96354	HMPREF0623_0405	non-canonical purine NTP pyrophosphatase	1,42	1,96E-08	1,71	1,12E-09	
EFL95772	HMPREF0623_0808	accessory Sec system protein Asp1	1,41	1,46E-07	1,39	1,63E-06	S
EFL95050	HMPREF0623_1556	hypothetical protein	1,36	9,09E-07	0,91	1,49E-03	S
EFL95774	HMPREF0623_0810	accessory Sec system translocase SecA2	1,35	8,20E-14	1,31	4,19E-12	U
EFL95336	HMPREF0623_1073	hypothetical protein	1,30	2,33E-13	1,50	1,02E-14	
EFL95332	HMPREF0623_1069	hypothetical protein	1,27	5,32E-12	1,40	4,99E-13	S
EFL95770	HMPREF0623_0806	accessory Sec system protein Asp3	1,25	2,24E-07	1,37	1,48E-07	S

EFL95966	HMPREF0623_0017	deoxyadenosine kinase	1,25	1,34E-09	0,76	3,16E-04	F
EFL95463	HMPREF0623_1200	diguanylate cyclase	1,24	1,76E-06	0,91	1,12E-03	T
EFL95038	HMPREF0623_1544	hypothetical protein	1,24	3,35E-08	1,58	2,12E-10	S
EFL95442	HMPREF0623_1179	nucleoside transporter	1,21	3,94E-05	1,07	8,72E-04	F
EFL95340	HMPREF0623_1077	hypothetical protein	1,19	8,80E-06	1,19	4,99E-05	
EFL95441	HMPREF0623_1178	ribonucleoside hydrolase	1,13	1,57E-08	1,01	1,15E-06	F
EFL95335	HMPREF0623_1072	hypothetical protein	1,12	1,37E-08	1,26	2,90E-09	S
EFL96066	HMPREF0623_0117	cAMP-binding protein	1,10	6,41E-03	1,16	9,93E-03	T
EFL94857	HMPREF0623_1725	fructose 2,6-bisphosphatase	1,10	9,14E-06	0,76	5,62E-04	G
EFL95873	HMPREF0623_0909	Uric acid/xanthine permease	1,09	1,35E-10	0,60	1,34E-04	F
EFL94733	HMPREF0623_1601	hypothetical protein	1,04	9,39E-12	1,19	1,09E-12	G
EFL95768	HMPREF0623_0804	hypothetical protein	1,03	1,48E-06	1,06	4,81E-06	S
EFL94747	HMPREF0623_1615	cysteine desulfurase	1,02	4,21E-06	0,84	5,67E-04	E
EFL95334	HMPREF0623_1071	hypothetical protein	1,02	1,70E-09	1,28	1,10E-11	
EFL95644	HMPREF0623_0680	isochorismatase	1,01	3,29E-08	0,97	3,44E-08	Q
EFL95333	HMPREF0623_1070	hypothetical protein	1,00	4,85E-06	1,09	4,85E-06	
EFL96207	HMPREF0623_0258	hypothetical protein	0,99	6,66E-07	0,95	8,56E-06	
EFL96255	HMPREF0623_0306	hypothetical protein	0,99	9,28E-04	0,88	1,09E-02	
EFL95771	HMPREF0623_0807	accessory Sec system protein Asp2	0,98	7,16E-05	1,05	1,03E-04	S
EFL95462	HMPREF0623_1199	EAL domain-containing protein	0,97	1,15E-08	0,78	7,06E-06	T
EFL94856	HMPREF0623_1724	amino acid permease	0,96	4,82E-09	0,96	8,24E-08	E
EFL96409	HMPREF0623_0460	hypothetical protein	0,96	1,62E-08			
EFL95342	HMPREF0623_1079	hypothetical protein	0,96	1,08E-05	1,18	8,70E-07	S
EFL95338	HMPREF0623_1075	hypothetical protein	0,96	1,38E-07	1,19	1,51E-09	
EFL96067	HMPREF0623_0118	transcriptional regulator, ArgR family	0,95	4,84E-05	0,86	8,94E-04	K

8.2.4 Table S9. Top 50 most down-regulated genes in DSM 20284 and A28 grown in MRS with and without furfural, sorted by logFC.

			Without furfural		With furfural		
Systematic Name	**Locus Tag**	**Annotation**	**logFC**	**Adj. p.value**	**logFC**	**Adj. p.value**	**COG group**
EFL95384	HMPREF0623_1121	alkaline-shock protein	-3,76	9,47E-32	-3,14	8,31E-26	S
EFL95383	HMPREF0623_1120	alkaline-shock protein	-3,76	4,28E-26	-3,02	5,59E-20	S
EFL95387	HMPREF0623_1124	hypothetical protein	-3,54	3,67E-22	-2,99	2,41E-17	S
EFL95385	HMPREF0623_1122	hypothetical protein	-3,47	1,62E-27	-2,80	3,09E-21	
EFL95612	HMPREF0623_1349	carbamate kinase	-3,46	8,23E-28	-3,13	1,26E-23	E
EFL95189	HMPREF0623_1501	peptidase	-3,30	1,56E-23	-2,80	1,39E-18	M
EFL95613	HMPREF0623_1350	arginine deiminase	-3,22	2,82E-32	-2,75	1,20E-26	E
EFL95611	HMPREF0623_1348	ornithine carbamoyltransferase	-3,19	1,58E-24	-2,98	2,04E-21	E
EFL95386	HMPREF0623_1123	hypothetical protein	-3,11	8,20E-25	-2,86	2,39E-21	
EFL95388	HMPREF0623_1125	membrane protein	-3,07	8,10E-36	-2,58	6,19E-30	S
EFL96296	HMPREF0623_0347	DUF378 domain-containing protein	-2,91	1,21E-23	-2,53	3,83E-19	S
EFL95614	HMPREF0623_1351	amino acid permease	-2,68	5,51E-24	-2,36	8,49E-20	E
EFL95071	HMPREF0623_1577	hypothetical protein	-2,53	2,18E-17	-2,13	5,00E-13	S
EFL95511	HMPREF0623_1248	hypothetical protein	-2,18	1,07E-15	-2,21	2,05E-14	S
EFL94911	HMPREF0623_1779	hypothetical protein	-2,07	5,94E-22	-1,94	3,25E-19	S
EFL95123	HMPREF0623_1435	hypothetical protein	-2,06	1,74E-18	-1,85	5,72E-15	S
EFL95094	HMPREF0623_1406	manganese catalase	-2,03	1,50E-14	-1,98	7,85E-13	P
EFL95122	HMPREF0623_1434	oxidoreductase	-1,99	1,00E-16	-1,75	1,65E-13	S
EFL95190	HMPREF0623_1502	hypothetical protein	-1,94	2,27E-13	-1,56	3,58E-09	
EFL94727	HMPREF0623_1882	hypothetical protein	-1,78	9,84E-12	-1,59	3,81E-09	M
EFL96537	HMPREF0623_0588	hypothetical protein	-1,74	3,10E-09	-0,97	1,49E-03	
EFL95087	HMPREF0623_1593	glutathione reductase	-1,71	5,73E-17	-1,33	1,58E-11	C
EFL94861	HMPREF0623_1729	arginine ABC transporter ATP-binding protein	-1,68	6,02E-17	-1,57	1,84E-14	E
EFL94928	HMPREF0623_1796	hypothetical protein	-1,63	4,88E-09	-1,01	5,37E-04	S

EFL95871	HMPREF0623_0907	hypothetical protein	-1,63	7,53E-10	-1,74	7,38E-10	S
EFL94860	HMPREF0623_1728	glutamine ABC transporter substrate-binding protein	-1,62	1,98E-13	-1,57	1,24E-11	E
EFL96295	HMPREF0623_0346	tryptophan-rich sensory protein	-1,53	2,44E-11	-1,31	2,90E-08	T
EFL96513	HMPREF0623_0564	glucose starvation-inducible protein B	-1,51	1,22E-10	-1,72	3,41E-10	R
EFL96391	HMPREF0623_0442	NAD(FAD)-dependent dehydrogenase	-1,46	4,05E-09	-1,07	5,14E-05	R
EFL95366	HMPREF0623_1103	magnesium-transporting ATPase	-1,46	4,57E-10	-1,35	3,21E-08	P
EFL94859	HMPREF0623_1727	glutamine ABC transporter permease	-1,40	3,77E-15	-1,19	6,09E-11	E
EFL94866	HMPREF0623_1734	hypothetical protein	-1,39	2,49E-11	-0,98	1,68E-06	S
EFL96029	HMPREF0623_0080	hypothetical protein	-1,38	2,65E-08	-1,42	8,92E-08	S
EFL94868	HMPREF0623_1736	amino acid permease	-1,37	2,50E-11	-0,93	2,75E-06	E
EFL96524	HMPREF0623_0575	catalase	-1,37	3,14E-07	-1,42	1,01E-06	P
EFL96242	HMPREF0623_0293	phosphoenolpyruvate synthase	-1,35	1,29E-07	-1,20	1,04E-05	
EFL96040	HMPREF0623_0091	PTS mannitol transporter subunit IIA	-1,31	6,21E-03	-1,40	3,11E-03	G
EFL96394	HMPREF0623_0445	ATP-dependent Clp protease ATP-binding subunit, clpL	-1,31	9,89E-08	-1,01	1,09E-04	O
EFL94826	HMPREF0623_1694	sigma-54 modulation protein	-1,30	5,81E-05	-0,99	5,14E-03	J
EFL95761	HMPREF0623_0797	peptidoglycan-binding protein, LysM	-1,22	8,49E-06	-1,21	5,14E-05	M
EFL95655	HMPREF0623_0691	chemotaxis protein	-1,22	6,32E-08	-1,16	1,44E-06	R
EFL94760	HMPREF0623_1628	glucose-1-dehydrogenase	-1,20	1,13E-12	-0,86	3,52E-07	S
EFL96131	HMPREF0623_0182	hypothetical protein	-1,17	1,06E-10	-0,85	3,06E-06	S
EFL95983	HMPREF0623_0034	hypothetical protein	-1,16	6,18E-03			
EFL95075	HMPREF0623_1581	hypothetical protein	-1,15	3,46E-06	-1,17	1,60E-05	
EFL96243	HMPREF0623_0294	hypothetical protein	-1,15	1,85E-05	-1,16	1,35E-04	
EFL96428	HMPREF0623_0479	hypothetical protein	-1,14	4,80E-05	-0,72	1,10E-02	
EFL96375	HMPREF0623_0426	tyrosine recombinase XerC	-1,13	5,35E-05			L
EFL95808	HMPREF0623_0844	hypothetical protein	-1,08	4,15E-05	-0,70	1,48E-02	K
EFL95759	HMPREF0623_0795	hypothetical protein	-1,05	1,93E-03	-0,81	4,27E-02	S