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Development of metabolically engineered *Saccharomyces cerevisiae* strain towards lactic acid production

Master thesis

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STATUTORY DECLARATION

I declare that I have authored this thesis independently, that I have not used other than the declared sources / resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

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Abstract

Lactic acid has received increasing attention especially for the use as a monomer for the production of the biodegradable polymer polylactic acid, that has the future potential to replace petroleum based polymers. Currently it is mainly produced by the use of lactic acid bacteria, but with limited success. The major disadvantage is the need of neutralization of the fermentation process because those hosts are not able to produce lactic acid at low pH. Since *S. cervisiae* exhibits high acid tolerance, lactic acid fermentation might be accomplished without neutralization, significantly simplifying downstream processing and thus reducing the production cost. Thus, recently *S. cervisiae* has become of great interest as alternative production host. This study is the first example that deals with the development of a genetically engineered yeast strain that is capable to convert efficiently both glucose and xylose into lactic acid. The xylose fermenting *S. cervisiae* strain IBB10B05 was used as background strain for the genetic engineering. At first the coding region of pyruvate decarboxylase 1 gene (*pdc1*) was deleted, in order to suppress ethanol formation. Double deletion of *pdc1* and *adh1* genes was also attempted, however, the resulting mutant was not viable probably because of the accumulation of acetaldehyde to toxic levels as a result of *adh1* deletion. To facilitate lactic acid production two lactate dehydrogenase genes from two different organism (*R. oryzae* and *P. falciparum)* were expressed in the single *pdc1* mutant from a 2µm multi copy plasmid under regulation of a strong constitutive promoter (TEF1). Despite their strong expression, activity and protein concentration as well as lactic acid production (maximal 3 %) from both, glucose and xylose, was poor. Alternatively we substituted the coding region of *pdc1 gene* with that of lactate dehydrogenase from *P. falciparum*, enabling expression from the native *pdc1* promoter, while *pdc1* is completely disrupted (BI-*Pfldh* strain). As a result, high activity (1.24 U/mg) and elevated protein levels could be detected, indicating that metabolic regulation of protein expression is important for the success of lactic acid production. Fermentation studies further showed, that strain BI-*Pfldh*, is capable of converting high amounts of glucose (44 g/L) to lactic acid within a short time frame (12 h) at high yield (0.39 g/g). However xylose fermentation with BI-*Pfldh* was inhibited severely, and thus only minor amount was converted to lactic acid within a time frame of 166 h. The additional pH decline as a result of lactic acid production resulted in lower substrate consumption rates as compared to the background strain.

Kurzfassung

Milchsäure hat insbesondere zunehmend an Bedeutung gewonnen, aufgrund des steigenden Bedarfes des biologisch abbaubaren Polymers, Polymilchsäure. Von dem ausgegangen wird, das es zukünftig erdölbasierende Polymere ersetzen könnte. Die Herstellung von Milchsäure erfolgt derzeit hauptsächlich durch mikrobieller Fermentation mittels Milchsäurebakterien. Da diese jedoch keine effiziente Produktion bei niedrigen pH Werten erlauben, ist eine Neutralisierung des Fermentationsprozesses erforderlich. Aufgrund der hohen Säuretoleranz hat der Hefestamm *S. cervisiae* großes Interesse als alternativer Produktionsstamm geweckt. Die Milchsäureproduktion könnte dadurch ohne Neutralisierung durchgeführt werden, was eine vereinfachtere Aufreinigung und somit eine Verringerung der Produktionskosten zur Folge hätte. Diese Arbeit beschäftigt sich mit der Entwicklung von gentechnisch veränderten Hefestämmen, die eine effiziente Umsetzung von Glukose als auch Xylose zu Milchsäure ermöglichen. Als Ausgangsstamm diente der Xylose vergärende Hefestamm *S. cervisiae* IBB10B05. Um die Ethanol Produktion zu unterdrücken, wurden vorerst die Gene codierend für Pyruvate Decarboxylase 1 (*pdc1*) und Alkohol Dehydrogenase 1 (a*dh1*) deletiert. Die Deletion von *adh1* führte jedoch zur vermehrten Bildung von Acetaldehyd, das sich letal auf das Wachstum auswirkte. Um die Milchsäureproduktion letztendlich zu ermöglichen wurden in der *pdc1* Mutante zwei Lactate Dehydrogenase Gene aus unterschiedlichen Organismen (*R. oryzae* und *P. falciparum*) durch die Verwendung eines 2µm multicopy Plasmids unter der Kontrolle eines starken konstitutiven Promoters (TEF1) exprimiert. Trotz der starken Expression konnte jedoch kaum Aktivität und Produktion an Milchsäure (maximal 3 %) nachgewiesen werden. Alternativ dazu wurde das Gen codierend für Lactate Deyhdrogenase von *P. falciparum* durch homologe Rekombination am *pdc1* Lokus integriert, um die Expression unter der Kontrolle des nativen *pdc1* Promoters zu steuern. Demzufolge konnte eine hohe Aktivität (1.24 U/mg) und Milchsäureproduktion durch fermentative Umsetzung aus Glukose mit einer Ausbeute von 0.39 g Milchsaure pro g Glukose detektiert werden. Daraus lässt sich schließen, dass die metabolische Regulation der Proteinexpression essentiell für eine erfolgreiche Milchsäureherstellung ist. Jedoch eine effiziente Umsetzung aus Xylose konnte nicht erzielt werden, da vermutlich der starke pH Abfall resultierend aus der Milchsäurebildung sich stark inhibierend auf die Substrataufnahme auswirkte.

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1 Introduction

1.1 Lactic acid and its applications

Lactic acid, chemically classified as 2-hydroxypropanoic acid (CH_3CHOH) is the most common occurring hydroxycarboxylic acid. Two optically active isomers are existing, L (+) and D (-)- lactic acid and they both represent metabolic intermediates occurring in many organisms, from prokaryotes to humans [1]. It is now considered to be one of the most useful platform chemicals with potential applications in food, cosmetic, pharmaceutical and chemical industries. Traditionally it is mainly used in food and food-related application, which in the USA accounted for approximately 85% of the demand. The rest of the uses are for specialized nonfood industrial application. Lactic acid has the GRAS status (generally recognized as safe) and consistently its major application in the food and food-related industry is e.g. as flavoring, pH regulator, antimicrobial and acidulant agent [2]. Moreover it is used as preservative in the cosmetic industry and as biodegradable polymer in medical applications especially for prostheses and drug release control. Based on its chiral character and the reactive functional groups (carboxylic- and hydroxyl group) lactic acid has potential for future high-end applications in the pharmaceutical and fine chemical industry. Thus, it could be used as building block for the synthesis of drugs, agro-chemicals and other added-value products such as propylene oxide, propanoic acid and acrylic acid [3,4]. Further application as platform for bulk products e.g. are polymers and solvents, are considered to be possible breaking new ground in large scale industries [3]. Most recently lactic acid has particularly called attention as a monomer for the production of the biodegradable plastic polylactic acid (PLA) [5] and the environmentally friendly solvent ethyl lactate [6]. The worldwide production of lactic acid is roughly 100.000 metric tons per year [7]. However lactic acid consumption is expected to expand 19 % per year especially due to the increased demand for PLA [8] which has the potential to replace petrochemical based polymers [9]. PLA is synthesized either by polycondensation of lactic acid or by ring-opening polymerization of the lactide. PLA can either be composed of solely one stereoisomer (homopolymer) or combination of L- and D- lactic acid in various ratios (heteropolymer). Additionally other co monomers e.g. trimethylene carbonate, 1.5-dioxepan-2-one, can be incorporated (copolymer). The ratio and the distribution of the monomers is largely affecting the thermo physical and mechanical properties. Thus it is crucial that enantiomeric pure lactic acid is available. In addition PLA is

biodegradable and biocompatible and during the last decade environmental issues have become of increasing importance. Thus PLA is well suited for many applications ranging from packaging to various medical products due to the possibility to tailor the product properties within a wide range [10].

1.2 Current state of industrial scale lactic acid production

On industrial scale, lactic acid is produced chemically from petrochemical feedstocks by synthesis or biologically from carbohydrates by microbial fermentation (Figure 1.1) [2,11]. The chemical synthesis involves the base catalyzed addition of hydrogen cyanide to acetaldehyde to form lactonitrile, which is then hydrolized to lactic acid catalyzed by sulfuric acid. After esterification with methanol to produce methyl lactate, it is recovered by distillation, subsequently hydrolized to lactic acid by water under acid catalysts and finally concentrated by purification. The biotechnological process includes the conversion of readily available sugars to lactic acid by fermentation. During the process calcium carbonate is added to neutralize the acid and keep the pH constant. Downstream processing includes the purification of calcium lactate from fermentation by filtration. The filtrate is then carbon treated, evaporated and acidified with sulfuric acid to convert the salt into lactic acid. The byproduct calcium sulfate is removed by filtration and the filtrate is further purified by carbon columns and ion exchange to get pure lactic acid [2]. The main advantage of the biotechnological over the chemical route is that enantiomeric pure (either L- or D-) lactic acid can be produced [2,11]. Because of the need of stereoisomeric pure lactic acid for most applications e.g. for PLA production (as described in 1.1) [10] manufactures are commonly based on microbial fermentation [3].

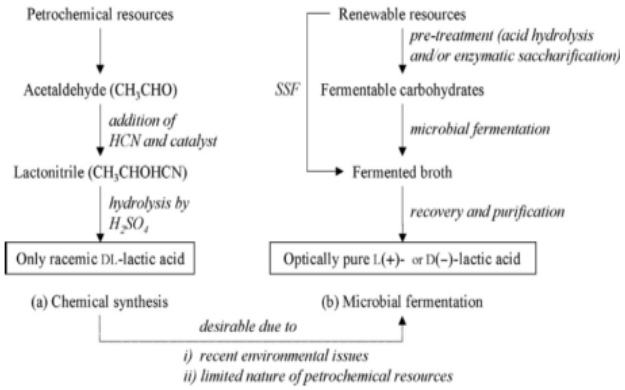

Figure 1.1: Overview of the production process for lactic acid [11]**.**

Chemical synthesis (a) and microbial fermentation (b). Simultaneous saccharification and fermentation (SSF).

Currently, bacteria e.g. lactic acid bacteria (LAB) or *E. coli* are the most widely used hosts for industrial scale production [12]. These strains are naturally capable of fermenting sugars to lactic acid via the Emden-Meyerhof Pathway. Thus, lactic acid is formed from pyruvate by simultaneous oxidation of NADH to NAD^+, catalyzed by the NAD-dependent lactate dehydrogenase (*ldh*, E.C.1.1.1.27). Two types of *ldh* are present in biological systems: L- and D- *ldh*, resulting either in the formation of L- or D- lactic acid [13]. A large number of *ldh* from different organisms (bacterial, fungal and mammalian) have already been well characterized [14–21], and nucleotide as well as amino acid sequences are readily available on protein and genbank databases. The availability of production strains that exhibit high productivities and product yields of enantiomeric pure lactic acid is a prerequisite for industrial scale production in terms of economical competitiveness [22]. However, most wildtype strains do not meet these requirements, particularly because by-products such as ethanol, acetate, format, succinate or glycerol are produced at the expense of lactic acid. Thus, lowering the yield of lactic acid which is theoretically 1 g per g glucose. Moreover many of these strains produce a racemic mixture of lactic acid because they encode both L- and D- *ldh* [12]. To overcome these bottlenecks, strain improvement – mostly through genetic engineering – has been accomplished [23]. Examples are the *L. lactis* strain constructed by Davidson et al [24] with increased yield of lactic acid as a result from an increase of *ldh* copy number or the *L. plantarum* strain that produces exclusively optically pure D (+)- lactic acid as a result of chromosomal deletion of the corresponding L-*ldh* gene [25]. Further strains are the recombinant *E. coli* [26] and *L. helveticus* [27] producing optically pure L- lactic acid. Another example is the recombinat *E. coli* strain utilizing hexose as well as pentose sugar as described by Dien et al [28]. In fact, engineered bacteria are currently capable of producing lactic acid in high purity and yields of more than 0.9 g per g glucose [11]. Even though advances towards improved production through metabolic engineering have been made, there are still some limitations in lactic acid fermentation by the use of bacteria. One major problem is the need of complex nutritional requirements due to their limited ability to synthesize their own growth factors such as B vitamins and amino acids, which add up to 35 % of the production cost [29]. In addition at low pH cell growth is inhibited, resulting in a significant decrease in volumetric productivity of lactic acid. The addition of bases or carbonates for maintenance of a neutral pH can lead to reduced product solubility and requires

complex downstream processing e.g. for regeneration of precipitated lactate salt or removal of gypsum as by-product [12]. Due to the increased demand and future potential of lactic acid there is a great interest to develop alternative host organisms to overcome these limitations in lactic acid production associated with bacteria.

1.3 Relevance of yeast in lactic acid production

A promising organism for lactic acid fermentation is the yeast strain *S. cervisiae*. Due to its high inhibitor tolerance, low nutrient requirement and high acid tolerance, it is since centuries a favored production organism for industrial scale applications. Based on its low pH optimum, lactic acid production utilizing *S. cerevisiae* could be accomplished without neutralization, significantly simplifying downstream processing and thus, reducing the production costs [12]. However, *S. cervisiae* predominately produces ethanol under anaerobic fermentations (Figure 1.2) and is naturally unable to ferment glucose to lactic acid. Therefore introduction of the pathway for NADH-dependent reduction of pyruvate to lactic acid by expression of heterologous *ldh* is necessary (shown as dashed square in Figure 1.2) [30].

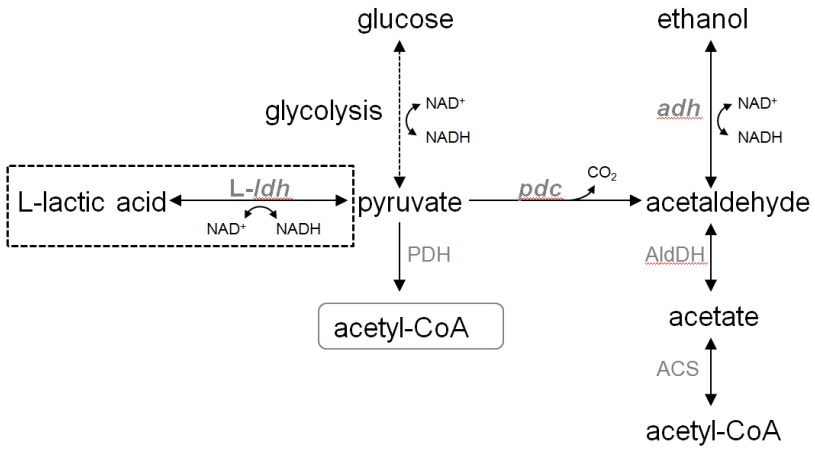

Figure 1.2: Pyruvate metabolism in *S. cervisiae* expressing heterologous L-*ldh*.

Pyruvate decarboxylase (*pdc*, EC 4.1.1.1), alcohol dehydrogenase (*adh*, EC 1.1.1.1), acetaldehyde dehydrogenase (AldDH), acetyl-CoA synthetase (ACS) and pyruvate dehydrogenase complex (PDH). Pyruvate is converted to acetaldehyde by *pdc* and ethanol is formed by reduction of acetaldehyde including simultaneous regeneration of cellular NAD^+, catalyzed ADH. Casually, pyruvate can be converted to acetyl-CoA (Ac-CoA) either by pyruvate dehydrogenase complex (PDH) located in the mitochondrial matrix or via an indirect route involving the enzymes *pdc*, AldDH and ACS [31]. The dashed square represents the reaction of introduced heterologous L-lactate dehydrogenase (L-*ldh*).

The first genetically engineered *S. cerevisiae* was reported by by Dequin et al [32]. This strain transformed up to 20 % of glucose into lactic acid as a result of expression of the bacterial L-(+)-*ldh* from *L. casei* from a multi copy plasmid. However, ethanol was still produced concurrently and has become a major problem in lactic acid fermentations ever since. Therefore several studies have focused on metabolic engineering approaches to increase lactic acid production by simultaneously suppressing ethanol formation. One approach is the deletion of pyruvate decarboxylase (*pdc*) or alcohol dehydrogenase (*adh*) genes. *S. cervisiae* encodes three structural genes for *pdc* (*pdc1*, *pdc5* and *pdc6*) [32,33] and five for *adh* (*adh1*, *adh2*, *adh3*, *adh4* and *adh5*) [35]. Though *pdc* and *adh* activity is mainly due to *pdc1* and *adh1* [32,34]. A single *adh1* mutant, that expresses fungal *ldh* from *R. orzyae* from *adh1* promoter on a multi copy plasmid was constructed by Skory et al [36]. However *adh1* deletion led to high accumulation of acetaldehyde, which is toxic for the cell and leads to significant reduction of growth and lactate yield on glucose medium. The single *pdc1* mutant combined with expression of bovine L- *ldh* from a multi copy plasmid reported by Adachi et al [37] improved lactate yield from 0.155 to 0.20 g/g. Inactivation of all three *pdc* genes might theoretically repress alcoholic fermentations completely. However, it was reported that such triple inactivation [33, 38] and even double inactivation of *pdc1* and *pdc5* [39,40] strongly impairs the growth on glucose medium. Thus the PHD complex is unable to provide sufficient amount of acetyl-CoA for growth (Figure 1.2), some *pdc* activity is necessary, since acetyl-CoA is needed as an building block for a number of biosynthetic processes including lipids [31]. However, so far the highest lactate yield of 0.815 g/g was achieved with a *S. cerevisiae* mutant strain, where *pdc1* and *pdc5* genes where each replaced by two copies of the bovine L-*ldh* gene. However the production rate was very low and it took 216 h to produce 80.7 g/l lactic acid [40].

It is considered that lactate production efficiency with *ldh* expressing *S. cervisiae* depends strongly on the source of the heterologous *ldh* gene and on the yeast strain background [41].

In order to realize a sustainable, economically and environmentally friendly process, the substrates for the lactic acid production must be cheap, readily available and renewable. In recent years lignocellulosic biomass form agricultural, municipal and forestry waste streams have called attention as sustainable and renewable source of carbohydrates. However lignocellulosic hydrolyzates contain both glucose and xylose

sugars as the main components. To our best knowledge, no example of a xylose-fermenting *S. cerevisiae* strain, enable to lactic acid production, has been described so far [30].

This study is the first example that deals with the development of a genetically engineered *S. cervisiae* strain that is able to ferment both glucose and xylose to lactic acid. As host background the xylose fermenting strain *S. cervisiae* IBB10B05, that is a descendant of the previously described *S. cerevisiae* strain BP10001 was used [42]. It was altered by laboratory evolution including continuous cultivation on xylose as sole carbon source under strictly anaerobic conditions. Strain selection was based on maximal growth and specific xylose uptake rates. To minimize ethanol formation it was attempted to disrupt the *pdc1* and *adh1* genes sequentially. It was considered that decreasing *adh* activity in the single Δ *pdc1* background would reduce the accumulation of acetaldehyde because metabolic flux from pyruvate is reduced through *pdc1* deletion. In order to facilitate lactic acid production, an eukaryotic and bacterial L-ldh gene, derived from *R. oryzae* and *P. falciparum* were expressed from the strong yeast TEF1 promoter on a 2µm multi copy plasmid. Additionally *ldh* from *P. falciparum* was expressed under the control of the native *pdc1* promoter by substitution of the coding region of *pdc1* with the *ldh* gene. The fermentation properties towards lactic acid production from both glucose and xylose among strains including the wildtype were compared.

2 Materials and Methods

2.1 Strains and Media

The xylose fermenting strain *S. cervisiae* IBB10B05, henceforth termed as wildtype B, was used as host strain for genetic engineering towards lactic acid production. As bacterial host for molecular subcloning *E. coli* BL 21 (Novagen Vienna, Austria) was used. Isolation of fungal lactate dehydrogenase (LDH) gene from genomic DNA was performed using *R. oryzae* NRRL 395 [20].

Media components were all derived from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). Yeast Peptone Dextrose (YPD) medium (10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose) was used for cultivation of *S. cervisiae*. The culture medium for *E. coli* was Luria-Bertani (LB) consisting of 10 g/L yeast extract, 16 g/L peptone and 5 g/L NaCl. *R. oryzae* was cultivated in yeast extract dextrose (YD) medium with 50 g/L glucose and 5 g/L yeast extract. YPD and LB agar additionally contained 15 g/L and 20 g/L agar, respectively. For selection of transformants and prevention of plasmid loss during cultivation agar and liquid media were supplemented with the appropriate antibiotics, which were filtrated sterile prior to use (115 µg/ml ampicillin (amp), 200 µg/ml geniticin sulphate (G418), 200 µg/ml hygromycin B (hph) and 100 µg/ml nourseothricin (nat)).

2.2 Plasmids, Genes and Primers

All plasmids used in this study are listed in Table 2.1. For detailed information, vector maps of the plasmids p427TEF, pUG75 and pUG74 can be found in the supplementary information (see 7.1). The recombinant vector pTXB3-*Pfldh* contained the lactate dehydrogenase gene from *Plasmodium falciparum* as fusion protein with an intein/chitin binding domain adjacent to the C-terminal end. This vector was kindly provided by David K. Wilson (Section of Molecular and Cell Biology, University of California, Davis, U.S.A.). The primers used for the genetic engineering are shown in Table 2.2, and were all purchased from Sigma Aldrich (St. Louis, USA). Two lactate dehydrogenase genes from different source organisms were used for expression in *S. cervisiae* (Table 2.3). The enzymes will be henceforth termed as *Roldh* (*Rhizopus oryzae* lactate dehydrogenase) and *Pfldh* (*Plasmodium falciparum* lactate dehydrogenase).

Table 2.1: Plasmids

Plasmid	Features	Source
p427TEF (Yeast 2micron expression plasmid)	TEF1 promotor; multiple cloning site; CYC1 terminator; ampicillin and kanamycine resistance gene; 2micron and pBluescript origin of replication	Dualsystems Biotech (Zurich, Switzerland)
pUG75 (Yeast deletion marker plasmid)	hygromycin B resistance cassette, which is flanked by loxP sites; ampicillin resistance gene and *E. coli* origin of replication	Euroscarf-EUROpean Saccharomyces Cervisiae ARchive for Functional Analysis (Institute for Molecular Bioscience, Johann Wo lfgang Goethe-University Frankfurt, Germany)
pUG74 (Yeast deletion marker plasmid)	nourseothricin resistance cassette, which is flanked by loxP sites; ampicillin resistance gene and *E. coli* origin of replication	Euroscarf-EUROpean Saccharomyces Cervisiae ARchive for Functional Analysis (Institute for Molecular Bioscience, Johann Wolfgang Goethe-University Frankfurt, Germany)
pTXB3-*Pfldh (E. coli* expression plasmid containing *ldh from P. falciparum* (Table 2.3))	IPTG inducible T7 promotor; lac operator; shine dalgarno sequence; multiple cloning site; C-terminal intein/chitin binding domain (27kDa) for purification; ampicillin resistance gene; *E. coli* origin of replication	Provided by David K. Wilson (Section of Molecular and Cell Biology, University of California, Davis, U.S.A.)

Table 2.2: Primers

Oligo # [A]	Sequence (5`- 3`)	N[B]	Tm [C]
A	TCTACTCATAACCTCACGCAAAATAACACAGTCAAAC AATCAAA*CTGAAGCTTCGTACGCTGC*	64	85.3
B	AAAAATGCTTATAAAACTTTAACTAATAATTAGAGATTA AATCGC*CGACTCACTATAGGGAGACCG*	66	77.9
C	TGAGATAAGCACACTGCACC	20	62.0
D	TCAGAAACTTCTCGACAGACG	21	62.7
E	TCTATCAGAGCTTGGTTGACG	21	62.4
F	CAATTGCAGAGTCGAATTCG	20	63.7
G	CAACAACTTAGCTGGGACG	19	61.4
H	ATATTTCAAGCTATACCAAGCATACAATCAACTATCTC ATATACA*CTGAAGCTTCGTACGCTGC*	64	80.8
I	TATAACTTATTTAATAATAAAAATCATAAATCATAAGAA ATTCGC*CGACTCACTATAGGGAGACCG*	66	77.1
J	CTCGTCATTGTTCTCGTTCC	20	62.4
K	TTGACGTTGGTGACCTCC	18	62.9
L	CGTGGTCGTCTCGTACTCC	19	63.9
M	CCTGAGAAAGCAACCTGACC	20	64.2
N	AACCTAGACCACCAGCAGC	19	62.4
O	ATAGGATCCATGGTATTACACTCAAAGGTCG	31	70.3
P	ATActcgagTCAACAGCTACTTTTAGAAAAGG	32	67.1
Q	ATAGGATCCATGGCACCAAAAGCAA	25	71.7
R	ATActcgagTTAAGCTAATGCCTTCATTCTC	32	67.8
S	TCTCTTTCGATGACCCTCC	19	62.3
T	TTTTCAGTATAATGTTACATGCG	23	58.7
U	TCTACTCATAACCTCACGCAAAATAACACAGTCAAAT CAATCAAA*ATGGCACCAAAAGCAA*	61	84.7
V	AAAAATGCTTATAAAACTTTAACTAATAATTAGAGAT TAAATCGC*CTCCTTGACAGTCTTGACG*	64	77.6
W	TGAGATAAGCACACTGCACC	20	62
X	TCCATTCTTTGTCACTCTTTCC	22	62.4

Oligo name used in this study (A), overall number of nucleotides (B), melting temperature of the primers determined with the online web tool (http://www.thermoscientificbio.com/fermentas/).

Table 2.3: Genes

Gene	Organism	Accession Nr. [A]	(N)[B]	Abr.[C]	Reference
ldh	*R. oryzae*	AF226154	963	*Roldh*	[20]
ldh	*P. falciparum*	M93720.1	951	*Pfldh*	see Table 2.1

Genbank accession number (A), number of nucleotides (B), abbreviation for enzymes used in this study (C).

2.3 Enzymes

The enzymes used for genetic work are shown in Table 2.4.

Table 2.4: Enzymes

Name	Source
Phusion™ Hot Start II High-Fidelity DNA Polymerase (2 U/µl)	Thermo Scienctific Inc., Wilmington, USA
Fast™ Thermosensitive Alkaline Phosphatase (1 U/µl)	
T4 DNA Ligase (1 U/µl)	Fermentas International Inc., Burlington, Canada
BamHI restriction enzyme (10 U/µl)	
XhoI restriction enzyme (10 U/µl)	
Lyticase from *Athrobacter luteus* (619 U/mg)	Sigma Aldrich, St. Louis, USA

2.4 Deletion of *pdc1* gene in *S. cervisiae*

The genomic pyruvate decarboxylase 1 gene (*pdc1*) on chromosome XII (genbank accession number NC_001144; gene ID: 850733) in the wildtype *S. cervisiae* B was deleted by replacing the open reading frame (ORF) by a linear deletion cassette consisting of the hygromycin B resistance gene, which further was used as screening system. An overview of the strategy is depicted in Figure 2.1. The cassette was synthesized by applying polymerase chain reaction (PCR) from the deletion marker plasmid pUG75, prior amplified in *E. coli*. As forward and reverse primer Oligo A and B were used (Table 2.2). To allow site specific target integration through homologous

recombination, both primers were designed to contain a 45 nucleotide sequence in length that were either homologous to the *pdc1* promotor upstream of the ATG startcodon (Oligo A, underlined in Table 2.2) or homologous to the *pdc1* terminator downstream of the TAA stop codon (Oligo B, underlined in Table 2.2). In addition Oligo A bound to the sequence region on pUG75 located 19 Bp upstream and Oligo B 54 Bp downstream of the loxP sites (shown in italics in Table 2.2). Deletion strains were verified by obtaining fragments of expected size from colony PCR screening, using pairs of oligonucleotide primers matching the sequence region within and up- or downstream of the integrated deletion cassette.

2.4.1 Amplification of pUG75 in *E. coli*

Electrocompetent cells were prepared according to the protocol described by Miller et al [43]. Electroporation was carried out using Micro PulserTM (Bio-Rad, Vienna, Austria) and 2 mm electroporation cuvettes (PEQLAB Biotechnologie GMBH, Erlangen, Germany) with 50 µl of electrocompetent cells and 1µl (100 ng/µl) of salt free plasmid DNA. After electroporation the cells were immediately transferred to 1 ml of pre-heated (37 °C) LB medium and incubated for 1 hour at 37 °C and 300 rpm using the Thermomixer Comfort (Eppendorf AG, Hamburg, Germany). Afterwards adequate amount of cells (50 µl, 100 µl and the rest of the onset, which was obtained as pellet after centrifugation for 30 sec and 13000 rpm) were plated on LB-agar plates supplemented with 115 µg/ml amp and incubated overnight at 37°C. To produce sufficient biomass single transformants were transferred to fresh LB-agar plates and again incubated overnight at 37 °C. Plasmids were isolated using Wizard® Plus SV Minipreps DNA Purification Kit (Promega Corporation, Madison, USA) according to the manual with the exception that cells from one colony were directly resuspended in the respective solution. The plasmid concentration was determined with a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, USA) and then stored at -70 °C.

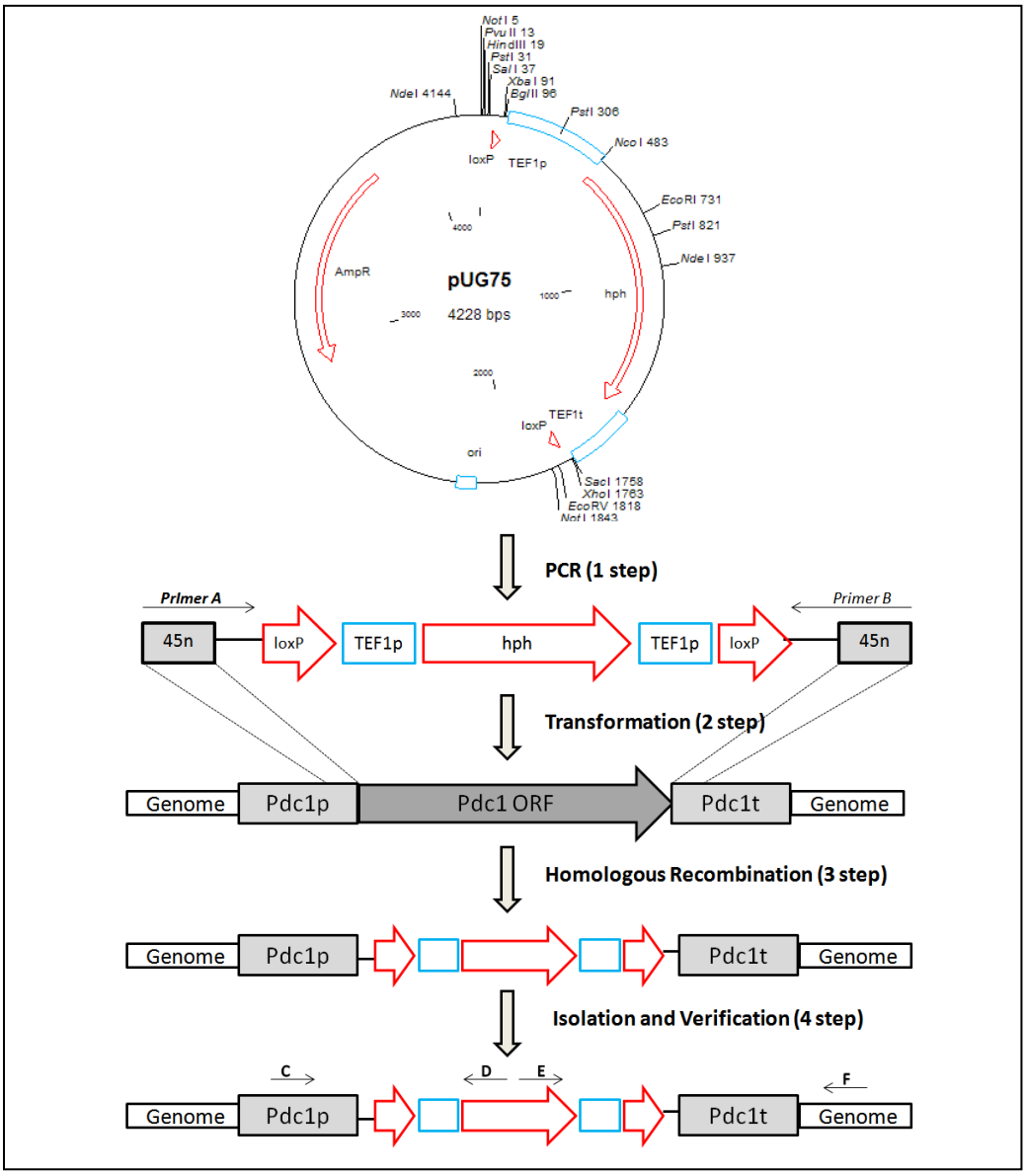

Figure 2.1: Deletion of *pdc1* in *S. cervisiae* wildtype B.

2.4.2 Synthesis of *pdc1* deletion cassette

The PCR mixture with a final volume of 50 µl was assembled as follows: 32.5 µl nuclease free water (NFW), 10 µl 5x Phusion HF Buffer (Thermo Scienctific Inc., Wilmington, USA) 1 µl dNTP´s (10 mM, Fermentas International Inc., Burlington, Canada), 2.5 µl forward and reverse primer (Oligo A and B, Table 2.2), respectively, 1 µl template plasmid pUG75 (10 ng) and 0.5 µl DNA Polymerase (2 U/µl). PCR was carried out with the Thermal Cycler iCycler™ (Bio-Rad Laboratories, Hercules, USA) using the following temperature profile: initial denaturation at 98 °C for 2 min; 2 cycles of denaturation at 98 °C for 10 sec, annealing at 64 °C for 30 sec and

extension at 72 °C for 40 sec; 28 cycles at 98 °C for 10 sec, 70 °C for 30 sec and 70 °C for 40 sec; and final extension at 72 °C for 7 min. After PCR, a linear deletion cassette 1954 Bp in length was expected, which is composed of the hygromycin B resistance cassette including loxP sites flanked by the 45 Bp homologous region (*pdc1* target region) (Figure 2.1, 1 step). To confirm the size of the fragments an aliquot of 2 µl was analyzed via agarose gel electrophoresis (AGE) with 1% (w/v) agarose gel in a Gel-Doc 2000 chamber (Biorad Laboratories, Hercules, USA). The running buffer was 1x TAE (40 mM Tris, 1 mM EDTA, 20 mM acetic acid, pH 8, from Carl Roth GmbH + Co. KG, Karlsruhe, Germany). The standard ladder was O`GeneRuler™ 1kb DNA Ladder (Fermentas International Inc., Burlingtion, Canada). For visualization the gel was treated with DNA Gel Red™ Nucleic Acid Gel Stain (Biotium Inc.,Hayward, California). Prior to transformation into *S. cervisiae* IBB10B05, the generated deletion cassette was purified with Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, Madison, USA) according to the protocol recommended by the manufacturer. DNA concentration after purification was determined using Nanodrop 2000 UV-Vis Spectrophotometer.

2.4.3 Transformation of deletion cassette into *S. cervisiae*

Transformation of deletion cassette was accomplished by the Lithium Acetate (LiAc) method described by Gietz et. al 1992 [44]. Yeast cells from an overnight culture were used to inoculate 50 ml pre-warmed YPD medium to an initial optical density at 600nm wavelength (OD_{600}) of ~ 0.2. The culture was incubated at 30 °C and 182 rpm until an OD_{600} of ~ 0.8 to 1. Optical density was determined with spectrophotometer DU800 (Beckman Coulter Inc., Fullerton, USA). Cells were harvested by centrifugation at 5000 rpm and room temperature for 5 min (Centrifuge 5810R, Eppendorf AG, Hamburg, Germany). The pellet was washed with 25 ml of sterile water and resuspended in 1 ml of 100 mM LiAc (Sigma Aldrich, St. Louis, USA) pH 8.4. Afterwards the cell suspension was transferred into a 1. 5 ml Eppendorf tube and centrifuged for 20 sec, 13000 rpm and room temperature (Centrifuge "Eppifuge" 5810R Eppendorf AG, Hamburg, Germany). The pellet was resuspended in 100 mM LiAc (pH 8.4) to a final volume of 0.5 ml by vortexing vigorously. The cells were spun down (20 sec at top speed) and 100 µl were mixed with the transformation mixture, which was assembled as follows: 240 µl polyethylene glycol (PEG) 4000 (Fermentas

International Inc., Burlington, Canada), 36µl 1 M LiAc (pH 8.4), 50 µl of SS carrier DNA (2 mg/ml, Sigma-Aldrich, St. Louis, USA), boiled for 10 min at 99 °C, and 1.5 µg DNA dissolved in sterile water. The mixture was carefully mixed by pipetting up and down and incubated at 30 °C, 350 rpm for 30 min followed by heat shock at 42 °C, 350 rpm for 25 min. Afterwards YPD medium was added to a final volume of 1.2 ml and incubated for 1 h, 350 rpm at 30 °C (recovery period). Cells were harvested by centrifugation (4 °C, maximum speed, 20 sec). The pellet was resuspended in 1 ml of sterile water and aliquots of 50 µl, 100 µl and the rest were plated on YPD agar plates containing the antibiotic hph (200 µg/ml). The plates were incubated at 30 °C for 2 days until colonies were clearly visible.

2.4.4 Verification of *pdc1* deletion strains

Homologous recombination leads to a replacement of the *pdc1* gene by the deletion cassette (Figure 2.1, step 2 and 3). Correct integration was verified by the appearance of PCR products of expected size using primers that span the left and right junctions of the deletion module within the genome (Figure 2.1, step 4). A few selected transformants were analyzed by colony PCR and AGE as described below. To attain the genomic DNA, cell disruption was performed as follows: one single colony was mixed with 20 µl lyticase solution (500 U/ml) and incubated at 37°C for 30 min. Afterwards the suspension was heated to 99°C for 10 min, The cell debris was removed by centrifugation (10 sec, maximum speed), and 5 uL of the supernatant was directly used as template for the PCR. PCR was performed with primer pairs C-D and E-F, respectively (Table 2.2, Figure 2.1, step 4). Oligo C and F were designed to anneal 927 Bp upstream and 403 Bp downstream of the start- and stop codon of the *pdc1* ORF. Oligo D and E annealed to the sequence region, which is located within the coding region of the *hph* selection marker. In addition PCR with primer pairs A-G was performed. Oligo G (not shown in Figure 2.1) was designed to be homologous to the *pdc1* ORF located 525 Bp downstream of ATG. If integration was successful, no fragment can be detected by AGE, further proving the replacement of the *pdc1* gene. PCR and AGE was carried out as described previously (see 2.4.2) except PCR temperature profile was as follows: initial denaturation at 98°C for 2 min; 30 cycles of denaturation at 98 °C for 10 sec, annealing at 62 °C for 30 sec and extension at 72 °C for 40 sec; and final extension at 72 °C for 7 min. The *pdc1*

deletion strain was stored in 60 % glycerol stocks at - 70 °C and henceforth is termed as B Δ*pdc1*.

2.5 Deletion of *adh1* gene in *S. cervisiae*

Deletion of genomic alcohol dehydrogenase 1 gene (*adh1*) on chromosome XV (genbank accession number NC_001147.6; gene ID: 854068) in B Δpdc1 strain was performed applying the same strategy described in 2.4. The deletion cassette was amplified from plasmid pUG74 (Table 2.1) using Oligo H and I as forward and reverse primer. The 45 nucleotide sequences of both primers (underlined in Table 2.2) were designed to be homologous to the *adh1* promotor and terminator sequence located up- and downstream of ATG and TAA, respectively. The PCR amplified deletion cassette containing the nourseothricin resistance gene including loxP sites flanked by the homologous regions was expected to consist of 1498 Bp in overall. Selection of transformants was carried out on YPD agar plates containing 100 µg/ml nourseothricin. As described above (see 2.4.4) verification of integration was accomplished by colony PCR and AGE with the primer pairs: J-K (J binds 74 Bp upstream of ATG), L-M (M binds 129 Bp downstream of TAA) and J-N (Table 2.2) giving product sizes of 1012 Bp, 806 Bp and 646 Bp, respectively.

2.6 Construction of *ldh* expression vectors

The coding region of *Roldh* and *Pfldh* (Table 2.3) were amplified by PCR designed to additionally introduce a BamHI and XhoI restriction site adjacent to the start- and stop codon. For amplification of *Roldh*, genomic DNA, isolated from *R. oryzae*, was used as template and Oligo O-P as primer pairs. *Pfldh* was obtained from the recombinant vector pTXB3 using primer pairs Q-R. The BamHI (underlined) and XhoI (lowercase) restriction sites of the primers, additionally contained an ATA overhang at the 5`prime end, are shown in Table 2.2). Amplicons were treated by double digestion with the respective restriction enzyme and cloned into the multiple cloning site (MCS) of the yeast expression vector p427TEF (Table 2.1). The latter was previously digested using the same restriction enzymes. Prior to transformation of the newly constructed vectors into the wildtype B and B Δ*pdc1* strains, respectively to express *ldh* proteins from the TEF1 promoter, the constructs, termed as p427TEF-*Roldh* and p427TEF-

Pfldh were sent to sequencing (LGC Genomics GmbH, Berlin, Germany) to confirm proper frame insertion and to exclude mutations. As sequencing primers Oligo S and T (Table 2.2) designed to bind to the TEF1 promoter and CYC1 terminator region located 145 Bp and 188 Bp up- and downstream of the start- and stop codon, were used. Transformation of the recombinant vectors were carried out as described previously (2.4.3) and strains henceforth termed as B-*Roldh*, B Δ*pdc1-Roldh*, B-*Pfldh* and B Δ*pdc1-Pfldh*.

2.6.1 Isolation of genomic DNA from *R. oryzae*

Fungal spores of *R. oryzae*, which were freshly prepared from mycelium grown on PEG agar plates, were used for inoculation of 50 ml YD medium and cultivated for 24 h at 30 °C and 200 rpm. After cultivation the mycelium was centrifuged for 10 min at room temperature and 4000 rpm. The supernatant was discarded and 100 mg of wet biomass was used for isolation of genomic DNA. Isolation was carried out using GeneJET Plant Genomic DNA Purification Kit (Fermentas International Inc., Burlington, Canada) according to the protocol of the manufacturer except disruption of the mycelia was performed as follows: 100 mg of wet biomass, 100 mg of ø 0.5 mm Soda Lime glass beads (Carl Roth GmbH + Co. KG, Karsruhe, Germany) and 350 µl Lysis Buffer A were mixed and vortexed for 10 sec. Afterwards the mixture was cooled down on ice for 10 sec. The procedure was repeated five times. Concentration of genomic DNA was determined with NanoDrop.

2.6.2 Amplification and double digestion of *ldh* sequences

Amplification of *ldh* sequences by PCR was performed as described previously (see 2.4.2), with an adapted temperature profile. For *Roldh* sequence temperature profile was chosen to be: 98 °C for 2 min; 30 cycles of 98 °C for 10 sec, 62 °C for 30 sec and 72 °C for 30 sec; and final extension at 72 °C for 7 min. The *Pfldh* sequence was amplified at 98 °C for 2 min; 2 cycles at 98 °C for 10 sec, 59 °C for 30 sec and 72 °C for 15 sec; 28 cycles at 98 °C for 10 sec, 67 °C for 30 sec and 72 °C for 15 sec; and final extension at 72 °C for 7 min. The size of PCR products (981 Bp; *Roldh*) and (969 Bp, *Pfldh*) was verified by AGE using as standard either MassRuler™ DNA Ladder Mix or O`GeneRuler™ 1kb DNA Ladder (Fermentas International Inc.,

Burlingtion, Canada). Prior to double digestion with the restriction enzymes BamHI and XhoI the fragments were purified and DNA concentration was determined with Nanodrop. The restriction mixture with a total volume of 20 µl contained 4 µl 10 x restriction buffer 2 x Tango™ (Fermentas International Inc., Burlington, Canada), 1 µl BamHI (10 U/µl), 0.5 µl XhoI (10 U/µl) and 1 µg purified DNA dissolved in 14.5 µl NFW. After incubation overnight at 37 °C and 300 rpm the reaction was stopped by heat at 80 °C for 20 min. The total amount of restriction onset was then separated by size utilizing AGE. The target bands were cut out and the fragments purified with PCR Clean-Up System. Finally DNA concentration was determined using NanoDrop.

2.6.3 Cloning of the recombinant vector

Double digestion of p427TEF vector with the restriction enzymes BamHI and XhoI, followed by AGE and purification steps were carried out with the same protocol as described previously (see 2.6.2). The linearized fragment exhibiting a size of 6650 Bp was expected. Prior to ligation the fragment was dephosphorylated with Fast Alkaline Phosphatase (Fast AP) to avoid self-ligation. The reaction mixture with a total volume of 20 µl was chosen as follows: 2 µl Fast AP buffer (Thermo Scienctific Inc., Wilmington, USA), 1 µl Fast AP (1 U/µl), 1µg DNA dissolved in 17 µl NFW. Incubation was carried out at 37 °C for 10 min and enzymes were inactivated by heat at 75 °C for 5 min. The purified dephosphorylated vector was then used for ligation with the purified restricted *ldh* sequences (see 2.6.2). The total volume of the ligation mixture was 20 µl, made up of 2 µl 10 x T4 DNA Ligase buffer (Fermentas International Inc., Burlington, Canada), 1 µl T4 DNA Ligase (1U/µl) and 158 ng restricted fragment and 106 ng linearized vector DNA dissolved in NFW. Incubation was at 22 °C for 2 h and enzyme inactivation at 65 °C for 10 min. The mixture was desalted using 0.025 µm MF™ Membrane Filter (Millipore, Billerica, USA) and 5 µl thereof were transformed into electrocompetent *E. coli* cells. Transformation and purification of the plasmids was accomplished as described earlier (see 2.4.1).

2.7 Integration of *Pfldh* at *pdc1* gene locus

The coding region of *Pfldh* was genomically integrated at *pdc1* gene locus into *S. cervisiae* B Δ*pdc1* strain as described previously (see 2.4). The sequence was

amplified by PCR from p427TEF-*Pfldh* vector (see 2.6) using as primer pair Oligo U-V (Table 2.2). Oligo U was designed to bind to the 5`end of the ORF of *Pfldh* including ATG (shown in italics) and Oligo V to the sequence region located upstream of the ORF of the antibiotic gene G418 (shown in italics). In addition primers contained the 45 nucleotide sequence homologous to the *pdc1* promoter or terminator region (underlined). The PCR temperature profile was as follows: initial denaturation at 98 °C for 2 min; 2 cycles of denaturation at 98 °C for 10 sec, annealing at 59 °C for 30 sec and extension at 72 °C for 60 sec; 28 cycles at 98 °C for 10 sec, 70 °C for 30 sec and 72 °C for 60 sec; and final extension at 72 °C for 7 min. After PCR a product consisting of the ORF of *Pfldh*, CYC1 terminator and the ORF of G418 flanked by the homologous sequences was expected to show a size of 3454 Bp. Primary selection of transformants was done on YPD agar plates containing the antibiotic G418 (200 µg/ml). After incubation of plates at 30 °C for 2 days, a few colonies were picked and streaked out again on YPD agar containing hph (200 µg/ml). Cells grown on G418 but not on hph were analyzed for site specific target integration by colony PCR using primer pair W-X (Table 2.2) that were designed to bind to the *pdc1* promoter region located 630 Bp upstream and to the coding region of *Pfldh* located 259 Bp downstream of the start codon, respectively. The PCR fragment was expected to consist of 930 Bp and the strain is henceforth termed as BI-*Pfldh*.

2.8 Measurement of *Pfldh* activity in *E. coli* cell- free extract

To ensure homogeneous biomass, *E. coli* cells were cultivated in 50 mL LB medium at 37 °C and 130 rpm (Certomat® BS 1 incubator from Sartorius) overnight. This preculture was then used for inoculation of the main culture containing 250 ml LB medium to an initial OD_{600} of 0.1. The cells were incubated until an OD_{600} of 0.8 was reached. After induction of protein expression with 0.1 mM isopropyl β-D-thiogalactopyranosid (IPTG, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) cells were further incubated for 3 h under same conditions. Cells were harvested by centrifugation for 30 min, at 5000 rpm and 4 °C. The cell pellet was resuspended in 5 ml 0.1 M sodium phosphate buffer, pH 7.5 ($NaH_2PO_4H_2O$ and Na_2HPO_4, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) supplemented with protease inhibitor cocktail tablets (SPIC buffer) (Roche Diagnostics, Mannheim, Germany). Afterwards cell

disruption was performed using the French Pressure Cell Press (Travenol Laboratories Inc., Deefield, USA) operated at a pressure of 1500 psi. Cell debris was separated by centrifugation for 30 min at 5000 rpm and 4 °C and the supernatant immediately used for *ldh* activity measurements and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) analysis. *Ldh* activity was measured spectrophotometrically by measuring first order change in absorbance at 340 nm, resulting from oxidation of NADH to NAD+. Activity measurement was performed at 25 °C and pH 7.5 in SPIC buffer using Beckman DU-800 spectrophotometer equipped with a temperature control. The protein concentration was adjusted to ensure that change in absorbance followed first order kinetics for at least 3 min. The reaction volume was in overall 500 µl and contained an initial concentration of 10 mM pyruvate (Sigma Aldrich, St. Louis, USA) and 0.1 mM NADH (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). The reaction was started with 50 µl of diluted crude cell extract. The molar extinction coefficient of NADH was assumed to be 6220 M^{-1} cm^{-1} [45]. Enzyme activity was calculated in Units (U) pro mg protein of crude cell extract. One Unit of enzyme activity is defined as conversion of 1 µmol of NADH to NAD+ per minute. Protein concentration in the crude cell extract was determined with Bradford assay [46]. Bovine serum albumin (BSA, Sigma Aldrich, St. Louis, USA) with a concentration of 0.1 to 1 mg/ml was used for standard calibration. Measurement was carried out by mixing 20 µl of crude cell extract with 1 ml of Roti®-Quant solution (Carl Roth GmbH, Karsruhe, Germany). After an incubation time of 10 min at room temperature absorbance at 595 nm was measured. SDS PAGE analysis of crude cell extract was obtained by separation of 1 µg protein samples either from supernatant or pellet. NuPAGE® Novex Bis-Tris Mini Gel (Novex, country) and as SDS running buffer 1x MOPS were used. Sample preparation and running condition were applied according to the protocol available at the website of invitrogen (www.invitrogen.com/manuals, accessed at December 2012). Protein bands were visualized using silver staining, which was carried out as follows. Gel was at first soaked for 20 min in fixation (10 % acetic acid, 40% ethanol) and sensitizing solution (0.2 % natriumthiosulfate, 0.5 M natrium acetate, 30 % ethanol), respectively. It was washed four times with H_2O for 5 min, each, and incubated for 15 min in silver solution (0.2 % silver nitrate). The gel was again washed for 1 min with H_2O and finally kept in developing (3 % sodium carbonate, 0.01 % formaldehyd) and stopping solution (1.5 % EDTA) for 10 min, respectively.

2.9 Measurement of *Roldh* and *Pfldh* activity *in S. cervisiae* cell-free extract

S. cervisiae strains were aerobically cultivated overnight in 50 ml preculture (YPD medium) at 30 °C and 182 rpm (Incubator-Infors RS-T, Infors AG, Bottmingen, Swizerland). The main culture, incubated at the same conditions as preculture, was inoculated to an initial OD_{600} of 0.5. Cells grown to an OD_{600} of 4 and 8 were harvested by centrifugation (4 °C, 5000 rpm for 5 min). The cell pellet was weight out and dissolved in two times its weight of buffer used for activity measurement (as described below). Additionally, the same weight of glass beads (ø 0.5 mm Soda Lime, Carl Roth GmbH + Co.KG, Karlsruhe, Germany) was added. Cell disruption was accomplished by vortexing the mixture for 45 sec and keeping it on ice for another 45 sec. This procedure was repeated 10 times. Cell debris was separated by centrifugation for 5 min at 5000 rpm and 4 °C and the supernatant immediately used for *ldh* activity measurement and SDS PAGE analysis (as described in 2.8).

Activity of *Roldh* was measured at 30 °C and pH 7.2 in 0.1 M SPIC buffer (see 2.8) and alternatively in the same buffer containing additionally 10 % glycerol and 1 mM dithiothreitol, 4 mM pyruvate and 0.256 mM NADH. *Pfldh* activity was measured with 1 mM instead of 10 mM pyruvate. In addition the SDS gel, obtained after separation of proteins via SDS PAGE was stained with PageBlue™ Protein Staining solution following the fast staining protocol from manufacturer (Fermentas International Inc., Burlington, Canada).

2.10 Fermentations

To ensure homogenous cell cultures, pre- and main cultures were accomplished.

Precultures (as described in 2.8) were used for inoculation of the main culture (250 ml YPD) with an starting OD_{600} of 0.05 and incubated aerobically at 30 °C and 182 rpm overnight. Cells were harvested in the exponential phase (OD_{600} was ~ 4) by centrifugation (4 °C, 5000 rpm for 20 min) with Sorvall® RC-5B Refrigerated Superspeed Centrifuge (Thermo Fisher Scientific, Waltham, USA). The cell pellet was washed twice with physiological 0.9 % sodium chloride solution (NaCl),finally resuspended therein and directly used as inoculum for the fermentation process.

Fermentation experiments were carried out anaerobically in glass bottles (90 ml working volume) tightly sealed with rubber septa at 30 °C and 182 rpm (Certomat

BS-1, Sartorius mechatronics, Germany). The fermentation set up was chosen to be 60 % (v/v) yeast extract-peptone, 30 % (v/v) carbohydrate solution using either glucose or xylose and 10 % (v/v) for inoculation. The fermentation media consisted of 20 g/L yeast extract, 10 g/L peptone, 50 g/L glucose or xylose. The initial OD_{600} was 5 (corresponds to 1.85 g/L) for conversion experiments and 0.1 (corresponds to 0.037 g/L) for growth analysis (see 2.11). The initial pH of the fermentation media was chosen to be 6.5, adjusted with 1 M NaOH (Carl Roth GmbH + Co.KG, Karsruhe, Germany). To ensure anaerobic conditions, the flasks were purged with molecular nitrogen (N_2) for approximately 15 min prior to and for 5 min after inoculation. During the fermentation process samples of 1.5 ml were taken periodically from flasks through rubber septa using sterile syringe. Samples were immediately processed by centrifugation for 10 min at 13000 rpm and 4°C with Eppendorf Centrifuge 5415R (Eppendorf AG, Hamburg, Germany) and the supernatant stored at -20 °C for further analysis.

2.11 HPLC measurement and data processing

Substrates (glucose, xylose) and extracellular metabolites (ethanol, acetate, glycerol, lactic acid and xylitol) were quantitatively analyzed by high performance liquid chromatography (HPLC) using LaChrom HPLC System (Merck-Hitachi) equipped with an L-7400 UV detector, an L-7490 RI detector and a thermo-stated column oven (Jones Chromatography, Hengoed, U.K.). As column for separation Aminex® HPX-87H, 300 ×7.8 mm (Biorad, Hercules, USA) was used. Prior to analysis HPLC was optimized by varying operation conditions (temperature, flow rate and eluent). Samples of the chromatograms can be found in the supplementary information (see 7.3) The system was operated at 50 °C with a flow rate of 0.6 ml/min using 5 mM sulphuric acid (H_2SO_4, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) as eluent. Lactic acid and glycerol showed the same retention time in the RI. Since lactic acid could be detected in the UV detector, concentration of glycerol was calculated by abstraction of the amount detected in RI from that in UV. Prior to measurement samples were diluted 1:1 in sterile water. As standards, compounds in the following concentration range were used: glucose, xylose and lactic acid (27 - 0.27 g/L), ethanol (14 - 0.14 g/L), xylitol (10 - 0.1 g/L), acetate (5 - 0.05 g/L) and glycerol applied as external standard (5 - 0.05 g/L). The yield coefficients were defined as g

metabolite produced per g substrate consumed. The C- recovery was calculated by taking all measured compounds including biomass and CO_2, into account. Biomass (g/L cell dry weight, CDW) was calculated from OD_{600} measurements based on the ratio of cell dry weight to OD_{600} of a value 0.37 and the amount of carbon transformed into biomass of a value 26.4 g/C mole biomass. Furthermore it was assumed that 1 mole CO_2 was formed from 1 mole of ethanol and acetate. The specific growth rates (µmax) of the strains denoted per hour were determined from the slope of the linear phase by plotting natural logarithm of OD_{600} over time. The glucose uptake rates (g glucose consumed per hour) were determined by plotting concentration of substrates consumed against reaction time. The data were fit using the software program SIGMAPLOT™ with the two parameter function $y=y_0 +at +bt^2$. The letters used in the equation representing substrate uptake in g/L (y), time in h (t) and the values of the calculated parameters (a and b). The first derivatives was then used to calculate substrate uptake rate relating to g substrate ultilized per hour and per g CDW at the time of withdrawal of samples.

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3 Results and Discussion

3.1 Strain construction

3.1.1 Deletion of *pdc1* and *adh1* genes

Prior to introduction of the heterologous *ldh* genes into the *S. cervisiae* wildtype B strain, the coding region of pyruvate decarboxylase 1 gene (Δ*pdc1*) was deleted in order to minimize ethanol formation. Deletion was accomplished by introducing the hph resistance gene at *pdc1* locus. Site specific replacement was verified by obtaining the expected fragment sizes of ~ 1167 (lane 1) and ~ 994 Bp (lane 2) from colony PCR with primer pair C-D and E-F (Figure 3.1). Since no fragments were detected in the control strain (lane 4, 5), it can be assumed that *pdc1* deletion was successful. As depicted in Fig 3.1. the upper bands of the marker were not separated accurately. An example of it including complete sizes of fragments can be found in the supplementary information (see 7.1).

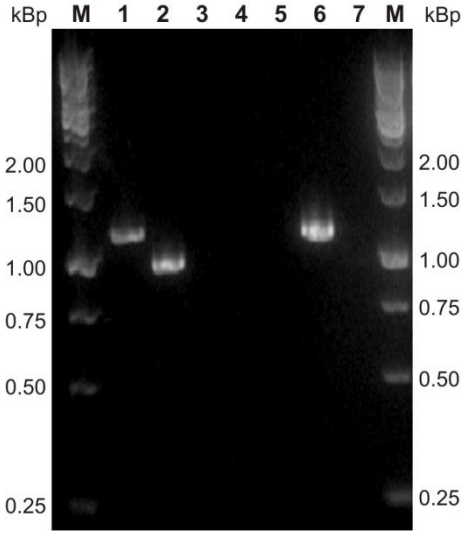

Figure 3.1: Verification of Δ*pdc1* deletion.
O`GeneRuler™ 1 kb DNA Ladder (M) including marker sizes (kBp). Obtained fragments from PCR screening of B Δ*pdc1* strain using primer pair C-D (1), E-F (2), A-G (3) and those from the wildtype B with primer pair C-D (4), E-F (5) and A-G (6).

Further, we attempted to delete alcohol dehydrogenase 1 gene (Δ*adh1*) in the B Δ*pdc1* mutant following the same strategy as described above. Apart from background growth, only few single clones grew within three days of incubation, which did not show site specific integration. Most likely these transformants integrated the resistance gene into the genome unspecifically, otherwise strains would not been able to grow on the selective media. We suppose that further growth of the potentially positive background colonies was prevented because acetaldehyde accumulated to toxic levels as a result of *adh1* deletion as observed by Skory et al [36]. In the study of Ishida et al. [47], disruption of the *adh1* gene in a Δ*pdc1* mutant

was performed after the *ldh* (bovine) was introduced into the genome. This double mutant strain was reported to be capable of growth on glucose, leading to the assumption that the *ldh* gene prevents channels most of the pyruvate towards lactic acid production, preventing severe accumulation of acetaldehyde. However, growth rates were still reduced by 50%.

3.1.2 Recombinant *ldh* expressing strains

In order to facilitate lactic acid production five different yeast strains based on either the wildtype B or B Δ*pdc1* strain have been developed (Table 3.1). Strains (B-*Roldh*, B-*Pfldh*, B Δ*pdc1-Roldh* and B Δ*pdc1-Pfldh*) were transformed with the recombinant plasmids p427TEF-*Roldh* or p427TEF-*Pfldh* to express the heterologous *ldh* gene from *R. oryzae* (*Roldh*) or *P. falciparum* (*Pfldh*) from the TEF1 promoter on the 2µm multi copy plasmid. Substitution of the coding region of *pdc1* by the *Pfldh* gene allowed expression of lactate dehydrogenase from *P. falciparum* under the control of the native *pdc1* promoter (BI-*Pfldh* strain). Site specific substitution was confirmed by sustaining the fragment of the expected size of ~ 930 Bp (lane 1) from colony PCR screening with primer pair W-X (Figure **3**.**2**). Apart from yeasts, *E. coli* was transformed with the recombinant vector pTXB3-*Pfldh* (Ec-*Pfldh strain,* not shown in Table 3.1) to express *Pfldh* from the inducible tac promoter. But this strain was solely used for comparison of activity with that obtained from the corresponding *Pfldh* expressing yeast strains.

Table 3.1: S. cervisiae strains constructed in this study.

S. cervisiae	**Genotype**	**Plasmid (p) / Integration (i)**	***Ldh* gene**[A]
B-*Roldh*	wildtype	p427TEF-*Roldh* (p)	*R. oryzae*
B-*Pfldh*	wildtype	p427TEF-*Pfldh* (p)	*P. falciparum*
B Δ*pdc1-Roldh*	Δ*pdc1*	p427TEF-*Roldh* (p)	*R. oryzae*
B Δ*pdc1-Pfldh*	Δ*pdc1*	p427TEF-*Pfldh* (p)	*P. falciparum*
BI-*Pfldh*	Δ*pdc1*	*Pfldh* (i)	*P. falciparum*

The gene sequence of l*dh* from *R. oryzae* and *P. falciparum* can be withdrawn from genbank accession number AF226154 and M93720.1 (A).

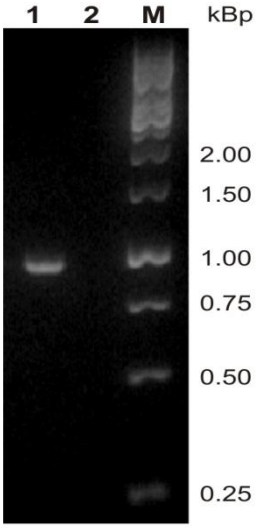

Figure 3.2**: Verification of site specific integration of *Pfldh* gene.**

O`GeneRuler™ 1 kb DNA Ladder (M) including marker sizes (kBp). Obtained fragments from PCR screening of BI-*Pfldh* (1) and those for the control strain wildtype B (2) using primer pair W-X.

3.2 Activity measurement of *ldh*

The specific activity of *ldh* was determined as Units per mg of crude cell extract (Table 3.2). The values are presented as net activity of *ldh*, and are calculated by abstracting the simultaneously measured wildtype activity. An example of the activity measurement can be found in the supplementary information (see 7.2). Independent from the time point of cell harvesting (OD600 of either 4 or 8), the activity of *Roldh* in B-*Roldh* strain in SPIC buffer was determined to be 0.1 U/mg. The activity increased two fold (0.2 U/mg) by the addition of the protein stabilizing agents (10 % glycerol, 1 mM DDT), but that was still 7.5 times lower than that obtained by Skory et al (1.5 U/mg) [36]. In contrast to our study, *Roldh* was expressed under regulation of a strong constitutive promoter, gene expression in the study of Skory et al, was performed with the native *adh1* promoter. The latter allows regulation of gene expression at a level that is accustomed to the precedent pathway that is glycolysis. Further, despite overexpression with the TEF1 promoter no protein band (each subunit 36 kDa [48]) was detected on SDS gel (Figure 3.3). Thus, we assume that elevated level of protein expression led to premature protein degradation induced by cell stress, and thus only low levels of activity could be detected. Consistently, we found that single copy integration of *Pfldh* at the *pdc1* locus led to a significantly increased *ldh* activity as compared to the *Pfldh* under regulation of the strong TEF1 promoter. Expression of *Pfldh* from the multi copy plasmid resulted in an activity of 0.15 U/mg (B-*Pfldh) and* 0.17 U/mg (B ∆*pdc1-Pfldh*). In both strains *Pfldh* (33 kDa [49]) was not detected by SDS PAGE (Figure 3.4). The activity (1.24 U/mg) was

seven fold higher by expression of *Pfldh* from the native *pdc1* promoter (BI-*Pfldh*) than compared to that from the multi copy plasmid.

No significant activity of *Pfldh* in Ec-*Pfldh* was detected (0.01 U/mg), even though the enzyme was expressed from the multi copy plasmid pTXB3, which was already optimized for expression in *E. coli*. Therefore protein samples from both the supernatant and the pellet were analyzed by SDS PAGE (Figure 3.5). The clear protein band of *Pfldh* (60 kDa) from the pellet (lane 4) implies that the protein precipitated in the form of inclusion bodies and, thus explaining the low activity obtained. To improve activity by avoiding formation of inclusion bodies, it will be necessary to reduce expression speed of *Pfldh*, which can be achieved by lowering the expression temperature.

Table 3.2: Specific activity of ldh from the various recombinant strains.

Strain	**Specific activity +/- SD[A] [U/mg]**
B-*Roldh*	[B]0.10 +/- 0.01, [C]0.10 +/- 0.01, [D]0.20 +/- 0.01
Ec-*Pfldh*	0.01 +/-0.00
B-*Pfldh*	0.15 +/-0.00
B Δ*pdc1-Pfldh*	0.17 +/-0.01
BI-*Pfldh*	1.24 +/-0.00

The specific activity and standard deviation (SD) from two independent experiments, respectively (A). Activity of *ldh* from *R. oryzae* were determined in SPIC buffer by harvesting cell at an optical density at 600nm (OD_{600}) of ~ 4 (B) and ~ 8 (C) and alternatively in SPIC supplemented with 10 % (v/v) glycerol and 1 mM DDT harvested at OD_{600} of ~ 4 (D)

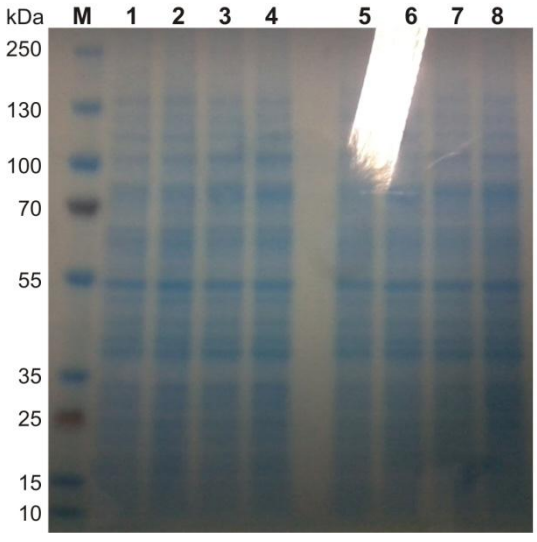

Figure 3.3: SDS PAGE analysis of *Roldh* expressed in B-*Roldh* strain.

PAGE Ruler™ Plus Prest. Prot. Ladder (M) including marker sizes (kDa). Proteins obtained from supernatant of crude cell extract of the wildtype strain from cells harvested at OD_{600} of 4 (1, 2) and 8 (5, 6) and those for B-*Roldh* strain at OD_{600} of 4 (3,4) and 8 (7,8).

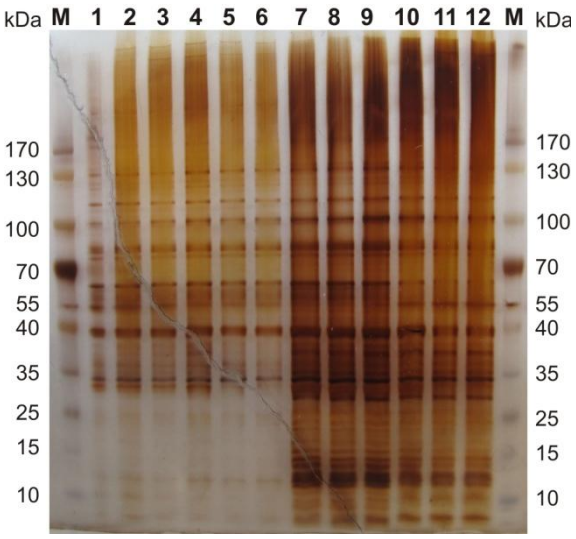

Figure 3.4: SDS PAGE analysis of *Pfldh* expressed in B-*Pfldh* and B Δ*pdc1-Pfldh* strain.

PAGE Ruler™ Prest. Prot. Ladder (M) including marker sizes (kDa). Proteins obtained from supernatant and pellet of the wildtype B (1,2 and 7,8), of B-*Pfldh* (3,4 and 9,10) and B Δ*pdc1-Pfldh* (5,6 and 11, 12).

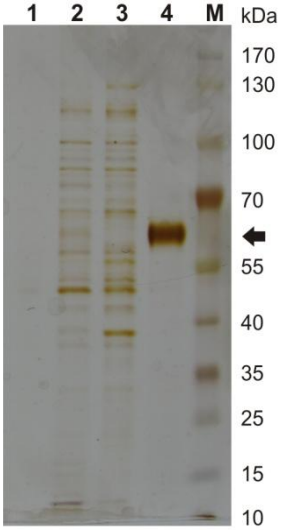

Figure 3.5: SDS PAGE analysis of *Pfldh* expressed in Ec-*Pfldh* strain.

PAGE Ruler™ Prest. Prot. Ladder (M) including marker sizes (kDa). Proteins obtained from supernatant (1) and pellet (3) from crude cell extract of the wildtype and those for Ec-*Pfldh* obtained from supernatant (2) and pellet (4).

3.3 Physiological characterization of strains

The specific growth rate (μmax) under anaerobic conditions was determined for the wildtype B, B-*Roldh* and B Δ*pdc1-Roldh* strain on both, glucose and xylose medium (Table 3.3). On glucose the wildtype B showed a two times higher μmax than B-*Roldh* (0.260 versus 0.132 h^{-1}). Deletion of *pdc1* (B Δ*pdc1-Rold*) leads to a further decrease from 0.132 to 0.103 h^{-1}. In xylose fermentations, differences between the three strains were not as significant. The growth rate for B-*Roldh* and B Δ*pdc1-Roldh* strain was 10 and 24 % lower than compared to that of the wildtype B.

Table 3.3: Growth rates for wildtype and Roldh expressing strains on glucose and xylose.

Strain	μmax [h^{-1}]	
	Glucose[A]	Xylose[B]
wildtype B	0.260	0.034
B-*Roldh*	0.132	0.031
B Δ*pdc1-Roldh*	0.103	0.026

The specific growth rates were determined from the linear phase by plotting natural logarithm of OD_{600} over time from 1 to 9 h after inoculation (A) and from 1 to 20 h (B).

Yield coefficients for glucose and xylose fermentation are depicted in Table 3.4 and Table 3.5, respectively, and are consistent due to reasonably closed carbon balances. The course of fermentation as well as the pH change over time are depicted in Figure 3.6 and Figure 3.7 for fermentation with the wildtype B and BI-*Pfldh* strain.

The strains (B-*Roldh*, B-*Pfldh*, B Δ*pdc1-Roldh* and B Δ*pdc1-Pfldh*) that expressed *Roldh* and *Pfldh* from the multi copy plasmid showed no significant difference in fermentation characteristics (supplementary information, see 7.2) as compared to the wildtype B. Except that little amounts of lactic acid (maximal 3 %) were detected after complete depletion of both substrates (~ 50 g/L, respectively), the yields within limits of experimental errors were the same. The wildtype B fermented 53.6 g/L of glucose to the corresponding metabolites ending up with a final pH of 4.9 after a reaction time of 10 h. On the contrary the BI-*Pfldh* strain produced concurrently relative high amounts of lactic acid with a yield of 0.39 g/g from glucose. In comparison to the wildtype, the yield of ethanol decreased from 0.37 to 0.22 g/g (~ 40 %) and that for glycerol from 0.04 to 0.01 g/g. In contrast the yield of acetate increased from 0.1 to 0.3 g/g. The initial concentration of glucose was 43.8 g/L and 77 % thereof (33.9 g/L) were consumed and being transformed into lactic acid (13.1 g/L), ethanol (7.5 g/L), acetate (0.8 g/L) and glycerin (0.3 g/L) after a reaction time of 12 h. Alongside with lactic acid formation, the pH dropped from 5.9 to 3.1. In comparison to the wildtype, it might be assessed that the BI-*Pfldh* strain utilized glucose less efficiently because an 1.5 times lower concentration of glucose was utilized. To investigate this in more detail, we determined for both strains the specific uptake rate normalized on the

actual amount of cell dry weight at defined points of time of fermentation (Table 3.6). The corresponding plots can be found in the supplementary information (see 7.2). The data show that $q_{glucose}$ in both strains decreased over time, while a faster decrease was observed for the BI-*Pfldh* strain. Moreover we asserted that there is a clear correlation between $q_{glucose}$ and the actual pH present. Thus we assume that the substrate uptake rate depends not only on the actual amount of substrate [50], but also strongly on the actual pH present. Skory et al [36] observed as well that the production rate of lactic acid decreases by lowering the pH. The comparison of lactic acid yield with other recombinant *S. cervisiae* strains that have been developed so far is shown in Table 3.7. In comparison to studies were *ldh* was expressed from a multi copy plasmid, the BI-*Pfldh* strain in this study showed with 0.39 g/g a higher or similar (0.445 g/g) [36] lactic acid yield. Higher yields were only reported when *ldh* gene was integrated into the genome multiple times. Thus, Ishida et al [40, 47, 51] reported the so far highest yields of 0.647, 0.690 and 0.815 g/g. In addition it was shown that the recombinant strain with two genome integrated copies of *ldh* showed higher yield of lactic acid than that containing only one copy (data not shown). However, glucose conversion rates as well as growth rates of the double mutated *pdc1*/*pdc5* and *pdc1*/*adh1* strains were significantly lower as compared to BI-*Pfldh*, where it was possible to convert 44 g/L of glucose to lactic acid within a short time frame (12 h). Similar to a decrease in glucose fermentation, xylose fermentation by BI-*Pfldh* also was significantly slower as compared to the wildtype. The data obtained were not sufficient to determine useful yields from xylose and are therefore not shown in Table 3.5. Only small amounts of xylose (8 g/L) were transformed into lactic acid (2.27 g/L) with 166 h of fermentation. Whereas the wildtype strain utilized 6 times higher concentration of xylose (46. 6 g/L) within 99 h (Figure 3.6). Even though less xylose was consumed by the *BI-Pfldh strain*, the final pH was significantly lower than compared to that of the wildtype (pH of 4 versus pH of 4.6). This might indicate that the lower pH resultant from lactic acid production strongly impaired the xylose consumption rate. However this limitation in lactic acid production from xylose by the use of BI-*Pfldh* strain might be overcome through neutralization of the fermentation process.

Table 3.4: Physiological parameters for strains from batch fermentations on glucose (~ 50 g/L) medium.

Yield [g/g]	wildtype B[A]	B-*Roldh*[A]	B Δ*pdc1*-*Roldh*[A]	B-*Pfldh*[B]	B Δ*pdc1*-*Pfldh*[B]	BI-*Pfldh*[C]
$Y_{lactic\ acid}$	0.00	0.02	0.01 +/- 0.00	0.01	0.01	0.39 +/- 0.00
$Y_{ethanol}$	0.37	0.38	0.38 +/- 0.00	0.39	0.39	0.22 +/- 0.00
$Y_{glycerol}$	0.04	0.04	0.03 +/- 0.00	0.04	0.03	0.01 +/- 0.00
$Y_{acetate}$	0.01	0.02	0.01 +/- 0.00	0.01	0.01	0.03 +/- 0.00
$Y_{biomass}$	0.05	0.05	0.05 +/- 0.00	0.05	0.05	0.05 +/- 0.00
C-recovery [%]	84	87	85 +/- 0.32	87	88	92 +/- 0.07

Yield coefficients (g/g glucose consumed) were calculated using data obtained after a reaction time of 10 h (A), 28 h (B) and 12 h (C). Values including standard deviation (SD) represents mean values derived from two independent experiments.

Table 3.5: Physiological parameters for strains from batch fermentations on xylose (~ 50 g/L) medium.

Yield [g/g]	wildtype B[A]	B-*Roldh*[A]	B Δ*pdc1*-*Roldh*[A]	B-*Pfldh*[B]	B Δ*pdc1*-*Pfldh*[B]	BI-*Pfldh*
$Y_{lactic\ acid}$	0.00	0.02	0.01 +/- 0.00	0.03	0.01	ND
$Y_{ethanol}$	0.32	0.31	0.32 +/- 0.01	0.34	0.34	ND
$Y_{glycerol}$	0.03	0.03	0.03 +/- 0.00	0.03	0.03	ND
$Y_{acetate}$	0.04	0.04	0.03 +/- 0.00	0.04	0.03	ND
$Y_{xylitol}$	0.29	0.29	0.28 +/- 0.01	0.23	0.23	ND
$Y_{biomass}$	0.06	0.04	0.03 +/- 0.00	0.04	0.05	ND
C-recovery [%]	100	102	102 +/- 0.00	102	102	ND

Yield coefficients (g/g xylose consumed) were calculated using data obtained after a reaction time of 99 h (A) and 142 h (B). Not determined (ND). Values including standard deviation (SD) represents mean values derived from two independent experiments.

Table 3.6: Glucose uptake rates at defined points of time from fermentation of the wildtype B and BI-*Pfldh* strain.

Time [h]	Wildtype B		BI-*Pfldh*	
	$q_{glucose}$ [g/gCDW/h]	pH	$q_{glucose}$ [g/gCDW/h]	pH
1	1.80	5.92	ND	ND
2	1.71	5.68	2.13	4.06
4	1.41	5.40	1.55	3.46
6	1.37	5.19	ND	ND
8	1.32	4.96	0.88	3.25
10	1.29	4.95	ND	ND
12	ND	ND	0.60	3.12

Not determined (ND).

Table 3.7: Lactate yields from transgenic yeasts.

***Ldh* gene**	**Genotype**	**Expression (copy number)**	**Promoter**	**$Y_{lactate}$ (%)**	**Ref.**
Bovine	wildtype	2µm plasmid	*adh1*	15.5	[37]
Bovine	Δ*pdc1*	2µm plasmid	*pdc1*	20.0	[37]
Bovine	Δ*pdc1*	2µm plasmid	*pdc1*	19.6	[52]
L. casei	wildtype	2µm plasmid	*adh1*	24.0	[32]
R. oryzae	wildtype	2µm plasmid	*adh1*	44. 5	[36]
L. plantarum	Δ*gal7*	genome (1)	*adh1*	29.0	[52]
Bovine	Δ*pdc1*	genome (2)	*pdc1*	64.7	[51]
Bovine	Δ*pdc1*, Δ*adh1*	genome (4)	*pdc1*, *adh1*	69. 0	[47]
Bovine	Δ*pdc1*, Δ*pdc5*	genome (4)	*pdc1*, *pdc5*	81.5	[40]

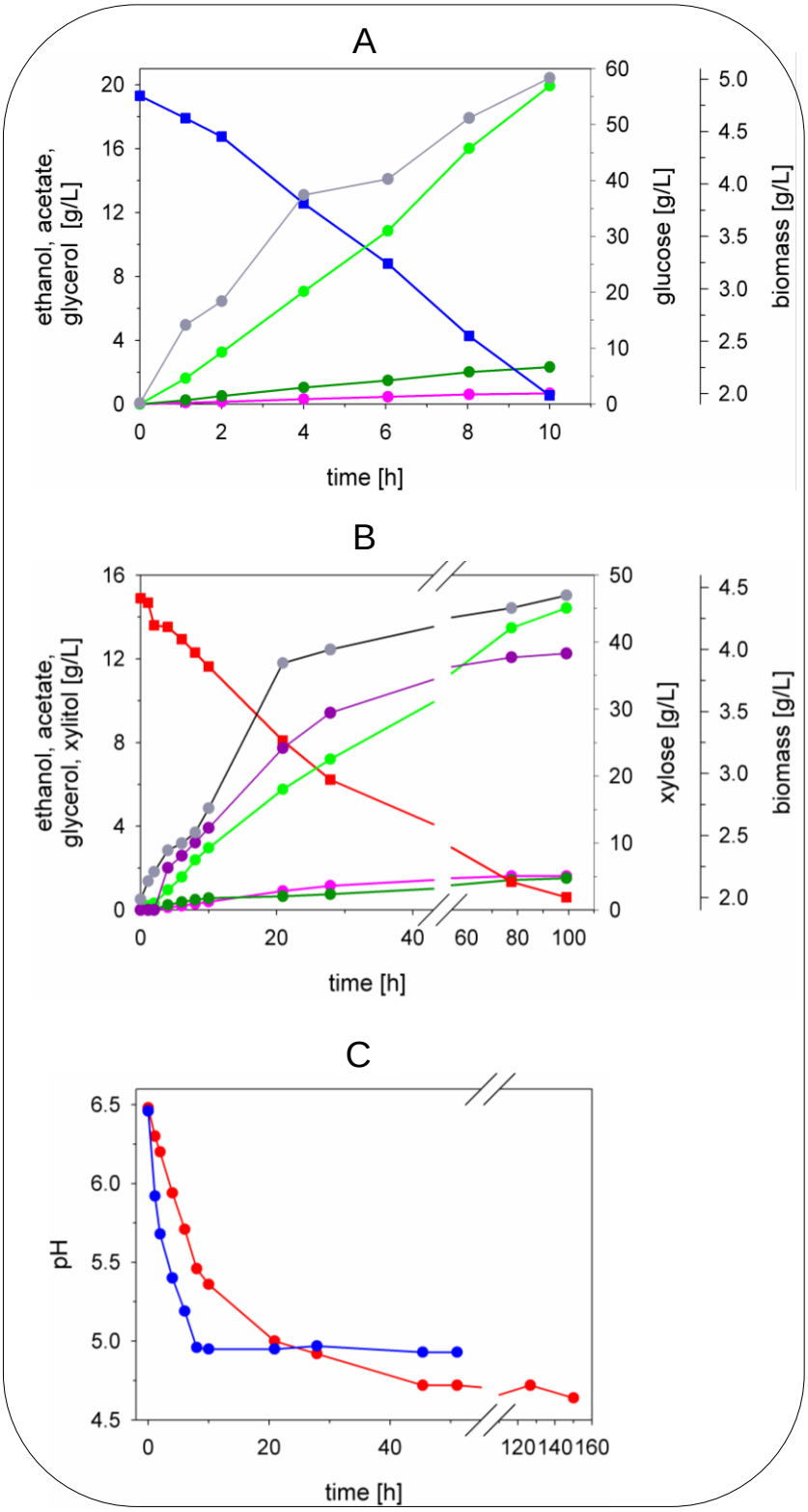

Figure 3.6: Course of fermentation for wildtype B.

Conversion of glucose (panel A) and xylose (panel B) and pH change for both substrates (panel C). Symbols: glucose (blue square), xylose (red square), ethanol (green circle), glycerin (dark green circle), actate (pink circle), xylitol (dark pink circle), and CDW (dark grey circle). Change of pH for glucose (blue circle) and xylose (red circle)

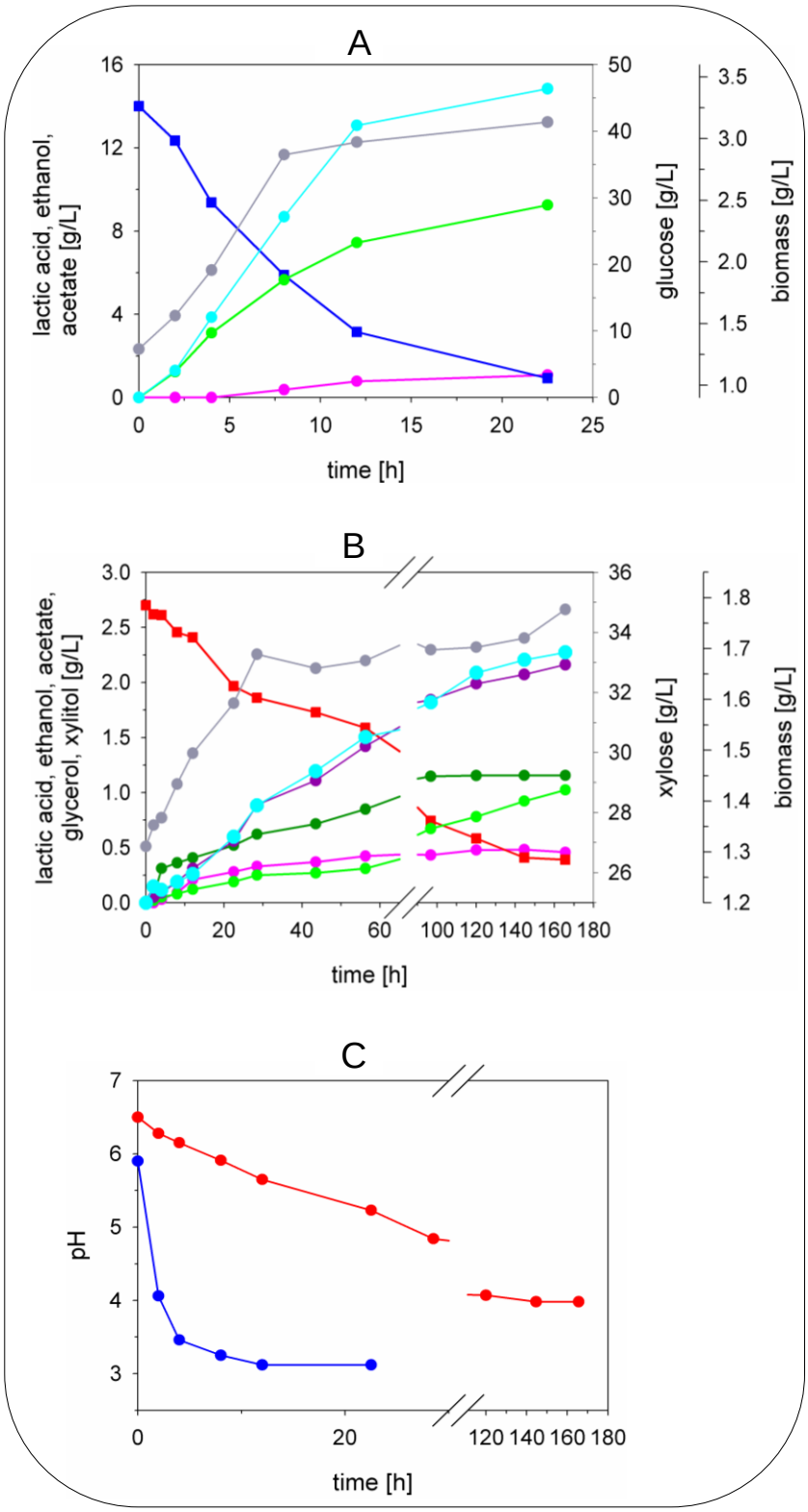

Figure 3.7: Course of fermentation for BI-*Pfldh* strain.

Conversion of glucose (panel A) and xylose (panel B) and pH change for both substrates (panel C). Symbols: glucose (blue square), xylose (red square), lactic acid (cyan circle), ethanol (green circle), glycerin (dark green circle), actate (pink circle), xylitol (dark pink circle), and CDW (dark grey circle). Change of pH for glucose (blue circle) and xylose (red circle).

4 Conclusion

Aim of this study was to establish a robust xylose fermenting *S. cerevisiae* strain for efficient lactic acid production. At first the genetic background of wildtype B was altered by deleting the coding region of *pdc1* gene, in order to supress ethanol formation. Double deletion of *pdc1* and *adh1* was also attempted, however, the resulting mutant was not viable probably because of the accumulation of acetaldehyde to toxic levels as a result of *adh1* deletion. Afterwards, two *lactate dehydrogenase* genes from two different organism (*R. oryzae* and *P. falciparum)* were expressed from a multi copy plasmid under regulation of a strong constitutive promoter (TEF1), and their expression level and *ldh* activity compared in the Δ*pdc1* mutant. Despite the strong expression, activity as well as protein concentration was poor in both cases, probably caused by premature protein degradation in the cell induced by cells stress. However, we additionally transformed a single copy of the more promising lactate dehydrogenase of *P. falciparum* into the *pdc1* locus (BI-*Pfldh* strain). As a result, high activity (1.24 U/mg) and elevated protein levels could be detected, indicating that metabolic regulation of protein expression from the preceding pathways (glycolysis) is important for the success of lactic acid fermentation. Fermentation studies further showed, that strain BI-*Pfldh*, is capable of converting high amounts of glucose (44 g/L) to lactic acid within a short time frame (12 h) at high yield (0.39 g/g). Further, we noticed that glucose conversion rates were attained under the difficult substrate conditions (down to pH 3.1), which is in contrast to previously published studies. Xylose fermentation in strain BI-*Pfldh* was inhibited severely, and thus only minor amount was converted to lactic acid within a time frame of 166 h. The pH control or further strain adaptation e.g. laboratory evolution might be possible strategies to overcome this bottleneck. Overall, the recombinant yeast strain of this study seems to be suitable for efficient production of lactic acid from both, glucose and xylose and there is the possibility to improve lactic acid production by integration of additional copies of *ldh* genes into the genome.

5 References

1 Datta R (2005) Hydroxy carboxylic acids. *Kirk-Othmer encyclopedia of chemical technology*, 114–134.

2 Datta R, Tsai S-P, Bonsignore P, Moon S-H et al (1995) Technological and economic potential of poly (lactic acid) and lactic acid derivates. *FEMS microbiology reviews* **16**, 221–231.

3 Datta R & Henry M (2006) Lactic acid: recent advances in products, processes and technologies—a review. *Journal of chemical technology and biotechnology* **81**, 1119–1129.

4 Varadarajan S & Miller DJ (1999) Catalytic upgrading of fermentation-derived organic acids. *Biotechnology progress* **15**, 845–854.

5 Hester A (2000) IB market forecast. *Ind Bioprocess* **22**, 3–5.

6 Watkins K (2002) A solvent business. *Chemical and engineering news* **80**, 6–15.

7 Hester A (2000) IB market forecast. *Ind Bioprocess* **22**, 4–5.

8 Jarvis L (2003) Properties for lactic acid are healthy as demand for all end uses grows. *Chemical market reporter* **263**, 12.

9 Tullo A (2000) Plastic found at the end of the maize. *Chemical and engineering news* **78**, 13–13.

10 Södergård A & Stolt M (2002) Properties of lactic acid based polymers and their correlation with composition. *Progress in polymer science* **27**, 1123–1163.

11 Wee Y-J, Kim J-N & Ryu H-W (2006) Biotechnological production of lactic acid and its recent applications. *Food technology and biotechnology* **44**, 163.

12 Litchfield JH (1996) Microbiological production of lactic acid. *Advances in applied microbiology* **42**, 45–95.

13 Garvie EI (1980) Bacterial lactate dehydrogenases. *Microbiological reviews* **44**, 106–139.

14 Kim SF, Baek SJ & Pack MY (1991) Cloning and nucleotide sequence of the Lactobacillus casei lactate dehydrogenase gene. *Applied and environmental microbiology* **57**, 2413–2417.

15 Kochhar S, Hottinger H, Chuard N et al (1992) Cloning and overexpression of Lactobacillus helveticus D-lactate dehydrogenase gene in Escherichia coli. *European journal of biochemistry / FEBS* **208**, 799–805.

16 Savijoki K & Palva A (1997) Molecular genetic characterization of the L-lactate dehydrogenase gene (ldhL) of Lactobacillus helveticus and biochemical characterization of the enzyme . *Applied and environmental microbiology* **63**, 2850–2856.

17 Taguchi H & Ohta T (1991) D-lactate dehydrogenase is a member of the D-isomer-specific 2-hydroxyacid dehydrogenase family. Cloning, sequencing, and expression in Escherichia coli of the D-lactate dehydrogenase gene of Lactobacillus plantarum. *Journal of biological chemistry* **266**, 12588–12594.

18 Lee JH, Choi MH & Park JY (2004) Cloning and characterization of the lactate dehydrogenase genes from Lactobacillus sp. RKY2. *Biotechnology and bioprocess engineering* **9**, 318–322.

19 Duncan MJ & Hillman JD (1991) DNA sequence and In vitro mutagenesis of the gene encoding the fructose-1,6-diphosphate-dependent Lö(+)-lactate dehydrogenase of Streptococcus mutans. *Infection and immunity* **59**, 3930–3934.

20 Skory CD (2000) Isolation and expression of lactate dehydrogenase genes from Rhizopus oryzae. *Applied and environmental microbiology* **66**, 2343–2348.

21 Ishiguroa N, Osames S, Kagiyab R et al (1990) Primary structure of bovine lactate dehydrogenase-A isozyme and its synthesis in Escherichia coli. *Gene* **91**, 281–285.

22 Meussen BJ, de Graaff LH, Sanders JPM et al (2012) Metabolic engineering of Rhizopus oryzae for the production of platform chemicals. *Applied microbiology and biotechnology* **94**, 875–86.

23 Singh SK, Ahmed SU & Pandey A (2006) Metabolic engineering approaches for lactic acid production. *Process biochemistry* **41**, 991–1000.

24 Davidson BE, Llanos RM & Cancilla MR (1995) Current research on the genetics of lactic acid production in lactic acid bacteria. *International dairy journal* **5**, 763–784.

25 Ferain T, Garmyn D, Bernard N et al (1994) Lactobacillus plantarum ldhL gene: overexpression and deletion. *Journal of bacteriology* **176**, 596–601.

26 Chang D, Jung H & Rhee J (1999) Homofermentative Production of D- or L-lactate in metabolically engineered Escherichia coli RR1. *Applied and evironmental microbiology* **65**, 1384–1389.

27 Kylä-Nikkilä K, Hujanen M, Leisola M et al (2000) Metabolic engineering of Lactobacillus helveticus CNRZ32 for production of pure L-(+)-lactic acid. *Applied and environmental microbiology* **66**, 3835–3841.

28 Dien BS, Nichols NN & Bothast RJ (2001) Recombinant Escherichia coli engineered for production of L-lactic acid from hexose and pentose sugars. *Journal of industrial microbiology & biotechnology* **27**, 259–264.

29 Oh H, Wee YS & Yun JS (2003) Lactic acid production through cell-recycle repeated-batch bioreactor. *Applied biochemistry and biotechnology* **105**, 603–613

30 Nevoigt E (2008) Progress in metabolic engineering of Saccharomyces cerevisiae. *Microbiology and molecular biology reviews* **72**, 379–412.

31 Dequin S & Barre P (1994) Mixed lactic acid-alcoholic fermentation by Saccharomyces cervisiae expressing the Lactobacillus casei L (+)- LDH. *Nature biotechnology* **12**, 173–177.

32 Hohmann S (1991) Characterization of PDC6, a third structural gene for pyruvate decarboxylase in Saccharomyces cerevisiae. *Journal of bacteriology* **173**, 7963–7969.

33 Schaalf I, Green JB & Gozalbo D (1989) A deletion of the PDC1 gene for pyruvate decarboxylase of yeast causes a different phenotype than previously isolated point mutations. *Current genetics* **15**, 75–81.

34 Leskovac V, Trivic S & Pericin D (2002) The three zinc-containing alcohol dehydrogenases from baker's yeast, Saccharomyces cerevisiae. *FEMS Yeast research* **2**, 481–494.

35 Skory CD (2003) Lactic acid production by Saccharomyces cerevisiae expressing a Rhizopus oryzae lactate dehydrogenase gene. *Journal of industrial microbiology & biotechnology* **30**, 22–27.

36 Adachi E, Torigoe M, Sugiyama M et al (1998) Modification of metabolic pathways of Saccharomyces cerevisiae by the expression of lactate dehydrogenase and deletion of pyruvate decarboxylase genes for the lactic acid fermentation at low pH value. *Journal of fermentation and bioengineering* **86**, 284–289.

37 Pronk JT, Steensma HY & Duken JPV (1996) Pyruvate metabolism in Saccharomyces cervisiae. *Yeast* **12**, 1607–1633.

38 Steensmati H, Vandijkent JP & Pronkt JT (1996) Pyruvate decarboxylase: an indispensable enzyme for growth of Saccharomyces cervisiae on glucose. *Yeast* **12**, 247–257.

39 Hohmann S & Cederberg H (1990) Autoregulation may control the expression of yeast pyruvate decarboxylase structural genes PDC1 and PDC5. *European journal of biochemistry* **188**, 615–621.

40 Ishida N, Saitoh S, Onishi T et al (2006) The effect of pyruvate decarboxylase gene knockout in Saccharomyces cerevisiae on L-lactic acid production. *Bioscience, biotechnology, and biochemistry* **70**, 1148–53.

41 Branduardi P, Sauer M, De Gioia L et al (2006) Lactate production yield from engineered yeasts is dependent from the host background, the lactate dehydrogenase source and the lactate export. *Microbial cell factories* **5**, 4.

42 Petschacher B & Nidetzky B (2008) Altering the coenzyme preference of xylose reductase to favor utilization of NADH enhances ethanol yield from xylose in a metabolically engineered strain of Saccharomyces cerevisiae. *Microbial cell factories* **7**, 9.

43 Miller EM & Nickoloff JA (1995) Escherichia coli electrotransformation. *Electroporation protocols for microorganisms*, 105–113.

44 Gietz RD & Woods RA (2002) Transformation of yeast by theLiac/SS carrier DNA/PEG method. *Methods in enzymology* **350**, 87–96.

45 Hald E, Lehmann P & Ziegenhorn J (1975) Molar absorptivities of ß-NADH and ß-NAD at 260 nm. *Clinical chemistry* **21**, 884–887.

46 Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry* **72**, 248–54.

47 Tokuhiro K, Ishida N, Nagamori E, Saitoh S et al (2009) Double mutation of the PDC1 and ADH1 genes improves lactate production in the yeast Saccharomyces cerevisiae expressing the bovine lactate dehydrogenase gene. *Applied microbiology and biotechnology* **82**, 883–90.

48 Yu R & Hang YD (1991) Purification and characterization of NAD-dependent lactate dehydrogenase from Rhizopus oryzae. *Food chemistry* **41**, 219–225.

49 Bzik DJ, Fox B & Gonyer K (1993) Expression of Plasmodium falciparum lactate dehydrogenase in Escherichia coli. *Molecular and biochemical parasitology* **59**, 155–166.

50 Krahulec S, Petschacher B, Wallner M et al (2010) Fermentation of mixed glucose-xylose substrates by engineered strains of Saccharomyces cerevisiae: role of the coenzyme specificity of xylose reductase, and effect of glucose on xylose utilization. *Microbial cell factories* **9**, 16.

51 Ishida N, Saitoh S, Tokuhiro K et al (2005) Efficient production of L -lactic acid by metabolically engineered Saccharomyces cerevisiae with a genome-integrated L -lactate dehydrogenase gene. *Applied and environmental microbiology* **71**, 1964–1970.

52 Colombié S, Dequin S & Sablayrolles J. (2003) Control of lactate production by Saccharomyces cerevisiae expressing a bacterial LDH gene. *Enzyme and microbial technology* **33**, 38–46.

6 Abbreviations

adh	Alcohol dehydrogenase
AGE	Agarose gel electrophoresis
amp	Ampicillin
Bp	Base pairs
BSA	*Bovine* serum albumin
CDW	Cell dry weight
DNA	Deoxyribonucleic acid
dNTP`s	Nucleoside triphosphate
E. coli	*Escherichia coli*
Fast AP	Fast Alkaline Phosphatase
G418	Geniticin sulphate
HF buffer	High fidelity buffer
hph	Hygromycin B
HPLC	High performance liquid chromatography
IPTG	Isopropyl β-D- thiogalactopyranosid
LAB	Lactic acid bacteria
LB	Luria-bertani
ldh	Lactate dehydrogenase
LiAc	Lithium acetate
MCS	Multiple cloning site
nat	Nourseothricin
NFW	Nuclease free water
OD_{600}	Optical density at 600nm
ORF	Open reading frame
P. falciparum	*Plasmodium falciparum*
PCR	Polymerase chain reaction
pdc	Pyruvate decarboxylase
PEG	Polyethylene glycol
Pfldh	Lactate dehydrogenase from *P. falciparum*
PLA	Poly lactic acid
psi	Pounds per square inch
R. oryzae	*Rhizopus oryzae*

RI	Refractive index
Roldh	Lactate dehydrogenase from *R. oryzae*
rpm	Rotation per minutes
S. cervisiae	*Saccharomyces cerevisiae*
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPIC buffer	Sodium phosphate buffer
SS carrier DNA	Deoxyribonucleic acid sodium salt type III from salmon testes
U	Units
UV	Ultraviolet
YD	Yeast extract dextrose
YPD	Yeast peptone dextrose

7 Supplementary Information

7.1 Plasmid maps and ladders

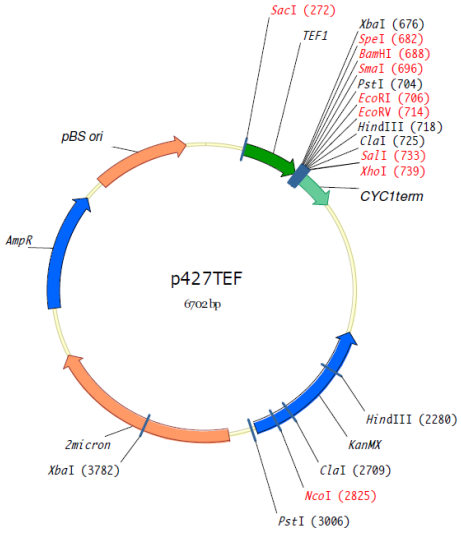

Figure 7.1: Yeast expression plasmid p427TEF.

The plasmid contains the following features: TEF1 promotor (272-675), multiple cloning site (675-743), CYC1 terminator (739-1000), geniticin (kanMX) resistance gene allows selection in *S. cervisiae* (1973-2976), 2micron origin of replication allows propagation of plasmid in yeast at high copy numbers (3173-4540), ampicillin (AmpR) resistance gene allows selection in *E. coli* (4896-5756) and pBluescript origin (pBS ori) of replication allows propagation of plasmid in *E. coli* at high copy numbers (5904-6571).

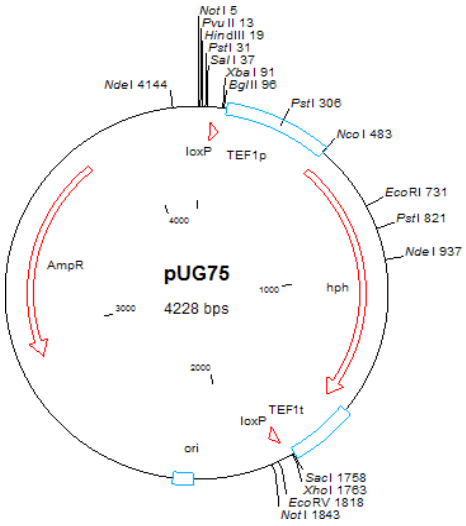

Figure 7.2: Yeast deletion marker plasmid pUG75.

The plasmid contains the following features: hygromycin B resistance cassette (106-1753) consisting of TEF1 promotor (TEF1p), hygromycin B (hph) resistance gene and TEF1 terminator (TEF1t), which is flanked by loxP sites (53-86, 1779-1812) that allow removing of resistance marker by expression of cre recombinase, *E. coli* origin of replication (ori) ColE1 (2126-2198) and ampicillin (AmpR) resistance gene (2901-3761).

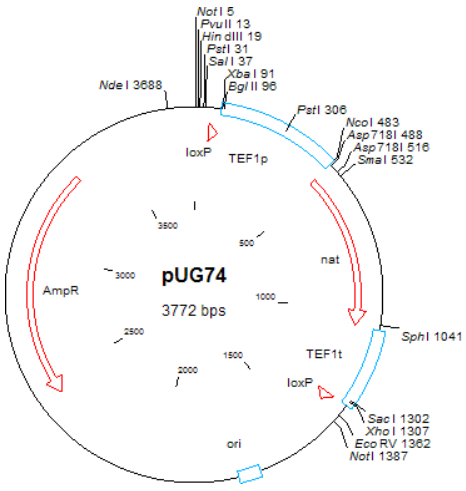

Figure 7.3: Yeast deletion marker plasmid pUG74.

The plasmid contains the following features: nourseothricin resistance cassette (87-1322) consisting of TEF1 promotor (TEF1p), nourseothricin (nat) resistance gene and TEF1 terminator (TEF1t), which is flanked by loxP sites (53-86, 1323-1356) that allow removing of resistance marker by expression of cre recombinase, *E. coli* origin of replication(ori) ColE1 (1670-1742) and ampicillin (AmpR) resistance gene (2445-3305).

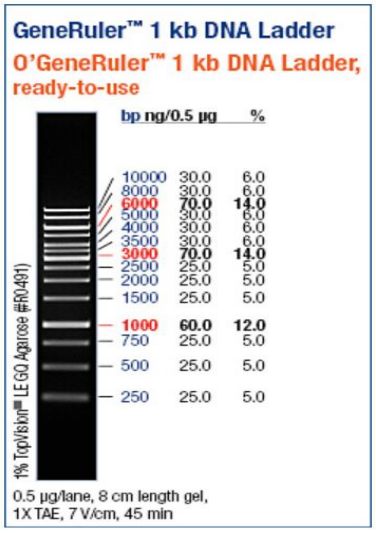

Figure 7.4: O`GeneRuler™ 1 kb DNA Ladder.

From Fermentas International Inc., Burlington, Canada.

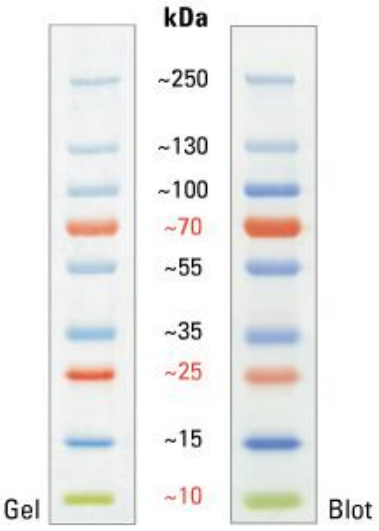

Figure 7.5: PAGE Ruler™ Plus Prest. Prot. Ladder.

From Fermentas International Inc., Burlington, Canada.

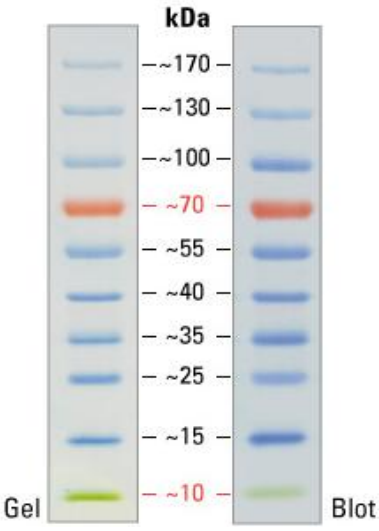

Figure 7.6: PAGE Ruler™ Prest. Prot. Ladder.

From Fermentas International Inc., Burlington, Canada

7.2 Activity measurement and fermentations

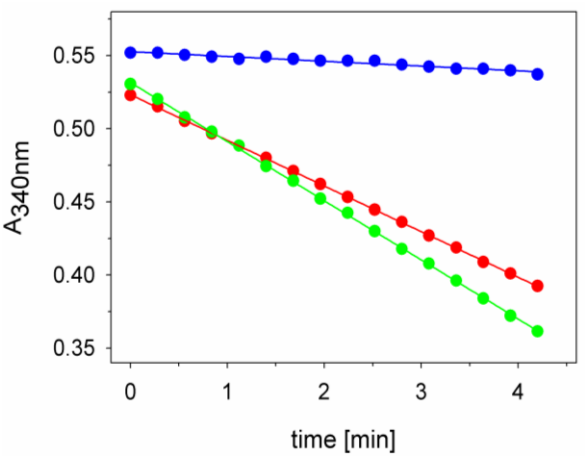

Figure 7.7: Activity measurement of *ldh* by monitoring decrease of absorbance at 340nm over time.

Measurement was performed with crude cell extract from the wildtype B (blue circle), B-*Pfldh* red circle) and B Δ*pdc1-Pfldh* (green circle) strain. Conditions: 25 °C, pH 7.5, 0.1 M sodium phosphate buffer, 0.1 mM NADH and 1 mM pyruvate.

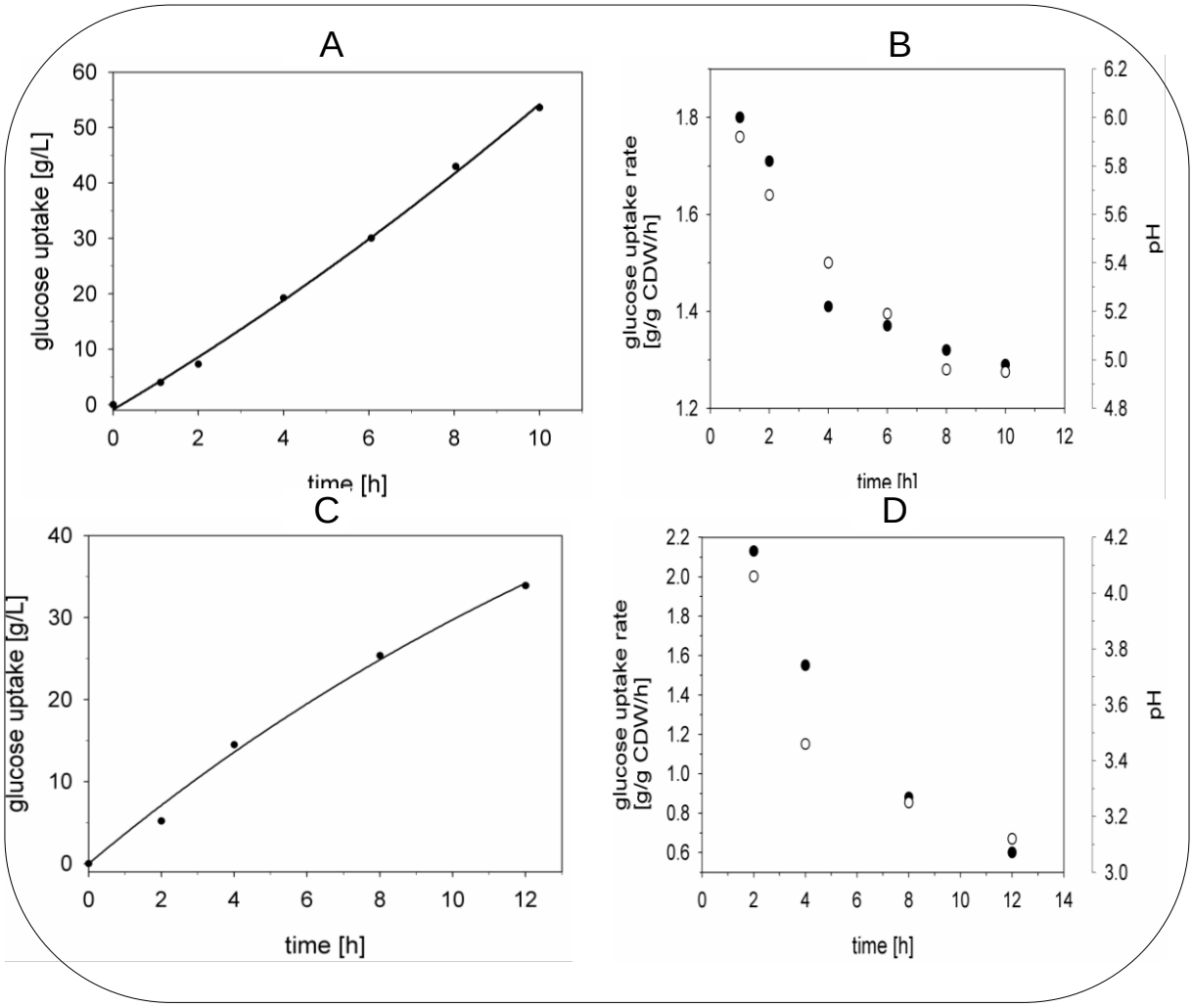

Figure 7.8: Glucose uptake rates for the wildtype B (panel A, B) and for BI-*Pfldh* (panel C, D) strain. Equation: y=y0 + ax + bt2. Parameters: a= 4.5519, b=0.0963, R2= 0.9991 (panel A) and a= 3.3920, b=-0.0864, R2= 0.9950 (panel B).

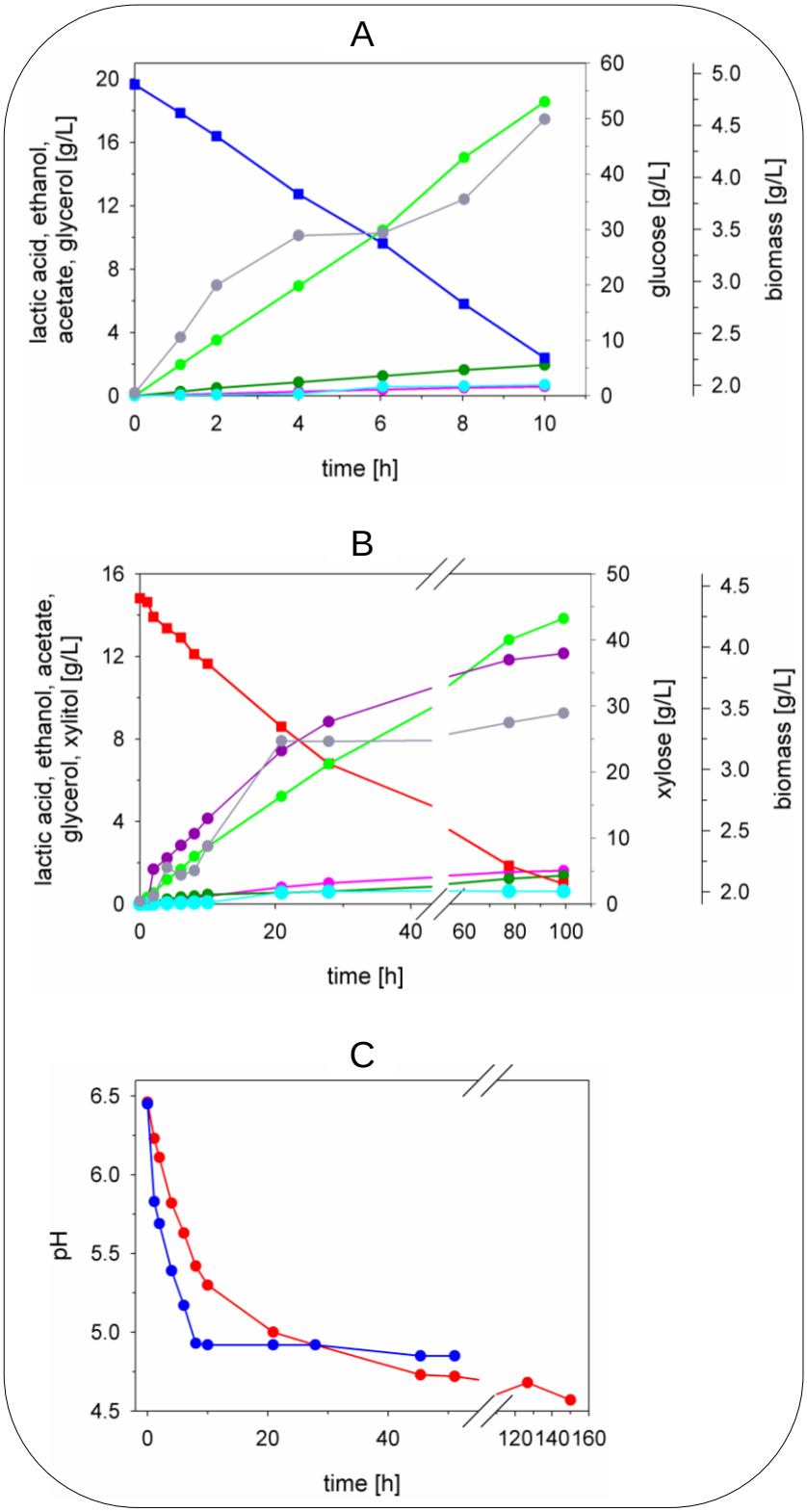

Figure 7.9: Course of fermentation for B-*Roldh* strain.

Conversion of glucose (panel A) and xylose (panel B) and pH change for both substrates (panel C). Symbols: glucose (blue square), xylose (red square), lactic acid (cyan circle), ethanol (green circle), glycerin (dark green circle), actate (pink circle), xylitol (dark pink circle), and CDW (dark grey circle). Change of pH for glucose (blue circle) and xylose (red circle).

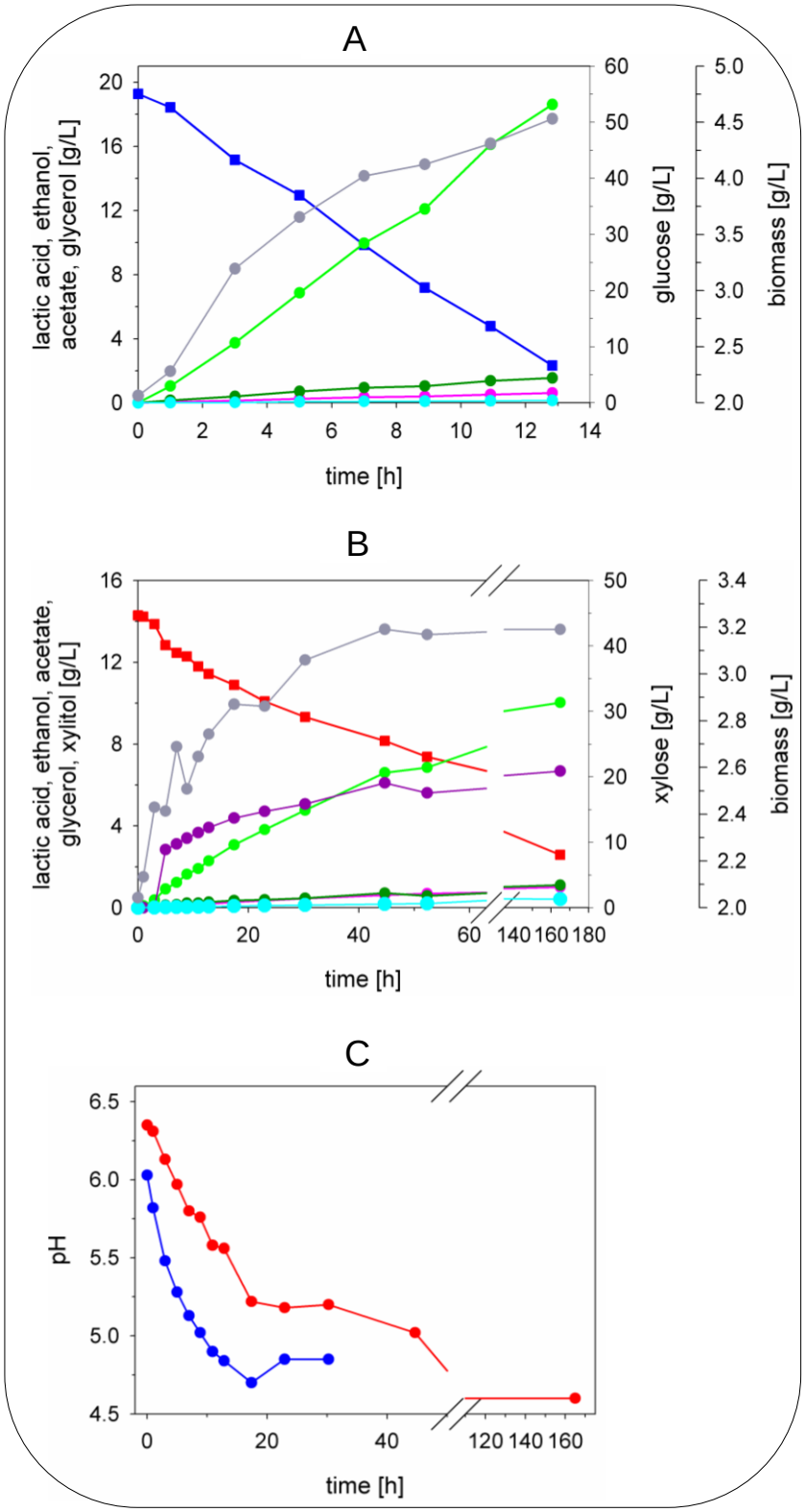

Figure 7.10: Course of fermentation for B Δ*pdc1-Roldh* strain.

Conversion of glucose (panel A) and xylose (panel B) and pH change for both substrates (panel C). Symbols: glucose (blue square), xylose (red square), lactic acid (cyan circle), ethanol (green circle), glycerin (dark green circle), actate (pink circle), xylitol (dark pink circle), and CDW (dark grey circle). Change of pH for glucose (blue circle) and xylose (red circle).

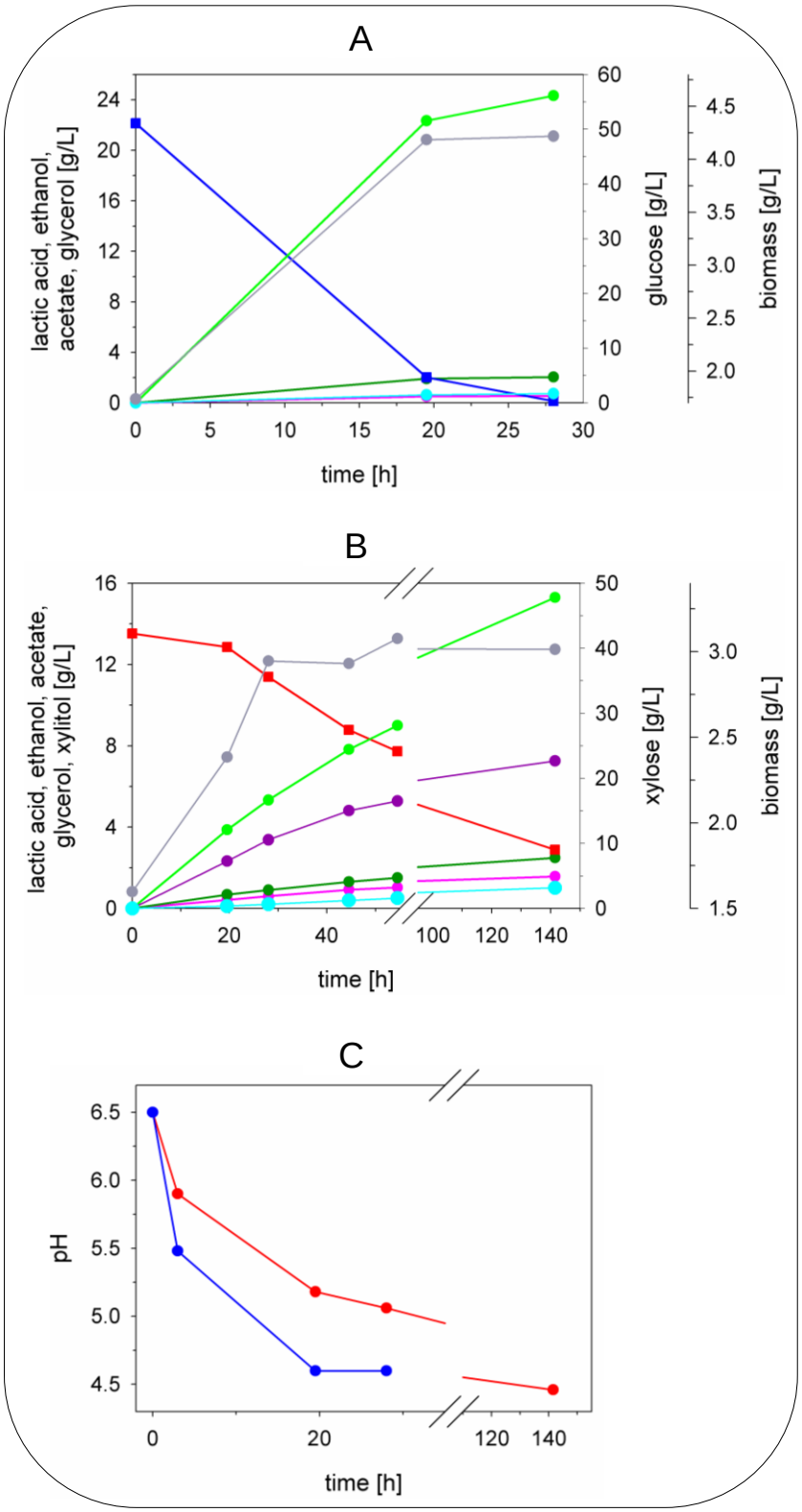

Figure 7.11: Course of fermentation for B-*Pfldh* strain.

Conversion of glucose (panel A) and xylose (panel B) and pH change for both substrates (panel C). Symbols: glucose (blue square), xylose (red square), lactic acid (cyan circle), ethanol (green circle), glycerin (dark green circle), actate (pink circle), xylitol (dark pink circle), and CDW (dark grey circle). Change of pH for glucose (blue circle) and xylose (red circle).

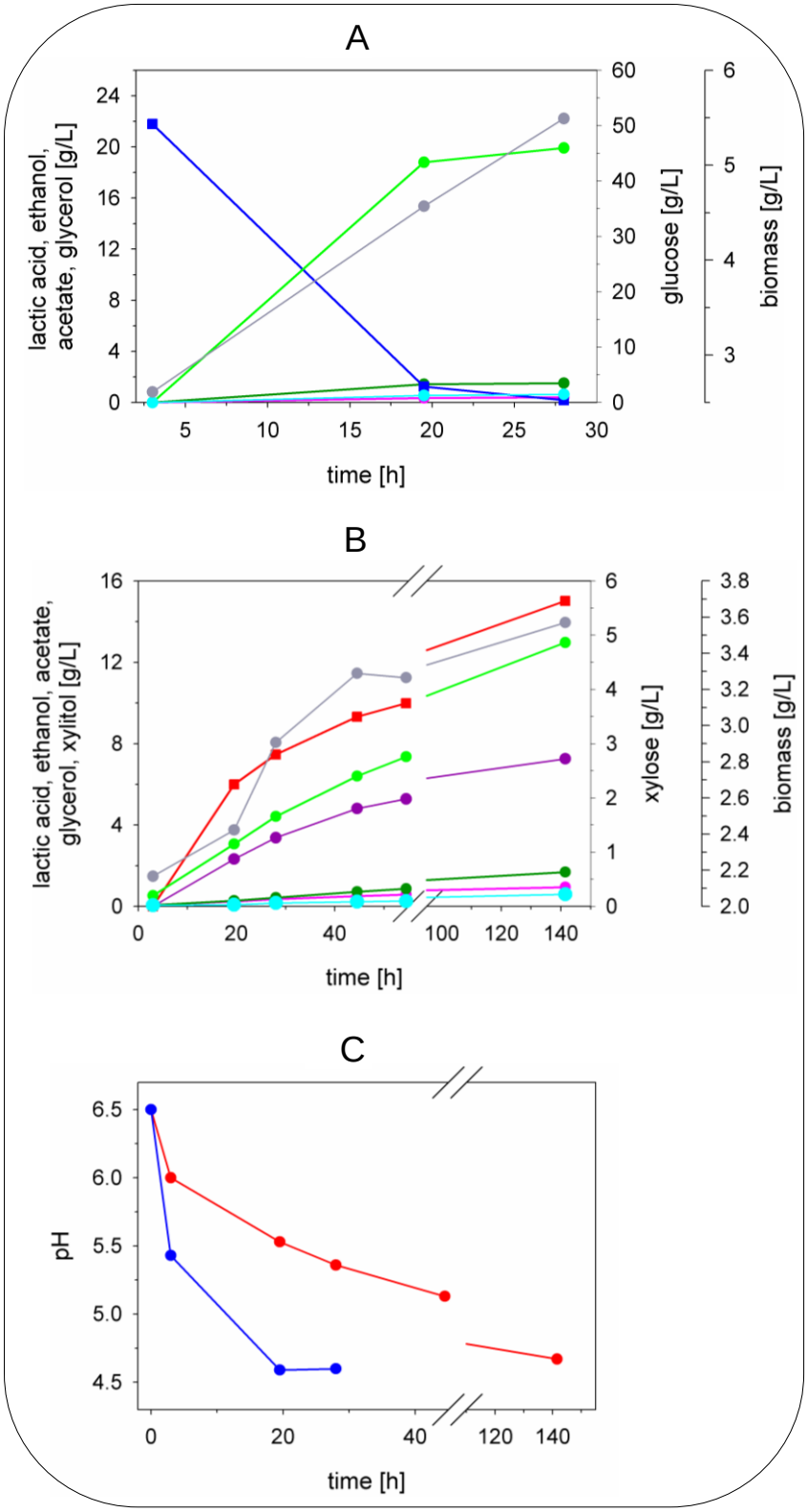

Figure 7.12: Course of fermentation for B Δ*pdc1-Pfldh* strain.

Conversion of glucose (panel A) and xylose (panel B) and pH change for both substrates (panel C). Symbols: glucose (blue square), xylose (red square), lactic acid (cyan circle), ethanol (green circle), glycerin (dark green circle), actate (pink circle), xylitol (dark pink circle), and CDW (dark grey circle). Change of pH for glucose (blue circle) and xylose (red circle).

7.3 Chromatograms

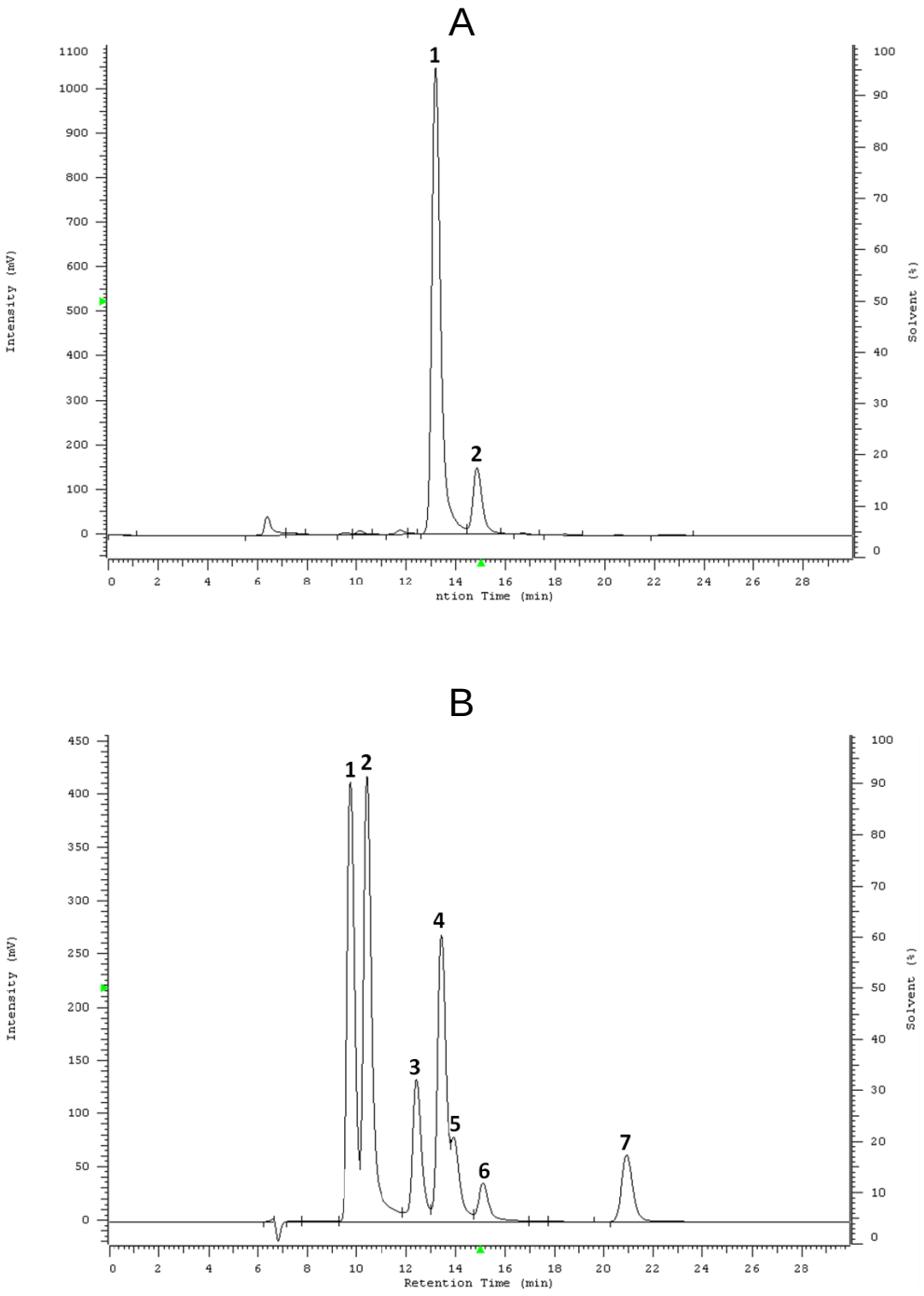

Figure 7.13: Chromatogram from UV (panel A) and RI (panel B) detection of HPLC operated at 65 °C with a flow rate of 0.6 ml/min using as eluent 5 mM H_2SO_4.

UV retention times: lactic acid 13.19 sec (1) and acetate 14.85 sec (2). RI retention times: glucose 9.76 sec (1), xylose 10.42 sec (2), xylitol 12.44 sec (3), lactic acid 13.43 sec (4), glycerol 13.93 sec (5), acetate 15.11 sec (6) and ethanol 20.92 sec (7).

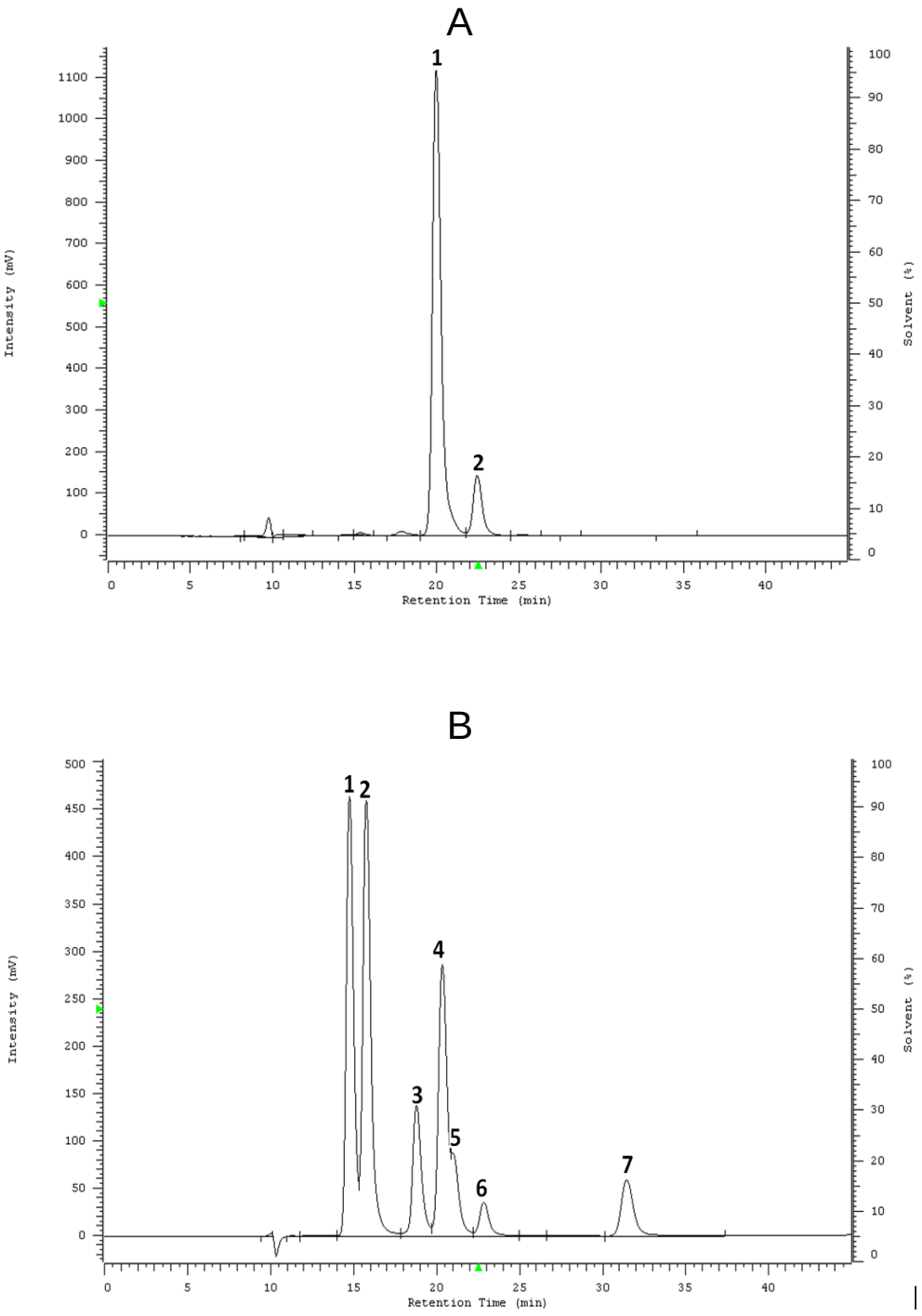

Figure 7.14: Chromatogram from UV (panel A) and RI (panel B) detection of HPLC operated at 65 °C with a flow rate of 0.4 ml/min using as eluent 5 mM H_2SO_4.

UV retention times: lactic acid 19.97 sec (1) and acetate 22.46 sec (2). RI retention times: glucose 14.76 sec (1), xylose 15.77 sec (2), xylitol 18.79 sec (3), lactic acid 20.35 sec (4), glycerol 20.97 sec (5), acetate 23.81 sec (6) and ethanol 31.44 sec (7).

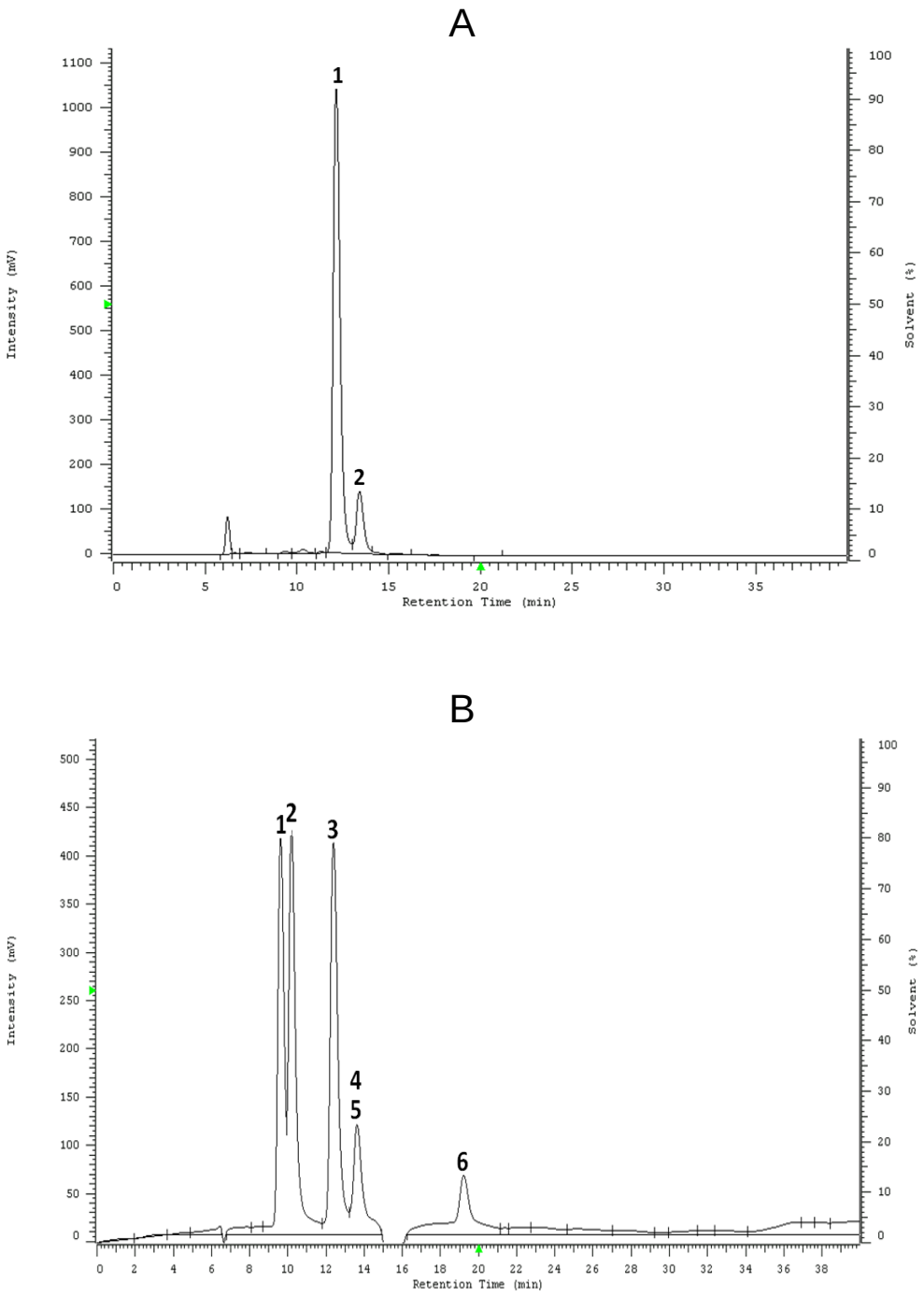

Figure 7.15: Chromatogram from UV (panel A) and RI (panel B) detection of HPLC operated at 65 °C with a flow rate of 0.6 ml/min using as eluent 5 mM H_2SO_4 supplemented with 10 % (v/v) acetonitrile.

UV retention times: lactic acid 12.15 sec (1) and acetate 13.43 sec (2). RI retention times: glucose 9.64 sec (1), xylose 10.21 sec (2), xylitol 12.40 sec (3), lactic acid and glycerol 13.64 sec (4 and 5) and acetate 19.23 sec (6). Ethanol peak was not detected in that time frame.

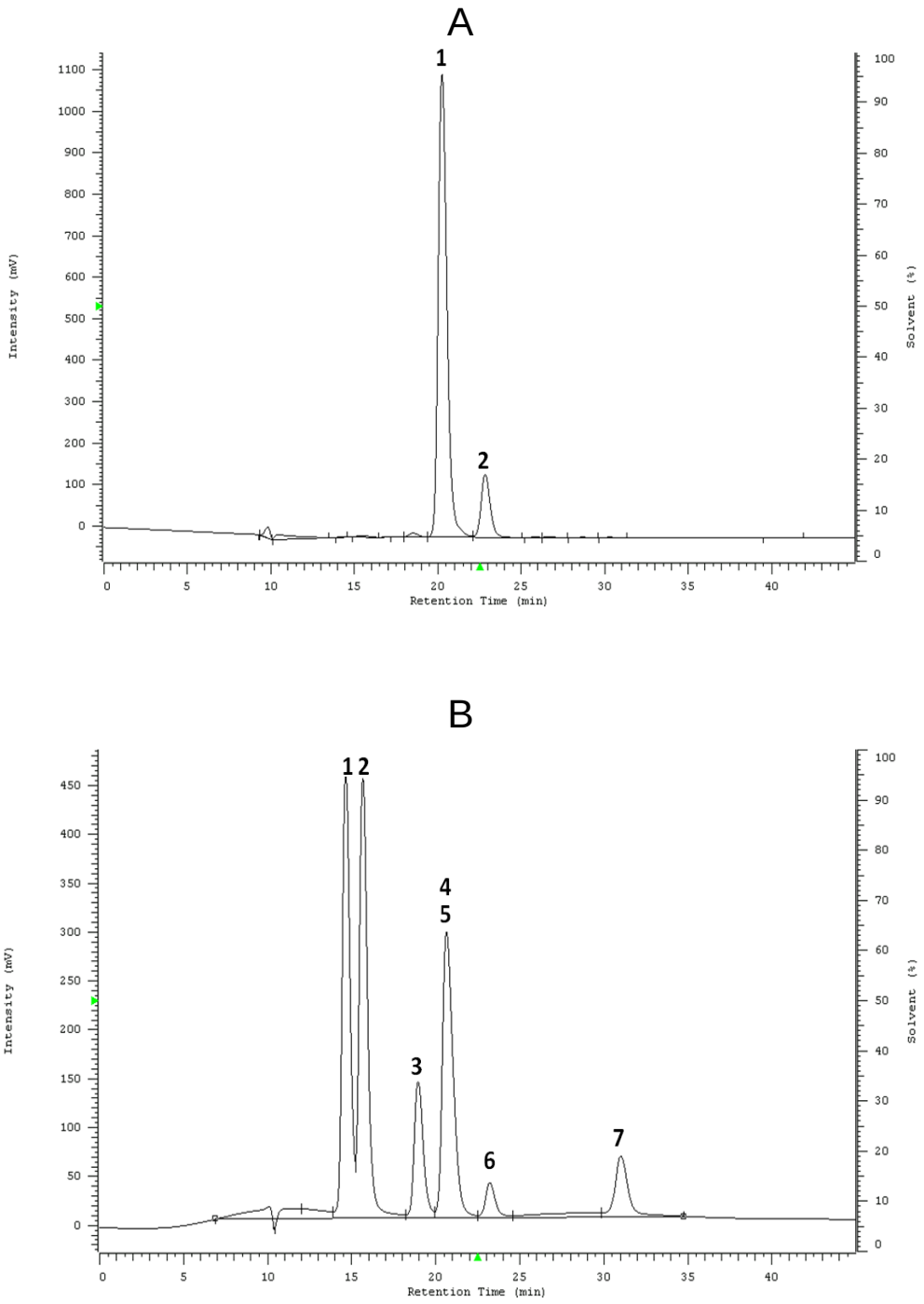

Figure 7.16: Chromatogram from UV (panel A) and RI (panel B) detection of HPLC operated at 50 °C with a flow rate of 0.4 ml/min using as eluent 5 mM H_2SO_4.

UV retention times: lactic acid 20.25 sec (1) and acetate 23.83 sec (2). RI retention times: glucose 14.65 sec (1), xylose 15.67 sec (2), xylitol 18.95 sec (3), lactic acid and glycerol 20.64 sec (4 and 5), acetate 23.22 sec (6) and ethanol 31.00 sec (7).

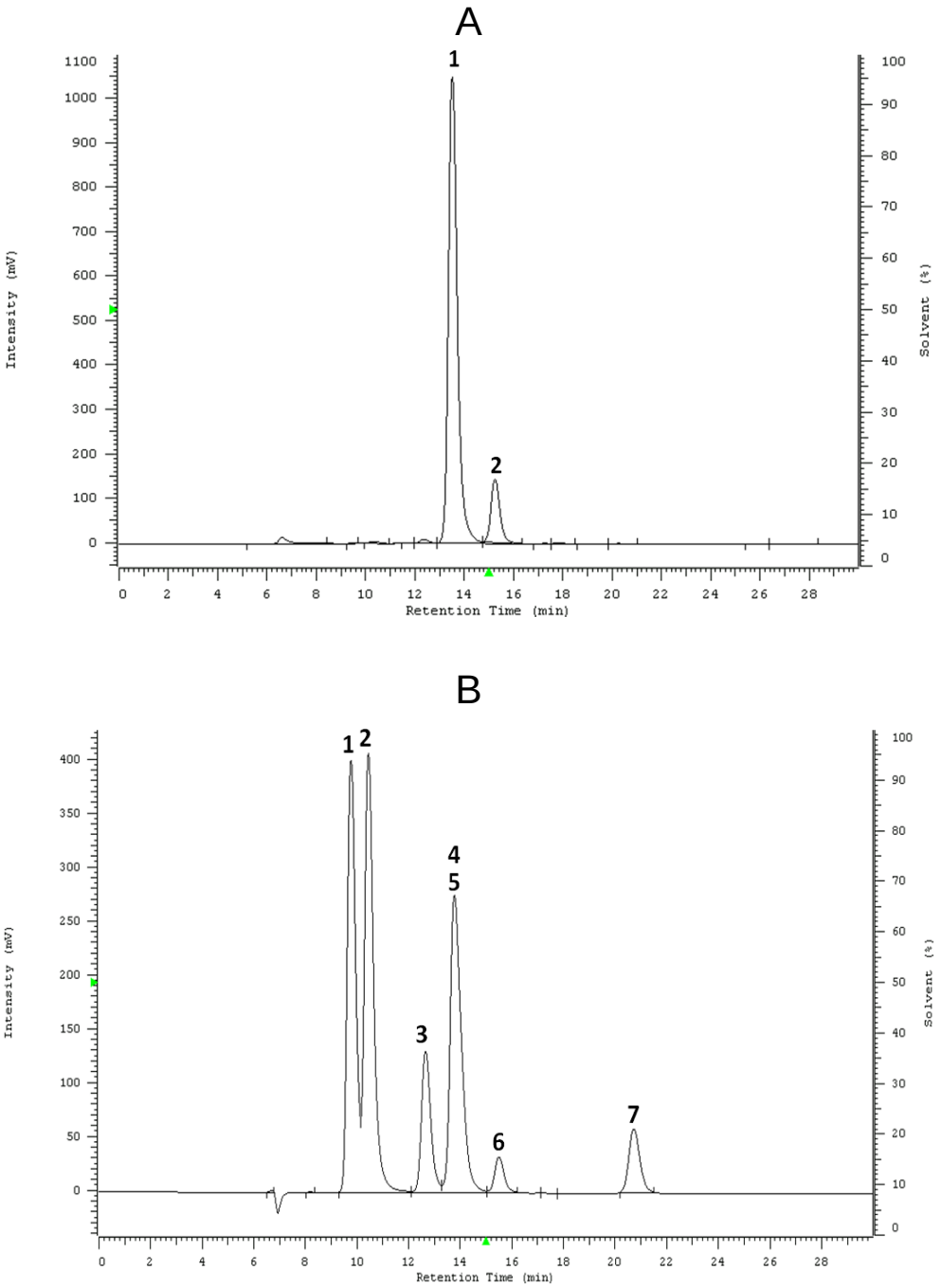

Figure 7.17: Chromatogram from UV (panel A) and RI (panel B) detection of HPLC operated at 50 °C with a flow rate of 0.6 ml/min using as eluent 5 mM H_2SO_4.

UV retention times: lactic acid 13.52 sec (1) and acetate 15.25 sec (2). RI retention times: glucose) 9.77 sec (1), xylose 10.45 sec (2), xylitol 12.66 sec (3), lactic acid and glycerol 13.78 sec (4 and 5), acetate) 15.50 sec (6) and ethanol 20.72 sec (7).