

**Osmotic stress response in the
industrially important bacterium
*Gluconobacter oxydans***

Dissertation

to obtain the degree of

Doctorate (Dr. rer. nat.)

from the

Faculty of Mathematics and Natural Sciences

of the

Rheinische Friedrich-Wilhelms University of Bonn, Germany

submitted by

Nageena Zahid

from

Lahore, Pakistan

Bonn, November 2016

Finalized with the permission from the Faculty of Mathematics and Natural Sciences of the
Rheinische Friedrich-Wilhelms University of Bonn, Germany

First Referee: Prof. Dr. Uwe Deppenmeier

Second Referee: Prof. Dr. Erwin A. Galinski

Day of Promotion: 07.02.2017

Year of Publication: 2017

List of parts of this thesis that have already been published.

Zahid N., Schweiger P., Galinski E., Deppenmeier U. (2015). Identification of mannitol as compatible solute in *Gluconobacter oxydans*. *Appl Microbiol Biotechnol* **99**, 5511-5521.

Zahid N. and Deppenmeier U. (2016). Role of mannitol dehydrogenases in osmoprotection of *Gluconobacter oxydans*. *Appl Microbiol Biotechnol* **100**, 9967-9978.

For my Son

TABLE OF CONTENTS

1. INTRODUCTION	1
1.1. Acetic acid bacteria	1
1.2. The genus <i>Gluconobacter</i>	2
1.2.1. Respiratory chain of <i>G. oxydans</i>	3
1.2.2. Intracellular carbohydrate metabolism in <i>G. oxydans</i>	6
1.2.3. Types and function of sugars and polyols metabolizing enzymes in <i>Gluconobacter</i>	7
1.2.4. Biotechnological applications of <i>G. oxydans</i> and its limitations	8
1.3. Aims of the work	10
2. MATERIALS AND METHODS	12
2.1. Chemicals and Enzymes	12
2.2. Bacterial strains, plasmids, primers	12
2.2.1. Bacterial strains	12
2.2.2. Oligonucleotides and Plasmids	13
2.3. Antibiotic stock solutions	18
2.4. Microbiology methods	18
2.4.1. Media and culture conditions	18
2.4.2. Measurement of osmolalities of growth media	22
2.4.3. Measurement of growth parameters	22
2.4.4. Preparation of stock cultures	23
2.5. Molecular biology methods	23
2.5.1. Isolation and purification of DNA	23
2.5.2. Isolation and purification of plasmid DNA	23
2.5.3. Isolation and purification of RNA	24
2.5.3.1. RNA extraction using the Trizol reagent method	24
2.5.3.2. RNA extraction with the Ribopure-Bacterial Kit	24
2.5.4. Spectrophotometric quantifications of DNA and RNA samples	25
2.5.5. Restriction digestion of DNA	25
2.5.6. Ligation	26

2.5.7. Polymerase chain reaction (PCR)	26
2.5.8. Reverse transcription quantitative PCR (RT-qPCR)	27
2.5.9. Agarose gel electrophoresis	28
2.5.10. Denaturing agarose gel electrophoresis	29
2.5.11. Staining of agarose gels	29
2.5.12. Evaluation of RNA integrity with Bioanalyzer	29
2.5.13. DNA sequencing	30
2.5.14. Illumina Next Generation Sequencing (NGS) and data analysis	30
2.5.15. Transformation of <i>E. coli</i> and <i>G. oxydans</i>	32
2.5.16. Generation of <i>G. oxydans</i> strains carrying in-frame deletions	33
2.5.17. Plasmid-based expression of genes	35
2.6. Biochemical methods	35
2.6.1. Protein overproduction and purification	35
2.6.1.1. Heterologous overproduction of proteins in <i>E. coli</i>	35
2.6.1.2. Heterologous overproduction of proteins in <i>G. oxydans</i>	36
2.6.1.3. Cell disruption and extraction of crude cell extract	36
2.6.1.4. Protein purification by Strep-Tactin Affinity chromatography	36
2.6.2. Extraction of cell cytoplasm	37
2.6.3. Quantification of protein concentration	37
2.6.4. PolyAcrylamide Gel Electrophoresis (PAGE)	38
2.6.4.1. Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis	38
2.6.4.2. Native PAGE	38
2.6.5. Silver staining	39
2.6.6. Immunoblot	39
2.6.7. Activity staining	40
2.6.8. Determination of enzymatic activities	41
2.6.8.1. Measurement of activities of NAD(P)H dependent oxidoreductases	41
2.6.8.2. Glucose isomerase: enzyme assay with auxillary enzymes	43
2.7. Analytical methods	44
2.7.1. Sample preparation for the determination of intracellular solutes	44
2.7.2. High Performance Liquid Chromatography	45
2.7.3. Photometric determination of glucose and fructose concentration	46
2.7.4. ¹³ C-Nuclear magnetic resonance spectroscopy	46

2.7.5. Microscopy	47
2.8. Internet tools used for bioinformatic analysis	47
3. RESULTS	49
3.1 Osmotic stress responses in <i>G. oxydans</i>	50
3.1.1. Choice of substrate and osmolyte	50
3.1.2. Expression studies of osmotically regulated genes in <i>G. oxydans</i>	52
3.1.3. Analysis of osmotic stress responses in <i>G. oxydans</i> through genome-wide transcriptome analysis	54
3.1.3.1. Quality testing of RNA samples	55
3.1.3.2. Differential gene expression under osmotic stress analyzed by transcriptome sequencing	57
3.1.3.3. Validation of the transcriptome data and selection of candidate genes	63
3.1.3.4. Generation of <i>G. oxydans</i> strains carrying in-frame deletions for <i>gox1118</i> and <i>gox1119</i>	64
3.1.3.5. Characterization of Gox1849: an uncharacterized oxidoreductase	68
3.2. Mannitol as a major intracellular metabolite and osmolyte in <i>G. oxydans</i>	70
3.2.1. ¹³ C-NMR spectroscopy of total cellular metabolites from <i>G. oxydans</i>	70
3.2.2. Effect of mannitol on cellular catalytic activity	72
3.2.3. De novo synthesis of mannitol in <i>G. oxydans</i> under reduced water activity	74
3.2.4. Effect of carbon sources and osmolytes on mannitol accumulation	79
3.2.5. Effect of exogenous mannitol on growth and morphology of osmotically stressed cells	80
3.2.6. Effect of polyols on growth and substrate oxidation rates of <i>G. oxydans</i>	83
3.3. Enzymatic routes for the biosynthesis of mannitol in <i>G. oxydans</i>	84
3.3.1. Identification and bioinformatic analysis of the mannitol dehydrogenases from <i>G. oxydans</i>	85
3.3.2. Characterization of the mannitol dehydrogenases from <i>G. oxydans</i>	87
3.3.3. Transcript abundance of genes coding for D-fructose reductases in <i>G. oxydans</i>	91
3.3.4. Characterization of fructose reductase deletion mutants	92
3.3.4.1. Effect of the deletion of fructose reductases on growth of <i>G. oxydans</i>	92
3.3.4.2. Intracellular mannitol formation and activity of cytoplasmic fructose reductases	97

3.3.4.3. Effect of the deletion of fructose reductases on cellular catalytic efficiency	101
3.4. Metabolic engineering of <i>G. oxydans</i> for enhanced osmotolerance	102
3.4.1. Overproduction of D-fructose reductase (Gox1432) in <i>G. oxydans</i>	103
3.4.2. Heterologous overproduction of glucose isomerases in <i>G. oxydans</i>	104
4. DISCUSSION	108
4.1. Osmotic stress responses in <i>G. oxydans</i>	111
4.1.2. Response of <i>G. oxydans</i> to osmotic stress at transcriptional level	113
4.1.3. Genome-wide transcriptome analysis of osmotically stressed cells of <i>G. oxydans</i>	113
4.2. Mannitol as a major intracellular metabolite and osmolyte in <i>G. oxydans</i>	116
4.2.1. De novo synthesis of mannitol in <i>G. oxydans</i> under reduced water activity	118
4.2.2. Osmodependent accumulation of mannitol in <i>G. oxydans</i>	119
4.2.3. Effect of carbon sources and osmolytes on mannitol accumulation in <i>G. oxydans</i>	120
4.2.4. Protective effect of mannitol on cell physiology	121
4.2.5. Effect of polyols on growth and substrate oxidation rates of <i>G. oxydans</i>	125
4.3. Biosynthesis of mannitol in <i>G. oxydans</i>	126
4.4. Characterization of the relative contribution of Gox1432 and Gox0849 in cellular osmoprotection	131
4.5. Characterization of fructose reductase deletion mutants	133
4.6. Metabolic engineering of <i>G. oxydans</i> for enhanced osmotolerance	134
4.6.1. Overproduction of D-fructose reductase (Gox1432)	135
4.6.2. Heterologous overproduction of glucose isomerases in <i>G. oxydans</i>	136
4.7. Gox1432: key player for osmotolerance of <i>G. oxydans</i>	138
5. SUMMARY	140
6. REFERENCES	142
7. CURRICULUM VITAE	165
8. ACKNOWLEDGEMENTS	167

ABBREVIATION LIST

ACN	Acetonitrile
ADP	Adenosine diphosphate
α	Alpha
Amp	Ampicillin
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	Base pair
β	Beta
cDNA	Complementary DNA
cdw	Cell dry weight
$^{\circ}\text{C}$	Degree centigrade
δ	Chemical shift
ddH ₂ O	Double distilled water
DMSO	Dimethyl sulfoxide
DNase	Deoxyribonuclease
dNTP	Desoxyribonucleotide triphosphate
DSMZ	German Collection of Microorganisms and Cell cultures
<i>e.g.</i> ,	<i>exempli gratia</i> (For example)
EDP	Entner-Doudoroff pathway
EDTA	Ethylenediaminetetraacetic acid
EMP	Embden-Meyerhof-Parnas glycolytic pathway
<i>et al.</i>	<i>et alii</i> (and others)
FAD	Flavin adenine dinucleotide
5-FC	5-fluorocytosine
For	Forward
<i>g</i>	Gravitational acceleration (9.8 m/s ²)
GI	Glucose isomerase
Gox	<i>Gluconobacter oxydans</i>
h	Hour
H ₂ O ₂	Hydrogen peroxide
H ₂ O _{dest}	Destillata (Distilled water)
H ₂ SO ₄	Sulfuric acid
HABA	4-hydroxyazobenzen-2-carbonic acid
HCl	Hydrogen chloride
HEPES	2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethanesulfonic acid
HPLC	High performance liquid chromatography
K _m	Kanamycin
kb	Kilobase
<i>K</i> _{cat}	Turnover number
kDa	Kilodalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
kg	Kilogram
<i>K</i> _M	Michaelis Menten constant
K-phosphate	Potassium phosphate
LB	Lysogeny broth
M	Molar (mol L ⁻¹)
mbar	Millibar

Mbp	Megabase pair
MDH	Mannitol dehydrogenase
MgCl ₂	Magnesium chloride
MgSO ₄	Magnesium sulphate
μ	Micro
min	Minute
MOPS	3-(N-morpholino)-propansulfonic acid
mRNA	Messenger RNA
ms	Millisecond
NaCl	Sodium chloride
NAD ⁺	Nicotinamide adenine dinucleotide
NADP ⁺	Nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology information
ng	Nanogram
NGS	Next generation sequencing
NMR	Nuclear magnetic resonance
OD	Optical density
Osm	Number of osmoles
ox. PPP	Oxidative pentose phosphate pathway
P	Phosphate
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
%	Percent (grams per 100 mL or mL per 100 mL)
pg	Picogram
P _i	Inorganic phosphate
PPP	Pentose phosphate pathway
PQQ	Pyrroloquinoline quinone
R ²	The Pearson correlation coefficient of determination
rev	Reverse
rpm	Revolutions per minute
RNA-Seq	RNA sequencing
<i>RPKM</i>	Reads Per Kilobase of transcript per Million mapped reads
rRNA	Ribosomal RNA
RT-qPCR	Real Time quantitative reverse transcription PCR
SDS	Sodium dodecyl sulphate
SOC	Super optimal broth with catabolite repression
sp.	Species
TCA	Tricarboxylic acid cycle
TEMED	<i>N,N,N',N'</i> -Tetramethylethane-1,2-diamine
TMSP	Trimethylsilylpropanoic acid
TRI-reagent	Trizol reagent
Tris	Tris(hydroxymethyl)-aminomethane
U	Unit
UV	Ultraviolet
V	Volt
v/v	Volume per volume
V _{max}	Maximal reaction rate
w/v	Weight per volume
XI	Xylose isomerase

1. INTRODUCTION

1.1. Acetic acid bacteria

Acetic acid bacteria are Gram-negative, rod-shaped, and obligate aerobic microorganisms, which belong to the family *Acetobacteraceae*, within the class of Alphaproteobacteria (Gillis and De Ley 1980, Yamada *et al.* 1997). At present, the family *Acetobacteraceae* is divided into 17 genera (*Acetobacter*, *Gluconobacter*, *Gluconacetobacter*, *Asaia*, *Komagataeibacter*, *Acidomonas*, *Kozakia*, *Swaminathania*, *Saccharibacter*, *Neoasaia*, *Granulibacter*, *Tanticharoenia*, *Ameyamaea*, *Neokomagataea*, *Endobacter*, *Nguyenibacter*, and *Swingsia*). The first five genera contain a large number of species while the remaining 12 genera have only one species (monotypic) except the genus *Neokomagataea*, which consists of two species (Yamada 2016). Acetic acid bacteria have been known for decades to thrive in acidic and sugar-rich environments such as fruit juices, flowers, nectars, alcoholic and soft drinks (Asai 1935, Gillis and De Ley 1980, Gupta *et al.* 2001, Battey and Schaffner 2001). Recently, some bacteria have been isolated from other sources including human patients, mosquitoes, soil rhizosphere, nodules, and activated sludges (Yamashita *et al.* 2004, Greenberg *et al.* 2006, Komagata *et al.* 2014). With the exception of the genus *Granulibacter*, all other acetic acid bacteria are non-pathogenic to humans and animals (De Ley and Swings 1984, Greenberg *et al.* 2006).

Acetic acid bacteria perform an overflow metabolism which is characterized by the phenomenon of incomplete oxidation of growth substrates (alcohols, sugars, or sugar acids). The corresponding partially-oxidized products are excreted and accumulate in the culture medium. As a result of incomplete oxidation of growth substrates, low amounts of energy are conserved by the organisms, resulting in low growth yields (Olijve and Kok 1979, De Ley and Swings 1984, Deppenmeier and Ehrenreich 2009). The most conventional process is the oxidation of ethanol to acetic acid, carried out by almost all genera of acetic acid bacteria (Sievers and Swings 2005). Due to rapid and incomplete oxidative fermentations of sugars, sugar alcohols, and sugar acids, acetic acid bacteria are widely used in various biotechnological processes such as the production of vinegar and the synthesis of precursors of vitamin C (Tsukada and Perlman 1972, Hoshino *et al.* 1990, Sugisawa *et al.* 1995, Saeki *et al.* 1997, Macauley *et al.* 2001, Adachi *et al.* 2003). Furthermore, they are used for the production of the anti-diabetic drug miglitol, shikimate, and dihydroxyacetone (Claret *et al.* 1994, Schedel 2000, Gupta *et al.* 2001, Asano 2003, Deppenmeier *et al.* 2002, Bauer *et al.* 2005, Adachi *et al.* 2006, Raspor and Goranovič 2008, Mamlouk and Gullo 2013).

On the basis of acetate utilization, acetic acid bacteria are broadly classified into two groups referred to as acetate oxidizers and non-acetate oxidizers. Acetate oxidizers assimilate acetic acid by oxidation to CO₂ through the TCA and the glyoxylate cycles. These include members of the genera *Acetobacter*, *Gluconacetobacter*, *Kozakia*, *Acidomonas*, and *Asaia*. Non-acetate oxidizers include the genus *Gluconobacter*. These organisms are unable to oxidize acetate to CO₂ due to the lack of the glyoxylate cycle and presence of an incomplete TCA cycle (Greenfield and Claus 1972, Chinnawirotpisan *et al.* 2003, Prust *et al.* 2005, Yamada and Yukphan 2008). An acetate oxidation test is routinely used as a conventional phenotypic test for the identification of closely related genera *Acetobacter* and *Gluconobacter* (Yamada and Yukphan 2008). At the genetic level, the differentiation between the genera is possible through 16S rRNA gene sequence analysis (Sievers *et al.* 1995, Komagata *et al.* 2014).

1.2. The genus *Gluconobacter*

The generic name *Gluconobacter* was introduced in the family *Acetobacteraceae* by Asai (1935) for the strains of acetic acid bacteria that are capable of producing large amounts of gluconates from glucose. These strains proliferate in sugar-rich niches such as flowers, honey bees, and fruits and oxidize glucose more efficiently than ethanol. According to the NCBI Taxonomy Browser (<https://www.ncbi.nlm.nih.gov/Taxonomy/Browser>), the genus *Gluconobacter* splits into 15 species (*G. albidus*, *G. cerevisiae*, *G. kondonii*, *G. morbifer*, *G. sphaericus*, *G. kanchanaburiensis*, *G. roseus*, *G. uchimurae*, *G. cerinus*, *G. nephelii*, *G. wancherniae*, *G. thailandicus*, *G. frateurii*, *G. japonicus*, and the type species *G. oxydans*).

G. oxydans is a Gram-negative, strict aerobic, and rod-shaped bacterium, which can be present as single cells, in pairs, or in short chains (Fig. 1). Under oxygen limitations, the bacterium is motile with the formation of 3-8 polar flagella (Gupta *et al.* 2001, De Muyneck *et al.* 2007, Deppenmeier and Ehrenreich 2009). The organism grows optimally in highly concentrated sugar environments at 25-30°C. The optimum pH for growth is in range of 5.5-6.0 (Olijve and Kok 1979). However, most of the strains can grow even at acidic pH (≤ 4.0). During cultivation on complex medium with glucose as substrate, *G. oxydans* cells can grow with reasonable growth rates at a constant pH of 2.5 (Olijve and Kok 1979). In addition, the organism can grow to higher cell densities in complex media containing yeast extract and high amounts of sugars or polyols in comparison to chemically defined media (Olijve and Kok 1979, Raspor and Goranovič 2008).

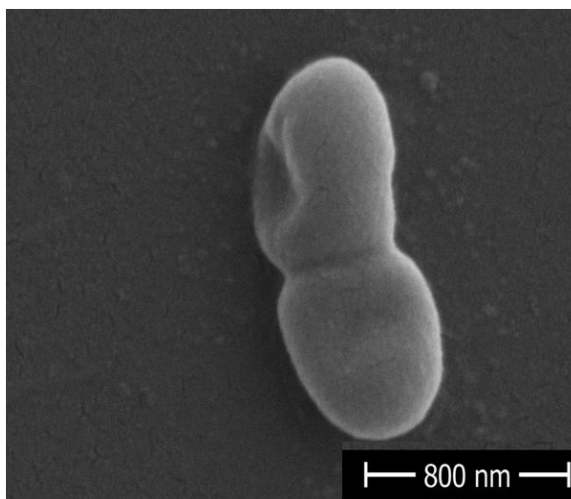


Figure 1: Scanning electron microscope image of *G. oxydans*

(The micrograph was kindly provided by K. Kosciow from the Institute for Microbiology and Biotechnology of University Bonn, and was developed by H. J. Ensikat at the Nees-Institute for biodiversity of plants of University Bonn, Germany).

The complete genome of *G. oxydans* 621H was sequenced in 2005 by Prust *et al.* The genomic data provide extensive insights into the overall metabolic pathways of the organism and is helpful to understand its physiology during growth under extreme conditions. The genome consists of a circular chromosome (2.7 Mbp) and 5 plasmids (0.22 Mbp) with 2735 open reading frames (Prust *et al.* 2005). Genome analysis revealed the presence of many genes encoding membrane-bound dehydrogenases, which are involved in rapid and incomplete oxidation of a multitude of sugars and sugar alcohols such as glucose, sorbose, ethanol, mannitol, sorbitol, and glycerol (De Ley *et al.* 1984, Prust *et al.* 2005, De Muyneck *et al.* 2007). The active centers of the enzymes are oriented towards the periplasm, facilitating the rapid oxidation of substrates at the cell membrane level. During the oxidation process, electrons are released that are transferred into the membrane-integral respiratory chain (Fig. 2). Here, electron carriers (ubiquinone-10) receive these electrons and transfer them via quinol oxidases to oxygen (a terminal electron acceptor). During this transfer process, protons are pumped across the cell-membrane, resulting in the generation of the electrochemical proton gradient that is a driving force for ATP production in a cell (Kulhanek 1989, Ameyama *et al.* 1987).

1.2.1. Respiratory chain of *G. oxydans*

The respiratory chain of the organism is branched at the ubiquinone level with a KCN-sensitive and an insensitive terminal oxidases of the *bo*₃- and the *bd*-type, respectively (Fig. 2). Both terminal oxidases transfer electrons from ubiquinol to molecular oxygen as terminal acceptor (Ameyama *et al.* 1987, Matsushita *et al.* 1989, Matsushita *et al.* 1994). Accordingly,

in the genome of *G. oxydans*, genes encoding the cytochrome *bd* (*cydAB*) and the cytochrome *bo*₃ (*cyoBCAD*) type quinol oxidase were identified (Prust *et al.* 2005). Among these two oxidases, the ubiquinol *bo*₃-type was found to be important for growth of the organism. It has higher affinity for oxygen in comparison to the *bd*-type oxidase. Deletion of the *bo*₃-type quinol oxidase resulted in severe growth retardation of *G. oxydans*, while the deletion of the *bd*-type oxidase had no impact on cell growth (Richhardt *et al.* 2013a). Moreover, the genome sequence revealed the presence of genes coding for a cytochrome *bc*₁ complex (*qcrABC*) and for a soluble cytochrome *c* (*cycA*). However, an exact function of these proteins could not be attributed because the genes encoding a cytochrome *c* oxidase (complex-IV) were not found. In addition, genes coding for the proton-translocating NADH: ubiquinone oxidoreductase (complex-I) were also missing. Rather than complex-I, *G. oxydans* possesses a gene encoding the type II NADH dehydrogenase, which does not translocate protons (Prust *et al.* 2005).

Due to the absence of complex-I and complex-IV, the ability of the respiratory chain to translocate protons across the membrane is limited. Only the cytochrome *bo*₃-type quinol oxidase is able to pump protons during the transfer of electrons from ubiquinol to terminal oxygen molecule (Matsushita *et al.* 1987). The quinol oxidase of *bd*-type does not pump protons, but participates in the generation of proton motive force (Miller and Gennis 1985) through the oxidation of quinol near the periplasmic surface and the uptake of protons from the cytoplasm to form water (Miller and Gennis 1985, Dueweke and Gennis 1991, Borisov *et al.* 2011). Proton translocation might also be carried out by another route involving a membrane-bound nicotinamide dinucleotide transhydrogenase. The genome data revealed the presence of three genes coding for such a transhydrogenase. In other organisms, these enzymes perform a reversible hydride transfer between NADPH + H⁺ and NAD⁺, along with the transfer of protons across the membrane (Cotton *et al.* 2001, Bizouarn *et al.* 2002). In *G. oxydans*, this membrane-bound transhydrogenase may contribute to the generation of an electrochemical proton gradient, which is required for ATP synthesis by the F₁F_o-type ATP synthase (Prust *et al.* 2005).

In comparison to its enormous potential for rapid oxidation of substrates, the ability of *G. oxydans* for energy transduction is limited. This is probably due to the inadequate coupling of the electron transport with proton translocation that results in low H⁺/e⁻ ratios of 0.5-1.0. Therefore, only a small amount of energy is conserved by the organism, which limits its growth rates and cell yields, while most part of the energy is lost in the form of heat (Matsushita *et al.* 1989).

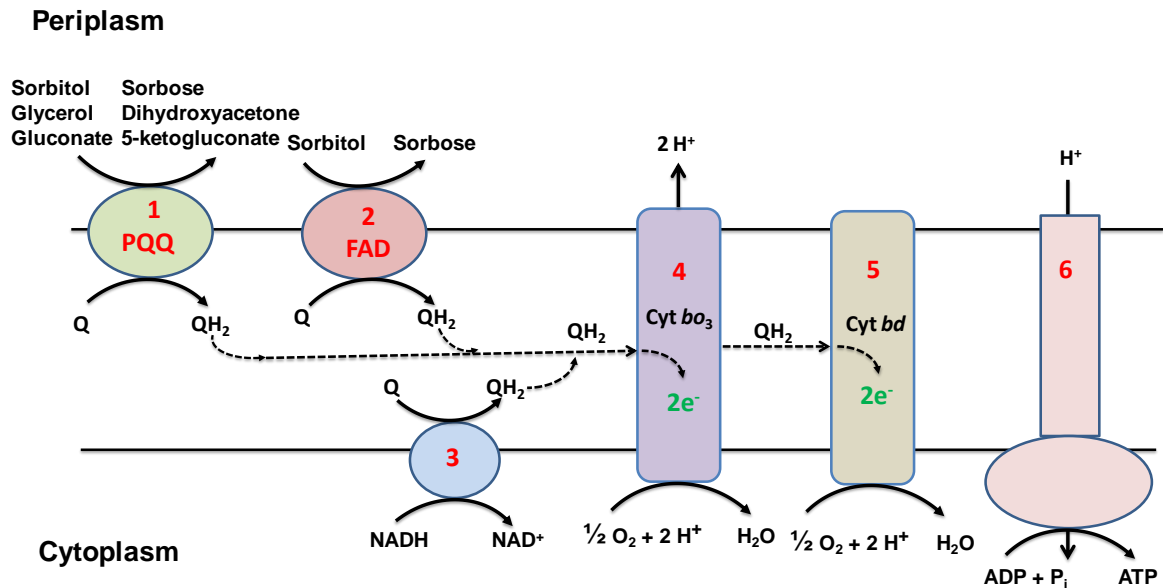


Figure 2: Schematic representation of the respiratory chain of *G. oxydans*. (1) PQQ-dependent polyol dehydrogenase, (2) FAD-dependent sorbitol dehydrogenase, (3) NADH-dehydrogenase (Type-II), (4) quinol oxidase (*bo*₃-type), (5) quinol oxidase (*bd*-type), (6) F₁F₀-ATP-synthase. Q: Ubiquinone (electron acceptor), QH₂: Ubiquinol (electron carrier). (Modified from Deppenemeier and Ehrenreich 2009).

In several aerobic bacteria, the production of cell biomass correlates with the respiration-driven proton extrusion ratio (H^+/O). For example, *Acetobacter pasteurianus* has a cell yield of 13.1 g_{cdw} per mole ethanol with a H^+/O ratio of 1.9 ± 0.1 (Luttik *et al.* 1997). Likewise, cultivation of *G. oxydans* under pH and aeration control conditions leads to a cell biomass of 16.2 g_{cdw} per mole glucose with a H^+/O ratio of 1.8 ± 0.3 (Olijve and Kok 1979, Matsushita *et al.* 1989, Krajewski *et al.* 2010, Richhardt *et al.* 2012, Richhardt *et al.* 2013a). The H^+/O ratio of *G. oxydans* is almost 50 % lower than that of *E. coli* (H^+/O ratios 3.0 - 4.9). Moreover, the *E. coli* culture also attains higher cell yields of 68-90 g_{cdw} per mole glucose than that of *G. oxydans* (Harrison and Loveless 1971, Lawford and Haddock 1973, Minohara *et al.* 2002, Soini *et al.* 2008, Richhardt *et al.* 2013a). These observations suggest that the poor growth yield of *G. oxydans* is linked to the low H^+/e^- and H^+/O ratios. The presence of such an intriguing and inefficient energy transduction mechanism might have an ecological relevance for the organism. In natural sugar-rich environments, *G. oxydans* co-exists with other microorganisms and competes for available growth substrates. The presence of membrane-bound dehydrogenases provide benefit to the organism for rapid oxidation and utilization of the substrates at the periplasmic or the cell membrane level, without any delay for intracellular transport. This may limit the availability of the substrates to other

microorganisms. Meanwhile, *G. oxydans* excrete the oxidized products to the environment, which might be difficult to assimilate by the others. Thus, *G. oxydans* probably outcompetes the nutritional competitors in ecological niches. Moreover, in the context of less efficient proton translocation, the rapid oxidation of substrates is also mandatory for the organism to generate the sufficient electrochemical proton gradient across the membrane for energy conservation and cellular maintenance. Thus, the simple respiratory chain facilitates such uninterrupted and accelerated incomplete oxidation of carbon substrates via membrane associated dehydrogenases (McNeil and Harvey 2005, Komagata *et al.* 2014).

1.2.2. Intracellular carbohydrate metabolism in *G. oxydans*

Gluconobacter strains transport only very small amount of substrates or oxidized products into the cell cytoplasm for assimilation. The intracellular metabolism of the oxidized products is carried out either by soluble NAD(P)⁺-dependent oxidoreductases, by the enzymes of the oxidative pentose phosphate pathway (PPP), or the Entner-Doudoroff pathway (EDP) (Olijve and Kok 1979, Shinjoh *et al.* 1990, Matsushita *et al.* 1994, Sievers and Swings 2005, Rauch *et al.* 2010). The genome data of *G. oxydans* 621H depict the presence of a complete set of genes for the PPP and the EDP (Prust *et al.* 2005). In contrast, the Embden-Meyerhof-Parnas pathway (EMP) and the TCA cycle are incomplete due to the absence of genes encoding a 6-phosphofruktokinase and a succinate dehydrogenase, respectively. In addition, other pathways for carbohydrate metabolism such as the glyoxylate cycle and the gluconeogenesis are also absent as none of the key genes of these pathways are encoded on the genome of *G. oxydans* (Prust *et al.* 2005, De Ley and Swings 1984, Greenfield and Claus 1972, Deppenmeier and Ehrenreich 2009).

Between the two functional pathways for intracellular sugar catabolism, the PPP is of major importance for *G. oxydans* rather than the EDP. Mutant studies revealed that the deletion of the PPP gene *gox1705* (6-phosphogluconate dehydrogenase) resulted in reduced growth of the organism with mannitol and glucose as carbon sources. Moreover, the deletion of the gene induced a strong selection pressure for a second mutation of gene (*gox0145*) encoding a glucose-6-phosphate dehydrogenase. The resulting double mutant was inefficient to grow on fructose as well as on gluconates. In contrast, the deletion of the EDP genes *gox0430* (6-phosphogluconate dehydratase) and *gox0431* (2-keto-3-deoxy-6-phosphogluconate aldolase) had a relatively beneficial effect on growth and substrate utilization efficiency of the mutant strain in comparison to the reference strain with mannitol as substrate. While on glucose, the growth characteristics of the mutant strain were unaltered and comparable to the wild type.

These observations highlight the importance of the PPP for *G. oxydans* to assimilate sugar, sugar alcohols, and their oxidized products inside the cell. In such context, the EDP pathway is of minor importance or is dispensable for the organism (Richhardt *et al.* 2012, Richhardt *et al.* 2013b).

1.2.3. Types and function of sugars and polyols metabolizing enzymes in *Gluconobacter*

Gluconobacter strains have two different enzyme systems for the oxidation and utilization of various substrate molecules. These enzyme systems vary from one another with respect to their sub-cellular location, cofactor specificity, and function within the cell (Fig. 2 and Fig. 3). One set, comprising membrane-bound enzymes, utilizes PQQ, FAD, or heme c as prosthetic groups (Adachi *et al.* 1978, Ameyama *et al.* 1981, Shinagawa *et al.* 1984, Choi *et al.* 1995, Hölscher and Görisch 2006). These dehydrogenases perform the oxidation of substrates in a regio- and stereoselective manner at a pH range of 3.0-6.0 (Adachi *et al.* 2001b). Furthermore, the enzymes catalyze the oxidation of a broad range of substrates and some of them even share overlapping substrate spectra (De Muynck *et al.* 2007, Peters *et al.* 2013a). Some examples of such enzymes are the glucose dehydrogenase (Gox0265), the alcohol dehydrogenase (Gox0756, Gox1067 and Gox1068), the aldehyde dehydrogenase (Gox0585 and Gox0587), the gluconate dehydrogenase (Gox1230 and Gox1232), and the polyol dehydrogenase (Gox0854 and Gox0855) (Adachi *et al.* 1978, Adachi *et al.* 1980, Ameyama *et al.* 1981, Shinagawa *et al.* 1984, Adachi *et al.* 2001b, Matsushita *et al.* 2003, Prust *et al.* 2005). These enzymes enable the organism to catalyze the incomplete oxidation of substrates in a single step. The oxidized products are released into the culture medium in almost equal amounts. Due to the catalytic robustness and nearly quantitative excretion of products, whole cells of *Gluconobacter* sp. are commercially used in several biotransformation process (Shinjoh and Toyama 2016).

The second set of enzymes is confined to the cytoplasm of cells and referred to as cytoplasmic or soluble oxidoreductases (Fig. 3). These enzymes can catalyze both oxidation as well as reduction reactions and require NAD(P)⁺ or NAD(P)H as cofactors. They are involved in intracellular assimilation of oxidized products, biosynthesis of precursor molecules, and in maintenance of cellular functions (Adachi *et al.* 1991a, Adachi *et al.* 1991b, Adachi *et al.* 2001a, Parmentier *et al.* 2005). Examples of such alcohol-sugar oxidoreductases are the NADPH dependent L-sorbose reductase (Gox0849), the NAD⁺-specific xylitol dehydrogenase (Gox0865), the NADP⁺-dependent glucose-6-phosphate

dehydrogenase (Gox0145), and the NAD⁺-specific 6-phosphogluconate dehydrogenase (Gox1705) (Shinjoh *et al.* 2002, Sugiyama *et al.* 2003, Rauch *et al.* 2010).

Both enzyme systems operate side by side at their respective cellular levels and enable *Gluconobacter* sp. for efficient conversion and utilization of growth substrates. As an example, the metabolic pathways involved in the oxidation and intracellular assimilation of D-sorbitol are indicated in Figure 3. D-sorbitol is oxidized by the membrane bound D-sorbitol dehydrogenase to L-sorbose, which is further oxidized to L-sorbosone by the membrane-bound L-sorbose dehydrogenase. A major portion of both L-sorbose and L-sorbosone is excreted by into the culture medium. Only a small fraction of the substrates is transported into the cell, where L-sorbosone is reduced to L-sorbose by the catalytic activity of cytosolic L-sorbosone reductase. L-sorbose is further reduced to D-sorbitol by the soluble L-sorbose reductase. Then, D-sorbitol is oxidized to D-fructose by the catalytic activity of cytoplasmic D-sorbitol dehydrogenase. D-fructose is then channeled after phosphorylation into the PPP or the EDP by a cascade of enzymatic reactions (Reactions 6-11, Fig. 3) (Shinagawa *et al.* 1982, Sugisawa *et al.* 1991, Adachi *et al.* 1999a, Adachi *et al.* 1999b, Shibata *et al.* 2000, Shinjoh *et al.* 2002, Toyama *et al.* 2005, Soemphol *et al.* 2007). In the similar way, metabolism of other sugars or polyols is carried out by the organisms using both type of enzyme systems. However, a major part of the substrates is oxidized at the periplasmic level by the membrane-integral enzymes, while a minor portion is taken up by the cells and catalyzed by the soluble oxidoreductases (Hanke *et al.* 2013).

1.2.4. Biotechnological applications of *G. oxydans* and its limitations

G. oxydans has tremendous potential for oxidative fermentation of sugars, sugar acids, and polyols. The special feature is attributed to the organism due to the presence of membrane-bound enzymes that oxidize the substrates in an enantio- and regioselective manner. Moreover, the organism utilizes a small amount of the substrate for biomass production and excrete almost 90% of the oxidized products into the culture media (Hanke *et al.* 2013). These properties make *G. oxydans* an ideal organism for various bio-based applications on industrial-scale (Macauley *et al.* 2001). For example, intact cells of the organism are commercially used as biosensors for detection of different alcohols, sugars, and sugar derivatives (Reshetilov *et al.* 1997, Reshetilov *et al.* 1998, Lusta and Reshetilov 1998, Svitel *et al.* 1998). Moreover, the intact cells or the purified proteins are also used in several biotechnological process for production of valuable compounds. Examples for economically-important chemicals produced by *G. oxydans* are intermediates for the synthesis of vitamin C

and the antidiabetic drug miglitol (Saito *et al.* 1997, Saito *et al.* 1998, Schedel 2000). Furthermore, the cells are used for the production of gluconates (sequestering agents in food and pharmaceutical industry), ketogluconates (precursors of isoascorbic acid and tartaric acid), and the tanning agent dihydroxyacetone (Prescott and Dunn 1959, Meiberg and Spa 1983, Weenk *et al.* 1984, Klasen *et al.* 1995, Svitel and Sturdik 1994, Gupta *et al.* 2001, Tkac *et al.* 2001, Deppenmeier *et al.* 2002, Bauer *et al.* 2005, Elfari *et al.* 2005, Merfort *et al.* 2006).

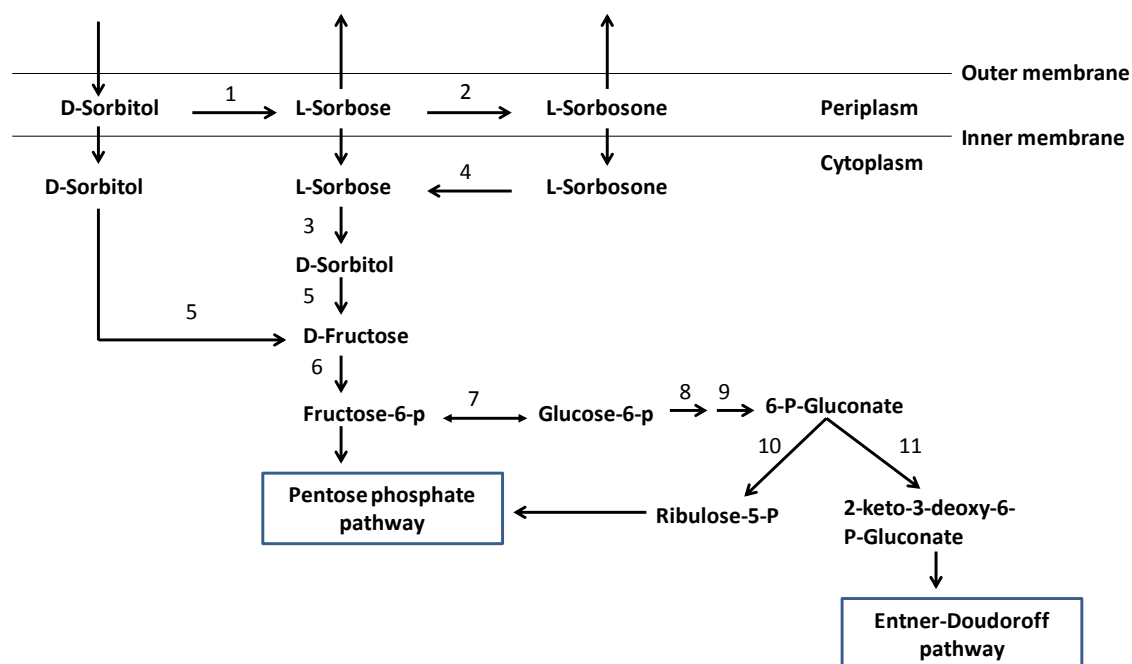


Figure 3: Schematic representation of metabolic pathways for the oxidation and the intracellular assimilation of D-sorbitol and its derivatives in *Gluconobacter* sp. (1) membrane-bound D-sorbitol dehydrogenases, (2) membrane-bound L-sorbose dehydrogenase, (3) NAD(P)H-dependent L-sorbose reductase, (4) NAD(P)H-dependent L-sorbosone reductase, (5) NAD(P)⁺-dependent D-sorbitol dehydrogenase, (6) fructose kinase, (7) glucose-6-phosphate isomerase, (8) glucose-6-phosphate dehydrogenase, (9) 6-phosphogluconolactonase, (10) 6-phosphogluconate dehydrogenase, (11) phosphogluconate dehydratase. (Modified from Shinjoh *et al.* 2002, Deppenmeier *et al.* 2002).

Despite its great biotechnological importance, the use of *G. oxydans* in whole-cell biotransformation processes is often limited due to low biomass production. In industrial-scale fermentations, high-cell densities of bacterial cultures are required for volumetric production of valuable compounds in a cost effective manner. Many studies have been carried out to enhance the cell density of *Gluconobacter* species by cultivation under aeration

and pH control conditions (Mori *et al.* 1981, Rosenberg *et al.* 1993). In fed-batch cultures, continuous supply of nutrients plus oxygen result in high biomass production. For example, the maximum L-sorbose concentration of 628 g L⁻¹ and the biomass concentration of 6 g L⁻¹ were achieved during fed-batch cultivation of *G. oxydans* with D-sorbitol. In contrast, the productivity in batch-cultures was relatively low with 190 g L⁻¹ of sorbose and 2 g L⁻¹ of biomass (Yamada *et al.* 1979, Mori *et al.* 1981).

However, growth of cultures in fed-batch settings is often subjected to the inhibitory osmotic stress, imposed by high concentrations of substrates and products that accumulate in the media. The accumulation of L-sorbose up to a concentration of 510 g L⁻¹ inhibits growth of *G. oxydans* and decreases the sorbitol oxidation rate (Mori *et al.* 1981). Likewise, another important parameter, having an impact on growth and productivity of *G. oxydans*, is the pH value of fermentation medium which is often controlled either by the addition of highly concentrated buffers (for shake flask cultures) or by titration (Olijve and Kok 1979, Silberbach *et al.* 2003). However, the addition of highly concentrated soluble buffers or salts to the growth medium increases its osmotic pressure that limits culture proliferation and may inhibit product formation (Kumar *et al.* 2004, Jeude *et al.* 2006). It has been studied that the growth of *G. oxydans* is reduced at an osmolality value of > 0.5 Osm kg⁻¹, raised by the addition of soluble buffers or salts, in the culture medium (Luchterhand *et al.* 2015).

1.3. Aims of the work

Due to its industrial relevance, *G. oxydans* has been extensively studied for many decades, particularly after the publication of the complete genome sequence (Prust *et al.* 2005). Most of the studies deal with the identification and characterization of its potential dehydrogenases and oxidoreductases (Adachi *et al.* 1999a, Adachi *et al.* 1999b, Hoshino *et al.* 2003, Schweiger *et al.* 2007, Schweiger *et al.* 2010, Meyer *et al.* 2015). Some studies focus on the investigation of the intracellular metabolic routes of the organism (Rauch *et al.* 2010, Richhardt *et al.* 2012, Richhardt *et al.* 2013b). Recently, some other aspects such as expansion of the substrate spectrum of *G. oxydans* (Kosciow *et al.* 2014, Kosciow *et al.* 2016), and the construction of molecular tools for its metabolic engineering have gained considerable interests (Kallnik *et al.* 2010, Kostner *et al.* 2013).

So far, very little attention has been paid to understand the survival mechanisms of the organism under osmotic stress conditions. *G. oxydans* frequently encounters the osmotic stress during growth in natural habitats or under controlled conditions in fermenters. In the

natural environment, the variable precipitation rates, desiccation periods, and the seasonal effects exert osmotic pressure on the living organisms. Analogously, the use of high-sugar concentrations in industrial-scale fermentation decreases the water activity of the growth media and imposes osmotic stress to the cultures. Therefore, there is a need to investigate the osmoprotective mechanisms present in *G. oxydans* to elucidate its physiology and response under osmotic stress.

The aim of the present work is the investigation of the effects of external osmotic pressure on growth and cellular functions of *G. oxydans*. To achieve these targets, two initial strategies are designed. In the first instance, the difference in global gene expression pattern of the organism under osmotic stress conditions in comparison to non-stress conditions is planned to be examined by Next-Generation Sequencing (NGS). The second strategy focuses on the identification of potential compatible solutes, accumulate in the cells under osmotic stress. Therefore, experiments are designed targeting the analysis of total cellular extracts by ¹³C-NMR spectroscopy and HPLC.

Further objectives include the identification and characterization of genetic elements responsible for the synthesis of compatible solutes in *G. oxydans*. To achieve these tasks, experiments are planned to generate in-frame deletion mutants lacking the osmoprotectant synthesizing genes, and to analyze the effect of deletions on the osmotic tolerance of the mutants. It is expected that the data will provide a plethora of information regarding the osmoprotection of *G. oxydans*, and will be useful for the rational engineering of osmotolerant strains for future biotechnological applications.

2. MATERIALS AND METHODS

2.1. Chemicals and Enzymes

All media components, chemicals, and antibiotics were purchased from Sigma-Aldrich (München, Germany), Merck KGaA (Darmstadt, Germany), Carl Roth GmbH (Karlsruhe, Germany), or Fluka (Sigma-Aldrich München, Germany). Enzymes, PCR reagents, and other biochemicals were obtained from Thermo Fisher Scientific Biosciences GmbH (Schwerte, Germany). Phusion DNA polymerase was purchased from Biozym Scientific GmbH (Oldendorf, Germany). NAD⁺, NADPH, and NADH were obtained from Carl Roth GmbH (Karlsruhe, Germany), while NADP⁺ was ordered from Merck KGaA (Darmstadt, Germany).

2.2. Bacterial strains, plasmids, primers

2.2.1. Bacterial strains

Organisms used in the present work were *Escherichia (E.) coli* (strains K12, NEB 5- α , BL21, and ECG18), *Gluconobacter (G.) oxydans* 621H (Δ *hsdR*), and *Streptomyces (S.) griseus*. The genotype of the organisms is listed in Table 1.

Table 1: Bacterial strains used in the present study and their genotypes

Strain	Genotype	Reference
<i>E. coli</i> (NEB 5- α)	Derivative of DH α <i>fhuA2</i> , Δ (<i>argF-lacZ</i>)U169, <i>phoA</i> , <i>glnV44</i> , ϕ 80 Δ (<i>lacZ</i>)M15, <i>gyrA96</i> , <i>recA1</i> , <i>relA1</i> , <i>endA1</i> , <i>thi-1</i> , <i>hsdR17</i>	New England Biolabs, Frankfurt am Main, Germany
<i>E. coli</i> BL21 (DE3)	F ⁻ , <i>ompT</i> , <i>gal</i> , <i>dcm</i> , <i>hsdSB</i> (<i>rB</i> ⁻ <i>mB</i> ⁻)	Invitrogen, Carlsbad, USA
<i>G. oxydans</i> 621H Δ <i>hsdR</i>	Δ <i>gox2567</i> derivative of <i>G. oxydans</i> 621H (DSM 2343), <i>Cef</i> ^R , referred to as wild type throughout the work	S. Bringer-Meyer, research center Juelich GmbH, Germany
<i>E. coli</i> K12	Wild type	(DSM 498)
<i>E. coli</i> ECG18 (pRK 2013)	HB 101, <i>lamB</i> pRK2013 (<i>Km</i> ^R , <i>oriColE1</i> , <i>Mob</i> , <i>RK2</i> , <i>Tra</i> ⁺)	(DSM 5599)
<i>S. griseus</i>	Type strain	(DSM 40236)

2.2.2. Oligonucleotides and Plasmids

Primers used in the present study were generated with an online program Primer3 (<http://bioinfo.ut.ee/primer3-0.4.0/primer3/>), the software Primer D'Signer (IBA GmbH, Göttingen, Germany), or designed manually. Synthesis of the oligonucleotides was done by Eurofins MWG Operon (Ebersberg, Germany). The nucleotide sequences of these primers are given in Table 2 with sites of restriction enzymes (underlined) and homologous sequences for fusion PCR (in bold).

Table 2: List of primers used in the study.

Primer	Sequence (5' → 3')	Restriction enzyme
Primers used for RT-qPCR		
Gox0352_fw	CATCATTGCCGGTGTTTTCG	
Gox0352_rev	GCGACACCTGCGAGAATG	
Gox0378_fw	AATACCGTCATGAACGCACA	
Gox0378_rev	TGGTTACGCTCGGAAAGAAG	
Gox0540_fw	GTCAACAACATCCTGGACCG	
Gox0540_rev	CAGAAATCCACCCGCAGC	
Gox0676_fw	GGAAGCCTATATCACCCGCA	
Gox0676_rev	AGATATCTTCCTGGGACGGC	
Gox0707_fw	TCCAGCATCGATGGCTTCTC	
Gox0707_rev	CGCTGTTCTTGGCAACATCC	
Gox0717_fw	AAGACGCTGAAAACCGGTTC	
Gox0717_rev	CGTCATGCCCAATTCGACAT	
Gox0820_fw	TCGAGGCTCTTGAAGCACAG	
Gox0820_rev	CACGGGCGAATTTCTGGATG	
Gox0849_fw	GACCGAACATCCTGCCATTG	
Gox0849_rev	ATTGACCTCGATCCACTCCG	
Gox0857_fw	GTTCTGGCTGACGAGTTCA	
Gox0857_rev	GGAGGACAGCTCGATCTTGG	

Gox1119_fw	CTCAAGGAAGAGCTGGACGG
Gox1119_rev	ACAAACCCCACATCTGCCAT
Gox1205_fw	ACGCCGATGGTCTGGAATAT
Gox1205_rev	CATGCCATCCAGTTTCTGCA
Gox1332_fw	TTCTCTACCGACGCTTTCCG
Gox1332_rev	GATCTTCGAGTTCGTCGGG
Gox1372_fw	GGTGTGCTGCTTCTTGGATC
Gox1372_rev	GATTTTCGGTTGTCCTGCCAC
Gox1432_fw	CATTGCGTGACTACCTGCTC
Gox1432_rev	CCTTTACTGCCGCATTCTCC
Gox1600_fw	CCTGACGCTTGTCTTCCACA
Gox1600_rev	GTCGCCAAGCCATTCAATCC
Gox1709_fw	AAACGCAGATGGGTCTGAAG
Gox1709_rev	CTGACCGGTGCTCATCTTCT
Gox1790_fw	TCCTGATGCTCGAGTCGGTA
Gox1790_rev	GGAGGCGGATTTCGTCTTCA
Gox1822_fw	CGTTCTCGTGGATGGCTACA
Gox1822_rev	TCACGGATGACGGTATTGGC
Gox1849_fw	TTGTGATGATGGGGCTGGAT
Gox1849_rev	CCGCCAGCTCTCACAAATAC
Gox2164_fw	GGGAGGAAGAGACCAGACTG
Gox2194_rev	GTTCTCGCTGTTTACAAGGG
Gox2182_fw	GGGTGGTGGTACAGTTCGAG
Gox2182_rev	CGCCACCACGATCTATGTGA
Gox2217_fw	CCATACCAGATTGCGTGCTG
Gox2217_rev	CACCAGCCTCATGAAATCCG
Gox2220_fw	ATGGCTGCGTCTGAAACTGA
Gox2220_rev	AAGCCCAGGATTTCTCCACG

Gox2667_fw	AAATGGATGGATGCGAACCG	
Gox2667_rev	GCCACTTGATAAACCGTCCC	
Primers used for generation of deletion mutants		
Up_gox0849_fw	ATAGGTACCGGCCCGGATGTTTCGAGCTGTC	<i>KpnI</i>
Up_gox0849_rev	CCAAAACTTGGACAGGATCTTTCAGGC GAAAGGACCGGC	
Do_gox0849_fw	GATCCTGTCCAAGTTTTTGGTGAAGCTTT TTCCAAAAAGC	
Do_gox0849_rev	GCGTCTAGAAGGGGCTTGAGACGCTGGGC GC	<i>XbaI</i>
Up_gox1118_fw	ATAGGTACCGTCTGGCAGATGAGCCCTGA	<i>KpnI</i>
Up_gox1118_rev	GCCACGAGGTGAAGCTGTCATTTCTTCCT GTTTCTGGGTC	
Do_gox1119_fw	TGACAGCTTCACCTCGTGGCCAAGGAGT TCATCGCGGCGC	
Do_gox1119_rev	GCGTCTAGAGCTACTGACAAAGGAGACAG	<i>XbaI</i>
Up_gox1432_fw	ATAGGTACCTGACGACGGCGCGGGACCAG	<i>KpnI</i>
Up_gox1432_rev	GAAGAAGAGGAGGAATTCTCCGTGGTTT CGGGCCGGTCG	
Do_gox1432_fw	GGAGAATTCCTCCTCTTCTTCAGGGCAC AAAAAACCGCGC	
Do_gox1432_rev	GCGTCTAGATGGAGCGAGCGTTTTCCCGCG	<i>XbaI</i>
Primers used for sequencing		
pASK_fw	GAGTTATTTTACCACTCCT	
pASK_rev	CGCAGTAGCGGTAAACG	
pBBR.p264_fw	GTGCAGAGTCTCTGCAGGAT	
CodA_fw	TGTGGAGGCTAACAGTGTCG	
CodA_rev	GATAATCAGGTTGGCGCTGT	
Up_gox0849_int_fw	AATCATGTCCGTCTCGGTCT	
Do_gox0849_int_rev	CGAGACGATCTTCGAGAACC	
Up_gox1117_fw	GGTGTTGAAGCCTGTGTCAG	

Do_gox1120_rev	GATCGTGACAATCCCAAGCC
Up_gox1432_int_fw	AGATGCGCTTTGTGATGTCC
Do_gox1432_int_rev	ACCGGCAGCTTTGATCTTTC

Primers used for cloning of genes into expression vectors

pASK5_gox0849_fw	ATGGTAGGTCTCAGCGCCATGATCCTGACT TCCCAGACCC	<i>Eco31I</i>
pASK5_gox0849_rev	ATGGTAGGTCTCATATCATAACGTTTGTGAC CAGATCCTTCAG	<i>Eco31I</i>
pASK3_gox1432_fw	ATGGTAGGTCTCAAATGATTACGCGCGAA ACCCTCAAGTC	<i>Eco31I</i>
pASK3_gox1432_rev	ATGGTAGGTCTCAGCGCTGGCCGGAATAG CGGCCTTCAC	<i>Eco31I</i>
pASK3_gox1849_fw	ATGGTAGGTCTCAAATGCGTACAAATTTTT CCGTAGAGGGC	<i>Eco31I</i>
pASK3_gox1849_rev	ATGGTAGGTCTCAGCGCTTTTACGGAACCG CCGCCAGCT	<i>Eco31I</i>
pBBR.p264_gox1432_fw	GCGGATATCGGCCCGAAACCACGGAGAAT TCCATG	<i>EcoRV</i>
pBBR.p264_gox1432_rev	ATAGGCGCGCCGGCCGGAATAGCGGCCTT C	<i>AscI</i>
pBBR.p264_ <i>E. coli</i> .GI_fw (for <i>E. coli</i> gene <i>b3565</i>)	GCAGGAATTCTGGAGTTCAATATGCAAGC C	<i>EcoRI</i>
pBBR.p264_ <i>E. coli</i> .GI_rev (for <i>E. coli</i> gene <i>b3565</i>)	TCCAGGGCGCGCCTTTGTCTGAACAGATAAT G	<i>AscI</i>
pBBR.p264_ <i>S. gr.</i> GI_fw (for <i>S. griseus</i> gene <i>SGR_RS05195</i>)	TTATGGAATTCAAGGCGGTCGGACGACGA TGACGGA	<i>EcoRI</i>
pBBR.p264_ <i>S. gr.</i> GI_rev (for <i>S. griseus</i> gene <i>SGR_RS05195</i>)	TATAAGGCGCGCCGCGCACGGCGAGCAGG TGG	<i>AscI</i>

Up: upstream, Do: downstream

A list of plasmids used in the work is provided in Table 3.

Table 3: List of plasmids used in the present work

Plasmid	Description	Source
pASK-IBA.3	C-terminal Strep-tag II sequence, <i>tetA</i> promoter/repressor system, <i>Amp</i> ^R	IBA GmbH
pASK-IBA.5	N-terminal Strep-tag II, <i>tetA</i> promoter/repressor system, <i>Amp</i> ^R	IBA GmbH
pBBR.p264.ST	Derivate of pBBR1MCS-2 with the promoter of gene <i>gox0265</i> and a C-terminal Strep-tag II from pASK-IBA.3 vector	Kallnik <i>et al.</i> (2010), Meyer <i>et al.</i> (2015)
pKos6b	Derivative from pAJ63a, Δupp , <i>codBA</i> integrated, <i>Km</i> ^R , FC ^S	Kostner <i>et al.</i> (2013)
pASK5- <i>gox0849</i>	<i>Amp</i> ^R , Derivate of pASK-IBA.5, contains a 1476 bp long PCR fragment of <i>gox0849</i>	This work
pASK3- <i>gox1432</i>	<i>Amp</i> ^R , Derivate of pASK-IBA.3, contains a 1458 bp long PCR fragment of <i>gox1432</i>	This work
pASK3- <i>gox1849</i>	<i>Amp</i> ^R , Derivate of pASK-IBA.3, contains a 1023 bp long PCR fragment of <i>gox1849</i>	This work
pBBR.p264. <i>gox1432</i> .ST	<i>Km</i> ^R , Derivate of pBBR.p264.ST, contains a 1476 bp long PCR fragment of <i>gox1432</i>	This work
pBBR.p264- <i>E. coli</i> .GI_ST	<i>Km</i> ^R , Derivate of pBBR.p264.ST, contains a 1323 bp long PCR fragment of <i>b3565</i> from <i>E. coli</i>	This work
pBBR.p264- <i>S. gr.</i> .GI_ST	<i>Km</i> ^R , Derivate of pBBR.p264.ST, contains a 1167 bp long PCR fragment of <i>SGR_RS05195</i> from <i>S. griseus</i>	This work
pKos6b $\Delta gox1118\Delta gox1119$	<i>Km</i> ^R , Deletion vector based on pKos6b, for deletion of <i>gox1118</i> and <i>gox1119</i>	This work
pKos6b $\Delta gox1432$	<i>Km</i> ^R , Deletion vector based on pKos6b, for deletion of <i>gox1432</i>	This work
pKos6b $\Delta gox0849$	<i>Km</i> ^R , Deletion vector based on pKos6b, for deletion of <i>gox0849</i>	This work

2.3. Antibiotic stock solutions

Ampicillin: Ampicillin stock solution (100 mg mL^{-1}) was prepared in 50 % (v/v) ethanol and stored at -20°C after filter sterilization.

Kanamycin: Kanamycin stock solution (50 mg mL^{-1}) was prepared in autoclaved distilled water and stored at -20°C after filter sterilization for further use.

Cefoxitin: Cefoxitin stock solution (50 mg mL^{-1}) was prepared in autoclaved distilled water and stored at -20°C after filter sterilization for further use.

2.4. Microbiology methods

2.4.1. Media and culture conditions

Unless mentioned specifically, all growth media were sterilized at 121°C for 20 min and used for culture inoculation. Reducing sugars (glucose, fructose) were separately autoclaved and then added to the media. Sucrose and PEG-6000 solutions were used after filter sterilization through $0.22 \mu\text{m}$ pore size filters (Carl Roth GmbH, Karlsruhe, Germany).

E. coli cells were cultured in lysogeny broth (LB) at 37°C and 120-150 rpm. 1 % (v/v) overnight grown cultures were used for inoculation of main cultures in 50-200 mL LB medium.

LB medium (Miller 1972)

Yeast extract	5 g
Peptone	10 g
NaCl	10 g
H ₂ O _{dest}	ad 1000 mL

For agar-plates, 15 g Bacto-agar was added in 1000 mL LB medium.

For protein overproduction, *E. coli* transformants were cultivated in 100-200 mL sterile maximal induction (MI) medium. The medium was supplemented with M9 salts, which were separately autoclaved and added before inoculation. In addition, some other salts such as CaCl₂, MgSO₄, and FeNH₂ citrate were also added in trace amounts after filter sterilization to the sterile medium. For induction, anhydrotetracyclin was added at a concentration of 200 ng mL^{-1} .

MI medium (Mott *et al.* 1985)

Yeast extract	20 g
Trypton	32 g
20 x M9 salts	50 mL
CaCl ₂	1 mM
MgSO ₄	1 mM
FeNH ₂ citrate	1 μM
H ₂ O _{dest}	ad 1000 mL

20 x M9 salts

NH ₄ Cl	20 g
Na ₂ HPO ₄ × 2 H ₂ O	120 g
KH ₂ PO ₄	60 g
NaCl	10 g
H ₂ O _{dest}	ad 1000 mL

To avoid contamination and for the cultivation of transformants, antibiotics such as ampicillin or kanamycin were added in sterile media to final concentrations of 100 μg mL⁻¹ or 50 μg mL⁻¹, respectively. For the regeneration of *E. coli* cells after transformation, SOC medium was used, which was provided by New England Biolabs (Frankfurt am Main, Germany).

Streptomyces griseus was cultured in 10 mL Tryptic Soya Broth (TSB) and incubated at 28°C and 180 rpm.

TSB medium (Shepherd *et al.* 2010)

Tryptone	10 g
Soytone	3.0 g
Glucose	2.5 g
NaCl	5.0 g
K ₂ HPO ₄	2.5 g
H ₂ O _{dest}	ad 1000 mL

G. oxydans was cultivated aerobically in baffled flasks (Duran, Germany) at 30°C and 180 rpm on a rotary shaker HT-minitron (INFORS GmbH, Bottmingen, Germany). Precultures were grown in Yeast Mannitol (YM), Yeast Glucose (YG), or Yeast Fructose (YF) media. For maintenance of pH, 100 mM phosphate buffer (pH 6.8) was added under sterile conditions to some media. Throughout the text, the presence of phosphate buffer in a medium is indicated by the symbol "P" along with the name of medium such as YGP (YG medium with phosphate buffer). *G. oxydans* is naturally resistant to ceftiofur, thus to avoid other microbial contamination, the antibiotic was added in growth media at a concentration of 50 µg mL⁻¹. Precultures were grown in 250 mL baffled flasks (filled with 20 % of their volume) and incubated for 2-3 days under the same conditions as mentioned above.

YM medium (Yeast Mannitol medium)

Yeast extract	6 g
Mannitol	100 mM
H ₂ O _{dest}	ad 1000 mL

YG medium (Yeast Glucose medium)

Yeast extract	6 g
Glucose	100 mM
H ₂ O _{dest}	ad 1000 mL

YF medium (Yeast Fructose medium)

Yeast extract	6 g
Fructose	50 mM
H ₂ O _{dest}	ad 1000 mL

YGP medium

Yeast extract	6 g
Glucose	50 mM
Potassium phosphat buffer	100 mM
H ₂ O _{dest}	ad 1000 mL

For agar-plates, 15 g Bacto-agar was added to 1000 mL medium.

To induce **osmotic stress**, the osmolality of growth media was raised with different metabolizable osmolytes such as polyols (mannitol, glycerol), sugars (glucose, fructose), or with metabolically inert organic compounds such as PEG-6000 or sucrose. The osmolytes were added in variable amounts or concentrations to media (Table 4). All cultures were grown in 500-2000 mL baffled flasks (filled with 20-25 % of their volume) and incubated at 30°C and 180 rpm on a rotary shaker. 3-5 % (v/v) of precultures, grown in YGP medium, were added to the main cultures as primary inocula.

Table 4: List of media used to study the response of *G. oxydans* to osmotic stress

Medium	Description
YM _{high}	YM medium containing 1000 mM mannitol
YG _{high}	YG medium containing 1000 mM glucose
YGP _{high}	YGP medium supplemented with 450 mM to 1000 mM glucose
YF _{high}	YF medium containing 500 mM fructose
YGP + sucrose	YGP medium supplemented with 150 mM to 600 mM sucrose
YGP + PEG	YGP medium supplemented with 10 % (v/v) PEG-6000
YFP + PEG	YF medium with 100 mM phosphate buffer and 10 % (v/v) PEG-6000
YM + PEG	YM medium supplemented with 2.5 % to 10 % (v/v) PEG-6000
YGly	medium containing yeast extract (6 g L ⁻¹) and glycerol (500 mM)

To examine the protective effect of mannitol against osmotic stress, *G. oxydans* was cultured in YGP medium with 300 mM or 600 mM sucrose in the presence of 2.5 mM to 10 mM mannitol. Moreover, the stimulatory effects of exogenous polyols on growth, rate of glucose consumption, and gluconate formation were analyzed by the addition of 5 mM sorbitol, mannitol, or arabitol to YGP_{high} medium containing 1000 mM glucose. All the cultures were grown in 500 mL baffled flasks (filled with 20 % of their volume) and incubated at 30°C and 180 rpm. 3-5 % (v/v) of precultures, grown in YGP medium, were used as primary inocula.

For **transformation** of *G. oxydans*, following media were used

Mating Medium (MM)

Yeast extract	6 g
Mannitol	100 mM
NaCl	10 mM
H ₂ O _{dest}	ad 1000 mL

Electroporation medium (EP medium)

Mannitol	80 g
Yeast extract	15 g
Glycerol	0.5 g
MgSO ₄ x 7H ₂ O	2.5 g
CaCl ₂	1.5 g
H ₂ O _{dest}	ad 1000 mL

The pH was adjusted to 6.0 with HCl. Salt solutions such as MgSO₄ and CaCl₂ were prepared separately and added to the sterile medium after filter sterilization.

For the cultivation of transformants, *G. oxydans* cultures were supplemented additionally with 50 µg mL⁻¹ kanamycin.

2.4.2. Measurement of osmolalities of growth media

The osmolality of a growth medium was measured with a Freezing-point osmometer (Osmomat 030, Gonotec GmbH, Berlin, Germany) at the Institute of Cellular and Molecular Botany (IZMB) of University Bonn. Prior to sample analysis, the osmometer was calibrated using NaCl solution of 300 mOsm kg⁻¹ (150 mM). 15 µL sample volume was used for single measurement. Each media sample was measured at least twice, using two different biological replicates. A mean of all the values was taken to determine the osmolality of a medium.

2.4.3. Measurement of growth parameters

Growth of bacteria in liquid cultures was determined by measuring the optical density at 600 nm (OD₆₀₀) with a Helios Epsilon Photometer (Thermo Scientific, Schwäbisch-Gmünd, Germany). OD₆₀₀ values of a culture were recorded at regular intervals against the respective blank solution (medium without inoculation). At higher cell densities (OD₆₀₀ value above 0.3), appropriate dilutions of cell cultures were made with corresponding blank solutions and used for measurement. Growth parameters of a culture such as doubling time (t_d) and growth rate (μ) were determined by plotting the OD₆₀₀ values on a logarithmic scale against time. In a linear region of exponential growth phase, slope of the graph was calculated which indicated the growth rate (μ) of the culture. With the help of the growth rate, the doubling time of the culture was calculated as $\ln(2)/\mu$.

G. oxydans cultures were also analyzed for other parameters such as change in pH, substrate, and product concentrations. Briefly, 1 mL sample volume was obtained from a growing culture at regular intervals and centrifuged in 1.5 mL eppendorf cups at 10,000 x g for 5 min. The upper supernatant was transferred to a new 1 mL cup and used for further analysis. The pH was measured with a pH 210 Microprocessor (Hanna Instruments GmbH, Vöhringen, Germany). Different metabolites concentrations were quantified by High Performance Liquid Chromatography (HPLC). All growth experiments were performed in triplicates with at least two biological replicates. Representative growth curves are shown in the present work.

2.4.4. Preparation of stock cultures

To prepare stock culture, 900 µL of an exponentially grown bacterial culture were mixed with 100 µL of sterile DMSO (10 % (v/v)) in a sterile cryogenic vial (Carl Roth GmbH, Karlsruhe, Germany) and stored at -70°C.

2.5. Molecular biology methods

2.5.1. Isolation and purification of DNA

For the isolation of chromosomal DNA from *E. coli*, *G. oxydans*, or *S. griseus*, 1 mL of the exponentially grown cultures was centrifuged at 10,000 x g for 5 min at room temperature. The supernatant was discarded and the cell pellet was processed according to the protocol of the GenJET™ Genomic DNA Purification Kit (Thermo Scientific, Schwerte, Germany). The purified DNA samples were dissolved in sterile water and stored at 4°C.

The Purification of DNA fragments from PCR amplifications was performed using the GenJET™ PCR Purification Kit (Thermo Scientific, Schwerte, Germany) according to the manufacturer's instructions. Likewise, the purification of DNA fragments from agarose gels was done using the GenJET™ Gel Extraction Kit (Thermo Scientific, Schwerte, Germany).

2.5.2. Isolation and purification of plasmid DNA

The isolation of plasmid DNA from *E. coli* cultures was done by using the GenJET™ Plasmid Miniprep Kit (Thermo Scientific, Schwerte, Germany). Cell pellets were obtained by centrifugation of 2 mL exponentially grown cultures at 10,000 x g for 5 min at room temperature. The pellets were processed for DNA extraction following the manufacturer's instructions. The isolated plasmids were eluted in sterile water and stored at 4°C.

2.5.3. Isolation and purification of RNA

Total cellular RNA was isolated from 50-100 mL culture volume of *G. oxydans*, grown to the mid log-phase (OD₆₀₀ 0.4-0.6). The cells were harvested by centrifugation at 8,000 x g, 4°C for 15 min. All the plastic-wares, used for RNA isolation, were autoclaved twice. The total RNA from the cell pellets was extracted following two different methods:

2.5.3.1. RNA extraction using the Trizol reagent method (Chomczynski 1993)

The cell pellets were dissolved in 5 mL of TRI-reagent (Sigma-Aldrich, Munich, Germany) and kept at room temperature for 15 min. Samples were distributed as 1 mL aliquots in sterile 2 mL eppendorf cups and processed for phase separation. Phase separation was carried out by the addition of 100 µL of 1-bromo-3-chloropropane per mL of TRI-reagent (used). The samples were vigorously stirred and kept at room temperature for 15 min. The mixtures were centrifuged at 12,000 x g, 4°C for 10 min and the upper colorless aqueous phases (containing RNA) were transferred to new, sterile eppendorf cups (1.5 mL). Following phase separation, 500 µL of 2-propanol was added per mL of TRI-reagent. The mixtures were gently stirred for 5-10 sec and kept at -20°C for 10-15 min. Then, the samples were centrifuged at 12,000 x g, 4°C for 10 min to pellet down the RNA. The supernatants were discarded and the RNA pellets were washed with 1 mL of 75 % ethanol per mL of TRI-reagent. Following washing, the RNA pellets were air-dried and dissolved in 90 µL of sterile double distilled water.

2.5.3.2. RNA extraction with the Ribopure-Bacterial Kit (Ambion™, Life technologies)

The cell pellets were processed according to the manufacturer's instructions for RNA extraction. The extracted RNA was dissolved in 90 µL of sterile double distilled water.

RNA samples, prepared by both methods, were treated with DNase I (Thermo Scientific, Schwerte, Germany) for DNA digestion. The reaction mixture, containing 90 µL RNA, 10 µL DNase I buffer, and 2 µL DNase I, was incubated at 37°C for 2-3 h. The RNA was purified and concentrated using the Hi-Yield RNA pure Kit (Süd-Laborbedarf GmbH, Gauting, Germany). The purified RNA samples were dissolved in sterile double distilled water and stored at -80°C. Purity of the RNA samples was determined by denaturing agarose gel electrophoresis and by PCR. The quality of the samples was evaluated by Bioanalyzer (Chapter 2.5.12).

2.5.4. Spectrophotometric quantifications of DNA and RNA samples

The quantity of DNA and RNA samples was determined with a NanoDrop device (Eppendorf AG, Hamburg, Germany) following the instructions provided by the company. Briefly, DNA or RNA samples were diluted in double distilled water (1:100). 4 μL of the diluted samples was used for spectrophotometric quantification at 260 nm against pure water (blank). Concentration of DNA or RNA was calculated using the following equations (1 and 2):

$$\text{DNA } (\mu\text{g mL}^{-1}) = A_{260} \times \text{Dilution factor} \times 50 \quad (\text{Equation 1})$$

$$\text{RNA } (\mu\text{g mL}^{-1}) = A_{260} \times \text{Dilution factor} \times 40 \quad (\text{Equation 2})$$

The purity of a sample was determined by recording the absorption at 280 nm (for proteins) and 320 nm (for salts and organic solvents) and by calculating the A_{260}/A_{280} and A_{260}/A_{280} ratios. Pure DNA or RNA samples usually have A_{260}/A_{280} and A_{260}/A_{280} ratios of 1.8-2.0 (Gallagher and Desjardins 2007).

2.5.5. Restriction digestion of DNA

The DNA samples were digested using restriction endonucleases that cut the samples at specific positions (restriction sites). Digestion of DNA results in the generation of fragments with either sticky- or blunt-ends. Cloning of a DNA fragment into a vector (plasmid) is facilitated due to the formation of such complementary ends on both DNA molecules. For single digestion, 5-10 μg DNA was used in 20 μL total reaction volume, with 1 μL of a restriction enzyme, and 2 μL of 10 x buffer (recommend by manufacturer). For double digestion, 1 μL each of two different restriction enzymes was used in the same reaction volume with a suitable buffer (compatible for both enzymes). The reaction mixtures were incubated at 37°C for 3-16 h. At the end of digestion, the plasmid DNA samples were further treated with fast alkaline phosphatase (Thermo Scientific, Schwerte, Germany) to remove phosphate moieties in order to prevent self ligation. Therefore, 0.5 U of the enzyme was added in the digestion mixture, which was incubated for another 30 min at 37°C. At the end of the reaction, the enzymes were denatured by heating the samples to 65 - 70°C for 10 min, and the digested DNA fragments were purified as described in chapter 2.5.1.

2.5.6. Ligation

The restricted DNA fragments and the dephosphorylated plasmid molecules were joined using the T4 DNA Ligase (Thermo Scientific, Schwerte, Germany). The reaction mixture (20 μ L) was composed of 10-15 μ L insert DNA, 2 μ L plasmid DNA, 1 μ L T4 Ligase, and 2 μ L of 10 x ligase buffer. The ligation mixture was incubated at room temperature for 1-2 h. A final incubation was provided at 65°C for 10 min to denature the Ligase.

2.5.7. Polymerase chain reaction (PCR)

PCR is a routine-based method, performed in molecular biology labs for the amplification of DNA fragments (Mullis and Faloona 1987). The amplified fragments can be used to construct expression vectors or to generate deletion mutants (Rabinow 1996). Genomic or plasmid DNA was used as template for DNA polymerase mediated amplification. The specific oligonucleotides (primers) were used for the amplification of a target DNA (Table 2). During PCR, the annealing temperature was adjusted to 5°C lower than the melting temperature of the primers. Two types of DNA polymerases were used, depending on the future use of the amplified DNA product. For non-preparative applications such as colony PCR, the Dream Taq polymerase (Thermo Scientific, Schwerte, Germany) was used (reaction mixture as in Table 5). The enzyme extends 1 kb DNA fragment in 1 min (per cycle). For preparative applications such as cloning, DNA amplification was performed with the Phusion polymerase (Biozym Scientific GmbH, Oldendorf, Germany). The enzyme has high replication fidelity and elongates 1 kb DNA in 30 sec (reaction mixture as in Table 6). The elongation of DNA fragments was carried out at 72°C with 30 cycles for amplification (denaturation (94-98°C), annealing (55-60°C), and extension (72°C)).

Table 5: Composition of PCR reaction mixture with the Taq-polymerase

Component	μ L / reaction	Final concentration
10 x Dream Taq Buffer	2.0	1 x
Forward primer (10 μ M)	0.5	0.25 μ M
Reverse primer (10 μ M)	0.5	0.25 μ M
dNTPs (25 mM)	0.1	125 μ M
DMSO (100 %)	1.0	5 %
Taq-polymerase	0.25	1.25 U
DNA material	5.0	
ddH ₂ O	ad 20 μ L	

Colony PCR was performed to quickly screen mutants (clones) for the presence of absence of desired DNA fragments. A whole colony was picked with the help of a sterile tooth-pick and resuspended in 10 μL sterile water. 3-5 μL of the mixture was used in a PCR reaction as a source of template DNA for the amplification of a specific DNA fragment. Positive clones were recovered and cultured by adding 100 μL of culture medium in the remaining corresponding colony mixtures. PCR amplifications were performed with the MyCycler (Bio-Rad, München, Germany) or the C1000 Thermal cycler (Bio-Rad, München, Germany).

Table 6: Composition of PCR reaction mixture with the Phusion polymerase

Component	μL / reaction	Final concentration
5 x HF Buffer	4.0	1 x
Forward primer (10 μM)	1.0	0.5 μM
Reverse primer (10 μM)	1.0	0.5 μM
dNTPs (25 mM)	0.2	250 μM
DMSO (100 %)	1.0	5 %
Phusion polymerase	0.25	0.5 U
DNA material	0.5	
ddH ₂ O	ad 20 μL	

2.5.8. Reverse transcription quantitative PCR (RT-qPCR)

RT-qPCR was performed to quantify the relative transcript abundance for a gene of interest in relation to a constitutively expressed (reference) gene. Gene specific primers were used (Table 2) to amplify a short stretch of nucleotides (maximum 200 bp). The efficiency of primers was analyzed by quantitative PCR (qPCR) using serial dilutions of template DNA (Stahlberg *et al.* 2003). For RT-qPCR, a single step SYBR Green RT-PCR Kit (Qiagen, Hilden, Germany) was used according to the manufacturer's instructions. Each reaction mixture contained 200-300 ng RNA, 1 pmol μL^{-1} of each primer, QuantiTect SYBR Green RT-PCR Master Mix, and QuantiTect RT Mix. Control reactions were also performed without adding reverse transcriptase to check DNA contamination in each RNA sample. As reference, the transcript abundance of two genes, *gox0378* and *gox1709*, was used to quantify the relative amount of transcripts of genes of interest. The transcript abundance was quantified with the master cycler gradient (Eppendorf, Hamburg, Germany) or the iCycler (Bio-Rad, Munich, Germany) using SYBR Green as the fluorescent dye. The program set up for the machines is depicted in Table 7. The data was analyzed using the software of the

cyclers as per manufacturer's recommendations. A Ct (cycle threshold) value was calculated for each gene. To analyze the expression of a target gene in relation to the reference gene, the Ct value of the target gene was subtracted from the reference gene to get a ΔCt value. The ΔCt value represents the relative cDNA concentration of the target gene in relation to the reference gene in a reaction mixture. From these values, the fold change of a given target gene was calculated and expressed as $2^{\Delta\text{Ct}}$. Moreover, for evaluation of the effect of osmotic stress on the expression of a target gene (A), the $\Delta\Delta\text{Ct}$ value of the gene was calculated (Equation 3) and utilized to express the fold change as $2^{(\Delta\Delta\text{Ct})}$.

$$\Delta\Delta\text{Ct}_{(\text{gene A})} = \Delta\text{Ct}_{1(\text{gene A})} - \Delta\text{Ct}_{2(\text{gene A})} \quad (\text{Equation 3})$$

$\Delta\text{Ct}_1 = \Delta\text{Ct}$ of gene A under osmotic stress

$\Delta\text{Ct}_2 = \Delta\text{Ct}$ of gene A under non-osmotic stress

Table 7: RT-qPCR program set up

Step	Time (min)	Temperature (°C)	Repeats
Reverse transcription	30	50	1
Activation of DNA polymerase	15	95	
Denaturation	00:15	95	40
Annealing	00:30	55	40
Elongation	00:30	72	40
Melt curves	00:10	35 + 1 every cycle	60

2.5.9. Agarose gel electrophoresis

The separation of DNA fragments of variable sizes was done with agarose gel electrophoresis. 1-2 % (w/v) agarose gels were prepared in 1 x TAE buffer (40mM Tris-HCl, 10 mM EDTA, pH 8.0, 20 mM glacial acetic acid). DNA samples were mixed with 6 x sample loading dye (Carl Roth GmbH, Karlsruhe, Germany) in 6:1 ratio and loaded onto the gels. Appropriate DNA standards (Thermo Scientific, Schwerte, Germany) were also loaded to estimate the size of the sample DNA fragments. Electrophoresis was performed in 1 x TAE buffer (electrophoresis buffer) at 80-100 V for 40 min.

2.5.10. Denaturing agarose gel electrophoresis

The quality of RNA samples was determined with formaldehyde containing (denaturing) agarose gels (Sambrook and Russell 2001). Formaldehyde prevents the intramolecular base pairing and the secondary structure formation in RNA. The linear RNA molecules can be separated according to their sizes during electrophoresis. The quality of any RNA preparation can be estimated on the basis of separation of two most prominent bands corresponding to the 23S and 16S rRNA and their intensity ratio. 1 % agarose gels were prepared by dissolving 1 g agarose in 72 mL ddH₂O water with 10 mL of 10 x MOPS buffer (0.4 M MOPS, pH 7.0, 0.1 M sodium acetate, 10 mM EDTA) and 18 mL of 37 % formaldehyde solution. 1-2 µg of RNA were mixed with 2 x RNA loading dye (95 % (v/v) formamide, 0.025 % SDS, 18mM EDTA, 0.02 % bromophenol blue, 0.02 % xylene cyanol), incubated at 65°C for 10 min, and loaded immediately onto the gels. The electrophoresis was carried out with 1 x MOPS buffer (running buffer) at 80 V for 30 min. For calibration and size estimation, Riboruler high range RNA ladder (Thermo Scientific, Schwerte, Germany) was also loaded onto the gels.

2.5.11. Staining of agarose gels

After electrophoresis, agarose gels were incubated in a GelRed-solution (Biotium, Hayward, USA) for 5-10 min at room temperature. The stained DNA or RNA fragments were visualized under UV-light with the INTAS Gel Imager (INTAS GmbH Digital Imaging and Microscopy, Göttingen, Germany).

2.5.12. Evaluation of RNA integrity with Bioanalyzer

The quality and integrity of the RNA preparation was determined more precisely with a highly sophisticated electrophoretic system known as Bioanalyzer (Agilent 2100) using a RNA600 LabChip Kit (Agilent Technologies, Waldbronn, Germany). Like traditional gel electrophoresis, separation with the Bioanalyzer is based on the principle of capillary electrophoresis to resolve the molecules according to their size. However, the chip format of Bioanalyzer requires less sample volume and saves plenty of time. The Labchips were run according to the manufacturer's instructions. Briefly, the RNA samples were applied to a small 16 wells containing glass chip (Fig. 4A). The chip is an integrated electrical circuit, where each well (micro channel) contains a sieving polymer matrix and a fluorescent dye. The fluorescent dye intercalates the RNA molecules and the mobility pattern of these complexes is detected by a laser-induced fluorescence during electrophoresis. The current was provided to each well using the 16 pin electrode cartridge. At the end of electrophoresis,

the data of each RNA sample was automatically displayed as electropherogram (Fig. 4B) and as a gel like picture (Fig. 4C). As an internal reference, the RNA ladder was also employed along with the samples. The migration time of each fragment of the RNA ladder was plotted against its size to get a standard curve. On the basis of standard curve, the sizes of 23S and 16S rRNA fragments in the sample preparations were calculated. According to the 23S/16S rRNA ratio, the integrity of each RNA sample was determined. The Agilent Expert 2100 software was used to assign the RNA integrity number (RIN) to each sample (Schroeder *et al.* 2006). The software calculates the RIN value on the basis of the electrophoretic traces of degraded and intact molecules, present in each RNA preparation. The value varies between 1 (highly degraded RNA) to 10 (intact RNA), depending upon the quality of each RNA sample.

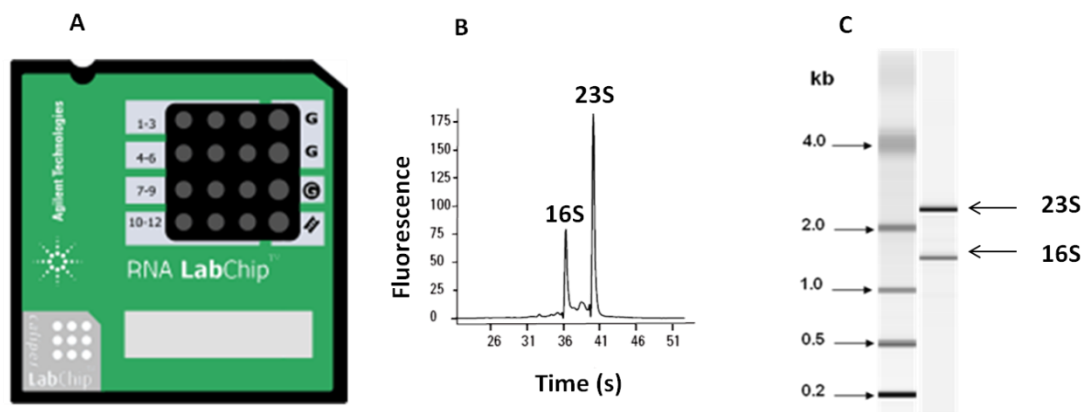


Figure 4: Evaluation of RNA quality with Bioanalyzer. (A) Nano LabChip to analyze RNA samples. (B) Electropherogram of total RNA showing the peaks of the 23S and 16S rRNAs. (C) Densitometry plot showing both rRNA bands. (Modified from Kuschel 2000).

2.5.13. DNA sequencing

DNA samples were sent to StarSeq GmbH (Mainz, Germany) for sequencing. Samples were prepared according to the standard guidelines of the company. Briefly, each reaction mixture contained 1 μL of DNA, 1 μL primer (10 $\text{pmol } \mu\text{L}^{-1}$), and 5 μL ddH₂O. The sequencing data was analyzed using the Technelysium DNA sequencing software Chromas 2.6.1 (South Brisbane, Australia).

2.5.14. Illumina Next Generation Sequencing (NGS) and data analysis

Illumina NGS was performed to analyze the effect of osmotic stress on the transcription profile of *G. oxydans*. High quality RNA samples were prepared from osmotically stressed

and non-stressed cells of the organism and sent to BGI Tech Solutions Co., Ltd (Hongkong) for sequencing with Illumina HiSeq-2000. Prior to sequencing, the RNA samples were processed by the company in a standard format (Fig. 5). Briefly, out of the total cellular RNA, the mRNA was isolated using the magnetic oligo (dT) beads and then fragmented into small chunks (maximum 200 bp). The cDNA was synthesized using the mRNA fragments as template. The small cDNA fragments were purified and washed with buffer for the addition of single nucleotide adenine (A). These fragments were processed for the preparation of a sequencing library. The sequencing library is generated by ligating short sequencing adapters to the fragments, which were purified by agarose gel electrophoresis. Each fragment was then used as template to generate multiple identical copies (clonal clusters) by a process known as bridge amplification (Adessi *et al.* 2000). All the sample libraries were used for sequencing via Illumina HiSeq-2000 platform. The NGS is a sequencing by synthesis (SBS) technology that enables the identification of every newly incorporated fluorescently labelled dNTPs during amplification of the template DNA strands. The SBS technology is based on reversible terminator-based methods to track the incoming nucleotide during chain elongation (Canard and Sarfati 1994). The incoming nucleotides are labeled with fluorescent tags, emit fluorescence upon incorporation, and can be therefore easily detected. The sequencing data was aligned against the reference genome of *G. oxydans* (GB: CP000004-9) and analyzed with the DNA sequence visualization and annotation tool Artemis (Wellcome Trust Sanger Institute, England).

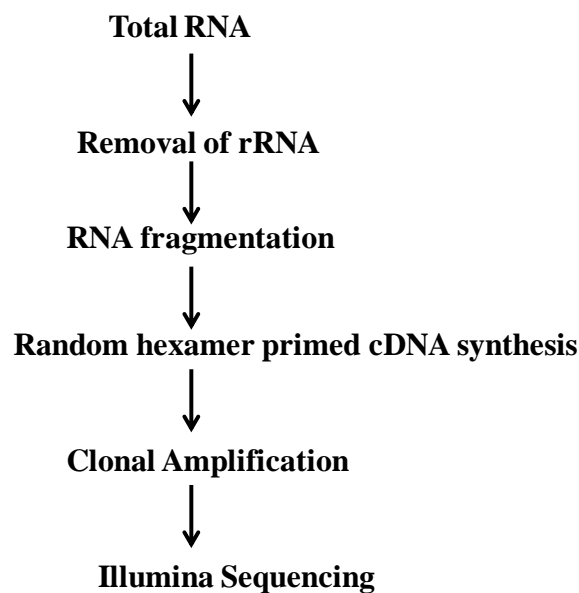


Figure 5: Flow chart to represent all steps of RNA- Seq using the Illumina HiSeq platform.

From the sequencing data, the expression level of each gene was calculated using the *RPKM* method (Mortazavi *et al.* 2008). *RPKM* (Reads Per Kilobase per Million mapped reads) is a method for the quantification of gene expression from RNA-Seq data. The expression of each gene was normalized for the total number of sequencing reads and the total length of the gene (Equation 4). Data normalization eliminates the influence of difference in gene length and sequencing discrepancy on the quantification of gene expression. Thus, the obtained values were directly used to examine the difference in gene expression among control and osmotic stress samples of *G. oxydans*.

$$RPKM_{(\text{gene A})} = 10^6 \times C / N \times L \times 10^3 \quad (\text{Equation 4})$$

C = number of reads that are uniquely aligned to gene A

N = total number of reads that are uniquely aligned to all genes

L = number of bases of gene A

The differentially expressed gene were further categorized with respect to their biological functions (pathway enrichment) using the KEGG database (Kanehisa *et al.* 2008). The analysis enables the identification of the significantly enriched or important metabolic pathways, induced upon osmotic stress, in the context of the whole genome of *G. oxydans*.

2.5.15. Transformation of *E. coli* and *G. oxydans*

Super-competent *E. coli* cells (New England Biolabs, Frankfurt am Main, Germany) were transformed by the heat shock method (Hanahan 1983). 25 μ L cells were mixed with 5 μ L ligation mixture and incubated on ice for 30 min. The mixture was heated to 42°C for 1 min and immediately transferred to ice for a short incubation of 2 more min. Then, 1 mL SOC medium was added and the cells were incubated at 37°C for 1-2 h. Transformants were selected by spreading the cells on LB agar plates containing appropriate antibiotics for selection. The plates were incubated at 37°C for 18-24 h, and the transformants were screened by colony PCR.

The cells of *G. oxydans* were transformed via electroporation or triparental mating (Condon *et al.* 1991, Kallnik *et al.* 2010, Choi *et al.* 2006). For electroporation, competent cells of *G. oxydans* were generated by a modified method of Mostafa *et al.* (2002). Cells were grown to an OD₆₀₀ of 0.8-1.2 in 40 mL EP medium. The culture was centrifuged at 8,000 x g, 4°C for 10 min. The cell pellets were washed at least twice with 1 mM HEPES-buffer (pH 7.0) and resuspended in 800 μ L of 1 mM HEPES- buffer. 40 μ L competent cells were mixed with 10

μL sterile glycerol (75 %) and 1 μL of a purified plasmid. The electroporation of the cells was performed with a MicroPulser (Bio-Rad, München, Germany) using pre-cooled cuvettes with 1 mm electrode distance (Bio-Budget Technologies GmbH, Krefeld, Germany). A pulse of 2.2 kV was provided for 4-5 ms, and the cells were transferred to 15 mL sterile falcon tube with 1 mL fresh EP medium. After regeneration at 30°C and 120 rpm for 16 h, the cells were plated on selective agar plates and incubated at 30°C for 2-3 days.

For the transformation of *G. oxydans* by triparental mating, *E. coli* (ECG18) was used as a helper strain. The strain contains the plasmid pRK2013 which carries transfer genes (*tra*) to assist the transfer of a mobilizable plasmid from another *E. coli* strain (donor) to *G. oxydans* (acceptor). Therefore, both *E. coli* strains (helper and donor) were cultured in LB medium to OD₆₀₀ of 0.6, and *G. oxydans* was grown in YM medium to an OD₆₀₀ of 0.8-1.2. Cell pellets were obtained by centrifugation of 2 mL of each cell culture at 10,000 x *g* for 5 min. The pellets were washed at least twice with mating medium (MM) and mixed in 1:1:1 ratios. 200-300 μL cell suspensions were pipetted on sterile filter paper discs, which were placed on non-selective MM agar plates. After incubation at 30°C for 16 h, the cells were scraped and resuspended in sterile YM medium. Appropriate dilutions of the suspensions were plated on YM agar plates containing cefoxitin and kanamycin as selection markers. Plates were incubated at 30°C for 2-3 days. Only transformants of *G. oxydans* (containing the mobilizable plasmid) could grow on these selective plates.

2.5.16. Generation of *G. oxydans* strains carrying in-frame deletions

G. oxydans strains having in-frame deletions in their chromosomes for target genes were generated using the *codAB* marker-less deletion system (Kostner *et al.* 2013). The system is based on the counter-selection of deletion mutants, lacking the *codAB* genes, during growth on a medium with 5-fluorocytosine (5-FC). 5-FC is a non-toxic pyrimidine analogue that is converted by the cytosine deaminase (encoded by *codA* gene) to a toxic compound 5-fluorouracil (5-FU) (Neuhard 1983, Martinussen and Hammer 1994). Cells lacking the *codA* gene can grow on the marker 5-FC containing plates. *G. oxydans* does not possess the cytosine deaminase encoding gene. Therefore, a deletion system was designed by Kostner *et al.* (2013) to transfer the *E. coli codA* and *codB* genes into *G. oxydans* using a suicidal plasmid pKos6b. The plasmid can autonomously replicate in *E. coli*. Replication in *G. oxydans* is only carried out after integration of the plasmid into the chromosome via homologous recombination. The resulting transformants of *G. oxydans* cannot grow in a medium containing 5-FC (> 60 $\mu\text{g mL}^{-1}$). The sensitivity of the transformants to 5-FC is ~ 8-

fold increased in the presence of *codB* gene, cloned along with *codA* in the vector. The gene *codB* encodes for a cytosine permease that enhances the penetration of 5-FC inside the cell. Culturing of transformants under a non-selective environment facilitates the events of second homologous recombination to excise the integrated plasmid. The plasmid excision will result in the generation of 5-FC resistant strains of *G. oxydans* that can easily be selected on a growth medium containing 5-FC (as counter selection marker).

To generate in-frame deletion mutants of *G. oxydans* for the target genes (*gox0849*, *gox1118-gox1119*, or *gox1432*), flanking regions (1 kb up- and downstream) of the genes were amplified and cloned into the pKos6b vector. The recombinant plasmids can integrate at the homologous positions on chromosome. For cloning, the flanking regions of a target gene were amplified using special sets of primers with complementary overhangs (Table 2) via the phusion-polymerase mediated PCR (Chapter 2.5.7). For example, for the generation of Δ *gox0849*, 1 kb of the up- and downstream regions of *gox0849* were amplified using primers Up_*gox0849_fw*/Up_*gox0849_rev* and Do_*gox0849_fw*/Do_*gox0849_rev* (Table 2). Then, a crossover PCR was performed to ligate both fragments into a single 2 kb fragment using primers Up_*gox0849_fw* and Do_*gox0849_rev* (Peters *et al.* 2013b, Wach 1996). The amplified fused fragment was digested with *Kpn1* and *Xba1* and cloned into the MCS region of pKos6b vector, yielding pKos6b Δ *gox0849* vector that was transformed into *G. oxydans* via triparental mating (Chapter 2.5.15). Analogously, the other recombinant plasmids pKos6b Δ *gox1432* and pKos6b Δ *gox1118* Δ *gox1119* were generated for the deletion of gene *gox1432* or an operon *gox1119-gox1118*, respectively. The transformation of the plasmids resulted in the generation of kanamycin-resistant and 5-FC-sensitive mutants, which were screened by colony PCR. For plasmid excision, the positive transformants were selected and cultured on non-selective, liquid YM medium for 1-2 days at 30°C. The excision of the plasmids would lead to the generation of kanamycin-sensitive and 5-FC-resistant mutants that were further selected on selective YM agar plates with 5-FC (60 $\mu\text{g mL}^{-1}$). Appropriate dilutions of the cultures were plated on the marker plates that were incubated at 30°C for 2-3 days. Plasmid excision will generate two types of mutants having either the wild type copy of chromosome or carrying in-frame deletion for the target genes. The distribution of both types of mutants is a random process and requires additional testes for confirmation. The deletion of desired genes was verified by amplification of the flanking regions of the genes with subsequent sequencing.

2.5.17. Plasmid-based expression of genes

Three genes of *G. oxydans* (*gox0849*, *gox1432* or *gox1849*) were cloned into pASK-IBA vectors for overexpression in *E. coli* (host). For cloning, the genes were amplified with gene specific primers (Table 2) and cloned into the respective vectors after restriction digestion and ligation (Chapter 2.5.5 and 2.5.6). The resulting plasmids pASK5_*gox0849*, pASK3_*gox1432*, and pASK3_*gox1849* (Table 3) were transformed into *E. coli* via the heat-shock method (Chapter 2.5.15).

Likewise, for the expression of genes in *G. oxydans*, the pBBR.p264.ST vector system was used. The expression vector contained a strong, constitutively expressed promoter p264 from *G. oxydans* and a C-terminal Strep-tag sequence of the IBA vector pASK3 (Kallnik *et al.* 2010, Meyer *et al.* 2015). For protein production, a desired gene was cloned in-frame to the C-terminal Strep-tag sequence. The strong promoter allows a constitutive expression of the cloned gene, while the C-terminal Strep-tag facilitates the purification of the target protein. In the present study, the expression of three genes was analyzed in *G. oxydans*. These included the gene *gox1432* (from *G. oxydans*) and the *xylA* genes *b3565* (from *E. coli*) and *SGR_RS05195* (from *S. griseus*). All the genes were amplified with their native ribosomal binding sites and cloned into the pBBR.p264.ST vector by restriction digestion and ligation of the DNA fragments (Chapter 2.5.5 and 2.5.6). The resulting plasmids pBBR.p264.*gox1432*.ST, pBBR.p264_*E. coli*.GI_ST, and pBBR.p264_*S. gr*.GI_ST were transformed into *G. oxydans* by electroporation or triparental mating (Chapter 2.5.15).

2.6. Biochemical methods

2.6.1. Protein overproduction and purification

2.6.1.1. Heterologous overproduction of proteins in *E. coli*

Gox0849, Gox1432, and Gox1849 were overproduced in *E. coli* cells using pASK-IBA vectors (Table 3). *E. coli* precultures were grown in 50 mL LB medium with ampicillin (100 $\mu\text{g mL}^{-1}$) at 37°C. For the overproduction of the proteins, 1 % (v/v) of the exponentially grown precultures was inoculated in 100 mL MI medium containing ampicillin (100 $\mu\text{g mL}^{-1}$) and incubated at 37°C under shaking at 180 rpm. At OD₆₀₀ of 0.4-0.5, the expression of the genes was induced with the addition of anhydrotetracyclin (200 ng mL⁻¹). The cultures were further incubated overnight at 16°C. The cells were harvested by centrifugation at 6,000 x g

for 10 min at 4°C and resuspended in 15-20 mL buffer W (100 mM Tris-Cl, 100 mM NaCl, pH 8.0).

2.6.1.2. Heterologous overproduction of proteins in *G. oxydans*

Xylose/Glucose isomerase of *E. coli* and *S. griesus* were overproduced in *G. oxydans* using pBBR.p264.ST vector (Table 3). Precultures of *G. oxydans* were grown in 50 mL YM medium with cefoxitin (50 µg mL⁻¹) and kanamycin (50 µg mL⁻¹) at 30°C and 180 rpm. For the overproduction of the proteins, 500 mL YGP medium containing MgSO₄ (20 mM), cefoxitin (50 µg mL⁻¹), and kanamycin (50 µg mL⁻¹) was inoculated with 3 % (v/v) of the exponentially grown preculture and incubated at 30°C and 180 rpm. At an OD₆₀₀ of 0.8-1.2, the cells were harvested by centrifugation at 10,000 x g for 10 min at 4°C. The pellets were resuspended in 20 mL Buffer W and processed for the extraction and purification of proteins using Strep-Tactin affinity chromatography.

2.6.1.3. Cell disruption and extraction of crude cell extract

E. coli and *G. oxydans* cells were disrupted by sonication. Prior to lysis, the cell suspensions were treated with the addition of 5 µL protease inhibitor cocktail (Sigma-Aldrich, Munich, Germany), a pinch of lysozyme (Serva, Heidelberg, Germany), and of DNase I (Thermo Scientific, Schwerte, Germany), and incubated on ice for 15 min. The cell lysis was performed by sonication (Branson Sonifier Cell Disruptor with Branson Ultrasonics converter, Danbury USA) at 50 % amplitude with cooling (Colora Messtechnik GmbH, Lorch/Württ, Germany) for 1.5 min mL⁻¹. To obtain crude cell extracts, the lysed samples were centrifuged at 12,000 x g, for 10 min at 4°C to remove cell debris and unlysed cells.

2.6.1.4. Protein purification by Strep-Tactin Affinity chromatography

Recombinant protein, containing a Strep-tag, was purified by Strep-Tactin Superflow® affinity chromatography as described by the manufacturer (IBA GmbH, Göttingen, Germany). Briefly, the cell lysate was applied to a Strep-Tactin containing column that was pre-equilibrated with buffer W. After filtration of the lysate, the column was washed with 6 x 1 column volume (CV) buffer W. Then, the protein was eluted with 6 x 0.5 mL buffer E (buffer W and 2.5 mM desthiobiotin). The used column was regenerated with 2 x 5 mL buffer R (buffer W and 1 mM HABA) and stored at 4°C in buffer W. The extracted protein was quantified by the Bradford method (Bradford 1976).

2.6.2. Extraction of cell cytoplasm

To determine the activities of soluble fructose reductases, the cytoplasmic fractions were extracted from stressed and non-stressed cultures of wild type and mutant strains (devoid of fructose reductases) of *G. oxydans*. Therefore, the cultures were grown to the mid log-phase in 100 mL YGP medium (control cultures) and 250 mL YGP medium with 450 mM sucrose (stressed cultures). The cells were harvested by centrifugation of the samples at 10,000 x *g* for 15 min at 4°C and resuspended in 10 mL cell suspension buffer (100 mM potassium phosphate buffer, pH 7.0, 5 mM MgSO₄·7H₂O, 0.75 mM dithiothreitol). Lysis was performed by sonication of the cell suspensions (Chapter 2.6.1.3), and the lysed samples were centrifuged at 15,000 x *g* for 15 min at 4°C to remove unlysed cells and debris. For the extraction of cytoplasm, the crude cell extracts were centrifuged twice at 45,000 x *g* for 1 h at 4°C to remove cellular membranes. The clear supernatants (cytoplasmic fractions) were used to determine the activities of NADPH and NADH-dependent fructose reductases. The concentration of protein in each fraction was determined by the Bradford test (1976).

2.6.3. Quantification of protein concentration

The protein concentration of a sample was quantified by the Bradford test (1976). Therefore, a calibration curve was prepared using the known concentrations of BSA protein solutions (0-1 mg mL⁻¹) for each fresh bottle of Bradford reagent (Sigma-Aldrich, Munich, Germany). For that, 20 µL of the each protein solution was mixed with 980 µL of Bradford reagent and incubated under dark for 10 min at room temperature. Then, the absorption of each sample was recorded at 595 nm against blank solution (Bradford reagent with buffer) to draw a standard curve. From a straight region of the curve, the best fit of the data was obtained to get an equation with R^2 value near to 1. Analogously, the absorption of an unknown protein sample was recorded and used for the calculation of an exact protein concentration in the sample (Equation 5):

$$y = mx \quad \text{(Equation 5)}$$

y = absorption of a sample at 595 nm

m = slope of standard curve

x = concentration of an unknown protein sample

For an exact quantification, the concentration of each sample was determined at least twice.

2.6.4. PolyAcrylamide Gel Electrophoresis (PAGE)

2.6.4.1. Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was used to separate proteins according to their molecular mass by electrophoresis in polyacrylamide gel matrix with SDS as denaturant (Laemmli 1970). The gel matrix was composed of 5 % (v/v) collecting gel and 12.5 % (v/v) resolving gel (Table 8), and the electrophoresis was carried out in vertical chambers (Bio-Rad, München, Germany). The protein samples were mixed (1:1) with 2 x sample loading buffer (2 % (w/v) SDS, 0.001 % (w/v) bromophenol blue, 5 % (v/v) β -mercaptoethanol, 50 % (v/v) glycerol, 20 % (v/v) collecting buffer (600 mM Tris, pH 6.8)), cooked at 95°C for 10 min, and loaded onto the gel. PageRuler prestained protein ladder (Thermo Scientific, Schwerte, Germany) was used as an internal calibrator for the estimation of the molecular mass of protein samples. Gels were run with 1 x electrode buffer (20 mM Tris, 190 mM Glycin, 0.1 % (w/v) SDS, pH 8.3) at 20 mA for 30 min and then at 30 mA for 1-2 h. After electrophoresis, proteins were visualized by silver staining.

Table 8: Composition of collecting and resolving gels used for SDS-PAGE

Component	Collecting gel (5 %)	Resolving gel (12.5 %)
40 % acrylamide solution (Rotiphorese Gel 40)	250 μ L	2.0 mL
Collecting Buffer (600 mM Tris, pH 6.8)	400 μ L	
Separating Buffer (1.8 M Tris, pH 8.8)		1.2 mL
0.5 % (w/v) SDS	400 μ L	1.2 mL
10 % (w/v) APS	30 μ L	30 μ L
TEMED	5 μ L	5 μ L
ddH ₂ O	950 μ L	1.6 mL

2.6.4.2. Native PAGE

Native or non-denaturing PAGE was performed to separate proteins according to their charge and oligomeric state. For native PAGE, 4–20 % gradient gels (Mini-Protean Precast Gels TGX) were purchased from Bio-Rad (München, Germany). Gels were run in 1 x electrode buffer (0.12 % (w/v) Tris base, 0.576 % (w/v) glycine). Prior to loading, the protein samples

were mixed in 1:1 ratio with the native sample loading buffer (0.001 % (w/v) bromophenol blue, 40 % (w/v) sucrose, 20 % (v/v) electrode buffer, pH 8.3). Standard proteins of known molecular masses (669 kDa thyroglobulin, 440 kDa ferritin, 232 kDa catalase, 140 kDa lactate dehydrogenase, 66 kDa bovine serum albumin) were used for size estimation of sample proteins. These standard proteins (high-molecular-weight calibration kit for native electrophoresis) were obtained from GE, Health care (Buckinghamshire, UK). Before loading, the standard protein mixture was also diluted in 1:50 with the electrode buffer. Electrophoresis was carried out at 20 mA for 2-3 h under cold conditions.

2.6.5. Silver staining

After electrophoresis, proteins were visualized by silver staining (Blum *et al.* 1987). The method is quite sensitive and can detect up to 1 ng protein. All chemicals used for silver staining were freshly prepared before use. Proteins were fixed by the incubation of the gels in a fixer solution (50 % (v/v) methanol, 12 % (v/v) glacial acetic acid, and 0.05 % (v/v) formaldehyde) for 20 min. Then, the gels were washed with 50 % (v/v) methanol for 10 min, followed by additional 10 min washing with ddH₂O. To increase the sensitivity for color development, the gels were treated for 1 min with 0.02 % (w/v) thiosulphate solution, which was removed by subsequent washing with ddH₂O (2 x 1 min). After that, the gels were exposed to 0.1 % (w/v) silver nitrate solution for 20 min in the dark at 4°C. During this step, silver ions bind to the negatively charged side chains of amino acids. Later on, the silver ions were reduced to metallic silver by formaldehyde during incubation of the gels with the developer solution (3 % (w/v) Na₂CO₃, 0.1 % (v/v) formaldehyde) which resulted in the staining of protein bands. The reaction was stopped by successive washing of the gels with ddH₂O.

2.6.6. Immunoblot

Immunoblot (western blot) was used to detect specific tagged proteins from the crude cellular extracts. The sensitivity of the method is quite high, with a detection limit of 500 pg for proteins. During the process, the proteins, resolved by PAGE, were transferred to a membrane by a modified method of Towbin *et al.* (1979). In the present study, the recombinant Strep-tag proteins were transferred to a nitrocellulose membrane (4.5 µM, Bio-Rad, München, Germany) by electroblotting with a Semi-Dry Electroblotter (Biozym Scientific GmbH, Oldendorf, Germany). All the components for the western blot (whatman filter-paper and nitrocellulose membrane) were dipped in Towbin buffer (0.34 % (w/v) Tris

base, 1.44 % (w/v) glycine, 20 % (v/v) methanol) before use. The blotting was done at 15 V for 30-40 min.

After electroblotting, the Strep-tag proteins were chromogenically detected with horseradish peroxidase (HRP) conjugate (IBA GmbH, Göttingen, Germany) according to the manufacturer's instructions. The membrane was blocked overnight at room temperature with PBS-blocking buffer (0.5 % (v/v) Tween-20, 5 % (w/v) milk powder, PBS buffer (0.8 % NaCl, 0.02 % KCl, 0.025 % Na₂HPO₄, 0.02 % KH₂PO₄, pH 7.4)). After blocking, the membrane was washed (3 x) with PBS-tween buffer (0.1 % Tween-20 in PBS buffer) at room temperature. Then, the membrane was incubated with 20 mL PBS-tween buffer with 5 µL of horseradish peroxidase (HRP) conjugate for 1-16 h at room temperature under gentle shaking. To remove unbound, excessive conjugate, the membrane was washed with 20 mL PBS-tween buffer (2 x) and with 20 mL PBS buffer (2 x). To detect proteins, the membrane was treated with 20 mL PBS buffer with 200 µL chloronaphthol solution (3 % (v/v) in methanol) and 20 µL H₂O₂ solution (30 %). During incubation under dark conditions, the HRP catalyzes the oxidation of the chromogenic substrate 4-chloro-1-naphthol, in the presence of peroxide, to an insoluble purple color product 4-chloro-1-naphthol, which precipitates in a region of protein bands (Hawkes *et al.* 1982).

2.6.7. Activity staining

The activity of xylose/glucose isomerases were detected in native gels by a modified method of Yamanka (1975) following electrophoresis. Activity staining is based on the principle of the oxidation of a colorless compound triphenyltetrazolium chloride by xylulose to a colored compound formazan in an alkaline environment (Sapunova *et al.* 2004). Xylulose was obtained by the isomerization of xylose, catalyzed by the xylose isomerase enzymes (loaded on the gels). Thus, the gels were soaked in 50 mL 0.1 M Tricine-NaOH buffer containing 100 mM D-xylose and 30 mM MgCl₂, and incubated at 37°C for 30 min. During this step, the xylose could be converted to xylulose by the active xylose isomerases. After washing with dH₂O, the gels were incubated in dark with 50 mL 1 M NaOH solution containing triphenyltetrazolium chloride (1 mg mL⁻¹). The isomerized xylulose would oxidize the compound to a pink color formazan that stains the protein bands in the gels.

2.6.8. Determination of enzymatic activities

In the present study, activities of different purified and cytoplasmic enzymes were determined with the Jasco V-600 Spectrophotometer (Jasco, Gross-Umstadt, Germany). For the control of temperature, the Jasco ETC-717 Peltier-Thermostats was used. All the enzymes were assayed for catalyzing substrate dependent changes in the absorption of cofactors (NAD(P)H /NAD(P⁺)). The rate of a reaction was determined spectrophotometrically at 340 nm in 1 mL cuvette at 30°C. The reactions were started by the addition of substrates, and the change in extinction of cofactors was recorded for 1-2 min using the Spectra Manager III software. Following formulae were used to calculate volume activities and specific enzyme activities:

$$\text{Volume activity (U mL}^{-1}\text{)} = \Delta E \text{ min}^{-1} \times V / d \times \varepsilon \times v \quad (\text{Equation 6})$$

$$\text{Specific activity (U mg}^{-1}\text{)} = \text{Volume activity} / c \quad (\text{Equation 7})$$

$\Delta E \text{ min}^{-1}$ = change in extinction min^{-1}

V = total volume of assay (mL)

d = thickness of the cuvette (cm)

ε = molar extinction coefficient ($\text{mM}^{-1} \text{cm}^{-1}$)

v = volume of the sample or probe (mL)

c = protein concentration (mg mL^{-1})

One unit (U) of enzyme activity corresponded to the amount of enzyme catalyzing the conversion of 1 μmol of pyridine dinucleotide (NAD(P)/H) per minute. For the accurate measurements, the enzymatic activities were determined from at least two biological replicates of a sample using different dilutions.

2.6.8.1. Measurement of activities of NAD(P)H dependent oxidoreductases

The activities of purified enzymes Gox0849 and Gox1432 were determined in the presence of NAD(P)/H ($\varepsilon = 6.22 \text{ mM}^{-1}\text{cm}^{-1}$) at 340 nm as described by Adachi *et al.* (1999a). Both enzymes primarily catalyzed the reversible reduction of fructose to mannitol (Reaction 1). Other substrates were also tested to determine the substrate spectrum of both proteins.



The reduction of substrates was analyzed at acidic to neutral pH values (5.0-7.0), while the oxidation reactions were monitored at pH 9.0-10.0. The optimum buffers for catalysis were selected by quantifying the reaction rates at different pH values. Following buffers were used for enzymatic reactions: potassium phosphate buffer (pH 6.0-7.0), sodium acetate buffer (pH 5.0-5.5), Tris-Cl buffer (pH 7.0-9.0), and sodium carbonate buffer (pH 10.0-10.5). The reaction mixture contained 125 μ M NAD(P)/NAD(P)H, 100 mM substrate, enzyme solution, and 50 mM appropriate buffer in a final volume of 1 mL. The reduction of different substrates (D-fructose, L-sorbose, or 5-keto D-gluconate) was monitored in the presence of 50 mM potassium phosphate buffer, pH 7.0, while the oxidation of substrates (D-mannitol, D-sorbitol, D-arabitol or gluconate) was recorded with 50 mM Tris-Cl, pH 9.0 (for Gox0849) and 50 mM sodium carbonate buffer, pH 10 (for Gox1432).

To determine enzyme kinetics, assays were performed at optimum pH conditions with variable substrate concentrations (5 - 1000 mM) or co-factor concentrations (2.5 - 250 μ M). Kinetic parameters such as Michaelis Menten constant (K_M) and maximum reaction velocity (V_{max}) were determined by applying nonlinear regression to the Michaelis-Menten data with GraphPad Prism program 6.0 (San Diego, California, USA). Moreover, a linear regression was also calculated from the data using LineWeaver Burk Plot. The K_M value refers to the substrate concentration at which an enzyme attained a half-maximal reaction velocity ($1/2 V_{max}$). V_{max} denotes to the maximal velocity of an enzymatic reaction irrespective of the substrate concentration. Other kinetic parameters such as turn over number (K_{cat}) for an enzyme was calculated according to Equation 8. From the turn over numbers, the catalytic efficiency K_{cat}/K_M ($s^{-1}M^{-1}$) of enzymes was calculated to compare the relative rates of different enzymes acting on same substrate, or of a single enzyme acting on different competing substrates.

$$K_{cat} (s^{-1}) = V_{max} / \mu\text{mol mg}^{-1} \text{ enzyme} \times 1/60 \quad (\text{Equation 8})$$

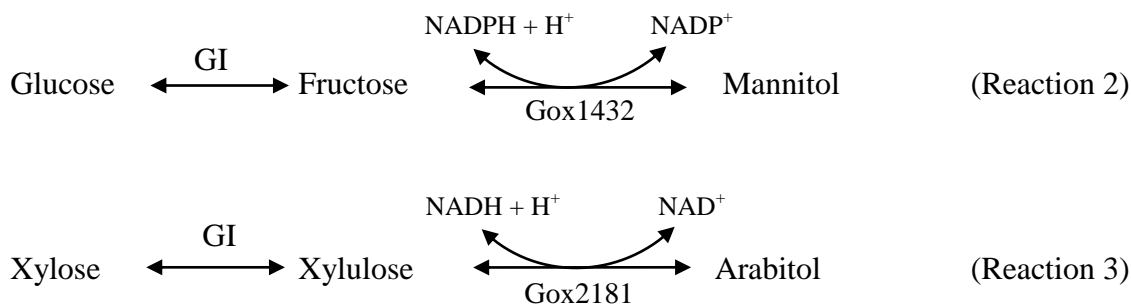
Besides measurements with the purified enzymes, the activities of NAD(P)/H oxidoreductases were also determined in the cell cytoplasm of different strains of *G. oxydans* (lacking one or both of the fructose reductases). Therefore, the cell cytoplasm was extracted from the strains, grown under control and osmotic stress conditions (Chapter 2.6.2) and assayed directly for the reduction of fructose as well as for the oxidation of mannitol at physiological pH value (7.0). As a reference, cytoplasmic fractions of the wild type strain of *G. oxydans* were also extracted and tested under the same conditions.

In addition, the activity of a purified putative NADH oxidoreductase (Gox1849) was also tested in the similar way with different substrates including: **ketones** (acetoin, 2-hexanone, 2-butanone, pentandion, heptandion, 3-hexanone); **aldehydes** (glycolaldehyde, butyraldehyde, acetaldehyde, glyoxal, formaldehyde, p-tolualdehyde, 3-methyl-benzaldehyde, methylvaleraldehyde, benzaldehyde); **amino acids** (glycin, L-serin, L-valin, lysin, valin, alanine, tyrosin); **sugars and polyols** (glycerol, xylose, D-galactose, arabitol, sorbitol, erythritol, butandiol, pentanol, 2-hexanol, cis/trans-1,2 cyclopentandiol, 2-phenylethylamine, methanol, ethanol, ethyleneglycol, propanol, 1,4 cyclopentandiol), and **mixed substrates** (2-ketogluconate, ethylpyruvate).

2.6.8.2. Glucose isomerase: enzyme assay with auxillary enzymes (Gao *et al.* 2002)

The glucose isomerase overproducing strains of *G. oxydans* were grown on YGP_{high} medium containing 450 mM glucose with 20 mM MgSO₄ till the mid log-phase. The cell cytoplasm was extracted from the cultures (Chapter 2.6.2) and used for assaying the glucose isomerization activity. The assay mixture containing 50 mM phosphate buffer (pH 7.0), 100 µl cytoplasm, 10 mM MgCl₂ and 250 mM D-glucose was incubated at 30°C for 2-18 h. Glucose isomerase isomerizes glucose to fructose that can be quantified using Gox1432 as an auxiliary enzyme (Reaction 2). Thus, the assay mixture was supplemented with 125 µM NADPH and the reaction was started with the addition of purified Gox1432 (5-10 U). The rate of the reduction of fructose to mannitol was calculated by recording the decrease in absorption of NADPH at 340 nm and 30°C.

In addition, the isomerase activity of the purified xylose/glucose isomeases was also determined with glucose or xylose as substrates using Gox1432 (fructose reductase) or Gox2181 (xylulose reductase) as auxiliary enzymes, respectively. The reaction mixtures contained 50 mM phosphate buffer (pH 7.0), 10 mM MgCl₂, 100-250 mM substrates, and 1-5 µg purified isomerase. The isomerases were activated by heating the assay mixture at 70°C for 15 min. The concentration of the isomerized products (fructose or xylulose) was determined using the corresponding auxiliary enzymes (in excess) with 125 µM NAD(P)H (Reaction 2 and 3). Prior to the addition, the reaction mixtures were cooled on ice for 2 min.



2.7. Analytical methods

2.7.1. Sample preparation for the determination of intracellular solutes

For the determination of intracellular osmolytes, control and stressed cells were harvested at the late log-phase from 200-2000 mL cultures by centrifugation at 11,300 x g for 15 min. The cell pellets were frozen at -20°C for 2-3 h. After that, the frozen pellets were freeze-dried in a lyophilizer (Heraeus Christ, Alpha 1-6) with a vacuum of 0.05 mbar at 20-30°C for 24-48 h. The pellets were weighed for the cell dry mass measurement and then processed for the extraction of intracellular osmolytes by a modified Bligh and Dyer method (1959).

The Bligh and Dyer extraction procedure is routinely employed for the separation of cellular components using a mixture of water, methanol, and chloroform. During extraction, the cellular components are separated into two layers, the chloroform layer containing cellular lipids, and the upper methanol layer with all soluble, non-lipid components. Meanwhile, cellular proteins and cell wall fragments are precipitated at an interface of both layers. Briefly, 30-60 mg of freeze-dried cell material was transferred into a 1.5 mL eppendorf cup. 500 µL of Bligh and Dyer solution (methanol:chloroform:water 10:5:4) was added in the tube, and the mixture was vortexed for 5-10 min at room temperature. Then, the mixture was extracted with the addition of 260 µL of chloroform and water mixture (1:1) and vortexed for 5 min at room temperature. Following vortexing, the mixture was processed for the phase separation by centrifugation at 9,300 x g for 5 min. The upper polar layer of water and methanol was transferred into a new 1.5 mL tube and analyzed by HPLC. The remaining mixture was dried under the fume-hood to evaporate chloroform, and the left over organic phase was hydrated with 10 mM NaOH solution and used for the quantification of protein content by the Bradford method (Chapter 2.6.3). Different dilutions of the mixture were used for the accurate quantification of the protein content.

2.7.2. High Performance Liquid Chromatography (HPLC)

HPLC is an analytical method to separate the compounds, present in a sample mixture or analyte, via column chromatography. Different types of columns (stationary phase) and solvents (mobile phase) are employed for the separation. Different compounds in a mixture interact differentially with both phases and have unique migration rates (retention times) on the columns. The substances can be identified by comparing the retention times to commercial standards. Moreover, the amount of a substance can be quantified by applying different known concentrations of the compound to the column. The area of the signal-peaks was measured for each concentration and used to draw a calibration curve. From a straight region of the curve, the best fit of the data was obtained in the form of an equation with R^2 value near to 1. Likewise, the concentration of a substance of interest in a sample mixture can be quantified by measuring the area of its signal-peak and comparing the value to calibration curve.

In the present study, the solute spectra in the Bligh and Dyer mixtures were analyzed by isocratic HPLC system. The samples were diluted with 80 % (v/v) acetonitrile and separated on a LiChrospher 100 NH₂ column (125 × 4 mm, 5 μm) (Merck, Darmstadt, Germany) using 80 % (v/v) acetonitrile as solvent. Substances were eluted at a flow rate of 1 mL min⁻¹. The elution pattern of the substances was dependent on their selective interactions with the terminal NH₂ groups of the silica matrix of the column. With 80 % acetonitrile, the resin acts as weak anion exchanger, thus the elution of the hydrophilic compounds is decelerated in comparison to the hydrophobic substances. The separation of sugars (*e.g.*, glucose, fructose, sucrose) and polyols (*e.g.*, mannitol, arabitol) were monitored by a refractive index detector. The concentration of sugars and polyols were quantified by their calibration curves. The HPLC data were analyzed using the ChromQuest 4.2.34 software (Thermo Fisher Scientific Inc, USA).

The concentration of glucose and gluconate in a glucose grown culture medium of *G. oxydans* were detected by HPLC. Therefore, the samples were prepared as described in chapter 2.4.3. The supernatants were diluted with 5 mM H₂SO₄ and analyzed by HPLC with Aminex-HPX87H column (Bio-Rad, Munich, Germany, 300 mm × 7.8 mm) using 5 mM H₂SO₄ as eluent. Substances were eluted at 65°C with a flow rate of 0.3 mL min⁻¹. Gluconate, 2-ketogluconate, and 5-ketogluconate were detected at 210 nm by a UV-vis detection system, while the separation of glucose was monitored by a refractive index detection. The concentration of the substances were quantified using the respective

calibration curves. The HPLC data were analyzed using the ChromGate Client 3.1.7 software (Knauer Wissenschaftliche Geräte GmbH, Berlin, Germany).

2.7.3. Photometric determination of glucose and fructose concentration

The cytoplasmic fractions of the glucose isomerase overproducing strains of *G. oxydans* were tested for the isomerization of glucose to fructose (Chapter 2.6.8.2). The assay mixture contained 50 mM phosphate buffer (pH 7.0), 100 μ l cytoplasm, 10 mM $MgCl_2$, and 250 mM D-glucose. After incubation at 30°C for 18 h, the mixture was filtered with a 0.22 μ m filter paper. Glucose and fructose concentrations in the filtrate were determined using the D-Glucose/D-Fructose UV-Kit (R-Boehringer AG Darmstadt, Germany) according to the manufacturer's instructions. The assay was performed in 1.5 mL plastic-cuvette with 760 μ L total reaction volume. The detection limit of the kit is of approximately 4-8 mg L⁻¹ for glucose and fructose, respectively. As positive control, 1.4 mM glucose and 2.8 mM fructose solutions were used along with test samples.

2.7.4. ¹³C-Nuclear magnetic resonance spectroscopy (¹³C-NMR)

¹³C-NMR is an analytical technique used to identify carbon atoms present in an organic compound and to elucidate the chemical structure of the compound. It detects only the magnetically active ¹³C isotope of carbon, present in a substance. These isotopes resonate at a unique frequency in a magnetic field and appear at a specific chemical shifts (δ) in NMR spectrum. Upon comparing the NMR spectra of unknown samples with standard NMR spectra or spectral libraries, chemical structure of all compound present in the samples could be solved. The technique was used to identify compatible solutes in *G. oxydans*. Therefore, the total cellular metabolites were extracted from 300 mg dried cell material of control, 10 % PEG-6000 stressed, and 300 mM sucrose stressed cultures by a modified Bligh and Dyer method (Chapter 2.7.1). The upper polar, water and methanol containing layers were dried and dissolved separately in 500 μ L of D₂O. Each mixture was supplemented with 5 mg trimethylsilyl propionate sodium salt (TMSP) and 10 μ L acetonitrile (ACN), added as internal standards. The NMR spectra were recorded on a Bruker Avance 300DPX operating at 75 MHz at the Institute of Pharmaceutical Biology of University Bonn. The spectra were processed using the Bruker TonSpin 1.3 software and calibrated to the residual solvent peaks of TMSP ($\delta_c=0$ ppm) and ACN ($\delta_c = 3.61$ and 121.8 ppm) (Lewis *et al.* 2009). The ¹³C-NMR standard shifts of other pure compounds are mentioned below:

Mannitol- δ :	73.61, 72.04, and 66.05 ppm
Fructose- δ :	104.33, 100.90, 83.50, 78.17, 77.26, 72.51, 72.03, 70.36, 66.70, 66.19, 65.45, and 65.22 ppm
Gluconate- δ :	181.46, 76.92, 75.41, 74.02, 73.80, and 65.46 ppm
Sucrose- δ :	106.30, 99.77, 84.00, 79.02, 76.58, 75.18, 75.01, 73.68, 71.83, 64.96, 63.97, and 62.73 ppm
Glucose- δ :	97.95, 94.18, 78.01, 77.74, 76.23, 74.76, 73.61, 73.43, 71.74, 62.87, and 62.75 ppm

2.7.5. Microscopy

The morphological examinations of *G. oxydans* cultures were done using a Zeiss Axio inverted microscope combined with a high-resolution fluorescence image system. Phase contrast imaging was performed to compare the morphological variations of cells in control and osmotically stressed cultures. Therefore, 1 μ L of the cultures was placed on thin agarose layers mounted on microscopic glass slides. Thin agarose layers were prepared by dissolving 1 % (w/v) agarose in phosphate-buffer saline. Microscopic examinations were performed with a Zeiss Axio Observer Z1 microscope using a 100x objective (1.46 oil, phase contrast 3). Moreover, the effect of osmotic stress was also observed on viability of the culture by staining the cells with SYTOX green dye (Molecular Probes, Life Technologies, Eugene, Oregon). Therefore, 20 μ L of the cultures was mixed with 1 μ L of 5 mM SYTOX green dye. 1 μ L of the mixture was imaged within 100 ms exposure time using an Axiocam MRM camera. A 38HE filter set was used at 470 nm and 524 nm excitation and emission maxima, respectively. The captured images were analyzed using the ZEN 2012 software.

2.8. Internet tools used for bioinformatic analysis

In the present study, a couple of online tools were employed for the ease of data analysis. For example, the NCBI BLAST programs (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) were used for the comparison of gene and protein sequences among all orthologous sequences, available in the databank (Altschul *et al.* 1997). Likewise, the KEGG database was used to access gene and protein sequences of *G. oxydans* (<http://www.genome.jp/kegg/>). The molecular mass of proteins was computed using the ExPASy tools (http://web.expasy.org/compute_pi/). Likewise, the identification of extracellular or periplasmic proteins was carried out by analyzing the amino acids sequences using Phobius (<http://phobius.sbc.su.se>). The prediction of protein secondary structure was done using Psipred (<http://bioinf.cs.ucl.ac.uk/psipred>) and

I-TASSER (<http://zhanglab.ccmb.med.umich.edu/I-TASSER/>). Protein motifs and conserved domains were identified by analyzing the amino acid sequences with the online EMBL-EBI tools such as Interpro (<https://www.ebi.ac.uk/interpro/>) and pfam (<http://pfam.xfam.org/>). For the comparison of gene or protein sequences, an EMBL-EBI tool Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) was used. The complete gene sequence of *G. oxydans* was obtained using the NCBI Genome database (<https://www.ncbi.nlm.nih.gov/genome>).

3. RESULTS

Members of the genus *Gluconobacter* are well known for their potential to survive in environments containing high amounts of sugars and polyols. One interesting and still unknown feature is the survival of these organisms in their natural osmotically enriched habitats. It has been reported that *G. oxydans* can grow in media containing as high as 30 % glucose (1.7 M) (Sievers and Swings 2005). Therefore, there was a need to analyze the behaviour of this organism under osmotic stress conditions to understand its physiology and to improve its catalytic efficiency.

In the present study, the physiological behavior of *G. oxydans* was explored in response to various osmotic stress conditions. In the first part, genome-wide-transcriptome analysis was performed to understand the global transcriptional response of *G. oxydans* induced by hyperosmotic stress. The transcriptome data were analyzed on the basis of previously described gene annotations, and two candidate genes were selected for further analysis including gene deletion and expression studies.

The second part comprises the results obtained from the analysis of intracellular metabolites of *G. oxydans* under osmotic stress by ¹³C-NMR spectroscopy and HPLC. These experiments were performed to identify a potential compatible solute helping the cells to survive in such adverse growth conditions. These experiments led to the identification of mannitol as an osmoprotectant in *G. oxydans*.

The third part focuses on the role of enzymes involved in the biosynthesis of mannitol and the osmotolerance of *G. oxydans*. These enzymes (fructose reductases) were characterized for their kinetic parameters. Furthermore, a closer look was taken to understand their importance for the survival of the cells under osmotic stress.

The final part of the results deals with some engineering strategies to further enhance the osmotolerance of *G. oxydans*. These experiments include the overexpression of gene *gox1432* (encoding a fructose reductase) and the *xylA* genes from *E. coli* and *S. griesus* (encoding glucose isomerases) in *G. oxydans*. The effect of overexpression of these genes on growth of the organism under osmotic stress was also investigated.

3.1 Osmotic stress responses in *G. oxydans*

3.1.1. Choice of substrate and osmolyte

In this study, the major aim was to examine the physiological and transcriptional responses induced by osmotic stress in *G. oxydans*. Initially, the composition of growth media was optimized to select suitable osmotic stress conditions. Therefore, two different media containing glucose (YG) or mannitol (YM) as carbon sources as well as osmolytes were compared for cultivation of *G. oxydans*. Control experiments were performed using 100 mM glucose or mannitol as carbon sources, resulting in an osmolality of about 170 mOsm kg⁻¹. The concentrations of both substrates were raised to 1000 mM (1100 mOsm kg⁻¹) to induce osmotic shock to the cultures. These osmotically enriched media were referred to as YG_{high} (containing 1 M Glucose) and YM_{high} (containing 1 M mannitol), respectively (Table 4). To measure the effect of osmotic stress on *G. oxydans*, growth parameters including growth rate (μ), doubling time (t_d), and final optical density of the cultures were evaluated. Moreover, the pH of the cultures was recorded at the end of the cultivation. It was observed that under non-stress conditions, both cultures had no apparent lag phase, divided exponentially with doubling times of 1.5 ± 0.1 h, and reached final optical densities of 1.4 with glucose and of 2.0 with mannitol as substrates (Fig. 6A and 6B).

The pH of the culture grown in YG medium dropped to 3.0 due to the formation of gluconic acid (Pronk *et al.* 1989). The cells grown in YM medium formed small amounts of acetate, resulting in a pH of 4.5 (Richhardt *et al.* 2012). In contrast to the control cultures, remarkable differences in growth parameters were observed between the cultures grown in YG_{high} and YM_{high} media. It was found that growth of *G. oxydans* in YG_{high} medium was highly impaired as compared to YM_{high} medium. In YG_{high} medium, the culture had an increased lag phase of 10 h, followed by slow growth with a doubling time of 4.2 h, and reached a final optical density of only 0.1 (Fig. 6A). In contrast, growth parameters in YM_{high} medium were only slightly affected with respect to the control. It was found that the doubling time of the stressed culture increased from 1.5 h to 2 h and the final OD₆₀₀ declined to 1.5 (Fig. 6B). Moreover, consistent with the control cultures, the final pH of the stressed cultures grown on YG_{high} and YM_{high} media was below 3.0 and 4.8, respectively. It has been reported that the metabolic activities of *G. oxydans* cease below the optimum pH value of 5.0 and results in poor growth yields (Olijve and Kok 1979). Therefore, it was concluded that the *G. oxydans* cultures were subjected not only to osmotic, but also to pH shock in YG_{high} medium. Hence, in the absence of a suitable buffering system, it was not possible to study exclusively

osmoinduced transcriptional response of *G. oxydans* grown on YG_{high} medium. Thus, glucose was not selected as growth substrate and osmolyte for further studies.

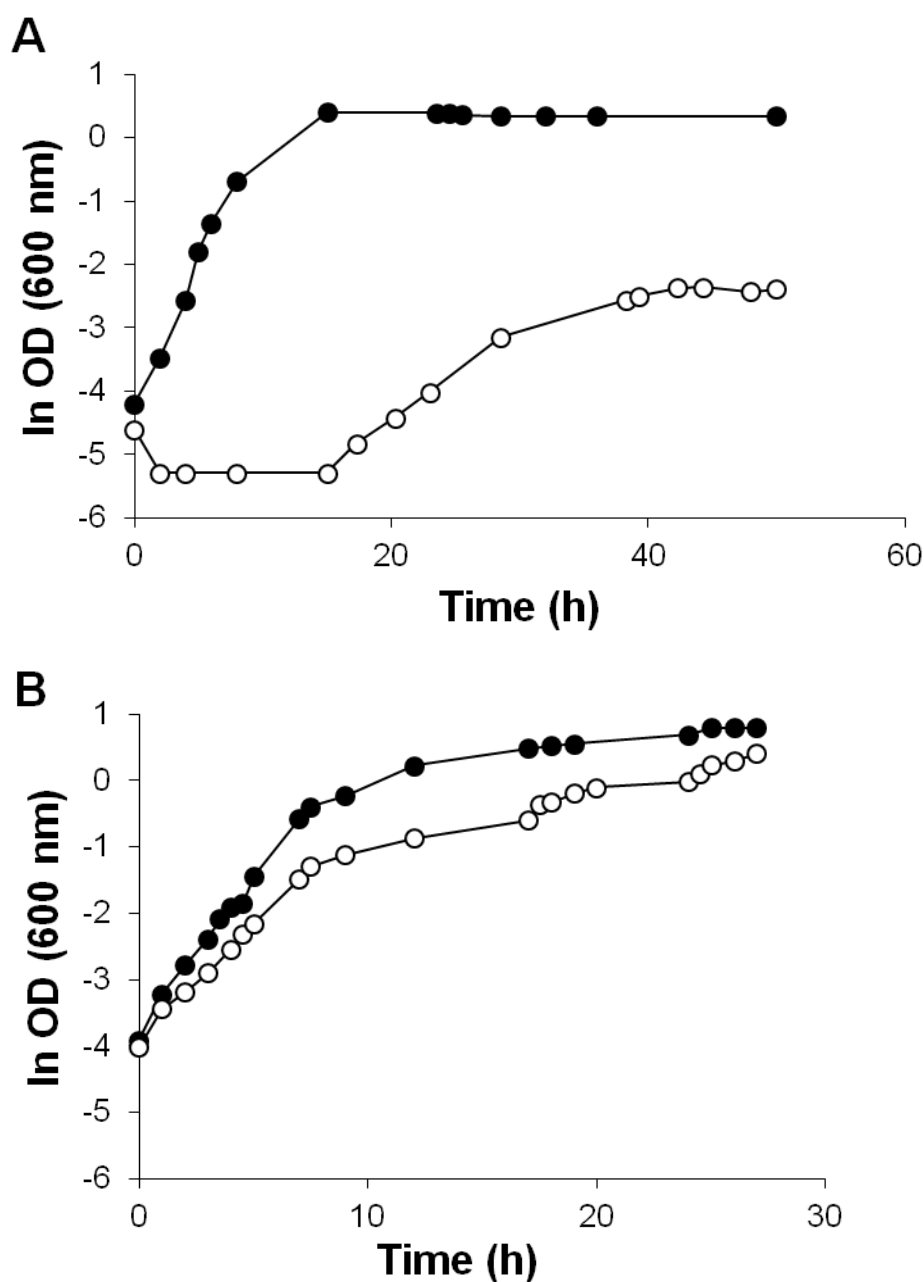


Figure 6: Effect of osmotic stress on the growth of *G. oxydans*. (A) Cultivation in YG (●) medium and YG_{high} (○) medium. (B) Growth in YM (●) medium and YM_{high} (○) medium.

Mannitol was a suitable carbon source that supported growth of *G. oxydans* and did not cause pH stress in un-buffered cultures. However, it was observed that in the presence of mannitol, the growth difference of the stressed culture was less pronounced in comparison to the control culture. This could be due to the fact that mannitol provided nutritional benefits to the culture, a process that is described in chapter 4. The alternative choice was a non-permeating osmolyte, such as polyethylene glycol (PEG) that decreases the water potential of growth

media similar to other osmolytes (Busse and Bottomley 1989, Kets *et al.* 1996a). It has been reported that the extent of decrease in water activity depends on the concentration of PEG applied to the medium (Michel 1983). Therefore, growth experiments were performed in the presence of PEG-6000 which was added in different amounts to YM medium. The concentration of PEG-6000 was varied from 25 g L⁻¹ (2.5 %) to 100 g L⁻¹ (10 %). It was observed that PEG-6000 in high concentrations had a negative effect on the growth parameters of the cultures. The growth rate and the final optical density decreased with increasing osmolality of the medium (Table 9). Noticeably, the decrease of the growth rate was most pronounced with 7.5 % and 10 % PEG-6000, which led to 65 % and 45 % reduction in the cell biomass and an increase of the doubling time from 1.6 h to 2.0 h, respectively. Thus, it was found that PEG-6000 reduced the water potential of the medium and negatively affected the growth of *G. oxydans*.

Table 9: Growth parameters of *G. oxydans* during cultivation under non-osmotic and osmotic stress conditions.

Media type	Growth rate (h ⁻¹)	Doubling time (h)	End optical density
YM (control)	0.43	1.6	2.0
YM + 2.5 % PEG-6000	0.41	1.7	1.5
YM + 5 % PEG-6000	0.39	1.8	1.4
YM + 7.5 % PEG-6000	0.37	1.9	1.3
YM + 10 % PEG-6000	0.35	2.0	0.9

3.1.2. Expression studies of osmotically regulated genes in *G. oxydans*

The osmotic stress responses of *G. oxydans* induced by mannitol or PEG-6000 were further compared at the transcriptional scale to select a suitable osmolyte for genome-wide transcriptome studies. Therefore, total RNA was extracted from exponentially grown cultures cultivated in three different media: 1) YM medium (170 mOsm kg⁻¹), 2) YM_{high} medium (1100 mOsm kg⁻¹), and 3) YM medium containing 5 % PEG-6000 (240 mOsm kg⁻¹). The extracted RNA preparations were checked for the contamination of DNA by conventional PCR. Only the pure samples, free of DNA contamination, were used for RT-qPCR analysis. To confirm the transcriptional regulation under osmotic stress, 10 different genes of *G. oxydans* were selected (Table 10) on the basis of their homology to osmoinduced genes found in *E. coli* and *P. aeruginosa* strains (Gunasekera *et al.* 2008, Andrés-Barrao *et al.* 2012). In these organisms, the osmoinduced genes were categorised into various groups on the basis of their functions such as involvement in the synthesis of compatible solutes, influx and efflux of solute molecules, regulation of transcription, and signal transduction. The putative

homologues of some of these genes in *G. oxydans* were identified using the NCBI non-redundant protein blast database (Table 10). The corresponding nucleotide sequences were used to generate gene specific primers (Table 2). After performing RT-qPCR, the transcript abundance of these genes was calculated in reference to the house keeping gene *gox0378* encoding the ribosomal protein L23. Genes with a fold change of above 2 were considered as up-regulated and less than 0.5 as down-regulated in the present study.

Table 10: List of selected genes of *G. oxydans* for RT-qPCR analysis

Gene ID	Annoation	Function
<i>gox0707</i>	DNA starvation/stationary phase protection protein	Cellular process (adaptation and protection)
<i>gox1119</i>	alpha,alpha-trehalose-phosphate synthase	
<i>gox1332</i>	Alkyl hydroperoxide reductase	
<i>gox1822</i>	UDP-N-acetylglucosamine acyltransferase	
<i>gox0820</i>	Molecular chaperone GrpE	Chaperon
<i>gox0857</i>	Molecular chaperone DnaK	
<i>gox1600</i>	Two component response regulator	Cell signalling and response
<i>gox1790</i>	Integrase/recombinase XerD	DNA replication, repair, recombination
<i>gox2182</i>	Mannitol/sorbitol ABC transporter permease	ABC transporters
<i>gox2220</i>	Ribose ABC transporter ATP-binding protein	

RT-qPCR analysis revealed that the expression of genes encoding a trehalose 6-phosphate synthase (*Gox1119*) and a two component response regulator (*Gox1600*) was up-regulated, whereas the gene encoding a DNA integrase/recombinase (*Gox1790*) was down-regulated in both osmotically stressed cultures (Fig. 7). However, a gene *gox1332* coding for an antioxidant like AhpC protein (alkyl hydroxyperoxide reductase) did not show any differential expression under both stress conditions. Expression of the other six genes was uniquely induced depending upon the type of osmolyte. For example, the genes encoding chaperonin proteins (*Gox0820*, *Gox0857*) and Dps proteins (*Gox0707*) were up-regulated (> 3 fold) in the culture containing PEG-6000, whereas the expression of these genes was either down-regulated or remained unchanged in YM_{high} culture. Molecular chaperonins are known to protect cells during hyperosmotic stress and heat shock by preventing the aggregation of denatured proteins (Wu *et al.* 1996). Similarly, Dps proteins protect cellular DNA from various stress situations like stationary and starvation phase, oxidative, desiccation, and acid base conditions (Almirón *et al.* 1992). Likewise, two other genes (*gox2182* and *gox2220*) encoding ABC transporter proteins were also down-regulated in YM_{high} culture. However, gene *gox2182* exhibited strong induction (4-fold), while the gene *gox2220* did not show any regulation in PEG-6000 containing culture. In addition, only one gene *gox1822* was up-

regulated in the culture grown in YM_{high} medium. The protein Gox1822 has been annotated as UDP-N-acetylglucosamine acyltransferase and is likely to be involved in lipopolysaccharide biosynthesis (Prust *et al.* 2005).

Hence, it was concluded from RT-qPCR results that the osmotic stress exerted by PEG-6000 induced significant transcriptional regulation in *G. oxydans* compared to that induced by mannitol. Therefore, pure RNA samples were extracted from the cultures stressed with PEG-6000 and utilized for Next Generation Sequencing (NGS).

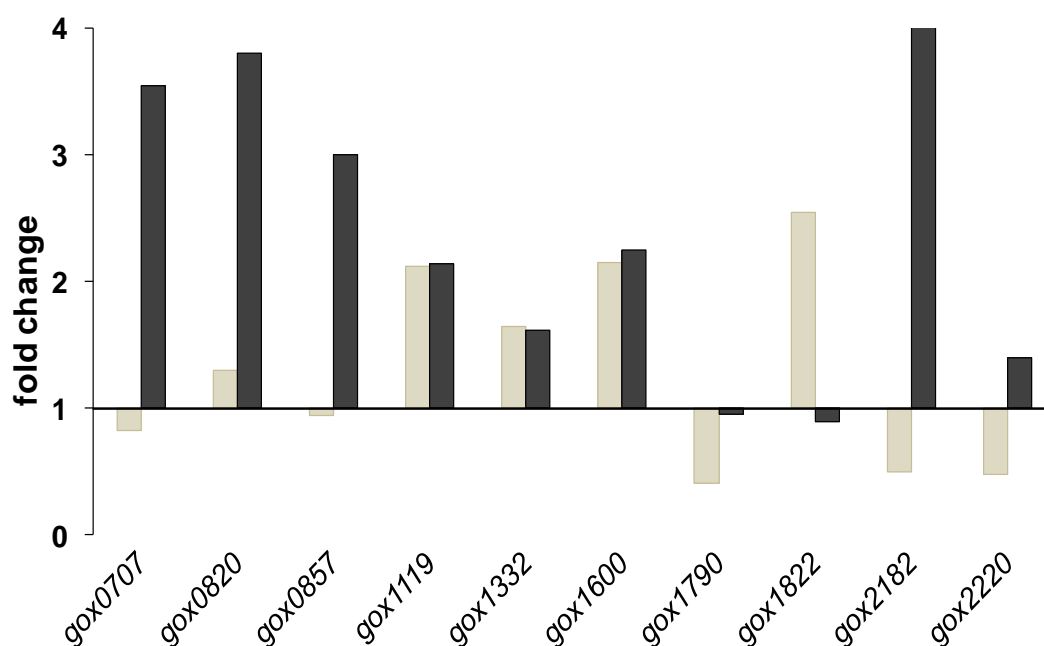


Figure 7: Differential gene expression in *G. oxydans* under osmotic stress. Gray and black bars represent the change of target genes expression under stress induced by 1 M mannitol and 5 % PEG-6000, respectively. The fold change was calculated as described in chapter 2.5.8.

3.1.3. Analysis of osmotic stress responses in *G. oxydans* through genome-wide transcriptome analysis

To have an insight into the global pattern of osmotic stress induced responses in *G. oxydans*, genome-wide transcriptome analysis was performed. Thus, high quality RNA samples were prepared from non-stressed and 5 % PEG-6000 stressed cultures and sent to BGI Tech Solutions Co., Ltd (Hongkong) for Illumina sequencing.

3.1.3.1. Quality testing of RNA samples

For Illumina sequencing, highly pure and intact RNA samples (free of DNA and protein contaminations) are recommended. Therefore, total cellular RNA was isolated from stressed and control cultures using the Trizol reagent method (Chapter 2.5.3.1) or the Ribopure™-Bacterial Kit (Ambion, Life Technologies; Chapter 2.5.3.2). The extracted RNA samples were treated twice with DNase I and subsequently purified using the Hi-Yield RNA pure Kit (Süd-Laborbedarf GmbH, Gauting, Germany; Chapter 2.5.3). The quality of the RNA samples was examined by performing denaturing agarose gel electrophoresis (Fig. 8). Therefore, 2-3 µg of each RNA sample was loaded onto the gel after spectroscopic quantification by a NanoDrop device (Chapter 2.5.4). The quality of the samples was analyzed by the estimation of the band intensities of the 23S and 16S rRNAs at 3 kb and 1.5 kb, respectively. The ratio of these bands for high quality RNA should be in the range of 1.5-2.5 (Ishikawa 1977). However, in the RNA samples of *G. oxydans*, two distinct bands with sizes of 1.6 kb and 1.4 kb were observed. Only a very faint band of the 23S rRNA was detected. Therefore, it was assumed that the 23S rRNA probably denatured during the extraction or purification procedure. However, no apparent degradation products were observed in the gel and all RNA samples, extracted by two different methods, revealed the similar results.

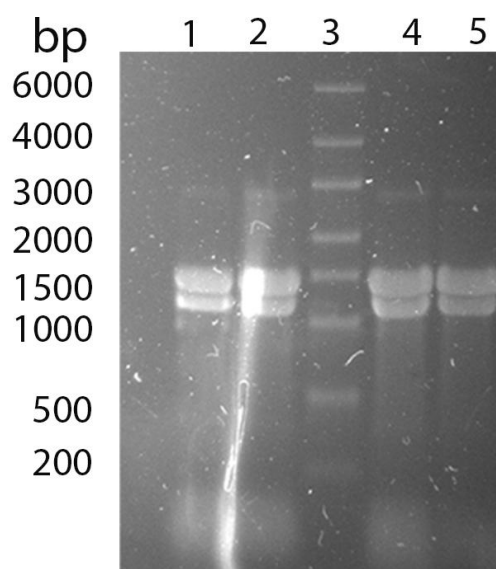


Figure 8: RNA samples separated by denaturing agarose gel electrophoresis. Lane 1 and 2: RNA samples were extracted from the non-stressed and stressed cultures, respectively, using the Trizol reagent method. Lane 4 and 5: RNA samples were prepared using the Ribopure™-Bacterial Kit from the non-stressed and stressed cultures, respectively. Lane 3: Riboruler high range RNA ladder (Thermo Scientific).

To further confirm the integrity of RNA, the samples were run on an automated electrophoretic system known as Bioanalyzer (Agilent technologies, Waldbronn, Germany). The intactness of RNA samples was calculated in a numerical form, referred to as RNA integrity number (RIN) (Chapter 2.5.12). Bioanalyzer results showed that all RNA samples were of high quality with a RIN value of above 7.0 (Fig. 9). Moreover, it was observed that the RNA samples extracted by the Trizol reagent method were more intact and pure in comparison to the samples extracted using the RibopureTM-Bacterial Kit. The RNA samples prepared by the Trizol reagent method showed high RIN values as well as high 260/230 nm and 260/280 nm absorbance ratios (Table 11). On the basis of these results, the total RNA extracted by the Trizol reagent method from non-stressed and 5 % PEG-6000 stressed culture were selected for sequencing.

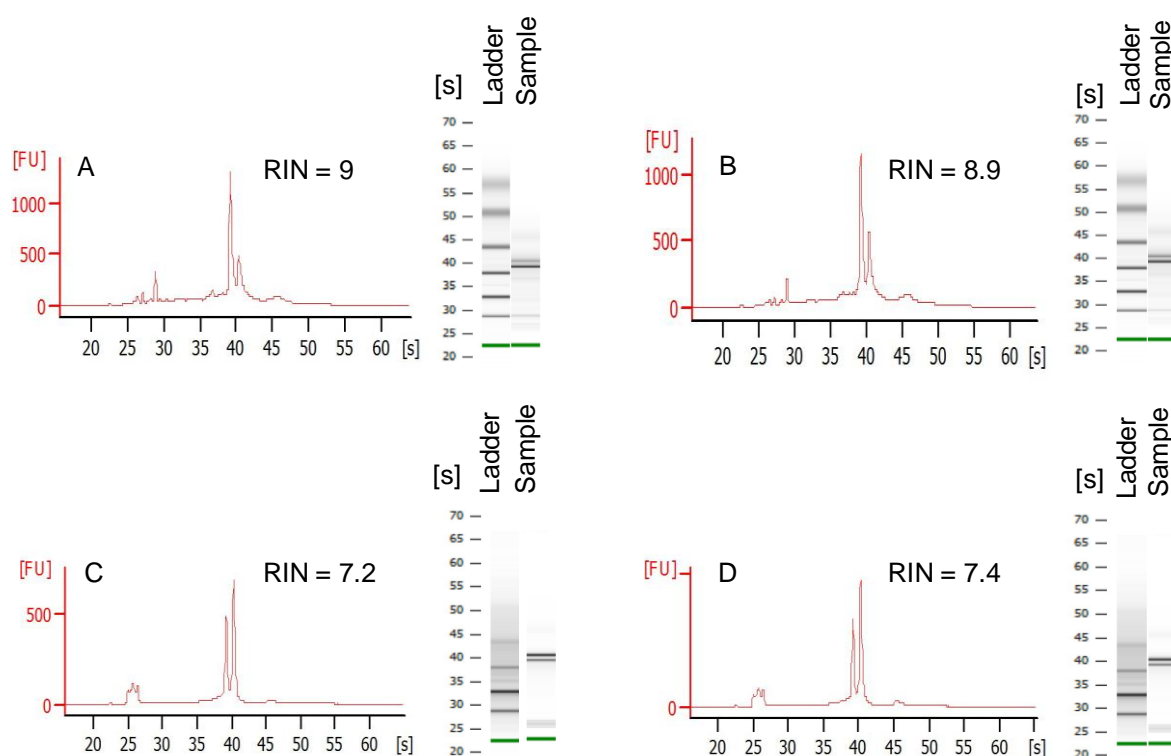


Figure 9: Agilent bioanalyzer based electropherograms and densitometry plots of the total RNA samples extracted from *G. oxydans*. (A) and (B) total-RNA extracted from non-stressed and stressed cultures, respectively, using the Trizol reagent method. (C) and (D) total-RNA isolated by using the RiboPure-Bacterial Kit from non-stressed and stressed cultures, respectively. [s] represents migration time of RNA fragments in seconds.

Table 11: Quality testing data of RNA samples analyzed by Bioanalyzer and NanoDrop

Name of RNA sample	Sample type	Extraction method	260/280 ^(a)	260/230 ^(a)	Concentration (ng/μl) ^(b)	RIN ^(b)
A	Non-stressed	Trizol based	2.12	2.13	2001	9.0
B	Stressed		2.12	2.16	1836	8.0
C	Non-stressed	RiboPure-Bacterial Kit	2.15	1.69	1071	7.2
D	Stressed		2.17	2.03	708	7.4

(a) Absorptions at 260 nm, 280 nm, and 230 nm were measured by NanoDrop

(b) Concentration and RIN values were determined by Agilent 2100 Expert software

3.1.3.2. Differential gene expression under osmotic stress analyzed by transcriptome sequencing

The osmoinduced differential gene expression in *G. oxydans* was analyzed at the transcriptional level by sequencing the RNA samples using Illumina sequencing technology. For NGS, a cDNA library of each RNA sample was prepared by BGI Tech Solutions Co., Ltd (Hongkong) as described in chapter (2.5.14). The library was then processed for cluster generation through bridge amplification, followed by Illumina HiSeq-2000 sequencing step (Meyer and Kircher 2010). The obtained sequencing data were aligned against the reference genome of *G. oxydans* 621H (GB: CP000004-9) and analyzed with the help of the DNA sequence viewer tool "Artemis" (Rutherford *et al.* 2000). From the raw sequencing data, the *RPKM* (Reads Per Kilobase of transcript per Million mapped reads) value of each gene was calculated (Equation 4), which indicated the transcript abundance of the corresponding gene. To compare the effect of osmotic stress, a ratio of the *RPKM* values of each gene from the stressed and control sample's RNA-Seq data was calculated and transformed into the logarithm base 2 (\log_2) ratio. The threshold value of these \log_2 ratios was arbitrarily set to + 1.0 and – 1.0 to select up- and down-regulated transcripts, respectively. It was found that 105 genes, out of the 2735 open reading frames (ORFs), showed differential expression (DE) under osmotic stress condition in comparison to non-stress condition. Amongst them, 103 genes were up-regulated and only two were down-regulated (Table 12). These differentially expressed genes were further categorized into 19 groups (Fig. 10, Table 13) according to their putative functions (annotations) as described by Prust *et al.* (2005). The selected categories are explained in the following paragraphs (i-vii).

(i). Genes involved in transport: A total of 14 genes encoding proteins involved in the transport of molecules across the cell were differentially expressed during osmotic up-shock. The phenomenon of water efflux across the cell membrane upon hyperosmotic shock deals

with the activity of transporter proteins like aquaporins, mechanosensitive channels, and other transporters for osmolyte uptake, etc. (Mager *et al.* 2000). In *G. oxydans*, most of the up-regulated genes encode transporter proteins that are members of the family of TonB-dependent receptor proteins. In addition, genes coding for ABC transporter proteins were also up-regulated, indicating the significance of active transport of solutes in osmotically stressed cells of *G. oxydans*.

(ii). Genes involved in signal transduction and regulation: Among other DE genes, an increased expression of four genes coding for transcription regulators was observed. Three of these proteins (Gox0676, Gox0717, Gox2164) belong to the family of single component transcription regulators, while one protein Gox1946 is a member of two component response regulator system.

Table 12: Number of up- and down-regulated genes of *G. oxydans* after osmotic up-shock.

Functional group of genes	Number of osmotic stress regulated genes	
	Up	Down
Metabolism	7	0
Respiration and energy metabolism	1	0
Carbohydrate metabolism	1	0
Glycolysis and gluconeogenesis	1	0
Pentose phosphate pathway	1	0
Amino acid metabolism	1	0
DNA modification	2	0
Lipid metabolism	1	0
Biosynthesis of lipopolysaccharide	1	0
Biosynthesis of cofactor	1	0
Regulation and signal transduction	4	0
Translation machinery	1	0
Transport	13	1
Motility	3	0
Stress link protein	1	0
Detoxification	2	0
Transposon function	11	0
Prophage function	10	0
Hypothetical proteins	41	1

(iii). Genes involved in metabolism: A total of 14 genes, up-regulated under osmotic stress, encode proteins that are probably involved in diverse cellular metabolic processes such as metabolism of sugars, amino acids, lipids, biosynthesis of lipopolysaccharides and cofactors, modification and repair of nucleic acid molecules, as well as biosynthesis of the secondary metabolite (trehalose). Trehalose is known for its function as an osmoprotectant in certain organisms *e.g.*, bacteria, yeast, and plants (Crowe *et al.* 1992, Garg *et al.* 2002).

(iv). Genes involved in motility: Flagella and pili are the extracellular appendages that protrude from cell surface of bacteria and participate in locomotion of cells. In *G. oxydans*, there are 29 genes that encode potential proteins involved in flagella biosynthesis and assembly. However, the transcriptomic data indicated that expression of only one of these genes (*gox0420*) was up-regulated, when the cells were grown in YM medium supplemented with 5% PEG-6000. Likewise, only two genes encoding type IV pilus proteins (PilL and PilN) showed enhanced expression under osmotic stress.

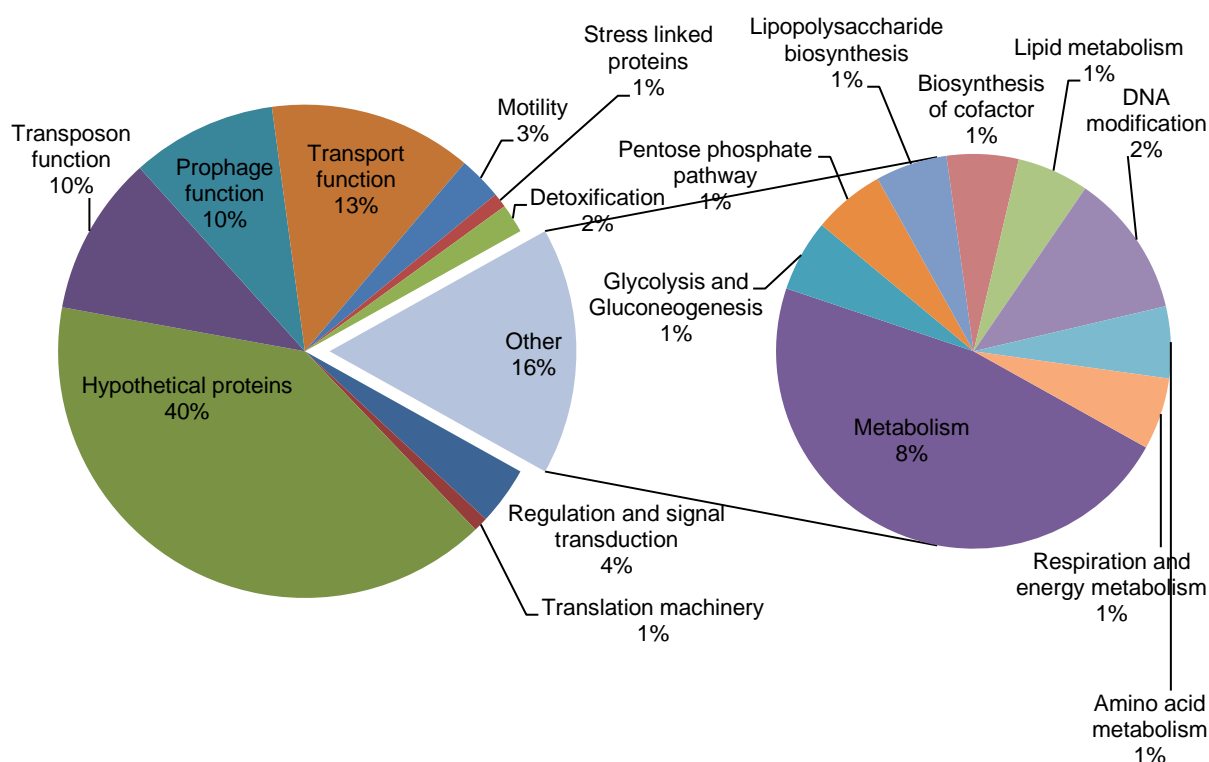


Figure 10: Pie charts showing the classification of osmotic stress regulated genes (fold change > 2) of *G. oxydans* (Prust *et al.* 2005).

(v). Genes involved in detoxification and stress responses: In the transcriptomic data, three genes, *gox0573*, *gox2163*, and *gox2559*, revealed higher expression under osmotic stress. Two of them, *gox0573* and *gox2559*, encode a metallo-beta-lactamase and a RND-type multidrug efflux pump, respectively. Both proteins are involved in resistance against antibiotics (Nikaido 1998). Whereas gene *gox2163* is involved in cold shock response. As the culture of *G. oxydans* was not subjected to cold and drug shock during cultivation, the elevated expression of these genes under osmotic stress might be a result of cross regulation among general stress response pathways (Gunasekera *et al.* 2008).

(vi). Genes with predicted functions: In this category, three genes (*gox1849*, *gox2594*, and *gox0201*) encoding putative oxidoreductase were up-regulated under osmotic stress condition. The oxidoreductases contain the NAD(P)⁺ binding domains and may be involved in cellular metabolic pathways, respiration, or biogenesis.

(vii). Genes with unknown functions: A high proportion of differentially expressed genes in the present data encode proteins with unknown functions. Among the 42 such DE genes, only one gene (*gox1696*) was down-regulated while all others were up-regulated.

Table 13: Differentially expressed genes of *G. oxydans* (fold change > 2) during cultivation on YM + 5 % PEG-6000 medium (osmotic stress culture) in comparison to YM medium (non-stress culture)

Locus Tag	Annotation	log ₂ ratio	p-value	Function
GOX0543	Xaa-Pro dipeptidase	1.24	1.15E ⁻⁰⁵	Metabolism
GOX0994	Acid phosphatase	1.06	0.00	
GOX1372	Glutathione S-transferase	1.44	6.11E ⁻¹³	
GOX1628	Protease	1.02	0.00	
GOX1849	Putative oxidoreductase	1.65	5.43E ⁻¹⁰	
GOX2594	Putative oxidoreductase	1.14	7.66E ⁻¹³	
GOX2711	Conjugal transfer protein, TraD	1.08	3.28E ⁻¹³	
GOX0201	NAD(P)H-flavin oxidoreductase	1.05	1.76E ⁻¹²	Respiration and energy metabolism
GOX1118	Trehalose-phosphatase	1.07	0.00	Carbohydrate metabolism
GOX2217	Triosephosphate isomerase	1.5	2.07E ⁻¹³	Glycolysis/Gluconeogenesis
GOX2218	Ribose-5-phosphate isomerase B	1.08	1.10E ⁻¹³	Pentose phosphate pathway
GOX0859	Shikimate 5-dehydrogenase	1.12	0.00	Amino acid metabolism
GOX2274	CDP-diacylglycerol- glycerol-3-phosphate 3- phosphatidyltransferase / cardiolipin synthase	1.30	0.00	Lipid metabolism
GOX1908	Uracil-DNA glycosylase	1.05	1.02E ⁻¹³	DNA modification
GOX2523	DNA helicase II UvrD	1.21	6.34E ⁻¹²	
GOX1205	Folylpolyglutamate synthase / dihydrofolate synthase	1.74	2.28E ⁻¹⁰	Biosynthesis of cofactor

GOX1610	Putative D-glycero-D-manno-heptose 1,7-bisphosphate phosphatase	1.12	1.12E ⁻¹⁰	Biosynthesis of lipopolysaccharide
GOX0676	Iron-regulated sigma factor	1.08	5.34E ⁻⁰⁵	Regulation and signal transduction
GOX0717	Transcriptional regulator LysR family	1.93	5.04E ⁻¹²	
GOX1946	Two component response regulator	1.30	0.00	
GOX2164	Transcriptional regulator	1.51	0.00	
GOX1175	Translation initiation inhibitor	1.07	3.16E ⁻⁰⁶	Translation machinery
GOX0541	TonB-dependent outer membrane receptor	1.21	3.37E ⁻¹³	Transport
GOX0542	Hypothetical metabolite transport protein	1.50	6.09E ⁻⁰⁵	
GOX0972	Cation efflux system protein CzcB	1.30	0.00	
GOX1075	TonB-dependent outer membrane receptor	1.25	0.00	
GOX1173	TonB-dependent outer membrane receptor	1.00	0.00	
GOX1184	ABC transporter sugar-binding protein	1.15	1.99 E ⁻⁰⁴	
GOX1188	Outer membrane receptor protein	1.56	1.42E ⁻¹⁴	
GOX1277	Na ⁺ /H ⁺ antiporter	1.05	1.26E ⁻¹²	
GOX1341	TonB-dependent outer membrane receptor	1.20	8.17E ⁻¹⁴	
GOX1886	Putative translocase transmembrane protein	1.20	5.92E ⁻¹³	
GOX1924	Putative permease	-1.02	5.34E ⁻²²	
GOX2219	Ribose ABC transporter, periplasmic binding protein	1.32	0.00	
GOX2220	Ribose ABC transporter, ATP-binding protein	1.30	0.00	
GOX2654	Integral membrane protein	1.10	7.21E ⁻⁰⁵	
GOX0420	Flagellar biosynthesis protein A	1.05	4.88E ⁻¹²	Motility
GOX2624	PilL	1.10	2.50E ⁻⁰⁵	
GOX2625	PilN	1.20	0.00	
GOX2163	Cold shock protein	1.84	7.86E ⁻¹²	Stress link protein
GOX0573	Metallo-beta-lactamase superfamily protein	1.06	0.00	Detoxification
GOX2559	RND-type multidrug efflux pump	1.10	0.00	
GOX0419	Transposase (class II)	1.14	1.65E ⁻¹³	Transposon function
GOX0463	Transposase	1.82	3.57E ⁻⁰⁷	
GOX1325	Transposase (Class III)	2.18	1.04E ⁻⁰⁸	
GOX1472	Transposase	1.20	2.84E ⁻¹³	
GOX1566	Transposase (class IV)	1.33	1.90E ⁻¹²	
GOX2201	Transposase (class II)	1.01	1.53E ⁻¹³	
GOX2593	Transposase (class V)	1.81	1.84E ⁻⁰⁵	
GOX2659	Transposase	1.40	0.00	
GOX2677	Transposase (class I)	1.20	1.67E ⁻¹³	
GOX2719	Transposase	1.53	4.41E ⁻¹²	
GOX2728	Truncated transposon gamma-delta resolvase	1.03	2.84E ⁻¹³	

GOX1213	Phage minor structural protein GP20	1.60	1.34E ⁻⁰⁷	Prophage function
GOX1218	Phage tail sheath protein	1.33	9.36E ⁻⁰⁸	
GOX1221	Phage-recombinase-like protein	1.03	1.76 E ⁻⁰⁴	
GOX2346	Putative phage protein	1.20	3.41E ⁻⁰⁶	
GOX2352	Bacteriophage tail protein	1.01	1.50E ⁻¹⁰	
GOX2356	Bacteriophage tail protein	1.26	4.10E ⁻¹³	
GOX2357	Bacteriophage tail fiber protein	1.08	1.03E ⁻⁰⁸	
GOX2447	Hypothetical protein	1.02	2.17E ⁻¹³	
GOX2465	Putative phage-related protein	1.07	1.54E ⁻¹³	
GOX2479	Phage-related protein	1.37	2.42E ⁻¹³	
GOX0247	Hypothetical protein	1.27	0.00	Hypothetical proteins
GOX0266	Hypothetical protein	1.03	1.48E ⁻¹⁰	
GOX0326	Hypothetical protein	1.31	0.00	
GOX0347	Hypothetical protein	1.20	0.00	
GOX0352	Hypothetical protein	2.21	0.00	
GOX0353	Hypothetical protein	1.40	2.75E ⁻¹³	
GOX0540	Hypothetical protein	1.90	2.54E ⁻⁰⁶	
GOX0650	Hypothetical protein	1.35	8.17E ⁻¹⁴	
GOX0904	Hypothetical protein	1.08	9.89E ⁻⁰⁷	
GOX1209	Hypothetical protein	1.30	3.54E ⁻⁰⁷	
GOX1210	Hypothetical protein	2.40	1.50E ⁻⁰⁵	
GOX1217	Hypothetical protein	2.40	4.03E ⁻⁰⁶	
GOX1222	Hypothetical protein	2.25	1.18 E ⁻⁰⁴	
GOX1309	Hypothetical protein	1.40	0.00	
GOX1391	Hypothetical protein	1.25	0.00	
GOX1401	Hypothetical protein	1.40	2.69E ⁻¹³	
GOX1509	Hypothetical protein	1.14	3.90E ⁻⁰⁶	
GOX1696	Hypothetical protein	-1.26	3.13E ⁻¹⁶	
GOX1850	Hypothetical protein	2.10	4.50E ⁻¹³	
GOX1890	Hypothetical protein	1.08	3.18E ⁻¹²	
GOX2060	Hypothetical protein	1.22	2.01E ⁻¹¹	
GOX2212	Hypothetical protein	1.11	0.00	
GOX2424	Hypothetical protein	1.04	1.72E ⁻⁰⁵	
GOX2461	Hypothetical protein	1.15	0.00	
GOX2463	Hypothetical protein	1.24	0.00	
GOX2482	Hypothetical protein	1.20	1.06E ⁻¹³	
GOX2506	Hypothetical protein	1.30	0.00	
GOX2533	Hypothetical protein	1.34	2.07 E ⁻⁰⁴	
GOX2536	Hypothetical protein	1.25	4.57E ⁻¹⁴	
GOX2571	Hypothetical protein	1.25	4.52E ⁻¹³	
GOX2573	Hypothetical protein	1.70	0.00	
GOX2600	Hypothetical protein	1.20	2.10E ⁻⁰⁹	
GOX2614	Hypothetical protein	1.23	4.30E ⁻⁰⁴	
GOX2666	Hypothetical protein	1.03	8.75E ⁻¹³	
GOX2667	Hypothetical protein	1.70	6.11E ⁻¹³	
GOX2672	Hypothetical protein	1.40	1.40E ⁻⁰⁷	
GOX2688	Hypothetical protein	1.10	0.00	
GOX2695	Hypothetical protein	1.60	8.67E ⁻⁰⁹	
GOX2698	Hypothetical protein	1.00	4.35E ⁻¹⁴	
GOX2712	Hypothetical protein	2.23	0.00	
GOX2720	Hypothetical protein	1.21	1.61E ⁻¹²	
GOX2723	Hypothetical protein	1.04	0.00	

3.1.3.3. Validation of the transcriptome data and selection of candidate genes

For the validation of the transcriptomic data, 12 out of 105 DE genes were randomly selected and their expression profile was analyzed by RT-qPCR. Therefore, fresh RNA samples were prepared from non-stressed and 5 % PEG-6000 induced stressed cultures. The RT-qPCR results revealed that the expression of nine genes (*gox0352*, *gox0676*, *gox1119*, *gox1205*, *gox1849*, *gox2164*, *gox2217*, *gox2220*, *gox2667*) was significantly up-regulated (fold change > 1.5) under hyperosmolal conditions. These results were consistent with the data obtained by RNA-Seq (NGS) technique (Table 14). However, in contrast to the NGS results, the expression of the other three genes (*gox0540*, *gox0717*, *gox1372*) was found to be unchanged by RT-qPCR. These observations indicate that most of the results obtained by RNA-Seq were consistent with the RT-qPCR values. However, as indicated above, some slight discrepancies existed. This result can be due to many reasons. One possible reason could be that the data obtained by NGS were affected by certain nuisance factors, referred to as technical effects or technology specific effects in the RNA-Seq literature (Fang and Cui 2011). However, the results obtained by RT-qPCR were validated by both biological and technical replicates, which confirmed the reliability of the data.

Table 14: Comparison of differential expression of selected genes under osmotic stress, examined by RNA-Seq and RT-qPCR.

Locus tag	Gene Annotation	Fold change by RNA-Seq	Fold change by RT-qPCR
GOX0352	Hypothetical Protein	4.6	2.3 ± 0.23
GOX0540	Hypothetical protein	3.7	1.1 ± 0.16
GOX0676	Iron-regulated sigma factor / transcriptional regulator	2.1	1.6 ± 0.23
GOX0717	LysR family transcriptional regulator	3.8	1.2 ± 1.1
GOX1119	Trehalose-phosphate synthase	1.3	2.2 ± 0.27
GOX1205	Folylpolyglutamate synthase/dihydrofolate synthase	3.4	1.5 ± 0.51
GOX1372	Glutathione S-transferase	2.7	0.7 ± 0.1
GOX1849	NADH oxidoreductase	3.2	3.3 ± 1.35
GOX2164	Transcriptional regulator	2.8	1.8 ± 0.25
GOX2217	Triosephosphate isomerase	2.8	1.5 ± 0.15
GOX2220	Ribose ABC transporter ATP-binding protein	2.5	1.4 ± 0.06
GOX2667	Hypothetical protein	3.3	3.2 ± 0.08

For further studies, two genes (*gox1119*, *gox1849*), which showed enhanced expression following osmotic shock, were selected and their role in osmoadaptation of *G. oxydans* was investigated. The gene *gox1119* encodes a trehalose-6-phosphate synthase, and forms an operon with gene *gox1118* that codes for a trehalose-6-phosphatase. Both proteins participate in the biosynthesis of trehalose, which is reported as compatible solute in different prokaryotic and eukaryotic organisms (Welsh and Herbert 1999). It was assumed that *G. oxydans* might synthesize and accumulate this osmoprotectant to combat osmotic stress. Therefore, deletion mutants lacking both *gox1118* and *gox1119* were generated and analyzed for their growth behavior under osmotic up-shock conditions in comparison to the wild type. The second gene *gox1849*, which was significantly up-regulated (> 3-fold) under osmotic stress, encodes a putative oxidoreductase. To examine the function of Gox1849, the protein was heterologously produced in *E. coli* for in vitro analysis.

3.1.3.4. Generation of *G. oxydans* strains carrying in-frame deletions for *gox1118* and *gox1119*

To generate deletion mutants of *G. oxydans* for the putative trehalose biosynthesis genes (*gox1118*, *gox1119*), 1 kb DNA fragments from the up- and downstream region of *gox1118-1119* were amplified and cloned into the multiple cloning site (MCS) of pKos6b vector, yielding pKos6bΔ*gox1118*Δ*gox1119* (Fig. 11). The construct was checked by double digestion and by sequencing of the isolated plasmid. As shown in Figure 12, double digestion of the plasmid resulted in DNA fragments with sizes of 6 kb for the plasmid backbone and 2 kb for the fused up- and down-fragment. After electroporation, the vector was integrated into the genome of *G. oxydans* by homologous recombination which resulted in kanamycin resistant mutants. Mutants carrying in-frame deletion of *gox1118* and *gox1119* were generated by plating *G. oxydans* transformants on 5-fluorocytosine containing agar plates (Chapter 2.5.16). The mutants were screened for deletion of the genes by colony PCR using a primer pair (Up_ox1117_fw/ Do_ox1120_rev), which binds in the up- and downstream region of the deleted fragments. PCR fragments of 964 bp corresponded to the deletion mutant, whereas 2733 bp fragments represented the wild type (Fig. 13).

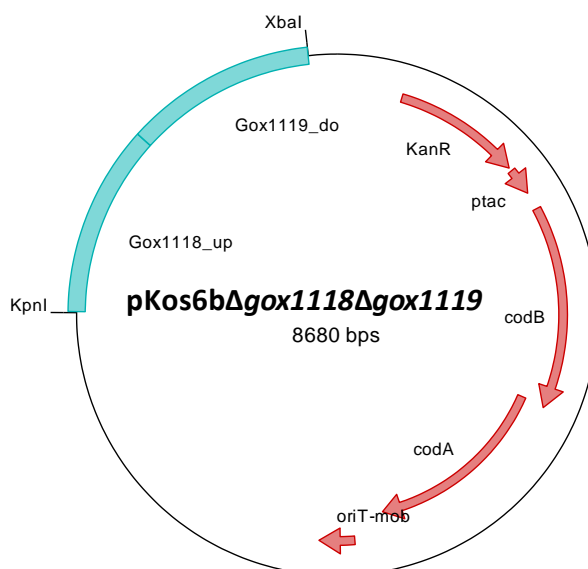


Figure 11: Vector map for the generation of knock out mutants of *G. oxydans* for genes *gox1118* and *gox1119*.

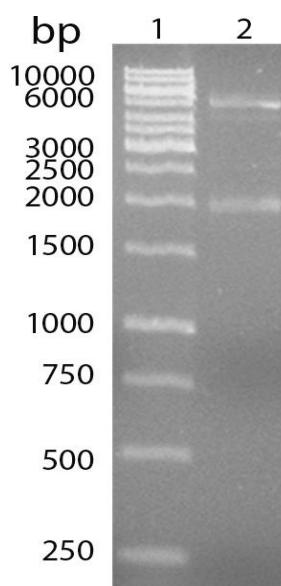


Figure 12: Restriction digestion of plasmid pKos6bΔgox1118Δgox1119. Lane 1: GeneRuler 1 kb DNA ladder (Thermo Scientific). Lane 2: Double digestion of the plasmid with *Xba*I and *Kpn*I.

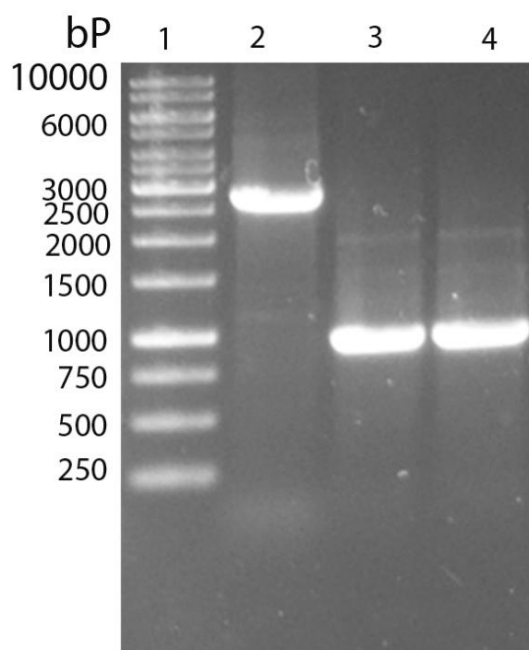


Figure 13: Colony PCR of *G. oxydans* to screen for mutants carrying deletion of *gox1118-gox1119*. Lane 1: 1 kb DNA ladder. Lane 2: PCR product of the wild type. Lanes 3-4: PCR products of the $\Delta gox1118\Delta gox1119$ mutants.

To investigate whether the deletion of genes *gox1118-gox1119* had an impact on osmotic tolerance of the cells, growth of the mutant strain (*G. oxydans* $\Delta gox1118 \Delta gox1119$) was compared with the wild type strain during cultivation on YM medium and YM medium with 10 % PEG-6000. The growth experiments clearly indicated that the deletion of *gox1118-1119* had no impact on growth of *G. oxydans*. Growth parameters of the mutant strain were comparable to the wild type under both conditions (Fig. 14 A and B). In YM medium, both cultures grew with the doubling time of 1.6 h and reached a final optical density of 2. In comparison, the doubling time of both cultures was slightly increased to 1.85 h and the final optical density was reduced to 0.9 in YM medium with 10 % PEG-6000. These results depicted that the genes *gox1118* and *gox1119* were apparently not involved in osmoregulation of *G. oxydans*.

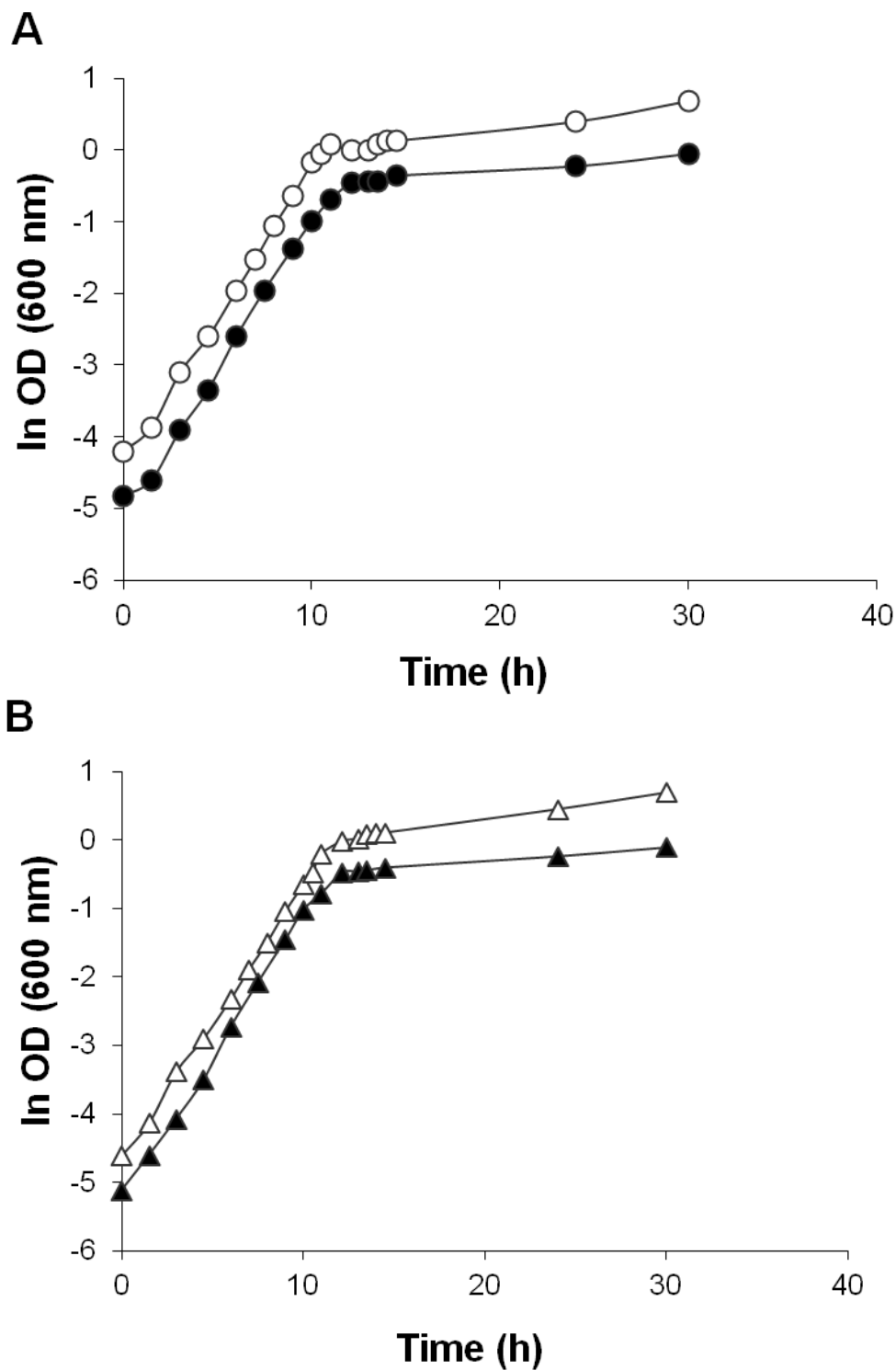


Figure 14: Effect of deletion of *gox1118-gox1119* on growth of *G. oxydans*. (A) Growth of the wild type culture on YM (○) medium and YM with 10 % PEG-6000 (●) medium. (B) Growth profile of *G. oxydans* $\Delta gox1118\Delta gox1119$ on YM (△) medium and YM with 10 % PEG-6000 (▲) medium.

3.1.3.5. Characterization of Gox1849: an uncharacterized oxidoreductase

Protein Gox1849 was annotated as putative oxidoreductase, consisting of 340 amino acids. The amino acid sequence of the polypeptide chain was analyzed by the Pfam protein database, indicating the presence of a Rossmann fold NAD(P)⁺ binding motif (G-X₁₋₂-G-X-V-G) (Rossmann *et al.* 1974, Brakoulias and Jackson 2004).

For the characterization of the enzyme, gene *gox1849* was cloned into an expression vector with a tetracycline inducible promoter (*tet* promoter), yielding pASK3_*gox1849* (Fig. 15). After transformation into *E. coli* (BL21), the recombinant protein was overproduced using anhydrotetracyclin as an inducer for the *tet* promoter. The expressed protein was purified by Strep-Tactin affinity chromatography and analyzed by SDS-PAGE. In total 4 mg protein was obtained from 1L *E. coli* culture. SDS-PAGE and western blot analysis revealed a molecular mass of 37.4 kDa for Gox1849, which is in accordance with the expected mass of 37.2 kDa for the recombinant tagged protein (Fig 16A and B).



Figure 15: Vector map for the heterologous overproduction of protein Gox1849 in *E. coli*.

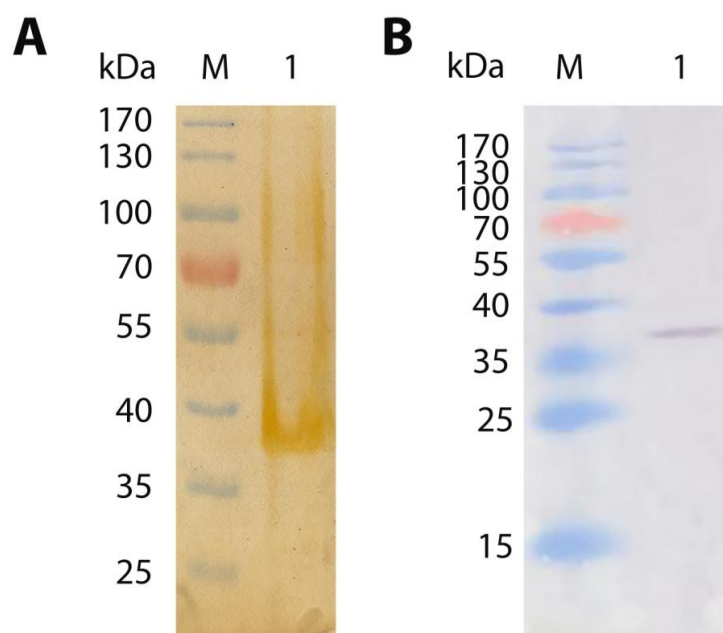
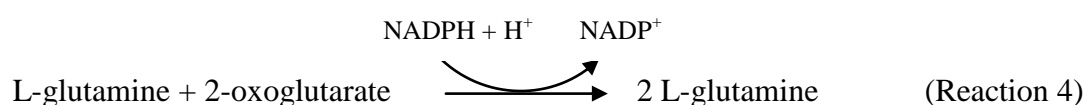


Figure 16: SDS-PAGE and western blot of protein Gox1849. (A) SDS-PAGE visualized by silver staining. (B) Immuno Blot. Lane M (A/B): PAGE ruler prestained protein ladder. Lane 1 (A/B): Gox1849.

The activity of the purified protein was tested with different substrates (polyols, aldehydes, ketones, amino acids, sugars and derivatives) in the presence of NAD(P)H or NAD(P)⁺ as electron donor or acceptor. However, the protein did not react with any of these substrates. Nevertheless, on close inspection of the genome of *G. oxydans*, it was found that gene *gox1849* clustered with the genes coding for a glutamate synthase (*gox1851* and *gox1852*) and the hypothetical gene *gox1850* (Fig. 17). It was assumed that all these four genes may constitute a functional operon for the biosynthesis of glutamate inside the cell. In bacteria, the glutamate synthase is a heterodimeric protein, constitutes of two subunits ($\alpha\beta$). The alpha-subunit contains a FMN binding domain and an amidotransferase domain to catalyze the conversion of 2-oxoglutarate and L-glutamine to L-glutamate (Reaction 4). The beta-subunit is a NADPH dependent oxidoreductase, containing FAD as a cofactor. This small subunit transfers reducing equivalents from NADPH to the FMN center of the alpha-subunit (Van den Heuvel *et al.* 2004).



Motif scan analysis using the InterPro protein database revealed that protein Gox1852 contains the glutamate synthase amidotransferase domain, while protein Gox1851 has a glutamate synthase FAD/NAD(P)⁺ binding domain. Due to the presence of a NADPH binding domain, it is tempting to speculate that Gox1849 could be a part of the electron transfer chain involving the β -subunit (Gox1851) for the reduction of 2-ketoglutarate as catalyzed by the α -subunit of the glutamate reductase. In such context, enhanced expression of gene *gox1849* provided an indication of a potential role of glutamate as an osmoprotectant in osmotically stressed cells of *G. oxydans*. Therefore, the presence and the osmoindependent accumulation of glutamate in *G. oxydans* was needed to be examined. This issue will be addressed in chapter 3.2.

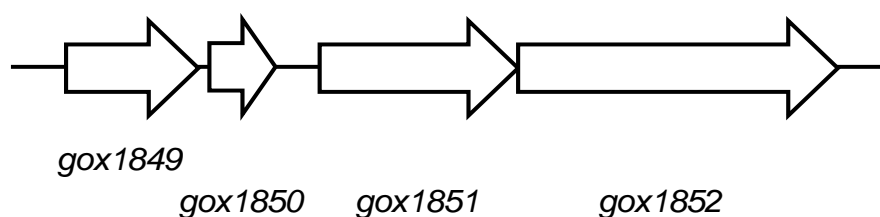


Figure 17: Genomic organization of a putative glutamine synthase encoding genes in *G. oxydans* 621H.

3.2. Mannitol as a major intracellular metabolite and osmolyte in *G. oxydans*

3.2.1. ¹³C-NMR spectroscopy of total cellular metabolites from *G. oxydans*

To address the question of glutamate and trehalose production in *G. oxydans* under osmotic stress, total cellular metabolites were extracted by a modified method of Bligh and Dyer (1959) from cultures grown on YM medium with 10 % PEG-6000 and analyzed by ¹³C-NMR. It was found that neither trehalose nor glutamate were accumulated in *G. oxydans* under osmotic stress. Therefore, no further studies were performed to investigate the involvement of the above mentioned genes (Chapter 3.1.3.5.) in the osmoregulation of *G. oxydans*. However, to our surprise, in the ¹³C-NMR spectrum, the most prominent signals were detected at chemical shifts of 73.6, 72.02, and 66.02 ppm that were identified as mannitol by comparison to its commercial standard. Similarly, the second highest resonances at 104.29, 100.87, 83.46, 78.22, 77.21, 72.46, 72.22, 70.33, 66.66, 66.15, 65.21, and 64.00 ppm were identified as D-fructose (Fig. 18). Intracellular accumulation of these two compounds provided evidence that *G. oxydans* might accumulate D-mannitol from the medium as a compatible solute to compensate the externally applied osmotic stress. D-

fructose is a potential precursor molecule for intracellular mannitol biosynthesis (discussed in detail in chapter 4 of the present study). Mannitol, which was provided as a carbon source, was oxidized to fructose by the cells via the membrane-bound polyol dehydrogenase (Gox0854 and Gox0855). Therefore, mannitol and fructose were present in excess in the medium, which probably remained in part in the external water space of the cell pellet after centrifugation of the culture for NMR analysis. Moreover, mannitol and fructose can also be taken up by the cells for intracellular assimilation as mentioned by Richhardt *et al.* (2012). Therefore, the signals observed in the ^{13}C -NMR spectrum derived obviously from both the extracellular medium and the intracellular space of the cells. In agreement to this finding, polyols such as D-mannitol have been reported as an osmoprotectant in some other organisms such as *Pseudomonas (P.) putida*, *Acinetobacter (A.) baylyi*, and *Zymomonas (Z.) mobilis* (Empadinhas and da Costa 2008, Sand *et al.* 2013). Regardless of being a major intracellular metabolite, the osmoprotective role of mannitol in *G. oxydans* was needed to be investigated for confirmation.

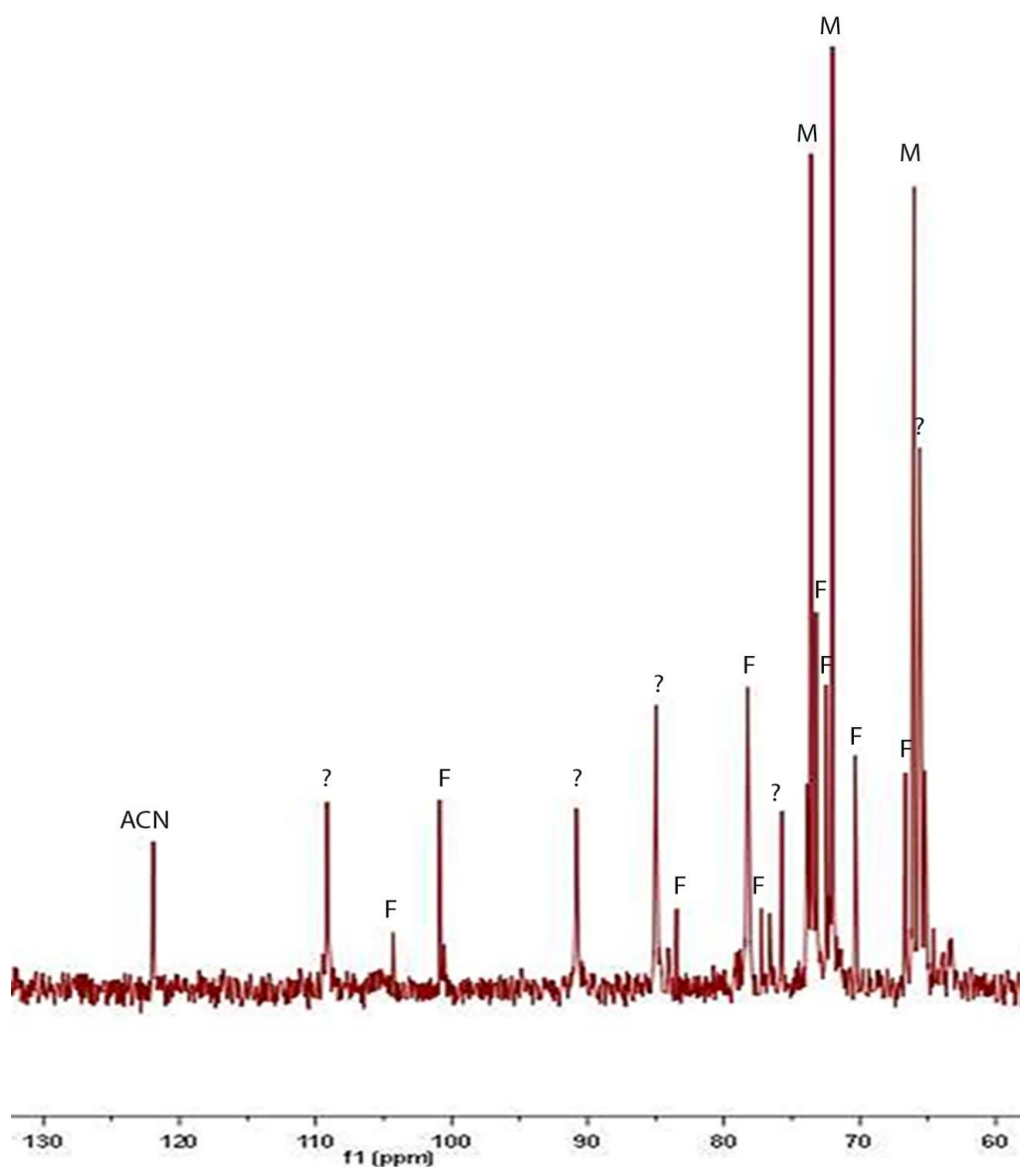


Figure 18: ^{13}C -NMR spectrum of total cellular metabolites from *G. oxydans*, cultivated in YM medium with 10 % PEG-6000. The spectrum was calibrated to the residual solvent peak of TMSP. Signals observed were from fructose (F), mannitol (M), internal standard acetonitrile (ACN), and from unknown compounds (?).

3.2.2. Effect of mannitol on cellular catalytic activity

The question arose, whether mannitol was able to protect *G. oxydans* from deleterious osmotic effects. To shed light on this question, the substrate consumption rate of the control culture was compared to the 10 % PEG-6000 stressed culture. In *G. oxydans*, most of the substrates are oxidized in the periplasm and their products are released into medium via outer

membrane porin proteins (Deppenmeier and Ehrenreich 2009). Thus, offline media samples were collected at different points during cultivation of both cultures and analyzed for metabolite concentrations by HPLC. In this experiment, the substrate mannitol was added at concentration of 20 g L^{-1} in the control and stressed cultures. Oxidation of mannitol to fructose is catalyzed by the membrane-bound polyol dehydrogenase (SldAB), which requires PQQ as a prosthetic group for catalysis and oxidizes a broad range of substrates such as mannitol, glycerol, sorbitol, arabitol, and gluconate (Matsushita *et al.* 2003). HPLC analysis revealed that the mannitol oxidation rates of the control and stressed cultures were identical during cultivation. It was observed that both cultures completely oxidized mannitol to fructose ($15\text{-}19 \text{ g L}^{-1}$) in the first 10-14 h of cultivation. Accordingly, the cells grew exponentially in the first 10 h with the growth rates of 0.4 h^{-1} (control culture) and 0.32 h^{-1} (stressed culture). At the end of cultivation, the final optical density of the control and stressed culture was 1.8 and 1.0, respectively. However, both cultures accumulated almost equal amount of fructose (16 g L^{-1}) in the media (Fig. 19). Hence these results indicated that growth of *G. oxydans* was only slightly affected under osmotic up-shock. Moreover, the cultures could oxidize mannitol with comparable efficiencies.

In summary, these observations further supported the assumption that mannitol had an osmoprotective role in *G. oxydans* to counteract osmotic stress imposed by PEG-6000. This phenomenon could also explain the weak transcriptional response of the organism towards the applied osmotic stress (Chapter 3.1.3.2). Because of the protective effect of mannitol, osmotic stress imposed by PEG-6000 could have been overcome by the cells, resulting in minor modifications of gene expression. Evidence in favor of this hypothesis came from the NGS data that manifested a relative low change in expression level of transcripts in response to PEG-6000 induced osmotic stress (Chapter 3.1.3.2). Moreover, the degree of protection of cellular functions by an osmoprotectant strongly depends upon the magnitude of external osmotic stress (Amezaga *et al.* 1995, Kets *et al.* 1996b). It was examined that 5 % PEG-6000 raised the osmolality of YM medium only from 170 to 240 mOsm kg^{-1} . Furthermore, the presence of mannitol as putative compatible solute might have led to experimental conditions which were insufficient to trigger a stringent osmotic stress response in *G. oxydans*.

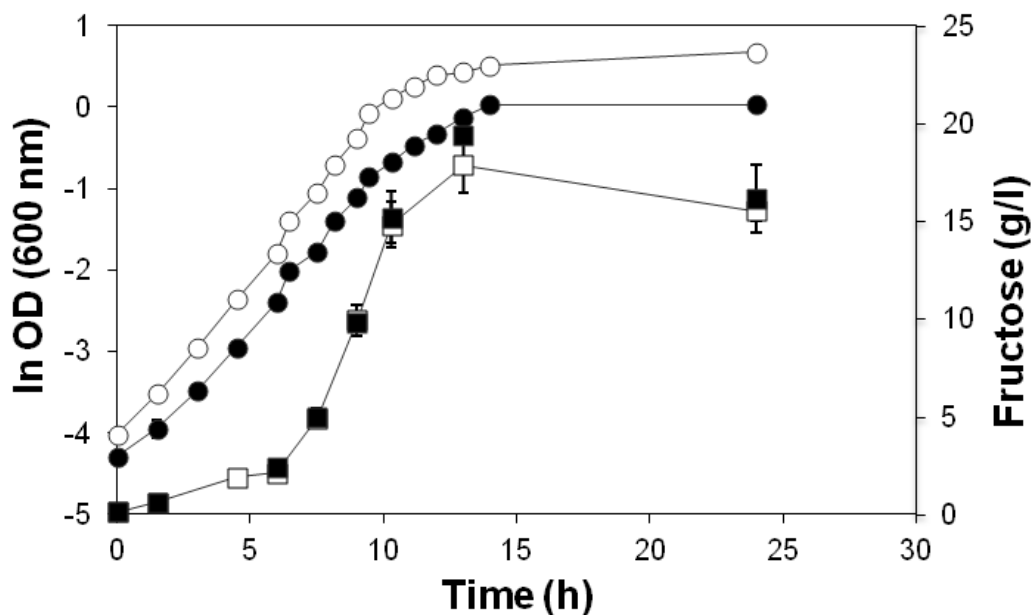


Figure 19: Effect of osmotic stress on growth and fructose production of *G. oxydans*. Cells were cultivated in YM medium (open symbols) or YM medium with 10 % PEG-6000 (filled symbols). Correlation between growth (○,●) and D-fructose formation (□,■) of the cultures.

3.2.3. De novo synthesis of mannitol in *G. oxydans* under reduced water activity

In the previous experiments it was shown that *G. oxydans* proliferates in media with low water activity by accumulation of exogenously supplied D-mannitol. However, there was still the question whether *G. oxydans* can synthesize this osmoprotectant de novo. To address this question, the microorganism was cultivated in YGP medium (310 mOsm kg^{-1}) containing yeast extract (0.6 g L^{-1}), glucose (50 mM) as a carbon source, and 100 mM phosphate buffer ($\text{pH } 7.0$) for pH control. To increase the osmolality, the medium was supplemented with 300 mM sucrose which resulted in a total osmolality of 680 mOsm kg^{-1} . Sucrose is a natural osmolyte, present in the ecological niches of *G. oxydans*. It constitutes almost 40-60 % of the total sugar content of nectar (Johnson and Nicolson 2008). Moreover, *G. oxydans* cannot metabolize this sugar due to a lack of hydrolysing enzymes (Prust *et al.* 2005, Peters *et al.* 2013a). In the presence of this non-metabolizable osmolyte, cells were grown till the early stationary phase and processed for the examination of intracellular osmolytes by ^{13}C -NMR spectroscopy. In the ^{13}C -NMR spectra, three distinct resonances at 73.42, 71.85, and 65.87 ppm were identified as mannitol. These signals were much more pronounced in the spectrum

of stressed cultures in comparison to the non-stressed cultures (Fig. 20 A and B), indicating the osmotic relevance of the de novo-synthesized D-mannitol. Moreover, in the metabolite pool of stressed cultures, additional resonances at 104.12, 100.70, 83.27, 78.04, 77.05, 72.28, 71.93, 70.15, 66.49, 65.98, 65.44, 65.23 ppm and 106.30, 99.77, 84.00, 79.02, 76.58, 75.18, 75.01, 73.68, 71.83, 64.96, 63.97, 62.73 ppm were identified as fructose and sucrose, respectively. Whereas, other signals corresponded to glucose (98.5, 94.7, 78.54, 78.36, 76.75, 75.37, 74.09, 74.06, 72.29, 63.74, 63.37 ppm) and gluconate (181.24, 76.75, 75.37, 74.05, 73.74, 65.44 ppm). All these compounds were identified by comparison of their resonances with the ^{13}C -NMR spectra of pure standards. Glucose and sucrose were present in the medium while gluconate derived from the oxidation of glucose in the periplasm. Fructose could be an intermediary compound for mannitol production. These results depicted that *G. oxydans* could synthesize mannitol de novo with glucose as a substrate for biosynthesis.

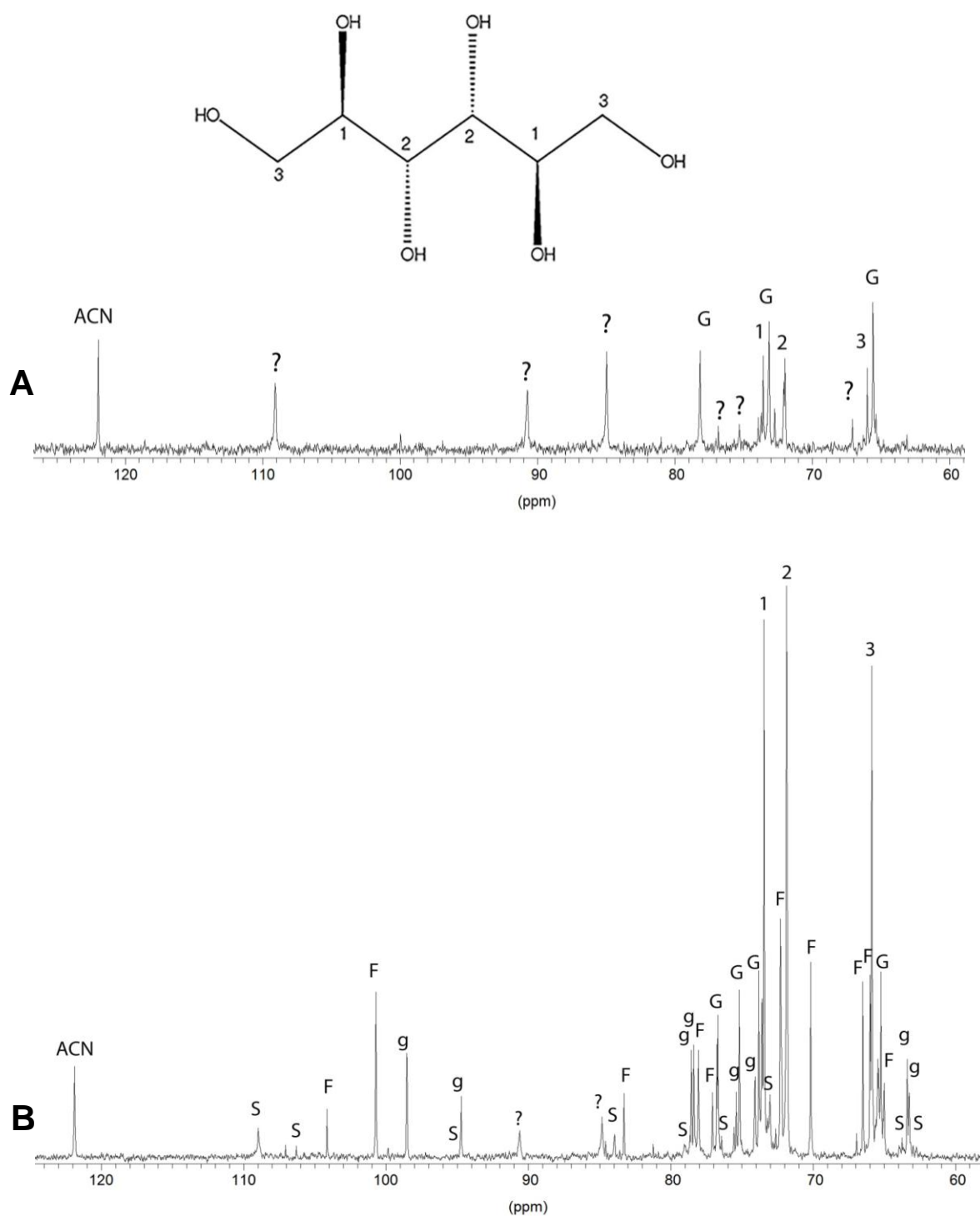


Figure 20: ^{13}C -NMR spectra of the total cellular metabolites of *G. oxydans*. Results are shown for extracts of cells cultivated in YGP medium (A) and YGP medium supplemented with 300 mM sucrose (B). The spectra were calibrated to the residual solvent peak of TMSP. Signals were from fructose (F), glucose (g), gluconate (G), sucrose (S), mannitol (1,2,3), internal standard acetonitrile (ACN), and from unknown compounds (?). *Inset:* chemical structure of mannitol and assignment of the carbon atoms. (Modified from Zahid *et al.* 2015).

For further analysis and quantification of the mannitol accumulation pattern under osmotic stress, *G. oxydans* was grown in YGP medium with 0-600 mM sucrose (310 mOsm kg⁻¹ to 1160 mOsm kg⁻¹) as osmolyte. It was found that under non-osmotic stress conditions, *G. oxydans* grew with the doubling time of 1.5 h and reached a final optical density of 1.8 after 24 h of cultivation. Under stress conditions, the doubling time of the culture gradually increased from 1.5 to 3.6 h, while the optical densities decreased from 1.8 to 0.5 with increasing sucrose concentrations from 0-600 mM (Fig. 21). The effect of osmotic stress was more pronounced at sucrose concentrations of 450 mM and 600 mM (900-1160 mOsm kg⁻¹) that led to a sharp increase of the doubling times of the cultures up to 2.5 and 3.6 h, respectively. Likewise, the final optical densities of the cultures were severely reduced to 0.7 and 0.5, respectively.

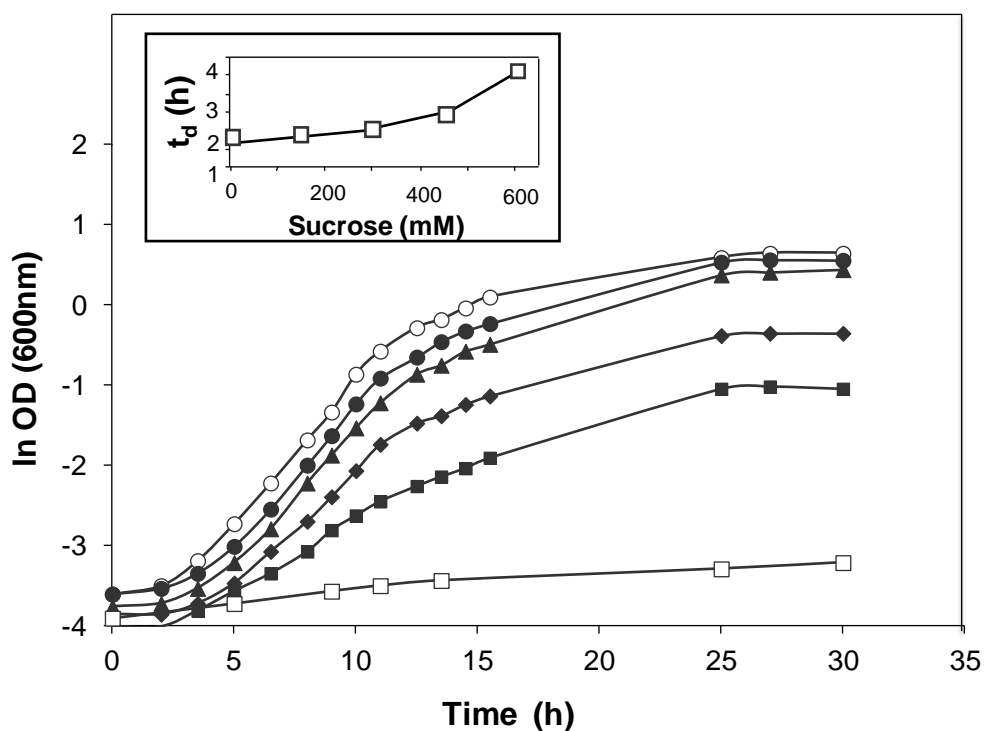


Figure 21: Effect of increasing sucrose concentrations on growth of *G. oxydans*. Cells were cultivated in YGP medium (○), supplemented with 150 mM sucrose (●), 300 mM sucrose (▲), 450 mM sucrose (◆), and 600 mM sucrose (■). The YGP medium contained yeast extract (0.6 g L⁻¹), glucose (50 mM), and 100 mM phosphate buffer (pH 6.8). Control cultures were cultivated in yeast extract-sucrose (300 mM) without glucose (□). The *inset* represents the effect of the sucrose concentration on the doubling time (t_d). (Modified from Zahid *et al.* 2015).

To correlate the effect of osmotic stress with the intracellular mannitol accumulation pattern, total cellular metabolites from control and stress cultures were extracted and analyzed by HPLC. Concordant with the ^{13}C -NMR results, HPLC analysis revealed the accumulation of both mannitol and fructose in osmotically stressed cells of *G. oxydans* that were grown on YGP medium in the presence of different amounts of sucrose (Fig. 22). The levels of the internal mannitol and fructose content increased linearly with the increase of the external sucrose concentration and reached saturation limits of up to 2.7 and 1.4 $\mu\text{mol mg}^{-1}$ cell protein at 450 mM sucrose (906 mOsm kg^{-1}), respectively. Further increment in the sucrose concentration (600 mM) only led to a slight increase in the mannitol content up to 2.8 $\mu\text{mol mg}^{-1}$ protein without affecting the fructose concentrations. Moreover, the amount of intracellular glucose remained constant, with a maximum of up to 0.4 $\mu\text{mol mg}^{-1}$ protein at 300 mM to 600 mM external sucrose. These results corresponded to the growth profile of *G. oxydans* and indicated that higher sucrose concentrations in the media (450-600 mM) impaired cell division, biomass formation, and mannitol accumulation. In comparison to stressed cells, mannitol was detected in very low concentrations (0.08 $\mu\text{mol mg}^{-1}$ protein) in non-stressed cells. These observations indicated that the intracellular accumulation of mannitol and fructose is influenced by the extracellular osmolality of the medium. In addition, it seems that mannitol, being a primary osmolyte, plays an important role in *G. oxydans* under osmotic stress conditions.

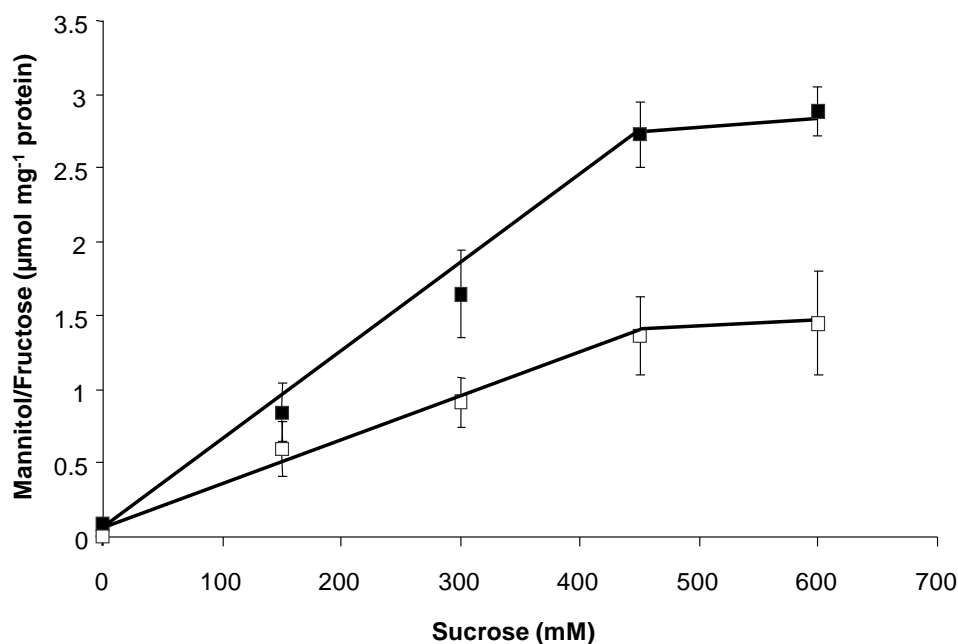


Figure 22: Effect of extracellular sucrose on intracellular mannitol and fructose accumulation of *G. oxydans*. Cells were cultured in YGP medium with the addition of sucrose (0-600 mM). Mannitol (■) and fructose concentration (□) were determined by HPLC. The figure represents the mean of three independent experiments. Error bars indicate standard deviation. (Modified from Zahid *et al.* 2015).

3.2.4. Effect of carbon sources and osmolytes on mannitol accumulation

In microorganisms, the synthesis and the accumulation of osmoprotectants are influenced by the carbon sources and osmolytes present in the medium. For example, in *P. putida* intracellular levels of compatible solutes (glycine betaine, mannitol, glutamate, and NAGGN) were sensitive to the change in water activity of minimal medium (Kets *et al.* 1996a, Kets *et al.* 1996b). To examine such effects on the levels of intracellular mannitol accumulation, *G. oxydans* was cultivated in media containing permeating and non-permeating osmolytes such as glucose, fructose, glycerol, and PEG-6000. It was observed that permeating and metabolizable osmolytes such as 0.5 M (610 mOsm kg⁻¹) fructose or 1 M (1580 mOsm kg⁻¹) glucose induced the osmodependent accumulation of mannitol up to 0.82 ± 0.01 and 2.0 ± 0.4 μmol mg⁻¹ protein, respectively. The intracellular levels of mannitol were slightly lower but comparable to those obtained with the non-metabolized osmolyte sucrose (Table 15). On contrary, mannitol accumulation was not observed when 0.5 M (682 mOsm kg⁻¹) glycerol

was used as carbon source and osmolyte in the medium. Rather mannitol, the cells accumulated glycerol in high amounts ($5 \mu\text{mol mg}^{-1}$ protein) to combat external osmotic stress. A clear fluctuation in the intracellular mannitol content was observed when the osmolality of the growth media was raised with the non-permeating osmolyte PEG-6000. Addition of 10 % PEG-6000 in media containing 50 mM glucose or fructose (YGP or YFP) as carbon sources resulted in the accumulation of mannitol up to only 0.3 and 0.9 $\mu\text{mol mg}^{-1}$ protein, respectively. These results indicated that osmodependent accumulation of mannitol in *G. oxydans* was substantially affected by the change of the carbon sources and osmolytes present in the growth media.

Table 15: Effect of carbon sources and osmolytes on the intracellular levels of mannitol in osmotically stressed cells of *G. oxydans*. (Modified from Zahid and Deppenmeier 2016).

Medium	Osmolality (mOsm kg ⁻¹)	Intracellular mannitol ($\mu\text{mol mg}^{-1}$ protein)
YGP	310	0.08 ± 0.03
YF _{high} (0.5 M fructose)	610	0.82 ± 0.01
YFP + 10 % PEG-6000	665	0.9
YGP + 10 % PEG-6000	665	0.3
YGP + 600 mM sucrose	1160	2.80 ± 0.2
YGP _{high} (1 M glucose)	1580	2.00 ± 0.4

3.2.5. Effect of exogenous mannitol on growth and morphology of osmotically stressed cells

As mentioned above (Chapter 3.2.1 and 3.2.3), *G. oxydans* could synthesize mannitol during cultivation under osmotic stress conditions. It was assumed that the bacterium might use this compound as an osmoprotectant to survive under reduced water activity situations. To investigate such osmoprotective role, growth of the organism was examined during cultivation in osmotically enriched media in the presence of extracellular mannitol. Therefore, *G. oxydans* was cultivated in YGP medium supplemented with either 300 mM or 600 mM sucrose (680 or 1160 mOsm kg⁻¹, respectively) and 10 mM mannitol. As expected, significant improvement in growth of the stressed cultures was observed with exogenous mannitol. Addition of 10 mM mannitol restored the normal growth pattern of the culture exposed to 300 mM sucrose stress. The doubling time of the culture decreased from 2 h to 1.4 h and the final optical density increased from 1.5 to 1.7. In addition, 10 mM exogenous mannitol could partially alleviate the inhibitory osmotic effect of 600 mM sucrose. However, the growth pattern of the culture was comparable to that of *G. oxydans* cells exposed to 300

mM sucrose stress in the absence of extracellular mannitol. The doubling time of culture in the presence of mannitol reduced from 3.5 h to 2 h and the final optical density increased from 0.5 to 1.5 (Fig. 23A). Next, the osmoprotective effect of mannitol was more exclusively examined when applied in low concentrations (2.5 mM and 5 mM) to *G. oxydans* cells exposed to 300 mM sucrose stress. Like the addition of 10 mM mannitol, both 2.5 and 5 mM mannitol concentrations were equally efficient to restore normal growth behavior of the cells. Doubling times of the cultures reduced to 1.86 h and 1.85 h and the final ODs reached 109% and 112% of the non-stressed control cultures (Fig. 23B). In accordance with osmoindependent biosynthesis and accumulation of mannitol, these results clearly depicted the osmoprotective role of this polyol in *G. oxydans*. Moreover, these data indicate that very low amounts of exogenously supplied mannitol are sufficient for osmoprotection against 300 mM sucrose (680 mOsm kg⁻¹).

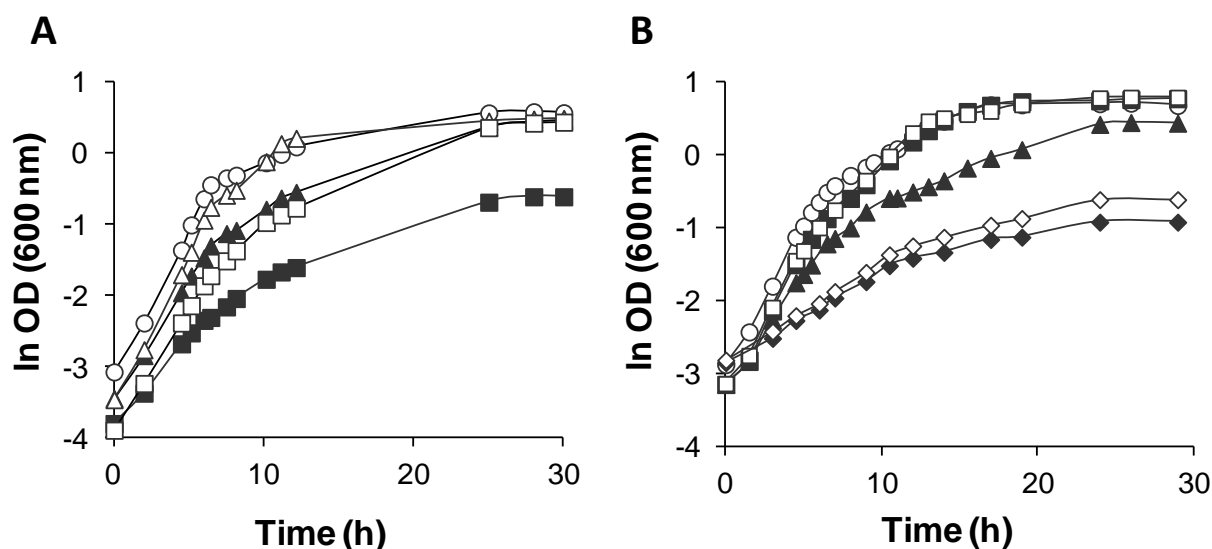


Figure 23: Effect of extracellular mannitol on growth of *G. oxydans*. (A) Growth curves in YGP medium supplemented with 0 mM sucrose (○), 300 mM sucrose (▲), 300 mM sucrose + 10 mM mannitol (△), 600 mM sucrose (■) and 600 mM + 10 mM mannitol (□). (B) Growth curves in YGP media with 0 mM sucrose (○), 300 mM sucrose (▲), 300 mM sucrose + 2.5 mM mannitol (■), and 300 mM sucrose + 5 mM mannitol (□). Control cultures were cultivated in yeast extract (0.6 g L⁻¹) with 2.5 mM mannitol (◆) or 5 mM mannitol (◇). (Zahid *et al.* 2015).

It was reported that like other acetic acid bacteria, *Gluconobacter* sp. form involutions during unfavorable growth conditions (Asai 1971, De Muynck *et al.* 2007). These irregular cell shapes can be of various forms such as cylindrical or filamentous cells, enlarged swollen

cells, or other aberrant forms. The presence of such involution forms was examined in *G. oxydans* cultures subjected to 600 mM sucrose stress. *G. oxydans* cells, cultivated under control (non-stressed) conditions were of rod- or oval-shapes (Fig. 24A). Upon addition of 600 mM sucrose, various unusual cells with swollen ends or cylindrical cell filaments along with some normal cells were observed (Fig. 24B). The deformity of the cells was considerably reduced upon addition of 10 mM mannitol to cells exposed to 600 mM sucrose (Fig. 24C).

To determine the viability of such aberrant cell forms, SYTOX green dye was used. The dye stains DNA of cells having compromised/porous cell membranes, and is used to distinguish dead cells from live cells in a culture. Upon staining, all irregular or involuted cell forms were found to be senescent, and comprised almost 20 % of the total cells in the culture with 600 mM sucrose (Fig. 24D). Addition of 10 mM mannitol to the stress culture resulted in an increased viability of the cells regardless of their morphology (Fig. 24E). The stimulatory effects of exogenously supplied mannitol on growth, viability, and morphology of the osmotically stressed cells clearly underlines the function of mannitol as an osmoprotectant in *G. oxydans*.

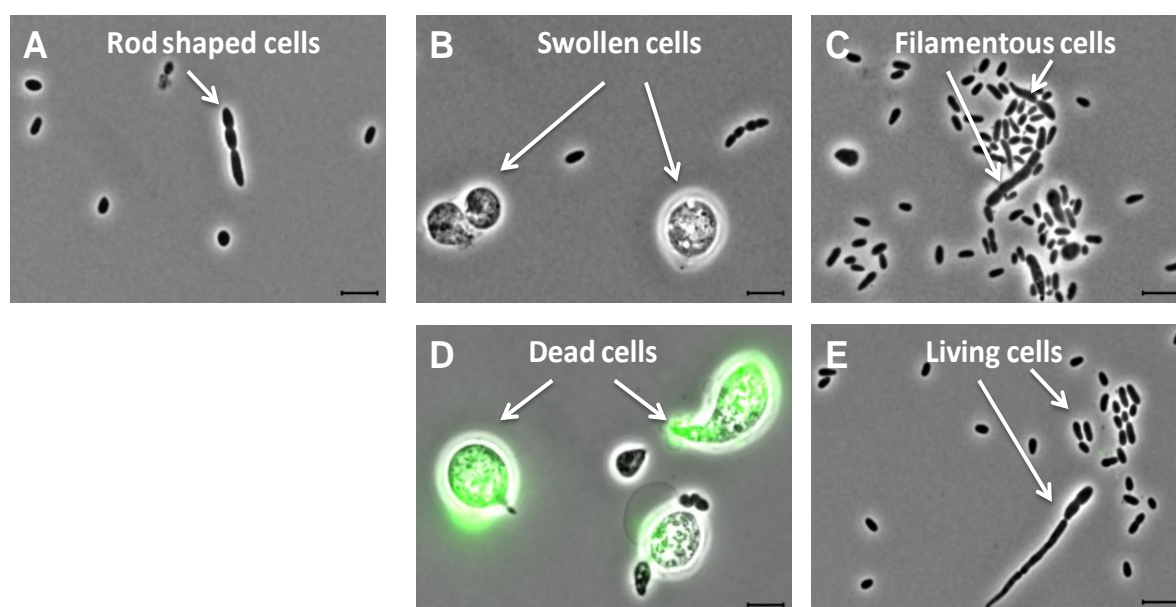


Figure 24: Microscopic examination of *G. oxydans* cells. Phase contrast microscopy of *G. oxydans* cells grown in the YGP medium (A), YGP medium with 600 mM sucrose (B), YGP medium with 600 mM sucrose + 10 mM mannitol (C). Fluorescent microscopy of SYTOX green-stained cells with 600 mM sucrose (D) and with 600 mM sucrose + 10 mM mannitol (E). Scale bar = 5 μ m. (Modified from Zahid *et al.* 2015).

3.2.6. Effect of polyols on growth and substrate oxidation rates of *G. oxydans*

The osmoprotective effects of mannitol on cellular morphology and growth behavior of *G. oxydans* raised an interesting question whether other polyols could equally protect the strain from deleterious osmotic effects. To address this question, *G. oxydans* was cultivated in YGP_{high} media containing 1 M glucose (1580 mOsm kg⁻¹) supplemented with either 5 mM mannitol, sorbitol, or arabitol. It was observed that growth of *G. oxydans* was highly impaired in YGP_{high} medium with a doubling time of 6 h and a final optical density of only 0.7. In comparison to the same medium supplemented with 5 mM mannitol, sorbitol, or arabitol, the growth retardation of osmotically stressed cultures was restored, indicated by doubling times of 2 h and final optical densities of 2.0 (Fig. 25A). In addition, the effect of these polyols on glucose oxidation rates of the cultures was investigated. Glucose was oxidized to δ -gluconolactone in the periplasm by the membrane-bound glucose dehydrogenase (mGDH), and the hydrolyzed product (gluconate) was excreted, leading to a drop of the pH value of the medium. During growth, samples were collected and analyzed for pH and metabolite concentrations. Analysis of the samples revealed that the pH of polyol supplemented cultures decreased very sharply from 7.0 to 3.5 within 12 h of cultivation, whereas in the media without polyol addition, the same drop was observed within 25 h. Furthermore, the gluconate content of these cultures was determined by HPLC. As expected, the rates of glucose oxidation and gluconate formation were higher in the cultures containing 5 mM polyols in comparison to the culture devoid of polyols. At the end of the exponential phase ($t = 25$ h), the osmotically stressed culture (on YGP_{high} medium) produced 305 mM gluconate, while the cultures with 5 mM polyols supplementation produced about 560-580 mM gluconate (Fig. 25B). These results were concordant with the total amount of gluconate measured at the end of cultivation (48 h) which showed that about 700 mM gluconate accumulated in the polyol containing cultures and 380 mM in the polyol-free stress cultures. Overall the cultures with polyols consumed 800 mM glucose whereas the polyol-free cultures utilized only 480 mM of the substrate. From these results, it can be concluded that polyols (in general) play an important osmoprotective role in *G. oxydans* as growth and catalytic efficiency of the cultures significantly improved in their presence.

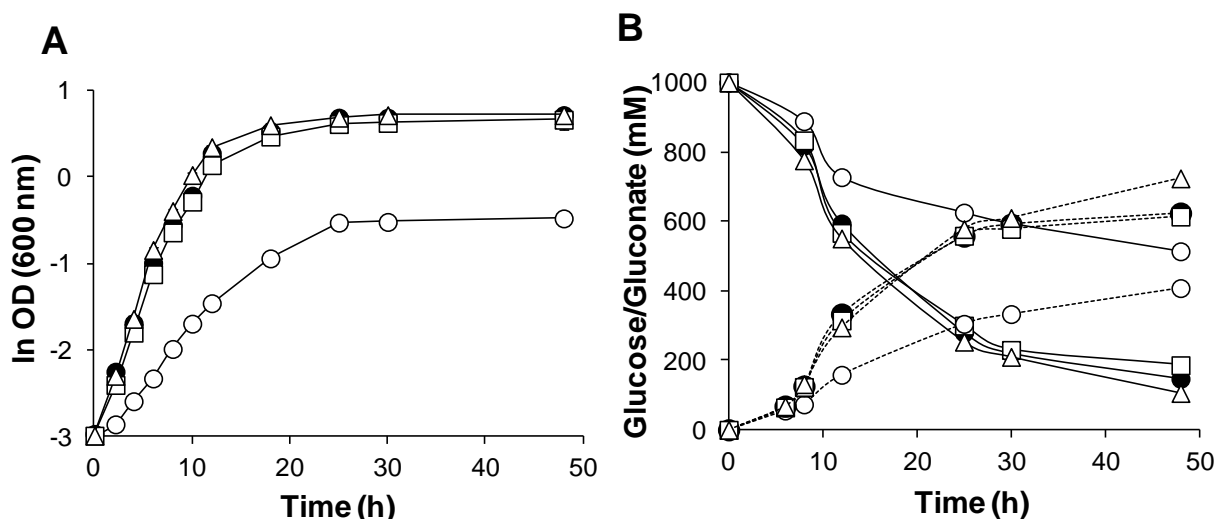


Figure 25: Effect of polyols on growth and gluconate production of 1 M glucose stressed cultures. (A) Correlation between optical densities of the cultures grown in YGP_{high} medium (○) and YGP_{high} medium supplemented with 5 mM mannitol (●), sorbitol (□), or arabinol (Δ). (B) Correlation between glucose consumption (symbols with solid lines) and gluconate formation (symbols with dashed lines) rates of the cultures on YGP_{high} medium (○), and YGP_{high} medium with 5 mM mannitol (●), 5 mM sorbitol (□), or 5 mM arabinol (Δ).

3.3. Enzymatic routes for the biosynthesis of mannitol in *G. oxydans*

As shown above, polyols including mannitol behave as osmoprotective compounds in *G. oxydans*. The accumulation of mannitol as compatible solute has been examined during growth of the organism in media containing high concentrations of sucrose, fructose or glucose (Zahid and Deppenmeier 2016). However, until now the enzymes and pathways involved in mannitol biosynthesis in *G. oxydans* were not studied. In general, three possible routes for the biosynthesis of mannitol are known in different microorganisms. One is the conversion of fructose-6-phosphate to mannitol by the mannitol-1-phosphate dehydrogenase and the mannitol-1-phosphate phosphatase. This two-step conversion pathway is present in homofermentative lactic acid bacteria (Wisselink *et al.* 2002). However, in *Acinetobacter baylyi* a single polypeptide chain catalyzes both the dehydrogenase as well as the phosphatase reaction. This enzyme is a unique bifunctional mannitol dehydrogenase containing an N-terminal phosphatase domain and a C-terminal mannitol-1-phosphate dehydrogenase domain (Sand *et al.* 2015). The second route is the direct reduction of fructose to mannitol via a mannitol-2-dehydrogenase, and is frequently present in heterofermentative lactic acid bacteria (Saha 2004, Wisselink *et al.* 2002). The third pathway is based on the conversion of mannose-6-phosphate to mannitol that is catalyzed by a mannose-6-phosphate reductase and

a phosphatase. Such type of mannitol biosynthetic route is most prevalent in certain species of yeasts and plants (Loescher *et al.* 1992, Onishi and Suzuki 1968).

3.3.1. Identification and bioinformatic analysis of the mannitol dehydrogenases from *G. oxydans*

In *G. suboxydans* IFO 12528, two types of mannitol dehydrogenases have been reported that differ in their co-factor specificity (Adachi *et al.* 1999a). They are known as NADP⁺-dependent mannitol dehydrogenase (EC 1.1.1.138) and NAD⁺-dependent mannitol dehydrogenase (EC 1.1.1.67); however, due to the unavailability of the amino acid sequences of these polypeptide chains, the respective genes could not be identified. Inspection of the genome of *G. oxydans* 621H using the NCBI non-redundant BLAST database revealed the presence of two putative homologues (Gox1432 and Gox0849) of potential mannitol-2-dehydrogenases. Gox1432 and Gox0849 were annotated as NADP⁺-D-sorbitol dehydrogenase and NADPH-dependent L-sorbose reductase, respectively (Prust *et al.* 2005). The amino acid sequences of these proteins were highly identical to other putative oxidoreductases from acetic acid bacteria. Homologues of Gox1432 were polyol: NADP⁺ oxidoreductases from *Gluconobacter oxydans* (A0A149UG77, 99.4 % identity, 100 % query coverage), *Gluconobacter frateurii* NBRC 103465 (A0A0S6TGQ1, 86.2 % identity, 100 % query coverage), and from *Gluconobacter japonicus* (A0A149S712, 85.8 % identity, 100 % query coverage) as well as sorbitol dehydrogenases from *Gluconobacter oxydans* (Q9KWR5, 95.5 % identity, 100 % query coverage) and from *Gluconobacter morbifer* G707 (G6XMR1, 86 % identity, 100 % query coverage). Likewise, Gox0849 was homologous to NADH oxidoreductases from *Gluconobacter oxydans* (A0A149U9H1, 99 % identity, 100 % coverage) and *Gluconobacter roseus* (A0A149T6L7, 94 % identity, 100 % query coverage), as well as to NADPH dependent L-sorbose reductases from *G. morbifer* G707 (G6XJZ3, identity 74.8 %, query coverage 100 %), *G. oxydans* H24 (K7SVB4, 75.1 % identity, 100 % identity), *G. thailandicus* NBRC 3255 (M9MJR6, 75.1 % identity, 100 % query coverage), and to the mannitol dehydrogenase from *Gluconobacter oxydans* DSM 3504 (A0A067Z2Z1, 99 % identity, 100 % query coverage). Alignment of the amino acid sequences of Gox1432 and Gox0849 with the respective homologous enzymes is shown in Figure 26. As all these homologues, mentioned above, were not characterized with respect to their catalytic efficiency and substrate spectrum; therefore, it was necessary to check the prediction function of both Gox1432 and Gox0849. An additional BLAST search using the SwissPort database indicated a low homology of both Gox1432 and Gox0849 to other biochemically characterized proteins. These included mannitol-2-dehydrogenase from *Pseudomonas*

fluorescens (IM2W_B, 41-42 % identity and 86-98% query coverage; Brünker *et al.* 1997, Kavanagh *et al.* 2002) and from *Rhodobacter sphaeroides* (P33216, 38-44 % identity and 89-98 % query coverage; Schneider *et al.* 1993), as well as a D-arabinitol-4 dehydrogenase from *Klebsiella pneumoniae* (O52720, 32-34 % identity and 83% coverage; Heuel *et al.* 1998). However, since the homology between these enzymes and Gox1432/Gox0849 was low, it was not possible to predict the function of the *G. oxydans* enzymes based on a blastp search.

The genes *gox1432* and *gox0849* are 1458 and 1476 bp long and code for polypeptide chains of 485 and 491 amino acids, respectively. The calculated molecular masses of Gox1432 and Gox0849 proteins were 53.6 kDa and 54.1 kDa, respectively. The secondary structure of both proteins was predicted by PSIPRED (Buchan *et al.* 2013, Jones 1999) that indicated that Gox1432 contains 19 alpha-helices with eight-parallel beta sheets, while Gox0849 has 20 alpha-helices and eight parallel beta-sheets. An additional analysis with the InterPro database (<http://www.ebi.ac.uk/interpro>) revealed that Gox1432 and Gox0849 contain N-terminal NAD⁺/NAD(P)⁺ binding Rossmann fold domains (Rossmann *et al.* 1974) and C-terminal mannitol dehydrogenase signature sequence motifs (Fig. 26). The amino acid sequences of both proteins share 61 % similarity and 46 % identity to each other with a query coverage of 99 %.


```

Gox1432 -----MITRETLKSLPANVQAPPYDIDGIKPGIVHFGVGNFFRAHEAFYVEQIL- 49
Gox0849 -----MILTSQTLShLPDSILTSSYDRDAVTPGIVHLSVGNFHRAHQAVYTDRALA 51
G6XMR1 -----MITRETLKSLPADVQAPPYDIDGIRPGIVHFGVGNFFRAHEAFYVEQIL- 49
A0A0S6TGQ1 -----MITHEILKSLPAGVQAPPYDINGIKPGIVHFGVGNFFRAHEAFYVEQIL- 49
A0A149S712 -----MITHEILKSLPVGVQAPPYDINGIKPGIVHFGVGNFFRAHEAFYVEQIL- 49
Q9KWR5 -----MITRETLKSLPANVQAPPYDIDGIKPGIVHFGVGNFFRAHEAFYVEQIL- 49
A0A149UG77 -----MITRETLKSLPANVQAPPYDIDGIKPGIVHFGVGNFFRAHEAFYVEQIL- 49
A0A149T6L7 -----MILTSQTLSDLPESILTSFYDRDGVTAGIVHLSVGNFHRAHQAVYTDRALA 51
A0A067Z2Z1 -----MILTSQTLShLPDSILTSSYDRDAVTPGIVHLSVGNFHRAHQAVYTDRALA 51
A0A149U9H1 -----MILTSQTLShLPDSILTSSYDRDAVTPGIVHLSVGNFHRAHQAVYTDRALA 51
G6XJZ3 -----MILTPETLAQLPESILTLFYDRKVTTPGIAHLSVGNFHRAHQAVYVDRALA 51
K7SVB4 MGLSFGRTIMKLTSETLAHLPETILTSPYDRQKVTAGIAHLSVGNFHRAHQAVYTDRALA 60
M9MJR6 MGLSFGRTIMKLTSETLAHLPETILTSPYDRQKVTAGIAHLSVGNFHRAHQAVYTDRALA 60
          : * : * ** : : ** . : ** * : **** : * * : *
Gox1432 RRRRDAGGKAFTVMSCDNLRHNGNVARKAFLGYAKARDPELAKWIEENATFPNGMVDRI 233
Gox0849 RQRRDAGVAPFTILSCDNPANGDVARNVAVLSFARLRDAELAEWIEVNVAFPSCMVDRI 234
G6XMR1 RRRREAGGKAFTIMSCDNLRHNGNVARKAFLGYAKARDPELARWIEENATFPNGMVDRI 233
A0A0S6TGQ1 RRRRDAGGKAFTIMSCDNLRHNGNVARKAFLGYAKARDPELAKWIEENATFPNGMVDRI 233
A0A149S712 RRRRDAGGKAFTIMSCDNLRHNGNVARKAFLGYAKARDPELAKWIEENATFPNGMVDRI 233
Q9KWR5 RRRWDAGGKAFTVMSCDNLRHNGNVARKAFLGYAKARDPELAKWIEENATFPNGMVDRI 233
A0A149UG77 RRRRDAGGKAFTVMSCDNLRHNGNVARKAFLGYAKARDPELAKWIEENATFPNGMVDRI 233
A0A149T6L7 RQRRDAGITPFTILSCDNPVSNVDVARNVAVLSFARLRDPELATWIEANVAFPSCMVDRI 234
A0A067Z2Z1 RQRRDAGVAPFTILSCDNPANGDVARNVAVLSFARLRDAELAEWIEANVAFPSCMVDRI 234
A0A149U9H1 RQRRDAGVAPFTILSCDNPANGDVARNVAVLSFARLRDAELAEWIEANVAFPSCMVDRI 234
G6XJZ3 RLRRDAGTAPFTILSCDNPANGDIARNVAVLSFARLRDATLAEWIGQNVAFPSCMVDRI 234
K7SVB4 RLREAGVNAFTILSCDNPVSNGEIARRAVLSFARLKDELADWISRNVAFPSCMVDRI 243
M9MJR6 RLREAGVNAFTILSCDNPVSNGEIARRAVLSFARLKDELADWISRNVAFPSCMVDRI 243
          * * : ** ** : **** : ** : * * * . : : * * * * : * * * * *
Gox1432 DWEHVKIRMLNAGHITLFCFPGLVGYENVDDAIEDKDLRGNLENYLNKDVIP 348
Gox0849 PYEQVKLRMLNASHSTLALPGVLMGYRLVDEAMGDEHLAALLEQFLRHDVEP 349
G6XMR1 DWEQVKRMLNAGHIMLCFPAIVGYENVVDQAVQSDLRNLENFLNRDVI 348
A0A0S6TGQ1 DYEHVKIRMLNAGHIMLCFPGVLAGYENVVDQALADADLRNLENFLNKDVIP 348
A0A149S712 DYEHVKIRMLNAGHIMLCFPGVLAGYENVVDQALADADLRNLENFLNKDVIP 348
Q9KWR5 DWEYVKIRMLNAGHVMLCFPGILVGYENVDDAIEDSELLGNLKNYLNKDVIP 348
A0A149UG77 DWEHVKIRMLNAGHITLFCFPGLVGYENVDDAIEDKDLRGNLENYLNKDVIP 348
A0A149T6L7 PYEQVKLRMLNASHSTLALPGVLMGYRLVDEAMGDEHLAALLEQFLRHDVEP 349
A0A067Z2Z1 PYEQVKLRMLNASHSTLALPGVLMGYRLVDEAMGDEHLAALLEQFLRHDVEP 349
A0A149U9H1 PYEQVKLRMLNASHSTLALPGVLMGYRLVDEAMGDEHLAALLEQFLRHDVEP 349
G6XJZ3 PYERVKLRMLNASHSMLSLPGVLMGYRFVSEAMQDDHLFRLLQQFLGWDAEP 349
K7SVB4 PYEQVKLRMLNASHSMLALPAVLMGYRFVSEAMEDENLFRLLQQFLGFDSE 358
M9MJR6 PYEQVKLRMLNASHSMLALPAVLMGYRFVSEAMEDENLFRLLQQFLGFDSE 358
          : * ** : **** * * : * : * * * . : : * * * * : * * * * *

```

Figure 26: Multiple sequence alignment of important regions of Gox1432 and Gox0849 from *G. oxydans* 621H with homologous proteins from other *Gluconobacter* species. The amino acids marked in red indicate the NAD⁺/NADP⁺ binding motifs (IPR016040) and in yellow the mannitol dehydrogenase catalytic domains.

3.3.2. Characterization of the mannitol dehydrogenases from *G. oxydans*

For characterization, both putative mannitol dehydrogenases were heterologously produced in *E. coli*. Therefore, the corresponding genes were amplified by PCR and cloned into pASK-IBA vectors. Both constructs were sequenced and transformed into *E. coli* for overproduction of the proteins. The resulting recombinant proteins were purified from crude cellular extract using Strep-Tactin affinity chromatography, yielding 1-2 mg of the target proteins from 1 L *E. coli* cultures. The purity of both proteins was determined by SDS-PAGE and western blot analysis, which revealed that the proteins were apparently homogenous and formed single

bands of 54.9 kDa and 55.7 kDa (Fig. 27), which were in good agreement with the deduced masses of 54.6 kDa and 55.0 kDa for the recombinant tagged proteins Gox1432 and Gox0849, respectively. Native PAGE revealed that Gox1432 and Gox0849 exhibited apparent molecular mass of 56.3 kDa and 134.4 kDa, respectively, indicating that Gox1432 was a monomeric protein, while Gox0849 possessed a homodimeric structure (Fig. 27). However, small amounts of dimers at 100 kDa for Gox1432 and trimers at 160 kDa for Gox0849 were also observed. Similarly, Adachi *et al.* (1999a) has reported that among two types of mannitol dehydrogenases, purified from *G. suboxydans* IFO 12528, the NADP⁺-dependent enzyme (EC 1.1.1.138) was a 50 kDa monomeric protein, whereas the NAD⁺-dependent enzyme (EC 1.1.1.67) was a dimeric protein with native molecular mass of 130 kDa. According to similarity of molecular masses, the proteins Gox1432 and Gox0849 from *G. oxydans* 621H can be considered as the homologues of NADP⁺- and NAD⁺-dependent mannitol dehydrogenases from *G. suboxydans* IFO 12528, respectively.

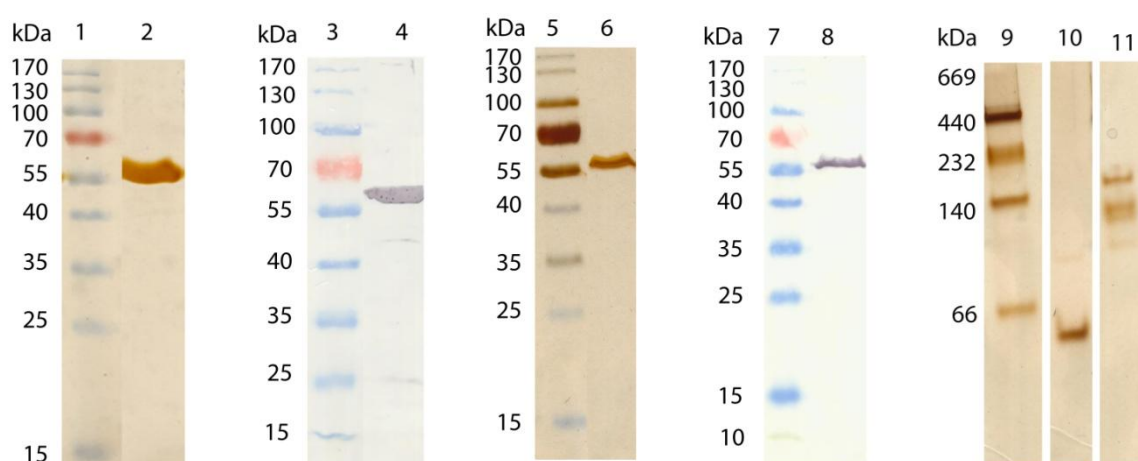


Figure 27: SDS- and native PAGE of purified mannitol dehydrogenases (Gox1432, Gox0849) from *G. oxydans*. Lane 1,3,5,7: SDS-PAGE of PageRuler prestained protein ladder (ThermoFisher Scientific). Lane 2: SDS-PAGE of purified Gox1432 (silver staining). Lane 4: Western blot of purified Gox1432. Lane 6: SDS-PAGE of purified Gox0849 (silver staining). Lane 8: Western blot of purified Gox0849. Lane 9: Native PAGE of high molecular weight standard (HMW Native marker kit, GE, Healthcare). Lane 10: Native PAGE of purified Gox1432. Lane 11: Native PAGE of purified Gox0849. (Modified from Zahid and Deppenmeier 2016).

To confirm that Gox1432 and Gox0849 are indeed mannitol dehydrogenases, their catalytic activities were determined by detecting the rate of change in absorbance of NAD(P)⁺ or NAD(P)H at 340 nm in the presence of various sugars and polyols (Fig. 28, Table 16).

Gox1432 catalyzed the NADP^+ -dependent oxidation of various polyols such as D-mannitol, D-sorbitol, D-arabitol, and D-glycerol. The highest activity was measured with D-mannitol as substrate with a $K_{\text{cat}}/K_{\text{M}}$ of $7.8 \times 10^3 \pm 1.5 \text{ s}^{-1} \text{ M}^{-1}$ and K_{M} of $11.7 \pm 2.5 \text{ mM}$. The protein did not show any activity with NAD^+ as electron acceptor. Moreover, the enzyme was able to catalyze the NADPH-dependent reduction of various sugars (*e.g.*, D-fructose, L-sorbose, and 5-keto D-gluconate). The highest reduction rate was obtained for D-fructose with a K_{M} of $55.7 \pm 8.3 \text{ mM}$ and a $K_{\text{cat}}/K_{\text{M}}$ of $2.8 \times 10^3 \pm 0.35 \text{ s}^{-1} \text{ M}^{-1}$. NADH did not serve as electron donor. In comparison, Gox0849 had a limited substrate spectrum and catalyzed only the NADH-dependent reduction of D-fructose to D-mannitol with a $K_{\text{cat}}/K_{\text{M}}$ of $2.9 \times 10^3 \pm 0.027 \text{ s}^{-1} \text{ M}^{-1}$ and a K_{M} of $38 \pm 0.4 \text{ mM}$. A reduction of other sugars was not observed. It was noticed that Gox0849 had clear preference for NADH as electron donor and showed 90 % decrease in activity with NADPH as cofactor. In addition, Gox0849 oxidized only D-mannitol in the presence of NAD^+ as electron acceptor with an apparent $K_{\text{cat}}/K_{\text{M}}$ of $6.4 \times 10^3 \pm 1.05 \text{ s}^{-1} \text{ M}^{-1}$ and a K_{M} value of $8.6 \pm 1.9 \text{ mM}$.

The activity of both purified enzymes was tested over a wide pH range of 5-10.5. It was found that the optimum pH for the reduction of D-fructose was 7.0 for both proteins, whereas the optimum pH for the oxidation of D-mannitol was 10.0 and 9.0 for Gox1432 and Gox0849, respectively. A substantial decrease in the activities of both proteins was observed when the oxidation of D-mannitol was tested at the cellular pH-value. It was noted that at physiological pH of 7.0, the rate of mannitol oxidation was 4-fold reduced for Gox1432 and 3-fold reduced for Gox0849. Taken together, the kinetic data suggested that the proteins were able to oxidize mannitol at alkaline pH, but their major *in vivo* function was the reduction of D-fructose to D-mannitol. Therefore, the proteins Gox1432 and Gox0849 can be classified as D-fructose reductases rather than D-mannitol dehydrogenases. From here on this nomenclature will be used in this study.

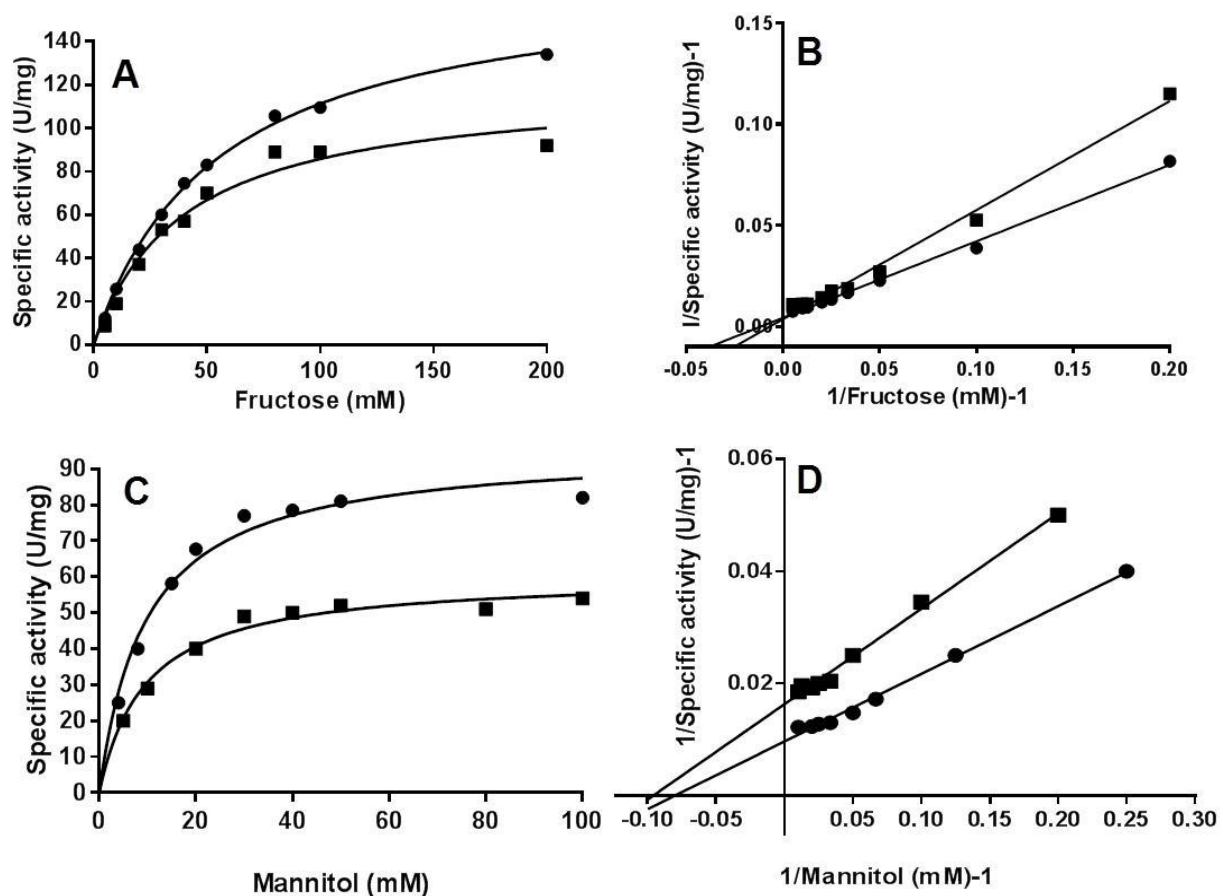


Figure 28: Kinetic parameters of Gox1432 (●) and Gox0849 (■). (A, C) Michaelis-Menten curves. (B, D) Lineweaver Burk curves. (A and B) Kinetic parameters for the reduction of D-fructose at pH 7.0. (C and D) Kinetic parameters for the oxidation of D-mannitol at pH 9.0 (for Gox0849) and 10.0 (for Gox1432). The graphs were prepared using GraphPad Prism 6.

Table 16: Apparent kinetic parameters of Gox1432 and Gox0849

Enzyme	Substrates	pH	K_M (mM) *	V_{max} * (U/mg)	K_{cat} (s ⁻¹)	K_{cat}/K_M (s ⁻¹ M ⁻¹)
Gox1432	D-Fructose	7	55.7 ± 8.3	167.8 ± 4.0	153.8 ± 3.0	2.8 x 10 ³ ± 0.35
	L-Sorbose	7	507.0 ± 5.4	209.3 ± 3.0	192.0 ± 3.0	3.8 x 10 ² ± 0.02
	D-Mannitol	10	12.0 ± 2.5	98.0 ± 2.0	90.0 ± 2.0	7.8 x 10 ³ ± 1.5
	D-Sorbitol	10	195.0 ± 28	185.5 ± 22	170.0 ± 20	8.9 x 10 ² ± 0.2
	D-Mannitol	7	19.0 ± 1.5	13.2 ± 4.0	12.1 ± 3.0	6.6 x 10 ² ± 2.4
Gox0849	D-Fructose	7	38.0 ± 0.4	120.0 ± 2.4	110.0 ± 2.2	2.9 x 10 ³ ± 0.027
	D-Mannitol	9	9.0 ± 2.0	59.0 ± 3.1	54.0 ± 3.0	6.4 x 10 ³ ± 1.05
	D-Mannitol	7	15.0 ± 3.2	17.0 ± 3.0	16.2 ± 3.0	1.1 x 10 ³ ± 0.064

* K_M and V_{max} were determined using nonlinear regression of the Michaelis–Menten data (GraphPad Prism 6).

3.3.3. Transcript abundance of genes coding for D-fructose reductases in *G. oxydans*

The proteins Gox1432 and Gox0849 could reduce D-fructose to mannitol with comparable catalytic efficiencies. To analyze their importance for the metabolism of *G. oxydans*, the transcript abundance of the corresponding genes (*gox1432* and *gox0849*) was compared by RT-qPCR analysis. Moreover, the effect of externally applied osmotic stress on the expression rates of the genes was monitored. Therefore, total RNA was extracted from *G. oxydans* cells cultivated under non-stress (YGP medium) and osmotically stress conditions (YGP medium + 450 mM sucrose). The expression levels of *gox1432* and *gox0849* were compared in relation to the constitutively expressed gene *gox1709* encoding the gluconokinase (Rauch *et al.* 2010). RT-qPCR analysis revealed that the transcription of both genes was not regulated because significant differences in their quantification cycle (Ct) values were not observed under control and osmotic stress conditions (Fig. 29). However, it was found that *gox1432* was expressed at a higher level than *gox0849*. Transcript abundance of *gox1432* gene was 3.5 fold higher than the reference gene, highlighting its importance for the cellular metabolism. In contrast, the transcripts from *gox0849* were eight fold less abundant than the reference gene. In summary, these results indicated that the expression levels of these genes in *G. oxydans* were not affected by a change in osmolality of the growth medium, and an evidence was provided that the major fructose reductase in *G. oxydans* was Gox1432.

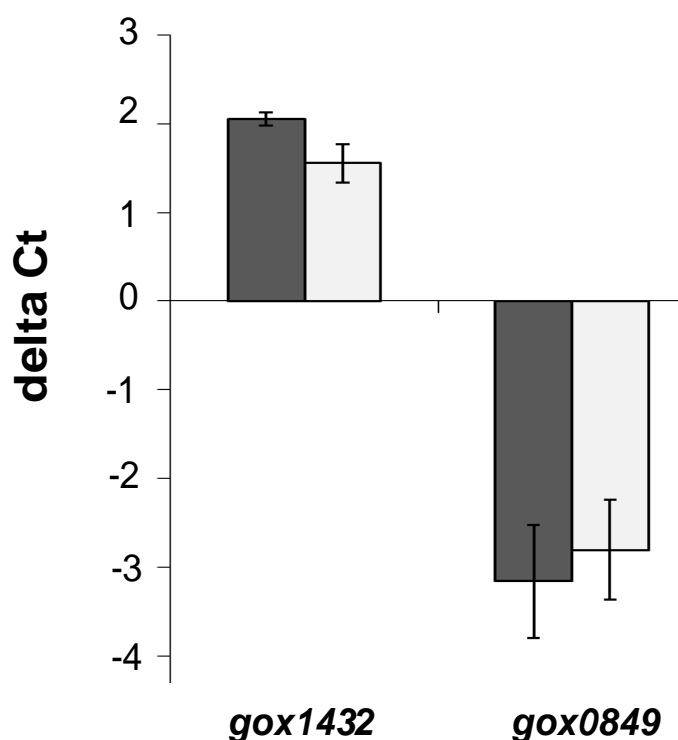


Figure 29: Transcript abundance of genes *gox1432* and *gox0849* encoding fructose reductases in comparison to the house-keeping gene *gox1709*. Dark and light gray bars represent the transcript abundance of the genes under non-stress and osmotic stress conditions, respectively. Δ Ct values were obtained by subtracting average Ct values of the genes of interest from the reference gene *gox1709*. (Modified from Zahid and Deppenmeier 2016).

3.3.4. Characterization of fructose reductase deletion mutants

3.3.4.1. Effect of the deletion of fructose reductases on growth of *G. oxydans*

To investigate the physiological role of the two fructose reductases in *G. oxydans*, the single deletion mutants Δ *gox1432* and Δ *gox0849* as well as the corresponding double mutant (Δ *gox1432* Δ *gox0849*) were generated as described by Kostner *et al.* 2013 (Chapter 2.5.16). The deletion mutants were screened by colony PCR, and gene deletion was confirmed by sequencing of the PCR fragments. As shown in Figure 30A, a PCR fragment of 559 bp was generated with the primer pair Up_*gox1432*_int_fw/Do_*gox1432*_int_rev and corresponded to the *gox1432* deletion mutant, while the DNA fragment had a size of 2017 bp in the wild type. Likewise, DNA amplification using the primer pair Up_*gox0849*_int_fw/

Do_gox0849_int_rev resulted in the expected band sizes of 714 bp for the deletion mutant Δ gox0849 and 2190 bp for the wild type (Fig. 30B).

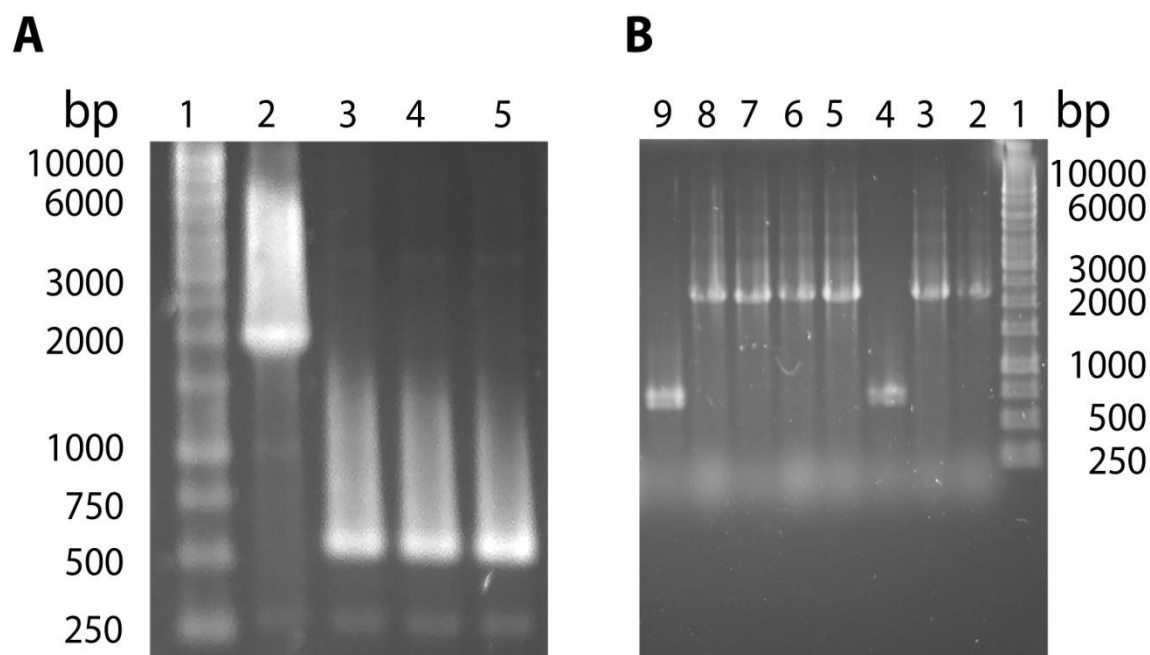


Figure 30: Screening of *G. oxydans* Δ gox1432 and Δ gox0849 deletion mutants by colony PCR. (A) Screening of colonies with a deletion of gene *gox1432*. Lane 1: 1 kb DNA ladder; Lane 2: fragment of the wild type colony; Lanes 3-5: fragments of the deletion mutant Δ gox1432. (B) Screening of colonies with a deletion of gene *gox0849*. Lane 1: 1 kb DNA ladder; Lanes 2-3 and 5-8: wild type colonies; Lanes 4 and 9: the deletion mutants Δ gox0849.

The respective single and double deletion mutants for fructose reductases were analyzed for growth, osmotolerance, intracellular mannitol content, and enzymatic activities. Therefore, cells were cultured in YGP medium or YGP medium with 450 mM sucrose. It was found that growth, doubling time, and optical density of all mutants were identical compared to the wild type culture under non-osmotic stress conditions. All cultures grew with a doubling time of 2.2 ± 0.1 h and reached final optical densities of 2.0 (Fig. 31A). In the presence of 450 mM sucrose, growth parameters of the wild type were comparable to the Δ gox0849 mutant with doubling times of 3.2 h and 3.3 h, respectively. Similarly, the final optical densities of these two cultures were identical *i.e.*, 0.96 for the wild type strain and 0.91 for the Δ gox0849 mutant (Fig. 31B). Contrarily, growth of *G. oxydans* Δ gox1432 was significantly impaired under osmotic stress conditions because the doubling time of the culture increased to 6 h and the final optical density decreased by 45 % in comparison to the parental strain (Fig. 31B). To confirm whether the growth defect observed in this mutant was solely due to the deletion of

gene *gox1432*, a plasmid borne allele of the gene was introduced into the deletion mutant via the broad host range vector pBBR1-MCS containing the constitutively expressed promoter p264 from *G. oxydans*. Therefore, gene *gox1432* was amplified from genomic DNA of *G. oxydans* using the primer pair pBBR.p264.gox1432_fw/pBBR.p264.gox1432_rev and was cloned into the *EcoRV* and *AscI* sites of the pBBR. p264. ST vector. The recombinant plasmid (pBBR.p264.gox1432.ST) was transformed into the Δ *gox1432* mutant by triparental mating. Colony PCR was performed to screen for positive transformants using the primer pair pBBR.p264_gox1432_fw/pASK_rev. A PCR fragment of 1.5 kb was obtained from the complemented strains which confirmed the presence of the full length gene *gox1432* (Fig. 32). The transformants were selected and monitored for their growth behavior under osmotic stress conditions. It was found that the complementation of *gox1432* led to improve growth of the culture in comparison to the mutant culture. As shown in Figure 31C, the complemented strain grew with a doubling time of 3.1 h and reached a final optical density of 1.4, which was slightly higher than the wild type ($t_d = 3.2$ h, final $OD_{600} = 0.96$). These finding confirmed that gene *gox1432* is important for proper growth and survival of *G. oxydans* under osmotic stress.

Furthermore, severe growth defects, caused by osmotic stress, were observed for the double mutant (*G. oxydans* Δ *gox1432* Δ *gox0849*), lacking both of the fructose reductase encoding genes. In YGP medium with 450 mM sucrose, the double deletion mutant had an extended lag phase of 8-10 h, followed by slow growth with a doubling time of 6 h, and reached a final optical density of only 0.4 within 32 h (Fig. 31B). It was assumed that the double mutant could not synthesize sufficient mannitol due to the deletion of both fructose reductases that led to impaired growth of the culture under osmotic stress. To confirm this hypothesis, growth parameters of the double deletion mutant were monitored in YGP + 450 mM sucrose medium supplemented with 5 mM exogenous mannitol. As expected, growth of the culture was significantly improved and was comparable to the wild type non-stressed culture with the doubling time of 2.1 h and a final optical density of 2.0 (Fig. 31C). These observations further strengthen our previous findings that mannitol functions as an osmoprotectant in *G. oxydans*, and low mannitol concentrations in a medium are sufficient for the cells to cope with osmotic challenges (Zahid *et al.* 2015).

In summary, these results indicated that both fructose reductases were not essential for growth under non-osmotic stress environment. In contrast, *G. oxydans* required gene *gox1432*, encoding the NADPH-dependent fructose reductase, for efficient proliferation in media with low water activities. Gene *gox0849*, encoding the NADH-dependent fructose

reductase, was important for efficient proliferation of the cells under osmotic stress when *gox1432* was deleted. Moreover, the growth defect observed for the Δ *gox1432* mutant and the double mutant under osmotic stress was due to a defect in intracellular mannitol biosynthesis. However, it is not clear yet whether *G. oxydans* produces only two fructose reductases for mannitol synthesis and osmoprotection. In this context, it is to note that in the absence of exogenous mannitol, the double deletion mutant grew slowly with an extended lag phase in the medium containing 450 mM sucrose. This result points towards the presence of a yet unknown enzyme that is able to produce low amounts of intracellular mannitol under osmotic stress conditions (Discussed in chapter 4).

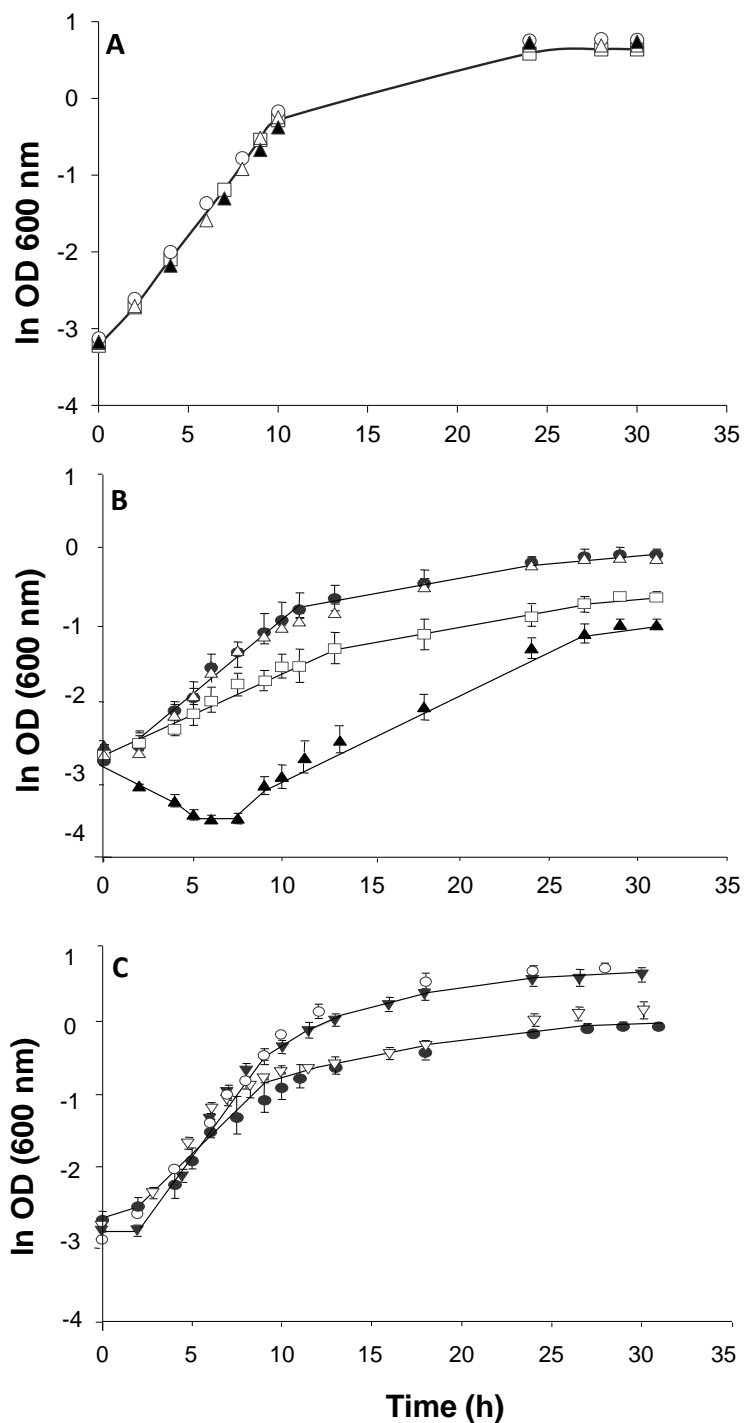


Figure 31: Growth of the wild type and the mutant strains of *G. oxydans* on YPG medium (A) and on YGP medium supplemented with 450 mM sucrose (B and C). Wild type non-stressed strain (○), wild type stressed strain (●), mutant strain $\Delta gox0849$ (Δ), mutant strain $\Delta gox1432$ (□), double mutant $\Delta gox1432 \Delta gox0849$ (▲), $\Delta gox1432$ complemented strain (▽), double mutant $\Delta gox1432 \Delta gox0849$ with 5 mM mannitol (▼). (Modified from Zahid and Deppenmeier 2106).

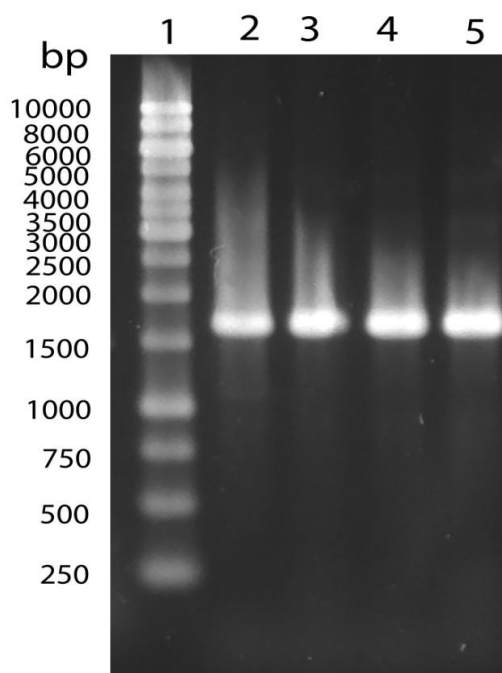


Figure 32: Screening of *G. oxydans* $\Delta gox1432$ strain complemented with pBBR. p264. *gox1432*. ST. Lane 1: 1 kb DNA ladder. Lane 2-5: DNA fragments with the expected size of 1.5 kb amplified from four different positive transformants.

3.3.4.2. Intracellular mannitol formation and activity of cytoplasmic fructose reductases

Since growth of the $\Delta gox1432$ mutant and the double mutant $\Delta gox1432 \Delta gox0849$ was sensitive to increased osmolarity of the medium, all mutants were analyzed for the production of intracellular mannitol in the presence of 450 mM sucrose in YGP medium. Therefore, all cultures were harvested in the late exponential phase, freeze dried, and analyzed by HPLC after Bligh and Dyer (1959) extraction. It was noted that regardless of having no apparent effect on growth, deletion of *gox0849* resulted in a slight reduction of the intracellular mannitol level *i.e.*, $1.7 \pm 0.1 \mu\text{mol mg}^{-1}$ protein, which was 1.3 fold lower than that of the wild type ($2.25 \pm 0.3 \mu\text{mol mg}^{-1}$ protein). In contrast, mannitol accumulation was decreased by 60 to 70 % in $\Delta gox1432$ and the double deletion mutants, respectively (Fig. 33). These results were concordant with the findings of growth experiments (shown above) and confirmed that reduced cell proliferation of the mutants under osmotic stress was due to impaired intracellular mannitol synthesis. Moreover, the presence of low amounts of mannitol ($0.4 \pm 0.1 \mu\text{mol mannitol mg}^{-1}$ protein) in the double mutant provided first evidence for the presence of another intracellular fructose reductase with limited catalytic activity that enabled slow growth of the culture under osmotic stress conditions.

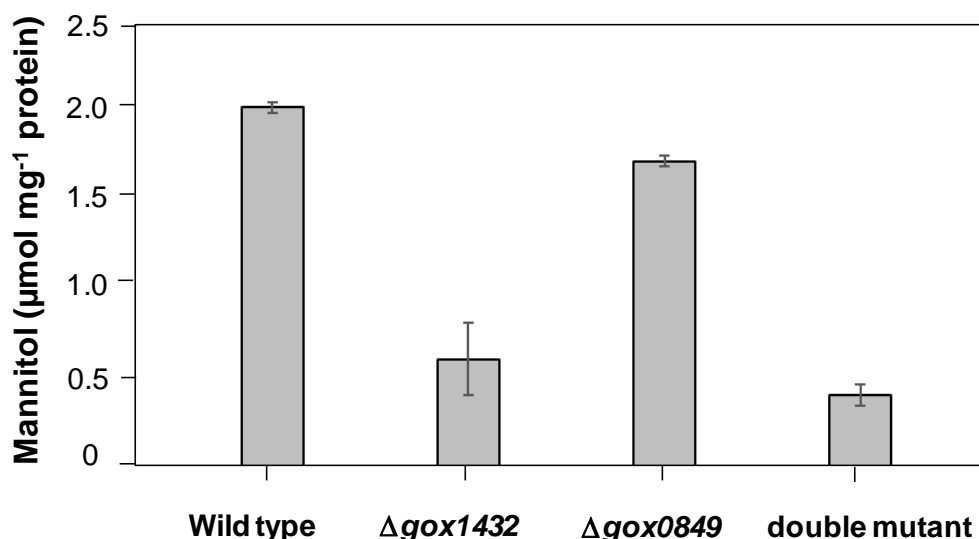


Figure 33: Effect of the deletion of fructose reductases on intracellular mannitol content in the wild type and the mutant strains of *G. oxydans*. Cells were cultivated in YGP medium containing 450 mM sucrose. Compatible solutes were extracted and analyzed by HPLC. Y-axis represents the intracellular mannitol concentrations ($\mu\text{mol mg}^{-1}$ protein) in all strains of *G. oxydans*. (Modified from Zahid and Deppenmeier 2016).

For the confirmation of these results, cytoplasmic fractions from the wild type, both single mutants, and the double mutant were extracted and assayed for fructose reduction and mannitol oxidation activities at the physiological pH 7.0 (Adachi *et al.* 1999a). At this pH, both purified proteins had maximum catalytic activity for the reduction of D-fructose rather than the oxidation of D-mannitol (Table 16). Hence, it became evident that both enzymes function as fructose reductases *in vivo*. For activity analysis, cytoplasmic fractions were prepared from cultures grown on YGP medium (control) and on YGP medium supplemented with 450 mM sucrose (osmotic stress).

As shown in Figure 34A, the highest fructose reduction rates were $3.0 \pm 0.4 \mu\text{mol min}^{-1} \text{mg}$ of cytoplasmic protein⁻¹, obtained with NADPH as a cofactor. These rates were determined with cytoplasmic fractions of the osmotically stressed wild type and $\Delta\text{gox0849}$ mutant cells, and represented the activity of the NADPH-dependent fructose reductase Gox1432. The activity of this enzyme was 2.4 fold lower in the cytoplasm of cells grown in the absence of sucrose. Under non-stress conditions, the maximum reduction rate of fructose was $1.25 \pm 0.2 \mu\text{mol min}^{-1} \text{mg}$ cytoplasmic protein⁻¹. In contrast, the cytoplasmic fractions of the $\Delta\text{gox1432}$ mutant and the double mutant did not reduce D-fructose in the presence of NADPH. These

results indicated that Gox1432 is the sole NADPH-dependent D-fructose reductase present in the cytoplasm of *G. oxydans*.

Besides NADPH-dependent reduction of D-fructose, the same cytoplasmic fractions were also tested for NADH-specific reduction activities (Fig. 34A). Remarkably, the reduction rates of fructose in the cytoplasm of the wild type with NADH were 10-folds lower ($0.3 \pm 0.03 \mu\text{mol min}^{-1} \text{mg cytoplasmic protein}^{-1}$) compared to the rates with NADPH as electron donor. Moreover, it was observed that osmotic stress had no influence on NADH-dependent fructose reduction because comparable rates were detected in the cytoplasm of the wild type, extracted under stress and non-stress conditions. Likewise, similar rates of NADH-specific D-fructose reduction were found in the cytoplasm of the $\Delta\text{gox1432}$ mutant *i.e.*, $0.3 \pm 0.04 \mu\text{mol min}^{-1} \text{mg of cytoplasmic protein}^{-1}$. In contrast, the NADH-dependent reduction of fructose was two-fold reduced in the cytoplasm of $\Delta\text{gox0849}$ and the double mutant. These results confirmed the presence of a third unknown intracellular NADH-dependent D-fructose reductase in *G. oxydans*. The cytoplasmic fractions were also tested for the oxidation of D-mannitol at pH 7.0. It was noticed that the rates of NADP⁻- and NAD⁺-dependent D-mannitol oxidation were 10-fold lower in the stressed and non-stressed cultures in comparison to the rates of D-fructose reduction (Fig. 34B). These observations confirmed that both types of oxidoreductases primarily work as fructose reductases in the cytoplasm of *G. oxydans* at physiological conditions.

In summary, there are three types of soluble D-fructose reductases present in *G. oxydans*. One is the NADPH-dependent enzyme Gox1432 whose activity is up-regulated by an increase in osmolality of the growth medium. The other two are NADH-specific D-fructose reductases, and their catalytic efficiencies are unaltered under stress and non-stress conditions. The activities of these two enzymes for fructose reduction constituted only 24 % ($0.3 \mu\text{mol min}^{-1} \text{mg of cytoplasmic protein}^{-1}$) of the activity of the NADPH-dependent enzyme ($1.25 \mu\text{mol min}^{-1} \text{mg of cytoplasmic protein}^{-1}$). One of these two NADH-specific D-fructose reductases is Gox0849, constituting almost 60 % of the total NADH-dependent D-fructose reduction rate in the cytoplasm of wild type *G. oxydans*. However, the second enzyme, catalyzes the remaining 40 % of the reduction of fructose, is yet unknown.

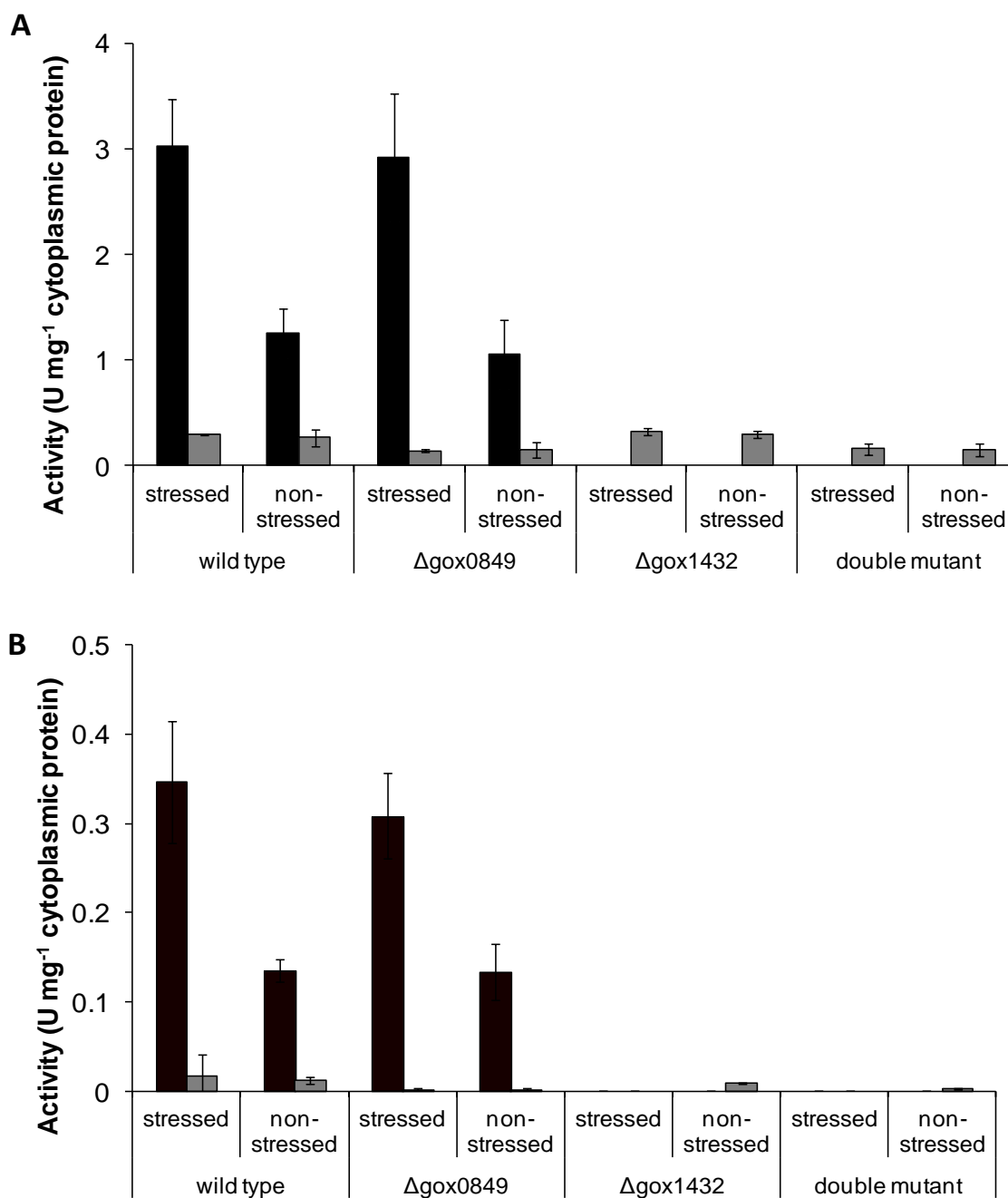


Figure 34: Comparison of the activities of fructose reductases in the cytoplasmic fractions of the wild type and the mutant strains of *G. oxydans* grown on YGP medium (non-stressed) or YGP medium + 450 mM sucrose (stressed) at pH 7.0. Activities were determined with D-fructose (A) and D-mannitol (B) in the presence of NADP(H) (black bars) and NAD(H) (gray bars). (Modified from Zahid and Deppenmeier 2016).

3.3.4.3. Effect of the deletion of fructose reductases on cellular catalytic efficiency

As mentioned above, two fructose reductases Gox1432 and Gox0849 participate in vivo to reduce D-fructose to D-mannitol that acts as an osmoprotectant in *G. oxydans*. The corresponding deletion mutant could not efficiently grow under osmotic stress conditions due to the decrease of the intracellular mannitol concentration. Here, the glucose oxidation efficiencies of the wild type and the double deletion mutant were compared during cultivation in YGP_{high} medium containing 1 M glucose. As expected, a severe effect of glucose-induced osmotic stress was observed on growth, gluconate formation, and pH value of the double mutant in comparison to the wild type. The double mutant had an increased lag phase of about 5 h, followed by slow growth with doubling time of 10 h, and reached a final optical density of 0.2. In comparison, the wild type divided with a doubling time of 6 h and reached a final optical density of 0.7 (Fig. 35A). Likewise, the glucose oxidation rates of both cultures were different during cultivation. It was examined that the double mutant produced only 83 mM gluconate till the end of exponential phase (24 h), whereas the wild type produced 334 mM within the same time period. Similarly, the total amount of gluconate accumulated at the end of the cultivation (30 h) was 3.5-fold lower (100 mM) in the double mutant in comparison to the wild type that formed 350 mM gluconate.

Moreover, the pH of both cultures dropped accordingly due to the production of variable amounts of gluconate under osmotic stress. The pH of the double mutant culture decreased from 6.5 to 3.5 within 30 h, while the same drop in pH was observed in only 14 h in the wild type (Fig. 35B). For further investigation, the intracellular mannitol contents of both cultures were determined under the same growth conditions. Once again, it was observed that the deletion mutant could accumulate mannitol only up to a concentration of $0.4 \pm 0.2 \mu\text{mol mg}^{-1}$ protein in comparison to $2.0 \pm 0.4 \mu\text{mol mg}^{-1}$ protein in the wild type. These results clearly highlight the direct influence of the deletion of fructose reductase encoding genes on cellular growth and catalytic efficiencies of *G. oxydans*. Thus, it can be concluded that *G. oxydans* requires these fructose reductases for survival in medium with reduced water activity, and for efficient product formation.

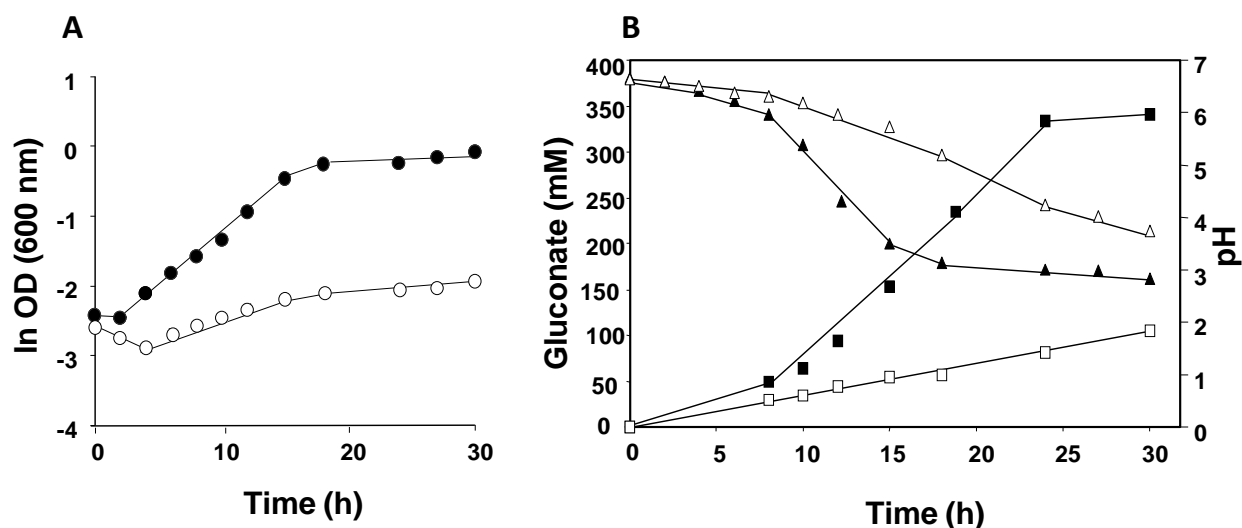


Figure 35: Effect of the deletion of fructose reductases encoding genes on growth and gluconate formation of *G. oxydans* strains. The cells were cultured on YGP_{high} medium containing yeast extract (0.6 g L⁻¹), glucose (1000 mM) and 100 mM K-phosphate buffer (pH 6.8). (A) Correlation between the growth profile of the wild type (●) and the $\Delta gox1432 \Delta gox0849$ double mutant (○). (B) Comparison between gluconate formation (■, □) and decrease in pH (▲, △) of the wild type (filled symbols) and the $\Delta gox1432 \Delta gox0849$ double mutant (open symbols). (Modified from Zahid and Deppenmeier 2016).

3.4. Metabolic engineering of *G. oxydans* for enhanced osmotolerance

The last part of this thesis deals with various approaches to increase the osmotolerance of *G. oxydans*, a bacterium that has vast applications in pharmaceutical and food industries. It has been shown that mannitol and other polyols have an osmoprotective effect on growth, cell morphology, and catalytic potential of *G. oxydans* when the cells are grown in high osmotic media (Chapter 3.2). Therefore, it was postulated that the biotechnological potential of this organism can be enhanced by increasing intracellular mannitol synthesis. To achieve this goal two different strategies were followed (Fig. 36). The first strategy was the homologous overexpression of gene *gox1432* encoding the NADPH-dependent D-fructose reductase in *G. oxydans* to enhance intracellular mannitol production (Fig. 36, Reaction 4). The second approach was the heterologous overproduction of glucose isomerases in *G. oxydans* to increase the carbon flux from glucose towards fructose, a precursor of the compatible solute mannitol (Fig. 36, Reaction 5).

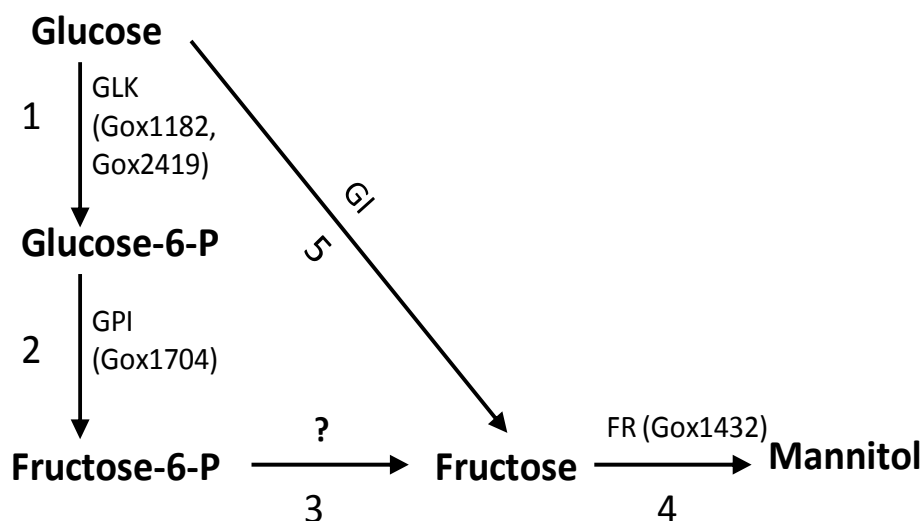


Figure 36: Scheme for metabolic engineering in *G. oxydans* for enhanced intracellular mannitol biosynthesis from glucose. Steps 1-4 represent the potential reactions involved in carbon efflux towards mannitol production. Reaction step 5 indicates a bypass reaction to increase the intracellular fructose content by isomerization of glucose using recombinant isomerases. The question mark indicates an unknown phosphatase. GLK - glucokinase (Gox2419, Gox1182), GPI - glucose-6-phosphate isomerase (Gox1704), FR - fructose reductase (Gox1432), GI - glucose isomerase.

3.4.1. Overproduction of D-fructose reductase (Gox1432) in *G. oxydans*

The first approach to increase the osmotic tolerance was the homologous overproduction of the D-fructose reductase (Gox1432) in *G. oxydans*. As mentioned above, deletion of *gox1432* resulted in a severe growth defect that was recovered upon complementation of the corresponding gene in *G. oxydans*. The idea was that the reduction of fructose to mannitol could be a rate-limiting step, and the overproduction of protein Gox1432 might be an appropriate approach to increase the intracellular D-mannitol content. To confirm this hypothesis, a plasmid construct (pBBR.p264.*gox1432*.ST) carrying *gox1432* was transformed and overexpressed in the wild type *G. oxydans*. The effect of overproduction of the protein on cellular growth was examined during cultivation in YGP_{high} medium containing 500 mM glucose as carbon source and osmoticum (790 mOsm kg⁻¹). As shown in Figure 37A, overexpression of gene *gox1432* did not result in an improvement in the growth parameters of *G. oxydans* when cultivated in YGP_{high} medium. Both, the wild type and the *gox1432* overexpression mutant revealed a doubling time of 2.8 h and reached a final optical density of 1.5. However, when the strains were grown in YF_{high} medium containing 500 mM fructose (610 mOsm kg⁻¹), growth of the *gox1432* overexpression strain improved in comparison to

the wild type (Fig. 37B). The doubling time of *G. oxydans* harboring the plasmid-borne gene *gox1432* was 3.6 h in contrast to 5.3 h for the wild type. Likewise, the optical density of the *gox1432* overexpression mutant was 1.6 at the end of cultivation ($t = 48$ h), a value that was 1.35 times higher than that of the wild type. In accordance, the intracellular mannitol content of the Gox1432 overproducing strain was 1.3 fold higher ($1.05 \mu\text{mol mg}^{-1}$ protein) in comparison to the wild type. These results indicate that the overexpression of gene *gox1432* had a positive influence on growth and intracellular mannitol content of *G. oxydans*, cultivated in the presence of fructose. However, this growth stimulatory effect was missing during cultivation on YGP medium. It is tempting to speculate that limited amounts of free fructose inside the glucose-grown cells hampered efficient mannitol production in the Gox1432 overproducing strain. Hence, it can be concluded that both, the production of Gox1432 (D-fructose reductase) and the availability of free fructose, are rate-limiting steps for in vivo mannitol production in *G. oxydans*.

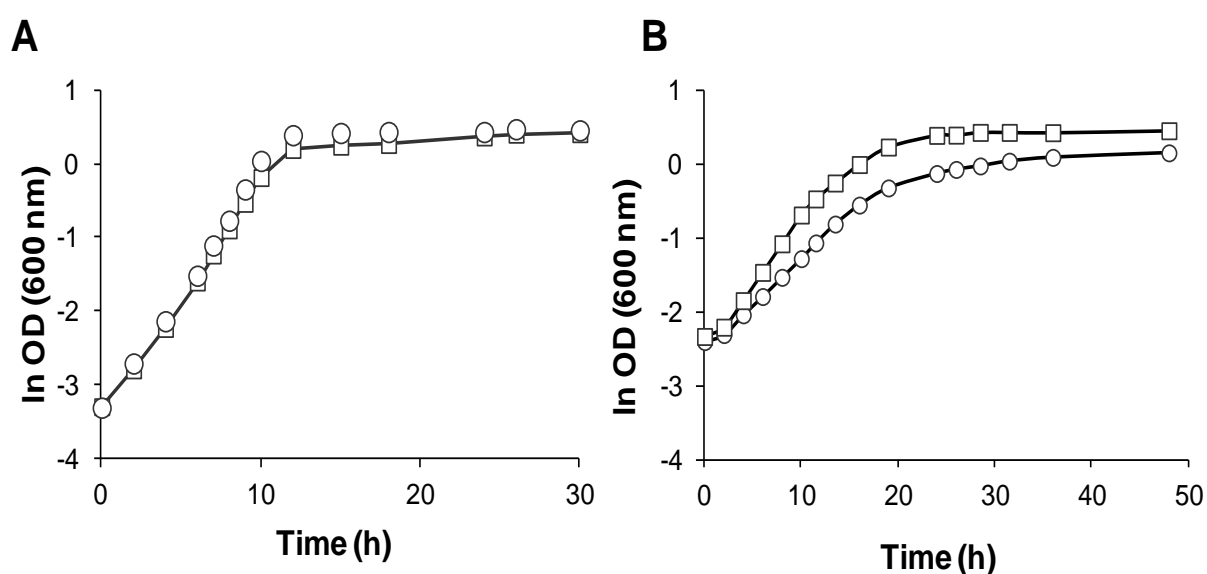


Figure 37: Effect of overexpression of *gox1432* on growth of *G. oxydans*. Growth profiles of the wild type *G. oxydans* (○) and the Gox1432 overproducing strain (□) grown on YGP_{high} medium containing 500 mM glucose (A) or YF_{high} medium with 500 mM fructose (B).

3.4.2. Heterologous overproduction of glucose isomerases in *G. oxydans*

An alternative strategy for the production of sufficient intracellular fructose in *G. oxydans* was the direct conversion of glucose to fructose by the catalytic activity of heterologously expressed glucose isomerases (GI). For this purpose, the *xylA* genes encoding xylose/glucose isomerases (XI/GI) (EC 5.3.1.5) from *E. coli* K12 (*b3565*) and *Streptomyces griseus*

(*SGR_RS05195*) were cloned into pBBR.p264.ST vectors and were expressed in the wild type *G. oxydans* 621H. The *xylA* expression mutants were cultured on YGP media with 20 mM MgSO₄. The proteins were purified from crude cell extracts using Strep-Tactin affinity chromatography and analysed by SDS- and native PAGE (Fig. 38A and 38B). SDS-PAGE revealed that both proteins were apparently homogenous and formed single bands of 50.7 kDa and 44 kDa, which were in agreement with the deduced masses of 49.7 (for *E. coli*) and 43.1 kDa (for *S. griseus*) of the recombinant tagged proteins, respectively. Native PAGE revealed that the glucose isomerases from *E. coli* and *S. griseus* exhibited apparent molecular masses of 151 kDa and 86 kDa which indicated that the former protein was homotrimeric, while the latter one possessed homodimeric structure. The enzyme xylose/glucose isomerase catalyzes the isomerization of C-5 or C-6 aldo-sugars like xylose and glucose to their corresponding keto-sugars xylulose and fructose, and is frequently used in industry for production of valuable high fructose corn syrups from starch hydrolysates (Lobanok *et al.* 1998). Both of the purified proteins were tested for the isomerization of xylose in native gels by a modified method of Yamanka (1975). Staining of the protein bands was performed by using alkaline solution of 2,3,5-triphenyltetrazolium which was oxidized by xylulose to the pink color compound formazan. As shown in Figure 38C, pink bands were observed around protein bands in native gels, and indicated the successful isomerization of xylose to xylulose by both purified proteins *in vitro*.

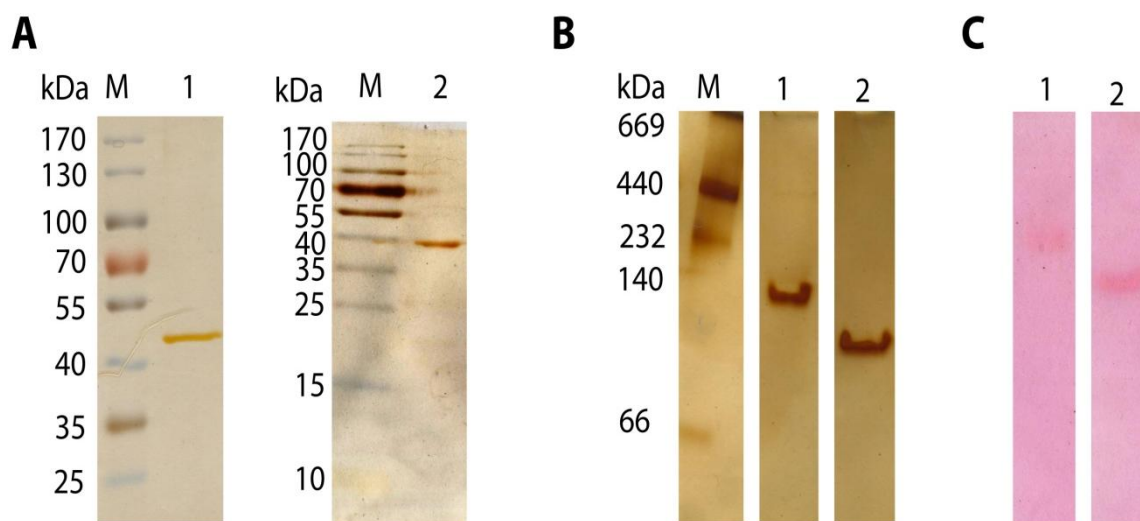


Figure 38: SDS- and native PAGE of glucose isomerases from *E. coli* and *S. griseus* heterologously produced in *G. oxydans*. (A) SDS-PAGE. (B) Native PAGE. (C) Activity staining of both purified glucose isomerases. A/B/C: Lane 1 represents the glucose isomerase from *E. coli*; Lane 2: from *S. griseus*; Lane M: Molecular weight standards.

On the basis of activity staining, it was evident that both glucose isomerases could catalyze the conversion of glucose to fructose *in vivo*. The glucose isomerase producing strains of *G. oxydans* were compared for their osmotolerance during cultivation in YGP_{high} medium containing 450 mM glucose as a substrate and osmoticum. *G. oxydans* wild type was used as a control strain. Unexpectedly, no significant difference in the growth parameters of the glucose isomerase overexpressing mutants and the wild type cultures were observed (Fig. 39). All the cultures grew with the doubling time of 3.2 h and reached a final optical density of 1.2 after 30 h of cultivation. Similarly, the drop in pH was identical, which decreased from 6.8 to 3.2 within 30 h.

To examine the activities of heterologously produced glucose isomerases, cytoplasmic fractions were prepared from all *G. oxydans* cultures, grown to the mid-log phase, and were tested for the isomerization of glucose into fructose. The assay mixture contained 50 mM phosphate buffer (pH 7.0), 100 μ l cytoplasm, 10 mM MgCl₂, and 250 mM D-glucose. The concentrations of glucose and fructose in the filtered assay mixtures were determined by a photometric test using a D-Glucose/D-Fructose UV-Kit (Chapter 2.7.3). It was found that glucose was not isomerized to fructose with any of the cytoplasmic fractions. In all assay mixtures, only glucose was detected which was provided as a substrate. The results indicated the catalytic inactivity of glucose isomerases in the cytoplasm of the cells expressing *xylA* genes from *E. coli* or *S. griseus*. Marshall *et al.* (1957) had reported that isomerization of glucose to fructose using cellular extracts of *Pseudomonas hydrophila* was dependent on the incubation time and initial glucose concentrations. To test the effect of incubation time, the assay mixtures were kept at 30°C for 18 h and tested for the presence of fructose. It was found that the increase in incubation time had no positive effect on the isomerization process. Furthermore, similar results were obtained when the same assay mixtures were tested for D-fructose production by an alternate method using fructose reductase (Gox1432) as described by Gao *et al.* (2002). The purified isomerases were also tested for isomerization of D-glucose to D-fructose or D-xylose to xylulose using the fructose reductase Gox1432 and the xylulose reductase Gox2181 as auxiliary enzyme (Chapter 2.6.8.2). The isomerase activity of both proteins was detected only at high temperatures. It was observed that at 70°C, the glucose isomerases from *E. coli* and from *S. griseus* isomerized glucose to fructose with specific activities of 0.22 U mg⁻¹ and 0.34 U mg⁻¹ protein, respectively. Likewise, xylose isomerization was also catalyzed by both proteins at high temperatures with specific activities of 0.5 U mg⁻¹ (for *E. coli* isomerase) and 0.7 U mg⁻¹ (for *S. griseus* isomerase). Wovcha *et al.* (1983) had reported that the glucose isomerase from *E. coli* isomerized glucose optimally at

60°C. Similarly, the optimum temperature for the catalytic activity of glucose isomerases from *Streptomyces* sp. has been reported as 70°C (Chen *et al.* 1979). However, the optimum temperature for growth of *G. oxydans* is in range of 25-30°C which is much lower than that of the isomerases. This might also be a reason why the cell cytoplasm of glucose isomerase producing strains of *G. oxydans* were devoid of the required isomerase activity. Therefore, the proposed stimulatory effect of these isomerases on growth of *G. oxydans* under osmotic stress could not be observed.

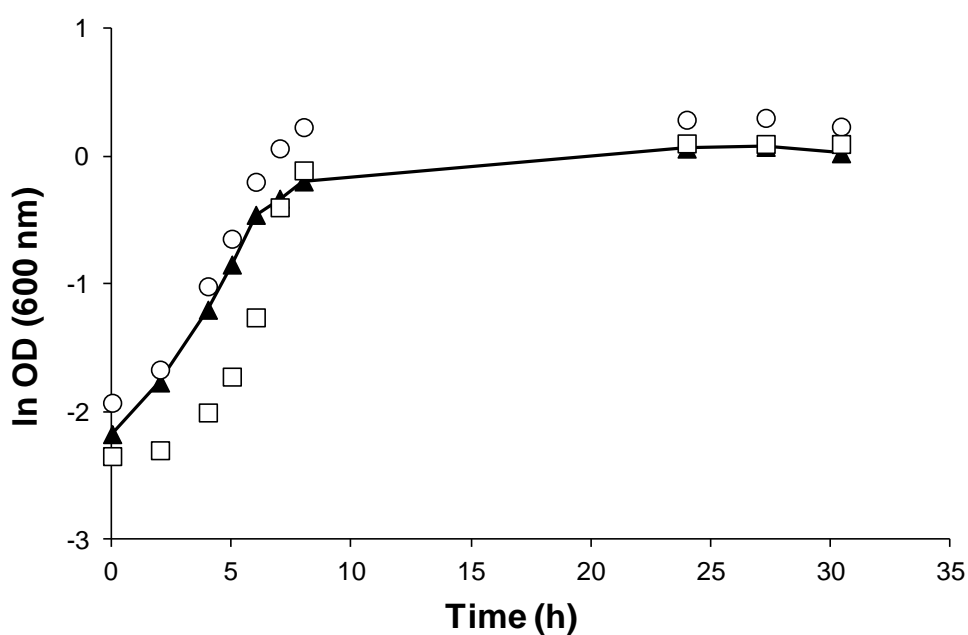


Figure 39: Effect of overexpression of glucose isomerases on growth of *G. oxydans*. Growth profiles of the wild type *G. oxydans* (○), the mutant strains overexpressing glucose isomerases from *E. coli* (▲) and from *S. griseus* (□), grown on YGP_{high} media with 450 mM glucose and 20 mM MgSO₄.

4. DISCUSSION

Among different abiotic factors, water is the most important component that affects growth of organisms in their natural habitats. Despite an abundance of water in different geographical areas such as oceans, its availability for living organisms is limited in many environments. The amount of dissolved solutes in water is the driving force that affects its colligative properties such as osmotic pressure, freezing, and boiling points. For example oceanic water contains a high percentage of salt (NaCl) that makes it unsuitable for consumption. Due to variable rates of rainfall and evaporation, the concentration of dissolved solutes in water, referred to as osmolality, is continuously fluctuating in ecosystems that imparts osmotic stress on the local biota. Living organisms undergo physiological changes to overcome the detrimental effects of limited water availability. The organisms maintain a positive intracellular turgor pressure that guarantees cell expansion and division (Wood 2015). Gram-positive bacteria are known to have a very high turgor pressure of approximately 20 bar while Gram-negative bacteria have only 3-10 bars (Whatmore and Reed 1990, Ingraham and Marr 1996, Csonka and Epstein 1996).

Hence, the questions arise as how microorganisms maintain such a high turgor inside the cell. Furthermore, it is of interest to know how the organisms sense and respond to changes in their environment. Many studies have been published concerning osmoadaptation mechanisms in organisms living in halophilic, osmophilic, and xerophilic conditions. So far, it has been reported that only two types of strategies prevail among organisms to cope with the osmotic pressure exerted by their environment. One is the salt-in strategy, and the other is the synthesis or uptake and accumulation of organic molecules (Galinski and Trüper 1994, da Costa *et al* 1998, Kempf and Bremer, 1998). The salt-in strategy, where salts especially inorganic ions (K^+ , Cl^-) are transported and accumulated inside the cell to balance the osmolality across the cytoplasmic membrane, is commonly present in halophilic archaea and halophilic bacteria (Galinski 1995, Oren 2008). These cations stabilize DNA molecules by interacting with the negatively charged phosphate backbone that results in an increase in its melting temperature (Kurz 2008). The second strategy, the accumulation of compatible solutes, is widely distributed in all three lineages of life, including Archaea, Bacteria, and Eukarya (Pflüger *et al.* 2003, da Costa *et al.* 1998, Loos *et al.* 1994, Grant 2004). In general, compatible solutes protect the cells by osmobalancing, stabilizing cellular proteins and their expression, as well as protecting them from denaturation in case of osmotic up-shifts, repeated freeze-thaw cycles, desiccation, or high temperatures (Welsh 2000, Carpenter 1993). The spectrum of these protective small organic molecules is quite broad, comprising sugar

molecules, polyols, amines, or their derivatives (Le Rudulier *et al.* 1984). Interestingly, it is known that these compounds can be accumulated within the cell in molar concentrations without affecting the cellular metabolism (Brown 1976).

As these compounds are compatible with cell physiology, compatible solutes have been intensively used *in vitro* to stabilize proteins and to increase their efficiencies such as in PCR mixtures. Compatible solutes are also used to stabilize nucleic acids, nucleoprotein complexes, to purify nucleic acids, and to improve cell free transcription processes (Kurz 2008). Mostly these solutes are non-polar (glycerol, mannitol, trehalose) or present as zwitter ions (proline, ectoine, glycine betaine), and do not interfere with the charge balance of cell cytoplasm. However, exceptions do exist in the domain of Archaea where the compatible solutes are anionic derivatives of carbohydrate molecules, such as mannosylglycerate, 2-sulfotrehalose, and galactopyranosyl-hydroxylysine (Desmarais *et al.* 1997, Lamosa *et al.* 1998, Martins *et al.* 1999). The negative charge of these molecules is conferred due to the presence of carboxylate, phosphate, or sulphate moieties (Martins *et al.* 1999). The plausible reason for the use of these anionic molecules as compatible solutes is to balance cationic charge (K^+), present in abundance in the cell cytoplasm of archaeal organisms (Roeßler and Müller 2001). Both of these osmoadaptive strategies are not confined to a discrete group of microorganisms as some bacteria share both of these osmoadaptive responses during osmotic up-shock. For example, Gram-positive lactic acid bacteria initially respond to a decrease in water activity of the growth medium by the uptake of K^+ ions (transient response), followed by the accumulation of compatible solutes (secondary response) (Le Marrec 2011). Such temporal sequence of events for the regulation of cellular functions upon osmotic shock have also been extensively studied in *E. coli*, *C. glutamicum*, and *M. thermolithotrophicus* (Roeßler and Müller 2001, Galinski 1995).

The present study deals with the investigation of such response mechanisms in the industrially important bacterium *Gluconobacter oxydans*. This organism has the ability to incompletely oxidize various substrates (sugars and polyols) to biotechnologically important compounds. Therefore, *G. oxydans* is extensively used in several biotransformation processes to obtain valuable sugar derivatives that are used as flavoring agents (aliphatic and aromatic carboxylic acids), pharmaceutical products (miglitol: an antidiabetic drug, L-ribose), food additives (gluconate, vinegar), and tanning agents (dihydroxyacetone) (Rabenhorst *et al.* 2001, Schedel 2000, Adachi *et al.* 2003). The economical importance of these compounds can be estimated by their annual production rate. As an example, gluconate is produced at the rate of almost 10,000 tons per annum, and is one of the top-ten organic chemicals derived

from sugar sources (Lichtenthaler 2006). Gluconate chelates metal ions and is used in various cleaning products to prevent mineral deposition. In the form of salts, gluconate is used as an oral or injectable metal ion supplement for humans and animals. Sodium gluconate is utilized in the cement industry to impede the process of cement polymerization under reduced moisture conditions (Pollard *et al.* 1991).

The aerobic microorganism *G. oxydans* oxidizes most of the substrates at the cytoplasmic membrane or in the periplasm by the catalytic activity of a number of dehydrogenases. In the course of the oxidation of substrates, electrons are channeled into the respiratory chain for energy conservation. Most of the oxidized products are excreted into the media via outer membrane porin proteins. Only a very small amount of substrate is taken up by the cells and metabolized for biomass production (Siever and Swings 2005, Richhardt *et al.* 2013a). Due to economic aspects, this bacterium has been under extensive research for many decades regarding the identification and characterization of novel enzymes and their metabolic relevance. However, very little attention has been paid to understand the physiological response of this organism towards reduced water activity. In industrial-scale fermentations, many bacteria encounter osmotic stress provided by high concentrations of substrates, products, or salts (*e.g.*, buffers for pH control) resulting in reduced bacterial activities (Riesenberg *et al.* 1991). Recently, a study was carried out to target the effects of concentrated soluble buffers, added to the media for pH control, on growth and product formation rates of *G. oxydans*. It was found that osmolality values of more than 0.5 Osm kg⁻¹ inhibited growth and catalytic efficiency of the organism. Similar results were obtained when osmolalities of the growth media were raised with salts rather than buffers (Luchterhand *et al.* 2015). However, limited information is available with respect to the osmotic sensitivity of the organism towards organic substrates (sugars, polyols, and their derivatives), present in abundance in its natural habitats. *G. oxydans* thrives naturally in sugar-rich niches such as fruits, honey-bees, cider, wines (Gupta *et al.* 2001) and can survive in highly concentrated sugar solutions containing as high as 20-30 % glucose, sorbose, or sorbitol (Sievers and Swings 2005, Mori *et al.* 1981, Rosenberg *et al.* 1993). Nevertheless, information regarding protective mechanisms, which prevail in the organisms to counteract osmotic stress, is missing. Therefore, it is necessary to investigate these osmoprotective mechanisms to understand the physiology of *G. oxydans* in a better way. In the present study, experimental evidences were provided for the role of polyols especially mannitol as an osmoprotectant in *G. oxydans*. Moreover, the molecular mechanisms for mannitol biosynthesis were explored in detail to get an overview of the osmoadaptive strategies prevailing in this organism.

4.1. Osmotic stress responses in *G. oxydans*

Due to the lack of a suitable mineral medium, *G. oxydans* is routinely cultured on complex media containing yeast extract using monosaccharides (glucose, mannitol, glycerol, fructose) as carbon and energy sources. In order to select suitable conditions for osmotic stress studies, *G. oxydans* was initially cultivated in complex media containing glucose (YG) or mannitol (YM) as a growth substrate. For control experiments, both sugars were provided in concentration of 100 mM (170 mOsm kg⁻¹) and analyzed for their effect on growth of *G. oxydans*. No significant effect of these sugars was observed on growth of the cultures (Chapter 3.1.1). Both cultures grew exponentially with a doubling time of 1.5 ± 0.1 h. However, the final optical density of the culture grown with glucose was slightly lower in comparison to the culture with mannitol as a substrate. Growth of *G. oxydans* with glucose resulted in the production of gluconates (Olijve and Kok 1979), which dropped the pH of the growth medium below 3.0 and ultimately reduced the cell proliferation. Whereas growth in the presence of mannitol led to production of fructose and trace amounts of acetate (Richhardt *et al.* 2012), thus the pH of the culture remained in a normal range to support cell growth. The same sort of growth behavior was observed by Peters *et al.* (2013a) during cultivation of *G. oxydans* on complex media in the presence of 50 mM glucose or mannitol. The culture had a doubling time of 1.77 h on mannitol and of 1.82 h on glucose. In addition, the mannitol-grown culture attained a higher final optical density of 1.94 in comparison to 1.42 of the glucose-grown culture. Thus, it can be concluded that *G. oxydans* can efficiently utilize both sugars for energy conservation and biomass formation. However, in un-buffered growth media, mannitol is a better substrate for growth rather than glucose.

For osmotic studies, different permeating and non-permeating osmolytes were used in this study to increase the osmolality of the growth media. These included glucose and mannitol as permeating and metabolizable osmotica that were applied to yeast extract containing media in concentrations of 1000 mM (1100 mOsm kg⁻¹). *G. oxydans* cultures, cultivated on these sugar rich media with comparable osmolalities, showed remarkable differences in their osmotolerance levels. It was found that growth in 1 M glucose medium (YG_{high}) was highly impaired in comparison to the 1 M mannitol containing medium (YM_{high}). With glucose, the culture had a prolonged lag phase of 10 h, followed by slow growth with a doubling time of 4.2 h, and reached a final optical density of only 0.1 (Chapter 3.1.1). This effect was probably due to the pH of the medium which dropped to below 3.0 and inhibited metabolic activities especially the pentose phosphate pathway, leading to a poor growth of the culture (Olijve and Kok 1979). In contrast, growth of the culture stressed with isosmotic concentrations of

mannitol was significantly better and nearly resembled the control culture (Chapter 3.1.1). In addition, pH values of the mannitol-grown culture remained in a range of 4.5-5.0 which was close to the optimal range (5.0) for growth of this organism (Matsushita *et al.* 1994, Gupta *et al.* 2001). These results were concordant with the findings of Luchterhand *et al.* (2015) that in an un-buffered medium, a low pH-value of the culture rather than osmolality was the responsible factor for poor growth of *G. oxydans*. Therefore, further experiments were performed in the presence of mannitol as a carbon source that has been reported as preferred substrate for growth of this organism (Prust *et al.* 2005, Richhardt *et al.* 2012).

The sensitivity of *G. oxydans*, with respect to increased osmolalities of growth media, was further investigated in the presence of an inert organic polymer PEG-6000, which is a non-permeating osmolyte in Gram-negative bacteria (Nikaido and Vaara 1985). Growth experiments showed that the growth rates and final optical densities of the cultures decreased with an increase in the concentrations of PEG-6000 in the growth medium (Chapter 3.1.1). This effect was due to the fact that PEG-6000 in increasing concentrations (25 - 100 g L⁻¹) raised the osmolality of the growth medium (170 to 385 mOsm kg⁻¹), which imparted osmotic stress to the cultures and negatively affected their growth parameters (Mille *et al.* 2005, Luchterhand *et al.* 2015). In comparison, it was observed that growth of the *G. oxydans* culture subjected to osmotic stress with the permeating osmolyte mannitol was better than with the non-permeating PEG-6000; although, the osmolality of the medium were higher in the presence of the former osmolyte. A possible reason for this difference could be the interaction between the osmolyte PEG-6000 and the carbon source mannitol. Such cooperative interaction among solutes significantly lowers the water potential of a solution (Michel 1983) and might be inhibitory for growth of cells. These results suggest that the growth behavior of osmotically stressed cultures of *G. oxydans* depends on the nature of osmolytes added to the media. Similar types of growth profiles have been observed in certain other microorganisms. For example, cultivation of *Pseudomonas putida* S12 under hyperosmolal conditions showed that at identical water activity (a_w), hyperosmotic stress imposed by the addition of PEG or sucrose in the growth medium resulted in a strong decrease of the growth rate rather than by the glycerol or sodium chloride (Kets *et al.* 1996a). Likewise, different strains of lactic acid bacteria have been reported for manifestation of variable osmosensitivity levels induced by isosmotic concentrations of salts or organic osmolytes (Glaasker *et al.* 1996, Prasad *et al.* 2003).

4.1.2. Response of *G. oxydans* to osmotic stress at transcriptional level

Apart from growth studies, a closer look was taken at the transcriptional level to understand the differential response of *G. oxydans* towards mannitol and PEG-induced osmotic stress. The availability of the complete genome sequence of *G. oxydans* provided benefit for the identification of potential genetic elements regulated by osmotic stress. Thus, a comparative analysis of the differential expression of these potentially osmoinduced genes was performed using cultures grown in YM_{high} medium and YM medium supplemented with 5 % PEG-6000 (Chapter 3.1.2). The data from RT-qPCR analysis, which were in accordance with the results of the growth experiments, showed that the expression of potentially osmoinduced genes was significantly enhanced in the presence of PEG-6000 rather than with 1 M mannitol as osmoticum in the growth medium. The genes, induced by osmotic stress with PEG-6000, were most likely involved in the protection of cellular homeostasis from non-favorable growth conditions. These include genes encoding a DNA starvation and stationary phase protection protein (Gox0707), a trehalose phosphate synthase (Gox1119), molecular chaperons (Gox0820, Gox0857), and ABC transporter proteins (Gox2182, Gox2220). Thus, the results of both, RT-qPCR analysis and growth experiments, collectively supported the hypothesis that *G. oxydans* undergoes different physiological and genetic changes in response to osmotic shock elicited by PEG-6000 rather than mannitol. Therefore, genome-wide transcriptome analysis of the PEG-induced osmotically stressed culture was performed to get a comprehensive picture of osmotic stress responses in this organism.

4.1.3. Genome-wide transcriptome analysis of osmotically stressed cells of *G. oxydans*

Next-generation sequencing (NGS) analysis indicated that from the 2735 ORFs of *G. oxydans*, 105 genes were differentially expressed (more than two-fold) in response to osmotic up-shift (Chapter 3.1.3.2). Analysis of the transcriptome data revealed that various cellular processes linked to metabolism, cell signaling, transport, and transposition were affected in osmotically stressed *G. oxydans* cultures. These differentially expressed genes were categorized into 18 groups according to their role in cellular functions (Chapter 3.1.3.2). A major percentage (40 %) of these differentially expressed genes encode hypothetical proteins and are classified as hypothetical genes with unknown cellular functions. It was noted that the expression of only a few genes encoding proteins involved in cellular metabolism, transport, transcription regulation, motility, and detoxification were induced under hyperosmotic conditions. Moreover, the NGS data revealed that in *G. oxydans*, genes encoding putative transposases and phage proteins were significantly up-regulated in response to osmotic shock.

These included the genes *gox1325*, *gox0463*, *gox2593*, and *gox1213*, whose expression was more than three-fold increased under osmotic stress. These findings can be explained by the fact that bacteria are prompted to undergo genetic variability during unfavorable growth conditions (Foster 2007). Thus, the activation of transposition could be part of various stress responses to generate genetic diversity or to transfer genetic information from one cell into another cell. This genetic diversity may assist the bacterial population to withstand harsh environmental conditions (Kleckner 1990, Capy *et al.* 1997, Capy *et al.* 2000).

Furthermore, the genome-wide transcriptome analysis revealed an increased expression of genes encoding transporter proteins in osmotically stressed cells of *G. oxydans*. Most of these genes belong to the TonB type outer membrane receptor proteins and the ABC type sugar transporter proteins. A number of studies showed that microorganisms undergo a rapid efflux of water and plasmolysis on exposure to hypertonic conditions. To prevent the excessive dehydration, cells tend to maintain their turgor by increasing cytoplasmic concentrations of osmotically active solutes. These solutes can be transported from the environment by means of various transporter proteins. For example, *E. coli* cells actively transport compatible solute (proline, glycine betaine, choline) under osmotic up-shift, and the transporters responsible for acquisition of these osmoprotectants (*e.g.*, ProP and ProU) belong to the major facilitator superfamily and the ABC transporter superfamily (Paulsen *et al.* 1996, Gouesbet *et al.* 1994). Additionally, TonB proteins are known for the active transport of solutes (vitaminB₁₂, iron, siderophores) into the periplasm, via interaction with outer membrane receptor proteins (Kadner 1990). Also, an increased expression of the transcriptional regulator Gox0676, belonging to the group of iron-regulated sigma factors, was examined. Such types of regulators control the expression of genes involved in iron transport and homeostasis. The increased expression of these transport protein encoding genes in *G. oxydans* highlights the significance of the active transport of solutes and essential ions under osmotic stress conditions.

In addition, expression of certain genes involved in the metabolism of sugars, amino acids, and lipids was also induced. This group includes genes encoding a glycolytic enzyme, the triose phosphate isomerase (Gox2217) and a PPP enzyme, the ribose-5-phosphate isomerase (Gox2218). Both pathways are involved in the assimilation of sugars for biomass generation and synthesis of precursor molecules for anabolic processes. For example, the ribose-5-phosphate isomerase is involved in the isomerization of ribulose-5-phosphate to ribose-5-phosphate, which is required for the synthesis of nucleic acid molecules. Likewise, the expression of the gene encoding a dihydrofolate synthase (Gox1205) was up-regulated. This

enzyme participates in the de novo-synthesis of tetrahydrofolic acid, which is involved in the synthesis of purines and in the interconversion of glycine and serine molecules (Shane 1989, Ravanel *et al.* 2001). Probably, nucleic acid biosynthesis could be impaired in *G. oxydans* under osmotic stress conditions. Thus, acceleration of the pathways involved in the synthesis of such precursor molecules could be the plausible solution. Supporting evidences came from an increased expression of the genes encoding enzymes for DNA repair such as the uracil-DNA glycosylase (Gox1908) and the DNA helicase II UvrD (Gox2523). These types of cellular stress responses are very common and prevalent among prokaryotes and eukaryotes (Kültz 2003). For example, an overexpression of the pea DNA helicase 45 gene in tobacco plants or bacteria resulted in an enhanced tolerance of the mutant strains towards salinity stress situations (Sanan-Mishra *et al.* 2005, Tajrishi *et al.* 2001). Among other differentially expressed genes in *G. oxydans*, expression of three putative oxidoreductase (Gox0201, Gox1849, Gox2594) encoding genes was two-fold increased under osmotic stress conditions. As the specific functions of these putative enzymes are not known, their link to osmotic stress could not be established.

As stated above, the change of intracellular osmolytes concentrations is one of the well-investigated physiological responses in osmotically stressed organisms. A closer inspection of the transcriptome data revealed that the transcription of a genes encoding the putative trehalose-6-phosphate phosphatase (Gox1118) was two-fold increased in *G. oxydans* following osmotic up-shock. Likewise, RT-qPCR analysis showed that the expression of a gene coding for a trehalose-6-phosphthate synthase (Gox1119) was significantly induced under osmotic shock (Chapter 3.1.3.3). Both genes *gox1118* and *gox1119* are organized in an operon in the genome of *G. oxydans*. Moreover, blast analysis using the NCBI non-redundant protein database indicated that the amino acid sequence of Gox1118 shared weak homology (34 % identity, 78 % query coverage) to the trehalose phosphatase (OtsB: NP_416411) of *E. coli*. While Gox1119 was homologous to the *E. coli* protein trehalose-6-phosphate synthase (OtsA: NP_416410) with 41% identity (68 % query coverage). Both OtsA and OtsB are osmoregulatory proteins and involved in the synthesis of the compatible solute trehalose in *E. coli* (Giaever *et al.* 1988, Klein *et al.* 1991). In addition, a number of other studies showed that the production of trehalose as well as the production of the corresponding enzymes for trehalose synthesis was increased following desiccation or limited water activity situations in different microorganisms (Cytryn *et al.* 2007, Tsuzuki *et al.* 2011). These observations led to the assumption that both genes *gox1118* and *gox1119* could possess a similar function and could participate in the biosynthesis of trehalose in osmotically stressed *G. oxydans* cells.

Giaever *et al.* (1988) reported that mutants of *E. coli*, unable for trehalose biosynthesis, showed compromised growth on osmotically enriched mineral medium. Similarly, deletion mutants of *Rhizobium leguminosarum* for trehalose biosynthesis genes displayed an osmosensitive phenotype under drying conditions (McIntyre *et al.* 2007). Thus, the role of *gox1118* and *gox1119* in *G. oxydans* was studied by generating deletion mutants of these genes and by analyzing their growth profile in YM medium with 10% PEG-6000 as osmoticum. Intriguingly, growth of the mutant strain was not affected and was comparable to the wild type (Chapter 3.1.3.4). These observations indicated that the genes *gox1118* and *gox1119* were not essential for growth of *G. oxydans* under osmotic stress conditions. Moreover, ^{13}C -NMR analysis of the intracellular metabolites of osmotically stressed cells of *G. oxydans* indicated that trehalose is not produced as a potential compatible solute in this organism (Chapter 3.2.1). Thus, it is tempting to speculate that the genes *gox1118* and *gox1119* do not synthesize trehalose under given conditions. However, an enhanced expression under osmotic stress suggests the participation of these genes in some other unknown osmoregulatory mechanism in *G. oxydans*.

4.2. Mannitol as a major intracellular metabolite and osmolyte in *G. oxydans*

To identify compatible solutes accumulated in *G. oxydans* for the compensation of externally applied osmotic pressure, ^{13}C -NMR studies were performed. Therefore, total cellular metabolites of PEG-6000 osmotically stressed cells were extracted and analyzed by NMR spectroscopy. ^{13}C -NMR analysis revealed the accumulation of mannitol as a major osmolyte in osmotically stressed cells of *G. oxydans* (Chapter 3.2.1). This observation led to the assumption that under osmotic stress, cells take up mannitol from the media to maintain their osmotic homeostasis with the external environment. It has been reported that various polyols like mannitol have an osmoprotectant role in different organisms under osmotic stress conditions such as bacteria, yeasts, fungi, and plants (Grant 2004, Wisselink *et al.* 2002, Sand *et al.* 2013, Zahid *et al.* 2015). Thus, the accumulation of polyols in these organisms might reflect an evolutionary convergent osmoadaptive mechanism. Moreover, microbes mostly rely on the uptake of compatible solutes (if available), from their environment because the *de novo* synthesis of these compounds is energetically expensive (Oren 1999). In such paradigms, it is tempting to speculate that in the presence of mannitol, *G. oxydans* takes up this compound and does not reveal any other physiological response to the osmotic stress, exerted by PEG-6000. However, the growth rate and the final optical density of *G. oxydans* was affected by the addition of PEG-6000 to the growth medium. This could be caused by the high molecular mass of PEG that slowly crosses the outer membrane of Gram-negative

bacteria and imposes a gradient across the cytoplasmic membrane which may result in the release of free cytoplasmic water and in the decrease of the growth rate (Nikaido and Vaar 1985, Record *et al.* 1998). A similar phenomenon was observed in *Pseudomonas putida*, where the addition of PEG-4000 to the medium could not trigger any physiological response in the osmotically stressed cells. Although PEG decreased the a_w and reduced the growth rate of the culture, the accumulation of K^+ ions and glycine betaine was not observed. Accumulation of potassium ions and glycine betaine was examined when the water activity of the growth medium was decreased to comparable levels with other osmolytes such as sucrose and sodium chloride (Kets *et al.* 1996a). Therefore, it was suggested that the composition of growth medium has a great influence on osmotolerance of each organism (Galinski 1995).

In *E. coli*, an increase in osmolarity of the growth medium leads to the release of free cytoplasmic water and a change of the cell turgor pressure. This change of the cell turgor triggers the activation and an increase of the production of various transport proteins for the uptake of osmoprotectants like proline, trehalose, and glycine betaine (Record *et al.* 1998, Lucht and Bremer 1994). In the present study, the transcriptome data and RT-qPCR results revealed an increased expression of genes coding for transport proteins, indicating an enhanced active transport of various solutes and sugar molecules. An increased expression of ABC transporters and TonB proteins in connection with the accumulation of mannitol highlights the active transport of sugar molecules and other solutes as an osmoinduced response in stressed cells of *G. oxydans*. Interestingly, RT-qPCR analysis revealed a four-fold enhanced expression of gene *gox2182* which encodes a mannitol/sorbitol ABC transporter permease (Chapter 3.1.2). For further investigations on the role of this transport system for the uptake of mannitol, multiple attempts were performed to generate deletion mutants for the putative mannitol/sorbitol transport proteins (Gox2183-2185). However, it was not possible to create such deletion mutants indicating that the transporter proteins are essential for the survival of *G. oxydans* (data not shown).

Compatible solutes have intrinsic properties to protect the cellular proteins and other macromolecular structures against denaturing stress conditions (Santoro *et al.* 1992, Knapp *et al.* 1999, Crowe *et al.* 1984, Zahid *et al.* 2015). For example, trehalose and hydroxyectoine confer protection to *E. coli* K12 against freeze drying processes (Manzanera *et al.* 2004). Similarly, glycine betaine and proline provide protection to lactic acid bacteria against salinity and thermal denaturation (Caldas *et al.* 1999). For the investigation of such an osmoprotective effect of mannitol in *G. oxydans*, cellular catalytic activities of both the

control and 10 % PEG-6000 applied stressed cultures were compared (Chapter 3.2.2). Despite a decrease in the optical density of the stressed culture, the rates of the oxidation of mannitol (substrate) to fructose (product) were identical under both growth conditions. These observations were concordant with the fact that compatible solutes protect cellular functions and especially stabilize the structure and activity of enzymes under adverse conditions (Jennings 1984, Kets 1997). It has been reported that the activity of the purified α -amylase of *Bacillus* sp. was significantly recovered in the presence of sorbitol as an osmoprotectant during temperature and chemical induced denaturation (Chi *et al.* 2012). Likewise, the addition of low levels of glycine betaine to a osmotically rich medium of *Lactobacillus casei* increased lactic acid production by stabilization of cellular proteins including the lactate dehydrogenase (Zou *et al.* 2013).

4.2.1. De novo synthesis of mannitol in *G. oxydans* under reduced water activity

Mannitol production and accumulation in *G. oxydans*, subjected to osmotic stress, was further investigated in the absence of the exogenous supply of this osmoprotectant. Therefore, *G. oxydans* was cultivated in YGP medium containing 50 mM glucose as growth substrate. To control the pH of the culture, 100 mM soluble phosphate buffer was added to the medium. The osmolality of this basal (control) medium was ~ 300 mOsm kg^{-1} . Osmotic stress was applied by adding 300 mM sucrose to the medium that resulted in the total osmolality of 680 mOsm kg^{-1} . *G. oxydans* cannot metabolize the disaccharide sucrose due to the lack of hydrolyzing enzymes (Prust *et al.* 2005, Peters *et al.* 2013a); therefore, it is considered an inert osmolyte. Moreover, it is present in the natural habitats of *G. oxydans* such as nectar where sucrose constitutes 40-60% of the total sugar content (Johnson and Nicolson 2008). ^{13}C -NMR analysis revealed the presence of mannitol in great abundance in the intracellular metabolite pool of osmotically stressed *G. oxydans* cultures in comparison to the non-stressed cultures (Chapter 3.2.3). Additional compounds such as fructose, glucose, gluconates, and sucrose were also detected at low levels in the ^{13}C -NMR spectrum. These findings clearly demonstrated that mannitol is synthesized and accumulated as a compatible solute in *G. oxydans* under osmotic stress. Fructose could be a precursor molecule for mannitol biosynthesis and gluconate is produced by the oxidation of glucose (carbon source). Glucose and sucrose were medium components which remained in part in the periplasmic and external water space of the cells during sample preparation for spectroscopic analysis.

So far, few microorganisms are reported for the accumulation of polyols as osmoprotectant under osmotic stress. These include *Z. mobilis*, *P. putida*, *A. baylyi*, and heterofermentative

lactic acid bacteria (Barrow *et al.* 1984, Kets *et al.* 1996b, Sand *et al.* 2013, Wisselink *et al.* 2002). In these microorganisms, the synthesis and accumulation pattern of such osmoprotectants are dependent on the concentrations of osmolytes applied in the media. In the present study, it was found that the growth behavior and the mannitol accumulation pattern of osmotically stressed *G. oxydans* cells were influenced by the change in osmolality of the growth media (Zahid *et al.* 2015, Zahid and Deppenmeier 2016). An increase in the concentration of sucrose in the YGP medium negatively affected the growth of the organism (Chapter 3.2.3). The growth rate of the culture gradually decreased from 0.4 to 0.19 h⁻¹ with the increase in external osmolality from 310 to 1160 mOsm kg⁻¹ (0-600 mM external sucrose), respectively. The effect of osmotic stress on the growth was pronounced at very high osmolalities (exceeding 680 mOsm kg⁻¹) of the growth medium. Sucrose concentrations of 450 mM and 600 mM provided severe osmotic shock to the cells, resulting in considerably reduced growth rates and low final optical densities of the cultures. From these data, it was concluded that *G. oxydans* can optimally grow till an external osmolality of 680 mOsm kg⁻¹ (Zahid *et al.* 2015). These optimum osmolality values were slightly higher in comparison to those reported by Luchterhand *et al.* (2015) who determined the optimum osmolality value of 400 mOsm kg⁻¹ for *G. oxydans*. This difference in the osmolality threshold could be due to the different type of osmolytes included in the media for osmotic studies. Growth of *G. oxydans* was inhibited at osmolality value of more than 500 mOsm kg⁻¹, raised with salts or buffer compounds. Whereas, the herein determined optimum osmolality limit of 680 mOsm kg⁻¹ was detected in the presence of organic osmolytes in the growth medium. Comparably, the optimum osmolality for growth of *E. coli* is 280 mOsm kg⁻¹ (Record *et al.* 1998), which is significantly lower than that for *G. oxydans*. *E. coli* grew efficiently in complex medium with an external osmolality of ~ 0.3 Osm kg⁻¹. The growth rate decreased linearly and reached 0 h⁻¹ at an external osmolality of 2.7 to 3.0 Osm kg⁻¹ with NaCl (Record *et al.* 1998, Mclaggan *et al.* 1990). Like *G. oxydans*, the growth rate of the closely related bacterium *Z. mobilis* decreased from 0.54 to 0.12 h⁻¹ when the external sucrose concentration increased from 200 to 300 g L⁻¹ (Sootsuwan *et al.* 2013).

4.2.2. Osmodependent accumulation of mannitol in *G. oxydans*

The modulation of the intracellular osmolyte pool of microorganisms is actively controlled by external osmotic fluctuations. In this study, a linear increase in the intracellular mannitol and fructose concentration was observed with increasing external sucrose concentrations (Zahid *et al.* 2015). The maximum intracellular concentrations of 2.7 μmol mg⁻¹ and 1.4 μmol mg⁻¹ were obtained for mannitol and fructose in the presence of 450 mM external sucrose,

respectively (Chapter 3.2.3). A further increase of the sucrose concentration had no significant effect on the intracellular osmolyte concentration. The reason for this could be the saturation limit of the cells for the accumulation of these solutes and the reduced growth rate of the cultures under conditions of high osmotic stress. Analogously, Amezaga *et al.* (1995) observed an inverse correlation between growth rate and intracellular levels of compatible solutes during growth of the Gram-positive microorganism *Listeria monocytogenes* under osmotic stress conditions.

The osmodependent increase of the concentration of cytoplasmic solutes has been well-investigated in different microorganisms. For example, in *Z. mobilis*, sorbitol is produced and accumulated intracellularly during growth on either sucrose or a mixture of glucose and fructose. It was observed that the accumulation pattern of sorbitol depends on changes of the concentration of these sugars in the growth media. Sorbitol was accumulated intracellularly up to concentrations of 700 mM with 1 M sucrose in the medium. Moreover, the bacterium can accumulate exogenously supplied sorbitol in response to an osmotic up-shock by external glucose concentrations exceeding 0.83 M. At higher glucose concentrations (> 1.3 M), the added sorbitol was accumulated inside the cells up to a concentration of 1 M (Loos *et al.* 1994). Likewise, in *P. putida* and *A. baylyi*, mannitol was produced and accumulated during growth on mineral media containing glucose and succinate as carbon substrates, respectively. Intracellular mannitol concentrations in both organisms were also dependent on the external osmolyte concentrations. It was observed that the concentration of mannitol increased from 0 to 170 $\mu\text{mol g}^{-1}$ cell dry weight in *P. putida* S12 due to an increase of the NaCl concentration from 0 to 0.5 M (Kets *et al.* 1996b). Likewise, in *A. baylyi*, the cellular mannitol concentration increased five-fold from 0.3 $\mu\text{mol mg}^{-1}$ protein at 300 mM NaCl to 1.6 $\mu\text{mol mg}^{-1}$ at 900 mM (Sand *et al.* 2013).

4.2.3. Effect of carbon sources and osmolytes on mannitol accumulation in *G. oxydans*

The concentration of intracellular osmolytes in osmotically stressed organisms is dependent on the carbon source as well as on the osmolytes included in the growth medium. In this study, *G. oxydans* was subjected to osmotic stress exerted by different permeating and non-permeating osmolytes such as glucose, fructose, glycerol, and PEG-6000. Mannitol was accumulated with all these osmotica with the exception of glycerol (Zahid and Deppenmeier 2016). However, the intracellular concentration of mannitol varied with these osmolytes regardless of the osmolality of the media (Chapter 3.2.4). Maximum levels of mannitol accumulation were observed with glucose as carbon source along with sucrose or glucose (in

higher concentrations) as osmolytes. While under osmotic stress elicited by PEG-6000, higher levels of mannitol were accumulated with fructose as growth substrate in comparison to glucose. In the presence of glycerol as a sole carbon and osmotic source, cells accumulated only glycerol in high amounts. This finding could be explained by the fact that glycerol is a permeating polyol which can cross the cytoplasmic membrane simply by diffusion as shown by Alemohammed and Knowles (1974). Thus, osmotically stressed *G. oxydans* cells can accumulate this small polyol to counteract externally applied osmotic stress. As stated by Oren (1999) and Galinski (1995), in the presence of small organic osmolytes in the surrounding medium, their up-take and accumulation is preferred by organisms over in vivo synthesis to acquire osmotolerance. These results indicate that not only the osmolality of medium, but also the nature of osmolyte has great influence on cytoplasmic solutes of osmotically stressed organisms. Analogous results were obtained in *P. putida*, where a comparable decrease in water activity of a minimal medium with different osmolytes (NaCl, KCl, Na₂SO₄, K₂SO₄ and sucrose) resulted in variable intracellular accumulation pattern of compatible solutes (Kets *et al.* 1996b). For example at a medium a_w value of 0.983, mannitol accumulation was 1.25- and 1.52-fold higher with Na₂SO₄ and K₂SO₄ in comparison to NaCl, respectively. Likewise, *P. putida* cells accumulated variable levels of intracellular mannitol in the presence of different carbon sources in a mineral medium containing 0.5 M NaCl as osmolyte. With glucose, the mannitol accumulation was 4.74-fold higher in the cells in comparison to equimolar amounts of fructose (Kets *et al.* 1996b).

4.2.4. Protective effect of mannitol on cell physiology

Protective effects of osmolytes can be multifaceted. Three different modes of protective mechanism have been suggested:

- 1) Maintenance of the cell turgor under reduced water activity conditions. Microbes respond to external osmotic stress by the release of free cytoplasmic water which results in an increase of the concentrations of cytoplasmic solutes. Moreover, microorganisms actively maintain their cellular hydration level by the uptake of water molecules due to the accumulation of intracellular osmolytes (Jennings 1984, Kets *et al.* 1997)
- 2) Stabilization of membrane lipids and proteins (Crowe *et al.* 1992, Leslie *et al.* 1995)
- 3) Protection of cellular systems from oxidative damage which is in some organisms activated in response to osmotic stress (Smirnoff 1993). Mannitol is known as a scavenger of hydroxyl radicals and is an effective antioxidant (Chaturvedi *et al.* 1997, Gilmour *et al.* 1995,

Shen *et al.* 1997). Mannitol has also therapeutic effects (Li *et al.* 1996), especially protects the human retinal pigment epithelial cells against H₂O₂-induced oxidative stress (Liu *et al.* 2010).

The present study revealed the osmoprotective effects of mannitol on cell growth, physiology, and morphology in *G. oxydans*. It was noticed that very low concentrations of mannitol (2.5-10 mM) in the growth medium could completely alleviate osmotic stress induced by 300 mM sucrose as the doubling times of stressed cultures were similar to those of unstressed cultures (Chapter 3.2.5, Zahid *et al.* 2015).

The osmoprotective effect of mannitol on growth was also observed in 600 mM sucrose stressed cultures, but to a lesser extent (Zahid *et al.* 2015). It was found that the addition of 10 mM mannitol to the medium promoted growth of the culture as the doubling time of the cells was decreased from 3.5 to 2 h but the final optical density was lower than in cultures grown in the presence of 300 mM sucrose (Chapter 3.2.5). This effect might be due to the higher concentration of sucrose (600 mM) that results in a high osmolality shift of the growth medium and limits the activity of mannitol transporter proteins. In other bacteria (such as *E. coli*, *C. glutamicum*), the activity of compatible solute transporters (ProP, ProU, BetP) is sensitive to changes in the osmolality of the surrounding medium (Culham *et al.* 1993, Lucht and Bremer 1994). The transporter proteins require a certain threshold of external osmolality for optimal activity that could be decreased or inhibited at very high external osmolalities (Poolman and Glaasker 1998, Rübenhagen *et al.* 2000).

Similar effects were observed during growth of *Z. mobilis* under osmotic stress in the presence of exogenous compatible solutes. The addition of 50 mM sorbitol to a medium containing 1.39 M glucose resulted in a better growth of the cultures in comparison to a medium with 1.66 M glucose. With 1.39 M glucose, the cells divided without any apparent lag phase and reached a final optical density of 6. Whereas the culture without sorbitol supplementation had a lag phase of more than 72 h and a final optical density of only 2. However, at increased concentration of glucose (up to 1.66 M), no growth was observed without the addition of sorbitol. With 50 mM sorbitol, the culture grew after a lag period of 90 h and reached a final optical density of 4 (Loos *et al.* 1994). Such stimulatory effects of exogenous compatible solutes (osmoprotectants) on growth were also examined in other organisms subjected to varying osmotic stress conditions. For example, in *L. plantarum*, the addition of 0.6 M NaCl to a chemically defined medium reduced the growth rate from 0.25 h⁻¹ to 0.17 h⁻¹ and decreased the final optical density from 1.4 to 0.2. The effect of 0.6 M NaCl

was mitigated by the addition of 2 mM betaine as the growth rate and the final optical density restored to 0.22 h^{-1} and 1.0, respectively (Kets and de Bont 1994).

Osmoprotectants are known for their potential to increase the survival of cells that depends on the integrity of the macromolecular structures of the organism under denaturing stress conditions. It has been postulated that osmoprotectants stabilize cellular membranes and proteins either by water replacement or by the preferential exclusion phenomenon. In the water replacement mechanism, sugar and polyols replace or substitute water molecules between the lipid headgroups of membranes and maintain the liquid-crystalline (LC) state of biological membranes (Crowe and Crowe 1992). In the preferential exclusion process, stabilizing solutes do not bind to macromolecules; indeed, they are excluded from the water-protein interface which results in more compact folding and maintenance of the native state of proteins (Arakawa and Timasheff 1985, Timasheff 1992). This phenomenon was recently termed, the osmophobic effect (Bolen and Baskakov 2001). The present study revealed the protective effect of mannitol on the survival rate of osmotically stressed cells of *G. oxydans* (Chapter 3.2.5). Microscopic examination and SYTOX green staining indicated that mannitol enhanced the viability of the cultures (Zahid *et al.* 2015). SYTOX green is a nucleic acid binding, membrane-impermeable fluorescent dye that stains the dsDNA of nonviable cells due to their disintegrated membranes (Lebaron *et al.* 1998). In the absence of mannitol, the morphology and viability of cells stressed with 600 mM sucrose was severely compromised. Upon staining, it was found that almost 20 % of the total cells in the medium containing 600 mM sucrose were involuted and stained with the dye. However, in the presence of mannitol, the degree of deformity of the cells was greatly reduced which led to almost 98 % viability of the total cells. Although some aberrant shaped cells were still observed but they were not stained with SYTOX green indicating the intactness of the cell membranes and viability of the culture with mannitol supplementation (Chapter 3.2.5). Analogously, in lactic acid bacteria, subjected to drying conditions, the addition of mannitol enhanced the viability of dried cells (Efiuvwevwere *et al.* 1999). Sorbitol is also reported as a stabilizer of freeze-dried lactic acid bacteria during long-term storage (Carvalho *et al.* 2003). Moreover, sorbitol also acts as an osmoprotectant in eukaryotic organisms such as mammalian kidney medullary cells cultured in a hyperosmotic medium (Yancey *et al.* 1990).

As stated above, the stimulatory effect of extracellular mannitol on the growth of osmotically stressed cells of *G. oxydans* highlights the significance of an efficient transport system which helps the cells to up-take mannitol and to maintain cellular homeostasis. Richhardt *et al.* (2012) shed light on the mannitol metabolism in *G. oxydans*. Mannitol is oxidized to fructose

at the cytoplasmic membrane by the catalytic activity of the PQQ-dependent polyol dehydrogenase (Gox0854-Gox0855). Fructose is transported into the cell and is further metabolized by the pentose phosphate pathway and the Entner Doudoroff pathway. Under osmotic stress situations, fructose is probably taken up and part of this compounds is reduced to mannitol to protect cellular functions from deleterious external osmotic pressure. The PQQ-dependent polyol dehydrogenase (EC 1.1.99.22) is a versatile enzyme, which oxidizes a broad range of polyols such as arabitol, glycerol, mannitol, sorbitol, meso-erythritol etc. Analysis of the catalytic and kinetic properties have revealed that the purified enzyme catalyzed the oxidation of various polyols with comparable activities (28-50 U mg⁻¹) but with different K_M values (K_M mannitol = 113 mM, K_M arabitol = 20 mM, K_M sorbitol = 40 mM, K_M glycerol = 50 mM) (Adachi *et al.* 2001b). In the present study, mannitol was provided in concentrations of 2.5 - 10 mM which were much lower than the K_M value of the enzyme. Thus, it is tempting to speculate that mannitol was directly transported into the cell via the putative mannitol/sorbitol ABC transport system (Gox2182-Gox2185) without prior oxidation to fructose (Richhardt *et al.* 2012, Prust *et al.* 2005).

In natural ecosystems, the availability of compatible solutes is generally low; transporters are usually efficient and have a high affinity for their substrates with very low K_M values, usually in the micromolar range (Kempf and Bremer 1998). Moreover, the transport of compatible solutes is an energy-driven process, and is carried out by different sets of transport systems in microorganisms. For example, it has been shown that the transport of mannitol in *P. fluorescens* and *Phaeobacter inhibens* DSM17395 is accomplished by specific ABC transporters (Wiegmann *et al.* 2014). Whereas, in *Z. mobilis*, sorbitol is transported through an energy-dependent carrier system that follows Michaelis-Menten kinetics and uses the electrochemical ion gradient as a driving force for the transport rather than ATP. In certain other organisms, such as *E. coli*, *Bacillus subtilis*, and *Lactococcus lactis*, mannitol up-take is mediated by a mannitol-specific phosphotransferase system (PTS) (Groisillier *et al.* 2015). However, in the genome of *G. oxydans*, homologues of ECIICD transport proteins were not present (Prust *et al.* 2005); therefore, it is very unlikely that a PTS system participates in the transport of mannitol in this bacterium. In *G. oxydans*, a couple of putative sugar/polyol-dependent ABC transporters (Gox1179-1184, Gox2182-2185, Gox2219-2221) and sugar/polyol-proton symporters (*e.g.*, Gox0649, Gox0925, Gox1047, Gox1971) have been identified that might be involved in the up-take of mannitol (Prust *et al.* 2005).

4.2.5. Effect of polyols on growth and substrate oxidation rates of *G. oxydans*

Compatible solutes are of different types and widespread in all kingdoms of life. For example, glycine betaine is found in every lineage of life, whereas polyols are mostly present in terrestrial organisms that undergo desiccations or freezing conditions. Similarly, taurine is widespread in marine organisms (Yancey 2005). Due to the limited availability of these osmoprotectants in natural ecosystems, most organisms can use more than one compound as compatible solutes for the protection of cellular functions against osmotic stress. For example, growth of the Gram-positive bacterium *Lactobacillus plantarum* under osmotic stress was efficiently restored with different structurally-related quaternary compounds such as carnitine and betaine (Kets *et al.* 1994). Likewise, a variety of osmolytes can recover growth of *E. coli* under salinity stress (Nagata *et al.* 2002, Nagata *et al.* 2005). Similarly, osmoprotective effects of different polyols on the growth of *G. oxydans* were observed during cultivation under high glucose concentrations (Chapter 3.2.6). It was found that both C-6 and C-5 polyols (sorbitol, mannitol, and arabitol) equally restored normal growth rates and yields of glucose stressed cultures. From these results, it can be concluded that some osmolytes, which belong to the same chemical category, are functionally interchangeable. This could be a plausible strategy, adopted by microbes, to survive in ecological niches where the availability of a particular type of compound could be limiting under different environmental conditions.

The use of osmoprotectants in biotechnology, agriculture, molecular biology, and medicine is gaining great attention to increase product yield, treat diseases, increase efficiency of enzymatic reactions (PCR), and to engineer drought tolerant plants and microbes. Industrially important microorganisms are usually cultured under osmotically stressful conditions, imposed by high substrate concentrations, to achieve high cell densities parallel to maximum product yields (Shiloach and Fass 2005, Brown 1990, Mille *et al.* 2005). However, such stress factors significantly decrease growth yields, biocatalytic rates, and often result in mutations in the cells (Winkler *et al.* 2014, Dragosits and Mattanovich 2013, Riesenber *et al.* 1991). Therefore, the use of osmoprotectants to confer resistance to microorganisms and for catalytic robustness is essential for industrial-scale fermentations. *G. oxydans* is an industrially important organism for the production of valuable products such as gluconates, ketogluconates, vitamin C, dihydroxyacetone, 6-amino-L-sorbose, etc. (Claret *et al.* 1994, Hommel and Ahnert *et al.* 2000, Macauley *et al.* 2001, De Muynck *et al.* 2007). The increase in osmolality of fermentation media impose osmotic stress to the cultures and result in reduced growth and product formation (Gao *et al.* 2014, Mori *et al.* 1981, Luchterhand *et al.*

2015, Zahid *et al.* 2015). Hence, an increase in osmotic tolerance and the maintenance of high-cell densities are prerequisites for the synthesis of bio-based products in high amounts. In the present study, it was observed that growth and gluconate production decreased during growth of *G. oxydans* in a medium containing up to 1 M glucose (Zahid *et al.* 2015). However, the addition of trace amounts of polyols (mannitol, sorbitol, or arabitol) increased the growth rates and doubled the gluconate yield (Chapter 3.2.6). Similarly, the addition of sorbitol to the growth medium of *Z. mobilis* increased the growth rates and enhanced the fermentation capabilities of the cultures under osmotic, heat, and ethanol stress (Sootsuwan *et al.* 2013). Likewise, other short-chain polyols such as arabitol, glycerol, and sorbitol serve as compatible solutes in different microorganisms under osmotic as well as cold and oxidative stress conditions (de Barros and Celligoi 2006, Efiuvwevwere *et al.* 1999, Hocking 1988). These findings suggest that polyols, in general, protect the cellular functions and enhance the cellular catalytic efficiencies under different stress situations. Therefore, the addition of polyols to growth media during fermentation processes could be a suitable option to increase the cell densities and the yield of bio-based products. Such an increase in the biocatalytic potential of osmotically stressed cultures will definitely reduce the cost of fermentation processes for efficient production of valuable substances. For example, gluconate, produced at a rate of 10,000 tons per annum, has wide applications in many industries such as food and pharmaceutical industries (Lichtenthaler 2006, Prescott *et al.* 1953). As shown in this thesis, the production rate of gluconate can be increased by the supplementation of polyols in small amounts in the fermentation media. Thus, the information regarding the role of polyols (*e.g.*, mannitol) as osmoprotectant solutes in *G. oxydans* could be important to understand the physiology of the organism and to improve its catalytic robustness during fermentation processes.

4.3. Biosynthesis of mannitol in *G. oxydans*

G. oxydans is a strict aerobic microorganism and performs oxidative fermentation. During this process, a great variety of substrates (sugars, alcohols, and polyols) are incompletely oxidized by membrane-bound dehydrogenases (Deppenmeier *et al.* 2002). The oxidized products are released into the periplasmic space and are further excreted into the medium. Only very low amounts of the substrates are transported into the cells and are utilized for biomass production. Thus mannitol is not produced from primary metabolism and it is not an end product of fermentative processes. Moreover, mannitol is not a product of glucose consumption in the periplasm or an intermediate of the general catabolism in the cytoplasm

(Richhardt *et al.* 2013b, Hanke *et al.* 2013). Thus, it is obvious that mannitol is produced as a secondary metabolite in the cytoplasm.

In other organisms, mannitol is produced either by the reduction of fructose-6-phosphate to mannitol-1-phosphate by a mannitol-1-phosphate dehydrogenase or by direct reduction of fructose to mannitol via a mannitol-2-dehydrogenase (Wisselink *et al.* 2002, Saha 2004, Saha and Racine 2008, Saha and Racine 2011, Sand *et al.* 2015). Hence, fructose or fructose-6-phosphate is probably the direct precursor for mannitol biosynthesis in *G. oxydans* under osmotic stress. Intracellularly, fructose-6-phosphate could be formed from glucose by two different routes (Fig. 40). In the first pathway, glucose is converted to fructose-6-phosphate in two steps (Reaction 1 and 10, Fig. 40) by the catalytic activities of glucokinases (Gox1182, Gox2419) and the glucose-6-phosphate isomerase (Gox1704). Moreover, the production of fructose-6-phosphate is possible through the oxidative pentose phosphate pathway (Reactions 2-9, Fig. 40) (Richhardt *et al.* 2013b, Rauch *et al.* 2010). In this metabolic pathway, intracellular glucose is firstly oxidized to gluconate by a glucose dehydrogenase (Gox2015), and then phosphorylated to 6-phosphogluconate by the catalytic activity of a gluconokinase (Gox1709). In addition, the production of 6-phosphogluconate is also possible through the direct oxidation of glucose-6-phosphate, carried out by a glucose-6-phosphate dehydrogenase (Gox0145), which is then channeled into the oxidative pentose phosphate pathway.

In the PPP, 6-phosphogluconate is decarboxylated by a 6-phosphogluconate dehydrogenase (Gox1705) to yield ribulose-5-phosphate. This conversion is followed by an interwoven cascade of enzymatic reactions (epimerization, isomerization, transketolation, and transaldolation) which result in the production of fructose-6-phosphate from the precursor molecule ribulose-5-phosphate (Reactions 6-9, Fig. 40). In such ways, a sufficient amount of fructose-6-phosphate is generated inside the cell. Under osmotic stress conditions, fructose-6-phosphate might undergo a dephosphorylation process to form free fructose (Reaction 11, Fig. 40). This reaction is probably carried out by a still unknown phosphatase. Possible candidates are Gox1036, Gox1190 or Gox2002. As shown in this thesis, free fructose is then reduced to mannitol via the fructose reductases Gox0849 or Gox1432 (Reaction 12, Fig. 40). Another pathway for the production of fructose is the direct conversion of intracellular glucose by the catalytic activity of glucose isomerases (EC 5.3.1.5) as present in some fungi (Sayyed *et al.* 2010). However, the genes encoding such isomerases are not present in the genome of *G. oxydans* (Prust *et al.* 2005).

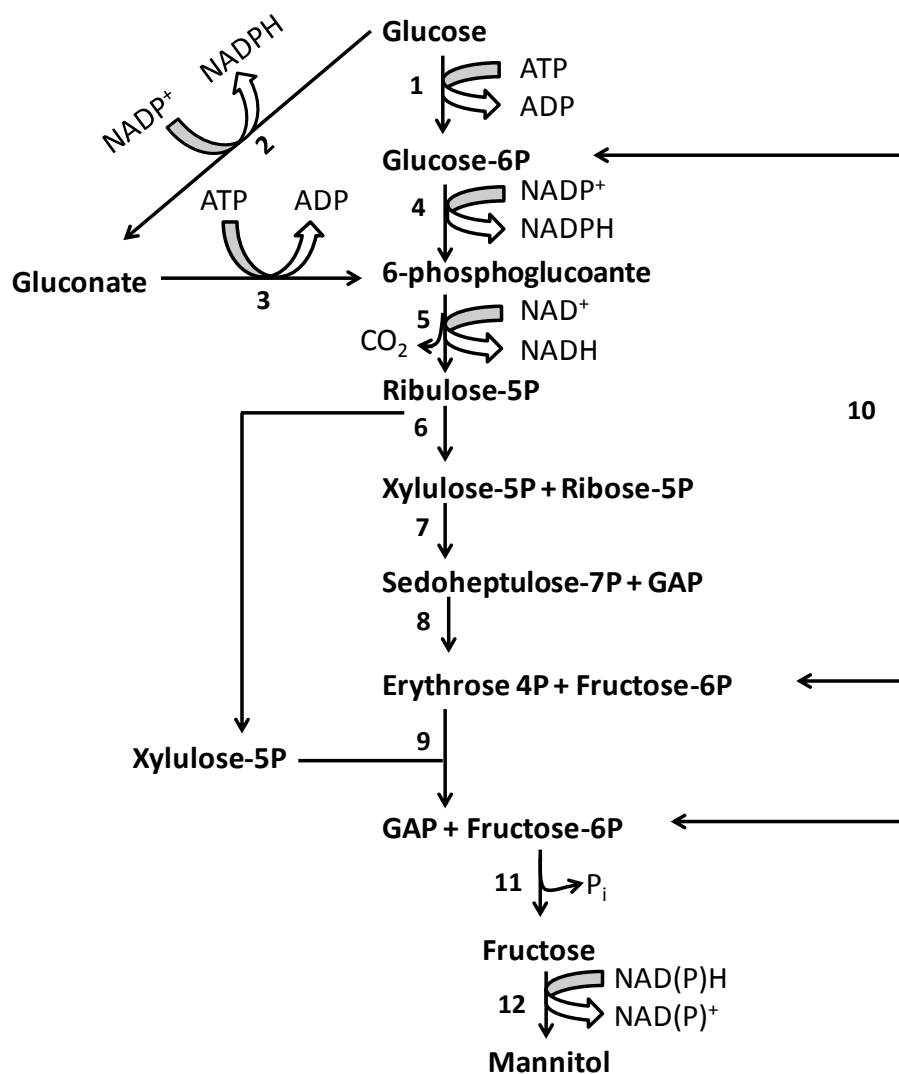


Figure 40: Intracellular routes for the synthesis of mannitol from glucose in *G. oxydans*. P = phosphate, GAP = glyceraldehyde phosphate. (1) glucokinase (Gox2419, Gox1182), (2) glucose dehydrogenase (Gox2015), (3) gluconokinase (Gox1709), (4) glucose-6-phosphate dehydrogenase (Gox0145), (5) 6-phosphogluconate dehydrogenase (Gox1705), (6) ribulose-phosphate-3-epimerase (Gox1352) and ribose-5-phosphate isomerase (Gox1708), (7) transketolase (Gox1703), (8) transaldolase (Gox1704), (9) transketolase (Gox1703), (10) glucose-6-phosphate isomerase (Gox1704), (11) unknown phosphatase, (12) fructose reductase (Gox0849 or Gox1432). (Modified from Deppenmeier and Ehrenreich 2009).

In *Gluconobacter* species, four different types of polyol dehydrogenases were identified that utilize either mannitol or fructose as substrates. These enzymes can be differentiated from each other with respect to their molecular masses, cofactor specificity, and cellular localization (Zahid and Deppenmeier 2016). Type 1 are the membrane-bound polyol dehydrogenases that require PQQ as cofactor for catalysis. These enzymes have different

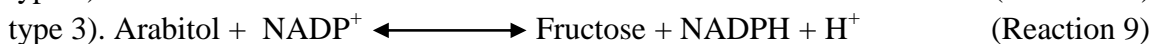
molecular masses in different *Gluconobacter* strains and perform incomplete oxidations of a variety of sugars and alcohols (Sugisawa and Hoshino 2002). For example, the enzyme (Gox0854-0855) from *G. oxydans* 621H has two subunits of 80 and 14 kDa and catalyzes the oxidation of polyols and aldoses (mannitol, glycerol, sorbitol, arabitol, ribitol, erythrose, threose, ribose, lyxose, glyceraldehyde) as well as secondary alcohols (isopropanol, 2-hexanol) and diols (1,2-pentanediol) (Peters *et al.* 2013a). This enzyme is also responsible for the production of L-sorbose (an intermediate in vitamin C production) by the oxidation of D-sorbitol (Matsushita *et al.* 2003, Hoshino *et al.* 2003). The following equations represent some reactions catalyzed by such type of enzymes:



Type 2 enzymes comprise NADP⁺-dependent mannitol dehydrogenases (EC 1.1.1.138) that consist of a single subunit of 50 kDa and catalyze the reversible oxidation of only mannitol to fructose (Reaction 7, Adachi *et al.* 1999a).



Type 3 proteins are NADP⁺-dependent mannitol dehydrogenases with molecular masses of 50 kDa per single subunit and catalyze the reversible oxidation of different polyols such as mannitol, arabitol, and sorbitol (Sugisawa *et al.* 1991, Klasen 1994, Shibata *et al.* 2000, Shinjoh *et al.* 2002). These proteins are named D-sorbitol dehydrogenase in *G. oxydans* G624, L-sorbose reductase in *G. melanogenus* N44-1 and *G. suboxydans* IFO 3291, and polyol: NADP⁺-5-oxidoreductase in *G. oxydans* DSM3503. According to the molecular mass, the number of subunits, and the homology of the amino acid sequence, it was found that Gox1432 of *G. oxydans* 621H belongs to this group of mannitol dehydrogenase. Two examples of reactions catalyzed by this group of mannitol dehydrogenase are shown below:



Type 4 represent NAD⁺-dependent mannitol dehydrogenases (EC 1.1.1.67) that were found in the cell extracts of *G. oxydans* LMG 1489 (Parmentier *et al.* 2005) and *G. suboxydans* IFO 12528 (Adachi *et al.* 1999a). These mannitol dehydrogenases have a molecular mass of 130 kDa as reported by Adachi *et al.* (1999a). In the present study, the first enzyme belonging to type 4 was purified which is encoded by gene *gox0849*. This enzyme catalyzes the following reaction:



The present study depicted that both purified enzymes (Gox1432 and Gox0849) from *G. oxydans* 621H catalyze the reversible reduction of fructose to mannitol with comparable catalytic efficiencies at pH 7.0 (Chapter 3.3.2). Gox1432 requires NADPH as cofactor for catalysis while Gox0849 prefers NADH. Besides the reduction of D-fructose, both purified enzymes were also capable of efficient oxidation of mannitol but at non-physiological alkaline conditions. The pH of the cell cytoplasm is usually around 7.0, which in case of *G. oxydans* is optimal for the reduction of fructose rather than the oxidation of mannitol. It was found that at cellular pH (7), the oxidative activities of both purified enzymes decreased more than three-fold indicating that the proteins work intracellularly as reductases and can be classified as fructose reductases instead of mannitol dehydrogenases (Zahid and Deppenmeier 2016).

For the elucidation of the intracellular routes used by *G. oxydans* for mannitol synthesis, the activity of both purified proteins was also tested with fructose-6-phosphate. However, it was found that none of the proteins could reduce fructose-6-phosphate to mannitol-1-phosphate. Therefore, in the context of mannitol biosynthesis, it is proposed that fructose is the precursor for mannitol synthesis, produced by dephosphorylation of fructose-6-phosphate (Fig. 40). The enzyme responsible for such a dephosphorylation is yet unknown. Sand *et al.* (2015) had identified a unique bifunctional enzyme mannitol-1-phosphate dehydrogenase/phosphatase that is responsible for production of mannitol in *A. baylyi* under osmotic stress conditions. This unique enzyme has a N-terminal haloacid dehalogenase (HAD) superfamily like domain that catalyzes the dephosphorylation of mannitol-1-phosphate to mannitol. The C-terminal domain is responsible for fructose reduction. A close inspection of the genome of *G. oxydans* revealed that the amino acid sequence of protein Gox1036 shares 23-25 % homology with the HAD-like domain of mannitol-1-phosphate dehydrogenases from *A. baylyi* (ACIAD1672) and *P. putida* (PP_2052) (Fig. 41). Moreover, sequence alignment indicates that despite having a high sequence divergence, the members of the HAD superfamily share four conserved sequence motifs that constitute a main catalytic scaffold for the transfer of the phosphoryl group. These include the motifs: DXD (motif 1), T/S (motif 2), K/R (motif 3), and E/DD (motif 4) (Caparros-Martin *et al.* 2013). On the basis of the presence of the conserved motifs, it is tempting to speculate that Gox1036 might serve as a potential phosphatase during mannitol biosynthesis in *G. oxydans* 621H. However, further studies are required to confirm the phosphatase activity of this enzyme.

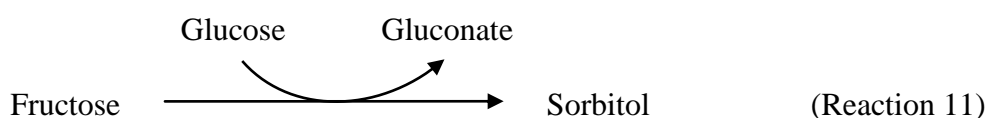
			motif 1		
GOX1036	MTTALDPAGQLKLVIFDCDGV	LVLDSEGPS	SCRATAEFAR	-SKGLSISDDEAHKRFAGKALP	59
PP_2052	--MLFFHGKQIFSAIFDMDG	TLFDTERLRFR	TLKQASLEIFGKALSEQTLIGSL	-GLSAK	57
ACIAD1672	--MLIFHNSVQGALFDM	DTMFDTERLR	FTLQKQASQELLGQTFSDDYLMQCL	-GLSAT	57
	:	:	..** **..*:*	:: : : * :*:: : * :	
			motif 2		
GOX1036	QIVTELEQELGHS	LPEETAFLKREN	-LVQMMRKS	AEP-VSGALEMLAGV	RALGMPVRVGS 117
PP_2052	KAEALAKAHNGED	FPYAQIRQRADE	LELEYVRNHGVP	IKAGLLEVLERLRK	SGLTMAVAT 117
ACIAD1672	TAEQLAKSQYGDD	VPKAIRKCADD	LELEWVRRD	GVP IKKGLVQVLER	LRKSGLRMAVAT 117
	:	.	*...*	: :: ::* . * * :*:* :* *:: :* :	
			motif 3	motif 4	
GOX1036	NSSVREMEAKFKRT	GMSQYFPENRI	HSANDMGCP	KPAPDVVLYAAKA	ESVLPENCVVIED 177
PP_2052	SSRRAIAEEYLIN	ANVLKYFD--IT	VCGEVGGQKPH	EIFLKAARALNCE	PGQCFMVED 175
ACIAD1672	SSRRAIAEEYLIN	ANVYKFFD--V	LVCGDEVQGGK	PHPEIFEKAAQKL	NLDPAQCLMFED 175
	.*	*	: .: : :*	::* *::: ** : . * :*::**	
GOX1036	SNTGAEAAWKAG	MACVLLRPDD	VPLPPFPVEG	-----SVRITHL	DELVPLLRRTLESQ 231
PP_2052	SENGMLSAMRAE	GQAILIEDIK	PPAPEIKAGAL	KAYRSMHEFLAD	LSECVPDLGMPALSE 235
ACIAD1672	SENGISSAHAAG	GITILLKDIK	SPNEHMLSKAN	FYFETMYDCLLN	LDDYVPIMDMPLIQE 235
	:.	:* *	:*:. . *	:	: .*:: ** : .:
GOX1036	KHA-----				234
PP_2052	PPFASMNQF	SVGIHGFGA	IGGGYLTQVF	SHWDGYTRPRE	IIAATRSRMLRESVSAFGYS 295
ACIAD1672	TFPQTLNQL	TVGIHGFGA	IGGGYIAQIL	SHWDGYTRPQR	IIASTRNSLYREAVNAFGYS 295

Figure 41: Conserved sequence motifs of HAD-like domains from different organisms. Multiple sequence alignment of the putative phosphatase (Gox1036) from *G. oxydans* 621H, the N-terminal domains of mannitol-1-phosphate dehydrogenases/phosphatases from *A. baylyi* (ACIAD1672) and *P. putida* (PP_2052). Identical or similar amino acid residues are indicated with asterisks and dots, respectively. The conserved motifs of HAD domains are marked in yellow.

4.4. Characterization of the relative contribution of Gox1432 and Gox0849 in cellular osmoprotection

For the resolution of the relative contribution of the two fructose reductases for mannitol biosynthesis and cellular osmoprotection, expression levels of the corresponding genes (*gox1432* and *gox0849*) were compared (Chapter 3.3.3). It was found that both genes were constitutively transcribed as their expression levels were not affected by changes in osmolality of the growth media. However, the transcript abundance of gene *gox1432* was much higher in comparison to gene *gox0849* (Zahid and Deppenmeier 2016). These results depicted that Gox1432 is of major importance for the production of mannitol and the protection of the cell against osmotic stress. Evidences in favor of this hypothesis came from the test of activities of the NADPH-dependent fructose reductase (Gox1432) in the cell cytoplasm, which were four-fold higher than the activity of the NADH-dependent enzyme (Gox0849) (Chapter 3.3.4.2). Moreover, the activity of the NADPH-dependent fructose reductase within the cells was almost 2.5-fold higher in vivo under osmotic stress whereas the

activity of the NADH-specific enzyme remained unchanged (Zahid and Deppenmeier 2016). This effect could be due to the K_M value (56 mM) of Gox1432 for fructose which is higher than that of other orthologous enzymes such as 0.24 mM in *P. fluorescens* and 16.3 mM in *R. sphaeroides* (Klimacek and Nidetzky 2010, Schneider and Giffhorn 1989). In the current study, it was shown that besides mannitol, fructose concentrations progressively increased in the cellular extracts with the increase in external sucrose concentrations (Chapter 3.2.3). Therefore, such an increase in free fructose concentration under osmotic stress might regulate the activity of Gox1432. Similarly, the osmodependent synthesis of sorbitol in stressed cells of *Z. mobilis* is carried out by an enzyme referred to as glucose-fructose oxidoreductase (GFOR, EC 1.1.99.-). This enzyme requires the presence of both glucose and fructose for sorbitol formation (Barrow *et al.* 1984). During catalysis, the reduction of fructose to sorbitol is accompanied by the oxidation of glucose to gluconate (Leigh *et al.* 1984). The enzyme GFOR catalyzes the following reaction:



The affinity of GFOR for fructose is very low with a K_M of 400 mM to 1 M (Hardman and Scopes 1988, Zachariou and Scopes 1986). Hence, the formation of sorbitol is accomplished at considerable rates only during cultivation of the organism with high concentrations of sugars (Loos *et al.* 1994, Nidetzky *et al.* 1997). Normally, the intracellular availability of free fructose is limited because of fructokinases that have a very high affinity for free fructose (K_M in micromolar ranges) and channel the substrate into the main cellular metabolic pathways after phosphorylation. For example, in *Z. mobilis*, the K_M value of the fructokinase (EC 2.7.1.4-) for fructose is 70 μM , which is very low in comparison to the K_M value of the GFOR for fructose (Doelle 1982). Therefore, under normal growth conditions, the synthesis of sorbitol cannot be accomplished. However, under osmotic stress conditions, fructose was accumulated by growing cells due to the inhibition of the fructokinase. Hence, fructose could be reduced to sorbitol by the GFOR oxidoreductase (Barrow *et al.* 1984). Moreover, in the polyol producer yeast *Candida zeylanoides*, a change of the activity of transketolase, an enzyme of the pentose phosphate pathway, resulted in an alteration from erythritol to mannitol fermentation due to variations in the levels of erythrose-4-phosphate and fructose-6-phosphate (Hattori and Suzuki 1975). Similarly, homofermentative lactic acid bacteria produce small amounts of mannitol during growth on glucose. Mannitol is formed by reduction of the glycolytic intermediate fructose-6-phosphate to mannitol-1-phosphate and

finally to mannitol by the catalytic activities of the mannitol-1-phosphate dehydrogenase (MPDH) and phosphatase (MPase), respectively (Loesche and Kornman 1976, Bhatt *et al.* 2013). It has been shown that an alteration of the pattern of glycolytic end products, by the disruption of lactate dehydrogenase (LDH) encoding genes, resulted in an increased expression of MPDH and MPase. Thus the LDH-deficient strains accumulate mannitol as an end product of glucose metabolism (Ferain *et al.* 1996, Neves *et al.* 2000, Gaspar *et al.* 2004). The PPP is the main route for growth of *G. oxydans* (Olijve and Kok 1979, Richhardt *et al.* 2013b) which is negatively affected by the increase in osmolality of the growth medium. It is tempting to speculate that in *G. oxydans*, osmotic stress might affect the activity of the PPP which results in a reduced proliferation of the cells and an accumulation of fructose that is further reduced to mannitol (osmoprotectant) by fructose reductases (Zahid and Deppenmeier 2016). These results might also explain the survival of the organism in sugar rich habitats such as honey, fruit saps, nectars. In these sugary niches, surplus amount of free fructose might be available to the cells to form mannitol in order to counteract the high-sugar stress.

4.5. Characterization of fructose reductase deletion mutants

The importance of the fructose reductases (Gox1432 and Gox0849) for *G. oxydans* became even more evident by analyzing the growth pattern and the compatible solute accumulation of the corresponding deletion mutants. Under osmotic stress conditions, the deletion of *gox1432* resulted in a 37 and 45 % reduction of the growth rate and the final optical density of the mutant culture in comparison to the wild type strain, respectively. In addition, NADPH-dependent fructose reduction was completely abolished in the cytoplasm of the Δ *gox1432* strain. However, the NADH-dependent reduction of fructose was unaltered. These results clearly indicated that Gox1432 is the sole enzyme, catalyzing the intracellular NADPH-specific reduction of D-fructose. Likewise, the intracellular mannitol concentration was decreased by 60 % in osmotically stressed Δ *gox1432* cells, depicting the significance of Gox1432 for the osmoadaptation of *G. oxydans*. Concordant effects were observed in *Z. mobilis* when the GFOR encoding gene (*gfo*) was disrupted, which catalyzes the synthesis of the osmoprotectant sorbitol under osmotic stress conditions. The cells became osmosensitive and had decreased growth rates from 0.18 (without stress) to 0.08 h⁻¹ in high-sugar media containing 300 g L⁻¹ sucrose. The defect in growth of the *Z. mobilis* Δ *gfo* strain was completely rescued by complementation of the *gfo* gene or by the addition of the osmolyte to the sugar-rich media (Sootsuwan *et al.* 2013). In a similar manner, supplementation of low amounts of mannitol to high-sugar media or complementation with the full length gene

gox1432 led to complete recovery of the *G. oxydans* Δ *gox1432* strain from osmoinduced growth defects (Chapter 3.3.4.1).

In contrast to the Δ *gox1432* strain, deletion of the gene *gox0849* had no impact on the growth of *G. oxydans* as the doubling time and the final optical density of the mutant culture were comparable to the wild type culture under osmotic stress conditions (Chapter 3.3.4.1). Only the intracellular mannitol content was slightly reduced (1.3-fold) in comparison to the wild type cells (Chapter 3.3.4.2), which indicated a minor participation of the gene *gox0849* in the osmoprotection of *G. oxydans* (Zahid and Deppenmeier 2016). Moreover, the deletion of the gene *gox0849* did not result in a complete loss of the NADH-dependent D-fructose reduction activity in cellular extracts of the stressed and non-stressed cultures. The results clearly depict the presence of another, yet unknown, soluble fructose reductase with low catalytic activity. These results were further supported by the analysis of the double deletion mutant strain (*G. oxydans* Δ *gox1432* Δ *gox0849*) which revealed the same residual potential for NADH-mediated reduction of D-fructose (Chapter 3.3.4.2) under stress and non-stress conditions. However, the third unknown fructose reductase could not enable the double deletion mutant to completely restore its cellular turgor and to proliferate under osmotic stress. The culture had a long lag phase and reached an optical density of only 0.4, which was 50 % of the parental strain. Moreover, the intracellular mannitol content was also reduced by 70 % in the double deletion strain. The defect in the growth pattern as well as the reduction of the internal mannitol content in the double deletion strain strengthen the notion that fructose reductase Gox1432 is a key player in the osmoprotection of *G. oxydans* (Zahid and Deppenmeier 2016). As stated above, *G. oxydans* contains an unknown enzyme of limited catalytic activity for the reduction of fructose to mannitol. Sugiyama *et al.* (2003) had reported that the NAD⁺-specific xylitol dehydrogenase (Gox0865) from *G. oxydans* ATCC 621 can oxidize mannitol, but the oxidative activity is less than 1 % compared to the rate of xylitol oxidation (113 U mg⁻¹). Unfortunately, information is not available regarding the reductive activity of the enzyme with fructose as substrate. However, it is tempting to speculate that this xylitol dehydrogenase may reduce fructose to mannitol with the same weak catalytic efficiency and might serve as a third fructose reductase in *G. oxydans*.

4.6. Metabolic engineering of *G. oxydans* for enhanced osmotolerance

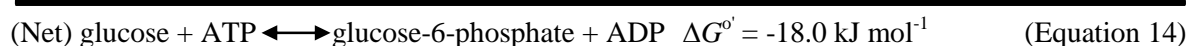
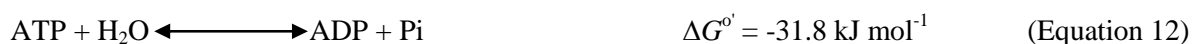
G. oxydans is often subjected to osmotic stress conditions during industrial fermentation processes. For example, the organism is routinely cultivated in fed-batch cultures for the volumetric production of industrially important compounds. However, during such

cultivations, cells are often exposed to osmotic stress conditions due to the presence of high amounts of substrates or products that result in substantial reduction in growth and product yield (Mori *et al.* 1981, Gao *et al.* 2014, Zahid *et al.* 2015). The present study depicted that mannitol is an effective protectant during cultivation of the organism under these hostile conditions. Hence, it was proposed that the osmotolerance of the organism may be enhanced by increasing intracellular mannitol biosynthesis. The strain *G. oxydans* 621H was amenable to genetic alterations due to the availability of the complete genome sequence and the availability of genetic tools for protein expression and the generation of deletion mutants. Therefore, this strain was considered as suitable model organism for metabolic engineering approaches. To achieve an increased mannitol synthesis in the organism, two different strategies were attempted as described in chapter 3.4.

4.6.1. Overproduction of D-fructose reductase (Gox1432)

Among two different fructose reductases, it was found that Gox1432 plays a central role for the intracellular mannitol formation and osmotolerance of *G. oxydans* (Zahid and Deppenmeier 2016). Therefore, the production of the enzyme was increased inside the cell by homologous overexpression of the gene *gox1432*. It was assumed that the overproduction of Gox1432 might result in an increase in the carbon flow from glucose to mannitol formation. Growth studies indicated that the overproduction of Gox1432 was not sufficient to direct the hexose flux to mannitol synthesis as the growth parameters of the overexpression strain were comparable to the wild type during cultivation on YGP_{high} medium with 500 mM glucose as carbon source and osmoticum (Chapter 3.4.1). However, substantial improvement of the growth parameters was observed when glucose was replaced with fructose in the growth medium (Chapter 3.4.1). These results indicated that during glucose metabolism, the intracellular level of fructose-6-phosphate might be very low and hamper efficient mannitol biosynthesis. The major part of fructose-6-phosphate was probably directed towards the oxidative pentose phosphate pathway leading to a reduced supply for mannitol biosynthesis. However, in the presence of fructose in the medium, cells might have a sufficient supply of intracellular fructose that could be directly reduced to mannitol by the enzyme Gox1432. Hence, it became evident that an alternative strategy to increase mannitol production during glucose metabolism is the increase of the intracellular fructose concentration. This could be achieved by the overproduction of either a putative phosphatase for dephosphorylation of fructose-6-phosphate to free fructose or of a glucose isomerase for the direct conversion of glucose to fructose. From a thermodynamic point of view, the isomerization of glucose to fructose was a suitable option to increase the carbon flux from glucose to mannitol

production. The bypass reaction could contribute to save cellular energy expenditure, spent on the activation of glucose for intracellular assimilation. Phosphorylation of glucose is an endergonic process with ΔG° of 13.8 kJ mol^{-1} (Equation 13, Cornish-Bowden 1981). Intracellular phosphorylation of glucose is coupled with the hydrolysis of one phosphoanhydride bond of ATP. Under standard conditions, hydrolysis of ATP to ADP and inorganic phosphate results in the release of $-31.8 \text{ kJ mol}^{-1}$ energy (Equation 12, Thauer *et al.* 1977, Mayer and Müller 2014). This free energy change of the hydrolysis of ATP is higher than that required for the phosphorylation of glucose, and results in the net change in free energy of $-18.0 \text{ kJ mol}^{-1}$ (Equation 14).



Glucose-6-phosphate is further isomerized to fructose-6-phosphate, which in *G. oxydans*, should partly undergo dephosphorylation to yield a sufficient amount of free fructose for mannitol biosynthesis under osmotic stress (Fig. 40). Overall, one ATP equivalent is probably consumed during conversion of one mole glucose to one mole of fructose. The net loss of one ATP equivalent could be saved by direct conversion of glucose to fructose. In such context, the overproduction of glucose isomerases in *G. oxydans* should be a rational strategy.

4.6.2. Heterologous overproduction of glucose isomerases in *G. oxydans*

Xylose isomerases catalyze the reversible isomerization of C-5 or C-6 aldo-sugars like xylose and glucose to their corresponding keto-sugars xylulose and fructose (Fig. 42). These enzymes have a much higher affinity for xylose than for glucose (Bhosale *et al.* 1996, Batt *et al.* 1990). Despite having a low affinity for glucose, glucose isomerases are used in industry for the production of high fructose corn syrups from corn starch hydrolysates. They are commercially available under various trade names such as Sweetzyme (from *B. coagulans*) of Novo Nordisc industries, Maxazyme and Ketozyme (from *Actinoplanes missouriensis*) of Gist Brocades N V and Universal Oil Products, respectively (Verhoff *et al.* 1985).

In the present study, two different glucose isomerase encoding genes *xylA* from *E. coli* (b3565) and *S. griseus* (SGR_RS05195) were cloned into the broad host range vector pBBR.p264.ST and were heterologously overexpressed in *G. oxydans*. Both proteins were successfully produced and purified to apparent homogeneity from cellular extracts of *G.*

oxydans strains expressing the *xylA* genes (Chapter 3.4.2). Activity staining indicated that the recombinant proteins could catalyze the isomerization of xylose to xylulose (Chapter 3.4.2). However, the overexpression of both glucose isomerases in *G. oxydans* did not result in an improvement of growth parameters of the organism during cultivation under osmotic stress conditions (Chapter 3.4.2). This might be due to the fact that the enzymes are highly active only at high temperatures (optimum temperatures above 50°C) and have high K_M values for free glucose with poor catalytic efficiency. For example, the glucose isomerases from *E. coli* K12, *Streptomyces viridobrunneus* and *Streptomyces* sp. 32 had K_M values of 500 mM, 178 mM, and 288 mM, for glucose respectively (Rozanov *et al.* 2009, Sapunova *et al.* 1999). However, glucose is usually not present in such a high concentration inside the cell. Mostly glucose is phosphorylated and fed into the intracellular metabolic pathways such as glycolysis. Phosphorylation is carried out by the phosphotransferase system as well as by the cytoplasmic glucokinases (Postma and Lengeler 1985, Bramley and Kornberg 1987, Meyer *et al.* 1997). The enzymes catalyzing such phosphorylations require very low glucose concentrations (K_M in micromolar range) for activation. For example, the glucokinases from *E. coli* has a K_M of 150-780 μ M for free glucose (Arora and Pedersen 1995, Meyer *et al.* 1997). On the basis of these facts, it is evident that the glucose isomerases, produced in *G. oxydans*, might be inactive in the absence of sufficient amount of substrates. Furthermore, it was found that the cytoplasm of the glucose isomerase producing strains of *G. oxydans* were devoid of glucose isomerase activity when tested in vitro. Due to the lack of stimulatory effects of the heterologously produced glucose isomerases on the osmotolerance of *G. oxydans*, further studies were not carried out with these mutant strains.

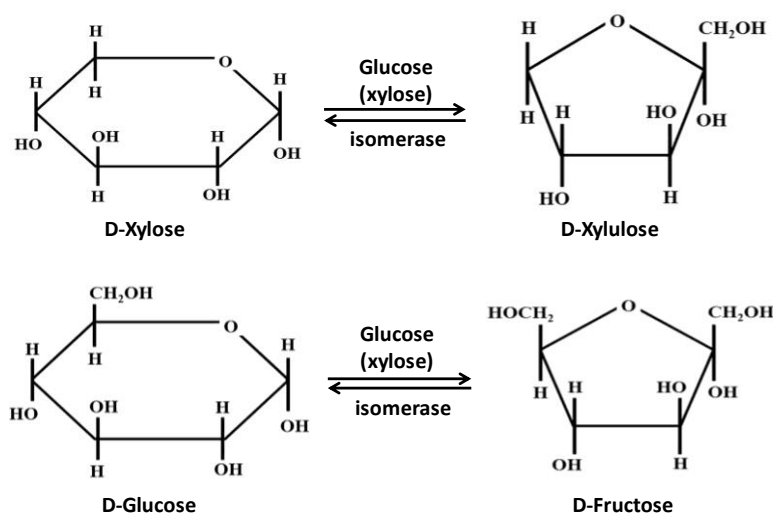


Fig. 42: Isomerization of xylose and glucose to xylulose and fructose by glucose (xylose) isomerase, respectively. (Modified from Cho *et al.* 2013).

4.7. Gox1432: key player for osmotolerance of *G. oxydans*

It was found in the course of this work that the NADPH-dependent fructose reductase Gox1432 is pivotal for an efficient proliferation of *G. oxydans* under osmotic stress. However, studies on the orthologous enzymes in different *Gluconobacter* strains revealed that Gox1432 is essential for the utilization of partially oxidized sugar derivatives such as L-sorbose and 5-ketogluconate (Sugisawa *et al.* 1990, Sugisawa *et al.* 1991, Shinjoh *et al.* 2002, Soemphol *et al.* 2007). The disruption of the orthologous gene of *gox1432* in *G. suboxydans* IFO 3291 and *G. frateurii* THD 32 resulted in a growth defect of the cultures in media containing 500 mM L-sorbose (Shinjoh *et al.* 2002). In *G. oxydans* DSM 3503, the enzyme catalyzes the reduction of 5-ketogluconate to gluconate (Klasen 1994) which can be used as carbon- and energy source. Analogously, it was found that the *G. oxydans* Δ *gox1432* strain was not able to grow in the presence of L-sorbose or 5-ketogluconate (data not shown). These observations led to the speculation that Gox1432 participates in intracellular reduction of partially oxidized products that are formed by the membrane-bound polyol dehydrogenase (Gox0854-0855) (Fig. 43). When the major substrates (sorbitol or gluconate) are consumed, the corresponding oxidized products are taken up by the cells and are reduced by the catalytic activity of Gox1432. These reduced compounds are channeled into the central metabolic pathways (the PPP or the EDP). L-sorbose is reduced to D-sorbitol that is converted to fructose and finally phosphorylated to fructose-6-phosphate by the putative sugar kinase (Gox0284) (Fig. 3, Shinjoh *et al.* 2002, Richhardt *et al.* 2012). Likewise, 5-ketogluconate is reduced to gluconate and phosphorylated to 6-phosphogluconate by a gluconokinase (Gox1709) (Rauch *et al.* 2010, Richhardt *et al.* 2013b). Both, fructose-6-phosphate and 6-phosphogluconate, are then catabolized in the pentose phosphate pathway. Thus, the present study revealed that Gox1432 has a dual physiological role in *G. oxydans*. On one side, it protects the cell from osmotic up-shock by participating in the synthesis of the osmoprotectant mannitol; whereas, on other side, it enables the cell to assimilate various oxidized substrates. Hence, it is suggested that Gox1432 is the key player for the survival of the organism in sugar-rich habitats and in mixed microbial populations. This information is especially useful for designing strategies to engineer *G. oxydans* strains with enhanced osmotolerance for bio-based industrial applications.

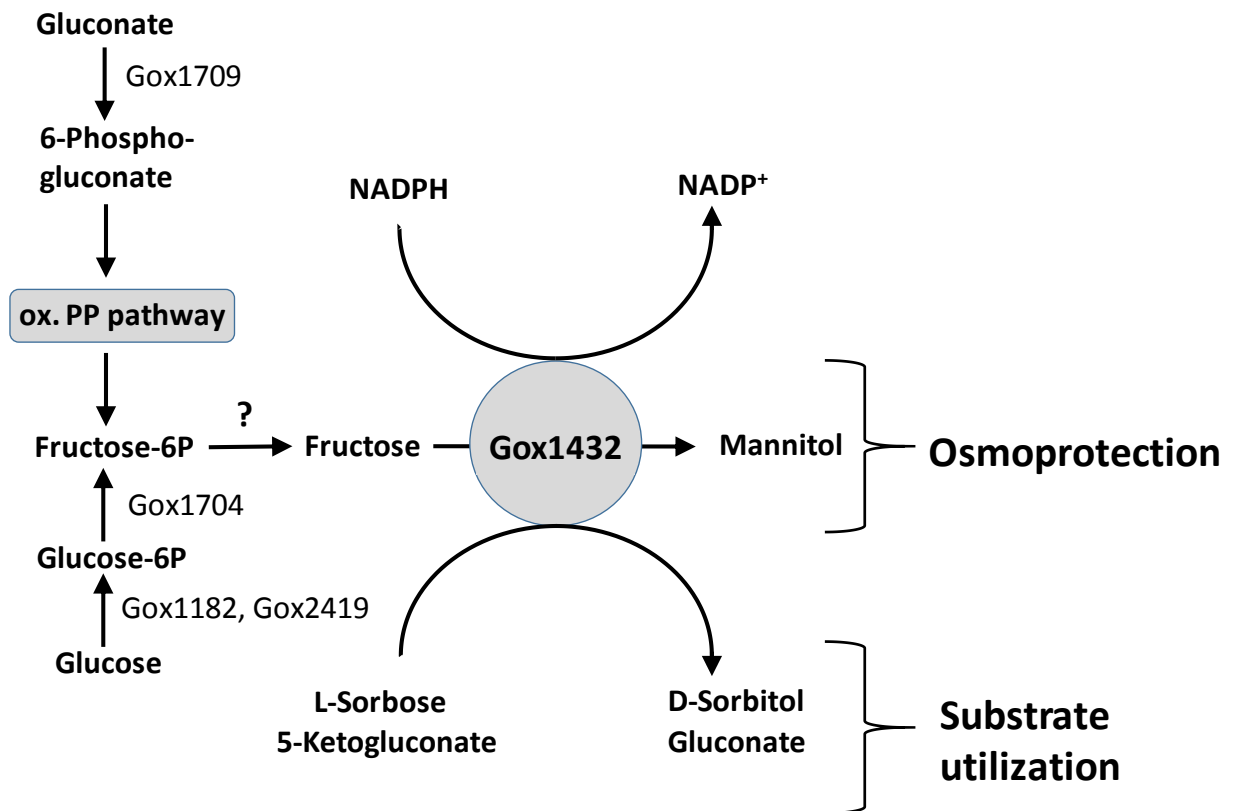


Figure 43: Function of Gox1432 and putative pathways of mannitol production. Question mark indicates an unknown phosphatase. ox PP pathway: oxidative pentose phosphate pathway. (Zahid and Deppenmeier 2016).

5. SUMMARY

G. oxydans is commercially used for the oxidative fermentation of various sugars, sugar acids, and alcohols. The oxidation of substrates, catalyzed by the organism, is generally incomplete, stereo- and regiospecific. Moreover, the resulting biotechnologically valuable products are almost completely excreted into the surrounding medium. Due to these unique metabolic features, the bacterium has been exploited in industry for decades, and has been under extensive research for many years. However, the question regarding the response of the organism to osmotic stress has not yet been investigated. Therefore, the current study was conducted to explore the mechanisms of osmoprotection in *G. oxydans*.

One common strategy to cope with osmotic stress is the intracellular accumulation of compatible solutes. For the identification of the presence of compatible solute in *G. oxydans*, total cellular metabolites were extracted from control and osmotic stress cultures and analyzed by ^{13}C NMR spectroscopy and HPLC. The analysis of the total intracellular metabolites revealed that *G. oxydans* accumulated mannitol as compatible solute in response to osmotic stress to a maximum concentration of $2.7 \mu\text{mol mg}^{-1}$ total cell protein. The intracellular mannitol concentration varied and depended on the type of osmoticum and the carbon source in the growth medium. The osmoprotective role of mannitol was further investigated by the addition of this polyol in small amounts to osmotically stressed cultures. It was found that in the presence of external mannitol, the growth rates of the stressed cultures were comparable to the control culture. In addition, the viability of stressed culture was increased in the presence of external mannitol. Thus, it was concluded that mannitol assisted the cells to alleviate osmotic stress and to maintain their proliferation rates. Another line of evidence came from analyzing the stimulatory effects of polyols on the cellular catalytic efficiencies. Addition of small amounts of polyols (sorbitol, arabitol, or mannitol) to YGP_{high} media (containing 1 M glucose) not only improved the cellular growth, but also doubled the rate of glucose consumption and gluconate formation. Gluconate, for example is a biotechnologically desirable product which is synthesized at the rate of 10,000 ton per annum. Thus, the increment of the product formation rate of *G. oxydans* upon addition of minute amounts of polyols to high-sugar containing fermentation media is important for biotechnological applications. Hence, the finding of the present study that polyols especially mannitol function as compatible solute in *G. oxydans* could have great economic impacts.

In addition to the analysis of polyols as compatible solute, the role of two cytoplasmic fructose reductases was elucidated with respect to intracellular mannitol formation and

osmotolerance of *G. oxydans*. It was found that the organism possesses two genes (*gox0849* and *gox1432*) that encode potential fructose reductases. Enzyme assays revealed that both purified proteins (Gox0849 and Gox1432) catalyzed the reversible reduction of fructose to mannitol. Gox1432 (EC 1.1.1.138) catalyzed a NADPH-specific fructose reduction, while Gox0849 (EC 1.1.1.67) preferred NADH as cofactor. Both proteins could oxidize mannitol to fructose; however, at physiological pH 7.0, the rate of fructose reduction was 3-4 fold higher than the rate of mannitol oxidation. Thus, it was inferred that both proteins in vivo function as D-fructose reductases.

The role of Gox0849 and Gox1432 in osmotolerance of *G. oxydans* was elucidated by the deletion of the corresponding genes and the analysis of the deletion mutants under non-stress and osmotic-stress growth conditions. During cultivation in high-sugar media, the deletion mutant lacking *gox1432* showed retarded growth, while the growth of strain Δ *gox0849* was unaffected. The sensitivity of the cells towards osmotic stress was increased when both fructose reductases were simultaneously deleted. Likewise, the intracellular mannitol content was reduced in the mutants lacking *gox1432* during growth under osmotic stress. These results indicated the importance of Gox1432 in intracellular mannitol formation and osmotolerance of *G. oxydans*. Other evidence came from the plasmid-based overexpression of *gox1432* in the Δ *gox1432* deletion mutant that enabled the cells to recover from osmotic stress.

The importance of *gox1432* was also evident from the transcript abundance of the gene which was 30-fold higher than that of *gox0849*. Analogously, the activity of the NADPH-dependent enzyme Gox1432 was 10-fold higher in comparison to the NADH-dependent enzyme Gox0849 in the cytoplasmic fractions of osmotically stressed cells. These findings provided detailed insights into the molecular mechanisms of mannitol-mediated osmoprotection in *G. oxydans*. Based on these results a pathway was proposed to indicate putative routes involved in intracellular mannitol production from potential precursor molecules. Additionally, strategies were designed targeting the possible bottlenecks of the pathway to increase the intracellular mannitol production and osmotolerance of *G. oxydans*.

6. REFERENCES

- Adachi O., Ano Y., Toyama H., Matsushita K. (2006). High shikimate production from quinate with two enzymatic systems of acetic acid bacteria. *Biosci Biotechnol Biochem* **70**, 2579-2582.
- Adachi O., Fujii Y., Ano Y., Moonmangmee D., Toyama H., Shinagawa E., Theeragool G., Lotong N., Matsushita K. (2001a). Membrane-bound sugar alcohol dehydrogenase in acetic acid bacteria catalyzes L-ribulose formation and NAD-dependent ribitol dehydrogenase is independent of the oxidative fermentation. *Biosci Biotechnol Biochem* **65**, 115-125.
- Adachi O., Fujii Y., Ghaly M. F., Toyama H., Shinagawa E., Matsushita K. (2001b). Membrane-bound quinoprotein D-arabitol dehydrogenase of *Gluconobacter suboxydans* IFO 3257: a versatile enzyme for the oxidative fermentation of various ketoses. *Biosci Biotechnol Biochem* **65**, 2755-2762.
- Adachi O., Moonmangmee D., Toyama H., Shinagawa E., Matsushita K. (2003). New developments in oxidative fermentation. *Appl Microbiol Biotechnol* **60**, 643-653.
- Adachi O., Tayama K., Shinagawa E., Matsushita K., Ameyama M. (1978). Purification and characterization of particulate alcohol dehydrogenase from *Gluconobacter suboxydans*. *Agric Biol Chem* **42**, 2045-2056.
- Adachi O., Tayama K., Shinagawa E., Matsushita K., Ameyama M. (1980). Purification and characterization of membrane-bound aldehyde dehydrogenase from *Gluconobacter suboxydans*. *Agric Biol Chem* **44**, 503-515.
- Adachi O., Toyama H., Matsushita K. (1999a). Crystalline NADP-dependent D-mannitol dehydrogenase from *Gluconobacter suboxydans*. *Biosci Biotechnol Biochem* **63**, 402-407.
- Adachi O., Toyama H., Theeragool G., Lotong N., Matsushita K. (1999b). Crystallization and properties of NAD-dependent D-sorbitol dehydrogenase from *Gluconobacter suboxydans* IFO 3257. *Biosci Biotechnol Biochem* **63**, 1589-1595.
- Adessi C., Matton G., Ayala G., Turcatti G., Mermoud J. J., Mayer P., Kawashima E. (2000). Solid phase DNA amplification: characterization of primer attachment and amplification mechanisms. *Nucleic Acids Res* **28**, E87.
- Alemohammed M. M. and Knowles C. J. (1974). Osmotically induced volume and turbidity changes of *Escherichia coli* due to salts, sucrose and glycerol, with particular reference to rapid permeation of glycerol into the cell. *J Gen Microbiol* **82**, 125-142.
- Almirón M., Link A. J., Furlong D., Kolter R. (1992). A novel DNA-binding protein with regulatory and protective roles in starved *Escherichia coli*. *Genes Dev* **6**, 2646-2654.
- Altschul S. F., Madden T. L., Schäffer A. A., Zhang J., Zhang Z., Miller W., Lipman D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389-3402.

- Ameyama M., Matsushita K., Shinagawa E., Adachi O. (1987). Sugar-oxidizing respiratory chain of *Gluconobacter suboxydans*. Evidence for a branched respiratory chain and characterization of respiratory chain-linked cytochromes. *Agric Biol Chem* **51**, 2943-2950.
- Ameyama M., Shinagawa E., Matsushita K., Adachi O. (1981). D-Glucose dehydrogenase of *Gluconobacter suboxydans*: solubilization, purification and characterization. *Agric Biol Chem* **45**, 851-861.
- Amezaga M.-R., Davidson I., Mclaggan D., Verheul A., Abee T., Booth I. R. (1995). The role of peptide metabolism in the growth of *Listeria monocytogenes* ATCC 23074 at high osmolarity. *Microbiology* **141**, 41-49.
- Andrés-Barrao C., Saad M. M., Chappuis M. L., Boffa M., Perret X., Pérez R. O., Barja F. (2012). Proteome analysis of *Acetobacter pasteurianus* during acetic acid fermentation. *J Proteomics* **75**, 1701-1717.
- Arakawa T. and Timasheff S. N. (1985). The stabilization of proteins by osmolytes. *Biophys J* **47**, 411-414.
- Arora K. K. and Pedersen P. L. (1995). Glucokinase of *Escherichia coli*: induction in response to the stress of overexpressing foreign proteins. *Arch Biochem Biophys* **319**, 574-578.
- Asai T. (1935). Taxonomic studies on acetic acid bacteria and allied oxidative bacteria isolated from fruits. A new classification of the oxidative bacteria. *Nippon Nogeikagaku Kaishi* **11**, 674-708.
- Asai T. (1971). The classification and biochemistry of acetic acid bacteria. In: K. Sakaguchi., T. Vemuna. and S. Kinoshito (Eds.), *Biochemical and industrial aspects of fermentation*. Tokyo, Japan: Kadansha Ltd. pp. 201-232.
- Asano N. (2003). Glycosidase inhibitors: update and perspectives on practical use. *Glycobiology* **13**, 93R-104R.
- Barrow K. D., Collins J. G., Leigh D. A., Rogers P. L., Warr R. G. (1984). Sorbitol production by *Zymomonas mobilis*. *Appl Microbiol Biotechnol* **20**, 225-233.
- Batt C. A., Jamieson A. C., Vandeyar M. A. (1990). Identification of essential histidine residues in the active site of *Escherichia coli* xylose (glucose) isomerase. *Proc Natl Acad Sci U S A* **87**, 618-622.
- Bathey A. S. and Schaffner D. W. (2001). Modelling bacterial spoilage in cold-filled ready to drink beverages by *Acinetobacter calcoaceticus* and *Gluconobacter oxydans*. *J Appl Microbiol* **91**, 237-247.
- Bauer R., Katsikis N., Varga S., Hekmat D. (2005). Study of the inhibitory effect of the product dihydroxyacetone on *Gluconobacter oxydans* in a semi-continuous two-stage repeated-fed-batch process. *Bioprocess Biosyst Eng* **5**, 37-43.
- Bhatt S. M., Mohan A., Srivastava S. K. (2013). Challenges in enzymatic route of mannitol production. *ISRN Biotechnol* Article ID 91418. **DOI:** 10.5402/2013/914187

- Bhosale S. H., Rao M. B., Deshpande V. V. (1996). Molecular and industrial aspects of glucose isomerase. *Microbiol Rev* **60**, 280-300.
- Bizouarn T., Althage M., Pedersen A., Tigerstrom A., Karlsson J., Johansson C., Rydstrom J. (2002). The organization of the membrane domain and its interaction with the NADP(H)-binding site in proton-translocating transhydrogenase from *E. coli*. *Biochim Biophys Acta* **1555**, 122-127.
- Bligh E. G. and Dyer W. J. (1959). A rapid method of lipid extraction and purification. *Can J Biochem Physiol* **37**, 911-917.
- Blum H., Beier H., Gross H. J. (1987). Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* **8**, 93-99.
- Bolen D. W. and Baskakov I. V. (2001). The osmophobic effect: natural selection of a thermodynamic force in protein folding. *J Mol Biol* **310**, 955-963.
- Borisov V. B., Murali R., Verkhovskaya M. L., Bloch D. A., Gennis R. B., Verkhovsky M. (2011). Aerobic respiratory chain of *Escherichia coli* is not allowed to work in fully uncoupled mode. *Proc Natl Acad Sci U S A* **108**, 17320-17324.
- Bradford M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248-254.
- Brakoulias A. and Jackson R. M. (2004). Towards a structural classification of phosphate binding sites in protein-nucleotide complexes: An automated all-against-all structural comparison using geometric matching. *Proteins* **56**, 250-260.
- Bramley H. F. and Kornberg H. L. (1987). Sequence homologies between proteins of bacterial phosphoenolpyruvate-dependent sugar phosphotransferase systems: Identification of possible phosphate-carrying histidine residues. *Proc Natl Acad Sci U S A* **84**, 4777-4780.
- Brown A. D. (1976). Microbial water stress. *Bacteriol Rev* **40**, 803-846.
- Brown A. D. (1990). Microbial water stress physiology. Principles and perspectives. New York: John Wiley and Sons Inc.
- Brünker P., Altenbuchner J., Kulbe K. D., Mattes R. (1997). Cloning, nucleotide sequence and expression of a mannitol dehydrogenase gene from *Pseudomonas fluorescens* DSM 50106 in *Escherichia coli*. *Biochim Biophys Acta* **1351**, 157-167.
- Buchan D. W., Minneci F., Nugent T. C., Bryson K., Jones D. T. (2013). Scalable web services for the PSIPRED Protein Analysis Workbench. *Nucleic Acids Res* **41** (Web Server Issue): W349-W357.
- Busse M. D. and Bottomley P. J. (1989). Growth and nodulation responses of *Rhizobium meliloti* to water stress induced by permeating and nonpermeating solutes. *Appl Environ Microbiol* **55**, 2431-2436.

- Canard B. and Sarfati R. S. (1994). DNA polymerase fluorescent substrates with reversible 3'-tags. *Gene* **148**, 1-6.
- Caparros-Martin J. A., McCarthy-Suarez I., Culiarez-Macia F. A. (2013). HAD hydrolase function unveiled by substrate screening: enzymatic characterization of *Arabidopsis thaliana* subclass I phosphosugar phosphatase AtSgpp. *Planta* **237**, 943-954.
- Capy P., Bazin C., Higuete D., Langin T. (1997). Dynamic and evolution of transposable elements. Austin, Texas, USA: R.G. Landes.
- Capy P., Gasperi G., Biemont C., Bazin C. (2000). Stress and transposable elements: co-evolution or useful parasites? *Heredity* **85**, 101-106.
- Carpenter J. F. (1993). Stabilization of proteins during freezing and dehydration: application of lessons from nature. *Cryobiology* **30**, 220-221.
- Carvalho A. S., Silva J., Ho P., Teixeira P., Malcata F. X., Gibbs P. (2003). Protective effect of sorbitol and monosodium glutamate during storage of freeze-dried lactic acid bacteria. *Le Lait* **83**, 203-210.
- Chaturvedi V., Bartiss A., Wong B. (1997). Expression of bacterial *mtlD* in *Saccharomyces cerevisiae* results in mannitol synthesis and protects a glycerol-defective mutant from high-salt and oxidative stress. *J Bacteriol* **179**, 157-162.
- Chen W.P., Anderson A.W., Han Y.W. (1979). Production of glucose isomerase by *Streptomyces flavogriseus*. *Appl Environ Microbiol* **37**, 324-331.
- Chi M.-C., Wu T.-J., Chen H.-L., Lo H.-F., Lin L.-L. (2012). Sorbitol counteracts temperature- and chemical-induced denaturation of a recombinant α -amylase from alkaliphilic *Bacillus* sp. TS-23. *J Ind Microbiol Biotechnol* **39**, 1779-1788.
- Chinnawirotpisan P., Matsushita K., Toyama H., Adachi O., Limtong S., Theeragool G. (2003). Quinoprotein alcohol dehydrogenase is involved in catabolic acetate production, while NAD-dependent alcohol dehydrogenase in ethanol assimilation in *Acetobacter pasteurianus* SKU1108. *J Biosci Bioeng* **96**, 564-571.
- Cho J.-W., Han B.-G., Park S. Y., Kim S. J., Kim M.-D., and Lee B. I. (2013). Overexpression, crystallization preliminary X-ray crystallographic analysis of a putative xylose isomerase from *Bacteroides thetaiotaomicron*. *Acta Crystallogr Sect F Struct Biol Cryst Commun* **69**, 1127-1130.
- Choi E.-S., Lee E.-H., Rhee S.-K. (1995). Purification of a membrane-bound sorbitol dehydrogenase from *Gluconobacter suboxydans*. *FEMS Microbiol Lett* **125**, 45-50.
- Choi K. H., Kumar A., Schweizer H. P. (2006). A 10 min method for preparation of highly electrocompetent *Pseudomonas aeruginosa* cells: Application for DNA fragment transfer between chromosomes and plasmid transformation. *J Microbiol Methods* **64**, 391-397.
- Chomczynski P. (1993). A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *Biotechniques* **15**, 532-537

- Cladas T., Demont-Caulet N., Ghazi A., Richarme G. (1999). Thermoprotection by glycine betaine and choline. *Microbiology* **145**, 2543-2548.
- Claret C., Salmon J. M., Romieu C., Bories A. (1994). Physiology of *Gluconobacter oxydans* during dihydroxyacetone production from glycerol. *Appl Microbiol Biotechnol* **41**, 359-365.
- Condon C., FitzGerald R. J., O’Gara F. (1991). Conjugation and heterologous gene expression in *Gluconobacter oxydans* ssp. *suboxydans*. *FEMS Microbiol Lett* **80**, 173-177.
- Cornish-Bowden A. (1981). Thermodynamic aspects of glycolysis. *Biochem Educ* **9**, 133-137.
- Cotton N. P. J., White S. A., Peake S. J., McSweeney S., Jackson J. B. (2001). The crystal structure of an asymmetric complex of the two nucleotide binding components of proton-translocating transhydrogenase. *Structure* **9**, 165-176.
- Crowe J. H. and Crowe L. M. (1992). Membrane integrity in anhydrobiotic organisms: toward a mechanism for stabilizing dry cells. In: G. N. Somero., C. B. Osmond., C. L. Bolis (Eds.), *Water and life: comparative analysis of water relationships at the organismic, cellular and molecular levels*. New York: Springer . pp. 87-103.
- Crowe J. H., Crowe L. M., Chapman D. (1984). Preservations of membranes in anhydrobiotic organisms: the role of trehalose. *Science* **223**, 701-703.
- Crowe J. H., Hoekstra F. A., Crowe L. M. (1992). Anhydrobiosis. *Annu Rev Physiol* **54**, 579-599.
- Csonka L. N. and Epstein W. (1996). Osmoregulation. In: F. C. Neidhardt (Ed.), *Escherichia coli and Salmonella. Cellular and molecular biology*. 2nd ed. Washington, DC: ASM Press. pp.1210-1223.
- Culham D. E., Lasby B., Marangoni A. G., Milner J. L., Steer B. A., van Nues R. W., Wood J. M. (1993). Isolation and sequencing of *Escherichia coli* gene *proP* reveals unusual structural features of the osmoregulatory proline/betaine transporter, ProP. *J Mol Biol* **229**, 268-276.
- Cytryn E. J., Sangurdekar D. P., Streeter J. G., Franck W. L., Chang W. S., Stacey G., Emerich D. W., Joshi T., Xu D., Sadowsky M. J. (2007). Transcriptional and physiological responses of *Bradyrhizobium japonicum* to desiccation-induced stress. *J Bacteriol* **189**, 6751-6762.
- da Costa M. S., Santos H., Galinski E. A. (1998). An overview of the role and diversity of compatible solutes in *Bacteria* and *Archaea*. *Adv Biochem Eng Biotechnol* **61**, 117-153.
- de Barros M. and Celligoi M. A. P. C. (2006). Synthesis of sorbitol by *Zymomonas mobilis* under high osmotic pressure. *Braz J Microbiol* **37**, 324-328.
- De Ley J. and Swings J. (1984). Genus *Gluconobacter*. In: N. R. Krieg. and J. G. Holt (Eds.), *Bergey’s Manual of Systematic Bacteriology*. vol 1. London: Williams and Wilkins.pp. 267-278.

- De Muynck C., Pereira C. S. S., Naessens M., Parmentier S., Soetaert W., Vandamme E. J. (2007). The genus *Gluconobacter oxydans*: comprehensive overview of biochemistry and biotechnological applications. *Crit Rev Biotechnol* **27**, 147-171.
- Deppenmeier U. and Ehrenreich, A. (2009). Physiology of acetic acid bacteria in light of the genome sequence of *Gluconobacter oxydans*. *J Mol Microbiol Biotechnol* **16**, 69-80.
- Deppenmeier U., Hoffmeister M., Prust C. (2002). Biochemistry and biotechnological applications of *Gluconobacter* strains. *Appl Microbiol Biotechnol* **60**, 233-242.
- Desmarais D., Jablonski P. G., Fedarko N. S., Roberts M. I. (1997). 2-Sulfotrehalose, a novel osmolyte in haloalkaliphilic archaea. *J Bacteriol* **179**, 3146-3153.
- Doelle H. W. (1982). Kinetic characteristics and regulatory mechanisms of glucokinase and fructokinase from *Zymomonas mobilis*. *Eur J Appl Microbiol Biotechnol* **14**, 241-246.
- Dragosits M. and Mattanovich D. (2013). Adaptive laboratory evolution-principles and applications for biotechnology. *Microb Cell Fact* **12**, 64. DOI: 10.1186/1475-2859-12-64.
- Dueweke T. J. and Gennis R. B. (1991). Proteolysis of the cytochrome *d* complex with trypsin and chymotrypsin localizes a quinol oxidase domain. *Biochemistry* **30**, 3401-3406.
- Efiuvwevwere B. J. O., Gorris L. G. M., Smid E. J., Kets E. P. W. (1999). Mannitol enhanced survival of *Lactococcus lactis* subjected to drying. *Appl Microbiol Biotechnol* **51**, 100-104.
- Elfari M., Ha S. W., Bremus C., Merfort M., Khodaverdi V., Herrmann. U., Sahm H., Görisch H. (2005). A *Gluconobacter oxydans* mutant converting glucose almost quantitatively to 5-keto-D-gluconic acid. *Appl Microbiol Biotechnol* **66**, 668-674.
- Empadinhas N. and da Costa M. S. (2008). Osmoadaptation mechanisms in prokaryotes: distribution of compatible solutes. *Int Microbiol* **11**, 151-161.
- Fang Z. and Cui X. (2011). Design and validation issues in RNA-Seq experiments. *Brief Bioinform* **12**, 280-287.
- Ferain T., Schanck A. N., Delcour J. (1996). ¹³C nuclear magnetic resonance analysis of glucose and citrate end products in an *ldhL-ldhD* double-knockout strain of *Lactobacillus plantarum*. *J Bacteriol* **178**, 7311-7315.
- Foster P. L. (2007). Stress-induced mutagenesis in bacteria. *Crit Rev Biochem Mol Biol* **42**, 373-397.
- Galinski E. A. (1995). Osmoadaptation in bacteria. *Adv Microb Physiol* **37**, 272-328.
- Galinski E. A. and Trüper H. G. (1994). Microbial behaviour in salt stressed ecosystems. *FEMS Microbiol Rev* **15**, 95-108.

- Gallagher P. R. and Desjardins P. R. (2007). Quantitation of DNA and RNA with absorption and fluorescence spectroscopy. *Curr Protoc Hum Genet* Appendix 3D. DOI: 10.1002/0471142727.mba03ds76.
- Gao L., Hu Y., Liu J., Du G., Zhou J., Chen J. (2014). Stepwise metabolic engineering of *Gluconobacter oxydans* WSH-003 for the direct production of 2-keto-L-gulononic acid from D-sorbitol. *Metab Eng* **24**, 30-37.
- Gao Q., Zhang M., McMillan J. D., Kompala D. S. (2002). Characterization of heterologous and native enzyme activity profiles in metabolically engineered *Zymomonas mobilis* strains during batch fermentation of glucose and xylose mixtures. *Appl Biochem Biotechnol* **98-100**, 341-355.
- Garg A. K., Kim J.-K., Owens T. G., Ranwala A. P., Choi Y. D., Kochian L. V., Wu R. J. (2002). Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. *Proc Natl Acad Sci U S A* **99**, 15898-15903.
- Gaspar P., Neves A. R., Ramos A., Gasson M. J., Shearman C. A., Santos H. (2004). Engineering *Lactococcus lactis* for production of mannitol: high yields from food-grade strains deficient in lactate dehydrogenase and the mannitol transport system. *Appl Environ Microbiol* **70**, 1466-1474.
- Giaever H. M., Styrvold O. B., Kaasen I., Strom A. R. (1988). Biochemical and genetic characterization of osmoregulatory trehalose synthesis in *Escherichia coli*. *J Bacteriol* **170**, 2841-2849.
- Gillis M. and De Ley J. (1980). Intra- and intergeneric similarities of the ribosomal ribonucleic acid cistrons of *Acetobacter* and *Gluconobacter*. *Int J Syst Bacteriol* **30**, 7-27.
- Gilmour P., Beswick H. P., Donaldson K. (1995). Surface free radical activity of a range of respirable industrial fibres assessed using ϕ x 174 RF1 plasmid DNA. *Carcinogenesis* **16**, 2973-2979.
- Glaasker E., Konings W. N., Poolman B. (1996). Glycine betaine fluxes in *Lactobacillus plantarum* during osmostasis and hyper and hypo-osmotic shock. *J Biol Chem* **271**, 10060-10065.
- Gouesbet G., Jebbar M., Talibart R., Bernard T., Blanco C. (1994). Pipecolic acid is an osmoprotectant for *Escherichia coli* taken up by the general osmoporters ProU and ProP. *Microbiology* **140**, 2415-2422.
- Grant W. D. (2004). Life at low water activity. *Philos Trans R Soc Lond B Biol Sci* **359**, 1249-1266.
- Greenberg D. E., Porcella S. F., Stock F., Wong A., Conville P. S., Murray P. R., Holland S. M., Zelazny A. M. (2006). *Granulibacter bethesdensis* gen. nov., sp. nov., a distinctive pathogenic acetic acid bacterium in the family *Acetobacteraceae*. *Int J Syst Evol Microbiol* **56**, 2609-2616.

- Greenfield S. and Claus G. W. (1972). Non-functional tricarboxylic acid cycle and the mechanism of glutamate biosynthesis in *Acetobacter suboxydans*. *J Bacteriol* **112**, 1295-1301.
- Groisillier A., Labourel A., Michel G., Tonon T. (2015). The mannitol utilization system of the marine bacterium *Zobellia galactanivorans*. *Appl Environ Microbiol* **81**, 1799-812.
- Gunasekera T. S., Csonka L. N., Paliy O. (2008). Genome-wide transcriptional responses of *Escherichia coli* K-12 to continuous osmotic and heat stresses. *J Bacteriol* **190**, 3712-3720.
- Gupta A., Singh V. K., Qazi G. N., Kumar A. (2001). *Gluconobacter oxydans*: Its biotechnological applications. *J Mol Microbiol Biotechnol* **3**, 445-456.
- Hanahan D. (1983). Studies on transformation of *E. coli* with plasmids. *J Mol Biol* **166**, 557-580.
- Hanke T., Nöh K., Noack S., Polen T., Bringer S., Sahm H., Wiechert W., Bott M. (2013). Combined fluxomics and transcriptomics analysis of glucose catabolism via a partially cyclic pentose phosphate pathway in *Gluconobacter oxydans* 621H. *Appl Environ Microbiol* **79**, 2336-2248.
- Hardman M. J. and Scopes R. K. (1988). The kinetics of glucosefructose oxidoreductase from *Zymomonas mobilis*. *Eur J Biochem* **173**, 203-209.
- Harrison D. E. F. and Loveless J. E. (1971). The effect of growth conditions on respiratory activity and growth efficiency in facultative anaerobes grown in chemostat culture. *J Gen Microbiol* **68**, 35-43.
- Hattori K. and Suzuki T. (1975). Enzymatic studies on the conversion of erythritol fermentation to D-mannitol fermentation in n-alkane-grown yeast *Candida zeylanoides*. *Agric Biol Chem* **39**, 57-61.
- Hawkes R., Niday E., Gordon J. (1982): A dot-immunobinding assay for monoclonal and other antibodies. *Anal Biochem* **119**, 142-147.
- Heuel H., Garakani A. S., Turgut S., Lengeler J. W. (1998). Genes for D-arabinitol and ribitol catabolism from *Klebsiella pneumoniae*. *Microbiology* **144**, 1631-1639.
- Hocking A. D. (1988). Strategies for microbial growth at reduced water activities. *Microbiol Sci* **5**, 280-284.
- Hölscher T. and Görisch H. (2006). Knockout and overexpression of pyrroloquinoline quinone biosynthetic genes in *Gluconobacter oxydans* 621H. *J Bacteriol* **188**, 7668-7676.
- Hommel R. and Ahnert P. (2000). *Gluconobacter*. In: R. K. Robinson., C. A. Batt., P. Patel (Eds.), *Encyclopedia of Food Microbiology*. London: Academic Press. pp. 955-961.
- Hoshino T., Sugisawa T., Shinjoh M., Tomiyama N., and Miyazaki T. (2003). Membrane-bound D-sorbitol dehydrogenase of *Gluconobacter suboxydans* IFO 3255 enzymatic and genetic characterization. *Biochim Biophys Acta Prot Proteomics* **1647**, 278-288.

- Hoshino T., Sugisawa T., Tazoe M., Shinjoh M., and Fujiwara A. (1990). Metabolic pathway for 2-keto-Lgulonic acid formation in *Gluconobacter melanogenus* IFO 3293. *Agric Biol Chem* **54**, 1211-1218.
- Ingraham J. L. and Marr A. G. (1996). Effect of temperature, pressure, pH and osmotic stress on growth. In: F. C. Neidhardt (Ed.), *Escherichia coli and Salmonella typhimurium: cellular and molecular biology*. 2nd ed. Washington, DC: ASM Press. pp.1570-1578.
- Ishikawa H. (1997). Evolution of ribosomal RNA. *Comp Biochem Physiol* **58**, 1-7.
- Jennings D. H. (1984). Polyolmetabolism in fungi. *Adv Microb Physiol* **25**, 149-193.
- Jeude M., Dittrich B., Niederschulte H., Anderlei T., Knocke C., Klee D., Büchs J. (2006). Fed-batch mode in shake flasks by slow-release technique. *Biotechnol Bioeng* **95**, 433-445.
- Johnson S. D. and Nicolson S.W. (2008). Evolutionary associations between nectar properties and specificity in bird pollination systems. *Biol Lett* **4**, 49-52.
- Jones D. T. (1999). Protein Secondary Structure Prediction Based on Position-specific Scoring Matrices. *J Mol Biol* **292**, 195-202.
- Kadner R. J. (1990). Vitamin B12 transport in *Escherichia coli*: energy coupling between membranes. *Mol Microbiol* **4**, 2027-33.
- Kallnik V., Meyer M., Deppenmeier U., Schweiger P. (2010). Construction of expression vectors for protein production in *Gluconobacter oxydans*. *J Biotechnol* **150**, 460-465.
- Kanehisa M., Araki M., Goto S., Hattori M., Hirakawa M., Itoh M., Katayama T., Kawashima S., Okuda S., Tokimatsu T., Yamanishi Y. (2008). KEGG for linking genomes to life and the environment. *Nucleic Acids Res* **36**, D480-484.
- Kavanagh K. L., Klimacek M., Nidetzky B., Wilson D. K. (2002). Crystal structure of *Pseudomonas fluorescens* mannitol 2-dehydrogenase binary and ternary complexes: specificity and catalytic mechanism. *J Biol Chem* **277**, 43433-43442.
- Kempf B. and Bremer E. (1998). Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environments. *Arch Microbiol* **170**, 319-330.
- Kets E. P. W. (1997). Compatible solutes in lactic acid bacteria subjected to water stress. Ph.D. Thesis, Wageningen Agricultural University, Wageningen, The Netherlands.
- Kets E. P. W. and de Bont J. A. M. (1994). Protective effect of betaine on survival of *Lactobacillus plantarum* subjected to drying. *FEMS Microbiol Lett* **116**, 251-256.
- Kets E. P. W., de Bont J. A. M., Heipieper H. J. (1996a). Physiological response of *Pseudomonas putida* S12 subjected to reduced water activity. *FEMS Microbiol Lett* **139**, 133-137.
- Kets E. P. W., Galinski E. A., de Witt M., de Bont J. A. M., Heipieper H. J. (1996b). Mannitol, a novel compatible solute in *Pseudomonas putida* S12. *J Bacteriol* **178**, 6665-6670.

- Klasen R. (1994). Molecularbiologische Untersuchungen zur 5-Ketoglukonatbildung bei *Gluconobacter oxydans*. Thesis, Heinrich-Heine University of Duesseldorf, Germany.
- Klasen R., Bringer S., Sahm H. (1995). Biochemical characterization and sequence analysis of the gluconate-NADP-5-oxidoreductase gene from *Gluconobacter oxydans*. *J Bacteriol* **177**, 2637-2643.
- Kleckner N. (1990). Regulation of transposition in bacteria. *Annu. Rev. Cell Biol* **6**, 297-327.
- Klein W., Ehmman U., Boos W. (1991). The repression of trehalose transport and metabolism in *Escherichia coli* by high osmolarity is mediated by trehalose-6-phosphate phosphatase. *Res Microbiol* **142**, 359-371.
- Klimacek M. and Nidetzky B. (2010). The oxyanion hole of *Pseudomonas fluorescens* mannitol 2-dehydrogenase: a novel structural motif for electrostatic stabilization in alcohol dehydrogenase active sites. *Biochem J* **425**, 455-63.
- Knapp S., Ladenstein R., Galinski E. A. (1999). Extrinsic protein stabilization by the naturally occurring osmolytes β -hydroxyectoine and betaine. *Extremophiles* **3**, 191-198.
- Komagata K., Lino T., Yamada Y. (2014). The Family *Acetobacteraceae*. In: E. F. DeLong., S. Lory., E. Stackebrandt., F. Thompson (Eds.), *The Prokaryotes. Alphaproteobacteria and Betaproteobacteria*. Berlin Heidelberg: Springer. pp. 3-78.
- Kosciow K., Domin C., Schweiger P., Deppenmeier U. (2016). Extracellular targeting of an active endoxylanase by a TolB negative mutant of *Gluconobacter oxydans*. *J Ind Microbiol Biotechnol* **43**, 989-999.
- Kosciow K., Zahid N., Schweiger P., Deppenmeier U. (2014). Production of periplasmic trehalase in *Gluconobacter oxydans* and growth on trehalose. *J Biotechnol* **189**, 27-35.
- Kostner D., Peters B., Mientus M., Liebl W., Ehrenreich A. (2013). Importance of *codB* for new *codA*-based markerless gene deletion in *Gluconobacter* strains. *Appl Microbiol Biotechnol* **97**, 8341-8349.
- Krajewski V., Simić P., Mouncey N. J., Bringer S., Sahm H., Bott M. (2010). Metabolic engineering of *Gluconobacter oxydans* for improved growth rate and growth yield on glucose by elimination of gluconate formation. *Appl Environ Microbiol* **76**, 4369-4376.
- Kulhanek M. (1989). Microbial dehydrogenations of monosaccharides. *Adv Appl Microbiol* **34**, 141-181.
- Kültz D. (2003). Evolution of the cellular stress proteome: from monophyletic origin to ubiquitous function. *J Exp Biol* **206**, 3119-3124.
- Kumar S., Wittmann C., and Heinzle E. (2004). Review: minibioreactors. *Biotechnol Lett* **26**, 1-10.
- Kurz M. (2008). Compatible solute influence on nucleic acids: Many questions but few answers. *Saline Systems* **4**, 6.

- Kuschel M. (2000). Analysis of total RNA using the Agilent 2100 Bioanalyzer and the RNA 6000 LabChip® kit. (<http://www.genomics.liv.ac.uk/tryps/papers/AGILENTR.PDF>).
- Laemmli U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.
- Lamosa P., Martins L. O., da Costa M. S., Santos H. (1998). Effects of temperature, salinity and medium composition on compatible solute accumulation by *Thermococcus* spp. *Appl Environ Microbiol* **64**, 3591-3598.
- Lawford H. G. and Haddock B. A. (1973). Respiration-driven proton translocation in *Escherichia coli*. *Biochem J* **136**, 217-220.
- Le Marrec C. (2011). Responses of lactic acid bacteria to osmotic stress. In: E. Tsakalidou. and K. Papadimitriou (Eds.), *Stress responses of lactic acid bacteria*. Heidelberg London: Springer US. pp. 67-90.
- Le Rudulier D., Strøm A. R., Dandekar A. M., Smith L. T., Valentine R. C. (1984). Molecular biology of osmoregulation. *Science* **224**, 1064-1068.
- Lebaron P., Catala P., Parthuisot N. (1998). Effectiveness of SYTOX green stain for bacterial viability assessment. *Appl Environ Microbiol* **64**, 2697-2700.
- Leigh D., Scopes R. K., Rogers P. L. (1984). A proposed pathway for sorbitol production in *Zymomonas mobilis*. *Appl Microbiol Biotechnol* **20**, 413-415.
- Leslie S. B., Israeli E., Lighthart B., Crowe J. H., Crowe L. M. (1995). Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. *Appl Environ Microbiol* **61**, 3592-3597.
- Lewis I. A., Schommer S. C., Markley J. L. (2009). rNMR: open source software for identifying and quantifying metabolites in NMR spectra. *Magn Reson Chem* **47**, 123-126.
- Li X. Y., Gilmour P. S., Donaldson K., Macnee W. (1996). Free radical activity and proinflammatory effects of air pollution (PM-10) in vivo and in vitro. *Thorax* **51**, 1216-1222.
- Lichtenthaler F. W. (2006). The key sugar of biomass: Availability, present non-food applications and potential industrial development lines. In: B. Kamm., M. Kamm., P. Gruber (Eds.), *Biorefineries-biobased industrial processes and products*. Weinheim: Wiley-VHC. pp. 3-59.
- Liu J. H., Chen M. M., Huang J. W., Wann H., Ho L. K., Pan W. H., Chen Y. C., Liu C. M., Yeh M. Y., Tsai S. K., Young M. S., Ho L. T., Kuo C. D., Chuang H. Y., Chao F. P., Chao H. M. (2010). Therapeutic effects and mechanisms of action of mannitol during H₂O₂-induced oxidative stress in human retinal pigment epithelium cells. *J Ocul Pharmacol Ther* **26**, 249-257.
- Lobanok A.G., Sapunova L. I., Dikhtievski Y. O., Kazakevich I. O. (1998). Screening of glucose isomerase-producing microorganisms. *World J Microbiol Biotechnol* **14**, 259-262.

- Loesche W. J. and Kornman K. S. (1976). Production of mannitol by *Streptococcus mutans*. *Arch Oral Biol* **21**, 551-553.
- Loescher W. H., Tyson R. H., Everard J. D., Redgwell R. J., Bielecki R. L. (1992). Mannitol synthesis in higher plants: evidence for the role and characterization of a NADPH-dependent mannose 6-phosphate reductase. *Plant Physiol* **98**, 1396-1402.
- Loos H., Kramer R., Sahm H., Sprenger G. A. (1994). Sorbitol promotes growth of *Zymomonas mobilis* in environments with high concentrations of sugar: evidence for a physiological function of glucose-fructose oxidoreductase in osmoprotection. *J Bacteriol* **176**, 7688-7693.
- Lucht J. and Bremer E. (1994). Adaptation of *Escherichia coli* to high osmolarity environments: osmoregulation of the high-affinity glycine betaine transport system. *FEMS Microbiol Rev* **14**, 3-20.
- Luchterhand B., Fischöder T., Grimm A. R., Wewetzer S., Wunderlich M., Schlepütz T., Büchs J. (2015). Quantifying the sensitivity of *G. oxydans* ATCC 621H and DSM 3504 to osmotic stress triggered by soluble buffers. *J Ind Microbiol Biotechnol* **42**, 585-600.
- Lusta K. A. and Reshetilov A. N. (1998). Physiological and biochemical features of *Gluconobacter oxydans* and prospects of their use in biotechnology and biosensor systems (review). *Appl Biochem Microbiol* **34**, 307-320.
- Luttik M., Van Spanning R., Schipper D., Van Dijken J. P., Pronk J. T. (1997). The low biomass yields of the acetic acid bacterium *Acetobacter pasteurianus* are due to a low stoichiometry of respiration-coupled proton translocation. *Appl Environ Microbiol* **63**, 3345-3351.
- Macauley S., McNeil B., Harvey L. M. (2001). The genus *Gluconobacter* and its applications in biotechnology. *Crit Rev Biotechnol* **21**, 1-25.
- Mager W. H., de Boer A. H., Siderius M. H., Voss H.-P. (2000). Cellular responses to oxidative and osmotic stress. *Cell Stress Chaperones* **5**, 73-75.
- Mamlouk D. and Gullo M. (2013). Acetic acid bacteria: physiology and carbon sources oxidation. *Indian J Microbiol* **53**, 377-384.
- Manzanera M., Vilchez S., Tunnacliffe A. (2004). High survival and stability rates of *E. coli* dried in hydroxyectoine. *FEMS Microbiol Lett* **233**, 347-352.
- Marshall R. O., Kooi E. R., Moffett G. M. (1957). Enzymatic conversion of D-glucose to D-fructose. *Science* **125**, 648-649.
- Martins L. O., Empadinhas N., Marugg J. D., Miguel C., Ferreira C., da Costa M. S., Santos H. (1999). Biosynthesis of mannosylglycerate in the thermophilic bacterium *Rhodothermus marinus*. *J Biol Chem* **274**, 35407-35414.
- Martinussen J. and Hammer K. (1994). Cloning and characterization of *upp*, a gene encoding uracil phosphoribosyltransferase from *Lactococcus lactis*. *J Bacteriol* **76**, 6457-6463.

- Matsushita K., Fujii Y., Ano Y., Toyama H., Shinjoh M., Tomiyama N., Miyazaki T., Sugisawa T., Hoshino T., Adachi O. (2003). 5-Keto-D-gluconate production is catalyzed by a quinoprotein glycerol dehydrogenase, major polyol dehydrogenase, in *Gluconobacter* species. *Appl Environ Microbiol* **69**, 1959-1966.
- Matsushita K., Nagatani Y., Shinagawa E., Adachi O., Ameyama M. (1989). Effect of extracellular pH on the respiratory chain and energetics of *Gluconobacter suboxydans*. *Agric Biol Chem* **53**, 2895-2902.
- Matsushita K., Shinagawa E., Adachi O., Ameyama M. (1987). Purification, characterization and reconstitution of cytochrome *o*-type oxidase from *Gluconobacter suboxydans*. *Biochim Biophys Acta* **894**, 304-312.
- Matsushita K., Toyama H., Adachi O. (1994). Respiratory chains and bioenergetics of acetic acid bacteria. *Adv Microb Physiol* **36**, 247-301.
- Mayer F. and Müller V. (2014). Adaptation of anaerobic archaea to life under extreme energy limitation. *FEMS Microbiol Rev* **38**, 449-472.
- McIntyre H. J., Davies H., Hore T. A., Miller S. H., Dufour J. P., Ronson C. W. (2007). Trehalose biosynthesis in *Rhizobium leguminosarum* bv. *trifolii* and its role in desiccation tolerance. *Appl Environ Microbiol* **73**, 3984-3992.
- McLaggan D., Logan T. M., Lynn D. G., Epstein W. (1990). Involvement of gamma-glutamyl peptides in osmoadaptation of *Escherichia coli*. *J Bacteriol* **172**, 3631-3636.
- McNeil B. and Harvey L. (2005). Energy well spent on a prokaryotic genome. *Nat Biotechnol* **23**, 186-187.
- Meiberg J. B. M. and Spa H. A. (1983). Microbial production of gluconic acids and gluconates. *J Microbiol* **49**, 89-90.
- Merfort M., Herrmann U., Bringer S., Sahm H. (2006). High yield 5-keto-D-gluconic acid formation is mediated by soluble and membrane-bound gluconate-5-dehydrogenases of *Gluconobacter oxydans*. *Appl Microbiol Biotechnol* **73**, 443-451.
- Meyer D., Schneider-Fresenius C., Horlacher C., Peist R. Boos W. (1997). Molecular characterization of glucokinase from *Escherichia coli* K-12. *J Bacteriol* **179**, 1298-306.
- Meyer M. and Kircher M. (2010). Illumina sequencing library preparation for highly multiplexed target capture and sequencing. *Cold Spring Harb Protoc* **DOI: 10.1101/pdb.prot5448** pmid:20516186.
- Meyer M., Schweiger P., Deppenmeier U. (2015). Succinic semialdehyde reductase Gox1801 from *Gluconobacter oxydans* in comparison to other succinic semialdehyde-reducing enzymes. *Appl Microbiol Biotechnol* **99**, 3929-3939.
- Michel B. E. (1983). Evaluation of the water potentials of solutions of polyethylene glycol 8000 both in the absence and presence of other solutes. *Plant Physiol* **72**, 66-70.
- Mille Y., Beney L., Gervais P. (2005). Compared tolerance to osmotic stress in various microorganisms: towards a survival prediction test. *Biotechnol Bioeng* **92**, 479-484.

- Miller J. H. (1972). Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor.
- Miller M. J. and Gennis R. B. (1985). The cytochrome *d* complex is a coupling site in the aerobic respiratory chain of *Escherichia coli*. *J Bacteriol Chem* **260**, 14003-14008.
- Minohara S., Sakamoto J., Sone N. (2002). Improved H⁺/O ratio and cell yield of *Escherichia coli* with genetically altered terminal quinol oxidases. *J Biosci Bioeng* **93**, 464-469.
- Mori H., Kobayashi T., Shimizu S. (1981). High density production of sorbose from sorbitol by fed-batch culture with DO-stat. *J Chem Eng Jpn* **14**, 65-70.
- Mortazavi A., William B. A., McCue K., Schaeffer L., Wold B. (2008). Mapping and quantifying mammalian transcriptome by RNA-Seq. *Nat Method* **5**, 621-628.
- Mostafa H. E., Heller K. J., Geis A. (2002). Cloning of *Escherichia coli lacZ* and *lacY* genes and their expression in *Gluconobacter oxydans* and *Acetobacter liquefaciens*. *Appl Environ Microbiol* **68**, 2619-2623.
- Mott J. F., Grant R. A., Ho Y. S., Platt T. (1985). Maximizing gene expression from plasmid vectors containing the λ PL promoter: strategies of overproducing termination factor ρ . *Proc Natl Acad Sci U S A* **82**, 88-92.
- Mullis K. B. and Faloona F. A. (1987). Specific synthesis of DNA in vitro via a polymerase catalyzed chain reaction. *Methods Enzymol* **155**, 335-350.
- Nagata S., Maekawa Y., Ikeuchi T., Wang Y. B., Ishida A. (2002). Effect of compatible solutes on the respiratory activity and growth of *E. coli* K-12 under NaCl stress. *J Biosci Bioeng* **94**, 384-389.
- Nagata S., Sasaki H., Oshima A., Takeda S., Hashimoto Y., Ishida A. (2005). Effect of proline and K⁺ on the stimulation of cellular activities in *Escherichia coli* K12 under high salinity. *Biosci Biotechnol Biochem* **69**, 740-746.
- Neuhard J. (1983). Utilization of preformed pyrimidine bases and nucleosides. In: A. Munch-Peterson (Ed.), *Metabolism of nucleotides nucleosides and nucleobases in microorganisms*. London: Academic Press. pp. 95-148.
- Neves A. R., Ramos A., Shearman C., Gasson M. J., Almeida J. S., Santos H. (2000). Metabolic characterization of *Lactococcus lactis* deficient in lactate dehydrogenase using in vivo ¹³C-NMR. *Eur J Biochem* **267**, 3859-3868.
- Nidetzky B., Furlinger M., Gollhofer D., Haug I., Haltrich D., Kulbe K. D. (1997). Simultaneous enzymatic synthesis of gluconic acid and sorbitol: production, purification, and application of glucose-fructose oxidoreductase and gluconolactonase. *Appl Biochem Biotechnol* **63-65**, 173-188.
- Nikaido H. and Vaara M. (1985). Molecular basis of bacterial outer membrane permeability. *Microbiol Rev* **49**, 1-32.

- Nikaido H., Basina M., Nguyen V., Rosenberg E. Y. (1998). Multidrug efflux pump AcrAB of *Salmonella typhimurium* excretes only those beta-lactam antibiotics containing lipophilic side chains. *J Bacteriol* **180**, 4686-4692.
- Olijve W. and Kok J. J. (1979). Analysis of growth of *Gluconobacter oxydans* in glucose containing media. *Arch Microbiol* **121**, 283-290.
- Onishi H. and Suzuki T. (1968). Production of D-mannitol and glycerol by yeast. *Appl Microbiol* **16**, 1847-1852.
- Oren A. (1999). Bioenergetic aspects of halophilism. *Microbiol Mol Biol Rev* **63**, 334-348.
- Oren A. (2008). Microbial life at high salt concentrations: phylogenetic and metabolic diversity. *Saline Systems* **4**, 2.
- Parmentier S., Beauprez J., Arnaut F., Soetaert W., Vandamme E. (2005). *Gluconobacter oxydans* NAD-dependent, D-fructose reducing, polyol dehydrogenases activity: screening, medium optimisation and application for enzymatic polyol production. *Biotechnol Lett* **27**, 305-311.
- Paulsen I. T., Brown M. H., Skurray R. A. (1996). Proton-dependent multidrug efflux systems. *Microbiol Rev* **60**, 575-608.
- Peters B., Mientus M., Kostner D., Junker A., Liebl W., Ehrenreich A. (2013a). Characterization of membrane-bound dehydrogenases from *Gluconobacter oxydans* 621H via whole-cell activity assays using multideletion strains. *Appl Microbiol Biotechnol* **97**, 6397-6412.
- Peters B., Junker A., Brauer K., Mühlthaler B., Kostner D., Mientus M., Liebl W., Ehrenreich A. (2013b). Deletion of pyruvate decarboxylase by a new method for efficient markerless gene deletions in *Gluconobacter oxydans*. *Appl Microbiol Biotechnol* **97**, 2521-2530
- Pflüger K., Baumann S., Gottschalk G., Lin W., Santos H., Müller V. (2003). Lysine-2,3-aminomutase and β -lysine acetyltransferase genes of methanogenic archaea are salt induced and are essential for the biosynthesis of N^{ϵ} -acetyl- β -lysine and growth at high salinity. *Appl Environ Microbiol* **69**, 6047-6055.
- Pollard S. J. T., Montgomery D. M., Sollars C. J., Perry R. (1991). Organic compounds in the cement-based stabilisation/solidification of hazardous mixed wastes-mechanistic and process considerations. *J Hazard Mater* **28**, 313-327.
- Poolman B. and Glaesker E. (1998). Regulation of compatible solute accumulation in bacteria. *Mol Microbiol* **29**, 397-407.
- Postma P. W. and Lengeler J. W. (1985). Phosphoenolpyruvate: carbohydrate phosphotransferase system of bacteria. *Microbiol Rev* **49**: 232-269.
- Prasad J., McJarrow P., Gopal P. (2003). Heat and osmotic stress responses of probiotic *Lactobacillus rhamnosus* HN001 (DR20) in relation to viability after drying. *Appl Environ Microbiol* **69**, 917-925.

- Prescott F. J., Shaw J. K., Bilello P., Cragwall G. O. (1953). Gluconic acid and its derivatives. *Ind Eng Chem* **45**, 338-342.
- Prescott S. C. and Dunn C. G. (1959). *Industrial Microbiology*. New York, NY: McGraw-Hill.
- Pronk J. T., Levering P. R., Olijve W., Van Dijken J. P. (1989). Role of NADP-dependent and quinoprotein glucose dehydrogenase in gluconic acid production by *Gluconobacter oxydans*. *Enzyme Microb Technol* **11**, 160-164.
- Prust C., Hoffmeister M., Liesegang H., Wiezer A., Fricke W. F., Ehrenreich A., Gottschalk G., Deppenmeier U. (2005). Complete genome sequence of the acetic acid bacterium *Gluconobacter oxydans*. *Nat Biotechnol* **23**, 195-200.
- Rabenhorst J., Gatfield I., Hilmer J. M. (2001). Natural, aliphatic and thiocarboxylic acids obtainable by fermentation and a microorganism therefore. *EP Patent 1,078,990*.
- Rabinow P. (1996). *Making PCR: A story of biotechnology*. University of Chicago Press.
- Raspor P. and Goranovič D. (2008). Biotechnological Applications of Acetic Acid Bacteria. *Crit Rev Biotechnol* **28**, 101-124.
- Rauch B., Pahlke J., Schweiger P., Deppenmeier U. (2010). Characterization of enzymes involved in the central metabolism of *Gluconobacter oxydans*. *Appl Microbiol Biotechnol* **8**, 711-718
- Ravanel S., Cherest H., Jabrin S., Grunwald D., Surdin-Kerjan Y., Douce R., Rébeillé F. (2001). Tetrahydrofolate biosynthesis in plants: molecular and functional characterization of dihydrofolate synthetase and three isoforms of folylpolyglutamate synthetase in *Arabidopsis thaliana*. *Proc Natl Acad Sci U S A* **98**, 15360-15365.
- Record M. T., Courtenay E. S., Cayley D. S., Guttman H. J. (1998). Responses of *E. coli* to osmotic stress: large changes in amounts of cytoplasmic solutes and water. *Trends Biochem Sci* **23**, 143-148.
- Reshetilov A. N., Donova M. V., Dovbnaya D. V., Il'yasov P. V., Boronin A. M., Leasers T., Green R. (1998). Membrane-bound dehydrogenases of *Gluconobacter oxydans*: sensors for measuring sugars, alcohols, and polyoles. *Bull Exp Biol Med* **126**, 702-704.
- Reshetilov A. N., Iliasov P. V., Donova M. V., Dovbnaya D. V., Boronin A. M., Leathers T. D., Greene R. V. (1997). Evaluation of a *Gluconobacter oxydans* whole cell biosensor for amperometric detection of xylose. *Biosens Bioelectron* **12**, 241-247.
- Richhardt J., Bringer S., Bott M. (2012). Mutational analysis of the pentose phosphate and Entner–Doudoroff pathways in *Gluconobacter oxydans* reveals improved growth of a Δ edd Δ eda mutant on mannitol. *Appl Environ Microbiol* **78**, 6975-6986.
- Richhardt J., Luchterhand B., Bringer S., Büchs J., Bott M. (2013a). Evidence for a key role of cytochrome *bo*₃ oxidase in respiratory energy metabolism of *Gluconobacter oxydans*. *J Bacteriol* **195**, 4210-4220.

- Richhardt J., Bringer S., Bott M. (2013b). Role of the pentose phosphate pathway and the Entner-Doudoroff pathway in glucose metabolism of *Gluconobacter oxydans* 621H. *Appl Microbiol Biotechnol* **97**, 4315-4323.
- Riesenberg D., Schulz V., Knorre W. A., Pohl H. D., Korz D., Sanders E. A., Ross A., Deckwer W. D. (1991). High cell density cultivation of *E. coli* at controlled specific growth rate. *J Biotechnol* **20**, 17-28.
- Roeßler M. and Müller V. (2001). Osmoadaptation in bacteria and archaea: common principles and differences. *Environ Microbiol* **3**, 743-754.
- Rosenberg M., Svitel J., Rosenbergova I., Sturdik E. (1993). Optimization of sorbose production from sorbitol by *Gluconobacter oxydans*. *Acta Biotechnol* **35**, 269-274.
- Rossmann M. G., Moras D., Olsen K. W. (1974). Chemical and biological evolution of a nucleotide-binding protein. *Nature* **250**, 194-199.
- Rožanov A. S., Zagrebely S. N., Beklemishev A. B. (2009). Cloning of *Escherichia coli* K12 xylose isomerase (glucose isomerase) gene and studying the enzymatic properties of its expression product. *Appl Biochem Microbiol* **45**, 31-37.
- Rübenhagen R., Rönsch H., Jung H., Krämer R., Morbach S. (2000). Osmosensor and osmoregulator properties of the betaine carrier BetP from *Corynebacterium glutamicum* in proteoliposomes. *J Biol Chem* **275**, 735-741.
- Rutherford K., Parkhill J., Crook J., Horsnell T., Rice P., Rajandream M. A., Barrell B. (2000). Artemis: sequence visualization. *Bioinformatics* **16**, 944-5.
- Saeki A., Theeragool G., Matsushita K., Toyama H., Adachi O. (1997). Development of thermotolerant acetic acid bacteria useful for vinegar fermentation at higher temperatures. *Biosci Biotechnol Biochem* **61**, 138-145.
- Saha B. C. (2004). Purification and characterization of a novel mannitol dehydrogenase from *Lactobacillus intermedius*. *Biotechnol Prog* **20**, 537-542.
- Saha B. C. and Racine F. M. (2008). Production of mannitol by lactic acid bacteria: a review. In: C. T. Hou. and J. F. Shaw (Eds.), *Biocatalysis and bioenergy*. Hoboken: Wiley. pp. 391-404.
- Saha B. C. and Racine F. M. (2011). Biotechnological production of mannitol and its applications. *Appl Microbiol Biotechnol* **89**, 879-891.
- Saito Y., Ishii Y., Hayashi H., Imao Y., Akashi T., Yoshikawa K., Noguchi Y., Soeda S., Yoshida M., Niwa M., Hosoda J., Shimomura K. (1997). Cloning of genes coding for L-sorbose and L-sorbose dehydrogenases from *Gluconobacter oxydans* and microbial production of 2-keto-L-gulonate, a precursor of L-ascorbic acid, in a recombinant *G. oxydans* strain. *Appl Environ Environ Microbiol* **63**, 454-460.
- Saito Y., Ishii Y., Hayashi H., Yoshikawa K., Noguchi Y., Yoshida S., Soeda S., Yoshida M. (1998). Direct fermentation of 2-keto-L-gulonic acid in recombinant *Gluconobacter oxydans*. *Biotechnol Bioeng* **58**, 309-315.

- Sambrook J. and Russell D. W. (2001). *Molecular Cloning: A Laboratory Manual*. 3rd ed. Cold Spring Harbor New York: Cold Spring Harbor Laboratory Press.
- Sanan-Mishra N., Pham X. H., Sopory S. K., Tuteja N. (2005). Pea DNA helicase 45 overexpression in tobacco confers high salinity tolerance without affecting yield. *Proc Natl Acad Sci U S A* **102**, 509-514.
- Sand M., Mingote A. I., Santos H., Müller V., Averhoff B. (2013). Mannitol, a compatible solute synthesized by *Acinetobacter baylyi* in a two-step mechanism including a salt-induced and salt-dependent mannitol-1-phosphate dehydrogenase. *Environ Microbiol* **15**, 2187-2197.
- Sand M., Rodrigues M., González J. M., de Crécy-Lagard V., Santos H., Müller V., Averhoff B. (2015). Mannitol-1-phosphate dehydrogenases/phosphatases: a family of novel bifunctional enzymes for bacterial adaptation to osmotic stress. *Environ Microbiol* **17**, 711-719.
- Santoro M. M., Liu Y., Khan S. M. A., Hou L., Bolen D. W. (1992). Increased thermal stability of proteins in the presence of naturally occurring osmolytes. *Biochem* **31**, 5278-5283.
- Sapunova L. I., Lobanok A. G., Kazakevich I. O., Evtushenkov A. N. (2004). A plate method to screen for microorganisms producing xylose isomerase. *Microbiology* **73**, 107-112.
- Sapunova L. I., Parakhnya E. V., Kazakevich I. O., Lobanok A. G. (1999). Biosynthesis and some properties of the xylose (glucose) isomerases of *Streptomyces viridobrunneus* GI-97 and *Streptomyces sp.* 1 and 32. *Microbiology* **68**, 270-275.
- Sayyed R. Z., Shimpi G. B., Chincholkar S. B. (2010). Constitutive production of extracellular glucose isomerase by an osmophilic *Aspergillus sp.* under submerged conditions. *J Food Sci Technol* **47**, 496-500.
- Schedel M. (2000). Regioselective oxidation of aminosorbitol with *Gluconobacter oxydans*, key reaction in the industrial synthesis of 1-deoxynojirimycin. In: D. R. Kelly (Ed.), *Biotechnology: Biotransformations II*. vol 8b. 2nd ed. Weinheim: Wiley-VCH. pp. 296-308.
- Schneider K. H. and Giffhorn F. (1989). Purification and properties of a polyol dehydrogenase from the phototrophic bacterium *Rhodobacter sphaeroides*. *Eur J Biochem* **184**, 15-19.
- Schneider K. H., Giffhorn F., Kaplan S. (1993). Cloning, nucleotide sequence and characterization of the mannitol dehydrogenase gene from *Rhodobacter sphaeroides*. *J Gen Microbiol* **139**, 2475-2484.
- Schroeder A., Mueller O., Stocker S., Salowsky R., Leiber M., Gassmann M., Lightfoot S., Menzel W., Granzow M., Ragg T. (2006). The RIN: an RNA integrity number for assigning integrity values to RNA measurements. *BMC Mol Biol* **7**, 3.
- Schweiger P., Gross H., Deppenmeier U. (2010). Characterization of two aldo-keto reductases from *Gluconobacter oxydans* 621H capable of regio- and stereoselective α -ketocarbonyl reduction. *Appl Microbiol Biotechnol* **87**, 1415-1426.

- Schweiger P., Volland S., Deppenmeier U. (2007). Overproduction and characterization of two distinct aldehyde-oxidizing enzymes from *Gluconobacter oxydans* 621H. *J Mol Microbiol Biotechnol* **13**, 147-155.
- Shane B. (1989). Folylpolyglutamate synthesis and role in the regulation of one-carbon metabolism. *Vitam Horm* **45**, 263-335.
- Shen B., Jensen R. G., Bohnert H. J. (1997). Mannitol protects against oxidation by hydroxyl radicals. *Plant Physiol* **115**, 527-532.
- Shepherd M. D., Kharel M. K., Bosserman M. A., Rohr J. (2010). Laboratory maintenance of *Streptomyces* species. *Curr Protoc Microbiol* **10**, DOI: 10.1002/9780471729259.mc10e01s18.
- Shibata T., Ichikawa C., Matsuura M., Takata Y., Noguchi Y., Saito Y., Yamashita M. (2000). Cloning of a gene for D-sorbitol dehydrogenase from *Gluconobacter oxydans* G624 and expression of the gene in *Pseudomonas putida* IFO3738. *J Biosci Bioeng* **89**, 463-468.
- Shiloach J. and Fass R. (2005). Growing *E. coli* to high cell density: a historical perspective on method development. *Biotechnol Adv* **23**, 345-357.
- Shinagawa E., Matsushita K., Adachi O., Ameyama M. (1982). Purification and characterization of D-sorbitol dehydrogenase from membrane of *Gluconobacter suboxydans* var. *α*. *Agric Biol Chem* **46**, 135-141.
- Shinagawa E., Matsushita K., Adachi O., Ameyama M. (1984). D-gluconate dehydrogenase, 2-keto-D-gluconate yielding, from *Gluconobacter dioxycetonicus*: purification and characterization. *Agric Biol Chem* **48**, 1517-1522.
- Shinjo M. and Toyama H. (2016). Industrial application of acetic acid bacteria (vitamin C and others). In: K. Matsushita., H. Toyama., N. Tonouchi., A. Okamoto-Kinuma (Eds.), *Acetic Acid Bacteria: Ecology and Physiology*. Japan: Springer. pp. 321-338.
- Shinjo M., Setoguchi Y., Hoshino T. (1990). Sorbose dissimilation in 2-keto-L-gulonic acid producing mutant UV10 derived from *Gluconobacter melanogenus* IFO 3293. *Agric Biol Chem* **54**, 2257-2263.
- Shinjo M., Tazoe M., Hoshino T. (2002). NADPH-dependent L-sorbose reductase is responsible for L-sorbose assimilation in *Gluconobacter suboxydans* IFO 3291. *J Bacteriol* **184**, 861-863.
- Sievers M. and Swings J. (2005). Family II. *Acetobacteraceae* Gillis and De Ley 1980. In: G. Garrity (Ed.), *Bergey's Manual of Systematic Bacteriology*. 2nd ed. New York: Springer. pp. 41-95.
- Sievers M., Gaberthüel C., Boesch C., Ludwig W., Teuber M. (1995). Phylogenetic position of *Gluconobacter* species as a coherent cluster separated from all *Acetobacter* species on the basis of 16S ribosomal RNA sequences. *FEMS Microbiol Lett* **126**, 123-126.

- Silberbach M., Maier B., Zimmermann M., Buchs J. (2003). Glucose oxidation by *Gluconobacter oxydans*: characterization in shaking flasks, scale-up and optimization of the pH profile. *Appl Microbiol Biotechnol* **62**, 92-98.
- Smirnoff N. (1993). The role of active oxygen in the response of plants to water deficit and desiccation. *New Phytol* **125**, 27-58.
- Soemphol W., Toyama H., Moonmangmee D., Adachi O., Matsushita K. (2007). L-sorbose reductase and its transcriptional regulator involved in L-sorbose utilization of *Gluconobacter frateurii*. *J Bacteriol* **189**, 4800-4808.
- Soini J., Ukkonen K., Neubauer P. (2008). High cell density media for *Escherichia coli* are generally designed for aerobic cultivations-consequences for large-scale bioprocesses and shake flask cultures. *Microb Cell Fact* **7**, 26. DOI: 10.1186/1475-2859-7-26.
- Sootsuwan K., Thanonkeo P., Keeratirakha N., Thanonkeo S., Jaisil P., Yamada M. (2013). Sorbitol required for cell growth and ethanol production by *Zymomonas mobilis* under heat, ethanol, and osmotic stresses. *Biotechnol Biofuels* **6**, 180-193
- Stahlberg A., Aman P., Ridell B., Mostad P., Kubista M. (2003). Quantitative real-time PCR method for detection of B-lymphocyte monoclonality by comparison of κ and λ immunoglobulin light chain expression. *Clin Chem* **49**, 51-59.
- Sugisawa T. and Hoshino T. (2002). Purification and properties of membrane-bound D-sorbitol dehydrogenase from *Gluconobacter suboxydans* IFO 3255. *Biosci Biotechnol Biochem* **66**, 57-64.
- Sugisawa T., Hoshino T., Fujiwara A. (1991). Purification and properties of NADPH-linked L-sorbose reductase from *Gluconobacter melanogenus* N44-1. *Agric Biol Chem* **55**, 2043-2049.
- Sugisawa T., Hoshino T., Masuda S., Nomura S., Setoguchi Y., Tazoe M., Shinjoh M., Someha S., Fujiwara A. (1990). Microbial production of 2-keto-L-gulonic acid from L-sorbose and D-sorbitol by *Gluconobacter melanogenus*. *Agric Biol Chem* **54**, 1201-1209.
- Sugisawa T., Ojima S., Matzinger P., Hoshino T. (1995). Isolation and characterization of a new vitamin C producing enzyme (L-gulono- γ -lactone dehydrogenase) of bacterial origin. *Biosci Biotechnol Biochem* **59**, 190-196.
- Sugiyama M., Suzuki S.-i., Tonouchi N., Yokozeki K. (2003). Cloning of the xylitol dehydrogenase gene from *Gluconobacter oxydans* and improved production of xylitol from D-arabitol. *Biosci Biotechnol Biochem* **67**, 584-591.
- Svitel J. and Sturdik E. (1994). Product yield and by-product formation in glycerol conversion to dihydroxyacetone by *Gluconobacter oxydans*. *J Ferment Technol* **78**, 351-355.
- Svitel J., Curilla O., Tkac J. (1998). Microbial cell-based biosensor for sensing glucose, sucrose or lactose. *Biotechnol Appl Biochem* **27**, 153-158.
- Tajrishi M. M., Vaid N., Tuteja R., Tuteja N. (2011). Overexpression of a pea DNA helicase 45 in bacteria confers salinity stress tolerance. *Plant Signal Behav* **6**, 1271-1275.

- Thauer R. K., Jungermann K., Decker K. (1977). Energy conservation in chemotrophic anaerobic bacteria. *Bacteriol Rev* **41**, 100-180.
- Timasheff S. N. (1992). A physicochemical basis for the selection of osmolytes by nature. In: G. N. Somero., C. B. Osmond., C. L. Bolis (Eds.), *Water and life: a comparative analysis of water relationships at the organismic, cellular, and molecular levels*. Berlin: Springer. pp. 70-84.
- Tkac J., Navratil M., Sturdik E., Gemeiner P. (2001). Monitoring of dihydroxyacetone production during oxidation of glycerol by immobilized *Gluconobacter oxydans* cells with an enzyme biosensor. *Enzyme Microb Technol* **28**, 383-388.
- Towbin H., Staehelin T., Gordon J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* **76**, 4350-4354.
- Toyama H., Soemphol W., Moonmangmee D., Adachi O., Matsushita K. (2005). Molecular properties of membrane-bound FAD-containing D-sorbitol dehydrogenase from thermotolerant *Gluconobacter frateurii* isolated from Thailand. *Biosci Biotechnol Biochem* **69**, 1120-1129.
- Tsukada Y. and Perlman D. (1972). The fermentation of L-sorbose by *Gluconobacter melanogenus*. III. Investigation of the metabolic pathway from sorbose to 2-keto-L-gulonic acid. *Biotechnol Bioeng* **14**, 1035-1038.
- Tsuzuki M., Moskvina O. V., Kuribayashi M., Sato K., Retamal S., Abo M., Zeilstra-Ryalls J., Gomelsky M. (2011). Salt stress-induced changes in the transcriptome, compatible solutes, and membrane lipids in the facultatively phototrophic bacterium *Rhodobacter sphaeroides*. *Appl Environ Microbiol* **77**, 7551-7559.
- van den Heuvel R. H., Curti B., Vanoni M. A., Mattevi A. (2004). Glutamate synthase: a fascinating pathway from L-glutamine to L-glutamate. *CMLS Cell Mol Life Sci* **61**, 669-681.
- Verhoff F. H., Boguslawski G., Lantero O. J., Schlager S. T., Jao Y. C. (1985). Glucose isomerase. In: M. Moo-Young., C. L. Cooney., A. E. Humphrey (Eds.), *Comprehensive Biotechnology*. vol 3. New York: Pergamon Press. pp. 837-859.
- Wach A. (1996). PCR-synthesis of marker cassettes with long flanking homology regions for gene disruptions in *S. cerevisiae*. *Yeast* **12**, 259-265.
- Weenk G., Olijve W., Harder W. (1984). Ketogluconate formation by *Gluconobacter* species. *Appl Microbiol Biotechnol* **20**, 400-405.
- Welsh D. T. (2000). Ecological significance of compatible solute accumulation by microorganisms: from single cells to global climate. *FEMS Microbiol Rev* **24**, 263-290.
- Welsh D. T. and Herbert R.A. (1999). Osmotically induced intracellular trehalose, but not glycine betaine accumulation promotes desiccation tolerance in *Escherichia coli*. *FEMS Microbiol Lett* **174**, 57-63.

- Whatmore A. M. and Reed R. H. (1990). Determination of turgor pressure in *Bacillus subtilis*: a possible role of K⁺ in turgor regulation. *J Gen Microbiol* **136**: 2521-2526.
- Wiegmann K., Hensler M., Wöhlbrand L., Ulbrich M., Schomburg D., Rabus R. (2014). Carbohydrate catabolism in *Phaeobacter inhibens* DSM 17395, a member of the marine *Roseobacter* clade. *Appl Environ Microbiol* **80**, 4725-4737.
- Winkler J. D., Garcia C., Olson M., Callaway E., Kao K. C. (2014). Evolved osmotolerant *Escherichia coli* mutants frequently exhibit defective N-acetylglucosamine catabolism and point mutations in cell shape-regulating protein MreB. *Appl Environ Microbiol* **80**, 3729-3740.
- Wisselink H. W., Weusthuis R. A., Eggink G., Hugenholtz J., Grobben G. J. (2002). Mannitol production by lactic acid bacteria: a review. *Int Dairy J* **12**, 151–161.
- Wood J. M. (2015). Bacterial responses to osmotic challenges. *J Gen Physiol* **145**, 381-388.
- Wovcha M. G., Steuerwald D. L., Brooks K. E. (1983). Amplification of D-xylose and D-glucose isomerase activities in *Escherichia coli* by gene cloning. *Appl Environ Microbiol* **45**, 1402-1404.
- Wu B., Wawrzynow A., Zylicz M., Georgopoulos C. (1996). Structure-function analysis of the *Escherichia coli* GrpE heat shock protein. *EMBO J* **15**, 4806-4816.
- Yamada Y., Hoshino K., Ishikawa T. (1997). The phylogeny of acetic acid bacteria based on the partial sequences of 16S ribosomal RNA: the elevation of the subgenus *Gluconoacetobacter* to the generic level. *Biosci Biotechnol Biochem* **61**, 1244-1251.
- Yamada Y., Wada M., Chibata I. (1979). Oxygen transfer as a parameter of automatic control of the continuous cultivation for the conversion of sorbitol to sorbose by *Acetobacter suboxydans*. *J Ferment Technol* **57**, 210-214.
- Yamada Y. (2016). Systematics of Acetic Acid Bacteria. In: K. Matsushita., H. Toyama., N. Tonouchi., and A. Okamoto-Kinuma (Eds.), *Acetic Acid Bacteria: Ecology and Physiology*. Japan: Springer. pp. 1-50.
- Yamada Y. and Yukphan P. (2008). Genera and species in acetic acid bacteria. *Int J Food Microbiol* **125**, 15-24.
- Yamanka K. (1975). D-xylose isomerase from *Lactobacillus brevis*. *Methods Enzymol* **41**, 466-471.
- Yamashita S., Uchimura T., Komagata K. (2004). Emendation of the genus *Acidomonas* Urakami, Tamaoka, Suzuki and Komagata 1989. *Int J Syst Evol Microbiol* **54**, 865-870.
- Yancey P. H. (2005). Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. *J Exp Biol* **208**, 2819-2830.
- Yancey P. H., Haner R. G., Freudenberger T. (1990). Effects of an aldose reductase inhibitor on osmotic effectors in rat renal medulla. *Am J Physiol* **259**, 733-738.

- Zachariou M. and Scopes R. K. (1986). Glucose-fructose oxidoreductase, a new enzyme isolated from *Zymomonas mobilis* that is responsible for sorbitol production. *J Bacteriol* **167**, 863-869.
- Zahid N. and Deppenmeier U. (2016). Role of mannitol dehydrogenase in osmoprotection of *Gluconobacter oxydans*. *Appl Microbiol Biotechnol* **100**, 9967-9978.
- Zahid N., Schweiger P., Galinski E., Deppenmeier U. (2015). Identification of mannitol as compatible solute in *Gluconobacter oxydans*. *Appl Microbiol Biotechnol* **99**, 5511-5521.
- Zou H., Wu Z., Xian M., Liu H., Cheng T., Cao Y. (2013). Not only osmoprotectant: betaine increased lactate dehydrogenase activity and L-lactate production in lactobacilli. *Bioresour Technol* **148**, 591-595.

Publications

Zahid N. and Deppenmeier U. (2016). Role of mannitol dehydrogenases in osmoprotection of *Gluconobacter oxydans*. *Appl Microbiol Biotechnol* **99**, 5511-5521.

Zahid N., Schweiger P., Galinski E., Deppenmeier U. (2015). Identification of mannitol as compatible solute in *Gluconobacter oxydans*. *Appl Microbiol Biotechnol* **99**, 5511-21.

Kosciow K., **Zahid N.**, Schweiger P., Deppenmeier U. (2014). Production of a periplasmic trehalase in *Gluconobacter oxydans* and growth on trehalose. *J Biotechnol* **189**, 27-35.

Bukhari D. A., **Zahid N.**, Shakoori A. R. (2013). Molecular characterization of *cry4B* gene from local isolates of *Bacillus thuringiensis* encoding 70 kDa crystal protein. *Pak J Zool* **45**, 779-791.

Zahid N., Zulfiqar S., Shakoori A. R. (2012). Functional analysis of *cus* operon promoter of *Klebsiella pneumoniae* using *E. coli lacZ* assay. *Gene* **495**, 81-88.

Conferences / Workshops / Symposia (Presented and Attended)

Annual Conference of the Association for General and Applied Microbiology March **2016**, Jena, Germany. (Poster presentation).

Annual Conference of the Association for General and Applied Microbiology **2015** Marburg, Germany. (Poster presentation).

Annual Conference of the Association for General and Applied Microbiology **2014** Dresden, Germany. (Poster presentation).

International Pakistan Congress of Zoology **2011** Muzaffarabad, Pakistan. (Oral presentation).

International Pakistan Congress of Zoology **2010** Faisalabad, Pakistan. (Oral Presentation).

International Pakistan Congress of Zoology **2009** Jamshoro, Pakistan. (Oral Presentation).

8. ACKNOWLEDGEMENTS

I am highly thankful to **Prof. Dr. Uwe Deppenmeier** for allowing me to come from Pakistan to work in his lab. Being a supervisor, he guided me with his valuable suggestions and ideas throughout my work period. It was due to his moral support and encouragement that I could complete my tasks successfully.

My special thanks to **Prof. Dr. Erwin A Galinski** to be a second referee of my work. Moreover, I am deeply indebted to him for providing me time to time guidance, spending his worthy time to discuss experimental problems, and to share his experience to resolve NMR spectra and physiology of *G. oxydans*.

I am thankful to **Prof. Dr. Lukas Schreiber** and **Prof. Dr. Sigurd Höger** for showing interest in my work and for being a part of my Ph.D defense committee. I am also obliged to **Prof. Dr. Florian MW Grundler** for granting me permission to use Bioanalyzer in his lab.

I am grateful to **Higher Education Commission of Pakistan** and their German collaborators **DAAD** for providing me a continuous financial support (scholarship) to stay in Germany and to complete my present work in a peaceful way.

I am gratified to all the members of the Institute for Microbiology and Molecular Biology, University Bonn. There is an endless list of persons whom I want to acknowledge. At first I am grateful to **Dr. Elisabeth Witt** and **Dr. Kati Wassman** for their help especially in dealing with HPLC instruments, **Dr. Fabian Grein** for assisting me in microscopic examinations of cell cultures, **Dr. Stefanie Berger** and **Dr. Maria Meyer** for providing moral and personal support. I am thankful to all my group members especially to Dipl. Biol. **Sarah Refai** for being so friendly and for explaining all the rules and regulations regarding lab work and thesis write up. My special thanks to two friends and colleagues M.Sc. **Lena Kröniger** and M. Sc. **Anna Siemen** for helping me time to time and for proofreading some parts of my write up. I am also grateful to M.Sc. **Konrad Kosciow** for providing me an electron micrograph of *G. oxydans* and for investing efforts to keep a harmony among lab fellows. In addition, I am thankful to M.Sc. **Thomas Franke** for being so understanding and sharing his new ideas regarding science and technology.

I want to pay special thanks to Dipl. Biol. **Elisabeth Schwab**, a person whom I cannot forget. You helped me not only in lab to deal with all the technical stuff regarding

8. Acknowledgements

instrumentation, but also invested energy and time to solve all my personal issues including translation of letters, filling formulae, finding kindergarten, and house etc. For all these, I am deeply indebted. You will always be in my memories. I also want to pay my deep regards to Ms. **Brigit Amendt** and Mrs. **Marlene Hecker** for providing technical assistance.

Last but not the least, I am thankful to my husband Mr. **Muhammad Shahzad Anjam** and my son **Hassan Shahzad** to be a backbone of my life. Without you two, it was nearly impossible for me to do my work. You people strengthened me to pass through all the ups and downs of life in a graceful way. May you live long and prosperous life ever. Of course, I cannot forget my **parents, brothers, family**, and my **teachers** from Pakistan who spent efforts to bring me to this point. I am always be indebted to you. Thank you.