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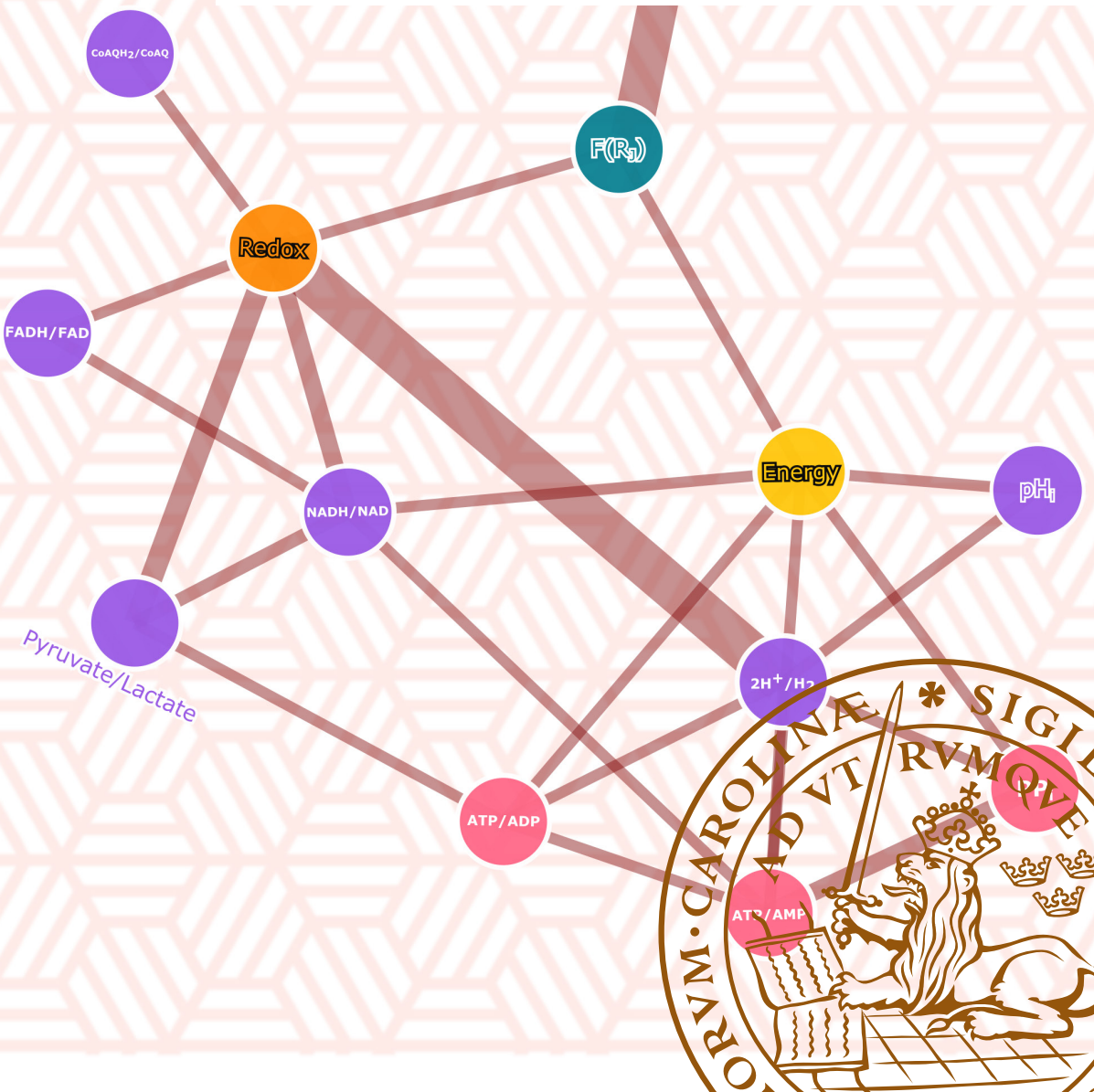

Explorations of interlinked energy and redox metabolism in two industrially applied microorganisms

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LUND
UNIVERSITY

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Title and subtitle: Explorations of interlinked energy and redox metabolism in two industrially applied microorganisms

Abstract: Anaerobic fermentation, compared to aerobic processes, remains the most scalable method for bioproduction of compounds. This is due to that aerobic microbial processes require large amounts of energy and are unable to satisfy the oxygen demand of a high-density microbial biomass. Thus, anaerobic microbes are readily scalable to large volumes. It was found that most anaerobic microbes have evolved with the Embden-Meyerhof-Parnas pathway for efficient and luxurious growth under these conditions due to adequate energy production provided by the pathway. It is also found that most of these microbes are able to establish a balanced redox and energy metabolism either by the use of anaerobic respiration or other forms of ATP synthesis.

Some, microbes however are of industrial relevance but are not capable of luxurious growth without the use of external electron acceptors or other external conditions. Among these microbes, the suspected issues are usually attributed to redox or energy imbalances. Thus, the thesis expands on our understanding of the regulatory mechanisms and explores possible engineering methods to improve their growth rates by focusing on two specific microbes, namely the bacterium *Limosilactobacillus reuteri* and the yeast *Saccharomyces cerevisiae.*

Oxygen tolerance of *Lb. reuteri* is important as it is one of the mechanisms to alleviate the redox imbalance. This study expands on the variations of oxygen tolerance between strains and shows that *Lb. reuteri* DSM 17938 does not necessarily produce more peroxide per biomass but has greater resistance than its counterparts. In parallel the various lactate dehydrogenases present in *Lb. reuteri* DSM 17938 were enzymatically characterised to explore the presence of alternative control mechanisms that may be present due to the simultaneous utilisation of two different central carbon pathways. The impact of overexpression of the native phosphofructokinase candidates which are predicted to be from a minor family revealed issues related to protein burden in lean media.

The introduction of a proton pumping pyrophosphatase (H^+-PPase) to supplement the proton pumping ATPase (H^+-ATPases) in *S. cerevisiae* was also explored. Under stressful conditions, the study revealed that the H^+-PPase could improve the growth rate and successfully act in restoring pH homeostasis. The H^+-PPase improved growth of *S. cerevisiae* in high acetic acid concentrations and showed that there may be more limiting factors in xylose engineered *S. cerevisiae*. The study also revealed new avenues for improving productivity for ethanol production using lignocellulosic biomass as well as possible alternative methods that could be implemented to increase production of existing compounds that are currently ATP limited.

Key words: Redox and energy balance, Embden-Meyerhof-Parnass pathway (EMP), Oxygen tolerance, Adenosine Triphosphate (ATP), Proton pumping pyrophosphatase (H^+-PPase), Proton pumping ATPase (H^+-ATPase)

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Popular Science Summary

Microorganisms form the backbone of life on this planet. They are responsible for key global elemental cycles (such as the carbon, nitrogen and phosphorus cycle) that maintain the balance of biologically available forms of nitrogen and phosphorus in the form of ammonium and phosphate which are essential for the sustenance of small and large organisms. The carbon cycle is also maintained by microorganisms to a large extent as they account for the conversion of the majority of carbon di oxide into sugars and vice versa.

Due to the variety of microorganisms present they are capable of producing and breaking down most organic material except for some synthetic materials such as teflon and plastics. These synthetic materials are causing long term damage to the environment as well as to our health. To enable the discovery and production of alternative materials for replacing these synthetic compounds, as well as to degrade existing synthetic compounds that have entered the environment, we need to widen the variety of microbes that we can cultivate. As of now we can cultivate only a small fraction of the microbes that are around us, and to expand this we need a better understanding of what limits their growth.

To expand our ability to cultivate a wider variety of microbes, there is a need to understand alternative central carbon pathways. The central carbon metabolism is a series of chemical reactions in which various forms of carbon are fixed or broken down to form energy carriers for growth and reproduction of the microbes. For microbial growth, the energy carrier is in the form of adenosine triphosphate (ATP) which is a molecule that releases energy when the phosphate bonds are broken. It can also be in the form of electrons that can be utilised in reactions where electrons are transferred between molecules by cofactors such as nicotinamide adenine dinucleotide (NAD^+), in what is called oxido-reductive reactions or 'redox reactions'. The amount of redox cofactors (NAD^+) and energy currency (ATP) formed is determined by the central carbon metabolism. This is well understood for the most common pathway which is the Embden-Meyerhoff-Parnas pathway but has to be expanded when microbes that use less-well studied pathways like the phosphoketolase pathway in *Limosilactobacillus reuteri*, which is a common probiotic microbe being grown industrially.

This thesis focuses on expanding our understanding in the relationship between redox carriers and energy in two specific microbes. One is *Limosilactobacillus reuteri* that prefers the use of a pathway that produces less energy (phosphoketolase pathway) even when a fully functional EMP pathway is present. This organism has evolved to colonise the gastrointestinal tract of various animals and thus understanding the physiology of its central carbon pathway can shed light into how this microbe can successfully compete with other gut microbes even when it has less energy available.

The second organism in focus is the yeast *Saccharomyces cerevisiae* that has been engineered with a xylose-utilising pathway. Xylose is a substantial part of lignocellulose, which is a promising substrate for biosynthesis of various value-added compounds. The pathway that was introduced by engineering reduces the total energy production rate while imposing a redox imbalance when grown on xylose. This is caused by a disconnect between the reduced and oxidised forms of NAD^+. The thesis focuses on using pyrophosphate as an additional energy carrier, which is a byproduct of cell growth and metabolism. My research showed that it increased robustness of the yeast when metabolizing in stressful environmental conditions as often seen in industrial fermentation processes.

Populärvetenskaplig sammanfattning

Mikroorganismer utgör grunden för liv på vår planet. De ansvarar för viktiga kretslopp av olika grundämnen (såsom kol-, kväve- och fosforcykeln) som upprätthåller balansen av biologiskt tillgängliga former av kväve och fosfor i form av ammonium och fosfat, vilket är helt avgörande för små och stora organismers överlevnad. Kolcykeln upprätthålls också till stor del av mikroorganismer eftersom de står för majoriteten av omvandlingen av koldioxid till socker och vice versa.

På grund av deras stora mångfald så kan mikroorganismer producera och bryta ned nästan allt organiskt material förutom vissa syntetiska material såsom teflon och plast. Dessa syntetiska material orsakar långsiktig skada på miljön och vår hälsa. För att möjliggöra upptäckt och produktion av alternativa material för att ersätta dessa syntetiska föreningar, samt för att bryta ned befintliga syntetiska föreningar som har släppts ut i miljön, behöver vi en bredare variation av mikroorganismer som vi kan odla. För närvarande kan vi bara odla en bråkdel av de mikroorganismer som finns runt omkring oss, och för att expandera detta behöver vi en bättre förståelse över vad som begränsar deras tillväxt.

För att öka vår förmåga att odla en bredare variation av mikrober finns det ett behov av att förstå alternativa vägar för kolmetabolism. Den centrala kolmetabolismen är en serie kemiska reaktioner där olika former av kol fixeras eller bryts ned för att bilda energibärare som behövs för mikrobernas tillväxt. För mikrobiell tillväxt bärs energin i form av adenosintrifosfat (ATP), vilket är en molekyl som frigör energi när dess fosfatbindningar bryts. Energin kan också vara i form av elektroner som kan användas i reaktioner där elektroner överförs mellan molekyler med hjälp av cofaktorer såsom nikotinamidadenindinukleotid (NAD^+), i vad som kallas 'redoxreaktioner'. Mängden redox-cofaktorer (NAD^+) och energivaluta (ATP) som bildas bestäms främst av den centrala kolmetabolismen. Detta är välstuderat för den vanligaste vägen som är Embden-Meyerhoff-Parnas-vägen (EMP) men måste utforskas mer när det gäller mikroorganismer som använder mindre studerade vägar så som fosfoketolasvägen i *Limosilactobacillus reuteri*, vilket är en vanligt förekommande probiotisk mikroorganism som odlas i industriell skala.

Denna avhandling fokuserar på att fördjupa vår förståelse för förhållandet mellan redoxbärare och energi i två specifika mikroorganismer. Den ena är *Limosilactobacillus reuteri* som föredrar att använda en väg som producerar mindre energi (fosfoketolasvägen) även när en fullt fungerande EMP-väg är tillgänglig. Denna organism har utvecklats för att kolonisera mag-tarmkanalen hos olika djur och förstå fysiologin för dess centrala kolmetabolismen kan därför belysa hur denna mikrob framgångsrikt kan konkurrera med andra tarmmikrober även när den har mindre energi tillgänglig.

Den andra organismen i fokus är en stam av jästen *Saccharomyces cerevisiae* som har modifierats för att kunna använda xylos. Xylos utgör en väsentlig del av

lignocellulosa, vilket är ett lovande substrat för biologisk produktion av olika värdefulla ämnen. Den metabola vägen som har introducerats minskar den totala energiproduktionshastigheten samtidigt som den inför en redox-obalans när jästen odlas på xylos. Detta beror på att jämvikten mellan de reducerade och oxiderade formerna av NAD^+ påverkas. Avhandlingen fokuserar på att använda pyrofosfat som ytterligare en energibärare. Pyrofosfat är en biprodukt från celltillväxt och metabolism. Min forskning visade att det ökade jästens robusthet när den odlas under stressiga miljöförhållanden, vilket ofta ses i industriella jäsningsprocesser.

Abstract

Anaerobic fermentation, compared to aerobic processes, remains the most scalable method for bioproduction of compounds. This is due to that aerobic microbial processes require large amounts of energy and are unable to satisfy the oxygen demand of a high-density microbial biomass. Thus, anaerobic microbes are readily scalable to large volumes. It was found that most anaerobic microbes have evolved with the Embden-Meyerhof-Parnas pathway for efficient and luxurious growth under these conditions due to adequate energy production provided by the pathway. It is also found that most of these microbes are able to establish a balanced redox and energy metabolism either by the use of anaerobic respiration or other forms of ATP synthesis.

Some microbes, however, are of industrial relevance but are not capable of luxurious growth without the use of external electron acceptors or other external conditions. Among these microbes, the suspected issues are usually attributed to redox or energy imbalances. Thus, the thesis expands on our understanding of the regulatory mechanisms and explores possible engineering methods to improve their growth rates by focusing on two specific microbes, namely the bacterium *Limosilactobacillus reuteri* and the yeast *Saccharomyces cerevisiae.*

Oxygen tolerance of *Lb. reuteri* is important as it is one of the mechanisms to alleviate the redox imbalance. This study expands on the variations of oxygen tolerance between strains and shows that *Lb. reuteri* DSM 17938 does not necessarily produce more peroxide per biomass but has greater resistance than its counterparts. In parallel the various lactate dehydrogenases present in *Lb. reuteri* DSM 17938 were enzymatically characterised to explore the presence of alternative control mechanisms that may be present due to the simultaneous utilisation of two different central carbon pathways. The impact of overexpression of the native phosphofructokinase candidates which are predicted to be from a minor family revealed issues related to protein burden in lean media.

The introduction of a proton pumping pyrophosphatase (H^+-PPase) to supplement the proton pumping ATPase (H^+-ATPases) in *S. cerevisiae* was also explored. Under stressful conditions, the study revealed that the H^+-PPase could improve the growth rate and successfully act in restoring pH homeostasis. The H^+-PPase improved growth of *S. cerevisiae* in high acetic acid concentrations and showed that there may be more limiting factors in xylose engineered *S. cerevisiae*. The study also revealed new avenues for improving productivity for ethanol production using lignocellulosic biomass as well as possible alternative methods that could be implemented to increase production of existing compounds that are currently ATP limited.

List of Papers

The thesis is based on the following papers and manuscripts, which are referred to by roman numerals.

I. Variation in oxygen tolerance among *Limosilactobacillus reuteri* strains

Larsson C., Sreenivas K., Linares-Pastén J.A., Artsanchorn P., Koendjbiharie J., Håkansson S., van Niel E.W.J

II. A comparison of different mechanisms for lactate dehydrogenase regulation at protein level from three Gram-positive bacteria

Sreenivas K, Mohsin D., Linares-Pastén J.A., van Niel E.W.J.

III. Manipulation of EMP flux and its impact on growth of *Limosilactobacillus reuteri*

Sreenivas K., Musaj Rangel T., Carlquist M., van Niel E.W.J.

IV. Evaluation of pyrophosphate-driven proton pumps in *Saccharomyces cerevisiae* under stress conditions

Sreenivas K., Eisentraut L., Brink D.P., Persson V.C., Carlquist M., Grauslund M.G., van Niel E.W.J. *Microorganisms*. 2024; 12(3):625. https://doi.org/10.3390/microorganisms12030625

Author's contribution to the papers

Paper I

I participated in experimental work and contributed to the bioinformatic analysis. I performed enzyme assays and data analysis. I also contributed to the drafting of the manuscript.

Paper II

I participated in the experimental design with my supervisor. I carried out the planning and execution of the experiments together with my students Dima mohsin. I performed the data analysis and first draft of the manuscript.

Paper III

I engaged in the experimental design with my supervisor and co-supervisor. I carried out the planning and execution of the experiments. I trained a bachelor's thesis student Teuta Musaj who performed part of the experiments. I trained a project student Anirudh Kulkarni who optimised the staining protocol used. I supervised Teuta Musaj on her data analysis and conducted further analysis. I drafted the manuscript.

Paper IV

I was engaged in the experimental design with my supervisor and other authors. I supervised a master's student Leon Eisentraut who performed batch cultivations with pHluorin. I performed the remaining batch cultivations. I performed the data analysis and drafted the manuscript.

Other scientific contributions

This is a list of my scientific contributions that are not part of the thesis.

- Bhattacharya, A., Majtorp, L., Birgersson, S., Wiemann, M., Sreenivas, K., Verbrugghe, P., Van Aken, O., van Niel, E.W.J., Stålbrand, H., 2022. Cross-Feeding and Enzymatic Catabolism for Mannan-Oligosaccharide Utilization by the Butyrate-Producing Gut Bacterium *Roseburia hominis* A2-183. Microorganisms 10, 2496. https://doi.org/10.3390/microorganisms10122496
- Byrne, E., Björkmalm, J., Bostick, J.P., Sreenivas, K., Willquist, K., van Niel, E.W.J., 2021. Characterization and adaptation of *Caldicellulosiruptor* strains to higher sugar concentrations, targeting enhanced hydrogen production from lignocellulosic hydrolysates. Biotechnology for Biofuels 14, 210. https://doi.org/10.1186/s13068-021-02058-x
- Trauger, M., Hile, A., Sreenivas, K., Shouse, E.M., Bhatt, J., Lai, T., Mohandass, R., Tripathi, L., Ogden, A.J., Curtis, W.R., 2022. CO_2 supplementation eliminates sugar-rich media requirement for plant propagation using a simple inexpensive temporary immersion photobioreactor. Plant Cell Tiss Organ Cult 150, 57–71. https://doi.org/10.1007/s11240-021-02210-3
- Vongkampang, T., Sreenivas, K., Engvall, J., Grey, C., van Niel, E.W.J., 2021. Characterization of simultaneous uptake of xylose and glucose in *Caldicellulosiruptor kronotskyensis* for optimal hydrogen production. Biotechnology for Biofuels 14, 91. https://doi.org/10.1186/s13068-021-01938-6
- Vongkampang, T., Sreenivas, K., Grey, C., van Niel, E.W.J., 2023. Immobilization techniques improve volumetric hydrogen productivity of *Caldicellulosiruptor* species in a modified continuous stirred tank reactor. Biotechnology for Biofuels and Bioproducts 16, 25. https://doi.org/10.1186/s13068-023-02273-8

Abbreviations

EMP	Embden-Meyerhof-Parnas pathway
PKP	Phosphoketolase pathway
AcP	Acetyl phosphate
ATP	Adenosine Triphosphate
NAD^+	Nicotinamide Adenine dinucleotide
NADH	Nicotinamide Adenine dinucleotide (reduced)
PP_i	Inorganic pyrophosphate
pH_i	Intracellular pH
pH_c	Cytosolic pH
LDH	Lactate dehydrogenase
PYK	Pyruvate kinase
PFK	Phosphofructokinase
XR	xylose reductase
XDH	xylitol dehydrogenase
XK	xylulokinase
H^+-PPase	proton pumping pyrophosphatase
H^+-ATPase	ATP dependent proton pumping phosphatase

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1 The need for expanding industrially relevant microbes

A select number of microorganisms retain a large preference for industrial production of various compounds [1]. These microbes have the advantage of being optimised for production by the long use through history, and later through established toolboxes for engineering [2]. These industrial processes are now faced with new challenges because of various factors, such as limitations in the metabolic pathways present in the microbes, ability of current microbes to produce new pharmaceutical compounds and so on [2].

Any limitation in the metabolic pathways present in existing industrial microbes hinders their use in upscaling bioprocesses for production of value-added compounds. These processes are essential to tackle climate change and to introduce a cyclic economy. Some examples of this are the production of biofuels, such as methane, ethanol and butanol from compost waste, animal husbandry waste and agricultural/forestry waste [3]. Other examples are recycling plastic waste [4], production of microbial polymeric matrices for alternative clothing material and leather [5].

Recently, a few companies are focussing on using nonconventional microbes for production of various metabolites and compounds [6]. One such company is Lanza biotech, that pioneers the use of C1 compounds (CO_2) for production of biofuels using *Clostridium autoethanogenum* [7].

The ease of use of the microbes currently applied in industry is enabled by the understanding of their central carbon pathways that are required for producing energy and redox equivalence for the biochemical reactions that enable growth. To use waste streams for production of high-value compounds, alternative central carbon pathways are required. To this effect, an understanding of limitations in organisms that do not use the Embden-Meyerhoff-Parnas (EMP) pathway could shed light in the adoption of new microbes for industry as well as engineering existing microbes for new metabolic avenues.

1.1 Diversity in regulation of EMP Pathway

The collective works of numerous scientists over a period of almost a century, led to the elucidation of the most canonical pathway for glucose metabolism, the Embden-Meyerhof-Parnas pathway. The studies elucidating the EMP pathway were finalised in 1940, and it remains the most well studied of the central carbon pathways [8]. This pathway (Figure 1) converts the 6-carbon sugar, glucose to two 3-carbon moieties (pyruvate).

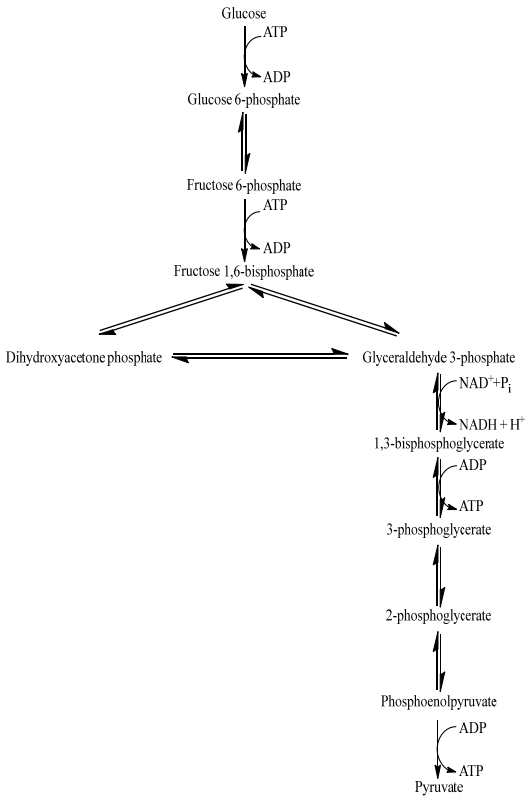


Figure 1: The Embden Mayerhoff parnas pathway.

The process begins with phosphorylating the sugar in two individual steps. The initial step after the facilitated uptake of glucose is phosphorylation. In higher eukaryotes this is facilitated by ATP-dependent kinases [9]. However, in at least a third of bacteria this is facilitated by a sugar phosphotransferase (PTS) system [10]. This is followed by phosphorylation of the other end of the 6-carbon compound to make the entire sugar usable. The resultant fructose 1,6 bisphosphate is split into glyceraldehyde 3 phosphate (G3P) and dihydroxyacetone phosphate. The latter is

converted to G3P, and a cascade of reaction leads to the formation of pyruvate with the net production of 2 molecules of ATP and 2 molecules of NADH per glucose molecule.

Microbes that are capable of aerobic (O_2) or anaerobic respiration (NO_3^-, SO_4^{2-}) can convert the redox equivalence (NADH) into energy (ATP) via a proton motive force [11], [12]. The involved electron transport chains can be regulated to establish a redox balance and elevated energy levels [13]. Microbes that lack an electron transport chain, rely on substrate level phosphorylation to recover the redox equivalence, and are termed as fermenters. Some aerobic microbes are capable of fermentation in anaerobic conditions and these microbes can be subject to redox imbalances when under additional stress conditions. This is the case for *S. cerevisiae* that is grown under anaerobic conditions in the presence of phenolic compounds [14].

Due to the ubiquitous nature of the EMP, it is regulated at the transcription, translation, and protein levels [15], [16], [17]. The nature and quantity of regulation are varied and based on the overall metabolism of the microorganisms.

1.1.1 Regulation of the EMP pathway using energy carriers

The breakdown of sugars leads to the production of energy in the form of phosphate bonds (ATP) and electron donors (NADH + H^+, and NAD(P)H + H^+) [18]. The reactions generating or using these cofactors are key points as these reactions are considered irreversible in the EMP pathway [18]. These molecules are used to drive biochemical reactions towards the direction favourable for microbes. The concentration of these molecules can regulate the activity of the enzymes in the EMP pathway [19].

Energy in biology is mainly obtained by breaking phosphate bonds [18]. This is primarily carried out using adenosine triphosphate (ATP), which is a small molecule made of covalently bonded nucleoside (adenine), sugar (ribose) and three phosphate groups. Some organisms are also capable of using adenosine diphosphate (ADP) as well as inorganic pyrophosphate (PP_i). These molecules act as a phosphate donor to drive phosphorylation reactions such as sugar phosphorylation, protein phosphorylation, etc. ATP is also involved in active uptake or excretion of some compounds as well as cations. It can function as an intracellular response/signalling molecule in addition to maintenance of intracellular pH (pH_i), and intracellular cation concentrations (Na^+, Mg^{2+}, Ca^{2+}).

Redox equivalence or redox cofactors (NAD^+, $NADP^+$, FAD^+) are involved in oxidation-reduction reactions, which are a major part of biological processes. These cofactors can store electrons released by the oxidation of sugars and drive the reactions that require energy in the form of electrons. Examples are reactions involving detoxification and reactive oxygen species response as well as substrate

level regeneration (e.g. reduction of pyruvate to lactate). These cofactors share the ADP nucleotide, and ribose with the addition of either nicotinamide or flavine moieties instead of a regular nucleic base in the second nucleotide.

In all organisms the concentrations of the energy carriers (ATP, ADP, AMP) and redox cofactors (NADH, NAD^+), among other metabolites, regulates the activity of the enzymes in the EMP pathway [20], [21], [22], [23]. These enzymes have variations in their amino acid sequences making them susceptible to detect and respond to variations in the intracellular conditions based on the concentration or formation fluxes of these cofactors [21]. This response is especially important under stress conditions that lead to a depletion of ATP or redox cofactors. For instance, cell death becomes likely if ATP production and depletion through regulation of the glycolytic pathway is not maintained, because ATP is required for the first half of EMP pathway.

1.1.2 Regulation using intermediate metabolites

The concentrations of the intermediate metabolites of the EMP also regulates the activity of the downstream reactions. A classic example is the feed forward activation of pyruvate kinase and lactate dehydrogenase (LDH) by the concentration of fructose 1,6 bisphosphate (FBP) [24], [25], [26]. This method of regulation is also conserved in higher organisms. In *Saccharomyces cerevisiae* only the pyruvate kinase is regulated by this mechanism as no native LDH is present [27], [28].

1.1.3 Other regulatory mechanisms

The response of the enzymes involved in the EMP pathway is related to their enzymatic properties at optimal conditions. These optimal conditions are established by a combination of numerous factors, such as intracellular pH (pH_i), substrate, product, and cofactor concentrations [29], [30]. The extracellular conditions also affect the intracellular environment and thus the enzyme efficiency. The enzyme activity efficiencies for the various reactions in the pathway defines the total flux through that pathway [16].

The pH_i is assumed to be at near neutral conditions. This is supported by strong evidence showing that the catalytic activity of the EMP pathway enzymes are directly affected by changes in pH beyond the neutral range [16], [31]. However, some microbes, such as *Lactobacillus acidophilus,* can maintain enzyme activity in the EMP pathway even when the pH_i approaches 4.5 [32].

1.2 Scope of the Thesis

This thesis focuses on chemo-organo-heterotrophic microbes. These microbes require organic compounds such as sugars for its growth. Of all chemo-organo-heterotrophs many microbes still elude cultivation in the laboratory environment [33], [34], [35]. Of this small subset of cultivable microbes, the most successful laboratory and industrial microbes are likely to use the EMP pathway making it the most well understood pathway. This pathway is also used as a benchmark to understand other pathways [36], [37], [38]. This thesis focuses on two microorganisms possessing alternative pathways as case studies.

The first organism *Limosilactobacillus reuteri* uses the phosphoketolase pathway (PKP) as its main central carbon pathway and uses the EMP pathway as a shunt. This organism is of interest due to its ability to produce industrially relevant compounds as well as its probiotic properties. To understand the probiotic properties a clearer picture of the metabolic limitations of the unique central carbon metabolism must be obtained. **Paper I, II** and **III** elucidates the various characteristics of *Lb. reuteri* and variations among its strains. Most research with this microbe uses rich media such as Man-Rogosa Sharpe (MRS) medium. This medium has various electron acceptors in addition to the substrate, which might interfere with our study focus. We are interested in understanding the mechanisms involved in mitigating the various restrictions posed by its central carbon metabolism, and thus, defined and semi defined media are essential to avoid multiple interpretations.

Another organism in focus is an engineered strain of *Saccharomyces cerevisiae* that is capable of fermenting xylose using the oxido-reductive xylose pathway. This organism has limitations in its ATP formation flux on xylose under anaerobic conditions. **Paper IV** reports on an alternative method for restoring pH homeostasis that can relieve the ATP burden under stressful conditions.

2 Applying redox and energy balances to understand growth

A comprehensive genomic analysis revealed that anaerobic microbes overwhelmingly rely on the adequate ATP yield of the EMP pathway (Figure 2) [39]. This begs the question as to why some microbes use alternative pathways under the same constraints.

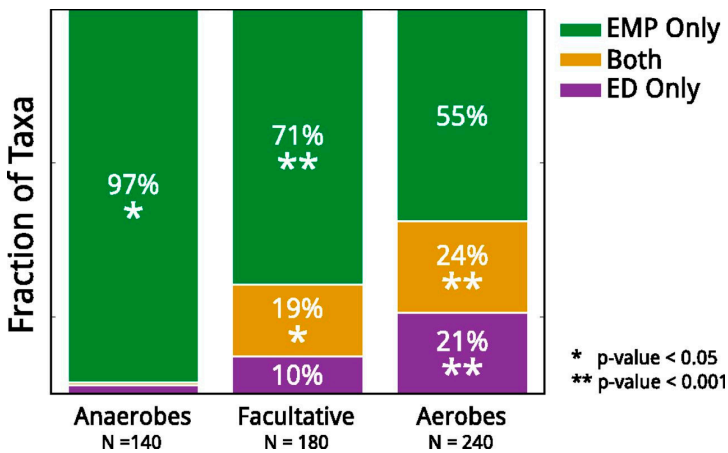


Figure 2: Microbial glycolytic pathway in relation to its oxygen requirement. Adapted from Flamholz et al., (2013)[39]

To expand our understanding of microbial growth under anaerobic conditions, a simplified toolbox based on redox and energy formation fluxes could be employed. The toolbox utilizes only the formation fluxes as this is mainly correlated to the central carbon metabolism, whereas the consumption of energy and redox occurs in many more reactions making it difficult to follow. This difficulty becomes more prevalent when organisms are under stress as these mechanisms are generally upregulated only during exposure to such stress conditions [37], [40], [41]. This is because ATP is generally considered as the driving force due to the free energy (ΔG) of 50 kJ/mol produced by breaking the phosphate bonds under physiological conditions [42]. If we consider the redox cofactors, microbes with aerobic or anaerobic respiration chains can convert this into energy. In the case of microbes

that lack a respiratory chain, they still require the redox cofactors for oxido-reductive reactions. If we take a closer look at the oxido-reductive reactions the reductive potential of the reaction is proportional to the ΔG required for the reaction and thus these cofactors can be interpreted as a form of potential energy. Since oxido-reductive reactions involve a redox couple each half of the couple is provided a standard reduction potential ($\Delta E_0'$) and ΔG can be calculated from a formula (Figure 3, Equation 1).

$$\Delta G^{\circ\prime} = -nF \cdot \Delta E_0' \quad (1)$$

Where ΔG°' is the Gibbs free energy, n is the number of electrons being transferred, and F is the Faraday constant (96.5 kJ/mol). An example being the free energy released by the substrate level reduction of pyruvate to lactate produces -25.1 kJ/mol [43].

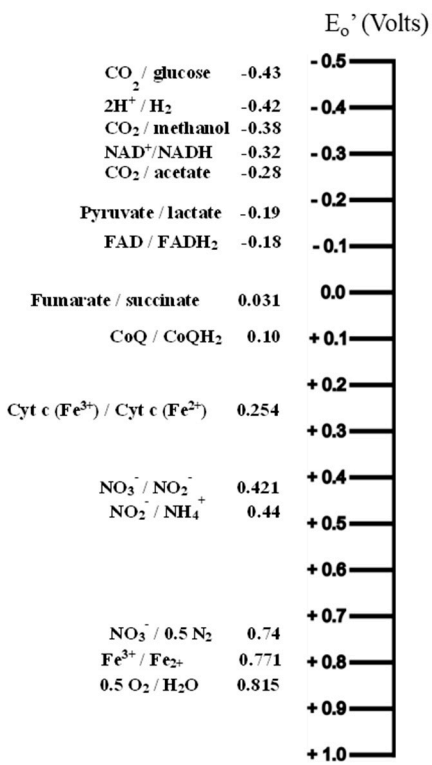


Figure 3: The redox tower: The reduction potentials measured in electon volts (E$_o$') with the difference of electron potential for the most common redox couples.

By considering ATP as kinetic and redox as the potential energy we can consider the growth rate as a function of a ratio of these fluxes (Equation 2):

$$\mu = F(R_J) = F(\frac{J_{NAD(P)H}}{J_{ATP}}) \quad (2)$$

where, R_J is the dimension less formation flux ratio, J are the formation fluxes in mmol·h^{-1}·g^{-1}, *NAD(P)H* is the summation of the redox equivalences. The equation captures kinetics and thus requires flux balance analysis to determine these fluxes [44].

2.1 Formation flux ratios in well elucidated central carbon pathways

Taking only the formation fluxes of energy and redox in the EMP pathway, it is noted that a total of 2 moles of NADH and 4 moles of ATP are produced per mole of glucose (Figure 4A). This will give, according to equation 2, a R_J-value of 0.5. This low value is considered as the most optimal situation, i.e. the most efficient route for a balanced redox and ATP. We can compare it now with the Entner-Doudoroff (ED) (Figure 4B) and phosphoketolase pathways (Figure 4C) for which we find the R_J-values being higher: 1 and 1.5, respectively.

In most microbes there are always a minimum of two central carbon pathways operating simultaneously and thus the R_J-value will be liable to changes in the fluxes through these pathways, and this may depend on the microbe and the environmental condition. Taking only the EMP and the PPP pathways as an example, *L. lactis* has a very small (<2%) carbon flux through the PPP pathway [45]. However, the flux through the PPP in *S. cerevisiae* ranges from 0.9 to 20% depending on the cell phase and whether glucose is oxidised or not [46].

2.2 Implications of formation flux ratio

This convenient formation flux ratio (R_J) can be used as a ballpark for the direction of engineering for increasing growth rates. Reducing the R_J value improves the growth rate and performance. Some organisms that showcase metabolisms with high R_J values are *Leuconostoc* sp., *Limosilactobacillus reuteri*, *Zymomonas mobilis* and xylose-fermenting *Saccharomyces cerevisiae*. The last organism mentioned has usually a low R_J value, like during aerobic or anaerobic growth on glucose. *Lb. reuteri* and *S. cerevisiae* are discussed in greater detail in the subsequent chapters, whereas a brief observation is provided here for evidence of the hypothesis in the other organisms mentioned.

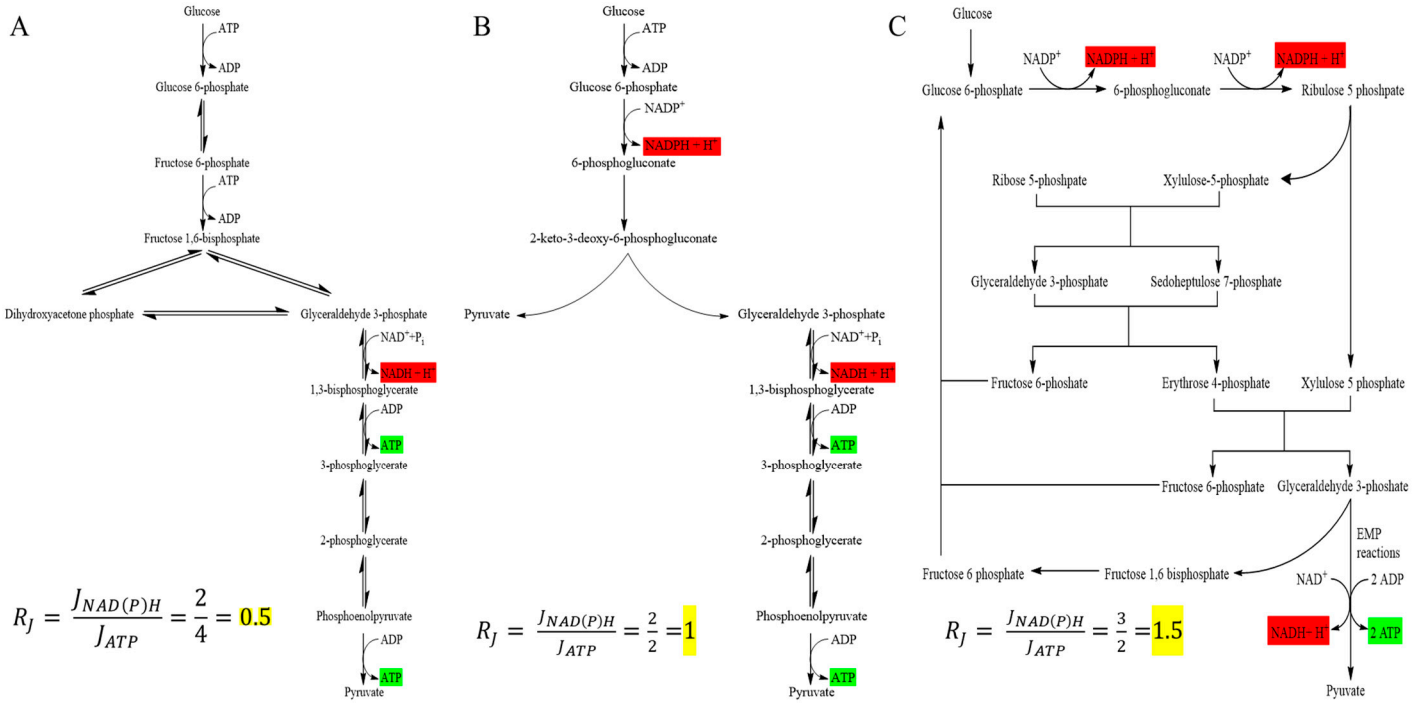


Figure 4: The R_J calculations for the three pathways, $J_{NAD(P)H}$ is highlighted in red, J_{ATP} is highlighted in green. (A) is the Embden-Meyerhof-Parnas pathway. (B) is the Entner-Doudoroff pathway. (C) is the Phosphoketolase pathway

Leuconostoc mesenteroides is an obligate heterofermentative LAB that relies solely on the phosphoketolase pathway for growth and assimilation of carbon [47]. The phosphoketolase pathway converts one mole of glucose to one mole of acetyl phosphate and one mole of glyceraldehyde 3 phosphate. Only the latter metabolite leads to the formation of ATP, and acetyl phosphate is converted to ethanol to regenerate NADH, if the organism is grown anaerobically [38], [47]. The organism can produce one mole of acetate per mole of glucose if oxygen or electron acceptors, such as fructose, are provided [47]. As a result, the ATP yield increases, thereby decreasing the R_J value significantly. It has been argued that microorganisms possessing the PKP pathway thrive best in environments where electron acceptors are present [47], [48].

Even though microorganisms such as *Zymomonas mobilis* have been characterised well, there are still large gaps in understanding how it copes with utilising only the Entner-Doudoroff pathway. Overexpression of most glycolytic proteins in this bacterium leads to a decrease in growth rate due to the burden on protein expression [49]. This was, however, further clarified by other studies and it revealed that the glycolytic control was ATP dependent and increasing ATP formation is a viable engineering strategy for the microbe [50].

2.3 Methods for following intracellular cell states

The goal to manipulate the formation flux ratio (R_J) is to understand the impact of attempts to restore the redox balance as well as the energy balance of the cells in relation to its growth rate. This is a dynamic system that has far reaching consequences as the redox (NAD(P)H) levels define the reductive power of the cells that remains available for other metabolic processes and the energy carrier ATP is important for proliferation and maintenance of vital systems, such as intracellular pH and DNA synthesis.

Since the aim is to disrupt the redox and energy as a means to gain insight in their interconnected metabolism, measurement of these cofactors is useful for elucidating the cell state and determine the direction of engineering. This has been facilitated by numerous methods. However, the measurement of the redox cofactors is affected by various factors, such as the estimation of the concentration of NADH and NADPH in the protein-bound form [51].

The traditional methods used to estimate the concentration of intracellular redox couples are limited in their scope due to their time consuming and expensive nature [51]. Most of these methods are also limited to offline measurement, for example mass spectrometry, NMR, enzymatic assays or fluorescent lifetime imaging [51]. These methods are subject to various considerations due to the rapid turnover of redox couples within the cell. To address the rapid turnover rates quenching of the

metabolic activity is required which usually involves the addition of methanol at -40°C or the use of liquid nitrogen and recent advances have reduced the time required for this [52]. This is followed by cell disruption and then the estimation of the concentration of the redox couples.

The estimation of the concentration of redox couples can be done directly with spectrophotometry at a wavelength of 340 nm using cell extracts by the use of enzymatic cycling (ethanol and alcohol dehydrogenase for determination of NAD^+ and NADH; glucose 6-phosphate and glucose 6-phosphate dehydrogenase for $NADP^+$ and NADPH) [53], [54]. However, this method is subject to various inaccuracies caused by variations in the handling of cells, activity of the enzymes used in the cell extract, and pH. The use of mass spectrometry is promising due to its low limit of detection, but they are restricted to use after HPLC systems which operate at a pH that leads unfortunately to analyte degradation [55], [56], [57]. The use of ^{13}C-NMR is robust for the detection of the redox couple concentrations, but they usually require high cell concentrations for reliable measurements and the cost of ^{13}C-labelled metabolites and the use of NMR are rather expensive for routine estimation [58], [59], [60]. Fluorescence life time imaging for the estimation of redox couple concentrations would be a good alternative, but this method is rather limited in scope for scale up of bioreactors which may be subject to population variances [61], [62].

Alternatively, flux balance analysis can also be used to estimate the redox balance. However, without the use of ^{13}C-NMR, this method is subject to overestimations [63]. Nonetheless this method is also not capable of providing the immediate live state of the cells growing in bioreactors as they require HPLC to estimate the concentrations of the metabolic substrates and products.

An online method is beneficial for direct observation of these factors. This can be facilitated using GFP-based fluorescence proteins that place very little burden on the cell and facilitate a direct non-invasive method of following intracellular cell states [64], [65], [66]. The application of these proteins has been used extensively with live cell imaging to understand the metabolic processes on a single cell level and their adoption for assessing the cell states in larger scale cultivations using flow cytometry is slowly growing.

2.3.1 Fluorescence Biosensors

Unlike the autofluorescence of the redox couples (NADPH and flavins), cellular structures (mitochondria and lysosomes) and other proteins (proteins containing high amounts of tryptophan, tyrosine and phenylalanine) [67], [68], [69], [70], induced fluorescence can be enabled by the use of engineered fluorescent proteins [71], [72], [73], [74], [75], [76], [77]. This can be due to changes in intracellular states such as the concentration of small molecules, metabolites as well as changes

in the expression of various genes. Some fluorescence biosensors/reporters use the existing promoters that respond to various cell stresses to induce expression of fluorescent proteins [78], [79]. This enables the identification of the global cell state or the time/growth phase at which a particular gene or promoter is induced [74], [80], [81]. Other fluorescent biosensors are chimeric proteins, which is a mix of a fluorescence protein with a protein that is involved in a particular function, that changes its fluorescence intensity based on the quaternary structure of the bound protein [66], [76], [77], [82]. A few fluorescence proteins of the latter variety are highlighted here (pun not intended).

2.3.2 ATP biosensors

Estimating the relative ATP abundance of the cell during the various growth phases enables a deeper understanding of cellular energetics [83], [84], [85]. The concentration of ATP is dynamic during the various phases of the cell and following this in real time has been challenging using traditional methods [86], [87]. However, the development of numerous ATP binding biosensors has enabled the real time measurement of ATP concentrations in living cells [64], [76], [86]. Biosensors in combination with live cell imaging can provide variations of ATP concentrations between cells but the combination of these biosensors with flow-cytometry can provide real-time data from a heterogenous population that can be present in bioreactors.

2.3.3 Redox sensors

The intracellular redox state is a driving force for the oxido-reduction reactions of a cell. However, there are multiple redox couples in a microbe ($NADH/NAD^+$, $NADPH/NADP^+$, $FAD/FADH_2$) in addition to certain biological indicators (production of H_2O_2) [88], [89], [90]. Thus, the redox biosensors should be very specific for a particular type of redox couple. Some redox biosensors that are specific are peredox-mCherry, SoNar ($NADH/NAD^+$), ObaQ (glutathione redox balance), HyPer, and rxRFP (H_2O_2) [66], [82], [90], [91].

2.3.4 Intracellular pH

The intracellular pH (pH_i) is also an important factor that determines the growth rate of microbes. The various glycolytic enzymes cannot function efficiently beyond a certain pH range [16]. The pH_i is also related to the quinary structure of the proteins in the microbe [92]. Metabolic engineering attempts may affect this by the interference with the native pH homeostasis mechanisms, and thus the observation of intracellular pH can provide vital information on the intracellular state of the cell. Some well-known intracellular pH sensors are pHTomato, mNectarine, and

pHluorin [65], [75], [93], [94]. In addition to the use of fluorescence proteins, this can also be performed with certain dyes, without the requirement of strain engineering, such as pHrodo®, BCECF and CFDA-SE, with each method having its own benefits and drawbacks [95], [96], [97].

2.4 Flow cytometry, a tool for live monitoring

The biosensors stated above has been used extensively in conjunction with microscopy. This is, however, not feasible for monitoring the cell states in large bioreactors due to the variations inherent to fermentation in larger volumes [62], [98]. A relatively recent adoption of online flow cytometry aims to target this issue. A flow cytometer, by the use of lasers in conjunction with photodiodes through a narrow capillary flow chamber aims to distribute and observe singular cells passing through the laser beam and observe its phenotype and fluorescence profile. Usually, a blue laser (488 nm) is used to obtain the forward scatter ((FSC) shadows) and side scatter ((SSC) scatter at 90°) profiles of the cells passing the laser. The use of lasers also allows the capture of fluorescence data by the use of dichroic mirrors at the same time [99], [100]. The device samples a small volume and collect the fluorescence profiles of more than 10,000 individual cells per second which gives us a snapshot of a small population in the bioreactor. By altering the number of samples and the number of events captured we can obtain a representative idea of the intracellular state of all the cells in the fermentation.

3 Understanding *Limosilactobacillus reuteri*

Lactobacillus reuteri, reclassified as *Limosilactobacillus reuteri* is a gram positive, exopolysaccharide forming, catalase negative and non-sporulating species in the lactobacillus family. This organism has been isolated from various niches but is particularly abundant in the gastrointestinal tract of humans, sheep, chicken, pigs and rodents [101], [102], [103], [104], [105]. The organism is classified as a facultative anaerobic microbe, but they lack an aerobic respiration chain [106], [107], [108], and most strains can grow in micro aerobic conditions and not fully aerobic conditions [109], [110]. These microbes grow optimally at 37°C in still shake flasks but have better growth characteristics under anaerobic conditions. The microbe as a part of the LAB species is generally regarded as safe and has been shown numerous health benefits when provided as supplements in humans [111]. The health benefits range from preventing bone loss in the elderly mice to regulation of bowel movements in germ free mice [112], [113], [114]. Thus, this microbe is considered as a probiotic bacterium and is currently being commercially sold as such. *Lb. reuteri* shows many other potential applications ranging from antibiotic production (reuterin, reutericyclin), vitamin production, medical treatment (treatments for leaky gut, obesity, immunomodulation) and many more [111].

Lb. reuteri is an obligate heterofermentative microbe as opposed to the facultative heterofermentative profiles seen in other lactobacillus species such as *Lactiplantibacillus plantarum*. This is due to the possession of the phosphoketolase pathway (PKP), resulting in the formation of lactate, ethanol, and CO_2. However, *Lb. reuteri* has the interesting physiological feature of having the simultaneous utilization of two glycolytic pathways, the EMP pathway and the PKP pathway, whereas the PKP pathway is active in *Lp. plantarum* only under stress conditions [38], [48], [115]. The type-strain and certain other closely related strains have been characterised to use the PKP pathway as the main central carbon pathway and the EMP pathway acts only as a shunt with a ceiling of 30% carbon flux (Figure 5A) [115]. The optimal medium for growing these microbes is De Man-Rogosa-Sharpe (MRS) medium which is a very rich medium and thus limits the exploration of the physiology of the central carbon metabolism. This is due to the presence of digests of beef extract, yeast extract in addition to fatty acids (Tween 80), vitamins and amino acids [116]. Sodium acetate and ammonium citrate are also part of the

medium that may act as alternative carbon source and electron acceptors, respectively [116].

3.1 Central carbon metabolism

As stated above *Lb. reuteri* prefers the phosphoketolase pathway even in strains that contain and express all the genes for the EMP pathway. This pathway introduces the preference for the presence of acetate in the growth media which boosts the growth of the microbe, though the mechanism is not yet well elucidated.

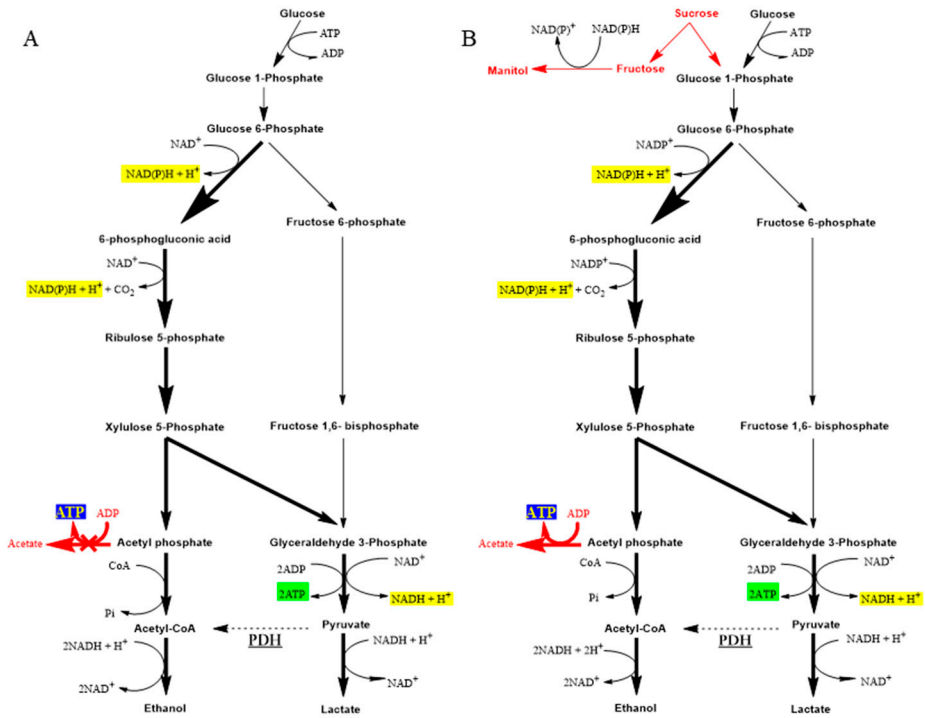


Figure 5: The central carbon pathway in *limosilactobacillus reuteri*. (A) The pathway in the absence of an external electron acceptor. (B) The pathway in the presence of an external electron acceptor (In this case Furctose)

This distribution of flux towards a less energy productive pathway produces certain challenges for the microbe which includes a 50% reduction in total ATP production (1 mol of ATP/mol of glucose) since, the PKP pathway produces one 3-carbon compound that is converted to pyruvate in a 3-carbon pathway similar to the one in the EMP pathway. In the upper part of the PKP, two molecules of $NAD(P)^+$ are reduced that can give rise to a redox imbalance. Usually, these redox cofactors are

re-oxidised via the reduction of acetyl phosphate (AcP) to ethanol in the 2-carbon branch of the PKP. This means that only from the 3-carbon branch ATP can be extracted. Production of ethanol being the only mechanism for achieving a redox balance limits the total glucose consumption rate and making the microbe grow slower in media without electron acceptors. When an electron acceptor such as fructose is provided, the redox cofactors are re-oxidised in the conversion of fructose to mannitol. This allows the AcP flux to be diverted towards acetate which increases the total ATP yield.

The dual central carbon pathways in *Lb. reuteri* raises questions such as (1) How does the regulation of the enzymes in the EMP pathway work, (2) Are there strain variations in other aspects of growth in lean media, (3) How is the metabolism of *Lb. reuteri* affected when a functional EMP pathway is introduced.

3.2 Idea behind engineering

Due to the dependence of *Lb. reuteri* on the two distinct central carbon pathways, it may provide the involvement of unique potential alternative metabolic control mechanisms. By manipulating the flux through each pathway, it may become possible to compare the effects on these two pathways. The PKP pathway has a higher -ΔG for the reactions whereas the EMP pathway has a higher ATP yield but a lower -ΔG for the cumulative reactions in the pathway [47].

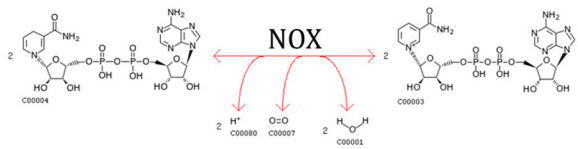
It enables the exploration of diminishing the redox burden by the addition of electron acceptors or increasing and decreasing the formation flux of ATP by manipulating the flux through the EMP pathway. This can be done by creating knock out strains for the native phosphofructokinase, which is predicted to be from the minor phosphofructokinase family (*pfkB*), as well as introducing an inducible phosphofructokinase with known kinetic properties from the major family of phosphofructokinases (*pfkA*).

3.3 Role of electron acceptors

Electron acceptors are compounds that cannot be used a carbon or energy source, but instead they are reduced in oxido-reductive reactions to recycle redox cofactors (NAD(P)H). Compounds such as citrate, fructose, glycerol, and oxygen are electron acceptors for *Lb. reuteri*. The addition of these compounds in the medium improves growth and total biomass produced in *Lb. reuteri* [48], [117], [118]. This is due to the recycling of redox by means other than the reduction of AcP to ethanol. It allows the formation of acetate combined with an ATP as a fermentation product and thus

increases the total ATP production (Figure 5B). This is especially evident when this organism is grown in a mixture of glucose and fructose or sucrose as the total ATP yield per biomass increases [48].

Each electron acceptor performs to different extents in the various strains of *Lb. reuteri*. This is for instance seen when oxygen is used as an electron acceptor. *Lb. reuteri* lacks a proton translocating electron transport chain making it incapable of aerobic respiration [106], [107], [108]. Nevertheless, it reduces oxygen to water catalysed by NAD(P)H oxidases (Figure 6). Still *Lb. reuteri* is sensitive when exposed to oxygen due to the lack of complete protection mechanisms against reactive oxygen species.



$$2NADH + H^+ + O_2 \leftrightarrow 2NAD^+ + 2H_2O$$

Figure 6: NADH oxidase (NOX) catalysing the reduction of oxygen with NADH.

In the two strains used in this thesis the PTA 4659 is more sensitive to oxygen compared to DSM 17938, which is caused by hydrogen peroxide, which sounds peculiar since both strains were isolated from breast milk [117]. The increased sensitivity to peroxide in the PTA 4659 is investigated in **Paper I** as the strain produces lethal quantities of hydrogen peroxide upon aeration and this in turn led to cell apoptosis.

3.4 Lactate dehydrogenase

Lb. reuteri DSM 17938 has a total of 6 predicted lactate dehydrogenases (EC 1.1.1.27). This enzyme is regulated at the enzyme level by the concentrations of redox cofactors, ATP and ADP [19], [119], [120], [121], which is seen with many dehydrogenases in other microorganisms [23]. This enzyme is also assumed to be activated by of fructose 1,6-bisphosphate (FBP), thus enabling the feed forward activation of the enzyme when the culture is exposed to glucose [121]. This may be questioned for *Lb. reuteri* as its concentration of FBP is very low [48]. Additionally, very few LDH's that lack FBP activation have been characterised [119], [122]. Thus, **Paper II** aims to expand our understanding on the regulatory mechanism of this enzyme in *Lb. reuteri* and compare it with lactate dehydrogenases of other Gram-positive bacteria.

3.5 Engineering *Lb. reuteri*

Since the discovery of *Lb. reuteri* in 1962, it has been a subject for strain engineering to understand molecular mechanisms involved in gut interaction and expression of its antimicrobial compounds. The initial strain improvement was through random mutagenesis, adaptive evolution and other traditional non-targeted methods. The increased understanding of targeted mutation and the reduced cost for custom synthesis of various DNA elements has led to the adoption of methods such as Cre-LoxP, single strand recombineering and CRISPR-Cas9 in *Lb. reuteri*. The Cre-LoxP system has been used in the laboratory strain *Lb. reuteri* DSM 20016 for the deletion of two putative propanediol dehydrogenases and this lead to the characterisation of the mutant phenotypes [123]. Single strand recombineering was initially developed and implemented for the point mutation of targeted genes using single stranded oligo nucleotides with efficiencies ranging from 0.4% to 19% in *Lb. reuteri* PTA 6475 [124]. This was further improved by the implementation of the CRISPR-Cas9 system to introduce double stranded breaks and thereby increasing the transformation efficiency as well as reducing non-transformed colonies [125]. The CRISPR-Cas9 system has also been used successfully in editing and studying many other mutations in *Lb. reuteri* PTA 6475 as well as *Lb. reuteri* DSM 20016 [95].

Other methods of engineering *Lb. reuteri* is by the use of overexpression plasmids with various inducible systems. The nisin-controlled gene expression system (NICE) was discovered in *L. lactis* and then adapted to *Lb. reuteri* DSM 20016 with a successful linear induction of α-amylase between the range of 0 to 50 $ng \cdot \mu l^{-1}$ [126]. Concentrations above this range proved toxic for the growth of the microbe with the minimum inhibitory concentration at 75 $ng \cdot \mu l^{-1}$ for nisin [126]. Another expression system that has a linear induction mechanism is the sakacin induction system identified in *Lactobacillus sakei* [127]. This is a native system used for the production of a bacteriocin (sakacin) by the microbe but through promoter engineering the system has been adopted for inducible expression by the use of a 36 Dalton peptide [128]. This system has been used in *Lb. reuteri* ATCC 55730 as well as PTA 6475 successfully [129], [130].

3.6 Metabolic flux and pathway variations

Saulnier et al., (2011) illustrated the variations in the expression of the glycolytic enzymes, overtime, between strains. The study showed that the pyruvate dehydrogenase (PDH) is upregulated in the early log phase in *Lb. reuteri* ATCC 55730 whereas the PTA 6475 strain expressed PDH in the early stationary phase [117]. This was further expanded in subsequent studies showing that the ratio of flux between the pathways also changes based on the strains used [115]. These

variations between strains and the control of the PDH enzyme being redox ratio dependent warrants clarification of the PDH expression overtime as well as their activity [131].

The engineering targeting the manipulation of the flux through the EMP pathway was attempted with three goals. (1) the elucidation of the PDH gene in the strains DSM 17938 and PTA 6475, (2) identification and deletion of the native *pfkB*, (3) expression of an exogenous *pfkA* under an inducible promoter. The latter two are explored in **Paper III**.

For the elucidation of the PDH activity as well as to eliminate any possible interference of it in the overexpression of the EMP pathway, a knockout strategy with the replacement of each of the coding regions in the PDH operon replaced by a GFP protein was attempted (Figure 7) using the double-crossover integration method previously demonstrated in *Lb. reuteri* [132], [133]. This strategy did not lead to any mutants in the PTA 6475 strain, whereas some mutants were obtained in the DSM 17938 strain.

Figure 7: Schematic representation of the attempted double-crossover integration. (Black = PDH operon upstream and downstream regions; Blue = the genes in the PDH operon; Yellow = selection pressure with Cre-LoxP sites; Green = pNZ5319 backbone)

From the obtained mutants attempts to remove the antibiotic resistance gene (Chloramphenicol (Cm^r)) by Cre-Lox recombination were not successful due to microbial lysis observed one hour after electroporation with the Cre-recombinase plasmid obtained from Lambert et al., (2007). Further analysis of the mutant strains also showed both unmutated as well as mutated genotypes in a single colony. This might indicate a duplication of the operon during trials of genetic engineering or integration of the entire plasmid at some other region. It is also noted that no fluorescence was observed in the mutant strains under any of the tested conditions

(growth in aerobic baffled shake flasks, still cultures and anaerobic conditions). Attempts of the same engineering with a CRISPR-Cas9 system, developed by another research group [134], also led to undefined genotypes. The lack of fluorescence upon expression of a GFP using a PDH promoter could be attributed to low levels of expression, misfolded proteins or the high background fluorescence of *Lb. reuteri,* and further investigation was not performed.

The observed difficulty in engineering may be due to variations between the strains regarding the number of restriction systems present as well as the differences in the expression of the glycolytic enzymes. It is also noted that similar engineering attempts for deletion of various other genes worked in the parent strain of DSM 17938, namely ATCC 55730 [135], [136]. The difference between the two strains being the loss of two plasmids [137], and a brief look at the REBASE database revealed a larger variety of restriction systems present in the ATCC 55730 strain with one restriction system being present in one of the plasmids (Figure 8) [138].

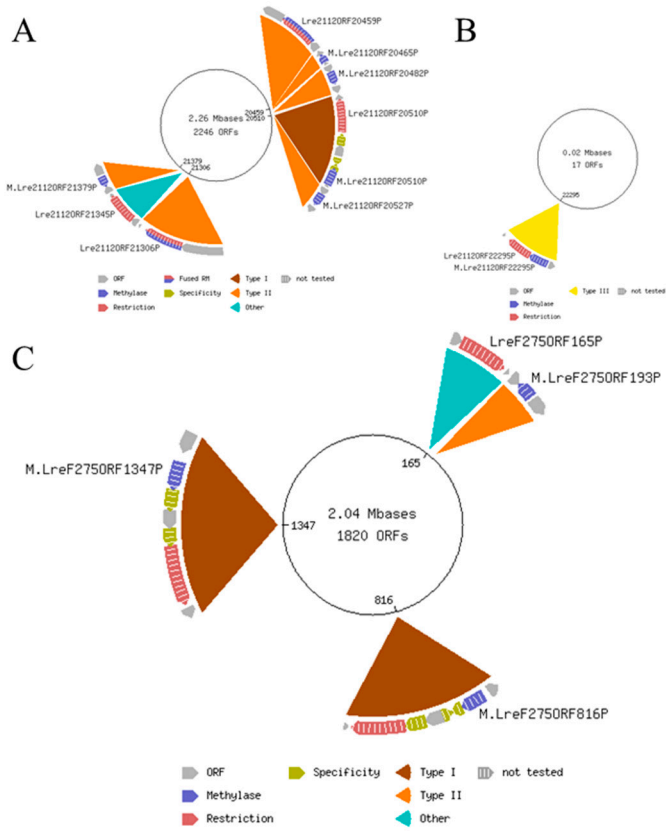


Figure 8: Restriction modification systems recognised by REBASE database. (A) the restiction modification system present in the genome of DSM 17938 and ATCC 55730. (B) the restriction

modification system present in the plasmid of ATCC 55730. (C) The restriction modification system present in *Lb. reuteri* JCM1112 (Analogous to PTA 6475).

Furthermore, some studies have been successful with the expression of GFP in *Lb. reuteri* isolates from the environment so genetic experiments can be done with *Lb. reuteri*, but it requires further investigation for the DSM 17938 strain. The PTA 6475 strain is also subjected to various genome rearrangements based on the origin of replication present in the plasmids and thus a comprehensive genome sequencing may be required after deletion of genes [139].

3.7 Phosphofructokinase activity

Lb. reuteri DSM 17938 and PTA 6475 strains have three and two probable hits for functional complementation of phosphofructokinase activity respectively. These hits are orthologous to phosphofructokinase B of *Escherichia coli*. The genes are expressed at relatively high levels in transcriptome data (not published) in DSM 17938 but their enzyme activity is relatively low and thus the effect of overexpression was of interest. The lack of pyruvate dehydrogenase activity in *Lb. reuteri* DSM 17938 during logarithmic growth provides an ideal platform for metabolic flux analysis to follow the effects of overexpression. However, our attempts at engineering this strain failed. Previous attempts at increasing the flux through the EMP pathway used a truncated *pfkA* from *Aspergillus niger*, in the parent strain of *Lb. reuteri* DSM 17938, namely *Lb. reuteri* ATCC 55730, showed improved growth rates and up to 60% carbon flux through the EMP pathway when grown in rich media [140].

We wanted to follow and characterise the variations in redox balance and energy production by using the controlled expression of *pfkA* from *Lactococcus lactis.* This is to elucidate the variations in regulation of the EMP pathway in *Lb. reuteri* as well as to explore the effect of the *L. lactis*'s feedforward control mechanism. The *pfkA* in *L. lactis* is part of the *las* operon which contains the genes for pyruvate kinase (PYK) and lactate dehydrogenase in addition to the phosphofructokinase [141]. The expression of the operon would allow the control of the flux through the EMP as PYK and LDH are regulated by the concentration of fructose bisphosphate. Alternatively, expression of only the phosphofructokinase would elucidate if the second half of the EMP pathway would remain a bottleneck. This is explored in **paper III**.

4 Industrial application of *Saccharomyces cerevisiae*

Fungal species have played an important role in the industrial production of a wide variety of compounds and have instigated an increased interest for their applications in waste management and valorisation of various waste streams into value added compounds [142], [143]. This is attributed to their high tolerance to numerous stressful conditions, their capability to produce a wide variety of compounds and their scalability attributed to their robustness [144]. A few examples of the various genera in industrial application as well as their uses are listed in Table 1.

Table 1: Fungal families and their industrial applications.
Fungal families were found for some of organisms found in the the lists present in the references and simplified [142], [143], [144], [145], [146], [147], [148], [149], [150], [151].

Fungal families	Fungal genera	Compounds produced									
		Pharmaceutcals and antimicrobials	Enzymes							Agriculture	Food
			Amylase	Protease	Pectinase	Galactosidase	Chitinase	Lipase	Lignocellulolytic		
Trichocomaceae	Aspergillus, Penicillium, Talaromyces	Clavatol, Lovastatin, Aspergillomarasmine	•	•	•	•	•	•	•		Tea, chocolate, miso, sake, cheese, sausages
Sclerotiniaceae	Botrytis				•				•		sweet wines (aszú, sauternes)
Saccharmoycetacea	Saccharomyces, Candida	Insulin, Human serum albumin, hepatitis vaccines	•	•	•	•	•	•			bread, cheese, chocolate, alcohol
Hypocreaceae	Trichoderma	Peptaibolis		•	•		•		•	•	

Of the numerous species, *Saccharomyces cerevisiae* is the most relied upon due to its ease of engineering and long history of use [152]. It is currently used for a wide range of products ranging from human enzymes and vaccines to bread and alcohol [149], [152], [153]. Engineering this organism for new functions enable the transfer of existing petrochemical processes to a more environmentally friendly production and reduced reliance on fossil fuels. A few of these new functions is the valorisation of lignocellulosic biomass to biofuels and production of various amine compounds.

4.1 Lignocellulosic biomass

Lignocellulose is a cheap substrate that is promising for production of value-added compounds due to its abundance [154]. Lignocellulose refers to the remnants of various industries as listed in brackets for a few.

1. Agriculture – crops for consumption (wheat straw, corn stover, sugarcane bagasse [155]),
2. Forestry – timber for building material, furniture, etc. (wood chips, saw dust [155])
3. Industrial waste streams – brewery waste, paper mills etc. (brewer's Spent grain, black liquor [156])

These waste streams are unattractive for conventional conversion processes and are thus either burnt or discarded [154]. Although some of the waste from food crops can be used as feed stocks for industrial processes, a majority is simply discarded due to their low value as a source of nutrition, and the lack of cost-benefit for processing this waste [154], [157]. Some of these waste streams that have been adopted in industrial processes are paper production from sugarcane bagasse (Vincent corporation, TNPL), and bioethanol production from lignocellulose (Granbio, Beta renewables) but they are not yet widespread, culminating in that production of this waste far outpaces its conversion. Thus, it is recognized as a potential source for biorefineries for the production of biofuels, biochemicals, bioenergy and biomaterials.

The main polymers that remain in lignocellulose are cellulose, hemicellulose, and lignin. Cellulose in isolation can be fermented by most microbes since it is a linear chain of β1→4 linked glucose molecules [158], [159], [160]. Cellulose can constitute anywhere from 40 to 60% of the residual waste but is inaccessible to microbes due to the substrate being embedded in a matrix of hemicellulose and lignin [161], [162], [163].

Hemicellulose is a polymer consisting of a combination of various five or six carbon sugars (e.g. arabinose, galactose, glucose and xylose) in chains of β1→ 4 and β1→3 linkages. Their specific combination of sugars can vary based on plant species but their total composition ranges from 15 to 30% of the lignocellulosic biomass [160],

[161], [163]. There are many fungal species that can degrade this compound, but they are not in use in industry for various reasons ranging from difficulty of strain engineering, unavailability of genetic toolboxes, difficulty faced during scaleup and so on [164].

Lignin can take 5 - 30% of the lignocellulosic biomass based on plant species [161], [163], [165], [166]. This fraction of lignocellulose is composed of highly heterogenous mixture of aromatic compounds that form a polymer using carbon-carbon or carbon-oxygen bonds [165]. This fraction also makes the outer structure of the lignocellulosic biomass, thereby preventing industrial microbes from accessing their sugar substrates [161], [166]. Therefore, pretreatment that degrades is necessary to make these sugars accessible.

4.2 Lignocellulose pretreatment – How and drawbacks

Lignocellulose can be broken down using different chemical and biological methods [167], [168]. Each of these methods have their advantage and disadvantages. Some of the methods, such as the biological degradation using enzymes is environmentally friendly, but due to the long processing time for hydrolysis, combined with low hydrolysis efficiency, scale up of this method proves difficult [168]. The current most widely adopted method for lignocellulose biomass pretreatment of hard wood is steam explosion with an acid catalyst [167]. This physiochemical method involves the addition of acids, such as dilute sulphuric acid to assist the autohydrolysis caused by water at high temperature (190°C) and pressure (1-3.5 MPa) [169], [170].

The autohydrolysis results in the release of monomeric glucose, xylose and other sugars in various ratios depending on the starting material [169]. This method is relatively environmentally friendly as it does not involve the use of stronger acids and bases [169]. However, this method does have drawbacks such as production of fermentation inhibiting compounds (week acids, phenolic compounds and furaldehyde) [171], [172].

S. cerevisiae has a relatively high tolerance to the various inhibitors present in this waste stream. This compounded with three decades worth of research enabling xylose utilization in this species, makes it an ideal organism for making value-added compounds from pretreated lignocellulose. However, these inhibitors and the rate of xylose utilisation by *S. cerevisiae* requires further optimisation for the process to be cost-effective and competitive to fossil fuel-based production.

4.3 Xylose fermentation

Catabolism of xylose has been engineered in *Saccharomyces cerevisiae* by the integration of various pathways. Of these pathways, two pathways have been expressed in yeast and improved upon by following an iterative processes for optimal xylose fermentation with variable results [173]. Among the two pathways, the oxido-reductive pathway is found in other yeast species, whereas the xylose isomerase pathway is found in bacteria. Other notable pathways are the Weimberg pathway and the Dahms pathway [174], [175], [176].

Although progress has been made for the efficient catabolism of xylose by *S. cerevisiae*, they are still not a viable replacement for petroleum-based processes. One of the limiting factors for each of these pathways is hypothesised to be xylose not being sensed as a fermentable sugar on top of the inherent nature of catabolic repression [173], [177]. This thesis focuses on the oxido-reductive pathway, as this pathway faces a redox imbalance coupled with a limited ATP formation flux under anaerobic conditions (Figure 9)[178].

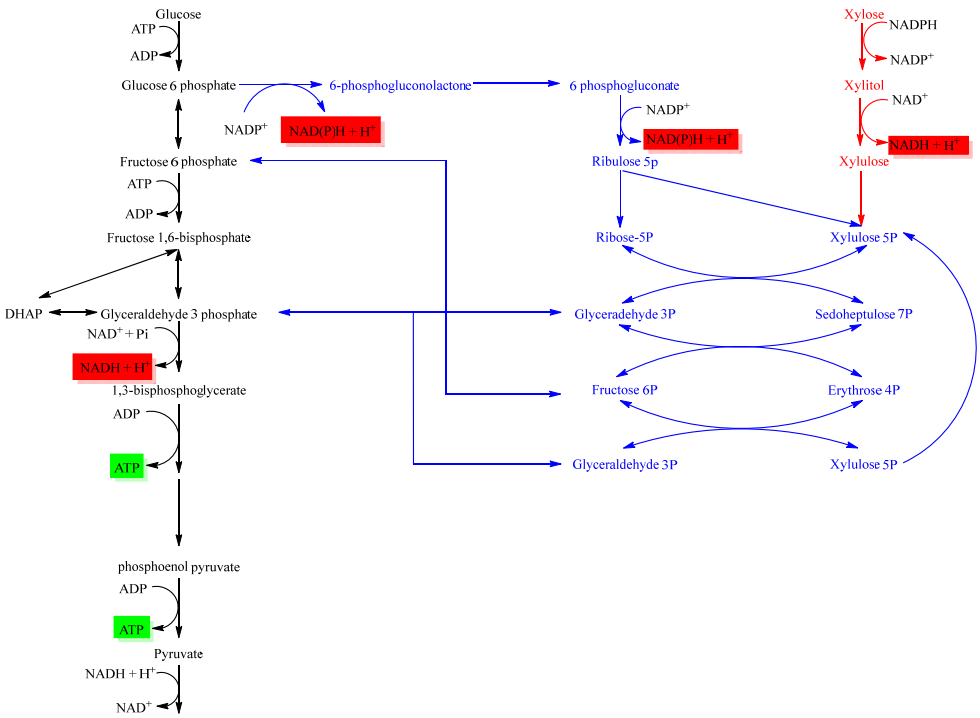


Figure 9: The oxido-reductive pathway (In red) introduced into *S. cerevisiae*. The pentosephosphate pathway is represented in blue and the EMP pathway is represented in black. The formation fluxes of redox ($J_{NAD(P)H}$) is represented in red and the formation fluxes of energy (J_{ATP}) is represented in green.

Strains developed with this pathway have a high xylose uptake rate and are preferred for anaerobic xylose fermentation [179], [180]. It is also the preferred pathway for production of xylitol, which is a common sugar substitute in the industry [181]. The xylose reductase (XR) is predominantly NADPH dependent, whereas the xylose dehydrogenase (XDH) uses NAD^+ as the sole cofactor [173]. This causes a redox imbalance that has been tackled by various means, such as addition of an external electron acceptor, protein engineering, and using alternative xylose reductases [182], [183]. This study builds on the culmination of all these engineering attempts and uses TMB 3504, which is one of the fastest growing oxido-reductive strains developed by our group [180], [183], [184]. This strain uses the XR from *Spathaspora passalidarum,* which reduces the redox burden as the enzyme has a greater reliance on NADH rather than NADPH under anaerobic conditions [183].

As stated before (Chapter 1), the uptake of substrates such as glucose causes feed forward activation of the EMP pathway. This creates a competitive advantage for the organism. In the case of *S. cerevisiae* that is engineered to ferment xylose, this effect is absent. The heterologous xylulokinase (XK) gene has a high expression level and upon the availability of xylulose ends up using large quantities of ATP [185]. We hypothesise that this instigates a decrease in the ATP pool available for biomass production and cell maintenance such as pH homeostasis.

4.4 pH homeostasis mechanism

In *S. cerevisiae* the pH homeostasis machinery is dependent on ATP [186]. This is attributed to the use of ATP-dependent proton translocating pumps [187]. There are two classes of these enzymes in *S. cerevisiae*. One is the P-type H^+-ATPase encoded by *pma1* that is targeted to the cell membrane [187]. This is the most abundant protein found on the cell membrane. Mutations of this protein become lethal if the activity of the protein drops below 20% [188]. The other type of H^+-ATPases are V-ATPases [187]. There are numerous subunits in this type of H^+-ATPase, and these are targeted towards the vacuole and cell organelles. Deletion of this protein is not lethal in *S. cerevisiae* due to Pma1p substituting the V-ATPase function but the same mutation in other eucaryotes is lethal [189]. This enzyme is responsible for vacuolar acidification which is an important part of cell growth in *S. cerevisiae* [186]. The two enzymes combined account for the majority of the ATP consumption during growth [190].

The ATPases in *S. cerevisiae* are regulated by glucose at the transcription, translation and protein level [186], [190]. It is seen that the H^+-ATPases dissociates in the absence of glucose due to direct interaction at the enzyme level. This causes the enzyme activity to remain reduced in sugars other than glucose [190], [191]. Further research has shown that the sugar signalling pathway also influences the pH

homeostasis machinery [192]. Thus when *S. cerevisiae* was engineered for xylose consumption there was uncertainty on whether the pH homeostasis machinery was activated in a proper manner.

4.5 Idea behind engineering

In prokaryotes, and plants an alternative energy carrier in the form of pyrophosphate (PP_i) can be utilised for pH homeostasis [193], [194]. This has not been found in fungal species and in the case of *S. cerevisiae* the majority of the PP_i formed is hydrolysed by the cytosolic pyrophosphatase (*ipp*1) [195], [196], [197], [198]. **Paper IV** evaluates if the pH homeostasis can be offloaded to a proton pumping pyrophosphatase to reduce the ATP consumption flux for pH homeostasis, and thus be redirected to increasing glucose and/or xylose consumption flux.

This is especially relevant when *S. cerevisiae* is grown in the presence of weak acids, such as acetic acid and levulinic acid, at low pH [163], [199]. These acids can pass into the cytoplasm through diffusion [200], [201]. Upon arrival at the cytoplasm which is maintained at a neutral pH the weak acids dissociate into protonated forms, thus decreasing the intracellular pH. The mechanism for dealing with weak acid stress is reliant on the pH homeostasis machinery, and thus increases the ATP demand. This is apparent in the reduced growth rates seen on glucose at low pH. This is compounded on xylose fermentations since the higher NAD(P)H formation flux over ATP formation flux limits the availability of ATP for the active export of protons to retain cytosolic pH (pH_c) [44].

The formation flux ratio (R_J) was adapted to use PP_i as a potential additional energy carrier that expands the total available energy pool in *S. cerevisiae* when grown anaerobically on xylose to combat higher ATP demand that limits the growth rate of the microbe [178]. Thus, the formation flux ratio (equation 2) is modified to add the energy obtained from PP_i (Equation 3)

$$\mu = F(R_J) = F\left(\frac{J_{NAD(P)H}}{J_{ATP} + J_{PP_i}}\right) \quad (3)$$

4.6 Fluorescence proteins as reporters

Most GFP based biosensors require oxygen for fluorescence [202]. The oxygen requirement is usually satisfied during the sampling from the bioreactor in most cases [203], [204], [205], enabling the use of GFP proteins under anaerobic conditions. This is also the case for the biosensors (mQueen-2m and pHluorin) used in **paper IV**. Literature shows that GFP can be activated to a certain extent with

brief exposure to oxygen, so these biosensors were expressed under the same assumption [205]. To compensate for their lower fluorescence levels compared to the yeast enhanced GFP that is regularly used under anaerobic conditions, very high expression levels were forced.

It was observed that the pHluorin and the mQueen-2m biosensors used were able to fluoresce when observed in the flow cytometer, 5 minutes of sampling from the anaerobic bioreactors but the fluorescence values did increase 1 hour after sampling. The wait for the increased fluorescence value was avoided in to obtain the fluorescence value closest to the sampling condition as the ATP and intracellular pH can also vary within minutes of sampling. pHluorin is a GFP protein based on the wild type *Aequorea victoria* GFP and thus the maturation time for 50% (t_{50}) fluorescence is ~58 min [203], [206]. The newer variety of pHluorin based on the superfold GFP may be better suited for the application of observing intracellular pH at anaerobic conditions since it has a much shorter maturation time (t_{50} ~19 min). The mQueen-2m biosensor used a Circularly permuted green fluorescent protein (cpGFP) for which the maturation times are not readily available, but we observed that the fluorescence values increased only for the 1st hour after exposure to air [86], [207].

As stated in chapter 2, the metabolic flux analysis for the strains is of interest but further optimisation and understanding of the modifications is required. **Paper IV** shows that the addition of pHluorin in xylose fermentations improved the strain performance. The specific conditions causing this is of interest.

5 Conclusions and outlook

The main conclusions of this thesis are:

- Hydrogen peroxide is the chief reactive oxygen species that is produced by the *Lb. reuteri* species tested. **(Paper I)**
- *Lb. reuteri* DSM 17938 adapts and can grow with up to 500 $mL \cdot min^{-1}$ of air sparging, whereas the PTA 6475 and PTA 4659 can only grow with a rate of up to 50 $mL \cdot min^{-1}$ of air. **(Paper I)**
- *Lb. reuteri* DSM 17938 does not necessarily produce less H_2O_2 than its counterparts, but its resistance mechanism against H_2O_2 is more resilient in the strain. **(Paper I)**
- The NADH oxidases of the *Lb. reuteri* species are capable of utilising NADPH in addition to NADH and do not produce H_2O_2, but only H_2O. **(Paper I)**
- The lactate dehydrogenase enzyme (LDH) of *Lb. reuteri* is not activated by FBP. **(Paper II)**
- The lactate dehydrogenase enzyme (LDH) of *Lb. reuteri* does not have any other known regulatory mechanisms at a protein level. **(Paper II)**
- There is a competitive inhibition by ATP and ADP on the LDH enzyme in *Lb. reuteri*, which has been observed for dehydrogenases of other bacteria. **(Paper II)**
- Overexpression of the predicted native phosphofructokinase candidates from the minor family led to loss of fitness of the *Lb. reuteri* PTA 6475 strains in lean media. **(Paper III)**
- The overexpression of the *L. lactis* las operon revealed a possibly active pyruvate dehydrogenase enzyme in *Lb. reuteri* PTA 6475. **(Paper III)**
- Expression of a H^+-PPase at the vacuolar membrane improves the growth of *S. cerevisiae* in media at low pH and high acetic acid concentrations (pH 3.7, 6 $g \cdot L^{-1}$ acetic acid). **(Paper IV)**
- The expression of the H^+-PPase at the vacuolar membrane under normal conditions on glucose or xylose leads to elevated ATP levels. **(Paper IV)**

- The H^+-PPase expression at the vacuolar membrane acidifies the cytosol when grown on 20 $g \cdot L^{-1}$ glucose at pH 5. **(Paper IV)**
- The pH homeostasis machinery is likely running in engineered strains of *S. cerevisiae* even though this machinery requires protein level activation by glucose. **(Paper IV)**
- The expression of pHluorin in addition to the vacuolar H^+-PPase led to increased growth rate and volumetric productivity of ethanol. **(Paper IV)**
- The H^+-PPase targeted to the vacuole in *S. cerevisiae* is capable of maintaining the intracellular pH when pHluorin is expressed. **(Paper IV)**

As usual, research generates more questions than it can answer. The work described here is not different and has not find all answers to previous research questions. The outlook for further research based on this thesis is as follows:

1. The mechanism of hydrogen peroxide formation requires further investigation to pinpoint the protein responsible for the increased tolerance of *Lb. reuteri* DSM 17938 compared to that of PTA 4659.
    a. This is especially relevant due to applications of *Lb. reuteri* to counter fungal infections. [208]
2. This work has revealed that, for the microbes studied, the kinetic mechanism of lactate dehydrogenase enzyme regulation is related to regulation of metabolic shifts in the catabolism, which is dependent on environmental conditions. This needs to be further verified in other organisms that produce lactate under normal or only under stress conditions.
3. The characterisation of the native enzyme responsible for the phosphofructokinase activity in *Lb. reuteri* is of interest for the understanding of the loss of the PFK-A from a practical and an evolutionary perspective. The real function of the two assigned genes for the PFK-B type in *Lb. reuteri need* to be further investigated. A possible experiment would be a knock-out approach for each gene and overexpressing it in another microorganism.
4. A full characterisation of *S. cerevisiae* expressing the vacuolar H^+-PPase with is required for a complete elucidation of the mechanism behind the observed improvement of growth under stressful conditions. It could include strains producing other heterologous proteins and testing them for growth on xylose.
5. The effect of the vacuolar H^+-PPase expression in *S. cerevisiae* on fermentation in lignocellulosic hydrolysates is of interest to establish whether it improves the resistance to inhibitory compounds.

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