Genetic engineering of lactic acid bacteria to produce optically pure lactic acid and to develop a novel cell immobilization method suitable for industrial fermentations

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Cover page photo: Trade card for Lactart Milk Acid, Avery Lactate Company, Boston, Massachusetts, 1884.
To my family
# Table of contents

Table of contents 4  
List of original publications 6  
Abbreviations 8  
Abstract 11  
1. Introduction 13  
  1.1 Lactic acid bacteria – interesting observations from the history 13  
  1.2 Current definition of LAB 15  
  1.3 The Gram-positive cell envelope 16  
    1.3.1 Structure of peptidoglycan backbone and interconnecting peptide crosslinks 17  
    1.3.2 Secondary cell wall polymers 18  
    1.3.3 Lipoteichoic acids 21  
    1.3.4 S-layers 22  
  1.4 Cell retention mechanisms for secreted and excreted proteins in LAB 24  
    1.4.1 Protein secretion mechanisms among LAB 24  
    1.4.2 Autolysins and excretion of cytosolic proteins 25  
    1.4.3 Various cell envelope retention mechanism for proteins in LAB 26  
  1.5 Fermentation pathways in LAB 37  
    1.5.1 Fermentation and respiration in LAB 37  
    1.5.2 Typical LAB fermentations 37  
    1.5.3 Other fates of pyruvate 40  
    1.5.4 Classification of LAB according to isomer of lactic acid produced 41  
  1.6 Lactic acid – usage, applications and production 42  
    1.6.1 Synthesis of PLA 44  
    1.6.2 Conventional LAB based industrial scale fermentation processes for lactic acid 45  
    1.6.3 Modern technologies in industrial bacterial fermentations – immobilization of cells for extracellular production of metabolites 48  
  1.7 Genetic engineering of Gram-positive bacteria to achieve whole cell immobilization with industrial potential 53  
    1.7.1 Immobilization on collagen surfaces by non-covalent binding 55  
    1.7.2 Immobilization on starch surfaces by non-covalent binding 55  
    1.7.3 Immobilization on cellulose surfaces by non-covalent binding 55
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.7.4 Immobilization on chitin surfaces by non-covalent binding</td>
<td>56</td>
</tr>
<tr>
<td>1.7.5 Immobilization to inorganic or synthetic surfaces by non-covalent binding</td>
<td>57</td>
</tr>
<tr>
<td>1.8 Genetic engineering of LAB to improve optical purity of the produced lactic acid</td>
<td>58</td>
</tr>
<tr>
<td>1.8.1 Various biosynthetic routes leading to D(-)- and L(+)lactic acid formation in LAB</td>
<td>58</td>
</tr>
<tr>
<td>1.8.2 Use of genetic engineering to produce lactic acid with enhanced optical purity</td>
<td>62</td>
</tr>
<tr>
<td>2. Aims of the study</td>
<td>67</td>
</tr>
<tr>
<td>3. Materials and methods</td>
<td>68</td>
</tr>
<tr>
<td>3.1 Plasmids</td>
<td>68</td>
</tr>
<tr>
<td>3.2 Bacterial strains, growth conditions and media</td>
<td>69</td>
</tr>
<tr>
<td>3.3 Oligonucleotides</td>
<td>71</td>
</tr>
<tr>
<td>3.4 Methods used in this study</td>
<td>73</td>
</tr>
<tr>
<td>4. Results and discussion</td>
<td>76</td>
</tr>
<tr>
<td>4.1 Production of PLA grade L(+)-lactic acid by genetically modified homofermentative <em>Lactobacillus</em> strain (publication I)</td>
<td>77</td>
</tr>
<tr>
<td>4.1.1 Construction of D-LDH negative <em>L. helveticus</em> strain by inactivation of <em>ldhD</em> by gene replacement</td>
<td>77</td>
</tr>
<tr>
<td>4.1.2 Construction of D-LDH negative <em>L. helveticus</em> strain by replacing the structural gene of <em>ldhD</em> with that of <em>ldhL</em></td>
<td>77</td>
</tr>
<tr>
<td>4.1.3 Growth and fermentation characteristics of the wild type strain and D-LDH negative strains</td>
<td>78</td>
</tr>
<tr>
<td>4.2 Development of whole cell immobilization method for LAB by surface engineering</td>
<td>81</td>
</tr>
<tr>
<td>4.2.1 Studying the potential of S-layer protein (<em>SlpA</em>) of <em>L. brevis</em> for anchoring peptides for cell surface display (publication II)</td>
<td>81</td>
</tr>
<tr>
<td>4.2.2 Indicating the potential of whole cell immobilization of <em>L. lactis</em> by cell surface displayed binding domain (publication III and IV)</td>
<td>83</td>
</tr>
<tr>
<td>5. Conclusions and future prospects</td>
<td>90</td>
</tr>
<tr>
<td>6. Acknowledgements</td>
<td>92</td>
</tr>
<tr>
<td>7. References</td>
<td>93</td>
</tr>
</tbody>
</table>

Publications I-IV
List of original publications

This thesis is based on the following publications, which are referred to in the text by their Roman numerals. The original articles are reprinted with the kind permission of the publishers. In addition, some unpublished data are presented.


The author’s contribution in the above mentioned publications:

Publication I
- construction of the recombinant strains and verification of the resulted strains (except sequencing)
- enzymatic assays
- transcriptional analysis
- Biostat B2 fermentations
- interpretation of the results and writing the publication in collaboration with the other authors

Publication II
- construction of pKTH5006, pKTH5007 and pKTH5008 plasmids
- transformation of these plasmids to Lactococcus lactis and Lactobacillus brevis in co-operation with the 1st author
- detection of the surface exposure of the VP1 epitope by whole cell ELISA in the Lactobacillus brevis strains carrying the above mentioned plasmids in co-operation with the 1st author
Publication III
- all genetic constructs
- major part of the adhesion testing and the method development
- major responsibility in the interpretation of the results and writing the publication in collaboration with the other authors

Publication IV
- all genetic constructs
- developing the adhesion testing method
- minor contribution in the interpretation of the results and writing the publication
**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>ABC</td>
<td>ATP binding cassette</td>
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<tr>
<td>CBD</td>
<td>cellulose-binding domain</td>
</tr>
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<td>CBM</td>
<td>cellulose-binding module</td>
</tr>
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<td>ChBD</td>
<td>chitin-binding domain</td>
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<tr>
<td>CPS</td>
<td>capsular polysaccharide</td>
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<tr>
<td>CWBD</td>
<td>cell wall binding domain</td>
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<tr>
<td>DEAE</td>
<td>diethylaminoethyl</td>
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<tr>
<td>DHAP</td>
<td>dihydroxyacetone phosphate</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<td>ECP</td>
<td>excretion of cytosolic enzymes</td>
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<td>EFSA</td>
<td>European food safety authority</td>
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<td>EPS</td>
<td>exopolysaccharide (sugar)</td>
</tr>
<tr>
<td>FDP (or FBP)</td>
<td>fructose-1,6-diphosphate (or fructose-1,6-biphosphate)</td>
</tr>
<tr>
<td>FEA</td>
<td>flagella export apparatus</td>
</tr>
<tr>
<td>FPE</td>
<td>fimbrilin-protein exporter (or pseudopilin export pathway)</td>
</tr>
<tr>
<td>GAP</td>
<td>glyceraldehyde-3-phosphate</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GLP</td>
<td>glycerol intrinsic proteins (glycerol facilitators)</td>
</tr>
<tr>
<td>HePS</td>
<td>heteropolysaccharides</td>
</tr>
<tr>
<td>HicDH</td>
<td>2-hydroxyisocapronate dehydrogenase</td>
</tr>
<tr>
<td>HoPS</td>
<td>homopolysaccharides</td>
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<tr>
<td>iLDH</td>
<td>NAD-independent LDH</td>
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<tr>
<td>LAB</td>
<td>lactic acid bacteria</td>
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<td>LDH</td>
<td>lactate dehydrogenase enzyme</td>
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<tr>
<td>LTA</td>
<td>lipoteichoic acid</td>
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<tr>
<td>MCS</td>
<td>multiple cloning site</td>
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<tr>
<td>MG</td>
<td>methylglyoxal</td>
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<td>MGS</td>
<td>methylglyoxal synthase</td>
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<tr>
<td>MLF</td>
<td>malolactic fermentation</td>
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<tr>
<td>nLDH</td>
<td>NAD dependent LDH</td>
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<tr>
<td>PDLA</td>
<td>poly(D-lactic acid)</td>
</tr>
<tr>
<td>PET</td>
<td>polyethylene terephthalate</td>
</tr>
<tr>
<td>PG</td>
<td>peptidoglycan layer</td>
</tr>
<tr>
<td>PLA</td>
<td>poly(lactic acid)</td>
</tr>
<tr>
<td>PLLA</td>
<td>poly(L-lactic acid)</td>
</tr>
<tr>
<td>PS</td>
<td>polystyrene</td>
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<tr>
<td>PTS</td>
<td>phosphoenolpyruvate-dependet phosphotransferase system</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>QPS</td>
<td>qualified presumption of safety</td>
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<tr>
<td>R-PCR</td>
<td>recombinant PCR</td>
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<tr>
<td>S-layer</td>
<td>surface layer</td>
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<tr>
<td>SBD</td>
<td>starch-binding domain</td>
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<tr>
<td>scPLA</td>
<td>stereocomplex PLA</td>
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<tr>
<td>SCWP</td>
<td>secondary cell wall polymers</td>
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<tr>
<td>Sec pathway</td>
<td>the general secretory pathway</td>
</tr>
<tr>
<td>SLH</td>
<td>surface layer homology domain</td>
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<tr>
<td>TA</td>
<td>teichoic acid</td>
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<tr>
<td>Tat</td>
<td>twin-arginine translocation pathway</td>
</tr>
<tr>
<td>TMD</td>
<td>transmembrane domain</td>
</tr>
<tr>
<td>TMS</td>
<td>transmembrane segment</td>
</tr>
<tr>
<td>TUA</td>
<td>teichuronic acid</td>
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<tr>
<td>WPS</td>
<td>cell wall polysaccharides</td>
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<tr>
<td>Wss</td>
<td>WXG100 secretion system</td>
</tr>
<tr>
<td>WTA</td>
<td>wall teichoic acid</td>
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</table>
Abstract

In this study *Lactobacillus helveticus* CNRZ32 was genetically engineered to produce pure L(+) -lactic acid. Naturally, this organism produces mixture of D(-) - and L(+) -lactic acid during fermentation of sugars. Production of D(-) - and L(+) -lactic acid is based on the activity of LdhD and LdhL, respectively. In this work, LdhD enzyme activity was removed by using two different genetic engineering approaches. In the first approach, *ldhD* gene expression and thus the LdhD activity was prevented by deleting the *ldhD* promoter region. In the second approach, the structural gene of *ldhD* was replaced with that of *ldhL* of CNRZ32. In both cases, a stable chromosomal mutant, neither requiring any additional selection for maintenance of the mutation nor carrying any foreign DNA in its genome, was achieved. Based on the fermentation tests, these two strains were demonstrated to be potential candidates for production of L(+)-lactic acid for the manufacturing of high melting temperature biodegradable poly(lactic acid) based plastics, as the optical purity of the L(+)-lactic acid produced by these strains exceeded the criteria (optical purity > 95 \%) recommended for this process.

Also, the surface display and anchoring of heterologous peptides and cellulose binding domain were studied with *Lactobacillus brevis* and *Lactococcus lactis*, respectively. In the *Lactobacillus brevis* study, the main interest was to demonstrate the surface display of poliovirus epitope VP1 by using the *L. brevis* S-layer as the anchoring system. In *Lactococcus lactis*, different types of anchors were studied to facilitate the whole cell immobilization of *Lactococcus lactis* with unmodified cheap cellulosic carrier. Most typically, continuous fermentation with immobilized micro-organism is the preferred choice, whenever possible, to run an industrial-scale fermentation in which the product is secreted to the surrounding medium. In this work, the whole cell immobilization of lactococcal cells on chemically unmodified cellulosic material was demonstrated by using two different cell wall anchors in combination with cellulose binding domain of *Cellvibrio japonicus*. 
1. Introduction

1.1 Lactic acid bacteria – interesting observations from the history

Lactic acid bacteria (hereafter referred as LAB), plants and animals (including also Homo sapiens) share a long and common history and interdependent relationship. The Neolithic revolution, so transition from nomadic way of living (hunting, gathering) to more sedentary way of living with agriculture and pastoralism, seems also to be a starting point for the development of many food fermentations (Teuber, 2000; Douillard and de Vos, 2014). Old evidences found in Mesopotamia supports that dairying was highly developed already about 6000 B.C. (Prajapati and Nair, 2008). Furthermore, from the era right after the invention of writing (about 3200 B.C by Sumerians of Mesopotamia), also written evidences have been found that contains signs for dairy products, such as milk, butter, fat, and cheese (Teuber, 2000).

About five thousands years after (1780) from the first documented evidences of food fermentations, Swedish chemist Carl Wilhelm Steele refined and studied lactic acid from sour milk (Teuber, 1993). About three decades later, 1808, another Swedish scientist, Jöns Jacob Berzelius found lactic acid in meat and other animal products (Benninga, 1990). One motive for his research with lactic acid was to prove its individual nature – at that time especially some French chemists were unwilling to acknowledge the individuality of this acid discovered by Berzelius famous fellow countryman, Scheele (Jorpes, 1970). Indeed, this individuality question remained open for a long time. In 1833, French scientists Gay-Lussac and Pelouzed tackled to this same question in their work and made and investigated 15 different salt of lactic acid. During these experiments, they found sublimation of pure lactic acid and found that the sublimate had almost completely lost its acid taste but regains acidic properties after prolonged boiling in water. Indeed, this was the first document record of a polymeric derivate of lactic acid (polylactide), although these scientists did not realize that in their time (Benninga, 1990). About 100 years later (1932), a process to polymerize lactide (cyclic di-ester of lactic acid) was developed and later patented by DuPont (1954) (Auras et al., 2010). However, it took several decades before the first large-scale production of this new biodegradable plastic started (Taskila and Ojamo, 2013).

In 1848 Engelhardt noticed in his study with calcium and zink salts, that the racemic lactate from sour milk and lactate originated from muscle had different solubility, water of crystallization, and decomposition points. Later, the concept of isomerism in lactate was introduced in 1873 by Wislicenus after he evaluated the optical rotation of the various lactate salts and concluded that structure of lactic acid needs to be \( \alpha \)-hydroxypropionic acid in order to comply with isomerism property of lactic acid molecule (Benninga, 1990).
He also stated that this geometrical isomerism must somehow be explained by “the
different arrangements of their atoms in space”, and his discoveries were also promoting
the invention of “asymmetric carbon atom” by another scientist, van Hoff (1874) (Rocke,
2010).

In the middle of 19th century, Louis Pasteur, another scientist with passion to study crystal
structures of compounds and their optical activity, got interested about fermentations.
Pasteur was convinced that only living processes could produce optically active
compounds (Simon, 2013). He also started to study the spoilage of wine and beer and
discovered new form of yeast that was responsible of the sour taste in beverages. In 1857
Pasteur coined this yeast as lactic yeast, because it had a different size and shape when
compared to commonly known brewer’s yeast and the yeast involved in alcoholic
fermentation. In addition, Pasteur’s studies with alcoholic and lactic fermentations
confirmed his original hypothesis - ‘the ferment is life’, meaning that the fermentation
takes place due to the activity of living organisms (Debré, 1998; Barnett, 2003). Later
(1873), Lister was the first scientist who isolated a pure culture of bacteria by using liquid
laboratory techniques. He coined this species as Bacterium lactis (currently known as
Lactococcus lactis). In this work, the original driving force for Pasteur was to model the
theory that infectious diseases of humans are the result of the growth of specific living
microscopic organisms in the human host. To support this theory, he constructed a model
system for which he isolated pure culture of bacterium from milk sample by dilution
method and inoculated that into pre-heated milk, resulting in lactic acid fermentation
(Santer, 2010).

Industrial scale lactic acid production started in 1883 in USA by Avery Lactate Co.
(Boston) and couple of years later (1885) by Albert Boehringer Chemische Fabrik in
Ingelheim, Germany (van Velthuijsen, 1994; Benninga, 1990). At that time, most of the
lactic acid produced was used by dyeing, leather, textile, and food industry (Anonymous,
2016b). As reviewed by Josephsen and Jespersen (2006), commercial production of
various starter cultures was initiated during the late 1880s by the Danish pharmacist Chr.
D.A. Hansen and the use of dairy pure cultures in the milk fermentations began in late 19th
century almost simultaneously in Denmark, Germany and United States. As reviewed by
Porto de Souza Vandenberghe et al. (2013), in the long run the progress with the dairy
starter cultures contributed to the development of the whole dairy chain and led to rise of
various specified standard products to fulfill different demands of market.

Interestingly, association of lactic acid fermentation products to beneficial health impacts
was used in marketing already in the early days of industrial production of lactic acid (see
cover page picture: Trade card for Lactart Milk Acid, Avery Lactate Company, Boston,
Mass., 1884). Couple of decades later, more scientific publications came out from
scientist originated from Russia, Elie Metchnikoff. During the first decade of new century,
he theorized that the human health could be enhanced and also senility delayd, by
manipulating the intestinal microbiome with host-friendly bacteria found in yogurt. One of
the things behind this theory was his observations that long-lived Bulgarian citizens
consumed Lactobacillus-fermented dairy products (Mackowiak 2013; Bibel 1988). His
theories got a lot of publicity and his texts were also sometime exaggerated in different
public papers or magazines – like using phrase ‘prolongation of life’ in headlines. As
reviewed by Bibel (1988), this kind popularization of science also effected to the growing
demand for different kind lactobacillus cultures and fermented milk products in the early
20th century. Notably, this early enthusiasm towards fermented milk products allowed the word yoghurt to enter in the common language (Baglio, 2014).

Metchnikoff’s hypothesis for the connection between senility and intestinal flora must have gained a lot of scientific attention in this era, because still four decades later (1949), famous Danish scientist Orla-Jensen in his late days re-visited this theory with his colleagues and found that it is not the undesirable flora found in the aged which causes impaired digestion, but rather the impaired digestion resulting in undesirable intestinal flora (Olsen, 1950). However, in the scientific literature Orla-Jensen is best known from his work for classification of lactic acid bacteria. As reviewed by his contemporary (Heineman, 1920) and later by Murray and Holt (2006), he made an extensive study with various types of lactic acid-producing bacteria and delimited genera and species on the basis of characteristics such as fermentation of various sugars, by-products from these fermentations, morphology and temperature ranges for growth. In his work published in 1942, he described LAB as Gram-positive, non-motile, non-spore-forming, rod- or coccus-shaped organisms that ferment carbohydrates and higher alcohols to form mainly lactic acid (Franz and Holzapfel, 2011). Orla-Jensen’s work with classification was far-reaching, because his basic scheme for the classification remained useful for a long time. After the 2nd World War and the development of modern biochemical and molecular methods, it was found out that this traditional indentification scheme was not anymore completely correlating with phylogenetic relationships learned by using these new tools (Stackebrandt and Teuber, 1988). Pot and Tsakalidou (2009) have reviewed the development of the classification of the lactic acid bacteria (and especially that of the genus Lactobacillus) by reviewing the most popular phenotypic fermentation characteristics that were in use for the classification of LAB before the development of modern genotyping tools and the current polyphasic approach for the taxonomic classification of LAB.

1.2 Current defnition of LAB

Although no unequivocal definition exists for LAB, some usual characteristics for a typical member of LAB can be listed (Axelsson, 2004). The general description for lactic acid bacteria refers typically to group of non-sporulating, Gram-positive bacteria which are devoid of functional cytochromes, non-respiring but aerotolerant anaerobes, catalase-negative (some may have pseudo-catalase), acid tolerant fastidious cocci, coccobacilli or rods with low G+ C content of DNA (less than 50 mol %) and which produce lactic acid as the major end product during the fermentation of carbohydrates (Axelsson, 2004; Vandamme et al., 2014).

Due to their fastidious nature and other typical characteristics, LAB are usually associated with nutrient rich niches like plant and animal raw materials, environments and products in which fermentation of these materials occurs. Also, some species of LAB may occur in the respiratory, intestinal and genital tracts of humans and animals (Giraffa, 2014).

The traditional definition of LAB is not a taxonomic one, but rather a definition for functional characteristics that food microbiologists have used to describe harmless bacteria
that produce lactic acid and which occurs spontaneously in traditional lactic acid fermented foods (Molin, 2008). As presented by Axelsson (2004), LAB comprise around 20 genera, out of which the main genera of food related, ‘principal’, LAB are: *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella*. As summarized in more recent review by Vandamme et al. (2014), the genuine members of LAB belong to the order Lactobacillales, in class Bacilli in phylum Firmicutes. Under this order, there are six families: Aerococcaceae, Carnobacteriaceae, Enterococcaceae, Lactobacillaceae, Leuconostocaceae and Streptococcaceae. Taxonomy under these families is evolving constantly – in 2014, total 38 genera and about 400 species were classified under the order Lactobacillales.

1.3 The Gram-positive cell envelope

In general, Gram-positive bacteria carry only one membrane (plasma membrane) around the cell, but have a relatively thick peptidoglycan layer (30-100 nm) covering the plasma membrane. Instead, Gram-negative bacteria have a relatively thin peptidoglycan layer, but these organisms possess an additional membrane (outer membrane) on top of the peptidoglycan layer. In some cases, bacteria may also carry an S-layer, a monomolecular layer composed of identical subunits, which shows as the outermost layer in the cell envelope. The combined structure that is formed by the above mentioned cellular components is known as cell envelope. The major structures in the envelope, membrane(s) and peptidoglycan layer, are decorated with various molecules, like proteins, polymers and other macromolecules, which are important contributors not only for the envelope structure and function (Silhavy et al., 2010), but ultimately for the survival of the whole cell, too. Also, S-layers may function as scaffolds to display functional proteins and/or carbohydrate moieties on the cell surface (Fagan and Fairweather, 2014; Schuster and Sleytr, 2015). An artist’s view of the cell envelope of a Gram-positive bacterium is presented in Fig. 1.

The main function of the peptidoglycan layer (or peptidoglycan macromolecule) is to maintain cell integrity by withstanding the internal osmotic pressure. It has also important role in maintaining the defined cell structure and it is intimately involved in the cell division process (Nanninga, 1998). This peptidoglycan (PG) layer (sacculus) formed around the cell is the structural equivalent of the exoskeleton of insects. Actually, one of the best term to describe the PG layer is that of a fisherman’s net - so a structure that wraps the bacterial cellular contents.
In this net structure, the mesh is formed by two segments of parallel, rather inextensible glycan threads, held together by two small elastic peptide crosslinks. These crosslinks allow the net to expand or shrink. In Gram-positive bacteria, several concentric PG layers are covalently staggered by radial half-crosslinks, expanding the 3D architecture of the peptidoglycan.

1.3.1 Structure of peptidoglycan backbone and interconnecting peptide crosslinks

In LAB, like in all eubacteria, the glycan thread in the peptidoglycan structure is a polymer of the disaccharide N-acetyl-glucosamine-\(\beta(1\rightarrow4)\)-N-acetyl-muramic acid, so it has alternating N-acetyl-muramic acid (MurNAc) and N-acetyl-glucosamine subunits (GlcNAc) (Delcour et al., 1999). There are only minor variations in the chemistry of the glycan chains among all the bacteria. Instead, more considerable variation is found in the composition of stem peptides which are linked to the carboxyl group of the MurNac (Scheffers and Pinho, 2005). Typically, the consensus amino acid sequence of this stem peptide among LAB is L-Ala-\(\gamma\)-D-Glu-X-D-Ala (see Fig. 2). The third amino acid (X) is a di-amino acid, most often L-Lys (like in \textit{L. lactis} and most lactobacilli) but can also be mesodiaminopimelic acid (mDAP) or lornithine (Chapot-Chartier and Kulakauskas, 2014). Interestingly, although the occurrence of amino acids with the D configuration (like those found in stem consensus sequence) in nature is quite rare, review published by Martínez-Rodríguez et al. (2010) reveals that D-amino acids are often, but not always,
found in various self-defence related molecules, like those of antibiotics, venoms, bacteriocins or defensive structures like cell wall peptidoglycan layer. Another interesting discovery related to the structure of stem peptides of some LAB, is the presence of D-Lac as the last residue in the stem peptide among the vancomycin resistance LAB species, like *Lactobacillus plantarum* or *Lactobacillus casei*. As pointed out by Chapot-Chartier and Kulakauskas (2014), most often D-Ala predominates at this position in the newly synthesized PG of a typical (and vancomycin sensitive) LAB strain.

Variation exists also in the interpeptide bridge connecting the stem peptides elongating from different glycan strands. Quite often, the interpeptide bridge is composed out of single D-amino acid (most typically D-Asp or D-Asn), as it the case with many lactobacilli (Chapot-Chartier and Kulakauskas, 2014), but it is also possible that it contains several L-amino acids as is the case with *Streptococcus thermophilus* and with many other streptococci, too, as reported by Schleifer and Kandler (1972). Indeed, these two researchers (1972) developed specific nomenclature and rules to classify different variations found in the PG structure. According this system, the primary structure of PG is divided into two main classes depending on the anchorage point of the crossbridge in the stem peptide, and further divided into several subclasses depending on the presence/type of the connecting interpeptide bridge and the amino acid in the third position of the stem peptide (Šimelytė et al., 2003).

**Figure 2.** Schematic structure of peptidoglycan typically found among many LAB presented with some possible variations (modified from Chapot-Chartier, 2014).

### 1.3.2 Secondary cell wall polymers

According to classification developed during 1980s, the cell wall polysaccharides of Gram-positive organisms can be classified under three distinct structural related groups: (i) teichoic acids, (ii) teichuronic acids, and (iii) other neutral or acidic polysaccharides not classified in the former groups (Schäffer and Messner 2005, Araki ja Ito 1989, Munson and Glaser, 1981). As all of these compounds are not primarily related to the main
function of the cell wall, the term of secondary cell wall polymers (SCWP) has been assigned to them. The first two groups of these polysaccharides are often referred as ‘classical’ SCWPs. Although, the exact biological functions of these ‘classical’ SCWPs are not fully understood, several different functions have been attributed to them, like the following ones reported by Schäffer and Messner (2005):

(i) binding of divalent cations  
(ii) role in the balance of metal ions for membrane functionality  
(iii) binding of proteins  
(iv) role in folding of extracellular metallo-proteins  
(v) providing a source of phosphate under phosphate starvation conditions  
(vi) interaction with cell wall lytic enzymes  
(vii) formation of a barrier to prevent diffusion of nutrients and metabolites

More information for the biological functions of various cell wall glycopolymers in LAB is available in the recent review by Chapot-Chartier and Kulakauskas (2014). In the following section the main focus is on introducing the prevalence and basic structure of these molecules among LAB.

1.3.2.1 Classical secondary cell wall polymers

1.3.2.1.1 Teichoic acids

Different polyanionic cell wall carbohydrates are found to be important determinants to bacterial electrostatic charge and hydrophobicity and play pivotal role in bacterial attachment to abiotic surfaces and eukaryotic cells (Theilacker and Hübner, 2009). For Gram-positive bacteria, teichoic acids (TAs) represent the most abundant polyanionic compounds in the cell envelope (Fischer, 1994). The polyanionic characteristic of TAs is due to the negatively charged phosphodiester bonds located in the polyol-phosphate backbone of these molecules (Theilacker and Hübner, 2009). Indeed, most teichoic acids have also zwitterionic properties. This is due to the presence of negatively charged phosphate groups and modifications with free amino groups that are contained in residues such as D-alanine (Neuhaus and Baddiley, 2003; Fischer, 1997; Weidenmaier and Peschel, 2008). The cell envelope related TAs are usually divided into two different categories: wall teichoic acids (WTA) and lipoteichoic acids (LTA). As lipoteichoic acids are not cell wall bound but attached to the cell membrane via glycolipid anchor, those will be introduced in more detail in other section in this work.

WTAs are covalently bound to peptidoglycan. Most commonly, the structure of WTA is composed by glycerol or ribitol groups that are connected by phosphodiester bonds. More complex repeating units, consisting of trioses or hexoses or glycosylpolyolphosphate monomers, exists also, e.g in Streptococcus pneumoniae and in Enterococcus faecalis (Fischer et al., 1993; Theilacker and Hübner, 2009). WTAs are not found in every species; L. casei and Lactoccus lactis subsp. cremoris being known examples among LAB that
lack these compounds (Delcour et al., 1999). However, in some bacterial species WTAs may constitute up to half of cell wall total dry weight (Weidenmaier and Peschel, 2008; Chapot-Chartier and Kulakauskas, 2014).

1.3.2.1.2 Teichuronic acids

Many Gram-positive bacteria produce other polyanionic cell wall glycoligopolymers which lack phosphate groups in their polymer backbone and are not, therefore, classified as TAs. One compound belonging to this group is teichuronic acid (TUA) (Weidenmaier and Peschel, 2008). Structurally TUAs are consisted of glycosidically linked sugar monomers and uronic acid residues. The negative charge of TUA is due to the carboxyl groups of the uronic acid residues (Delcour et al., 1999). If grown under phosphate starvation some species like *Bacillus subtilis* replaces the phosphate containing WTA with TUA (Neuhaus and Baddiley, 2003). Furthermore, under these conditions a phosphate loss from the walls has been detected, proposing the usage of the WTA bound phosphate for the necessary cell metabolism (Grant, 1979). It is speculated that this is an indication from the physiological need to have an anionic polymer present in the cell wall (Neuhaus and Baddiley, 2003). The interdependency between WTA and teichuronic acid synthesis observed by Grant (1979), seems to ensure a constant level of anionic cell wall charge and reserve phosphate source for the cell. So far, the presence of cell wall related TUA have not been described for any LAB. However, as the phylogenetic distance between species of the genera *Bacillus* and LAB is rather short, the existence of teichuronic acids in the PG is possible in LAB, too (Delcour et al., 1999). In fact, part of the genes needed for TUA synthesis were detected during a whole genome sequencing project of *L. lactis* (Bolotin et al., 2001). Also, according to Poxton (2014) the presence of teichuronic acid in *Streptococcus bovis* has been reported in early 1970s.

1.3.2.2 Other secondary cell wall related polymers

The last third group of SCWPs (so the group ‘others’) contains different neutral and acidic polysaccharides. This group is usually divided into two other subclasses: capsular polysaccharides (CPSs), which form a thick outermost shell around the bacteria and are often covalently bound to the cell wall, and *sensu stricto* group of cell wall polysaccharides (WPS), which decorate the envelope and are either covalently bound or loosely associated with the peptidoglycan (Delcour et al., 1999).

Some LAB strains, like *L. lactis* and *Streptococcus agalactiae*, are covered by pellicle. Pellicles are polysaccharide structures covalently linked to peptidoglycan and are therefore qualified as WPS (Chapot-Chartier et al., 2010; Bessaurt et al., 2014). In *L. lactis* this structure has predicted to confer a protective barrier against host phagocytosis by murine macrophages whereas the pellicle detected in *S. agalactiae* seems to allow access to underlying peptidoglycan. Interestingly, in some lactococci these pellicle polysaccharides seem to act as a receptor for certain phages of this species, too (Mahony et al., 2013). In general, as reviewed by Chapot-Chartier (2014), WPS in LAB appear to be omnipresent components of the cell surfaces of these organisms and seems to display high structural variety even in a strain level.
As mentioned above, CPSs are typically bound covalently to PG and form a thick outer layer surrounding the cell. In addition to these polymers, there is a distinct group of bacterial polysaccharides found outside the cell, exopolysaccharides (EPSs), which do not permanently remain attached to cell walls but remain loosely related to the cell or are released into the medium as a slime (Sutherland, 1972). Differentiation and proper nomenclature between CPS and EPS may sometimes be rather complicated, as it is possible under specific conditions that CPSs are released in the growth medium and EPS are associated with the cell surface (Cescutti, 2009). Also, in many occasions microbial exopolysaccharides (EPS) are referred to be either capsular or slime associated (de Vuyst and Degeest, 1999), which further blur the border between CPS and EPS.

According to Chapot-Chartier (2014), members of all above mentioned cell wall polysaccharides (WPS, CPS and EPS) may be produced by the same bacterium, although it may be sometime difficult to make difference between the members of these three groups. Most often, these polysaccharides are neutral, but some may be acidic due the branching with anionic substituents (Delcour et al., 1999; Kleerebezem et al., 2010). Structurally, CPS and EPS can be divided into homopolysaccharides (HoPS) and heteropolysaccharides (HePS), depending whether the structure in their repeating unit is composed of polymers with single or multiple type of monosaccharides, respectively (Cescutti, 2009; Ryan et al., 2015). Because of their abundant presence on the outer surface of the cell wall, it is expected that extracellular and cell-wall associated polysaccharides determine to a large extent the surface properties of microorganisms (Schaer-Zammaretti and Ubbink, 2003). Production of HoPS is typical for many Streptococcus, Leuconostoc and Pediococcus strains, whereas HePS are typically produced by mesophilic or thermophilic LAB strains that are often utilized in dairy industry, where the EPS producing ‘ropy’ strains are important in developing the proper consistency of fermented milks and yoghurts (de Vuyst and Degeest, 1999, Ruas-Madiedo and Reyes-Gavilán, 2005). In addition to beneficial properties in food industry, it seems that microbial EPS possess promising therapeutic potential in terms of anti-oxidation, hypocholesterolemia, immunomodulation and promotion of a functional digestive tract through prebiotic activity (Ryan et al., 2015).

Interestingly, Schäffer and Messner (2005) proposed that the glycoconjugates that mediate the non-covalent attachment of some glycosylated S-layer proteins to the underlying cell wall in Bacillaceae should be classified under the ‘other’- group of secondary cell wall polymers. Furthermore, authors coined this type of SCWPs as ‘non-classical’ SCWPs to differentiate those from teichoic acids and teichuronic acids (classical SCWPs). Also the same authors divided the ‘non-classical’ SCWPs into three groups according to common features found among the representatives of these groups.

1.3.3 Lipoteichoic acids

Interestingly, the most common type of membrane-anchored anionic polymers, lipoteichoic acids (LTAs), were first extracted from a lactic acid bacterium strain, namely Lactobacillus fermentum NCTC6991 (formerly Lactobacillus fermenti NCTC6991) (Wicken and Knox 1970; Delcour et al., 1999). These membrane bound polymers are
usually constituted of glycerol-phosphate repeating units connected to glycolipid and are
usually less diverse in structure than cell wall glycopolymers due to their peculiar
biosynthetic pathway (Weidenmaier and Peschel, 2008). However, more complex
structures have been presented for some strains, including Lactococcus garviae as an
example among LAB (Greenberg et al., 1996). In LAB, LTAs have been detected at least
in enterococci, lactobacilli, lactococci, leuconostocci, and streptococci (Fischer, 1994). In
fact, LTAs are quite abundant in some LAB species. It has been estimated that glycolipid
anchor of LTAs in L. lactis constitutes every fifth lipid molecule of the outer leaflet on the
cytoplasmic membrane of this strain (Fischer, 1981).

1.3.4 S-layers

S-layers are monomolecular isoporous crystalline lattice layers, that are usually found as
the most outermost cell envelope structure in many prokaryotic cells and represent the
most simplest form of biological membranes developed during evolution. Most typically,
these structures are composed of a single protein or glycoprotein species and the formed
S-layer covers the surface of the whole cell (Sleytr et al., 2001). As a result, if present, S-
layers are among the most abundant proteins in a given cell, and represent typically 10-15
% of total cellular proteins (Boot and Pouwels, 1996). Indeed, the biosynthesis of the
lattice subunits needs to be very effective, because a typically rod-shaped prokaryotic cell
with generation time of 20 min, requires synthesis of 500 subunits in a second to keep the
whole cell covered (Sleytr et al. 1999).

The S-layer subunits are held together by non-covalent interactions. Morphological units
found in different S-layer lattices may consist of one or two (obliques), four (squares), or
three or six (hexagonal) identical protein subunits and isolated S-layer (glyco)proteins
possess remarkably intrinsic property for recrystallization in suspension and at a broad
range of surfaces and interfaces (Egelseer et al., 2010; Sleytr et al., 2001). Most typically,
anchoring of S-layer proteins to the cell takes place via non-covalent interactions with
cell wall structures; like lipopolysaccharides (LPSs) in Gram-negative bacteria and cell
wall polysaccharides in Gram-positive bacteria (Fagan and Fairweather, 2014). In addition
to domain (or region) responsible for the binding to the cell wall, another conserved
domain exists in S-layer protein which is related to the self-assembly of S-layer monomers
(Sleytr et al., 2014).

As S-layers are usually the most outermost envelope structure, it is not surprising that the
discovery of these structures was linked to the development of microscopy. Indeed, the
first observation from S-layers was made over sixty years ago when studying the electron
micrographs of shadowed preparations (Houwink, 1953; Sleytr and Messner, 1983). With
the development of instrumentation and procedures used in microscopy and in other
analytics (like gene sequencing), the number of organisms that are known to carry S-layer
protein is currently counted in hundreds (Sleytr and Messenr, 1983; Messner et al., 2010).
In Fig. 3A, protein dense S-layer from Lactobacillus crispatus is shown as a thin layer
around the cell. In Fig. 3B, examples of S-layers composed out of different morphological
units are presented.
It seems that S-layers are typically found only in one genus among LAB - in *Lactobacillus*. In a recent review by Hynönen and Palva (2013), various S-layers of lactobacilli are reviewed. Inside this genus, S-layers are present on many but not in all species. Typical characteristics of the S-layers of lactobacilli are: protein monomers are secreted through the general secretary pathway, S-layer proteins are usually small (25-71 kDa) and form lattices with oblique or hexagonal symmetry, and have high predicted overall pI value (isoelectric point): 9.4-10.4. In contrast to most other S-layers, lactobacillar S-layer proteins do not possess surface layer homology domain (SLH) that is in many organism responsible for attaching the S-layer lattice to the peptidoglycan layer by means of non-covalent binding. Instead, it seems that lactobacillar S-layers typically possess two repeated amino acid motifs (showing high predicted pI) in their cell wall binding area. Most typically, the cell wall binding domain is C-terminal, but at least in *Lactobacillus brevis* and *Lactobacillus hilgardii*, it is located at the N-terminus (Åvall-Jääskeläinen et al., 2008; Dohm et al., 2011). As the positive charge of lactobacillar S-layer is especially concentrated into same area where the cell wall binding region exists, it has been proposed that electrostatic interaction occurs between the negatively charged cell wall polymers and S-layer cell wall binding region (Hynönen and Palva, 2013; Antikainen et al., 2002).

As reviewed by Dohm et al. (2011), various post-translational modifications, such as linking of glycan strands, lipids, sulphate or phosphate groups with the S-layer protein scaffold, have been reported, indicating adaptation of the corresponding S-layers to their specific environment. One of these modifications is O-glycosylation, which has been detected in few lactobacillar S-layers. In a recent study reported by Anzengruber et al. (2014b), the O-glycosylation of S-layer and the presence of related protein O-glycosylation mechanism in *Lactobacillus buchneri* were proposed.
Although various functions for different S-layers have been proposed and demonstrated, no common general function for these structures has been found, yet (Sleytr et al., 2014). Among *Lactobacillus*, S-layers have often been considered to mediate bacterial adherence to various targets, and currently most of the S-layer research among LAB is focused to study the interactions between S-layers or S-layer possessing strains with human or animal derived cells, receptors or macromolecules. Other putative roles suggested for lactobacillar S-layers include: protection of cell towards lytic enzymes, stress conditions (e.g. tannic acid and copper in wines) and role as putative phage receptor (Hynönen and Palva, 2013). Also, it has been demonstrated that S-layers are able to defend host cell by binding metal ions (Dohm et al., 2011, Schut et al., 2011; Gerbino et al., 2012, Gerbino et al., 2015). Interestingly, murein hydrolase activity (e.g. towards cell wall peptidoglycan of *Salmonella enterica*) has been detected in the C-terminal part of the S-layer protein SA of *L. acidophilus* ATCC 4356 as reported by Prado-Acosta et al. (2008). The same research group detected later the synergestic relationship between nisin and S-layer of this same species in inhibition of the growth of pathogenic Gram-negative *S. enterica* and potential pathogenic Gram-positive bacteria *Bacillus cereus* and *Staphylococcus aureus* (Prado-Acosta et al., 2010).

Genetically engineered S-layers have a great potential in many applications. As recently reviewed by Sleytr et al. (2014), recombinant S-layers proteins have been engineered to be used in wide range of applications, like in vaccine development, biochip applications, bioremediation, purification/downstream processing, building blocks of nanoparticle arrays, biosensors, protective barriers, and as a platform for immobilized biocatalysts.

### 1.4 Cell retention mechanisms for secreted and excreted proteins in LAB

#### 1.4.1 Protein secretion mechanisms among LAB

In Gram-positive bacteria, proteins synthesized in cytoplasm may remain in this compartment, or are sorted to the cytoplasmic membrane, to the cell wall or to the surrounding medium (Tjalsma et al., 2000). Sorting of proteins to their relevant subcellular compartment or surrounding environment is essential as proteins of different final location might have very unrelated functions (Bendtsen and Woolbridge, 2009). Most often, proteins which need to be transported to an extracytoplasmic location contain an N-terminal signal peptide. With this signal, the newly synthesized protein from the ribosome is targeted to certain protein transport pathway. After the translocation, specific signal peptidases cleave the signal sequence, resulting in possible detachment of the protein from the membrane. In Gram-positive bacteria (including LAB), the majority of the secreted proteins are exported from the cytoplasm through the cytoplasmic membrane via the general Secretory (Sec) pathway (Sibbald and van Dijl, 2009). According to Desvaux et al. (2009) other characterized protein secretion systems translocating proteins with N-terminal signal peptides are those of Twin-arginine translocation pathway (Tat) and Fimbrillin-protein exporter (FPE, also known as pseudopilin export pathway).
Furthermore, small antibacterial peptides, bacteriocins, with N-terminal leader sequence are typically secreted by specific ABC exporters (Bendtsen and Woolbridge, 2009; Kleerebezem et al., 2010).

As reviewed by Sibbald and van Dijl (2009), it seems that the proportion of secreted proteins without any known secretion signal varies per organism. When considering *B. subtilis*, the model organism of Gram-positive secretion, this number is relatively low, whereas for group A *Streptococcus*, it seems to be much higher. Some of the proteins without any known export signal are actively transported while others are proposed to be released during cell lysis. In Gram-positive bacteria, the former group includes at least proteins which are transported via the Wss pathway, holin transport system, flagella export apparatus (FEA) and in some cases via the accessory Sec system (Desvaux and Hébraud, 2006; Desvaux et al., 2009). In some case, part of the cell’s plasmamembrane may be reformed into vesicles carrying proteins or other macromolecules (like DNA) inside. Indeed, active lysis-independent vesicle formation and subsequent release of these vesicles into external medium is a conserved phenomenon among Gram-negative bacteria, including pathogenic and non-pathogenic species, but detected also among Gram-positive bacteria (MacDonald, 2012), also including some streptococci (Liao et al., 2014; Olaya-Abril et al., 2014).

1.4.2 Autolysins and excretion of cytosolic proteins

The very ultimate mechanism for any single cellular organisms, like LAB, is to deliver proteins to surrounding medium by induced partial or complete autolysis. In bacteria, autolysis or self-digestion is a process, which can be defined as a breakdown (or lysis) of the cell, and which results from the cell’s own hydrolytic enzyme activity towards various and specific bonds in its cell wall peptidoglycan (Shockman et al., 1996; Crouigneau et al., 2000). Enzymes that are responsible for this lytic activity are called autolysins. Although, the exact mechanism and regulation of the extend of autolysis among LAB is not fully understood, yet, the functional effects of this phenomenona, like the release of internal peptidases inside starter cultures during cheese ripening, are widely acknowledged and employed in modern dairy business. Probably the best know autolysin among the dairy related species, is the major autolysin of *L. lactis*, AcmA, which is covered in more detail in later in this study.

As reviewed by Chapot-Chartier (2010), autolysins have been proposed and demonstrated to be involved in several different functions during the bacterial growth cycle:

- Septation and separation of daughter cells
- Cell expansion
- Peptidoglycan turn-over
- Protein secretion

Also, it has been speculated that they possess roles in more specific functions, which are related to: competence for genetic transformation, flagellar morphogenesis, spore formation and germination, biofilm formation, pathogenicity, waking up dormant bacteria and autolysis and programmed cell death (Chapot-Chartier, 2010). The role of autolysins
in the regulated excretion of cytosolic enzymes (ECP), during which cell’s viability remains secured, remains to be a hot topic in scientific debate until the beauty of this putative non-classical excretion mechanism has been completely revealed (Götz et al., 2015).

1.4.3 Various cell envelope retention mechanism for proteins in LAB

Desvaux et al. (2009) have reviewed the different fates of translocated proteins for Gram-positive bacteria. According these authors, after translocation a single protein may remain to be anchored to the membrane, associate covalently or non-covalently with cell-wall components, assemble into macromolecular structures on the cell surface (e.g. flagellum, pilus and S-layer), be injected into a host cell, or be released in the surrounding medium. Here, the main interest is to study various cell envelope retention mechanisms that provide surface display of the target protein (or domain).

In most cases, translocated (or excreted) proteins are secreted or diffused to the surrounding medium unless they do not possess any retention signals (or domains) which keep them bound with the cell envelope constituents. However, protein localization within the cell envelope does not necessarily guarantee its surface exposure. Vice versa, translocation of the whole protein is not always required for the surface display. Typical example for this is membrane proteins which contains one or several hydrophobic transmembrane domains (TMD).

As reviewed by Desvaux et al. (2006), four major types of cell surface displayed proteins are generally recognized: proteins which are anchored to the cytoplasmic membrane by hydrophobic transmembrane domain(s), lipoproteins which are covalently attached to membrane lipids, proteins which are covalently linked to peptidoglycan via C-terminal LPXTG-like motif, and proteins attaching to cell wall by specific cell wall binding domains. Together, this subset of surface exposed proteins constitutes the surfaceome of the bacterial cell (Cullen et al., 2005, Desvaux et al., 2006). As stated by Cullen et al. (2005), identification of the members belonging to this group is critical to understanding the interactions of bacteria with their environments.

In the following sections, the mechanism of various cell membrane and cell wall retention mechanisms are presented among LAB. Specially, the main focus is on introduction of the non-covalent retention mechanisms in LAB.

1.4.3.1 Binding of proteins to membrane

Intrinsic (or integral) membrane proteins are tightly associated with biomembranes and do not detach from those by washing (change in pH or in ionic strength). Indeed, detachment during this kind of washing is the characteristic feature for the extrinsic (or peripheral) membrane proteins, which are membrane associated. Most typically, the binding of the peripheral proteins take place via electrostatic and hydrophobic interactions, whereas integral proteins remain membrane bound by one (bitopic proteins) or several (polytopic
proteins) transmembrane spans. In addition, monotopic integral proteins penetrate membranes partially but do not span the entire membrane, or are anchored in the membrane by covalent bonding with the membrane lipids (Luckey, 2014). In the following sections, binding mechanisms of the transmembrane proteins and lipid anchored proteins are introduced in more detail.

1.4.3.1.1 Anchoring by membrane spanning

Transmembrane regions in proteins are structurally either $\beta$-barrels or $\alpha$-helical sheets, out of which the latter type of structure is typically found also among the membrane spanning proteins of Gram-positive bacteria. The average length of a transmembrane $\alpha$-helix is more than 25 amino acids and is not typically oriented perpendicular to the membrane plane but is more or less tilted (Schneider et al., 2007). Indeed, there exist several different topological variants within the group of $\alpha$-helical membrane proteins. According to Higy et al. (2004) these proteins may be divided into sub categories depending on presence/absence of cleavable signal sequence and subcellular location of the N- and C-terminus (when anchored). Also, classification may also be based on the number of passes through the membrane.

The actual biogenesis of $\alpha$-helical membrane proteins is often described by the model proposed by Popot and Engelman (2000). This model includes two energetically distinct stages: formation and subsequent side-to-side association of independently stable transbilayer helices. During the first stage, membrane proteins are synthesized by the ribosomes and their transmembrane domains are integrated into the target membrane, which leads to adoption of their topologies (topogenesis). During the second stage, membrane proteins fold and adopt their native structures. The stability of the individual domains is a consequence of the hydrophobic effect and main-chain hydrogen bonding, whereas other interactions drive to side-to-side helix association. As this model is very rough, it is not always possible to clearly separate the second stage from the topogenesis.

According to Zweers et al. (2008), there are enormous variation among the number, hydrophobicity and the membrane topology of the transmembrane segments (TMSs) between different membrane proteins. Most typically, the topology of membrane proteins follows the positive-inside rule (von Heijne, 1986). According this rule, the positively charged residues are more likely found in the cytoplasmic loops rather than in periplasmic loops. However, alteration of the charge distribution in the regions flanking a TMS may result in reversion of the topology. Also, the length and the mean hydrophobicity of the TMS affect the membrane insertion. Typically, the first TMS of a polytopic membrane protein determines the orientation of subsequent TMSs, as there is need to alternate the direction for the membrane spanning (Driessen and Nouwen, 2008).

1.4.3.1.2 Covalent anchoring of lipoproteins

Bacterial lipoprotein biogenesis was established by Wu and co-workers (Braun and Wu, 1994) in Gram-negative model organism Escherichia coli, in which the related enzymes have also been found to be indispensable for survival (Hutchings et al., 2009). As discovered in E. coli, prolipoproteins carry Type II signal peptides, which direct these molecules through Sec- (or Tat-) system to the lipoprotein biogenesis machinery. The type II signal peptides include a conserved motif (the ‘lipobox’), which is typically
L-[A/S/T]_{2}-[G/A]_{1}-C_{1+1} with the +1 cysteine absolutely conserved in all bacterial lipoproteins. Lipid anchoring is initiated by formation of a covalent linkage between the membrane phospholipid derived diacylglyceride group and the indispensable cysteine residue found in the lipoprotein signal peptide. This process is catalyzed by prolipoprotein diacylglycerol transferase (Lgt) enzyme. Interestingly, the Lgt of \textit{L. lactis} was the first Gram-positive prolipoprotein diacylglycerol transferase that has been biochemically characterized (Banerjee and Sankaran, 2013). The next step in the lipoprotein biogenesis includes the cleavage of the signal peptide by a dedicated Type II lipoprotein signal peptidase (Lsp) at the conserved cleavage site in the lipobox. As a result, the lipid-modified cysteine remains as the first N-terminal amino acid in the mature lipoprotein. Finally, the lipoprotein N-acetyl transferase (Lnt) catalyzes the addition of third fatty acid in an amide linkage to the free amino group of the lipidated cysteine. According to Buddelmeijer (2015), the first two enzymes in the lipoprotein biogenesis, so Lgt and Lsp, are conserved in all bacteria species, but Lnt has only been identified in proteobacteria and actinomycetes. The general model for bacterial lipoprotein biogenesis is presented in the Fig. 4.

![Figure 4](image.jpg)

**Figure 4.** Lipoprotein biogenesis pathway in bacteria (modified from Buddelmeijer, 2015). Abbreviations: C, cytoplasm; CM, cytoplasmic membrane; LB, lipobox; Lgt, phosphatidylglycerol:prolipoprotein diacylglycerol transferase; Lnt, apolipoprotein N-acetyltransferase; Lsp, prolipoprotein signal peptidase; SP, signal peptide; PG, phosphatidylglycerol; PE, phosphatidylethanolamine.

As reviewed by Buddelmeijer (2015), triacylated lipoproteins and new sub groups for diacylated lipoproteins have recently been detected also in some firmicutes and terenicutes (that include mollicutes), suggesting the presence of Lnt-like enzymes and/or novel non-canonical lipidation pathways in these organisms. One of the novel diacylated lipoprotein structures that was discovered by Kurokawaka et al. (2012) was coined by the authors as “lyso” form. Authors found this type of lipoprotein in some low-GC Gram-positive
species, including also LAB species of *E. faecalis*, *Streptococcus sanguis* and *Lactobacillus bulgaricus*. As suggested by the authors, the lyso form of lipoprotein may be well distributed among wider group of low-GC content Gram-positive bacteria.

1.4.3.1.3 Sec-attached proteins

Sec-attached proteins are special group of proteins carrying a predicted cleavage sites for signal peptidase in their signal sequence, but which for some unknown reason avoid the cleavage and remain N-terminally anchored to the cell membrane (Tjalsma and van Dijl, 2005). When studying the S-layer associated proteins of *Lactobacillus acidophilus*, Johnson et al. (2013) predicted with Hidden Markov Model, which was developed by using the sequences of putatively sec-attached bacillus proteins, that some of the S-layer associated (extracted) proteins were capable for sec-attachment. However, this was not confirmed by further experimental work and researchers finally classified these candidates as extracellular proteins that are predicted to be sec-attached.

1.4.3.2 Binding of proteins to cell wall by covalent anchoring

In early 70s’ the first evidence for covalent anchoring of protein A to the peptidoglycan of *S. aureus* was presented (Sjöquist et al., 1972). In late 80’s, Pancholi and Fischetti (1988) studied the anchoring mechanism of the M protein of *Streptococcus pyogenes* and suggested that enzyme mediated cleavage of the C-terminal anchor of this protein takes place between proline- and glycine-rich region and hydrophobic region. A year later, this same group reported that an endogenous membrane anchor cleaving enzyme is able to to release M protein from isolated streptococcal protoplasts (Pancholi and Fischetti, 1989). In the following study Fischetti et al. (1990) reported that the LPXTG hexapeptide, preceding the hydrophobic C-terminal domain, is conserved in anchor region of several surface proteins originated from Gram-positive cocci (Fischetti et al., 1990). Navarre and Schneewind (1994) proposed couple of years later a novel hypothesis for the cell wall linkage of surface proteins in Gram-positive bacteria. In this model, after the translocation across the cytoplasmic membrane, the polypeptide chain with C-terminal cell wall sorting signal (including the LPXTG-motif) was suggested to be recognized (or sorted by) by sortase enzyme, following the subsequent cleavage of the precursor between threonine and glycine of the LPXTG motif and linkage of the resulting N-terminal fragment to the cell wall. The structural characteristics for the sortase A dependent proteins are shown in Fig. 5.

During the following years more evidence and details were gathered supporting the key role of the sortase in this process. Mazmanian et al. (1999) managed to screen and sequence the DNA fragment from *S. aureus* that seemed to be responsible for reducing the accumulation of uncleaved precursor form of the reporter polypetide in this organism. They also reported the presence of homologs (based on database search) of this gene in several other Gram-positive bacteria. As reviewed by Navarre and Schneewind (1999) and Maraffini et al. (2006), this sortase dependent cell wall sorting pathway (see Fig. 5) is universal in many Gram-positive bacteria.
Pallen et al. (2001) studied the presence of sortase-like proteins by database search. Genomic data available at that time indicated that sortase homologous were present in almost all Gram-positives studied and also the one of the very first observations of the presence of sortase like enzymes in Gram-negative bacteria and archae was reported. Authors also reported that there are usually more than one sortase-like protein encoded in each Gram-positive genome, and proposed that this could be an indication of specificity to different substrates. Because of the increasing number of reports on the identification and/or characterization of paralogous sortase genes during the very first years of this millenium, the need for classification and nomenclature system for sortases was evident.

Figure 5. Sortase A (SrtA) dependent surface display of proteins (modified from Schneewind and Missiakas, 2014). Structures described in picture: P1, full length protein precursor (with signal peptide); P2, protein precursor harboring only the C-terminal sorting signal; AI, an acyl enzyme intermediate; P3 protein precursor that resulted from the linkage of the C-terminal threonine of acyl intermediate with Lipid II (after nucleophilic attack by free amino group of Lipid II) and subsequent regeneration of sortase; M, mature covalently anchored surface protein. MN and GN denote N-acetylmuramic acid and N-acetylglucosamine, respectively.

Dramsi et al. (2005) gave their proposal for classification by dividing different sortases to four main classes (classes A, B, C and D). This classification proposal was based on detailed analysis of sixty-one sortases from complete Gram-positive genomes. Out of these classes, the sortases belonging into group A (SrtA) are required for anchoring the majority or all of the LPXTG-containing proteins of a given bacteria. Without few exceptions, genes expressing SrtA like housekeeping enzymes seem to be present as single copy in genomes of all low GC% Gram-positive bacteria (Dramsi et. al., 2008). Sortases in other classes (B, C and D) are accessory and appear to anchor more dedicated substrates (Schneewind and Missiakas, 2014). Several, but not all sortases (via their substrates) in class B are related to iron acquisition, whereas enzymes belonging to SrtC–class are polymerizing enzymes that are involved in the formation of pili or fimbria in several Gram-positive bacteria, and finally some members belonging to SrtD-class have shown to
posses role under specific developmental processes (mycelium formation in *Streptomyces coelicor*, sporulation in *Bacillus anthracis*) (Dramsi et al., 2008; Schneewind and Missiakas, 2014). Recent review of the relevance and application of sortases in LAB has been published by Call and Klaenhammer (2013).

### 1.4.3.3 Most typical non-covalently attached cell wall domains among LAB

For LAB proteins and LAB related phages endolysins, many different non-covalent binding mechanisms to the cell wall have been proposed. The most relevant and best known binding domains have been characterized and assigned with a Pfam (Finn et al., 2014) and InterPro (Mitchell et al., 2015) identification codes.

**Table 1.** The most relevant non-covalent cell wall binding domains in LAB (with Pfam identification codes).

<table>
<thead>
<tr>
<th>non-covalent CWBD</th>
<th>Short description of the domain</th>
<th>Direct citations from the InterPro databases are in italics (Mitchell et al., 2015)</th>
<th>Proposed binding (recognition) ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PG Binding_1</strong></td>
<td><em>This entry represents peptidoglycan binding domain (PGBD), as well as related domains that share the same structure. PGBD may have a general peptidoglycan binding function. It has a core structure consisting of a closed, three-helical bundle with a left-handed twist. It is found at the N or C terminus of a variety of enzymes involved in bacterial cell wall degradation.</em></td>
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<tr>
<td><em>PF01471</em></td>
<td><em>Li et al. (2011) discovered that proline-rich PG Binding_1 domains (PF01471) have a highly conserved pocket(s) that is not present in the eukaryotic domains belonging to this same group. Authors suggested that conserved amino acid residues in this pocket are required for the function and/or the folding of the pocket for peptidoglycan binding.</em></td>
<td><em>Peptidoglycan (Li et al., 2011)</em></td>
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<tr>
<td><strong>LysM</strong></td>
<td><em>The LysM (lys mott) domain is about 40 residues long and it is found in a variety of enzymes involved in bacterial cell wall degradation. It contains a beta-alpha-alpha-beta conformation, with the beta strands forming an antiparallel beta sheet and the two alpha helices packing on one side of this sheet. It may have a general peptidoglycan binding function.</em></td>
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<tr>
<td><em>PF01476</em></td>
<td><em>Note, this domain is described in more detail in the following section.</em></td>
<td><em>N-acetylglucosamine (Buist et al., 2008, Messmang et al., 2014)</em></td>
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<tr>
<td><strong>CW Binding_1</strong></td>
<td><em>According to Desvaux et al. (2006), choline-binding domains are also called as cell wall binding domain of Type 1 (here referred as CW Binding_1). These repeats of about 20 amino acids are characterised by conserved aromatic residues and glycines, and exists in proteins in multiple tandem copies. Several choline binding proteins are related to virulence, although choline binding proteins have been found in commensal bacteria, also (Hakenbeck et al., 2009).</em></td>
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<tr>
<td><em>(choline-binding domain) PF01473</em></td>
<td><em>These repeats are essentially found in two bacterial Gram-positive protein families: the choline binding proteins and glycosyltransferases (EC 2.4.1.3). In choline-binding proteins cell wall binding repeats bind to choline moieties of both teichoic and lipoteichoic acids; two components peculiar to the cell surface of Gram-positive bacteria.</em></td>
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<td></td>
<td><em>In glycosyltransferases, CW Binding_1 is often spanned by COG2523 (YG rich glucan binding domain). Typically, these enzymes do not show choline dependent binding (Shah et al., 2004).</em></td>
<td><em>Choline moieties of both teichoic and lipoteichoic acids (Fernández-Torrero et al., 2001)</em></td>
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<tr>
<td><strong>WxL</strong></td>
<td><em>The WxL motif appears in two or three copies in these bacterial proteins and confers a cell surface localisation function. It seems likely that this region is the cell wall-binding domain of Gram-positive bacteria, and may interact with the peptidoglycan.</em></td>
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<tr>
<td><em>PF13731</em></td>
<td><em>This domain was originally coined by Klerebezem et al. (2003) during the genomic sequencing project of <em>Lactobacillus plantarum</em> WCFS1. The name originates from the conserved repeating motif that is found twice in this domain. Later, Sozen et al. (2006) demonstrated the cluster-like presence of WxL genes in <em>Lactobacillus plantarum</em> WCFS1 but also in some other LAB.</em></td>
<td><em>Peptidoglycan (Brünster et al., 2007)</em></td>
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<tr>
<td>non-covalent CWBD</td>
<td>Short description of the domain. Direct citations from the InterPro databases are in italics (Mitchell et al., 2015)</td>
<td>Proposed binding (recognition) ligands</td>
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<tr>
<td><strong>G5</strong> PF07501</td>
<td>The G5 domain (named after its conserved glycose residues) is a module of ~80 residues that is found in a variety of enzymes such as Streptococcal IgA peptidases and various glycosyl hydrolases in bacteria. The G5 domain contains a few highly conserved residues. None of these conserved residues are the polar type of amino acids found in active sites, so it seems unlikely this region has an enzymatic function. However, in nearly all cases the G5 domain is associated with a known enzymatic domain. As a common feature of the proteins containing G5 domains is N-acetylglicosamine binding. It has been suggested that this function might be attributed to the G5 domain. Other alternative functions could be allosteric regulation of the enzymatic domain or cofactor binding. It has also been suggested that G5 domains may promote biofilm formation (Bateman et al., 2005).</td>
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<tr>
<td><strong>SH3b</strong> - bacterial SH3 domains (SH3_3 PF08239)</td>
<td>SH3b domains are prokaryotic counterparts to well known eukaryotic SH3 domains (Ponting et al., 1999, Whisstock and Lesk 1999). The SH3 domain has a characteristic fold which consists of five or six beta-strands arranged as two tightly packed anti-parallel beta sheets. The linker regions may contain short helices. The surface of the SH3-domain bears a flat, hydrophobic ligand-binding pocket which consists of three shallow grooves defined by conservative aromatic residues in which the ligand adopts an extended left-handed helical arrangement. The ligand binds with low affinity but this may be enhanced by multiple interactions. The biggest difference in structure between eukaryotic SH3 domains and bacterial SH3b domains is in the RT-loop region (RT loop is named after the arginine and threonine pair) of the domain. In SH3b domains long insertions are detected in this region (Kamitori and Yoshida, 2015). A homologue of the SH3 domain has been found in a number of different bacterial proteins including glycyl-glycine endopeptidase, bacterocin and some hypothetical proteins. In the literature, the definition of bacterial type of SH3 varies, and one of the the most widespread definition includes 5 subgroups: SH3_3, SH3_4, SH3_5, SH3_6 and SH3_8 (Kamitori and Yoshida, 2015). In the InterPro database, SH3b consists only SH3_3 and SH3_5 subgroups.</td>
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<tr>
<td><strong>SH3_8</strong> PF13457</td>
<td>See SH3b. In many cases this domain is classified under SH3b. However, in the InterPro database a specific code has been assigned for this bacterial type of SH3 domain. As reported by Marino et al. (2002), this domain has high similarity with the well known GW domain. Initially, GW domain was discovered by Braun et al. (1997) from the C-terminal anchoring region of ImB of Listeria monocytogenes (ImB is a surface protein of the internalin multigene family). This domain was coined because of characteristic GW dipeptide found in the beginning of this repeat. Surprisingly, Marino et al. (2002) noticed that GW domains resemble SH3 domains and bacterial SH3b domains in their structure. Despite this similarity, authors speculated that the GW domains do not mimic typical functionality of SH3 domains, since their potential proline-binding sites are blocked or destroyed (Marino et al., 2002). Currently, The InterPro database recognizes three SH3_8 domains in the C-terminal anchoring region of ImB - so within the same region in which Braun et al. (1997) found the GW repeats, earlier.</td>
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**Table 1 continued.**
In literature, some good reviews exist that covers these domains among the main species of industrial related LAB (Zadravec et al., 2015; Chapot-Chartier and Kulakauskas, 2014; Sengupta et al., 2013; Tarahomjoo, 2013; Kleerebezem et al., 2010). Furthermore, in some studies cell wall binding domains of specific strain is compared to broader range of LAB strains - see studies published by Kankainen et al. (2009) and van Pijkeren et al. (2006) as examples. Out of these proposed non-covalent binding domains in literature, the most important for LAB are presented in the Table 1.

### 1.4.3.4 LysM domain

Among LAB, LysM is the most studied non-covalent cell wall binding domain. In the literature, several excellent reviews covering the LysM domain and its potential
applications have been published. One of the most recent one of these is the review published by Visweswaran et al. (2014).

Also, the binding mechanism of LysM domain is one of the best known among all the non-covalent binding domains. Steen et al. (2003) demonstrated that cA domain of AcmA of *L. lactis* is responsible for binding this autolysin enzyme to peptidoglycan. This C-terminal cA domain contains three separate LysM domains which are separated by non-homologous sequences. Steen et al. (2003) noticed that other cell wall constituents, most probably lipoteichoic acid in *Lactococcus*, hindered the interaction between peptidoglycan and cA domain. Binding test with S-layer bearing *Lactobacillus helveticus* ATCC 15009 demonstrated that also S-layer seemed to hinder efficient binding. It was also found that these C-terminal LysM domains bind to specific sites on the bacterial surface, i.e. near the poles and septum of *L. lactis* cells. In *L. lactis* SK110 these locations were demonstrated to be typical binding sites for galactosyl-decorated LTAs that are typical for this phase resistant strain (Sijtsma et al., 1990). In conclusion, Steen et al. (2003) suggested that the repetitive disaccharide component in peptidoglycan, composed out of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) molecules, is most probably the ligand for the binding as it is the only common part in A- and B-type peptidoglycans (which were both demonstrated to bind cA).

As reviewed by Buist et al. (2008), in some eukaryotes LysM modules are present in chitinases, suggesting that LysM is also able to bind chitin (polymer of GlcNAc capable to form hydrogen bonding between the adjacent polymer chains). According to Buist et al. (2008) it remains unanswered whether GlcNAc is the sole moiety recognized by LysM. Wong et al. (2014) proposed after experiments with LysM domains from CwIS (NlpC/P60 D,L-endopeptidase) of *B. subtilis* that bacterial LysM domains do not discriminate between peptidoglycan and chitin GlcNAc polymers but recognize both of these substrates with similar affinity, which is not usual within hydrolase activity related LysM domains in plants. In this study, they indicated by microscale thermophoresis assay that at least two LysM domains are necessary for efficient binding to long chitin polymers whereas three LysM domains are required for proper binding to peptidoglycan fragments. For comparison, study conducted by Steen et al. (2005) with AcmA of *L. lactis* MG1363 indicated that at least two domains are needed for peptidoglycan binding, but three domains provides optimal peptidoglycan binding and biological functioning. More recently, Mesnage et al. (2014) proposed molecular basis for bacterial peptidoglycan recognition by LysM domains. This study was based on multimodular LysM domain from AtlA from *E. faecalis*. The data from this study suggested that LysM module recognizes GlcNAc-X-GlcNAc moieties, in which X is GlcNAc or MurNAc, and peptide stems act as negative discriminants that modulate the binding but do not prevent it. In this same study, the earlier observations (Steen et al., 2005, Wong et al., 2014) suggesting that individual LysM modules bind in cooperative manner to long glycan chains present in cell wall was confirmed. Most recently, Wong et al. (2015) proposed model for intermolecular dimerization of P60_tth (NlpC/P60 endopeptidase containing two N-terminal LysM modules) of *Thermus thermophiles* with peptidoglycan substrate.
The Lc-LysBD-domain was recently characterized in the C-terminal part of prophage endolysins (Lc-Lys and Lc-Lys2) of \textit{L. casei} BL23 (Regulski et al., 2013). Authors demonstrated that this domain binds to PG and is highly specific for amidated D-Asp cross-bridge present in the peptidoglycan of \textit{L. casei}. So far, this binding domain has also been reported to be found in \textit{L. lactis} phage 949 endolysin and in \textit{L. casei} phage endolysins A2 and PL-1 (Regulski et al., 2013; Chapot-Chartier 2014).

In some cases, no anchor or binding domain has been detected or does not exist for a specific protein although it seems obvious that the protein is attached to cell envelope. Also, in some cases slow diffusion through peptidoglycan layer or inner wall zone temporary reservoirs of proteins may lead to wrong conclusion when detecting cell attached proteins. These last examples hint that diffusion through the peptidoglycan is not necessarily an unregulated event. As reviewed by Forster and Marquis (2012), also protein size and form with many other factors, like the average length of glycan chains, the level of crosslinking between glycan chains, the presence or absence of bridges between the crosslinking peptides, electrostatic interactions, and the mechanical tension imposed by cell turgor pressure may influence the cell wall permeability.

The charge of the Gram-positive cell wall is most typically negative, due to presence of polyanionic teichoic acids (TAs) in the cell envelope (Neuhaus and Baddiley, 2003). Under physiological conditions, secreted proteins with high overall pI values remain positively charged, which may make their journey through the peptidoglycan layer slow (Forster and Marquis, 2012) or even bind transiently these proteins to the peptidoglycan layer. Among LAB, an example of this kind of binding is that of the cystine-binding protein (CuyC) of \textit{Lactobacillus reuteri} BR11 (formerly referred as BspA of \textit{L. fermentum} BR11) (Turner et al., 1997). More recently, Anzenburger et al. (2014a) characterized homologous N-acetylmuramidases from two different \textit{L. buchneri} strains and demonstrated their binding to \textit{L. buchneri} cell walls \textit{in vitro}. In both of these above mentioned examples, as no obvious cell wall binding domains were detected and high pI value was predicted for both of these proteins, authors proposed that the cell wall binding was due to electrostatic interactions between positively charged proteins and negatively charged cell wall constituents. Turner et al. (1997) also demonstrated that the electrostatic attachment was abolished and BspA released, when whole cells of \textit{L. fermentum} were treated with LiCl-solution. Earlier, LiCl- treatment was demonstrated to release S-layer of \textit{L. helveticus}, another cationic high pI protein from the cell surface (Lortal et al., 1992).

Some surface displayed enzymes are called as moonlighting proteins. The peculiar name of moonlighting proteins is due to their dual life-style –the performance of more than one function by a single protein (Copley, 2012). As summarized by Jeffery (2003), these enzymes are single proteins which have multiple functions, but this dualism is not due to gene fusions, splice variants or multiple proteolytic fragments. As reviewed by Kainulainen and Korhonen (2014), moonlighting proteins often perform their canonical and moonlighting functions in separate cell compartments, and in some cases, like in many proteomic studies, this localization limited more narrow criterium for identification for bacterial moonlighting proteins has remained in use. In these cases, moonlighting proteins have more than one function but these functions need also to be displayed in
different cell compartments. Most typically moonlighting proteins are canonically cytoplasmic proteins, but are found also on the surface of the cell or in the surrounding medium, although they do not possess any recognizable sequence motifs for secretion or anchoring to the bacterial or to the eukaryotic cell surface (Copley, 2012; Kainulainen and Korhonen 2014). First bacterial moonlighting proteins were found from group A streptococci by Pancholi and Fischetti (1992). These researchers demonstrated that, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), one of the key enzymes in cytosolic glucose metabolism, is found on the surface of group A streptococci and is capable to bind to fibronectin. As stated by the authors, this property suggests a putative role for GAPDH in the colonization of the pharynx. This type of dual role for the glycolytic enzymes is not rare, as practically all enzymes in the bacterial Embden-Mayerhof glycolytic pathway have been assigned with putative adhesive moonlighting function (Kainulainen and Korhonen, 2014). Indeed, adhesion to host epithelial cells, mucus and extracellular matrix (ECM) components, and interaction with circulating host components and modulation of host immune responses, are the most typical moonlighting functions found among bacteria (Kainulainen and Korhonen, 2014; Mangiapane et al., 2015) – in good (supporting probiotic action) or in bad (supporting virulence).

As reviewed by Kainulainen and Korhonen (2014), it seems that ionic interactions with lipoteichoic acids and cell division sites seems to be critical for the surface localization for many moonlighting proteins. Also, authors speculated that moonlighting proteins in commensal lactobacilli and in pathogenic species are functionally different because it seems that the nature of anchoring to the bacterial surface is often different: several pathogenic species are able to retain moonlighting proteins on cell surface at neutral pH, whereas among lactobacilli these proteins are released in many cases. As an supporting example for this generalization, Terrasse et al. (2015) demonstrated that peptidoglycan serves as binding ligand for the GAPDH of S. pneumoniae and this binding is not dependent on the presence of the teichoic acids component, which is the case with some lactobacilli – GAPDH of L. crispatus as an example (Antikainen et al., 2007, Kainulainen et al., 2012). Regardless of the nature of the binding, observations suggesting that some moonlighting proteins may be released from a single bacterial species and then reassociate onto the surface of another species, or bind directly after secretion/cell lysis with an universal ligand (like peptidoglycan) of another species, represents a novel mechanism in bacteria-bacteria interactions and may have importance in bacteria-host interactions (Oliveira et al., 2012; Kainulainen and Korhonen, 2014; Terrasse et al., 2015).

Interestingly, Waśko et al. (2014) suggested that S-layer proteins of L. helveticus T159 binds some moonlighting proteins, which makes the surface of this strain more hydrophobic and improves the adhesion and aggregation capacity of this strain. Authors also observed the S-layer associated presence of some technologically important non-moonlighting proteins, such as L-lactate dehydrogenase, glucose-1-phosphate adenlyltransferase, and glycerol-3-phosphate ABC transporter. Earlier, Johnson et al. (2013) studied S-layer associated proteins (which they coined as SLAPs) of L. acidophilus NCFM. In this study, 37 proteins were solubilized from the S-layer wash fraction and closer study suggested that four of these proteins were putative moonlighting proteins and 17 were putative SLAPs. One of the SLAP was studied closer (LBA1029), and it it was demonstrated that it contributes to a pro-inflammatory TNF-α response from murine DCs. Based on their observations authors suggested that SLAPs appear to impart important surface display features and immunological properties to S-layer coated microbes.
Notably, SLAP term used here does not refer to pfam SLAP domain (*Lactobacillus* S-layer protein –domain), although some SLAPs reported by Johnson et al. (2013) may also carry SLAP pfam domains.

1.5 Fermentation pathways in LAB

1.5.1 Fermentation and respiration in LAB

Lactic acid bacteria are chemotrophic organisms that gain the energy for their metabolism by oxidation of chemical compounds (Ribéreau-Gayon et al., 2006). According to the final acceptor of electrons, metabolism of a cell is classified either as fermentative or respirative. During fermentation, the oxidation and phosphorylation (energy generation by formation of ATP) are not coupled, as is the case with respiration. For fermentative organisms, like LAB, the final reduced molecule is characteristically an endogenous compound. For LAB, this molecule is typically pyruvate. However, depending on the presence of alternative electron acceptors, growth conditions and metabolic balance, alternative pyruvate-utilizing pathways are possible for some LAB strains (von Wright and Axelsson, 2011).

In rare cases, functional oxidative respiration has been observed in some LAB species under special circumstances (heme, or heme and menanqueine present in the growth medium). The best documented and understood respiration process is that of *L. lactis*. Respiration in this species has demonstrated to have positive impact on bacterial biomass, resistance to oxygen, and long-term survival (Pedersen et al., 2012).

1.5.2 Typical LAB fermentations

In general, the generation of metabolic energy among LAB primarily occurs through the substrate level phosphorylation (Konings, 2002). In this process, the oxidation of substrate generates energy rich intermediates, which subsequently can be used for ATP generation. This process results in the formation of NADH from NAD\(^+\), which has to be regenerated in order for cells to continue the fermentation. In the case of LAB, pyruvate is the most typical final electron (or hydrogen) acceptor, resulting in formation of the reduced end product, lactate (Axelsson, 2004).

\[
\text{Pyruvate} + \text{NADH} + \text{H}^+ \rightarrow \text{lactate} + \text{NAD}^+
\]

The above sum reaction can be divided as follows:

Oxidation (NADH loses e\(^-\)): \( \text{NADH} + \text{H}^+ \rightarrow \text{NAD}^+ + 2\text{H}^+ + 2e^- \)

Reduction (pyruvate gains e\(^-\)): \( \text{Pyruvate} + 2\text{H}^+ + 2e^- \rightarrow \text{lactate} \)

In general, LAB as a group exhibits an enormous capacity to utilize different carbohydrates and related compounds. Furthermore, as LAB are able to adapt their
metabolism in response to various conditions, this may result to different end product patterns even with the case of single substrate (Axelsson, 2004).

In the literature the most common metabolic pathway described for LAB is the fermentation of glucose (a hexose sugar, see Fig. 6). However, also other hexose sugars such as mannose, galactose, and fructose are readily fermented by many LAB, too.

**Figure 6.** Most relevant metabolic pathways of selected sugars among LAB (Abdel-Rahman et al., 2013; Li and Cui, 2010; Wang et al., 2015). Most important enzymes for homolactic and heterolactic fermentation are identified with number: 1, fructose-1,6 diphosphate aldolase; 2, lactate dehydrogenase; 3, transketolase; 4, transaldolase; 5, glucose-6-phosphate dehydrogenase; 6, 6-phosphogluconate dehydrogenase; 7, phosphoketolase; 8, acetate kinase; 9, alcohol dehydrogenase. The numbers for the key enzymes for homolactic and heterolactic fermentation are presented with yellow background.
Depending of the specific uptake mechanism of a given sugar (permease-/PTS-system), the sugar molecule may be intact or phosphorylated after entering into cell. If needed, the sugar is further processed, most typically phosphorylated or isomerized, before joining the glycolytic pathway usually at the level of the glucose-6-phosphate or fructose-6-phosphate. However, deviations to the above described route exist and one of those is the galactose metabolism in some LAB. As an example, the galactose uptake in *L. lactis*, *E. faecalis*, and *L. casei* happens through the PTS system, resulting in simultaneous phosphorylation of the sugar. The resulting galactose-6-phosphate in these species is processed through tagatose-6-phosphate pathway which coincides with the glycolytic pathway at the level of glyceraldehyde-3-phosphate (GAP, see Fig. 6).

Under the normal conditions (excess of sugar, limited access to oxygen), LAB rely on two principal pathways for catabolism of hexose sugars: homolactic pathway and heterolactic pathway (Fig. 6). Theoretically, in homolactic pathway sugar is completely converted to lactic acid. For hexose sugars, this means production of two mol of lactate per one mol of consumed hexose with the concomitant production of 2 mol of ATP. More practically, fermentation is often regarded as homolactic when more than 90% of the hexoses are converted to lactic acid and 2 mol of ATP is generated per mole of hexose (Hutkins, 2001). As lactic acid is virtually the only end product, this type of sugar metabolism is referred to as homolactic fermentation (Axelsson, 2004). This pathway is typical among genera of *Carnobacterium*, *Aerococcus*, *Enterococcus*, *Lactococcus*, *Vagococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, and in some lactobacilli (Axelsson, 2004). Characteristics for this pathway (see Fig. 6) is the formation of fructose-1,6-diphosphate (FDP) and subsequent branching of the pathway (by FDP aldolase) to glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP). In the course of conversion of glyceraldehyde-3-phosphate to lactate, four moles of ATP per mole of hexose are generated, resulting in net generation of 2 moles of ATP per mole of hexose over the whole pathway (two moles of ATP were consumed before generation of FDP). The very last enzymatic reaction, so the reduction of pyruvate to lactic acid by a NAD+ dependent lactate dehydrogenase (nLDH) concomitantly converts NADH to NAD+, providing the electron sink required to maintain the vital redox balance in cell’s metabolism (Hutkins, 2001). For pentoses, the characteristic feature in homolactic pathway is the conversion of xylulose-5-P to fructose-6-P and glyceraldehyde-3-P through series of reactions catalyzed by two key enzymes: transaldolase and transketolase. This branch of the homolactic fermentation presented in Fig. 6 is known as pentose phosphate pathway (Endo and Dicks, 2014). By this route, pentoses are converted completely to lactic acid with the theoretical yield of 1.67 mol of both lactic acid and ATP from one mole of pentose sugar (Tanaka, 2002). Homolactic pentose fermentation is not common among LAB, but has been demonstrated with few strains – *L. lactis* IO-1 (Tanaka et al., 2002) and *Lactobacillus vini* Mont 4T (Rodas et al., 2006) as examples.

In heterolactic pathway (or 6-phosphogluconate/phosphoketolase-pathway, 6-PG/PK), approximate equimolar amounts of lactate, acetate, ethanol and CO2 are generated with 1 mol of ATP per mole of hexose (Hutkins, 2001). As this metabolic pathway results in multiple end products (usually in equimolar amounts) it is referred as heterolactic fermentation. This fermentation pathway is typical among genera of *Leuconostoc*, *Oenococcus*, *Weissella* and some lactobacilli. Characteristics for this pathway are the initial dehydrogenation steps with the formation of 6-phosphogluconate, followed by decarboxylation reaction (release of CO2). After the decarboxylation, the remaining
pentose-5-phosphate is split by phosphoketolase enzyme, resulting in formation of glyceraldehyde-3-phosphate and acetyl phosphate. The former of these intermediates is then processed to lactate by the same pathway that is employed during the homolactic fermentation, whereas the latter is reduced to ethanol via acetyl CoA and acetaldehyde with concomitant regeneration of NADH to NAD\(^+\) (Axelsson, 2004). When compared to the homolactic pathway, less ATP (one mole per one mol of hexose) is generated from the hexose substrate by this pathway. However, in some cases when an external electron acceptor is present, LAB using the heterolactic fermentation may gain an additional ATP by reducing an external electron acceptor instead of reducing acetyl-phosphate to ethanol. As a result, acetate kinase is able to dephosphorylate acetylphosphate, resulting in formation of acetate and concomitant phosphorylation of ADP to ATP (Axelsson, 2004). In the case of pentoses, heterolactic fermentation yields equimolar amounts of lactate and acetate. Ethanol is not produced as the final product, because no NADH is formed during the preceding 5-carbon metabolism. As an extra benefit, one additional mol of ATP is generated when compared to heterolactic hexose fermentation.

Additionally, members belonging to genus lactobacillus have been traditionally divided into three sub-groups based on their glucose fermentation characteristics: (i) the obligately homofermentative lactobacilli, (ii) the facultatively heterfermentative lactobacilli, and (iii) the obligately heterofermentative lactobacilli (Hammes and Vogel, 1995). This physiological classification relies on the presence or absence of fructose-1,6-diphosphate aldolase and phosphoketolase, that are the key enzymes for the homo- and heterofermentative sugar metabolism, respectively (Axelsson, 2004).

1.5.3 *Other fates of pyruvate*

Under certain conditions some homolactic LAB strains may shift to mixed-acid metabolism during the fermentation on sugars. Transition to this metabolism is strain specific, but typically factors that initiate it may include: carbon limitation, excess of slowly metabolized sugars (such as galactose), presence of oxygen or respiratory lifestyle (Mayo et al., 2010; Neves et al., 2005). Typical characteristic of this mixed-acid metabolism is production of formate, acetate, ethanol and/or CO\(_2\) in addition of lactate. The formation of these products is orginated from pyruvate by the key enzymes presented in the Fig. 7. Out of these enzymes, pyruvate formate lyase is operational only under anaerobic conditions, whereas pyruvate oxidase is oxygen dependent. Pyruvate dehydrogenase has catabolic role under aerobic conditions, so backing up the pyruvate formate lyase in the presence of oxygen. Under anaerobic conditions, pyruvate dehydrogenase mainly serves anabolism by providing acetyl CoA for lipid biosynthesis (Axelsson, 2004).
Figure 7. Alternative reaction pathways for pyruvate (Lorquet et al., 2004, von Wright and Axelsson, 2011). Most important enzymes in these pathways are identified with number: 1, pyruvate formate lyase; 2, pyruvate oxidase; 3, pyruvate dehydrogenase; 4, phosphotransacetylase.

In addition to the mixed acid fermentation, pyruvate may also be converted to alanine by alanine dehydrogenase or serve as starting substrate for diacetyl production (Endo and Dicks, 2014). Diacetyl is known as the butter aroma compound in dairy products and it is typically produced in very low amounts during lactic fermentations, as most of the flux from pyruvate through this alternative metabolic pathway is directed to form acetoin. This type of metabolism requires a surplus of pyruvate relative to the need of NAD+ regeneration, and is typically found in leuconostocs and some lactococci that can gain that surplus by citrate metabolism in milk (von Wright and Axelsson, 2011). The formed acetoin is chemically quite similar to diacetyl, but is does not give the desired butter aroma. Acetoin is formed by decarboxylation of α-acetolactate, which in turn is formed from pyruvate by the acetolactate synthase activity. Some dairy strains lack functional α-acetolactate decarboxylase, which results in accumulation of α-acetolactate and enables its subsequent chemical conversion to diacetyl in the presence of oxygen (Hugenholtz and Kleerebezem, 1999).

1.5.4 Classification of LAB according to isomer of lactic acid produced

As an end product of catabolic reactions from sugars, lactic acid with either a slight positive or negative specific optical activity is formed. More commonly, these chiral molecules are known as L(+) and D(-)-lactic acid, respectively. The optical isomer(s) of lactic acid produced depends on the presence of D-nLDH, L-nLDH and lactate racemase enzymes in the cell. As reviewed by Goffin et al. (2005), L-lactate induced lactate racemase, enzyme catalyzing the racemization of L(+) lactic acid to D(-)-lactic acid, has been detected only in few species, including Lactobacillus sakei, Lactobacillus curvatus, and Lactobacillus paracasei ssp. paracasei (formerly L. casei ssp. pseudoplantarum) and L. plantarum. Most typically, if both D(-)-lactic and L(+) lactic acids are detected in the
cell, these end products are formed by the activity of single D-nLDH and L-nLDH enzyme, respectively, without the contribution of lactate racemase. As a result of the enzymatic diversity among LAB, different enantiomers of lactic acid can be produced either exclusively (L(+)-lactic acid or D(-)-lactic acid, alone), approximately equal amounts of both, or predominately one form but measurable amounts of the other (Axelsson, 2004). The characteristics pathway used for glucose fermentation and optical isomer of lactic acid produced by different LAB genus are presented in Table 2.

**Table 2.** Fermentation pathway for glucose utilization and optical isomer of lactic acid produced of main LAB genus (adapted from Axelsson, 2004).

| Mode of fermentation | Arabinobacterium | Carbohydractrum | Enterococcus | Lactobacillus | Lactococcus | Lactococcus lactis | Lactosoccus | Oenococcus | Peptococcus | Pediococcus | Peptostreptococcus | Streptococcus | Torulaspora | Weissella |
|----------------------|-----------------|-----------------|--------------|--------------|-------------|------------------|-------------|------------|-------------|-------------|-----------------|----------------|-------------|
| Lactic acid produced | L (+)           | L (+)           | L (+)        | L (+)        | D (+)       | D (+) and L (+)² | L (+)       | L (+)      | L (+)       | D (+)       | D (+) and L (+)² | L (+)         | L (+)       | L (+)       |

1. Small amounts of CO₂ can be produced, depending on media
2. Varies between species

### 1.6 Lactic acid – usage, applications and production

Lactic acid is a versatile molecule and is used in various applications in several industries as demonstrated by the following examples (Vijayakumar et al., 2008, Wee et al., 2006):

- **food industry:** acidulant, flavouring agent, preservative
- **chemical industry:** base chemical for variety of chemical conversions, like conversion to acrylic acid via dehydration
- **pharmaceutical industry:** electrolyte in many parenteral/I.V. (intravenous) solutions, ingredient in various mineral preparations and formulations (especially lactate salts)
- **cosmetic industry:** moisturizing agent, pH regulator
- **polymer industry:** raw material for the production of different grades of poly(lactic acid) (PLA)
- **other functions in various industries:** dyeing of various textiles, mordant in the printing of woolens, bating and plumping of leathers, deliming of hides, tanning of vegetables, flux for soft soldering, green solvent in several applications

Relevant chemical characteristics and most typical applications of the most commonly known commercial lactic acid grades are presented in Table 3.
According to recent market study (Anonymous, 2014), markets of lactic acid have been steadily and rapidly growing since 2008 and the 800 000 ton/year milestone was reached in 2013. According to this study, three major manufacturer dominated the markets (PURAC, Cargill and Henan Jindan Lactic Acid Technology Co., Ltd), manufacturing together about 505 000 ton of lactic acid. Production of PLAs was evaluated to be one of the most potential applications of lactic acid, and during 2013 the global PLA production was estimated to be about 320 ton/year, from which a single company (Natureworks) represents almost half (45.2 %) of the total market share.

Although the chemical manufacturing of lactic acid started to expand already in early 1960s mostly due to demand for heat stable stearoyl lactylates in the baking industry (Benninga, 1990), almost all lactic acid currently available on the market is produced by fermentation (Groot et. al, 2010). One of the main reason for this is the fact, that chemical synthesis yields always an racemic mixture of D(-)- and L(+) -lactic acid. In some cases, manufacturing of thermostabile PLA grade as an example, this kind of end product mixture is not the preferred choice for the further processing of the lactic acid (Lunt, 1998; Södergård and Stolt, 2002).

Indeed, the promising market of PLAs has been the main driving force during the last decades to develop microbial fermentation processes that are able to deliver pure enantiomeric forms of lactic acid - either L(+) or D(-). The beauty of PLAs is that they are bio-based, bioreorable, and biodegradable under industrial composting conditions. In addition, thermoplastic properties with rigidity and clarity, make some of the PLA grades good alternative for many oil-based polymers. Indeed, because of these polymer properties together with attractive price and commercial availability, PLA became the first mass-produced bio-based polyester in the market and is the front runner in replacing the oil-based polymers with bio-based alternatives (Groot et al., 2010).

In many applications, it does not make any difference whether the optical structure of the used lactic acid is D(-), L(+) or mixture of both enantiomers, but especially food and pharmaceutical industries prefer to use pure L(+)-lactic acid, since elevated levels of the D(-)-isomer are harmful to humans (Anonymous, 1967). However, the D(-)-lactic acid
plays also an important role in novel polylactic acid formulations, as the polymer blend of pure L(+)- and D(-)-polylactic acids (stereocomplex, scPLA) seems to possess superior temperature tolerance when compared to polymers made from pure L(+)- or D(-)-poly(lactic acids) (Tsuji and Fukui, 2003). As the melting temperature of both pure homopolymers falls between 170-180 °C, the melting temperature of the 50:50 (%) blend is about 230 °C (Ikada et al., 1987). If the single polymer chain is not pure homopolymer, pure poly(L(+)-lactic acid) polymer (PLLA) or pure poly(D(-)-lactic acid) polymer (PDLA), but is made out of both enantiomers, the melting temperature will drop and crystallization behavior will change dramatically. According to Groot et al. (2010), already D(-)-content higher than 12-15 % in mainly L(+)-based PLA, is enough to make the resulting PLA amorphous. According to Lunt (1998), in many aspects, the basic properties of typical PLA polymers lie between those of crystal polystyrene (PS) and polyethylene terephthalate (PET).

Before the start of mass production of PLAs, the most relevant use of these polymers was typically focused to high value applications, like biomaterials in medicine (Lasprilla et al., 2012), but currently various PLA formulations are used in much broader range - even in single use packing applications (Hazarika et al., 2015). This indicates that the economics related to PLA production has improved substantially.

1.6.1 Synthesis of PLA

Pure solution of lactic acid at equilibrium conditions is indeed a mixture of monomeric lactic acid, dimeric lactic acid, higher oligomers of lactic acid and lactide (cyclic dimer). The explanation for this kind of mixture lies in the nature of lactic acid molecule – it has a hydroxyl and an acid functional group. Due to this specific chemistry, intermolecular and intramolecular esterification reactions may happen. First, a linear dimer (lactoyl lactic acid) is formed and this condensation reaction can further proceed to higher oligomers. This reaction is promoted by removal of water. Also, lactide, a cyclic dimer, is formed in small amounts by intramolecular breakdown of higher oligomers or esterification of lactoyl lactic acid.

Although this kind of polycondensation process is possible without modern process control and catalysts, as accidentally demonstrated by Gay-Lussac and Pelouze in the first half of the 19th century (Benninga, 1990), the benefit gained by the usage of optimized catalyst and polymerization conditions is crucial to extend the reaction and gain polyesters with higher molecular weights (Hiltunen et al., 1997). Most typically, manufacturing of PLA from the purified lactic acid solution takes place via one of the three alternative routes described in the Fig. 8.
Currently, the above described conventional polycondensation process is not the primary mechanism for industrial high molecular weight PLA production, as in the early 1990s Cargill Inc. developed a process based on ring opening reaction (ROP) of lactide for this purpose. Indeed, ROP of cyclic esters was already invented before 2nd world war by Carothers and later patented by Lowe (1954) for DuPont, but Cargill was the first company that managed to adopt it into profitable large scale PLA process in mid 1990s (Masutani and Kimura, 2014).

1.6.2 Conventional LAB based industrial scale fermentation processes for lactic acid

Typical industrial scale microbial lactic acid process can be divided into three main processing phases: fermentation, recovery and purification. These phases can further be divided into other process steps or unit operations, as shown in example presented in Fig. 9. Depending on production site, this core process is integrated to longer value chain with various ways. In literature, a good example of this is available in the report describing the whole production chain of PLAs from cradle (starting from the cultivation of raw materials for the fermentation) to gate in one of the PURAC’s manufacturing site (Groot and Borén, 2010).
In lactic acid fermentation, among the most relevant decisions to be made are: choice of the substrate, choice of the production organism, mode of the fermentation and management of the cell density, and choice of the fermentation conditions (pH and temperature). Indeed, from fermentor design point of view, conventional LAB based lactic acid production processes are simple, as fermentations with these organisms do not usually require aeration, gas exchange or gas mass transfer (Taskila and Ojamo, 2013) and have low power and cooling needs (Datta et al., 1995). As reviewed by Taskila and Ojamo (2013), typical lactic acid fermentation choices other than simple batch are repeated batch, fed-batch and use of continuous fermentation either with free cell-recycling or with immobilized cells. Success of fermentations is typically measured with parameters like: titer (g lactic acid /dm³), productivity (g lactic acid/(dm³ x h)) and yield (g lactic acid/g carbon substrate). Enantiometric purity of the lactic acid produced is also verified, if necessary. Datta et al. (1995) proposed that, over 90% yield from the carbohydrate substrates, final product concentration of at least 1M (90 g/l) and high stable productivity (> 2 g/l h) are criteria for a successful fermentation process.

As reviewed by Idler et al. (2015), the most important lactic acid LAB producers and the optical isomer(s) of lactic acid produced by these species are listed in the Table 4.

Table 4. Typical LAB species used for lactic acid production (adapted from Idler et al., 2015).

<table>
<thead>
<tr>
<th>L(+)-lactic acid producers:</th>
<th>D(-)-lactic acid producers:</th>
<th>D(-)- and L(+) -lactic acid producers:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus amylophilus</td>
<td>Lactobacillus delbrueckii</td>
<td>Lactobacillus acidophilus</td>
</tr>
<tr>
<td>Lactobacillus casei</td>
<td></td>
<td>Lactobacillus helveticus</td>
</tr>
<tr>
<td>Lactobacillus paracasei ssp. paracasei</td>
<td></td>
<td>Lactobacillus brevis</td>
</tr>
<tr>
<td>Lactobacillus rhamnosus</td>
<td></td>
<td>Lactobacillus fermentum</td>
</tr>
<tr>
<td>Lactobacillus manihotivorans</td>
<td></td>
<td>Lactobacillus reuteri</td>
</tr>
<tr>
<td>Enterococcus faecium</td>
<td></td>
<td>Lactobacillus plantarum</td>
</tr>
<tr>
<td>Lactococcus lactis</td>
<td></td>
<td>Pediococcus acidilactici</td>
</tr>
<tr>
<td>Streptococcus thermophilis</td>
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</tbody>
</table>
typically, the selection of a specific carbohydrate feedstock depends on the price and purity of the various carbohydrate sources locally available. Traditionally, a wide variety of carbohydrate sources, e.g. whey, molasses, corn syrup, dextrose and cane or beet sugar, have been used. Typical nitrogen and nutrient sources are corn steep liquor, yeast extract, soy hydrolysate, etc. (Datta and Henry, 2006). Most often, pH during the fermentation is kept constant with addition of NH₃, NH₄OH, KOH, NaOH, NaHCO₃, Ca(OH)₂ or CaCO₃ as a neutralizing agent. Most commonly used out of these are Ca(OH)₂ and CaCO₃. As the fermentations are typically run at pH value above the pKA of lactic acid (3.8), this leads to precipitation of lactic acid as lactate salt in high performance batch fermentations. This kind of trapping of fermentation end product reduces the toxic effects of lactic acid on the growth and morphology of microbial cells (Juturu and Wu, 2015). As the pKa of the lactic acid is 3.8, the higher the fermentation pH is the more calcium lactate is generated due to result of addition of pH adjustment chemical. In a typical homolactic lactobacillar lactic acid batch fermentation temperature is around 40°C and pH varies between 5.4 and 6.4 (Domenek et al., 2011). Running the process under these conditions requires large quantities of neutralizing agents and results in high amounts of insoluble lactate salt.

During the recovery, cells of production strain are removed and calcium lactate is regenerated with addition of sulfuric acid (see Fig. 9), resulting in generation of free lactic acid and formation of insoluble gypsum waste (Upadhyaya et al., 2014). Depending on the final usage of the lactic acid, solution is also purified and concentrated with various additional processing steps during the recovery and purification. Also, if significant amounts of by-products are formed during the fermentation, those are separated during the downstream processing and used for further processing, rejected or recycled. According to Jem et al. (2010) most typical purification steps in lactic acid manufacturing include those of chromatography, esterification, and/or distillation. In addition, membrane filtration, crystallization and/or evaporation may also be employed to purify and/or concentrate product to meet the desired end product specifications.

Good example of the product specifications is the requirement for the heat stability. As stated by Groot et al. (2010), the formation of color upon heating prohibits the use of crude acid in foods that need to undergo heating, like sterilization or pasteurization, during the manufacturing process. Also, heat stability is crucial for the monomer grade lactic acid, because during the step-growth polymerization processes, lactic acid is typically heated to between 150-190°C (Södergård and Inkinen, 2011). Formation of the color upon the heating is most often result of the presence of excess residual sugar and nitrogen compounds in the solution. Thus, production of heat stable lactic acid requires some additional processing steps to get rid of these compounds (Groot et al., 2010).

Another important criterium for plastic grade lactic acid is the optical purity of the lactic acid. According to Jem et al. (2010), plastic grade lactic acid needs to be heat stable, and typically the optical purity needs to be at least 98%. In the patent application filed by Purac Biochem B.V (EP 2748256 A2, Noordegraaf and De Jong, 2012), optical purity of at least 95 %, but preferable at least 99.5 % was set for qualification criteria for manufacturing PLA grade plastics. As stated by Jem et al. (2010), also the processing time with high temperatures should be minimized during recovery and purification, as it has been observed that racemization of D(-)-lactic acid to L(+-)-lactic acid, and vice versa, is prone to progress under these conditions. As described in Cargill Dow LLC’s patent application (WO2001038284 A1, Quarderer et al., 2000), high temperature alone (180°C
or higher) or the presence of suitable catalyst may start the racemization. As an example of catalyst, salts of group I and group II were mentioned (e.g. sodium, potassium, calcium and magnesium salts). These two factors operate also together, so the exposure of lactate material to temperatures above 100°C with the catalyst present can cause significant racemization.

1.6.3 Modern technologies in industrial bacterial fermentations– immobilization of cells for extracellular production of metabolites

As defined in the literature by Karel et al. (1985), whole cell immobilization refers to the localization of intact cells to defined region of space with the preservation of catalytic activity, whereas Abbott (1977) pointed out that immobilization is physical confinement or localization of a microorganism that permits the economical reuse of the microorganisms. As reviewed by Abbott (1977), one of the first examples of the usage of immobilized whole cells was the “quick-vinegar” production process developed by Schuetzenbach in 1823. In this process, wooden vat with perforated bottom was filled with wood shavings and the substrate, ethanol solution, was trickled through this vat, resulting in outflow of acetic acid solution. At that time, it was believed that the conversion of ethanol to acetate was due to a chemical reaction. Only after a couple of decades later, Pasteur found the connection between fermentations and the presence of micro-organisms.

End and Schöning (2004) have weighted the potential advantages and disadvantages of use of immobilization technology as follows:

<table>
<thead>
<tr>
<th>Potential advantages:</th>
<th>Potential disadvantages:</th>
</tr>
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<tbody>
<tr>
<td>Ease of developing continuous processes</td>
<td>The necessity of developing an immobilization process</td>
</tr>
<tr>
<td>Fast reaction rate due to high catalyst concentration (for certain reactor types)</td>
<td>The existence of mass transfer resistances (diffusion limitations)</td>
</tr>
<tr>
<td>Higher activity by better availability of catalytic centers</td>
<td>Additional cost caused by support and additional reagents</td>
</tr>
<tr>
<td>Repetitive use of biocatalysts</td>
<td></td>
</tr>
<tr>
<td>Higher resistance to shear stress and contamination</td>
<td></td>
</tr>
<tr>
<td>Higher stability with regard to temperature, pH, and catalyst poisoning</td>
<td></td>
</tr>
<tr>
<td>Easy separation from reaction media (easier downstream processing)</td>
<td></td>
</tr>
</tbody>
</table>

Methods for the whole cell immobilization have been reviewed by Pilkington et al. (1998) and Kourkoutas et al. (2004) and those are usually categorized based on the physical mechanism employed for the immobilization:

(a) attachment or adsorption on solid carrier surfaces  
(b) entrapment within a porous matrix  
(c) self-aggregation by flocculation (natural) or with crosslinking  
(d) cell containment
Figure 10 illustrates typical solutions and related mechanisms how the above mentioned four physical immobilization methods may be applied for various immobilization systems. Also, simplified model for the biofilm formation and natural flocculation is presented.

Figure 10. Most typical whole cell immobilizations methods and dynamics of biofilm formation and deformation. Adapted from Fukuda 1994; Kourkourtas et al., 2004; Ploux et al., 2010; Hall-Stoodley et al., 2004; Wingender and Flemming 1999.

Adsorption is generally regarded as the initial attachment of cells to solid surface (or deposition), whereas aggregation is one mechanism for the subsequent expansion of the initial adhesion population. If aggregates are not attached to a surface, term ‘floc’ has been used to describe these cellular aggregates (Hall-Stoodley et al., 2004). The importance of autoaggregation, reversible aggregation of similar cells to form discrete groups (Dube, 2005), in beer brewing was first understood by Pasteur 1876 when studying alcoholic yeasts fermentations. In these fermentations, liquid suspended aggregates are most typically formed on the top (surface yeast) or on the bottom (bottom yeast) of the fermentation vessel. Bacterial cells may also be cross-linked together with chemicals. This kind of chemical treatment leads to formation of artificial flocs. Cross-linking agents can also be used to bind cells to an organic or inorganic support by creating covalent bonds between cells and support material. However, covalent bonding is not the preferred way to immobilize living cells, since many of the chemicals used in these methods are highly toxic for the cells (Genisheva et al., 2014). Alternatively, cells can be artificially bound to solid support by applying various bioaffinity binding strategies. Many of these systems require cumbersome and costly modifications (surface engineering of bacteria and/or need of special functional groups attached to binding carrier) and are not viable for large scale industrial productions. However, in the scientific literature there are some promising reports available that are based on genetically modified bacteria which are able to adhere by bioaffinity mechanism to cheap immobilization supports without any significant pretreatment steps of the support material. As an example, Wang et al. (2001) demonstrated the cellulose binding capacity of genetically engineered *E. coli* strain and
Şimşek et al. (2013) managed to construct genetically engineered *L. lactis* strain that is capable to bind to chitin.

Immobilization of whole cells is also possible by enclosing bacteria inside a layer which permits the necessary nutrient and product flow for the biocatalysis. Genişheva et al. (2014) have reviewed the positive and negative aspects of this technology in their recent publication. Among LAB, the most widely used immobilization method in various food fermentations is based on cell entrapment using natural polymers like alginate, agarose, carrageenan, chitosan, and pectin. These natural gelling polysaccharides are non-toxic, biocompatible, cheap, and offer versatile techniques for biocatalyst preparation. The most common drawbacks of this immobilization method are the limited cell proliferation and activity (due to mass-transfer limitations in matrix) and lack of regeneration method for this kind of immobilization (Kosseva et al., 2011).

Adsorption of a bacterial cell to solid surface and the following putative biofilm formation is a complex process, which involves several steps and is affected by several variables. The initial bacterial adsorption to a surface is a reversible process, allowing also desorption from the surface if the net repulsive forces are greater than the net attractive forces (Garret et al., 2008). As summarized by Araújo et al. (2010), adhesion of bacteria to a solid surface is dependent upon van der Waals, electrostatic, and acid–base interactions. Furthermore, the physiochemical properties of surface and the characteristics of the bacterial surface, like hydrophobicity, surface charge, and electron donor–electron acceptor properties, are influencing the above mentioned interactions.

First task for a planktonic cell (a single cell floating in a bulk phase) is to get closer to the surface. This task can be initially fueled by Brownian motion, sedimentation due to differences in specific gravity between the bacteria and the bulk liquid, or by movement of the bulk fluid (convective mass transport). Also, some bacteria may move actively using flagella to move toward target surface (Palmer et al., 2007). When getting closer to the surface, typical long range forces like van der Waals and electrostatic interactions, but later also and hydrophobic/hydrophilic interactions may effect the movement of bacterial cells towards the surface. If the bacterial cell is able to overcome the energetic barrier surrounding the binding substrate, it may get a step closer to irreversible adsorption. In this battle, the above mentioned forces are taken into account, but also short range interactions, such as covalent and hydrogen bonding interactions are part of the equation. These interactions are receptor/ligand-type of interactions and are due to specific properties present on the bacterial cell surface but also include the specific properties of the binding substrate. Examples of specific bacterial surface properties which may promote adhesion with the target surface are bacterial flagella and pili (Ploux et al., 2010). In some cases, irreversible binding never happens and bacteria detach from the surface and return back to the planktonic form of life. This was also pointed out in the review written by Kosseva et al. (2011), as the low binding force between the cell and immobilization substrate was considered to be one of the weaknesses of the adsorption based immobilization systems. However, the other side of the coin in this matter is the relative easy regeneration of the carrier.

If irreversible adsorption is initiated, in some bacteria it is enhanced by cell’s capability to sense the surface (“surface sensing”) and to adapt rapidly its adhesive arsenal (e.g. by modifying the cell wall composition by expressing adhesive appendages or receptors)
(Ploux et al., 2010). Soon, this initial adhesion of first bacteria to cell surface is followed by colonization of the immobilization support, leading to the potential formation of a biofilm (Junter and Jouenne, 2004).

According to Saxena (2015), most typically the cellular growth in artificial immobilization systems is restricted. However, as reviewed by Junter and Jouenne (2004), in some cases growth in immobilized system may be ever faster than in the corresponding free cell system. In most cases, steady state conditions are preferred in continuous fermentation processes. When considering the immobilized production strain in this respect, it usually means that the total mass of bacterial cells inside the system remains close to constant after the ramp up and stabilization of the continuous fermentation process. The thickness of biofilm in steady state situation is function of growth and senescence (temperature, nutrient supply etc) and biofilm degradation (shear forces, film stability) (Patching and Fleming, 2003). Furthermore, several bacteria are able to produce extracellular polymeric substances (EPS) and even use specific signalling molecules to promote the growth and stability of the biofilms and cell-cell communication in this environment. In general, cells in biofilms are physiologically distinct from planktonic cells and in many cases possess a better stress tolerance than their planktonic relatives (Davies, 2003).

1.6.3.1 Potential solid supports for industrial scale immobilized fermentations

When selecting an immobilization carrier for a specific industrial process, the next aspects should be considered case by case as proposed by Virkajärvi and Linko (1999):

- type of immobilization
- price of material
- ease of immobilization
- cell load
- mass transfer
- channeling or blocking of reactors
- stability
- rigidity
- regeneration
- sterilization
- binding characteristics of contaminating microorganisms
- freedom to use various reactor designs
- possibility of fluidization (also for regeneration)
- approval for food use

As reviewed by End and Schöning (2004), typical solid support used for adsorption based immobilization include those of:

<table>
<thead>
<tr>
<th>Organic supports:</th>
<th>Inorganic supports:</th>
</tr>
</thead>
<tbody>
<tr>
<td>cellulose, chitine (chitosan), wood,</td>
<td>(porous) glass, clay, bentonite,</td>
</tr>
<tr>
<td>lignin</td>
<td></td>
</tr>
<tr>
<td>nylon, polypropylene fibres, polymer</td>
<td>zeolites, ceramics, mesoporous silica</td>
</tr>
<tr>
<td>nets, membranes</td>
<td></td>
</tr>
<tr>
<td>polyurethane foam, polyacrylamide</td>
<td>metal oxides (Fe, Ti, Mg), metal</td>
</tr>
<tr>
<td>ion-exchange resins</td>
<td>phosphates</td>
</tr>
<tr>
<td></td>
<td>mineral powder</td>
</tr>
</tbody>
</table>
1.6.3.2 The potential of new raw materials and whole cell immobilization in industrial scale LAB fermentations

The first industrial large scale application using immobilized cells was the *E. coli* based continuous L-aspartic acid manufacturing process that was successfully started in 1973 by Tanabe Seiyaku Co. Ltd. (Chibata et al., 1979; Linko and Linko, 1983). Currently, probably the most significant commercial application that is based on the use of whole cell immobilization technology is the beer manufacturing process with immobilized yeast strains. Within this industry, commercialization of immobilization technology has been in focus already for several decades and it has been utilized with various degrees of success for a number of purposes, like those of continuous primary fermentation, low-alcohol beer production and secondary maturation (Nedović et al., 2015). In wine industry, the potential of immobilized cells has not only been studied for the alcoholic fermentation (typical yeast fermentation), but also for the subsequent malolactic fermentation (MLF). In the wine manufacturing process, malolactic fermentation (MLF) is a secondary fermentation, which is needed in some wine grades to decrease the acidity and for subtle modification of the aroma. During the malolactic fermentation, L-malic acid is transformed into L-lactic acid and carbon dioxide. So, MLF starts when alcoholic fermentation has finished and it is typically a result of the fermentation by lactic acid bacteria that most typically are member of genus *Lactobacillus*, *Pediococcus*, *Leuconostoc* or *Oenococcus* (Genisheva et al., 2014). *Oenococcus oeni* (formerly known as *Leuconostoc oenos*) is one of the most typical strains found in the wine during spontaneous MLF (Lafon-Lafourcade, 1983; Solieri et al., 2010). Indeed, it is also available as commercial freeze-dried bacterial culture, readily used by the wineries, to gain more control over this challenging process (Nielsen and Richelieu, 1999). As reviewed by Nedović et al. (2015), several potential industrial whole cell immobilization processes for wine making have been proposed, including also those based on LAB - like the use of *O. oeni* for MLF. However, significant breakthrough in commercialization of these processes in large scale is still pending.

During the last decades, several articles have been published in which the use of whole cell immobilization of lactic acid bacteria in lactic acid (see reviews of), dairy and starter industries have been reviewed (Abdel-Rahman et al., 2013; Kosseva et al., 2009; Kosseva et al., 2011). In spite of the high scientific interest in this field, it seems that the final breakthrough in the commercial scale has not occurred, yet. As reviewed by Thongchul (2013), the reduction of product formation and instability of the immobilized cells during long-term fermentation are the main challenges to be resolved in order to make the immobilized (whole cell) lactic acid process commercially more attractive.

Another key element for sustainable and economical attractive lactic acid fermentation is the source, quality and cost of the substrates. As presented in the objectives of the major lactic acid producers in the world, technology to use the 2nd generation feedstock (non-food cellulosic raw materials) in industrial scale has been estimated by both Corbion (Purac) and NatureWorks to reach the maturity between 2015-2016. Indeed, according to Lin and Tanaka (2006) cellulose materials represent the most abundant global source of biomass. Over 90% of this biomass is in the form of lignocellulose. Clearly, there is a lot of potential in these raw materials, but the commercial use of lignocellulose in lactic acid production is still challenging because physicochemical pretreatments and multi-enzymatic reactions are required to yield monomeric hexoses and pentoses for the lactic
acid fermentation (Abdel-Rahman et al., 2011). Starchy non-edible raw materials, like agro-industrial waste and food waste, provide an easier solution for the carbon source for the lactic acid fermentations. However, as pointed out by Reddy et al. (2008) and Mazzoli et al. (2014), relative few LAB have been reported to be capable to ferment starch directly to lactic acid. As reviewed by Kosseva et al. (2009), the use of cheese whey as a substrate for the lactic acid fermentation has been studied widely. Although a part of the cheese whey can be utilized (e.g. by food and pharmaceutical industries), a significant amount of whey is disposed with dairy wastewaters – increasing the nutrient loads in waste water treatment plants. Economically, one of the most attractive scenario for utilizing whey is to separate the whey protein and sell it as high value protein isolates products and then to find relevant use for the remaining lactose fraction. As lactose is the natural substrate for many milk related LAB, many scientists have focused on studying the use of this two-carbon sugar in LAB based lactic acid fermentations. Not surprisingly, major lactic acid producers do not reveal details from their processes, but small Irish start-up company, Cellulac, has opened the door for some process details. As claimed on their www-pages (http://cellulac.co.uk/en/design-objectives/, accessed 15th of October 2015), company has developed technology to flexible utilize whey and lignocellulosic biomass (wheat straw, spent brewery grains from beer production, dried distilled grains from ethanol production) for manufacturing of optically pure D(-)-lactic acid by Lactobacillus strains capable to hydrolyze disachharides like lactose, but also pentoses and hexoses. Furthermore, in May 2014 they claimed to complete the world’s first ever industrial level continuous production of lactic acid from deproteinized lactose whey.

In scientific literature, there are some good reviews of non-industrial scale lactic acid fermentations based on the use of immobilized LAB. As an example, Kosseva et al. (2009) reviewed the whey based fermentations, whereas Abdel-Rahman et al. (2013) widely reviewed not only the use of pure sugars but also the use of different starchy and lignocellulosic carbon sources for various fermentation approaches.

1.7 Genetic engineering of Gram-positive bacteria to achieve whole cell immobilization with industrial potential

Although several researches studying the whole cell immobilization of Gram-positive bacteria have been published, only a small fraction of the proposed solutions are suitable for large scale industrial applications. Due to small number of LAB related publications in this area, the scope here is widened to cover also some other Gram-positive bacteria. In this section, examples are given from studies (Table 5) in which genetic engineering has been applied to modify the surface properties of given host strain and in which the nature of the materials used for immobilization is close enough to those that could be potentially used also in the industrial scale. Primarily, the focus is on those solutions in which bacteria are able to immobilize to a solid organic or inorganic carrier by direct non-covalent binding.
<table>
<thead>
<tr>
<th>Year</th>
<th>Technique</th>
<th>Methodology</th>
<th>Result</th>
<th>Industrial Interest</th>
</tr>
</thead>
<tbody>
<tr>
<td>2007</td>
<td>Genetic</td>
<td>Selection of strains with a specific enzyme</td>
<td>Cell membrane anchored enzyme (Glu-4)</td>
<td>Not mentioned</td>
</tr>
<tr>
<td>2007</td>
<td>Genetic</td>
<td>Construction of recombinant bacteria (Escherichia coli)</td>
<td>Cell membrane anchored enzyme (Glu-4)</td>
<td>Not mentioned</td>
</tr>
<tr>
<td>2007</td>
<td>Genetic</td>
<td>Expression of enzymes from different microorganisms</td>
<td>Cell membrane anchored enzyme (Glu-4)</td>
<td>Not mentioned</td>
</tr>
<tr>
<td>2007</td>
<td>Genetic</td>
<td>Use of genetically engineered cells</td>
<td>Cell membrane anchored enzyme (Glu-4)</td>
<td>Not mentioned</td>
</tr>
<tr>
<td>2007</td>
<td>Genetic</td>
<td>Expression of enzymes from different sources</td>
<td>Cell membrane anchored enzyme (Glu-4)</td>
<td>Not mentioned</td>
</tr>
</tbody>
</table>

**Table 5** Use of genetic engineering to achieve whole cell immobilization (Gram-positive organisms) with industrially interesting catalysts.
1.7.1 Immobilization on collagen surfaces by non-covalent binding

Antikainen et al. (2002) studied the collagen binding properties of the assembly region of CbsA (S-layer protein) of L. crispatus. In this work, authors studied which regions in this protein are required for binding to collagens and laminin, anchoring to the bacterial cell wall and self-assembly of the S-layer protein. For this task, authors made several constructs by inserting DNA fragments encoding various parts of CbsA in to pLPMSSA3 vector under an inducible α-amylase promoter. In this vector, the cbsA fragments were inserted in frame with the LPXTG cell wall-anchoring motif derived from the surface protease (PrtP) of L. casei. The resulting constructs were expressed in L. casei and the surface display of the N-terminal CbsA fragments was confirmed by ELISA. Successful binding of cells to type IV of collagen, but also to laminin, was demonstrated for several constructs. Most efficient binding to both collagen and laminin was observed with strain expressing CbsA residues 28-287. As no S-layer structure was observed in these cells, authors speculated that the above described binding was putatively achieved by the LPXTG bound CbsA peptides displayed on the bacterial surface.

1.7.2 Immobilization on starch surfaces by non-covalent binding

Tarahomjoo et al. (2008) fused the C-terminal LysM domain of peptidoglycan hydrolase (CPH) of L. lactis IL1403 with the linker region and the starch-binding domain (SBD) of α-amylase of S. bovis 148. The DNA construct expressing this fusion protein was expressed in E. coli, and the resulted protein was isolated and purified from the cell lysate. Purified fusion protein (designated as CPH-SBD) was first mixed with L. casei ssp. casei NRRL B-441 to allow binding of the fusion protein with cells, followed by the immobilization study with the corn starch granules. Measurement of the optical density was used to calculate the adhesion percentage to corn starch. The adhesion percentage under the optimal conditions for the cells was 32% whereas the same result for the control (free cells) was 4 %. Authors also discovered by phase contrast microscopy observation that CPH-SBD bound bacteria mediated the crosslinking of starch granules, resulting in aggregate formation in the solution. As estimated by the authors, in average 6 ×10^4 molecules of CPH-SBD fusion protein were bound per one L. casei cell.

1.7.3 Immobilization on cellulose surfaces by non-covalent binding

Lehtiö et al. (2001) studied the immobilization of genetically modified Staphylococcus carnosus on cotton fibres. Authors constructed an expression vector, in which the gene region encoding the cellulose binding domain (CBD) of Trichoderma reesei cellulase Cel6A of was fused with the promoter, signal sequence, propeptide encoding region of lipase gene from Staphylococcus hyicus. This propeptide encoding DNA fragment was included in the construct, as it was known to promote the secretion of heterologous proteins in S. carnosus when co-expressed with lipase signal. In order to enable cell wall anchoring, the above described DNA construct was fused with gene fragments encoding
the serum albumin binding region (ABP) of protein G of Streptococcus sp. G148 and the cell surface-anchoring regions X and M of protein A of S. aureus. The final expression construct was transformed into S. carnosus host by protoplast transformation and the resulting strain was used for immobilization studies with cotton fibers. Quantification of the cellulose-bound cells was based on the addition of biotinylated human serum albumin (binds with ABP tag) to the cotton fibers, followed by the addition of the streptavidin-alkaline phosphatase conjugate. Finally, the amount of specific binding of the streptavidin-alkaline phosphatase conjugate with ABP tag was visualized by addition of chromogenic substrate. Authors demonstrated that the immobilization efficiency of the strain expressing the CBD-fusion protein was more than double of the negative control. This result was also confirmed by light microscopy.

Recently, there has been a lot of scientific interest to express minicellulosomes on the surface of Gram-positive bacteria. Minicellulosomes, or designer cellulosomes, are composed of a chimeric scaffoldin which displays an optional cellulose binding module (CBM) and cohesins of different specificities which bind selectively to the cellulolytic enzymes appended with the matching dockerings (Mingardon et al., 2007). By using this approach, CEM (cellulose-enzyme-microbe) complexes are formed (You et al., 2012), in which the cellulose binding domain thether the microbe-cellulosome complex with the cellulosic substrate and allows it to progressively degrade cellulose by crawling along its strands (Wieczorek et al., 2013). Cell wall attached minicellulosomes with cellulose binding module attached within the scaffold have already been constructed for several industrially attractive Gram-positive micro-organisms, like those of B. subtilis, L. lactis and Corynebacterium glutamicum (You et al., 2012; Wieczorek and Martin; 2010; Kim et al., 2014). In these studies different kind of cell wall anchors were applied for anchoring of the scaffolding to the cell wall: You et al. (2010) used the LysM domain of cell wall hydrolase (LytE) of B. subtilis, Wieczorek and Martin (2010) used LPXTG anchor of M6 protein of S. pyogenes and Kim et al. (2014) utilized the transmembrane structures of the mechanosensitive channel (Msc) of C. glutamicum ATCC. The cellulose binding domains in these scaffolds were originated either from Clostridium thermocellum or Clostridium cellulovorans (see Table 5 for details). As the primary target for these studies was not to characterize the cellulose binding efficiency of the related recombinant cells but to demonstrate the enhanced cellulolytic activity of these strains, no specific data to evaluate the cellulose binding efficiency of the different recombinant strains was published.

1.7.4 Immobilization on chitin surfaces by non-covalent binding

Şimşek et al. (2013) constructed L. lactis strains capable to be immobilized on chitin. The chitin binding property of these strains was achieved by surface display of the ChBD (chitin binding domain) of chitinase A1 of B. subtilis on the surface of L. lactis nisin producing strains N8 and LL27. In the fusion constructs, ChBD was fused with PrtP anchors of three different lengths, and also with an AcmA anchor. All these anchors were derived from L. lactis. The gene constructs coding these anchor-ChBD fusions were expressed under the P45 constitutive promoter and usp45 signal sequence. Surface display of the expressed ChBD in L. lactis was verified with whole cell ELISA and the whole cell binding with chitin flakes was tested in an immobilization study. The nisin production
capability of the engineered strains was also evaluated by repeated cyclic fermentation test with immobilized chitin flakes. According to the results obtained from all of these experiments, the strains carrying either the longest or the second longest PrtP anchors, 800 and 344 C-terminal amino acids, respectively, outperformed the strains displaying the LysM based immobilization construct (242 amino acids) and the shortest PrtP immobilization construct (153 amino acids). In cyclic nisin production test, the highest productivity was measured with the strain carrying the longest PrtP anchor, although it was not statistically different from that obtained with the strain carrying the second longest PrtP anchor. The PrtP-ChBd fusion construct with the longest PrtP anchor demonstrated also the highest immobilization percentage on chitin, being 91 and 94 %, when using N8 and LL27 hosts, respectively.

1.7.5 Immobilization to inorganic or synthetic surfaces by non-covalent binding

Basically, the strategies to enhance immobilization of bacteria on a surface can be divided into specific methods and non-specific ones. The former include methods based on specific affinity between the binding ligand and the binding substrate (as is the case with CBD and cellulose), whereas the latter methods are based on more general interactions and characteristics, like to the overall surface charges or hydrophobicities, expression of protruding appendages on the bacterial surface or roughness of the immobilization material. Most often, the adsorption type of bacterial immobilization on solid surfaces like glass or plastic, has been based on these non-specific interactions, although during the recent years efforts to generate more specific synthetic peptide adhesins also for these materials have been increased (Karaca et al., 2014). Examples within this are the development of bioadhesive peptides for myocardial tissue engineering with polymeric materials (Rosellini et al., 2015) and construction of peptides with high affinity to bone-like apatite-based minerals (Segvich et al., 2009). However, it seems that the technological maturity to use these very specific peptides for industrial scale whole cell immobilization has not been reached, yet. Instead, during the recent years some promising results have been published about the use of peptide adhesins with a wider binding spectrum for immobilization of Gram-negative bacterial cells on various inorganic or synthetic solid supports. As an example, Park et al. (2014) evaluated the use of mussel’s adhesive catecholamine moiety to generate a sticky E. coli strain.

When considering Gram-positive bacteria, cell wall binding domains of lactococcal cell surface proteinases (most typically PrtP) are one of the most widely used anchors in Gram-positive surface display systems. Interestingly, PrtP is also known of its capability to enhance bacterial adhesion to some solid surfaces, like glass or PTFE. The biological role of this protein in milk related LAB is to initiate the proteolytic degradation of the milk casein to smaller peptides (Siezen, 1999). Habimana et al. (2007) studied the affinity of the lactococcal cells displaying various alleles of PrtP to solvents with different physico-chemical properties, and cell adhesion of the resulted variant strains to solid glass and tetrafluoroethylene surfaces. In these test three different strains of L. lactis ssp. cremoris were used: MG1363: PRTP+ (PrtP anchored and active, pGKV2-based construct), PRTP* (PrtP anchored and inactive, pGKV2-based construct) and PRTP- (MG1363 carrying vector plasmid pGKV2 without the prtP gene) as control strain.
Researchers found that the exposure of an anchored cell wall proteinase PrtP, undependent of its biological activity, was responsible for greater cell hydrophobicity and adhesion. When compared to the negative control (PRTP-), adhesion to both solid supports was enhanced with cells displaying either active or inactive form of PrtP on their surface. As the properties of these solid supports were very different (PTFE: apolar and hydrophobic, glass: polar and hydrophilic) and affinity to all various solvents (apolar, Lewis-acid or Lewis-base) increased, Habimana et al. (2007) proposed that the increased immobilization to glass or PTFE was due to surfacial presence of PrtP, which results in cell’s increased capacity to exchange attractive van der Waals interactions. As a result of this phenomena, authors suggested that bioadhesion of PrtP displaying lactococci is increased to various types of surfaces (e.g., inert, polar or apolar, or organic). Interestingly, the highest immobilization on solid surfaces was achieved with the surface display of the inactive PrtP. Inactivation of the PrtP was achieved by two point mutations (Asp-30 to Asn-30) in a catalytic site (Haandrikman et al., 1991). Habimana et al. (2007) speculated that the weaker immobilization capacity of the strain dipalying the active PrtP was due to degradation of AcmA (AcmA activity increases bacterial adhesion as demonstrated by Mercier et al. (2002)) and/or by self-cleavage of the exposed surface proteins by the active PrtP. The 100 x change in the salt concentration (NaCl: 1.5 mM to 150 mM) did not considerably affect to the binding with solid substrates and within the tested salt concentrations the PRTP* strain showed about six to eight times more efficient binding to PTFE and about eight to ten times more efficient binding to glass when compared to that of the control strain (PRTP-).

1.8 Genetic engineering of LAB to improve optical purity of the produced lactic acid

1.8.1 Various biosynthetic routes leading to D(-)- and L(+) -lactic acid formation in LAB

In LAB, lactic acid is most typically generated by lactate dehydrogenase which reduces the end product of glycolysis, pyruvate, with the concomitant regeneration of NAD+ (NADH oxidation). As described earlier, lactic acid is a chiral molecule and depending on the LAB strain, D(-), L(+) or both enantiomers are produced by D(-)- and/or L(+) - stereospecific lactate dehydrogenases (LDHs). In addition to lactate dehydrogenases, some LAB produce these lactate isomers also by stereospecific 2-hydroxyisocaproate dehydrogenases. Production of L-lactate may also take place by malolactic enzyme, which is capable to produce this lactate isomer by decarboxylation of L-malate. Finally, in some LAB lactic acid may putatively be produced through methylglyoxal detoxification pathways. All these pathways are described in Fig. 11.

In lactic acid bacteria, lactate dehydrogenases that catalyze the reduction of the pyruvate to lactic acid are typically NAD-linked (or NAD dependent) enzymes (nLDHs), which makes sense as the reduced nicotinamide adenine dinucleotide molecules (NADHs) generated during glycolysis need to be regenerated to maintain the fermentative life style.
(Garvie, 1980). Furthermore, some of the L-nLHDs are allosterically activated by fructose 1,6-diphosphate (FDP dependent L-nLDHs) – by one of the key intermediates of the glycolysis. More recently, Feldtmann-Salit et al. (2013) have studied the regulation of some L-LDHs of lactic acid bacteria more closely. According to Garvie (1980) allosteric regulation is especially typical for many streptococcal L-nLDHs, although the most extensively studied allosteric L-nLDH is that of L. casei.

Figure 11. Various potential metabolic pathways for lactic acid production and utilization among LAB. Reactions shown with bold white arrows are not described in detail. *Cofactors vary for different iLDHs (NAD-independent lactate dehydrogenases).

According to Garvie (1980), the other major group among LDHs is NAD-independent lactate dehydrogenases, i.e. iLDHs. These enzymes do not need NAD/NADH as a coenzyme, and typically catalyze only lactate oxidation to pyruvate or acetate (lactate utilization). Recently, Jiang et al. (2014) have reviewed the different types of bacterial iLDHs. These enzymes are also stereospecific, and that property is beneficial for enantiomeric purification of mixtures of D(-)- and L(+)-lactates – other optical enantiomer of lactic acid is oxidized while the other enantiomer remains intact. Also, many D-nLDHs and some FDP-independent L-nLDHs are capable to catalyze reaction between pyruvate and lactate in both directions (Garvie, 1980; Goffin et al., 2004). Capability to catalyze both oxidation and reduction reactions have also been confirmed for 2-hydroxyisocaproate...
dehydrogenases (Hummel and Kula, 1989). The main functional difference between lactate dehydrogenases and 2-hydroxyisocaproate dehydrogenases is the much wider substrate specificity of the latter group. Most lactate dehydrogenases accept only pyruvate and in minor degree 2-oxobutyrate, the closest longer structural homologue for pyruvate, as substrates (Hummel and Kula, 1989). However, 2-hydroxyisocaproate dehydrogenases (HicDHs) are usually able to convert also 2-keto acids with branched or aromatic side chains to corresponding 2-hydroxy acids (Hummel, 1999). Evolutionarily, D-LDHs and D-HicDHs are related (Kochhar et al., 1992), as is the case with L-LDHs and L-HicDHs (Lerch et al., 1989), too.

Not much is known about the putative methylglyoxal detoxification pathways in LAB. Most of the cellular methylglyoxal is synthesized by methylglyoxal synthase (MGS) from dihydroxyacetone phosphate (DHAP) by the removal of the phosphate group under conditions of carbon excess or phosphate limitation (Booth et al., 2003). However, the resulting metabolic intermediate, methylglyoxal (MG), is toxic for the cell because it is structurally a reactive α-ketoaldehyde (Murata-Kamiya and Kamiya, 2001). In bacteria, the main role of this alternative non-ATP generating glycolytic branch is to restore inorganic phosphate levels in stress conditions. As bacteria can also encounter MG in the environment, too, the detoxification capability of MG is an advantage in evolutionary competition (Chakraborty et al., 2015).

Currently, there are several different routes described in the literature for methylglyoxal (MG) detoxification (Fig. 11). Out of these, the glyoxalase I & II or III activity dependent routes and the route based on glycerol dehydrogenase and lactaldehyde dehydrogenase lead to generation of the D(-)-lactate, while the route catalyzed by methylglyoxal reductase and lactaldehyde dehydrogenase leads to production of L(+)lactate. Out of these enzymes, glycerol dehydrogenase and lactaldehyde dehydrogenase are most common among LAB, whereas glyoxylase I and II–like enzymes have been reported so far only in few LAB. Furthermore, the presence of the glyoxylase III-like enzymes in LAB has not been reported, yet. In the following paragraphs, there are few examples of LAB research papers, which include data of the presence and functions of the catabolic glyoxalase enzymes among LAB.

In general, MG metabolism seems to be most abundantly detected, or at least best known, among enteric bacteria. In accordance with this, an enteric LAB strain, *E. faecalis* V583 is one of those few LABs in which the genes related to MG metabolism has been putatively detected and expression level studied (Yan et al., 2015). In this strain, nine putative genes have been indentified to encode proteins belonging to the glyoxalase family. As detected by the authors, the abundance of transcripts from several transport systems in this strain was increased under oxidative stress conditions. However, transcription of many other genes, like that of the putative MG synthase and those five sharing the sequence identity with genes of the glyoxalase I–II system, was decreased under the same stress conditions. As suggested by the authors, the reduction in the abundance of transcripts encoding cellular glyoxalases demonstrates the importance of the fine tuning of the intracellular MG content for the oxidative stress adaptation for *E. faecalis*. As reviewed by Kant et al. (2010), Glyoxylase I –like enzyme has been putatively identified in silico among some LAB strains. One of these is *Streptococcus mutans*, in which the glyoxylase I–like enzyme has been functionally characterized by Korithoski et al. (2007). As observed by these authors, the lactoylglutathione lyase (LGL) in this strains (a glyoxalase I enzyme)
functions to detoxify methylglyoxal, resulting in increased aciduricity. However, the authors did not further identify the nature of the acid produced by this strain. These authors also observed some glyoxalase II activity in *S. mutans*, although they could not identify glyoxalase II genes *in silico*.

Finally, production of lactate may take place through lactaldehyde intermediates. Oxidation of the L-lactaldehyde or D-lactaldehyde by NAD-dependent lactaldehyde dehydrogenase leads to generation of L(+)lactate or D(-)lactate, respectively. In the case of L(+)lactate, the reduction of methylglyoxal to L-lactaldehyde is catalyzed by oxidoreductase enzyme. Chaillou et al. (2005) detected genes in *L. sakei* 23K for methylglyoxal bypass: These genes encoded putative enzymes capable to synthesize MG from dihydroxyacetone phosphate (by methylglyoxal synthase) and to convert it to L(+)lactate (by oxidoreductase and aldehyde dehydrogenase). In a later study, McLeod et al. (2011) observed up-regulation of the first two genes in this pathway (methylglyoxal synthase and putative oxidoreductase) after the change of carbon source from glucose to ribose. However, no induction was observed for the putative iron-containing aldehyde dehydrogenase, suggesting that reduction of L(+)lactaldehyde to lactate was not catalyzed by this putative enzyme. Among LAB, lactaldehyde dehydrogenase activity has also been proposed to function in the other direction (catalyzing lactate reduction), especially in some silage related LAB. These strains are able to reduce lactate first to lactaldehyde and then further to 1,2-propanediol with the concominant production of acetic acid and ethanol from lactate (Elferink et al., 2001; Saxena et al., 2010).

It is known that the NAD-dependent lactaldehyde dehydrogenase is also able to catalyze the oxidation of D-lactaldehyde to D(-)lactate, although reactions with this optical enantiomer are usually slower when compared to that of L-lactaldehyde (Purich and Allison, 2002). In case of *E. coli*, the production of D-lactaldehyde from methylglyoxal by glyoxalase I & II or III independent route has been proposed to happen by glycerol dehydrogenase activity (Altaras and Cameron, 1999). As stated by authors, this kind of reaction seems to be possible because the wide substrate specificity displayed by many known glycerol dehydrogenases. In the lactate production by this route, the ketone group of methylglyoxal is first stereospecifically reduced to give D-lactaldehyde, followed by oxidation by lactaldehyde dehydrogenase to yield D(-)-lactate (Altaras and Cameron, 1999).

Racemases catalyze the inversion of stereochemistry in biological molecules (Desguin et al., 2014). In case of lactate, lactate racemase activity has so far been detected only in few species among LAB, including of those of *L. plantarum* (Ferain et al., 1996), *L. sakei* (Hiyama et al., 1968), *L. curvatus* (Stetter and Kandler, 1973) and *L. paracasei* ssp. *paracasei* (formerly *L. casei* ssp. *pseudoplan tum*) (Stetter and Kandler, 1973). The best characterized lactate racemase system is that of *L. plantarum* WCFS1 (Goffin et al., 2005; Bienert et al., 2013; Desguin et al., 2014; Desguin et al., 2015). This strain is able to produce both D(-)- and L(+)lactate from sugars without lactate racemase, but it is also capable of interconversion of lactate isomers by lactate racemase (Lar) that is induced by the L(+)lactate and transcriptionally controlled by the L(+)/D(-) lactate ratio. It has been suggested that the physiological role of the Lar in *L. plantarum* is to secure the availability of D(-)-lactate for the cells, as the structure of the *L. plant arum* peptidoglycan precursors ends with D(-)lactate (instead the more frequently met D-alanine, see section 1.3.1) and cells lacking the capability to synthesize this lactate isomer are not viable. Indeed, this
uncommon peptidoglycan structure leads also to vancomycin resistance of *L. plantarum* cells (Ferain et al., 1996). Reports published by Bienert et al. (2013), Desguin et al. (2014) and Desguin et al. (2015) provide also *in silico* prediction for the presence of lar-related genes in other LAB, too.

Malolactic enzyme is able to convert L-malate (natural isomer of malate) directly to lactic acid and carbon dioxide without any other enzymatic activity. Indeed, the two other routes for malolactic fermentation, decarboxylation of malate to pyruvate or oxidation first to oxaloacetate and then subsequent decarboxylation of the formed oxaloacetate to pyruvate, are both dependent on the L-lactate dehydrogenase activity when considering the production of lactate (Schümann et al., 2013; Korkes and Ochoa; 1948; Flesch, 1969). As reviewed by Matthews et al. (2004), malolactic fermentation in wine-borne LAB is most typically catalysed by the malolactic enzyme. As mentioned in section 1.6.3.2, *O. oeni* (formerly known as *L. oenos*) is one of the most typical LAB strain found in the wine during spontaneous MLF (Lafon-Lafourcade, 1983; Solieri et al., 2010) and it also sold for that purpose as a commercial product. In 1990’s Labarre et al. (1996) were the first to identify and sequence the gene cluster carrying the genes responsible for expression of malate carrier and malolactic enzyme in *O. oeni*. During the preceding decade, Caspritz and Radler (1983) proposed the overall reaction equation for the L(+)-lactate synthesis by malolactic enzyme. What they observed was that this reaction is dependent on the presence of NAD+ and manganese. However, as no reduction of NAD+ or detection of free reaction intermediates was detected, the exact mechanism for this reaction remained unsolved (Caspritz and Radler, 1983; Groisillier and Lonvaud-Funel, 1999). According to Arthurs and Lloyd (1999), malolactic enzyme is present in many species belonging to the genera *Lactobacillus, Leuconostoc, Oenococcus,* and *Pediococcus.* However, this enzyme has been detected also in some other LAB genera. As an example of this, it has been proposed that malolactic enzyme has important role against lethal intracellular acidification and it also protects cells against oxidative and starvation damage in oral streptococci (Sheng et al., 2010).

When considering other putative biosynthetic routes for lactic acid, it is also possible that some other enzymatic side activity or totally undiscovered enzymes are responsible of lactate production. As an example, Aarnikunnas et al. (2002) could not find explanation for the D(-)-lactate production in *L. fermentum* strain in which both stereospecific *ldh*-genes were inactivated by deletion. Authors speculated that the marginal formation of D(-)-lactate in this strain was due to some other enzyme, with D-lactate dehydrogenase side activity.

### 1.8.2 Use of genetic engineering to produce lactic acid with enhanced optical purity

In this section, the main emphasis is on examples from LAB studies, which involve the use of modern methods of genetic engineering to modify the very last step in lactic acid biosynthesis, resulting in enhanced optical purity of the (main) lactic acid isomer produced. Furthermore, the focus is especially on studies in which the enhanced optical purity meets the criterium recommended for the raw materials for production of high melting point PLA-grades: optical purity at least 95 %, preferably at least 99.5 % (EP
Results of these studies are presented in Table 6.
The optical purity in Table 6 is expressed as enantiomeric excess (Okano et al., 2009) and yields are expressed as the conversion ratio (g/g) of the produced optical enantiomer from the initial amount of the sugar added in the fermentation medium.

1.8.2.1 Genetic engineering of LAB to improve the optical purity of D(-)-lactate

Ferain et al. (1994) were among the first scientists to study the consequences of the perturbation of lactate dehydrogenase activities in *L. plantarum*, and how this effects on the total production and ratio of L(+)- to D(-)-lactate in this strain. In this study, one of the strains constructed was TF101, in which the *ldhL* gene was inactivated by chromosomal deletion. This was achieved by constructing an unstable integration vector carrying an erythromycin resistance gene and flanking DNA fragments of the desired deletion site, followed by using this vector for two-step homologous recombination. Later, Goffin et al. (2005) used this strain in another research to study the role of *ldhL* expression and the role of L(+)-lactate for the function of the lactate racemase operon (*lar* operon) in *L. plantarum*. In this later study, it was confirmed that TF101 strain produce only D(-)-lactate and the lactate racemization activity detected in the host strain is positively regulated by L(+)-lactate (as no lactate racemase activity could be detected in the TF101 strain). There was no difference in the total lactate production between the TF101 and wild type strain.

Okano et al. (2009) constructed also an *ldhL* – deletion strain (referred as Δ*ldhL1*) of *L. plantarum* by using a plasmid with a temperature sensitive operon and an antibiotic resistance gene for homologous recombination. For the homologous recombination events, plasmid carried also two 1 kb regions flanking the *ldhL1* of *L. plantarum*. After the double-crossover homologous integration (and excision of the plasmid), screening of the LdhL1-negative strain was carried out with PCR amplification. Finally, pCUSαA -plasmid expressing the α-amylase (AmyA) of *S. bovis* 148 was introduced into *L. plantarum* host and Δ*ldhL1* strain, resulting in the strains referred as WT/pCUSαA (hereafter referred as reference strain) and Δ*ldhL1/*pCUSαA, respectively. Both of these strains were able to grow in raw corn starch as the sole carbon carbon source, which was not possible for the original wild type host. Authors demonstrated that the Δ*ldhL1/*pCUSαA -strain produced comparable amount of total lactic acid with the reference strain, but the optical purity of the produced D(-)-lactate was improved significantly (4.0 % vs 99.6 %).

Chae et al. (2013) employed a different strategy to enhance the D(-)-lactate optical purity in *Leuconostoc citreum* 95. They constructed a new *E. coli-Leuconostoc* shuttle vector, which was engineered to overexpress D-lactate dehydrogenase of *L. citreum* 95. This was accomplished by cloning the *ldhD* of *L. citreum* 95 into this vector, following transformation of the resulted plasmid by electroporation into the wild type host. The resulting strain demonstrated stable growth and lactic acid production under the erythromycin selection. However, the control strain (wild type + shuttle vector without *ldhD* gene) showed impaired growth in the fermentation test. As stated by the authors, the observed significant difference in cell growth between these two strains suggests that the overexpression of *ldhD* in *L. citreum* 95 putatively help cells to maintain the glucose metabolism and NADH/NAD⁺ recycling in balance during the growth at high
concentration of glucose. The optical purity of D(-)-lactate produced by the ldhD overexpressing strain was at least 99.8 % when expressed as enantiomeric excess (authors reported purity higher than 99.9 % when calculated as ratio of D(-)-lactate to the total lactate). In this study, no data for the optical purity of the wild type cells was provided. In an other study reported by Jin et al. (2009), the optical purity for the D(-)-lactate was lower (90.5 %) when the same wild type strain was used in fermentation tests. Thus, it seems possible that overexpression of ldhD resulted in a minor improvement in the optical purity in the study of Chae et al. (2013). However, these numbers are not directly comparable, because of the differences in the fermentation conditions of these two studies.

1.8.2.2 Genetic engineering of LAB to improve the optical purity of L(+)-lactate

Bhowmik and Steele (1994) cloned and characterized the ldhD of L. helveticus CNRZ32. This strain is industrially attractive due its homofermentative metabolism and high acid tolerance. Also, it produces lactate with good yield, but the produced lactate is mixture of D(-)- and L(+)-isomers. To improve the industrial attraction of this strain, authors inactivated its ldhD gene by gene disruption. This was accomplished by homologous recombination by using a SA3-based integration vector carrying an erythromycin-resistance gene for selection and being unable to replicate at high temperatures. The resulting recombination of this plasmid in to chromosome under high temperature led to disruption of the native ldhD gene and generation of erythromycin resistant strain that produced only L(+)-lactate. The total amount of L(+)-lactate produced by the mutant strain was only marginally less than that of the wild type. However, the maximum lactate concentration was reached somewhat earlier with the mutant strain.

Lapierre et al. (1999) focused on to develop a non-D(-)-lactate producing variant for the well known and widely used probiotic L. acidophilus La1 strain. In typical milk fermentations, this strain produces D(-)- and L(+)-lactates in ratio of 60:40 (%). As reviewed by the authors, in some diseases the accumulation of D(-)-lactate in blood may lead to a manifestation of D-acidosis and encephalopathy. In addition, food ingredients containing D(-)-lactate are not recommended to infants and young children (until 3 years age), because their liver may not be mature to metabolize D(-)-lactate completely. Lapierre et al. (1999) truncated the ldhD gene in vitro by PCR (8 bp deletion), resulting in the formation of a translational stop signal in the middle of the gene. The modified DNA sequence was then used to construct integration vector in Lactococcus. The integration vector was transferred to Lactobacillus johnsonii via conjugation on solid agar plates. After homologous recombination events in L. johnsonii, growth on non-selective medium gave raise of desired mutants in which the gene replacement and plasmid excision had taken place. In the following fermentation test, it was demonstrated that one of these mutants (designated as La1 ldhD-2) was able to produce L(+)-lactate with optical purity at least 98.9 %. However, although the yield of L(+)-lactate from glucose for the mutant strain was reasonable good (86.5 %), it was still lower when compared with that reported for the wild type strain.

In the study reported by Aarnikunnas et al. (2002), the main focus was to enhance the production of mannitol in combination with pure L(+)-lactic acid or pyruvate in L.
fermentum. One of the strains constructed to meet this goal was a LdhD negative strain (designated as GRL1030), in which the *ldhD* gene was inactivated by introducing a 0.4 kb deletion by gene replacement technique in the promoter and 5’ end region of the *ldhD* of *L. fermentum*. Fermentation tests demonstrated that the lack of the D(-)-lactate dehydrogenase activity in *L. fermentum* resulted in only minor changes in primary sugar metabolism (and mannitol production), but the growth and sugar consumption of the mutant was slightly slower when compared to those of the wild type cells. More surprisingly, the L(+)-lactate dehydrogenase activity was also decreased when compared to wild type host. However, the mutant was still able to produce high levels of pure L(+)lactate without a slowdown in mannitol production.

Viana et al. (2005) studied the effects of genetic inactivation of D-hydroxyisocaproate dehydrogenase (HicDH) to the end-product formation in *L. casei*. Although *L. casei* is most often referred to as an L(+-)lactate producer, about 5 % of the total lactate produced by the wild type cells of *L. casei* strain BL23 was detected to be D(-)-lactate in this study. Inactivation of the gene encoding HicDH of BL23 was accomplished by single cross-over integration with plasmid a carrying a gene region coding for a C-terminal truncated form of the native HicDH. The resulting mutant did not produce any D(-)-lactate, but otherwise the fermentation end-product pattern was practically identical to that of the wild type strain. Moreover, the amount of L(+-)lactate produced by the mutant cells was comparable with the total lactate production measured for the wild type cells.

Finally, Goffin et al. (2005) utilized the TF102 strain of *L. plantarum* (Ferain et al., 1996) to study its lactate racemase. Although the *ldhD* in this strain is disrupted and non-functional, Ferain et al. (1996) demonstrated that the production of D(-)-lactate was not blocked during growth on glucose and almost equimolar amounts of D(-) and L(+)-lactate were measured from the fermentation supernatant. According to these authors, this kind of distribution of the optical enantiomers of lactic acid suggested the presence of L(+-)lactate inducible lactate racemase in this strain. Earlier, it had been proposed that racemization is dependent on the L(+-)-lactate induction (Stetter and Kandler, 1973), so Goffin et al. (2005) decided to utilize this information for the identification of genes responsible for the putative lactate racemization activity in *L. plantarum*. Authors managed to identify a locus (lar) involved in lactate racemization and observed that it is composed of six genes that are organized in an operon. Deletion of this operon in the D(-)-lactate negative TF102 strain resulted in complete loss of D(-)-lactate production but also in loss of growth in media without supplemented D(-)-lactate. As demonstrated by Ferain et al. (1996), D(-)-lactate is needed for the peptidoglycan synthesis (and vancomycin resistance) of *L. plantarum*. Goffin et al. (2005) observed that when the concentration of supplemented D(-)-lactate was higher than 0.5 mM, the growth characteristics between the wild type cells (strain NCIMB8826) and the *ldhD lar* double mutant were essentially the same. For the fermentation test with *ldhD lar* double mutant, MRS broth containing 20 mM added D(-)-lactate was used. As expected, no net increase of D(-)-lactate was found in the supernatant after the fermentation test with the mutant strain and the produced amount of L(+-)-lactate was comparable with the total lactate produced by the wild type cells.
2. Aims of the study

Industrial production of thermostable PLA requires lactic acid of high enantiomeric purity. Often, industrial fermentations are carried out with thermotolerant microorganisms, like that of *L. helveticus*, in order to minimize contamination risk during fermentation. During these fermentations, the cell density is one of the most important factors affecting the total productivity. One of the strategies used to increase cell density is immobilization. However, many of the current whole cell immobilization methods are either too costly or too cumbersome, or those are not robust enough to be used in industrial fermentations of commodity chemicals with high dilution rates.

In this work, the focus was to (roman numbers refers to related publications):

1) Develop a LAB strain that produces PLA grade L(+)-lactic acid efficiently also in the stationary phase (I).

   The main hypothesis for this aim: production of L(+)-lactic acid by *L. helveticus* CNRZ32 is more efficient over the whole fermentation cycle if both native *ldh*-promoters drive the synthesis of this isomer.

2) Develop a novel whole cell immobilization method for LAB that would be potentially viable to be used also in industrial scale lactic acid fermentations. This goal was divided into two phases.

   2.1) Studying of various cell surface anchors for successful surface display in LAB. First, potential of *L. brevis* S-layer as a platform for surface display was studied (II). Moreover, the potential of various cell wall anchors (type/length) for surface display in *L. lactis* was compared (III, IV).

   The main hypothesis for this aim: it is possible to construct a cell envelope anchored cell surface display system in LAB that provides measurable surface exposure for the fused target molecule.

   2.2) Indicating the potential of whole cell immobilization of *L. lactis* to cellulose by cell surface displayed binding domain (III, IV).

   The main hypothesis for this aim: it is possible to construct a cell envelope anchored cell surface display system that enables the fused cellulose binding domain to bind with chemically unmodified cellulose and enhance the immobilization of mutant lactococci cells to a solid cellulosic carrier.
3. Materials and methods

3.1 Plasmids

The plasmids used in this work are introduced in Table 7. The detailed use of the plasmids is described in publications I-IV.

Table 7. Plasmids used in this work.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant properties</th>
<th>Reference/source</th>
<th>Used in</th>
</tr>
</thead>
<tbody>
<tr>
<td>pZeRO-2</td>
<td>Km(^r), lacZalpha-ccdB-based E. coli cloning vector</td>
<td>Invitrogen Co. Carlsbad, USA</td>
<td>I</td>
</tr>
<tr>
<td>pSA3</td>
<td>Em(^r), Tc(^r), Cm(^r), Ts replicon, Streptococcus-E. coli shuttle vector</td>
<td>Dao and Ferretti, 1985</td>
<td>I</td>
</tr>
<tr>
<td>pJDC9</td>
<td>Em(^r), E. coli cloning vector</td>
<td>Chen and Morrison, 1988</td>
<td>I</td>
</tr>
<tr>
<td>pUC19</td>
<td>Ap(^r), E. coli cloning vector</td>
<td>Yanisch-Perron et al., 1985</td>
<td>I</td>
</tr>
<tr>
<td>pKTH2153</td>
<td>Upstream region of the L. helveticus ldhD gene in pZeRO-2</td>
<td>This work</td>
<td>I</td>
</tr>
<tr>
<td>pKTH2154</td>
<td>ΔldhD construction in pSA3</td>
<td>This work</td>
<td>I</td>
</tr>
<tr>
<td>pKTH2155</td>
<td>slpA transcription terminator and downstream region of the ldhD gene in pSA3</td>
<td>This work</td>
<td>I</td>
</tr>
<tr>
<td>pKTH2156</td>
<td>Promoter region of the ldhD gene and structural gene of ldhL in pJDC9</td>
<td>This work</td>
<td>I</td>
</tr>
<tr>
<td>pKTH2157</td>
<td>ldhD::ldhL construction in pSA3</td>
<td>This work</td>
<td>I</td>
</tr>
<tr>
<td>pNZ9530</td>
<td>Em(^r), nisRK cloned in pIL252, expression of nisRK driven by rep readthrough</td>
<td>Kleerebezem et al., 1997</td>
<td>II</td>
</tr>
<tr>
<td>pLET1</td>
<td>Cm(^r), Km(^r), lactococcal expression vector with T7 RNA polymerase promoter</td>
<td>Wells et al., 1993</td>
<td>II</td>
</tr>
<tr>
<td>pKTH2151</td>
<td>Cm(^r), Km(^r), pLET1 derivative carrying the L. brevis slpA gene</td>
<td>This work</td>
<td>II</td>
</tr>
<tr>
<td>pKTH2152</td>
<td>Cm(^r), Km(^r), pKTH 2151 derivative; slpA fused with a poliovirus VP1 epitope insert in insertion site I</td>
<td>This work</td>
<td>II</td>
</tr>
<tr>
<td>pNZ8032</td>
<td>Cm(^r), pNZ8008 derivative carrying the gusA gene translationally fused to the nisA promoter (P(_{nisA}))</td>
<td>de Ruyter et al., 1996</td>
<td>II</td>
</tr>
</tbody>
</table>
3.2 Bacterial strains, growth conditions and media

The bacterial strains used in this study are shown in Table 8. The *E. coli* strains were grown in Luria-Bertani (LB) medium (Miller, 1972) at +37°C on agar plates or in liquid...
with shaking. The *L. lactis* strains were grown in M17 (Terzaghi and Sandine, 1975) at +30°C medium supplemented with 0.5% (wt/vol) glucose (M17G) on agar plates or in liquid without shaking. The *L. helveticus* strains were grown in MRS medium (de Man et al., 1960) at 37°C (or at 42 °C) on agar plates or in liquid without shaking. The *L. brevis* strains were also grown in MRS medium at 37°C on agar plates or in liquid without shaking. *S. aureus* was grown in LB medium at 37°C on agar plates or in liquid with shaking.

When appropriate, *E. coli* medium was supplemented with kanamycin (50 μg ml⁻¹), chloramphenicol (100 μg ml⁻¹), tetracycline (10 μg ml⁻¹), ampicillin (50 or 100 μg ml⁻¹), and erythromycin (200 or 300 μg ml⁻¹), *L. helveticus* medium was supplemented with erythromycin (4 μg ml⁻¹), *L. brevis* medium was supplemented with chloramphenicol (7.5 μg ml⁻¹) and erythromycin (7.5 μg ml⁻¹), and *L. lactis* medium was supplemented with chloramphenicol (2.5 or 10 μg ml⁻¹) and erythromycin (5 or 7.5 μg ml⁻¹).

**Table 8.** Bacterial strains used in this study.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant properties</th>
<th>Reference/source</th>
<th>Used in</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> Top10F⁺</td>
<td>Host strain of pZERO-2</td>
<td>Invitrogen, Carlsbad, USA</td>
<td>I</td>
</tr>
<tr>
<td><em>Escherichia coli</em> DH5a</td>
<td>Transformation host</td>
<td>Hanahan, 1983</td>
<td>I</td>
</tr>
<tr>
<td><em>Escherichia coli</em> DH5αF⁺</td>
<td>Transformation host</td>
<td>Hanahan, 1983</td>
<td>I</td>
</tr>
<tr>
<td><em>Escherichia coli</em> ERF573</td>
<td><em>E. coli</em> Top10F⁺ with pKTH2153</td>
<td>This work</td>
<td>I</td>
</tr>
<tr>
<td><em>Escherichia coli</em> ERF574</td>
<td><em>E. coli</em> DH5αF⁺ with pKTH2154</td>
<td>This work</td>
<td>I</td>
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<tr>
<td><em>Escherichia coli</em> ERF575</td>
<td><em>E. coli</em> DH5αF⁺ with pKTH2155</td>
<td>This work</td>
<td>I</td>
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<td><em>Escherichia coli</em> ERF576</td>
<td><em>E. coli</em> DH5αF⁺ with pKTH2156</td>
<td>This work</td>
<td>I</td>
</tr>
<tr>
<td><em>Escherichia coli</em> ERF577</td>
<td><em>E. coli</em> DH5αF⁺ with pKTH2157</td>
<td>This work</td>
<td>I</td>
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<tr>
<td><em>Lactobacillus helveticus</em> CNRZ32</td>
<td>Wild-type strain, L-LDH⁺, D-LDH⁺</td>
<td>Centre National de Recherches Zootechniques, Jouy-en-Josas, France.</td>
<td>I</td>
</tr>
<tr>
<td><em>Lactobacillus helveticus</em> GRL86</td>
<td>ΔldhD mutant of CNRZ32</td>
<td>This work</td>
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<td><em>Lactobacillus helveticus</em> GRL89</td>
<td>ldhD::ldhL mutant of CNRZ32</td>
<td>This work</td>
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<tr>
<td><em>Lactobacillus brevis</em> GRL1</td>
<td><em>L. brevis</em> ATCC 8287</td>
<td>American Type Culture Collection</td>
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<tr>
<td><em>Lactobacillus brevis</em> GRL1001</td>
<td><em>L. brevis</em> GRL1 with pNZ9530</td>
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<td><em>Escherichia coli</em> TG1</td>
<td>Transformation host</td>
<td>Gibson, 1984</td>
<td>III</td>
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</tbody>
</table>
### Table 8 continued.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Description</th>
<th>Reference</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactococcus lactis</em></td>
<td>N8 Nisin producer. Source of chromosomal DNA for <em>nisP</em> anchor amplification</td>
<td>Graeffe et al., 1991; Immonen et al., 1995</td>
<td>III, IV</td>
</tr>
<tr>
<td><em>Lactococcus lactis</em></td>
<td>MQ421 Carries pLP763 (55 kb plasmid). PrtP&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Kiwaki et al., 1989</td>
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</tr>
<tr>
<td><em>Lactococcus lactis</em></td>
<td>MG1363 derivative carrying a deletion in <em>acmA</em></td>
<td>Buist et al., 1995</td>
<td>III</td>
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<tr>
<td><em>Lactococcus lactis</em></td>
<td>IL1403 htrA <em>L. lactis</em> IL1403 derivate containing disrupted <em>htrA</em> gene; <em>htrA</em>::pVE8039, Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Poquet et al., 2000</td>
<td>III, IV</td>
</tr>
<tr>
<td><em>Lactococcus lactis</em></td>
<td>LAC236 (strain identification code unpublished) <em>L. lactis</em> MG1363 strain carrying pLEB595. Used for whole-cell ELISA. Em&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
<td>IV</td>
</tr>
<tr>
<td><em>Lactococcus lactis</em></td>
<td>LAC242 <em>L. lactis</em> MG1363 strain carrying pLEB606. Used for whole-cell ELISA. Em&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
<td>III</td>
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<tr>
<td><em>Lactococcus lactis</em></td>
<td>LAC243 <em>L. lactis</em> MG1363 strain carrying pLEB607. Used for immobilization test. Em&lt;sup&gt;R&lt;/sup&gt;</td>
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<td><em>Lactococcus lactis</em></td>
<td>LAC247 <em>L. lactis</em> MG1363 strain carrying pLEB596. Used for whole-cell ELISA. Em&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>III</td>
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<tr>
<td><em>Lactococcus lactis</em></td>
<td>LAC248 <em>L. lactis</em> MG1363 strain carrying pLEB597. Used for whole-cell ELISA. Em&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
<td>III, IV</td>
</tr>
<tr>
<td><em>Lactococcus lactis</em></td>
<td>LAC252 <em>L. lactis</em> MG1363*acmA&lt;sup&gt;AI&lt;/sup&gt; strain carrying pLEB597. Used for immobilization test. Em&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
<td>III</td>
</tr>
<tr>
<td><em>Lactococcus lactis</em></td>
<td>LAC257 <em>L. lactis</em> IL1403 htrA strain carrying pLEB597. Used for immobilization test. Em&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
<td>IV</td>
</tr>
<tr>
<td><em>Lactococcus lactis</em></td>
<td>LAC351 <em>L. lactis</em> MG1363*acmA&lt;sup&gt;AI&lt;/sup&gt; strain carrying pLEB685. Used for immobilization test. Em&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>III</td>
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<tr>
<td><em>Lactococcus lactis</em></td>
<td>LAC352 <em>L. lactis</em> MG1363*acmA&lt;sup&gt;AI&lt;/sup&gt; strain carrying pLEB596. Used for immobilization test. Em&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>III</td>
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<tr>
<td><em>Lactococcus lactis</em></td>
<td>LAC353 <em>L. lactis</em> MG1363*acmA&lt;sup&gt;AI&lt;/sup&gt; strain carrying pLEB606. Used for immobilization test. Em&lt;sup&gt;R&lt;/sup&gt;</td>
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<tr>
<td><em>Lactococcus lactis</em></td>
<td>LAC355 <em>L. lactis</em> MG1363*acmA&lt;sup&gt;AI&lt;/sup&gt; strain carrying pLEB686. Used for immobilization test. Em&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
<td>III</td>
</tr>
<tr>
<td><em>Lactococcus lactis</em></td>
<td>LAC357 <em>L. lactis</em> MG1363*acmA&lt;sup&gt;AI&lt;/sup&gt; strain carrying pLEB687. Used for immobilization test. Em&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
<td>III</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>Cowan I (NCTC 8530) Source of chromosomal DNA for <em>srtA</em> amplification.</td>
<td>Steidler et al., 1998</td>
<td>IV</td>
</tr>
</tbody>
</table>

### 3.3 Oligonucleotides

Oligonucleotide primers used for the PCR amplifications done in this work are listed in Table 9. The detailed use of the primers is described in publications I-IV.
<table>
<thead>
<tr>
<th>Oligonucleotides</th>
<th>Nucleotide sequence (5' =&gt; 3')</th>
<th>Use/site of hybridization</th>
<th>Used in</th>
</tr>
</thead>
<tbody>
<tr>
<td>O1</td>
<td>CTTGAAGAGTAGAATCTGCAAGTGATTC</td>
<td><em>ldhD</em> mRNA 5'-end mapping</td>
<td>I</td>
</tr>
<tr>
<td>O2</td>
<td>ATTACGAAATCAGTTAAGCCGATTC</td>
<td>Amplification of <em>ldhD</em> upstream region</td>
<td>I</td>
</tr>
<tr>
<td>O3</td>
<td>CTTAGGATCCGCTGGAATGATTATCGTG</td>
<td>Amplification of <em>ldhD</em> upstream region</td>
<td>I</td>
</tr>
<tr>
<td>O4</td>
<td>GATAGGATCCATAGCTATTCGAAAGACG</td>
<td>Amplification of <em>ldhD</em> region</td>
<td>I</td>
</tr>
<tr>
<td>O5</td>
<td>TGTCTGGGAATTCCTTACCTTC</td>
<td>Amplification of <em>ldhD</em> region</td>
<td>I</td>
</tr>
<tr>
<td>O6</td>
<td>CCCGCGATCCCTAAATATATATATAGTGTA</td>
<td>Amplification of transcription termination region of <em>slpA</em></td>
<td>I</td>
</tr>
<tr>
<td>O7</td>
<td>GCATATCGATGTTTTTCTCTAACAAGGCG</td>
<td>Amplification of transcription termination region of <em>slpA</em></td>
<td>I</td>
</tr>
<tr>
<td>O8</td>
<td>GACCATAGAATCTGGGATTTTCGCG</td>
<td>Amplification of <em>ldhD</em> region</td>
<td>I</td>
</tr>
<tr>
<td>O9</td>
<td>TGCACGCAACTTAGTCTCTG</td>
<td>Amplification of <em>ldhD</em> upstream region</td>
<td>I</td>
</tr>
<tr>
<td>O10</td>
<td>GATAATTAAAAACCATTCTTATACCTTC</td>
<td>R-PCR for <em>ldhD-ldhL</em> mRNA joint</td>
<td>I</td>
</tr>
<tr>
<td>O11</td>
<td>TGAGGAATGTTAAATTATCCTAATAAAAAG</td>
<td>R-PCR for <em>ldhD-ldhL</em> mRNA joint</td>
<td>I</td>
</tr>
<tr>
<td>O12</td>
<td>GAACGGGATCTTTATAGCAGAAGCTTACGCA</td>
<td>Amplification of <em>ldhL</em> region</td>
<td>I</td>
</tr>
<tr>
<td>p1</td>
<td>CGATCCGTCTTTAACGCTGCGAACTGCT</td>
<td>VP1 epitope sequence</td>
<td>II</td>
</tr>
<tr>
<td>p2</td>
<td>CGATAGTACCGAGTTTTCAACACAGCAGTTAACGAT</td>
<td>VP1 epitope sequence</td>
<td>II</td>
</tr>
<tr>
<td>p3</td>
<td>CTGGAATTACGGTTACAGCAACCAGC</td>
<td>Amplification of transcription termination region of <em>slpA</em></td>
<td>II</td>
</tr>
<tr>
<td>p4</td>
<td>TTTAAAGCTTTTTTCTCTAAACAGGCG</td>
<td>Amplification of transcription termination region of <em>slpA</em></td>
<td>II</td>
</tr>
<tr>
<td>p5</td>
<td>CCATGGTCAATCAAGTTAAAGAAATCTC</td>
<td>Amplification of <em>slpA</em> region</td>
<td>II</td>
</tr>
<tr>
<td>p6</td>
<td>CCTGCTTTAACTGTGTTGAAACTGCTGCACTGTACCAG</td>
<td>R-PCR, site II/VP1 in <em>slpA</em></td>
<td>II</td>
</tr>
<tr>
<td>p7</td>
<td>AGTAGCACCAGTTTTCAACACAGCAATACGCCTTACCTTTAGGAACCTG</td>
<td>R-PCR, site II/VP1 in <em>slpA</em></td>
<td>II</td>
</tr>
<tr>
<td>p8</td>
<td>CCTGCTTTAACTGTGTTGAAACTGCTGCACTGTACCAG</td>
<td>R-PCR, site III/VP1 in <em>slpA</em></td>
<td>II</td>
</tr>
<tr>
<td>p9</td>
<td>AGTAGCACCAGTTTTCAACACAGCAATACGCCTTACCTTTAGGAACCTG</td>
<td>R-PCR, site III/VP1 in <em>slpA</em></td>
<td>II</td>
</tr>
<tr>
<td>p10</td>
<td>TACCGAATTCCGGGACAGGCTTACAGAGAC</td>
<td>Amplification of <em>slpA</em> region</td>
<td>II</td>
</tr>
<tr>
<td>p11</td>
<td>AGTAGCACCAGTTTTCAACACAGCAATACGCCTTACCTTTAGGAACCTG</td>
<td>R-PCR, site IV/VP1 in <em>slpA</em></td>
<td>II</td>
</tr>
<tr>
<td>p12</td>
<td>CCGTCTTTAACTGTGTTGAAACTGCTGCACTGTACCAG</td>
<td>R-PCR, site IV/VP1 in <em>slpA</em></td>
<td>II</td>
</tr>
<tr>
<td>p13</td>
<td>GTATGAAATTGGCAATGTGACTTCAAGAAAGGG</td>
<td>Amplification of <em>slpA</em> region</td>
<td>II</td>
</tr>
<tr>
<td>p14</td>
<td>GACAGGATCCATAGAAGAAGAAGGCG</td>
<td>Amplification of <em>slpA</em> region</td>
<td>II</td>
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<tr>
<td>p15</td>
<td>CACAAAGGCTTTGAAGAAGCAGCAGCTGCTC</td>
<td>Amplification of <em>slpA</em> region</td>
<td>II</td>
</tr>
<tr>
<td>NIS195</td>
<td>TGCTTCTAGAGGATCCACTTGGTATGCGGA CATTGC</td>
<td>Used for constructing <em>prtP</em>&lt;sub&gt;135aa&lt;/sub&gt;</td>
<td>III</td>
</tr>
<tr>
<td>NIS196</td>
<td>ATGGGGGCCCTATTTCTACGGTTTCCG</td>
<td>Used for constructing <em>prtP</em>&lt;sub&gt;135aa&lt;/sub&gt; and <em>prtP</em>&lt;sub&gt;144aa&lt;/sub&gt;</td>
<td>III</td>
</tr>
<tr>
<td>NIS197</td>
<td>TGGCTCTAGAGGATCCAAGCTGACTTT ATACGG</td>
<td>Used for constructing <em>prtP</em>&lt;sub&gt;144aa&lt;/sub&gt;</td>
<td>III</td>
</tr>
<tr>
<td>NIS216</td>
<td>CTACGGGATCCAGTTATGGCGCTTCACTG</td>
<td>Used for constructing <em>acmA</em>&lt;sub&gt;242aa&lt;/sub&gt;</td>
<td>III</td>
</tr>
<tr>
<td>NIS217</td>
<td>GTAATGGGCCCCTATTATTTATCTGAGTAATCT GAC</td>
<td>Used for constructing <em>acmA</em>&lt;sub&gt;242aa&lt;/sub&gt;</td>
<td>III</td>
</tr>
<tr>
<td>NIS265</td>
<td>GCACGAGCTCTGATAAAATATGA</td>
<td>Used for constructing immobilization control plasmid</td>
<td>III</td>
</tr>
<tr>
<td>NIS266</td>
<td>GCGAGGATCCAGCATAACCCTGACAAC</td>
<td>Used for constructing immobilization control plasmid</td>
<td>III</td>
</tr>
<tr>
<td>NIS191 (previously unpublished)</td>
<td>TGGCTAGAGGATCCCGGAATAAAGCTTTT AGC</td>
<td>Amplification of <em>nisP</em> anchor region</td>
<td>IV</td>
</tr>
<tr>
<td>NIS192 (previously unpublished)</td>
<td>ATATGGGGACCCTTATTCTCTCCTTTT T C</td>
<td>Amplification of <em>nisP</em> anchor region</td>
<td>IV</td>
</tr>
<tr>
<td>NIS218 (previously unpublished)</td>
<td>GTGAGGGCCCTAAAAGGAGCCTAACGTAT G</td>
<td>Amplification of <em>srtA</em> region</td>
<td>IV</td>
</tr>
<tr>
<td>NIS219 (previously unpublished)</td>
<td>GCGTCCCGGGTATTGACTTCTGAGCTAC</td>
<td>Amplification of <em>srtA</em> region</td>
<td>IV</td>
</tr>
</tbody>
</table>

### 3.4 Methods used in this study

During this work well-established DNA isolation, hybridization, detection, manipulation, characterization, amplification and transformation protocols were applied (see Table 10). Also, RNA isolations, hybridizations and detections were based on earlier documented methods (see Table 10). Methods used for protein quantification and detection, or for enzymatic activity assays, are also included in this same table.

The whole cell enzyme-linked immunosorbent assay (ELISA) and cellulose binding assay were in the key role in this work. The whole cell ELISA-assy applied in this work was used for detection of the surface exposure of anchor-fused molecules and was partially carried out as described earlier by Laitinen et al. (2002). More detailed description for the whole cell ELISA-assay used in this work is available in publications II and III.

The cellulose binding assay was developed for this work (applied in publications III and IV) and this method is described in detail in publication III but is shortly covered here, also. In this assay, chemically unmodified Whatman No. 1 filter paper (Ø 42.5 mm) was used as immobilization support. Before the immobilization test with the filter paper, the Petri dishes (Ø 55 mm, made out of polystyrene) in which the filter paper assay
(immobilization test) was later done were first ‘saturated’ with pre-prepared cell suspension (in PBST: PBS + Tween 20 (0.5 %, v/v), pH7.4), in order to minimize the unspesific binding of bacterial cells to polystyrene during the actual filter paper immobilization test. After this saturation phase, the optical density of the cell suspension was assayed (at 600 nm wavelength, later referred as OD600) by taking a small sample from the Petri dish and leaving the remaining cell suspension in the dish. After this, immobilization test was initiated by adding the filter paper into the cell suspension. Adhesion of cells to filter paper was promoted by shaking the Petri dish for 1 h at 28°C (100 rev min⁻¹). After the adhesion, cell suspension was completely collected from the Petri dish by pipette for OD600 measurement, and washing solution (PBST) was added into Petri dish. After the washing period of 10 min (100 rev min⁻¹ shaking at 28°C), the optical density of the washing solution was measured and that result was used to calculate the robustness of the cellulose binding (‘washing loss’).

**Table 10.** Methods used in this study.

<table>
<thead>
<tr>
<th>Methods</th>
<th>Principle of the method described by</th>
<th>Used in</th>
</tr>
</thead>
<tbody>
<tr>
<td>General methods</td>
<td></td>
<td></td>
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<tr>
<td>DNA isolation, manipulation and amplification methods.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentrations and conditions in enzyme catalyzed reactions were adjusted to meet the recommendations set by the enzyme manufacturers</td>
<td>Sambrook et al., 1989</td>
<td>I, II, III, IV</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Specific recombinant DNA technology methods</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Gene replacement method</td>
<td>Bhowmik et al., 1993</td>
<td>I</td>
</tr>
<tr>
<td>Recombinant PCR technique</td>
<td>Higuchi, 1990</td>
<td>I, II</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Specific DNA isolation and purification methods</th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. helveticus</em> chromosomal DNA isolation.</td>
<td>Vidgren et al., 1992</td>
<td>I</td>
</tr>
<tr>
<td><em>E. coli</em> plasmid DNA isolation</td>
<td>Wizard Miniprep (Promege), FlexiPrep (Pharmacia)</td>
<td>I</td>
</tr>
<tr>
<td><em>L. lactis</em> and <em>L. brevis</em> plasmid isolation</td>
<td>QIAfiter Plasmid Midi Kit (Qiagen)</td>
<td>II</td>
</tr>
<tr>
<td><em>L. lactis</em> plasmid isolation</td>
<td>O’Sullivan and Klaenhammer, 1993</td>
<td>III, IV</td>
</tr>
<tr>
<td><em>L. lactis</em> chromosomal DNA isolation</td>
<td>Marmur, 1961</td>
<td>III, IV</td>
</tr>
<tr>
<td>DNA gel extraction with kit</td>
<td>E.Z.N.A.® Gel Extraction Kit (Omega Bio-tek)</td>
<td>III, IV</td>
</tr>
<tr>
<td>DNA purification with kit</td>
<td>QIAquick PCR Purification Kit (Qiagen), E.Z.N.A.® Cycle-Pure Kit (Omega Bio-tek)</td>
<td>III, IV</td>
</tr>
</tbody>
</table>
### Table 10 continued.

<table>
<thead>
<tr>
<th>Methods related to DNA hybridizations</th>
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</thead>
<tbody>
<tr>
<td>Digoxigenin (DIG) labelling of DNA probes</td>
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<tr>
<td>Southern transfer</td>
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<tr>
<td>Southern hybridization and DIG-detection</td>
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<tr>
<td>Colony hybridization</td>
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<table>
<thead>
<tr>
<th>DNA transformation methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli transformation</td>
</tr>
<tr>
<td>L. helveticus / L. brevis transformation</td>
</tr>
<tr>
<td>L. lactis transformation</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>RNA methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total RNA isolation</td>
</tr>
<tr>
<td>mRNA start site analysis</td>
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<tr>
<td>RNA dot blot</td>
</tr>
<tr>
<td>RNA hybridization</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Methods used for protein quantification or detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein quantification</td>
</tr>
<tr>
<td>Immunofluorescence microscopy</td>
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<tr>
<td>Whole cell ELISA</td>
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<tr>
<td>Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)</td>
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<td>Western blotting</td>
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<td>Western detection</td>
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<table>
<thead>
<tr>
<th>LDH activity and lactic acid kits</th>
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</thead>
<tbody>
<tr>
<td>Screening of D(-)-lactic acid negative strains</td>
</tr>
<tr>
<td>D(-)-LDH and L(+) - LDH activity measurements</td>
</tr>
<tr>
<td>L(+) - and D(-)-lactic acid assay</td>
</tr>
<tr>
<td>HPLC assay for total lactic acid quantification</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Whole cell immobilization test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose binding assay</td>
</tr>
</tbody>
</table>
4. Results and discussion

The first goal in this work was to construct a thermostable LAB strain capable of efficient PLA grade L(+)-lactic acid production. *L. helveticus* was chosen for lactic acid producer due to its homofermentative metabolism and thermo- and acid-tolerant nature. Also, it has been regarded as a efficient lactate producer (Roy et al., 1986; Idler et al., 2015) and it possess the QPS status (Qualified Presumption of Safety) granted by EFSA (European Food Safety Authority) (Anonymous, 2013). High optical purity of lactic acid is required to produce thermostable PLA grades (over 95 %, preferable over 99.5 %), as enantiomeric impurities decrease the melting point of the produced PLA. However, the selected host strain, *L. helveticus* CNRZ32, is not as such the preferred organism for production of PLA grade lactate, as it produces mixture of D(-)- and L(+)lactic acid during lactic acid fermentation. Therefore, the first focus in this work was to genetically modify *L. helveticus* CNRZ32 to produce only L(+) –lactic acid. In the resulting strains, the L-LDH expression took place under either native promoter (in GRL86 strain) or in combination of native promoter and *ldhD* promoter (in GRL89 strain). In the former case, *ldhD* was inactivated, whereas in the latter case the structural gene of *ldhD* was replaced by that of *ldhL*. Both of these strains were achieved by applying homologous recombination method. Furthermore, the relationship between growth of biomass and lactic acid production in the D-LDH deficient mutant strains was also studied. In many conditions, growth and lactic acid production are coupled in lactic acid bacteria. From the fermentation point of view this is a potential drawback; when the cells are entering to the stationary growth phase, production of lactic acid gradually ceases. This kind of characteristic, although often unavoidable, is not usually desirable for high cell density industrial fermentations, especially if the cells are immobilized and fermentation is operated at a continuous mode.

Another key focus area in this work was to study the potential of various anchoring motifs for surface display and eventually for whole cell immobilization. First, the gene *slpA*, encoding the S-layer protein subunit of *L. brevis*, was studied to detect the best locations within this gene to allow expression of the poliovirus VP1 epitope on the cell surface of this bacterium. The learnings from the VP1 surface display experiments were successfully applied to develop an expression system for c-Myc epitopes from human c-myc proto-oncogene. The primary purpose of the original publication II was to develop a novel whole cell platform for displaying vaccine antigens. For this thesis, the main interest is not focused to vaccines itself, but to evaluate possibilities of S-layer as a platform for surface display and ultimately for whole cell immobilization. Finally, the whole cell immobilization of *L. lactis* was studied with cellulosic material. In this work, different anchoring motifs and cell wall spanning sequences were fused with cellulose binding domain (CBD) of *Cellvibrio japonicus*. The surface exposure of the expressed fusion proteins was tested with whole cell ELISA by using CBD specific antibody and the functionality of these fusion proteins was evaluated by immobilization test with filter paper.
4.1 Production of PLA grade L(+) -lactic acid by genetically modified homofermentative \textit{Lactobacillus} strain (publication I)

4.1.1 Construction of D-LDH negative \textit{L. helveticus} strain by inactivation of \textit{ldhD} by gene replacement

A \textit{L. helveticus} CNRZ32 derived strain with \textit{ldhD} promoter deletion was constructed by using the gene replacement method described by Bhowmik et al. (1993). The integration vector (pKTH2154) used in this gene replacement was first constructed in \textit{E. coli} and then transferred to \textit{L. helveticus} by electroporation. This vector was pSA3-based \textit{Streptococcus-E. coli} shuttle vector with thermosensitive replicon, carrying fused homologous DNA fragments from both side of the \textit{ldhD} promoter region of \textit{L. helveticus} CNRZ32, but missing the actual promoter region (0.6 kb deletion). The integration of this plasmid into chromosome took place by homologous recombination after the temperature shift from 37 °C to 45°C under erythromycin selection. The second homologous recombination and excision of integration plasmid was achieved by growing the cells for 100 generations at 37°C without any antibiotic selection. As a result, 5% of the resulting clones were erythromycin-sensitive. Out of these, one clone (designated as GRL86) out of the fifteen showed D(-)-lactic acid negative phenotype.

4.1.2 Construction of D-LDH negative \textit{L. helveticus} strain by replacing the structural gene of \textit{ldhD} with that of \textit{ldhL}

Another \textit{L. helveticus} CNRZ32 derived strain, in which the structural gene of \textit{ldhD} was replaced by that of \textit{ldhL}, was constructed also by gene replacement method. First, the transcription initiation site of the \textit{ldhD} gene was determined by primer extension. This site, base G in position -34 upstream of the first nucleotide of the start codon, was used as a joint between the homologous \textit{ldhD} region and homologous \textit{ldhL} region when constructing the DNA fragment for homologous recombination (see Fig. 12). The beginning of the homologous \textit{ldhL} fragment was set to start from the transcription initiation site of this gene as determined by Savijoki and Palva (1997). In practise, this DNA fragment (\textit{ldhD\textsubscript{upstream region}} - \textit{P\textsubscript{ldhD}} - \textit{ldhL\textsubscript{structural gene}}) was built with recombinant PCR technique (R-PCR, Higuchi, 1990). The amplified \textit{ldhL} region did not include the transcription termination loop of \textit{ldhL}, instead the transcription terminator region from \textit{slpA} of \textit{L. brevis} was fused to the 3’ end of coding region of \textit{ldhL}. In the final integration vector (pKTH2157), this terminator was preceeding the other homologous \textit{ldhD} region needed for the gene replacement (see Fig. 12). This ′ldhD fragment started from the \textit{ClaI} restriction site located in the 5’-end of the corresponding structural gene.
The integration of pKTH2157 into L. helveticus CNRZ32 chromosome and the subsequent gene replacement events were achieved essentially as described above. However, an extra verification by PCR after the first recombination was needed in order to be sure that recombination had taken place only in the region of the \textit{ldhD} gene. After growing the cells for approximately 100 generations at 37°C without any selection, about 5\% of clones were erythromycin sensitive. Out of these, one clone (designated as GRL89) out of 25 tested showed D(-)-lactic acid negative phenotype.

4.1.3 Growth and fermentation characteristics of the wild type strain and D-LDH negative strains

First, growth, lactic acid production and transcriptional and enzymatic levels of LDHs of \textit{L. helveticus} CNRZ32 wild type strain were studied during pH controlled (pH 5.9) small scale (1.5 liter) bioreactor fermentation at 42°C (see Fig. 13). It was noticed that the production of L(+)lactic acid took place during the exponential growth phase and rapidly ceased when cells entered to the stationary phase. However, it seemed that L-LDH activity remained at a relatively high level even through out the stationary phase, too. The bulk of the D(-)-lactic acid was produced between the late exponential phase and the early stationary phase. In contrast to L-LDH activity, D-LDH activity decreased rapidly after having its maximum between the late exponential phase and the early stationary phase. Similar pattern was also observed for the relative intensities of \textit{ldh} mRNAs – mRNA for \textit{ldhL} reached its maximum to somewhat earlier than mRNA for \textit{ldhD}. Interestingly, at the time when production of L(+)lactic acid suddenly ceased, the amount of L-LDH activity was very near its maximum and there were still plenty of detectable \textit{ldhL} mRNA present. Based on these results it seems that the preference for D(-)-lactate production during the growth phase in which cells are entering to the stationary phase is not a consequence of limited presence of L-LDH enzyme but putatively due to changes in intracellular conditions in such a way that either the affinity of L-LDH for pyruvate is diminished or the catalytic activity of L-LDH is inhibited, resulting in a flow of pyruvate through D-LDH.
When the wild type host and D-LDH negative strains were compared with the above described small scale fermentation set-up, minor but insignificant differences were observed in the growth rate. Also, the total amount of lactic acid produced by all these strains was essentially the same. The optical purity (or enantiomeric excess) for L(+) – lactic acid was 16.0 % for the wild type strain but 99.8 % for the D-LDH negative mutants as measured by enzymatic assay (unpublished results). So, practically both D-LDH deletion mutants created were pure L(+)-producers and well qualified for production of L(+)-lactic acid for thermostable PLA manufacturing process. As expected, no D-LDH activity could be detected with the mutant strains GRL86 and GRL89. The highest L-LDH activities measured for GRL86 and GRL89 were 53% and 93%, respectively, higher when compared with that of CNRZ32 strain. All these strain reached their maximum L-LDH activity during the late exponential/early stationary phase (at 9 h time point). The difference between the L-LDH activities of mutant strains indicates that ldhD promoter was functional in GRL89. Notably, the production period of L(+)-lactic acid in the mutant strains was prolonged when compared to that observed in the wild type strain. In mutant strains, L(+)-lactic acid production ceased approximately at the same time point (15 h, in the stationary phase) in which the D(-)-lactic acid production stopped with the wild type strain. Indeed, there was no decrease in the rate of L(-)-lactate synthesis at a lactic acid concentration at which L(+)-lactate excretion ceased completely in the wild-type strain. This observation suggests that the rate of L-LDH catalysis is not likely dependent on the lactic acid concentration. Thus the change of flow from pyruvate to D(-)-lactate observed in the wild type strain may be due to changes in substrate binding between D- and L-
LDHs under these conditions. However, no other data to support this hypothesis was generated during this work.

The activity of two different \textit{ldh}-promoters were also studied by a fermentation experiment ran at higher temperature (44°C) but at lower pH (5.4). As observed by Armane and Prigent (1999), at low pH values growth and lactic acid production in \textit{L. helveticus} are uncoupled. Interestingly, this kind of phenomenon was putatively also observed with GRL89, but not with GRL86, at pH 5.4. With GRL86 both growth (biomass accumulation) and lactic acid production ceased simultaneously, whereas with GRL89, lactic acid production continued despite the biomass accumulation was levelled (Fig. 14). However, as the lactose seemed to be depleted at the same time when the biomass reached its maximum, it remained unanswered what was the reaction mechanism supporting the late lactic acid synthesis in GRL89.

![Figure 14](image)

\textbf{Figure 14.} Batch fermentations of genetically engineered \textit{L. helveticus} strains GRL86 (open symbols) and GRL89 (solid symbols), data from two parallel cultivations at pH 5.4 and 44°C. Concentrations of lactose (circles) and lactic acid (squares), and changes in dry weight as a function of time are presented in panels A and B, respectively.

Under these conditions, yield from lactose based media (initial lactose content about 80 g/l) with GRL89 strain was 91.6% with the maximum productivity rate of 3.21 g liter\(^{-1}\)h\(^{-1}\), whereas the same numbers for GRL86 were 76.2% and 3.34 g liter\(^{-1}\)h\(^{-1}\), respectively. Thus, in GRL89 in which two different promoters drove the lactic acid production, the total lactic acid production was approximately 20% higher when compared to GRL86 strain. When compared to criteria set by Datta et al. (1995) for a potential industrial scale
lactic acid fermentation, both the yield (91.6 vs. >90 %) and productivity (3.21 g liter⁻¹h⁻¹ vs. >2 g liter⁻¹h⁻¹) targets were met with the GRL89 strain. As the amount of sugar added into fermentation medium was less than the criteria set by Datta et al. (1995) for the desired end product concentration (>90 g/l), the true performance of this strain in this sense remained unanswered.

It seems that the \( ldhD \) promoter is capable to drive the L-LDH-synthesis longer (vs. growth phase) than the native \( ldhL \) promoter. Other explaining reason for the difference observed in lactic acid production, however quite unlikely, is the inactivation of putative fructosamine-3-kinase gene product in the GRL86 strain (unpublished information). When the \( ldhD \) in this strain was inactivated by promoter deletions, this deletion also inhibited the potential transcription of this gene. However, the putative enzymatic activity lost due to the gene replacement in this strain is not directly affecting the lactose catabolism in \( L. helveticus \), as this homofermentative species possess lactose permease and \( \beta \)-galactosidase activity and it metabolizes galactose via the Leloir pathway (Mollet and Pilloud, 1991; Fortina et al., 2003).

In addition, the optimum conditions for lactic acid production for GRL89 strain were studied by using statistical experimental design and response surface methodology. As a result of this study, pH 5.9 and 41°C were predicted to be optimum conditions for lactic acid production in this strain.

### 4.2 Development of whole cell immobilization method for LAB by surface engineering

#### 4.2.1 Studying the potential of S-layer protein (SlpA) of \( L. brevis \) for anchoring peptides for cell surface display (publication II)

Four insertion sites for the poliovirus VP1 epitope within the \( slpA \) gene of \( L. brevis \) ATCC 8287 (designated as \( L. brevis \) GRL1 below), were selected by using the hydrophilicity profile of the SlpA protein. In these regions (most hydrophilic parts of the SlpA protein), an 11-amino-acid immunodominant region of the VP1 capsid protein of enteroviruses (Hovi and Roivainen, 1993) was expressed to verify the surface accessibility of the chosen sites. The construction of the DNA sequence coding the VP1 epitope was carried out by hybridization, and the resulting fragment was used to construct the modified \( slpA \)-genes by conventional DNA manipulation techniques or by R-PCR technique. In the resulting constructs, the VP1 epitope in the expressed fusion proteins was displayed between amino acid residues Asp362 and Thr363 (insertion site I), Lys249 and Ala250 (insertion site II), Ala313 and Asn314 (insertion site III) and Ala49 and Lys50 (insertion site IV). Each of these insertions was constructed separately and the resulting \( slpA \) constructs were expressed under the regulated \( nisA \) promoter (\( P_{nisA} \)) in a controlled manner in a two-plasmid system. In this system, modified \( slpA \) genes were expressed under \( nisA \) promoter (found in pNZ8032), and the \( nisR \) and \( nisK \) genes needed for the nisin-induced transcription from \( P_{nisA} \) (Kleerebezem et al., 1997) were expressed by pNZ9530 plasmid. The plasmids expressing modified \( slpA \) genes with VP1 epitope coding sequence in
location I, II, III and IV were designated as pKTH5006, pKTH5007, pKTH5008 and pKTH5063, respectively. These plasmids were used to transform *L. brevis* ATCC 8287 strain carrying pNZ9530 plasmid (hereafter referred as GRL1001) under erythromycin and chloramphenicol selection, resulting to strains referred as GRL1001+epi-I, GRL1001+epi-II, GRL1001+epi-III and GRL1001+epi-IV, respectively. As a results, four *L. brevis* strains were obtained, which expressed not only the native *slpA* but also VP1-modified *slpA*. The GRL1001 strain was used as a control strain (possess only native *slpA*) when testing the surface exposure of these above mentioned VP1-modified *slpA* variant strains.

The surface accessibility of the VP1 epitope in the above mentioned strains was tested by whole-cell enzyme-linked immunosorbent assays (ELISA) with anti-VP1 antibody raised in rabbit. According to whole-cell ELISA, the strongest color response within the strains carrying VP1-modified S-layer was obtained with the recombinant strain in which the insertion was in site II (GRL1001+epi-II). Also, the strain expressing VP1 in site I (GRL1001+epi-I) gave a positive color response, whereas the response for the strains expressing VP1 in sites III (GRL1001+epi-III) and IV (GRL1001+epi-IV) was at the same level with that measured for the control strain (GRL1001). In addition to the whole-cell ELISA assay, surface exposure of the VP1 in these recombinant strains was analysed by immunofluorescence microscopy using anti-VP1 antibody and FITC-conjugated secondary antibody. However, none of the recombinant strains tested gave visible signal in these tests. Cells for both of these tests (whole-cell ELISA and immunofluorescence microscopy) were induced with 10 ng/ml of nisin. With this nisin concentration, both the induction of *PnisA* and cell growth after the induction was at reasonable level for subsequent immunological detection of VP1.

In the above described mutant *slpA* strains there is competition between the expression of native *slpA* and VP1-modified *slpA*. Because the expression levels of these *slpA*-epitope constructs were still low when compared to the high-level expression of the native chromosomal *slpA* gene, epitopes were not able to be seen by immunofluorescence microscopy. In order to increase the expression level of the epitope-SlpA protein, different expression strategy was employed with another epitope (c-Myc). This epitope was incorporated at site II in *slpA* and the resulting recombinant *slpA* was used for gene replacement in *L. brevis* (see publication II for details) to replace the native chromosomal *slpA* gene with the modified one. As a result, a recombinant strain (GRL1046, see publication II) was achieved that displayed uniform S-layer on its surface with the desired antigen in all of the S-layer protein subunits and which was easily detected by the immunofluorescence microscopy, also. Further studies indicated that the S-layer lattice structure was not affected by the presence of the additional c-Myc epitope in the S-layer subunits.

As a summary, two insertion sites out of total four tested gave a positive signal in whole-cell ELISA, which demonstrated that these insertion sites in native *slpA* can be used for various cell surface display applications in which S-layer functions as a cell wall anchor. The VP1 epitope used in this study was short (11 amino acid residues), so the potential of displaying longer motifs in these insertion sites remained unverified in this study. As the S-layer is usually the most outermost layer in cells in which it exists, the peptides anchoring to it do not need to span through the peptidoglycan layer. As a comparison, the estimated minimum length needed for the cell wall spanning and to allow proper surface exposure of the target protein is usually at least 90–100 residues (Fischetti et al., 1990; Strauss and Götz, 1996).
The advantage of the S-layer for surface display is the high number of protein subunits present on the cell surface. As estimated by Sleytr and Messner (1988), an average-sized cell consists of approximately $5 \times 10^5$ S-layer monomers on its surface. As it was demonstrated by c-Myc epitope (10 aa), it is possible to incorporate small peptides into every single S-layer subunit proteins without interfering the assembly and structure of the S-layer. Regardless of the incorporated peptide and the binding mechanism involved (mediated by this peptide), it is possible that already a weak interaction could provide a whole cell binding with carrier. So far scientists have not managed to construct a genetically engineered LAB strain which could be robustly immobilized to an industrially viable carrier material by its genetically modified S-layer. However, in several cases S-layer mediated whole cell binding with various receptors, cells and biological matrixes has been detected (Hynönen and Palva, 2013; Sleytr et al., 2014). This seems to be consequence of direct interactions between various S-layer components and target structures.

4.2.2 Indicating the potential of whole cell immobilization of L. lactis by cell surface displayed binding domain (publication III and IV)

The final goal of this work was to develop a L. lactis strain capable to be immobilized to a cellulotic carrier by CBD-cellulose bio-affinity. The typical properties required from an industrial immobilization carrier have been reviewed earlier in this work (see section 1.6.3.1). Out of these, among the most important properties are good availability, chemical and physical robustness, nontoxicity, high immobilization capacity and low price. Cellulosic materials, like wood chips, have been used in many traditional fermentations processes – especially in those in which mixing is not needed or the shear forces applied are very low. In order to increase the binding force between bacterial cell and cellulose, chemical pretreatments have been used to modify cellulose. As the surface charge of most microorganisms is negative, basic derivates of cellulose, such as modification with DEAE-groups (diethylaminethyl), make it a more efficient carrier for the whole cell immobilization (Phillips and Poon, 1988). However, these kinds of treatments require the use of additional processing time and chemicals and are costly. Thus, transition from DEAE-based carriers to unmodified cellulotic carriers is advantageous in many cases.

In this work, different kind of anchoring motifs and cell wall spanning sequences were tested for both cell surface exposure and whole cell immobilization efficiency when fused with the cellulose-binding domain (CBD) of XylA of Cellvibrio japonicus, referred hereafter as CBDXylA. The XylA of C. japonicus is an endo-1,4-beta-xylanase A and was originally characterized by Hall et al. (1989) from Pseudomonas fluorescens subspecies cellulosa (reclassified as C. japonicus by Humphry et al., 2003). This enzyme has an N-terminal carbohydrate binding module, classified under CBM_2 family in the Conserved Domain Database (CDD) (Marchler-Bauer et al., 2015). This kind of cellulose binding domain is typically found among bacteria.
4.2.2.1 Construction of different cell wall anchors for whole cell immobilization of \( L. \) \( lactis \)

During this work, both covalent and non-covalent cell wall anchors were tested to display \( CBD_{XylA} \) for cellulose binding (see Table 11). Covalent cell wall anchors of LPXTG-type (Fischetti et al., 1990) included those of PrtP of \( L. \) \( lactis \) MQ421 (Kiwaki et al., 1989) and NisP of \( L. \) \( lactis \) N8 (Immonen et al., 1995). In addition to LPXTG-based anchors, the non-covalent LysM anchor region (containing three repeats) from AcmA of \( L. \) \( lactis \) MG1363 was tested to anchor \( CBD_{XylA} \). Also, the length of the sequence between the \( CBD_{XylA} \) and the conserved LPXTG recognition sequence and the effect of the heterologues sortase A (SrtA, Mazmanian et al., 1999) overexpression for the covalent anchoring were studied with the PrtP anchor of \( L. \) \( lactis \) MQ421. The sortase gene for this construct was amplified from chromosomal DNA of \( Staphylococcus \) \( aureus \) Cowan I (NCTC 8530).

Table 11. Anchors used to display \( CBD_{XylA} \) for cellulose binding.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Cell envelope anchor fused with ( CBD_{XylA} )</th>
<th>Type of anchoring</th>
<th>Length of the anchor</th>
<th>Other relevant information</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLEB597</td>
<td>PrtP of ( L. ) ( lactis ) MQ421</td>
<td>LPXTG  (covalent)</td>
<td>344 aa</td>
<td>Anchor part covers AN-, W-, H-domains and also the C-terminal part of the B-domain of PrtP (as described by Siezen (1999))</td>
</tr>
<tr>
<td>pLEB607</td>
<td>PrtP of ( L. ) ( lactis ) MQ421</td>
<td>LPXTG  (covalent)</td>
<td>344 aa</td>
<td>( srtA ) of ( S. ) ( aureus ) transcriptionally fused to prtP anchor-sequence</td>
</tr>
<tr>
<td>pLEB196</td>
<td>PrtP of ( L. ) ( lactis ) MQ421</td>
<td>LPXTG  (covalent)</td>
<td>153 aa</td>
<td>Anchor part covers AN- and W-domains and also the C-terminal part from the H-domain of PrtP (as described by Siezen (1999))</td>
</tr>
<tr>
<td>pLEB606</td>
<td>AcmA of ( L. ) ( lactis ) MG1363</td>
<td>LysM  (non-covalent)</td>
<td>242 aa</td>
<td>Anchor part covers three repeated regions (LysM-domains)</td>
</tr>
<tr>
<td>pLEB595</td>
<td>NisP of ( L. ) ( lactis ) N8</td>
<td>LPXTG  (covalent)</td>
<td>121 aa</td>
<td></td>
</tr>
</tbody>
</table>

The DNA region encoding the \( CBD_{XylA} \) was provided by Cultor Ltd. in a form of plasmid (cloned in pN5). In this plasmid, the 5’end of the region encoding the \( CBD_{XylA} \) was fused with the sequences coding the Usp45 secretion signal of \( L. \) \( lactis \) (van Asseldonk et al., 1990), whereas the 3’end of the \( CBD_{XylA} \) encoding region was fused with sequence coding the cell wall anchor (SPA) of Protein A of \( S. \) \( aureus \) (Shuttleworth et al., 1987; Steidler et al., 1998). This fusion construct was cut out from pN5 and transferred into pLEB124 under the constitutive \( ss45 \) promoter (P45, Sibakov et al., 1991) and partial secretion signal. The pLEB124 vector was constructed by Ra et al. (1996), and this lactococcal expression plasmid is also able to replicate in \( E. \) \( coli \). To allow further clonings, one \( XbaI \) restriction site (located in the upstream region of P45) in the resulting pLEB124 based plasmid was removed and the resulting plasmid was designated as pLEB594. In this plasmid, the remaining \( XbaI \)-site is located at the seam between DNA fragments coding for \( CBD_{XylA} \) and SPA anchor. This \( XbaI \)-site was directly used for constructing the most
CBD-anchor fusions during this work just by directly replacing the SPA anchor with other C-terminal anchors (as XbaI-ApaI fragments). This approach was used to constructs plasmids with fragments encoding NisP 121 aa, PrtP 153 aa and PrtP 344 aa anchors, resulting in plasmids pLEB595, pLEB596 and pLEB597, respectively.

Cloning of AcmA 242 aa anchor utilized BamHI site built between the DNA fragments encoding the CBDXylA and NisP anchor. In practice, the NisP 121 aa anchor was replaced (as BamHI-ApaI fragment) with the region coding for the AcmA 242 aa anchor, resulting in generation of plasmid pLEB606. Finally, the pLEB607 plasmid expressing CBDXylA -PrtP 344 aa –SrtA was built in the pLEB597 based vector. The DNA fragment for expression of SrtA in this construct is transcriptionally fused with the C-terminal end of the fragment encoding the PrtP 344 aa, and possesses also the original ribosome binding site of the srtA of S. aureus. In detail, the construction of this plasmid started with ApaI linearization of pLEB597, followed by ligation with an ApaI digested srtA fragment (generated by PCR). This ligation mixture was used as template for PCR amplification of the region, containing the P45 promoter region and the DNA fragment coding for CBDXylA –PrtP 344 aa –SrtA. The resulting PCR-product was digested with BamHI and XmaI, generating fragments coding the fusion of PrtP 344 aa –SrtA for the final ligation with similarly digested pLEB597 vector. As mentioned above, the resulting plasmid was designated as pLEB607.

All the different pLEB124-based CBDXylA-anchor constructs were used to transform L. lactis hosts by electroporation. L. lactis MG1363 host was used to verify the surface exposure of CBDXylA when fused with different anchors. For cellulose binding tests (publication III), the expression host for the CBDXylA-anchor fusion was MG1363acmAΔ1. In this strain, the major autolysin of the host (MG1363) is inactivated. As noticed by Buist et al. (1995), the loss of AcmA activity was seen as a change in growth characteristics. As observed by these authors, mutants did not show growth as single cells as typically observed with the wild type strain, but seemed to grow as long chains, indicating that AcmA is required for cell separation in this strain. The main reason why this strain was preferred to be the host in immobilization tests instead of the MG1363 strain, was the lower unspecific binding with the filter paper under the conditions used for the immobilization test. This observation, suggesting the promoting effect of active AcmA on the adhesion of cells to solid surfaces, is in agreement with the results reported by Mercier et al. (2002). However, in publication IV the MG1363 strain was used as the host strain also in the cellulose binding tests, as the main target was not to quantify the exact efficiency of the binding, but rather to verify is there any difference in cellulose binding with or without overexpression of sortase (with the same anchor construct).

4.2.2.2 Immunological verification of the surface exposure of CBDXylA when anchored to cell with different anchors

The surface exposure of CBDXylA in constructed L. lactis MG1363 strains expressing CBDXylA-fusion proteins with either the NisP 121 aa-, PrtP 153 aa-, PrtP 344 aa- (with or without SrtA) or AcmA 242 aa -anchor was tested by whole-cell ELISA with the CBD-antibody raised in rabbit. As indicated by the result (Fig. 15), the highest positive response was measured for fusion constructs with the longer PrtP anchor (344 aa), but also
the response measured for the AcmA 242 aa anchor construct clearly exceeded that measured for the wild type strain (MG1363). However, the response with shorter PrtP anchor fusion was weak.

**Figure 15.** Selected results from whole-cell ELISA-assays. Lactococcal cells expressing CBD-anchor fusions were tested with CBD-specific antibody. The volume of the cell suspension used in the detection is indicated.

With the NisP 121 aa-anchor fusion, the response from the assay was not clearly distinguishable from that of the wild type strain (see publication IV). Also, it seemed that there were no differences in the surface exposure of the CBD<sub>XylA</sub> between the strains in which the CBD<sub>XylA</sub> - PrtP 344 aa was expressed with or without transcriptionally coupled heterologous *srtA* (see publication IV).

Based on these results, all the above strains except the one expressing CBD<sub>XylA</sub>-NisP 121 aa fusion protein were used for the immobilization test with Whatman no.1 filter paper. In these tests, the strain expressing CBD<sub>XylA</sub>-PrtP 344 aa – SrtA was not included in the quantitative immobilization testing (that was performed for all the other strains), but instead it was used in one to one comparative study against the strain expressing the same CBD<sub>XylA</sub>-anchor construct but not the SrtA enzyme.

The most probable reason for the poor surface exposure of the CBD<sub>XylA</sub>-NisP 121 aa-fusion protein was the significant degradation of this protein, which was clearly seen by the Western analyses. In contrast the CBD<sub>XylA</sub> –PrtP 153 aa fusion protein seemed to suffer from weak anchoring, as the only clearly detectable band of right size for this fusion protein in Western analysis was found from the supernatant fraction (results not shown). Also, significant part of the longer PrtP fusion protein and major part of the AcmA (result not shown) fusion protein were found in the supernatant fraction. These observations indicate restricted binding capacity of these anchors under the conditions used for preparing the cells for this testing.

**4.2.2.3 Verification of whole cell immobilization of genetically modified *L. lactis* by immobilization tests**

Finally, to evaluate the immobilization capability of the selected recombinant strains, a simple filter paper-based (with Whatman filter paper no.1) immobilization assay was developed. In this assay, immobilization was quantified by measuring the optical density of cell suspension at 600nm before and after the filter paper was incubated with the cells.
(cells were suspended in PBST buffer). The actual immobilization phase with the filter paper took 1h at 28 °C (with 100 rev min⁻¹ shaking). Optical measurement was also applied to estimate the robustness of the binding of the cells with filter paper by measuring the optical density from the liquid phase after the immobilized filter paper was washed with pure PBST buffer. In order to take into account the effect of unspecific binding caused by the host strain and the anchor, pLEB124 based control strains were constructed. These control strains carried plasmids otherwise similar to CBD₅₁₅₄(anchor –fusion constructs, but lacked the CBD₅₁₅₄ sequence completely. The unspecific binding measured for these controls were then compared with the corresponding CBD fusion proteins to evaluate whether the binding measured for the CBD₅₁₅₄-anchor –fusions were statistically different from those measured for the control strains.

According to results obtained from the immobilization tests (see Fig. 16), the PrtP 153 aa anchor was not able to secure whole cell binding with the fused CBD₅₁₅₄ -domain. Although an anchor peptide with 90-100 residues in the extended loop (between the target protein and LPXTG box) may already be long enough for surface display (Fischetti et al., 1990; Strauss and Götz, 1996), it seemed that this 153 aa anchor (with over 100 residues in the extended loop) was too short in this case to extend the CBD domain for proper position and distance from the cell surface for the cellulose binding. Instead, the immobilization efficiency with the longer PrtP anchor (PrtP 344 aa) fused with the CBD₅₁₅₄ -domain was significantly improved when compared to the wild type strain. Indeed, the nature of this binding was so strong that the washing loss measured for these cells was very low (only about 1%). Also, the immobilization of the L. lactis cells expressing the CBD-AcmA 242 aa fusion protein was also efficient, although not comparable with that measured for the CBD₅₁₅₄–PrtP 344 aa fusion protein expressing strain. Interestingly, the nature of the binding between the strains expressing CBD-AcmA 242 aa and CBD-PrtP 344 seems to be somewhat different, as the proportion of the washing loss to the final adhesion was quite different between these two strains. However, this observation is in line with that detected in the above described Western result (see section 4.2.2.2). It seems that the nature of binding with AcmA anchor is not as strong as that observed with properly attached PrtP (LPXTG) –anchor. Also, it is possible that this variation is partially dependent on the number of potential anchoring ligands presented in the cell surface. Indeed, instead of uniform distribution on the cell surface, the occurrence of the AcmA on the cell surface is putatively localized as suggested by Steen et al. (2003). When the immobilization efficiency was compared with the host strain (MG1363), only the strains expressing CBD-AcmA 242 aa and CBD-PrtP 344 aa fusion proteins showed statistically significant difference.
Figure 16. Filter paper immobilization tests of *L. lactis* strains. Legends for the bars indicate the fusion protein expressed in MG1363*aemAA1* host. Blue color indicates the final immobilization % of the strains on the filter paper and purple color indicates the calculated washing loss value for each strain. Statistical significance between the strain expressing the CBD-anchor fusion and the corresponding control strain (expressing the anchor only) is marked with one asterisk (*), whereas two asterisks (**) indicates statistical significance between the strain expressing CBD-anchor fusion and the host strain ($P < 0.05$, unpaired double-tailed t-test).

During the development of the immobilization test we noticed that addition of 0.5 % (v/v) of Tween 20 into immobilization solution prevented the nonspecific binding of the lactococcal cells. Also, we noticed that the immobilization efficiency of the strain displaying CBD-AcmA 242 aa fusion protein increased significantly when compared to the control strains (results not shown). Steen et al. (2003) have proposed that LTAs (lipoteichoic acids) may potentially hinder the binding of AcmA in lactococci. As it is known that some nonionic surfactants are able to release cell-bound LTAs (Ohta et al., 2000) and LTAs are potentially more concentrated in lactococci in those areas in which autolysin-anchored proteins are not able to bind, it can be speculated whether the improved binding with the CBD-AcmA 242 aa fusion protein with Tween 20 was a consequence of reduced number of cell-bound LTAs.

As proposed by Habimana et al. (2007), the presence of PrtP on the bacterial surface may increase the cell’s capacity to exchange attractive van der Waals interactions. Thus, as suggested by the authors, bioadhesion of PrtP displaying lactococci is increased to various types of surfaces. In this work, this kind of effect with the PrtP constructs was not observed (see Fig. 16). Indeed, the adhesion with the PrtP-anchor- control strains were at the same level with the host strain. Notably, in this thesis the used PrtP anchors covered
only C-terminal part of the whole PrtP protein, whereas Habimana et al. (2007) used in their study full length PrtP alleles. Notably, Habimana et al. (2007) did not use cellulose in their test, but instead used solid glass and tetrafluoroethylene surfaces with different salt concentrations.

As extensive degradation was detected during the Western analysis of many CBD-fusion proteins, htrA negative Lactococcus-strain was tested as expression host. As reported by Poquet et al. (2000), HtrA is a unique extracellular housekeeping protease in L. lactis, and it is involved in propetide processing, maturation of native proteins and degradation of recombinant proteins. The HtrA negative lactococcus strain L. lactis IL1403 htrA (Poquet et al., 2000) was transformed with pLEB597, and the resulting strain expressing CBDXylA–PrtP 344 aa fusion protein, was designated as LAC257. The expressing of CBDXylA–PrtP 344 aa fusion protein in this strain was compared with LAC248 strain (HtrA positive MG1363 host for pLEB597) with Western analysis. The immunological detection with CBD antibody from rabbit indicated that in HtrA negative host strain the CBD XylA –PrtP 344 aa expressed was less degraded and more of the fusion protein could be found on the cell wall fraction when compared with the HtrA positive host strain carrying the same expression plasmid. Based on these results, it seems most likely that HtrA activity, at least partially, is responsible for the degradation of the CBDXylA–PrtP 344 aa fusion protein in MG1363 based host.

Finally, the effect of heterologous SrtA activity to the whole cell immobilization of L. lactis with cellulosic material was studied. Simply, the immobilization test method described earlier was used to compare the immobilization efficiency of strain expressing CBDXylA–PrtP 344 aa fusion protein in MG1363 (LAC248) with that measured for LAC243 strain, in which the DNA fragment coding this same fusion protein was transcriptionally fused with DNA fragment including the RBS and structural gene of srtA of S. aureus. The result from this one to one comparision indicated that the LAC243 did not gain any substantial advantage from the additional heterologous SrtA activity and the difference in the immobilization efficiency between these two strains was statistically non-significant. Because the production of this heterologues SrtA was hard to verify due to lack of specific antibodies, it remains unanswered whether the potential increase of sortase activity was too low to have an effect on the proportion of cell bound CBD-PrtP/secerted CBD-PrtP, or was this heterologous SrtA incompatible with the machinery attaching LPXTG type cell wall anchor proteins to the cell wall in L. lactis. Also, as the covalent anchoring of cell wall surface exposed protein is composed of a series of steps including protein exporting, retention, sortase cleavage and cell wall linkage by transpeptidation, the effect resulted from boosting only one of step in this process may not lead to substantial difference for the efficiency of the whole process. After the genomic sequencing of L. lactis MG1363 (Wegmann et al., 2007), it was found that the amino acid sequence identity between the sortases of S. aureus and L. lactis is low and even within the sortase domain (PF04203) the identity is just around 30 % (unpublished results). Thus, it is quite probable that the Staphylococcus-originated SrtA is not fully effective when anchoring LPXTG proteins in L. lactis.
5. Conclusions and future prospects

In this work, one of the main targets was to genetically engineer a pure L(+)-lactic acid producer by inactivation of the gene responsible for the synthesis of D-lactate dehydrogenase in *L. helveticus* CNRZ32. This goal was achieved and two different strains capable to produce polylactic acid grade L(+)-lactic acid were constructed. Indeed, polylactic acid (PLA) is one of the most abundant bioplastic grades in the market. For PLA, it has been demonstrated that the enatiomeric purity of lactic acid monomers within the polymeric PLA molecule is directly affecting to the thermostability of the produced plastic.

When profiles of L(+)-lactic acid production and bacterial growth were studied in detail, it was found that the production phase of L-(+)-lactic acid in the mutant strains was prolonged (continued also in stationary phase) when compared to the wild type strain. Furthermore, the first experiments studying the connection between growth and lactic acid production putatively indicated that the L(+)–lactate production and growth are uncoupled in GRL89 strain under the conditions studied. In this strain, the structural gene of *ldhD* was replaced with that of *ldhL*, enabling synthesis of L-LDH enzyme by two different promoters. This putative uncoupling is very interesting observation but needs to be verified with new set of experiments. As a conclusion, the hypothesis set in section 2, suggesting that the L(+)-lactic acid fermentation is more efficient if the both *ldh*-promoters drive the L(+)-lactic acid synthesis, can’t be overruled.

One of the most effective ways to run a continuous fermentation process, in which the product of interest is extracellular, is to use immobilized production strain. Most typically, chemicals and/or expensive modifications are needed to achieve an immobilization system which is capable to provide efficient and robust production of desired metabolite. The other main goal of this work was to develop a *Lactococcus* strain capable to efficient immobilization on unmodified cellulosic materials. In more details, we wanted to achieve efficient immobilization on cellulose without the need of specific pre-treatment procedures, such as the modification of cellulose with DEAE-groups (diethylaminethyl). To reach this goal and to learn to how to achieve a proper surface display, different genetically modified lactic acid bacteria strains with different types of cell wall anchors were constructed and tested. The goal was to study and to be able to confirm (see hypothesis in section 2) proper surface display with various types of cell envelope anchors. This goal was met and the hypothesis confirmed. Clearly, the type of cell envelope anchor, length of the anchor-fusion protein, but also the location of anchoring point within the supporting molecule (in case of SlpA-study), affected the signal level detected in whole-cell ELISA assays.

Although the whole cell immobilization (for industrial fermentations) was not the original focus in the S-layer study, this kind of application for S-layers remains to be interesting
Finally, the whole cell immobilization of *Lactococcus* on cellulosic material was successfully demonstrated with two different types of cell wall anchors (LPXTG and LysM) fused with cellulose binding domain of *C. japonicus*. In these immobilization studies, statistical significant difference to the control strain was measured for two different anchor-fusion proteins, confirming the hypothesis set in section 2. In this work, the purpose was to prove the feasibility of this concept in lactococci. However, more work is needed to develop a robust industrial scale process based on the concept developed in this study. Indeed, this kind of work has already progressed, as surface display anchor constructs developed in this work have been used to develop *L. lactis* strains for continuos nisin production with immobilized cells (Şimşek et al., 2013, Şimşek 2014). In these constructs, chitin binding domain was utilized instead of cellulose binding domain and the length of the LPXTG anchor proteins (PrtP of *L. lactis*) in the fusion construct was further optimized. As reported by Şimşek (2014), the optimized system yielded the highest nisin production ever reported and demonstrated its potential to be used in industrial scale.

As the whole cell immobilization systems developed in this study are plasmid based and the P45 promoter has been tested to be functional in some other LAB, too, the next logical step in the context of this work would be to combine the immobilization capability and the capability to produce either PLA grade D(-)- or L(+) lactic acid within the same LAB strain. Indeed, the current host species for the above mentioned constructs, *L. lactis*, is already filling these conditions as it is known to be a L(+) lactic acid producer. Also, it would be interesting to see how other affinity domains or other functional domains apart from cellulose binding domain and chitin binding domain would work when fused with the anchors constructed in this work, and what kind of new applications these new constructs would facilitate.
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