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Surface display of cellobiose 2-epimerases on lactic acid bacteria for conversion of lactose to epilactose

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Abstract

An increasing body of research elicits the many health benefits imparted by the gut-associated microbiota to their host. These beneficial strains are referred to as probiotic organisms. One way to promote growth and increase activity of probiotic strains is ingestion of prebiotic compounds. Several promising prebiotics are under development, such as epilactose. The methods for epilactose production have previously been prohibitively expensive and wasteful for sufficient production for it to be used as a prebiotic. This is changing, as several cellobiose 2-epimerases (CEases) have been shown to convert lactose into epilactose, making large-scale production of epilactose possible.

The bacterial CEases utilized in this thesis are derived from *Caldicellulosiruptor bescii* (CbCEP) and *Roseburia faecis* (RfCEP). The pSIP system was used to fuse each CEase with a lipoprotein anchor (Lipo) and a LysM domain anchor (LysM), allowing for construction of inducible plasmids for surface-display of the CEases. The plasmids were transformed to *Limosilactobacillus reuteri* and *Lactiplantibacillus plantarum* and the recombinant strains were characterized in enzyme activity assays for their ability to convert lactose to epilactose.

The assay samples underwent High Performance Anion Exchange Chromatography (HPAEC) with Pulsed Amperometric Detection (PAD) for carbohydrate separation and analysis using the ICS-6000. The analysis showed two recombinant *L. reuteri* and all recombinant *L. plantarum* produced epilactose. The *L. plantarum* harboring Lipo-Rf and LysM-Rf for surface-display of RfCEP were selected for more in-depth analysis. The best epilactose producer was *L. plantarum* harboring Lipo-Rf, yielding an epilactose fraction of 30 %, even when bacteria had been frozen for up to 48 hours at -20 °C. *L. plantarum* harboring LysM-Rf yielded epilactose fractions of 16 %, which was reduced to 8 % after freezing, showing how the anchoring strategy affects RfCEPs tolerance to freezing. Attempts to reuse bacteria for multiple reactions proved unsuccessful, as the production of epilactose declined in reactions with recycled cells, proving all strains were unfit for multiple cycles.

The pSIP system was used successfully for surface display of CbCEP and RfCEP in *L. plantarum*, using a lipoprotein- and a LysM domain anchor. The recombinant strains were used as biocatalysts in enzyme activity assays, producing epilactose from lactose.

Sammendrag

En økende mengde forskning peker ut helsegevinster forbundet med den kommensale mikrofloraen i tarmen. De gunstige artene kalles gjerne probiota og en måte å øke deres vekst og aktivitet i tarmen er å innta prebiotiske midler. Flere lovende prebiotika er under utvikling, for eksempel epilaktose. Produksjon av epilaktose har tidligere vært svært kostbar, lite miljøvennlig og for ineffektiv til å produsere tilstrekkelige mengder til bruk som prebiotika. Dette ble endret da det ble oppdaget at flere cellobiose 2-epimeraser (epimeraser) kan konvertere laktose til epilaktose, noe som muliggjør en industriell produksjon av epilaktose.

De bakterielle epimerasene undersøkt i denne oppgaven er isolert fra *Caldicellulosiruptor bescii* (CbCEP) og *Roseburia faecis* (RfCEP). Det plasmidbaserte pSIP systemet ble brukt til å konstruere plasmider hvor hver epimerase ble fusjonert med et lipoprotein-anker (Lipo) og et LysM domene-anker (LysM). Plasmidene ble transformert til *Limosilactobacillus reuteri* og *Lactiplantibacillus plantarum* for å utrykke epimerasene på celleoverflaten.

De rekombinante stammene undersøkt i aktivitets-forsøk for deres evner til å konvertere laktose til epilaktose. Prøver høstet fra aktivitetsforsøk ble analysert ved High Performance Anion Exchange Chromatography (HPAEC) med Pulsed Amperometric Detection (PAD) i en ICS-6000 for å måle innhold av karbohydrater.

Resultatet av analysene viste at to rekombinante *L. reuteri*-stammer og alle rekombinante *L. plantarum*-stammer produserte epilaktose. *L. plantarum* med plasmidene Lipo-Rf og LysM-Rf, som begge ankret RfCEP, ble valgt ut for videre analyser. Den beste produsenten av epilaktose viste seg å være *L. plantarum* med Lipo-Rf, denne stammen produserte en epilaktose-fraksjon på 30 %, selv etter nedfrysning ved -20° i opptil 48 timer. *L. plantarum* med LysM-Rf oppnådde en epilaktose-fraksjon på 16%, men etter nedfrysning sank produksjonsevnen til 8%, noe som viser at ankermetoden påvirker RfCEPs toleranse til nedfrysning. Forsøk der bakteriene ble høstet og gjenbrukt i flere reaksjoner viste tydelig at stammene ikke var egnet til dette formålet.

pSIP-systemet lot seg anvende for overflateankring av CbCEP og RfCEP og de rekombinante stammene ble brukt i biokatalytiske reaksjoner for å konvertere laktose til epilaktose.

Abbreviations

BHI	Brain-heart infusion
bp	Base pair
BSA	Bovine serum albumin
CbCEP	CEase derived from *Caldicellulosiruptor bescii*
CEase	Cellobiose 2-epimerase
CFU	Colony forming units
FITC	Fluorescein isothiocyanate
GIT	Gastrointestinal tract
GMO	Genetically modified organism
GRAS	Generally recognized as safe
HPAEC	High Performance Anion Exchange Chromatography
HPLC	High Performance Liquid Chromatography
HRP	Horseradish peroxidase
ICS-6000	Dionex Ion Chromatography System-6000
LAB	Lactic acid bacteria
Lipo	Lipoprotein anchor (Lp_1261)
LysM	LysM domain anchor (Lp_3014)
MRS	De Man, Rogosa, Sharpe
OD	Optical density
PA	Primary antibody
PAD	Pulsed Amperometric Detection
PCR	Polymerase Chain Reaction
RfCEP	CEase derived from *Roseburia faecis*
SA	Secondary antibody
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TCA	Trichloroacetic acid

Table of contents

1 Introduction 1

1.1 Lactic acid bacteria 1

1.2 Inducible gene expression systems 3

1.3 Secretion and anchoring of heterologous proteins 4

1.4 Enzyme-displaying bacteria as biocatalytic factories 7

1.5 Prebiotics 8

1.6 Cellobiose 2-epimerases 11

1.7 Aim of this study 12

2 Materials and Methods 13

2.1 Equipment and instruments 13

2.2 Chemicals 16

2.3 DNA, proteins, and enzymes 18

2.4 Kits 19

2.5 Primers 20

2.6 Bacterial strains 22

2.7 Plasmids 23

2.8 Buffers, solutions, and gels 24

2.9 Growth media 25

2.10 Cultivation of bacteria 27

2.11 Bacterial glycerol stocks 28

2.12 DNA isolation 28

2.12.1 Plasmid isolation from bacterial culture 28

2.12.2 Determination of DNA concentration 28

2.12.3 Digestion of DNA with restriction enzymes 29

2.12.4 Agarose Gel Electrophoresis 29

2.12.5 Isolation of DNA from agarose gels 30
2.13 Polymerase Chain Reaction - PCR 30
2.13.1 PCR by Q5® Hot Start High Fidelity 2x Master Mix 31
2.13.2 Colony PCR with VWR Red Taq DNA Polymerase Master Mix 33
2.14 Cloning 34
2.14.1 In-Fusion cloning 34
2.14.2 ElectroLigase 35
2.15 Sequencing plasmids and PCR products 36
2.16 Preparation of competent cells 36
2.16.1 Electrocompetent *L. lactis* 36
2.16.2 Electrocompetent *L. plantarum* 36
2.16.3 Electrocompetent *L. reuteri* 37
2.17 Transformation of competent cells 38
2.17.1 Transformation of chemically competent *E. coli* 38
2.17.2 Transformation of electrocompetent bacteria 38
2.18 Sample preparation for gene product analysis 40
2.18.1 Cultivation and harvesting of recombinant bacteria 40
2.19 Growth curve analysis of recombinant bacteria 41
2.20 Cell lysate preparation 41
2.21 Protein precipitation with TCA 42
2.22 Protein gel electrophoresis 43
2.23 Western blot 44
2.23.1 Blotting with iBlot™ Dry Blot System 44
2.23.2 SNAP i.d.® immunodetection 45
2.23.3 Detection of proteins by chemiluminescence 46
2.24 Detection of surface-located proteins 46
2.24.1 Flow cytometry 47

2.25 Determination of colony forming units (CFU) 48
2.26 Enzyme activity assays 48
2.26.1 Setup of enzyme activity assay experiments 48
2.26.2 Sample preparation for analysis by HPAEC 49
2.27 High-Performance Anion Exchange Chromatography 50
2.27.1 Sample analysis with the ICS-6000 51
3 Results 52
3.1 Plasmid construction 52
3.2 Growth curve experiments 57
3.2.1 Selecting strains for enzyme production 57
3.2.2 Growth curves for recombinant strains harboring relevant plasmids 57
3.3 Analysis of epimerase production by Western blot analysis 58
3.4 Detection of surface-anchored proteins by flow cytometry 60
3.5 Enzyme activity assays 61
3.5.1 Standard conditions 61
3.5.2 Initial enzyme activity assays 62
3.5.3 The effect of shaking frequency on epilactose production 66
3.5.4 Time effect on epilactose production 66
3.5.5 The effect of the number of bacteria on substrate conversion 67
3.5.6 Freezing bacteria and its effect on substrate conversion 68
3.6 Reusing bacteria in multiple enzyme activity assays 69
3.6.1 Analysis of *L. plantarum* after enzyme activity assays 70
3.6.2 Western blot of reaction solution 71
3.7 Combined effects of temperature, pH, and time 72
3.8 Quantification and reporting by ICS-6000 75
4 Discussion 76
4.1 Plasmid construction 76

4.2 Growth rates of recombinant strains 77
4.3 CEase expression by Western blot in recombinant strains 77
4.4 Detection of surface anchored CEases in recombinant strains 78
4.5 Enzyme activity assays 79
4.5.1 Epimerase characterization standard condition bias 79
4.6 Enzyme activity assays with recombinant *L. reuteri* 80
4.7 Enzyme activity assays with recombinant *L. plantarum* 81
4.7.1 Freezing and reusing bacteria for multiple cycles 82
4.7.2 Optimization of temperature- and pH conditions for RfCEP 85
4.8 Conclusion and future work 86
5 References 87
6 Appendix i
6.1 Appendix A i
6.2 Appendix B iii

1 Introduction

Lactic acid bacteria comprise a group of Gram-positive bacteria found in a wide range of environments such as on plants, food and in the gastrointestinal tract (GIT). Many of these bacteria have probiotic properties and confer beneficial health effects to the host organism. A diet rich in prebiotic compounds such as fructo- and galacto-oligosaccharides can promote growth of probiotic strains. In addition to diet, prebiotic supplements can be ingested to promote growth of certain probiotic strains. Several new, promising prebiotics are under development, such as epilactose. The chemical production of epilactose has so far been challenging, expensive and wasteful. This changed with the discovery that several cellobiose 2-epimerases (CEase) can produce epilactose by using lactose as a substrate.

Utilizing the pSIP system, the CEases can be fused with anchor proteins that enable surface display of CEases in food-grade recombinant strains. These strains can be used as enzyme-displaying particles in biocatalytic reactions to produce compounds safe for human consumption. An added benefit of surface display is that the enzyme-displaying bacteria may be reused which can reduce the need to add fresh enzyme to catalyze multiple reactions.

1.1 Lactic acid bacteria

Lactic acid bacteria (LAB) are a group of Gram-positive, non-sporulating, non-respiring, aerotolerant cocci- or rod-shaped bacteria. Energy is gained directly through substrate phosphorylation of hexoses, either by homolactic fermentation yielding lactic acid as a byproduct, or by heterolactic fermentation, yielding lactic acid, CO_2, acetic acid and/or ethanol as byproducts (Cocaign-Bousquet et al., 1996; Zaunmuller et al., 2006).

Several LABs, such as *Limosilactobacillus reuteri* and *Lactiplantibacillus plantarum*, were previously grouped in the *Lactobacillus* genus. Recently, the *Lactobacillus*-genus underwent a massive taxonomic reorganization, dividing the genus into 25 new genera grouping each organism based on their phylogenetic position, all under the *Lactobacillales* order (Zheng et al., 2020). The species found in the *Lactobacillales* order are extremely diverse and are found in food products, on plants and animals, particularly on mucosal surfaces and the gastrointestinal tract (GIT).

Many LABs have been used throughout history to preserve food, because the pH of the food is reduced as the bacteria grow and produce lactic acid and other metabolites. The lowered food pH is an important factor that decreases the risk of colonization by pathogenic microorganisms and thereby increases shelf-life (Jans et al., 2016; Singh, 2018). In more recent times, artisanal- and industrial-scale food production use fermentation largely as a method to enhance the flavor and texture of foods and beverages, not as a conservation method (Marco et al., 2017; Park et al., 2014; Shiby & Mishra, 2013).

In addition to the health benefits associated with diets that have a naturally high fiber content, fermented fruits and vegetables can be included in the diet as an efficient way to introduce probiotic strains as well as prebiotic compounds to the gut (Roberfroid et al., 2010; Sanlier et al., 2019). These prebiotic compounds promote growth of probiotic strains, which in turn provide beneficial effect in the gut such as generating short-chained fatty acids as nutrients for colonocytes and aiding the immune system (Holscher, 2017; Slavin, 2013). Medical use of probiotics is limited to treatment of diarrhea and pouchitis (Islam, 2016). However, an increasing number of studies suggest probiotic and prebiotic supplements may increase general health and well-being (Islam, 2016; Pineiro et al., 2008; Roberfroid et al., 2010; Sarao & Arora, 2017).

Lactiplantibacillus plantarum, previously known as *Lactobacillus plantarum*, is one of the best studied species in the *Lactobacillales* order. The genome of *L. plantarum* was sequenced in 2003, revealing a large variety of proteases, transport- and regulatory proteins, including a high number of extracellular proteins (Kleerebezem et al., 2003). The higher-than-average variety of expressible proteins can explain how *L. plantarum* is able to adapt and remain flexible in a wide variety of environments, as it is frequently found in food, plants, and the GIT (Boekhorst et al., 2006; Kleerebezem et al., 2003). *L. plantarum* is one of a handful species in the *Lactobacillales* order that has the ‘Generally Recognized As Safe’ (GRAS) status (Burdock & Carabin, 2004). *L. plantarum* is used in food production, industrial fermentations (Rodriguez et al., 2009), and has been investigated as a potential delivery vector for vaccines (Fredriksen et al., 2012; Kuczkowska et al., 2019a).

Limosilactobacillus reuteri, previously known as *Lactobacillus reuteri* has a ubiquitous presence in a variety of environments and is often found in meat- and dairy products. *L. reuteri* can colonize different body sites of vertebrates, and is considered a probiotic bacteria

of the GIT in humans (Mu et al., 2018). *L. reuteri* is not considered pathogenic, has a GRAS status, and is used in food production for making sourdough bread (Zheng et al., 2015). Research suggests that *L. reuteri* impart many health benefits in the GIT, such as inhibiting colonization by pathogenic bacteria, strengthening the intestinal barrier and boosting the hosts immune system (Mu et al., 2018).

1.2 Inducible gene expression systems

Inducible gene expression systems enable controlled expression of heterologous genes. The gene expression in these systems are initiated by extraneous addition of a chemical inducer or change in a specific physical parameter such as pH or temperature (Diep et al., 2009). The pSIP expression system (Sørvig et al., 2003; Sørvig et al., 2005) (Figure 1) is based on the pheromone-like class II bacteriocins sakacin A and sakacin P, which requires an inducer peptide to be present in order to start the gene expression (Sørvig et al., 2005).

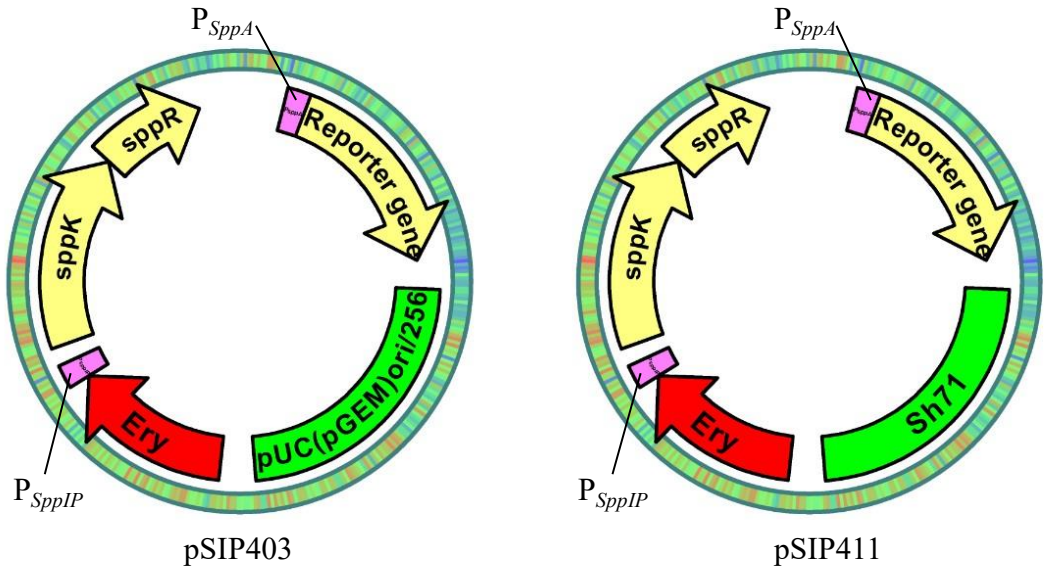


Figure 1. Representation of vectors pSIP403 and pSIP411. P_{SppA}: Inducible promotor; Reporter gene: cloning site for heterologous gene; pUC(pGEM)ori/256: Replicons in pSIP403 vector; Sh71: Replicon in pSIP411 vector; Ery: Erythromycin resistance marker; P_{SppIP}: inducible promotor; sppK: Histidine protein kinase; sppR: response regulator.

The pSIP vector components are assembled using cassettes with multiple restriction sites that simplify exchange of vector components by using restriction digestion and ligation (Sørvig et al., 2003). The primary genes composing the pSIP vectors are the gene *sppK*, encoding a

histidine protein kinase (HK), the gene *sppR*, encoding a response regulator protein (RR), and the *sppIP* promoter, controlling expression of these genes. The original gene operon also contained the s*ppIP* gene, encoding the inducer peptide (SppIP) itself. In the pSIP system, the s*ppIP* gene is deleted to allow for exogenous control of expression of the target proteins. Exogenous expression of the target genes is achieved by adding exogenous SppIP. Inducing the system leads to phosphorylation of the HK protein which transfers a phosphate group to the RR protein. The phosphorylated RR protein binds to the inducible promotors P_{SppA} and P_{sppIP} (Figure 1). Binding of the RR protein to the inducible promotors results in expression of the target genes, as well as production of more HK and RR proteins, also resulting in a massive production of the target protein encoded by the Reporter gene (Figure 1).

The pSIP system has received several improvements and modifications since it's conception to optimize expression and surface display of heterologous proteins (Halbmayr et al., 2008; Mathiesen et al., 2020; Sørvig et al., 2005). Figure 1 shows the pSIP403 version with replicon pUC(pGEM)ori, for replicating in *Escherichia coli* as well as the 256-replicon for replicating in *L. plantarum* and *Latilactobacillus sakei*. Figure 1 also shows the pSIP411 version with the Sh71-replicon which replicates in a broad range of hosts, but necessitates sub-cloning in *Lactococcus lactis,* as it cannot replicate in *E. coli*.

1.3 Secretion and anchoring of heterologous proteins

The cell membranes of Gram-positive bacteria are covered by a thick peptidoglycan cell wall. Secretion and anchoring of proteins is simpler in Gram-positive bacteria than in Gram-negative bacteria, as the proteins only need to cross one membrane before they can be released or anchored on the bacterial surface (van Roosmalen et al., 2004). Most secreted proteins follow the Sec pathway (Anne et al., 2017; Schneewind & Missiakas, 2014). Proteins destined for secretion by the Sec pathway are synthesized with an N-terminal signal sequence that enable chaperons to guide them to membrane-bound channel proteins where the proteins are translocated. As the proteins are translocated, they are digested by a Signal Peptidase, and depending on the N-terminal signal sequence in the proteins, they are either released to the surrounding environment, or anchored to the surface.

Several mechanisms are used by Gram-positive bacteria for anchoring proteins to their outer surfaces. Figure 2 shows four protein classes which are utilized as anchor-mechanisms for surface display of heterologous proteins and illustrates how the anchors offer varying degrees of exposure and protection for surface-displayed proteins, as they anchor at different locations on the bacterial surface.

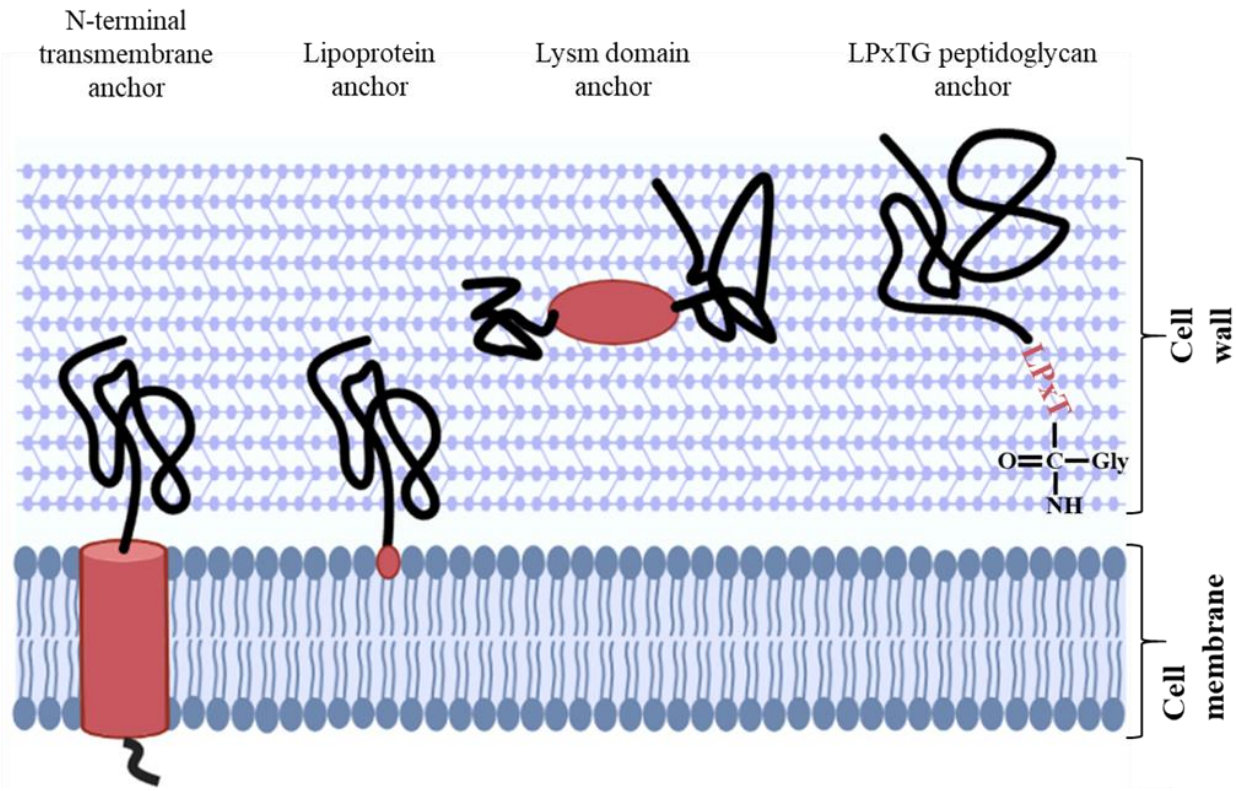


Figure 2. Schematic Overview of various anchoring mechanisms in Gram-positive bacteria. The anchor mechanisms are based on either covalent or non-covalent binding to the cell wall or the phospholipid membrane. Red color indicates the binding motifs and domains that are used to anchor the displayed proteins, indicated in black. Created with BioRender.com.

Many proteins destined for secretion have a signal peptidase cleavage site. This site is cleaved by signal peptidase, resulting in secretion of the protein. If this site is absent, a central stretch of hydrophobic amino acid residues in the protein will act as an N-terminal transmembrane helix, anchoring the protein in the membrane (Michon et al., 2016).

Lipoproteins have the lipobox motif, L-X-X-C, in the C-terminal part of their signal peptide. Following secretion by the Sec pathway, the enzyme diacylglycerol transferase couples the cysteine (C) in the lipobox motif to a membrane phospholipid by transfer of a diacyl-group from the phospholipid to the SH-group on the cysteine (Braun & Hantke, 2019). This is

followed by cleaving of the signal peptide by a lipobox-specific signal peptidase, SPase II, forming the N-terminal part of the lipoprotein covalently joined to the cell membrane. Lipoprotein anchors have been used for surface display of antigens on *L. plantarum* in development of mucosal vaccines (Fredriksen et al., 2012; Kuczkowska et al., 2019a). The *L. plantarum* derived lipoprotein Lp_1261 has been used for surface display of a heterologous beta-mannanase and a chitosanase on recombinant *L. plantarum*, as a step towards developing whole-cell biocatalysts for prebiotic production (Nguyen et al., 2016).

The LPxTG-like proteins are named from their conserved sequence motif in their C-terminal region. The LPxTG sequence is followed by hydrophobic amino acids and a short positively charged tail. The N-terminal portion of the protein carries the signal sequence, ensuring export through the Sec pathway (Fischetti, 2019). After translocation by the Sec system, the LPxTG motif is cleaved by a transpeptidase sortase enzyme between the Thr and Gly residues. The cleaving results in covalently binding of the protein to the peptidoglycan cell wall by its threonine carboxyl group (Michon et al., 2016). By this mechanism, proteins are anchored by their C-terminal, with the N-terminal protruding from the anchor point. Covalent cell wall anchors have been used to surface display beta-mannanase and chitosanase on recombinant *L. plantarum* (Nguyen et al., 2016). The results presented by Nguyen et al., showed their enzyme-displaying strains could be reused in four separate reactions without losing more than ~30% enzyme activity in surviving cells.

Non-covalent cell wall protein anchors, use non-covalent interactions to attach proteins to the cell wall. The proteins interact directly with surface proteins or with surface layer homology domains, such as choline binding domains, SH3 domains and LysM domains (Michon et al., 2016). The lysine motif domain, LysM, is often located in the N- or C-terminal of a protein and can be used to anchor heterologous proteins non-covalently to the peptidoglycan cell wall (Visweswaran et al., 2014). The anchor is believed to interact strongly with the N-acetylglucosamine (NAG) monomers of the peptidoglycan (Buist et al., 2008). The LysM domain anchor Lp_3014 (LysM), derived from an extracellular transglycosylase from *L. plantarum* has previously been used for surface display of two beta-galactosidases in recombinant strains for conversion of lactose in production of galacto-oligosaccharides (Pham et al., 2019). Pham et al., showed their recombinant *L. plantarum* could be reused in up to four separate biocatalytic reactions for production of prebiotics without drastic loss of catalytic ability.

1.4 Enzyme-displaying bacteria as biocatalytic factories

Enzyme-display on the surface of bacteria enable bacteria to be used as biocatalytic factories in large-scale production of specific compounds. This strategy has several potential advantages compared to using the free form of the enzyme for biocatalytic production.

Surface-displayed enzymes and enzymes in their free form will interact with their specific substrate in a solution, converting the substrates into product. Using the free form of the enzyme is generally considered an efficient method but it is also wasteful as the added enzyme usually cannot be easily recollected and reused (Robinson, 2015). In contrast, enzymes anchored to the surface of bacteria can be separated from the solution simply by centrifugation or filtration (Schuurmann et al., 2014). Another advantage of using bacteria as biocatalytic factories is the added protection provided by the cell wall, partially hiding the enzyme in the cell wall which may reduce their risk of proteolytic degradation (Guoyan et al., 2019). However, if the enzymes are too deeply embedded in the cell wall, their catalytic sites may be blocked, lowering the overall activity and crippling production.

Previously, *E. coli* was the default choice for heterologous protein expression. However, *E. coli* does not have the GRAS status and is a diderm bacteria, which limits its use concerning creation of food-grade compounds and complicates surface expression. In later years, an increasing interest has been given to use recombinant lactic acid bacteria in biocatalytic reactions . Many lactic acid bacteria, such as *L. plantarum*, have the GRAS status and can be utilized with less restrictions, which is one reason this species may prove suitable for surface-display of enzymes and production of food-grade compounds such as prebiotics (Sewalt et al., 2016).

Studies using *L. plantarum* for surface-displaying cellulosomal complexes showed promising results in biocatalytic reactions where they were used to break down lignocellulosic biomass (Ben-David et al., 2019; Stern et al., 2018). Another study used *L. plantarum* for surface display of a β-mannanase and a chitosanase, fusing the enzymes with a lipoprotein anchor (Lp_1261) and a covalent cell wall anchor (Nguyen et al., 2016). The *L. plantarum* strains used for surface-display of the fusion proteins created by Nguyen et al., produced manno- and chito-oligosaccharides, respectively, proving they could be used for production of prebiotic compounds. A study used the non-covalent LysM domain anchor (Lp_3014) to create a fusion

protein with two different β-galactosidases (Pham et al., 2019). The fusion protein was successfully surface-displayed on the surfaces of several LAB species and used in multiple rounds of reactions with lactose as substrate to produce galacto-oligosaccharides.

Most countries have stringent laws that prohibits use of GMOs in food production (Bruetschy, 2019). Depending on regulations, methods can be developed to circumvent prohibitive legislation. For example, fusion proteins can be created by splicing an enzyme and a non-covalent cell wall anchor protein such as a LysM-domain anchor. This fusion protein can be expressed in large amounts by recombinant *E. coli*. These fusion proteins can be separated from *E. coli* and attached to lactic acid bacteria with a GRAS status, such as *L. plantarum.* The *L. plantarum*, now surface-displaying the fusion protein, can be utilized in biocatalytic production of valuable compounds or nutrients intended for human consumption (Visweswaran et al., 2014).

1.5 Prebiotics

Prebiotics are compounds that induces growth and activity of beneficial microbes, most commonly beneficial species residing in the GIT (Pineiro et al., 2008; Sarao & Arora, 2017). Prebiotics must not be confused with probiotics, which are live microorganisms conferring health benefits to their host (Murakami et al., 2015; Pineiro et al., 2008; Sanlier et al., 2019; Sarao & Arora, 2017). Many probiotic species are found in the genera *Bifidobacterium*, *Bacteroides* and *Eubacterium*, including many members of the recently re-organized *Lactobacillales* order (Sarao & Arora, 2017; Zheng et al., 2020). Probiotic bacteria serve multiple functions in the GIT, such as producing short chained fatty acids for colonocytes, improving digestion, modulating the immune system and help fight infections of the GIT (Holscher, 2017; Islam, 2016; Sarao & Arora, 2017).

In recent years the interest in prebiotic compounds have increased, as they may improve health and well-being and may also be utilized in disease treatments (Guarino et al., 2020). The two most important prebiotic groups are the galacto- and fructo-oligosaccharides, naturally found in certain foods (Davani-Davari et al., 2019). Modern diets are considered to contain insufficient amounts of prebiotics to induce their desired health benefits (Holscher, 2017; Singh et al., 2017). This has spurred research into development of efficient production

methods for promising prebiotics, many using lactose as a substrate. Lactose (β-D-Galactopyranosyl-(1→4)-D-glucose) is a common disaccharide derived from the condensation of galactose and glucose, forming a β-1→4 glycosidic bond. Lactose is not a prebiotic but is used as a substrate in enzymatic reactions for production of other valuable prebiotics, such as lactulose and epilactose (Xiao et al., 2019).

Lactulose (4-O-β-D-Galactosyl-D-fructose) is a non-digestible isomer of lactose classified as a prebiotic and is used in the medical field for treating ailments such as constipation and hepatic encephalopathy (Mukherjee & John, 2021; Schumann, 2002). Lactulose is generally produced by alkaline chemical isomerization of lactose (Figure 3) (Seo et al., 2016), with more advanced methods, such as electroisomerization of lactose being developed (Karim & Aider, 2020b). These chemical production methods require substantial resources and produce much waste. A potentially superior method of lactulose production may have been discovered, as several CEases have been used in biocatalytic reactions for production of lactulose (Karim & Aider, 2020a; Rentschler et al., 2015).

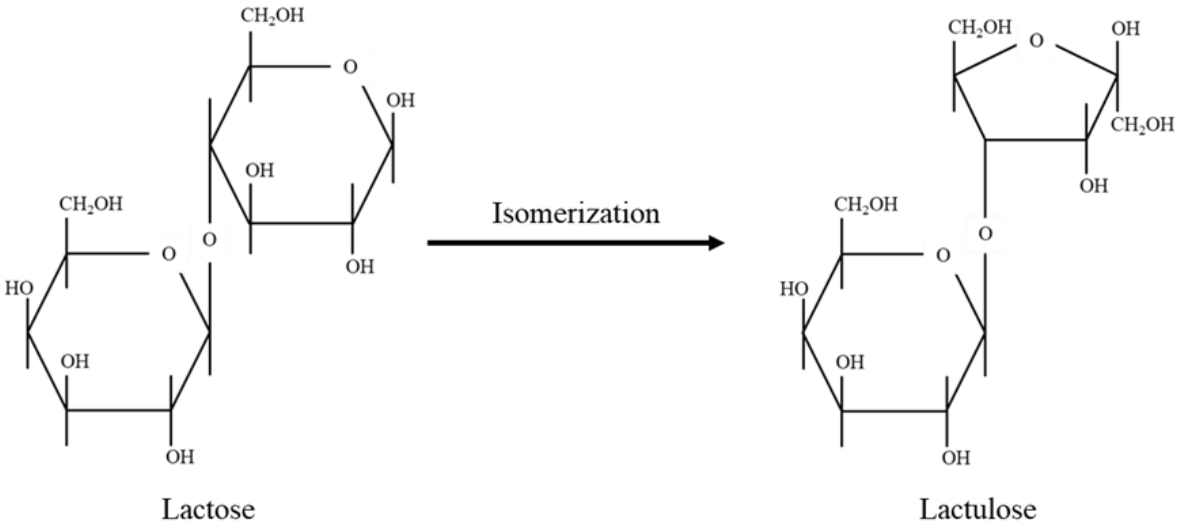


Figure 3. Schematic showing isomerization of lactose to lactulose.

Epilactose (4-O-β-D-galactopyranosyl-D-mannose) is a non-digestible epimer of lactose and classified as a prebiotic. Epilactose has shown powerful prebiotic effects in animal trials, were ingestion of epilactose increased the number of beneficial members from the *Lactobacillales* order and the *Bifidobacterium* genus (Seki & Saito, 2012), as well as having an inhibitory effect in secondary bile acid formation (Watanabe et al., 2008). Additionally, animal experiments showed ingestion of epilactose increased intestinal calcium absorption and

lowered plasma total cholesterol, implying epilactose may reduce the risk of atherosclerosis, and could potentially be useful for weight loss treatments (Mu et al., 2013; Murakami et al., 2015; Nishimukai et al., 2008; Watanabe et al., 2008).

Epilactose has until recently been produced by expensive and wasteful processes requiring use of harmful chemicals, yielding low amounts of epilactose requiring extensive purification (Moreno et al., 2003; Mu et al., 2013; Olano et al., 1989) . Recently, several CEases have been used for biocatalytic conversion of lactose in milk or whey filtrate for production of valuable potential prebiotics, such as lactulose and epilactose (Xiao et al., 2019). These CEases epimerize lactose to epilactose (Figure 4) and prove to be a superior method of production (Jameson et al., 2021; Krewinkel et al., 2015; Wang et al., 2021).

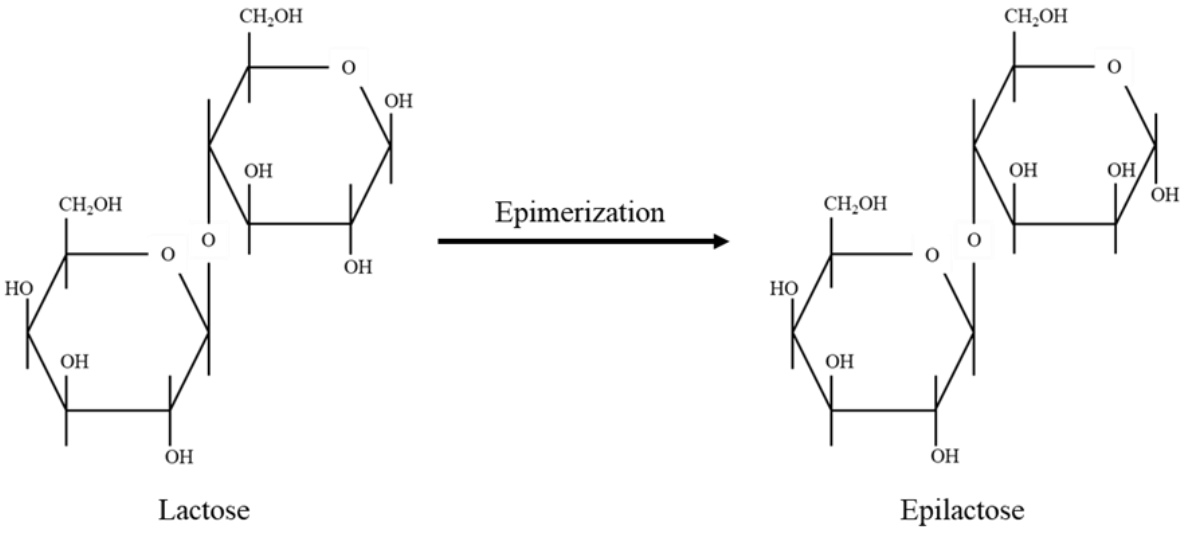


Figure 4. Schematic showing epimerization of lactose to epilactose.

Epilactose can be produced in large quantities by enzymatic catalysis, potentially providing a new source of revenue for the dairy industry (Krewinkel et al., 2014; Krewinkel et al., 2015). This could lead to development of dairy products enriched with epilactose, as well as mass production and isolation of epilactose for use as a food additive, on par with the established prebiotic fructo-oligosaccharides (FOS). Food manufacturers use FOS as sweeteners and for their prebiotic properties, as they promote growth of probiotic bacteria in the lower GIT (Slavin, 2013).

1.6 Cellobiose 2-epimerases

Carbohydrate isomerases and epimerases are crucial enzymes involved in carbohydrate metabolism. Cellobiose 2-epimerases (CEases) belong to a large family of isomerases that catalyze epimerization of cellobiose, specifically they target the reducing end of D-glucose in β-(1-4)-linked disaccharides, converting them into D-mannose (Figure 3) (Saburi, 2016).

Most CEases exclusively catalyze epimerization reactions. Certain CEases can also catalyze isomerization reactions (Figure 4). In reactions using lactose as a substrate, these CEases, will produce both epilactose and lactulose, by epimerization and isomerization respectively (Saburi, 2016). The pH optimum for most CEases is close to neutral, while the temperature optimum ranges based on growth temperature favored by the source organism. CEases have been isolated from both anaerobe and aerobe microorganisms, though most CEases are derived from thermophilic strains such as *Caldicellulosiruptor saccharolyticus*, *Caldicellulosiruptor bescii* and *Rhodothermus marinus* (Jameson et al., 2021; Yang et al., 2010). The CEase isolated from *C. saccharolyticus* has been used for conversion of lactose to epilactose in milk at 8 °C, a temperature considered industrially relevant for milk processing (Rentschler et al., 2015). Promising CEases derived from mesophilic bacteria include the CEases from *Treponema brennaborense* (Chen et al., 2021) and *Roseburia faecis* (Jameson et al., 2021).

Two bacterial CEases are investigated in this thesis. The CEase derived from *Caldicellulosiruptor bescii* is named CbCEP. *C. bescii* is a thermophilic, cellulolytic, anaerobic bacteria originally isolated from geothermally heated freshwater pools (Yang et al., 2010). CbCEP has exceptional pH- and temperature stability, and is able to produce both epilactose and lactulose by adjusting the temperature of the reaction (Jameson et al., 2021). The CEase derived from *Roseburia faecis* is named RfCEP and converts lactose to epilactose (Jameson et al., 2021). *R. faecis* is a mesophilic, Gram-negative (or Gram-variable), anaerobic rod-shaped bacteria originally isolated from human feces (Duncan et al., 2006).

1.7 Aim of this study

The aim of this study was to use an inducible plasmid expression system to construct vectors for surface display of cellobiose 2-epimerases in recombinant strains. These strains were characterized in enzyme activity assays for their ability to produce epilactose using lactose as a substrate.

The selected cellobiose 2-epimerases were derived from *Caldicellulosiruptor bescii* (CbCEP) and *Roseburia faecis* (RfCEP). The cellobiose 2-epimerases was cloned in the pSIP system and fused with a covalent lipoprotein anchor, a non-covalent cell wall anchor and a covalent cell wall anchor, all derived from *L. plantarum*. The resulting plasmids were transformed to *L. plantarum* WCFS1 and *L. reuteri* DMS20016. The recombinant strains were investigated in enzyme activity assays for their ability to produce epilactose with lactose as a substrate. The samples collected from the assays were analyzed by High Performance Anion-Exchange Chromatography and Pulsed Amperometric Detection to measure the fractional content of epilactose and lactose. The most promising strains were selected for more in-depth characterization to ascertain their potential use as enzyme-displaying biocatalytic factories for epilactose production.

2 Materials and Methods

2.1 Equipment and instruments

Laboratory equipment	**Supplier**
96-well plate	Thermo Scientific
Acid washed glass beads	Sigma
Borosilicate glassware	VWR
Cellstar® tubes, 15 and 50 mL	Greiner bio-one
Cryotube, 1.5 mL	Sarstedt
Electroporation cuvette, Gene Pulser®, 0.2 cm	Bio-Rad
Eppendorf tube, 1.5 and 2.0 mL	Axygen
Falcon 2059 polypropylene, round bottom tube, 14 mL	Becton Dickinson
FastPrep® tubes and lids	Fisher Scientific
Filter tips for pipette, 1000 µL	Sarstedt
Micro test plate 96 well, sterile	Sarstedt
Multiscreen® 0.45 µm hydrophilic, Low Protein Binding Durapore® Membrane	Millipore
Next Generation Tip Refill, 10, 100 and 1000 µL	VWR
Nitrile gloves, large	VWR
Parafilm	CURWOOD
PCR strips, 0.2 mL	Axygen
Petri dishes	Heger
Plastic beakers, 50 to 500 mL	VWR
Plastic cuvettes, 1.5 mL	Brand
Pre-sterile pipette tips, 100 µL	Thermo Scientific
Sealing tape	Thermo Scientific
Serological pipettes; 5, 10 and 25 mL	Sarstedt
Slide glasses and cover slips	Thermo Scientific
Steritop® 45mm Neck Size Millipore Express® PLUS 0.22 µm PES, 250mL	Millipore®
Syringes, 5.0 to 50.0 mL	Plastipac

Volumetric cylinders, 10 to 100 mL	VWR
Volumetric flasks, 10 to 100 mL	Duran

Laboratory instruments and tools	**Supplier**
Centrifuges	
Allegra X-30R Centrifuge	Beckman Coulter™
Centrifuge 5418 R	Eppendorf
Galaxy 14D	VWR
Heraeus Pico™ 21 microcentrifuge	Thermo Scientific
Mini Star Silverline	VWR
Dionex ICS-6000 DP	Thermo Scientific
Analytical column:	
Dionex CarboPac™ PA210-Fast-4µm, 150 x 2 mm	
Dionex™ ICS-6000 w/ EGC and PAD detection	
Guard column:	
Dionex CarboPac™ PA210, 30 x 2 mm	
Electrophoresis chambers	
PowerPac Basic	Bio-Rad
PowerPac 300	Bio-Rad
Electroporation devices	
GenePulser® II	Bio-Rad
Pulse Controller Plus	Bio-Rad
Incubators	
Innova® 44 Incubator Shaker Series	New Brunswick Scientific
Termaks Incubator	Termaks
Other instruments	
913 pH Meter	Metrohm
Bransonic® Ultrasonic	Sigma-Aldrich
CertoClav Sterilizer	CertoClav
FastPrep® - 24 Tissue and Cell Homogenizer	MP Biomedicals
Multiskan™ FC Microplate photometer	ThermoFisher

PCR-machines	
SimpliAmp Thermal Cycler	Applied Biosystems
SensoQuest Labcycler	SensoQuest
Mastercycler gradient	Eppendorf
Photo- and optical equipment	
Azure c400	Azure Biosystems
Gel Doc™ EZ Imager	Bio-Rad
QuBit™ Fluorometer	Invitrogen
Transilluminator, UV-light source	VWR
Ultrospec 10 Cell Density Meter	BioChrom US
Pipettes	
Finnpipette F1 Multichannel pipette	Thermo Scientific
Finnpipette® F2	Thermo Scientific
Pipetboy comfort	Integra
Scales	
Sartorius	VWR International
Shaking or mixing devices	
IKA® MS 3 basic	IKA
IKA® RCT classic	IKA
ThermoMixer® C, w/ ThermoTop	Eppendorf
Sterile benches and fume hoods	
AV-100	Telstar
SAFE 2020	VWR
Vacuum devices	
Multiscreen® Vacuum Manifold	Millipore
VCP 80 vacuum pump	VWR
Water and ice systems	
Milli-Q Direct 16 – Automatic Sanitization Module	Millipore
Water baths	
Isotemp® GPD 05	Fisher Scientific
SBB Aqua 5 Plus	Grant
Water bath	Julabo

Software	Supplier
AzureSpot Analysis	Azure Biosystems
FlowJo	Flowjo
ImageLab	Bio-Rad
pDraw32	Acaclone
Photoshop CC	Adobe Systems
Office 365	Microsoft
SKANIT Software 2.5.1	Thermo Scientific
CLC DNA Main Workbench 7	Qiagen
Jamovi 2.0.0	Jamovi.org

2.2 Chemicals

Chemicals	Supplier
Acetone	Merck
Agar powder	VWR
Ammonium citrate dibasic	VWR
Ammonium citrate tribasic	VWR
Ascorbic acid	Sigma
Bacto™ Yeast Extract	Gibco
Brain Heart Infusion (BHI)	Oxoid
Cysteine-HCl	Sigma-Aldrich
De Man, Rogosa, Sharpe (MRS)	Oxoid
D-Glucose, anhydrous	VWR
Epilactose	Sigma-Aldrich
Erythromycin	Sigma-Aldrich
Ethanol	Sigma-Aldrich
Glycerol	Merck
Glycine	Duchefa Biochemie
Hydrochloric acid (HCl)	Sigma
Kanamycin	Sigma-Aldrich
Lab-Lemco powder	Oxoid

Lactose monohydrate	VWR
Lactulose	Sigma-Aldrich
M17	Oxoid
Magnesium sulphate heptahydrate, $MgSO_4$ x 7x dH_2O	Sigma-Aldrich
Manganese sulphate tetrahydrate, $MnSO_4$ x 4x dH_2O	Sigma-Aldrich
Mannose	Sigma
Meat extract	Fluka
MOPS, sodium salt (3-[N-Morpholino]propanesulfonic acid)	Sigma-Aldrich
MRS	Oxoid
Peptone from meat, bacteriological	Sigma-Adrich
Polyethylene glycol, PEG_{1450}	Aldrich
Polyethylene glycol, PEG_{1500}	Aldrich
Potassium chloride, KCl	Merck
Potassium phosphate dibasic, K_2HPO_4	Alfa Aesar
Potassium phosphate monobasic, KH_2PO_4	VWR
S.O.C. medium	Invitrogen
SeaKem® LE Agarose	Lonza
Sodium acetate, anhydrous	Sigma-Aldrich
Sodium acetate, trihydrate	Sigma-Aldrich
Sodium chloride	VWR
Sodium deoxycholate	Sigma-Aldrich
Sodium dihydrogen phosphate, NaH_2PO_4	Merck
Sodium hydroxide	Sigma
Sodium monohydrogen phosphate, Na_2HPO_4	Merck
Sucrose	Sigma-Aldrich
Trichloroacetic acid (TCA)	Sigma
Tris-acetate-EDTA buffer, 50X (TAE)	Thermo Scientific
Tris-Glycine-SDS buffer, 10X (TGS)	Bio-Rad
Trypticase peptone	Oxoid
Tryptone	Oxoid

Tryptose	Oxoid
Tween-20	Sigma-Aldrich
Tween-80	Sigma-Aldrich

2.3 DNA, proteins, and enzymes

Components	Supplier
Antibodies	
Anti-Myc, Mouse Monoclonal IgG	Invitrogen
Anti-Mouse IgG, FITC Polyclonal	Sigma
Anti-Mouse IgG, HRP Polyclonal	Sigma
Ligation/Cloning enzymes and buffers	
5X In-Fusion HD Enzyme Premix	Takara Bio
ElectroLigase®	NEB
ElectroLigase® Reaction Buffer	NEB
Polymerase Master Mix	
Q5® Hot Start High-Fidelity	NEB
DNA Polymerase Master Mix	
VWR Red Taq	VWR
DNA Polymerase Master Mix	
Protein Standard	
MagicMark® XP Western Protein Standard	Invitrogen
Restriction enzymes and buffers	
HindIII	NEB
MLuI	NEB
MLuI-HF®	NEB
NEB Buffer 2.1 (10x buffer)	NEB
NEB Buffer 3.1 (10x buffer)	NEB
SalI	NEB
SalI-HF®	NEB
Various components	
Bovine Serum Albumin (BSA)	Sigma-Aldrich

GeneRuler™ 1 kb DNA ladder	Thermo Scientific
Inducer Peptide, SppIP	Caslo
DNA Gel Loading Dye, 6X	NEB
Lysozyme	Sigma
PeqGreen	Peqlab

2.4 Kits

All kits and devices were used according to the manufacturer's instructions unless otherwise stated.

Kits	**Components**	**Supplier**
iBlot® Dry Blotting system		Invitrogen
	iBlot® Gel Transfer Device	
	iBlot® Gel Transfer Stack, regular	
	iBlot® Cathode Stack, top and bottom	
	iBlot® Disposable sponge	
	iBlot® Filter paper	
	Blotting roller	
NucleoSpin® Gel and PCR Clean-up		Macherey-Nagel
	Binding Buffer, NTI	
	Collection tubes, 2 mL	
	Elution Buffer, NE	
	NucleoSpin® Gel and PCR Clean-up columns	
	Wash Buffer, NT3	
NucleoSpin® Plasmid Kit		Macherey-Nagel
	Buffers A1, A2, A3 and A4.	
	Collection tubes, 2 mL	
	Nucleospin® Plasmid/Plasmid (NoLid) column	
Novex® NuPAGE® SDS-PAGE Gel system		Invitrogen
	NuPAGE® LDS Sample Buffer (4x)	
	NuPAGE® Sample Reducing Agent	

	Mini-PROTEAN® TGX™ Precast Gels, 15 wells	Bio-Rad
	Mini-PROTEAN Tetra Cell	Bio-Rad
Qubit™ dsDNA BR Assay		Thermo Scientific
	Qubit™ dsDNA BR Reagent	
	Qubit™ dsDNA BR Buffer	
	Qubit™ dsDNA BR Standard #1	
	Qubit™ dsDNA BR Standard #2	
	Qubit™ Assay tubes	
SNAP i.d. Protein Detection System		Millipore
	SNAP i.d.® Single Well Blot holder	
	SNAP i.d.® Spacer	
	Filter paper	
SuperSignal® West Pico Chemiluminescent Substrate		Thermo Scientific
	Luminol/enhancer	
	Stable Peroxide Buffer	

2.5 Primers

All primers used for PCR and sequencing is listed in Table 1. The In-Fusion primers were designed according to the manufacturer's specifications.

Table 1. Primers used for PCR amplification and DNA sequencing.

Name	Sequence	Description
CBCep_2F	CAACGTCGACATCACCAAGTTT AAAGAGGATCTGA	Additional sequencing primer for CbCEP.
CbCEP_F	GATTGCGGCGGTCGACATCACC AAGTTTAAAGAGGAT	In-Fusion primer for amplification of CbCEP.
CbCEP_R	CTGTAATTTGAAGCTTTTACAGA TCCTCTTCTGAGATGAGTTTTTG TTCGCCCACACGTTTAATGATT	In-Fusion primer for amplification of CbCEP, adds Myc-tag.
CbCEP_Sek2F	GCGTTTGGTATCTATGGCC	Additional sequencing primer for CbCEP.

CbCEP-cwa2_F	GGCCTCCAAGGTCGACGAACAAAACTCATCTCAGAAGAGGATCTGGACATCACCAAGTTTAAAGAGGA	In-Fusion primer for amplification of CbCEP with cwa2-anchor, adds Myc-tag.
CbCEP-cwa2_R	TCCTGGTTCAGTGACACGCGTGCCCACACGTTTAATGATTTC	In-Fusion primer for amplification of CbCEP with cwa2-anchor.
CbCEP-cwa2-step2	TCATTAAACGTGTGGGCACGCGTGTCACTGAACCA	Second step in process for cloning fragment myc-CbCEP-cwa2 into Sh71-containing plasmid.
CbCEP-sekvensering_R	TCCTTATCCCAGAACTTGTTCT	Reverse sequencing primer binds CbCEP.
RfCep_2F	GCATGTCGACAACAAGAGCATGATGAAAGAGGAA	Additional sequencing primer for RfCEP.
RfCEP_F	GATTGCGGCGGTCGACAACAAGAGCATGATGAAA	In-Fusion primer for amplification of RfCEP.
RfCEP_R	CTGTAATTTGAAGCTTTTACAGATCCTCTTCTGAGATGAGTTTTTGTTCCAGACGACGAATGATTTCCAT	In-Fusion primer for amplification of RfCEP, adds Myc-tag.
RfCEP_Sek2F	CGGAAAAAGGCTGCATCC	Additional sequencing primer for RfCEP.
RfCEP-cwa2_F	GGCCTCCAAGGTCGACGAACAAAACTCATCTCAGAAGAGGATCTGAACAAGAGCATGATGAAAGAGGAA	In-Fusion primer for amplification of RfCEP with cwa2-anchor, adds Myc-tag.
RfCEP-cwa2_R	TCCTGGTTCAGTGACACGCGTCAGACGACGAATGATTTCCAT	In-Fusion primer for amplification of RfCEP with cwa2-anchor.
RfCEP-cwa2-step2	TGGAAATCATTCGTCGTCTGACGCGTGTCACTGAACCA	Second step in process for cloning fragment myc-RfCEP-

		cwa2 into Sh71-containing plasmid.
RfCEP-sekvensering_R	TAGGTGTGTTTGGTGGTATC	Reverse sequencing primer, binds RfCEP.
Sek_cwa2_R	CCTGGTTCATCCGGTTTACTT	Reverse sequencing primer, binds cwa2.
SekCbCEP_F	GTGGTTGGCTTCTTTAACGC	Additional sequencing primer for CbCEP.
sekF	GGCTTTTATAATATGAGATAATGCCGAC	Forward sequencing primers for pSIP vectors.
sekR	CCTTATGGGATTTATCTTCCTTATTCTC	Reverse sequencing primer for pSIP vectors.
SekRfCEP_F	TTATCAAGGAACACGTGATTGAC	Additional sequencing primer for RfCEP.

2.6 Bacterial strains

All bacterial species used in thesis work is presented in Table 2.

Table 2. Bacterial species used in present study

Bacterial strain	**Source**
Escherichia coli HST08 (Stellar Competent Cells)	Clontech
Escherichia coli TOP10	Invitrogen
Lactiplantibacillus plantarum WCFS1	(Kleerebezem et al., 2003)
Lactococcus lactis IL1403	(Bolotin et al., 2001)
Limosilactobacillus reuteri DSM20016	DSMZ

2.7 Plasmids

Plasmids used in this study are shown in Table 3.

Table 3. Plasmids used in this study

Plasmid name (abbreviation)	Description	Source
pET-28a(+)CbCEP	Plasmid source for amplification of CbCEP sequence, codon optimized for expression in *E. coli*.	(Jameson et al., 2021)
pET-28a(+)RfCEP	Plasmid source for amplification of CbCEP sequence, codon optimized for expression in *E. coli*.	(Jameson et al., 2021)
pEV	Empty vector, pSIP411 derivative without target genes, with replicon Sh71.	(Fredriksen et al., 2012)
pLp_1261_AE6-DC_Sh71	pSIP derivative with replicon Sh71, source for amplification of lipoprotein anchor.	(Kuczkowska et al., 2019b)
pLp_1261-Ag85B-E6-DC	pSIP derivative with replicon pUC(pGEM)ori/256, source for amplification of lipoprotein anchor.	(Øverland, 2013)
pLp_1261-CbCEP-myc_Sh71 (Lipo-Cb)	pSIP411 derivative with replicon Sh71, for surface display of CbCEP by lipoprotein anchoring.	This study
pLp_1261-RfCEP-myc_Sh71 (Lipo-Rf)	pSIP411 derivative with replicon Sh71, for surface display of RfCEP by lipoprotein anchoring.	This study
pLp_3014-AgE-DC-Sh71	pSIP derivative with replicon Sh71, source for amplification of non-covalent cell wall anchor.	(Mathiesen et al., 2020)
pLp_3014-CbCEP-myc_Sh71 (LysM-Cb)	pSIP411 derivative with replicon Sh71, for surface display of CbCEP by a non-covalent LysM domain anchor.	This study
pLp_3014-RfCEP-myc_Sh71 (LysM-Rf)	pSIP411 derivative with replicon Sh71, for surface display of RfCEP by a non-covalent LysM domain anchor.	This study

pLp_3050-DC-Ag85B-E6-cwa2	pSIP derivative with replicon pUC(pGEM)ori/256, source for amplification of covalent cell wall anchor.	(Kuczkowska et al., 2017)
pLp_3050-DC-AgE-cwa2-Sh71	pSIP derivative with replicon Sh71, source for amplification of covalent cell wall anchor.	(Mathiesen et al., 2020)
pLp3014Ag85B:ESAT6_DC	pSIP derivative with replicon pUC(pGEM)ori/256, source for amplification of non-covalent cell wall anchor.	(Målbakken, 2014)

2.8 Buffers, solutions, and gels

Components used to make buffers, solutions and gels are listed in section 2.2.

Phosphate Buffered Saline (PBS)

8.0 g NaCl

0.2 g KCl

1.44 g Na_2HPO_4

0.24 g KH_2PO_4

Add dH_2O up to 1.0 L, adjust to pH = 7.4. Sterilized by CertoClav at 121 °C for 15 minutes

Tween-20 + PBS (TBPS)

PBS + 0.1 % (w/v) Tween-20

Tris-buffered saline (TBS)

8.0 g/L NaCl

0.2 g/L KCl

3.0 g/L Tris-HCl

Add dH_2O up to 1.0 L, adjust to pH = 7.4. Sterilized by CertoClav at 121 °C for 15 minutes.

Tween-20 + TBS (TTBS)

TBS + 0.1 (w/v) Tween-20

Agarose gel for electrophoresis (1.2 % agarose)

6.0 g Seakem® LE Agarose powder + 500 mL TAE buffer, 1 X.

Mixed, then sterilized by Certoclav at 121°C for 15 minutes. Solution is stored in a heated locker ready to use as needed.

MOPS, 100 mM solution

23.13 g MOPS sodium salt, dH_2O added to final volume of 1.0 L.
MOPS buffer pH is dependent on temperature. The buffer solution is warmed to the correct temperature for its intended use before pH-adjustments are made with HCl/NaOH as needed. The buffer solution is sterilized by filtration through a 0.22 µm pore filter.

Substrate solution for enzyme activity assays

50.0 g/L lactose in pH-adjusted MOPS buffer. Solution is mixed, then sterile filtered through a 0.45 µm pore plate filter. The substrate solution is kept on ice and used the same day.

2.9 Growth media

Certain growth media were made from base ingredients for cultivation of bacteria. Ready-made growth media were also used.

Brain Heart Infusion (BHI) liquid medium

18.6 g BHI dissolved in dH_2O for a total volume of 500 mL. Solution sterilized in CertoClav at 121 °C for 15 minutes.

BHI agar medium

BHI liquid medium supplemented with 1.5 % (w/v) agar. Solution sterilized by CertoClav at 121°C for 15 minutes. Solution cooled to ~50 °C before addition of antibiotics if needed, then poured into sterile plates and let cool until hard before storing the plates at 4 °C.

De Man, Rogosa, Sharpe (MRS) liquid medium

26 g MRS. dH_2O to 500 mL. Sterilized in CertoClav at 115°C for 10 minutes.

MRS agar medium

MRS liquid medium + 1.5% (w/v) agar. Sterilized by CertoClav at 115 °C for 10 minutes. Solution cooled to ~50 °C before addition of antibiotics if needed, then poured into sterile plates and let cool until hard before storing the plates at 4 °C.

MRS + sucrose + magnesium chloride (MRSSM)

5.2 g MRS + 17.1 g sucrose (0.5 M) + 2.0 g $MgCl_2$ x $6H_2O$ (0.1 M). dH_2O to 100 mL. Sterilized by filtration through a 0.22 µm pore filter. Stored in Eppendorf tubes at -20 °C.

M17

18.63 g M17 dissolved in dH_2O to 500 mL. Solution was sterilized by CertoClav at 121 °C for 10 minutes.

M17 + glucose (GM17)

M17 + sterile glucose to concentration 0.5%.

M17 + sucrose + glucose (SGM17)

GM17 + 0.5 M sterile sucrose.

Super Optimal Catabolite medium (S.O.C.)

Premade and ready to use from manufacturer.

MRS w/o tween-20 and w/o sugar (called MRS w/o sugar) (J. C. De Man, 1960).

To make MRS w/o sugar the following components were added into a 1.0 L bottle:

10.0 g peptone

8.0 g Lab Lemco powder

4.0 g yeast extract

2.0 g dipotassium hydrogen phosphate

5.0 g sodium acetate x $3H_2O$

2.0 g triammonium citrate

0.2 g magnesium sulphate x $7H_2O$

0.05 g manganese sulphate x $4H_2O$

dH_2O added to 1.0 L and mixed. The solution was pH-adjusted to 6.2 +/- 0.2 and sterilized by filtration through a 0.22 µm pore filter.

***Lactobacillus* Carrying Medium + Glucose (LCMG)**

100.0 g trypticase peptone

10.0 g glucose

5.0 g yeast extract

3.0 g tryptose

2.7 g dipotassium hydrogen phosphate

2.0 g diammonium hydrogen citrate

1.0 g sodium acetate

0.2 g cysteine-HCl

1.0 mL tween-80

10.0 mL mineral solution

dH_2O added to 1.0 L. Sterilized by CertoClav at 121 °C for 15 minutes.

Mineral solution used in LCMG

5.8 g $MgSO_4$ x $7H_2O$

2.8 $MnSO_4$ x H_2O

0.5 g ascorbic acid

dH_2O added to 1.0 L. Sterilized by CertoClav at 121 °C for 15 minutes.

2.10 Cultivation of bacteria

Bacteria were cultivated in suitable liquid media or spread on agar plates. Appropriate antibiotics were added to cultures as described in Table 4. *E. coli* was cultivated in BHI and incubated at 37 °C in a shaking incubator overnight. *L. lactis* was cultivated in GM17 at 30 °C without shaking overnight. *L. plantarum* and *L. reuteri* were both cultivated in MRS at 37 °C without shaking overnight.

Table 4. Overview of antibiotic concentrations for different media

	E. coli		Lactic acid bacteria	
Antibiotic	Agar media	Liquid media	Agar media	Liquid media
Erythromycin	200 µg/mL	200 µg/mL	10 µg/mL	10 µg/mL
Kanamycin		100 µg/mL		

2.11 Bacterial glycerol stocks

Glycerol stocks were made to enable long-term storage of bacteria. Stocks are kept at -80 °C.

Materials

Overnight bacterial culture, sterile glycerol solution, 1.5 mL cryovial.

Method

1.0 mL of a bacterial culture grown overnight and 300 µL 87% (w/v) glycerol was combined in a 1.5 mL Cryovial. The contents were carefully mixed by inverting a few times, before stored in a freezer at -80 °C. To cultivate bacteria from a frozen glycerol stock, a small sample was taken by a sterile toothpick. This toothpick was dropped into a suitable growth medium with appropriate antibiotic and cultivated overnight, yielding a fresh culture.

2.12 DNA isolation

2.12.1 Plasmid isolation from bacterial culture

Plasmid DNA was isolated from cultivated bacteria using the NucleoSpin® Plasmid Kit following the manufacturer's protocol. Protocol 5.2 was used for all plasmid isolation.

For plasmid isolation from gram-positive *L. lactis*, 50 µL of a 100 mg/mL lysozyme solution was added to buffer A1. The mixture was incubated for 30 minutes at 37 °C, following manufacturers protocols. Lysozyme was added to aid in lysis of the bacterial cell wall.

To improve plasmid yield from *L. lactis*, the elution procedure in protocol 5 was repeated up to 4 times. The elution buffer was changed to dH_2O. To concentrate the plasmid content, eluate was evaporated by centrifugation in a vacuum centrifuge at 37 °C for 30 minutes. After evaporation, 10 µL dH_2O was added to rehydrate the plasmid DNA.

2.12.2 Determination of DNA concentration

The concentration of eluted DNA was determined using the Qubit™ Fluorometer, following the manufacturers protocols.

2.12.3 Digestion of DNA with restriction enzymes

DNA was either single or double digested by restriction enzymes in optimal buffer. Restriction enzymes cleave specific nucleotide sequences in double stranded DNA. DNA cleaved by such enzymes leave an overhang of a few nucleotides at the cleavage site, called sticky ends. If a vector and an insert are digested with the same enzymes, their overhangs will be compatible for ligation.

Materials

DNA sample, restriction enzyme and buffer depending on digestion, dH_2O.

Method

The components in Table 5 and target DNA, was added to an Eppendorf tube, gently mixed by finger-flicking, and quickly spun in a centrifuge to collect components in the bottom of the tube. The reaction mixture was incubated in a water bath at 37 °C for 2 hours. After incubation, loading dye was added and the mixture was loaded onto an agarose gel to separate the fragments by electrophoresis.

Table 5. An example of a double digest using SalI-HF and HindIII.

Components	50 µL reaction
DNA	< 1 µg
SalI-HF	1.0 µL (20 units) *
HindIII	1.0 µL (20 units) *
10X NEBuffer 2.1	5.0 µL (1x)
dH_2O	Up to 50 µL

2.12.4 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to separate DNA fragments according to their size. The negatively charged DNA fragments from the negative pole towards the positive pole when an electric current is applied. The agarose gel has a pore-structure which enables smaller fragments to move faster than larger fragments. A DNA ladder with fragments of known sizes was used to identify the fragment size of samples.

Materials

1.2 % agarose solution, 1 x TAE buffer, PeqGREEN, Loading Dye, GeneRuler™ 1 kb DNA Ladder.

Method

To prepare a gel for electrophoresis, 60 mL of the liquid agarose solution was poured into a beaker together with 2.5 µL PeqGREEN. The components were mixed by stirring before pouring into a plastic mold. A comb was placed to make indentations for wells. The gel was left to solidify for minimum 20 minutes before removing the comb and placing the gel into the electrophoresis chamber. The electrophoresis chamber was filled with sufficient 1x TAE buffer to submerge the gel. The ladder and the DNA sample were loaded into the wells of the gel. The gels were run at 90 volts for 30 to 45 minutes, depending on fragment sizes.

2.12.5 Isolation of DNA from agarose gels

To extract DNA fragments agarose gels the NucleoSpin® Gel and PCR Clean-up kit was used. The DNA in the pre-run gel was visualized on a UV-light emitting table. Fragments of interest were excised by a scalpel and transferred to an Eppendorf tube, before following the extraction steps detailed in the manufacturers protocol 5.2: ‘DNA Extraction from agarose gels’. Eluted DNA was kept at -20 °C.

2.13 Polymerase Chain Reaction - PCR

The Polymerase Chain Reaction (PCR) is a vital technique for in vitro amplification of specific DNA sequences. Performing a PCR requires a thermostable DNA polymerase, template DNA, deoxynucleotide triphosphates (dNTPs), forward and reverse primers (oligonucleotides) as well as a suitable buffer solution. After all necessary components are mixed in a PCR tube, it is inserted into a PCR thermocycler machine. The machine performs cyclic temperature changes and can be programmed to accommodate different template sizes and melting temperatures. The main steps of a PCR cycle are denaturation, annealing and elongation.

In the denaturation step, also called melting step, the machine uses high temperature to separate the double stranded template DNA, yielding single-stranded DNA. The single strands are used as templates for synthesis of new double strands.

The annealing step is performed at a lower temperature, allowing the forward and reverse primer to bind to their complementary nucleotide sequences on the single-stranded template DNA. The temperature is adjusted according to the primers used.

In the elongation step, the primers annealed to the template DNA will act as binding sites for the DNA polymerase. The polymerase will synthesize DNA based on the complementary strand by incorporating dNTPs present in the reaction solution. The temperature and time depend on the DNA polymerase used, as they vary in speed (kb/min) and temperature optimum. These three steps are repeated for 25 to 35 cycles, each cycle doubles template amount, ensuring an exponential amplification of the DNA.

2.13.1 PCR by Q5® Hot Start High Fidelity 2x Master Mix

The Q5® Hot Start High Fidelity 2x Master Mix contains a DNA polymerase with a very low error rate. As DNA fragments amplified for cloning must be virtually error-free this polymerase is the preferred choice. The polymerase is delivered ready to use in a Master Mix.

Materials

Q5® Hot Start High Fidelity 2x Master Mix, template DNA, primers from Table 1 and dH_2O.

Method

The manufacturers protocol for Q5® Hot Start High Fidelity 2x Master Mix was followed when preparing all PCR reactions. Components were added to PCR tubes as seen in Table 6. The PCR tubes were closed, gently mixed, and quickly centrifuged to collect components to the bottom of the tubes.

Table 6. Typical set up for PCR by Q5® Hot Start High Fidelity 2x Master Mix

Components	Volume (µL)	Final concentration
PCR by Q5® Hot Start High Fidelity 2x Master Mix	25	1x
10 µM Forward primer	2.5	0.5 µM
10 µM Reverse primer	2.5	0.5 µM
Template DNA	0.5-1.0	<1 µg
dH_2O	Until 50	

Components were always kept on ice until transferred to a thermocycler and run according to the program seen in Table 7.

Table 7. Thermocycler program **for Q5 PCR.**

Step	Temperature (°C)	Time	Cycles
Initial denaturation	98	30 seconds	1
Denaturation	98	10 sec	25-35
Annealing	51-77*	30 seconds	25-35
Elongation	72	20-30 seconds/kb	25-35
Final elongation	72	2 minutes	1
Hold	4	∞	

***Annealing temperature depends on what primers are added. The general rule is an annealing temperature approximately 3 °C lower than the melting temperature (Tm) of the primer with the lowest Tm.**

The amplified fragments were loaded onto an agarose gel to verify the correct fragment size before they were utilized in further work.

2.13.2 Colony PCR with VWR Red Taq DNA Polymerase Master Mix

Colony PCR was used to verify whether the correct DNA insert had been successfully cloned into a plasmid. VWR Red Taq DNA Polymerase Master Mix contains dNTPs, $MgCl_2$ and a suitable buffer.

Materials

Template DNA from bacterial colony, VWR Red Taq DNA Polymerase Master Mix, primers from Table 1 and dH_2O. All set up according to Table 8.

Table 8. Components used for PCR by Red Taq DNA Polymerase

Components	Volume (μL)	Final concentration
Taq 2X Master Mix Red	25	1X
10 μM Forward primer	1	0.2 μM
10 μM Reverse primer	1	0.2 μM
dH_2O	23	
Template DNA	Variable	

Method

The manufacturers protocol and suggestions were followed for colony PCR. Using tweezers and a sterile toothpick, a small quantity of a colony was picked from an agar plate and placed into a suitable growth medium with appropriate antibiotics. This culture served as backup if the colony PCR proved successful. After securing the backup, a small number of bacteria from the same colony was stuck to the inside of a 0.2 μL PCR tube using a toothpick. For colony PCR on Gram-positive strains, the PCR-tubes were put into a microwave for 30 seconds on full effect to crack the cell wall. All samples were kept on ice as components from Table 8 were added into the PCR-tubes. The thermocycler settings were adjusted according to Table 9. After PCR, the samples were mixed briefly then loaded onto an agarose gel to verify successful cloning of fragment.

Table 9. Thermocycler program for Red Taq PCR

Step	Temperature (°C)	Time	Cycles
Initial denaturation	95	2 minutes	1
Denaturation	95	20-30 sec	35
Annealing	51-77*	20-40 sec	35
Elongation	72	1 min/kb	35
Final elongation	72	5 minutes	1
Hold	4	∞	

***Annealing temperature depends on what primers are added. The general rule is an annealing temperature approximately 3 °C lower than the melting temperature (Tm) of the primer with the lowest Tm.**

2.14 Cloning

Cloning recombinant DNA is normally done by a DNA ligase enzyme which forms a covalent link between two complementary DNA fragments. Another method, the In-fusion method, works in a different manner and uses special primers that adds complementary regions to the insert that are complimentary to the vector used to clone into the linearized vector.

2.14.1 In-Fusion cloning

The In-Fusion Cloning Kit is used for cloning a DNA fragment into a linearized vector. The In-Fusion cloning method requires specifically designed primers that add 15 base pair (bp) overhangs on the amplified insert that are homologous to the end of the linearized vector. The PCR amplified insert and the remaining components in Table 10 are mixed and incubated. The In-Fusion® enzyme recognize the 15 bp overhangs and fuses the insert and linearized vector together.

Materials

5X In-Fusion HD Enzyme Premix, purified PCR product, linearized vector, dH_2O.

Method

All components listed in Table 10 are added into a microcentrifuge tube. The purified PCR product was added in a 2-fold excess to the linearized vector according to the online tool; ‘In-

Fusion molar ratio calculator'. All components were carefully mixed by finger flicking, then spun down to collect in the bottom of the tube. The mixture was incubated at 50 °C for 15 minutes, then placed on ice. The finished ligation mixture was either transformed into competent cells, or stored at -20 °C.

Table 10. In-Fusion® cloning components

Component	Volume (µL)
5X In-Fusion HD Enzyme Premix	2
Linearized vector	50-200 ng
Purified PCR fragment (insert)	10-200 ng
dH_2O as needed	X
Total volume	10

2.14.2 ElectroLigase

ElectroLigase from NEB® combines T4 DNA ligase with a ready-to-use 2X reaction buffer. ElectroLigase ligates both sticky and blunt end, works well for low concentrations of DNA, and the ligation mixture can be used to directly transform electrocompetent cells. The manufacturers ElectroLigase® 'Ligation Protocol for Subcloning' was used for transforming electrocompetent *L. lactis*.

Materials

ElectroLigase®, ElectroLigase Reaction Buffer®, purified insert, linearized vector, dH_2O.

Method

20-100 ng vector DNA was transferred to an Eppendorf tube and combined with a 3-fold molar excess of purified insert. The total volume of vector and insert was adjusted with dH_2O to reach 5 µL, into which 5 µL ElectroLigase Reaction Buffer and 1 µL ElectroLigase was added. The components were mixed by pipetting up and down 7-10 times before a 30-minute incubation at room temperature. After incubation, the resulting reaction mixture was moved to 65 °C for 15 minutes, to inactivate the ligase. The ligation mixture was transformed into electrocompetent *L. lactis* cells or stored at -20 °C for later use.

2.15 Sequencing plasmids and PCR products

To confirm the DNA sequence in plasmids and PCR fragments, samples were sent to Eurofins Genomics for Sanger sequencing. Depending on the length of the sequence sent for analysis several samples were prepared, each with a unique sequencing primer to ensure adequate cover.

Materials

400-500 ng DNA template, 25 pmol primer, dH_2O up to 11 µL total volume.

Method

Purified DNA was added to microcentrifuge tubes in the amount specified, together with one primer per tube, dH_2O was added as needed. Every sample was given a unique barcode for identification. Sequencing results were analyzed with CLC DNA Main Workbench 7.

2.16 Preparation of competent cells

Chemically competent *E. coli* and electrocompetent *L. lactis*, *L. plantarum* and *L. reuteri* was utilized in this thesis. Some were delivered ready-to-use by the manufacturer while others had to be made competent.

2.16.1 Electrocompetent *L. lactis*

Sofie Kristensen (PhD student at KBM) kindly provided all electrocompetent *L. lactis* used in transformation experiments.

2.16.2 Electrocompetent *L. plantarum*

Electrocompetent *L. plantarum* was prepared by the following protocol. The competent cells can be stored at -80 °C until needed.

Materials

Overnight culture of *L. plantarum* WCFS1, MRS, MRSSM, MRS + 1 % glycine, 20 % glycine, 30 % PEG_{1450} (made fresh), 50 mL tubes, dry ice.

Method

A glycerol stock of *L. plantarum* WCFS1 was grown overnight in 10 mL MRS at 37 °C. The next day 1 mL of this culture was used to make a serial dilution from 10^{-1} to 10^{-10} in MRS + 1.0 % glycine. These new cultures were incubated over night at 37 °C. The next morning, 1 mL of a culture with an OD_{600} of 2.5 +/- 0.5 was diluted with 20 mL MRS + 1.0 % glycine, then incubated at 37 °C for 3 hours.

After this time, the culture had reached a logarithmic growth phase marked by an OD_{600} of 0.7 +/- 0.07. The culture was removed from the incubator and placed on ice for 10 minutes. The chilled culture was centrifuged at 5000 x g for 10 minutes at 4 °C. supernatant was discarded after centrifugation. 5 mL fresh, ice-cold 30 % PEG_{1450} was used to resuspend the cell pellet. An additional 20 mL PEG_{1450} was added for a total volume of 25 mL. The tube was capped, then inverted gently a few times to mix the solution before placing it back on ice to cool. The solution was centrifuged at 5000 x g for 10 minutes at 4 °C before discarding the supernatant. The resulting cell pellet was resuspended in 400 µL of 30 % PEG_{1450}. Fresh microcentrifuge tubes had been placed on dry ice in advance to chill them sufficiently. 40 µL of the resuspended cells were pipetted into each microcentrifuge tube. competent *L. plantarum* were placed at -80 °C until needed.

2.16.3 Electrocompetent *L. reuteri*

Electrocompetent *L. reuteri* were prepared by using the following, internal protocol. The competent cells created by this method must be used quickly as they are not made to be stored at -80 °C.

Materials

Overnight culture of *L. reuteri*, LCM + 1 % glucose (LCMG), 30 % PEG_{1500} and dH_2O.

Method

L. reuteri was grown overnight in LCMG at 37 °C. The following day, a small quantity of the overnight culture was added to a fresh, pre-heated LCMG solution, reaching an OD_{600} of ~0.20. The culture was incubated at 37 °C until OD_{600} of ~0.80. The culture was put on ice for 10 minutes, then centrifuged at 5000 x g for 5 minutes at 4.0 °C. The supernatant was discarded, and the cell pellet washed with 10 mL dH_2O. The centrifugation and washing step

were repeated once. After the last washing step, the cell pellet was resuspended in chilled 30 % PEG_{1500}. The cells were used for transformation experiments immediately, according to protocol.

2.17 Transformation of competent cells

2.17.1 Transformation of chemically competent *E. coli*

Chemically competent *E. coli* TOP10 or Stellar Competent *E. coli* was used for transformation experiments together with ligation mixture from In-Fusion reaction described in section 2.14.1.

Materials

Competent *E. coli* (TOP10 / Stellar Competent Cells), In-Fusion Reaction mixture, S.O.C. medium, Falcon 2059 Polypropylene Round Bottom tube 14 mL, BHI agar plates + 200 µg/mL erythromycin.

Method

The manufacturers recommendations were followed for transformation of competent *E. coli.* After transformation was done, 100 µL of the transformation mixture was spread on BHI agar plates with 200 µg/mL erythromycin and incubated at 37 °C overnight. Several colonies were selected for colony-PCR (section 2.13.2).

2.17.2 Transformation of electrocompetent bacteria

A total of three electrocompetent species were used in transformation experiments for work related to this thesis. Electroporation uses an electric pulse to disrupt the cellular membrane of specially prepared electrocompetent bacteria to enable uptake of foreign DNA, such as plasmids. The strains used can be seen in Table 2 and electroporation settings and post-electroporation conditions used is listed in Table 11.

Table 11. Electroporation settings and relevant information

Bacterial strain	Electroporation setting	Post-electroporation recovery medium	Agar and incubation
L. lactis	Cuvette: 0.2 cm		GM17 + 10 µg/mL
	Capacitance: 25 µF	700 µL SGM17	erythromycin
	Voltage: 2.0 kV	2-4 hours, 30 °C	2-3 days, 30 °C
	Resistance: 200 Ω		
L. plantarum	Cuvette: 0.2 cm		MRS agar plates + 10
	Capacitance: 25 µF	450 µL MRSSM	µg/mL erythromycin
	Voltage: 1.5 kV	2-4 hours, 37 °C	2-3 days, 37 °C
	Resistance: 400 Ω		
L. reuteri	Cuvette: 0.2 cm		MRS agar plates + 10
	Capacitance: 25 µF	900 µL LCMG	µg/mL erythromycin
	Voltage: 2.5 kV	2-4 hours, 37 °C	2-3 days, 37 °C
	Resistance: 200 Ω		

Materials

Electrocompetent bacteria: *L. lactis*, *L. plantarum* and *L. reuteri*, purified Plasmid DNA or ElectroLigase® Ligation-mix, media, and agars from Table 11, Electroporation cuvette, Bio-Rad GeneRuler® II and Bio-Rad Pulse Controller Plus.

Method

The electroporator settings was adjusted according to Table 11. Electrocompetent strain was thawed on ice and pipetted into a pre-chilled electroporation cuvette. The cuvette was placed securely into the electroporation device before administering a brief electric pulse through the cuvette. After which the cuvette was quickly opened to administer ice-cold recovery medium to the transformed bacteria.

The resulting suspension was mixed by careful pipetting up and down a few times before pouring the suspension with the transformed bacteria into sterile microcentrifuge tubes. The transformed cells were allowed to recover before a small volume of the bacterial suspension was spread on agar with suitable antibiotics and incubated for up to 2 days. After incubation,

several colonies were selected for colony-PCR according to section 2.13.2. Promising transformant strains were isolated for further work.

2.18 Sample preparation for gene product analysis

The genes cloned into the pSIP system are expressed when the inducer peptide, SppIP, is introduced to a culture in the exponential growth phase. Recombinant *L. plantarum* and *L. reuteri* were induced to competence and harvested, their gene products investigated by Western blot analysis, flow cytometry and enzyme activity assays.

2.18.1 Cultivation and harvesting of recombinant bacteria

Bacteria intended for growth curve-, Western blot- or flow cytometry analysis were cultivated in MRS and washed in PBS buffer after harvest. Bacteria intended for use in enzyme activity assays were cultivated in MRS w/o tween-20 and washed in MOPS buffer after harvest.

Materials

Recombinant strains, inducer peptide (SppIP), MRS or MRS w/o tween-20, erythromycin, PBS buffer or MOPS buffer.

Method

1. Bacteria were cultivated overnight in suitable in conditions (section 2.10). The overnight cultures were diluted in 50 mL tubes containing pre-heated growth medium with 10 µg/mL erythromycin to an OD_{600} of 0.10-0.15. The diluted cultures were incubated at 37 °C until reaching an OD_{600} of 0.28-0.33.
2. When the cultures reached an OD_{600} of 0.28-0.33, they were induced with 25 ng/mL SppIP.
3. The induced cultures were incubated for an additional 3 hours at 37 °C.
4. After the incubation, the cultures were placed on ice to cool down before the cells were harvested by centrifugation at 4700 x g for 5 minutes at 4 °C. The supernatant was discarded.
5. The cell pellet was resuspended and washed in 10 mL buffer, then centrifuged 4700 x g for 5 minutes at 4 °C. Supernatant was discarded. This washing process was repeated two times, making the bacteria ready for further use or analysis.

2.19 Growth curve analysis of recombinant bacteria

Growth curves of induced and non-induced recombinant *L. plantarum* and *L. reuteri* were analyzed.

Materials

Bacterial cultures (section 2.18.1), sterile 96-well plate, Multiscan FC, sterile sealing film, SkanIt Software 2.5.1.

Method

Induced and non-induced bacterial cultures described in step 2, section 2.18.1 were pipetted in technical triplicate, into the wells of a 96-well plate. After loading all samples to the 96-well plate, the plate was sealed using sterile film and placed in the Multiscan FC. The SkanIt software was set to incubate the samples at 37 °C and capture the OD_{595} value of samples every 10 minutes for 24 hours. The results were exported as an Excel-file and analyzed.

2.20 Cell lysate preparation

To investigate protein production using Western blot analysis, the induced bacteria were homogenized into a protein-rich cell lysate with FastPrep. FastPrep uses glass beads and intense shaking to destroy the cell wall. The resulting protein-rich lysate is used in protein gel electrophoresis and Western blotting, described in sections 2.22 and 2.23.

Materials

1.0 mL harvested cells in buffer solution from section 2.18, FastPrep® tubes and lids, glass beads, PBS, FastPrep® - 24 Tissue and Cell Homogenizer.

Method

A 1.0 mL bacteria suspension was transferred to a FastPrep® tube containing ~0.5 g glass beads. The tubes were places in the FastPrep® - 24 Tissue and Cell Homogenizer and run at 6.5 m/s for 45 seconds to extract proteins. This step was repeated a total of three times with breaks of five minutes in between for cooling samples on ice.

After homogenization, the tubes were centrifuged at 16000 x g at 4 °C for 1 minute. Protein extract was carefully pipetted into fresh Eppendorf tubes. The samples were centrifugated one

additional time to ensure removal of cell debris and glass beads. The protein extract was stored at -20 °C until needed.

2.21 Protein precipitation with TCA

This method was used to precipitate proteins present in reaction solutions at the end of enzyme activity assays, described in section 2.26. By precipitating proteins from a large sample volume, a larger amount of protein could be used to prepare SDS-PAGE and a stronger signal obtained in Western blot analysis.

Materials

600 µL sample from enzyme activity assay, fume hood, pH-measurement sticks, NaOH, Na-Deoxycholate, TCA, acetone, dH_2O.

Method

1. Samples were adjusted to pH ≈ 7.0 by addition of NaOH where necessary using pH-measurement sticks. Each sample was placed in fresh 2 mL Eppendorf tubes. Na-Deoxycholate was added to a final concentration of 0.2 mg/mL. The samples were mixed and incubated on ice for 30 minutes. TCA was added to the samples to a final concentration of 16 %. The samples were mixed, then incubated overnight at 4 °C to precipitate the proteins.
2. The following day, the precipitated proteins were pelleted by centrifugation at 16000 x g for 25 minutes at 4 °C. The supernatant was removed before adding 600 µL ice-cold acetone. The samples were briefly mixed before centrifugation at 16000 x g for 20 minutes at 4 °C. Supernatant was discarded. This step was performed twice.
3. After the last acetone wash, the Eppendorf tubes were left open inside the fume hood to evaporate remaining acetone from samples. After 10 minutes a visual inspection of sample tubes concluded no remaining TCA or acetone. 20 µL dH_2O was added to rehydrate the precipitated proteins, a small pipette was used to facilitate this.
4. The samples were now ready and treated the same way as protein extracts intended for SDS-PAGE described in section 2.22.

2.22 Protein gel electrophoresis

The method Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used to separate denatured proteins according to their molecular weight. The denaturation of proteins in a sample is caused by addition of an anionic detergent, a reducing agent and boiling the mixture in a 100 °C water bath. SDS binds regularly to the amino acid chain of denatured proteins. Every SDS molecule bound to the amino acid chain increases the negative charge of the denatured protein. In this way the negative charge is based on the proteins molecular weight.

The denatured samples are loading onto the gel and an electric current is applied. The current causes negatively charged proteins to migrate through the gel towards the positive anode. Proteins with a lower molecular weight will travel faster than high molecular weight proteins. After some time, the proteins will be separated throughout the gel according to size. By including a protein standard of known fragment sizes, the molecular weight of unknown samples can be estimated.

Materials

Protein samples from section 2.20, Mini-PROTEAN® TGX Stain-Free ™ Precast gels, 15 well type, NuPAGE® LDS Sample Buffer (4X), NuPAGE® Reducing agent (10X), TGS Buffer, MagicMark™ XP Western Protein Standard.

Method

All samples were denatured according to the manufacturers protocol provided with Mini-PROTEAN® TGS™ Precast Gels, using NuPAGE® components. To enable comparison between samples, the OD_{600} value of each sample at the time of harvest was used to adjust the protein extract amount.

Every sample was adjusted to a total volume of 20 µL with dH_2O, before addition of 7.5 µL sample buffer and 3 µL reducing agent. The components were mixed and placed in a 100 °C water bath for 10 minutes. The prepared samples were loading onto a gel together with the MagicMark™ XP Western Protein Standard. The gels were run for 23 minutes at 200 Volts. Using a GelDoc®, images were captured before the gels went on to the blotting procedure described in section 2.23.

2.23 Western blot

The Western blot analysis uses antibody hybridization and chemiluminescence to visualize target proteins. The first step involves the transfer of proteins from an SDS-gel to be blotted onto a membrane. Prior to antibody hybridization, the blotted membrane is treated with a blocking solution to avoid non-specific binding reactions. A primary antibody binds to the target protein, this primary antibody has a binding site to which a secondary antibody can bind. The secondary antibody is conjugated with the enzyme horseradish peroxidase (HRP). HRP oxidizes luminol which gives off detectable light that is used to visualize the target proteins using special image capture equipment.

2.23.1 Blotting with iBlot™ Dry Blot System

The iBlot ™ Dry Blot System was used to blot proteins onto a nitrocellulose membrane from an SDS-PAGE gel.

Materials

Pre-run SDS-PAGE gel from section 2.22, iBlot™ Dry Blotting system (section 2.4), TBS, dH_2O.

Method

The pre-run gel was washed with dH_2O for 5 minutes. The gel was assembled in the iBlot™ Dry Blot System according to Figure 5. The filter paper was soaked with dH_2O before placed and any air bubbles were carefully removed by using the botting roller. After all components were in place, a disposable sponge was added before closing the lid. The blotting program settings was 20 V for 8 minutes. After blotting, the membrane was carefully removed and placed in TBS awaiting antibody hybridization.

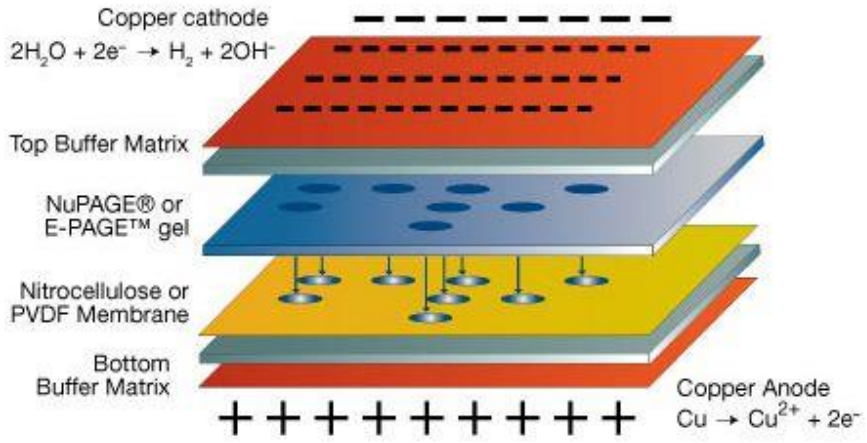


Figure 5. Assembled components when using the iBlot™ Dry Blot System. The figure is taken from the iBlot® Dry Blotting manual.

2.23.2 SNAP i.d.® immunodetection

The SNAP i.d.® Protein detection system was used to hybridize antibodies to membrane-blotted proteins from section 2.23.1. The system uses a vacuum pump to pull the added solutions through the membrane.

Materials

Blotted membrane, SNAP i.d.® Protein detection system (section 2.4), TTBS, TTBS + 1% BSA, vacuum-pump, primary antibody: Anti-Myc Mouse Monoclonal IgG (1 mg/mL) and secondary antibody: Anti-Mouse IgG, HRP Polyclonal (1:20000) (section 2.3).

Method

1. Blot holders were moistened with dH_2O before the blotted membrane was placed protein side down inside blot holder. A moistened filter paper was added on top of the membrane before the blot holder was closed. Any air bubbles were removed by a blot roller. The blot holder was inserted into the plastic casing of the SNAP i.d.® protein detection system device.
2. The membrane was covered with 30 mL TTBS + 1% BSA (blocking solution), by adding 10 mL each time the vacuum had pulled the solution through. The primary antibody (PA) was added in a mixture composed of 3 mL TTBS + 3% BSA + 3 µL

PA. The PA-mixture is poured over the membrane with care to ensure all parts of the membrane is covered by the solution, then incubated for 10 minutes.

3. After incubation, the membrane was washed with 10 mL TTBS for a total of three times. The added TTBS was pulled through the membrane by vacuum.
4. After washing, the secondary antibody (SA) was added in a mixture composed of 3 mL TTBS + 1% BSA + 0.25 μL SA. The membrane was incubated with the mixture for 10 minutes.
5. Step 3 was repeated. After the final washing step, the membrane was removed from the blot holder and placed in a plastic container. Further work on the membrane is described in section 2.23.3.

2.23.3 Detection of proteins by chemiluminescence

Materials

Membrane from section 2.23.2, SuperSignal® West Pico Chemiluminescent Substrate: Luminol Solution, Stable Peroxide Buffer.

Method

5 mL Luminol and 5 mL Stable peroxide buffer was mixed thoroughly. The mixture was poured onto the membrane residing in a plastic container. The container was wrapped with aluminum foil to block light exposure and incubated in the dark for 5 minutes. After incubation, excess mixture was removed, and the membrane was placed inside an Azure c400 photosystem for visualization and image capture of target proteins.

2.24 Detection of surface-located proteins

For detection of surface located proteins, specific primary antibodies that target the protein must be used. After the primary antibody binds to the target, a secondary antibody binds to the primary antibody. The secondary antibody, conjugated with a fluorochrome such as fluorescein Isothiocyanate (FITC), will emit light when excited by a laser-beam.

2.24.1 Flow cytometry

Flow cytometry is a technique in which bacteria are suspended in a solution and move individually through a thin capillary tube. As each bacterium move through the capillary, it is struck by a laser beam. Some of the laser-light will be reflected. The quality of reflected light is registered by special sensors. The registered signals can be seen as characteristic scatter signals such as Forward Scatter (FSC) and Side Scatter (SSC), which give information about cells size and surface. For cells bound to a FITC-conjugated antibody, the laser-beam hitting the FITC will give of a specific fluorescence signal. The signals make it possible to compare relative fluorescence between different populations of bacteria. A strong fluorescent signal indicates the presence of a FITC-conjugated protein on the surface.

Materials

Induced recombinant strains, PBS, PBS + 2 % BSA, primary antibody: Anti-Myc Mouse Monoclonal IgG (1 mg/mL), and secondary antibody: Anti-Mouse IgG, FITC Polyclonal (section 2.3).

Method

1. Recombinant *L. plantarum* and *L. reuteri* were prepared according to section 2.18.1 following steps 1 through 3.
2. After incubation, the number of cells in 500 µL of a culture with an OD_{600} = 1 was pipetted into an Eppendorf tube, centrifuged at 5000 x g for 2 minutes to remove supernatant. The cell pellet was resuspended and washed with 1000 µL PBS and centrifuged to remove the buffer.
3. After washing and centrifugation, each cell pellet was resuspended in 50 µL PBS + 2 % BSA + 0.2 µL primary antibody solution. The samples were incubated in a water bath at 37 °C for 30 minutes. The cell suspension was centrifuged at 5000 x g for one minute to remove supernatant.
4. The cell pellet was resuspended and washed three times with 600 µL PBS + 2 % BSA with centrifugation at 5000 x g 2 minutes to remove supernatant and excess antibodies.
5. After the washing steps, the cell pellet was resuspended in 50 µL PBS + 2 % BSA + 0.3 µL secondary antibody (FITC-conjugated). Samples were packed in aluminum foil

and incubated at room temperature for 30 minutes, then centrifuged for 1 minute to remove supernatant.

6. Step 4 was repeated, this time to remove excess secondary antibodies.
7. After washing, the cell pellet was resuspended in 1.0 mL PBS and further diluted 1/10 with PBS in a test tube before flow cytometry analysis. The results from the flow cytometry were analyzed using MacsQuantify™ and Flowjo® software.

2.25 Determination of colony forming units (CFU)

The following method was used to determine CFU/mL recombinant *L. plantarum* before and after a 24-hour enzyme activity assay (section 2.26).

Materials

Recombinant *L. plantarum*, MRS-agar plates, 100 mM MOPS buffer pH = 7.0, multichannel pipette, sterile 96-well plate.

Method

20 µL sample of a bacterial solution was diluted in 180 µL MOPS buffer in a 96-well plate, 10-fold dilutions were made up to 10^{-7}. 20 µL of the diluted samples was spread in lines across MRS-agar plates using a multichannel pipette. The plates were incubated at 37 °C overnight and colonies were counted in stripes with appropriate dilutions. The CFU/mL was determined by the formula: CFU/mL = colony count x dilution factor.

2.26 Enzyme activity assays

2.26.1 Setup of enzyme activity assay experiments

Recombinant *L. plantarum* and *L. reuteri* harboring plasmids for surface display of CbCEP and RfCEP were characterized in enzyme activity assays.

The experimental setup of the enzyme activity assays was based on the standard conditions seen in Table 12 and investigating how changing one of these conditions affected epilactose

production. Some experiments also introduced novel conditions intended to explore commercially relevant properties, such as reusing bacteria for multiple rounds of biocatalysis.

Materials

Induced strains prepared according to section 2.18.1, 2.0 mL microcentrifuge tubes, substrate solution (100 mM MOPS buffer at pH = 7.0 with 50.0 g/L lactose) (section 2.8), ThermoMixer™.

Method

A certain number of bacteria were pipetted into 2.0 mL Eppendorf tubes. The bacteria were washed with MOPS buffer, then centrifuged to remove remaining growth medium before resuspending the cell pellet in freshly made substrate solution. These mixtures were incubated with shaking using a ThermoMixer for a set amount of time, then placed on ice to stop the enzyme activity, before samples were prepared for HPAEC (section 2.26.2).

2.26.2 Sample preparation for analysis by HPAEC

All samples collected from the enzyme activity assay undergo filtration and dilution before they analysis by HPAEC. Samples are always kept on ice. First, the assay samples are centrifuged and filtered to remove bacteria and debris. After filtration the sample is referred to as reaction solution and is diluted 4000× with dH_2O making it ready for analysis.

Materials

Reaction solution samples from assays, HPLC vials and caps, Internal Standard (mannose), 96-well plate, 0.45 µm pore filter-plate, plate vacuum-pump, dH_2O.

Method

Samples from assays were prepared according to section 2.26.1 and placed on ice for 10 minutes before the bacteria were removed from the reaction solution by centrifugation at 10.000 x g for 2 minutes. 200 µL of reaction solution from each sample was pipetted into a well on a 0.45 µm pore filter plate which filtered the samples onto a fresh 96-well plate using vacuum. The filtered reaction solutions were pipetted into fresh Eppendorf tubes, and kept at -20 °C, or diluted for analysis by HPAEC.

Dilution of a sample was done in two stages: First, 25 µL of the filtered reaction solution was diluted in 975 µL dH_2O, making a 40× dilution. This 40× dilution was rigorously mixed, placed on ice for five minutes and mixed again before the last dilution. 10 µL of the 40x diluted sample was diluted in 990 µL dH_2O, yielding a total dilution of 4000x. If mannose (internal standard) was included in the sample, 10 µL of the 40x diluted sample was diluted with 980 µL dH_2O + 10 µL of a 0.5 g/L mannose solution, yielding the final 4000× dilution of the sample with an appropriate mannose concentration of 0.0050 g/L.

After filtration and dilution, 100 µL of each sample was pipetted into HPLC vials in technical triplicate and capped. Bubble-formation would occasionally occur in the sample vials and may be detrimental to HPAEC analysis. Bubbles were removed by carefully tapping the capped vial containing the sample down onto a smooth surface, releasing the bubbles. The capped samples were analyzed by HAPEC (section 2.27.1).

2.27 High-Performance Anion Exchange Chromatography

High-Performance Anion Exchange Chromatography (HPAEC) with Pulsed Amperometric Detection (PAD) is an extensively used analytical method used to separate and analyze carbohydrates of interest. The carbohydrates of interest in this thesis are lactose, lactulose, epilactose and mannose (IS). HPAEC is performed at high pH, ensuring deprotonation of hydroxyl- and carboxyl groups present on the carbohydrates in the injected sample. As the now negatively charged carbohydrates travels through the mobile phase, they react with the positively charged stationary phase of the column. The inherent column affinity of each carbohydrate is caused by the high pH environment and their spatial configurations. As the carbohydrates are not identical, they are separated as they move at different speeds through the column stationary phase. However, because of their relatively similar charge and spatial configuration, the eluting conditions must remain weak to ensure proper separation of analytes.

2.27.1 Sample analysis with the ICS-6000

Samples were prepared according to section 2.26.2 and analyzed by HPAEC, using the Dionex Ion Chromatography System-6000 (ICS-6000) (section 2.1).

The system is largely automated and analyses the samples based on set parameters. Analysis begins with the autosampler withdrawing the volume required from the capped vial containing the prepared sample, injecting it into the column for analysis. The system was run isocratically with a flow rate of 0.200 mL/min and an eluent concentration of 12 mM KOH, using 18 minutes per sample. The column temperature was kept at 30.0 °C. Commercial lactose, lactulose and epilactose were used as external standards, mannose was added as an internal standard. Analysis of internal- and external standards with known concentrations was used by the system to create standard curves for quantification of the unknown assay samples. The results were analyzed using Chromeleon® 7 Chromatography Data System software.

3 Results

The goal of this thesis was construction of plasmids for surface-display of two cellobiose 2-epimerases for conversion of lactose to epilactose in recombinant strains. The cellobiose 2-epimerase sequences were derived from *Caldicellulosiruptor bescii* (CbCEP) and *Roseburia faecis* (RfCEP), both codon-optimized for use in *E. coli* (Table 3). Three anchors derived from *L. plantarum* were selected for surface display of the epimerases: A lipoprotein anchor (Lipo), a non-covalent cell wall anchor (LysM), and a covalent cell wall anchor (cwa2).

The constructed plasmids were transformed to *Lactiplantibacillus plantarum* and *Limosilactobacillus reuteri* and were investigated by Western blot analysis for detection of heterologous gene products and flow cytometry analysis to investigate the presence of surface displayed cellobiose 2-epimerase. The recombinant strains were characterized in enzyme activity assays to investigate their utility as biocatalytic factories for production of epilactose using lactose as a substrate.

3.1 Plasmid construction

The initial plan used plasmids derived from the pSIP403-series with 256-replicon (Sørvig et al., 2003; Sørvig et al., 2005), which uses *E. coli* for sub-cloning. Figure 6 shows the construction strategy for surface display of CbCEP and RfCEP using a lipoprotein anchor (Lp_1261) and a non-covalent cell wall anchor (Lp_3014). The original inserts present in the vectors pLp_1261-Ag85B-ESAT6-DC and pLp_3014Ag85_E6_DC, were removed by double digestion using SalI and HindIII (Figure 6).

New inserts were prepared by PCR amplification with primers found in Table 1 and plasmids found in Table 3. PCR amplification of CbCEP used pET-28a(+)CbCEP as template and the primer-pair CbCEP_F / CbCEP_R. PCR amplification of RfCEP used pET-28a(+)RfCEP as template and the primer-pair RfCEP_F / RfCEP_R). The reverse primers both add downstream Myc-tags to the CEase-sequences, used to detect expressed CEase in Western blot analysis and flow cytometry. The inserts made by PCR were combined with the digested vectors by In-Fusion® cloning (section 2.14.1), and the new plasmids were transformed to chemically competent *E. coli* (section 2.17.1).

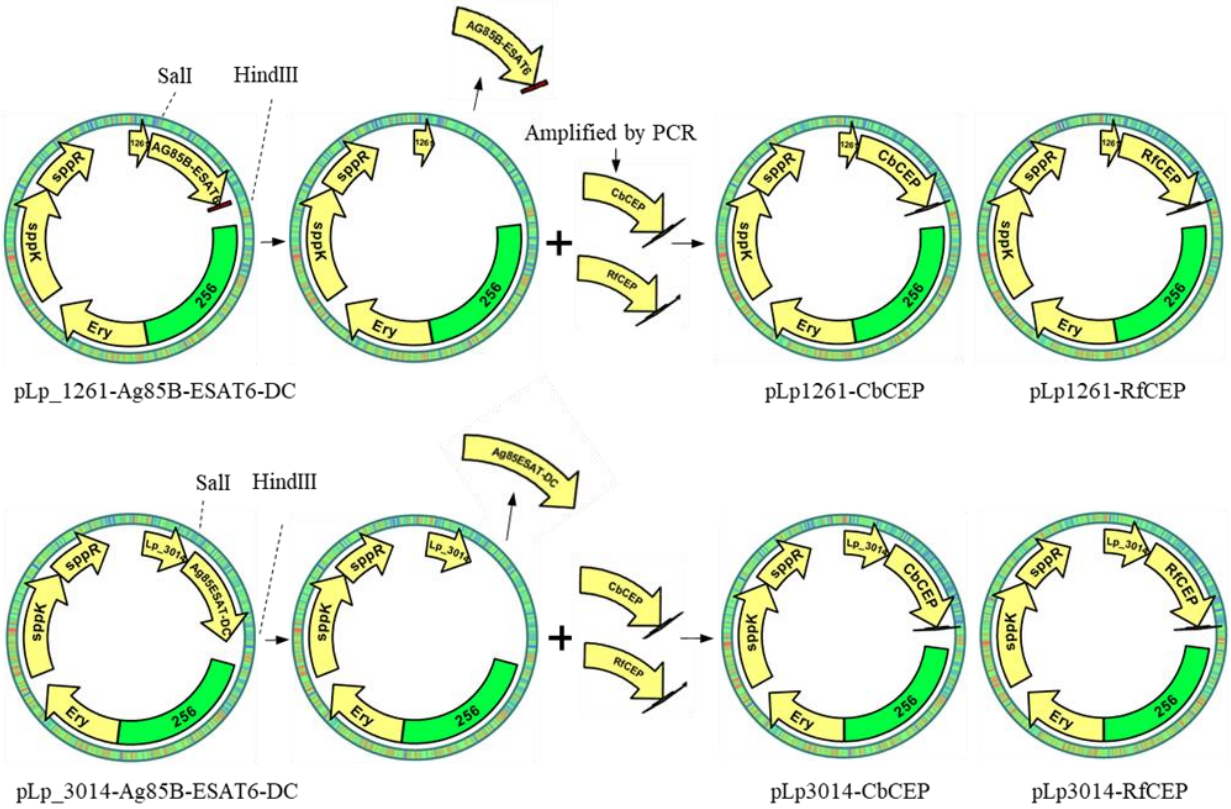


Figure 6. Strategy for construction of the plasmids pLp1261-CbCEP, pLp1261-RfCEP, pLp3014-CbCEP and pLp3014-RfCEP. The vectors pLp_1261-Ag85B-ESAT6-DC and pLp_3014-Ag85B-ESAT6-DC were digested with restriction enzymes SalI and HindIII to linearize the vectors. PCR amplification of CEases used primers CbCEP_F and CbCEP_R for amplification of CbCEP, and the primers RfCEP_F and RfCEP_R for amplification of RfCEP. In-Fusion® cloning was used to fuse the PCR products and linearized vectors, yielding the plasmid constructs. Schematic abbreviations: Erythromycin resistance marker (Ery), histidine proteinase (sppK), response regulator (sppR). 1261 (lipoprotein anchor), 3014 (LysM domain anchor), CbCEP (CEase), RfCEP (CEase), Myc (Myc-tag downstream of CEase), 256* (256-replicon). *(Also contains the pUCori replicon).

Figure 7 shows the plasmid construction strategy for covalent cell wall anchoring of CEases. The plasmid vector pLp_3050-DC-Ag85B-E6-cwa2 was double digested with SalI and MLuI (Figure 7) to remove the previous insert (DC-Ag85B-E6) and linearize the vector.

PCR amplification was used to create inserts for cloning, using primers and plasmids listed in Table 1 and Table 3. Amplification of CbCEP used pET-28a(+)CbCEP as the template, and the primer-pair CbCEP-cwa2_F / CbCEP-cwa2_R. Amplification of RfCEP used pET-28a(+)RfCEP as template with primer-pair RfCEP-cwa2_F / RfCEP_R. The digested vector and PCR amplified inserts were combined using the In-Fusion® cloning protocol and transformed to chemically competent *E. coli*.

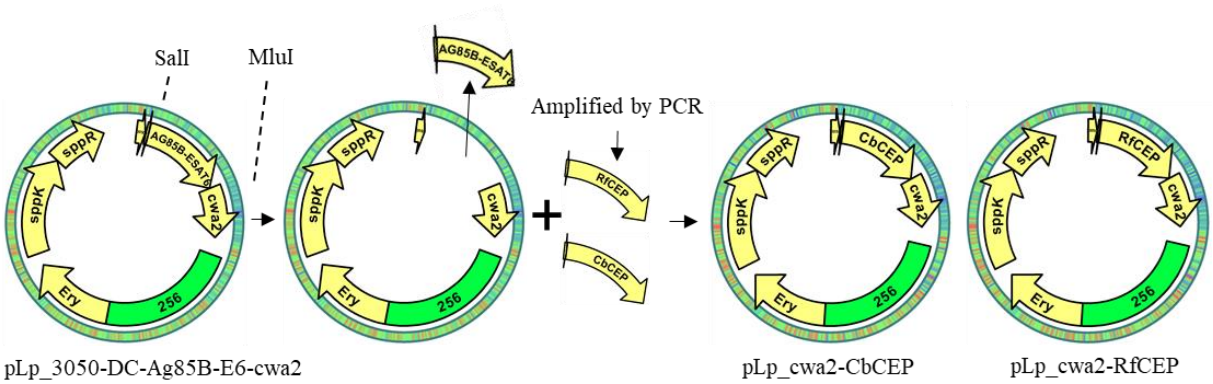


Figure 7. Strategy for construction of the plasmids pLp_cwa2-CbCEP and pLp_cwa2-RfCEP.
The vector pLp_3050-DC-Ag85B-E6-cwa2 was digested with restriction enzymes SalI and MLuI to linearize it. The epimerase genes were amplified by PCR using primers CbCEP-cwa2_F / CbCEP-cwa2_R for amplification of CbCEP, and primers RfCEP-cwa2_F / RfCEP_R for amplification of RfCEP. The linearized vector and PCR products underwent In-Fusion® cloning, yielding the plasmid constructs. Schematic abbreviations: Erythromycin resistance gene (Ery), histidine proteinase gene (sppK), response regulator gene (sppR), covalent cell wall anchor (cwa2), CbCEP (epimerase), RfCEP (epimerase), Myc (Myc-tag on epimerases), 256* (256-replicon). *(Also contains the pUCori replicon).

Colony PCR (section 2.13.2) with the primer-pairs CbCEP_F/CbCEP_R and RfCEP_F/RfCEP_R, was used to investigate the presence of CbCEP or RfCEP in recombinant *E. coli*. The colony PCR results indicated presence of CEase in the recombinant strains. Surprisingly, sequencing of isolated plasmid DNA from the recombinant strains revealed the presence of point mutations in all plasmids, which introduced a frameshift in the target gene. Figure 8 is an example of such a mutation and shows the sequencing results from an *E. coli* transformant harboring pLp1261-CbCEP (Figure 6), the arrow marked 'Conflict' points towards the point mutation where one nucleotide is deleted.

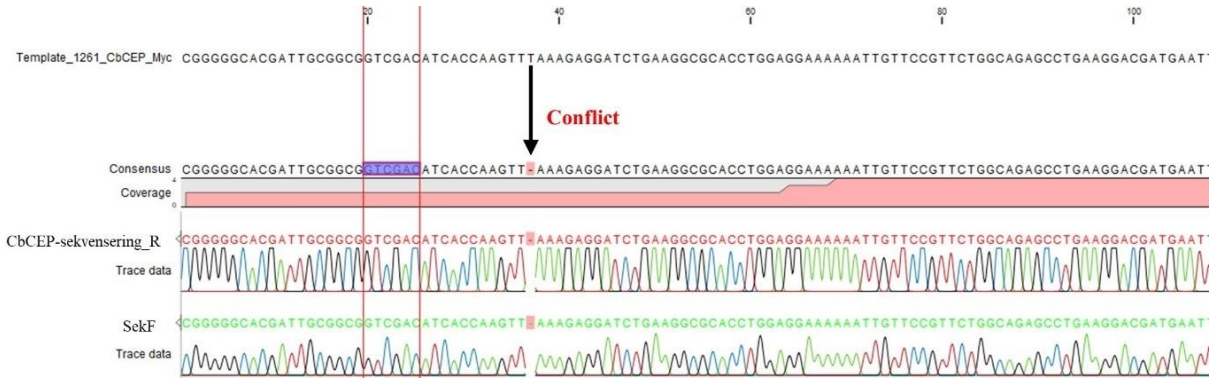


Figure 8. Chromatograms of the sequencing results from plasmid isolated from *E. coli* harboring pLp1261-CbCEP. The six nucleotides comprising the SalI restriction site are highlighted in blue and is located between the anchor- and CEase sequences of the plasmid constructs in Figure 6. The chromatograms reveal sequencing data based on two sequencing primers: CbCEP-sekvensering_R and SekF (Table 1). The black arrow indicates the conflict site where sequencing results revealed a point deletion. Chromatogram analyzed by DNA Main Workbench software.

Several cloning attempts were undertaken with fresh PCR products and vector backbones. Every experiment resulted in frameshift mutations comparable to the mutation in Figure 8. The recurring mutations could be the result of a toxic gene effect in *E. coli*, which can occur in recombinants expressing heterologous genes (Bienick et al., 2014; Schlegel et al., 2013).

To solve the issue, sub-cloning in *E. coli* with the 256/pUCori-replicon was changed to sub-cloning in *L. lactis* using the pSIP411-variant with the Sh71-replicon (Sørvig et al., 2005). The pSIP403 variant used for sub-cloning in *E. coli* has the pUCori- and 256-replicon, enabling replication in *E. coli*, *L. plantarum* and *L. sakei* (Sørvig et al., 2003; Sørvig et al., 2005). The pSIP411 variant enables sub-cloning in *L. lactis* and has the Sh71-replicon, replicating in a broad range of hosts, but not *E. coli* (Sørvig et al., 2005).

The pSIP403 and pSIP411 variants are identical apart from their replicons. Because of this, the cloning strategy showed in Figure 6 was reused for construction of plasmids with the lipoprotein anchor and the LysM-domain anchor, both with the Sh71-replicon.

For construction of vectors with the Sh71-replicon, pLp_1261_AE6-DC_Sh71 and pLp_3014-AgE-DC-Sh71 (Table 3) were used as vector backbones, both identical to the original backbones seen in Figure 6, apart from their replicon type. The vectors were digested by SalI and HindIII to remove the previous inserts and linearize the vectors. PCR amplification of inserts was done using the same primers as described in Figure 6, and were

digested with SalI and HindIII as well. The digestion of the PCR-amplified inserts removed the 15 bp overhangs used for In-Fusion cloning and created compatible ligation sites between the linearized vectors and amplified PCR products, necessary for ligation by ElectroLigase® (section 2.14.2). The resulting ligation mixtures were transformed to *L. lactis*. The recombinant *L. lactis* were investigated by colony PCR using CEase-specific primers to verify presence of both CbCEP and RfCEP. Gel electrophoresis was used to visualize the amplified products which indicated successful transformation of the recombinant into *L. lactis*. Sequencing results from plasmid DNA of recombinant *L. lactis* harboring plasmids revealed no mutations and they were transformed to electrocompetent *L. plantarum* and *L. reuteri*.

Figure 9 shows the four plasmid constructs with the Sh71-replicon, their full names can be seen in Table 3 and only their nicknames are used in the text.

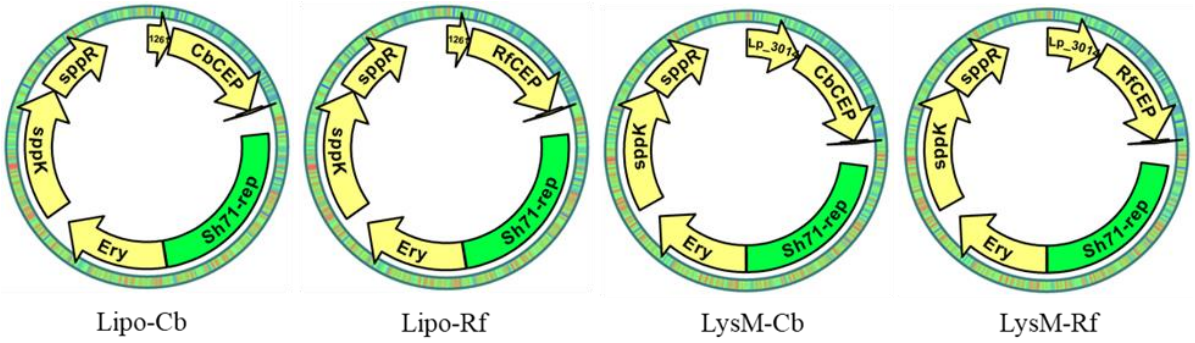


Figure 9. Overview of plasmids harbored by *L. lactis*, *L. plantarum* and *L. reuteri*.

The plasmid construction strategy for covalent cell wall anchoring of CEases in *E. coli* (Figure 7) cannot be used in *L. lactis*, as it requires digestion of the vector and PCR-amplified insert by SalI and MLuI. Both CbCEP and RfCEP have naturally occurring MLuI restriction sites, making digestion by MLuI impossible. This necessitated a new strategy for construction of plasmids and can be seen in the Appendix section 6.1 (Figure 25 and Figure 26). The cloning strategy yielded transformants, which where cultured overnight and subsequently plasmid DNA was isolated and sequenced. The sequencing results revealed mutations leading to frameshifts that knocked out expression of the target gene. The cloning procedure was attempted once.

3.2 Growth curve experiments

3.2.1 Selecting strains for enzyme production

The strains selected as hosts for production and surface display of CEase must be unable to metabolize epilactose. *L. plantarum* WCFS1 and *L. reuteri* DSM20016 were considered promising strains for surface display of enzymes. A growth experiment undertaken by Kamilla Wiull, PhD, showed that *L. plantarum* did not metabolize epilactose (data not shown).

The growth experiment to investigate if *L. reuteri* could metabolize epilactose was performed according to steps seen in section 2.18, with these changes: *L. reuteri* cultivated overnight in biological triplicate, the overnight cultures were diluted in prewarmed MRS w/o sugar to OD_{600} ~0.10. From the diluted cultures, 1.0 mL were pipetted into fresh Eppendorf tubes and centrifuged to remove residual sugar. Each cell pellet was resuspended in normal MRS, MRS w/o sugar, or one of the blends created by supplementing sugar-free MRS with glucose, lactose, lactulose and epilactose to a total sugar concentration of 2.0 g/L.

200 µL of each resuspended culture was loaded onto a 96-well plate and incubated in a Multiskan FC microplate reader at 37 °C for 24 hours, measuring the OD_{595} every 10 minutes. The growth analysis showed that *L. reuteri* did not metabolize epilactose (data not shown).

3.2.2 Growth curves for recombinant strains harboring relevant plasmids

Studies have shown that heterologous protein production may reduce bacterial growth (Bienick et al., 2014; Karlskas et al., 2014; Mathiesen et al., 2020). To investigate growth reduction of recombinant strains, their growth curves were followed for 10 hours (Figure 10).

Overnight cultures of recombinant bacteria were diluted in prewarmed MRS to OD_{600} ~0.10 and incubated until OD_{600} ~0.30. 200 µL of the growing cultures were transferred to separate wells of a 96-well plate. The remaining culture was induced by adding 25 ng/mL of the inducer peptide. 200 µL of the induced cultures were transferred into separate wells on the same plate to compare growth patterns between induced and non-induced cultures. The 96-well plate was placed in a Multiskan FC microplate reader. The samples were incubated at 37

°C for 10 hours. The growth curve was made by measuring the OD_{595} value every 10 minutes (Figure 10).

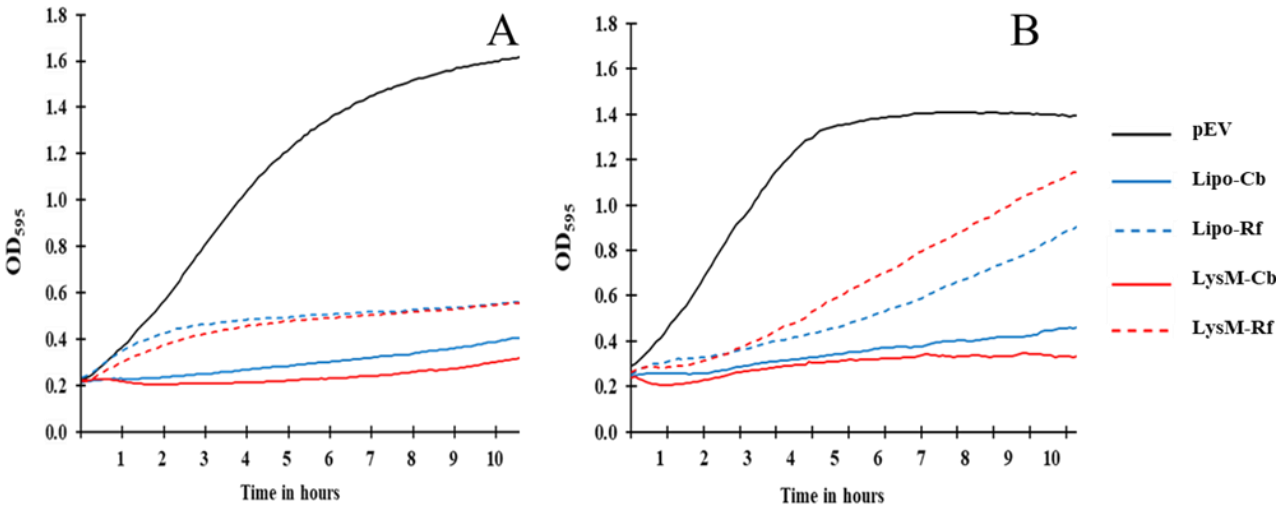


Figure 10. Growth curves of bacteria harboring relevant plasmids induced at time 0 and incubated at 37 °C. OD_{595} values were measured every 10 minutes for a total of 10 hours. All uninduced strains showed similar growth pattern as the control strain (pEV) and are not included in the figure. Figure 10A. Growth curves of induced *L. plantarum* harboring relevant plasmids. Graph data based on biological triplicates. Figure 10B. Growth curves of induced *L. reuteri* harboring relevant plasmids. Graph data based on technical triplicates.

Figure 10A shows a reduced growth rate for all induced *L. plantarum* transformants harboring epimerase-expressing plasmids. The decrease in growth is most pronounced in the two *L. plantarum* harboring CbCEP-expressing plasmids. Figure 10B illustrates growth of induced *L. reuteri* transformants. The induced *L. reuteri* all show a reduced growth rate. Figure 10B show a higher growth rate for *L. reuteri* harboring the RfCEP-displaying plasmids. All uninduced *L. plantarum* and *L. reuteri* showed similar growth pattern as their pEV.

3.3 Analysis of epimerase production by Western blot analysis

Western blot analysis was used to investigate if epimerase production was present in induced, recombinant bacteria (details in sections 2.18, 2.20, 2.22 and 2.23). Overnight cultures of recombinant bacteria were diluted in prewarmed MRS and grown until OD_{600} ~0.30 and induced by adding 25 ng/mL SppIP. The induced bacteria were incubated for three hours, harvested by centrifugation, and disrupted using FastPrep®, creating cell-free protein extract. The volume of protein extract used for SDS-PAGE analysis was adjusted based on the OD_{600}

value at the point of harvest, ensuring all samples were represented by an equal number of cells.

Figure 11 shows the Western blot of protein extracts from induced *L. plantarum* and *L reuteri* harboring relevant plasmids. Surprisingly, only samples from *L. plantarum* harboring Lipo-Rf and LysM-Rf gave detectable signals. Samples from *L. reuteri* yielded no signal for neither of the epimerases. To verify the results, the blotting procedure was repeated once for *L. plantarum* recombinants, with similar results. The procedure was not repeated for *L. reuteri*.

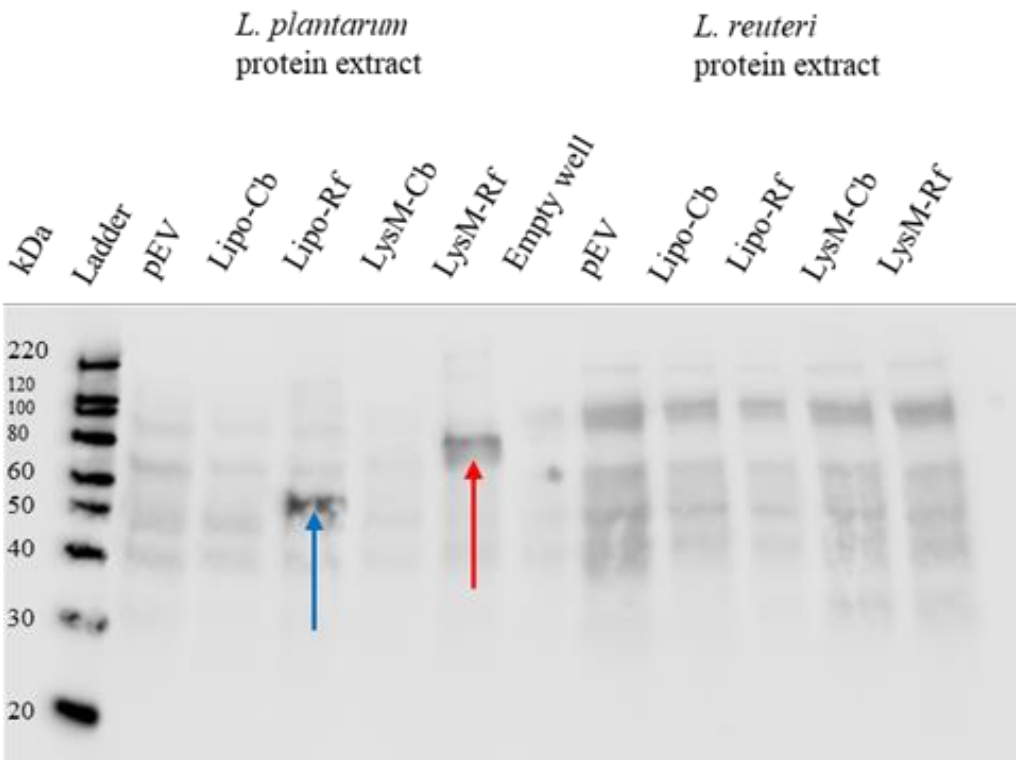


Figure 11. Western blot comparison analysis of protein extracts from induced *L. plantarum* and *L. reuteri* harboring relevant plasmids. First lane contains ladder MagicMark® Western Protein Standard. The theoretical molecular masses of the translated anchor-epimerase products are ~56.0 kDa for Lipo-Cb (no signal) and Lipo-Rf (blue arrow), and ~69.0 kDa for LysM-Cb (no signal) and LysM-Rf (red arrow). The protein gel used for the Western blot can be found in section 6.2 of the Appendix.

3.4 Detection of surface-anchored proteins by flow cytometry

Flow cytometry analysis was used to investigate the presence of surface-displayed CEases on induced, recombinant strains (section 2.24). The data gathered by flow cytometry analysis is presented as histograms where each peak represents a cell population or sample. The y-axis represents cell quantity in a normalized, modulated form and the x-axis represents the relative fluorescence of the fluorochrome FITC, measured by the cytometer. The histogram of samples with increased fluorescence are shifted towards the right on the x-axis in the diagram. Induced cultures of recombinant *L. plantarum* and *L. reuteri* were harvested after three hours. The harvested bacteria were stained by a primary antibody against the Myc-tag, then with a FITC-conjugated antibody that hybridized to the Myc-bound primary antibody (section 2.24.1).

Figure 12A shows *L. plantarum* harboring relevant plasmids. The *L. plantarum* harboring Lipo-Rf, (lipoprotein anchor) and LysM-Rf (non-covalent LysM-domain cell wall anchor) both show a slight fluorescence shift that could indicate the presence of epimerase on the surface. To strengthen the signal of the flow cytometry analysis, the amount of primary- and secondary antibodies used for staining were adjusted. The strongest shift is seen in Figure 12A.

No fluorescent signal was detected for any of the *L. reuteri* samples (Figure 12B). An additional attempt to increase the signal by varying the amount of primary- and secondary antibodies was done, with no effect.

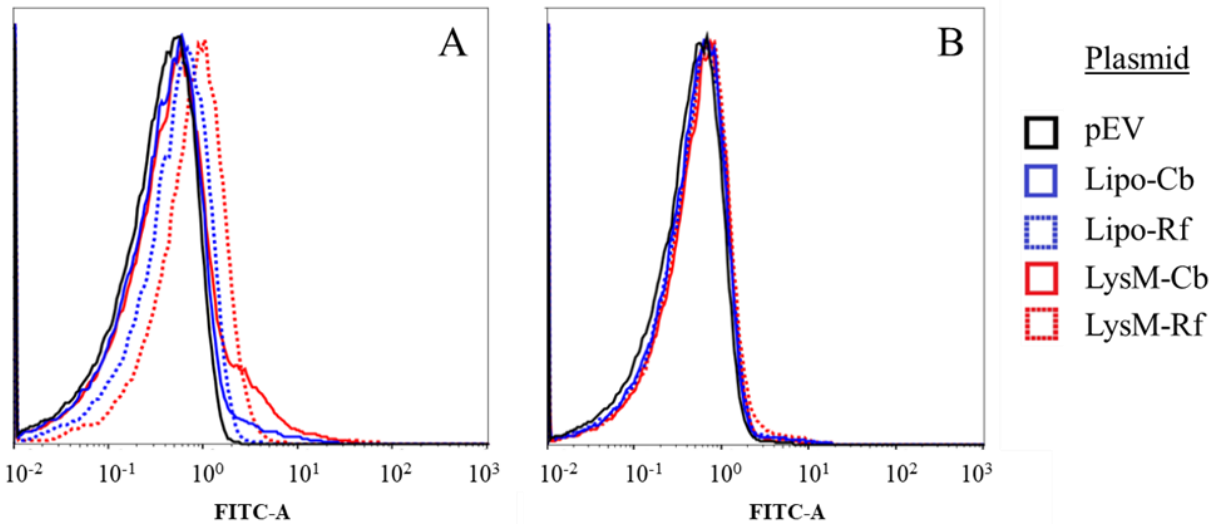


Figure 12. Flow cytometry results of FITC-stained proteins on the cell surface of recombinant strains. The results are depicted as normalized histograms with relative fluorescence (x-axis) plotted against number of fluorescent cells (y-axis). Each curve is color-coded and represents the unique plasmid construct harbored by the recombinant strains. pEV display no fluorescence and is used as a negative control. Figure 12A. Results from *L. plantarum* samples. Figure 12B. Results from *L. reuteri* samples.

3.5 Enzyme activity assays

The recombinant strains harboring CEase-displaying plasmids constructed in this thesis were used in enzyme activity assays to investigate their ability to produce epilactose with lactose as a substrate.

3.5.1 Standard conditions

The work presented in this thesis uses recombinant bacteria that surface-display CbCEP or RfCEP by a lipoprotein anchor or a non-covalent cell wall anchor. The purified forms of these CEases were recently characterized by Jameson et al. (2021), and their findings served as a guide to establish the standard conditions used in this thesis as the starting point for enzyme activity assays. Table 12 shows the standard conditions that apply for all assays, unless stated otherwise in the text.

All assays are set up with two negative control reactions: One control containing only substrate solution and the other containing both substrate solution and the recombinant strain harboring pEV. Analysis of all control samples and real samples shows that a similar fraction

of lactulose is present in every sample. In this case, the lactulose is produced by chemical isomerization (Jameson et al., 2021) by the buffer. As this phenomenon is constant, control samples are only shown in Figure 15.

Table 12. Standard conditions for enzyme activity assays.

Condition	Value
Temperature	37.0 °C
Time	24 hours
ThermoMixer speed	350 RPM
Buffer and pH	100 mM MOPS, pH = 7.0
Lactose concentration	50.0 g/L
CFU / mL	2.7×109

Statistical analysis of assay samples was done using the t-test in selected experiments (Jamovi 2.0.0). Differences were considered significant at $p < 0.05$.

3.5.2 Initial enzyme activity assays

The first enzyme activity assays used recombinant *L. plantarum* and *L. reuteri* to investigate their potential use as biocatalysts for production of epilactose using lactose as a substrate.

Bacteria intended for use in assays were cultivated in MRS w/o tween-20. Overnight cultures were diluted in 50.0 mL prewarmed MRS w/o tween-20, to OD_{600} ~0.10. The diluted cultures were incubated to OD_{600} ~0.30 before they were induced and incubated for three hours. The induced bacteria were collected by centrifugation and washed with pH-adjusted MOPS buffer (section 2.18), preparing them for use in enzyme activity assays.

Samples were set up for enzyme activity assays by resuspending 2.7×10^9 CFU of a prepared strain in 1.0 mL substrate solution (50.0 mg/mL lactose in 100 mM MOPS buffer, pH = 7.0). The reaction mixtures were incubated at 37.0 °C for 24 hours with a shaking speed of 350 RPM. After incubation, the samples were centrifuged at 8000× g for 60 seconds to separate

the bacteria from the supernatant (reaction solution). The reaction solution was sterile filtrated to remove any remaining bacteria and cell debris. The filtered samples were diluted 4000× with dH_2O and prepared in technical triplicate for analysis by the HPAEC (section 2.27.1).

Figure 13 shows a typical chromatogram of a sample analyzed by HPAEC. This example is from a reaction solution that used *L. plantarum* harboring Lipo-Rf. The three distinct peaks indicate the retention times of mannose (IS), lactose and epilactose in the column, proving successful conversion of lactose to epilactose.

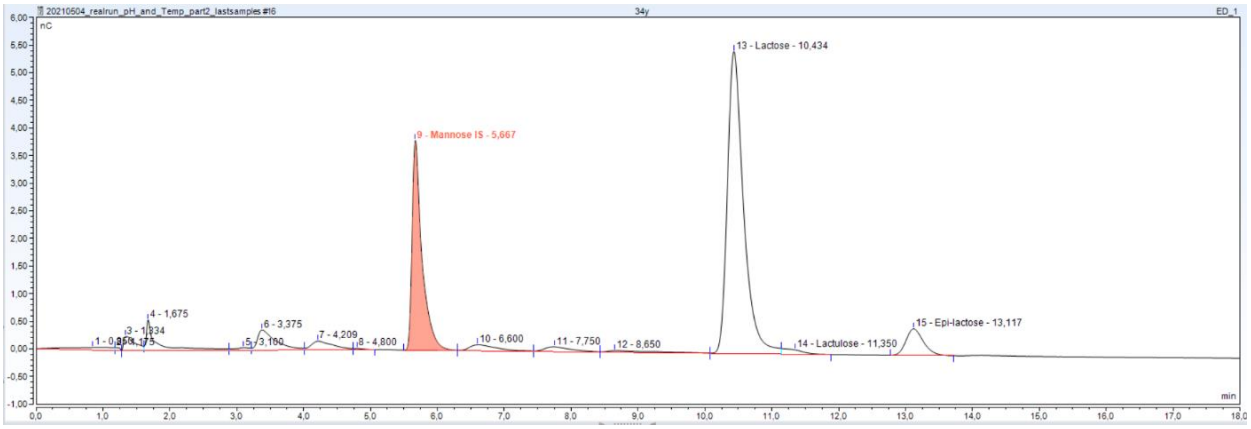


Figure 13. A representative chromatogram showing retention times of a sample analyzed by HPAEC. The x-axis of the chromatogram shows the retention times for various compounds, seen as peaks. The names of important compounds are seen on top of the curves: Mannose IS (Internal Standard), lactose, lactulose, and epilactose. The sample analyzed is the reaction solution from an assay using *L. plantarum* harboring Lipo-Rf to convert lactose to epilactose.

Results from the enzyme activity assay using recombinant *L. reuteri* is seen in Figure 14, and shows the relative fractions of lactose, lactulose and epilactose in each sample. Only two samples using *L. reuteri* yielded detectable epilactose. The *L. reuteri* harboring Lipo-Rf yielded ~7 % epilactose, while the strain harboring LysM-Rf yielded ~13 % epilactose.

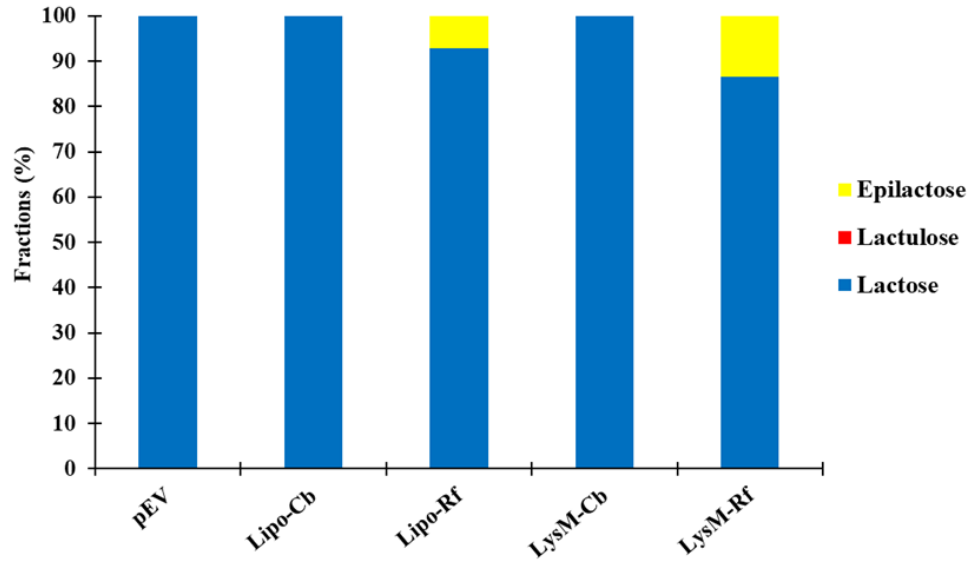


Figure 14. Enzyme activity assay at standard conditions using recombinant *L. reuteri* harboring relevant plasmid constructs. Sample contents of lactose, lactulose and epilactose are presented as fractions. Data presented are based on single samples, no biological replicates.

Figure 15 shows the assay results using recombinant *L. plantarum*. As expected, no epilactose is detected for *L. plantarum* harboring pEV. The experiment shows that all recombinant *L. plantarum* harboring the epimerase-displaying plasmids successfully produced epilactose. The epilactose yield ranged from 20 % in *L. plantarum* harboring Lipo-Cb, to 30 % for *L. plantarum* harboring Lipo-Rf. All samples contained roughly the same fraction of lactulose, including the negative controls.

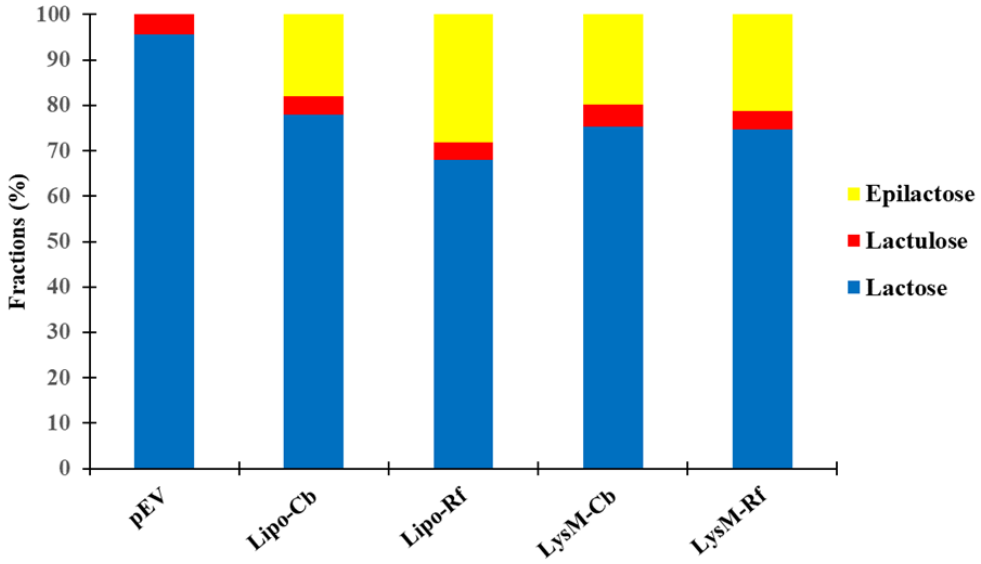


Figure 15. Enzyme activity assay at standard conditions using recombinant *L. plantarum* harboring relevant plasmid constructs. Sample contents of lactose, lactulose and epilactose are presented as fractions. Data presented are average values of three biological replicates.

The initial assay results were used to select strains for more in-depth characterization. Based on the disparity of epilactose production between recombinant *L. reuteri* (Figure 14) and recombinant *L. plantarum* (Figure 15), none of the *L. reuteri* recombinants were selected for further experimentation. All recombinant *L. plantarum* had an epilactose fraction over 20 %. *L. plantarum* harboring Lipo-Rf produced 30 % epilactose, clearly the most effective producer and was selected for further experimentation. *L. plantarum* harboring LysM-Rf was included to investigate how the anchor type would affect RfCEPs ability to produce epilactose.

The reduction in recombinant strains allowed for more in-depth characterization in enzyme activity assays with *L. plantarum* harboring Lipo-Rf and LysM-Rf.

3.5.3 The effect of shaking frequency on epilactose production

To investigate how shaking frequency affected epilactose production, three RPM settings of the ThermoMixer were compared: 350, 500 and 800 RPM. The results showed that the RPM setting did not influence the epilactose production (Figure 16).

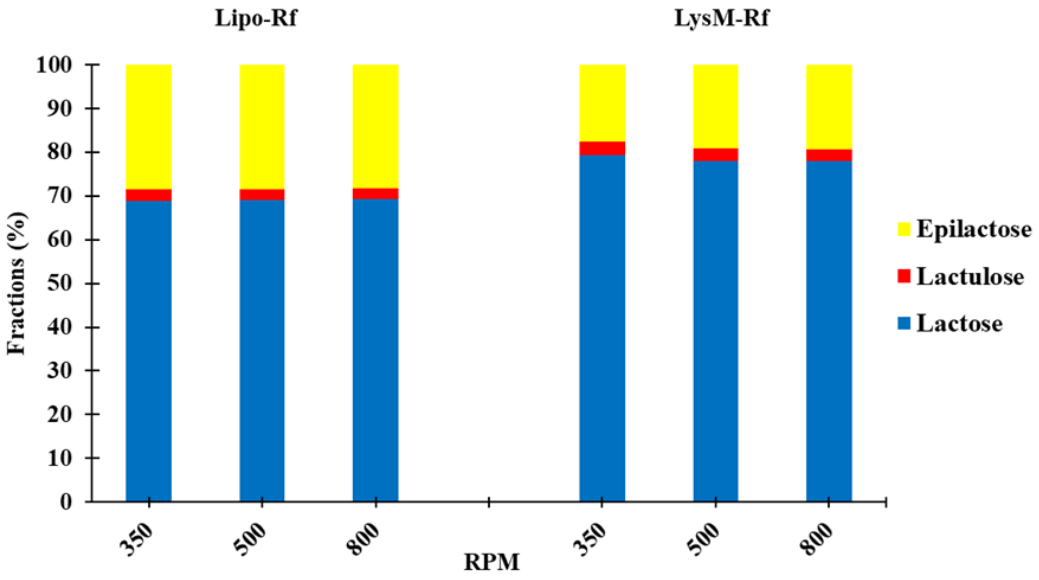


Figure 16. Shaking effect on epilactose production using recombinant *L. plantarum* harboring plasmid constructs Lipo-Rf and LysM-Rf. Results presented as fractions, based on sample data from two biological replicates. The RPM setting is the ThermoMixer® speed setting used for sample incubation.

3.5.4 Time effect on epilactose production

To investigate how time affected epilactose production, assays were set up with four reactions per recombinant strain. Each reaction was removed from incubation after a predetermined amount of time. Figure 17 shows that a substantial fraction of epilactose was produced after only 30 minutes of incubation. The epilactose fraction increases steadily up to the 14.5-hour mark. The epilactose fraction increases only slightly from 14.5 to 24 hours of incubation, indicating that the reaction has stopped, or slowed considerably.

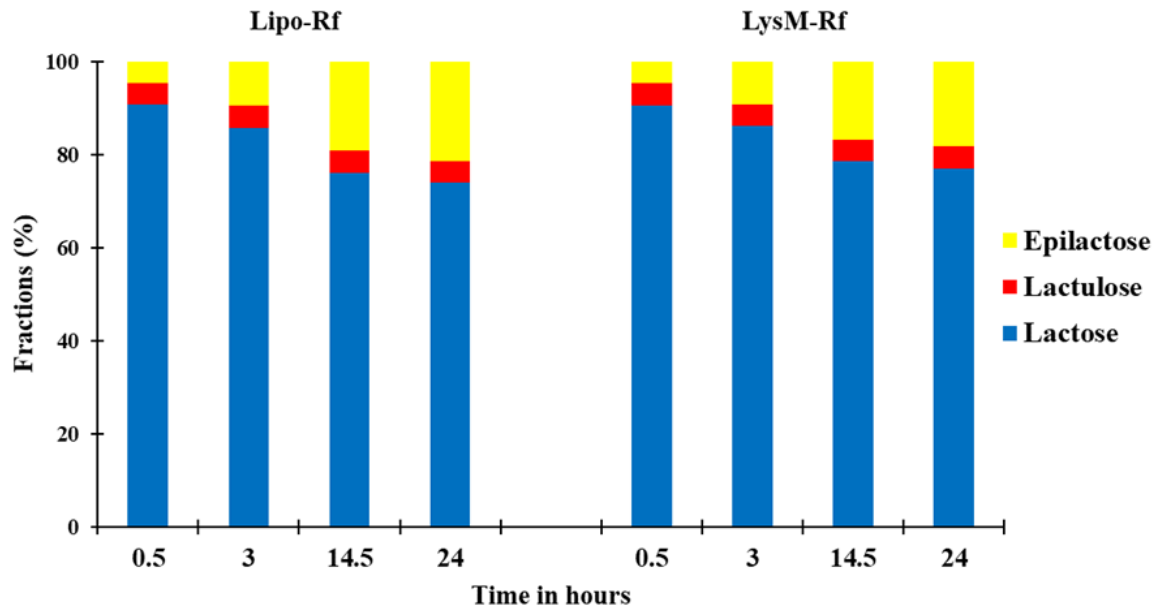


Figure 17. Time effect on epilactose production in assays using recombinant *L. plantarum* harboring Lipo-Rf and LysM-Rf. Results presented as fractions, based on sample data from one biological replicate.

3.5.5 The effect of the number of bacteria on substrate conversion

The following experiment was performed to investigate how the number of bacteria in a reaction (CFU/mL) affected the epilactose production. The assay reactions were set up with standard conditions and included three reactions with reduced number of recombinant *L. plantarum*. Figure 18 shows how a high CFU/mL is linked to high epilactose production.

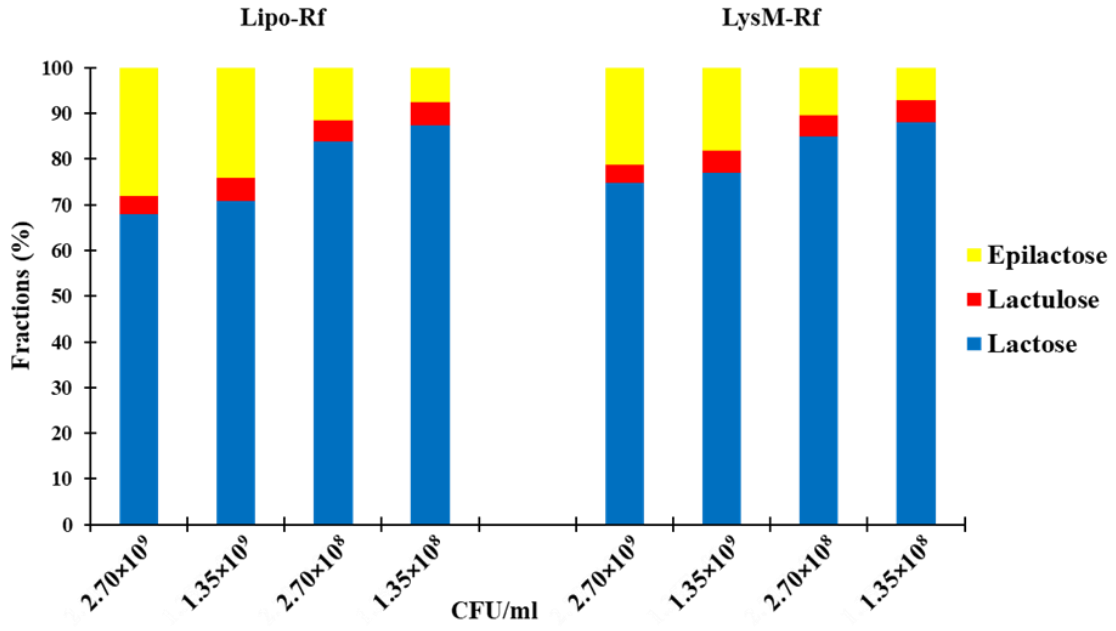


Figure 18. Effect of CFU/mL on epilactose production using recombinant *L. plantarum* harboring plasmid constructs Lipo-Rf and LysM-Rf. Results presented as fractions, based on sample data from one biological replicate.

3.5.6 Freezing bacteria and its effect on substrate conversion

For commercial reasons it would be beneficial if the bacteria retained their epilactose producing capabilities after freezing. Cultivating and freezing large batches, kept on hand for use when needed would conserve resources and negate the need to continuously produce fresh cultures to catalyze reactions.

To investigate the effects of freezing, recombinant strains were harvested as usual (section 2.18). After centrifugation, the cell pellets were frozen immediately at -20 °C, for 24 and 48 hours. Figure 19 shows the epilactose from assay samples with fresh bacteria (Not frozen), and bacteria frozen for 24 and 48 hours. In samples using *L. plantarum* harboring Lipo-Rf, there is no discernible difference in the fractions between frozen and non-frozen samples. Interestingly, assay samples from *L. plantarum* harboring LysM-Rf shows reactions using frozen bacteria has a decreased epilactose fraction compared to the reaction with fresh bacteria. This points out a variation dependent on the anchoring method (Figure 19).

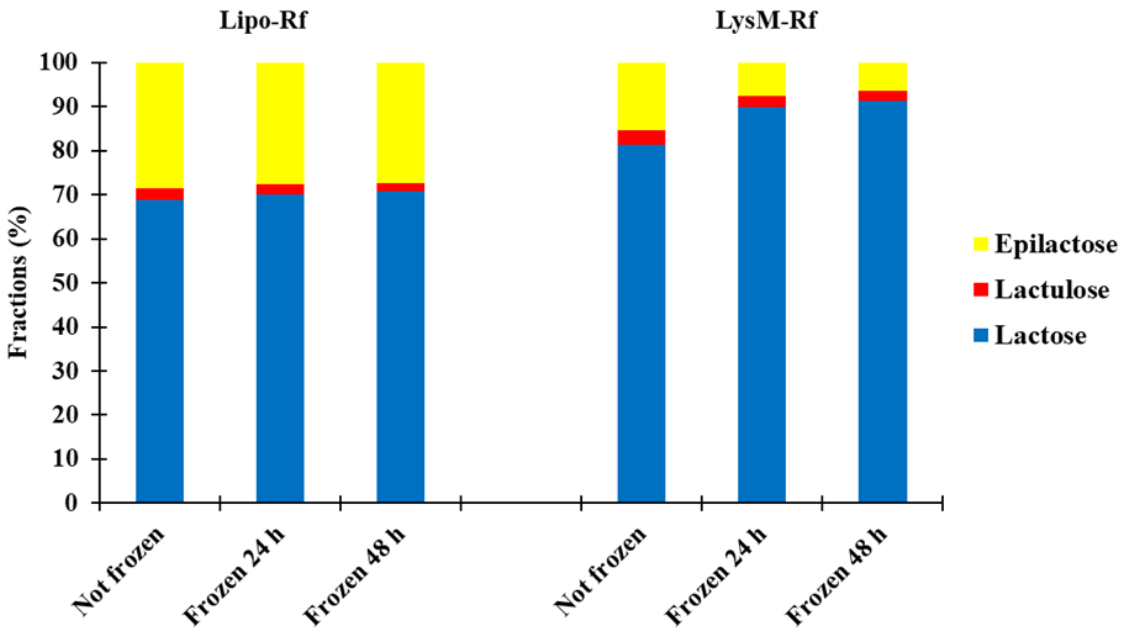


Figure 19. Freezing effect on epilactose production using recombinant *L. plantarum* harboring Lipo-Rf and LysM-Rf. Results presented as fractions, based on sample data from two biological replicates.

3.6 Reusing bacteria in multiple enzyme activity assays

In an industrial setting, one benefit of using surface displayed enzymes on bacteria is the ability to separate the bacteria at the end of a reaction and reuse them for multiple rounds of biocatalysis, increasing the total yield from the same number of bacteria.

To investigate the possibility of reusing bacteria, the assays were set up as normal (Table 12) and after the end of the 24-hour assay, the bacteria were harvested and resuspended in fresh substrate solution for another assay round. The fresh cells were reused for two additional assay rounds. Bacteria frozen for 24 hours (described in section 3.5.6) were included in the experiment and reused in one additional assay round. Figure 20 shows a sharp decline in epilactose production when recombinant strains were reused. Both strains proved unsuitable for reuse, as did the frozen strains.

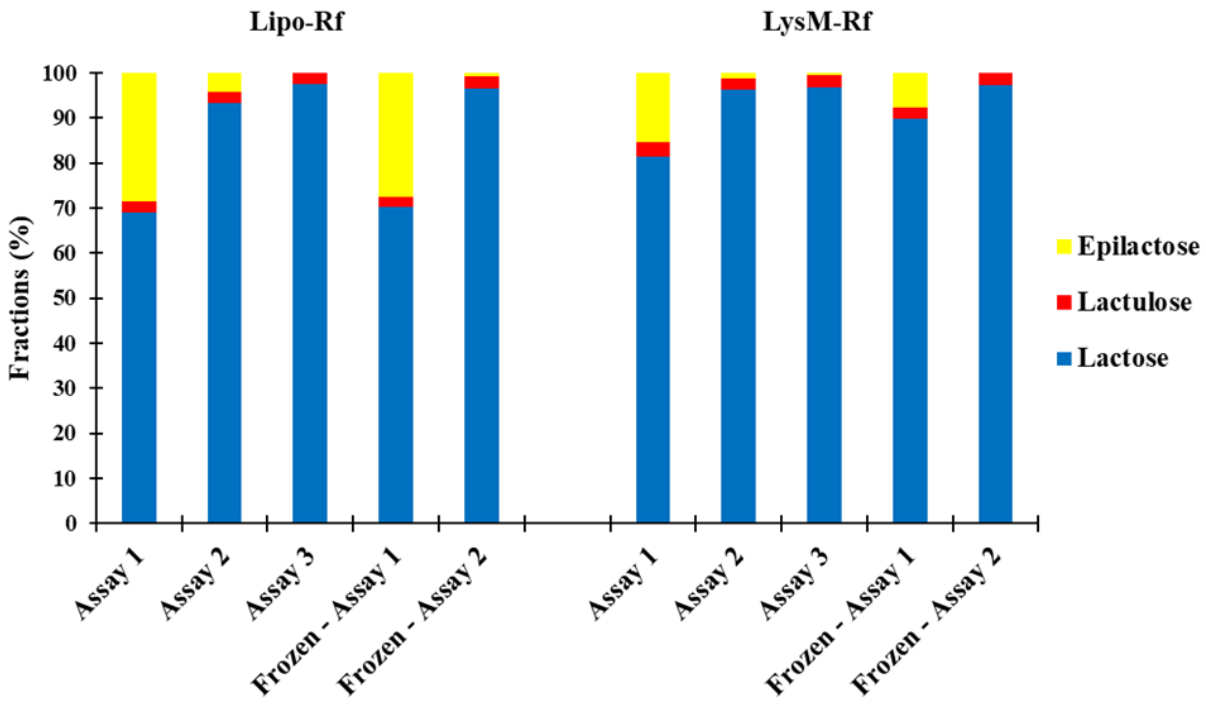


Figure 20. Activity assay results with non-frozen and frozen recombinant *L. plantarum* harboring Lipo-Rf and LysM-Rf, in which the bacteria have been reused in multiple assays. Assay 1 indicate freshly harvested cells, and Assay 2 and Assay 3 indicating the number of reuses. Frozen – Assay 1 indicate frozen cells used for the first assay reaction, Frozen – Assay 2 indicate reuse of frozen cells. All results are presented as fractions, using data from two biological replicates.

3.6.1 Analysis of *L. plantarum* after enzyme activity assays

The results presented in section 3.6, shows that reusing recombinant *L. plantarum* harboring Lipo-Rf and LysM-Rf is not feasible. Investigation as to why the strains lost most of their epilactose production ability after reuse were undertaken. Possible hypotheses for the reduction in epilactose production was thought to be degradation or lysis of the bacteria or shedding of anchored RfCEP from the bacterial surface.

To begin the investigation, the CFU/mL of recombinant strains were measured before and after activity assays at standard conditions (Table 13) (Section 2.25). The results showed that most bacteria did not survive incubation. The most pronounced decrease in CFU/mL was found in *L. plantarum* harboring pEV and LysM-Rf, with a decrease of 99.5 % and 94.0 % CFU/mL respectively (Table 13). The decrease in CFU/mL for *L. plantarum* harboring Lipo-Rf was 86.3 %.

Table 13. Reduction in CFU/mL for induced *L. plantarum* before and after a 24-hour enzyme activity assay. Results are based on the average values of two biological replicates.

Plasmid	CFU/mL before activity assay	CFU/mL after activity assay	Decrease in CFU/mL after 24 hours (%)
pEV	1.28×1012	6.60×109	99.5 %
Lipo-Rf	1.90×1010	2.60×109	86.32 %
LysM-Rf	4.00×109	2.40×108	94.00 %

3.6.2 Western blot of reaction solution

The results from section 3.6.1 revealed a low survival rate of recombinant *L. plantarum* after enzyme activity assays. In addition, the strains lost most of their enzyme activity after only one reuse (Figure 20). A likely hypothesis was shedding of anchored RfCEP from the bacterial surface into the solution.

To investigate the presence of shed RfCEP in the reaction solution, bacteria was first separated from the reaction solutions by centrifugation. The resulting cell-free reaction solution was prepared for Western blot analysis by two methods. The first method used 20 µL of the cell-free reaction solution directly for sample preparation and SDS-PAGE (section 2.22). The second method used TCA to precipitate the proteins in 600 µL of the cell-free reaction solution (section 2.21). TCA precipitation was used to increase the protein concentration before SDS-PAGE, to provide a stronger signal in the Western blot analysis. The SDS-PAGE included protein extracts of *L. plantarum* harboring pEV as a negative control, and *L. plantarum* harboring Lipo-Rf and LysM-Rf as positive controls.

The Western blot results in Figure 21 shows two distinct bands for the positive controls, marked by a blue arrow for Lipo-Rf, and a red arrow for LysM-Rf. The lanes with proteins from the assay reaction solutions shows no bands that correspond with either control sample, implying that neither CEase is present.

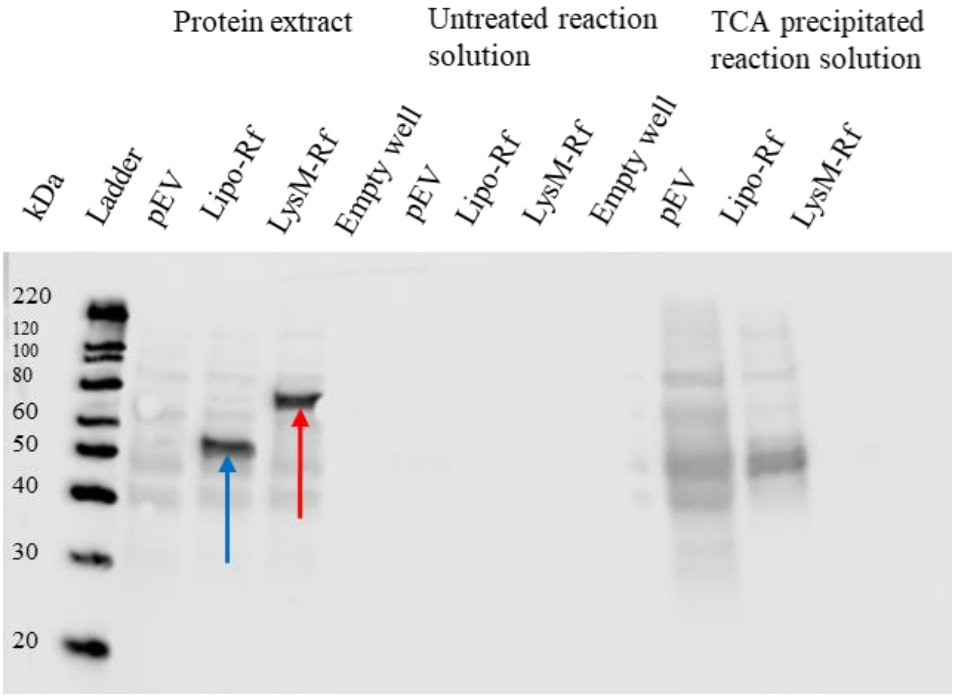


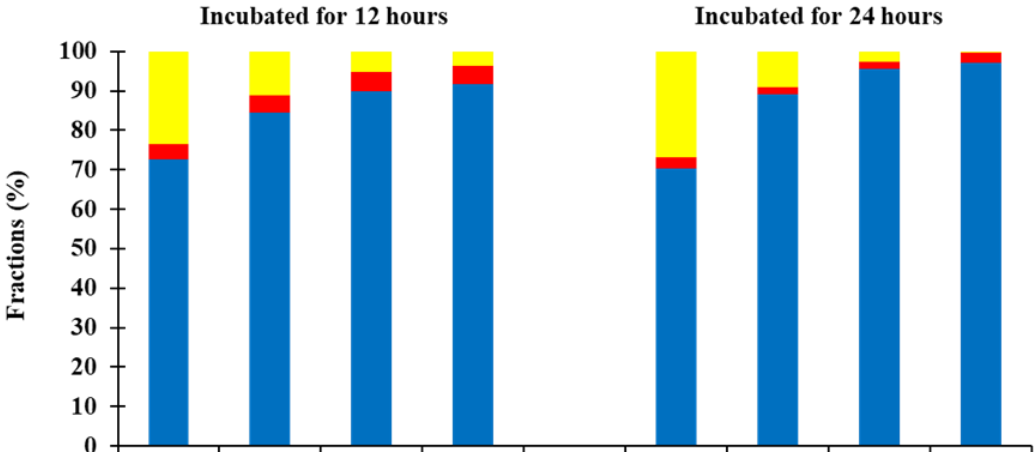
Figure 21. Western blot analysis of samples from reaction solution with recombinant *L. plantarum*. Protein extracts of *L. plantarum* harboring pEV, Lipo-Rf (blue arrow) and LysM-Rf (red arrow) included as control samples. Samples named 'untreated reaction solution', represent protein from 20 µL reaction solution after an activity assay. Samples named 'TCA precipitated reaction solution' represent TCA-precipitated protein from 600 µL of reaction solution after an activity assay. The protein gel used for the Western blot can be found in section 6.2 of the Appendix.

3.7 Combined effects of temperature, pH, and time

Temperature and pH are important conditions that influence enzyme activity. The standard conditions (Table 12) were selected to accommodate both CbCEP and RfCEP. The free form of RfCEP had been characterized earlier, which established their optimum conditions (Jameson et al., 2021). To investigate if these optimum conditions held true for the surface-displayed form of RfCEP, assays were set up with *L. plantarum* harboring Lipo-Rf and LysM-Rf which changed the temperature and pH conditions to investigate their individual effect on epilactose production. Samples were incubated for 12 and 24 hours.

Figure 22 shows how epilactose production was influenced by temperature, pH and time in assays using *L. plantarum* harboring Lipo-Rf. Interestingly, the samples incubated at standard conditions showed the highest epilactose fraction, after both 12 and 24 hours. In theory, the samples incubated at 50.0 °C and pH = 8.0, which is the theoretical optimum conditions found

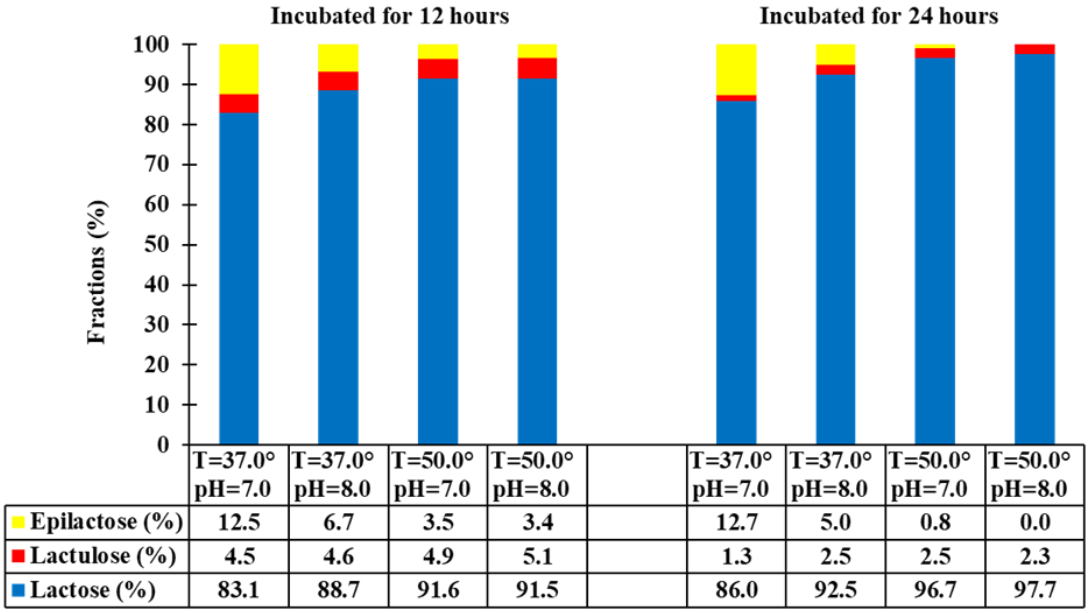
by Jameson et al., were expected to outperform the samples incubated at standard conditions. Conversely, the samples incubated at the theoretical optimal conditions yielded the lowest epilactose fraction (Figure 22). The experiment revealed that temperature influenced epilactose production more than the pH value, as both samples incubated at 37 °C yielded a higher epilactose fraction than samples incubated at 50 °C. The experiment shows the standard conditions are superior for epilactose production, however, incubation for 24 hours leads only to a minor increase in the epilactose fraction compared to incubation for 12 hours.



	T=37.0° pH=7.0	T=37.0° pH=8.0	T=50.0° pH=7.0	T=50.0° pH=8.0		T=37.0° pH=7.0	T=37.0° pH=8.0	T=50.0° pH=7.0	T=50.0° pH=8.0
Epilactose (%)	23.5	11.1	5.3	3.6		26.7	9.2	2.6	0.4
Lactulose (%)	3.9	4.3	4.7	4.8		3.0	1.7	1.8	2.4
Lactose (%)	72.6	84.5	90.0	91.7		70.2	89.1	95.7	97.1

Figure 22. Assay results showing the effects of pH, temperature, and time, using recombinant *L. plantarum* harboring Lipo-Rf. The results are presented as fractions, based on sample data from two biological replicates. Effects of temperature, pH, and time on epilactose production were all significantly different from standard conditions ($p < 0.05$).

Figure 23 shows how epilactose production was influenced by temperature, pH and time in assays using *L. plantarum* harboring LysM-Rf. The results show that the standard conditions yielded the greatest epilactose fractions, with temperature as the most important condition.



	T=37.0° pH=7.0	T=37.0° pH=8.0	T=50.0° pH=7.0	T=50.0° pH=8.0		T=37.0° pH=7.0	T=37.0° pH=8.0	T=50.0° pH=7.0	T=50.0° pH=8.0
Epilactose (%)	12.5	6.7	3.5	3.4		12.7	5.0	0.8	0.0
Lactulose (%)	4.5	4.6	4.9	5.1		1.3	2.5	2.5	2.3
Lactose (%)	83.1	88.7	91.6	91.5		86.0	92.5	96.7	97.7

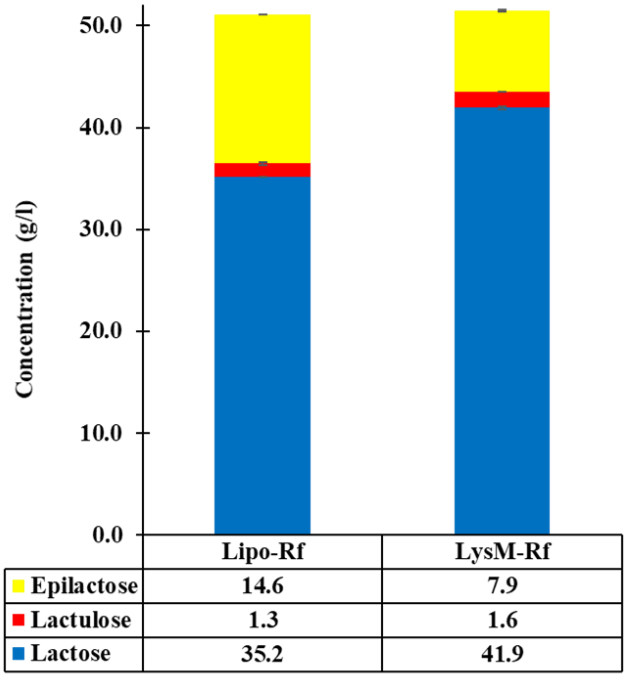
Figure 23. Assay results showing the effects of pH, temperature, and time, using recombinant *L. plantarum* harboring LysM-Rf. The results are presented as fractions, based on sample data from two biological replicates. Effects of temperature, pH, and time on epilactose production were all significantly different from standard conditions ($p < 0.05$).

Comparing the results in Figure 22 and Figure 23 shows that the epilactose production of *L. plantarum* harboring Lipo-Rf and LysM-Rf are similarly affected when incubated at the same specific temperatures and pH conditions, with no discernible benefit given either anchor strategy.

3.8 Quantification and reporting by ICS-6000

Samples analyzed by HPAEC were quantified and reported in grams per Liter. Unfortunately, the system initially underreported concentrations in samples making it impossible to use quantification data directly to compare samples. Converting the reported values from g/L into fraction values made sample comparison possible. Analysis by HPAEC was improved gradually, particularly by the inclusion of mannose as an internal standard.

Eventually, the reported quantitative data could be used directly for sample comparison. Figure 24 shows quantitative values reported in g/L from analysis of assay reaction solutions using *L. plantarum* harboring Lipo-Rf and LysM-Rf using standard conditions (Table 12) and illustrates the superior production capability of the strain harboring Lipo-Rf.



	Lipo-Rf	LysM-Rf
Epilactose	14.6	7.9
Lactulose	1.3	1.6
Lactose	35.2	41.9

Figure 24. Results from enzyme activity assays presented in g/L using *L. plantarum* harboring Lipo-Rf and LysM-Rf incubated at standard conditions. The results are based on two biological replicates. The standard deviations are based on mean values of the technical triplicates from each biological replicate.

4 Discussion

The free form of the cellobiose 2-epimerases CbCEP and RfCEP was characterized in 2021 by Jameson et al. and showed that both CEases had potential for use in epilactose production using lactose as a substrate. In this thesis, the CEases were fused with anchor proteins and cloned in inducible pSIP vectors for surface display of the CEases in recombinant *L. plantarum* and *L. reuteri*. The recombinant strains were investigated in growth experiments, Western blot, and flow cytometry analysis as well as enzyme activity assays. Due to time constraints, only *L. plantarum* harboring Lipo-Rf and LysM-Rf were selected for in-depth characterization in enzyme activity assays.

4.1 Plasmid construction

The initial plan for surface display of CEases utilized existing pSIP403 vectors with the 256/pUCori-replicons as backbones for sub-cloning in *E. coli*. The sequencing results of plasmids isolated from recombinant *E. coli* showed mutations in the target genes (Figure 8), leading to out of frame mutations. The fact that all sequenced transformants had point mutations knocking out target gene expression, strongly indicate that expression of both CbCEP and RfCEP are toxic in *E. coli*.

Toxic gene effects are not uncommon when expressing heterologous proteins. The SppIP promotor with the 256-replicon has a small leakage in *E. coli*, the leak can induce a minor expression of the target genes without exogenous addition of the inducer peptide (Sørvig et al., 2003; Wiig, 2020). If expression of the target protein is toxic to *E. coli*, the effect could be lethal. This toxic effect may explain why all the isolated *E. coli* transformants showed target gene mutations.

After multiple failed attempts at sub-cloning in *E. coli* with the pSIP403 system, the choice was made to switch to the pSIP411 system with the Sh71-replicon and sub-cloning in *L. lactis*. Sub-cloning in *L. lactis* quickly established strains harboring Lipo-Cb, Lipo-Rf, LysM-Cb and LysM|-Rf (Figure 9), plasmids were subsequently isolated and transformed to *L. reuteri* and *L. plantarum*. Sequencing of the plasmids for covalent cell wall anchored CEases (cwa2) transformed to *L. lactis* revealed point mutations causing frameshift mutations in the target sequence. The mutations may indicate a toxic gene effect by the covalent cell wall

anchor (cwa2) used to express the CEases in *L. lactis* and may be influenced by the fact that both RfCEP and CbCEP are codon-optimized for expression in *E. coli*.

4.2 Growth rates of recombinant strains

All recombinant strains harboring plasmids for surface display of CEases (Figure 9) show reduced growth after being induced compared to pEV (Figure 10). This growth reduction is a common stress response observed when using the pSIP system for overproduction of heterologous proteins (Kuczkowska et al., 2019a; Mathiesen et al., 2020).

The growth rates of recombinant *L. reuteri* (Figure 10B) shows the strains harboring Lipo-Cb and LysM-Cb are most affected by inducing to competence, implying that expression of CbCEP is more detrimental to the growth rate. Growth rates for induced *L. reuteri* harboring Lipo-Rf and LysM-Rf are both reduced compared to pEV, but to a lesser degree than *L. reuteri* harboring Lipo-Cb and LysM-Cb and implies that expression of RfCEP is more tolerable.

Induced *L. plantarum* (Figure 10A) harboring Lipo-Rf and LysM-Rf show high growth rates from the point of inducing until ~2-3 hour after inducing, at which point the growth rates stagnate, likely due to cell stress caused by high levels of expressed target protein. The lowest growth rates are found in *L. plantarum* harboring Lipo-Cb and LysM-Cb (Figure 10A), implying that expression of CbCEP is more stressful for *L. plantarum* than expression of RfCEP.

4.3 CEase expression by Western blot in recombinant strains

The Western blot analysis of cell free protein extracts (Figure 11) from recombinant *L. plantarum* showed successful expression of Lipo-Rf and LysM-Rf. No signal was detected from *L. plantarum* expressing Lipo-Cb and LysM-Cb, indicating the absence of expressed target proteins. In contrast, results from flow cytometry and enzyme activity assays with *L. plantarum* revealed the presence of expressed CbCEP. The lack of a signal from protein extracts from *L. plantarum* harboring Lipo-Cb and LysM-Cb in the Western blot analysis could be due to a low degree of binding between the Myc-tag and antibodies. This is unlikely,

as a clear signal was observed in recombinant strains harboring Lipo-Rf and LysM-Rf. However, the anti-Myc primary antibodies used for staining had passed their expiration date, potentially reducing their binding ability. If the strains harboring Lipo-Cb and LysM-Cb have a reduced degree of expression of the target proteins, as indicated by the low growth rate discussed in section 4.2, the low degree of antibody hybridization could yield a signal that was too weak to register compared to Lipo-Rf and LysM-Rf. Another possible cause for the lack of signal may be due to a technical error committed when preparing the protein extracts from *L. plantarum* harboring Lipo-Cb and LysM-Cb.

The Western blot results from recombinant *L. reuteri* (Figure 11) did not show presence of any expressed CEase. The Western blot results for *L. plantarum* and *L. reuteri* (Figure 11) both show protein bands that do not correspond to the sizes of the anchored CEases, caused by non-specific staining.

4.4 Detection of surface anchored CEases in recombinant strains

Flow cytometry of the *L. reuteri* strains (Figure 12B) yielded no fluorescent signal, implying absence of anchored CEase on the bacterial surfaces. This finding reinforced the results from the Western blot analysis which did not detect any expressed CEases in the *L. reuteri* strains.

Figure 12A shows weak fluorescence signals for all recombinant *L. plantarum*. The signals from Lipo-Cb and LysM-Cb was surprising, as the Western blot analysis (Figure 11A) did not reveal their presence. *L. plantarum* harboring LysM-Rf and Lipo-Rf showed the strongest fluorescence shifts. This is expected, as the LysM domain anchors the heterologous proteins on the cell wall, increasing exposure.

The fluorescent signals obtained from flow cytometry with recombinant *L. plantarum* (Figure 12A) can be considered weak, but not uncommonly so, as similar flow results can be seen in other studies where heterologous proteins are anchored to the surface of bacteria (Mathiesen et al., 2020; Nguyen et al., 2019). One explanation for the weak signals may be due to the Myc-tag being embedded in the cell wall. To investigate this hypothesis, the induced strains could have been treated with mutanolysin or lysozyme prior to staining for flow cytometry exposing embedded Myc-tags would lead to increased antibody hybridization, and thereby potentially increasing the signal strength.

A more likely explanation is that the primary antibody (Anti-Myc) used in the Western blot analysis and flow cytometry has a poor degree of hybridization as it is past its expiration date. This hypothesis is backed by the fact that signal strength is generally poor or non-existent in all Western blots and flow cytometry results, except for results using *L. plantarum* harboring Lipo-Rf and LysM-Rf. The results from enzyme activity assays with all recombinant *L. plantarum* and the recombinant *L. reuteri* harboring Lipo-Rf and LysM-Rf proved these strains expressed and surface-displayed the heterologous gene products based on their epilactose-producing ability.

4.5 Enzyme activity assays

Based on the results from initial enzyme activity assays, *L. plantarum* harboring Lipo-Rf and LysM-Rf were selected for further analysis.

4.5.1 Epimerase characterization standard condition bias

The standard conditions in Table 12 are largely based on characterization of the free form of CbCEP and RfCEP done by Jameson et al. in 2021 and intended to give suitable conditions for both CEases.

The incubation time chosen was 24 hours and was picked as the default based on characterization by Jameson et al. (2021). The temperature was set to 37 °C, which is closest to the RfCEP optimum. The temperature was deliberately set this low to minimize production of lactulose by CbCEP, which occur at higher temperatures.

Sodium phosphate buffers affect chemical lactulose conversion more than the MOPS buffer does (Jameson et al., 2021). Therefore, a 100 mM MOPS buffer was used. The buffer pH of the standard conditions (Table 12) are not optimal for either CbCEP (optimum pH = 7.5) or RfCEP (optimum pH = 8.0). However, low pH values significantly decrease chemical isomerization of lactose into lactulose in this buffer system (Aider & Halleux, 2007; Jameson et al., 2021), which is why pH = 7.0 was selected for epilactose production.

All assays were set up using 50.0 g/L lactose and yielded the highest epilactose production, as observed by Jameson et al. (2021) Another reason for keeping the lactose concentration high

was the propensity of CbCEP to shift towards lactulose production at lower lactose concentrations (Jameson et al., 2021).

The shaking speed was set to 350 RPM. This setting was deliberately higher than the minimum 180 RPM, and lower than the maximum 800 RPM used for characterizations of the free form of CbCEP and RfCEP by Jameson et al. (2021) The shaking speed was set at 350 RPM with the intention to avoid exposing bacteria overly stressful conditions while also ensuring sufficient movement to properly mix the contents.

The number of bacteria added to each reaction (2.7×10^9 CFU/mL) was selected for practical reasons. Each recombinant strain was grown and induced in 50.0 mL cultures (section 2.18.1), this ensured each culture yielded enough bacteria to set up of seven or eight assay reactions at minimum. If the experiment required more reactions, additional 50.0 mL cultures were cultivated.

4.6 Enzyme activity assays with recombinant *L. reuteri*

The recombinant *L. reuteri* showed no sign of expressed CEase in Western blot analysis. Similarly, flow cytometry with recombinant *L. reuteri* did not indicate presence of surface displayed CbCEP or RfCEP. Interestingly, growth experiments showed that inducing recombinant *L. reuteri* decreased their growth rate (Figure 10), a typical sign of cell stress caused by heterologous gene expression.

Enzyme activity assays using *L. reuteri* harboring Lipo-Rf and LysM-Rf produced detectable epilactose (Figure 14). The presence of epilactose serves as qualitative proof that Lipo-Rf and LysM-Rf is expressed and functional in *L. reuteri*. The lack of epilactose production in *L. reuteri* harboring Lipo-Cb and LysM-Cb may be caused by the bacteria breaking down the expressed gene products. This can be inferred from the lack of signal in both the Western blot analysis and the flow cytometry. The growth curve analysis of induced strains shows cell stress in the form of growth reduction, and likely the bacteria breaking down the expressed heterologous proteins. *L. reuteri* harboring Lipo-Rf and LysM-Rf also display reduced growth when induced, a sign that expression of the target genes causes the bacteria to break down the heterologous gene product. However, enough expressed heterologous protein evade

degradation, proven by the strains' ability to produce epilactose in enzyme activity assays (Figure 14).

4.7 Enzyme activity assays with recombinant *L. plantarum*

All recombinant *L. plantarum* produced epilactose. Because of time constraints, only *L. plantarum* harboring Lipo-Rf and LysM-Rf were selected for more in-depth characterization.

The experiment investigating the effect of shaking speed (Figure 16) showed no difference in epilactose production in reactions incubated at different RPM settings. However, the experimental setup is flawed as the samples were only analyzed after the full 24 hours and does not reveal if the different RPM settings influenced the epilactose production rate over time. If the experiment had been set up to include earlier points in time the RPM effect on epilactose production may have been more apparent, as the higher RPM increase the chances for interaction between the enzymes and substrate. Enzyme characterization studies show that shaking speed is an important factor (Ingesson et al., 2001; Mais et al., 2002). However, studies using bacteria with surface-displayed enzymes for biocatalysis regularly incubate samples at shaking intensities lower than 350 RPM (Nguyen et al., 2019; Pham et al., 2019), which is the shaking intensity used in this thesis (Table 12).

The experiment investigating the time effect on epilactose production (Figure 17) showed a marked relationship between incubation time and epilactose production. *L. plantarum* harboring Lipo-Rf and LysM-Rf showed a distinctly rising epilactose fraction in samples incubated for 30 minutes up to samples incubated for 14.5 hours. However, the epilactose fraction only increases between 1-2 % from the 14.5 to the 24-hour mark, marking a stagnation of epilactose production in both strains, suggesting inactivation of RfCEP.

Experiments investigating how the bacterial number affected epilactose production (Figure 18) showed that a higher CFU/mL yielded increased epilactose fractions. This experiment would have benefited from inclusion of additional reactions with higher bacterial numbers to investigate if the epilactose production could be improved. The separate experiments investigating the effects of shaking, time and bacterial amount may have provided more useful data if combined into one single experiment. In so doing, the effects of the shaking intensity in the earlier stages of an incubation could have been determined by including time

as a factor, as it is likely that an increased shaking intensity may improve epilactose production yields in the early stages of the reaction. Additionally, the experiment may have benefited by including assay reactions with higher CFU to find the optimal bacteria number per reaction. By investigating these factors simultaneously, an incubation time, RPM and CFU/mL could have been found for optimal epilactose production.

4.7.1 Freezing and reusing bacteria for multiple cycles

The assay experiment using frozen *L. plantarum* (Figure 19) showed production of epilactose in strains frozen for up to 48 hours. The produced epilactose fraction from *L. plantarum* harboring Lipo-Rf was not affected by freezing if not reused. *L. plantarum* harboring LysM-Rf, showed a ~50 % decrease in its epilactose production ability after freezing. The strains harboring LysM-Rf uses non-covalent LysM-domain anchors for surface-display that may anchor RfCEP in way that increased exposure and degradation by freezing damage, which could potentially deactivate a portion of the enzymes and decrease epilactose production. Another hypothesis may be that freezing contributes to shedding of LysM-Rf from the bacterial surface. If correct, the LysM-Rf shed from the bacterial surface may retain its catalytic properties in the solution. Unfortunately, assay samples were prepared from bacterial pellets made by centrifugation of induced 50.0 mL cultures. The pellets were frozen, then resuspended in 1.0 mL MOPS buffer. This 1.0 mL solution was used to prepare all samples by pipetting appropriate volumes to fresh Eppendorf tubes, removing the excess buffer by centrifugation before resuspending the cell pellet in substrate solution before incubation. If freezing cause shedding of LysM-Rf, removal of the buffer solution used to resuspend the cell pellet may remove much of the shed LysM-Rf and may be the cause of the reduced epilactose fraction seen in frozen strains harboring LysM-Rf (Figure 19). The bacteria were frozen by putting cell pellets directly in the freezer at -20 °C and is likely a suboptimal method that may be more damaging to the bacteria than freeze-drying (Wang et al., 2020), which could not be attempted due to time limitations. The effects of freezing the bacteria may cause degradation of LysM-Rf itself and be the reason for the fall in epilactose production. Freeze-drying is considered a gentler method to freeze *L. plantarum* (Wang et al., 2020), and this method could potentially allow *L. plantarum* harboring LysM-Rf to retain more epilactose production capability after freezing, if the activity loss is caused by shedding of the enzyme.

Reusing the recombinant *L. plantarum* for multiple assay cycles proved neither strain was suitable for reuse after 24 hours incubation (Figure 20). The epilactose production was drastically diminished in the second cycle and close to zero after the third cycle for both strains, the frozen strains proved even less suitable for reuse (Figure 20). This is a major drawback, as reusing bacteria for multiple epilactose production cycles would be advantageous in an industrial setting, negating the need to add fresh bacteria for catalyzing multiple reactions.

To investigate the decline in epilactose production after reuse, the viability of bacteria before and after one assay cycle discovered that less than 15 % of bacteria survived, even in the most resilient strains (Table 13). This high death rate is surprising, as the MOPS buffer is considered a gentle physiological buffer . However, the buffer was solely chosen based on its qualities to ensure a good environment for the CEases, while the effect it could have on the survivability of bacteria may have been underestimated. One reason for the low survival rate may be due to the 100 mM MOPS buffer was prepared from MOPS sodium salt. If the MOPS buffer is prepared from the MOPS sodium salt, it requires several mL of concentrated HCl for pH-adjustment to reach pH = 7.0. This causes formation of NaCl, which can be detrimental to survivability of bacteria in sufficient quantities, even to salt-tolerant strains such as *L. plantarum* (Yao et al., 2020). This issue could have been further investigated by creating the MOPS buffer using both the MOPS sodium salt and the MOPS free acid versions, which creates an almost neutral buffer that requires only slight adjustment with strong acid or base to reach pH = 7.0. Other factors may contribute to the high death rates seen after incubation, such as the long incubation time, lack of nutrients and the shaking intensity, or a combination of these factors. The lowest survival rate after a 24-hour activity assay is seen in strains harboring pEV, implying that surface-display of RfCEP by both Lipo and LysM confers some survival benefit when incubated at the standard conditions. However, inducing the strains to competence leads to cell stress which potentially changes the cell morphology, and the cells individual OD_{600} value. This makes comparison between strains harboring pEV, Lipo-Rf and LysM-Rf using their OD_{600} values a point of contention.

The recombinant strains lost most of their epilactose producing ability after their first reuse (Figure 20). One possible hypothesis for this may be that anchored CEase is shed from the cell surface. This could have been investigated by separating the protein fraction from the cell fraction after the first assay cycle, then reusing both the isolated protein fraction and cell

fraction in new activity assays for epilactose production. Successful production of epilactose using the separated protein fraction could explain the low production seen when reusing bacteria or cell fraction. Production of epilactose by the protein fraction would lend credibility to the hypothesis that anchored CEases are shed from the bacterial surface but remain active in the protein fraction. Instead of this method, a Western blot analysis was performed to determine the presence of Lipo-Rf and LysM-Rf in cell-free reaction solution samples after the first assay cycle (Figure 21). The Western blot analysis of the reaction solutions could not detect bands corresponding to Lipo-Rf or LysM-Rf, not even from the 600 µL TCA precipitated samples. Non-specific staining reveals bands in the TCA-precipitated samples, potentially representing degradation products of Lipo-Rf or LysM-Rf. However, the presence of bands representing proteins with higher molecular weights than Lipo-Rf and LysM-Rf (Figure 21) makes it difficult to point out any smaller bands as potential degradation products with certainty. Only the protein fractions from the reaction solutions were investigated by Western blot (Figure 21). This was an oversight, the bacteria separated from the reaction solution should have been included in the Western blot analysis to detect presence of Lipo-Rf and LysM-Rf. Despite this, the experiments that reused bacteria shows a very low residual enzymatic activity and proves that the recombinant strains retain some anchored CEase after reuse but that the production capability is severely reduced. To further investigate, a semiquantitative Western blot analysis may have been helpful to illustrate the relative amounts of Lipo-Rf and LysM-Rf present on bacteria before and after an assay reaction, as well as the amount present on bacteria after they are reused. Such an analysis would benefit from including serial dilutions of the free form of RfCEP for comparison. However, this method of analysis depends on the fact that surface displayed RfCEP remains intact for staining by antibodies and is not degraded during the activity assay.

Based on the decline in epilactose production after 14.5 (Figure 17), the poor epilactose production observed when reusing bacteria and the lack of signal observed in Western blot analysis of reaction solutions strongly suggests that the anchored CEases are inactivated or degraded in the enzyme activity assays. One hypothesis to explain the degradation of the CEases may be that one or more variables in the standard conditions cause degradation of the anchored CEases. Another hypothesis is that the assay conditions cause cell lysis of the bacteria, releasing one or more of *L. plantarum*s 19 intracellular proteases (Kleerebezem et al., 2003). These released proteases may be active in the MOPS buffer and able to degrade

anchored CEases. It is not certain if the assay conditions cause cell lysis or cell death without lysis, as the CFU-experiment did not investigate this (Table 13). Regardless, the decrease in enzymatic activity and the lack of a signal from Western blot analysis of reaction solutions point to degradation of the CEases as a likely hypothesis to explain the loss of enzymatic activity.

4.7.2 Optimization of temperature- and pH conditions for RfCEP

The experiment intended to increase epilactose production in assays using *L. plantarum* harboring Lipo-Rf and LysM-Rf by using the theoretical temperature- and pH optima discovered by Jameson et al. (2021), which found RfCEPs temperature optimum to be 50 °C, and its pH optimum to be 8.0. The assays were set up with both the optimum and the standard conditions (Table 12), as well as intermediate reactions set up with combinations of the theoretical optimum or standard conditions (Figure 22, Figure 23). Surprisingly, each change from the standard conditions toward the optimum conditions proved detrimental to the epilactose production. The results collected in this experiment point out that the characteristics of RfCEP changes when it is anchored by the lipoprotein and the LysM-anchor, rather than being in its free form.

An alternate hypothesis may be that the increase in pH or temperature creates a harsher environment for the bacteria, increasing the rate of cell lysis, and subsequent release of intracellular proteases, breaking down the surface-displayed CEases. To further investigate this hypothesis, different conditions may have been helpful to create an environment that would be more appropriate for survival of *L. plantarum*.

4.8 Conclusion and future work

The work presented in this thesis established recombinant *L. plantarum* for successful surface-display of RfCEP and CbCEP. The recombinant strains were used in enzyme activity assays for production of epilactose from lactose and can be considered as a small, but successful first step in developing recombinant *L. plantarum* for use in large-scale, biocatalytic production of epilactose.

One challenge of the established strains is their inability for reuse in multiple reactions, a feature that would be beneficial in an industrial setting. The problem with reusing the bacteria may be caused by suboptimal conditions in the enzyme activity assays, such as the shaking intensity, buffer type, pH, and a long incubation time. Further characterization is necessary to optimize conditions and exploring additional strategies such as covalent cell wall anchoring of the cellobiose 2-epimerases may aid in improving enzyme stability.

The recombinant *L. plantarum* established in this thesis should be tested in realistic conditions approximating an industrial setting, using milk or whey filtrate as the substrate for epilactose production. Testing the bacteria in realistic conditions may aid in determining if they can be used for commercial epilactose production.

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6 Appendix

6.1 Appendix A

Covalent cell wall anchoring (Figure 2) of CbCEP and RfCEP required an elaborate construction strategy. Figure 25 shows the 3-step PCR reaction used to create the amplified CbCEP-insert, and Figure 26 shows digestion of the vector backbone and the amplified insert. The digested components are ligated by ElectroLigase, yielding the finished plasmid for covalently cell wall anchored CbCEP. The construction strategy for covalent cell wall anchored RfCEP is identical but uses pET-28a(+)RfCEP (Table 3) for amplification of RfCEP and all exchanges CbCEP-specific primers to RfCEP-specific primers (Table 1).

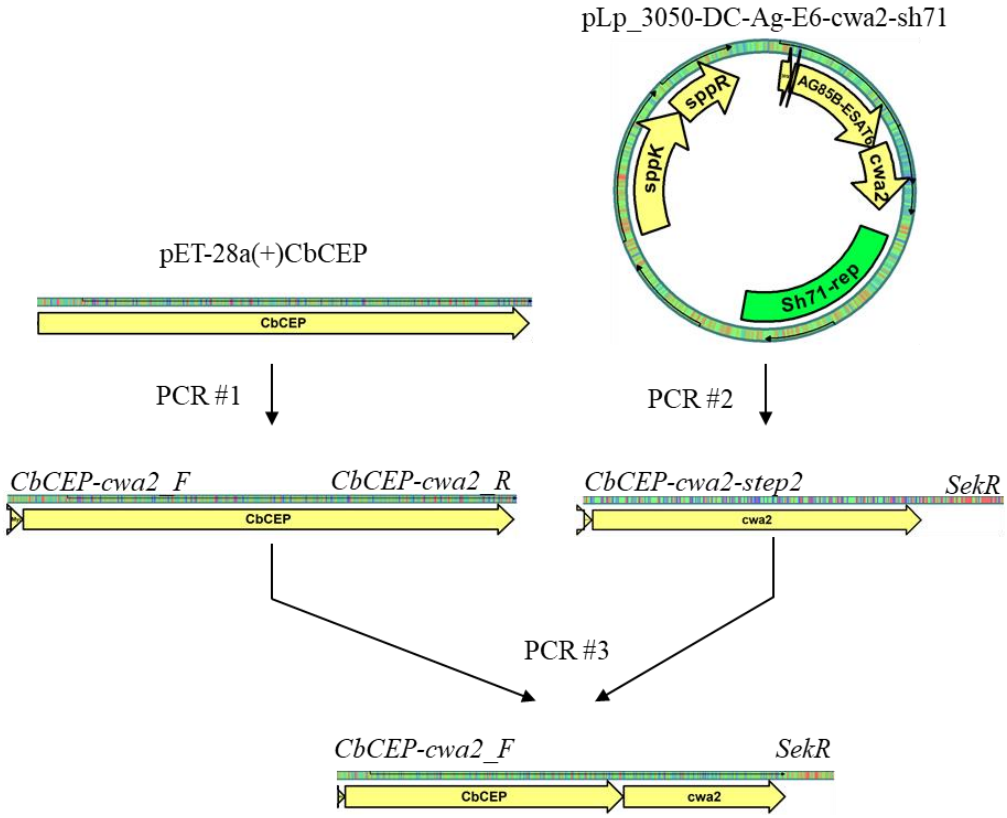

Figure 25. PCR-strategy for creating the insert for cloning the plasmid for covalent cell wall-anchoring of CbCEP in *L. lactis*. Primers are marked by cursive font. PCR #1 amplifies the CbCEP sequence, adding an upstream Myc-tag. PCR #2 amplifies the cwa2 sequence and includes a sequence overlap complementary to CbCEP. In PCR #3 the products from PCR #1 and PCR #2 are combined, creating a sequence that contains the Myc-tag, CbCEP and cwa2 (anchor).

The amplified product created in Figure 25 and the vector backbone are both digested by SalI and HindIII in Figure 26. Following digestion the insert and linearized vector are ligated by ElectroLigase (2.14.2), yielding the plasmid for covalent cell wall-anchored CbCEP (Figure 26).

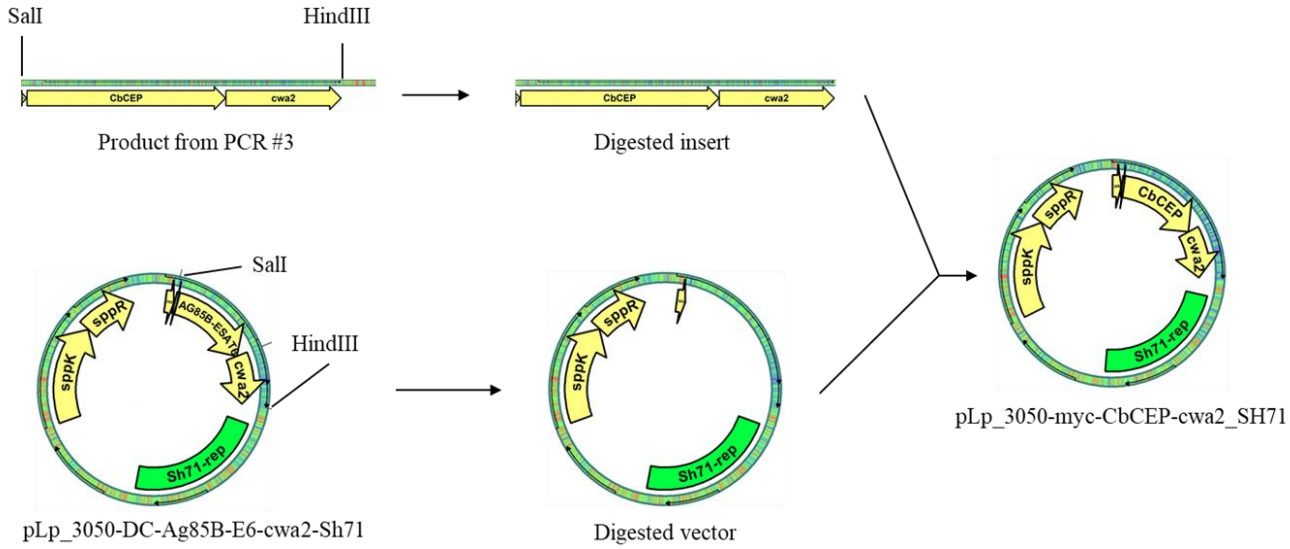

Figure 26. Digestion of vector backbone and the amplified PCR-product from PCR #3 (Figure 25) by SalI and HindIII. The digested vector and insert are ligated by ElectroLigase, yielding the new plasmid.

6.2 Appendix B

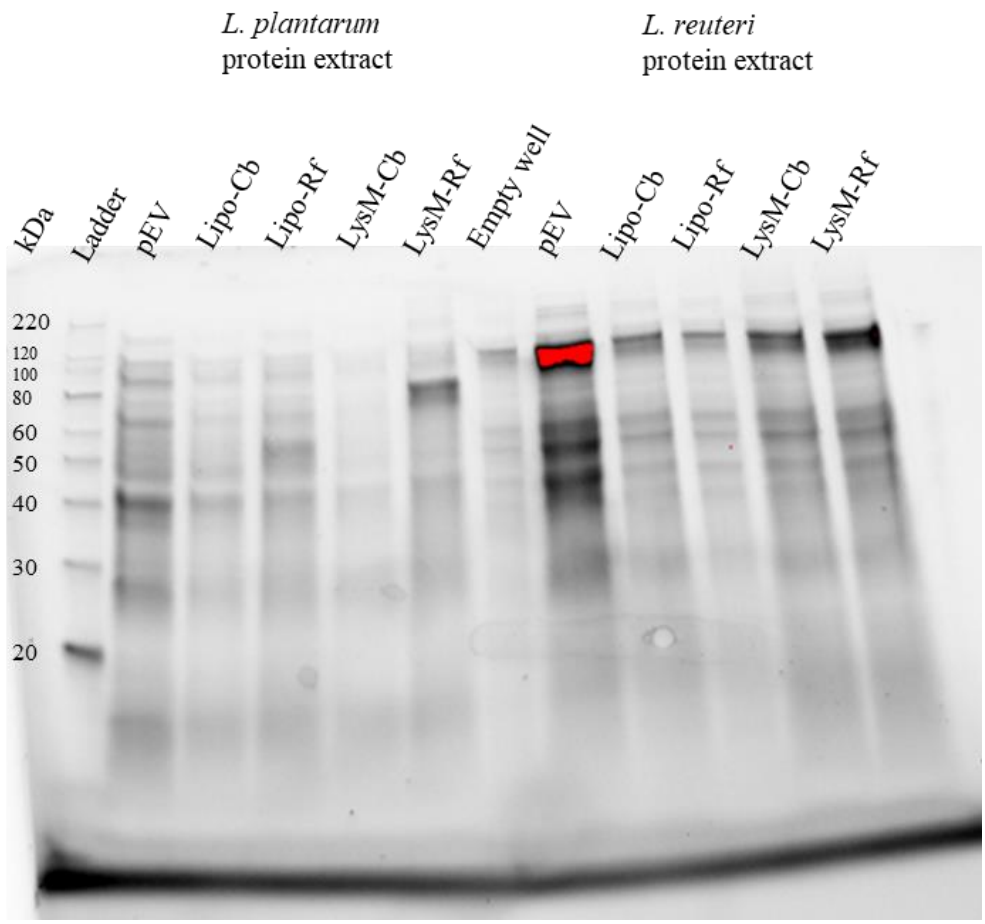

Figure 27. SDS-PAGE of protein extracts from induced *L. plantarum* and *L. reuteri* used for Western blot analysis (Figure 11). First lane contains ladder MagicMark® Western Protein Standard. The theoretical molecular mass of the translated anchor-epimerase products is ~56.0 kDa for Lipo-Cb and Lipo-Rf, and ~69.0 kDa for LysM-Cb and LysM-Rf. Image captured by GelDoc.

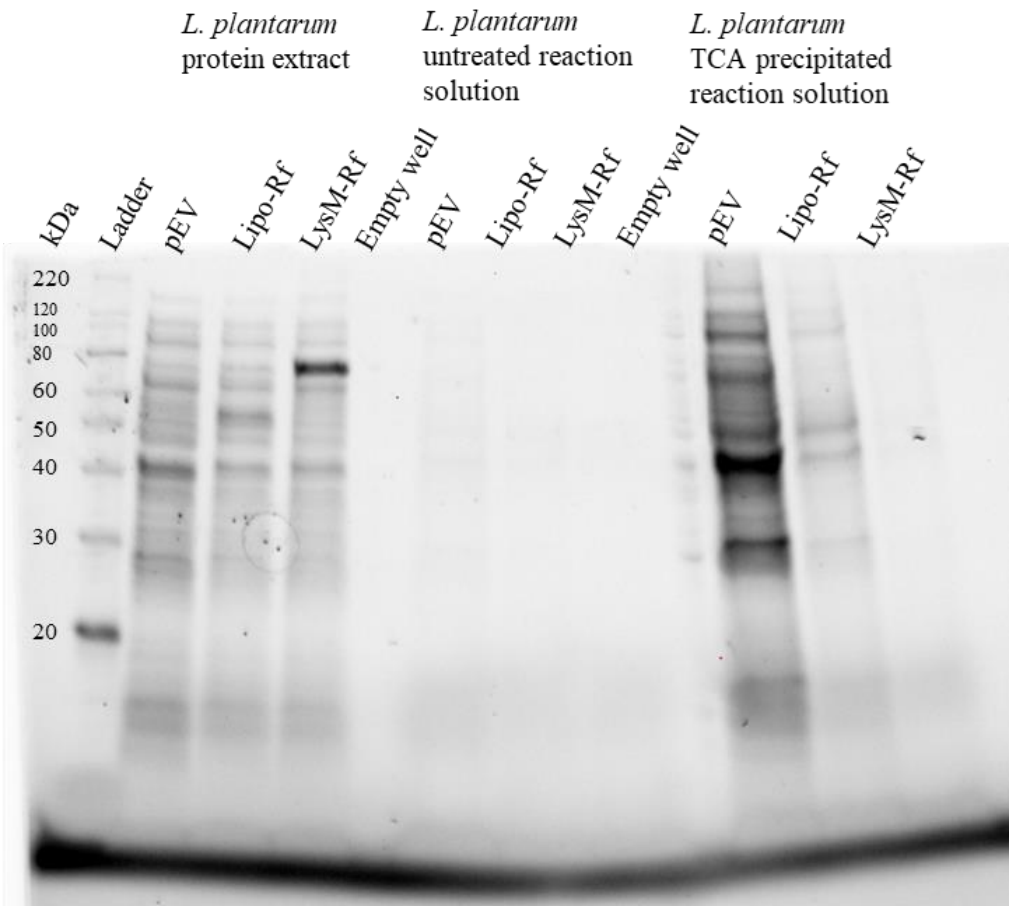

Figure 28. SDS-PAGE of proteins used for Western blot analysis (Figure 21). Protein extracts of *L. plantarum* harboring pEV, Lipo-Rf, and LysM-Rf are included as control samples. Samples named 'untreated reaction solution', represent protein from 20 µL reaction solution after an activity assay. Samples named 'TCA precipitated reaction solution' represent TCA-precipitated protein from 600 µL of reaction solution after an activity assay. Image captured by GelDoc.

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