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Master Thesis

Constitutive expression of a β -mannanase in *Lactobacillus plantarum*

Submitted by
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II. ABSTRACT

Lactobacillus plantarum has been known as a probiotic with GRAS (generally regarded as safe) status and it has been used in various fermentation processes. Due to the potential use of *L. plantarum* as a whole cell biocatalyst, the surface display as well as the constitutive expression of a target enzyme has been studied. Based on the inducible lactobacillal expression system, so called pSIP, a constitutive expression system was developed to express, secret and display the target gene that encodes a β -mannanase (ManB) from *Bacillus licheniformis* strain DSM13 on the surface of *L. plantarum* cells. The promotor regions of the genes, that encode a S-layer protein (SlpA) from *L. acidophilus* ATCC 4356 and a phosphoglycerate mutase (Pgm) from *L. acidophilus* NCFM, were used to replace the inducible pSIP promotor. To achieve the cell surface display an N-terminal lipoprotein-anchor (Lp1261 from *L. plantarum* WCFS1) was additionally fused to the constitutive promotor.

Furthermore, the application of *L. plantarum* as whole-cell biocatalyst in food products and *in-situ* applications remains unaccomplished due to an erythromycin resistance gene (*erm*) used as selection marker. Therefore, within the present work, the erythromycin resistance gene (*erm*) was replaced by an alanine racemase gene (*alr*) and the newly constructed vectors were transformed into *L. plantarum* cells with an alanine racemase knockout in their genome.

Immunofluorescence Microscopy and Flow Cytometry was used to confirm the localization of the enzyme on the cell surface and the expression of β -mannanase was determined using enzymatic activity assays and Western Blot. Furthermore the comparison of the β -mannanase activity obtained with the *alr*- and *erm*-based expression plasmids was conducted. The presence of ManB on the cell surface was confirmed for the strains harboring the *erm*-based constitutive vectors for secretion and anchoring, whereas the *L. plantarum* strains harboring the *alr*-based constitutive vectors showed relatively low surface displayed activity. Further research needs to be conducted with *alr*-based constitutive system to obtain improved secretion efficiencies.

III. KURZFASSUNG

Lactobacillus plantarum ist sehr bekannt als Probiotikum mit GRAS (generally regarded as safe) Status. Dieser Stamm wurde bereits in verschiedensten Fermentationsprozessen eingesetzt und wegen der potentiellen Nutzungsmöglichkeit von *L. plantarum* als „whole cell biocatalyst“ wurde der Zelloberflächen-Display sowie die konstitutive Expression eines Enzymes untersucht. Basierend auf dem induzierbaren *Lactobacillus spp.* Expressionssystem, dem sogenannten pSIP, wurde ein konstitutives Expressionssystem entwickelt, welches das Enzym, β -Mannanase (ManB) von *Bacillus licheniformis* DSM13, exprimiert, sekretiert und auf der Zelloberfläche verankert. Die Promotor Region eines S-layer Proteins (SlpA) aus *L. acidophilus* ATCC 4356 und eine phosphoglycerate mutase (Pgm) aus *L. acidophilus* NCFM wurden verwendet, um den induzierbaren pSIP Promotor auszutauschen. Der Zelloberflächen-Display wurde, durch Fusion eines N-terminalen Lipoprotein-Ankers (Lp1261 aus *L. plantarum* WCFS1) an den konstitutiven Promotor, ermöglicht.

Die Anwendung von *L. plantarum* als „whole cell biocatalyst“ bleibt weiterhin jedoch unerfüllt, da das Erythromycin Resistenzgen (*erm*) als Selektionsmarker verwendet wurde. Aus diesem Grund wurde, in der vorliegenden Arbeit, das Erythromycin Resistenzgen (*erm*) durch das Alanine Racemase Gen ersetzt und der neu konstruierte Vektor wurde in *L. plantarum* Zellen mit einem Alanine Racemase Knockout in ihrem Genom transformiert.

Immunofluoreszenz Mikroskopie und Flow Cytometry wurden verwendet, um die Lokalisation des Enzyms an der Zelloberfläche zu bestätigen. Western Blot und eine spezifische enzymatische Aktivitätsprüfung wurde durchgeführt, um die Expression von β -Mannanase zu bestimmen. Des Weiteren wurde die β -Mannanase Aktivität des *alr*- und *erm*-basierenden Vektorensystems verglichen. Die Anwesenheit von ManB auf der Zelloberfläche konnte für die *erm*-basierten Vektoren bestätigt werden wohingegen *L. plantarum*, welche die *alr*-basierenden Vektoren beherbergten, sehr geringe Zelloberflächen Enzymaktivität zeigten. Weitere Untersuchungen müssen durchgeführt werden, um mit den *alr*-basierenden konstitutiven Systemen verbesserte Leistungen zu erhalten.

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VII. LIST OF ABBREVIATIONS

AB	antibody
BSA	bovine serum albumin
CFU	colony forming units
CM	cell membrane
<i>Erm</i>	erythromycin resistance gene
DNS	3,5-dinitrosalicylic acid
Fw	forward
GRAS	generally regarded as safe
HF	harvesting factor
HK	histidine kinase
IN	intracellular
IP	induction pheromone
LAB	lactic acid bacteria
LBG	locust bean gum
Man	β -mannanase
MOS	manno-oligosaccharides
PCR	polymerase chain reaction
PBS	phosphor buffered saline
QPS	qualified presumption of safety
RBS	ribosome binding bite
RR	response regulator
Rw	reverse
TBS/-T	tris buffered saline/-Tween 20
ORF	open reading frame

1 Introduction

1.1 Lactic acid bacteria

Lactic acid bacteria (LAB), Gram- positive, non-sporing cocci, coccobacilli or rods, have been used in the food and beverage industry as starter cultures in the production of fermented meats, cheese, fermented plant foods, wine, beer, sourdough bread and silage. Over the past few decades LAB have also been used as producers of flavoring enzymes, antimicrobial peptides or metabolites that contribute to the flavor, texture and safety of food products (de Vos 1999b; de Vos 1999a; Kuipers et al. 1997). LAB are able to ferment hexose sugars to produce primarily lactic acid. Acidification caused by the production of lactic acid is the main reason for the preservative effect due to the resulting low-pH condition which inhibits the growth of microorganisms causing food spoilage. *Lactobacillus spp.* are generally the most acid tolerant of the LAB and will therefore end many spontaneous fermentations (Sørvig et al. 2003). Many LAB, such as *Lactobacillus spp.*, *Enterococcus spp.*, as well as *Bifidobacterium spp.*, have been recognized as potentially health promoting microorganisms in the human gastrointestinal tract, also known as probiotics. Besides their food-grade status and probiotic characteristics, their beneficial roles include maintaining the normal microflora, pathogen interference, exclusion and antagonism, immuno-stimulation and immuno-modulation, anti-carcinogenic and anti-mutagenic activities, deconjugation of bile acids and lactase presentation in vivo (Klaenhammer 1998). Therefore several LAB such as *Lactococcus lactis*, *Lactobacillus sakei* and *Lactobacillus plantarum* are considered as safe and effective food expression hosts for food application purposes (de Vos 1999a; Kuipers et al. 1997). Additionally, they are used as attractive alternatives to the well-known bacterial expression systems such as *Escherichia coli* or *Bacillus subtilis*. *Lc. lactis*, *L. sakei* and *L. plantarum* are the best-studied LAB for the purpose of developing effective food-grade expression systems. Based on the ability of *L. plantarum* to persist longer in the intestinal tract and survive the passage through the gastrointestinal tract (Collins et al. 1998), they are more likely to be used in term of *in-situ* production than *Lc. lactis* or *L. sakei*.

1.2 *Lactobacillus plantarum*

L. plantarum has been widely used in food because of its probiotic characteristic and the qualified presumption of safety (QPS) as well as the general regarded as safe (GRAS) status. The gram-positive aerotolerant strain can be found in many habitats such as the gastrointestinal tract and the oral cavity of humans, and in a variety of fermented food products (Axelsson & Ahrné 2000). The used strain in this study is a single colony isolate of strain NCIMB8826, which was originally isolated from human saliva (Kleerebezem et al. 2003).

L. plantarum has been known as a host for the production of heterologous proteins as well as a carrier of proteins that are of interest for food and therapeutic applications (E. Sørvig et al. 2005; E Sørvig et al. 2005; Halbmayer et al. 2008; T. T. Nguyen et al. 2011). This well-studied member of the lactobacilli has been also used as a host for the efficient secretion of heterologous proteins (Mathiesen et al. 2008; Sak-Ubol et al. 2016). Furthermore, *L. plantarum* has been recently exploited as a host for cell-surface display of heterologous proteins, particularly for proteins with medical interests and applications relevant for the food industry (Michon et al. 2016; Fredriksen et al. 2012; Fredriksen et al. 2010; Nguyen et al. 2016).

1.3 Gene expression in *Lactobacillus plantarum*

Overexpression of several enzymes has been done in *Lactobacillus spp.* using the inducible promoter-based pSIP expression system and its derivatives. In the following sections the different expression systems will be explained in detail.

1.3.1 pSIP expression vectors for inducible gene expression

The pSIP expression system for use in lactobacilli is based on the presence of secreted peptide pheromone (Eijsink et al. 2002), also called induction peptide (IP), which regulates the production of some class II bacteriocins by quorum sensing mechanisms. The IP pheromone is ribosomal synthesized, modified and exported to the outside of the cells at low levels (Ennahar et al. 2000). When its concentration outside of the cell reaches a certain threshold level (Nes 1999), the IP activates a two-component regulatory system consisting of a membrane bound histidine kinase HK receptor (encoded by *sapK/sppK*) and a cognate response regulator RR (encoded by *sapK/sppR*) (E. Sørvig et al. 2005). HK senses the pheromone and activates the RR, which induces the cognate promoter of the class II bacteriocins sakacin A (*sap* gene cluster) or sakacin P (*spp* gene cluster) by binding to the promoter at a specific site (Nes 1999).

The pSIP expression system (Sørvig et al. 2003 and Sørvig et al. 2005) is a one-plasmid system developed for inducible gene expression in *Lactobacillus sakei* and *Lactobacillus plantarum*. It consists of two operons, one for the expression of the target gene (originally sakacin A or sakacin P) and one for the two-component regulatory system responding to a separately produced and secreted peptide pheromone (Mathiesen et al. 2004). The operon of the two-component regulatory system consist of a promoter (P_{sapIP}/P_{sppIP}) which is induced by externally added IP, or a native promoter of erythromycin resistance gene via read through. The regulated promoter of the bacteriocin operon, found in *L. sakei*, derived either from sakacin A (P_{sapA}) or from the sakacin P (P_{sppA}/P_{sppQ}) structural gene (E. Sørvig et al. 2005), is responsible for the expression of a target gene. The vector also contains the gene of interest

(target gene), the antibiotic resistance marker (*erm*) and a replicon region. This replicon region encodes the replicon determinants for *E. coli* (pUCori) and the narrow host replicon 256rep, which works in *L. plantarum* and *L. sakei*, respectively (Sørvig et al. 2003). To avoid the losing of plasmid, erythromycin is supplemented in cultivation media with the concentration of 200 µg/ml or 5 µg/ml for *E. coli* or *L. plantarum*, respectively.

Several pSIP vectors with different properties have been designed and previous studies indicate that the sakacin P-based vector pSIP401 is one of the most promising (E. Sørvig et al. 2005). The genes of interest are inserted using the 5'-end *NcoI* site that allows translational fusion to the sakacin P promoter (P_{sppA}), and the 3'-end *EcoRI* or *Acc65I* sites found in a multiple cloning site (MCS) downstream of the *NcoI* (Mathiesen et al. 2008).

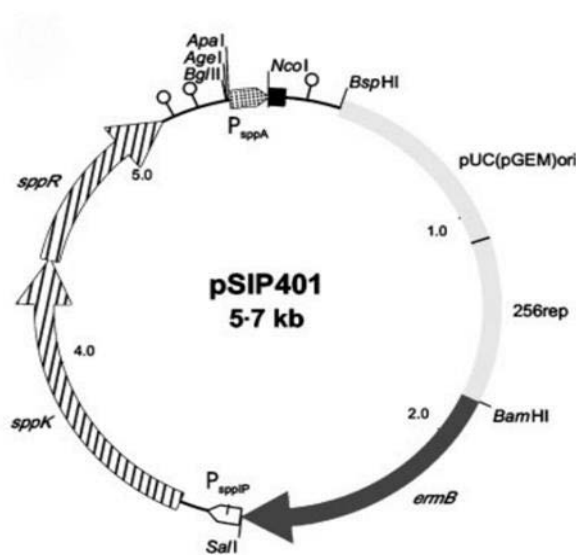


Figure 1: Schematic overview of pSIP401 plasmid. The dotted region determines the inducible P_{sppA} promoter; black box, multiple cloning site; light grey area, replication determinants (pUCori for *E. coli* and 256rep for *L. plantarum*); dark-grey area, erythromycin resistance gene; white region, inducible P_{sppIP} promoter; vertically hatched regions, histidine protein kinase (*sppK*) and response regulator (*sppR*) genes; lollipop structure, transcriptional terminator. The figure is taken from Sørvig et al. 2005.

Mathiesen et al. (2008) adapted the modular pSIP system, precisely the pSIP401 vector to create derivatives which incorporate homologous signal peptides (SP) from *Lactobacillus plantarum* WCFS1 downstream of the P_{sppA} promoter. The *NcoI* restriction site was replaced by *NdeI*, using site-directed mutagenesis, allowing construction of translational fusions without the need to change the second codon of the gene of interest. In addition a *SalI* restriction site in the nonessential region of the vector was removed to create a unique *SalI*, (encoded by valine and aspartic acid) restriction site between the SP and the gene of interest. Thus, the

easy exchange of SP themselves, but also of promoters and target gene can be permitted (Mathiesen et al. 2008).

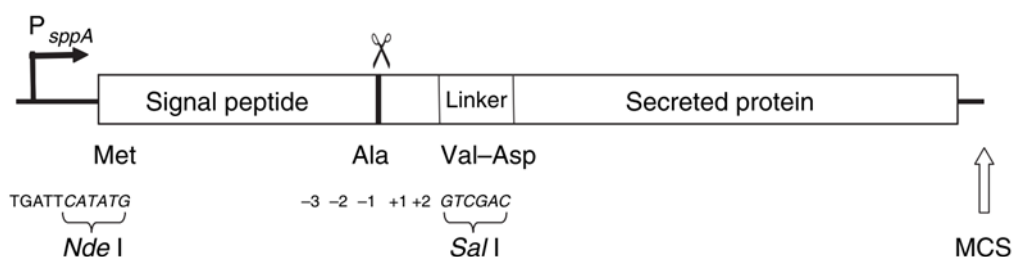


Figure 2: Schematic overview of the secretion cassette. The signal peptide (SP) is translationally fused to the P_{sppA} promoter and includes the first two amino acids of its native gene product. A 6 nucleotides-linker comprising the *SalI* restriction site, encoding valine and aspartic acid was used to fuse the SP to the reporter gene site. The scissor indicates the signal peptide cleaving site and MCS stand for the multiple cloning site downstream of the secreted protein site. The figure was taken from Mathiesen et al. 2008.

The strong inducible pSIP vectors have been proven to be tightly controlled by the addition of IP and give high expression level of recombinant proteins, if necessary at a specific moment of the fermentation. An alternative to these inducible expression vectors would be the use of constitutive expression vectors, which enables the development of LAB as deliverer of therapeutic or enzymatic compounds in the human body.

1.3.2 Constitutive Gene Expression

As mentioned before, the development of constitutive promoter-based displaying vectors enables the *in situ* production of target enzymes in the human body, using *Lactobacillus plantarum* WCSF1 as whole-cell biocatalyst. Additionally, since no synthetic and expensive induction pheromone is needed, the change from inducible to constitutive gene expression is desirable.

1.3.2.1 S-Layer Protein of *Lactobacillus acidophilus* ATCC 4356

The S-layer is a crystalline structure of S-layer proteins, which is considered as a membrane like structure on the outside of several species of archaea and bacteria (Boot et al. 1996). The S-layer protein subunits are linked to the underlying cell and to each other by non-covalent forces (Mobili et al. 2010).

The occurrence of S-layer proteins has been reported for many species of lactobacilli and some are considered as probiotic strains, e.g. *L. plantarum* (Möschl et al. 1993), *L. acidophilus*, *L. helveticus*, *L. casei* and *L. brevis* (Messner & Sleytr 1992). Several specific functions have been linked to the S-layer, such as adhesion, aggregation or pathogen inhibition (Mobili et al.

2010). In fact, these previous listed functions have been also related to some probiotic properties of certain bacteria (Mobili et al. 2010). For example, the interaction of those bacteria with components of the epithelial cell layer of the intestinal tract and in that way the protection from pathogenic bacteria and damages (Mobili et al. 2010)

The wild-type S-layer of *L. acidophilus* ATCC 4356 is build-up of the S_A- protein, which is encoded by the *slpA* gene. This *slpA* gene has been cloned and sequenced previously by Boot et al. 1993, and sequence analysis of the promotor region of the *slpA* expression site revealed two potential promoters, P-1 (-228 to -198) and P-2 (-335 to -303) (Boot et al. 1996).

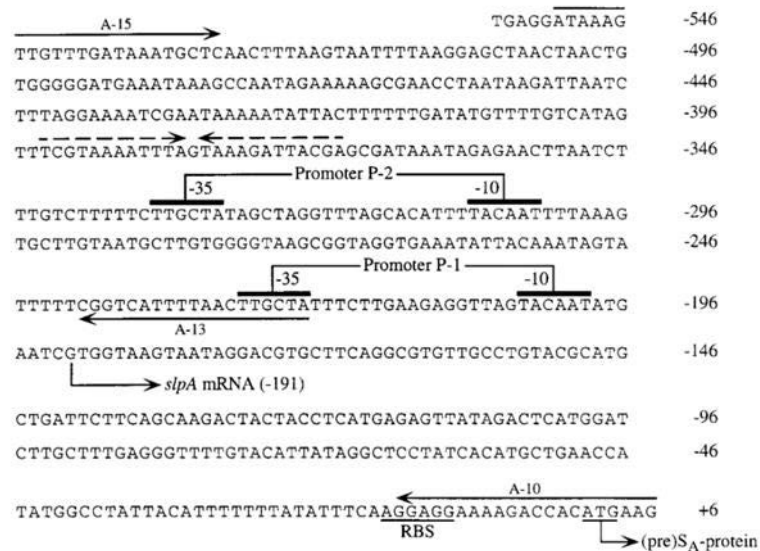


Figure 3: Nucleotide sequence of the promoter region of the *slpA* expression site of *L. acidophilus* ATCC4356. Two potential promoter structures (P-1 and P-2) and the transcription start point of the S_A-protein are indicated. The ribosomal binding site (RBS) before the *slpA* ORF is also indicated. Figure taken from Boot et al. 1996

Boot et al. (1996) successfully transformed a plasmid containing the SlpA promoter and chloramphenicol acetyltransferase (CAT) as reporter protein into *L. casei* ATCC 393, which does not possess a native S-layer. The reporter gene activity was found to be three times higher when using the SlpA promoter than when using the most active homologous promoter known in *L. casei* ATCC 393 (Boot et al. 1996). Furthermore, they found out that both, the P-1 and P-2, -10 and -35 promoter regions are followed by an A+T-rich region, which is known as the UP element. This UP element is interacting with the α subunit of the RNA polymerase in *E. coli* and by doing so the transcription of the target enzyme is stimulated (Ross et al. 1993). Analysis of the transcripts of both promoters during all growth conditions showed that only the P-1 promoter was active whereas no transcript could be detected from the more distinct P-2 promoter. This finding indicates, that the P-2 promoter was not active under the given conditions (Boot et al. 1996).

Computer analysis predicted the folding of the untranslated leader sequence of *slpA* mRNA into an extensive hair-pin like structure. This folding at the 5'-end of the untranslated leader sequence is known to protect mRNA against degradation (Petersen 1992). In the predicted secondary structure the ribosomal binding site (RBS) is exposed in a loop, which is necessary for the hybridization of RBS with the complementary sequences of the 16S rRNA subunit of the ribosome (Selker & Yanofsky 1979). Boot et al. (1996) further found out that the untranslated leader sequence plays an important role in the efficient production of target proteins, since deletion of part of the sequence reduces the activity of the reporter protein by nearly twofold.

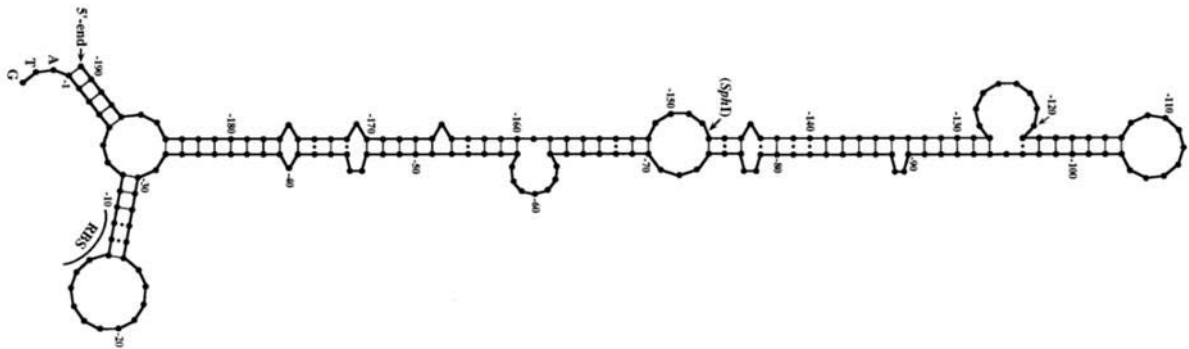


Figure 4: Computer prediction of the secondary structure of the untranslated leader sequence of the *S_A*-protein mRNA. Figure taken from Boot et al. (1996)

1.3.2.2 Phosphoglycerate Mutase (*pgm*) gene of *Lactobacillus acidophilus* NCFM

The promoter for phosphoglycerate mutase (*pgm*) is a strong constitutive promoter derived from *L. acidophilus* NCFM, a probiotic culture widely used in nutritional supplements, dairy products and infant formulas. Duong et al. (2010) used the *pgm* promoter to construct a constitutive expression system with β -glucuronidase (GusA3) as reporter protein. Furthermore, they compared the constitutive promoter (P_{pgm}) to other putative promoters from fructooligosaccharides (FOS), lactose (*lac*), trehalose (*tre*), which were induced by various carbohydrates. Expression levels of GusA3 expressed by the constitutive construct, were approximately 10-fold over the levels observed with the induced constructs (Duong et al. 2010).



Figure 5: Nucleotide sequence of native *pgm* promoter region. Figure taken from Duong et al (2010)

1.3.3 Food grade system for constitutive gene expression

The use of the pSIP vector and its derivations for the expression and secretion of high amounts of heterologous proteins, such as β -mannanase, β -glucuronidase, β -galactosidase, amylase and aminopeptidase in lactobacilli (Sørvig et al. 2003; E. Sørvig et al. 2005; Halbmayer et al. 2008; Mathiesen et al. 2008), has been limited to non-food application, due to the use of an erythromycin antibiotic selection marker. Therefore, the development of constitutive expression and display system based on food-grade selection marker would be the preferred choice towards developing expression systems for the use in food and medical applications.

The classification of food-grade selection markers is done, based on their effects on the host strain, into dominant and complementation markers (de Vos 1999b). Dominant markers usually add a new ability to the host strain and have the advantages that they may be used in a wide variety of strains (T. T. Nguyen et al. 2011). However, those dominant markers are dependent on the supplementation of e.g. carbohydrates or bacteriocins to the cultivation media. Contrariwise, complementation marker do not require supplements in the cultivation medium, though, specific adapted host strain, with deleted or mutated genes on the chromosome are needed for the cultivation under selective conditions (T. T. Nguyen et al. 2011). A wild-type copy of the mutated gene is inserted into the expression vector, therefore only cells hosting the vector are able grow in media without any supplementation.

Alanine racemases (EC.5.1.1.1) are pyridoxal 5'-phosphate-depending enzymes involved in the interconversion of D-alanine and L-alanine (Bron et al. 2002). D-Alanine plays a crucial role in the cell wall assembly of prokaryotes, as it is a necessary precursor in the biosynthesis of peptidoglycan of *L. plantarum* (Palumbo et al. 2004). Furthermore it is needed for the cross-linking of neighboring peptidoglycan strands and it is the major substituent of *L. plantarum* wall teichoic acids and lipoteichoic acids, which are exclusively found in Gram-positive bacteria,

either covalently linked to peptidoglycan or anchored to membrane glycolipid (Palumbo et al. 2004). Palumbo et al. (2003) constructed a stable mutant of *L. plantarum* with a knockout of the alanine racemase and observed a stable and unaffected growth and viability of the cells when the mutant was supplemented with D-alanine. No rapid lysis of the *alr*-deficient *L. plantarum* strain was observed under the shortage of D-alanine during exponential growth phase, as it was observed with *alr*-deficient *E. coli* or *B. subtilis*. However the *L. plantarum* cells were gradually affected in viability (decrease of colony forming units over 99% in less than 24h) and the loss of cell envelope integrity (Palumbo et al. 2004).

Nguyen et al. (2011) constructed an inducible food-grade pSIP vector by exchanging the erythromycin resistance gene with the alanine-racemase gene (Fig. 4). *Lactobacillus plantarum* TLG02 was used as host strain with an alanine-racemase knockout in the chromosome.

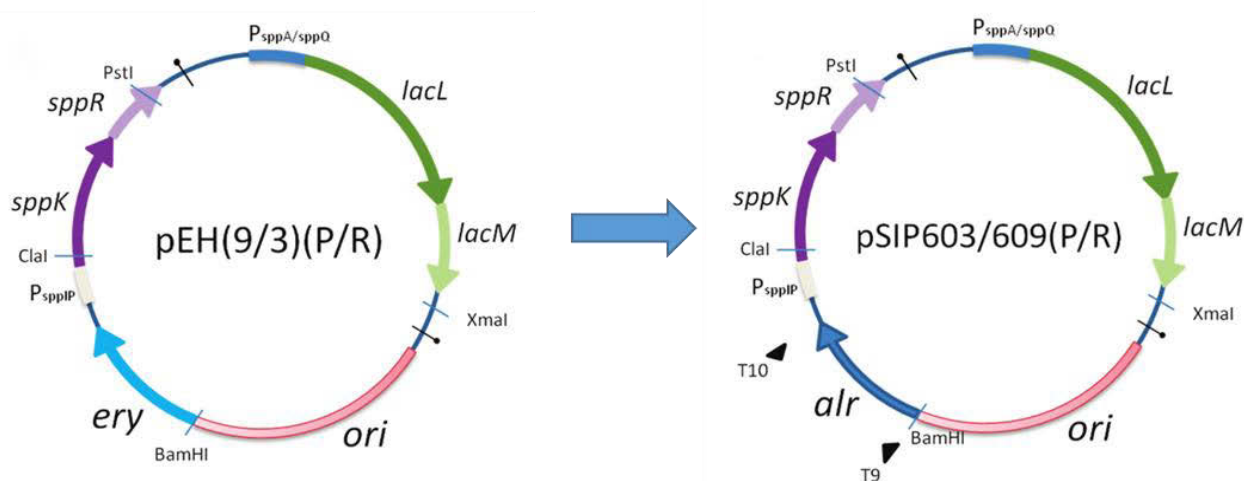


Figure 6: Schematic overview of the expression vectors for LacLM based on the erythromycin resistance gene (*erm*; light blue) and the alanine racemase (*alr*; dark blue) gene as selection marker. P_{sppIP} regulates the *sppK* (histidine kinase) and *sppR* (response regulator). P_{sppA} or P_{sppQ} encodes the inducible promoter which is regulating the *lacLM* gene. Ori stands for the replication determinants for *E. coli* and *Lactobacillus plantarum*. Figure taken from Nguyen et al. (2011).

The obtained expression level of a reporter enzyme, β -galactosidases from *L. plantarum* WCFS1 (P) and *L. reuteri* L103 (R), encoded by two overlapping genes called *lacLM* (Halbmayer et al. 2008), using the food-grade system were compared to the expression levels obtained with the conventional pSIP system. were generally comparable to the levels obtained with *erm*-based vectors (T. T. Nguyen et al. 2011). Fermentation experiments showed that the faster growth of *L. plantarum* TLG02 compared to *L. plantarum* WCFS1 resulted in higher wet biomass and higher volumetric activity (U/mL) of the food-grade system (T. T. Nguyen et al. 2011). Overall it can be confirmed that the new system, using alanine racemase as selection marker, is suitable for the production of ingredients and additives for the food industry.

1.4 Secretion of proteins in Gram-positive bacteria

Secretion typically describes the complete release of a protein from the cell to the extracellular milieu yet proteins can also become inserted in a membrane and function as transporters, channels in energy conversion, sensory transducers, etc. Proteins might also be cell-wall anchored lipoproteins, extracellularly surface displayed or residents of the periplasm (Chatzi et al. 2013). In Gram-positive bacteria seven main protein secretion mechanisms have been characterized by Kleerebezem et al. (2010), whereas the Sec dependent pathway is the most important one.

1.4.1 Sec dependent pathway (I)

Depending on the folding state, the nature and the final destination of the protein, different secretion systems have been evolved by the bacterial cells, whereupon the Sec system is by far the most understood one. It mediates the secretion of most of the expressed proteins and the membrane-embedded Sec-channel (Sec YEG) is conserved in all three domains of life (Van den Berg et al. 2004). Polypeptides that cross the membrane via the Sec-channel are synthesized as preproteins and possess an N-terminal signal sequence of conserved physiochemical properties. The latter will be proteolytically removed in a post-translocation step which leaves the native polypeptide on the *trans* side of the membrane. Crucial for the transportation through the membrane embedded Sec YEG channel is that preproteins remain unfolded and maintain in a non-native state (Chatzi et al. 2013). In addition to the ability to differentiate between secretory and cytoplasmic proteins, signal peptides also function as 'ribosome-to-membrane' address tags (Hegde & Bernstein 2006) and act as allosteric keys at the membrane which activates the Sec-channel (Gouridis et al. 2009). Membrane embedded proteins rarely possess signal peptide sequences, however they are still transported into the membrane via the Sec-pathway. Post- and co-translational translocation is possible within the bacterial cell whereas the vast majority of secretory proteins in bacteria reach the Sec-channel post-translational.

1.4.2 The twin-arginine translocation (Tat) export pathway (II)

The Tat pathway identifies characteristic amino acid motifs, including two consecutive arginine residues, in the signal peptide of the corresponding precursor proteins. In contrast to the Sec pathway, it has the remarkable ability to export completely folded proteins across the cytoplasmic membrane (Freudl 2013). This pathway is most important for proteins which have to recruit a cofactor in the cytosol and therefore need to be exported in a folded state. Also

cofactor-less proteins with rapid folding kinetics are transported via the Tat pathway, because of their exclusion from the Sec pathway (Freudl 2013).

1.4.3 Other secretion pathways in *Lactobacillus* spp.

The VII/WXG100 secretion system (III) is a unique and specialized protein export system which some pathogenic Gram-positive bacteria possess, to secrete their virulence factors, belonging to the WXG 100 or to the PE/PPE families of proteins. It is not yet clear why those proteins cannot be transported via the classical export systems (Sec or Tat) (Freudl 2013). The flagella export apparatus (FEA) (IV) and the fimbriin-protein exporter (FPE) (V). The peptide efflux ABC- ATP- binding cassette- transporters (VI), are primarily involved in the export of antimicrobial peptides, such as bacteriocins. ATP is used to drive the export through the bacterial cell membrane. Hollins (VII) are integrated into the cell membrane and can be found in the genome of *Lactobacillus* genome although they are frequently encoded by bacteriophage genome. The small membrane proteins play a role in the autolysis of the cells and are primarily involved in the secretion of murein hydrolases lacking a signal peptide. (Kleerebezem et al. 2010)

1.5 Anchoring of proteins in Gram-positive bacteria

In nature, cell surface localized molecules are common among bacterial cells. Attached molecules are fundamental to many biological phenomena, such as cell-cell recognition, signal transduction, colonization, immunoreactions, binding and degradation of complex nutrients (e.g. polysaccharides) or nutrient uptake and surface adherence to the host cell (Boekhorst et al. 2006; Samuelson et al. 2002). Generally speaking, many extracellular proteins are attached covalently or non-covalently to the components of the bacterial cell wall, such as teichoic acids or peptidoglycan whilst others are anchored to the membrane (Boekhorst et al. 2006). The membrane anchoring can be obtained through membrane-spanning helices or covalently bound to lipids (Boekhorst et al. 2006).

To obtain the cell surface display of a protein on the microbial cell membrane or cell wall, the protein of interest is fused N- or C-terminally to various anchoring motifs. These anchoring motifs are usually cell surface proteins or their fragments. Also sandwich fusion techniques can be considered for the fusion of target proteins with anchor motifs. (Lee et al. 2003)

Four essential requirements should be covered by a carrier to ensure the successful secretion and subsequent display of the target protein on the cell surface: I) it should encode for a strong signal peptide to ensure the transportation of a premature fusion protein through the inner membrane, II) the anchoring motif should be strong to obtain a tight bond between the target protein and the cell surface, allowing no detachment, III) the carrier should not become

unstable i.e. the compatibility of the carrier and the target protein is inevitable and IV) the sequence should be resistant to proteases in the surrounding periplasmic space. (Lee et al. 2003)

Various biotechnological and industrial applications are possible with the microbial cell surface display. Lee et al. (2003) summarized in their review 'Microbial cell-surface display' following potential purposes: 'live vaccine development; screening-displayed peptide libraries by sequential binding and elution or, more efficiently, by fluorescence-activated cell sorting; antibody production by expressing surface antigens to raise polyclonal antibodies in animals; bioadsorbents for the removal of harmful chemicals and heavy metals; whole-cell biocatalysts by immobilizing enzymes; biosensor development by anchoring enzymes, receptors or other signal sensitive components for diagnostic, industrial or environmental purposes and detection of single amino acids changes in the target peptide after random mutagenesis.'

1.5.1 Extracellular proteins and anchor types in *Lactobacillus plantarum*

The genome of *Lactobacillus plantarum* contains 3018 protein encoding genes, of which 237 genes are predicted to belong to the secretome and are secreted to the outside of the cell. Updated annotations of the secretome of *L. plantarum* as well as the domain composition can be found at http://www.cmbi.ru.nl/lab_secretome/index.php.

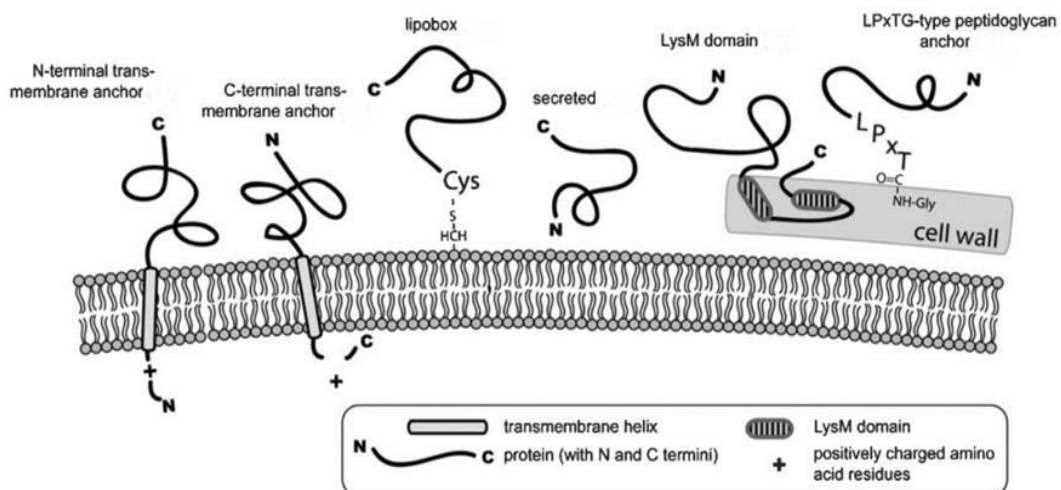


Figure 7: Overview of extracellular anchored proteins in *L. plantarum*. Figure taken from Boekhorst et al. (2006)

These 237 assumed extracellular proteins are secreted to the outside of the cell, released into the media or surface-associated. The latter group can be divided, based on their binding mechanisms, into 4 subgroups: (I) membrane anchored proteins, through a single hydrophobic N- or C- terminal domain, (II) lipoproteins, which are N-terminally anchored to fatty acids of the membrane, (III) C-terminal LPxTG motif proteins, anchored to the peptidoglycan and (IV) noncovalently bound proteins (Kleerebezem et al. 2010).

1.5.2 Lipoprotein anchors

Lipoproteins hold an N-terminal signal peptide, containing the lipobox motif in its C-terminal part, and are therefore compatible for the transport via the Sec pathway. Covalent binding of the lipoprotein to the lipid membrane is achieved through a diacylglycerol transferase, which covalently links a conserved cysteine in the lipobox to a phospholipid in the membrane, while the signal peptide is removed by a lipobox-specific peptidase (Kleerebezem et al. 2010). Those steps lead to the thioether linkage between the mature protein and the membrane (Hutchings et al. 2009). Anchoring of a recombinant protein to the cell membrane can thus be achieved by fusing a heterologous protein downstream of a suitable lipoprotein anchoring gene.

The predicted proteins to hold lipoproteins in *Lactobacillus spp.* mainly encompass the substrate binding proteins of the ABC transporter and also some proteins that are involved in antibiotic resistance, cell-envelope homeostasis and protein secretion, folding and translocation, sensory processes or adhesion (Kleerebezem et al. 2010). Fredriksen et al. (2012) used the 75 residue Lp_1261 lipoprotein, derived from the *L. plantarum* oligopeptide ABC transporter, to display a long (Inv) and a short (InvS) fragment of invasion, from *Yersinia pseudotuberculosis* on the cell surface of *L. plantarum*. Immunofluorescence microscopy and flow cytometry analysis confirmed the presence of the fragments on the cell surface (Fredriksen et al. 2012).

In this study, the lipoprotein Lp_1261 from *L. plantarum* was used for achieving the covalent binding of β -mannanase to the cell surface of the host strain towards the development of *L. plantarum* as whole-cell biocatalyst.

1.4. β - mannanase

β -Mannanase (EC 3.2.1.78), also endo-1,4- β -mannanase or mannan endo-1,4- β -mannosidase, is an enzyme that catalyzes random hydrolysis of β -1,4-mannosidic linkages in the main chain of β -1,4-mannan, glucomannans and galactomannans. Thus, heteromannans are transferred into manno-oligosaccharides and small amounts of mannose, glucose and galactose (Songsiriritthigul et al. 2010). Mannans or heteromannans can be widely found in nature, as key component of lignocellulose, which is part of hemicelluloses in the primary cell

wall of plants. Mannanases were found in several animals, plants, fungi, bacteria and invertebrates.

The production of potential prebiotic manno-oligosaccharides (MOS) from food industry waste materials or agricultural by-products such as coffee bean, palm kernel or copra meal gained significant interest (Songsiriritthigul et al. 2010; Yamabhai et al. 2014). Other applications of β -mannanases have been developed recently in the industrial fields of pulp and paper, food and feed, pharmaceuticals as well as oil and textile. For example, improvement in the gas and oil drilling (Gübitz et al. 2001), pre-treatment of lignocellulosic biomass for the production of biofuel (Sanchez 2009), clarification of juices (Moreira & Filho 2008), improving the digestibility and providing more nutrients to low nutritional value feed (Daskiran et al. 2004), cleaning composition in laundry detergent and other cleansing reagents (Bettiol et al. n.d.; Kirk et al. 2002). (Yamabhai et al. 2014; Songsiriritthigul et al. 2010)

In this study β -mannanase (ManB) derived from *Bacillus licheniformis* DSM13 is the enzyme of interest. *B. licheniformis*, Gram-positive, endospore-forming, non-pathogenic microorganism belonging to the *B. subtilis* group within the genus *Bacillus*, has already been extensively used in large scale enzyme production (Schallmey et al. 2004). Amino acid sequencing of the target enzyme, β -mannanase, revealed a theoretical molecular mass of 41 kDa and showed the affiliation to the glycosyl hydrolase family GH26 (according to the Carbohydrate-Active EnZYme databank, www.cazy.org/) (Songsiriritthigul et al. 2010).

1.6 Aim of this study

The main aim of this master project was to investigate the expression, secretion and display of ManB on the cell surface of *L. plantarum* using the lipoprotein anchor Lp_1261 and two strong constitutive promoters (SlpA and Pgm) towards the development of *L. plantarum* as whole-cell biocatalyst. The second part of the project focused on the construction of novel food-grade anchoring systems based on the above mentioned system. The development of food-grade plasmids is of great interest for the possible applications of those strains in the field of food technology and processing as well as the potential use for medical purposes.

2 Materials and Methods

All present work was done at the Food Biotechnology Laboratory, Department of Food Science and Technology, University of Natural Resources and Life Sciences, Vienna, Austria.

2.1 Materials

2.1.1 Equipment

- C100 Thermal Cycler (Biorad)
- Gel Doc/ Chemie Doc (Biorad)
- Autoclave (Thermo Scientific, Advantage-Lab)
- Power Pac 300 (Biorad)
- Power Pac HC (Biorad)
- Centrifuge 5424 (Eppendorf)
- Centrifuge 5412 (Eppendorf)
- Thermomixer Comfort (Eppendorf)
- Balances (Sartorius)
- Pipettes (Gilson Pipetman; Eppendorf Research)
- Mini Sub Cell GT (Biorad)
- Mini Protean Tatra Cell (Biorad)
- pH and conductivity electrodes (Metrohm)
- Micro Pulser (Biorad)
- Heraus Incubator (Thermo Scientific)
- Shaking Incubator
- Precellys 24 glass bead mill (PEQLAB Biotechnology)
- French Pressure Cell Press (AMINCO)
- Sonoplus HD 60 Sonicator (Bandelin)

2.1.2 Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Restriction enzymes and restriction buffers were purchased from Thermo Fisher Scientific (Waltham, MA, USA) and Biorad (Berkeley, California, USA).

DNS solution

DNS solution was prepared as described previously with small modification (Chaplin & Kennedy 1994).

1 g of 2-hydroxy-3,5-dinitrobenzoic acid (DNS) was added to 20 mL dH₂O stirred and heated at 50°C for 1h hour. Light was avoided by covering the bottle with aluminum foil. After 1 h of heating 20 mL of 2 M NaOH were slowly added. The solution was further mixed with 50 mL KNaC₄H₄O₆·4H₂O. Storage was in dark condition and at room temperature.

PMSF

Phenylmethylsulfonylfluorid (PMSF) used as a protease inhibitor was dissolved in Isopropanol to final concentration of 1 mM

2.1.3 Antibiotics

Stock solutions of erythromycin (5 mg/ml) and ampicillin (100 mg/ml) were prepared with ethanol (96%) and dH₂O. Storage of the stock solutions was at -20°C.

2.1.4 Media

Broth media were prepared as described below and autoclaved with standard conditions at 121°C and 15 minutes prior to cooling at 60°C in a water bath. For the preparation of selective media, erythromycin or ampicillin was added after cooling to the final concentrations as described in 2.2.1. Preparation of media agar plates was done by adding 1.5% (w/v) Agar-Agar Kobe I (Carl-Roth, Germany) to the solution before autoclaving. The cooled, sterile agar media solution, if necessary containing erythromycin or ampicillin, was poured into sterile Petri dishes and storage was possible for several weeks at 8°C.

MRS (de Man, Rogosa, Sharpe)

MRS-Broth was purchased from Sigma-Aldrich and Carl Roth (Karlsruhe, Germany). The media was prepared according to the supplier's manual.

- Sigma-Aldrich: dissolve 51 g of MRS in 1 L of H₂O; add 1mL/L Tween 80
- Carl Roth: dissolve 52 g of MRS in 1 L of H₂O

After dissolving, the MRS broth was sterilized by autoclaving at 121°C for 15 minutes.

LB (Luria-Bertani)

For 1 L LB media

- 10 g peptone from casein
- 5 g yeast extract
- 10 g NaCl (Carl-Roth, Germany)

All components were dissolved in dH₂O.

MRSSM

Preparation for 100 mL:

- 5.2 g MRS
- 0.5 M Sucrose
- 0.1 M MgCl₂ · 6 H₂O

All components were dissolved with dH₂O and sterile filtrated (filter membrane: 0.2µm).

SOC (Super Optimal Broth with Catabolite repression)

Preparation for 100 mL:

- 2 g Bacto-Tryptone
- 0.5 g Bacto-Yeast extract
- 0.01 M NaCl
- 2.55 mM KCl
- 20 mM MgSO₄

All components were dissolved in 60 mL dH₂O and the solution was autoclaved. After cooling down, 1 mL of 2M sterile-filtrated glucose solution was added and the volume was adjusted to 100 mL with sterile H₂O.

2.1.5 Buffers**PBS (phosphate buffered saline) buffer (pH 7.4)**

- 0.14 mM KH₂PO₄
- 0.8 mM Na₂HPO₄
- 1.40 mM NaCl
- 0.27 mM KCl

TBS (Tris-buffered saline) buffer (pH 7.4)

- 0.15 M NaCl
- 0.01 M Tris-Base (pH 7,4)

TBS-T: TBS + 0.05% Tween 20

Lysis buffer (pH 8.0)

- 20 mM Tris-Base
- 150 mM NaCl

Running buffer for SDS- PAGE

Preparation of 1 L running buffer 10x:

- 500 mM 3-(N-Morpholino)propansulfonsäure (MOPS)
- 500 mM Tris-Base
- 1% Sodium Dodecyl Sulfate (SDS)
- 10 mM Ethylenediaminetetraacetic acid (EDTA)

2.1.6 Kits

- Pure Yield Plasmid Miniprep System (Promega, Madison, Wisconsin, USA)
- Illustra GFX PCR DNA and Gel band purification Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK)

2.1.7 Bacterial strains and plasmids

Table 1: Bacterial strains used in this study

Strain	Source of reference
<i>E.coli</i> Neb5 α	New England Biolabs, USA
<i>E.coli</i> MB2159	Strych et al., 2001
<i>Lactobacillus plantarum</i> WCFS1	Kleerebezem et al., 2003
<i>Lactobacillus plantarum</i> TLG02	Nguyen et al., 2011

Table 2: Plasmids used in this study

Plasmid	Description	Reference
pSIP_1261ManB	<i>erm</i> ; inducible pSIP promoter; lipoanchor sequence from Lp_1261 and <i>man-myc</i>	Nguyen et al., 2016
pPgm_ManB	<i>erm</i> ; pSIP_1261ManB derivative with fragment of constitutive promoter <i>pgm</i> instead of inducible pSIP promoter, no lipoanchor sequence from Lp_1261	HM Nguyen*, unpublished
pSlpA_ManB	<i>erm</i> ; pSIP_1261ManB derivative with fragment of constitutive promoter <i>slpA</i> instead of inducible pSIP promoter, no lipoanchor sequence from Lp_1261	HM Nguyen*, unpublished
pPgm_1261ManB	<i>erm</i> ; pSIP_1261ManB derivative with fragment of constitutive promoter <i>pgm</i> instead of inducible pSIP promoter and lipoanchor sequence from Lp_1261	EM Stelzer*, unpublished
pSlpA_1261ManB	<i>erm</i> ; pSIP_1261ManB derivative with fragment of constitutive promoter <i>slpA</i> instead of inducible pSIP promoter and lipoanchor sequence from Lp_1261	EM Stelzer*, unpublished
pJet pgm_1261ManB	<i>amp</i> ; pJET derivative with fragment of constitutive promoter <i>pgm</i> fused with lipoanchor (Lp_1261) and <i>man-myc</i> .	EM Stelzer*, unpublished
pJet slpA_1261ManB	<i>amp</i> ; pJET derivative with fragment of constitutive promoter <i>slpA</i> fused with lipoanchor (Lp_1261) and <i>man-myc</i> .	EM Stelzer*, unpublished
pSIP609gusA	pSIP_1261ManB derivative; <i>erm</i> replaced by <i>alr</i>	Nguyen et al., 2011
pSlpA_1261ManB_alr	<i>alr</i> , pSIP609 derivative with constitutive promoter <i>slpA</i> , lipoanchor sequence from Lp_1261 and <i>man-myc</i> .	This work
pPgm_1261ManB_alr	<i>alr</i> , pSIP609 derivative with constitutive promoter <i>pgm</i> , lipoanchor sequence from Lp_1261 and <i>man-myc</i>	This work

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Figure 8: Expression vectors based on pSIP system with SlpA and Pgm as constitutive promoters, β -mannanase as the target gene, lipoprotein-anchor Lp1261 and selection marker based on erythromycin-resistant gene. (Constructed by Elena-Maria Stelzer)

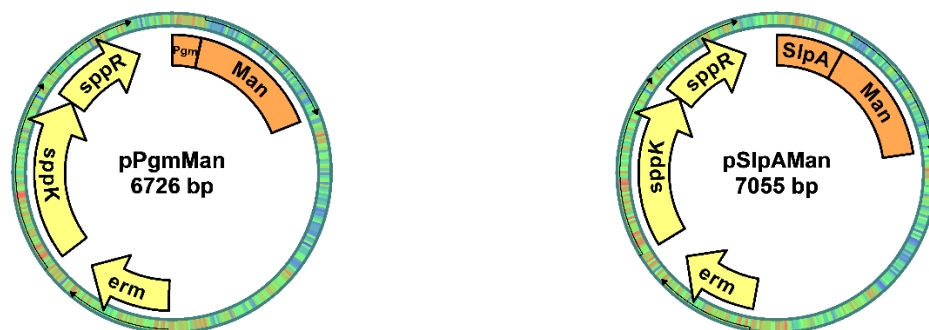


Figure 9: Intracellular expression vector based on pSIP system with SlpA and Pgm as constitutive promoters, β -mannanase as target enzyme and selection marker based on erythromycin-resistant gene. (Constructed by Hoang-Minh Nguyen)

2.2 General microbiological methods

2.2.1 Cultivation of bacterial cultures

Recombinant bacterial cells harboring plasmids consisting of an antibiotic resistance gene or an alanine racemase gene were cultivated in selective media with or without antibiotics, respectively. Single colonies were isolated by spreading the bacteria on plates containing medium solidified with 1.5% (w/v) agar. The following incubation of the plates was done at 37°C, overnight for 18h. A pre-culture was prepared by inoculating the specific media broth with a single colony from the agar plate and cultivation was done at 37°C for 16-18h. To detect the cell density, as well as monitoring the bacterial growth, the optical density of the cell solution was measured with the spectrophotometer (Beckmann- Coulter, Indianapolis, Indiana, USA) at 600 nm (OD₆₀₀).

Escherichia coli

E. coli strains harboring plasmids encoding an ampicillin or erythromycin resistant gene were cultivated at 37°C with shaking in 3 mL of LB media broth containing 100 µg/ml of ampicillin or 200 µg/ml of erythromycin, respectively. The recombinant strains carrying the food-grade plasmids were grown in the LB medium without antibiotics.

Lactobacillus plantarum

L. plantarum strains harboring *erm*- or *alr*-based plasmids were cultivated at 37°C for 16-18 hours without shaking in 10 mL of MRS media broth with or without adding 5 µg/ml of erythromycin, respectively. Such overnight cultures were used as inoculum for subsequent fermentations.

2.2.2 Long-term storage

Bacterial cells used in this study were either stored at 4°C on specific agar plates for maximum 3 weeks or at -80°C in their specific media broth with 20% (v/v) glycerol for several months. Agar plates were sealed with parafilm to prevent drying out of the agar during the storage.

Plasmids were stored at -20°C.

2.2.3 Preparation of electro-competent *Escherichia coli* TLG02

Escherichia coli cells must be made electro-competent to permit transformation of plasmids into the cells by electroporation. Via an electric impulse DNA is transformed into *E. coli* cells. The following protocol describes a method for preparing electro-competent *E. coli* with high efficiencies.

Materials:

- Baffled, sterile, shaking flasks (1L)
- Centrifuge beaker (500 mL)
- sterile 50 mL Greiner tube
- sterile Eppendorf tubes (1.5 mL)
- sterile tips
- measuring cylinder 250 mL and 50 mL
- 10% glycerol
- sterile LB media

Procedure:

Glycerol, all equipment and tubes were kept at 4°C during the harvesting of the cells.

1. 1/100 volume of a fresh overnight *E. coli* culture was inoculated in 300 mL of LB media.
2. Cells were grown at 37°C, with shaking at 160 rpm, to an OD₆₀₀ of approximately 0.6-0.7.
3. The cell culture was transferred to a cool, sterile centrifuge beaker and chilled on ice for 20 minutes. Harvesting of the cells was done by centrifugation at 4°C, maximum 4000 rpm for 15 minutes.
4. Carefully the supernatant was poured off and discarded.
5. The cell pellet was suspended carefully in 250 mL of ice-cold, sterile 10% glycerol and centrifuged again at 4°C, maximum 4000 rpm for 15 minutes.
6. Again the supernatant was poured off and further the pellet was suspended in 125 mL of ice-cold, sterile 10% glycerol. Followed by centrifugation with the previous parameters and discard of the supernatant.
7. 20 mL sterile, ice-cold 10% glycerol were used to suspend the pellet, followed by centrifugation and pouring off the supernatant.
8. Finally the cell pellet was suspended in 1 mL sterile, cold 10% glycerol and this suspension was aliquoted in 50 µL portions in sterile, cold Eppendorf tubes.
9. Storage of the electro-competent cells is possible for up to 6 month at -80°C.

2.3 General Methods in Molecular Biology –DNA manipulation

Plasmids (pPgm_1261ManB, pSlpA_1261ManB, and pSIP609GusA) were isolated from *E. coli* cells using the Pure Yield Plasmid Miniprep System from Promega. The procedure was performed according to the manufacturer protocol. Double digestion of the plasmids was done by using the restriction enzymes *EcoRI* and *BglII* and 10x Tango Buffer (Thermo Fisher Scientific, USA) (Table 3), which lead to digested fragments with sticky ends. The digestion reaction was done at 37°C for 3h.

Table 3: Restriction enzymes used in this study

Enzyme	Restriction site	Buffer
EcoR I	5'-G [▼] AATTC-3'	Tango
Bgl II	5'-A [▼] GATCT-3'	Tango

Agarose gel electrophoresis was done to separate the digested fragments of DNA according to size. The digestion reaction mixture obtained after 3 h incubation was thoroughly mixed with 6x loading dye (New England Biolabs, USA) before application into each well of a 0.8 % agarose gel containing PEQ- green (PEQlab, Erlangen, Germany). Electrophoresis was operated at 90V for 40 minutes. Correct sized DNA bands were cut from the gel and purified with the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, UK) DNA concentration was relatively estimated based on the thickness of each fragment band compared to the DNA ladder bands, of which concentrations are known.

Ligation of DNA fragments was performed using the T₄ Ligase (Thermo Fisher Scientific, USA) which can join cohesive ends of a DNA insert and vector backbone together. A ratio of these DNA fragments was calculated by using the bioinformatic software In-Fusion Molar Ratio Calculator (<http://bioinfo.clontech.com/infusion/molarRatio.do>). Incubation of the ligation mixture was done at room temperature for 3h. In order to test the efficiency of ligation, the ligation mixture was transformed into *E.coli* MB2159 by electro-poration. Therefore 5 µL of ligation mixture was added to 50 µL of electro-competent *E.coli* MB2159 cells and incubated on ice for 5 min. The cell ligation mixture was then transferred into cooled, sterilized 1mm transformation cuvettes and electroporation was done with the electroporation device, Micro Pulser (Bio-rad, USA), using the *E.coli* protocol. Next, 900 µL of SOC media was added into the cuvettes right after applying the electric pulse and incubation at 37°C, with shaking, for 1h was accomplished. Selection of transformants was done on LB only plates. Certain amounts of the cell cultures (200 or 500 µL) were incubated on LB agar plates, at 37°C, overnight. The

consequently formed colonies were selected for colony PCR to confirm the presence of plasmids in the cells.

Table 4: Sequence of primers used for colony PCR

Primer	Sequence (5'->3')	Restriction site
Fw-slpA	ATGCAGATCTATAAAGTTGTTTGATAAATGCTCAAC	BglII
Rv-slpAMan	ATGCGAATTCTTACAGATCCTCTTCTGAGAT	EcoRI
Fw-pgm	ATGCAGATCTTGCGACAAGTAATAAACTAAAC	BglII
Rv-pgmMan	ATGCGAATTCTTACAGATCCTCTTCTGAGATG	EcoRI

For colony PCR, a picked colony was transferred into 100 μ L of dH₂O and heated at 99°C, for 7 minutes to lyse the cells. After centrifugation at maximum speed for 1 min, 1 μ L of the collected supernatant was mixed with Phusion Master Mix (Thermo Fisher Scientific, USA), and a pair of forward and reverse primers (Table 4). PCR was operated according to the Phusion Polymerase instruction manual and the product of colony PCR was visualized by agarose gel electrophoresis method as described above. The positive *E.coli* clones, chosen according to the result of colony PCR, were stored at -80°C.

To generate the *L. plantarum* expression strains, two plasmids, pSlpA_1261ManB_alr and pPgm_1261ManB_alr, isolated from the *E. coli* recombinant strains were transformed into electro-competent *Lactobacillus plantarum* TLG02. Briefly, 5 μ L of the plasmid solution was gently mixed with 40 μ L of electro-competent *L. plantarum* TLG02. The ligation mixture was kept on ice for 5 minutes before transferring it into a cooled sterile 2 mm electroporation cuvette. The electroporation parameters were set as following: voltage 1500V, capacitance 25 μ F, and resistance 400W. After the electric pulse was applied, 900 μ L of MRSSM media were added, followed by a 3 hours incubation at 30°C. Transformants were selected on MRS only plates. Incubation of the plates was done at 37°C. The positive colonies were checked with colony PCR before a cultural cryo stock with 20% glycerol was prepared. DNA sequencing was performed by a commercial provider (Microsynth, Vienna, Austria) to verify the cloned plasmid.

2.4 Expression of β -mannanase in *Lactobacillus plantarum*

For gene expression, overnight cultures of *L. plantarum* harboring the *erm*- or *alr*-based plasmids were diluted in fresh MRS broth to an OD₆₀₀ of ~0.1. 5 μ g/ml erythromycin were added to the MRS broth containing the *erm*-based strains. Incubation was carried out at 37°C, without agitation and cells were harvested 4h after inoculation by centrifugation at 4000 \times g,

for 10 min at 4°C. The obtained cell pellet was washed twice with PBS buffer, and re-suspended in PBS containing 2% of 50 mM PMSF, if cell disruption followed.

For the intracellular expression of ManB, the recombinant strains harboring pPgm_ManB and pSlpA_ManB were refreshed in 300 mL MRS broth containing 5 µg/ml erythromycin, and were incubated at 37°C. The pH of fermentation was kept constant at 6.5 by using 3M NaOH. Cultivation was carried out until OD₆₀₀ reached values between 5- 7.

2.5 Cell disruption methods

The chosen method of cell disruption is dependent on the amount of cell to be disrupted. For small cell pellets, sonication or glass bead mill is the method of choice yet for bigger cell pellets, French Press will be used for disruption.

2.5.1 Sonicator

Disruption of the *L. plantarum* cells was operated with the Bandelin Sonoplus HD 60 Sonicator (Bandelin electronics, Berlin, Germany) and parameters were set to cycle 20%, power 70% and operation time of 2 minutes. If the sonication was repeated, the sample was stored on ice in between the runs for a minimum of 2 minutes.

2.5.2 Glass bead mill

To disrupt the cells with the method of glass bead mill, 1 mL of cell-suspension was transferred to a tube containing 1g of 0,17-0,18mm glass beads. Disruption parameters for the Precellys 24 glass bead mill (PEQLAB Biotechnology GmbH, Erlangen, Germany) were set to 6000 × *g* and 30 seconds. Two runs were performed and in between, the tubes were cooled on ice for 10 minutes. Cell-free extracts were obtained by separation of glass beads, cell debris and supernatant through centrifugation with 14000 × *g* for 10 minutes, at 4°C.

2.5.3 French Pressure Cell Press

Cell disruption was performed by using the French Pressure Cell Press (French Press; AMINCO, USA). Therefore the cell pellet was re-suspended with the ratio biomass to lysis buffer of approximately 1:3 and the following cell disruption was performed at least for three times. Separation of the cell debris and the supernatant was done by ultracentrifugation at 10000 × *g* for 20 minutes, at 4°C.

2.5.4 Bradford method

Protein concentration [mg/mL] was determined using the method of Bradford (Bradford 1976) with bovine serum albumin (BSA) as standard. 600 μ L of Bradford solution was mixed with 15 μ L of protein solution, and then incubated in the dark for 15 minutes before measuring the absorbance at 595nm.

2.6 SDS- PAGE

Proteins in cell-free extracts were separated by running on a 10% Mini-Protean TGX gels (Bio-rad, Hercules, California, USA). 20 μ L of cell-free extract and laemmli buffer (ratio 1:1 with protein concentration between 20-30 μ g/well) were loaded into the wells and electrophoresis was operated with 120 Volt for 50 minutes. The gel was stained with a ready-to-use Coomassie staining solution (Bio-rad, USA), for 1h. Destaining was proceeded with deionized- water, overnight. The stained gel was further scanned with the Bio-rad Chemidoc XRS+ imaging system to obtain a digital picture.

2.7 Western Blot method

Proteins separated on the SDS gel were transferred to a nitrocellulose membrane (Mini Format 0.2 μ m nitrocellulose; Bio-rad, USA) by using the Trans-Blot Turbo Transfer system (Bio-rad; USA) according to manufacturer' s manual. After electro-blotting for 7 minutes, the membrane was incubated in the blocking buffer (Tris-buffered saline with 0.05 % Tween 20 [TBS-T] and 1 % BSA), for 1 h, at room temperature, with shaking. In the detection process, the Myc tag was probed with a primary Mouse Anti-Myc Monoclonal Antibody (diluted 1:5000 in TBS-T/ BSA 0.5%; Invitrogen, USA) at 4°C, overnight, with shaking. Washing of the membrane was performed 3 times with TBS-T, for 10 minutes. The secondary antibody mixture, containing Polyclonal Rabbit Anti- Mouse Immunoglobulin/ horseradish peroxidase (HRP) (diluted 1:2000; Dako, Glostrup, Denmark), and Precision Protein Strep Tactin- HRP Conjugate (Bio-rad, USA), was incubated with the membrane for 1 h, at room temperature, with shaking. The membrane was washed 3 times with TBS-T before incubating for 5 minutes with a substrate solution (Supersignal West Pico Chemiluminescent Substrate; Thermo Science, USA). Upon the addition of the substrate, the conjugated HRP produced the signal which can be visualized by Chemidoc XRS+ imaging system (Bio-rad, USA).

2.8 Flow Cytometry and Indirect Immunofluorescence Microscopy

Flow Cytometry and Indirect Immunofluorescence Microscopy were conducted to confirm the surface localization of the protein through the 3'-end fused myc-tag as specific antibody target. Mouse Anti-Myc Monoclonal Antibody (Invitrogen, USA) and Goat Anti-Mouse IgG H&L- Alexa Fluor 488 antibody (Abcam, Cambridge, UK) were used as primary and secondary antibodies, respectively.

Cultivation of the sample was done as previously described. After 4h inoculation, one mL of cell culture ($OD_{600} \sim 0.5$) were harvested, and cells were washed two times with 500 μ L PBS buffer before being re-suspended in 50 μ L PBS containing 1% BSA and 0.6 μ L Mouse Anti-Myc Monoclonal Antibody (Invitrogen; diluted 1:5000 PBS-B). After incubation at room temperature for 30 minutes, the cells were centrifuged at $4000 \times g$, for 5 min, at 4°C and were washed three times with 500 μ L PBS-B. The cells were subsequently incubated with 50 μ L PBS-B and 1.5 μ L Goat Anti-Mouse Alexa Fluor 488 (IgG H&L) (Abcam; Cambridge, UK; diluted 1:2000 in PBS-B) for 30 min, in the dark, at room temperature. After collecting the cells by centrifugation ($4000 \times g$, 4°C for 5 min) and washing five times with 500 μ L PBS buffer, before re-suspending the cells in 100 μ L of PBS buffer. The stained cells were analyzed by flow cytometry using a Gallios Flow Cytometer (Beckmann- Coulter, USA) at 488nm, following the manufacturer's instructions. For indirect immunofluorescence microscopy, the stained bacteria were visualized under a Leica SP5 II laser scanning confocal microscope (Leica Microsystems, Mannheim, Germany) using the 488-nm argon laser line to excite fluorescein immobilized to the secondary antibody.

2.9 Enzymatic activity assay

β -Mannanase activity was determined following the method described previously (Nguyen et al. 2016), with some modifications. The reaction mixtures consisted of 100 μ L of a suspension of enzyme-displaying cell in PBS and 900 μ L of a 0.5 % (w/v) galactomannan solution (locust bean gum, LBG; Megazyme, Bray, Ireland). The galactomannan solution was prepared by dissolving LBG in 50 mM sodium citrate buffer (pH 6.0) at 50°C for 30 min.

Enzyme-displaying cells were collected from the cultures, 4 h after inoculation, by centrifugation at $4000 \times g$, for 5 min, at 4°C. Obtained cell pellets were washed twice with PBS and were re-suspended in 200 μ L of PBS. 100 μ L of the mannanase-displaying cells were incubated with LBG at 37°C, with mixing at 600 rpm for 5 min. The cells were removed by centrifugation ($5000 \times g$, for 2 min, at 4°C,) and the amount of the released reducing sugars in the supernatant of the enzymatic reaction were detected using the 3,5-dinitrosalicylic acid (DNS) method, as previously described (Miller 1959), with some modifications. Briefly, 100 μ L

of the reaction supernatant were mixed with 100 μ l of DNS solution, followed by heating at 99°C for 10 min, cooling on ice for 5 min, and dilution with 800 μ l of de-ionized water, before measuring the absorbance at 540 nm using 1–5 μ mol/ml of D-mannose as standard. One unit (U) of mannanase activity was defined as the amount of enzyme releasing 1 μ mol of reducing sugars per minute under the given conditions.

The dry weight of cells, used for the enzymatic mannanase assay, was further determined. Therefore, the cell pellet used for the enzymatic assay was re-suspended with 100 μ L of buffer or water, applied on a weighted filter paper and dried in a drying chamber at 50°C. After overnight incubation and completely drying of all volatile substances, the weight of the papers was measured again. The dry cell weight was inferred from the obtained differential weight and converted into mg dry cell weight/ mL.

2.10 Growth curve and colony forming units

For the analysis of growth characteristics, cells were cultivated in 50 mL MRS broth containing erythromycin at 37°C for 24 hours. During the incubation period, samples were taken every hour for measuring turbidity at 600 nm (OD_{600}).

Additionally at the time points 0, 4, 6 and 8 the colony forming unit (cfu) was determined by spreading 100 μ L of dilution series on MRS agar plates containing erythromycin. To be able to simultaneously spread several plates, sterile glass beads were used to distribute the cell solution on the surface through shaking. Incubation was followed at 37°C for 18 hours. Plates were taken into account if 30- 300 colonies were countable. Calculation of cfu was done, following the equation of the arithmetic mean. All steps were performed in duplicates.

2.10.1 Analyses of β -mannanase production

The harvesting factor ($HF = OD_{600} \cdot V$ [mL]) was used to ensure that the same amount of cells would be utilized for the activity measurements. Enzymatic activity assay was therefore performed with the HF of 20, 10, 8, 6, 4 and 2. In brief, the OD_{600} of the culture broth was measured 4h after inoculation and a culture volume was harvested respectively to the desired HF. All harvesting factors were tested in triplicates.

The production of proteins in the recombinant *L. plantarum* strains were analyzed by studying protein extracts from surface display, intracellular (IN), supernatant (SN), and cell-membrane (CM) fractions. The harvested cell pellet was re-suspended in 300 μ L of lysis buffer and disruption was performed by sonication. Separation of supernatant and cell debris was gained by centrifugation (4000 x g, for 5 min, at 4°C). The obtained cell debris (CM fraction) was washed once with PBS buffer before re-suspending in 100 μ l of PBS. The cell-free extract (IN)

and CM fractions were used for the enzymatic activity assay and the protein concentration [mg/mL] was determined by the Bradford method.

In order to test the production of β -mannanase in the supernatant, 20 mL of cell free cultural broth were dialyzed in 1L of 10mM Tris-HCl pH 8.0, at 4°C with slow stirring. The dialysis buffer was exchanged twice (after 2 hours and 4 hours). The final dialysis run was performed overnight before the cultural broth was used for the enzymatic activity assay.

3 Results and Discussion

3.1 Construction of *alr*- based plasmids

Double digestion of the plasmids pSlpA_1261ManB, pPgm_1261ManB and pSIP609gusA were performed using the restriction enzymes *EcoRI* and *BglII*, to obtain the insert fragments and the backbone fragment harboring the alanine racemase gene, respectively. The obtained fragments were visualized by gel electrophoresis as shown in Figure 10.

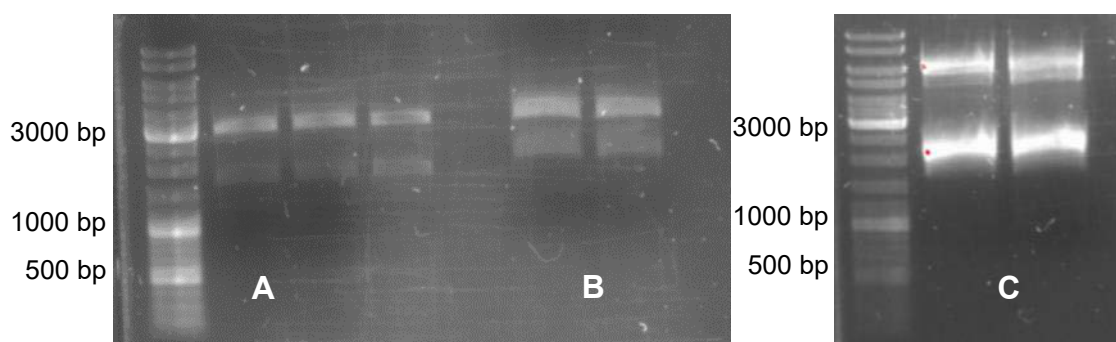


Figure 10: Gel electrophoresis of double digested plasmids. (A) *pgm_1261ManB* fragment with ~1633bp; (B) *slpA_1261ManB* fragment with ~1845bp and (C) backbone fragment with the *alr* gene ~5847bp.

The fragments, *pgm_1261ManB* and *slpA_1261ManB*, were ligated with the backbone fragment of pSIP609 (*alr*) and the resulting ligated products were transformed into *E.coli* MB2159. Screening of the correct clones was done by colony PCR technique and results are shown in Figure 11.

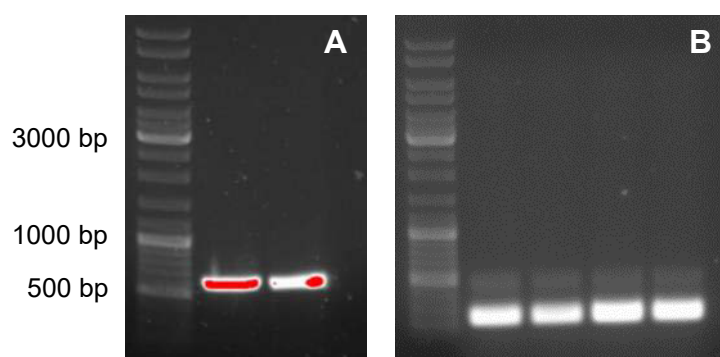


Figure 11: Gel-electrophoresis of colony PCR. (A) *slpA* promoter sequence (566 bp) (B) *pgm* promoter sequence (237 bp)

The inserted fragments were subsequently checked by DNA sequencing to confirm the presence of the right insert. After confirmation of the correctness of the inserts, transformation of the constructed plasmids into *Lactobacillus plantarum* TLG02 was carried out. Screening for correct clones was again performed with colony PCR. All DNA manipulation steps were done as described in chapter 2.3.

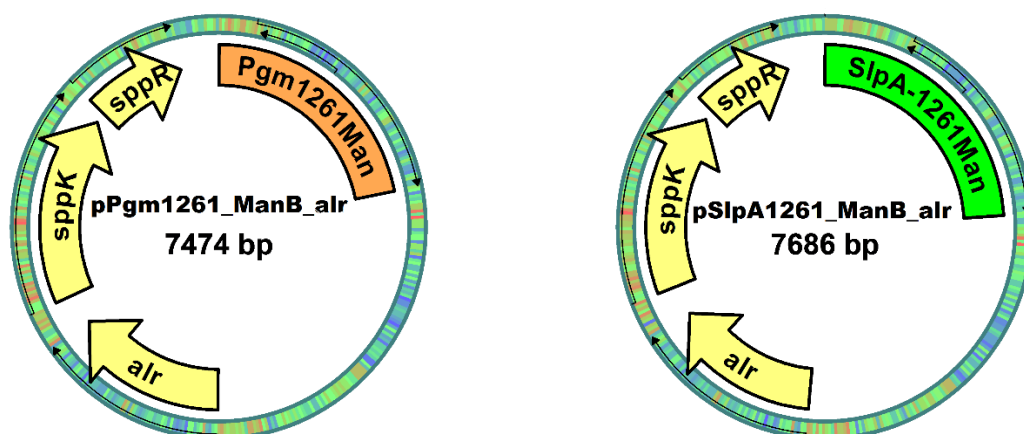


Figure 12: Expression vectors based on pSIP system with SlpA and Pgm as constitutive promoters, β -mannanase as the target gene, lipoprotein-anchor Lp1261 and selection marker based on alanine racemase gene (this work).

3.2 Conformation of ManB expression

3.2.1 ManB activity

Table 5 presents the activity (U/g dry cell weight) and the volumetric activity of surface displayed β -mannanase in *L. plantarum* harboring pPgm_1261ManB_alr, using different amount of cells for the enzymatic assay, which were obtained by using 5 different harvesting factors. The highest enzyme activity per g dry cell weight and the volumetric activity was found when using the harvesting factor of 6, which equals to the amount of cells of ~25 mg/mL. The activity of *L. plantarum* ManB-displaying cells harboring the constitutive expression vector pSlpA_1261ManB_alr was not detectable since the absorbance readings were below the lower range of the calibration curve. These findings were not to our expectation, as high enzymatic activity of 2500 U/g dry cell weight and 1000 U/g dry cell weight were obtained with the *erm*-based constitutive expression vectors pSlpA_1261ManB and pPgm_1261ManB, respectively (data not published; manuscript in preparation, personal communication with H.M Nguyen, PhD student at Food Biotechnology Lab, BOKU).

Table 5: Maximum volumetric activity [U/L] and enzymatic activity related to dry cell weight of displayed β -mannanase in *L. plantarum* using the constitutive food grade system harboring pPgm_1261ManB_alr.

Harvesting Factor	Enzymatic activity [U/g dry cell weight]	Volumetric activity [U/L fermentation]
20	141.1 \pm 6.7	66.0 \pm 1.2
10	138.6 \pm 2.7	116.7 \pm 2.9
8	133.2 \pm 18.2	104.8 \pm 17.3
6	204.3 \pm 20.5	174.7 \pm 19.0
4	82.2 \pm 1.6	92.1 \pm 5.4

To further investigate whether the ManB was expressed but not sufficiently secreted out and anchored on the cell surface, the mannanase activities were measured in different cell fractions and the results are given in Table 6.

Table 6: Volumetric mannanase activity of surface displayed, intracellular and cell membrane fraction, in U/L. ND- not detectable.

Volumetric activity [U/L]	Surface	Intracellular	Cell membrane fraction
pSlpA_1261ManB	207.5 \pm 3.8	891.7 \pm 152	153.4 \pm 12.8
pPgm_1261ManB	160.8 \pm 9.3	595.0 \pm 7.3	157.5 \pm 38.6
pSlpA_1261ManB_alr	ND	285.4 \pm 10.5	ND
pPgm_1261ManB_alr	174.7 \pm 19.0	1065.5 \pm 79.6	122.9 \pm 20.2
pSlpA_ManB	-	300.9 \pm 7.6	-
pPgm_ManB	-	ND	-

As already mentioned before, the mannanase activity on the cell surface of *L. plantarum* using the *alr*-based vectors could only be detected for the construct using the *pgm* promotor, and the obtained volumetric activity was comparable to the volumetric activity of the *erm*-based systems (Table 6). Concerning the enzymatic activity of the cell membrane fraction, the obtained results indicate a slight loss of activity, which could be explained with the possible release of the enzyme from the cell membrane during the cell disruption.

The enzymatic activity in the culture medium was further detected (results not shown). It is not yet clear, (I) if the found enzymatic activity is due to enzyme that has been secreted to the outside of the cell without being covalently bound to the cell membrane or, (II) if not all reducing sugars present in the MRS media have been removed by dialysis. A closer look needs to be taken and the results need careful evaluation before an assumption of the enzymatic activity

in the cultural broth can be drawn. However, it can be concluded that these extracellular activity were relatively low.

When testing the enzymatic activity of the intracellular expressed ManB using a constitutive expression system without a signal peptide and N-terminal anchoring motif, no activity was detectable for the pPgm_ManB construct. As seen in Figure 13, *L. plantarum* harboring pSlpA_ManB showed an intracellular enzymatic activity comparable to the intracellular activity of the pSlpA_1261ManB_alr strain.

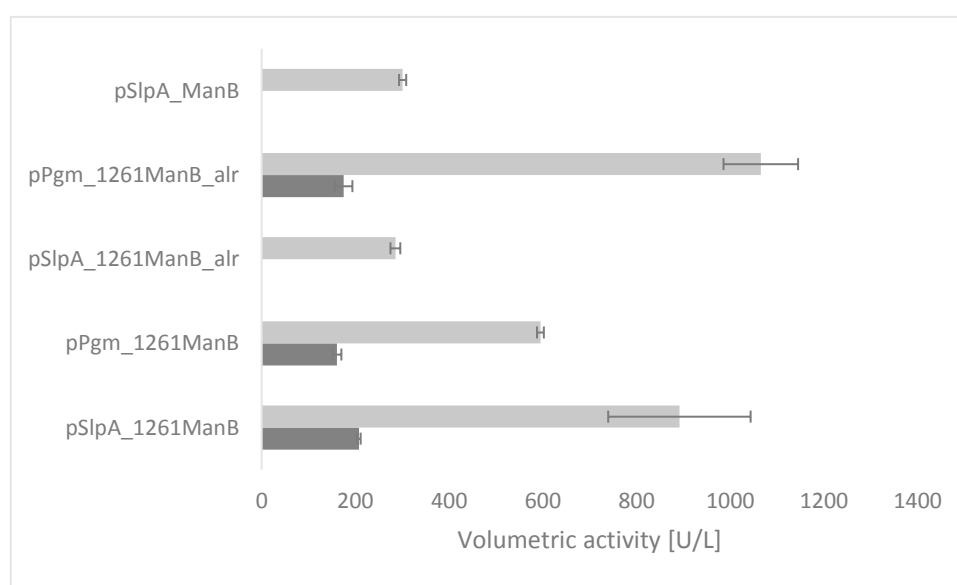


Figure 13: Enzyme activity on the cell surface (dark grey) and intracellular activity (light grey) of different *L. plantarum* strains expressing recombinant ManB after 4 h of inoculation.

Surprisingly, pPgm_1261ManB_alr showed the highest intracellular activity, which was ~2 times higher than the intracellular activity obtained with the pPgm_1261ManB and ~4 times higher than the activity obtained with the *alr*-based slpA strain. The results further indicate that the strain harboring pPgm_1261ManB_alr successfully expresses ManB intracellularly in high amount but only ~20% of the produced enzyme is secreted and surface-displayed. For the strain harboring the plasmid pSlpA_1261ManB_alr no surface-displayed mannanase activity nor in CM fraction could have been detected and only low activity of ManB was detected intracellularly. Hence, it can be presumed that the constitutive slpA promotor, with its strong transcription efficiency is hindered when used in *alr*-based vectors in this study, although we do not have a clear explanation for this observation. Surprisingly, the results also show that intracellular production of ManB using constitutive expression vector in the absence of signal peptide was also hindered.

These findings on the expression efficiencies reveals that more studies need to be conducted to fully understand the Sec-dependent secretion pathway in *L. plantarum*, to be able to identify possible limiting factors and to further develop constitutive surface displaying enzyme systems with improved expression and secretion efficiencies.

3.3 Growth characteristics

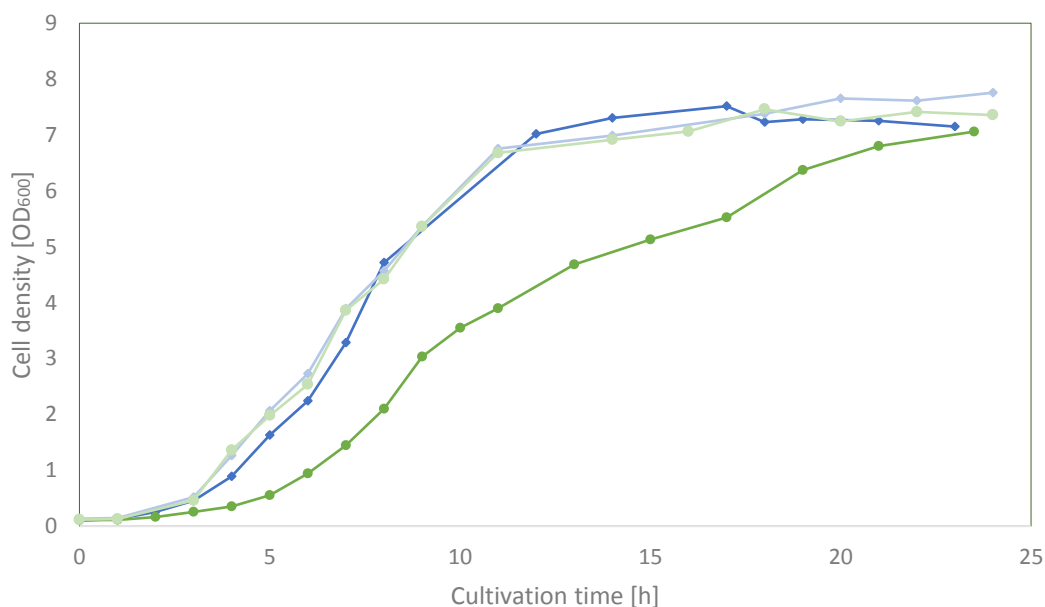


Figure 14: Growth curve of *L. plantarum* harboring (—●—) pPgm_1261ManB, (—●—) pPgm_1261ManB_alr, (—●—) pSlpA_1261ManB, (—●—) pSlpA_1261ManB_alr in the time course of 24 hours.

Figure 14 shows the growth curve of *L. plantarum* strains harboring the plasmids pPgm_1261ManB, pSlpA_1261ManB, pPgm_1261ManB_alr and pSlpA_1261ManB_alr, after refreshing the overnight culture in fresh MRS media, with or without erythromycin, resulting in a starting OD₆₀₀ of ~0.1. The strain harboring pSlpA_1261ManB showed a slower growth in comparison with the other strains. This might be due to the newly constructed constitutive promotor, which might cause a higher stress level to the growing cells, leading to inhibited cell growth. The other strains showed a similar growth curve profile, indicating that the cells might be exposed to less stress resulting to faster growth.

To further understand the effect of the growth on the production of the expressed proteins and thus the activity of displayed ManB, the colony forming unit [CFU/mL] during the cell cultivation was determined. Due to the low surface activity of ManB expressed by pPgm_1261ManB_alr (results shown in Table 5) and no detectable ManB activity using pSlpA_1261ManB_alr, the colony forming unit were not determined for those strains.

Table 7: Colony forming units [CFU/ mL] of different *L. plantarum* strains in MRS media.

Time [h]	pSlpA_1261ManB [CFU/mL]	pPgm_1261ManB [CFU/mL]
0	$5.5 \cdot 10^7$	$1.2 \cdot 10^8$
4	$1.8 \cdot 10^9$	$6.2 \cdot 10^{10}$
6	$1.1 \cdot 10^{10}$	$2.2 \cdot 10^{11}$
8	$3 \cdot 10^{11}$	$3 \cdot 10^{12}$

The CFU/ mL was detected as described in 2.10 and the results are presented in Table 7. At the time point 0, the overnight culture was refreshed in MRS broth to an OD₆₀₀ of ~0.1 and 100 µL of a dilution series were immediately used for detecting the colony forming units. The detected CFU indicates that although diluting the *L. plantarum* strains to the same OD₆₀₀, pSlpA_1261ManB overnight culture seem to have lower viability, hence less cells with the ability to reproduce were transferred into the fresh cultivation broth.

These findings indicate that the slower growth of *L. plantarum* pSlpA_1261ManB is caused by the lower CFU at the start of the fermentation process. Still, it is not yet known if the promotor strength has as well an impact on the CFU and how the found activity of ManB (for pSlpA_1261ManB) is affecting the CFU/ growth curve. To be able to pronounce a valid hypothesis on the correlation of CFU, growth curve and promotor strength/ManB activity, the CFU must be checked additionally at several time points, ideally every hour over the time span of 24 hours.

3.3.1 SDS PAGE

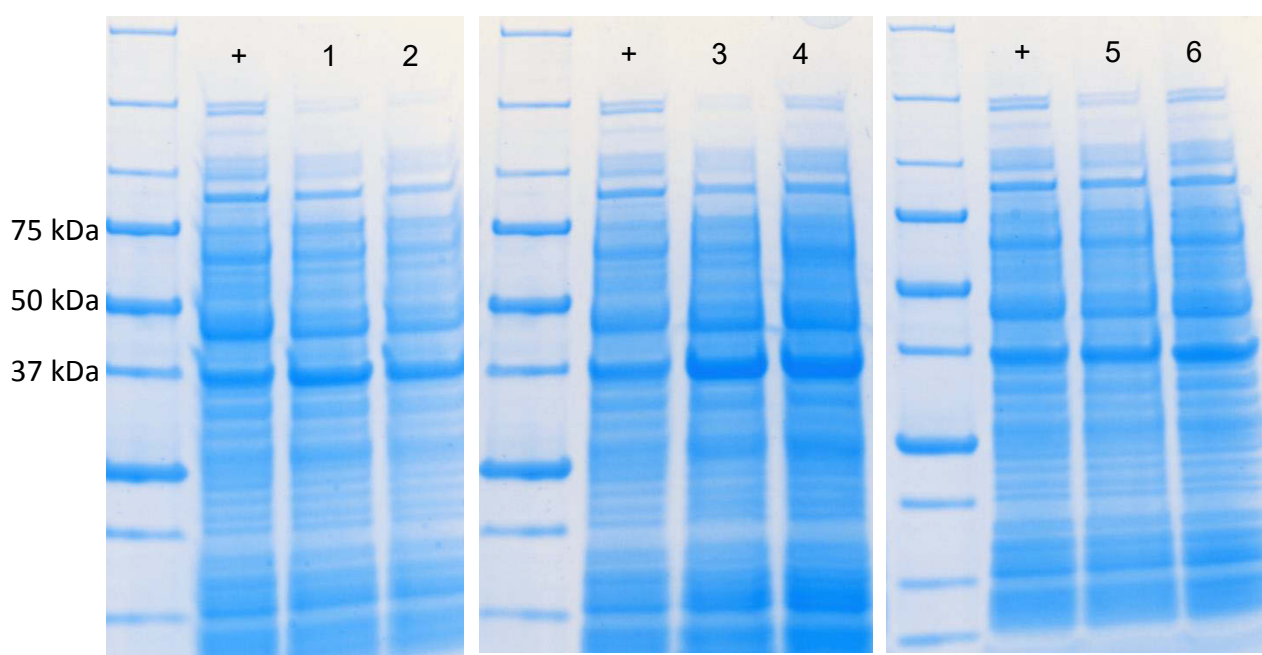


Figure 15: Coomassie stained SDS-PAGE showing intracellular expressed recombinant protein. Protein obtained with cell disruption of strains harboring the constitutive plasmids (1) pSlpA_1261ManB, (2) pPgm_1261ManB; (3) pSlpA_1261ManB_alr, (4) pPgm_1261ManB_alr; (5) pSlpA_ManB; (6) pPgm_ManB and the inducible (+) pSIP1261_ManB plasmid.

The expected protein size of the recombinant ManB, consisting of the lipoprotein anchor motif, the target protein and the C-terminal myc-tag is ~50kDa. As it is shown in Figure 15, the confirmation of the expressed protein cannot solely be based on the SDS-PAGE, due to the presence of many other protein bands at around 50 kDa. Western Blot was then used for the confirmation of the target protein expression.

3.3.2 Western Blot

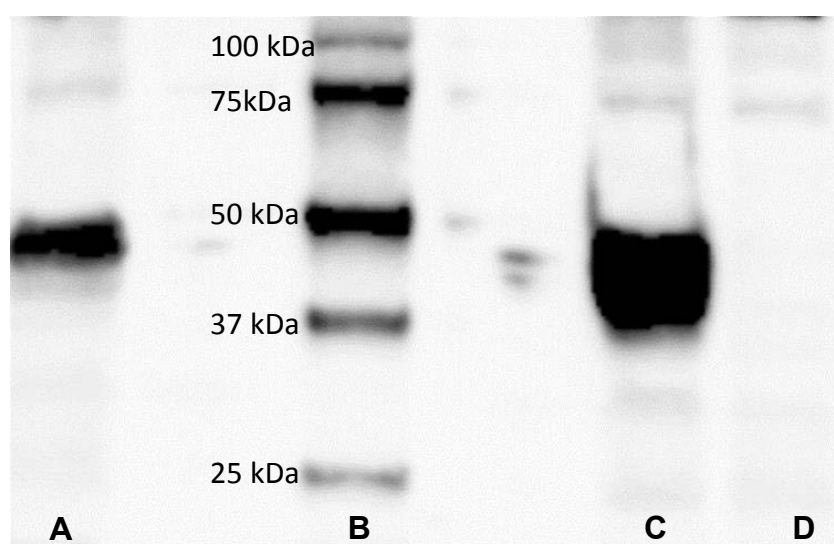


Figure 16: Western Blot. (A) pSlpA_1261ManB; (B) Precision Protein ladder (C) positive control-pSlP_1261ManB; (D) negative control- pEV.

Figure 16 presents the Western Blot result for the strain harboring the plasmid pSlpA_1261ManB. Therefore, the proteins in the SDS-PAGE gel were transferred to a nitrocellulose membrane and ManB was detected by specific antibody probing, as described in 2.7. Surprisingly, even after several attempts, a positive result could only be obtained for the strain harboring *erm*-based vector with the constitutive S-layer protein promotor slpA. It remains unclear, why no band could be detected in Western blot for the strains harboring the vectors (both *erm*- and *alr*-based) containing the pgm promotor, given the high intracellular activities obtained as shown in Figure 13.

3.4 Confirmation of ManB surface localization

Antibodies are only able to detect epitopes that are exposed to the outside of the cell, since they are not able to pass through the cell membrane. Therefore, surface displayed enzyme could be probed with the antibodies. Again, the *alr*-based expression vectors were not used in this part for the confirmation of the ManB surface localization, due to very low or no detectable ManB activity on the cell surface. Only the *erm*-based vectors were used for this purpose.

3.4.1 Flow Cytometry

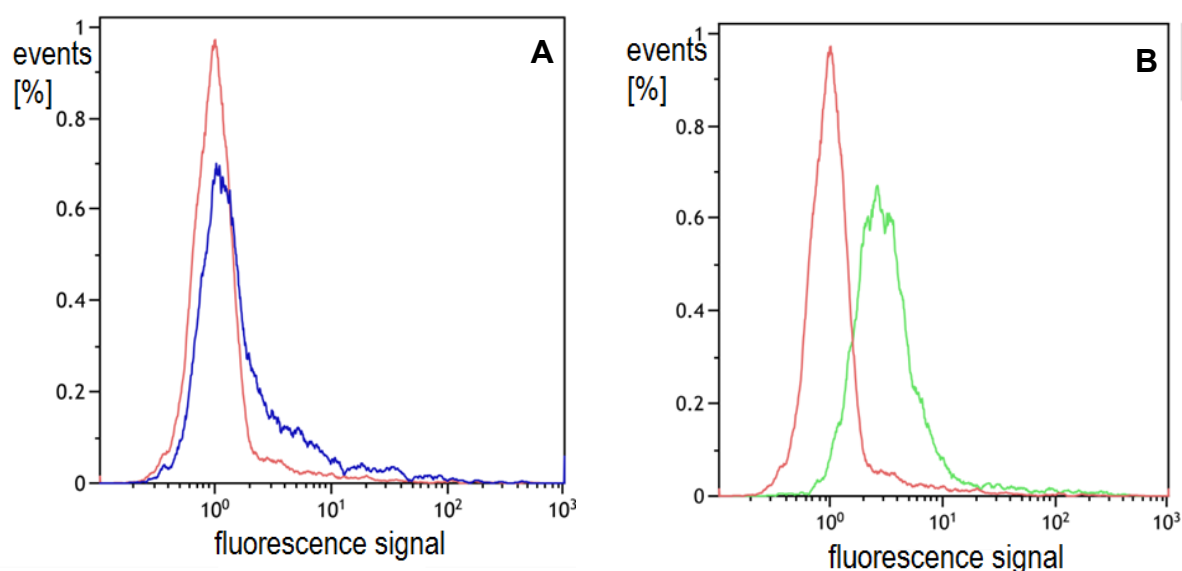


Figure 17: Flow Cytometry. Fluorescence signal obtained from *L. plantarum* harboring (A) pPgm_1261ManB (blue), (B) pSlpA_1261ManB (green) and the negative control pSIP_ManB (red).

The obtained fluorescence signal, as shown in can be used to verify the localization of ManB on the cell surface of the host strain. *L. plantarum* cells with intracellular expressed protein, lacking both, a signal peptide and the myc-tag (which is necessary for the antibody probing) were used as negative control. As can be seen in the flow cytometry histogram, (B) pSlpA_1261ManB shows a clear shift in the fluorescence intensity in comparison to the used negative control, whereas *L. plantarum* harboring pPgm_1261ManB (A) shows only a slight shift. This represents a confirmation for the localization of ManB, derived with the slpA based constitutive expression system, on the cell surface of the host strain. To be able to confirm the presence of ManB derived from the pgm promotor system, Immunofluorescence Microscopy was further conducted.

3.4.2 Indirect Immunofluorescence Microscopy

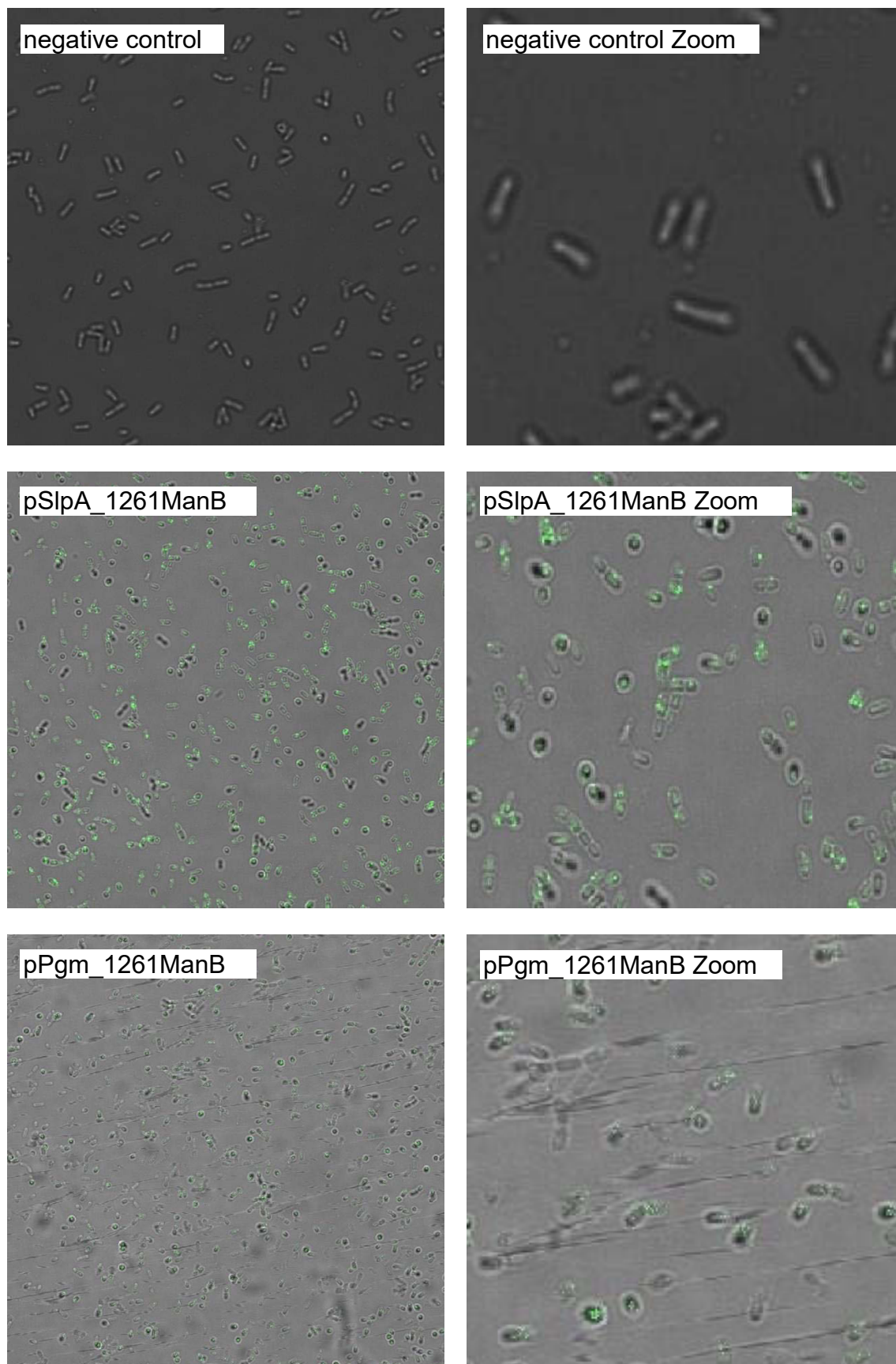


Figure 18: Results from indirect immunofluorescence microscopy of the modified *L. plantarum* cells.

Figure 18 shows the obtained immunofluorescence microscopy pictures of *L. plantarum* harboring different constitutive expression plasmids. As for the Flow Cytometry analysis, *L. plantarum* with intracellular expressed protein was used as the negative control and as expected, it shows no fluorescence signal. For the other constructs harboring pSlpA_1261ManB and pPgm_1261ManB, a green fluorescence signal could be seen on the outline of the cells, which indicates the presence of the expressed protein on the cell surface. Comparing the fluorescence intensity of the two strains, it appears that the cells harboring the construct with the slpA promoter give stronger fluorescence signal than the cells harboring the pgm promoter. The results confirm the presence of ManB on the surface of *L. plantarum* cells harboring the *erm*-based vectors with both constitutive promoters.

3.5 Conclusion

The present study confirms the secretion and the surface display of ManB on the cell surface of *L. plantarum* harboring the constitutive *erm*-based expression vectors, pSlpA_1261ManB and pPgm_1261ManB. Immunofluorescence Microscopy and Flow Cytometry results confirmed the localization of ManB on the cell surface, whereas Western Blot and activity measurements confirmed the expression and the functionality of the displayed enzyme.

Furthermore, the erythromycin resistance gene was successfully replaced with a food-grade selection marker, the alanine racemase gene (*alr*). This was done with the aim to develop a food grade, whole-cell biocatalyst that could be used for different applications. Although the successful exchange of the selection marker was achieved, activity of surface displayed ManB was rather low or not at all detectable for pPgm_1261ManB_alr and pSlpA_1261MaB_alr, respectively. This finding was somehow unexpected in comparison to the high activity obtained with the *erm*-based constitutive expression systems using the same Lp1261 anchoring motif and ManB as target protein. Further studies should be conducted to gain more understanding about the secretion pathway in *L. plantarum* to be able to improve protein secretion efficiency.

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