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Sakacin – based expression systems – application for overproduction of beta-galactosidase in *Lactobacillus*

Dissertation

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Contents

Abstract

The purpose of this PhD thesis was to study pheromone-inducible sakacin-based expression pSip systems applying for food-grade overproduction of several lactobacillal β-galactosidases in *Lactobacillus* cell factories. The inducible overexpression of β-galactosidase encoding genes *lacLM* from different *Lactobacillus* species in pSip, one hand were investigated at mRNA level with quantitative real-time Polymerase Chain Reaction (Real Time PCR) method, and on the other hand were optimized by changing the cultivation condition or by using molecular biology approaches to get higher yield of food-grade product.

The expression levels of two-component regulatory systems, histidine kinase gene (*sppK*) and response regulator gene (*sppR*), and *lacLM* target genes from *Lactobacillus reuteri* and *L. acidophilus* in pEH9R and pEH9A vector, respectively, were determined by using the quantitative Real Time PCR. The results indicated that the plausible differences in nucleotide sequence of these target genes were likely to affect both mRNA synthesis rates and mRNA stability, therefore resulted in the significant differences in mRNA level and activity of β-galactosidases. Several parameters like sugar content, temperature, induction or pH were verified for fermentation of strain *L. plantarum* WCFS1 harboring pEH9R plasmid with *lacLM* genes from *L. reuteri*. By that way, the yield of the enzyme production could raise 4 to 5–fold higher in optimized conditions compared to non-optimized fermentation. In order to eliminate erythromycin from cultivation media of *Lactobcillus* host carrying prototype of pSip which relying on antibiotic resistance marker (*erm*), an alternative selection marker, i.e the alanine racemase encoding gene (*alr*) from *L. plantarum*, was replaced for the *erm* in pSip vector resulting in the new complementary expression system. The *alr*-based vectors were applied for the overexpression of β-galactosidase genes from *L. reuteri* L103 *and L. plantarum* WCFS1 in an *alr* deletion mutant of *L. plantarum* WCFS1. The expression levels obtained in this way, i.e. without the use of antibiotics, were comparable to the levels obtained with the conventional system based on selection for erythromycin resistance. The new system is suitable for the production of ingredients and additives for the food industry.

The pSip expression vectors were reported as effective systems for some *lacLM* genes in term of high expression level of target protein with low concentration of inducer pheromone used vs. low basal expression level. However, the effectiveness of pSip systems is unpredictable for a particular gene and the induction protocol is laborious, thus the application of pSip systems, especially in the industrial scale, is limited. Further works should be done to improve the systems and make them more controllable and feasible for the food industry.

Zusammenfassung

Das Ziel dieser Arbeit war, das Pheromon-induzierbare pSip Expressionssystem auf die Eignung zur „food grade" Produktion von bakteriellen β-Galactosidasen mit *Lactobacillus* als „Zellfabrik" zu untersuchen. Die induzierbare Überexpression verschiedener β-Galactosidase codierender Gene (*lacLM*) wurde mittels quantitativer real-time PCR (real time-PCR) auf mRNA-Ebene erforscht. Weiters wurden Lactobacillen unter unterschiedlichen Wachstumsbedingungen kultiviert und einige molekularbiologische Ansätze zur Modifikation des pSip-Systems angewandt, um eine höhere Ausbeute von β - Galactosidasen zu erzielen.

Real time-PCR Analyse der Expression des regulatorischen 2-Komponenten-Systems bestehend aus Histidin-Kinase-Gen (*sppK*) und Response-Regulator (*sppR*), sowie der *lacLM* Gene aus *Lactobacillus reuteri* und *L. acidophilus* im pSip409 Expressionsystem zeigte, dass die Unterschiede in den Nucleotidseqenzen der oben erwähnten Gene offensichtlich sowohl Produktionsrate als auch Stabilität der mRNA beeinflußen. Somit entstehen signifikante Unterschiede in mRNA Gehalt und Ausbeute der exprimierten β-Galactosidasen. Bei der Expressionssoptimierung von *L. plantarum* WCFS1 (transformiert mit pSip409, *lacLM* aus *L. reuteri* enthaltend), wurden Parameter wie Zuckergehalt im Medium, Temperatur, Induction oder pH variert. Auf dieser Weise konnte die Ausbeute der Enzymproduktion um das 4 bis 5 fache erhöht werden.

Um eine Antibiotikafreie Kultivierung von *Lactobcillus* zu ermöglichen, wurde der Erythromycin Resistenz-Marker (*erm*) aus dem Vektor pSip gegen das Alanin Racemase Gen (*alr*) aus *L. plantarum* ausgetauscht. Diese neuen Vektoren wurden für die Überexpression von ß-Galactosidasen aus *L. reuteri* L103 und *L. plantarum* WCFS1 in eine (*alr*-negative) *L. plantarum* WCFS1 Mutante eingesetzt. Dadurch wurde ein neues Expressionssystem entwickelt, dessen Expressionsrate mit jener des konventionellen Expressionssystem (mit Erythromycin-Resistenz) vergleichbar war. Das neue Konstrukt ist somit gegeignet für die Produktion von Nahrungsmittelbestandteilen und Lebensmittelsatzstoffen.

Das pSip Expressionssystem ist ein effizientes System für die Herstellung von ß-Galactosidasen bei dem Einsatz einer geringen Induktorkonzentrationen. Allerdings ist

der Arbeitsaufwand noch hoch und limitiert dadurch den Einsatz des pSip- Systems in industriellen Maßstab. Zukünftige Arbeiten sollten sich mit der weiteren Optimierung des Systems sowie dessen Kontrollierbarkeit befassen um einen breiten Einsatz in der Lebensmittelindustrie zu ermöglichen.

Objectives and outline of thesis

Lactic acid bacteria (LAB) are important micro-organisms in the food and beverages industry, in which they have been playing crucial roles as starter culture and producers of flavoring enzymes, antimicrobial peptides or metabolites that contribute to the flavor, texture and safety of food products. In addition, because of their food-grade status and probiotic characteristics, several LAB, especially lactobacilli, have been considered as safe and effective cell-factories for food-application purposes. Among variety of constitutive or inducible gene expression systems developed for LAB hosts, the systems pSIP which based on promoters and genes from Sakacin A/P bacteriocin operon from *Lactobacillus sakei* are a well-known pheromone-inducible lactobacillal expression system beside the Nisin-controlled expression system.

β-galactosidases are known as important enzymes in the dairy industry. A part from lactose hydrolysis ability, this enzyme possesses another beneficial ability is the trans-galactosylation which co-occurs during lactose hydrolysis resulting in the formation of prebiotic galacto-oligoscharides. Beta-galactosidase can be obtained from several sources, however the enzyme from *Lactobacillus* probiotics have been received a great attention with the expectation that the enzyme from these species can produce a specific GOS product for probiotics. Some industrially potential candidates of lactobacillal β-galactosidases such as from *L. reuteri*, *L. plantarum* have been isolated, characterized and successfully overexpressed in *Escherichia coli*, then in *Lactobacillus* hosts due to the food grade status of this expression host with pSip systems. Several pSip vectors have been indicated as effective expression systems and resulted in high level of target protein with high induction factor (low basal expression level in non-induced condition) using rather low inducer pheromone. However, the effectiveness of particular target gene such as *lacLM* genes from *Lactobacillus* in pSip vectors has been reported as followed an unpredictable manner. The functionality of pSip depends on host strain, promoter, genes of interest, plasmid copy numbers.

The main objective of this thesis is to study in detail the expression pSip systems with encoding gene *lacLM* of lactobacillal beta-galactosidases used as reporter genes in food-grade *Lactobacillus*.

Chapter 1 is a short literature review discribing genomic origins, i.e the genes involving in sakacin bacteriocin synthesis from several *Lactobacillus sakei* strains, the development of sakacin-based expression systems, in particularly pSip systems, and the application of these systems for heterologous and homologous overexpression of diverse target proteins in *Lactobacillus* host.

The question for chapter 2 is derived from previous work, in which *lacLM* β-galactosidase encoding genes from several *Lactobacillus* strain have been over expressed in *Lactobacillus plantarum* and *Lactobacillus sakei* using pSip403 and pSip409 systems. In spite of high similarity on sequence of these β-galactosidases, a significant difference of respective enzyme activities were observed. In chapter 2, the reasons for such difference between two *L. plantarum* strains harbouring pSip409 vector carrying *lacLM* genes from *L. reuteri* and *L. acidophilus* are investigated, mainly focused on comparision of plasmid copy number, expression level of target genes and regulatory genes of expression systems by using a reproducible quantitative real time PCR method.

In order to obtain higher yield of recombinant β-galactosidase from *L. reuteri* expressed in *L.plantarum* with pSip system, in Chapter 3 the optimization of fermentation is discribed. Several varified conditions for the fermentation of strain *L. plantarum* WCFS1 harboring pSIP409 with *lacLM* from *L. reuteri* have been applied such as concentration of C-source (glucose), cell optical densitiy for induction, concentration of inducer pheromone, temperature and pH control. Meantime, the copy number of interested plasmid has been studied to see the relationship with expressed β-galactosidase activity.

In chapter 4, the effectiveness of pSip systems was confirmed when successfully applied for overexpression of β-galactosidase *lacZ* from *L. bulgaricus*, resulting in high expression level of recombinant enzyme. Subsequently, the recombinant *lacZ* β-galactosidase was purified and further characterized as well as applied for lactose hydrolysis and transgalactosylation.

pSIP systems, even though appearing as effective expression system for case of some β-galactosidases from *Lactobacillus* species in food-grade *Lactobacillus* host, the food-application potential of products is limited due to the nessesity of erythromycin included in fermentation media to avoid the loosing of plasmid. In chapter 5, the new pSip series have been developed which based on alternative food-grade selection marker, i.e alanine

racemase encoding gene (*alr*) as complementary selection marker. The new expression systems have been applied for overexpression of some β-galactosidase from *Lactobacillus* and resulting in comparable expression level to antibiotic-based. Moreover, no antibiotic is needed for new systems, thus the recombinant products can be used for food application.

Chapter 1

Sakacin A/P bacteriocin: from genome to expression vectors

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Abstract

Sakacin which are produced by several *Lactobacillus sakei* are classified into the anti-listeria Pediocin-like class II of bacteriocin. Among this group, sakacin A and P are well-studied in both characteristics and biosynthesis. The quorum-sensing-based-regulatory genes involved in the sakacin A/P synthesis has been successfully used for development of constitutive and inducible systems which are specifically applied for heterologous and homologous over-expression of diverse target proteins in lactobacilli host. In this mini review, we first discuss about the genomic determants responsible for the sakacin A and P production, and second the exploitation and the application of the sakacin-based expression prototype and derivatives based on these regulator genes, especially focusing in pSip series.

Background

Lactic acid bacteria (LAB), generally recognized as safe (GRAS), have a long history of using in food processing especially in fermented food due to the primarily function of synthesis lactic acid bacteria from sugar [1]. Many LAB are known as producers of processing enzymes, antimicrobial peptides, or metabolites that contribute and improve the quality of food products [1, 2]. Moreover, several lactobacilli have been referred to probiotics which are commensals in the gastro intestinal tract and have health beneficial effects to human by attributing to modulate the immune response of host and inhibiting the pathogenic microorganism [3, 4]. Among many products from LAB, antimicrobial peptides, known as bacteriocin, have been received the considerable attention for many years. Bacteriocin of LAB are ribosomally synthesized via the quorum sensing mechanism [5]. Several bacteriocin have a high potential lethal function to closely related to the producers strain at nanomolar concentration [6], therefore they are promising antimicrobial substances for food preservation.

With the aim of food applications, several of bacterial or fungal species such as *E. coli*, *Bacillus subtilis*, *B. licheniformis*, *Aspergillus oryzae* or *Asp. Niger,* have been safely used as sources for native or recombinant products [7]. However, in certain special conditions, these strains can produce secondary metabolites which are considered as toxin for human like mycotoxin (for fungi), or endotoxin (*E. coli*) [7, 8]. One of the most potential applications of LAB is to use as safe "cell factories" for the recombinant technology applying in food industry to overcome the disadvantages of fungi, *E. coli* or *Bacillus* hosts [8]. As a consequence, several expression systems which derived from the genes and promoters involving in, for example, sugar utilization, heat shock resistance and bacteriocin production [2, 9, 10] from LAB have been developed and specifically applied for LAB cell factories. Among these expression system, the two most well-known inducible expression systems are NICE [11] and pSIP system [12-14] which are based on the quorum sensing mechanism in the bacteriocin productions. The "NICE" stands for NIsin Controller gene Expression system, which contains genes and promoters involving the nisin bacteriocin synthesis of *Lactococcus lactis* [15]. The latter system have been derived from genes of sakacin production, induced by secreted peptide pheromone and specific for *Lactobacillus* host [13]. In this minireview, the involved genes and the

development as well as the application of sakacin-based systems, especially pSIP, are in detail described and discussed.

Sakacin bacteriocin

Bacteriocins from LAB have been classified into 3 classes: the lanthibiotics (class I), the non-modified heat-stable bacteriocins (class II) and the large heat labile bacteriocins (class III) [16, 17]. The term of "Sakacin" has been used to identify the bacteriocins which are produced by *Lactobacillus sakei* species. To date, several sakacins have been discovered from different *Lactobacillus sakei* species such as sakacin A [18], B [19], M [20], G [21], K [22], P [23], Q [24], 674 [25], T, X [26]. Major of sakacin bacteriocins in general are classified into the pediocin-like class IIa of bacteriocin which are highly active against *Listeria* strains, and has a typical amino acid sequence motif of YGNGV(X)C(X)$_4$C(X)V in their N terminal [16, 27, 28]. As belong to the class II bacteriocin, sakacins are non-lanthibiotic which does not go through the extensive posttranslational modification, except the cleavage of 15-30 residues leader peptide at C-terminal side of a double glycin motif to form the mature and active structure. A few sakacins such as type Q and T are classified into class IIb [24, 26].

Like other bacteriocins, the action mode on target cells of class II bacteriocin in general and sakacin in particular is supposedly based on the pore formation mechanism on the cytoplasm membrane. The pores reduce the Proton Motive Force (PMF) and facilitate the leakage of nutrient and metabolites, resulting in the death of target cells [28]. To form the pores, firstly bacteriocin binds to the cystoplasm membrane via a receptor locating on membrane. The C-terminal domains of bacteriocin were indicated that it greatly affect on specificity of pediocin-like class IIa bacteriocin [29]. The N-terminal conserved sequence of YGNGV is considered as the "hook" to link with the receptors of susceptible cells and the β-sheet structure with positive charge at physiological pH provides the flexibility for bacteriocin molecules to interact with molecular on surface of target cells [28]. It is well-known that lipid II play a such role for the lanthibiotic nisin [30-32]. It has recently shown that the components of the mannose phosphotransferase systems (man-PTS) of sensitive

cells are receptors for lactococcin A as the representative of class IIa bacteriocin [33, 34], while the lipid II has shown as the "harbor" for class IIb lactococcin 972 [35].

Genes involving sakacin A/P biosynthesis

Among sakacin group from several *Lactobacillus sakei (*or *L. sake* as named by the source of first isolated "sake" [36]) strains, Sakacin A and P from *L. sakei* Lb706 [18] and *L. sakei* LTH673 [23], respectively, have been received more consideration from microbiologists [3]. Since their introduction then, the characteristics as well as the biosynthesis and the genes involving in production of sakacin A/P from those strains were gradually elucidated.

The genes for the sakacin biosynthesis, immunity, processing and exporting are located in plasmid (for sakacin A from *Lactobacillus sakei* Lb706) [37, 38] or chromosome (for sakacin P from *L. sakei* LTH673) [39]. However, the arrangements of these genes for both sakacins are similarly organized in two or three operons (Figure 1). For sakacin A-Lb706, the structural gene (encoding for sakacin A) *sapA* and the immunity gene *sapiA* are in one operon, whereas the genes encoding for the histidine kinase *sapK*, the response regulator *sapR*, the inducer peptide *sapIP* and the transporter proteins *sapTE* are in second operon. Two operons are separated by an unknown-function sequence IS1163 [37]. For sakacin P-LTH673, two transporter protein genes *sppT* and *sppE* are regulated by a separated promoter in the third operon (figure 1).
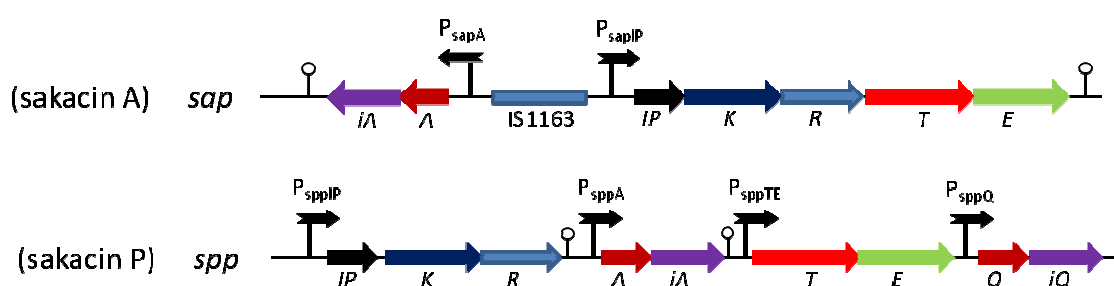
Figure 1. **The *sap* (for sakacin A from *L. sakei* Lb706) and *spp* regulon (for sakacin P from *L. sakei* LTH673)**. A, Q, iA and iQ encode for bacteriocin and immunity protein. IP, K, R encode for peptide pheromone, histidine kinase (HK) and response regulator (RR). T and E encode for ABC transporter and an accessory protein. IS1163 denotes for unknown function sequence. Lollipops-like symbols indicate the terminators.
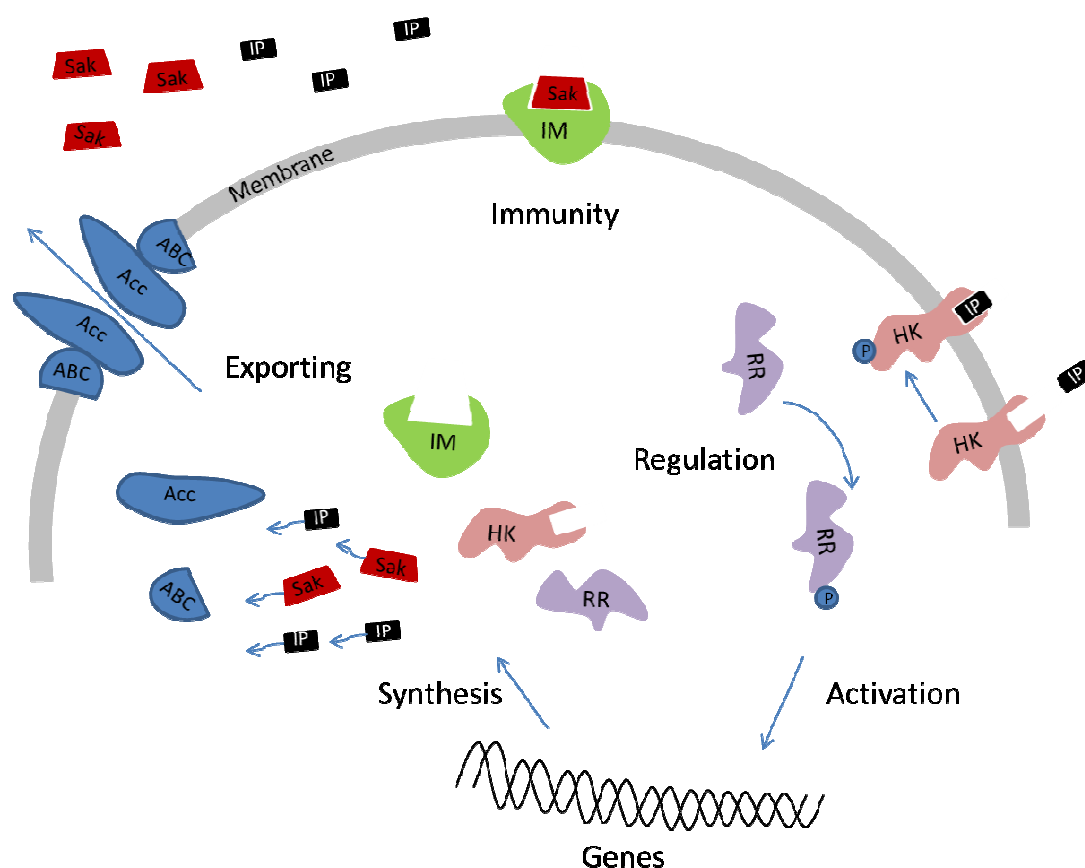
Figure 2. Schematic overview of regulation, synthesis, excretion and immunity for sakacin production (adapted from Enhanar, 2000). The pheromone (IP) is synthesized and exported to outside of bacteria cells. IP activates two-component system consisting of histine kinase (HK) and cognate response regulator (RR). Activated RR then turns on the transcription of relevant genes to produce precursors of bacteriocin (Sak) and IP with leading sequence, immunity protein (IM), HK, RR, ABC transporter (ABC) and accessory protein for exporting (Acc).

The biosynthesis of sakacin A and P are regulated via the quorum sensing mode (Figure 2), in which a secreted peptide plays as inducer, so call induction peptide (IP) or peptide pheromone [16, 40]. The pheromone is ribosomally synthesized, modified and exported to outside of the cells at low levels [28]. At certain level of the cell density of bacteria in medium, the concentration of IP reaches the threshold value, thus activate the two-component system consisting of histine kinase HK (encoded by *sapK/sppK*) and cognate response regulator RR (encoded by *sapK/sppR*) [27]. The IP pheromone binds to the transmembrane histidine kinase (HK) leading the autophosphorylation of HK at conserved histidine residue [41]. The activated histidine kinase subsequently phosphorylates its cognate reponse regulator RR, in which the phosphate group is transferred to conserved

Asp residue of the response regulator [41]. Finally, the active response regulator RR activates the relevant promoters in sakacin operon by binding to the promoter at specific site [41]. The RR binds to the promoters at inverted repeated region RIR and LIR [37] of *sap* operon or at repeated sequences found in -80 to -40 region of the promoters of *spp* operons [42].

Regarding to the sakacin P production genes from *L. sakei* LTH673, the promoters in front of the bacteriocin operon was indicated as strict regulated by IP inducer [42, 43] and the induction factor, which is calculated by the ratio of reporter protein from induced cells to that from non-induced cells, for instance around 1000-fold with chloramphenicol resistance gene as reporter gene [42]. Whereas the promoter of regulatory operon P_{sppIP} was less strict regulated, thus a low expression level of regulator protein HK and RR (SppKR protein) could be detected in the non induction condition [43]. The part upstream of -80 to -40 region in the promoters is responsible for the constitutive expression regulatory and the transporter relevant genes [42]. The forth operon in *spp* regulon (figure 1), latterly were shown that encodes for sakacin Q and its immunity gene [24]. The promoter P_{sppQ} (formerly P_{orfX}) from this operon has been reported as the strongest among 4 promoters in sakacin P/Q regulon [24, 42], which then is very useful for construction of expression vector (see below). The leaky of P_{sppIP} promoter compared to bacteriocin promoters (such as P_{sppQ}) recently has been quantitatively confirmed by Real time PCR technique. In the condition without inducer, the expression levels of the regulatory genes *sppKR* under regulation of P_{sppIP} were observed 2-fold higher than the transcription level of target gene controlled by P_{sppQ} [44]. A similar observation was previously reported by Vaughan and co-worker who have also used Real Time PCR to quantitative analysis the transcription of three genes involving the production of sakacin T encoding for inducer IP (*stxP*), bacteriocin (*sakT*$_\beta$) and histidine kinase (*stxK*) located in different operons with the regulation of different promoters from malt isolated *L. sakei* 5. Vaughan et al. have observed the relation of these genes in bacteriocin production [45]. During the early of log phase, all three genes were transcribed constitutively, in which the transcription levels of *stxP* and *stxK* were similar in pattern and higher than that of *sakT*$_\beta$. The expressions level of two regulatory genes make the peaks at 7 hours of cultivation, meanwhile the expression level of the bacteriocin gene *sakT*$_\beta$ increased to higher than the

expression level of histidine kinase gene *stxK* and kept constant from 9h of cultivation [45].

The mature form of inducer pheromone (IP pheromone) for the production of sakacin A or sakacin P are small cationic peptides consisting of 23 or 19 amino acids, respectively [46-48]. They have no antimicrobial activity. Similar to bacteriocin, their precursors are ribosomally synthesis and simultaneously cleaved the leader sequence (signal peptide) and exported into medium. Pheromone precursors are encoded by *sapIP* or *sppIP*, under the regulation of promoters P$_{sapIP}$/P$_{sppIP}$, therefore these peptides have other names as Sap-IP or Spp-IP, respectively. Due to the "little leakiness" of these promoters, IPs are produced "constitutively" at low level and accumulated in medium. When IP amount reach a threshold level, they activate the regulation loop of sakacin production. The difference in amino acid sequence between two IPs indicates the specific interaction of an IP to its cognate histidine kinase [3]. The synthetic IP can induce the sakacin synthesis and themselves of Bac- culture (unable to produce bacteriocin) which can be obtained by using an extreme dilution from the Bac+ phenotype culture of cognate strain [47].

The quorum sensing mechanism for sakacin production can be influenced by the changes of environment condition such as temperature and oxygen concentration [3]. The sakacin A production of *L. sakei* Lb706 were shown as remarkably reduced when cultivated at 33°C or higher temperature compared to at 20-30°C [46]. The sakacin P production ability of *L. sakei* LTH673 can be restored from Bac- cells of this strain by changing to the anaerobic condition from aerobic condition or by using other types of media [43]. However, it has been shown that such effects of environment factors may be ignored in the presence of synthetic IP with sufficient concentration [3].

Sakacin based-expression systems

Similar to Nisin controlled expression systems (NICE) which include the promoters and regulatory genes of *Lactococcus* nisin production [15], the sakacin-based expression systems have been constructed with the genes and promoters in the auto-reglulation loop of sakacin clusters from *Lactobacillus sakei*.

The first version of sakacin-based expression system was introduced by Axelsson and co worker (1998) which consists of two plasmids [12]. One plasmid, named pSAK20, contains the promoters and genes necessary for the regulatory (*sapIP-K-R*), exporting and processing of bacteriocin precursor (*sapT-E*) of sakacin A from *L. sakei* Lb706 with chloramphenicol resistance gene (*cat*) as selection marker [37]. The other plasmid which was constructed with the "backbone" of small, stable and shuttle *E. coli-Lactobacillus* vector pLPV111 with erythromycin resistance (*erm*) selection [37] contains reporter genes regulated by P$_{sapA}$ promoter [12]. These two-plasmid systems were applied for the heterologous expression of structural as well as immunity genes of three bacteriocins including sakacin P (*sppA* and *sppiA*), pediocin PA-1 (*pedA*, *pedB*) and piscicolin 61 (*psc61*, *orf$_X$*). For that purpose, the pSAK20 plasmid was firstly introduced into a bacteriocin-negative strain *L. sakei* Lb790. Secondly, the plasmids with target genes were transformed into *L. sakei* Lb790(pSAK20). Because the intact gene encoding for peptide pheromone (*sapIP*) was included in pSAK20 vector, these systems were activated without supplementation of Sap-IP into medium, and resulted in equal or slightly higher of sakacin P and Pediocin PA-1 yield compared to from the wild type [12]. The native promoters for these target genes were also used, however the regulatory systems encoded in pSAK20 did not recognize native promoters of these reporter genes therefore resulted in a low bacteriocin yield [12]. As the first version, the two- plasmid systems have some drawback such as: "bulky" (2 different plasmids), constitutive, two antibiotics needed to avoid the loosing of plasmids. The two-plasmid system has been used as tool for studies of bacteriocin like site-directed mutagenesis, but not for production of non-bacteriocin protein [3].

To overcome the disadvantages of the two-plasmid system, Axelsson and co-worker continued to create a new inducible expression one-plasmid pKRV3 in 2003. Generally, this plasmid pKRV3 was constructed as a combination of two previous plasmids with some modifications. Three components of regulatory systems with frame-shift mutated *sapIP* obtained from pSAK20E [46] under the regulation of P$_{sapIP}$ promoter were combined with *erm* gene, two origin replicons *256rep* and *pUCori* (for *Lactobacillus* and *E. coli* respectively) and *lacZ* containing multi-cloning site (MCS) for blue-white screening for cloning in *E. coli* and *Lactobacillus* [13]. The promoterless reporter gene was fused with

promoter P$_{sapA}$ and cloned into plasmid by using appropriate restriction sites in MCS. The pKRV3 plasmid is considered as the starting point for further development of lactobacillal inducible expression systems.

A similar work has been reported by Mathiesen and co worker (2004) when based on *spp* regulon to develop expression vectors. With the expectation that the highest production level can be obtained if all necessary genes involving in sakacin P synthesis with nature arrangement are included in expression vector, the plasmid pMLS114 containing all *spp* genes [39] were used as starting point [49]. Two different reporter genes, aminopeptidase *pepN* from *Lactococcus lactis* or β-glucuronidase *gusA* from *E. coli*, were replaced to *sppAiA* in pMLS114 (with and without deletion mutation of *sppIP*), yielding non-inducible and inducible vectors pGM1, pGM4 and pGM4gusA [49]. Despite the fact that pMLS114 derivatives gave high expression levels, some drawbacks could be observed such as: large in size (13.7 kb), difficult to "engineer", unstable in one of two used *Lactobacillus* strains and high background level [49, 50].

pSip vector series

The prototype pSip have been firstly introduced and named by Sorvig in 2003. To date, the pSip series are the most well-known and widely used inducible sakacin-based expression vectors for lactobacilli. Several derivatives of pSip have developed by modifying the components in the prototype vectors such as origin replicon, promoters and selection marker.

General construction of pSip

In general, the prototype pSip vectors have a similar construction to pKRV3 vector, including pSip300 and pSip400 series based on *sap* (sakacin A) and *spp* (sakacin P) regulon, respectively [3, 14, 50]. These vectors were composed of replication origin, selection marker, regulatory components cassettes separated by appropriate restriction sites, thus allow to easy exchange of these parts using standard digestion and ligation (Figure 3).

Table 1 - *sap* and *spp*-based expression systems

Expression systems	Relevant characteristics	Target gene	Host expression	Reference
1st plasmid: pSAK20 2nd plasmid - pSPP1/pSPP2 - pPED1/pPED2 - pPSC1/pPSC2	Two-plasmid systems *sapKRTE ΔsapA ΔsaiA*, Cm^r Shuttle vector for *E.coli-L.plantarum-L.sake*; Em^r, *lacZ*, target gene are regulated by: - native promoter or P$_{sapA}$ promoter - native promoter or P$_{sapA}$ promoter - native promoter or P$_{sapA}$ promoter	*sppA, spiA* *pedA, pedB* *psc61, orfX*		[12]
pKRV3 derivatives	P*sapIP-ΔsapIP-sapKR*-T*saiA*, *E.coli-L.plantarum-L.*sake replicons; Em^r, *lacZ*	*gusA*	*L. sakei* Lb790 *L. plantarum* NC7, NC8	[13]
pGM4 series	Shuttle vector for *E.coli-L.plantarum-L.sake*; Em^r, *lacZ*, *sppKRTE ΔsppIP,* target gene under control of P$_{sppA}$	*pepN or gusA*	*L. plantarum* NC8	[49]

pSip300: *sap*-based vectors
  pSip301
 	
  pSip304
	pUC(pGEMori), *p256rep* and *erm* and T$_{pepN}$ terminator, *sapKR* expression driven by *erm* read-through.
	sapKR was preceded by non functional version of *sapIP* with P$_{sapIP}$ promoter.
	
	p256rep replaced by *SH71rep*
		gusA, pepN	*L. sakei* Lb790
			L. plantarum NC8
				[4, 14, 50-52]
				[53]

pSip400: *spp*-based vectors
  pSip401
  pSip404
 	
  pSip409
	pUC(pGEMori), *p256rep* and *erm* and T$_{pepN}$ terminator, *sppKR* expression driven by *erm* read-through.
	sppKR was preceded by non functional version of *sppIP* with P$_{sppIP}$ promoter.
	p256rep replaced by *SH71rep*
	
	P$_{sppA}$ replaced by P$_{sppQ}$
		lacLM, l-ri
		gusA, pepN
		
		lacZ, oxdc
			L.plantarum BPT232
			L. plantarum WCFS1,
			L. plantarum NC8
			L.sakei Lb790	

Signal peptide pSip vectors	pSip401 derivative, signal peptide located upstream of target gene	*nucA, amyA*	*L. plantarum* WCFS1	[54]
Intein vector	pSip401 derivative, intein tag in MCS, used for purification improvement	*lacLM*	*L. plantarum* WCFS1	[55]
pSIP600	Food-grade pSip400 derivatives with alanine racemase (*alr*) complementary marker	*lacLM*	*L. plantarum* WCFS1	[56]

The promoters used to regulate the expression of target gene are P_{sapA} or P_{sppA}/P_{sppQ} separated to reporter gene by *Nco*I, therefore the reporter gene can be cloned into vectors downstream by using *Nco*I restriction site (or used alternative REs such as *BsmB*I or *Bsa*I) and another appropriate site in MCS. Several synthetic promoters could be used to regulate the expression of target gene, instead of sakacin promoters [57]. Although they are constitutive, some of them can give the higher expression level than P_{sppQ} which is considered as the strongest promoter among used [57]. The regulatory components *sapKR* and *sppKR* downstream of non-functional *SppIP* were regulated by cognate promoters (P_{sapIP}/P_{sppIP}) or by native promoter of *erm* gene (via read-through). The cluster for regulatory gene is separated to *erm* cluster by *Sal*I (Figure 3) (in some derivatives this non-sense *Sal*I is mutated, see *signal peptide vectors*).

The replicon region of pSip consists of two different replication determants,
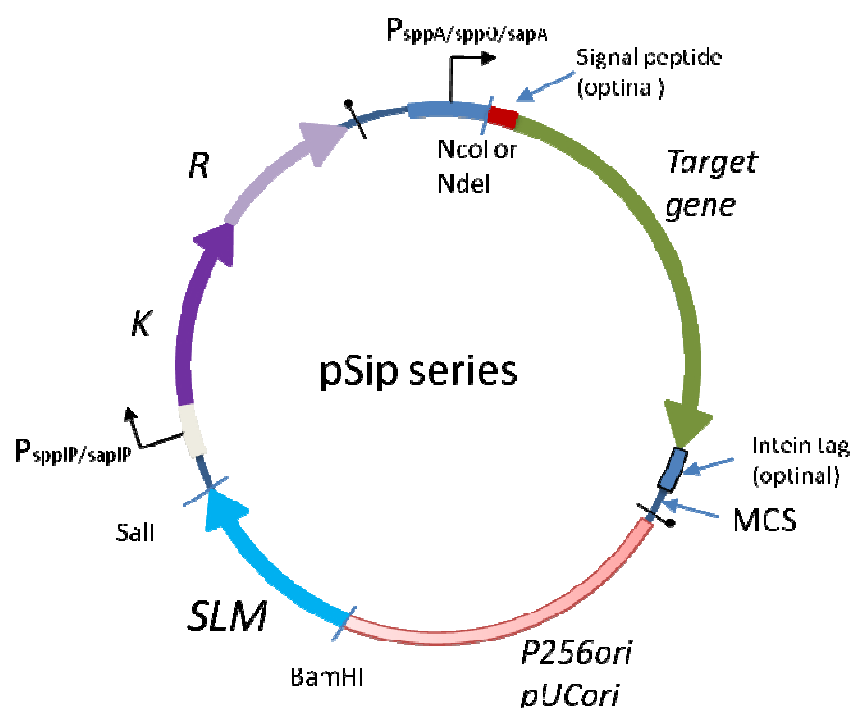
Figure 3. Schematic overview of the modular prototype and derivatives of pSip. The *K* and *R* regulated by P_{sppIP} or P_{sapIP} encode for histidine kinase and response regulator, respectively. The target genes with either optional leading sequence (signal peptide) or intein tag is cloned downstream of the bacteriocin promoter by *Nco*I site (or *Nde*I in case of signal peptide vector) (see text for detail). The replicon region contains two replication determants; *p256ori* and *pUCori* for *Lactobacillus* and *E. coli* respectively. SLM denotes for selection marker which is erythromycin resistance gene (*erm*) in prototype pSip or alanine racemase gene (*alr*) in food-grade pSip derivatives. Lollipops indicate transcription terminators.

pUC(pGEM)ori and *p256ori* which help the cloning of pSip in *E. coli* and *Lactobacillus*, respectively. The *pUC(pGEM)ori* is high copy number determant specific for *E. coli* [14], permitting to get high amount of plasmid facilitating for further step in molecular practical works. The plasmid copy number is considered as key factors effect on efficiency of heterologous expression since influencing on dose of target genes. The plasmid copy number is decisively affected by replication determant [58]. However, *p256ori* is specific for narrow - host range of *Lactobacillus* strains [59, 60], resulting in the low copy number, i.e. three in *L. sakei* and six in *L. plantarum* NC8 [50] or four in *L. plantarum* WCFS1 [61]. Another broad-host range, high copy number replicon *pSH71ori* [62] were replaced for p256*ori* in pSip, thus increased the copy number of plasmid to 3-to 8-fold upon host and vectors [50]. Moreover, with the lactococcal *pSH71ori*, pSip could be replicated in *Lactococcus* host.

pSip series rely on erythromycin resistance gene *erm* regulated by native promoter from *Lactobacillus reuteri* [63] as selection marker. To avoid the loosing of plasmid, erythromycin is supplemented in cultivation media with the concentration of 200 µg/ml or 5 µg/ml for *E. coli* or *Lactobacillus*, respectively. However, using antibiotic is drawback of not only pSip but also other expression systems which are intended to use for food-application [9]. In the attempt to develop the food grade systems based on pSip, recently Nguyen and co-workers have replaced *erm* with alanine racemase encoding gene (*alr*) from *L. plantarum* WCFS1, resulting in new food-grade versions of pSip. This new version was found that more stable in host *L. plantarum* compared to original version pSip with *erm* marker [56].

pSip derivatives

Signal peptide-vectors

Signal peptides are amino acid sequences located at N-terminal of a protein which can be excreted out of bacterial cell by Sec-dependent system [64]. After driving the secretion of protein, signal peptide is cleaved by peptidase releasing protein for further translocation process [65]. To test certain putative signal peptides from *L. plantarum* WCFS1 and develop a new protein targeting system which can be used both for overexpression and secretion of protein, Mathiesen and co workers have modified pSip401 vector by changing *Nco*I site which normally used for digestion and ligation of a particular target gene in pSip to *Nde*I and

removing the *Sal*I site in non-sense region of pSip vector. The N-terminus of several signal peptide sequences were fused downstream of P_{sppA} promoter, whereas the C-terminus were followed by reporter gene with 6-nucleotide linker (*Sal*I) in between. By that, the authors have developed a new modular vectors, so called signal-peptide pSip vectors, allowing easy exchange of signal peptide as well as target gene [54].

Food-grade vectors

The erythromycin antibiotic resistance gene (*erm*) selection marker in pSip does limit the food-application potential of pSip systems. It is necessary to use the food-grade selection marker instead of antibiotic marker. Several food-grade selection markers have been used in development of expression systems such as: sugar utilization genes [66-72] or bacteriocin resistance/immunity genes [73, 74]. Certain attempt has been done in our group to investigate a new food-grade selection marker for pSip. Nisin have been well-known as GRAS food additive for several decades due to antimicrobial effect on gram positive bacteria such as *Clostridium*, *Staphylococcus*, and *Lactobacillus*. Therefore, nisin immunity gene *nisI* from *Lactococcus* could be a potential dominant marker for system which applied in *Lactobacillus* [74]. Unfortunately, this strategy was fail with the case of *L. plantarum* WCFS1 as host because it was found that this strain is inducible resistant to nisin by produce proteinase (unpublished data) which could be putatively encoded by Lp_1420 coding sequence [75]. An alternative option, *alr* gene which solely encodes for crucial enzyme alanine racemase in *L. plantarum* [76-78] has been used as complementary marker for the same purpose. The new pSip derivative relying on *alr* gene combining with respective *alr* deletion mutant of *L. plantarum* WCFS1 yield the new complementary expression systems for food application [56].

Intein-vectors

In another approach, Nguyen and co-workers have cloned the intein tag, which is a combination of inducible self-cleavage intein [79] with chitin binding domain sequence of chitinase [80, 81] or with six-histidine tag, in MCS of pSip403 vector yielding new derivatives of pSip, so called intein-vectors. These vectors were used for overexpression of "precursor" of target protein followed by intein tag. The chitin binding domain or histidine tag facilitates the specific binding of "precursor protein" on chitin or Ni^{2+} metal column, respectively. Due

to the inducible self-cleavage ability of intein in presence of DTT [82, 83], the tags can be excised, thus release target protein in native form. Thus, intein- pSip vectors can be used for heterologous overexpression in *Lactobacillus* host [55], as similarly to IMPACT vectors from New England Biolab [84].

Heterologous and homologous protein expression in *Lactobacillus* host strains using sakacin–based systems

To date sakacin –based systems, in particular pSip series have been successfully used for mainly heterologous expression in *Lactobacillus* host of diverse target proteins from different sources such as *E. coli*, *Lactococcus*, *Lactobacillus*, *Acinetobacter*, *Bacillus* and *Corynerbacterium*. The expression hosts have been mostly belong to *Lactobacillus* species such as *L. sakei* Lb790, 23K, and *L. plantarum* NC7, NC8, BPT197 and well-known WCFS1. Some of them have been reported that contains homologues of *sppKR* on expression vector

The inducible expression of target protein with pSip vectors are activated by synthetic peptide pheromone. It was indicated that in batch culture, the induction was most effective in exponential growth phase (OD_{600} from = 0.3-0.5) and the maximum target protein level was obtained in the late of growth phase when OD_{600} = 1.5 − 2.0 [13, 49]. This observation has been supported by transcript analysis results of *sppA* reported by Bruberg et al., in which the maximum amount of transcript were found approximately after 4h of induction (at early stationary phase) [43]. However, the time for peak of expression level can be changed depending on target gene, expression host and condition for cultivation. For example, the pH control fermentation can result in higher maxima expression level at later time after induction compared to un-controlled pH condition [61].

It has been shown that to obtain high expression level of target gene, the amount of IP supplemented in culture should be corresponding to cells density of induction, e.g. at the higher OD_{600} of induction, the more IP amount needed. By control the pH of cultivation at optimal value (for example at 6.5 for *Lactobacillus* host) or by supplying more carbon source sugar in fermentation medium, the log phase can be prolonged, thus the induction can be

induced at higher cell density with relative amount of pheromone to obtain maximum yield of target protein [61].

Several pSip vectors have exhibited as effective expression systems and resulted in high level of target protein with high induction factor (low basal expression in non-induced condition) using rather low IP pheromone (25 ng/ml) [3]. However, the effectiveness of particular target gene in pSip vectors has been reported as followed an unpredictable manner. The functionality of pSip depends on host strain, promoter, genes of interest, plasmid copy numbers [3, 50]. A clear example has been published recently by Halbmayr and coworker (2008) when authors used pSip403 and pSip409 to overexpress β-galactosidase (*lacLM*) from four *Lactobacillus* species in *L. plantarum* WCFS1 and *L. sakei* Lb790 [4]. Even though the amino acid sequences of these β-*galactosidases* are highly similar (approximately 60 – 70%) and the closed phylogenetic relationship between those strains, the activities of β-galactosidase were obtained as significantly different depending the source of target, promoter and host. Two high similar *lacLM* genes from *L. acidophilus* and *L. reuteri* gave different expression level even with the same promoter and expression host. The target protein of *lacLM* from *L. reuteri* yielded approximately 55% of intracellular protein, c.a 18-fold higher than the yield of *lacLM* from *L. acidophilus*. The difference at mRNA level corresponding for two target genes was recently indicated as reason for the difference in activity [44]. Another unexpected result observed in this study was the low level of homologous expression of *L. sakei lacLM* genes in *L. sakei* [4].

Aminopeptidase N (*pepN*) from *Lactococcus lactis* and glucoronidase (*gus A*) from *E. coli* have been widely used as reporter genes for development of new expression vectors. Despite of the origin of these reporters, their expressions with different sakacin-based systems/ in *Lactobacillus* host are successful, for example the PepN protein yielded c.a. 40 - 46% of intracellular protein [49, 50]. *L. bulgaricus lacZ* also were successfully overexpressed in *Lactobacillus* with pSip systems which yielded approximately 47% of intracellular protein [53]. Amylase (*amyA*) from *Lactococcus amylovorus* and nuclease (*nucA*) from *Staphylococcus aureus* have been used for testing the signal peptide vector by Mathiesen and co-worker. It has been shown that the expression of these two genes in those pSip401 derivatives were rather high, and a fraction of target protein was secreted out of bacterial cells with the level depending on the signal peptide used [54]. Some other examples for the

effectiveness of pSip series were recently reported with the target gene from different origins such as oxalate decarboxylase (*oxdc*) gene from *Bacillus subtilis* [52], L-ribose iromerase (*l-ri*) from *Acinobacter* [51] or 2,5-diketo-gluconic acid-reductase *(DKR)* from *Corynerbactericum glutamicum* [85].

Conclusion

Bacteriocin from lactic acid bacteria does provide for food industry the potential preservatives. Moreover, the knowledge about the genomic determents and synthesis of LAB bacteriocin has exploited the development of several vectors used for heterologous or homologous expression in LAB. The sakacin-based systems, in particular pSip expression systems based on sakacin bacteriocin regulation has been developed and specifically and successfully applied in lactobacilli. Because of GRAS and probiotic characteristics of lactobacilli host, the sakacin based systems has been used not only for food application but also in medical field as the delivery vehicles in mammals.

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Chapter 2

Quantitative transcript analysis of the inducible expression system pSIP: comparison of the overexpression of *Lactobacillus* spp. β-galactosidases in *Lactobacillus plantarum*

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Abstract

Two sets of overlapping genes, *lacLMReu* and *lacLMAci*, encoding heterodimeric β-galactosidases from *Lactobacillus reuteri* and *Lactobacillus acidophilus,* respectively, were previously cloned into the pSIP vector for inducible lactobacillal expression and successfully expressed in *Lactobacillus plantarum* WCSF1. Despite the high similarity between these *lacLM* genes and the use of identical cloning and expression strategies, strains harboring *lacLMReu* produced about twenty-fold more β-galactosidase than strains containing *lacLMAci*.

In this study, the plasmid copy numbers (PCN) of expression vectors pEH9R (*lacLMReu*) and pEH9A (*lacLMAci*) as well as the transcription levels of both *lacLM* genes were compared using a quantitative PCR methods. Analyses of parallel fermentations of *L. plantarum* harboring either pEH9R or pEH9A showed that the expression plasmids had similar copy numbers. However, transcript levels of *lacLM* from *L. reuteri* (pEH9R) were up to 18 times higher than those of *lacLM* from *L. acidophilus* (pEH9A). As a control, it was shown that the expression of regulatory genes involved in pheromone-induced promoter activation were similar in both strains.

The use of identical expression strategies for highly similar genes led to very different mRNA levels. The data indicate that this difference is primarily caused by translational effects that are likely to affect both mRNA synthesis rates and mRNA stability. These translational effects thus seem to be a dominant determinant of the success of gene expression efforts in lactobacilli.

Background

Lactic acid bacteria (LAB) are important micro-organisms in the food and beverages industry. Over the past few decades, LAB have been used not only as starter culture but also as producers of flavoring enzymes, antimicrobial peptides or metabolites that contribute to the flavor, texture and safety of food products [1-3]. Moreover, because of their food-grade status and probiotic characteristics, several LAB, especially lactobacilli, considered as safe and effective cell-factories for food-application purposes [2, 3]. As a consequence, a variety of constitutive or inducible gene expression and protein targeting systems for LAB hosts have been developed, including sugar-inducible, thermo-inducible and pH-dependant expression systems [1, 2, 4].

Two well-known inducible expression systems for LAB employ promoters from bacteriocin operons, the Nisin-controlled expression system (NICE) [5] and the pheromone-inducible system pSIP [6]. The NICE system employs genes and promoters involved in the production of the antimicrobial peptide (lantibiotic) nisin by *Lactococcus lactis* and the inducing substance is nisin itself [5]. Similarly, the pSIP systems were developed based on promoters and regulatory genes involved in the production of the class II bacteriocins sakacin A [7] and sakacin P [8, 9] in *Lactobacillus sakei*. In these lactobacilli, bacteriocin production is regulated by a three-component system, consisting of a secreted peptide pheromone (IP) which interacts specifically with a cognate membrane embedded histidine protein kinase (HPK). A response regulator (RR) encoded in the same operon as IP and HPK (Figure 1) is activated by the HPK, leading to induction of all the promoters in the bacteriocin operons [8]. The pSIP systems have been used to over-produce enzymes such as β-glucuronidase and aminopeptidase N in several *Lactobacillus* hosts [6, 10, 11].

β-galactosidases (lactase, EC 3.2.1.23) are known as important enzymes in the dairy industry [12-14]. β-Galactosidases are used to cleave lactose into galactose and glucose to prevent the crystallization of lactose, to improve sweetness, to increase the solubility of milk products, and to produce lactose-free food products [15]. Another beneficial ability of β-galactosidases is the trans-galactosylation reaction which co-occurs during lactose

hydrolysis [12] resulting in the formation of galacto-oligoscharides (GOS). Similarly to fructo-ologosaccharides (FOS), GOS possess prebiotic properties [13, 14, 16, 17].

Almost all β-galactosidases of lactobacilli, including the enzymes from *L. reuteri* and *L.*
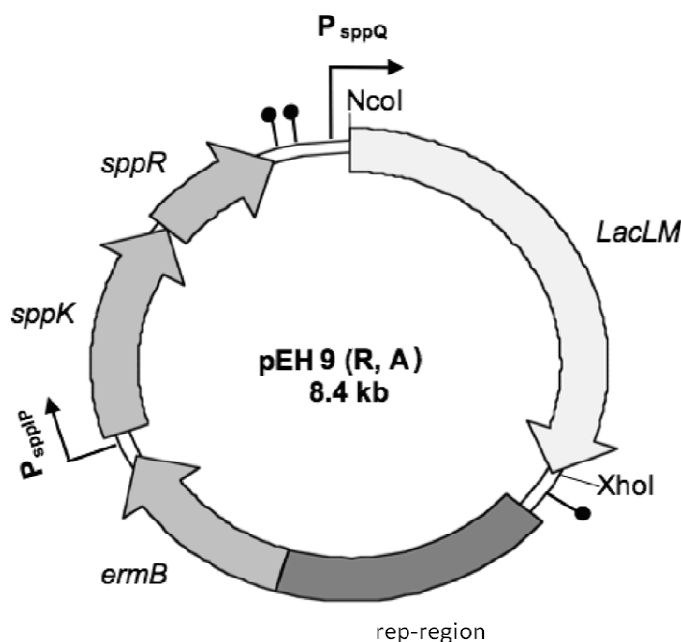
Figure 1. Schematic representation of the pEH9 (R/A) plasmids. These plasmids are based on the pSIP409. R and A refer to the *lacLM* genes from *L. reuteri* L103 and *L.acidophilus* R22, respectively. Two inducible promoters P_{sppIP} and P_{sppQ} (P_{orfX}) control the expression of *sppKR* and *lacLM* genes, respectively. The *lacLM* genes are fused to the P_{sppQ} promoter by translational coupling. The regulatory *sppKR* operon, whose gene products are needed to convert the signal form the externally added peptide pheromone into activation of the inducible promoters (see text), differs from the original *sppIPKR* operon in that the *sppIP* gene carries a major in-frame deletion.

acidophilus, consist of two subunits, one large and one small, which are encoded by two overlapping genes, *lacL* and *lacM*, respectively [13]. In a previous study, we have overexpressed the β-galactosidases from *L. reuteri* L103 and *L. acidophilus* R22 (as well as β-galactosidases from *L. sakei* and *L. plantarum*) by cloning the *lacLM* genes into pSIP vectors [10]. Two of the resulting expression vectors, pEH9R and pEH9A, are based on pSIP409 [10] and contain *lacLMReu* from *L. reuteri* L103 and *lacLMAci* from *L. acidophilus* R22, respectively (Fig. 1). The *lacLM* genes are under the control of the strong promoter P_{sppQ} (previously known as P_{orfX}) [6, 11], to which they are translationally fused, and over-expressions of these β-galactosidases in the well-studied food-grade strain *Lactobacillus*

plantarum WCSF1 was successful. However, even though the amino acid sequences of these β-galactosidases are highly similar (around 70%) [18], both SDS-PAGE analyses of cell extracts and activity measurements showed that the two enzymes had very different production levels, with *lacLMReu* being expressed about twenty times more efficiently than *lacLMAci* [10].

The observed expression levels are the end-product of transcription, translation and post-translational processes, which all may be influenced by a large number of factors, including the gene dose, which is determined by the plasmid copy number (PCN). Messenger-RNA (mRNA) levels are a decisive factor for the final amount of active protein. In the present study we have used RT-PCR to verify whether the different expression efficiencies of *lacLMAci* and *lacLMReu* correlate with differences in mRNA levels. Furthermore, we used RT-PCR to check for possible differences between the plasmid copy numbers (PCN) of pEH9A and pEH9R. Since identical cloning strategies had been used for highly similar genes, substantial differences were not a priori expected. However, interestingly, large differences in mRNA levels were found.

Material and Methods

Bacterial strains and media

Lactobacillus plantarum WCFS1 harboring pEH9R or pEH9A containing the overlapping genes (*lacLM*) encoding β-galactosidase of *Lactobacillus reuteri* L103 and *Lactobacillus acidophilus* R22, respectively, were maintained in MRS (Merck, Germany) containing 5 µg/ml erythromycin at – 70°C.

Fermentations

Strains were activated from frozen stock in 5 ml of MRS with 5 µg/ml erythromycin at 37°C for 16-18h. These cultures were used to inoculate 400ml MRS medium (40 g/l glucose, 5 µg/ml erythromycin). Cultivations were done in an HT-Multifors system (Infors HT, Switzerland) with pH control at pH 6.5, at 37°C. Sodium hydroxide was used for maintaining the pH. A low speed (200 rpm) of agitation was set to ensure the

homogeneity of medium and other parameters as well as to ensure continuous contact between bacterial cells and nutrient. Induction of gene expression was achieved by added a 19 amino acid synthetic peptide pheromone, IP673, with a sequence identical to the sequence of the pheromone as originally isolated from *Lactobacillus sakei* LTH673 [19].

Growth of bacteria was monitored via the optical density at 600 nm (OD_{600}). After six hours, when OD_{600} had reached around 3.0, IP673 was added to a final concentration of 80 ng/ml to induce *lacLM* gene transcription. Samples were collected at intervals for OD_{600} measurements, enzyme assays, and DNA and RNA isolation.

For β-galactosidase measurements, cells from 1 ml of fermentation broth were pelleted by centrifugation at 13200 rpm for 3 min. Cells were re-suspended in buffer P [16], then disrupted by sonication (4 x 1 min at 100 % power, interrupted by 1 min breaks and constant cooling on ice, using a Bandelin Sonopuls HD60, Germany). Subsequently, cell debris was removed by centrifugation at 13200 rpm for 10 min at $4^{o}C$. The obtained crude extract was used for measuring β-galactosidase activity as well as protein concentration.

For DNA or RNA isolation, cells were pelleted as described, shock-frozen by liquid nitrogen and stored at $-80^{o}C$ until further use.

Enzyme assays

β-Galactosidase activity was determined using *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG) as previously described [13]. Protein concentration was determined using the method of Bradford [20] with bovine serum albumin as standard.

Bacterial DNA isolation and purification for PCN estimation

1 ml of culture at an OD_{600} of 10 was used for DNA isolation. For cells harvested at lower values of OD_{600}, correspondingly higher culture volumes were collected (e.g., 2 ml of a culture with an OD_{600} of 5). DNA was isolated and purified using the phenol-chloroform extraction method as described in literature [21]. The purified bacterial DNA was stored at $-20^{o}C$ until use.

RNA isolation and purification

Total RNA was isolated using the peqGOLD Bacterial kit (Peqlab) according to the supplier's instructions without DNA on-column digestion. The concentration of total RNA was determined spectrophotometrically at 260nm (A_{260}) (Beckman DU80). RNA integrity was examined by agarose gel electrophoresis (2% agarose). DNA contaminations in total RNA samples were completely removed by digestion with 1 U/µl of DNAse (PeqLab) in a total volume of 20 µl using DNAse reaction buffer as recommended [22]. After 10 min at 37°C, 30 mM EDTA solution was added to a final concentration of 3 mM. The mixture was heated at 70°C for 15 min to inactivate DNAse and stored at -70°C. The absence of residual DNA contamination was confirmed by normal PCR with 16S primer pair (not shown).

Reverse transcription and real time quantitative PCR

Reverse transcription

Table 1- Oligonucleotide primers used in this study

Primer	Sequence 5´ - 3´	T_a^a (^{o}C)	Concentration[b] (mM)	Product size (bp)	Tm product (^{o}C)
16S_f	TGATCCTGGCTCAGGACGAA		250		
16S_r	TGCAAGCACCAATCAATACCA	60	250	81	81
Ery^R_f	CCGTGCGTCTGACATCTAT		250		
Ery^R_r	TGCTGAATCGAGACTTGAGTG	60	250	108	79
LacReu_f	CCA GAT TCC GTG GTA TTA CCT TTG TG		250		
LacReu_r	TAC TACT ACG TCA CGC CAT TGA GGA AC	60	500	154	80
LacAci_f	TCTAGTTCACTACGAAGGTGTCG		500		
LacAci_r	GTCATGCATGTATTCACACTCC	60	500	154	76,5
SppKR_f	CAAGCCGTTCAAGAAACCGAT		250		
SppKR_r	AGCGCCTTTCGTTGAATAGCC	60	500	144	78,5

[a] optimized annealing temperature as described in the Materials Section, [b] optimized concentration as described in the Materials Section

Messenger RNA in total RNA samples was reverse-transcribed using the First Strand cDNA Synthesis kit (Fermentas) with random hexamer priming and 100 units of reverse transcriptase (RevertAidTM M-MuLV Reverse Transcriptase, Fermentas). The resulting cDNAs were stored at $-20^{o}C$. For control samples, dimethylpyrocarbonate (DEPC) treated water was used to replace reverse transcriptase.

Oligonucleotide primers

Oligonucleotide primers used in this study are listed in Table 1. For plasmid copy number estimation, the erythromycin resistance gene *ermB* and *16SrRNA* were chosen as representatives for plasmid DNA and genomic DNA, respectively. From the sequences of these genes, two primer pairs called Ery^{R} and 16S were designed (Table 1).

For relative mRNA quantification of three genes *lacLMReu*, *lacLMAci* and *sppKR*, 3 primer pairs called LacReu, LacAci and SppKR, respectively, were designed. The two former primer pairs were designed so that their characteristics as well as the length of amplicons were similar. The chromosomal*16SrRNA* gene was used as reference gene.

RT- PCR reaction using SYBR Green I dye

The thermal cycling system iCycler and myIQ single Color Real-Time PCR Detection system (BioRad) were used for qPCR amplification and detection. The qPCR reactions (25 µl total volume) were prepared in duplicates in 96 wells plates (BioRad) that were sealed with optical adhesive covers (Microseal 'B' film, BioRad). Each reaction included an optimized concentration for each of forward and reverse primers (see Table 1), 12.5 µl of Perfecta SYBR Green Super mix of IQ (Quanta Biosciences), and 2.5 µl of DNA template. Negative controls (no template control), prepared by replacing the DNA template with DEPC water, were included in each run to confirm the absence of DNA contaminations in the reagents. Before setting up the experiments described in the Results section, primer concentrations, annealing temperatures and DNA template concentrations were optimized according to procedures and criteria described in [22] and the final optimized reaction parameters are shown in Table 1.

The qPCR reactions were conducted as follows: initial denaturation at 95°C for 3 min followed by 50 cycles of 20 s at 95°C, 20s at 60°C, and 72°C for 10s. The fluorescence signal was collected at the end of each extension step at 72°C. Afterwards, the

temperature was increased from 55°C to 95°C at a rate of 0.2^{o}C/s to establish the melting curve.

The threshold cycle values (C$_t$) were automatically determined by the software Biorad MyIQ optical system Version 2.0.

Calculation of the PCN value

Base on the PCN definition, which is the number of copies of a plasmid present per chromosome in bacteria [23, 24] the PCN can be calculated by the following equation [25]: $PCN = \dfrac{E_c^{C_{tc}}}{E_p^{C_{tp}}}$ (1), here, E_c, C_{tc} and E_p, C_{tp} are the amplification efficiency and the threshold cycle value of the amplicon representing chromosome and plasmid, respectively. The equivalence between the amplification efficiency (E) of plasmid and chromosome amplicons was confirmed in validation experiments as recommended [26]. In addition, to compare the PCN between two recombinants, the relative PCN values were calculated using the comparative C$_t$ method (ΔΔC$_t$), in the following equation: $\dfrac{pEH9R}{pEH9A} = 2^{-\Delta\Delta C_t}$ (2), where ΔΔC$_t$ = ΔC$_t$ of the sample corresponding to pEH9R − ΔC$_t$ of sample corresponding to pEH9A, and ΔC$_t$ = average C$_t$ value of target (for erythromycin resistance gene) − average C$_t$ value of reference gene.

Calculation of the expression ratio

The relative expression level between the two genes (e.g. A and B) was also estimated as described in equation (2), where ΔΔC$_t$ = ΔC$_t$ corresponding to gene A - ΔC$_t$ corresponding to gene B and ΔC$_t$ = average C$_t$ of target genes (A or B) − average C$_t$ of reference gene (*16S rRNA*).

The relative expression level of each gene of interest compared to the time point before induction (here after 6 hours of cultivation) was estimated accordingly, but ΔΔC$_t$ = ΔC$_t$ of genes of interest at different time points - ΔC$_t$ of genes of interest after 6 hours of cultivation. In the present work, the genes of interest were *lacLMReu, lacLMaci* and *sppKR.*

Codon usage and mRNA secondary structure analysis

The codon usage of the *lacLM* genes was compared to the codon usage of *Lactobacillus plantarum* WCFS1 using the software Graphical Codon Usage Analyzer (http://gcua.schoedl.de/index.html). The codon usage table of *L. plantarum* WCFS1 is estimated based on 3057 CDS's (934462 codons) (from http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=220668).

Messenger-RNA (mRNA) secondary structure for both *lacLM* genes was analyzed using mfold (mobile.pasteur.fr/cgi-bin/portal.py?form=mfold) from the transcription start point (65 nt upstream of ATG) to 150 nt (50 codons).

Results

Optimization and validation of the RT-PCR reaction

An optimization step is necessary to achieve reproducibility in quantitative PCR reactions [22]. Annealing temperature and primer concentration are objects for variation and optimization and were tested. Parameters resulting in a lower C_t value and a higher fluorescence signal were considered optimal and are listed in Table 1 for all primer combinations. As a quality control, experiments were done to analyze to what extent ΔC_t (i.e. the difference in C_t between two amplicons amplified from the same template) depended on the dilution of the template. The validation experiments were conducted between primer pairs 16S (corresponding to reference gene *16SrRNA*) and each of the four primer pairs, Ery[R], LacReu, LacAci and Spp, representing the target genes. A series of 10 fold dilutions of cDNA or plasmid (for Ery[R]) was used to obtain C_t values, and the ΔC_t between the primer pairs were calculated. ΔC_t values were plotted against the logarithm of initial amount of plasmid DNA or cDNA. The slopes of all regression lines were lower than 0.1 (data not shown), which implies that the $\Delta\Delta C_t$ method can safely be applied for each of the four amplicons [26].
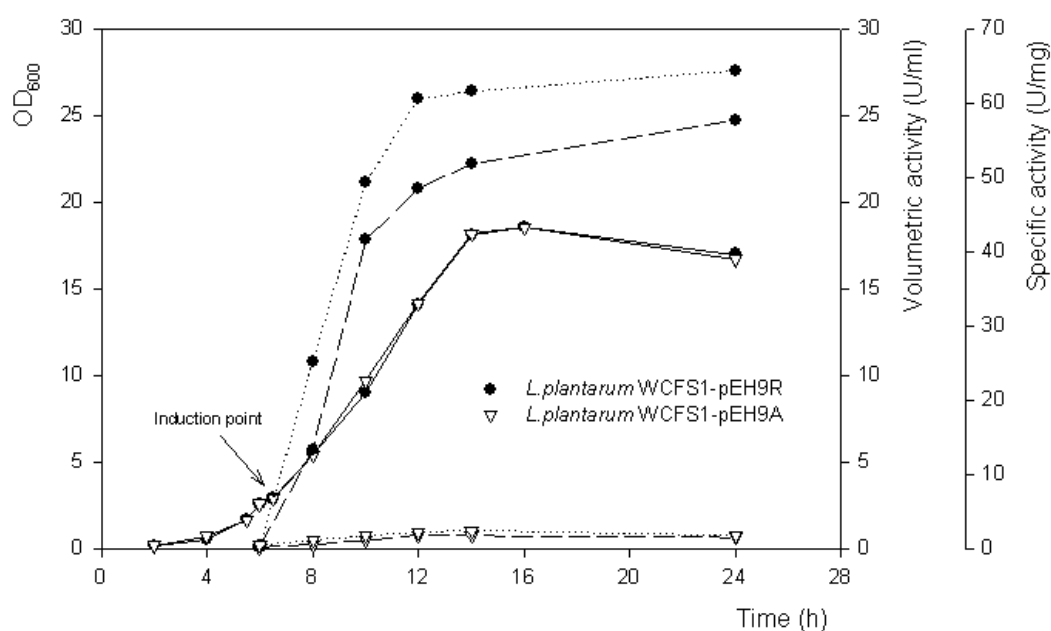
Figure 2. Time course for growth of *L. plantarum* WCFS1 carrying pEH9R and pEH9A. Cultivations were carried out with pH control at pH 6.5 in 400 ml laboratory fermentors at 37°C using MRS medium (40 g/l glucose). The graph shows OD$_{600}$ (solid lines), β-galactosidase activity (units per millilitre of fermentation broth) (dashed lines) and specific activity (units per milligram protein) (dotted lines). Cultures were induced with 80 ng/ml of pheromone after six hours of growth, i.e. at an OD$_{600}$ of approximately 3.0.

Fermentations

L. plantarum WCFS1 carrying either pEH9R or pEH9A was fermented with pH control using conditions that had previously been determined to yield high enzyme. Gene expression was induced by adding the peptide pheromone IP-673 6 hours after the start of the fermentation (at an OD$_{600}$ of approximately 3.0). Figure 2 shows that growth of the two strains was nearly identical over the whole period of the fermentation. In contrast, β-galactosidase yields (in terms of both both units per volume of fermentation broth and units per mg protein) were considerably different. *L. plantarum* WCFS1 carrying pEH9A showed a maximum activity around 0.8 U/ml and 2.5 U/mg, whereas with pEH9R maximum activities amounted to about 22 U/ml and 62 U/mg (Figure 2). SDS-PAGE experiments (not shown) confirmed that these activity differences are correlated with large differences in protein production levels. Previous studies have shown that the

purified β-galactosidases from *L. reuteri* and *L. acidophilus* have similar specific activities [13, 16].

Table 2 – Ratios of β-Galactosidase activity, plasmid copy number and expression level of *sppKR* and *lacLM* for *L. plantarum* WCFS1 carrying pEH9R versus *L. plantarum* WCFS1 carrying pEH9A

Time (h)	Time after induction (h)	Activity [a]	Plasmid copy number	*lacLM* expression level	*sppKR* expression level
6 [b]	0	0.90	1.39 ± 0.25	3.88 ± 0.27	0.76 ± 0.12
8	2	20.81	0.81 ± 0.07	13.25 ± 3.34	1.00 ± 0.06
12	6	27.03	0.79 ± 0.01	18.13 ± 4.23	0.93 ± 0.22
24	18	36.17	1.34 ± 0.06	-	-

The presented values are obtained by dividing the average value for the pEH9R-harboring strain with the average value for the pEH9A-harboring strain as described in Material and Methods.

[a] Based on specific activity (U/mg protein), [b] Just before induction

Table 3 - β-Galactosidase activity and transcript levels of *sppKR* and *lacLM* at different time points, expressed relative to the levels at the induction point

Time (h)	Time after induction (h)	pEH9R			pEH9A		
		Activity [a]	*lacLM* expression level	*sppKR* expression level	Activity [a]	*lacLM* expression level	*sppKR* expression level
6 [b]	0	1	1.00 ± 0.07	1.00 ± 0.16	1	1.00 ± 0.13	1.00 ± 0.21
8	2	56.1	59.92 ± 15.65	1.88 ± 0.11	2.42	17.77 ± 4.26	2.49 ± 0.04
12	6	135.2	55.40 ± 13.50	1.67 ± 0.39	4.48	11.92 ± 1.97	2.00 ± 0.20
24	18	143.7	16.44 ± 2.57	nd[c]	3.56	0.46 ± 0.12	nd

[a] Specific activity (U/mg protein), [b] Just before induction, [c] non-determined

Table 4 - Ratio of expression levels of *sppKR* versus *lacLM* in *L. plantarum* WCFS1 carrying pEH9R and *L. plantarum* WCFS1 carrying pEH9A

Time (h)	Time after induction (h)	pEH9R	pEH9A
6[a]	0	4.87 ± 0.78	14.32 ± 2.96
8	2	0.15 ± 0.01	2.01 ± 0.03
12	6	0.15 ± 0.03	2.40 ± 0.23

Values were obtained by dividing the average transcript number of *sppKR* with the average transcript number of *lacLM* as described in Material and Methods

[a] Just before induction

Plasmid copy numbers

The PCN of pEH9R was compared to that of pEH9A in *L. plantarum* WCFS1 at several time points including 6 hours (point of induction), 8 hours, 12 hours and 24 hours of fermentation. As shown in table 2, the PCN ratio for pEH9R versus pEH9A varied between 1.39 and 0.79, i.e. close to one in all cases. Thus, both plasmids had similar copy numbers throughout the fermentation. Additionally, the PCN for pEH9R in *L. plantarum* WCFS1 was determined to be approximately three to four (not shown).

Transcription level of *lacLM* genes

To study mRNA levels and to analyze the effect of induction on the expression of *lacLM* genes, the relative expression of *lacLMReu* and *lacLMAci* at several time points after induction (2, 6 and 18 hours after induction, i.e. 8, 12 and 24 hours after start of the fermentation) was compared to the expression of these genes just before induction (6 hours after start of the fermentation). Table 3 shows strongly increased expression of the *lacLM* genes 2 and 6 hours after induction. Messenger-RNA levels for the *lacLM* genes from *L. reuteri* showed an approximately 60-fold increase after 8 hours, whereas mRNA levels for *lacLM* from *L. acidophilus* was increased about 18-fold. Subsequent to this immediate response, mRNA levels decreased and they did so faster for *lacLMAci* than for *lacLMReu*. After 24 hours of fermentation (18 hours after induction) mRNA levels for

lacLMReu were still considerably elevated, whereas mRNA levels for *lacLMAci* now were even lower than before induction (Table 3).

Table 2 shows a direct comparison of mRNA levels of *lacLMReu* on pEH9R and *lacLMAci* on pEH9A after 6, 8 and 12 hours of fermentation (0, 2 and 6 hours after induction). The mRNA levels for *lacLM* from *L. reuteri* were generally higher, i.e. both before (about four-fold) and after induction (13-fold and 18-fold after 2 and 6 hours, respectively).

Transcription levels of *sppKR*

Adding peptide pheromone to the growth culture will induce the expression of *sppK* and *sppR* (Fig. 1), and this autoinduction phenomenon will increase the expression of *lacLM*. Although not likely, the strength of the expression of *sppKR* may vary between the two plasmids. Therefore, we analyzed mRNA levels for *sppKR* in the two recombinant strains harboring pEH9R or pEH9A, before and after induction (Table 2 and Table 3). At 0, 2 and 6 hours after induction, the ratio between the two *sppKR* transcripts was within the range 0.76 – 1, i.e. close to 1 (Table 2). This shows that expression levels of *sppKR* were essentially identical in both strains. Interestingly, while expression of *sppKR* indeed was increased after induction, this increase amounted to not more than a doubling of the expression level (Table 3).

For both fermentations (pEH9R and pEH9A), the transcript level of *sppKR* was compared to that of the reporter genes, *lacLM* (Table 4). Before induction, the mRNA level of *sppKR* was higher than the level of *lacLM* mRNA (approximately five-fold and 14-fold for pEH9R and pEH9A, respectively). However, after induction these ratios decreased about 0.15 for pEH9R and 2.0 – 2.4 for pEH9A (Table 4). This reflects the much higher apparent induction of the promoter preceding the *lacLM* genes (see below for further discussion).
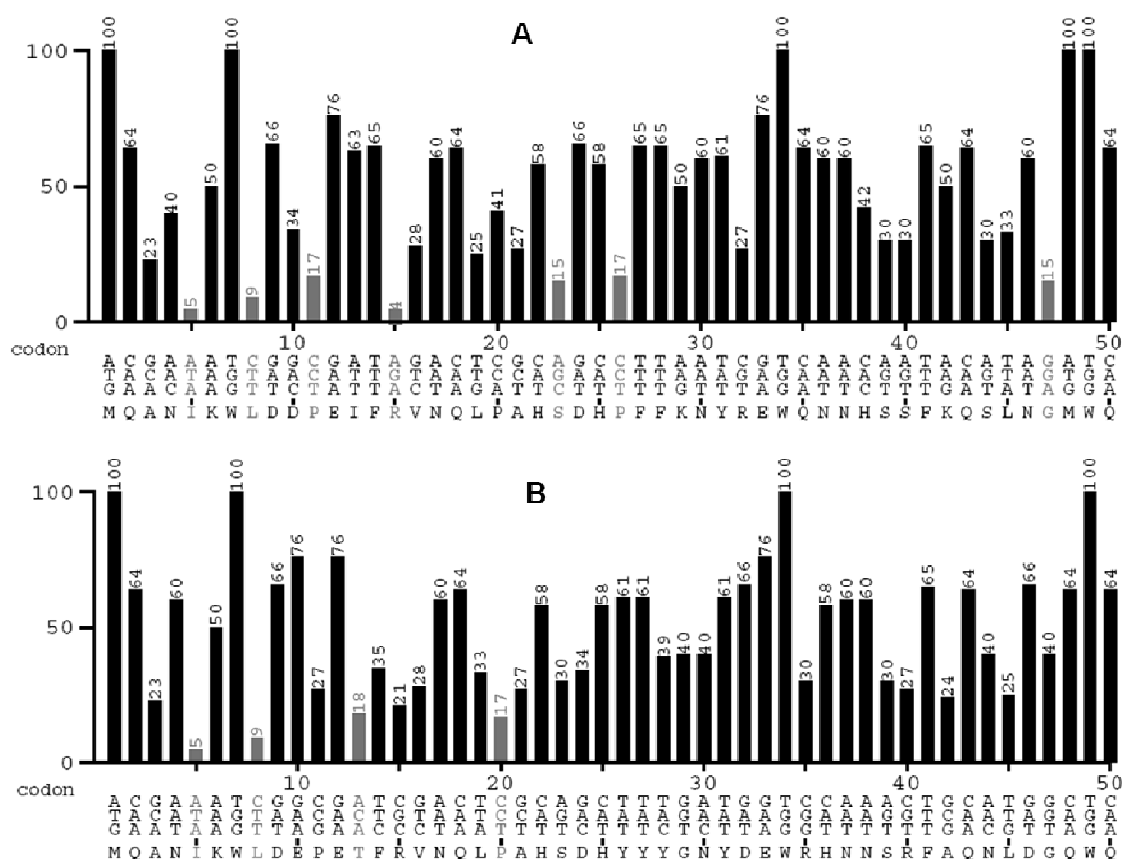
Codon usage analysis

The mean difference of codon usage in the *lacLM* genes from *L. reuteri* and *L. acidophilus* compared to the codon usage of *L. plantarum* WCFS1 was 16.48% and 23.45% (for *lacL*) and 18.22 % and 25.75% (for *lacM*), respectively. The difference of codon usage between *L.reuteri* and *L.acidophilus* was calculated as 5.39% using Graphical Codon Usage

Analyzer with 586361 codons for *L.acidophilus* [27] and 568715 codons for *L. reuteri* (http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=299033&aa=1&style=N).

Discussion

We had previously shown that lactobacillal *lacLM* genes can be overexpressed in *Lactobacillus plantarum* WCFS1 using the inducible pSIP expression system [10]. In this previous work we observed remarkably large differences in expression levels of β-galactosidases from different *Lactobacillus* strains. In the present study, we have used optimized conditions (unpublished observations) in parallel fermentations of *Lactobacillus plantarum* WCFS1 expressing different *lacLM* genes. Under conditions of pH control (pH 6.5) and high sugar content (see **Materials and Methods** section), the highest β-galactosidase activities were observed in the late exponential phase, where *lacLMReu* yielded 65 U/mg protein compared to only 2.5 U/mg obtained with *lacLMAci*.

Plasmid copy numbers may have significant effects on the synthesis of recombinant proteins encoded by a plasmid-borne gene [24]. Plasmids pEH9R and pEH9A were constructed using identical strategies based on a pSIP409 backbone that contains the 256rep replication determinants derived from the *Lactobacillus plantarum* NC7 plasmid p256 [28]. Copy numbers of expression vectors with this origin of replication are rather low, and were determined to be approximately three in *L. sakei* Lb790 and six in *L. plantarum* NC8, using slot-blot hybridization [11, 28]. In agreement with these reports, quantification with RT-PCR yielded a copy number for pEH9R in *L. plantarum* WCFS1 of 3 to 4. The ratio of plasmid copy numbers of pEH9R- and pEH9A-harboring strains of *L. plantarum* WCFS1, respectively, was close to one during the fermentation; i. e. the copy number is approximately identical in both strains. Clearly, differences in copy number and thus gene dose are negligible and cannot explain the large differences in the production levels for the two β-galactosidases.

Figure 3. Codon usage analysis of 50 first codons in *lacLM* of *L.acidophilus* (A) and *L.reuteri* (B). The vertical axis indicates the codon usage frequency (%) of triplet codons in *L.plantarum* WCFS1 (from http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=220668). The rare codons show the frequency < 20% (grey color).

The pSIP409 vector system comprises an autoinduction loop, because induction by the peptide pheromone also induces transcription of the *sppKR* operon, via the inducible P_{sppIP} promoter (Figure 1) [6, 29, 30]. From earlier studies, it is known that the transcription levels of the two components of the regulatory system (histidine kinase and response regulator) influence the transcription of the reporter gene [6, 29, 30]. Studies with reporter genes have shown that the P_{sppIP} promoter differs from e.g. the P_{sppQ} promoter in that it is more leaky, i.e. it displays more activity under non-inducing conditions [31]. This is supported by our comparative data on the transcription of *sppKR* and *lacLM* (Fig. 4), showing that before induction the former operon has higher transcription levels (Table 4). Somewhat surprisingly, transcription of *sppKR* increased only approximately two-fold upon induction, compared to an up to 60-fold increase for the *lacLM* genes controlled by P_{sppQ} (in strains harboring pEH9R) (Table 3). Previous

studies suggest that the *sppKR* transcript is unstable [8]. It is thus conceivable that transcription of these regulatory genes transiently increased to much higher levels immediately after addition of the IP, and was already decreasing again after two hours, when samples were taken. This may also explain de discrepancy with the results of Risøen et al [31], who found a much higher apparent degree of induction using reporter genes. Reporter protein activity can remain unaltered for some time even after transcription of the encoding gene has ceased and the corresponding mRNA is already degraded. For the purpose of this study, the most important conclusion is that transcription of the *sppKR* genes in strains harboring either pEH9R or pEH9A is essentially equal, both before and after induction (Table 2). Variations in the transcription levels of *sppKR* are therefore not responsible for the large differences in the production levels of the two β-galactosidases.

In previous studies of gene regulation in the natural sakacin P producer [8] transcripts for the operon under the control of the P_{sspQ} promoter could be detected as early as 15 minutes after induction, whereas maximum levels were observed 4 hours after induction. Northern blots [8] showed that transcript levels were close to the maximum 2 – 4 hours after induction. In our study, transcription of *lacLM* in pEH9R and pEH9A yielded maximum transcription levels two hours after induction (Table 3). The mRNA levels were slightly lower 6 hours after induction, i.e. at the start of the stationary phase. The highest activity of β-galactosidase was observed 6 hours after induction (12 hours of cultivation), showing the accumulation of the enzyme after induction (Figure 2).

After 24 hours of cultivation, well into the stationary phase, mRNA of *lacLMReu* was still detected at an 18-fold higher level than before induction (Table 3). In contrast, mRNA levels for *lacLMAci* after 24 hours were lower than before induction, indicating that *lacLMReu* mRNA is more stable than *lacLMAci* mRNA in *L. plantarum* WCFS1.

The present data clearly show that the large differences in protein production observed for *lacLMReu* and *lacLMAci* correlate with different mRNA levels. It is unlikely that this is due to differences in the frequency or efficiency of transcription initiation, since the two constructs are identical up to their start codons. Thus, translational effects on mRNA production or stability must be the main cause of the large difference in mRNA levels. Translational effects on mRNA levels are often ascribed to the impact of translation on mRNA stability, the main idea being that naked untranslated mRNA is prone to

degradation by ribonucleases. It should be noted though that low translation levels also will affect mRNA synthesis directly, either because longer stretches of nascent naked mRNA will be prone to premature Rho-mediated transcription termination [32, 33] and because a lack of ribosomes promotes "back-tracking" of the RNA polymerase complex and thus delays transcription, as recently shown by Proshkin et al [34].

One obvious potential cause of variation in the amount of ribosomes on an emerging mRNA concerns variation in translation initiation frequencies due to variation in the sequence and accessibility of the ribosome-binding site (Shine-Dalgarno-sequence) [35, 36]. For example, mRNAs with stable secondary structures near the translational start can hinder ribosome access to the translational initiation region (TIR) (= the ribosome binding site, the start codon and adjacent up- and downstream regions) [37, 38]. Analyses using the Mfold web server [39] showed only small differences between the two predicted mRNA structures in this region (not shown), but we cannot exclude that these differences play a role.

Another potential cause for slow translation is the presence of rare codons, in particular in the 5´ region of the gene [40]. Interestingly, in their recent landmark study on RNA polymerase backtracking [34], Proshkin et al. showed that rare codons not only reduce the speed of translation but also the speed of transcription. Over the entire length of the genes, the two *lacLM*, genes used in this study have similar amounts of rare codons, but the number of unfavorable codons among the first 50 triplets is considerably higher in *lacLMAci* (seven) than in the better expressed *lacLMReu* (four) (Figure 3), especially the 15th triplet in *lacLMAci* (AGA for arginine) shows the low frequency of 4%.

Conclusion

The results clearly indicate that the much higher β-galactosidase levels obtained in *L. plantarum* harboring *lacLM* from *L.reuteri* (on pEH9R) as compared to *L. plantarum* harboring *lacLM* from *L. acidophilus* (on pEH9A) are caused by higher mRNA levels in the former strain. This is remarkable, since the two operons are expressed using identical transcription and translation machineries and start sequences. This shows the importance

of translational effects on mRNA levels. Our data so far indicate that these translational effects are caused by subtle sequence variations at the level of one or a few rare codons or by minor variation in the secondary structure of the TIR, each of which would affect both mRNA synthesis rates and mRNA stability.

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Chapter 3

Optimization of the over-expression of a recombinant β–galactosidase in *Lactobacillus plantarum*

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Abstract

The two overlapping genes *lacLM* encoding for β-galactosidase from *Lactobacillus reuteri* were cloned and successfully over-expressed in food-grade host strain *Lactobacillus plantarum* WCFS1 using the inducible lactobacillal expression system pSIP which were constructed based on the genes involving in the production of the class II bacteriocins sakacin A and sakacin P. The overexpression of the recombinant β-galactosidase in *L. plantarum* WCFS1 harboring the plasmid pEH9R containing these genes from *L. reuteri* was optimized. The copy number of pEH9R plasmid which was speculated to be an important factor in the production of recombinant β-galactosidase, was estimated using quantitative real time PCR method.

By optimizing the growth conditions such as pH, growth temperature, amount of carbon source and level of antibiotic as the selective marker in the cultivation medium as well as the conditions for induction of the cultures such as cell optical densities at induction and amount of peptide pheromone as an inducer used, the yield of recombinant β-glactosidase is considerably higher (4 to 5-fold) than the yield reported for non-optimized conditions. The plasmid copy number of pEH9R in *L. plantarum* WCFS1 was estimated to be relatively low (approximately 4).

The overexpression of recombinant β-galactosidase in *L. plantarum* WCFS1 harboring the plasmid pEH9R was successfully optimized resulting in efficient overproduction of β-galactosidase in food grade host strain which can be of great interest for applications in food industry. The presented results suggested the tightly controlled and regulated expression systems using lactobacillal expression vectors pSIP for the overproduction of enzymes and proteins of interest.

Background

Lactic acid bacteria (LAB) have been known for a long time as important micro-organisms in the preparation and processing of a wide range of different foods, beverages and animal feed [1-3]. Being capable of rapidly converting glucose to lactic acid, LAB have been used as starter cultures in the production of a number of fermented foods in e.g., the meat and dairy industries, and thus have played an important role in human nutrition. Some lactic acid bacteria are known as producers of processing enzymes, antimicrobial peptides, or metabolites that contribute to flavor, conservation or texture of various foods. Furthermore, some LAB, in particular *Lactobacillus* spp., have been used as commercial probiotic cultures with health-promoting functions [2-4]. These bacteria are therefore generally recognized as safe (GRAS) for human consumption.

Because of their GRAS status and long use in food industry, LAB are increasingly considered as safe and attractive expression hosts and cell-factories, especially for food-application purposes [2, 3] and as vehicles for *in situ* delivery of antigens or other bioactive compounds in the GI-tract [5]. As a consequence, a variety of constitutive or inducible gene expression and protein targeting systems have been developed for LAB [1, 2, 5-7]. One of the most widely used gene expression systems derived from LAB is the NIsin-Controlled gene Expression system (NICE), which is based on the autoregulatory properties and the genes involved in the synthesis of nisin, an antimicrobial peptide produced by certain strains of *Lactococcus lactis* [8]. This system has been adapted to lactobacilli, but this approach has not always been straightforward or successful [9] [10]. An alternative expression system, the so-called pSIP system [11], was constructed for *Lactobacillus* spp. based on the promoter and regulatory genes involved in the production of the class II bacteriocins sakacin A [12] and sakacin P [13, 14]. The production of these two bacteriocins is regulated via quorum sensing mechanisms that are based on secreted peptide hormones with little or no bacteriocin activity [5, 15, 16]. The peptide pheromone (IP-673) activates a two-component regulatory system consisting of a membrane-bound histidine kinase sensing the pheromone, and an intracellular response regulator that, upon activation by the histidine kinase, induces the promoters of the operons involved in bacteriocin synthesis. Expression of the gene of interest is placed under control of a strong inducible bacteriocin promotor in the vectors of the pSIP systems, and gene

expression is induced by the addition of the peptide pheromone. An advantage of these systems is that they are highly efficient and strongly regulated. The applicability of these sakacin-based expression systems was shown for the over-production of enzymes such as β-glucuronidase and aminopeptidase in several *Lactobacillus* hosts [7, 11, 17].

β-Galactosidases (lactases, EC 3.2.1.23) catalyse the hydrolysis of lactose into galactose and glucose, and are important enzymes for applications in the dairy industry [18-20], where they can be used to produce low lactose or lactose-free products, prevent crystallization of lactose especially at low temperatures, to name a few [21]. Moreover, β-galactosidases can catalyse transgalactosylation reactions, transferring galactosyl moieties from e.g. lactose to a suitable acceptor molecule [18]. When lactose is the primary acceptor, galacto-oligosacharides (GOS) are obtained, which are physiologically important and health-promoting prebiotic sugars [19, 20, 22, 23]. Especially β-galactosidases obtained from known probiotic bacteria such as bifidobacteria or lactobacilli are of interest for the synthesis of these prebiotic GOS [24, 25] Nguyen et al. (2006) screened a number of *Lactobacillus* isolates and found that a strain of *L. reuteri* exhibited high β-galactosidase activity with significant transferase activity [19], yet the activity levels obtained with the wild-type strain (~2300 U per L of cultivation medium, corresponding to 14 mg of β-galactosidase protein per L) are too low to be attractive from an applied point of view. To improve these low yields, the coding regions of the two overlapping genes *lacL* and *lacM* (*lacLM)* were cloned and over-expressed in a standard expression host, *Escherichia coli* [26]. Heterologous expression in *E. coli* resulted in efficient over-expression of β-galactosidase (~110 kU/L of fermentation broth), yet *E. coli* might not be the preferred host for food-related enzymes. Recently, we reported the overproduction of this enzyme in the food-grade expression host *Lactobacillus plantarum* WCSF1 [17]. The *lacLM* genes from *L. reuteri* were cloned into the expression vectors pSIP403 and pSIP409, which are based on the sakacin operon of *L. sakei* [7, 11] and differ in the promotors P_{sppA} and P_{sppQ} (formerly P_{OrfX}), respectively. This resulted in the two expression plasmids, pEH3R and pEH9R [17]. When over-expressed in the host *L. plantarum* WCFS1, cultivations yielded of up to ~23 kU of β-galactosidase activity per L with a specific activity of 60 U/mg protein were obtained [17], albeit without any optimisation of this fermentation process. The P_{sppQ} promoter seemed to be more tightly

regulated than the P_{sppA} promotor, since hosts carrying pSIP409-derived constructs showed lower basal induction when no inducer was added.

In this study, we studied various cultivation and induction conditions in order to get a more detailed insight into these lactobacillal expression systems and to obtain higher expression levels of the recombinant β-galactosidase LacLM in the host strain *L. plantarum* WCFS1 carrying the plasmid pEH9R. Amongst other methods, real-time quantitative PCR was used to determine the plasmid copy number (PCN) of the pEH9R plasmid during growth of this strain in order to study the correlation between PCN and protein expression.

Materials and Methods

Bacterial strain and media

Lactobacillus plantarum WCFS1 harbouring the plasmid pEH9R, which contains the overlapping genes (*lacLM*) coding for β-galactosidase of *Lactobacillus reuteri* L103 [17, 19], was maintained at -70°C in MRS medium (Merck, Germany) containing 5 μg/ml erythromycin.

Fermentations

Strains were activated from frozen cultures at $37^{°}$C in 5 ml of MRS containing 5 μg/ml erythromycin for 16–18h. These fresh cultures were used as inocula for subsequent cultivations.

Table 1- Sequences of the primers of qPCR for PCN determination used in this study

Target gene	Primers	Sequence (5'-3')	Reference
Plasmid DNA (Erythromycin resistant gene)	Ery^R_f	CCGTGCGTCTGACATCTATC	This study
	Ery^R_r	TGCTGAATCGAGACTTGAGTG	
Genomic DNA (16S-rRNA)	16s_f	TGATCCTGGCTCAGGACGAA	[27]
	16s_r	TGCAAGCACCAATCAATACCA	

f denotes forward primers, r denote reverse primers

For batch fermentations without pH control, 1% (v/v) of inoculum was added to 50 ml of medium, and the cultures were grown in tightly closed bottles at 37°C. Batch fermentations with pH control were carried out in 400 ml medium in the HT-Multifors system (Infors HT, Switzerland), inoculating these cultivations again with 1% (v/v) of a preculture. The pH was controlled at pH 6.5 using sodium hydroxide, and agitation was set at 200 rpm. Glucose concentrations in the MRS medium were varied as indicated. The expression levels of β-galactosidase were studied by inducing the cultures with varying levels of the inducer, which was added at different phases of the growth curve. The synthetic pheromone IP673, a 19-amino acids peptide synthesised according to the sequence of the original pheromone from *Lactobacillus sakei* LTH673 [28], was used as the inducer.

Samples were taken periodically to measure optical density at 600 nm (OD_{600}), β-galactosidase activity and the plasmid copy number (PCN). For β-galactosidase measurements, cells from 1 ml of culture were harvested by centrifugation at 13200 rpm for 3 min, cell pellets were re-suspended in sodium phosphate buffer (buffer P) [22], and then disrupted by sonication (Bandelin Sonopuls HD60, Germany). Subsequently, debris was removed by centrifugation at 13200 rpm for 10 min. This crude cell extract was used to determine β-galactosidase activity and protein concentration. For PCN estimation, an appropriate volume of sample was taken depending on the densities of the cultures (OD_{600}) to ensure sufficent biomass for DNA isolation. Cells were pelleted by centrifugation and stored at –80°C until further use.

β-Galactosidase assay

β-Galactosidase activity was determined using *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG) as the substrate as described previously [19]. In brief, the assay was performed at an *o*NPG concentration of ~20 mM *o*NPG, pH 6.5, and 30°C. One unit of *o*NPG activity is defined as the amount of enzyme releasing 1 μmol of *o*NP per minute under these conditions. Protein concentration was determined by the method of Bradford using bovine serum albumin as the standard.

DNA isolation and purification for PCN estimation

DNA from bacterial cells was isolated and purified using the phenol-chloroform extraction method as described previously [29]. Purified bacterial DNA was stored at −20°C until further use.

Quantitative real-time PCR (qPCR)

Oligonucleotide primers

The erythromycin resistance gene *ermB* and *16 SrRNA* were chosen as representatives for plasmid DNA and genomic DNA, respectively. The degenerated oligonucleotides Ery[R]-f, Ery[R]-r, 16s-f and 16s-r (Table 1) were used for qPCR. All primers were obtained from VBC-Biotech (Vienna, Austria).

qPCR using SYBR Green I

The thermal cycling system iCycler together with the myIQ single Color Real-Time PCR Detection system (Biorad) were used for qPCR amplification and detection. The qPCR reactions were carried out in duplicates of 25-µl reaction mixtures in 96-well plates (iCycler, Biorad) sealed with optical adhesive covers (Microseal 'B' film, Biorad). Each reaction contained 250 nmol/l of each primer, 12.5 µl of Perfecta SYBR Green Super mix of IQ (Quanta Biosciences) and 2.5 µl of template DNA. Negative controls (no template control, NTC), prepared by replacing template DNA with DEPC water, were included in each run to ensure the absence of DNA contaminants in the reagents. The concentration of primers, annealing temperature and template DNA concentrations had been optimized before the actual experiments as previously described [30]. The qPCR reactions were conducted as follows: initial denaturation at 95°C for 3 min followed by 50 cycles of 20 s at 95°C, 20 s at 60°C, and 72°C for 10 s. Fluorescence was measured at the end of each extension step at 72°C. Subsequently, the temperature was increased from 55°C to 95°C at a rate of 0.2°C per s to establish the melting curve. The threshold cycle values (C_t) were automatically determined by the software MyIQ optical system version 2.0 (Biorad).

Calculation of the PCN value
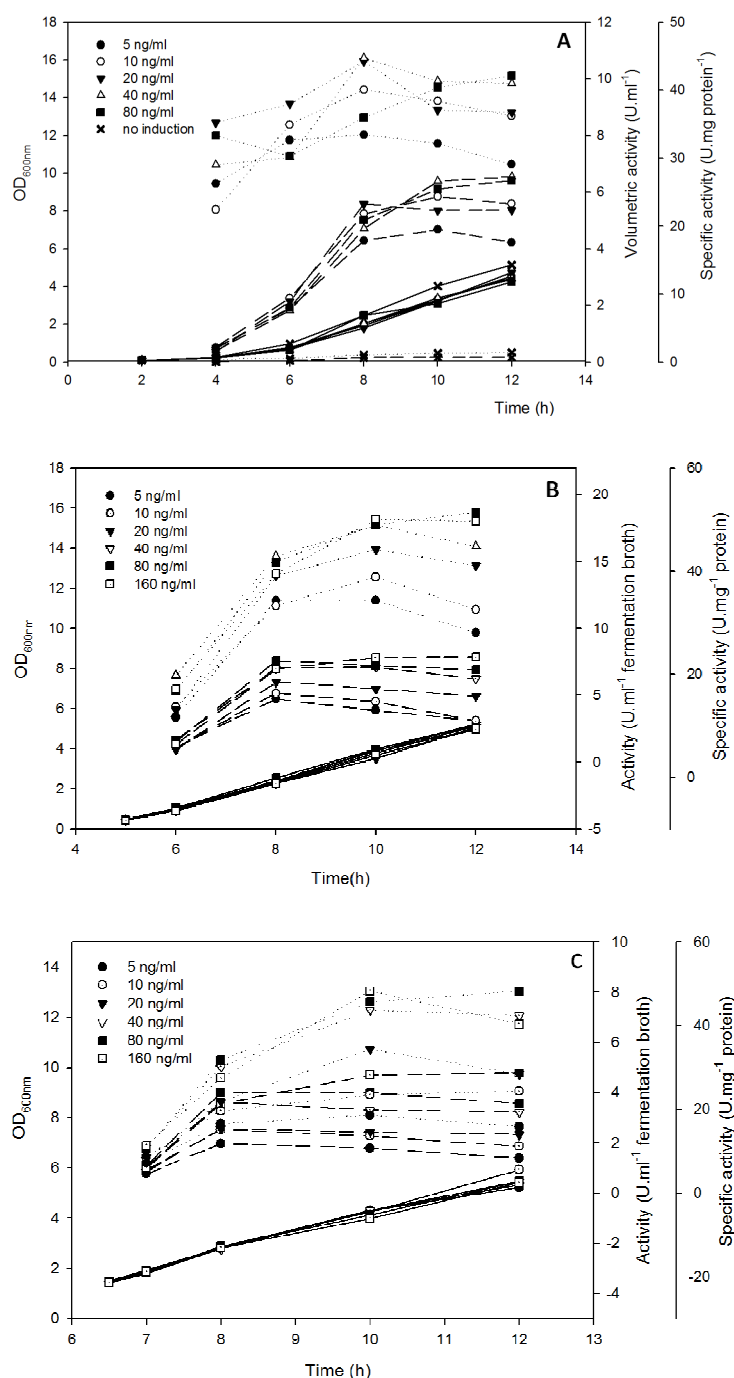
Figure 1. Time course of cultivations of *L. plantarum* over-expressing β-galactosidase LacLM without pH control. *L. plantarum* WCFS1 harbouring the pEH9R plasmid, which contains the two overlapping genes of *lacLM* encoding β-galactosidase from *L. reuteri,* was cultivated on 50-ml scale using MRS medium with 20 g/l glucose at 37°C. Recombinant protein expression was induced by the addition of varying amounts of the inducing pheromone IP (ng/ml fermentation) at different phases of the cultivation, i.e., different OD_{600} values: from the beginning immediately after inoculation of the strain (a); at OD_{600} of 0.4–0.5 (b); at OD_{600} of 1.5 (c). Symbols: OD_{600} (solid lines), volumetric β-galactosidase activity in units per ml of fermentation broth (dashed lines) and specific β-galactosidase activity in units per mg protein (dotted lines).

Based on PCN definition, which is the number of copies of a plasmid present per

chromosome in bacteria [31, 32], the PCN can be calculated using equation (1) as previously reported [33]:

$$ PCN = \frac{E_c^{C_{tc}}}{E_p^{C_{tp}}} \qquad (1) $$

where E_c, C_{tc} and E_p, C_{tp} are the amplification efficiencies and the threshold cycle values of the amplicon representing chromosome and plasmid, respectively. The equivalence between the amplification efficiency (E) of plasmid and chromosomal amplicons was confirmed in validation experiments as recommended [34]. Moreover, the relative PCN values were calculated to examine the variation of the PCN value over the cultivation by using the comparative C_t method ($\Delta\Delta C_t$) as expressed by the following equation (2):

$$ Relative\ PCN = 2^{-\Delta\Delta C_t} \qquad (2) $$

where $\Delta\Delta C_t = \Delta C_t$ of a sample from a certain time point $- \Delta C_t$ of the sample from hour 2; and ΔC_t = average C_t value of target (for the erythromycin resistance gene) $-$ average C_t value of the reference gene.

High-performance liquid chromatography

Concentrations of glucose and lactic acid in fermentation samples were determined by HPLC (Dionex, USA) with an Aminex HPX87-H column and 0.005 M sulphuric acid as eluent at a flow rate of 0.6 ml/min.

Results

Effect of inducer concentration and time of induction

L. plantarum WCFS1 harbouring the plasmid pEH9R, which contains the *lacLM* gene under control of the pheromone-inducible P$_{sppQ}$ promoter, was grown with and without pH-control under various induction conditions. To this end, the concentration of the inducer IP was varied and the inducer was added at different growth phases of the host organism.

Batch cultivations without pH control

Cultivations were performed without pH control at 37°C using MRS medium containing 20 g/l glucose. Despite of varying induction conditions, growth of the organism was in all cases very similar and reached an OD_{600} of ~4.5 after 12 h of cultivation (Figure 1). However, the enzyme yields obtained were significantly affected both by the time of induction as well as the pheromone concentrations employed. The volumetric activity of β-galactosidase (U per ml of fermentation broth) increased to around 2–8 U/ml upon induction, depending on the conditions employed, and remained at these production levels after OD_{600} reached 2.0–3.0 (Figure 1). One factor affecting enzyme yields is the time of addition of the inducer or better the growth phase of the culture. When induction was performed at higher cell densities such as at OD_{600} of 1.5 (late growth phase), lower volumetric activities (2–4 U/ml fermentation) were observed (Figure 1c), whereas induction either immediately after inoculation or at an OD_{600} of 0.4–0.5 resulted in increased enzyme activities of approximately 5–8 U/ml (Figure 1a, 1b). Induction during the early phase of growth gave slightly higher specific β-galactosidase activity of up to 50 U/mg.
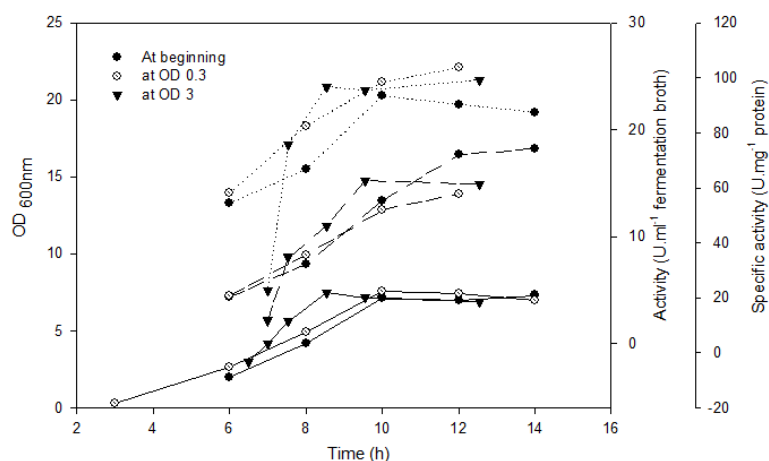
Figure 2. Time course of cultivations of *L. plantarum* over-expressing β-galactosidase LacLM with pH control and using 20 g/l glucose MRS medium. *L. plantarum* WCFS1 harbouring the pEH9R plasmid was cultivated in 400-ml laboratory fermentations at 37°C using MRS medium with 20 g/l glucose and pH control at pH 6.5. Expression of LacLM was induced by the addition of 20 ng/ml pheromone at different OD_{600} points: at the beginning immediately after inoculation (●); at OD_{600} of 0.3 (○) and at OD_{600} of 3.0 (▼). Symbols: OD_{600} (solid lines), volumetric β-galactosidase activity in units per ml of fermentation broth (dashed lines) and specific β-galactosidase activity in units per mg protein (dotted lines).
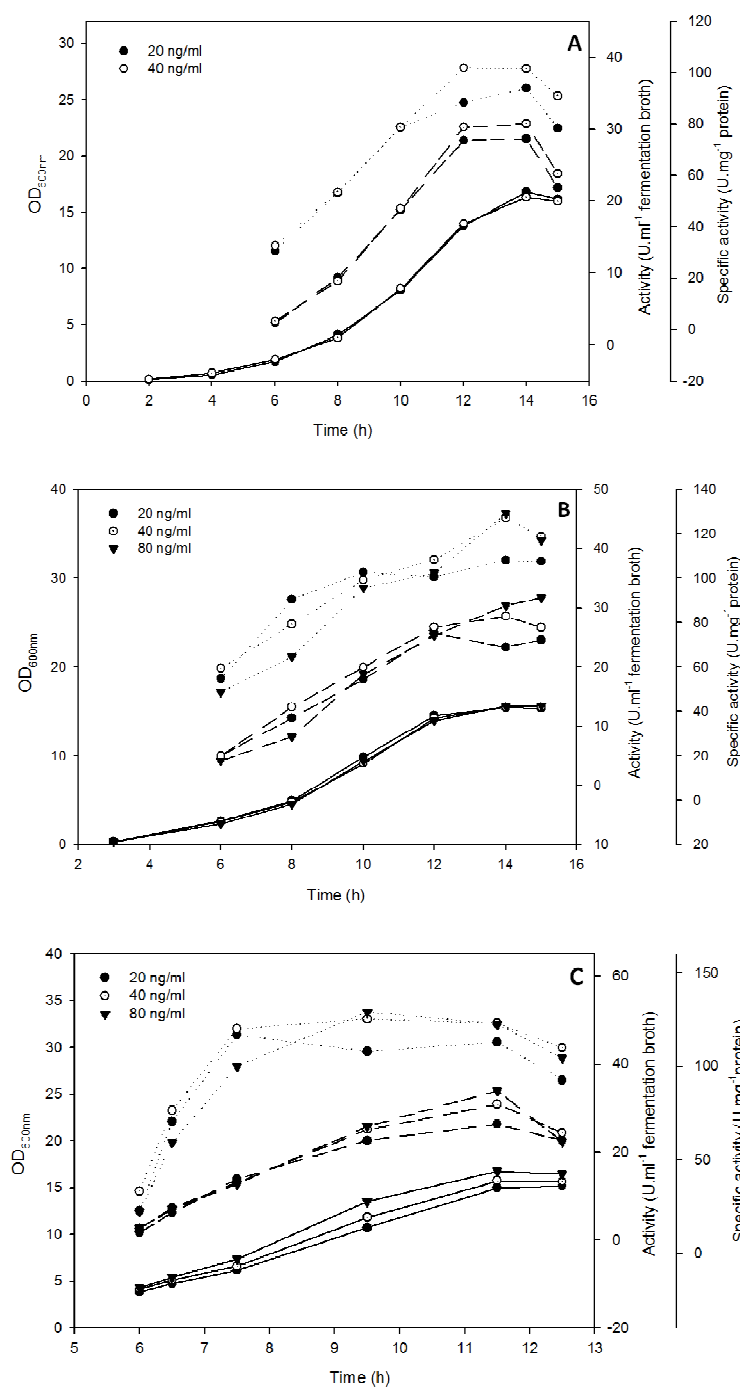
Figure 3 Time course of cultivations of *L. plantarum* over-expressing β-galactosidase LacLM with pH control and using 40 g/l glucose MRS medium. *L. plantarum* WCFS1 harbouring the pEH9R plasmid was cultivated in 400-ml laboratory fermentations at 37°C using MRS medium with 40 g/l glucose and pH control at pH 6.5. Expression of LacLM was induced by the addition of varying amounts of pheromone (ng/ml fermentation broth) at different OD_{600} values: at the beginning immediately after inoculation (a); at OD_{600} of 0.3 (b) and at OD_{600} of 3.0 (c). Symbols: OD_{600} (solid lines), volumetric β-galactosidase activity in units per ml of fermentation broth (dashed lines) and specific β-galactosidase activity in units per mg protein (dotted lines).

Furthermore, we observed a concentration-dependent behaviour of induction, with increased IP concentration having positive effects on enzyme formation (Figure 1), even though this effect was not linear or very pronounced. An increase in the IP concentration positively affected volumetric β-galactosidase activity to some extent, and showed a stronger effect on the specific activity obtained, thus increased induction levels (ratio of specific activity under induced and non-induced conditions) were obtained with higher IP concentrations. Recombinant enzyme synthesis of non-induced cultures was very low with only approximately 0.2 U/ml of fermentation broth or 1.3 U/mg of specific activity (Figure 1a). The acid lactic was produced and accumulated during the fermentation, resulted in the reducing of pH to approximately 5.2 or 4.3 after 7h or 12 h of cultivation, respectively which can retard the growth of bacteria.

Batch cultivations with pH control

Lactic acid, which typically is formed during growth of lactic acid bacteria, lowers the pH of the medium, and therefore limits growth as well as protein synthesis. In order to study this effect on recombinant protein production, the pH value of the cultivation was set at pH 6.5 by the addition of sodium hydroxide during growth of *L. plantarum* WCFS1 harbouring pEH9R. pH control had a significant positive effect on both growth and protein expression. After 12 h of cultivation at 37°C using again MRS medium with 20 g/l glucose, OD_{600} values of around 7 were reached regardless of induction time (Figure 2), which is significantly higher than those obtained for growth under non-controlled pH conditions (OD_{600} of 5). Likewise, recombinant enzyme formation was improved, and increased until the cells reached the early stationary phase to yield volumetric activities of 15–19 U/ml, which is a 2.5–3 fold increase compared the batch fermentation without pH control. Specific β-galactosidase activities were increased to values of around 90–100 U/mg. Induction right after inoculation resulted in slightly higher yields (~19 U per ml of fermentation broth) than induction at later time (at OD_{600} of either 0.3 or 3), even though identical pheromone concentrations were used (Figure 2).

Subsequently, we studied the effect of varying glucose concentrations on recombinant enzyme production under pH-controlled conditions. When using MRS medium with 40 g/l glucose, optical densities of the cultures increased to values of 15–18 (Figure 3), which is significantly higher than observed for cultures grown on MRS medium containing 20 g/l

glucose (OD$_{600}$ of 7). Concomitantly, the recombinant enzyme production improved and continuously increased to reach a maximum when the stationary growth phase was reached. The maximum yield obtained under these conditions was between 25 to 35 U/ml fermentation, depending on the amount of IP used (Figure 3). Under these conditions, a later time of induction seems favourable since highest activities (both volumetric and specific) were found when induction was performed at OD ~3.0 (volumetric and specific β-galactosidase activities of ~35 U/ml and ~125 U/mg, respectively). It is evident that an increase in glucose concentrations results in prolonged cultivation times, which in turn gives sufficient time for recombinant protein synthesis even when inducing at a higher cell density. Varying the inducer concentration in the range of 20 to 80 ng/ml under these cultivation conditions did not significantly affect
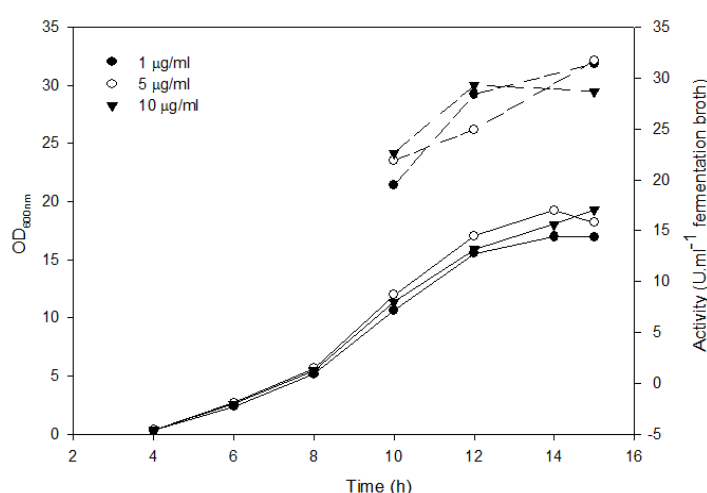

Figure 4. Effect of varying erythromycin concentrations on recombinant protein expression. *L. plantarum* WCFS1 harbouring the pEH9R plasmid harbouring the *lacLM* genes as well as the antibiotic resistance marker *ermB* was cultivated in 400-ml laboratory fermentations at 37°C using MRS medium with 40 g/l glucose and the pH was controlled at pH 6.5. Expression of β-galactosidase LacLM was induced by the addition of 20 ng/ml inducing pheromone at an OD$_{600}$ of 0.3. Growth (OD$_{600}$) and formation of β-galactosidase (units per ml of fermentation broth) are represented as solid lines and dashed lines, respectively.

recombinant protein expression, even though somewhat lower results were obtained for 20 ng/ml IP (Figure 3). The higher concentration of glucose (120 g/l) was also applied for fermentation, however except the higher cell densities, no significant increasing of enzyme yield was observed.

Effect of antibiotic concentrations

The plasmid pEH9R carries the *ermB* gene coding for erythromycin ribosomal methylase, which confers resistance to erythromycin. Hence erythromycin resistance was used as a selection marker, and was added at a concentration of 5 µg/ml in all of the previous experiments. To examine the effect of different antibiotic concentrations on recombinant enzyme production, erythromycin concentrations of 1 µg/ml, 5 µg/ml and 10 µg/ml (final concentration in the cultivation medium) were tested. As is obvious from Figure 4, these different erythromycin concentrations did not exert any significant difference on growth as well as recombinant protein production. Interestingly, when no antibiotic was added to the culture medium, the obtained yield of enzyme activity was much lower than with antibiotic (approximately 2 U/ml of volumetric activity) indicating the absolute necessity to maintain the selection pressure on the expression system. In our recently observation about segregational stability of pEH9R in *L.plantarum* WCFS1,
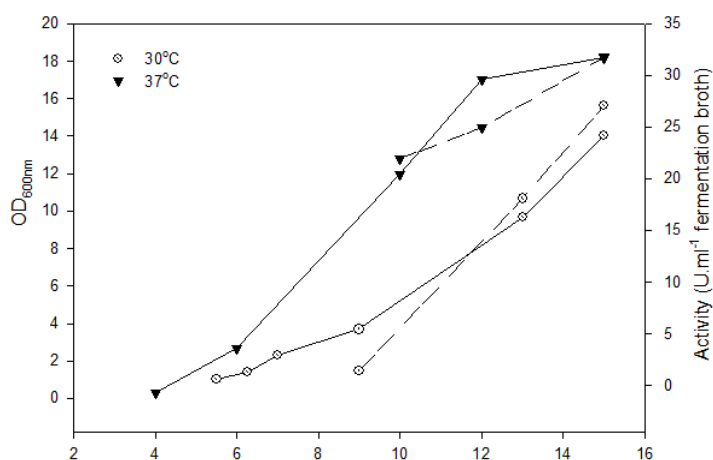
Figure 5. Effect of cultivation temperature on growth of *L. plantarum* WCFS1 harbouring pEH9R and recombinant protein expression. The cultivations were carried out in 400-ml laboratory fermentations at 37°C using MRS medium with 40 g/l glucose and with the pH value controlled at 6.5. Expression of β-galactosidase LacLM was induced by the addition of 80 ng/ml pheromone at an OD_{600} of 3.0. Growth (OD_{600}) and formation of β-galactosidase (units per ml of fermentation broth) are represented as solid lines and dashed lines, respectively.

we found that the absence of antibiotic in cultivation resulted in the decreasing of

number of cells harbouring the plasmid pEH9R, thus affected on gene-dose and finally reduced the enzyme production yield (unpublished data).

Effect of temperature

Finally, we studied the effect of two different cultivation temperatures, 30°C and 37°C, on growth as well as over-expression of β-galactosidase LacLM. When *L. plantarum* WCFS1 carrying pEH9R was grown in 40 g/l glucose MRS medium and pH control at 6.5, growth and enzyme production were faster at 37°C than at 30°C (Figure 5). After 15 h of growth, OD$_{600}$ values were approximately 18 and 14 for cultivations at 37 and 30°C, respectively. This difference in cell densities also resulted in differences in volumetric β-galactosidase activity, which ranged from 32 U/ml for 37°C to 27 U/ml for 30°C, respectively, after 15 h. However, when comparing identical cell densities during these cultivations, the β-galactosidase yields are again comparable (Figure 5), indicating that temperature mainly affects the growth rate while the level of recombinant protein formed is not influenced.

Validation of the RT-PCR reaction

The variation of ΔC$_t$ (i.e., the difference in the threshold cycle value C$_t$ between two amplicons amplified from the same template) with template dilution was studied for the Ery^R and 16s primers to examine whether amplification efficiencies depend on template dilution. A series of 10-fold dilutions of template DNA was used to run real-time PCR reactions in order to estimate C$_t$ values and to subsequently calculate the ΔC$_t$ values between two primer pairs. Figure 6a shows the plots of C$_t$ versus the logarithm of DNA dilution. The amplification efficiency (E) was determined based on the slope of the regression line for each primer. The amplification efficiencies for the 16s and Ery^R primer were found to be equivalent, i.e. 0.96 and 0.97, respectively. This is also corroborated by the plot of ΔC$_t$ versus log$_{10}$(DNA dilution) shown in Figure 6b, where a slope of the regression line of 0.04 was obtained. This indicates that the ΔΔC$_t$ method can be used in this study for the two primers 16s and Ery^R [34].

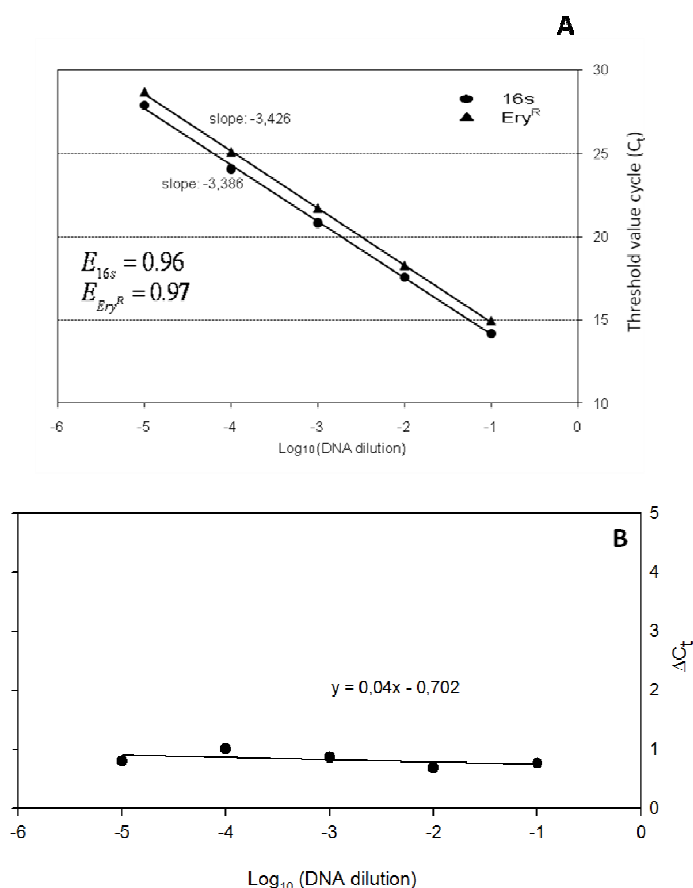**Variation of the plasmid copy number (PCN) during growth**

The variation of the PCN and relative PCN during a cultivation of *L. plantarum* WCFS1 harbouring the plasmid pEH9R using 400 ml of MRS medium containing 45 g/L of initial glucose concentration and controlling the pH at 6.5 was studied (Figure 7). The change in

glucose consumption and lactic acid production during the cultivation was also followed (Figure 7a). Induction of recombinant protein expression with 80 ng/ml of pheromone at an OD_{600} of 3 led to a rapid increase in LacLM formation, and values for the volumetric and specific β-galactosidase activity of approximately 40 U/ml and 90 U/mg were obtained after 12 h of cultivation when growth reached stationary phase (Figure 7b). This also coincided with the time when glucose was almost completely exhausted from the medium and lactic acid production stopped at around 40 g/l (Figure 7a).

The copy number of the plasmid pEH9R in its host *L. plantarum* WCFS1 was determined to be rather low. As shown in Figure 7b, a maximum value of the PCN of ~4 was measured. The variation of the plasmid copy number over the course of the cultivation was found to be uncorrelated to growth of the organism. Interestingly, at 10 h of cultivation, both PCN and relative PCN decreased and then increased again to stay approximately constant at the end of the cultivation.

Discussion

The inducible pSIP expression systems were developed based on promoters and regulatory genes involved in the production of the class II bacteriocins sakacin A (*sap* gene cluster) or sakacin P (*spp* gene cluster). The expression of the gene of interest is placed under control of a strong inducible bacteriocin promoter and gene expression is induced by an addition of peptide pheromone [11]. This study again confirms the important role of pheromone in the expression of β-galactosidase in *L. plantarum* WCFS1. Without induction of the cultures the yield of enzyme production was found to be very low (figure 1a), which reached the highest levels at the optical densities of the cultures (OD_{600}) of around 2 – 3 after 8 – 10 hours of fermentation. This is also in agreement with the previous studies of pSIP expression systems [7, 11].

Figure 6. Efficiency validation for the primers Ery[R] and 16s used in real-time PCR for the determination of the plasmid copy number. (a) The amplification efficiencies E of the erythromycin resistance gene from plasmid DNA (E_{EryR}) and *16srRNA* from genomic DNA (E_{16s}) were calculated based on the slope of the regression lines of the threshold cycle versus $\log_{10}$(DNA dilution) as: $E = 10^{-\frac{1}{slope}} - 1$.   (b)   The regression line for validation of the $2^{-\Delta\Delta Ct}$ method was plotted based on $\log_{10}$(DNA dilution) versus ΔC_t = ($C_{t,EryR}$ - $C_{t,16s}$).

The time at induction is also an important factor which affects the obtained yield of enzyme production. In the case of the cultivations without pH control, the later the induction time point was, the lower the enzyme yield was obtained. When the cultures were induced at OD_{600} ~ 1.5, only 2 – 4 U/ml of fermentation broth was obtained (figure 1c), which was only about half of the yield obtained with the cultures induced at OD_{600} ~ 0.3 (4 – 7 U/ml of fermentation) (figure 1b). Induction at OD_{600} ~ 0.3 was also recommended in previous studies for the cultivations without pH control [7, 11]. In contrast to the differences in volumetric activities (U/ml of fermentation broth), maximum enzyme specific activities of 30 – 50 U/mg of protein depending on the amount of pheromone added were similar with all three induction time points tested (figure 1a, b,

c). Halbmayer et al. also reported similar yield of a recombinant β-galactosidase expressed in *L. plantarum* WCFS1 (54.5 U/mg protein) in a recent study [17].

In pH-controlled fermentations, the expression of β-galactosidase in *L. plantarum* WCFS1 carrying pEH9R plasmid was found to be at significantly higher level yielding 15 – 20 U/ml of fermentation broth and 90 – 100 U/mg protein (figure 2). Induction of the cultures right after inoculation of the strain resulted in slightly higher yield than inducing at two other OD_{600} points (0.3 and 3.0). The improved yield of the expressed β-galactosidase in controlled-pH fermentations showed that pH control has the positive effects not only on the growth of the strain but also on the expression of the target gene. It is known that lactic acid is produced during fermentations of lactic acid bacteria hence lowering the pH of the cultures and the change in pH during cultivations might have the effects on the growth as well as the production of protein of interest. Moreover, it was indicated that at the low pH, erythromycin is unstable and can be degraded [35, 36] and thus somewhat affected on the plasmid segregation stability and as consequence influenced on enzyme production yield. In this study, pH was maintained at pH 6.5 during cultivations based on the profiles of pH optimum and stability of this enzyme [17, 19, 26]. Even higher yield of enzyme production was obtained under pH-controlled conditions when initial glucose concentration in the culture medium was increased from 20 to 40 g/l. The maximum yields of 35 U/ml of fermentation and approximately 120 U/mg proteins were obtained under these conditions.

Diep and co-workers have indicated that temperature can influence on the quorum sensing mechanism, thus on expression level [37]. It have been confirmed that at 37°C the lower expression level as well as the higher background level (non-induced) were observed when compared to those at 30°C (unpublished data). Despite of that, in this study, with pH control, there was no significant difference in production yield of enzyme between 2 temperatures (at the identical cells densities). Moreover, the growth of the host strain *L.plantarum* WCFS1 is better at 37°C. For that reason and regardless the background activity, the temperature of 37°C combining with pH control condition can be used in industry to reduce time-cost.
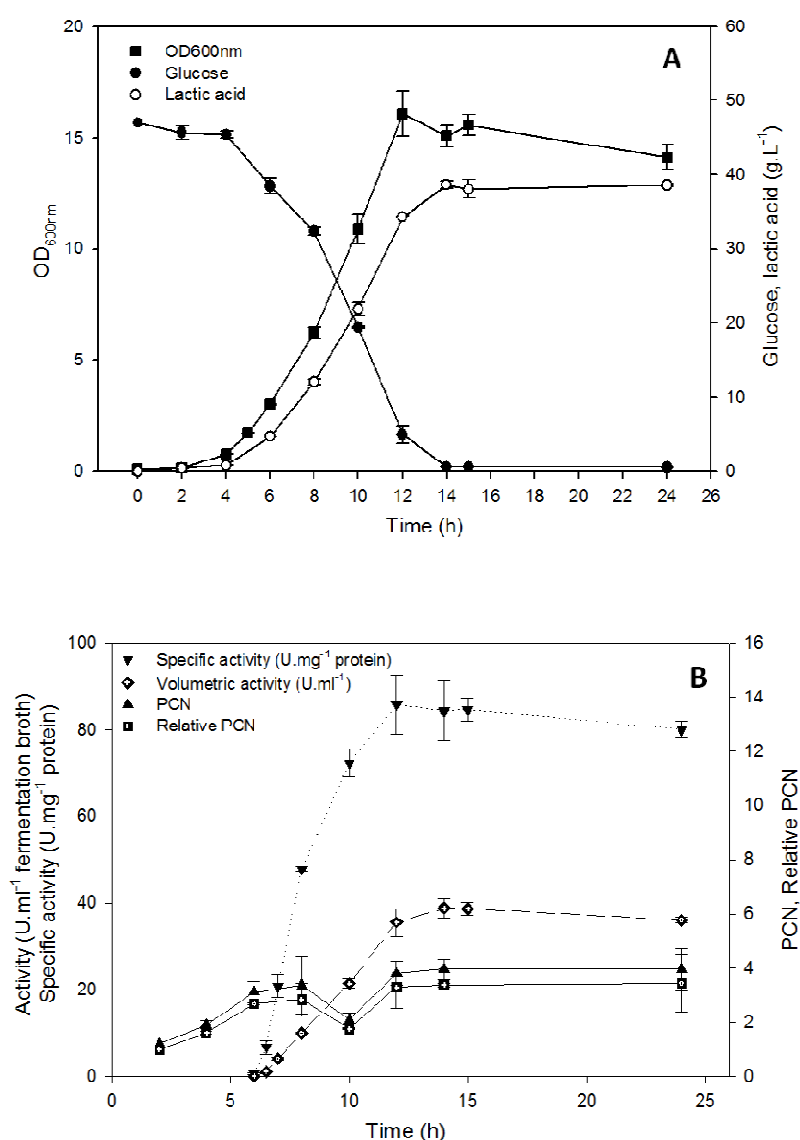
Figure 7. Cultivation of *L. plantarum* WCFS1 harbouring pEH9R under optimized conditions. The cultivation was performed using MRS medium containing 45 g/L glucose at 37°C with pH control at 6.5 and the cells were induced at OD ~ 3 with 80 ng/µl peptide pheromone. (a) OD$_{600}$, glucose consumption and lactic acid production; (b) β-galactosidase overproduction (U/ml of fermentation and U/mg protein) and plasmid copy number (PCN) of pEH9R. All data were average values from 2 separate runs.

It is necessary to use a selective marker to prevent the release of plasmid and antibiotics are generally used for that purpose, however, their use is limited or even restricted for food application processes. Varied concentrations of erythromycin were tested in this study to investigate the effect of antibiotic on the expression level of enzyme. It was found that there was no significant differences in the yields of expressed protein obtained with all concentrations of erythromycin tested, also even at low

concentration of 1 μg/ml erythromycin. However, without adding erythromycin to the cultivation medium, very low yield of enzyme production was obtained. Therefore, the presence of erythromycin in the cultivation medium is of importance for the high yield of the expressed protein even though its use is yet a hurdle for food applications. Recently, in another work, we have successfully developed the new expression system pSip which relies on food-grade alanine racemase encoding gene (*alr*) [38, 39] as complimentary selection markers. The new *alr*-based expression systems could not only overcome the disadvantage of antibiotic-based systems, but only give comparable production level of enzyme (unpublished data).

In contrast to wide variations of plasmid copy number in *Escherichia coli* which were extensively reported [31, 33], the copy number of pEH9R in *L. plantarum* WCFS1 varied slightly during fermentation. This plasmid appeared to have low copy number (around 2 – 4) and this is in accordance with the findings that 256_{rep} replicon is a low-copy-number replicon [40]. The time-course profile of PCN during fermentation in our experiment was found to be different to the results about PCN in *E. coli* reported previously [31, 33, 41]. PCN of pEH9R expressed in *L. plantarum* WCFS1 increased at the beginning of fermentation and then reduced to the lowest value after 10 hours of cultivation. This might due to fast duplication of the cells, therefore two daughter cells from one mother cell contain less PCN. When the growth rate is lower, PCN increased again and was stable during growth stationary phase. The patterns of PCN and enzyme production during fermentation were found to be different which suggested that protein production do not strictly correlate to PCN. The yield of the expressed protein of interest encoding by one or more genes in a plasmid also depends on the transcription and translation of those genes [32].

Conclusion

We here described the optimization in terms of growth and induction conditions for the over-expression of a recombinant β-galactosidase using pSIP409 expression vector in *Lactobacillus plantarum* WCFS1. Under optimized conditions the production of the recombinant β-galactosidase with specific activity of 84 U/mg protein increased to nearly

40 U/ml of fermentation broth which was 5-fold higher than the yield obtained under non-optimized conditions. The plasmid copy number of the expression vector was also estimated and found to be low-copy-number plasmid.

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Chapter 4

Beta galactosidase from *Lactobacillus bulgaricus* DSM20081: High overexpression in *Lactobacillus plantarum* and characterization

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Abstract

In this study, a *lacZ* gene (3027 bp) encoding for β – galactosidase from *Lactobacillus bulgaricus* DSM 20081 was cloned and successfully expressed in the host strain *Lactobacillus plantarum* WCFS1 using the lactobacillal inducible expression vectors pSIP403 and pSIP409 which are based on the sakacin P operon of *L. sakei*. The high expression level of the recombinant enzyme was obtained in laboratory cultivations with a production yield of approximately 43 kU/L fermentation. The β- galactosidase levels amounted to 47% of the total intracellular protein of the host organism. The histidine-tagged recombinant enzyme was purified to apparent homogeneity and further characterized. As judged by SDS-PAGE and native-PAGE, β-galactosidase from *L. bulgaricus* DSM 20081 is a homodimer consisting of two identical subunits of approximately 110 kDa. The K_m, K_{cat}/K_m values for o-nitrophenyl β-D-galactopyranoside and lactose were 1.2 mM, 785 $mM^{-1}.s^{-1}$ and 20 mM, 20.35 $mM^{-1}.s^-1$, respectively. The optimum temperature and pH for *o*NPG and lactose was 45°C, pH 6.5- 8 and 65°C, pH 7.5 to 8, respectively. The recombinant enzyme was a thermostable enzyme with the half-life time of 60 hours at 50°C. The *L. bulgaricus* β-galactosidase was used for lactose conversion and showed a high activity of transgalactosylation. From 600 mM of lactose, the maximum yield of GOS was approximately 50% (w/w) of total sugar at 90% of converted lactose. In the GOS mixture, four main products were β-D-Gal*p*-(1→6)-D-Glc (allolactose), β-D-Gal*p*-(1→6)-Lac, β-D-Gal*p*-(1→3)-Lac and β-D-Gal*p*-(1→3)-D-Glc, in which two first components were yielded at 17.1 % (w/w) and 12.5 % (w/w) of total sugar, respectively.

Background

The term of "prebiotic" was firstly introduced and defined by Gibson and co-worker as "nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" [1]. Due to the special chemical structure, prebiotics can be not digested by enzyme in small intestine of mammalian, but bacteria in colon [1, 2]. Among many potential candidates of prebiotics such as nondigestible carbohydrate, peptides and protein, and lipids, oligosaccharides have been received the most attention and research [1-3]. Based on the chemical constituents and the degree of polymerization, prebiotic oligosccharides can be classified into several group including manno-oligosccharides, pectin oligosaccharides, pectic-oligosaccharides, isomalto- and xylo- oligosaccharides [4]. Galactose-oligosacharide (GOS) have been reported as the most attractive prebiotic oligosaccharides because it presents with large amount (1 g/l) compared to other ingredients in human milk [4, 5]. As a consequence, the production processes and characteristics of this prebiotic have been extensively studied both in laboratory and industrial scale. Moreover, it is reported that GOS is one of the top commercial prebiotics products [5].

GOS is defined as a mixture consisting of di-, tri-, tetra- and up to 10 monomers oligosaccharides with a terminal galactose obtained from lactose via co-transgalactosylation and hydrolysis reaction catalyzed by β-galactosidases [4].

Over last few decades, β-galactosidase, the most important factor in galacto-oligosaccharide production, has been continuously exploited from many sources of plant, animal and microorganism [6]. Recently, β-galactosidase from *Lactobacillus* species have been increasingly concerned with the expectation that the enzyme from probiotics strains might produce the GOS which is specific for probiotic strains [7]. Many native and recombinant β-galactosidases from *Lactobacillus* spp., such as *L. reuteri* [5, 8], *L. acidophilus* [9], *L. plantarum* [10], *L. pentosus* [11], were either isolated or over-expressed, characterized and applied for lactose hydrolysis and transgalactosylation. Most of these enzymes are composed of 2 different sub-units, LacL (large subunit) and LacM (small subunit), encoded by two overlapping genes *lacL* and *lacM* in chromosome,

respectively [6]. *Lactobacillus bulgaricus* have been widely used as the starter culture in the fermentation of dairy products [12]. *L. bulgaricus* even though is classified into *Lactobacillus* genus, β-galactosidase of this species is encoded by single gene *lacZ* [13] . Since the long history use of *L. bulgaricus* in food industry, the β-galactosidase from this species, especially in cell extract form, has been in consideration as a key factor for lactose hydrolysis and transgalactosylation [14, 15].

The goals of this study were firstly to overexpress β-galactosidase from *L. bulgaricus* in food-grade host *Lactobacillus plantarum* WCFS1 using inducible Sakacin-based expression systems pSip [16], which reported as effective systems for overproduction of several proteins [17]. Secondly, the recombinant enzyme were over produced, purified, characterized and further applied for lactose hydrolysis as well as transglactosylation. The carbohydrate components in the GOS products were analyzed.

Materials and Methods

Bacterial strains and media

The bacterial strains used in this study are listed in Table 1. *Lactobacillus* strains were cultivated in MRS media at 37°C, without agitation. *Escherichia coli* NEB5α (New England Biolab) strains was grown at 37°C in Luria Bertani (LB) media with shaking of 120 rpm. When needed, antibiotic erythromycin was supplemented in medium with concentration of 5 μg/ml for *Lactobacillus* or 200 μg/ml for *E.coli*, whereas ampliciline was used with concentration of 100 μg/ml for *E.coli.*

DNA manipulation

Total DNA of *Lactobacillus bulgaricus* DSMZ 20081 was isolated by a lysis protocol with chloroform extraction as described by Nguyen et al. [5] with some modifications. The cell pellets from 3 ml overnight culture were re-suspended and incubated at 37°C for 1 h in 400 μl 1mM Tris-EDTA buffer pH of 8 (TE buffer) with 50 μl of lysozyme (100 mg/ml) and 50 μl mutanolysin (480 U/ml). The mixture was supplemented with 50 μl SDS (10 %) and 10 μl proteinase K (20 mg/ml) and consequently incubated at 60°C for 1h. After the inactivation of proteinase K (at 75°C for 15 min), 2 μl RNAse (2 mg/ml) was added in the

mixture and incubated for 30 min at 37°C. The genomic DNA were extracted and purified by using phenol-chloroform, precipitated with 3M sodium acetate pH 3.8 and cold isopropanol. The DNA precipitate was washed with cold (-20°C) ethanol 70%. The DNA pellets were dissolved in 50 µl of TE buffer pH 7.5 at room temperature with gently shaking.

Table 1 - Bacterial strains and plasmids used in this work

Strains and plasmids	Relevant characteristics and purpose	References
Strains		
Lactobacillus		
L. bulgaricus DSMZ 20081	*lacZ* source	DSM
L. plantarum WCFS1	Host strain, plasmid free	[18]
E.coli NEB5α	Cloning host	New England Biolab
Plasmids		
pJET12	For sub-cloning	Fermentas
pSIP403	Spp-based expression vector; *sppKR* expression driven by *ermB* read through; 256rep; Em^{R}; *gusA* as reporter gene, controlled by P$_{sppA}$	[16]
pSIP409	Spp-based expression vector; *sppKR* expression driven by *ermB* read through; 256rep; Em^{R}; *gusA* as reporter gene, controlled by P$_{sppQ}$	[16]
pTH101	pSIP403 derivative, *lacZ* replaces *gusA*	This study
pTH102	pSIP403 derivative, *lacZ* with 6-histidine tag replaces *gusA*	This study
pTH103	pSIP409 derivative, *lacZ* replaces *gusA*	This study
pTH104	pSIP409 derivative, *lacZ* with 6-histidine tag replaces *gusA*	This study

Table 2 - Sequence of primers used in this study*

Primers	Restriction enzyme	Sequence (5'-3')	Source/ref. sequence accession no.
F1	*Bsmb*I	GCTG<u>CGTCTC</u>CCATGAGCAATAAGTTAGTAAAAG	M23530
R1	*Xho*I	C GCG<u>CTC GAG</u>TTATTTTAGTAAAAGGGGCTG	M23530
R2	*Xho*I	*C* GCG <u>CTC GAG</u>TTA*GTGGTGGTGGTGGTGGTG* TTT TAG TAA AAG GGG C	M23530

* Underlined sequences indicate the nuclease restriction sites. The 6-histidine tag sequence is shown in italic.

The primers used for PCR amplification of *lacZ* from genomic DNA of *L. bulgaricus* DSMZ20081 (Gene-bank accession number M23530) were supplied by VBC-Biotech Service GmbH (Vienna, Austria) (Table 1). The appropriate endonuclease restriction sites were introduced in forward and reverse primer.

The DNA amplification was performed with Phusion High-Fidelity DNA polymerase (Finnzymes, Espoo, Finland) as recommendation by the supplier. Plasmid DNA from *E.coli* was purified using Gene Elute TM plasmid miniprep KIT (Sigma). DNA was purified with Wizard^R SV Gel and PCR clean-up system KIT (Promega). When needed, pJET12 plasmid (CloneJET TM PCR cloning KIT, Fermentas) was used for sub-cloning.

Plasmids construction and transformation

The *Bsmb*I – *Xho*I fragments of *lacZ* with or without six-histidine tag were ligated the 5.6 kb *Nco*I –*Xho*I fragments of pSip403 or pSip409 resulted in 4 plasmids named pTH101, pTH102, pTH103 and pTH104 (Table 1). The resulting plasmids were electroporated into competent cells of *L. plantarum* WCFS1 which was prepared following a previously described procedure [19].

β-Galactosidase assay

β-galactosidase activity was determined using *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG) or lactose as substrates following Nguyen et.al (2006) [8].

Protein measurement

Protein concentration was determined by using the method of Bradford [20] with bovine serum albumin (BSA) as standard.

Expression of the recombinant β-galactosidases

The overnight cultures (around 16h) of *Lactobacillus plantarum* WCFS1 harboring various plasmids (table 1) were added into 15 ml of fresh MRS broth containing erythromycin to initial OD_{600nm} ~ 0.1. The cultivations were incubated at $30^{o}C$ without agitation. The cells were induced at OD_{600nm} of 0.3 by adding the inducer IP pheromone to the concentration of 25 ng/ml. The bacteria cells from 10 ml of the cultivations were harvested at OD_{600nm} between 1.8 to 2, washed twice with buffer P [8], and resuspended in 0.5 ml of the same buffer. The cells were disrupted by 1 gram glass bead (Precellys 24, PEQLAB Biotechnologie GMBH, Germany). The cell-free extracts were obtained after a centrifugation step at $9000 \, ^{x} g$ /15 min, at $4^{o}C$ and further used for enzyme assay, protein and SDS-PAGE analysis.

Fermentation and purification of the recombinant β-galactosides

L. plantarum WCFS1 harboring pTH102 plasmid was cultivated in 1 liter fermentation to obtain large amount of the recombinant enzyme. The bacterial cells were induced at OD_{600nm}~ 0.3 and harvested at OD_{600nm} ~ 6. The cell pellets were disrupted with French press. The whole cell-free extract was clarified by ultra–centrifugation at 30000 rpm/20 min, $4^{o}C$ (Beckman). The purification of the recombinant enzyme was performed in a single step with Ni Sepharose column (GE healthcare).

SDS-PAGE and Native PAGE

To compare the expression level of β-galactosidase in *L.plantarum* WCFS1 with different plasmids, the cell-free extracts were applied in SDS-PAGE. In order to see the intact form of the recombinant β-galactosidase, the purified enzyme was analyzed by Native PAGE with Phast System (Pharmacia Biotech). Coomassie Brilliant Blue was used to stain the bands on SDS- and Native- PAGE. The active staining was carried out using 4-methylumbelliferyl β-D-galactoside as previously described [8].

Steady-state kinetic measurements

To estimate the kinetic parameters of the recombinant enzyme with lactose and *o*NPG as nature and synthesis substrate, respectively, a series of substrate concentrations (1 mM to 25 mM for *o*NPG, 10mM to 600 mM for lactose) was used. The enzyme assays were performed for 10 min at $30^{o}C$ in 50 mM sodium phosphate buffer (pH6.5). The

kinetic parameters were calculated by using Henri-Michaelis-Menten model (SigmaPlot, SPSS Inc., Chicago, USA).

The influence of pH and temperature on activity and stability of the recombinant enzyme

The pH dependence of the recombinant enzyme was evaluated in Briton-Robinson buffer [19] with pH between 3 and 10. To determine the pH-stability, the purified enzyme was incubated at 37^oC in different pH buffers. At time of intervals, the residual activities were measured with oNPG as substrate.

The optimum temperature for hydrolysis activity of enzyme with both substrates lactose and o-NPG were determined between 20^oC and 90^oC. Whereas, the thermo-stability was evaluated by incubating the pure enzyme in 50mM sodium phosphate buffer (pH 6.5) at several temperatures from 20^oC to 60^oC. The residual activities were measured regularly with o-NPG as substrate. In addition, to study the effect of Mg^{2+} and K^+ cations on the thermo-stability of the recombinant enzyme, $MgCl_2$ or KCl (in 50 mM sodium phosphate buffer pH6.5) was supplemented in the enzyme reactions to final concentration of 1mM or 10 mM.
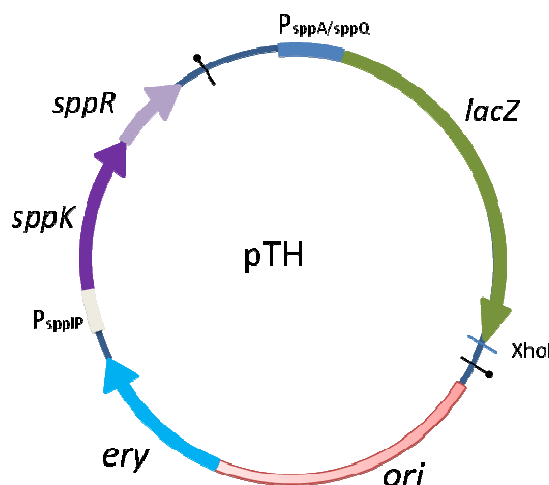
Figure 1. **Scheme of plasmids developed in this study**. Two inducible promoters P_{sppIP} and P_{sppQ} (P_{orfX}) control the expression of regulatory involved genes *sppKR* and *lacZ* genes (with and without histidine tag), respectively. Ori region contains replicon determant.

Influence of cations

The effects of cations on the enzyme activity were evaluate by performing the enzyme assays in 10 mM Bis-Tris pH 6.5 including interested cations at different concentrations of 1, 10, 50 and 100 mM with oNPG as substrate

Lactose hydrolysis and transgalactosylation

To study the lactose hydrolysis as well as transgalactosylation of the recombinant β-galactosidase from *L. bulgaricus*, 2ml of 600 mM lactose solution including MgCl$_2$ 10 mM in 50 mM sodium phosphate buffer were incubated at 30°C with ~ 1.5 U$_{lactose}$/ml of enzyme. Regularly, the samples were collected from the reactions to determine the residual activity and the carbohydrates content in the reaction mixtures including lactose, glucose, galactose and galacto-oligosaccharides by thin layer chromatography (TLC), high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and capillary electrophoresis (CE) as previously described [21].

Results

Plasmids construction and expressions of β-galactosidase from *L. bulgaricus* in *L. plantarum* WCFS1

In the present work, four expression vectors called pTH101, pTH102, pTH103 and pTH104 was constructed by exchanging the *lacZ* (with or without histidine tag) to *gusA* in pSip403 and pSip409 (Table 1). In these plasmids, the transcription of *lacZ* is regulated by the promoters P$_{sppA}$ or P$_{sspQ}$ for pSip403 or pSIP409 derivatives, respectively (Figure 1). These plasmids were successfully electroporated into *L. plantarum* WCFS1. The expressions of the *lacZ* β-galactosidase in *L. plantarum* WCFS1 with these vectors were studied.

As can be seen in the SDS PAGE for cell free extracts of *L. plantarum* WCFS1 carrying various vectors, the expressions of *lacZ* were relatively high as demonstrated by the strong density bands at approximately 100 kDa (Figure 2). The activity data from the induced cells show the quantitative evaluation for these expression levels, in which the β-galactosidase were produced around 15-23 U/ml fermentation broth with the specific activity between 160 – 200 U/mg protein (Table 3). The activity of enzyme was considerably high in the non-induced cells, especially with the cultivation of *L. plantarum* WCFS1 harbouring the pSIP403 derivative plasmids, for instance 10.25 U/mg and 11.74 U/mg respective to pTH101 and pTH102. The non-induced cells of *L. plantarum* WCFS1

carrying pSIP409 derivatives yielded lower background activity, around 3.43 - 4.11 U/mg (Table 3). The expression levels of *lacZ* adding histidine tag (in plasmids pTH102 and pTH104) were around 160-167 U/mg, whereas the expression levels of *lacZ* without tag (in plasmid pTH101 and pTH103) were in range of 192-198 U/mg (Table 3)

Table 3 - β-galactosidase activity in cell-free lysate of the induced and non-induced cells of *L. plantarum* WCFS1 carrying various plasmids*

Plasmid	volumetric activity (U/ml fermentation broth)		specific activity (U/mg protein)		Induction factor
	Induced	Non-induced	Induced	Non-induced	
pTH101	22.52 ± 0.77	1.50 ± 0.04	196.05 ± 2.86	10.25 ± 1.13	19
pTH102	15.51 ± 0.63	1.62 ± 0.13	158.42 ± 2.61	11.74 ± 0.53	13
pTH103	22.04 ± 1.26	0.63 ± 0.03	192.76 ± 10.10	4.11 ± 0.18	47
pTH104	18.00 ± 0.49	0.51 ± 0.04	167.98 ± 3.99	3.43 ± 0.13	49

*The data are expressed in mean ± standard deviation from three independent cultivations. The induction factors are calculated as division of mean specific activity (U/mg) between induced and non-induced results.

Table 4 - Kinetic parameters for the recombinant β-galactosidase

Substrate	Method for determination of enzyme activity	Kinetic parameter	
Lactose	Release of D-glucose	$V_{max,Lac}$ ($\mu mol.min^{-1}.mg^{-1}$)	111
		$K_{m,Lac}$ (mM)	20
		K_{cat} (s^{-1})	407
		K_{cat}/K_m ($mM^{-1}.s^{-1}$)	20.35
o-NPG	Release of *o*-NP	$V_{max,o\text{-}NPG}$ ($\mu mol.min^{-1}.mg^{-1}$)	257
		$K_{m,o\text{-}NPG}$ (mM)	1.20
		K_{cat} (s^{-1})	942
		K_{cat}/K_m ($mM^{-1}.s^{-1}$)	785
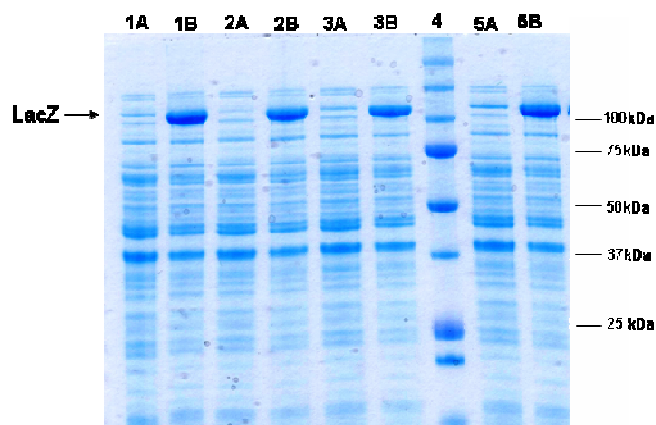
Figure 2. SDS-PAGE analysis of cell free extract of non-induced cells and induced cells of *L.plantarum* WCFS1 harbouring various plasmids. An "A" stands for the non-induced cells and "B" stands for the induced cells. Lanes 1A and 1B, pTH101; lane 2A and 2B, pTH103; lane 3A and 3B, pTH104; lane 5A and 5B, pTH102. Lane 4 shows the Precision Plus Protein standard (Biorad).
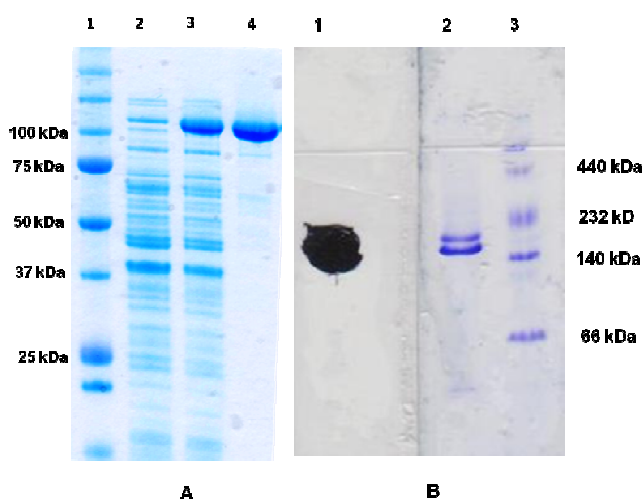
Figure 3. **SDS PAGE (A) and Native PAGE (B) of the purified recombinant β-galactosidase from *L. plantarum* WCFS1 carrying pTH102**. (A) On SDS-PAGE, the purified recombinant enzyme exhibited a single band (lane 4) with the size around 110 kDa as confirmed by The Precision plus Protein standard Ladder (Biorad) (lane 1). (B) Lane 1 indicates active staining of β-galactosidase with 4-methylumbelliferyl β –D-galactoside; lane 2, β-galactosidase; lane 3, high molecular mass protein ladder (Amersham TM HMW, GE healthcare).

Fermentation and purification of recombinant enzyme with 6-histidine tag

For further characterization, the recombinant enzyme with 6-histidines tag was expressed and purified from the cultivation of *L. plantarum* harboring pTH102. From 1

litter cultivation of this strain, approximately 7.7 g wet biomass and 43 kU of β-galactosidase were obtained (with the specific activity around 100 U/mg). Due to the 6-histidine tag, the recombinant enzyme was purified by single step to apparent homogeneity with Ni Sepharose column. The specific activity of this purified recombinant enzyme was measured approximately 215 U/mg.

Molecular characteristics of lacZ β-galactosidase

As judged by the SDS PAGE and the Native PAGE (Figure 3), the β–galactosidase from *L. bulgaricus* could be a homodimer (lane 2 in Figure 3B) consisting of two identical subunits of 110 kDa (lane 4 in Figure 3A). On the Native PAGE, the purified enzyme appeared in two bands. Both of them exhibited the functionality with active staining reagent (lane 1, Figure 3B)

Kinetic parameters

Table 4 shows the kinetic parameters of the recombinant β-galactosidase with two substrates lactose and *o*NPG. The K_m and the catalytic efficiency K_{cat}/K_m values are 20 mM and 20.35 mM^{-1}.s^{-1} and 1.2 mM and 785 mM^{-1}.s^{-1} for lactose and *o*NPG, respectively.

Temperature and pH dependence of the recombinant enzyme

The recombinant enzyme showed the highest activity at 45°C with *o*NPG as substrate and in range between 55°C and 65°C with lactose as substrate (Figure 4A). The higher temperature (>70°C) resulted in the rapid decreasing of the activity. At 90°C, the recombinant β-galactosidase was almost inactivated.

The thermo-stability of the recombinant enzyme was tested for 60 h of incubation at different temperatures in buffer sodium phosphate pH 6.5. At 50°C, approximately 50% of initial activity was remained, meanwhile at 30°C or 40°C, the activity of enzyme was approximately 80% of initial activity. However, at 60°C the enzyme was lost almost activity after only 30 min of incubation. The influence of cations K$^+$ and Mg^{2+} on the thermostability at 50°C of enzyme was also evaluated. The residual activity (% compared to initial activity) of enzyme stored in 50 mM sodium phosphate buffer pH 6.5 was around

70 to 80 % of after 36 h or 50 to 60 % after 72 h in the presence of K^+ or Mg^{2+} with concentration 1 mM or 10 mM.
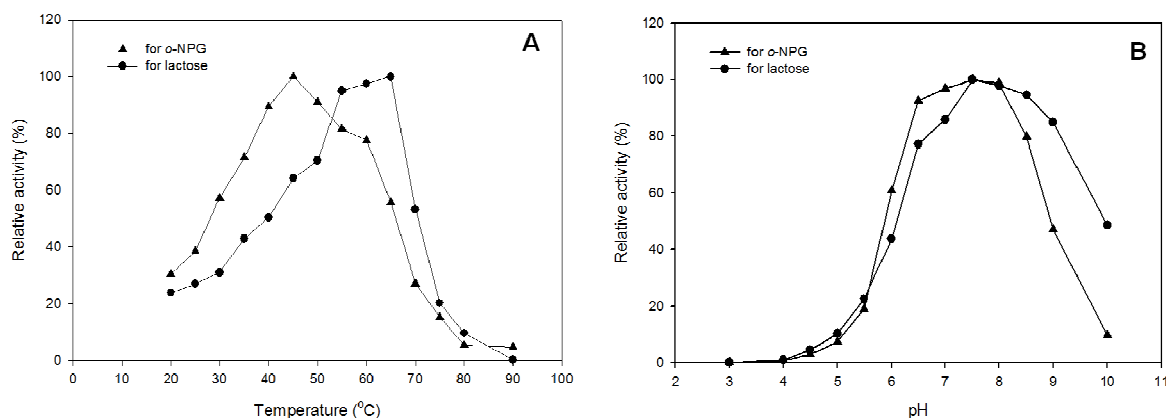
Figure 4. **Optimal temperature (A) and pH (B) of recombinant β-galactosidase from *L.bulgaricus* DSMZ20081 with lactose and *o*NPG as substrates**.

The enzyme has the pH optimum range between 7.5 to 8 for both substrates lactose and *o*NPG (Figure 4B). When stored in different pH buffers, the enzyme was stable in range of pH close to optimum value. The enzyme nearly remained the 100 % activity when stored in buffer 50 mM sodium phosphate buffer pH 7 at 37^oC for 3 days.

Table 5- Effect of cations on activity of enzyme in 10 mM Bis-Tris buffer 6.5

Cation	Concentration (mM)	Relative activity (%)[b]
Control[a]	-	100
Na$^+$	1	722
	10	1029
	50	1188
K$^+$	1	365
	10	536
	50	507
Mg^{2+}	1	85
	10	31
Zn^{2+}	1	3
	10	0.55
Ca^{2+}	1	77
	10	38

[a] without the presence of cation

Table 6 - Effect of cations on activity of enzyme in 50 mM sodium phosphate buffer

Cation	Concentration (mM)	Relative activity (%)[b]
Control[a]	-	100
K$^+$	1	115
	10	136
Mg^{2+}	1	148
	10	149

[a] without the presence of cation
[b] calculated based on activity of control sample

Effect of metal cations on activity of enzyme

In Bistris buffer pH 6.5, the activity of enzyme can be inhibited or activated by cation presenting in buffer. In the presence of monovalent cation like K$^+$ or Na$^+$ with difference concentration, the enzyme activity were 3 to 5-fold or 7 –to ~12-fold, respectively, higher than the enzyme activity of control sample (no cation) (Table 5). In contrast, the activity of enzyme was lowered when divalent ions Mg^{2+}, Ca^{2+} and Zn^{2+} were added in the reaction mixtures. Among the tested cations, Zn^{2+} showed the strongest inhibition, approximately 97% of enzyme activity lost in the presence of only 1 mM of this ion (Table 5).

Interestingly, in 50 mM sodium phosphate buffer pH6.5, the presence of Mg^{2+} resulting in 1.5-fold increasing of the activity of enzyme compared to in the same buffer without Mg^{2+} (Table 6). Monovalent cation K$^+$ had similar effect on the β-galactosidase activity and increased 1.1- 1.4 times of activity (Table 6).

Table 7- Oligosaccharide components (% of total sugar) and lactose conversion (%) in the GOS mixture at the time corresponding to the maximum yield of total GOS obtained at 3 temperatures.

Components	Temperature (^{o}C)		
	30[a]	40[b]	50[c]
Glucose	28.7	31.0	32.5
Galactose	11.9	13.5	14.2
Total GOS	49.5	48.7	48.2
β-D-Gal*p*-(1→6)-D-Glc	17.1	15.5	15
β-D-Gal*p*-(1→3)-D-Gal	0.6	0.6	0.6
β-D-Gal*p*-(1→6)-Lac	12.5	12.5	13.2
β-D-Gal*p*-(1→3)-D-Glc	3.8	4	3.9
β-D-Gal*p*-(1→3)-Lac	5.6	5.1	4.5
β-D-Gal*p*-(1→6)-D-Gal	1.0	1.3	1.1
Unknown OS	8.9	9.7	9.9
Lactose conversion	90.1	93.2	94.9

[a] at 12h; [b] at 8h; [c] at 5h

Lactose hydrolysis and transgalactosylation of recombinant β-galactosidase

The conversions were catalyzed by approximately 1.5 $U_{lactose}$ /ml of recombinant enzyme at 30°C with the initial concentration of lactose of 600 mM in 50 mM sodium phosphate buffer for 24 h. The carbohydrate components in the reaction mixtures were analyzed at interval times by thin layer chromatography (TLC), HPCL and CE. As can be seen in TLC plate (Figure 5), apart from glucose, galactose and remained lactose, some oligosaccharides were synthesized and appeared in some visible bands in region corresponding to GOS. The conversion rate of lactose was relative high resulting in approximately 96 % of lactose converted after 24h (Figure 6A). The maximum GOS yield
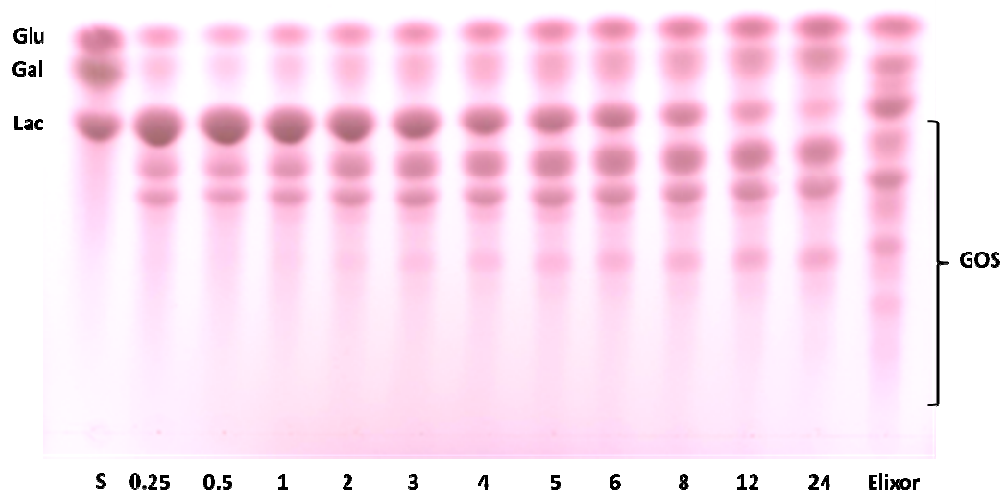
Figure 5. Thin layer chromatography of reaction mixture at 30°C at interval time. The lactose conversions was parallel conducted in 2 ml of 600 mM lactose in 50 mM sodium phosphate buffer containing 10 mM $MgCl_2$ with ~ 1.5 $U_{lactose}$/ ml of recombinant β-galactosidase. S indicates the standard mixture (20 g/l) including galactose, glucose and lactose. Elixor indicates the commercial GOS from Vivian (20 g/l) as reference.

was c.a. 50 % (w/w) (of total sugar) corresponding to 90 % of lactose conversion (Figure 6B) after 12h incubation. The HPLC data show major new synthesized oligosaccharides were β-D-Gal*p*-(1→6)-D-Glc, β-D-Gal*p*-(1→6)-Lac and β-D-Gal*p*-(1→3)-Lac (Figure 6B). Two trisaccharides β-D-Gal*p*-(1→6)-Lac and β-D-Gal*p*-(1→3)-Lac were formed in higher amount than other component at low lactose conversion and were degraded to the end of conversion, whereas the allolactose was synthesized slowly but yielded in higher amount at the end (Figure 6B). At higher temperature 40°C or 50°C, in general the profile of carbohydrates was similar to that obtained at 30°C, except the conversions were faster (data not shown). The maximum yields of GOS for the conversions at 40°C and 50°C were obtained at 8h and 5h, respectively. In the GOS mixtures corresponding to maximum yield, the proportion of β-D-Gal*p*-(1→6)-D-Glc, β-D-Gal*p*-(1→3)-D-Gal, β-D-Gal*p*-(1→3)-D-Glc, β-D-Gal*p*-(1→6)-Lac and β-D-Gal*p*-(1→3)-Lac in GOS were comparable for 3 temperatures (Table 7), in which β-D-Gal*p*-(1→6)-D-Glc (allolactose) and β-D-Gal*p*-(1→6)-Lac were observed in the higher amount than other components (around 15-17.1% and 12.5 to 13.2% of total carbohydrate in the mixture, respectively) (Table 7). The capillary electrophoresis chromatograph shows that GOS mixture consists of mono-, di-, tri- and tetra-saccharides (Figure 7).

Discussions

In the present work, β-galactosidase from *L. bulgaricus* has been successfully overexpressed in *Lactobacillus plantarum* using the inducible expression systems pSip. The pSIP expression system was developed based on the genes involving the production of bacteriocin class IIa sakacin (A and P). These systems have been successfully used for overproduction of β-glucuronidase (*gusA*) from *E. coli*, aminopeptidase N (*pepN*) from *Lactococcus lactis* and β-galactosidase *LacLM* from several *Lactobacillus* strains in *Lactobacillus* host [16, 17, 19, 22]. In this study, the advantages of lactic acid bacterial cell factories as well as the sakacin-based systems pSIP have been demonstrated again when were effectively used for over-production of the β-galactosidase *lacZ* from *Lactobacillus bulgaricus* DSMZ20081.

The expressions of *lacZ* (3027 bp) in 4 vectors (Table 1) were studied in both non-induced and induced cells. The SDS-PAGE (Figure 2) and the data of activity (Table 3) indicate the high expression of *lacZ* in pSip systems. Regardingless the promoter used, the considerable background activity (between 3.43 U/mg to 11.74 U/mg with the non-induced cells) obtained in this work show that the regulation of promoters depends on reporter gene, since before the lower background activity of this systems were observed but different target genes [19]. In agreement with previous reports that the promoter P_{sspA} is less tightly regulated then the promoter P_{sspQ} [16, 19], therefore the induction factor estimated for pSip409 derived vectors were higher than that for pSIP403-derived vectors (around 47-49 vs. 13-19, respectively) (Table 3). The introduction of the 6-histidine tag in *LacZ,* one hand facilitates for purification process, on the other hand lead the longer products which could result in lower amount of product from a determined amount "material and energy" for protein synthesis, therefore yielded a lower expression level as can be seen with pTH102 and pTH104 plasmids (Table 3).

In 1 liter scale, approximately 43 kU of β-galactosidase activity were produced by *Lactobacillus plantarum* carrying pTH102 vector. Since the cells were harvested at OD_{600} of 6, therefore the specific activity were lower than that with the cells harvested at OD_{600}
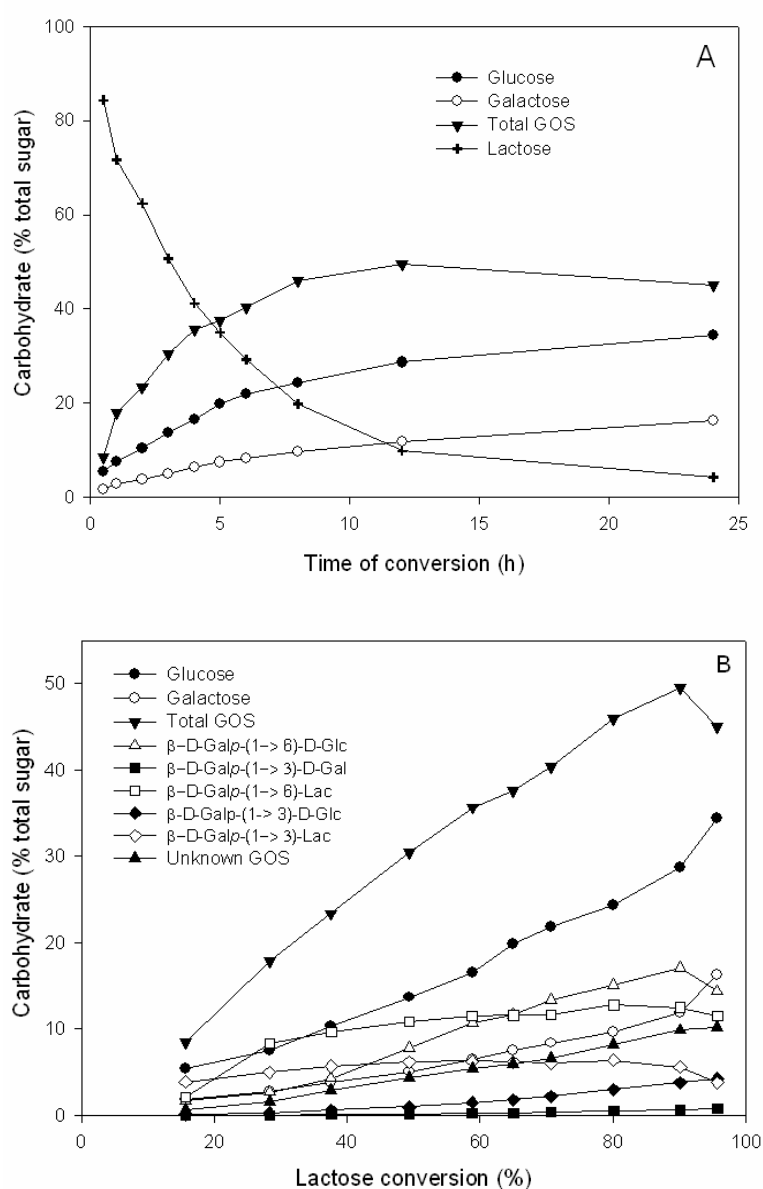
Figure 6. Hydrolysis and production of galacto-oligosaccharides during the lactose conversion catalyzed by recombinant β-galactosidase from *L.bulgaricus*. The reaction was carried out with initial concentration of 600 mM of lactose in 50 mM sodium phosphate buffer pH 6.5 in the presence of 10 mM $MgCl_2$ at 30^oC using ~1.5 $U_{lactose}$/ml of enzyme. (A) The time course of conversion. (B) The formation and degradation of carbohydrate during the lactose conversion.

between 1.8 to 2, which has been reported as the cell optical density to obtain maximum specific activity [23]. The β-galactosidase protein did amount approximately 47% of total intracellular protein of *L. plantarum* WCFS1 (compared to the specific activity of the purified recombinant enzyme of 215 U/mg). The enzyme production from the wild type *L.*

bulgaricus in MRS medium at 37°C with 20 g/L lactose as carbon source was also estimated. It was shown that the yield of the recombinant enzyme expressed with
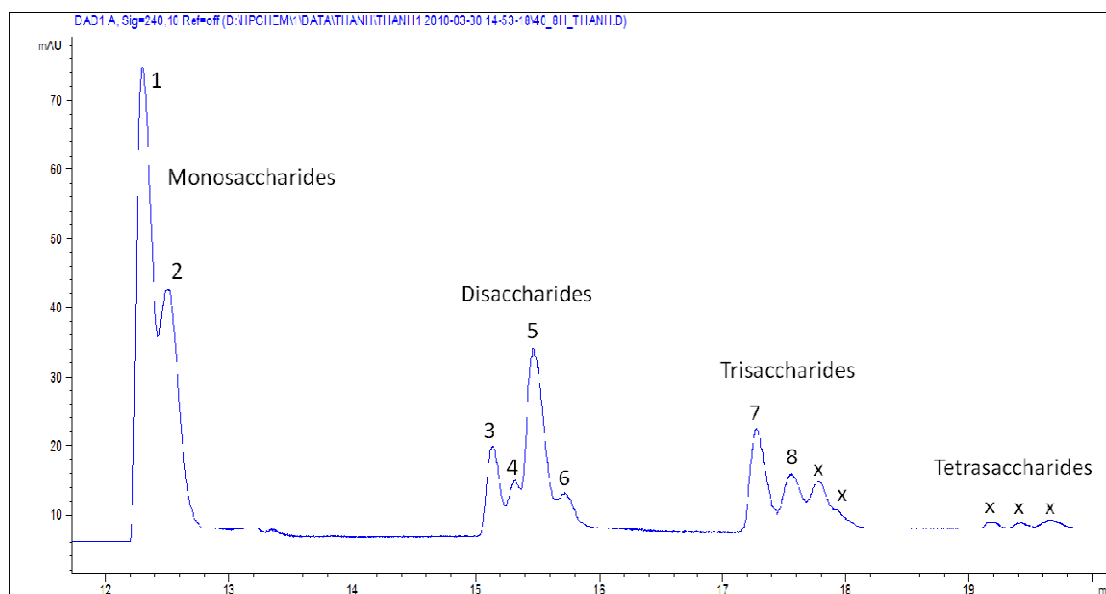
Figure 7. **Capillary electrophoresis chromatograph of mixtures at 8h from lactose conversion at 40°C**. The reaction was performed in sodium phosphate buffer pH6.5 with initial lactose concentration of 600 mM. The identified components are indicates as follows: (1), glucose; (2), galactose; (3), lactose; (4) D-Gal*p*-(1→3)-D-Glc; (5), D-Gal*p*-(1→6)-D-Glc (allolactose) with D-Gal*p*-(1→3)-D-Gal; (6), D-Gal*p*-(1→6)-D-Gal; (7), D-Gal*p*-(1→6)-Lac; and (8), D-Gal*p*-(1→3)-Lac. Unknown compounds are indicated by x symbol.

pTH102 was nearly 11-fold higher compared to the activity of enzyme from the wild type strain. Moreover, the recombinant enzyme includes the 6-histidine tag which simplified the purification of recombinant enzyme as well as increased the recovery yield of purification process (data not shown).

The amino acid sequence of β-galactosidase from *L. bulgaricus* was reported as approximately 34% similar to the β-galactosidase enzyme from *E. coli* [13]. Beta-galactosidase from *E.coli* has been known as a tetramer with 4 identical subunits. The recombinant enzyme in this study, as judged by the SDS PAGE and the Native PAGE (Figure 3), is likely to be a homodimer consisting of 2 identical subunits of approximately 110 kDa. This is in accordance to the calculation based on the nucleotide sequence of *lacZ* and previously observations [13, 24]. However, this is in contrast with recently report of Rhimi and co-workers, in which the recombinant enzyme was over expressed in *E. coli* and shown as a tetramer [24]. The possible explanation for this difference might be on

the expression host used for overexpression of the enzyme, which can influence on the folding of target protein, i.e *E. coli* versus *L. plantarum* in this study. The appearance of 2 bands on the Native PAGE (Figure 3B) indicates that the recombinant enzyme could make some isoforms [25] which also have the catalytic function therefore exhibited the activity when stained with substrate (Figure 3B).

As demonstrate by the kinetic parameters such as K_m and catalytic efficiency, β-galactosidase from *L. bulgaricus* prefers the artificial substrate *o*NPG to the nature substrate lactose (Table 4). The K_m value with lactose of the recombinant enzyme is of 20 mM that comparable with the K_m value of enzyme from some other lactobacilli such as *L. reuteri*, *L. plantarum* or *L. acidophilus* [8, 9]. However, this K_m value is much higher than value of the same enzyme reported previously [24].

Even though the recombinant enzyme shows the highest lactose hydrolysis activity at 65°C (Figure 4A), it is quickly inactivated at this temperature. However, the enzyme is stable at 50°C, especially in presence of Mg^{2+}. This could be beneficial for speeding up the rate of the lactose conversion process in industrial scale. Our observation about thermostability of enzyme is in accordance to the thermophile phenotype of *L.bulgaricus* and with observation of Itoh and co-worker [26].

The monovalent ions such as Na^+ and K^+ have a strong influence on the activity of the recombinant β-galactosidase (Table 5). Whereas, divalent ions have an inhibiting effect on the enzyme activity, especially the ions of heavy metal as Zn^{2+}. Mg^{2+} has been reported as important for the lacZ β-galactosidase [6, 25], but this ion alone reduces the activity of β-galactosidase from *L. bulgaricus* (Table 5), and only has the increasing effect when combining with Na^+ (Table 6). The synergistic influence of the monovalent and divalent on LacZ β-galactosidase has been observed previously [6, 25].

The conversion of lactose catalyzed by the recombinant β-galactosidase from *L. bulgaricus* yielded maximum GOS of approximately 50% (w/w) (Figure 6A) which is higher than typical yield (30-40% of total sugar) of maximum GOS produced by LacLM β-galactosidase from other *Lactobacillus* and *Bifidobacterium* [27]. The increasing of temperature even though increase the conversion rate of lactose, can be lead the slightly reducing of maximum GOS with simultaneous increasing of monosaccharides (Table 7). This means that at higher temperature the hydrolysis activity of the LacZ β-galactosidase

is more dominant than the transgalactosylation activity. The GOS mixture contains mono-, di-, tri-, and tetra-saccharide (Figure 7) which are compared favorably to the GOS products produced by LacLM β-galactosidase from *L. reuteri* and *L. plantarum* [10]. In the GOS mixture, β-D-Gal*p*-(1→6)-D-Glc (allolactose), β-D-Gal*p*-(1→6)-Lac, β-D-Gal*p*-(1→3)-Lac and β-D-Gal*p*-(1→3)-D-Glc are main components, and β-D-Gal*p*-(1→6)-D-Glc (allolactose) and β-D-Gal*p*-(1→6)-Lac amounted higher yield than others (Table 7). Similar to LacLM β-galactosidase, the β-galactosidase from *L. bulgaricus* shows a high specificity for the formation of β 1-6 than β 1-3. However, allolactose is predominant component in GOS and β-D-Gal*p*-(1→6)-D-Gal was formed in little amount (Table 7). It has been shown that the oligosaccharides containing the 1-6 linkage can promote the growth of bifidobacteria [28], thus GOS mixture produced by β-galactosidase from *Lactobacillus bulgaricus* can be considered as potential prebiotics.

In conclusion, the present study reported the high overexpression of β-galactosidase from *Lactobacillus bulgaricus* in *Lactobacillus plantarum* with inducible lactobacillal expression systems. Adding to previous reports, this study have shown the molecular and biochemical characteristics of this enzyme. To the best of our knowledge, there is little systematic research about lactose conversion ability of β-galactosidase from *L.bulgaricus*. Therefore, this study might be the first consideration in details on major components of galacto-oligosaccharide produced by this enzyme.

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Chapter 5

A food-grade system for inducible gene expression in *Lactobacillus plantarum* using an alanine racemase-encoding selection marker

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Abstract

Food-grade gene expression systems for lactic acid bacteria are useful for applications in the food industry. We describe a new food-grade host/vector system for *Lactobacillus plantarum* based on pSIP expression vectors and the use of the homologous alanine racemase gene (*alr*) as selection marker. A new series of expression vectors were constructed by exchanging the erythromycin resistance gene (*erm*) in pSIP vectors by the *L. plantarum* WCFS1 *alr* gene. The vectors were applied for the overexpression of β-galactosidase genes from *L. reuteri* L103 *and L. plantarum* WCFS1 in an *alr* deletion mutant of *L. plantarum* WCFS1. The expression levels obtained in this way, i.e. without the use of antibiotics, were comparable to the levels obtained with the conventional system based on selection for erythromycin resistance. The new system is suitable for the production of ingredients and additives for the food industry.

Background

Bacteria from the genera *Lactococcus* and *Lactobacillus* have considerable potential as safe cell factories of ingredients for food applications [2, 3]. Several gene expression systems have been developed based on genes and promoters involving sugar utilization, heat shock resistance and bacteriocin production [4-7]. Two well-known inducible expression systems developed for lactic acid bacteria (LAB) are based on quorum-sensing mechanisms involved in regulation of bacteriocin production. These systems are derived from the genes involved in the production of nisin in *Lactococcus lactis* [7] or sakacin A and P (pSIP vectors) in *Lactobacillus sakei* [8, 9]. The pSIP vectors have been used to express high amounts of heterologous proteins such as β-glucuronidase, aminopeptidase, amylase and β-galactosidases in lactobacilli [1, 8-11] . However, due to the use of an erythromycin antibiotic resistance gene (*erm*) as selection marker, the potential of the pSIP system for food applications has been limited.

Food-grade selection markers can be classified as dominant markers or complementation markers [4]. Dominant markers usually confer a new ability to the host strain such as bacteriocin immunity/resistance [12-14], heat-shock resistance [15], or sugar utilization abilities [16, 17]. Dominant markers have the advantage that they potentially may be used in a wide variety of strains, but the number of suitable food-grade markers is limited [4]. Furthermore, these markers have certain potential disadvantages such as a narrow spectrum of application (e.g bacteriocin immunity genes [12, 13]), necessary manipulation of the growth medium (carbohydrate source, bacteriocins) or the necessity to use unfavorable cultivation conditions for the host strain (e.g. elevated temperature, [15]). Selection markers based on complementation do not require supplements in the cultivation medium; their disadvantage is that they require the use of specially adapted host strains.

In order to develop a food-grade complementation-based host/marker system, a gene on the host chromosome is mutated or deleted, and a wild-type copy is inserted into the expression vector. A gene which is chosen as complementation marker usually has a decisive influence on the growth of host strain. Examples of such genes include the thymidylate synthase gene (*thyA*) [18], and genes involved in lactose conversion, such as

lactose phosphotransferase (*lacF)* [19, 20] or phospho-β-galactosidase (*lacG)* [21]. The enzyme alanine racemase converts L-alanine to D-alanine, which is crucial for cell wall biosynthesis, and is thus an essential enzyme for growth of prokaryotic cells [22]. In lactococci and lactobacilli, alanine racemase activity is encoded by a single gene, *alr* [22, 23]. D-alanine is not a common ingredient of large-scale fermentation media and previous studies have shown that the *alr* gene has considerable potential as a food-grade selection marker in lactic acid bacteria [24].

The goal of the present study was to develop a food-grade expression system based on the pSIP expression vectors [9], using the *alr* gene as selection marker in *L. plantarum* WCFS1. The new host/vector systems were applied to overproduce β-galactosidases from *L. plantarum* WCFS1 and *L. reuteri* L103, both dimeric proteins encoded by two over-lapping genes called *lacLM* [1, 25]. The expression levels obtained with the newly developed food-grade system were compared to the levels obtained using the conventional pSIP system, which is based on the use of an erythromycin resistance gene for selection [1]. These new vectors open up the possibility to use the pSIP expression system for food-related applications.

Materials and Methods

Bacterial strains and media

The bacterial strains, plasmids and primers used in this study are listed in Tables 1 and 2. *L. plantarum* WCFS1 [26] was grown in MRS medium (Oxoid, Basingstoke, UK) at 37°C without agitation. *E. coli* Top10 (Invitrogen, Carlsbad, CA) or *E. coli* MB2159 (D-alanine auxotroph) [27] were used as cloning hosts and cultivated in Luria-Bertani (LB) medium at 37°C with shaking at 200 rpm. Solid media were prepared by adding 1.5% agar to the respective media. Unless otherwise stated, the antibiotic concentrations were 10 μg/ml of chloramphenicol (Cm) for *Lactobacillus* and *E. coli*, and 5 μg/ml or 200 μg/ml of erythromycin (Erm) for *Lactobacillus* or *E. coli*, respectively. *E. coli* MB2159 as well as *L. plantarum* strains TLG01 and TLG02 (Table 1) were cultivated as the wild types except for addition of 200 μg/ml D-alanine (Sigma, St. Louis, MO) to the respective media.

DNA manipulation

Genomic DNA of *L. plantarum* WCFS1 was isolated by using the E.Z.N.A bacterial DNA kit (Omega Bio-Tek Inc., Doraville, GA) according to the instructions of the manufacturer. Plasmids were isolated using NucleoSpin® Plasmid Kit (Mini-Prep) (Macherey-Nagel GmbH & Co., Düren, Germany) or Jetstar (Midi-Prep) plasmid purification system (Genomed GmbH, Löhne, Germany) as recommended by the supplier. PCR amplification of DNA was performed with the hot start KOD polymerase (Toyobo, Osaka, Japan) and primers purchased from Operon Biotechnologies GmbH (Cologne, Germany). All amplified sequences were verified by DNA sequencing. PCR products and restriction enzyme digested DNA were purified with Nucleo-Spin Extract II kit (Macherey-Nagel GmbH & Co) and the DNA concentration was measured using the Quant-it® assay (Invitrogen).

L. plantarum competent cells were prepared and electroporated as described by Josson and co-workers [28]. Chemically competent cells of *E.coli* TOP10 were supplied by Invitrogen. Competent cells of *E.coli* MB1259 were prepared and transformed according to the method of Inoue et al. [29].

Construction of the *alr* deletion mutant

The integrative vector for deletion of the alanine racemase gene (*alr*) from *L. plantarum* WCFS1 genome was constructed as described by Lambert and co-workers [30]. The two flanking fragments upstream and downstream (~ 1 kb) of the *alr* gene were PCR amplified using genomic DNA from *L. plantarum* as template and the two primer pairs, T2 & T3 and T4 & T5, respectively (Table 2). The *lox66*-P$_{32}$-*cat-lox71* cassette (~ 1.2 kb) was amplified from the pNZ5319 template [30] using the primers is128 and is129 (Table 2). The flanking regions were fused with the *lox66*-P$_{32}$-*cat-lox71*-fragment by overlap extension PCR (SOE-PCR) using primer pair T2 and T5. Subsequently, the fused fragment was ligated to a ~ 2.7 kb fragment obtained from *Swa*I-*Ecl*136II digestion of pNZ5319, yielding pTLG01 (Table 1).

Table 1 - Strains and plasmids used in this work*

Strains or plasmids	Relevant characteristics	Reference (s)
Strains		
Lactobacllus plantarum		
WCFS1	Wild type	[26]
TLG01	Double cross over mutant Δalr, Cm^r, D-alanine auxotroph	This work
TLG02	Δ*alr*, D-alanine auxotroph	This work
E.coli		
MB2159	D-alanine auxotroph, cloning host	[27]
TOP10	Cloning host	Invitrogen
Plasmids		
pNZ5319	Cm^r, Em^r, containing *lox66*-P$_{32}$-*cat-lox71* fragment	[30]
pNZ5348	Em^r, *Cre*-recombinase expression	[30]
pTLG01	Cm^r, Em^r, pNZ5319 derivative for knocking out *alr* from *L. plantarum* WCFS1 genome	This work
pSIP403	Em^r, pSIP401 derivative, *gusA* controlled by P$_{sppA}$	[9]
pSIP409	Em^r, pSIP401 derivative, *gusA* controlled by P$_{sppQ}$	[9]
pLp_0373sNuc	pSIP401 derivative, Em^r	[10]
pEH9R	Em^r, pSIP409 derivative, containing *lacLM* from *L. reuteri* L103	[1]
pEH9P	Em^r, pSIP409 derivative, containing *lacLM* from *L. plantarum* WCFS1	[1]
pEH3R	Em^r, pSIP403 derivative, containing *lacLM* from *L. reuteri* L103	[1]
pEH3P	Em^r, pSIP403 derivative, containing *lacLM* from *L. plantarum* WCFS1	[1]
pSIP609	pSIP409 derivative, *alr* replaced with *erm*	This work
pSIP609R	pSIP409 derivative, *alr* replaced with *erm*, *gusA* replaced by *lacLM* from *L. reuteri* L103	This work
pSIP609P	pSIP409 derivative, *alr* replaced with *erm*, *gusA* replaced by *lacLM* from *L. plantarum* WCFS1	This work
pSIP603R	pSIP403 derivative, *alr* replaced with *erm*, *gusA* replaced by *lacLM* from *L. reuteri* L103	This work
pSIP603P	pSIP403 derivative, *alr* replaced with *erm*, *gusA* replaced by *lacLM* from *L. plantarum* WCFS1	This work

*Cm^r, Erm^r : chloramphenicol and erythromycin resistance; *cre*: cre-recombinase encoding gene; *alr*: alanine racemase encoding gene; *erm*: erythromycin resistance gene

Table 2 - Primers used in this study*

Primer	Sequence (5′- 3′)
T1	CAGCACTTGTGCGTCCTATGC
T2	CGAGCATTGCCTAGCCACC
T3	GCATACATTATACGAACGGTAGATTT AACCATCACAAATTGCCTCTTTC
T4	CGGTACAGCCCGGGCATGAG ATAGATTAAGTTTTATCAGACGATAGGTT
T5	TCAACTAGCTTACCTAATCGTCGTC
T6	AGCGATATTCACGATTTACAGCC
is128	AAATCTACCGTTCGTATAATGTATGC
is129	CTCATGCCCGGGCTGTACCG
T7	TATGCGTGCGGGATCCC (*Bam*HI)
T8	GCGGTGCTCCCCAATTACAACCATGTAATCTCTCCTGAAGTTAAACGATT
T9	ATGGTTGTAATTGGGGAGCAC
T10	TTAATCTATATAAACTCTCGGCACTCGT
T11	GAGTGCCGAGAGTTTATATAGATTAATTCTATGAGTCGCTTTTTTAAATT
T12	CACTTTTGATAATCGATATGGTAAACT (*Cla*I)

* The restriction sites are underlined

Plasmid DNA was purified by phenol-chloroform extraction and sodium acetate/iso-propanol precipitation from the cloning host (*E. coli* TOP 10) and re-suspended in 50µl nuclease-free ddH$_2$O. Four micrograms (4 µg) of plasmid DNA was used for transformation into *L. plantarum.* Selection of the double crossover mutant (named *L. plantarum* TLG01), marker excision by Cre-dependent recombination of the *lox*-sites and selection of the *alr* deletion mutant (*L. plantarum* TLG02) was done using the previously described method [30]. Successful double crossover plus subsequent marker excision should result in a deletion of the entire *alr* coding sequence in the chromosome of *L. plantarum.* Selected progeny was checked by PCR amplification with the primers T1 and T6 primers (Table 2) and named *L. plantarum* TLG02.

Construction of β-galactosidase expression vectors

The food-grade expression vector was constructed based on the pSIP vector series (Figure 1A) [1] by replacing the erythromycin resistance gene (*erm*) with the alanine racemase gene (*alr*) from *L. plantarum* WCFS1 genome. For that purpose, the pSIP derivative pLp_0373sNuc [10] was used as template to amplify upstream and downstream flanking regions of *erm* with primer pairs T7 & T8 and T11 & T12, respectively (Table 2). The *alr* gene was amplified using primers T9 and T10. In order to allow seamless replacement of the *erm* gene, the *alr* gene was fused by SOE-PCR to the two fragments located upstream and downstream of the *erm* gene. Using the In-Fusion™ Advantage PCR Cloning Kit (Clontech, Mountain View, CA), this fused fragment was ligated to a ~ 5.8 kb fragment obtained upon cleavage of pSIP409 [9] (Table 1) with *Bam*HI-*Cla*I, resulting in plasmid pSIP609 (Table 1). The net effect of these manipulations is an exchange of the coding region of the *erm* gene in pSIP409 with the coding region of the *alr* gene, without any additional changes in upstream our downstream sequences.

Fragments containing the *lacLM* genes from *L. reuteri* L103 and *L. plantarum* WCFS1 fused to promoters P_{sppQ} (pSIP409) or P_{sppA} (pSIP403) were obtained by digesting plasmids pEH9R, pEH9P, pEH3R and pEH3P [1] with *Pst*I and *Xma*I (Figure 1A). These fragments were ligated into the ~ 5.5 kb *Pst*I-*Xma*I digested fragment of pSIP609 yielding 4 plasmids named pSIP609R, pSIP609P, pSIP603R and pSIP603P (Figure 1B and Table 1). These expression vectors were constructed in *E. coli* MB2159 before electroporation into the D-alanine auxotroph expression host *L. plantarum* TLG02.

Segregational stability of *alr*- and *erm*–based plasmids

The wild type *L. plantarum* WCFS1 harbouring pEH9R and mutant *L. plantarum* TLG02 harbouring pSIP609R were cultivated in non-selective and selective medium (MRS with and without 5 µg/ml erythromycin or 200 µg/ml D-alanine, respectively) at 37°C without agitation. Under these conditions, about 7 generations of growth passed in 12 hours. The number of cells that still contained the expression plasmids after 12 hours was estimated by plating dilution series of the culture on selective plates [31]. The effect of induction on the segregational stability of the plasmids was tested by supplying SppIP (25 ng/ml; CASLO Laboratory, Lyngby, Denmark) to the culture. The percentage of plasmid-harboring cells and the β-galactosidase activity from these samples were determined regularly.

Expression of β-galactosidases with *alr*-based vectors

Overnight cultures of L. plantarum harbouring the pSIP derived novel plasmids were diluted in fresh prewarmed MRS medium (for *erm*-based systems, 5 μg/ml of erythromycin was added) to an OD_{600nm} ~ 0.1 and incubated at 30°C. The cells were induced at an OD_{600nm} ~ 0.3 by adding the peptide pheromone (SppIP) to a final concentration of 25ng/ml. Cells were harvested at OD_{600nm} of 1.8 to 2.0. Bacterial cells were pelleted from 10 ml of culture by centrifugation at 3500 ×g for 10 min at 4°C, washed with buffer P (50mM of sodium phosphate buffer, pH 6.5, 20% of glycerol and 1 mM DTT) [32], and resuspended in 500 μl of the same buffer. The cells were disrupted by ~1 g glass beads (0.5 mm) using a Precelly 24 glass bead mill (PEQLAB Biotechnologie GmbH, Erlangen, Germany). Cell-free extracts obtained after 5 min of centrifugation at 9000 ×g (4°C) were used for activity assays, protein concentration determination.

Enzyme assay and protein determination

β-galactosidase activity was determined using *o*-nitrophenyl-β-D-galactopyranoside (*o*-NPG) as described previously [32]. Protein concentration was determined by using the method of Bradford [33] with bovine serum albumin (BSA) as standard.

Results

Construction *alr⁻* mutant strain

After transforming *L. plantarum* WCSF1 with the (non-replicating) vector pTLG01 chloramphenicol-resistant progeny (on MRS-agar + D-alanine) was replica-plated on MRS-agar containing chloramphenicol (without D-alanine) and MRS-agar with D-alanine and erythromycin to check for D-alanine-auxotrophy and erythromycin-sensitivity (indicating replacement of the genomic *alr* gene by the chloramphenicol-resistance gene as well as loss of the plasmid-backbone. Six out of ten tested transformants displayed the desired phenotype; two of these were checked by PCR and confirmed as gene replacements. Marker excision of the *lox*-sequence-flanked chloramphenicol-resistance gene was done by transformation with pNZ5348 containing the gene for the Cre-recombinase. About 20 % of the transformants displayed the desired Ery[R], Cm[S] phenotype and marker excision was confirmed by PCR. Loss of pNZ5348 was then induced by cultivation in the absence of

erythromycin. The resulting strain, *L. plantarum* TLG02, was unable to grow within 24h at 37°C in both liquid and solid MRS medium. In contrast, growth of the mutant strain in the presence of D-alanine was identical to the wild type *L. plantarum* WCFS1 (data not shown).

pSIP-derived expression vectors for the new host strain.
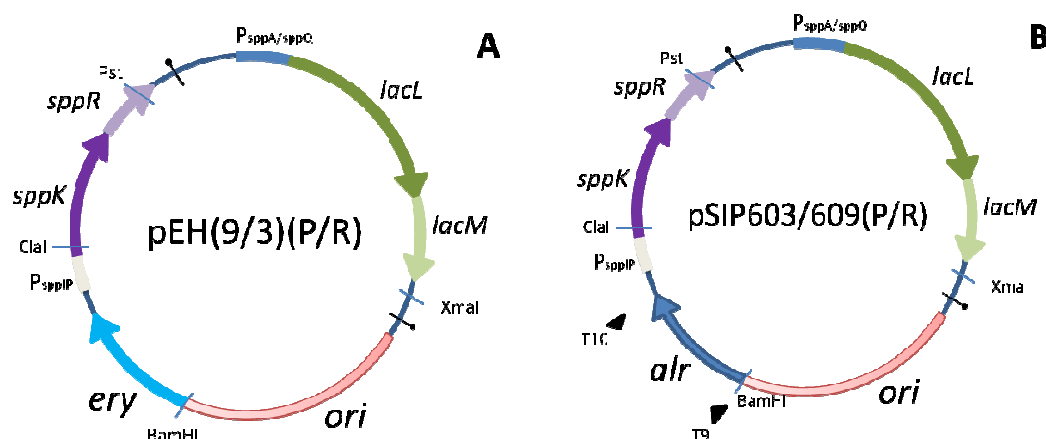
Figure 1. **Scheme of expression vectors used in this study.** Expression vectors for *lacLM* based on the erythromycin resistance gene (*erm*) [1] (A) and the alanine racemase (*alr*) gene (B) as selection markers. *SppK* and *sppR*, denoting a histidine kinase and a response regulator, respectively, are regulated by P$_{sppIP}$. The structural genes (overlapping genes *lacLM* from *L. plantarum*, indicated by "P", or *L. reuteri*, indicated by "R") are controlled by the inducible promoter P$_{sppA}$ (pSIP403 derivatives) or P$_{sppQ}$ (pSIP409 derivatives).

New expression vectors were constructed based on the pSIP-vector series by exchanging the *erm* gene with the genomic *alr* gene (Figure 1A, 1B) as described in Material and Methods. The expression of *alr* is controlled by the native erythromycin promoter which is derived from a *Lactobacillus reuteri* strain [34].

The *lacLM* genes from *L. plantarum* and *L. reuteri* [1] were cloned into these *alr*-based plasmids resulting in 4 plasmids in which the transcription of *lacLM* genes is controlled by the promoters P$_{sppA}$ (pSIP603P and pSIP603R, respectively) or P$_{sppQ}$ (pSIP609P and pSIP609R, respectively) (Figure 1B).

Plasmid segregational stability

Table 3- Segregational stability of *alr*-based and *erm*-based plasmids in different media*

| Plasmid/medium* | Cells remaining plasmid (%) and specific activity (U/mg) after different generations** | | | | | |
| | 7 | | 42 | | 84 | |
	%	(U/mg)	%	(U/mg)	%	(U/mg)
pEH9R/MRS	-	-	37	-	3	-
pEH9R/MRS+SppIP	-	26.5	14	0.6	0	0.1
pEH9R/MRS+ery	-	-	89	-	82	-
pEH9R/MRS+ery+SppIP	-	32.0	88	27.7	79	16.1
pSIP609R/MRS	-	-	100	-	100	-
pSIP609R/MRS+SppIP	-	27.9	100	23.9	100	20.3
pSIP609R/MRS+D-alanine	-	-	90	-	76	-
pSIP609R/MRS+D-alanine+ SppIP	-	26.0	56	17.5	17	5.0

* *L. plantarum* WCFS1 harbouring pEH9R and *L. plantarum* TLG02 harbouring pSIP609R were cultivated in selective and non-selective medium with and without SppIP (the final concentration of SppIP was 25 ng/ml). The D-alanine concentration was 200 µg/ml, the erythromycin concentration was 5 µg/ml.

* *The strains were cultivated at 37°C without agitation. Every 12h, 7 generations passed. The fraction of plasmid containing cells was calculated by dividing the number of CFU on selective medium with the number of CFU on non-selective medium. A symbol "-" stands for non-determined

The segregational stability of the *alr*-and *erm*-based vectors was tested using pSIP609R(*alr*) in *L. plantarum* TLG02 and pEH9R(*erm*) in *L. plantarum* WCFS1 as representatives. The strains were cultivated for an estimated 84 generations (144 hours) at 37°C in non-selective and selective medium, followed by replica plating of diluted cultures in order to determine the fractions of cells retaining the plasmid. The fraction of cells retaining pEH9R after 84 generations in non-selective medium (MRS without erythromycin) was 3%, whereas in selective medium 82% of the colonies still contained the plasmid. Interestingly the pSIP609R plasmid showed better segregational stability: after 84 generations it was retained in approximately 76 % and 100 % of cells of *L. plantarum* TLG02 under non-selective and selective conditions, respectively (Table 3). Induction of expression of the *lacLM* genes generally led to reduced plasmid stability, a phenomenon that is most clearly visible under non-selective conditions (Table 3). Table 3 also shows that plasmid loss is reflected in gradual loss of the β-galactosidase activity in

cell extracts. For example, for *L. plantarum* TLG02/pSIP609R grown in non-selective medium and in the presence of SppIP, only 17% of the cells still harboured the plasmid after 84 generations, resulting in a β-galactosidase activity amounting to production of 5.0 U/mg of, as compared to 26.5 U/mg after 7 generations.

Table 4 - β-galactosidase activity in cell-free lysates of induced and non-induced *L. plantarum* WCFS1 and *L. plantarum* TLG02 carrying various plasmids.

Strain/plasmid	Specific activity (U/mg protein)*		Induction factor**
	Induced	Non-induced	
TLG02/pSIP603P	87.50 ± 7.38	1.15 ± 0.09	76
WCFS1/pEH3P	99.35 ± 3.04	1.67 ± 0.03	59
TLG02/pSIP603R	61.25 ± 3.17	0.51 ± 0.10	119
WCFS1/pEH3R	109.29 ± 2.09	2.51 ± 0.11	44
TLG02/pSIP609P	79.84 ±2.86	0.51 ± 0.16	158
WCFS1/pEH9P	90.57 ± 3.86	0.45 ± 0.02	200
TLG02/p SIP609R	64.74 ± 3.57	0.50 ± 0.10	129
WCFS1/pEH9R	71.68 ± 1.10	0.72 ± 0.01	99

*Data are expressed as means ± standard deviation from three independent cultivations.

**The induction factors are calculated by dividing the mean specific activities (U/mg) of induced and non-induced cultures.

Expression of *Lactobacillus* β-galactosidases in *alr*-based vectors and comparison with *erm*-based vectors.

The high expression levels were indicated by measured enzyme activities (Table 4). In general, the obtained specific enzyme activities (U/mg protein) were slightly higher with the conventional system (*erm* based vectors in *L. plantarum* WCFS1) and the magnitude of this difference varied between the pairs of constructs. The largest difference was observed for pSIP403 derivatives used to express *L. reuteri lacLM*, where the conventional system (WCFS1/pEH3R) yielded a specific activity that was almost twice as high as the activity obtained with the TLG02/pSIP603R system (Table 4). Non-induced cells harbouring the *alr*-based vectors generally yielded lower background activities than non-induced cells harbouring the *erm*-based vectors in three out of four cases (Table 4) and
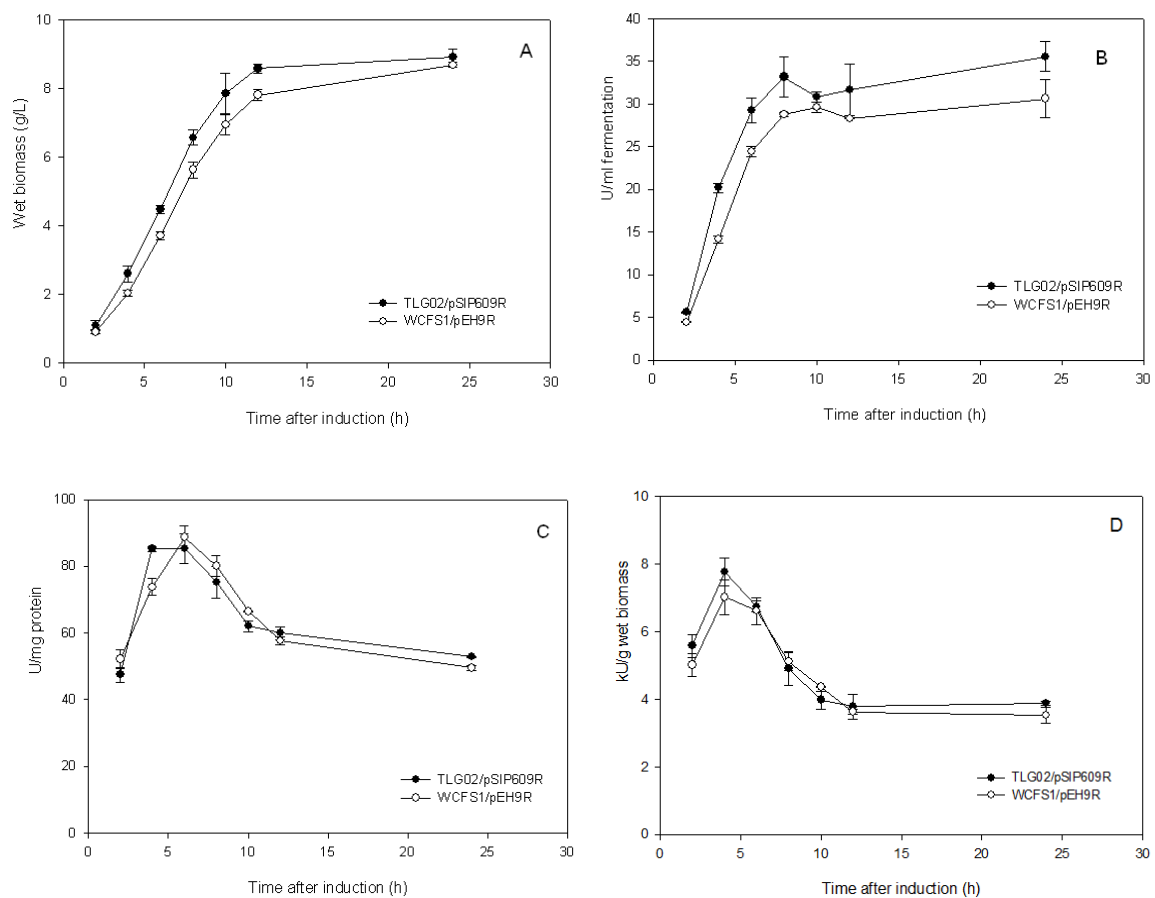
Figure 2. Fermentations of *L. plantarum* TLG02/pSIP609R (closed circles) and *L. plantarum* WCFS1/pEH9R (open circles) *L. plantarum* in MRS at 30°C. For *L. plantarum* WCFS1/pEH9R the medium contained Erythromycin (5 µg/ml). The cells were induced at OD$_{600}$ ~0.3. The wet biomass g/L fermentation (A), the volumetric activity U/ml fermentation (B), specific activity U/mg protein (C), and specific biomass activity (kU/g) were determined for the interval after induction. The data are the mean value of 3 cultivations and error bars indicate the standard deviations.

this may be beneficial in cases strict control is desirable. To get better insight into the fermentation characteristics of the two systems, two of the strains, *L. plantarum* WCFS1 carrying pEH9 and *L. plantarum* TLG02 carrying pSIP609R, respectively, were cultivated in 200 ml of MRS broth at 30°C without agitation. The medium for the former strain contained 5 mg/ml erythromycin; both cultures were induced at an OD$_{600}$ of 0.3 with 25 ng/ml SppIP. After induction, cells were sampled every two hours for activity analysis and biomass determination. Figure 2 gives an overview of the wet biomass (g/l), β-galactosidase activity (U/ml fermentation and U/mg protein), and specific biomass activity (activity in 1 g of wet biomass) (kU/g) during the fermentation period for both

systems. The growth rate of bacteria harboring the *erm*-vector (pEH9R) was slightly lower than of those containing an *alr*-vector (pSIP609R). For example, at 12 hours after induction WCFS1/pEH9R yielded 7.8 g/l wet biomass, while TLG02/pSIP609R yielded 8.6 g/l at the same sampling time (Fig 2A). The amount of enzyme increased from the induction time and reached maximum values at 8 – 10 h after induction. Cells carrying the *alr*-based vector gave slightly higher yields compared to cells carrying the *erm*-based vector (approximately 33 and 29 U/ml fermentation, respectively; Figure 2B). However, the specific activity of the enzyme was similar in both systems and reached the highest value approximately 6 h after induction (~ 85 U/mg; Figure 2C). The relationship between volumetric activity (U/ml) and biomass (g/L) which is expressed as specific biomass activity is shown in Figure 2D. The specific biomass activities were almost identical for the two systems and reached maxima of 7.8 kU/g and 7 kU/g for for *alr*-based and *erm*-based systems, respectively, 4h after induction (Figure 2D).

Discussion

We have constructed a new host/vector system for inducible gene expression in *Lactobacillus* using pSIP-type vectors and the *alr* gene as a selection marker. The Δ*alr* mutant *L. plantarum* TLG02 was developed using the Cre-*lox* system for complete *alr* gene replacement in wild type *L. plantarum* WCFS1. This approach prevents the re-integration of the *alr* marker back into the host chromosome as has been observed during early work on *alr*-based selection in lactic acid bacteria where the gene was only partially deleted from the host chromosome [24]. The segregational stability of one of the *alr*-based vectors was evaluated for 84 generations and compared to that of the corresponding *erm*-based vector. The results (Table 3) showed that the *alr*-based vector was more stable than the *erm*-based vector, both under selective and non-selective conditions. Interestingly, when the expression system was activated by SppIP pheromone, the *erm*-based plasmid was lost from the bacterial cells more quickly (Table 3), indicating that expression of the target gene exerts a considerable metabolic load on the cells. Most interestingly, this effect was not observed for the *alr*-based plasmid in the mutant strain

under selective conditions, meaning that in this case, all cells maintained the plasmid. Since selective conditions are easy to maintain for a complementation marker, the *alr*-based plasmids offer clear advantages for heterologous expression under industrial conditions.

We have previously overexpressed *lacLM* genes from four *Lactobacillus* species in the closely related hosts *L. plantarum* WCFS1 and *L. sakei* Lb790 using *erm*-based pSIP vectors [1]. The highest expression levels were obtained for the *lacLM* genes from *L. reuteri* L103 and *L. plantarum* WCFS1, hence these genes were used to test β-galactosidase overproduction in the new *alr*-based expression systems. The specific activity data (Table 4) show that the expression levels of *lacLM* genes with *alr*-based vectors were comparable to the levels obtained with the *erm*-based vectors, although they generally were slightly lower. The β-galactosidase activities in non-induced cultures were also higher using the conventional system, especially with the pSIP603-series, where *lacLM* expression is controlled by the most leaky of the two promoters, P_{sppA} (Table 4). It is conceivable that this difference between the *alr*-based and *erm*-based systems is due in part to slight differences in plasmid copy number. In the wild type strain, a single copy of the *alr* gene is required for growth, and additional copies may not offer any advantage to the cells. In contrast, in an antibiotic-containing environment a higher copy number of the resistance gene may benefit the cells, which could exert selection pressure towards keeping multiple copies of the *erm*-based vectors, resulting in a higher background activity.

In the fermentation experiments, *L. plantarum* TLG02 harbouring pSIP609R (*alr*) grew slightly faster than *L. plantarum* WCFS1 harbouring pEH9R (*erm*), and the former strain showed a higher wet biomass yield (Figure 2A) and higher volumetric activity (U/ml) (Figure 2B). It is possible that the synthesis of the *erm*-gene product and/or the resulting methylation of 23S rRNA puts a higher physiological stress on the bacteria than synthesis of alanine racemase and racemization of L-alanine (i.e., normal, wild-type metabolism).

Recent studies on the use of β-galactosidases from probiotic lactobacilli, in particular *L. reuteri*, for application in the production of prebiotic galacto-oligosaccharides (GOS) have shown promising results [32, 35]. We show here that high amounts of *L. reuteri* β-galactosidases may be produced using the new *alr*-based system (~ 33 U/ml

fermentation). While this production level is slightly higher than the level obtained with a corresponding *erm*-based pSIP vector (Figure 2B), it is still considerably lower than the yields obtained with *E. coli*/pET21 systems (which may amount to as much as 110 U/ml [25]). However, as the *erm*-based pSIP systems, this latter system depends on the use of antibiotics in the fermentation medium [25]. Recently, Maischberger and co-workers showed that expression of the same *L. reuteri lacLM* gene with the food grade *lacF*-based NICE system yielded production levels of approximately 8 U/ml of fermentation [36], which is much lower than the yields obtained with the *alr*-based system in this study. The *lacF*-based expression systems require that lactose must be used as carbon source, whereas *alr*-based systems are carbon source independent, providing more options for fermentation in industrial scale. A potential additional disadvantage of the *erm*-based systems is that the selective agent, erythromycin, can be degraded at acidic pH [37, 38], which is unfavorable in fermentations of lactic acid bacteria without pH control.

Summarizing, we present in this study a new version of the pSIP expression vector series for lactic acid bacteria based on using alanine racemase as a food grade complementation marker. When tested for overexpression of lactobacillal β-galactosidases, these systems showed high production levels. Overcoming certain drawbacks of the original *erm*-based systems, these new *alr*-based host/vector systems have a potential to be used on an industrial scale for the production of recombinant proteins with food applications. Currently, we are working on development of an economical and effective medium for industrial fermentation using these new systems.

Acknowledgements

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Appendix

Article:

Iqbal S, Nguyen TH, **Nguyen TT**, Maischberger T, Haltrich D: beta-Galactosidase from *Lactobacillus plantarum* WCFS1: biochemical characterization and formation of prebiotic galacto-oligosaccharides. *Carbohydr Res* 2010, 345(10):1408-1416.

Abstract:

Tien-Thanh Nguyen, Thu-Ha Nguyen, Dietmar Haltrich. Improvement of purification and immobilization of the recombinant β-galactosidase from *Lactobacillus reuteri*

β-Galactosidase from *Lactobacillus plantarum* WCFS1: biochemical characterization and formation of prebiotic galacto-oligosaccharides

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ABSTRACT

Recombinant β-galactosidase from *Lactobacillus plantarum* WCFS1, homologously over-expressed in *L. plantarum*, was purified to apparent homogeneity using *p*-aminobenzyl 1-thio-β-D-galactopyranoside affinity chromatography and subsequently characterized. The enzyme is a heterodimer of the LacLM-family type, consisting of a small subunit of 35 kDa and a large subunit of 72 kDa. The optimum pH for hydrolysis of its preferred substrates *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG) and lactose is 7.5 and 7.0, and optimum temperature for these reactions is 55 and 60 °C, respectively. The enzyme is most stable in the pH range of 6.5–8.0. The K_m, k_{cat} and k_{cat}/K_m values for *o*NPG and lactose are 0.9 mM, 92 s^{-1}, 130 mM^{-1} s^{-1} and 29 mM, 98 s^{-1}, 3.3 mM^{-1} s^{-1}, respectively. The *L. plantarum* β-galactosidase possesses a high transgalactosylation activity and was used for the synthesis of prebiotic galacto-oligosaccharides (GOS). The resulting GOS mixture was analyzed in detail, and major components were identified by using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) as well as capillary electrophoresis. The maximal GOS yield was 41% (w/w) of total sugars at 85% lactose conversion (600 mM initial lactose concentration). The enzyme showed a strong preference for the formation of β-(1→6) linkages in its transgalactosylation mode, while β-(1→3)-linked products were formed to a lesser extent, comprising ∼80% and 9%, respectively, of the newly formed glycosidic linkages in the oligosaccharide mixture at maximum GOS formation. The main individual products formed were β-D-Gal*p*-(1→6)-D-Lac, accounting for 34% of total GOS, and β-D-Gal*p*-(1→6)-D-Glc, making up 29% of total GOS.

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1. Introduction

The lactose-hydrolyzing enzyme β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) has long been used as an important biocatalyst in the dairy industry as well as in synthetic glycochemistry. β-Galactosidase can catalyze two basic reactions, hydrolysis of the milk sugar lactose and structurally related galactosides and transglycosylation reactions, resulting, for example, in a mixture of galacto-oligosaccharides when lactose is the starting material for the latter reaction. Possible sources of the enzyme are plants, animal organs, bacteria, yeasts, fungi and moulds.[1] Amongst them microbial sources are clearly preferable and are mostly used for both reactions because of their ease of fermentative production, high activities and generally good stability.[2,3]

Lactic acid bacteria (LAB), which constitute a diverse group of lactococci, streptococci and lactobacilli, have been studied intensively with respect to their enzymes for various different reasons including

their generally recognized as safe (GRAS) status. Hence, enzymes derived from these organisms can be used without extensive purification in various food-related applications.[4,5] *Lactobacillus plantarum* is a versatile lactic acid bacterium, which is encountered in a range of environmental niches including dairy, meat and especially vegetable fermentations. Moreover, it is commonly found in the human gastrointestinal tract.[6] Recently, the complete genome sequence of *L. plantarum* WCFS1, a single colony isolate of *L. plantarum* NCIMB 8826 from human saliva, was determined and annotated[7] and this achievement has opened new ways in the comprehensive study of a variety of biological processes of this organism.

Prebiotics are defined as a 'selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health',[8] and typically these prebiotic compounds are oligosaccharides of various composition. Galacto-oligosaccharides (GOS) together with fructo-oligosaccharides are amongst the most important and best-studied groups of prebiotic oligosaccharides. GOS have been classified as one of the few proven prebiotics fulfilling the three criteria (i) resistance to gastric

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acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption; (ii) fermentation by intestinal microflora and (iii) selective stimulation of the growth and/or activity of intestinal bacteria associated with health and well-being.[8] GOS are principally formed from lactose by biocatalytic reactions employing the transgalactosylation activity of various retaining β-galactosidases.[1] In the reaction of lactose hydrolysis, a galactosyl–enzyme intermediate is formed, and instead of transferring this galactose unit to water, the enzyme transfers it to a suitable nucleophile acceptor such as a sugar. In the synthesis of GOS, acceptor sugars can be lactose, especially at the start of the reaction when its concentration is high, but also the various reaction products of the β-galactosidase-catalyzed reaction, either glucose or galactose formed as primary hydrolysis products or the various oligosaccharides formed through transgalactosylation (Scheme 1). Furthermore, these oligomeric products are only transiently formed as kinetic intermediates, and are in turn hydrolyzed by β-galactosidase. As a result, GOS are very complex mixtures consisting of numerous different oligosaccharides varying in their DP and glycosidic linkage. These mixtures depend strongly on the source of the enzyme used for their production as well as on the progress of the reaction. Relatively few studies have dealt with a detailed analysis of the composition of these GOS mixtures.[9,10] GOS typically have a degree of polymerization between 2 and 10 with a terminal glucose moiety. Very little is known about the structure–function relationship of prebiotic oligosaccharides, although there has been recent interest in this area.[11]

GOS are stable at high temperatures in acidic environments and the caloric value of these oligosaccharides is only 1.7 kcal g^{-1}, which makes them of particular interest for the food and drink industry, for both their prebiotic properties and their use as sweeteners, especially in confectionery, acidic beverages and fermented milk products.[12] Since the GOS products currently available on the market were not designed rationally, there is still need to find good microbial sources of β-galactosidases showing high transgalactosylation activities, and to characterize the individual components of the GOS mixtures obtained with these biocatalysts.[13]

In the present study, β-galactosidase from L. plantarum WCFS1 homologously over-expressed in its original source by using a Sakacin P-based expression system[14] was purified to apparent homogeneity, characterized and analyzed with respect to the transgalactosylation of lactose. Major components of the GOS formed by this enzyme were analyzed by high performance anion exchange chromatography with pulsed amperometric detection and capillary electrophoresis.

2. Results and discussion

2.1. Purification of β-galactosidase

The β-galactosidase from L. plantarum WCFS1 was homologously over-expressed using its original source strain as expression host together with a lactobacillal Sakacin P-based expression system,[14] and subsequently purified to apparent homogeneity using a single-step purification procedure based on p-aminobenzyl 1-thio-β-D-galactopyranoside affinity chromatography. The specific activity of the purified enzyme was 154 U mg^{-1} of protein, using standard assay conditions and o-nitrophenyl β-D-galactopyranoside (oNPG) as substrate, which is about threefold higher than that of the crude extract. A total of 265 U$_{oNPG}$ β-galactosidase activity were obtained from the initial activity (1040 U) in the crude cell extract, corresponding to a purification recovery of ∼25%. The homogeneity of the purified β-galactosidase was verified by SDS–PAGE and native PAGE (Fig. 1).

2.2. Determination of molecular mass

The L. plantarum β-galactosidase is a heterodimeric enzyme of a molecular mass of ∼107 kDa, consisting of a larger subunit of ∼72 kDa and a smaller subunit of ∼35 kDa as estimated under denaturing conditions of SDS–PAGE (Fig. 1A) and by native PAGE (Fig. 1B). These results are in agreement with the theoretical molecular masses of 35,223 Da and 72,180 Da for the smaller and larger subunits, respectively, as calculated (http://www.expasy.ch/tools/pi_
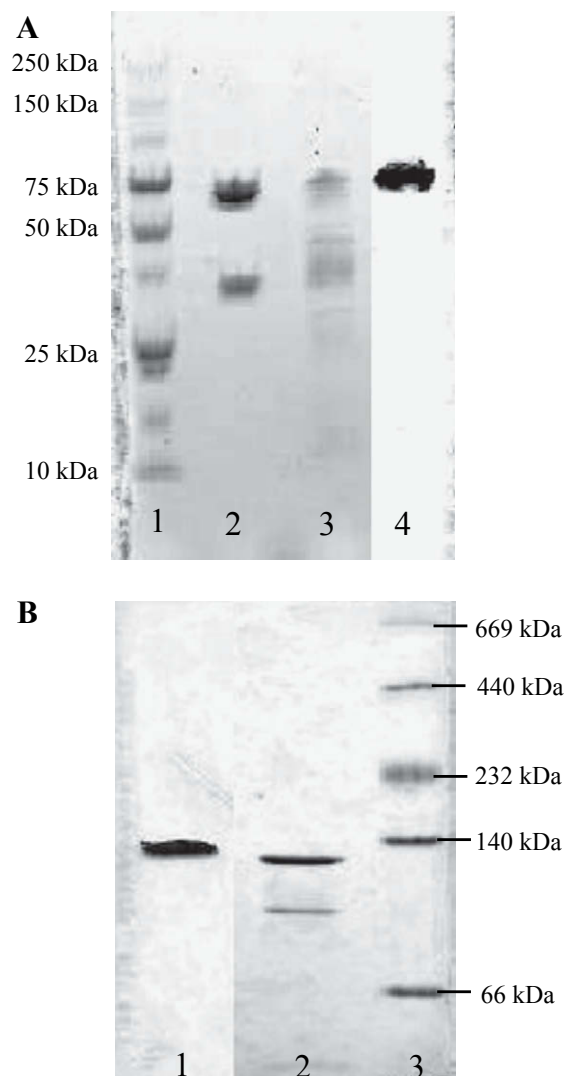
Figure 1. SDS–PAGE (A) and native PAGE (B) analysis of β-galactosidase from L. plantarum WCFS1, applying approx. 1–2 µg of total protein per lane. (A) Lanes 1–3 were Commassie blue stained, showing the Dual colour standard (Amersham) (Lane 1), purified β-galactosidase (Lane 2) and crude cell extract (Lane 3) while Lane 4 shows active staining with 4-methylumbelliferyl β-D-galactoside (MUG) of purified β-galactosidase. (B) Lane 1 shows active staining with MUG of purified β-galactosidase, Lanes 2 and 3 are Commassie blue stained showing purified enzyme and high molecular weight marker (Amersham), respectively.
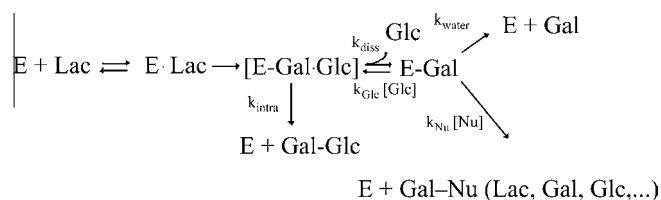
Scheme 1. Hydrolysis and galactosyl transfer reactions, both intra- and intermolecular, during the β-galactosidase-catalyzed conversion of lactose. E, enzyme; Lac, lactose; Gal, galactose; Glc, glucose; Nu, nucleophile.[1]

tool.html) from the amino acid sequences of the encoding *lacL* and *lacM* genes (GenBank accession number AL935262). Very similar molecular masses have been reported for the heterodimeric β-galactosidases of the LacLM type from *Lactobacillus sakei* 23 K,[15] *Lactobacillus acidophilus*,[16] *Lactobacillus reuteri*[17] and *Lactobacillus helveticus*.[18] Active staining directly on the SDS–PAGE gel, after pre-incubation of the enzyme with denaturing Laemmli buffer at 60 °C for 5 min, showed that only one band, corresponding to the larger subunit LacL, exhibited activity with 4-methylumbelliferyl-β-D-galactoside (MUG) while the smaller subunit LacM did not show any activity. Active staining on the native PAGE gel, using MUG as the substrate, showed one single band with activity of approximately 115 kDa, corresponding to the intact enzyme. A second, smaller band of ∼85 kDa that is visible on the native gel after Commassie blue staining is apparently a degradation product of the intact protein or corresponds to the larger subunit LacL.[17] It was furthermore observed that heating the enzyme with Laemmli buffer at 99 °C for 5 min results in the complete denaturation of the heterodimeric enzyme and loss of activity.

The isoelectric point (p*I*) of β-galactosidase from *L. plantarum* WCFS1 was determined by isoelectric focusing and a compact band was observed in the range of 4.7–4.9 (not shown) which is in excellent agreement with the theoretical calculated value for the p*I* of 4.74.

2.3. Kinetic measurements

The kinetic behaviour of β-galactosidase from *L. plantarum* WCFS1 with its natural substrate lactose and the artificial substrate *o*NPG was studied, and results are summarized in Table 1. The K_m values calculated for *o*NPG (0.9 mM) and lactose (29 mM) are in accordance with β-galactosidases from a number of different sources, for which it was shown that Michaelis constants for the artificial substrate *o*NPG are significantly lower than those for lactose.[19] The K_m value of 29 mM determined for lactose is comparable with the reported values for other β-galactosidases from *Lactobacillus* spp., for example, 31 mM for *L. reuteri* L461 and 13 mM for *L. reuteri* L103[17] and 14 mM for *Lactobacillus crispatus*.[20] The K_m value from *L. plantarum* β-galactosidase compares furthermore very favourably with the reported values for some commercial β-galactosidases (36–180 mM for *Aspergillus oryzae*; 54–100 mM for *Aspergillus niger*; 15–52 mM for *Kluyveromyces fragilis* and 35 mM for *Kluyveromyces lactis*)[21,22] which is important when turning over lactose in lower concentrations. The k_{cat} values were calculated on the basis of theoretical v_{max} values obtained by non-linear regression analysis using Sigma Plot (SPSS Inc.). The specificity constants (k_{cat}/K_m) for two substrates again indicate that the hydrolytic activity of β-galactosidase towards *o*NPG is better than that towards lactose. This is mainly the result of a lower Michaelis constant (K_m) for *o*NPG (Table 1).

2.4. Effects of various chemicals and cations on β-galactosidase activity

The effects of various chemicals and cations on the activity of β-galactosidase, each added in final concentrations of 1–100 mM to the standard assay mixture, are shown in Table 2. The addition of the monovalent cations K+ and Na+ showed positive effects on β-galactosidase activity, when added in the selected concentration range. The divalent ions Mg^{2+} and Mn^{2+} showed strong activation of β-galactosidase activity for all concentrations considered. The lower concentrations (1–10 mM) of urea, DTT and 2-mercaptoethanol showed a stimulating effect, while at a higher concentration (100 mM) these compounds inhibit the enzyme activity to a varying extent. EDTA was found to strongly inactivate the enzyme even at the lowest concentration that was tested (1 mM), indicating the necessity of various metal ions for the activity of this enzyme.

2.5. Effect of pH and temperature

The pH dependence of β-galactosidase activity from *L. plantarum* WCFS1 was determined using both lactose and *o*NPG as substrates (Fig. 2A) and the optimum pH values were found to be 7.0 and 7.5, respectively. The activity with lactose shows a rather narrow optimum (pH 6.5–7.5), while a broader optimum (pH 6.0–8.5) was observed when *o*NPG was the substrate. The enzyme was found to be most stable in the neutral pH range of 6.5–8.0, and retained more than 75% of its activity when stored at these pH values at 30 °C for 5 h.

Optimum temperatures for the hydrolysis of *o*NPG and lactose were found to be 55 and 60 °C, respectively (Fig. 2B) under otherwise standard assay conditions. The enzyme needs MgCl$_2$ for its stability, retaining 73% and 85% of its activity after an incubation at 37 °C for 5 h in the presence of 1 and 10 mM MgCl$_2$, respectively, as compared to residual 45% activity without MgCl$_2$. The Arrhenius plot of the temperature dependence of β-galactosidase from *L. plantarum* WCFS1 was found to be linear in the range of 30–55 °C for *o*NPG and 20–55 °C for lactose. The energy of activation (E_a) was calculated as 39.6 and 42.7 kJ mol^{-1} for *o*NPG and lactose, respectively.

2.6. Synthesis of galacto-oligosaccharides

Several batch conversion experiments were carried out at 2 mL scale and 37 °C using an initial lactose concentration of 600 mM prepared in 50 mM sodium phosphate buffer (pH 6.5) containing 1 mM MgCl$_2$ and 1 U$_{Lac}$ of β-galactosidase activity from *L. plantarum* WCFS1 per mL reaction mixture to investigate the formation of galacto-oligosaccharides (GOS) and individual components of this GOS mixture in detail. During lactose conversion, the relative amount of GOS increased constantly up to ∼85% lactose conversion, and reached a maximum of 41% of total sugars at this conversion rate. Subsequently, hydrolysis prevailed over transgalactosylation,

Table 1
Kinetic parameters for β-galactosidase from *L. plantarum* WCFS1 for the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG) and lactose

Kinetic parameters	Substrates	
	Lactose	*o*NPG
K_m (mM)	29 ± 1	0.9 ± 0.4
v_{max} (µmol min^{-1} mg^{-1})	12.9 ± 0.5	153 ± 2
k_{cat} (s^{-1})	98	92
k_{cat}/K_m (mM^{-1} s^{-1})	3.3 ± 0.5	130 ± 50

Table 2
Effect of various chemicals and cations on the activity of purified β-galactosidase from *L. plantarum* WCFS1[a]

| Reagents	Relative activity (%)		
	1 mM	10 mM	100 mM
Blank (none)	100	100	100
K+	115	326	197
Mn^{2+}	294	341	nd
Na+	158	165	145
Mg^{2+}	262	414	399
EDTA	17	22	13
Urea	107	105	48
2-Mercaptoethanol	110	113	91
1,4-Dithiothreitol	157	148	84

nd, not determined.
[a] Enzyme activity was determined under standard assay conditions using *o*NPG as the substrate with the respective chemical/cation added to give the stated final concentration.
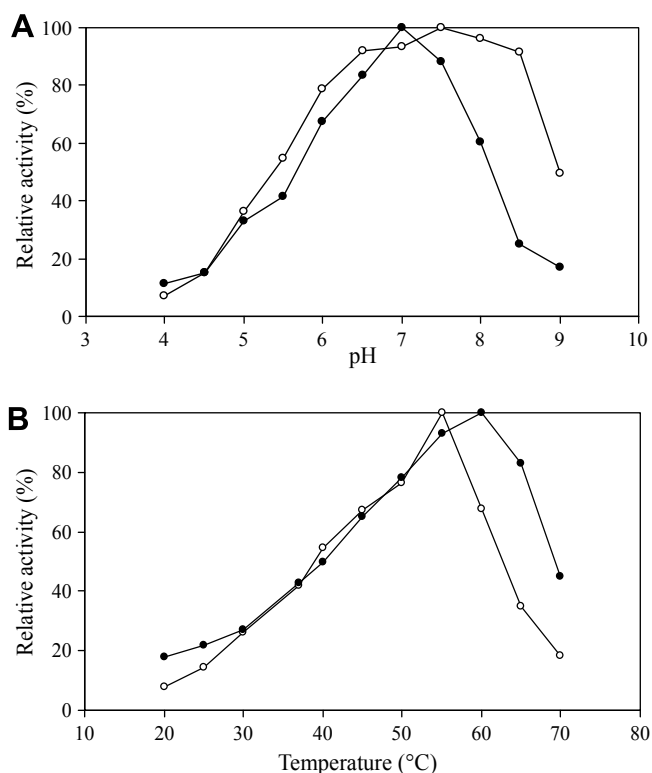
Figure 2. pH (A) and temperature optimum (B) of the activity of β-galactosidase from *L. plantarum* WCFS1. Symbols: (○), *o*NPG as the substrate; (●), lactose as the substrate.

which finally resulted in the complete degradation of these newly formed oligosaccharide products as well as lactose. After 8 h of reaction (corresponding to 85% lactose conversion), GOS reached a maximum level of 84 g L^{-1} of total sugars, and this GOS mixture was composed of 19% non-lactose disaccharides, 21% trisaccharides and 1.3% tetrasaccharides, relative to the total sugars in the reaction mixture (Fig. 3). This yield of GOS (41% w/w of total sugars) produced by β-galactosidase from *L. plantarum* compares favourably with the yields reported for other bacterial β-galactosidases (36% for β-galactosidase from *L. reuteri* L103,[23] 38.5% for β-galactosidase from *L. acidophilus* R22[16] and 36–43% for β-galactosidase from *Bifidobac-*
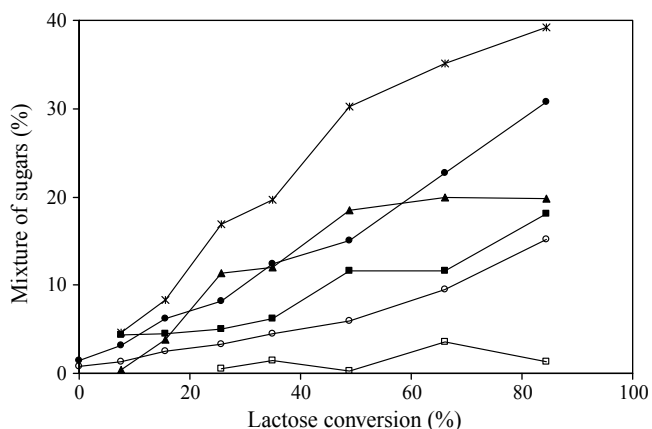
Figure 3. Analysis of total sugars during lactose conversion catalyzed by β-galactosidase from *L. plantarum* WCFS1. The reaction was performed at 37 °C with 205 g L^{-1} lactose as substrate in 50 mM sodium phosphate buffer (pH 6.5) and 1 mM MgCl$_2$. Symbols: (●), glucose; (○), galactose; (■), disaccharides other than lactose; (▲), trisaccharides; (□), tetrasaccharides; (*), total galacto-oligosaccharides.

terium bifidum NCIMB 41171[24]) and very favourably with yields given for fungal β-galactosidases (e.g., β-galactosidase from *A. oryzae* 36%[25] or 22%[26]). In a recent review[27] it has been stated that typical optimized yields for the production of GOS are in the range of 30–40%. Hence, the β-galactosidase from *L. plantarum* shows a very pronounced transgalactosylation potential, especially when considering that the initial lactose concentration of 205 g L^{-1} (corresponding to the solubility of lactose at ambient temperature) used in our experiments is relatively low compared to that in other studies, and that the lactose concentration is considered as one of the main factors affecting maximum GOS concentrations that can be achieved.[27]

In a previous study,[23] we produced GOS using β-galactosidase from *L. reuteri* L103, and major components of this GOS mixture were characterized. In order to analyze the GOS components obtained by transgalactosylation using β-galactosidase from *L. plantarum* in more detail, these GOS were similarly characterized using authentic standards and the standard addition technique and compared with the GOS mixture produced by β-galactosidase from *L. reuteri* using thin layer chromatography (TLC), high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and capillary electrophoresis. The thin layer chromatogram (Fig. 4) as well as the chromatograms from HPAEC-PAD (Fig. 5) and capillary electrophoresis (Fig. 6) indicates that the major GOS components produced by *L. reuteri* L103 and *L. plantarum* WCFS1 are quite similar. Furthermore, a comparison by TLC showed that the GOS mixture obtained with the lactobacillal enzymes is considerably different from a commercially available GOS preparation, Elix'or (Friesland Foods Domo), which is composed mainly of β-(1→4)-linked oligosaccharide products.[9] The *L. plantarum* β-galactosidase shows a strong preference for the formation of β-(1→6) linkages in its transgalactosylation mode, while β-(1→3)-containing compounds are formed to a lesser extent, with approximately 80% and 9% of the newly formed transgalactosylation products containing a β-(1→6) or β-(1→3) linkage, respectively. The main components of this GOS mixture were identified as D-Gal*p*-(1→6)-D-Glc (allolactose), D-Gal*p*-(1→3)-D-Glc, D-Gal*p*-(1→6)-D-Gal, D-Gal*p*-(1→3)-D-Gal, D-Gal*p*-(1→6)-Lac and D-Gal*p*-(1→3)-Lac.

As mentioned above, the GOS mixtures produced by the two lactobacillal enzymes are quite similar. Distinct differences are that the *L. plantarum* β-galactosidase has a stronger preference for the formation of the main trisaccharide compounds D-Gal*p*-(1→6)-Lac and D-Gal*p*-(1→3)-Lac (Fig. 5B, peaks 7 and 9, respectively, and Table 3) as compared to *L. reuteri* β-galactosidase, while on the other hand the yet unidentified component with retention time (RT) of ∼25 min and the individual non-lactose disaccharides are formed in significantly lesser amounts (Table 3). A careful study of the products formed by individual β-galactosidases thus gives the opportunity for more selective production of specific structural motifs in GOS products through choice of an individual biocatalyst as well as reaction engineering.[27]

Figure 7 shows a detailed analysis of the individual main components formed during the transgalactosylation reaction of β-galactosidase from *L. plantarum* using lactose as substrate. After only 10% lactose conversion the two main transgalactosylation products were D-Gal*p*-(1→6)-D-Gal and D-Gal*p*-(1→6)-D-Glc. During this initial phase of the reaction the concentrations of these two sugars D-Gal and D-Glc, which serve as galactosyl acceptors, are relatively low. This indicates that both monosaccharides are excellent acceptor molecules for transgalactosylation, or that at least D-Gal*p*-(1→6)-D-Glc is formed by intramolecular transgalactosylation, that is, the D-Gal moiety is transferred onto D-Glc before it can leave the active site of β-galactosidase and another acceptor molecule or water can enter (Scheme 1). Up to a lactose conversion of approximately 50%, the formation of D-Gal*p*-(1→6)-Lac proceeds
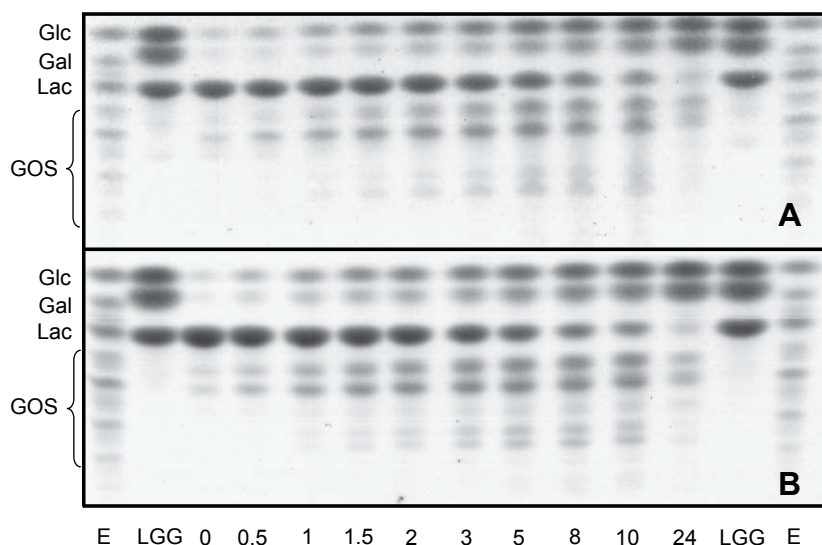
Figure 4. Hydrolysis of lactose catalyzed by β-galactosidase from *L. plantarum* WCFS1 (A) and *L. reuteri* L103 (B) as analyzed by thin layer chromatography (TLC) on pre-activated silica plates (eluent: *n*-butanol–*n*-propanol–ethanol–water = 2:3:3:2). The reaction was carried out at 37 °C with an initial lactose concentration of 205 g L^{-1} in 50 mM sodium phosphate buffer (pH 6.5) containing 1 mM MgCl$_2$ and 1.0 U$_{Lac}$ mL^{-1} of recombinant β-galactosidase. The samples were taken at regular time intervals during the reaction. A commercially available GOS preparation, Elix'or (Friesland Foods Domo), was added for comparison (indicated by E). LGG indicates a standard mixture of lactose, glucose and galactose.

almost linearly, while the formation of D-Gal*p*-(1→3)-Lac is far less pronounced and the potential galactosylation product D-Gal*p*-(1→4)-Lac was not identified at all. This again confirms the strong preference of β-galactosidase from *L. plantarum* to form (1→6) linkages in its transgalactosylation reaction. At maximal GOS production, β-D-Gal*p*-(1→6)-D-Lac is the most abundant oligosaccharide formed comprising 34% of total GOS, followed by β-D-Gal*p*-(1→6)-D-Glc as second most important GOS component formed (29% of total GOS). While current commercial GOS products contain structures with predominant β-(1→4) linkages,[9,10] the lactobacilli enzymes form mainly β-(1→6) linkages in their transgalactosylation products. It has been suggested that the formation of a (1→6) bond is favoured since the C6–OH group is the most sterically accessible alcohol group of the galactosyl acceptor, and that several other β-galactosidases also preferentially produce β-(1→6) linkages.[27] In a study on the structure–function relationship of various disaccharides with respect to their prebiotic effect it was shown that amongst a group of galactose-containing disaccharides, those containing a (1→6) linkage supported growth of bifidobacteria best in mixed culture populations. Because of this strong bifidogenic effect of these compounds, a GOS mixture produced with β-galactosidase from *L. plantarum* that is rich in these β-(1→6)-linked oligosaccharides should therefore be of considerable interest as a prebiotic mixture.[28]

## 3. Experimental

3.1. Chemicals and enzymes

All chemicals used in this study were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated, and were of the highest quality available. Phenylmethanesulfonyl fluoride (PMSF) 99% and glucose oxidase (GOD) from *A. niger* (lyophilized, 205 U mg^{-1} enzyme preparation) were from Fluka (Buchs, Switzerland), while 1,4-dithiothreitol (DTT) was from Roth (Karlsruhe, Germany). The test kit for the determination of D-glucose and D-galactose was purchased from Megazyme (Bray, Ireland). All chromatographic materials were from Amersham Biosciences (Uppsala, Sweden).

3.2. Bacterial strains and culture conditions

L. plantarum WCFS1 was originally from the National Collection of Industrial and Marine Bacteria (Aberdeen, UK), and the construction of recombinant *L. plantarum* WCFS1 carrying the expression plasmid pEH3P for the homologous overexpression of the genes *lacLM* encoding β-galactosidase from *L. plantarum* WCFS1 was recently described.[14] The strain was grown anaerobically overnight at 37 °C in MRS broth containing 10 g L^{-1} peptone, 10 g L^{-1} meat extract, 5 g L^{-1} yeast extract, 2 g L^{-1} di-potassium hydrogen phosphate, 2 g L^{-1} di-ammonium hydrogen citrate, 5 g L^{-1} sodium acetate, 0.2 g L^{-1} magnesium sulfate, 1 g L^{-1} Tween 80 and 0.025 g L^{-1} manganese sulfate. Glucose 2% (w/v) served as the C-source (*Lactobacillus* broth[29]). Whenever necessary, the MRS medium contained 5 µg mL^{-1} erythromycin to stably maintain the plasmid in recombinant *L. plantarum*. For the homologous over-expression of the β-galactosidase genes *lacLM* from *L. plantarum* WCFS1, an overnight culture of *L. plantarum* WCFS1 harbouring plasmid pEH3P was diluted in 50 mL of fresh MRS medium containing erythromycin to an OD$_{600}$ of <0.1 and incubated at 37 °C to an OD$_{600}$ of 0.3. The cultures were then induced with 25 ng mL^{-1} of the peptide pheromone IP-673 (supplied by the Molecular Biology Unit, University of Newcastle-upon-Tyne, UK).

3.3. Purification of β-galactosidase from *Lactobacillus plantarum* WCFS1

Cells were harvested by centrifugation (8000*g*, 20 min at 4 °C) when the stationary phase of microbial growth was reached (OD at 600 nm of approx. 1.8). The cells were washed twice with 50 mM sodium phosphate buffer (pH 6.5) and resuspended in the same buffer, containing 1 mM of both DTT and PMSF. Cell disruption was carried out with a French Press (Aminco, Maryland) and cell debris was removed by centrifugation (25,000*g*, 25 min, 4 °C) to obtain the crude cell extract. A single-step purification of β-galactosidase was performed using affinity chromatography on a 10-mL column of *p*-aminobenzyl 1-thio-β-D-galactopyranoside immobilized on cross-linked 4% beaded agarose (Sigma). The column was pre-equilibrated with buffer A (50 mM sodium
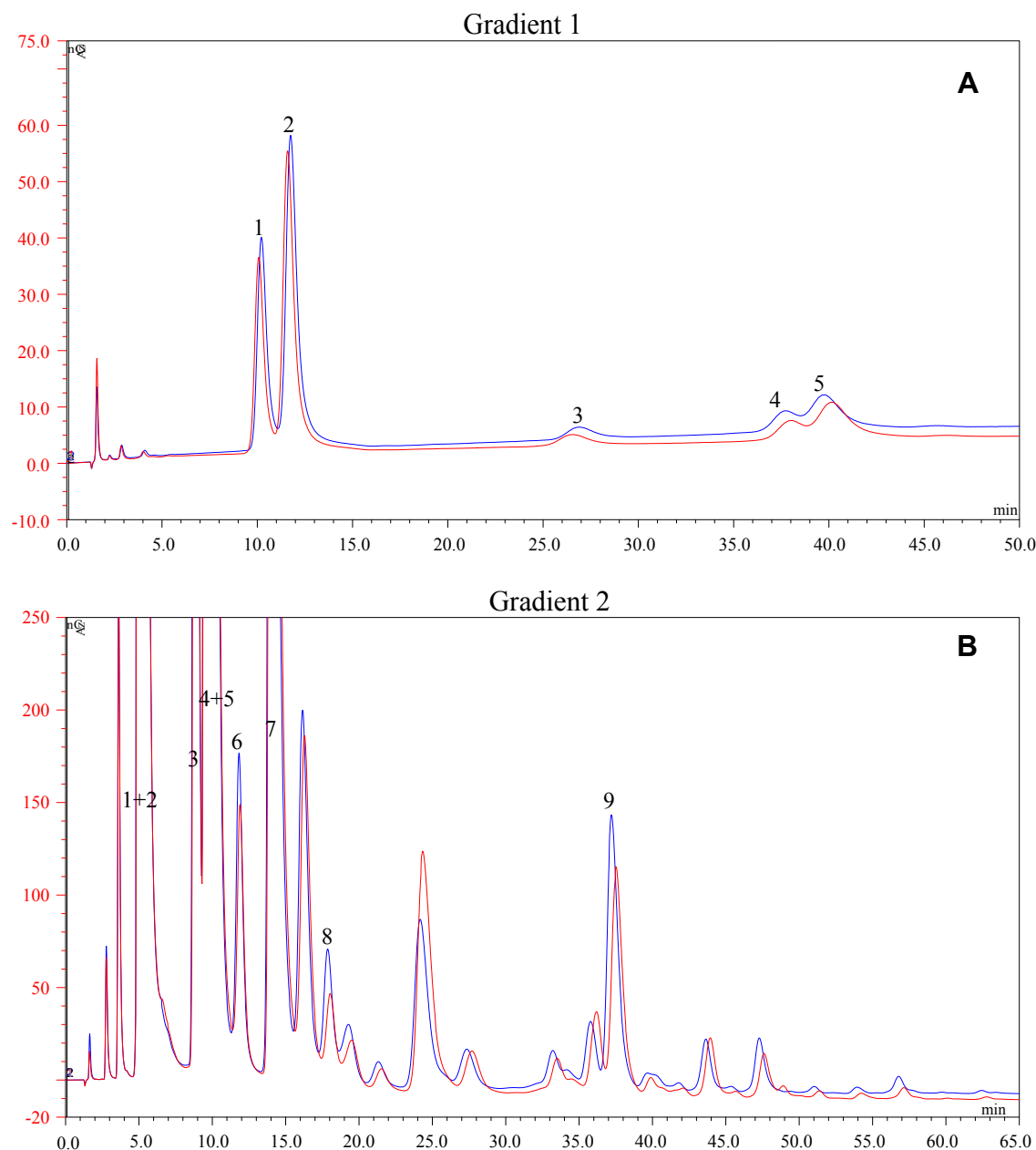
Figure 5. Separation and quantification by HPAEC-PAD of individual galacto-oligosaccharides (GOS) produced by transgalactosylation with lactose as substrate catalyzed by β-galactosidase from *L. plantarum* (blue/black) and compared with well-characterized GOS[10] produced by *L. reuteri* L103 β-galactosidase (red/grey). Two different gradients were used in HPAEC-PAD as give in Section 3. Initial lactose concentration was 205 g L^{-1} in 50 mM sodium phosphate buffer (pH 6.5). The extent of substrate conversion was approximately 85%. The identified compounds are indicated: (1), galactose; (2), glucose; (3), D-Gal*p*-(1→6)-D-Gal; (4), D-Gal*p*-(1→6)-D-Glc (allolactose); (5), lactose; (6), D-Gal*p*-(1→3)-D-Gal; (7), D-Gal*p*-(1→6)-Lac; (8), D-Gal*p*-(1→3)-D-Glc; and (9), D-Gal*p*-(1→3)-Lac. Peaks marked with an × are not-yet-identified GOS. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

phosphate buffer, pH 6.5) and then the enzyme was eluted with buffer B (50 mM sodium phosphate and 2 M NaCl, pH 6.5) using a linear gradient from 0% to 100% buffer B in 15 column volumes. The flow rate was adjusted to 0.5 mL min^{-1}. The purified enzyme was desalted, concentrated (Amicon Ultra Centrifugal filter tubes; 10 kDa cut-off; Millipore, Billeria, MA, USA) and stored at 4 °C in 50 mM sodium phosphate buffer, pH 6.5.

3.4. Enzyme assays

β-Galactosidase activity was determined using both *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG) and lactose.[17] When chromogenic *o*NPG was used as the substrate, 20 μL of the enzyme solution was added to 480 μL of 22 mM *o*NPG prepared in 50 mM sodium phosphate buffer (pH 6.5) to start the reaction, which was stopped after 10 min of incubation at 30 °C and shaking at 600 rpm by adding 750 μL of 0.4 M Na$_2$CO$_3$. The release of *o*-nitrophenol (*o*NP) was measured by determining the absorbance at 420 nm. One unit of *o*NPG activity (U$_{oNPG}$) was defined as the amount of enzyme liberating 1 μmol *o*-nitrophenol (*o*NP) per minute under the described conditions.

When lactose was used as the substrate, 20 μL of enzyme solution was added to 480 μL of 600 mM lactose solution in 50 mM sodium phosphate buffer, pH 6.5. After 10 min of incubation at 30 °C and shaking at 600 rpm, the reaction was stopped by heating the reaction mixture at 99 °C for 5 min. The reaction mixture was cooled
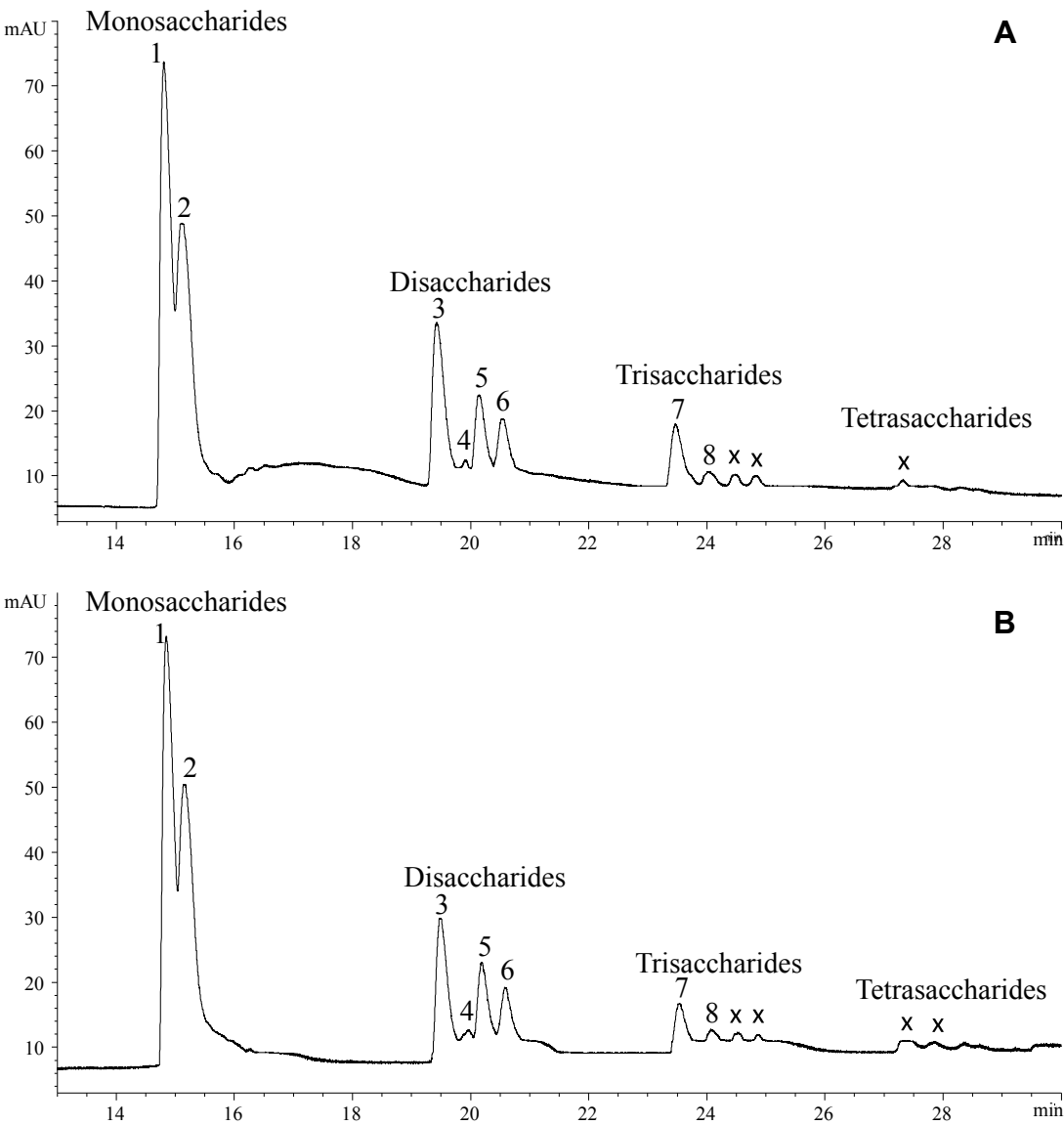
Figure 6. Separation and quantification by capillary electrophoresis of individual galacto-oligosaccharides produced during the lactose conversion catalyzed by β-galactosidase from *L. plantarum* WCFS1 (A) and compared with GOS produced by the enzyme from *L. reuteri* L103 (B). The samples are mixtures of sugars obtained after the reaction of β-galactosidase with 205 g L^{-1} initial concentration of lactose. The extent of substrate conversion was approximately 85%. The identified compounds are indicated as follows: (1), glucose; (2), galactose; (3), lactose; (4), D-Gal*p*-(1→3)-D-Glc; (5), D-Gal*p*-(1→6)-D-Glc (allolactose) with D-Gal*p*-(1→3)-D-Gal; (6), D-Gal*p*-(1→6)-D-Gal; (7), D-Gal*p*-(1→6)-Lac; and (8), D-Gal*p*-(1→3)-Lac. Products marked with an × are minor components and were not identified. Peaks appearing at a retention time ∼27 min are tetrasaccharides.

Table 3
Concentrations of individual GOS components produced by the transgalactosylation reaction with lactose as substrate and catalyzed by β-galactosidases from *L. plantarum* WCFS1 (A) and *L. reuteri* L103 (B)

GOS components	Concentration of individual GOS components (g L^{-1}) at different lactose conversion levels													
	8%		15%		35%		50%		66%		85%		95%	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B
D-Gal*p*-(1→6)-D-Gal	6.10	2.90	5.80	4.52	5.76	5.32	10.6	11.4	8.82	12.9	13.5	18.8	4.24	3.37
D-Gal*p*-(1→6)-D-Glc (allolactose)	2.90	2.10	1.60	3.78	5.26	8.04	11.32	14.5	14.3	22.1	24.1	27.4	nd	3.94
D-Gal*p*-(1→3)-D-Gal	nd	nd	0.35	0.33	0.50	0.69	0.83	1.04	1.37	1.59	1.98	2.18	1.78	1.29
D-Gal*p*-(1→6)-Lac	0.81	3.97	6.54	8.18	21.5	17.5	29.3	19.6	29.7	24.9	28.3	22.3	5.26	5.02
D-Gal*p*-(1→3)-D-Glc	nd	1.10	1.90	1.78	2.12	2.42	1.88	4.70	0.54	1.42	1.02	3.02	0.88	0.60
D-Gal*p*-(1→3)-Lac	1.17	nd	4.62	1.54	9.79	2.97	11.2	7.33	9.18	4.85	6.15	4.59	0.49	0.08
Unidentified compound[a]	nd	0.55	0.36	0.76	0.95	1.93	1.52	3.00	2.57	4.41	3.06	4.51	0.24	0.38

nd, not determined due to low concentration.

[a] Unidentified compound, corresponding to the peak with retention time ∼25 min in Figure 5.
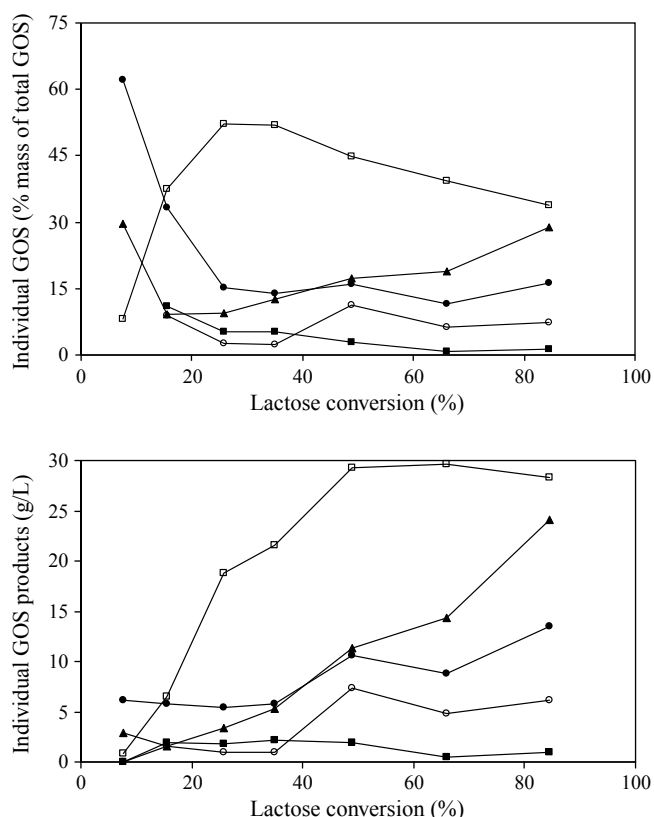
Figure 7. Formation of individual galacto-oligosaccharides during lactose conversion by *L. plantarum* WCFS1 β-galactosidase. The reaction was performed at 37 °C at an initial concentration of 205 g L^{-1} lactose in 50 mM sodium phosphate buffer (pH 6.5) + 1 mM MgCl$_2$. Symbols: (■), D-Gal*p*-(1→3)-D-Glc; (▲), D-Gal*p*-(1→6)-D-Glc; (●), D-Gal*p*-(1→6)-D-Gal; (□), D-Gal*p*-(1→6)-Lac; (○), D-Gal*p*-(1→3)-Lac.

to room temperature, and the release of D-glucose was determined colourimetrically using the glucose oxidase–peroxidase (GOD–POD) assay.[30] One unit of lactase activity (U$_{Lac}$) was defined as the amount of enzyme releasing 1 µmol of D-glucose per minute under the given conditions. Protein concentrations were determined by the dye-binding method according to Bradford using the Biorad reagent and bovine serum albumin (BSA) as a standard.

3.5. Gel electrophoresis analysis and active staining

Native polyacrylamide gel electrophoresis (PAGE) and denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) were performed on a PhastSystem unit (Amersham, Uppsala, Sweden) using precast polyacrylamide gels (PhastGel 8–25, Amersham). For SDS–PAGE, the enzyme was pre-incubated with Laemmli buffer for 5 min at 90 °C for Commassie blue staining and 60 °C for active staining. Commassie brilliant blue R-250 was used for the visualization of protein bands, and active staining was performed by using 4-methylumbelliferyl-β-D-galactoside (MUG)[17] as substrate, which was added directly onto the gel. Samples that were applied to the individual lanes contained approximately 1–2 µg of the total protein.

Isoelectric focusing was performed in the range of pH 3–10 using the Multiphor System (Amersham) and precast, dry polyacrylamide gels (CleanGel IEF, Amersham) rehydrated with carrier ampholytes as recommended by the supplier with some modifications. The broad p*I* marker protein kit, pH 3–10, (Amersham) was used to compare and determine the p*I* value. Protein bands were visualized with Coomassie blue staining following the instructions of the manufacturer.

3.6. Steady-state kinetic analysis

All steady-state kinetic measurements were performed at 30 °C using *o*NPG and lactose as the substrates in 50 mM sodium phosphate buffer, pH 6.5, with concentrations ranging from 0.1 to 22 mM for *o*NPG and from 0.1 to 600 mM for lactose. The kinetic parameters were calculated by non-linear regression and the observed data were fit to the Henri–Michaelis–Menten equation using SigmaPlot (SPSS Inc., Chicago, IL, USA).

3.7. Temperature and pH profile of β-galactosidase activity

The temperature optimum of the hydrolytic activity of β-galactosidase from *L. plantarum* was determined by assaying the enzyme samples over the range of 20–70 °C for 10 min using *o*NPG and lactose as substrates. Thermal stability was determined by incubating aliquots of the enzyme in 50 mM sodium phosphate buffer (pH 6.5) at the desired temperatures. Aliquots were removed at various times and assayed under standard *o*NPG assay conditions to determine the residual activity. The effect on stability of different concentrations of MgCl$_2$ was also studied at different temperatures. Arrhenius plots were prepared for the determination of energy of activation (*E*$_a$) for both substrates.

For the determination of the optimum pH of the enzyme activity, *o*NPG and lactose assays were performed for a pH range of 4–9 using Britton–Robinson buffer (containing 20 mM each of phosphoric, acetic and boric acids adjusted to the required pH with NaOH). To determine the pH stability of *L. plantarum* β-galactosidase, enzyme samples were incubated at pH values ranging from 4.0 to 9.0 at 30 °C for up to 24 h, and the remaining enzyme activity was measured at different time intervals using *o*NPG standard assay conditions.

3.8. Effect of various cations and reagents

To study the effect of various cations and reagents on β-galactosidase activity, the enzyme samples were assayed at 30 °C for 10 min with 22 mM *o*NPG (in 50 mM sodium phosphate buffer, pH 6.5) as the substrate in the presence of various cations and reagents added in final concentrations of 1, 10 and 100 mM. The measured activities were compared with the activity blank of the enzyme solution determined under identical conditions but without added cations/reagents.

3.9. Enzymatic synthesis of galacto-oligosaccharides

A 600-mM lactose solution was prepared in 50 mM sodium phosphate buffer, pH 6.5, containing 1 mM MgCl$_2$. Transgalactosylation reactions were performed on a 2-mL scale at 37 °C using 300 rpm agitation and 1 U$_{Lac}$ mL^{-1} final concentration of a homogenous preparation of β-galactosidase from *L. plantarum*. Samples were withdrawn at specific time intervals and immediately transferred to 99 °C for 5 min to inactivate the enzyme. The samples were stored at −18 °C for subsequent analysis. High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), capillary electrophoresis (CE) and thin layer chromatography (TLC) were used to determine the composition of GOS mixtures, following previously described methods.[10] Two different combinations of four eluents were used in HPAEC–PAD for effective separation of GOS. Eluent A (100 mM NaOH), eluent B (100 mM NaOH and 1 M NaOAc), eluent C (water) and eluent D (100 mM NaOH and 50 mM NaOAc) were mixed to form two different gradients. Gradient 1, 15% A and 85% C from 0 to 50 min and gradient 2, 100% A from 0 to 20 min and from 0 to 100% D from 20 to 70 min. For eluent preparation, MilliQ water, 50% (w/v) NaOH and NaOAc (BDH, Prolabo, Belgium) were used. After each run, the

column was washed with 100% B for 10 min and re-equilibrated for 15 min with the starting conditions of the employed gradient. Individual GOS compounds were identified and quantified by using authentic standards and the standard addition technique.[10]

Acknowledgements

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Abstract

Improvement of purification and immobilization of the recombinant β-galactosidase from *Lactobacillus reuteri*

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Inteins are internal protein sequences which can be induced to excise themselves from host proteins and concomitantly ligate the flanking sequences with a peptide bond. Inteins have been applied in commercial kits with chitin binding domain for affinity purification of fusion protein by single chromatographic step on a chitin resin without using protease. In order to facilitate the purification and the immobilization of the hetero-dimeric enzyme β-galactosidase from *Lactobacillus reuteri*, the encoding genes *lacLM* were fused with the intein sequence from *Mycobacterium xenopi*, the chitin binding domain sequence (ChBD) from *Bacillus circulans* as well as six-histidine tag. Using the inducible lactobacillal Sakacin P- based expression systems pSip (SØrvig et al. *Microbiology 2005, 151,* 2439-2449) as backbone, four plasmids including *lacLM* genes combined with four constructs such as intein + ChBD, intein + polyhistidine tag, ChBD + polyhistidine tag and ChBD + intein + polyhistidine tag were developed and expressed in well-known food-grade host *Lactobacillus plantarum*. The obtained recombinant enzymes corresponding to four constructs were shown able to be purified via single step or immobilized by using chitin beads.

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