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Production of Recombinant β-Galactosidase in *Lactobacillus plantarum*, using a pSIP-Based Food-Grade Expression System

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Abstract. Food-grade expression systems based on using food-grade microorganisms have been developed for the production of recombinant enzymes used in food applications. Lactic acid bacteria (LAB), especially Lactobacilli, have been widely used for various purposes in food and recognized as a promising host of food-grade enzyme production. In this study, the pSIP409 vectors, originally containing the *erm* gene, were used to replace this selection marker by the *alr* gene resulting in the production of the pSIP609 expression vector in *L. planatarum*. This vector could express high amounts of β-galactosidases, showing both high volumetric as well a specific enzymatic activity. Thus, the food-grade recombinant enzyme production in *L. planatarum* harboring pSIP609 was very fruitful and useful for food industries.

Introduction

Lactic acid bacteria (LAB) play important roles in foods, amongst others because they have been recognized as a food-grade additive. Several LAB, and especially Lactobacilli, have been developed as cell factories relating to an inducible gene expression for food applications in which they are used for the production of a range of interesting proteins. Such enzyme production can be obtained using the Nisin-Controlled Expression (NICE) or the pheromone-inducible (pSIP) systems, which are the well-known overexpression systems for LAB [1-5]. Furthermore, the vectors in the pSIP system permit all parts of the plasmid, to be easily exchanged and to be used amongst different LAB, especially *Lactobacillus* spp., while the plasmids in the NICE system are suitable mainly for *Lactococcus* spp. [1, 6]. Moreover, it was shown that the pSIP system resulted in higher levels of overexpressed enzyme than the NICE system. Thus, recently, the use of the pSIP system has been increasingly attractive in food applications. Yet, the pSIP system still has a major limitation when it is applied in foods because the erythromycin antibiotic resistance gene (*erm*) is used as a selection marker in the original system. Consequently, the erythromycin used for the cell selection stage of enzyme production might affect the microflora in human body, and the *erm* gene might be transferred to other organisms resulting in resistance to this antibiotic. To avoid the addition of undesirable antibiotics to the enzyme expression process, the alanine racemase gene (*alr*) can be used as a selection marker instead of the *erm* gene, which is safer in terms of human food consumption. Using pSIP vectors carrying the *alr* gene as selection marker have been successfully applied in complementation approaches both in Lactococci and Lactobacilli [7]. The alanine racemase enzyme is important for cell wall biosynthesis, especially in LAB. It converts L-alanine to D-alanine, which is an essential component for the growth and cell wall biosynthesis of prokaryotic cells [8]. Thus, using the *alr* gene as a selection marker together with the lacZ gene,

coding for β-galactosidase, on an expression plasmid, might be useful and safer than using *erm* and might be a practical and new method to produce recombinant β-galactosidase in a completely food-grade manner.

β-Galactosidase (lactase, EC 3.2.1.23) is an important enzyme in the dairy industry [9]. This enzyme hydrolyzes lactose into glucose and galactose and can be used to prevent lactose crystallization in dairy products such as ice cream and condensed milk, to increase the solubility of milk products, and to produce lactose-free food products [10]. Moreover, β-galactosidase can produce galacto-oligosaccharides (GOS), which co-occurs during lactose hydrolysis [11]. Thus, even though use of the pSIP vectors with the *erm* gene in Lactobacilli as a host can express high yields of β-galactosidase [9, 12], there still is a problem of antibiotic resistance in such systems. Up to date, only the work of Nguyen and coworkers from 2011 [12] dealt with this problem having expressed an expression vector carrying the *alr* gene with as suitable *alr*-deletion strain to overexpress a β-galactosidase of the LacLM type [8]. This lacLM protein is heterodimeric and is encoded by two partially overlapping genes, which might be more complicated to express and study as a model system. Thus, using the *lacZ* gene coding for the homodimeric lacZ β-galactosidase together with the *alr* gene in a suitable pSIP vector, known as a recombinant system, might be more beneficial in terms of being more economic, less time consuming, and easier for using in food products than the heterodimeric system.

The objective of this study was to prove that such a system could provide the possibility of overexpression for recombinant β-galactosidase in *L. plantarum*.

2. Materials and Methods

2.1 Bacterial Strains and Media.

The wild-type strain *L. plantarum* WCFS1 and TLG02 (Δ*alr*, D-alanine auxotroph) [7] were chosen and grown in MRS medium (Oxoid, Basingstoke, U.K.) at 37 °C without agitation. *E. coli* MB2159, harboring pSIP409(*erm*)-lacZ, *erm*–lacZ-histag, or pSIP409_*alr,*was used as cloning host and was cultivated in Luria-Bertani (LB) medium at 37°C with shaking at 200 rpm. The antibiotic concentrations used for selection were 5 μg/mL or 200 μg/mL of erythromycin for *L. plantarum* and *E. coli*, respectively.

2.2 Construction of β-Galactosidase Expression Vectors.

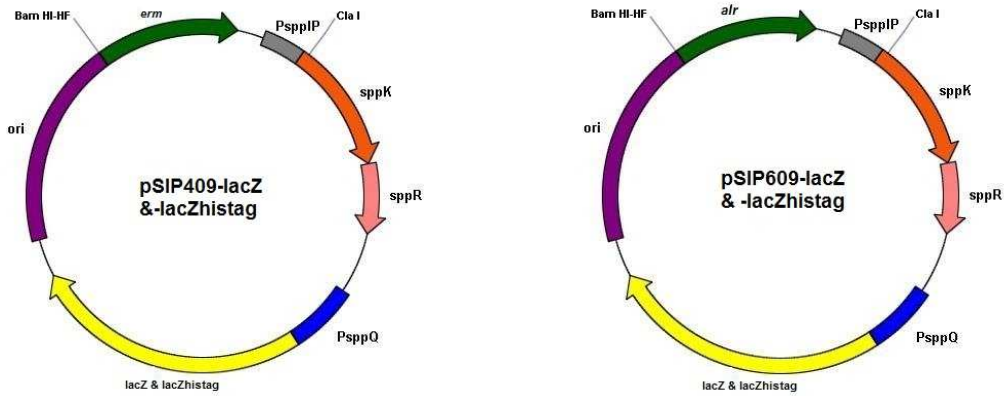
The food-grade expression vector was constructed based on the pSIP vectors (Fig. 1) by replacing the erythromycin resistance gene (*erm*) with the alanine racemase gene (*alr*) from the *L. plantarum* genome as described by Nguyen and coworkers (2011) [12]. This could change the plasmid from pSIP409(*erm*) to pSIP609(*alr*).

2.3 Expression and Confirmation of β-Galactosidases with *alr*-Based Vectors.

Overnight cultures of *L. plantarum* harboring pSIP609(*alr*) were diluted in fresh pre-warmed MRS medium (for *erm*-based systems, 5 μg/mL of erythromycin was added) to get a cell concentration $OD_{600nm}$of ~0.1 and then the mix was incubated at 30 °C until OD_{600nm} of ~0.3 was reached. Then, 25 ηg/mL peptide pheromone (IP-673) was added to the mix and the cultivation was continued until a final cell culture concentration of OD_{600nm}1.8 to 2.0 was reached. Ten mL of cell culture solutions were pelleted by centrifugation at 3500×g for 10 min at 4 °C. The pelleted cells were washed with buffer P (50 mM sodium phosphate buffer, pH 6.5, 20% of glycerol and 1 mM DTT) and resuspended in 500 μL of the same buffer. The pelleted cells were disrupted to get the lysate of cells as described by Nguyen and coworkers (2011) [12]. This suspension was centrifuged at 9000×g (4 °C) for 5 min to get the cell-free extracts used for activity assays and protein concentration determination using SDS-PAGE.

2.4 Enzyme Assay and Protein Determination.

β-Galactosidase activity was determined using o-nitrophenyl-β-D-galactopyranoside (o-NPG) following Nguyen and coworkers (2011) [12]. Protein concentration was determined by using the method of Bradford using bovine serum albumin (BSA) as a standard.



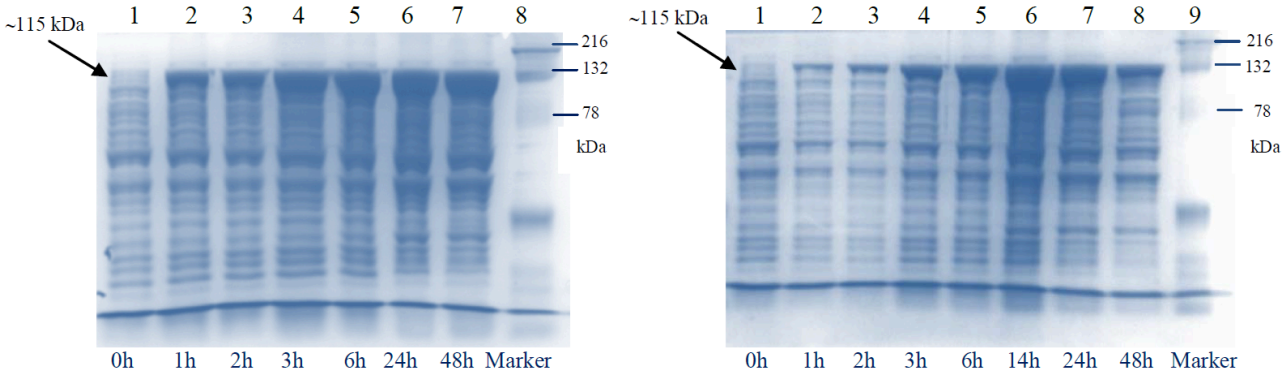
(A) (B)

Figure 1. Expression vectors for lacZ and lacZ-histag based on (A) the erythromycin resistance gene (*erm*) and (B) the alanine racemase (*alr*) gene as the selection markers. SppK and sppR, denoting a histidine kinase and a response regulator, respectively, are regulated by PsppIP, a promoter which drives their expression. In addition, the structural genes were controlled by the inducible promoter PsppQ, which is switch on by the peptide pheromone IP-673.

3. Results and Discussion

3.1 Expression of pSIP409-lacZ and pSIP409-lacZ-histag in *L. plantarum*.

L. plantarum WCFS1 was used as a host for the expression of pSIP409-lacZ and –lacZ-histag. The results of protein determination are shown in Fig. 2A and B for pSIP409-lacZ and –lacZ-histag, respectively. β-Galactosidase from *L. bulgaricus* is a homodimer consisting of two identical subunits which have a molecular weight ~115 kDa [9]. The unique bands of proteins in *L. plantarum* cells induced by IP-673 from this study are visible in SDS-PAGE as indicated by the arrow (Fig. 2).



(A) (B)

Figure 2. SDS-PAGE analysis of cell-free extracts from induced cells of *L. plantarum* WCFS1 harboring pSIP409 at different times: (A) lacZ (Lane 1, noninduced by IP-673 counted as 0 h; Lane 2, induced for 1 h; Lane 3, induced for 2 h; Lane 4, induced for 3 h; Lane 5, induced for 6 h; Lane 6, induced for 24 h; Lane 7, induced for 48 h; and Lane 8, Precision plus Protein standard ladder (Bio-Rad)); and (B) lacZ-histag (Lane 1, noninduced by IP-673 counted as 0 h; Lane 2, induced for 1 h; Lane 3, induced for 2 h; Lane 4, induced for 3 h; Lane 5, induced for 6 h; Lane 6, induced for 14 h; Lane 7, induced for 24 h; Lane 8, induced for 48 h; Lane 9, Precision plus Protein standard ladder (Bio-Rad)).

3.2 Confirmation of Plasmid pSIP609 and the *alr* Gene

The pSIP409-lacZ and –lacZ-histag were used for the construction of the food-grade systems by replacing the *erm* gene with the *alr* gene from *L. plantarum*. The lacZ and lacZ-histag genes were cloned into this *alr*-based plasmid, resulting in plasmid pSIP609. The size of the *alr* gene was about 1.8-2.0 kb [8]. The integrity of this plasmid (pSIP609-lacZ) was confirmed by digesting plasmids with *Bam*H1-HF and *Cla*1 restriction enzymes. In addition, pSIP609-lacZ-histagalso had the same size as pSIP609-lacZ (data not shown). The bands with the size of the *alr* gene are indicated by an arrow (Fig. 3). The results were comparable to those of Nguyen and coworkers [7].

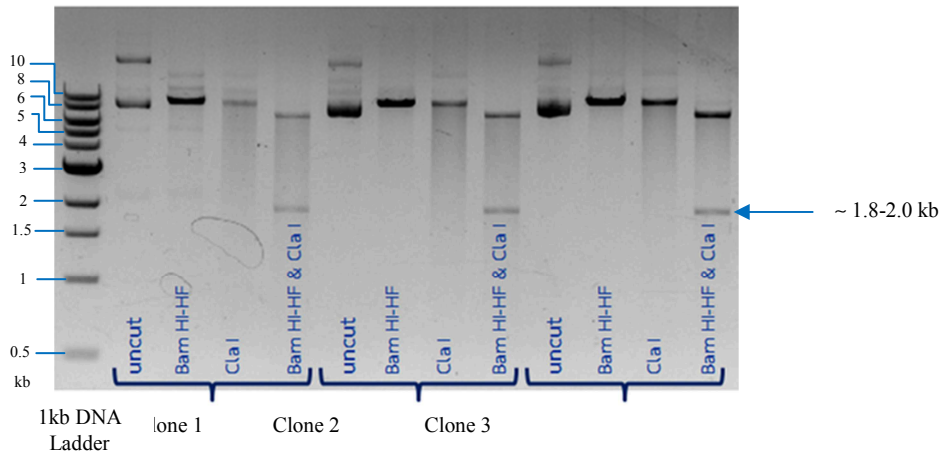


Figure 3. DNA restriction analysis: *alr* gene with *Bam*H1-HF and *Cla*1 of pSIP609-lacZ.

3.3 Expression of *Lactobacillus bulgaricus* β-Galactosidase in *alr*-Based Vectors

The constructed plasmids (pSIP609-lacZ and–lacZ-histag) were transformed into D-alanine auxotroph *L. plantarum* TLG02 using electro-competent cells. Then the organisms were cultivated for overproduction of β-galactosidases with *alr*-based vectors. The results showed that cell-free extracts obtained at different cell concentrations provided high production levels of β-galactosidases in *alr*-based vectors (Fig 4). These high expression levels are also confirmed by measuring the volumetric activity of β-galactosidases. The volumetric activity values of pSIP609-lacZ and –lacZ-histag were approximately 307 and 81 U/mL of cultivation medium, respectively, while the volumetric activity values when using the plasmids pTH103 and pTH104, which are both based on pSIP409, for the overexpression of lacZ in *L. plantarum* were approximately 193±10 and 168±4 U/mL, respectively [9]. The volumetric activity value of approx. 300 U/mL correspond to roughly 1 mg of recombinant protein produced per mL of medium as calculated from the specific activity of the purified enzyme [9]. The specific activity of pSIP609-lacZ was higher than that of pTH103. This might be due to the substitution of the *erm* gene in pTH103 by the *alr* gene in pSIP609-lacZ, which might give a better selection pressure on plasmid-containing cells since erythromycin is known to be slowly degraded at low pH values as typically found in Lactobacillus cultivations [13]. From this study, the specific activity of pSIP609–lacZ-histag was lower than that of pTH104. This can be attributed to the C-terminal His-tag, which can interfere and reduce the activity of lacZ by reducing its active sites as has been shown previously [9, 14].

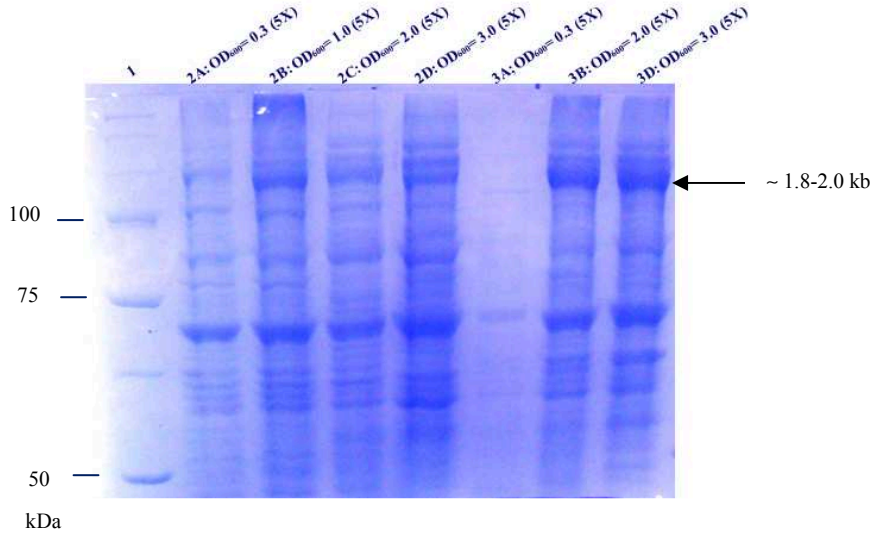


Figure 4. SDS-PAGE analysis of cell-free extracts at different cell concentrations (OD_{600nm}~0.3, 1.0, 2.0, and 3.0) of *L. plantarum* TLG02 harboring pSIP609-lacZ (Lanes 2A, 2B, 2C, 2D), and pSIP609-lacZ-histag (Lanes 3A, 3B, 3C, 3D), Lane 1 shows the Precision plus Protein standard (Bio-Rad). The arrow indicates the band representing the expressed protein β-galactosidase. The gel was stained with Coomassie blue.

Conclusion

The expression plasmids pSIP609-lacZ and –lacZ-histag were constructed by replacing the *erm* gene with *alr* gene from *L. plantarum,* which as a gene derived from a GRAS organism and is an essential step towards food-grade production of proteins in *L. plantarum*. These constructed plasmids resulted in efficient overproduction of β-galactosidases when using the *alr*-based vectors under appropriate inducing conditions. These crude enzymes from pSIP609-lacZ and –lacZ-histag showed a high potential, which might be applied to food industries for reduced-lactose dairy products further.

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