Food-grade *Lactobacilli* expression systems for recombinant enzymes

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Summary

Lactobacilli are Gram-positive bacteria used throughout the food industry as traditional starters for various fermented foods. *Lactobacilli* would be superior for recombinant enzyme production regarding the food safety demands since most of them are Generally Recognised As Safe (GRAS) organisms. The major advantages of *Lactobacilli* as food-associated microorganisms used for recombinant enzyme production are their safe and sustainable use as overall safety food-grade expression systems. In the work presented, *Lactobacilli* were studied in detail as food-grade expression systems for recombinant enzyme production. In a first analysis, the two pSIP expression systems, pSIP403 and pSIP409, were investigated to produce a hyper-thermophilic *β*-glycosidase (CelB) from *Pyrococcus furiosus* in *Lactobacillus plantarum* NC8 and *Lactobacillus casei* as hosts, respectively. Both *Lactobacilli* harbouring the pSIP409-celB vector produced active CelB in batch bioreactor cultivations, while the specific CelB activity of the cell-free extract was about 44% higher with *Lb. plantarum* (1,590 ± 90 $nkat_{pNPGal}/mg_{protein}$) than with *Lb. casei* (1,070 ± 66 $nkat_{pNPGal}/mg_{protein}$). A fed-batch bioreactor cultivation of *Lb. plantarum* NC8 pSIP409-celB resulted in a specific CelB activity of 2,500 ± 120 $nkat_{pNPGal}/mg_{protein}$. A basal whey medium with supplements was developed as an alternative to the cost intensive MRS medium used. About 556 ± 29 $nkat_{pNPGal}/mg_{protein}$ of CelB activity was achieved in bioreactor cultivations using this medium. It was shown that both *Lactobacilli* were potential expression hosts for recombinant enzyme production.

An additional approach was performed to produce a metagenome-*β*-galactosidase using *Lb. plantarum* NC8 with the pSIP expression system. Using this system, a quite low maximal galactosidase activity of only 0.18 $nkat_{oNPGal}/mg_{protein}$ was detected. A 13 times higher activity of 2.42 $nkat_{oNPGal}/mg_{protein}$ was produced after the knock out of the interfering native *Kluyveromyces lactis β*-galactosidase in the well-known food-grade *K. lactis* pKLAC2 expression system. Nevertheless, the best performing expression system for the recombinant production of the metagenome-derived enzyme was the *Escherichia coli* BL21 strain with a pET vector, resulting in the highest *β*-galactosidase of 82.01 $nkat_{oNPGal}/mg_{protein}$.

Beside the use of the pSIP expression system, a novel expression system for *Lb. plantarum* was developed. This system is based on the manganese starvation-inducible promoter from the specific manganese transporter of *Lb. plantarum* NC8

which was cloned for the first time. The expression of CelB was achieved by cultivating *Lb. plantarum* NC8 at low manganese concentrations with MRS medium and the pmntH2-celB expression vector. A CelB activity of 8.52 μkat_{oNPGal}/L was produced in a bioreactor. The advantages of the novel expression system are that no addition of an external inducing agent was required, and additionally, no further introduction of regulatory genes was necessary. The new promoter meets the general demands of food-grade expression systems.

The glutamic acid racemase of *Lb. plantarum* NC8 was cloned and characterized in this work for the first time as a possible target for a food-grade selection system for this species. Glutamic acid racemases (MurI, E.C. 5.1.1.3) catalyse the racemisation of L- and D-glutamic acid. MurIs are essential enzymes for bacterial cell wall synthesis, which requires D-glutamic acid as an indispensable building block. Therefore, these enzymes are suitable targets for antimicrobial drugs as well as for the potential design of auxotrophic selection markers. A high expression system in *E. coli* BL21 was constructed to produce and characterize the biochemical properties of the MurI from *Lb. plantarum* NC8. The recombinant, tag-free MurI was purified by an innovative affinity chromatography method using L-glutamic acid as the relevant docking group, followed by an anion exchange chromatography step (purification factor 9.2, yield 11%). This two-step purification strategy resulted in a MurI sample with a specific activity of 34.06 $\mu kat_{D\text{-}Glu}/mg_{protein}$, comprising a single protein band in SDS-PAGE. The purified MurI was used for biochemical characterization to gain in-depth knowledge about this enzyme. Only D- and L-glutamic acid were recognised as substrates for the MurI with similar k_{cat}/K_m ratios of 3.6 sec^{-1}/mM for each enantiomer.

The findings in this study may contribute to further development and implementation of food-grade *Lactobacilli* expression systems for recombinant enzyme production. Furthermore, the results obtained may help to optimise and select hosts and expression systems for industrial enzyme production for the needs of the food industry.

Zusammenfassung

Lactobacilli sind Gram-positive Bakterien, deren Einsatz als traditionelle Starterkulturen für verschiedenste Nahrungsmittel in der Lebensmittelindustrie weit verbreitet ist. Als GRAS-Organismen (``generally recognised as safe´´) eignen sie sich hinsichtlich Lebensmittelsicherheit hervorragend zur rekombinanten Enzymproduktion. Sie bieten als lebensmittelassoziierte Mikroorganismen große Vorteile für die rekombinante Enzymproduktion, da sie als sogenannte ``food-grade´´ Expressionssysteme sicher und nachhaltig eingesetzt werden können. Ziel der vorliegenden Arbeit war es, den Einsatz von *Lactobacilli* als food-grade Expressionssysteme für rekombinante Enzymproduktionen detailliert zu analysieren.

In ersten Untersuchungen wurde dafür das pSIP Expressionssystem mit den Vektoren pSIP403 und pSIP409 zur rekombinanten Produktion der hyper-thermophilen *β*-Glucosidase (CelB) aus *Pyrococcus furiosus* in *Lactobacillus plantarum* NC8 und *Lactobacillus casei* als Wirtsorganismen genutzt. Mit beiden Organismen konnte mit dem Vektor pSIP409-celB in Bioreaktor-Kultivierungen im Batch-Verfahren aktives Enzym produziert werden. Mit *Lb. plantarum* wurde dabei eine um 44% höhere CelB-Aktivität (1.590 ± 90 $nkat_{pNPGal}/mg_{Protein}$) im Vergleich zu *Lb. casei* (1.070 ± 66 $nkat_{pNPGal}/mg_{Protein}$) generiert. Durch ein Fed-Batch-Verfahren gelang es, die spezifische CelB-Aktivität im Bioreaktor mit *Lb. plantarum* auf ein Maximum von 2.500 ± 120 $nkat_{pNPGal}/mg_{Protein}$ zu steigern. Als Alternative zu dem genutzten kostenintensiven MRS-Medium wurde ein basales Molkemedium mit Zusätzen entwickelt. Unter Verwendung dieses Mediums wurde eine maximale spezifische CelB-Aktivität von 556 ± 29 $nkat_{pNPGal}/mg_{Protein}$ im Bioreaktor erzielt. Somit konnte gezeigt werden, dass sich *Lactobacilli* grundsätzlich als potentielle food-grade Expressionssysteme zur rekombinanten Enzymproduktion anbieten.

In weiteren Untersuchungen erfolgte die Produktion einer Metagenom-*β*-Galactosidase mit *Lb. plantarum* und dem pSIP409 Expressionssystem. Unter Nutzen dieses Systems konnte eine geringe maximale *β*-Galactosidase-Aktivität von nur 0,18 $nkat_{oNPGal}/mg_{Protein}$ detektiert werden. Eine 13-fach höhere Aktivität der Metagenom-*β*-Galactosidase konnte in dem food-grade Expressionssystem *Kluyveromyces lactis* unter Verwendung des pKLAC2 Vektors, nach knock-out der störenden nativen *β*-Galactosidase, generiert werden. Als leistungsfähigstes Expressionssystem zur rekombinanten Produktion dieses Enzyms stellte sich in

weiteren Experimenten *Escherichia coli* BL21 mit pET Vektor heraus, dessen Einsatz zu der höchsten *β*-Galactosidase-Aktivität von 82,01 $nkat_{oNPGal}/mg_{Protein}$ führte.
Zusätzlich zu dem genutzten pSIP System erfolgte die Entwicklung eines neuen Expressionssystem für *Lb. plantarum* NC8. Dieses Expressionssystem basiert auf dem Manganmangel-induzierbaren Promotor eines spezifischen Mangantransporter-Proteins von *Lb. plantarum*, welcher erstmalig zur rekombinanten Proteinproduktion genutzt wurde. Eine Expression der CelB konnte bei Kultivierung von *Lb. plantarum* mit dem pmntH2-celB Expressionsvektor in MRS-Medium bei niedrigen Mangankonzentrationen nachgewiesen werden. Es wurde in Bioreaktor-Kultivierungen eine maximale CelB Aktivität von 8,52 $\mu kat_{oNPGal}/L$ erzielt. Die Vorteile dieses neuen Promotorsystems bestehen darin, dass keine Zugabe eines externen Induktors notwendig ist und dass keine weiteren regulatorischen Gene in den Wirtsorganismus eingebracht werden müssen. Somit erfüllt dieser neue Promotor die generellen Anforderungen an ein food-grade Expressionssystem.
In weiteren Arbeiten wurde die Glutaminsäure-Racemase von *Lb. plantarum* als möglicher food-grade Selektionsmarker in der vorliegenden Studie erstmalig kloniert und charakterisiert. Glutaminsäure-Racemasen (MurI, E.C. 5.1.1.3) katalysieren die Racemisierung von L- und D-Glutaminsäure und sind essentielle Enzyme für den bakteriellen Zellwandaufbau. Daher eignen sie sich als mögliche Ziele für antimikrobielle Arzneimittel sowie zur Konstruktion möglicher auxotropher Selektionsmarker. Nach rekombinanter Expression der MurI von *Lb. plantarum* NC8 in *E. coli* BL21 wurde das rekombinante Enzym durch eine Affinitätschromatographie mit L-Glutaminsäure als Kopplungsgruppe und folgender Anionenaustausch-chromatographie gereinigt (Reinigungsfaktor 9.2, Ausbeute 11%). Dies resultierte in einer spezifischen MurI-Aktivität von 34,06 $\mu kat_{D\text{-}Glu}/mg_{Protein}$, und einer Einzelbande in der SDS-PAGE-Analyse. Eine detaillierte biochemische Charakterisierung zeigte unter anderem, dass nur D- und L-Glutaminsäure als Substrate mit gleichem k_{cat}/K_m von 3,6 sec^{-1}/mM von der MurI akzeptiert werden.
Die Resultate dieser Arbeit können für eine Weiterentwicklung und Implementierung von food-grade *Lactobacilli* Expressionssystemen von rekombinanten Enzymen beitragen. Außerdem können die erzielten Erkenntnisse zukünftig helfen, Wirts- und Expressionssysteme für die industrielle Enzymproduktion gemäß den Anforderungen der Lebensmittelindustrie auszuwählen und zu optimieren.

Chapter 1

1. General Introduction and Thesis Outline

Abstract

In addition to the well-established recombinant microbial expression systems, such as *Escherichia coli, Bacillus subtilis* and others, increased attention has been paid to lactic acid bacteria, such as *Lactobacilli*, as expression host systems in the last few years. *Lactobacillus* spec. are a diverse genus of Gram-positive lactic acid bacteria which are widely used in the food industry. Different species of this genus have been applied since the 1990s as recombinant expression hosts. They have been used as so-called food-grade cell factories for the production of recombinant proteins, such as enzymes and antigens, as well as other pharmaceuticals. The major advantages of *Lactobacilli* as food-associated microorganisms used for recombinant protein production are the safe and sustainable use as overall safety food-grade expression systems. Some progress has been made over the past few years in the development of these promising bacterial expression systems, mostly on a laboratory scale. In this general introduction, an overview of some well-characterized tools for heterologous gene expression in *Lactobacilli* (host organisms, promoters, selection markers) is presented with a particular regard to food-grade approaches.

Lactic acid bacteria

Lactic acid bacteria (LAB) are extensively used in the food industry for the production and the preservation of fermented foods. The LAB group includes about 20 different genera; those mainly important for technical applications in the food industry are the genera *Lactococcus, Lactobacillus, Leuconostoc, Oenococcus, Pediococcus*, and *Streptococcus.* LAB are a phylogenetically heterogeneous group of Gram-positive, facultative anaerobic microorganisms that are clustered due to similarities in their metabolic pathway and physiological characteristics [Canchaya et al. 2006; Claesson et al. 2007; Hugenholtz and Smid 2002; Mozzi 2010]. The genus *Bifidobacteria,* as another important genus for industrial application, also has some of their typical features, but is phylogenetically unrelated to them [Sonomoto and Yokota 2011]. All of those mentioned are characterized due to a good degradation of different kinds of carbohydrates, which are metabolized to the predominant end-product – lactic acid. Because of that, they have been used for thousands of years for the acidification of food and feed, which has a preservative effect. This results in a good growth and viability even at low pH values of about 3.5 to 4 [de Vries et al. 2006]. They are inhabitants in different environments with a high level of carbohydrates, such as different kinds of fermented foods, as well as plant-derived substrates. LAB are used for the production of a variety of food and feed raw materials and are added as starter or adjunct cultures in different food products, such as yogurt, cheese, sausages, and fermented vegetables [Bron and Kleerebezem 2011; Pedersen et al. 2005]. They are involved in effecting the texture, flavour and shelf life of this food. Therefore, they are a common part of the human diet and generally harmless to humans. They do not generate endotoxins and are non-sporulating. Thus, they have a long history of safe use and many LAB have obtained the GRAS status [Bernardeau et al. 2008]. Additionally, specific LAB strains, mainly of the genus *Lactobacillus*, are known for their health-promoting capacity by the production of specific metabolites with a beneficial effect on healthy human gut microbiota and are used in probiotic products.

The genus *Lactobacillus* belongs to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales*, and family *Lactobacillaceae*. They are rod-shaped bacteria with either homo- or heterofermentative lactic acid production. Thereby, the major fermentation pathway of glucose resulting in the end-product lactate or lactate, ethanol and/or acetic acid in equimolar amounts. The genus *Lactobacillus* contains

over 100 recognised species and is characterized by a high level of diversity [Canchaya et al. 2006]. This diversity is reflected by the G + C content of the genomic DNA of species included in the genus. This range is twice the span usually accepted for one genus and between 32-55% [Axelsson 2004]. The high diversity of the genus *Lactobacillus* is also obvious in the different kinds of habitat of various species. Some *Lactobacillus* sp. appear to be highly specialized only in a limited number of habitats, such as *Lactobacillus delbrueckii* which is found only in dairy environments. Other species, such as *Lactobacillus plantarum*, have been isolated from a diverse range of habitats including vegetables, meat, fish, and dairy products, as well as the gastro-intestinal tract of humans. The high adaption to habitats rich in carbohydrates and other nutrients leads to a poor biosynthetic capability and to high nutrient requirements for the cultivation of *Lactobacilli*.

Due to a broad range of carbon sources which can be metabolised, *Lactobacillus* sp. have been used for the production of lactic acid from whey for decades [Kulozik and Wilde 1999]. A lot is known about the cultivation of *Lactobacilli* in a bioreactor [Schiraldi et al. 2003]. The industrial importance of *Lactobacilli* leads to the fact that this LAB has been extensively studied and is nowadays one of the best investigated microorganisms. A huge amount of knowledge has been obtained in research fields, such as microbiology, physiology, genetics, gene modification, and metabolic engineering, by applying biochemical, molecular biological and bioinformatic techniques.

For these reasons, interest in *Lactobacilli* as suitable production hosts for recombinant proteins has increased over the last two decades. Different kinds of applications are conceivable using recombinant LAB. They have, for example, potential as delivery systems for valuable proteins, such as antibodies and antigens, and are used as live vaccines [Diep et al. 2009]. Additionally, due to the particular demands of the food industry, *Lactobacilli* are desirable cell factories for the production of food-grade enzymes [Garcia-Fruitos 2012; Peterbauer et al. 2011]. All these reasons explain why different *Lactobacilli* expression systems have been developed recently.

Food-grade concept: Consideration of regulatory framework

Novel applications of recombinant food-grade *Lactobacilli* as production hosts of proteins, enzymes or other molecules need a regulatory framework for the safe use of this organism [de Vos 1999a; Salminen et al. 1998; Sybesma et al. 2006]. Nevertheless, recombinant *Lactobacilli* are, even as a food-grade organism, a genetically modified organism (GMO) and their use in the food industry is, therefore, strongly regulated. Besides regulatory rules, different public opinions have been established globally about the use of GMO in food [Gruère and Rao 2007; Renault 2002]. Within the United States of America there is only slight public resistance against it. In other areas of the world, such as Europe and Oceania, contrary public attitudes to using GMO for food production have been observed. The definition of “genetic modification” and GMO differs according to the geographic location and the legislative authority involved. In the USA, for example, a “genetic modification” is any alteration of the DNA sequence using any technique, and the approval depends on the characteristics of the resulting strain [FDA 2001]. The foods produced by organisms modified using recombinant DNA technology are indicated as “bioengineered foods” and the term GMO is not used [Pedersen et al. 2005]. In USA, genetically modified LAB are able to obtain GRAS status and have been on the market for several years. In contrast to that, no genetically modified LAB are on the market in Europe and the definition of GMO is quite different as well. In Europe, all organisms in which genetic material has been modified *in vitro* in a way that does not occur naturally are designated as GMO [EC 2001]. Contrary to that, GMOs in Oceania are defined as organisms in which any genetic material has been modified using *in vitro* techniques [Hobbs 2001]. Labelling is also quite varied in the different regions. Table 1 shows an overview of the different formalities in the context of the food industry.

Table 1: Guidelines for the use of GMO in food products in several regions [Carter and Gruère 2007]

Area	Definition GMO	Product Labelling	Authority
China	Organism in which DNA has been modified using any recombinant technology	Yes, also if no novel DNA or recombinant protein is detectable	Chinese Ministry of Science and Technology (MOST)
Europe	Organism in which DNA has been modified in a way that does not occur naturally by mating and/or natural recombination	Yes, also if no novel DNA or recombinant protein is detectable, product threshold 0.9%	European Food Safety Authority (EFSA)
Oceania	Organism in which DNA has been modified by any *in vitro* technique	Only when novel DNA or recombinant protein is detectable, otherwise not required	Food Standards Australia New Zealand (FSANZ)
United States	Term not used; GMO foods are designated as bioengineered	Only if food differs significantly from conventional equivalent	Food and Drug Administration (FDA)

In addition to the regulatory fundamentals of GMO and their application, some extra recommendations for the use of certain microorganisms in food production processes have to be fulfilled to ensure product safety. The GRAS status of the US FDA was the leading standard for this purpose in the past. All organisms used for food processing had to hold GRAS status. A long history of safe use was the basic requirement of the microorganism to get this status. In the last decade, the European Union introduced a similar system to the GRAS status of the FDA. Due to the fact that the term “long history of safe use” is indistinct, the EU’s system is unambiguously based on state-of-the-art microbiological, biochemical and molecular biological techniques [EFSA 2007]. This generic approval system, called Qualified Presumption of Safety (QPS), provides a more scientific, generic approval tool for use within the EFSA and is obligatory in the EU. All qualified microorganisms, without any properties that may be harmful for human health or the environment, and which have been introduced into the food chain are announced on a list of organisms which have obtained QPS status. This list, first established in 2007, is reviewed and modified by the EFSA annually via its panel on Biological Hazards (BIOHAZ) [EFSA 2011].

Due to the fact that *Lactobacilli* are non-toxic, some of them are even probiotics with health promoting effects, and their usage in food production processes have existed for thousands of years, they have obtained either GRAS status and/or are a QPS organism. This has lead to an increased usage of *Lactobacilli* as recombinant expression hosts over the last decade. While the model LAB *Lactococcus lactis* was predominantly used in the early-1990s, in the last decade, more and more *Lactobacilli*-based systems have been developed [Kleerebezem et al. 1997; Konings et al. 2000; Mierau and Kleerebezem 2005].

The use of GM-LAB-based expression systems requires the consideration of various specifications that should be met for a safe use in food. These considerations are summarised under the so-called “food-grade” concept. This term was established in the 1990s when the first LAB expression systems were developed. The theory behind the term “food-grade” describes what kind of genetic engineering in LAB could be appropriate for an intended usage in food. A strict definition of food-grade expression systems was published by Johansen [1999]. The most important criterion of these expression systems is primarily that the host strains used for genetic engineering should be safe, highly characterized, stable, and have the GRAS or QPS status. Only food-compatible selection markers should be used. Antibiotic resistance genes or markers have to be avoided, and any harmful, toxic or allergenic compounds should not be produced by the organism or be applied with the expression system. All genetic modifications should fulfil the criteria of self-cloning. Another important characteristic of food-grade systems is the ability for large scale industrial application for biotechnological purposes or in food production. Furthermore, food-grade modifications of LAB should result in chromosomal integrations in microorganisms that are stable in the gastro-intestinal tract if they might be consumed by humans. The strict food-grade definition declares that genes introduced in food-grade LAB need to be based on either genes obtained from species of the same genus, or from other genera of food microorganisms with GRAS or QPS status. In a broader definition, it is acceptable to use genes from non-GRAS or QPS donors during the construction process, if the DNA is removed completely in the final food-grade strain. This is in clear contrast to the current EC regulation about food enzymes and the guidance of the EFSA [EC 2008; EFSA 2009]. The EC regulation about food enzymes states clearly that, after accurate and detailed

evaluation of safety, each enzyme can be added to food after approval via a community list.

Based on this food-grade concept, a large number of systems have been developed in the last two decades, beginning with the LAB model organism *L. lactis*, and were further applied for recombinant *Lactobacilli* as food-grade expression systems. Different kinds of *Lactobacilli* hosts, promoters and food-grade selection markers were developed [Diep et al. 2009; Morello et al. 2008]. All this work was focusing mainly on the application of *Lactobacilli* on a laboratory scale for the recombinant production of enzymes and on the use of recombinant *Lactobacilli* as live vaccines.

Lactobacilli hosts for recombinant protein production

The first step in genetic modification of an organism and its use as a recombinant host is the introduction of foreign genes into the genetic material of the microorganism by transformation. Since the first reports about transformation of *Lactobacilli* in the late-1980s and the early-1990s, several *Lactobacilli* have been used as host organisms for recombinant protein production [Aukrust and Blom 1992; Aukrust and Nes 1988; de Vos 1987; Natori et al. 1990]. Electroporation emerged as the most successful method for the introduction of foreign genes into different *Lactobacilli*. Additionally, protocols were also developed for the generation of several recombinant hosts using protoplast formation or even conjugation methods, both of them with only limited efficiency. Using these techniques, some *Lactobacilli*, such as *Lb. acidophilus, Lb. casei, Lb. helveticus, Lb. plantarum Lb. reuteri, Lb. sakei*, and *Lb. pentosus*, were analysed as organisms for heterologous protein production, beside the popular expression hosts, such as *E. coli* or others like the Gram-positive *Bacilli* strains, for example, *Bacillus subtilis* or *L. lactis* [Hashiba et al. 1992; Kok 1996; Pouwels and Leer 1993; Wanker et al. 1995]. Many different proteins were successfully expressed in the different strains (Table 2). Different kinds of antigens from various origins were also produced in some *Lactobacillus* sp., which were summarized in recently published reviews and, therefore, are not listed here [Bermudez-Humaran et al. 2011; Tarahomjoo 2012; Wells and Mercenier 2008].

Table 2: *Lactobacilli* which have been used for the recombinant expression of proteins

Strain	Recombinant protein	Yield*	Induction	Reference
Lb. plantarum	*α*-Amylase	1,154 nkat/L	constitutive	[Fitzsimons et al. 1994]
	Aminopeptidase N	n. d.	constitutive	[Takala and Saris 2002]
	Aminopeptidase N	53 nkat /$mg_{protein}$	sppIP	[Sorvig et al. 2005]
	Chitinase	0.42 nkat/$mg_{protein}$	sppIP	[Nguyen et al. 2012]
	Cholesterol Oxidase	0.06 nkat/$mg_{protein}$	constitutive	[Kiatpapan et al. 2001]
	β-galactosidase	1,017 nkat/$mg_{protein}$	sppIP	[Halbmayr et al. 2008]
	β-glucosidase	2,500 nkat/$mg_{protein}$	sppIP	[Böhmer et al. 2012]
	β-glucuronidase	1680 Miller Units	sppIP	[Sorvig et al. 2005]
	Green fluorescent protein	n. d.	Nisin	[Geoffroy et al. 2000]
	Oxalat Decarboxylase	43.3 nkat/$mg_{protein}$	sppIP	[Kolandaswamy et al. 2009]
	Malolactic enzyme	368 nkat/$mg_{protein}$	sppIP	[Schümann et al. 2012]
	Green fluorescent protein	n. d.	Nisin	[Geoffroy et al. 2000]
Lb. casei	Cholesterol Oxidase	0.015 nkat/$mg_{protein}$	constitutive	[Kiatpapan et al. 2001]
	β-glucosidase	1,070 nkat/$mg_{protein}$	sppIP	[Böhmer et al. 2012]
	Levanase	8,335 nkat/L	Inulin	[Wanker et al. 1995]

Strain	Recombinant protein	Yield*	Induction	Reference
	Xylose-isomerase	n. d.	D-Xylose	[Posno et al. 1991]
Lb. sakei	Aminopeptidase N	80 nkat/$mg_{protein}$	sppIP	[Sorvig et al. 2005]
	β-glucuronidase	1590 Miller Units	sppIP	[Sorvig et al. 2005]
	β-galactosidase	492 nkat/$mg_{protein}$	sppIP	[Halbmayr et al. 2008]
Lb. reuteri	α-Amylase	82 nkat/mL per OD_{600nm}	Nisin	[Wu et al. 2006]
	β-glucanase / Xylanase	14.3/25.0 nkat/mL	Lactose	[Liu et al. 2007]
Lb. acidophilus	*β*-galactosidase	35 nkat/mL	Lactose	[Lin et al. 1996]
Lb. brevis	Alcohol dehydrogenase / Pyruvate decarboxylase	30/58 nkat/mg	Lactose	[Liu et al. 2007]
Lb. bulgaricus	Nuclease	1250 µg digested DNA/mL	constitutive	[Chouayekh et al. 2009]
Lb. helveticus	*α*-Amylase	n. d.	Lactose	[Hashiba et al. 1992]
Lb. paracasei	scFv antibody fragment	500 µg/L	constitutive	[Martin et al. 2011]
Lb. paracasei	Mannanase	90 nkat/mL	constitutive	[Yoon 2012]
Lb. pentosus	Chloramphenicol-acetyltransferase	n. d.	Xylose	[Lokman et al. 1994]

* denoted in nkat in order to make them comparable, if no conversion possible similar to that presented in the original publications
sppIP: sakacin P inducing peptide; inducing peptide of 19 amino acids

Lactobacillus plantarum

After *L. lactis, Lb. plantarum* seems to be the most popular lactic acid bacterium used for recombinant protein production with Gram-positive, food-grade *Bacilli* [Axelsson et al. 2012]. It is a highly versatile, facultative anaerobic and heterofermentative organism. During the last decade, *Lb. plantarum* has developed into one of the best characterized *Lactobacilli* and is considered as a model organism in the research of this genus [de Vries et al. 2006; Siezen and van Hylckama Vlieg 2011; Sturme et al. 2007]. The high phenotypic diversity of this species is caused by the different kinds of habitat from which they were isolated, such as decaying plant materials, and also from the human vaginal and intestinal tracts, and faeces. Additionally, they were found in fermented foods, such as sauerkraut, sourdough, olives, kimchi, and fermented dairy products. They dominate in the later phase of the fermentation processes of these foods due to their high acid tolerance. The probiotic attribute of *Lb. plantarum* and the high survival rate during passage through the human gastro-intestinal tract was studied, focusing on the beneficial effects on human health and possible use for delivery of pharmaceutical proteins [Pouwels et al. 2001]. *Lb. plantarum* WCFS1, originally isolated from human saliva, was, in 2003, the first *Lactobacillus* whose complete genome was fully sequenced and published [Kleerebezem et al. 2003; Siezen et al. 2012]. The 3.3 Mb genome is still the largest of any organism of this genus. The complete genomes of the strains *Lb. plantarum* NC8, JDM1, Stm-III, and ATCC14917 have been sequenced and show high homology > 98% [Axelsson et al. 2012; Wang et al. 2010]. Detailed genomic and proteomic studies were possible after the genome sequences became available. The most extensive model strains of *Lb. plantarum* are the strains WCFS1 and NC8, which have been used for the development of genetic tools, metabolic engineering and studies of bacteriocin production, as well as general fermentation.

Lb. plantarum NC8 was originally isolated from grass silage in the 1980s [Aukrust and Blom 1992]. Nowadays, it is the most widely applied *Lactobacillus* strain for heterologous protein production [Diep et al. 2009; Peterbauer et al. 2011]. It has a number of favourable features as an expression host, including the fact that it is naturally plasmid free, shows a low protease activity and has the ability to secrete proteins. Additionally, it is known for good growth using different substrates due to a high metabolic capacity. The first expression of heterologous genes was reported in the 1990s [Fitzsimons et al. 1994] and has become even more popular in the 2000s.

Over the years, a number of promoters as well as selection systems have been constructed. In some cases, the recombinant protein level amounted to approximately 55-60% of the total intracellular protein of *Lb. plantarum*, which is one of the highest expression levels ever obtained with gene expression systems in lactic acid bacteria [Halbmayr 2008]. Other studies showed that the yields of recombinant protein were almost the same as those obtained in *E. coli* [Böhmer et al. 2012; Kolandaswamy et al. 2009]. Therefore, *Lb. plantarum* seems to be the most promising host organism and an interesting species for industrial and pharmaceutical protein production.

Lactobacillus casei

Lb. casei is not as well-studied and characterized as *Lb. plantarum,* but it is also a promising host for recombinant protein production. *Lb. casei* is facultative anaerobic, homofermentative and known as a good L-lactic acid producer [Ding and Tan 2006; Rodriguez-Diaz et al. 2012]. It is found in the human intestine and is most commonly used as a probiotic in the dairy industry. Because of that, there is good knowledge about the cultivation of this organism on an industrial scale. Its viability at a broad pH range is a reason, therefore, that it persists for a long time in the digestive tract. Some studies were performed with recombinant *Lb. casei* as live vaccines and vehicles for the delivery of medically relevant proteins to mucosal surfaces [Bermudez-Humaran et al. 2011]. The most widely used strain is the well-known *Lb. casei* BL23, which is a plasmid-free strain, whose complete genome sequence has been available with 3.1 Mb since 2010 [Maze et al. 2010]. BL23 is easily transformable and widely used for physiological, genetic and biochemical studies, and is a kind of model organism of the species *Lb. casei*.

There are different kinds of expression systems described for the use of *Lb. casei* using constitutive and inducible promoters, as well as several selection markers [Binishofer et al. 2002; Böhmer et al. 2012; Hazebrouck et al. 2007; Rochat et al. 2006; Takala et al. 2003]. Heterologous expressed proteins can be localized intracellularly or linked to the cell surface and, thereby, presented on the cell wall. Furthermore, recombinant proteins can be secreted directly out of the cell. However, it should be mentioned that the yields of recombinant proteins obtained produced with *Lb. casei* were most frequently very low and obtained only µg/L extracellularly or 1-1.5% of total soluble protein intracellularly [Maassen et al. 1999].

Lactobacillus sakei

Lb. sakei is a facultative anaerobic and heterofermentative food-associated and important LAB. The genome of the strain 23k was the first one sequenced completely, and revealed, with 1.8 Mb, a smaller size than that of *Lb. plantarum* or *Lb. casei*. Regarding the gene products, the *Lb. sakei* genome shares the highest level of conservation with *Lb. plantarum* [Chaillou et al. 2005]. *Lb. sakei* was isolated and described for the first time from Japanese rice wine [Katagiri et al. 1934], and it is found most commonly in fresh meat and fish, and also on fermented plants. Nowadays, it is widely used as a starter and adjunct culture in the meat industry, tolerating harsh conditions, such as high salt, low water activity, and low temperature and pH, and producing antimicrobial bacteriocins as biopreservation [McLeod et al. 2010]. The bacteriocins produced by *Lb. sakei* belong to the so-called class II of bacteriocins and are small, heat-stable and antimicrobial peptides. An overview of some class II bacteriocins from *Lactobacilli* is given in Table 3.

Table 3: Class II bacteriocins produced by *Lactobacilli* [Ennahar et al. 2006].

Bacteriocin	*Lactobacillus*	size	sequence
Sakacin A	*Lb. sakei*	41 aa	ARSYGNGVYC NNKKCWVNRG EATQSI IGGM ISGWASGLAG M
Sakacin P	*Lb. sakei*	37 aa	KYYGNGVHCG KHSCTVDWGT AIGNIG NNAA ANWATGGNAG WNK
Bavaricin A	*Lb. sakei*	37 aa	KYYGNGVHXG KHSXTVDWGT AIGNIG NNAA ANXATGXNAG G
Pediocin PA-1	*Lb. plantarum*	44 aa	KYYGNGVTCG KHSCSVDWGK ATTCIIN NGA MAWATGGHQG NHKC
Plantaricin-A	*Lb. plantarum*	23 aa	AYSLQMGATA IKQVKKLFKK WGW
Curvacin A	*Lb. curvatus*	41 aa	ARSYGNGVYC NNKKCWVNRG EATQSI IGGM ISGWASGLAG M

The production of these peptides is strictly controlled and highly induced by a mechanism based on *quorum-sensing* controlled by a secreted peptide pheromone. The mechanisms and promoters of the gene clusters coding for the bacteriocins sakacin A and sakacin P from *Lb. sakei* have been used for recombinant protein production since the beginning of the 2000s [Axelsson et al. 2003]. The so-called pSIP expression system developed for recombinant enzyme production is one of the

strongest expression systems known for *Lactobacilli*, and is used not only with *Lb. sakei*, but even more often with *Lb. plantarum* as host. Different kinds of proteins have been produced, usually with somewhat higher yields in *Lb. plantarum* compared to *Lb. sakei* [Diep et al. 2009]. Nevertheless, *Lb. sakei* also has a great potential and interest for the food industry and especially from the meat industry as a food-grade recombinant production host.

Promoters used for recombinant expression in LAB

It is necessary to obtain high expression levels of the desired recombinant proteins for an efficient industrial application of food-grade *Lactobacilli*. High-level production of recombinant proteins in *Lactobacilli* were obtained with either constitutive or inducible promoters and their regulatory elements [Axelsson et al. 2003; Pavan et al. 2000; Pouwels and Leer 1993; Rud et al. 2006; Sorvig et al. 2005].

Generally, inducible expression is preferable in applications where the aim is the overproduction of a desired protein at high levels at a defined moment during fermentation, e.g. when toxic proteins are to be produced. In other cases, inducible expression systems are less suitable, e.g. during *in situ* production of recombinant proteins by *Lactobacilli* as live vaccines in the human body or when steady-state gene expression is required in metabolic engineering approaches. The expression system of choice for such applications may be constitutive promoters, even if they are usually characterized by lower expression levels.

Constitutive Promoters

Various expression systems using constitutive promoters have been described to date for different *Lactobacilli* [Gasson et al. 1994; Lizier et al. 2010; Pouwels and Leer 1993]. Research has been done to identify and isolate strong constitutive promoters from housekeeping genes. The level of expression from these constitutive promoters was demonstrated to be organism- and promoter-dependent [Chen and Steele 2005]. The use of native promoters was often beneficial, as the endogenous transcriptional signals are guaranteed to be recognised by the host strain [Stephenson et al. 2011]. A general functional promoter for *Lactobacilli* is as yet unknown because of the high diversity of the genus.

One of the widely used constitutive promoters is the highly efficient promoter of the lactate dehydrogenase genes (*ldh*) from different *Lactobacillus* species [Gory et al.

2001; Pouwels et al. 1996]. Pouwels et al. showed a high expression level of the *ldh* promoter even in different kinds of *Lactobacilli*. The *ldh* gene is constitutively highly expressed in *Lactobacilli* due to the key role in the fermentation of lactic acid. Therefore, the *ldh* promoter was one of the first promoters used for recombinant protein expression in different kinds of *Lactobacilli* and is still today one of the strongest constitutive promoters known [Rud et al. 2006]. Several other promoters have been identified over the last decade for strong constitutive expression, such as the one of the surface layer protein A (P*slpA*) [Kahala and Palva 1999; McCracken et al. 2000] or the clpC gene coding for an ATPase (P*cpl*C). Those constitutive promoters achieved amounts of recombinant protein up to 28% of total intracellular protein. Different synthetic promoters were also constructed and showed a good expression in *Lb. plantarum.* [Stephenson et al. 2011].

Inducible Promoters

Extensive studies have been done on inducible promoters of *Lactobacilli*. These promoters express proteins when there is a specific stimulation from the environment. Regulated promoters are clearly favourable in industrial fermentations because of the possibility of controllable overproduction of proteins at any desired moment of cultivation [Diep et al. 2009; Kuipers et al. 1997; Peterbauer et al. 2011]. This specific induction of recombinant protein production may be advantageous, for example, when the protein is toxic or harmful for the production host. An additional benefit is the option to vary the intracellular amount of protein to reduce inclusion bodies or for detailed studies and control of metabolic pathways.

Inducible Promoters without externally added inducing agents

Considering the food-grade concept for gene expression systems in *Lactobacilli*, the specific stimulation or inducing agent used has to be non-toxic, safe and food-approved. Therefore, it is highly desirable to obtain expression systems which may be inducible without adding an inducing agent, for example, by thermal or pH-shifts during cultivation [D'Souza et al. 2012; De Vos 1999b]. Different examples of these systems have been described in the last few decades, mainly for the well-known *L. lactis* system [Nauta et al. 1997; Sanders et al. 1997]. Similar approaches in recombinant protein production by stress conditions without the adding of external inducers are the so-called auto-inducing promoters. Using that kind of promoter,

recombinant expression starts during cultivation after limiting media components are depleted and starvation conditions are obtained. Auto-inducing expression systems have been developed for *L. lactis* and *B. subtillis* in recent years. These are based on phosphate or metal starvation transporter systems [Kerovuo et al. 2000; Sirén et al. 2008]. Because phosphate is an essential component for bacterial growth, the promoters of these transport proteins are turned on under starvation conditions, and this can be used for recombinant protein expression. A similar expression system based on regulatory elements of high specific Zn^{2+} uptake during Zn^{2+} starvation was developed for recombinant protein production in *L. lactis* [Lull and Poquet 2004]. The only starvation inducible expression system applied in *Lactobacilli* is the auto-inducing expression system based on the manganese starvation-inducible promoter from the specific manganese transporter of *Lb. plantarum* NC8 [Böhmer et al. 2013b].

Inducible Promoters based on externally added agents

Due to the fact that sugar utilisation has been extensively studied in *Lactobacilli*, and that the genes involved in sugar metabolism are strongly expressed and controlled, different kinds of sugar-inducible expression systems have been developed. Nowadays, induction of recombinant protein production can be obtained with several promoters and sugars as the inducer, such as lactose, xylose or trehalose [Duong et al. 2010; Lokman et al. 1994; Perez-Arellano and Perez-Martinez 2003].

The strongest inducible promoters known for LAB up to now are based on *quorum-sensing* systems and need specific peptides as inducers. So far, only the lactoccocal-based so-called Nisin-controlled-expression (NICE) system has found widespread use as an inducible expression system and has also been realised on an industrial scale [de Ruyter et al. 1996; Diep et al. 2009; Kuipers et al. 1997; Mierau and Kleerebezem 2005; Mierau et al. 2005]. It has been derived from the molecular characterization of the production of the anti-microbial peptide nisin consisting of 34 amino acids. Nisin is a food-grade peptide and can be used to induce the P*nisA* promoter, which has been used for successful recombinant expression in lactococci and other LAB, such as *Lb. plantarum* [Hazebrouck et al. 2007; Pavan et al. 2000].

Another food-grade expression system based on *quorum-sensing* and used for induced gene expression in *Lactobacilli* is the so-called pSIP system. The first application of these *quorum-sensing* based expression system with the promoters of

the Sakacin gene cluster was described by Axelsson et al. [1998]. Subsequently, a series of versatile expression vectors based on regulatory elements of Sakacin A (pSIP300) and Sakacin P (pSIP400) was developed by the same group [Axelsson et al. 2003; Sorvig et al. 2003; Sorvig et al. 2005]. Figure 1 gives a schematic overview of the pSIP vectors. The functionality of the pSIP vectors have been analysed using several homologous and heterologous genes of interest, e.g. a *β*-glucoronidase from *E. coli*, an aminopeptidase from *L. lactis*, *β*-galactosidases from *Lactobacilli*, a chitinase from *Bacillus licheniformis*, or a *β*-glucosidase from *Pyrococcus furious* [Böhmer et al. 2012: Halbmayr et al. 2008; Nguyen et al. 2012; Sorvig et al. 2005]. Expression studies have been conducted with *Lb. plantarum, Lb. sakei* and *Lb. casei* as host strains. It was shown that the promoters are tightly controlled and very high expression levels were reached upon induction. Levels of recombinant protein up to 46% of total intracellular protein were obtained with the P*sppQ* promoter of the Sakacin P gene cluster. This is almost twice as high as the highest level of recombinant protein obtained with constitutive promoters in *Lactobacilli*, which distinguishes the superiority of these inducible promoters. Additionally, the amount of inducing peptide (sppIP, a peptide consisting of 19 amino acids) needed for induction was extremely low, in the range of only 25-50 ng/L. A clear dose-response effect was observed at lower sppIP concentrations. A lesser yield of recombinant enzyme as well as lower control of the promoter's tightness were analysed under the same conditions, using a nisin-based analogous (pSIP500) [Sorvig et al. 2003]. Nevertheless, it has to be mentioned that the pSIP vectors applied for recombinant expressions are non-food-grade expression systems due to the antibiotic resistance used for selection. An advantage of the pSIP vectors is the modular cassette system from which they are composed, which enables a fast and easy exchange of the non-food-grade antibiotic resistance.

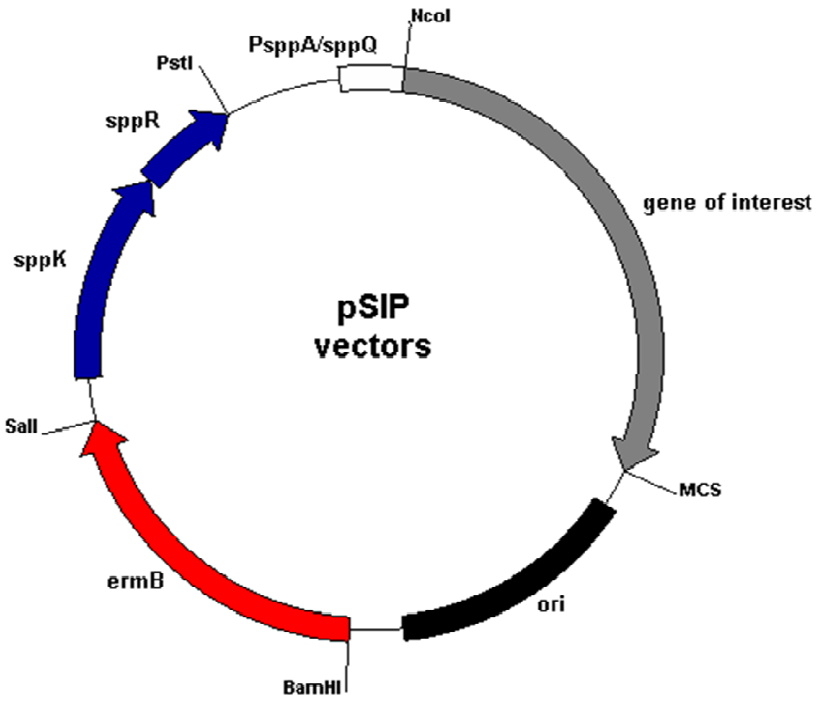

Figure 1: Schematic overview of the pSIP vector expression system (modified after Sorvig et al. 2005)

Figure 1 illustrates the pSIP vector series map and the modular cassette system. The sppK and sppP genes encode a histidin kinase and response regulator, which are proteins of the regulatory system. The gene of interest is under the control of either the PsppA or PsppQ promoter from the sakacin A (sppA) or sakacin P (sppQ) gene cluster. The ori is derived from the pUC ori for *E. coli* and the 256rep for *Lactobacilli*. Restriction sites for an easy exchange of different modules are indicated. The selection marker ermB is the resistance to the antibiotic erythromycin used in those vectors.

Food-grade selection markers

A fundamental aspect of the concept of food-grade expression systems is avoiding antibiotic resistance markers, because of the risk of the transfer of antibiotic resistance to the human intestine microbiota [De Vos 1999b; Peterbauer et al. 2011]. Several potential selection markers have been developed that fulfil the requirements of the food-grade definition and avoid the use of any harmful or toxic substances. Resistance markers used in food-grade approaches can be classified, based on the method of selection, into dominant or complementation selection markers; this is explained in detail below.

Dominant selection markers

Dominant selection markers share the benefit that they can often be used species independently in different kind of *Lactobacilli*, similar to antibiotic selection markers.

They allow the direct selection of positive transformants and the stable integration of plasmids in host organisms as long as selection pressure is obtained. A widely applied approach of dominant selection is based on the capacity of *Lactobacilli* to utilise a large range of rare sugars. In addition to glucose, several *Lactobacilli* are capable of fermenting sugars such as D-xylose, inulin and others. Characterization of the genes and enzymes responsible for this unusual sugar fermentation were studied extensively and later on employed as dominant selection markers. This was possible due to the fact that not all species are able to use that sugar and the genes involved in their utilisation can be introduced in non-fermenting hosts without any homologues of these genes. The ability of D-xylose catabolism of *Lb. pentosus*, for example, is known to be coded by the genes *xylRAB* (D-xylose isomerase, D-xylose kinase and D-xylose catabolism regulatory protein). These three genes were successfully implemented in hosts like *Lb. plantarum* and *Lb. casei* and were used as selection markers similar to the antibiotic erythromycin [Posno et al. 1991]. A similar selection approach based on the *scrA/scrB* genes from *Pediococcus pentosaceus* coding for a sucrose transporter system can be used for dominant selection of recombinant LAB with sucrose [Leenhouts et al. 1998].

Another dominant selection marker applied in *Lactobacilli* is the *nisI* gene coding for the NisI protein responsible for nisin immunity of *L. lactis* [Takala and Saris 2002]. NisI is a lipoprotein and protects the cell against the antimicrobial peptide nisin. The actual mechanism of NisI-mediated nisin immunity is not fully clear. The constructed plasmid containing *nisI* was originally used for *L. lactis* and was constructed entirely of food-grade lactococcal DNA. It was also successfully transformed and applied in *Lb. plantarum.* The food-grade preservative agent nisin was used as a dominant selection marker. Among others, there is also a food-grade dominant selection marker based on a bile salt hydrolase gene *bsh* from *Lb. plantarum* which was demonstrated to be functional in *Lactobacilli* in a recent study. Within this approach, transformed cells showed normal growth in the presence of bile salt, while growth of wild-type *Lactobacilli* was significantly inhibited [Yin et al. 2011].

Complementation selection markers

Food-grade complementation selection markers are based on specific mutations or deletions in a chromosomal gene of the host organism encoding an essential step in a metabolic pathway. A copy of the knocked out gene can then be inserted in an

expression vector and, thereby, used as a selection marker after transformation. Due to the fact that a mutant with a specific knock out must be generated to use complementation selection markers, followed by the construction of particular expression vectors, a two-step protocol is always required. As a consequence, these selection markers are only applicable in specific host-vector combinations. The limited use and high effort of construction are clear drawbacks of that type of marker system. However, a benefit of selection by complementation is that no supplements in the cultivation medium are required to maintain selective pressure during fermentation, e.g. in food production processes [Hansen 2002].

The first expression systems described for this class of selection markers at the beginning of the 1990s are based on lactose complementation. In *Lb. helveticus,* a vector-based *β*-galactosidase was able to complement a chromosomal mutation in the gene coding for the *β*-galactosidase for more than 100 generations when cultivated in milk [Hashiba et al. 1992]. In another approach, a mutation in the *lacF* gene, coding for an essential protein of the lactose transport system in *Lactobacilli*, was used as a selection marker. Lactose-deficient mutants containing a complete deletion of the 0.3 kb *lacF* gene or even only a missense mutation in this gene were complemented by a vector-borne wild-type *lacF* gene [De Vos 1999b]. Several proteins were produced recombinantly using this marker with expression systems that comply with all food-grade requirements.

Another example of a food-grade complementation selection marker in *Lactobacilli* is the *alr* gene encoding an alanine racemase [Bron et al. 2002; Nguyen et al. 2011a; Renault 2002]. The alanine racemase catalyses the interconversion of L-alanine to D-alanine, which is crucial for the cross-linking of peptidoglycan and, therefore, for cell wall biosynthesis. Thus, alanine racemase is described as an essential enzyme for the growth of prokaryotic cells. Knock out of the alanine racemase, encoded by a single *alr* gene in *Lactobacilli*, resulted in *Lb. plantarum* being strictly able to grow only in the presence of D-alanine [Hols et al. 1997; Palumbo et al. 2004]. Plasmids carrying a heterologous *alr* were able to complement the D-alanine auxotrophy in *Lb. plantarum* and selection was found to be highly stringent and stable over more than 200 generations [Bron et al. 2002]. D-alanine is no common ingredient of complex substrates, which are often used in large-scale fermentation media, and therefore this expression system is a potential alternative for industrial application of recombinant food-grade *Lactobacilli*. Similar to the *lacF* marker, an expression

system that complies with all food-grade requirements was developed based on the pSIP vectors by an exchange of the antibiotic resistance with the alanine racemase as the selection marker [Nguyen et al. 2011a]. The usability of the markers was confirmed by overexpression of heterologous *β*-galactosidases and by comparison of the new vectors with the alanine racemase selection with the conventional pSIP vectors with antibiotic resistance. Another possible selection marker is the glutamic acid racemase, an enzyme similar to the alanine racemase essential for the cross-linking of peptidogylcan. In a recent study, the glutamic acid racemase of *Lb. plantarum* NC8 was overexpressed, purified and characterized [Böhmer et al. 2013a]. This enzyme may be another target to create auxotroph *Lactobacilli* strains for food-grade approaches in the future.

Examples and prospects for application of recombinant enzyme production in *Lactobacilli*

Lactobacilli are receiving increased attention for their application as expression systems for the recombinant production of food-related industrial enzymes; this is additional to their usability to produce pharmaceutical proteins. In this section, some examples for the production of food-related enzymes will be presented and further prospect applications will be discussed. The usage of recombinant *Lactobacilli* in pharmaceutical applications has been reviewed in detail in already published literature and is not the topic of this work [Bermudez-Humaran et al. 2011; Wells and Mercenier 2008]. Different enzymes were produced in the last two decades using recombinant *Lactobacilli* (see Table 1). However, many of these examples are not fully food-grade as described above, for example, antibiotic resistance markers have often been used. Nevertheless, due to the fact they present proof of principle studies, these examples are presented here.

The first recombinant enzymes produced in *Lactobacilli* were mainly so-called reporter genes, such as the *β*-glucoronidase GusA from *E. coli* or the aminopeptidase PepN from *L. lactis* [Kahala and Palva 1999; Mathiesen et al. 2004; Pouwels and Leer 1993]. These enzymes were used mainly because they have been studied intensively and enzyme activity can be easily determined with chromogenic substrates. In addition, they have the advantage that their activity is usually not present in *Lactobacilli*. Accessorily, they were expressed well in this genus using

different expression systems with high yields up to 30% of total intracellular protein. After analysing the expression performance and applicability of new expression systems using reporter genes, more recombinant produced enzymes were mostly food- or feed-related. An example of this is the expression of the phytase from *B. subtillis* in *Lb. plantarum* described by Kerouvo and Tynkynnen [1999]. Phytases are enzymes hydrolysing phytate, the major storage form of phosphorus in cereals and legumes. Due to the fact that different animals, like pigs and poultry, have no phytate-degrading enzymes in their intestine, it is important for the feed industry to increase the nutritive value of feed by use of phytases. An ancillary effect of the degrading of phytate in feed is the reduction of the environmental phosphate pollution.

The expression of a *B. subtillis* phytase was carried out using a *Lb. plantarum* host and an inducible amylase promoter. The recombinant phytase was secreted and after overnight cultivation, 2 mg/L catalytically active phytase was present in the culture supernatant. In comparison to the expression levels needed for industrial enzyme production, this is a quite low amount of enzyme produced in *Lb. plantarum*. However, it is an interesting example of the production of an industrial enzyme in *Lactobacilli*. By using the recombinant host strain as a starter culture in fermented plant materials like silage, it may be applicable even with the low amount of recombinant enzyme.

In a recent study, the enzyme produced was a 2,5-diketo-D-gluconic acid reductase from *Corynebacterium glutamicum* [Kaswurm et al. 2013]. The 2,5-diketo-D-gluconic acid reductase catalyses the reduction of 2,5-diketo-D-gluconic acid to 2-keto-L-gulonic acid, an important precursor of L-ascorbic acid, and is thus essential for the biotechnological production of this important food-supplement. The 2,5-diketo-D-gluconic acid reductase is usually produced recombinantly, mainly with *E. coli* as the host organism [Banta et al. 2002]. Kaswurm et al. analysed the alternative food-grade host systems *L. lactis* with the NICE system and *Lb. plantarum* with the pSIP vector system, and evaluated the effectiveness of both systems by cultivation in stirred-tank reactors of a 0.5 L scale. Both expression systems were able to produce the recombinant reductase, while the better production performance was observed with *Lb. plantarum* TLG02, a WCFS1 derivative, and the pSIP system. Reductase activity of about 262 $U/L_{fermentation\ broth}$ was detected in *Lb. plantarum*, which was a 2.5 times higher level than in *L. lactis* and is the highest heterologous expression level so

far reported for this enzyme. It was shown by optimisation of the fermentation processes that the yields of recombinant enzymes produced by *Lactobacilli* with the pSIP expression system can be increased further [Böhmer et al. 2012]. Accordingly, *Lb. plantarum*/pSIP might be an interesting alternative to *E. coli* expression systems for industrial 2,5-DKG reductase production. Due to the use of an alanine racemase complementation-based selection system, the experiments presented fulfilled all the requirements of food-grade recombinant enzyme production. Several other enzymes applicable in the food industry were produced in *Lactobacilli*. Examples are the heterologous production of a catalase by *Lb. casei*, which functions as protection against oxidative damage, the production of malolactic enzyme by *Lb. plantarum*, important for the ageing and stability of wine, or even the metabolic engineering of *Lb. plantarum*, which resulted in a strain producing high yields of the low calorie sugar sorbitol [Ladero et al. 2007; Rochat et al. 2006; Schümann et al. 2012].

Most of the studies about food-grade *Lactobacilli* as a production host for recombinant enzymes focused on *β*-galactosidases as proteins of interest. Different *β*-galactosidases were produced with high yields [Halbmayr et al. 2008; Nguyen et al. 2007; Nguyen et al. 2011a; Nguyen et al. 2011b]. In a recent work published by Nguyen et al., a full food-grade expression system was used for the recombinant production of *Lb. reuteri β*-galactosidase in the host organism *Lb. plantarum* using an expression vector based on the selection of alanine racemase with highest activities up to 109 U/mg [Nguyen et al. 2011a]. A clear difference in the yields obtained is described for *β*-galactosidases from different microbial sources. The expression level of the *β*-galactosidases from *Lb. reuteri* and *Lb. acidophilus*, for example, differed about 18 fold when using the same expression vectors and cultivation strategies, resulting in 144 U/mg for the *Lb. reuteri β*-galactosidase and significantly lower 3.94 U/mg for the *Lb. acidophilus β*-galactosidase. The reason, therefore, was different mRNA stabilities resulting in different mRNA levels and translational effects. Nevertheless, *Lactobacilli* showed good expression levels and high yields in most cases when used for recombinant *β*-galactosidase production. The highest amount of recombinant intracellular protein ever produced with *Lactobacilli* was reported in a published study with up to 63% of total intracellular protein when producing the *β*-galactosidase from *Lb. delbrueckii* in *Lb. plantarum* resulting in 196 U/mg [Nguyen et al. 2011b].

β-Galactosidases are enzymes belonging to the class of glycoside hydrolases (EC. 3.2.1.23) catalysing the hydrolysis of terminal non-reducing *β*-D-galactose residues in *β*-D-galactosides [Oliveira et al. 2011]. They are ubiquitous enzymes produced by a vast majority of microorganisms. *β*-Galactosidases are industrially important enzymes with a widespread use in the dairy industry. They are applied for the hydrolysis of lactose, the main carbohydrate in milk or milk-derived products. A significant part of the worldwide population is lactose intolerant, resulting from absent or reduced *β*-galactosidase activity in the small intestine, and so are not able to consume milk products. Additionally, due to their transgalactosylation activity, they can be applied for the synthesis of prebiotic sugars, such as galacto-oligosaccharides (GOS) [Gosling et al. 2010]. Most of the commercially available *β*-galactosidases used today in industry are derived from *Kluyveromyces* sp. or *Aspergillus* sp., which are known to produce high yields, making these enzymes cost effective, for example, by a rapid growth on lactose as a carbon source [Nor et al. 2001]. *β*-Galactosidases can be produced on a large scale with a high yield giving them an important commercially acceptable price for application in the food industry. Beside the *β*-galactosidases of this species, the use of recombinant technologies for the production of *β*-galactosidases of different origin with high yields has emerged over the last few years.

Several expression systems and host organisms, such as *E. coli*, *Pichia pastoris* and *B. subtilis*, or food-grade organisms, such as *K. lactis* or *Lactobacilli*, were applied for the recombinant production of *β*-galactosidases [Halbmayr et al. 2008; Oliveira et al. 2011]. New *β*-galactosidases from the metagenome with superior properties have, by the use of recombinant production processes, also become available for the dairy industry [Wang et al. 2010]. Due to the drawbacks of commercially available *β*-galactosidases, for example, an inhibition by galactose, sodium or calcium, or less activity at low temperatures, new enzymes are highly desirable for industrial application.

Metagenomics have emerged among classical microbial screening over the last decade, as an alternative approach to screen for new, up-to-date completely unknown enzymes with favourable characteristics [Lorenz and Eck 2005; Streit and Schmitz 2004]. Metagenomics concerns the extraction, cloning and analysis of the entire genetic complement of a habitat [Handelsman et al. 1998]. Only about 1% of all bacteria in the environment are cultivable under laboratory conditions, for which

reason, 99% of all microorganisms in the environment are unknown. The direct cloning of DNA from environmental probes for generating gene libraries, first described by Torsvik et al. in the 1980s, was the key to access these uncultivable organisms for biotechnological purposes [Torsvik et al. 1980]. Thus, metagenomics have developed since the 1990s to identify novel enzymes with superior characteristics for industrial applications. Since its introduction, metagenomics has identified a lot of formerly unknown biocatalysts with high potential for use in industrial processes. Different kinds of enzymes have been identified in metagenome-derived DNA libraries, e.g. dehydrogenases, oxioreductases, lipases, esterases, proteaeses, and *β*-galactosidases [Lorenz and Eck 2005; Wang et al. 2010]. Screening approaches, therefore, were mainly function-based. These screening approaches used the detection of specific activities detectable in metagenomic libraries, for example, by the use of artificial chromogenic substrates. In the last few years, using next-generation sequencing techniques, a huge number of metagenome sequences have been made available and sequence homology-based screening approaches developed using *in silico* analysis [Simon and Daniel 2010]. Consequently, the metagenome has also developed as an interesting source of new enzymes for the food industry. However, it is mandatory to produce metagenomic-derived enzymes by the use of recombinant expression techniques with genetically engineered organisms. As described above, *Lactobacilli* may be the ideal host candidates for the recombinant production of that kind of new biocatalysts for food applications.

Thesis Outline

The research described in this thesis was initiated to gain a better understanding of *Lactobacillus* sp. as food-grade expression systems for recombinant enzymes. Better cultivation strategies as well as new promoters and food-grade selection markers may lead to more efficient and economic approaches for recombinant enzyme production regarding the food-grade concept. All in all, the development and deep knowledge of *Lactobacilli*-based expression systems offer unprecedented opportunities for new products derived from GMO organisms. The results described in this thesis may aid new processes for recombinant enzyme production for food applications.

Chapter 1 provides an introduction to the thesis and gives an overview of general aspects of lactic acid bacteria food-grade expression systems. Throughout this thesis, new processes were designed, promoters analysed and possible new selection markers investigated mainly with *Lb. plantarum* as the host organism. Almost all studies of *Lactobacilli* as recombinant expression systems were conducted to date only on a laboratory scale by shaking-flask cultivation, and there is still limited knowledge about the cultivation and application of food-grade *Lactobacilli* expression systems on a bioreactor scale. In **chapter 2**, a detailed study about process optimisation in bioreactors with recombinant *Lactobacillus* sp. using the well-known pSIP expression system was carried out. About 60% higher yields of recombinant enzyme were obtained using fed-batch cultivation strategies. A comparison of the *Lb. plantarum*/pSIP409 expression system with the well-known *E. coli* BL21/pET expression system and the *K. lactis*/pKLAC2 expression system with a new metagenome-*β*-galactosidase as the target enzyme is presented in **chapter 3**. Growth of *Lb. plantarum* under manganese-limiting conditions was investigated in **chapter 4**, resulting in the development of a new kind of manganese starvation-inducible promoter system for *Lb. plantarum.* This auto-inducing promoter system enables food-grade recombinant enzyme production without the need of the addition of specific inducers. In **chapter 5**, the glutamic acid racemase (MurI) of *Lb. plantarum* NC8 was expressed, purified and characterised. This first study showed that the assigned *murI* gene of *Lb. plantarum* NC8 codes for a glutamic acid racemase. The racemase, after generations of knock out mutants, may be a food-grade complementation selection marker.

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Chapter 2

2. Recombinant production of hyperthermostable CelB from *Pyrococcus furiosus* in *Lactobacillus* sp.

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Abstract

Lactic acid bacteria (LAB) are used widespread in the food industry as traditional starters for various fermented foods. For recombinant protein production, LAB would be superiour with view from the food safety demands since most of them are Generally Recognized As Safe (GRAS) organisms. We investigated the two pSIP expression systems, pSIP403 and pSIP409 (Sørvig et al. 2005), to produce a hyperthermophilic *β*-glycosidase (CelB) from *Pyrococcus furiosus* in *Lactobacillus plantarum* NC8 and *Lactobacillus casei* as hosts, respectively. Both lactobacilli harbouring the pSIP409-celB vector produced active CelB in batch bioreactor cultivations (MRS medium) while the specific CelB activity of the cell free extract was about 44% higher with *Lb. plantarum* (1,590 ± 90 nkat/$mg_{protein}$) than with *Lb. casei* (1,070 ± 66 nkat/$mg_{protein}$) using *p*-nitrophenyl-*β*-galactoside (pNPGal) as the substrate. A fed-batch bioreactor cultivation of *Lb. plantarum* NC8 pSIP409-celB resulted in a specific CelB activity of 2,500 ± 120 nkat $_{p\text{NPGal}}$ /$mg_{protein}$ after 28 h. A repeated dosage of the inducer spp-IP did not increase the enzyme expression further. As alternative for the cost intensive MRS medium, a basal whey medium with supplements (yeast extract, Tween 80, NH_4-citrate) was developed. In bioreactor cultivations using this medium 556 ± 29 nkat $_{p\text{NPGal}}$ /$mg_{protein}$ of CelB activity was achieved. It was shown, that both LAB were potential expression hosts for recombinant enzyme production. The pSIP expression system can be applied in *Lb. casei*.

Introduction

Lactobacillus sp. are gram positive, facultative anaerobic microorganisms belonging to the diverse group of lactic acid bacteria (LAB) whose primary fermentation end product is lactic acid when grown on sugars. The genus *Lactobacillus* contains more than 100 species (Canchaya et al. 2006), and is used extensively for fermentation in the food industry. *Lactobacillus* sp. are added as starter or adjunct cultures in different food products such as yogurt, cheese, sausages and fermented vegetables (Konings et al. 2000). Additionally, several *Lactobacillus* sp. are applied as probiotic strains with a beneficial effect on healthy human gut microbiota. They do not generate endotoxins and are nonsporulating. Thus, many lactobacilli obtained the generally recognized as safe (GRAS) status (Bernardeau et al. 2008). Due to a broad range of carbon sources, which can be metabolized, LAB were used for the production of lactic acid from whey for decades (Kulozik and Wilde 1999). There is a good knowledge about the cultivation of lactobacilli in a bioreactor (Schiraldi et al. 2003). Therefore, the interest to employ lactobacilli for biotechnological applications, like production hosts for recombinant proteins, increased over the last decade. Here, recombinant lactobacilli demonstrated potential as delivery systems for pharmaceutical proteins like truncated antibodies or antigens and, especially to the needs of the food industry, as cell factories for the production of so called “food grade” enzymes (Renault 2002; de Vos 1999). All these reasons explain why different lactobacilli expression systems have been developed recently. Several of the expression systems for lactobacilli are based on the regulatory system of antimicrobial peptides called bacteriocins. The production of bacteriocins by lactobacilli is strictly controlled by strong inducible promoters and, regulated via quorum sensing mechanism based on a secreted peptide pheromone. A two-component system is activated by action of this peptide pheromone. The first step of this control mechanism is the binding of the pheromone on a histidine kinase which is located in the cell membrane. By this the signal is transmitted to an intracellular response regulator, which gets phosphorylated and enhances the transcription of the operons for bacteriocin production. The best known bacteriocin based expression system is the so called NIsin Controlled Expression (NICE) system from *Lactococcus lactis* (Mierau and Kleerebezem 2005; de Ruyter et al. 1996). Another of these systems is the so called pSIP expression system (Mathiesen et al. 2004; Sørvig et al. 2003). The pSIP system is one of the best performing expression system for

lactobacilli. It is derived from *Lb. sakei* and uses the regulatory elements from the class IIb bacteriocins, sakacin A when using the pSIP403 vector and sakacin P when using the pSIP409 vector. The production of the heterologous proteins is induced by an externally added peptide pheromone. Different kinds of enzymes like aminopeptidase N from *Lb. lactis,* *β*-glucoronidase from *Escherichia coli* and several *β*-galactosidases from *Lactobacillus* sp. were recombinantly expressed with high yields in *Lb. plantarum* and *Lb. sakei*, employing the pSIP systems (Halbmayr et al. 2008; Sørvig et al. 2005). However, most expression studies were performed in shaking flask experiments and only limited results exist about the performance of the pSIP system in controlled bioreactor cultivations.

In our work the ability of recombinant *Lactobacillus* sp. harbouring the pSIP expression system was investigated for the production of CelB (EC 3.2.1.21; *β*-glucosidase) from the thermophilic archae *Pyrococcus furiosus* in bioreactor cultivations. The recombinant production of CelB was already done previously in *E. coli* (Lebbink et al. 2001). CelB has a very high *β*-galactosidase side activity of about 60% compared to its glucosidase activity. Therefore it can be used for the enzymatic formation of the prebiotic sugar lactulose by galactosylation of fructose via the transgalactosylation mechanism (Fischer et al. 1996; Mayer et al. 2010). Lactulose is a sugar with higher sweetness and better solubility than lactose. It is added as a prebiotic ingredient to infant formula products and has several possible applications in the pharmaceutical and food industry. It can be deployed in the treatment of chronic constipation and portal systemic encephalopathy and can be given to different milk products (Strohmaier 1998). For an application of CelB in the food industry it is important to use so called “food grade” expression hosts and vector systems as it is the case with *Lactobacillus* sp. and the pSIP system.

Material and methods

Chemicals and Enzymes

All chemicals were of analytical grade or higher and supplied from Sigma-Aldrich (Seelze, Germany) or Carl Roth (Karlsruhe, Germany). Bayolan PT ultra-filtrated whey powder was obtained from BMI Bayerische Milchindustrie eG (Landshut, Germany). T4 DNA Ligase and Hexokinase/Glucose-6-Phosphate Dehydrogenase (HK/G6P-DH) were purchased from Roche (Mannheim, Germany). All restriction enzymes were from New England Biolabs (Frankfurt, Germany). HotStar HiFidelity Polymerase was purchased from Qiagen (Hilden, Germany).

Bacterial Strains and Culture Conditions

Bacteria used in this study are listed in table 1. *Escherichia coli* XL1-blue was purchased from Stratagene (Santa Clara, USA), and grown in Luria-Bertani media at 37℃ with shaking (120 rpm). *Lactobacillus plantarum* NC8 was obtained from the culture collection of the Norwegian University of Life Science (Ås, Norway) and grown in MRS media according to De Man Rogosa and Sharp (De Man et al. 1960) or in whey based medium, developed in this work, at 30℃ with shaking (90 rpm). *Lactobacillus casei* 2421 was obtained from the culture collection of the Department of Food Microbiology, Institute of Food Science and Biotechnology, University of Hohenheim. *Lb. casei* 2421 is originating from sauerkraut, and was grown in MRS media at 37℃ with shaking (90 rpm). Agar plates we re solidified by adding 1.5% (w/v) agar. When required, erythromycin was added as follows: 200 µg/mL for *E. coli* and 10 µg/mL for *Lb. plantarum* and *Lb. casei*. Ampicillin was added when required with a concentration of 100 µg/mL for *E. coli*. The sakacin P inducing peptide (spp-IP) used for induction studies was obtained from EMC Microcollections (Tübingen, Germany) and it was added to the growth medium to accomplish a final concentration of 50 ng/mL.

Table 1 Bacterial strains and plasmids

Strains and plasmids	Characteristics	Source or reference
E. coli XL1	Host strain	Novagene
Lb. plantarum NC8	Host strain, silage isolate, plasmid free	(Axelsson et al. 2003)
Lb. casei 2421	Host strain, sauerkraut isolate, plasmid free	Our institute
pLUW511	pET9d derivate carrying CelB gene	(Lebbink et al. 2001)
pLUW511_KpnI	Source of CelB gene with NcoI and KpnI sites	This work
pSIP403	p256rep/pUC(pGEM)ori; P_{sppA}::gusA; Em^R	(Sørvig et al. 2005)
pSIP409	p256rep/pUC(pGEM)ori; P_{orfX}::gusA; Em^R	(Sørvig et al. 2005)
pSIP403-celB	p256rep/pUC(pGEM)ori; P_{sppA}::celB; Em^R	This work
pSIP409-celB	p256rep/pUC(pGEM)ori; P_{orfX}::celB; Em^R	This work

Cultivation in microtiter plate scale

A whey based medium for *Lb. plantarum* was developed by using a microtiter plate equipped Bioscreen C automatic cultivator (LabSystems, Finland). Ultra-filtrated whey powder was used as carbon source instead of glucose. The solubilized ultra-filtrated whey (UFW) powder was dissolved in water, the solution heated up for 15 min to 90℃ and centrifuged at 8000 *g* in order to separate from the denaturated protein. After that, the other supplements were added to the UFW-medium and it was autoclaved. The following components and concentrations were used: Ultra filtrated whey (30-100 g/L), yeast extract (0-5 g/L), $MnSO_4$ (0-0.05 g/L), TWEEN 80 (0-1 g/L) and NH_4-citrate (0-2 g/L). For details of media composition see results section. Cultivation was done at 30℃ with continuous shaking in microtiter plates. Determination of growth was done in 250 µL scale. 240 µL of the autoclaved medium

were inoculated with 10 µL of an overnight starting tube culture in the respective medium. Four wells were used as quadruplicate testing of each media. Optical density at 600 nm (OD_{600nm}) was measured and readings for each well were taken every 15 min for 16 h.

Preparation of Plasmids, Cloning and Transformation

The lactobacilli expression vectors pSIP403 and pSIP409 were kindly provided by Dr. Lars Axelsson, MATFORSK Norwegian Food Research Institute (Ås, Norway). Construction of the expression vectors pSIP403-celB and pSIP409-celB was done using *E. coli* XL1-blue and standard molecular biology techniques (Ausubel 1994). Plasmids used in this study are listed in table 1. The celB gene was translationally cloned to the promoters of the pSIP403 and pSIP409 vectors using the *Nco*I site. Cloning of the CelB gene was done using *Nco*I and *Kpn*I restriction sites. The pLUW511 vector, kindly provided by Dr. Kengen, University of Wageningen, was the source of the celB gene. The *Kpn*I restriction site was constructed in the pLUW511 vector by exchange of the *Bam*HI site through quick change PCR using pLUW_KpnI_fw (5'-CGGGCTTTGTTAGCAGCCGGTACCCTACTTTCTTGTAAC) and pLUW_KpnI_rev (5'-GTTACAAGAAAGTAGGGTACCGGCTGCTAACAAAGCCCG) Primer. The PCR was done with an initial denaturation step of 95 ℃ for 5 min, followed by 14 cycles of denaturation at 95℃ for 1 5 sec, an annealing at 52℃ for 1 min and elongation at 68℃ for 12 min. This result ed in the pLUWKpnI plasmid after *Dpn*I digestion and transformation. It was used as source of the CelB gene to construct pSIP403-celB and pSIP409-celB expression plasmids. The plasmids were transformed into *E. coli*. Plasmid DNA was purified from *E. coli* by the QIAprep Spin Miniprep Kit from Qiagen (Hilden, Germany) and subsequently transformed into lactobacilli like described in literature (Aukrust and Blom 1992). After electroporation positive clones were selected by plating on MRS agar containing 10 µg/mL erythromycin and incubation for 24-48 h at the required temperatures under anaerobic atmosphere using an anaerobic jar and Anaerocult A reagent from Merck (Darmstadt, Germany). Plasmid DNA was purified from overnight cultures of lactobacilli after centrifugation (13000 *g,* 10 min, 4℃) by incubation of the cells for 1 h at 37 ℃ with 1 ng/mL Lysozyme and 50 U/mL Mutano lysin. Lactobacilli DNases were denaturated for 20 min at 70℃, following by t he QIAprep Spin Miniprep Kit from Qiagen according to the manufacturer.

Expression experiments in shaking flask

First expression experiments were done in baffled shaking flasks (500 mL) under aerobic conditions. Freshly prepared lactobacilli clones were grown over night in test tubes (5 mL volume) and transferred to 50 mL MRS Medium, pH 6.4, with erythromycin to an OD_{600nm} about 0.1 at 30℃ like described above.
Induction was done with 50 ng/mL spp-IP at different optical densities to find the best expression conditions. The best performing expression times were analyzed by harvesting the cells at different times. After centrifugation (10 min, 8000 *g*) the cells were washed and resuspended in sodium acetate buffer (50 m*M*, pH = 5.0) before disruption. Cells were disrupted by grinding with glass beads (diameter 0.1-0.11 mm) using a bead mill from Rentsch (Haan, Germany). Cell free extract (supernatant) was obtained after separation the cell debris by centrifugation (10 min, 8000 *g*, 4℃).

Fermentation experiments in bioreactor

Expression experiments were continued in a Multifors parallel bioreactor system from Infors (Bottmingen, Switzerland) in 500 mL working volume. The lactobacilli were cultivated at 100 rpm, N_2 gassing < 0.1 vvm ($volume_{gas}$ per $volume_{medium}$ and min). The pH was controlled at pH = 6.45 by addition of 1 *M* NaOH. The experiments were performed in batch and fed-batch mode. The latter with addition of glucose to a final concentration of ≥10 g/L at different periods of cultivation when the glucose concentrations depleted below 2 g/L. Induction was done at OD_{600nm} = 0.3 with 50 ng/mL spp-IP. Samples were withdrawn throughout the fermentation to analyse biomass, carbon source as well as CelB activity. Lactobacilli precultures were grown over night in test tubes (5 mL volume), then transferred into 50 mL appropriate medium in shaking flasks and grown over night (MRS-Ery or UFW-Ery). The bioreactor was inoculated with 50 mL of this overnight cultures. The cells were harvested and disrupted like described above.

Analytical Methods

Cell growth was monitored by measuring the optical density at 600 nm. Biomass was quantified gravimetrically as cell dry weight of cells (CDW). Samples were centrifuged, twicely washed with saline, and dried in pre-weighted tubes at 40℃ at 10 mbar in a RVC 2-33 IR vacuum centrifuge from Christ (Osterode, Germany).

Protein concentrations were determined by the method of Bradford with bovine serum albumin as standard (Bradford 1976). Expression of CelB was analyzed by SDS-PAGE using 12% polyacrylamid gel according to the method of Laemmli (Laemmli 1970). Therefore 5 µg of protein were loaded onto a gel, separated, and stained with Coomassie blue R250 like described by Fairbanks (Wong et al. 2000).
The glucose concentrations were enzymatically determined with HK/G6P-DH-test kit by a photometric assay at 340 nm in microtiter plates, following the manufacturers protocol for the D-glucose/D-fructose test kit (R-Biopharm AG, Darmstadt, Germany; product code 10 139 106 035). Lactose concentrations from ultra filtrated whey based medium were analyzed by HPLC. This was done using a ThermoFinigan Surveyor system (degaser, LC pump, autosampler) equipped with a Ca^{2+} column (300 mm x 7.8 mm Rezex, Phenomenex, Aschaffenburg, Germany) at 85℃ with a Sedex 75 evaporative light scattering detector (Sedere, France). Elution was done isocratic with water and a flow rate of 0.5 mL/min.

Enzyme activity measurements

CelB activity (EA) was determined in 50 m*M* sodium acetate buffer, pH 5.0, using *p*-nitrophenyl-*β*-D-galactopyranoside (*p*NPGal) as substrate at 75 ℃ (1 mL scale) as described previously (Mayer et al. 2004). Prior activity determination, the cell free extracts (see above) were heat denaturated for 15 min at 75 ℃ and the interfering heat-labile host proteins were precipitated by centrifugation (10 min, 8000 *g*, 4℃). Afterwards 100 µL of the clear enzyme solution was added to the substrate solution (25 m*M* *p*NPGal). Both solutions were pre-heated for 10 min at 75 ℃ separately. The release of *p*-nitrophenol was detected photometrically over a time period of two minutes at 405 nm using a temperature controlled cuvette in a spectrophotometer (Ultrospec 3000, GE Healthcare, Freiburg, Germany). CelB activity was calculated from the slope of the straight line with the molar absorption coefficient of 0.523 L/mmol/cm and was done at least three times. One nanokatal was defined as the amount of enzyme that catalyzed the release of 1 nmol of *p*-nitrophenol from *p*NPGal per second.

Results

Construction of expression vectors and generation of recombinant lactobacilli

In this study the CelB gene was cloned in the pSIP403 and pSIP409 vectors by exchanging the gusA gene, present in these vectors, to obtain the pSIP403-celB and pSIP409-celB vectors (see table 1). The source of the CelB gene was the pLUW511 *E. coli* expression vector. The required *Nco*I restriction site was present in the pLUW511 vector, the missing *Kpn*I site was constructed by a quick change PCR resulting in pLUW511_KpnI vector. Out of this vector the CelB gene was cloned in the pSIP expression vectors. Construction and storage of the vectors was done initially in *E. coli* XL1. Lactobacilli competent cells were transformed by electroporation and were plated for selection on MRS-Erythromycin medium. Positive clones were analyzed by plasmid isolation and restriction with *Nco*I and *Kpn*I resulting in a 1.4 kb fragment of the CelB gene. These positive clones were chosen for further expression experiments.

Expression of CelB in shaking flask experiments

First expressions of CelB were done using *Lb. plantarum* NC8 (Halbmayr et al. 2008; Sørvig et al. 2005) and either the vector pSIP 403-celB or pSIP409-celB under the same cultivation conditions in 50 mL scale (MRS medium, 30℃, starting pH 6.45, aerobic). Both vectors contained different promoters P_{sppA} and P_{orfX}, respectively, which were investigated for recombinant CelB production. The expressions were induced by 50 ng/mL spp-IP at an OD_{600nm} of 0.3, followed by cell harvest at an OD_{600nm} of 1.8. The cells harbouring the pSIP409-celB plasmid reached a higher specific *β* -galactosidase activity of 170 $nkat_{pNPGal}$ / $mg_{protein}$ in comparison to the cells harbouring pSIP403-celB with 151 $nkat_{pNPGal}$ / $mg_{protein}$. Thus, for further expression experiments the pSIP409-celB vector was chosen. The moments of induction and of harvesting were varied (table 2). The best result was obtained when the time between induction (OD_{600nm} 0.3) and harvesting (OD_{600nm} 6.0) was longest (6 h). Here, the specific CelB activity reached 426 $nkat_{pNPGal}$ / $mg_{protein}$.

Table 2 Specific *β*-galactosidase activities of CelB in cell free extracts of *Lb. plantarum* NC8 harbouring pSIP409-celB plasmid

OD_{600nm}			
induction	harvest	period of induction [h]	specific activity [$nkat_{pNPGal}/mg_{protein}$]
0.3	1.8	2.5	169
0.3	6.0	6	426
1.0	6.0	4	245

Production of CelB in bioreactor experiments

For the bioreactor cultivations of the lactobacilli a parallel bioreactor system (Multifors, 500 mL working volume, 100 rpm) with pH control (pH 6.45) and anaerobic gassing (0.1 vvm N_2) was used.

First the CelB production using the recombinant *Lb. plantarum* NC8 pSIP409-celB was assigned from shaking flask (see above, 30℃) to the anaerobic bioreactor system. The induction with 50 ng/mL spp-IP was done at an optical density of 0.3 again. The batch bioreactor cultivation led to an increase of biomass up to an OD_{600nm} ~12, equal to 4.8 g cell dry weight/L after 15 hours, when the glucose was consumed and the stationary growth phase was reached. This was a two-fold increase of biomass in comparison to the shaking flask experiments. The maximal specific growth rate for *Lb. plantarum* was determined with 0.42 h^{-1}, equal to a doubling time of 1.6 h. The cells were harvested, disrupted and, the obtained cell free extract reached a specific CelB activity of about 1590 ± 90 nkat $_{p\text{NPGal}}$ /$mg_{protein}$. This was ca. 3.7-fold of CelB activity in comparison to the shaking flask experiments.

As a novel and alternative expression host for the recombinant production of enzymes *Lb. casei* was implemented with the pSIP-vector system, to our best knowledge for the first time. *Lb. casei* was selected because of good results in preliminary expression studies in our laboratory (data not shown). The *Lb. casei* species is used in many food applications for dairy products as starter and probiotic. The cultivations of *Lb. casei* pSIP409-celB were done accordingly to *Lb. plantarum* in a bioreactor (see above), but at 37℃, that is the optimum temperature for *Lb. casei*. Figure 1 illustrates the cultivation of *Lb. casei* pSIP409-celB. After 4 hours cultivation time CelB activity was detected in the cell free extract prepared (see material and

methods). The highest specific CelB activity of 1070 ± 66 nkat $_{pNPGal}$ /$mg_{protein}$ was measured after 10 h cultivation time, which was about 69% compared to the *Lb. plantarum* host system. However, the functionality of the pSIP-vector system in *Lb. casei* was successfully demonstrated. Compared with the *Lb. plantarum* NC8 pSIP409-celB batch cultivation (see above), *Lb. casei* pSIP409-celB reached the stationary growth phase faster after ca.10 h of cultivation (see figure 1). The OD_{600nm} came up to a value of ~13, equal to the same biomass of 4.8 g cell dry weight/L as with *Lb. plantarum* pSIP409-celB in the standard MRS medium. Thus, in difference to *Lb. plantarum*, the maximal specific growth rate of *Lb. casei* was 0.59 h^{-1} (doubling time of 1.2 h). Because in batch cultivations the higher CelB activities were achieved with the *Lb. plantarum* pSIP409-celB expression system we investigated this expression system further.

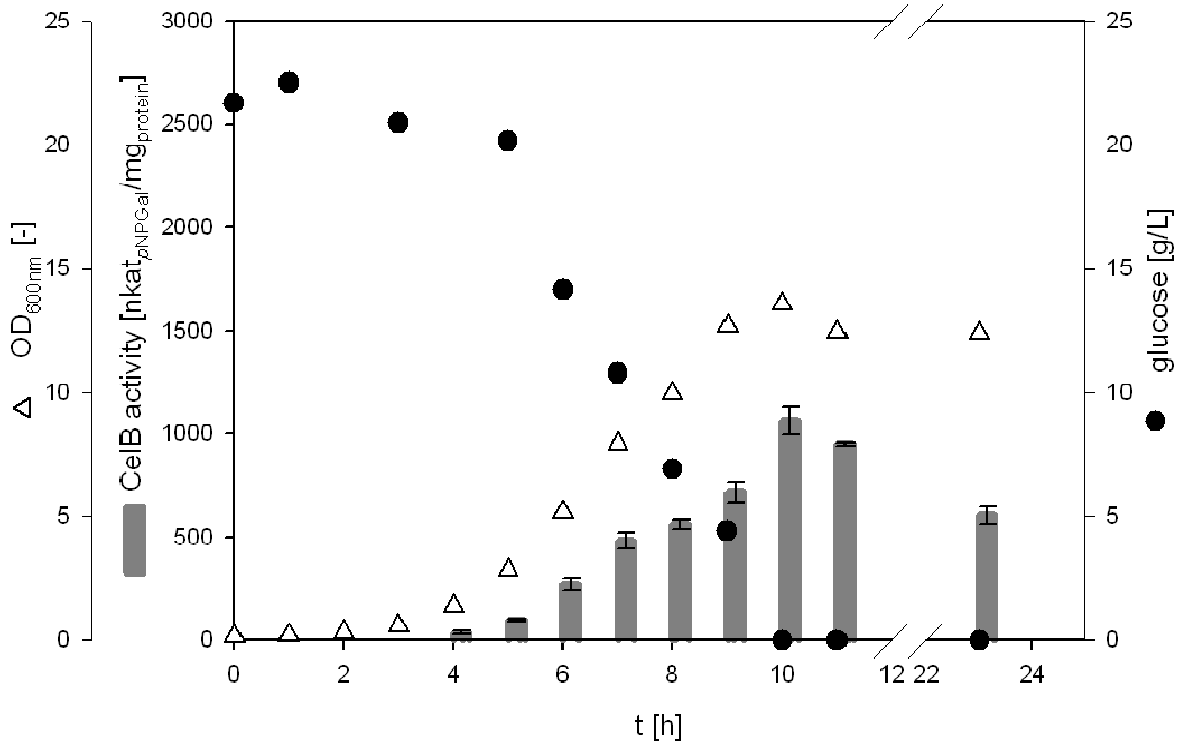

Figure 1 Recombinant production of CelB from batch fermentation of *Lb. casei* pSIP409-celB with MRS medium at 37℃

(Open triangle: OD600nm; filled circle: glucose concentration; bars: specific CelB activity, error bars indicate the standard deviation)

Due to the fact, that the highest CelB activities were obtained at the beginning of the stationary growth phases of the recombinant lactobacilli, we presumed a better performance of the recombinant CelB production by an extended growth phase and

at higher biomasses. Thus we cultivated *Lb. plantarum* pSIP409-celB using a fed-batch strategy. The conditions were as described before (see above, 30℃). Now, a sterile glucose solution (400 g/L) was injected into the bioreactor when the glucose concentration depleted to 2 g/L in order to raise the glucose concentration of above 10 g/L again. The fed-batch cultivation of *Lb. plantarum* pSIP409-celB is shown in figure 2.

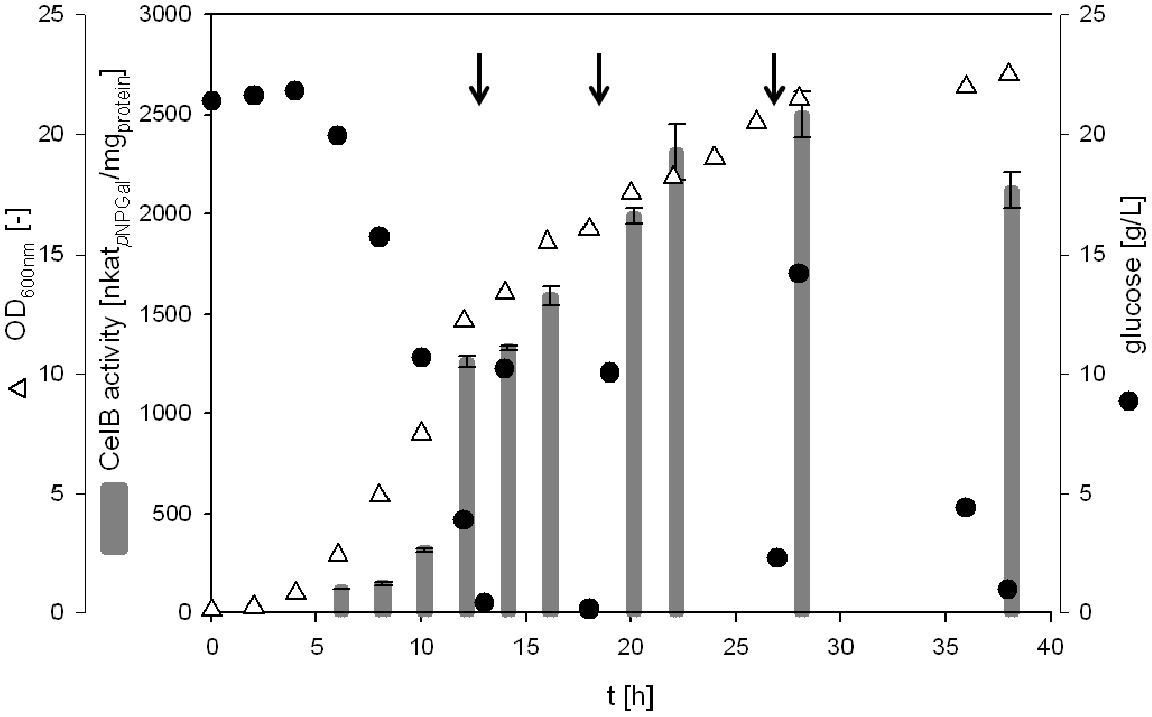

Figure 2 Recombinant production of CelB from fed-batch fermentation of *Lb. plantarum* pSIP409-celB with MRS medium at 30℃

(Open triangle: OD_{600nm}; filled circle: glucose concentration; bars: specific CelB activity, error bars indicate the standard deviation, arrows indicate glucose feed)

The stationary growth phase was reached after 28 hours although the glucose concentration was still at about 14 g/L. So, one or more of the essential complex medium components (*e.g.* amino acids, vitamins) of the MRS medium was consumed and further growth of the cells was stopped. At this time a biomass of about 7.0 g cell dry weight/L and an OD_{600nm} = 22.5 was determined. The highest specific CelB activity of 2500 ± 120 nkat $_{p\text{NPGal}}$ /$mg_{protein}$ was measured at the beginning of the stationary growth phase after 28 hours. This value was about 57% more compared to the batch cultivation. After 38 hours of cultivation the glucose was nearly consumed and the specific CelB activity of the cells decreased to 2210 ± 90 nkat $_{p\text{NPGal}}$ /$mg_{protein}$.

The high level of CelB expression by *Lb. plantarum* pSIP409-celB was also proven by gel electrophoresis experiments (SDS-PAGE). Figure 3 shows the SDS-PAGE from samples of the cell free extracts of the wild type *Lb. plantarum* (lane 1) and of the *Lb. plantarum* pSIP409-celB (lanes 2 and 3). The overexpressed CelB was the visible protein band at the expected molecular mass of about 56 kDa.

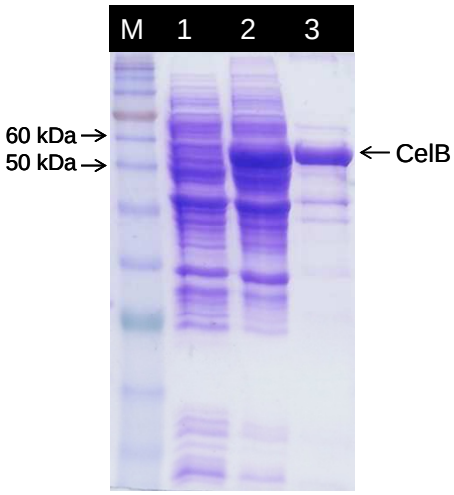

Figure 3 SDS-PAGE of intracellular protein production of *Lb. plantarum*
M: Molecular weight marker; lane 1: *Lb. plantarum* wild type (5 µg); lane 2: *Lb. plantarum* pSIP409-celB from fed batch cultivation after 28 h (5 µg); lane 3: *Lb. plantarum* pSIP409-celB heat denaturated crude extract (5 µg).
CelB protein (molecular mass 56 kDa).

In addition, the influence of a repeated induction with the inducer spp-IP on the CelB production was tested in the cultivation experiments with glucose feeding. The peptide spp-IP was added to the glucose stock solution and co-injected after 13 h (final concentration of 50 ng/mL spp-IP). This action decreased the maximum specific CelB activity to a value of 1860 ± 90 nkat $_{p\text{NPGal}}$ /mg$_{\text{protein}}$ after 28 h of cultivation. So, the highest CelB activity with *Lb. plantarum* pSIP409-celB was obtained using pure glucose feeding resulting in a calculated volumetric CelB activity of 625 μkat/L$_{\text{culture}}$.

Development of a whey based medium

Due to the auxotrophic metabolism of lactobacilli, the MRS standard medium is a very reliable complex medium accredited with diverse essential salts and organic N-sources. However, the MRS medium is quite expensive. So, we investigated ultra-filtrated whey powder, which was supplemented with particular additives, as a potential low cost medium for the cultivation, first with the wild-type of *Lb. plantarum* NC8 (see table 3).

Table 3 Growth of *Lb. plantarum* in microtiter plate scale in different whey based media (250 µL, 30°C, continuous shaking, quad ruplicate test for each media, standard deviation < 2%)

#	UFW [g/L]	YE [g/L]	$MnSO_4$ [g/L]	T [g/L]	NH_4-C [g/L]	OD_{600nm} [-]
1	55	-	-	-	2	0.03
2	55	-	0.05	-	2	0.03
3	55	-	0.05	1	2	0.03
4	55	2.5	0.02	-	-	0.03
5	55	-	-	-	-	0.04
6	55	-	0.05	1	-	0.04
7	55	-	-	1	-	0.05
8	30	5.0	0.05	1	-	0.10
9	55	5.0	-	-	-	0.24
10	100	-	0.05	-	-	0.39
11	55	5.0	0.02	-	-	0.69
12	55	5.0	0.05	-	-	1.10
13	55	5.0	0.01	-	-	1.10
14	100	5.0	0.05	-	-	1.27
15	55	5.0	0.05	1	-	1.43
16	MRS medium					2.17

UFW = ultra-filtrated whey powder; YE = yeast extract; T = TWEEN 80; NH_4-C = NH_4-citrate

The medium screening was accomplished in microtiter plate cultivations (total volume 250 µL). The media with a concentration of 55 g ultra-filtrated whey powder/L resulted in finally 45 g lactose/L. The added yeast extract was used as organic N-source and complex nutrient source, respectively. NH_4-citrate was tested as an additional N-source. Tween 80 was known as important source for fatty acids when cultivating lactobacilli. Also manganese was known for optimal growth of *Lb. plantarum* and it was added to the medium as $MnSO_4$. The standard MRS medium was used in parallel as reference (table 3). The highest biomass estimated by OD_{600nm} measurement was achieved with a medium containing 55 g ultra-filtrated whey powder/L, 5 g yeast extract/L, 1 g Tween 80/L and 0.05 g $MnSO_4$/ L (OD = 1.43). This was about 66% compared to the standard MRS medium (OD = 2.17).

Production of CelB with whey based medium in a bioreactor

The ascertained whey based medium (see above) was used for cultivation experiments of *Lb. plantarum* pSIP409-celB in a bioreactor (500 mL scale, pre-cultures were also grown in the same whey based medium). This cultivation is shown in figure 4.

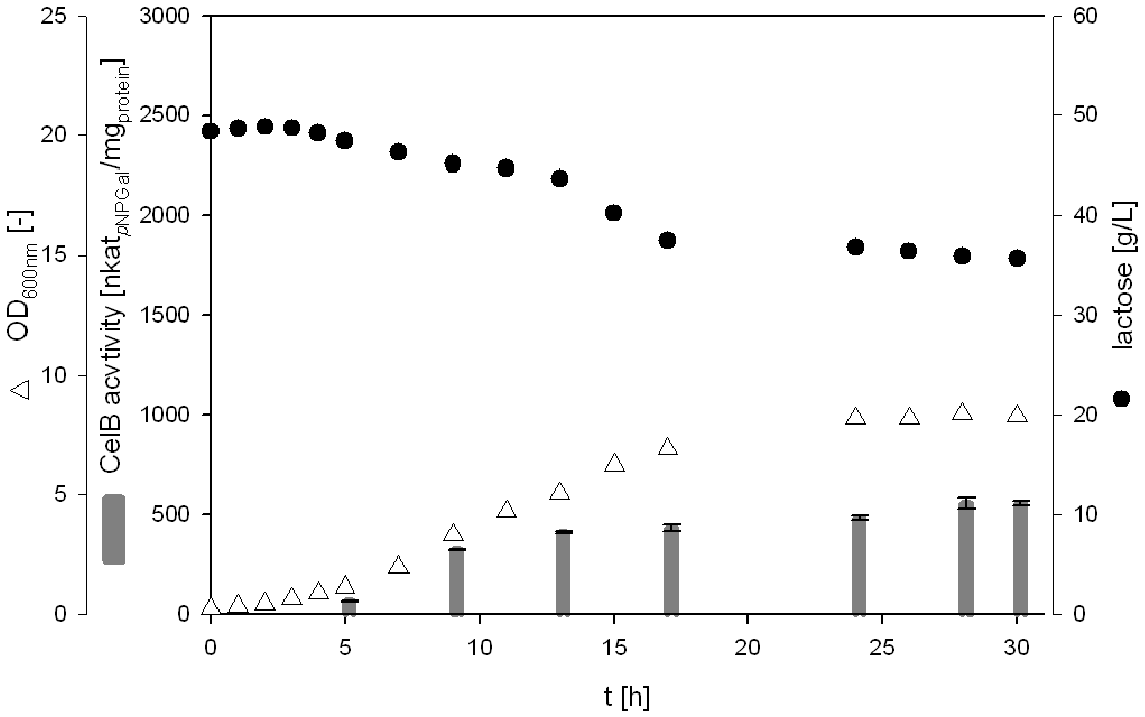

Figure 4 Recombinant production of CelB from batch fermentation of *Lb. plantarum* pSIP409-celB with whey based medium at 30℃

(Open triangle: OD_{600nm}; filled circle: lactose concentration; bars: specific CelB activity, error bars indicate the standard deviation)

Induction with spp-IP occurred at an OD_{600nm} = 0.3 again. As expected from the microtiter experiments cell growth was slower and biomass less when compared with the MRS medium (see bioreactor results above). The stationary growth phase was reached after 24 h of cultivation with an OD_{600nm} = 8.3 (about 65% in comparison to MRS medium). The lactose concentration decreased from 48 g/L to 35 g/L in this time, indicating a limitation of one of the needed supplements. Also the maximal CelB activity achieved after 28 h of cultivation (556 ± 29 nkat $_{pNPGal}$ /$mg_{protein}$) was significantly lower than in MRS medium (1590 ± 90 nkat $_{pNPGal}$ /$mg_{protein}$ after 15 h, see above). This was in accordance with SDS-PAGE experiments of the cell free extracts from whey based medium grown cells (data not shown). Thus, if a whey based

medium will be considered for recombinant protein production in *Lb. plantarum* further improvements are necessary. Nevertheless, the proof of principle using a whey based medium for recombinant *Lb. plantarum* as host organism was demonstrated.

Discussion

The application of new expression hosts such as lactic acid bacteria (LAB) propagated over the last decade, so nowadays not only the well known prokaryotic *E. coli* and *Bacillus* hosts are used for recombinant protein production. Particularly for the recombinant production of food relevant enzymes new expression systems based on food-grade microorganisms, which are generally recognized as safe, were generated as cell factories (Peterbauer et al. 2011). Also the use of recombinant LAB, such as *Lb. plantarum,* as potential delivery vehicles for mucosal vaccines and other therapeutic applications was discussed in the literature (Wells and Mercenier 2008).

In our studies two expression vectors, pSIP403 and pSIP409, with different promoters, were tested for CelB expression in *Lb. plantarum*. The pSIP409-celB vector showed a higher CelB expression, which was also ascertained by Sørvig et al. (2005) when comparing both vectors/promoters for *β*-glucoronidase expression in *Lb. plantarum* and *Lb. sakei*. However, Sørvig et al. (2005) harvested the cells with highest enzyme activities at an OD_{600nm} 1.8 while in our case longer cultivation periods up to OD_{600nm} 6.0 (shaking flask) or 22.5 (bioreactor), respectively, were favourable.

In bioreactor cultivations of the recombinant *Lb. plantarum* pSIP409-celB the *β*-galactosidase activity was maximum using a fed-batch strategy with glucose. Herewith a CelB activity of about 675 $\mu kat_{pNPGal}/L_{culture}$ was achieved. This was a much higher amount of enzyme activity (ca. 67-fold) then it was described for the recombinant expression of a thermophilic *α*-glucosidase from *Sulfolobus solfataricus* in *Lactococcus lactis* by batch cultivation (Giuliano et al. 2004). It has to be mentioned, that Giuliano et al. (2004) applied a NICE-system (NIsin Controlled Expression system) with *L. lactis* and that the activities of the thermophilic *α*-glucosidase were detected with thermo-treated whole cell biocatalysts. The NICE-system is the best known expression system for *L. lactis* and comparable to the pSIP system based on a bacterocin induction. Another study from Halbmayr et al. (2008) described the recombinant production of *β*-galactosidases from *Lactobacillus* sp. in *Lb. plantarum* and *Lb. sakei*. The highest expression levels were found with *Lb. plantarum* WCFS1 harbouring pSIP403 containing *β*-galactosidase genes from *Lb.*

reuteri with 383 μkat_{oNPGal}/L (Halbmayr et al. 2008). In our study the expression level of CelB acting as a *β*-galactosidase was 1.8 times higher.

Nevertheless it must be mentioned, that the wild-type *P. furiosus* produced 300 $nkat_{pNPGal}/mg_{protein}$ CelB in shaking flasks (Kengen et al. 1993), corresponding to two-third of these activities in recombinant *Lb. plantarum* in shaking flasks. Furthermore, when CelB was recombinantly expressed in the *E. coli* pET system, one of the strongest known expression systems, the *β*-galactosidase activity reached values up to 4300 $nkat_{pNPGal}/mg_{protein}$ (Lebbink et al. 2001). That is 1.7 times more than in our expression system *Lb. plantarum* pSIP409-celB.

The recombinant production of CelB was done in our studies also in *Lb. casei* 2421. To our best knowledge that is the first time, that the pSIP expression system was succesfully deployed in *Lb. casei*. There are different other expression systems described for the use in *Lb. casei.* For example the promoter system based on the phage ΦFSW regulatory elements or the constitutive promoter of pepR from *Lb. rhamnosus* (Binishofer et al. 2002; Takala et al. 2003). The functionality of the pSIP vector system in *Lb. casei* may be due to the coding for the regulatory elements on the pSIP vector. So the histidine kinase and response regulator, coding on the expression vector and acting as regulatory elements, can be expressed in *Lb. casei*. There they may operate regulatory like in the native host *Lb. sakei* and in *Lb. plantarum*. It was also described, that a *Lb. casei* strain, named CRL 705, has a class IIb bacteriocin producing system, which is quite similar to the sakacinP system from *Lb. sakei*. Even the inducing peptides spp-IP and Lactocin705*β* from *Lb. casei* share 50% similarity (Cuozzo et al. 2000). So it is also possible, that a native *Lb. casei* regulation mechanism for bacteriocin production recognizes and interacts with the vector based system additionally. In our studies the CelB activities of the *Lb. casei* expression host were not as high as with *Lb. plantarum* as host, so modifications of the cultivation strategies were done for *Lb. plantarum* only. Anyhow the pSIP-system should be considered as system, which is functional in *Lb. casei*, too. Other recombinant proteins may be expressed as well or even better in *Lb. casei* then in *Lb. plantarum*.

In this study supplemented whey based medium for the cultivation of *Lb. plantarum* and recombinant expression of CelB was investigated. The standard MRS medium is a very costly one (lab price about 6.40 €/L), which was replaced for several applications by low cost media (Sawatari et al. 2006; Trinetta et al. 2008). It was

known, that lactobacilli grew well on whey, a huge by-product of the dairy industry (Brinques et al. 2010; Kulozik and Wilde 1999). The recombinant protein production in *E. coli* was also carried out using whey and ultra filtrated whey (Viitanen et al. 2003). We used ultra filtrated whey powder as basal low cost medium supplemented with other essential nutrients for LAB. A similar approach was done in order to cultivate *Lb. plantarum* UG1 for bacteriocin production.

The best performing medium mixture consisting of ultra-filtrated whey permeate powder, yeast extract, Tween 80 and $MnSO_4$ was quite inexpensive (lab price about 0.65 €/L) compared to the MRS medium. This medium fulfils the food grade requirements. The final biomass obtained with this medium was 65% of the biomass achieved in MRS medium. For commercial considerations, the partly lower activity of recombinant enzyme gained with this medium probably would be negligible due to the much lower medium costs. So, this whey based medium might be a starting point for the development of a low cost alternative in recombinant enzyme production using *Lb. plantarum* in the future. The overall results confirmed that lactobacilli are an interesting host organism in the recombinant production of enzymes for the food industry.

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Chapter 3

3. Recombinant production of a Metagenome-*β*-Galactosidase using three different expression host systems

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Abstract

β-Galactosidases are industrially important enzymes with manifold applications in the food industry. *β*-galactosidases are necessarily produced on a large scale with a high yield giving them a commercially acceptable price for industrial applications. We have compared the expression of a new metagenome-*β*-galactosidase on a bioreactor scale in the well-known *E. coli* BL21/pET expression system, the food-grade *Klyuveromyces lactis*/pKLAC2 expression system and the food-grade *Lb. plantarum*/pSIP409 expression system. The highest *β*-galactosidase activities of 82.01 $nkat_{oNPGal}/mg_{protein}$ were obtained with the *E. coli* expression system, which was also used as the host organism during the metagenome screening. Much lower activity levels of 0.2% (*Lb. plantarum*) to 2.5% (*K. lactis*) were observed in the food-grade expression systems. Therefore, the *E. coli* expression system used for the screening approach is also the preferable system for further expression of the metagenome-*β*-galactosidase for industrial applications.

Keywords

Metagenome-β-Galactosidase; *Escherichia coli*; *Lactobacillus plantarum*; *Kluyveromyces lactis*; recombinant production; comparison

Introduction

β-Galactosidases (E.C. 3.2.1.23) catalyse the hydrolysis of the disaccharide lactose to glucose and galactose. They are industrially important enzymes with a widespread use in the dairy industry (Harju et al. 2012). They are applied for the hydrolysis of lactose in milk or derived products and for the utilisation of lactose in whey as a by product from the cheese industry. In addition to the hydrolysis of lactose, the synthesis of prebiotic galacto-oligosaccharides is also performed by these enzymes with transgalactosylation activities (Gosling et al. 2010). Production of the β-galactosidases on a large scale and with a high yield is important to achieve a commercially acceptable price for application in the food industry. Therefore, most of the commercially available β-galactosidases used today in the industry are derived from *Kluyveromyces* ssp. or *Aspergillus* ssp., which are known to produce high yields of these enzymes cost effectively, e.g. by a rapid growth on lactose as a carbon source. Beside the β-galactosidases of this species, the use of recombinant technology for the production of β-galactosidases of different origin with high yields has emerged over the last few years (Oliveira et al. 2011). Thus, new β-galactosidases from the metagenome with superior properties have also become available for the dairy industry (Niehaus and Eck 2012; Wang et al. 2010). Due to the drawbacks of commercially available β-galactosidases, such as an inhibition by galactose, sodium or calcium, or less activity at low temperatures, new enzymes are highly desirable.

Several expression systems and host organisms, such as *Escherichia coli*, *Pichia pastoris, Bacillus subtilis*, or *Lactobacillus* ssp. have been applied for the recombinant production of β-galactosidases. At present, it is impossible to predict on a rational basis which host strain may be the best choice for the production of a given heterologous protein (Terpe 2006). Therefore, performance of different expression systems has to be analysed. The *E. coli* expression system is usually the first choice for laboratory investigations and initial development due to the good knowledge and handling over decades (Sorensen and Mortensen 2005). Other expression systems used for the demands of the food industry, such as *K. lactis* or food-grade *Lactobacillus* ssp., are the more desirable host systems. While the *K. lactis* system has been used for more than 20 years for the recombinant production of recombinant enzyme preparations, also on a large scale, *Lactobacillus* ssp. has received more

attention in the last decade as an alternative expression host (Peterbauer et al. 2011; van Ooyen et al. 2006).

In the present study, we examined the production of a metagenome-β-galactosidase in three different expression host systems (*E. coli, Lb. plantarum* and *K. lactis*), analysed the performance of the different systems and optimised the production in *E. coli,* the best one of those compared.

Materials and methods

Strains, vectors, reagents, and media

E. coli XL1-blue (Stratagene, Waldbronn, Germany) was used for the propagation and manipulation of plasmids. *E. coli* BL21 and the pET20b(+) expression vector from Novagene (Darmstadt, Germany). *E. coli* were grown in either LB or 2YT medium at 20-37℃ with shaking (120 rpm). *Lactobacillus plantarum* NC8 and the pSIP409 expression vector were provided by Dr. Lars Axelsson, MATFORSK Norwegian Food Research Institute (Ås, Norway). *Lb. plantarum* was grown in MRS medium at 30℃ with shaking (90 rpm). *Kluyveromyces lactis* GG799 and the pKLAC2 expression vector were from New England Biolabs (Frankfurt, Germany). *K. lactis* was grown either in YCB or YPGal medium at 30℃ with shaking (120 rpm). The pUG6 and pSH65 knock-out vectors with the cre/lox-system were obtained from EUROSCARF (Frankfurt, Germany). All chemicals were of analytical grade or higher. T4 DNA Ligase and Hexokinase/Glucose-6-Phosphate Dehydrogenase (HK/G6P-DH) were purchased from Roche (Penzberg, Germany). All restriction enzymes, Klenow polymerase and Mung bean nuclease were obtained from New England Biolabs. HotStar HiFidelity Polymerase was purchased from Qiagen (Hilden, Germany).

Construction of the expression vectors

Construction of the expression vectors was performed using *E. coli* XL1-blue and standard molecular biology techniques (Sambrook and Russel 2001). In order to construct the pET20-M1 expression vector for *E. coli*, the M1 β-galactosidase was amplified by PCR using primers M1_for_NdeI, M1_rev_XhoI (see Table 1) and the PCS-M1 screening vector as template DNA (Niehaus and Eck 2012). The PCR

product and the pET20b vector were digested with *Nde*1 and *Xho*1 and ligated with T4-Ligase to generate pET20b-M1. The pSIP409-M1 expression vector for *Lb. plantarum* was constructed using the pET20b-M1 vector as a template. A PCR was performed using the M1_for_NdeI and M1_rev_Xho primers to amplify the M1 gene (see Table 1). The PCR product was digested with *Nde*I and blunted with Klenow polymerase. The pSIP409 plasmid was digested with *Nco*I and blunted using Mung bean nuclease. After blunting, the vector and the PCR product were purified and digested with *Xho*I to get compatible ends. Afterwards, a ligation with T4-Ligase was performed and the expression vector pSIP409-M1 was transformed in *E. coli* XL1-blue.

The pKLAC2-M1 expression vector for *K. lactis* was constructed using the pET20b-M1 vector as a template. A PCR was performed using the M1_for_NdeI and M1_rev_SalI primers (see Table 1) to amplify the M1 gene. Additionally, the pKLAC2 vector was digested with *Hind*III and *Xho*I, blunted with Klenow polymerase and ligated by T4-Ligase to generate pKLAC2Δmf with a removed α-mating factor secretion signal for an intracellular expression of M1. The PCR product and the pKLAC2Δmf vector were digested with *Nde*1 and *Sal*1 and ligated with T4-Ligase, whereby the PCR product was cloned downstream of the Lac4 promoter, yielding the pKLAC2-M1 expression vector.

Table 4: Primers used in this study

Primer	Sequence
M1_fw_NdeI	5’-agagtctgcatatgcgacaaaagcttgttta
M1_rev_XhoI	5’-actaatctcgagaatggtgcgaaacgtaaag
M1_fw_SalI	5’-ctgagtgtcgacttaaatggtgcgaaacgtaaagtc
lac4_loxP_fw	5’-gattgcctactagggcttactactatgatcaggatattttcgaatcagctgaagcttcgtacgc
lac4_loxP_rev	5’-cttattcaaaagcgagatcaaactcaaagttgaaatcttgagcttgcataggccactagtggatctg

Deletion of the native *β*-galactosidase *lac4* in *K. lactis* GG799

The deletion of the *lac4* gene was carried out using a loxP marker cassette and a Cre-mediated gene knockout for the removal of the geneticin antibiotic resistance

(Gueldener et al. 2002). The *lac4* disruption cassette was generated by PCR using the pUG6 plasmid as a template of lox-sites and kan^R, and the primers lac4_loxP_rev and lac4_loxP_fw (see Table 1). The primers were designed to disrupt the lac4 gene from +55 to +3066 (Genbank accession number M84410.1) without changing the promoter sequence of the lac4. *K. lactis* GG799 competent cells were transformed with a 2 µg *lac4* disruption cassette using the electroporation method. Transformants were grown on YPD plates containing geneticin 418 (G418) and transferred to YPGal plates containing 40 µg X-Gal ml^{-1} to analyse the remaining *β*-galactosidase activity resulting in *K. lactis* GG799Δlac4. The *loxP*-Kan^R integrated in the genome was removed by transformation of *K. lactis* GG799Δlac4 with 2 µg of the pSH65 plasmid by electroporation, such as described elsewhere, to generate a food-grade *K. lactis* GG799Δlac4 expression host.

Expression of the *β*-galactosidase M1 in *E. coli* BL21

E. coli BL21 (DE3) was transformed with pET20-M1 and used for recombinant expression of the *β*-galactosidase. First expression experiments were carried out aerobically in baffled shaking flasks using 2YT in 100 ml scale at 37℃ at 120 rpm. Induction was carried out at an OD_{600nm} = 0.5 with a final concentration of 0.5 mM Isopropyl-β-D-thiogalactopyranoside (IPTG). After induction, the temperatures were lowered to 30℃. The cells were harvested by centri fugation (8000 *g,* 10 min, 4℃) after the stationary growth phase was reached, resuspended with 100 mM potassium-phosphate buffer, pH 6.75 with 5 mM $MgCl_2$ (30% w/v). Cells were disrupted by sonification. Crude extract was obtained after separation of the cell debris by centrifugation (8000 g, 10 min, 4℃).

Expression experiments were continued in a bioreactor with a 500 ml working volume. The *E. coli* pET20-M1 were cultivated in 2YT-medium with different glucose concentrations at 500 rpm, air gassing vvm = 2 (pO_2 > 30%) and pH = 7.0, controlled with 12.5% v/v NH_4 and 0.66 M H_3PO_4. Induction was carried out at OD_{600nm} = 5 with 0.5 mM IPTG; after that, the temperature was lowered to 20-30℃. Cells were harvested after the stationary growth phase was reached and centrifuged, as described above.

Expression of the *β*-galactosidase M1 in *Lb. plantarum* NC8

The pSIP409-M1 vector was used for the electroporation of *Lb. plantarum* NC8 to generate *Lb. plantarum*-pSIP409-M1 after selection with MRS-Erythromycin plates at 30℃ in an anaerobic jar. First expression experiments were carried out aerobically in baffled shaking flasks at 30℃ at 90 rpm. Freshly prepared *Lb. plantarum* clones were grown in 100 ml MRS-Erythromycin medium. Induction was carried out with 50 ng/ml^{-1} spp-IP at OD_{600nm} = 0.3. The cells were harvested by centrifugation (8000 *g,* 10 min, 4℃) after the stationary growth phase was reached, resuspended with 100 mM potassium-phosphate buffer, pH 6.75 with 5 mM $MgCl_2$ (30% w/v). Cells were disrupted by sonification. Crude extract was obtained after separation of the cell debris by centrifugation (8000 g, 10 min, 4℃). Expression experiments were continued in a bioreactor with a 500 ml working volume. The *Lactobacilli* were cultivated at 100 rpm, N_2 gassing < 0.1 vvm. The pH was controlled at pH = 6.45 by the addition of 1 M NaOH. Induction was carried out at OD_{600nm} = 0.3 with 50 ng/ml^{-1} spp-IP. The cells were harvested and disrupted as described above.

Expression of the *β*-galactosidase M1 in *K. lactis* GG799Δlac4

The pKLAC2-M1 vector was linearised with *Sac*II and 2 µg were used for the electroporation of *K. lactis* GG799Δlac4. Selection of *K. lactis* GG799Δlac4-pKLAC2-M1 was carried out with YCB-plates containing 5 mM acetamide, according to the supplier's instructions (NEB). A first analysis of M1 expression was carried out by plating the transformants on YPGal plates containing X-Gal.

First expression experiments were carried out aerobically in baffled shaking flasks at 30℃ at 120 rpm in 100 ml YPGal medium. After 36 h, the cells were harvested by centrifugation (8000 *g*, 10 min, 4℃). Cell-free crude extract was obtained by centrifugation after vortexing (5 x 2 min) a 30% w/v cell suspension in 100 mM potassium-phosphate buffer, pH 6.75 with 5 mM $MgCl_2$ with glass beads (diameter 0.75-1.0 mm). Expression experiments were continued in a bioreactor with a 500 ml working volume. The *K. lactis* GG799Δlac4-pKLAC2M1 were cultivated at 750 rpm, air gassing 1 vvm ($volume_{gas}$ per $volume_{medium}$ and min). The pH was controlled at pH = 6 by the addition of 12.5% NH_4 and 0.66 M H_3PO_4. The experiments were performed in a fed-batch mode with the addition of galactose to a final concentration of ≥ 20 g/l^{-1} at different periods of cultivation when the galactose concentrations were depleted below 1 g/l^{-1}. The cells were harvested and disrupted, as described above.

Analytical methods and enzyme assay

Protein concentrations were determined by the Bradford method with bovine serum albumin as a standard. Expression of M1 was analysed by SDS-PAGE using 12% polyacrylamid gel, according to the Laemmli method. Cell growth was monitored by measuring the optical density at 600 nm. Biomass was quantified gravimetrically as the cell dry weight. The glucose concentrations were enzymatically determined with an HK/G6P-DH test kit by a photometric assay at 340 nm in microtitre plates. Galactose concentrations were analysed by capillary electrophoresis, according to Deak et al. (2003). The β-galactosidase activity (EA) was determined in 100 mM potassium-phosphate buffer, pH 6.75 with 5 mM $MgCl_2$, using *o*-nitrophenyl-β-D-galactopyranoside (*o*NPGal) as a substrate at 30℃ (1 ml scale). Therefo re, 100 µl of the enzyme solution was added to the substrate solution (25 mM *o*NPGal). Both solutions were pre-heated for 10 min at 30℃ separa tely. The release of *o*-nitrophenol was detected photometrically over 2 min at 405 nm using a temperature-controlled cuvette in a spectrophotometer. β-galactosidase activity was calculated from the slope of the straight line with the molar absorption coefficient of 1.667 l mM^{-1}/cm^{-1} and was carried out at least three times. One nanokatal was defined as the amount of enzyme that catalysed the release of 1 nM of *o*-nitrophenol from *o*NPGal per second.

Results and discussion

Production of M1 in *E. coli* BL21

First expressions of the metagenome-β-galactosidase M1 were performed in the well-known *E. coli* BL21 production host. Different kinds of β-galactosidases have been produced with high yields using this host in the past (Oliveira et al. 2011). Additionally, the metagenome screening was carried out in an *E. coli* host resulting in an active metagenome-derived enzyme (Niehaus and Eck 2012). Therefore, we assumed a good production performance in *E. coli* BL21. The M1 gene was cloned in the pET20b vector resulting in pET20b-M1. After transformation in *E. coli*, basic expression experiments were carried out in a shaking flask (data not shown). Further expression experiments were performed in a 0.5 l bioreactor as batch cultivations. The highest M1 activities up to 82.01 $nkat_{oNPGal}/mg_{protein}$ were obtained after 16 h of cultivation using 2YT medium supplemented with 2% w/v glucose and lowering the

temperature after induction to 30°C. A detailed overview of the cultivation is given in Figure 1.

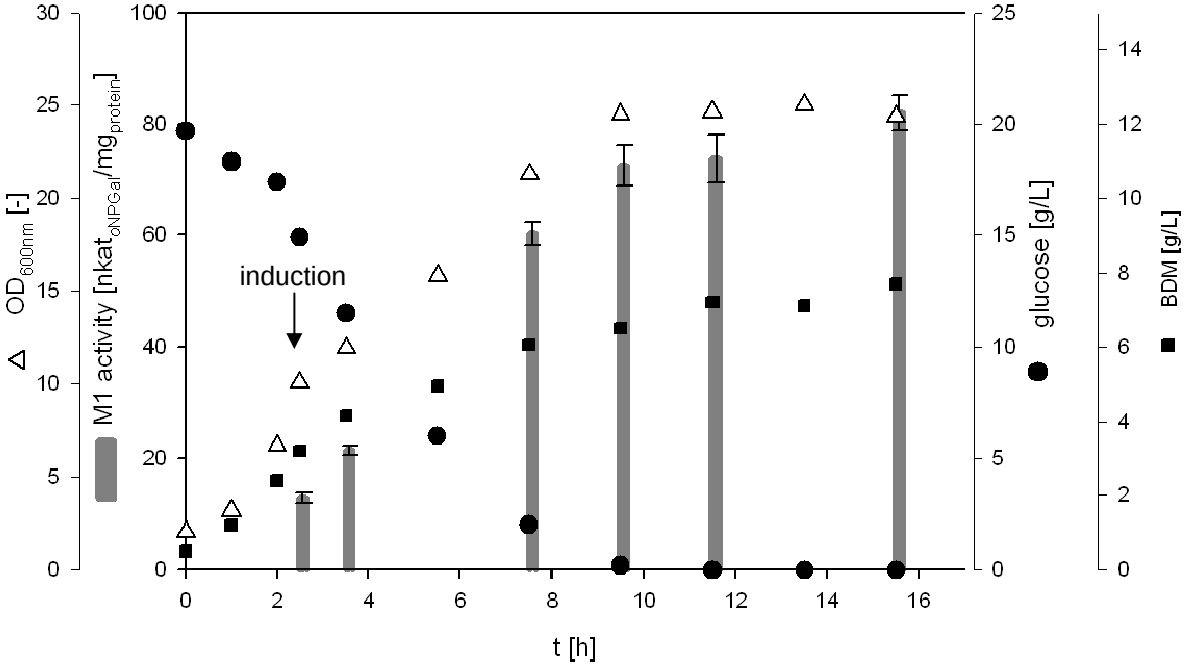

Figure 1: Recombinant production of M1 with *E. coli* BL21 pET20b-M1 (2YT medium + 2% w/v glucose; 0.5 l scale bioreactor cultivation)

(open triangles OD_{600nm}; filled circles: glucose concentration; filled squares: bio dry mass; bars: specific M1 activity, error bars indicate the standard deviation; arrow: addition of IPTG for induction and lowering of the temperature from 37 to 30°C)

The high level of M1 expression by *E. coli* BL21 pET20b-M1 was also proven by gel electrophoresis experiments (SDS-PAGE). Figure 2 shows the SDS-PAGE from samples of the cell-free extracts of *E. coli* BL21 wild-type and *E. coli* BL21 pET20b-M1 after different cultivation times. The overexpressed M1 appeared as a visible band on SDS-PAGE with an apparent mass of ~ 120 kDa (see Figure 2). This result is in agreement with the predicted mass of 122.4 kDa calculated *in silico.*

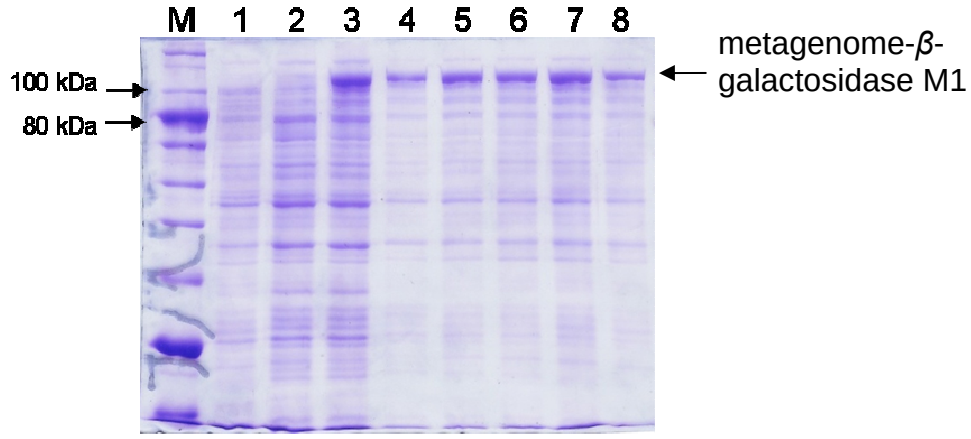

Figure 2: 12.5% SDS-PAGE analysis of the expression of M1 in *E. coli* BL21

M: Protein Ladder 10-250 kDa; 1: *E. coli* BL21 wild-type ;2: *E. coli* BL21 pET20b-M1 before induction;
3-8: *E. coli* BL2 pET20b-M1 2 h, 5 h, 9 h, 11 h, 13 h, and 15 h after induction;
5 µg total protein loaded per lane, Coomassie-stained.

Production of M1 in *Lb. plantarum* NC8

Lactobacilli are receiving increased attention concerning their application as expression systems for the recombinant production of food-related industrial enzymes. Therefore, *Lb. plantarum* NC8 was used with a strong pSIP expression system for the production of M1 in a food-grade system. Different *β*-galactosidases were produced with high yields using this expression system (Halbmayr et al. 2008; Nguyen et al. 2011a; Nguyen et al. 2011b). After construction of the pSIP409-M1 vector and transformation of *Lb. plantarum*, the production of M1 was performed in a 0.5 l bioreactor system using a standard expression procedure (Böhmer et al. 2012; Sorvig et al. 2005). A detailed overview of the cultivation of *Lb. plantarum* NC8 pSIP409-M1 is given in Figure 3.

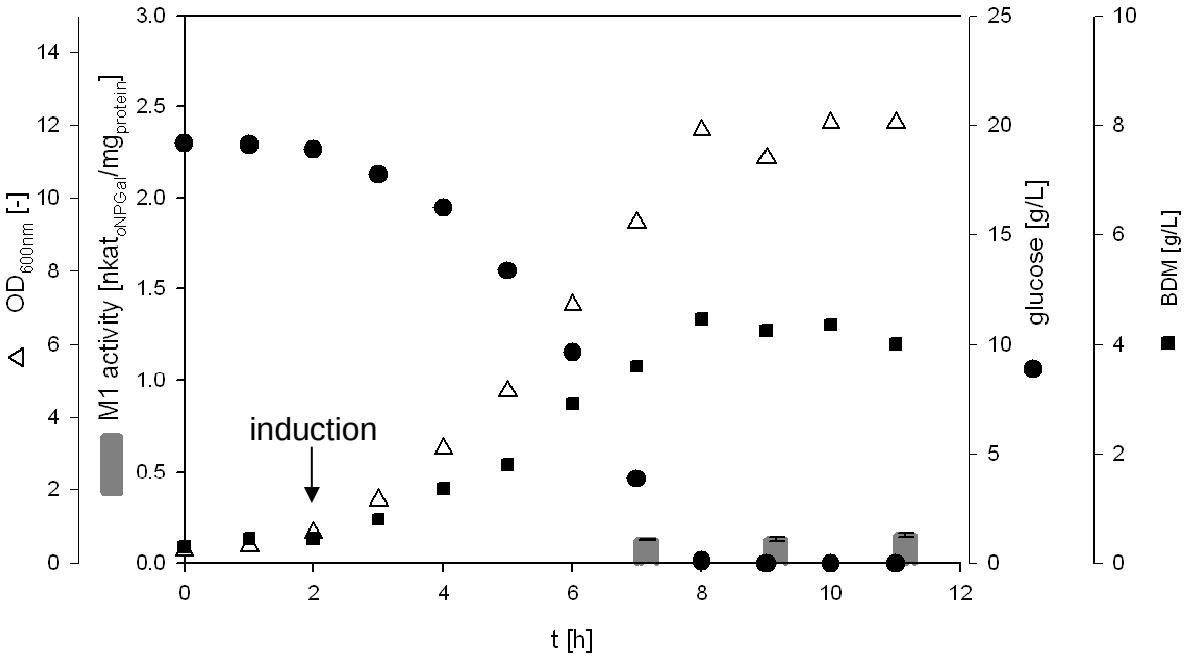

Figure 3: Recombinant production of M1 with *Lb. plantarum* pSIP409-M1 (MRS medium; 0.5 l scale bioreactor cultivation)

(open triangles: OD_{600nm}; filled circles: glucose concentration; arrow: addition of IP for induction; filled squares: bio dry mass; bars: specific M1 activity, error bars indicate the standard deviation)

Only very low amounts of M1 with an activity of 0.18 $nkat_{oNPGal}/mg_{protein}$ (0.01 U/mg) were obtained at the beginning of the stationary growth phase. This is about 450 times less when compared to *E. coli* BL21 pET20b-M1. No overexpression of the M1 was detectable by gel electrophoresis. A clear difference in the yields obtained, even from closely related species, is described for the recombinant expression of β-galactosidases from different microbial sources in *Lb. plantarum*. The expression level of the β-galactosidases from *Lb. reuteri* and *Lb. acidophilus*, for example, differed about 18-fold when using the same expression vectors and cultivation strategies, resulting in 144 U/mg for the *Lb. reuteri* β-galactosidase and significantly lower 3.94 U/mg for the *Lb. acidophilus* β-galactosidase (Nguyen et al. 2011b). The reason, therefore, was different mRNA stabilities resulting in different mRNA levels and translational effects. Codon usage as well as the folding and stability of mRNA are also known to hamper the expression performance of foreign genes (Kudla et al. 2009).

Construction of the *K. lactis* GG799Δlac4 expression strain

The recombinant expression of the M1 β-galactosidase in *K. lactis* with the use of the pKLAC2 expression system is controlled by the lac4 promoter and induced by galactose. Thereby, the production of the native *K. lactis* β-galactosidase lac4 is also induced. Because of that, the chromosomal lac4 was knocked out in the expression strain *K. lactis* GG799 before transformation with the pKLAC2-M1 expression vector. The lac4 gene was disrupted from bases +55 to +3066 without any change in the promoter sequence. Therefore, the integration sites for the pKLAC2-M1 expression construct were not affected. A deletion of the *lac4* gene was analysed by plating on YPGal-X-Gal agar-plates and visualised by white colonies. In our study, we obtained about 20% gene targeting efficiency using the cre/lox system with 45-50 bp of homologue targeting regions. In previous studies, an integration efficiency of 0% using small homologue targeting regions of 50 bp for *K. lactis* was reported, which is contrary to our results (Kooistra et al. 2004; Ribeiro et al. 2007). Additionally, cultivation in shaking flasks was carried out with YPGal medium without the detection of any β-galactosidase in the cell-free extracts (data not shown).

Production of M1 in *K. lactis* GG799Δlac4

Due to the inefficient expression performance of the food-grade *Lb. plantarum* NC8 pSIP409-M1, the M1 was produced in *K. lactis,* another food-grade expression host for recombinant enzyme production. After transformation with the expression plasmid pKLAC2-M1, stable integrations of the expression construct in the genome of *K. lactis* resulting in *K. lactis* GG799Δlac4-pKLAC2-M1 were generated. Correct integration was analysed by PCR (data not shown). An intracellular localization of the recombinant M1 was performed, because it is known that *K. lactis* secretes only low amounts of β-galactosidases in the culture supernatant even when homologous secretion signals are used (Rodriguez et al. 2006). A first analysis of β-galactosidase activity was carried out by plating the *K. lactis* transformants on YPGal plates containing X-Gal. All of the transformants checked showed an expression of the recombinant M1 resulting in blue colonies. The production of the M1 in *K. lactis* was performed after first experiments in a shaking flask on 0.5 l bioreactor scale using a standard expression procedure (van Ooyen et al. 2006). A detailed overview of the cultivation of *K. lactis* GG799Δlac4-pKLAC2-M1 is given in Figure 4.

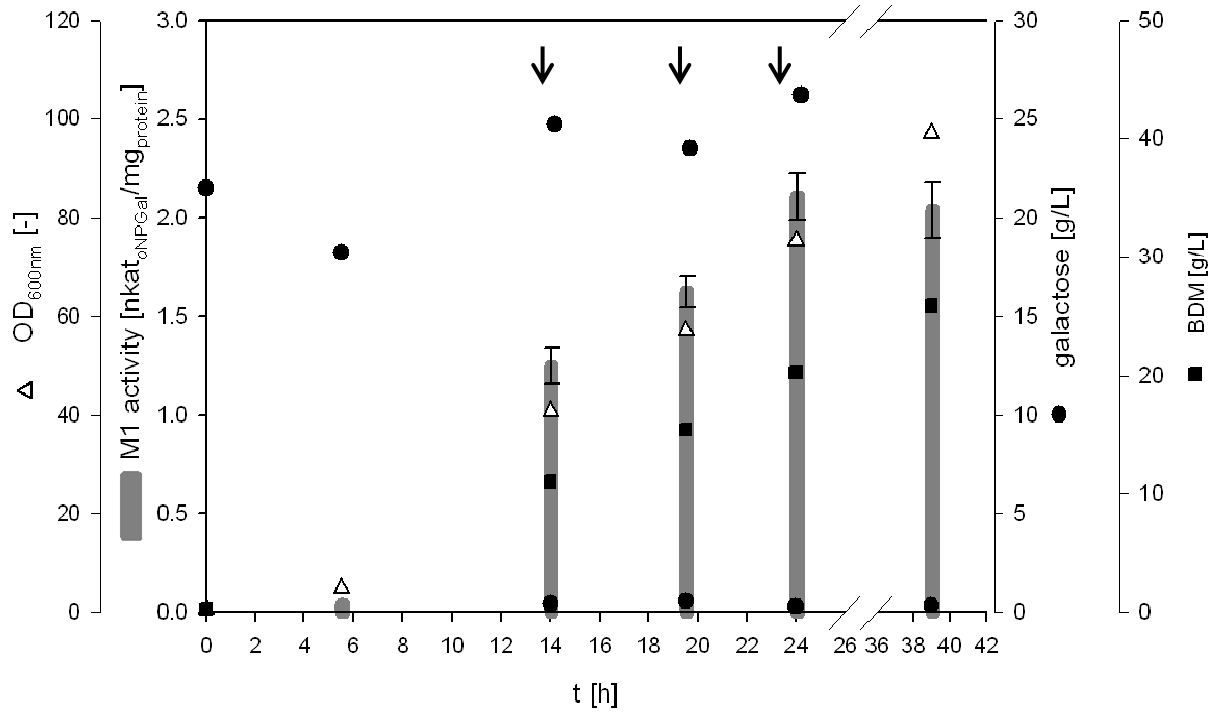

Figure 4: Recombinant production of M1 with *K. lactis* pKLAC2-M1 (YPGal medium; 0.5 l scale bioreactor cultivation)

(open triangles: OD_{600nm}; filled circles: galactose concentration; filled squares: bio dry mass; bars: specific M1 activity, error bars indicate the standard deviation; arrows: galactose feed)

Maximal M1 activity of 2.42 $nkat_{oNPGal}/mg_{protein}$ (0.01 U/mg) were obtained after 24 h of cultivation and repeated feed of the inducer galactose. The yield of M1 produced with the food-grade system *K. lactis* is 13 times higher when compared to the food-grade *Lb. plantarum* expression system. Nevertheless, the highest activities were obtained with the *E. coli* BL21 pET20b-M1 expression system. A comparison of the yields of recombinant M1 using different expression systems is given in Table 2.

Table 2: *β*-galactosidase activities obtained with different expression systems

Expression system	Specific activity [$nkat_{oNPGal}/mg_{protein}$]	Volumetric activity [$nkat_{oNPGal}/ml_{bioreactor}$]
Lb. plantarum NC8 pSIP409-M1	0.18	0.5
K. lactis Δlac4 pKLAC2-M1	2.42	18.8
E. coli BL21 pET20b-M1	82.01	1090

Another study from Wang et al. (2010) describes the recombinant production of a metagenome-*β*-galactosidase in the yeast *Pichia pastoris*. Comparable to our work, the *β*-galactosidase screening was performed using *E. coli* as the host organism. Afterwards, the expression of the enzyme using *P. pastoris* as the host and secretion

of the recombinant enzyme in the culture supernatant resulted in the highest expression level of ca. 300 mg/l. Contrary to that, in our work, the best expression performance for the metagenome-*β*-galactosidase M1 was observed in the screening host *E. coli*.

The efficient production of a functional metagenome enzyme does not only depend on the promoter strength and gene dosage, but also on the availability of respective precursors and cofactors, on putative codon bias, type and amount of chaperones, posttranscriptional and posttranslational modifications, and unwanted protein degradation (McMahon et al. 2012; Troeschel et al. 2012). It is known that *E. coli* as a screening host is often problematic because of insufficient and biased expression of metagenomic DNA (Aakvik et al. 2009; Uchiyama and Miyazaki 2009). Similar challenges might be the reason for a low expression efficiency of the metagenome-*β*-galactosidase M1 in expression systems other than the screening host. Therefore, if a specific expression system is desired for industrial scale production of metagenome-derived enzymes at competitive prices, the expression system should be considered at the beginning of the screening approach (Lorenz and Eck 2005). The use of a novel broad host range of shuttle vectors is also a promising approach to enable and optimise the production of functional proteins for the rapid and easy comparison of expression systems (Troeschel et al. 2012).

The present study shows that different expression systems for the production of metagenome enzymes lead to wide differences in the yields obtained. Moreover, the best expression performance of the metagenome-*β*-galactosidase M1 was gained with the screening host *E. coli* and the pET20b-M1 vector. This is the preferable system for further expression of the M1 for industrial applications such as the production of lactose-depleted milk products.

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Chapter 4

4. A novel manganese starvation-inducible expression system for *Lactobacillus plantarum*

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Abstract

A novel expression system for *Lactobacillus plantarum* was developed. This system is based on the manganese starvation-inducible promoter from specific manganese transporter of *Lb. plantarum* NC8 which was cloned for the first time. The expression of a *β*-glucosidase from *Pyrococcus furiosus* (CelB) was achieved by cultivating *Lb. plantarum* NC8 at low manganese concentrations with MRS medium and the pmntH2-celB expression vector. A CelB activity of 8.52 $\mu kat_{oNPGal}\, L^{-1}$ was produced in a bioreactor (4 L). The advantages of the novel expression system are that no addition of an external inducing agent was required, and additionally, no further introduction of regulatory genes was necessary. The new promoter meets the general demands of a food-grade expression system.

1 Introduction

Lactic acid bacteria (LAB) are gram-positive, facultative anaerobic bacteria that are widely used in the food industry to produce various fermented foods (Konings et al., 2000). Because they do not generate endotoxins and are non-sporulating, they are *Generally Recognized As Safe* (GRAS) by the U.S. Food and Drug Administration. There is widespread knowledge about the cultivation of LAB at bioreactor scale. *Lactococcus lactis* was considered as a model organism for a long time, in the last decade also other *Lactobacilli* became more deeply studied. For instance *Lactobacillus plantarum* was established and used for metabolism studies as well as for the development of genetic tools (Siezen et al., 2011). For these reasons, interest in *Lb. plantarum* as a suitable production host for recombinant proteins has increased over the last decade. Here, the LAB have potential as delivery systems for valuable proteins like antibodies and antigens (Diep et al., 2009). To the particular demands of the food industry, *Lactobacilli* are desirable cell factories for the production of food-grade enzymes (Peterbauer et al., 2011).

Several different types of promoters from *Lb. plantarum* have been isolated to produce recombinant proteins. Some of them are constitutive, while a few of them are inducible (Rud et al., 2006). The most common expression systems are the NICE and pSIP expression systems (de Ruyter et al., 1996; Sørvig et al., 2003). The promoters of these systems are based on the regulatory system of antimicrobial peptides and the quorum sensing mechanism. Thus, for the induction of recombinant proteins in *Lb. plantarum*, an inducing peptide must be added during cultivation. The pSIP system is the most extensively used inducible system. It is derived from *Lb. sakei* and uses the regulatory elements from the class IIb bacteriocins, either Sakacin A when using the pSIP403 vector, or Sakacin P for the pSIP409 vector (Sørvig et al., 2003; Sørvig et al., 2005). The recombinant overproduction of heterologous proteins is induced by an externally added peptide pheromone. When these systems were employed in previous studies, different types of enzymes such as aminopeptidase N from *L. lactis,* *β*-glucuronidase from *Escherichia coli*, *β*-galactosidases from *Lactobacillus* sp. and a *β*-glucosidase from *Pyrococcus furiosus* were recombinantly expressed in *Lb. plantarum* and *Lb. sakei* with high yields (Sørvig et al., 2003; Halbmayr et al., 2008; Böhmer et al., 2012). Other types of auto-inducing expression systems were developed for *L. lactis* and *Bacillus subtillis* in recent years. These are based on phosphate or metal starvation transporter systems (Sirén et al., 2008;

Kerouvo et al., 2000). Because phosphate is an essential component for bacterial growth, the promoters of these transport proteins are turned on under starvation conditions, and this can be used for recombinant protein expression. A similar system based on regulatory elements of high specific Zn^{2+} uptake during a Zn^{2+} starvation was developed for recombinant protein production in *L. lactis* (Llull and Pouqet, 2004). Such systems can be more convenient for the recombinant production of proteins in some cases because they require no addition of any inducing agents. This will extend the tools for recombinant protein expression in *Lactobacilli*, and it completes the well established systems with external inducers.

In addition to phosphate and Zn^{2+}, trace amounts of manganese are of physiological importance in a variety of ways for almost all bacteria, including its need as cofactors of enzymes. Therefore manganese ions are present in microorganism in very low amounts. However, in *Lb. plantarum* and some other lactic acid bacteria, high amounts of manganese up to 30 mM accumulate intracellularly due to the absence of superoxide-dismutase (SOD) in these species (Archibald & Fridovich, 1981a/b; Archibald & Duong, 1984). The Mn^{2+} ions are used instead of the SOD to scavenge the toxic product superoxide (O_2^-) when the bacteria are grown in the presence of oxygen or during fermentative growth (Horsburgh et al., 2002). In the presence of Mn^{2+} ions, superoxide is chemically converted to hydrogen peroxide (H_2O_2), which is further enzymatically converted by a catalase. Thus, *Lb. plantarum* requires high concentrations of manganese in the standard MRS medium to be able to grow successfully. The uptake of Mn^{2+} ions occurs by different types of cation transporters with an active Mn^{2+} ion transport system (Hantke, 2005). The transporters were identified and described after the genome sequencing of *Lb. plantarum* WCFS1 in previous work by *in silico* analysis as ABC (ATP-binding cassette) transporter type (called mntH2) and Nramp (natural resistance associated macrophage protein) transporter (called mtsCBA) (Groot et al., 2005). Expression of these transporters during manganese starvation was verified by Northern blot analysis. In our work presented here, the promoter of the specific transport protein MntH2 was cloned and investigated for its use as novel promoter for recombinant protein production.

2 Material and Methods

2.1 Chemicals and Enzymes

All chemicals were of analytical grade or higher and were purchased from Sigma-Aldrich (Seelze, Germany) or Carl Roth (Karlsruhe, Germany). T4 DNA Ligase and Hexokinase/Glucose-6-Phosphate Dehydrogenase (HK/G6P-DH) were purchased from Roche (Mannheim, Germany). All restriction enzymes were from New England Biolabs (Frankfurt, Germany). HotStar HiFidelity Polymerase was purchased from Qiagen (Hilden, Germany).

2.2 Bacterial Strains and Culture Conditions

Escherichia coli XL1-blue was purchased from Stratagene (Santa Clara, USA), and was grown in Luria-Bertani medium at 37 °C with shaking (120 rpm). *Lb. plantarum* NC8 was obtained from culture collection by the Norwegian University of Life Science (Ås, Norway) and grown in MRS medium according to De Man, Rogosa and Sharp (1960) or in MRS media with different $MnSO_4$ concentrations at 30 °C. Agar plates were solidified by adding 1.5% (w/v) agar. When required, erythromycin was added as follows: 200 µg mL^{-1} for *E. coli* and 10 µg mL^{-1} for *Lb. plantarum*.

2.3 Cultivation at microtiter plate scale

A microtiter plate-based Bioscreen C automatic cultivator (LabSystems, Finland) was used for growth analysis of *Lb. plantarum* at different $MnSO_4$ concentrations (0-296 µM). Cultivation was performed in microtiter plates at 30 °C with continuous shaking. Determination of growth was performed in 250 µLs of solution, and 240 µL of each media was inoculated with 10 µL of an overnight culture in the respective medium. Four wells were used for quadruplicate testing of each medium. Optical density at 600 nm (OD_{600nm}) was measured and readings for each well were taken every 15 min for 16-24 h.

2.4 Preparation of Plasmids, Cloning and Transformation

Construction of the expression vector pmntH2-celB was performed using *E. coli* XL1-blue and standard molecular biology techniques (Sambrook et al., 2001).

The promoter was amplified using the genomic DNA of *Lb. plantarum* NC8 as template. Isolation of genomic DNA was performed according to Sørvig (Sørvig et al.,

2005). Primers (Table 1) were constructed using the sequenced genome from *Lb. plantarum* WCFS1 (Kleerebezem et al., 2003, GenBank: AL935263.2).

Table 5: Primers used in this study

Primer	Sequence
mntH2fw_*Sal*I	5'-gtcgacttcacacctccaagcacatcgtac
mntH2rev_*Nco*I	5'-ccatggcaattaaaagaccacctttctatatc

The resulting PCR product of approximately 500 bp in length was cloned in the pSIP409-celB vector using *Sal*I and *Nco*I restriction sites to remove the P_{orfX} promoter and regulator elements of this induction system. This generated the expression plasmid pmntH2-celB. The plasmid was transformed into *E. coli.* Plasmids used in this study are shown in Table 2.

Table 6: Plasmids used and constructed in this study

Plasmid	Characteristics	Source
pSIP409-celB	p256rep/pUC(pGEM)ori; P_{orfX}::celB; Em^R	Böhmer et al., (2012)
pmntH2-celB	p256rep/pUC(pGEM)ori; P_{mntH2}::celB; Em^R	This work

Plasmid DNA was purified from *E. coli* by the QIAprep Spin Miniprep Kit from Qiagen (Hilden, Germany) and subsequently transformed into *Lb. plantarum* by electroporation (Aukrust and Blom, 1992). Positive clones were selected by plating on MRS agar containing 10 µg mL^{-1} Erythromycin and incubation for 24-48 h at 30℃ under anaerobic atmosphere using an anaerobic jar and Anaerocult A reagent from Merck (Darmstadt, Germany).

2.5 Expression experiments in shaking flasks

Initial expression experiments were performed aerobically in baffled shaking flasks at 30 ℃. Freshly prepared *Lb. plantarum* clones were grown over night in test tubes (5 mL scale) and transferred to 100 mL MRS media with erythromycin and different $MnSO_4$ concentrations. The cells were harvested by centrifugation (10 min, 8000 *g*) after the stationary growth phase was reached, washed, and resuspended with sodium acetate buffer (50 mM, pH = 5.0). Cells (30% w/v) were disrupted by

sonification using an Ultrasonic Processor UP 200S (Hielscher Ultrasonic, Teltow, Germany). Crude extract was obtained after separating the cell debris by centrifugation (10 min, 8000 *g*, 4 ℃). The best performing $MnSO_4$ concentrations for recombinant expression of CelB were determined by analysis of the CelB activity.

2.6 Fermentation experiments in the bioreactor

Expression experiments were continued in a Biostat E fermenter (B. Braun, Melsungen, Germany) with 4 L working volume. *Lb. plantarum* was cultivated at 100 rpm, anaerobic with N_2 gassing < 0.1 vvm or aerobic with O_2 gassing with pO_2 > 30%, 0.5 vvm, and pH = 6.45, controlled with 2 M NaOH at 30 ℃. The experiments were run in batch mode. Samples were withdrawn throughout the fermentation to determine biomass, glucose concentration, CelB activity and manganese concentrations. Before bioreactor inoculation precultures were first grown overnight at 5 mL, followed by precultures at 50 mL scale over night and finally, were grown overnight at 400 mL scale in baffled shaking flasks in the appropriate medium under aerobic conditions. The cells were harvested and disrupted as described above.

2.7 Analytical Methods

Cell growth was monitored by measuring the optical density at 600 nm (OD_{600nm}). Biomass was quantified gravimetrically as cell dry weight of cells (CDW). Samples were centrifuged, twice washed with saline, and dried in pre-weighted tubes at 40℃ at 10 mbar in a RVC 2-33 IR vacuum centrifuge from Christ (Osterode, Germany). Protein concentrations were determined by the method of Bradford (Bradford, 1976). The glucose concentrations were measured in microtiter plates by a photometric assay at 340 nm. HK/G6P-DH was used as the coupling enzyme for the reaction, and the technique was based on the manufacturers protocol for the D-glucose/D-fructose test kit (R-Biopharm AG**,** Darmstadt, Germany; product code 10 139 106 035). The total Mn^{2+} content was measured with a Perkin-Elmer model 2380 atomic absorption spectrophotometer. For intracellular Mn^{2+} determination lyophilized cells were digested overnight in 70% nitric acid at 37℃. The digestion mixture was diluted with water to a final nitric acid concentration of 10% before measurement with atomic absorption spectrophotometer.

2.8 Enzyme activity measurements

CelB activity (EA) was determined using *o*-nitrophenyl-*β*-D-galactopyranoside (*o*NPGal) as a substrate at 75 °C with 50 mM sodium ac etate buffer, pH 5.0, in 1 mL of solution as described previously (Mayer et al., 2010). One nanokatal is defined as the amount of enzyme that catalyzes the release of 1 nmol of *o*-nitrophenol from *o*NPGal per second.

The native protein in cell free crude extracts used for the enzyme solution was heat-denaturated for 15 min at 75 °C. After that the he at-denaturated, interfering native *Lactobacillus* protein was precipitated by centrifugation (10 min, 8000 *g*, 4°C). CelB activity was calculated with an absorption coefficient of 0.495 L $mmol^{-1}$ cm^{-1}. All measurements were performed in triplicate.

3 Results and Discussion

3.1 Growth analysis of *Lb. plantarum* at different Mn^{2+} concentrations

As previously reported, due to the absence of a SOD, *Lb. plantarum* needs a relative high concentration of manganese ions for optimal growth (Archibald & Fridovich, 1981a/b; Archibald & Duong, 1984). *Lb. plantarum* NC8 was grown in MRS media supplemented with decreasing Mn^{2+} concentrations (0-296 µM) in order to determine the limiting concentration for this ion. The growth investigations by optical density (OD_{600nm}) were performed in microtiter plate-based cultivations as described above. The results are shown in Figure 1.

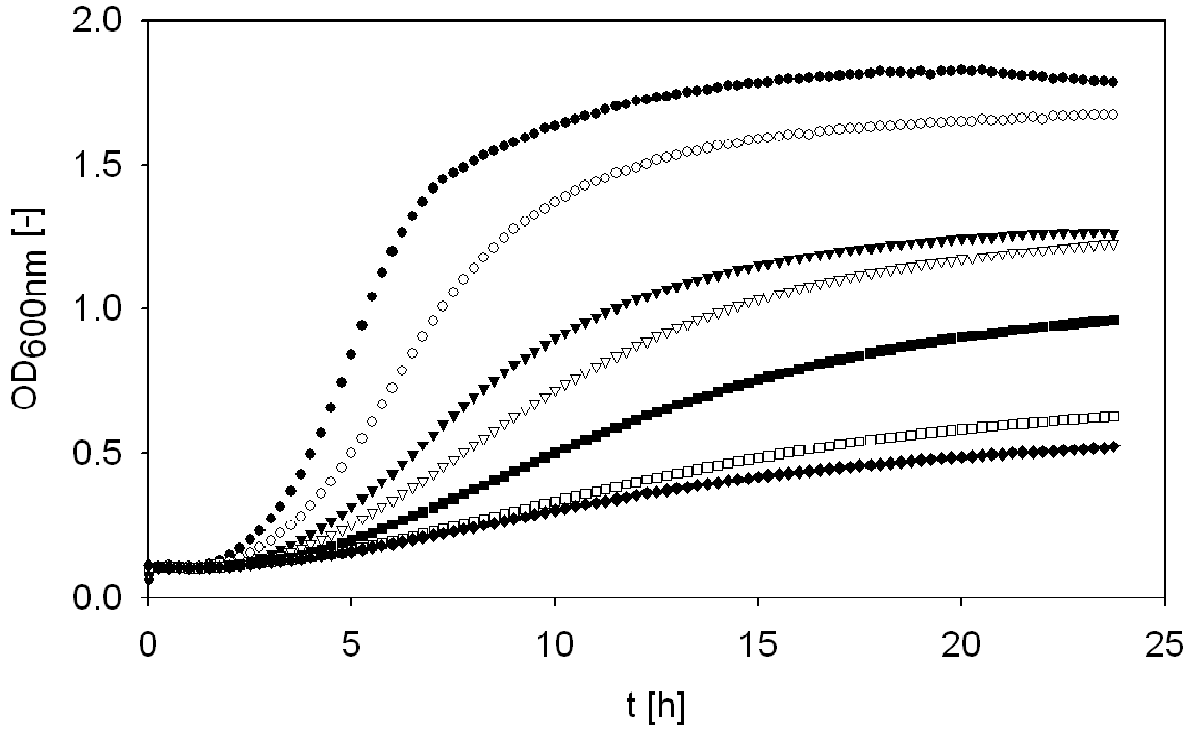

Figure 2: Growth curve of *Lb. plantarum* NC8 at different $MnSO_4$ concentrations in microtiter cultivations (MRS medium, 30℃, 250 µL s cale, aerobic)
♦ 0 µM $MnSO_4$, □ 1.5 µM $MnSO_4$, ■10 µM $MnSO_4$, Δ 20 µM $MnSO_4$, ▼ 50 µM $MnSO_4$, ○100 µM $MnSO_4$, ● 296 µM $MnSO_4$, at least four replicates of all cultivations were performed.

The highest OD_{600nm} of 1.78 was reached in microtiter scale with the highest Mn^{2+} ion concentration tested (296 µM). Up to Mn^{2+} ion concentrations of 100 µM, a clear limitation of growth was observed. These results illustrated that the growth of *Lb. plantarum* NC8 was directly correlated to the amount of Mn^{2+} ions in the medium. It was in accordance with previously published data, when the extracellular

concentration of $MnSO_4$ was below 150 µM, its intracellular concentration became dependent on the quantity of Mn^{2+} ions added in the culture medium (Archibald & Duong, 1984). In chemically defined media, no growth was observed without manganese salt supplementation (Hao et al., 1999; Groot et al., 2005), but in complex media, such as the MRS medium used in the present study, slight growth was detectable even without adding any $MnSO_4$ into the medium. This observation was also described in previous literature (Watanabe et al., 2012) and is due to trace amounts of manganese in the complex compounds of MRS medium, such as yeast and meat extracts or tryptone.

3.2 Performance of the promoters in MRS medium without $MnSO_4$

The promoter region of the gene encoding the manganese transporters MntH2 from *Lb. plantarum* WCFS1 have been studied (Groot et al., 2005). As the gene is induced by manganese starvation, we amplified a 496 bp DNA fragment encompassing of the mntH2 promoter region. This promoter sequence included the transcription start site and Shine-Dalgarno sequence. Additionally, parts of the promoter sequence (19 bp) are similar to target regions for the metalloregulator MntR from *B. subtillis* (Que & Hellmann, 2000). The binding site of the ScaR regulator protein from *Streptococcus gordonii* was a part of the promoter sequence of P_{mntH2}, as well (Jakubovics et al., 2000). The promoter was cloned upstream from the celB gene as reporter gene in the pSIP409-celB vector replacing the P_{orfX} promoter and regulator elements. This resulted in the plasmid pmntH2-celB. The vector backbone of the pSIP409-celB vector was used, which consists of replication origins for *E. coli* (pUCori) and *Lactobacilli* (256rep), an erythromycin resistance marker (ermL) and the pepN terminator (Böhmer et al 2012, Sørvig et al 2003). As described by Sørvig et al. (2005) the used minimal replicon 256rep results in a copy number of about 6 and is known to replicate via a theta mechanism.

The expression performance of P_{mntH2} was investigated using the thermophilic glucosidase (CelB) from *Pyrococcus furiosus* (Voorhorst et al., 1995) as reporter gene. Initial expression experiments with *Lb. plantarum* transformants were performed in baffled shaking flasks in MRS medium without any $MnSO_4$ (aerobically, pH 6.45, 30℃). *Lb. plantarum* pmntH2-celB achieved a CelB activity of 17 $nkat_{oNPGal}$ $mg_{protein}^{-1}$. Thus, the proof of principle for recombinant protein production using a manganese starvation based promoter was demonstrated. For the next expression

experiments, different amounts of $MnSO_4$ were added to the MRS medium in order to analyze the dependency of PmntH2-celB expression of Mn^{2+} concentration in the growth medium (see Table 3). The highest specific CelB activity of 20.27 $nkat_{oNPGal}$ $mg_{protein}^{-1}$ was reached at 10 µM $MnSO_4$.

Table 7: Growth and CelB activity of *Lb. plantarum* NC8 pmntH2-celB at different concentrations of $MnSO_4$ (baffled shaking flasks, anaerobic conditions, 100 mL medium, 30°C)

$MnSO_4$ concentration [µM]	Final OD_{600} [-]	CelB activity [$nkat_{oNPGal}$ $mg_{protein}^{-1}$]
0	0.78	17.55 ± 0.43
1.5	0.96	19.04 ± 0.12
10	2.01	20.27 ± 0.51
20	2.85	16.36 ± 0.18
50	4.72	9.60 ± 0.10
100	7.45	7.67 ± 0.17
150	8.44	5.63 ± 0.15
296	11.85	2.63 ± 0.07

Our results demonstrated that in addition to the reported phosphate starvation promoter system for *Lactococcus* and *Bacillus* species (Sirén et al., 2008; Kerouvo et al., 2000) another starvation promoter system based on manganese ions can be applied to suitable hosts such as *Lactobacilli*. A similar system was developed for *L. lactis* using promoter and repressor protein of a zinc uptake system (Llull and Poquet, 2004). Using the Zn^{2+} starvation inducible system induction factors of ~ 50 were reached after zinc consumption during cell growth. In our study, an induction factor of ~ 10 was detected comparing the medium with 10 µM $MnSO_4$ to the medium with 296 µM $MnSO_4$. Induction factors obtained with some other promoters using LAB systems are quite in the same order of magnitude (de Vos, 1999). To the best of our knowledge, this is the first time a starvation promoter expression system was used successfully in a “food-grade” *Lactobacillus* host, that did not need any addition of an inducing agent. It may also be possible to use this food-grade promoter P_{mntH2} for the production of live vaccines in *Lb. plantarum* as delivery vehicle, as was previously discussed with different type of lactic acid bacteria as host organisms, and for the expression of therapeutic proteins or vaccines (Diep et al., 2009; Renault, 2002; Wells & Mercenier, 2008).

3.3 Bioreactor experiments with *Lb. plantarum* pmntH2-celB

The performance of the CelB production of *Lb. plantarum* pmntH2-celB in the bioreactor using MRS medium with 20 µM $MnSO_4$ is shown in Figure 2.

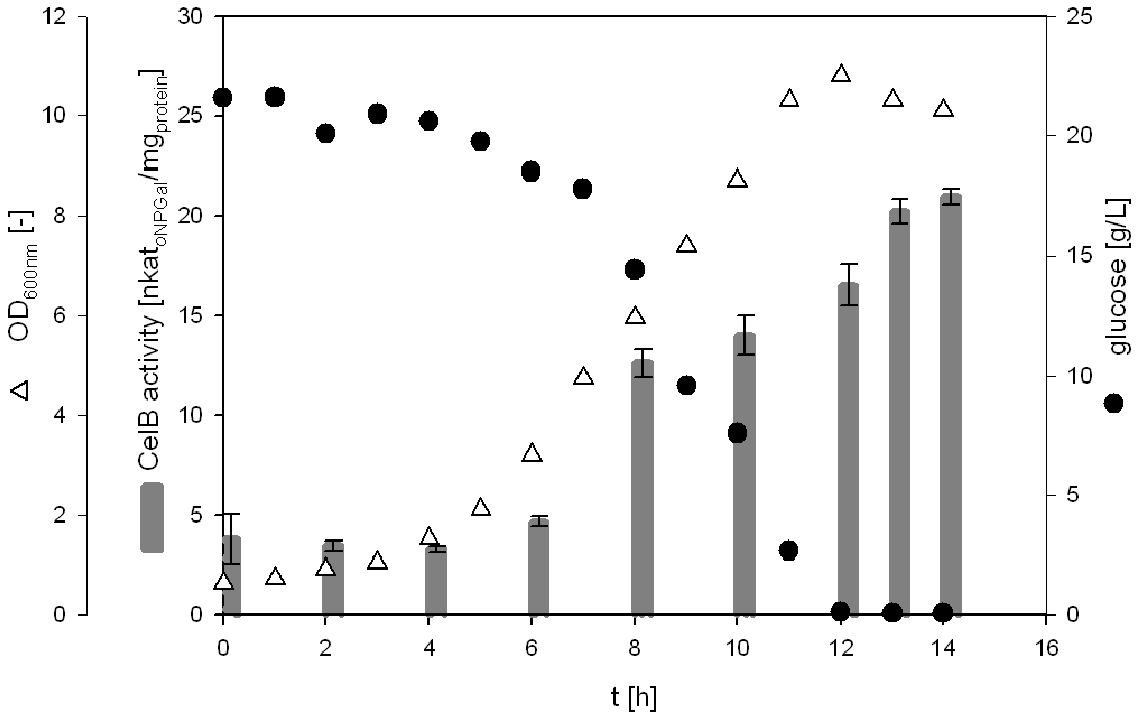

Figure 2 Recombinant production of CelB from anaerobic batch cultivation of *Lb. plantarum* pmntH2-celB (4 L scale, MRS medium with 20 µM $MnSO_4$, 30℃, 100 rpm)
(Open triangle: OD_{600nm}; filled circle: glucose concentration; bars: specific CelB activity, error bars indicate the standard deviation)

To enhance the amount of biomass formation, and still have a good induction level of P_{mntH2}, 20 µM $MnSO_4$ was chosen as limiting concentration of Mn^{2+}. Contrary to the shaking flasks experiments described above, the cultivation was done anaerobic (N_2 gassing) to avoid unwanted oxidative damage by superoxide under Mn^{2+} limiting conditions. Figure 2 illustrates the bioreactor cultivation of *Lb. plantarum* pmntH2-celB under N_2 -gassing conditions. The maximal achieved biomass of 4.0 g L^{-1} cell dry weight is equal to an OD_{600nm} = 10.8 under manganese starvation conditions (20 µM $MnSO_4$). This is about 11% lower than the biomass obtained by bioreactor cultivation in standard MRS medium (296 µM $MnSO_4$), were a cell dry weight of 4.5 g L^{-1}, equal to an OD_{600nm} = 12.3, was reached. A bioreactor cultivation was also performed under aerobic (air gassing) conditions using the same medium. Using this technique, we determined whether the expression rate of the promoter P_{mntH2} was

influenced by oxygen. The biomass as well as the expression performance of P_{mntH2} by aerobic cultivation (data not shown) was quite the same to the cultivation with N_2 gassing. Therefore the presented system may be easily applicable due to less technical demands in kind of N_2-gassing. In other studies it was shown that *Lb. plantarum*, grown aerobically in standard MRS medium (with 296 µM $MnSO_4$), resulted in higher OD_{600nm} values than when grown under anaerobic conditions (Brooijmanns et al., 2009). However, Watanabe et al. (2012) ascertained that this growth difference did not occur when the cultivation was performed without the addition of $MnSO_4$ into the MRS medium. This was also the case in our studies in which we used a very low $MnSO_4$ supplementation of 20 µM.

Maximal specific CelB activities were obtained in aerobic and anaerobic bioreactor cultivations with 22.4 ± 0.9 $nkat_{oNPGal}\ mg_{protein}^{-1}$ and 20.9 ± 0.5 $nkat_{oNPGal}\ mg_{protein}^{-1}$, respectively (anaerobic see Figure 2). So, the induction of P_{mntH2} did not depend on oxygen. In the bioreactor cultivations, the specific CelB activities were approximately as high as in the shaking flask experiments (see above). The biomass was four times higher in the bioreactor, and a maximal volumetric activity of 8.52 $\mu kat_{oNPGal}\ L^{-1}$ was obtained after 14 hours of cultivation under N_2-gassing conditions.

3.4 Kinetic of P_{mntH2} induction during manganese depletion

Analysis of the induction kinetics of *Lb. plantarum* pmntH2-celB in medium with 20 µM $MnSO_4$ is shown in Figure 3 and was done by quantification of the extracellular and intracellular manganese concentrations by AAS.

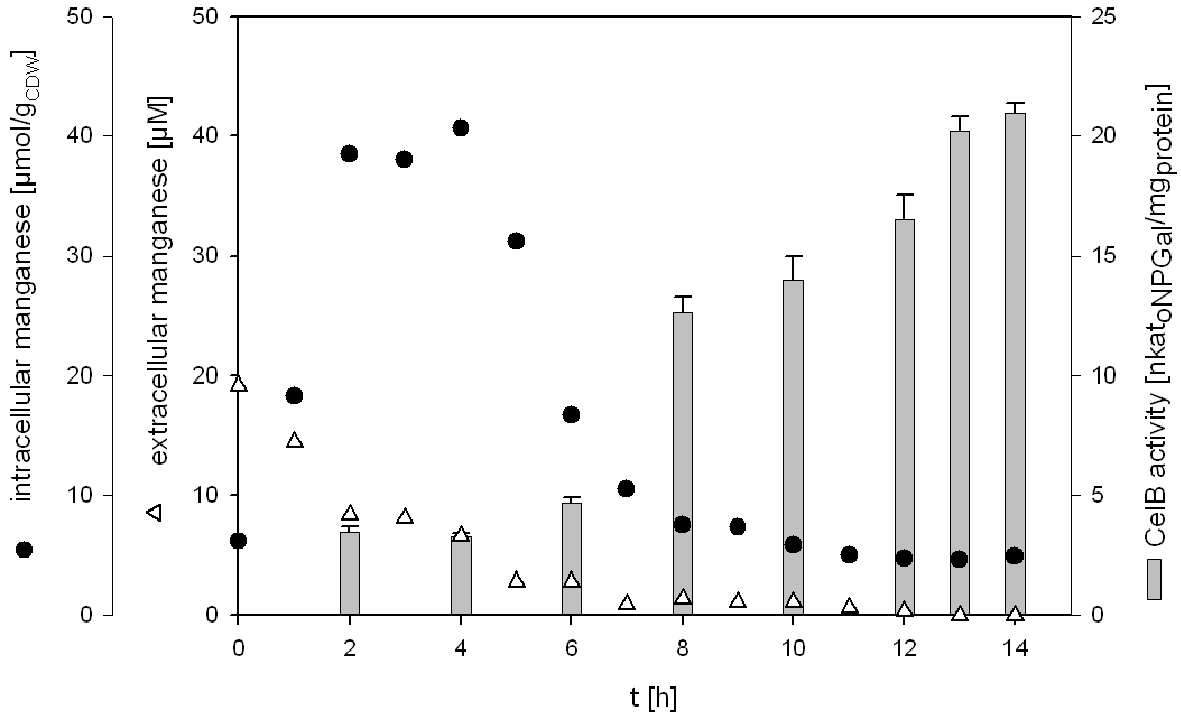

Figure 3 Manganese concentrations and kinetic of P_{mntH2} induction from anaerobic batch cultivation of *Lb. plantarum* pmntH2-celB
(Open triangle: extracellular manganese; filled circle: intracellular manganese; bars: specific CelB activity, error bars indicate the standard deviation)

In the beginning of the cultivation the manganese accumulates intracellular up to concentrations of about 40 µmol g_{cdw}^{-1} due to an uptake of manganese. In standard MRS medium with 296 µM $MnSO_4$ the intracellular manganese accumulates with 76 µmol g_{cdw}^{-1} almost twice as high. An intracellular enrichment of manganese due to transport systems as protection mechanism against the damaging effect of oxygen radicals in *Lb. plantarum* is described in the literature (Archibald & Fridovich, 1981a/b; Archibald & Duong, 1984; Groot et al. 2005). The mntH2-promoter was induced by manganese depletion due to the bacterial growth in the medium with 20 µM $MnSO_4$ by auto-induction. An increase of CelB activity was visible after 8 hours of cultivation in the mid exponential phase of growth when extracellular manganese decreases less than 1.5 µM and the intracellular manganese decreases due to starvation conditions less than 10 µmol g_{CDW}^{-1}. No increase of CelB activity was recognised in standard MRS medium, where a 20 times lower activity of 1.1 $nkat_{oNPGal}$ $mg_{protein}^{-1}$ was detected. The intracellular manganese concentration did not reduce below values of 31 µmol g_{cdw}^{-1}, also the extracellular concentration did not decrease below 19.7 µM. So, no inducing concentrations were reached due to an

excess of $MnSO_4$ resulting in low induction of the mntH2 promoter. In the literature, no expression of the MntH2 transporter was described also at Mn^{2+} concentrations of about 100 µM or higher (Groot et al. 2005).

The proof of principle of the recombinant protein expression by manganese starvation in *Lb. plantarum* was successfully demonstrated, although further improvement to the expression system will be needed before an industrial application will become economically feasible. Nevertheless it has to be mentioned, that in comparison to the established pSIP409-celB system the activity was 60 times lower, in this system a specific CelB activity of 675 $\mu kat_{pNPGal}\ L^{-1}$ was achieved (Böhmer et al, 2012). The presented auto-inducing pmntH2 expression construct may be beneficial in applications, were no high yields of recombinant proteins are necessary e.g. *Lactobacilli* as food-grade live vaccines.

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Chapter 5

5. Recombinant expression, purification and characterisation of the native glutamate racemase from *Lactobacillus plantarum* NC8

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Abstract

Glutamic acid racemases (MurI, E.C. 5.1.1.3) catalyse the racemisation of L- and D-glutamic acid. MurIs are essential enzymes for bacterial cell wall synthesis, which requires D-glutamic acid as an indispensable building block. Therefore these enzymes are suitable targets for antimicrobial drugs as well as for the potential design of auxotrophic selection markers. A high expression system in *Escherichia coli* BL21 (DE3) was constructed to produce and characterise the biochemical properties of the MurI from *Lactobacillus plantarum* NC8. In a 4-L-bioreactor cultivation, 3,266 $nkat_{D\text{-}Glu}/mg_{protein}$ of specific enzyme activity was produced. The recombinant, tag-free MurI was purified by an innovative affinity chromatography method using L-glutamic acid as the relevant docking group, followed by an anion exchange chromatography step (purification factor 9.2, yield 11%). This two-step purification strategy resulted in a MurI sample with a specific activity of 34,060 $nkat_{D\text{-}Glu}/mg_{protein}$, comprising a single protein band in SDS-PAGE. The purified MurI possessed an assay temperature optimum of 50℃, but it was not stable at this temperature. The half-lives of the purified MurI were 162 h at 20℃ and only 1.9 h at 40℃. The MurI activity was maximum between pHs 7 and 10, resulting in a maximal half-life of 287 h at pH 7. Only D- and L-glutamic acid were recognised as substrates for the MurI with similar k_{cat}/K_m ratios of 3.6 sec^{-1}/mM for each enantiomer.

Highlights

- Recombinant expression of the MurI from *Lb. plantarum* in *E. coli* for the first time
- Use of a novel, L-Glu affinity chromatography for purification to a single band wihtout use of His-tag or other affinity tags
- First enzymological characterisation of the MurI from *Lb. plantarum* at this level of detail

Keywords

Lactobacillus plantarum,

glutamic acid racemase,

MurI,

over-expression,

enzyme purification,

kinetics

Introduction

Glutamic acid racemases (E.C. 5.1.1.3) catalyse the racemisation of L- and D-glutamic acid. They are cofactor-independent enzymes with two cysteines acting as the catalytic residues [1]. Bacteria use this racemase for direct generation of D-glutamic acid from the proteinogenic amino acid L-glutamic acid. The product D-glutamic acid is an important feature that is incorporated into all bacterial cell walls. With D-alanine, it is used in the peptidoglycan layer as part of the cross-linking pentapeptides, that result in a strong, elastic polymer, encapsulating the bacterial cell and serving as protection against osmotic lysis [2]. The gene for glutamic acid racemase was first described in *Escherichia coli* and designated *murI* [3]. Currently, the genes encoding amino acid racemases have been identified as essential genes in many bacteria [4, 5]. For this reason, these enzymes have emerged as targets for the design of new antibiotics. In addition, there is an industrial application of glutamic acid racemases in the biotransformation of L-glutamic acid to the D,L-form [6]. A further conversion of the racemic mixture to pure D-glutamic acid can be obtained by selective decarboxylation. The resulting D-glutamic acid is an important intermediate for the production of pharmaceuticals.

For industrial applications of glutamic acid racemase, it is important to obtain the enzyme in sufficient amounts and with high specific activities. Generally, the amounts of glutamic acid racemase from wildtype organisms are limited due to their low activities in most cellular metabolisms. The existence of glutamic acid racemase was first demonstrated in diverse lactobacilli, including *Lb. fermenti, Lb. brevis, Lb. casei* and *Lb. plantarum,* but with low activities [7-10]. Recombinant production of MurIs from different organisms was performed in *E. coli* to gain higher activities, and mainly reported as using shaken flask cultivations [5, 11, 12]. Recombinant production of MurI from *Lb. fermenti* in *E. coli* gave approximately 3000-fold higher yields compared to the wildtype *Lb. fermenti* [13]. For accurate biochemical characterisation or structure analysis, it is necessary to make the recombinantly expressed enzymes, such as glutamic acid racemases in native, active and highly purified form. Recently, the glutamic acid racemases from pathogenic as well as non-pathogenic bacteria were recombinantly expressed in an active form in *E. coli,* purified by precipitation, ion exchange chromatography and His-tag affinity chromatography and subsequently characterised [5, 12, 14]. With the use of affinity chromatography, the purification of proteins to homogeneity can often be achieved in fewer purification steps [15].

Nevertheless the use of His-tags and other added affinity tags sometimes causes significant effects on the enzymatic activities of some proteins. Thus, the use of a tag-free purification system prevents this disadvantage. [16]. Therefore, it is desirable to generate a tag-free recombinant MurI. Enzymes can be purified by affinity chromatography using the enzymes substrates, products or even inhibitors as ligands [15]. With this strategy, a recombinant enzyme should be efficiently purified in a tag-free, native form. In the present study, the glutamic acid racemase from *Lb. plantarum* NC8 was investigated for recombinant production in a bioreactor cultivation of *Escherichia coli* BL21 (DE3) and tag-free purified by substrate affinity chromatography. Additionally, the purified, tag-free enzyme was biochemically characterised.

Materials and Methods

Chemicals and enzymes

All chemicals were obtained from Sigma-Aldrich (Seelze, Germany) or Carl Roth (Karlsruhe, Germany) in analytical grade or higher. L-glutamic acid dehydrogenase from bovine liver was purchased from Sigma-Aldrich. T4-DNA Ligase and hexokinase/glucose-6-phosphate dehydrogenase (HK/G6P-DH) were purchased from Roche (Mannheim, Germany). All restriction enzymes were from New England Biolabs (Frankfurt, Germany). HotStar HiFidelity polymerase was purchased from Qiagen (Hilden, Germany).

Bacterial strains, media, cultivation conditions and plasmids

Escherichia coli XL1-blue was purchased from Stratagene (Santa Clara, USA), *Escherichia coli* BL21 (DE3) and the pET20b(+) expression plasmid from Merck KGaA (Darmstadt, Germany). Both were grown in Luria-Bertani media or 2YT media + 1 % (w/v) glucose at 37℃ with shaking (120 rpm). *Lactobacillus plantarum* NC8 (CCUG 61730) was obtained from the culture collection of the Norwegian University of Life Science (Ås, Norway) and grown in MRS media according to De Man Rogosa and Sharp [17] at 30℃ with shaking (90 rpm) . Agar plates were solidified by adding 1.5% (w/v) agar. Ampicillin was added when required to a concentration of 100 µg mL^{-1}.

Analytical methods

Cell growth was monitored by measuring the optical density at 600 nm (OD_{600nm}). Glucose concentrations were measured by a photometric assay at 340 nm with HK/G6P-DH as a coupling enzyme reaction in microtiter plates, based on the manufacturer's protocol for the D-glucose/D-fructose test kit (R-Biopharm AG**,** Darmstadt, Germany; product code 10 139 106 035). Protein concentrations were determined using the method of Bradford with bovine serum albumin as the standard [18]. Expression of the racemase was analysed by SDS-PAGE using a 12.5% polyacrylamide gel according to the method of Laemmli [19]. For this procedure 5 µg of protein was loaded onto a gel, separated and stained with Coomassie blue R250 as described by Fairbanks [20].

Cloning of the *murI* from *Lb. plantarum* NC8 and construction of the pET20b-murI expression vector

The glutamic acid racemase gene (*murI*) from *Lb. plantarum* NC8 was amplified from genomic DNA by PCR. Genomic DNA was isolated as described [21]. The primers were designed using the genome data for *Lb. plantarum* WCFS1 [22], according to the DNA sequence of the *murI* gene (NCBI GeneID: 1062262), and ordered from biomers.net (Ulm, Germany). The primers, murI_fw (cga cat atg gca aat gaa cat gca att ggc) and murI_rev (tag gaa ttc tta gtc att cgc ttc act ccc taa atc), carried *Nde*I and *Eco*RI restriction sites (underlined) for cloning into the corresponding sites of pET20b. The amplification was carried out using genomic DNA (100 ng) as the template and 100 pmol of each primer. After an initial denaturation of the DNA at 95℃ for 5 min, 35 cycles of denaturation (95℃ for 15 s), annealing (52℃ for 60 s) and extension (72℃ for 90 sec) were executed, with a final extension at 72℃ for 10 min. The expression plasmid was constructed using standard molecular biology techniques and designated pET20b-murI [23].

The PCR fragment obtained was sequenced to confirm the full length DNA sequence of the *murI* gene using a Long ReadIR 4200 DNA sequencer (LI-COR Corporation, Lincoln, USA) with the Thermo Sequenase™ Cycle Sequencing Kit (Affymetrix, Santa Clara, USA). The pET20b-murI construct was used for the production of MurI in *E. coli* BL21 (DE3) cells.

Recombinant expression of MurI in *E. coli*

E. coli BL21 (DE3) was transformed with pET20b-murI and used for recombinant expression of MurI. Initial expression experiments were performed in baffled shaking flasks using 2YT medium at the 100 mL scale at 37℃. Induction was done at an OD_{600nm} = 0.5 with a final concentration of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After induction, the temperatures were lowered to 30℃ or 20℃ and the cells were harvested by centri fugation (8,000xg, 10 min, 4℃) after 16 h.

Large-scale cultivation was performed using a Biostat E fermenter (B. Braun, Melsungen, Germany) with 4 L of the above-mentioned medium at 500 rpm, air gassing (pO_2 > 30%) and pH 7.0, controlled with 12.5% (v/v) NH_4 and 0.66 M H_3PO_4. An initial preculture was grown over night in test tubes (5 mL) and second (50 mL) and third precultures (400 mL) were grown overnight in shaking flask in the

appropriate medium before inoculation of the bioreactor. Induction was initiated at OD_{600nm} = 10 with 0.4 mM IPTG, after which the temperature was lowered to 20℃. Cells were harvested after the stationary growth phase was reached and centrifuged as described above. Samples were withdrawn throughout the fermentation to analyse biomass, glucose and MurI activity.

Purification of MurI

A 30% (w/v) cell suspension in 25 mM potassium phosphate buffer with 0.5 mM EDTA (pH 7.5, buffer A) was prepared for the purification of MurI. The cells were lysed by sonification using an Ultrasonic Processor UP 200S (Hielscher Ultrasonic, Teltow, Germany). A cell-free extract was obtained after separation of the cell debris by centrifugation (8,000xg, 10 min, 4℃).

The purification was performed using an Äktapurifier system (GE Healthcare, Munich, Germany). As the first step, a glutamic acid affinity chromatography was used with L-glutamic acid coupled onto pre-activated BioFox 40ACT agarose (Wissenschaftliche Gerätebau Dr. Ing. Herbert Knauer GmbH, Berlin, Germany). To accomplish this step, 25 g of BioFox 40ACT agarose was washed with 10 volumes (w/v) of distilled H_2O. Next, the agarose was resuspended in 25 mL of 50 mM Na_2CO_3 (pH = 8), 10 mM L-Glu and agitated gently overnight at room temperature. Unreacted L-Glutamic acid was removed by washing with distilled H_2O. Remaining active groups were blocked by resuspending in 1 M ethanolamine in distilled H_2O (pH = 8) and reacted overnight. Afterwards, derivatised BioFox 40ACT-L-Glu was washed with ddH_2O.

After filtration (0.45 µm), the cell-free extract was loaded onto the L-Glu-Affinity column (1 column volume (CV) = 18 mL) with a flow rate of 0.1 mL min^{-1}. Unbound protein was eluted with 3 CV of buffer A and a flow rate of 0.1 ml min^{-1}. MurI was eluted with a linear gradient (0-1,000 mM NaCl in buffer A) of 5 CV and a flow rate of 0.5 mL min^{-1}. Fractions containing MurI activity were pooled and dialysed overnight against 25 mM potassium phosphate buffer with 0.5 mM EDTA and 1 mM L-Glu (pH 7.5, buffer B) at 4℃. The sample was then loaded onto a BioFox 40Q anion exchange column (Wissenschaftliche Gerätebau Dr. Ing. Herbert Knauer GmbH, Berlin, Germany) and washed with 2 CV (1 CV = 20 mL) of buffer B. MurI was eluted with a linear gradient (0-1,000 mM NaCl in buffer B) of 10 CV. Load, wash and elution were performed with a flow rate of 2 ml min^{-1}. Finally, the pooled fractions with MurI activity were dialysed as described above.

Enzyme assay for MurI activity

MurI activity was assayed enzymatically for the substrate D-glutamic acid using a Ultrospec 3000 spectrophotometer (GE Healthcare, Munich, Germany), as described by Glaser [7] with minor modifications. The enzyme assay consisted of two consecutive reactions. The first MurI reaction mixture contained 1 mL 25 mM potassium phosphate, pH = 7.5, 0.5 mM EDTA and, as the substrate 12 mM D-glutamic acid. The enzyme was added to a final volume of 1.5 mL. The reaction was started by addition of the enzyme and incubated at 37℃ with vigorous shaking. After 120 sec, the reaction was stopped by addition of 0.1 mL 3 M perchloric acid, and denatured protein was removed by centrifugation (13,000xg, 5 min, 4℃). The supernatant was decanted and neutralised by addition of 0.1 mL 3 M potassium hydroxide and incubation for 15 min on ice. The precipitate was removed by centrifugation (13,000xg, 5 min, 4℃) and the supernatant, containing 0.03-0.15 µM L-glutamic acid, was subsequently analysed with L-glutamic acid dehydrogenase in the second reaction mixture. The second reaction mixture contained 0.2 mL 0.5 M TRIS-HCl (pH = 9.5), 0.1 mL 0.1 M NAD^+ and 0.1 mL of the first reaction mixture in a total volume of 1 mL. The reaction was started by addition of 0.03 mL of L-glutamic acid dehydrogenase and was incubated at 25℃ with vigorous shaking for 1 h. Finally, the rate of the NAD^+ reduction was measured spectrophotometrically at 340 nm.

One nkat of enzyme was defined as the amount of enzyme that produced 1 nmol of L-glutamic acid from D-glutamic acid per sec.

Circular Dichroism (CD) assay for MurI activity

The kinetic parameters of MurI were analysed for the substrates D- and L-glutamic acid, for which the enzymatic assay for MurI activity was not suitable. A circular dichroism assay was used, whereby the change in ellipticity was monitored using a Jasco J-715 CD spectrophotometer (JASCO Germany GmbH, Gross-Umstadt, Germany), according to the method described by Potrykus [12] with slight modifications. Reactions were conducted in 10 mM potassium phosphate buffer (pH 8.0) at 37℃ with substrate concentrations of 0.5-5 mM in a final volume of 800 µL in a 0.5 cm quartz cuvette. The reaction was started by addition of the purified MurI (dialysed against 10 mM potassium phosphate buffer (pH = 8) and at a final concentration of 0.6 µg mL^{-1}). The change in ellipticity at 202 nm was measured over

a period of 5 min. The assay mixture was agitated by a magnetic stirrer after addition of MurI. Velocities were determined using a molar ellipticity ([θ]) of 34.8 mdeg mM^{-1} cm^{-1} for L-glutamic acid and 34.4 mdeg mM^{-1} cm^{-1} for D-glutamic acid. The values of k_{cat} were calculated by dividing v_{max} values by total MurI concentrations using 59,424 Da as the MW value for the dimer.

Effect of temperature and pH on MurI activity

The effect of temperature on the activity of the recombinant MurI was investigated using the enzymatic assay at 20, 30, 35, 40, 45, 50, 55 and 60℃ in 25 mM potassium phosphate, 0.5 mM EDTA,1 mM L-Glu (pH 7.5) after pre-incubation of the substrate solution for 5 min. The effect of pH on the activity of the recombinant MurI was examined with different buffers in the pH range of 5.0 - 11.0. The buffers 25 mM potassium phosphate (pH 5.0 – 7.0), 25 mM TRIS-HCl (pH 7.0 – 9.0) and 25 mM glycine-NaOH (pH 9.0-11.0) were used. The relative activity was calculated using the sample with the highest activity as 100%.

Thermal stability was investigated by incubating the recombinant MurI over 24 h at 0, 10, 20, 30 and 40℃ in 25 mM potassium phosphate, 0 .5 mM EDTA, 1 mM L-Glu (pH 7.5). The pH stability was investigated by incubating the recombinant MurI over 22 h at pH 7.0, 8.0, 9.0 (TRIS-HCl) and 10.0 (NaOH) at 0℃. The residual enzyme activity at various time points was measured under enzyme assay conditions. The relative activity was calculated using the 0 h sample activity as 100%.

Determination of the substrate specifity

A preliminary survey of the substrate specificity of MurI was performed in potassium phosphate buffer, 0.5 mM EDTA (pH 7.5) by incubating MurI with 1 mM of amino acid (D/L-glutamic acid, D/L-glutamine and D/L-aspartic acid) at 37℃ for 10 min in a volume of 1 mL. The products were analysed after derivatisation with BOC-L-Cys-OPA according to Hashimoto [24] by HPLC using a Thermo SpectraSYSTEM (degasser, P2000 LC pump, AS1000 Autosampler, UV1000 UV-Vis detector, Thermo Fisher Scientific, Dreieich, Germany) with a RP C18 column (Gromsil 120-ODS-3, 125 x 4.6 mm, 3 µM, GROM Analytik + HPLC GmbH, Herrenberg, Germany).

Determination of the molecular weight

The molecular weight and the quaternary structure of the recombinant MurI were examined under native conditions by gel filtration using a Superdex 75 10/300 GL column with an Äkta FPLC (GE Healthcare, Munich, Germany). Purified MurI and standard protein mix (Gel Filtration Calibration Kit LMW, GE Healthcare) were eluted under isocratic conditions using 25 mM potassium phosphate buffer + 150 mM NaCl, 0.5 mM EDTA, 1 mM L-Glu (pH 7.5) with a flow rate of 0.5 mL min^{-1} and a detection at 280 nm. The molecular weight of the subunits was analysed by SDS-PAGE.

Results and Discussion

Cloning and expression of *murI*

The *murI* of the complete genome of *Lb. plantarum* WCFS1 was found in GenBank (NCBI GeneID: 1062262) [22]. In a recent study, complete resequencing of the *Lb. plantarum* WCFS1 genome and analysis were performed by InterProScan. The *murI* gene was afterwards 'inferred by homology' as the glutamic acid racemase gene [25]. The important cysteine residues of the active centre were conserved in *Lb. plantarum* MurI as Cys^{74} and Cys^{185}, as described for the *Lb. fermentum* MurI and the *B. subtilis* RacE [1, 9].

Based on this sequence information an 837 bp *Nde*I-*EcoR*I fragment containing the 822 bp *murI* gene was amplified with genomic DNA from *Lb. plantarum* NC8 as the template and ligated in pET20b. The plasmid obtained (pET20b-murI) was used for the recombinant production of the enzyme in *E. coli* BL21 (DE3) after sequencing of the *murI* insert. The cloned *murI* gene from *Lb. plantarum* NC8 showed complete homology to the *murI* gene from *Lb. plantarum* WCFS1 (NCBI GeneID: 1062262). Recently, the genome of *Lb. plantarum* NC8 was sequenced [26]. The cloned gene also showed complete homology to the now available *murI* gene sequence from *Lb. plantarum* NC8 (NCBI AGRI01000006.1).

In the literature the formation of inclusion bodies has been described as occuring when recombinant glutamic acid racemases were produced in *E. coli* [27-29]. The formation of inclusion bodies resulted in misfolded, insoluble and inactive MurI. A widely used strategy to overcome this problem is the cultivation of the recombinant *E. coli* at low temperatures, even as low as 6 – 10℃ [30]. In our case, preliminary expression experiments were performed in baffled shaking flasks with *E. coli* BL21 (DE3) pET20b-murI at 20℃. At this temperature, rea sonable growth of the cells was obtained, an overexpressed protein band occurred in SDS-PAGE of the clear cell-free extract, and glutamic acid racemase activity could be detected (data not shown). Therefore, a subsequent bioreactor cultivation (4 L-scale) was performed over 21 h at 20℃ (Figure 1). The highest MurI activity of $3,266 \pm 13$ $nkat_{D\text{-}Glu}/mg_{protein}$ was obtained at the end of the cultivation when the cells reached the stationary growth phase, and the glucose was completely consumed.

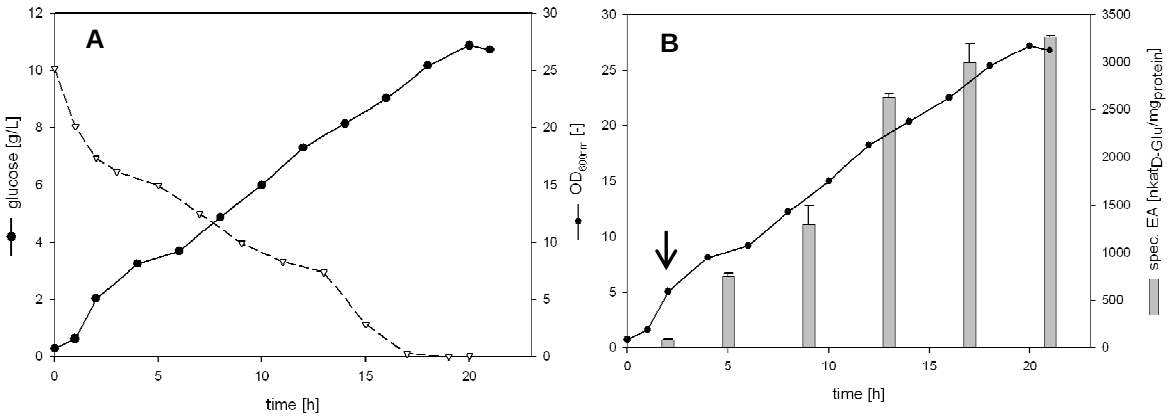

Figure 1: Recombinant production of MurI with *E. coli* BL21 pET20b-murI (2YT medium + 1 % (w/v) glucose; 4-L-scale bioreactor cultivation)
A: Cell growth and glucose consumption; B: cell growth and recombinantn MurI expression. (filled circles: OD_{600nm}; open triangles: glucose concentration; arrow: addition of IPTG for induction and lowering of the temperature from 37 to 20℃; bars: specific MurI activity, error bars indicate the standard deviation)

To our knowledge this is the first time that a tag-free, “native” glutamate racemase (MurI) from *Lb. plantarum* NC8 has been recombinantly expressed at such high activity levels. Our maximum MurI activity value was approximately 62,000-fold higher than that previously described for native MurI in the original wildtype *Lb. plantarum* [31]. In other studies the MurI from *Lb. fermenti* was recombinantly expressed in *E. coli* DH5α [13], and a specific activity of 621 $nkat_{D\text{-}Glu}/mg_{protein}$ was achieved. This value is 5-fold less than our result. In addition, the MurI from *Lactobacillus brevis* ATCC8287 was expressed in *E. coli* TM93 producing a specific activity of 153 $nkat_{L\text{-}Glu}/mg_{protein}$ [9]. This value is approximately 21-fold lower than in our study.

Purification of the recombinant MurI

MurI was purified 9.16-fold by L-glutamic acid affinity chromatography and anion exchange chromatography (BioFox 40Q), with an overall yield of 11% (Table 1).

Table 1: Purification of recombinant MurI from *Lb. plantarum* NC8

	Total protein [mg]	Specific activity [$nkat_{D\text{-}Glu}$ /mg]	Total activity [$nkat_{D\text{-}Glu}$]	Yield [%]	Purification [fold]
Crude extract	32.5	3,720	121,110	100	1
L-Glu affinity chromatography	4.6	11,700	53,710	44	3.15
Biofox40Q chromatography	0.4	34,060	13,450	11	9.16

Most common purification procedures published for native MurIs are based on ammonium sulphate precipitation, followed by hydrophobic interaction chromatography and ion exchange chromatography and finally by hydroxyapatite chromatography [31, 32]. The affinity chromatography resulted in a purification factor of 3.15, with an activity yield of 44% (see Table 1). After the first purification step, further buffers used for MurI purification required supplementation with 1 mM L-/D-Glu, otherwise the enzyme became unstable. This requirement was also recognised for the native MurI from *Lb. plantarum* [7]. After the BioFox 40Q chromatography, the purified MurI appeared as a homogenous single band on SDS-PAGE with an apparent mass of 30 kDa (see Figure 2).

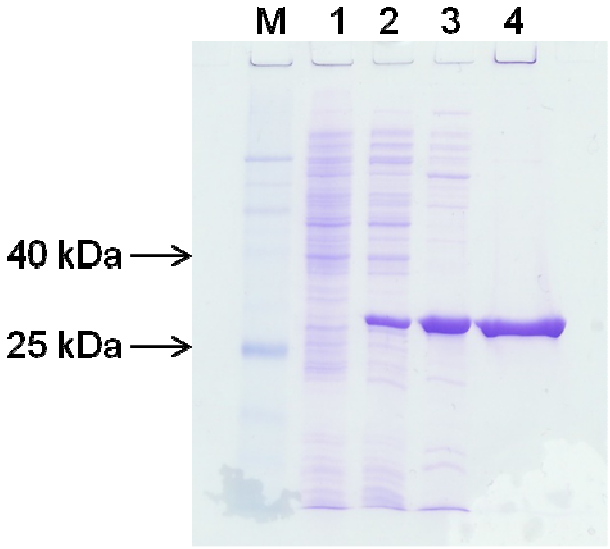

Figure 2: 12.5% SDS-PAGE analysis of the expression and purification of MurI in *E. coli* BL21
M: Molecular weight markers; lane 1: *E. coli* BL21-pET20, crude extract; lane 2: *E. coli* BL21-pET20-murI crude extract after 21 h cultivation; lane 3: MurI after L-Glu affinity chromatography; lane 4: MurI after Biofox40Q chromatography; 5 µg total protein loaded per lane, Coomassie stained.

This result is in agreement with the predicted mass of 29.7 kDa calculated *in silico.* As negative control crude extract of *E. coli* BL21 pET20b was used, where no activity of the *E. coli* MurI was detectable using the assay under standard parameters indicating a very low level of expression of the *E. coli* MurI in the stationary growth phase.

To determine the molecular weight and the quaternary structure of the active MurI, size exclusion chromatography (SEC) was performed, and the molecular weight was calculated according to the retention times of calibration standards (Fig. 3).

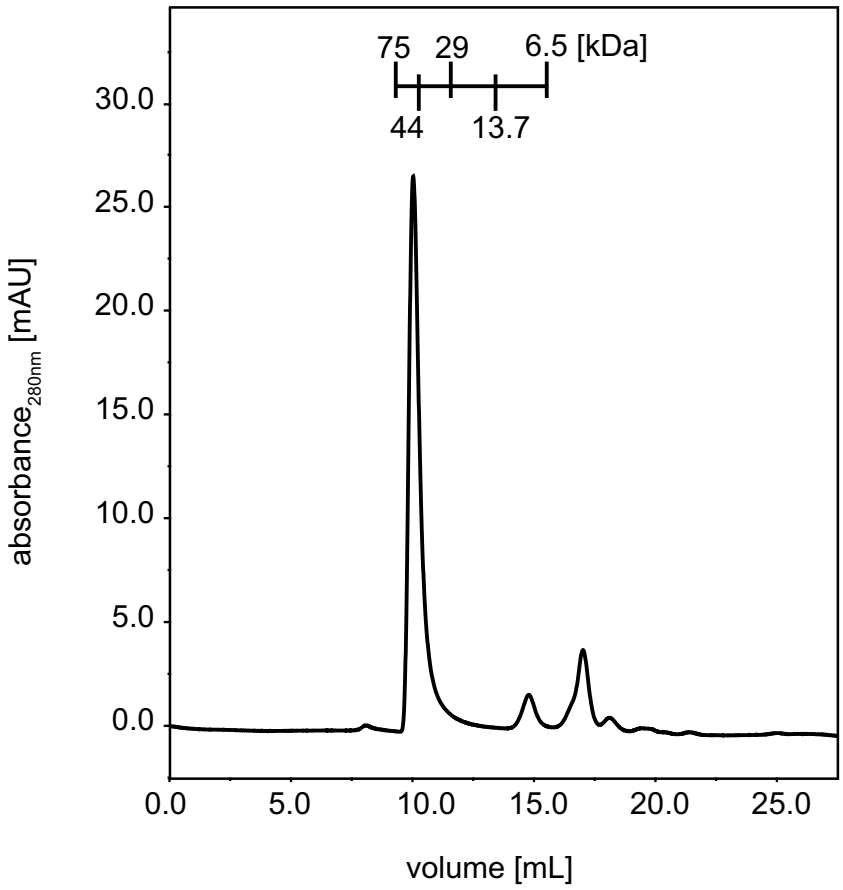

Figure 3: SEC of the purified recombinant MurI from *Lb. plantarum* NC8.
(25 mM potassium phosphate buffer + 150 mM NaCl, 0.5 mM EDTA, 1 mM L-Glu (pH 7.5); flow rate = 0.5 mL min^{-1}; detection at 280 nm)

Using this method, a homodimeric structure with an apparent molecular weight of 55.8 kDa was determined for MurI, which is in accord with the 30 kDa monomer size determined using SDS-PAGE (Fig. 2). Additionally, no aggregated soluble MurI is present after purification. This result was contrary to the monomeric form of the active MurI from *Lb. fermentum*, analysed and described by Gallo et al. [13]. However, some glutamic racemases from Gram-positive bacteria, such as *Bacillus subtilis*, *Staphylococcus aureus* and *Enterococcus faecalis* are known to assemble into homodimeric structures similar to the MurI of *Lb. plantarum* [5]. The MurI from *E. coli* is also described as a monomer [33].

Characterisation of the recombinant MurI

Substrate specificity

Because glutamic acid racemases are highly conserved enzymes and supply the bacterium with the essential D-glutamic acid for cell wall synthesis, they are known to

be very specific for glutamic acid [10, 12, 34]. An HPLC analysis was performed to investigate whether the structurally similar amino acids glutamine and aspartic acid were substrates for MurI. No enzymatic racemisation reaction was detected for either D- or L-glutamine or for D- or L-aspartic acid. Therefore, the substrate specificity of MurI was the same as for other glutamic acid racemases. A similar strict substrate specificity is described also for the alanine racemases, which catalyse only the racemisation of D- or L-alanine [32].

Determination of kinetic parameters

The kinetic parameters of the purified MurI were analysed using CD spectroscopy. This technique is common found in the literature because it allows the kinetic parameters for both of the substrates, D- and L-Glu, to be determined [12, 13, 35]. The resulting Hanes linearisation is shown in figure 4, and the kinetic parameters are listed in table 2.

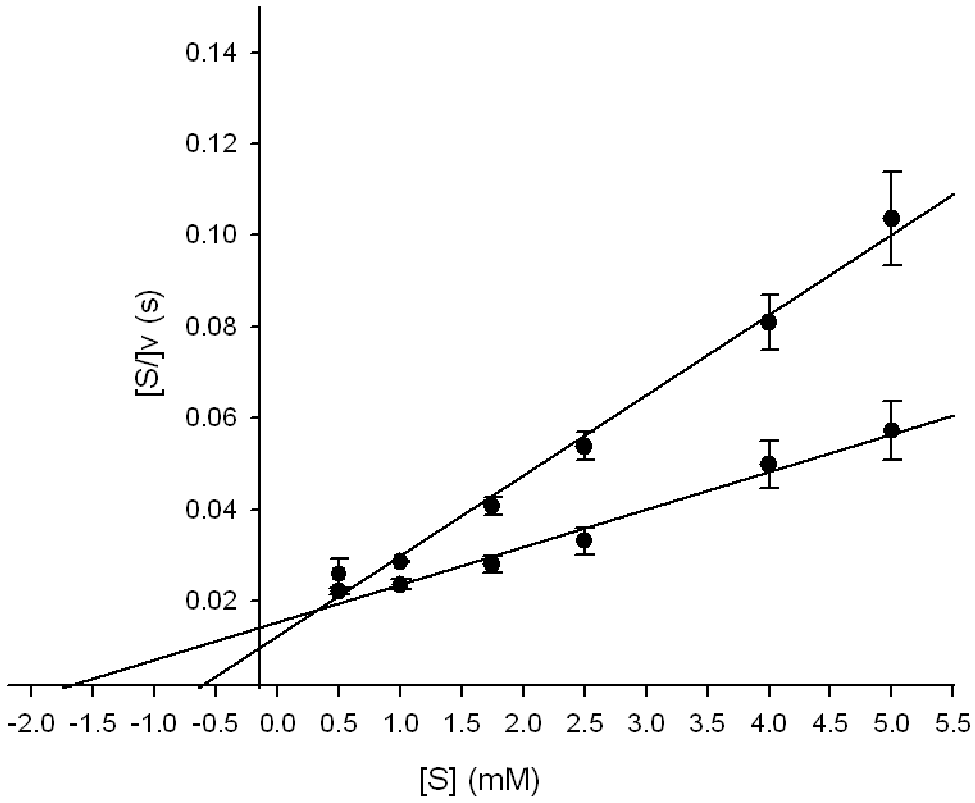

Figure 4: Calculation of K_m and v_{max} for MurI from *Lb. plantarum* NC8 with D-Glu (filled circles) and L-Glu (open circles) as substrates using a Hanes plot.

The K_m for D-Glu was 1.64 mM and for L-Glu 0.84 mM. While the $K_{m,\ D\text{-}Glu}$ is approximately 2-fold higher than the $K_{m,\ L\text{-}Glu}$, the efficiency ratio k_{cat}/K_m is similar for both substrates (see table 2).

Table 2: Kinetic parameters for purified MurI from *Lb. plantarum* NC8

Substrate	k_M [mM]	v_{max} [nkat/mL]	K_{cat} [sec^{-1}]	k_{cat}/K_m [sec^{-1}/mM]
D-glutamic acid	1.64 ± 0.12	60.4 ± 4.9	5980	3.64
L-glutamic acid	0.84 ± 0.14	30.6 ± 5.9	3029	3.61

The same observation was described for the MurI from *Fusobacterium nucleatum* [12]. Therefore, the MurI from *Lb. plantarum* NC8 seems to exhibit pseudosymmetry for the racemisation of Glu in both directions. Generally, the K_m and k_{cat} values determined for the MurI from *Lb. plantarum* NC8 were in the same ranges as those described for MurIs from *Lactobacillus* sp. and other bacteria [5, 9, 13].

Effect of temperature and pH of MurI Activity

The analyses to determine MurI's temperature optimum, its pH optimum and its stability were performed with D-Glu as the substrate and are illustrated in figure 4. The recombinant MurI showed a temperature optimum of 50℃ (Fig. 5 A). At a temperature of 55℃, MurI was completely inactive. The temperature optimum for another recombinant glutamic acid racemase [9] from *Lb. brevis* was significantly lower at 37℃. The more important temperature stability of the MurI from *Lb. plantarum* NC8 is shown in Fig. 5 B. After an incubation time of 22 h, a residual MurI activity of 77% was estimated at 30℃ (half-life of 54 h). At lower temperatures, the enzyme was quite stable over this time frame (half-life of 162 h at 20℃).

MurI was similarly active between pH 7 and 10 (see Fig. 5 C), whereas the enzyme was stable only at pH 7 to 8 (see Fig. 5 D), resulting in residual activities of 97% after 22 h. The half-lives were estimated by extrapolation of the data and equalled 287 h at pH 7, 154 h at pH 8, 25 h at pH 9 and 6 h at pH 10. The purified native MurI from *Lb. plantarum* was described by Glaser [7]. The pH stability of this enzyme was maximal at pH 6.5 to 7.5, and at pH 8.5, only 70% residual activity was reported. The native MurI from *Lb. brevis* ATCC 8287 possessed a pH optimum between 8.0 and 9.0 [9].

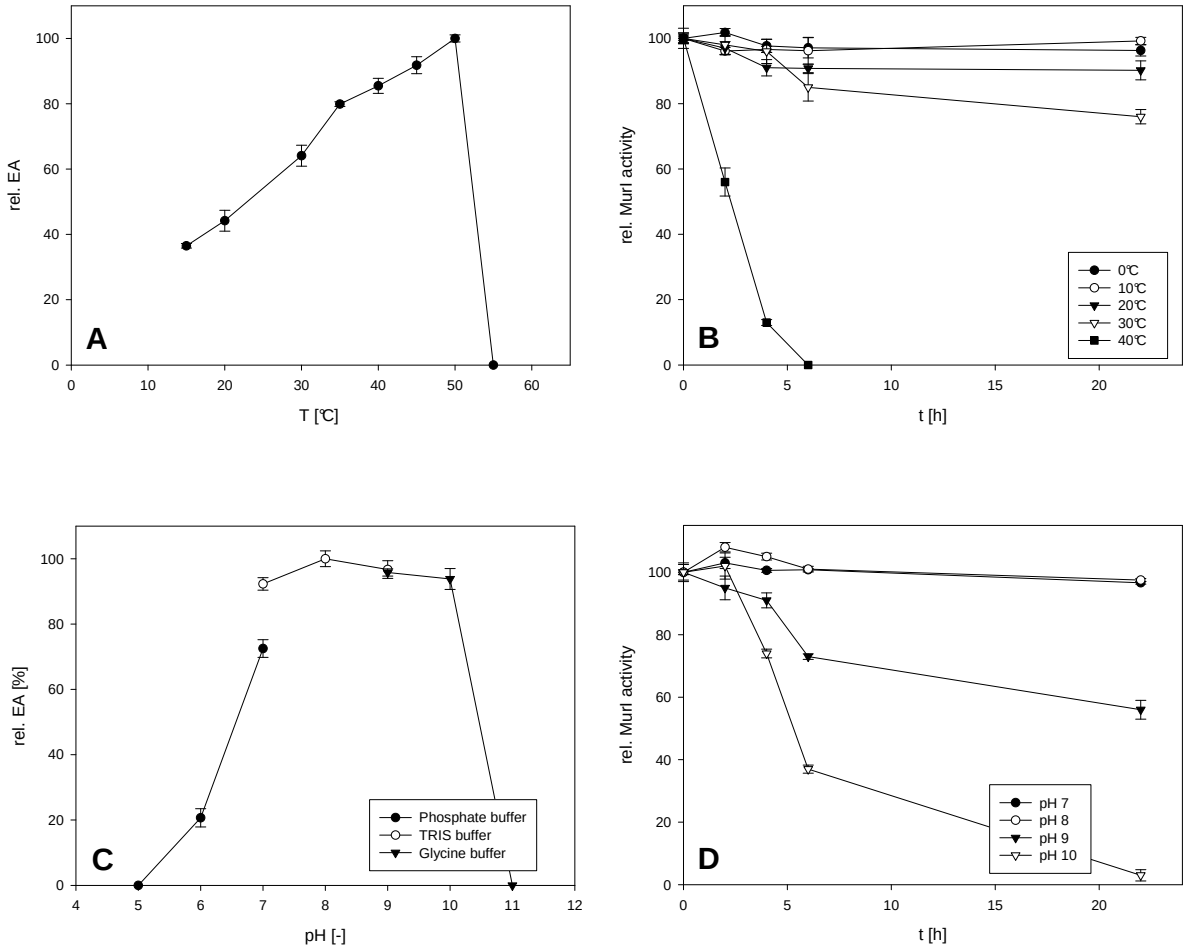

Figure 5: Characterisation of recombinant purified MurI from *Lb. plantarum* NC8 analysed with D-Glu as substrate.
(A: temperature profile; B: temperature stability; C: pH profile; D: pH stability)

Acknowledgements

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6. Own publications

- Nico Böhmer, Sabine Lutz-Wahl and Lutz Fischer (2012) Recombinant production of hyperthermostable CelB from *Pyrococcus furiosus* in *Lactobacillus* sp., *Applied Microbiology and Biotechnology* 96(4):903-12

- Nico Böhmer, Saskia König and Lutz Fischer (2013) A novel manganese starvation-inducible expression system for *Lactobacillus plantarum, FEMS Microbiology Lett*ers (2013) 342(1):37-44

- Nico Böhmer, Andreas Dautel, Thomas Eisele and Lutz Fischer (2013) Recombinant expression, purification and characterisation of the native glutamate racemase from *Lactobacillus plantarum* NC8, *Protein Expression and purification* 88 (1):54-60

7. Additional scientific publications

Beside the presented publications and manuscripts additional scientific results were prepared and are not elements of this thesis.

- Erich, S., Böhmer, N., Meyer S. und Fischer, L.: Laktosefreie Milchprodukte durch *β*-Galaktosidasen der nächsten Generation (BIOspektrum, 6/2012, S. 668)

- Böhmer, N.; Gnam, A. und Fischer, L.: „Galactooligosaccharide (GOS) mit neuen *β*-Galactosidasen aus *Bacillus megaterium*“ 6. Kongress Lebensmitteltechnologie der GDL (2012), Dresden

- Böhmer, N.; Gulan, S.; Meyer, S. and Fischer, L.: „Production of a Metagenome-*β*-Galactosidase” 1. European Congress of Applied Biotechnology (2011), Berlin

- Böhmer, N., Lutz-Wahl, S. and Fischer, L: „Recombinant production of a thermophilic *β*-glycosidase in *Lactobacillus plantarum*” 28. DECHEMA-Jahrestagung der Biotechnologen (2010), Aachen

- Böhmer, N., Liu, L., Lutz-Wahl, S. and Fischer, L: „Production of *β*-Galactosidases in food grade *Lactobacillus plantarum*” 4. Wissenschaftliche Kolloquium (2009), Hohenheim

Personal details:
Date of birth: 31.01.1985
Place of birth: Aachen, Germany
Marital status: Single
Nationality: German

Curriculum Vitae

Professional

01/13-present Development Scientist at the Sandoz GmbH, Anti-Infectives, Kundl, Austria.

- Application of state-of-the-art fermentation technologies for development of new and improvement of existing processes
- Scale-up of fermentation processes from pilot plant (up to 3 m^3) and transfer to production site
- Scientific supervising, documentation and coordination of research and development projects

10/08-12/12 PhD student at the University of Hohenheim, Institute of Food Science and Biotechnology, Department of Biotechnology (Professor Lutz Fischer, dissertation title: „Development of a food grade expression system for the recombinant production of β-galactosidases“)

Scientific Experience:
- Development and recombinant enzyme production in different expression systems (*Escherichia coli, Lactobacillus* spec*., Kluyveromyces lactis*)
- Scale-up and process development up to 50 L
- Purification of recombinant and native enzymes using ultrafiltration, different precipitation and also chromatographic methods (HIC, IEX, SEC, His-tag- and substrate affinity by FPLC)
- Development of assays and characterization of different enzymes (galactosidases, glucosidases, racemases) by diverse analysis techniques

Project Management:
- Cooperation in the FEI-project: „Screening und Bereitstellung neuer, industrietauglicher Beta-Galactosidasen für die Milchindustrie (AIF 15801)“

Teaching and Advising:
- Elaboration, advising and teaching in the master module „Bioreaktortechnik“ and „Überexpression und Mutagenese“ of the master program Enzyme Biotechnology at the University Hohenheim
- Co-Advisor of some bachelor- and diploma thesis’s

Education

09/04-08/08 Studies of Bioengineering, University of Applied Science Aachen, Germany
Best of the year, grade 1.2 (approximately equivalent: A)
Badge of Honour of the FH Aachen for best graduation
Majors: bioprocess engineering, genetic engineering, enzyme technology
Diploma thesis: „Expression und Charakterisierung von Proteasen aus *Nicotiana tabacum*“

8/95-6/04 St. Michael Gymnasium Monschau
Abitur (High School Diploma): June 2004, grade 2.1

Internship

10/07-08/08 Internship and diploma thesis at the Fraunhofer Institute of Molecular Biology and Applied Ecology IME, Aachen, Department Plant Biotechnology
Cooperation in an international team (laboratory speech, meetings and progress reports in English)

Scientific Experience:

- Recombinant enzyme production in different expression hosts (*Pichia pastoris, Nicotiana tabacum*)
- Optimization of expression and extraction methods
- Purification of recombinant enzymes using ultrafiltration and different affinity chromatographies (His-tag, Strep-tag®II) by FPLC
- SDS-PAGE, Dot Blot/ Western Blot and ELISA
- Generation of polyclonal antibodies in rabbits

Advanced Training

08/10 Äkta™ design systems and UNICORN™ control software, GE Healthcare, Munich, Germany

Language Skills

German (native)
English (fluent)

Computer Skills

MS Office (very good)
UNICORN™ (very good)
Vector NTI® 10 (good)
Clone Manager (basic)
SigmaPlot (basic)

UNIVERSITÄT HOHENHEIM

Anlage 2 zur Promotionsordnung der Universität Hohenheim zum Dr. rer. nat.

Eidesstattliche Versicherung gemäß § 7 Absatz 7 der Promotionsordnung der Universität Hohenheim zum Dr. rer. nat.

1. Bei der eingereichten Dissertation zum Thema

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   handelt es sich um meine eigenständig erbrachte Leistung.

2. Ich habe nur die angegebenen Quellen und Hilfsmittel benutzt und mich keiner unzulässigen Hilfe Dritter bedient. Insbesondere habe ich wörtlich oder sinngemäß aus anderen Werken übernommene Inhalte als solche kenntlich gemacht.

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Ort und Datum Unterschrift