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MASTER THESIS

Submitted by

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Immobilization of β-Galactosidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* using chitin-binding domain and biochemical characterization of immobilized enzyme for lactose hydrolysis

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Food Sciences and Technology

Supervised by

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and

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Tatjana Leister

I ACKNOWLEDGMENTS

It is not the possession of truth, but the success which attends the seeking after it, that enriches the seeker and brings happiness to him.

Max Plank

I would like to express my sincere gratitude to my advisor, Dr. Thu-Ha Nguyen, for her attentive guidance during this work. Without her scientific insight and her generous support, this work would not have been possible.

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II ABSTRACT

With the great use of β-galactosidases in the food industry, immobilization of these enzymes gains increasing interest as this technique enables reutilization, continuous operation, and occasionally increased stability. Furthermore, β-galactosidases show transgalactosylation reaction forming galacto-oligosaccharides which are non-digestible oligosaccharides with prebiotic function. As conventionally immobilization techniques usually come with restrictions in diffusion and mechanical stability, or severe reaction conditions, therefore their applications in industrial scale are often limited. With the development of recombinant DNA technology, new approaches for enzyme immobilization via affinity tags are of great interest. In this study, the β-galactosidase gene *lacZ* from *Lactobacillus delbrueckii* subsp. *bulgaricus* was fused C-terminally to the chitin-binding domain (ChBD) of chitinase A1 from *Bacillus circulans* WL-12 and subsequently expressed in *L. plantarum*. The resulting fusion protein was then immobilized on insoluble chitin and characterized for its biochemical properties, including optimal operation pH and temperature, thermal stability and kinetic parameters. The application potential of this biocatalyst in lactose transformation was also confirmed using lactose solutions and milk samples.

III KURZFASSUNG

Aufgrund des großen Einsatzes von β-Galactosidase in der Lebensmittelindustrie, gewann die Immobilisierung dieses Enzyms großes Interesse. Neben der Wiederverwendbarkeit, kontinuierlicher Betriebsweise bietet diese Methode oftmals erhöhte Stabilität. Darüber hinaus zeigt β-Galactosidase sogenannte Transgalactosylierungs-Aktivität auf, wobei unverdauliche, präbiotische Galactooligosaccharide synthetisiert werden. Herkömmliche Immobilisierungsmethoden eignen sich weniger für den Großeinsatz, da diese oft mit Einschränkungen in Produkt- sowie Substrat-Diffusion und mechanischer Stabilität oder mit scharfen Reaktionsbedingungen einhergehen. Mit rekombinanter DNA-Technologie wurde in vorliegender Arbeit ein neuer Ansatz untersucht, wobei die Immobilisierung mittels Affinitäts-Tag erfolgt. In dieser Weise wurde das für β-Galactosidase codierende Gen *lacZ* aus *Lactobacillus delbrueckii* subsp. *bulgaricus* am C-Terminus mit der Chitin-Bindungs-Domäne (ChBD) der Chitinase A1 aus *Bacillus circulans* WL-12 verknüpft. Das entstehende Hybrid-Protein wurde in *Lactobacillus plantarum* exprimiert und anschließend auf Chitin immobilisiert. Die immobilisierte β-Galactosidase wurde biochemisch hinsichtlich ihrer Optima und Stabilität über pH- und Temperatur sowie ihrer Kinetik charakterisiert. Die Anwendbarkeit für die Umwandlung von Laktose wurde mittels Laktose-Lösungen und Milch-Proben getestet.

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β-gal	β-galactosidase
AR%	activity retention
ChBD	chitin binding domain
DA	Degree of acetylation
DD	Degree of deacetylation
DTT	Dithiothreitol
EDTA	Ethylene Diamine Tetraacetic Acid
ery	Erythromycin
GH	Glycoside hydrolase
Glu	Glutamic acid residue
GlcNAc	N-acetyl-D-glucosamine
GOD	Glucose oxidase from Aspergillus niger
GOS	galacto-oligosaccharide(s)
GRAS	generally regarded as safe
HPLC	High-peformance liquid chromatography
IY%	immobilization yield
k_{cat}	catalytic constant
k_{cat}/K_M	catalytic efficiency
K_M	Michaelis-Menten constant
LAB	Lactic acid bacteria
LAU	Lactose activity Unit
Mw	Molecular weight
NaPP	Sodium phosphate
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
POD	peroxidase from horseradish
oNPG	o-Nitrophenyl-β-D-galactopyranoside
*o*NP	o-Nitrophenol
SDS	sodium dodecyl sulfate
TAE	Tris acetate EDTA
$t_{1/2}$	Half life time of activity
TLC	Thin-layer chromatography
V_{max}	maximal reaction velocity

MANUSCRIPT PREPARED FOR PUBLICATION

Immobilization of β-Galactosidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* using chitin-binding domain and biochemical characterization of immobilized enzyme for lactose hydrolysis

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1 ABSTRACT

The β-galactosidase gene *lacZ* from *Lactobacillus delbrueckii* subsp. *bulgaricus* was cloned and fused C-terminally with the chitin-binding domain (ChBD) of chitinase A1 from *Bacillus circulans* WL-12. The resulting fusion protein (LacZ-ChBD) was exprssed in *Lactobacillus plantarum* yielding 31000 U of β-galactosidase activity per liter of medium. The fusion protein was then immobilized onto chitin resin with a high immobilization yield (IY%) ranging between 90 and 95%. The maximum resulting immobilized activity was found to be 2585 ± 205 U/g of chitin with high activity retention (AR%) of 55%. The immobilized enzyme was then characterized for its biochemical properties including optimal operating pH and temperature, thermal stability and kinetic parameters (K_m and V_{max}). The optimal pH values were found to be 8 and 6.5, while the optimal operating temperatures were 55 – 60 and 60 − 65 °C, when using o-nitrophenyl β-D-galactopyranoside (oNPG) and lactose as substrates respectively. The immobilized fusion enzyme showed a high half-life time at 37 °C of 8.5 days. At 55 °C, the immobilized enzyme showed a half-life time of 7 h. Reusability of the immobilized enzyme was investigated by subsequent hydrolysis of oNPG revealing over 40% of residual activity after ten batches. Immobilized β-galactosidase was used for lactose conversion and for 50 g/L and 205 g/L initial lactose concentrations the maximum lactose conversions of 81% and 70% respectively, were obtained. The maximum yield of galacto-oligosaccharides (GOS) was approximately 25% when using an initial concentration of 600 mM lactose, indicating that the enzyme can be of interest for the production of GOS.

KEYWORDS: β-galactosidase, lactase, immobilization, chitin, galacto-oligosaccharides

2 INTRODUCTION

The naturally occurring enzyme β-galactosidase (EC.3.2.1.23) hydrolyses lactose into its monomers glucose and galactose. As lactase deficiency is increasing in human population, β-galactosidase is of major importance for applications in the food processing industry (SHUKLA and WIERZBICKI, 1975; MAHONEY, 1997). Besides lactose hydrolysis the enzyme shows also transglycosylation activity synthesizing galacto-oligosaccharides (GOS) by transferring galactosyl residues to lactose (ARONSON, 1952; PAZUR, 1953). These GOS are non-digestible oligosaccharides and show prebiotic properties as they promote the growth of beneficial bacteria in the digestive tract (FULLER and GIBSON, 1997; SPLECHTNA et al., 2006; SPLECHTNA et al., 2007).

The β-galactosidases encoded by the *lacZ* gene belong to glycoside hydrolase family GH2 (CAZy nomenclature) and thus form di- or oligomeric GH2 β-galactosidases. These β-galactosidases can be found in several lactic acid bacteria (LAB) including *L. delbrueckii* subsp. *bulgaricus*, but also some *Streptococcus* sp. or bifidobacteria (NGUYEN et al., 2012; CANTAREL et al., 2009; LAPIERRE et al., 2002).

LAB are Gram-positive, non-spore forming, strictly fermentative and anaerobic bacteria. They found widespread application in traditional conservation and as starter cultures in food fermentation. Some *Lactobacillus* species such as *L. crispatus*, *L.gasseri*, and *L. plantarum* can even be found in human gut (HOLZAPFEL et al., 2001; CATALOLUK et al., 2004). With *L. plantarum* and *L. delbrueckii* subsp. b*ulgaricus* amongst others as microorganisms with probiotic properties affecting the microflora of the host in a beneficial manner, they attracted major attention regarding the application in food and nutrition (HOLZAPFEL et al., 1998; HAVENAAR et al., 1992).

β-Galactosidase have numerous applications in the food and dairy industries, thus the immobilzed variants gained great interest because of its potential benefits, such as reutilization of enzyme, increased stability, and continuous operation (KLIBANOV, 1979). Various methods have been described for immobilization such as physical absorption, entrapment, and covalent binding method on different support materials (BICKERSTAFF, 1996). In previous studies, β-galactosidases were immobilized using different microbial sources and methods. Immobilized β-

galactosidases were also implemented in large-scale processes in food industry (GROSOVÁ et al., 2008; PANESAR et al., 2010). However, the great expense of carrier material and the drawbacks of prevailing immobilization methods such as detaching or diffusion limitations make up-scaling difficult (WANG and CHAO, 2006; TANAKA and KAWAMOTO, 1999). Improvements were achieved by using protein engineering techniques such as fusion of affinity tags from heterologous sources to proteins enabling very specific binding to support materials. Affinity tag immobilization features with valuable properties such as strong and reversible binding, mild immobilization conditions, and oriented attachement (SALEEMUDDIN, 1999; ANDREESCU et al., 2006). Chitin-binding domain (ChBD) of chitinase A1 from *Bacillus circulans* WL-12 was previously studied and showed high affinity to chitin, reversible binding, and stability over a wide pH range (HASHIMOTO et al., 2000). Due to its strong and specific binding to its substrate chitin and its compact structure and small size it has been described as a very useful tool for immobilization of cells and enzymes (WANG and CHAO, 2006; CHERN and CHAO, 2005; CHIANG et al., 2009).

The characteristics of the immobilized enzymes are associated with those of the support materials (TISCHER and WEDEKIND, 1999). In any case the carrier material must show high protein affinity, hydrophilicity, regenerability, and mechanical stability and rigidity. For applications related to food and pharmaceuticals, additional requirements such as nontoxicity and biocompatibility of the support material are playing a great role. Furthermore, industrial scale applications demand for inexpensive raw materials. Chitin and chitosan obtained as a by-product from the seafood industry fulfill all these criteria (PETER, 1995; HUDSON and SMITH, 1998; SHAHIDI et al., 1999).

The aim of this study was to fuse the *lacZ* gene from *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 20081 encoding for a β-galactosidase to a chitin-binding domain and heterologously expressed in a food grade microorganism *L. plantarum* WCFS1. The purified fusion enzyme was immobilzed onto a chitin resin in order to study its biochemical properties and performance during batch lactose hydrolysis and GOS production.

3 MATERIALS AND METHODS

3.1 Chemicals and Enzymes

All chemicals were purchased from Sigma (St. Louis, MO., USA) unless otherwise stated. MRS broth powder was obtained from Merck (Darmstadt, Germany). Restriction enzymes and T4 DNA Ligase were obtained from New England Biolabs (Frankfurt, Germany) whereas *Pfu* DNA polymerase was from Promega (Mannheim, Germany). Agarose was purchased from Roth (Karlsruhe, Germany) and the chitin beads used for the immobilization of the β-galactosidase were obtained from New England Biolabs.

3.2 Bacterial Strains and Culture Conditions

Lactobacillus plantarum WCFS1 was obtained from the culture collection of Food Biotechnology Lab, University of Natural Resources and Applied Life Sciences and was grown in MRS media at 30 °C, without agitation. *Escherichia coli* NEB5α (New England Biolabs, Ipswich, MA) was grown at 37 °C in Luria-Bertani (LB) medium (10 g/l peptone, 5 g/l yeast extract, 10 g/l NaCl) with shaking at 120 rpm. When needed, erythromycin was supplemented to media in concentrations of 5 µg/mL for *L. plantarum* or 200 µg/mL for *E. coli*.

3.3 DNA Manipulation and Cloning

The expression vector pSip403GusA-F1R1 containing the chitin binding domain (ChBD) of chitinase A1 from *Bacillus circulans* WL-12 fused C-terminal to the *gusA* gene was obtained from the Food BioTechnology Lab, University of Applied Life Sciences and Natural Resources, Vienna. The vector was a result of previous work (oral communication with Thu-Ha Nguyen). The previously constructed vector pJETlacZ harboring the *lacZ* gene from *Lactobacillus delbrueckii subsp. bulgaricus* DSM 20081 (NCBI reference sequence no. NC_008054) (NGUYEN et al., 2012) was used as template for amplifying the target gene.

The degenerated oligonucleotides were designed based on the sequence for *lacZ* gene from pJETlacZ for a subsequent direct fusion of the *lacZ* gene to ChBD in the pSip403GusA-F1R1-vector. The restriction sites *BsmB*I and *Xho*I were introduced to the forward and the reverse primer, respectively (Table 1). The primers used for PCR amplification of *lacZ* were supplied by VBC-Biotech Service (Vienna,

Austria). In previous work with respective pSip vector a stop codon was added downstream of ChBD.

Table 1. Primers used for amplification of *lacZ* gene from *L. bulgaricus*[a].

primer	restriction enzyme	sequence (5'→3')
Fw	*BsmB*I	GCTGCGTCTCCCATGAGCAATAAGTTAGTAAAAG
Rv	*Xho*I	GAAGCTCGAGTGATTTTAGTAAAAGGGG

[a]Underlined nucleotides indicate the restriction sites

DNA amplification was performed with Phusion High-Fidelity DNA polymerase (Finnzymes, Espoo, Finland) using standard procedures. The total reaction volume of 25 µL contained 0.2 mM of each deoxynucleotide triphosphate, 0.5 µM of each primer, 10 µL of 5x Phusion HF buffer, 1 U of Phusion DNA Polymerase, and approximately 50 ng of genomic DNA. The initial denaturation step at 98 °C for 30 s was followed by 35 cycles of denaturation at 98 °C for 10 s, annealing at 60 °C for 20 s, and extension at 72 °C for 1 min. The final cycle was followed by a further 5-min elongation step at 72 °C. The amplified products were visualized by gel electrophoresis at 7 V/cm in a 1.2% agarose gel (containing PEQgreen 0.2 µg/mL) in 1x TAE (Tris-Acetate) electrophoresis buffer (Tris base 4.8 g/L, acetic acid 1.2 g/L, 1 mM EDTA, pH 8.0). The PCR products were purified from the agarose gel using the Wizard® SV Gel and PCR Clean-Up System (Promega) and double-digested with *BsmB*I and *Xho*I. The vector pSip403GusA-F1R1 was double-digested using *Nco*I and *Xho*I restriction enzymes and visualized by gel electrophoresis as described above. The empty vector without the *gusA* gene was purified using above-mentioned method. The digested fragments were ligated for 16 h at 4 °C using T4 DNA Ligase. The constructed plasmid pTL01 was transformed into electrocompetent *E. coli* NEB5α cells and positive clones were selected on LB-Ery plates. The newly constructed vector was confirmed by restriction digestion of the extracted plasmid-DNA with *Sal*I and *Xho*I followed by agarose gel electrophoresis and DNA sequencing (Microsynth Austria GmbH, Austria). For overexpression the pTL01-vector containing *lacZ* fused to ChBD was transformed into electrocompetent cells of *L. plantarum* WCFS1 and positive clones screened using MRS-Ery plates.

3.4 Expression of Recombinant β-Galactosidase

For the heterologous overexpression of the *lacZ*-ChBD gene from *L. bulgaricus*, overnight cultures (~16 h) of *L. plantarum* WCFS1 harboring the expression plasmid pTL01 were added to 15 mL of fresh MRS medium containing erythromycin to an OD_{600} of ~0.1 and incubated at 30 °C without agitation. The cells were induced at an OD_{600} of 0.3 by adding 25 ng/mL of the inducing peptide pheromone IP-673 (supplied by the Molecular Biology Unit, University of Newcastle-upon-Tyne, U.K.). Cells were harvested at an OD_{600} of 1.8−2 by centrifugation at 4000 rpm for 30 min at 4 °C, washed twice by 50 mM sodium phosphate (NaPP) buffer, pH 6.5, and resuspended in 0.5 mL of the same buffer. Cells were disrupted in a bead beating homogenizer using 1 g of glass beads (Precellys 24; PEQLAB, Germany). Cell-free extracts were obtained after a centrifugation step at 9000 g for 20 min at 4 °C.
For large scale production of the fusion enzyme, *L. plantarum* WCFS1 harboring pTL01 was cultivated in 1 L fermentations. The fermentation conditions and the induction protocol were identical to those of the small-scale cultivations. Expression of LacZ-ChBD was induced at OD_{600} of 0.3, and the cells were harvested at OD_{600} ~6 by centrifugation at 6000 rpm for 30 min at 4 °C, washed twice by 50 NaPP (pH 6.5), containing 1 mM DTT and resuspended in the same buffer. After centrifugation, cells were disrupted by using a French press (Aminco, Silver Spring, MD), and cell debris was removed by ultra-centrifugation (25000 rpm, 30 min, 4 °C).

3.5 Immobilization and Binding Capacity

For determination of the binding capacity during immobilization, different amounts of oNPG activity Units (ranging from 25 to 500 Units) of fusion β-galactosidase in 50 mM NaPP buffer, pH 6.5 were added to about 10 mg of chitin in a column. The preparations were left at 4 °C under continuous and gentle rotation (9 rpm) for one hour. The column was then washed with 50 mM NaPP (pH 6.5) until no more activity could be detected in the washing fractions. Optimum conditions for immobilization were determined preliminary, showing no or marginal difference by varying the incubation time from 1 to 18 h (data not shown). This may be due to the strong binding of the chitin-binding domain to the chitin beads.

The purification fractions were analyzed via SDS-PAGE. The enzyme was denatured by incubation with SDS buffer (47 mM Tris-HCl, pH 6.8 containing 34 mg/ml

SDS, 0.1 mg/ml bromphenol blue, 5% v/v mercaptoethanol and 15% v/v glycerol) at 99°C for 5 minutes. Protein bands on the SDS-PAGE were visualized with Coomassie blue staining.

Preliminary tests were performed measuring β-galactosidase activity of the bound enzyme. Certain amounts of the crude extract (25 to 500 oNPG activity Units) were added to the chitin beads, to obtain the appropriate enzyme dilutions needed for the characterization of the recombinant β-galactosidase in its immobilized form. The chitin bead slurry (in 20% ethanol) was washed in 50 mM NaPP-buffer (pH 6.5) and then incubated with the crude extract. The different enzyme bead suspensions were rotated at 4 °C for one hour to obtain sufficient binding of the protein hybrid. The liquid was separated from the bead bed by filtration and the chitin beads were subsequently washed twice and resuspended in NaPP buffer (50 mM, pH 6.5) to obtain the purified immobilized enzyme.

The amount of enzyme which was effectively bound on the beads P_g (mg/g) was calculated using the eq. 1:

$$P_g = \frac{C_0V_0-(C_fV_f+C_{w1}V_{w1}+C_{w2}V_{w2}+C_{w3}V_{w3})}{w} \quad (1)$$

Where C_0 is the initial protein concentration (mg/mL), C_f the protein concentration of the filtrate (mg/mL), C_w the protein concentration of particular wash fractions (mg/mL), V_0 the initial volume of the enzyme solution (mL), V_f the volume of filtrate (mL), V_w the volume of the wash fractions (mL), and w the weight of carrier material used (g) (ELNASHAR et al., 2009).

The immobilized activity was calculated taking the difference between the applied and the recovered activities in the pooled supernatant and washing fractions. The immobilization yield (IY) and the activity retention (AR) was calculated according to the following equations (KLEIN et al., 2012).

$$IY\% = \frac{immobilized\ activity}{applied\ activity} x100 \quad (2)$$

$$AR\% = \frac{activity\ measured\ on\ the\ support}{immobilized\ activity} x100 \quad (3)$$

3.6 β-Galactosidase Assays and Protein Determination

β-Galactosidase activity was determined using o-nitrophenyl β-D-galactopyranoside (oNPG) and lactose as the substrates, as described previously (NGUYEN et al., 2006). When chromogenic oNPG was used as the substrate, the determination of β-galactosidase activity was carried out at 30 °C with 22 mM oNPG in 50 mM NaPP (pH 6.5). For the free enzyme reaction was initiated by adding 20 µL of enzyme solution to 480 µL of the substrate solution, and then the mixture was incubated for 10 min using an Eppendorf (Hamburg, Germany) thermomixer compact. Agitation was carried out at 600 rpm. After the incubation time, the reaction was stopped by adding 750 µL of 0.4 M Na_2CO_3.

To start the reaction using the immobilized enzyme 20 µL of enzyme-chitin-slurry was mixed with 1480 µL of oNPG solution, incubated for 5 min as described above. After the incubation time, different dilutions of the converted oNPG solution were mixed with NaPP (500 µL volume in total) and 750 µL Na_2CO_3 to stop the reaction and achieve an appropriate dilution for spectrophotometric measurements. Calculating back from the detected absorbance, oNPG still was available in abundance. The release of o-nitrophenol (oNP) was assessed by determining the absorbance at 420 nm. One unit of oNPG activity was defined as the amount of enzyme releasing 1 µmol of oNP per minute under the described conditions.

When lactose was used as the substrate, 20 µL of either a free or an immobilized enzyme solution was added to 480 µL of a 600 mM lactose solution in 50 mM NaPP (pH 6.5). The reaction mixture was incubated at 30 °C and 600 rpm agitation using an Eppendorf thermomixer compact. After 10 min, the reaction was stopped by heating the reaction mixture at 99 °C for 5 min. After the sample had been cooled to room temperature and appropriately diluted, the release of D-glucose was assessed colorimetrically using the GOD/POD assay by adding 60 µL of this reaction mixture to 600 µL of a solution containing GOD (21.5 U/mL), POD (1.35 U/mL), 4-aminoantipyrine (157 µg/mL), and phenol (11 mmol/L) in 0.1 potassium phosphate buffer (pH 7.0). This assay mixture (660 µL) was incubated in the dark at room temperature for 40 min, and the absorbance at 505 nm was measured. One unit of lactase activity (LAU) was defined as the amount of enzyme releasing 1 µmol of D-glucose per minute under the given conditions.

The protein concentration was determined by the method of Bradford using bovine serum albumin as a standard (BRADFORD, 1976). The determination of the immobilized protein amount was estimated on the basis of the difference between the amount of protein added to the beads and that recovered in the flow through and washing fractions.

3.7 Steady-state Kinetic Measurements

For determination of the steady-state kinetic constants (V_{max}, K_m, k_{cat}) for lactose and oNPG hydrolysis the activity of the immobilized enzyme was measured under standard conditions (30 °C, 10 min, in 50 mM NaPP-buffer, pH 6.5). The substrate concentrations were varied from 0.5 to 50 mM for oNPG and from 1 to 600 mM lactose, respectively, and the kinetic parameters were obtained by nonlinear regression. Sigma Plot (SPSS Inc., Illinois, USA) was used to fit observed data to the Henri-Michaelis Menten equation.

3.8 Determination of pH and Temperature Profiles

The pH and temperature optima of the bound β-gal-ChBD were determined using lactose and oNPG as susbtrates. Sodium hydroxide was used to adjust the pH in the range from 4 to 9 in Britton Robinson buffer (20 mM boric acid, 20 mM acetic acid, 20 mM phosphoric acid) and the activities were measured in steps of at least 0.5 pH units under standard conditions for immobilized enzyme (30 °C, 5 min using 22 mM oNPG or 30 °C, 10 min using 600 mM lactose). The temperature optima were determined under standard assay conditions for immobilized enzyme, keeping the pH constant at 6.5 but varying the temperature from 23 to 75 °C.
The pH stability was tested by incubating the enzyme samples at different pH values in Britton Robinson buffer at 37 °C up to 23 days and measuring the residual activity of each sample at time intervals under standard assay conditions for immobilized enzyme with oNPG as substrate. For assessing temperature stability the enzyme samples were incubated in 50 mM NaPP, pH 6.5 at certain temperatures and residual activity was measured using oNPG as substrate. To study the effect of Mg^{2+}on thermal stability of β-galactosidase activity, the immobilized enzyme was stored in 50 mM NaPP, pH 6.5 containing 10 mM Mg^{2+} and activity was assayed using oNPG as substrate. To determine thermal stability, the assayed time intervals ranged up to 16 days. Residual activities were plotted versus the incuba-

tion time. The inactivation constants k_{in} were obtained by linear regression of ln(residual activity) versus time. The half-life values of thermal inactivation $\tau_{1/2}$ were calculated using $\tau_{1/2} = \ln(2)/ k_{in}$ (POLIZZI et al., 2007).

3.9 Recycling Test of the Immobilized Enzyme

To assess the possibility of recycling of the immobilized enzyme, two samples of bound β-gal-ChBD were assayed repeatedly under standard conditions for immobilized enzyme with the substrate oNPG. Other than in the standard assay the reaction was not stopped immediately after 5 minutes of incubation in the thermal mixer but the reaction mixture was separated from the beads via filtration. The flow through was diluted appropriately and 500 µL out of that dilution was mixed with 750 µL of 0.4 M Na_2CO_3 and absorption at 420 nm was measured (reaction number 1). Resulting enzyme activity was defined as 100% relative activity obtained for the immobilized enzyme. To start a further reaction oNPG solution (1480 µL) was added to the remaining chitin bead suspension and again incubated. Up to 10 reactions were performed and residual activities of the immobilized enzyme were determined.

3.10 Lactose Hydrolysis and Transgalactosylation

The transformation of lactose was carried out in discontinuous mode using immobilized recombinant β-galactosidase from *L. bulgaricus*. The immobilized enzyme was applied in different amounts ranging from 1.7 to 9.7 LAU/mL to 50 g/L as well as 205 g/L initial lactose concentration in sodium phosphate buffer (50 mM, pH 6.5) containing 10 mM $MgCl_2$, and to ultra heat treated unskimmed cow milk, respectively. The incubation temperature was varied from 37 to 60 °C and continuous agitation was applied at 300 rpm. Samples were withdrawn periodically, heated at 99 °C for 5 minutes and further analyzed for their contents of lactose, galactose, glucose and galacto-oligosaccharides (GOS).

Qualitative sugar analysis was performed by TLC using butanol-propanol-ethanol-water (2:3:3:2, v/v/v/v) as the mobile phase. Visualizing was achieved by dipping the HPTLC Lichrospher® plates (Thomas Scientific, NJ, USA) into 0.5% (w/v) thymol in 5% (v/v) sulfuric acid and heating for 2 minutes at 100 °C.

Lactose and conversion products were analyzed by HPLC (Dionex, Germany) equipped with refractor index and Aminex HPX-87K (300 mm x 7.8 mm) carbohy-

drate analysis column (Bio-Rad, CA, USA). Ultra-pure water was used as eluting solvent at a flow rate of 0.5 mL min^{-1}, at 80 °C. The concentration of saccharides was calculated by interpolation from external standards. GOS concentration was calculated as the difference between initial and remaining quantified saccharides (lactose, glucose, glactose). The GOS yield (%) was defined as the percentage of GOS produced compared with the weight of initial lactose in the sample.

4 RESULTS AND DISCUSSION

4.1 Cloning and Expression

The *lacZ* gene derived from L. bulgaricus was amplified from the vector pJETlacZ. As visualized by agarose gel electrophoresis the PCR product had a size of ~ 3 kbp confirming a successful amplification of *lacZ* (3027 bp). The expression plasmid pTL01 was constructed by replacing *gusA*, which originally was used as a reporter gene in the pSIP plasmid series, in the vector pSip403GusA-F1R1 (containing the chitin binding domain fused C-terminal to the *GusA* gene) by *lacZ*. In the pSIP plasmid series, the transcription of the reporter gene is regulated by the inducible promoter P_{sppA} for the pSIP403 derivative (Figure 1). The expression of the fusion enzyme LacZ-ChBD was subsequently studied in *L. plantarum* WCFS1 as host, using an inducer concentration of 25 ng/mL of the inducing peptide pheromone IP-673. Induced and noninduced cells were harvested in the late stationary phase (OD_{600} of ~1.8), and the intracellular cell-free extracts were analyzed by SDS-PAGE, which showed unique bands of ~115 kDa in induced *L. plantarum* cells (Figure 2). Analysis of the crude cell extract gave a volumetric activity in the range of 1.76 ± 0.04 U/mL of cultivation medium and a specific activity of 40 ± 2 U/mg. Noninduced cells of *L. plantarum* harboring the expression vector were also cultivated and tested for basal expression from the promoters (Table 2).

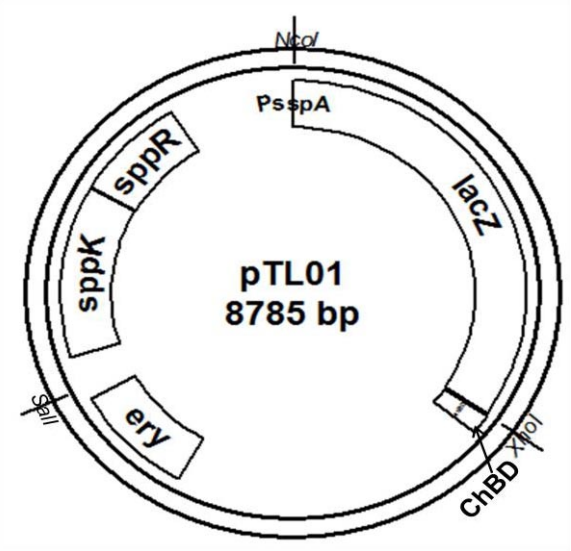

Figure 1. Schematic overview of the pTH01 plasmid developed in this study. The structural gene *lacZ* fused to chitin-binding domain is controlled by the inducible promoter P_{sppA} (pSIP403 derivative). *Ery* indicates the erythromycin resistance marker, and restriction sites are marked by dashes.

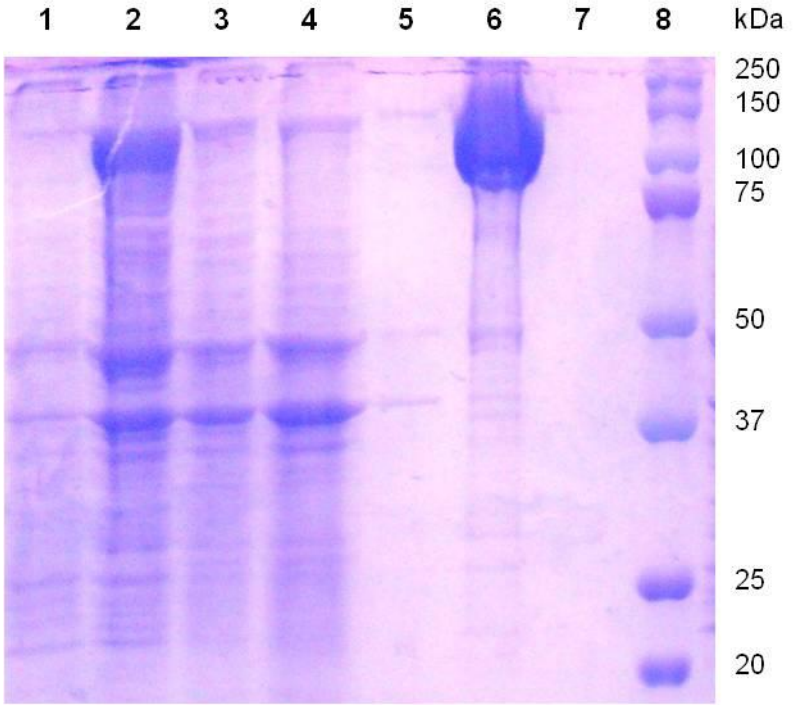

Figure 2. SDS PAGE analysis of expression and immobilization of β-galactosidase from *L. bulgaricus*. Samples were mixed with SDS and heated for 5 minutes at 99°C before loading in following order: crude lysate at OD ~0.3 (**lane 1**), crude lysate 16 h after induction (**lane 2**), flow through (**lane 3**), washing fractions (**lane 4 and 5**), washed beads with immobilized β-gal (**lane 6**), chitin beads slurry (**lane 7**) and molecular mass marker (**lane 8**).

Table 2. β-Galactosidase activity in cell-free extracts of induced and noninduced cells of *L. plantarum* WCFS1 carrying the expression plasmid TL01[a].

	volumetric activity [U/mL]	specific activity [U/mg protein]	induction factor
induced	65.83 ± 1.53	40.27 ± 2.36	4
noninduced	16.77 ± 1.02	9.43 ± 0.14	

[a] Data given are the average and standard deviation of two experiments. The induction factor was calculated from the specific β-galactosidase activity obtained under inducing conditions divided by the activity under noninduced conditions in cells harvested at OD_{600} of ~1.8

4.2 Fermentation of Recombinant β-Galactosidase

L. plantarum harboring pTL01 was cultivated on a larger scale (1 L cultivation volume). Typical yields obtained in 1 L laboratory cultivations were approximately 10.5 ± 0.1 g wet biomass and 31 ± 3 kU of β-galactosidase activity. The specific activity of the crude cell extract was determined as 151 ± 2 U/mg for fusion enzyme. With ~20% decreased specific activity of the fusion enzyme compared to the non-tagged LacZ reported by NGUYEN et al. (2012) the chitin binding domain is speculated to affect the activity somehow.

4.3 Purification by Immobilization and Binding Capacity

For immobilization, the crude lysate containing the fusion enzyme was incubated with 10 mg of chitin as described above. After washing the column the different fractions of immobilization were analyzed by SDS-PAGE. (Figure 2) Here the purification can be confirmed by a strong band in the induced crude lysate as well as in the washed fraction containing the immobilized recombinant enzyme at ~115 kDa, which fitted with expected mass of the fusion protein. These results show the strong binding of chitin-binding domain to chitin which was already proven by CHERN and CHAO (2005).

The results in Figure 3 show the binding capacity, where the activity retention AR% (active remaining relative activity after immobilization) was plotted versus the amount of applied β-galactosidase activity Units. The graph shows that with increasing load of enzyme, the activity retention is decreasing. However, no significant higher activity could be detected in the flow through, meaning that the enzyme could bind in large part on the chitin surface. This may result by the fact that with higher enzyme amount and correspondingly higher protein amount, the bound enzymes may interfere and lose their activity by blocking the active sites due to protein overload.

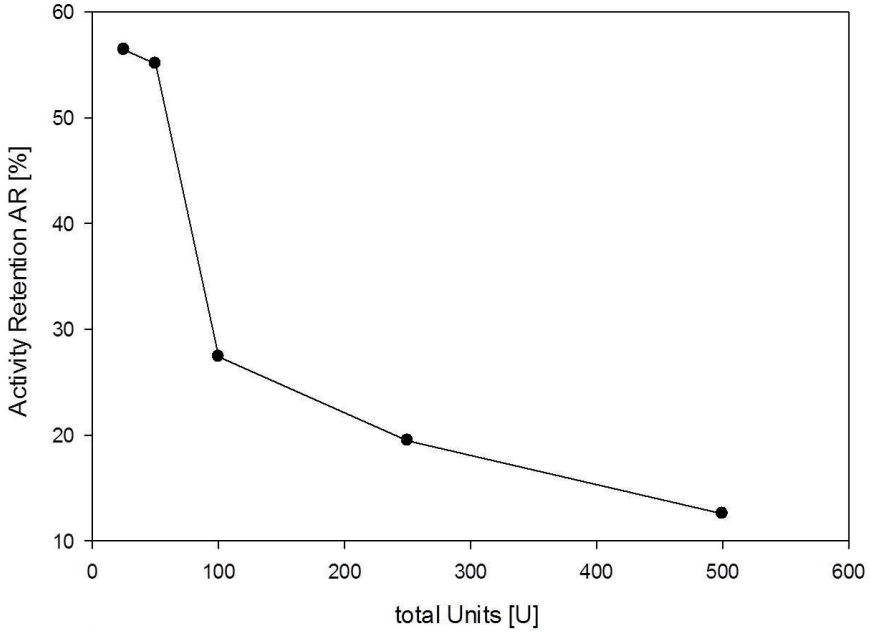

Figure 3. Binding capacity expressed as activity retention. Active remaining immobilized activity (*oNPG* units) plotted versus total applied activity units per 0.5 mL chitin slurry containing 20 mg dry chitin per mL.

The immobilization yield, expressing the bound protein after loading and washing the packed column two times, ranged between 90 and 95% for all experiments. The highest retention of activity was achieved by loading 25 to 50 Units per 10 mg dry chitin yielding 50 – 60 AR%. The resulting immobilized activity was found to be 1275 ± 42 and 2585 ± 205 U/g of chitin for 25 Units and 50 Units loading on 0.5 mL chitin slurry (~ 10 mg chitin), respectively. In previous work LU et al. (2012) immobilized β-gal from *L. bulgaricus* L3 fused to cellulose-binding domain on cellulose and achieved similar AR% (61%), whereas binding capacity was considerably lower with 97.6 U/ g of cellulose.

Due to the strong binding of the chitin binding domain to its substrate chitin, the immobilization step results in purification of the fusion β-galactosidase. The recovery of the fusion enzyme by elution of the chitin resin was not possible as elution conditions require pH < 3 or pH > 9 (HASHIMOTO et al., 2000). Under these conditions the enzyme denatures and suffers the loss of β-galactosidase activity. The chitin resin supplied by New England Biolabs, Frankfurt, Germany was used as chitin source for the immobilization of the fusion enzyme via its chitin binding domain. However, fusion of a chitin binding domain to β-galactosidase facilitates immobilization and application as the immobilized enzyme can be recovered and reused during operation. For our later application of using immobilized enzyme, the recovery of the fusion enzyme by elution of the chitin resin is not necessary.

4.4 Kinetic Analysis of the Immobilized Fusion Enzyme

The steady-state kinetic constants for the hydrolysis of the natural substrate lactose as well as for the chromogenic substrate *o*-nitrophenol-β-D-galactopyranoside (oNPG) for the immobilized β-galactosidase from *L. bulgaricus* are summarized in Table 3. The k_{cat} values were calculated on the basis of the theoretical V_{max} values experimentally determined by nonlinear regression and using a molecular mass of 120 kDa for the catalytically active subunit. β-Galactosidase from *L. bulgaricus* is not inhibited by its substrates lactose in concentrations of up to 600 mM or oNPG in concentrations of up to 25 mM as is evident from the Michaelis−Menten plots (not shown). Kinetic analysis of immobilized enzyme yielded a higher theoretical reaction velocity (87 µmol min^{-1} mg^{-1}) and affinity (K_m ~ 14 mM) for oNPG than for lactose (V_{max} ~ 40 µmol min^{-1} mg^{-1}; K_m ~ 46 mM). The calculated value for k_{cat} and

the resulting catalytic efficiencies (k_{cat}/K_m) thus again show that oNPG is the preferred substrate for the immobilized β-galactosidase.

Compared to the kinetic parameters for the free soluble LacZ by NGUYEN et al. (2012), catalytic efficiency of the immobilized enzyme is found to be 50-fold and 7-fold lower for oNPG and lactose, respectively. This may be due to internal structural changes and restricted access to the active site.

Table 3. Kinetic parameters for immobilized fusion β-galactosidase LacZ from *L. bulgaricus* for the hydrolysis of lactose and o-nitrophenyl β-D-galactopyranoside (oNPG).

kinetic parameter	substrate	
	lactose	oNPG
v_{max} [µmol min^{-1} mg^{-1}]	39.9 ± 0.9	87.1 ± 2.8
K_m [mM]	45.5 ± 4.2	13.7 ± 1.1
k_{cat} [s^{-1}]	79.8 ± 1.8	174.2 ± 5.6
k_{cat}/K_m [M^{-1} s^{-1}]	1754	12715

4.5 Temperature and pH Profiles

The temperature optima of the activity of immobilized β-galactosidase from *L. bulgaricus* were determined to be in the range of 55 – 60 and 60 − 65 °C for oNPG and lactose hydrolysis, respectively (Figure 4A). The pH optimum of immobilized LacZ-ChBD activity is pH 6.5 and pH 8 for lactose and oNPG as substrate, respectively (Figure 4B). Overall, the pH curves show broad peaks with more than 80% of maximal β-galactosidase activity in the pH range of 6.5 − 7.5 for lactose and 6 – 8 for oNPG (Figure 4B).

Thermal stability that is the length of time the enzyme remains active of immobilized β-galactosidase from *L. bulgaricus* was measured in 50 mM NaPP at a constant pH of 6.5 while the temperature was varied from 37 to 65 °C. In addition to check the impact of Mg^{2+} cations on enzyme stability, the immobilized enzyme was incubated in 50 mM NaPP containing 10 mM $MgCl_2$. Immobilized LacZ-ChBD activity showed first-order inactivation kinetics when analyzed in the plot of ln(residual activity) versus time (not shown). Data for the inactivation constants k_{in} and half-life times of activity $\tau_{1/2}$ are summarized in Table 4. Immobilized LacZ-ChBD was well stable at 37 °C with a half-life time of 8.5 days. When the temperature was increased to 55 °C or even 65 °C, activity was, however, lost rapidly (Table 4). Whilst incubating the immobilized enzyme in NaPP containing Mg^{2+} ions, the half-life time found to be increased 2-fold up to 10-fold for 65 and 55 °C,

respectively. It was also reported previously that Mg^{2+} appears to enhance thermal stability and activity of GH2 β-galactosidases (NGUYEN et al. (2012), NGUYEN et al. (2006) and MAISCHBERGER et al. (2010)).

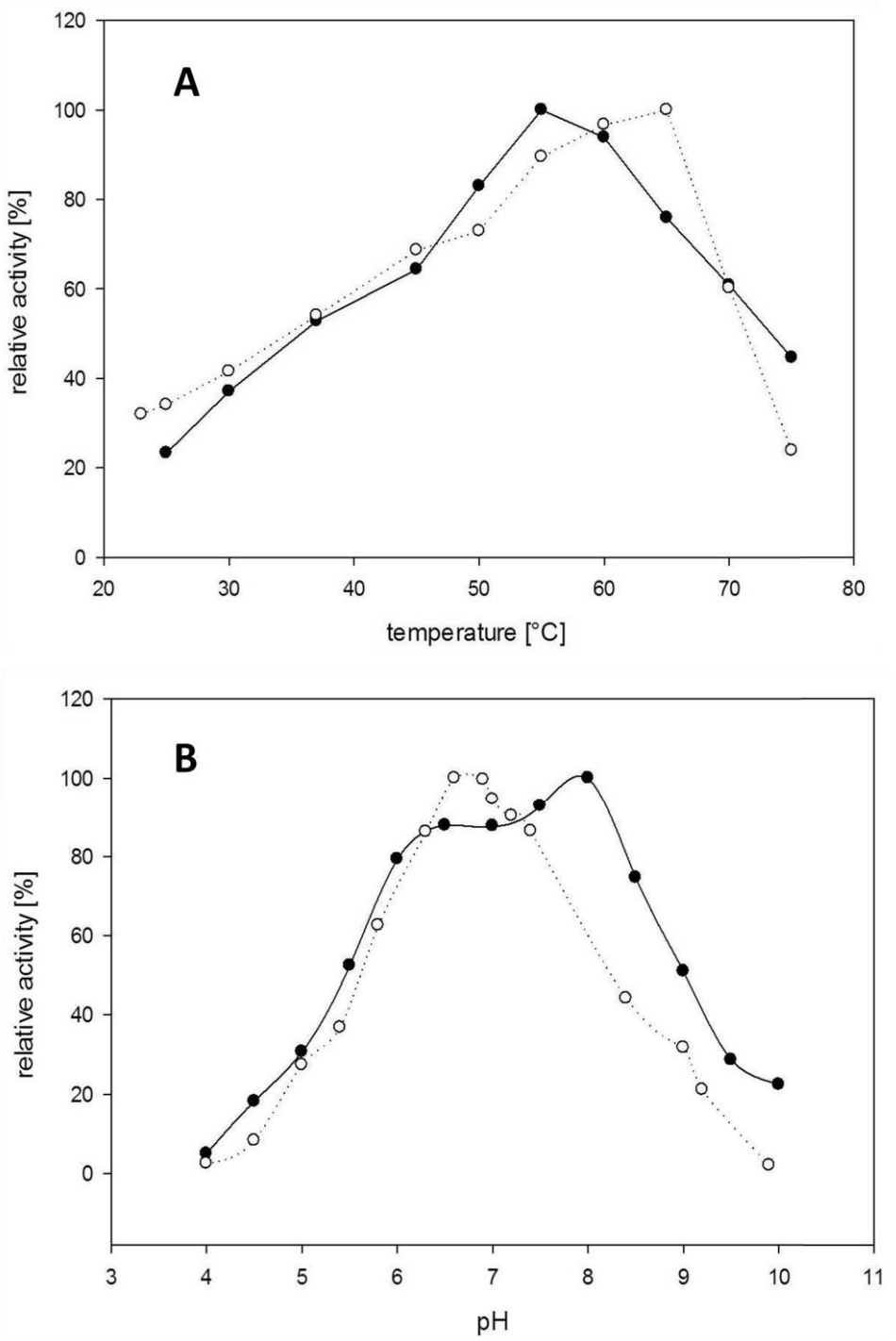

Figure 4. Temperature (**A**) and pH optima (**B**) of immobilized β-galactosidase from *L. bulgaricus*. Enzyme activity was measured under standard conditions with oNPG (●) and lactose (○) as substrates, respectively.

Table 4. Thermal stability of recombinant immobilized β-galactosidase from *L. bulgaricus.*

	NaPP, pH 6.5		NaPP, pH 6.6 + 10 mM $MgCl_2$	
temperature [°C]	k_{in} [h^{-1}]	$\tau_{1/2}$ [h]	k_{in} [h^{-1}]	$\tau_{1/2}$ [h]
37	0.0034	203.867	n.d.	n.d.
55	0.9543	0.726	0.0996	6.959
60	n.d.	n.d.	0.9862	0.703
65	8.3593	0.083	4.5943	0.151

During this study the effect of pH on the stability of the immobilized LacZ-ChBD was observed by incubating the enzyme at different pH values in Britton Robinson buffer at 37 °C for up to 23 days. Residual activities were measured at certain time intervals. Half-life times of activity ($\tau_{1/2}$) were calculated as described above and are summarized in Table 5. In general the immobilized enzyme shows highest stability in the neutral pH range from 6 to 6.5, at which the half-life time was found to be approximately 48 days. Interestingly, the immobilized enzyme displayed higher stability in Britton Robinson Buffer compared to the residual activity when stored at 37°C in NaPP, pH 6.5 containing $MgCl_2$.

Table 5. Effect of pH on enzyme stability. Half-life times of immobilized β-galactosidase were determined by incubating enzymes at 37 °C at different pH values.

pH	4	4.5	5	5.5	6	6.5	7	7.5	8	8.5	9
$\tau_{1/2}$ [h]	7.0	15.4	198.0	770.2	1155.2	1155.2	866.4	239.0	79.7	37.7	2.3

4.6 Repeated Use of the Immobilized Enzyme

Figure 5 shows a reuse study of the immobilized β-gal, using the subsequent hydrolysis of oNPG as a model reaction. β-Galactosidase activity measured after the first reaction was determined as 100% relative activity. Residual activity determined for the following reactions were plotted versus the number of reactions. Each measurement was performed in duplicate. In this work, the immobilized enzyme was reused for 10 cycles with a good stability as after four batches there was still more than 80% of initial enzyme activity observed, and 40% of the initial activity was retained after the tenth cycle. One cycle is defined as one batch of oNPG hydrolysis at 30 °C and pH 6.5, lasting 5 min. These results demonstrate the operational stability of the immobilized enzyme and its feasibiity for industrial application.

These results are comparable with previously reported results. LU et al. (2012) immobilized β-gal from *L. bulgaricus* on cellulose via cellulose-binding domain which showed a residual enzyme activity of 85% over 20 cycles at 45 °C. CHIANG et al. (2009) immobilized levansucrase on chitin using chitin-binding domain which retained approximately half of its initial activity at the end of the seventh cycle. Immobilized β-gal from *Kluyveromyces lactis* on chitosan by LIMA et al. (2013) showed 70% residual activity retained after the tenth cycle at 37 °C.

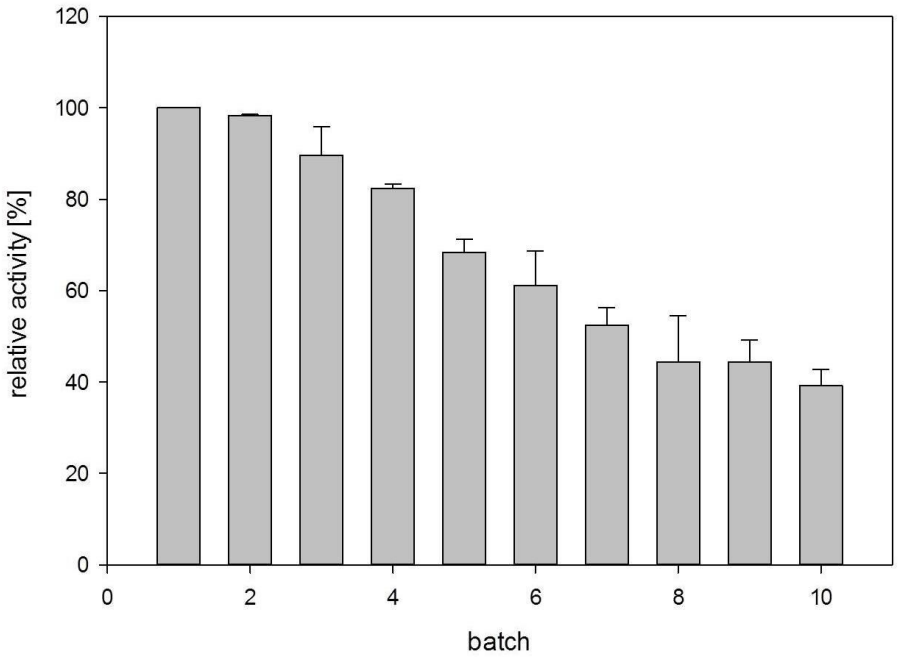

Figure 5. Reuse and stability of immobilized β-gal when hydrolyzing oNPG at 30 °C.

4.7 Lactose Transformation

Figure 6 shows the products formed during lactose conversion, using different initial lactose concentrations (50 g/L or 205 g/L) in 50 mM sodium phosphate buffer with 10 mM $MgCl_2$, pH 6.5, with different amounts of lactose activity units (LAU), and varying temperatures (55 – 60 °C) as analyzed by thin layer chromatography.. A GOS mixture supplied by Vivinal (Amersfoort, Netherlands) was used as 'standard'. During the initial reaction phase, galacto-oligosaccharides (GOS) formation together with the hydrolysis products D-galactose and D-glucose can be observed. Lactose hydrolysis of commercial whole milk sample (~ 5% w/v lactose) using immobilized recombinant β-galactosidase was also investigated (Figure 6A, B).

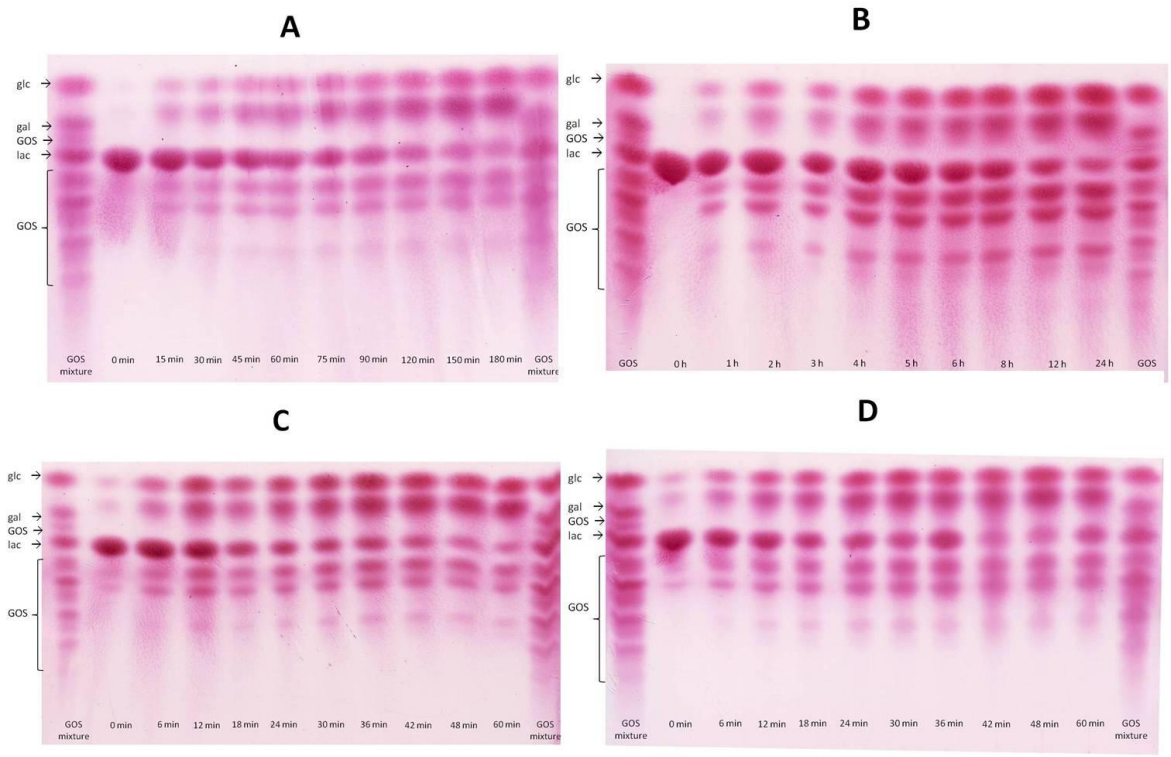

Figure 6. Thin layer chromatography (TLC) analysis of different batches of lactose conversion by immobilized β-galactosidase from *L. bulgaricus*. The batch conversions were carried out under various conditions: 50 g/L initial lactose concentration in 50 mM NaPP, pH 6.5, 10 mM $MgCl_2$, 3 h at 55 °C using 3.2 LAU/mL (**A**), 205 g/L initial lactose concentration in 50 mM NaPP, pH 6.5, 10 mM $MgCl_2$, 24 h at 55 °C using 1.7 LAU/mL (**B**), 50 g/L initial lactose concentration in 50 mM NaPP, pH 6.5, 10 mM $MgCl_2$, 1 h at 60 °C using 9.7 LAU/mL (**C**), commercial whole milk, 1 h at 60 °C using 9.7 LAU/mL (**D**)

The samples were then quantitatively analyzed using high-performance liquid chromatography (HPLC), with the time course and the summarized results shown in Figure 7 and Table 6, respectively. A maximum lactose conversion of 81% was achieved after 1 h at 60 °C when 9.7 LAU/mL was applied to 50 g/L initial lactose solution. The yield of total GOS reached a maximum of 25% after 12 h at 55 °C when 1.7 LAU/mL was applied to 205 g/L initial lactose solution, while 62% of initial lactose was converted. Afterwards, the total GOS amount decreased as they get also hydrolyzed by the immobilized β-galactosidase. Lactose conversion then reached 70% after further 12 h of the reaction. The hydrolysis of GOS observed above, however, proceeds very slowly as significant amount of lactose (~ 60 g/L) still present in the reaction mixture. This may also due to the end product inhibition by D-galactose, which occurs in notable concentrations at this stage of batch conversion. This end product inhibition leads to very slight breakdown of present

GOS, thus only approximately 4% of total GOS are degraded when the reaction proceeds for another 12 h. However, when lactose is still present in reasonable amount, it is the preferred substrate compared to other species in the mixture.

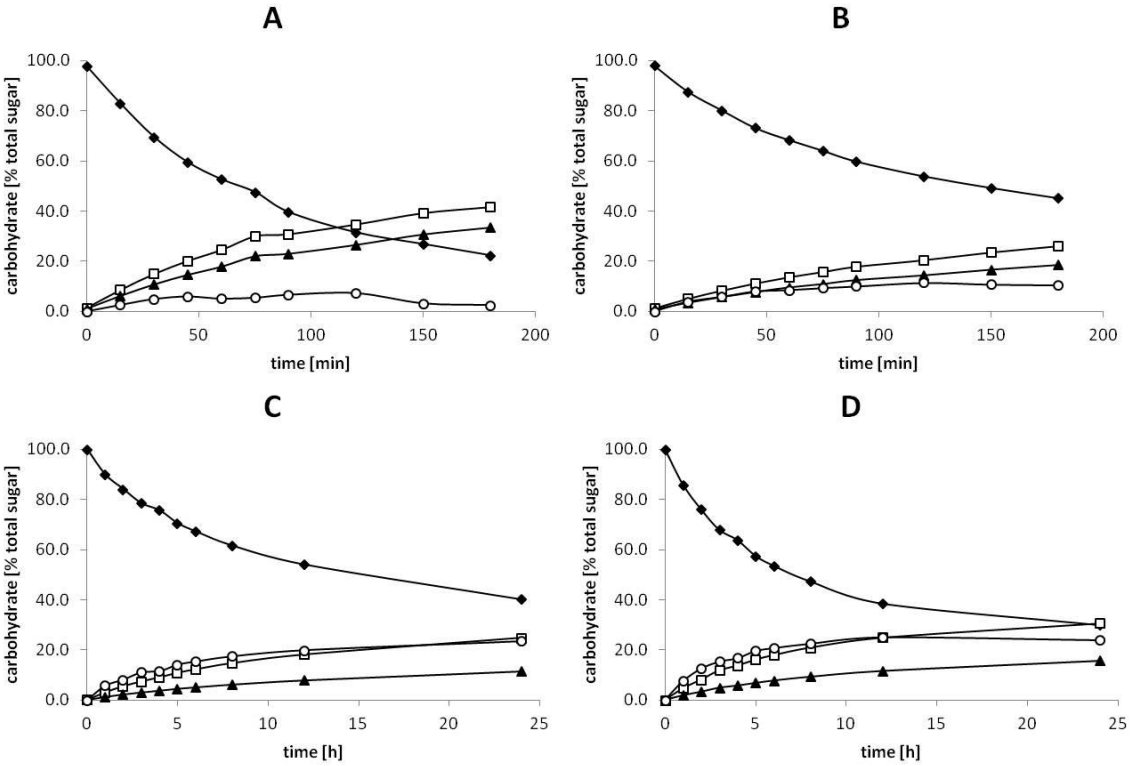

Figure 7. Carbohydrate composition during lactose transformation by immobilized β-galactosidase from *L. bulgaricus* determined by HPLC. The batch conversions were carried out under following conditions: 50 g/L initial lactose concentration in 50 mM NaPP, pH 6.5, 10 mM $MgCl_2$, 3 h at 55 °C using 3.2 LAU/mL (**A**), 50 g/L initial lactose concentration in 50 mM NaPP, pH 6.5, 10 mM $MgCl_2$, 3 h at 37 °C using 3.2 LAU/mL (**B**),205 g/L initial lactose concentration in 50 mM NaPP, pH 6.5, 10 mM $MgCl_2$, 24 h at 37 °C using 1.7 LAU/mL (**C**), 205 g/L initial lactose concentration in 50 mM NaPP, pH 6.5, 10 mM $MgCl_2$, 24 h at 55 °C using 1.7 LAU/mL (**D**), showing the time course of the conversion: lactose (♦), glucose (□), galactose (▲), and total galacto-oligosaccharides (○).

The effect of temperatures during the batch conversions using *L. bulgaricus* LacZ immobilized on chitin was investigated by performing lactose conversion at three different temperatures that were 37, 55 and 60 °C. Table 6 lists these results of the reaction mixtures, showing faster lactose conversion at higher temperatures, for instance, the time needed to obtain 60% lactose conversion was reduced from 24 h of reaction time at 37 °C to 12 h at 55 °C. Other researchers observed increased transgalactosylation reaction at higher temperatures using β-gal from

Bifidobacteria (HSU et al., 2007; OSMAN et al., 2010). However, for *L. bulgaricus* LacZ NGUYEN et al. (2012) did not observe the effect of reaction temperature on the maximum GOS yield.

Table 6. Lactose conversions using immobilized β-galactosidase from *L. bulgaricus*. Table shows the various conditions and obtained results at the end of each batch (run time).

	initial lactose concentration				
	50 g/L			205 g/L	
temperature	37 °C	55 °C	60 °C	37 °C	55 °C
run time	3 h	3 h	1 h	24 h	24 h
glucose	26.0%	41.7%	39.3%	24.8%	30.6%
galactose	18.5%	33.6%	32.8%	11.6%	15.7%
total GOS	10.4%	2.5%	10.4%	23.5%	23.9%
lactose conversion	54.2%	77.2%	81.2%	59.8%	70.2%

5 CONCLUSIONS

This work described the β-galactosidase from *L. bulgaricus* immobilized onto chitin via chitin-binding domain, which provides a promising and efficient approach for lactose hydrolysis and formation of prebiotic galacto-oligosaccharides. The immobilized fusion enzyme can be re-used for several cycles for lactose hydrolysis and transformation as it retaines over 40% of its activity after 10 batches. This study could be of considerable interest for industrial scale applications such as production of low-lactose milk and milk products.

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IX APPENDIX: REVIEW OF LITERATURE

1 β-GALACTOSIDASE

1.1 Generalities and Reaction Mechanism

The enzyme β-galactosidase (EC 3.2.1.23) catalyzes the hydrolysis of D-galactosides, such as lactose into its monomers D-glucose and D-galactose (Figure 8). β-Galactosidases can be found in large a variety of sources including microorganisms, plants and animals. For commercial and biotechnological applications microbial β-galactosidases gained considerably importance (RICHMOND et al., 1981). For lactose hydrolysis in milk and whey, the enzyme found application in food industry. In the early 1950s the so-called transgalactosylation reaction by β-gal was reported. During that reaction galacto-oligosaccharides (GOS) can be formed which attracted great interest due to their prebiotic properties promoting the growth of beneficial bacteria in the host (MAHONEY, 1997; ARONSON, 1952).

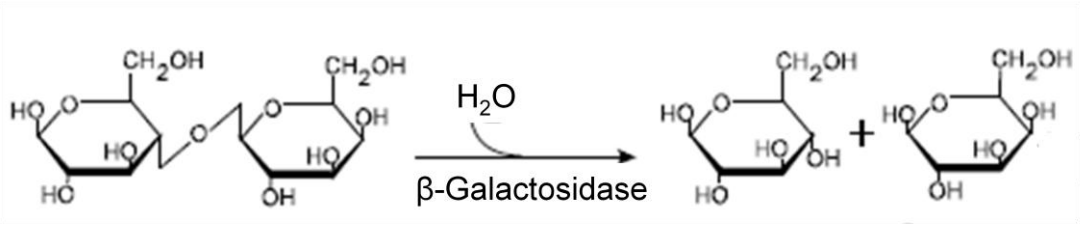

Figure 8. Hydrolysis activity of β-galactosidase

The enzyme belongs to the glycoside hydrolase family 2 (GH2) of carbohydrate active enzymes (http://www.uniprot.org). Glycosyl hydrolases catalyze the hydrolysis of the glycosidic linkage between carbohydrates or between a carbohydrate and a non-carbohydrate residue. Glycosyl hydrolases and related enzymes have been classified into now 133 families on the basis of amino-acid-sequence similarities (HENRISSAT, 1991). This databank continuously gets updated by the carbohydrate active enzymes (CAZy) web server (http://www.cazy.org/Glycoside-Hydrolases.html). WALLENFELS (1960) described the enzymatic hydrolysis of the glycosidic bond of lactose by general acid catalysis which requires a proton donor and a nucleophilic acceptor. In this described hydrolysis reaction the cystine and histidine residues were supposed to act as proton donor and nucleophile site, respectively. Recently, two glutamic acid residues (Glu^{482} and Glu^{551}) were identified

as the proton donor and the nucleophile/base of the β-galactosidase from a variety of microbial origins (ZHOU and CHEN, 2001).

As the enzyme shows both reactions, hydrolysis and transgalactosylation, there can be observed competing reactions when incubated with lactose. Generally, GOS are the initial products as transgalactosylation dominates in the beginning reaction. During that conversion process the hydrolysis reaction then increases resulting in monosaccharides (PRENOSIL et al., 1987). GOS have also been reported to contribute to the reduction of serum cholesterol and lipid level, the synthesis of B-complex vitamins and enhancing the absorption of dietary calcium (BHATTACHARJEE et al., 2006; NAKAO et al., 1988). However, these health promoting properties vary depending on the origin of the β-galactosidase (MLICHOVA and ROSENBERG, 2006).

Figure 9 shows the hydrolysis and transgalactosylation reaction of β-galactosidases when lactose is the substrate. The reaction proceeds in two steps, where in the beginning step an enzyme–galactoside complex is formed liberating glucose at the same time. In the second step the enzyme–galactosyl complex is transferred to a nucleophilic acceptor which carries a hydroxyl group. If water acts as nucleophilic acceptor hydrolysis takes place producing glucose and galactose. If the the enzyme–galactosyl is transferred to another sugar, di-, tri-, tetra- and higher galacto-saccharides are produced (BIALKOWSKA et al., 2009).

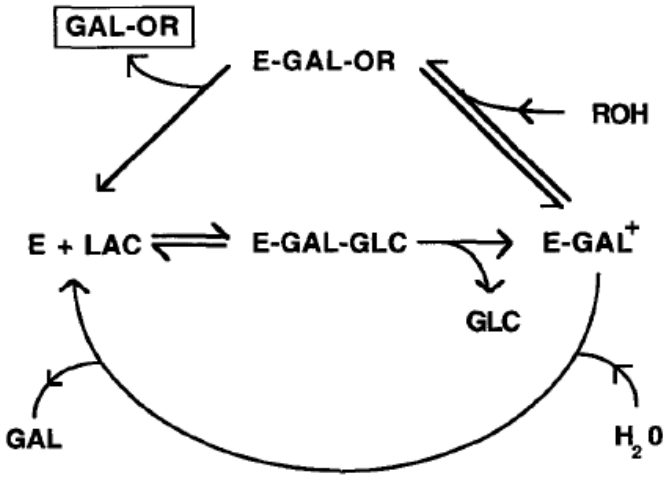

Figure 9. Reaction mechanism of β-galactosidase (CUPPLES et al., 1990).

In general the hydrolysis of lactose occurs predominantly. But at high concentrations of lactose it can be observed that the reaction is shifted towards transgalactosylation. Not only lactose concentration, but other factors such as

temperature, pH and the presence of inhibitors or activators specific for the enzyme influence the reaction (LI, Q. et al., 1992). For GOS production from lactose glycosyltransferases (EC 2.4) or glycoside hydrolases (EC 3.2.1) have been tested (HATZINIKOLAOU et al., 2005). As glycosyltransferases are hardly available they did not get used in the synthesis of GOS. Instead, glycoside hydrolases are used, because of showing minor stereoselectivity than glycosyltransferases (GUIMARAES et al., 2008). Glycoside hydrolases from archeas, bacteria (including *Bifidobacterium* sp., *Thermus* sp., *Bacillus* sp., *Geobacillus* sp., *Lactobacillus* sp., *Streptococcus* sp., *Enterobacter* sp., *Escherichia* sp.) and yeasts (such as *Aspergillus* sp., *Penicillinum* sp., *Kluyveromyces* sp.) have been tested for GOS synthesis (Njipels, 1981; HATZINIKOLAOU et al., 2005).

Depending on the supposed application of an enzyme, different parameters, such as temperature and pH optima should be considered. For instance β-galactosidases from bacterial and yeast origins show neutral pH optima in the range from 6.0 to 7.0, and therefore appear applicable for the lactose hydrolysis in milk and sweet whey, whereas fungal β-galactosidases demonstrate higher performance at acidic pH values around pH 4.0 to 5.0. Thus the latter is preferably used for the hydrolysis of acidic whey and the industrial production of GOS (RICHMOND et al., 1981). For commercial application, β-galactosidases have been isolated mainly from the yeasts *Kluyveromycs lactis, Kluyveromycs marxianus* and *Kluyveromyces fragilis*, as well as from fungi such as *Aspergillus oryzae* and *Aspergillus niger.* They have been studied for long time and proved to be suitable for food biotechnological purposes as they represent safe sources for native and recombinant enzymes ensuring efficient enzyme production (OLEMPSKA-BEER et al., 2006). For food and pharmaceutical applications, bacterial sources which are "generally regarded as safe" (GRAS) have gained great interest. In terms of lactose hydrolysis and transgalactosylation, bacteria such as *Streptococcus thermophilus* and *Lactobacillus bulgaricus* have been studied (NGUYEN et al., 2012; GREENBERG, 1983). Furthermore thermostable β-galactosidases, for instance from *Sulfolobus solfataricus* and *Thermotoga maritime,* have been tested. As they show higher temperature optima they bring in a great benefit for processes running at increased temperatures (PARK et al., 2008). β-Galactosidases from probiotic strains such as *Lactobacillus* spp. gained great attention for application in lactose

hydrolysis and GOS production (SPLECHTNA et al., 2006; SPLECHTNA et al., 2007; NGUYEN et al., 2006).

1.2 β-Galactosidase from *L. bulgaricus* DSM 20081

β-Galactosidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 20081 (LacZ) is a dimeric enzyme consisting of two identical subunits of approximately 110,000 Da each (NGUYEN et al., 2012). By cloning the *lacZ* gene in an inducible expression vector in *Lactobacillus plantarum* WCFS1, NGUYEN et al. (2012) obtained high expression levels of 53000 U of β-galactosidase activity per liter of medium.The specific activity of purified enzyme is 306 U/mg when using the standard *o*NPG assay. β-Galactosidase fom *L. bulgaricus* showed high catalytic efficiencies (k_{cat}/K_m) of 12300 M^{-1} s^{-1} and 655000 M^{-1} s^{-1} for lactose and oNPG, respectively. The enzyme was not inhibited by its substrates lactose in concentrations of up to 600 mM or *o*NPG in concentrations of up to 25 mM. Furthermore the inhibition by the reaction products D-glucose and D-galactose was only moderate. The temperature optima were found to be 45 – 50 °C and 55 – 60 °C for *o*NPG and lactose, respectively. β-Galactosidase fom *L. bulgaricus* worked best at pH 7.5 for both substrates. In terms of stability, LacZ showed a high half-life time at 37 °C of more than 6 days. In this study, activity was considerably increased in the presence of Na^+ and Mg^{2+} ions. It was reported that Mg^{2+} enhances thermal stability of β-galactosidases (NGUYEN et al., 2012; JUERS et al., 2009).β-Galactosidase from *L. bulgaricus* was used for lactose conversion and showed very high transgalactosylation activity. The maximum yield of galacto-oligosaccharides (GOS) was approximately 50% when using an initial concentration of 600 mM lactose, indicating that the enzyme can be of interest for the production of GOS.. The GOS mixture composed different GOS which are similar in structure to those produced by other β-galactosidases from LAB. The properties and high transgalactosylation activity make the β-galactosidase fom *L. bulgaricus* an attractive biocatalyst for food applications. (NGUYEN et al., 2012)

2 APPLICATION OF β-GALACTOSIDASES

Microbial β-galactosidase plays a great role in the industrial production of various products such as biosensor, lactose-hydrolyzed milk, ethanol, and galacto-oligosaccharides. Furthermore it has been used in the field of medical and immunology research.

2.1 Production of Lactose-hydrolyzed Milk and Whey

β-Galactosidase from *Arthrobacter* sp. 32c was studied and showed cold–stable properties and therefore appeared to be feasible for industrial lactose hydrolysis in milk (BIALKOWSKA et al., 2009). It has been reported that β-galactosidase extract produced by *Lactobacillus* ssp. *bulgaricus* CHR Hansen Lb 12 was applied in sterile milk for hydrolyzing lactose. Optimum amount of crude β-galactosidase extract for producing lactose-hydrolyzed milk was observed to be 0.418 U/mL during 6 h of processing. Using more than 418 U/L of crude β-galactosidase extract showed undesirable acidity what significantly increased at temperatures of between 15 and 17 °C (LI, Q. et al., 1992). The challenging enzyme extraction and poor permeability of cell membranes to lactose was solved when *Kluyveromyces marxianus* NCIM 3465 cells were permeabilized using ethanol and subsequently applied on milk. Those yeast cells yielded 89% of lactose conversion under optimized conditions (PANESAR et al., 2007).

Whey as a waste by-product of the dairy industry generates with the large volume incurring by cheese factories severe disposal problems. However, whey proved to be suitable for various applications in food industry, notably in confectionery. For this reason research has been emphasized on membrane separation technology, enabling the separation of milk protein and lactose in a large scale (CUI, 2005; BHATTACHARJEE et al., 2006; NAKAO et al., 1988). Further promising approaches so as to use whey in full capacity, such as GOS synthesis, as well as alcohol production have been reported (MLICHOVA and ROSENBERG, 2006; HATZINIKOLAOU et al., 2005). In this way, application of β-galactosidase on whey provides promising strategies for recycling waste- and/or by-products.

2.2 Function of β-Galactosidase in Ethanol Fermentation

The alcoholic fermentation of lactose using a recombinant strain of *Saccharomyces cerevisiae* NCYC869-A3/T1, expressing on the one hand β-galactosidase, on the other hand lactose permease from *Kluyveromyces lactis,* was studied. The lactose was completely converted during fermentative processes and even showed increased ethanol production when enhancing the initial lactose concentration (5 to 200 g/L) (GUIMARAES et al., 2008). Kinetic analysis of biomass growth, lactose hydrolysis, and ethanol production using β-galactosidase produced by *Kluyveromyces* sp. was performed using whey permeate and cheese whey as growth medium (SILVEIRA et al., 2005; OZMIHCI and KARGI, 2007). A membrane recycled bioreactor using immobilized *S. cerevisiae* and β-galactosidase was developed for ethanol production in semi-continuous fermentation processes with whey permeate and whey powder as medium (MEHAIA and CHERYAN, 1990).

2.3 Galacto-oligosaccharide Production

In recent years, research has been emphasized in the field of prebiotics, with oligosaccharides among them as functional food ingredients. Oligosaccharides are composed of three to ten sugar units linked via glycosidic bonds. Several classes of oligosaccharides have been reported with GOS attracting great attention as they are naturally existent in human breast milk. These galacto-oligosaccharides are non-digestible, oligosaccharides composed of galactose-units chains with a terminal glucose unit, synthesized by transgalactosylation in presence of β-galactosidases. Depending on the source of enzyme and conditions of reaction, various glycosidic linkages, such as β-(1,2), β-(1,3), β-(1,4), and β-(1,6), are formed in the end product (GOS) (GOSLING et al., 2010). The amount of GOS production from lactose was shown to be depended on the initial lactose concentration. Studies have shown that GOS in human milk promote the growth of *Bifidobacteria* in the gastrointestinal tract of newly born and breast-fed infants. It has been also demonstrated that GOS fraction in cow's milk also provide health benefits (JUNG et al., 2008). Non caries-producing GOS pass through the small intestine without being digested for low caloric value (MARTINEZ et al., 2013).

2.4 Use of β-Galactosidase in Biosensors

For quantitative detection of lactose in milk, a biosensor arranged with the two enzymes β-galactosidase and glucose peroxidase has been developed by MARRAKCHI and others (MARRAKCHI et al., 2008). It was also reported about the use of β-galactosidase for detection and quantification of toxoflavin in toxoflavin-contaminated foods and environmental samples (OKHEE et al., 2013).

2.5 Applications in Medical Application

A recombinant endo-β-galactosidase (ABase) was found to be capable of degrading the blood group A and B antigenicity of human type A and B erythrocytes by realeasing A-Trisaccharide and B-Trisaccharide from blood group A+ and B+ containing glycoconjugates. The specificity of β-galactosidase enabled studying the structure and function of blood group A-containing and B-containing glycoconjugates (ANDERSON et al., 2005). The recombinant ABase releasing A/B antigen was developed in 2009, showing removal of 82% of A antigen and 95% of B antigen from human A/B red blood cells. This approach is believed to be suitable for minimizing antibody removal and anti-B cell immunosuppression as a promising therapy of ABO incompatible kidney, liver, and possibly heart transplantation (KOBAYASHI et al., 2009). A β-galactosidase from the mesoacidophilic fungus *Bispora* sp. MEY-1 demonstrated higher stability and lactose hydrolysis yield under simulated gastric conditions than commercially available β-galactosidase from *Aspergillus oryzae* ATCC 20423. Thus, this β-galactosidase may be a better supplement for people with lactase deficiency (WANG et al., 2009).

3 IMMOBILIZATION OF ENZYMES

Enzymes display a number of advantages, such as catalytic efficiency and high specificity. Additionally, unlike chemical catalysts, they accomplish chemical conversions under mild environmental conditions. Therefore these biocatalysts are used in a wide range of industry, including food and feed industry but they also find application in the medical, diagnostical, and clinical field. Over the last few decades research in the field of enzyme technology was emphasized approaching a resource- and energy-saving application of enzyme. Even tough enzymes as biocatalysts bring in various features they bear drawbacks in practical application,

such as high-priced extraction methods, as well as their instability in terms of process conditions. Facing these issues immobilization of enzymes emerged as a promising technique (D'SOUZA, 1999; TISCHER and WEDEKIND, 1999, MATEO et al., 2007).

By fixing the biocatalysts on or within solid supports, a natural mode of occurrence of enzymes in living cells can be imitated, as enzymes often operate attached to the cellular membranes. Immobilized enzyme systems bring in various features, such as possible recovery of enzyme and product, higher product purity, and increased stability making it more durable to environmental changes. Subsequently multiple reuse of the enzyme and continuous process is feasible (KRAJEWSKA, 2003).

Immobilization of biocatalysts can be performed with either the isolated enzyme or the whole cells or cellular organelles. When whole cells have been immobilized the expensive enzyme purification can be avoided and the enzyme remains in its natural environment and therefore is sheltered from inactivation. On the other hand immobilization of whole cells can bear limited diffusion of substrates and products through the cell wall and undesirable reactions due to the presence of other enzymes. Mainly extracellular enzymes produced by microbes are used at industrial scale as they can be easily obtained as crude extract from the fermentation broth. Indeed, in spite of high costs due to enzyme purification, 90% of the enzymes produced are intracellular (TAMPION and TAMPION, 1987).

3.1 Immobilization Methods

A large variety of immobilization techniques and supports have been reported. Depending on the nature of the enzyme, its substrate and its designated application, an appropriate immobilization method and matrix can be approached. The methods can be broadly classified into three categories: (i) adsorption, (ii) entrapment, (iii) and covalent binding and cross-linking, or a combination of these methods (Figure 10) (SHELDON, 2007).

Adsorption and entrapment as physical methods are based on weak interactions between the enzyme and the support. In contrast chemical methods such as covalent binding and cross-linking are marked by stronger bonds, therefore being more resistant but more expensive and often affected with loss of enzyme activity. There have been studied that combinations of two or more methods yield the most ap-

propriate immobilization system depending on the chemical characteristics of the enzyme, the properties of the substrate and products, and the intended use. The aim is to ensure maximum retention of activity and operational stability of the immobilized enzyme (KRAJEWSKA, 2004).

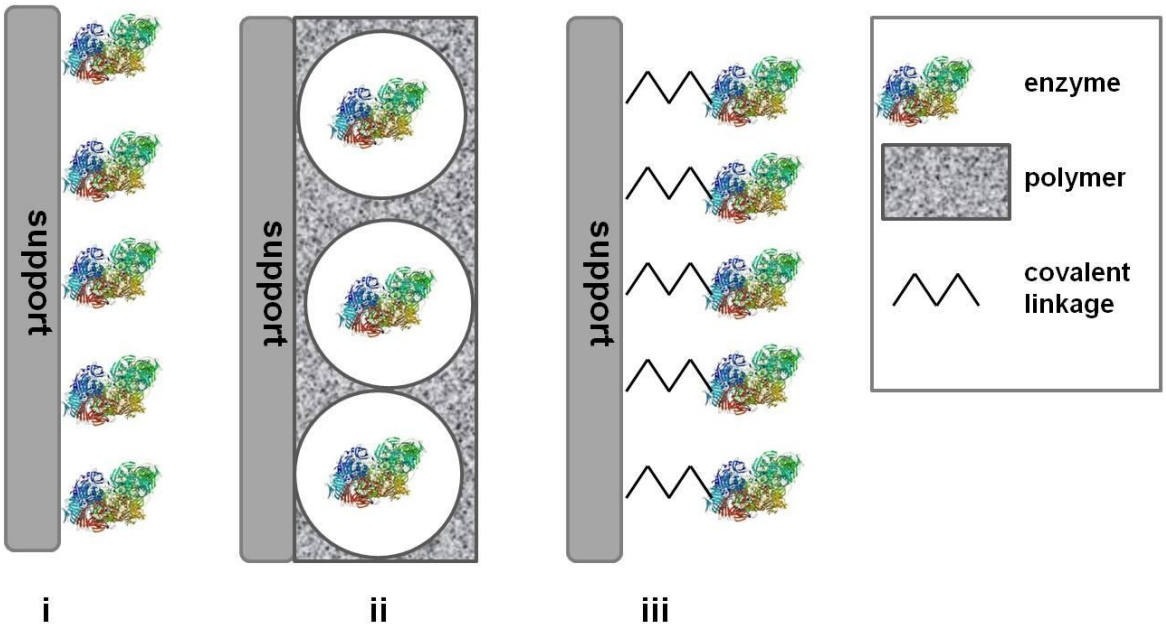

Figure 10. Various immobilization methods using different support materials: adsorption (**i**), entrapment (**ii**), and covalent binding (**iii**).

During immobilization the enzyme undergoes changes in physical and chemical properties due to the binding process and the support material. In that way the surface matrix may account different benefits such as retention of the enzyme tertiary structure or the formation of electron transition complexes. The kinetic properties as well as the stability of the enzyme can be favorably modified due to a different microenvironment created by the support matrix and formed products. For instance the retention of the tertiary structure was observed to enhance stability of the enzyme regarding temperature and pH compared to the soluble enzyme. Due to the new microenvironment the pH optimum of the immobilized enzyme gets altered and the enzyme may even work in a wider pH range. Immobilization also prevents the enzyme from aggregation, autolysis or proteolysis by proteolytic enzymes. However, immobilization also comes along with several drawbacks affecting the performance of the enzyme. The support material, the different microenvironment and the structural changes of the enzyme often interfere with the activity and substrate affinity resulting in lower activity and increased Michaelis constant

compared to the free enzyme (KRAJEWSKA, 2004). The microenvironment can limit the the diffusion of the substrate and therefore the interaction with the enzyme. This often occurs when using the entrapment method for enzyme immobilization (RICHMOND et al., 1981).

3.1.1 Adsorption

Adsorption is considered as the simplest and most inexpensive immobilization method by non-covalent adsorption, mainly due to hydrophobic and van der Waals interactions, and ionic interactions. Since these bindings to the surface of a water-insoluble carrier do not harm the active confirmation of enzymes, usually no significant loss of activity can be observed (SHELDON, 2007).

However, physical adsorption is very weak, the enzyme easily detach and often additional chemical methods such as cross-linking are required to stabilize the immobilized enzyme. Doing so bi- or multifunctional cross-linking reagents are used, such as glutaraldehyde, dimethyladipimate and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (GROSOVÁ et al., 2008). Due to their reactive functional groups covalent bonds to the carrier matrix are formed allowing inter- and intramolecular crosslinking of the protein (TANAKA and KAWAMOTO, 1999).

Ionic binding on support carrier with ionic exchange groups is generally stronger than hydrophobic adsorption. The location and charge of the binding residues on the protein surface and the carrier strongly affect the effectiveness of the binding. Immobilization via ionic interactions can be enhanced if factors which affect ionization like pH, counter-ion identity, hydrophobicity and ionic strength are optimized (LALONDE and MARGOLIN, 2002). Ionic adsorption was the oldest immobilization method that has been used at industrial scale (CHIBATA, 1978).

3.1.2 Entrapment

Another method for immobilization is entrapment technique which is based on physical embedding of an enzyme in a polymer network or membrane allowing the substrate to penetrate whereas the enzyme cannot leak. As basic material polymer matrices such as organic polymer and silica sol-gel or membrane devices like microcapsule and hollow fiber found to be suitable for the confinement of enzymes

(SHELDON, 2007). Entrapment bears several drawbacks as this method is expensive and shows high diffusion limitations.

The choice of carrier material plays a great role in the case of matrix entrapment. The ratio of the pore size of the carrier and the size of the enzyme has to be considered as it is decisive for the entrapment procedure. Thus large pores may cause enzyme leakage and small pores merely allow adsorption on external surfaces (GÓRECKA and JASTRZĘBSKA, 2011). Apart from these disadvantages, matrix entrapment is regarded as a straightforward and effective immobilization technique in the same time. For the immobilization step no harsh conditions are required and along with that no significant loss of activity can be observed (RODGERS et al., 2006). Furthermore, the new environment can be similar to that of the protein in the bulk reaction media, so that the catalytic activity persists during utilization (LALONDE and MARGOLIN, 2002). Even multi-enzyme reactions by co-immobilization of coupled enzyme systems like co-encapsulation of a redox enzyme and a cofactor regeneration system in silica particles has been reported (BETANCOR et al., 2006). As polymeric matrix polyacrylamide gels were mostly applied, however they are not suitable for use in food applications due to their toxicity. Therefore hydro gels and thermo reactive water-soluble polymers gained great attraction. Additionally the environment created by these gels is close to that of the soluble enzyme hence diffusion limitations are diminished (D'SOUZA, 1999).

The microencapsulation of enzymes in membrane devices refers to the restriction of the protein by the membrane walls forming a capsule. At the same time the biocatalyst can diffuse within this capsule. Again mild immobilization conditions and application in multi-enzyme systems feature the microencapsulation method with the addition that leakage of the biocatalysts does not occur and the catalytic activity is preserved during exploitation. Despite these advantages the pore size of the membrane has to be supervised accurately, what may be an issue when small size enzymes are used for microencapsulation (GÓRECKA and JASTRZĘBSKA, 2011).

3.1.3 Covalent Binding and Cross-linking

Covalent binding and cross-linking are chemical immobilization methods where enzymes are covalently attached to a solid support material. Due to the binding of

amino acid side chain residues of the enzyme to reactive functional groups of the carrier covalent binds are formed (LALONDE and MARGOLIN, 2002). Notably, nucleophilic amino (lysine, histidine and arginine), thiol (cysteine), and hydroxyl groups (serine threonine and tyrosine), as well as electrophilic carboxylate groups (aspartic acid and glutamic acid) maintain covalent bonds (BRADY and JORDAAN, 2009). As the functional groups of both, the enzyme and the support matrix, are not active enough for ensuring efficient immobilization, these groups often have to be activated. The method of covalent attachment enables efficient immobilization allowing no or marginal enzyme leakage and therefore a stable enzyme system (LALONDE and MARGOLIN, 2002). Indeed the severe immobilization conditions lead to conformational changes and thus to loss of catalytic activity (ZHAO et al., 2006).
During cross-linking three-dimensional enzyme aggregates or crystals due to covalent linking of enzyme molecules are formed. This process occurs by application of bifunctional reagent leading to carrier-free immobilized enzyme systems, named cross-linked enzyme crystals (CLECs) and cross-linked enzyme aggregates (CLEAs). Cross-linking immobilization offers increased enzyme activity due to enzyme concentration, lower costs by reason of no use of support material, and enhanced enzyme stability. However, this technique bears difficult aggregate size control and hindered substrate diffusion as main disadvantages (ZHAO et al., 2006).

3.2 Support Materials for Enzyme Immobilization

In the last decades immobilization techniques have been studied intensively therefore a large number of support materials and methods have been investigated. As the properties of immobilized enzyme are determined by the characteristics of both, the enzyme and the support matrix, the selection of the appropriate carrier is crucial. Thereby, parameters such as activity retention and increased stability, but also economic aspects have to be taken into account. Depending on the enzyme and application of the immobilized enzyme system chemical and physical properties of support materials are considered. Regarding physical characteristics numerous parameters are important, such as hydrophilic character, adequate surface area, insolubility in the reaction medium, and thermal stability. With respect to chemical properties, carrier materials are commonly divided into organic and inorganic materials. Organic carrier materials are usually classified as natural macro-

molecules or synthetic polymers (KENNEDY and CABRAL, 1987). Amongst them organic support materials include mainly polysaccharides (such as modified celluloses, dextran, chitosan and agarose), vinylic and acrylic polymers (such as polyacrylamide and poly(vinyl alcohol)). Also polyamides, like nylon, have been reported to be suitable for enzyme immobilization (ZUCCA and SANJUST, 2014).

Considering inorganic materials, several silica-based and other oxide-based materials have been studied (HARTMANN and KOSTROV, 2013). Inorganic carriers present several advantages, such as high mechanical stability, increased stability regarding temperature and pH, durability to organic solvents and microbial contamination (ZUCCA and SANJUST, 2014).

Table 7. Important large-scale applications of immobilized enzymes (adapted from KRAJEWSKA, 2004).

enzyme (EC number)	substrate	product
glucose isomerase (5.3.1.5)	glucose	fructose (high-fructose corn syrup)
β-galactosidase (3.2.1.23)	lactose	glucose and galactose (lactose-free milk and whey)
lipase (3.1.1.3)	triglycerides	cocoa butter substitutes
nitrile hydratase (4.2.1.84)	acrylonitrile	acrylamide
	3-cyanopyridine	nicotinamide
	adiponitrile	5-cyanovaleramide
aminoacylase (3.5.1.14)	D,L-aminoacids	L-amino acids (methionine, alanine, phenylalanine, tryptophan, valine)
raffinase (3.3.1.22)	raffinose	galactose and sucrose (raffinose-free solutions)
invertase (3.2.1.26)	sucrose	glucose/fructose mixture (invert sugar)
aspartate ammonia-lyase (4.3.1.1)	ammonia + fumaric acid	L-aspartic acid (used for production of synthetic sweetener aspartame)
thermolysin (3.4.24.27)	peptides	aspartame
glucoamylase (3.2.1.3)	starch	D-glucose
papain (3.4.22.2)	proteins	removal of "chill haze" in beers
hydantoinase (3.5.2.2)	D,L-amino acid hydantoins	D,L-amino acids
penicillin amidase (3.5.1.11)	penicillin G and V	6-aminopenicillanic acid (precursor of semi-synthetic penicillins, e.g. ampicillin)
β-tyrosinase (4.1.99.2)	pyrocatechol	L-DOPA

3.3 Application of Immobilized Enzymes in Industrial Scale

Numerous immobilized enzyme-systems have been implemented in large-scale processes. With fructose corn syrup production by glucose isomerase or lactose hydrolysis in milk and whey by β-galactosidase, immobilized biocatalysts found major utilization in food industry. But also in chemical and pharmaceutical processes immobilized enzyme systems proved to manufacture economically mainly optically pure substances, such as L-DOPA and precursors of penicillins

(SENGUPTA and DASGUPTA, 2006). Further important applications in food industry are summarized in Table 7. To date, research and industrial application of immobilized enzyme systems for medical devices and biosensors have been emphasized. (SENGUPTA and DASGUPTA, 2006; KRAJEWSKA, 2004)

3.4 Immobilization of β-Galactosidase

As mentioned before, microbial β-galactosidases found great interest and hence application for the hydrolysis of lactose in milk and whey. With the feature of transgalactosylation reaction resulting in synthesis of prebiotic galacto-oligosaccharides, β-galactosidase gained major attraction (PRENOSIL et al., 1987).
Despite often reported drawbacks of enzyme immobilization like drop of catalytic activity, immobilized β-galactosidase proved to show increased operational stability, enhanced half-life time, higher thermal stability and less inhibition by end products (mainly galactose). Furthermore, in respect to large-scale utilization the binding of biocatalysts on a carrier allows recovery of the immobilized enzyme system, enabling repeated and continuous operation (GROSOVÁ et al., 2008; JUNG et al., 2008; MARTINEZ et al., 2013; ANDERSON et al., 2005).
To date, industrial lactose conversion is mainly performed with free enzyme preparations (GERMAN, 1997). As soluble enzyme systems bear numerous drawbacks such as low operating temperature and stability, increased product inhibition and less substrate affinity, immobilized β-galactosidase for large-scale applications was implemented. In the 1970s the *K. lactis* β-galactosidase was entrapped in cellulose triacetate fibres by SnamProgetti (Italy) and subsequently applied in food industry (MARCONI and MORISI, 1978). Sumitomo Chemicals (Japan) immobilised β-galactosidase from *A. oryzae* by covalent binding to phenol-formaldehyde resin for industrial utilization in milk and whey (GEKAS and LOPEZ-LEIVA, 1985; KATCHALSKI, 1993). In the following years immobilized β-galactosidase found increased application in industrial scale. Thus, a large variety of enzyme sources, immobilization techniques, and carrier materials have been investigated (Table 8).

Table 8. Different enzyme sources and techniques for immobilization of β-galactosidase (adapted from PANESAR et al., 2010).

Source of β-galactosidase	Immobilization method	Immobilizing agents
A. niger	Physical adsorption	Porous ceramic monolith
	Covalent Binding	Magnetic polysiloxane-polyvinyl alcohol
A. oryzae	Entrapment	Spongy polyvinyl alcohol Cryogel
	Physical adsorption	Celite and chitosan
	Covalent Binding	Silica gel activated with TiCl3 and FeCl3
	Covalent Binding	Amino-epoxy sepabead
	Covalent Binding	Polyvinylalcoheol hydrogel and magnetic Fe3O4-chitosan as supporting agent
	Physical adsorption	Phenol-formaldehyde resin
	Physical adsorption	Polyvinyl chloride and Silica gel membrane
	Entrapment	Nylon-6 and zeolite
	Covalent Binding	Chitosan bead and nylon membrane
	Covalent Binding	Cotton cloth and activated with tosyl chloride
	Covalent Binding	Silica
B. circulans	Physical adsorption	Polyvinyl chloride and Silica
	Covalent Binding	Eupergit C (Spherical acrylic polymer)
B. stearothermophilus	Physical adsorption	Chitosan
E. coli	Physical adsorption	Chromosorb-W
	Entrapment	Polyacrylamide gel
	Covalent Binding	Hen egg white
	Covalent Binding	Polyvinyl alcohol
	Covalent Binding	Gelatin
	Covalent Binding	Cyanuric chloride-activated cellulose
K. bulgaricus	Entrapment	Alginate using BaCl3
K. fragilis	Physical adsorption	Chitosan bead
	Physical adsorption	Chitosan
	Physical adsorption	Cellulose beads
	Covalent Binding	Silica-alumina
K. fragilis and *K. lactis*	Physical adsorption	Chitosan
K. lactis	Physical adsorption	CPC-silica and agarose
	Covalent Binding	Corn grits
	Covalent Binding	Thiosulfinate/thiosulfonate
	Covalent Binding	Graphite surface
	Covalent Binding	Cotton fabric
K. lactis, A. oryzae, Saccharomyces cerevisiae	Entrapment	Poly(vinylalcohol) hydrogel
L. bulgaricus	Covalent Binding	Egg shells
Penicillium expansum F3	Entrapment	Calcium alginate
Pisum sativum	Physical adsorption	Sephadex G-75 and chitosan beads
S. anamensis	Covalent Binding	Calcium alginate
Thermus aquaticus YT-1	Entrapment	Agarose bead
Thermus sp. T2	Physical adsorption	PEI- sepabeads, DEAE-agarose

4 CHITIN AND CHITOSAN

4.1 Structure, Source and Properties

Being widely distributed in nature like as supporting material of crustaceans, insects, and certain fungi, chitin is a naturally abundant polysaccharide. Chitin is composed of β-(1→4)-linked 2-acetamido-2-deoxy-β-D-glucose forming a long chain linear polymer and is highly insoluble. The polysaccharide is inelastic, white and due to acetamide groups a nitrogen source. Chitosan, a linear copolymer of β-(1–4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose and 2-amino-2-deoxy-β-D-glycopyranose, is the *N*-deacetylated derivative of chitin (MUZZARELLI, 1973; ZIKAKIS, 1984). The schematic structure and deacetylation of chitin to chitosan are shown in Figure 11.

Chitin mainly derives from the shell waste of shrimps, lobsters, krills and crabs with annual yields of several millions tons of chitin. Therefore this biological macromolecule is regarded as a cheap and easily available material (ROBERTS, 1992; RHA et al., 1984; STRUSZCYK et al., 1992). However, chitosan only occurs naturally in some fungi (*Mucoraceae*) (ROBERTS, 1998). Potential and usual applications of the biopolymers chitin, chitosan and their derivatives are estimated to be more than 200 (KUMAR, 2000).

The structure of chitin as well as chitosan shows great similarity to cellulose, as the C-2 hydroxyl residues are replaced by acetamide groups. Chitin can be found with different degree of acetylation depending on the origin and processing conditions. For instance, in crab chitin counts 5000 to 8000 N-acetyl-D-glucosamine (GlcNAc) residues, whereas in yeast only around 100 residues. The separate chitin chains are linked by hydrogen bonds (SYNOWIECKI et al., 2000). These chains can be arranged in different ways resulting in various polymorphic conformations, whereat the α-chitin composed of antiparallel sheets is mainly formed (KRAJEWSKA, 2004). The α-conformation indicates higher stability due to strong hydrogen bonds and hinders swelling in water. β-chitin with its parallel arrangement of chitin chains can be irreversibly converted to the α-form. It can swell in water and shows higher permeability. The proportion of these two chain alignments strongly influences hardness, permeability, flexibility and solubility of the shell (ARANAZ et al., 2009). In γ-chitin, every third chitin chain is aligned in reverse orientation compared to the two preceding chains (ROBERTS, 1998).

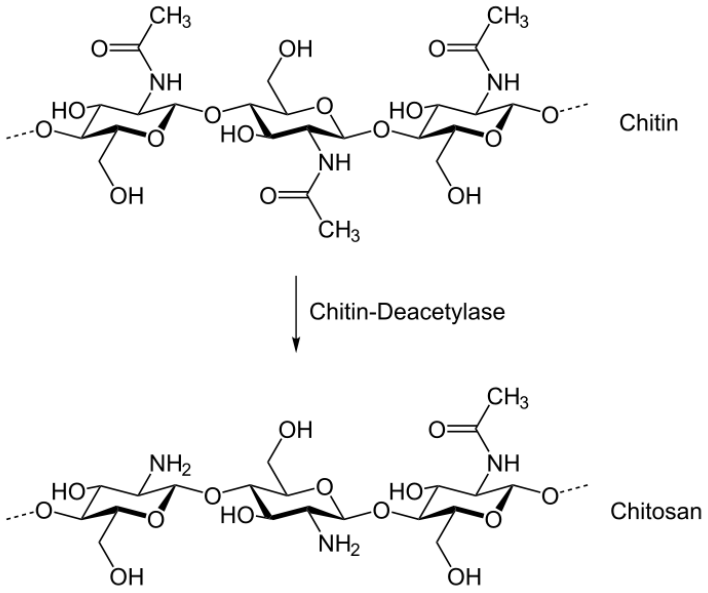

Figure 11. Schematic illustration of enzymatic chitosan synthesis out of chitin.

The three different configurations are shown in Figure 12. Principally, chitin is insoluble in water and organic solvents, due to its increased amount of hydrogen bonds. In contrast to chitin, the presence of free amine groups along the chitosan chain allows this macromolecule to dissolve in diluted aqueous acidic solvents due to the protonation of these groups. Chemically modified chitin and chitosan structures resulting in improved solubility in general organic solvents have been reported (SYNOWIECKI et al., 2000; KUMAR, 2000).

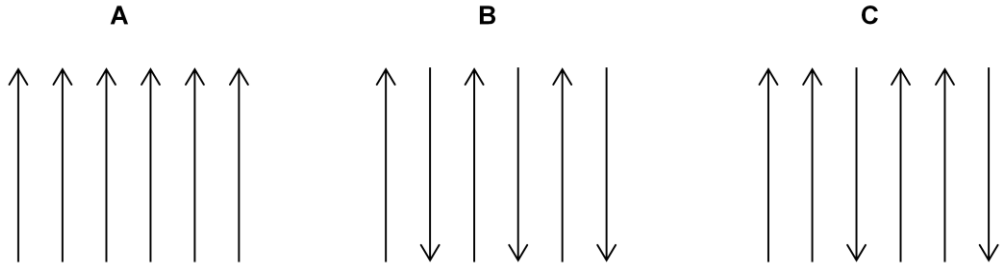

Figure 12. Three different configurations of chitin chains: α-chitin (**A**), β-chitin (**B**), and γ-chitin (**C**).

As most of the polymers used nowadays are synthetic materials, natural polymers such as cellulose, chitin, chitosan and their derivatives gained great interest as they show higher biocompatibility and biodegradability. Additionally chitin and chitosan are feasible functional materials since non-toxicity and excellent adsorption properties are counted among their characteristics (NISHIMURA et al., 1991).

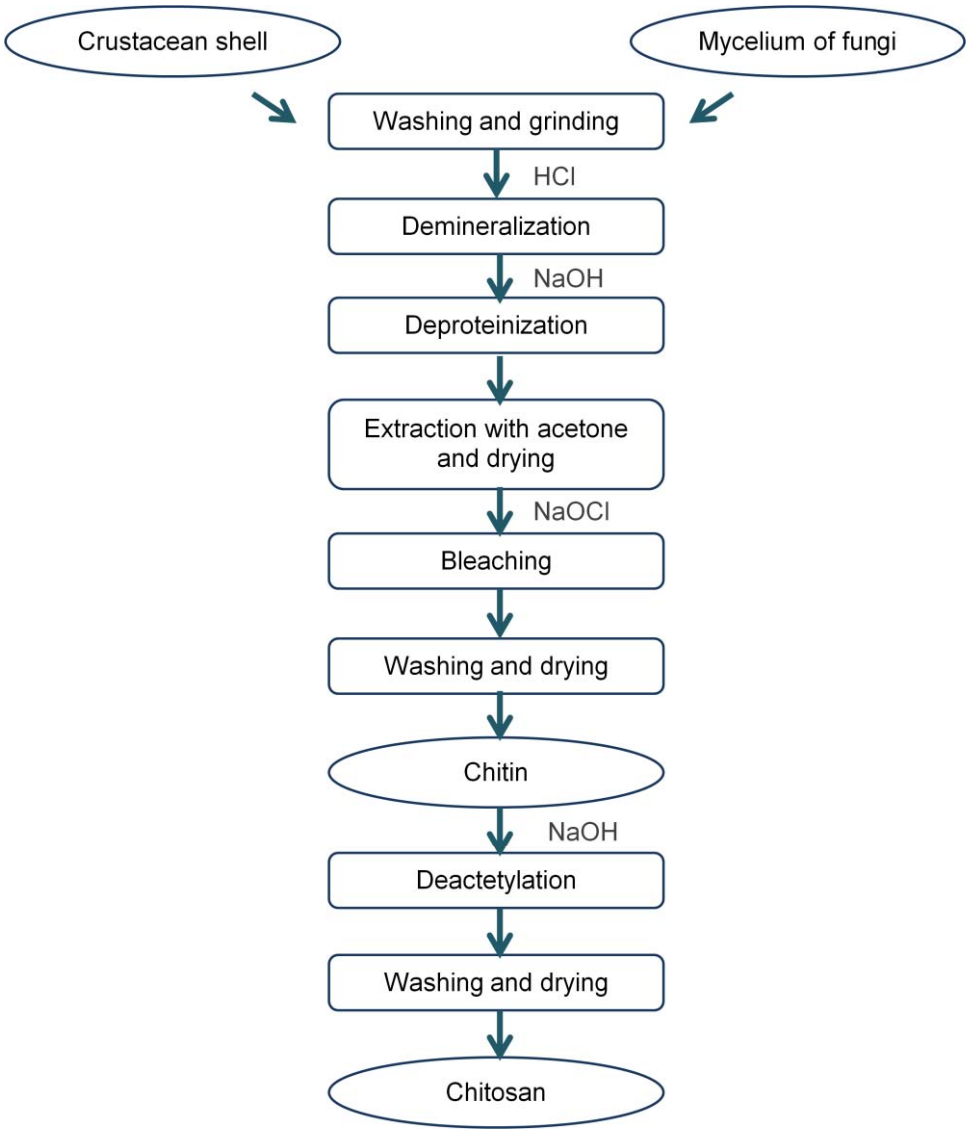

Figure 13. Shematic flow chart of chitin and chitosan preparation.

4.2 Preparation Methods

As mentioned above, chitins mainly get isolated from marine crustaceans, since a large amount of shell waste occurs during sea food processing. Besides proteins (30-40%), calcium carbonate (30-50%), and pigments such as carotenoids, crustacean shells also contain 20-30% chitin, depending on source and season. In Figure 13 the process to prepare chitin and chitosan from raw material is demonstrated. By means of acid treatment present calcium carbonate in the raw resources can be dissolved. Subsequent treatment with alkali dissolves the proteins enabling the extraction of chitin. In order to obtain a colorless product mainly astaxantine but also pigments such as astathin, canthaxanthin, lutein and β-carotene can be removed by a following bleaching step (ACOSTA et al., 1993).

The preparation of chitosan is performed by hydrolysis of acetamide groups of chitin using strong alkali (HORTON and LINEBACK, 1965). Generally, for deacetylation of chitin sodium or potassium hydroxides (30-50% w/v) are used in combination with increased temperature (80-140 ºC) and long processing times between 1 and 80 hours. Considering the concentration of alkali, particle size and pretreatment of chitin, the dregree of deacetylation (DD) can be controlled. Two methods can be distinguished in heterogeneous deacetylation of solid chitin, mainly applied in industrial scale, and homogeneous deacetylation of pre-swollen chitin under vacuum. The maximal attainable DD in a single alkaline treatment is about 75-85% (ROBERTS, 1998).

4.3 Properties and Applications

The properties of chitin and chitosan vary depending on their characteristics and hence enable different applications. It was reported that the source of chitin affects its crystallinity and purity as well as its polymer chain conformation, and hence its properties (RINAUDO, 2006). Also the surficial area of the polymer depends on the origin, so was shown that chitin derived from crab exhibit major surface that lobster and shrimp. Mainly the parameters molecular weight (Mw), polydispersity, crystallinity, and DD influence the polymer properties. Amongst them DD has been reported to greatly affect the chemical properties (LI Q. et al., 1992; BAXTER et al., 1992). The average Mw plays a great role regarding the viscosity in aqueous solutions, which may be respected in biochemical and biopharmacological application of chitosan (THARANATHAN et al., 2003). For utilization in food and medical applications, the purity, the moisture, the heavy metal content, endotoxin, and proteins must be considered.

As mentioned above chitin and chitosan are currently gaining major attraction for the use in medical and pharmaceutical applications due to their biocompatibility, biodegradability and non-toxicity. In previous studies further properties such as analgesic, antitumor, hemostatic, hypocholesterolemic, antimicrobial, and antioxidant properties have been reported (KUMAR, 2000; KUMAR et al., 2004). Due to its poor solubility the use of chitin is limited and more attention has been paid to the investigation and modification of the soluble derivative chitosan (ZOHURIAAN-MEHR, 2005). The polymer has proved to be more feasible for different utilizations, since it shows excellent gel forming and adsorption properties, as well as polyelectrolyte

properties enabled by its reactive amino and hydroxyl residues (SYNOWIECKI et al., 2000). In the field of food production chitosan showed promising qualifications as a food preserving agent due to its anti-microbiological and antioxidant properties. Thus for the extension of shelf life of fruits, vegetables and marine food coatings with semi-permeable membranes and films were developed (BHADRA et al., 2012; SYNOWIECKI et al., 2000). Furthermore chitosan shows characteristics of dietary fibers and functional food ingredients. In this scope fat and cholesterol binding properties as well as effects on pancreatic lipase activity were reported decreasing the gain in weight or plasma lipid content (ACOSTA et al., 1993). Because of its moisturizing and protective effects, chitosan found application in cosmetics as ingredient in creams and toothpaste. Other applications of chitin and chitosan are in textile industry and water treatment. As fibers they enable an improvement in water vapor permeability in textiles and in waste water treatment chitosan proved to remove hardness, phosphorous, heavy metal ions, and oils (BHADRA et al., 2012; SYNOWIECKI et al., 2000).

In some Asian countries chitosan has been already approved as functional food whereas in the EU chitin and chitosan have been considered by the Codex Alimentarius Commission but not yet authorized as food ingredient (BERGER et al., 2004; MUZZARELLI, 1989).

4.4 Application of Chitin and Chitosan in Biocatalysis

Chitin and chitosan have been widely used as supports for enzyme and cell immobilization due to their appropriate characteristics. Hence, these biocompatible and biodegradable polymers are commercially available as powders, flakes, resins, and gels (KRAJEWSKA, 2004). Table 9 shows an overview of investigated chitin- and chitosan-based materials for immobilization of β-galactosidase.

Chitin shows neutral charge due to the low amount of free amino groups on its surface and therefore poor interaction through H-bonding or ionic forces are able. In this respect enzyme immobilization on chitin via adsorption is only possible at their isoelectric point. In contrast, chitosan as a positively charged polymer at pH lower than 6.5 negatively charged proteins can easily adsorb on its surface (STRAND et al, 2003). Immobilization using covalent binding involves the formation of a covalent bond between the biocatalyst and the support material. These bonds

are promoted by functional groups on the support and the enzyme (amino, carboxyl, hydroxyl and sulphydryl groups). Functional groups on the support material can be chemically modified to form bonds between the enzyme and the support. For chitin and chitosan commonly a bifunctional dialdehyde (most frequently glutaraldehyde) is applied for covalent immobilization. When chitin is applied as support material, the low number of free amino groups on the surface enables the enzyme molecule to form only single point attachments. Contrary, when chitosan is used as support the biocatalyst can bind to several residues achieving a multipoint immobilization. With entrapment as another method for enzyme immobilization, alginate-chitosan polyelectrolyte complexes have been used to encapsulate proteins (ARANAZ et al., 2009).

Gel and bead formation from various chitin solutions and non-solvents have been studied, finding out that ethanol is the optimal non-solvent for homogeneous bead formation from chitin solutions (YILMAZ and BENGISU, 2003). PEREIRA et al (2003) described that porous chitosan beads were more suitable for the immobilization of lipases than chitosan flakes. Other researchers immobilized four different hydrolases on chitosan micro particles by covalent binding using glutaraldehyde as a cross-linking and activating agent. They found out that the molecular weight and quantity of glutaraldehyde had a significant effect on residual enzyme activity, also depending on the specific enzyme (ZUBRIENE et al., 2004). Furthermore different krill chitin supports were studied for their feasibility as carrier material. In this study enzyme activity was influenced by several parameters, such as the degree of deproteinization of chitin, availability of amino groups, content of minerals, mesh size, structure of the surface, and conformation of the chitin molecules (SYNOWIECKI et al., 1981). The degree of deacetylation (DD) was also shown to affect significantly the hydrophobic modification of chitosan and, as a consequence, the activity of the immobilized enzyme. In this scope, an increased DD provided optimal enzyme immobilization properties but on the other hand the pretreatment for deacetylation reduced its polymer molecular weight resulting in loss of enzyme activity (SJOHOLM et al., 2009).

Table 9. β-Galactosidases immobilized on chitin- and chitosan-based materials (adapted from KRAJEWSKA, 2004).

support material	immobilization technique	reference
chitin powder	covalent binding of enzyme to glutaraldehyde-activated support	ILLANES et al., 1998; ILLANES et al., 2001
chitosan powder	covalent binding of enzyme to glutaraldehyde-activated support	CASTRO et al., 1997
chitosan beads	covalent binding of enzyme to glutaraldehyde-activated support	REIJKUMAR and DEVI, 1995; REJIKUMAR and Devi, 2001
chitosan beads	adsorption of enzyme to support; covalent binding of enzyme to glutaraldehyde-activated support	SHIN et al., 1998; SHEU et al., 1998
chitosan-polyphosphate beads	gel inclusion	GHANEM and SKONBERG, 2002
chitosan precipitate	adsorption of enzyme to support followed by crosslinking with glutaraldehyde	PORTACCIO et al., 1998
chitin beads	adsorption of enzyme using affinity tag (chitin-binding domain)	this work

5 SUBSTRATE-BINDING DOMAINS FOR PROTEIN IMMOBILIZATION

The immobilization methods described above are often involved with various drawbacks such as diffusion limitations of substrates, mechanical fragility of materials, or difficulty in stably retaining cells or enzymes (WANG and CHAO, 2006). However, using covalent binding methods in order to achieve strong and robust attachment of enzymes chemical modification of support materials is often required. The severe and harsh reaction conditions during that chemical treatment usually derogate the enzyme's catalytic activity (ZHAO et al., 2006). Therefore genetic engineering was used allowing the fusion of a protein with an affinity tag which specifically binds to an unnatural cognate ligand. Several advantages such as strong and reversible binding of enzymes to the support, proper exposure of active domains of immobilized enzymes, mild adsorption conditions, and the lack of diffusion constraints come along with the immobilization of enzymes via affinity tags (SALEEMUDDIN, 1999). There have been reported on the use of some peptide tags with FLAG, poly-His, c-myc, and glutathione S-transferase being the most commonly used (TERPE, 2003). Substrate-binding domains are regions of an enzyme enabling binding to specific oligo- or polysaccharide such as cellulose, chitin or xylan, which are cheap and easily available. High binding specificity towards their substrates, autonomy, and absence of influence on the enzymatic activity of a target recombinant protein make them valuable tools for the immobilization or iso-

lation and purification of proteins. Also the size plays an important role for the choice of a substrate-binding domain with amino acid sequences varying from 30 to 200 amino acid residues. Domains of large amino acid sequences are more difficult to incorporate into a structure of a recombinant protein so that this substrate binding region marginally affects three-dimensional structure of the protein and its enzymatic activity (KUREK et al., 2009).

Major disadvantage of the affinity tags mentioned above is represented by their corresponding cost-intensive ligand materials. Hence peptides binding to low cost and easily available materials gained great interest. Among such substrate-binding domains, the cellulose-binding domains (CBD) are the most studied (SHOSEYOV, 2002). The group of the chitin-binding domains (ChBDs), as a further class of substrate-binding domains, was found to be involved in the catalytic activity of chitinases which degrade chitin to oligosaccharides. Its removal from the enzyme structure results in a decrease in the rate of chitin degradation, proving the strong interaction talking place between the substrate and the binding domain (KUREK et al., 2009).

5.1 Chitin-Binding Domain of *Bacillus circulans* WL-12 Chitinase A1

In the presence of chitin, *Bacillus circulans* WL-12 was found to produce up to 10 various chitinases, whereof chitinase A1 (EC 3.2.1.14) is responsible for highly efficient chitin degradation due to a particular catalytic domain. The C-terminal chitin-binding domain (ChBD) of chitinase A1 derived from the gene *chi*A is a very small peptide of 45 amino acids located between Ala^{655} and Gln^{699}. Besides the ChBD the enzyme further consists of two fibronectin type III (ALAM et al., 1996).

The ChBD of chitinase A1 from *Bacillus circulans* WL-12 ($ChBD_{ChiA1}$) was detected to bind very specific to insoluble chitin but not to chito-oligosaccharides, soluble derivatives of chitin, or other insoluble polysaccharides. It was studied that ChBD recognizes a structure that is present only in insoluble or crystalline forms of chitin thus exhibiting a binding mechanism different from that found in other polysaccharide binding domains (HASHIMOTO et al., 2000). The structure of ChBD is compact and globular showing the topology of a twisted β-sandwich similar to that of the cellulose-binding domain (CBD) of *Erwinia chrysanthemi* endoglucanase Z (CBD_{EGZ}) (Figure 14). Whereas CBD_{EGZ} only consists of two β-sheets (β1, β2), one composed by two, the $ChBD_{ChiA1}$ shows additionally another, 45 °C twisted

sheet, made up by three strands (β3, β4, β5) (IKEGAMI et al., 2000). BRUN et al. (1997) suggested that $ChBD_{ChiA1}$ does not exhibit the stWWst (small-turn-aromatic-aromatic-small-turn) motif which is a highly conserved sequence among chitinases and endoglucanases also present in CBD_{EGZ}.. In CBD_{EGZ} the aromatic residues Trp^{43}, Tyr^{44} from the motif generate a loop together with Trp^{18} as an additional residue interacting with cellulose via hydrophobic bonds. ChBD does not show this region but other residues being on the one hand exposed on the surface and on the other hand hydrophobic or aromatic and therefore supposed to bind to chitin (IKEGAMI et al., 2000).

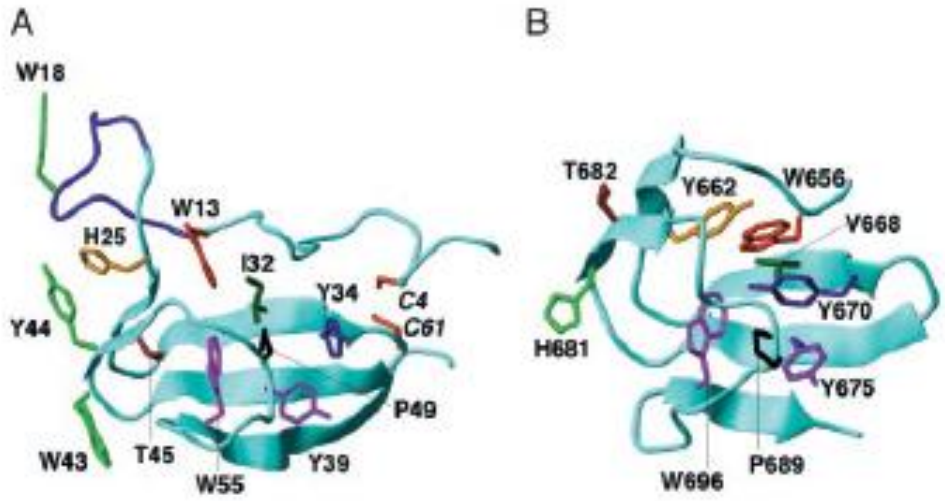

Figure 14. Tertiary structures of cellulose-binding domain of endoglucanase Z of *E. crysanthemi* (**A**) and chitin-binding domain of *Bacillus circulans* WL-12 chitinase A1 (**B**) (IKEGAMI et al., 2000).

5.2 Immobilization of Enzymes via ChBD

Immobilization of enzymes using the chitin-binding domain of chitinase A1 from *Bacillus circulans* WL-12 was first described by CHONG et al. (1997) for the purification of recombinant proteins in a single chromatographic step. Thereby the desired protein is fused to a tag consisting of the ChBD and a self-splicing element, the intein. Due to the specific and strong binding only the recombinant protein remains immobilized on chitin whereas other unspecifically bound proteins are washed away. Subsequent elution is then mediated by treatment with a reducing agent such as DTT to induce the self cleavage of intein obtaining the desired protein in its native form.

CHIANG et al. (2009) explored the utilization of the mesophilic bacteria *Bacillus circulans* WL-12 ChBD ($ChBD_{ChiA1}$) as an affinity tag to retain *Zymomonas mobiliz*

levansucrase on chitin beads, leading to an enhanced production of levan. CHERN and CHAO (2005) managed to immobilize D-hydantoinase-$ChBD_{ChiA1}$ on chitin for continuous production of N-carbamoyl-D-hydroxyphenylglycine. WANG and CHAO (2006) immobilized *Escherichia coli* with surface-displayed $ChBD_{ChiA1}$ on chitin within a wide range of pH (5 to 8) and temperatures (15 to 37 °C). KOJI et al. (2004) reported a hybrid protein glutaryl-7-aminocephalosporic acid acylase (GLA)-$ChBD_{ChiA1}$ stably immobilized on the chitin carrier, displaying physical and chemical stabilities. CHIANG et al. (2009) reported that evolved *Agrobacterium radiobacter* carbamoylase with $ChBD_{ChiA1}$ on chitin beads could be recycled 16 times with the achievement of 100% conversion yield in the efficient production of D-HPG from D,L-HPH. Recently, WANG et al. (2013) fused the chitin-binding domain of chitinase A1 from *Thermoanaerobacterium thermosaccharolyticum* DSM571 as thermophilic strain as well as ChBD from *Bacillus circulans* WL-12 as mesophilic strain to xylosidase-arabinosidase from *T. ethanolicus*. They found that target proteins with ChBD from *T. thermosaccharolyticum* exhibited stronger affinity to colloidal chitin at temperatures ranging from 65 °C to 75 °C. These results suggest a new approach for immobilization of enzymes designated for high temperature applications.

In conclusion, it was found that enzymes immobilized via ChBD showed higher operational and long-term stability compared with the souluble enzyme. Moreover, product isolation and enzyme reutilization was facilitated.

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