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ß-Galactosidase of *Bacillus licheniformis:* Immobilization on chitin using a chitin binding domain and biochemical characterization

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Abstract

ß-galactosidase (ß-gal) also known as the lactose hydrolyzing enzyme is a widely known catalysator for a variety of applications in food industry such as the production of lactose-free dairy products and of so-called galacto-oligosaccharides (GOS) for new carbohydrate-based functional foods. For the optimization of industrial processes ß-galactosidases have already been tested in several enzyme immobilization systems, though high costs of material and the immobilization method itself often limited the use in industrial scale applications. Now, recombinant DNA technology allows the use of substrate binding domains for the purification and immobilization of proteins. In this study the chitin binding domain (*ChBD*) of the chitinase A1 from *Bacillus circulans* WL-12 was used as affinity tag for the immobilization of the ß-gal from *Bacillus licheniformis* DSM 13 (*LacA*) on chitin. C-and N-terminal fusions of the *ChBD* to the *LacA* gene were tested as well as fusions of *LacA* with the *ChBD* with and without linker at the C-terminus of *LacA*, assessing possible changes in ß-galactosidase activity depending on the nature of the fusion enzyme. All of the tested recombinant proteins exhibited ß-gal activity and showed remarkably strong and specific binding to chitin though elution was only possible at a pH < 3, which are denaturing conditions for the ß-gal not enabling enzyme purification using a chitin column. Therefore, the soluble form of the fusion enzyme was purified using affinity chromatography and gel filtration. Subsequently both, the free as well as the immobilized enzyme, were biochemically characterized and compared regarding kinetic parameters, pH- and temperature stability and profiles as well as stability in the presence of various chemical reagents.

Kurzfassung

ß-Galaktosidase (ß-Gal), auch als lactosehydrolysierendes Enzym bekannt, ist ein wichtiger Katalysator für eine Vielzahl von Anwendungen in der Lebenmittelindustrie, vor allem für die Herstellung von lactosefreien Milchprodukten wie auch für die Produktion von sogenannten Galactooligosacchariden (GOS), die als Präbiotika wichtige Zusatzstoffe von funktionellen Lebensmitteln darstellen. Zur Optimierung von industriellen Prozessen wurden ß-Galaktosidasen unterschiedlichen Ursprungs in verschiedenen Enzymimmobilisierungssystemen getestet, jedoch limitieren oft hohe Materialkosten und die Immobilisierungsmethode selbst eine Anwendung in industriellem Maßstab. Heutzutage ermöglicht rekombinante DNA Technologie die Nutzung von Substratbindungsdomänen als Affinitäts-Tags für die Immobilisierung von Enzymen.

In dieser Arbeit wurde die ß-Galaktosidase aus *Bacillus licheniformis* DSM13 mit der Chitinbindungsdomäne (*ChBD*) der Chitinase A1 aus *Bacillus circulans* WL-12 verknüpft um eine Immobilisierung auf Chitin zu ermöglichen. Die Chitinbindungsdomäne wurde sowohl am C-als auch am N-terminalen Ende der ß-Galaktosidase kloniert, wie auch mit dazwischen eingefügter Linker-domäne, um mögliche Einflüsse auf die Enzymaktivität zu testen. Alle Fusionsproteine hatten unveränderte ß-Galaktosidase-Aktivität und zeigten starke, fast irreversible Bindung an Chitin, denn die Elution der rekombinanten Proteine war nur in stark saurem Bereich (pH < 3) möglich, in dem eine Inaktivierung des Enzyms unvermeidbar war und eine Reinigung mittels Chitinmatrix unmöglich machte. Die Reinigung des löslichen ß-Gal-ChBD Proteins erfolgte somit mittels Affinitätschromatographie und Gelfiltration. Anschließend wurde das rekombinante Protein bestehend aus ß-Galaktosidase und direkt am C-Terminus fusionierter Chitinbindungsdomäne (ohne Linker) auf Chitinküglchen gebunden. Das Enzym wurde in dieser Art immobilisierten, wie auch in ungebundener Form biochemisch charakterisiert und hinsichtlich ihrer Kinetik, der pH- und Temperaturoptima, wie auch der Stabilität bei unterschiedlichen pH und Temperaturwerten und in Anwesenheit verschiedener Chemikalien verglichen.

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LIST OF ABBREVIATIONS

ß-gal	ß-galactosidase
ABTG	p-Aminobenzyl-1-Thio-ß-Galactopyranose
BME	ß-mercaptoethanol
ChBD	chitin binding domain
CIP	Calf Intestine Phosphatase
DD	Deacetylation degree
DTT	Dithiothreitol
EDTA	Ethylene Diamine Tetraacetic Acid
GH	Glycoside hydrolase
Glu	Glutamic acid residue
GOD	Glucose oxidase from *Aspergillus niger*
GOS	galacto-oligosaccharide(s)
GRAS	generally regarded as safe
IPTG	Isopropyl-ß-D-Thio-Galactopyranoside
k_{cat}	catalytic constant
k_{cat}/K_m	catalytic efficiency
K_m	Michaelis-Menten constant
MUG	4-methylumbelliferyl-ß-D-galactopyranoside
MU	4-methylumbelliferone
NaPP	Sodium phosphate
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
POD	peroxidase from horseradish
*o*NPG	*o*-Nitrophenyl-ß-D-galactopyranoside

*o*NP	*o*-Nitrophenol
SDS	sodium dodecyl sulfate
TAE	Tris acetate EDTA
$t_{1/2}$	Half life time of activity
V_{max}	maximal reaction velocity
X-Gal	5-Brom-4-chlor-3-indolyl-ß-D-galactopyranoside

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ß-Galactosidase of *Bacillus licheniformis:* Immobilization on chitin via chitin binding domain and biochemical characterization

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Abstract

Immobilized enzyme systems allow easy recovery of both, enzyme and product, multiple re-use of enzymes, continuous operation of enzymatic processes, rapid termination of reactions and greater variety of bioreactor designs. ß-galactosidase as extensively used catalyst for the hydrolysis and transgalactosylation of lactose has already been immobilized using a variety of methods and support matrices, though its application in industrial scale is limited by a number of factors including high material costs and the immobilization method itself. Recently, the chitin binding domain of chitinase A1 from *Bacillus circulans* WL-12 (*ChBD*) has gained increased attention as affinity tag for immobilization purposes. Its small size and strong binding to chitin together with the favorable properties and easy availability of the substrate being the second most abundant natural polymer after cellulose suggested its use for the immobilization of enzymes. In this study the ß-galactosidase of *Bacillus licheniformis* DSM 13 was fused C-terminally to the ChBD and immobilized on chitin beads. The immobilized ß-galactosidase had higher activity at its temperature optimum than its soluble form when *o*-Nitrophenyl-ß-D-Galactopyranoside (*o*NPG) was used as substrate. Furthermore, the decrease in ß-gal activity was less influenced by the end product glucose, as well as by the presence of chemical reagents. However, the pH and temperature stability was not enhanced by binding the recombinant enzyme to the solid support as expected from previous immobilization studies. Most importantly, recovery of the bound ß-galactosidase was possible enabling repeated use of the enzyme over several reaction cycles where it maintained 60 % of its initial activity. Thus, the minimal interference of the ChBD with the enzyme, the strong and nearly irreversible binding being stable over a wide pH range and the possibility of enzyme recycling, confines it as a useful tool for protein immobilization. With chitin being not only cheap and easily available but as well non-toxic, biodegradable and biocompatible, a ChBD-based enzyme immobilization system can be considered for large scale applications in food industry.

1 Introduction

ß-galactosidases (ß-gal, EC 3.2.1.23) catalyze the hydrolysis of D-galactosides, as for example lactose, into monosaccharides. Estimated 75 % of the world's population is lactose intolerant in adulthood, and thus the enzyme has become of major industrial importance for the cleavage of lactose in milk and other dairy products. The much sweeter reaction products galactose and glucose are known for better solubility and easier fermentation and other than lactose they do not have to be indicated on food products (García-Garibay and Gómez-Ruiz 1996). Furthermore, the hydrolysis of lactose is important for the prevention of lactose crystallization in dairy products (Patocka and Jelen 1988; Matak 1999), to reduce cheese whey waste (Rech *et al.* 1999), to improve several milk proteins and to produce ethanol. Another important application of ß-galactosidases in food industry is the use of their transgalactosylation activity. During transgalactosylation of lactose galacto-oligosaccharides (GOS) are formed, which are specific types of dietary carbohydrates and thus gain increased attention as food additives in new carbohydrate-based functional foods. Being non-digestible for humans and mono-gastric animals but fermentable by certain beneficial microorganisms, they promote the growth of desirable bacteria in the colon and therefore provide favorable effects on human health (Park and Oh 2009b). ß-galactosidases have been isolated and characterized from numerous different sources including microorganisms, plants and animals, of which microbial ß-galactosidases have attracted most attention for biotechnological applications (Mahoney 1998). One of the most important criteria for food-related applications and the production of food-relevant enzymes is the safety of microorganisms. Therefore, the main producers of ß-galactosidase are the so-called "generally regarded as safe" (GRAS) organisms, such as *Aspergillus niger, Aspergillus oryzae*, *Kluyveromyces lactis* and *Klyuveromyces fragilis*. Now, ß-galactosidases from probiotic strains such as *Lactobacillus spp.* gain more attention for the production of prebiotic galacto-oligosaccharides (GOS) due to their high transgalactosylation activity of their host cell ß-galactosidases and their good growth on GOS itself (Nguyen *et al.* 2006; Nguyen *et al.* 2007). Some *Bacillus*

species are also listed on the Food and Drug Administration's GRAS list, most notably *B. licheniformis* and *B. subtilits.* Recently, the genome of the strain *Bacillus licheniformis* ATCC 14580 (or DSM 13) has been sequenced and the genome section Bli00447 was identified as the sequence encoding a ß-galactosidase, the *lacA* gene (Veith *et al.* 2004). *Bacillus licheniformis* is a gram-positive, spore-forming and facultative anaerobic soil bacterium, an industrial organism widely used for enzyme over-production, antibiotics, and chemicals that has never been reported to be pathogenic (Salkinoja-Salonen *et al.* 1999). Furthermore, it is taxonomically related to *B. subtilis,* the second most important model organism after *Escherichia coli* (Rey *et al.* 2004) and its known ability to produce large amounts of extracellular proteins (20-25g/l) makes it a useful expression hosts for food-related applications (Schallmey M. 2004).

However, the currently used commercial enzyme preparations have met some limitations for the use in industrial applications, which are low substrate affinity, bad pH and temperature stability, inhibition by hydrolysis products etc. resulting in high demands of enzyme and time, in short, in high production costs. Optimization of enzymatic processes can be achieved by immobilization, where the enzyme is bound on or within a solid support. Enzyme immobilization has already been reported in a number of studies using different methods and matrices which can be generally sorted in four different categories which are (i) adsorption, (ii) covalent binding, (iii) entrapment, (iv) cross-linking or combinations of one or more of these techniques (Illanes *et al.* 1990; Ovsejevi *et al.* 1998; Yang and Bednarcik 2001; Albayrak and Yang 2002a; Albayrak and Yang 2002b; Ladero *et al.* 2003; Pessela *et al.* 2003; Mariotti *et al.* 2008). One of the beneficial effects resulting from binding the enzyme to a support matrix is the stabilization of its tertiary structure making it more robust to environmental changes such as pH, temperature and organic solvents (D'Souza 1999). Moreover, the main advantage of immobilized enzyme systems is the easier recovery of product and enzyme enabling continuous operation and multiple reuse of the enzyme (Krajewska 2003). Immobilization of ß-galactosidase is one of the best studied immobilized enzyme systems and some of the immobilized-enzyme based processes could even be successfully implemented in food industry (Grosová *et al.* 2008). Still,

up-scaling is made difficult by the high costs of material and by limiting factors of the currently used immobilization strategies such as leakage, desorption or diffusion constraints (Wang and Chao 2006). An alternative approach for binding the enzyme on a suitable support was found in the modification of proteins using recombinant DNA technology for subsequent immobilization of the protein via a substrate binding domain. Cellulose binding domains are the most intensively studied protein domains, which have already been used for the isolation and purification of proteins as well as for immobilization purposes. However, over the last decade the chitin binding domain of *Bacillus circulans* WL-12 chitinase A1 (ChBD) has gained increased attention (Chong *et al.* 1997; 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 very useful tool for the immobilization of cells and enzymes (Blank *et al.* 2002; Chern and Chao 2005; Wang and Chao 2006; Chiang *et al.* 2009). In food-related processes not only the immobilized enzyme but also the support matrix has to meet special requirements which are non-toxicity, biocompatibility and biodegradability (Krajewska 2003). Chitin and its derivatives have proven to provide all those health and environmental concerns as well as all other common characteristics of currently used support matrices such as high affinity to proteins, availability of functional groups, mechanical stability and rigidity amongst others. Besides, chitin is a natural polymer and one of the world's most plentiful, renewable organic resources what makes it cheap and easily available for immobilization purposes in industrial scale (Dutta *et al.* 2004).

The aims of this study were to fuse the *LacA* gene of *Bacillus licheniformis* DSM13 with the chitin binding domain (ChBD) of *Bacillus circulans* WL-12 chitinase A1 and to establish an immobilized enzyme system using chitin as support matrix. Furthermore, the soluble as well as the immobilized recombinant fusion protein were purified and biochemically characterized.

2 Materials and Methods

2.1 Chemicals

All chemicals were purchased from Sigma (St. Louis, MO., USA) unless otherwise stated. Restriction enzymes, as well as calf intestine phosphatase (CIP) and T4 DNA Ligase were obtained from New England Biolabs (Frankfurt, Germany) whereas *Pfu* DNA polymerase was from Promega (Mannheim, Germany). Isopropyl-ß-D-galactopyranoside (IPTG) and agarose were purchased from Roth (Karlsruhe, Germany). The chitin beads used for the immobilization of the ß-galactosidase were obtained from New England Biolabs. According to the manufacturer they were prepared from emulsified chitosan and subsequently acetylated to chitin.

2.2 Strain and culture conditions

Bacillus licheniformis DSM 13 was obtained from the culture collection of the Suranaree University of Technology and was grown aerobically on nutrient broth (NB) media (5 g/l peptone, 3 g/l meat extract, 10 mg/l $MnSO_4$) at 37°C. For sub-cloning and therewith chemical transformation of ligated reaction products the strain *E. coli* DH5α (Invitrogen Corporation, Carlsbad, CA, USA) was used. *E. coli* TOP 10 (Invitrogen) was used as expression host. Both strains have a mutation in the *lacZ* gene encoding for *E. coli* ß-galactosidase what enabled the selection of positive clones by blue-white screening. *E. coli* strains were grown in Luria Bertani (LB) medium (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl) containing 100 µg/ml ampicillin for clone selection and plasmid maintenance.

2.3 DNA amplification and cloning of *ChBD*

All described cloning steps were carried out at the Suranaree University of Technology (SUT) in Nakhon Ratchasima, Thailand. The chitin binding domain of chitinase A1 from *Bacillus circulans* WL-12 was amplified from the pTXB1– vector of the Impact Kit purchased from New England Biolabs (NEB; Frankfurt,

Germany), which was originally manifested for the single-step purification of recombinant proteins (Chong *et al.* 1997; Chong *et al.* 1998). The expression vector pOJ01 was obtained from the Department of Food Biotechnology, University of Applied Life Sciences and Natural Resources, Vienna. The vector was a result of previous work on the pFlag-CTS vector from Sigma-Aldrich (oral communication Onladda Juajun) containing the *LacA* gene of *Bacillus licheniformis* DSM 13 in its multiple cloning site.

Table 1. Primer sequences for the amplification of the chitin binding domain (ChBD). Underlined nucleotides represent the respective restriction sites.

Primer	Sequence (5' --> 3')
ChBD fw	CTGTGCTCGAGACGACAAATCCTGGTGTATCCGCTTGGC
ChBD rv	CTGTGCGGTACCTCATTGAAGCTGCCACAAGGCAGG

The degenerated oligonucleotides were designed based on the given sequence for *ChBD* from pTXB1 and for a subsequent direct fusion of the ChBD to the *LacA* gene in the pOJ01-vector. The restriction sites *Xho*I and *Kpn*I were introduced to the forward and the reverse primer, respectively (**Table 1**). For repression of His-Tag and flag expression a stop codon was added downstream of *ChBD* so that any interaction could be excluded. The primers were obtained from Sigma (Korea) and PCR amplification was carried out in a Peltier thermo cycler PTC-200 using 6 U of *Pfu* DNA polymerase. The total reaction volume of 100 µl contained 200 µM of each deoxynucleotide triphosphate, 0.5 µM of each primer, 100 ng of diluted plasmid DNA and 10 µl of 10x *Pfu* buffer. The PCR was performed under standard conditions (listed in **Table 2**) and the resulting 159 bp PCR product was visualized by gel electrophoresis (2 % agarose gel containing 1 µg/ml ethidium bromide in 1xTAE (Tris acetate, EDTA) buffer). The amplified *ChBD* gene was purified using the Wizard® SV Gel and PCR Clean-Up System (Promega), double-digested with *Xho*I and *Kpn*I, treated with 1 U of calf intestine phosphatase (CIP) and purified again on a 2 % agarose gel. The vector pOJ01 was double-digested using the same restriction enzymes and purified on a 1 % agarose

gel. The digested fragments were ligated for 16 h at 16 °C using 200 U of T4 DNA Ligase. Positive clones were selected on LB-Amp plates and the new vector pCM01 was confirmed by restriction digest of the extracted plasmid-DNA with *BamH*I followed by agarose gel electrophoresis and DNA sequencing (Macrogen, Korea).

Table 2. PCR conditions for the amplification of the chitin binding domain

Step	Temperature	Time	Cycles
Initial denaturation	95 °C	2 min	1
Denaturation	95 °C	45 sec	30
Annealing	55 °C	45 sec	
Extension	72 °C	30 sec	
Final Extension	72 °C	10 min	1

2.4 Expression of the ß-gal fusion protein

E. coli TOP 10 carrying the recombinant pCM01 were grown in TB medium containing 100 µg/ml ampicillin at 37°C until the optical density measured at 600 nm (OD_{600}) reached 0.6. Gene expression was induced at optimized conditions using 0.01 mM IPTG at 25°C and cultures were further incubated for 17 h until an OD_{600} of 20 was reached (see A.4.3). The cells were harvested at 4°C by centrifugation (4000 g, 20 min) washed twice with 50 mM sodium phosphate buffer (NaPP; pH 6.5) and disrupted using a French Press (Aminco, Maryland, USA). Cell debris was removed by ultra-centrifugation (15000 g, 4°C) and the obtained crude extract was frozen at -20°C.

2.5 Protein purification

The purest form of the immobilized ß-galactosidase was obtained by the immobilization step itself due to the strong and selective binding of the chitin binding domain to its substrate chitin. However, a successful purification to regain the fusion enzyme (ß-gal-ChBD) in its free form was prevented by the fact that elution was only possible at a pH < 3 (Chern and Chao 2005). In preliminary

assays it was shown that the enzyme denatures at this pH and ß-galactosidase activity could not be restored (see A 4.2.3). Since His-Tag expression had been disabled by the introduction of a stop codon after the *ChBD* gene sequence, purification of the free fusion enzyme was realized using affinity chromatography (ÄKTA Prime system, GE Healthcare, Biosciences, Uppsala, Sweden) followed by a buffer exchange step and gel filtration (ÄKTA Explorer system, GE Healthcare). For affinity chromatography p-Aminobenzyl-1-Thio-ß-Galactopyranose (ABTG) media from Sigma (Frankfurt, Germany) was used. The column was equilibrated in buffer A (50 mM NaPP, pH 6.5), the crude extract loaded at a flow rate of 30 cm h^{-1} and eluted at the same rate with a linear gradient from 0 to 100 % buffer B (50 mM NaPP, 2 M NaCl, pH 6.5) over 15 column volumes (CV). The fractions with the highest specific activity were pooled, concentrated by membrane centrifugation (Amicon Ultra-15 Cell, Millipore; Billerica Massachusetts, USA) and transferred into the gel filtration buffer (20 mM NaPP, 150 mM NaCl). The second step of purification was realized using Superose 12 prep grade media (GE Healthcare) equilibrated in gel filtration buffer. The flow rate was set at 40 cm h^{-1} and again the fractions with the highest specific activity were pooled and desalted for further analysis.

2.6 Immobilization of the fusion protein on chitin

The chitin beads (New England Biolabs, Frankfurt, Germany) were the most appropriate chitin source for the immobilization of the recombinant enzyme via its chitin binding domain (ChBD) (see A 4.2.4). The capacity of the chitin beads were found to be approximately 5 mg of protein hybrid per ml of chitin bead bed (Chern and Chao 2005). Furthermore, preliminary tests were performed measuring ß-galactosidase activity of several dilutions of the bound enzyme. Reproducible results could only be obtained of those bead suspensions in dilutions ranging from 1:5 to 1:20 (see A 4.2.5).Therefore certain dilutions of the crude extract (1:1-, 1:10- and 1:100) were added to the chitin beads, to obtain the appropriate enzyme dilutions needed for the characterization of the recombinant ß-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 10 ml of the respective dilution of the crude extract (1:1, 1:10 or 1:100). The different enzyme-bead suspensions were shaken at 4°C for at least one hour to obtain sufficient binding of the protein hybrid. The supernatant was separated from the bead bed by centrifugation and the chitin beads were subsequently washed twice 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 was calculated from the protein concentration measured in the crude extract and in the supernatant after the enzyme had been immobilized on the chitin beads.

2.7 Characterization of the bound and the unbound enzyme

2.7.1 Molecular mass determination and activity staining

The purified fusion protein was analyzed via PAGE in terms of molecular mass and its activity using the PhastSystem unit (Amersham, Uppsala, Sweden) with precast polyacrylamide gels (PhastGel 8-25, Amersham) and subsequent staining. For the sodium dodecyl sulfate polyacrylamide gel electrophoresis (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. On the native polyacrylamide gel a second lane was loaded with the purified protein for subsequent activity staining. Protein bands on the SDS-PAGE and the native PAGE were visualized with Coomassie blue staining whereas the activity of the fusion enzyme in its native form was visualized with the substrate ß-methylumbelliferyl-ß-D-galactopyranoside (3.5 mg MUG in 1 ml 50 mM NaPP, pH 6.5). The second lane of the native PAGE was therefore separately incubated with 1 ml of the substrate at room temperature for 20 minutes and protein bands displaying enzyme activity were detected under UV light releasing fluorescent 4-methylumbelliferone.

2.7.2 Protein determination

Protein concentration was determined using the Bradford Assay (Bradford 1976). 20 µl of diluted enzyme sample were incubated 15 min with 1 ml Bradford Reagent and absorption at 595 nm (OD_{595}) was subsequently measured on the spectrophotometer. With the help of a calibration curve the protein concentration of the soluble ß-gal-ChBD was calculated from the OD_{595} in the linear range between 0.3 and 0.6. Protein concentration of the immobilized form could not be determined using Bradford.

2.7.3 Activity determination

Besides blue-white-screening and activity staining, the hydrolysis activity of the fusion enzyme in its free as well as in its immobilized form was determined with the synthetic substrate *o*-nitrophenyl-ß-D-galactopyranoside (*o*NPG), displayed in **Figure 1**, as well as with the natural substrate lactose (**Figure 2**). All measurements were performed at least in duplicate. Activity of the free enzyme was determined in units per ml of protein solution whereas for the immobilized enzyme activity was calculated in units per gram of chitin beads.

2.7.3.1 ß-galactosidase assay with chromogenic glycoside

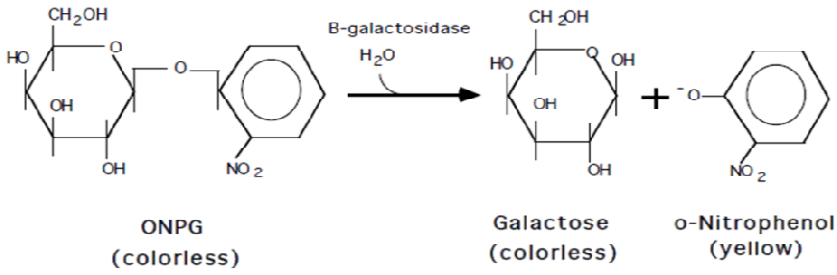


Figure 1. Hydrolysis of *o*-Nitrophenyl-ß-D-galactopyranoside

For activity determination with the chromogenic *o*NPG 480 µl of a 22 mM *o*NPG solution in 50 mM NaPP (pH 6.5) were pre-warmed at 30°C in an Eppendorf thermo mixer compact (Hamburg, Germany). The reaction was initiated by the addition of 20 µl appropriate enzyme dilution (in 50 mM NaPP, pH 6.5) and the

reaction mixture was incubated for 10 minutes at 30°C and an agitation speed of 600 rpm. To stop the reaction 750 µl of sodium carbonate (Na_2CO_3; 0.4 M) were added and the amount of formed *o*-nitrophenol (*o*NP) was determined measuring the absorbance at 420 nm. One unit of *o*NPG activity was defined as the amount of enzyme releasing 1 µmol of *o*NP per minute under the described conditions (Nakayama and Amachi 1999).

2.7.3.2 ß-galactosidase assay with lactose

In this assay 20 µl enzyme solution were used for the initiation of the reaction in 50 mM NaPP-buffer containing 600 mM lactose. After 10 minutes of agitation at 30°C the reaction was stopped by heat inactivation at 99°C for 5 minutes. The immobilized ß-galactosidase fusion enzyme was inactivated by a pH-shift from 6.5 to 10.5 as conducted in the *o*NPG assay with addition of the sodium carbonate. This was realized adding 20 µl of 1 M NaOH. One unit of enzyme activity was defined as the amount of ß-galactosidase releasing 1 µmol of D-glucose per minute what was determined by the GOD/POD-assay. Thereby the sample was cooled down (in the case of heat-inactivation) and a new reaction was started by mixing 60 µl of the sample with 600 µl of a solution containing GOD (94 µg/ml), POD (6.1 µg/ml), 4-aminoantipyrine (157 µg/ml) and phenol (1.95 % v/v) in 0.1 M potassium phosphate buffer (pH 7.0). The absorbance at 546 nm was measured giving the amount of D-glucose released after an incubation of 45 minutes at room temperature in the dark (Kunst *et al.* 1988).

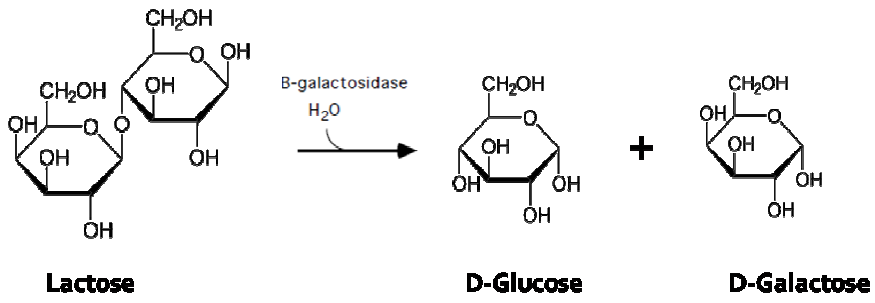


Figure 2. Hydrolysis of lactose catalyzed by ß-galactosidase

2.7.4 Steady-state kinetic measurements

For determination of the steady-state kinetic constants (V_{max}, K_m, k_{cat}) for lactose and *o*NPG hydrolysis the activity of the free and the immobilized enzyme were measured under standard conditions (30°C, 10 min, in 50 mM NaPP-buffer, pH 6.5). The substrate concentrations were varied from 0.01 to 22 mM for *o*NPG 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.

2.7.5 Determination of pH and Temperature profiles

The pH and temperature optima of the bound and unbound protein hybrid were as well determined for lactose and *o*NPG hydrolysis, respectively. Sodium hydroxide was used to adjust the pH in the range from 4 to 9 in Britton Robinson buffer (20mM boric acid, 20 mM acetic acid, 20 mM phosphoric acid) and the activities were measured in steps of 0.5 pH units under standard conditions (30°C, 10 min, 22 mM *o*NPG or 600 mM lactose). The temperature optima were as well determined under standard assay conditions, keeping the pH constant at 6.5 but varying the temperature from 20 to 70°C. pH stability was tested by incubating the enzyme samples at different pH values in Britton Robinson buffer at 30°C up to 5 days and measuring the remaining activity of each sample after certain time intervals under standard assay conditions with *o*NPG. For assessing temperature stability the enzyme samples were incubated in 50 mM sodium phosphate buffer, pH 6.5 at certain temperatures and residual activity was as well determined using *o*NPG as the substrate. The assayed time intervals ranged up to 24 and 72 hours for the free and the immobilized enzyme, respectively.

2.7.6 Effects of various chemicals on enzyme stability

For studying the possible effects of various chemicals on the ß-galactosidase-ChBD fusion protein samples of free and immobilized enzyme were incubated at 4°C up to 8 days with different concentrations of the desired chemical. Representatives of different groups of chemicals were chosen to test their influence on the recombinant enzymes. EDTA as a chelating agent mainly forms

complexes with divalent cations such as Mn^{2+}, Ca^{2+} and Co^{2+} amongst others. Dithiothreitol and ß-mercaptoethanol were chosen as reducing agents therefore influencing the formation of disulfide bridges, whereas Urea represents the group of chaotropic agents. From the salts only $MgCl_2$ was tested since it was known to enhance ß-galactosidase activity of *Lactobacillus* (Nguyen *et al.* 2006). To assess any activating effect of the chosen chemicals the activity was determined under standard assay conditions with the substrate *o*NPG in the presence of these agents. Subsequently, the samples were incubated with the respective chemical agents and enzyme activities were determined in certain time intervals. Additionally, the effect of 10 mM EDTA on the stability was tested at an incubation temperature of 42°C.

2.7.7 Recycling test of the immobilized enzyme

To assess the possibility of recycling of the immobilized enzyme, two samples of bound ß-gal-ChBD were assayed repeatedly under standard conditions with the substrate *o*NPG. Other than in the standard assay the reaction was not stopped immediately after 10 minutes of incubation in the thermal mixer. Instead, the reaction mixture was centrifugated (4000 g, 2 min) to separate the supernatant from the beads with the bound enzyme. 400 µl of supernatant were taken off and mixed with 750 µl of 0.4 M Na_2CO_3 to stop the reaction. The remaining 100 µl of chitin bead suspension were again used in the standard activity assay using 480 µl *o*NPG. The samples were centrifugated after 10 min of reaction time, 480 µl of supernatant were mixed with 750 µl 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. Up to six reactions were performed, each using 100 µl of the remaining bead suspension from the previous reaction and residual activities of the immobilized enzyme were determined, respectively. Between the reactions all samples were kept on ice.

3 Results

3.1 Cloning and Expression

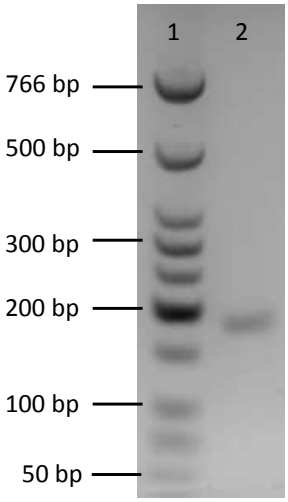


Figure 3. Gel electrophoresis of the amplified ChBD. A 2 % agarose gel was used for visualization of the PCR product (lane 2) and a low molecular weight standard ladder (lane 1)

The chitin binding domain was amplified from the pTXB1-vector (Impact Kit, New England Biolabs). As visualized by agarose gel electrophoresis (**Figure 3**) the PCR product had a size of 159 bp confirming a successful amplification of the chitin binding domain (ChBD). Calculation using Expasy translate tool (http://www.expasy.ch/tools/dna.html) yielded in 52 amino acids. Since the ChBD in the pTXB1-vector was derived from *Bacillus circulans* WL-12 chitinase A1 consisting only of 45 amino acids (Hashimoto *et al.* 2000), the result suggests that the residual seven amino acids represent a small linker domain which might ease the construction of a C-terminal ChBD fusion protein and prevent any mutual interaction.

3.2 Purification of the fusion enzyme

As previously described affinity chromatography followed by buffer exchange using filter membranes (Amicon Ultra-15 Cell, Millipore) and size exclusion chromatography was used to obtain the purest form of the soluble fusion enzyme. As represented in **Table 3** a 4-fold isolation of the fusion enzyme and an overall yield of 13% was achieved. The specific activity of 129 units/mg protein was determined under standard assay conditions with *o*NPG as substrate and the purification yield was further judged by SDS-PAGE analysis.

Table 3. Summary of purification data of enzyme isolation

purification step	total activity (units)	total protein (mg)	specific activity (units/mg)	purification factor	Recovery (%)
crude extract	1841	60.8	30.3	1.0	100
affinity chromatography	922	14.3	64.7	2.1	50
gelfiltration	232	1.8	129.2	4.3	13

3.3 Properties of the fusion enzyme

SDS PAGE and native PAGE analysis of the purified enzyme were performed to reveal the purification yield of the enzyme on the one hand and to determine size and molecular mass on the other hand. On both PAGEs only one protein band was observed suggesting a successful purification of the fusion enzyme. It was calculated with Expasy Tools (http://au.expasy.org/tools/protparam.html), that the recombinant ß-galactosidase consists of 738 amino acids resulting in a calculated molecular mass of 84,768 kDA what was confirmed by SDS PAGE analysis. 79 kDa are derived from the native ß-galactosidase of *Bacillus licheniformis* DSM 13 and approximately 5 kDa represent the small chitin binding domain.

In the native PAGE a band of about 170 kDa was found (**Figure 4B,** lane 1 and 3). Activity staining with MUG confirms the band as ß-galactosidase since MUG was converted into fluorescent 4-methylumbelliferone. On the denaturing SDS PAGE one band with half of the molecular mass from the band observed on the native PAGE was detectable (**Figure 4A**). The result suggests that the

ß-galactosidase of *Bacillus licheniformis* DSM 13 is a homodimer formed by two identical monomers. As a consequence of treatment with SDS-buffer at 99°C the enzyme dissociated into its two subunits of the same size resulting in only one band on the PAGE. The homodimer exhibited ß-galactosidase activity whereas with the monomeric form activity staining was not performed.

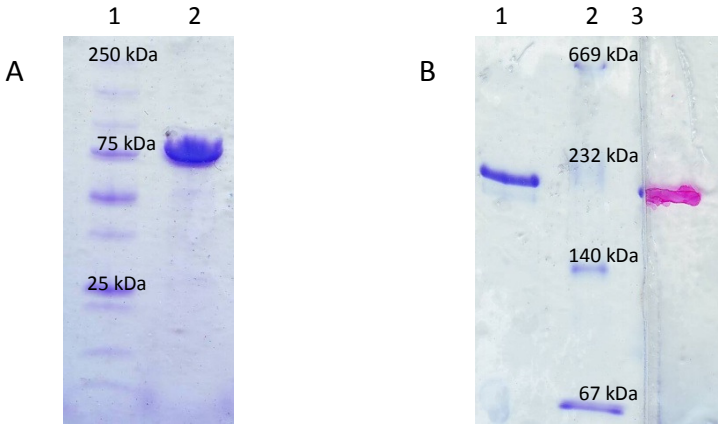


Figure 4. SDS PAGE (**A**) and native PAGE (**B**) of the purified recombinant ß-galactosidase. (**A**) Lane 1: molecular mass marker (Precision Plus All Blue, Bio-Rad Laboratories, Vienna); Lane 2: purified fusion enzyme . (**B**) Lane 1 and 3: purified ß-gal-ChBD stained with coomassie blue and 4-methylumbelliferyl-ß-D-galactopyranoside (MUG), respectively. Lane 2: molecular mass marker (HMW-native, Amersham, GE Healthcare).

3.4 Kinetic analysis of free and immobilized fusion enzyme

Steady-state kinetic constants of the free and the immobilized fusion enzyme are summarized in **Table 4**. Standard *o*NPG and lactose hydrolysis assays were performed and the obtained data were analyzed using Sigma Plot (SPSS Inc.). The Michaelis-Menten regression could be successfully applied for the immobilized enzyme. Kinetic analysis of the free enzyme yielded a higher theoretical reaction velocity (109 µmol min^{-1} mg^{-1}) and lower affinity (11mM) for *o*NPG than for

lactose (V_{max}: 15 µmol min^{-1} mg^{-1}; K_m: 156 mM) stating a higher affinity for the synthetic substrate. k_{cat} and resulting catalytic efficiencies (k_{cat}/K_m) were calculated on the basis of theoretical V_{max} and illustrated as well that *o*NPG is the preferred substrate for the ß-galactosidase. The steady-state kinetic constants obtained with the enzyme in its free and immobilized form can only be compared for lactose hydrolysis. Whereas the K_m-values are nearly the same for both enzymes (156 and 158 mM), the theoretical maximal velocity of the hydrolysis reaction (V_{max}) obtained for the free enzyme is higher (15 compared to 10 µmol min^{-1} mg^{-1} for the immobilized enzyme), as well resulting in a slightly higher catalytic efficiency.

Table 4. Kinetic constants for lactose and *o*NPG hydrolysis. The soluble and immobilized fusion enzyme were assayed independently with varying substrate concentrations and data was fitted to the Michaelis-Menten equation using Sigma Plot.

	free enzyme		immobilized enzyme	
Kinetic parameter	*o*NPG	Lactose	*o*NPG	Lactose
V_{max} (µmol min^{-1} mg^{-1})	109 ± 18	15 ± 0.5	n.d.	10 ± 0.6
K_m (mM)	11 ± 4	156 ± 11		158 ± 21
k_{cat} (s^{-1})	308	42		28
k_{cat}/K_m (mM^{-1} s^{-1})	28.0	0.3		0.2

3.5 Temperature and pH profiles

The pH optima with the substrates *o*NPG (**Figure 5A**) and lactose (**Figure 5B**) were both found to be at pH 7.0, for the free as well as for the immobilized enzyme. The relative activity of the immobilized ß-galactosidase was approximately 20 % higher at its optimum than the free enzyme when *o*NPG was used as substrate and both enzyme formulations showed more than 50 % activity in the pH range from 5.5 to 8 compared to standard assay conditions (pH 6.5). For lactose hydrolysis at least 80 % of the enzyme activity obtained at pH 6.5 was measured in the range from pH 5 to 7.5. The optimum temperature of the free

enzyme was found to be 45°C for both substrates whereas for the immobilized enzyme the optimum was determined to be 50°C for *o*NPG (**Figure 5C**) and 45°C for the substrate lactose (**Figure 5D**).

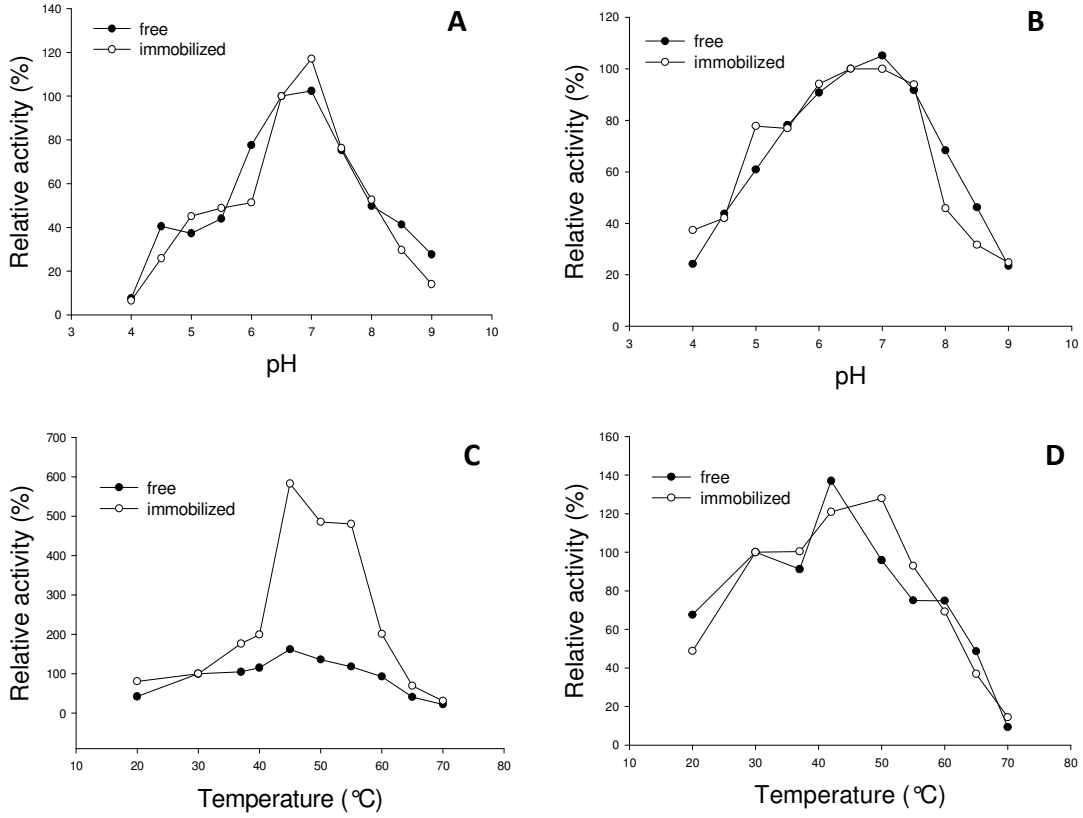


Figure 5. pH and temperature optima of the free and the immobilized ß-galactosidase. Enzyme activity was measured under standard conditions with varying pH using *o*NPG (**A**) and lactose (**B**) as substrates or with varying temperature with *o*NPG (**C**) and lactose (**D**), respectively.

With both substrates the activity of the free enzyme was increased to approximately 130 % at its optimum temperature (45°C) compared to standard assay conditions at 30°C. The relative activity of the immobilized ß-galactosidase measured with *o*NPG as substrate was even increased up to approximately 600 % at its optimum indicating that it is more stable and therefore more active at higher

temperatures than the free enzyme. When lactose was used as substrate, the increase of relative activity was not different from the free fusion enzyme with calculated 130 % compared to standard assay conditions. The result can be explained by the event of end product inhibition in the case of lactose hydrolysis which will be discussed later.

Table 5. Influence of pH and temperature on enzyme stability. Half life times of activity of the free and the immobilized ß-galactosidase were determined at different pH values (**A**) and temperatures (**B**).

(A) Effect of pH on the stability of the enzyme

	Half-life time of activity (h)										
pH	4	4.5	5	5.5	6	6.5	7	7.5	8	8.5	9
free enzyme	0.5	3.6	36	198	368	230	678	239	198	203	203
immobilized enzyme	0.4	0.4	3.9	20	120	134	129	226	214	200	146

(B) Effect of temperature on the stability of the enzyme

	Half-life time of activity (h)			
Temperature (°C)	55	42	25	4
free enzyme	4.6	24	62	92
immobilized enzyme	5.0	9	62	883

For observing the pH stability of the free and the immobilized ß-galactosidase, the enzymes were incubated at different pH values for up to one week at 30°C. Half-life times of activity ($t_{1/2}$) were calculated based on the logarithm of residual relative enzyme activity measured after certain time intervals up to 5 days (**Table 5A)**. The immobilized enzyme generally showed lower stability than the unbound ß-gal and was most stable in the alkaline pH range from 7.5 to 8.5 with $t_{1/2}$ of approximately 9 days. The soluble enzyme showed the same stability in a wider range from 6.0 to 9.0 with a significant maximum at pH 7.0, and was as well more stable compared to the immobilized form at low pH values. The temperature stability of the enzymes was determined by incubating both forms of the enzymes at various temperatures (4, 25, 42 and 55°C) at pH 6.5. Maximal half-life time of ß-galactosidase activity was achieved at 4°C (**Table 5B**) and both

enzymes showed decreasing stability with an increase of temperature. Only at 4°C the immobilized ß-galactosidase showed a10-fold higher stability compared to the soluble form.

3.6 Effects of various chemicals

Activity and stability of the soluble and the immobilized fusion enzyme was tested in the presence of various chemicals representing different groups of chemical reagents (**Table 6A and B**). All chemical supplements enhanced the activity of the enzyme after one hour of incubation, regardless if bound or unbound, though not to any notable extent. Only the addition of 10 mM EDTA had a significant effect on the activity of the free fusion enzyme. An approximately 3 times higher activity was found after one hour of incubation with 10 mM EDTA compared to the sample incubated without any chemical agent. The enhancing effect of EDTA could not be observed for the immobilized ß-galactosidase. However, incubation with 10 mM EDTA, neither any other chemical agent, did not show any stabilizing effect on the fusion enzyme at 4°C.

The influence of 10 mM EDTA on enzyme stability was further tested at 42°C. The recombinant ß-galactosidase is less stable at higher temperatures so that the effect of the chemical is more significant. As illustrated in **Figure 6**, EDTA enhanced the stability of both enzymes, especially of the soluble form which maintained 90 % of its initial activity over 6 days of incubation at 42°C. Without EDTA no activity of the soluble enzyme was detected anymore after 6 days. The immobilized fusion enzyme was generally less stable at 42°C than the soluble form. Even in the presence of EDTA it already lost its activity after 6 days, whereas the bound enzyme without EDTA was already inactivated after 3 days of incubation.

Table 6. Influence of various chemicals on enzyme stability. The soluble (**A**) and the immobilized (**B**) recombinant ß-galactosidase were incubated with different chemical reagents for up to one week.

(**A**) Effect on the free enzyme

			relative activity (%)				
			Incubation time (h)				
chem. agent	name	conc. (mM)	0	1	24	48	168
none			100 ± 3.5	100 ± 3.5	111 ± 3.4	117 ± 1.7	111 ± 6.8
EDTA	Ethylene-Diamine-Tetraacetic acid	0.1		128 ± 5.5	138 ± 0.3	148 ± 1.3	122 ± 3.8
		1		114 ± 1.4	107 ± 1.5	123 ± 0.1	115 ± 0.0
		10		278 ± 2.1	273 ± 2.8	269 ± 2.1	253 ± 4.7
DTT	Dithiothreitol	0.1		142 ± 0.5	134 ± 1.0	143 ± 1.8	123 ± 0.7
		1		141 ± 0.0	139 ± 4.7	146 ± 0.7	138 ± 0.9
		10		137 ± 0.6	144 ± 3.7	139 ± 1.8	147 ± 2.8
Urea		1		132 ± 2.1	133 ± 2.1	143 ± 0.2	141 ± 0.7
		10		132 ± 4.3	121 ± 5.2	111 ± 4.9	127 ± 1.7
BME	ß-mercapto-ethanol	1		130 ± 0.7	131 ± 11.5	128 ± 0.5	129 ± 0.9
		10		146 ± 0.2	136 ± 3.6	134 ± 2.1	125 ± 9.0
$MgCl_2$		1		141 ± 1.4	125 ± 1.4	127 ± 0.9	123 ± 4.6
		10		132 ± 0.1	133 ± 0.3	127 ± 1.2	115 ± 0.0

(**B**) Effect on the immobilized enzyme

			relative activity (%)					
			incubation time (h)					
chem. agent	name	Conc. (mM)	0	1	24	72	144	192
none			100 ± 8.1	100 ± 8.1	142 ± 16.9	168 ± 0.0	128 ± 5.1	99 ± 16.5
EDTA	Ethylene-Diamine-Tetraacetic acid	0.1		134 ± 1.6	165 ± 6.7	144 ± 14.5	147 ± 23.0	177 ± 35.1
		1		142 ± 10.6	151 ± 0.5	155 ± 30.2	150 ± 9.8	147 ± 13.5
		10		134 ± 4.7	91 ± 8.7	173 ± 18.2	111 ± 2.2	125 ± 25.3
DTT	Dithio-threitol	0.1		134 ± 14.1	178 ± 6.7	101 ± 25.4	131 ± 7.2	130 ± 5.6
		1		152 ± 28.0	138 ± 0.4	86 ± 3.7	125 ± 16.9	209 ± 31.1
		10		134 ± 10.4	153 ± 10.1	126 ± 2.4	173 ± 13.1	133 ± 4.2
Urea		1		116 ± 10.9	134 ± 12.8	105 ± 4.4	190 ± 31.0	163.1 ± 18.1
		10		158 ± 35.8	127 ± 13.9	118 ± 0.8	154 ± 3.4	153 ± 31.2
BME	ß-mercapto-ethanol	1		147 ± 2.1	89 ± 14.1	95 ± 13.5	164 ± 24.9	171 ± 21.0
		10		135 ± 27.1	120 ± 10.8	141 ± 7.7	154 ± 28.1	153 ± 31.2
$MgCl_2$		1		123 ± 0.1	146 ± 3.7	149 ± 55.4	141 ± 28.4	115 ± 26.2
		10		156 ± 11.0	98 ± 8.0	100 ± 1.2	122 ± 13.6	167 ± 12.9

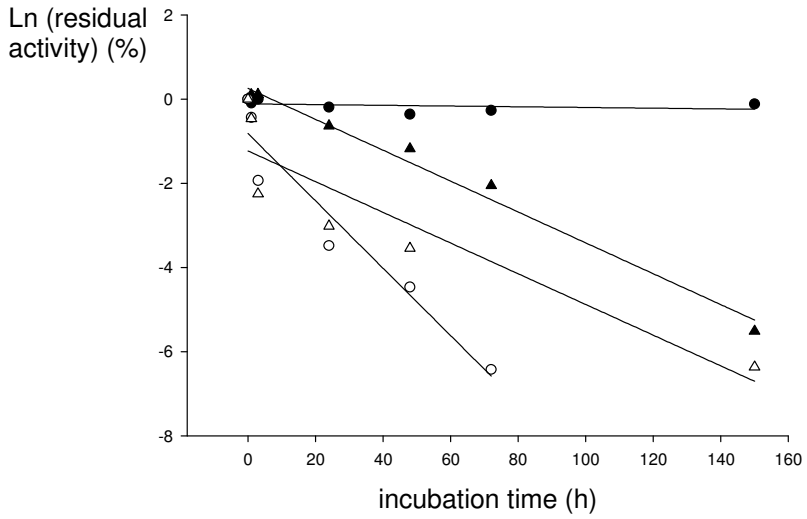


Figure 6. Influence of 10 mM EDTA on enzyme stability. Logarithmic function of residual relative activities of the free fusion enzyme with (▲) and without (●) EDTA and of the immobilized protein with (△) and without (○) EDTA, measured after certain time intervals up to 6 days of incubation at 42°C, are plotted versus incubation time.

3.7 Repeated use of the immobilized enzyme

Recycling of the recombinant ß-galactosidase immobilized on chitin was tested by performing up to six reactions with the same bead. ß-gal 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. The test was realized in duplicate (sample A and sample B) as displayed in **Figure 7.** After three times of recycling there was a significant loss of approximately 35 % of initial enzyme activity observed. However, 60 % of ß-galactosidase activity detected in the first reaction could be maintained over the consecutive cycles, what proves the possibility of reusability of the immobilized ß-galactosidase.

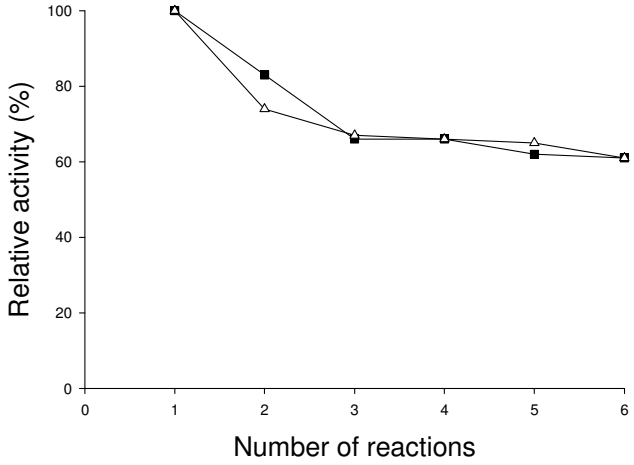


Figure 7. Residual activity of the immobilized ß-galactosidase after repeated use. Two samples (■ sample A, △ sample B) of the bound enzyme were assayed repeatedly in 6 reaction cycles.

4 Discussion

Over the past decades, the use of immobilized enzyme systems has become more relevant for the use in industrial applications with the purpose to improve enzyme properties such as pH and temperature stability, kinetic parameters, reduction of product inhibition etc. (D'Souza 1999; Schallmey M. 2004). ß-galactosidase is one of the most studied enzymes concerning immobilization, where it was mainly used in large scale processes for the hydrolysis and transgalactosylation of lactose in whey permeate (Grosová *et al.* 2008). Recently, ß-galactosidases have become of great interest for the production of galacto-oligosaccharides (GOS) having favorable effects on human health and thus being used as additives in functional foods (Parvez *et al.* 2006). The currently preferred hosts for the production of recombinant proteins include species such as *Bacillus spp.*, *Caulobacter crescentus* and most importantly *E. coli* providing adequate promoter systems (Terpe 2006). However, for food applications only certain organisms can be used

which are "generally regarded as safe" (GRAS) excluding *E. coli lacZ* as the best characterized ß-galactosidase from application in food-related processes. Several ß-galactosidases from *Lactobacillus* and *Bacillus spp.* have been isolated and characterized due to their GRAS status which showed different properties (Chakraborti *et al.* 2000; Nguyen 2006; Halbmayr *et al.* 2008). Moreover, ß-galactosidases from different microbial origins have been immobilized using a variety of matrices and methods. Though up scaling of such immobilization systems has met some limitations such as high costs of material or difficult application in industrial scale (Grosová *et al.* 2008). Therefore, chitin and moreover chitosan as cheap, biodegradable, non-toxic and biocompatible polymers have proven to be very suitable as support matrices in food-related and pharmaceutical applications (Chong *et al.* 1997; Blank *et al.* 2002; Krajewska 2003; Bernard *et al.* 2004; Wang and Chao 2006; Kurek *et al.* 2009). Furthermore, the good availability of the substrate in combination with the strong and specific binding of the chitin binding domain (ChBD) of chitinases suggest its application for the immobilization of enzymes and cells (Chern and Chao 2005; Wang and Chao 2006; Chiang *et al.* 2009).

In this study, ß-galactosidase of *Bacillus licheniformis* DSM 13 was fused to the ChBD of chitinase A1 of *Bacillus circulans* WL-12 using recombinant DNA technology to enable immobilization of the modified enzyme on chitin beads. The chitin binding domain was cloned at the C-terminus of the ß-galactosidase in the pOJ01-vector and expressed in *E. coli* TOP10. The expressed recombinant fusion enzyme showed unchanged or even better ß-galactosidase activity compared to the native enzyme confirming minimal interference of the chitin binding domain with the catalytic activity of the enzyme as it has been already predicted from the analysis of its compact crystal structure by Ikegami *et al*. (Ikegami *et al.* 2000). Depending on the incubation time and protein concentration in the crude lysate up to 6 mg protein could be bound per g chitin beads (data not shown). A similar result has been reported for the ChBD-based immobilization of D-hydantoinase using the same chitin binding domain and chitin beads (Chern and Chao 2005). Furthermore, it was determined that binding efficiency as well as specific activity of the enzyme per gram beads was higher using less concentrated enzyme

solutions for immobilization purposes avoiding a maximal load of the beads. The result was consistent with a study where lipase was bound on chitosan beads by physical adsorption yielding better efficiency at lower loadings (Pereira *et al.* 2003). In any case the recombinant ß-galactosidase showed very strong, nearly irreversible binding to chitin due to the attached ChBD as it was expected. The specificity of the binding enabled at the same time the isolation of the recombinant ß-galactosidase from other *E. coli* host proteins obtaining the immobilized fusion enzyme with high purity. Hashimoto *et al.* further reported that the specific binding of the chitin binding domain of *B. circulans* chitinase A1 is mediated by hydrophobic interactions and controllable by a pH shift (Hashimoto *et al.* 2000). Desorption of the bound fusion enzyme was only possible at a $pH < 3$. At these acidic conditions, the ß-galactosidase of *B. licheniformis* DSM 13 showing better stability at neutral or alkaline pHs, did not exhibit any activity anymore. Therefore purification of the recombinant fusion enzyme for biochemical characterization of its soluble form could not be realized using a chitin based matrix. Instead, affinity chromatography and gel filtration were carried out to isolate the fusion enzyme from the crude lysate (obtaining a specific activity of 129.2 U/mg of protein). SDS and native PAGE analysis revealed that the fusion enzyme forms a homodimer with a calculated molecular mass of 168 kDa. Formation of the homodimer and correct folding of the enzyme was not hindered by the fused chitin binding domain as the fusion enzyme still exhibited ß-galactosidase activity. However, it remained unclear if only the dimeric or as well the monomeric form of the enzyme is active. Furthermore, it would be interesting to study the binding mechanism of the ß-gal-ChBD fusion enzyme with chitin, since it was not obvious, if only one or both chitin binding domains of the homodimeric protein bound on chitin.

The kinetic constants of the soluble and the immobilized fusion ß-galactosidase could be obtained with Michaelis-Menten regression using the substrates *o*-nitrophenyl-ß-D-galactoside (*o*NPG) and lactose. For the soluble enzyme a lower K_m-and higher V_{max} - value obtained with *o*NPG compared to lactose was consistent with previously reported results showing that *o*NPG is the preferred substrate for ß-galactosidases in general (Dickson *et al.* 1979; Kim *et al.* 1997;

Nguyen *et al.* 2006; Nguyen *et al.* 2007). The synthetic substrate is converted with about 10 times higher catalytic efficiency than lactose. Furthermore, the affinity of the recombinant protein to *o*NPG determined with 11 mM is nearly the same as it was reported for the native ß-galactosidase with a K_m-value of 13 mM (data not shown). Also the affinity for lactose is comparable for the native and the recombinant (free and immobilized) enzyme with 169, 158 and 156 mM, respectively. Similar results were also found independently for the reaction rates of lactose hydrolysis where 13 µmol (native enzyme), 15 and 10 µmol (free and immobilized fusion enzyme) of Glucose per min and mg protein were released. Only the reaction rates obtained for *o*NPG conversion found for the native and the recombinant enzyme showed high discrepancy with 299 and 109 µmol *o*NP per min per mg protein. Also the temperature profile differed for lactose in the case of the immobilized enzyme, where maximal activity was measured at 50°C, whereas the soluble enzyme had its optimum at 45°C for lactose and *o*NPG hydrolysis. The better operational stability of the immobilized enzyme at higher temperatures using *o*NPG as substrate has to be remarked. Due to the increased reaction velocity at higher temperatures, activity was increased up to 6 times at 50°C compared to standard assay conditions (30°C). The highest relative activity of the soluble enzyme was only 130 %, signifying that the immobilized ß-galactosidase is more stable in its active form compared to the soluble enzyme. At higher temperatures (65 and 70°C) denaturation of the enzyme takes place maintaining less than 50 % relative activity. For lactose hydrolysis both enzymes showed similar temperature profiles and reached 130 % relative activity at their optima. In general, the catalytic efficiency for *o*NPG and lactose hydrolysis of the ß-galactosidase of *Bacillus licheniformis* DSM 13 is much lower than those found for other ß-galactosidases. Analysis of inhibition constants by Juajun (oral communication) revealed significant inhibition by galactose for lactose hydrolysis ($K_{i,Gal}/K_{m,lac}$ = 0.0055) and to a lesser extent by glucose for *o*NPG hydrolysis ($K_{i,Glu}/K_{m,\ oNPG}$ = 6.073) (data not shown). End product inhibition of ß-galactosidases of different origins has already been reported in a number of studies and inhibition constants were summarized by Jurado in 2002 and by Park and Oh in 2009 (Jurado *et al.* 2002; Park and Oh 2009b). Ladero *et al.* further

reported that immobilization of a ß-galactosidase reduced inhibition by its end product glucose, but still being inhibited by galactose (Ladero *et al.* 2003). Also Pessela *et al.* obtained less inhibition for the immobilized ß-galactosidase on sepabead supports (Pessela *et al.* 2003). Since in our study the activity of the immobilized fusion enzyme showed a much higher increase for *o*NPG than for lactose hydrolysis at higher temperatures, whereas this is not the case for the soluble enzyme, the result of Ladero *et al.* seems to be confirmed.

The pH profiles were unchanged with both substrates for the immobilized enzyme as well as for the soluble form of the fusion enzyme with measured maximal activity at pH 7.0. The result differs from other studies where the pH optimum was shifted by up to 2 steps (Pereira *et al.* 2003) or the pH profile was broadened (Chen *et al.* 2009) due to immobilization or support. However, this was mostly due to the use of a cross-linking reagent, a polyelectrolyte or entrapment where the microenvironment of the enzyme is significantly changed. Chern and Chao as well using the chitin binding domain for immobilization purposes obtained similar results, where pH profiles for immobilized and soluble D-hydantoinase were the same. The immobilized enzyme also had a 10°C higher temperature optimum and even maintained high relative activity at temperatures up to 70°C (Chern and Chao 2005). However, when the soluble and immobilized ß-galactosidase was incubated at different pH values and temperatures, unexpectedly the stability of the immobilized form was not better or even worse compared to the free fusion enzyme. pH stability of the bound ß-galactosidase was better in the alkaline environment suggesting that the chitin beads had some influence at long term storage since the unbound enzyme showed much higher stability in the neutral and acidic environment. Also temperature stability of the immobilized ß-galactosidase differed from results described in other studies. As predicted from increased enzyme activity at higher temperatures in the immobilized form, the ChBD-immobilized D-hydantoinase maintained 90 % of its initial relative activity for more than one week whereas the free form already lost 70 % after 4 days (Chern and Chao 2005). Higher temperature stability of immobilized ß-galactosidase has also been reported using other binding strategies (Ovsejevi *et al.* 1994; Albayrak and Yang 2002b; Zhang *et al.* 2009) as well as for other immobilized enzymes

(Mateo *et al.* 2007). In this study the immobilized enzyme only showed higher stability at 4°C, with a calculated half life time of more than one month. With increasing temperatures it did not differ from the soluble enzyme, both showing reduced stability at higher temperatures.

The addition of various chemicals did not show any notable effect on enzyme activity except from EDTA, but contrary to the result obtained by Nguyen *et al.* where EDTA strongly inhibited the ß-galactosidases of *Lactobacillus* (Nguyen *et al.* 2006), it significantly enhanced enzyme activity of the recombinant ß-galactosidase of *Bacillus licheniformis* DSM 13. Furthermore the presence of 10 mM EDTA stabilized both, the soluble and the immobilized form. It was reported previously that several cations, such as Na^+, K^+, Mg^{2+}, Ca^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+} had different effects on ß-galactosidases depending on its origin and the substrate used (Kim *et al.* 1997; Nguyen *et al.* 2006; Nguyen *et al.* 2007; Kreft and Jelen 2000). EDTA as a complexing agent reduces metal ion content in the solution thereby affecting enzyme activity. ß-galactosidases of *Lactobacillus* were inhibited by most divalent cations except from Mg^{2+} and Mn^{2+}, which showed to activate the enzyme. This effect was also observed for other ß-galactosidases (Dickson *et al.* 1979; Chakraborti *et al.* 2000), and in most cases Na^+ or K^+ was necessary for enzyme activation depending on the substrate used (Dickson *et al.* 1979; Kreft and Jelen 2000). Juers *et al.* proposed that the metal ions Mg^{2+} and Na^+ play an important role for the catalytic activity and substrate binding of *E. coli* ß-galactosidase, belonging to the same glycoside hydrolase family (GH-2) as the enzyme of *Lactobacillus.* The ß-galactosidase of *Bacillus licheniformis* DSM 13 is a member of GH-42; its activity was not enhanced by Mg^{2+}. Chen *et al.* studied the effect of several cations on a soluble and immobilized ß-galactosidase of *Bacillus stearothermophilus* stating that none of both forms of the enzyme was influenced by the presence of Na^+, Mg^{2+} or K^+. Furthermore, the thermophile ß-gal was inhibited by all of the tested divalent cations, whereas the immobilized form maintained its activity in their presence (Chen *et al.* 2009). In our study, EDTA had a favorable effect on enzyme activity, though only on the free fusion enzyme. The result suggests that EDTA forming a complex with the cations present in the buffer also binds Na^+ which might inhibit enzyme activity of

B. licheniformis ß-galactosidase. Therefore, EDTA significantly enhances its activity, whereas the immobilized enzyme being less influenced by Na^+ as well does not show any change in activity in the presence of EDTA. Only long term storage of both enzymes with EDTA also affected the immobilized ß-galactosidase being slightly more stable when incubated at 42°C. However, the soluble enzyme generally showed much better stability at 42°C. In the presence of EDTA it maintained 90 % of activity compared to a total loss of enzyme activity when incubated without EDTA for 6 days. Still, the major advantage of immobilization is its reusability, what could successfully be proved in our study. The ß-galactosidase immobilized on chitin beads was assayed repeatedly in 6 reaction cycles and maintained 60 % of its initial activity. It was significant that most of its activity (30 %) was lost in the first 3 reaction cycles suggesting that the enzyme settled down to an equilibrium retaining its activity over the following reaction cycles. Another explanation is a loss of enzyme due to handling. Since the ChBD is known for its strong binding character, detachment of the enzyme from the chitin beads can be excluded. It is more likely that the chitin beads themselves were lost in the supernatant after centrifugation.

5 Conclusion

ß-galactosidase has already been proven to work properly in several immobilization systems, however, depending on the strategy used mostly showed reduced activity, desorption or leakage after several reaction cycles (Grosová *et al.* 2008). Although cross-linking reagents like glutaraldehyde showed to improve binding efficiency and operational stability of ß-galactosidase, in most cases its activity was also reduced significantly compared to the free form due to the denaturing reagent (Illanes *et al.* 1990; Carrara and Rubiolo 1994; Grosová *et al.* 2008). Covalent binding on chitosan gel membranes often had inhibitory effects on enzyme activity resulting from structural, diffusion limitation-related and micro-environmental factors, the latter mainly causing pH-dependant inhibition due to the electrostatic potential of the poly-cationic support (Krajewska and Piwowarska 2005). In contrast, the immobilization of enzymes via the chitin

binding domain has never been reported to reduce enzyme activity (Bernard *et al.* 2004; Chern and Chao 2005; Chiang *et al.* 2009). Chitin as uncharged natural polymer and ChBD having a compact structure do not seem to influence the catalytic activity of the enzyme. Moreover, pH and temperature stability could be improved and multiple reuse of the enzyme enabled increased productivity since time consuming steps for the recovery of product and enzyme were not needed. In this study ChBD-based immobilization of ß-galactosidase did not increase enzyme stability, though the bound enzyme showed higher activity compared to the soluble form due to higher purity and less inhibition by the end products glucose and galactose. The kinetic behavior of the immobilized enzyme obeyed the Michaelis-Menten equation and catalytic efficiency for lactose hydrolysis was not reduced in comparison to its soluble counterpart. Furthermore, the enzyme could easily be recycled and maintained 60 % of initial relative activity after 6 reaction cycles, still being the major advantage of immobilized enzyme systems in general. The specific, strong and nearly irreversible binding of the chitin binding domain of *Bacillus circulans* WL-12 chitinase A1 to chitinous supports could be proven as valuable tool for the immobilization of ß-galactosidase. Chitin provides several merits, being a biodegradable, cheap, non-toxic, and yet under-utilized biopolymer, and thus ChBD-enzyme immobilization on chitin can be considered as suitable system for a variety of food applications such as lactose hydrolysis or GOS production in industrial scale.

APPENDIX

A 1 Immobilization of Enzymes

In the past decades conventional methodologies of chemical processes have been partly replaced by alternative methodologies to allow production, separation and analytical determination of products. One of the most promising strategies being not only efficient and safe but as well resource- and energy-saving is the utilization of enzymes (D'Souza 1999). Enzymes in general find a variety of applications in industry and can be classified into different major sectors according to their application, which are (i) food enzymes, (ii) enzymes for animal feed, (iii) enzymes for technological applications, (iv) enzymes for medical applications and (v) enzymes for diagnostical and clinical applications. Unlike chemical catalysts they accomplish chemical conversions under mild environmental conditions with high specificity discriminating not only between reactions but also between substrates, similar parts of molecules and optical isomers, preventing the formation of undesirable products. As a result manufacturing and material costs could be significantly reduced due to the higher reaction yields and operation at mild environmental conditions. However, practical application of biocatalysts in industry has met several limiting factors, mainly the high cost of isolation and purification of enzymes, their instability, and their sensitivity as well as possible inhibition by products and other substances. Additionally, most enzymes operate dissolved in water not only contaminating the product but making it impossible to recover them for reuse (Mateo *et al.* 2007). Many approaches have been made to overcome these limitations and the most successful of the newly developed technologies was found to be the immobilization of enzymes offering a wider and more economical exploitation of applications in industry.

Immobilization is achieved by fixing the biocatalysts on or within solid supports mimicking a natural mode of occurrence in living cells, where they often operate attached to the cellular membranes. Immobilized enzymes systems proved to have a lot of advantages compared to the free enzyme, most notably their heterogeneity allowing easy recovery of both, enzyme and product. Thus, higher product purity can be achieved what is crucial in food processing and pharmaceutical industry. Furthermore, the structure of the enzyme is stabilized by immobilization making it more robust and resistant to environmental changes allowing multiple reuse of the enzyme and continuous operation of enzymatic processes (Krajewska 2003). Other major advantages include greater control over enzymatic reactions and a greater variety of bioreactor designs.

Depending on the application and nature of the support and the desired biocatalyst binding can be done using either the isolated enzyme or the whole cells or cellular organelles. Most of the enzymes used at industrial scale are extracellular enzymes produced by microbes due to their ease of isolation as crude extract from the fermentation broth being as well more stable to external environmental conditions. However, 90 % of the enzymes produced are intracellular but their economical exploitation is limited by the high cost involved in enzyme isolation. (D'Souza 1999).

A 1.1 Preparation techniques

Biocatalysts can be immobilized using a variety of techniques and supports, the choice depending on the nature of enzyme, nature of substrate and its application. The methods can be broadly classified into four categories: (i) adsorption, (ii) entrapment, (iii) covalent binding and (iv) cross-linking, which are further distinguished by the nature of the fixation. Physical methods, such as adsorption and entrapment are based on weak interactions between the enzyme and the support whereas chemical ones including covalent binding and cross-linking are determined by stronger covalent bonds, therefore being more durable and effective but more expensive and easily worsening enzyme performance (Krajewska 2003). Combinations of one or more techniques are as well applied to

achieve the most appropriate immobilization system for the biocatalyst. Since there is no optimal immobilization condition determined for each enzyme and its application, it is important to choose in a way ensuring maximum retention of activity and operational stability of the immobilized enzyme, which in turn determines productivity and economy of a process.

Under the physical methods, adsorption is the simplest way of preparing immobilized enzymes. No reagents and only a minimum of steps is required, it is cheap and effective, but reversible. Because of the weak interaction forces protein desorption occurs easily so that additional chemical methods such as cross-linking are often required to stabilize the immobilized enzyme. Thereby bi- or multifunctional cross-linking reagents are used, such as glutaraldehyde, dimethyladipimate and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Grosová *et al.* 2008). Their reactive functional groups form covalent bonds with the support matrix allowing inter- and intramolecular cross-linking of the protein (Tanaka and Kawamoto 1999).

Another physical fixation method is the gel-entrapment using natural polymers like agar, agarose and gelatin which are thermo reversal polymerized, or alginate and carrageen where enzymes are entrapped by ionotropic gelation. Synthetic polymers include photo-crosslinkable resins, polyurethane prepolymers and acrylic polymers (Godbole *et al.* 1983). Limitations for the use of gel entrapment are due to diffusional problems causing slow leakage of the enzyme during continuous use as well as to steric hindrance, especially when macromolecular substrates are used. Polyacrylamide gels are the widest spread matrices but cannot be used in food technology in view of its toxicity. Recently developed hydro gels and thermo reactive water-soluble polymers provide an attractive alternative, not only for food-related processes but as well in the field of biotechnology. The microenvironment of such gels with a water content of about 96 % is close to that of the soluble enzyme so that diffusional restrictions are reduced (D'Souza 1999).

Chemical fixation can be achieved by covalent attachment to a water-insoluble matrix or by cross-linking, of which the first is the most extensively studied of the binding techniques. Covalent bonds are formed between functional groups of the

enzymes and the support matrix or the cross-linking agent, respectively. Thereby the active site of the enzyme must be unaffected by the reagents to prevent any loss of activity. The major problem of cross-linking is that enzyme activity is often worsened due to harsh environmental conditions whereas a limitation of covalent binding is the availability of suitable functional groups and the involved high costs of a suitable matrix (Tanaka and Kawamoto 1999).

A 1.2 Properties of immobilized enzymes

For the choice of an appropriate immobilization technique it is crucial to understand the changes in physical and chemical properties the enzyme would be expected to undergo in the course of binding. The surface on which the enzyme is fixed has several vital roles to play such as retention of the tertiary structure of the enzyme or the formation of electron transition complexes. Thus, attachment of the enzyme must involve solely groups outside the active catalytic binding site. Essential groups at the active site can be protected by e.g. a substrate of the enzyme or a competitive inhibitor, as long as the binding is reversible and the protective group can be removed without any loss in enzyme activity (Sengupta and Dasgupta 2006). Due to a different microenvironment imposed upon the enzyme by the support matrix and by the formed products, the kinetic properties as well as the stability of the enzyme can be altered. The retention of the tertiary structure usually leads to increased thermal stability of the enzyme, as well as a general higher stability compared to the soluble enzyme when stored at a certain temperature and pH. Furthermore, the different microenvironment can cause a displacement of the pH optimum up to two pH units and at best, the pH range in which the enzyme can work efficiently is even broadened. Immobilization also protects the enzyme from auto-digestion by proteolytic enzymes.

In spite of the numerous advantages described above, immobilization involves as well several effects which can worsen the performance of the enzyme. Depending on the nature of support, on the reaction taking place and on the design of the reactor, a microenvironment different to the bulk solution and the structural changes of the enzyme often result in lowered activity and increased Michaelis

constant compared to the free enzyme (Goel 1994; Krajewska 2003). The reaction rate can be limited by the diffusion of the substrate from the bulk solution to the microenvironment, especially in the case of entrapment. The substrate passes over the insoluble particle with a specific rate affecting the thickness of the diffusion film and as a result the substrate concentration in the vicinity of the enzyme. The diffusion in turn is limited by the molecular weight of the substrate so that low molecular weight substrates are generally easier converted. However, high molecular weight substrates could be an advantage in some cases, protecting the immobilized enzyme from attack by larger inhibitor molecules (Richmond *et al.* 1980).

A 1.3 Applications of immobilized enzymes

For the implementation of an immobilized enzyme system in a commercial process all beneficial and detrimental effects have to be weighed including economical viability. To date, several immobilized enzyme-based processes have proved economic and have been realized in a larger scale. They have been implemented mainly in food industry and in the manufacture of fine speciality chemicals and pharmaceuticals, particularly for the production of optically pure products, e.g. enantiopure L-DOPA with an annual production of 10^2 t. In food industry, the commercially most important process regarding the scale of manufacture is the isomerisation of glucose to fructose with glucose isomerase. Granules, fibrous or amorphous forms of immobilized enzyme are used to produce up to 10^6 t per year of high fructose corn syrup (Sengupta and Dasgupta 2006). Other important processes in food industry include the aminoacylase system for amino acid production and immobilized lactase for hydrolysis of whey lactose. Present and potential future applications of immobilized enzyme systems other than industrial include laboratory scale organic synthesis, analytical applications (chiefly in biosensors) and medical applications for the diagnosis and treatment of diseases (Krajewska 2003).

A 2 ß-galactosidase

A 2.1 Generalities

ß-galactosidase (EC 3.2.1.23) is a hydrolase enzyme that catalyzes the hydrolysis of D-galactosides, such as lactose, into monosaccharides (**Figure 8**) and is found widespread in nature. ß-galactosidases have been isolated and characterized from a variety of sources including microorganisms, plants and animals, of which microbial ß-galactosidases have attracted most attention for biotechnological applications (Richmond *et al.* 1980).

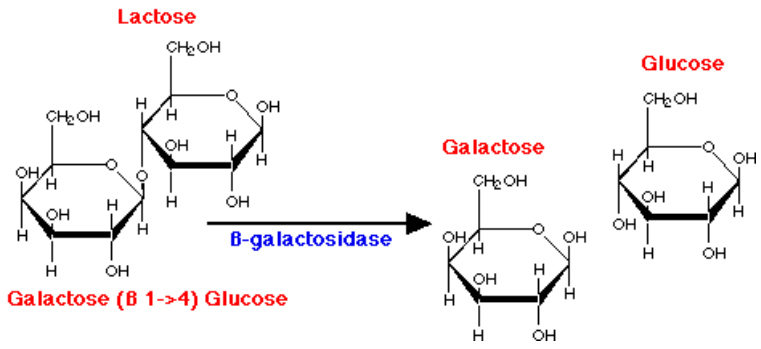


Figure 8. Catalytic activity of ß-D-galactosidase

The investigation of the production, properties and methods of application of this enzyme has been greatly intensified over the past two decades. One of the major applications of the enzyme in industry is the preparation of lactose-hydrolyzed milk and whey, resulting in a number of benefits in the areas health, food technology and environment. Later it was discovered that the enzyme does not only hydrolyze glycosidic bonds but could as well make those bonds between two saccharides by removing water in a so-called transgalactosylation (Juan Ignacio Sanz Valero 2009). In this way galacto-oligosaccharides (GOS) can be formed which are considered as prebiotics because of their functional effects on human health. The choice of suitable enzymatic preparations depends on its properties and the purpose of its application as reviewed by Richmond *et al.* For industrial

production, parameters such as temperature and pH optima are crucial factors which have to be taken into account. Whereas ß-galactosidases from bacterial and yeast origins have nearly neutral pH optima of about 6.0 to 7.0, being more suitable for the hydrolysis of lactose in milk and sweet whey, fungal ß-galactosidases are more active at acidic pH values in the range from 4.0 to 5.0, therefore being preferably used for the hydrolysis of acidic whey and the industrial production of GOS (Richmond *et al.* 1980). At the current moment, the enzymes of the yeasts *Kluyveromycs lactis* and *Kluyveromyces fragilis,* as well as from fungal origins such as *Aspergillus oryzae* are used due to their long history as safe sources of native and recombinant enzymes in food biotechnology and their efficient growth under industrial production conditions (Olempska-Beer *et al.* 2006).

Also some bacterial sources which are "generally regarded as safe" (GRAS) have been used for the catalysis of the transgalactosylation and hydrolysis of lactose such as *Streptococcus thermophilus* (Greenberg and Mahoney 1983), and *Cryptococcus laurentii.* Besides, thermostable ß-galactosidases have gained increased attention due to their higher temperature optima (70°C - 90°C). They have the economic advantage of being tolerant to pasteurization and immobilization but are only poorly produced by their original host. ß-galactosidases were analyzed from strains such as *Sulfolobus solfataricus, Thermotoga maritime* (Kim *et al.* 2004)*, Caldicellulosiruptor saccharolyticus* (Park and Oh 2009a) and *Thermus sp.* (Ohtsu *et al.* 1998). Recently, ß-galactosidases from probiotic strains such as *Lactobacillus spp.* have become more interesting for the production of prebiotic GOS in new carbohydrate-based functional food and thus a number of strains showing good growth on GOS and high ß-gal activity have been isolated and characterized (Nguyen *et al.* 2006; Nguyen *et al.* 2007; Splechtna *et al.* 2007; Halbmayr *et al.* 2008). Some *Bacillus* species are as well on the Food and Drug Administration's GRAS list, most notably *Bacillus subtilis* and *Bacillus licheniformis. Bacillus licheniformis* is a gram-positive, spore-forming and facultative anaerobic soil bacterium, an industrial organism widely used for the manufacture of enzymes, antibiotics, and chemicals that has never been reported to be pathogenic (Salkinoja-Salonen *et al.*

1999). Sequencing of the genome of *B. licheniformis* DSM 13 revealed Bli00447 the gene encoding a ß-galactosidase (*lacA*) (Veith *et al.* 2004). Furthermore, it is taxonomically related to *B. subtilis* 168, the second most important model organism after *E. coli* and its known ability to produce and secrete large amounts of extracellular proteins (20-25g/l) makes it a useful expression host as well for food-related applications (Rey *et al.* 2004; Schallmey M. 2004).

A 2.2 Classification and catalytic mechanism of ß-galactosidase

According to http://www.ncbi.nlm.nih.gov/protein/52787954 ß-galactosidase of *Bacillus licheniformis* DSM 13 (*LacA*) belongs to the glycoside hydrolase family 42 (GH-42) of carbohydrate active enzymes. ß-galactosidases are the most common activity of this family also consisting of α-L-arabinosidase and β-D-fucosidase activity amongst others. Other ß-galactosidases belonging to the GH-42 family were found in *Arthrobacter sp.* (Gutshall *et al.* 1997), *Bacillus subtilis* (Shaikh *et al.* 2007) and *circulans* (Bakken *et al.* 1992), various *Bifidobacteria* (Van Laere *et al.* 2000; Möller *et al.* 2001; Hinz *et al.* 2004), and *Thermus sp*. (Ohtsu *et al.* 1998).

Glycosyl hydrolases (EC 3.2.1-3.2.3), in general, are a widespread group of enzymes hydrolyzing the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. A decade ago 301 glycosyl hydrolases and related enzymes corresponding to 39 EC entries of the I.U.B. classification system have first been classified into 35 families on the basis of amino-acid-sequence similarities (Henrissat 1991). Over the years the number of families of glycoside hydrolases has grown steadily and currently there are 115 families which are available on the continuously updated carbohydrate active enzymes (CAZy) web server (http://www.cazy.org/fam/GH.html).

Glycosidases and glycosyl transferases fall into two major mechanistic classes, those that hydrolyze the glycosidic bond with retention of anomeric configuration and those that do so with inversion. Retaining glycosidases and the transglycosylases use a two-step, double-displacement mechanism in which a

covalent glycosyl-enzyme intermediate is formed and hydrolyzed via oxocarbenium ion-like transition states as shown in **Figure 9** (Withers 2001).

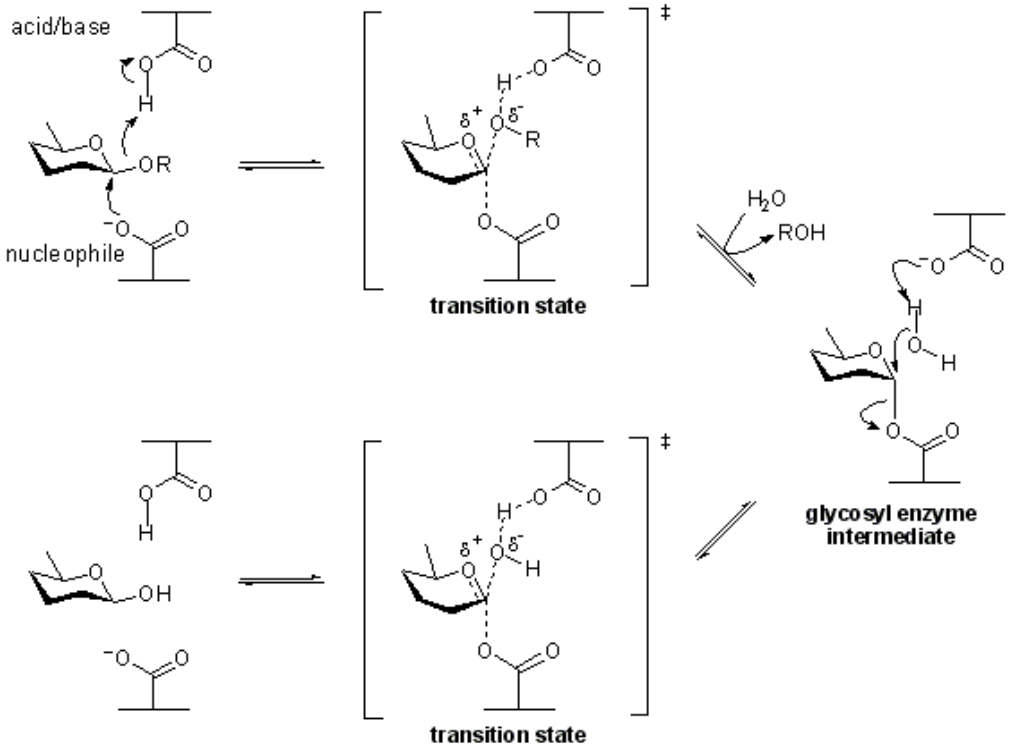


Figure 9. Retaining mechanism of a retaining ß-glycosidase

Two glutamic acid residues (Glu) act as proton donor and nucleophile and are located in the active site of the enzyme, the so called catalytic domain. They are responsible for the hydrolytic activity as following: The catalytic nucleophile attacks the sugar anomeric centre and becomes covalently attached to the galactosyl moiety forming a covalent glycosyl enzyme intermediate. Then the acid/base residue protonates the aglycone oxygen which diffuses away and deprotonates the water molecule attacking the anomeric carbon so that the covalent glycosyl enzyme intermediate is hydrolyzed (McCarter and Withers 1994). The structure of the catalytic domain of retaining glycosidases was determined to be a $(\beta/\alpha)_8$-barrel first found in triose-phosphate isomerase (TIM-barrel) (Henrissat *et al.* 1995). Since the two residues emanate from strands 4 and

7 of the barrel, this clan is sometimes referred to as the 4/7 super family or super family A (Rojas *et al.* 2004). ß-galactosidases belong to the GH-A family and can be further distinguished in terms of their key carboxylic residues which are contained in the active site of the enzyme, separating them into 4 different subgroups: GH-1, GH-2, GH-35 and GH-42.

The main approach for the analysis of these important residues involved in substrate binding and catalysis is the site-specific mutagenesis. Thereby, the residues are systematically substituted by other residues and the effect is studied kinetically and physically. The most extensively studied enzyme is ß-gal of *Escherichia coli* (LacZ) which was found to be a 464 kDa homotetramer (Fowler and Zabin 1978). It is used as a model of enzymes that hydrolyze disaccharides in general. Gebler *et al.* provided evidence that Glu-537 is a nucleophile in the active site of the enzyme (Gebler *et al.* 1992). Furthermore, the central role of Glu-461 in binding and as a general acid/base catalyst in the overall catalytic mechanism could be determined (Juers *et al.* 2009). Other residues that have been found to be important for ß-galactosidase activity are His-418, Tyr-503, Gly-794, Glu-416, His-357, His-391, His-540, Trp-999 and Trp-568. LacZ of *E.coli* belongs to glycosyl hydrolase family 2, which also includes ß-galactosidases of *Kluyveromyces lactis, Lactobacillus spp.*, *Lactococcus and some Streptococcus* species. They show high sequence similarities with ß-glucoronidases of bacterial, animal and human sources (http://www.cazy.org/fam/GH2.html). Glycosyl hydrolase family 1 (GH-1) and family 35 (GH-35) contain only a few number of bacterial ß-galactosidases, isolated from *Pyrococcus woesei* and *Sulfolobus solfataricus* which belong to GH-1 and in GH-35 most notably from *Bacillus circulans* and *Xanthomonas campestris and manihotis*. Human ß-galactosidase as well as most fungal ß-galactosidases (*Aspergillus oryzae, A. niger, Penicillum sp., Arabidopsis thaliana*) were assigned to GH-35.

While the key carboxylic acids have been experimentally defined for the ß-galactosidases from GH-families 1, 2 and 35 previously, for ß-galactosidases belonging to GH-42 these residues have just lately been determined. Shaikh *et al.* identified the catalytic nucleophile Glu-295 in the *B. subtilis* YesZ β-galactosidase

as well being present in other members of the GH-42 family as illustrated in **Figure 10** (Shaikh *et al.* 2007). Di Lauro *et al*. further determined Glu-157 to be the acid/base catalyst of the reaction (Di Lauro *et al.* 2008). The first study about the three dimensional structure of a GH-42 family β-galactosidase was from the extreme thermophile, *Thermus thermophilus* A4 (A4-β-Gal) (Hidaka *et al.* 2002). Other 3D-structures that have been solved include *E. coli* lacZ of GH-2 (Jacobson *et al.* 1994; Juers *et al.* 2000) and of *Penicillium sp.* belonging to GH-35 (Rojas *et al.* 2004). Structural comparison revealed that the environment around the catalytic nucleophile of A4-ß-gal was found to be similar to that of *E. coli* ß-galactosidase (Hidaka *et al.* 2002).

Thermus sp. A4 β-Galactosidase	306	GRFWVM**E**QQPGPVNWAPHNPS	326
B. Subtilis β-Galactosidase	289	RPFWIL**E**TSPSYAASLESSAY	309
C. perfringens β-Galactosidase	299	KPFMMM**E**SSPSSTNWQPVAKL	319
B. circulans β-Galactosidase	309	QPFLLM**E**STPSSTNWQEVSKL	329
G. stearothermophilus β-Galactosidase	297	QPFILM**E**QVTSHVNWRDINVP	317
T. maritima β-Galactosidase	110	GRFWVM**E**QQAGPVNWAPYNLW	130
T. neapolitana β-Galactosidase	308	GRFWVM**E**QQAGPVNWAPYNLW	328
Thermus sp. T2 β-Galactosidase	306	GRFWVM**E**QQPGPVNWAPHNPS	326
H. lucentense β-Galactosidase	305	KPFWVM**E**QQPGDINWPPQSPQ	325

Figure 10. The region around the catalytic nucleophile residue (in bold). Partial multiple sequence alignment of representative family 42 enzymes (adapted from Shaik *et al.*)

Figure 11 shows the hydrolysis and transgalactosylation reaction of ß-galactosidases when lactose is the substrate. According to the mechanism of retaining enzymes as described above, the reaction proceeds in two steps: In the first step a enzyme–galactosyl complex is formed with simultaneous glucose liberation and in the second step the enzyme–galactosyl complex is transferred to a nucleophilic acceptor containing a hydroxyl group. In case of lactose hydrolysis the galactosyl-transfer is mediated to water, resulting in the end products glucose and galactose. If the acceptor is another sugar, di-, tri-, tetra- and higher galactosyl-saccharides are produced (Neri *et al.* 2009). The hydrolysis of lactose

occurs predominantly. Only at high concentrations of lactose the reaction is shifted towards transgalactosylation and the galactose is transferred to the substrate resulting in galactosyllactose (disaccharide). Another transgalactosylation yields galactosylgalactosyllactose (trisaccharide) and so on. Also other sugars such as glucose and galactose can serve as acceptors creating a large pool of so called galacto-oligosaccharides (GOS). Apart from lactose concentration, other factors as well influence the occurrence of the reaction including reaction conditions, temperature, pH and the presence of inhibitors or activators specific for the enzyme (Park and Oh 2009b).

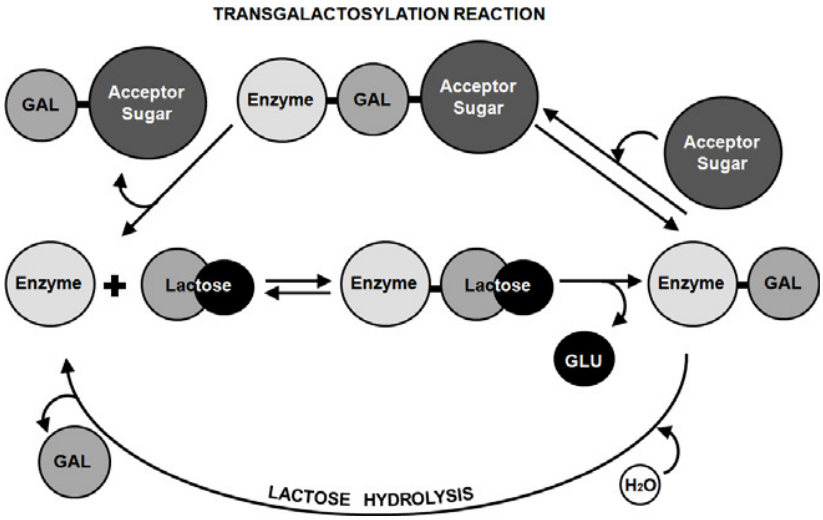


Figure 11. Reaction mechanism of ß-galactosidase
(adapted from Neri *et al*. 2008)

A 2.3 Applications of ß-galactosidases

Lactose hydrolysis has become of major importance due to the high prevalence of lactose intolerance in humans. To be absorbed lactose needs to be hydrolyzed by a ß-galactosidase in the small intestine, also called lactase, which splits lactose into glucose and galactose. These monosaccharides are easily absorbed resulting in an elevation of blood glucose (Savaiano and Levitt 1987). About two thirds of the world population is lactase deficient, meaning that they cannot digest significant

quantities of lactose because of enzyme insufficiency (Vesa *et al.* 2000). In the severe case of lactose intolerance the consumption of lactose may lead to cramps, flatulence, diarrhea and abdominal pain. The addition of microbial ß-galactosidase or lactose hydrolysis in dairy products can circumvent those problems of maldigestion (De Vrese *et al.* 1992). Thus, numerous enzyme preparations from different microbial sources are still investigated as potential supplement in dairy products for the alleviation of lactose intolerance (O'Connell and Walsh 2009).

Another important application of ß-galactosidases in food technology is the lactose hydrolysis in other dairy products such as ice-cream, frozen milks, whey spreads and condensed milk to improve the technological and sensorial quality of dairy foods (Martinez *et al.* 1990). Since lactose causes a sandy, mealy or gritty taste at high concentrations, soluble enzyme preparations of either the acid (*Aspergillus*) or the neutral (*Kluyveromyces*) type ß-galactosidase are used to reduce the lactose content to an acceptable value (Patocka and Jelen 1988; Martinez *et al.* 1990).

Simultaneously to lactose hydrolysis galacto-oligosaccharides (GOS) are formed, preferably at high lactose concentrations. Being indigestible for human digestive enzymes they act as dietary fibers promoting the growth of intestinal bifidobacteria. The predominance of those probiotic bacteria in turn exert favorable effects on human health, such as the suppression of potentially harmful bacteria, strengthening of innate immunity, control of irritable bowel syndrome, prevention of colon cancer, alleviation of food allergy symptoms, and reduction of serum cholesterol (Parvez *et al*. 2006). Due to these beneficial properties, they are currently utilized as low-calorie sweeteners in fermented milk products, confectioneries, breads, and beverages (Park and Oh 2009b).

In cheese industry lactose is considered as waste being one of the main by-products of cheese production. The worldwide production of cheese whey is estimated to be about 4×10^7 tons per year causing several economical and environmental problems (Rech *et al.* 1999). Furthermore, it has been estimated that cheese production increases more than 3% annually and almost half of the entire production is disposed of (Juan Ignacio Sanz Valero 2009). Hydrolysis of

lactose present in whey generates useful products such as sweet syrup which can be used in the dairy, confectionary, baking and soft drinks industries. Lactose from cheese whey can also be used for the manufacture of GOS using fungal ß-galactosidases (Illanes *et al.* 1990; Grosová *et al.* 2008).

A 2.4 Immobilization of ß-galactosidase

Whether the interest is the removal of lactose from milk and whey or the production of prebiotic GOS for functional foods, in either case the process has to be economically feasible. However, commercial enzyme preparations currently used in food industry are limited by several factors as for example low substrate affinity, low temperature optima, inhibition by hydrolysis products etc. resulting in higher demands of enzyme and time and thus increased production costs (Richmond *et al.* 1980). Furthermore the price of ß-galactosidase is rather high compared to the low value of the waste product whey making direct addition of the enzyme economically unacceptable. Therefore immobilized enzyme systems for large scale production have gained increased attention. Several techniques and matrices for ß-galactosidase immobilization have been investigated which are summarized in **Table 7**. They include entrapment, adsorption, cross-linking and covalent binding of the enzyme as already explained above resulting in different, mainly reduced relative activity compared to the free enzyme (Grosová *et al.* 2008). The drop of activity after immobilization highly depends on the method and source of ß-galactosidase used. However, this can be compensated by a number of advantages that have been reported for immobilized ß-galactosidase systems such as are better operational stability, increased half-life time, higher thermal stability and less inhibition by end products (mainly galactose). Moreover, binding the enzyme on a solid support allowed recovery of both, immobilized ß-gal and product, enabling repeated use of the immobilized ß-gal and continuous operation as described earlier (Illanes *et al.* 1990; Carrara and Rubiolo 1994; Nakkharat and Haltrich 2006; Chen *et al.* 2009).

Table 7. Reported ß-galactosidase immobilization systems (adapted from Grosovà *et al.*)

Source of enzyme	Immobilization method	Recovery of activity [%]	References
K.fragilis	covalent binding on corn grits	8	Siso et al (1994)
	covalent binding on cellulose beads	82	Roy & Gupta (2003)
	covalent binding on porous silanised glass modified by glutaraldehyde	90	Sczodrak (2000)
	entrapment in alginate-carrageen gels	-	Mammarella & Rubiolo (2005)
	adsorption in phenol-formaldehyde resin	23	Woudenberg van Oosterom *et al.* (1998)
	adsorption onto bone powder	83	Carpio et al (2000)
K.lactis	covalent binding onto glutaraldehyde-agarose	36-40	Giacomini *et al.* (2001)
	covalent binding onto thiosulfate-agarose	60	
	covalent binding on graphite surface	0.01	Zhou & Chen (2001a)
K.marxianus	covalent binding on oxides supports: alumina, silica, silicated alumina	< 5	Di Serio *et al.* (2003)
E.coli	covalent binding onto glutaraldehyde-agarose	39	Giacomini *et al.* (2001)
	covalent binding onto thiosulfate-agarose	75-85	
	entrapment in liposomes	28	Rodriguez-Nogales & Delgadillo-Lopez (2006)
	covalent binding onto gelatin cross-linked with chromosorb (III) acetate	25	Sungur & Alkbult (1994)
	covalent binding onto gelatin cross-linked with glutaraldehyde	22	
	adsorption on chromosorb-W	-	Bodalo *et al.* (1991)
B.circulans	adsorption onto a ribbed membrane made from polyvinylchloride and silica	-	Bakken *et al.* (1992)
A.oryzae	fibers composed of alginate and gelatin cross-linking with glutaraldehyde	56	Tanriseven & Dogan (2002)
	carbodiimide coupling to alginate beads	76	Dominguez *et al.* (1988)
	entrapment in a spongy polyvinyl alcohol cryogel	-	Rossi *et al.* (1999)
	entrapment in cobalt alginate beads cross-linked with glutaraldehyde	83	Ates & Mehmetoglu (1997)
	microencapsulation in alginate beads	64	Dashevsky (1998)
	encapsulation into gelatin and cross-linking with transglutaminase	8-46	Fuchsbauer *et al.* (1996)
	adsorption on phenol-formaldehyde resin	54	Woudenberg van Oosterom *et al.* (1998)
	adsorption on polyvinylchloride (PVC)	-	Bakken *et al.* (1990)
	adsorption on silica gel membrane		
	adsorption on celite	2	
	covalent binding to chitosan	18.4	Gaur *et al.* (2006
	cross-linked aggregation by glutaraldehyde	13.5	
	covalent binding in polyurethane foams	-	Hu *et al.* (1993)
	covalent binding to the tysolated cotton cloth	55	Albaryrak & Yang (2002c)
A.niger	adsorption on a porous ceramic monolith	80	Papayannakos & Markas (1993)
Chicken bean	immobilized on cross-linked polyacrylamide gel	72	Sun *et al.* (1999)

A 3 Chitin and chitosan – structure and properties

Chitin is a polysaccharide composed from ß-1,4-linked N-acetyl-D-glucosamine (GIcNAc) residues which naturally occurs in exoskeletons and cuticles of many invertebrates and in the cell walls of some fungi, green algae and yeasts. Although it is the second most abundant natural biopolymer after cellulose, much less attention has been paid to chitin, primarily due to its inertness. Also structurally it exhibits great similarity to cellulose, the only difference being the replacement of the C-2 hydroxyl residues by acetamide groups as shown in **Figure 12**. Depending on the source and isolation conditions chitin has a different degree of acetylation and length of chitin molecules varying from 5000 to 8000 GIcNAc residues in crab down to 100 residues in yeast. Furthermore the chitin chains are associated with one another by hydrogen bonds between the >NH groups of one molecule and the >C=O groups of the adjacent chain (Synowiecki and Al-Khateeb 2003). Depending on the arrangement of chains either the more common polymorphic α-chitin is formed, which is composed of antiparallel sheets, or ß-chitin with parallel arrangement of the adjacent chains. A third rather controversial form of γ-chitin has been reported which is formed by parallel chains with every third chain arranged in opposite direction (↑↑↓) (Krajewska 2003). α-chitin has higher stability being composed of stronger fixed hydrogen bonds nearly rendering it impossible for swelling in water and limiting its permeability. ß-chitin can be irreversibly converted to the α-form, is easier to swell and more permeable. Therefore proportions of the two existent forms in the shell strongly influence hardness, permeability, flexibility and solubility (Inmaculada *et al.* 2009) In general, chitin does not dissolve in water, neither in any organic solvent, diluted acid nor base solutions because of its high density of hydrogen bonds so that chemical modifications were applied to form more soluble derivatives. This is mainly achieved by cleaving inter- and intramolecular hydrogen bonds without disrupting the glycosidic linkages (Synowiecki and Al-Khateeb 2003).

Chitosan naturally occurs only in the cell walls of some *Zygomycetes* species and therefore is usually chemically synthesized by *N*-deacetylation of chitin. Due to its

reactive amino and hydroxyl groups emerging from the hydrolysis of the acetamide groups of chitin, chitosan possesses distinct chemical and biological properties (**Figure 12**). As a cationic polyelectrolyte it is soluble in the aqueous acidic media at pH < 6.5 and when dissolved possesses high positive charge on the protonated amino groups so that it adheres to negatively charged surfaces (Krajewska 2003). Due to those physicochemical characteristics along with the biological properties being biodegradable, non-toxic and biocompatible with animal and human cells and tissues, chitosan is mostly the preferred substrate to chitin for a variety of applications. It has been approved ass additive in functional foods in some Asian countries, though it is not yet listed in the General Standard for Food Additives nor has it been authorized as a food ingredient in the EU since no long-term studies of human safety have been reported. In the field of medical applications only the use of a derivative of chitosan, namely chitosan hydrochloride, has been approved and was included in the European Pharmacopeia in 2002 (Inmaculada *et al.* 2009).

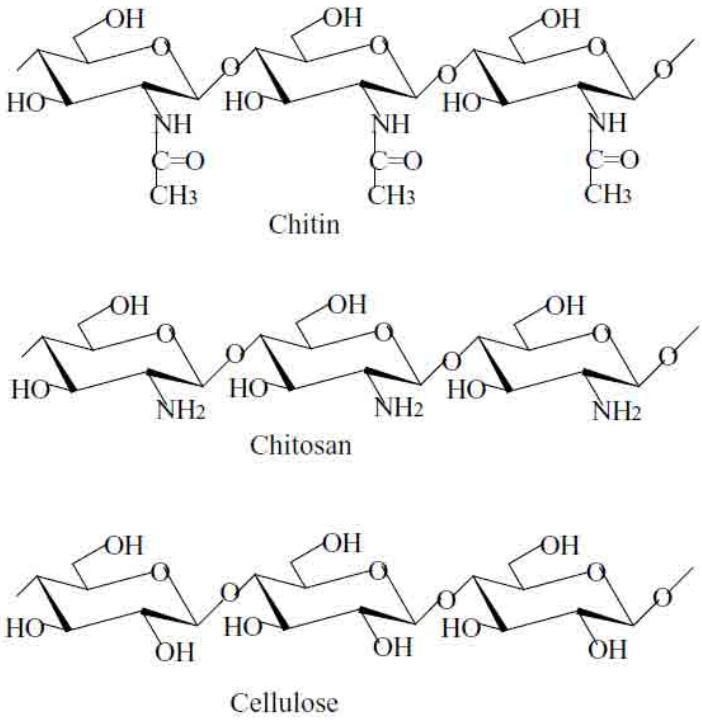


Figure 12. Biochemical structure of chitin and related substrates

A 3.1 Resources of chitin

Commercial chitin is usually produced from crustaceans, mainly because a large amount of waste is available as a by-product of food-processing with about 600 and 1600 million tons obtained from freshwater and marine ecosystems, respectively. Among them shellfish, oyster and squid are the best characterized, their chitin contents being estimated as 2 to 12 % of the whole body mass depending highly on peeling conditions during processing, species, state of nutrition and stage of reproductive cycle (Synowiecki and Al-Khateeb 2003). The type of species is also a determining factor for the form of chitin obtained, with α-chitin being the more exploited form in marine crustaceans and ß-chitin being the product of squid pen extraction. In the United States up to 39 000 tons of crab and pacific shrimp offal are yearly generated and in Canada even up to 50 000 tons with chitin contents of 13 to 24 % and 14 to 42 % of dry weight, respectively. The resulting environmental pollution of shrimp and crab processing led to increased interest in chitin and chitosan production originating from Japan and now being produced worldwide, in the US, Norway, Australia, India and Poland (Pradip Kumar Dutta 2004).

As microbial sources for chitin isolation the mycelia of various lower fungi such as *Allomyces, Aspergillus, Penicillium, Fusarium, Mucor, Rhisopus,* etc. has been reported. Furthermore the cell walls of the mold mycelia contain significant quantities of chitosan (from 2 % up to 60 % of dry weight) and other polysaccharides. The enhanced use of those microorganisms in the industrial production of citric acid, enzymes, vitamins, antibiotics, hormones and others has brought many advantages with it: Fungal cell walls for the utilization of chitin and chitosan have become readily available, cultivation of the fungi was optimized having high growth rates, the substrates (cellulose) are inexpensive and isolation costs for chitin are lower compared to processing of shellfish waste. The most commonly studied microorganism as a source of chitin is *Mucor rouxii* which has been tested for chitin and chitosan production as well as protein content under varying conditions yielding maximal chitin and chitosan contents of 8.9 and

7.3 % on a dry basis, respectively (Synowiecki and Al-Khateeb 1997; Galed *et al.* 2005).

A 3.2 Preparation and characterization of chitin and chitosan

Chitin and chitosan characteristics have a great effect on their properties and hence, on their possible application making a complete characterization of the samples indispensable. The main parameters affecting the polymer properties are degree of deacetylation, molecular weight, polydispersity and crystallinity. For food and medical applications other properties such as ash content (purity), moisture and content of heavy metals, endotoxin and proteins must be determined. The major factors influencing crystallinity and purity as well as polymer chain arrangement of chitin and chitosan, are the origin of chitin (usually type of crustacean) and chitin isolation (Galed *et al.* 2005).

A 3.2.1 Isolation of chitin

Apart from chitin, the crustacean shell consists mainly of protein (30-40 %) and mineral salts (Ca_2CO_3, 30-50 %) and pigments of lipidic nature such as carotenoids, which have to be effectively removed for a successful isolation and purification of chitin. Chitin is extracted by acid treatment to dissolve the calcium carbonate, by alkaline treatment to dissolve the proteins and by a depigmentation step to obtain the colourless product (Acosta *et al.* 1993).

Figure 13 shows a flow sheet of chitin and chitosan preparation from raw material. Typically commercial chitins are prepared by a first step of deproteinization followed by a second step of demineralization resulting in a so called “collapsed chitin” because of the loss in the native structure. Performing the steps in reverse order, the native chain and fibrous structures are retained giving “compacted chitin” (Inmaculada *et al.* 2009) and a higher degree of deacetylation can be achieved by the subsequent steps, both being crucial for the use of chitin as enzyme support or adsorbent. Demineralization is achieved by 1 to 3 h extraction with diluted hydrochloric acid, for deproteinization dilute sodium hydroxide (1 to 10 %) is used at elevated temperatures from 65 to 100°C. The base concentration and base ratio to shells, as well as temperature determine the

effectiveness of protein solubilization. However, prolonged alkali treatment (> 6 h) or the use of too high amounts of hydrochloride for demineralization can lead to chitin depolymerization affecting its crystallinity, which is one of the main parameters with high impact on the properties of chitin, as well as chitosan (Rinaudo 2006). Pigment residues can be removed by extraction with acetone, chloroform, ethyl acetate, ethanol or either mixture. Decolourization is usually carried out by a bleaching treatment with NaOCl or H_2O_2 solutions as well damaging the chitin structure (Synowiecki and Al-Khateeb 2003).

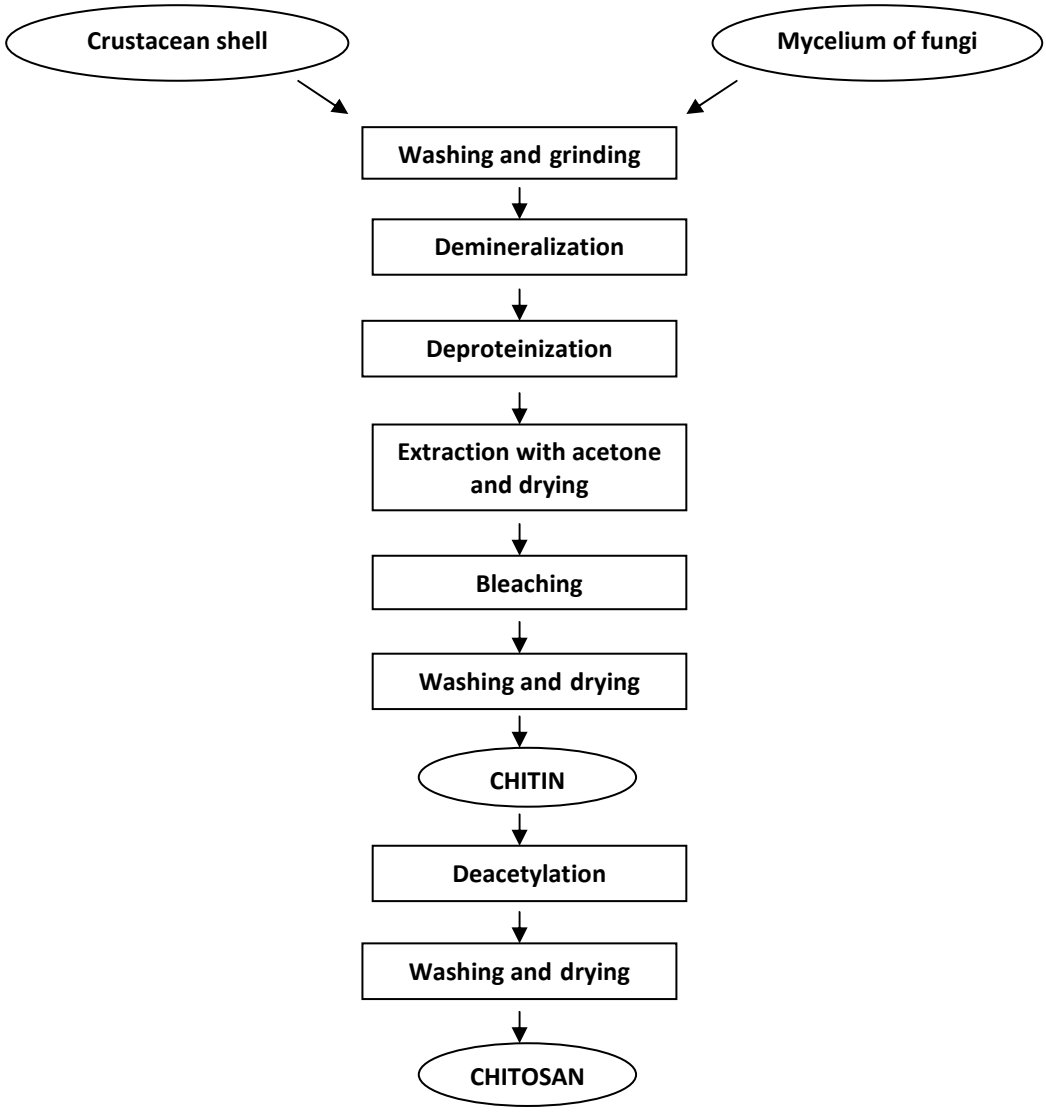


Figure 13. Flow sheet of chitin and chitosan preparation

A 3.2.2 Chitosan production

Chitosan, the major soluble derivative of chitin, is usually prepared by hydrolysis of the acetamide groups of chitin from crab and shrimp offal. It is the simplest chemical modification of chitin, usually conducted by severe alkaline treatment (30-60% w/v sodium or potassium hydroxide solution) at different combinations of temperatures (80-140°C). Alkali concentration, time and temperature have to be strictly controlled because they significantly influence its main physicochemical characteristics such as degree of deacetylation (DD), molecular weight (Mw), molecular weight distribution and distribution of acetyl groups along the polysaccharide chain (Synowiecki and Al-Khateeb 2003), which in turn have a great impact on its properties and hence applications. Chitin has to be sufficiently deacetylated (DD>70 %) to be defined as chitosan and a DD of 80-85 % has to be achieved to obtain a soluble product (Krajewska 2003).

A 3.3 Properties and Applications of chitin and chitosan

The reason that chitin and chitosan are currently receiving a great deal of interest is mainly due to their biological properties, of which the most important are biodegradability, biocompatibility and non-toxicity. Although they are absent from mammals they can be degraded *in vivo* by several proteases, especially by non-specific lysozymes which are present in all mammalian tissues. The products of degradation are non-toxic oligosaccharides of variable lengths, which are either incorporated or excreted. The rate of degradation highly depends on the degree of deacetylation (DD), the distribution of acetyl groups and molecular weight and also affects the biocompatibility of chitin and chitosan. At a low DD, degradation takes place at high rate, what is not favorable due to the accumulation of amino sugars produced evoking an inflammatory response. In general, chitin and chitosan both show good compatibility, though they are not bioavailable since they could not be absorbed after oral administration (Inmaculada *et al.* 2009). LD_{50} of chitosan in laboratory mice was determined to be 16g/kg body weight, close to that of salt and sugar (Singh and Ray 2000). However the low toxicity is only represented by chitosan with a degree of deacetylation higher than 35 %

while a DD under 35 % causes dose dependant toxicity (Inmaculada *et al.* 2009). Chitosan has a higher cytocompatibility than chitin due to the free amino groups their positive charge being responsible for an increased interaction with the cells. It could be shown that chitosan with a high DD strongly stimulated fibroblast proliferation while samples with lower levels of deacetylation showed less activity (Howling *et al.* 2001).

Other biological properties of chitin and chitosan, which make them specifically interesting for pharmaceutical and medical applications, include haemostatic, analgesic and permeation enhancing effects, antitumor, antioxidative and antimicrobial activity and remarkable affinity to proteins amongst others (Dutta *et al.* 2004; Pradip Kumar Dutta 2004). Thus, chitin and chitosan in particular have been successfully employed in drug delivery systems, implantable and injectable systems such as orthopedic and periodontal composites, as wound healing accelerators and blood coagulants, in haemodialysis membranes, as scaffolds in tissue engineering, in artificial skin and as bacteriostatic and fungistatic agents (Singh and Ray 2000).

A 3.3.1 Applications in Industry

The utilization and investigation of chitin is highly limited by its poor solubility and the reactions under heterogeneous conditions are accompanied by various problems such as the poor extent of reaction, difficulty in selective substitution, structural ambiguity of the products, and partial degradation due to severe reaction conditions (Zohuriaan-Mehr 2005). As already mentioned above, more attention has been paid to the modification of the soluble derivative chitosan. Due to its reactive amino and hydroxyl groups, its polyelectrolyte properties, gel forming ability and adsorption capacity it is more versatile allowing the formation of useful derivatives and therefore being more suitable for a variety of applications (Synowiecki and Al-Khateeb 2003).

In food industry chitosan could be successfully introduced as a food preserving agent because of its anti-microbiological action against food spoilage microorganisms and antioxidant properties. Coatings with semi-permeable membranes and films were used to protect fruits and vegetables, fish, oysters,

eggs amongst others. Also for plants chitosan has a protective effect against pathogens inhibiting their growth and inducing resistance to fungal, viral or viroid infections (Synowiecki and Al-Khateeb 2003). On the other hand chitosan can act as a dietary fiber and as functional food ingredient. Fat and cholesterol binding as well as effects on pancreatic lipase activity were reported resulting in a significant reduction in body weight gain or plasma lipid content of humans and animals (Inmaculada *et al.* 2009). Other applications of chitosan include cosmetics where it is added in creams, hair-care products and toothpaste due to its moisturizing and protective effects. In textile industry chitin and chitosan are used for the production of fibers or coatings improving water vapor permeability in textiles and in waste water treatment chitosan showed to possess good sorption capacity and selectivity for the reduction of contaminants such as heavy metal ions (Synowiecki and Al-Khateeb 2003).

A 3.3.2 Chitin and chitosan for enzyme immobilization

For enzyme immobilization purposes chitin and its derivatives are used in form of powders, flakes and gels of different geometrical configurations (Krajewska 2003). Chitosan with its pH dependant solubility and the advantage of forming complexes with anionic polyelectrolytes can be easier manufactured so that chitinous supports are often formed by acetylation of chitosan preparations, as it is described for the chitin beads of the New England Biolabs. Physical bulk gel and bead formation from various chitin solutions and non-solvents were reported by Yilmaz and Bengisu. They found that ethanol is the optimal non-solvent for homogeneous spherical bead formation from chitin solutions. Furthermore, differences in bead morphology, crystallinity, and thermal degradation depended on the achieved balance between attractive hydrogen bonding in the chitin gel network and segment–non-solvent interactions (Yilmaz and Bengisu 2003).

Different chitin and chitosan preparations can have distinctive effects on the immobilized enzymes. Pereira et al described that porous chitosan beads were more suitable for the immobilization of lipases than chitosan flakes (Pereira *et al.* 2003). Zubriene *et al.* immobilized four different hydrolases on chitosan micro particles by covalent binding using glutaraldehyde (GA) as a cross-linking and

activating agent showing that molecular weight and quantity of GA had a significant effect on residual enzyme activity, also depending on the specific enzyme (Zubriene *et al.* 2004). In another study different krill chitin supports were tested for their suitability as immobilization supports. Enzyme activity was influenced by several factors: 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). According to a report from Sjoholm *et al*., the degree of deacetylation (DD) has as well significant effects on the hydrophobic modification of chitosan and, as a consequence, on the activity of the immobilized enzyme. A high DD provided optimal enzyme immobilization properties but in turn required treatment for deacetylation reduced its polymer molecular weight what negatively affected immobilized enzyme activity (Sjoholm *et al.*).

A 3.3.3 Immobilization of enzymes via substrate binding domains

As already mentioned above traditional enzyme immobilization methods meet some limitations concerning their application. Gel entrapment is frequently made difficult by the problem of limited transport of substrates and the mechanical fragility of materials, while physical adsorption has difficulty in stably retaining cells or enzymes, particularly in the presence of hydrodynamic shear (Wang and Chao 2006). To achieve long-standing retention of enzymes chemical modification of support materials followed by fixing the enzymes on the matrix surface via covalent binding is often required. However, chemical treatment is usually harsh to most of the enzymes to be immobilized and frequently increases the probability of more than one functional site of enzymes binding to the matrix surface resulting in heterogeneous preparation of immobilized enzymes (Chern and Chao 2005).

An alternative approach for the immobilization of enzymes is the fusion of a protein with an affinity tag using recombinant DNA technology so that it specifically binds to an unnatural cognate ligand. This approach has shown to provide several advantages, which are: 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. Among the peptide tags previously developed, FLAG, poly-His, c-*myc*, and glutathione *S*-transferase are the most commonly used (Terpe 2003). However, these peptides are not useful for immobilization due to the economic infeasibility of their corresponding ligand materials (Wang and Chao 2006). 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 isolation 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).

Among the substrate binding domains, the cellulose-binding domains (CBD) are the most studied. It has been first demonstrated to be an attractive alternative with cellulose as a low cost and easily available support material (Shoseyov 2002). More than 200 amino acid sequences of such domains have already been determined. However, the CBD-fused protein expressed in *E. coli* formed aggregates, leading to the requirement for protein refolding prior to the administration of enzyme immobilization. Another class of substrate binding domains is the group of the chitin binding domains (ChBDs). They are involved in the catalytic activity of chitinases which degrade chitin to oligosaccharides. Chitin binding domains are subdivided into three families according to their amino acid sequences. Family 1 contains domains of fungal and plant chitinases. Family 2 mainly involves the chitinase domains of insects and viruses. Family 3 includes domains of chitinases of microorganisms. 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).

A 3.3.4 Chitin binding domain of *Bacillus circulans* WL-12 chitinase A1

The WL-12 *Bacillus circulans* bacterium can produce up to 10 various chitinases in the presence of chitin of which exochitinase A1 (EC 3.2.1.14) is the most effective for chitin degradation. Its chitin binding domain (ChBD) is a very small peptide of 45 amino acids located at the C-terminal end between Ala655 and Gln699 of the ChiA1-enzyme which belongs to family 3 of the carbohydrate-binding regions. Apart from the ChBD the enzyme further consists of two fibronectin type III domains and the N-terminal catalytic domain.

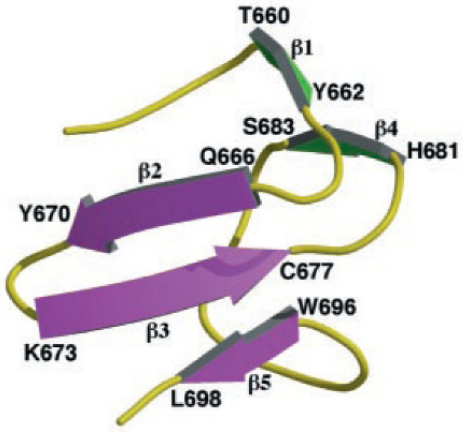


Figure 14. Tertiary structure of the chitin binding domain

Hashimoto *et al*. studied the properties of the ChBD of chitinase A1 from *Bacillus circulans* without influence from the other domains and found highly specific binding to insoluble chitin but not to chito-oligosaccharides, soluble derivatives of chitin, or other insoluble polysaccharides. He suggested that the chitin binding domain of chitinase A1 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 ChBD has a compact and globular structure with 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**).

As CBD_{EGZ}, it also consists of two ß-sheets (β1, β2) , one composed by two, the other, 45°C twisted sheet, by three strands (β3, β4, β5) (Ikegami *et al.* 2000). However, the ChBD 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} (Hardt 2002). Furthermore, in CBD_{EGZ} the aromatic residues Trp43, Tyr44 from the motif form a loop together with an additional residue (Trp18), this loop being responsible for the hydrophobic interaction with cellulose. ChBD lacks this region but has other residues indicated in **Figure 15**, which are (i) exposed on the surface, (ii) conserved in the ChBD family and (iii) hydrophobic or aromatic and therefore thought to participate in the chitin binding (Ikegami *et al.* 2000).

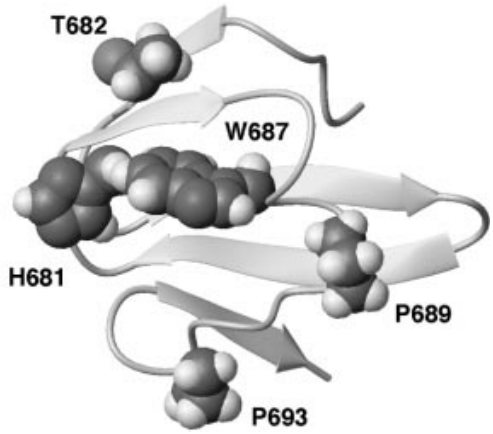


Figure 15. ChBD structure with marked amino acid residues. The labeled residues are thought to be responsible for the hydrophobic interaction with insoluble chitin.

A 3.3.5 Immobilization of enzymes via ChBD

Though the exact binding mechanism is still unclear, the chitin binding domain of chitinase A1 from *Bacillus circulans* WL-12 could already been proven as suitable polypeptide for the immobilization of enzymes and cells, as well as for isolation and purification of proteins. Immobilization of enzymes using the chitin

binding domain of chitinase A1 from *Bacillus circulans* WL-12 was first described by Chong *et al.* for the purification of recombinant proteins in a single chromatographic step (Chong *et al.* 1997; Chong *et al.* 1998). 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.

Enzyme immobilization via ChBD for a purpose other than the single-step purification of proteins has been reported by Chern and Chao and by Chiang *et al.* amongst others. In their studies, they found higher operational and long-term stability of a ChBD-based immobilized enzyme compared with its unbound form. Moreover, product isolation and enzyme reutilization was facilitated. Chern and Chao showed that the binding to chitin was mediated by strong hydrophobic interactions being applicable in a wide range of pHs (4-12). Strongest binding was achieved in the vicinity of its isoelectric point at 9.0 and elution only in a highly acidic environment (pH < 3) (Chern and Chao 2005; Chiang *et al.* 2009).

Cell immobilization on chitin using the chitin binding domain has as well been investigated. Wang and Chao constructed in-frame fusions of ChBD to *lpp* and *ompA,* subsequently expressed in *E.coli.* The resulting ChBD-displaying cells showed high binding to chitinous substrates such as colloidal chitin, chitin beads and flakes. The degree of deacetylation (DD) significantly influenced the degree of adsorption with determined 92 % on colloidal chitin (DD of 8 %), 61 % on chitin beads (DD of 18 %), and 53 % on chitin flakes (DD of 22 %) confirming the better affinity of ChBD to insoluble chitin forms (Wang and Chao 2006).

A 4 Accessorily Information to Manuscript

A 4.1 Preliminary Experiments

A 4.1.1 C- and N- terminal cloning

In order to assess whether a C- or a N-terminal fusion of the chitin binding domain to the ß-galactosidase of *Bacillus licheniformis* DSM 13 (*LacA*) was preferable for its immobilization and resulting enzyme activity, both fusions were tested in a preliminary assay using the Impact Kit (New England Biolabs, Germany). Based on a self splicing protein element (intein) the Impact Kit was created for the purification of recombinant proteins in a single chromatographic step (Chong *et al.* 1997; Chong *et al.* 1998). The vectors pTXB1 and pTYB11 provided by the Kit both contained the chitin binding domain of chitinase A1 from *Bacillus circulans* WL-12, an intein tag and a multiple cloning site, so that cloning of a target protein (*LacA* in this study) into the respective vector would yield a C-or N-terminal fusion protein as illustrated in **Figure 16.**

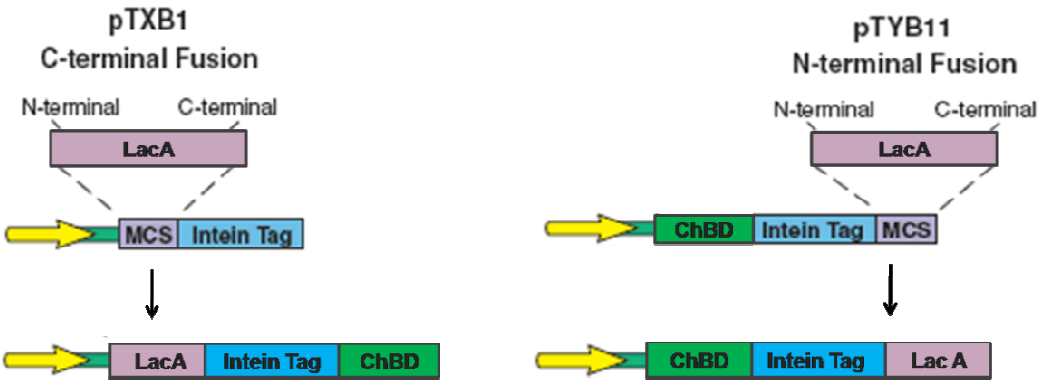


Figure 16. C-and N-terminal cloning of the LacA gene. The ß-galactosidase of *Bacillus licheniformis* DSM 13 was cloned into the vectors pTXB1 and pTYB11 of the Impact Kit.

LacA was amplified from the vector pOJ01 using the degenerated oligonucleotides summarized in **Table 8**. The respective restriction sites were included in the primer sequences for subsequent cloning into the vectors pTXB1 and pTYB11.

Table 8. Forward and reverse primer for the amplification of the *LacA* gene of *Bacillus licheniformis* DSM 13 with different restriction sites (underlined) for subsequent cloning into pTXB1 and pTYB11, respectively

Vector	Primer	Sequence (5' --> 3')
pTXB1	Bli*NdeI*fw	CTGTGCATATGCCAAAAATTTATACGACCCAAGCAAGATACATG
	Bli*SapI* rv	CTGTGCGCAGGAAGAGCCTTCTTTTGCTTTTACCGCTATTCTGGCCTC
pTYB11	Bli*SapI*fw	CTGTGAACAGAAGAGCTATGCCAAAAATTTATACGACCCAAGCAAGATACATG
	Bli*EcoRI*rv	CTGTCGAATTCCTATTCTTTTGCTTTTACCGCTATTCTGGCCTC

PCR reactions were set up in a total volume of 100 µl containing approximately 1 µg plasmid DNA, 0.5 µM of the forward and the reverse primer, 200 µM of each deoxynucleotide triphosphate, 6 U of *Pfu* DNA Polymerase and 10 µl of 10x *Pfu* Buffer. The amplification was carried out in a Peltier thermo cycler PTC-200 under the conditions listed in **Table 9**. For visualization of the appropriate amplification, the PCR products were visualized on a 1 % agarose gel containing 1 µg/ml ethidium bromide in 1xTAE (Tris acetate EDTA) buffer. The 2055 bp -bands representing the two LacA genes, each with different restriction sites were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) and double-digested overnight with *Nde*I, *Sap*I and *EcoR*V respectively. The pTXB1- and the pTYB11-vectors were as well treated with the appropriate restriction enzymes and with 1 U of CIP. Together with the gene products, they were again visualized on a 1 % agarose gel, excised and purified using the Clean-Up System. Ligation reactions were carried out at 25°C for 1 hour using T4 DNA Ligase and transformed into *E. coli* DH5α. For expression and blue-white screening the plasmids were subsequently transformed into *E. coli* ER2566 (Impact Kit, NEB).

Table 9. PCR conditions for the amplification of LacA

Step	Temperature	Time	Cycles
Initial denaturation	95 °C	2 min	1
Denaturation	95 °C	45 sec	35
Annealing	56 °C	45 sec	
Extension	72°C	4.5 min	
Final Extension	72°C	10 min	1

A 4.1.2 Blue-White Screening

The vectors pTXB1 and pTYB11 of the Impact Kit both have a T7 promoter and the *lacI* gene to provide stringent control of the fusion gene expression. To verify the activity of the ß-galactosidase fusion proteins, blue-white-screening was realized on LB-Amp. 2 % X-Gal (5-bromo-4-chloro-3-indolyl-ß-D-galacto-pyranoside) in DMSF were used as substrate and 0.5 mM IPTG induced expression of the fusion proteins. The empty vector pTXB1 served as negative control and pOJ01 as positive control.

A 4.1.3 Immobilization of the fusion proteins

Expression of the fusion proteins in *E. coli* ER2566 was induced with 0.4 mM IPTG for 3 hours at 37°C. The cells were harvested by centrifugation at 4000 g at 4°C for 30 min and resuspended in lysis buffer containing 500 mM NaCl, 20 mM Tris-HCl and 1 mM EDTA. Crude lysates were obtained by sonication (amplitude 50, 2 min, 10 s pulse, 10 s off) and subsequent centrifugation (10000 g, 30 min) to separate the cell debris. The over-expression of the recombinant ß-galactosidases was verified using SDS PAGE analysis.

Binding of the fusion proteins was tested in small scale assays using the chitin beads provided by the Impact Kit. 20 µl chitin bead slurry (corresponding to 10 µl chitin bead bed) were washed with column buffer, incubated with 200 µl of the crude lysates for 1 hour at 4°C under rotation and then again 2 times washed with 20 bed volumes of column buffer to get rid of all un-specifically bound proteins.

The chitin beads were resuspended in 100 µl of column buffer to enable the determination of ß-galactosidase activity using the *o*NPG assay. Activities of the C- and of the N-terminal fusion proteins were measured in their free, as well as in their immobilized form. Protein concentration using the Bradford Assay could only be assessed for the free fusion enzymes, since the reagent binds to the protein, which itself is bound to the chitin beads so that no homogenous coloration of the solution was achieved. Therefore protein concentration of the immobilized forms of the fusion enzymes was calculated from the protein concentration differences measured in the crude lysates and the flow through after immobilization.

A 4.1.4 Cloning of the ChBD with and without linker domain

Since there was no difference in ß-galactosidase activity observed between the C-and the N-terminal fusion, the chitin binding domain of *Bacillus circulans* WL-12 chitinase A1 (*ChBD*) was cloned C-terminally to the *LacA* gene into the vector pOJ01 as already described in the manuscript. To assess the possibility of interference between chitin binding domain and ß-galactosidase activity, the proteins were not only fused directly, but as well indirectly by incorporation of a small linker domain upstream of the *ChBD*. The linker was a glycine- and serine-rich domain consisting of 16 amino acids, which promised great flexibility due to the small amino acids permitting the independent movement of the fused proteins. The degenerated oligonucleotides for the creation of the two fusion constructs are listed in **Table 10**.

Table 10. Primer for amplification of the chitin binding domain of *Bacillus circulans* chitinase A1 with and without linker, containing the restriction sites *Xho*I and *Kpn*I (underlined) in the forward and the reverse primer, respectively.

Primer	Sequence (5' --> 3')
ChBD fw	CTGTGCTCGAGACGACAAATCCTGGTGTATCCGCTTGGC
ChBDlinkfw	CTGTGCTCGAGGGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGGTGGCGG ATCGACGACAAATCCTGGTGTATCCGCTTGGC
ChBD rv	CTGTGCGGTACCTCATTGAAGCTGCCACAAGGCAGG

For the amplification of both fragments (the ChBD with linker and the one without) the same reverse primer was used (ChBD rv). For subsequent cloning into the vector pOJ01 the restrictions sites *Xho*I and *Kpn*I were as well introduced into forward and reverse primer sequences, respectively. The PCR reaction and all following cloning steps were carried out as described in chapter 2.3. The DNA sequences of the two constructs were analyzed by sequencing (Macrogen Korea) and expression was induced by 0.01 mM IPTG in 30 ml TB-media at 18°C for 40 hours. The native enzyme was expressed under the same conditions to compare ß-galactosidase activity. After harvesting of the cells crude lysates were obtained by sonication as previously. 3.5 ml of ß-galactosidase-ChBD fusions with and without linker were immobilized on 1 ml of chitin beads and protein concentration and ß-gal activity were determined of each fraction. Since the strong binding of the ChBD to its substrate can only be cleaved at a pH < 3 (Chern and Chao 2005), elution was mediated by the addition of 1.5 ml of 0.3 M NaOH. After 6 h of incubation the ß-galactosidase activity of the eluted fraction was measured and all fractions were analyzed using SDS PAGE.

A 4.1.5 Binding on different columns for purification

Since expression of the His-tag sequence in the original vector pOJ01 was suppressed in the new constructs pCM01 and pCM02 by the stop codon inserted after the sequence encoding the chitin binding domain, purification of the fusion enzyme using IMAC (immobilized metal ion chromatography) was not possible anymore. Therefore several columns packed with different materials were tested in small scale for the purification of the fusion protein (LacA-ChBD) from the crude lysate. The first contained 1 ml of chitin bead bed, the second 1 ml colloidal chitin (1.53 g/ml) and the third was filled with p-Aminobenzyl-1-thio-ß-galactopyranose (ABTG). All of the columns were equilibrated in 50 mM sodium phosphate buffer (NaPP; pH 6.5) and 1 ml of protein solution was applied to each column and incubated over night. The columns were washed 3 times with 50 mM NaPP-buffer and elution was conducted with 2 M NaCl and alternatively with 0.3 M NaOH for chitin-based materials. ß-galactosidase activity and protein

concentration were measured in each fraction to estimate protein recovery from the different packing materials.

A 4.1.6 Assessment of appropriate dilution of immobilized enzyme

Determination of ß-galactosidase activity for *o*NPG as well as for lactose hydrolysis with the immobilized enzyme was made difficult by the suspension of chitin beads causing a significant error probability. Therefore preliminary experiments were carried out using different dilutions and amounts of immobilized enzyme. ß-galactosidase activity was measured in duplicate using 20 and 40 µl of enzyme-bead suspension diluted 1:2, 1:5, 1:10, 1:20 and 1:40. The results and statistical errors obtained with the *o*NPG-assay were compared.

A 4.2 Results of preliminary experiments

A 4.2.1 ß-galactosidase activities of C-and N-terminal fusion proteins

After cloning of the fusion constructs into *E. coli* ER2566, colonies carrying the modified pTXB1 and pTYB11 were streaked out on LB-Amp plates containing X-Gal and IPTG to screen for ß-galactosidase activity. Colonies carrying the unmodified vector pTXB1 served as negative control. The vector pOJ01 in *E. coli* TOP 10 was used as positive control. The blue staining of the colonies confirmed successful expression of active fusion proteins as displayed in **Figure 17.** In the case of ß-galactosidase activity 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-Gal) is cleaved into galactose and 5-bromo-4-chloro-3-hydroxyindole, latter being further oxidized into 5,5'-dibromo-4,4'-dichloro-indigo, an insoluble blue product. Colonies carrying the empty pTXB1 do not contain the *LacA* gene for ß-galactosidase expression and thus colonies remained white. The positive control (colonies containing pOJ01) as well showed blue staining of the colonies, confirming ß-galactosidase activity.

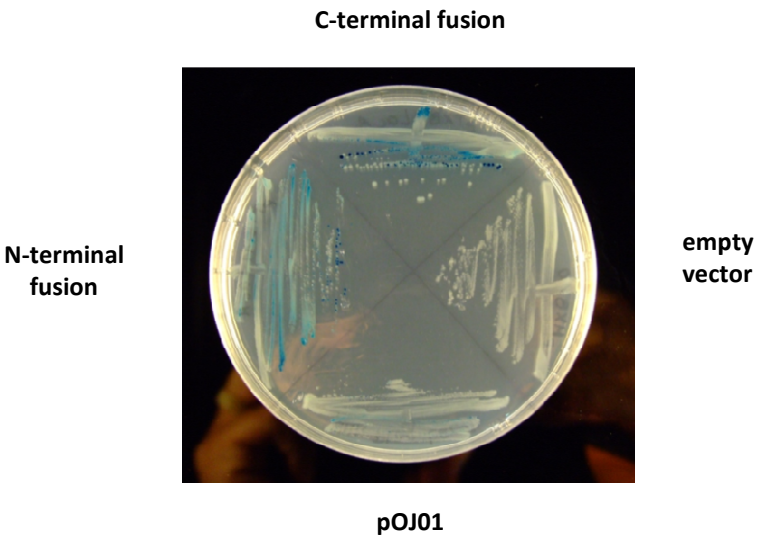


Figure 17. Blue-white screening of C- and N-terminal fusion proteins.

ß-galactosidase activity of the crude lysates obtained after expression in *E. coli* ER2566 and sonication was further determined and compared using the *o*NPG-assay. The native ß-galactosidase showed lower expression (data not shown) than the recombinant enzymes, what could already be seen in the blue-white screening. The specific activity of the C-and the N-terminal fusion enzymes was nearly the same and they both bound to chitin. Thus they could be successfully isolated from the rest of the proteins present in the crude lysate, which is illustrated by the significant increase of specific activity (**Table 11**).

Table 11. Isolation of C-and N-terminal ß-galactosidase fusion proteins due to specific binding to chitin via the chitin binding domain.

LacA-ChBD fusion proteins		activity [U/ml]	protein conc. [mg/ml]	spec. act. U/mg
crude lysate	C-terminal	32.761	8.136	4.027
	N-terminal	15.683	4.012	3.909
immobilized	C-terminal	17.015	0.145	117.343
	N-terminal	7.355	0.064	114.929

A 4.2.2 C-terminal cloning of ChBD with and without linker

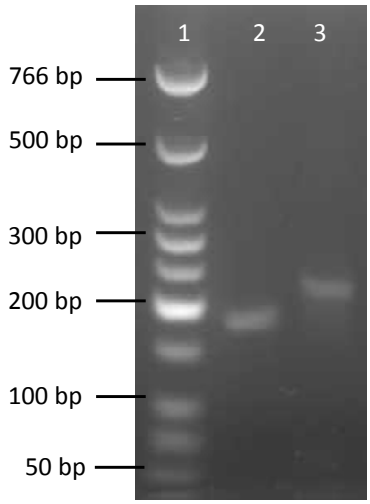


Figure 18. Agarose gel electrophoresis of amplified PCR-products. Lane 2 displays the amplified ChBD and lane 3 the ChBD with linker compared to the low molecular weight marker in lane 1.

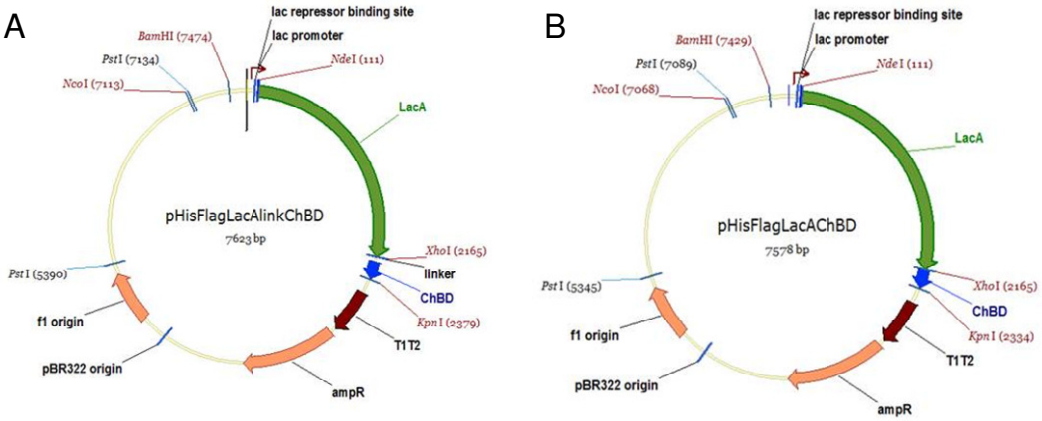


Figure 19. Vector maps of the two plasmids pCM02 and pCM01 with inserted *ChBDs* with (A) and without linker (B), respectively, created with Vector NTI Program from Invitrogen. Both fragments were cloned C-terminally of the ß-galactosidase gene (*LacA*).

Chitin binding domain with and without linker were amplified from the pTXB1-vector of the Impact Kit and successful amplification was confirmed by agarose gel electrophoresis (**Figure 18**). The chitin binding domain has 159 bp, the linker 48 bp, resulting in a band of 207 bp representing the linker-ChBD. **Figure 19** shows the two resulting plasmids after insertion of the two PCR products into the vector pOJ01 between the restriction sites *Xho*I and *Kpn*I. Apart from the restriction sites the vector maps also display inducible lac promoter and repressor binding site, T1T2-terminator, the ampR gene encoding ampicillin-resistance and bacterial (pBR322) and bacteriophage (f1) origins of replication.

A 4.2.3 Binding and elution using a chitin column

Table 12. Data of binding and elution assay using a chitin column. The ß-gal fusion proteins were applied on a column packed with chitin beads.

Protein	fraction	activity [U/ml]	protein conc. [mg/ml]	specific activity [U/mg]
LacA	crude lysate	181.878	5.046	36.044
LacA-ChBD	crude lysate	338.709	6.117	55.372
	supernatant	1.612	4.528	0.356
	immobilized	267.057	1.589	168.066
	elution	0.185	n.d.	n.d.
LacA-linkChBD	crude lysate	221.931	6.188	35.865
	supernatant	1.729	4.752	0.364
	immobilized	246.449	1.436	171.622
	elution	0.786	n.d.	n.d.

ß-galactosidase activity of the LacA-ChBD representing the enzyme which is directly fused to the chitin binding domain showed higher activity than the fusion protein with the linker (LacA-linkChBD) and the native enzyme (LacA). Approximately the same amount of fusion enzyme (approximately 1.5 mg/ml) was bound on the chitin beads resulting in comparable specific activities as illustrated in **Table 12**. Elution of the fusion enzymes was conducted using 0.3 M NaOH. At the resulting low pH the ChBD should undergo conformational changes and detach from chitin. However, no ß-galactosidase activity could be detected in the eluted fraction. SDS PAGE analysis was performed to reveal if the protein was eluted from the column (**Figure 20**).

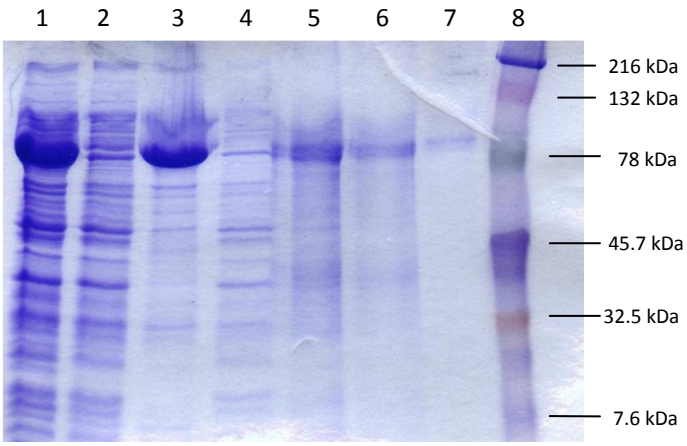


Figure 20. SDS PAGE analysis of binding and elution assay. Samples of the different fractions were mixed with SDS and boiled 3 minutes at 99°C before applying them to the slots in the following order: Crude lysate (lane 1), flow through (lane 2), ß-gal-ChBD bound on chitin beads (lane 3), wash (lane 4), elution (lane 5 and 6), beads after elution (lane7) and molecular mass marker (lane 8).

A 4.2.4 Result of purification assay

The specific binding of the free ß-galactosidase – chitin binding domain fusion protein was tested on differently packed columns in small scale in order to select the best strategy and obtain the purified fusion enzyme. Specific binding could not only be achieved using the strong interaction of the chitin binding domain with its substrate but as well by the use of ß-galactosidase affinity to p-Aminobenzyl-1-thio-ß-galactopyranose (ABTG). Although most of the protein bound to the matrix, the binding was not as strong and specific as with chitin so that more than 50 % were washed out together with other unspecific proteins. However, the fusion protein could be successfully eluted from the column using 2 M NaCl and resulting specific activity was increased by 30 % (**Table 13**). When chitin beads were used as a substrate, most of unwanted proteins were already separated in the first step showing no ß-galactosidase activity. All other unspecific proteins were eliminated in the subsequent washing steps. Elution of the LacA-ChBD fusion

protein from the beads was first tested with NaCl but showed no promising results. As already proved in previous immobilization assays, the chitin binding domain could only be released from its substrate in acidic environment. Since ß-galactosidase is not stable at a pH < 3, elution with immediate neutralization of the protein solution using phosphoric acid was as well tested but did not result in ß-galactosidase activity (data not shown). Also colloidal chitin was inapplicable for purification, not only because of the fact, that the fusion protein was only released under denaturing conditions, but moreover because of its abrasive structure. Thus, the only appropriate packing material for the purification of the ß-gal fusion enzyme was found to be ABTG.

Table 13. Purification assay using different columns. The efficiency of binding and elution of the recombinant ß-galactosidase was analyzed.

column	fraction	Activity [U/ml]	protein conc. [U/mg]	specific activity [U/mg]
	crude lysate	408.27	11.75	34.74
ABTG	flow through	4.84	1.24	3.90
	wash	163.32	7.45	21.92
	elution with 2 M NaCl	89.21	1.94	45.98
chitin beads	flow through	0.00	7.05	0.00
	wash	0.00	2.26	0.00
	elution with 0.3 M NaOH	0.00	2.65	0.00
colloidal chitin	flow through	69.01	4.85	14.24
	wash	21.07	1.47	14.30
	elution with 0.3 M NaOH	0.00	1.58	0.00

A 4.2.5 Appropriate dilutions of immobilized enzyme

Table 14 shows ß-galactosidase activities of an immobilized enzyme sample using different dilutions (1:2 – 1:40) and amounts (20 and 40 µl) of enzyme-bead suspension and the deviation from the theoretical value calculated. *o*NPG was the substrate and the theoretical number of units bound on the beads was calculated from weighed bead bed and measured protein concentration in the crude lysate assuming that all of the fusion protein was bound on the beads after an incubation time of at least 1 hour. The obtained units per gram beads varied significantly

with errors from 5 to more than 50 %. In general, smaller errors occurred when a bigger volume of enzyme-bead suspension was employed in the *o*NPG assay. The lowest standard deviation could be achieved with 1:10 - and 1:20 - dilutions for both volumes of enzyme solution used in the assay. Thus, for the characterization of the immobilized enzyme, ß-galactosidase activity was measured employing only dilutions of the enzyme-bead suspensions ranging from 1:4 until 1:20. Furthermore, if a higher dilution than 1:20 was needed to determine enzyme activity, a volume of 40 µl was used, whereas for lower dilutions reproducible results could be rather achieved using 20 µl. Measurements for the characterization of the immobilized enzyme were carried out at least in triplicate to obtain reproducible values with low standard deviations.

Table 14. Errors obtained using different dilutions of immobilized enzyme. ß-galactosidase activity was assessed with *o*NPG as substrate and compared to calculated theoretical activity.

volume [µl]	dilution	activity [U/g beads]	error [%]	**volume [µl]**	dilution	activity [U/g beads]	error [%]
	theoretical	**15.25**			**theoretical**	**15.25**	
20	1:2	13.80 ± 2.01	9.52	**40**	1:2	7.43 ± 2.14	51.3
	1:5	18.69 ± 3.66	22.57		1:5	14.47 ± 3.96	5.1
	1:10	19.32 ± 1.15	26.64		1:10	13.64 ± 0.51	10.5
	1:20	14.25 ± 1.86	6.59		1:20	13.48 ± 0.27	11.60
	1:40	23.15 ± 8.69	51.81		1:40	16.77 ± 3.06	9.93

A 4.3 Optimization of protein over-expression

For optimal over-expression of the recombinant ß-galactosidase, temperature, incubation time and concentration of the inducer IPTG (Isopropyl-ß-D-galactopyranoside) were varied. *E. coli* TOP10 were cultured in 100 ml TB expression medium until an optical density of 0.6 was reached and expression of LacA-ChBD was induced with IPTG for up to 45 hours. In **Figure 21** the optimized expression, achieved at 25°C compared to the expression at 18°C with 0.01 mM IPTG for 45 hours is illustrated. Samples were taken every 3 h until 24

hours of incubation, then only every 10 h. Crude lysates were obtained by sonication and ß-galactosidase activity of each sample was determined using the *o*NPG assay.

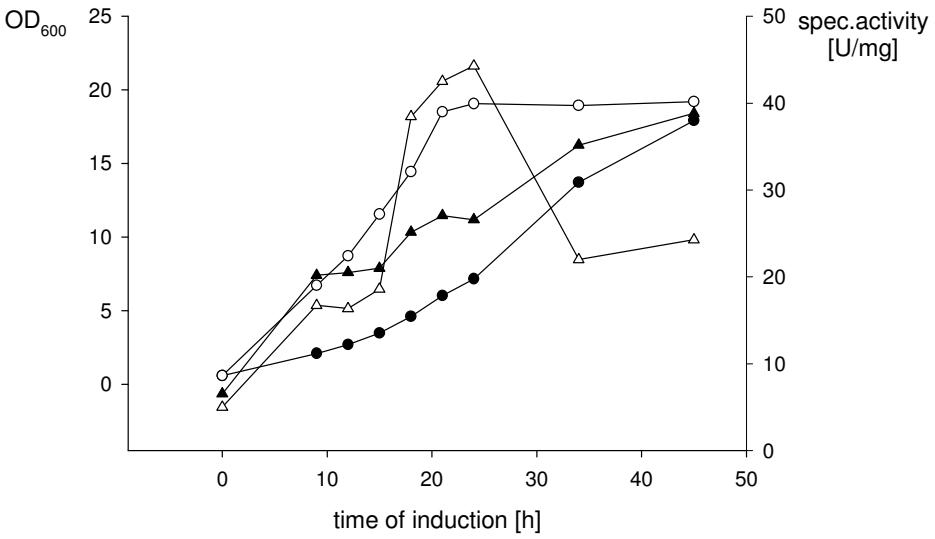
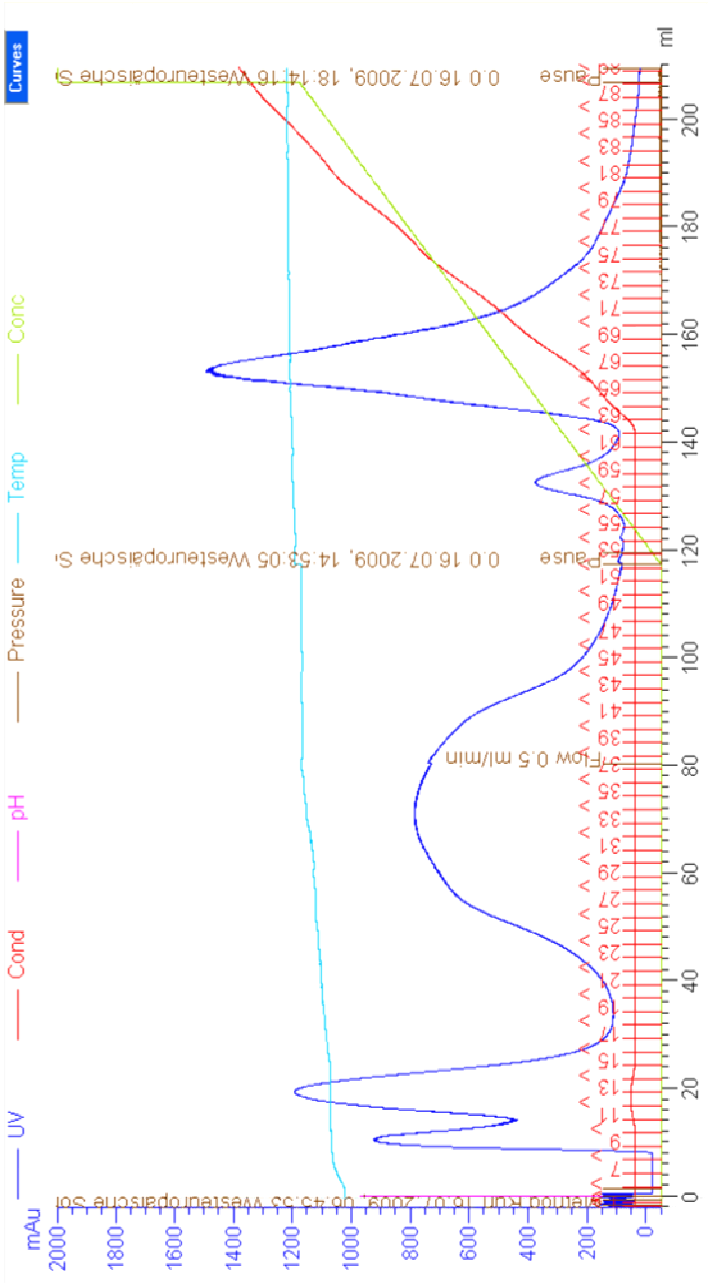


Figure 21. Expression of the ß-gal-ChBD fusion protein. Growth curves of cultivated *E.coli* TOP 10 at 18°C (●) and 25°C (○) and specific activities at 18°C (▲) and 25°C (△) with induction of 0.01 mM IPTG for 45 hours are illustrated.

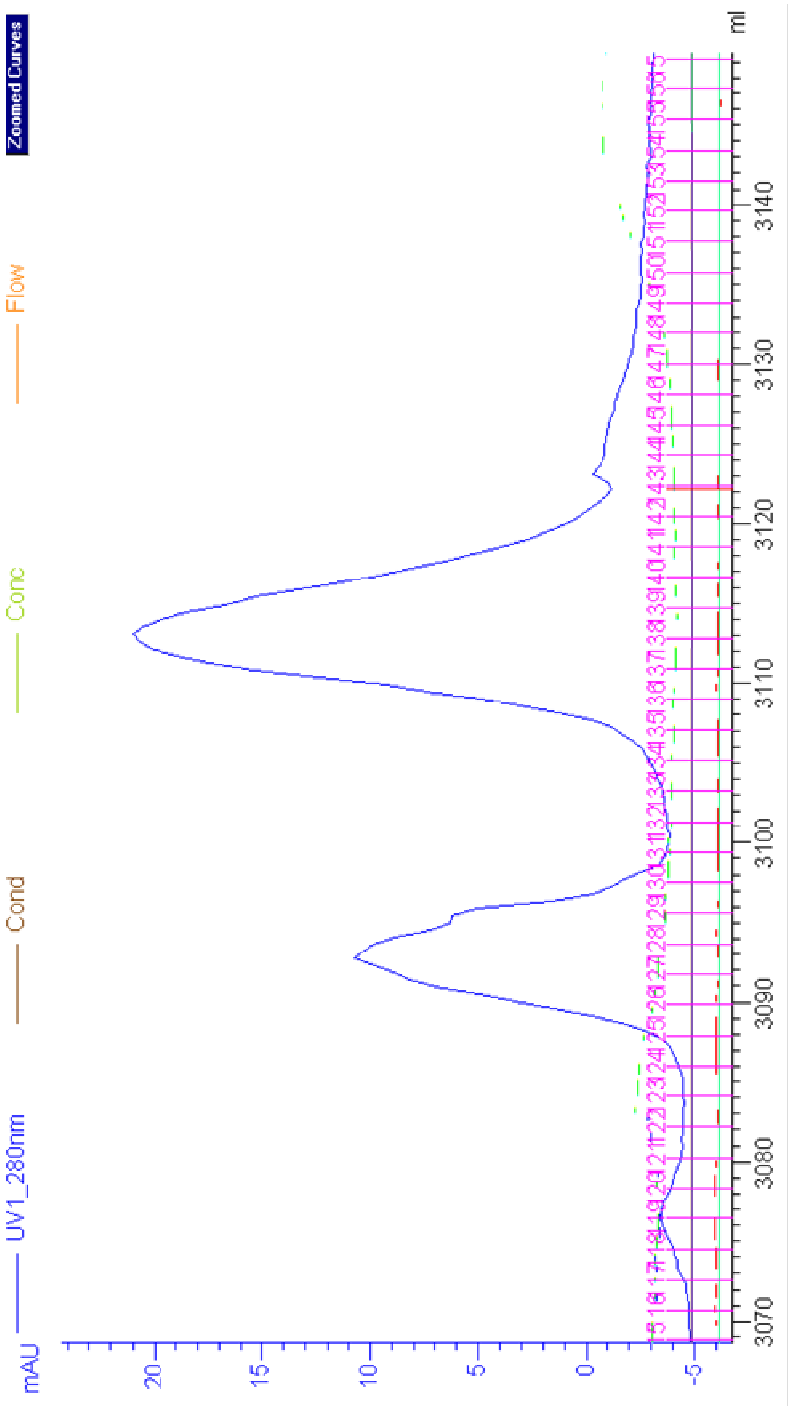
The cells cultivated at 25°C were growing about twice as fast compared to those incubated at 18°C. Maximal optical density (OD_{600}) at 25 °C was reached after 24 h and remained constant signifying the cessation of exponential growth. The specific activity of expressed recombinant ß-galactosidase increased proportionally to cell density with a maximum at 45 U/mg observed after 24 h of incubation where an OD_{600} of about 20 was reached. Cultures grown at 18°C grew constantly reaching maximal OD_{600} and maximal specific activity only after 45 hours. The result suggests that specific activity of the expressed fusion enzyme depends on the optical density (OD) of the cell culture so that optimized conditions were determined to be induction with 0.01 mM IPTG, incubation at 25°C, and induction time until an OD_{600} of 20 was achieved.

A 4.4 Accessorily information to protein purification

A 4.4.1 Purification on ABTG-column



A 4.4.2 Gel filtration



A 5 Media and Solutions

A 5.1 Media

Media components were dissolved in deionized water and autoclaved 20 minutes at 121°C. Solid media were cooled down to approximately 50°C and poured into sterile Petri dishes. The plates were aseptically prepared in a sterile bench. The plates were stored at 4°C.

NB medium:	Peptone	5 g/l
	Meat extract	3 g/l
	$MnSO_4$	10 mg/l
LB medium:	Tryptone	10 g/l
	Yeast extract	5 g
	NaCl	10 g/l
TB medium:	Peptone from casein	12 g/l
	Yeast extract	24 g/l
	glycerol	4 ml
	1 M KH_2PO_4 pH 7,5	
SOB medium:	Tryptone	20 g/l
	Yeast extract	5 g/l
	NaCl	0.5 g/l
	250 mM KCl, pH 7.0	10 ml/l
	2 M $MgCl_2$	5 ml/l

A 5.2 Buffer and other solutions

All buffer components were dissolved in deionized (DI) water filled up to 90% to the volume and titrated pH with NaOH or HCl before deionized water was added to reach the final volume.

List of buffers and chemicals

MUG	3.5 mg/ml in NaPP-buffer
ß-mercaptoethanol stock solution	1 M in 50 mM NaPP
Ampicillin	100 µg/ml
Britton Robinson buffer	20mM boric acid 20 mM acetic acid 20 mM phosphoric acid
Buffer A	50 mM NaPP
Buffer B	2 M NaCl in 50 mM NaPP
Dithiothreitol stock solution	1 M DTT in 50 mM NaPP
Ethylendiaminetetraacetic acid stock sol.	1 M EDTA in 50 mM NaPP
Hydrochloride	1 M HCl
Gel filtration buffer	150 mM NaCl in 20 mM NaPP
Glucose stock solution	1g/L
GOD/POD assay solution	21.5 U/ml GOD 1.35 U/ml POD 157 µg/ml 4-aminoantippyrine 11.5 µmol/ml phenol 0.1 M potassium phosphate buffer (pH 7.0)
Lactose solution	600 mM in 50 mM NaPP
Magnesium chloride	1 M $MgCl_2$ in 50 mM NaPP

o-Nitrophenyl-ß-D-galactopyranoside	22 mM in 50 mM NaPP
o-Nitrophenol stock solution	20 mM *o*NP
Potassium phosphate buffer (7.0)	0.1 M KPP
Sodium carbonate solution	0.4 M Na_2CO_3
Sodium hydroxide	0.3 mM and 1 M NaOH
Sodium phosphate buffer (pH 6.5)	50 mM NaPP
Urea stock solution	1 M Urea in 50 mM NaPP

Agarose Gel electrophoresis

1 % /2 % Agarose	1 g / 2 g in 100 ml 1 x TAE-buffer
50 x TAE (Tris acetate EDTA) buffer	242 g Tris Base 57.1 ml Glacial acetic acid 100 ml 0.5 M EDTA (pH 8.0)
Ethidium bromide	1 µg/ml

SDS PAGE:

12 % separating gel:	total volume	**5 ml**	**10 ml**
	H_2O	1.60	3.30
	30% acryl amide mix	2.00	4.00
	1.5 M Tris (pH 8.8)	1.30	2.50
	10% SDS	0.05	0.10
	10% ammonium persulfate	0.05	0.10
	TEMED	0.002	0.004
5 % stacking gel:	total volume	**2 ml**	**4 ml**
	H_2O	1.40	2.70
	30% acryl amide mix	0.33	0.67
	1.5 M Tris (pH 8.8)	0.25	0.50

10% SDS	0.02	0.04
10% ammonium persulfate	0.02	0.04
TEMED	0.002	0.004

1 x Tris-glycine electrophoresis buffer

250 mM glycine
0,1% v/v SDS

SDS buffer

47 mM Tris-HCl, pH 6.8
34 mg/ml SDS
0.1 mg/ml bromphenol blue
5 % v/v mercaptoethanol
15 % v/v glycerol

A 5.3 Equipment

Table 15. List of instruments used for the experiments

Instrument	Company	Application
French press	Aminco	Cell disruption
Äkta Explorer system	GE Healthcare	Protein purification
Äkta Purifier system	GE Healthcare	Protein purification
Spectrophotometer Beckman DU-62	Beckman	Protein concentration ß-gal activity assays
Centrifuge, Sorvall (RC 26 Plus, MC 12V, Evolution RC)	Du Pont	centrifugation
Ultracentrifuge, Beckman L8-55	Beckman	centrifugation
Omnifuge 2.0 RS	Heraeus	centrifugation
Eppendorf Centrifuge 5415R	INULA	centrifugation
Laboratory Autoclave, Certoclave EL 10L	Kelomat	Sterilisation
Autoclave, Varioclave 500	H+P Labortechnik	Sterilisation
Sterile bench HS-P 12/2 (LF-Werkbank)	Heraeus	all aseptic steps
Membrane vacuum pump CVC 2	Vacuubrand	filtration of buffers
Culture orbital shaker	SZT	fermentation
pH-instruments	WTW and Orion	buffer preparation
SDS-PAGE: Hoefer Mighty Small II SE250	Amersham Bioscien.	Protein purity
Ultra filtration polysulfone membrane	Pall Corporation	Concentration, Desalting
Shaker Infors Unitron	Infors	Small scale fermentation
Shaker HAT	Infors	Small scale fermentation
Thermo mixer compact	Eppendorf	ß-gal activity assays
Sonicator	Ultrasonic	sonication
Balances	Sartorius	Media / buffer preparations

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