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β-Galactosidases from *Lactobacillus pentosus* and *Lb.* L662: Purification, characterization and galacto-oligosaccharide production

Masterarbeit zur Erlangung des akademischen Grades Diplomingenieur

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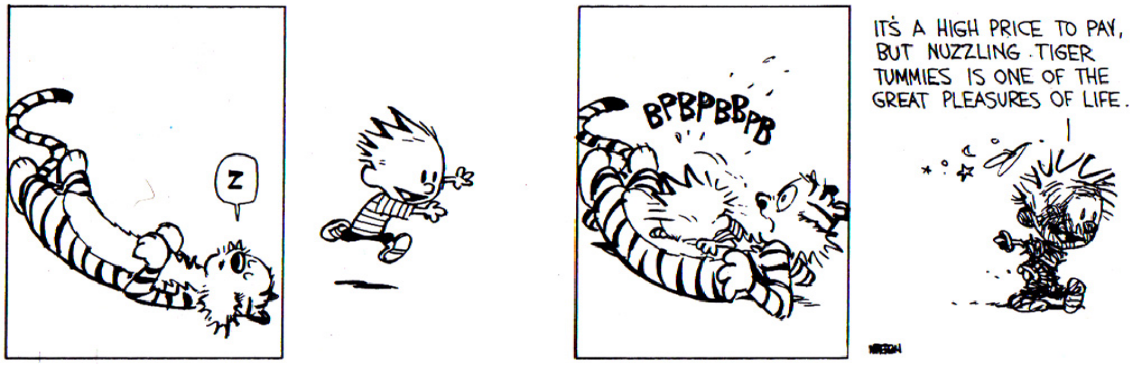
Functional foods:

On their way to being a great pleasure of life



Vorwort

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LIST OF ABBREVIATIONS / ABKÜRZUNGSVERZEICHNIS

AC	affinity chromatography
API 50 CH	API test, 50 chambers filled with different carbohydrates
APTG	*p*-aminobenzyl-1-thio-β-D-galactopyranoside
ASP	ammonium sulphate precipitation
β-gal	β-galactosidase
CBA	Corynebacterium Agar
CE	capillary electrophoresis
CH	Pool for the characterization measurements
DTT	Dithiothreitol
EC	Pool for the determination of enzymatic conversion ability
EDTA	ethylenediaminetetraacetic acid
GOD	glucose oxidase from *Aspergillus niger*
GOS	Galacto-oligosaccharides
HIC	hydrophobic interaction chromatography
HPLC	high performance liquid chromatography
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
Lb. L662	*Lactobacillus* sp.
LAB	lactic acid bacteria
LB	Luria Bertani Media
ME	2-mercapto ethanol
OD_{600}	optical density measured at 600 nm
*o*NP	*o*-nitrophenol
*o*NPG	*o*-nitrophenyl-β-D-galactopyranoside
PMSF	Phenyl methyl sulfonyl fluoride
POD	horseradish peroxidase
SCFA	short-chain fatty acids
SDS	Sodium dodecyl sulphate

1. Introduction

The aim of this work was the biochemical characterization of the enzyme β-galactosidase, purified from two different *Lactobacillus* strains. Beside the hydrolysis of the milk sugar lactose this enzymes are able to form galacto-oligosaccharides (GOS). GOS are prebiotic sugars which are interesting for the production of food with a certain nutritional purpose. To produce such functional foods it is of high interest to choose enzymes from food-grade microorganisms. Lactobacilli have been used in food technology (yoghurt, sauerkraut, sausages) for thousands of years and are therefore generally recognized as safe (GRAS). Additionally they are naturally found in the human intestinal flora of adults, as well as of breast milk fed infants which makes them, apart from their ability of prebiotic sugar production, interesting for the use as probiotics in dairy products.
In this work the β-galactosidase from the strains *Lactobacillus pentosus* and *Lb.* L662, a not further identified *Lactobacillus* strain, were characterised determining different biochemical parameters like temperature-, pH-optimum and temperature-, pH-stability. The kinetic properties, the specificity for different substrates and the effect of different ions or reagents were also investigated. Furthermore, the characteristics of GOS production during lactose hydrolysis were analysed.

1.1 Functional Foods

Japan is the birthplace of any “functional food”. In the early 1980s research programmes were launched and funded by the Japanese government. One of the research projects was the “Analysis of functional foods and molecular design”. Foods for Specified Health Use (FOSHU) were established in 1991 in order to reduce the escalating costs of health care. FOSHU is expected to have specific health effects resulting from relevant constituents and contents. Also allergens free foods belong to this group.
Due to increasing costs of the health care the industrialized world faces new challenges in the 21st century. Furthermore, the longer life

expectancy, improved scientific knowledge, development of new technologies, and major changes in lifestyles bring a possibility and maybe also a need for improved food. Nutrition scientists try to manage these new challenges by the idea of “optimal nutrition”. An idea which focuses on optimising the quality of the daily diet, concerning its content of nutrients and reduction of non-nutrients, as well as providing food with properties that assure the maintenance of health. This is where the development of functional foods starts becoming interesting (Ashwell, 2002).

Jones (2002) described functional foods as “a food or beverage that imparts a physiological benefit that enhances overall health, helps to prevent or treat a disease/condition, or improves physical or mental performance via an added functional ingredient, processing modification or biotechnology”. The Ministry of Health in China defined functional foods in 1996 as “a food that has special health functions. It is suitable for consumption by special groups of people and has the function of regulating human body functions but is not needed for therapeutic purposes” (reviewed by Yang, 2008). Another definition is given by Chadwick *et al.* (2003): “a food can be regarded as “functional” if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease. Functional foods must remain foods, mostly composed of bulk ingredients and they must demonstrate their effects in amounts that can be expected for normal consumption patterns: they are not pills or capsules but part of a normal diet”. Nevertheless, a world wide definition has not been found yet.

Ashwell summarized in 2002 possible forms and technologies to obtain functional foods:

- Natural enhancement of components through special growing conditions (e.g. lycopene in tomatoes).
- Addition of components to provide benefits for the consumer (e.g. probiotic bacteria in dairy products).
- Removal of components in order to reduce adverse health effects (e.g. Saturated Fatty Acids in snacks).
- A food with one or more chemically modified components to improve health (e.g. hydrolysed protein in infant formulas to reduce the likelihood of allergenicity).
- Increase of the bioavailability of one or more components to provide greater absorption of a beneficial component.

A combination of the mentioned groups is possible.

1.1.1. Fortified foods

1.1.1.1 Unsaturated fatty acids

These fats are naturally found in fish and fish oils and have gained interest and publicity playing a role in prevention and management of cardiovascular diseases and prevention of atherosclerosis. Omega-3 fatty acids which are found mostly in fish oil have been recognized to play important roles in the nervous system, particularly during the development of children (Holub, 2002).

Fish fatty acids like Eicosapentaenoic acid (EPA) and Docosahexaenonic acid (DHA), both omega-3 fatty acids, are transferred into fatty products containing none of these (margarine) to widen the spectrum of unsaturated fats in these products.

1.1.1.2 Phytosterols

Phytosterols are a group of chemical compounds that occur naturally in plants and are structurally related to mammalian cholesterol. The addition of plant sterols and stanols, as well as their esters, to food products is aiming at the goal of lowering the cholesterol level in human blood plasma. This reflects a major development in the functional food sector in Europe and North America, as cholesterol-derived diseases are increasing in modern societies. The plant sterols and stanols act in the small bowel by completely inhibiting intestinal absorption of cholesterol. This can therefore lower the circulating total and LDL cholesterol levels by suppressing the cholesterol absorption and thus lowers the blood cholesterol level (Jones *et al.,* 2000).

Normally occurring in large quantities in fruit, vegetables, nuts, seeds and other foods, they are transferred to certain oil-containing products such as milk, yogurt or margarine. Esterification allows the sterols and stanols to be solubilised in the matrix of food fat for easier incorporation.

1.1.1.3 Vitamins and minerals

Fortified foods with vitamins and minerals are already very common, as well as the fortification with antioxidants which often belong to the group of vitamins, like ascorbic acid.

Folate is mentioned here representatively for added vitamins. This vitamin, belonging to the group of B-vitamins, is nowadays found in dry products like flour, cereals, and bread. It normally occurs in form of folic acid and is able to reduce a neural tube defect in newborn infants. Addition of calcium, here mentioned representatively for the nutrient group of minerals, improves the bone mineral density and should help to prevent the establishment of osteoporosis in old age (Ashwell, 2002; National Osteoporosis Prevention, USA). Calcium is often added to milk products or cereals.

1.1.1.4 Probiotics

Another large group of functional food is the group of probiotic products. The word *probiotic* is deduced from Greek, meaning “for life” and is defined as a “viable microbial dietary supplement that beneficially affects the host through its effects in the intestinal tract” (Fuller, 1989). Probiotic bacteria are widely used in the fermentation of dairy products such as yogurt and yogurt drinks. The bacteria genera most often used as probiotics are *Lactobacillus* and *Bifidobacterium*. After passing through the stomach and the small intestine, some probiotics survive and become established transiently in the large bowel, competing with pathogenic bacteria in the gut. Animals treated with the probiotic bacterium *Lb. acidophilus* showed that this strain can delay colon tumour formation by prolonging the induction of the latent period (Goldin and Gorbach, 1980). Probiotic Lactobacilli (e.g. *Lb. acidophilus, Lb. casei*) are used either as single species or in mixed culture with other bacteria when applied to food or feed. Bifidobacteria, among them *B. bifidum* or *B. longum*, seem to have positive effects for the consumer suffering from diarrhoea, constipation, recolonization by pathogens, flatulence and gastric acidity (Goldin and Gorbach, 1992; Gibson and Roberfroid, 1995).

Both Lactobacilli and Bifidobacteria are known to inhibit the growth of exogenous and/or harmful bacteria like *Clostridium difficile* or *C. perfringens*, stimulate immune functions, aid in digestion and/or absorption of food ingredients/minerals and synthesise vitamins (Gibson and Roberfroid, 1995). Some are even capable of producing antibiotic material (Tomomatsu, 1994).

Probiotics must meet the following criteria (Fuller, 1992):

1. Possible production in large scale fermentations
2. Viable, stable and storable
3. Survive in the intestinal ecosystem
4. Positive effect on the animal

Market situation for functional food products in Austria

Actually, a wide range of functional food products are available on the Austrian market, being enriched with different types of ingredients ranging from unsaturated fatty acids to prebiotics. Some examples are given here:

- Unsaturated fatty acids from fish, like DHA, are added into spreads and can be found in the product *Rama Idee!* (Unilever).
- Plant sterols are found in the spread *Becel pro activ* (Unilever) or in the drink *Danacol* (Danone).
- Several cereal products from *Kellogg's* are enriched with folic acid, and calcium can be found in *Knüsperli* muesli (Bioquell), or in the children's product *Fruchtzwerge* (Danone).
- Different kinds of probiotic products can be found in the supermarket, from *Acidophilusmilch* (NÖM) with cultures of *Lb. acidophilus* to *Yakult* (Yakult) containing *Lb. casei Shirota*.

More information concerning probiotic bacteria in available products can be found on http://www.food-info.net/de/ff/probiotics.htm, an initiative on Food Information from the University of Wageningen.

Also a synbiotic product is found at the market, being a nutritional supplement: *Omni Biotic* (Allergosan). This product contains six probiotic strains (*Lb. acidophilus, L. lactis, Enterococcus faecium, B. longum, Lb. casei and Lb. salvarius*) and additionally fructo-oligosaccharides (FOS) which belong to the group of prebiotica.

1.2 Prebiotica

Diet can regulate the following key processes in digestive physiology: satiety (the rate and extent of macronutrient breakdown and absorption from the small bowel), sterol metabolism (the colonic microflora, fermentation), mucosal function and bowel habit, and the gut immune system (Salminen *et al*., 1998). A possible approach is the usage of prebiotics in food. A prebiotic is a "non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or

activity of one or a limited number of bacteria in the colon, and thus improves host health" (Gibson and Roberfroid, 1995).

Roberfroid (2007) defined three aims a prebiotic compound must meet:

1. Resistance to gastric acidity, to hydrolysis by mammalian enzymes, and to gastrointestinal absorption
2. Fermentation by intestinal microflora
3. Selective stimulation of the growth and/or activity of those intestinal bacteria that contribute to health and well-being

The following groups of foods can be enriched with prebiotics (Gibson *et al*., 2004):
Dairy products, beverages, spreads, infant formulae and weaning foods, cereals, bakery products, confectionery, chocolates, chewing gum, savoury products, soups, sauces and dressings, meat products, dried instant foods, canned foods, and food supplements.

Prebiotics are also used in combination with probiotics and are then called synbiotics. The concept behind the synbiotics is the stimulation of the growth of probiotics using prebiotic sugars and giving these organisms a competitive advantage over indigenous species.

Not all non-digestible carbohydrates, including miscellaneous compounds such as resistant starch, non-starch polysaccharides (plant cell wall polysaccharides, hemicellulose, pectins, and gums) fulfil all three criteria and are thus not regarded as prebiotics (Gibson and Roberfroid, 1995). Resistance, the first criterion, does not necessarily mean that the prebiotic has to be completely indigestible but it should guarantee the availability of a significant amount of the compound in the intestine, especially in the large bowel, to work as a fermentation substrate (Roberfroid, 2007).
The main products from the fermentation of dietary residues, mainly carbohydrates, are short-chain fatty acids (SCFA), such as acetic, propionic and butyric acid. Beneficial changes in the gut lumen include a lowering of the pH, increase in faecal water, a decrease in its toxicity, and

sometimes- laxative properties- including softening of faeces. Stimulation of colonic mineral absorption of magnesium and calcium has also been described (Coudray, 1997).
Prebiotics have great potential to improve or maintain a balanced intestinal microflora, to enhance health and well-being. They are discussed to be a more practical and efficient way to manipulate the gut microflora than probiotics, unless the health-promoting species are present in the bowel. In 2007, Roberfroid designates three groups of carbohydrates with prebiotic activities: Inulin and oligofructose, galacto-oligosaccharides, and lactulose.

Studies on prebiotics have focused on inulin, fructo-oligosaccharides (FOS) and galacto-oligosaccharides/transgalactosylated oligosaccharides (GOS/TOS). In particular, the latter group of carbohydrates, has a history of safe commercial use, and is not classified under the category of novel foods (Macfarlane *et al.*, 2008).

Food that has not been used in this form before 5th May 1997 in the European Union or that is produced using a new kind of process, for example food (components) isolated or made from microorganisms, fungi or algae, or with a new or directly modified molecular structure are classified as Novel Food (EU Regulation Nr. 258/97).

Oligosaccharides can be purified and produced from different natural sources as displayed in **Figure 1**.

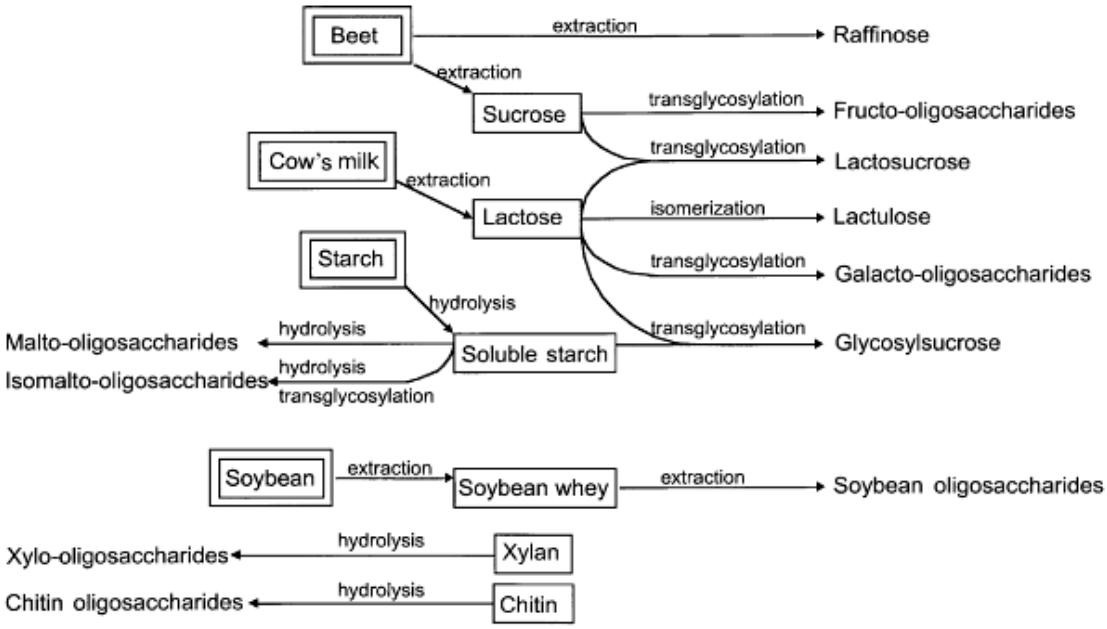

Figure 1: Schematic display of production processes of non-digestible oligosaccharides. (Sako *et al*., 1999)

GOS are derived from the milk sugar lactose which is found in the industrial bulk product whey, the by-product from cheese making. The production of these prebiotically active carbohydrates is becoming an important branch of the dairy industry.

1.2.1 Galacto-oligosaccharides

Galacto-oligosaccharides are found in different natural sources like human milk, fruits, vegetables, and honey and are formed by catalytic activities of many glycosidases and β-galactosidases. These enzymes not only hydrolyze the disaccharide lactose into the monosacchrides glucose and galactose, but also show a transferase activity which leads to the formation of oligosaccharides. For the commercial production of GOS from lactose the galactosyl-transferase activity of β-galactosidase is used (Sako *et al*., 1999).

A schema of the production of different possible oligosaccharides from lactose is shown in **Figure 1**. A specific scheme for galacto-oligosaccharide production is displayed in **Figure 2**.

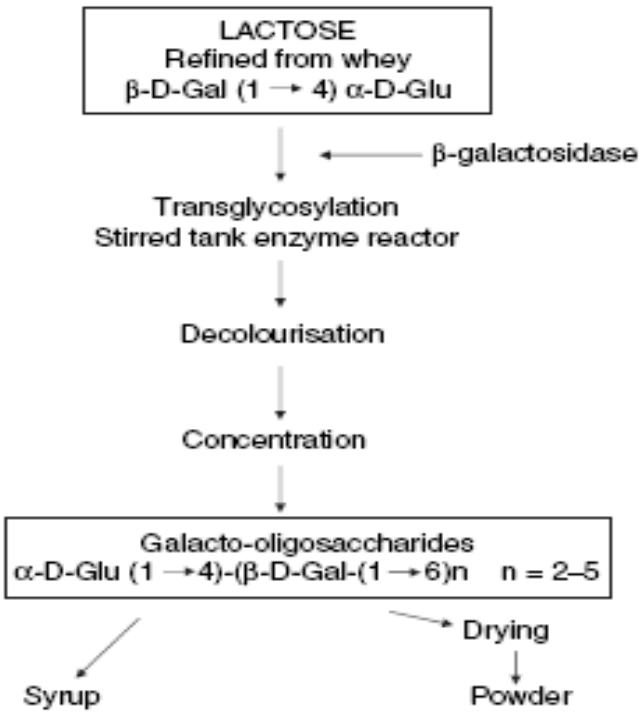

Figure 2: Production of galacto-oligosaccharides from lactose (Macfarlane *et al*., 2008). (Gal: galactose; Glu: glucose; Fru: fructose)

GOS are natural compounds of human milk and traditional yoghurt.

1.2.1.1 Characteristics and health effects of oligosaccharides

Due to the numerous outstanding qualities (water solubility or mild sweetness without being caryogen) oligosaccharides, including fructo-oligosaccharides, GOS and soybean oligosaccharides, are becoming more and more popular in the food industry.

Streptococcus mutans, a causative agent for dental caries, can not metabolize GOS. Their relative low sweetness (0.3 - 0.6 times as sweet as sucrose) is useful in food production when a bulking agent with reduced sweetness is desirable to enhance other food flavors. Compared with mono- and disaccharides, the higher molecular weight of oligosaccharides provides increased viscosity which leads to improved mouthfeeling. They can also be used to alter the freezing temperature and to control the amount of browning by Maillard reactions in heat-processed foods. Furthermore they provide a high moisture-retaining capacity, preventing excessive drying, and a low water activity which is convenient in controlling microbial contamination (reviewed by Crittenden and Playne, 1996). Bread is a suitable candidate for GOS inclusion. During

fermentation with yeast and baking of bread, GOS are not broken down, and complement the bread in excellent taste and texture (Sako *et al.*, 1999).

In 1994, Tomomatsu reviewed health effects of oligosaccharides. One large benefit of oligosaccharide ingestion comes from the increased population of indigenous bifidobacteria. They suppress the activity of putrefactive bacteria and reduce their formation of toxic fermentation products. Lactobacilli show similar effects, mainly in the upper gut. Toxic fermentation products can be:
ammonia (liver toxin), amines (liver toxin), nitrosamines (carcinogens), phenols and cresols (cancer promoters), estrogens (suspected carcinogens or breast cancer promoters) and secondary bile acids (carcinogens or active colon cancer promoters). The faeces consists for a large part of bacterial mass, the amount of toxic metabolites formed by colonic fermentation cannot be ignored. Further health effects are the prevention of constipation (production of SCFA stimulate the intestinal peristalsis), reduction of total serum cholesterol and increased ration of HDL-cholesterol in females (due to the niacin forming ability of bifidobacteria).

Macfarlane *et al.* (2008) reviewed health related effects of GOS and other prebiotics. For example, GOS were shown to circumvent the attachment of EPEC (enteropathogenic *E. coli*) to tissue culture cells more effective than inulin or FOS. The pathogen would bind to the soluble oligosaccharides and be displaced or flushed away from the gastrointestinal tract, rather than binding to surface oligosaccharides of host cells and initiating the infection process (Shoaf *et al*., 2006).

1.1.2.2 Structure of GOS

The linkage between galactose units, the efficiency of transgalactosylation and the compounds in the final products depend on the enzymes and the conditions used in the reaction. The different possibilities were reviewed by Sako *et al*. (1999): Glycoside bonds between two galactose units are mainly β(1→4) bonds when the β-gal derived from *Bacillus circulans* or *Cryptococcus laurentii*, and β1→6 bonds when enzymes derived from *Aspergillus oryzae* or *Streptococcus thermophilus* are used.

Production of GOS by β-galactosidases from *Lactobacillus reuteri* L103 and L461 resulted in the products with mainly β(1→6) and β(1→3) linkages (Splechtna *et al*., 2006), GOS production by *Sterigmatomyces elviae*, a yeast, produces mainly β(1→4) structures (Onishi *et al*., 1995). Due to the β-glycosydic linkage GOS are indigestible and stable to hydrolysis by α-amylase of human saliva and human gastric juices.

1.2.2 Inulin and Lactulose

There are more prebiotics apart from GOS which differ in their molecular structure. Two further ones are described here: inulin and lactulose a fructo-oligosaccharide and disaccharide, respectively.

1.2.2.1 Inulin

Inulin**,** belonging to the fructo-oligosaccharide group, is used by some plants as energy source and is therefore often found in roots. Plants like onions and garlic synthesize inulin and do not store energy in form of starch. Even though there are several inulin-producing plants like wheat, onion, banana, and garlic, only chicory (*Cichorium intybus*) is used for industrial inulin production. Chicory inulin and oligofructose are officially recognized as natural food ingredients in most European countries, and they have a self-affirmed GRAS status in the United States. In Europe, fructans are classified as Novel foods (Roberfroid, 2000).

Inulin is defined as a polydisperse carbohydrate mainly consisting of β(2→1)-fructosyl-fructose linkages (Roberfroid, 2000).

Inulin-type fructans are used as sugar substitutes, fat replacers (inulin), and as a means of providing texture, stabilizing foam. They are also used for improving the mouthfeeling in different products such as fermented dairy products; desserts such as jellies and ice creams; bakery products such as cookies, breads, and pastries; spreads; and infant formulas (Roberfroid, 2000).

1.2.2.2 Lactulose

Lactulose is a synthetically manufactured disaccharide made from fructose and galactose (β-linkage), showing a prebiotic effect. Lactulose is a specific substrate for lactic acid bacteria growing in the gut. Today, this disaccharide has found widespread application in the medical world as a laxative. The disaccharide can not be absorbed in the gut and draw therefore water osmotically into the bowel and can so lead to softening of the faeces, helping when suffering from constipation (Duphalac).
Ballongue *et al*. (1997) found evidence for the prebiotic effect of lactulose: the number of probiotic bacteria was increased, whereas the population of putrefactive bacteria and potential pathogens were significantly minimized. This resulted in a reduced activity of pro-carcinogenic enzymes, a global increase of short-chain fatty acids and an effect on pH and moisture of faeces. Tuohy *et al.* (2002) proved the increase of bifidobacteria in faeces after feeding lactulose.

1.3 Lactic acid bacteria and their β-galactosidases

1.3.1 Lactic acid bacteria

Lactic acid bacteria (LAB) are differentiated by biochemical characteristics belonging to the Gram positive, low in GC-content, generally not sporulating, non-respiring, in form of rod or cocci bacteria. They produce lactic acid as a major metabolic end product of carbohydrate fermentation.

They are divided into two large groups:

- homofermatative LAB catabolize glucose in the glycolyse pathway via pyruvate to lactic acid (for example *Lb. acidophilus*).
- Heterofermentative LAB use the pentose phosphate way and produce apart from lactic acid also acetic acid, CO_2, and ethanol (for example *Leuconostoc mesenteroides*).

LAB are historically linked to food conservation (acid production) and fermentation (flavour, texture). They are very important in food industry, as they are GRAS and are found as natural inhabitants in the human gut. LAB include the genera of *Lactobacillus, Leuconostoc, Pediococcus, Lactococcus, Streptococcus* and more.
Most probiotic strains belong to the genus *Lactobacillus,* such as *Lb. acidophilus, Lb. rhamosus, Lb. casei, Lb. johnsonii*. But also often used as probiotics are *B. lactis, B. longum* and *B. animalis* (Food info, University of Wageningen, NL. For the weblink refer to page 6).
The beneficial roles of LAB are summarized by Klaenhammer (1998) including immuno-stimulation and immuno-modulation, pathogen interference, exclusion and antagonism, production of bioactive materials, anti-carcinogenic and anti-mutagenic activities and general intestinal health.

1.3.2 β-Galactosidase

The enzyme β-galactosidase (β-gal, EC 3.2.1.23) belongs to the group of hydrolases, forming two products from one substrate. The enzyme catalyzes the hydrolysis and trans-galactosylation reaction of β-D-galactopyranosides. These enzymes are found widespread in nature: in microorganisms, plants, and animals. Microbial β-galactosidases are of major interest due to their easy application in bioprocess technology. The microbial enzymes have long been used for the hydrolysis of lactose, for increasing the digestibility of milk, or for improving the functional properties of dairy products. During the past decades, the β-galactosidase-catalyzed transgalactosylation has proved to be useful for

structural and functional modification of food materials, medicines, and other biologically active compounds. Additionally, microbial β-galactosidases are taken as a marker enzyme for coliforme bacteria which are indicators of the faecal pollution of water (Nakayama and Amachi, 1999).

1.3.2.1 Characteristics of β-galactosidases

Henrissat classified in 1991 glycosyl hydrolases into four families based on sequence similarities. According to this system, β-galactosidases are grouped into four families: 1, 2, 35 and 42. The members of family 2 include the β-galactosidase from *E. coli* (*lacZ*), the most extensively studied β-galactosidase. Unfortunately, the *E. coli* enzymes can not be used in the food industry, mainly because of the unacceptability of the bacterium (Nakayama and Amachi, 1999).

1.3.2.2 Substrates

Many chromogenic, fluorogenic, and luminogenic substrates that are specific for β-galactosidase have been developed. A summary can be found by Nakayama and Amachi (1999).

In nature, the substrate for β-galactosidases is lactose. In **Figure 3** both substrate structures together with their respective reaction products are displayed.

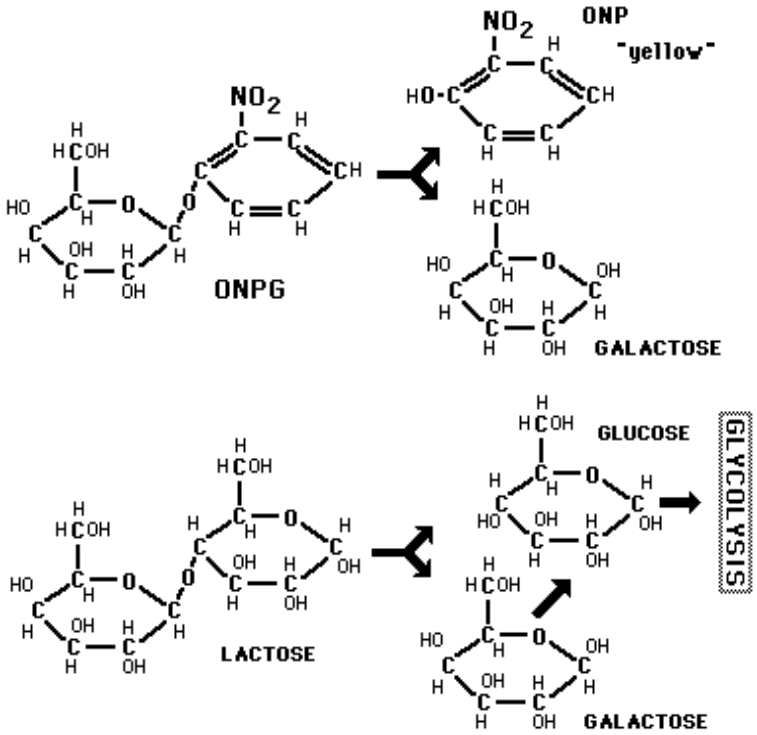

Figure 3: Substrate degradation by β-galactosidase:
2-Nitrophenyl-β-D-galactoside (*o*NPG) in comparison to the natural substrate lactose.
(http://www.science-projects.com/Lactase.htm)

Figure 4 shows the formation of GOS. During intramolecular transgalactosylation lactose is cleaved and the glucose molecule will bind immediately again with a different glycosidic linkage to the galactose-moiety, which is not replaced from the active site by a water molecule. This is how allolactose is formed. When high concentrations of lactose are present, lactose can act as a galactosyl acceptor (Nu), and the transfer product is galactosyllactose. The galactosyllactose can act again as galactosyl acceptor to produce tetrasaccharides (intermolecular transgalactosylation). Due to this transgalactosylation reaction a mixture of oligosaccharides constisting of di-, tri-, and higher oligosaccharides is produced from lactose (Nakayama and Amachi, 1999).

It appears that the enzyme is inhibited by its reaction products galactose and glucose (Nguyen *et al*., 2006; Onishi *et al*., 1995).

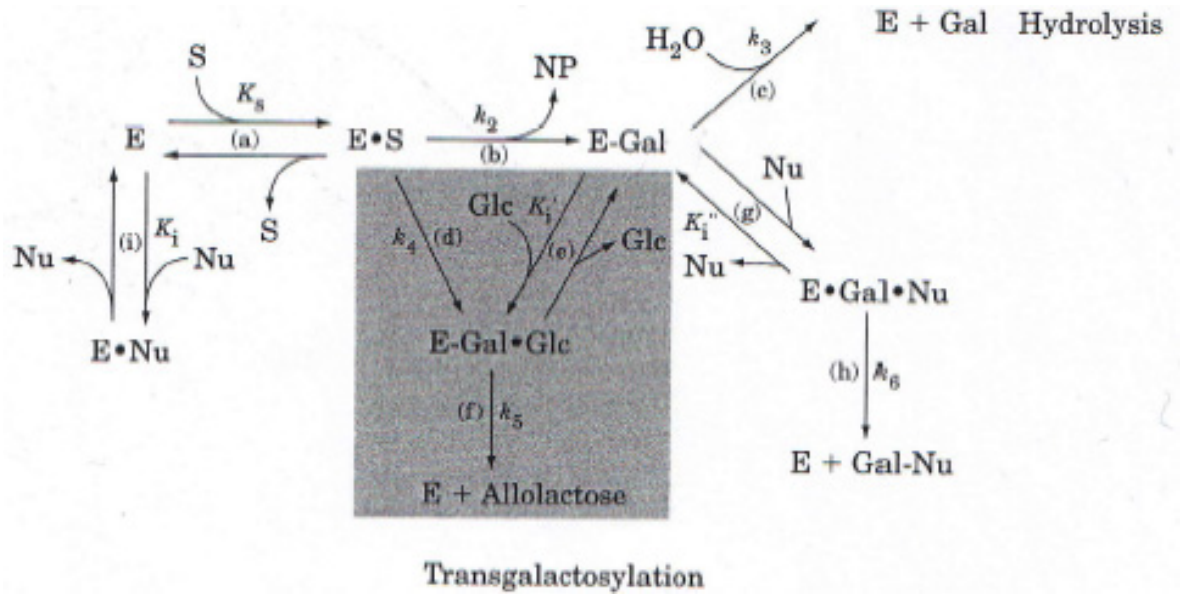

Figure 4: Hydrolysis and transgalactosylation of lactose catalyzed by *Escherichia coli lacZ* β-galactosidases
E: enzyme (β-galactosidases); S: substrate (lactose); Lac: lactose; Gal: galactose; Glc: glucose; Nu: nucleophil. (Nakayama and Amachi, 1999).

1.3.3 Lactose

Lactose (**Figure 5)** is the natural substrate for β-galactosidases. It is a disaccharide, consisting of a D-glucose and a D-galactose unit, linked by a β(1→4) glycosidic linkage.

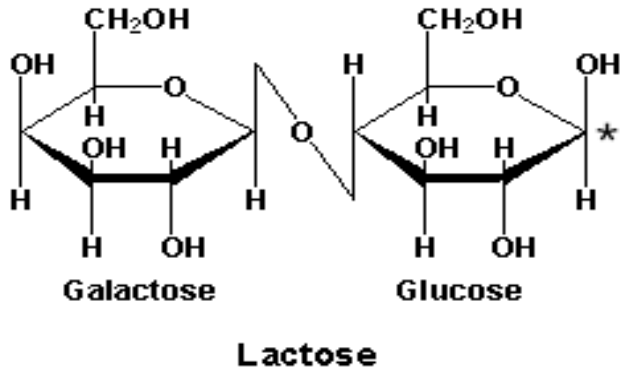

Figure 5: Chemical structure of lactose.

Lactose is naturally found in milk. Cow milk contains about 4.5 - 5.0 % (w/v), whereas more than 70 % of whey's dry matter is lactose. During cheese production almost all the lactose in milk remains in the whey and therefore hard cheese contains nearly no lactose. Remaining lactose is fully converted into lactic acid by fermentation reactions of starter bacteria. Lactose is an important product of the whey industry. It is used in feed and food, like baby food, cakes, biscuits, chocolate, sugar confectionary, soups and sauces (Schaafsma, 2008). Due to the very high lactose content in whey its disposal is difficult.

Lactose is not an unproblematic raw material. Most of the world's population is intolerant to lactose (**Figure 6**).

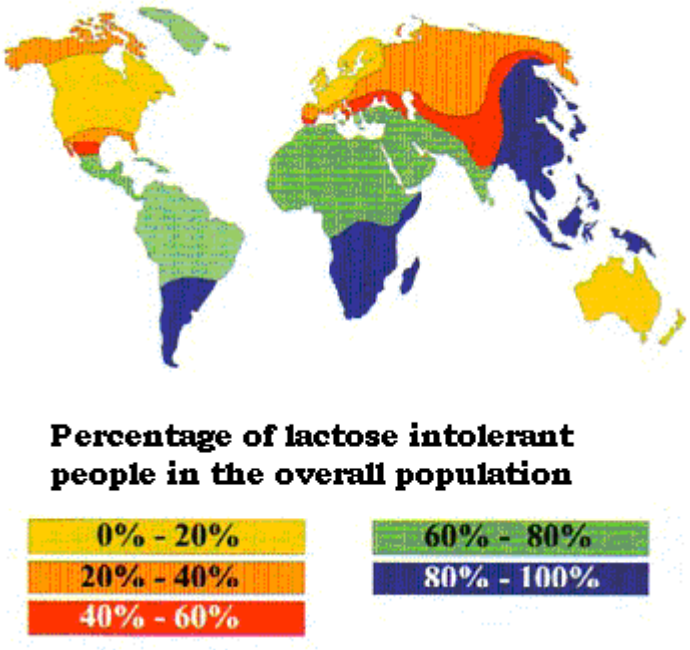

Figure 6: World's distribution of lactose intolerance
http://sigridgramlow.de/Sigrid%20Gramlow/Histamin.html

The β-gal, bound to the mucosal membrane of the small intestine, is totally active in infants but activity decreases during life time. Remarkably, humans belonging to the West European Caucasian race and to some isolated Indian and African tribes maintain a high lactase activity throughout life (lactase persistence). The decrease in activity is independent from lactose ingestion, still after the weaning period. Then, consumption of small amounts of lactose can lead to diarrhoea, intestinal

gas, cramps and bloating, caused by undigested lactose going through the gastrointestinal tract and serving as substrate for the colonic bacteria. They excrete gas, a process known as anaerobic respiration. Galactosemia, if untreated, can lead to fatal liver damage and blindness, resulting from osmotic effects in the lens of the eye, caused by accumulation of galactitol. Deficiency in one or more of the enzymes involved in lactose digestion and galactose metabolism can lead to metabolic disturbances known as lactose intolerance and galactosemia and lactose has to be eliminated from the diet completely (Schaafsma, 2008). But milk is one of the best calcium providers. Problems can occur when stopping consuming milk products, like increased risk of osteoporosis. A possible way out of this problem is consuming lactose free milk products which are available in the supermarket nowadays.

2. Material and Methods

2.1. Material

All chemicals used for this work were purchased in the highest quality available from Fluka (Buchs, Switzerland), Merck (Darmstadt, Germany), Roth (Graz, Austria) and Sigma-Aldrich (St. Louis, USA), unless otherwise stated. Glucose oxidase (GOD) from *Aspergillus niger* (23 U/mg) was purchased from Sigma-Aldrich and horse radish peroxidase (POD) with a specific activity of 181 U/mg was obtained from Fluka.

2.1.1 Media

Media components solved in the appropriate amount of water and autoclaved at 121°C with 1 bar overpressure for 20 minutes. 15 g/L agar was added to solidify the medium. The agar plates were stored at 4°C.

MRS medium (*Lactobacillus* agar according to De Man, Rogosa and Sharpe)

Casein peptone, tryptic digest	10.0 g/L
Meat extract	10.0 g/L
Yeast extract	5.0 g/L
Dipotassium hydrogen phosphate (K_2HPO_4)	2.0 g/L
Diammonium hydrogen citrate	2.0 g/L
Sodium acetate	2.0 g/L
Magnesium sulphate ($MgSO_4$ x $7H_2O$)	0.2 g/L
Manganese sulphate ($MnSO_4$ x H_2O)	0.5 g/L
(Agar	15.0 g/L)

Adjusted to a pH of 6.5 using NaOH

Glucose (1 %) or lactose (2 %) was added separately after autoclaving.

Corynebacterium Agar

Casein peptone, tryptic digest	10.0 g/L
Yeast extract	5.0 g/L
NaCl	5 g/L
(Agar	15.0 g/L)

Adjusted to a pH of 6.5

Glucose (1 %) or lactose (2 %) was added separately after autoclaving.

M17 Agar (DIFCO)

Casein peptone, tryptic digest	5.0 g/L
Soybean peptone	5.0 g/L
Meat extract	5.0 g/L
Yeast extract	5.0 g/L
Ascorbic acid	0.5 g/L
Magnesium sulphate ($MgSO_4$ x $7H_2O$)	0.25 g/L
Disodium-β-glycerophosphate	19.0 g/L
(Agar	11 g/L)

2.1.2 Solutions

Glucose/Lactose stock solution

- 20 % glucose solution
- 12.5 % lactose solution

Solutions for the *o*NPG assay

- 22 mM *o*NPG solution (*o*-Nitrophenyl-β-D-galactopyranoside, Sigma-Aldrich): 0.06628 g/L were dissolved in 50 mM sodium phosphate buffer. This solution was divided into aliquots, wrapped in foil and stored at -20°C.
- 0.4 M Na_2CO_3 solution: 42.396 g/L Na_2CO_3 were dissolved in water.

- 2 mM *o*NP (*o*-Nitrophenol) solution for determining the *o*NP standard curve: 0.00278 g/L were dissolved in 50 mM sodium phosphate buffer.

Solutions for the GOD/POD assay

- Solution1: 2.77 g KH_2PO_4 and 32.1 mg 4-amino-antipyridine were dissolved in 160 mL water and adjusted with 4 M NaOH to a pH of 7.0. 21 mg GOD (glucose oxidase from *Aspergillus niger*, 23 U/mg); and 1.5 mg POD (peroxidase type II from Horseradish, 181 U/mg) were added and filled up to a final volume of 200 mL. The bottle was wrapped in foil and stored at 4°C. This solution is stable for about 3 weeks.
- Solution2: 211 mg phenol was dissolved in 4 mL water (560 mmol/L), wrapped in foil and stored at 4°C. This solution is stable indefinitely.
- Assay solution: 50 mL of Solution1 was mixed with 1 mL of Solution2. This solution was wrapped in foil and is stable for about 1 week at 4°C.
- For the glucose standard curve 0.1 g glucose was dissolved in 100 mL water to obtain the glucose standard solution (1 g/L).
- The 600 mM lactose solution was prepared by dissolving 21.6 g lactose in 100 mL sodium phosphate buffer (50 mM)

Solutions for pH-optimum measurements

20 mM acetic acid buffer: 0.3 g acetic acid was dissolved in 250 mL water and adjusted to the appropriate pH using NaOH (pH 4.0, 5.0, 5.5, 6.0).

20 mM sodium phosphate buffer: 0.78 g sodium phosphate was dissolved in 250 mL water and adjusted to the appropriate pH using NaOH (pH 6.0, 6.5, 7.0, 7.5).

20 mM glycerine buffer: 0.46 g glycerine was dissolved in 250 mL water and adjusted to the appropriate pH using NaOH (pH 7.5, 8.0, 8.5, 9.0).

Solution for the activation measurements:

All chemicals were dissolved in water.

NaCl	1M, 100 mM, 10 mM, 1 mM
KCl	1M, 100 mM, 10 mM, 1 mM
$MgCl_2$	100 mM, 10 mM, 1 mM
$MnSO_4$	100 mM, 50 mM 10 mM, 1 mM
2-Mercaptoethanol	100 mM, 10 mM, 1 mM
DTT	100 mM, 10 mM, 1 mM
Urea	100 mM, 10 mM, 1 mM
EDTA	100 mM, 10 mM, 1 mM

Starting from a 1 M stock solution, the other concentrations were obtained by dilution into the *o*NPG standard solution (22 mM).

Other solutions:

- 50 mM sodium phosphate buffer: 7.8 g/L NaH_2PO_4 were dissolved in water and adjusted to a pH of 6.5 with 4 M NaOH.
- 5 mg/mL PMSF (phenylmethylsulfonylfluoride) stock solution: 0.005 g were dissolved in 800 µL Ethanol (96 %) and 200 µL sterile H_2O.
- 1 M DTT (Dithiothreitol) stock solution: 0.152 g were dissolved in 1 mL sterile H_2O.

2.2. Methods

2.2.1. Standard assays and standard procedures

β-galactosidase activity measurement using the *o*NPG standard assay

The activity assay was carried out as described by Nguyen (Nguyen *et* al., 2006). 480 µL *o*NPG (22 mM *o*NPG in 50 mM sodium phosphate buffer) was incubated at 30 °C for 1 minute using a thermomixer. 20 µL of the enzyme solution was added and incubated at 30°C for 10 minutes under shaking at 600 rpm. The reaction was stopped by adding 750 µL 0.4 M Na_2CO_3 solution. The absorption of the yellow colour was measured photometrically at 420 nm.

This measurement was done in duplicates.

For the blank 20 µL buffer instead of enzyme solution was used.

The calculation of the enzyme activity is shown in **Equation 1**.

$$U/mL = \frac{Abs(420nm) - blank}{slope(o\text{NPstandardcurve})} * \frac{1}{t(\text{reaction})[\text{min}]} * \frac{V(\text{enzyme}) + V(o\text{NPG})}{V(\text{enzyme})} * (\text{enzymedilutionfactor})$$

Equation 1: Calculation of the β-gal activity using *o*NPG (22 mM) as substrate

One unit (U) of *o*NPG activity is defined as the amount of enzyme releasing 1 µmol of *o*NP per minute under the specific assay conditions.

This measurement was done to determine the activity of the enzyme β-galactosidase before and after the protein purification steps, and for the characterization measurements.

Determination of the *o*NP standard curve

Concentrations of 0, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mM *o*NP were prepared in sodium phosphate buffer, and the extinction at 420 nm was measured, as described by Nguyen (Nguyen, 2006). The standard curve was determined:

$$y = 1.7017x + 0.0285$$

The measurement was done in duplicates.

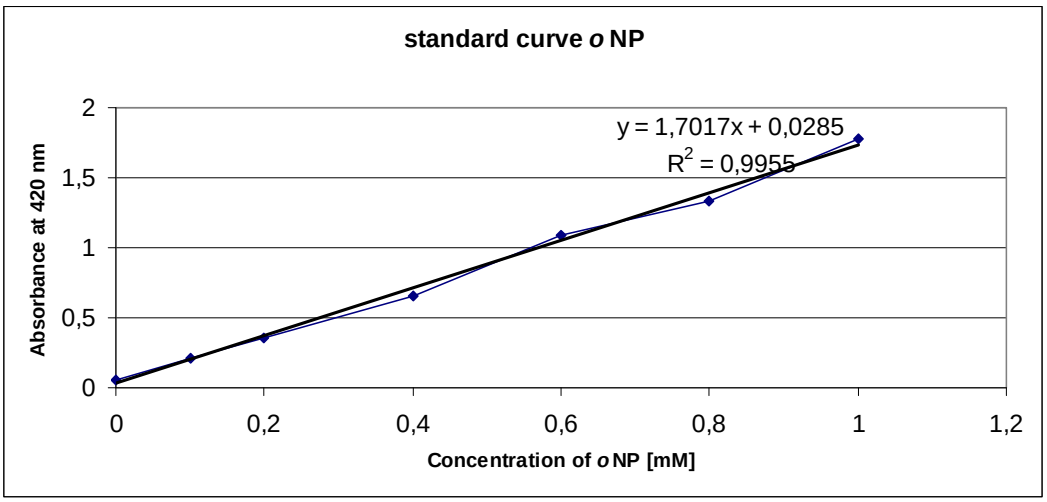

Figure 7: Standard curve of *o*-nitrophenol (*o*NP) for the determination of the β-galactosidase activity; *o*NP concentrations from 0 to 1.0 mM.

Lactose assay

β-galactosidase activity was determined using lactose as substrate, as described previously (Nguyen *et* al., 2006). 20 µL enzyme solution was added to 480 µL lactose solution (600 mM, dissolved in 50 mM sodium phosphate buffer, pH 6.5) and incubated at 30°C under shaking at 600 rpm using a thermomixer. After 10 min the reaction was stopped by heating the reaction mixture up to 99°C for 5 min. When the mixture has cooled to room temperature, the release of D-glucose is determined photometrically by using the standard GOD/POD assay (Kunst *et al*., 1988).

The measurement was done in duplicate. **Equation 2** was used to calculate the enzyme activity.

$$U/mL = c(\text{glucose}[mM]) * \left(\frac{1}{t(\text{reaction}[\text{min}])}\right) * \left(\frac{V(\text{enzyme}) + V(\text{lactose})}{V(\text{enzyme})}\right) * (\text{enzymedilutionfactor})$$

Note: $c_{glucose}$ [mM] = $c_{glucose}$[µmol/mL]

Equation 2: calculation of the β-galactose activity using lactose as substrate (600 mM)

One unit (U) of β-galactosidase activity is defined as the amount of enzyme releasing 1 µmol of D-glucose per minute under the described conditions.

Determination of the glucose concentration (GOD/POD standard assay)

For the determination of the glucose concentration the GOD/POD standard assay was used (Kunst *et al*., 1988). 60 µL enzyme solution and 600 µL assay solution (50 mL Solution1 and 1 mL Solution2; refer to page 22) were incubated in the dark at room temperature for 40 min and the absorbance of the mixture was measured photometrically at 546 nm. For the blank 60 µL buffer instead of enzyme solution was used. This measurement was done in duplicate.

The glucose concentration was calculated using **Equation 2** and **3**

$$c[g/L] = \left(\frac{Abs(546nm) - blank}{slope(\text{glucose standardcurve})}\right) * (\text{sampledilutionfactor})$$

$$c[mM] = \left(\frac{c[g/L]}{Mr(\text{glucose})}\right) * 1000$$

Equation 3: Calculation of the glucose content

Determination of the glucose standard curve

Six different glucose concentrations (0.0 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 g/L glucose) were prepared in water. 60 µL glucose solution and 600 µL Assay solution (50 mL Solution1 and 1 mL Solution2) were incubated in the dark at room temperature for 40 min and the absorbance of the mixture was measured photometrically at 546 nm. For the blank 60 µL buffer was used. This measurement was done in duplicate. The standard curve was determined:

$$y = 2.3982x + 0.0007$$

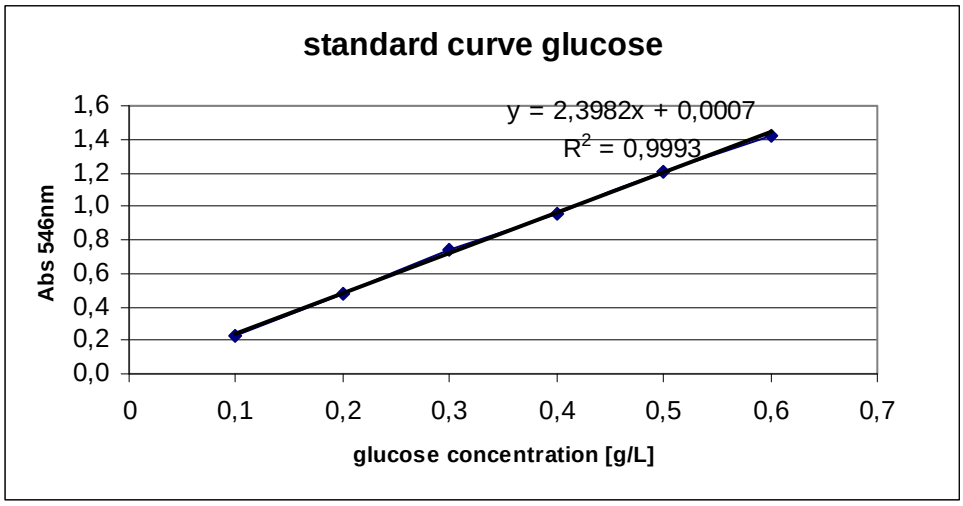

Figure 8: Standard curve of glucose for the determination of the glucose concentration; concentrations from 0.1 to 0.6 g/L glucose.

Cell disruption

Cells were disrupted using the french press, the sonicator, or the homogenizer.

French Press

French pressing was carried out 3 times/sample at 1000 psi.

Sonicator

To find the best conditions for disrupting Lactobacilli, the following combinations were tried out:

15 sec on / 45 sec off, 3 times, 6 times, or 12 times, 50 % power

30” on / 90” off, 3 times, 6 times, or 12 times, 50 % power

15” on / 45” off, 3 times, 6 times, or12 times, 80 % power

30” on / 90” off, 3 times, or 6 times, 80 % power

90” on / 90” off, 3 times, or 6 times, 50 % power

90” on / 90” off, 3 times, or 6 times, 80 % power

For each combination, protein and activity was measured. Best disruption efficiencies were achieved with the method 90” on/90” off, 6 times, 80 % power.

Homogenizer (APV System Mod 200)

Disruption of the cells was done at 0°C and pressures between 900-1000 psi. For one sample three disruption cycles were performed.

Protein determination

Protein concentrations were determined by the method of Bradford (Bradford, 1971) using bovine serum albumin as standard.

Therefore, 1 mL of Bradford solution was mixed with 20 µL enzyme solution and after 15 minutes incubation time the absorption was measured at 595 nm.

This measurement was done in duplicates.

Enzyme concentration by centrifugation

Fractions containing β-galactosidase activity were poured into an Amicon tube (Amicon Ultra 15 Centrifugal Filter Devices, Millipore), concentrated, and washed with buffer. The cut off size of the membrane was 10 kDa. Centrifugation was done at 4000 rpm for 10 min at 4°C. For washing, the chamber was filled with buffer again and centrifuged. This step was done three times. The volume after the last washing step was reduced to less than 1 mL.

Gel Elektrophoresis and active staining

SDS-PAGE

To control the purification progress SDS-PAGEs were carried out using the PhastGel System (Amersham). 2 times concentrated Laemmli buffer was added to the samples. After heating at 95°C for 5 minutes and a short centrifugation step the samples were applied on the gel following the PhastGel manual. Coomassie Blue staining was performed for visualization of the protein bands. Activity staining was carried out using 4-methylumbelliferyl-β-D-galactoside as the substrate as previously described (Nguyen *et al*., 2006).

Native-PAGE

Native PAGE was performed using the PhastGel System. Samples were prepared as described above with slight modifications (samples were not heated). Coomassie Blue staining was performed for visualization of the protein bands. Activity staining was carried out using 4-methylumbelliferyl-β-D-galactoside as the substrate as previously described (Nguyen *et al*., 2006).

2.2.2. Screening of lactic acid bacteria (LAB)

First screening

6 different LAB were screened on their ability to produce β-galactosidase. The strains were obtained from the culture collection (Food-biotechnology/BOKU). The first recultivation step was done on MRS agar plates at 37°C (*Lactobacillus rhamnosus* 241, *Lb. plantarum* 268, *Lb. plantarum* 322), on M17 agar plates at 30°C (*L. lactis* HA, *L. lactis* NZ9700), and on Corynebacterium agar plates at 37°C (*Enterococcus faecium*). All cultivation steps were performed under anaerobic conditions.

After the second recultivation step on agar-plates the plates were sealed with parafilm and stored at 4°C.

Inoculum for a 300 mL fermentation was done in the appropriate medium using 2 % glucose as carbohydrate source. 6 mL from the overnight culture was used to inoculate the 300 mL fermentation. The three lactobacilli strains were harvested after 24h fermentation time, reaching OD_{600} values of around 13. The cell pellet was harvested by centrifugation at 6000 rpm (SORVALL Evolution RC centrifuge) for 15 minutes at 4°C and was resuspended in 20 mL buffer (50 mM sodium phosphate buffer, pH 6.5). 5 mg/mL PMSF were added. After cell disruption the cell debris was separated by centrifugation and activity and protein content was measured from the cell free enzyme crude extract.

L. lactis HA, *L. lactis* NZ9700 and the *E. faecium* did not grow very well. The fermentation time was prolonged for another 24 hours. Sample treatment was performed as described above.

Non of the screened strains (*Lb. plantarum* 268*, Lb. plantarum* 322*, Lb. rhamnosus* 241*, L. lactis* HA*, L. lactis* NZ9700*, Ec. faecium*) showed high β-galactosidase activity and were therefore not chosen for further experiments.

Second screening

Three lactobacilli *(Lb. pentosus* KVBST10-1, *Lb. bulgaricus* TISTR451, *Lb. sakei* TISTR911*)* obtained from the Kasetsart University (Bangkok, Thailand) were screened on their ability to produce β-galactosidase activity.

The recultivation was done at 37°C under anaerobic conditions in two steps on MRS media using 2 % lactose as carbohydrate source. *Lb. sakei* was grown in M17 media (2 % lactose), too. 3 mL from the overnight culture was taken as inoculum for a 900 mL fermentation in MRS media (2 % lactose, *Lb. pentosus* and *Lb. bulgaricus*). *Lb. sakei* was fermented in both media, MRS and M17 (both 1 % lactose). *Lb. sakei* did not grow well, neither on MRS nor on M17, so its fermentations started not until 48 hours incubation of the preculture.

For a first activity check 1 mL of the overnight culture was sonicated (90 seconds on/90 seconds off, 6 times, 80 % power). After cell disruption the cell debris was separated by centrifugation and activity and protein content was measured from the cell free enzyme crude extract. *Lb. pentosus* showed *o*NPG activity, *Lb. bulgaricus* did not show activity for this substrate. Testing *Lb. bulgaricus* for β-galactosidase activity when grown at different incubation temperatures (25°C, 30°C, 37°C and 42°C) it did not show any activity either (data not shown).

After 24 h the pH values during *Lb. pentosus* and *Lb. bulgaricus* fermentations decreased from 6.5 to 4.0. Optical density measurements (OD_{600}) proofed a better growth of *Lb. pentosus* than *Lb. bulgaricus*. *Lb. sakei* grew better on MRS than on M17.

The cell pellets were harvested by centrifugation at 5000 rpm for 10 min at 4°C and the wet biomass were resuspended in about 45 mL buffer.

An aliquot of 1 mL was sonicated after addition of PMSF. The debris was discarded by centrifugation and the β-galactosidase activity of the crude extract was measured using the *o*NPG standard assay. *Lb. pentosus* was the only strain which showed activity and was therefore chosen for further experiments. The *Lb. pentosus* cell suspension was french pressed. The β-gal activity after french press was much lower than after sonication. So, in this case french pressing is not the appropriate way disrupt lactobacilli.

Third screening

Three lactobacilli *(Lb. rhamnosus* 177*, Lb. amylovorus and Lb.* L662*)* were screened on their ability to produce β-galactosidase activity. The strains were obtained from the culture collection (Food-biotechnology/BOKU). The recultivation was done in two steps on MRS media using 2 % lactose as carbohydrate source, at 37°C under anaerobic conditions. The strain *Lb.* L662 could not be identified by an API 50 CH test (Biomérieux).

For a rough overview for β-galactosidase activity 1 mL of each culture was sonicated (90"/90", 80 %/80 cycles), and activity of the enzyme crude extract was measured after discarding the cell debris by centrifugation. *Lb.* L662 showed highest activity and was chosen for further experiments.

2.2.3. Cultivation of *Lb. pentosus* and *Lb.* L662

Cultivation of *Lb. pentosus*

To obtain enough biomass *Lb. pentosus* was cultivated at 37°C under anaerobic conditions in MRS medium containing 2 % lactose. Biomass was harvested and resuspended in 50 mM sodium phosphate buffer (pH 6.5). After homogenizing (APV System Mod 200) the sample for three times the cell debris was separated by ultracentrifugation (30.000 rpm at 4°C, 30 min). The supernatant was used for enzyme purification (ammonium sulphate precipitation, hydrophobic interaction chromatography and affinity chromatography).

This fermentation was done three times. Biomass was homogenized, cell debris was separated by ultracentrifugation and each cell free crude extract was purified separately.

Cultivation of *Lb.* L662

Lb. L662 was cultivated in MRS medium at 37°C under anaerobic conditions containing 2 % lactose. Biomass was harvested and resuspended in 50 mM sodium phosphate buffer (pH 6.5). After homogenizing the sample for three times the cell debris was separated by ultracentrifugation. The supernatant was used for enzyme purification (ammonium sulphate precipitation, hydrophobic interaction chromatography and affinity chromatography).

2.2.4. Purification

The purification steps were identical for the enzymes from both strains (*Lb. pentosus* and *Lb.* L662).

2.2.4.1 Ammonium sulphate precipitation

Groups of similar proteins precipitate when the salt concentration of a solution changes. Each protein precipitate is dissolved in fresh buffer and assayed for the amount of β-galactosidase using the *o*NPG standard assay and for total protein content using the Bradford method.

The first purification step was precipitation using ammonium sulphate.

The ammonium sulphate precipitation was done as described in literature (Cutler, 2004). In the first trial 5 different fractions were obtained (0 – 30 %, 30 – 50 %, 50 – 65 %, 65 – 80 % and 80 – 90 %). After β-gal activity measurement only 2 fractions were collected (0 – 50 %, 50 – 70 % ammonium sulphate saturation).

Ammonium sulphate was slowly added in small portions to the crude extract. The extract was stirred during the precipitation and was cooled by ice water. The precipitate was obtained by centrifugation (6500 rpm, 25 min at 4°C) and solved again in the smallest amount of sodium phosphate buffer (pH 6.5). More ammonium sulphate was added to the supernatant to obtain another fraction.

Fractions collected were 0 – 50 %, and 50 – 70 % saturation. This was tested in a first trial as described above.

2.2.4.2 Hydrophobic Interaction Chromatography

A soluble protein in the “mobile phase” passes the “stationary phase” (column-material) and binds to the hydrophobic parts of the column. The binding happens in the presence of high salt concentrations and elution is done in the absence of salt. This allows a separation of differently charged proteins.

The second purification step was the purification by hydrophobic interaction chromatography (HIC). Ammonium sulphate and the column material phenyl sepharose gel was used. The column had a volume of 5 mL. For each run, 5 mL enzyme solution were applied using 100 % buffer A (50 mM sodium phosphate buffer with 2 M ammonium sulphate). Buffer B was 50 mM sodium phosphate buffer. The buffers were degased before usage and the column was washed with buffer A before the enzyme was applied to the column.

Lb. pentosus:

For the first run the fraction size was set to 8 mL, flow rate to 3 mL/min and the elution gradient from 100 % buffer A to 100 % buffer B was reached after 10 times of the column volume (50 mL). For further runs the conditions were slightly changed: 1.5 mL/min flow rate, 3 mL/fraction size.

Each fraction was tested for β-galactosidase activity and protein content using the *o*NPG standard assay and the Bradford method, respectively. The appropriate fractions where then pooled, washed and concentrated by centrifugation. The different pools were further purified by affinity chromatography.

Lb. L662

The flow rate was 1.5 mL/min, fraction size was set to 3 mL/fraction, the elution gradient from 100 % buffer A to 100 % buffer B was reached after 10 times of the column volume.

Depending on their activity and protein content appropriate fractions were pooled, washed and concentrated by centrifugation. For the following characterization measurements or lactose conversion experiments the β-gal was not further purified due to low protein content. Only for the

determination of the K_m-value the enzyme was applied to an affinity chromatography column.

2.2.4.3 Affinity chromatography

Affinity chromatography is another chromatographic method to separate biochemical substances. This method is based on a highly specific biologic interaction such as between enzyme and substrate, antigen and antibody, or receptor and ligand. This chromatography step was used to purify the β-gal to homogeneity. The process can be seen as a specific binding action of the enzyme of interest to a solid or stationary phase. Other molecules, which do not show the desired property, will not bind and are therefore eluted immediately.

The third purification step was the purification by affinity chromatography.

Buffer A was 50 mM sodium phosphate buffer, buffer B was 50 mM sodium phosphate buffer + 2 M NaCl. Both buffers were set to pH 6.0, and were degased before usage.

For each run, 4-5 mL were applied to 4 % crosslinked beaded agarose immobilized with *p*-aminobenzyl-1-thio-β-D-galactopyranoside. The column volume was 2 mL and was washed with buffer A before the enzyme was loaded.

Lb. pentosus:

For the first run, flow rate was set to 1 mL/min and fraction size was 1.5 mL. The elution gradient from 100 % buffer A to 100 % buffer B was reached after 9 times of the column volume. For further purifications, the conditions were slightly changed to 0.5 mL/min flow rate. The elution gradient was reached after 10 times of the column volume.

The obtained fractions were tested for β-galactosidase activity and protein content (*o*NPG standard assay and Bradford). Depending on their activity and protein content, different pools were formed (for characterization measurements, conversion test, determination of the K_m-value). Each pool was separately washed and concentrated by centrifugation.

Lb. L662:

Before applying the enzyme to the column, the solution was centrifuged at 5500 rpm for 10 minutes to get rid of precipitated protein, as the enzyme was stored at 4°C before this chromatography step for more than a month. About 3.5 mL were applied.

The flow rate was set to 0.4 mL/min, fraction size to 1.5 mL. The elution gradient from 100 % buffer A to 100 % buffer B was set to 15 times of the column volume.

The obtained fractions were tested for β-gal activity and protein content (*o*NPG standard assay and Bradford). The pool after washing and concentration by centrifugation was taken for K_m-value measurements.

2.2.5. Characterization

The following parameters for the two β-galactosidases from **Lb. pentosus** and *Lb.* L662 were determined: temperature- and pH- optimum, temperature- and pH- stability, steady state kinetic parameters (K_m, V_{max}, k_{cat}), activity on five other substrates than lactose and *o*NPG, galacto-oligosaccharide (GOS) formation ability and the effect of different ions and chemicals.

2.2.4.1 Determination of the temperature optimum

For oNPG

The activity of the β-galactosidase at different temperatures was determined. Therefore the *o*NPG standard assay was performed using different incubation temperatures:

20°C, 25°C, 30°C, 37°C, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C and 70°C.

For each temperature a blank sample was tested, too. As reference for activity calculation the standard assay at 30°C was set to 100 % activity.

For lactose

The lactose assay was performed as described on page 25, using the same temperature range as for the chromogenic substrate *o*NPG:

20°C, 25°C, 30°C, 37°C, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C and 70°C. In general, a ten times lower activity for lactose compared to *o*NPG was assumed.
For each temperature a blank sample was assayed, too. As reference for activity calculation the standard assay at 30°C was set to 100 % activity.

2.2.4.2 Determination of the pH optimum

For the determination of the pH optimum 3 different buffers were prepared. Each buffer was set to 4 different pH values (using 4 M and 1 M NaOH). In total 12 different pH values were tested to find the optimum pH for the β-galactosidase.
Acetic acid buffer, pH 4.0, 5.0, 5.5, 6.0
Sodium phosphate buffer, pH 6.0, 6.5, 7.0, 7.5
Glycerine buffer, pH 7.5, 8.0, 8.5, 9.0

For oNPG
22 mM *o*NPG was dissolved in the appropriate buffer and the *o*NPG standard assay was performed using these new solutions. As reference for activity calculation the standard assay in sodium phosphate buffer (pH 6.5) was set to 100 % activity.

For lactose
Lactose was dissolved in the appropriate buffer and the lactose assay was performed using these new solutions. As reference for activity calculation the standard assay in sodium phosphate buffer at pH 6.5 was set to 100 % activity.

2.2.4.3 Determination of the temperature stability

For the determination of the temperature stability the enzyme was stored in 50 mM sodium phosphate buffer (pH 6.5) at different temperatures. The activity was measured using the *o*NPG standard assay before and after storage:

Lb.pentosus β-gal: 0.5, 2.5, 4.5 h, 1 day, 1.5 days, 2 days, 2.5 days, 3 days, and 4 days

Lb. L662 β-gal: 0.5, 2, 4, 6 h, 1 day, 1.5 days, 2 days, 2.5 days, 3 days, and 4 days

The following temperatures were tested:

4°C, room temperature (about 20-22°C), 30°C, 37°C and 40°C; at the higher temperatures (30°C, 37°C, 40°C) 2 mM $MgCl_2$ was added for better enzyme stability (later marked as 30°C*, 37°C*, 40°C*).

2.2.4.4 Determination of the pH stability

For the determination of the pH stability the enzyme was stored at 37°C in the same buffers as used for the pH optimum:

Acetic acid buffer, pH 4.0, 5.0, 5.5, 6.0

Sodium phosphate buffer, pH 6.0, 6.5, 7.0, 7.5

Glycerine buffer, pH 7.5, 8.0, 8.5, 9.0

Activity measurements for the *Lb. pentosus* β-gal using the *o*NPG standard assay were done after 0.75, 1.5, 3.0 and 5.15 hours, 1 day, 1.5 days, 2 days, 2.5 days, and 3 days. Residual activity of the *Lb.* L662 β-galactosidase was measured after 0.5, 2.0, 4.0, 6.0 hours, 1 day, 1.5 days, 2 days, and 3 days.

2.2.4.5 Determination of the K_m-value

The K_m-value is the Michaelis-Menten constant calculating the enzyme-substrate relation. This constant describes the substrate concentration for which the reaction velocity is half of the maximum.

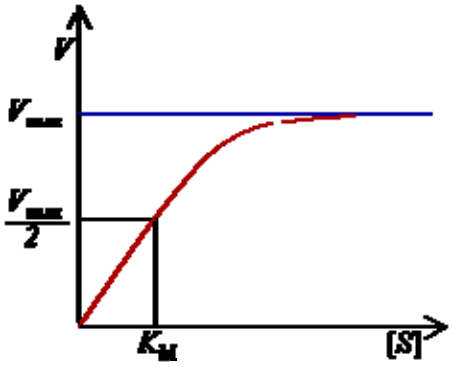

Figure 9: Michaelis-Menten Plot: reaction rate v_0 (velocity) versus substrate concentration [S]

For the determination of the K_m-value the β-galactosidase activity was measured using different substrate concentrations (either *o*NPG or lactose).

Starting from the standard 22 mM *o*NPG solution lower concentrations were prepared: 22 mM, 15 mM, 12 mM, 8 mM, 5 mM, 3 mM, 1 mM, 0.1 mM and 0 mM *o*NPG (in sodium phosphate buffer, pH 6.5).

Starting from the standard 600 mM lactose solution lower concentrations were prepared: 600 mM, 300mM, 150 mM, 100 mM, 75 mM, 50 mM, 20 mM, 10 mM, 1 mM, 0.1 mM and 0 mM lactose (in sodium phosphate buffer, pH 6.5)

For oNPG

The *o*NPG standard assay was done using these newly prepared substrate solutions and the enzyme activity was determined. As reference for activity calculation the standard assay using 22 mM *o*NPG was set to 100 % activity.

For lactose

The lactose assay was performed using the newly prepared lactose solution and the activity of the enzyme was determined. As reference for activity calculation the standard assay using 600 mM lactose was set to 100 % activity.

2.2.4.6 Determination of the activity of β-gal for 5 other substrates

This test was performed to test the enzyme ability to convert other substrates than *o*NPG, a β-D-galactopyranoside. Standard solutions (22 mM) of 4-N-phenyl-β-D-cellubioside, 4-N-phenyl-α-D-galactopyranoside, 4-N-phenyl-β-D-mannopyranoside, 4-N-phenyl-β-D-xylopyranoside, and 4-N-phenyl-β-D-glucopyranoside were prepared and β-gal activity was measured using the standard assay protocol.

As reference for activity calculation the standard assay using β-D-galactopyranoside (*o*NPG) was set to 100 % activity.

2.2.4.7 Transgalactosylation activity

This test should proof the enzyme ability of converting lactose into galacto-oligosaccharides (GOS). The initial lactose concentration should decrease during the reaction time due to lactose hydrolysis into the monosaccharides glucose and galactose and a parallel formation of GOS. Purified β-galactosidase from the two lactobacilli (5 *o*NPG units per mL reaction mixture) were used for the discontinuous formation of galacto-oligosaccharides (GOS) at 30°C using 205 g/L lactose in 50 mM sodium phosphate buffer (pH 6.5, 2 mM $MgCl_2$) as substrate.

Samples were taken every hour, heated up to 99°C and centrifuged. The supernatant was stored at -20°C.

In total, 10 samples were collected (0, 1, 2, 3, 4, 5, 6, 7, 8, and 24 hours). The samples were used for further measurements: determination of the glucose content by GOD/POD standard assay, D-galactose determination (Lactose and D-Galactose Rapid KIT, Megazyme), high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and capillary electrophoresis (CE) for determining the

composition of the GOS mixture. For HPAEC-PAD, the samples were diluted in high quality water.
The galactose content determination was carried out following the protocol of the manufacturer.

Samples for CE were derivatised as described previously (Splechtna *et* al. 2006). 10 µL sample in the appropriate dilution were evaporated using the Speed Vac at 60°C for 2 hours and derivatised with 20 µL aminopyridine (1 g aminopyridine in 600 µL methanol and 470 µL acetic acid). The samples were incubated for 15 minutes at 90°C and dried again, under the same conditions as described above. After addition of 25 µL borohydride (59 mg $NaCNBH_3$ in 1 mL acetic acid (30 %)) and incubation for 30 min at 90°C the sample was dried again. The obtained derivatised sample was resuspended in 200 µL water and applied to the CE.

2.2.4.8 Effects of various reagents and cations

Na: 1M, 100 mM, 10 mM, 1 mM (using NaCl)
K: 1M, 100 mM, 10 mM, 1 mM (using KCl)
Mg: 10 mM, 1 mM (using $MgCl_2$). The only possible measurements were 1 mM and 10 mM as the other solutions gave precipitations after addition of the enzyme solution.
Mn: 1 mM (using $MnSO_4$). The only possible measurement was 1 mM as the other solutions gave precipitations after addition of enzyme solution.
2-Mercaptoethanol: 100 mM, 10 mM, 1 mM
DTT: 100 mM, 10 mM, 1 mM
Urea: 100 mM, 10 mM, 1 mM
EDTA: 100 mM, 10 mM, 1 mM

Standard solutions were prepared and appropriate volumes of these solutions were added to the *o*NPG standard solution. A example is given here: 500 µL from a 1 M stock solution was added to 5 mL *o*NPG solution, giving a final concentration of 100 mM.

As reference for activity calculation the standard *o*NPG assay was set to 100 % activity.

3. Results

3.1. Screening of LAB

A first screening was carried out to test the lactic acid bacteria on high β-galactosidase activity. No β-galactosidase activity was found when *o*NPG was used as substrate. Probably these strains are using a phospho-β-galactosidase to use the phosphorylated lactose as carbon source.
A summary of the activities, both for the cell crude extract obtained after cell disruption using the french press and for the cell free supernatant obtained after cell disruption using the french press and centrifuging the cell debris off, is displayed in **Table1**.

Table 1: Summary of the measured activities on *o*NPG in the cell free crude extract extract and in the crude extract after french pressing for *Lb. plantarum* 268*, Lb. plantarum* 322*, Lb. rhamnosus* 241*, Lactococcus lactis* HA*, L. lactis* NZ9700 and *Enterococcus feacium*.

strains	Activity from the cell crude extract (U/mg)	Activity from the cell free supernatant (U/mg)
Lb. plantarum 268:	10.4	0.26
Lb. rhamnosus 241*:*	14.1	0.13
Lb. plantarum 322*:*	10.9	0.11
Lactococcus lactis HA	-	-
L. lactis NZ9700	-	-
Enterococcus faecium	-	-

The activity should be found in the cell free supernatant and not in the cell crude extract, french pressing was proven not to be the appropriate method for disrupting these two lactobacilli, so sonication or homogenizing was used for cell disruption.

In the second screening three LAB (*Lb. pentosus* KVBST10-1, *Lb. bulgaricus* TISTR451, *Lb. sakei* TISTR911) from Kasetsart University were screened for β-galactosidase activity. Only *Lb. pentosus* showed β-gal activity (6 U/mg) for the *o*NPG substrate. By measuring the activity of the media supernatant from *Lb. pentosus* and *Lb. bulgaricus* it was proven that the enzyme was not secreted into the media, as no activity was determined (data not shown).

In a third screening *Lb. rhamnosus* 177*, Lb. amylovorus, and Lb.* L662 were tested for β-galactosidase activity and the β-gal from *Lb.* L662 was found most active. This *Lactobacillus* strain could not be identified by an API 50 CH test (Biomérieux).

A summery of growth characteristics and β-galactosidase activity from all screened *Lactobacillus* strains is displayed in **Table 2**.

Table 2: Growth characteristics and β-galactosidase activity of *Lactobacillus* strains grown on lactose. Activity was determined from the cell free supernatant after cell disruption by sonication and centrifugation.

Strain	Growth on lactose[a]	β-galactosidase activity[b]	
		Volumetric activity (U/mL)	Specific activity (U/mg)
Lb. plantarum 268	+	0.15	0.26
Lb. plantarum 322	+	0.06	0.11
Lb. rhamnosus 241	+	0.06	0.13
Lb. rhamnosus 177	+	0.03	0.003
Lb. amylovorus	+	0.15	0.04
***Lb.* L662**	**+**	**30**	**4**
***Lb. pentosus* KVBST10-1**	**+**	**30.8**	**5.6**
Lb. bulgaricus TSTR451	+/-	0.22	0.5
Lb. sakei TSTR911	-	-	-

[a]All strains were grown on MRS medium containing 2 % (w/v) lactose for 24 h at 37°C. The growth was evaluated as no growth (-), moderate growth (+/-) and good growth (+).
[b]β-Galactosidase activity was measured at 30°C with *o*NPG as the substrate under standard assay conditions from cells harvested after 24 h of growth in MRS medium supplemented with lactose as sole carbon source.

3.2. Lactobacillus pentosus

3.2.1. Cultivation

The cultivation of *Lb. pentosus* was done in three steps. Each following purification step was done separately. A summary of measured β-galactosidase activities is displayed in **Table 3**.

Table 3: Summery of the three fermentations of *Lb. pentosus* with their corresponding activities for β-galactosidase

fermentation volume [L]	crude extract volume [mL]	specific activity (U/mg)	volumetric activity/ total units (U)
0.9	100	4.7	382
4.5	150	6.0	3190
4.0	100	8.7	2287
Total			**5477**

The enzyme crude extract obtained from the first fermentation was used for the first tryouts of ammonium sulphate precipitation.

3.2.2. Purification

In the first step five different fractions were collected (0 – 30 %, 30 – 50 %, 50 – 65 %, 65 – 80 % and 80 – 90 % ammonium sulphate saturation) and the activity was determined by the standard *o*NPG assay (data not shown). In further purification steps only 2 fractions were collected: 0 – 50 % and 50 – 70 % saturation which were then further purified by hydrophobic interaction chromatography and affinity chromatography.

The last two fermentations of *Lb. pentosus* yielded in about 5500 units in total in the cell free crude extract after cell disruption and ultracentrifugation. After the three purification steps (ammonium sulphate precipitation, hydrophobic interaction chromatography and affinity chromatography) 110 units in three different pools were left. The

purification yield was calculated to be 2.0%. The specific activity increased from about 10 U/mg to 97 U/mg (K_m-pool, pool of the best purified fractions). An overview over the β-galactosidase activities is given in **Table 4**.

Table 4: Overview over the three pools obtained after affinity chromatography (β-galactosidase *Lb.pentosus*)

Pool	Specific activity (U/mg)	total units
Crude extract	10	5500
EC	37.8	41.0
CH	74.0	31.1
K_m	97.0	38.6
Total		110.7

EC: pool for enzymatic lactose conversion experiments; CH: pool for β-gal characterization; K_m: pool for steady state kinetic measurements

3.2.3. Gel Electrophoresis

The following PAGEs proof that the β-galactosidase from *Lb. pentosus* is a hetero-dimer with a molecular mass of 107 kDa. The large subunit showed a protein band at approximately 72 kDa, the smaller one at 35 kDa. Active-staining proofed that the large subunit was also active without building up the hetero-dimer complex (**Figure 10A and 10B**).

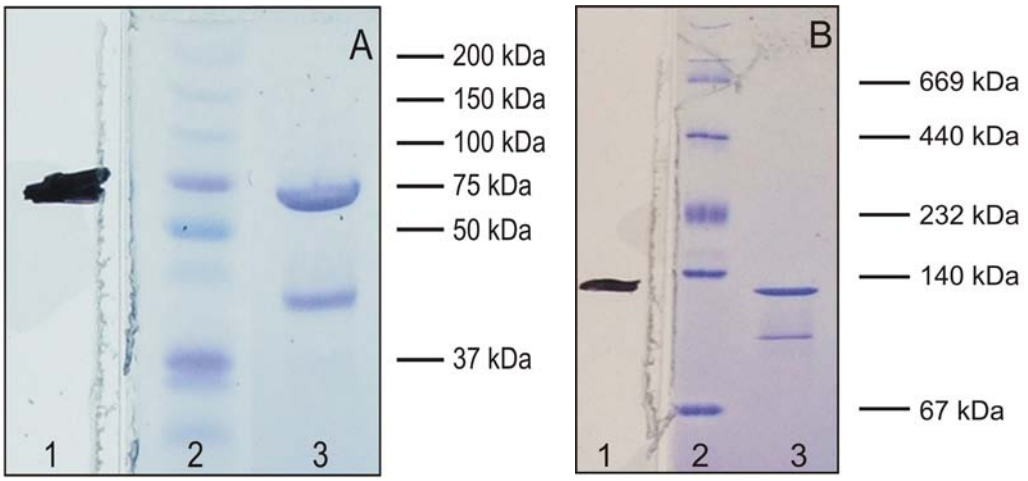

Figure 10: SDS-PAGE (A) and native PAGE (B) of purified β-galactosidase from *Lb. pentosus*.
A: lane 1, active staining with 4-methylumbelliferyl β-D-galactoside; lane 2, molecular weight marker (Precision Plus Protein Dual Color, Biorad); lane 3, Coomassie Blue staining. B: lane 1, active staining with 4-methylumbelliferyl β-D-galactoside; lane 2, molecular weight marker (High Molecular Weight Calibration Kit for electrophoresis, Amersham), lane 3, Coomassie Blue staining.

3.2.4. Characterization

3.2.3.1 Temperature optimum

For determining the temperature optimum the β-gal from the characterization pool (CH) was used. The standard assay at 30°C was used as reference and set to 100 % activity for the substrates *o*NPG and lactose.

For the chromogenic substrate *o*NPG the temperature optimum was 55°C (465 %) and for lactose 60°C (335 %) as displayed in **Figure 11**.

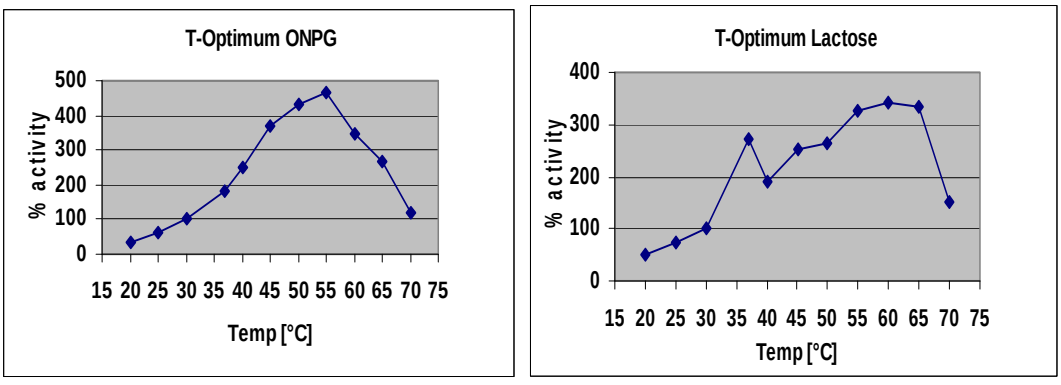

Figure 11: Temperature optimum of β-gal from *Lb. pentosus* using either *o*NPG or lactose as substrate.

3.2.3.2 pH Optimum

For the pH-optimum *o*NPG was dissolved in different buffers at different pH values (acetic acid buffer, pH 4.0, 5.0, 5.5 and 6.0; sodium phosphate buffer, pH 6.0, 6.5, 7.0, and 7.5; glycerine buffer, pH 7.5, 8.0, 8.5, and 9.0)
For determining the pH optimum the β-gal from the characterization pool was used. The standard assay in sodium phosphate buffer at pH 6.5 was set to 100 % (**Figure 12**).
The highest activity was found in glycerine buffer at a pH value of 7.5 for *o*NPG (320 %) and 8.0 (glycerine buffer) for lactose (300 %).

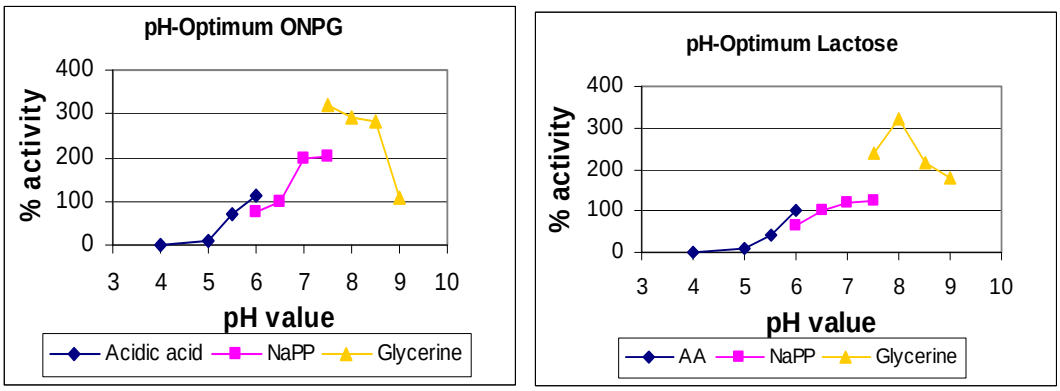

Figure 12: pH optimum of β-gal from *Lb. pentosus* using either *o*NPG or lactose as substrate. NaPP: sodium phosphate buffer

3.2.3.3 Temperature stability

For determining the temperature stability the β-gal from the characterization pool was used. The β-galactosidase was stored at five different temperatures and activity was measured at several time points, more often in the beginning of this test.
At the higher temperatures (30°C, 37°C and 42°C) 2 mM $MgCl_2$ for stabilization was added. This was marked with a * (30°C*, 37°C* and 42°C*). Standard activity measurement with *o*NPG was set to 100 % activity for the storage at 4°C.

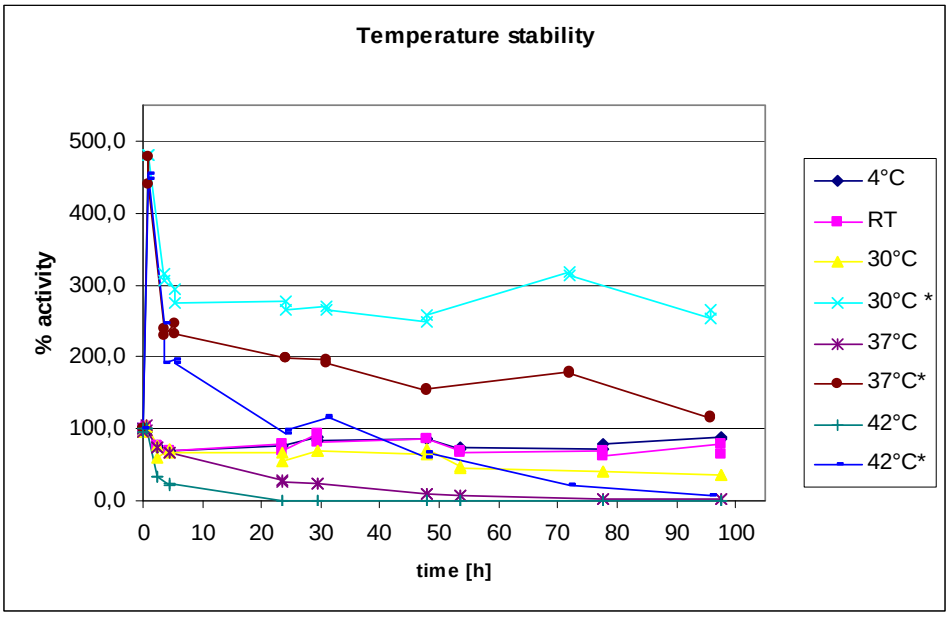

Figure 13: Temperature stability of β-gal from *Lb. pentosus* at five different temperatures with (*) and without $MgCl_2$
RT: room temperature (about 20-22°C)

The divalent ion Mg^{2+} stabilized and activated the enzyme at all tested temperatures up to an activity of 450 % for a few hours.

A maximum of activity (450 % at 42°C*) was reached after 3.5 hours, before decreasing again. At the lower temperatures (4°C, room temperature and 30°C without $MgCl_2$) the enzyme was quite stable and did not loose much of its activity. Activity was lost rapidly when stored at 37°C and 42°C without adding $MgCl_2$. At 30°C*, a constant level of activity was reached (about 250 %). β-Gal stored at 42°C* reached high activity too, but decreased quite fast after 24 hours (**Figure 13**).

3.2.3.4 pH stability

For investigating the pH stability the β-gal from the characterization pool was used. The β-galactosidase was stored at constant temperature (37°C) in different buffers at different pH values and activity was measured at several time points, more often in the beginning of this test.

Sodium phosphate buffer at pH 6.5 was set to 100 % activity.

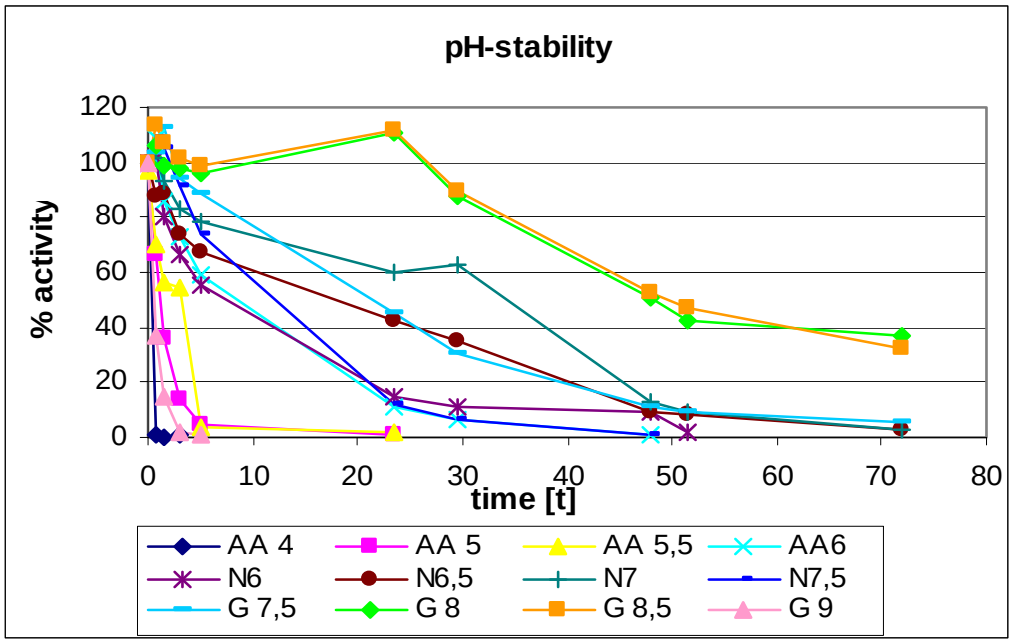

Figure 14: pH stability of β-gal from *Lb. pentosus*. Storage in 12 different buffer environments at 37°C
AA: Acetic acid buffer; N: Sodium phosphate buffer; G: Glycerine buffer.

The β-galactosidase was not stable in acetic acid buffer at any pH value that was tested, and was not stable at the highest tested value (pH 9.0) in glycerine buffer. Best stability was found in glycerine buffer at pH 8.0 and 8.5: the enzyme activity increased up to 120 % for 24 hours, before decreasing again (**Figure 14**).

3.2.3.5 K_m-value for lactose

For determining the steady state kinetics the β-gal from the K_m pool was used. Steady-state kinetics were determined for the hydrolysis of lactose and *o*NPG by calculating kinetic constants like V_{max}, K_m, and k_{cat}. The k_{cat} values were calculated on the basis of theoretical V_{max} values obtained by nonlinear regression using SigmaPlot (SPSS Inc.). Data are summarized in **Table 5** and **Figure 15**.

Table 5: Kinetic properties of β-gal from *Lb. pentosus* for the hydrolysis of lactose and *o*-nitrophenyl-β-D-galactopyranoside

substrate	method for determination of enzyme activity	kinetic parameters	*Lb. pentosus* β-galactosidase
lactose	release of D-Glucose	V_{max} (µmol min^{-1} mg^{-1})	11.3 +/- 0.75
		K_m (mM)	37.8 +/- 9.41
		k_{cat} (s^{-1})	20.1
		k_{cat}/K_m ($mM^{-1}s^{-1}$)	0.53
*o*NPG	release of *o*NP	V_{max} (µmol min^{-1} mg^{-1})	304 +/- 24.6
		K_m (mM)	1.67 +/- 0.64
		k_{cat} (s^{-1})	543
		k_{cat}/K_m ($mM^{-1}s^{-1}$)	325

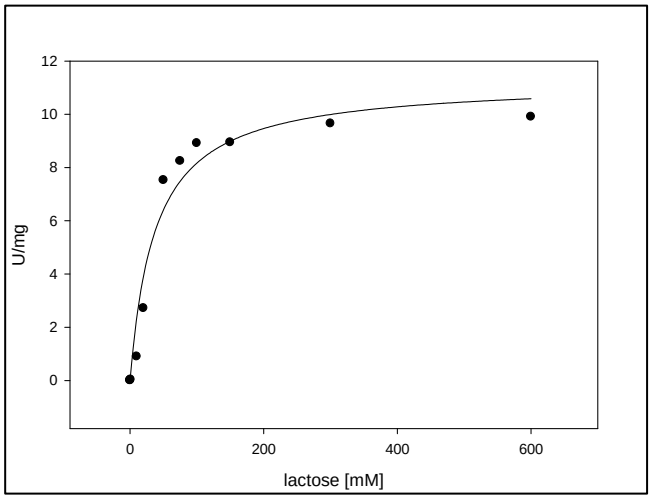

Figure 15: K_m-value of β-gal from *Lb. pentosus* using lactose as substrate. Concentrations measured: 600, 300, 150, 100, 75, 50, 20, 10, 1, and 0.1 mM lactose.

The K_m-value for lactose was determined to be 38 mM, the K_m-value for *o*NPG was 1.67 mM and the $k_{cat,oNPG}$ was 543 s^{-1}. The catalytic efficiencies (k_{cat}/K_m) for the two substrates, *o*NPG and lactose, indicate that *o*NPG is the highly preferred substrate, because of the more favourable K_m and much higher k_{cat} value.

3.2.3.6 Determination of the β-galactosidase activity for five other substrates

For determining the enzyme specificity the β-gal from the characterization pool was used testing the following 5 substrates: β-cellobioside, β-glucopyranoside, β-xylopyranoside, β-mannopyranoside and α-galactopyranoside.

Activity with *o*NPG was set to 100 %. The enzyme from *Lb. pentosus* gave nearly 50 % residual-activity on 4-nitrophenyl-α-D-galactopyranosid. However, the assay solution showed a slightly green shade, so the absorption measurement might have been affected and the measured value is not correct.

No activity could be determined for the other four substrates.

3.2.3.7 Transgalactosylation activity

This experiment was done to investigate the transgalactosylation activity of the *Lb. pentosus* β-gal. **Figure 16** shows the sugar composition during GOS formation over the conversion rate.

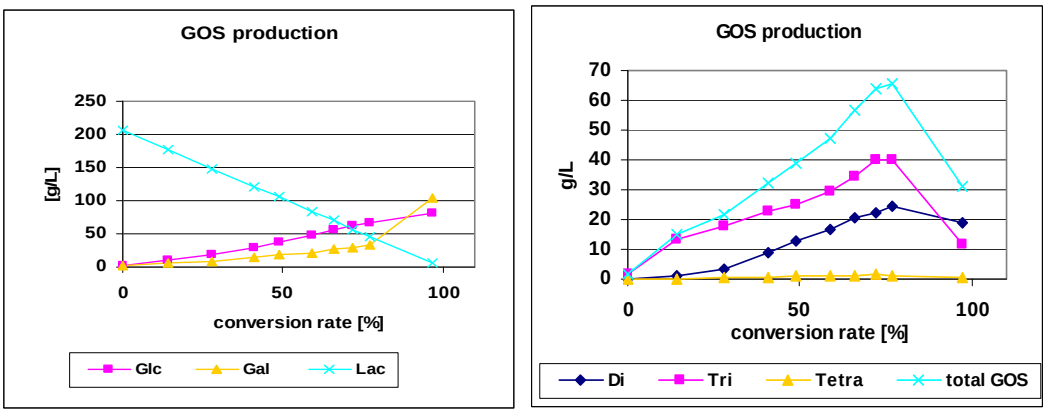

Figure 16: Developement of GOS production at 30°C by *Lb. pentosus* β-gal (5 U_{oNPG}/mL); addition of 2 mM $MgCl_2$. Left: developement of monosaccharides and lactose, right: developement of Oligo-saccharides.
Glc: glucose, Gal: galactose, Lac: lactose, Di: disaccharides (apart from lactose), Tri: trisaccharides, Tetra: tetrasaccharides.

The glucose and the galactose concentration were constantly increasing for 8 hours, as well as the concentrations of the newly formed galacto-oligosaccharides. At 77 % (w/v) lactose conversion rate the maximum GOS yield (31 % of total sugar) was obtained. The newly formed sugar mixture contained 31.5% glucose, 15.9 % galactose, 21.9 % lactose,

11.4% non-lactose disaccharides, 18.7 % trisaccharides and 0.6 % tetrasaccharides (**Table 6**). After 8 h conversion time the newly formed GOS were hydrolysed again.

Table 6: Lactose conversion by *Lb. pentosus* β-galactosidase (5U/ml) and formation of GOS

Time [h]	Conversion rate [%]	% total GOS [g/g]	Glc[a]	Gal[a]	Lac[a]	Di[a]	Tri[a]	Tetra[a]	total GOS[a]
0	0	0.08	1.64	1.16	205.39	0	1.6	0	1.6
1	14	7.1	10.19	5.82	176.8	1.16	13.5	0.2	14.86
2	28	10.8	19.48	9.36	148.74	3.55	17.58	0.41	21.54
3	41	16.4	28.25	15.5	121.34	9.03	22.6	0.6	32.23
4	49	19.4	37.33	18.97	105.51	12.97	25.16	0.877	39.007
5	59	23.7	47.64	21.09	83.8	16.811	29.45	1.21	47.471
6	66	27	56.96	26.07	70.66	20.83	34.7	1.2	56.73
7	72	30.1	63.06	29.11	57	22.08	40.11	1.91	64.1
8	**77**	**31**	**66.98**	**33.79**	**46.57**	**24.22**	**39.89**	**1.31**	**65.42**
24	97	12.2	80.58	103.98	5.6	18.87	11.7	0.77	31.34

Glc: glucose; Gal: galactose; Total GOS: % GOS of total sugars; Lac: lactose; Di: disaccharides apart from lactose; Tri: trisaccharides; Tetra: tetrasaccharides
[a] Values in g/L

3.2.3.8 Effects of various reagents and cations

Due to low β-gal amount the following measurements were done with only partially purified enzyme (after HIC purification). Standard activity measurement with *o*NPG was set to 100 %. The effect on the β-gal activity was tested using four different ions (Na^+, K^+, Mg^{2+} and Mn^{2+}) and four different reagents (2-mercaptoethantol, DTT, Urea, and EDTA). All tested cations and reagents were in concentrations of 100 mM, 10 mM and 1 mM, except Mn^{2+} (1 mM) and Mg^{2+} (10 mM and 1 mM). **Table 7** summarises the relative β-gal activities.

Table 7: Effect of various cations and reagents on the β-gal activity from *Lb. pentosus*

Ions/reagents	Relative β-galactosidase activity (%)			
	1 mM	10 mM	100 mM	1000 mM
none	100	100	100	100
Na^+	187	137	124	32
K^+	113	151	201	110
Mg^{2+}	544	596		
Mn^{2+}	642			
EDTA	19.6	18.6	10.1	
urea	143	138	136	
DTT	132	106	129	
2-mercaptoethanol	30.8	162	89.7	

DTT: 1,4-dithiotreitol

Best activation was reached by addition of $MnSO_4$ (1 mM). Activity was enhanced more than sixfold to 642 %. Nearly the same effect had the addition of 10 mM $MgCl_2$ (596 %). Na^+ ions enhanced the activity by 80 % when added in a concentration of 1 mM, K^+ by 100 % (100 mM).

Only EDTA reduced the activity at all tested concentrations to 10 % (100 mM) and 20 % activity (10 mM and 1 mM).

3.3. Lactobacillus L662

3.3.1. Cultivation

Biomass of this lactobacillus was produced in 10 x 0.8 L MRS medium containing 2 % lactose. 200 mL cell free crude extract after homogenizing and ultracentrifugation gave 2650 total β-gal units.

3.3.2. Purification

Fermentation of *Lb.* L662 yielded in about 2650 total units. After two purification steps (ammonium sulphate precipitation and hydrophobic interaction chromatography) 1150 units in total were left. The yield was 43.4 %. Specific activity increased to 23 U/mg. This was the pool for the characterization measurements.

Even though the enzyme was stored for more than one month at 4°C before using affinity chromatography for further purification, activity did not decrease. After affinity chromatography only 3.6 units were left with a specific activity of 3.8 U/mg. This pool was finally used for steady state kinetic measurements. **Table 8** displays a summary of the β-gal activity after purification steps.

Table 8: Purification of β-gal from *Lb*. L662

	Specific activity (U/mg)	total units (U)
crude extract	(0.1)	2650
0-50 % ASP	0.35	3360
After HIC	23	1150
AC 1st pool (K_m-pool)	3.8	3.6
2nd pool[a]	40.7	69.2

[a] unfortunately this pool was lost.
ASP: ammonium sulphate precipitation; HIC: hydrophobic interaction chromatography; AC: affinity chromatography

3.3.3. Gel Elektrophoreses

The β-galactosidase expressed by *Lb*. L662 is a hetero-dimer, consisting of two subunits: 72 kDa and 35 kDa. This was proven on a SDS-PAGE by Coomassie Blue staining.

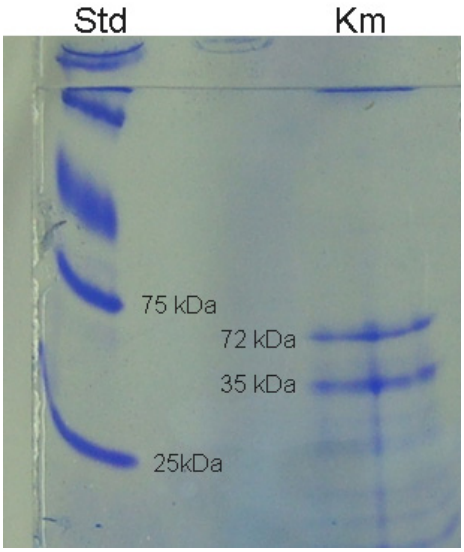

Figure 17: SDS-PAGE of β-galactosidase (K_m pool from *Lb.* L662*),* lane STD, molecular weight marker (Precision Plus Protein Dual Color, Biorad); lane Km, Coomassie Blue staining.

3.3.4. Characterization

For the characterization measurements of the enzyme from *Lb.* L662 the enzyme was purified by ammonium sulphate precipitation and HIC.

3.3.3.1 Temperature optimum

Standard assay activity with *o*NPG was set to 100 %.

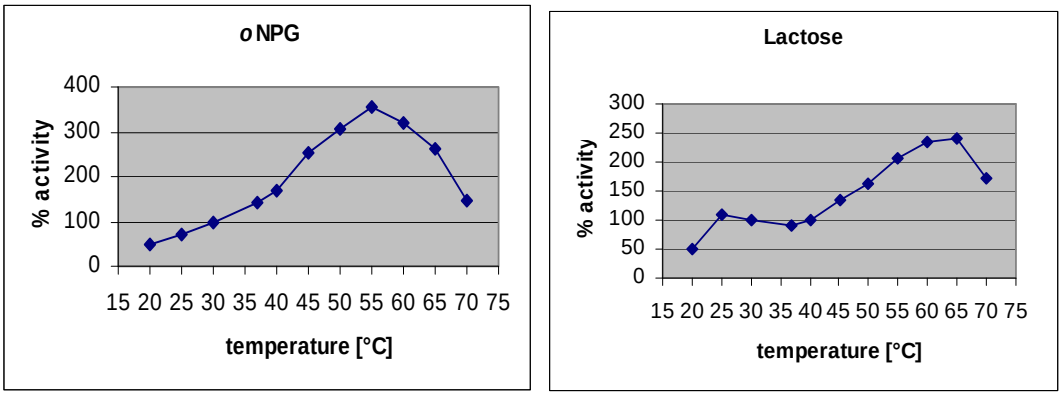

Figure 18: Temperature optimum of β-gal from *Lb.* L662 using either *o*NPG (55°C) or lactose (65°C), as substrate.

The optimum temperature measured under standard assay conditions (10 min) for lactose and *o*NPG hydrolysis was 55°C and 65°C, respectively (**Figure 18**).

3.3.3.2 pH optimum

For the pH-optimum *o*NPG was dissolved in different buffers at different pH values (acetic acid buffer, pH 4.0, 5.0, 5.5 and 6.0; sodium phosphate buffer, pH 6.0, 6.5, 7.0, and 7.5; glycerine buffer, pH 7.5, 8.0, 8.5, and 9.0) For determining the pH optimum the β-gal from the characterization pool was used. The standard assay in sodium phosphate buffer at pH 6.5 was set to 100 %. In other buffer environments other activities were scored and their percentage of activity was calculated.

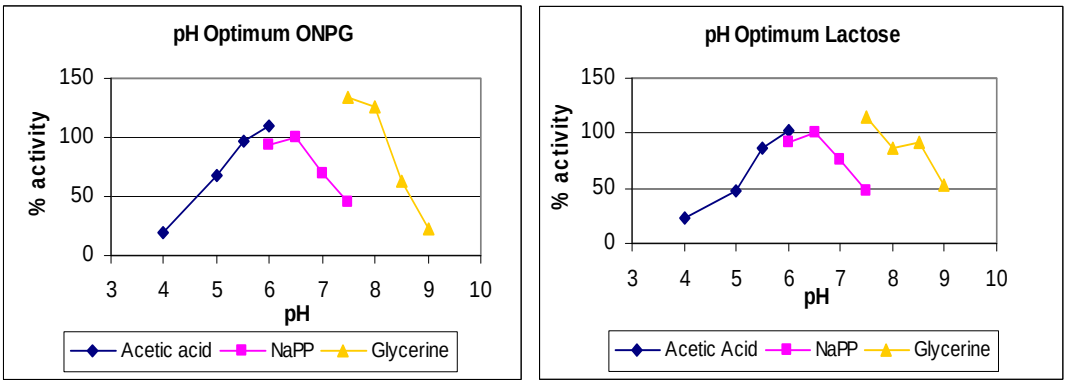

Figure 19: pH optimum of β-gal from *Lb.* L662 in glycerine buffer, using either *o*NPG or lactose as substrate (both optima at pH 7.5).
NaPP: sodium phosphate buffer

The pH optimum of *Lb.* L662 β-galactosidase was pH 7.5 in glycerine buffer when using lactose and *o*NPG as substrate (**Figure 19**).

3.3.3.3 Temperature stability

To determine the temperature stability the *Lb*. L662 β-gal was stored at five different temperatures and activity was measured at several time points. At higher temperatures (30°C*, 37°C* and 42°C*) 2 mM $MgCl_2$ for stabilization was added. The standard assay in sodium phosphate buffer (pH 6.5) at 30 °C was set to 100 %

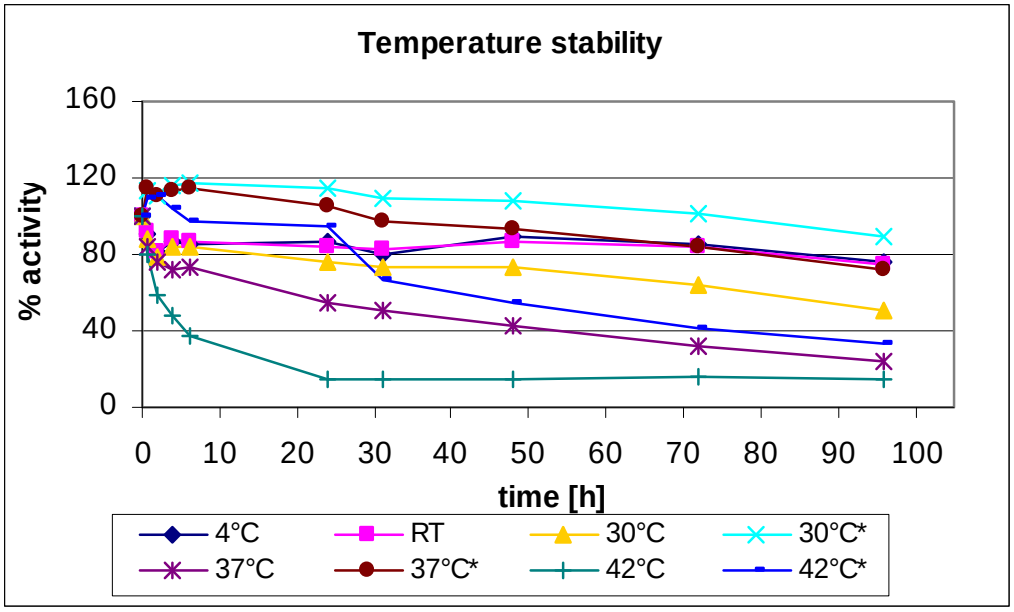

Figure 20: Temperature stability of β-gal from *Lb.* L662 at five different temperatures with (*) or without $MgCl_2$
RT: room temperature (about 20-22°C)

The enzyme was quite stable at all temperatures; a significant loss in activity was measured at higher temperature (42°C). This high inactivation rate was reduced by adding $MgCl_2$. Slight activity increase could be achieved at 30°C*, 37°C*, and 42°C*, each supplemented with $MgCl_2$ (**Figure 20**).

3.3.3.4 pH stability

For investigating the pH stability the β-gal from the characterization pool was used. The β-galactosidase was stored at constant temperature (37°C) in different buffers at different pH values and activity was measured at several time points, more often in the beginning of this test. β-Gal activity in sodium phosphate buffer at pH 6.5 was set to 100 %.

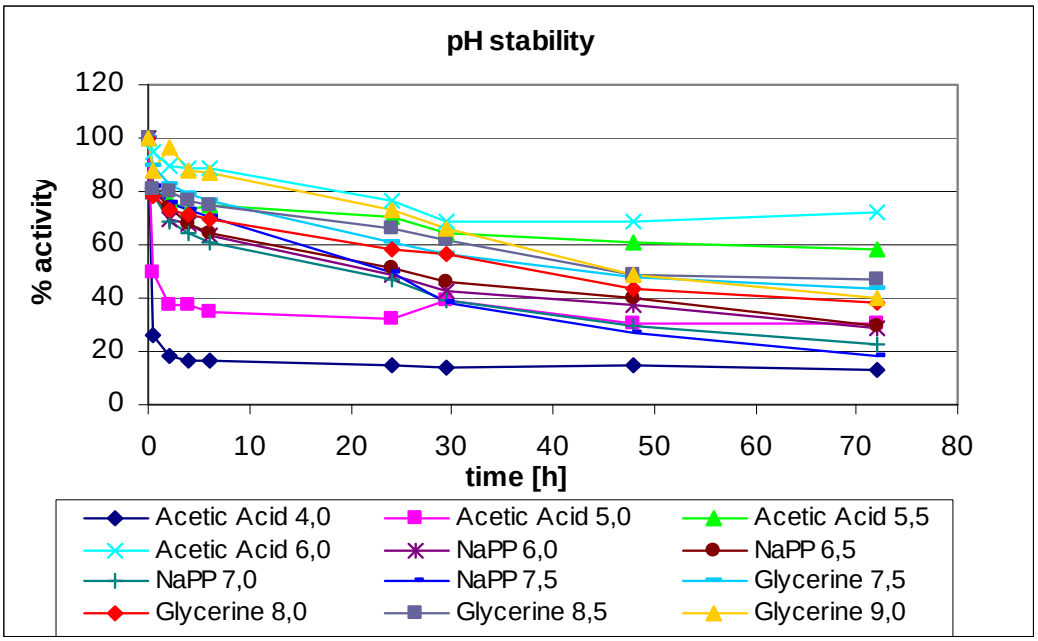

Figure 21: pH stability of β-gal from *Lb.* L662. Storage in 12 different buffer environments at 37°C.
NaPP: sodium phosphate buffer

The enzyme was most stable in acetic acid buffer (pH 6.0). Over 72 hours only 30 % of activity was lost. Activity was reduced in all buffers quite constantly. The enzyme lost most of its activity in acetic acid 4.0, (**Figure 21**). But it never lost activity completely, like the β-galactosidase from *Lb. pentosus*.

3.3.3.5 K_m-value for lactose

For determining the steady state kinetics the β-gal from the after a affinity chromatography step was used. Steady-state kinetics were determined for the hydrolysis of lactose by calculating kinetic constants like V_{max}, K_m, and k_{cat}. The k_{cat} values were calculated on the basis of theoretical V_{max} values obtained by nonlinear regression using SigmaPlot (SPSS Inc.). Data are summarized in **Table 9**.

Table 9: Kinetic properties of β-gal from *Lb.* L662 for the hydrolysis of lactose

substrate	method for determination of enzyme activity	kinetic parameters	*Lb.* L662 β-galactosidase
lactose	release of D-Glucose	V_{max} (µmol min^{-1} mg^{-1})	1.3 +/- 0.16
		K_m (mM)	17.4 +/- 8.6
		k_{cat} (s^{-1})	2.40
		k_{cat}/K_m ($mM^{-1}s^{-1}$)	0.14

The K_m-value for lactose was found to be 17 mM. Due to the lack of enzyme the dilution was too high and too few concentrations were measured and steady state kinetic measurements were not determined properly. So measurements should be repeated.

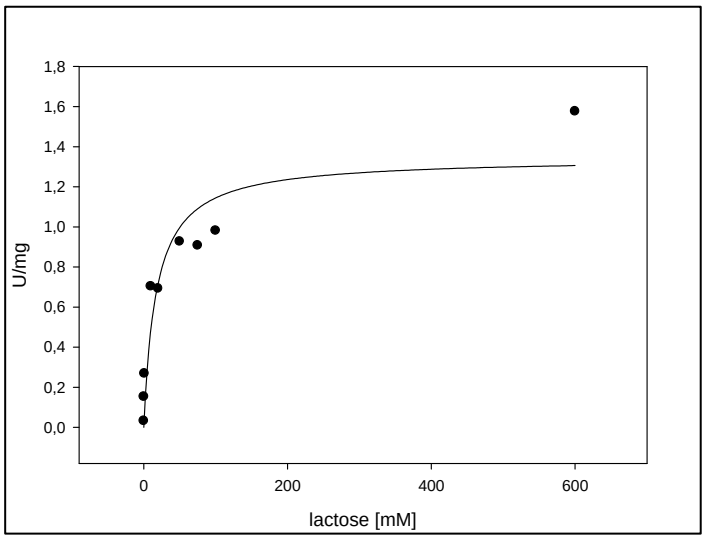

Figure 22: K_m-value of β-gal from *Lb.* L662 using lactose as substrate. Concentrations: 600, 100, 75, 50, 20, 10, 1, and 0.1 mM lactose.

3.3.3.6 Determination of the β-galactosidase activity for five other substrates

To test the enzyme specifity five different chromogenic substrates were tested for β-galactosidase activity: β-cellobioside, β-glucopyranoside, β-xylopyranoside, β-mannopyranoside and α-galactopyranoside.

The β-galactosidase from this *Lactobacillus* L662 was active with two of the tested substrates: 4-ntrophenyl-α-D-galactopyranoside and 4-ntrophenyl-β-xylopyranoside giving 77 % and 2.5 % residual activity, respectively. However, both solutions were of a green shade, so the absorbance measurement might have been influenced and the real activities could differ from the determined ones. No activity was determined for the other three substrates.

3.3.3.7 Transgalactosylation activity

To confirm the potential of the novel β-galactosidase for the production of GOS a discontinuous lactose conversion process at 30°C, using 205 g/L initial lactose concentration in 50 mM sodium phosphate buffer (+ 2 mM $MgCl_2$, pH 6.5) and 5 U_{oNPG}/mL of β-galactosidase were used to produce GOS. The conversion rate was very slow and after 24 hours incubation time only 14 % of the lactose had been converted and only 7.7 g/L (3.8 %) GOS were built up (**Table 10**, **Figure 23**). The majority of the obtained GOS mixture were the trisaccharides (6.5 g/L).

Table 10: Lactose conversion by *Lb.* L662 β-gal (5U/ml) and formation of GOS

Time [h]	Conversion rate [%]	% total GOS [g/g]	Glc[a]	Gal [a]	Lac[a]	Di[a]	Tri[a]	Tetra[a]	Total GOS[a]
0	0	0.08	1.64	1.16	205.39	0	1.6	0	1.6
24	14	3.8	11.7	9.7	177	0.86	6.5	0.3	7.66

Glc: glucose; Gal: galactose; Total GOS: % GOS per total sugars; Lac: lactose;
Di: disaccharides apart from lactose; Tri: trisaccharides; Tetra: tetrasaccharides
[a] Values in g/L

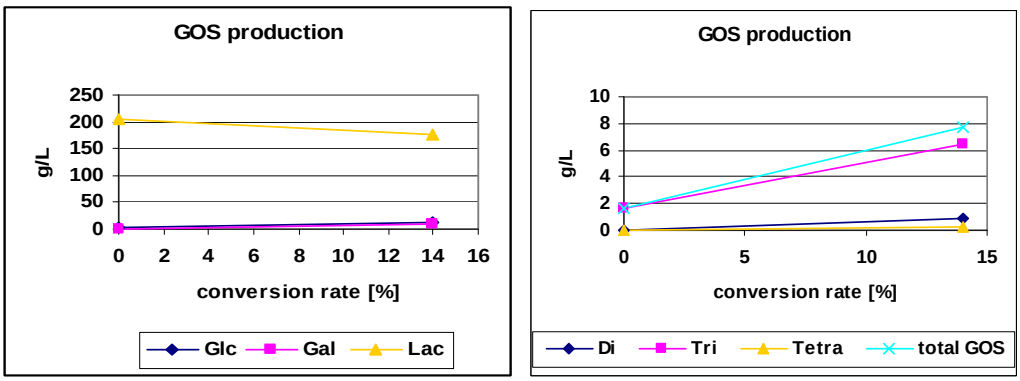

Figure 23: Developement of the GOS production at 30°C by *Lb.* L662 β-gal (5 U_{oNPG}/mL); addition of 2 mM $MgCl_2$. Left: developement of monosaccharides and lactose, right: developement of Oligo-saccharides.
Glc: glucose, Gal: galactose, Lac: lactose, Di: disaccharides (apart from lactose), Tri: trisaccharides, Tetra: tetrasaccharides.

3.3.3.8 Effects of various reagents and cations

Various reagents and cations were tested with respect to a possible stimulating or inhibitory effect on the β-galactosidase activity (**Table 11**). Each agent was added in concentrations of 100 mM, 10 mM and 1 mM, except Mg (10 mM and 1 mM) and Mn (1 mM). The effect of these four ions (Na^+, K^+, Mg^{2+} and Mn^{2+}) and of these four reagents (2-mercaptoethantol, DTT, Urea and EDTA) were tested on the β-gal activity.

Table 11: Effect of various cations and reagents on the β-gal activity from *Lb.* L662

Ions/reagents	Relative β-galactosidase activity (%)			
	1 mM	10 mM	100 mM	1000 mM
none	100	100	100	100
Na^+	138	131	123	71
K^+	124	129	123	92
Mg^{2+}	157	167		
Mn^{2+}	177			
EDTA	82	88	91	
urea	104	100	101	
DTT	96	95	122	
2-mercaptoethanol	89	81	79	

DTT: 1,4-dithiotreitol

Best activation was reached by adding 1 mM $MnSO_4$ (176 %). $MgCl_2$ in a concentration of 10 mM resulted in an activity increase to 166 %. The reagents (2-mercaptoethantol, urea and EDTA) had nearly no effect on the enzyme activity, neither had DTT.

On the one hand this enzyme was very stable as it was nearly not deactivated by any reagent, but on the other hand it was also not activated by ions.

4. Discussion

Special lactic acid bacteria, so called probiotics, play an important role in the health of the human intestinal tract. Probiotics are viable microorganisms that beneficially affect the host through its effects in the intestinal tract (Fuller, 1989). After passage through the stomach and the small intestine some probiotics survive and become established transiently in the large bowel, competing in the gut with pathogenic bacteria. *Lactobacillus acidophilus* treated animals showed that this strain can delay colon tumour formation by prolonging the induction of the latent period (Goldin and Gorbach, 1980). Prebiotics are the “food” for probiotic bacteria. These carbohydrates are metabolised by the intestinal microflora, due to its β-structure. Because of the transgalactosylation activity the enzyme β-galactosidase is able to form such prebiotics, galacto-oligosaccharides (GOS) in special. Therefore, there is great interest in finding and biochemical characterization of novel β-galactosidases.

The substrate specifity and molecular properties of the microbial β-galactosidase differ significantly by the enzyme source. The catalytic efficiency and specificity of the transgalactosylation reactions depend on the source of the enzyme, as well as on the native molecular weights and subunit structures of the β-galactosidases. The β-gal structures range from monomer, e.g. the *Saccharopolyspora rectivirgula* enzyme, to hetero-octamere, the *E.coli ebg* enzyme (Nakayama and Amachi, 1999). The β-galactosidase from lactobacilli often show a dimeric structure, like the one from *Lb. reuteri.* This enzyme is a hetero-dimer with a molecular weight of 107 kDa, consisting of the small subunit with approximately 35 and the large one with 72 kDa (Nguyen *et al*., 2006).

The bacterial β-galactosidases generally show optimum activities at neutral pH values. Some representatives of the β-galactosidase family require trace amounts of mono- and/or divalent ions for maximum activity. The β-gal from *Lb. reuteri* is strongly activated by monovalent ions like Na^+

and K^+, as well as divalent ions like Mg^{2+} and Mn^{2+} (Nguyen *et al*., 2006). A recent structural study showed binding sites close to the active site of for Na^+ and Mg^{2+}, which are thought to be corresponding to stability and activity of the enzyme (Juers *et al*., 2000). On the contrary, the β-gal from *Lb. acidophilus* R22 does not show specific requirements for metal ions, with the exception of Mg^{2+} which enhances both activity and stability (Nguyen *et al*., 2007). These observations strongly suggest the structural diversity of the microbial β-galactosidases (Nakayama and Amachi, 1999).

During the screening of several *Lactobacillus* strains for β-galactosidase activity *Lb. pentosus* and *Lb.* L662 were found to be active for the substrates *o*NPG and lactose and were then characterized for the following biochemical parameters:
Temperature and pH optimum, temperature and pH stability at different temperatures and in different buffer systems, substrate specificity, and steady state kinetic constants for lactose and *o*NPG. Furthermore, the transgalactosylation activity and the effect of ions and reagents on the two β-galactosidases were investigated.
Before these characterization measurements were carried out, the enzyme was purified by ammonium sulphate precipitation, hydrophobic interaction chromatography, and affinity chromatography. The two substrates *o*NPG and lactose were used for standard activity measurements.

4.1. Screening

Several lactic acid bacteria were screened for their ability to grow on MRS media containing 2 % lactose as only carbone source. The cell free crude extract after cell disruption and centrifugation was assayed on β-gal activity using the chromogenic substrate *o*NPG. The following strains were screened: *Lb. plantarum* 268 and *Lb. plantarum* 322, *Lb. rhamnosus* 241 and *Lb. rhamnosus* 177, *L. lactis* HA and *L. lactis* NZ9700, *Enterococcus faecium*, *Lb. pentosus* KVBST10-1, *Lb. bulgaricus* TISTR451, *Lb. sakei*

TISTR911, *Lb. amylovorus* and *Lb.* L662, which could not be identified by an API 50 CH test.
Lb. pentosus and *Lb.* L662 were found to produce high amounts of active β-galactosidase and were then cultivated, purified and characterized.

4.2. Purification

First purification step was the fractionation by ammonium sulphate precipitation. Highest activity was found in the fraction that contained the precipitate of 0 – 50 % ammonium sulphate saturation. The obtained pellet was dissolved in 50 mM sodium phosphate buffer (pH 6.5) and applied to a hydrophobic interaction chromatography column, containing phenyl-sepharose. The separation capacity was too small so that the enzyme solution was applied in several runs which increased the loss of enzyme. For fewer losses in activity the column should be in a dimension that is large enough for all the cell free crude extract being applied in one step.
Fractions with the highest β-gal activity were further purified by affinity chromatography using crosslinked (4%) beaded agarose immobilized with *p*-aminobenzyl-1-thio-β-D-galactopyranoside.
Most of the activity was lost during this last purification step; maximal 2 % of the initial β-galactosidase activity were yielded at the end of the purification. It was reported that the last affinity purification step was a very critical one (oral communications). To solve this problem new column material has to be ordered.

4.3. Structure

Even though the enzymes from *Lb. pentosus* and *Lb.* L662 differ in their need for metal ions, they are both expressed as hetero-dimers. The larger subunit is about 72 kDa, the smaller one about 35 kDa large, the whole enzyme shows a size of approximately 107 kDa. This fact was proven by separation on native PAGEs and SDS-PAGEs. Also the β-galactosidase from the strains *Lb. acidophilus* R22, *Lb. reuteri* and *Lb. helveticus* show this hetero-dimeric structure which is not encountered frequently among

bacteria (Nguyen *et al*., 2006; Nguyen *et al*., 2007, Fortina *et al*., 2003). Active staining with 4-methylumbelliferyl-β-D-galactoside as substrate proofed that the larger subunit is active without building up the hetero-dimer with the small subunit. This was already observed by Nguyen *et al*. (2006, 2007) for the β-galactosidases from *Lb. acidophilus* R22 and *Lb. reuteri*.

4.4. Steady state kinetic

The K_m-value was determined using lactose (both β-galactosidases) and *o*NPG (*Lb. pentosus* β-gal) measuring the β-gal activity by the release of D-glucose and *o*NP, respectively.

For the enzyme of *Lb. pentosus* the K_m-value was determined to be 37.8 +/- 9.41 mM lactose, compared to 1.67 +/-0.64 mM *o*NPG. This preference for *o*NPG is indicated in the catalytic efficiency term (k_{cat}/K_m) too: 325 $mM^{-1}s^{-1}$ for *o*NPG, but only 0.53 $mM^{-1}s^{-1}$ for lactose. Similar results were described by Nguyen and co-workers (Nguyen *et al*., 2006). For the enzyme of *Lb.* L662 only the K_m-value for lactose was determined as it was assumed to be comparable for *o*NPG to the one of the *Lb. pentosus* β-gal. For lactose the K_m value was 17.4 +/- 8.6 mM. But this value has to be tested again, because due to lack of enzyme this measurement was not carried out in duplicate.

The K_m-value of 37.8 mM lactose (β-gal *Lb. pentosus*) is comparable to the value of 31 mM lactose for *Lb. reuteri* L461; 17 mM (*Lb.* L662) lactose is comparable to 13 mM lactose by *Lb. reuteri* L103 (β-gal), as determined by Nguyen *et al.* (2006). On the other hand, the K_m-value of the β-galactosidase from *Lb. acidophilus* R22 was only 4.04 mM lactose or 0.73 mM *o*NPG (Nguyen *et al.*, 2007), a difference between natural and synthetic substrate that is not as big as measured for the β-gals from *Lb. pentosus* or *Lb. reuteri*. That low K_m-value of the β-gal from *Lb. acidophilus* R22 can be useful for a complete lactose hydrolysi, whereas the other enzymes do not show that useful characteristic.

4.5. Temperature optimum

The optimum for the β-gal of *Lb. pentosus* as well as for *Lb.* L662 was 55°C when tested on *o*NPG. For lactose the optimum shifted to 60°C (*Lb. pentosus*) and 65°C (*Lb.* L662). The 65°C for lactose are quite high, when compared to the optimum at 50°C from *Lb. reuteri* (Nguyen *et al*., 2006).

4.6. pH optimum

The optimum pH value for the *Lb. pentosus* β-galactosidase was found at neutral pH regions: pH of 7.5 for the tested synthetic substrate *o*NPG and pH of 8.0 for the natural substrate lactose. These values were achieved in 20 mM glycerine buffer. For *Lb.* L662 the optimum was at pH 7.5, also in glycerine buffer for both substrates.
Generally, it is described in literature that β-galactosidases show pH optima around a neutral pH range (Nakayama and Amachi, 1999).

4.7. Temperature stability

2 mM $MgCl_2$ was added for the temperature stability experiment at 30°C, 37°C, and 42°C. β-Gal activity was highly increased at all three temperatures when Mg^{2+} ions were present. The β-galactosidase from *Lb. pentosus* was most stable when stored at 30°C (addition of 2 mM $MgCl_2$ to the reaction mixture). The activity was increased to 280 % by the addition of Mg^{2+} ions compared to 80 % without Mg^{2+}. Highest activity was reached during the storage at 42°C with 2 mM $MgCl_2$, increasing the activity by the factor of 4.8. At the lower tested temperatures of 4°C and room temperature (about 20-22°C) the enzyme did not loose much activity but due to the lack of Mg^{2+} ions, no increased activities were determined.
This phenomenon of activation and stabilization by Mg^{2+} ions was already described in literature (Nakayama and Amachi, 1999; Nguyen *et al*., 2006, Juers *et al*., 2000). Juers and co-workers found binding sites for Mg^{2+} near the active site in the β-galactosidase from *E. coli*. Even though the β-galactosidase from *Lb. pentosus* was not very stable in unfavourable pH

values or suboptimum conditions it was therefore highly stabilized and activated by addition of Mg^{2+} ions.

Best stability for the β-galactosidase from *Lb.* L662 was determined at 30°C with 2 mM $MgCl_2$, although only an increase of 10 % was measured after 24 h. This enzyme could not be activated by Mg^{2+} ions like the one from *Lb. pentosus*.

It is interesting that the temperature optimum was higher (55°C) than the temperature at which the enzyme showed best stability (30°C), especially because best stabilities could be achieved by addition of Mg^{2+} ions to the reaction mixture. They had not been added when tested for the T-optimum. This was also observed by Nguyen *et al.* (2006): the two β-galactosidases from *Lb. reuteri* (L103 and L461) were most active at 50°C but stable for only 3 h at 45°C.

Furthermore, activities were further increased when the buffer environment in which the β-gals were stored was changed from the standard 50 mM sodium phosphate buffer pH 6.5 to the optimal buffers which waere determined for these two β-galactosidases (20 mM glycerine buffer pH 8.0 or 8.5 for *Lb. pentosus* ,or pH 6.0 20 mM acetic acid buffer for *Lb.* L662*)*.

4.8. pH stability

The experiment for pH stability was carried out at 37°C without addition of 2 mM $MgCl_2$.

At these conditions, the enzyme from *Lb. pentosus* was active for 24 hours in 20 mM glycerine buffer, pH 8.0 and 8.5, the buffer which was also determined to be the optimal buffer system. The enzyme also remained active in 20 mM sodium phosphate buffer pH 7.0 but in all other tested buffer systems the enzyme activity decreased to less than 50 % after 24 hours incubation time.

Best activity for *Lb.* L662 was measured in 20 mM acetic acid buffer (pH 6.0). During three days storage at 37°C, only 15 % of activity was lost. So it may be possible that higher activity and longer stability is reached when the enzyme is stored and reacts in these optimal buffer environments.

4.9. Effect of ions and reagents

The enzyme activation by Mg^{2+} ions was tested in another experiment, investigating the effect of different ions and reagents on the activity of the two β-galactosidases. Mg^{2+} and Mn^{2+}, the two tested divalent ions, increased the activity of the enzyme from *Lb. pentosus* six-fold when added in a concentration of 1mM, Mg^{2+} also in a concentration of 10 mM. On the other hand Na^{+} and K^{+}, both monovalent ions, did not lead to such an increase in activity (K^{+}: +100 %). The phenomenon of Mg^{2+} was already described in literature (Nakayama and Amachi, 1999; Nguyen *et al*., 2006; Juers *et al*., 2000). The enzyme stayed quite active towards the tested detergent agents, 2-mercaptoethanol, DTT and Urea. Only EDTA lowered the activity extremely (minus 90 %).

Unlike the β-galactosidase from *Lb. pentosus*, the enzyme from *Lb.* L662 could not be activated that high by any tested ion. But it was already described in literature that not all β-galactosidases show a requirement for ions due to structural diversities. *Lb. acidophilus* R22 did not show a requirement for metal ions either (Nakayama and Amachi, 1999; Nguyen *et al*., 2007). The highest increase in activity of 65 % was achieved when $MgCl_2$ in a concentration of 10 mM was added. Therefore, the enzyme did not loose much activity and showed high stability when it was tested with different reagents. A maximum loss of 20 % activity was reached by addition of 10 and 100 mM 2-mercaptoethanol. Even EDTA, which ruined the β-galactosidase from *Lb. pentosus* nearly totally, did not show such a inactivating effect.

On the one hand, the enzyme from *Lb.* L662 is really stable towards reagents or suboptimal pH values; on the other hand activity was not increased by addition of any ions, neither mono- nor divalent ions.

None of the two enzymes were highly activated in the presence of K^+ ions like the β-galactosidase gained from *Klyveromyces marxianus,* a yeast strain. This enzyme yielded in highest lactose hydrolysis when K^+ ions where available in the reaction mixture (Rajakala and Karthigai Selvi, 2006). Also the enzymes from *Lb. reuteri* L103 and L461 showed a requirement for Na^+ and K^+ (Nguyen *et al*., 2006)

4.10. Substrate specifity

The β-galactosidases from *Lb. pentosus* and *Lb.* L662 were able to convert the structurally different α-galactopyranoside to a certain amount of 50 % and 75 %, respectively. But the reaction mixture showed a slightly green shade instead of the normal yellow colour. So these percentages might be wrong as the absorbance of a green liquid is different to a yellow one when β-galactopyranoside (*o*NPG) was used.

4.11. Transgalactosylation activity and GOS production

Lb. pentosus β-galactosidase was able to degrade lactose and form GOS. After 8 hours reaction time at 30°C, 77 % of the lactose were converted and galacto-oligosaccharides were formed. At this lactose conversion rate 65 g/L (31 % of the reaction mixture) GOS (includes non-lactose disaccharides, tri- and tetrasaccharides) were formed, consisting mainly of trisaccharides (40 g/L). GOS are not the endproducts of this transgalactoslyation reaction. They are formed transiently (see **Figure 4**). After the “critical” point of 80 % lactose conversion more GOS were hydrolysed again.

The β-galactosidase from *Lb.* L662 converted only 14 % of the lactose in 24 h and only 3.8 % of GOS were formed. The reason for the very low transgalactosylation activity could be the very low k_{cat}/K_m value and/or very low inhibition kinetic values for glucose and/or galactose, which were not measured. Therefore more units should have been used in this conversion experiment to get comparable results.

The GOS production of the β-galactosidase from *Lb. pentosus* is similar to the one of *Lb. reuteri*. After reaction times between 8 and 20 hours, a maximum of 70 g/L GOS were formed when 80 % of lactose was converted. The fact that GOS were formed transiently was also shown for the *Lb. reuteri* β-galactosidase. (Splechtna *et al.*, 2006). These 65 and 70 g/L GOS for *Lb. pentosus* and *Lb. reuteri,* respectively, are the same when compared to the yield of GOS by the β-galactosidase from *Lb. acidophilus* R22 which formed 38.5 % GOS after 75 % lactose conversion (Nguyen *et al*., 2007). When recombinant β-galactosidase from *Lb. reuteri* was cloned into *E. coli*, only 25.5 % of GOS (14.7 % trisaccharaides) were formed after 73 % of lactose conversion (reaction was carried out at 23 °C and not at 30 °C) (Maischberger *et al*., 2008).

Although, *Lb. pentosus* is not a probiotic bacterium its enzyme β-galactosidase shows similar properties compared to the enzymes of the two probiotic strains *Lb. acidophilus* R22 and *Lb. reuteri* L103. It is therefore possible that this enzyme is able to build up galacto-oligosaccharides with prebiotic qualities which would be an interesting application for the β-galactosidase of *Lb. pentosus* in the food-industry.

5. Summary

This work presents two β-galactosidases from *Lactobacillus pentosus* and *Lb.* L662, a strain which could not be further identified using an API 50 CH test. After purification by ammonium sulphate precipitation, hydrophobic interaction chromatography and affinity chromatography the two enzymes were biochemically characterized. Both enzymes are hetero-dimers consisting of two subunits of 35 kDa and 72 kDa, which were determined by gel electrophoresis. The activity was measured using the synthetic chromogenic substrate *o*NPG (22 mM) and the natural substrate lactose (600 mM). The standard buffer environment was 50 mM sodium phosphate buffer (pH 6.5).

The β-galactosidases from *Lb. pentosus* and *Lb.* L662 had temperature optima at 55°C and 60°C, respectively, when tested on *o*NPG (10 min assay) and 65°C when tested on lactose. The buffer and pH optima for both enzymes was found to be 20 mM glycerine buffer (pH 7.5). The enzymes were most stable at 30°C in the presence of 2 mM $MgCl_2$. Testing the enzymes for pH stability (storage in different buffers at 37°C), the β-galactosidase from *Lb. pentosus* was most stable in 20 mM glycerine buffer (pH 8.0 and 8.5) and the one from *Lb.* L662 in 20 mM acetic acid buffer (pH 6.0). The determination of the pH stability was performed in the absence of Mg^{2+} ions. The K_m-value was determined for lactose (both β-galactosidases), and for *o*NPG (*Lb. pentosus* β-gal). The K_m and V_{max} values for lactose and *o*NPG were 37.8 ± 9.41 mM, 11.3 ± 0.75 µmol D-glucose released per min per mg protein and 1.67 ± 0.64 mM, 304 ± 24.6 µmol *o*-nitrophenol released per min per mg protein, respectively for the *Lb. pentosus* β-galactosidase. The $K_{m,lactose}$ with a value of 17.4 mM and the $V_{max,lactose}$ with a value of 1.34 µmol was measured for the β-gal of *Lb*. L662.

The β-gal from *Lb. pentosus* was highly activated (6 fold) in presence of the divalent ions Mg^{2+} and Mn^{2+} when added in concentration of 1 mM. Monovalent ions like Na^+ or K^+ did not increase the enzyme activity in

such a high way (K^+: +100 %). On the other hand, the enzyme from *Lb.* L662 did not show that high activity increase in the presence of mono- or divalent ions.

Testing the enzymes on the substrate specificity only α-galactopyranoside was hydrolysed. 50 % and 75 % residual activities were measured when using the β-galactosidases from *Lb. pentosus* and *Lb.* L662, respectively. The colour of the reaction mixture became a slight green instead of a yellow colour (*o*NPG hydrolysis).

Finally the ability of degrading lactose (600 mM) and forming galacto-oligosaccharides (GOS), prebiotic molecules, was tested. A maximum of 65 g/L GOS (31 % of total sugar) were obtained after 8 hours incubation time at 30°C with 2 mM $MgCl_2$. At this time point 77 % of the lactose was converted (*Lb. pentosus* β-gal). GOS were formed only transiently. Only 14 % of lactose was converted in 24 hours when using the β-galactosidase from *Lb.* L662, and 3.8 % GOS were formed. The reason for the very low transgalactosylation activity could be the very low k_{cat}/K_m value and/or very low inhibition kinetic values for glucose and/or galactose.

In general, the enzyme of *Lb.* L662 showed a higher affinity to the natural substrate lactose than the β-galactosidase from *Lb. pentosus* but both enzymes preferred the synthetic substrate *o*NPG over lactose.

6. Zusammenfassung

In dieser Arbeit werden zwei β-Galaktosidasen beschrieben, eine isoliert aus *Laktobacillus pentosus* und eine aus *Lb.* L662, ein Stamm der mittels API 50 Test nicht näher identifiziert werden konnte. Die beiden Enzyme wurden biochemisch charakterisiert, nachdem sie mittels Ammonsulfatfällung, hydrophober Interaktionschromatographie und Affinitätschromatographie gereinigt worden waren. Beide Enzyme sind Heterodimere: die große Untereinheit zeigt eine Größe von 72 kDa, die kleine eine Größe von 35 kDa. Dies wurde mittels Gelelektrophorese nachgewiesen. Die Aktivität der beiden β-Galaktosidasen wurde mit Hilfe des chromogenen Substrates *o*NPG (22 mM) und des natürlichen Substrates Laktose (600 mM) gemessen. Die Messungen wurden in 50 mM Natriumphosphat Puffer (pH 6.5) durchgeführt.

Die β-Galaktosidasen von *Lb. pentosus* und *Lb.* L662 haben beide ihr Temperaturoptimum bei 55°C für *o*NPG (10 min Assay) und bei 60°C (*Lb. pentosus* β-gal), beziehungsweise 65°C (*Lb.* L662 β-gal) für Laktose. Das pH Optimum liegt für beide Enzyme in 20 mM Glycerinpuffer bei pH 7.5. Die Stabilität der β-Galaktosidase von *Lb. pentosus* war am Größten bei 30°C in Anwesenheit von 2 mM $MgCl_2$. Die pH Stabilität wurde über einen Zeitraum von bis zu vier Tagen getestet, indem die beiden β-Galaktosidasen in verschiedenen Puffern bei 37°C aufbewahrt wurden. Die β-Galaktosidase von *Lb. pentosus* war in 20 mM Glycerinpuffer mit einem pH Wert von 8.0 und 8.5 am Stabilsten, die β-Galaktosidase von *Lb.* L662 in 20 mM Essigsäurepuffer (pH 6.0). Dieser Test wurde in Abwesenheit von Mg^{2+} Ionen durchgeführt.
Der K_m und V_{max} Werte für Laktose und *o*NPG waren 37.8 ± 9.41 mM, 11.3 ± 0.75 µmol D-Glukose pro Minute pro mg Protein und 1.67 ± 0.64 mM, 304 ± 24.6 µmol *o*-Nitrophenol pro Minute pro mg Protein für die β-Galaktosidase von *Lb. pentosus*. Der K_m-Wert von 17.4 mM und der V_{max}-Wert von 1.34 µmol wurde für die β-Galaktosidase von *Lb.* L662 für das Substrat Laktose bestimmt.

Die β-Galaktosidase aus *Lb. pentosus* konnte durch die Anwesenheit von 1 mM Mg^{2+} und Mn^{2+}, beides divalente Ionen, sechsfach aktiviert werden. Monovalente Ionen wie Na^{+} oder K^{+} (+ 100 %) zeigten jedoch keine dermaßen große Steigerungen in der Aktivität, verglichen zu den beiden divalenten Ionen. Anders die β-Galaktosidase aus *Lb.* L662: weder mono- noch divalente Ionen hatten einen besonderen Einfluss auf die Aktivität des Enzyms.

Um die Substratspezifität der beiden β-Galaktosidasen zu testen wurde der Standard Aktivitätstest mit vier dem *o*NPG sehr ähnlichen Substraten durchgeführt. Lediglich ein Substrat, nämlich 4-Nitrophenyl-α-D-Galactopyranosid, konnte von den beiden β-Galaktosidasen hydrolysiert werden. Allerdings war die Substrat Affinität im Vergleich zum *o*NPG erniedrigt, was zu einem Aktivitätsverlust von 50 % (*Lb. pentosus* β-Galaktosidase) und 25 % (*Lb*. L662 β-Galaktosidase) führte. Des Weiteren ist hier zu berücksichtigen, dass das Reaktionsgemisch eine grüne anstatt einer gelben Farbe aufwies.

Abschließend wurde noch getestet in wie weit Laktose (600 mM) hydrolysiert wird und aus den Hydrolyseprodukten (Glukose und Galaktose) Galakto-oligosaccharide (GOS), die präbiotische Aktivität besitzen können, gebildet werden. Nach 8 Stunden Reaktionszeit waren 77 % der Laktose hydrolysiert und insgesamt 65 g/L GOS, das sind 31 % des Reaktionsgemisches, gebildet worden (*Lb. pentosus* β-Galaktosidase). Die Reaktion wurde bei 30°C durchgeführt und die Zugabe von 2 mM $MgCl_2$ gewährleistete gute Enzymstabilität. Aufgrund von noch ungeklärten Wechselwirkungen konnte die β-Galaktosidase aus *Lb*. L662 nach 24 Stunden Reaktionszeit lediglich 14 % der Laktose bei einer GOS Ausbeute von nur 3.8 % umsetzten. Eine mögliche Ursache in diesem Fall könnte einerseits der niedrige kcat/Km Wert und anderseits eine starke Hemmung des Enzyms durch die Endprodukte (Glukose und Galaktose), die bei der Hydrolyse der Laktose entstehen, sein.

Im Allgemeinen zeigte die β-Galaktosidase, gereinigt aus *Lb. pentosus,* eine etwas geringere Affinität zum natürlichen Substrat Laktose als das Enzym aus *Lb.* L662, beide Enzyme jedoch zeigten eine höhere Affinität zu *o*NPG als zu Laktose.

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