Oligosaccharide Production with Thermophilic Enzymes

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Chapter 1

Introduction

Introduction

Non-digestible oligosaccharides can be used as prebiotics in food. In this chapter we describe currently available prebiotic oligosaccharides and their routes for production. A short overview on the concept of prebiotics is given. These products are often not pure oligosaccharides, but mixtures. In this thesis focus is on the production of oligosaccharides of higher purity. One route of production is the synthesis from monosaccharides or disaccharides, using glycosidases as a catalyst. The biochemical production route and the enzyme that is used are described. The choice for a thermophilic enzyme is further discussed. Finally an overview of the aim and contents of this thesis will be given.

Oligosaccharides

Prebiotics

The human colon is host of 10^{11}-10^{12} bacterial cells per gram of gut contents. These can be pathogenic, benign or beneficial. Pathogens can produce toxic compounds and carcinogens; overgrowth of these bacteria can lead to diarrhoea. In addition, there are chronical effects like inflammatory bowel disorders and colon cancer. Beneficial bacteria can aid in the adsorption and digestion of food ingredients and minerals, protect against chronic gut disorders, stimulate immune functions and produce short chain fatty acids (Gibson & Collins, 1999; Gibson *et al.*, 2000). The stimulation of the growth of these beneficial bacteria can be achieved by introducing these bacteria in food (probiotics) or by indigestion of components that specifically promote the growth of these beneficial bacteria (prebiotics), thus feeding the bacteria “in-situ”. The non-digestible oligosaccharides discussed in this thesis can be used as prebiotics. Table 1 shows some commercial prebiotic oligosaccharides.

The Market

Functional foods have a potentially beneficial effect on health when consumed as part of a varied diet on a regular basis. They are generally more established in Japan and the rest of Asia than in the European countries. This is reflected by the use of prebiotics in many functional foods and drinks in Japan and the regulatory situation in that country. In Japan foods can be classified as Food for Special Health Use (FOSHU), allowing some degree of health claim on the product. For prebiotics this can be: promotes bifidobacteria growth in the gut. Prebiotics in Japan are predominantly incorporated in soft drinks and tabletop sweeteners, but also in candy and deserts. The European market is far less advanced in this field. While a range of oligosaccharides is available on the Japanese market, in Europe products are predominantly based on fructo-oligosaccharides.

Table 1 Some commercial prebiotic oligosaccharides and their structure. The major manufacturers of this type of oligosaccharides are given with the product names. (Gibson, *et al.* 2000; Playne & Crittenden 1996) Production figures are based on literature estimates for 1995.

Oligosaccharide	Structure	Production	Product	Manufacturer
Lactulose	Galβ1→4Fru	20,000 tons	MLS-50, MLP-40, MLC-A, MLC-H	Morinaga Milk Industry Co. (Japan)
			Lactulose	Solvay (Germany)
Galacto-oligo's	Tri to pentasaccharides with	15,000 tons	TOS syrup	Borculo Whey Products (Netherlands)
	Galβ1→6Gal and Galβ1→3Gal linkages		Oligomate and TOS-100	Yakult Honsha Co. Ltd (Japan)
			Cup-Oligo	Nissin Sugar Manufacturing Co. Ltd (Japan)
Fructo-oligo's	$Fru(\beta2\rightarrow1Fru)_n$ n=2-5	12,000 tons	Raftilose	Orafti (Belgium)
			Actilight	Beghin-Meiji Industries (France)
			Meioloigo	Meiji Seika Kaisha (Japan)
			NutraFlora	Golden Technologies Co. (USA)
			Oligo-Sugar	Cheil Foods and Chemicals (Korea)
Isomalto-oligo's	$Glc(\alpha1\rightarrow6Glc)_n$ n=1-4	11,000 tons	Isomalto	Showa Sangyo Co. (Japan)
			Panorup*	Hayashibara Shoji Inc. (Japan)
			Panorich* and Biotose	Nihon Shokuhin Kako Co. (Japan)
Soybean oligo's	Galα1→6Glcα1↔2βFru	2,000 tons	Soya-oligo	The Calpis Food Industry Co. (Japan)
Lactosucrose	Galβ1→4Glcα1↔2βFru	1,600 tons	Nyuka-Origo and Pet Oligo	Ensuiko Sugar Refining Co. (Japan)
			Newka-Oligo	Hayashibara Shoji Inc. (Japan)
Xylo-oligo's	Xylβ1→4Xyl	300 tons	Xylo-oligo	Suntory Ltd. (Japan)
Inulin	Fruβ2→1Fru	na	Raftiline	Orafti (Belgium)
Gentio-oligo's	Glcβ1→6Glc	400 tons	Gentose	Nihon Shokuhin Kako Co. (Japan)

* Includes > 25% panose

Production

Oligosaccharide synthesis

Oligosaccharides can be manufactured through extraction, hydrolysis or synthesis. Production of prebiotic oligosaccharides by fermentation is an ill-explored area. Although many organisms produce extracellular polysaccharides (EPS), few are known to produce oligosaccharides (Gibson *et al.*, 2000).

Non-enzymatic processes are based on extraction or chemical synthesis. Extraction is currently performed commercially by Orafti (Belgium) with inulin extracted from chicory and by Calpis Food industry Co. (Japan) with raffinose and stachyose extracted from soybeans. The only chemical synthesis is the isomerisation of lactose to lactulose by Morinaga Milk Industry (Japan) and Solvay (Europe) (Timmermans, 1996).

All other processes follow an enzymatic route to produce oligosaccharides. Examples of the hydrolysis of polysaccharides are partial chicory inulin hydrolysis by a fungal inulase to fructo-oligosaccharides (Orafti, Belgium) (de Leenheer, 1996) and hydrolysis of (arabino) xylan from corncobs, oat spelt or wheat to xylo-oligosaccharides (Suntory Ltd., Japan) (Playne & Crittenden, 1996).

Several oligosaccharides are manufactured through enzymatic synthesis. Most processes use glycosidases for transglycosylation of disaccharides. Important processes are the transglycosylation of sucrose to fructo-oligosaccharides by fructosyltransferase (Meiji Seka, Japan) and the transglycosylation of lactose to transgalacto-oligosaccharides (TOS) by β-galactosidases (Yakult Honsha and Snow Brand, both in Japan, Borculo Whey products, The Netherlands). Hayashibara Shoji Inc. and Ensuiko Sugar Refining Co. (both in Japan) produce lactosucrose from a combination of lactose and sucrose incubated with β-fructofuranosidase. Isomalto-oligosaccharides are first hydrolysed from starch by α-amylase and pullulanase after which a α-glucosidase is added to convert the α1→4 linked malto-oligosaccharides into α1→6 linked isomalto-oligosaccharides (Kohmoto *et al.*, 1988; Gibson *et al.*, 2000).

The final oligosaccharide preparations are usually a powder or highly concentrated sugar solution (70-75% solids). The variation in oligosaccharide content is wide, 20-95% oligosaccharides per total dry weight. (Gibson *et al.*, 2000)

Oligosaccharide recovery

Membrane separation or chromatographic techniques can be used to purify oligosaccharides. Crystallisation is used only for recovery of sugars from watery solutions.

Ion-exchange chromatography can be applied for the purification of saccharide mixtures (Mills, 1961; Saunders, 1968). Cation-exchange resins have a low affinity for oligosaccharides and high affinity for monosaccharides and therefore trisaccharides (or

higher) are the first to elute from the column. Due to a low absolute affinity, elution can be performed with water. It can be applied semi-continuously by using a simulated moving bed (SMB). Ion-exchange chromatography has been applied on an industrial scale for e.g. the production of high-fructose syrup. Here, glucose and fructose are separated after enzymatic isomerisation.

Another chromatographic method is adsorption on active carbon. Affinity is reversed compared to ion exchange chromatography. This separation method is often coupled to an enzymatic reaction. Enzymatic production of oligosaccharides with continuous removal of oligosaccharides from the reaction mixture by adsorption on activated carbon was studied with monosaccharides (Ajisaka *et al.*, 1987) or disaccharides (Boon *et al.*, 2000) as a substrate. A batch process using lactose as substrate with in-line product removal showed a yield improvement of 30% compared to the batch process without product removal. No yield improvement was given for the galactose condensation. Activated carbon has a high affinity for the produced oligosaccharides and product recovery has to be done off-line by 10-50% ethanol elution.

Membrane separations allow continuous purification of saccharide mixtures. Most sugar streams are rather concentrated and it is therefore necessary to work at higher temperatures to prevent unwanted solidification. In contrast to the described chromatographic techniques, most membrane materials can not be used above 60-75°C and sugar solutions have to be diluted (Pedersen, 1999). Recently, nanofiltration membranes with a suitable molecular weight cut-off that can be operated at elevated temperatures were tested for sugar separations. Continuous diafiltration was successfully used for the removal of monosaccharides from an oligosaccharide mixture (Goulas *et al.*, 2002). Ion exchange membranes are used for electro-osmosis in the separation of fructose and glucose. This separation is based on the complexation of fructose with cations (Jain & Giuffrida, 1981).

The enzyme

Usually, glycosidases are applied in oligosaccharide synthesis. They are readily available and use cheap substrates (e.g. sugars). Glycosyltransferases may be applied when high product specificities are necessary. However, these enzymes use (too) expensive sugar nucleotides as substrates. (Bucke, 1996)

β-Glycosidase from Pyrococcus furiosus

The enzyme that is used in the research described in this thesis is a β-glycosidase from *Pyrococcus furiosus*. It was chosen for its high thermostability. Higher temperatures enable solubility of high sugar concentrations, which can benefit oligosaccharide production.

The β-glycosidase from *P. furiosus* was discovered and purified by Kengen *et al.* (1993) and expressed in *E. coli* (Voorhorst *et al.*, 1995), which allows easy purification by heat treatment. The enzyme shows maximal activity at 102-105°C and can be used as a β-glucosidase or β-galactosidase. It also exhibits high β-xylosidase activity.

Enzyme mechanism

β-Glycosidases hydrolyse the β-glycolytic bond between two sugars or between a sugar and a non-sugar (aglycon). Hydrolysis can occur via two mechanisms; inverting or retaining. Hydrolysis via the inverting mechanisms changes the OH-group on the C1 atom of the accepting sugar molecule from an α to a β or vice versa.
The β-glycosidase from *P. furiosus* is a retaining enzyme. When hydrolysis occurs via this mechanism, the configuration of the OH-group on the C1 atom of the accepting sugar molecule is retained. This type of enzyme can be used to synthesise oligosaccharides. (Withers, 2001)

Aim of the thesis

The aim of this thesis is to develop a process concept for the production of a specific set of functional oligosaccharides. Process optimisation is done by performing the synthesis at high substrate concentration. Elevated temperatures are required to dissolve the substrate and subsequently a thermophilic enzyme is used. Enzyme kinetics, equilibrium position and inactivation of the enzyme have been studied. A method for product removal that is applicable at higher temperatures is integrated with the enzymatic reaction.

Outline of the thesis

For a process at elevated temperatures, we used a thermophilic β-glycosidase from *Pyrococcus furiosus*. Other thermophilic enzymes and their industrial applications are reviewed in chapter 2.
The β-glycosidase is used in the enzymatic oligosaccharide synthesis from monosaccharides or disaccharides. Monosaccharides can be condensated to disaccharides and disaccharides can be transglycosylated to trisaccharides. The enzyme kinetics of these reactions with various substrates is studied and modelled in chapter 3.
In chapter 4 the occurrence of the Maillard reaction is related to an increase in enzyme inactivation. The Maillard reaction occurs in sugar-protein (in our case enzyme) systems at higher process temperatures. Maillard products are brown pigments that colour the reaction mixture. This brown colour formation can be modelled in time and an attempt is made to couple it to the enzyme inactivation.

Further reaction optimisation requires a down-stream processing method for oligosaccharide separation. In chapter 5, chromatographic experiments are described and a simulated moving bed (SMB) is modelled. This system is compared to a system with nanofiltration for separation.
In chapter 6 various aspects of the process are discussed further. Emphasis is on the specific influence of temperature on the process and on further optimisation of the downstream processing of oligosaccharides.

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Chapter 2

Thermozymes and their applications

Abstract

Enzymes from thermophilic microorganisms, thermozymes, have unique characteristics such as high temperature-, chemical- and pH stability. They can be used in several industrial processes, where they replace mesophilic enzymes or chemicals. Application of thermozymes is often found when the enzymatic process is compatible with existing (high temperature) process conditions.

The main advantages of performing processes at higher temperatures are the reduced risk of microbial contamination, lower viscosity, improved transfer rates and improved solubility of substrates. However, co-factors, substrates or products might be unstable or other side reactions may occur.

Recent developments show that thermophiles are a good source of novel catalysts that are of great industrial interest. Thermostable polymer degrading enzymes like amylases, pullulanases, xylanases, proteases and cellulases are expected to play an important role in food, chemical, pharmaceutical, paper, pulp and waste-treatment industries.

Considerable research effort is put into a better understanding of the stability of thermozymes. There are no major conformational differences with mesophilic enzymes and a small number of extra salt bridges, hydrophobic interactions or hydrogen bounds seem to confer the extra degree of stabilization.

Nowadays overexpression of thermozymes in standard *Escherichia coli* allows the production of much larger enzyme quantities, which are easy to purify by heat treatment. With wider availability and lower cost, thermophilic enzymes will be more and more applied in industry.

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Introduction

Since the discovery of thermophiles in the 1970's by Thomas Brock, the study of thermophilic and hyperthermophilic microorganisms has become a major domain of research (Brock, 1985). Increasingly attention is paid to these microorganisms because of their unusual properties. Their enzymes (thermozymes) have unique characteristics such as temperature, chemical and pH stability. Thermozymes are more and more used in several industrial processes.
In this review we will focus on thermozymes and their applications as described in literature. The background of their high (thermo)stability and production of thermozymes in mesophiles will be discussed. Furthermore, the application of thermozymes in industrial processes and their benefits over mesophilic enzymes will be considered. Examples of current and future processes using various enzymes will be reviewed.

Thermophilic organisms

Early characterizations of thermophiles were limited to spore-forming aerobes such as *Bacillus stearothermophilus* and anaerobes such as *Clostridium thermosaccharolyticum*. These organisms were not thought to possess inherently stable enzymes and were assumed to have evolved from mesophiles. More recently, microorganisms that grow optimally above 60°C such as *Thermus aquaticus* and *Methanobacterium thermoautotrophicum*, a member of the Archaea, have been isolated which do possess thermostable enzymes. Furthermore, hyperthermophiles have now been isolated such as *Pyrococcus furiosus* and *Thermotoga neopolitana* and *Thermotoga maritima* which grow from 80°C to above 100°C. These types of microbes are thought to be amongst the first forms of life to have evolved on earth. A phylogenetic tree, showing hyperthermophiles and their place in the three-domain model, is depicted in figure 1. With the exception of Thermotogales and *Aquifex*, all organisms which grow optimally above 80°C are Archaea (Vieille *et al.*, 1996a).

Thermozymes

Enzymes from thermophiles and hyperthermophiles are thermostable and only display irreversible protein denaturation at high temperatures. Thermozymes also feature a high temperature for maximum activity. In this chapter we define thermozymes as enzymes that have their maximum activity above 60°C. Hyperthermophilic enzymes have their maximum activity above 80°C.
Thermophilic enzymes can be used in several industrial processes, where they can replace mesophilic enzymes or chemicals. This opens new possibilities for process optimization.

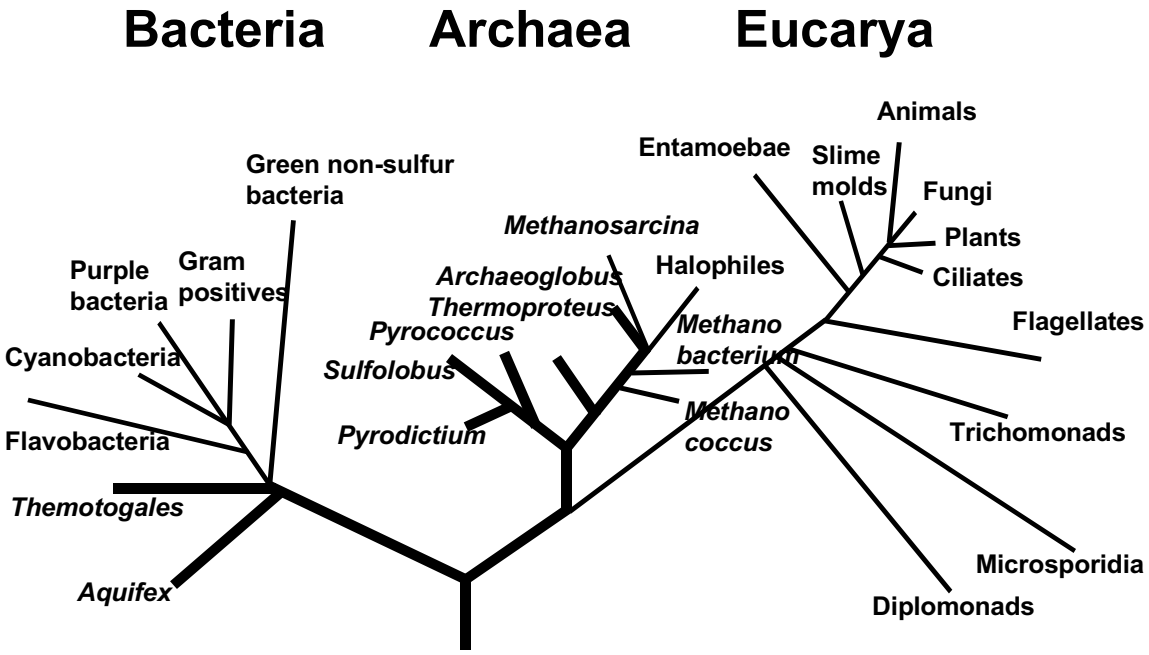

Figure 1 Phylogenetic tree according to Woese *et al.* (1990). The tree has been rooted by analysis of duplicates in protein sequences (Iwabe *et al.*, 1989). Hyperthermophilic genera are depicted as thick lines.

These enzymes, particularly those that are active and stable at temperatures up to 100°C are used to study protein adaptation to high temperatures and to obtain a better understanding of protein stability in general (D'Auria *et al.*, 1998).

Stability of thermozymes

The stability of proteins is the result of a delicate balance between large stabilizing and large destabilizing forces (Brandts, 1967; Jaenicke, 1991; Jaenicke, 1996). Consequently, relatively small changes in either the stabilizing or destabilizing forces can result in large changes in stability. A small number of extra salt bridges, hydrophobic interactions or hydrogen bounds can confer this extra degree of stabilization. There are therefore no systematic structural differences between extremely stable and normal proteins (Matthews, 1993).
The stability of enzymes is interesting from a fundamental as well as from an industrial point of view. Thermozymes can function as examples for improved stability. A better understanding of the stability of thermozymes might reveal ways to stabilize other enzymes. Engineering of mesophilic enzymes might be an option when specific functionality can not be found amongst thermozymes.

The first studies on thermostability focused on amino acid sequences (Nosoh & Sekiguchi, 1988). More recently, crystal structures of thermozymes have been compared with those of their mesophilic counterparts. This gives extra insight into their mechanism of stability (Maes *et al.*, 1999).
Conformational stability of proteins is a result of compromise between two opposing factors: flexibility, for the catalytic function of the enzyme, and rigidity, for conformational stability. Thermozymes are significantly more rigid than their mesophilic counterparts at room temperature. Their high rigidity protects them from unfolding and preserves their catalytically active structure. Therefore they can be optimally active under more denaturing conditions (e.g. higher temperatures).
Enzymes that have been characterized and/or cloned from (hyper) thermophilic organisms have been listed by Vieille *et al.* (1996a). They included thermostability properties of these enzymes, where available.

Mechanisms of enzyme stability

Basic mechanisms of stabilization are high packing density, optimum charge patterns or ion-pairs, minimization of accessible hydrophobic surface area, helix stabilization and subunit assembly (Jaenicke, 1996; Maes *et al.*, 1999; Vieille & Zeikus, 1996b). Oligomer formation and other environmental factors can further stabilize the enzyme. These mechanisms will be illustrated below.

Internal stabilization

Maximum packing efficiency of an enzyme can be achieved by filling cavities in the molecular structure and increasing core hydrophobicity. Higher levels of isoleucine, alanines and prolines are suggested, which should provide tighter packing in hydrophobic cores and extra stability to loops. Vieille and Zeikus (1996b) listed several thermozymes with an increased hydrophobicity, but only two of them have an increased packing efficiency.
Enhanced helix stabilization can contribute to thermostability in various ways. Most important is the loss of conformational entropy of amino acid residues in helical arrangements. Amino acids that are branched on C^{β} (i.e. valine, isoleucine and threonine) cause more conformational restraints and therefore they occur less frequently in thermozymes (Facchiano *et al.*, 1998). Additionally, specific amino acid compositions at the helical ends can influence the stability, but this phenomena is not unique to thermozymes (Maes *et al.*, 1999).
Another way to stabilize enzymes is by increasing the number of stabilizing interactions in the folded protein. Disulfide bridges can stabilize the enzyme by increasing the conformational rigidity. Hydrogen bonds might also stabilize the enzyme (Nosoh & Sekiguchi, 1990). Lately,

the increasing numbers of known 3D structures have made it possible to see the importance of ion-pairs, organized in large networks. It is believed that these networks at the surface of proteins are a major stabilizing factor for thermostability (D'Auria *et al.*, 1998). Arginines frequently occur in ion-pair networks and indeed they occur in higher numbers in enzymes from hyperthermophiles (Mrabet *et al.*, 1992).
Stabilizing interactions between domains and subunits also contribute significantly to the intrinsic stability of proteins. Enzymes from (hyper)thermophilic organisms are known to exist as higher order association states compared with their mesophilic analogues, suggesting that the formation of oligomers is one way of increasing thermostability (Jaenicke, 1996).
At temperatures above 100°C inactivation of enzymes is mainly caused by chemical modification of the protein, rather than by irreversible unfolding. Examples of non-enzymatic irreversible reactions or irreversible covalent modifications are deamidation of asparagine and glutamine or oxidative degradation of cysteine, methionine and tryptophan (Ahern & Klibanov, 1985; Daniel, 1996). Enzymes with lower levels of asparagine, glutamine, cysteine, methionine and tryptophan are less susceptible to degradation by chemical modification.

Environmental factors

Although most enzymes from (hyper)thermophilic organisms are intrinsically very stable, some intracellular enzymes obtain their thermostability from intracellular environmental factors. The presence of salts, high protein concentrations, co-enzymes, substrates, activators or general stabilizers such as thermamine, sorbitol or cyclic polyphosphates can stabilize the enzyme. By manipulating the environmental conditions one can sometimes achieve higher thermostabilities than by application of genetical engineering (Daniel, 1996). Cell-bound thermozymes like saccharidases and proteases, are active at temperatures (far) above the optimal growing temperature of the organism and are, as a rule, highly stable.
Other environmental factors that can be important are for instance chemical crosslinking, immobilization and glycosylation. Glycosylation gives various effects only in some cases protein stability is improved (Jenkins & Curling, 1994). Glycosylation can also improve the efficiency of tertiary protein assembly. The latter effect involves the stabilization of the nascent rather than the native protein (Jaenicke, 1996).
A last method for enzyme stabilization is the use of ultra high pressure (up to 50 MPa), which may lead to more compact structures and thus higher thermostability (Michels & Clark, 1997; Clark *et al.*, 1996).

Protein engineering

Research into the mechanisms of thermostability provides fundamental knowledge on enzyme structure. It allows us to specifically modify enzymes to improve their stability. Enzymes with specific catalytic functions that can not be found amongst thermozymes may be engineered from the appropriate mesophilic enzymes.
Different approaches can be used to stabilize an (mesophilic) enzyme against inactivation by unfolding. Strategies that are promising are often directed at protein surface loops and turns. Surface residues are typically involved in fewer intermolecular interactions than internal residues and are less likely to create volume interferences.
Stabilizing or creating ion-pairs and reduction of conformational strain in helices can be successful. Additional disulfide bridges have been introduced with varying success. They should not be introduced on places where they put too much strain on the protein structure. Also, cysteine residues are not stable around and above 100°C, which renders them less suitable for this purpose (Ahern & Klibanov, 1985). Various strategies to improve core packing such as stabilization of α-helices have shown varying results and the gain in stability is usually small.
To further stabilize thermophilic enzymes, the inactivation mechanism must first be identified. As discussed before, inactivation above 100°C is often caused by covalent modification rather than by unfolding, as is the case with mesozymes. This type of inactivation may be prevented by substitution of specific surface amino acids like asparagine, glutamine, cysteine, methionine and tryptophan with residues that are less susceptible to degradation (Vieille & Zeikus, 1996b; Nosoh & Sekiguchi, 1990).
Until now protein engineering of thermozymes has mainly focused on improving and understanding (thermo)stability. The main goal is to improve activity of (mesophilic) enzymes at higher temperatures and other extreme conditions. Less attention is given to engineering thermozymes for the purpose of changing activity or enlarging selectivity.

Production of thermozymes in mesophilic organisms

In early purification schemes of enzymes from thermophilic bacteria, the growth of large quantities of these thermophiles under unconventional conditions was required (Chien *et al.*, 1976). Nowadays overexpression of enzymes from thermophiles in standard *E. coli*, or other organisms such as *Saccharomyces cerevisiae* (Morana *et al.*, 1995), allows the production of much larger enzyme quantities, which are easy to purify by heat treatment. Thermal denaturation will only affect the host's mesophilic enzymes and the precipitated proteins can simply be eliminated by centrifugation leaving the thermophilic enzyme in solution.

Despite of being synthesized in a mesophilic host, recombinant thermophilic enzymes usually have kinetic and thermal characteristics identical to those of the native protein (Morana *et al.*, 1995).

Reasons for using thermozymes

The main reason for selecting enzymes from thermophiles is their high stability, which makes them attractive for several industrial processes. It is not only their thermostability but also their greater stability under other extreme conditions like high pH or low water concentrations, which is useful in many applications. Stable enzymes are also more likely to allow the use of organic solvents and detergents and are more resistant to proteolytic attack. Proteases are for instance used as additive to household detergents for laundering, where they have to be resistant to denaturation by surfactants and alkaline conditions (Niehaus *et al.*, 1999). Thermophiles to be used as biocatalyst in organic solvents have been reviewed by Sellek and Chaudhuri (1999).

A further advantage of using higher temperatures is the reduced risk of contamination. Temperatures above 70°C are sufficiently high to kill almost all pathogenic bacteria and greatly reduce the numbers of the bacteria most likely to cause troublesome contamination of food processes.

A third factor for applying thermostable enzymes for production purposes is lower viscosity of process fluids. At higher temperatures viscosity is usually reduced, which lowers shear and consequently costs of pumping, filtration and centrifugation, or allowing the use of lower water levels during processing.

Related to this are the improved rates of heat and mass transfer. At higher temperatures diffusion rates will be higher and mass transfer is less limiting (Peek *et al.*, 1992).

More substrate will dissolve at higher temperatures, which can shift the equilibrium to a higher product yield. This is for instance the case in reversed hydrolysis reactions in water (Wilkinson *et al.*, 1997).

It is often speculated that thermozymes should have higher maximal catalytic rates (e.g. Zeikus, 1996) since classical reaction rate theory states that the rate of a chemical reaction increases with temperature. These higher maximal catalytic rates are however not found experimentally. Despite their activity at high temperatures, thermophilic enzymes catalyze reactions at these temperatures with K_m and V_{max} values similar to their mesophilic counterparts at their respective optimal temperature (Cowan *et al.*, 1987). As expected thermozyme activity is driven by temperature-dependent substrate kinetic energy variation alone. If the enzyme structure would change significantly with different temperatures one would expect to find non-linear Arrhenius plots, which is not the case (More *et al.*, 1995). However thermozymes do have a broader temperature range in which they are active (Peek *et al.*, 1992).

An important disadvantage of enzymatic processes at higher temperatures is the loss of selectivity and formation of byproducts. Further, possible required co-factors, substrate (Wilkinson *et al.*, 1997) or products might be unstable and side reactions, like Maillard reactions in sugar-enzyme mixtures, may occur.
Some processes will have higher costs due to additional heating, while other processes are less likely to need cooling (Peek *et al.*, 1992). It depends on the overall process and its implementation whether operation at higher temperatures is beneficial or not. Often applications with thermozymes are found when the enzymatic process is compatible with existing (high temperature) process conditions.

Applications

Recent developments clearly show that thermophiles are a good source of novel catalysts that attract great industrial interest. Thermostable polymer degrading enzymes like amylases, pullulanases, xylanases, proteases and cellulases are expected to play an increasingly important role in food, chemical, pharmaceutical, paper, pulp or waste-treatment industries. A short summary of thermozymes and their (future) applications is given in table 1.

Proteases

Proteases hydrolyze proteins into amino acids and peptides. Thermostable proteases are already used in several industrial processes. In most cases they are used for their stability at higher pH's or in organic solvents rather than their stability at elevated temperatures. Daniel *et al.* (1995) listed several thermostable proteases. The best-characterized thermostable protease is probably Thermolysin produced by *Bacillus thermoproteolyticus*, first described by Endo (1962).
The use of proteases at higher temperatures gives rise to a specific problem: heat enhances autolysis, that is, self-digestion of proteases. The resulting conformational unfolding leads to much faster autolysis, because the (partially) unfolded form of the enzyme is a particularly good substrate for the protease molecules that are still active. The better susceptibility of the substrate to proteolytic attack, results in higher specific activities for the proteases from thermophilic origin (Cowan *et al.*, 1987; Daniel *et al.*, 1982). Since most commercial substrates for proteases are mesophilic proteins it has a high industrial potential. A method to prevent autolysis might be immobilization of the enzyme (Wilson *et al.*, 1994a).
Thermostable proteases are used in peptide synthesis, mainly because of their compatibility with organic solvents (Wilson *et al.*, 1994b). Major current use of a thermostable protease is Thermolysin, used in the synthesis of the dipeptide, N-CBZ-L-Asp-L-Phe methyl ester, which is the precursor in the preparation of the sweetener Aspartame (Isowa *et al.*, 1979). In the process that employs immobilized Thermolysin the compatibility of the enzyme to organic

solvents is crucial to the process rather than its use at high temperature (Nakanishi *et al.*, 1990).
Another actively marketed thermostable protease is a *Thermus* protease, Pretaq, used to clean up DNA before amplification in the polymerase chain reaction (PCR). The commercial success of this application may be due to the relatively cost intensive nature of the application.
Proteases that are resistant to alkalinity and anionic or nonionic surfactants are used as additives to domestic detergents for laundering. Improved stability is desirable here, but thermostable proteases are not yet being produced in high enough quantities to be cost competitive. Moreover, current trends in laundering processes towards lower temperatures may present another barrier. The use of thermozymes in laundry detergents for institutional (e.g. hospital) use may well be a future application since in those processes particularly high temperatures are required.
A larger application might be the use of thermophilic enzymes in (domestic) dishwashing detergents. Here, much lower pHs are applied (around 9), combined with temperatures of around 60°C. A thermostable protease would well be able to withstand this pH and temperatures above 60°C.
Proteases showing high keratinolytic and elastolytic activities are used for soaking in the leather industry (Niehaus *et al.*, 1999). Thermophilic proteases could be better applicable at the bating of hides and skins at acid (pH 2.5-5) and alkaline (pH 12-13) conditions.
Coolbear *et al.* (1992) tested several proteases for application in the cleaning of ultrafiltration membranes. Thermostable proteases at high temperatures (and low viscosity's) may allow these products to be re-used, and this may lead to a reduction in chemical use and waste production. Presently, no thermophilic proteases are available for this application.
Other potential applications are found in meat tenderizing, where the ideal meat tenderizing enzyme would be active only during preparation, and not during storage (Wilson *et al.*, 1992).

Starch converting enzymes

Starch is a mixture of amylose and amylopectin. Amylose consists solely of α-1,4 linked glucose polymers, where amylopectin also has α-1,6 linkages.
Several enzymes are involved in the conversion of starch; endo- and exoacting amylases that act primarily on α-1,4-linkages, debranching enzymes, like pullulanases, that act on α-1,6-linkages and cyclodextrin glycosyltransferases that degrade starch by catalyzing cyclization and disproportionation reactions. The industrial conversion of starch is a multistage process, which involves a range of enzymes in successive steps and is illustrated in figure 2. Enzymes from hyperthermophiles that can act at the boiling point of water and preferably at lower pH’s

Table 1 Summary of thermozymes, their possible applications and the main reason why a specific thermophilic enzyme is of interest for an industrial process.

Enzyme	Applications	Reason for usage	Reference
Alcohol dehydrogenase	Chiral Synthesis	Improved stability	WO 9921971
Amylase	Production of high glucose syrup	Compatibility with an existing high temperature process	Antranikian *et al.*, 1995, Wind, 1997, US 5714369
Cellulase	Pulp and paper processing	Compatibility with an existing high temperature process	WO 9744361, WO 9714804
	Laundry detergents	Stable at high pH	WO 9744361, WO 9743381, WO 9714804
Cyclodextrin glycosyl transferases	Cyclodextrin production	Compatibility with an existing high temperature process	Wind, 1997, Starnes, 1990, Kim *et al.*, 1997, JP 10234387
DNA polymerase	DNA amplification, reverse transcription	Reaction at high temperature	Kotewitz *et al.*, 1988, US 6054301, WO 0020629, WO 9953074, WO 9845452, WO 9814590, WO 9735988
	DNA labeling	Reaction at high temperature	-
	DNA sequencing, Cycle sequencing method	Reaction at high temperature	Innis *et al.* 1988, EP 892058, WO 9814588
Glucoamylase	Starch conversion	Compatibility with an existing high temperature process	Wind, 1997
Glycosidase	Hydrolysis of lactose	Less microbial growth at high temperature	Petzelbauer *et al.*, 1999
	Oligosaccharide synthesis	Better substrate solubility at high temperature	Wilkinson *et al.*, 1997, Boon *et al.*, 1998, Reuter *et al.*, 1999

Continuation of table 1

Enzyme	Applications	Reason for usage	Reference
	Synthesis of alkyl glycoside detergents	Compatible with organic solvents	Fischer *et al.*, 1996
Laccase	Textile bleaching	-	WO 9725469
	Laundry detergents	Stable at high pH	WO 9743381
Ligase	Ligase chain reaction	Reaction at high temperature	Barany, 1991, WO 0026381, US 5830711
	Construction of sequencing primers	Reaction at high temperature	Kaczorowski & Szybalski, 1996
Lipase	-	Melting of substrate at high temperature	-
	Detergents	Stable at high pH	-
Protease	Synthesis of aspartame precursor	Compatibility with organic solvents	Endo, 1962, Isowa *et al.*,1979, Nakanishi *et al.*, 1990
	Clean up DNA before PCR	Compatibility with an existing high temperature process	-
	Meat tenderization	High temperature	Wilson *et al.*, 1992
	Peptide synthesis	Compatibility with organic solvents	Wilson *et al.*, 1994b
	Detergents	Stable at high pH	US 5714373
	Leather soaking	Stable at extreme pH	Niehaus *et al.*, 1999
	Membrane cleaning	High temperature: low viscosity	Coolbear *et al.*, 1992
Pullulanases	High glucose syrup production	Compatibility with an existing high temperature process	Wind, 1997, US 5714369
Xylanase	Bleaching	Compatibility with an existing high temperature process	Chen *et al.*,1997, US 5922579, EP 828002, WO 9736995, WO 9722691, WO 9714803
Xylose/Glucose isomerase	High fructose syrup production	High temperature shifts equilibrium	Zeikus, 1996, Liu *et al.*, 1996

are regarded as interesting candidates for starch conversion. Several thermophilic archaeal amylolytic enzymes have been listed by Lévêque *et al.* (2000).

α-Amylases

α-Amylases are endoacting enzymes that cleave α-1-4 bonds. They are used in the first stage of starch conversion, called liquefaction.
Raw starch consists of insoluble semi-crystalline granules on which most enzymes are poorly active. The first process step consists of heating the granules in water, which causes swelling and gelatinization (Nigam & Singh, 1995). Then starch is liquefied by e.g. the thermostable α-amylase from *Bacillus licheniformis* (Thermamyl) or *Bacillus amyloliquefaciens*. Various enzymes can be used depending on the desired product (Antranikian *et al.*, 1995). Several thermostable α-amylases have already been characterized (e.g. Koch *et al.*, 1991; Wind *et al.*, 1994; Lee *et al.*, 1996; Canganella *et al.*, 1994; Liebl *et al.*, 1997; Kwak *et al.*, 1998) The most thermostable α-amylase to date is from *Pyrococcus woesei*. It remained active after autoclaving for 4 h at 120°C (Antranikian, 1991).

Glucoamylases

Glucoamylases are exoacting enzymes that cleave both α-1-4 and α-1-6 bonds. The branching points are however hydrolyzed at a rather slow rate. These enzymes are currently used in the second step in starch conversion where they are used for the production of high glucose syrup from starch-originated polysaccharides. For industrial production the glucoamylase from *Aspergillus niger* is generally used. Due to the lack of commercially available thermostable enzymes, the process conditions after starch liquefaction have to be changed. For the production of glucose the pH and temperature have to be lowered. Due to the pH variation large amounts of salts are added, which need to be removed again.
Improvement of the process by introduction of new and more efficient, e.g. thermostable, enzymes would significantly lower the costs. Only few thermostable glucoamylases have been found up to now. Recently, a thermostable glucoamylase has been purified from *Thermoanaerobacterium thermosaccharolyticum* DSM 571 (Ganghofner *et al.*, 1998).

Pullulanases

Pullulanase type I cleaves α-1-6 bonds in starch and is an example of a debranching enzyme. It is used in the production of high glucose syrup from polysaccharides. For industrial purposes the pullulanase from *Bacillus acidopullulyticus* is often used, in combination with the glucoamylase from *A. niger* (Wind, 1997). Thermostable pullulanase type I from

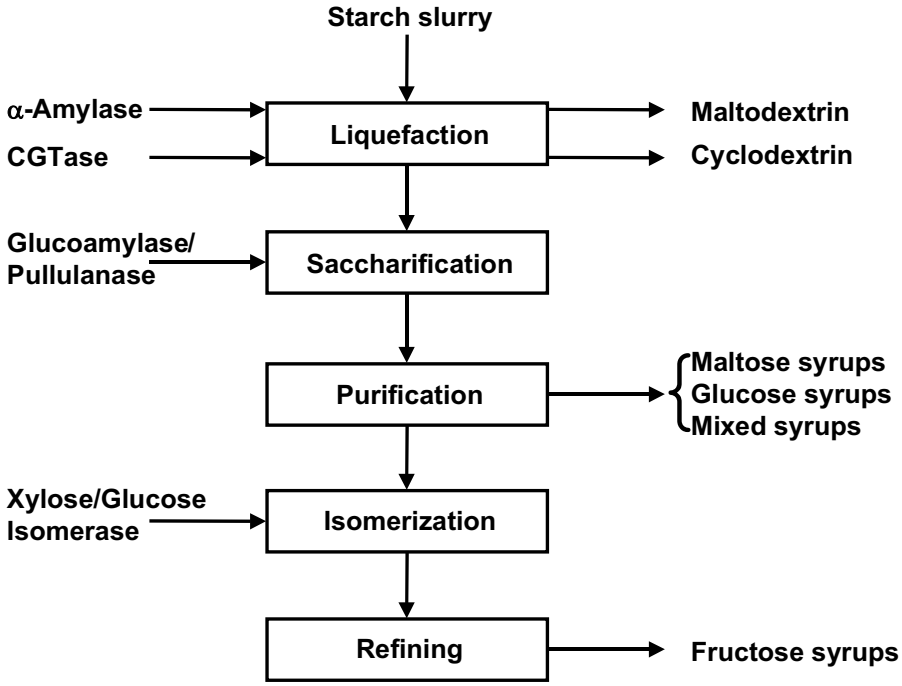

Figure 2 Enzymatic starch conversion into various sugars (adapted from Olsen, 1995).

Thermus caldophilus and *Fervidobacterium pennavorans* (Koch *et al.*, 1997) have been characterized and the latter has recently been cloned into *E. coli* by Bertoldo *et al.* (1999).
Pullulanase type II cleaves in addition to α-1-6 bonds also α-1-4 bonds and is therefore able to saccharify starch directly into maltotetraose, maltotriose and maltobiose. Pullulanase type II is usually referred to as amylopullulanase (Zeikus, 1996).
Several thermostable type II pullulanases have already been described (Canganella *et al.*, 1994; Duchiron *et al.*, 1997) and purified (Brown & Kelly, 1993; Gantelet & Duchiron, 1998). Two hyperthermophilic pullulanases type II, from *P. woesei* (Rudiger *et al.*, 1995) and from *P. furiosus* (Dong *et al.*, 1997), have been expressed in *E. coli*.

Cyclodextrin glycosyl transferases

Cyclodextrin glycosyl transferases (CGTases) attack α-1-4 linkages in polysaccharides and convert starch by transglycosilation to α-, β-, and γ-cyclodextrins. Cyclodextrins can form inclusion complexes with small hydrophobic molecules and this ability has provided a number of applications in the food, cosmetic, pharmaceutical and agrochemical industry as well as in analytical chemistry (Starnes, 1990).
Normally cyclodextrin production is a two-stage process. The first step consists of starch liquefaction by a heat stable α-amylase followed by the cyclization reaction by a CGTase from a *Bacillus* sp. Because of the low stability of this enzyme, the second process step is carried out at a lower temperature than the previous liquefaction step (Niehaus *et al.*, 1999).

New thermostable CGTases have been found that are able to solubilize starch without the need for α-amylase pretreatment (Kim *et al.*, 1997). By using thermostable CGTases, overall cyclodextrin production time and cost can be reduced significantly. This is required to open up the application of cyclodextrins on a commercially attractive scale (Wind, 1997).
Thermostable CGTases have been found in *Thermoanaerobacter* sp. (Kim *et al.*, 1997) and *Thermoanaerobacterium thermosulfurogenes* (Wind *et al.*, 1995).

Other important sugar converting enzymes

Xylose isomerase and glucose isomerase

Xylose isomerase is used for the isomerization of glucose into fructose. This transfers high glucose corn syrup into high fructose corn syrup, which is used as a sweetener.
The current process is operated at 58°C with mesophilic xylose isomerases and yields syrup with 40-42% fructose. This necessitates an additional chromatographic step to obtain the required 55% syrup concentration.
Since the conversion is endothermal, the equilibrium for the isomerization reaction is shifted to fructose at higher temperatures. The same concentration of 55% fructose syrup can be obtained by performing the reaction at 95°C with a thermophilic xylose isomerase without the chromatographic concentration step (Zeikus, 1996; Quax, 1993).
Thermophilic xylose isomerases have been characterized from a *Thermoanaerobacterium* strain (Liu *et al.*, 1996) and from *Thermus flavus* (Park *et al.*, 1997).
Isomerization of glucose into fructose can also be done by a glucose isomerase. The most acid-stable and thermostable glucose isomerase available is the glucose isomerase of *Bacillus coagulans* (Wind, 1997).

β-Glycosidases

Glycosidases hydrolyze sugar bonds. Bauer *et al.* (1996) listed several glycosidases from hyperthermophiles. The best-known β-glycosidases are from *P. furiosus* (Kengen *et al.*, 1993) and *Sulfolobus solfataricus* (Pisani *et al.*, 1990).
Most industrial applications of β-glycosidases have employed thermostable glycosidases for hydrolytic purposes. β-Glycosidase is for instance used in the hydrolysis of lactose into glucose and galactose. The monosaccharides that are formed from this reaction have a higher solubility and sweetening effect than lactose and are more easily digested by humans. The main reasons for using thermozymes in this process are increased substrate and product solubility and a reduced risk of microbial growth (Petzelbauer *et al.*, 1999).
Glycosidases are also used in the reverse reaction of oligosaccharide synthesis. The thermostable *P. furiosus* β-glucosidase produces tri- and tetrasaccharides with lactose as a

substrate. These oligosaccharides may be used as prebiotics in functional foods. The increase of the substrate solubility at higher temperatures and the subsequent lower water activity improves the oligosaccharide yield. The occurrence of Maillard reactions between the sugar and the enzyme hinders this reaction at higher temperature (Boon *et al.*, 1998).
The low acceptor specificity of glycosidases can be further exploited for the synthesis of alkyl glycoside detergents and other novel products. The β-glucosidase of *P. furiosus* can accept primary and secondary alcohols, diols and cyclic diols and sometimes tertiary alcohols (Fischer *et al.*, 1996).
Lowering the water activity for specific synthesis may also be achieved by using solvents, since thermozymes are often compatible with organic solvents. They further permit higher concentrations of hydrophobic donor substrates (Reuter *et al.*, 1999).

Xylanases

Xylanases catalyze the hydrolysis of xylan. Kulkarni *et al.* (1999) listed some xylanases from thermophilic bacteria. Xylan is the main polymeric compound of hemicellulose. Xylanases can be used in the biobleaching of wood or bagasse pulp. Because of environmental concerns, this is of great interest since the use of chlorine chemicals can be reduced. Minimal attack on the cellulose fiber is a prerequisite and for this reason the xylanases should be cellulase free. During the enzymatic bleaching process, the polysaccharide chains attached to the lignin hydrolyze and the pulp structure opens up. In this way, the consumption of bleaching chemicals can be reduced while a higher final optical brightness is obtained.
Commercial enzymes like Pulpzyma and Cartazyme are not thermostable. In the treatment of pulp, several stages before the chlorine bleaching step are performed at 70°C and higher. Consequently, pulp must be cooled before the enzymatic treatment and reheated for subsequent processing steps (Chen *et al.*, 1997). In addition to improved thermostability it is also desirable to have enzymes that are active at the alkaline pH of the pulp (Garg *et al.*, 1996).

DNA-processing enzymes

DNA polymerases

DNA polymerases are the key enzymes in the replication of DNA. Nowadays thermostable polymerases play a major role in several molecular biological applications like DNA amplification, sequencing and labeling. A major advance in molecular biology is the development of the polymerase chain reaction (PCR). In the first PCR reaction a heat-labile *E. coli* DNA polymerase was used, which had to be added during each cycle following the denaturation and primer hybridization steps. The availability of thermostable DNA

polymerases facilitated the automation of the thermal cycling enormously. The polymerase from *T. aquaticus*, Taq polymerase, is the first characterized thermophilic polymerase (Chien *et al.*, 1976) and is still widely applied in PCR.
Thermostable DNA polymerases can also be used for reverse transcription, where RNA is used as a starting template by first converting RNA to cDNA. This can then be used for generating cDNA libraries. The original mesophilic reverse transcriptase that was used at low temperatures gave the problem of the formation of stable secondary RNA structures (Kotewitz *et al.*, 1988). However many thermostable polymerases can also use RNA as a template. The DNA polymerase from *Thermus thermophilus* HB8 is commonly used for this reaction.
The introduction of thermostable DNA polymerases in sequencing was a major step forward leading to the cycle sequencing method. This is a PCR-like amplification of the sequencing products. The advantages are that less template DNA is required, no separate primer annealing step is needed and dissolution of unwanted secondary structures within the template can be achieved by high temperature elongation (Innis *et al.*, 1988).

Ligases

DNA ligase catalyzes the linking of polynucleotides. The first thermostable ligase was discovered in *T. thermophilus* HB8 in 1984 (Takahashi *et al.*, 1984). Several thermostable DNA ligases have been discovered since then (Starnes, 1990). An important application for thermophilic ligases is the ligase chain reaction. In this reaction DNA ligase amplifies DNA, but a single-base substitution prevents ligation and is thus distinguished. It can therefore be used in DNA diagnostics for the detection of single-base mismatches. The specificity of this reaction is highest at the melting point of the primers (Barany, 1991), which is typically 50-65°C. Another application lies in the construction of sequencing primers by high temperature ligation of hexameric primers (Kaczorowski & Szybalski, 1996).

Lipases

Lipases hydrolyze tri- di- and monoglycerides into fatty acids and glycerol. Several (hyper)thermophilic lipases have been isolated and characterized. Biological treatment of fats at high temperatures is advantageous, since fats above their melting point, in the liquid state, become more accessible to the enzyme. However, not many specific applications for thermophilic lipases are mentioned in literature. Although oil modification is often performed under virtually anhydrous conditions, and at temperatures of up to 70°C most processes still use mesophilic enzymes (Quinlan & Moore, 1993).

Is there a future for thermozymes?

Thermozymes are already found in several existing processes and have the potential for much wider application. They can replace enzymes from mesophiles in processes that benefit from a higher temperature of operation. At higher temperatures synthesis processes can be operated at other, beneficial conditions, such as higher substrate concentrations.
Thermozymes can be applied as biocatalysts in existing industrial processes to replace presently used, often polluting, chemical reagents. This is especially true for the pulp and paper industry that has problems with aggressive waste from the bleaching process.
Thermozymes play a central role in newly developed processes, such as PCR, that could not efficiently be performed before the discovery of thermozymes.
Daniel (1996) states that the theoretical upper limit of enzyme thermal stability might be well above 130°C. If these enzymes cannot be found in nature, then they might be developed by engineering. This, and the enhanced stability under other extreme conditions, will further broaden the field in which thermozymes can be used.
The production of thermozymes in mesophilic organisms makes large scale production and easy purification of these enzymes possible and it is therefore expected that with wider availability and lower costs thermophilic enzymes will be increasingly applied in industry.

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Chapter 3

Enzyme kinetics and modelling

Abstract

Oligosaccharides can be synthesised from monosaccharides or disaccharides, using glycosidases as a catalyst. To investigate the potential of this synthesis with β-glycosidase from *Pyrococcus furiosus* we determined kinetic parameters for substrate conversion and product formation from cellobiose, lactose, glucose and galactose.
The obtained parameters for initial rate measurements of disaccharide conversion were also used for the interpretation of experiments in time. The model for cellobiose gave a good description of the experiments. The enzyme was found to be uncompetitively inhibited by cellobiose and competitively inhibited by glucose. Lactose conversion however, could not be modelled satisfactorily; apparently additional reactions take place. Monosaccharide condensation also yielded oligosaccharides, but much slower.
The use of a hyperthermostable enzyme was found to be positive. More substrate could be dissolved at higher temperatures, which benefited all reactions.

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Introduction

Glycosidases can be used as a biocatalyst in oligosaccharide synthesis. They are inexpensive when compared to transglycosidases and do not need complex expensive substrates, like sugar nucleotides. With disaccharides as a substrate, oligosaccharides can be formed in a kinetically controlled reaction that shows an optimum in time. Oligosaccharide (trisaccharides or higher) yields are typically around 40% (w/w). However, the number of available substrates is limited. Up to date, commercialised processes are based on lactose and sucrose. Equilibrium synthesis from monosaccharides might be an alternative to the transglycosylation from disaccharides. Equilibrium yields are much lower (around 10-25% (w/w) disaccharides and only a few percentages trisaccharides or higher), but there are no hydrolytic side products and when combined with an effective separation process, a hypothetical 100% yield is possible.

In this chapter we describe oligosaccharide synthesis from cellobiose, lactose, glucose and galactose, with the aid of β-glycosidase. The oligosaccharides that are produced can be used as prebiotic food ingredients. Non-digestible oligosaccharides have a positive influence on the growth of essential microorganisms in the human gut flora. This has been proven for transgalactosyl-oligosaccharides, but also β-gluco-oligosaccharides may proof useful. Novel oligosaccharides continue to get patented as bifidogenic factors, but further research is required (Crittenden, 1999).

The β-glycosidase used in this research is from *Pyrococcus furiosus*, a hyperthermostable organism. This type of enzyme has an increased stability and enables us to perform experiments at elevated temperatures. The main advantages hereof are the reduced risk of microbial contamination, lower viscosity, improved transfer rates and improved solubility of substrates and products. For oligosaccharide production the main benefit of using higher reaction temperatures lies in the increase of the substrate concentration that improves the oligosaccharide yield. Possible disadvantages in the use of thermozymes may be found in the instability of co-factors, substrates or products and the occurrence of side reactions (chapter 2). In the process considered here the occurrence of Maillard reactions between the sugar and the enzyme might lead to increased enzyme inactivation that limits applicability of glycosidases at higher temperatures (chapter 4).

In this chapter we describe the synthesis of oligosaccharides from disaccharides and alternatively from monosaccharides. The kinetic parameters for these processes will be determined and a comparison between them will be made.

Theory

Synthesis with disaccharides as a substrate

The enzymatic synthesis of oligosaccharides is usually catalysed by glycosidases with disaccharides as a substrate. This reaction is carried out as a kinetically controlled reaction that shows an optimum in time. A substrate-enzyme complex is formed, which can either be attacked by another substrate molecule or by water, yielding a trisaccharide or a monosaccharide respectively. In diluted systems, where the formation of oligosaccharides is negligible, it is sufficient to model disaccharide hydrolysis (Santos *et al.*, 1998; Ladero *et al.*, 2001). When using higher substrate concentrations, incorporation of the back and forth reaction to trisaccharides is necessary. This has been done for cellobiose conversion by several authors (Gusakov *et al.*, 1984; Calsavara *et al.*, 1999). Other authors modelled the formation of oligosaccharides from lactose (Prenosil *et al.*, 1987; Nakayama & Amachi, 1999; Boon *et al.*, 1999). More complex models also incorporate the formation of higher oligosaccharides than trisaccharides, the formation of galactose-galactose disaccharides (Bakken *et al.*, 1992), mutarotation of galactose (Bakken *et al.*, 1992), product inhibition, substrate inhibition and the formation of allolactose due to intramolecular transfer reactions (Huber *et al.*, 1976; Petzelbauer *et al.*, 2000).

Kinetic modelling of disaccharide conversion

Characterisation of an enzyme usually includes the measurement of initial conversion rates for several substrates. A classical Michaelis-Menten mechanism (Michaelis & Menten, 1913) is often chosen to describe the results. This was done by several authors for cellobiose and/or lactose conversion by β-glycosidase from *P. furiosus* (Petzelbauer *et al.*, 2000). Unfortunately, most of these measurements were performed at low substrate concentrations. Besides the conversion rate of the substrate, it is also vital to know the rate of transglycosylation when considering the synthesis of oligosaccharides. In most studies there is no separate measurement of the reaction rate for transglycosylation. Therefore in this work special attention was paid to the oligosaccharide formation rates. To be able to give a good description of our data we propose a reaction mechanism that includes both hydrolysis and transglycosylation.
The rate expressions were derived with the King-Altman method (King & Altman, 1956). Figure 1a depicts the models that are used for initial rate modelling, figure 1b for reactions in time. The equations for the models are given in figure 2. For the description of the initial conversion of disaccharides four models will be tested. The simplest model to be used is depicted by the solid arrows in figure 1a. It uses three kinetic rate constants: k_1, a measure for the overall reaction rate, and k_2 and k_3 as a measure for hydrolysis and synthesis respectively.

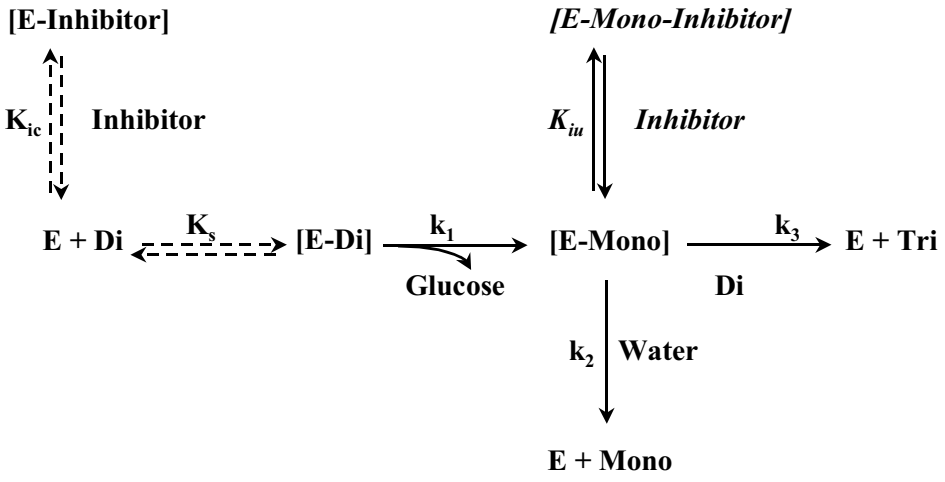

Figure 1a Schematic model for initial disaccharide conversion. The disaccharide can be either cellobiose or lactose, the corresponding monosaccharide in the enzyme complex glucose or galactose. Basic model, as used for lactose is in normal font, cellobiose extensions are in italic, unnecessary extensions are dotted. (mono = monosaccharide, di = disaccharide, tri = trisaccharide, E = enzyme, k_1, k_2, k_3 and k_4 = kinetic rate constants, K_{ic} and K_{iu} = kinetic parameter for competitive and uncompetitive substrate inhibition, K_s = dissociation constant)

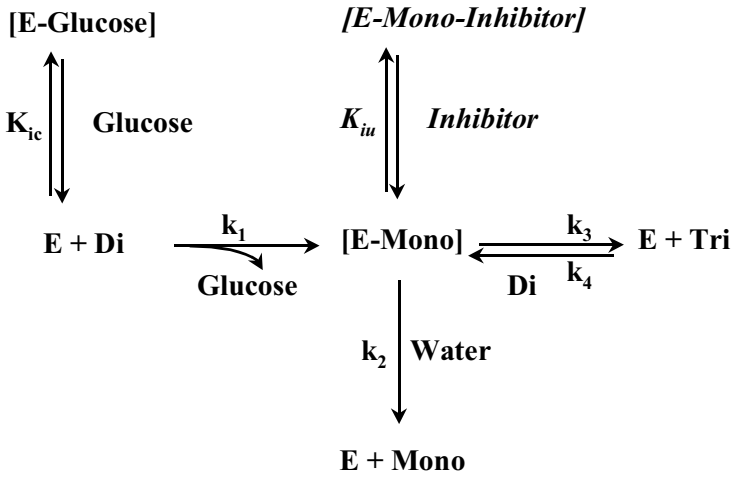

Figure 1b Schematic model for disaccharide conversion in time. Symbols according to figure 1a.

Rate equations for initial cellobiose conversion as used in the results:

$$\left.\frac{d[glu]}{dt}\right|_{ini} = \frac{(2k_1k_2[H_2O][C] + k_1k_3[C]^2)E_0}{k_2[H_2O] + (k_3 + k_1)[C] + \mathrm{K_{iu}k_1[C]^2}} \quad \text{C1}$$

$$\left.\frac{d[tri]}{dt}\right|_{ini} = \frac{k_1k_3[C]^2 E_0}{k_2[H_2O] + (k_3 + k_1)[C] + \mathrm{K_{iu}k_1[C]^2}} \quad \text{C2}$$

Rate equations for cellobiose conversion in time as used in the results:

$$\frac{d[glu]}{dt} = \frac{(2k_1k_2[H_2O][C] + k_1k_3[C]^2 + k_2k_4[H_2O][tri])E_0}{k_2[H_2O] + (k_3 + k_1)[C] + \mathrm{K_{iu}}k_1[\mathrm{C}]^2 + \mathrm{K_{ic}}k_2[H_2O][glu] + \mathrm{K_{ic}}k_3[C][glu] + k_4[tri]} \quad \text{C3}$$

$$\frac{d[tri]}{dt} = \frac{(k_1k_3[C]^2 - k_2k_4[H_2O][tri])E_0}{k_2[H_2O] + (k_3 + k_1)[C] + \mathrm{K_{iu}}k_1[\mathrm{C}]^2 + \mathrm{K_{ic}}k_2[H_2O][glu] + \mathrm{K_{ic}}k_3[C][glu] + k_4[tri]} \quad \text{C4}$$

Balance:

$$2\frac{d[C]}{dt} = -3\frac{d[tri]}{dt} - \frac{d[glu]}{dt} \quad \text{C5}$$

Rate equations for initial lactose conversion as used in the results:

$$\left.\frac{d[gal]}{dt}\right|_{ini} = \frac{k_1k_2[H_2O][L]E_0}{k_2[H_2O] + (k_3 + k_1)[L]} \quad \text{L1}$$

$$\left.\frac{d[tri]}{dt}\right|_{ini} = \frac{k_1k_3[L]^2 E_0}{k_2[H_2O] + (k_3 + k_1)[L]} \quad \text{L2}$$

Rate equations for lactose conversion in time as used in the results:

$$\frac{d[gal]}{dt} = \frac{(k_1k_2[H_2O][L] + k_2k_4[H_2O][tri])E_0}{k_2[H_2O] + (k_3 + k_1)[L] + \mathrm{K_{ic}}k_2[H_2O][glu] + \mathrm{K_{ic}}k_3[L][glu] + k_4[tri]} \quad \text{L3}$$

$$\frac{d[tri]}{dt} = \frac{(k_1k_3[L]^2 - k_2k_4[H_2O][tri])E_0}{k_2[H_2O] + (k_3 + k_1)[L] + \mathrm{K_{ic}}k_2[H_2O][glu] + \mathrm{K_{ic}}k_3[L][glu] + k_4[tri]} \quad \text{L4}$$

Balances:

$$\frac{d[glu]}{dt} = \frac{d[gal]}{dt} + \frac{d[tri]}{dt} \quad \text{L5}$$

$$\frac{d[L]}{dt} = -\frac{d[gal]}{dt} - 2\frac{d[tri]}{dt} \quad \text{L6}$$

Figure 2 Equations that describe cellobiose (upper half) and lactose (lower half) conversion. (glu = glucose, gal = galactose, L = lactose, C = cellobiose, tri = trisaccharide, E = enzyme, k_1, k_2, k_3 and k_4 = kinetic rate constants, K_{ic} and K_{iu} = kinetic parameter for competitive and uncompetitive substrate inhibition).

The model can be extended with uncompetitive (K_{ic}) or competitive substrate inhibition (K_{iu}) or with the addition of the noncovalent Michaelis (disaccharide-enzyme) complex (K_s). These additions are depicted in italic and with dotted arrows in figure 1a.
The kinetic rate constants derived from the initial rate data can be used to predict the reaction in time. However, in a reaction in time other reactions have to be taken into account that initially do not occur. Most important are the hydrolysis of the formed trisaccharides (k_4) and enzyme inhibition by glucose (Boon *et al.*, 1998). In long time experiments enzyme inactivation can also become important. Other examples are the formation of higher oligosaccharides than trisaccharides, the formation of disaccharides from monosaccharides and intramolecular transfer reactions.
The four suggested models give the same balances for the formation and conversion rates of the sugars. An overall balance can be made as shown in figure 2 by equation C5 for cellobiose conversion. By using overall mass balances, measurements of two reaction rates are enough for a description of the whole system.
An important difference between lactose and cellobiose conversion is the production of respectively two or one type(s) of monosaccharide. For lactose as a substrate the separate production of galactose and glucose makes it easier to distinguish between the different reaction routes. With cellobiose as a substrate, glucose is the only monosaccharide produced. This formation rate cannot be used directly for the description of the hydrolytic activity, because glucose is also formed when oligosaccharides are produced.

Synthesis with monosaccharides as a substrate

Another way to produce oligosaccharides with the aid of glycosidases, is the condensation reaction of monosaccharides (Ajisaka *et al.*, 1987a; Bucke, 1996; Rastall & Bucke, 1992a). This reaction is slower and for a single reaction step the equilibrium oligosaccharide yield is lower, but in a next step the substrate can be recycled. When combined with a good method of separation a 100% conversion can be obtained theoretically. Enzymatic glucose condensation can yield one disaccharide (Nunoura *et al.*, 1997), often gentiobiose, or several disaccharides (Fujimoto *et al.*, 1987; Ajisaka *et al.*, 1987a) depending on the enzyme used. Process conditions influence the composition of the mixture (Fujimoto *et al.*, 1987).
Glucose condensation can also be achieved by using β-glucanase (Rastall *et al.*, 1992b) or glucoamylase (Fujimoto *et al.*, 1987; Rastall *et al.*, 1991). Other options for oligosaccharide synthesis from monosaccharides are with e.g. galactose and fructose, using a β-galactosidase (Ajisaka *et al.*, 1987b) or mannose, using α-mannosidase (Rastall *et al.*, 1992c; Johansson *et al.*, 1989).

Equilibrium description for monosaccharide condensation

The equilibrium reaction in which 2 monosaccharides (mono) react to a disaccharide (di) and water can be described by equation 1, in which K_{eq} is the equilibrium constant.

$$K_{eq} = \frac{[di]\cdot[water]}{[mono]^2} \qquad [1]$$

Material and Methods

Materials

All chemicals were from Sigma (St Louis, USA). β-Glycosidase from *P. furiosus* was prepared from an *E. coli* lysate and heated to denature proteins other than the hyperthermostable enzyme (Voorhorst *et al.*, 1995).

Determination of protein concentration

Protein was determined with Coomassie brilliant blue G250 as described by Bradford (1976). Bovine Serum Albumin was used as a standard.

Enzyme activity measurements

β-D-Glycosidase was assayed using pNPG (*p*-nitrophenyl-β-D-glucopyranoside) as an artificial substrate at 80°C. The increase in absorbance at 405 nm as a result of *p*-nitrophenol formation was measured. A standard reaction mixture contained 2.0 m*M* pNPG in 0.2 mol/kg citrate buffer at pH 5.0.

HPLC analysis of sugars

The reaction mixture was analysed by HPLC to detect the different sugars present. Samples from the reaction mixture were treated with $Pb(NO_3)_2$ (final concentration in the samples 0.1 $mol \cdot l^{-1}$) to precipitate the citrate that would otherwise interfere in the analysis and stored in the freezer for at least 1 hour to accelerate precipitation. Before analysis the samples were centrifuged at 13,000 rpm and diluted.
The samples were analysed on HPLC using a RSO Oligosaccharide Column (Phenomenex, Amstelveen, the Netherlands) at 80°C. The column was eluted with Milli-Q water (purged with helium) at a flow rate of 0.3 $ml \cdot min^{-1}$. The eluent was monitored with a refractive index

detector. It was assumed that the response was independent on the degree of polymerisation. Lactose, glucose, galactose and oligosaccharides, such as tri-, tetra-, and pentasaccharides were detected and measured as percentages of total sugar on weight base.

Initial rates measurements

The initial conversion of cellobiose and lactose was measured at substrate concentrations varying from 0.01 to 1.5 mol·kg^{-1}. Glucose and galactose condensation was only measured at 1.5 and 1.7 mol·kg^{-1} respectively to find the maximal rate of conversion. Experiments were performed at 80°C and the sugar was dissolved in 0.2 mol·kg^{-1} citrate buffer at pH 5.0. Enzyme was added and the reaction was followed for approximately 10-15 minutes. Samples (50 µl) were taken at regular intervals. All sugars were measured via HPLC.

The reaction in time

The conversion of cellobiose and lactose was also measured for a longer period in time. The initial disaccharide concentration was 745 mmol·kg^{-1} with an enzyme concentration of 1.3 mg·kg^{-1} under similar conditions as the other experiments in which the initial conversion rates were measured. Conversion was measured for approximately 2 days. Samples were treated as in the initial rate experiments and analysed on HPLC.

Condensation equilibrium

Glucose and galactose condensation was measured at varying substrate concentrations. The solution consisted of 0.2 *M* citrate buffer, pH 5.0, 80°C, in which 0.2 ml of the enzyme solution and different amounts of monosaccharides, varying from 0.2 to 1.2 gram were added to the total amount of 2 gram reaction mixture. The reaction was left for 6 hours to 1 week incubation. Samples were taken for HPLC analysis. Putting the samples on ice for 15 minutes stopped the reaction.

Results and Discussion

Kinetic analysis of initial disaccharide hydrolysis and transglycosylation

The initial product formation rates and substrate conversion rate were determined for cellobiose and lactose conversion by β-glycosidase from *P. furiosus* at different substrate concentrations. The results are shown in figure 3 and 4.

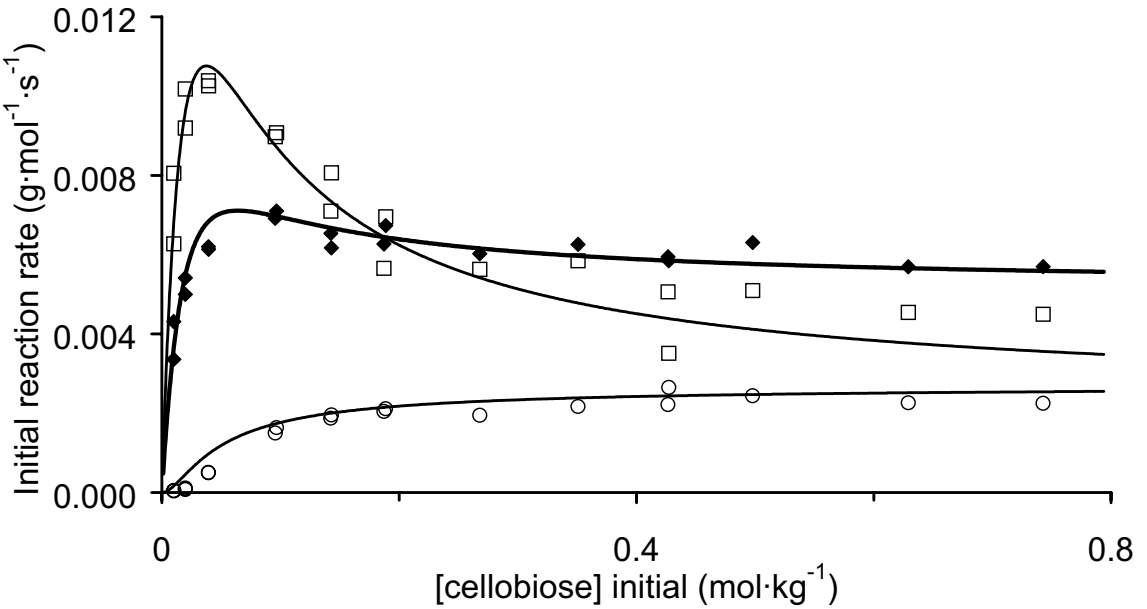

Figure 3 Initial rates (in moles of sugar per second per gram enzyme) for the conversion of cellobiose (◆) and the formation rates of glucose (□) and trisaccharides (○) at different initial cellobiose concentrations by β-glycosidase of *P. furiosus* at 80°C, pH5. Lines are according to equations C1, C2 and C5 (figure 2).

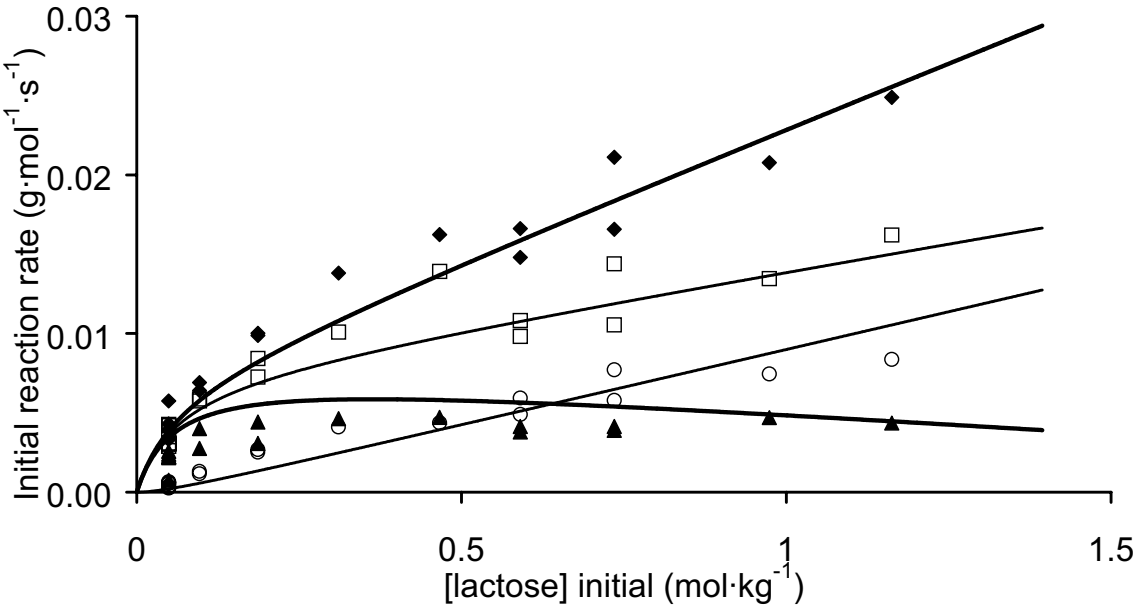

Figure 4 Initial rates (in moles of sugar per second per gram enzyme) for the conversion of lactose (◆) and the formation rates of glucose (□), galactose (▲), and trisaccharides (○) at different initial lactose concentrations by β-glycosidase of *P. furiosus* at 80°C, pH5. Lines are according to equations L1, L2, L5 and L6 (figure 2).

Initial disaccharide conversion with cellobiose as substrate

Figure 3 shows the initial rate of glucose and trisaccharide formation and cellobiose conversion for the kinetically controlled reaction with cellobiose as a substrate. Cellobiose was transglycosylated to tri- and tetrasaccharides, and in addition hydrolysed to glucose. The graph shows an optimum in the conversion rate of cellobiose and in the hydrolysis rate of cellobiose to glucose between 0.04 and 0.1 $mol \cdot kg^{-1}$, while the trisaccharide formation rate remains constant at high substrate concentrations. The maximum initial oligosaccharide formation rate is $2.5 \cdot 10^{-3}$ $mol \cdot g^{-1} \cdot s^{-1}$. The decrease in conversion and hydrolysis rates is probably due to substrate inhibition. Cellobiases often exhibit substrate inhibition (Gusakov *et al.*, 1984; Cascalheira & Queiroz, 1999; Calsavara *et al.*, 1999).
We fitted the four models for initial conversion and formation rates as described in the introduction and depicted in figure 1 and 2 to our data. All data points were fitted simultaneously in a program written in SAS (Cary, North Carolina, USA). The only model that could describe the downward movement of the conversion rate for cellobiose at higher concentrations was the model in which uncompetitive substrate inhibition was included, depicted in solid arrows, including the italic symbols, in figure 1. This was described by two rate equations and the overall mass balance (equations C1, C2 and C5 in figure 2). The kinetic parameters that were thus obtained are given in table 1. k_1 Determines the overall reaction rate, while k_2 and k_3 determine the rate of hydrolysis and transglycosylation. k_3 Is much larger than k_2, but the rate of transglycosylation is not faster than the hydrolysis rate since formation rates are not only dependent on the reaction rate constant, but also the concentration of the reactant. Since water is more abundant that the accepting sugar, transglycosylation is often slower than hydrolysis.

Table 1 Kinetic parameters obtained from the data in figure 3 and 4 by modelling according to the equations in figure 2. k_1, k_2 And k_3 are kinetic rate constants, K_{iu} is the kinetic parameter for uncompetitive substrate inhibition.

Substrate	k_1 (s^{-1})	k_2 (s^{-1})	k_3 (s^{-1})	K_{iu} ($g \cdot mol^{-1} \cdot s^{-1}$)
Cellobiose	$4.9 \cdot 10^2$	0.27	75	$2.8 \cdot 10^4$
Lactose	$1.3 \cdot 10^2$	0.15	10	

Initial disaccharide conversion with lactose as substrate

Lactose is transglycosylated to tri and tetrasaccharides, and hydrolysed to glucose and galactose. Figure 4 shows that the glucose and oligosaccharide formation and the lactose conversion rates are positively related to the initial substrate concentration. The maximal initial oligosaccharide formation rate is $8.4 \cdot 10^{-3}$ $mol \cdot g^{-1} \cdot s^{-1}$ at the highest substrate

concentration measured. This suggests that even higher substrate concentrations will benefit the oligosaccharide production rate. The galactose formation rate shows a Michaelis-Menten type curve. This is in good agreement with the derived rate equation for galactose formation, which can be rewritten to fit Michaelis-Menten kinetics.

All four models for initial conversion and formation rates as described in the introduction were fitted to our data. All data points were fitted simultaneously. The models showed fits of comparable quality and therefore the one with the least parameters was chosen. It was described by two rate equations and two mass balances (equations L1, L2, L5 and L6 in figure 2). The model gives a good fit for lactose conversion and galactose and glucose formation, but only a reasonable fit for the formation of oligosaccharides. This hinders predictions for production efficiency. The predicted initial oligosaccharide production rate is smaller at lower substrate concentrations and larger at higher concentrations. This effect could not be explained by any knowledge that we have on lactose conversion. Many authors have described additional Michaelis-complexes in parts of the reaction. This might be better from a mechanistic point of view, but it does not change the shape of the curves and only adds an extra parameter. The same is valid for intramolecular transfer reactions (Petzelbauer *et al.*, 2000; Bakken *et al.*, 1992; Huber *et al.*, 1976).

The values of the kinetic parameters from the basic model that was used are given in table 1. All kinetic parameters for lactose as a substrate are lower than the equivalent parameters for cellobiose conversion. Based on these parameters a lower oligosaccharide production rate would be expected. However, above 150 $mmol \cdot kg^{-1}$ the effective substrate conversion as well as the oligosaccharide formation is higher in the case of lactose than when cellobiose was used as a substrate, since this reaction is not inhibited by its substrate.

A comparison to Michaelis-Menten kinetics

The overall reaction rate for cellobiose conversion is often estimated on the basis of glucose formation. Based on this we would obtain a K_m, K_i and k_{cat} of 7 $mmol \cdot kg^{-1}$, 232 $mmol \cdot kg^{-1}$ and 363 s^{-1} respectively. These estimates are unreliable since the assumption that solely hydrolysis takes place is not a valid one. With this method the overall reaction rate for cellobiose conversion is underestimated.

For lactose conversion, the overall reaction rate is also often taken from glucose formation rates. Clearly, this glucose formation rate does not obey Michaelis-Menten but continues to increase. The value of k_{cat} is therefore dependent on the measured concentration range. K_m and k_{cat} were calculated to be 185 $mmol \cdot kg^{-1}$ and 879 s^{-1} respectively. All values were similar to those previously reported in literature (Kengen *et al.*, 1993; Petzelbauer *et al.*, 2000; Lebbink *et al.*, 2000; Driskill *et al.*, 1999; Kaper, 2001). k_{cat} Was calculated using the subunit molecular mass of the enzyme as 54,580 Da (Lebbink *et al.*, 2000).

Kinetic analysis of disaccharide hydrolysis and transglycosylation in time

Next to measuring initial rates, we also followed cellobiose and lactose conversion in time. Reaction products were measured in time; the results are shown in figure 5 and 6 respectively. The curves were fitted according to the model for initial rates complimented with a reverse reaction from trisaccharides and glucose inhibition as depicted in figure 1b (see also Boon *et al.*, 1999 for lactose conversion). The equations are shown in figure 2. K_{ic} is the kinetic parameter for competitive substrate inhibition and k_4 is a measure for trisaccharide hydrolysis. k_1, k_2, k_3 and K_{iu} were obtained from the model for initial rates and were used as a constant. The fitted values for k_4 and K_{ic} are given in table 2.

Table 2 Parameters obtained from the data in figure 5 and 6 by modelling according to the equations in figure 2. k_4 Is a kinetic rate constant, K_{ic} is the kinetic parameter for competitive glucose inhibition.

Substrate	k_4 (s^{-1})	K_{ic} ($g \cdot mol^{-1} \cdot s^{-1}$)
Cellobiose	$4.6 \cdot 10^3$	$5.1 \cdot 10^7$
Lactose	$1.3 \cdot 10^2$	$3.1 \cdot 10^7$

Cellobiose conversion in time

Figure 5 displays the changes in sugar concentrations for the enzymatic conversion of 745 $mmol \cdot kg^{-1}$ cellobiose. Cellobiose was transglycosylated to tri- and tetrasaccharides, and in addition hydrolysed to glucose. The reaction showed an optimum in oligosaccharide production of 107 $mmol \cdot kg^{-1}$ around 5 hours. After this time the oligosaccharide concentration decreased again.
The parameters that were obtained from the initial rate data were used in the description of the reaction in time. The initial rates model was extended with the reverse reaction from trisaccharides and competitive glucose inhibition (equations C3, C4 and C5 in figure 2). This produces a good fit. The trisaccharides were hydrolysed quickly, which is evident from a large value of k_4.

Lactose conversion in time

Lactose was converted to tri- and tetrasaccharides by transglycosylation and additionally hydrolysed to glucose and galactose. This kinetically controlled reaction showed an optimum in oligosaccharide production of 135 $mmol \cdot kg^{-1}$ after approximately 1 day. The optimum was however not very pronounced, and the expected subsequent reduction in oligosaccharide concentration could not be identified unambiguously. Hydrolysis was faster than the transglycosylation and continued in time.

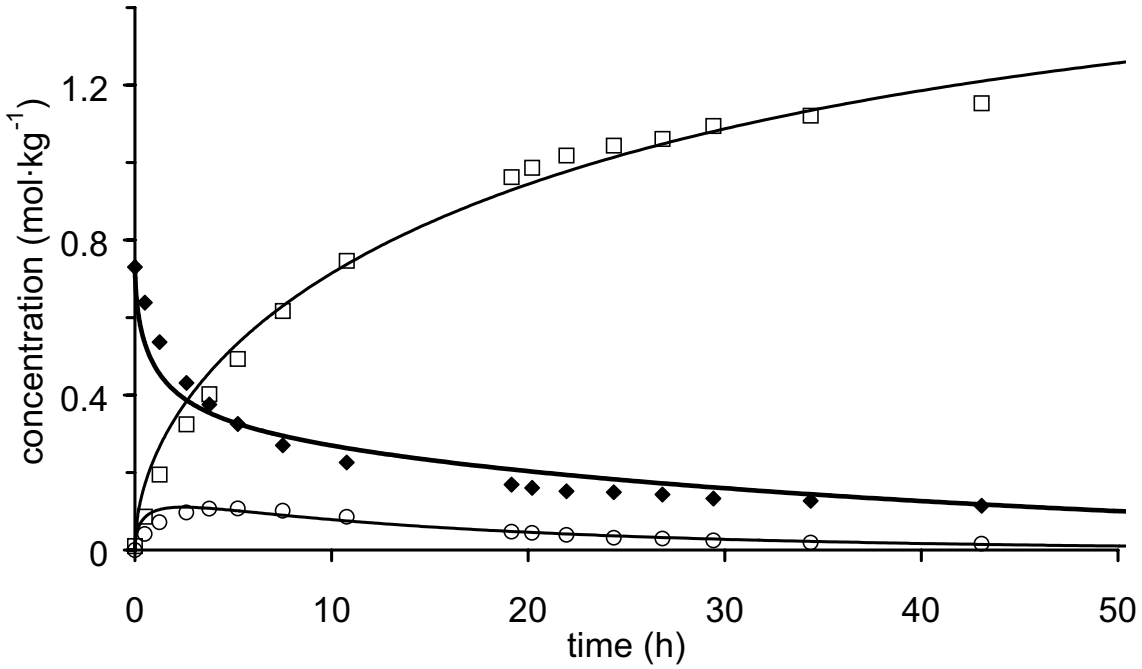

Figure 5 Conversion of cellobiose (◆) in time by β-glycosidase of *P. furiosus* at 80°C, pH 5, 0.745 mol·kg^{-1} cellobiose initially. Glucose (□) and trisaccharides (○) are formed. Lines are according to equations C3, C4 and C5 (figure 2).

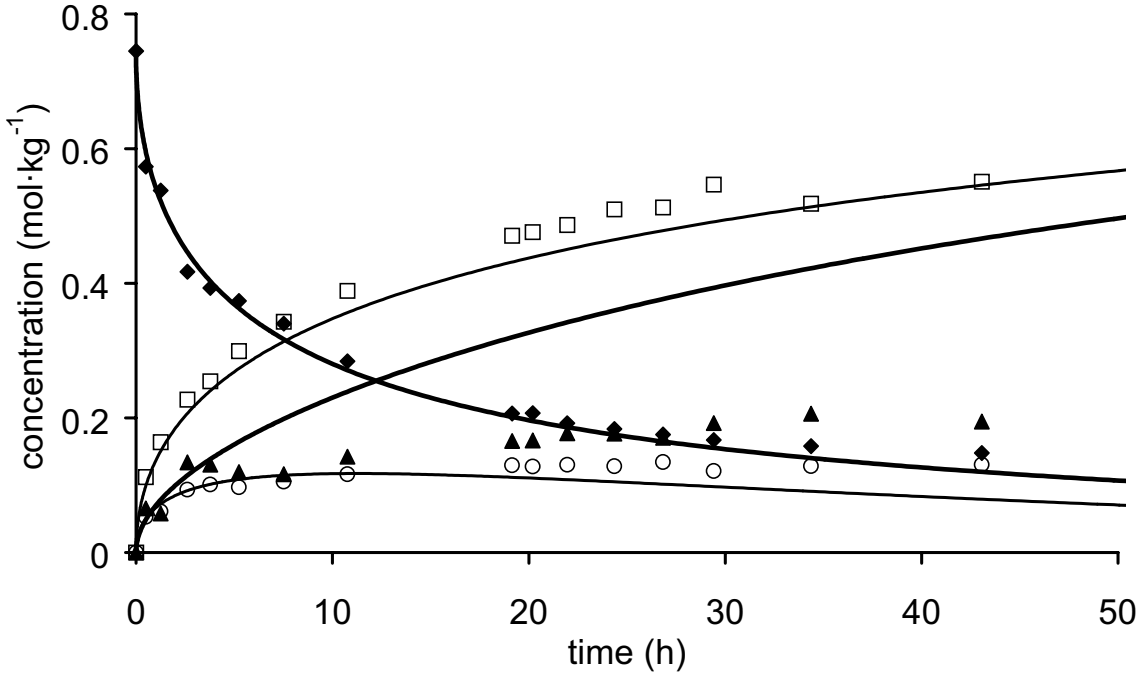

Figure 6 Conversion of lactose (◆) in time by β-glycosidase of *P. furiosus* at 80°C, pH 5, 0.745 mol·kg^{-1} lactose initially. Glucose (□), galactose (▲) and trisaccharides (○) are formed. Lines are according to equations L3, L4, L5 and L6 (figure 2).

The parameters that were obtained from the initial rate data (k_1, k_2 and k_3) were used in the description of the reaction in time. The initial rates model was extended with a reverse reaction from trisaccharides to the enzyme-galactose complex, described by k_4, and competitive glucose inhibition (K_{ic}) (equations L3, L4, L5 and L6 in figure 2). The value for competitive glucose inhibition should be similar for cellobiose and lactose conversion, which is approximately the case. k_4 Is much lower with lactose than when cellobiose is used as a substrate. This is corroborated by the absence of a decrease in the oligosaccharide concentration after the optimum. The model describes the glucose, lactose and oligosaccharide concentrations in time well, but the production of galactose is highly overestimated. This becomes more pronounced after 1 day of reaction time. We believe this could have been caused by the formation of galactose-disaccharides as has been reported for other β-galactosidases. From a glucose and galactose mass balance over the sugars the amount of galactose-disaccharides can be calculated. In our case the effect at the end of the experiment was more pronounced than for other β-galactosidases (Berger *et al.*, 1995; Boon *et al.*, 2000; Bakken *et al.*, 1992) and the galactose-disaccharides would account for 67 % of the disaccharides. However, since we could not differentiate between formed disaccharides with our HPLC system, the model was not modified accordingly.

Optimising oligosaccharide yields from disaccharides

In figure 7 the rate of oligosaccharide synthesis relative to substrate consumption for initial cellobiose and lactose conversion is plotted against the substrate concentrations. For the transglycosylation of disaccharides, an increase in the rate of oligosaccharide synthesis over substrate consumption was observed at higher substrate concentrations. For both substrates the optimal substrate concentration for oligosaccharide production is at the highest measured substrate concentration. This concentration was also used for the transglycosylation reactions in time. The lines through the data points are calculated according to the chosen models. The data for cellobiose is very well fitted, but for lactose conversion the oligosaccharide production rate is somewhat underestimated by the model. The oligosaccharide yield at 0.75 $mol \cdot kg^{-1}$ is 0.14 $mol \cdot mol^{-1}$ for cellobiose and 0.18 $mol \cdot mol^{-1}$ for lactose transglycosylation.
Literature showed that for the transglycosylation of lactose at even higher substrate concentrations, the oligosaccharide yield still increases, e.g. to 0.20 at 0.9 $mol \cdot kg^{-1}$(chapter 4). Raising the temperature is also beneficial from a kinetic point of view. At 95°C oligosaccharide yields are 0.22 at 0.9 $mol \cdot kg^{-1}$ lactose (chapter 4) and 0.26 at 2.0 $mol \cdot kg^{-1}$ lactose (Hansson & Adlercreutz, 2001).

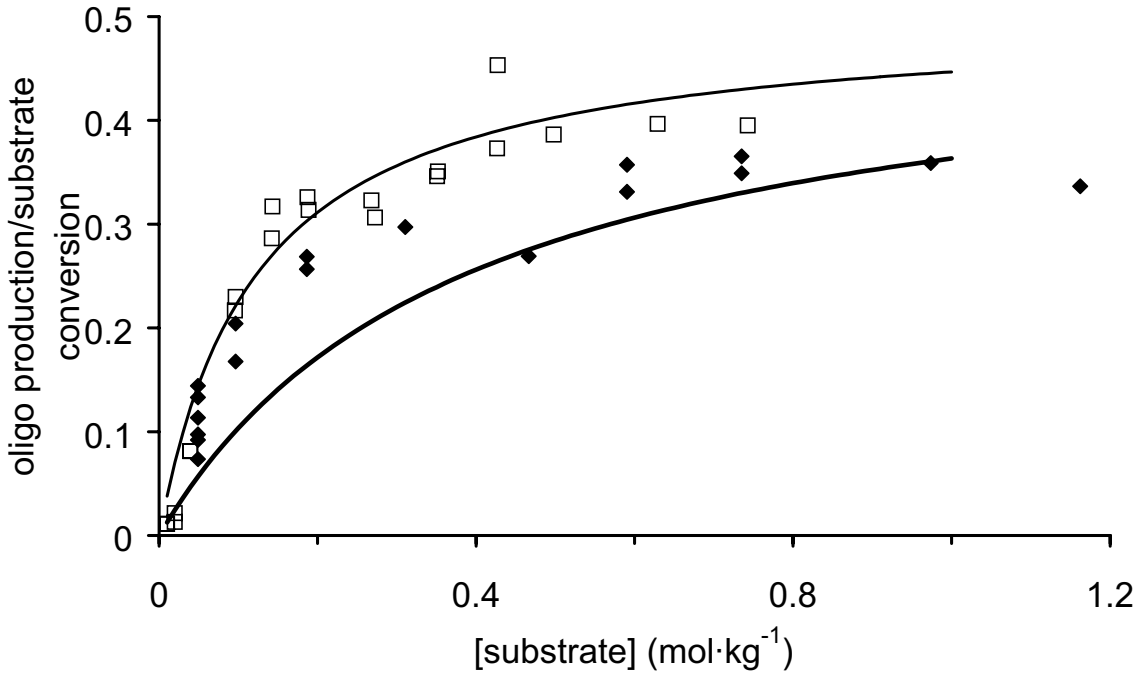

Figure 7 The initial rate of oligosaccharide synthesis over substrate conversion as a function of substrate concentration. Lines are fitted according to the model in figure 2 (C2 and C5, L2 and L6). Cellobiose (□) and lactose (◆)

Kinetic analysis of initial monosaccharide condensation

The initial rate of condensation of monosaccharides with the aid of with the β-glycosidase from *P. furiosus* was measured at high substrate concentration to find the maximal conversion rate. Glucose was condensated to disaccharides, with a disaccharide formation rate of $9.0 \cdot 10^{-5}$ $mol \cdot g^{-1} \cdot s^{-1}$. Disaccharide formation from galactose was slower; $3.9 \cdot 10^{-5}$ $mol \cdot g^{-1} \cdot s^{-1}$.
Compared to trisaccharide formation from disaccharides, the condensation of monosaccharides is much slower. Glucose condensation to di and trisaccharides is 28 times slower than cellobiose transglycosylation and the rate of galactose condensation is 215 times smaller than the transglycosylation of lactose.
Attempts to measure condensation rates at low substrate concentrations failed because synthesis was too slow to be measured without taking enzyme inactivation into account.

Equilibrium of monosaccharide condensation

By using the β-glycosidase from *P. furiosus*, equilibrium for monosaccharide condensation was reached within 3 days. Only the highest glucose concentration was incubated 5 days longer. Glucose and galactose were condensated to di-, tri- and tetrasaccharides. The results are depicted in figure 8 and are fairly similar for glucose and galactose. The oligosaccharide yield increases exponentially. K_{eq} can be calculated with equation 1 for both substrates (see

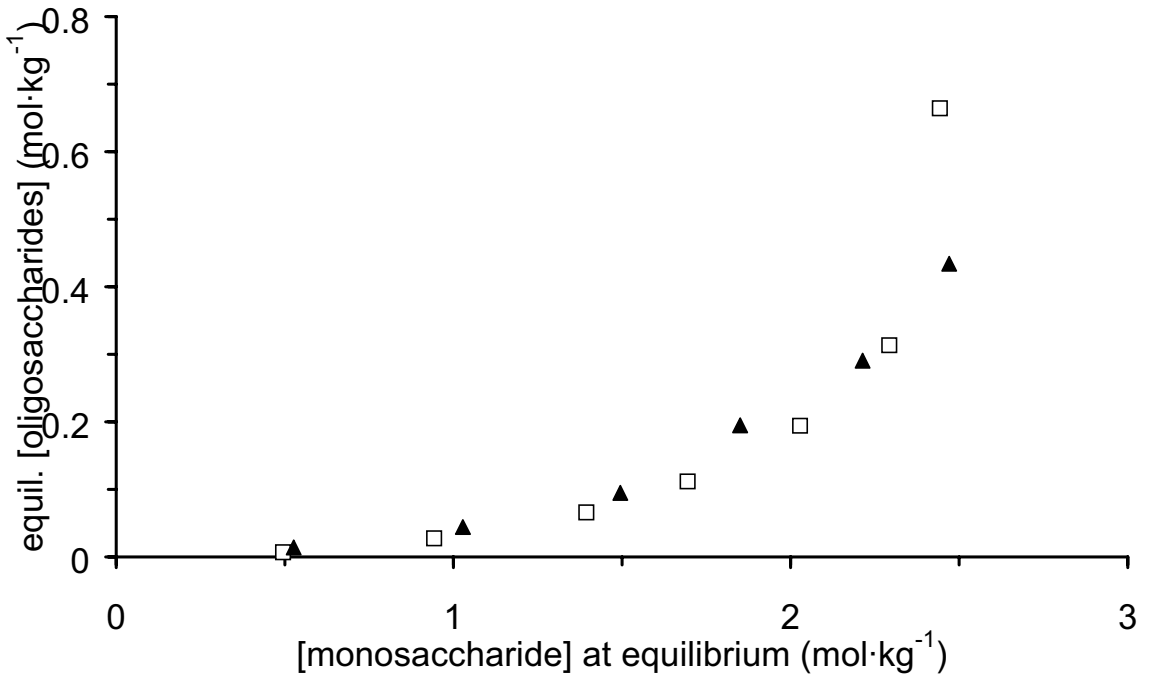

Figure 8 Oligosaccharide (=di, tri and tetrasaccharide) production by β-glycosidase of *P. furiosus* at 80°C, pH 5 and from different concentrations of monosaccharides. Concentrations are taken at equilibrium. (□) glucose (▲), galactose.

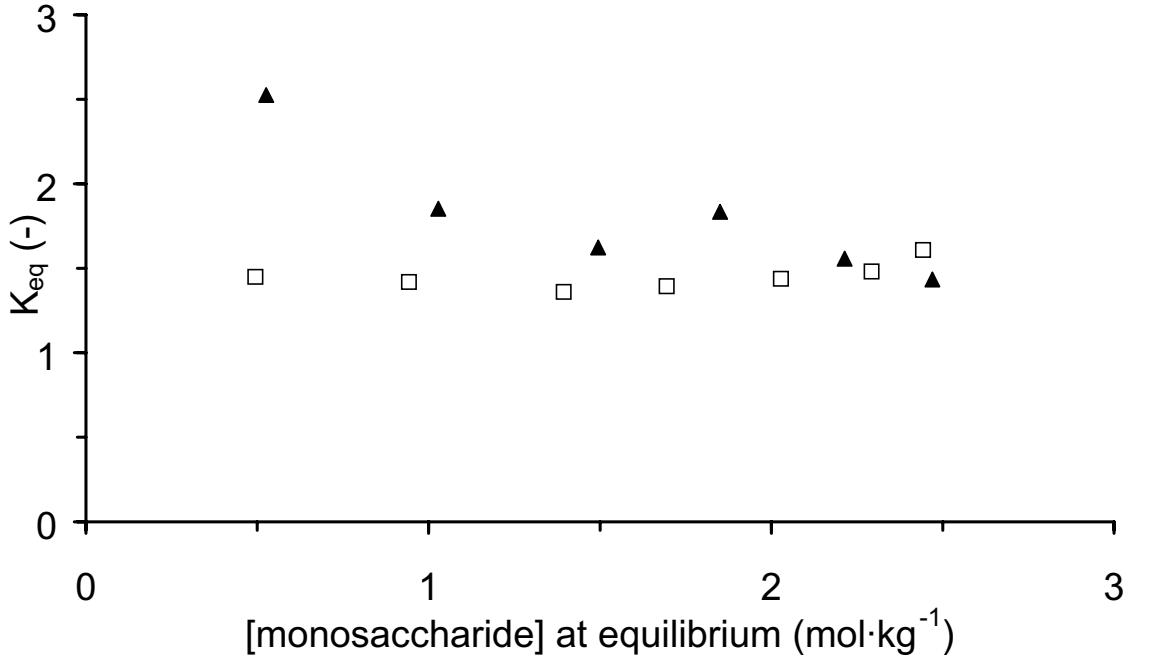

Figure 9 Equilibrium constants (K_{eq}) for different sugar concentrations. (□) glucose (▲), galactose.

figure 9). For glucose this is a constant value of 1.4, suggesting that equilibrium has been reached at all times. Galactose values are however declining from 2.5 to 1.4, which implies that equilibrium had not been reached after 3 days. Prolonged incubation did however not yield more oligosaccharides. Addition of extra enzyme was not feasible since it is produced in solution. Addition would dilute the reaction system.

Table 3 Maximum initial production rates and yields for oligosaccharide production with monosaccharides (glucose and galactose) or disaccharides (cellobiose and lactose) as a substrate. The maximal product yield is defined as the oligosaccharide amount in moles (either being defined as disaccharides and higher, or trisaccharides and higher) devided by the initial substrate concentration in moles.

Substrate	Maximal initial production rate ($mol \cdot g^{-1} \cdot s^{-1}$)	Maximal product* yield ($mol \cdot mol^{-1}$)	Maximal product** yield ($mol \cdot mol^{-1}$)
Glucose	$9.0 \cdot 10^{-5}$	0.27[a]	0.05[a]
Galactose	$3.9 \cdot 10^{-5}$	0.18[b]	0.02[b]
Cellobiose	$2.5 \cdot 10^{-3}$		0.14[c]
Lactose	$8.4 \cdot 10^{-3}$		0.18[c]

*disaccharides and higher, **trisaccharides and higher, [a]maximum yield at 2.4 $mol \cdot kg^{-1}$ substrate, [b]maximum yield at 2.5 $mol \cdot kg^{-1}$ substrate, [c]maximum yield at 0.75 $mol \cdot kg^{-1}$ substrate

Conclusions

Oligosaccharides can be produced using the β-glycosidase from *P. furiosus* with different substrates. The results are summarised in table 3. The main reason for choosing this enzyme was the possibility to work at elevated temperatures and thus being able to use high sugar concentrations. We showed that in this view thermophilic enzymes could be beneficial for synthesis reaction. For the condensation reaction of monosaccharides, like glucose and galactose, this gave an important increase in oligosaccharide yield. Also for the transglycosylation of disaccharides, oligosaccharide production increased at higher substrate concentrations, which was visible in an increase in the rate of oligosaccharide synthesis over substrate consumption.

The kinetic behaviour of the enzyme could be modelled for glucose, galactose and cellobiose conversion. The conversion of cellobiose to oligo- and monosaccharides could be modelled with good accuracy. The conversion of lactose shows a somewhat lesser fit. This is probably due to the parallel formation of different di- and oligosaccharides of variable composition, which was not explicitly accounted for in the model.

Acknowledgements

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Chapter 4

Maillard reactions and enzyme inactivation

Abstract

The thermostable *Pyrococcus furiosus* β-glycosidase was applied for oligosaccharide production from lactose in a kinetically controlled reaction. Our experiments showed that higher temperatures are beneficial for the absolute as well as relative oligosaccharide yield. However, at reaction temperatures of 80°C and higher, the inactivation rate of the enzyme in the presence of sugars was increased by a factor 2, compared to the inactivation rate in the absence of sugars. This increased enzyme inactivation was caused by the occurrence of Maillard reactions between the sugar and the enzyme. The browning of our reaction mixture due to Maillard reactions was modelled by a cascade of a 0^{th} and 1^{st} order reaction and related to enzyme inactivation. From these results we conclude that modification of only a small number of amino groups already gives complete inactivation of the enzyme.
Reduction of Maillard reactions can be done by altering process conditions or through modification of the enzyme, either chemically or by altering the enzyme structure through genetic modifications. In this work, chemical modification of the enzyme was chosen by covalent immobilisation on Eupergit. Unfortunately, the immobilisation did not reduce Maillard reactivity.

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Introduction

Enzymes from thermophilic microorganisms are highly stable which enables the performance of processes at higher temperatures. The main advantages are the reduced risk of microbial contamination, lower viscosity, improved transfer rates and improved solubility of substrates and products. (chapter 2)
Most industrial applications of β-glycosidases have employed thermostable glycosidases for hydrolytic purposes. Glycosidases can also be used in the reverse reaction for the production of oligosaccharides. These oligosaccharides may be used as prebiotics in functional foods (Crittenden & Playne, 1996; Crittenden, 1999; Gibson & Roberfroid, 1994). Using a disaccharide as substrate, glycosidases can produce oligosaccharides as shown in figure 1 for lactose. In this reaction a substrate-enzyme complex is formed, which can either be attacked by another substrate molecule or by water, yielding an oligosaccharide or a monosaccharide respectively. In this kinetically controlled reaction the oligosaccharide concentration shows an optimum in time. The β-glycosidase from *Pyrococcus furiosus* produces besides trisaccharides also tetrasaccharides with lactose as a substrate (Boon *et al.*, 1998; Petzelbauer *et al.*, 2000).
For the production of oligosaccharides the main benefit of using higher reaction temperatures lies in the increase of the substrate concentration and the subsequent decrease in water concentration, which suppresses hydrolysis and therefore improves the oligosaccharide yield. Possible disadvantages in the use of thermozymes may be found in the instability of co-factors, substrates or products and the occurrence of side reactions (chapter 2). In this chapter we investigate oligosaccharide production as a function of temperature including enzyme inactivation kinetics, with focus on inactivation by the Maillard reaction.

Maillard reaction

The Maillard reaction is the formation of N-glycosides upon heating. This chemical condensation reaction between amino acids and carbohydrates is strongly dependent on temperature, pH, salt concentration and water activity but also on the type of sugar, amount of reducing sugar groups, type of amino acid and their concentrations. In proteins it is assumed that the main amino acid in the Maillard reaction is the ε-amino group of lysine. It is the only reacting amino acid in milk, where other amino acids that contain a second amino group did not react (Finot *et al.*, 1981).
The Maillard reaction is commonly described as occurring in three stages (Hodge, 1953). In the first phase the condensation of the amino group of the amino acid or protein with the reducing end of the sugar takes place, followed by the Amadori rearrangement. This first phase of the Maillard reaction can be measured through the decrease in available lysine (Carpenter, 1960; Vigo *et al.*, 1992). In the second step of the Maillard reaction, colourless as

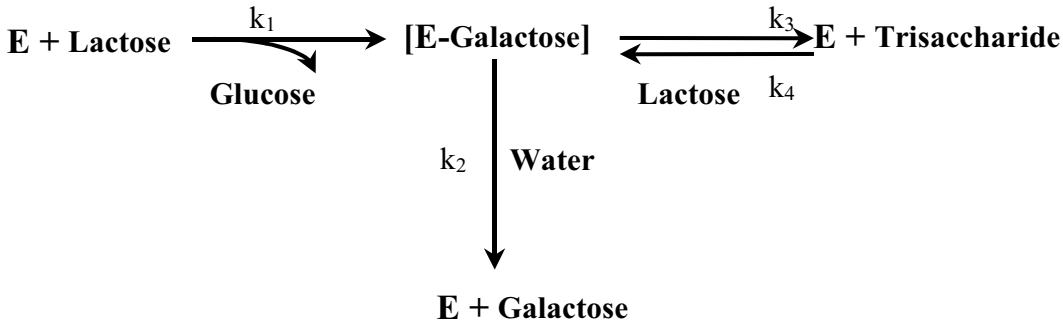

Figure 1 Mechanism of enzymatic lactose conversion in which E=enzyme.

well as fluorescent substances are formed. The final step of the Maillard reaction is where most of the colour is formed. This step is characterised by the formation of unsaturated, brown nitrogenous polymers and copolymers, which can be monitored by measuring browning at 420-490 nm.

Materials and methods

Materials

Lactose was obtained from Sigma (St Louis, USA). Other chemicals were obtained from Merck (Darmstadt, Germany). β-Glycosidase from *P. furiosus* was prepared from an *E. coli* lysate and heated to denature proteins other than the thermostable enzyme (Voorhorst *et al.*, 1995).

Determination of protein concentration

The BCA protein assay was used to determine protein concentrations according to the protocol of the supplier (Pierce, Rockford, USA). Bovine Serum Albumin was used as a standard.

Enzyme kinetics

The conversion of lactose was measured at different temperatures. Experiments were performed in a 200-ml vessel with 150 ml 0.2 mol·kg^{-1} citrate buffer at pH 5.5 in which the initial lactose concentration was 0.9 mol·kg^{-1}. The reaction was started by adding enzyme solution to the amount of 1.28 mg protein, which was equivalent to 1136 U enzyme. During 12 days, samples were taken at regular intervals. The reaction was stopped by putting these samples on ice for 15 minutes.

Browning

To monitor pigment formation caused by the Maillard reaction, browning of the mixture was measured spectrophotometrically at 420 and 470 nm.

Enzyme activity measurements

100 µl Of the reaction mixture was diluted 1:1 immediately after sampling. For the measurements under production conditions (with sugar) samples were diluted in standard buffer. In blank measurements (without sugar) samples were diluted in a 0.9 mol·kg^{-1} lactose solution to improve storage.
β-D-Glycosidase was assayed using pNPG (*p*-nitrophenyl-β-D-glucopyranoside) as an artificial substrate at 80°C. The increase in absorbance at 405 nm as a result of *p*-nitrophenol formation was measured. A standard reaction mixture contained 2.0 mmol·l^{-1} pNPG in 0.2 mol·kg^{-1} citrate buffer at pH 5.5. The influence of the sugars that were present in the samples was negligible and the results were corrected for browning of the reaction mixture.

HPLC analysis of sugars

Samples from the reaction mixture were treated with $Pb(NO_3)_2$ (final concentration in the diluted samples 0.1 mol·l^{-1}) to precipitate the citrate that would otherwise interfere in the analysis and stored in the freezer for at least 1 hour to accelerate precipitation. Before analysis the samples were centrifuged at 13,000 rpm and diluted ten times.
The samples were analysed on HPLC using a RSO Oligosaccharide Column (Phenomenex, Amstelveen, the Netherlands) at 80°C. The column was eluted with Milli-Q water (purged with helium) at a flow rate of 0.3 ml·min^{-1}. The eluent was monitored with a refractive index detector. All different sugars were detected and measured as percentages of total sugar on weight base.

Results and Discussion

In a set of experiments we measured different aspects of the system. Besides enzyme kinetics, we also measured browning caused by Maillard reactions and enzyme inactivation. Experiments were performed at 50, 60, 70, 80, 87.5 and 95°C.

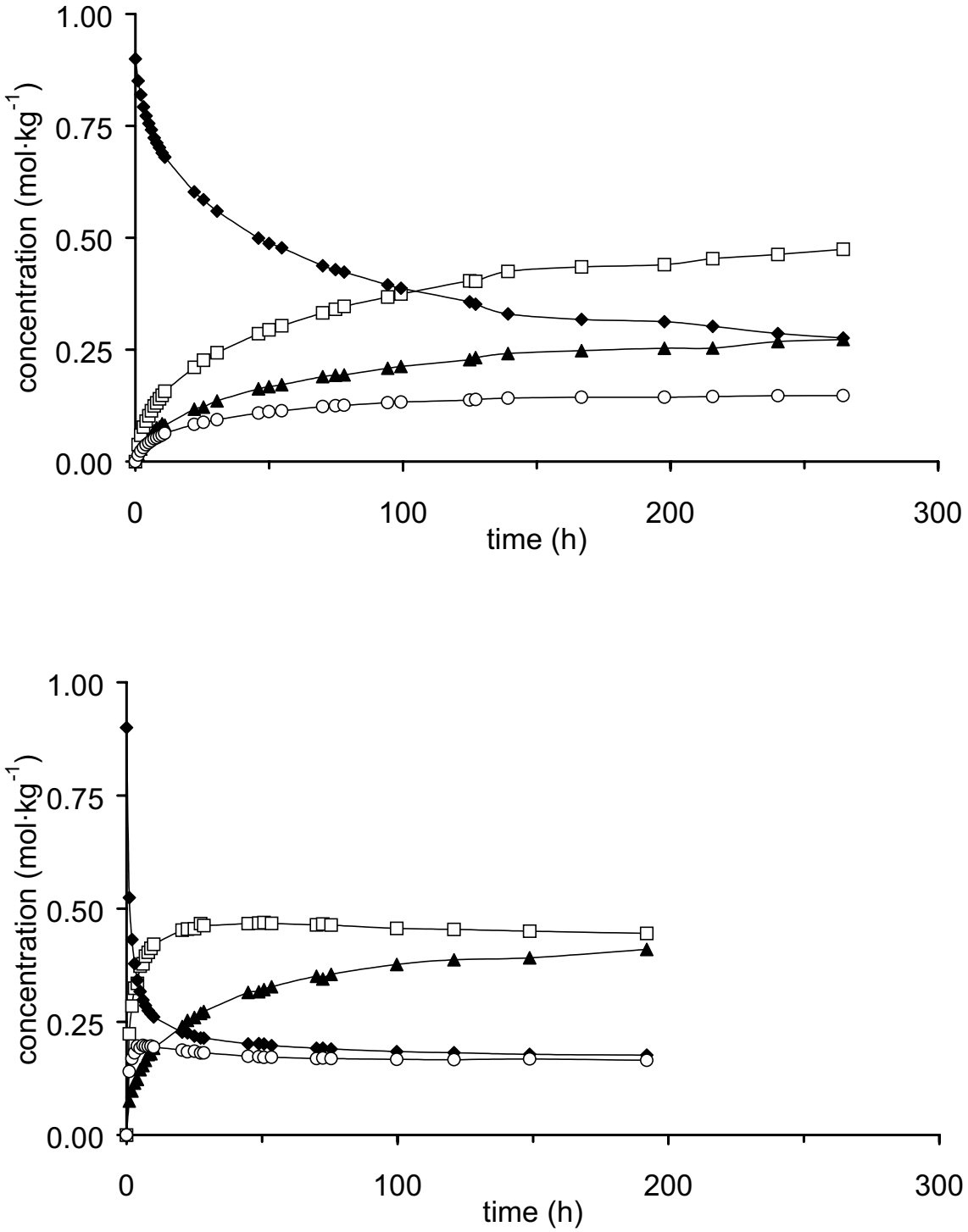

Figure 2 Lactose conversion by β-glycosidase of *P. furiosus* at 50 (upper figure) and 95°C (lower figure). Initial lactose concentration 0.9 $mol \cdot kg^{-1}$; enzyme concentration 8.6 $mg \cdot kg^{-1}$; pH 5.5. (◆, lactose; □, glucose; ○, oligosaccharides; ▲, galactose; lines for guidance) Oligosaccharides are the sum of tri-, tetra- and pentasaccharides.

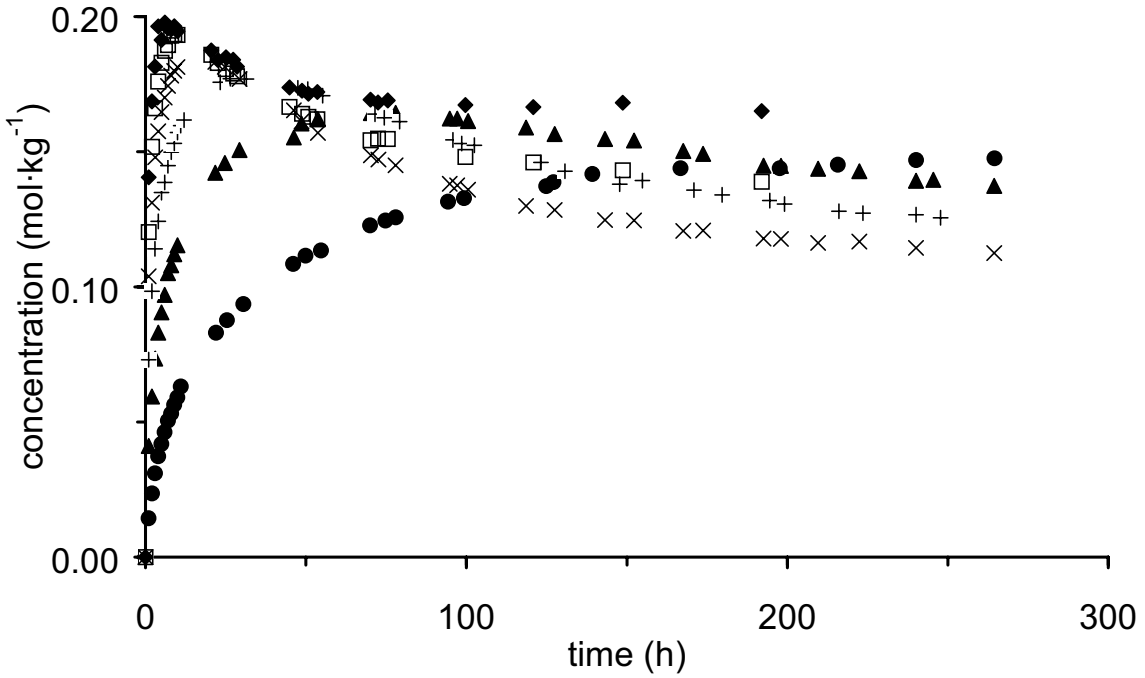

Figure 3 Oligosaccharide production in time by β-glycosidase (*P. furiosus*) at different temperatures. Initial lactose concentration 0.9 $mol \cdot kg^{-1}$; enzyme concentration 8.6 $mg \cdot kg^{-1}$; pH 5.5. (●, 50°C; ▲, 60°C; +,70°C; ×, 80°C; □, 87,5°C; ◆, 95°C).

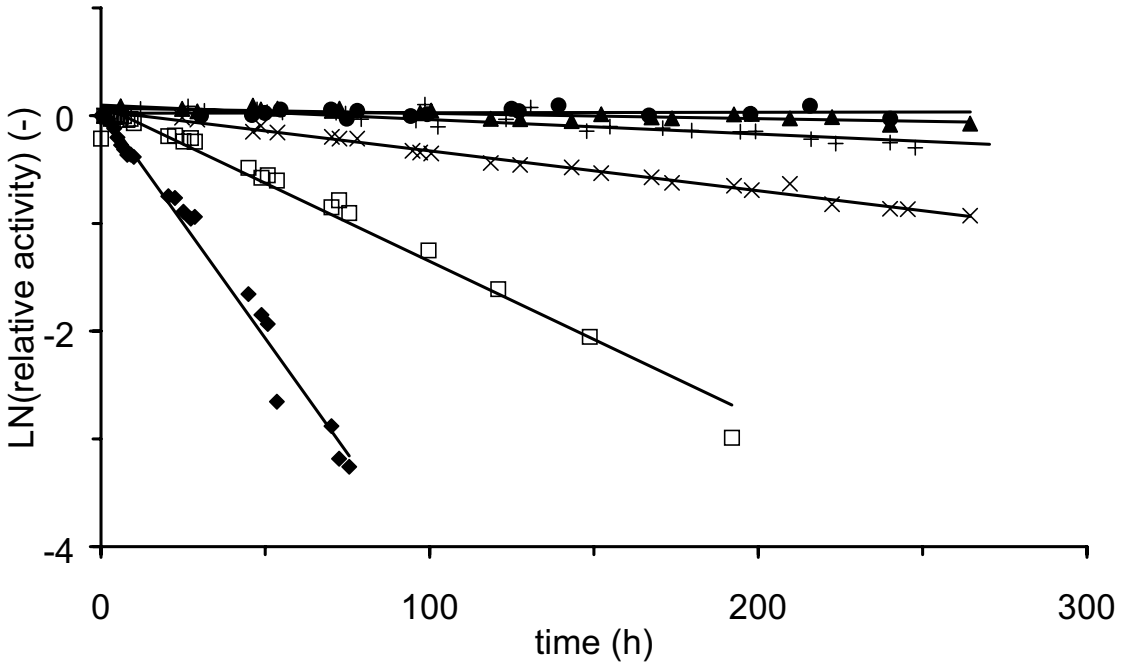

Figure 4 Inactivation of β-glycosidase (*P. furiosus*) during oligosaccharide synthesis at different temperatures. Initial lactose concentration 0.9 $mol \cdot kg^{-1}$; enzyme concentration 8.6 $mg \cdot kg^{-1}$; pH 5.5. (●, 50°C; ▲, 60°C; +,70°C; ×, 80°C; □, 87,5°C; ◆, 95°C).

Enzyme Kinetics

Conversion of lactose by β-glycosidase from *P. furiosus* was measured in time. Lactose was hydrolysed into glucose and galactose, and tri-, tetra- and pentasaccharides were synthesised. As an example, substrate and product concentrations in time at 50 and 95°C are shown in figure 2. The conversion rate for lactose increases with temperature directly resulting in an increase in both the rate of hydrolysis and the rate of oligosaccharide synthesis. Because the increase in production rate is faster for oligosaccharide synthesis than for hydrolysis, relatively more oligosaccharides are produced at higher temperatures. This is illustrated in figure 3. At 50°C the optimum in oligosaccharide concentration of which the occurrence is typical for a kinetically controlled reaction, is not yet reached after 250 hours. At 95°C this optimum was reached within 4 hours.
After 250 hours, concentrations still change in time at 50°C, which indicates that the enzyme is still active and the reaction is not finished. This is confirmed by the enzyme activity measurements. From these results we could further conclude that there is no enzyme activity left in the reaction mixture after a few days at 95°C.
However, sugar concentrations still change in this experiment. The galactose concentration is increasing, while all the other concentrations slightly decrease. This might be due to the Maillard reaction. In literature the liberation of galactose due to the reaction of lactose with a lysine has been described (van Boekel, 1998).

Enzyme inactivation

The total enzyme inactivation for the enzyme under production conditions (with sugar) and blank inactivation (without sugar) due to temperature were measured. Sample measurements were compared with the activity at the start of the experiments, which was set to be 100%.
The enzyme inactivation in the presence of sugars is depicted in figure 4. At 50°C no significant inactivation of the enzyme was found. At higher temperatures inactivation increases and at 87.5 and 95°C the enzyme was totally inactivated before the end of the experiment. From figure 4 it is clear that the enzyme inactivation follows first order kinetics; the first order inactivation constants are represented as an Arrhenius plot in figure 5. Here it has to be mentioned that the experiments at lower temperatures were less accurate, since enzyme inactivation was only approximately 10% of the total enzyme activity. Petzelbauer *et al.* (1999) measured enzyme inactivation of the β-glycosidase from *P. furiosus* at 70, 80 and 90°C in vials. The results at 80 and 90°C were in good agreement with our own. However, at 70°C enzyme inactivation was found to be lower than in our experiments, where we sampled a reaction mixture. Here, temperature inactivation might not be the main mechanism of enzyme inactivation and for example inactivation due to mechanical stress will be more important. This is corroborated by the observation that at these lower temperatures,

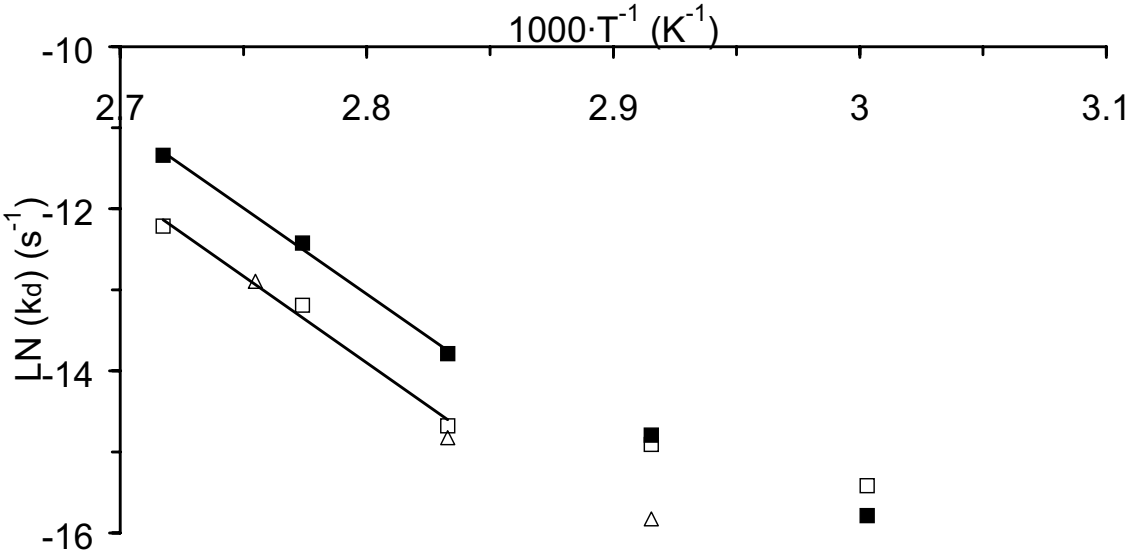

Figure 5 Arrhenius plot for inactivation of β-glycosidase (*P. furiosus*) at different temperatures. Enzyme concentration 8.6 $mg \cdot kg^{-1}$; pH 5.5. (■, Initial lactose concentration 0.9 $mol \cdot kg^{-1}$; □, Blank measurements with no added lactose; Δ, Blank literature values (Petzelbauer *et al.*, 1999))

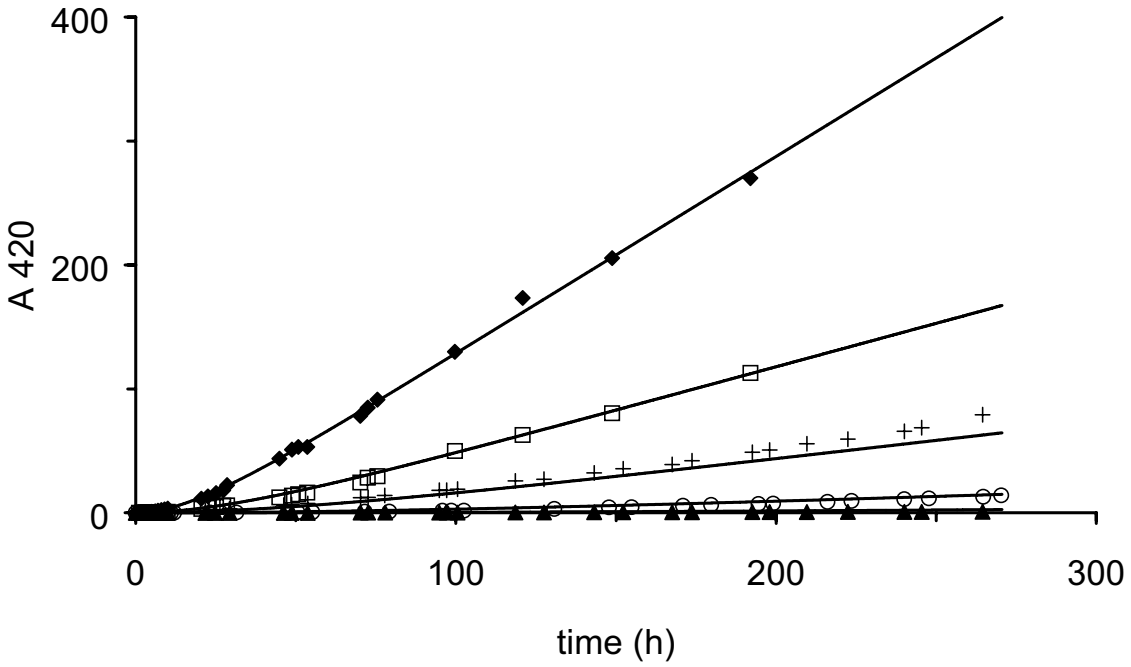

Figure 6 Browning of the reaction mixture during oligosaccharide synthesis by β-glycosidase (*P. furiosus*) at different temperatures. Initial lactose concentration 0.9 $mol \cdot kg^{-1}$; enzyme concentration 8.6 $mg \cdot kg^{-1}$; pH 5.5. Lines are fitted according to the 0^{th} - 1^{st} order model as shown in figure 7. (▲, 60°C; ○, 70°C; +, 80°C; □, 87.5°C; ◆, 95°C)

inactivation with and without sugars present appears to show the same rates. At the lowest temperature inactivation in the presence of sugars is even less than the blanc inactivation, probably due to the protecting force of the sugars.
From the blank experiments without sugar, it is possible to quantify the inactivation constant due to temperature ($k_{d,blank}$). The experiments under process conditions result in an overall inactivation constant ($k_{d,overall}$). By subtracting $k_{d,overall}$ with $k_{d,blank}$ it is possible to see the influence of sugars on the enzyme inactivation ($k_{d,Maillard}$). For this we only used the results at higher temperatures (95, 87.5 and 80°C), since these figures were more accurate.
The activation energies for enzyme inactivation due to Maillard reactions and due to temperature are very similar, 175 and 178 kJ/mol respectively. This might point to a similar inactivation mechanism. The activation energies were calculated from the straight lines in figure 5, using the Arrhenius equation. From the absolute values of the inactivation constants we can conclude for temperatures above 80ºC that in the presence of sugars enzyme inactivation increases with roughly a factor 2.

Browning

In literature absorptions varying from 420 to 490 are chosen to measure the browning caused by Maillard reactions (e.g. Manzocco *et al.*, 2000). Adsorption scans from 200-800 nm of our reaction mixture showed maxima at 420 and 470 nm, which we used as a measure for Maillard reactions. We used the data obtained at 420 nm since this value was always around 1.8 times higher than the adsorption measured at 470 nm.
Except for the reaction at 50°C, all reaction mixtures showed browning. At first the adsorption increases exponentially, in a later stage the browning became linear. In figure 6 the browning is shown for the various temperatures. It can be seen that the rate and amount of browning strongly increases with temperature.
As mentioned before, the Maillard reaction is greatly dependent on the composition of the reaction mixture, which is however constant in our experiments and for this reason the browning of the mixture will be mostly a function of temperature. However, since the enzyme is active in the reaction mixture, the concentrations and type of sugars change in time (see figure 2). Both will probably influence the rate of browning. Wedzicha and Kedward (1995) noticed that disaccharides react more slowly than mono and oligosaccharides and that browning was proportional to the reactivity of the reducing group. It is generally reported that disaccharides brown more slowly than monosaccharides, since they occur less in the open chain form (Naranjo *et al.*, 1998). This was confirmed by our own experiments in which we incubated Bovine Serum Albumin as non-catalytic protein with different sugars at 80°C. In these experiments, lactose gave less browning than glucose and galactose (results not shown). Furthermore, the number of reducing sugars increased from 0.9 $mol \cdot kg^{-1}$ at the start of the experiments to around 1.2 $mol \cdot kg^{-1}$ due to the enzymatic reaction. But although the sugar

concentrations varied in time in the individual experiments, the differences between the experiments at fixed times were small. Therefore we neglected possible differences caused by a different composition of the reaction mixtures.

Determining kinetic constants for the Maillard reaction

To be able to relate the browning of our reaction mixture to the degree of enzyme inactivation we calculated apparent rate constants from the light absorption experiments depicted in figure 6. Several reaction models for the Maillard reaction were compared. Two of these models are depicted in figure 7. In all cases we assumed the absorption due to browning to be linear to the concentration of Maillard products. This linear correlation is incorporated in the model as a constant (c). The initial concentration of amino groups (A_0) in our mixture is estimated to be the number of lysine groups (35 out of 472 amino acids in the enzyme) present at the start of the experiment and has a value of $5.2 \cdot 10^{-6}$ mol·kg^{-1}. The initial lactose concentration was 0.9 mol·kg^{-1}, which means there is a large excess of sugar, and therefore this concentration is taken as a constant in both models.
The first and most complete model is a cascade of two 1st order reactions, in which the amino group is the limiting substrate. In the model the amino acid (A) reacts via 1st order kinetics to an intermediate (I), which can then further react in a 1st order reaction to the brown end product of the reaction (M).
The second model is a simplification of the first model. It describes a cascade of a 0th and a 1st order reaction. This can be used when a large part of the amino groups has not yet reacted.
The kinetic rate constants (k_1 and k_2) for these models could be obtained by a single fit for each temperature, but since the kinetic constants showed a linear Arrhenius trend, we chose to use a multiple fit for all browning curves at different temperatures at the same time to yield k_2, k_1 and cA_0 for the 1st - 1st order model or k_2 and $ck_1[A_0]$ for the 0th - 1st order model at a specific temperature and their activation energies. This greatly reduced the number of parameters that had to be fitted and thus increased the accuracy of the obtained figures. The results of the 0th - 1st order model are represented in table 1 and these were used for the fit in figure 6. It was not possible to get independent parameters for the 1st - 1st order model. k_1 was linearly related to the inverse of cA_0 such that $ck_1[A_0]$ was the same as in the 0th - 1st model. The values of k_2 in the 1st - 1st order model were similar to those fitted with the 0th - 1st model. This implies that the assumption that the concentration of amino groups is not limiting in the reaction in the first model is justified and only a small part of the total amount of amino groups has already reacted.
In literature different models are often used to describe browning of sugar amino acid mixtures. A simple model that is often used describes the first (exponential) phase of browning by using a lag phase. A kinetic rate constant is then determined on the second, linear part of the browning curve, in which the reaction proceeds at a constant velocity

$$A \xrightarrow{k_1} I \xrightarrow{k_2} M$$

1^{st} - 1^{st} order, A is limiting substrate	0^{th} - 1^{st} order, no limiting substrate
$-\frac{d[A]}{dt} = k_1[A]$	$-\frac{d[A]}{dt} = k_1[A_0]$
$\frac{d[I]}{dt} = k_1[A] - k_2[I]$	$\frac{d[I]}{dt} = k_1[A_0] - k_2[I]$
$\frac{d[M]}{dt} = k_2[I]$	$\frac{d[M]}{dt} = k_2[I]$
Integration with A as a variable:	Integration with A as a constant:
$[M] = [A_0] \cdot \left(1 + \frac{\left(k_2 e^{-k_1 t} - k_1 e^{-k_2 t}\right)}{k_1 - k_2}\right)$	$[M] = k_1[A_0] \cdot t - \frac{k_1[A_0](1 - e^{-k_2 t})}{k_2}$
Browning is a function of the concentration of Maillaird products:	
$B = c[M]$	
$B = c[A_0] \cdot \left(1 + \frac{\left(k_2 e^{-k_1 t} - k_1 e^{-k_2 t}\right)}{k_1 - k_2}\right)$	$B = ck_1[A_0] \cdot t - \frac{k_1[A_0](1 - e^{-k_2 t})}{k_2}$
Parameters:	
$(c[A_0]), k_1, k_2$	$(ck_1[A_0]), k_2$

Figure 7 Method used for fitting the browning caused by Maillard reactions. The left part of the figure shows the 1^{st} - 1^{st} order model and on the right the 0^{th} - 1^{st} order model is shown. Integration gives an equation for the production of Maillard products as a function of time, which is used to fit data. (A=amino group, I=intermediate, M=Maillard product, k_1 and k_2 are reaction rate constants, B=browning measured at A_{420}, c=constant).

Table 1 Kinetic rate constants for the browning of β-glycosidase from *P. furiosus* with lactose reaction mixture in time as derived with the 0^{th} - 1^{st} order model and their subsequent apparent energy of activation. Initial lactose concentration 0.9 $mol \cdot kg^{-1}$; enzyme concentration 8.6 $mg \cdot kg^{-1}$; pH 5.5.

Model	60°C[b]	70°C[b]	80°C[a]	87.5°C[b]	95°C[b]	Ea[a] (kJ/mol)
$ck_1[A_0]$ (s^{-1})	$7.10 \cdot 10^{-6}$	$2.52 \cdot 10^{-5}$	$8.31 \cdot 10^{-5}$	$1.95 \cdot 10^{-4}$	$4.42 \cdot 10^{-4}$	120
k_2 ($kg \cdot mol^{-1} \cdot s^{-1}$)	$1.05 \cdot 10^{-6}$	$2.35 \cdot 10^{-6}$	$5.02 \cdot 10^{-6}$	$8.63 \cdot 10^{-6}$	$1.45 \cdot 10^{-5}$	77

[a] parameter fitted, [b] parameter derived by calculation

(Morales & van Boekel, 1998; Lerici *et al.*, 1990). The 0^{th} order rate constants that are a result of this method are the same as the 0^{th} order rate constants from our 0^{th} - 1^{st} model.
Another model, describing browning in glycine-glucose mixtures that are added in (roughly) equal amounts gives comparable results to our 0^{th} - 1^{st} order model. However, this model starts with a reducing sugar and uses 3 different rate constants to describe browning (Davies *et al.*, 1997; Leong & Wedzicha, 2000). The fact that with this model no better fit was obtained, resulted in preference for the 0^{th} - 1^{st} order model with only 2 parameters.

Inactivation and browning

In figure 8 the absorption at 420 nm is plotted against the relative amount of inactive enzyme found from the activity measurements. It shows that there is no clear temperature independent relationship between the enzyme inactivation and the formation of brown colour. At equal fraction of inactivated enzyme, most colour is formed at 80°C. There is less colour formation relative to the rate of enzyme inactivation at the higher (95 < 87.5 < 80°C) and lower temperatures (60 < 70 < 80°C). The differences in browning indicate the formation of different Maillard products. This is certainly true at higher temperatures where the inactivation due to Maillard reactions and inactivation due to other causes were shown to have similar activation energies. At 70 and 60°C, where there is no large increase in browning but significant enzyme inactivation, other factors become important for enzyme inactivation (see figure 5).
From figure 4 it is clear that at 95°C less than 1% of the initial activity was left after 75 hours. Figure 6 shows that the formation of the brown colour proceeds linearly till the end of the experiment at 200 hours. This means that the Maillard reaction continues, and that there are still sufficient free amino groups present in the reaction mixture. We can therefore conclude that glycosylation of only a few of the amino acids present in the enzyme already causes total inactivation. These results are in good agreement with the fact that the 0^{th} - 1^{st} order model fitted our data very well despite the necessary assumption of the presence of a large excess of amino groups in this model.

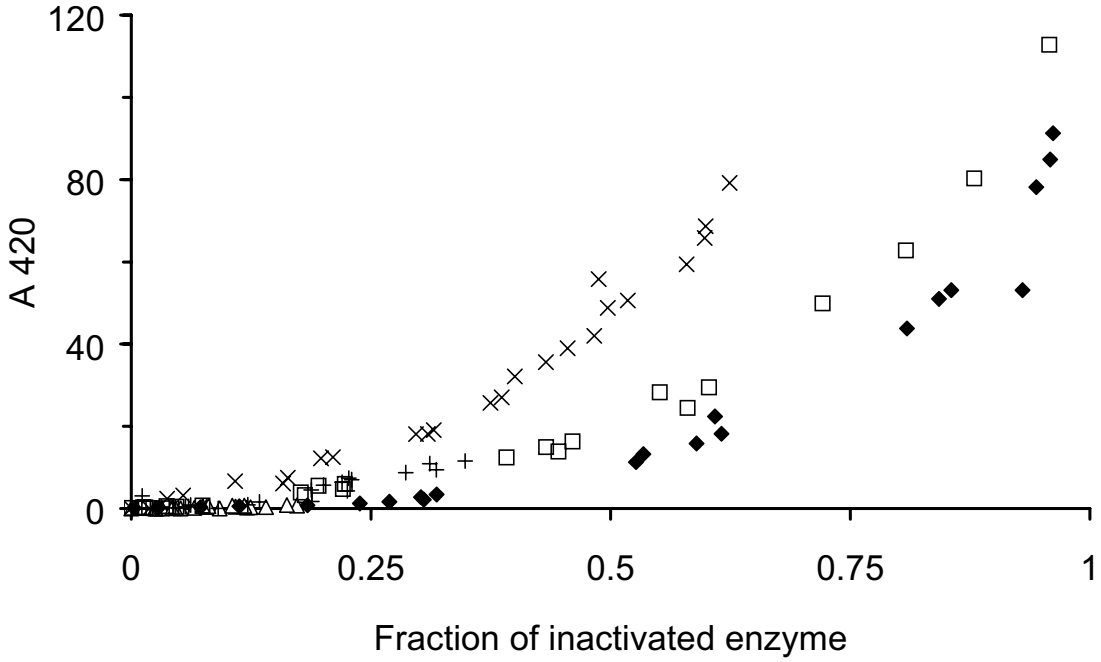

Figure 8 Browning during oligosaccharide synthesis as a function of the relative amount of inactivated enzyme. Initial lactose concentration 0.9 $mol \cdot kg^{-1}$; 8.6 $mg \cdot kg^{-1}$ β-glycosidase from *P. furiosus*; pH 5.5; (Δ, 60°C; +, 70°C; ×, 80°C; □, 87,5°C; ◆, 95°C)

Concluding remarks

Higher reaction temperatures lead to a better oligosaccharide production in the conversion of lactose by β-glycosidase from *P. furiosus*. A higher yield of oligosaccharide product is formed in shorter reaction times. However, enzyme inactivation also increases with temperature. At 80°C and higher, inactivation due to Maillard reactions roughly doubles the rate of enzyme inactivation. Further, the brown colour formation due to Maillard reactions may cause problems in the downstream processing of the oligosaccharide product.

Modelling of the Maillard reaction shows that only a relatively small fraction of the total amount of amino groups present were affected by the Maillard reaction. Since the inactivation of the enzyme at higher temperatures is fast, while the increase in browning remains gradual, we conclude that glycosylation of only a few of the amino acids present in the enzyme already gives complete inactivation of the enzyme.

This may have important implications for enzyme stabilisation by protein engineering. On the one hand it may imply that enzyme stabilisation by protein engineering is difficult to achieve. If modification of only a few amino groups is sufficient to inactivate the enzyme, modification of all lysines may be required, which is not a feasible option. On the other hand, if only the lysines that reacted can be modified it might be possible to obtain a more stable

enzyme by only a few changes (Eijsink *et al.*, 2001). However, it is not yet clear which amino groups are involved in the Maillard reaction.
Protein engineering is not the only solution when enzyme inactivation by Maillard reactions is a problem in the development of a reaction system. Reduction of Maillard reactions can also be achieved by altering process conditions or through chemical modification of the enzyme. In previous work, chemical modification of the enzyme was performed by covalent immobilisation on Eupergit. Unfortunately, the immobilisation did not reduce Maillard reactivity (see appendix).

Acknowledgements

The authors thank J. van Lieshout and J. van der Oost from Wageningen University for the supply and purification of the β-glycosidase from *P. furiosus*. This research is supported by the Dutch Technology Foundation STW, applied science division of NWO and the technology programme of the Ministry of Economic Affairs.

Appendix: Enzyme immobilisation.

Introduction

The extremely thermostable β-glycosidase from *P. furiosus* was used for the production of oligosaccharides with lactose as a substrate. Using a thermozyme made it possible to operate at higher reaction temperatures, and thus to increase the substrate concentration. This increased substrate concentration and the subsequent lower water concentration suppressed hydrolysis and therefore improved the oligosaccharide yield.
During the reaction brown pigments were formed, caused by Maillard reactions. This changes the structure of the enzyme and causes faster inactivation of the enzyme, compared to normal inactivation by temperature. In this appendix we discuss the prevention of Maillard reactions.

Possible solutions

It has become clear that for enzymatic reactions at elevated temperatures for the conversion of small sugars, it is important to solve the problems caused by Maillard reactions. Reduction of the extent of Maillard inactivation can be achieved by changing the process conditions or by modification of the enzyme.
Process conditions that are of importance are the type and the concentration of reducing sugars, the pH, temperature, oxygen concentration and water activity. Lowering the water activity below 0.6 will decrease Maillard reactions. This might not be possible in solely aqueous solutions, but the use of hydrophilic solvents, or ionic liquids could stabilise the enzyme against Maillard mediated inactivation. A low pH suppresses the Maillard reactions. In our experiments the pH was set at 5.0 or 5.5. Unfortunately, at lower pHs the enzyme become much less active. Oxygen is one of the substrates at the end of the Maillard reaction, so lowering the concentration of dissolved oxygen can have a positive influence on enzyme stability.
It is further possible to modify the enzyme, either by chemical modification, or by altering the enzyme structure by protein engineering. Specific replacement of lysines by e.g. arginines removes the amino groups that are available for the Maillard reaction, and thus will suppress the extend of Maillard reactions. These amino groups can however also be protected by chemical modification, e.g. by coupling to polyethylene glycol, by chemical crosslinking or by covalent immobilisation. For all these routes it is however not clear whether this will influence the activity of the enzyme significantly.

Immobilisation

We immobilised the β-glycosidase on Eupergit, using the method according to Fischer *et al.* (1996). The enzyme loading after immobilisation was measured to be 15 U·(g eupergit)$^{-1}$. The kinetics of the immobilised enzyme was compared to that of the free enzyme. Furthermore, the immobilised enzyme was re-used in three successive batches to test the enzyme stability.

Table 2 Conversion of lactose with β-glucosidase from *P. furiosus* as a free enzyme and immobilised, at 60 and 80°C. 0.66 U·(g solution)$^{-1}$ (pNPG test). The immobilised or free enzyme was incubated with 0.5 mol·kg^{-1} lactose in a 0.2 mol·kg^{-1} citrate buffer at pH 5.0. Initial conversion rates are given in mmol·kg^{-1}·min^{-1}.

	60°C free enzyme	60°C immobilised	80°C free enzyme	80°C immobilised
V_{ini} lactose	0.25	0.30	0.84	0.90
V_{ini} glucose	0.20	0.24	0.61	0.67
V_{ini} oligo	0.07	0.08	0.32	0.24
V_{ini} galactose	0.09	0.12	0.09	0.36
Synthesis/Hydrolysis[1]	0.8	0.7	3.6	0.7
Synthesis/Hydrolysis[2]	0.6	0.5	1.0	0.5

[1]Ratio of initial galactose and oligosaccharide formation,

[2]Ratio of galactose and oligosaccharide concentration after 1,500 minutes reaction

Comparison of the kinetics of the immobilised enzyme to that of the free enzyme was done at 60 and 80°C. The initial conversion and formation rates are given in table 2. For the reactions at 80°C, the sugar concentrations in time are shown in figure 9. Lactose was hydrolysed into glucose and galactose and oligosaccharides were synthesised in a kinetically controlled reaction. The oligosaccharides consisted mainly of trisaccharides but also tetra- and pentasaccharides were formed in later stages of the reaction. Although the amount of active enzyme was equal according to *p*-Nitrophenyl-β-D-Glucopyranoside (pNPG) testing (Kengen *et al.*, 1993), differences in initial conversion rates for the substrate lactose were found. At both 60 and 80°C, the conversion rates were higher for the immobilised enzyme. This may be attributed to different diffusion rates for pNPG and lactose and their products, which leads to different concentrations of the products at the surface, and thus to different conversion rates. The absolute and relative increase in oligosaccharide production with temperature is consistent with the results in this chapter.

Immobilisation however has a negative effect on oligosaccharide formation. This can be caused by diffusion limitations caused by molecular size differences in the small unwanted byproducts (monosaccharides) and the bigger oligosaccharides. At 80°C it even cancels out

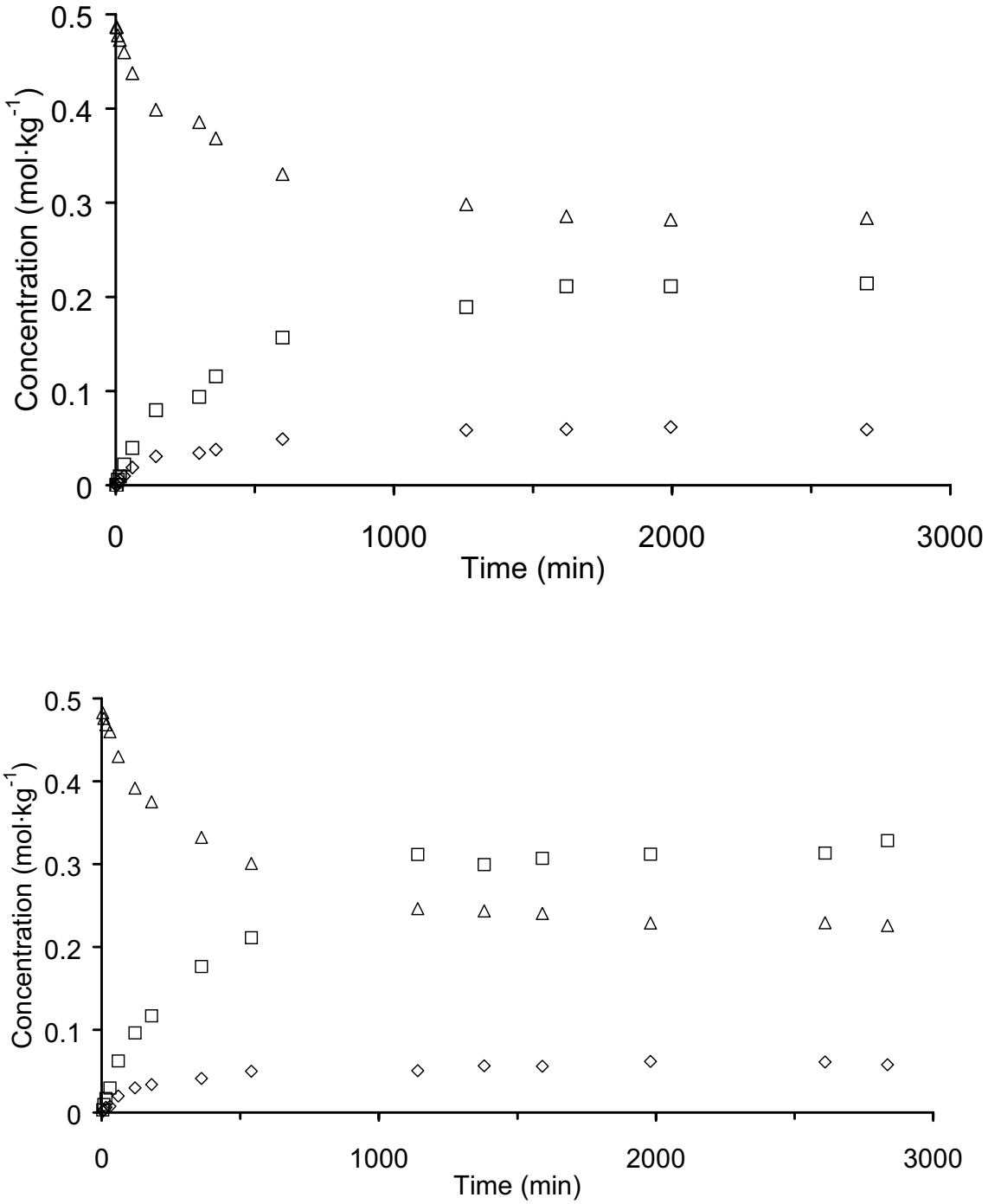

Figure 9 Conversion of lactose with β-glucosidase from *P. furiosus* as a free enzyme (upper graph) and immobilised (lower graph). For both the immobilised and the free enzyme 0.66 U·(g solution)$^{-1}$ were incubated with 0.5 mol·kg^{-1} lactose in a 0.2 mol·kg^{-1} citrate buffer at pH 5.0 and 80°C. (Δ, lactose; □, monosaccharides; ◊, oligosaccharides)

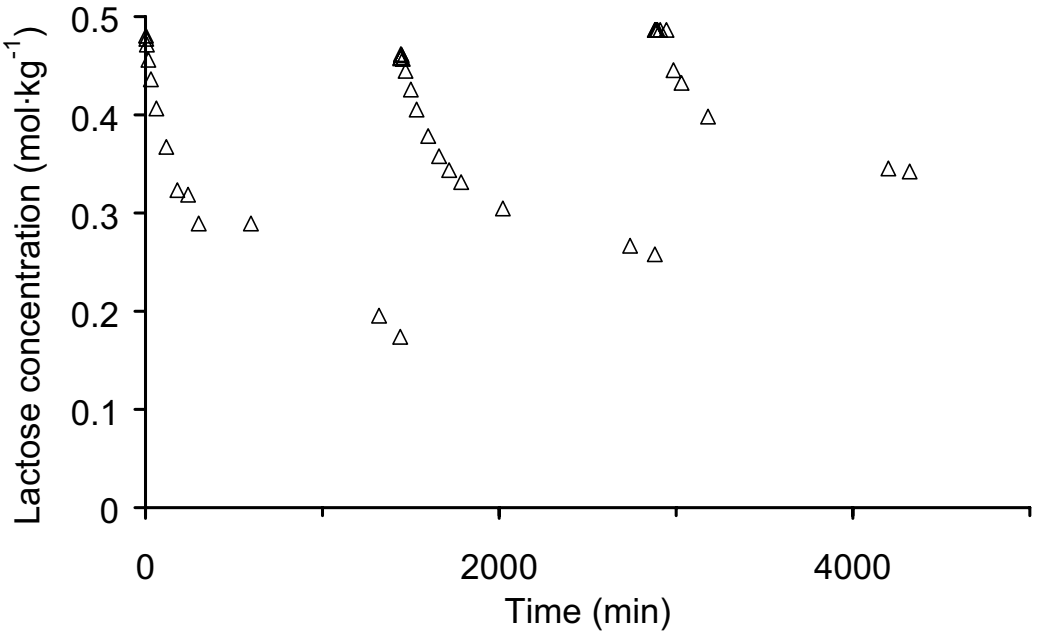

Figure 10 Three successive batches with immobilised enzyme from *P. furiosus* at 60°C, pH 5.0, with 0.5 mol·kg^{-1} lactose as a substrate.

the positive effect of temperature upon the synthesis-hydrolysis ratio.

Immobilisation may protect the enzyme from Maillard reactions. The brown end products of this reaction can be measured optically at 470 nm. At 80°C browning of the reaction mixture occurred and was measured in time for both the free and immobilised enzyme. Similar values of browning were found in both reaction mixtures. After prolonged incubation, more browning was found for the immobilised enzyme than for the free enzyme. To test the enzyme stability, the immobilised enzyme was re-used in three successive batches. These results are shown in figure 10. The enzyme was incubated with 0.5 mol·kg^{-1} lactose in a 0.2 mol·kg^{-1} citrate buffer at pH 5.0 and 60°C. Conversion of lactose was followed for 24 hours after which the immobilised enzyme was removed from the reaction mixture and put into a fresh lactose solution. This was repeated twice. The initial rate of lactose conversion decreased from 1.31 mmol·kg^{-1}·min^{-1} in the first batch to 0.59 mmol·kg^{-1}·min^{-1} in the second and to 0.37 mmol·kg^{-1}·min^{-1} in the third batch. This decrease in enzyme activity is considerable, since the free enzyme only shows a 10% decrease in activity after 5 days of use at 60°C.

Conclusions

Immobilisation of the thermostable β-glycosidase from *P. furiosus* was successfully achieved on Eupergit. Unfortunately, the immobilised enzyme was at least as sensitive to Maillard inactivation. Further, the inactivated enzyme exhibited a reduced synthesis-hydrolysis ratio, which indicates that transfer limitation of the substrates and products plays a role in such a system.

The results indicate that a reduction of the sensitivity to Maillard reactions has to be found in adaptation of process circumstances, or in biochemical engineering of the enzyme.

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Chapter 5

Downstream processing

Abstract

Oligosaccharides can be produced in a condensation reaction using β-glycosidase isolated from *Pyrococcus furiosus*. With a 60% (w/w) galactose solution as the substrate an oligosaccharide yield of 18% (w/w) was obtained. The feasibility of a Simulated Moving Bed (SMB) for downstream separation was investigated with column experiments. The components could be separated with an SMB into a 91% pure product stream and a 99% pure galactose stream, which can be recycled to the enzyme reactor. The results were compared to those that can be achieved with nanofiltration.
It is also possible to produce transgalacto-oligosaccharides in a more conventional way, with lactose as a substrate. This production and separation via SMB was also studied as an alternative to the galactose condensation.

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Introduction

Oligosaccharide production

Non-digestible oligosaccharides, which stimulate the growth of intestinal microorganisms (e.g. *Bifidobacteria*), are used as health-stimulating ingredient in foods since 1980. The production of oligosaccharides in 1995 was estimated to be 85,300 tons (Playne & Crittenden, 1996). Japan is the major market for prebiotics; the European market is significantly less developed. Nevertheless, also in Europe specific examples of prebiotic containing foods exist and prospects for the future are promising. Fructo-oligosaccharides, galacto-oligosaccharides and inuline are produced on a commercial scale (Gibson *et al.*, 2000; Playne & Crittenden, 1996). The principal producers of transgalacto-oligosaccharides in Japan are Yakult Honsha, Nissin Sugar Mfg. and Snow brand. In Europe oligosaccharides are produced by Borculo Whey Products. They are sold both as powder and as syrups with a >55% (w/v) solid content (Gibson *et al.*, 2000; Playne & Crittenden, 1996). The estimated global production of galacto-oligosaccharides in 1995 was 15,000 tons. The estimated production of more specific oligosaccharides, like lactosucrose, was 1,600 tons.

Enzymatic oligosaccharide synthesis

The commercial, enzymatic, production of galacto-oligosaccharides is achieved via transglycosylation from lactose. The process yields, besides the desired oligosaccharides, also galactose and glucose as unwanted side products. Most products are sold including these non-prebiotic sugars. Cup Oligo Syrup (Nissin Sugar Mfg.) contains about 25% mono and dimeric sugars (lactose, glucose, and galactose) as well as traces of larger components. Oligomate 50 (Yakult Honsha) consists of 50-52% galacto-oligosaccharides (36% transgalacto-oligosaccharides and 15% digalactose), 10-13% lactose and 36-39% monosaccharides. This mixture is produced via a two step enzymatic process without further purification. For a more specific product, further processing is necessary. Yakult Honsha also produces high grade transgalacto-oligosaccharides in a one step enzymatic synthesis followed by a chromatographic separation. The Yakult Honsha process is depicted in figure 1 (Matsumoto *et al.*, 1993).
In this chapter we discuss the design of a reactor concept that can be exploited for the production of tailor-made galacto-oligosaccharides, without glucose in the product. For this we chose galactose as a substrate. The specific oligosaccharides are synthesised in a condensation reaction by β-glycosidase isolated from *Pyrococcus furiosus* (Kengen *et al.*, 1993). This enzyme is chosen for its high thermostability. This enabled us to use higher temperatures, at which more sugar can be dissolved, which benefits oligosaccharide synthesis

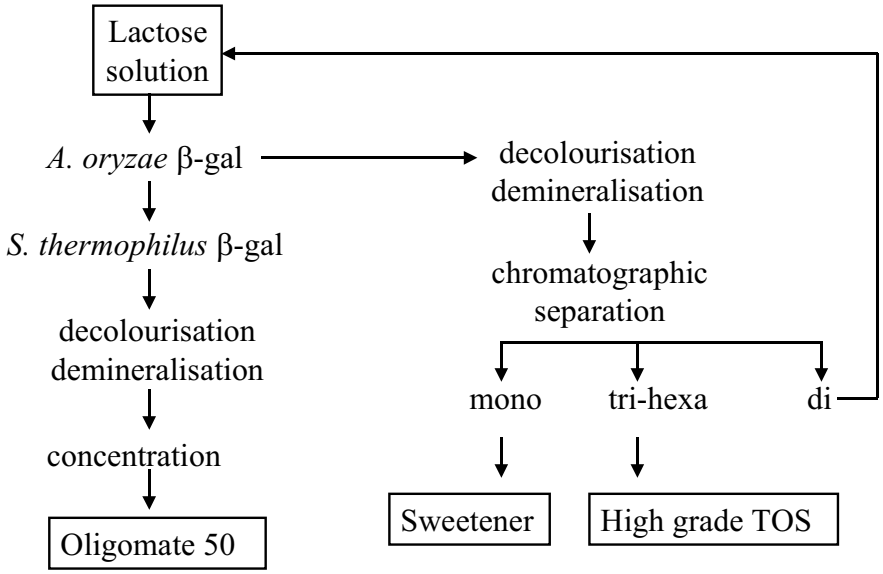

Figure 1 Yakult production process for transgalacto-oligosaccharides (TOS) with β-gal(actosidases).

(chapter 3). The coupling to a downstream purification process, and the subsequent recycling of monosaccharides, results in the production of specific high-purity oligosaccharides.

Methods of oligosaccharide recovery

Different separation processes have been described for the separation of oligosaccharides. Most processes are based on chromatographic techniques such as ion-exchange chromatography or chromatography with activated carbon.
Nanofiltration might be another option to purify saccharide mixtures (Goulas *et al.*, 2002; Wang *et al.*, 2002). A clear advantage is that it is readily applicable as a continuous process. It is however difficult to find membranes with the right pore-size that can be operated under higher temperatures. Since our product stream is highly concentrated, it has to be purified at elevated temperatures to avoid uncontrolled crystallisation, or the stream has to be diluted. Nanofiltration membranes with a molecular weight cut-off below 200-300 Da would be required. Until recently, the most suitable polymeric membranes could not be used above 75°C and sugar solutions had to be diluted to 30% (w/w) (Pedersen, 1999). Goulas *et al.* (2002) tested nanofiltration membranes that can be used at elevated temperatures for the separation of oligosaccharides. Continuous diafiltration using DS-5-DL membranes (Osmonics Desal, Le Mee sur Seine, France) gave yields of 98% galacto-oligosaccharides, 89% lactose and 18% glucose, making them suitable for the removal of monosaccharides from an oligosaccharide mixture.

Adsorption can be carried out with activated carbon. Activated carbon has a high affinity for oligosaccharides; product recovery has to be done off-line by 10-50% ethanol elution. Enzymatic production of oligosaccharides with continuous removal of oligosaccharides from the reaction mixture by adsorption on activated carbon was studied with monosaccharides (Ajisaka *et al.*, 1987) or disaccharides (Boon *et al.*, 2000) as a substrate. For oligosaccharide production from lactose, the batch process with in-line removal showed a yield improvement of 30% compared to the batch process without removal. For the galactose condensation, no yield improvement was reported.
Ion-exchange chromatography is most often used for sugar separations (Saunders, 1968; Jones *et al.*, 1960). Cation-exchange resins have higher affinity for monosaccharides and therefore trisaccharides (or higher) are the first to elute from the column. Elution is performed with water, which is a plus in a process that produces a food additive.

SMB and applications in the separation of small sugar molecules

Normal co-current elution chromatography is discontinuous and the products are diluted, resulting in low productivity and efficiency. Counter current processes have not been successful due to practical complications. Simulated counter current processes have been developed, where the ports of inlet and outlet, or in later designs the columns, are repeatedly switched to simulate flow of the solid phase (figure 2). The first Simulated Moving Bed (SMB) process was developed to separate para-xylene from a mixture of C8 hydrocarbons by Universal Oil Products (UOP) in the early 1960s (Broughton & Gerhold, 1961). Since then, SMB technology has been applied in various fields; petrochemistry, food, biotechnology and pharmacy.
For sugar separations, the technology was first applied to the separation of glucose and fructose in high fructose corn syrup production. This syrup contains 42% fructose and is introduced into the SMB system as a feed solution in a concentration of about 500 $kg \cdot m^{-3}$ monosaccharides. The separation is performed on a cation-exchange resin using warm water as eluent. The concentration of glucose and fructose in the product stream is about 200 $kg \cdot m^{-3}$, which is a small dilution compared to the single compound concentration in the feed. Fructose is recovered at 90-94% purity and a yield of over 90%. The glucose rich fraction is recycled for isomerisation (Bieser & de Rosset, 1977).
Sucrose recovery and purification from molasses is another process in which SMBs are implemented in factory scale processes. Here sucrose is separated from the other components like electrolytes, colorants and polysaccharides (Rearick *et al.*, 1999).
Some possible other applications for SMB in the separation of sugars are separation of trehalulose (a glucose-fructose disaccharide) and fructose (Nicoud, 1994) and the production of lactosucrose from lactose and sucrose (Kawase *et al.*, 2001).

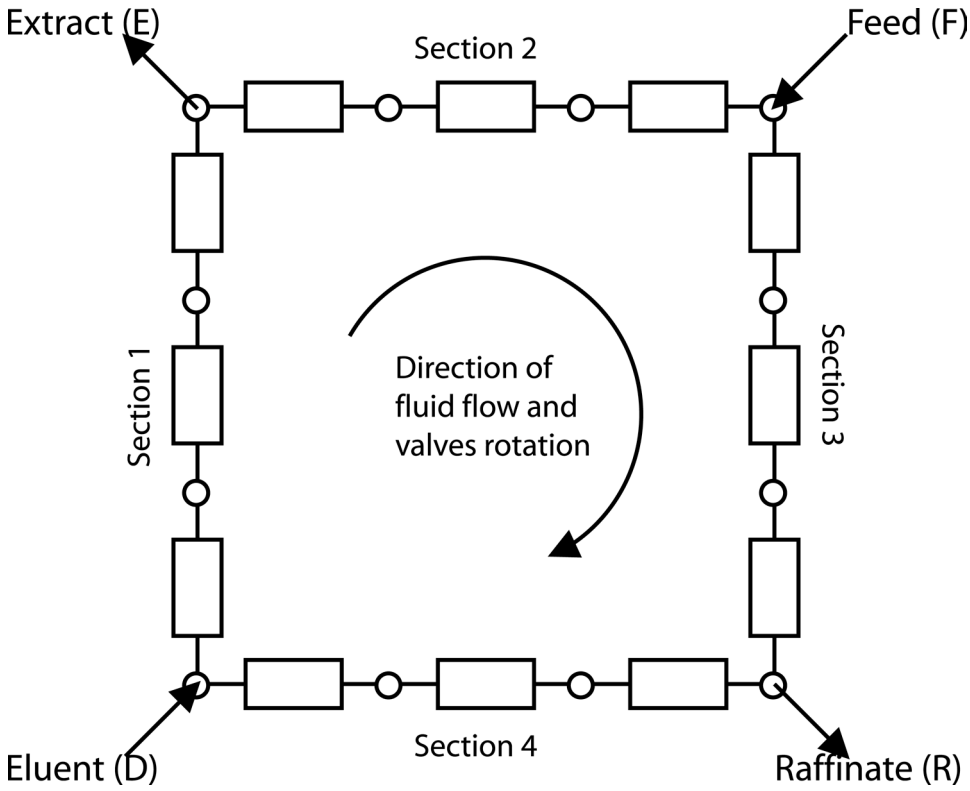

Figure 2 Representation of a simulated moving bed (SMB)

The fact that SMB's are used with concentrated product streams and the fact that many available resins can be used at elevated temperatures, make SMB an option for down-stream processing that is worth consideration.

Theory

Adsorption equilibrium and peak widening

The essential parameters for SMB design are those characterising adsorption equilibrium and mass transfer efficiency of the components that need to be separated (Ruthven & Ching, 1989). When dealing with the simplest case of linear (Henry-type) equilibrium, pulse experiments on a column on laboratory scale are a good method to obtain elution times (t), which enable the calculation of equilibrium parameters (K). This can be done by using equation 1.

$$\frac{v\varepsilon}{L_c}t = \varepsilon + (1-\varepsilon)K \qquad [1]$$

Literature values for equilibrium constants for sugars in size exclusion adsorbentia are given in table 1. K values decrease with increasing sugar size. Specific values differ due to the use of different materials.

Another important phenomena in characterising adsorption is peak widening. It was shown by van Deemter *et al.* (1956) that the effects of axial dispersion and mass transfer limitation and particle diffusion are additive in case of a linear isotherm. By adjusting the corresponding parameters, one can therefore simplify the model by considering only one of the effects while still capturing the effects of both phenomena. It has been shown indeed that the quality of the prediction of simpler models only considering (a lumped) mass transfer resistance does not differ significantly from those that consider both axial dispersion and mass transfer limitation explicitly (Golshan-Shirazi & Guiochon, 1992). In this work we chose to use a lumped mass transfer limitation approach. The overall volumetric mass transfer coefficient ($K_f a_v$) was estimated from literature correlations for fixed beds (table 1).

Table 1 Literature values for the overall volumetric mass transfer coefficient ($K_f a_v$) and the equilibrium constant (K) for sugars in ion exchange resins.

Sugar	$K_f a_v$ (s^{-1})	K (-)	Material	T (°C)	Reference
Glucose	0.02	0.31	Diaion UBK-350 resin	40	(Kim *et al.*, 1992)
Maltose	0.009	0.19	Na 100 mesh		
Maltotriose	0.004	0.10			
Glucose	0.027	0.49	Amberlite CR-1310 Na	50	(Kawase *et al.*, 2001)
Fructose	0.024	0.58			
Lactose	0.015	0.34			
Sucrose	0.012	0.32			
Lactosucrose	0.0105	0.25			
Glucose	0.015[a]	0.32	Lewatit MIDS 1368 Ca	25	(Beste *et al.*, 2000)
	0.018[a]	0.32	350 um	40	
	0.019[a]	0.324		60	
	0.019[a]	0.368		80	
Glucose	0.03	0.19	Zerolit-25SCR14 Ca	60	(Ching & Ruthven, 1984)

[a] also measured: axial dispersion constant of 0.00153 (m)

The SMB Model

The performance of an SMB system can be estimated approximately by modelling a True Moving Bed (TMB) as described by Ruthven and Ching (1989). In this work, four counter current sections were coupled (figure 3). While the raffinate and extract concentrations vary during the time of one cycle in a SMB, the TMB represents a steady state situation, which

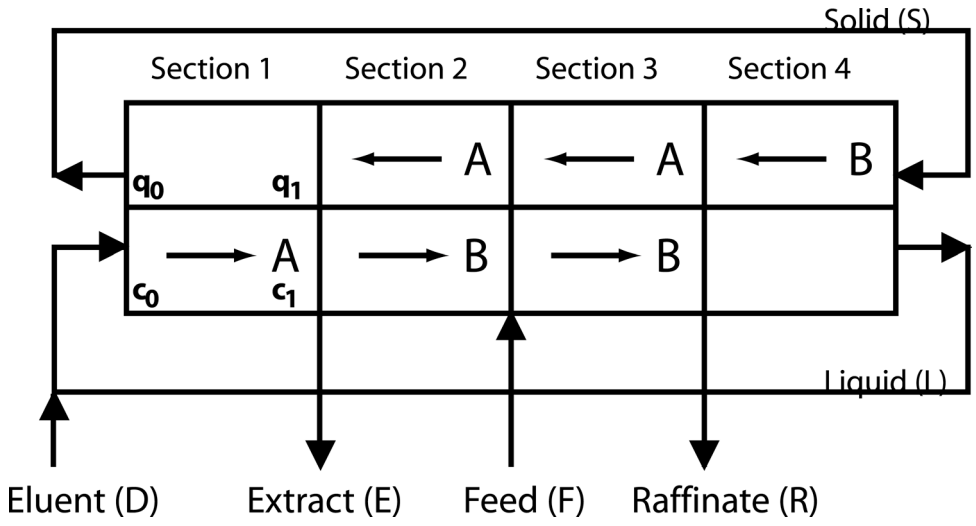

Figure 3 Representation of a true moving bed (TMB) with the relative motion of A and B as required for separation. A is the most retained and smaller sugar molecule.

approximates the time averaged situation in the SMB. The flow rate (u) of the solid phase in a TMB can be translated to a cycletime in SMB (t_{switch}) by using $t_{switch} = L_c/u$.

Axially dispersed flow was assumed for the fluid phase and plug flow for the solid phase. A linear equilibrium relationship (K) between the concentrations in the solid (q) and liquid phase (c) could be assumed since separation is based on size exclusion, which is characterised by a linear isotherm. The adsorption rate process is represented by a linear driving force approximation.

The concentrations of the components in the liquid at the end of each section (i) can be calculated with equation 2, in which γ represents the ratio of the downflow in the solid to the upflow in the fluid. A concentration profile can be obtained by varying the dimensionless axial distance (Z) from 0 to 1 (Navarro *et al.*, 1997).

$$\frac{c_{i+1}}{c_i} = \frac{1}{\gamma - 1}\left(\left(1 - \frac{q_i}{Kc_i}\right)\gamma \cdot e^{St(1-\gamma)Z} + \gamma\frac{q_i}{Kc_i} - 1\right) \qquad [2]$$

Since we chose to work with an overall volumetric mass transfer coefficient, the Stanton number (St) was incorporated in this equation. St can be calculated from $St = K_f a_v L_c/u = K_f a_v t_{switch}$.

Besides four equations for the concentrations in the bed given by equation 2, a mass balance for each section is required. Equation 3 was used for each section, yielding four extra equations for eight concentrations (for fluid (c_i) and solid (q_i) in each section) in total.

$$q_{i+1} = q_i + \frac{L_{i+1}}{S}(c_{i+1} - c_i) \quad [3]$$

Two additional equations (4 and 5) are needed to describe the concentration at the feed (c'_2) and eluent point (c_0).

$$c_2 L_2 + c_F F = c_2^{'}(L_2 + F) \quad [4]$$

$$c_0(D + L_4) = c_4 L_4 \quad [5]$$

Primary separation conditions

In order to achieve complete separation of a binary mixture it is necessary to fulfil the following relationships, which define the net flow of the components (Ruthven & Ching, 1989). The theoretical optimal design corresponds to the minimum value of the safety margin (α), i.e. maximum feed flow and minimal eluent flow, α=1. However, for a practical operating point α is somewhat larger to obtain a more robust system. An operating diagram or Morbidelli triangle (Storti *et al.*, 1993) can depict the complete region of separation. The equations which are then satisfied are depicted in bold, with γ being equal to 1.

Section 1:	$\gamma=K\cdot(S/L_1)/\alpha$	$\gamma_{mono}>1$	$\gamma_{di}>1$
Section 2:	$\mathbf{\gamma=K\cdot(S/L_2)/\alpha}$	$\gamma_{mono}>1$	$\mathbf{\gamma_{di}<1}$
Section 3:	$\mathbf{\gamma=K\cdot(S/L_3)\cdot\alpha}$	$\mathbf{\gamma_{mono}>1}$	$\gamma_{di}<1$
Section 4:	$\gamma=K\cdot(S/L_4)\cdot\alpha$	$\gamma_{mono}<1$	$\gamma_{di}<1$

These are used as boundary conditions in optimising the SMB process.

Nanofiltration

The enzymatic condensation of galactose yields a galacto-oligosaccharide mixture that can also be separated by membranes. A counter-current membrane system was modelled analogue to the SMB. Figure 4 shows the first membrane from the serie of units in this system. The permeate (P) from the first membrane unit is fed back into the enzymatic reactor, after removal of some of the water, e.g. by evaporation, and the addition of (solid) galactose. The permeates from the subsequent membrane units are recycled to the previous unit. The system was modelled by determining overall mass balances and component balances (equation 6 for the last membrane unit, equation 7 for all other units). Equal flows for permeates through the membrane units were taken.

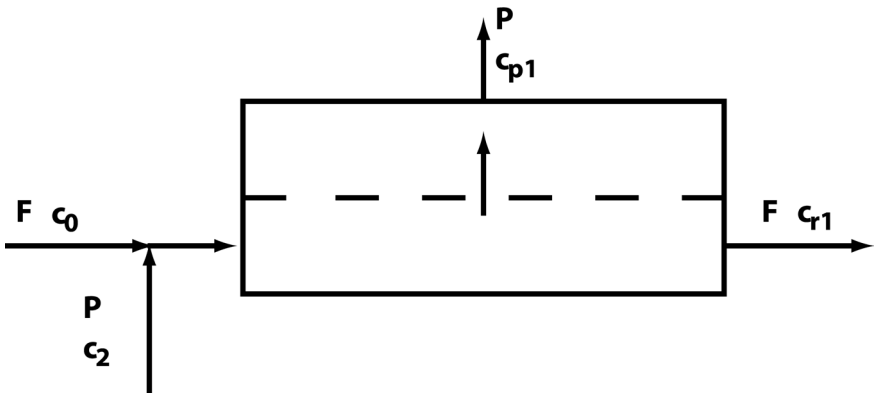

Figure 4 Representation of the first membrane unit in a counter-current operated membrane system in series.

$$Fc_{r(i+1)} + Pc_{p(i+1)} = Fc_{ri} \quad [6]$$

$$Fc_{r(i+1)} + Pc_{p(i+1)} = Fc_{ri} + Pc_{p(i+2)} \quad [7]$$

The rejection (R_m) is defined as 1- c_p/c_f, yielding equations 8 for the last membrane unit and equation 9 for all others.

$$Fc_{r(i+1)} + Pc_{ri}(1-R_m) = Fc_{ri} \quad [8]$$

$$Fc_{r(i+1)} + Pc_{ri}(1-R_m) = Fc_{ri} + Pc_{r(i+1)}(1-R_m) \quad [9]$$

Materials and methods

Materials

β-Glycosidase from *P. furiosus* was prepared from an *E. coli* lysate and heated to denature proteins other than the thermostable enzyme (Voorhorst *et al.*, 1995). The cation-exchange resin was Dowex 50W-X4, Na^+-form from Bio-Rad (St Louis, USA) with a particle diameter of 80-150 μm. All other chemicals were from Sigma (St Louis, USA).

HPLC analysis of sugars

Samples for HPLC analysis were treated with $Pb(NO_3)_2$ (final concentration in the samples 0.1 mol·l^{-1}) and stored in the freezer for at least 1 hour to accelerate precipitation of the citrate and the enzyme. Before analysis the samples were centrifuged at 13,000 rpm and diluted.

The samples were analysed on HPLC using a RSO Oligosaccharide Column (Phenomenex, Amstelveen, the Netherlands) at 80°C. The column was eluted with Milli-Q water (purged with helium) at a flow rate of 0.3 ml·min^{-1}. The eluent was monitored with a refractive index detector. It was assumed that the response was independent on the degree of polymerisation. Lactose, glucose, galactose and oligosaccharides, such as tri-, tetra-, and pentasaccharides were detected and measured as percentages of total sugar on weight base.

Enzymatic synthesis of oligosaccharides

Galactose condensation was followed during 1 week reaction time. The solution consisted of 0.6 ml 0.2 *M* citrate buffer, pH 5.0, 80°C, in which 0.2 ml of the enzyme solution and 1.2 gram galactose was added to the total amount of 2 gram. Samples were taken for HPLC analysis. Putting the samples on ice for 15 minutes stopped the reaction. The reaction mixture was then ready for column experiments. A similar reaction was done for glucose, to obtain a reaction mixture that could be used on column for comparison with other results.
Lactose transglycosylation was calculated for a 50% (w/w$_{mixture}$) lactose solution. The enzyme concentration was 3 mg·kg^{-1}. A full description of the calculations can be found in chapter 3.

Column experiments

The external void ratio of the column, ε=0.38, was estimated by pulse experiments using blue dextran as inert compound.
Pulse experiments were performed for determination of the equilibrium constant. A 0.5 ml pulse of the reaction mixture was injected through a four-way valve. The column (Pharmacia XK 16-40) was packed with the cation-exchange resin and thermostated by a water jacket kept at 80°C by a water bath. Elution was performed with 0.2 *M* citrate buffer, pH 5.0 at 0.5 to 0.7 ml·min^{-1}. A fraction collector was used to collect the samples (0.5 ml). Samples were analysed via HPLC. The elution times (t) of experimental peaks were calculated according to equation 10.

$$t = \frac{\sum_{i=1}^{n} c_i t_i \Delta t_i}{\sum_{i=1}^{n} c_i \Delta t_i} \qquad [10]$$

The elution time is needed to calculate the equilibrium constant (K), see equation 1 (Navarro *et al.*, 1997).

Optimisation of SMB operating conditions

For the optimisation of the SMB process, we assumed a column length of 1 meter, comparable to column lengths in glucose/fructose separations (Beste *et al.*, 2000; Strube *et al.*, 1998). We assume an annual oligosaccharide production of 1000 tons, based on current production of other oligosaccharides (Playne & Crittenden, 1996). Feed streams and concentrations are calculated from the concentrations of the sugars after the enzymatic reaction. The optimum operating point was chosen according to the theory.
Final optimisation was based on minimising the monosaccharide concentration in the raffinate. The SMB volume was optimised by minimising the diameter of the columns. Very small adjustments were made after that to minimise desorbent consumption.
The parameters characterising the quality of separation were defined as follows:

		compound in extract (a)	compound in raffinate (b)
Purity	(-)	$c_{a,e}/(c_{a,e}+c_{b,e})$	$c_{b,r}/(c_{b,r}+c_{a,r})$
Recovery	(-)	$(c_{a,e}E)/(c_{a,f}F)$	$(c_{b,r}R)/(c_{b,f}F)$
Productivity	$(kg \cdot m^{-3} \cdot s^{-1})$	$(c_{a,e}E)/(4L_cA(1-\varepsilon))$	$(c_{b,r}R)/(4L_cA(1-\varepsilon))$
Dilution	(-)	$c_{a,f}/c_{a,e}$	$c_{b,f}/c_{b,r}$

Results and Discussion

Enzymatic synthesis of oligosaccharides

Figure 5 shows the galactose condensation by β-glycosidase from *P. furiosus* in time. Galactose was converted to di-, tri- and tetrasaccharides. Table 2 shows the oligosaccharide production after 3 and 7 days for a 60% ($w/w_{mixture}$) galactose solution. The substrate concentration was chosen close to its maximum solubility to maximise the oligosaccharide yield. For comparison, we also show the calculated oligosaccharide production from lactose by the same enzyme (for details see chapter 3). This concentration was also chosen close to the maximum lactose solubility for oligosaccharide yield maximisation. The lactose transglycosylation reaction is much faster and produces more oligosaccharides. The main problem is however the production of large amounts of monosaccharides that cannot be recycled in this reaction. In the condensation reaction of galactose no glucose is produced; the remaining galactose can be recycled into the reaction after separation. This way no losses will occur. The disaccharides that are produced when galactose is used as a substrate obviously consist of only galactose sugar molecules. During the transglycosylation of lactose, dimer isomerisation and monomer condensation gives a whole range of different disaccharides consisting of both glucose and galactose, like lactose, or of two glucose or galactose sugar molecules. It depends on the industrial application whether this is positive or not.

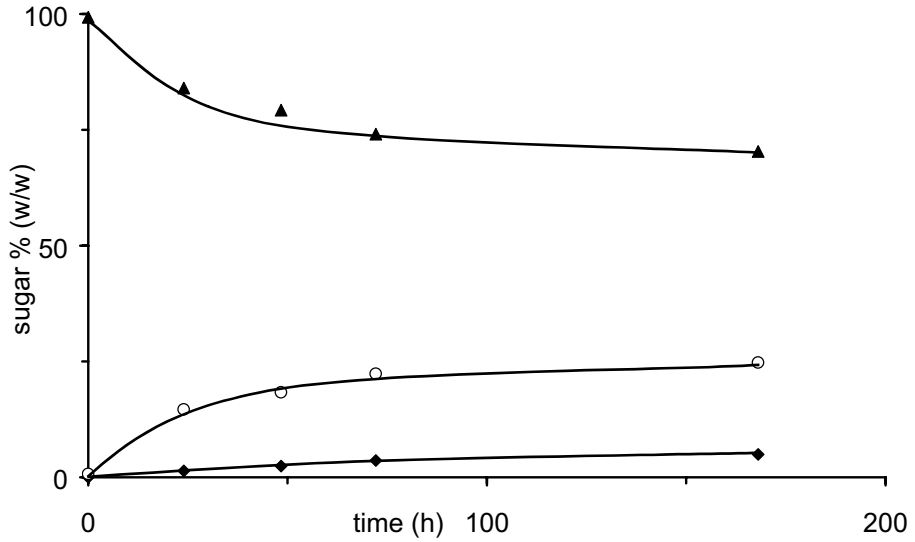

Figure 5 Condensation of galactose (▲) to oligosaccharides (=di-(○) and tri- and tetrasaccharides (◆)) by β-glycosidase from *P. furiosus* at 80°C, pH 5. Lines for guidance.

Table 2 Composition of the reaction mixture (% w/w) for various substrates, at different times.

Reactant	Time	Glucose	Galactose	Di	Tri+	Total
Galactose	3 days	-	44.4	13.4[a]	2.2	60
Galactose	7 days	-	42.2	14.8[a]	3.0	60
Lactose	8.1 h	12.2	5.6	13.7[b]	18.5	50

[a] solely digalactoses, [b] different disaccharides

Reactor optimisation

Based on the data in table 2, it is possible to make an estimate of the volume of a reaction vessel for an annual production of 1,000 tons of oligosaccharides. The synthesis reaction from galactose is equilibrium based and therefore a plug flow reactor is chosen. Alternatively, the reaction can be operated batch wise. Reactor sizes are calculated on a residence time of 8.1 hours for a lactose-based reaction and 7 days for a galactose based reaction. Decreasing the residence time for the galactose-based reaction would result in a significantly smaller reactor with a relatively small decrease in oligosaccharide production. This will however slightly increase the size of the SMB. Cost considerations will give an answer to whether the reaction or the separation unit should be minimised.

Table 3 Calculations for an enzymatic production of 1,000 tons oligosaccharides per year.

Reactant Minimal oligo size		Galactose n=2	Galactose n=3	Lactose n=2	Lactose n=3
Reactor size	(m^3)	105	637	2.8	4.9
Feed flow	($m^3 \cdot h^{-1}$)	0.65	3.8	0.35	0.61
c_a	($g \cdot g^{-1}$)	0.422	0.57	0.178	0.315
c_b	($g \cdot g^{-1}$)	0.178	0.03	0.322	0.185

Oligo's consist of minimal n sugar molecules. For n=2: a=monosaccharides and b=disaccharides and higher. For n=3: a= mono and disaccharides, b= trisaccharides and higher.

In our calculations we neglected downtimes. A pure product was assumed to be required. The results are shown in table 3. The relatively slow condensation reaction from galactose results in a much larger reactor volume than the transglycosylation from lactose. Based on the sugar composition at the end of the reaction an estimate for the feed rate has been made. From these calculations it can be seen that the galactose based production of trisaccharides and higher is relatively unfavourable.

Column experiments

Column experiments were performed to determine the equilibrium constants for all components. The column was used with the mixture from the condensation reaction from galactose at 40 and 80°C. For comparison a reaction mixture from a glucose condensation with the same enzyme was also applied. The results are depicted in table 4.

Table 4 Equilibrium adsorption constants (K) ($g \cdot g^{-1}$) for components from the galactose or glucose condensation reaction.

Temperature (°C)	Tri	Di	Mono	Reactant
40	0.30	0.43	0.56	Galactose
80	0.29	0.39	0.54	Galactose
80	0.26	0.35	0.49	Glucose

Ching and Ruthven (1984) showed that K for glucose is independent of temperature, because separation is based on size exclusion, which is an entropically determined process, and not on enthalpic adsorption. Galactose however does show some specific adsorption due to the formation of bidentate chelates. This is however a small effect as can be seen by the small dependence of K on temperature. The separation is mainly based on size exclusion but, as can be seen from the small influence of temperature and the differences with glucose adsorption, also partially based on complexation with the cations.

A glucose-fructose separation is based on a combination of mechanisms, although the same materials can be used. Since fructose can form tridentate chelates, monosaccharide separations such as glucose-fructose separations are based on adsorption, while a monosaccharide-disaccharide separation is based on size exclusion.

SMB optimisation

In our calculations we assumed that the amount of monosaccharides in the products should be low, to produce a specific high-grade galacto-oligosaccharide mixture. Therefore we minimised the concentration of monosaccharides in the end product. We calculated the size of the equipment and the flows through the system for all the cases mentioned in table 3. Galactose or lactose was used as reactant. As products either disaccharides and higher or trisaccharides and higher were considered. The feed flow and concentrations are taken from table 3; the results are presented in table 5.
In most cases product purities higher than 90% (w/w_{sugar}) could be obtained in the raffinate and in all cases the extract stream was 97-99% (w/w_{sugar}) pure. Separation was not adequate when galactose was used as a reactant and trisaccharides and higher are isolated. This was due to the low product concentration in the feed. The best result was found when lactose was used as a reactant and disaccharides and higher are the product. However, also in the two other cases separation was adequate. For both cases where trisaccharides and higher were defined as the final product, column diameters were significantly higher than when disaccharides and higher were the preferred product. In another large-scale separation, the glucose-fructose separations, industrial beds can have a diameter of 6.7 m and still perform as good as pilot plant beds with diameters of 10 cm. This is achieved by internal design of large adsorbent chambers to assure good distribution and uniform flow of the liquid (Bieser & de Rosset, 1977). Our results show that SMB is suitable for the separation of monosaccharides from di- and trisaccharides. However, for the separation of disaccharides from trisaccharides selectivity is much less.
When lactose is used, the extract will be a by-product stream. In the case of galactose condensation, the extract can be fed back straight into the enzyme reactor and used as a reactant. This is the main advantage of the galactose-based system. The flow of the extract stream is however larger than the feed flow and obviously more diluted. Therefore the recycle stream has to be concentrated by adding substrate and evaporating some of the water.
The productivity of the SMB for lactose mixtures is double that of the SMB for galactose based separations. This is due to the higher product concentration in the feed.

Table 5 Process parameters for a single SMB unit

Reactant Minimal oligo size		Galactose n=2	Galactose n=3	Lactose n=2	Lactose n=3
Parameters					
K_a	(-)	0.536	0.392	0.536	0.392
K_b	(-)	0.392	0.286	0.392	0.286
$K_f a_{v\,a}$	(s^{-1})	0.02	0.01	0.02	0.01
$K_f a_{v\,b}$	(s^{-1})	0.01	0.005	0.01	0.005
ε	(-)	0.38	0.38	0.38	0.38
L_c	(m)	1	1	1	1
α	(-)	1.01	1.01	1.01	1.01
Optimisation Results					
t_{switch}	(s)	$8.6 \cdot 10^3$	$1.8 \cdot 10^4$	$7.9 \cdot 10^3$	$1.7 \cdot 10^4$
St_A	(-)	172	176	157	171
St_B	(-)	86	88	79	85
d	(m)	4.7	19	3.3	7.5
Raffinate stream	($m^3 \cdot h^{-1}$)	1.3	1.9	0.6	0.8
Desorbent stream	($m^3 \cdot h^{-1}$)	1.4	2.3	0.7	0.9
Extract stream	($m^3 \cdot h^{-1}$)	0.8	4.3	0.4	0.7
Product purity	%	91	40	98	93
Product recovery	%	98	50	97	97
Product productivity	($kg \cdot m^{-3} \cdot h^{-1}$)	2.6	0.08	5.2	1.0
Product dilution	(-)	2.1	1.0	1.9	1.3
Substrate purity	%	99	97	95	98
Substrate recovery	%	96	96	96	96
Substrate productivity	($kg \cdot m^{-3} \cdot h^{-1}$)	6.1	3.0	2.8	1.7
Substrate dilution	(-)	1.2	1.1	1.3	1.2

Oligosaccharides consist of at least n sugar monomers. For n=2: a=monosaccharides and b=disaccharides and higher. For n=3: a= mono and disaccharides, b= trisaccharides and higher, F and c_{in} according to table 3.

Optimising SMB dimensions

The system with galactose as a reactant in combination with the separation of monosaccharides from the reaction mixture is interesting since there are no substrate losses and a specific glucose free galacto-oligosaccharide mixture can be obtained. We have used this system to examine further optimisation options.

In the galactose-based process a column diameter of 4.7 meters is needed and the time between column shifts is about two hours. In other applications the switch time is in the order

of minutes. These results are due to the high feed flow and the unfavourably low product concentration. Although in practice columns with these diameters are possible (Bieser & de Rosset, 1977), it can more effective to use several SMB's with a more standard geometry. For this we chose 1.6 meter as a diameter, being a standard size delivered by Novasep (Novasep, 2002). This way 3 SMB's in series or 9 parallel SMB would be necessary to maintain product purity (see table 6). The use of SMB units in parallel lowers column diameters, solely due to the decrease in feed per unit. Using several SMB units in series leads to a decrease in diameter due to easier oligosaccharide purification, which is then done in multiple steps. This will however lead to an eight times more dilute product. The best option is probably to use longer columns. This will also decrease the diameter of the columns. For a column diameter of 1.6 meter, the columns have to be enlarged to 8.5 meter. When a 12-column set-up is used in stead of 4 columns as used in the calculations, this will lead to a column height of 2.8 meter. Similar installations are used in the separation of fructose and glucose.

Table 6 Process parameters for different configurations with several SMB units in series or parallel. Galactose condensation products are disaccharide and higher

		1 SMB	9 SMB's parallel	3 SMB's serie	1 SMB 8.5 m long
Parameters					
Feed stream	($m^3 \cdot s^{-1}$)	$1.8 \cdot 10^{-4}$	$2.1 \cdot 10^{-5}$	$1.8 \cdot 10^{-4}$	$1.8 \cdot 10^{-4}$
L_c	(m)	1	1	1	8.5
Optimisation Results					
t_{switch}	(s)	$8.6 \cdot 10^{3}$	$8.6 \cdot 10^{3}$		$8.5 \cdot 10^{3}$
St_{mono}	(-)	172	172		170
St_{di}	(-)	86	86		85
d_c	(m)	4.7	1.6	1.6	1.6
Raffinate stream	($m^3 \cdot h^{-1}$)	1.3	0.14	5.4	1.3
Desorbent stream	($m^3 \cdot h^{-1}$)	1.4	0.15		1.4
Extract stream	($m^3 \cdot h^{-1}$)	0.8	0.09		0.8
Product purity	%	91	91	93	91
Product recovery	%	98	98	48	98
Product productivity	($kg \cdot m^{-3} \cdot h^{-1}$)	2.6	2.6	3.7	2.6
Product dilution	(-)	2.1	1.9	17.6	2.1
Substrate purity	%	99	99		99
Substrate recovery	%	96	96		96
Substrate productivity	($kg \cdot m^{-3} \cdot h^{-1}$)	6.1	6.1		6.1
Substrate dilution	(-)	1.2	1.3		1.2

c_{in} according to table 3, parameters according to table 5

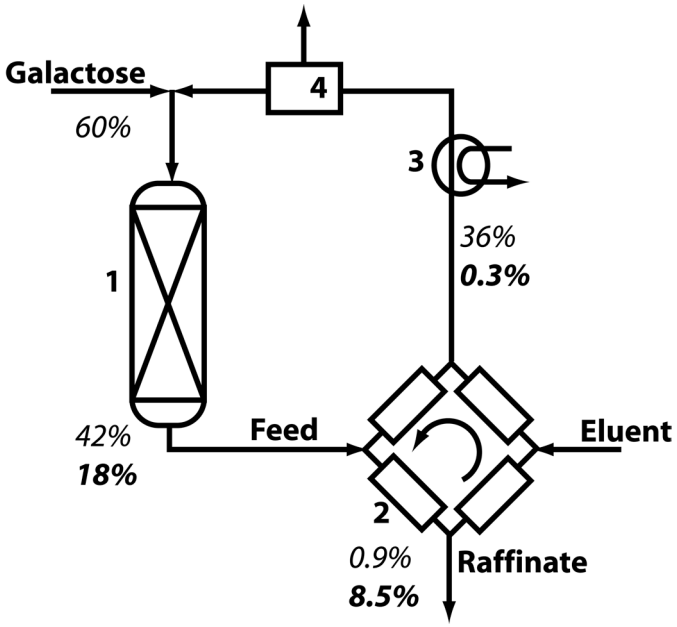

Figure 6 Schematic presentation of the oligosaccharide production process from galactose, using SMB for the separation of the product from (reusable) substrate. The whole process is operated at 80°C and β-glycosidase from *P. furiosus* is used as a biocatalyst in the conversion. ***Product*** concentrations in bold italic, *substrate* in italic, 1: reactor, 2: SMB unit, 3: heat-exchanger, 4: flash evaporator.

Overall process design

Based on the results discussed above a process scheme can be made. The enzymatic condensation of galactose yields a galactose-oligosaccharide mixture that can be separated by SMB. This is depicted in figure 6. The extract stream is fed back into the enzymatic reactor, after removal of some of the water (0.2 $m^3 \cdot h^{-1}$) e.g. by evaporation, and the addition of 130 $kg \cdot h^{-1}$ (solid) galactose. The water that is removed can be used for heating and as desorbent for the SMB. The raffinate contains the 91% (w/w_{sugar}) pure product. The concentration is 8.5% ($w/w_{mixture}$). It might be necessary to further concentrate the product and remove other, non-sugar, impurities. For instance in sugar beet processing, the sugar extraction is followed by demineralisation by anion and cation exchange chromatography and decolourisation by chromatography on activated carbon. The final product is then sterilised, concentrated and spray-dried (de Leenheer, 1996).

Further optimisation of SMB

Cost effectiveness of the SMB separation can be estimated by comparison with a glucose-fructose separation, since equipment and adsorbents costs in this type of purification are roughly the same. Productivities of this system are 8.5 times higher (22 $kg \cdot m^{-3} \cdot h^{-1}$ (Beste *et*

al., 2000)) than in our galacto-oligosaccharide separation. This is mainly due to a much larger difference between the equilibrium constants for glucose and fructose on the applied resin, compared to the equilibrium constants in our system. Selection of a better adsorbent is necessary for further optimisation of the galacto-oligosaccharide production system. In the glucose-fructose separation, the higher product concentration in the feed also contributes to a higher productivity.

For this study, we used a common adsorbent that is readily available (Dowex X4). However, the SMB process can be further optimised by selecting a better ion-exchange material. Resins with smaller pore sizes such as Dowex X8 might lead to a better separation of monosaccharides from disaccharides since its pore size is better suitable for this specific separation. For the separation between di- and trisaccharides Dowex X2 may be better suited. It is also possible to choose a different adsorbent, like Diaion UBK-350 (Kim *et al.*, 1992). When the adsorption equilibrium constants for this material are used in the calculations for the separation of mono and disaccharides the size of the SMB unit reduces considerably from 278 m^3 to 163 m^3.

A large decrease in the size of the separation unit can be obtained further when less pure products are required (figure 7). Decreasing the purity with only 1% from 91 to 90% (w/w_{sugar}) decreases the size of the SMB unit from 278 to 222 m^3. A 100 m^3 SMB unit will still give an 85% product purity. Figure 7 shows that for a product purity of 30%, the SMB size becomes zero, this is the concentration in the SMB feed.

Nanofiltration membranes as an alternative for oligosaccharide separation

The alternative to SMB separation would be nanofiltration. Goulas *et al.* (2002) recently evaluated nanofiltration membranes for the separation of oligosaccharides. We applied their results to our system. For the DS-5-DL nanofiltration membrane they measured rejection values of 0.99 for raffinose (a trisaccharide); 0.94 for sucrose and 0.28 for glucose. We used the feed flow from the galactose condensation reaction with the requirement that the retentate should be at least as pure as the one coming from the SMB (91% (w/w_{sugar})).

Based on this information a scheme for a process system could be made. Calculations with varying numbers of membrane units showed that two membranes were sufficient to separate the galactose-oligosaccharide mixture. This is depicted in figure 8.

In the substrate stream it is possible to use a 60% ($w/w_{mixture}$) sugar stream at 80°C. However the product itself is less soluble. This is not a problem in SMB separation, since the product stream is diluted. When using membranes the product is in the retentate and therefore concentrated in each step. To prevent the sugar mixture from crystallising, the sugar stream is diluted before separation. When a maximal final sugar concentration of 60% ($w/w_{mixture}$) is chosen, the feed stream has to be diluted almost 15 times. The oligosaccharide concentration in the retentate is then 55% ($w/w_{mixture}$).

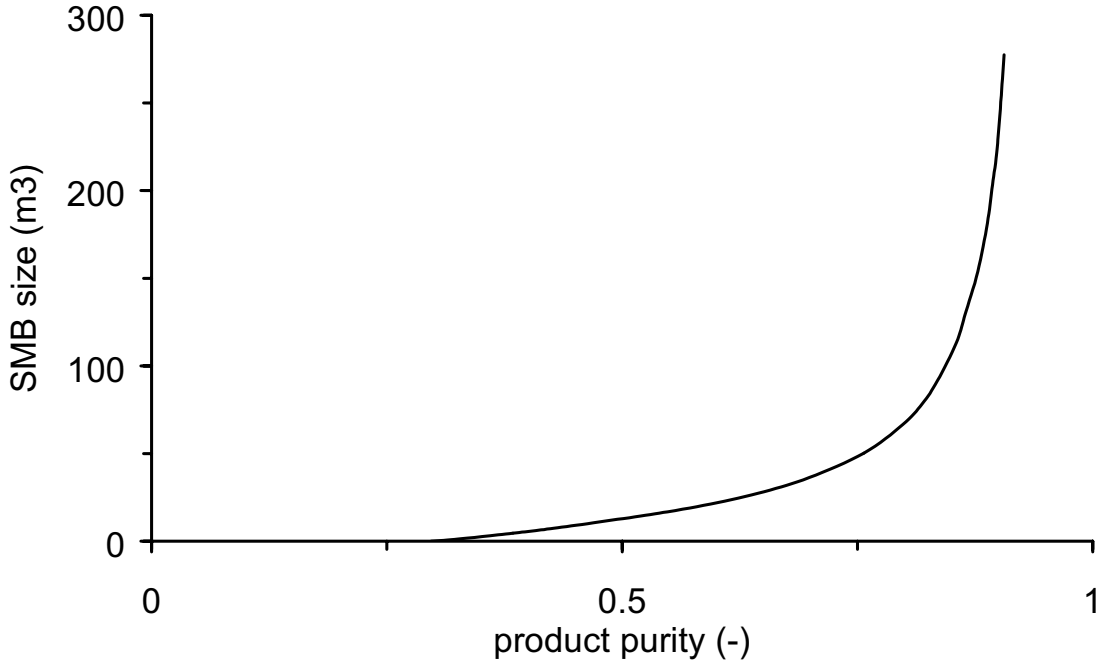

Figure 7 SMB unit size dependence on product purity. Note that the feed value corresponds to a value of 0.3. Therefore at this value, the required SMB size is reduced to zero.

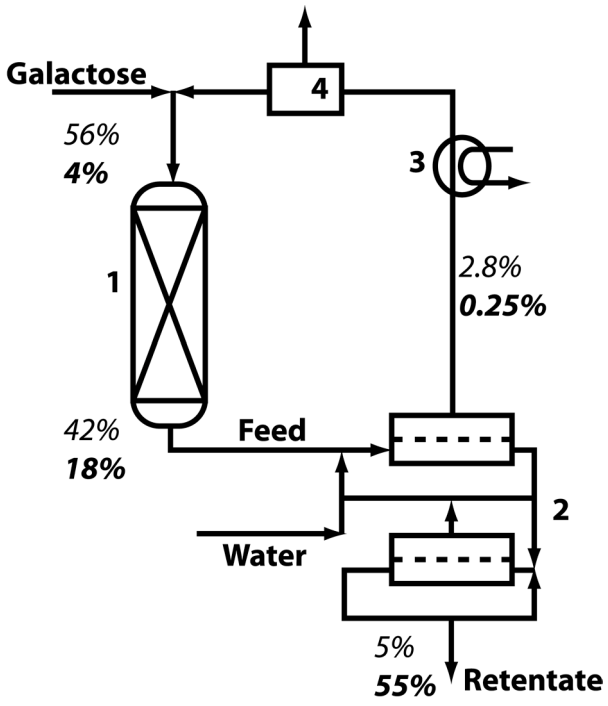

Figure 8 Schematic presentation of the oligosaccharide production process from galactose, using membranes for the separation of the product from (reusable) substrate.The whole process is operated at 80°C and β-glycosidase from *P. furiosus* is used as a biocatalyst in the conversion. ***Product*** concentrations in bold italic, *substrate* in italic, 1: reactor, 2: membrane units, 3: heat-exchanger, 4: flash evaporator.

Since the recovery of oligosaccharides in the final product is in this case only 78% (w/w_{sugar}), the productivity from the enzymatic reactor had to be increased to meet the annual assumed oligosaccharide production. Thus, the reactor volume has to be somewhat larger and subsequently the feed flow to the membrane separation unit increases. A third membrane unit that separates the permeate from the first membrane unit and recycles this retentate will increase the oligosaccharide recovery, but more membrane surface will be needed. The dilution of the feed results in a dilution of the permeate. This stream has to be concentrated before it can be re-used in the enzymatic reactor. For this a large part of the water (11.5 $m^3 \cdot h^{-1}$) has to be removed.
Using the values for the permeate flow (360 $g \cdot m^{-2} \cdot h^{-1}$) as given by Goulas *et al.* (2002), we find that a $6.8 \cdot 10^4$ m^2 surface area is needed for separation. If we assume that the membranes are capillary membranes with a radius of 2 mm, the surface to volume ratio is 900 $m^2 \cdot m^{-3}$, leading to a 76 m^3 separation unit.

Conclusion

In this chapter we reported a production process for a high-purity product of specific galacto-oligosaccharides based on galactose as a reactant and separation via SMB. The product is unique in the fact that it does not contain any traces of glucose and hardly any galactose. Commercial galacto-oligosaccharide products, produced with lactose as a reactant, often contain large amounts of non-prebiotic sugars like glucose, galactose and lactose. When more specific prebiotics are required this galactose-based process can be an alternative. The product separation is comparable to the lactose based process; however, the increase in reactor size from 3 m^3 for a lactose based process to over 100 m^3 may imply significant higher costs of the process.
Several methods for the production of galacto-oligosaccharides were tried and dependent on the required purity of the product, a suitable synthesis reaction with separation can be chosen. When product quality is not important, a fast and cost effective method is the production of transgalacto-oligosaccharides from lactose without any purification. When a more pure oligosaccharide product is required, condensation from galactose with an SMB separation can be used.
The main problem in an oligosaccharide production process based on nanofiltration is the dilution that is required and consequently the high costs of subsequent water removal. The amount of water that needs to be removed in the permeate is over 50 times as high as in the extract in the process with an SMB separation unit. An advantage is that the product itself is highly concentrated. The product stream from the SMB is 6.5 times more diluted. Although the costs of water removal are substantially, the membrane process is suited for product separation and has a smaller system volume when compared to an SMB system.

Notation

a_v	specific surface area ($m^2 \cdot m^{-3}$ bed)
c	fluid phase concentration ($g \cdot l^{-1}$)
d	column diameter (m)
D	desorbent flow in moving bed ($m^3 \cdot s^{-1}$)
E	extract flow in moving bed ($m^3 \cdot s^{-1}$)
F	feed flow ($m^3 \cdot s^{-1}$)
P	permeate flow in membrane unit ($m^3 \cdot s^{-1}$)
K	equilibrium constant (-)
K_f	overall mass transfer coefficient ($m \cdot s^{-1}$)
L	liquid flow in a true moving bed ($m^3 \cdot s^{-1}$)
L_c	column length (m)
q	solid phase concentration ($g \cdot l^{-1}$)
R	raffinate flow in moving bed, retentate flow in membrane unit ($m^3 \cdot s^{-1}$)
R_m	rejection: 1- c_p/c_f (-)
S	solid flow in a true moving bed ($m^3 \cdot s^{-1}$)
St	Stanton number (-)
t	time (s)
t_{switch}	time interval between column shifts (s)
u	solid interstitial velocity ($m \cdot s^{-1}$)
v	fluid interstitial velocity ($m \cdot s^{-1}$)
V	column volume (m^3)
Z	dimensionless axial distance (-)

Greek letters

α	safety margin (-)
ε	bed porosity (-)
γ	ratio of downflow in solid to upflow in fluid: KS/L (-)

Subscripts

1,2,3,4	referring to TMB zones
a	more retained component
b	less retained component
e,f,r,p	referring to effluent, feed, raffinate (or retentate) and permeate
i	section

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Chapter 6

Discussion

Introduction

The goal of the research reported in this thesis was to develop a process concept for the tailor made production of oligosaccharides. Our main interest was in a production process at elevated temperatures. This can have many advantages (chapter 2), amongst which is the possibility to increase the substrate concentration. With this we were able to increase oligosaccharide yields substantially (chapter 3). Unfortunately, the higher temperatures also led to faster enzyme inactivation, not only because of the direct denaturation of the enzyme due to temperature, but also because of the occurrence of Maillard reactions (chapter 4). Integration of the reaction with an adequate separation was essential to produce a pure oligosaccharide product. This was studied in detail in chapter 5.
In this chapter we will review various aspects of the process and identify aspects that need further study. Two important steps to process improvement will be discussed; using more extreme reaction conditions, e.g. high temperatures and high sugar concentrations, and using various routes towards (in-line) product isolation. Finally, a remark on the production of specific oligosaccharides will be made.

Enzyme inactivation during monosaccharide condensation

Although we did not measure actual inactivation constants during monosaccharide condensations there are indications that the enzyme inactivation during this reaction at elevated temperatures is considerable compared to enzyme inactivation during lactose transglycosylation.
First indications came from initial rates experiments with monosaccharides (chapter 3). Attempts to measure condensation rates at low substrate concentrations failed because synthesis was too slow to be measured without taking enzyme inactivation into account. However, it is difficult to differentiate between an increase in enzyme inactivation or strong substrate inhibition.
The browning of the reaction mixture and the related extra increase in enzyme inactivation due to Maillard reactions were described for lactose conversions in chapter 4. We mentioned that in the Maillard reaction mixtures of proteins with disaccharides brown more slowly than mixtures of proteins with monosaccharides, since these sugars are less present in the open chain form (Naranjo *et al.*, 1998). Browning is therefore more pronounced with processes based on galactose or glucose and enzyme inactivation might be more severe. Enzyme inactivation during monosaccharide condensation might be a problem in a continuous process.

The Maillard reaction

Process optimisation at higher process temperatures is possible by using a thermophilic enzyme. The only disadvantage of the use of elevated temperatures is an increase in enzyme inactivation due to Maillard reactions. Maillard reactions can be minimised by an optimisation in reaction conditions (chapter 4). Research into the molecular mechanism of this inactivation will hopefully lead to a better understanding on how the inactivation takes place. Present studies on Maillard reactions focus on the structural consequences of protein modification. Protein digestion and mass spectrometry reveal the modified amino acids (Chevalier *et al.*, 2001). In enzymes also stability and functionality can be influenced. Combining information might make directed design of a more stable enzyme possible. Our results indicated that the enzyme was already inactivated at relatively low Maillard conversions. This suggests that obtaining better resistance against Maillard inactivation through protein modification may be important.

Influence of temperature on enzyme kinetics and implications for the overall process

Several aspects will influence the oligosaccharide synthesis reaction at higher temperatures. There is an effect on the equilibrium of the reaction itself, an effect on the enzyme kinetics and an effect on sugar solubility. These effects were quantified to evaluate their effect on the production process of the oligosaccharides.

Monosaccharide condensations can be either endothermic or exothermic reactions, but generally, enthalpy changes are relatively small in the order of a few (0.5-4) $kJ \cdot mol^{-1}$ (Goldberg *et al.*, 1991; Tewari & Goldberg, 1991; Goldberg & Tewari, 1989; Tewari & Goldberg, 1989), therefore the direct influence of temperature on the equilibrium position of the reaction is also small. An enthalpy change of -4 $kJ \cdot mol^{-1}$ increases the oligosaccharide yield by 15% when the temperature increases from 60 to 95°C (calculated with the van 't Hoff isochore).

An additional effect takes place in the kinetically controlled transglycosylation from disaccharides. The relative rates of transglycosylation and hydrolysis determine the maximum oligosaccharide yield possible. Temperature affects these rates to a different extend. In the case of lactose conversion by the β-glycosidase from *Pyrococcus furiosus*, a temperature rise had a more positive effect on the transglycosylation reaction than on hydrolysis. A temperature increase from 60 to 95°C, shortened the reaction times from over 2 days to 6 hours and it also increased the oligosaccharide yield by 22% (see chapter 4).

By rising the temperature it is possible to increase the substrate concentration. The solubility of e.g. glucose increases from 75 to 85% (w/w) with a temperature rise from 60 to 95°C. This has an influence on both kinetically controlled reactions, as on equilibrium reactions. Assuming the equilibrium constant to be 1.45 for glucose condensation reactions at 80°C (as

described in chapter 3), the equilibrium oligosaccharide yield increases from 0.5 to 0.9 mol·kg^{-1}, an increase of 80%. The solubility of lactose increases roughly from 37 to 58% (w/w) with a temperature rise from 60 to 95°C. Using the model from chapter 3 to calculate the maximal oligosaccharide concentration at the kinetic optimum, the oligosaccharide yield increases from 0.22 to 0.48 mol·kg^{-1}, which is a 114% increase in oligosaccharide yield.
The effect of an increase in substrate concentration with higher temperatures is the most important aspect of the ones mentioned and an important improvement to the process. The effects of temperature on reaction equilibria and on enzyme kinetics can be positive or negative and are relatively small.

Other options for more substrate or less water

In general, substrate concentrations close to the maximal solubilities of the components were chosen to get the highest substrate concentrations and subsequently as little water as possible. A further increase in substrate concentration by using saturated solutions or a decrease in water concentration by the use of other solvents may look attractive.
It is possible to use saturated solutions with crystalline substrate present as reaction mixture. Reaction of monosaccharides can be followed by the simultaneous dissolution from the crystalline phase, thus ensuring a constant, maximal substrate concentration. In addition, it is possible to have a continuous removal of the product by precipitation when the equilibrium constant is sufficiently high compared to the product solubility. This results in a continuation of the reaction. (Ulijn *et al.*, 2001) However, the equilibrium for glucose and galactose condensation is mostly to the side of hydrolysis and more importantly their products are highly soluble. Therefore precipitation driven synthesis will not occur easily and this concept does not seem feasible for the enzymatic production of oligosaccharides in aqueous solutions. Studies reporting (trans)glycosylation in saturated solutions either use a combination of a sugar with an aglycon, like glucose and ethylene glycol (MillqvistFureby *et al.*, 1998a) or hexanol (de *et al.*, 2001) or use modified sugars, e.g. in transglycosylation with artificial substrates (MillqvistFureby *et al.*, 1998b).
Another method to draw the reaction to the side of condensation is to replace the water by alternative solvents. However, these solvents usually give a reduced solubility of both product and substrate, which reduces the overall productivity. Solvents giving higher solubilities, like glycerol and alcohols, can be glycosylated by the enzyme, leading to unwanted side-products. Recently, ionic liquids have been tried for transgalactolysation (Kragl *et al.*, 2002), which may offer an interesting route in the future. They offer higher substrate solubilities, but can not be used as a substrate. However the development of food-grade ionic liquids is still in its infancy.

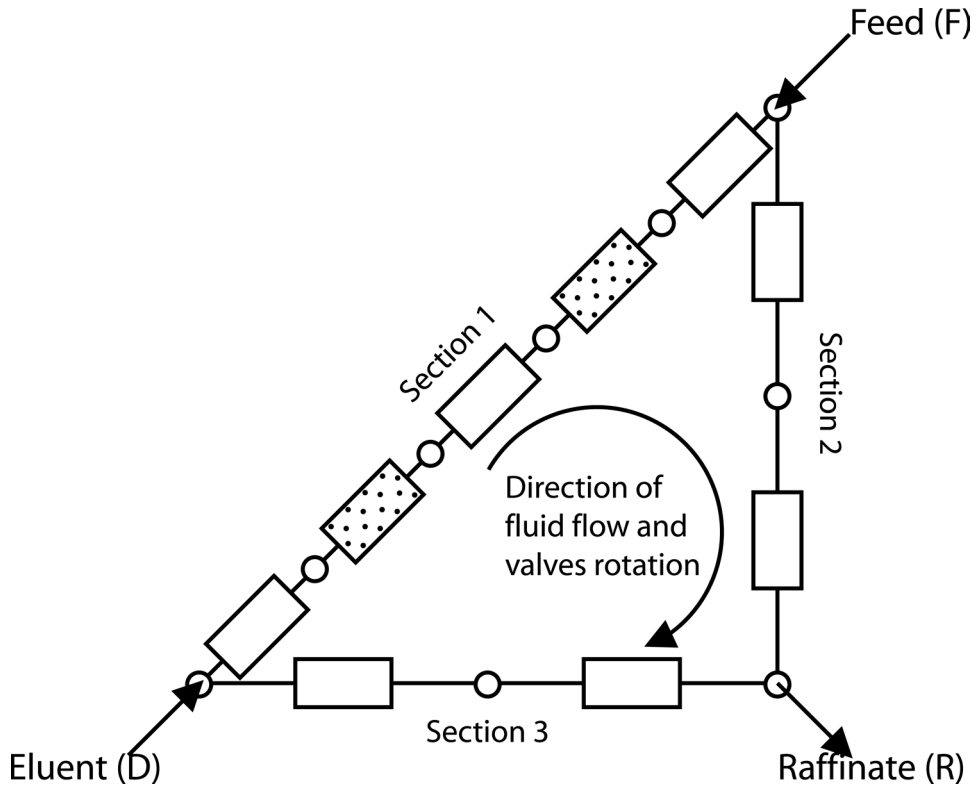

Figure 1 Representation of a simulated moving bed (SMB) with integrated columns with immobilised enzyme for the production of oligosaccharides. Clear columns for adsorption, dotted columns with immobilised enzyme, no adsorption. The columns with immobilised enzyme do not rotate and stay in section 1.

In-situ product removal

In-situ product removal offers the benefits of higher conversions and a continued use of the enzyme. It also prevents hydrolysis of the formed product (Schroen *et al.*, 2002). One route to in-situ product removal is the use of specific complexants. However, current complexants or adsorbents, like activated carbon are not very specific. Development of complexants, like antibodies (Vyas *et al.*, 2002) or lectins (Endo, 1996), that can specifically bind to one type of sugar instead of to a range of sugars, would enable the removal of a specific product, thereby forcing the reaction to produce more of this specific compound.

Another way to achieve in-situ product removal is the use of supersaturated solutions, as explained in the previous section. This is a clean physical method that would be preferential to chemical complexation, since no complexing agent is needed and subsequently no decomplexation of product and the complexing agent is necessary.

Nanofiltration and chromatographic methods can both be used for separation as discussed in chapter 5. However, in these sugar separation processes product removal is done in a separate unit.

In nanofiltration the product is the larger molecule when compared to the substrate and therefore always in the retentate. It is not possible to retain both the monosaccharides and the enzyme. The enzyme is excluded from the separation process by immobilisation or by use of an ultrafiltration membrane before the actual product separation takes place.
In chromatography, it should be possible to integrate the separation in the reaction. The integration of an enzymatic reaction in an SMB system was shown to be successful for glucose-fructose separations, with a glucose isomerase for the enzymatic conversion of glucose to fructose (Adachi, 1994). An integrated process, applicable to our system is drawn schematically in figure 1 (normal SMB in figure 2 chapter 5). The enzyme should be immobilised at the point in the SMB where the monosaccharide concentration is relatively high and the product concentration low (section 1). This shifts the reversible condensation reaction toward the favourable oligosaccharide product. The columns with the immobilised enzyme do not rotate like the columns for separation, buy remain in section 1.

Complete use of substrate in oligosaccharide production from lactose

Current commercial oligosaccharide production processes are based on lactose as a substrate, combining high conversion rates with a high oligosaccharide yield. The process as described in chapter 5 is based on galactose. This condensation reaction is slower with a much lower yield. Also substrate costs are higher for a galactose-based process than for a lactose-based one. The advantage of the condensation reaction is the lack of substrate loss. It is interesting to combine this efficiency of the equilibrium-controlled reaction with the speed of a kinetically controlled reaction. Possible systems for lactose-based oligosaccharide production with complete substrate use are schematically drawn in figures 2 and 3. In figure 2 a system for the production of disaccharide and higher oligosaccharides is depicted, while figure 3 represents the production of trisaccharides and higher.
For the calculations of the system size and sugar concentrations the parameters from chapter 5 (table 3 and 5) were used; the monosaccharide concentration in the raffinate was minimised. Monosaccharide condensation and separation is different from the galactose-based process in chapter 5, since also glucose is present. Initial synthesis of oligosaccharides from glucose is faster than from galactose, but equilibrium concentrations are in the same order (chapter 3). In mixtures of these, both sugars are used in the condensation reaction. The equilibrium adsorption constants in the SMB for glucose mixtures are comparable to those of galactose mixtures (table 4, chapter 5). Separation in this case is more complex, since glucose and its condensation products are more retained on the column material than the components from the galactose mixture. In practice this means that there is more overlap between mono, di and trisaccharide peaks. For the calculations presented here, the adsorption constants from the galactose experiments were taken.

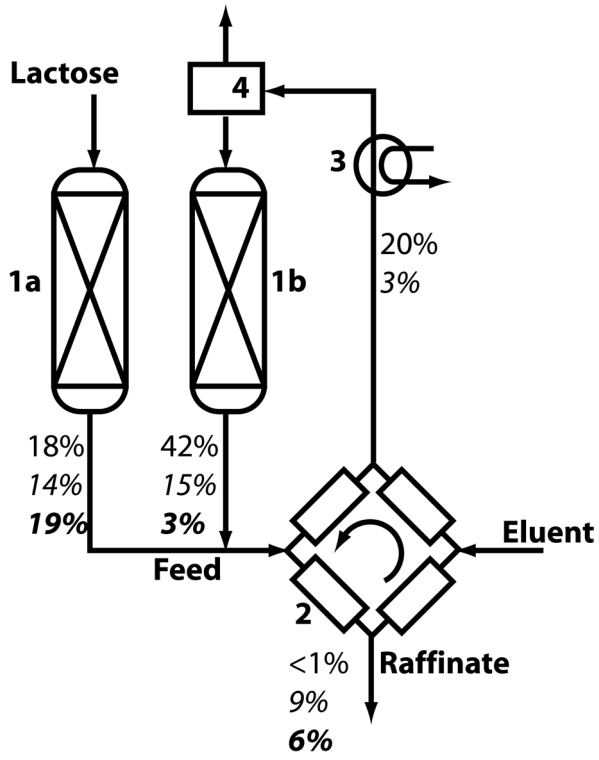

Figure 2 Schematic presentation of the oligosaccharide production process from lactose with SMB units for separation, when both di- and trisaccharides are the required product. Monosaccharide concentration in normal font, *disaccharide* concentration in italic, ***trisaccharide*** concentration in bold italic, 1a: transglycosylation reactor, 1b: condensation reactor, 2: SMB, 3: heat exchanger, 4: flash evaporator.

Production from lactose can be done with one separation step when disaccharides are included in the desired oligosaccharide product (figure 2). In this separation the monosaccharides are separated from the oligosaccharides and recycled to a second, condensation reactor. Some of the water in the recycle stream has to be removed to obtain a 60% ($w/w_{mixture}$) sugar mixture in the condensation reactor. The produced oligosaccharides, 15% ($w/w_{mixture}$), are in the raffinate. Only small amounts of monosaccharides are present (<1% ($w/w_{mixture}$)) giving a 95% (w/w_{sugar}) pure product. Due to the complete use of substrate the lactose reactor is reduced from 2.8 m^3 (chapter 5) to 1.8 m^3. Also the feed to the separation unit is reduced considerably. However, the recycle from the condensation reactor increases the feed flow for the SMB unit and at the same time lowers the product concentration in the feed resulting in an increase in the SMB unit size. The diameter of the SMB unit increases from 3.3 in a lactose based system without recycle (chapter 5) to 3.6 meter in a system with recycle. The recycle of monosaccharides does not reduce the total volume of the system and is therefore undesirable. It is more feasible to regard the monosaccharides stream from the SMB as a side-product as done in chapter 5.

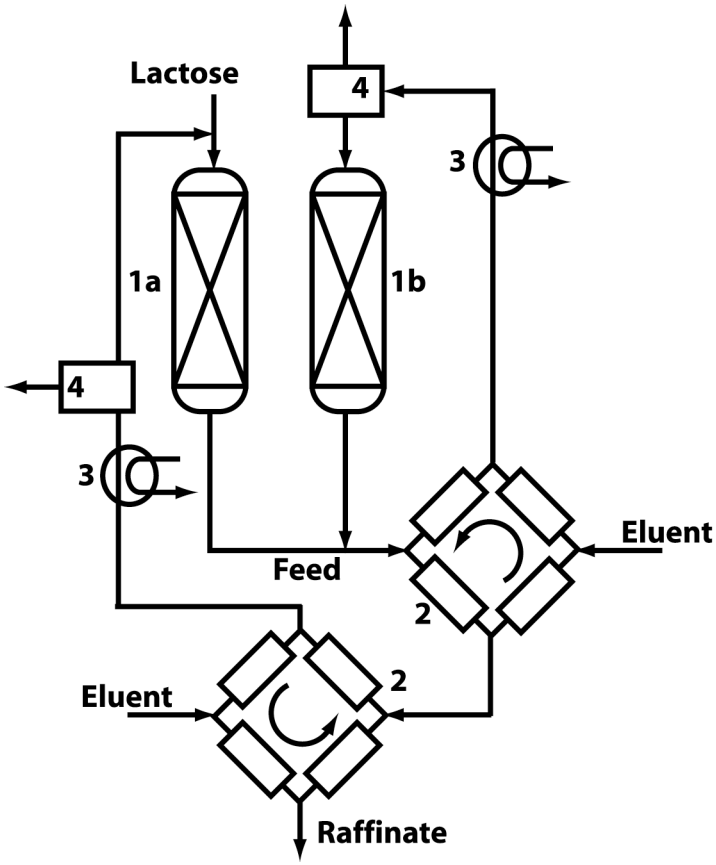

Figure 3 Schematic presentation of the trisaccharide production process from lactose with SMB units for separation. 1a: transglycosylation reactor, 1b: condensation reactor, 2: SMB, 3: heat exchanger, 4: flash evaporator.

The production of trisaccharides from lactose has to be done in two separation steps (figure 3). Research by Nicolaos *et al.* (2001) showed that the most difficult separation has to be performed by the second SMB. Therefore the first SMB is used to separate the monosaccharides that are a side-product from the transglycosylation reaction with lactose. The monosaccharides are fed to the condensation reactor, that is a recycle for this SMB. The oligosaccharides are further purified in a second SMB to yield the desired trisaccharides and higher. The results indicated that the recycling streams are too large when compared to the inflow of lactose (estimated at over 10 times). The size of the second SMB unit will be large since the separation between di and trisaccharides is relatively difficult. From the previous system we could already conclude that also the size of the first SMB would be larger than when a stream of monosaccharides as by-product is allowed. Also here, production without a recycle is therefore more cost-effective than a complete use of substrate.

Making more complex oligosaccharides

Current production processes make use of inexpensive sugar substrates and glycosidases are used as a biocatalyst. Finding new combinations of enzymes that are able to work together or

new combinations of substrates may enable the production of longer and/or more complex oligosaccharides. A successful new combination of substrates is in the production of lactosucrose from sucrose and lactose by β-fructofuranosidase (Fujita *et al.*,). Combinations of enzymes are not often used to make more complex oligosaccharides. In our experiments we attempted to find a combination of enzymes that could yield more complex or longer oligosaccharides. Combinations of the following enzymes were tested: β-glycosidase from *Pyrococcus furiosus*, β-galactosidase from *Aspergilles oryzae* and *Bacillus circulans*, β-glucosidase from almonds, amyloglucosidase from *Aspergillus niger*, α-galactosidase from *Bifidobacterium adolescentis* and α-glucosidase from yeast. Glucose or galactose were used as substrate. However, in all cases no additional products could be detected by combination of the enzymes.

Specific oligosaccharide synthesis reactions can be achieved by using e.g. glycosynthases instead of glycosidases for tranglycosylation (Moracci *et al.*, 2001), but this is not yet cost effective. The main bottleneck in making more complex oligosaccharides is the lack of diversity of the available catalysts. However, once more suitable catalysts are identified to produce more complex oligosaccharides, they can be readily implemented in a production process as described in this thesis.

Conclusions and recommendations

In this chapter we have reviewed various aspects of the process that merit further study.

The goal of the research described in this thesis was the design of an enzymatic production process for specific oligosaccharides. We were able to produce oligosaccharides from different substrates, using different approaches. The kinetically controlled transglycosylation of disaccharides proved to be fast and with high yield. The equilibrium-controlled condensation from monosaccharides is useful when more specific oligosaccharides have to be produced. Both reactions did benefit from higher substrate concentrations. The use of a thermophilic enzyme to facilitate a process at higher temperatures proved to be useful. Major further improvements on the synthesis part of the research are probably not feasible and further optimisation has to be found in optimising the downstream processing.

The major disadvantage of the high temperature process is the increased enzyme inactivation due to Maillard reactions. This can be a problem in continuous operation of the process. Further studies have to generate insight in what is happening on a molecular level to dissolve the problems on enzyme stability.

The separation of oligosaccharides was possible via ion-exchange chromatography and could be performed continuously in an SMB. This enabled the re-use of substrate and the production of high-grade oligosaccharides. Ideally separation takes place in-situ and the oligosaccharides are removed, pulling the reaction towards production. Specific in-situ removal of the produced oligosaccharides will further improve the process.

Acknowledgement

The authors thank Julia Meijer for performing the studies on enzyme combinations.

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Summary

The goal of the research reported in this thesis was to develop a process concept for the tailor made production of oligosaccharides. These specific non-digestible oligosaccharides can be used as prebiotics. They promote the growth of beneficial bacteria in the gastrointestinal (GI) tract. Commercial prebiotic oligosaccharides are often not pure oligosaccharides, but mixtures. In this thesis focus is on the production of oligosaccharides of higher purity.

Our main interest was in a production process at elevated temperatures. This can have many advantages, amongst which is the possibility to increase the substrate concentration. We used a thermophilic β-glycosidase from *Pyrococcus furiosus*. Enzymes from thermophilic microorganisms have unique characteristics such as high temperature-, chemical- and pH stability. Applications with thermophilic enzymes are summarised in chapter 2. The main advantages of performing processes at higher temperatures are the reduced risk of microbial contamination, lower viscosity, improved transfer rates and improved solubility of substrates. However, co-factors, substrates or products might be unstable or other side reactions may occur.

One route of oligosaccharide production is the synthesis from monosaccharides or disaccharides, using glycosidases as a catalyst. Monosaccharides can be condensated to disaccharides and disaccharides can be transglycosylated to trisaccharides. To investigate the potential of this synthesis with β-glycosidase from *P. furiosus* we determined kinetic parameters for substrate conversion and product formation from cellobiose, lactose, glucose and galactose. The obtained parameters for initial rate measurements of disaccharide conversion were also used for the interpretation of experiments in time. The model for cellobiose gave a good description of the experiments. The enzyme was found to be uncompetitively inhibited by cellobiose and competitively inhibited by glucose. Lactose conversion however, could not be modelled satisfactorily; apparently additional reactions take place. Monosaccharide condensation also yielded oligosaccharides, but much slower. The use of a hyperthermostable enzyme was found to be positive. More substrate could be dissolved at higher temperatures, which benefited all reactions. This research is described in chapter 3.

Besides the advantage of higher substrate solubility, temperature also influences enzyme kinetics. In chapter 4, the thermostable *P. furiosus* β-glycosidase was applied for oligosaccharide production from lactose in a kinetically controlled reaction. The experiments showed that higher temperatures are beneficial for the absolute as well as relative oligosaccharide yield.
However, at reaction temperatures of 80°C and higher, the inactivation rate of the enzyme in the presence of sugars was increased by a factor 2, compared to the inactivation rate in the

absence of sugars. This increased enzyme inactivation was caused by the occurrence of Maillard reactions between the sugar and the enzyme. The browning of our reaction mixture due to Maillard reactions was modelled by a cascade of a 0^{th} and 1^{st} order reaction and related to enzyme inactivation. From these results we conclude that modification of only a small number of amino-groups already gives complete inactivation of the enzyme.
Reduction of Maillard reactions can be done by altering process conditions or through modification of the enzyme, either chemically or by altering the enzyme structure through genetic modifications. Chemical modification of the enzyme was studied. The enzyme was covalently immobilised on Eupergit. Unfortunately, the immobilisation did not reduce Maillard reactivity.

Further reaction optimisation required a down-stream processing method for oligosaccharide separation. This was also essential for the production of a pure oligosaccharide product. Two methods for oligosaccharide purification are described in chapter 5.
Oligosaccharides were produced in a condensation reaction using the β-glycosidase from *P. furiosus*. With a 60% (w/w) galactose solution as the substrate and oligosaccharide yield of 18% (w/w) was obtained. The feasibility of a Simulated Moving Bed (SMB) for downstream separation was investigated by modelling. The required parameters were determined experimentally with column experiments. The components could be separated with an SMB into a 91% pure product stream and a 99% pure galactose stream. This galactose stream can be recycled to the enzyme reactor. Also nanofiltration can be used for oligosaccharide purification. This system was also modelled and the results were compared to those that can be achieved with SMB.
It is also possible to produce transgalacto-oligosaccharides in a more conventional way, with lactose as a substrate. Production is much cheaper when compared to a galactose-based process. Separation of oligosaccharides from this reaction via SMB was also studied.
The size of all separation units is still considerably large and further optimisation is necessary to make a process for the production of specific high purity galacto-oligosaccharides cost-effective.

Various aspects of the process are discussed further in chapter 6. Emphasis is on the specific influence of temperature on the process and on further optimisation of the downstream processing of oligosaccharides.

Samenvatting (voor iedereen)

In de darmen zitten veel bacteriën, meer dan tienduizend keer zo veel als er mensen zijn op aarde. Deze bacteriën kunnen goed of slecht zijn voor de mens. De groei van de goede bacteriën kan bevorderd worden door de inname van specifieke suikers die niet door het menselijk lichaam kunnen worden verteerd, maar die wel de darmflora kunnen stimuleren (prebiotica). Deze specifieke suikers die aan levensmiddelen toegevoegd kunnen worden zijn oligosachariden. Oligosachariden zijn suikers die opgebouwd zijn uit meerdere suiker eenheden (oligo=enkele, sacharide=suiker). Het doel van het onderzoek, zoals in dit proefschrift beschreven staat, was de ontwikkeling van een proces voor de enzymatische productie van oligosachariden.

We waren vooral geïnteresseerd in een productieproces bij hogere temperaturen. Dit heeft vele voordelen, waaronder de mogelijkheid van een verhoogde suikerconcentratie. Bij hogere temperaturen kan er namelijk meer suiker opgelost worden. Net zoals er in warme koffie of thee meer suiker oplost dan in koude. Dit is positief voor omzetting van het substraat in het oligosacharideproduct. Het substraat is de basisstof die moet worden omgezet, in ons geval een mono- (mono=een) of disacharide (di=twee). De omzettingsreactie verloopt onder normale omstandigheden (kamertemperatuur, neutrale zuurgraad, geen extra stoffen toegevoegd) uitermate traag. Om de reactie sneller te laten verlopen hebben wij een enzym toegevoegd. Een enzym is een eiwit dat zelf niet in de reactie verbruikt wordt, maar die de omzettingsreactie wel doet versnellen. Enzymen worden ook wel biokatalysatoren genoemd. Ze zijn afkomstig uit planten, dieren of micro-organismen. Om bij hogere temperaturen te kunnen werken gebruikten wij een enzym uit een warmteminnend (thermofiel) micro-organisme: *Pyrococcus furiosus*. Dit enzym werkt nog bij 100°C. We gebruikten een β-glycosidase, wat zowel in oligosacharide productie vanuit monosachariden als vanuit disachariden kan helpen. Het enzym zorgde ervoor dat monosachariden samengevoegd (gecondenseerd) konden worden tot een disacharide (di=twee). Vervolgens kan het enzym disachariden "transglycosyleren". Hierbij wordt er een suiker eenheid van het ene disacharide op het andere disacharide overgezet, wat een trisacharide (tri=drie) en een monosacharide oplevert. Deze langere suikers, zoals trisachariden, worden oligosachariden genoemd. De hogere substraatconcentraties en de hogere temperatuur verhoogden de oligosacharideopbrengst aanzienlijk.

Helaas veroorzaakten deze hogere temperaturen ook een versnelde enzyminactivatie. Dit gebeurde op twee manieren. Omdat enzymen eiwitten zijn kunnen ze onder invloed van bijvoorbeeld temperatuur hun structuur verliezen en ontvouwen. Denk hierbij aan het stollen van een ei als je het kookt. Dit wordt denaturatie genoemd en komt voor bij alle enzymen onder invloed van een (te) hoge temperatuur. De biologische functie van het enzym gaat dan verloren en de gewenste reactie blijft uit. Naast deze denaturatie door de temperatuur trad er ook nog een chemische reactie op die het enzym inactiveerde, de zogenaamde Maillard

reactie. De Maillard reactie komt voor in suiker-eiwit (in ons geval enzym) systemen bij hogere temperaturen en uit zich in de vorming van bruine pigmenten die het reactiemengsel kleuren. Deze reactie zie je ook bij het bakken van bijvoorbeeld vlees en brood. Met een spectrofotometer kan de bruine kleur gemeten worden. De mate van bruinkleuring is gekoppeld aan de mate van enzyminactivatie door tijd en temperatuur. Verschillende mogelijkheden om Maillard reacties te voorkomen zijn besproken.
Voor verdere optimalisatie is de zuivering van de oligosachariden noodzakelijk. Na de reactie is er namelijk nog steeds substraat aanwezig dat niet verder wordt omgezet. Overtollig substraat en product moeten dus gescheiden worden. Na scheiding kan het substraat hergebruikt worden.
Eén methode van scheiding is chromatografie. Hierbij worden kolommen gebruikt waarin een materiaal zit dat selectief bepaalde componenten tegenhoudt. In ons geval kunnen de oligosachariden veel sneller door de kolom heen dan het substraat. Dit verschil in snelheid wordt benut om beide componenten te scheiden. Aan het einde van de kolom wordt eerst het product en vervolgens het substraat opgevangen. Om dit proces continu te bedrijven zijn er meerdere kolommen nodig die om de beurt gebruikt worden. Het lijkt bij zo'n proces alsof het kolommateriaal (het "bed") beweegt, daarom wordt het een Simulated Moving Bed (SMB) genoemd. Dit proces is in de praktijk niet zo eenvoudig en relatief duur. Wij hebben experimenten gedaan op kleine schaal met één kolom en een wiskundig model gemaakt wat voorspelt hoe een groot SMB proces verloopt. Dit proces is vergeleken met een scheiding door een (nanofiltratie) membraan. Een membraan is een filter met gaatjes van een bepaalde grootte. Hierdoor zullen grotere moleculen (de oligosachariden) tegengehouden worden, terwijl andere kleinere moleculen (het substraat) wel doorgelaten worden. Met deze scheiding op grootte was het ook mogelijk de oligosachariden te zuiveren. Beide scheidingsmethoden zijn nu nog erg duur, omdat de benodigde apparatuur erg groot moet worden. Verdere optimalisatie is noodzakelijk om het proces winstgevend te maken.