Stellingen

1 Bij de enzymatische synthese van oligosacchariden is eigenlijk maar één ding van belang: het substraat lactose *(dit proefschrift)*.

2 Bonnin *et al.* (1996) bepalen ten onrechte de beste procescondities voor een kinetisch gecontroleerde reactie door het meten van de productopbrengst op een vast tijdstip.

Bonnin E, Thibault J-F. 1996. Galactooligosaccharide production by transfer reaction of an exogalactanase. Enzyme Microb Technol 19:99-106.

3 Bepaling van de kinetische parameters wordt veelal gedaan door het meten van initiële snelheden. Meer informatie wordt verkregen door het meten van de concentraties over een langere periode. Men moet dan niet vergeten dat nog steeds elk experiment één onafhankelijke waarneming is.

Bakken AP, Hill CG JR, Amundson CH. 1992. Hydrolysis of lactose in skim milk by immobilized ß-galactosidase (*Bacillus circulans*). Biotechnol Bioeng 39:408-417.

4 Parameters bepaald in een enkelvoudig adsorptie systeem moeten ook de adsorptie in een meervoudig systeem kunnen beschrijven. Het schatten van nieuwe parameters wijst op een onjuist model.

Lee JH, Song DI, Jeon YW. 1997. Adsorption of organic phenols onto dual organic cation montmorillonite from water. Sep Sci Technol 32:1875-1992.

5 Het publiceren van wetenschappelijke resultaten heeft soms iets weg van het herformuleren van eigen werk.

Bakken AP, Hill CG JR, Amundson CH. 1991. Use of novel immobilized ß-galactosidase reactor to hydrolyze the lactose constituent of skim milk. Appl Biochem Biotechnol 28/29:741-756.

Bakken AP, Hill CG JR, Amundson CH. 1992. Hydrolysis of lactose in skim milk by immobilized ß-galactosidase (*Bacillus circulans*). Biotechnol Bioeng 39:408-417.

6 De vereenvoudiging van de werkelijkheid in modellen leidt soms tot meer onduidelijkheid dan duidelijkheid.

7 Dyslexie is een moeilijk woord voor dyslexische mensen.

8 De kortste weg in Engeland is meestal ook de steilste weg.

9 Als je met meerdere mensen op een kamer zit zou lachen bij het lezen van de e-mail verboden moeten worden. Ook als het mailprogramma HappyMail heet.

10 Darmflora zou eigenlijk darmfauna moeten heten.

11 Rokers zijn meestal meer koffie dan theedrinkers.

12 Mensen die altijd tijd over hebben zijn net zo slecht in time-management als mensen die altijd tijd te kort komen.

13 De man heeft meer bijgedragen aan het woord emancipatie dan de vrouw.

14 Mensen met het Down syndroom (mongolen) zijn niet in één stelling te vatten.

Stellingen behorende bij het proefschrift

Enzymatic synthesis of oligosaccharides; kinetics, proces parameters, and reactor design

Floor Boon
Wageningen, 10 maart 2000

Enzymatic Synthesis of Oligosaccharides

kinetics, process parameters, and reactor design

Promotor: prof. dr. ir. Klaas van 't Riet
Voormalig hoogleraar in de levensmiddelenproceskunde

Co-promotor: dr. ir. Anja Janssen
Universitair docente, departement Levensmiddelentechnologie en Voedingswetenschappen

Floor Boon

Enzymatic Synthesis of Oligosaccharides

kinetics, process parameters, and reactor design

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
dr. C.M. Karssen,
in het openbaar te verdedigen
op vrijdag 10 maart 2000
des namiddags om half twee in de Aula.

The studies described in this thesis were part of the research program of the Graduate School VLAG (Food Technology, Agrobiotechnology, Nutrition and Health Sciences). These studies were supported by the Association of Biotechnological Schools (ABON) and Borculo Domo Ingredients.

ISBN 90-5805-161-3

All the modern things
Like cars and such
Have always existed

They've just been waiting in a mountain
For the right moment

Listening to the irritating noises
Of Dinosaurs and people
Dabbling outside

All the modern things
Have always existed
They've just been waiting

To come out
And multiply
And take over

It's their turn now

Björk

Contents

1

General introduction

Oligosaccharides as prebiotics

There is general agreement that the intestinal microflora, e.g. bifidobacteria, has a strong influence on the human health (Crittenden, 1999; Gibson *et al.*, 1996; Ziemer and Gibson, 1998). Some health claims attached to bifidobacteria are inhibition of the growth of potential pathogens e.g. by decreasing the pH, production of vitamins and enzymes, reduction of blood ammonia level, lowering of blood cholesterol level, and activation of the immune system (Gibson and Roberfroid, 1995). Consequently, there is an interest in the manipulation of the composition of this flora. Two approaches exist to increase the number or activity of health-promoting organisms in the gastrointestinal tract. Organism (probiotics) or a selective carbon source (prebiotics) can be added to the food. Combination of these two approaches (synbiotics) is also possible (Crittenden, 1999).

Organisms added to food as probiotics are mostly lactic acid bacteria and bifidobacteria. These bacteria must survive the gastrointestinal tract, establish, and be active in the new environment. Furthermore, the shelf life of the product has to be taken into account; the bacteria must stay viable and simultaneously the product must not be influenced negatively. Consumption of the product containing the probiotic is essential, when stopped, the added bacteria will disappear from the colon.

Prerequisites of prebiotics are that they must reach the colon intact and act there as a specific substrate for desired bacteria. To date almost all prebiotics described and those produced commercially in Japan and Europe are carbohydrates. They are added to the product as a 'functional food' ingredient. Examples are disaccharides (lactulose and lactitol), oligosaccharides (soybean, fructo- and galactooligosaccharides) and polysaccharides (inulin and resistant starch).

A lot of attention is focussed on oligosaccharides. They are naturally present in some food ingredients (soybeans, fruit, milk, and honey), in mother's milk and they are formed during food processing (beer brewing and bread baking). A great deal of research has been carried out to point out the positive effects of the indigestion of oligosaccharides. Alles (1998) tested several health claims of fructo- and transgalactooligosaccharides in healthy humans using placebo-controlled interventions. The oligosaccharides are completely fermented, but no evidence is found for the positive effect on the composition of the intestinal microflora, the glucose and lipid metabolism, and on colon cancer risk markers (such as stool weight, faecal concentration of protein degradation products, and the activity of several glycolytic enzymes). Hence, for healthy humans with a Western diet the health claims attached to oligosaccharides are not confirmed. We expect that oligosaccharides will be beneficial for humans with a disrupted microflora. Also the application of oligosaccharides in infant milk formulas seems to be promising.

The market for oligosaccharides

The popularity of functional food ingredients has developed rapidly in Japan and Europe. The 1996 list of 'foods for specified health use' (FOSHU) in Japan consisted of 58 approved foods, of which 34 contained oligosaccharides (Crittenden and Playne, 1996). On the other hand in the United States the Food and Drug Administration (FDA) has up till 1998 not allowed any health claims for prebiotics (Berner and O'Donnell, 1998). However the interest is large since health and diet are important trends (Sanders, 1998). The most important marketing point for oligosaccharides is the increase of bifidobacteria (Ziemer and Gibson, 1998).

The majority of companies producing and applying oligosaccharides are Japanese. Some examples of European companies are BioEurope (France), Borculo Domo Ingredients (the Netherlands) and Orafti Ltd. (Belgium) (Hartemink, 1999). The oligosaccharides are mainly produced using enzymatic processes. The product consists of a mixture of oligosaccharides of a different degree of polymerisation. Various sorts of oligosaccharides are commercially available on the Japanese market, such as transgalactosyl-, fructo-, and isomaltooligosaccharides.

Enzymatic synthesis of oligosaccharides

Chemical synthesis of oligosaccharides has been developed (Schmidt, 1986), but problems arise because of the need for multiple protection and deprotection steps and the lack of stereoselectivity of the glycosylation reactions. These problems can be avoided by enzymatic synthesis (Ichikawa *et al.*, 1992).

Enzymatic synthesis of oligosaccharides involves either the hydrolysis of polysaccharides or the synthesis from smaller sugars using glycosyltranferases or glycosidases. Glycosyltransferases catalyse the transfer of a monosaccharide from a sugar nucleotide to a glycosyl acceptor. The use of these enzymes is restricted because of the requirement of expensive nucleotide donors (Watt *et al.*, 1997) and their high specificity (Bucke, 1996).

Glycosidases besides hydrolase activity, also show transferase activity. In fact, hydrolysis of the glycosidic bond is a special case of transglycosylation in which the acceptor is water. Hence, oligosaccharides can be produced by enzymatic synthesis with a glycosidase as catalyst and a disaccharide as substrate (Crittenden and Playne, 1996). During transglycosylation, a glycosyl moiety is transferred from a donor to an acceptor molecule

$$\underset{(\mathit{Donor})}{\text{Glycosyl}-\text{O}-\text{R}} + \text{Enzyme} \Leftrightarrow \text{Glycosyl}-\text{Enzyme} + \text{R}-\text{OH}$$

$$\text{Glycosyl}-\text{Enzyme} + \underset{(\mathit{Acceptor})}{\text{R}'-\text{OH}} \Leftrightarrow \underset{(\mathit{Product})}{\text{Glycosyl}-\text{O}-\text{R}'} + \text{Enzyme}$$

Wallenfels and Malhotra (1961) first described this mechanism for lactose hydrolysis. Glycosidases are more abundant in nature but the oligosaccharide yields are generally low. Therefore, the application of glycosidases is restricted to the synthesis of small oligosaccharides (Watt *et al.*, 1997).

Kinetically controlled reactions

Oligosaccharide formation using the transferase activity of glycosidases is a kinetically controlled synthesis. The fundamentals of this approach were described and discussed by Kasche (1986). Other examples of kinetically controlled reactions by enzymes are the peptide synthesis by proteases and the β-lactam antibiotic synthesis (Nam *et al.*, 1985).

During enzymatic kinetically controlled synthesis a nucleophile attacks the substrate-enzyme complex to give the desired product. At the same time water also acts as a nucleophile and competitive hydrolysis of the substrate-enzyme complex takes places. The hydrolysis is thermodynamically favoured and the desired product is also liable to hydrolysis. Therefore, when following the reaction in time, an optimum in product concentration is observed. This has important implications for the reactor design.

The highest product concentration obtained is dependent on the initial transferase and hydrolase rate and the kinetic stability of the product (determined by the ratio between synthesis and hydrolysis of the product). These rates are properties of the enzyme. Hence, the right enzyme choice is very important. Also the nucleophile choice and concentration play an important role in the yield that can be obtained. Of course also other process properties such as pH, temperature, ionic strength, and medium composition influence the product yield (Kasche, 1986).

Aim of this thesis

Oligosaccharides are produced on larger scale in a batch process. The enzyme is added to a sugar solution and the mixture is stirred for a certain period of time. The aim of this research is to obtain more knowledge about the influence of process parameters as substrate concentration, temperature, time, enzyme origin, and reactor configuration on the oligosaccharide synthesis. This information can be used to improve the operating conditions for the batch process. Additionally this knowledge can be used to design a more appropriate reactor configuration for a batch or continuous process.

Furthermore, the enzymatic synthesis of oligosaccharide can serve as a model system for the synthesis of a kinetically controlled product. An extra complication in this model system is that the substrate and the nucleophile attacking the substrate-enzyme complex are identical.

Outline of this thesis

The model system used in this study is the oligosaccharide synthesis with lactose as a substrate and a β-galactosidase as catalyst. Since the synthesis is a kinetically controlled reaction, describing the concentrations of all reactants in time is essential for reactor design. Various models are proposed in chapter 2 and a procedure is developed to determine the model parameters and to select the most suitable model. The effect of temperature is implemented in the kinetic model and the model is used to describe the synthesis by β-galactosidases from various enzyme origins (chapter 3).

Model calculations indicated the positive effect of elevated temperatures. Furthermore, it has been reported that high initial lactose concentrations increase the oligosaccharide yields (Burvall *et al.*, 1979; Roberts and Pettinati, 1957). Higher initial lactose concentrations are possible at higher temperatures and this requires a thermostable and thermoactive biocatalyst. Therefore, in chapter 4 the oligosaccharide synthesis by the β-glucosidase from the hyperthermophilic archaeon *Pyrococcus furiosus* (Kengen *et al.*, 1993) is discussed.

In chapter 5 is shown that the production of a kinetically controlled product is increased when it is continuously removed from the reaction mixture. In the last chapter some aspects, such as enzyme inactivation, the application of water-miscible solvents, and alternative techniques to remove the product from the reaction mixture are discussed.

2

Model development and parameter estimation

Abstract

The aim of this research is to develop a model to describe oligosaccharide synthesis and simultaneously lactose hydrolysis. Model A (engineering approach) and model B (biochemical approach) were used to describe the data obtained in batch experiments with β-galactosidase from *Bacillus circulans* at various initial lactose concentrations (0.19-0.59 mol/kg). A procedure was developed to fit the model parameters and to select the most suitable model. The procedure can also be used for other kinetically controlled reactions.

Each experiment was considered as an independent estimation of the model parameters, and consequently, model parameters were fitted to each experiment separately. Estimation of the parameters per experiment preserved the time dependence of the measurements and yielded independent sets of parameters. The next step was to study by ordinary regression methods whether parameters were constant under the altering conditions examined.

Throughout all experiments, the parameters of model B did not show a trend upon the initial lactose concentration when inhibition was included. Therefore model B, a galactosyl-enzyme complex based model, was chosen to describe the oligosaccharide synthesis, and one parameter set was determined for various initial lactose concentrations.

This chapter has been published as: Boon MA, Janssen AEM, van der Padt A. 1999. Modelling and parameter estimation of the enzymatic synthesis of oligosaccharides by β-galactosidase from *Bacillus circulans*. Biotechnol Bioeng 64:558-567.

Introduction

There is general agreement that the intestinal microflora, e.g. bifidobacteria, has a strong influence on the human health (Minami *et al.*, 1983; Wijsman *et al.*, 1989). Consequently, there is an interest in the manipulation of the composition of this flora (Gibson and Roberfroid, 1995). The growth of bifidobacteria in the intestine can be enhanced by non-digestible oligosaccharides (Oku, 1996). Oligosaccharides reach the colon unaltered and thus can act as a specific substrate.

Chemical synthesis of oligosaccharides has been developed (Schmidt, 1986), but problems arise because of the need for multiple protection and deprotection steps and the lack of stereoselectivity of the glycosylation reactions. These problems can be avoided by enzymatic synthesis (Ichikawa *et al.*, 1992). This is possible with glycosidases because glycosidases, besides hydrolase activity, also show transferase activity. Hence oligosaccharides are formed during the hydrolysis of disaccharides (Crittenden and Playne, 1996).

The model system chosen in this study is the enzymatic synthesis of oligosaccharides catalysed by β-galactosidase from *Bacillus circulans* with lactose as a substrate. This enzyme was chosen since Nakanishi *et al.* (1983) found that β-galactosidase from *B. circulans* produces a relatively large amount of oligosaccharides during the hydrolysis of lactose. The aim of this study is to develop a model to describe oligosaccharide synthesis and lactose hydrolysis.

Theory

Two models (A and B) are proposed in this study. Both models are shown in Figure 1, the rate expressions are listed in Table I. In both models the synthesis of oligosaccharides is lumped together in the production of trisaccharides.

In model A, developed from an engineering perspective, the lactose hydrolysis and trisaccharide synthesis are considered as two parallel second-order reactions of which only the trisaccharide synthesis is reversible. Product inhibition by glucose and galactose is included.

Model B is proposed from a biochemical point of view. Investigation of β-galactosidase action on lactose has resulted in models with a galactosyl-enzyme complex. Nearly all models consider only lactose hydrolysis but some describe in addition oligosaccharide synthesis. In model B water or lactose can attack the galactosyl-enzyme

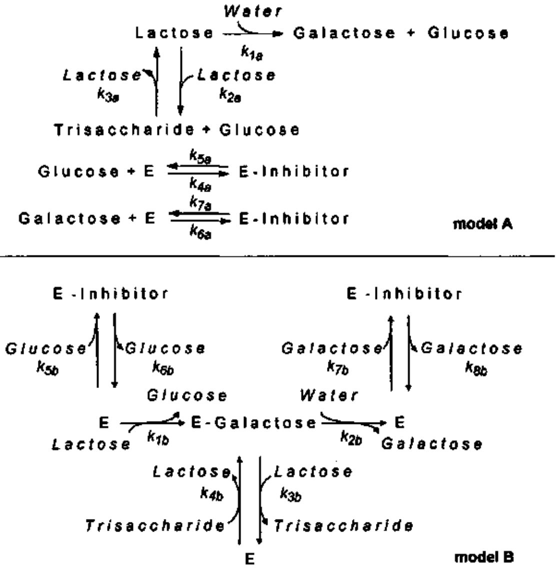

Figure 1 Models A and B proposed for the enzymatic synthesis of oligosaccharides.

complex. The synthesis of trisaccharides is assumed to be reversible. Again product inhibition by the monosaccharides is included. To derive the rate expressions for model B the approach of King and Altman (1956) has been used. Unlike other models, mutarotation of galactose (Bakken *et al.*, 1992), allolactose production (Huber *et al.*, 1976), and separate production of tri- and tetrasaccharides (Iwasaki *et al.*, 1996) are not taken into account. This prevents a further increase in the number of parameters. The reduction of parameters is a driving force for model development (so-called 'Ockham's razor', see e.g. McMeekin *et al.*, 1993).

The models for the enzymatic oligosaccharides synthesis from lactose (Bakken *et al.*, 1992; Huber *et al.*, 1976; Iwasaki *et al.*, 1996) show strong resemblance to models for the peptide synthesis (Gololobov *et al.*, 1988) and β-lactam antibiotics synthesis (Nam *et al.*, 1985). These models correspond to models for steady-state kinetics in the presence of added nucleophiles (Roberts, 1977). In these cases a nucleophile attacks the substrate-enzyme complex to give the desired product. At the same time water also acts as a nucleophile and competitive hydrolysis of the substrate-enzyme complex takes places. Consequently a mixture of several products is obtained. In case the nucleophile and substrate are identical, estimation of the parameters is complicated because studying hydrolysis in absence of synthesis is than impossible. Hence, all parameters can only be

Table I Rate expressions for models A and B.

	model A
lactose	$\frac{\mathrm{d[lac]}}{\mathrm{dt}} = \left(-k_{1a}[\mathrm{lac}][\mathrm{H_2O}] - 2k_{2a}[\mathrm{lac}]^2 + 2k_{3a}[\mathrm{glu}][\mathrm{tri}]\right)Y_a$
glucose	$\frac{\mathrm{d[glu]}}{\mathrm{dt}} = \left(k_{1a}[\mathrm{lac}][\mathrm{H_2O}] + k_{2a}[\mathrm{lac}]^2 - k_{3a}[\mathrm{glu}][\mathrm{tri}]\right)Y_a$
galactose	$\frac{\mathrm{d[gal]}}{\mathrm{dt}} = \left(k_{1a}[\mathrm{lac}][\mathrm{H_2O}]\right)Y_a$
trisaccharide	$\frac{\mathrm{d[tri]}}{\mathrm{dt}} = \left(k_{2a}[\mathrm{lac}]^2 - k_{3a}[\mathrm{glu}][\mathrm{tri}]\right)Y_a$
	with $Y_a = \frac{k_{5a}}{\left(k_{4a}[\mathrm{glu}] + k_{5a}\right)} \frac{k_{7a}}{\left(k_{6a}[\mathrm{gal}] + k_{7a}\right)}$
	model B
lactose	$\frac{\mathrm{d[lac]}}{\mathrm{dt}} = \left(-k_{1b}k_{2b}\frac{1}{k_{4b}}[\mathrm{lac}][\mathrm{H_2O}] - 2k_{1b}\frac{k_{3b}}{k_{4b}}[\mathrm{lac}]^2 + k_{2b}[\mathrm{tri}][\mathrm{H_2O}]\right)Y_b$
glucose	$\frac{\mathrm{d[glu]}}{\mathrm{dt}} = \left(k_{1b}k_{2b}\frac{1}{k_{4b}}[\mathrm{lac}][\mathrm{H_2O}] + k_{1b}\frac{k_{3b}}{k_{4b}}[\mathrm{lac}]^2\right)Y_b$
galactose	$\frac{\mathrm{d[gal]}}{\mathrm{dt}} = \left(k_{1b}k_{2b}\frac{1}{k_{4b}}[\mathrm{lac}][\mathrm{H_2O}] + k_{2b}[\mathrm{tri}][\mathrm{H_2O}]\right)Y_b$
trisaccharide	$\frac{\mathrm{d[tri]}}{\mathrm{dt}} = \left(k_{1b}\frac{k_{3b}}{k_{4b}}[\mathrm{lac}]^2 - k_{2b}[\mathrm{tri}][\mathrm{H_2O}]\right)Y_b$
	with $\frac{1}{Y_b} = k_{2b}\frac{1}{k_{4b}}[\mathrm{H_2O}] + \frac{k_{3b}}{k_{4b}}[\mathrm{lac}] + k_{1b}\frac{1}{k_{4b}}[\mathrm{lac}] + [\mathrm{tri}] +$ $k_{2b}\frac{1}{k_{4b}}\frac{k_{5b}}{k_{6b}}[\mathrm{glu}][\mathrm{H_2O}] + \frac{k_{3b}}{k_{4b}}\frac{k_{5b}}{k_{6b}}[\mathrm{lac}][\mathrm{glu}] +$ $k_{2b}\frac{1}{k_{4b}}\frac{k_{7b}}{k_{8b}}[\mathrm{gal}][\mathrm{H_2O}] + \frac{k_{3b}}{k_{4b}}\frac{k_{7b}}{k_{8b}}[\mathrm{lac}][\mathrm{gal}]$

estimated within one experiment.

When concentrations are measured in time, the measurements are dependent observations. As a result the deviation from the average of duplicate experiments are sometimes all positive or all negative (Figure 2). Naturally, it is possible to fit a model to the data from different experiments simultaneously by minimising the RSS of all concentrations of all experiments. However, the information that sets of data points belong

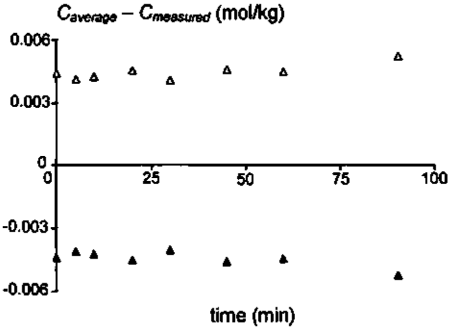

Figure 2 Deviation of the measured trisaccharide concentration of duplicate experiments at an initial lactose concentration of 0.42 mol/kg.

together will be neglected; therefore the overall least-square method is inappropriate. In this study each experiment is considered as an independent determination of the model parameters and the parameter estimation is done separately for each experiment. In this way independent sets of estimates are obtained which are the basis for further straightforward analysis. An example of modelling where time dependence between measurements is preserved, is the determination of initial rates for estimation of the Michaelis-Menten parameters.

Data treatment and statistical methods

Preliminary data screening

For some experiments the measurement error was considered to be too large. Hence, for all experiments the average glucose and galactose residue ratio was calculated, based on the sugar residue balance

$$\frac{[\text{glucose residue}]}{[\text{galactose residue}]} = \frac{[\text{lac}] + [\text{glu}] + [\text{tri}]}{[\text{lac}] + [\text{gal}] + 2[\text{tri}]}$$

All experiments start with lactose only, as a consequence the ratio should be 1. An error of 10% was excepted, therefore, all experiments were removed from the data set for which the average ratio was lower than 0.9 or higher than 1.1. Eventually, the data set consisted of 22 experiments at eight different initial lactose concentrations.

Estimation of model parameters

The kinetic parameters of models A and B were estimated by using data from separate batch experiments. By definition, the parameters should be positive since they are reaction constants in the given direction. The parameters were estimated by non-linear regression minimising the residual sum of squares (RSS) of the four concentrations using the Levenberg-Marquardt method.

The initial concentrations were determined directly after adding the enzyme to the lactose solution. The samples were heated up to 90°C to denaturate the enzyme prior to HPLC analysis. During the inactivation process, a certain amount of the lactose could react. There are two ways to correct for this error: estimation of three initial concentrations (the fourth could be calculated by using a mass balance) or estimation of a time shift. In this study is chosen for estimation of a time shift, assuming that initially only lactose was present. It is noteworthy that estimation of three initial concentrations resulted in approximately the same parameters.

Model selection

The aim is to find a model with one parameter set to describe the oligosaccharide synthesis at all initial lactose concentrations. Since models A and B are not hierarchical (in the sense that one can be obtained by putting restrictions on the parameters of the other) no formal test applies to distinguish between the models. In the hierarchical case (e.g., considering model B without inhibition(s) as a restriction of model B with inhibition) the approach of separate fits does allow for a formal test. However model B with inhibition is not the most general model and therefore the variance will not be a good estimation of the measurement error. Again no formal test for *Lack of Fit* can be applied.

The models will be judged separately and inadequacies will be traced by plotting parameter estimates against initial lactose concentration. The model for which the parameters were independent of the initial lactose concentration was considered the best model to describe the synthesis at various initial concentrations.

Estimation of one parameter set

When the decision was made for the most suitable model, one parameter set had to be determined from independent sets of parameters. An elegant route is to transform the parameters to stabilise the variance and determine the parameter by taking the average (Zwietering *et al.*, 1994). However, this method could not be used because the number of repeated experiments in this research was limited. Therefore, it was assumed that the variance was independent on the initial lactose concentration.

Since the dependency of the parameters on the initial lactose concentration was excluded (see *Model selection*), the average with its 95% confidence interval was taken as the estimate.

Materials and methods

Materials

Lactose was obtained from Sigma (St. Louis, USA) and the other chemicals were obtained from Merck (Darmstadt, Germany). The β-galactosidase from *Bacillus circulans* was obtained from Daiwa Kasai K. K. (Osaka, Japan). All were used without further purification.

Batch experiments

Lactose conversion was carried out in a standard stirred tank reactor with a reaction volume of 330 ml at 40°C. Four baffles (5.8 mm) were placed in the reactor, and a 0.02 M sodium-phosphate buffer at pH 5 was used as aqueous solution. The lactose concentration varied from 0.19 to 0.59 mol/kg (each concentration was at least one time repeated). The reaction was initiated by the addition of 0.4 g β-galactosidase dissolved in 2 ml water.

The reaction was followed for 90-240 min. Samples were taken at regular time intervals, and the enzyme was inactivated by heating the sample in a water bath at 90°C for 5 min. The samples were treated with $Pb(NO_3)_2$ (concentration in the sample 0.1 M) and stored in the freezer to accelerate precipitation (minimum time 1 h). Before analysis the samples were centrifuged for 10 min at 13000 rpm and diluted.

HPLC analysis

The samples were analysed by HPLC using a RSO oligosaccharide column (Phenomenex, the Netherlands) at 80°C. The column was eluted with double distilled water (filtered through a regenerated cellulose membrane of 0.45 μm and purged with helium gas) at a flow rate of 0.3 ml/min. The eluent was monitored by means of a refractive index detector, and it was assumed that the response was independent on the degree of polymerisation.

Lactose, glucose, galactose, and higher saccharides, such as tri- and tetrasaccharides, were measured as weight percentage.

Results and discussion

Determination of concentrations

Since no standard was used, HPLC measurements gave percentages as result. Hence, a mass balance and the initial lactose concentration were required to calculate concentrations. Three mass balances could be used: the monosaccharide, the glucose, or the galactose residue balance (in mol/kg)

$$[\text{monosaccharide residue}] = 2[\text{lac}] + [\text{glu}] + [\text{gal}] + 3[\text{tri}]$$
$$[\text{glucose residue}] = [\text{lac}] + [\text{glu}] + [\text{tri}]$$
$$[\text{galactose residue}] = [\text{lac}] + [\text{gal}] + 2[\text{tri}]$$

The concentrations calculated from percentages with these three balances differ slightly since the measured ratio between glucose and galactose residue varied over time. To eliminate the influence of the sample error on the chosen balance, one proportionality factor for each HPLC analysis (including four concentrations) can be introduced. These proportionality factors do not have a predictive value, should not differ too much from 1, and should not show a trend in time.

To determine which balance should be used and if the introduction of proportionality factors had the desired effect, model B including glucose inhibition was fitted to the data from five batch experiments. The batch experiments used were 0.19, 0.35 (two times), 0.49, and 0.59 mol/kg initial lactose concentration. The results with three different balances both including and excluding proportionality factors were determined (results not shown). The introduction of proportionality factors did not lead to the desired effect; the proportionality factors were dependent on time and varied from 0.92 up to 1.43, moreover, the difference between the estimated parameters using the different balances did not decrease by introducing proportionality factors. Without proportionality factors, the glucose residue balance usually gave the lowest RSS and therefore this balance was used.

It should be noted that in the system there is enough information to determine proportionality factors for each measurement. Each measurement consists of four concentrations (lactose, glucose, galactose, and trisaccharides); however, these four concentrations are not four independent observations. Since any reasonable kinetic model obeys the above three balances, only two concentrations are necessary to calculate all four concentrations. Therefore, with each measurement two degrees of freedom become available for parameter estimation. The number of model parameters determines the minimum amount of measurements required.

Inhibition

It is convenient to first determine whether product inhibition occurs because the absence of inhibition will lead to the elimination of parameters. Independent experiments with additional glucose (0.20 mol/kg) and galactose (0.10 mol/kg) in the reaction mixture were conducted to determine whether product inhibition took place. These sugar concentrations were chosen because during the oligosaccharide synthesis the galactose and glucose concentrations were never higher than these concentrations.

Figure 3 shows the lactose concentration in time, from which it is clear that both glucose and galactose inhibited the enzyme. In this study, contrary to literature (Bakken *et al.*, 1992; Mozaffar *et al.*, 1984), galactose inhibition was neglected because under conditions in our experiments it was measured to be less than the glucose inhibition. As a result the reaction constants for galactose inhibition (k_{6a}, k_{7a}, k_{7b}, and k_{8b}) were assumed to be zero. The glucose inhibition was considered to be competitive, which means that the glucose binds to or nearby the active site of the free enzyme to form an inactive complex (Deschavanne *et al.*, 1978). To our knowledge, glucose inhibition for β-galactosidase from *B. circulans* has not been reported before.

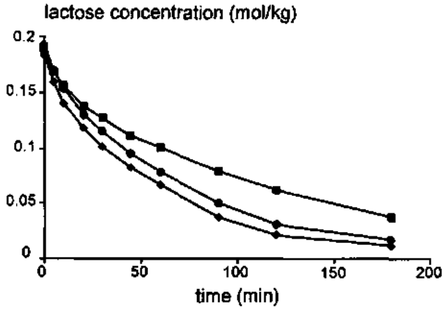

Figure 3 Lactose decrease as function of time at 0.20 mol/kg initial lactose (◆), with additional 0.20 mol/kg glucose (■) or with additional 0.10 mol/kg galactose (●) (lines for guidance).

Lumping of parameters

Model B includes a galactosyl-enzyme complex (E-galactose) that can be attacked by water or lactose (Figure 1). This galactosyl-enzyme complex cannot be measured resulting in an ill-conditioned parameter estimation problem, specifically for the reactions that have the concentration of this complex as driving force. Therefore, the six parameters were grouped into four parameters (k_{1b}, k_{3b}/k_{4b}, $1/k_{4b}$, and k_{5b}/k_{6b}) which were estimated and one fixed parameter (k_{2b}), which was assumed to be 1. It was checked that the value of parameter k_{2b} only very slightly influenced the parameter set found (see *Model evaluation*).

The inhibition parameters of model A were also grouped (k_{4a}/k_{5a}). Due to the restriction that all parameters had to be positive, the inhibition parameter of model A was zero for 17 out of 22 batch experiments. Therefore, only model A without inhibition will be discussed.

Model choice

Models A and model B (with and without inhibition) were fitted to the 22 batch experiments (at eight different initial lactose concentrations). Figure 4 shows examples of measurements and model predictions for four different batch experiments at various initial lactose concentrations with model B including inhibition. For each individual experiment, all models could describe the lactose, glucose, galactose, and trisaccharide concentrations in time. In other words it was possible by varying the parameters to produce nearly the same curves with the different models. It is worth to note that all models had the tendency to overestimate the galactose and trisaccharide concentrations at high initial lactose concentrations.

Figure 5 shows the residual variance (RSS divided by the degrees of freedom) for models A and B (with and without inhibition) as function of the initial lactose concentration. The variances for models A and B were in the same order of magnitude. At higher initial lactose concentrations the variance seemed to increase for all models. A possible reason is that at these higher initial concentrations almost no decrease in the trisaccharide concentration was measured, hence estimation of k_{3a}, k_{3b}/k_{4b}, and $1/k_{4b}$ was difficult.

The time shifts found were 3.0 ± 0.7, 9.9 ± 2.8, and 2.1 ± 0.3 min for model A and model B without and with inhibition, respectively. The time shift is a correction for the time needed for enzyme inactivation (should be << 5 min) and has no predictive value. Note that for model B without inhibition the time shift tends to be too long.

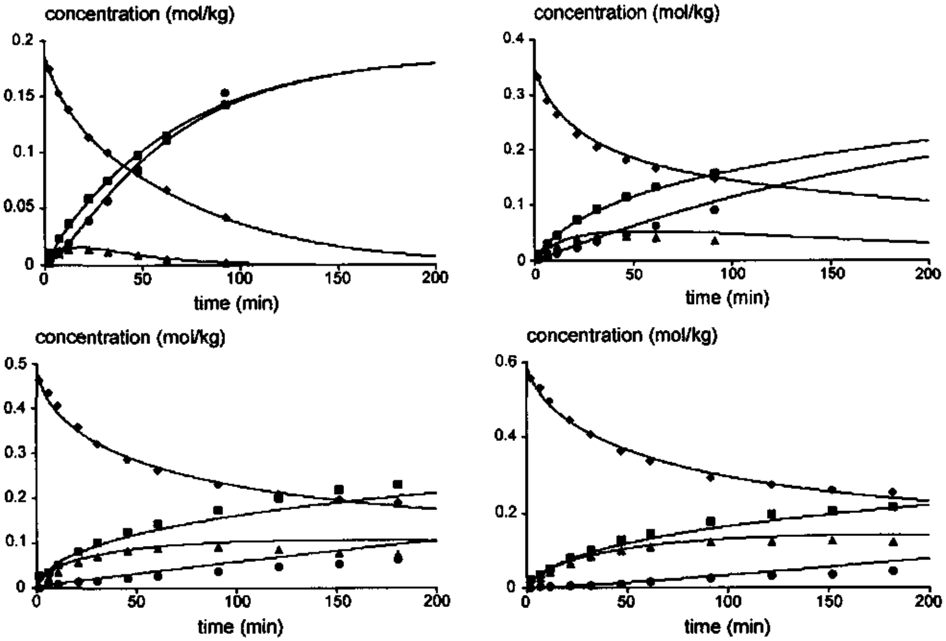

Figure 4 Measurements and model predictions with model B including inhibition (and with individually estimated time shifts) for 4 different batch experiments (◆, lactose; ■, glucose; ●, galactose, and ▲, oligosaccharide). Please note the different concentration scales.

The parameters of model A as function of the initial lactose concentration with the regression line are shown in Figure 6. Outliers which were of a different order of magnitude were left out when the slope or average parameter was determined. This implied that for model A the outliers for k_{2a} and k_{3a} (1.2 and 49.5, respectively, both at 0.19 mol/kg) were not included when the regression line was determined. All estimated parameters decreased upon increasing initial lactose concentration. This was confirmed for k_{1a} and k_{2a} by the 95% confidence interval of the slope, since zero was not included in the confidence interval (Figure 6 and Table II). Due to the dependency of the parameters on the initial lactose concentration, model A was rejected.

The parameters of model B (without and with inhibition) as function of the initial lactose concentration and the regression lines are shown in Figure 7. The 95% confidence interval of the slope is also shown in Table II. Due to the restriction that all parameters had to be positive, some parameters equalled zero. These values were not taken into account when determining the slope or average parameter because they were not fitted to the data points but were assumed to be zero. For the ratios k_{3b}/k_{4b} and k_{5b}/k_{6b} the logarithm was

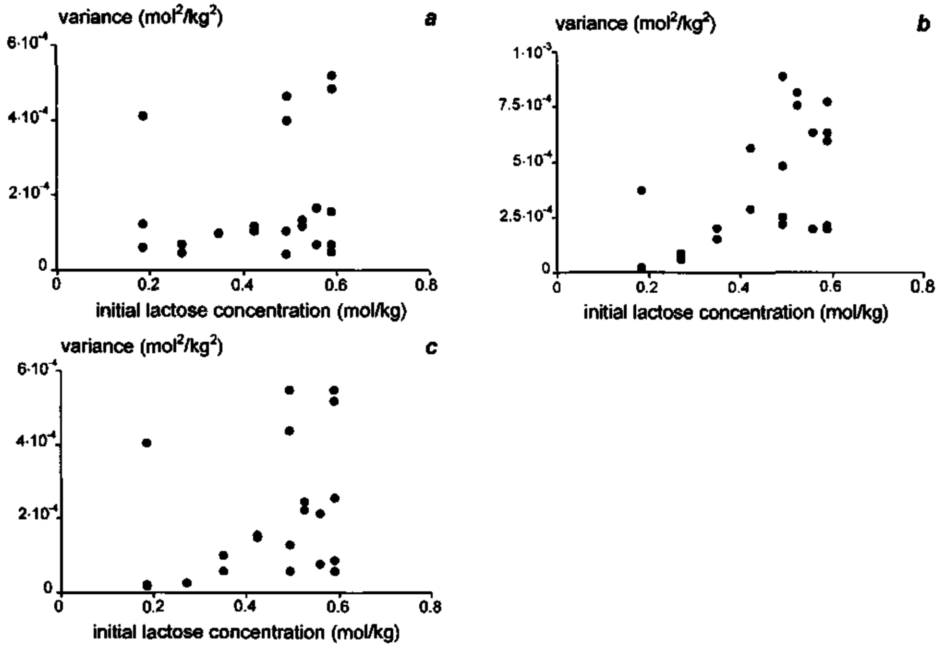

Figure 5 Variance of models A (a) and B without inhibition (b) and with inhibition (c) as function of the initial lactose concentration. Please note the different scale.

plotted. With and without inhibition, the values found for k_{3b}/k_{4b} and $1/k_{4b}$ were in the same order of magnitude. For the model without inhibition the value for k_{Ib} decreased upon increasing initial lactose concentrations, apparently to compensate for the absence of inhibition. Also log (k_{3b}/k_{4b}) was dependent on the initial lactose concentration. Therefore, model B without inhibition was rejected.

For model B with inhibition, no dependency of the parameters on the initial lactose concentration was found. For $1/k_{4b}$, two outliers (700 and 120 at 0.59 mol/kg) were not taken into account. Model B with inhibition was chosen to describe the oligosaccharide synthesis at various initial lactose concentrations, but first one parameter set had to be estimated.

Estimation of one parameter set

For model B with inhibition, the average of the parameters was taken to describe the oligosaccharide synthesis at various initial lactose concentrations. For the parameter $1/k_{4b}$, again the two outliers were not taken into account and for all parameters zero values were

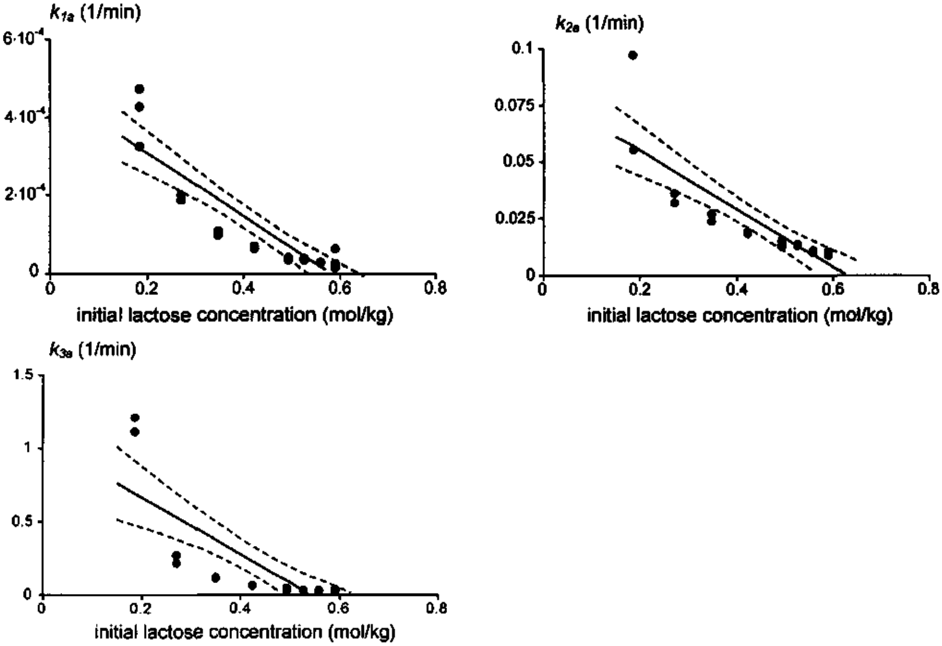

Figure 6 Parameters of model A as function of the initial lactose concentration with regression line and its 95% confidence interval. For k_{2a} and k_{3a} the outliers of 1.2 and 49.5, respectively, both at 0.19 mol/kg were ignored and not shown.

omitted. The parameter set found was $k_{1b} = 0.019 \pm 0.004$, $k_{3b}/k_{4b} = 4600$ (log $(k_{3b}/k_{4b}) = 3.7 \pm 0.1$), $1/k_{4b} = 11 \pm 5$, and $k_{5b}/k_{6b} = 21$ (log $(k_{5b}/k_{6b}) = 1.3 \pm 0.2$).

Unfortunately the parameter set can not be compared to sets from literature. On the one hand because usually experiments are fitted simultaneously. This results in erroneous parameters and comparison is useless. On the other hand because the models are different and therefore give rise to other parameters. Lumping of parameters in order to get the same set is often not possible.

Model evaluation

In model B, parameter estimation was very ill-conditioned; this problem was circumvented by fixing k_{2b} arbitrarily to 1. To evaluate that choice, the parameters per batch experiment were estimated for various values of k_{2b} (results not shown). The same RSS was found for all experiments varying k_{2b} from 0.01 up to 100. The parameters k_{1b}, $1/k_{4b}$, and k_{5b}/k_{6b} remained constant, while k_{3b}/k_{4b} changed nearly proportionally with k_{2b}. In case (k_{1b}/k_{4b})[lac]

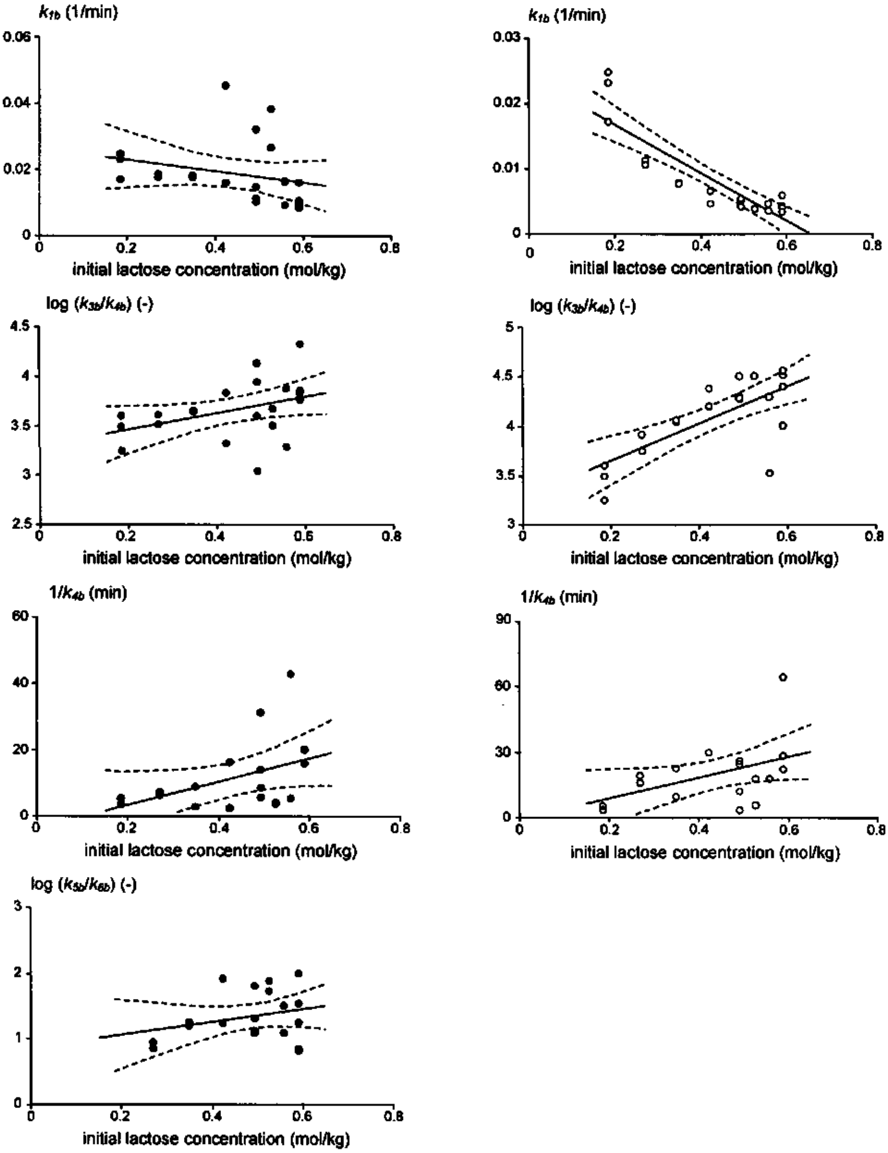

Figure 7 The parameters of model B as function of the initial lactose concentration (closed symbols with inhibition, open symbols without inhibition) with regression line and its 95% confidence interval. For $1/k_{4b}$ (with inhibition) the two outliers 700 and 120 at 0.59 mol/kg are ignored and not shown.

Table II 95% Confidence interval of the slope for parameters of models A and B as a function of the initial lactose concentration. The intervals including zero are given in bold.

		95% confidence interval of the slope	
		lower	upper
model A	k_{1a}	-0.001	-0.0006
	k_{2a}	-1.6	-0.08
	k_{3a}	**-62**	**0.2**
model B without inhibition	k_{1b}	-0.05	-0.03
	$\log(k_{3b}/k_{4b})$	1.0	2.8
	$1/k_{4b}$	**-20**	**75**
model B with inhibition	k_{1b}	**-0.05**	**0.01**
	$\log(k_{3b}/k_{4b})$	**-300**	**26000**
	$1/k_{4b}$	**-0.03**	**1.70**
	$\log(k_{5b}/k_{6b})$	**-0.80**	**2.70**

+ [tri] equalled zero, k_{3b}/k_{4b} would have been proportional to k_{2b}, since in that special case, all terms in numerator and denominator of the rate expressions would contain or k_{3b}/k_{4b} or k_{2b}. With the term (k_{1b}/k_{4b})[lac] + [tri] present in the model, the model is not over-parameterised. However, the nearly proportional increase in k_{3b}/k_{4b} with k_{2b} confirms that the parameter estimation of model B was ill-conditioned and fixation of e.g. k_{2b} was inevitable.

In Figure 8 four experiments and model predictions of model B with the general parameter set are shown. A time shift of 2 min was assumed for all experiments. Of main interest is the trisaccharide concentration in time. These concentrations, together with the lactose and glucose concentrations, were predicted very well. The galactose concentration was not predicted very well since the galactose concentration is excluded from the glucose balance. This balance was used to calculate concentrations from measured percentages.

By changing the parameters of the model it is possible to determine which parameters, and thereby which properties of the enzyme, are important during the synthesis of trisaccharides. The rate constants k_{1b}, k_{3b}, k_{4b}, and k_{5b}/k_{6b} were individually changed a factor 10 while the others were kept constant. For each situation the maximum trisaccharide yield was calculated. The trisaccharide yield was defined as mol trisaccharides measured per mol lactose initially present divided by the theoretical maximum (0.5 mol trisaccharides per mol lactose). The results are shown in Table III.

The trisaccharide yield was increased when the parameter k_{1b} was increased. A higher k_{1b} results in a higher concentration of the galactosyl-enzyme complex. The galactosyl-enzyme complex concentration is the driving force for the tranferase (k_{3b}[E-galactose]) and

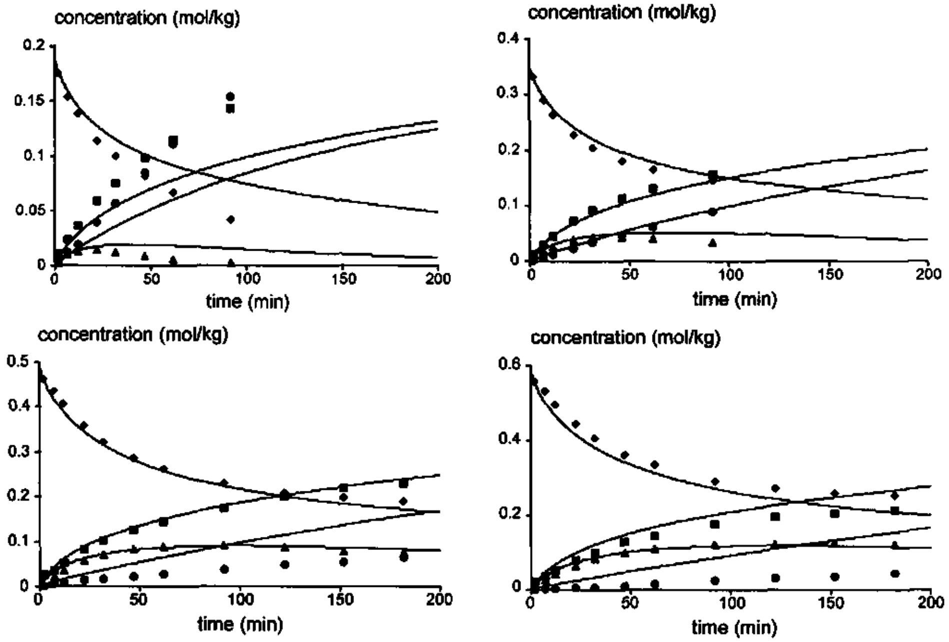

Figure 8 Measurements and model predictions with model B using parameter set found by fitting experiments separately (see text) and a time shift of 2 min, for 4 different batch experiment (◆, lactose; ■, glucose; ●, galactose, and ▲, oligosaccharide). Please note the different concentration scales.

hydrolase rate (k_{2b}[E-galactose]). Since k_{2b} was fixed only k_{3b} had to be taken into account. Table III shows that k_{3b}, and thereby the ratio k_{3b}/k_{2b}, has the largest influence on the trisaccharide yield, and therefore this is an important enzyme property for the trisaccharide synthesis. The parameter k_{4b} has the same influence on the trisaccharide yield as the parameter k_{1b}. The inhibition parameter (k_{5b}/k_{6b}) did not influence the trisaccharide yield, since it only determines the actual fraction of active enzyme. However it did influence the time necessary to reach the maximum yield.

The model parameters can be determined for various enzymes. By comparing the model parameters, the most suitable enzyme for oligosaccharide synthesis can be chosen.

Table III The influence of the model parameters of model B with inhibition on the yield in a batch reactor.

	factor (-)[*]	yield (mol/mol)
standard situation	-	0.37
k_{1b}	0.1	0.11
	10	0.61
k_{3b}	0.1	0.08
	10	0.71
k_{4b}	0.1	0.61
	10	0.11
k_{5b}/k_{6b}	0.1	0.37
	10	0.37

Note: standard situation; initial lactose concentration 0.5 mol/kg; water concentration 45.5 mol/kg; parameters as listed in text.

[*] Every kinetic parameter was changed a factor 0.1 or 10, while the others were kept constant.

Conclusions

To estimate kinetic parameters it is not only of importance to have good data, but every manipulation with the measured data has to be considered carefully because it will effect the estimates.

In experiments where concentrations are measured in time, the measurements are dependent observations. Hence, for fitting kinetic parameters each experiment must be considered as independent estimation of the parameters. When parameters are estimated per experiment, time dependence is preserved and it is possible to examine whether parameters are constant under the altering conditions studied. The dependence of parameters can be used to discriminate between models and facilitates the choice of the most suitable model. This fitting procedure can also be used for other kinetically controlled reactions.

Taking these considerations into account, for oligosaccharide synthesis one parameter set could be determined for a model describing the galactosyl-enzyme complex formation and subsequently hydrolysis or reversible product formation. The model contains inhibition by glucose, which has not been reported before for the used enzyme. The model with four parameters can describe the concentrations in time for a range of initial lactose concentrations. Important parameters for the trisaccharide yield are the first order constants for the galactosyl-enzyme complex formation (k_{1b} and k_{4b}), and the transferase over the hydrolase rate (k_{3b}/k_{2b}).

Acknowledgements

This work was carried out in collaboration with Albert Otten from the subdepartment Mathematics of the Wageningen Agricultural University. The Association of Biotechnological Schools (ABON), the Royal Netherlands Academy of Arts and Sciences (KNAW), and Borculo Domo Ingredients support this work.

3

The effect of temperature and enzyme origin

Abstract

The aim of this research is to quantify the effect of temperature and enzyme origin on the enzymatic synthesis of oligosaccharides. Quantification of these effects is important because temperature and enzyme origin are important process parameters. A kinetic model was used to describe the concentrations in time. The kinetic parameters were determined by using data obtained in batch experiments at various temperatures (20, 30, 40, and 50°C) and by using β-galactosidases from *Bacillus circulans*, *Aspergillus oryzae*, *Kluyveromyces lactis*, and *Kluyveromyces fragilis*.

The effect of temperature on the kinetic parameters could be described with the Arrhenius equation, except for the inhibition parameter. Slightly higher oligosaccharide yields were found at higher temperatures. However, the influence of the initial lactose concentration was much larger. The higher yield at higher temperatures is an additional advantage when operating at high initial lactose concentrations and consequently elevated temperatures. Clear differences between the β-galactosidases were found concerning amount, size, and type of oligosaccharides produced. The β-galactosidase from *B. circulans* produced the most abundant amount, the most different, and largest-size oligosaccharides. The β-galactosidases from *Kluyveromyces spp.* produced mainly trisaccharides. The kinetic parameters for the different enzymes were determined and differences were discussed.

This chapter has been accepted for publication as: Boon MA, Janssen AEM, van 't Riet K. 1999. Effect of temperature and enzyme origin on the enzymatic synthesis of oligosaccharides. Enzyme Microb Technol.

Introduction

Glycosidases, besides hydrolase activity, also show transferase activity. In fact, hydrolysis of the glycosidic bond is a special case of transglycosylation in which the acceptor is water. Hence, oligosaccharides can be produced by enzymatic synthesis with a glycosidase as catalyst and a disaccharide as substrate (Crittenden and Playne, 1996). The source of the enzyme and the operating conditions regulate the type and amount of oligosaccharides produced. The ingestion of these oligosaccharides encourages the proliferation of bifidiobacteria, which are considered to be beneficial for the human health (Minami *et al.*, 1983; Wijsman *et al.*, 1989).

The model system chosen in this study is the enzymatic synthesis of oligosaccharides catalysed by a β-galactosidase with lactose as a substrate. In the work presented in chapter 2 a kinetic model (Figure 1) was developed to describe the oligosaccharide synthesis and lactose hydrolysis.

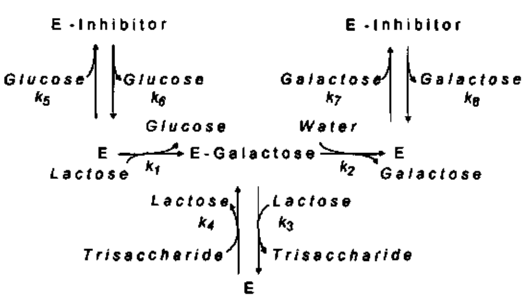

Figure 1 Kinetic model for the enzymatic synthesis of trisaccharides with lactose as a substrate and a β-galactosidase as catalyst.

The synthesis of oligosaccharides is lumped together in the production of trisaccharides. Competitive inhibition by the monosaccharides was included since Deschavanne *et al.* (1978) found that most β-galactosidases are inhibited by the produced monosaccharides. To derive the rate expressions, the approach of King and Altman (1956) was used. The preliminary data treatment and lumped model parameter determination were performed as described in chapter 2.

The model was used to describe the oligosaccharide synthesis at 40°C by the β-galactosidase from *B. circulans* (chapter 2). The aim of this study is to describe the oligosaccharide synthesis at various temperatures and by β-galactosidases from other origin. The effect of temperature and enzyme origin is implemented in the kinetic model.

The model can be used to design a suitable reactor for oligosaccharide synthesis taking the effect of these process parameters into account.

Effect of temperature on oligosaccharide synthesis

The effect of temperature on the oligosaccharide synthesis has been studied and different results have been reported. An increase of the disaccharide synthesis at higher temperatures was found for the β-glucosidase from almond (Ajisaka *et al.*, 1987a). Bakken, *et al.* (1991) described a lower oligosaccharide production at elevated temperatures by the β-galactosidase from *A. oryzae*, whereas others reported no effect (Iwasaki *et al.*, 1996; Prenosil *et al.*, 1987). For the β-galactosidases from *A. niger* (Bonnin and Thibault, 1996; Prenosil *et al.*, 1987; Yang and Okos, 1989) and from *K. fragilis* (Prenosil *et al.*, 1987) and *K. lactis* (Prenosil *et al.*, 1987) no effect of temperature was found.

In all these cases the effect of temperature was small compared to the influence of the initial substrate concentration. However, to describe the oligosaccharide synthesis at higher initial lactose concentrations and consequently elevated temperatures, the effect of temperature on the kinetic parameters has to be known.

Temperature dependency of reactions is often modelled with the Arrhenius equation. Usually the data of experiments at various temperatures are fitted simultaneously. However, the step-wise determination of the parameters at one temperature (chapter 2) results in a step-wise approach of the temperature dependency.

Effect of enzyme origin on oligosaccharide synthesis

β-Galactosidases from different origin synthesise various types and amounts of oligosaccharides. However, this knowledge has not been linked to kinetic parameters. In this study, the kinetic model of Figure 1 is used. It is studied whether differences between β-galactosidases from various origins can be supported by statistically different kinetic parameters.

In this research the β-galactosidases from *B. circulans*, *A. oryzae*, *K. lactis*, and *K. fragilis* were used. The β-galactosidase from *B. circulans* is known to produce a relatively large amount of oligosaccharides with a higher degree of polymerisation (tetra- and pentasaccharides) (Mozaffar *et al.*, 1984). The β-galactosidase from *A. oryzae* produces mainly trisaccharides (López Leiva and Guzman, 1995; Yanahira *et al.*, 1992) and a small amount of tetrasaccharides (Iwasaki *et al.*, 1996; Prenosil *et al.*, 1987). The β-galactosidases from *Kluyveromyces spp.* synthesise trisaccharides and only a limited amount of tetrasaccharides (Prenosil *et al.*, 1987).

Materials and methods

Materials

Lactose was obtained from Sigma (St. Louis, USA) and the other chemicals were acquired from Merck (Darmstadt, Germany). The β-galactosidase from *B. circulans* was obtained from Daiwa Kasai K. K. (Osaka, Japan). The β-galactosidase from *A. oryzae* was acquired from Sigma. The β-galactosidase from *K. lactis* ("Maxilact") was obtained from Gist brocades (Delft, the Netherlands). The β-galactosidase from *K. fragilis* ("Lactozym") was a gift from Novo Nordisk A/S (Bagsværd, Denmark). All were used without further purification.

Batch experiments effect temperature

Lactose conversion was carried out in stirred vessels with a reaction volume of 50 ml at 20, 30, 40, and 50°C. A 0.02 M McIlvaine standard citrate-phosphate buffer at pH 5.0 was used as aqueous solution. The lactose concentration varied from 0.20 to 0.60 mol/kg solution. The reaction was initiated by the addition of 62.5 mg β-galactosidase from *B. circulans* dissolved in 1 ml water, except in the experiments at 20°C 1.5 times this amount was added.

The reaction was followed for 360 min. Samples were taken at regular time intervals, and the enzyme was inactivated by heating the sample in a water bath at 90°C for 5 min. The samples were treated with $Pb(NO_3)_2$ (concentration in the sample 0.1 M) and stored in the freezer to accelerate precipitation (minimum time 1 h). Before analysis the samples were centrifuged for 10 min at 13000 rpm and diluted.

Batch experiments effect enzyme origin

For the other enzymes, the lactose conversion and sample treatment were carried out as described in the previous section. All experiments were carried out at 40°C. As aqueous solutions were used a 0.02 M McIlvaine standard citrate-phosphate buffer at pH 4.5 for β-galactosidase from *A. oryzae*, a 0.025 M potassium-phosphate buffer at pH 7.3 for β-galactosidase from *K. lactis* and at pH 6.5 for β-galactosidase from *K. fragilis*. For the enzymes from *K. lactis* and *K. fragilis*, 1.5 and 1 mM $MgCl_2$ was added, respectively, to improve the enzyme stability. The reaction was initiated by the addition of 62.5 mg β-galactosidase from *A. oryzae* dissolved in 1 ml water, 100 μl enzyme solution *K. lactis*, and 75 μl enzyme solution *K. fragilis*.

Analysis

The samples were analysed by HPLC using a RSO oligosaccharide column (Phenomenex, the Netherlands) at 80°C. The column was eluted with double distilled water (filtered through a regenerated cellulose membrane of 0.45 μm and purged with helium gas) at a flow rate of 0.3 ml/min. The eluent was monitored by means of a refractive index detector and it was assumed that the response was independent on the degree of polymerisation. Lactose, glucose, galactose, and higher saccharides, such as tri- and tetrasaccharides were measured as weight percentage.

To identify the oligosaccharides produced by the various enzymes, samples were also analysed by a Dionex LC system, equipped with a CarboPac PA-1 column (250 mm x 4 mm). The column was eluted with a gradient of NaOAc in 0.1 M NaOH at a flow rate of 1 ml/min. The pulsed amperometric detection (PAD) was carried out with a gold working electrode and triple pulse amperometry was used.

Estimation of model parameters

The estimation of the model parameters and the preliminary data treatment were carried out as described in chapter 2. The eight parameters were grouped into five parameters (k_1, k_3/k_4, $1/k_4$, and k_5/k_6 or k_7/k_8) which were estimated and one fixed parameter (k_2), which was assumed to be 1. Each experiment was considered as an independent determination of the model parameters. In this way independent sets of estimates were obtained. If the estimated parameters were constant under the altering conditions (meaning various initial lactose concentrations), one parameter was determined by taking the average.

Effect of temperature on model parameters

Temperature dependency was modelled with the Arrhenius equation. Inactivation was neglected for the duration of the experiments. To verify this, additional experiments were performed at 50, 55, 60, and 65°C (initial lactose concentration 0.30 mol/kg). The results are shown in Figure 2. It was concluded that at 50°C and 6 h reaction time, inactivation could be neglected.

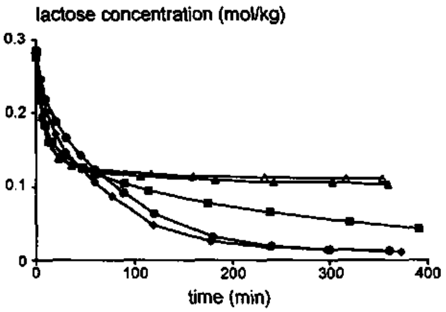

Figure 2 Measurements lactose conversion at 40 (●), 50 (◆), 55 (■), 60 (▲), and 65°C (Δ). Initial lactose concentration 0.30 mol/kg; enzyme β-galactosidase from *B. circulans* (batch 1) (lines for guidance).

Because the parameters at one temperature were determined step-wise, the temperature dependency was determined in the same way. When using the Arrhenius equation in a step-wise approach the 95% confidence interval of the parameters is neglected. Temperature dependence is therefore easily found. To take the 95% confidence interval of the parameters into account, also the t-test was used. The t-test compares the differences between two sample means. It was assumed that the variances of the two populations were equal. The following is calculated

$$t = \frac{\bar{y}_1 - \bar{y}_2}{s_p^2\sqrt{1/n_1 + 1/n_2}}$$

The subscript 1 and 2 denote the two populations, y is the sample means, n is the size of the population and s_p^2 denotes the pooled variance. The pooled variance is defined by

$$s_p^2 = \frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{(n_1 - 1) + (n_2 - 1)}$$

In which s^2 denotes the variance of the population. The t-value is compared to the 95% critical t-value at n_1+n_2-2. When the overlap of the 95% confidence interval is larger than 5%, the two values are identical. The t-distribution can be found in Perry and Green (1984 (a)).

Comparison of model parameters for β-galactosidases from various origin

To compare model parameters of different enzymes, parameters have to be corrected for the enzyme activity. The prerequisite is that the model parameters are linear with the enzyme concentration. Therefore, additional experiments with 30 mg and 80 mg β-galactosidase from *B. circulans* were performed. The model parameters were found to be linear with the enzyme concentration. All parameters were divided by the parameter, which describes the initial decrease of the lactose concentration, k_1.

Statistics were used to compare the parameters from the various β-galactosidases. The above mentioned t-test together with the *Lack of Fit* was used. The *Lack of Fit* compares the residual sums of squares (RSS) of various models to a general model. The general model is hierarchical, in the sense that the other models can be obtained by putting restrictions on the parameters of the general model. The difference between the RSS is the profit obtained by adding a parameter. It is tested if the introduction of a parameter is worthwhile. The following is calculated

$$f = \frac{(RSS - RSS_g)/(DF - DF_g)}{RSS_g / DF_g} \qquad \text{tested against } F_{DF_g}^{DF-DF_g}$$

The subscript g denotes the general model, DF is the degrees of freedom (number of independent experiments minus the number of parameters), RSS is the residual sums of squares. The F distribution can be found in Perry and Green (1984 (b)).

Results and discussion

Effect of temperature on oligosaccharide synthesis

Lactose conversion was followed for various initial lactose concentrations (0.20-0.60 mol/kg) at 20, 30, 40, and 50°C. Figure 3 shows the concentrations in time at an initial lactose concentration of 0.45 mol/kg at the four temperatures. The reaction rates increased with temperature. Therefore, at 20°C 1.5 time the amount of enzyme was added to be able to perform the reaction within 6 h.

The synthesis of oligosaccharides was lumped together in the production of trisaccharides. Only a slightly higher maximum trisaccharide yield was observed at higher temperatures. The trisaccharide yield was defined as mol trisaccharides measured per mol lactose initially present divided by the theoretical maximum (0.5 mol trisaccharides per

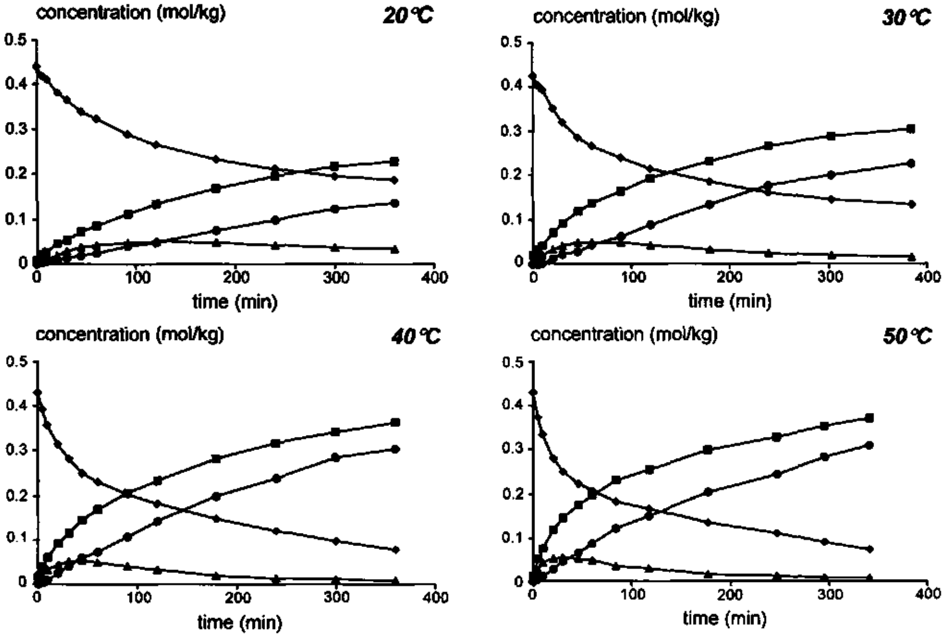

Figure 3 Measurements lactose conversion at 20, 30, 40, and 50°C. Initial lactose concentration 0.45 mol/kg; enzyme β-galactosidase from *B. circulans* (batch 1) (◆, lactose; ■, glucose; ●, galactose, and ▲, oligosaccharides) (lines for guidance).

mol lactose). For these four temperatures at an initial lactose concentration of 0.45 mol/kg, an experimental maximum yield of respectively, 0.23, 0.22, 0.24, and 0.24 mol/mol was found. There was no effect of the temperature on the production of higher oligosaccharides.

The model parameters were determined following the procedure described in chapter 2. For the β-galactosidases from *B. circulans* inhibition by glucose was found and therefore k_7 and k_8 were assumed to be equal to zero. To determine one parameter set for the various initial lactose concentrations, the parameters have to be independent of the initial lactose concentrations. Therefore, the slope and its 95% confidence interval of the parameters as function of the initial lactose concentration were determined (data not shown). When zero was included in the 95% confidence interval it was assumed that the parameter was independent of the initial lactose concentration.

A negative trend with the initial lactose concentration was encountered for k_1 at 20°C and for log (k_3/k_4) at 50°C. A positive trend for log (k_5/k_6) at 30 and 50°C was found. These trends were considered to be unimportant because not the same trend for parameters at one temperature or for one parameter at various temperatures was found. Therefore, the average parameter was taken. The effect of the temperature on the model parameters was modelled with the Arrhenius equation. In Figure 4 the parameters and the prediction of the Arrhenius equation are shown.

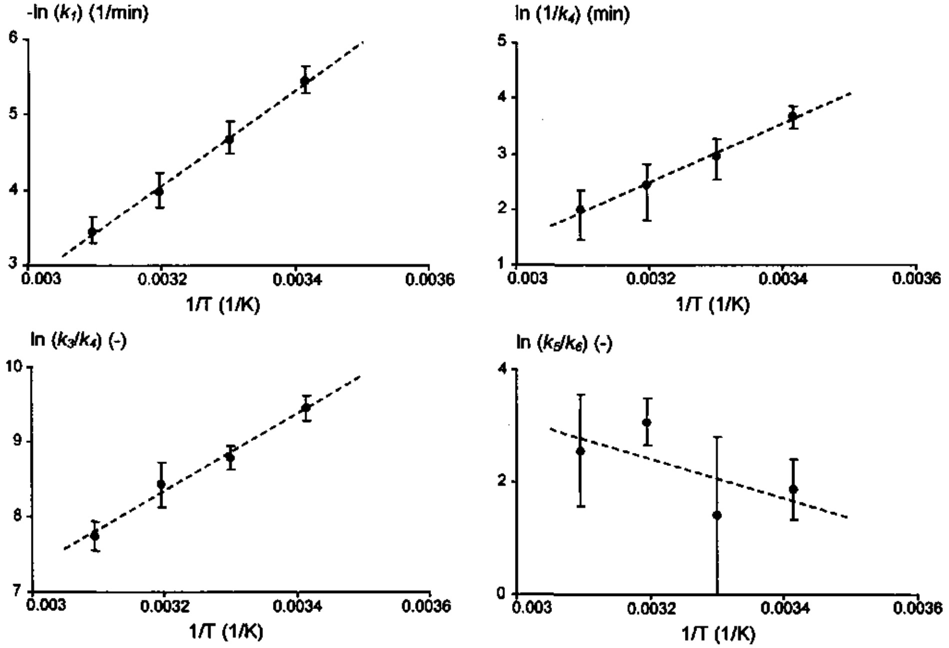

Figure 4 Natural logarithm of the model parameters (including its 95% confidence interval) as function of the temperature reciprocate, including the Arrhenius equation prediction. Enzyme β-galactosidase from *B. circulans* (batch 1).

For the parameters activation energies (E_A) of 53 ± 10, -43 ± 16, 44 ± 11, and 29 ± 103 kJ/mol for k_1, k_3/k_4, k_4, and k_5/k_6, respectively were determined. A negative activation energy was possible because the ratio of parameters was involved. For the inhibition parameters (k_5/k_6) and parameter k_3 the activation energies were not significantly different from zero. Hence, for these parameters no effect of temperature was found.

When using the Arrhenius equation in a step-wise approach (data for each temperature is fitted independently and subsequently to the Arrhenius equation) the error is underestimated and the 95% confidence interval of the parameters is neglected. Therefore also a t-test was performed. The Arrhenius equation has a physical background, contrary to the t-test. The t-test is a statistical tool and it determines solely if two values are different.

The t-test confirmed that the model parameters were different at various temperatures (Table I). Again no clear effect of the temperature on the parameter log (k_5/k_6) was found.

Table I Results t-test for temperature influence on the model parameters. Bold indicates that a difference between the parameters for two temperatures was found.

k_1	n	Estimate	s^2	versus	t	t_c	$1/k_4$	n	Estimate	s^2	versus	t	t_c
20°C	8	0.0043	$8 \cdot 10^{-7}$	20 ~ 30	**5.54**	2.62	20°C	8	39.6	75	20 ~ 30	**4.82**	2.62
30°C	8	0.0093	$6 \cdot 10^{-6}$	20 ~ 40	**4.18**	2.47	30°C	8	19.3	70	20 ~ 40	**6.42**	2.49
40°C	22	0.019	$9 \cdot 10^{-5}$	20 ~ 50	7.53	2.62	40°C	18	11.5	120	20 ~ 50	**9.50**	2.62
50°C	8	0.032	$1 \cdot 10^{-4}$	30 ~ 40	**2.72**	2.05	50°C	8	7.27	15	30 ~ 40	1.82	2.06
				30 ~ 50	**6.01**	2.62					30 ~ 50	**3.79**	2.62
				40 ~ 50	**3.12**	2.47					40 ~ 50	1.04	2.49
log (k_3/k_4)	n	Estimate	s^2	versus	t	t_c	log (k_5/k_6)	n	Estimate	s^2	versus	t	t_c
20°C	8	4.11	0.007	20 ~ 30	**6.81**	2.62	20°C	6	0.81	0.05	20 ~ 30	0.79	2.76
30°C	8	3.82	0.007	20 ~ 40	**4.21**	2.47	30°C	6	0.61	0.35	20 ~ 40	**3.17**	2.50
40°C	22	3.66	0.09	20 ~ 50	**12.8**	2.62	40°C	19	1.3	0.15	20 ~ 50	1.44	2.72
50°C	8	3.36	0.02	30 ~ 40	1.47	2.05	50°C	7	1.1	0.20	30 ~ 40	**3.57**	2.07
				30 ~ 50	**7.86**	2.62					30 ~ 50	1.72	2.72
				40 ~ 50	**2.76**	2.47					40 ~ 50	1.25	2.49

Note: n is the number of experiments; Estimate is the estimated parameter; s^2 is the variance; versus indicates the two temperatures compared; t is the calculated t-value; t_c is the 95% critical t-value.

To predict the effect of the temperature on the oligosaccharide production all the parameters have to be taken into account. Therefore, the concentrations in time were predicted and the maximum yield was calculated. This was done for various initial lactose concentrations and temperatures. The results are shown in Figure 5. The yield increased at higher temperatures and higher initial lactose concentrations. However, it is clear that the influence of the initial lactose concentration was much larger. The advantage of a higher yield at higher temperatures is an extra advantage when using high initial lactose concentrations at consequently elevated temperatures.

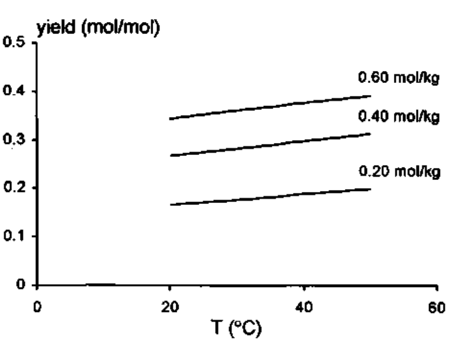

Figure 5 Calculated yield as function of temperature and initial lactose concentration. Enzyme β-galactosidase from *B. circulans* (batch 1).

Effect of enzyme origin on oligosaccharide synthesis

To determine the influence of the enzyme origin on the oligosaccharide synthesis and to link this knowledge to the kinetic parameters of the model, experiments were performed at various initial lactose concentrations (0.20-0.60 mol/kg) with four β-galactosidases. Two different batches of the β-galactosidase from *B. circulans* were used. The experimental data from the two batches were treated separately to determine if a statistical difference existed.

Figure 6 shows the concentrations in time at an initial lactose concentration of 0.40 mol/kg for four β-galactosidases. The production of oligosaccharides was divided in trisaccharides and in higher oligosaccharides. The production of higher oligosaccharides could be neglected for the β-galactosidases from *K. lactis* and *K. fragilis*.

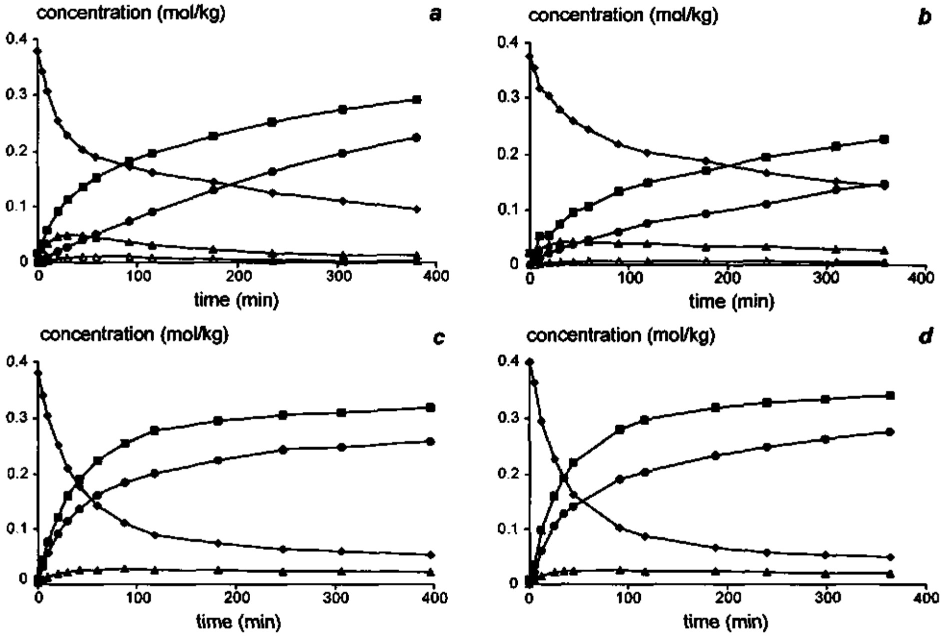

Figure 6 Measurements lactose conversion by β-galactosidases from *B. circulans* (batch 2) (a), *A. oryzae* (b), *K. lactis* (c) and *K. fragilis* (d). Initial lactose concentration 0.40 mol/kg (◆, lactose; ■, glucose; ●, galactose; ▲, trisaccharides, and Δ, tetrasaccharides and higher) (lines for guidance).

The β-galactosidases from *K. lactis* and *K. fragilis* produced mainly trisaccharides. The HPLC chromatogram showed that a considerable amount of tetra- and pentasaccharides were synthesised by the β-galactosidase from *B. circulans*. Also the enzyme of *A. oryzae* produced higher oligosaccharides, although in a smaller amount. The synthesis of higher saccharides (higher than trisaccharides) increased at higher initial lactose concentrations for the β-galactosidase from *B. circulans* and *A. oryzae*.

From the experimental data the maximum yield was calculated. Figure 7 illustrates that the yield increased at higher initial lactose concentrations for all enzymes and was dependent on the type of enzyme. The largest amount of oligosaccharides was produced by the β-galactosidase from *B. circulans*. The second batch of this enzyme had a slightly higher transferase activity compared to the first batch. The β-galactosidase of *A. oryzae* produced less oligosaccharides. The smallest amount was formed by the two β-galactosidases from *Kluyveromyces spp.*

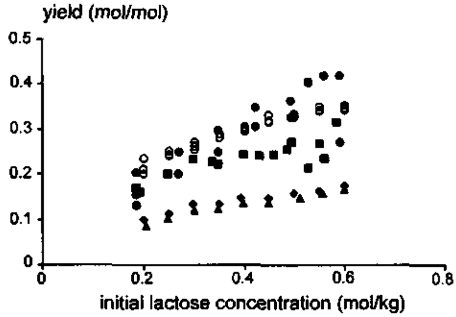

Figure 7 Measured yield as function of the initial lactose concentration and enzyme origin (O, *B. circulans* (batch 1); ●, *B. circulans* (batch 2); ■, *A. oryzae*; ◆, *K. lactis*, and ▲, *K. fragilis*).

To indicate the number of various oligosaccharides produced, samples were analysed by a Dionex LC system (Figure 8). The two batches from β-galactosidase from *B. circulans* gave the same oligosaccharide pattern. From the pattern it was clear that this enzyme produced the most different oligosaccharides. The oligosaccharides produced by the β-galactosidase from *B. circulans* were characterised by Fransen *et al.* (1998). The retention time of the oligosaccharides peaks in the chromatogram of the enzyme from *A. oryzae* were diverse compared to those of the β-galactosidases from *K. lactis* and *K. fragilis*. The oligosaccharide patterns of the enzymes from *K. lactis* and *K. fragilis* were identical.

Before the parameters of the kinetic model for the various enzymes were estimated, independent experiments with additional glucose and galactose (0.10 and 0.20 mol/kg) in the reaction mixture were conducted to determine whether product inhibition took place (data not shown). It is convenient to first determine whether product inhibition occurs because the absence of inhibition leads to the elimination of parameters.

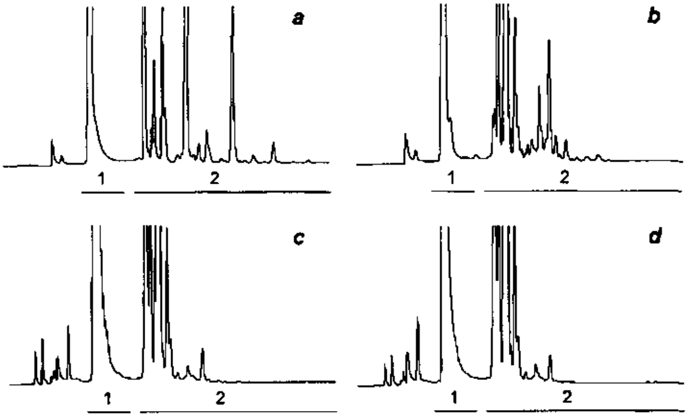

Figure 8 DIONEX profiles of the reaction products obtained with the β-galactosidase from *B. circulans* (a), *A. oryzae* (b), *K. lactis* (c), and *K. fragilis* (d) (1 indicates the monosaccharides, 2 indicates the higher saccharides).

For all enzymes inhibition by both monosaccharides was found but only the more pronounced one was included in the model. For the enzyme of *B. circulans* we have found inhibition by glucose (chapter 2). The β-galactosidase from *A. oryzae* was clearly more inhibited by galactose than glucose. This is in agreement with literature (Friend and Shahani, 1982; Ogushi *et al.*, 1980; Park *et al.*, 1979). The inhibition for the enzymes from *Kluyveromyces spp.* was less pronounced than for the β-galactosidases from *B. circulans* and *A. oryzae*. The β-galactosidase from *K. lactis* was slightly more inhibited by galactose. This is supported in the literature (Cavaille and Combes, 1995). For the enzyme from *K. fragilis* the inhibition by glucose was more distinct, whereas inhibition by galactose is reported in literature (Carrara and Rubiolo, 1996; Mahoney and Whitaker, 1977; Santos *et al.*, 1998).

As a result of these inhibition experiments the reaction constants for galactose inhibition (k_7 and k_8) were assumed to be zero for the enzymes of *B. circulans* and *K. fragilis*. The reaction constants for glucose inhibition (k_5 and k_6) were assumed to be zero for the enzymes of *A. oryzae* and *K. lactis*.

To be allowed to determine one parameter set for various initial lactose concentrations by taking the average, the parameters have to be independent on the initial lactose concentrations. The slope and its 95% confidence interval of the parameters as function of the initial lactose concentration were determined (data not shown). For the enzymes from

B. circulans (batch 1), *A. oryzae*, and *K. fragilis* the model can be applied and for none or only for one parameter a trend with the initial lactose concentration was found. For the second batch of the enzyme from *B. circulans* trends were found for all parameters except k_1. However, it was shown that the model could be applied to this enzyme (batch 1). For the β-galactosidase from *K. lactis* trends were found for k_1 and $1/k_4$. Because the experimental data at the various initial lactose concentrations for this enzyme could be described well by the average parameter, the set was not rejected.

The parameters for the various enzymes are shown in Table II. The parameter k_3 was the average of the calculated values for each batch experiment from the estimated k_3/k_4.

Table II Kinetic parameters for the β-galactosidases of various origins.

	B. circulans (1)	*B. circulans* (2)	*A. oryzae*	*K. lactis*	*K. fragilis*
k_1 (1/min)	0.019	0.041	0.024	0.042	0.036
k_3 (1/min)	400	740	340	49	43
k_4 (1/min)	0.088	0.28	0.094	$7.6 \cdot 10^{-3}$	$4.0 \cdot 10^{-3}$
k_5/k_6 (-)	21	33	-	-	15
k_7/k_8 (-)	-	-	95	11	-

In Figure 9 the parameters are shown including the 95% confidence intervals. The order in which the parameters were plotted on the x-axis is arbitrary. The parameters were corrected for the enzyme activity by dividing the parameters by k_1.

Figure 9 shows that the parameter log (k_3/k_4) was in the same order of magnitude for all β-galactosidases. This parameter is a measure of the equilibrium E-gal + lactose $\leftrightarrow$ E + trisaccharide (Figure 1). The value of the equilibrium constant seems to be independent on the type of enzyme.

Before equilibrium is reached, the values of the parameters k_3 and k_4 are important. A high k_3 will result in a high trisaccharide production rate and the parameter k_4 determines the velocity in which the trisaccharides are hydrolysed. Therefore, for the optimum yield not the ratio but the values of these two parameters are important. Figure 9 shows that for k_1/k_4 and k_3/k_1 clearly two groups are formed. The transferase activity of the β-galactosidases from *B. circulans* and *A. oryzae* was much larger than that of the β-galactosidases from *Kluyveromyces spp.* This was in accordance with the higher yield found in the experiments (Figure 7).

Statistics were used to test if the two batches from *B. circulans* (hypothesis 1) and if the enzymes from *Kluyveromyces spp.* (hypothesis 2) were identical. The results of the t-test and *Lack of Fit* are shown in Table III and IV. The general model used for the *Lack of Fit* was the possibility of five different parameters for the five enzymes, the most restricted

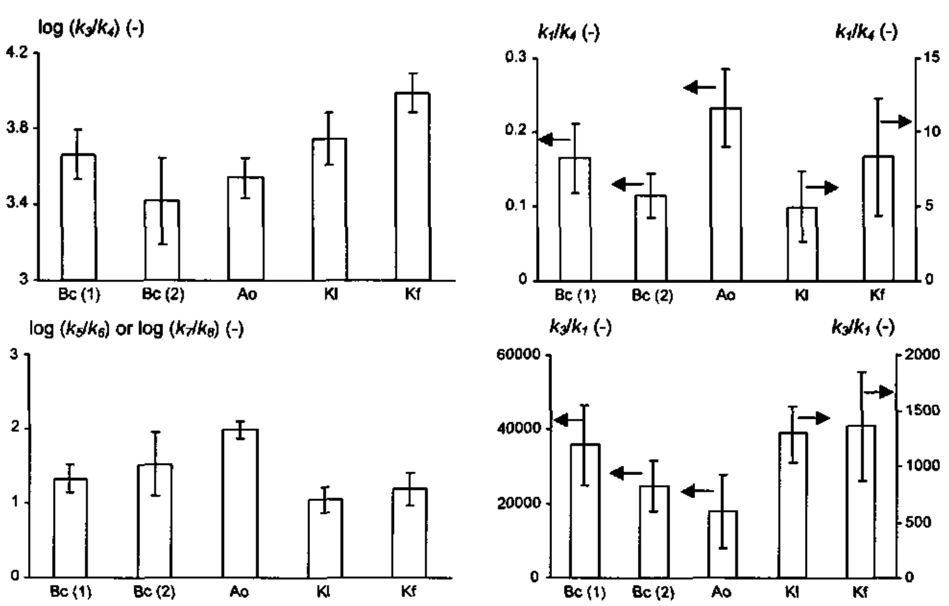

Figure 9 Model parameters (including its 95% confidence interval) for β-galactosidase from different enzyme origin; *B. circulans* batch 1 (Bc (1)); *B. circulans* batch 2 (Bc (2)); *A. oryzae* (Ao); *K. lactis* (Kl); *K. fragilis* (Kf). Please note the different scales.

model was that one parameter was sufficient. In total 26 different models were tested which included hypothesis 1 and/or 2. In Table IV only those models are shown which were accepted.

The *Lack of Fit* showed that the two batches of *B. circulans* were identical for the parameters k_1/k_4, log (k_5/k_6), log (k_7/k_8) and k_3/k_1. Only for the parameter log (k_3/k_4) this hypothesis was rejected. However, the t-test showed that all model parameters of the two β-galactosidases from *B. circulans* were identical. This was also clear from the overlap of the 95% confidence interval of the parameters as shown in Figure 9. Although the differences between the two batches from *B. circulans* seemed small, statistics could not give a conclusive answer if hypothesis 1 should be accepted.

The *Lack of Fit* and t-test were also used to test the second hypothesis, namely that the enzymes from *K. lactis* and *K. fragilis* were identical. The *Lack of Fit* showed that the parameters log (k_3/k_4), log (k_5/k_6), log (k_7/k_8), and k_3/k_1 from the enzymes of *Kluyveromyces spp.* were identical. Only for the parameter k_1/k_4 this hypothesis was rejected. The t-test showed that the two enzymes were dissimilar for the parameter log (k_3/k_4) (Table III). In Figure 9 is clear that the overlap of the 95% confidence interval was very small. Taking

Table III Results t-test for testing hypothesis 1 and 2. Bold indicates that no difference between the parameters for the two enzymes was found.

		n	Estimate	s^2	t	t_c
log (k_3/k_4)	*B. circulans* (1)	22	3.66	0.09	**2.00**	2.05
	B. circulans (2)	7	3.41	0.06		
	K. lactis	9	3.74	0.03	3.18	2.11
	K. fragilis	10	3.99	0.02		
k_1/k_4	*B. circulans* (1)	18	0.16	0.009	**1.38**	2.07
	B. circulans (2)	7	0.11	0.001		
	K. lactis	9	5.00	8.9	**1.89**	2.16
	K. fragilis	6	8.31	14		
log (k_5/k_6)	*B. circulans* (1)	19	1.33	0.14	**1.07**	2.06
	B. circulans (2)	7	1.52	0.21		
log (k_7/k_8)	*K. lactis*	9	1.05	0.05	**1.23**	2.16
log (k_5/k_6)	*K. fragilis*	6	1.19	0.04		
k_3/k_1	*B. circulans* (1)	18	36000	$5\cdot10^8$	**1.29**	2.06
	B. circulans (2)	7	25000	$5\cdot10^7$		
	K. lactis	9	1300	$1\cdot10^5$	**0.38**	2.16
	K. fragilis	6	1400	$2\cdot10^5$		

Note: n is the number of experiments; Estimate is the estimated parameter; s^2 is the variance; t is the calculated t-value; t_c is the 95% critical t-value.

Table IV Results *Lack of Fit* for testing hypothesis 1 and 2. Only the models that were not rejected are shown.

Parameter	Model	RSS	DF	f	F
log (k_3/k_4)	General: 5 different parameters	2.77	52		
	Kl = Kf, Ao = Bc (2)	3.12	54	3.15	3.18
	Kl = Kf, Ao = Bc (1)	3.11	54	3.11	3.18
k_1/k_4	General: 5 different parameters	140	44		
	Bc (1) = Bc (2) = Ao	140	46	0.00860	3.21
	Bc (1) = Bc (2)	140	45	0.00394	4.06
log (k_5/k_6)	General: 5 different parameters	4.65	45		
or log (k_7/k_8)	Bc (1) = Kl = Kf	5.16	47	2.44	3.20
	Bc (1) = Bc (2) = Kf	5.02	47	1.76	3.20
	Kl = Kf	4.73	46	0.697	4.06
	Bc (1) = Bc (2)	4.84	46	1.77	4.06
	Bc (1) = Bc (2), Kl = Kf	4.91	47	1.23	3.20
k_3/k_1	General: 5 different parameters	$9.7\cdot10^9$	44		
	Kl = Kf	$9.7\cdot10^9$	45	0.0001	4.06
	Bc (1) = Bc (2)	$1.0\cdot10^{10}$	45	2.74	4.06
	Bc (1) = Bc (2), Kl = Kf	$1.0\cdot10^{10}$	46	1.37	3.21
	Kl = Kf, Ao = Bc (2)	$9.9\cdot10^9$	46	0.42	3.21
	Kl = Kf, Ao = Bc (1)	$1.0\cdot10^{10}$	46	1.62	3.21

Note: β-galactosidases from various origin are indicated as: Bc (1), *B. circulans* batch 1; Bc (2), *B. circulans* batch 2; Ao, *A. oryzae*; Kl, *K. lactis*; Kf, *K. fragilis*. RSS is the residual sums of squares; DF is the degrees of freedom; f is the f-value; F is the 95% critical F-value.

into account that it is easier to prove that two parameters are similar when they are different, then visa versa, hypothesis 2 was rejected. Although the differences between the β-galactosidases from *K. fragilis* and *K. lactis* seemed small, statistically a difference was found.

Conclusions

The kinetic model can describe the oligosaccharide synthesis at various temperatures. The temperature effect can be modelled with the Arrhenius equation and the synthesis is enhanced at higher temperatures. The effect of temperature is small compared to the influence of the initial lactose concentration.

The same kinetic model can describe the synthesis by β-galactosidases that produced various amounts and types of oligosaccharides. The kinetic parameters describing the reversible oligosaccharide synthesis are of a different magnitude for the β-galactosidases from *B. circulans* and *A. oryzae*, compared to those from *K. fragilis* and *K. lactis*. The difference between the enzymes from *K. lactis* and *K. fragilis* is supported by statistics.

The model will be useful for design of a suitable reactor for oligosaccharide synthesis, taking the effect of temperature and enzyme origin into account.

Acknowledgements

The authors wish to thank Céline Malard for performing experiments. The Association of Biotechnological Schools (ABON), the Royal Netherlands Academy of Arts and Sciences (KNAW) and Borculo Domo Ingredients support this work.

4

Synthesis by the thermostable β-glucosidase from *Pyrococcus furiosus*

Abstract

The thermostable β-glucosidase from *Pyrococcus furiosus* has been shown to produce tri- and tetrasaccharides with lactose as a substrate (0.73-1.44 mol/kg) at elevated temperatures (75-95°C). The enzyme has a broad pH optimum (5.0-7.0), and was inhibited more by glucose than by galactose. An increase in the initial lactose concentrations led to a proportional increase of the ratio between the initial oligosaccharide production rate and the initial lactose conversion rate. Because of inhibition by Maillard components, the lowest temperature studied (75°C) is the best for oligosaccharide synthesis. The oligosaccharide yield obtained with this thermostable enzyme is independent of the initial lactose concentration and temperature and a factor 1.4 higher than reported for mesophilic enzymes.

This chapter has been published as: Boon MA, van der Oost J, de Vos WM, Janssen AEM, van 't Riet K. 1999. Synthesis of oligosaccharides catalyzed by thermostable β-glucosidase from *Pyrococcus furiosus*. Applied Biochem Biotechnol 75:269-278.

Introduction

Oligosaccharides are polymeric carbohydrates consisting of 2-10 monomers. The ingestion of these oligosaccharides encourages the proliferation of, e.g. bifidobacteria in the intestine, which is considered to be beneficial to the human health (Minami *et al.*, 1983; Wijsman *et al.*, 1989). There is a growing interest in the synthesis of oligosaccharides for application in functional foods; not only in Japan, but also in Europe, food manufacturers are producing and applying oligosaccharides (Crittenden and Playne, 1996).

Enzymatic synthesis of oligosaccharides is possible with glycosidases because these enzymes, besides hydrolase activity, also show transferase activity. Hence, oligosaccharides are formed during the hydrolysis of disaccharides (Crittenden and Playne, 1996). During transglycosylation, a glycosyl moiety is transferred from a donor to an acceptor molecule

$$\underset{(\mathit{Donor})}{\text{Glycosyl}-\text{O}-\text{R}} + \text{Enzyme} \Leftrightarrow \text{Glycosyl}-\text{Enzyme} + \text{R}-\text{OH}$$

$$\text{Glycosyl}-\text{Enzyme} + \underset{(\mathit{Acceptor})}{\text{R}'-\text{OH}} \Leftrightarrow \underset{(\mathit{Product})}{\text{Glycosyl}-\text{O}-\text{R}'} + \text{Enzyme}$$

Wallenfels and Malhotra (1961) first described this mechanism for lactose hydrolysis. Using lactose as donor, the acceptor can be a water molecule (hydrolysis), lactose, or a trisaccharide (both transglycosylation). This results in the products galactose, a tri- or tetrasaccharide, respectively. The ratio between hydrolase and transferase activity is important for maximum oligosaccharide yield.

Lactose is an industrial disaccharide that is widely used for food applications. It has been reported that high initial lactose concentrations increase the oligosaccharide yields (Burvall *et al.*, 1979; Roberts and Pettinati, 1957). Higher initial lactose concentrations are possible at higher temperatures, and this requires a thermostable and thermoactive biocatalyst. Therefore, the β-glucosidase from the hyperthermophilic archaeon *Pyrococcus furiosus* (Kengen *et al.*, 1993) was used in this study. The enzyme purified from *P. furiosus* exhibits high β-galactosidase activity and remarkable thermostability, with a half life of 85 h at 100°C and 13 h at 110°C. To produce larger quantities of enzyme, the corresponding gene has been identified and overexpressed in *Escherichia coli*. The β-glucosidase produced by *E. coli* exhibits similar kinetic properties and a comparable stability as the β-glucosidase purified from *P. furiosus* (Voorhorst *et al.*, 1995). Recently, this β-glucosidase has been used in transglycosylation reactions with various structurally different aglycones (Fischer *et al.*, 1996). The free and immobilised enzyme showed a broad substrate selectivity by accepting primary and tertiary organic alcohols.

The aim of this research is to investigate the oligosaccharide synthesis with the thermostable β-glucosidase from *P. furiosus*, with specific attention to the effect of enzyme concentration, pH, glucose and galactose concentration, initial lactose concentration, and temperature.

Materials and methods

Materials

Lactose was from Sigma (St. Louis, MO) and the other chemicals were from Merck (Darmstadt, Germany). β-Glucosidase from *P. furiosus* (13 or 15 ONPG-units/μl) was prepared from an *E. coli* lysate and heated to denature proteins other than the enzyme as described previously (Voorhorst *et al.*, 1995). Other chemicals were used without further purification.

Batch experiments

In a typical batch experiment, 0.04 mol lactose was added to 20 ml 0.02 M sodium-phosphate buffer at pH 5.0. The bottles were shaken by an end-over-end incubator (110 rpm) at 75°C. When the lactose was dissolved, 1500 U enzyme were added. The enzyme concentration was expressed in U/g solution and the initial lactose concentration in mol/kg solution.

The reaction was followed for approximately 50 h. Samples (80 μl) were taken at regular time intervals, and the reaction was stopped by cooling on ice for 15 min. The samples were treated with $Pb(NO_3)_2$ (concentration in the sample 0.1 M), and stored in the freezer to accelerate precipitation (minimum time 1 h). Before analysis, the samples were centrifuged for 10 min at 13000 rpm and diluted.

Initial lactose conversion rate was expressed in mmol lactose per kg solution per min and was determined over 10% initial lactose decrease. The initial oligosaccharide production rate was expressed in mmol oligosaccharides (sum of tri- and tetrasaccharides) per kg solution per min. The trisaccharide yield was defined as mol trisaccharides per mol lactose initially present divided by the theoretical maximum (0.5 mol trisaccharides per mol lactose).

HPLC analysis

The samples were analysed by HPLC, using a RSO oligosaccharide column (Phenomenex, the Netherlands) at 80°C. The column was eluted with double-distilled water (filtered through a regenerated cellulose membrane of 0.45 μm and purged with helium gas) at a flow rate of 0.3 ml/min. The eluent was monitored by means of a refractive index detector. Lactose, glucose, galactose, and higher saccharides, such as tri- and tetrasaccharides, were detected.

Results and discussion

Time-course lactose conversion and oligosaccharide synthesis

Figure 1 shows the lactose conversion and oligosaccharide synthesis by the *P. furiosus* β-glucosidase in a typical experiment. Lactose was hydrolysed to glucose and galactose, and, in addition, tri- and tetrasaccharides were produced. The synthesis of pentasaccharides was not observed under these conditions.

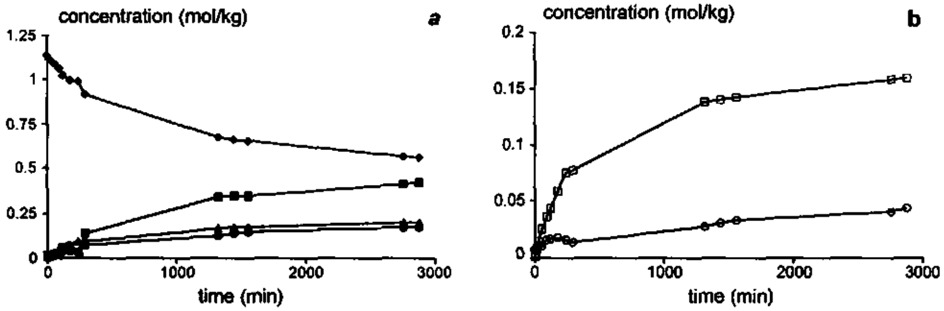

Figure 1 Time-course of lactose conversion (a) (◆, lactose; ■, glucose; ●, galactose, and ▲, oligosaccharide) and oligosaccharide synthesis (b) (□, trisaccharide and O, tetrasaccharide) (lines for guidance). Initial lactose concentration 1.16 mol/kg; enzyme concentration 75 U/g; sodium-phosphate buffer pH 5.0; temperature 75°C.

Enzyme concentration

The β-glucosidase concentration was varied to determine if the enzyme activity was linear with its concentration. Figure 2 shows that the initial lactose conversion rate and the initial oligosaccharide production rate were linear with the enzyme concentration. The ratio (initial oligosaccharide production rate divided by initial lactose conversion rate) was also linear with the enzyme concentration, and showed a minor decrease with the enzyme concentration. This suggests a dependence of the ratio on the enzyme concentration. Further experiments were carried out with an enzyme concentration of 75 U/g.

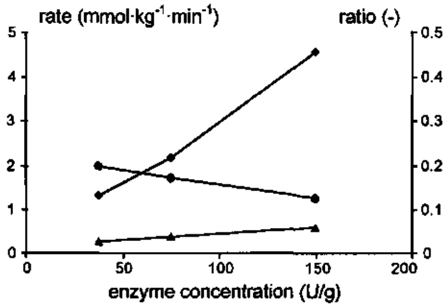

Figure 2 Initial lactose conversion rate (positive value) (◆), the initial oligosaccharide production rate (▲), and the ratio (initial oligosaccharide production rate divided by initial lactose conversion rate) (●) as function of the enzyme concentration (lines for guidance). Initial lactose concentration 1.16 mol/kg; sodium-phosphate buffer pH 5.0; temperature 75°C.

pH

Figure 3a shows that the initial lactose conversion rate was independent of the pH from 5.0-7.0, and was lower at pH 4.0. Kengen *et al.* (1993) found an optimum pH at 5.0 for the hydrolysis of GlcpβNp in a sodium-citrate buffer at 80°C. The ratio (initial oligosaccharide production rate divided by initial lactose conversion rate) did not depend on the pH in this range (data not shown). No decrease in the yield was measured within the time range of the experiments, except for pH 4.0. Hence, for the other experiments, the yield after 45 h was taken. The yield after 45 h was highest at pH 6.0 (Figure 3b).

It was observed that Maillard reactions occurred during the experiments. This is the reaction between a carbonyl group of a reducing sugar with a free, uncharged amine group

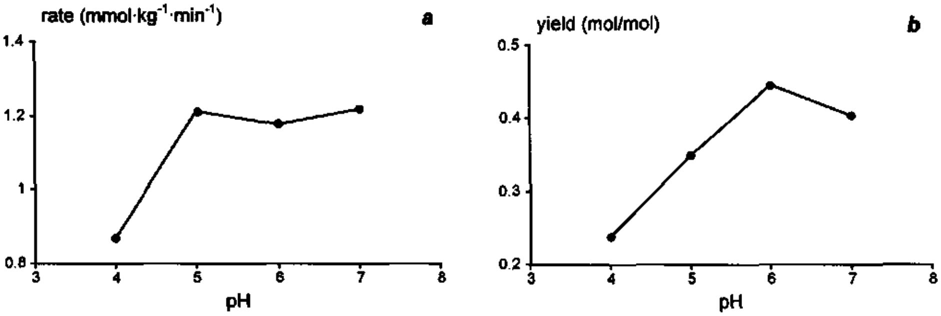

Figure 3 Initial lactose conversion rate (positive value) (a), and the yield after 45 h (b) as function of the pH (lines for guidance). Initial lactose concentration 1.16 mol/kg; enzyme concentration 75 U/g; 0.02 M sodium-phosphate buffer; temperature 75°C.

of an amino acid or protein (Labuza and Baisier, 1992). The yellow colour caused by these reactions intensified with increased pH. It is known that Maillard reactions are suppressed at low pH (Labuza and Baisier, 1992), and hence further experiments were performed at pH 5.0.

Glucose and galactose concentration

Deschavanne *et al.* (1978) found that most β-galactosidases are inhibited by the monosaccharides produced. To investigate product inhibition with the β-glucosidase from *P. furiosus*, experiments with additional glucose and galactose were done. Figure 4 shows that the enzyme was inhibited significantly by glucose, and to a lesser extent by galactose. Galactose inhibition will be of minor importance, because the galactose concentration is relatively low during oligosaccharide synthesis.

Initial lactose concentration and temperature

Table I shows the conversion rates and the ratio as functions of temperature and initial lactose concentration. As expected, the initial lactose conversion rate and the oligosaccharide production rate both increased with increasing temperature. The rates did not appear to depend on the initial lactose concentration, but the ratio did increase slightly with the initial lactose concentration. This confirmed the anticipated higher oligosaccharide yields at higher initial lactose concentrations (Burvall *et al.*, 1979; Roberts and Pettinati, 1957).

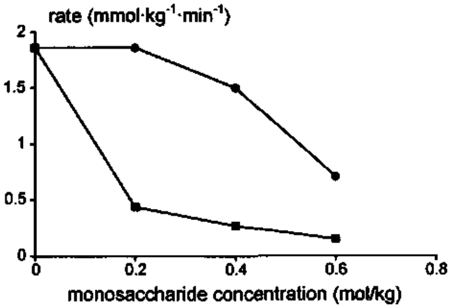

Figure 4 Initial lactose conversion rate (positive value) as function of the glucose (■) and galactose concentration (●) (lines for guidance). Initial lactose concentration 0.40 mol/kg; enzyme concentration 75 U/g; sodium-phosphate buffer pH 5.0; temperature 75°C.

Figure 5a shows the yield as function of the initial lactose concentration at different temperatures. The oligosaccharide yield was not very dependent on the initial lactose concentration and temperature. Onishi *et al.* (1995) compared the production of oligosaccharides at 30°C by several micro-organisms, and found yields below 0.33. The highest yield measured here for the β-glucosidase from *P. furiosus* is 0.46. This is about

Table I Initial lactose conversion rate (positive value), initial oligosaccharide production rate and ratio (initial oligosaccharide production rate divided by initial lactose conversion rate) as function of the initial lactose concentration and temperature.

initial lactose (mol/kg)	initial lactose conversion rate (mmol·kg⁻¹·min⁻¹)			initial oligosaccharide production rate (mmol·kg⁻¹·min⁻¹)			ratio (mol/mol)		
	75°C	85°C	95°C	75°C	85°C	95°C	75°C	85°C	95°C
0.73	1.6	-	-	0.32	-	-	0.20	-	-
0.93	1.4	2.8	3.4	0.31	0.68	1.1	0.23	0.24	0.32
1.01	1.6	-	-	0.40	-	-	0.25	-	-
1.09	1.1	-	-	0.30	-	-	0.28	-	-
1.16	0.89	1.9	3.3	0.45	0.65	1.3	0.50	0.35	0.39
1.23	-	2.0	-	-	0.71	-	-	0.36	-
1.29	-	2.1	-	-	0.79	-	-	0.37	-
1.37	-	1.5	2.4	-	0.65	0.79	-	0.42	0.33
1.44	-	-	2.2	-	-	0.94	-	-	0.43

Note: enzyme concentration 75 U/g; sodium-phosphate buffer pH 5.0.

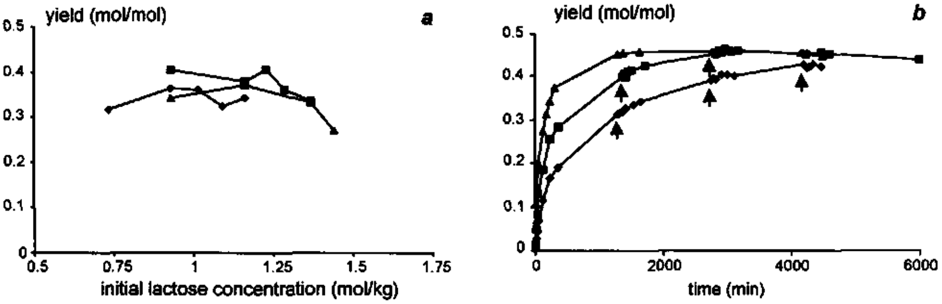

Figure 5 Yield as function of the initial lactose concentration at different temperatures (a) and time course of yield at different temperatures (b) (◆, 75°C; ■, 85°C, and ▲, 95°C) (lines for guidance). Experimental conditions in (a): enzyme concentration 75 U/g; sodium-phosphate buffer pH 5.0. Experimental conditions in (b): initial lactose concentration 1.16 mol/kg; enzyme concentration 75 U/g except at 95°C 150 U/g (arrows indicate addition of 750 U enzyme); sodium-phosphate buffer pH 5.0.

the same as reported for the thermostable β-galactosidase from *Sterigmatomyces elviae* (Onishi and Tanaka, 1995). Experiments with excess enzyme amounts did not result in higher yields (Figure 5b). This suggests enzyme inactivation.

The enzyme inactivation is most likely caused by Maillard products. The enzyme inactivation observed in sugar solutions was high compared to water, in which the half life was 85 h at 100°C and 13 h at 110°C (Kengen *et al.*, 1993). Inactivation caused to Maillard products (and inhibition by glucose) might be avoided in a continuous operating process. The stability of the β-glucosidase may also be further increased by immobilisation (Fischer *et al.*, 1996). This would result in higher oligosaccharide yields.

Conclusions

Synthesis of oligosaccharides by the *P. furiosus* β-glucosidase was observed under all reaction conditions studied in this research. With an enzyme concentration of 75 U/g, the enzyme activity is linear with the enzyme concentration, and lactose conversion is accomplished in an acceptable time. The *P. furiosus* enzyme has a broad pH optimum, which allows operation at a low pH (5.0), to diminish the production of Maillard components. The production of inhibiting Maillard components is more pronounced at higher temperatures, and therefore oligosaccharide synthesis appears to be best at the lowest temperature (75°C) studied. Increased initial lactose concentrations resulted in increased oligosaccharide concentrations, but did not affect the oligosaccharide yields. However, the oligosaccharide yield obtained with the *P. furiosus* β-glucosidase was a factor 1.4 higher than reported for mesophilic enzymes.

Acknowledgements

This work was supported by the Association of Biotechnological Schools (ABON), Borculo Domo Ingredients, and the European Union FAIR contract CT-96-1048.

5

Product removal during synthesis

Abstract

In this chapter, the enzymatic synthesis of oligosaccharides, which is an example of a kinetically controlled reaction, is studied. The aim is to show that the product yield can be enhanced by continuous removal of oligosaccharides from the reaction mixture. The oligosaccharides were removed by adsorption on activated carbon. The adsorption could be described by the multi-component Langmuir isotherm with different maximum saturation concentration for mono-, di-, and trisaccharides. The affinity for trisaccharides was larger (k_{tri} = 3.52 l/g) than for di- (k_{di} = 0.94 l/g) and monosaccharides (k_{mono} = 0.11 l/g).

A model combining kinetics, adsorption on activated carbon, and mass transfer in an adsorption column was developed. Model calculations for the batch process with removal showed a yield improvement of 23% compared to the batch process without removal. Experimentally a yield improvement of 30% was obtained. Model calculations for the continuous processes studied did not result in an increase of the yield. The advantages of removal were masked by the negative influence of re-circulation and the relative large time between formation and removal.

This chapter has been submitted as: Boon MA, van 't Riet K, Janssen AEM. 1999. Enzymatic synthesis of oligosaccharides: product removal during a kinetically controlled reaction.

Introduction

There is general agreement that the intestinal microflora, e.g. bifidobacteria, has a strong influence on the human health (Crittenden, 1999; Gibson *et al.*, 1996; Ziemer and Gibson, 1998). Consequently, there is an interest in the manipulation of the composition of this flora. Two approaches exist to increase the number or activity of health-promoting organisms in the gastrointestinal tract. Organisms (probiotics) or a selective carbon source (prebiotics) can be added to the food. Prerequisites of prebiotics are that they must reach the colon intact and act there as a specific substrate for desired bacteria. Oligosaccharides are a prebiotic.

Oligosaccharides can be produced by enzymatic synthesis with a glycosidase as catalyst and a disaccharide as substrate (Crittenden and Playne, 1996). Oligosaccharide formation using the transferase activity of glycosidases is a kinetically controlled reaction. The fundamentals of this approach were described and discussed by Kasche (1986). Other examples of kinetically controlled reactions by enzymes are the peptide synthesis by proteases, and the β-lactam antibiotics synthesis (Nam *et al.*, 1985).

During enzymatic kinetically controlled reactions, a nucleophile attacks the substrate-enzyme complex to give the desired product. At the same time water also acts as a nucleophile and competitive hydrolysis of the substrate-enzyme complex takes places. The hydrolysis is thermodynamically favoured. Therefore, when following the reaction in time, an optimum in the product concentration is observed. The synthesis of such a kinetically controlled product can be enhanced by continuous product removal.

In this research, the synthesis of trisaccharides is studied using the β-galactosidase from *Bacillus circulans* as a catalyst and lactose as a substrate. Lactose serves both as substrate and as nucleophile, which attacks the substrate-enzyme complex. The trisaccharides are removed during the synthesis by activated carbon. Both experimental results and model calculations are used.

Separation of sugars

For the removal of trisaccharides from the reaction mixture, is it important that the desired product (trisaccharides) is separated from the substrate (lactose) and the enzyme to prevent product hydrolysis. Sugars can be separated on a larger scale, using ion-exchange resins or activated carbon.

In chromatographic applications of ion-exchange resins to separate sugars, mainly cation-exchange resins are used (Jandera and Churácek, 1974). Cation-exchange resins have the highest affinity for monosaccharides and therefore trisaccharides are the first to elute from the column. Activated carbon has a higher affinity for trisaccharides, compared to mono-, and disaccharides. The separation of sugar classes by activated carbon is a technique used in industry (Whistler and Durso, 1950). Operation will be easier when the carrier has a larger affinity for the product than for the substrate since regeneration can take place off-line without large substrate losses. Hence, activated carbon is used in this study.

Activated carbon has also been used by Ajisaka *et al.* (1987b) who studied the synthesis of disaccharides with monosaccharides as a substrate and a β-galactosidase as a catalyst. This enzymatic synthesis is an equilibrium controlled reaction. Every small amount of disaccharides produced was eliminated from the reaction mixture by activated carbon. An improvement of the yield was obtained.

Theory

Mainly three processes are relevant in a system in which the enzymatic synthesis of trisaccharides is combined with continuous removal by activated carbon in a packed column; enzyme kinetics, adsorption, and mass transfer. These processes will be discussed next.

Enzyme kinetics

In our previous work (chapter 2) a kinetic model (Figure 1) was developed to describe the oligosaccharide synthesis and lactose hydrolysis. The synthesis of oligosaccharides was lumped together in the production of trisaccharides. To derive the rate expressions, the approach of King and Altman (1956) was used. The preliminary data treatment and lumped model parameters determination were described in chapter 2.

Adsorption

A monolayer at the surface is created when the adsorption is reversible and interaction between adsorbed molecules is neglected. The adsorption equilibrium for a single-component systems can than be described by the Langmuir equation. For component *i* the isotherm is as follows

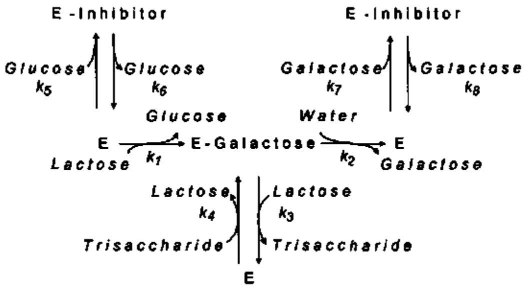

Figure 1 Kinetic model for the enzymatic synthesis of trisaccharides with lactose as a substrate and a β-galactosidase as catalyst.

$$q_{e,i} = \frac{q_{s,i} k_i C^*_{e,i}}{1 + k_i C^*_{e,i}} \tag{1}$$

in which		
	$q_{e,i}$	bound equilibrium concentration (mg/g)
	$C^*_{e,i}$	free equilibrium concentration (g/l)
	$q_{s,i}$	saturation concentration (mg/g)
	k_i	affinity constant (l/g)

The Langmuir equation was adapted for competitive adsorption by Butler and Ockrent (1930). The multi-component Langmuir isotherm for a mixture of three components (i, j, and k) with different saturation concentrations is given by

$$q_{e,i} = \frac{q_s k_i C^*_{e,i}}{1 + k_i C^*_{e,i} \frac{q_s}{q_{s,i}} + k_j C^*_{e,j} \frac{q_s}{q_{s,j}} + k_k C^*_{e,k} \frac{q_s}{q_{s,k}}} \tag{2}$$

in which		
	q_s	maximum saturation concentration of the components present in the mixture (mg/g)

The ratio of the saturation concentrations is a measure for the available surface area for the different components. The affinity constants and saturation concentrations can be obtained from single-component adsorption data.

Mass transfer

The amount adsorbed in a packed column is not only dependent on the adsorption capacity, but also on the column characteristics, such as the residence time distribution and operation time. Usually the rate-determining process on microscale is the diffusion of the component from the bulk liquid through the stagnant film to the surface.

A packed column can be modelled as a series of continuous-flow stirred-tank reactors (CSTR's). It was assumed that the number of transfer units was equal to the number of CSTR's in series. Hence, in every stage equilibrium between solution and activated carbon was assumed. A mass balance over the free phase and stationary phase for all components was used. The mass balances for component *i* in stage *m* are

$$\frac{\mathrm{d}C_{i,m}}{\mathrm{d}t} = \frac{(C_{i,m-1} - C_{i,m})}{\tau_m} - k_{lump}(C_{i,m} - C^{*}_{i,e,m}) \tag{3}$$

$$\frac{\mathrm{d}q_{i,m}}{\mathrm{d}t} = k_{lump}\frac{1-\varepsilon}{\varepsilon}(C_{i,m} - C^{*}_{i,e,m}) \tag{4}$$

in which	C	free concentration (g/l)
	q	bound concentration (g/l)
	ε	volume fraction carrier (-)
	$C^{*}_{i,e}$	free concentration that would be in equilibrium with the activated carbon (g/l)
	k_{lump}	lumped mass transfer coefficient (1/min)
	τ	residence time of the liquid phase (min)

the subscript *e* denotes equilibrium.

The driving force for mass transfer is assumed to be equal to the difference between the actual free concentration ($C_{i,m}$) and the free concentration that would be in equilibrium with the carbon ($C^{*}_{i,e,,m}$). An equation can be derived from the multi-component Langmuir isotherm in which the free equilibrium concentration in stage *m* is written as function of the bound equilibrium concentrations

$$C^{*}_{i,e,m} = \frac{q_{i,e,m}}{k_i\left(q_s - q_{i,e,m}\dfrac{q_s}{q_{i,s,m}} - q_{j,e,m}\dfrac{q_s}{q_{j,s,m}} - q_{k,e,m}\dfrac{q_s}{q_{k,s,m}}\right)} \tag{5}$$

The effects of axial mixing, mass transfer outside the particle, macropore and microparticle mass transfer are lumped in one mass transfer coefficient k_{lump}. The lumped mass transfer coefficient is coupled to the number of transfer units (n_c (-)) using

$$n_c = \frac{k_{lump} h}{\upsilon_s} \tag{6}$$

in which h height of the adsorption column (m)
v_s superficial velocity (m/min)
(Perry and Green, 1984 (c)).

Materials and methods

Materials

Lactose, galactose, and maltotriose were obtained from Sigma (St. Louis, USA), the activated carbon (Darco, 20-40 mesh, granular, density 1.8 g/ml) was acquired from Aldrich (Wisconsin, USA). All other chemicals were obtained from Merck (Darmstadt, Germany). The β-galactosidase from *Bacillus circulans* was obtained from Daiwa Kasai K. K. (Osaka, Japan). Eupergit C® beads were obtained from Röhm (Darmstadt, Germany). All were used without further purification.

Equilibrium experiments for determination Langmuir parameters

In a typical equilibrium experiment 1.6 g activated carbon was added to 8 ml 0.02 M McIlvaine standard citrate-phosphate buffer at pH 5.0 containing one or several sugars in various concentrations. For the mono-, di-, and trisaccharides respectively 66, 43, and 22 experiments were carried out in the concentration range of 6-108 g/l. For the mixture of several sugars 31 experiments were carried out in the concentration range of 3-75 g/l.

The bottles were shaken by an end-over-end incubator at room temperature. To estimate the time needed to reach equilibrium, adsorption was followed in time. After 1 h equilibrium was reached. To be certain after 24 h the equilibrium experiments were terminated. A sample was taken after precipitation of the activated carbon and a known amount of lactose, glucose, galactose, or maltotriose was added for concentration determination. The samples were treated with $Pb(NO_3)_2$ (concentration in the sample 0.1

M) and stored in the freezer to accelerate precipitation (minimum time 1 h). Before HPLC analysis the samples were centrifuged 10 min at 13000 rpm and diluted.

Breakthrough curves

Before the column (length 1 m, internal diameter 2.5 cm) (Pharmacia Biotech, Sweden) was packed with activated carbon (approximately 150 g), air was removed from the activated carbon macropores and filled with water. The packed column (74.5 cm) was pre-treated with a 0.02 M McIlvaine buffer (pH 5.0). The breakthrough curve of a sugar mixture containing lactose (5.9 g/l), galactose (5.8 g/l), and maltotriose (2.9 g/l) with a 0.02 M McIlvaine buffer at pH 5.0 as aqueous phase was determined. A second breakthrough curve was determined using a packed column (86.5 cm) pre-treated with a lactose solution (5.9 g/l). A sugar mixture containing lactose (5.9 g/l), galactose (5.9 g/l), and maltotriose (3.0 g/l) was used.

In both experiments the sugar solution was fed at the bottom of the column (3.12 ml/min) and samples of 25 ml were collected at the top. The sugars adsorbed to the activated carbon were recovered off-line using a 25% (v/v) ethanol-water solution. Elution took place over night (flow rate 1 ml/min) and one fraction of 2 l was collected. Prior to HPLC analysis ethanol was removed by distillation. The concentrations were determined in triplicate. With a mass balance was checked whether all sugars were regained in the outlet and in the ethanol fraction.

For all samples glucose was used as internal standard. Sample treatment was carried out as described in the previous section and were analysed on HPLC.

Continuous removal during synthesis

In Figure 2 the experimental set-up is shown schematically. Two experimental set-ups were used. One with the column filled with activated carbon (a) and the other one with the column filled with a lactose solution (reference system, b). The reference system was studied for comparison.

To prevent the enzyme from entering the column, β-galactosidase from *B. circulans* was immobilised as described by Fischer *et al.* (1996). 1.5 g β-galactosidase was incubated with 20 g Eupergit C® beads in 50 ml at 40°C for 6 h. During immobilisation the beads swelled and 75 g was obtained. To check the activity of the immobilised enzyme, 10 g of these beads were used for a kinetic experiment. In this experiment the lactose conversion in a stirred vessel (volume 50 ml) at 40°C with an initial lactose concentration of 0.25

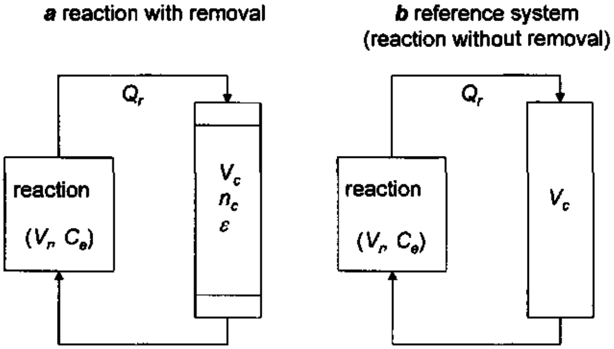

Figure 2 Scheme of continuously product removal (a) and the reference system without removal (b) for a batch process (V_r, volume reactor; C_e, enzyme concentration; V_c, volume column; n_c, number of transfer units; ε, porosity).

mol/kg was followed for 6 h. A 0.02 M McIlvaine buffer at pH 5.0 was used as aqueous solution.

Lactose conversion according to the set-ups in Figure 2 was carried out in a stirred vessel with a reaction mixture of 150 ml at 40°C with an initial lactose concentration of 0.25 mol/kg. The McIlvaine buffer mentioned before was used as aqueous solution. Immobilised enzyme (30 gram beads) was added to each reactor vessel. The reaction mixture was filtered and re-circulated over the column (flow rate 15 ml/min).

The column filled with activated carbon was packed as described in the previous section. The column was saturated with a lactose solution (0.30 mol/kg). The lactose concentration in the column of the reference system was 0.35 mol/kg. The total amount of lactose was identical in both systems and the same column height was used.

The reaction was followed for 6 or 12 h and samples were taken at regular time intervals. The enzyme was inactivated by heating the sample in a water bath at 90°C for 5 min. At the end of the experiment the yield in the total system was determined. The sugars adsorbed to the activated carbon were recovered off-line as described in the previous section. Concentrations were determined in duplicate.

A known amount of glucose was added for concentration determination. Sample treatment was carried out as described in *Equilibrium experiments for determination Langmuir parameters* and were analysed on HPLC.

HPLC analysis

The samples were analysed by HPLC using a RSO Oligosaccharide Column (Phenomenex, the Netherlands) at 80°C. The column was eluted with double distilled water (filtered through a regenerated cellulose membrane of 0.45 μm and purged with helium gas) at a flow rate of 0.3 ml/min. The eluent was monitored by means of a refractive index detector. An internal standard or the addition method were used for concentration determination. Response factors were determined for each series of samples.

Results and discussion

Estimation of Langmuir parameters

Equilibrium experiments in single-component systems were carried out to determine the Langmuir parameters for mono-, di-, and trisaccharides. Glucose and galactose were used to estimate the parameters for monosaccharides, maltodiose, and lactose for disaccharides, and maltotriose for trisaccharides. The measurements and fitted Langmuir isotherm (eq. 1) are shown in Figure 3, the parameters are listed in Table I. The Langmuir parameters were determined by minimisation of the quadratic difference between the measured and predicted free concentration.

Table I Langmuir parameters with 95% confidence interval for mono-, di-, and trisaccharides determined in single-component experiments.

	k (l/g)	q_s (mg/g)
monosaccharides	0.106 ± 0.012	92.0 ± 2.7
disaccharides	0.944 ± 0.31	117.7 ± 2.5
trisaccharides	2.05 ± 1.0	133.2 ± 3.1

The sugars could be grouped in mono-, di-, and trisaccharides. The affinity constant was highest for trisaccharides, which meant that the activated carbon had the highest affinity for trisaccharides. The lowest affinity was found for monosaccharides. The 95% confidence interval of k_{tri} was relatively large. This was the result of a high affinity and the necessity to measure at low free concentrations.

Three saturation concentrations were found. When every sugar moiety occupied the same space, one saturation concentration on weight basis would be found. When all molecules occupied the same space on the surface a ratio of one, two, and three between the saturation concentrations on weight basis for mono-, di-, and trisaccharides would be

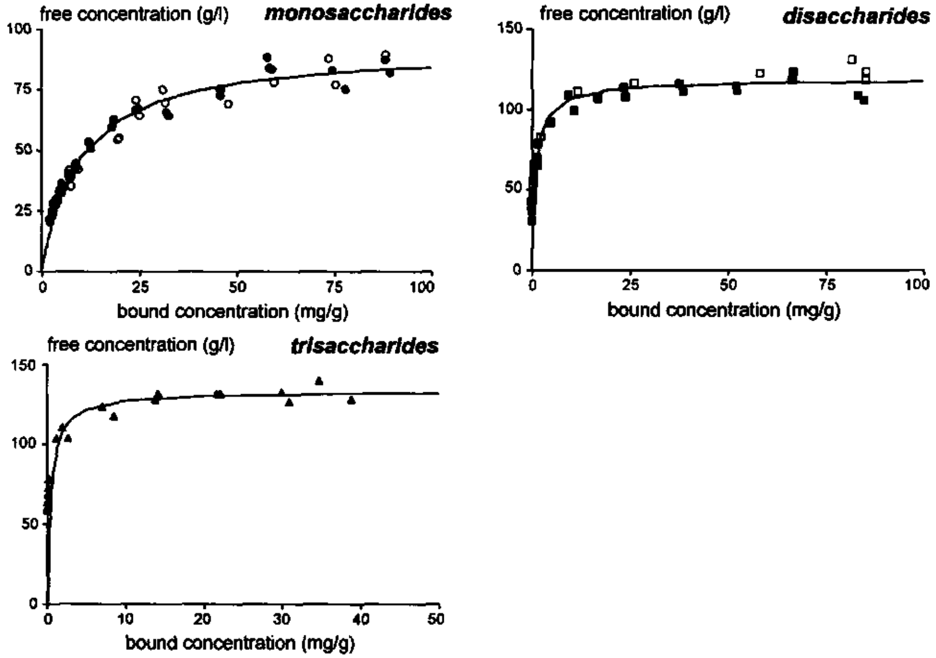

Figure 3 Data single-component equilibrium experiments with fitted Langmuir isotherm for mono-di-, and trisaccharides (O, glucose; ●, galactose; ■, lactose; □, maltodiose, and ▲, maltotriose). Experimental conditions as described in *Equilibrium experiments for determination Langmuir parameters*, Langmuir parameters are given in Table I.

found. The reality of the surface coverage was somewhere in between these extremes; the ratios found were 1, 1.3, and 1.4. When the saturation concentration had unity mg/g, q_s (eq. 2) equalled $q_{s,tri}$ (Table I).

To determine if the multi-component system could be predicted with the parameters determined in the single-component system, equilibrium experiments with sugar mixtures were carried out. The multi-component Langmuir isotherm (eq. 2) was used to predict the multi-component system. The parity plot of the equilibrium experiments of multi-component systems is shown in Figure 4. The results of the multi-component system were satisfactory predicted with the parameters determined in single-component systems. The absolute error with its 95% confidence interval was 1.06 ± 0.19 g/l. No structural deviation was found.

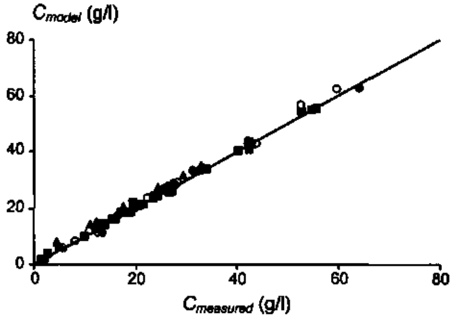

Figure 4 Parity plot data multi-component equilibrium experiments. Model predictions multi-component Langmuir isotherm with parameters as listed in Table I. C_{model} is predicted free concentration, $C_{measured}$ is measured free concentration (O, glucose; ●, galactose; ■, lactose, and ▲, maltotriose). Experimental conditions as described in *Equilibrium experiments for determination Langmuir parameters*.

Breakthrough curves

Adsorption in a packed column in combination with the enzymatic reaction in a vessel was used to show the advantages of continuous product removal. To link these two processes, breakthrough curves of multi-component systems were measured and modelled. The adsorption isotherm was combined with a model to describe the mass transfer processes in a packed-column (eq. 3 and 4).

The breakthrough curve was determined in two ways; one with the column pre-treated with buffer and another one with the column pre-treated with lactose. The fitted lines and the data of the breakthrough curve are shown in Figure 5. The fitted parameters and the mass balances are shown in Table II.

The breakthrough curve of the column pre-treated with buffer shows that the affinity was highest for tri- and lowest for monosaccharides. This was in agreement with the Langmuir isotherms. For mono- and disaccharides a peak of excess concentration was observed. This is a common phenomenon in multi-component adsorption in a packed column caused by a displacement effect (Huang *et al.*, 1995). The column pre-treated with lactose was not saturated at the beginning of the experiment; a lower lactose concentration was measured than the feed concentration. The breakthrough of monosaccharides was faster compared to the column pre-treated with buffer since less adsorption sites were available. The mass balance was satisfactorily met for all sugars (Table II).

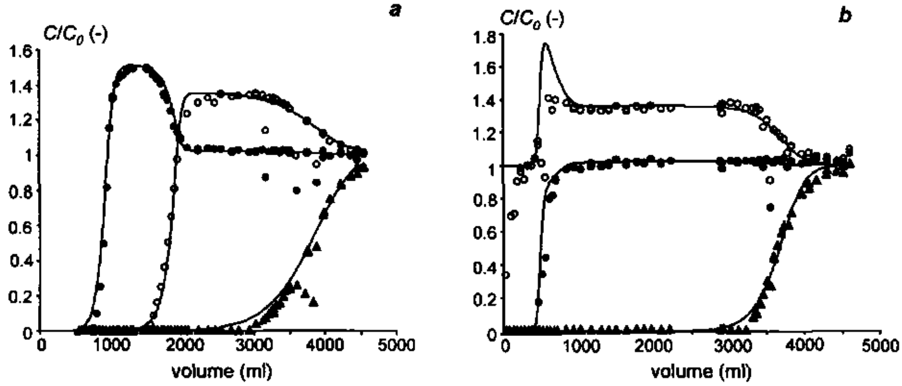

Figure 5 Experimental data and fitted model predictions of the breakthrough curves for columns pre-treated with buffer (a) and lactose (b). The mixture contained lactose (○), galactose (●) and maltotriose (▲). Fitted model parameters and mass-balances are listed in Table II. Experimental conditions as described in *Breakthrough curves*.

The excess peak of lactose from 500-1000 ml predicted by model was not measured in the second breakthrough curve, therefore, the measurements before 1500 ml were not taken into account. Otherwise the large deviation between these measurements and the predicted line would dominate the best fit. The curves could not be adequately described with k_{tri} determined with the Langmuir adsorption isotherms. It was decided to fit, next to the number of transfer units (n_c) and the porosity (ε), k_{tri} to the breakthrough data. The k_{tri}

Table II Fitted model parameters and mass-balances for the measured breakthrough curves of the columns pre-treated with buffer and lactose.

		column pre-treated with buffer	column pre-treated with lactose
mass-balance (%)	lactose	96.5	-
	galactose	98.4	99.1
	maltotriose	96.1	99.4
fitted parameters	n_c (-)	36	70
	ε (-)	0.210	0.185
	k_{tri} (l/g)	3.77	3.26

Note: the mass balance is the percentage of the sugars regained in the outlet and in the ethanol fraction. The mass balance for lactose was not determined for the experiment with the column pre-treated with lactose, since the amount of lactose in the column at the start of the experiment was not measured. It was not possible to determine the 95% confidence interval of the parameters since, according to the calculation method, n_c was a whole number.

determined with the breakthrough curves was higher than found in the equilibrium experiments of Table I. It should be noted that the determination of k_{tri} using equilibrium experiments was not very accurate, caused by its high value. The single-component equilibrium data could be described satisfactorily with a higher affinity constant. The RSS increased from 8.6 to 11.5. The average k_{tri} (3.52 l/g) of the breakthrough curves was used for further model calculations.

Whereas the pump stopped pumping during the experiment with the column pre-treated with buffer, diffusion of the sugars had taken place. Consequently a low n_c was found in comparison with the experiment with the column pre-treated with lactose (Table II).

Continuous removal during synthesis

Based on the model (combining synthesis and removal) calculations were made for a batch and semi-continuous system. Firstly the model calculations for the batch process are discussed. Secondly experiments for the batch system were carried out to check the model predictions. Thirdly model calculations for the semi-continuous process are presented.

Model calculations batch system

Figure 2a shows a scheme of the batch process with continuous removal of trisaccharides. The enzymatic conversion of lactose takes place in the reactor vessel. The reaction mixture is continuously led over an adsorption column containing activated carbon and the immobilised enzyme is kept in the reactor.

It was assumed that the column was saturated with lactose (concentration solution equal to concentration in reaction mixture) to avoid dilution of the system. The initial lactose concentration was 0.3 mol/kg throughout all model calculations. The kinetic parameters used were determined for β-galactosidase from *B. circulans* (chapter 2). The system with product removal was compared to a system without product removal.

To assess the best process configuration an overall yield was defined as the amount of trisaccharides in the total system (reactor vessel and column (free and bound)) divided by the amount of lactose initially present in the total system and multiplied by two because two lactose molecules are needed to form one trisaccharide molecule. The influence of the re-circulation flow (Q_r), the ratio between the reactor and column volume (V_r:V_c), the enzyme concentration (C_e), and the number of transfer units (n_c) on the oligosaccharide yield was studied. These parameters can be used to control the process. Also the influence of the affinity constants of di- and trisaccharides (k_{di} and k_{tri}) on the yield was studied. These parameters are properties of the activated carbon and are not control parameters. The oligosaccharide yield in time for various conditions is shown in Figure 6. Changes in the yield were compared to the standard conditions.

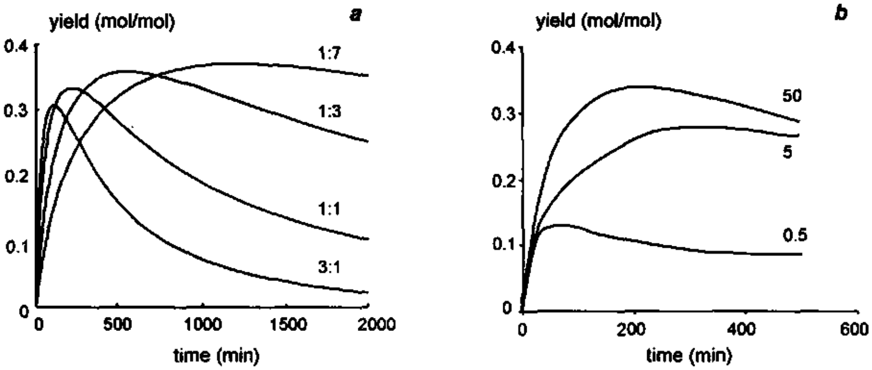

Figure 6 Calculated trisaccharide yield in a batch process as function of time for various volume ratios (V_r:V_c) (a) and re-circulation flows (Q_r) (b). Standard conditions: kinetic parameters k_1 = 0.019 1/min; k_2 = 1 1/min; k_3/k_4 = 4600; $1/k_4$ = 11.5 min; k_5/k_6 = 21.5; Q_r = 50 ml/min; V_t = 800 ml; volume ratio (V_r:V_c) 1:1; C_e = 1.25 g/l; ε = 0.225; n_c = 10; adsorption parameters as listed in Table I, except k_{tri} = 3.52 l/g.

The ratio V_r:V_c was varied at a constant total volume (V_t) of 800 ml (Figure 6a). A small reactor volume in combination with a large column volume was profitable since the adsorption capacity was large. A yield improvement of 11% was found for a ratio of 1:7 compared to a ratio of 1:1. At the same time the reaction time increased because a small reaction volume (with the same enzyme concentration) implicated less enzyme compared to the process with a larger reaction volume.

Figure 6b shows that the time for reaction (influenced by the enzyme concentration) should be small compared the time for re-circulation (Q_r). For an enzyme concentration of 1.25 g/l, the yield decreased 18% and 61% when Q_r was decreased a factor 10 and 100, respectively When Q_r is infinitely high, all trisaccharides produced are immediately removed.

A higher affinity constant for the substrate resulted in a lower trisaccharide yield because of a large loss of the substrate. A larger affinity constant for the product resulted in a better process performance. When the trisaccharide affinity was increased from 3.52 l/g to 100 l/g, the yield was improved from 0.33 to 0.43 mol/mol. The influence of column properties as ε and n_c on the yield was small, the adsorption capacity was more important (V_c) (data not shown).

The conditions chosen to compare this system with a reference system were Q_r = 50 ml/min; C_e = 1.25 g/l; V_t = 800 ml; V_r:V_c = 1:7; n_c = 10. The yield achieved in this system was 0.37 mol/mol. This yield will be compared with the yield in the reference system.

There are several possibilities to define the reference system without product removal. The reference system chosen here includes re-circulation, using an inert column (Figure 2b). The extra lactose introduced by the activated carbon column was assumed to be present in the total column volume of the reference system (concentration 0.35 mol/kg). The yield obtained in this system under the same conditions as chosen for the system with removal was 0.30 mol/mol.

The yield in the system with product removal (0.37 mol/mol) was higher than in the reference system (0.30 mol/mol). The difference was due to product removal. Therefore the product removal during a kinetically controlled reaction was advantageous. It was important that the rate of product removal was high in comparison with the production rate, that the affinity for the substrate was low and the affinity for the product was high, and that the adsorption capacity was large enough.

Experiments batch system

Two sets of experiments were carried out (Figure 2). Each set consisted of an experiment with a column filled with activated carbon (a) and a column filled with a lactose solution (b). To prevent the enzyme to enter the column, the enzyme was immobilised. Before the column experiments were performed the activity of the immobilised enzyme was determined four times in a batch experiment. Figure 7 shows the time course of one of these experiments. At the optimum trisaccharide concentration, the average oligosaccharide yield was 0.39 mol/mol.

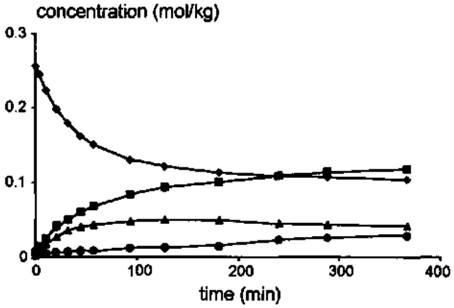

Figure 7 Experimental time-course for lactose conversion in batch reactor for immobilised enzyme (◆, lactose; ■, glucose; ●, galactose, and ▲, oligosaccharides) (lines for guidance).

The enzyme immobilisation method was reproducible. Unfortunately the concentrations in time could not be described with the kinetic parameters determined for the free enzyme. Even not when the data were corrected for the enzyme concentration. This might be caused by diffusion limitation in the beads or by a change of the enzyme conformation. The oligosaccharide yield measured with the immobilised enzyme was much higher than that predicted by the model. A substantial number of experiments would be needed to determine the kinetic parameters of the immobilised enzyme (chapter 2). Since immobilisation was just a tool to retain the enzyme in the reactor vessel, this behaviour was not studied in more detail.

The concentrations in the reactor of the two sets of experiments with a column filled with activated carbon (Figure 2a) and a column filled with a lactose solution (Figure 2b) are shown in Figure 8. In the beginning the additional lactose present in the columns

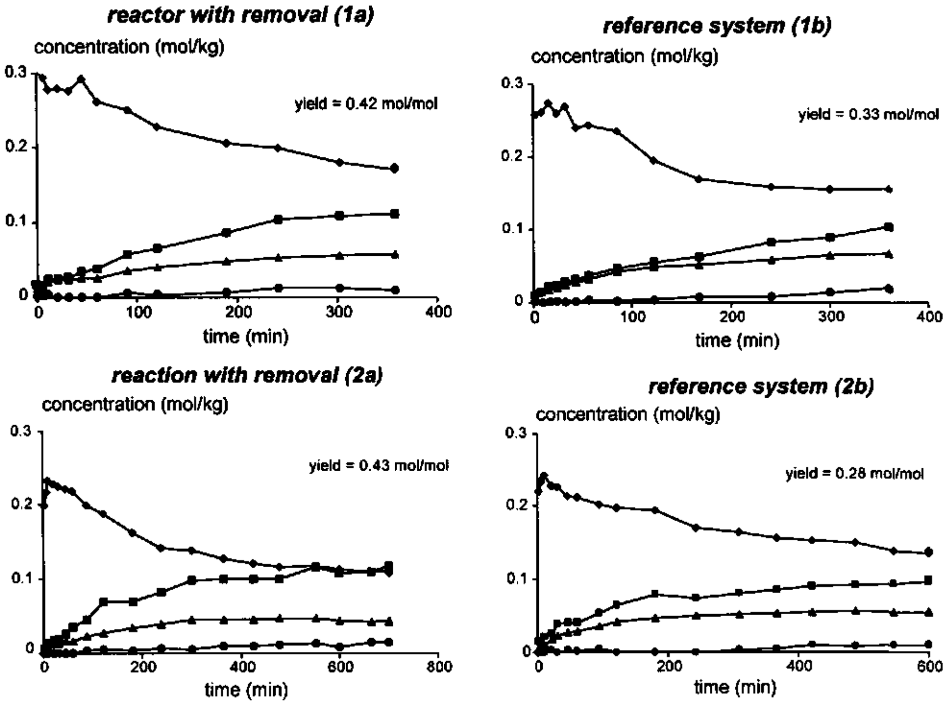

Figure 8 Measured concentrations in time in the reactor with removal (1a and 2a) and without removal (1b and 2b) (◆, lactose; ■, glucose; ●, galactose, and ▲, oligosaccharides). The yield at the end of the experiment is given in the graphs. Experimental conditions as described in *Continuous removal during synthesis* (lines for guidance).

resulted in an increase of the lactose concentration in the reactor, eventually conversion resulted in a decrease of the lactose concentration. The hydrolysis products glucose and galactose and the transferase product trisaccharides were formed. The oligosaccharide yields can only be determined at the end of the experiments and are also given in Figure 8.

In the experiments with activated carbon (1a and 2a) a higher yield was measured compared to the two experiments with the reference system (1b and 2b). The average yield determined in the experiments with activated carbon was 0.43 mol/mol (0.64 g/g). In the reference system a yield of 0.33 mol/mol (0.50 g/g) was found. For all these experiments it is uncertain if the maximum yield was obtained because only at the end of the experiment the yield can be determined. However the second experiment with activated column lasted 6 h longer than the first and no increase of the yield was measured.

The oligosaccharide yield measured with the immobilised enzyme was higher than measured with the free enzyme. Consequently model predictions and measurements for the experiments combining synthesis and removal could not be compared. However, the predicted yield improvement of 23% was in the same order of magnitude as the 30% improvement found in the experiments.

Model calculations semi-continuous system

Literature (Burvall *et al.*, 1979; Roberts and Pettinati, 1957) and calculations showed the advantage of using high initial lactose concentrations and therefore a plug-flow reactor (PFR) seemed to be a good configuration for a semi-continuous process. A PFR can be described as a series of CSTR's. When using three reactors in series, approximately 90% of the yield of a PFR could be achieved. By addition of a fourth the yield was only increased to 93%. Hence, three CSTR's in series were used. Furthermore calculations showed that an unequal distribution of V_r over three vessels had almost no influence on the performance of the reactor. For this reason the total volume was equally divided over the CSTR's. The residence time should be equal to the time needed to reach the maximum trisaccharide yield. The operation of three CSTR's on the maximum yield was chosen as starting point ($V_r = 400$ ml; $Q = 6.24$ ml/min; $C_e = 1.25$ g/l).

Model calculations were used to determine how the semi-continuous process should be operated. Assuming the availability of three vessels and two adsorption columns, two process configurations are possible (Figure 9). In system A two columns are placed in series, the outlet of the column is re-circulated and led back to the first CSTR. Re-circulation is essential for yield improvement. If there is no re-circulation the column is used as downstream processing. In system B the adsorption columns are placed between the vessels, re-circulation is not necessary. No column is placed at the outlet of the third vessel because this has no effect on the yield obtained in this system.

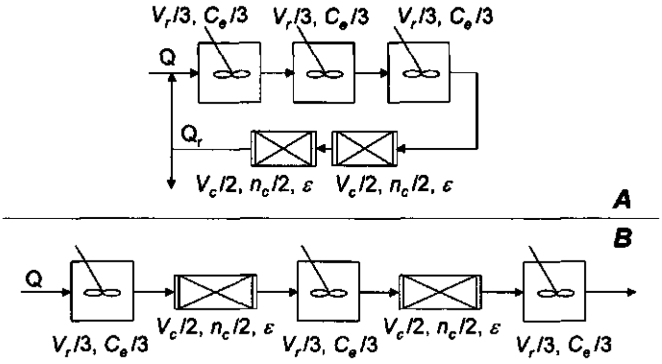

Figure 9 Scheme of two process configurations for continuously product removal in a semi-continuous process.

As in the batch system it was assumed that the columns were saturated with the initial lactose concentration (0.3 mol/kg). The same kinetic parameters and reference system were used as for the batch process. The reference system was operated under steady-state conditions since replacement of an inert column was useless.

To assess the best process configuration an overall yield was defined as the amount of trisaccharides in the total system (reactor vessel and column (free and bound)) and in the outlet of the system, divided by the amount of lactose initially present in the total system and in the feed, multiplied by two.

An assumption concerning the time for column replacement was made before the influences of process parameters were studied and the systems were compared to a reference system. The decision when the adsorption column should be regenerated is cost dependent. Here the columns were replaced when the yield in the column was highest (defined by the amount of trisaccharides in the column (free and bound) divided by the amount of lactose initially present in the column and in the feed of the system). The yield in the column should be as high as possible because the effect of the removal will then also be largest. In system A the second column replaced the first column when the yield in the first column was maximal, in system B the yield of the two columns were taken together and the columns were replaced simultaneously.

The influence of the re-circulation flow (Q_r), the reactor and column volume (V_r and V_c), the enzyme concentration (C_e) and the number of transfer units (n_c) on the oligosaccharide yield in the column was studied. In Figure 10 the time dependent oligosaccharide yield in the adsorption column under various conditions is shown.

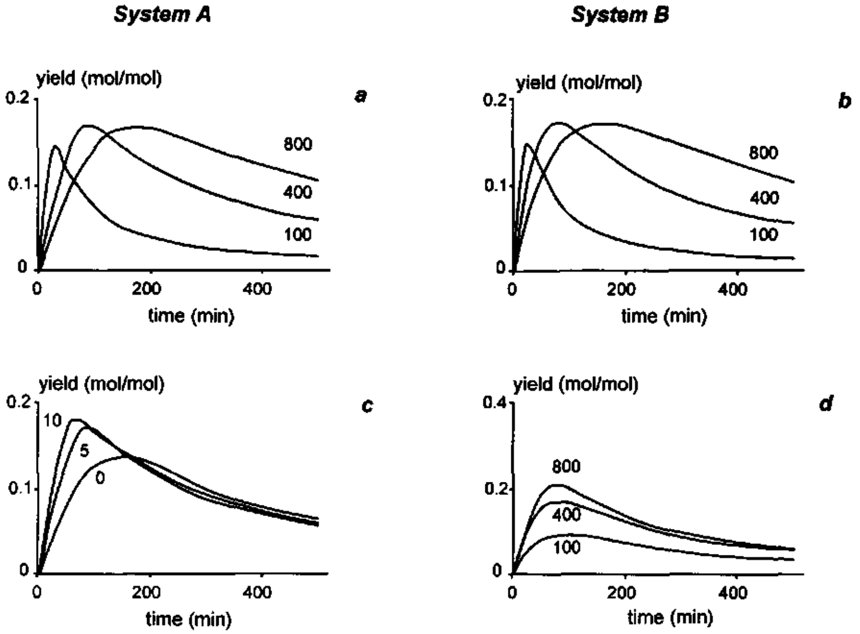

Figure 10 Calculated oligosaccharide yield in the adsorption column as function of time in semi-continuous processes A and B for various V_c (a and b), Q_r (c) and V_r (d). Standard conditions: kinetic parameters $k_1 = 0.019$ 1/min; $k_2 = 1$ 1/min; $k_3/k_4 = 4600$; $1/k_4 = 11.5$ min; $k_5/k_6 = 21.5$; $Q = 6.24$ ml/min; $Q_r = 5$ ml/min; $V_r = 400$ ml; ratio volumes (V_r:V_c) 1:1; $C_e = 1.25$ g/l; $\varepsilon = 0.225$; $n_c = 10$; adsorption parameters as listed in Table I, except $k_{tri} = 3.52$ l/g.

In system A an optimum column volume existed; for a V_c of 100, 400, and 800 ml a yield of 0.15, 0.17, and 0.16 mol/mol, respectively was found (Figure 10a). A small column had not enough adsorption capacity. A large column led to too much lactose loss when the re-circulation flow was not large enough. In system B the column should be large enough; for a V_c of 100, 400, and 800 ml a yield of respectively 0.15, 0.17, and 0.17 mol/mol was found (Figure 10b). In this system the flow through the adsorption column also passed through the next reaction vessel and no loss occurred. For all systems was found that the slope of the breakthrough curve (n_c) influenced the performance slightly.

Figure 10c shows that Q_r in system A should be as high as possible to obtain the largest yield in the column; the adsorption capacity in the column was used optimal. A two times as high Q_r resulted in a yield improvement of 7% (compared to the standard conditions). However, Q_r also influences the residence time distribution in the reaction vessels. When Q_r is much larger than Q the three CSTR's in series will behave as one CSTR. Therefore, the total yield in the system will drop at high Q_r caused by a change of the residence time distribution.

The trisaccharide concentration in the first reaction vessel is low and in system B this resulted in an inefficient use of the first column. Regarding the yield of both columns, a larger V_r was positive because a higher trisaccharide concentration in the vessels was obtained (Figure 10d). For a V_r of 400 ml, an optimum enzyme concentration of 4.4 g/l was found. This high enzyme concentration resulted in a lower trisaccharide yield in the reference system because the residence time was longer than the time needed to reach the maximum trisaccharide concentration.

The conditions chosen to compare system A with a reference system were Q_r = 10 ml/min; Q = 6.24 ml/min; C_e = 1.25 g/l; V_t = 800 ml; V_r:V_c = 1:1; n_c = 10. For system B the conditions were Q = 6.24 ml/min; C_e = 4.4 g/l; V_t = 800 ml; V_r:V_c = 1:1; n_c = 10. In Figure 11 the yield in the total system for the reference systems in steady state and the semi-continuous performance of system A and B are shown.

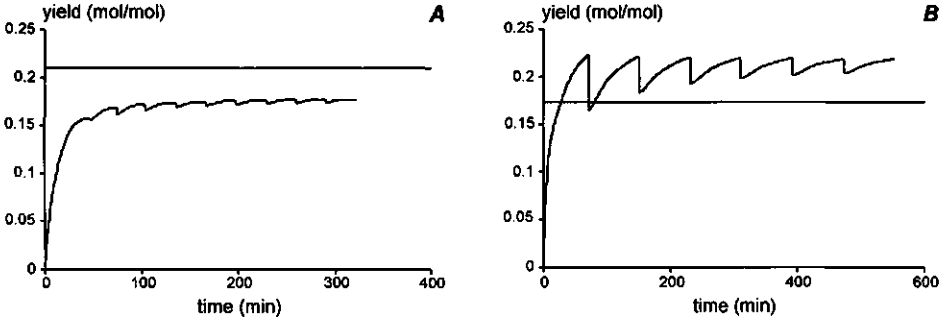

Figure 11 Calculated oligosaccharide yield in the total system as function of time for semi-continuous processes A and B. The horizontal line in both graphs indicates the steady state yield obtained in the reference system. Conditions: kinetic parameters k_1 = 0.019 1/min; k_2 = 1 1/min; k_3/k_4 = 4600; $1/k_4$ = 11.5 min; k_5/k_6 = 21.5; Q = 6.24 ml/min; Q_r = 5 ml/min; V_t = 800 ml; volume ratio (V_r:V_c) 1:1; C_e = 1.25 g/l (system A), 4.4 g/l (system B); ε = 0.225; n_c = 10; adsorption parameters as listed in Table I, except k_{tri} = 3.52 l/g.

The yield obtained in system A was 0.17 mol/mol and for the reference system 0.21 mol/mol. Re-circulation was necessary for yield improvement, but the yield of the system dropped caused by a change of the residence time distribution. For system B the yields were 0.21 and 0.17 mol/mol for the system with removal and the reference system, respectively. A positive effect of the product removal was found, however, the high enzyme concentration influenced the reference system negatively. The system with an optimum enzyme concentration and without re-circulation resulted in a yield of 0.24 mol/mol. This is a higher yield than found in the two postulated process configurations. For the semi-continuous systems studied here, it was not possible to prove that product removal was advantageous.

Conclusions

Trisaccharides were removed from the reaction mixture by activated carbon. The adsorption can be described by the multi-component Langmuir isotherm with three maximum saturation constants. The difference between the affinities for di- and trisaccharides is large enough to result in a yield improvement compared to a process without product removal. The overall yield shows an optimum in time when oligosaccharide synthesis and removal are combined in a batch process. In a system with removal this overall yield can only be determined at the end of the experiment. A good estimation of the time needed to reach the maximum yield is essential.

Model calculations show that in a batch system the yield is improved when trisaccharides are removed immediately after production. Experimentally this improvement is confirmed. In the semi-continuous processes studied, calculations could not prove the advantages of product removal. This is caused by the negative influence of re-circulation and the relative large time between formation and removal which masks the advantages of removal.

Acknowledgements

The authors wish to thank Jurgen Smeenk and Jos Sewalt for performing experiments. The Association of Biotechnological Schools (ABON), the Royal Netherlands Academy of Arts and Sciences (KNAW), and Borculo Domo Ingredients supported this work.

6

General discussion

Part of this chapter has been submitted as: Boon MA, van der Ven C, Janssen AEM. 1999. Effect of water-miscible solvents on the kinetics of lactose hydrolysis.

Introduction

Several aspects involved in the reactor design of the enzymatic synthesis of oligosaccharides were discussed in this thesis. A kinetic model was developed to describe the concentrations in time in chapter 2. The effect of two important process parameters, the enzyme origin and temperature, were discussed in chapter 3. Two possibilities to increase the oligosaccharide yield were investigated in chapter 4 and 5, namely the use of high temperatures in combination with a thermostable enzyme and the use of activated carbon to remove oligosaccharides from the reaction mixture during synthesis.

Some aspects that make this research more complete will be evaluated shortly in this final chapter. Firstly the inactivation of the thermostable β-glucosidase from *Pyrococcus furiosus* will be discussed. It was clearly shown that inactivation could not be neglected in chapter 4. To describe these experimental results with the kinetic model developed in chapter 2, first order enzyme inactivation was implemented. It was determined whether the extended model could describe the results satisfactorily.

Secondly, another possibility to increase the productivity, namely the use of organic solvents will be discussed. Organic solvents diminish the water concentration and thereby the hydrolysis activity. Since sugars have a preference for polar solvents, only the application of polar solvents was studied.

Thirdly, attention will be given to removal of the oligosaccharides from the reaction mixture. In chapter 5 it was shown that separation of trisaccharides from the reaction mixture increased the oligosaccharide yield. Besides activated carbon, other techniques are available to separate sugars, such as membranes and ion-exchange chromatography. Model calculations and experiments were performed to study these alternatives.

Inactivation of the thermostable β-glucosidase from *Pyrococcus furiosus*

Introduction

When using enzymes, the process conditions can be optimised regarding enzyme activation and inactivation. A high temperature results in a higher reaction rate but also in faster enzyme inactivation. The shorter reaction time can be used to minimise the needed amount of enzyme and reactor volume. However, owing to inactivation above a temperature to be determined, the enzyme costs will rise.

For the oligosaccharide synthesis with lactose as a substrate the operation at elevated temperatures is interesting. High initial lactose concentrations can be used, which will result in higher product yields. A thermostable enzyme is required to prevent enzyme inactivation at elevated temperatures.

In chapter 4 the oligosaccharide synthesis with the thermostable β-glucosidase from *P. furiosus* at elevated temperatures (75-95°C) and high initial lactose concentrations (0.73-1.51 mol/kg) was studied. It was clear that enzyme inactivation could not be neglected. This was probably caused by thermic inactivation or inactivation by Maillard products. Petzelbauer *et al.* (1999) also found inactivation for the same enzyme in lactose solutions besides the inactivation caused by elevated temperatures.

To describe the experimental data, inactivation should be included in the kinetic model developed in chapter 2. The aim of this paragraph is to determine whether the extended model could describe these concentrations in time.

Modelling

Maillard products are the result of the reaction between a reducing sugar and a reactive amine. The mechanism is very complex. However, neglecting back reactions and amine recycling, the loss of reactive amine can be described by a second order reaction. Assuming that the sugar is present in excess, the loss of enzyme can be modelled as a first order reaction (Labuza and Baisier, 1992). Since enzyme inactivation can also be described by first order kinetics, the lumped effect was modelled.

The experimental data obtained in chapter 4 were used to determine the first order constant (k_d) and the kinetic parameters for the β-glucosidase from *P. furiosus*. The method to determine the kinetic parameters was described in chapter 2. The enzyme was mainly inhibited by glucose and therefore k_7 and k_8 were assumed to be equal to zero (Figure 1, chapter 3). It was not possible to determine parameter k_4 since no decrease of the trisaccharide concentration was measured. Therefore, the parameters were grouped into k_1, k_3, k_5/k_6 and k_d. The results obtained should be considered as a first indication since only a limited amount of experimental data was available at each temperature.

The temperature dependency of the kinetic parameters was modelled with the Arrhenius equation (chapter 3). Enzyme inactivation is often modelled with the Arrhenius equation and the same is valid for the observed temperature dependence of the Maillard browning reaction (Labuza and Baisier, 1992). The t-test was used to determine whether there was a temperature effect on the parameters. This test takes the 95% confidence interval of the parameters into account (chapter 3).

Results and discussion

In Figure 1 the measured and predicted oligosaccharide synthesis at an initial lactose concentration of 0.93 mol/kg at 75, 85, and 95°C is shown. Both the reaction rates as the inactivation rate increased with temperature. It is clear that the extended model described the concentrations in time satisfactorily.

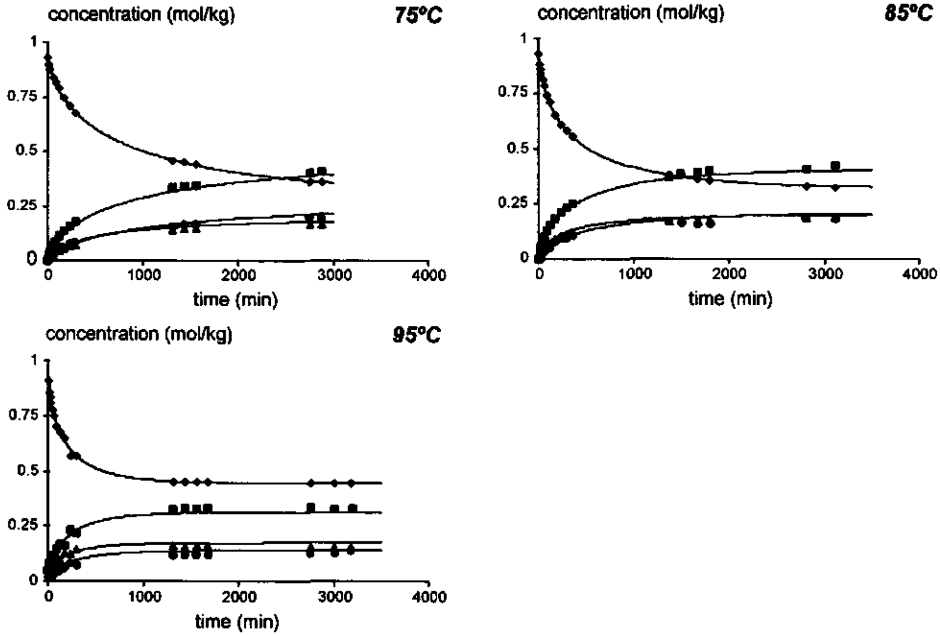

Figure 1 Measured and predicted lactose conversion at 75, 85, and 95°C. Initial lactose concentration 0.93 mol/kg, enzyme β-glucosidase from *P. furiosus* (◆, lactose; ■, glucose; ●, galactose, and ▲, oligosaccharides).

The model parameters were determined following the procedure described in chapter 2. To determine one parameter set for the various initial lactose concentrations, the parameters have to be independent on the initial lactose concentrations. A negative trend with the initial lactose concentration was encountered for k_1 at 95°C and for k_3 at 75 and 85°C. A positive trend for k_d at 95°C was found. These trends were considered to be unimportant because not the same trend for parameters at one temperature or for one parameter at various temperatures was found. Therefore, at each temperature the average parameter was taken.

The effect of the temperature on the model parameters was described with the Arrhenius equation. In Figure 2 the parameters and the prediction of the Arrhenius equation are shown. The activation energies (E_A) determined were 21 ± 140, 27 ± 220, -30 ± 38 and 105 ± 200 kJ/mol for respectively k_1, k_3, k_5/k_6, and k_d. A negative activation energy is possible because a ratio of parameters is involved. All activation energies were insignificant since zero was included in the 95% confidence intervals. Hence, no temperature dependency was found. However, since only three temperatures were studied and two parameters were determined, only one degree of freedom was left. This resulted in a high critical t (t_c) (12.706). When one temperature would be added t_c would drop (4.303), and a temperature dependency would be easier found. Furthermore is it possible that the temperature range studied was not wide enough to find a dependency; in chapter 3 the temperature range was 30°C.

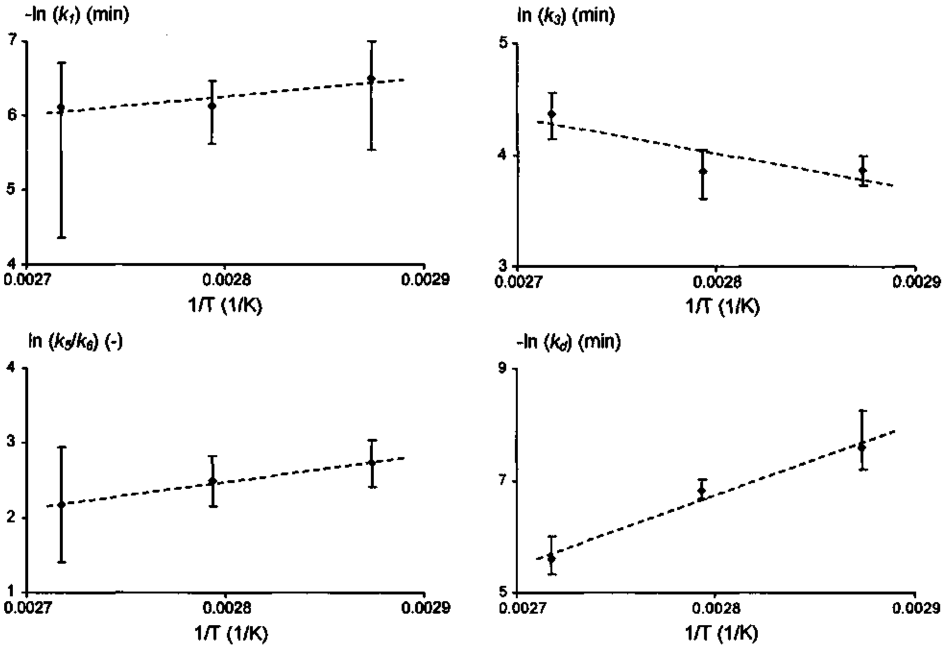

Figure 2 Natural logarithm of the model parameters (including its 95% confidence interval) as function of the temperature reciprocate, including the Arrhenius equation prediction.

The t-test was used to take the 95% confidence interval of the parameters into account (data not shown). As expected when observing Figure 2, for k_d an effect of the temperature was found. For parameter k_3 also an effect of the temperature was found, however this was solely due to the parameter at 95°C (Figure 2). Taking the results of the Arrhenius equation and the t-test into consideration it was concluded that as a first indication only the first order inactivation constant k_d was dependent on the temperature.

To predict the effect of the temperature on the oligosaccharide production all the parameters have to be taken into account. Therefore, the concentrations in time were calculated with the predicted parameters. The maximum oligosaccharide yield was determined for various initial lactose concentrations and temperatures (Figure 3). The yield increased at higher initial lactose concentrations. At higher temperatures the effect of inactivation was evident. The temperature, at which the highest yield was obtained, was higher at low than at high initial lactose concentrations.

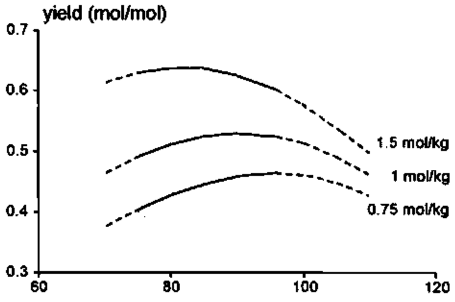

Figure 3 Calculated yield as function of temperature and initial lactose concentration.

Conclusions

The kinetic data obtained with the β-glucosidase from *P. furiosus* are described with the kinetic model including first order enzyme inactivation. The temperature dependency could not be determined well since only a small temperature range was used and a limited number of experiments per temperature were performed. As a first indication only the inactivation constant shows a dependency on temperature which can be described with the Arrhenius equation. The temperature, at which the highest yield was obtained, was higher at low than at high initial lactose concentrations.

The application of thermostable enzymes for the oligosaccharide synthesis is promising. Preventing of enzyme inactivation for application of these enzymes in sugar solutions is however essential.

Effect of water-miscible solvents on the kinetics of lactose hydrolysis

Introduction

The synthesis of oligosaccharides is mainly determined by the ratio between the transferase and hydrolase activity. The synthesis of a given enzyme can be influenced by the medium; the transferase activity is stimulated by a high nucleophile concentration and lowering the available water (water activity) diminishes the hydrolysis activity. The reaction is shifted to transferase activity and the oligosaccharide yield is enhanced. The water activity is only slightly influenced by the sugars in the solution, but can be decreased further by the use of solvents. Since the substrate and products prefer a polar environment, only the use of water-miscible solvents is studied here.

Mainly three difficulties arise when applying solvents during the enzymatic synthesis of oligosaccharides. Firstly, the solubility of the substrate, which is a sugar, decreases. A high substrate concentration is desired since it results in a higher transferase activity and thereby a higher yield. Secondly, the introduction of an additional process step is necessary to remove the solvent from the end product. Even when no trace of the solvent is present in the end product, the solvent has to be food-grade. This limits the choice of solvents that can be applied. Thirdly, a negative effect of the solvent on the enzyme activity can be possible. A small layer of water surrounds the enzyme. When a water-miscible solvent is used a one-phase system is formed. The enzyme comes in direct contact with the solvent and an effect on the enzyme configuration is likely.

In literature the synthesis of oligosaccharides in water-miscible solvents is reported. The effects of the solvent on the yield, the reaction rates, and the enzyme stability are studied. A higher yield compared to the aqueous system (Cantarella *et al.*, 1994; Finch and Yoon, 1997; Laroute and Willemot, 1989; Scheckermann *et al.*, 1997) is reported. The effect of the solvent on the enzyme stability is highly dependent on the solvent concentration. At low concentrations no effect is reported, at higher concentrations the enzyme stability decreases (Shubhada and Sundaram, 1995). This is often described to direct contact between solvent and enzyme.

In the previous studies the effect of solvation on the kinetic parameters was not taken into account. Solvation corrects for the partition of the substrates and products over the phases present. Also in a one-phase system this occurs because the solubility changes by introduction of the solvent. Correction for solvation should be made before the effect of the solvent on the enzyme activity can be determined (Dordick, 1992; Halling, 1994; Reimann *et al.*, 1994; van Tol *et al.*, 1995).

The aim of this paragraph is to determine the effect of various water-miscible solvents on the kinetic parameters of the oligosaccharide synthesis catalysed by the β-galactosidase from *Bacillus circulans*. The effect on the kinetic parameters of the model developed in chapter 2 would be laborious therefore as a first indication only the lactose hydrolysis is considered. Improvement of the transferase activity and enhancement of the oligosaccharide yield are not considered. Lactose hydrolysis can be described with Michaelis-Menten kinetics.

Theory

The effect of solvents on enzymes can be divided in the effect on the enzyme activity and on the kinetic parameters (solvation). The effect of solvents on the enzyme activity is caused by a disruption of the protein structure, by interfering with the hydrophobic interactions and hydrogen bonding, or by the removal of the water layer. The enzyme activity is especially influenced by the removal of the water layer (Zaks and Klibanov, 1988).

For comparison of the kinetic parameters in organic media, corrections for substrate solvation should be made. This can be done by using a transfer free-energy method (Dordick, 1992; Janssen and Halling, 1994; Wescott and Klibanov, 1993), by using activities instead of concentrations (van Tol *et al.*, 1995), or by using partition coefficients or solubilities (Halling, 1994; Reimann *et al.*, 1994).

For comparison of the kinetic parameters for lactose hydrolysis in mixtures of water and water-miscible solvents, it is convenient to use substrate solubilities. The solid substrate phase is taken here as the thermodynamic standard state. The K_m in the organic phase is related to the K_m in the standard or reference state as follows

$$K_{m,ref} = K_{m,org} / S_{org} \qquad (1)$$

in which S_{org} is the solubility of the substrate in the organic phase. Consequently the specificity constant V_m/K_m is corrected in the following way

$$(V_m / K_m)_{ref} = (V_m / K_m)_{org} S_{org} \qquad (2)$$

If the differences in the kinetic parameters in different organic media can be entirely explained by substrate solvation, the kinetic parameters in the reference state would have a constant value. If the value for $K_{m,ref}$, $(V_m/K_m)_{ref}$, and the solubility S_{org} are known, the expected parameters in every organic medium can be calculated

$$K_{m,org}^{expected} = K_{m,ref} S_{org} \qquad (3)$$

$$(V_m / K_m)_{org}^{expected} = (V_m / K_m)_{ref} / S_{org} \qquad (4)$$

These expected parameters can be compared to the measured kinetic parameters in de organic media. In this study $K_{m,ref}$ and $(V_m/K_m)_{ref}$ for the water phase are assumed to be correct and are used to calculate the expected kinetic parameters in the organic media.

In principles these corrections should always be made, before conclusions can be drawn on residual effects.

Materials and methods

Materials

Lactose was obtained from Sigma (St. Louis, USA), all other chemicals were obtained from Merck (Darmstadt, Germany). The β-galactosidase from *B. circulans* was obtained from Daiwa Kasai K. K. (Osaka, Japan). All were used without further purification.

Kinetic experiments

In a typical experiment, 25 mg enzyme was added to 50 g reaction mixture, which contained lactose, McIlvaine buffer at pH 5 (0.02 M), and a certain amount of solvent. The reaction mixture was kept at 30°C and mixed by magnetic stirring. The reaction was followed for 30 min and samples of 200 μl were taken every 5 min. The samples were heated 15 min at 90°C to inactivate the enzyme and to evaporate the main part of the solvent. After heating the samples were diluted and $Pb(NO_3)_2$ was added (concentration in the sample 0.1 M). Samples were stored in the freezer and analysed by HPLC. The initial reaction rates were determined by linear regression and expressed in mmol lactose per kg enzyme per s. The initial reaction rates were fitted to the Michaelis-Menten equation using nonlinear regression (statistical program SAS). For both fitting procedures the least-squares method was used.

Solubility experiments

The solubility of lactose was measured in various water-organic solvent mixtures in quadruplicate. These mixtures, with an excess of substrate, were incubated at 30°C in an end-over-end incubator (100 rpm) during 48 h. The mixture was filtered to remove solid substrate and a sample of the supernatant was taken. Galactose was added as internal standard. The sample was heated 30 min at 90°C to evaporate the solvent. After heating the samples were treated as described in the previous section and analysed by HPLC.

The solubilities of maltose and sucrose were measured using the same method. The incubation temperature, solvent and buffer concentrations were the same as in the kinetic experiments described by Ulbrich-Hofmann and Selisko (1993).

HPLC analysis

The samples were analysed by HPLC using a RSO oligosaccharide column (Phenomenex, the Netherlands) at 80°C. The column was eluted with double distilled water (filtered through a regenerated cellulose membrane of 0.45 μm and purged with helium gas) at a flow rate of 0.3 ml/min. The eluent was monitored by means of a refractive index detector.

Results and discussion

The lactose hydrolysis was followed in time in water, n-n-dimethyl formamide (DMF), aceton, tert-butanol, and acetonitrile. The mole fraction of water (x_w) was 0.92 (-) for all these mixtures. For acetonitrile the lactose hydrolysis was also followed for a x_w of 0.85, 0.80 and 0.75 (-). The initial reaction rates showed a Michaelis-Menten dependency on the initial lactose concentration (Figure 4).

Figure 4 shows that the type of solvent and the solvent concentration influenced the kinetics. The Michealis-Menten parameters are listed in Table I. The parameters K_m and V_m/K_m were fitted since the ratio V_m/K_m could be determined more accurately in our case. The K_m decreased in organic media compared to K_m in the water phase. A decrease of K_m was more pronounced at higher solvent concentrations. However, differences were not very clear since the 95% confidence intervals were large. The ratio V_m/K_m decreased in

Table I Kinetic parameters with their 95% confidence interval for lactose hydrolysis in different reaction mixtures. Also the experimental lactose solubility (S_{org}) in the reaction mixture is given.

solvent	x_w (-)	n (-)	K_m (mol/kg)	V_m/K_m ($g\cdot kg^{-1}\cdot s^{-1}$)	V_m ($mmol\cdot kg^{-1}\cdot s^{-1}$)	S_{org} (mol/kg)
water	1	20	0.199 ± 0.056	0.337 ± 0.058	0.067	0.602 ± 0.002
DMF	0.92	13	0.131 ± 0.086	0.190 ± 0.066	0.025	0.288 ± 0.005
aceton	0.92	19	0.128 ± 0.031	0.222 ± 0.032	0.028	0.278 ± 0.004
tert-butanol	0.92	15	0.119 ± 0.040	0.219 ± 0.040	0.026	0.214 ± 0.002
acetonitrile	0.92	19	0.123 ± 0.051	0.271 ± 0.071	0.033	0.380 ± 0.028
acetonitrile	0.85	13	0.152 ± 0.127	0.168 ± 0.059	0.026	0.239 ± 0.005
acetonitrile	0.80	10	0.129 ± 0.075	0.189 ± 0.046	0.024	0.144 ± 0.006
acetonitrile	0.75	6	0.074 ± 0.050	0.231 ± 0.069	0.017	0.099 ± 0.0004

Note: *n* is the number of lactose hydrolyse experiments in the given reaction mixture.

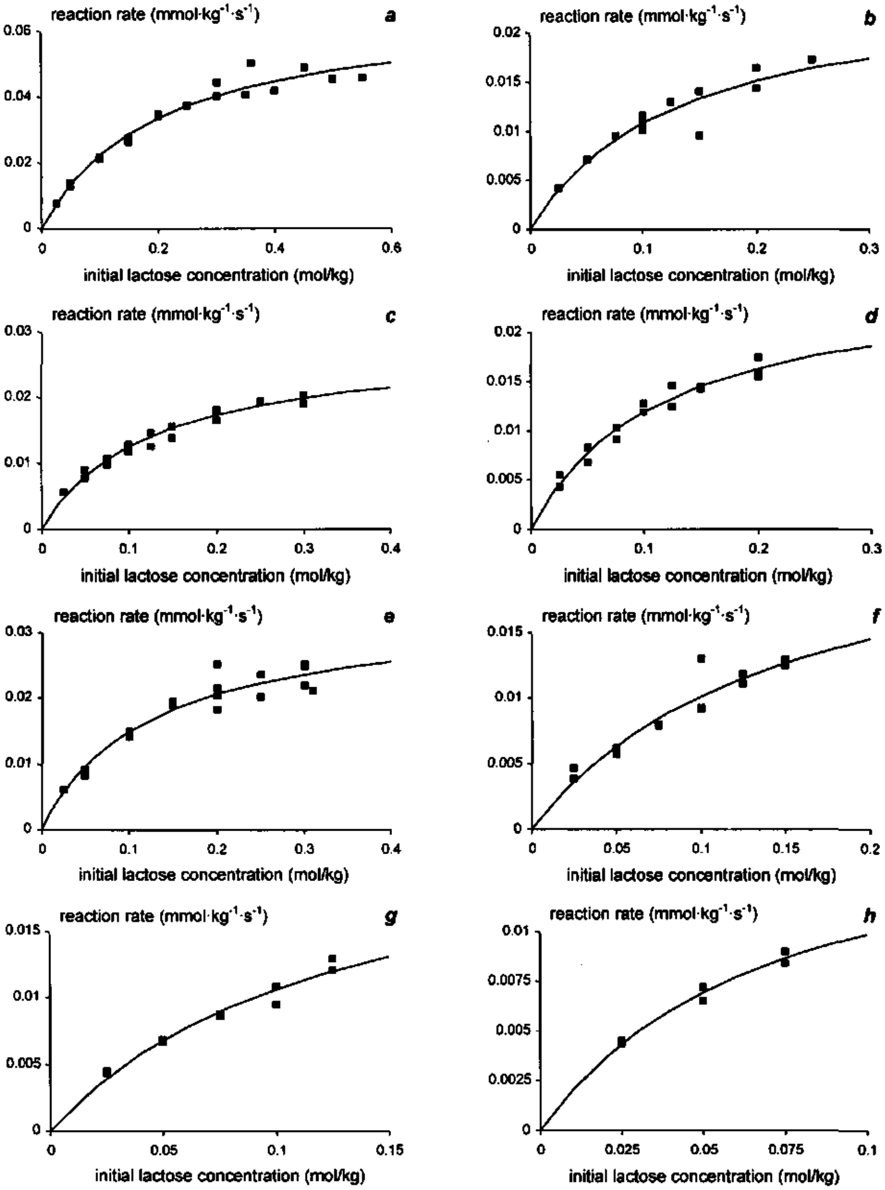

Figure 4 Initial hydrolysis reaction rate as function of the initial lactose concentration for water (a), DMF (b), aceton (c), tertair butanol (d), and acetonitrile ($x_w = 0.92$ (e), $x_w = 0.85$ (f), $x_w = 0.80$ (g), and $x_w = 0.75$ (h)). The fitted Michaelis-Menten equation is also shown.

organic media compared to the ratio V_m/K_m in the water phase. The decrease in V_m at high acetonitrile concentrations seemed to be more pronounced. However, ratios V_m/K_m for acetonitrile were within each other's 95% confidence interval.

To correct for the solvation, lactose solubility was determined (Table I). The kinetic parameters were corrected for substrate solvation as described in the theory. The parameters in the aqueous phase were calculated using eq. 1 and 2, the expected parameters in the organic media were calculated using eq. 3 and 4. The expected parameters are compared with the measured parameters in Figure 5.

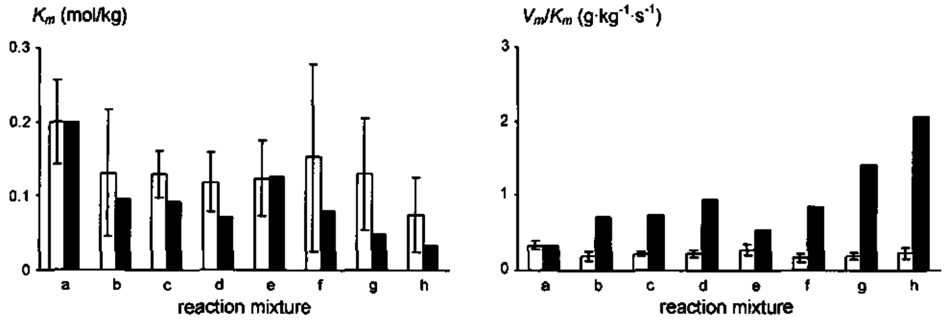

Figure 5 The measured (white) and expected (black) kinetic parameters for different reaction mixtures (water (a), DMF (b), aceton (c), tertair butanol (d), and acetonitrile (x_w = 0.92 (e), x_w = 0.85 (f), x_w = 0.80 (g), and x_w = 0.75 (h))). For the measured parameters the 95% confidence interval is included.

Based on the correction for solvation, a decrease of the K_m is expected. Trends were predicted considerably well on basis of solvation. For the ratio V_m/K_m it is clear that the effect of different solvents could not be explained with solvation, the expected value was higher than the measured ratio. According to the theory, the solvent does not effect V_m. This indicated that the solvent influenced the enzyme activity and a decrease in V_m was measured.

After the correction for solvation, an effect of the solvents on the enzyme activity remained unaccounted for and therefore the solvents influenced the enzyme activity. When using these solvents for the synthesis of oligosaccharides, a decrease of the activity should be taken into account.

Ulbrich-Hofmann and Selisko (1993) determined the kinetic parameters for the hydrolysis of maltose and sucrose catalysed by glucoamylase and invertase, respectively, in water-miscible organic solvents. To make corrections for solvations we have measured the necessary solubilities of maltose and sucrose. The data from Ulbrich-Hofmann and Selisko (1993) and the corrections made for solvation are shown in Table II.

Table II Kinetic parameters of glucoamylase and invertase and the solubility of the substrate in various reaction mixtures. K_m and V_m are from Ulbrich-Hofmann and Selisko (1993).

solvent	K_m (mM)	V_m ($mmol \cdot g^{-1} \cdot s^{-1}$)	S_s (mM)	expected K_m (mM)
glucoamylase in 50% (v/v) solvent				
water	1.16	0.0775	1.35 ± 0.02	1.16
glycerol	5.72	0.0735	0.65 ± 0.03	0.56
methanol	2.71	0.1	0.63 ± 0.01	0.55
dioxane	0.92	0.087	0.60 ± 0.01	0.52
ethanol	2.32	0.1015	0.58 ± 0.01	0.50
invertase in 30% (v/v) solvent				
water	36.6	6.98	1.81 ± 0.03	36.6
methanol	60.3	4.12	1.65 ± 0.01	33.4
ethanol	54.8	3.41	1.65 ± 0.03	33.4
dioxane	233.2	4.05	1.63 ± 0.07	33.0
DMF	141.8	2.06	1.61 ± 0.01	32.6

Note: substrate for glucoamylase was maltose and for invertase sucrose

As for the lactose hydrolysis, a decrease of the K_m is expected because the substrate solubility decreases when solvents are added to the reaction mixture. Instead, an increase of K_m was found. Therefore, the difference between the expected and measured parameters was enlarged by correction for substrate solvation and the effect of the solvent on the substrate affinity was even larger than concluded by Ulbrich-Hofmann and Selisko (1993). The K_m is more influenced by the solvents than the V_m. Solvation does not account for changes in V_m. Therefore, the differences between the V_m in solvents compared to the V_m water is solely due to the effect of the solvent on the enzyme. It seemed that the V_m of glucoamylase was less influenced by the solvents studied that the V_m of invertase.

Conclusions

When using solvents, a correction for solvation should always be made before the effect of the solvent on the enzyme activity is considered. Solvation also takes place when water-miscible solvents are used since the solubility is changed. The kinetic parameters which are expected if all the effects can be attributed to substrate solvation can be compared to the measured parameters.

For lactose hydrolysis was found that the effect on the K_m was mainly due to solvation. The value of V_m decreased for all solvents studied compared to the value of V_m in water, which means that an effect of the solvent on the enzyme activity was found. For an example from literature was found that as well the K_m as the V_m were influenced by the solvent.

After correction for solvation a large effect of the solvent on the enzyme activity is left unaccounted for. In one-phase systems the effect of the solvent on the enzyme is relatively large due to the direct contact between enzyme and solvent.

Other possibilities to remove oligosaccharides from reaction mixtures

Introduction

In chapter 5 oligosaccharides were continuously removed from the reaction mixture in a batch process. The yield was enhanced even if only a slight amount of oligosaccharides was withdrawn. In Figure 6 the continuous process is schematically shown. The feed containing lactose enters the system and the enzymatic conversion takes place in the reactor. The reaction mixture is continuously recycled over a separation process. The mono-, and disaccharides are fed back to the reactor and the trisaccharides are removed from the system. In chapter 5 was found that minimising the loss of the substrate lactose is important.

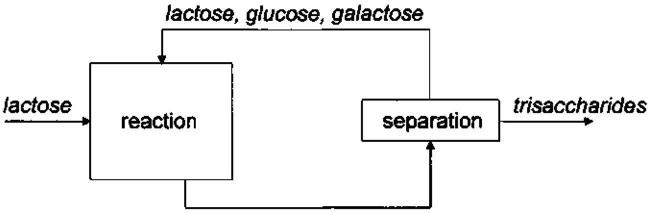

Figure 6 Product removal in a continuous process.

Activated carbon was used as a separation technique because activated carbon has the highest affinity for the product and recovery can take place off-line. However, also ion-exchange chromatography or membranes can be used. These alternatives will be discussed next.

In chromatographic applications to separate sugars mainly cation-exchange resins are used (Jandera and Churácek, 1974). Cation-exchange resins have the highest affinity for monosaccharides and therefore trisaccharides are the first to elute from the column. Elution is performed with water. Reaction and separation by chromatography are combined in several continuous apparatus. Shieh and Barker (1996) used a Simulated Counter-Current Chromatographic Bioreactor-Separator (SCCR-S) for the separation of glucose and galactose produced during the enzymatic hydrolysis of lactose. Sarmidi and Barker (1993) used the Continuous Rotating Annular Chromatograph (CRAC) for the separation of fructose and glucose produced during the enzymatic hydrolysis of sucrose. Utilisation of membranes for the separation of sugars was not found in literature.

Model calculations will be used to determine the potential and disadvantages of both separation processes. The results of two experiments with a membrane and ion-exchange resin will be presented.

Theory

Membranes

The continuous process for membranes is shown in Figure 7a. Synthesis of trisaccharides takes place in the first vessel (V_1). The trisaccharide concentration in the second vessel (V_2) is enhanced by cross-flow (Q_r) parallel to a selective membrane. The trisaccharides are retained and the other sugars (mono- and disaccharides) pass through the membrane (Q_p) and are led back to V_1. Part of Q_r leaves the system (Q).

The membrane is characterised by retention R (-) (Mulder, 1996)

$$R = 1 - \frac{C_p}{C_2} \tag{5}$$

in which C_2 the concentration in the incoming flow (see Figure 7a)
C_p the concentration in the permeate flow

R ranges between zero (no retention) and one (complete retention). A membrane with a high retention for trisaccharides and a low retention for mono- and disaccharides is preferred.

Mass balances over the reactor (eq. 6), the second vessel (eq. 7) and membrane (eq. 8) for all components were used to describe this process. All systems were compared to each other in steady state conditions. The mass balances are respectively

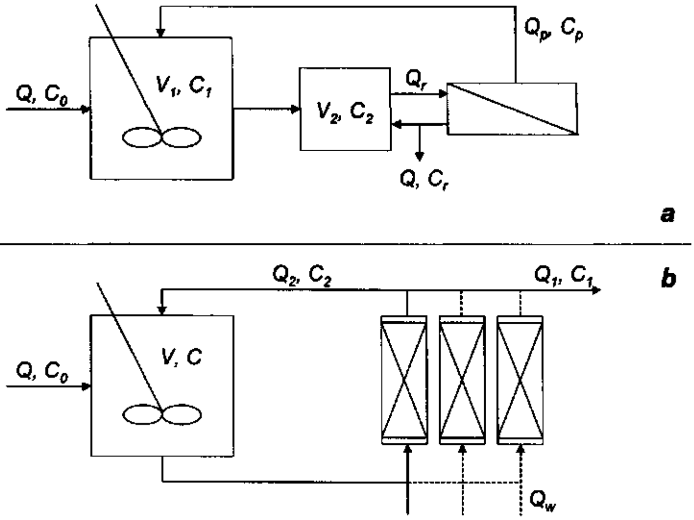

Figure 7 Product removal with a membrane (a) and ion-exchange columns (b) in a continuous process.

$$0 = QC_0 - (Q + Q_p)C_1 + Q_pC_p + rV_1 \tag{6}$$

$$0 = (Q + Q_p)C_1 - Q_rC_2 + (Q_r - Q - Q_p)C_r \tag{7}$$

$$Q_rC_2 = (Q_r - Q_p)C_r + Q_pC_p \tag{8}$$

The enzymatic reaction (r) is described with the kinetic model developed in chapter 2.

Ion-exchange chromatography

In Figure 7b the process including ion-exchange columns is shown. A series of columns is used to separate the sugars in the various pulses. The outlet pulse is divided in two parts. One part (Q_1) contains mainly oligosaccharides and is removed from the system. The other part (Q_2) contains mainly lactose, glucose and galactose and is returned to the reactor. Addition of water (Q_w) is necessary to elute the column.

A measure for the separation of two peaks 1 and 2 is the resolution $R_{1\text{-}2}$ (-) (Sarmidi and Barker, 1993)

$$R_{1-2}=\frac{\theta_2-\theta_1}{\frac{1}{2}(W_1+W_2)} \tag{9}$$

in which	W	the bandwidth for component 1 and 2
	θ	the retention of component 1 and 2

In Figure 8 is shown how the resolution can be determined from a chromatogram. R_{1-2} is a measure for the overlap between two peaks. For R_{1-2} larger than or equal to 1 the overlap is small and the separation therefore is good. Q_w influences the resolution since it changes both the retention and the bandwidth.

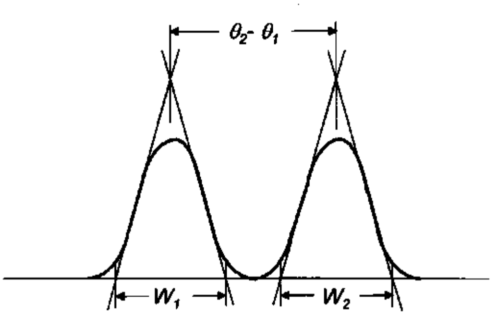

Figure 8 Definition of resolution and approximation of the peaks by triangles for calculation of peak percentages.

Mass balances over the reactor (eq. 10) and the ion-exchange column (eq. 11) for all components were used to describe this process. In steady state conditions the loading and elution of the columns were equal. The mass balances are respectively

$$0=QC_0-(Q+Q_2)C+Q_2C_2+rV \tag{10}$$

$$(Q+Q_2)C=Q_1C_1+Q_2C_2 \tag{11}$$

To obtain a constant reactor volume, it was assumed that Q_1 equalled the sum of Q and Q_w. The distribution of each component over Q_1 and Q_2 was described with a fraction

$$fraction\_1 = \frac{(Q+Q_w)C_1}{(Q+Q_2)C} \text{ and } fraction\_2 = \frac{Q_2C_2}{(Q+Q_2)C} \qquad (12)$$

To calculate the percentage of a sugar in fraction Q_1 and Q_2, the peaks were approximated by triangles (Figure 8). The dilution (D) was defined as the ratio between Q_w and Q.

Materials and methods

Materials

Lactose was obtained from Sigma (St. Louis, USA), all other chemicals were obtained from Merck (Darmstadt, Germany). The β-galactosidase from *Bacillus circulans* was obtained from Daiwa Kasai K. K. (Osaka, Japan). The desalt membrane was a kind gift from JM Separations (the Netherlands). The Dowex 50W-X4 resin (H^+, 100-200 mesh) was obtained from Aldrich (Wisconsin, USA). All were used without further purification.

Experimental set-up

A membrane and ion-exchange column were used to separate a sugar solution. The sugar solution used (lactose 46.0 mg/ml, glucose 7.6 mg/ml, and trisaccharides 18.7 mg/ml) was obtained by hydrolysis of lactose at 40°C by the β-galactosidase from *B. circulans*. For both separation experiments fractions of 1.5 ml were collected at the outlet. Sample treatment, concentration determination, and analysis were carried out as described in chapter 5.

Sugars were separated by dead-end membrane filtration in a stirred cell (Amicon, Millipore). The sugar retentions were determined using a desalt membrane at 5.0 bar and 40°C. The separation properties of the Dowex 50W-X4 resin were determined. A pulse of the sugar mixture (15 minutes, flow 0.3 ml/min) was led over a bed of 26 ml containing 20 g carrier. A column of 20 cm with a diameter 1.6 cm (Pharmacia Biotech, Sweden) was used.

Results and discussion

Experiments

Membranes

In Figure 9a the experimental results of the separation with the membrane is shown. The retention was high for lactose and trisaccharides and low for glucose. The average retentions measured were 0.26, 0.91, and 0.96 (-) for glucose, lactose, and trisaccharides respectively. The retention for lactose and glucose should be as low as possible, therefore especially the retention for lactose is too high. The high retention found for trisaccharides is desired. During the experiment a permeate flux of 1.6 $l{\cdot}m^{-2}{\cdot}h^{-1}$ was measured.

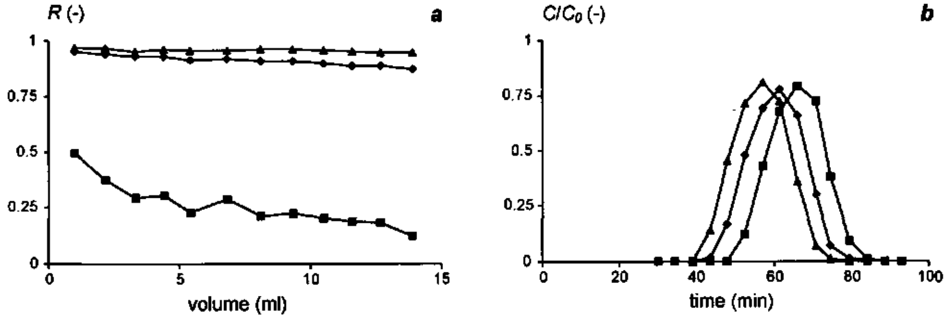

Figure 9 Measured sugar retention for the membrane (a) and concentrations in the outlet flow of the ion-exchange column (b) (◆, lactose; ■, glucose, and ▲, trisaccharides) (lines for guidance).

Ion-exchange chromatography

Figure 9b shows the results of the experiment with the ion-exchange resin. The resin had the lowest affinity for trisaccharides, since these were the first to elute from the column. Secondly the lactose and thirdly the glucose eluted from the column. The overlap between the peaks was large and the resolutions calculated were $R_{di\text{-}tri}$ 0.15, $R_{mono\text{-}di}$ 0.17 and $R_{mono\text{-}tri}$ 0.32. The pulse of 15 min was measured at the outlet as a pulse of 50 min, therefore the dilution was 3.3.

Model calculations

Model calculations were used to examine the performance of membrane and ion-exchange resins in general and of the ones studied above in specific. For all calculations the incoming flow (*Q*) (Figure 7) contained only lactose (0.3 mol/kg). The kinetic parameters determined for β-galactosidase from *B. circulans* (chapter 2) were used. All systems were compared to each other in steady state conditions. The system with product removal was compared to a system without product removal; in the reference system an inert separation process was present (chapter 5). The steady state condition for the reference system was equal for both separation processes.

Membranes

The oligosaccharide yield under steady state conditions for the membrane process for various membranes and residence times in the first reactor (τ_1) is given in Figure 10. The yield for the process without a membrane (which is a CSTR) showed an optimum in time; when τ_1 was too large, hydrolysis of trisaccharides took place. Using an ideal membrane (R_{lac} = 0 and R_{tri} = 1), the yield was increased; trisaccharides were trapped in V_2 and the concentration increased. A high Q_r parallel to the membrane (Q_r = 1000 ml/min compared to Q = 1 ml/min) was preferred under these conditions because then the membrane capacity was fully used.

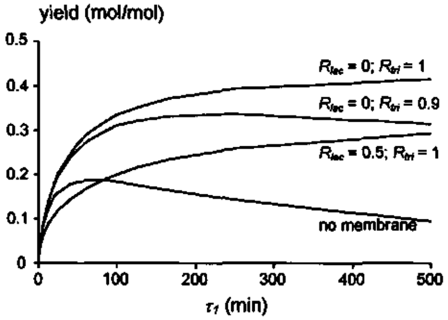

Figure 10 Model calculations for continuous product removal with a membrane. The oligosaccharide yield is plotted as function of residence time and retention. Standard conditions: initial lactose concentration 0.30 mol/kg; kinetic parameters k_1 = 0.019 1/min; k_2 = 1 1/min; k_3/k_4 = 4600; $1/k_4$ = 11.5 min; k_5/k_6 = 21.5; Q_r = 1000 ml/min; $Q_p = 0.1Q_r$; V_1 = 500 ml; V_2 = 500 ml; C_e = 1.25 mg/ml; R_{glu} = 0; R_{gal} = 0.

The yield decreased substantial when the retention of trisaccharides was changed from 1 to 0.9. The trisaccharides passed partly through the membrane and were not completely trapped once they enter V_2. When the retention of lactose increased (R_{lac} = 0.5), lactose was partly trapped in the second vessel and was not available as substrate. For short residence times in V_1 it was even observed that the yield was lower compared to the reference system. Owing to the high lactose concentration in V_1 and consequently also in V_2, high amounts of lactose left the system.

The high retention found for lactose for the desalt membrane that was tested in our lab will result in a high loss of substrate. Furthermore, a membrane area of 37.5 m^2 for a total reactor volume of 1 l ($Q_p = 0.1Q_r$) would be needed. This is an indication that the membrane is too dense for this application.

Ion-exchange chromatography

The effect of different ion-exchange resins was studied by varying $R_{di\text{-}tri}$. Also the effect of the process parameters Q_2 and dilution (D) were studied. The fraction of glucose and galactose in Q_1 and Q_2 was kept constant at a half. The monosaccharides could not accumulate in the reactor and in this manner only the effect of lactose and trisaccharides could be studied.

To calculate the fraction of a sugar in Q_1 and Q_2, the peaks were approximated by triangles. It was assumed that the bandwidth of both sugars was equal. To calculate the yield, the resolution, the bandwidth of the peaks (or the retentions), the ratio Q_2/Q and dilution had to be known. The relation between these variables is shown in Figure 11. It should be noted that the resolution by itself is also influenced by the dilution.

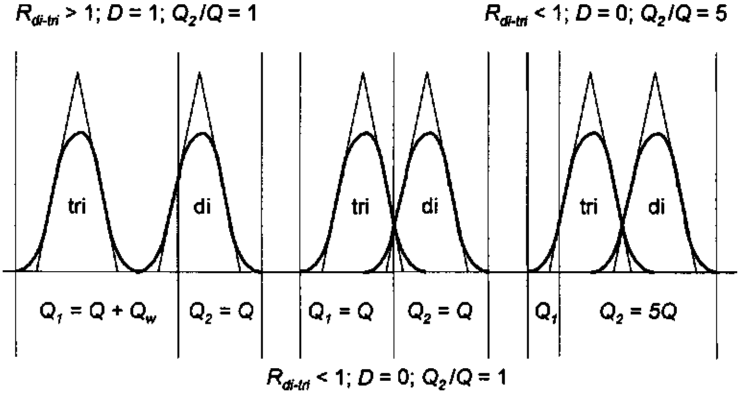

Figure 11 Effect of resolution, dilution and Q_2/Q on the sugar amounts in Q_1 and Q_2.

Q_1 and Q_2 are fixed when the dilution and the ratio Q_2/Q are fixed. The fraction $Q_1/(Q_1+Q_2)$ must be removed from the system to maintain a constant reactor volume. For a high dilution, a larger part of the peaks are removed from the system. On the other hand when Q_2 is much larger than Q_1 (high Q_2/Q), the main part of the peaks is returned to the vessel. The resolution determines the relative position of the peaks and for $R < 1$ the sugars can never be 100% separated.

The oligosaccharide yield under steady state conditions for the ion-exchange process as function of $R_{di\text{-}tri}$ (-) and D (-) is given in Figure 12a. The horizontal line indicates the yield in a process without removal under standard conditions. As expected, a high resolution and a low dilution were positive for the yield. A dilution of zero is not realistic but it gives the performance boundary of the system studied. When two times the volume of the pulse was needed to elute it from the column ($D = 2$), a $R_{di\text{-}tri}$ of minimal 0.6 (-) was needed for yield improvement.

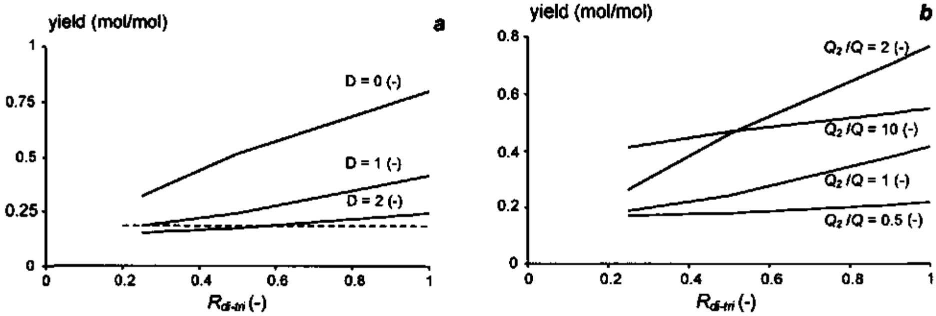

Figure 12 Results of model calculations for continuous product removal with ion-exchange column. Oligosaccharide yield as function of $R_{di\text{-}tri}$ and D with $Q_2/Q = 1$ (a) and as function of $R_{di\text{-}tri}$ and Q_2/Q with $D = 1$ (b). Standard conditions: initial lactose concentration 0.30 mol/kg; kinetic parameters $k_1 = 0.019$ 1/min; $k_2 = 1$ 1/min; $k_3/k_4 = 4600$; $1/k_4 = 11.5$ min; $k_5/k_6 = 21.5$; $Q = 10$ ml/min; $V = 1000$ ml; $C_e = 1.25$ mg/ml; 50 % of glucose and galactose in Q_1. Horizontal line in (a) is the yield in the reference system.

In Figure 12b the yield as function of $R_{di\text{-}tri}$ and the ratio Q_2/Q at $D = 1$ is shown. There was clearly an optimum ratio and the yield was increased substantial compared to $Q_2/Q = 1$. When the ratio was low, the main part of the eluted peaks left the system. When the ratio was high, the separated peaks were mainly returned to the vessel. For a high or low ratio the yield is less dependent on the resolution.

The experimental resolution and dilution (3.3) as shown in Figure 9b were used to calculate the percentages in Q_1 and Q_2. The high dilution together with the low resolutions could not result in an improvement of the yield for the studied process (Figure 12a).

Conclusions

Removal of trisaccharides from the reaction mixture leads to a higher yield when lactose loss is minimised, a small trisaccharide removal is already advantageous. When a good membrane is found, re-circulation is positive. In the ion-exchange process the dilution plays an important role. When the dilution is too high, a good resolution will not give the desired effect. As concluded in chapter 5 it is clear that loss and concentration of lactose play an important role in the synthesis of oligosaccharides with lactose as a substrate.

Concluding remarks

The enzymatic synthesis of oligosaccharides is studied in this thesis. A kinetic model is developed which is used to determine interesting research topics with the aim to increase the yield and to design a suitable reactor. This approach proves to be successful and a lot of insight in the reaction is obtained. Furthermore, the used approach and conclusions are also applicable for other kinetically controlled reactions.

Study of the oligosaccharide synthesis with lactose as a substrate and a β-galactosidase as catalyst is complicated by the fact that the nucleophile and substrate are identical. It is therefore not possible to study hydrolysis in absence of synthesis. It is found that the concentration of the nucleophile is very important. Experiments and model calculations emphasise that the loss of lactose or dilution of the reaction mixture should be avoided. Both result in an immediate decrease of the yield.

Since the synthesis is a kinetically controlled reaction, describing the concentrations in time is essential for reactor design. In this thesis each experiment is considered as an independent estimation of the parameters. In this way independent sets of estimates are obtained which are the basis for further straightforward analysis. Of course, is it possible to fit a model to the data from different experiments simultaneously by minimising the RSS of all concentrations of all experiments. This parameter set can describe the concentrations in time better than when the parameters are determined for each experiment separately and one parameter set is determined by taking the average. The overall least-square method is however inappropriate because of three reasons

1. The main reason is that the information that sets of data points belong together will be neglected. In experiments where concentrations are measured in time, the measurements are dependent observations. For fitting kinetic parameters each experiment must be considered as an independent estimation of the parameters. This is essential because an event in the beginning of the reaction influences the concentrations at the end of the reaction. An example of modelling where time dependence between measurements is preserved, is the determination of initial rates for estimation of the Michaelis-Menten parameters. Initial rates are used to determine the kinetic parameters and every experiment results in an independent estimation of an initial rate.
2. It is not possible to examine whether parameters are constant under the altering conditions studied and therefore whether in the first place it is allowed to determine one parameter set. In chapter 2 this dependency is used to discriminate between two models and the model for which the parameters are dependent on the initial lactose concentration is rejected.

3 The opportunity to check if all experiments result in similar parameters is not possible. One experiment can result in a different set of parameters indicating that something happened during this reaction. When a sensible reason is found for the discrepancy, this experiment can be removed from the data set. When the experiments are fitted simultaneously this information can not be traced and the experiment resulting in different parameters influences the parameter set found.

An effective method to increase the yield of the reaction proves to be the use of high temperatures and high initial lactose concentrations. Higher yields are found but the inactivation caused by Maillard products is considerable. The application of thermostable enzymes for the oligosaccharide synthesis is promising. Preventing of enzyme inactivation for application of these enzymes in sugar solutions is however essential. The reactive amine groups of the enzyme should be shielded. This can probably be done by enzyme immobilisation.

Another method to increase the yield is to remove oligosaccharides from the reaction mixture. This option is studied combining measurements and model calculations. Experiments gave an indication of the order of magnitude of separation parameters. Model calculations gave a lot of insight about the possibilities and disadvantages of the various techniques used. This approach proofs to be very effective.

The techniques studied were activated carbon, ion-exchange chromatography, and membranes. Adsorption on activated carbon was shown to be a good technique. On industrial scale however elution with ethanol can be a problem. Experimentally ion-exchange chromatography looked very promising but calculations pointed out that even for very good resolutions no yield improvement is found when the dilution is too high. Dilution of the reaction mixture must be prevented and therefore this technique is not promising. The use of membranes seems to be the most elegant technique. However, first a membrane with the appropriate retentions must be found. This is the main problem since the membrane must distinguish between di- and trisaccharides and these molecules are very alike.

Acknowledgements

The authors wish to thank Cornelly van der Ven, Rashmi Sahtoe, and Maartje Devriese for performing experiments and Pieter Overdevest for the helpful discussions. The Association of Biotechnological Schools (ABON), the Royal Netherlands Academy of Arts and Sciences (KNAW), and Borculo Domo Ingredients supported this work.

References

Ajisaka K, Nishida H, Fujimoto H. 1987a. The synthesis of oligosaccharides by the reversed hydrolysis reaction of β-glucosidase at high substrate concentration and at high temperature. Biotechnol Lett 9:243-248.

Ajisaka K, Nishida H, Fujimoto H. 1987b. Use of an activated carbon column for the synthesis of disaccharides by use of a reversed hydrolysis activity of β-galactosidase. Biotech Lett 9:387-392.

Alles MS. 1998. Physiological effects of consumption of fructo-oligosaccharides and transgalacto-oligosaccharides. Thesis Wageningen Agricultural University, the Netherlands.

Bakken AP, Hill CG Jr, Amundson CH. 1991. Use of novel immobilized β-galactosidase reactor to hydrolyze the lactose constituent of skim milk II Multiresponse nonlinear regression analysis of kinetic data. Appl Biochem Biotechnol 28/29:741-756.

Bakken AP, Hill CG Jr, Amundson CH. 1992. Hydrolysis of lactose in skim milk by immobilized β-galactosidase (*Bacillus circulans*). Biotechnol Bioeng 39:408-417.

Berner LA, O'Donnell JA. 1998. Functional foods and health claims legislation: applications to dairy foods. Int Dairy J 8:355-362.

Bonnin E, Thibault J-F. 1996. Galactooligosaccharide production by transfer reaction of an exogalactanase. Enzyme Microb Technol 19:99-106.

Bucke C. 1996. Oligosaccharide synthesis using glycosidases. J Chem Tech Biotechnol 67:217-220.

Burvall A, Asp NG, Dahlqvist A. 1979. Oligosaccharide formation during hydrolysis of lactose with *Saccharomyces lactis* lactase (Maxilact) Part I Quantitative aspects. Food Chem 4:243-250.

Butler JAV, Ockrent C. 1930. Studies in electrocapillarity Part III The surface tensions of solutions containing two surface-active solutes. J Phys Chem 34:2841-2859.

Cantarella L, Nikolov ZL, Reilly PJ. 1994. Disaccharide production by glucoamylase in aqueous ether mixtures. Enzyme Microb Technol 16:383-387.

Carrara CR, Rubiolo AC. 1996. Determination of kinetics parameters for free and immobilized β-galactosidase. Process Biochem 31:243-248.

Cavaille D, Combes D. 1995. Characterization of β-galactosidase from *Kluyveromyces lactis*. Biotechnol Appl Biochem 22:55-64.

Crittenden RG, Playne MJ. 1996. Production, properties and applications of food-grade oligosaccharides. Trends Food Sci Technol 7:353-361.

Crittenden RG. 1999. Prebiotics. In: Tannock GW. editor. Probiotics a critical review. Norfolk: Horizon scientific press.

Deschavanne PJ, Viratelle OM, Yon JM. 1978. Conformational adaptability of the active site of β-galactosidase. J Biol Chem 253:833-837.

Dordick JS. 1992. Designing enzymes for use in organic solvents. Biotechnol Prog 8:259-267.

Finch P, Yoon JH. 1997. The effects of organic solvents on the synthesis of galactose disaccharides using β-galactosidases. Carbohydr Res 303:339-345.

Fischer L, Bromann R, Kengen SWM, de Vos WM, Wagner F. 1996. Catalytical potency of β-glucosidase from the extremophile *Pyrococcus furiosus* in glucoconjugate synthesis. Bio/Technol 14:88-91.

Fransen CTM, van Laere KMJ, van Wijk AAC, Brüll LP, Dignum M, Thomas-Oates JE, Haverkamp J, Schols HA, Voragen AGJ, Kamerling JP, Vliegenthart JFG. 1998. α-D-Glc*p*-(1↔1)-β-D-Gal*p*-containing oligosaccharides, novel products from lactose by the action of β-galactosidase. Carbohydr Res 314:101-114.

Friend BA, Shahani KM. 1982. Characterization and evaluation of *Aspergillus oryzae* lactase coupled to a regenerable support. Biotechnol Bioeng 24:329-345.

Gibson GR, Roberfroid MB. 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J Nutr 125:1401-1412.

Gibson GR, Willems A, Reading S, Collins MD. 1996. Fermentation of non-digestible oligosaccharides by human colonic bacteria. Proc Nutr Soc 55:899-912.

Gololobov MY, Borisov IL, Belikov VM, Švedas VK. 1988. Acyl group transfer by proteases forming acyl-enzyme intermediate: kinetic model analysis. Biotechnol Bioeng 32:866-872.

Halling PJ. 1994. Thermodynamic predictions for biocatalysis in nonconventional media: theory, test, and recommendations for experimental design and analysis. Enzyme Microb Technol 16:178-206.

Hartemink R. 1999. Prebiotic effects of non-digestible oligo- and polysaccharides. Thesis Wageningen Agricultural University, the Netherlands.

Huang R-T, Chen T-L, Weng H-S. 1995. Binary adsorption in a fixed-bed column packed with an ion-exchange resin. Sep Sci Technol 30:2731-2746.

Huber RE, Kurz G, Wallenfels K. 1976. A quantitation of the factors which affect the hydrolase and transgalactosylase activities of β-galactosidase (*E. coli*) on lactose. Biochem 15:1994-2001.

Ichikawa Y, Look GC, Wong C-H. 1992. Enzyme-catalyzed oligosaccharide synthesis. Anal Biochem 202:215-238.

Iwasaki K, Nakajima M, Nakao S. 1996. Galacto-oligosaccharide production from lactose by an enzymic batch reaction using β-galactosidase. Process Biochem 31:69-76.

Jandera P, Churácek J. 1974. Ion-exchange chromotography of aldehydes, ketones, ethers, alcohols, polyols and saccharides. J Chromatogr 98:55-104.

Janssen AEM, Halling PJ. 1994. Specificities of enzymes 'corrected for solvation' depend on the choice of the standard state. J Am Chem Soc 116:9827-9830.

Kasche V. 1986. Mechanism and yields in enzyme catalysed equilibrium and kinetically controlled synthesis of β-lactam antibiotics, peptides and other condensation products. Enzyme Microb Technol 8:4-16.

Kengen SWM, Luesink EJ, Stams AJM, Zehnder AJB. 1993. Purification and characterization of an extremely thermostable β-glucosidase from the hyperthermophilic archaeon *Pyrococcus furiosus*. Eur J Biochem 213:305-312.

King EL, Altman C. 1956. A schematic method of deriving the rate laws for enzyme-catalyzed reactions. J Phys Chem. 60:1375-1378.

Labuza TP, Baisier WM. 1992. The kinetics of nonenzymatic browning. In: Schwartzberg HG, Hartel RW. editors. Physical chemistry of foods. New York: Marcel Dekker Inc. p 595-649.

Laroute V, Willemot R-M. 1989. Glucose condensation by glucoamylase in organic solvents. Biotechnol Lett 11:249-254.

López Leiva MH, Guzman M. 1995. Formation of oligosaccharides during enzymic hydrolysis of milk whey permeates. Process Biochem 30:757-762.

Mahoney RR, Whitaker JR. 1977. Stability and enzymatic properties of β-galactosidase from *Kluyveromyces fragilis*. J Food Biochem 1:327-350.

McMeekin TA, Olley JN, Ross T, Ratkowsky DA. 1993. Predictive microbiology: theory and application. Somerset: Research studies press Ltd.

Minami Y, Yazawa K, Tamura Z, Tanaka T, Yamamoto T. 1983. Selectivity of utilization of galactosyl-oligosaccharides by bifidobacteria. Chem Pharm Bull 31:1688-1691.

Mozaffar Z, Nakanishi K, Matsuno R, Kamikubo T. 1984. Purification and properties of β-galactosidases from *Bacillus circulans*. Agric Biol Chem 48:3053-3061.

Mulder M. 1996. Basic Principles of membrane technology. Dordrecht: Kluwer Academic Publishers.

Nakanishi K, Matsuno R, Torii K, Yamamoto K, Kamikubo T. 1983. Properties of immobilized β-D-galactosidase from *Bacillus circulans*. Enzyme Microb Technol 5:115-120.

Nam DH, Kim C, Ryu DDY. 1985. Reaction kinetics of cephalexin synthesizing enzyme from *Xanthomonas citri*. Biotechnol Bioeng 27:953-960.

Ogushi S, Yoshimoto T, Tsuru D. 1980. Purification and comparison of two types of β-galactosidases from *Aspergillus oryzae*. J Ferment Technol 58:115-122.

Oku T. 1996. Oligosaccharides with beneficial health effects: a Japanese perspective. Nutrition Reviews 54:S59-S66.

Onishi N, Yamashiro A, Yokozeki K. 1995. Production of galacto-oligosaccharide from lactose by *Sterigmatomyces elviae* CBS8119. Appl Environ Microbiol 61:4022-4025.

Onishi N, Tanaka T. 1995. Purification and properties of a novel thermostable galacto-oligosaccharide-producing β-galactosidase from *Sterigmatomyces elviae* CBS8119. Appl Environ Microbiol 61:4026-4030.

Park YK, de Santi MSS, Pastore GM. 1979. Production and characterization of β-galactosidase from *Aspergillus oryzae*. J Food Sci 44:100-103.

Perry RH, Green D. 1984. Perry's chemical engineers' handbook. New York: McGraw-Hill Book Company. Table 1-16 (a), Table 1-18 (b), chapter 14 (c).

Petzelbauer I, Nidetzky B, Haltrich D, Kulbe KD. 1999. Development of an ultra-high-temperature process for the enzymatic hydrolysis of lactose I The properties of two thermostable β-glycosidases. Biotechnol Bioeng 64:322-332.

Prenosil JE, Stuker E, Bourne JR. 1987. Formation of oligosaccharides during enzymatic lactose hydrolysis and their importance in a whey hydrolysis process Part II Experimental. Biotechnol Bioeng 30:1026-1031.

Reimann A, Robb DA, Halling PJ. 1994. Solvation of CBZ-amino acid nitrophenyl esters in organic media and the kinetics of their transesterification by subtilisin. Biotechnol Bioeng 43:1081-1086.

Roberts HR, Pettinati JD. 1957. Concentration effects in the enzymatic conversion of lactose to oligosaccharides. Agric Food Chem 5:130-134.

Roberts DV. 1977. Enzyme kinetics. Cambridge: Cambridge University Press.

Sanders ME. 1998. Overview of functional foods: emphasis on probiotic bacteria. Int Dairy J 8:341-347.

Santos A, Ladero M, García-Ochoa F. 1998. Kinetic modeling of lactose hydrolysis by a β-galactosidase from *Kluyveromices fragilis*. Enzyme Microb Technol 22:558-567.

Sarmidi MR, Barker PE. 1993. Simultaneous biochemical reaction and separation in a rotating annular chromatograph. Chem Eng Sci 48:2615-2623.

Scheckermann C, Wagner F, Fischer L. 1997. Galactosylation of antibiotics using the β-galactosidase from *Aspergillus oryzae*. Enzyme Microb Technol 20:629-634.

Schmidt RR. 1986. New methods for the synthesis of glycosides and oligosaccharides-Are there alternatives to the Koenigs-Knorr method? Angew Chem Int Ed Engl 25:212-235.

Shieh MT, Barker PE. 1996. Combined bioreaction and separation in a simulated countercurrent chromatographic bioreactor-separator for the hydrolysis of lactose. J Chem Tech Biotechnol 66:265-278.

Shubhada S, Sundaram PV. 1995. The role of pH change caused by the addition of water-miscible organic solvents in the destabilization of an enzyme. Enzyme Microb Technol 17:330-335.

van Tol JBA, Stevens RMM, Veldhuizen WJ, Jongejan JA, Duine JA. 1995. Do organic solvents affect the catalytic properties of lipase? Intrinsic kinetic parameters of lipases in ester hydrolysis and formation in various organic solvents. Biotechnol Bioeng 47:71-81.

Ulbrich-Hofmann R, Selisko B. 1993. Soluble and immobilized enzymes in water-miscible organic solvents: glucoamylase and invertase. Enzyme Microb Technol 15:33-41.

Voorhorst WGB, Eggen RIL, Luesink EJ, de Vos WM. 1995. Characterization of the *celB* gene coding for β-glucosidase from the hyperthermophilic archaeon *Pyrococcus furiosus* and its expression and site-directed mutation in *Escherichia coli*. J Bacteriol 177:7105-7111.

Wallenfels K, Malhotra OP. 1961. Galactosidases. Adv Carbohydr Chem 16:239-298.

Watt GM, Lowden PAS, Flitsch SL. 1997. Enzyme-catalyzed formation of glycosidic linkages. Curr Opin Structural Biol 7:652-660.

Wescott CR, Klibanov AM. 1993. Predicting the solvent dependence of enzymatic substrate specificity using semiempirical thermodynamic calculations. J Am Chem Soc 115:10362-10363.

Whistler RL, Durso DF. 1950. Chromatographic separation of sugars on charcoal. J Am Chem Soc 72:677-679.

Wijsman MR, Hereijgers JLPM, de Groote JMFH. 1989. Selective enumeration of bifidobacteria in fermented dairy products. Neth Milk Dairy J 43:395-405.

Yanahira S, Suguri T, Yakabe T, Ikeuchi Y, Hanagata G, Deya E. 1992. Formation of oligosaccharides from lactitol of *Aspergillus oryzae* β-D-galactosidase. Carbohydr Res 232:151-159.

Yang S-T, Okos MR. 1989. Effects of temperature on lactose hydrolysis by immobilized β-galactosidase in plug-flow reactor. Biotechnol Bioeng 33:873-885.

Zaks A, Klibanov AM. 1988. The effect of water on enzyme action in organic media. J Biol Chem 263:8017-8021.

Ziemer CJ, Gibson GR. 1998. An overview of probiotics, prebiotics and synbiotics in the functional food concept: perspectives and future strategies. Int Dairy J 8:473-479.

Zwietering MH, Cuppers HGAM, de Wit JC, van 't Riet K. 1994. Evaluation of data transformations and validation of models for the effect of temperature on bacterial growth. Appl Environ Microbiol 60:195-203.

Summary

There is general agreement that the intestinal microflora, e.g. bifidobacteria, has a strong influence on the human health. Two approaches exist to increase the number or activity of health-promoting organisms in the gastrointestinal tract. Organism (probiotics) or a selective carbon source (prebiotics) can be added to the food. Examples of prebiotics are oligosaccharides. These can be produced by enzymatic synthesis with a glycosidase as catalyst and a disaccharide as substrate. Oligosaccharide formation using the transferase activity of glycosidases is a kinetically controlled reaction. When following the reaction in time, an optimum in product concentration is observed. This has important implications for the reactor design. Furthermore, the enzymatic synthesis of oligosaccharide can serve as a model system for the synthesis of a kinetically controlled product.

Since the synthesis is a kinetically controlled reaction, describing the concentrations of all reactants in time is essential for a reactor design. Along with describing the concentrations in time, the aim of this research is to obtain more knowledge about the effect of process parameters such as substrate concentration, temperature, time, enzyme origin, and reactor configuration on the oligosaccharide synthesis. The model system used in this thesis is the reaction with lactose as a substrate and a β-galactosidase as catalyst.

In chapter 2 a procedure is developed to estimate the model parameters and to select the most suitable model. Two models are proposed, one from an engineering and one from a biochemical perspective. Batch experiments at various initial lactose concentrations (0.19-0.59 mol/kg) are performed. Each experiment is considered as an independent observation, and consequently, the kinetic parameters are fitted to each experiment separately. The parameters of the model from an engineering perspective show a trend upon the initial lactose concentration. The parameters of the galactosyl-enzyme complex based model (biochemical approach) are not dependent on the initial lactose concentration. Therefore, this model is chosen to describe the oligosaccharide synthesis at various initial lactose concentrations. The average parameter set describes the concentrations in time satisfactorily.

In chapter 3 the effect of enzyme origin and temperature on the synthesis of oligosaccharides is quantified. The kinetic parameters of β-galactosidases from *Bacillus circulans*, *Aspergillus oryzae*, *Kluyveromyces lactis*, and *Kluyveromyces fragilis* are determined. Clear differences between the β-galactosidases are found concerning amount, size, and type of oligosaccharides produced. Differences between the enzymes are supported by statistically different kinetic parameters. Also batch experiments at various temperatures (20-50°C) are performed. The effect of temperature on the kinetic parameters

can be described with the Arrhenius equation, except for the inhibition parameter. Although only slightly higher oligosaccharide yields are found at higher temperatures, these temperatures are interesting since higher initial lactose concentrations are possible. This requires a thermostable and thermoactive biocatalyst.

Therefore, in chapter 4 the oligosaccharide synthesis by the β-glucosidase from the hyperthermophilic archaeon *Pyrococcus furiosus* is discussed. This enzyme produces tri- and tetrasaccharides with lactose as a substrate at elevated temperatures up to 95°C. The oligosaccharide yield obtained with this thermostable enzyme is a factor 1.4 higher than reported for mesophilic enzymes. Inactivation by Maillard components is clearly visible. For application of thermostable enzymes in sugar solutions, it is essential to prevent enzyme inactivation.

In chapter 5 activated carbon is used to remove oligosaccharides from the reaction mixture and thereby increase the yield. The adsorption on activated carbon can be described by the multi-component Langmuir isotherm with different maximum saturation constants for mono-, di-, and trisaccharides. The affinity for trisaccharides is larger than for di- and monosaccharides. A model combining kinetics, adsorption, and mass transfer is developed. Model calculations for the batch process with removal show a yield improvement compared to the batch process without removal. Experimentally this is confirmed. Model calculations for the studied continuous processes do not result in an increase of the yield. The advantages of removal are masked by the negative influence of re-circulation and the relative long time between formation and removal.

In chapter 6 additional aspects are evaluated shortly. The inactivation of the thermostable β-glucosidase from *Pyrococcus furiosus* is discussed. First order enzyme inactivation is implemented in the kinetic model and the extended model can describe the results satisfactorily. Next the possibility to produce oligosaccharides in water-miscible solvents is discussed. Enhancement of the yield by addition of solvents is reported in literature. However, it is found that the lactose solubility and the observed reaction rates decrease. Decrease of the reaction rates is caused by solvation effects and enzyme inactivation. The solvation effects are quantified. Last other possibilities to remove oligosaccharides from the reaction mixture are studied. Model calculations for membranes and ion-exchange chromatography are used to determine if the yield can be improved compared to a process without removal. A membrane with a high retention for trisaccharides and a low retention for lactose, will result in a yield improvement. Calculations show that ion-exchange processes only result in a yield improvement when dilution is not too high.

Samenvatting

Er is consensis dat de darmflora, b.v. bifidobacteriën, een grote invloed heeft op de gezondheid van de mens. Er bestaan twee benaderingen om het aantal en de activiteit van gezondheidsbevorderende organismen in het maagdarmkanaal te beïnvloeden. Organismen (probiotica) of een specifieke koolstof bron (prebiotica) kunnen aan het eten worden toegevoegd. Een voorbeeld van prebiotica zijn oligosacchariden. Deze kunnen enzymatisch gevormd worden met een glycosidase als katalysator en een disaccharide als substraat. De oligosaccharidensynthese is een kinetisch gecontroleerde reactie. Dit wil zeggen dat een optimum in de productconcentratie wordt waargenomen als de reactie in de tijd wordt gevolgd. Dit heeft belangrijke gevolgen voor het reactorontwerp. De enzymatische synthese van oligosacchariden dient als modelsysteem voor de synthese van andere kinetisch gecontroleerde producten.

Voor het ontwerpen van een reactor voor een kinetisch gecontroleerde reactie is het beschrijven van de concentraties van alle reactanten in de tijd essentieel. Naast het beschrijven van de concentraties in de tijd, is het doel van dit onderzoek om meer inzicht te krijgen in de invloed van procesparameters zoals substraatconcentratie, temperatuur, tijd, oorsprong van het enzym en reactorconfiguratie op de oligosaccharidensynthese. Het modelsysteem dat gebruikt wordt is de reactie met lactose als substraat en β-galactosidase als katalysator.

In hoofdstuk 2 is een procedure ontwikkeld om de kinetische parameters te fitten en om het meest geschikte model te selecteren. Twee modellen zijn vergeleken, één vanuit een technologische en één vanuit een biochemische benadering. De experimenten zijn batchgewijs uitgevoerd bij verschillende initiële lactose concentraties (0.19-0.59 mol/kg). Elk experiment is beschouwd als een onafhankelijke waarneming en om die reden worden de kinetische parameters bepaald uit elk experiment afzonderlijk. De parameters van het eerste model (technologische aanpak) zijn afhankelijk van de initiële lactoseconcentratie. De parameters van het model gebaseerd op de vorming van een galactosyl-enzym complex (biochemische aanpak) zijn echter onafhankelijk van de initiële lactoseconcentratie. Daarom is dit model gekozen om de oligosaccharidensynthese te beschrijven bij verschillende lactoseconcentraties. De gemiddelde parameterset beschrijft de concentraties in de tijd goed.

In hoofdstuk 3 is de invloed van de oorsprong van het enzym en de temperatuur op de synthese van oligosacchariden gekwantificeerd. De kinetische parameters van de β-galactosidases van *Bacillus circulans*, *Aspergillus oryzae*, *Kluyveromyces lactis* en *Kluyveromyces fragilis* zijn bepaald. Er zijn duidelijke verschillen tussen de βgalactosidasen gevonden betreffende de hoeveelheid, grootte en typen geproduceerde oligosacchariden. Verschillen worden ondersteund door statistisch verschillende kinetische parameters. Ook zijn experimenten bij verschillende temperaturen (20-50°C) uitgevoerd. Het effect van de temperatuur op de kinetische parameters kan, met uitzondering van de inhibitieparameter, beschreven worden met de Arrhenius vergelijking. Hoewel slechts een geringe opbrengstverhoging bij hogere temperaturen is gevonden, zijn deze temperaturen toch interessant omdat hogere initiële lactoseconcentraties mogelijk zijn. Dit vereist een thermostabiele en thermoactieve biokatalysator.

Om deze laatste reden is in hoofdstuk 4 de oligosaccharidensynthese gekatalyseerd door de β-glucosidase van de hyperthermofiele archaeon *Pyrococcus furiosus* besproken. Met dit enzym zijn tri- en tetrasaccharides geproduceerd met lactose als substraat bij temperaturen tot 95°C. De oligosaccharidenopbrengst verkregen met dit thermostabiele enzym is een factor 1.4 hoger dan beschreven voor mesofiele enzymen. Inactivatie door Maillard componenten is duidelijk waarneembaar. Voor toepassing van thermostabiele enzymen in suikerhoudende oplossingen is het essentieel dat enzyminactivatie wordt voorkomen.

In hoofdstuk 5 is actief kool gebruikt om oligosacchariden uit het reactiemengsel te verwijderen en om op die manier de opbrengst te verhogen. De adsorptie op actief kool kan beschreven worden met de multi-component Langmuir isotherm met verschillende maximale verzadigingconstanten voor mono-, di-, en trisacchariden. De affiniteit voor trisacchariden is groter dan voor di- en monosacchariden. Een model is ontwikkeld waarin kinetiek, adsorptie en stofoverdracht zijn gecombineerd. Met modelberekeningen voor het batchgewijze proces is een toename van de opbrengst gevonden. Experimenteel is dit bevestigd. Voor de continue processen die bestudeerd zijn, is met modelberekeningen geen toename van de opbrengst aangetoond. De voordelen van verwijdering worden teniet gedaan door de negatieve invloed van recirculatie en de relatief lange tijd tussen vorming en verwijdering.

In de discussie (hoofdstuk 6) zijn enkele overige aspecten kort behandeld. De inactivatie van het thermostabiele enzym β-glucosidase van *Pyrococcus furiosus* is bestudeerd. Aan het kinetische model van hoofdstuk 2 is eerste orde enzyminactivatie toegevoegd. Met dit uitgebreide model kunnen de resultaten goed beschreven worden. Daarnaast is de mogelijkheid bestudeerd om oligosacchariden te produceren in watermengbare oplosmiddelen. Verhoging van de opbrengst door toevoeging van solvents is beschreven in de literatuur. Er is echter gevonden dat de oplosbaarheid van lactose en de reactiesnelheden afnemen. Lagere reactiesnelheden worden veroorzaakt door solvatatie en enzyminactivatie. Deze solvatatie effecten zijn gekwantificeerd. Als laatste zijn andere mogelijkheden om oligosacchariden uit het reactiemengsel te verwijderen bestudeerd.

Modelberekeningen voor membranen en ionenwisselingschromatografie zijn gebruikt om te bepalen of de opbrengst ten opzichte van een proces zonder verwijdering verhoogd kan worden. Een membraan met een hoge retentie voor trisacchariden en een lage retentie voor lactose, geeft een opbrengstverhoging. Berekeningen tonen aan dat ionenwisselaars-chromatografieprocesen alleen zullen leiden tot een opbrengstverhoging als de verdunning niet te hoog is.

Nawoord

Het nawoord is het laatste wat ik moet schrijven voordat het proefschrift af is. In dit nawoord wil ik mensen bedanken die de afgelopen vier jaar in welke vorm dan ook hebben bijgedragen aan het proefschrift of aan mijn goede gemoedstoestand. Ik heb die afgelopen 4 jaar als erg leuk en leerzaam ervaren. Natuurlijk heb ik ook perioden gehad dat het allemaal wat minder ging, maar nooit heb ik gedacht: was ik er maar niet aan begonnen. Dat wil echter niet zeggen dat ik nu niet blij ben dat het klaar is.

De mensen die ik wil bedanken zijn in te delen in twee groepen: werk en privé. Ik zal beginnen met het werk met als allereerste Anja. Als ik naar dit boekje kijk zie ik hoe sterk ik mijn eigen stempel daar op heb kunnen drukken. Je hebt me vrijheid gegeven om die kant op te gaan die mij het meeste interesseerde en ondertussen de kans gezien om mij goed te begeleiden. Dat is heel knap en ik heb met plezier met je samengewerkt. Klaas wil ik bedanken voor het feit dat hij mij heeft aangenomen. De AIO besprekingen die in het leven werden geroepen toen je eenmaal naar TNO was verhuisd heb ik als nuttig ervaren.

Dit project is deels gefinancierd door Borculo Domo Ingredients. De besprekingen die daaruit voort vloeiden heb ik als zeer motiverend ervaren. In het begin heb ik contact gehad met Peter Ekhart en Rob Kammelar, later met Jaap de Slegte en Miriam Heutinck. Ik hoop dat jullie met interesse het proefschrift zullen bekijken wat voor jullie ligt.

Mijn kamergenoot Pieter wil ik bedanken voor de vele discussies over zinnige en onzinnige onderwerpen zoals modelleren in het algemeen en modelleren in Matlab, films, videootjes, borrelen en de belevenissen in het weekend. Ik heb mij vaak verbaasd over het feit dat twee mensen die zo verschillend zijn, het toch zo goed met elkaar kunnen vinden. Ik heb met plezier vier jaar naast je gezeten. Na een jaar is Ed op de kamer gekomen. Ook Ed en ik zijn heel verschillend, maar een blik van verwondering hebben we vaak gewisseld achter Pieter zijn rug terwijl Pieter vertelde over zijn kroegbelevenissen.

De buurman Albert van kamer 608 wil ik bedanken voor zijn belangstelling op allerlei gebieden en voor zijn inbreng in hoofdstuk 2 maar ook in hoofdstuk 5. Als Anja afwezig was kon ik met mijn vragen vaak bij jou terecht.

De mensen die mij kennen weten dat mijn grote passie niet in het lab ligt. Ik ben daarom ook erg erkentelijk voor alle hulp die ik van Jos heb gehad. De keren dat ik iets niet kon vinden, een opstelling moest bouwen, de kolom waterdicht moest maken, de HPLC er geen zin meer in had of een schroef die te vast zat los moest draaien, en dat aan Jos heb gevraagd zijn ontelbaar. Op het eind heb je me ook nog met proeven geholpen. Dank je wel. Dit is ook een goede plek om Jos en Gerrit samen te bedanken voor de onvergetelijke Ardennen fietstochten. Met name hoe jullie weer terugkomen voor de langzamere fietsers en iedereen het gevoel geven dat jullie het leuk vinden dat ze meegaan is mooi om te zien.

Tijdens mijn promotie heb ik de hulp gehad van afstudeerders. Op deze plek wil ik Cornelly van der Ven, Rashmi Sahtoe, Maartje Devriese en Jurgen Smeenk vertellen dat zij voor mij heel motiverend waren en dat ik hun met plezier heb begeleid en mee samen heb gewerkt. Het is fijn om met mensen inhoudelijk te kunnen discusseren die met het zelfde onderwerp bezig zijn. I also hope I made the time in Wageningen for Elisabetta Fabbro and Céline Malard agreeable.

De sectie Proceskunde in het algemeen wil ik bedanken voor de lunches, labuitjes, dineetjes, AIO-reizen, filmavonden en promoties. Ik heb mij hier altijd erg thuis gevoeld en er heerst een goede en open sfeer op de sectie. Vooral de dagelijkse lunches heb ik als een leuke afwisseling van het werk ervaren.

De laatste persoon die ik wil bedanken voor ik overstap op de categorie privé is Esther. Esther ken ik al sinds mijn eerste jaar in Wageningen en ik heb het gevoel dat we elkaar steeds beter leren kennen. Ik was blij verheugd toen jij ook bij de sectie kwam werken. Het is prettig om iemand te hebben waar je af en toe gewoon even bij binnen kunt lopen.

Ik ben nu aangekomen bij de mensen die ik privé wil bedanken. Als eerste wil ik Afra en Rinus bedanken voor al hun steun, liefde en bezorgdheid. Mijn zussen Pol, Lot en Ploek voor de dagelijkse afleiding op de mail. Op de laatste plek komt traditie getrouw de partner, in dit geval is dat Eduard. Dank je wel voor het in stand houden van een goed evenwicht tussen werk en vrije tijd.

Floor

Curriculum vitea

Moniek Afra (Floor) Boon werd geboren op 2 juni 1972 te Den Haag. Zij bezocht de Rijksscholengemeenschap te Zierikzee en Vlissingen, waar zij bij de laatst genoemde in 1990 het VWO diploma behaalde. In dat zelfde jaar startte zij haar studie Milieuhygiëne aan de Landbouwuniversiteit te Wageningen. Zij deed afstudeervakken in de richtingen milieutechnologie en proceskunde. Haar stage liep zij bij de University of Trondheim in Noorwegen. In 1995 studeerde zij met lof af en begon met haar promotie onderzoek bij de sectie Proceskunde van de Landbouwuniversiteit te Wageningen, waarvan dit proefschrift het resultaat is. Momenteel is zij werkzaam bij de afdeling Processen en Applicaties van TNO-MEP te Apeldoorn.