Evaluation of current models for transgalactosylation catalysed by β-galactosidase

Master thesis

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Synopsis:

This aim of this project is to evaluate some of the current models to describe the transgalactosylation activity of β-galactosidase. Four models are compared in order to gain better understanding of the kinetics of β-galactosidase catalysed reactions. This is carried out by analysing the mechanistic and mathematical foundations of these models. The models are fitted to experimental data form a set of experiments using two concentrations of enzyme and three different initial lactose concentrations. The parameters were determined using nonlinear regression. It was shown that it is not possible to determine the rate parameters of the reaction with this methodology.

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1 Introduction

Food ingredients that has health promoting effects beyond what is normally associated with food are classified as functional foods [1, 2]. Prebiotics are a group of functional foods, a single and universally accepted definition of prebiotic substances does not exist. Roberfroid [3] has proposed a definition based on three properties 1) the substance should be resistant to gastric acidity, to hydrolysis by mammalian enzymes, and to gastrointestinal absorption, 2) it should be fermented by the intestinal microflora, and 3) it should selectively stimulate the growth and/or the activity of intestinal bacteria that contributes to the health and well-being of the host. So far only fructooligosaccharides, inulin, galactooligosaccahrides (*GOS*), and lactulose has been proven to fulfil all three of the criteria for prebiotics. Other oligosaccharides show promising properties, but still need further investigation [3].
Some of the main benefits of prebiotics are increased mineral uptake, alteration of the gut microbiota, and positive effects on the immune system[4, 5, 2, 1, 6]. Fermentation of prebiotics causes release of short chained fatty acids in the colon, resulting in lowering of the pH value, increasing the solubility of minerals and thereby thier bioavailability. Further, it has been shown that prebiotics alter the tissue in the large bowel causing the surface area to increase, thereby allowing increased transport into the body [6].
Prebiotics alters the proportion of the different bacteria present in the large bowel, resulting in a higher proportion of bifidobacteria and lactobacilli. These organisms has positive effects on health because they increase the gut resistance against colonisation of pathogens, and by altering the chemical environment e.g.through release of short chained fatty acids [6].

Use of prebiotics in infant formula has shown to decrease the number infections in infants less than 6 month of age, compared to infants fed with infant formula not containing prebiotics. The reason for reduction in infections is not completely known, but several mechanisms have been proposed, and there are most likely more of them working at the same time to result in lower infection rate [6].
Human milk has a high content of complex oligosaccharides, compared to other mammals the content of oligosaccharides is much higher and the structures are more complex. Most infant formula is based on bovine milk, which only has a oligosaccharide content of about a thousandth of human milk. Human milk oligosaccharides *(HMO)* have been shown to function as "decoys" for pathogenic bacteria. HMOs consists of a combination of up to five different monosaccarides: galactose; glucose; fucose;

N-acetylgucosamine; and sialic acid, figure 1.1 shows examples of the structures found in human milk oligosaccharides [7].

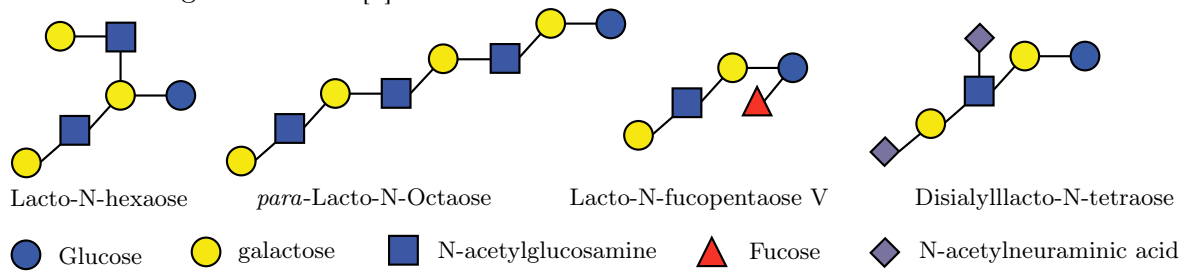


Figure 1.1: *Examples of HMO structures, adapted from Bode [7].*

Some of these oligosaccharides resemble the glycans found on the surface of the intestine, some pathogenic bacteria adhere to these surface glycans. By mimicking the structure of these glycans, HMOs block the receptors of the pathogenic bacteria, thereby functioning as an anti-adhesive/decoy [7].
Galactooligosaccharides are much less complex in composition and structure than HMOs. GOS consist of a chain of β linked galactosidase moieties with a terminal glucose moiety in the non-reducing end. A general formula of galactooligosaccharides is given is (1.1)

$$[Gal\beta 1 \rightarrow x]_n Gal\beta 1 \rightarrow 4Glu \tag{1.1}$$

Where Gal is galactose, Glu is Glucose, x indicates the position of the glycosidic linkage, it is most commonly 4 or 6, and n is number of galactose moieties added to lactose so that the degree of polymerisation (DP) is n plus 2, by current methods of production n is typically 1-3 [8]. Figure 1.2 shows an example of the structure of a GOS molecule.

GOS can be produced in an enzyme catalysed process using lactose as a substrate. This makes GOS an interesting product for the dairy industry due to the low cost of lactose which is found in large amounts as a by product from cheese production [8]. Thus converting lactose into GOS is a way of upgrading a low value product to higher value, both in terms of nutrition and price [8].

The enzyme used for production of GOS is a β-galactosidase (EC.3.2.1.23). β-galactosidase is a glycosyl hydrolase it hydrolyses the glycosidic bond of terminal β(1→3) and β(1→4)-D-galactose moites from the non-reducing end of β-galactosides, e.g. lactose [10]. β-galactosidase is found in many different organisms e.g. mammalian, fungal, and bacteria. Some β-galactosidases show tendency to perfom another reaction apart from hydrolysing the β glycosidic linkage to terminal D-galactose moiety, they also catalyse transgalactosylation [8]. Transgalactosylation is the reaction of transfer of a galactose moiety from one glycoside to another. This mechanism is described in more detail in section 2.1.

β-galactosidase has a low product specificity resulting in a mix of several products being produced. β-galactosidase is not specific with regards to the galactosyl acceptor, it can be water, with the result of hydrolysis, or it can be another sugar. The result is a product containing a mix of monosaccharides and oligosaccharides of various DP and regiochemistry [11]. The reaction mechanism of β-galactosidase is explained in detail in section 2.1.

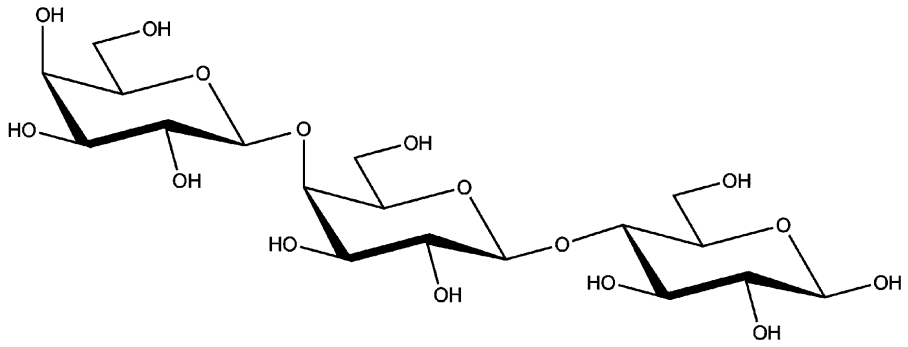


Figure 1.2: *Structure of a GOS trisaccharide, with a galactose moiety linked to lactose via a β(1→4) glycosidic bond, this is called a 4'-glactosyllactose. Adapted from Bultema et al. [9].*

The low specificity of β-galactosidase makes it hard to fully understand the kinetics of the reactions catalysed by β-galactosidase. To gain a better understanding of the kinetics of β-galactosidase several attempts has been made to formulate a model that can describe the progression of transgalactosylation [12, 13, 14]. Simulation provides a valuable tool understanding of chemical reactions and in process optimization. In spite of several published models, there have, to the best of my knowledge, not been establish a consensus on best model for β-galactosidase catalysed transglycosylation. The proposed models has many differences in terms of the underlying assumptions and the method of derivation, and no direct comparison between the models has been made.

1.1 Problem description

The aim of this project is to improve the understanding of the transgalactosylation reaction catalysed by β-galactosidase with lactose as substrate. This will be done by analysis of models proposed by Boon et al. [12], Vera et al. [13], and Palai et al. [14]. These models use quite rather different assumptions to describe the reaction, so they can not be directly compared, it is therefore difficult to determine if one of the models are superior. An assesment of the models will be carried out by using nonlinear regression to fit the models to the progression data obtain from experiments performed in the laboratory. A major factor in deciding the product yield in production of GOS is the initial lactose concentration [15], therefore focus will be on the models ability to simulate GOS production at different initial lactose concentration. It will be assumed that β-galactosidases follow the same reaction mechanism, therefore progression experiments of transgalactosylation will not be made with enzymes from different sources, but only with β-galactosidase from Aspergillus oryzae.

Theory 2

This chapter contains the theoretical backgound needed formulate a model for the reactions catalysed by β-galactosidase. The first part focuses on the structure and reaction mechanism of β-galactosidase. The second part focuses on the mathematical treatment of enzyme kinetics. Finally the third part introduces the basic concepts of nonlinear regression.

2.1 β-galactosidase: structure and function

β-galactosidase is a glycosyl hydrolase, for glycosyl hydrolases two independent systems of classification of glycosyl hydrolases exists. One is classification by the reactions they catalyse, this is the EC number classificatio. Another system of classification is based to sequence and structure similarities [16, 17, 18]. This system divides the glycosyl hydrolases into families, currently 135 families of glycosyl hydrolases exists. Related families are grouped into clans of which there are 14 [19, 20]. β-galactosidase is found in the clan GH-A, the members of GH-A are related by having a β/α_8 fold in the domain containing the active site, and the two catalytically active glutamic acid residues are located on the 4th and 7th β-strand[21]. Figure 2.1 shows the structure of *A. oryzae* β-galactosidase where the TIM-barrel structure of the catalytic domain can be seen. β-galactosidase is found in the families GHF-1, GHF-2, GHF-35, GHF-42, this reflects a

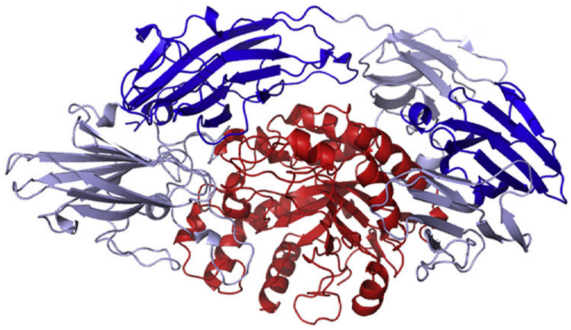
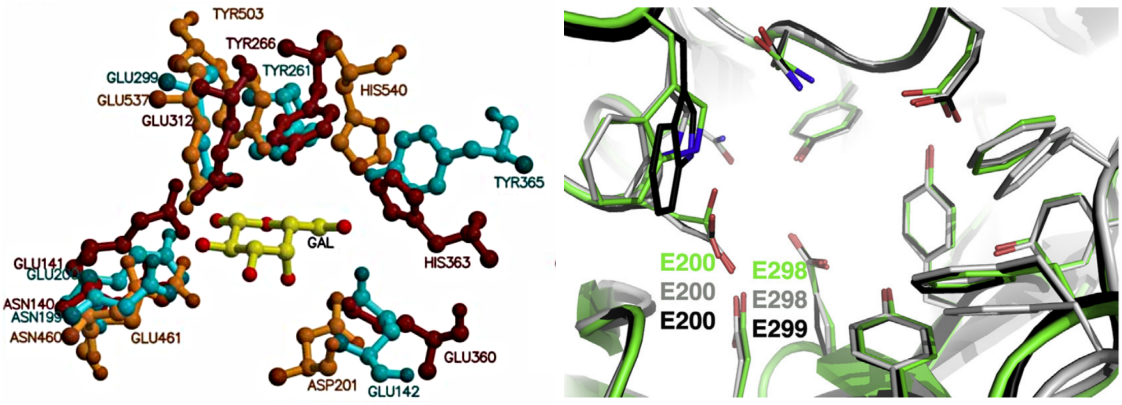


Figure 2.1: *The structure of A. oryzae β-galactosidase, the domain highlighted in red is the TIM-barrel domain. Adapted from Maksimainen et al. [10].*

large variation in the sequence and tertiary structure of the enzyme, but the TIM-barrel domain and especially the active site is somewhat preserved [21, 10]. The similarity of the active site across different β-galactosidases can be visualised by a superposition of

the active site from *Escherichia coli*, *Penicillicum sp.*, and *Thermus thermopilus*, (figure 2.2a), these belong to GHF-2, GHF-35, and GHF-42 respectively, although the primary and tertiary structures of these enzymes are very different, the active site is very preserved. Figure 2.2b shows a superposition of β-galactosidase from *Asperguillus oryzae*, *Trichoderma reesi*, and *Penicillicum sp.* which are all members of GHF-35 [10]. The high degree of similarity between the active sites, suggests that docking of the substrate follows similar mechanims for different β-galactosidases. The active site of β-galactosidase has a pocket type topology with room for one one saccharide ring [22, 23, 10, 21]. The active exhibit high selectivity of galactossyl moieties linked via a β-galactosyl linkage. As a consequence of the pocket type nature of the site, the specificity towards the galactosyl acceptor is broad [23, 24].



(a) **(b)**

Figure 2.2: ***(a)*** *Superposition of the active site of β-galactosidase from Escherichia coli (orange), Thermus thermopilus (brown), and Penicillicum sp. (cyan) shows the active site is highly preserved across different families glycosyl hydrolases. Adapted from Rojas et al. [21].* ***(b)*** *Superposition of the active site of β-galactosidase from Asperguillus oryzae (green), Trichoderma reesi (grey), and Penicillicum sp. (black). Adapted from Maksimainen et al. [10].*

β-galactosidase hydrolyses the glycosidic bond via general acid catalysis, with a glutamic acid residue acting as a proton donor and a glutamic acid residue acting a nucleophile [21]. When β-galactosidase binds to a substrate, galactose is positions so the glycosidic oxygen is within hydrogen bonding distance to the proton donor. The glycosidic oxygen then accepts the proton and the bond i broken. At the same time the nucleophile attacks the anomeric carbon which is thereby stabilised. A galactosyl acceptor then perfoms a nucleophile attack on the anomeric carbon and the product is released[22]. If the galacotosyl acceptor is water the result is hydrolysis, if the acceptor is a sugar the result is transgalactosylation. Figure 2.3 shows the mechanim of a retaining glycosyl hydrolase [22]. The distance between the catalytic residues determines the configuration of the anomeric carbon after hydrolysis. If the distance is approximately 5.5 Å the configuration of the anomeric carbon is retained, if the distance is approximately 10 Å the configuration is inverted [22]. The distance between catalytically active glutamic acid residues in β-galactosidase is a little bit smaller than 5.5 Å, β-galactosidase from *Penicillicum sp.* has the glutamic acid residues spaced at 4.5 Å [21].

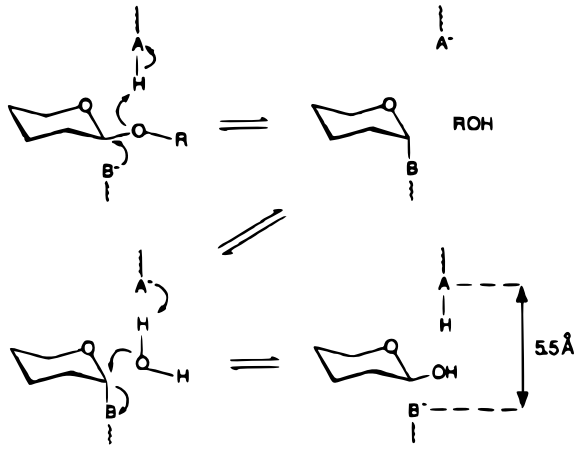


Figure 2.3: *The mechanism for hydrolysis of a retaining glycosyl hydrolases. Adapted from Davies and Henrissat [22]*

2.2 Derivation of rate laws for enzymatic reactions

2.2.1 Fundamentals of Enzyme Kinetics

Simple chemical reactions follow simple, integral order, rate laws. An irreversible unimolecular reaction of the form (2.1).

$$A \rightarrow P \tag{2.1}$$

With the rate law given by (2.3) is referred to as a first order reaction because the rate is dependent on reactants to the power of 1. An irreversible bimolecular reaction of the form (2.2).

$$A + B \rightarrow P + Q \tag{2.2}$$

where the rate law is given by (2.4) is a second order reaction because the rate is dependent on the product of the reactants. In the case where A and B are the same the rate is dependent on A to the power of two [25, chap. 2].

$$A \rightarrow P \frac{dP}{dt} = k[A] \tag{2.3}$$

$$\frac{dP}{dt} = k[A][B] \tag{2.4}$$

Catalytic reactions does not proceed through simpel uni- or bimolecular mechanisms, but through a series of intermediate steps, which results in overall rate laws that are not of integer order [25, chap. 3]. The most fundamental equation in enzyme kinetics is the Michaelis-Menten equation:

$$\frac{d[P]}{dt} = \frac{V_{max} * [A]}{[A] + K_M} \tag{2.5}$$

The Michaelis-Menten describes the reaction by (2.6). Where E represents free enzyme, A is the substrate, X is a enzyme substrate intermediate and P is the product. In the derivation of equation 2.5 that the revesible part of the reaction occurs much faster than

the irrevesible part, resulting in the reversible step of the reaction being in approximately thermodynamical equilibrium, this assumption that $k_3 \ll k_2$ [25].

$$\mathrm{E} + \mathrm{A} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{X} \xrightarrow{k_3} \mathrm{P} + \mathrm{E} \tag{2.6}$$

In equation 2.5 V_{max} represents the maximum rate of the reaction, where the concentration of A is so high that the reversible step of the reaction has been pushed all the way to the right. All the enzyme is bound in the intermediate state, in this case the reaction is a zeroth order reaction with regard to A and the rate is V_{max}. At the other limit of very small A the Michaelis-Menten equation transforms to a first order reaction with the pseudo first order rate constant $\frac{V_{max}}{K_M}$, K_M is the Michaelis-Menten constant which is equal to the concentration at half V_{max}. In terms of rate constants V_{max} and K_M are given by (2.7) and (2.8).

$$K_M = \frac{k_2}{k_1} \tag{2.7}$$

$$V_{max} = k_3 * E_{total} \tag{2.8}$$

The assumption of a rapid equilibrium made in the original treatment by Michaelis and Menten can not always be allowed. A more general treatment of enzyme kinetics is the steadys-state approximation formulated by Briggs and Haldane [26]. Briggs and Haldane [26] realised that for the most part of an enzyme catalysed reaction, the concentration of the enzyme-substrate intermediate remains approximately constant, figure 2.4 shows simulated progress curves of concentrations of A, P, and X, in the reaction 2.6, with $k_1 = k_2 = k_3$.

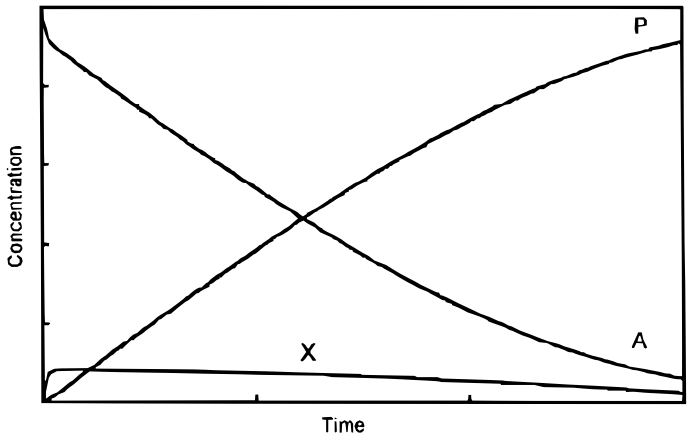


Figure 2.4: *Progress curves of the species in reaction 2.5 by numerical integration of the equations 2.9 with k_1, k_2, and k_3 set equal. Adapted from Leskovac [25, page 35].*

The assumption that $k_3 \ll k_2$ does not apply to the steady-state approximation, because the concentration of the enzyme intermediate will remain virtually constant for any k_3 and k_2. The steady-state approximation relies on the substrate concentration being substantially higher than the enzyme concentration to be valid.
The rate law of an enzyme catalysed reaction can be derived using the steady-state approximation by solving the equations for rate of change of each species in the reaction and using the equation for conservation of mass for E, for reaction 2.6 the equations are

given by (2.9a-e).

$$\frac{dA}{dt} = -k_1 * A * E + k_2 * X \tag{2.9a}$$
$$\frac{dX}{dt} = k_1 * A * E - k_2 * X - k_3 * X \tag{2.9b}$$
$$\frac{dE}{dt} = k_2 * X + k_3 * X \tag{2.9c}$$
$$\frac{dP}{dt} = k_3 * X \tag{2.9d}$$
$$E_0 = E + X \tag{2.9e}$$

By setting the $\frac{dX}{dt} = 0$ and using 2.9e to express E in terms of E_0 and X then substituting into equation 2.9b and solving for X we get 2.10.

$$X = \frac{k_1 * A * E_0}{k_2 + k_3} \tag{2.10}$$

Then substituting X in equation 2.9d to find the total rate (2.11.

$$v = \frac{dP}{dt} = \frac{k_1 * k_3 * E_0 * A}{k_1 * A + k_2 + k_3} \tag{2.11}$$

which can be rearranged to the form of the Michaelis-Menten as shown in 2.12

$$v = \frac{k_1 * k_3 * E_0 * A}{k_1 * A + k_2 + k_3} = \frac{k_3 * E_0 * A}{\frac{k_2 + k_3}{k_1} * A} = \frac{V_max * A}{K_M + A} \tag{2.12}$$

where:

$$V_{max} = k_3 * E_0 \tag{2.13}$$
$$K_M = \frac{k_2 + k_3}{k_1} \tag{2.14}$$

2.2.2 Complex Enzyme Reactions

For reactions with more than one substrate and multiple intermediates, the michaelis-menten equation is expanded with more Michaelis-Menten constants and more V_{max} terms by following the methods described by Cleland [27]. Describing the rate in terms of kinetic constants (K_M and V_{max}) makes it easier to measure the constants experimentally, because these can be determined by graphical methods e.g. lineweaver-burk plot. Cleland [27] calculated the rate equations of a number of common pathways, the calculation of rate constants into kinetics constants does however become increasingly difficult steps are added to the pathway and especially with branching of the reaction. For some reactions the kinetic constants cannot be determined directly, but has to be fitted using nonlinear regression [13]. In these cases the advantage of using kinetic constants over rate constants diminishes.

When an extra intermediate is introduced in the reaction mechanism solving of the rate equations by substitution becomes much harder. Therefore other methods has been developed. To calculate the distribution of enzyme between the three forms the reaction is first written with focus on the enzyme species:

$$\mathrm{E} + \mathrm{A} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{EA} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \mathrm{EP} \underset{k_6}{\overset{k_5}{\rightleftharpoons}} \mathrm{E} + \mathrm{P} \tag{2.15}$$

$$\mathrm{E} \underset{k_2}{\overset{k_1 * A}{\rightleftharpoons}} \mathrm{EA} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \mathrm{EP} \underset{k_6 * P}{\overset{k_5}{\rightleftharpoons}} \mathrm{E} \tag{2.16}$$

The rate equations for the enzyme species can then be written in the form a coefficient matrix:

$$\begin{pmatrix} \frac{dE}{dt} \\ \frac{dEA}{dt} \\ \frac{dEP}{dt} \end{pmatrix} = \begin{pmatrix} 0 \\ 0 \\ 0 \end{pmatrix} = \begin{pmatrix} -k_1 * A - k_6 * P & k_2 & k_5 \\ k_1 * A & -k_2 - k_3 & k_4 \\ k_6 * P & k_3 & -k_4 - k_5 \end{pmatrix} * \begin{pmatrix} [E] \\ [EA] \\ [EP] \end{pmatrix} \tag{2.17}$$

To write the concentration of each enzyme species in terms of coefficients, new matrices are generated for each species by deleting the row and column corresponding to that species and then taking the determinant of the new matrices. These solutions does not, however, satisfy the equation of conservation (2.9e) the solutions must be scaled by dividing with the sum of all the minors to get the solutions in (2.18 [28, 29]. Equations (2.18a) - 2.18) are the distribution terms of the enzyme, to calculate the concentration of each species the distribution terms are multiplied by the concentration E_0.

$$[E] = \frac{\begin{vmatrix} -k_2 - k_3 & k_4 \\ k_3 & -k_4 - k_5 \end{vmatrix}}{denominator} \tag{2.18a}$$

$$[EA] = \frac{\begin{vmatrix} -k_1 * A - k_6 * P & k_5 \\ k_6 * P & -k_4 - k_5 \end{vmatrix}}{denominator} \tag{2.18b}$$

$$[EA] = \frac{\begin{vmatrix} -k_1 * A - k_6 * P & k_2 \\ k_1 * A & -k_2 - k_3 \end{vmatrix}}{denominator} \tag{2.18c}$$

$$\begin{aligned} denominator = &\begin{vmatrix} -k_2 - k_3 & k_4 \\ k_3 & -k_4 - k_5 \end{vmatrix} + \begin{vmatrix} -k_1 * A - k_6 * P & k_5 \\ k_6 * P & -k_4 - k_5 \end{vmatrix} \\ &+ \begin{vmatrix} -k_1 * A - k_6 * P & k_2 \\ k_1 * A & -k_2 - k_3 \end{vmatrix} \end{aligned} \tag{2.18d}$$

To calculate the overall rate of reaction the concentration of the enzyme species can now be substituted into the rate equations for products and substrates [28].

$$\frac{dA}{dt} = -k_1 * E * A + k_2 * EA \tag{2.19}$$

$$\frac{dP}{dt} = k_5 * EP - k_6 * E * P \tag{2.20}$$

This procedure of calculating rate equation for enzyme catalysed reaction is easily implemented on a computer, Fromm and Fromm [28] demonstrated a method to implement the procedure in the mathematical software suite *Mathematica*, this method is easily translated to other programming languages like *matlab* or *python*. For this project the method has been implemented using *Sage* a *Python* based mathematical software package. Calculation of determinants of large matrices has only within the last 20 or so years become trivial thanks to the rapid increase in available computational power [28]. Before this alternative methods were developed to ease the calculations, one of the most widespread is the graphical method developed by King and Altman [29, 30].

Combination of the steady-state and rapid equilibrium approximations

Some reactions are best described by a combination of steady-state and rapid equilibrium approximations, to introduce simplifications on complex reaction pathways

[31]. Consider a monosubstrate reaction with two intermediates and competitive inhibition as depicted in figure 2.5a. This system can be treated with the approach described in section 2.2.2. The treatment can be simplified somewhat if the assumption in (2.21) can be made.

$$k_7 * I >> k_1 * A + k_6 * P \tag{2.21}$$

The calculations can than be simplified by assuming equilibrium between E and EI. One can then substitute the species in equilibrium with X, see figure 2.5, the rate constant going from X is multiplied with f_E, which is the fraction of X that is made up of free enzyme [31].

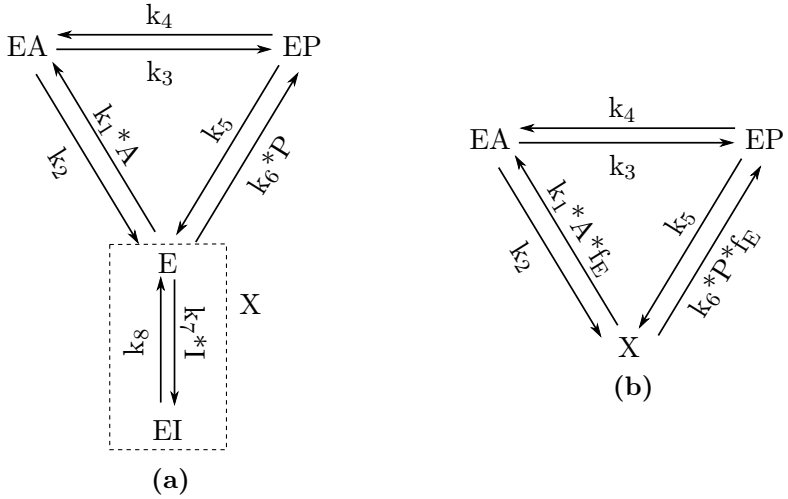


Figure 2.5: ***(a)*** *The boxed area encloses the species that are in equilibrium* ***(b)*** *The species in equilibrium has been replaced by X and the rate constants adjusted with* f_E.

2.3 Estimation of parameter by nonlinear regression

When the kinetic parameters of a chemical reaction cannot be measured directly, regression analysis can be used as a tool to estimate the value of the kinetic parameters. When using regression to estimate unknown parameters of a function the first step is to define a method of quantification of *goodnes of fit*. The standard method to quantify *goodness of fit* is the least squares method [32].

$$SSE = \sum_{i=1}^{n} (x_i - y_i(P))^2 \tag{2.22}$$

$$SSE = \sum_{i=1}^{n} \frac{1}{x_i^2} (x_i - y_i(P))^2 \tag{2.23}$$

The sum of the squared errors are calculated as shown in (2.22) where x_i is the measured value and y_i is the corresponding value obtained by simulation with the parameters P, SSE is the sum of the squared error. The magnitude of SSE does not by it self give much information about the accuracy of a simulation. For this it needs to be scaled e.g. by taking the mean and square root. The change in SSE, when solving for different values of P does however reveal if the new solution is better or worse than the

previous. When the data covers a large spread in magnitude it might be necessary to apply a weighting scheme. This is because, in many cases the variability of measurements scale with the magnitude of the measured values. Equation (2.23) a common weighting scheme known as relative weighting [32]. This weighting ensures that each data point carries the same weight for the same relative error.

To estimate the best values of the parameters an effective algorithm to minimisation of the SSE has to be used. There are many different algorithms for this type of minimisation problem. Most of these algorithms can be grouped into two categories: gradient based algoritms and stochastic optimisation algorithms.
The gradient based algorithms use the first or the second derivative of the error function to determine the direction to search for the minimum. The Levenberg-Marquardt algorithm is an example of a gradient based minimisation algorithm. The gradient based algorithms has the advantage of being very fast. These algorithms are however very sensitive to the initial guess for parameters, because they are not able to seach for a global minimum, the algorithm can only descent to the nearest minimum. For this reason the gradient based algorithms are most suited for smooth problems without multiple minimas or in cases where a starting guess can be made close to the real value. In case of problems with manyu local minimas or if an appropriate guess can not be made for the start value of the search, global optimisation algorithms are needed. For this task many stochastic optimisation algorithms have proven useful [33]. Stochastic optimisation algorithms does not direct their search on the basis of the gradients, but relays on the generation of random variables [34]. Many of these algorithms have been inspired by natural processes e.g. genetic algorithms and simulated annealing. A comparison of different stochastic optimisation algorithms have shown that for parameter estimation of rate constants of enzyme catalysed reactions, the particle swarm algorithm is very well suited [33, 35].

Analysis of transglycosylation models 3

This chapter will review the models for transgalactosylation catalysed by β-galactosidase proposed byby Boon et al. [12], Vera et al. [13] and Palai et al. [14]. The models will be analysed on the proposed reaction mechanism and on their mathematical treatment. Focus will be put on how simplifying assumptions are grounded in structural properties of β-galactosidase.

3.1 Model presented by Boon et al. [12]

The model proposed by Boon et al. [12] is based on the reaction pathway showed in Figure 3.1. In order to keep the number of parameters as low as possible several simplifications has been made to the model, most notably the model does not keep distinguish GOS with varying degree of polymerisation. This simplification can be justified if the production of GOS with a DP of 4 or higher is very low, otherwise the model will deviate too much from mass conservation.
Boon et al. [12] introduces two possible inhibition mechanisms, competitive inhibition by glucose and competitive inhibition by galactose. The inhibitior should be chosen according the the enzyme source, in their experimental work Boon et al. [12] used β-galactosidase from *Bacillus circulans* and showed inhibition from glucose, in this project β-galactosidase from *A.oryzae* has been used, therefore the model will be implemented with inhibition by galactose.

Boon et al. [12] claim to derive the rate expressions using the method of King-Altman arrive at the equations 3.1, these equations are general in that they treat both galactose and glucose as inhibitors before application the terms containing either k6/k7 or k8/k9 is set to zero. As described in section 2.2.2, the King-Altman method is a procedure to calculated the determinants required by the application of the steady-state approximation, is can therefore be assumed that Boon et al. [12] applies the steady-state approximation on the enzyme species.

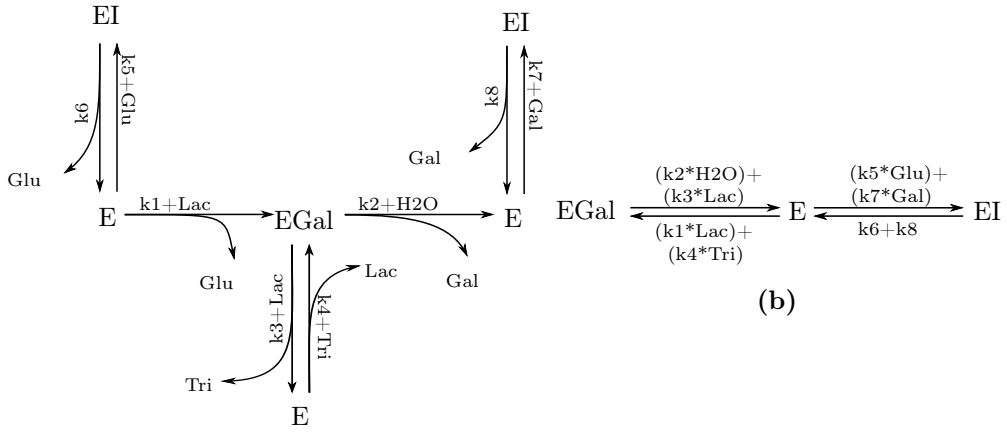


Figure 3.1: ***(a)*** *Schematic representation of the model presentened by Boon et al. [12];* ***(b)*** *Simplified schematic of the model presentened by Boon et al. [12]*

$$\frac{dLac}{dt} = (-k_1 \cdot k_2 \cdot Lac \cdot H_2O - 2 \cdot k_1 \cdot k_3 \cdot Lac^2 + k_2 \cdot k_4 \cdot Tri \cdot H_2O) \cdot Y \tag{3.1a}$$

$$\frac{dGlu}{dt} = (k_1 \cdot k_2 \cdot Lac \cdot H_2O + k_1 \cdot k_3 \cdot Lac^2) \cdot Y \tag{3.1b}$$

$$\frac{dGal}{dt} = (k_1 \cdot k_2 \cdot Lac \cdot H_2O + k_2 \cdot k_4 \cdot Tri \cdot H_2O) \cdot Y \tag{3.1c}$$

$$\frac{dTri}{dt} = (k_1 \cdot k_3 \cdot Lac^2 - k_2 \cdot k_4 \cdot Tri \cdot H_2O)Y \tag{3.1d}$$

$$\begin{aligned}\frac{1}{Y} = {} & k2 \cdot H_2O + k_3 \cdot Lac + k_1 \cdot Lac + k_4 \cdot Tri + \\ & \frac{k_2 \cdot k_5}{k_6} \cdot Glu \cdot H_2O + \frac{k_3 \cdot k_5}{k_6} \cdot Lac \cdot Glu + \\ & \frac{k_2 \cdot k_7}{k_8} \cdot Gal \cdot H_2O + \frac{k_3 \cdot k_7}{k_8} \cdot Lac \cdot Gal\end{aligned} \tag{3.1e}$$

If the steady-state approximation is applied to the reaction mechanism depicted in figure 3.1a, the first step is to rewrite the figure so that each enzyme species only occurs once, figure 3.1b. With the model redrawn the mass balances can be written (3.2a-g). The stady-state approximation can then be applied by setting (3.2e-g) equal to zero.

$$\frac{Lac}{dt} = -k_1 \cdot Lac \cdot E + k4 \cdot GOS \cdot E - k3 \cdot Lac \cdot EGal \tag{3.2a}$$

$$\frac{Glu}{dt} = k_1 \cdot Lac \cdot E - k5 \cdot Glu \cdot E + k \cdot EI \tag{3.2b}$$

$$\frac{Gal}{dt} = k_2 \cdot H2O \cdot EGal - k_7 \cdot Gal \cdot E + EI \cdot k_8 \tag{3.2c}$$

$$\frac{GOS}{dt} = k_3 \cdot Lac \cdot EGal - k4 \cdot GOS \cdot E \tag{3.2d}$$

$$\frac{dE}{dt} = 0 = -((k1 \cdot lac + k4 \cdot Tri) + k7 \cdot gal) \cdot E + (k3 \cdot lac + k2 \cdot H2O) \cdot Egal + (k8) \cdot EI \tag{3.2e}$$

$$\frac{dEGal}{dt} = 0 = (k1 \cdot lac + k4 \cdot Tri) \cdot E - k3 \cdot lac + (k2 \cdot H2O) \cdot Egal \tag{3.2f}$$

$$\frac{dEI}{dt} = 0 = (k7 \cdot gal) \cdot E - (k8) \cdot Ei \tag{3.2g}$$

$$E_{total} = E + EGal + EI \tag{3.2h}$$

By introducing the law of conservation of mass (3.2h) one can solve for *E, EGal,*and *EI*. The steady state expressions of *E, EGal,*and *EI* are given by (3.3a-c).

$$E = \frac{((k_2 \cdot k_6 + k_2 \cdot k_8) \cdot H2O + (k_3 \cdot k_8 + k_3 \cdot k_6) \cdot Lac) \cdot E_0}{Denominator} \tag{3.3a}$$

$$EGal = \frac{((k1 \cdot k6 + k1 \cdot k8) \cdot Lac + (k4 \cdot k6 + k4 \cdot k8) \cdot Tri) \cdot E_0}{Denominator} \tag{3.3b}$$

$$EI = \frac{((k2 \cdot k5 + k2 \cdot k7) \cdot H2O \cdot gal + (k3 \cdot k5 + k3 \cdot k7) \cdot Gal \cdot Lac) \cdot E_0}{Denominator} \tag{3.3c}$$

$$\begin{aligned} Denominator = {} & k_2 \cdot k_5 \cdot H2O \cdot Glu + k_2 \cdot k_7 \cdot H2O \cdot Gal + k_3 \cdot k_5 \cdot Glu \cdot Lac + \\ & k_3 \cdot k_7 \cdot Gal \cdot Lac + (k_2 \cdot k_6 + k_2 \cdot k_8) \cdot H2O + \\ & (k_1 \cdot k_6 + k_3 \cdot k_6) \cdot Lac + (k_1 \cdot k_8 + k_3 \cdot k_8) \cdot Lac + \\ & (k_4 \cdot k_6 + k_4 \cdot k_8) \cdot Tri \end{aligned} \tag{3.3d}$$

The rate expressions for *Lac, Glu, Gal* and *GOS* is then obtain by substituting (3.3a-c) into (3.2a-d). To test if the two mathematical representations of the model are equal the most convenient method is to insert values for k_{1-8} and the reactants and compare the result numerically. The expressions given by (3.1) are not equal to (3.2). When analysing eqations 3.1 the numerator of the rates has a pattern similar to results from application of the King-Altman method. When solving for the enzyme species using the King-Altman method, the numerator is the sum of the products of the different pathways that lead to the species being solved for. The denominator should be the sum of the numerators according to the King-Altman method. In the expressions from Boon et al. [12] I am not able to trace the calculation that lead to the denominator. Boon et al. [12] proposes a mechanistic model that provides simplifications that is well-founded in the structure and reaction mechanism of β-galactosidase. The m mathematical treatment by Boon et al. [12] seems, however, to be incorrect.

3.2 Model presented by Vera et al. [13]

The model presentend by Vera et al. [13] is based on the reaction pathway proposed by Boon et al. [12]. The model is extended to account for production of higher DP oligosaccharides because for some β-galactosidases, e.g. *Aspergillus oryzae*, GOS of DP 4 and DP 5 contributes to much to the total products to be neglected. Futher Vera et al. [13] extends the model with a step of reversible binding of substrate, the model also includes production of disaccharides consisting of two galactose moieties, thes are however not destinguised from lactose because they are assumed to appear in much

smaller quantities than the other products. The model considers the following reactions:

$$\mathrm{E} + \mathrm{Di} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{EDi} \xrightarrow{k_{cat}} \mathrm{EGal} + \mathrm{Glu} \tag{3.4a}$$

$$\mathrm{EGal} + \mathrm{Di} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \mathrm{EGalDi} \underset{k_6}{\overset{k_5}{\rightleftharpoons}} \mathrm{E} + \mathrm{Tri} \tag{3.4b}$$

$$\mathrm{EGal} + \mathrm{Tri} \underset{k_8}{\overset{k_7}{\rightleftharpoons}} \mathrm{EGalTri} \underset{k_10}{\overset{k_9}{\rightleftharpoons}} \mathrm{E} + \mathrm{Tet} \tag{3.4c}$$

$$\mathrm{EGal} + \mathrm{Tet} \underset{k_12}{\overset{k_11}{\rightleftharpoons}} \mathrm{EGalTet} \underset{k_14}{\overset{k_13}{\rightleftharpoons}} \mathrm{E} + \mathrm{Pen} \tag{3.4d}$$

$$\mathrm{EGal} + \mathrm{Gal} \underset{k_18}{\overset{k_17}{\rightleftharpoons}} \mathrm{EGalGal} \xrightarrow{k_17} \mathrm{E} + \mathrm{Di} \tag{3.4e}$$

$$\mathrm{EGal} + \mathrm{H_2O} \xrightarrow{k_{cat'}} \mathrm{E} + \mathrm{Gal} \tag{3.4f}$$

$$\mathrm{E} + \mathrm{Gal} \underset{}{\overset{K_I}{\rightleftharpoons}} \mathrm{EGal_I} \tag{3.4g}$$

Vera et al. [13] calculates the rate expressions by applying the steady-state approximation to *E, EDi, EGal, EGalTri, EGalTet,* and *EGalGal*. The treatment of the inhibition reaction is not stated clearly, but it is treated as a rapid equilibrium.
The reactions 3.4 (a,f) can be treated analogously to an irreversible reaction with one central complex see reaction 2.6, and the reactions reactions 3.4 (b, c, d) can be treated analogously to a reversible mechanism with one central complex:

$$\mathrm{E} + \mathrm{A} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{X} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \mathrm{E} + \mathrm{P}, \qquad \frac{dP}{dt} = \frac{\frac{V_1}{K_A} \cdot A - \frac{V_2}{K_P} \cdot P}{1 + \frac{A}{K_A} + \frac{P}{K_P}}$$

$$V_1 = k_3, \qquad V_2 = k_2$$

$$K_A = \frac{k_2 + k_3}{k1}, \qquad K_P = \frac{k2 + k3}{k4}$$

Obviously the rate expressions must account for the amount of enzyme distributed in every form therefore the final rate equations become somewhat more complicate, Vera et al. [13] derives the rate equations found in figure 3.2. The rate expressions can also be derived by the method described in section 2.2.2, this derivation is demonstrated in appendix A.1. I have not been able to fully understand the method of derivation used by Vera et al. [13], but the rate expressions presented by Vera et al. [13] has been numerically compared to the those given in appendix A.1 and the expressions have been found to be identical.

As explained in section 2.2.2 the advantage of expressing reaction rates in terms of kinetic parameters instead of rate constants is the possibility of measuring the kinetic constants directly, however in the case of a reaction as branched as the transgalactosylation reaction catalysed by β-galactosidase it is very difficult to set up initial rate experiments, only the constants K_M, K_I, and k_{cat} can be measured directly the remaining 15 parameters has to be found by nonlinear fitting [13]. It is recognized by Vera et al. [13] that fitting of 15 parameters when only 5 independent responses can be measured has an inherent risk of overfitting, the system is there for simplified by making the assumptions in (3.5).
Due to the pocket like nature of the active site in β-galactosidase it is reasonable to assume that the maximum rate and Michaels-Menten constants are virtually the same

$$v_{\mathrm{Glu}} = \frac{\mathrm{d(Glu)}}{\mathrm{d}t} = a \times \mathrm{Di} \times \frac{E_{\mathrm{L}}}{\alpha + \beta \times \gamma}$$

$$v_{\mathrm{Gal}} = \frac{\mathrm{d(Gal)}}{\mathrm{d}t} = (i - h \times \mathrm{Gal}) \frac{E_{\mathrm{L}} \times \gamma}{\alpha + \beta \times \gamma}$$

$$\begin{aligned} v_{\mathrm{Di}} &= \frac{\mathrm{d(Di)}}{\mathrm{d}t} \\ &= (b \times \mathrm{Tri} - a \times \mathrm{Di}) \frac{E_{\mathrm{L}}}{\alpha + \beta \times \gamma} \\ &\quad + (h \times \mathrm{Gal} - e \times \mathrm{Di}) \frac{E_{\mathrm{L}} \times \gamma}{\alpha + \beta \times \gamma} \end{aligned}$$

$$\begin{aligned} v_{\mathrm{Tri}} &= \frac{\mathrm{d(Tri)}}{\mathrm{d}t} \\ &= (c \times \mathrm{Tet} - b \times \mathrm{Tri}) \frac{E_{\mathrm{L}}}{\alpha + \beta \times \gamma} \\ &\quad + (e \times \mathrm{Di} - f \times \mathrm{Tri}) \frac{E_{\mathrm{L}} \times \gamma}{\alpha + \beta \times \gamma} \end{aligned}$$

$$\begin{aligned} v_{\mathrm{Tet}} &= \frac{\mathrm{d(Tet)}}{\mathrm{d}t} \\ &= (d \times \mathrm{Pen} - c \times \mathrm{Tet}) \frac{E_{\mathrm{L}}}{\alpha + \beta \times \gamma} \\ &\quad + (f \times \mathrm{Tri} - g \times \mathrm{Tet}) \frac{E_{\mathrm{L}} \times \gamma}{\alpha + \beta \times \gamma} \end{aligned}$$

$$\begin{aligned} v_{\mathrm{Pen}} &= \frac{\mathrm{d(Pen)}}{\mathrm{d}t} \\ &= -d \times \mathrm{Pen} \frac{E_{\mathrm{L}}}{\alpha + \beta \times \gamma} + g \times \mathrm{Tet} \times \frac{E_{\mathrm{L}} \times \gamma}{\alpha + \beta \times \gamma} \end{aligned}$$

$$\alpha = 1 + \frac{\mathrm{Di}}{K_{\mathrm{M}}} + \frac{\mathrm{Gal}}{K_{\mathrm{I}}} + \frac{\mathrm{Tri}}{K_{\mathrm{MTri}}{}'} + \frac{\mathrm{Tet}}{K_{\mathrm{MTet}}{}'} + \frac{\mathrm{Pen}}{K_{\mathrm{MPen}}{}'}$$

$$\beta = 1 + \frac{\mathrm{Gal}}{K_{\mathrm{MGal}}} + \frac{\mathrm{Di}}{K_{\mathrm{MDi}}} + \frac{\mathrm{Tri}}{K_{\mathrm{MTri}}} + \frac{\mathrm{Tet}}{K_{\mathrm{MTet}}}$$

$$\gamma = \frac{a \times \mathrm{Di} + b \times \mathrm{Tri} + c \times \mathrm{Tet} + d \times \mathrm{Pen}}{e \times \mathrm{Di} + f \times \mathrm{Tri} + g \times \mathrm{Tet} + h \times \mathrm{Gal} + i}$$

$$a = \frac{k_{\mathrm{cat}}}{K_{\mathrm{M}}} \quad b = \frac{k_{-2}}{K_{\mathrm{MTri}}{}'} \quad c = \frac{k_{-4}}{K_{\mathrm{MTet}}{}'} \quad d = \frac{k_{-6}}{K_{\mathrm{MPen}}{}'}$$
$$e = \frac{k_3}{K_{\mathrm{MDi}}} \quad f = \frac{k_5}{K_{\mathrm{MTri}}} \quad g = \frac{k_7}{K_{\mathrm{MTet}}} \quad h = \frac{k_9}{K_{\mathrm{MGal}}}$$
$$i = k_{\mathrm{cat}}{}' \times \mathrm{H_2O}$$

$$K_{\mathrm{M}} = \frac{k_{-1} + k_{\mathrm{cat}}}{k_1}, \quad K_{\mathrm{MDi}} = \frac{k_{-2} + k_3}{k_2},$$
$$K_{\mathrm{MTri}}{}' = \frac{k_{-2} + k_3}{k_{-3}}, \quad K_{\mathrm{MTri}} = \frac{k_{-4} + k_5}{k_4},$$
$$K_{\mathrm{MTet}}{}' = \frac{k_{-4} + k_5}{k_{-5}}, \quad K_{\mathrm{MTet}} = \frac{k_{-6} + k_7}{k_6},$$
$$K_{\mathrm{MPen}}{}' = \frac{k_{-6} + k_7}{k_{-7}}, \quad K_{\mathrm{MGal}} = \frac{k_{-8} + k_9}{k_8},$$
$$K_{\mathrm{I}} = \mathrm{E} \times \frac{\mathrm{Gal}}{\mathrm{EGal}*}$$

Figure 3.2

for tri-, tetra-, and penta saccharides. However the reaction between free the enxyme and lactose is assumed to be irreversible which implies that it is mechanistically different from the reactions between free enzyme and oligosaccharides.

$$K_M = K_{MTri'} = K_{MTet'} = K_{MPen'} \tag{3.5a}$$
$$K_{MDI} = K_{MTri} = K_{MTet} = K_{MGal} \tag{3.5b}$$
$$k_{-2} = k_{-4} = k_{-6} \tag{3.5c}$$
$$k_3 = k_5 = k_7 \tag{3.5d}$$

K_M can be determined directly, it is therefore odd that Vera et al. [13] chooses to disregard the experimentally determined value of K_M, and lump it together with $K_{MTri'} = K_{MTet'} = K_{MPen'}$ to be determined by regression. For this project the assumption will be that K_M should be determined indepently of $K_{MTri'} = K_{MTet'} = K_{MPen'}$.

3.3 Model presented by Palai et al. [14]

The model presented by Palai et al. [14] is much simpler than the two models described above. The model assumes the reactions shown in equations 3.6:

$$\mathrm{E} + \mathrm{Lac} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{ELac} \xrightarrow{k_3} \mathrm{EM} + \mathrm{M} \tag{3.6a}$$

$$\mathrm{EM} + \mathrm{Lac} \underset{k_6}{\overset{k_5}{\rightleftharpoons}} \mathrm{E} + \mathrm{GOS} \tag{3.6b}$$

$$\mathrm{EM} + \mathrm{GOS} \xrightarrow{k_7} \mathrm{E} + \mathrm{GOS} \tag{3.6c}$$

E represents free enzyme, $ELac$ is the enzyme lactose complex, EM is the enzyme galactose complex, M is monosaccharide no differentiation is made between galactose and glucose. Palai et al. [14] does not apply the steady state approximation on the system, instead the rate expressions are given by application of the law of mass action:

$$\frac{dE}{dt} = -k_1 \cdot E \cdot Lac + k_2 \cdot ELac + k_5 \cdot EM \cdot Lac - k_6 \cdot E \cdot GOS + k_7 \cdot EM \cdot GOS \tag{3.7a}$$

$$\frac{dELac}{dt} = k_1 \cdot E \cdot Lac - k_2 \cdot ELac - k_3 \cdot EM \cdot M \tag{3.7b}$$

$$\frac{dEM}{dt} = k_3 \cdot EM \cdot M - k_5 \cdot EM \cdot Lac + k_6 \cdot E \cdot GOS - k_7 \cdot EM \cdot GOS \tag{3.7c}$$

$$\frac{dM}{dt} = k_3 \cdot ELac \tag{3.7d}$$

$$\frac{dLac}{dt} = -k_1 \cdot E \cdot Lac + k_2 \cdot ELac - k_5 \cdot EM \cdot Lac + k_6 \cdot E \cdot GOS \tag{3.7e}$$

$$\frac{dGOS}{dt} = k_5 \cdot EM \cdot Lac - k_6 \cdot E \cdot GOS \tag{3.7f}$$

This approach is extremely simple in both reaction mechanism and in derivation, it is therefore remarkable that Palai et al. [14] are able to show excellent fits to their experimental data. Results are reported for three different initial lactose concentrations and with only one set of parameters excellent fit is achieved.

Materials and Methods 4

4.1 Chemicals and Enzyme

Glucose, galactose, and fucose were analytical grade obtained form Sigma-Aldrich. Lactose were HPLC grade from Sigma-Aldrich. The standards for GOS were supplied by Arla Foods Ingredient. Citric acid and sodium phosphate were analytical grade from J.T.Baker and VWR respectively. β-galactosidase was from *A. Oryzae* and obtained from Sigma-Aldrich.

4.2 measurement of enzyme content

In order to be able to express rate constants in molar the enzyme content of β-galactosidase was determined by spectroscopy, measuring the absorbance at 280 nm and using the extinction coefficient 191 950 $\text{M}^{-1}\,\text{cm}^{-1}$. This was calculated using the "ProtParam" tool on web.expasy.org.

4.3 Conversion of Lactose by β-galactosidase

Conversion of lactose was carried out as batch experiments in 15 ml centrifugal tubes. The reactors were placed on a shaking table fitted with a heating block controlled to 40 °C. To monitor the course of lactose conversion samples were taken regularly over a period of 24 hours. A citrate-phosphate buffer adjusted to pH 4.5 was prepared and used for preparation of stock solutions of lactose and enzyme. A lactose stock solution was prepared by dissolving 54.045 g lactose and 0.2048 g of L-fucose in a 250 ml volumetric flask and filling to the mark with citrate-phosphate buffer solution to give a solution of 0.6 M of lactose and 1.2 $\text{mmol}\,\text{dm}^{-3}$ of L-fucose. Fucose was added to the lactose stock as internal standard. Dry enzyme was stored at −18 °C, before use it was removed from the freezer allow to reach room temperature before opening to prevent precipitation of water. A solution of 9.695 µM enzyme in citrate-phosphate buffer were prepared and dispensed to the reactors to reach the desired concentration. A series of six different combinations of substrate and enzyme concentrations were prepared seen in table 4.1.

Aliquots of 50 µl were taken at regular intervals for 24 hours. To stop the reaction the samples were transferred to an Eppendorf tube containing 950 µl of deionised water preheated to 85 °C. The tube was then placed on a shaking table with a heating block

Table 4.1: *Table of the concentrations used. The large letter denotes the initial lactose concentration and the subscript denotes the enzyme concentration in* µM.

Enzyme\Lactose	0.6 M	0.3 M	0.15 M
48.48 µM	C_{48}	B_{48}	A_{48}
19.39 µM	C_{19}	B_{19}	A_{19}

set to 85 °C, the tube was shaken vigorously for 10 minutes before it was transferred to an ice bath to ensure rapid cooling. The samples were stored at 5 °C

4.4 Analysis of saccharide content by HPAE-PAD

Saccharide content was analysed by high performance anion exchange - pulsed amperometric detection *HPAE-PAD* on a Dionex-5000 system (Thermo Scientific) fitted with a Carbo-Pac PA-1 column (Thermo Scientific). Glucose and galactose were measured using an isocratic eluent of 4 mM sodium acetate and 18 mM sodium hydroxide, with a flow rate of $1\,\mathrm{mL\,min^{-1}}$. Lactose and oligosaccharides were measured using an isocratic eluent of 10 mM sodium acetate and 200 mM sodium hydroxide, with a flow rate of $1\,\mathrm{mL\,min^{-1}}$.
Samples were diluted 1250 times, 2500 times, 5000 times for the series *A, B,* and *C* respectively. The signal for all samples were corrected using the internal fucose standard. The concentration of glucose, galactose and lactose were quantified on the basis peak area of the corresponding peaks using calibration curves previously produced. Only qualitative standards for galactooligosaccharides were available, these were used to confirm the presence and position of the GOS peaks, this also proved that only oligosaccharides of DP 3 were present. Quantification of GOS were achieved by performing a mass balance on glucose, see (4.1)

$$Glu_{total} = Glu_{free} + Lac + GOS \Leftrightarrow GOS = Glu_{total} - (Glu_{free} + Lac) \tag{4.1}$$

Where *Glu, Lac,* and *GOS* denotes the concentration of glucose lactose and GOS respectively. The validity of this approach was tested by also performing a mass balance on galactose, arriving at the same result.

4.5 Parameter estimation

To estimate the parameters of the three models described in 3, the models were fitted to the data obtained experimentally. The models proposed by Boon et al. [12] were fitted in both the form presented in (3.1a)-(3.1e) and the form presented in (3.2a-f). The model presented by Vera et al. [13] was reduced by removing the tetra- and pentasaccharides terms, as only GOS with a DP of three were observed. The models based on Boon et al. [12] and Vera et al. [13] were fitted using a python program descibed below, the model presented by Palai et al. [14] was fitted using a specialised software package.

4.5.1 Fitting using a python program

The fitting was made to data from all six experiments simultaneously. For each data point the error was weighted using relative weighting as presented in (2.23) so that each

data point contributed by the same amount. Data points form the same experiment are not independent, because experimental errors will likely bias all the all points in the dataset in the same direction. To overcome this effect the contribution of each experiment to the total error was average of the weighted squared errors. So that each experiment was contributed the same to the error no matter the amount of data points. The error value fed to the minimization algorithm was calculated as shown in (4.2).

$$Total\ error = \sum_{i} \frac{(\sum_{j,k} \frac{1}{x_{i,j,k}} \cdot (x_{i,j,k} - y_{i,j,k}(P))^2)}{j \cdot k} \tag{4.2}$$

x denotes the measured value, y is the simulated value with the parameters P, the subscripts *i,j,k* are the experiment, species and sample number respectively.
The pyswarm library implementation of the particle swarm algorithm was used to estimate the best set of parameters [36]. The minimisation algorithm was set to a swarmsize of 100 particles the stopping criteria was set at a max number of iterations of 100 and a minimum improvement of 0.001, other settings were set to the default value. 20 runs were made with the search space constrained to 10^{-5} to 10^{5}. The worst ten solutions were then removed and constraints were changed to center the search around the best solutions. For each parameter the new constraints were calculated to narrow the search space and 10 more runs were made.

4.5.2 Fitting using COPASI

Parameter estimation of the model proposed by Palai et al. [14] was performed using the software package COPASI [37]. The rate expression for each reaction was set to *law of mass action*. The weighting scheme was set to "mean", which is equivalent to the scheme implemented for the other models. The *particle swarm* minimisation algorithm was chosen with the default settings. Minimisation was performed simultaneously on all six data sets. The constraints were refined as describe above.

Results and discussion 5

5.1 Conversion of Lactose by β-galactosidase

The chromatograms from HPAE-PAD analysis of the conversion products showed at least four products that eluted after lactose. Three of these peak were assigned as galactooligosaccharides with a DP of 3 by comparison with standards, the fourth peak was not investigated further but is assumed to be GOS of DP 3. Figure 5.1 show an enlargement of chromatograms of samples taken from experiment C_{48}.

The peaks located at 6.2 min, 9.6 min and 10.4 min are $Gal(1 \rightarrow 6)Gal(1 \rightarrow 4)Glu$, $Gal(1 \rightarrow 4)Gal(1 \rightarrow 4)Glu$, and $Gal(1 \rightarrow 3)Gal(1 \rightarrow 4)Glu$ respectively, the peak at 8.3 min is not known but is assumed to be a trisaccharide of a different structure. It is worth noting that also several peaks appear in front of lactose, the peak immediately in front of lactose at 4.7 min is likely to be a disaccharide, either allolactose or a di-galactose.

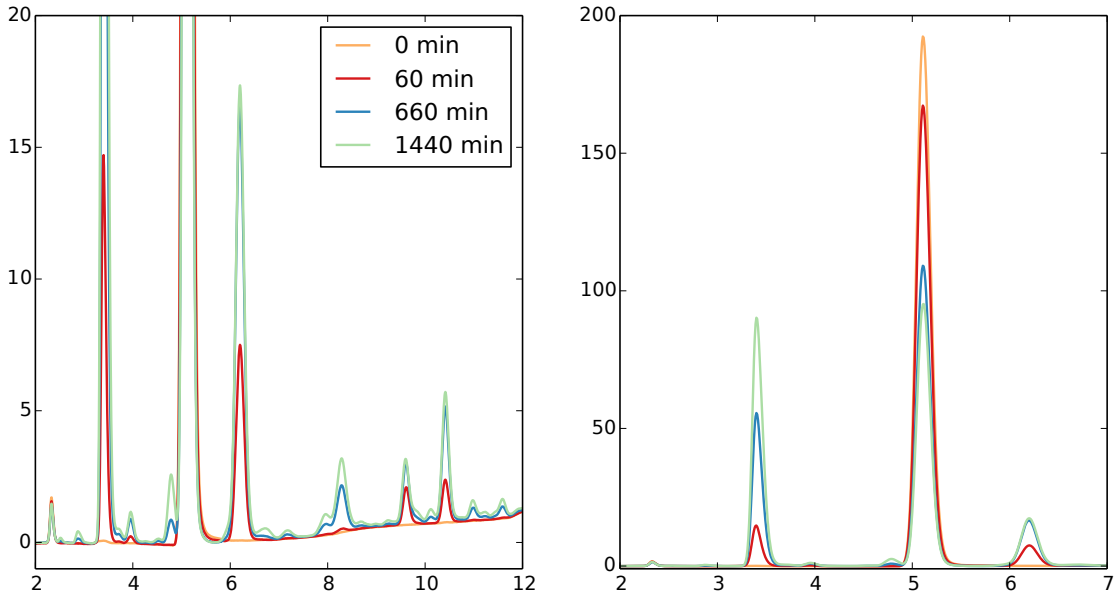


Figure 5.1: *An overlay of the chromatograms obtained with the method for quantification of lactose and oligosaccharides from experiment C_{48}. To the left is zoomed in on the region of GOS. On the right is zoomed in on monosaccharides and lactose. Galactose and glucose eluted together at* 3.4 min*, lactose eluted at* 5.1 min*, and the internal standard fucose eluted at* 2.3 min.

Glucose, galactose, and lactose were quantified by plotting the peak area a standard standard curve. It was attempted quantify the concentration of GOS by adding the area of all the peaks that eluted later than lactose and use the standard curve of lactose. This method was disregarded because the discrepancies in the mass balance were to big. Instead GOS were quantified by calculating a mass balance on glucose, see section 4.4. The full set of data experimental data is plottet along the simulation results in the figures 5.2, 5.3, 5.4, and 5.5. Some of the data sets suffer from quite a large amount of scatter, and the fact that it was not possible to quantify GOS directly on the basis of measured peak area reflect a data quality that is below what would be desired. Due to the limited time available on the HPAE-PAD dublicates were not produced. Because the noise in the measurements it not possible to do direct determination of Michaelis-Menten constant as suggested by Vera et al. [13], all parameters were therefore determined by regression.

5.2 Parameter estimation

The results of fitting are displayed in the figures 5.2 - 5.5 and in the accompanying tables 5.2 - 5.5. The figures display the result of simulation with the best parameter set obtained by regression. As can be seen in the plots and from the magnitude og the root mean squared error *(RMSE)* value. In all the regression very large variability was observed, for the solutions even for very similar error sums, parameters could be spread over more than five orders of magnitude. It it therefore unlikely that the optimal parameters were found.
The best objective values for the regressions (see 2.3) are given in table 5.1. The values can not be directly used to asses the accuracy of the fit, but the objective value is calculated in the same way for each of the models. The value can therefore serve as a basis of comparison between the models.

Table 5.1: *The smallest obtained objective value for regression of the four models.*

	Sum of weighted squared error
Recalculated model from Boon et al. [12]	0.056
Original model from Boon et al. [12]	3.86
Model by Vera et al. [13]	3.90
Model by Palai et al. [14]	59.96

Table 5.2: *The root mean square errors for each of the saccharides in each experiment fitted with the best obtained parametetes for the model represented by (3.2). The mean concentration is included for comparison of magnitude.*

		Galactose	Glucose	Lactose	Gos
A_{19}	RMSE	1.32×10^{-3} M	2.66×10^{-3} M	3.29×10^{-3} M	1.40×10^{-3} M
	Mean	1.88×10^{-2} M	3.11×10^{-2} M	1.24×10^{-1} M	1.23×10^{-2} M
A_{48}	RMSE	2.96×10^{-3} M	3.18×10^{-3} M	1.91×10^{-3} M	1.47×10^{-3} M
	Mean	3.19×10^{-2} M	4.62×10^{-2} M	1.14×10^{-1} M	5.32×10^{-3} M
B_{19}	RMSE	8.12×10^{-4} M	1.91×10^{-3} M	3.51×10^{-3} M	1.24×10^{-3} M
	Mean	1.69×10^{-2} M	4.17×10^{-2} M	2.64×10^{-1} M	2.49×10^{-2} M
B_{48}	RMSE	2.07×10^{-3} M	3.19×10^{-3} M	9.44×10^{-3} M	3.64×10^{-3} M
	Mean	3.16×10^{-2} M	6.49×10^{-2} M	2.36×10^{-1} M	3.33×10^{-2} M
C_{19}	RMSE	3.19×10^{-3} M	5.65×10^{-3} M	9.79×10^{-3} M	2.97×10^{-3} M
	Mean	1.79×10^{-2} M	6.51×10^{-2} M	5.58×10^{-1} M	4.73×10^{-2} M
C_{48}	RMSE	5.64×10^{-3} M	1.75×10^{-3} M	8.21×10^{-2} M	1.35×10^{-2} M
	Mean	3.16×10^{-2} M	6.49×10^{-2} M	2.36×10^{-1} M	3.33×10^{-2} M

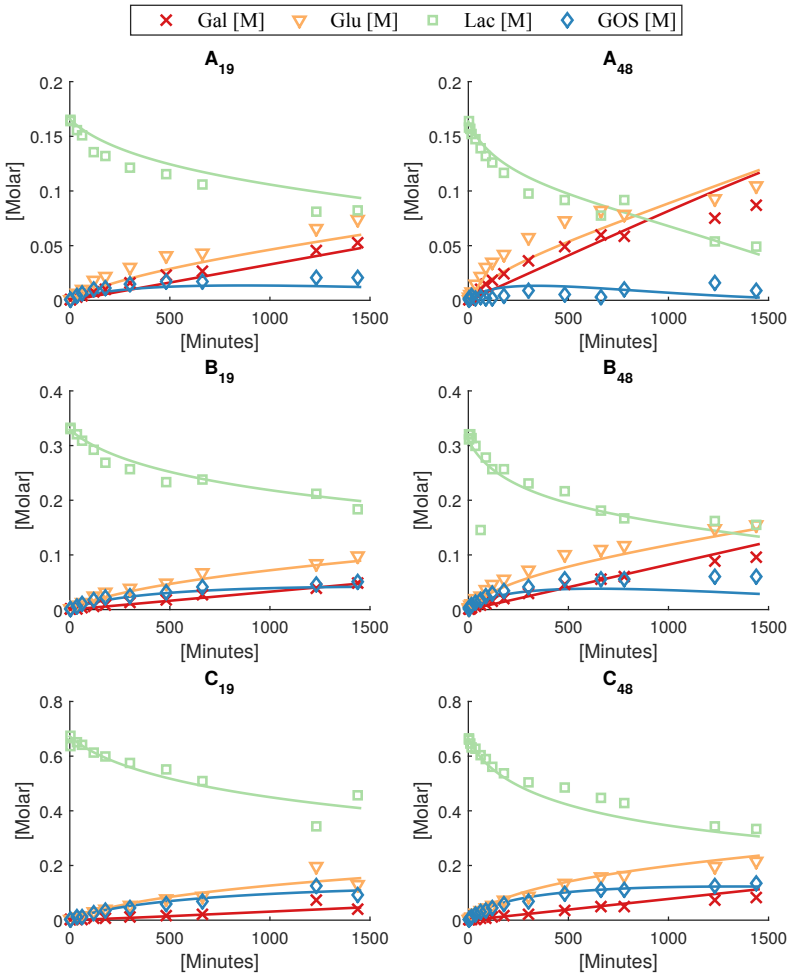


Figure 5.2: *Simulations of the recalculated model presented by Boon et al. [12] using the best parameter set found by regression.*

Table 5.3: *The root mean square errors for each of the saccharides in each experiment fitted with the best obtained parametetes for the model represented by (3.1a-e). The mean concentration is included for comparison of magnitude.*

		Galactose	Glucose	Lactose	Gos
A_{19}	RMSE	8.95×10^{-4} M	8.55×10^{-4} M	2.96×10^{-3} M	2.75×10^{-3} M
	Mean	1.88×10^{-2} M	3.11×10^{-2} M	1.24×10^{-1} M	1.23×10^{-2} M
A_{48}	RMSE	5.38×10^{-3} M	5.08×10^{-3} M	2.54×10^{-3} M	8.16×10^{-4} M
	Mean	3.19×10^{-2} M	4.62×10^{-2} M	1.14×10^{-1} M	5.32×10^{-3} M
B_{19}	RMSE	6.39×10^{-4} M	2.99×10^{-3} M	6.39×10^{-3} M	2.73×10^{-3} M
	Mean	1.69×10^{-2} M	4.17×10^{-2} M	2.64×10^{-1} M	2.49×10^{-2} M
B_{48}	RMSE	5.51×10^{-3} M	6.81×10^{-3} M	9.64×10^{-3} M	5.82×10^{-3} M
	Mean	3.16×10^{-2} M	6.49×10^{-2} M	2.36×10^{-1} M	3.33×10^{-2} M
C_{19}	RMSE	2.95×10^{-3} M	6.78×10^{-3} M	1.27×10^{-2} M	1.20×10^{-2} M
	Mean	1.79×10^{-2} M	6.51×10^{-2} M	5.58×10^{-1} M	4.73×10^{-2} M
C_{48}	RMSE	3.84×10^{-3} M	6.12×10^{-3} M	5.15×10^{-3} M	5.42×10^{-3} M
	Mean	2.61×10^{-2} M	8.74×10^{-2} M	5.29×10^{-1} M	6.13×10^{-2} M

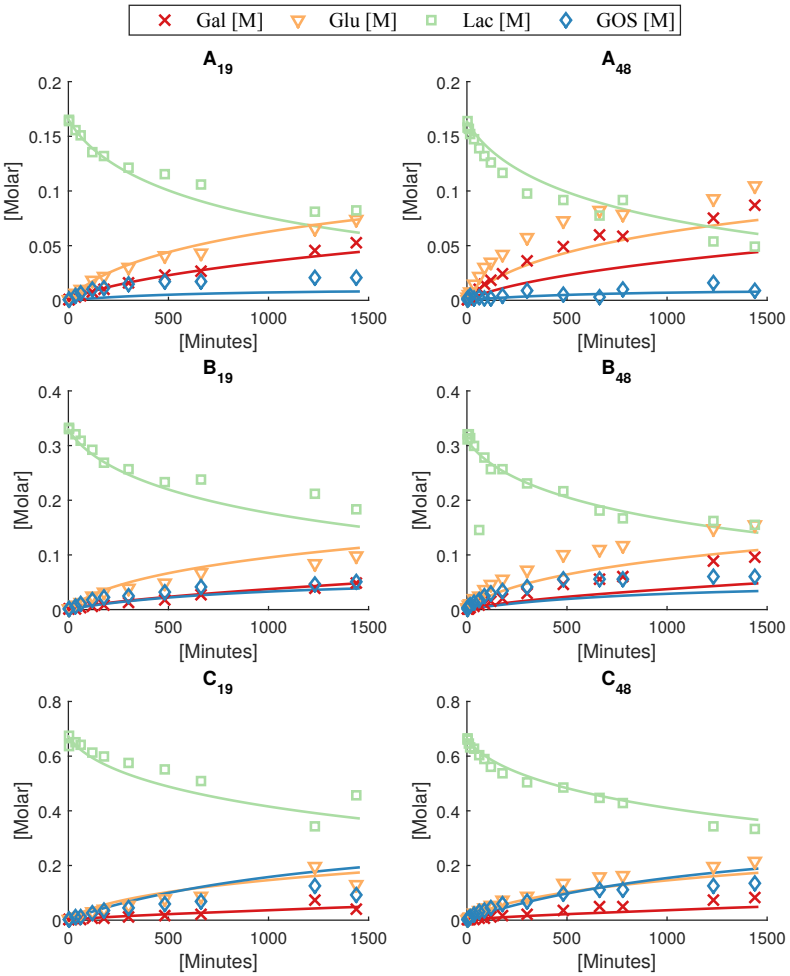


Figure 5.3: *Simulations of the model presented by Boon et al. [12] in its original form using the best parameter set found by regression.*

Table 5.4: *The root mean square errors for each of the saccharides in each experiment fitted with the best obtained parametetes for the model represented by equations 3.4. The mean concentration is included for comparison of magnitude.*

		Galactose	Glucose	Lactose	Gos
A_{19}	RMSE	3.38×10^{-3} M	4.16×10^{-3} M	1.62×10^{-2} M	2.03×10^{-3} M
	Mean	1.88×10^{-2} M	3.11×10^{-2} M	1.24×10^{-1} M	1.23×10^{-2} M
A_{48}	RMSE	5.69×10^{-3} M	5.01×10^{-3} M	4.78×10^{-3} M	1.10×10^{-3} M
	Mean	3.19×10^{-2} M	4.62×10^{-2} M	1.14×10^{-1} M	5.32×10^{-3} M
B_{19}	RMSE	1.79×10^{-3} M	2.78×10^{-3} M	6.76×10^{-3} M	3.02×10^{-3} M
	Mean	1.69×10^{-2} M	4.17×10^{-2} M	2.64×10^{-1} M	2.49×10^{-2} M
B_{48}	RMSE	5.18×10^{-3} M	4.81×10^{-3} M	9.79×10^{-3} M	3.76×10^{-3} M
	Mean	3.16×10^{-2} M	6.49×10^{-2} M	2.36×10^{-1} M	3.33×10^{-2} M
C_{19}	RMSE	3.58×10^{-3} M	5.69×10^{-3} M	1.09×10^{-2} M	3.71×10^{-3} M
	Mean	1.79×10^{-2} M	6.51×10^{-2} M	5.58×10^{-1} M	4.73×10^{-2} M
C_{48}	RMSE	2.81×10^{-3} M	2.89×10^{-3} M	3.59×10^{-3} M	1.76×10^{-3} M
	Mean	2.61×10^{-2} M	8.74×10^{-2} M	5.29×10^{-1} M	6.13×10^{-2} M

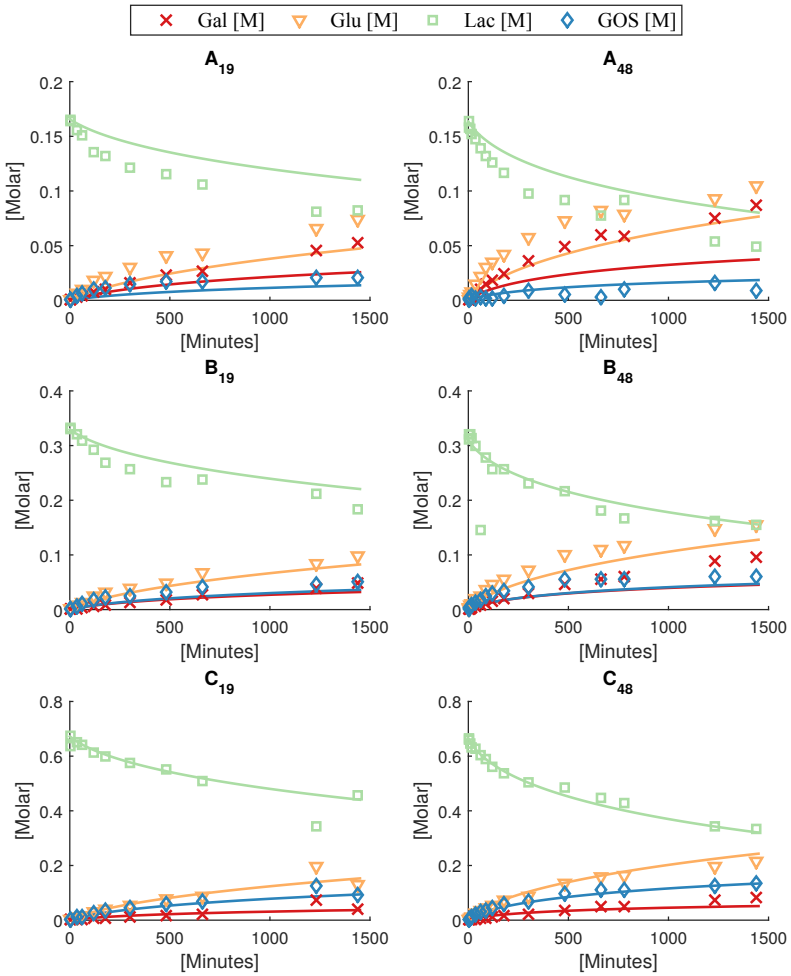


Figure 5.4: *Simulations of the model presented by Vera et al. [13] reduced to cover only trisaccharides and using the best parameter set found by regression.*

Table 5.5: *The root mean square errors for each of the saccharides in each experiment fitted with the best obtained parametetes for the model represented by equations (3.7a-f). The mean concentration is included for comparison of magnitude.*

		Monosaccharides	Lactose	Gos
A_{19}	RMSE	1.06×10^{-2} M	2.41×10^{-3} M	1.82×10^{-3} M
	Mean	4.54×10^{-2} M	1.28×10^{-1} M	1.12×10^{-2} M
A_{48}	RMSE	1.31×10^{-2} M	2.59×10^{-3} M	1.07×10^{-3} M
	Mean	7.29×10^{-2} M	1.17×10^{-1} M	4.97×10^{-3} M
B_{19}	RMSE	6.23×10^{-3} M	6.85×10^{-3} M	4.02×10^{-3} M
	Mean	5.33×10^{-2} M	2.70×10^{-1} M	2.26×10^{-2} M
B_{48}	RMSE	8.06×10^{-3} M	1.28×10^{-2} M	5.13×10^{-3} M
	Mean	9.00×10^{-2} M	2.41×10^{-1} M	3.11×10^{-2} M
C_{19}	RMSE	8.29×10^{-3} M	2.15×10^{-2} M	8.66×10^{-3} M
	Mean	7.55×10^{-2} M	5.68×10^{-1} M	4.30×10^{-2} M
C_{48}	RMSE	1.15×10^{-2} M	2.78×10^{-2} M	9.79×10^{-3} M
	Mean	1.06×10^{-1} M	5.38×10^{-1} M	5.72×10^{-2} M

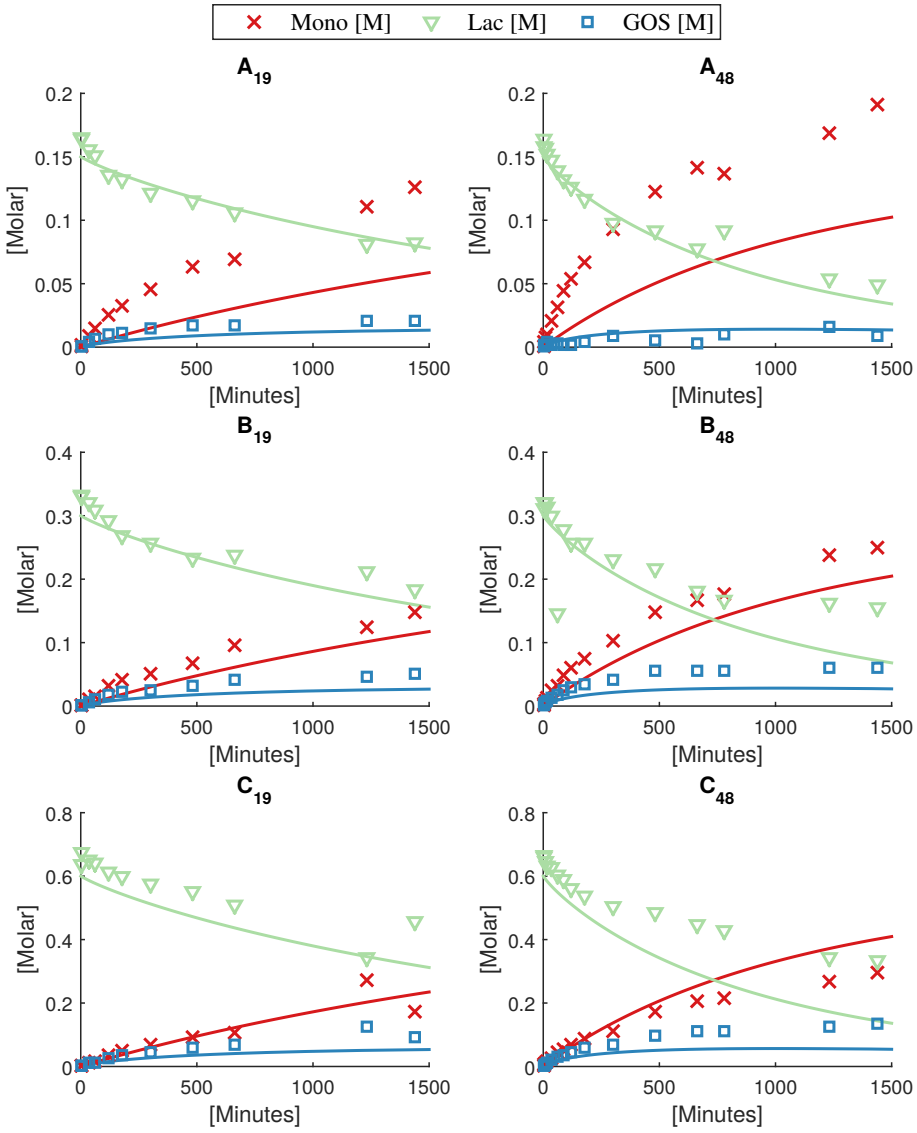


Figure 5.5: *Simulations of the model presented by Palai et al. [14] using the best parameter set found by regression.*

The model proposed by Palai et al. [14] was the simplest of the models, the simplicity of the model made it an interesting approach to describing the transgalactosylation reaction catalysed by β-galactosidase. This model perfomed worst of the models evaluated in this project, even though their published results demonstrate an excellent ability to describe thier data. The model does, however, contain some mechanistic flaws. The model does not consider hydrolysis of lactose, which seems to be an oversimplification. The experiments performed in this project has shown that at low substrate concentration the hydrolysis reaction is dominant, only at iniltial lactose concentrations of 0.6 M was the release of GOS higher than the release of galactose. The model proposed by Boon et al. [12] in both the unalterd and the recalculated form and the model proposed by [13] produced acceptable simulations, although especially the low substrate concetration simulation proved difficult for the models to handle. The difficulty in determining the correct parameter is illustrated by the variability of some parameters even at similar error values the tables 5.6 -5.8 show the best parameters for the models and the minimum and maximum values among the 10 best solutions. It is seen that some of the solution have converged very closely around some of the parameters e.g. the first column in 5.7 and other for other parameters there is no convergence e.g. the fourth column in 5.6 were the solutions span 7 orders of magnitude.

Table 5.6: *The span of solutions for parametes in the recalculated model based on the mechanism presented by Boon et al. [12].*

	k_1	k_2	k_3	k_4	k_7	k_8
Best	368.94	0.034	25.86	4648.15	0.0470	0.612
Min	31.64	0.034	10.68	0.0022	0.0140	0.425
Max	2659	0.0944	34.94	43766.8	55353.45	119.55

Table 5.7: *The span of solutions for parametes in the model derived by Boon et al. [12].*

	k_1	k_2	k_4	k_4	$\frac{k_7}{k_8}$
best	0.0039	$2.49 \cdot 10^{-6}$	0.170	163.69	119.50
min	0.0060	$8.05 \cdot 10^{-6}$	0.110	63.61	56.66
max	0.0014	10^{-6}	0.558	163.68	206.92

Table 5.8: *The span of solutions for parametes in the model derived by Vera et al. [13]*

	K_M	K_{MH}	K_{MT}	K_{MGal}	k_h	k_T	k_{cat}	$k_{cat'}$	k_9	KI
best	1667.13	9329.13	0.000011	0.000069	1.71	99.14	46286.12	54614.55	2256.58	0.0119
Min	0.00377	0.725	0.000011	0.00001	0.000013	0.02422	3.95	16928.96	665.56	0.00951
max	1667.13	9329.13	89705.63	0.00361	1557.13	83.70	46286.12	97594.3	58439.34	1477.89

5.3 Conclusion

For the models available currently available to describe the transgalactosylation of lactose catalysed by β-galactosidase it was not possible to determine the rate parameters with confidence as the solutions the minimisation problems that need to be solved have large unstable regions resulting in large variability in some of the parameters. The Model proposed by [14] is oversimplified and is not able to accurately simulate the progression of lactose conversion, especially at low substrate concentrations. The model proposed by Vera et al. [13] uses more parameters than the one proposed by [12], is not able to simulate the conversion of lactose with higer precision. The model proposed by

Vera et al. [13] might perform better if K_M, k_{cat} and KI were determined directly, that was however not possible.

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Appendix A

A.1 Rate Equations from

The rate equations for the model proposed by Vera et al. [13] was calculated by calculating the distribution of enzyme in the different fractions followed by substitution into the rate equations for glucose, galactose, di-, tri-, tetra-, and pentasaccharides.
The distribution of enzyme was found by computing the determinants of the reduced matrices that are found by removing the corresponding row and column of the coefficient matrix (A.1) in turn for each species.
After calulation of the destribution equations the concentration of free enzyme and inhibited enzyme can be calculated as an equilibrium. Then the expression for each enzyme species can be substituted into the elementary rate equation for glucose, galactose, di-, tri-, tetra-, and pentasaccharides:

$$\frac{dGlu}{dt} = k_{cat} * Edi \tag{A.1}$$

$$\frac{dGal}{dt} = k_h * H2O * EGal - k_{15} * Gal * EGal + k_{16} * EGalGal \tag{A.2}$$

$$\frac{dDi}{dt} = -k_1 * k_{cat} * E + k_2 * EDi - k3 * Di * EGal + k_4 * EGalDi + k_{17} * EGalGal \tag{A.3}$$

$$\frac{dTri}{dt} = k_5 * EGalDi - k_6 * Tri * E - k_7 * Tri * EGal + k_8 * EGalTri \tag{A.4}$$

$$\frac{dTet}{dt} = k_9 * EGalTri - k_{10} * Tet * E - k_{11} * Tet * EGal + k_{12} * EGalTet \tag{A.5}$$

$$\frac{dPen}{dt} = k_{13} * EGalTet - k_{14} * Pen * E \tag{A.6}$$

The expression for each enzyme species is shown on the following pages.

$\frac{d(E+EGal_I}{dt} =$	Di*k1 + Tet*k10 + Pen*k14 + Tri*k6)*f	k2	H2O*kH	k5	k9	k13	k17
$\frac{d(EDi}{dt} =$	Di*k1*f	-k2-kcat	0	0	0	0	0
$\frac{d(EGal}{dt} =$	0	kcat	-Tet*k11 - Gal*k15 - Di*k3 - Tri*k7 - H2O*kh	k4	k8	k12	k16
$\frac{d(EGalDi}{dt} =$	Tri*k6*f	0	Di*k3	-k4-k5	0	0	0
$\frac{d(EGalTri}{dt} =$	Tet*k10*f	0	Tri*k7	0	-k8-k9	0	0
$\frac{d(EGalTet}{dt} =$	Pen*k14*f	0	Tet*k11	0	0	-k12-k13	0
$\frac{d(EGalGal}{dt} =$	0	0	Gal*k15	0	0	0	-k16-k17

Table A.1: *f is the fraction undbound enzyme out of the sum of free enzyme and enzyme bound to inhibitor*

(E+EGal$_I$) =E_{total}*(Tet*k11*k13*k16*k2*k4*k8 + Tet*k11*k13*k17*k2*k4*k8 + Gal*k12*k15*k17*k2*k4*k8 + Gal*k13*k15*k17*k2*k4*k8 + Tet*k11*k13*k16*k2*k5*k8 + Tet*k11*k13*k17*k2*k5*k8 + Gal*k12*k15*k17*k2*k5*k8 + Gal*k13*k15*k17*k2*k5*k8 + Di*k12*k16*k2*k3*k5*k8 + Di*k13*k16*k2*k3*k5*k8 + Di*k12*k17*k2*k3*k5*k8 + Di*k13*k17*k2*k3*k5*k8 + Tet*k11*k13*k16*k2*k4*k9 + Tet*k11*k13*k17*k2*k4*k9 + Gal*k12*k15*k17*k2*k4*k9 + Gal*k13*k15*k17*k2*k4*k9 + Tet*k11*k13*k16*k2*k5*k9 + Tet*k11*k13*k17*k2*k5*k9 + Gal*k12*k15*k17*k2*k5*k9 + Gal*k13*k15*k17*k2*k5*k9 + Di*k12*k16*k2*k3*k5*k9 + Di*k13*k16*k2*k3*k5*k9 + Di*k12*k17*k2*k3*k5*k9 + Di*k13*k17*k2*k3*k5*k9 + Tri*k12*k16*k2*k4*k7*k9 + Tri*k13*k16*k2*k4*k7*k9 + Tri*k12*k17*k2*k4*k7*k9 + Tri*k13*k17*k2*k4*k7*k9 + Tri*k12*k16*k2*k5*k7*k9 + Tri*k13*k16*k2*k5*k7*k9 + Tri*k12*k17*k2*k5*k7*k9 + Tri*k13*k17*k2*k5*k7*k9 + Tet*k11*k13*k16*k4*k8*kcat + Tet*k11*k13*k17*k4*k8*kcat + Gal*k12*k15*k17*k4*k8*kcat + Gal*k13*k15*k17*k4*k8*kcat + Tet*k11*k13*k16*k5*k8*kcat + Tet*k11*k13*k17*k5*k8*kcat + Gal*k12*k15*k17*k5*k8*kcat + Gal*k13*k15*k17*k5*k8*kcat + Di*k12*k16*k3*k5*k8*kcat + Di*k13*k16*k3*k5*k8*kcat + Di*k12*k17*k3*k5*k8*kcat + Di*k13*k17*k3*k5*k8*kcat + Tet*k11*k13*k16*k4*k9*kcat + Tet*k11*k13*k17*k4*k9*kcat + Gal*k12*k15*k17*k4*k9*kcat + Gal*k13*k15*k17*k4*k9*kcat + Tet*k11*k13*k16*k5*k9*kcat + Tet*k11*k13*k17*k5*k9*kcat + Gal*k12*k15*k17*k5*k9*kcat + Gal*k13*k15*k17*k5*k9*kcat + Di*k12*k16*k3*k5*k9*kcat + Di*k13*k16*k3*k5*k9*kcat + Di*k12*k17*k3*k5*k9*kcat + Di*k13*k17*k3*k5*k9*kcat + Tri*k12*k16*k4*k7*k9*kcat + Tri*k13*k16*k4*k7*k9*kcat + Tri*k12*k17*k4*k7*k9*kcat + Tri*k13*k17*k4*k7*k9*kcat + Tri*k12*k16*k5*k7*k9*kcat + Tri*k13*k16*k5*k7*k9*kcat + Tri*k12*k17*k5*k7*k9*kcat + Tri*k13*k17*k5*k7*k9*kcat + H2O*k12*k16*k2*k4*k8*kh + H2O*k13*k16*k2*k4*k8*kh + H2O*k12*k17*k2*k4*k8*kh + H2O*k13*k17*k2*k4*k8*kh + H2O*k12*k16*k2*k5*k8*kh + H2O*k13*k16*k2*k5*k8*kh + H2O*k12*k17*k2*k5*k8*kh + H2O*k13*k17*k2*k5*k8*kh + H2O*k12*k16*k2*k4*k9*kh + H2O*k13*k16*k2*k4*k9*kh + H2O*k12*k17*k2*k4*k9*kh + H2O*k13*k17*k2*k4*k9*kh + H2O*k12*k16*k2*k5*k9*kh + H2O*k13*k16*k2*k5*k9*kh + H2O*k12*k17*k2*k5*k9*kh + H2O*k13*k17*k2*k5*k9*kh + H2O*k12*k16*k4*k8*kcat*kh + H2O*k13*k16*k4*k8*kcat*kh + H2O*k12*k17*k4*k8*kcat*kh + H2O*k13*k17*k4*k8*kcat*kh + H2O*k12*k16*k5*k8*kcat*kh + H2O*k13*k16*k5*k8*kcat*kh + H2O*k12*k17*k5*k8*kcat*kh + H2O*k13*k17*k5*k8*kcat*kh + H2O*k12*k16*k4*k9*kcat*kh + H2O*k13*k16*k4*k9*kcat*kh + H2O*k12*k17*k4*k9*kcat*kh + H2O*k13*k17*k4*k9*kcat*kh + H2O*k12*k16*k5*k9*kcat*kh + H2O*k13*k16*k5*k9*kcat*kh + H2O*k12*k17*k5*k9*kcat*kh + H2O*k13*k17*k5*k9*kcat*kh)/denominator

EDi = E_{total}*(Di*Tet*f*k1*k11*k13*k16*k4*k8 + Di*Tet*f*k1*k11*k13*k17*k4*k8 + Di*Gal*f*k1*k12*k15*k17*k4*k8 + Di*Gal*f*k1*k13*k15*k17*k4*k8 + Di*Tet*f*k1*k11*k13*k16*k5*k8 + Di*Tet*f*k1*k11*k13*k17*k5*k8 + Di*Gal*f*k1*k12*k15*k17*k5*k8 + Di*Gal*f*k1*k13*k15*k17*k5*k8 + Di*Di*f*k1*k12*k16*k3*k5*k8 + Di*Di*f*k1*k13*k16*k3*k5*k8 + Di*Di*f*k1*k12*k17*k3*k5*k8 + Di*Di*f*k1*k13*k17*k3*k5*k8 + Di*Tet*f*k1*k11*k13*k16*k4*k9 + Di*Tet*f*k1*k11*k13*k17*k4*k9 + Di*Gal*f*k1*k12*k15*k17*k4*k9 + Di*Gal*f*k1*k13*k15*k17*k4*k9 + Di*Tet*f*k1*k11*k13*k16*k5*k9 + Di*Tet*f*k1*k11*k13*k17*k5*k9 + Di*Gal*f*k1*k12*k15*k17*k5*k9 + Di*Gal*f*k1*k13*k15*k17*k5*k9 + Di*Di*f*k1*k12*k16*k3*k5*k9 + Di*Di*f*k1*k13*k16*k3*k5*k9 + Di*Di*f*k1*k12*k17*k3*k5*k9 + Di*Di*f*k1*k13*k17*k3*k5*k9 + Di*Tri*f*k1*k12*k16*k4*k7*k9 + Di*Tri*f*k1*k13*k16*k4*k7*k9 + Di*Tri*f*k1*k12*k17*k4*k7*k9 + Di*Tri*f*k1*k13*k17*k4*k7*k9 + Di*Tri*f*k1*k12*k16*k5*k7*k9 + Di*Tri*f*k1*k13*k16*k5*k7*k9 + Di*Tri*f*k1*k12*k17*k5*k7*k9 + Di*Tri*f*k1*k13*k17*k5*k7*k9 + Di*H2O*f*k1*k12*k16*k4*k8*kh + Di*H2O*f*k1*k13*k16*k4*k8*kh + Di*H2O*f*k1*k12*k17*k4*k8*kh + Di*H2O*f*k1*k13*k17*k4*k8*kh + Di*H2O*f*k1*k12*k16*k5*k8*kh + Di*H2O*f*k1*k13*k16*k5*k8*kh + Di*H2O*f*k1*k12*k17*k5*k8*kh + Di*H2O*f*k1*k13*k17*k5*k8*kh + Di*H2O*f*k1*k12*k16*k4*k9*kh + Di*H2O*f*k1*k13*k16*k4*k9*kh + Di*H2O*f*k1*k12*k17*k4*k9*kh + Di*H2O*f*k1*k13*k17*k4*k9*kh + Di*H2O*f*k1*k12*k16*k5*k9*kh + Di*H2O*f*k1*k13*k16*k5*k9*kh + Di*H2O*f*k1*k12*k17*k5*k9*kh + Di*H2O*f*k1*k13*k17*k5*k9*kh)/denominator

EGalDi = E_{total}*(Di*Tet*f*k10*k12*k16*k2*k3*k8 + Di*Tet*f*k10*k13*k16*k2*k3*k8 + Di*Pen*f*k12*k14*k16*k2*k3*k8 + Di*Tet*f*k10*k12*k17*k2*k3*k8 + Di*Tet*f*k10*k13*k17*k2*k3*k8 + Di*Pen*f*k12*k14*k17*k2*k3*k8 + Tet*Tri*f*k11*k13*k16*k2*k6*k8 + Tet*Tri*f*k11*k13*k17*k2*k6*k8 + Gal*Tri*f*k12*k15*k17*k2*k6*k8 + Gal*Tri*f*k13*k15*k17*k2*k6*k8 + Di*Tri*f*k12*k16*k2*k3*k6*k8 + Di*Tri*f*k13*k16*k2*k3*k6*k8 + Di*Tri*f*k12*k17*k2*k3*k6*k8 + Di*Tri*f*k13*k17*k2*k3*k6*k8 + Di*Pen*f*k12*k14*k16*k2*k3*k9 + Di*Pen*f*k12*k14*k17*k2*k3*k9 + Tet*Tri*f*k11*k13*k16*k2*k6*k9 + Tet*Tri*f*k11*k13*k17*k2*k6*k9 + Gal*Tri*f*k12*k15*k17*k2*k6*k9 + Gal*Tri*f*k13*k15*k17*k2*k6*k9 + Di*Tri*f*k12*k16*k2*k3*k6*k9 + Di*Tri*f*k13*k16*k2*k3*k6*k9 + Di*Tri*f*k12*k17*k2*k3*k6*k9 + Di*Tri*f*k13*k17*k2*k3*k6*k9 + Tri*Tri*f*k12*k16*k2*k6*k7*k9 + Tri*Tri*f*k12*k17*k2*k6*k7*k9 + Tri*Tri*f*k13*k17*k2*k6*k7*k9 + Di*Di*f*k1*k12*k16*k3*k8*kcat + Di*Tet*f*k10*k12*k16*k3*k8*kcat + Di*Di*f*k1*k13*k16*k3*k8*kcat + Di*Tet*f*k10*k13*k16*k3*k8*kcat + Di*Pen*f*k12*k14*k16*k3*k8*kcat + Di*Di*f*k1*k12*k17*k3*k8*kcat + Di*Tet*f*k10*k12*k17*k3*k8*kcat + Di*Di*f*k1*k13*k17*k3*k8*kcat + Di*Tet*f*k10*k13*k17*k3*k8*kcat + Di*Pen*f*k12*k14*k17*k3*k8*kcat + Tet*Tri*f*k11*k13*k16*k6*k8*kcat + Tet*Tri*f*k11*k13*k17*k6*k8*kcat + Gal*Tri*f*k12*k15*k17*k6*k8*kcat + Gal*Tri*f*k13*k15*k17*k6*k8*kcat + Di*Tri*f*k12*k16*k3*k6*k8*kcat + Di*Tri*f*k13*k16*k3*k6*k8*kcat + Di*Tri*f*k12*k17*k3*k6*k8*kcat + Di*Tri*f*k13*k17*k3*k6*k8*kcat + Di*Di*f*k1*k12*k16*k3*k9*kcat + Di*Di*f*k1*k13*k16*k3*k9*kcat + Di*Pen*f*k12*k14*k16*k3*k9*kcat + Di*Di*f*k1*k12*k17*k3*k9*kcat + Di*Di*f*k1*k13*k17*k3*k9*kcat + Di*Pen*f*k12*k14*k17*k3*k9*kcat + Tet*Tri*f*k11*k13*k16*k6*k9*kcat + Tet*Tri*f*k11*k13*k17*k6*k9*kcat + Gal*Tri*f*k12*k15*k17*k6*k9*kcat + Gal*Tri*f*k13*k15*k17*k6*k9*kcat + Di*Tri*f*k12*k16*k3*k6*k9*kcat + Di*Tri*f*k13*k16*k3*k6*k9*kcat + Di*Tri*f*k12*k17*k3*k6*k9*kcat + Di*Tri*f*k13*k17*k3*k6*k9*kcat + Tri*Tri*f*k12*k16*k6*k7*k9*kcat + Tri*Tri*f*k13*k16*k6*k7*k9*kcat + Tri*Tri*f*k12*k17*k6*k7*k9*kcat + Tri*Tri*f*k13*k17*k6*k7*k9*kcat + H2O*Tri*f*k12*k16*k2*k6*k8*kh + H2O*Tri*f*k13*k16*k2*k6*k8*kh + H2O*Tri*f*k12*k17*k2*k6*k8*kh + H2O*Tri*f*k13*k17*k2*k6*k8*kh + H2O*Tri*f*k12*k16*k2*k6*k9*kh + H2O*Tri*f*k13*k16*k2*k6*k9*kh + H2O*Tri*f*k12*k17*k2*k6*k9*kh + H2O*Tri*f*k13*k17*k2*k6*k9*kh + H2O*Tri*f*k12*k16*k6*k8*kcat*kh + H2O*Tri*f*k13*k16*k6*k8*kcat*kh + H2O*Tri*f*k12*k17*k6*k8*kcat*kh + H2O*Tri*f*k13*k17*k6*k8*kcat*kh + H2O*Tri*f*k12*k16*k6*k9*kcat*kh + H2O*Tri*f*k13*k16*k6*k9*kcat*kh + H2O*Tri*f*k12*k17*k6*k9*kcat*kh + H2O*Tri*f*k13*k17*k6*k9*kcat*kh)/denominator

EGalTri = E_{total}*(Tet*Tet*f*k10*k11*k13*k16*k2*k4 + Tet*Tet*f*k10*k11*k13*k17*k2*k4 + Gal*Tet*f*k10*k12*k15*k17*k2*k4 + Gal*Tet*f*k10*k13*k15*k17*k2*k4 + Tet*Tet*f*k10*k11*k13*k16*k2*k5 +

Tet*Tet*f*k10*k11*k13*k17*k2*k5 + Gal*Tet*f*k10*k12*k15*k17*k2*k5 + Gal*Tet*f*k10*k13*k15*k17*k2*k5 +
Di*Tet*f*k10*k12*k16*k2*k3*k5 + Di*Tet*f*k10*k13*k16*k2*k3*k5 + Di*Tet*f*k10*k12*k17*k2*k3*k5 +
Di*Tet*f*k10*k13*k17*k2*k3*k5 + Tet*Tri*f*k10*k12*k16*k2*k4*k7 + Tet*Tri*f*k10*k13*k16*k2*k4*k7 +
Pen*Tri*f*k12*k14*k16*k2*k4*k7 + Tet*Tri*f*k10*k12*k17*k2*k4*k7 + Tet*Tri*f*k10*k13*k17*k2*k4*k7 +
Pen*Tri*f*k12*k14*k17*k2*k4*k7 + Tet*Tri*f*k10*k12*k16*k2*k5*k7 + Tet*Tri*f*k10*k13*k16*k2*k5*k7 +
Pen*Tri*f*k12*k14*k16*k2*k5*k7 + Tet*Tri*f*k10*k12*k17*k2*k5*k7 + Tet*Tri*f*k10*k13*k17*k2*k5*k7 +
Pen*Tri*f*k12*k14*k17*k2*k5*k7 + Tri*Tri*f*k12*k16*k2*k4*k6*k7 + Tri*Tri*f*k13*k16*k2*k4*k6*k7 +
Tri*Tri*f*k12*k17*k2*k4*k6*k7 + Tri*Tri*f*k13*k17*k2*k4*k6*k7 + Tet*Tet*f*k10*k11*k13*k16*k4*kcat +
Tet*Tet*f*k10*k11*k13*k17*k4*kcat + Gal*Tet*f*k10*k12*k15*k17*k4*kcat + Gal*Tet*f*k10*k13*k15*k17*k4*kcat +
Tet*Tet*f*k10*k11*k13*k16*k5*kcat + Tet*Tet*f*k10*k11*k13*k17*k5*kcat + Gal*Tet*f*k10*k12*k15*k17*k5*kcat +
Gal*Tet*f*k10*k13*k15*k17*k5*kcat + Di*Tet*f*k10*k12*k16*k3*k5*kcat + Di*Tet*f*k10*k13*k16*k3*k5*kcat +
Di*Tet*f*k10*k12*k17*k3*k5*kcat + Di*Tet*f*k10*k13*k17*k3*k5*kcat + Di*Tri*f*k1*k12*k16*k4*k7*kcat +
Tet*Tri*f*k10*k12*k16*k4*k7*kcat + Di*Tri*f*k1*k13*k16*k4*k7*kcat + Tet*Tri*f*k10*k13*k16*k4*k7*kcat +
Pen*Tri*f*k12*k14*k16*k4*k7*kcat + Di*Tri*f*k1*k12*k17*k4*k7*kcat + Tet*Tri*f*k10*k12*k17*k4*k7*kcat +
Di*Tri*f*k1*k13*k17*k4*k7*kcat + Tet*Tri*f*k10*k13*k17*k4*k7*kcat + Pen*Tri*f*k12*k14*k17*k4*k7*kcat +
Di*Tri*f*k1*k12*k16*k5*k7*kcat + Tet*Tri*f*k10*k12*k16*k5*k7*kcat + Di*Tri*f*k1*k13*k16*k5*k7*kcat +
Tet*Tri*f*k10*k13*k16*k5*k7*kcat + Pen*Tri*f*k12*k14*k16*k5*k7*kcat + Di*Tri*f*k1*k12*k17*k5*k7*kcat +
Tet*Tri*f*k10*k12*k17*k5*k7*kcat + Di*Tri*f*k1*k13*k17*k5*k7*kcat + Tet*Tri*f*k10*k13*k17*k5*k7*kcat +
Pen*Tri*f*k12*k14*k17*k5*k7*kcat + Tri*Tri*f*k12*k16*k4*k6*k7*kcat + Tri*Tri*f*k13*k16*k4*k6*k7*kcat +
Tri*Tri*f*k12*k17*k4*k6*k7*kcat + Tri*Tri*f*k13*k17*k4*k6*k7*kcat + H2O*Tet*f*k10*k12*k16*k2*k4*kh +
H2O*Tet*f*k10*k13*k16*k2*k4*kh + H2O*Tet*f*k10*k12*k17*k2*k4*kh + H2O*Tet*f*k10*k13*k17*k2*k4*kh +
H2O*Tet*f*k10*k12*k16*k2*k5*kh + H2O*Tet*f*k10*k13*k16*k2*k5*kh + H2O*Tet*f*k10*k12*k17*k2*k5*kh +
H2O*Tet*f*k10*k13*k17*k2*k5*kh + H2O*Tet*f*k10*k12*k16*k4*kcat*kh + H2O*Tet*f*k10*k13*k16*k4*kcat*kh +
H2O*Tet*f*k10*k12*k17*k4*kcat*kh + H2O*Tet*f*k10*k13*k17*k4*kcat*kh + H2O*Tet*f*k10*k12*k16*k5*kcat*kh +
H2O*Tet*f*k10*k13*k16*k5*kcat*kh + H2O*Tet*f*k10*k12*k17*k5*kcat*kh +
H2O*Tet*f*k10*k13*k17*k5*kcat*kh)/denominator

EGalTet = E_{total}*(Tet*Tet*f*k10*k11*k16*k2*k4*k8 + Pen*Tet*f*k11*k14*k16*k2*k4*k8 +
Tet*Tet*f*k10*k11*k17*k2*k4*k8 + Pen*Tet*f*k11*k14*k17*k2*k4*k8 + Gal*Pen*f*k14*k15*k17*k2*k4*k8 +
Tet*Tet*f*k10*k11*k16*k2*k5*k8 + Pen*Tet*f*k11*k14*k16*k2*k5*k8 + Tet*Tet*f*k10*k11*k17*k2*k5*k8 +
Pen*Tet*f*k11*k14*k17*k2*k5*k8 + Gal*Pen*f*k14*k15*k17*k2*k5*k8 + Di*Pen*f*k14*k16*k2*k3*k5*k8 +
Di*Pen*f*k14*k17*k2*k3*k5*k8 + Tet*Tri*f*k11*k16*k2*k4*k6*k8 + Tet*Tri*f*k11*k17*k2*k4*k6*k8 +
Pen*Tet*f*k11*k14*k16*k2*k4*k9 + Pen*Tet*f*k11*k14*k17*k2*k4*k9 + Gal*Pen*f*k14*k15*k17*k2*k4*k9 +
Pen*Tet*f*k11*k14*k16*k2*k5*k9 + Pen*Tet*f*k11*k14*k17*k2*k5*k9 + Gal*Pen*f*k14*k15*k17*k2*k5*k9 +
Di*Pen*f*k14*k16*k2*k3*k5*k9 + Di*Pen*f*k14*k17*k2*k3*k5*k9 + Tet*Tri*f*k11*k16*k2*k4*k6*k9 +
Tet*Tri*f*k11*k17*k2*k4*k6*k9 + Pen*Tri*f*k14*k16*k2*k4*k7*k9 + Pen*Tri*f*k14*k17*k2*k4*k7*k9 +
Pen*Tri*f*k14*k16*k2*k5*k7*k9 + Pen*Tri*f*k14*k17*k2*k5*k7*k9 + Di*Tet*f*k1*k11*k16*k4*k8*kcat +
Tet*Tet*f*k10*k11*k16*k4*k8*kcat + Pen*Tet*f*k11*k14*k16*k4*k8*kcat + Di*Tet*f*k1*k11*k17*k4*k8*kcat +
Tet*Tet*f*k10*k11*k17*k4*k8*kcat + Pen*Tet*f*k11*k14*k17*k4*k8*kcat + Gal*Pen*f*k14*k15*k17*k4*k8*kcat +
Di*Tet*f*k1*k11*k16*k5*k8*kcat + Tet*Tet*f*k10*k11*k16*k5*k8*kcat + Pen*Tet*f*k11*k14*k16*k5*k8*kcat +
Di*Tet*f*k1*k11*k17*k5*k8*kcat + Tet*Tet*f*k10*k11*k17*k5*k8*kcat + Pen*Tet*f*k11*k14*k17*k5*k8*kcat +
Gal*Pen*f*k14*k15*k17*k5*k8*kcat + Di*Pen*f*k14*k16*k3*k5*k8*kcat + Di*Pen*f*k14*k17*k3*k5*k8*kcat +
Tet*Tri*f*k11*k16*k4*k6*k8*kcat + Tet*Tri*f*k11*k17*k4*k6*k8*kcat + Di*Tet*f*k1*k11*k16*k4*k9*kcat +
Pen*Tet*f*k11*k14*k16*k4*k9*kcat + Di*Tet*f*k1*k11*k17*k4*k9*kcat + Pen*Tet*f*k11*k14*k17*k4*k9*kcat +
Gal*Pen*f*k14*k15*k17*k4*k9*kcat + Di*Tet*f*k1*k11*k16*k5*k9*kcat + Pen*Tet*f*k11*k14*k16*k5*k9*kcat +
Di*Tet*f*k1*k11*k17*k5*k9*kcat + Pen*Tet*f*k11*k14*k17*k5*k9*kcat + Gal*Pen*f*k14*k15*k17*k5*k9*kcat +
Di*Pen*f*k14*k16*k3*k5*k9*kcat + Di*Pen*f*k14*k17*k3*k5*k9*kcat + Tet*Tri*f*k11*k16*k4*k6*k9*kcat +
Tet*Tri*f*k11*k17*k4*k6*k9*kcat + Pen*Tri*f*k14*k16*k4*k7*k9*kcat + Pen*Tri*f*k14*k17*k4*k7*k9*kcat +
Pen*Tri*f*k14*k16*k5*k7*k9*kcat + Pen*Tri*f*k14*k17*k5*k7*k9*kcat + H2O*Pen*f*k14*k16*k2*k4*k8*kh +
H2O*Pen*f*k14*k17*k2*k4*k8*kh + H2O*Pen*f*k14*k16*k2*k5*k8*kh + H2O*Pen*f*k14*k17*k2*k5*k8*kh +
H2O*Pen*f*k14*k16*k2*k4*k9*kh + H2O*Pen*f*k14*k17*k2*k4*k9*kh + H2O*Pen*f*k14*k16*k2*k5*k9*kh +
H2O*Pen*f*k14*k17*k2*k5*k9*kh + H2O*Pen*f*k14*k16*k4*k8*kcat*kh + H2O*Pen*f*k14*k17*k4*k8*kcat*kh +
H2O*Pen*f*k14*k16*k5*k8*kcat*kh + H2O*Pen*f*k14*k17*k5*k8*kcat*kh + H2O*Pen*f*k14*k16*k4*k9*kcat*kh +
H2O*Pen*f*k14*k17*k4*k9*kcat*kh + H2O*Pen*f*k14*k16*k5*k9*kcat*kh +
H2O*Pen*f*k14*k17*k5*k9*kcat*kh)/denominator

EGalGal = E_{total}*(Gal*Tet*f*k10*k12*k15*k2*k4*k8 + Gal*Tet*f*k10*k13*k15*k2*k4*k8 +
Gal*Pen*f*k12*k14*k15*k2*k4*k8 + Gal*Tet*f*k10*k12*k15*k2*k5*k8 + Gal*Tet*f*k10*k13*k15*k2*k5*k8 +
Gal*Pen*f*k12*k14*k15*k2*k5*k8 + Gal*Tri*f*k12*k15*k2*k4*k6*k8 + Gal*Tri*f*k13*k15*k2*k4*k6*k8 +
Gal*Pen*f*k12*k14*k15*k2*k4*k9 + Gal*Pen*f*k12*k14*k15*k2*k5*k9 + Gal*Tri*f*k12*k15*k2*k4*k6*k9 +
Gal*Tri*f*k13*k15*k2*k4*k6*k9 + Di*Gal*f*k1*k12*k15*k4*k8*kcat + Gal*Tet*f*k10*k12*k15*k4*k8*kcat +
Di*Gal*f*k1*k13*k15*k4*k8*kcat + Gal*Tet*f*k10*k13*k15*k4*k8*kcat + Gal*Pen*f*k12*k14*k15*k4*k8*kcat +
Di*Gal*f*k1*k12*k15*k5*k8*kcat + Gal*Tet*f*k10*k12*k15*k5*k8*kcat + Di*Gal*f*k1*k13*k15*k5*k8*kcat +
Gal*Tet*f*k10*k13*k15*k5*k8*kcat + Gal*Pen*f*k12*k14*k15*k5*k8*kcat + Gal*Tri*f*k12*k15*k4*k6*k8*kcat +
Gal*Tri*f*k13*k15*k4*k6*k8*kcat + Di*Gal*f*k1*k12*k15*k4*k9*kcat + Di*Gal*f*k1*k13*k15*k4*k9*kcat +
Gal*Pen*f*k12*k14*k15*k4*k9*kcat + Di*Gal*f*k1*k12*k15*k5*k9*kcat + Di*Gal*f*k1*k13*k15*k5*k9*kcat +
Gal*Pen*f*k12*k14*k15*k5*k9*kcat + Gal*Tri*f*k12*k15*k4*k6*k9*kcat +
Gal*Tri*f*k13*k15*k4*k6*k9*kcat)/denominator

denominator = (E+$EGal_I$) EDi + EGal + EGalDi + EGalTri + EGalTet + EGalGal