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ARAŞTIRMA MAKALESİ **RESEARCH ARTICLE**

Mathematical Modelling of Galactooligosaccharides Synthesis: Insights into *Aspergillus oryzae* Derived β-Galactosidase Kinetics*

Galaktooligosakkarit Sentezinin Matematiksel Modellenmesi: *Aspergillus oryzae* kaynaklı β-Galaktosidaz Kinetiğine İlişkin Bilgiler

Kadir CINAR[1*], Haci Ali GULEC[2], Gurbuz GUNES[3]

Abstract

The objective of this study was to adapt experimental data with a mathematical model based on transgalactosylation mechanisms identified in the literature for the synthesis of galactooligosaccharides and lactose hydrolysis. Galactooligosaccharides, synthesized from lactose through β-galactosidase-catalyzed transgalactosylation reactions, are acknowledged as specific bifidogenic factors among prebiotic carbohydrates. Modelling galactooligosaccharide synthesis is challenging due to simultaneous hydrolysis and transferase reactions with identical nucleophiles and substrates. Therefore, researchers tried to simplify the mechanism by ignoring intermediate reactions or inhibitory effects of glucose and galactose. This study explored various kinetic models, considering the complexity of the reaction mechanism and balancing accuracy with feasibility. Experimental data from galactooligosaccharide synthesis using a continuous stirred tank reactor was adapted to kinetic models (Models A and B) with modifications, incorporating glucose and galactose inhibitions. The COPASI software was used for testing fitness of reaction models. Eight models were evaluated for their suitability, utilizing six replicated experimental datasets under specific reaction conditions, including a temperature of 45°C, lactose concentration of 18.25°Brix, and enzyme concentration of 10-unit g lactose solution^{-1}. The analysis was conducted using the "random research" and "particle swarm" algorithms. Model evaluations revealed that Model B, augmented with glucose and galactose inhibitions, provided the closest alignment between experimental and simulated data. The study underscored the significance of glucose and galactose impacts on galactooligosaccharide synthesis efficiency. The Model B, with addition of glucose and galactose inhibitions, emerged as a valuable tool for predicting galactooligosaccharide yield, contributing to a deeper understanding of *A.oryzae*-derived β-galactosidase kinetics due to its significant influence on parameters such as productivity, conversion of lactose, and composition of product. The findings will guide optimization strategies for enhanced galactooligosaccharide production efficiency, advancing knowledge in enzymatic galactooligosaccharide synthesis.

Keywords: Kinetics, Transgalactosylation, Hydrolysis, Galactooligosaccharides, *A. oryzae*

[1*]**Sorumlu Yazar/Corresponding Author:** Kadir Cinar, Trakya University, Engineering Faculty, Department of Food Engineering, Edirne, Turkey. E-mail: kadircinar@trakya.edu.tr OrcID: 0000-0001-5488-652X

[2]Haci Ali Gulec, Trakya University, Engineering Faculty, Department of Food Engineering, Edirne, Turkey. E-mail: hacialigulec@trakya.edu.tr OrcID: 0000-0002-9525-6206

[3]Gurbuz Gunes, Istanbul Technical University, Faculty of Chemical and Metallurgical Engineering, Department of Food Engineering, Maslak, Istanbul, Turkey, Turkey. E-mail: gunesg@itu.edu.tr OrcID: 0000-0002-2948-3785

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Öz

Bu çalışmanın amacı, elde edilen deneysel verileri literatürde tanımlanmış laktoz hidrolizi ve galaktooligosakkaritlerin sentezi için belirlenmiş transgalaktosilasyon mekanizmalarına dayalı matematiksel bir modele uyumlu hale getirmektir. Galaktooligosakkaritler, laktozun β-galaktosidaz katalizli transgalaktosilasyon reaksiyonları aracılığıyla sentezlenen özel bifidojenik faktörler olarak bilinirler. Galaktooligosakkarit sentez mekanizmasının, aynı nükleofillere ve substratlara sahip hidroliz ve transferaz reaksiyonlarının eş zamanlı olarak gerçekleşmesi nedeniyle matematiksel modellemesi zorluklar içermektedir. Bu nedenle araştırmacılar, meydana gelen ara reaksiyonları veya glukoz ve galaktozun inhibe edici etkilerini göz ardı ederek mekanizmayı basitleştirmeye çalıştılar. Bu çalışma, reaksiyon mekanizmasının karmaşıklığını göz önünde bulundurarak doğruluk ile uygulanabilirlik arasında denge sağlamak amacıyla çeşitli kinetik modellerin uygunluğunu değerlendirmektedir. Sürekli karıştırmalı tank reaktörü kullanılarak elde edilen deneysel veriler, glukoz ve galaktoz inhibitörlerini içeren modifikasyonlarla kinetik modellere (Model A ve B) adapte edilmiştir. COPASI yazılımı, model denklemleri test etmek amacıyla kullanılmıştır. Toplam 8 adet model, 6 tekrarlı deneysel veri setinde (sıcaklık:45°C, laktoz konsantrasyonu:18.25 Briks ve enzim konsantrasyonu:10 ünite g laktoz çözeltisi^{-1}) uygunluğu test edilmiştir. Çözümleme sırasıyla "random research" ve "particle swarm" algoritmaları kullanılarak gerçekleştirilmiştir. Model değerlendirmeleri, glukoz ve galaktoz inhibitörlerini içeren Model B'nin deneysel ve simüle veriler arasında en yakın uyumu sağladığını göstermiştir. Çalışma, glukoz ve galaktozun galaktooligosakkarit sentezi verimliliği üzerindeki inhibitör etkilerinin önemini ortaya koymuştur. Glukoz ve galaktoz inhibitörlerini içeren Model B, galaktooligosakkarit veriminin öngörülmesi ve laktoz dönüşümü, üretkenlik, ürün bileşimi gibi parametreler üzerindeki önemli etkisi nedeniyle *A. oryzae* kaynaklı β-galaktosidaz kinetiğinin daha iyi anlaşılmasına katkı sağlayacak önemli bir araçtır. Çalışma sonucunda elde edilen bulgular, galaktooligosakkarit üretim verimliliğini artırmak için optimizasyon stratejilerine rehberlik edecek ve enzimatik galaktooligosakkarit sentezi konusunda literatüre katkı sağlayacaktır.

Anahtar Kelimeler: Kinetik, Transgalaktosilasyon, Hidroliz, Galaktooligosakkaritler, *A. oryzae*

1. Introduction

Galactooligosaccharides (GOS) are recognized as the most specific bifidogenic components among prebiotic carbohydrates (Uran et al., 2021). They are produced from lactose through transgalactosylation reactions facilitated by β-galactosidases. Various studies have been conducted in the literature to model the synthesis mechanism of GOS using different approaches (Chen et al., 2003; Mueller et al., 2018). The GOS synthesis mechanism is a complex process due to its simultaneous hydrolysis and transferase reactions, resulting from having identical nucleophiles and substrates. Consequently, evaluating the apparent rate constants is challenging. Researchers studying the GOS synthesis mechanism initially suggest a reaction pathway, allocating rate constants to each stage during catalysis, which are then experimentally determined. The comprehensive understanding of the reaction mechanism of β-galactosidase in existing literature adds attractiveness to this approach. Simplifications are made in the reaction mechanism to limit the number of experimentally predicted rate constants. While excessive simplifications negatively impact the model's accuracy, they may render the model construction more appropriate. Hence, achieving a balance between model accuracy and feasibility is crucial. Due to numerous unknown kinetic parameters, some authors have attempted to simplify the reaction mechanism by eliminating glucose (Vera et al., 2011) and galactose (Mueller et al., 2018) inhibition or both for reactions catalyzed by β-galactosidase from *A. oryzae.*

Iwasaki et al. (1996) expressed that the simultaneous occurrence of hydrolysis and transferase reactions in enzymatic GOS synthesis complicates the mathematical analysis. Therefore, they assumed that the GOS synthesis reaction could be represented by a stoichiometrically simple model. They constructed a GOS synthesis reaction model with a biochemical reaction chain consisting of thirteen equations, incorporating the formation of 3 and 4-chain GOS (GOS-3 and GOS-4, respectively) in their model. On the other hand, Boon et al. (1999) did not consider certain aspects, such as galactose mutarotation (Bakken et al., 1992), allolactose production (Huber et al., 1976), and the separate production of GOS-3 and GOS-4 (Iwasaki et al., 1996), in their proposed model, unlike some models in the literature. They obtained the rate parameters using the King-Altman method in their simplified reaction model. Kim et al. (2004) achieved good agreement with experimental data not only in lactose hydrolysis but also at various substrate concentrations using their proposed GOS synthesis reaction model. Their model includes the formation of allolactose, glucose's participation as a reactant, and enzyme concentration as parameters. In this model, lactose serves both as a substrate and a weaker glycosyl acceptor; however, at high concentrations, it has a higher chance of being an acceptor for GOS-3 synthesis. Galactose forms a complex with the free enzyme to create the galactosyl-enzyme complex, acting as an acceptor alongside glucose or lactose for subsequent transgalactosylation reactions. Notably, it does not bind to the galactosyl-enzyme complex. On the other hand, glucose, through interaction solely with the galactosyl-enzyme complex, functions as a more effective acceptor for transgalactosylation reactions, resulting in the formation of galactosyl-glucose disaccharides. The reaction mechanism assumes only one rate-limiting step and that all other steps are reversible. In their model, Neri et al. (2009) introduced competitive inhibition by glucose and galactose to trisaccharide synthesis and lactose hydrolysis reactions. The model comprises four dependent state variables (lactose, glucose, galactose, and trisaccharide concentration) and eight kinetic parameters. Additionally, this model assumes the reversibility of oligosaccharide synthesis, allowing water or lactose to interact with the galactosyl-enzyme complex.The model does not consider galactose mutarotation, tetrasaccharide formation, diffusion limitations, and time-dependent enzyme inactivation, focusing only on using lactose as a substrate. Moreover, Palai et al. (2012), Palai and Bhattacharya (2013), Palai et al. (2014), and Palai et al. (2016) used reaction models based on the Michaelis-Menten model to perform kinetic analysis for GOS synthesis. Palai et al. (2012) used a four-step model to simplify the reaction mechanism. In this model, synthesized GOS-3 and GOS-4 are expressed as a single product, GOS. No distinction is made between glucose and galactose; both being referred to as monosaccharides. Additionally, enzyme inhibition is neglected. Palai et al. (2014) and Palai et al. (2016) used a five-step reaction model in their studies. In this model, the formation of glucose and galactose is considered separately, and GOS hydrolysis and glucose's competitive inhibition are taken into account. 3, 4, and possible higher-chain GOS are expressed as GOS.

The complexity of the synthesis mechanism for GOS production, where transgalactosylation and hydrolysis reactions occur simultaneously, increases due to different reactions having different substrates and ratios (González-Delgado et al., 2016). Therefore, it is crucial to explore various methods for analysing and optimizing

the GOS synthesis process. The enzymatic synthesis of GOS is a reaction that is controlled kinetically and heavily relies on enzyme properties. Factors such as the enzyme source, lactose conversion, productivity, and product composition are affected by the choice of enzyme source (Özdinç and Velioğlu, 2022). *A. oryzae*-derived β-galactosidase stands out for its high specific activity, resistance to high temperatures, and cost-effectiveness. However, due to the low lactose conversion of this enzyme in GOS synthesis, it is vital to enhance the GOS yield by optimizing reaction conditions through optimization methods. For this reason, a specific focus on comprehending the kinetic mechanism of this enzyme is essential.

In light of this information, the aim of this study is to adapt experimental data to a mathematical model using the transgalactosylation mechanisms identified in the literature for GOS synthesis and lactose hydrolysis. Predicting the reaction rate constants will enable the estimation of the GOS amount at any given point in the reaction process, allowing for a more reliable assessment of GOS synthesis efficiency under different reaction conditions catalysed by *A. oryzae*-derived β-galactosidase.

2. Materials and Methods

2.1. Materials

β-galactosidase sourced from *A. oryzae*, with fungal lactase activity at 100000 U g^{-1}, was acquired from ENZECO® (Enzyme Development Corporation, New York, USA). Lactose (99.9%) and all other reagents with the highest purity were obtained from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). For the purpose of determining retention times in HPLC, glucose (Merck, Darmstadt, Germany) and galactose (Merck, Darmstadt, Germany) were utilized.

2.2. Methods

2.2.1. GOS Synthesis in the Continuous Stirred Tank Reactor

The synthesis of GOS was conducted using a temperature-controlled magnetic stirrer (MTops MS300HS, South Korea). Lactose solution (LS) by weighing 50 g was placed on the magnetic stirrer (Cinar et al., 2020). The lactose solution was formulated at the optimal operational pH for lactase enzyme (pH:4.5). Adjustments to the pH of reaction medium were made using 0.1 N HCl and 0.1 N NaOH solutions as required. To pinpoint the moment of peak GOS production during synthesis, 100 µl samples were extracted from the reaction medium at specific time intervals (5-30 minutes). Enzyme inactivation was accomplished by combining the samples with 900 µl of distilled water at 95°C. Subsequently, the samples were preserved at -18°C until further analysis, which included HPLC examination of sugar profiles when necessary. The outcomes derived from the HPLC sugar peaks were expressed as the percentage proportion relative to the total sugars.

2.2.2. HPLC Analysis

The Agilent 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA, USA) was used for analysing the product profile obtained after enzymatic reaction. The system comprised a column oven (Agilent, G1316A), autosampler (Agilent G1329B), pump (Agilent, G1311C), a refractive index detector (SpectraSystem RI-150), and a software package (Agilent Chem-Station) for system control and data evaluation. For determining the sugar content of the products obtained after GOS synthesis, samples were diluted in a 1:9 ratios (100 µl sample: 900 µl 95°C distilled water) and injected into the system. Separation conditions are summarized in Table 1 (Cinar et al., 2020).

Table 1 : Characteristics of the stationary and mobile phases for sugar analysis by HPLC.

Column	MetaCarb Ca Plus, 7.8 x 300 mm
Column temperature	80°C
Mobile phase	Deionized water (100%)
Injection volume	20 µl
Flow rate	0.5 mL min^{-1}
Detector	Refractive index (RI), 16X

In the HPLC analyses, no standard was employed. The concentrations of sugars (lactose, glucose, galactose, andtotal GOS) were calculated in terms of weight percentages (w/w) based on the peak areas corresponding to

these sugars (Boon et al., 2000).

2.2.3. Kinetic Modelling of Enzymatic Galactooligosaccharide Synthesis

The activity ratio between transgalactosylation and hydrolysis reactions varies based on the enzyme source and production method in studies related to GOS synthesis. In this study, experimental data were adapted to kinetic models using transgalactosylation mechanisms identified in the literature for lactose hydrolysis and GOS synthesis. In this context, reaction mechanism models for GOS synthesis, Model A (Manucci, 2009) and Model B (Kim et al., 2004) were employed. Also, these models were modified by adding glucose and galactose inhibitions. For the adaptation of experimental data to a kinetic model and the estimation of reaction rate constants, the COPASI 4.22 software package, enabling the modelling of biochemical systems, was utilized. The analysis was conducted using the "random research" and "particle swarm" algorithms. COPASI is extensively utilized for the kinetic modelling of biochemical reactions, offering essential features like various parameter estimation algorithms, support for metabolic control analysis, and the ability for users to export data in the SBML format (Costa and Vinga, 2016). The kinetic parameters of the proposed models were determined using nonlinear regression techniques with experimental data sets. For calculating molar concentrations, the molecular weights of lactose, galactose, and glucose were assumed to be 342.3 g moles^{-1}, 180.16 g moles^{-1}, and 180.16 g moles^{-1}, respectively. The molecular weight of synthesized GOS was assumed to be GOS-3, with a molecular weight of 504.32 g moles^{-1} (Warmerdam et al., 2013). The molecular weight of *A. oryzae*-derived β-galactosidase enzyme was considered as 105000 Da (Tanaka et al., 1975).

In this study, models identified in the literature for modelling GOS synthesis were applied to the obtained data. Subsequently, these models, along with modifications, were tested against experimental datasets to adapt them to GOS synthesis. A total of eight models were assessed for their appropriateness using six replicated experimental datasets at the specified reaction conditions: temperature (45°C), lactose concentration (18.25°Brix), and enzyme concentration (10 U g lactose solution^{-1}). The analyses and time-course simulations were conducted using COPASI 4.22 software, employing a deterministic LSODA solver (Hoops et al., 2006). The reaction mechanisms of the eight models used in the study are illustrated in Figure 3, i-viii. In these model equations, ‘E’ represents enzyme, ‘Glu’ stands for glucose, ‘Gal’ for galactose, ‘Lac’ for lactose, ‘GOS’ for total galactooligosaccharides, ‘E:Lac’ for enzyme-lactose complex, ‘E:Gal’ for enzyme-galactose complex, ‘Allo’ for allolactose, ‘E:IGlu’ signifies the glucose inhibited enzyme complex, ‘E:IGal’ signifies the galactose inhibited enzyme complex.

3. Results and Discussion

3.1. Evaluation of enzymatic synthesis

At the initial moment of the reaction (t=0), only lactose was present in the reaction medium. Therefore, at t=0, the entire component present in the medium constitutes lactose at 100% (Figure 1-2). The amount of lactose decreased throughout the reaction due to transgalactosylation and hydrolysis reactions. It was found that approximately 60% of initial lactose was converted into other reaction components in the 300-minute reaction time (*Figure 1*). The mean scores of six enzymatic synthesis experiments revealed that maximum GOS yield was achieved at the reaction time of 78.75±3.75 min. At this time obtained weight percentages of GOS-4, GOS-3 and GOS yield was 4.76±0.19, 16.75±0.28 and 21.51±0.12, respectively. As can be seen from Figure 1, after reaching peak values of GOS-3 and GOS-4, they began to degrade. At low lactose conversions, transgalactosylation is predominant in the early stages of the reaction, and when GOS yield reaches its maximum level, hydrolysis becomes dominant (Cinar et al., 2020). Moreover, Osman et al. (2016) explained that the rates of transgalactosylation and hydrolysis reactions reached equilibrium, and GOS Yield (GY) continued to increase up to a certain level. And after this point, GOS hydrolysis became dominant over transgalactosylation, and glucose and galactose became thermodynamically preferred products.

During GOS synthesis, the sequential enzymatic transfer of galactose to glucose and galactose, resulting in the formation of certain double-chain saccharides like allolactose and galactobiose, is possible (Neri et al., 2009). These reactions are considered intermediate steps in GOS synthesis and are transient. Throughout the reaction, they either act as galactosyl acceptors or are hydrolyzed by the enzyme into galactose and glucose (Huber et al., 1976). As observed from the obtained HPLC chromatograms (*Figure 2*), it is not possible to determine whether

these side reactions occur during the reaction. Additionally, it has been determined that *A. oryzae*-derived β-galactosidase produces negligible amounts of transgalactosyl disaccharides (Cheng et al., 2006; Huerta et al., 2011; Sanz-Valero, 2009; Vera et al., 2011). It has been reported that it predominantly produces GOS-3, a small amount of GOS-4, and negligible levels of GOS-2 and GOS-5 (Osman, 2016).

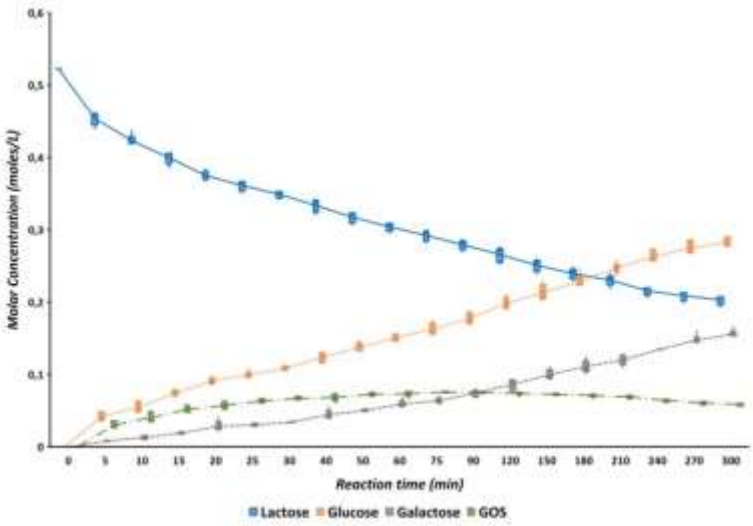

Figure 1. Time dependent change of molar concentrations of reaction components (Reaction conditions: temperature (45°C), lactose concentration (18.25°Brix), and enzyme concentration (10 U g lactose solution^{-1}).

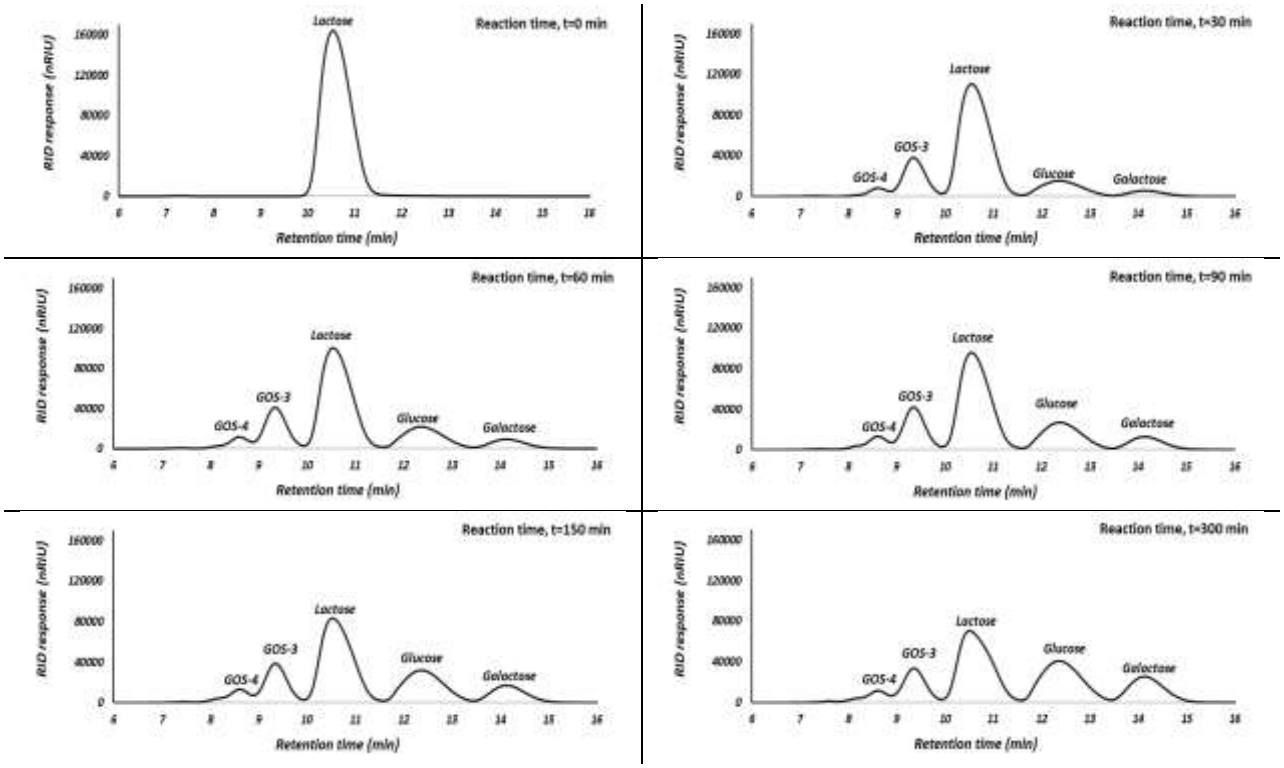

Figure 2. HPLC chromatograms showing the change of reaction components with time (Reaction conditions: temperature (45°C), lactose concentration (18.25°Brix), and enzyme concentration (10 U g lactose solution^{-1}).

The possibility of the formation of high-chain GOS with different structures during the reaction exists, and their absence in HPLC detection is also possible. In literature studies, double-chain GOS such as allolactose and galactobiose were assumed to be included in the lactose peak (Jenab et al., 2018; Manucci, 2009). In this study, it was also thought that the amount of allolactose is included in the lactose peak. Since allolactose, like lactose, acts as a galactosyl acceptor, this does not pose a problem in terms of the synthesis mechanism. Urrutia et al.

(2013) highlighted the significant contribution of disaccharides to the overall GOS content, an aspect often overlooked in the case of *A. oryzae* β-galactosidase. This oversight occurs because disaccharides are typically concealed by lactose when employing conventional analytical methods. They also added that the mechanism of allolactose formation is essentially an intermolecular reaction for this β-galactosidase. Furthermore, the molar balances of galactose and glucose throughout the reaction were monitored using the following equation, as done by Boon et al. (1999).

$$\frac{[glucose]}{[galactose]} = \frac{[lactose]+[glucose]+[GOS]}{[lactose]+[galactose]+2[GOS]} \quad \text{(Eq. 1)}$$

Since no standards were used in HPLC, and the quantities of reaction products were calculated as percentages, the molar ratio of glucose to galactose changes slightly during the reaction. The theoretical ratio, which should be "1," is considered to have an acceptable error margin of 10%, as evaluated by Boon et al. (1999)

3.2. Kinetic modelling

In an attempt to balance between an overly simplified model and inappropriately predicted rate constants, existing GOS synthesis models aim to match experimental data. The procedure commonly applied in studies to report kinetic parameters involves providing parameter, standard error, or standard deviation for each fit in each experimental set (Manucci, 2009). Statistical errors can be reported for each experimental set using multiple nonlinear regression techniques (Bates and Watts, 1988). Also, in this study the kinetic parameters of the proposed models were determined using the nonlinear regression technique with experimental datasets. The equation for the objective function used to predict kinetic rate constants is given below.

$$f(k) = \sum_{i,j} \frac{[\tilde{y}_{i,j} - y_{i,j}(k)]^2}{<\tilde{y}_i{}^2>} \quad \text{(Eq. 2)}$$

In this equation, "f(k)" represents the objective function of the rate constants, "$\tilde{y}$" denotes the experimental data, and "y" represents the simulated data. The subscripts "i" and "j" represent reaction elements and experimental data, respectively. The initial concentrations of reaction elements (glucose, galactose, GOS) and intermediate elements (E:Lac, E:Gal, Allo, E:IGlu, E:IGal) were taken as zero. Graphs depicting the time-dependent changes in reaction elements obtained from testing the appropriateness of eight models against six replicated experimental datasets are shown below (*Figure 3*). The graphs were directly obtained from the software; the x-axis represents time in minutes, and the y-axis represents molar concentration.

There is no good fit between experimental and simulated data in Model A (*Figure 3i*). It can be stated that up to 100 minutes of reaction time, there is conformity between the GOS and galactose values, but after this time, this trend collapses. However, the predicted individual fittings of lactose and glucose show poor matching with the experimental measurements throughout the reaction. Additionally, the weighted errors grow larger as the reaction continues. When galactose (*Figure 3ii*) and glucose (*Figure 3iii*) inhibitions are added to Model A, except for GOS, the fitness of lactose, glucose, and galactose almost shows a similar pattern with the simulated data of Model A (*Figure 3i*). It was observed that especially glucose exhibits extremely poor fitness between experimental and simulated data. Also, the fitness of simulated data of lactose was still better than the data of "Model A" and "Model A + glucose inhibition." Therefore, we can say that these models are not appropriate for making proper estimations of the experimental data. However, with the addition of glucose and galactose inhibitions to Model A (*Figure 3iv*), we can obtain better estimates than *Figures 3i-iii*. Especially, there was a good correlation between the time-dependent data of galactose and GOS and the simulated ones. The simulated data of lactose and glucose were well-fitted between the reaction times of 120 and 220; however, before and after these time ranges, the difference between the observations and the corresponding fitted values increased, so the appropriateness of the model to estimate reaction constants worsened. Hence, the ability of these models (*Figure 3i-iv*) to represent the time-dependent change in concentrations of reaction components during the GOS synthesis process was found to be incompatible.

When examining Model B (*Figure 3v*), it can be noted that there was an overlap for lactose, galactose, and GOS only until 100 minutes between the experimental and corresponding fitted data. However, this trend collapsed after this time. On the other hand, there was a marked difference between experimental and simulated data of glucose, as indicated by the increase in weighted errors over the reaction time (*Figure 3v*). The graph of

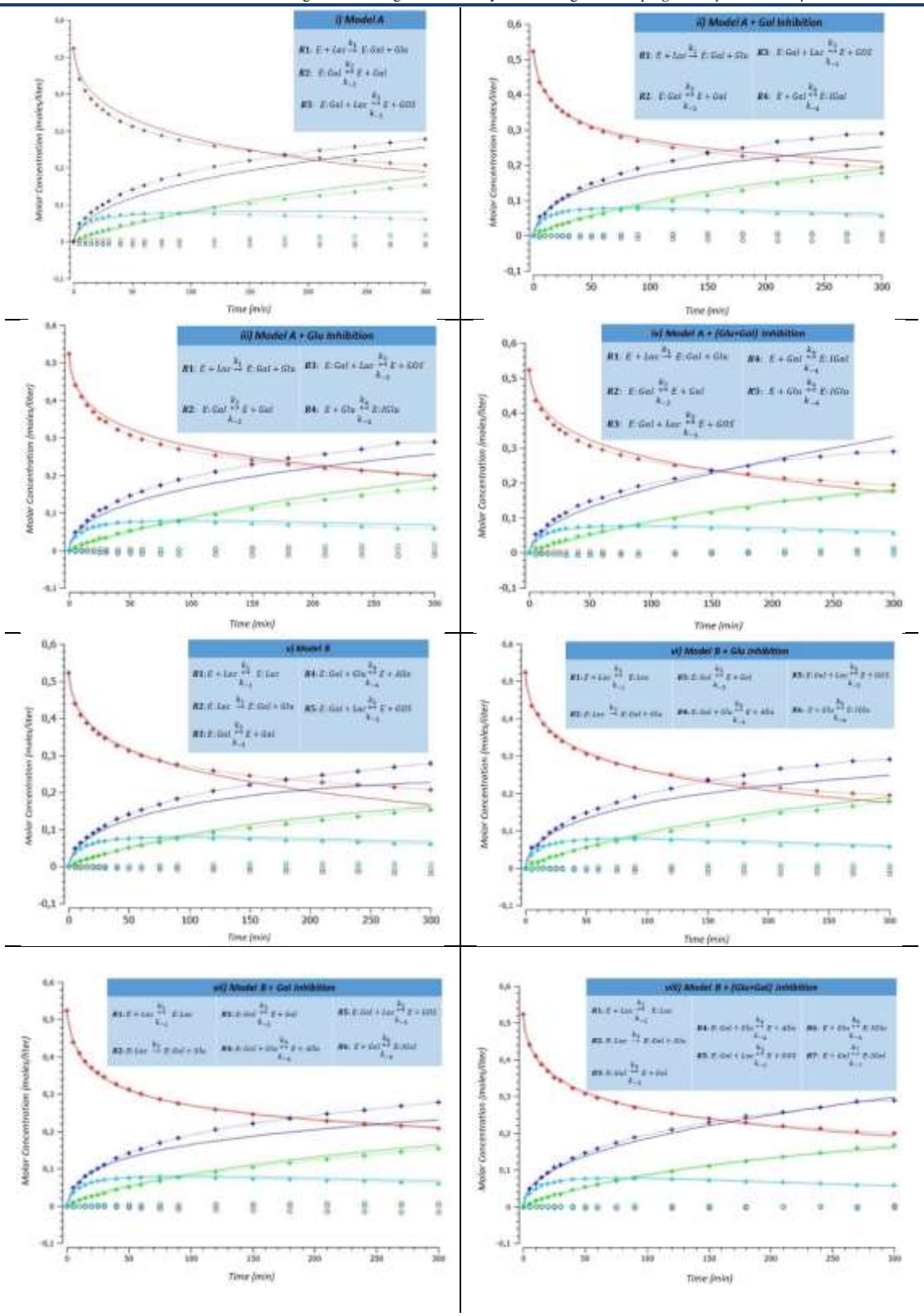

Figure 3. Schematic representations of experimental values and simulated values of eight models (- [GOS] Fitted Value, + [GOS] Measured Value, O [GOS] Weighted Value, - [Gal] Fitted Value, + [Gal] Measured Value, O [Gal] Weighted Value, - [Glu] Fitted Value, + [Glu] Measured Value, O [Glu] Weighted Value, - [Lac] Fitted Value, + [Lac] Measured Value, O [Lac] Weighted Value) (Reaction conditions: temperature (45°C), lactose concentration (18.25°Brix), and enzyme concentration (10 U g lactose solution^{-1}).

Model B, including glucose inhibition (*Figure 3vi*), indicated that although the fitness of lactose extended over 130 minutes, the same arguments for that of Model B (*Figure 3v*) were also valid. However, the addition of only galactose inhibition to Model B demonstrated a perfect fitness for lactose between the experimental and corresponding fitted data (*Figure 3vii*). Also, the fitness of galactose and GOS could be accepted as tolerable for a 300-minute reaction time, since the weighted errors of them were close to zero. However, the fitness of glucose broke the compatibility of the model, resulting in large residuals during the process (*Figure 3vii*). When the inhibition reactions of glucose and galactose were added to Model B (*Figure 3viii*), this new model provided optimal estimations for all of the reaction components. The fitness of lactose, glucose, galactose, and GOS was acceptable. The predicted individual fittings of galactose, GOS, and especially glucose showed the best matching with the experimental measurements throughout the reaction. This can be inferred from the data of weighted errors of reaction components, each of which was almost zero. Therefore, it becomes evident that the graph identified as "viii) Model B + glucose and galactose inhibition" in Figure 3 exhibits the closest alignment between experimental and simulated data.

The differential equations (Eq. 3-12) presented below illustrate the time-dependent alterations in each element within this seven-step model, where terms in square brackets denote the molar concentration of each element. The kinetic parameters in the reaction model describing GOS synthesis and lactose hydrolysis are expressed in the following units: k_1, k_{-3}, k_4, k_{-4}, k_5, k_{-5}, k_6, and k_7 have units of L moles^{-1} s^{-1}, while k_{-1}, k_2, k_3, k_{-6}, and k_{-7} have units of s^{-1}.

$$\frac{d[Lac]}{dt} = -k_1[E][Lac] + k_{-1}[E:Lac] - k_5[E:Gal][Lac] + k_{-5}[E][GOS] \quad \text{(Eq. 3)}$$

$$\frac{d[E]}{dt} = -k_1[E][Lac] + k_{-1}[E:Lac] + k_3[E:Gal] - k_{-3}[E][Gal] + k_4[E:Gal][Glu] - k_{-4}[E][Allo] + k_5[E:Gal][Lac] - k_{-5}[E][GOS] - k_6[E][Glu] + k_{-6}[E:IGlu] - k_7[E][Gal] + k_{-7}[E:IGal] \quad \text{(Eq. 4)}$$

$$\frac{d[E:Gal]}{dt} = k_2[E:Lac] - k_3[E:Gal] + k_{-3}[E][Gal] - k_4[E:Gal][Glu] + k_{-4}[E][Allo] - k_5[E:Gal][Lac] + k_{-5}[E][GOS] \quad \text{(Eq. 5)}$$

$$\frac{d[Glu]}{dt} = k_2[E:Lac] - k_4[E:Gal][Glu] + k_{-4}[E][Allo] - k_6[E][Glu] + k_{-6}[E:IGlu] \quad \text{(Eq. 6)}$$

$$\frac{d[Gal]}{dt} = k_3[E:Gal] - k_{-3}[E][Gal] - k_7[E][Gal] + k_{-7}[E:IGal] \quad \text{(Eq. 7)}$$

$$\frac{d[GOS]}{dt} = k_5[E:Gal][Lac] - k_{-5}[E][GOS] \quad \text{(Eq. 8)}$$

$$\frac{d[Allo]}{dt} = k_4[E:Gal][Glu] - k_{-4}[E][Allo] \quad \text{(Eq. 9)}$$

$$\frac{d[E:Lac]}{dt} = k_1[E][Lac] - k_{-1}[E:Lac] - k_2[E:Lac] \quad \text{(Eq. 10)}$$

$$\frac{d[E:IGlu]}{dt} = k_6[E][Glu] - k_{-6}[E:IGlu] \quad \text{(Eq. 11)}$$

$$\frac{d[E:IGal]}{dt} = k_7[E][Gal] - k_{-7}[E:IGal] \quad \text{(Eq. 12)}$$

This particular model that provide optimal fittings; accounts for the competitive inhibition of galactose and glucose, addresses the hydrolysis of GOS, treats the formation of glucose and galactose separately, represents all oligosaccharides with four chains, five chains, or more as GOS, assumes the irreversibility of the breakdown of the enzyme-lactose complex into the enzyme-galactose complex, disregards diffusional constraints, functions as either a substrate or glycosyl acceptor depending on lactose concentration, involves glucose reacting with the E:Gal complex to produce glucose-galactose disaccharides (allolactose), disregards the thermal deactivation of the enzyme. This study demonstrated that glucose and galactose inhibitions are important step for GOS synthesis mechanism. The primary reason for this phenomenon is believed to be the increased concentration of monosaccharides, especially galactose, exerting an inhibitory effect on the enzyme as the reaction progress. Indeed, galactose has been indicated as a competitive inhibitor in the literature in many studies on lactose hydrolysis reactions (Freitas et al., 2011; Rodriguez-Fernandez et al., 2011; Yin et al., 2017). Vera et al. (2011) have reported that the inhibitory effect of galactose is significantly stronger in transgalactosylation reactions

compared to lactose hydrolysis. Galactose is recognized as a competitive inhibitor for the enzyme's active site, competing with lactose (Hsu et al., 2007). This competitive inhibition is known to greatly slow down the enzyme's actions, leading to a decrease in reaction rate, particularly in reactions involving high lactose conversions (Iqbal et al., 2023; Sanz-Valero, 2009).

While some researchers have reported that glucose does not function as an inhibitor in the reaction kinetics of *A. oryzae*-derived β-galactosidases (Mueller et al., 2018; Shin and Ji-Won, 1998), others have explained the inhibitory effects of glucose (Palai et al., 2012; Vera et al., 2011). Neri et al. (2009) have expressed that the presence of free galactose in the reaction medium reduces the lactose consumption rate of β-galactosidase obtained from *A. oryzae* more than the presence of glucose and lowers the maximum GOS yield both individually and in combination with both glucose and galactose. The release of glucose in the reaction medium is inevitable during transgalactosylation. Therefore, it is imperative to remove the free galactose and glucose in the reaction medium to achieve a higher yield of GOS. The development of new strategies and approaches to alleviate the inhibitory effect of monosaccharides will further enhance the synthesis performance of GOS. Also, this study revealed that inhibitions of monosaccharides are important reaction steps for determination of appropriate kinetic reaction model for the synthesis of galactooligosaccharides catalysed by β-galactosidase from *A. oryzae.*

4. Conclusions

After rigorous analysis and comparison, it was found that Model B, enhanced with glucose and galactose inhibitions, demonstrated the closest agreement between experimental and simulated data. The proposed model effectively accounted for both lactose hydrolysis and GOS synthesis, providing a comprehensive understanding of the reaction pathway. The study highlighted the complexity of the GOS synthesis mechanism, where transgalactosylation and hydrolysis reactions occur simultaneously, necessitating a balance between model accuracy and feasibility. The incorporation of glucose and galactose inhibitions in Model B proved crucial in improving the model's ability to predict GOS synthesis efficiency under specified reaction conditions. Furthermore, the research emphasized the importance of understanding the kinetic mechanism of *A.oryzae*-derived β-galactosidase, considering its significant influence on factors such as conversion of lactose, productivity, and composition of product. The inhibitory effects of monosaccharides were identified as key factors influencing the enzyme's performance, leading to the development of a more accurate and reliable kinetic model. The adapted Model B with glucose and galactose inhibitions emerges as a valuable tool for predicting GOS yield at different time points during the reaction process. The findings contribute to the advancement of knowledge in enzymatic GOS synthesis and provide insights that can guide further optimization strategies for enhancing GOS production efficiency.

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Ethical Statement

There is no need to obtain permission from the ethics committee for this study.

Conflicts of Interest

We declare that there is no conflict of interest between us as the article authors.

Authorship Contribution Statement

Concept: Cinar, K., Gulec, H.A., Gunes, G.; Design: Cinar, K., Gulec, H.A.; Data Collection or Processing: Cinar, K..; Statistical Analyses: Cinar, K.; Literature Search: Cinar, K., Gulec, H.A.; Writing, Review and Editing: Cinar, K., Gulec, H.A., Gunes, G.

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