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Modeling of enzyme-catalysed processes: galacto-oligosaccharide formation and NAD(P)H oxidation as examples

Dissertation

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ABSTRACT

Ngoc Hung **Pham**. University of Natural Resources and Life Science; Vienna. 2015. **Modeling of enzyme-catalysed processes: galacto-oligosaccharide formation and NAD(P)H oxidation as examples**.

Supervisor: Univ.Prof. Dipl.-Ing. Dr.techn. Dietmar Haltrich

The aim of this research is to mathematically model enzymatic processes that are used in biocatalysis. This thesis comprises models for two different enzymatic reactions. The first model was used to describe lactose hydrolysis and transgalactosylation by β-galactosidase. The data used in this model were obtained in batch experiments in the previous work with β-galactosidase from two strains of *Lactobacillus reuteri,* L103 and L461; at various initial lactose concentrations ranging from 103 to 205 g L^{-1} and at temperatures of 25, 30 and 37^{0}C. The second model was used to describe an enzymatic regeneration system for NAD(P)H oxidation. This model also was used to simulate and optimize the employed enzyme, coenzyme and redox mediator concentrations.

The first model describes a single-enzyme process for lactose conversion by both hydrolysis and transgalactosylation reactions. In this model, a procedure was developed to fit the model parameters and to select the most suitable model. Each experiment was considered as an independent estimation of the model parameters, and consequently, model parameters were fitted to each experiment separately. The estimation of the parameters for each individual experiment preserved the time dependence of the set of measurements obtained by the experiment. The software package MATLAB was used in the numerical calculations. The parameters were estimated by the nonlinear least squares method with Genetic Algorithm until minimal error was achieved between experimental and calculated values. Each optimization simulation was run for 200 generations, with 100 as the population size of each generation.

The second model describes a bi-enzymatic process including an enzymatic regeneration system for NAD(P)H oxidation. The coenzyme regeneration system employs laccase and a number of various redox mediators to oxidize NAD(P)H. Reaction engineering by modeling was used to optimize the employed enzyme, coenzyme and redox mediator concentrations. Glucose dehydrogenase from *Bacillus* sp. served as a convenient example of the synthetic enzymes that depends either on NAD^+ or $NADP^+$. The capability of laccase from *Trametes pubescens* in combination with acetosyringone or syringaldazine as redox mediator was tested to regenerate (oxidize) the coenzymes. In a first step, pH profiles and catalytic constants of laccase for the redox mediators were determined. Then, second-order rate constants for the oxidation of NAD(P)H by the redox mediators were measured. In a third step, the rate equation for the whole enzymatic process was derived and used to build a Matlab model. After verifying the agreement of predicted vs. experimental data, the model was used to calculate different scenarios with varied concentrations of regeneration system components. The modeled processes were experimentally tested and the results compared to the predictions. It was found that the regeneration of NADH to its oxidized form was performed very efficiently, but that an excess of laccase activity leads to high concentrations of the oxidized form of redox mediator – a phenoxy radical - which presumably results in a coupling reaction (dimerization or polymerization) and enzyme deactivation.

ZUSAMMENFASSUNG

Ngoc Hung **Pham**. Universität für Bodenkultur Wien. 2015.

Modellierung von enzymkatalysierten Prozessen: Bildung von Galacto-Oligosacchariden sowie Oxidation von NAD(P)H als Beispiele

Betreuer: Univ.Prof. Dipl.-Ing. Dr.techn. Dietmar Haltrich

Die Zielstellung dieser Arbeit ist die mathematische Modellierung von Prozessen, die in der Biokatalyse eingesetzt werden und auf Enzymen basieren. Diese Dissertation beinhaltet Modelle für zwei verschiedene enzymatische Reaktionen: Das erste Modell wurde verwendet um die Hydrolyse sowie die Transgalactosylierung von Lactose durch β-Galactosidase zu beschreiben. Die Daten, auf denen das Modell basiert, stammen von Batch-Experimenten aus einer vorhergehenden Arbeit, in der β-Galactosidasen aus zwei Stämmen von *Lactobacillus reuteri* (L103 und L461) verwendet wurden. Die Experimente umfassten unterschiedliche Ausgangskonzentrationen von Lactose (103 bis 204 g L^{-1}) und Temperaturen (25, 30 und 37°C). Das zweite Modell wurde verwendet, um ein enzymatisches Regenerationssystem für die NAD(P)H Oxidation zu beschreiben. Dieses Modell wurde auch dazu verwendet, die eingesetzten Konzentrationen an Enzym, Coenzym und Redoxmediator zu simulieren und optimieren.

Das erste Modell beschreibt einen monoenzymatischen Prozess für die Lactose-Umsetzung durch Hydrolyse- und Transgalactosylierungsreaktionen. Dieses Modell wurde entwickelt, um die Modellparameter zu fitten und die jeweils passende Geschwindigkeitgleichung auszuwählen. Jedes Experiment wurde als unabhängige Abschätzung der Modellparameter betrachtet, wodurch die Modellparameter einzeln an jedes Experiment gefittet wurden. Da die Parameter für jedes Experiment einzeln abgeschätzt wurden, konnte die Zeitabhängigkeit der Datensätze, die im Verlauf des Experiments erhalten wurden, garantiert werden. Das Softwarepaket MATLAB wurde für numerische Berechnungen verwendet. Die Parameter wurden durch die nichtlineare

Methode der kleinsten Quadrate mit einem Genetischen Algorithmus, abgeschätzt bis ein minimaler Fehler zwischen experimentellen und berechneten Werten erhalten wurde. Jede Optimierungssimulation wurde 200 Generationen lang propagiert, wobei die Populationsgröße jeder Generation 100 war.

Das zweite Modell wurde für einen bienzymatischen Prozess entwickelt und beschreibt ein enzymatisches Regenerationssystem für die Oxidation von NAD(P)H. Durch die kinetische Charakterisierung der einzelnen Reaktionsschritte wurde ein kürzlich postuliertes Coenzym-Regenerationssystem näher untersucht, das Laccase und verschiedene Redoxmediatoren für die Oxidation von NAD(P)H beinhaltet. Die Konzentrationen von eingesetztem Enzym, Coenzym und Redoxmediator wurden mittels Reaction-Engineering optimiert. Glucose Dehydrogenase von *Bacillus* sp. diente als geeignetes Testsystem des synthetischen Enzyms, dass entweder von NAD^+ oder $NADP^+$ abhängt. Laccase aus *Trametes pubescens* in Kombination mit Acetosyringon oder Syringaldazin als Redoxmediator wurde auf ihre Fähigkeit untersucht das Coenzym zu regenerieren (oxidieren). Als Erstes wurden pH-Profile und die katalytischen Konstanten von Laccase mit den Redoxmediatoren bestimmt. Anschließend wurden die Geschwindigkeitskonstanten zweiter Ordnung für die Oxidation von NAD(P)H durch die Redoxmediatoren gemessen. In einem dritten Schritt wurde die Ratengleichung für den ganzen enzymatischen Prozess abgeleitet um letztlich ein Modell in MATLAB zu bilden. Nachdem die die Übereinstimmung zwischen vorhergesagten und experimentellen Daten verifiziert wurde, wurde das Modell dazu verwendet verschiedene Szenarien in Bezug auf unterschiedliche Konzentrationen von Komponenten des Regenerationssystem zu berechnen. Es konnte gezeigt werden, dass die Regenerierung von NADH zu seiner oxidierten Form sehr effizient war und dass ein Überschuss an Laccase-Aktivität zu hohen Konzentrationen an oxidiertem Redoxmediator führt – ein Phenoxy-Radikal, dass vermutlich zu einer Kopplungsreaktion und letztlich zur Enzyminaktivierung führt.

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I am grateful to my advisor, Professor Dietmar Haltrich, for his guidance, patience, advice, and generosity that he has provided me. This dissertation would not be possible without his support and dedication. I have gained a lot from his insights and I have greatly enjoyed my staying here as his student.

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A special thanks to my family. Words cannot express how grateful I am to my parents, my sister, for all of the sacrifices that you've made on my behalf. At the end, I would like express appreciation to my beloved wife Thu-Trang and my daughter Ngoc-Linh. Thank you!

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Ngoc Hung Pham

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NOMENCLATURE

eff	efficiency
k_d	deactivation-kinetic constant (h^{-1})
k_i	kinetic parameters (i=±1; ±2; ±3; ±4..)
K_I	inhibition constant (µM)
K_M	Michaelis-Menten constant (µM)
r	reaction rate (s^{-1})
r_2	second-order rate constant (M^{-1} s^{-1})
r_{hyd}	rate of D-glucono-1,5-lactone hydrolysis (s^{-1})
RM	redox mediator
t	time (h)
v_{max}	maximum enzymatic rate at infinite substrate concentration (U mL^{-1})

Greek symbol

ε	molar absorption coefficient (M^{-1} cm^{-1})

Abbreviations

GA	gluconic acid
GAL	Galactose
GL	D-glucono-1,5-lactone
GLC	D-glucose
GOS	Galacto-oligosaccharides
LAC	Lactose
NAD(P)H	nicotinamide adenine dinucleotide (phosphate)
O_2	molecular oxygen
STY	space-time yield (mM h^{-1})

1 Introduction

1.1 β-Galactosidases [1]

β-Galactosidases catalyze the hydrolysis and transgalactosylation of β-D-galactopyranosides (such as lactose) [1], [2] and are found widespread in nature. They catalyze the cleavage of lactose (or related compounds) in their hydrolysis mode, and are thus used in the dairy industry to remove lactose from various products. An attractive biocatalytic application is found in the transgalactosylation potential of these enzymes, which is based on their catalytic mechanism [3], [4]. Retaining β-galactosidases undergo a two-step mechanism of catalysis. First, this mechanism involves the formation of a covalently linked galactosyl-enzyme intermediate. Subsequently, the galactosyl moiety linked to the nucleophile is transferred to a nucleophilic acceptor. Water, as well as all sugar species present in the reaction mixture, can serve as a galactosyl acceptor. Hence, the resulting final mixture contains hydrolysis products of lactose, which are glucose and galactose, unconverted lactose as well as di-, tri- and higher oligosaccharides [5], [6]. **Figure 1** illustrates the possible lactose conversion reactions catalysed by β-galactosidases.

Transgalactosylation is described to involve intermolecular as well as intramolecular reactions. Intramolecular or direct galactosyl transfer to D-glucose yields regio-isomers of lactose. The glycosidic bond of lactose (β-D-Gal*p*-(1→4)-D-Glc) is cleaved and immediately formed again at a different position of the glucose molecule before it diffuses out of the active site. This is how allolactose (β-D-Gal*p*-(1→6)-D-Glc), the presumed natural inducer of β-galactosidases in certain microorganisms, can be formed even in the absence of

[1] Part of the following publications:

(1) Arreola, S. L.; Intanon, M.; **Pham, N. H.;** Haltrich, D.; and Nguyen, T.-H. Galacto-Oligosaccharides: Recent Progress on Research and Application As Prebiotics. *In Galactose: Structure and Function in Biology and Medicine*; Pomin, V. H., Ed.; Nova Science Publishers: New York, 2014;

(2) Intanon, M., Arreola, S. L., **Pham, N. H.,** Kneifel, W., Haltrich, D., and Nguyen, T.-H. Nature and biosynthesis of galacto-oligosaccharides related to oligosaccharides in human breast milk. *FEMS microbiology letters* 2014, 353, 89–97.

significant amounts of free D-glucose [5], [7]. By intermolecular transgalactosylation, various di-, tri-, tetrasaccharides and eventually higher oligosaccharides are produced. Any sugar molecule in the reaction mixture can be the nucleophile accepting the galactosyl moiety from the galactosyl-enzyme complex, which is formed as an intermediate in the reaction. The GOS produced are not the products of an equilibrium reaction, but must be regarded as kinetic intermediates as they are also substrates for hydrolysis, and hence transgalactosylation reactions are kinetically controlled [7], [8]. For these reasons GOS yield and composition change dramatically with reaction time, and the GOS mixtures thus obtained are very complex and can hardly be predicted.

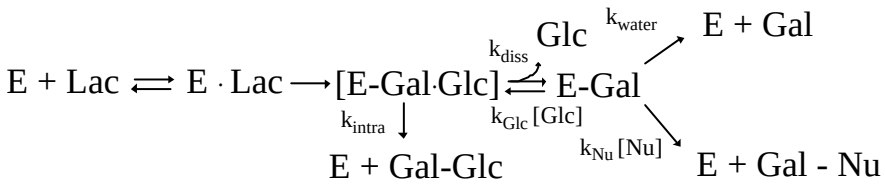

Figure 1. Hydrolysis and galactosyl transfer reactions, during the conversion of lactose catalysed by β-galactosidases. E, Enzyme; Lac, lactose; Gal, galactose; Glc, glucose; Nu, nucleophile

β-Galactosidases can be obtained from different sources including microorganisms, plants and animals. Microbial sources of β-galactosidase are of great biotechnological interest because of easier handling, higher multiplication rates, and production yield. **Table 1** presents some of the commercially available bacterial, fungal and yeast β-galactosidases.

Recently, a number of studies have focused on the use of the genera *Bifidobacterium* and *Lactobacillus* for the production and characterisation of β-galactosidases, including the enzymes from *L. reuteri, L. acidophilus, L. plantarum, L. sakei, L. pentosus, L. bulgaricus, L. fermentum, L. crispatus*, *B. infantis, B. bifidum, B. angulatum, B. adolescentis,* and *B. pseudolongum and B. breve* [9]–[21]. Bifidobacteria and lactobacilli have been studied intensively with respect to their enzymes for various different reasons, one of which is their 'generally recognized as safe' (GRAS) status and their safe use in food applications. It is

anticipated that GOS produced by these β-galactosidases will have better selectivity for growth and metabolic activity of these bacterial genera in the gut, and thus will lead to improved prebiotic effects [22]. An extensive list of bacterial and fungal sources of β-galactosidases, as well as the lactose conversion reaction conditions and GOS yields, are given in [23].

Table 1. Commercial β-galactosidases

Name	Manufacturer	Microorganism
BioLactase NTL-CONC	Biocon	*Bacillus circulans* [24], [25]
Lactozym pure 6500 L	Novozymes	*Kluyveromyces lactis* [25]
Lactase F "Amano"	Amano Enzyme Inc	*Aspergillus oryzae* [25], [26]
Biolacta FN5	Daiwa Fine Chemicals Co., Ltd.	*Bacillus circulans* [26], [27]
LACTOLES L3	Biocon Ltd., Japan	*Bacillus circulans* [26]
Maxilact	DSM Food Specialties	*Kluyveromyces lactis* [28]
Tolerase	DSM Food Specialties	*Aspergillus oryzae* [28]

Studies of thermostable glycosyl hydrolases have been conducted in pursuit of GOS production at high temperatures. These include β-glycosidases from *Pyrococcus furiosus* (F426Y), *Thermotoga maritima*, *Penicillum simplicissimum*, *Saccharopolyspora rectivirgula, Aspergillus niger*, *Bifidobacterium bifidum*, *Bacillus stearothermophilus*, *Alicyclobacillus acidocaldarius*, *Thermus aquaticus* YT-1, *Thermus thermophilus* KNOUC202, and *L. bulgaricus,* to name a few [29]–[34]. The GOS yields as influenced by transgalactosylation of lactose at different temperatures were given in recent reviews [23], [35]. Cold-active β-galactosidases have also attracted attention because their applications in the industrial processes of lactose hydrolysis and oligosaccharides synthesis can lower the risk of mesophiles contamination. Cold-active β-galactosidases were isolated from *P. haloplanktis* TAE 79, *Planococcus* sp., *Arthrobacter psychrolactophilus*, *Arthrobacter* sp. 32c,

Alkalilactibacillus ikkense, *Paracoccus* sp. 32d, *Halorubrum lacusprofundi,* and *Thalassospira frigidphilosprofundus* [36]–[42] and have been reported for their potential use to hydrolyze lactose in dairy products processed at low temperatures. Soluble cold-active β-galactosidases from *Paracoccus* sp. 32d and *Lactococcus lactis* IL 1403 were found to efficiently hydrolyse lactose in milk at 10℃ [41], [42].

It is a well-known fact that β-galactosidases from different species possess very different specificities for building glycosidic linkages, and therefore produce different GOS mixtures. For example, the β-galactosidase from *Kluyveromyces lactis* produced predominantly β-(1→6)-linked GOS [74], the β-galactosidase from *Aspergillus oryzae* produced mainly β-(1→3) and β-(1→6) linkages in their GOS [43], *Bacillus circulans* β-galactosidase forms β-(1→2), β-(1→3), β-(1→4), β-(1→6) linked GOS [76], whereas β-galactosidases from *Lactobacillus* spp. showed preference to form β-(1→3) and β-(1→6) linkages in transgalactosylation mode [7], [10], [13], [29].

1.2 Galacto-oligosaccharides [2]

Galacto-oligosaccharides (GOS), the products of transgalactosylation reactions catalyzed by β-galactosidases when using lactose as the substrate, are non-digestible carbohydrates meeting the criteria of 'prebiotics'. GOS are of special interest to human nutrition because of the presence of structurally related oligosaccharides together with different complex structures in human breast milk [44]–[46].

Production of GOS (or sometimes referred to as TOS, transgalactosylated oligosaccharides) typically employs lactose as galactosyl donor and the transfer of the galactosyl moiety of lactose to suitable acceptor carbohydrates or nucleophiles using either

[2] Part of the following publications:

(1) Arreola, S. L.; Intanon, M.; **Pham, N. H.;** Haltrich, D.; and Nguyen, T.-H. Galacto-Oligosaccharides: Recent Progress on Research and Application As Prebiotics. *In Galactose: Structure and Function in Biology and Medicine*; Pomin, V. H., Ed.; Nova Science Publishers: New York, 2014;

glycoside hydrolases (EC 3.2.1.) or glycosyltransferases (EC 2.4.) [47], [48]. Glycosyltransferases catalyze glycosidic bond formation employing sugar donors containing a nucleoside phosphate or a lipid phosphate leaving group, and are quite efficient as well as regio- and stereo-selective compared to glycoside hydrolases. However, due to limited supply, high price and necessity of specific sugar nucleotide as substrate of glycoside hydrolases, industrial GOS production favours the use of glycoside hydrolases [48].

Glycoside hydrolases (GH) are classified based on the stereochemical outcome of the hydrolysis reaction; they can be either *retaining* or *inverting* enzymes. Amino acid sequence similarities, hydrophobic cluster analysis, reaction mechanisms and the conservation of catalytic residues allow classification of β-galactosidases (β-gal; β-D-galactoside galactohydrolase E.C.3.2.1.23; lactase) in the GH families GH1, GH2, GH35, and GH42, indicating their structural diversity [47]. GH1 β-glycosidases are retaining enzymes of which the most commonly known enzymatic activities are myrosinases (thio-β-glucosidases), β-mannosidases, β-galactosidases, phospho-β-glucosidases and phospho-β-galactosidases. The GH2 family, to which most of the β-galactosidases belong, comprises the LacZ and LacLM β-galactosidases as isolated and described from *E. coli*, lactic acid bacteria and bifidobacteria. GH1 and GH2 β-galactosidases use only lactose, β-(1→3) and β-(1→6) linked galactosides as their substrates while those belonging to families GH35 and GH42 act on different galactose-containing glycosides including higher oligosaccharides and polysaccharides [47]. Owing to the different substrate specificities, β-galactosidases of GH2 and GH42 are often found in the same organism [49]–[51].

GOS are produced from lactose by microbial β-galactosidases employing different enzyme sources and preparations including crude enzymes, purified enzymes, recombinant enzymes, immobilized enzymes, whole-cell biotransformations, toluene-treated cells, and immobilized cells. The enzyme sources, the process parameters as well as the yield and the productivity of these processes for GOS production are summarised in detail in recent reviews [23], [35], [46], [52]. The highest GOS productivity, 106 g L^{-1} h^{-1}, was observed when

β-galactosidase from *Aspergillus oryzae* immobilized on cotton cloth was used for GOS production in a packed-bed reactor [53].

The choice of process technology either for lactose hydrolysis or GOS production depends on the nature of the substrate and the characteristics of the enzyme. The primary characteristic, which determines the choice and application of a given enzyme, is the operational pH range. Acid-pH enzymes, which are mainly from fungi, are suitable for processing of acid whey and whey permeate, while the neutral-pH enzymes from yeasts and bacteria are suitable for processing milk and sweet whey. Depending on the enzyme source, the pH value of the reaction mixture can be very acidic when using β-galactosidases from *A. oryzae* and *Bullera singularis* with optimum GOS yields at pH 4.5 and 3.7, respectively [54], [55]. Isobe and others studied the β-galactosidase from an acidophilic fungus, *Teratosphaeria acidotherma* AIU BGA-1, which was stable over the pH range of 1.5 to 7.0 and exhibited optimal activity at pH 2.5-4.0 and 70℃ [56]. The maximum yield of GOS was observed at neutral pH for most bacteria and fungi though [15]. The time required to get maximum GOS depends inversely on the amount of enzyme. The highest GOS yields are generally observed when the reaction proceeds to 45 - 90% lactose conversion [23].

1.3 The development of kinetic models

1.3.1 Enzymatic transgalactosylation and hydrolysis of lactose by β-galactosidase

Several kinetic mechanisms, either mechanistic, empirical or a combination of both, have been proposed to account for the transgalactosylation and lactose hydrolysis reactions and to subsequently define strategies to optimize the space-time yield for production. Most of kinetic models have been proposed based on the Michaelis-Menten equation [57]–[60], which describes the rate of product formation, as defined by the steady-state substrate affinity constant (K_M), and the maximum velocity for substrate conversion to product (v_{max})

[61] . Some kinetic models for enzymatic hydrolysis of lactose in the literatures are summarized in **Table 2**.

Table 2. Some kinetic models for enzymatic hydrolysis of lactose

Kinetic model proposed	References
Michaelis–Menten without inhibition by product (with enzyme–lactose complex formation)	[62]–[64]
Michaelis–Menten with competitive inhibition by product (total galactose)	[59], [63], [65]
Michaelis–Menten with non- competitive inhibition by product (total galactose)	[63]
Michaelis–Menten with competitive inhibition by product (glucose)	[59]
Di-, tri- and tetra-saccharides formation	[66]

The following models (model 1-7) were investigated in more detail. The models 1-3 were used to describe enzymatic hydrolysis of lactose. The models 4-7 was developed to describe oligosaccharide production from lactose hydrolysis by β-galactosidase. Based on these models, a model proposed for this research will be described in the section 2.1.4.

Model 1:

Michaelis–Menten without inhibition by product (galactose or glucose) was used to describe the model of lactose hydrolysis by β-galactosidase from *K. lactis* [64], [67], [68]. This standard model is obtained by the following equation:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (E{:}S) \overset{k_2}{\rightarrow} P + Q \quad (1)$$

$$r = -\frac{dS}{dt} = \frac{k_2 E_0 [S]}{K_m + [S]} \quad (2)$$

Where, *S, P, Q, E, and E:S* are lactose, galactose, glucose, enzyme and enzyme-lactose complex, respectively. k_1, k_{-1}, k_2, are primary reaction rate constants, E_0 is the initial enzyme concentration, *r* is the volumetric reaction rate and *t* is the reaction time. The

Michaelis-Menten constant K_m *is defined as* $(k_{-1}+k_2)/k_1$; $v_{max} = k_2.E_0$ is the maximum reaction rate.

Model 2:

Michaelis-Menten kinetics with competitive product inhibition by galactose has been widely used to study the kinetics of lactose hydrolysis. The products are instantaneously formed under the effect of the enzyme – substrate complex.

$$LAC + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (E{:}S) \overset{k_2}{\rightarrow} E + P + Q \quad (3)$$

$$(EP) \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E+P \quad (4)$$

$$r = -\frac{dS}{dt} = \frac{k_2 E_0 [S]}{K_m\left(1 + \frac{[P]}{K_i}\right) + [S]} \quad (5)$$

Assuming that the process described by Eq. (4) rapidly reaches its equilibrium state, and the products glucose and galactose are roughly similar ($[P] \approx [Q]$), the reaction rate can be calculated by Eq. (5), where $K_i = k_3/k_{-3}$ is the inhibition constant. The equations (3) and (4) indicate that glucose has no effect on the lactose hydrolysis reaction.

Jurado [69] also proposed this model with the assumption that $K_m = K_i$ for enzymatic hydrolysis of lactose by a β-galactosidase from *Kluyveromyces fragilis*.

Model 3:

Yang and Okos [57] presented Michaelis-Menten kinetics with competitive product inhibition by galactose and supposed that β-galactosidase-catalyzed reactions proceed through a chemical intermediate that occurs simultaneously with the liberation of the first product of the reaction. When lactose is the substrate, a galactosyl enzyme is one intermediate and glucose is the first product released from the hydrolysis reaction. The reaction mechanism is described by the following equations

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (E{:}S) \overset{k_2}{\rightarrow} E\text{-}P + Q \quad (6)$$

$$(E\text{-}P) \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + P \quad (7)$$

where *E-P* is galactosyl-enzyme complex; k_1, k_{-1}, k_2, k_3, k_{-3} are primary reaction rate constants.

Using the steady-state approximation, and suppose that $[P] \approx [Q]$, then the reaction rate can be calculated by:

$$r = -\frac{dS}{dt} = \frac{k_2 E_0 [S]}{K_m \left(1 + \frac{[P]}{K_i}\right) + \left(1 + K_p\right)[S]} \quad (8)$$

where $K_p = k_2/k_3$ is the rate constant for product. When $k_2 << k_3$ or $K_p << 1$, the reaction rate in equation (8) and in equation (5) are identical. This model indicates that glucose is released from the enzyme-substrate complex first, leaving the enzyme-galactose complex for further hydrolysis. This model also indicates that glucose has no effect on the reaction rate.

Model 4

It is well known that the enzymatic hydrolysis of lactose occurs at low lactose concentrations. Oligosaccharide conversion increased by transgalactosylation reaction on increasing the initial lactose concentration [54]. The reaction mechanism below describes galacto-oligosaccharide synthesis as well as simultaneous lactose hydrolysis while still including product inhibition and was proposed by Shou et al [70].

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (E{:}S) \overset{k_2}{\rightarrow} E\text{-}P + Q \quad (9)$$

$$(E\text{-}P) \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + P \quad (10)$$

$$S + E\text{-}P \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} (E{:}T) \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} E + T \quad (11)$$

where *T* and *E:T* are trisaccharides and the noncovalent enzyme–trisaccharide complex, respectively. In this model, lactose acts as both a substrate and an acceptor, while glucose is not used as an acceptor. Assuming the reactions described by Eqs. (9) and (10) are rapidly equilibrated, where the equilibrium constants are $K_i = K_3 = k_3/k_{-3}$, $K_4 = k_4/k_{-4}$ and $K_5 = k_5/k_{-5}$ and trisaccharides concentration is:

$$[T] = a[P][S] \tag{12}$$

where the constant $a = K_3K_4K_5 = k_3k_4k_5/\ k_{-3}k_{-4}k_{-5}$. The rate expression is:

$$r = -\frac{dS}{dt} = \frac{k_2 E_0 [S]}{K_m\left(1 + \frac{[P]}{K_i} + \frac{[T]}{K_T}\right) + [S]} \tag{13}$$

where $K_T = K_4K_5$ is inhibition constant for trisaccharides.

Model 5

A model has been developed by Kim *et al.* [71], in which lactose acts as both a substrate and a poorer glycosyl acceptor; but, at high concentrations, it has more chances to be the acceptor to make trisaccharides. Galactose binds to the free enzyme to make the galactosyl–enzyme complex for further transgalactosylation reactions with glucose or lactose as the acceptors, but does not bind to the galactosyl–enzyme complex. Glucose, however, acts as a better acceptor for transgalactosylation reactions, reacting only with the galactosyl–enzyme complex to form galactosyl–glucose disaccharides. The following equations were proposed:

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (E{:}S) \overset{k_2}{\rightarrow} E\text{-}P + Q \tag{14}$$

$$(E\text{-}P) \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + P \tag{15}$$

$$Q + E\text{-}P \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + D \tag{16}$$

$$S + E\text{-}P \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} E + T \tag{17}$$

where *D* is galactosyl–glucose disaccharides. Assuming that the reactions described by Eqs. (14)–(16) are rapidly equilibrated, the equilibrium constants for hydrolysis, disaccharides, and trisaccharides are $K_H = k_3/k_{-3}$, $K_D = k_4/k_{-4}$ and $K_T = k_5/k_{-5}$, respectively. The materials are conserved for the galactose moiety, $[S_0]-[S] = [P]+[D]+2[T]$, and for glucose moiety, $[S_0]-[S] = [P]+[D]+[T]$, where $[S_0]$ is the initial mole concentration of substrate. The reaction rate is:

$$r = -\frac{dS}{dt} = \frac{k_2 E_0 [S]}{K_m \left(1 + \frac{[P]}{K_H} + \frac{[D]}{K_D[G]} + \frac{[T]}{K_T[S]}\right) + [S]} \tag{18}$$

Model 6

Base on the model constructed by Kim *et al* [71], Palai proposed the six-step-eleven-parameter model. This model takes into account all the competitive reactions (inhibition by glucose and hydrolysis of GOS) and includes separate formations of glucose and galactose, and proposed dissociation of enzyme-lactose complex into enzyme-galactose complex as reversible reactions. The model is described by the following equations:

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (E{:}S) \tag{19}$$

$$(E{:}S) \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\text{-}P + Q \tag{20}$$

$$E\text{-}P \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + P \tag{21}$$

$$S + E\text{-}P \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E\text{-}GOS \tag{22}$$

$$E\text{-}GOS \overset{k_5}{\rightarrow} E + GOS \tag{23}$$

$$Q + E \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} E\text{-}Q \tag{24}$$

The model assumes the formations of tri-, tetra- and higher oligosaccharides into a single form of GOS.

Model 7

Vera *et al.* [72] proposed a model considering the synthesis of di (GOS-2), tri (GOS-3), tetra (GOS-4) and penta (GOS -5) saccharides with β-galactosidase and the estimation of its parameters by nonlinear multiresponse fitting. The model was described by the following equations:

$$S + Di \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EDi \overset{k_{cat}}{\rightarrow} EGal + GLC \quad (25)$$

$$EGal + Di \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EGalDi \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + Tri \quad (26)$$

$$EGal + Tri \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} EGalTri \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} E + Tet \quad (27)$$

$$EGal + Tet \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} EGalTet \underset{k_{-7}}{\overset{k_7}{\rightleftharpoons}} E + Pen \quad (28)$$

$$EGal + Gal \underset{k_{-8}}{\overset{k_8}{\rightleftharpoons}} EGalGal \overset{k_9}{\rightarrow} E + Di \quad (29)$$

$$EGal + H_2O \overset{k'_{cat}}{\rightarrow} E + Gal \quad (30)$$

$$E+Gal \overset{K_I}{\rightleftharpoons} EGal^* \quad (31)$$

This mechanism assumes that the enzyme is capable of producing transgalactosylated disaccharides formed by two galactose monomers, the enzyme does not discriminate between GOS-2 and lactose, the release of galactose to the reaction medium can be described as a first-order reaction with respect to the active galactosyl enzyme complex, the enzyme is competitively inhibited by galactose, and the effect of glucose on reaction kinetics is considered negligible.

By definition the dissociation constants as follows:

$$K_m = \frac{k_{-1} + k_{cat}}{k_1} \qquad K'_{mTri} = \frac{k_{-2} + k_3}{k_{-3}} \qquad K'_{mTet} = \frac{k_{-4} + k_5}{k_{-5}} \qquad K'_{mPen} = \frac{k_{-6} + k_7}{k_{-7}}$$

$$K_{mDi} = \frac{k_{-2} + k_3}{k_2} \qquad K_{mTri} = \frac{k_{-4} + k_5}{k_4} \qquad K_{mTet} = \frac{k_{-6} + k_7}{k_6} \qquad K_{mPen} = \frac{k_{-8} + k_9}{k_8}$$

then the reaction rate for each product can be obtained (equation not shown).

However, this model is proposed by assuming that the affinity of the free enzyme for different substrates can be represented by a unique average constant, and also a similar assumption was made for the affinity of the galactosyl-enzyme. Thus K_m, K'_{mTri}, K'_{mTet} and K'_{mPen} are close to each other. K_{mDi}, K_{mTri}, K_{mTet} and K_{mPen} are also approximately identical. This assumption has not been verified by an experiment.

The above kinetic mechanisms available in the literature have been proposed during the last two decades to account for the reactions of lactose hydrolysis and transgalactosylation. The model proposed by Boon [73], Iwasaki [54] or Kim [71] allow to define strategies of optimization the production of GOS. However, kinetic parameters of such mechanisms are difficult to estimate. The mechanism proposed by Boon [73] is one of the most frequently used. But this model only considers the production of trisaccharides. Besides, kinetic parameters are estimated using averaged values obtained by least square fitting of individual experimental data. A better model, which considers kinetics of each product [72] and estimates the affinity of the free enzyme and galactosyl-enzyme for different substrates, has been used in this work. In the present work, a simplified model will be proposed and described in detail in section 2.1.

1.3.2 Thermal stability of β-galactosidase

There are not many studies on the deactivation of β-galactosidase are available [74]–[77]. Deactivation mechanisms can be complex, since the enzymes have highly defined structures, and the slightest deviation in their native form can affect their specific activity. Better knowledge of enzyme stability under operating conditions could help to optimize the economic profitability of enzymatic processes [75].

The most widely used model describing the thermal deactivation of β-galactosidases is the first-order deactivation model:

$$(E) \xrightarrow{k_d} E_d \tag{32}$$

Where E is the concentration of native, active enzyme in the reaction medium, E_d is the concentration of deactivated enzyme and k_d is the deactivation kinetic constant. This model was used to simulate the deactivation of the enzyme activity in some literatures [57], [78]–[81]

Another serial deactivation model has been proposed to explain enzymatic deactivation. This model was described by [82]

$$(E) \xrightarrow{k_{d1}} (E_{d1}) \xrightarrow{k_{d2}} (E_{d2}) \tag{33}$$

This model considers two irreversible first-order steps and the presence of native enzyme (E) and modified enzymatic species (E_{d1},E_{d2}); k_{d1} and k_{d1} are the deactivation kinetic constants. This model has been applied to experimental results involving β-galactosidases by [77], [83].

Based on the model described in Eq. (33), Jurado et al. [75] also proposed another model in Eq. (34). In this model, the native enzyme (E) transforms into a non-active or less active damaged form (E_h) although it can transform again into the native enzyme. Afterwards E_h is denatured irreversibly to E_d.

$$(E) \underset{k_{h1i}}{\overset{k_{h1}}{\rightleftharpoons}} (E_h) \xrightarrow{k_{d2}} (E_d) \tag{34}$$

The model proposed in this work uses the model in equation (32) to describe the deactivation of enzyme.

1.4 Enzymatic Regeneration System for NAD(P)H Oxidation[3]

During the last years laccase/mediator systems have been proposed for the regeneration of NAD(P)+-dependent enzymatic processes in synthetic applications [84], [85]. These initial studies showed a high potential for up-scaling, but more detailed investigations

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are necessary to understand the strengths and weaknesses of these regeneration systems. In this work we studied the underlying principles for an efficient and stable enzymatic regeneration process of $NAD(P)^+$, which does not show the restrictions of alternative systems, e.g. electrochemical methods [86]. Coenzymes are costly [87] which makes them too expensive to employ more than the minimal amount that still guarantees fast conversion of the synthetic enzyme [88]. High costs have been an obstacle in the wider application of coenzyme-dependent oxidreductases, but this is also the strongest argument for applying efficient and economical coenzyme regeneration systems. Various methods such as chemical, biological, photochemical, electrochemical or enzymatic approaches have been suggested and reviewed for this purpose [89], [90]. Among them, the enzymatic methods seem to be the most convenient and useful. Such *in situ* regeneration reactions have been used in a number of oxidoreductase-catalyzed reactions, and some of them have been up-scaled to large-scale syntheses [84].

As suggested by Chenault and Whitesides [91], an ideal enzymatic regeneration system should meet the following criteria: (i) the enzymes should be inexpensive and stable, (ii) the enzymes should have high specific activity, (iii) simple and inexpensive reagents that do not interfere with the isolation of the product of interest or with enzyme stability should be employed, (iv) high turnover numbers should be obtained, (v) the total turnover number of the coenzyme should be at least between 10^2 and 10^4, and (vi) an overall equilibrium for the coupled enzyme system favorable to product formation should be reached. These criteria have been already partially met for NAD^+-reducing enzymes such as alcohol dehydrogenase, lactate dehydrogenase and glutamate dehydrogenase [90], [92], [93]. However, the enzymatic oxidation of NAD(P)H is not satisfactorily developed to date. The use of laccase for NAD(P)H oxidation seems to fulfil most of the postulated criteria: (i) Laccases are technical enzymes employed for decolorization or delignification processes, which can be produced recombinantly and inexpensively. (ii) Laccases, a member of the blue multicopper oxidase family, has a high specific activity for various substrates, which can reach up to several hundred per second. (iii) Most of the investigated redox mediators, which

typically are used in low concentrations, are inexpensive, but more work needs to be done on their removal from the product. Oxygen, the second substrate of laccase, can be easily provided to a biocatalytic process, and since water is produced by its reaction no purification of a by-product is required. (iv) It should be possible to obtain high turnover numbers for the coenzyme in a biocatalytic process when considering both the reported high stability and high specific activity of laccases, and (v) based on this high stability / high activity high total turnover numbers for the enzyme (laccase) should be achievable as well. (vi) The high redox potential of laccase of up to 800 mV vs. SHE allows to oxidize even redox mediators with high potentials [94], [95]. The high thermodynamic driving force of oxygen reduction makes processes irreversible and drives coenzyme-dependent reactions towards completion [85]. The ideal mediator in these reactions should be non-toxic, cheap and efficient, with stable oxidized and reduced forms that do not inhibit the enzymatic reaction [96].

Laccase/mediator systems have been reported to be applicable for NAD^+ regeneration [85], [97], [98] . The main advantages of such systems are high process stability, low co-substrate costs and tolerance towards co-solvents. Laccase substrates such as ABTS, Meldola's blue, acetosyringone, syringaldehyde, caffeic acid, *p*-coumaric acid, vanillin, acetovanillone, 3-hydroxyanthranilic acid, 4-hydroxybenzoic acid, hydroquinone, phenolsulfonphthalein [99] have been used as mediators, amongst others.

In this work a laccase from *Trametes pubescens* and acetosyringone are used as an enzyme/mediator system to regenerate the oxidized coenzyme $NAD(P)^+$ from NAD(P)H. Glucose dehydrogenase (GDH) from *Bacillus* sp. is here employed as the model synthesizing enzyme that uses the oxidized coenzyme $NAD(P)^+$, which is reduced to NAD(P)H, for the oxidation of D-glucose to D-glucono-1,5-lactone. The latter is further spontaneously hydrolysed to D-gluconic acid (**Figure 2**). Glucose oxidation catalysed by GDH is a popular model system, since it can use both NAD^+ and the phosphorylated form $NADP^+$ [100], [101]. The full rate equation of *Bacillus sp*. GDH, which is often applied for the regeneration of both NADPH and NADH, was recently elucidated [102]. Furthermore, modeling provides guidance in converting batch to continuous conversions as recently

demonstrated for lactobionic acid production [103]. Here we used modeling together with experimental approaches to obtain knowledge on enzyme and redox mediator stability under reaction conditions, as well as on the necessary activities of enzymes and minimum concentrations of redox mediator and coenzyme to design an efficient enzymatic process. Overall, we obtained detailed information on the strengths and possible limitations of the laccase/redox mediator regeneration system.

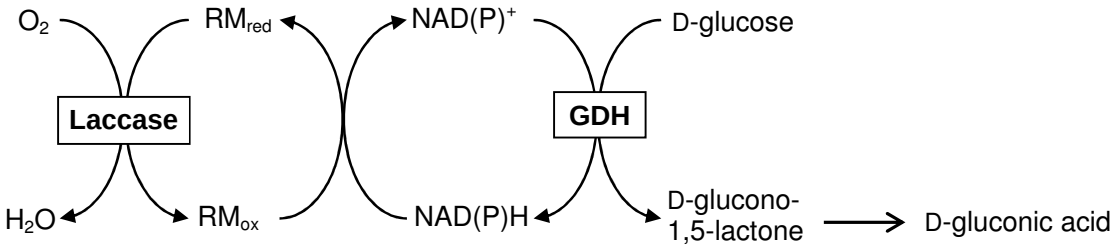

Figure 2. Reaction scheme for the bi-enzymatic system employing laccase as regenerating enzyme and glucose dehydrogenase (GDH) as synthetic enzyme. The redox mediator in its reduced form RM_{red} is oxidized by laccase to RM_{ox} with the rate r_1. The bimolecular rate observed for the reaction between RM_{ox} and the reduced form of the coenzyme NAD(P)H is given as r_2. NAD(P)H is reduced by GDH with the rate r_3. The concomitantly formed product is gluconolactone, an inhibitor of GDH. Its hydrolyzation rate to the non-inhibiting final product is r_{hyd}.

2 Materials and Methods

2.1 Modelling for lactose hydrolysis and transgalatosylation using β-galactosidase from *Lactobacillus reuteri*

2.1.1 Enzymes

β-galactosidases were produced using two strains of *Lactobacillus reuteri,* L103 and L461, obtained from Lactosan Starterkulturen. Enzymes were purified to homogeneity by hydrophobic interaction and affinity Chromatography. The method of enzymes production was described by Nguyen et al [104].

2.1.2 Enzyme activity assays

β-Galactosidase activity was determined using lactose as the substrates, 20 µL of an enzyme solution was added to 480 µL of a 600-mM lactose solution in 50 mM sodium phosphate buffer (pH 6.5). The reaction mixture was incubated at 30℃ using an Eppendorf thermomixer compact (600 rpm). After 10 min, the reaction was stopped by heating the reaction mixture at 99 ℃ for 5 min. After the sample had been cooled to room temperature, the release of D-glucose was assessed colorimetrically using the GOD/POD assay by adding 60 µL of this reaction mixture to 600 µL of a solution containing GOD (94 µg/mL), POD (6.1 µg/mL), 4-aminoantipyrine (157 µg/mL), and phenol (1.95%, v/v) in 0.1 potassium phosphate buffer (pH 7.0). This assay mixture (660 µL) was incubated in the dark at room temperature for 40 min, and the absorbance at 546 nm was measured. One unit of lactase activity was defined as the amount of enzyme releasing 1 µmol of D-glucose per minute under the given conditions.

The protein concentration was determined by the method of Bradford [105] using bovine serum albumin as a standard.

2.1.3 Time-course reactions for lactose hydrolysis and transgalactosylation

Time-course reactions for hydrolysis and transgalactosylation of lactose with β-galactosidase from two different isolates of *L. reuteri*, L103 and L 461, were carried out as described in Splechtna et al. [7]. **Table 3** shows time-course reactions for eight different batch reactions. These experimental data were supplied by Dr. Thu-Ha Nguyen [7], [104], and re-calculated in percent of initial lactose concentration. The standard batch condition is set at 30^0C and pH 6.0 for both enzymes L103 and L461. Enzyme activities for standard batches were 0.8 U mL^{-1} (batch 1, 2) and 1.6 U mL^{-1} (batch 5, 6) for L103 and L461, respectively. Enzyme activities for the batches at temperatures of 25^0C and 37^0C were estimated from the standard batch data using the Arrhenius equation of the temperature dependence of enzyme activity, with the activation energies being 30 and 15 kJ mol^{-1} for L103 and L461 β-galactosidase, respectively [104]. All batch transformations were carried out in 50 mM sodium phosphate buffer, 1 mM $MgCl_2$ at pH 6.5.

Table 3. Course of reactions for lactose conversions in discontinuous batch processes, initial lactose concentration, temperature, and enzyme activities used are shown for each reaction

Time (h)	Lactose (%)	Glucose (%)	Galactose (%)	GOS (%)
Batch 1: 103 g L^{-1} lactose; 30^0C; pH 6.5; 0.80 U_{LAC} /mL L103				
0	100	0	0	0
0.33	87.40	2.49	1.67	8.44
1.0	73.59	7.45	5.38	13.58
2.5	49.40	16.86	11.77	21.98
5.0	25.80	24.70	17.13	32.37
8.5	7.57	38.93	31.15	22.36
23.0	2.19	44.53	49.90	3.38
Batch 2: 205 g L^{-1} lactose; 30^0C; pH 6.5; 0.80 U_{LAC} /mL L103				
0	100	0	0	0.00
0.33	91.30	2.19	1.59	4.93
1.0	82.48	4.01	2.35	11.16
2.5	71.73	9.06	5.17	14.05
5.0	52.72	17.01	9.68	20.60
8.5	32.23	20.39	14.20	33.18
23.0	4.24	37.27	28.95	29.54
72.0	1.01	52.09	46.40	0.50
Batch 3: 205 g L^{-1} lactose; 25^0C; pH 6.5; 0.65 U_{LAC} /mL L103				
0	100	0	0	0
1.0	81.59	3.79	2.44	12.19
2.5	70.57	7.69	4.80	16.94
8.5	34.55	21.06	13.07	31.32
23.0	8.51	34.56	24.31	32.63
Batch 4: 205 g L^{-1} lactose; 37^0C; pH 6.5; 0.93 U_{LAC} /mL L103				
0	100	0	0	0
1.0	80.02	5.55	3.63	10.81
2.5	61.73	11.51	6.52	20.24
8.5	19.48	26.67	15.47	38.38
23.0	3.41	42.02	36.14	18.43
Batch 5: 103 g L^{-1} lactose; 30^0C; pH 6.5; 1.60 U_{LAC} /mL L461				
0	100	0	0	0
0.5	81.27	4.58	2.98	11.17
1.0	68.38	9.68	6.44	15.50
3.0	33.63	23.88	15.66	26.83
5.0	16.34	33.05	24.97	25.65
9.0	4.61	43.27	40.32	11.81

Time (h)	Lactose (%)	Glucose (%)	Galactose (%)	GOS (%)
24.0	1.84	45.44	53.62	0.90
Batch 6: 205 g L^{-1} lactose; 30^0C; pH 6.5; 1.60 U_{LAC} /mL L461				
0	100.00	0.00	0.00	0.00
1.0	80.54	5.66	4.53	9.27
2.0	66.57	9.85	5.35	18.23
4.0	47.90	17.65	9.21	25.24
6.0	31.12	23.01	13.75	32.12
9.0	17.80	27.63	17.42	37.15
24.0	1.80	38.12	37.08	23.00
48.5	0.00	40.75	48.12	11.12
Batch 7: 205 g L^{-1} lactose; 25^0C; pH 6.5; 1.45 U_{LAC} /mL L461				
0	100	0	0	0
1.0	84.19	4.75	2.89	8.17
4.0	52.44	14.48	8.05	25.03
9.0	25.79	26.10	16.71	31.41
24.0	5.14	37.56	32.01	25.28
Batch 8: 205 g L^{-1} lactose; 37^0C; pH6.5; 1.73 U_{LAC} /mL L461				
0	100	0	0	0
1.0	77.93	7.12	3.95	11.00
4.0	36.74	20.47	9.66	33.13
9.0	11.96	31.34	20.00	36.70
24.0	0.00	41.38	39.48	19.14

2.1.4 Proposed model for lactose conversion by hydrolysis and transgalactosylation reactions

The new model for hydrolysis and transgalactosylation reactions of β-galactosidase from *Lactobacillus reuteri* was proposed according to Michaelis-Menten kinetics with competitive product inhibition by galactose and a first-order deactivation model for enzyme activity, in which lactose acts as both a substrate and an acceptor, the enzyme is competitively inhibited by galactose, glucose is not considered as an acceptor, and the effect of glucose on reaction kinetics is considered negligible.

Galactose binds to the free enzyme to make the galactosyl-enzyme complex for further transgalactosylation reactions with lactose. At high concentrations, lactose is more likely to be the acceptor to make trisaccharides. The model combined tri- and tetra- and higher saccharides into a single species, GOS.

A first-order deactivation model, shown in equation (32), is used to describe thermal deactivation of the enzyme. During the reaction, the total enzyme is the sum of free and enzyme complexes. Based on this, a model simplification is proposed by assuming that the deactivation of free enzymes and all complexes enzyme are described by first-order deactivation with the same deactivation-kinetic constant k_d which is calculated from experimental data.

The following equations are proposed:

$$E + LAC \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (ELac) \overset{k_{cat}}{\rightarrow} EGal + GLC \tag{35}$$

$$E+GAL \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EGal \tag{36}$$

$$EGal + LAC \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} (EGos) \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + GOS \tag{37}$$

$$(E) \overset{k_d}{\rightarrow} E_d \tag{38}$$

Where *LAC, GLC, GAL, GOS* are lactose, glucose, galactose and galacto-oligosaccharides, respectively. *ELac, EGal, EGos* are the complexes of enzyme with substrate and products. E is the free enzyme.

Assuming that the materials are conserved, $[LAC]_0 - [LAC] = [GLC] + [GAL] + [GOS]$, where $[LAC]_0$ is the initial concentration of substrate, and for the enzyme $[E]_0 = [E] + [ELac] + [EGal] + [EGos]$, where $[E]_0$ is the initial concentration of enzyme.

The ordinary differential equations for the formation of each carbohydrate and lactose consumption derived from mass balances and the mass conservation law are further represented by the set of the following equations:

$$\frac{d[LAC]}{dt} = -k_1[E][LAC] + k_{-1}[ELac] - k_3[EGal][LAC] + k_{-3}[EGos] \tag{39}$$

$$\frac{d[GLC]}{dt} = k_{\text{cat}}[ELac] \tag{40}$$

$$\frac{d[GAL]}{dt} = -k_2[E][GAL] + k_{-2}[EGal] \tag{41}$$

$$\frac{d[GOS]}{dt} = k_4[EGos] - k_{-4}[E][GOS] \tag{42}$$

$$\frac{d[E]}{dt} = -k_1[E][LAC] + k_{-1}[ELac] + k_{-2}[EGal] - k_2[E][GAL] + k_4[EGos] - k_{-4}[E][GOS] - k_d[E] \tag{43}$$

$$\frac{d[ELac]}{dt} = k_1[E][LAC] - k_{-1}[ELac] - k_{\text{cat}}[ELac] - k_d[ELac] \tag{44}$$

$$\frac{d[EGal]}{dt} = k_{cat}[ELac] - k_{-2}[EGal] + k_2[E][GAL] - k_3[EGal][LAC] + k_{-3}[EGos] - k_d[EGal] \tag{45}$$

$$\frac{d[EGos]}{dt} = k_3[EGal][LAC] - k_{-3}[EGos] - k_4[EGos] + k_{-4}[E][GOS] - k_d[EGos] \quad (46)$$

The Michaelis constant and inhibition of the β-galactosidase for lactose are defined as follows:

$$K_M = \frac{k_{-1} + k_{cat}}{k_1} \quad (47)$$

$$K_i = \frac{k_{-2}}{k_2} \quad (48)$$

The Michaelis constants for the galactosyl-enzyme complex for lactose and GOS are defined as:

$$K_{M\_LAC} = \frac{k_{-3} + k_4}{k_3} \quad (49)$$

$$K_{M\_GOS} = \frac{k_{-3} + k_4}{k_{-4}} \quad (50)$$

To solve the set of ordinary differential equations, the ODE15s subroutine in the MATLAB R2009a software (The Mathworks Inc., Natick,MA, USA) was used, which is designed specifically to deal with stiff differential systems of equations. Initial conditions used for this model are $E = E_0$ (initial enzyme concentration), *[ELac] = [EGal] = [EGos] = 0; [GLU] = [GAL] = [GOS] = 0 and* $[LAC] = [LAC]_0$ (initial lactose concentration).

2.1.5 Kinetic constant of thermal deactivation

Enzyme inactivation caused by temperature was considered in the set of ordinary differential equations (Eqs. (39)-(46)) in proposed model. The kinetic constant k_d for thermal deactivation was estimated from [104].

Table 4. Deactivation constant of β-galactosidase L103 and L461 from *L.reuteri*

Enzyme	Temp (^{0}C)	k_d (h^{-1})
L103	25	4.134×10^{-3}
	30	1.064×10^{-2}
	37	2.374×10^{-2}
L461	25	4.210×10^{-4}
	30	7.431×10^{-3}
	37	1.284×10^{-2}

2.1.6 Nonlinear Adjustment of Model Parameters

The set of kinetic parameters (k_1, k_{-1}, k_2, k_{-2}, k_3, k_{-3}, k_4, k_{-4} *and* k_{cat}) of the model (Eqs. (35)-(38)) was estimated by using the data from discontinuous experiments (**Table 3**). These parameters were evaluated by using the residual *E(k)* defined as the sum of the squares of the differences between observed and calculated data points (Eq. (51)). Smaller residual *E(k)* indicates a better fit by this method.

$$E(k) = \sum_{i,j} \left(\tilde{y}_{i,j} - y_{i,j}(k) \right)^2 \qquad (51)$$

where, $\tilde{y}$ and y are experimental data and simulated data, respectively. Subscript i represents the reaction carbohydrate (lactose, glucose, galactose or GOS), subscript j represents the experimental points of the i^{th} reaction species, and k is the set of kinetic parameters.

The problem we encounter here is to estimate optimum parameters vector k^* as accurate as possible using the given experimental data. The objective here is mainly to find a coefficient vector k that minimizes the error function $E(k)$.

This type of problem is usually called parameter estimation in the literature and is often solved by deterministic optimization methods such as Nelder–Mead, Levenberg–Marquardt, Gauss–Newton etc. [106], [107]. Unfortunately, the solution to the problem using these methods is usually around the local minima if there are more than one minimum available.

In this study, the parameters were estimated by the nonlinear least squares method with Genetic Algorithm until a minimal error between experimental and calculated values was achieved. Each optimization simulation is run for 200 generations, with 100 as the population size of each generation.

The genetic algorithm method, the best-known stochastic optimization technique, imitates the natural evolution process [108], [109]. One of the main advantages of this method is that it requires no gradient of the objective function and additional information.

The genetic algorithm method is based on a computer simulation of biological evolution and initially works with a randomly generated population consisting of several individuals. A new population is built up by selecting individuals among members of the initial population according to their fitnesses through a fundamental genetic process of selection criterion. Once the selection is completed, the crossover operation is put into effect on the new population. Next, the mutation operation is implemented on the previous population. Thus, the first generation is completed and the genetic process is repeated until the termination criteria are satisfied. It should be noted that if the best individual is not the one as desired after completing the genetic process, advanced operators may be used.

In the recent literature, the genetic algorithm method was used to estimate kinetic parameters in several kinetic models [110]–[112]. Genetic Algorithm of COPASI was used to estimate the parameters on models by Palai et al. [27], [66].

2.2 Engineering an Enzymatic Regeneration System for NAD(P)H Oxidation[4]

2.2.1 Materials

Acetosyringone (4'-hydroxy-3',5'-dimethoxyacetophenone), syringaldehyde **(**4-hydroxy-3,5-dimethoxybenzaldehyde), NAD^+, $NADP^+$, NADH, NADPH, D-Glucose and D-glucono-1,5-lactone were purchased from Sigma–Aldrich (Steinheim, Germany). 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was purchased from Amresco (Ohio, USA). Stock solutions (200 mM) of acetosyringone and syringaldehyde were prepared in ethanol. All buffer reagents and other chemicals were of analytical grade. Water was purified by reversed osmosis and scavenger resins to a resistivity >18 MΩ cm. Laccase (EC: 1.10.3.2) from *Trametes pubescens* CBS 696.94 with a specific activity of 594 U/mg at pH 5.0 was produced by cultivation of the fungus in a bioreactor under copper induction following published procedures [113], [114]. Glucose dehydrogenase (GDH, EC 1.1.1.47) from *Bacillus* sp. with a specific activity of 18.5 U/mg at pH 5.0 was a gracious gift from Amano Enzyme Inc. (Nagoya, Japan).

2.2.2 Enzyme activity assays

To measure the deactivation of laccase in batch samples, laccase activity was determined with 2,2′-azino-bis (3-ethylbenz- thiazoline-6-sulfonate, ABTS) as colorimetric substrate. The assay mixture contained 1 mM ABTS in an air-saturated, 100 mM sodium-citrate solution buffer, pH 5.0, incubated for 15 min at 30°C before the measurement. After addition of a suitable amount of laccase, the oxidation of ABTS was monitored by following the increase of absorbance at 420 nm (ε_{420} =36.0 mM^{-1} cm^{-1}) for 180 s. One unit of laccase activity was defined as the amount of enzyme required to oxidize 1 µmol of ABTS per min.

[4] Part of the:
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GDH activity was followed by the increase in NAD(P)H absorbance at 340 nm (ε_{340}= 6.22 mM^{-1} cm^{-1}) for 180 s at 30°C. The assay contains glucose, the respective coenzyme (NAD^+ or $NADP^+$, 0.5 mM), 100 mM sodium-citrate solution buffer, pH 5.0 and a suitable amount of enzyme. One unit of GDH activity was defined as the amount of enzyme required to reduce 1 µmol of $NAD(P)^+$ per min. Measurements of enzymatic activities in samples taken from conversion experiments were affected by a small error introduced by the regenerating enzyme, but taken care of by performing reference measurements.

2.2.3 Determination of pH profiles, catalytic constants and rates for redox mediators

The optimum pH, pH profiles and the activity of laccase with redox mediators was determined in air-saturated, 100 mM phosphate-citrate buffer solution at vaious pH values (3.0 to 6.5). The assays were carried out at 30 ^{0}C using 1 mM of acetosyringone (ε_{400}= 1.7 mM^{-1} cm^{-1}) or syringaldehyde (ε_{380}= 1.6 mM^{-1} cm^{-1}). The molar absorption coefficients the two redox mediators were determined from standard curves (**Figure 3**). Catalytic constants of laccase for the redox mediators were calculated by non-linear least-squares regression, fitting the observed data to the Henri-Michaelis-Menten equation using SigmaPlot v.12 (Systat Software Inc, CA, USA). The K_M of laccase for oxygen (0.41 mM) was taken from [113]. The rate constant of the oxidation reaction of NAD(P)H by redox mediators was measured at 30°C in 100 mM phosphate-citrate buffer, pH 5.0 by following the reduction of the absorption band at 340 nm. The fast reaction of a 150 mM final concentration of both redox mediator and NAD(P)H concentration was recorded with an Applied Photophysics SX20 stopped-flow spectrophotometer. The results are averaged from four measurements.

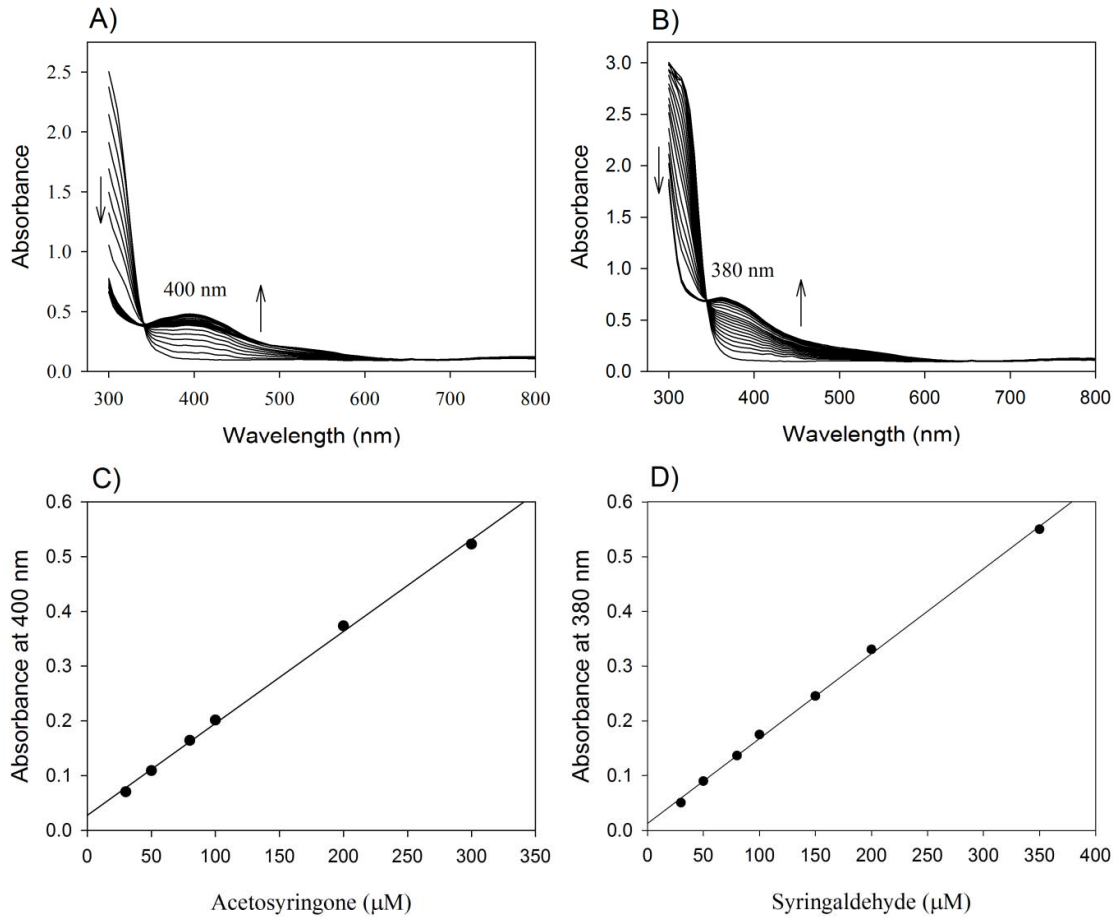

Figure 3. Determination of the peak wavelength and molar absorption coefficients of acetosyringone and syringaldehyde. Absorption spectra were recorded from a 200 µM solution of acetosyringone (A) and syringaldehyde (B) in 100 mM sodium-citrate buffer, pH 5.0. After addition of laccase the absobance increases at the indicated wavelengths. The linear relationship between concentration of the oxidized redox mediator and absorbance is shown for acetosyringone (C) and syringaldehyde (D).

2.2.4 Batch conversion experiments

All batch conversion experiments were performed in a parallel 0.5-L bioreactor system (Sixfors, INFORS HT, Bottmingen, Switzerland) with a working volume of 0.3 L. The reactions contained 200 mM D-glucose, 380–900 U L^{-1} laccase, 220–500 U L^{-1} GDH, 100–500 µM NAD^+ and 100–500 µM acetosyringone as redox mediator in 100 mM sodium citrate buffer, pH 5.0. Four batch conversion experiments (A, B, C and D) employing different

enzymatic activities, redox mediator and coenzyme concentrations were run. The reaction was prepared as follows: first glucose was dissolved in an appropriate amount of buffer. Then, the redox mediator, coenzyme and laccase were added. The redox mediator was dissolved in 1 mL ethanol (96%) before adding to the reactor. Finally, the reaction was started by the addition of GDH. The pH was regulated by automatic titration with an aqueous sodium carbonate solution (500 mM). The reaction solutions were thermostatted to 30°C, continuously stirred at 250 rpm and oxygenated using pure oxygen which was bubbled through a sparger. The dissolved oxygen concentration was measured by using an oxygen electrode (OxyFerm, Bonaduz, Switzerland) and the oxygen saturation was set to 21%, equal to air saturation. Samples were taken periodically and used immediately for enzymatic activity measurements or heated at 99°C for 5 min and then frozen at -18°C until HPLC analysis of glucose and gluconic acid.

2.2.5 HPLC analysis

The conversion of glucose was monitored from reaction samples by HPLC (Dionex Summit and Chromeleon software, Sunnyvale, CA, USA) using an Aminex HPX-87 H-column (BioRad Laboratories, CA, USA) and 5 mM H_2SO_4 as eluent with flow-rate of 0.5 mL min^{-1} at 60^0C. The components were detected by UV at a wavelength of 210 nm. Calibration curves was measured with glucose and gluconic acid in a concentration range from 5 to 20 mg L^{-1}. Samples were diluted to reach a concentration in this range. Due to the heat treatment of the sample the formed intermediate glucono-1,5-lactone is fully converted to gluconic acid until analysis begins and cannot be determined.

2.2.6 Rate equation and Matlab model

For the bi-enzymatic process employing GDH and laccase (**Figure 2**) a set of non-linear, differential equations was derived from mass balances and the mass conservation law. Laccase activity is modeled by a ping-pong *bi-bi* reaction mechanism [115] although this is only an approximation of the much more complicated reaction mechanism for which no

kinetic model is published. GDH activity was modeled a sequential ordered *bi-bi* mechanism kinetics with the coenzyme biding first [116]. The product of the laccase reaction, the oxidized redox mediator, reacts with the product of the GDH reaction, the reduced nicotinamide coenzyme, in a second order reaction [88] (this work). The reaction rates for laccase (r_1), the redox mediator/NAD(P)H (r_2) and GDH (r_3) are given by the equations (52) – (54):

$$r_1 = V_{max,LAC} \times \frac{[O_2]\times[RM_{red}]}{K_{M,RM}\times[O_2]+K_{M,O_2}\times[RM_{red}]+[O_2]\times[RM_{red}]} \tag{52}$$

$$r_2 = k_2 \times [RM_{ox}] \times [NADH] \tag{53}$$

$$r_3 = V_{max,GDH} \times \frac{[NAD]\times[GLC]}{\begin{array}{c} K_{I,NAD}\times K_{M,GLC}\times\left(1+\frac{[NADH]}{K_{I,NADH}}+[GL]\times\frac{K_{M,NADH}}{K_{I,NADH}\times K_{M,GL}}\right)+ \\ K_{M,GLC}\times[NAD]\times\left(1+[GL]\times\frac{K_{M,NADH}}{K_{I,NADH}\times K_{M,GL}}\right)+ \\ +K_{M,NAD}\times[GLC]\times\left(1+\frac{[NADH]}{K_{I,NADH}}\right)+[NAD]\times[GLC]\times\left(1+\frac{[GL]}{K_{I,GL}}\right) \end{array}} \tag{54}$$

The concentration of the redox mediator RM in oxidized and reduced state, NAD(P)$^+$, NAD(P)H, D-glucose (GLC), D-glucono-1,5 lactone (GL) and gluconic acid (GA) can be described by the set of differential equations (55) – (59):

$$\frac{d[GLC]}{dt} = -r_3 \tag{55}$$

$$\frac{d[NAD]}{dt} = -\frac{d[NADH]}{dt} = r_2 - r_3 \tag{56}$$

$$\frac{d[RM_{red}]}{dt} = -\frac{d[RM_{ox}]}{dt} = r_2 - r_1 \tag{57}$$

$$\frac{d[GL]}{dt} = -\frac{d[GLC]}{dt} - \frac{d[GA]}{dt} = r_3 - r_{hyd} \tag{58}$$

$$\frac{d[GA]}{dt} = k_{hyd} \times [GL] \tag{59}$$

The space-time yield (STY) for the sum of products (glucono-1,5-lactone plus gluconic acid) was calculated for the initial reaction to exclude enzyme deactivation and

redox mediator degradation and after 10 h (STY_{10}) when the reactions were stopped (Eq. (60)):

$$\mathrm{STY}[\mathrm{mol(GL+GA)L^{-1}h^{-1}}] = \frac{C^0_{GLC} - C^t_{GLC}}{t} \quad (60)$$

To solve the set of ordinary differential equations the ODE15s subroutine in the MATLAB R2009a software (The MathWorks, Natick, MA, USA) was used, which is designed specifically to deal with stiff differential systems of equations. For better comparison with the experimental results the predicted glucono-1,5-lactone and gluconic acid concentration are summed up and displayed in the **Figures 3** in one curve for the end product (gluconic acid).

3 Results

3.1 Modelling for lactose hydrolysis and transgalactosylation using β-galactosidase from *Lactobacillus reuteri*

3.1.1 Experimental kinetic constants

The steady-state kinetic constants of β-galactosidases L103 and L461 from *L.reuteri* for the hydrolysis of the natural substrate lactose including the catalytic constant of enzyme (k_{cat}), the galactose competitive inhibition constant (K_i) and the Michaelis constant for lactose (K_m) were determined at 30^0C, pH 6.5, and are shown in **Table 5**. These constants were calculated by non-linear least-squares regression, fitting the observed data to the Michaelis-Menten kinetics with competitive product inhibition by galactose according to equation (8) using SigmaPlot (Systat Software Inc, CA, USA) [104].

Table 5. Kinetic Parameters for β-galactosidases L103 and L461 from *L.reuteri*

Enzyme	$K_{m,LAC}$ (mM)	k_{cat} (s^{-1})	$K_{I,Gal}$ (mM)	Ref
L103	13 ± 2	60	188 ± 34	[104]
L461	31 ± 5	58	89 ± 27	[104]

3.1.2 Estimation of the kinetic constants in the model

Each set of experimental data shown in **Table 3** was used to run model simulation with GA to estimate its set of kinetic constant vector *k* including k_1, k_{-1}, k_2, k_{-2}, k_3, k_{-3}, k_4, k_{-4} *and* k_{cat}. To solve and consequently estimate these kinetic constants of the model, the following assumptions were made:

(i) The enzyme concentrations for batches with the same kind of enzyme are constant and calculated from experimental data in standard batches;

(ii) The initial concentration of lactose (LAC) for each batch is known; the enzyme activity is also known as described in the previous section;

(iii) The concentration of products (GLC, GAL, GOS) are zero at the beginning of the reaction, the formation of tri-, tetra- and higher oligosaccharides is combined to the single form of GOS;

(iv) The values of k_d, the thermal deactivation-kinetic constant for the enzymes and all enzyme complexes are identical, and were calculated and shown as in **Table** 4;

(v) In order to use GA to minimise the residual *E(k)* (Eq. (51)), the limitation (upper bound and lower bound) of Michaelis constants and the inhibition constant (Eqs. (47)-(50)) were set up according to the literature;

(vi) The set kinetic parameters *k* of the model were converted from the dissociation and the inhibition constant, and are used to solve the set of ordinary differential equations (Eqs. (39)-(46));

(vii) GA will find the optimum of the set kinetic parameter *k* and then recalculate the dissociation and the inhibition constant.

Two experimental batches (1, 2) were implemented at the same conditions (L103, 30^0C, pH 6.5), only data of batch 2 were used to estimate kinetic parameters. Experimental data of batch 1 will be used to evaluate model. This approach was also used for batches 5 and 6 with enzyme L461, here only data of the batch 6 were used to estimate kinetic parameters. Experimental data of batch 5 will be used to evaluate model. The estimated values of model parameters and inhibition constants of β-galactosidase L103 and L461 from *Lactobacillus reuteri* are listed in**Table 6**.

Table 6. Estimated kinetic parameters for 12 batches using the model with Genetic Algorithm

Batch no	K_m (mM)	$K_{m,LAC}$ (mM)	$K_{m,GOS}$ (mM)	$K_{s,LAC}$ (mM)	$K_{i,\ GAL}$ (mM)	k_{cat} (s^{-1})
1 2	13.6 (13 ± 2)[a)]	50.0	15.3	9.2	200.0 (188 ± 34) [a)]	60[a)]
3	10.7	44.9	10.0	9.6	131.8	48.8
4	15.5	80.0	31.9	14.9	269.8	69.8
5 6	30.3 (31 ± 5) [a)]	50.0	17.3	17.2	99.4 (89 ± 27) [a)]	58[a)]
7	24.1	36.4	10.0	14.2	82.2	52.6
8	33.2	62.0	22.2	12.4	181.4	62.7

[a)] data from Nguyen et al. [104]

3.1.3 Verification of the model validity

Using the mathematical models of the reaction (Eqs. (39)-(46)) and the estimated parameter values (**Table 6**), the time course of lactose hydrolysis of 12 experimental batches under defined conditions were predicted. **Figure 4** and **Figure 5** show the comparison of experimental measurements and model predictions for 12 different batch experiments at various initial lactose concentrations as well as various temperatures and pH values. For each individual experiments, the model could describe the lactose, glucose, galactose and total GOS concentration over time very well. For reasons of simplification, the value of R-square shown in **Table 7** is used to assesse the fit between experiments and models.

Table 7. R-square values assessing the fit of models

Batch no	Enzyme	Initial lactose ($g\ L^{-1}$)	Temp (^0C)	R^2
1	L103	103	30	0.9900
2		205	30	0.9935
3		205	25	0.9873
4		205	37	0.9943
5	L461	103	30	0.9901
6		205	30	0.9855
7		205	25	0.9957
8		205	37	0.9966

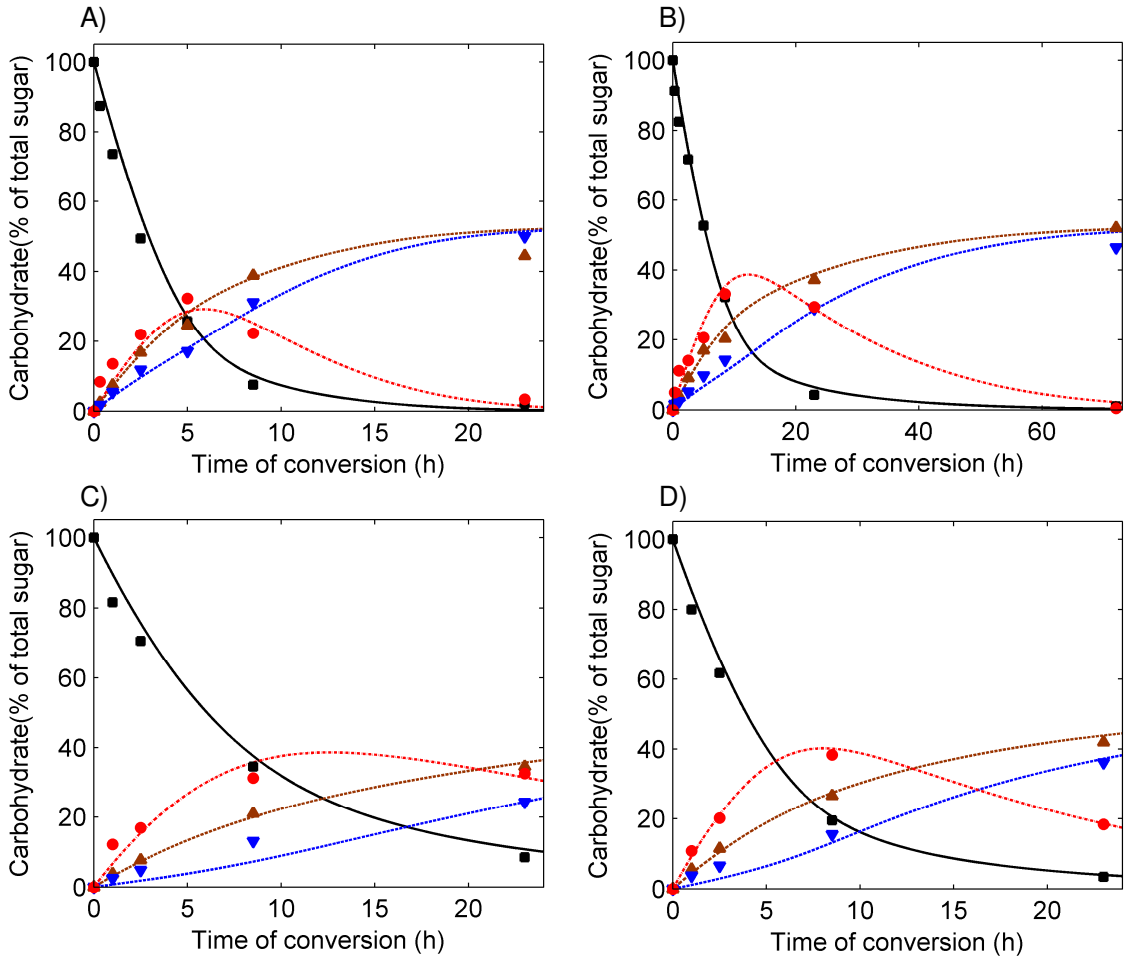

Figure 4. The time-course of lactose conversion by β-galactosidase from *L. reuteri* L103 with different initial concentration of lactose, temperature and pH as given for batches 1-6. Fig A-D simulate batch 1-4, respectively. Lactose (■), glucose (▲), galactose (▼), and total GOS (●). Symbols represent experimentally measured data, the lines indicate the values for substrate (solid line) and products (dashed lines) as calculated by the model.

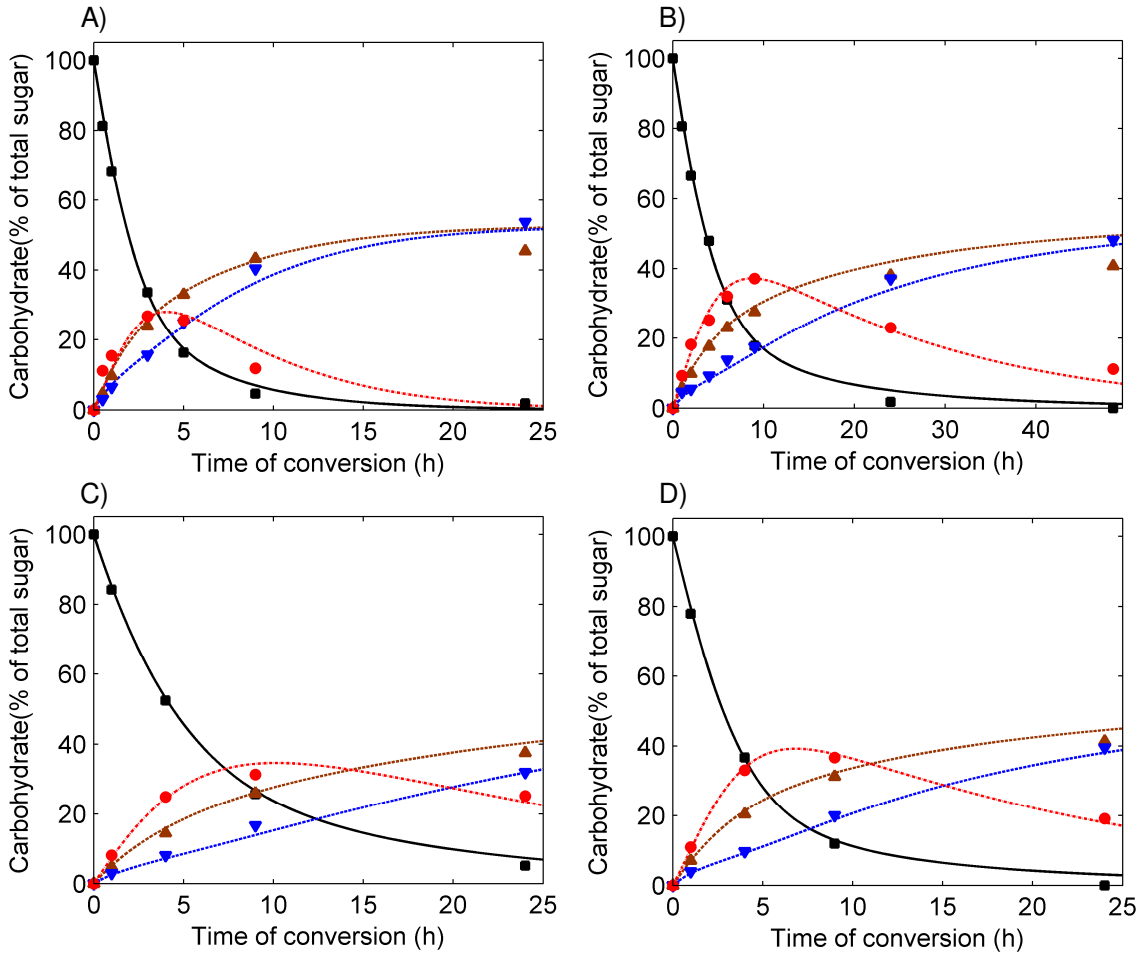

Figure 5. The time-course of lactose conversion by β-galactosidase from *L. reuteri* L461 with different initial concentration of lactose, temperature and pH using in batch 5-8. Fig A-D simulate batch 5-8, respectively. Lactose (■), glucose (▲), galactose (▼), and total GOS (●).Symbols represent experimentally measured data, thelines indicate the values for substrate (solid line) and products (dashed lines) as calculated by the model.

Figure 4 and **Figure 5** show the time course of lactose hydrolysis and GOS formation in reactions catalyzed by β-galactosidase L103 and L461 from *Lactobacillus reuteri.* The concentration of various sugars calculated from the model is compared with experimental data. At the beginning of the reaction, GOS, glucose, galactose increase, and then, GOS concentrations decrease while glucose and galactose concentrations continued to increase until lactose concentration is almost complete. At lower conversions, the galactose concentration is always much lower than glucose concentration. It can be explained by galactose being used in GOS formation. Early during the reaction, GOS are produced with a high yield while the lactose conversion proceeeds until the GOS concentration reached a maximum value. At higher conversion, there was a shift in the reactions from transgalactosylation to favor hydrolysis, which resulted in increased formation and release of hydrolysis products (glucose and galactose) and a decreased amount of GOS. The decrease in lactose hydrolysis and GOS cleavage at latter stages of the reaction can be attributed to competitive inhibition of the enzyme by galactose.

The simulated results show excellent agreement with the experimental values. This suggests that the proposed model follows the kinetics of GOS formation from enzymatic conversion of lactose using β-galactosidase.

3.1.4 GOS formation

The diagrams of the degree of lactose conversion versus GOS formation from an experiment at 30^{0}C and pH 6.5 for the enzymes L103 and L461 are shown in **Figure 6**.

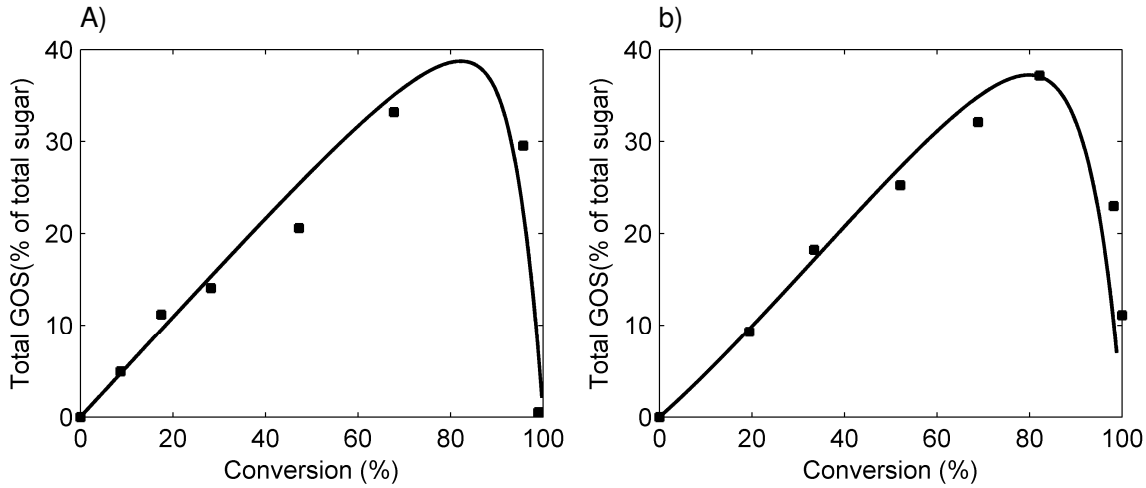

Figure 6. Model fit of total GOS concentration produced during synthesis with β-galactosidase from *Lactobacillus reuteri* A) L103 and B) L461 at pH 6.5, 30^{0}C, and initial lactose concentration is 205 g L^{-1}. Enzyme activity was 0.8 U mL^{-1} with L103 and 1.6 U mL^{-1} with L461.

3.1.5 Modeling the effect of initial lactose concentration on GOS formation

To determine the effect of different initial lactose concentrations on GOS production, GOS yields, and GOS formation rates were simulated as function of different initial lactose concentration for both enzymes L103 and L461 with initial activities of 0.8 U mL^{-1} and 1.6 U mL^{-1}, respectively, at a fixed temperature of 30^{0}C and pH 6.5. The outputs of these calculations, resulting from 200 simulations of initial lactose concentration from 40 to 700 g L^{-1}, are shown in **Figure 7** and **Figure 8**. In the lactose conversion with both L103 and L461, the maximum GOS yield increased rapidly in the range of initial lactose concentrations from 40 to 200 g L^{-1} and increased slightly when the initial lactose concentration was over 200 g L^{-1} (**Figure 7A**). The rate of GOS production also increases with the initial lactose concentration used in a way that is similar as for the maximum GOS yield. This maximum

rate achieved is 11 g L^{-1} h^{-1} at an initial lactose concentration of 240 g L^{-1} for L103, or 14 g L^{-1} h^{-1} at an initial lactose concentration of 240 g L^{-1} for L461. It should be noted that a higher initial enzyme concentration was used for L461. The degree of lactose conversion where the GOS maximum is achieved, is increased slightly with increasing initial lactose concentration. It is shifted from 74 to 86% with L103 and from 68 to 84% with L461. The maximum lactose converted can be achieved at 250 g L^{-1} for L103 and 380 g L^{-1} for L461 of initial lactose concentration and its values are 21 and 26 g L^{-1} h^{-1}, respectively.

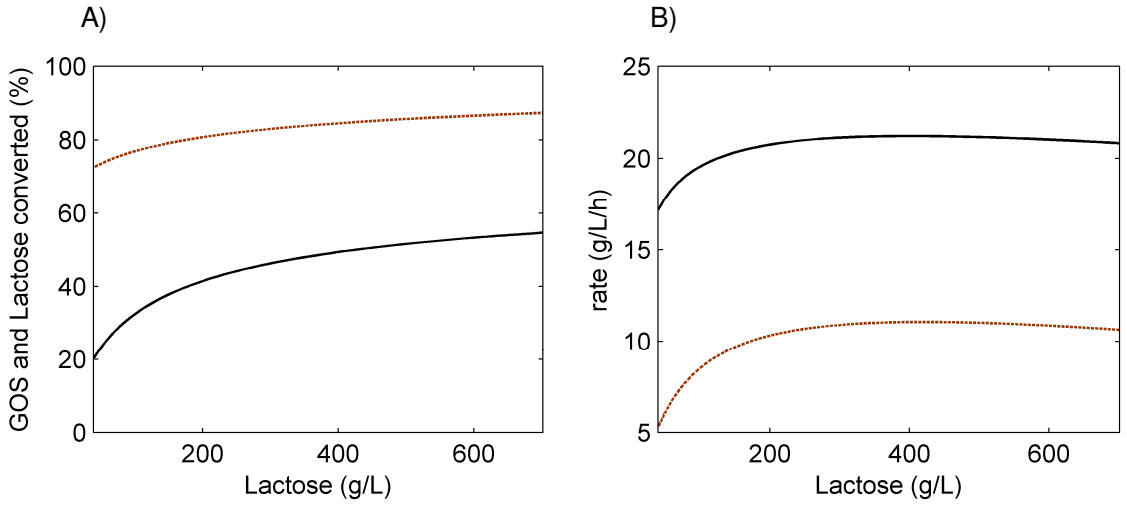

Figure 7. Effect of initial lactose concentrations on GOS production during lactose hydrolysis catalyzed by L103 with 0.8 U mL^{-1} enzyme activity at 30^0C and pH 6.5. Simulation: A) maximizing GOS yields (%; solid line) and lactose conversion (%, dashed line) where the GOS yield is maximum; B) rate of GOS formation and Lactose conversion.

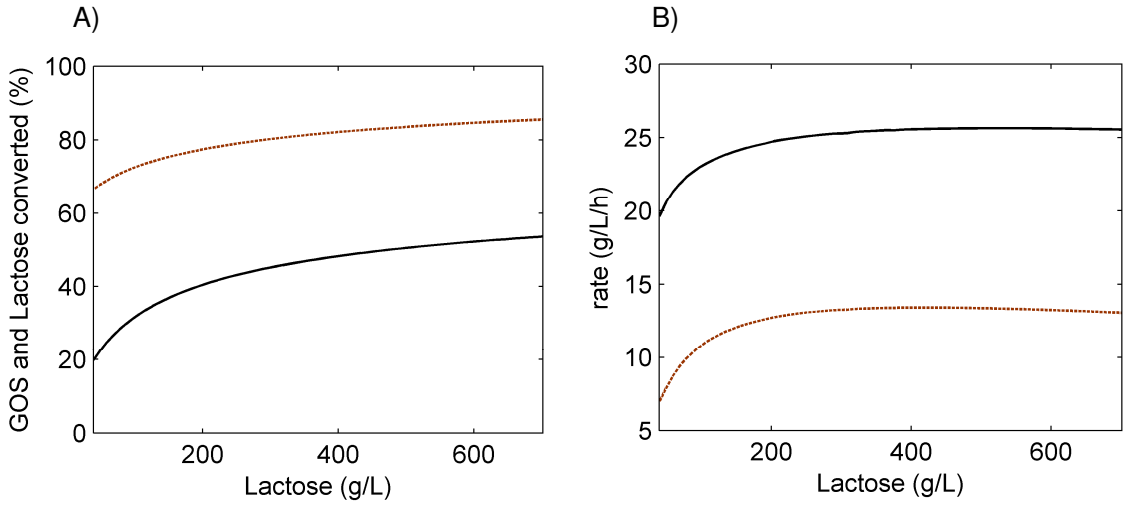

Figure 8. Effect of initial lactose concentrations on GOS production during lactose hydrolysis catalyzed by L461 with 1.6 U mL^{-1} enzyme activity at 30^0C and pH 6.5. Simulation: A) maximizing GOS yields (%; solid line) and lactose conversion (%, dashed line) where the GOS yield is maximum; B) rate of GOS formation and Lactose conversion.

3.1 Engineering an Enzymatic Regeneration System for NAD(P)H Oxidation [5]

3.1.1 Determination of catalytic constants and reaction rates

Laccase catalyzes the oxidation of acetosyringone and syringaldehyde to the corresponding phenoxy radicals at the expense of molecular oxygen, which is reduced to water, with a stoichiometry of 4:1. The pH optima of both reactions were determined photometrically using molar absorption coefficients determined at pH 5.0 (**Figure 3**). Laccase from *T. pubescens* exhibited a pH optimum at 3.5 for both redox mediators (**Figure 9**), with the activity towards acetosyringone being higher at less acidic pH values than syringaldehyde. At pH 5.0 the specific activity for acetosyringone was 243 U mg^{-1}, which is 1.9-fold higher than that for syringaldehyde (126 U mg^{-1}). To calculate r_1 in modeled processes, the catalytic constants of laccase were measured for both redox mediators. For

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measurements and batch reactions pH 5.0 was chosen as a compromise between the pH optima of laccase and GDH. The k_{cat} of laccase for acetosyringone is 2.6-fold higher and the K_M is 1.9-fold higher compared to syringaldehyde, which results in a 1.3-fold increased catalytic efficiency for acetosyringone at pH 5.0 (**Table 8**). The catalytic constants of GDH for glucose and NAD^+ and the inhibition constant for gluconolactone at pH 5.0 ($K_{I,GL}$ = 413 ± 12 mM) were also determined to model r_3 in batch conversion experiments. The last missing piece of kinetic information (to model r_2) was obtained by determining the second order rate constants (k_2) for the redox mediator/coenzyme couples. The second order reactions give the following rate constants: $k_{2\ NADH/acetosyringone}$ = 294 ± 5 M^{-1} s^{-1}, $k_{2\ NADPH/acetosyringone}$ = 152 ± 11 M^{-1} s^{-1}, $k_{2\ NADH/syringaldehyde}$ = 136 ± 5 M^{-1} s^{-1} and $k_{2\ NADPH/syringaldehyde}$ = 103 ± 8 M^{-1} s^{-1}.

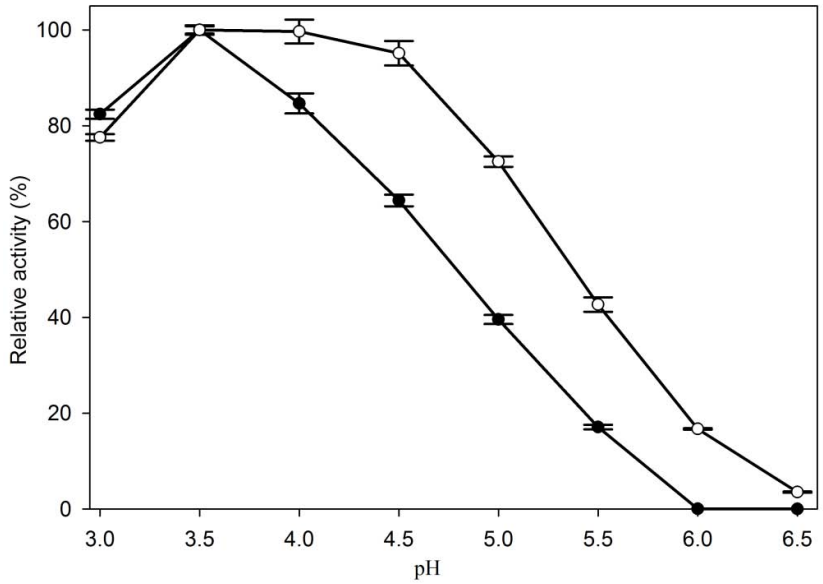

Figure 9. pH profile of the laccase activity with either acetosyringone as substrate (○) or syringaldehyde as substrate (●). Relative activities are shown for better comparison of the shape of the profile. At pH 3.5 the specific activitiy of laccase for acetosyringone and syringaldehyde were 334 and 318 U/mg, respectively.

Table 8. Catalytic constants of laccase and GDH for substrates and co-substrates were measured at 30^{0}C in air-saturated, 100 mM sodium-citrate buffer, pH 5.0.

Enzyme/Substrate	*Wavelength monitored*	*Extinction coefficient*	V_{max}	K_M	k_{cat}	k_{cat}/K_M
Laccase	*(nm)*	*(mM^{-1} cm^{-1})*	*(U mg^{-1})*	*(µM)*	*(s^{-1})*	*(M^{-1} s^{-1})*
Acetosyringone	400	1.7	289 ± 5	161 ± 10	289 ± 5	1.78 x 10^{-6}
Syringaldehyde	380	1.6	113 ± 2	85 ± 5	113 ± 2	1.34 x 10^{-6}
Oxygen*	420	36	2900		2900	7.0 x 10^6
GDH	*(nm)*	*(mM^{-1} cm^{-1})*	*(U mg^{-1})*	*(mM)*	*(s^{-1})*	*(M^{-1} s^{-1})*
Glucose	340	6.22	21.6 ± 0.2	16.7 ± 0.6	37.7± 0.3	2.26 x 10^{-3}
NAD^+	340	6.22	28.9 ± 0.4	0.26 ± 0.01	50.5 ± 0.8	194 x 10^{-3}

*) From [113], were determined at 25 ^{0}C using 1 mM ABTS as the electron donor

3.1.2 Experimental evaluation of the Matlab model

The first conversion experiment, **Batch A**, was performed with a 4.1-fold higher laccase activity than GDH activity (**Table 9**). The higher volumetric activity of the regenerating enzyme together with high concentrations of the redox mediator acetosyringone and the coenzyme NAD^+ (500 µM each) was chosen to ensure efficient NAD^+ regeneration. Because of experimental reasons, only the first 10 h of the process were followed by sampling. In these ten hours 30.2% of the glucose was converted, which correlates well with the predicted degree of conversion of 35.7% considering the observed enzyme deactivation. The measured and predicted data for Batch A are shown in **Figure 10A**. The averaged modeled NAD^+ concentration during this steady-state phase is ~485 µM (**Table 10**) and the acetosyringone concentration is ~33 µM (**Table 11**). During 10 h the measured laccase activity decreased by 20.8% and GDH activity by 26.7%, which led to a too big deviation from the initial settings to continue the experiment. At this point the total turnover number of laccase was 6.65×10^6 and of GDH 2.03×10^6. The averaged enzyme consumption numbers for laccase and GDH are 2.81 and 0.88 U $mmol^{-1}$ product, respectively.

Table 9. Batch conversion processes employing the system laccase/GDH/NAD(H)/acetosyringone. The reactions were performed with a working volume of 300 mL and an initial glucose concentration of 200 mM in 100 mM sodium-citrate buffer, pH 5.0. The measured and modeled data are taken after 10 h of reaction. Space-time yield (STY) and specific productivity are calculated for 10 h of reaction. Turnover numbers of enzymes, redox mediator and coenzyme were calculated by dividing the STY_{10} by the respective molar concentration.

Batch		**A**	**B**	**C**	**D**
Initial values					
Acetosyringone (µM)		500	500	500	100
NAD^+ (µM)		500	500	100	500
Laccase (U L^{-1})		900	380	900	900
GDH (U L^{-1})		220	480	300	500
Measured and modeled data					
Time to reach a conversion of 99% [h]	*modeled*	37.5	28.1	66.5	23.4
Conversion after 10 h [%]	*experiment*	30.2	37.7	17.7	13.0
	modeled	35.7	37.7	19.9	49.9
STY_{10} [mM h^{-1}]	*experiment*	6.04	7.69	3.61	2.65
	modeled	7.14	7.69	4.06	10.2
$Spec.productivity_{LAC}$ (mmol kU^{-1} h^{-1})	*experiment*	6.76	20.3	4.04	2.97
	modeled	7.98	20.2	4.54	11.4
$Spec.productivity_{GDH}$ (mmol kU^{-1} h^{-1})	*experiment*	27.3	16.0	11.9	5.29
	modeled	32.3	16.0	13.4	20.3
TN_{LAC} [h^{-1}]	*experiment*	6.65×10^5	1.99×10^6	3.97×10^5	2.92×10^5
	modeled	7.85×10^5	1.99×10^6	4.47×10^5	1.12×10^6
TN_{GDH} [h^{-1}]	*experiment*	2.03×10^5	1.19×10^5	8.86×10^4	3.93×10^4
	modeled	2.40×10^5	1.19×10^5	9.96×10^4	1.50×10^5
TN_{NAD^+} [h^{-1}]	*experiment*	12.1	15.4	36.1	5.31
	modeled	14.3	15.4	40.6	20.3
TN_{RM} [h^{-1}]	*experiment*	12.1	15.4	7.22	26.5
	modeled	14.3	15.4	8.12	102

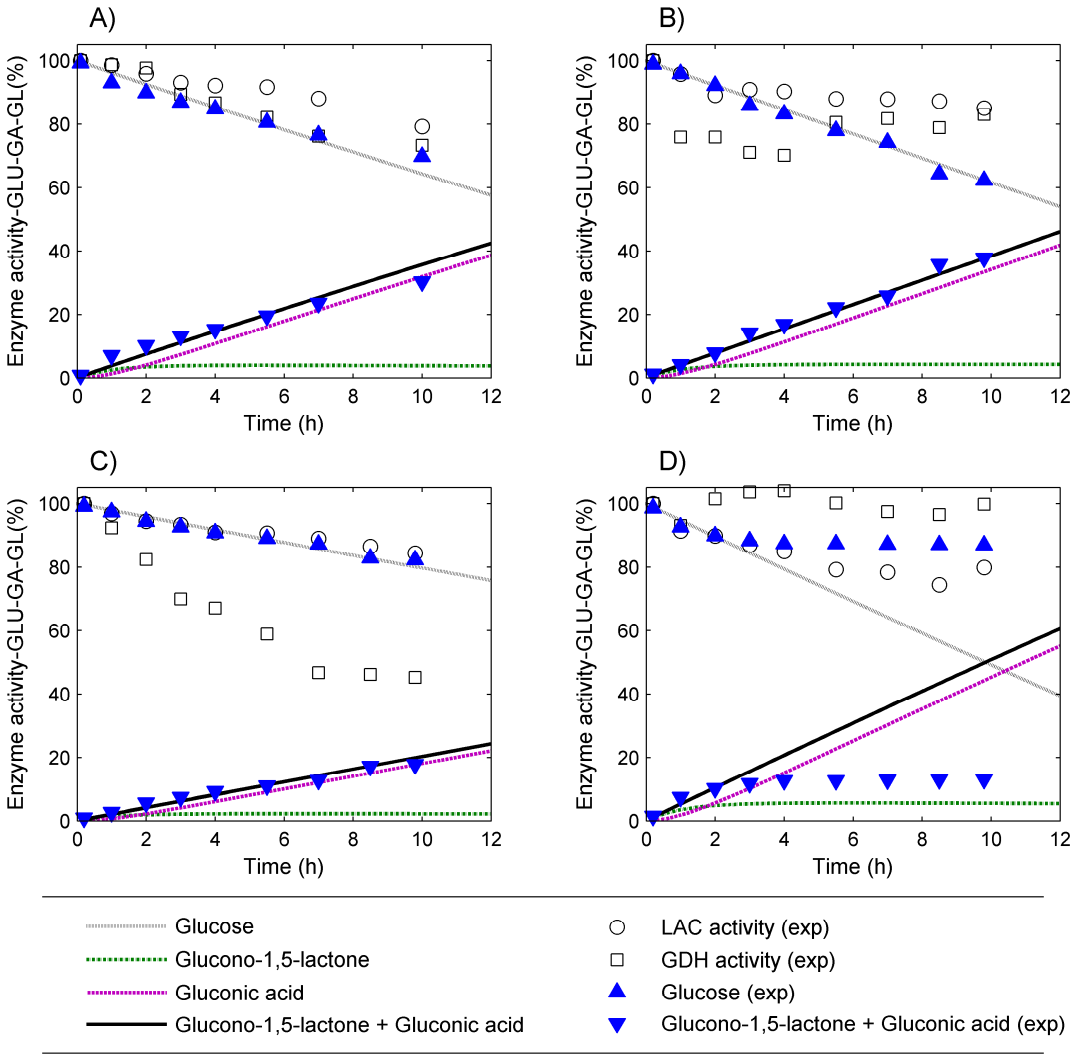

Figure 10. Batch conversions A–D. Measurements were stopped after 10 h of conversion. Measured values are indicated by data points, calculated values are indicated by lines. The Y-axis gives concentrations and activities as a percentage of their initial values to avoid multiple axes. Initial concentrations, activities and calculated performance numbers are given in Table 9.

3.1.3 Process modeling and engineering

To study the influence of different volumetric activities of laccase and GDH on the conversion rate in combination with three preselected, initial concentrations of redox mediator and coenzyme, productivity charts were generated from 1681 calculations per plot (**Figure 11A-C**).

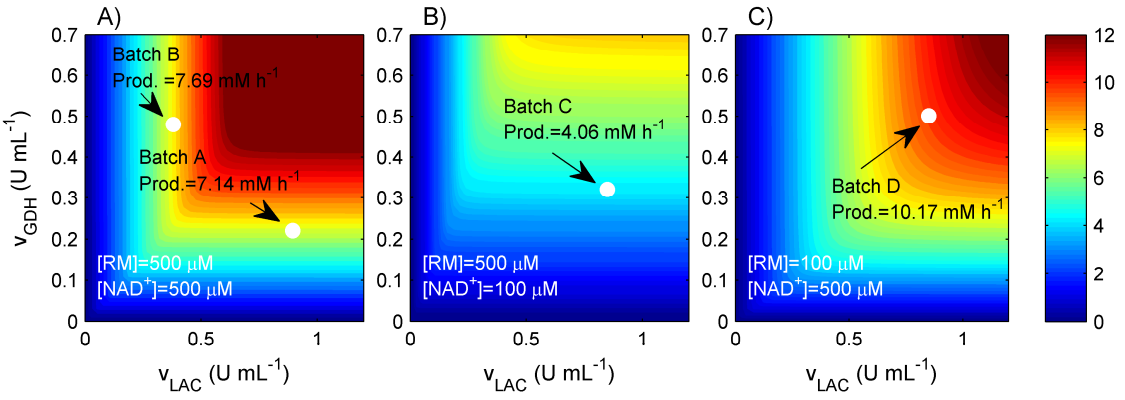

Figure 11. Isoproductivity charts calculated from space time yields after 10 h of reaction. The simulation was based on the following initial concentrations: 200 mM glucose and (A) 500 µM redox mediator and 500 µM coenzyme (NAD^+), (B) 500 µM redox mediator and 100 µM coenzyme (NAD^+), (C) 100 µM redox mediator and 500 µM coenzyme (NAD^+).

The availability (the concentration) of redox mediator and coenzyme is shown to be an important factor influencing STY. Three laccase/GDH activity ratios for Batches B, C and D were selected to test for the rate limiting step in the regeneration system, which are indicated in the plots. To investigate the influence of enzyme activity on the whole process in more detail, specific productivities for each enzyme were modeled for different combinations of the other enzyme's activity, redox mediator and coenzyme concentration. Isoproductivity plots for GDH and laccase gave a critical activity, below which the STY drops, and are given for Batches A and B (**Figure 12A,B**) and Batches C (**Figure 12C,D**) and D (**Figure 12E,F)**. Finally, the effect of the initial redox mediator and coenzyme concentration on the STY and turnover numbers for coenzyme and redox mediator was investigated. The simulations

shown in **Figure 13** and **Figure 14** illustrate the effect for the laccase/GDH activity ratio used in Batch A (900 and 220 U L^{-1}, respectively). Batches B (380 and 480 U L^{-1}, respectively) , C (900 and 300 U L^{-1}, respectively) and D (900 and 500 U L^{-1}, respectively).

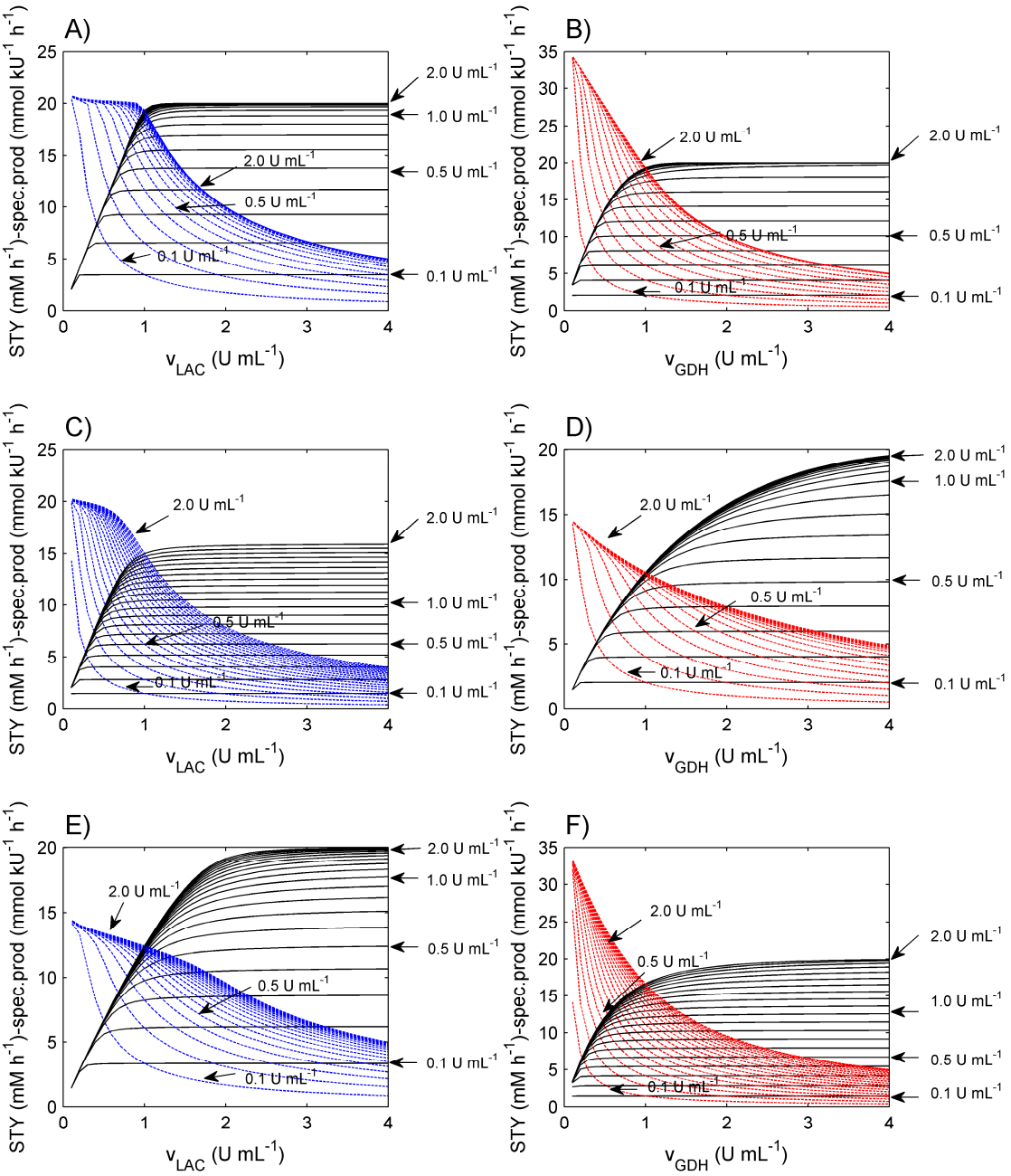

Figure 12. Isoproductivity plots based on the specific productivity after 10 h of reaction in regard to the laccase activity (A,C,E) and GDH (B,D,F) activity, simulated for the conditions in: Batch A and B (500 µM redox mediator and 500 µM coenzyme, Fig. A,B); Batch C (500 µM redox mediator and 100 µM coenzyme, Fig. C,D); Batch D (100 µM redox mediator and 500 µM coenzyme, Fig. E,F);. The activities indicated in the diagrams are the opposite enzyme as given on the X-axis. STY (straight lines), specific productivity (dashed lines).

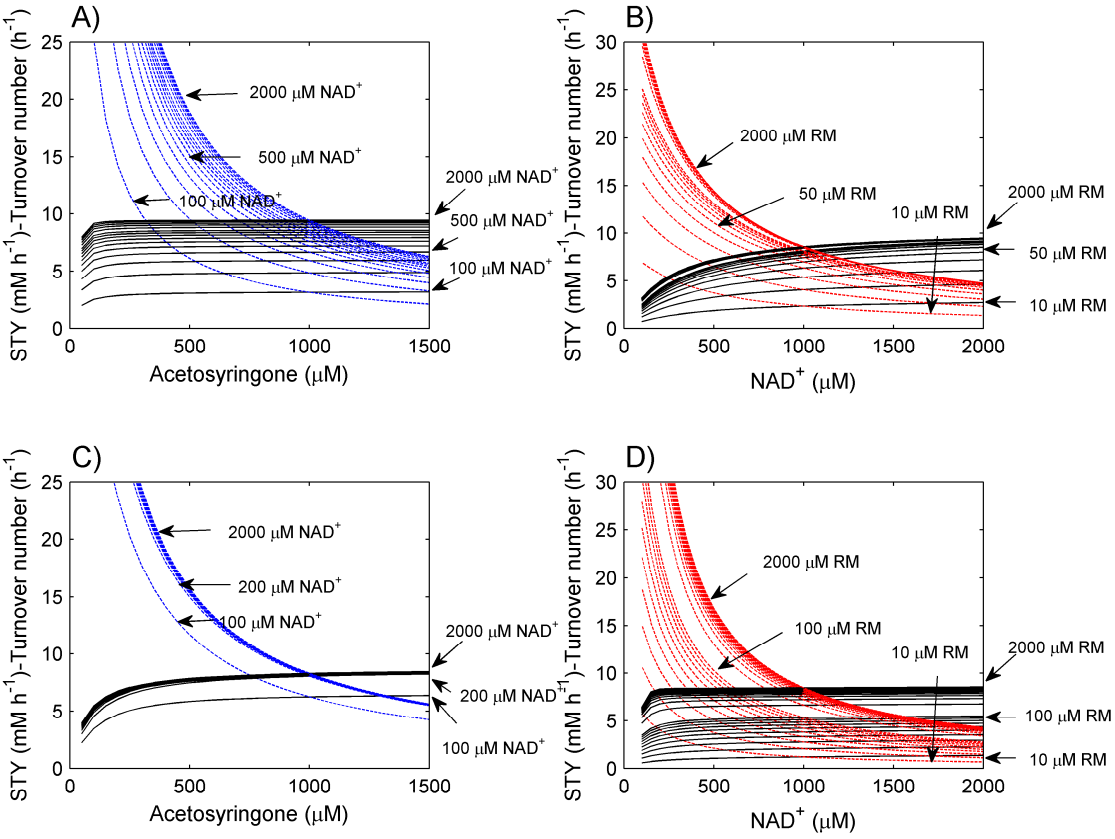

Figure 13. Effect of the initial redox mediator concentration (A, C) and coenzyme concentration (B,D) on STY and turnover numbers for the coenzyme and redox mediator after 10 h of reaction. The simulated laccase:GDH activity ratio corresponds to that used in: Batch A (900 U L^{-1} laccase and 220 U L^{-1} GDH, Fig A,B); Batch B (380 U L^{-1} laccase and 480 U L^{-1} GDH, Fig C,D). STY (straight lines), turnover numbers (dashed lines), NAD^+ stands for the total of applied coenzyme which was in the oxidized form.

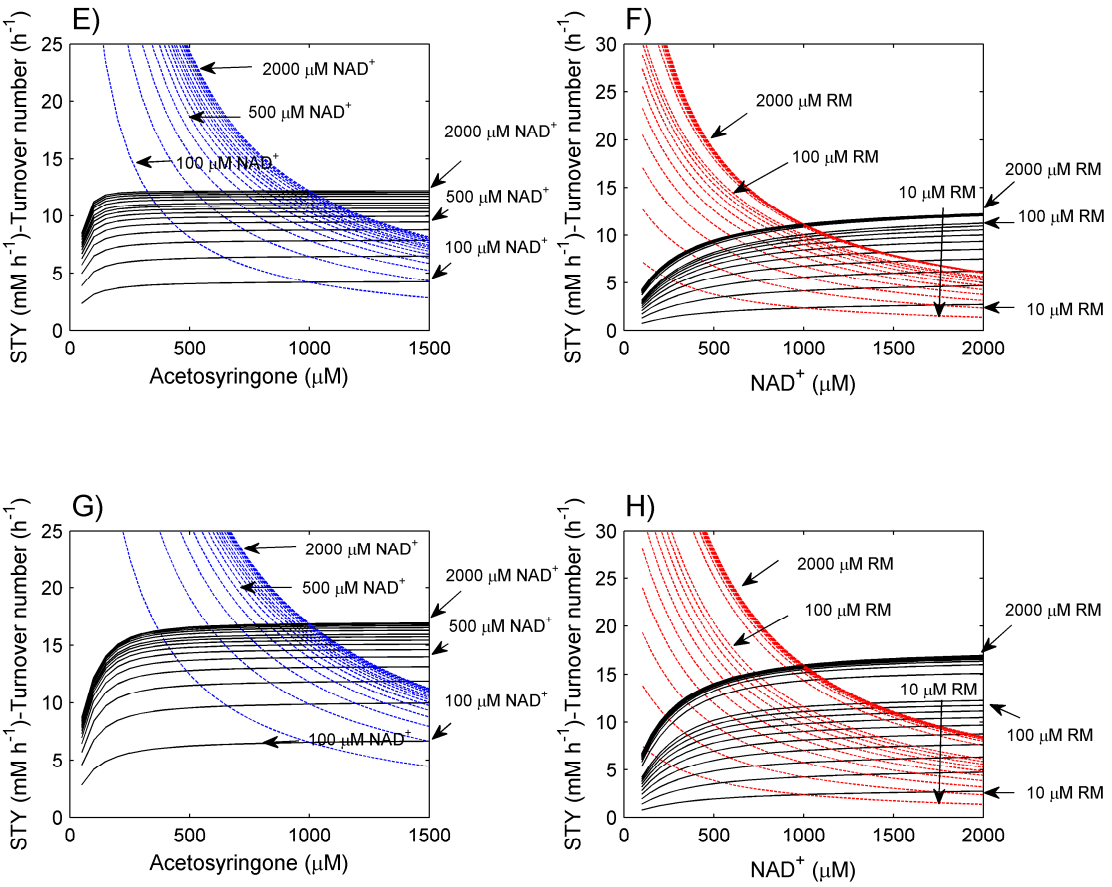

Figure 14. Effect of the initial redox mediator concentration (A, C) and coenzyme concentration (B, D) on STY and turnover numbers for the coenzyme and redox mediator after 10 h of reaction. The simulated laccase:GDH activity ratio corresponds to that used in: Batch C (900 U L^{-1} laccase and 300 U L^{-1} GDH, Fig A,B); Batch D (900 U L^{-1} laccase and 500 U L^{-1} GDH, Fig C,D). STY (straight lines), turnover numbers (dashed lines), NAD^+ stands for the total of applied coenzyme which was in the oxidized form.

3.1.4 Limiting activity of the regenerating enzyme

The settings for Batch B were selected to test the model of the regeneration system (laccase and redox mediator) and investigate the effect of a decreased efficiency of the regeneration system by making the laccase reaction rate limiting. The ratio of laccase:GDH volumetric activities was 0.8 while all other parameters were kept constant (**Table 9**). During 10 h 37.7% of the glucose was converted, which correlated very well with the predicted degree of conversion. The measured and predicted data for Batch B are shown in **Figure 10B**. The modeled, averaged NAD^+ concentration during time is ~170 µM (**Table 10**) and acetosyringone concentration is ~478 µM (**Table 11**). The measured activity decreased by 15% for laccase and 17% for GDH during these initial 10 h of reaction. The total turnover number after 10 h was 1.99×10^7 for laccase and 1.19×10^6 for GDH. The averaged enzyme consumption numbers for laccase and GDH were 0.76 and 1.08 U $mmol^{-1}$ product, respectively.

Table 10. Predicted NAD^+ concentration (µM) in the modeled batch conversion experiments. The initial coenzyme concentration is given at time 0. The coenzyme was added to the reactions in the form of NAD^+.

Time (h)	Batch			
	A	B	C	D
0	500.0	500.0	100.0	500.0
0.1	484.0	167.4	91.7	224.2
0.2	484.2	156.7	91.7	221.1
0.5	484.5	159.1	91.8	226.1
1	484.8	162.6	91.9	232.3
2	485.2	167.0	92.0	240.3
3	485.4	169.8	92.0	245.3
4	485.5	172.0	92.1	249.4
5	485.6	174.0	92.1	253.2
6	485.7	176.0	92.1	257.3
7	485.9	178.2	92.2	261.8
8	486.0	180.7	92.2	266.8
9	486.1	183.4	92.2	272.7
10	486.3	186.4	92.2	279.4

3.1.5 Limiting coenzyme concentration

Batch C was conducted to evaluate the effect of a decreased coenzyme concentration on the coenzyme regeneration and especially the synthetic reaction. In this experiment, the coenzyme concentration was 5-fold lower compared to Batches A and B, while keeping the laccase:GDH activity ratio high (3:1) (**Table 2**). The observed glucose conversion in 10 h was 17.7%. The correlation with the predicted degree of conversion of 19.9% is good, further measured and predicted data for Batch C are shown in **Figure 3C**. The modeled, averaged NAD^+ concentration during the first ten hours is very steady: ~92 µM (**Table 10**) and acetosyringone concentration is ~15 µM (**Table 11**). During the conversion the measured laccase activity decreased by 15.6% and GDH activity by 54.8%. The total turnover number after 10 h of laccase was 3.87×10^6 and of GDH 8.86×10^5. Both enzymatic activities decreased linearly. The averaged enzyme consumption numbers for laccase and GDH were 3.97 and 4.64 U $mmol^{-1}$, respectively.

Table 11. Predicted acetosyringone concentration (µM) in the modeled batch conversion experiments. The initial redox mediator concentration is given at time 0. The redox mediator was added to the reactions in its reduced form, the concentration of the oxidized form, the acetosyringone phenoxy radical, can be calculated by subtracting the indicated acetosyringone concentration from the applied concentration.

Time (h)	Batch			
	A	B	C	D
0	500.0	500.0	500.0	100.0
0.1	38.3	478.1	16.2	64.2
0.2	37.7	478.9	16.1	64.6
0.5	36.7	478.8	15.9	64.1
1	35.5	478.5	15.7	63.4
2	34.3	478.3	15.5	62.6
3	33.7	478.1	15.4	62.0
4	33.2	477.9	15.3	61.5
5	32.8	477.8	15.2	61.1
6	32.5	477.6	15.1	60.6
7	32.1	477.5	15.1	60.0
8	31.7	477.3	15.0	59.4
9	31.3	477.1	15.0	58.6
10	30.9	476.9	14.9	57.7

3.1.6 Limiting the redox mediator concentration

In the fourth experiment, Batch D, the initial redox mediator concentration was decreased 5-fold from standard conditions to evaluate the effect of decreased redox mediator concentration on both enzymatic cycles. The laccase:GDH activity ratio was 1.8:1 (**Table 2**). Glucose conversion was only 13% in 10 h, which was a result of an almost complete stop of the reaction after 4 h. The measured conversion after 1 h was 7.40%, which correlates roughly with the predicted value of 5.29% when considering the difference of actually added GDH activity and V_{max}. However, for later time points a much lower conversion is measured than predicted by the model. Data for Batch D are shown in **Figure 3D**. The modeled, averaged NAD^+ concentration during the first hour is ~240 µM (**Table 10**) and acetosyringone concentration is ~60 µM (**Table 11**). The measured specific productivity of GDH for the first hour is 29.5 mmol kU^{-1} h^{-1} and the second highest after Batch A.

However, after 1 h a dramatic drop in the specific productivity of GDH and laccase is observed. During the first 2the measured laccase activity decreases faster than afterwards, but the total loss of activity after 10 h is only 20.1%. Little GDH deactivation is observed in this reaction (~5%). The total turnover number after 10 h of laccase was 2.92×10^6 and of GDH 3.93×10^5. The averaged enzyme consumption numbers for laccase and GDH were 6.96 and 0.96 U $mmol^{-1}$, respectively. The model was used to calculate the theoretical, residual amount of redox mediator present in Batch D, based on the deviation of predicted and measured reaction rate for each hour. The reduction of the acetosyringone concentration was calculated to be 54, 15.6 and 7.3 µM after 2, 3 and 4 h, respectively.

4 Discussion

4.1 Modelling lactose hydrolysis and transgalactosylation using β-galactosidases from *Lactobacillus reuteri*

The proposed model explained well not only the lactose hydrolysis and galacto-oligosaccharide systhesis but also the thermal deactivation of the β-galactosidase from *L.reuteri* L103 and L461 at various initial concentration of substrate and various reaction conditions (**Figure 4, Figure** 5). The conversion data are in good agreement with the predictions of the derived reaction rate equation. In all case, the glucose concentration was higher than galactose concentration, indicating that some of the galactose formed during the hydrolysis of lactose was used in the transgalactosylation reaction for the formation of GOS.

The fitted Michaelis-Menten constant K_M (13.6 mM) of β-galactosidase L103 for the initial lactose concentration of 205 g L^{-1}, at the conditions of 30^0C and pH 6.5, is closed to the apparent K_M (13 mM) value of the enzyme for lactose obtained in measuring standard Michaelis-Menten kinetics varying different lactose concentrations up to 205 g L^{-1}, as determined by initial rate experiments (**Table 5**). This is also true for the enzyme β-galactosidase L461 for the same initial lactose concentration and the same conditions, here the fitted K_M estimated by the model (30.3 mM) is closed to the experimental value (31 mM). The fitted galactose inhibition K_i (200 mM) for β-galactosidase L103 was found to be somewhat higher than the its experimentally determined values (188 mM) for the initial lactose concentration of 205 g L^{-1}, at the condition of 30 ^{0}C and pH 6.5. And also, the fitted galactose inihibition for β-galactosidase L461 (99.4 mM) estimated by the model is higher than its experimental value for the same initial lactose concentration and at the same condition.

Lactose conversion was followed for various initial lactose concentrations at 25, 30 and 37^0C for both enzymes L103 and L461. **Figure 4B, C, D** and **Figure 5B, C, D** show the conversion at an initial lactose concentration of 205 g L^{-1}. The reaction rate increases with

temperature. The GOS yield was defined as percent of the g L^{-1} GOS per initial concentration g L^{-1} of initial lactose. For these 3 temperatures, the measured GOS yields maximum at 38.65, 38.74, 40.23 % for reaction with β-galactosidase from L103; 34.70, 37.23 39.27% for reaction with β-galactosidase from L461 at 25, 30 and 37^{0}C, respectively. The effect of the temperature on maximum GOS yield for β-galactosidase from L461 is more pronounced than for β-galactosidase from L103.

The approach of the Genetic Algorithm carried out by the Matlab R2009a program was applied to estimate the kinetic parameters. This algorithm has certain advantages: it can solve every optimisation problem which can be described with the chromosome encoding; it solves problems with multiple solutions; since the genetic algorithm execution technique is not dependent on the error surface, it can be used to solve multi-dimensional, non-differential, non-continuous, and even non-parametrical problems. Structural genetic algorithm gives us the possibility to solve the solution structure and solution parameter problems at the same time.

By using the simulation model, the K_M for β-galactosidase increases when temperature increases (10.7, 13.6, 15.5 mM for L103 and 24.1, 30.3, 33.2 mM for L461 at 25, 30, 37^{0}C, respectively). The coefficient of determination (R^2) in **Table 7** was higher than 0.985 for all 6 batches, indicating that the data by simulation fit well to experimental data.

4.2 Engineering an Enzymatic Regeneration System for NAD(P)H Oxidation [6]

To find the best pH for coupling acidic *T. pubescens* laccase and GDH, the pH optimum for the two previously published, NADH oxidizing redox mediators acetosyringone and syringaldehyde were determined [98]. The rate constants with the laccase at pH 5.0, were high enough to use the selected laccase together with GDH. The catalytic performance

[6] Part of the:
Article submitted to *Journal of Molecular Catalysis B:* **Pham, N.H.**, Hollmann,F., Kracher,D., Preims,M., Haltrich, D., Ludwig,R: Engineering an Enzymatic Regeneration System for NAD(P)H Oxidation.

of GDH at pH 5.0 compared to pH 8.0 [116] is still good enough to allow its application. One potential problem arising from employing GDH at low pH is the low hydrolysis rate of the reaction product gluconolactone, which results in product inhibition. Another critical factor is the supply of oxygen, which governs the regenerating laccase reaction. Therefore, the employed enzymatic activities were selected sufficiently low to avoid critical gluconolacone build-up or a decrease of the oxygen concentration in the reactor. Laccase from *T. pubescens* exhibits more favorable catalytic constants for acetosyringone than syringaldehyde at pH 5.0, which was one reason to study acetosyringone as redox mediator in this work. The other reason to employ acetosyringone was its higher rate constants with the coenzymes NADH and NADPH. The rate constants obtained for the reaction of syringaldehyde or acetosyringone with NADH were 1.3 – 2-fold higher than that for NADPH. The phosphorylation of the coenzyme reduces the re-oxidation rate. Considering the similar redox potential of both coenzyme forms (-320 mV vs. SHE) this is an unecpected result. The reason might be the additional negatively charged phosphate group in NADPH which may lead to increased electrostatic repulsion with the deprotonated phenolate in acetosyringone.

The experimental evaluation of the Matlab model for the GDH reaction was based on Batch A in which a high laccase activity was employed to ensure a high NAD^+ concentration. According to the model ~460 µM NAD^+ was present during the reaction. This allows the verification of the model for the synthetic GDH reaction without limitation from the laccase regeneration system. The difference of the observed and predicted substrate conversion during in first two hours shows that the activity obtained with the used assay does not reliably estimates the V_{max} of GDH, but underestimates it by a factor of ~1.6, because of the limited glucose (K_M = 16.7 mM, S = 100 mM), but especially the limited coenzyme concentration (K_M = 260 µM, S = 500 µM) in the assay. GDH deactivation during the process leads to a convergence of the actual and predicted glucose conversion.

The experimental evaluation of the Matlab model for the laccase reaction was based on Batch B, which employed a low laccase activity to keep the concentration of the reduced form of acetosyringone high. The model predicted a concentration of 478 µM

acetosyringone. The volumetric activity of laccase was well estimated by the enzymatic assay and its conversion from ABTS to acetosyringone activity by the estimated factor of 1:0.54. Since the oxygen concentration was kept constant, no deviation between the volumetric activity and V_{max} was found.

Process modeling and engineering was based on the verified Matlab model. The influence of different laccase:GDH activity ratios, redox mediator or coenzyme concentrations were modeled and useful activities and concentrations selected for the conversion experiments based on the model. The productivity plots show that the initial concentration of redox mediator and coenzyme together with the activity of regenerating and synthetic enzyme govern STY **(Figure 11**). At high concentrations of the coenzyme and redox mediator (500 µM each, **Figure 11A**), which are significantly above the K_M values of laccase and GDH, neither r_1 nor r_3 are limited. Also the bimolecular rate constant for the oxidized acetosyringone/NADH (294 M^{-1} $s^{-}1$) ensures that for the selected concentrations r_2 is high enough (73.5 µM s^{-1}) to support enzymatic activities up to 4400 U L^{-1}. With such a high efficiency of the mediator system, the activities of both enzymes and their ratio become dominant for the STY and specific productivities. Enzyme ratios for Batch A and B were selected to investigate the sensitivity of one enzyme from the other. For a reduced coenzyme concentration (100 µM, **Figure 11B**) a limiting effect of r_3 was predicted due to a NAD^+ concentration below the K_M of GDH for its coenzyme. This results in a low predicted STY (4.06 mM h^{-1}) and a reaction that is little sensitive towards changes in GDH activity and even less towards changes in laccase activity. The conditions or Batch C were chosen from this plot. To study the effect of a reduced redox mediator concentration (100 µM) the conditions for Batch D were derived from **Figure 11C**. The reduced redox mediator concentration shows only a moderate effect in the simulation which is based on the laccase activity of Batch A and the GDH activity of Batch B. The predicted STY for Batch D (10.17 mM h^{-1}) results from the optimized enzymatic activities. The relative insensitivity towards the reduced redox mediator concentration compared to the coenzyme is explained by the lower K_M value of laccase for acetosyringone than in the case of GDH for NAD^+.

Specific productivities of enzymes under the three selected redox mediator/coenzyme concentrations are plotted in **Figure 12**. By following the isoactivity curves for laccase and GDH the critical activities below which the STY drops can be found. The maximal specific productivity of laccase at 500 µM acetosyringone and 500 µM NADH is predicted to be 20.1 mmol kU^{-1} h^{-1} and the maximum specific productivity of GDH is 33 mmol kU^{-1} h^{-1}. A good compromise to achieve high specific productivities for laccase and GDH can be reached when the activity ratio of laccase:GDH is about 2:1. In the case of 500 µM acetosyringone and 100 µM NADH, the maximum specific productivity of laccase is unchanged, but the specific productivity of GDH is reduced to 14 mmol kU^{-1} h^{-1}, caused by the low possible NAD^+ concentration. Under these conditions, the best compromise to achieve high specific productivities for laccase and GDH is reached when the activity ratio of laccase:GDH is about 0.6:1. For the last case (100 µM acetosyringone, 500 µM NADH) the maximum specific productivity of laccase is lower (14 mmol kU^{-1} h^{-1}), whereas the specific productivity of GDH is the same as for Batch A and B (33 mmol kU^{-1} h^{-1}). The reason is the low possible concentration of reduced acetosyringone. Under these conditions, the best compromise to achieve high specific productivities for laccase and GDH is reached when the activity ratio of laccase:GDH is about 1.5:1.

For the activity ratios used in Batches A-D the following initial acetosyringone and NADH concentrations were predicted to be necessary to obtain a good STY (**Figure 13**, **Figure 14**). Batch A (activity ratio laccase:GDH 4.1:1): 100 µM acetosyringone and 1000 µM NADH; Batch B (activity ratio 0.8:1) 500 µM acetosyringone and 200 µM NADH; Batch C (activity ratio 3:1) 200 µM acetosyringone and 1000 µM NADH; Batch D (activity ratio 1.8:1) 300 µM acetosyringone and 1000 µM NADH. Of course, these concentrations were not applied, but given here to demonstrate that at activity ratios of laccase:GDH >1.5:1 the availability of the coenzyme governs the reaction rate, whereas in the case of a limiting laccase activity the redoxmediator concentration governs the reaction rate.

These predictions were tested by two Batch reactions: Batch C employing a limiting coenzyme concentration and Batch D with a limiting redox mediator concentration. For

Batch C, an available NAD^+ concentration of 92 µM from the initially added 100 mM was predicted. Despite the efficient regeneration of NADH the low coenzyme concentration is the rate limiting step in the reaction (r_3). In Batch D, the predicted concentration (~60 µM) of the oxidized redox mediator acetosyringone was sufficient to keep r_1 high. A reflection of the high catalytic efficiency of laccase for the oxidation of acetosyringone. However, in contrast to the model, the experiment showed an unexpected effect on the acetosyringone phenoxy radical. A polymerization reaction of the redox mediator occurred, which reduced the amount of the redox mediator drastically during the first 3 h and stopped the reaction.

The enzyme deactivation observed in the batch conversion processes is reltively low and high total turnover numbers of $>10^6$ were obtained. However, certain deactivation patterns are observed. In Batches A and B the measured enzymatic activities decreased linearly, which indicates that enzyme deactivation is proportional to product formation. The loss of activity was moderate. Enzyme consumption numbers of 0.76 – 1.08 U mM^{-1} formed product were observed. Only the inactivation of laccase in Batch A was higher (2.81 U mM^{-1}). Probably an effect of the higher concentration of the acetosyringone phenoxy radical. Under conditions which favor a high radical concentration even more (Batch C) the enzyme consumption number for both enzymes increases to 4 and 4.6 U mM^{-1}. The higher enzyme consumption is also observed for GDH and indicates that the deactivation caused by acetosyringone phenoxy radicals is concentration dependent and not connected with substrate turnover. Even more interesting results were found under a limiting redox mediator concentration in Batch D. After a short time a color change in the reaction vessel was observed, which indicates a degradation of the redox mediator acetosyringone. The most probable cause is a dimerization/polymerization by coupling of the formed phenoxy radicals or coupling to proteins. The low concentration of the redox mediator made this process obvious. A fast reduction of the acetosyringon phenoxy radical by NADH should stabilize the redox mediator in the process. Together with the destruction of the redox mediator also a deactivation of laccase slows down after the first hours and follows similar to GDH a time-dependent process.

5 Conclusions

The first developed model for a single-enzyme process based on β-galactosidase, can be used to describe galacto-oligosaccharides synthesis at various temperatures and lactose concentration. The effect of temperature is small compared to the influence of the initial lactose concentration. With one experimental time course, the model can estimate all significant parameters with high accuracy.

The second developed model for a bi-enzymatic process was used to model an optimized process based on the laccase/mediator system. A conversion process should fulfill the following criteria: i) A high concentration of NAD^+ to achieve a high specific GDH productivity. The NAD^+ concentration depends mostly on the initial coenzyme concentration and laccase activity. ii) A two-fold excess of laccase activity over GDH activity to ensure efficient regeneration of the coenzyme and high specific productivities of regenerating and synthetic enzymes. When the ratio drops, the NAD^+ concentration is low regardless of the added coenzyme concentration. iii) The enzyme activity ratio also affects the concentration of the oxidized redox mediator. High concentrations should be avoided to reduce enzyme deactivation. Also the redox mediator itself is susceptible to degradation and more stable in the reduced form. If necessary, the redox mediator has to be added during the reaction. iv) The enzyme activities can be increased to increase STY until the oxygen concentration becomes a limiting factor. v) Modeling can be elegantly used to optimize the enzymatic activities redox mediator and coenzyme concentrations to improve the productivity, stability and economics of the reaction.

6 References

[1] L. F. Pivarnik, A. G. Senecal, and A. G. Rand, “Hydrolytic and transgalactosylic activities of commercial beta-galactosidase (lactase) in food processing.,” *Adv. Food Nutr. Res.*, vol. 38, pp. 1–102, Jan. 1995.

[2] J. E. Prenosil, E. Stuker, and J. R. Bourne, “Formation of oligosaccharides during enzymatic lactose: Part I: State of art.,” *Biotechnol. Bioeng.*, vol. 30, no. 9, pp. 1019–25, Dec. 1987.

[3] T. Nakayama ; T.Amachi, *Encyclopedia of bioprocess technology: fermentation, biocatalysis, and bioseparation*. New York: John Willey and Sons, 1999, pp. 1291–1305.

[4] I. Petzelbauer, R. Zeleny, A. Reiter, K. D. Kulbe, and B. Nidetzky, “Development of an ultra-high-temperature process for the enzymatic hydrolysis of lactose: II. Oligosaccharide formation by two thermostable beta-glycosidases.,” *Biotechnol. Bioeng.*, vol. 69, no. 2, pp. 140–9, Jul. 2000.

[5] R. R. Mahoney, “Galactosyl-oligosaccharide formation during lactose hydrolysis: A review,” *Food Chem.*, vol. 63, no. 2, pp. 147–154, 1998.

[6] T. Sako, K. Matsumoto, and R. Tanaka, “Recent progress on research and applications of non-digestible,” *Int. Dairy J.*, vol. 9, pp. 69–80, 1999.

[7] B. Splechtna, T.-H. Nguyen, M. Steinbock, K. D.Kulbe, W. Lorenz, and D. Haltrich, “Production of Prebiotic Galacto-Oligosaccharides from Lactose Using β-Galactosidases from Lactobacillus reuteri,” *J. Agric. Food Chem.*, vol. 54, pp. 4999–5006, 2006.

[8] M. Boon, A. Janssen, and van ’t Riet K, “Effect of temperature and enzyme origin on the enzymatic synthesis of oligosaccharides.,” *Enzyme Microb. Technol.*, vol. 26, no. 2–4, pp. 271–281, Feb. 2000.

[9] T.-T. Nguyen, H. A. Nguyen, S. L. Arreola, G. Mlynek, K. Djinović-Carugo, G. Mathiesen, T.-H. Nguyen, and D. Haltrich, “Homodimeric β-galactosidase from Lactobacillus delbrueckii subsp. bulgaricus DSM 20081: expression in Lactobacillus plantarum and biochemical characterization.,” *J. Agric. Food Chem.*, vol. 60, no. 7, pp. 1713–21, Feb. 2012.

[10] S. Iqbal, T. Nguyen, H. A. Nguyen, T. T. Nguyen, T. Maischberger, R. Kittl, and D. Haltrich, “Characterization of a Heterodimeric GH2 β -Galactosidase from Lactobacillus sakei Lb790 and Formation of Prebiotic,” *J. Agric. Food Chem.*, vol. 59, no. 8, pp. 3803–3811, 2011.

[11] T.-H. Nguyen, B. Splechtna, M. Yamabhai, D. Haltrich, and C. Peterbauer, “Cloning and expression of the beta-galactosidase genes from Lactobacillus reuteri in Escherichia coli.,” *J. Biotechnol.*, vol. 129, no. 4, pp. 581–91, May 2007.

[12] T.-H. Nguyen, B. Splechtna, S. Krasteva, W. Kneifel, K. D. Kulbe, C. Divne, and D. Haltrich, “Characterization and molecular cloning of a heterodimeric beta-galactosidase from the probiotic strain Lactobacillus acidophilus R22.,” *FEMS Microbiol. Lett.*, vol. 269, no. 1, pp. 136–44, Apr. 2007.

[13] S. Iqbal, T.-H. Nguyen, T. T. Nguyen, T. Maischberger, and D. Haltrich, “beta-Galactosidase from Lactobacillus plantarum WCFS1: biochemical characterization and formation of prebiotic galacto-oligosaccharides.,” *Carbohydr. Res.*, vol. 345, no. 10, pp. 1408–16, Jul. 2010.

[14] G. X. Liu, J. Kong, W. W. Lu, W. T. Kong, H. Tian, X. Y. Tian, and G. C. Huo, “β-Galactosidase with transgalactosylation activity from Lactobacillus fermentum K4.,” *J. Dairy Sci.*, vol. 94, no. 12, pp. 5811–20, Dec. 2011.

[15] D. Gobinath and S. G. Prapulla, “Permeabilized probiotic Lactobacillus plantarum as a source of β-galactosidase for the synthesis of prebiotic galactooligosaccharides.,” *Biotechnol. Lett.*, vol. 36, no. 1, pp. 153–7, Jan. 2014.

[16] T. Maischberger, E. Leitner, S. Nitisinprasert, O. Juajun, M. Yamabhai, T.-H. Nguyen, and D. Haltrich, “Beta-galactosidase from Lactobacillus pentosus: purification, characterization and formation of galacto-oligosaccharides.,” *Biotechnol. J.*, vol. 5, no. 8, pp. 838–47, Aug. 2010.

[17] T. Goulas, A. Goulas, G. Tzortzis, and G. R. Gibson, “Comparative analysis of four beta-galactosidases from Bifidobacterium bifidum NCIMB41171: purification and biochemical characterisation.,” *Appl. Microbiol. Biotechnol.*, vol. 82, no. 6, pp. 1079–88, Apr. 2009.

[18] C. Nie, B. Liu, Y. Zhang, G. Zhao, X. Fan, X. Ning, and W. Zhang, “Production and secretion of Lactobacillus crispatus β-galactosidase in Pichia pastoris.,” *Protein Expr. Purif.*, vol. 92, no. 1, pp. 88–93, Nov. 2013.

[19] B. A. Rabiu, A. J. Jay, G. R. Gibson, and R. A. Rastall, “Synthesis and fermentation properties of novel galacto-oligosaccharides by beta-galactosidases from Bifidobacterium species.,” *Appl. Environ. Microbiol.*, vol. 67, no. 6, pp. 2526–30, Jun. 2001.

[20] K. Kawabata, Y. Sugiyama, T. Sakano, and H. Ohigashi, “Flavonols enhanced production of anti-inflammatory substance(s) by Bifidobacterium adolescentis: prebiotic actions of galangin, quercetin, and fisetin.,” *Biofactors*, vol. 39, no. 4, pp. 422–9, 2013.

[21] S. H. Yi, I. Alli, K. H. Park, and B. Lee, “Overexpression and characterization of a novel transgalactosylic and hydrolytic β-galactosidase from a human isolate Bifidobacterium breve B24.,” *N. Biotechnol.*, vol. 28, no. 6, pp. 806–13, Oct. 2011.

[22] F. Depeint, G. Tzortzis, J. Vulevic, K. I’anson, and G. R. Gibson, “Prebiotic evaluation of a novel galactooligosaccharide mixture produced by the enzymatic activity of Bifidobacterium bifidum NCIMB 41171, in healthy humans: a randomized, double-blind, crossover, placebo-controlled intervention study.,” *Am. J. Clin. Nutr.*, vol. 87, no. 3, pp. 785–91, Mar. 2008.

[23] D. P. M. Torres, M. D. P. F. Gonçalves, J. a. Teixeira, and L. R. Rodrigues, “Galacto-Oligosaccharides: Production, Properties, Applications, and Significance as Prebiotics,” *Compr. Rev. Food Sci. Food Saf.*, vol. 9, no. 5, pp. 438–454, Aug. 2010.

[24] Biocon.Española; S.A, “Documentros Biocon Biolactasa NTL Conc.” [Online]. Available: http://www.biocon.es/en/productos-documentos.php?id=36.

[25] B. Rodriguez-Colinas, L. Fernandez-Arrojo, A. O. Ballesteros, and F. J. Plou, “Galactooligosaccharides formation during enzymatic hydrolysis of lactose: towards a prebiotic-enriched milk.,” *Food Chem.*, vol. 145, pp. 388–94, Feb. 2014.

[26] “Amano Enzyme Japan Dictionary of Enzymes.” [Online]. Available: http://www.amano-enzyme.co.jp/eng/enzyme/23.html.

[27] T. Palai, S. Mitra, and P. K. Bhattacharya, “Kinetics and design relation for enzymatic conversion of lactose into galacto-oligosaccharides using commercial grade β-galactosidase.,” *J. Biosci. Bioeng.*, vol. 114, no. 4, pp. 418–23, Oct. 2012.

[28] P. S. Panesar, S. Kumari, and R. Panesar, “Potential Applications of Immobilized β-Galactosidase in Food Processing Industries.,” *Enzyme Res.*, vol. 2010, p. 473137, Jan. 2010.

[29] T.-T. Nguyen, H. A. Nguyen, S. L. Arreola, G. Mlynek, K. Djinović-Carugo, G. Mathiesen, T.-H. Nguyen, and D. Haltrich, “Homodimeric β-galactosidase from Lactobacillus delbrueckii subsp. bulgaricus DSM 20081: expression in Lactobacillus plantarum and biochemical characterization.,” *J. Agric. Food Chem.*, vol. 60, no. 7, pp. 1713–21, Feb. 2012.

[30] T. Hansson, T. Kaper, J. van Der Oost, W. M. de Vos, and P. Adlercreutz, “Improved oligosaccharide synthesis by protein engineering of beta-glucosidase CelB from hyperthermophilic Pyrococcus furiosus.,” *Biotechnol. Bioeng.*, vol. 73, pp. 203–210, 2001.

[31] J. B. Moore, P. Markiewicz, and J. H. Miller, “Identification and sequencing of the Thermotoga maritima lacZ gene, part of a divergently transcribed operon,” *Gene*, vol. 147, no. 1, pp. 101–106, Sep. 1994.

[32] W. Chen, H. Chen, Y. Xia, J. Zhao, F. Tian, and H. Zhang, “Production, purification, and characterization of a potential thermostable galactosidase for milk lactose hydrolysis from Bacillus stearothermophilus.,” *J. Dairy Sci.*, vol. 91, no. 5, pp. 1751–8, May 2008.

[33] B. Di Lauro, A. Strazzulli, G. Perugino, F. La Cara, E. Bedini, M. M. Corsaro, M. Rossi, and M. Moracci, “Isolation and characterization of a new family 42 beta-galactosidase from the thermoacidophilic bacterium Alicyclobacillus acidocaldarius: identification of the active site residues.,” *Biochim. Biophys. Acta*, vol. 1784, no. 2, pp. 292–301, Feb. 2008.

[34] J. Marín-Navarro, D. Talens-Perales, A. Oude-Vrielink, F. J. Cañada, and J. Polaina, “Immobilization of thermostable β-galactosidase on epoxy support and its use for lactose hydrolysis and galactooligosaccharides biosynthesis.,” *World J. Microbiol. Biotechnol.*, vol. 30, no. 3, pp. 989–98, Mar. 2014.

[35] D. O. Otieno, “Synthesis of β-Galactooligosaccharides from Lactose Using Microbial β-Galactosidases,” *Compr. Rev. Food Sci. Food Saf.*, vol. 9, no. 5, pp. 471–482, Aug. 2010.

[36] K. K. Pulicherla, P. S. Kumar, K. Manideep, V. P. B. Rekha, M. Ghosh, and K. R. S. Sambasiva Rao, “Statistical approach for the enhanced production of cold-active β-galactosidase from Thalassospira frigidphilosprofundus: a novel marine psychrophile from deep waters of Bay of Bengal.,” *Prep. Biochem. Biotechnol.*, vol. 43, no. 8, pp. 766–80, Jan. 2013.

[37] A. Hoyoux, I. Jennes, P. Dubois, S. Genicot, F. Dubail, J. M. François, E. Baise, G. Feller, and C. Gerday, “Cold-adapted beta-galactosidase from the Antarctic psychrophile Pseudoalteromonas haloplanktis.,” *Appl. Environ. Microbiol.*, vol. 67, no. 4, pp. 1529–35, Apr. 2001.

[38] J. M. Hu, H. Li, L. X. Cao, P. C. Wu, C. T. Zhang, S. L. Sang, X. Y. Zhang, M. J. Chen, J. Q. Lu, and Y. H. Liu, “Molecular cloning and characterization of the gene encoding cold-active beta-galactosidase from a psychrotrophic and halotolerant Planococcus sp. L4.,” *J. Agric. Food Chem.*, vol. 55, no. 6, pp. 2217–24, Mar. 2007.

[39] P. Hildebrandt, M. Wanarska, and J. Kur, “A new cold-adapted beta-D-galactosidase from the Antarctic Arthrobacter sp. 32c - gene cloning, overexpression, purification and properties.,” *BMC Microbiol.*, vol. 9, p. 151, Jan. 2009.

[40] T. Nakagawa, R. Ikehata, T. Myoda, T. Miyaji, and N. Tomizuka, “Overexpression and functional analysis of cold-active beta-galactosidase from Arthrobacter psychrolactophilus strain F2.,” *Protein Expr. Purif.*, vol. 54, no. 2, pp. 295–9, Aug. 2007.

[41] A. Wierzbicka-Woś, H. Cieśliński, M. Wanarska, K. Kozłowska-Tylingo, P. Hildebrandt, and J. Kur, “A novel cold-active β-D-galactosidase from the Paracoccus sp. 32d--gene cloning, purification and characterization.,” *Microb. Cell Fact.*, vol. 10, no. 1, p. 108, Jan. 2011.

[42] V. Vincent, N. Aghajari, N. Pollet, A. Boisson, S. Boudebbouze, R. Haser, E. Maguin, and M. Rhimi, “The acid tolerant and cold-active β-galactosidase from Lactococcus lactis strain is an attractive biocatalyst for lactose hydrolysis.,” *Antonie Van Leeuwenhoek*, vol. 103, no. 4, pp. 701–12, Apr. 2013.

[43] T. Toba, A. Yokota, and S. Adachi, “Oligosaccharide structures formed during the hydrolysis of lactose by Aspergillus oryzae β-galactosidase,” *Food Chem.*, vol. 16, no. 2, pp. 147–162, Jan. 1985.

[44] R. Crittenden, “Prebiotics and probiotics - the importance of branding.,” *Microb. Ecol. Health Dis.*, vol. 23, Jan. 2012.

[45] P. K. Gopal, P. A. Sullivan, and J. B. Smart, “Utilisation of galacto-oligosaccharides as selective substrates for growth by lactic acid bacteria including Bi x dobacterium lactis DR10 and Lactobacillus rhamnosus DR20,” *Int. Dairy J.*, vol. 11, pp. 19–25, 2001.

[46] V. Sangwan, S. K. Tomar, R. R. B. Singh, a K. Singh, and B. Ali, “Galactooligosaccharides: novel components of designer foods.,” *J. Food Sci.*, vol. 76, no. 4, pp. R103–11, May 2011.

[47] B. L. Cantarel, P. M. Coutinho, C. Rancurel, T. Bernard, V. Lombard, and B. Henrissat, “The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics.,” *Nucleic Acids Res.*, vol. 37, no. Database issue, pp. D233–8, Jan. 2009.

[48] B. M. De Roode, M. C. R. Franssen, A. Van Der Padt, and R. M. Boom, “Perspectives for the Industrial Enzymatic Production of Glycosides,” *Biotechnol. Prog.*, vol. 19, pp. 1391–1402, 2003.

[49] E. Yoshida, H. Sakurama, M. Kiyohara, M. Nakajima, M. Kitaoka, H. Ashida, J. Hirose, T. Katayama, K. Yamamoto, and H. Kumagai, “Bifidobacterium longum subsp. infantis uses two different β-galactosidases for selectively degrading type-1 and type-2 human milk oligosaccharides.,” *Glycobiology*, vol. 22, no. 3, pp. 361–8, Mar. 2012.

[50] T. Goulas, A. Goulas, G. Tzortzis, and G. R. Gibson, “Expression of four beta-galactosidases from Bifidobacterium bifidum NCIMB41171 and their contribution on the hydrolysis and synthesis of galactooligosaccharides.,” *Appl. Microbiol. Biotechnol.*, vol. 84, no. 5, pp. 899–907, Oct. 2009.

[51] C. Schwab, K. I. Sørensen, and M. G. Gänzle, “Heterologous expression of glycoside hydrolase family 2 and 42 β-galactosidases of lactic acid bacteria in Lactococcus lactis.,” *Syst. Appl. Microbiol.*, vol. 33, no. 6, pp. 300–7, Oct. 2010.

[52] A.-R. Park and D.-K. Oh, “Galacto-oligosaccharide production using microbial beta-galactosidase: current state and perspectives.,” *Appl. Microbiol. Biotechnol.*, vol. 85, no. 5, pp. 1279–86, Feb. 2010.

[53] N. Albayrak and S.-T. Yang, “Production of galacto-oligosaccharides from lactose by Aspergillus oryzae beta-galactosidase immobilized on cotton cloth.,” *Biotechnol. Bioeng.*, vol. 77, no. 1, pp. 8–19, Jan. 2002.

[54] S. N. K. Iwasaki, M. Nakajimab, “Galacto-oligosaccharide Production from Lactose by an Enzymic Batch Reaction Using β-Galactosidase,” *Process Biochem.*, vol. 31, no. 1, pp. 69–76, 1996.

[55] H. Shin, J. Park, and J. Yang, “Continuous production of galacto-oligosaccharides from lactose by Bullera singularis fi-galactosidase immobilized in chitosan beads,” *Process Biochem.*, vol. 33, no. 8, pp. 787–792, 1998.

[56] K. Isobe, M. Yamashita, S. Chiba, N. Takahashi, and T. Koyama, “Characterization of new β-galactosidase from acidophilic fungus, Teratosphaeria acidotherma AIU BGA-1.,” *J. Biosci. Bioeng.*, vol. 116, no. 3, pp. 293–7, Sep. 2013.

[57] S. T. Yang and M. R. Okos, “A new graphical method for determining parameters in Michaelis-Menten-type kinetics for enzymatic lactose hydrolysis.,” *Biotechnol. Bioeng.*, vol. 34, no. 6, pp. 763–73, Sep. 1989.

[58] C. Carrara and A. Rubiolo, “Determination of kinetics parameters for free and immobilized β-galactosidase,” *Process Biochem.*, vol. 31, no. 3, pp. 243–248, 1996.

[59] M. Di Serio, C. Maturo, E. De Alteriis, P. Parascandola, R. Tesser, and E. Santacesaria, “Lactose hydrolysis by immobilized β-galactosidase: the effect of the supports and the kinetics,” *Catal. Today*, vol. 79–80, pp. 333–339, Apr. 2003.

[60] M. Ladero, a Santos, and F. García-Ochoa, “Kinetic modeling of lactose hydrolysis with an immobilized beta-galactosidase from Kluyveromyces fragilis.,” *Enzyme Microb. Technol.*, vol. 27, no. 8, pp. 583–592, Nov. 2000.

[61] M. Yi and Q. Liu, “Michaelis–Menten mechanism for single-enzyme and multi-enzyme system under stochastic noise and spatial diffusion,” *Phys. A Stat. Mech. its Appl.*, vol. 389, no. 18, pp. 3791–3803, Sep. 2010.

[62] T. G.-V. J Berrueta-Jiménez, “Hidrólisis enzimática de lactosa en reactores de lecho fijo,” *Ing. Química*, vol. 246, pp. 141–146, 1988.

[63] E. A. F. Fontes, F. M. L. Passos, and F. J. V. Passos, “A mechanistical mathematical model to predict lactose hydrolysis by β-galactosidase in a permeabilized cell mass of Kluyveromyces lactis: validity and sensitivity analysis,” *Process Biochem.*, vol. 37, no. 3, pp. 267–274, Nov. 2001.

[64] D. C. D Cavaille, “Characterization of beta-galactosidase from Kluyveromyces lactis,” *Biotechnol. Appl. Biochem.*, vol. 22, no. 1, pp. 55–64, 1995.

[65] A. Santos, M. Ladero, and F. Garcıa-Ochoa, “Kinetic Modeling of Lactose Hydrolysis by a β-Galactosidase from Kluyveromices Fragilis,” *Enzyme Microb. Technol.*, vol. 0229, no. 97, pp. 558–567, 1998.

[66] T. Palai and P. K. Bhattacharya, “Kinetics of lactose conversion to galacto-oligosaccharides by β-galactosidase immobilized on PVDF membrane.,” *J. Biosci. Bioeng.*, vol. 115, no. 6, pp. 668–73, Jun. 2013.

[67] C. R. Carrara and A. C. Rubiolo, “Immobilization of .beta.-galactosidase on chitosan,” *Biotechnol. Prog.*, vol. 10, no. 2, pp. 220–224, Mar. 1994.

[68] M. . A. O Adali, T Baran, “Characterization of β-galactosidase immobilized into poly(hydroxyethyl) methacrylate membranes,” *Biochem. Arch.*, vol. 14, no. 2, pp. 123–129, 1998.

[69] E. Jurado, F. Camacho, G. Luzón, and J. . Vicaria, “A new kinetic model proposed for enzymatic hydrolysis of lactose by a β-galactosidase from Kluyveromyces fragilis,” *Enzyme Microb. Technol.*, vol. 31, no. 3, pp. 300–309, Aug. 2002.

[70] Q. Z. Zhou, X. D. Chen, and X. Li, “Kinetics of lactose hydrolysis by beta-galactosidase of Kluyveromyces lactis immobilized on cotton fabric.,” *Biotechnol. Bioeng.*, vol. 81, no. 2, pp. 127–33, Jan. 2003.

[71] C. S. Kim, E.-S. Ji, and D.-K. Oh, “A new kinetic model of recombinant beta-galactosidase from Kluyveromyces lactis for both hydrolysis and transgalactosylation reactions.,” *Biochem. Biophys. Res. Commun.*, vol. 316, no. 3, pp. 738–43, Apr. 2004.

[72] C. Vera, C. Guerrero, A. Illanes, and R. Conejeros, “A pseudo steady-state model for galacto-oligosaccharides synthesis with β-galactosidase from Aspergillus oryzae.,” *Biotechnol. Bioeng.*, vol. 108, no. 10, pp. 2270–9, Oct. 2011.

[73] M. Boon, A. Janssen, and van der Padt A, “Modelling and parameter estimation of the enzymatic synthesis of oligosaccharides by beta-galactosidase from bacillus circulans,” *Biotechnol. Bioeng.*, vol. 64, no. 5, pp. 558–67, Sep. 1999.

[74] D. Cavaille-Lefebvre and D. Combes, “Irreversible high pressure inactivation of β-galactosidase from Kluyveromyces lactis: Comparison with thermal inactivation,” *J. Biotechnol.*, vol. 61, no. 2, pp. 85–93, Apr. 1998.

[75] E. Jurado, F. Camacho, G. Luzón, and J. M. Vicaria, “Kinetic models of activity for β-galactosidases: influence of pH, ionic concentration and temperature,” *Enzyme Microb. Technol.*, vol. 34, no. 1, pp. 33–40, Jan. 2004.

[76] M. Ladero, R. Ferrero, a. Vian, a. Santos, and F. Garcia-Ochoa, “Kinetic modelling of the thermal and pH inactivation of a thermostable β-galactosidase from Thermus sp. strain T2,” *Enzyme Microb. Technol.*, vol. 37, no. 5, pp. 505–513, Oct. 2005.

[77] M. Ladero, a. Santos, and F. García-Ochoa, “Kinetic modelling of the thermal inactivation of an industrial β-galactosidase from Kluyveromyces fragilis,” *Enzyme Microb. Technol.*, vol. 38, no. 1–2, pp. 1–9, Jan. 2006.

[78] N. Papayannakos, G. Markas, and D. Kekos, “Studies on modelling and simulation of lactose hydrolysis by free and immobilized β-galactosidase from Aspergillus niger,” *Chem. Eng. J.*, vol. 52, no. 1, pp. B1–B12, Aug. 1993.

[79] C. R. Carrara and A. C. Rubiolo, “A method for evaluating lactose hydrolysis in a fixed bed reactor with β-galactosidase immobilized on chitosan,” *Chem. Eng. J.*, vol. 65, no. 2, pp. 93–98, May 1997.

[80] H. FUJIKAWA and T. ITOH, “Differences in the thermal inactivation kinetics of Escherichia coli .BETA.-galactosidase in vitro and in vivo.,” *Biocontrol Sci.*, vol. 2, no. 2, pp. 73–78, Jul. 1997.

[81] Q. Z. K. Zhou and X. D. Chen, “Effects of temperature and pH on the catalytic activity of the immobilized β-galactosidase from Kluyveromyces lactis,” *Biochem. Eng. J.*, vol. 9, pp. 33–40, 2001.

[82] J. P. Henley and A. Sadana, “A mathematical analysis of enzyme stabilization by a series-type mechanism: Influence of chemical modifiers.,” *Biotechnol. Bioeng.*, vol. 26, no. 8, pp. 959–69, Aug. 1984.

[83] C. Giacomini, G. Irazoqui, F. Batista-viera, and B. M. Brena, “Influence of the immobilization chemistry on the properties of immobilized β -galactosidases,” *J. Mol. Catal. B*, 2001.

[84] F. Hollmann, I. W. C. E. Arends, K. Buehler, A. Schallmey, and B. Bühler, “Enzyme-mediated oxidations for the chemist,” *Green Chem.*, vol. 13, no. 2, p. 226, 2011.

[85] E. E. Ferrandi, D. Monti, I. Patel, R. Kittl, D. Haltrich, S. Riva, and R. Ludwig, “Exploitation of a Laccase/Meldola’s Blue System for NAD + Regeneration in

Preparative Scale Hydroxysteroid Dehydrogenase-Catalyzed Oxidations," *Adv. Synth. Catal.*, vol. 354, no. 14–15, pp. 2821–2828, Oct. 2012.

[86] D. Monti, G. Ottolina, G. Carrea, and S. Riva, "Redox reactions catalyzed by isolated enzymes.," *Chem. Rev.*, vol. 111, no. 7, pp. 4111–40, Jul. 2011.

[87] R. Wichmann and D. Vasic-Racki, "Cofactor Regeneration at the Lab Scale," in *Technology Transfer in Biotechnology SE - 9*, vol. 92, U. Kragl, Ed. Springer Berlin Heidelberg, 2005, pp. 225–260.

[88] S. Kochius, A. O. Magnusson, F. Hollmann, J. Schrader, and D. Holtmann, "Immobilized redox mediators for electrochemical NAD(P)+ regeneration.," *Appl. Microbiol. Biotechnol.*, vol. 93, no. 6, pp. 2251–64, Mar. 2012.

[89] M. D. Leonida, "Redox enzymes used in chiral syntheses coupled to coenzyme regeneration.," *Curr. Med. Chem.*, vol. 8, no. 4, pp. 345–369, Mar. 2001.

[90] W. a van der Donk and H. Zhao, "Recent developments in pyridine nucleotide regeneration," *Curr. Opin. Biotechnol.*, vol. 14, no. 4, pp. 421–426, Aug. 2003.

[91] H. K. Chenault and G. Whitesides, "Regeneration of nicotinamide cofactors for use in organic synthesis," *Appl. Biochem. Biotechnol.*, vol. 14, no. 2, pp. 147–197, 1987.

[92] R. Lunzer, I. Ortner, D. Haltrich, K. D. Kulbe, and B. Nidetzky, "Enzymatic Regeneration of NAD in Enantioselective Oxidation of Secondary Alcohols: Glutamate Dehydrogenase Versus NADH Dehydrogenase," *Biocatal. Biotransformation*, vol. 16, no. 5, pp. 333–349, Jan. 1998.

[93] H. Keith Chenault and G. M. Whitesides, "Lactate dehydrogenase-catalyzed regeneration of NAD from NADH for use in enzyme-catalyzed synthesis," *Bioorg. Chem.*, vol. 17, no. 4, pp. 400–409, Dec. 1989.

[94] A. Martorana, R. Vazquez-Duhalt, S. a. Aguila, R. Basosi, and M. C. Baratto, "Spectroscopic characterization of 2,6-dimethoxyphenol radical intermediates in the Coriolopsis gallica laccase-mediator system," *J. Mol. Catal. B Enzym.*, vol. 107, pp. 100–105, Sep. 2014.

[95] K. Li, F. Xu, and K. Eriksson, "Comparison of fungal laccases and redox mediators in oxidation of a nonphenolic lignin model compound," *Appl. Environ. Microbiol.*, vol. 65, no. 6, pp. 2654–2660, 1999.

[96] O. V Morozova, G. P. Shumakovich, M. a Gorbacheva, S. V Shleev, and a I. Yaropolov, "'Blue' laccases.," *Biochem. Biokhimiia*, vol. 72, no. 10, pp. 1136–50, Oct. 2007.

[97] S. Aksu, I. W. C. E. Arends, and F. Hollmann, "A New Regeneration System for Oxidized Nicotinamide Cofactors," *Adv. Synth. Catal.*, vol. 351, no. 9, pp. 1211–1216, Jun. 2009.

[98] P. Könst, S. Kara, S. Kochius, D. Holtmann, I. W. C. E. Arends, R. Ludwig, and F. Hollmann, "Expanding the Scope of Laccase-Mediator Systems," *ChemCatChem*, vol. 5, no. 10, pp. 3027–3032, Oct. 2013.

[99] L. P. Christopher, B. Yao, and Y. Ji, "Lignin Biodegradation with Laccase-Mediator Systems," *Front. Energy Res.*, vol. 2, no. March, pp. 1–13, Mar. 2014.

[100] G. W. Huisman, J. Liang, and A. Krebber, "Practical chiral alcohol manufacture using ketoreductases.," *Curr. Opin. Chem. Biol.*, vol. 14, no. 2, pp. 122–9, Apr. 2010.

[101] H. Gröger, F. Chamouleau, N. Orologas, C. Rollmann, K. Drauz, W. Hummel, A. Weckbecker, and O. May, "Enantioselective reduction of ketones with 'designer cells' at high substrate concentrations: highly efficient access to functionalized optically

active alcohols.," *Angew. Chem. Int. Ed. Engl.*, vol. 45, no. 34, pp. 5677–81, Aug. 2006.

[102] V. Kaswurm, W. van Hecke, K. D. Kulbe, and R. Ludwig, "Engineering of a bi-enzymatic reaction for efficient production of the ascorbic acid precursor 2-keto-l-gulonic acid," *Biochem. Eng. J.*, vol. 79, pp. 104–111, Oct. 2013.

[103] W. Van Hecke, D. Haltrich, B. Frahm, H. Brod, J. Dewulf, H. Van Langenhove, and R. Ludwig, "A biocatalytic cascade reaction sensitive to the gas–liquid interface: Modeling and upscaling in a dynamic membrane aeration reactor," *J. Mol. Catal. B Enzym.*, vol. 68, no. 2, pp. 154–161, Feb. 2011.

[104] T. Nguyen, B. Splechtna, M. Steinböck, W. Kneifel, H. P. Lettner, K. D. Kulbe, and D. Haltrich, "Purification and Characterization of Two Novel β-Galactosidases from Lactobacillus reuteri," *J. Agric. Food Chem.*, vol. 54, no. 14, pp. 4989–4998, Jun. 2006.

[105] M. Bradford, "A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding," *Anal. Biochem.*, vol. 72, no. 1–2, pp. 248–254, May 1976.

[106] M. Bayram and B. Yildiz, "Parameter estimation of an enzyme kinetic system using computer algebra techniques," *Appl. Math. Comput.*, vol. 99, no. 1, pp. 93–98, Mar. 1999.

[107] N. Yildirim, F. Akçay, H. Okur, and D. Yildirim, "Parameter estimation of nonlinear models in biochemistry: a comparative study on optimization methods," *Appl. Math. Comput.*, vol. 140, no. 1, pp. 29–36, Jul. 2003.

[108] David A Coley., *Introduction to Genetic Algorithms for Scientists and Engineers*. London: Har/Dskt, 1999, p. 113.

[109] R. C. Mitsuo Gen, *Genetic Algorithms and Engineering Optimization*. New York: Wiley, 2000.

[110] S. Kar, K. Nag, A. Dutta, D. Constales, and T. Pal, "An improved cellular automata model of enzyme kinetics based on genetic algorithm," *Chem. Eng. Sci.*, vol. 110, pp. 105–118, May 2014.

[111] N. Tutkun, "Parameter estimation in mathematical models using the real coded genetic algorithms," *Expert Syst. Appl.*, vol. 36, no. 2, pp. 3342–3345, Mar. 2009.

[112] K. Muffler, M. Retzlaff, K.-H. van Pée, and R. Ulber, "Optimisation of halogenase enzyme activity by application of a genetic algorithm.," *J. Biotechnol.*, vol. 127, no. 3, pp. 425–33, Jan. 2007.

[113] C. Galhaup, S. Goller, C. K. Peterbauer, J. Strauss, and D. Haltrich, "Characterization of the major laccase isoenzyme from Trametes pubescens and regulation of its synthesis by metal ions.," *Microbiology*, vol. 148, no. Pt 7, pp. 2159–69, Jul. 2002.

[114] C. Galhaup and D. Haltrich, "Enhanced formation of laccase activity by the white-rot fungus Trametes pubescens in the presence of copper," *Appl. Microbiol. Biotechnol.*, vol. 56, no. 1–2, pp. 225–232, Jul. 2001.

[115] L. C. Petersen and H. Degn, "Steady-state kinetics of laccase from Rhus vernicifera," *Biochim. Biophys. Acta - Enzymol.*, vol. 526, no. 1, pp. 85–92, Sep. 1978.

[116] V. Kaswurm, W. Van Hecke, K. D. Kulbe, and R. Ludwig, "Guidelines for the Application of NAD(P)H Regenerating Glucose Dehydrogenase in Synthetic Processes," *Adv. Synth. Catal.*, vol. 355, no. 9, pp. 1709–1714, 2013.

APPENDIX A

Engineering an Enzymatic Regeneration System for NAD(P)H Oxidation.

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Engineering an Enzymatic Regeneration System for NAD(P)H Oxidation

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Abstract

A recently proposed coenzyme regeneration system employing laccase and a number of various redox mediators for the oxidation of NAD(P)H was studied in detail by kinetic characterization of individual reaction steps. Reaction engineering by modeling was used to optimize the employed enzyme, coenzyme as well as redox mediator concentrations. Glucose dehydrogenase from *Bacillus* sp. served as a convenient model of synthetic enzymes that depend either on NAD^+ or $NADP^+$. The suitability of laccase from *Trametes pubescens* in combination with acetosyringone or syringaldazine as redox mediator was tested for the regeneration (oxidation) of both coenzymes. In a first step, pH profiles and catalytic constants of laccase for the redox mediators were determined. Then, second-order rate constants for the oxidation of NAD(P)H by the redox mediators were measured. In a third step, the rate equation for the entire enzymatic process was derived and used to build a MATLAB model. After verifying the agreement of predicted vs. experimental data, the model was used to calculate different scenarios employing varying concentrations of regeneration system components. The modeled processes were experimentally tested and the results compared to the predictions. It was found that the regeneration of NADH to its oxidized form was performed very efficiently, but that an excess of laccase activity leads to a high concentration of the oxidized form of the redox mediator – a phenoxy radical - which causes a coupling (dimerization or polymerization) and enzyme deactivation.

1. Introduction

During the last years laccase/mediator systems have been proposed for the regeneration of $NAD(P)^+$-dependent enzymatic processes in synthetic applications [1,2]. These initial studies showed a high potential for up-scaling, but more detailed investigations are necessary to understand the strengths and weaknesses of these regeneration systems. In this work we studied the underlying principles for an efficient and stable enzymatic regeneration process of $NAD(P)^+$, which does not show the restrictions of alternative systems, e.g. electrochemical methods [3]. Coenzymes are costly [4], which makes them too expensive to employ more than the minimal amount that still guarantees fast conversion of the synthetic enzyme [5]. High costs have been an obstacle in the wider application of coenzyme-dependent oxidreductases, but this is also the strongest argument for applying efficient and economical coenzyme regeneration systems. Various methods such as chemical, biological, photochemical, electrochemical or enzymatic approaches have been suggested and reviewed for this purpose [6,7]. Among them, the enzymatic methods seem to be the most convenient and useful. Such *in situ* regeneration reactions have been used in a number of oxidoreductase-catalyzed reactions, and some of them have been up-scaled to large-scale syntheses [1].

As suggested by Chenault and Whitesides [8] an ideal enzymatic regeneration system should meet the following criteria: (i) the enzymes should be inexpensive and stable, (ii) the enzymes should have high specific activity, (iii) simple and inexpensive reagents that do not interfere with the isolation of the product of interest or with enzyme stability should be employed, (iv) high turnover numbers should be obtained, (v) the total turnover number of the coenzyme should be at least between 10^2 and 10^4, and (vi) an overall equilibrium for the coupled enzyme system favorable to product formation should be reached. These criteria have been already partially met for NAD^+-reducing enzymes such as alcohol dehydrogenase, lactate dehydrogenase and glutamate dehydrogenase [7,9,10]. However, the enzymatic oxidation of NAD(P)H is not satisfactorily developed to date. The use of

laccase for NAD(P)H oxidation seems to fulfil most of the postulated criteria: (i) Laccases are technical enzymes employed for decolorization or delignification processes, which can be produced recombinantly and inexpensively. (ii) Laccase, a member of the blue multicopper oxidase family, has a high specific activity for various substrates, which can reach up to several hundred per second. (iii) Most of the investigated redox mediators, which typically are used in low concentrations, are inexpensive, but more work needs to be done on their removal from the product. Oxygen, the second substrate of laccase, can be easily provided to a biocatalytic process, and since water is produced by its reaction no purification of a by-product is required. (iv) It should be possible to obtain high turnover numbers for the coenzyme in a biocatalytic process when considering both the reported high stability and high specific activity of laccases, and (v) based on this high stability / high activity high total turnover numbers for the enzyme (laccase) should be achievable as well. (vi) The high redox potential of laccase of up to 800 mV vs. SHE allows to oxidize even redox mediators with high potentials [11,12]. The high thermodynamic driving force of oxygen reduction makes processes irreversible and drives coenzyme-dependent reactions towards completion [2]. The ideal mediator in these reactions should be non-toxic, cheap and efficient, with stable oxidized and reduced forms that do not inhibit the enzymatic reaction [13].

Laccase/mediator systems have been reported to be applicable for NAD^+ regeneration [2,14,15]. The main advantages of such systems are high process stability, low co-substrate costs and tolerance towards co-solvents. Laccase substrates such as ABTS, Meldola's blue, acetosyringone, syringaldehyde, caffeic acid, *p*-coumaric acid, vanillin, acetovanillone, 3-hydroxyanthranilic acid, 4-hydroxybenzoic acid, hydroquinone, phenolsulfonphthalein [16] have been used as mediators, amongst others.

In this work a laccase from *Trametes pubescens* and acetosyringone are used as an enzyme/mediator system to regenerate the oxidized coenzyme $NAD(P)^+$ from NAD(P)H. Glucose dehydrogenase (GDH) from *Bacillus* sp. is here employed as the model

synthesizing enzyme that uses the oxidized coenzyme $NAD(P)^+$, which is reduced to NAD(P)H, for the oxidation of D-glucose to D-glucono-1,5-lactone. The latter spontaneously hydrolyses to D-gluconic acid (**Figure 1**). Glucose oxidation catalyzed by GDH is a popular model system, since it can use both NAD^+ and the phosphorylated form $NADP^+$ [17,18]. The full rate equation of *Bacillus sp*. GDH, which is often applied for the regeneration of both NADPH and NADH, was recently elucidated [19]. Furthermore, modeling provides guidance in converting batch to continuous conversions as recently demonstrated for lactobionic acid production [20]. Here we used modeling together with experimental approaches to obtain knowledge on enzyme and redox mediator stability under reaction conditions, as well as on the necessary activities of enzymes and minimum concentrations of redox mediator and coenzyme to design an efficient enzymatic process. Overall, we obtained detailed information on the strengths and possible limitations of the laccase/redox mediator regeneration system.

2. Materials and Methods

2.1 Materials

Acetosyringone (4'-hydroxy-3',5'-dimethoxyacetophenone), syringaldehyde **(**4-hydroxy-3,5-dimethoxybenzaldehyde), NAD^+, $NADP^+$, NADH, NADPH, D-glucose and D-glucono-1,5-lactone were purchased from Sigma–Aldrich (Steinheim, Germany). 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was purchased from Amresco (Ohio, USA). Stock solutions (200 mM) of acetosyringone and syringaldehyde were prepared in ethanol. All buffer reagents and other chemicals were of analytical grade. Water was purified by reversed osmosis and scavenger resins to a resistivity of >18 MΩ cm. Laccase (EC 1.10.3.2) from *Trametes pubescens* CBS 696.94 with a specific activity of 594 U/mg at pH 5.0 was produced by cultivation of the fungus in a bioreactor under copper induction following published procedures [21,22]. Glucose dehydrogenase (GDH, EC 1.1.1.47) from *Bacillus* sp. with a specific activity of 18.5 U/mg at pH 5.0 was a gracious gift from Amano Enzyme Inc. (Nagoya, Japan).

2.2 Enzyme activity assays

Laccase activity was determined with ABTS as colorimetric substrate. The assay mixture contained 1 mM ABTS in air-saturated, 100 mM sodium-citrate buffer, pH 5.0, incubated for 15 min at 30°C before the measurement. After addition of a suitable amount of laccase, the oxidation of ABTS was monitored by following the increase of absorbance at 420 nm (ε_{420} =36.0 mM^{-1} cm^{-1}) for 180 s. One unit of laccase activity was defined as the amount of enzyme required to oxidize 1 µmol of ABTS per min.

GDH activity was followed by the increase in NAD(P)H absorbance at 340 nm (ε_{340}= 6.22 mM^{-1} cm^{-1}) for 180 s at 30°C. The assay contained glucose (100 mM), the respective coenzyme (NAD^+ or $NADP^+$, 0.5 mM), 100 mM sodium-citrate buffer, pH 5.0 and a suitable amount of enzyme. One unit of GDH activity was defined as the amount of enzyme required to reduce 1 µmol of $NAD(P)^+$ per min. Measurements of enzymatic activities in

samples taken from conversion experiments were affected by a small error introduced by the regenerating enzyme, which was corrected by performing reference measurements.

2.3 Determination of pH profiles and catalytic constants

The pH profile of laccase activity with the redox mediators was determined in air-saturated, 100 mM phosphate-citrate buffer solution at various pH values (3.0 to 6.5). The assays were carried out at 30°C using 1 mM of acetosyringone (ε_{400}= 1.7 mM^{-1} cm^{-1}) or syringaldehyde (ε_{380}= 1.6 mM^{-1} cm^{-1}). The molar absorption coefficients of the two redox mediators were determined from standard curves (**Supplemental Figure S1**). Catalytic constants of laccase for the redox mediators were calculated by non-linear least-squares regression, fitting the observed data to the Henri-Michaelis-Menten equation using SigmaPlot v.12 (Systat Software Inc, CA, USA). The K_M value of laccase for oxygen (0.41 mM) was taken from [21]. The rate constants of the oxidation reaction of NAD(P)H by redox mediators were measured at 30°C in 100 mM phosphate-citrate buffer, pH 5.0 by following the reduction of the absorption band at 340 nm. The fast reaction of the redox mediator and NAD(P)H (both at 150 mM final concentration) was recorded with an Applied Photophysics SX20 stopped-flow spectrophotometer. Results are averaged from four independent measurements.

2.4 Batch conversion experiments

All batch conversion experiments were performed in a parallel 0.5-L bioreactor system (Sixfors, INFORS HT, Bottmingen, Switzerland) with a working volume of 0.3 L. The reactions contained 200 mM D-glucose, 380–900 U L^{-1} laccase, 220–500 U L^{-1} GDH, 100–500 µM NAD^+ and 100–500 µM acetosyringone as redox mediator in 100 mM sodium citrate buffer, pH 5.0. Four batch conversion experiments (A, B, C and D) employing different enzymatic activities, redox mediator and coenzyme concentrations were run. The reaction solution was prepared as follows: first glucose was dissolved in an appropriate amount of buffer. Then, the redox mediator, coenzyme and laccase were added. The redox

mediator was dissolved in 1 mL ethanol (96%) before adding to the reactor. Finally, the reaction was started by the addition of GDH. The pH was regulated by automatic titration with an aqueous sodium carbonate solution (500 mM). The reaction solutions were thermostatted to 30°C, continuously stirred at 250 rpm and oxygenated using pure oxygen which was bubbled through a sparger. The dissolved oxygen concentration was measured by using an oxygen electrode (OxyFerm, Bonaduz, Switzerland) and the oxygen saturation was set to 21%, equal to air saturation. Samples were taken periodically and used immediately for enzymatic activity measurements or heated at 99°C for 5 min and then frozen at -18°C until HPLC analysis of glucose and gluconic acid.

2.5 HPLC analysis

The conversion of glucose was monitored from reaction samples by HPLC (Dionex Summit and Chromeleon software, Sunnyvale, CA, USA) using an Aminex HPX-87 H-column (BioRad Laboratories, CA, USA) at 60°C and 5 mM H_2SO_4 as eluent with a flow rate of 0.5 mL min^{-1}. The components were detected by UV at a wavelength of 210 nm. Calibration was performed with authentic samples of glucose and gluconic acid in a concentration range from 5 to 20 mg L^{-1}. Because of the heat treatment of the sample the formed intermediate glucono-1,5-lactone was fully converted to gluconic acid and could not be determined.

2.6 Rate equation and MATLAB model

For the bi-enzymatic process employing GDH and laccase (**Figure 1**) a set of non-linear, differential equations was derived from mass balances and the mass conservation law. Laccase activity is modeled by a ping-pong *bi-bi* reaction mechanism [23] although this is only an approximation of the more complicated reaction mechanism for which no kinetic model is published. GDH activity was modeled by sequential ordered *bi-bi* mechanism kinetics with the coenzyme binding first [24]. The product of the laccase reaction, the

oxidized redox mediator, reacts with one of the products of the GDH reaction, the reduced nicotinamide coenzyme, in a second-order reaction [5] (this work). The reaction rates for laccase (r_1), the redox mediator/NAD(P)H (r_2) and GDH (r_3) are given by the equations (1) – (3):

$$r_1 = V_{max,LAC} \times \frac{[O_2]\times[RM_{red}]}{K_{M,RM}\times[O_2]+K_{M,O_2}\times[RM_{red}]+[O_2]\times[RM_{red}]} \quad (1)$$

$$r_2 = k_2 \times [RM_{ox}] \times [NADH] \quad (2)$$

$$r_3 = V_{max,GDH} \times \frac{[NAD]\times[GLC]}{\begin{array}{c} K_{I,NAD}\times K_{M,GLC}\times\left(1+\frac{[NADH]}{K_{I,NADH}}+[GL]\times\frac{K_{M,NADH}}{K_{I,NADH}\times K_{M,GL}}\right)+ \\ K_{M,GLC}\times[NAD]\times\left(1+[GL]\times\frac{K_{M,NADH}}{K_{I,NADH}\times K_{M,GL}}\right)+ \\ +K_{M,NAD}\times[GLC]\times\left(1+\frac{[NADH]}{K_{I,NADH}}\right)+[NAD]\times[GLC]\times\left(1+\frac{[GL]}{K_{I,GL}}\right) \end{array}} \quad (3)$$

The concentration of the redox mediator RM in the oxidized and reduced states, $NAD(P)^+$, NAD(P)H, D-glucose (GLC), D-glucono-1,5 lactone (GL) and gluconic acid (GA) can be described by the set of differential equations (4) – (8):

$$\frac{d[GLC]}{dt} = -r_3 \quad (4)$$

$$\frac{d[NAD]}{dt} = -\frac{d[NADH]}{dt} = r_2 - r_3 \quad (5)$$

$$\frac{d[RM_{red}]}{dt} = -\frac{d[RM_{ox}]}{dt} = r_2 - r_1 \quad (6)$$

$$\frac{d[GL]}{dt} = -\frac{d[GLC]}{dt} - \frac{d[GA]}{dt} = r_3 - r_{hyd} \quad (7)$$

$$\frac{d[GA]}{dt} = k_{hyd} \times [GL] \quad (8)$$

The space-time yield (STY) for the sum of products (glucono-1,5-lactone plus gluconic acid) was calculated for the initial reaction to exclude enzyme deactivation and redox mediator degradation and after 10 h (STY_{10}) when the reactions were terminated (9):

$$\mathrm{STY}[\mathrm{mol(GL+GA)L^{-1}h^{-1}}] = \frac{\mathrm{C^0_{GLC}} - \mathrm{C^t_{GLC}}}{\mathrm{t}} \quad (9)$$

To solve the set of ordinary differential equations the ODE15s subroutine in the MATLAB R2009a software (The MathWorks, Natick, MA, USA) was used, which is designed specifically to deal with stiff differential systems of equations. For better comparison with

the experimental results the predicted glucono-1,5-lactone and gluconic acid concentration are summed up and displayed in Figure 3 in only one curve for the end product (gluconic acid).

3. Results

3.1 Determination of catalytic constants and reaction rates

Laccase catalyzes the oxidation of acetosyringone and syringaldehyde to the corresponding phenoxy radicals at the expense of molecular oxygen, which is reduced to water, with a stoichiometry of 4:1. The pH optima of both reactions were determined photometrically using molar absorption coefficients determined at pH 5.0 (**Figure S1**). Laccase from *T. pubescens* exhibited a pH optimum at 3.5 for both redox mediators (**Figure 2**), with the activity towards acetosyringone being higher at less acidic pH values than syringaldehyde. At pH 5.0 the specific activity for acetosyringone was 243 U mg^{-1}, which is 1.9-fold higher than that for syringaldehyde (126 U mg^{-1}). To calculate r_1 in modeled processes, the catalytic constants of laccase were measured for both redox mediators. For measurements and batch reactions pH 5.0 was chosen as a compromise between the pH optima of laccase and GDH. The k_{cat} of laccase for acetosyringone is 2.6-fold higher and the K_M is 1.9-fold higher compared to syringaldehyde, which results in a 1.3-fold increased catalytic efficiency for acetosyringone at pH 5.0 (**Table 1**). The catalytic constants of GDH for glucose and NAD^+ and the inhibition constant for gluconolactone at pH 5.0 ($K_{I,GL}$ = 413 ± 12 mM) were also determined to model r_3 in batch conversion experiments. The last missing piece of kinetic information (to model r_2) was obtained by determining the second order rate constants (k_2) for the redox mediator/coenzyme couples. The second-order reactions give the following rate constants: $k_{2\ NADH/acetosyringone}$ = 294 ± 5 M^{-1} s^{-1}, $k_{2\ NADPH/acetosyringone}$ = 152 ± 11 M^{-1} s^{-1}, $k_{2\ NADH/syringaldehyde}$ = 136 ± 5 M^{-1} s^{-1} and $k_{2\ NADPH/syringaldehyde}$ = 103 ± 8 M^{-1} s^{-1}.

3.2 Experimental evaluation of the MATLAB model

The first conversion experiment, **Batch A**, was performed with a 4.1-fold higher laccase activity than GDH activity (**Table 2**). A higher volumetric activity of the regenerating enzyme together with high concentrations of both the redox mediator acetosyringone and the coenzyme NAD^+ (500 µM each) was chosen to ensure efficient NAD^+ regeneration.

Because of experimental reasons, only the first 10 h of the process were followed by sampling. In these ten hours 30.2% of glucose were converted, which correlates well with the predicted degree of conversion of 35.7% when considering enzyme deactivation. The measured and predicted data for Batch A are shown in **Figure 3A**. The averaged modeled NAD^+ concentration during this steady-state phase is ~485 µM (**Table S1**) and the acetosyringone concentration is ~33 µM (**Table S2**). The measured laccase activity decreased by 20.8% and the GDH activity by 26.7% during these 10 h of reaction, which led to a too big deviation from the initial settings to continue the experiment. At this point the total turnover number of laccase was 6.65×10^6 and of GDH 2.03×10^6. The averaged enzyme consumption numbers for laccase and GDH are 2.81 and 0.88 U $mmol^{-1}$ product, respectively.

3.3. Process modeling and engineering

To study the influence of different volumetric activities of laccase and GDH on the conversion rate in combination with three preselected, initial concentrations of redox mediator and coenzyme, productivity charts were generated from 1681 calculations per plot (**Figure 4A-C**). The availability (the concentration) of redox mediator and coenzyme is shown to be an important factor influencing STY. Three laccase/GDH activity ratios for Batches B, C and D were selected to test for the rate limiting step in the regeneration system, which are indicated in the plots. To investigate the influence of enzyme activity on the whole process in more detail, specific productivities for each enzyme were modeled for different combinations of the other enzyme's activity, redox mediator and coenzyme concentration. Isoproductivity plots for GDH and laccase gave a critical activity, below which the STY drops, and are given for Batches A and B (**Figure 5A**,**B**) and Batches C and D (**Figure S2A-D**). Finally the effect of the initial redox mediator and coenzyme concentration on the STY and turnover numbers for coenzyme and redox mediator was investigated. The simulations shown in **Figure 6** illustrate the effect for the laccase/GDH activity ratio used in Batch A (900 and 220 U L^{-1}, respectively). Simulations for the

conditions employed in Batches B, C and D are given in the Supplemental Information (**Figure S3A-F**).

3.4 Limiting activity of the regenerating enzyme

The settings for Batch B were selected to test the model of the regeneration system (laccase and redox mediator) and investigate the effect of a decreased efficiency of the regeneration system by making the laccase reaction rate limiting. The ratio of laccase:GDH volumetric activities was 0.8 while all other parameters were kept constant (**Table 2**). Glucose conversion was 37.7% during 10 h of reaction, which correlated very well with the predicted degree of conversion. The measured and predicted data for Batch B are shown in **Figure 3B**. The modeled, averaged NAD^+ concentration during time is ~170 µM (**Table S1**) and acetosyringone concentration is ~478 µM (**Table S2**). The measured activity decreased by 15% for laccase and 17% for GDH during these initial 10 h of reaction. The total turnover number after 10 h was 1.99×10^7 for laccase and 1.19×10^6 for GDH. The averaged enzyme consumption numbers for laccase and GDH were 0.76 and 1.08 U $mmol^{-1}$ product, respectively.

3.5 Limiting coenzyme concentration

Batch C was conducted to evaluate the effect of a decreased coenzyme concentration on coenzyme regeneration and especially the synthetic reaction. In this experiment, the coenzyme concentration was 5-fold lower compared to Batches A and B, while keeping the laccase:GDH activity ratio high (3:1) (**Table 2**). The observed glucose conversion was 17.7% within 10 h. The correlation with the predicted degree of conversion of 19.9% is good, further measured and predicted data for Batch C are shown in **Figure 3C**. The modeled, averaged NAD^+ concentration during the first ten hours is very constant with ~92 µM (**Table S1**) and the acetosyringone concentration is ~15 µM (**Table S2**). During the conversion the measured laccase activity decreased by 15.6% and GDH activity by 54.8%. The total turnover number after 10 h was 3.87×10^6 for laccase and 8.86×10^5 for GDH.

Both enzymatic activities decreased linearly. The averaged enzyme consumption numbers for laccase and GDH were 3.97 and 4.64 U $mmol^{-1}$, respectively.

3.6 Limiting the redox mediator concentration

In the fourth experiment, Batch D, the initial redox mediator concentration was decreased 5-fold from standard conditions to evaluate the effect of a decreased redox mediator concentration on both enzymatic cycles. The laccase:GDH activity ratio was 1.8:1 (**Table 2**). Glucose conversion was only 13% in 10 h, which was a result of an almost complete stop of the reaction after 4 h. The measured conversion after 1 h was 7.40%, which correlates roughly with the predicted value of 5.29% when considering the difference of actually added GDH activity and V_{max}. However, for later time points a much lower conversion is measured than predicted by the model. Data for Batch D are shown in **Figure 3D**. The modeled, averaged NAD^+ concentration during the first hour is ~240 µM (**Table S1**) and acetosyringone concentration is ~60 µM (**Table S2**). The measured specific productivity of GDH for the first hour is 29.5 mmol kU^{-1} h^{-1}, which is the second highest after Batch A. However, after 1 h a dramatic drop in the specific productivity of GDH and laccase is observed. During the first 2 h the measured laccase activity decreases faster than thereafter, but the total loss of activity after 10 h is only 20.1%. Little GDH deactivation is observed in this reaction (~5%). The total turnover number after 10 h was 2.92×10^6 for laccase and 3.93×10^5 for GDH. The averaged enzyme consumption numbers for laccase and GDH were 6.96 and 0.96 U $mmol^{-1}$, respectively. The model was used to calculate the theoretical, residual amount of redox mediator present in Batch D, based on the deviation of predicted and measured reaction rate for each hour. The reduction of the acetosyringone concentration was calculated to be 54, 15.6 and 7.3 µM after 2, 3 and 4 h, respectively.

4. Discussion

To find the best pH for coupling acidic *T. pubescens* laccase and GDH, the pH optima of laccase activity for the two previously published, NADH-oxidizing redox mediators acetosyringone and syringaldehyde were determined [15]. The rate constants with the laccase at pH 5.0, were high enough to use the selected laccase together with GDH. The catalytic performance of GDH at pH 5.0 compared to pH 8.0 [24] is still good enough to allow its application. One potential problem arising from employing GDH at low pH is the low hydrolysis rate of the reaction product gluconolactone, which results in product inhibition. Another critical factor is the supply of oxygen, which governs the regenerating laccase reaction. Therefore, the employed enzymatic activities were selected sufficiently low to avoid critical gluconolacone build-up or a decrease of the oxygen concentration in the reactor. Laccase from *T. pubescens* exhibits more favorable catalytic constants for acetosyringone than syringaldehyde at pH 5.0, which was one reason to study acetosyringone as redox mediator in this work. The other reason to employ acetosyringone was its higher rate constants with the coenzymes NADH and NADPH. The rate constants obtained for the reaction of syringaldehyde or acetosyringone with NADH were 1.3 – 2-fold higher than that for NADPH. The phosphorylation of the coenzyme reduces the re-oxidation rate. Considering the similar redox potential of both coenzyme forms (-320 mV vs. SHE) this is an unecpected result. The reason might be the additional negatively charged phosphate group in NADPH which may lead to increased electrostatic repulsion with the deprotonated phenolate in acetosyringone.

The experimental evaluation of the MATLAB model for the GDH reaction was based on Batch A, in which a high laccase activity was employed to ensure a high NAD^+ concentration. According to the model ~460 µM NAD^+ were present during the reaction. This allows the verification of the model for the synthetic GDH reaction without limitation from the laccase regeneration system. The difference of the observed and predicted substrate conversion during in first two hours shows that the activity obtained with the used

assay does not reliably estimates the V_{max} of GDH, but underestimates it by a factor of ~1.6, because of the limited glucose (K_M = 16.7 mM, S = 100 mM), but especially the limited coenzyme concentration (K_M = 260 µM, S = 500 µM) in the assay. GDH deactivation during the process leads to a convergence of the actual and predicted glucose conversion.
The experimental evaluation of the MATLAB model for the laccase reaction was based on Batch B, which employed a low laccase activity to keep the concentration of the reduced form of acetosyringone high. The model predicted a concentration of 478 µM acetosyringone. The volumetric activity of laccase was well estimated by the enzymatic assay and its conversion from ABTS to acetosyringone activity by the estimated factor of 1:0.54. Since the oxygen concentration was kept constant, no deviation between the volumetric activity and V_{max} was found.

Process modeling and engineering was based on the verified MATLAB model. The influence of different laccase:GDH activity ratios, redox mediator or coenzyme concentrations were modeled and useful activities and concentrations selected for the conversion experiments based on the model. The productivity plots show that the initial concentration of redox mediator and coenzyme together with the activity of regenerating and synthetic enzyme govern STY **(Figure 4**). At high concentrations of the coenzyme and redox mediator (500 µM each, **Figure 4A**), which are significantly above the K_M values of laccase and GDH, neither r_1 nor r_3 are limited. Also the bimolecular rate constant for the oxidized acetosyringone/NADH (294 M^{-1} s^{-1}) ensures that for the selected concentrations r_2 is high enough (73.5 µM s^{-1}) to support enzymatic activities up to 4400 U L^{-1}. With such a high efficiency of the mediator system, the activities of both enzymes and their ratio become dominant for the STY and specific productivities. Enzyme ratios for Batch A and B were selected to investigate the sensitivity of one enzyme from the other. For a reduced coenzyme concentration (100 µM, **Figure 4B**) a limiting effect of r_3 was predicted due to a NAD^+ concentration below the K_M of GDH for its coenzyme. This results in a low predicted STY (4.06 mM h^{-1}) and a reaction that is little sensitive towards changes in GDH activity

and even less towards changes in laccase activity. The conditions of Batch C were chosen from this plot. To study the effect of a reduced redox mediator concentration (100 µM) the conditions for Batch D were derived from **Figure 4C**. The reduced redox mediator concentration shows only a moderate effect in the simulation which is based on the laccase activity of Batch A and the GDH activity of Batch B. The predicted STY for Batch D (10.17 mM h^{-1}) results from the optimized enzymatic activities. The relative insensitivity towards the reduced redox mediator concentration compared to the coenzyme is explained by the lower K_M value of laccase for acetosyringone than in the case of GDH for NAD^+.

Specific productivities of enzymes under the three selected redox mediator/coenzyme concentrations are plotted in **Figure 5 and S2**. By following the isoactivity curves for laccase and GDH the critical activities below which the STY drops can be found. The maximal specific productivity of laccase at 500 µM acetosyringone and 500 µM NADH is predicted to be 20.1 mmol kU^{-1} h^{-1} and the maximum specific productivity of GDH is 33 mmol kU^{-1} h^{-1}. A good compromise to achieve high specific productivities for laccase and GDH can be reached when the activity ratio of laccase:GDH is about 2:1. In the case of 500 µM acetosyringone and 100 µM NADH, the maximum specific productivity of laccase is unchanged, but the specific productivity of GDH is reduced to 14 mmol kU^{-1} h^{-1}, caused by the low possible NAD^+ concentration. Under these conditions, the best compromise to achieve high specific productivities for laccase and GDH is reached when the activity ratio of laccase:GDH is about 0.6:1. For the last case (100 µM acetosyringone, 500 µM NADH) the maximum specific productivity of laccase is lower (14 mmol kU^{-1} h^{-1}), whereas the specific productivity of GDH is the same as for Batch A and B (33 mmol kU^{-1} h^{-1}). The reason is the low possible concentration of reduced acetosyringone. Under these conditions, the best compromise to achieve high specific productivities for laccase and GDH is reached when the activity ratio of laccase:GDH is about 1.5:1.

For the activity ratios used in Batches A-D the following initial acetosyringone and NADH concentrations were predicted to be necessary to obtain a good STY (**Figure 6**, **Figure**

S3). Batch A (activity ratio laccase:GDH 4.1:1): 100 µM acetosyringone and 1000 µM NADH; Batch B (activity ratio 0.8:1) 500 µM acetosyringone and 200 µM NADH; Batch C (activity ratio 3:1) 200 µM acetosyringone and 1000 µM NADH; Batch D (activity ratio 1.8:1) 300 µM acetosyringone and 1000 µM NADH. Of course, these concentrations were not applied, but given here to demonstrate that at activity ratios of laccase:GDH >1.5:1 the availability of the coenzyme governs the reaction rate, whereas in the case of a limiting laccase activity the redox mediator concentration governs the reaction rate.

These predictions were tested by two Batch reactions: Batch C employing a limiting coenzyme concentration and Batch D with a limiting redox mediator concentration. For Batch C, an available NAD^+ concentration of 92 µM from the initially added 100 mM was predicted. Despite the efficient regeneration of NADH the low coenzyme concentration is the rate-limiting step in the reaction (r_3). In Batch D, the predicted concentration (~60 µM) of the oxidized redox mediator acetosyringone was sufficient to keep r_1 high. However, in contrast to the model, the experiment showed an unexpected effect of the acetosyringone phenoxy radical. A polymerization reaction of the redox mediator occurred, which reduced the amount of the redox mediator drastically during the first 3 h and stopped the reaction.

The enzyme deactivation observed in the batch conversion processes is relatively low and high total turnover numbers of $>10^6$ were obtained. However, certain deactivation patterns are observed. In Batches A and B the measured enzymatic activities decreased linearly, which indicates that enzyme deactivation is proportional to product formation. The loss of activity was moderate. Enzyme consumption numbers of 0.76–1.08 U per mM formed product were observed. Only the inactivation of laccase in Batch A was higher (2.81 U mM^{-1}), which is probably an effect of the higher concentration of the acetosyringone phenoxy radical. Under conditions, which favor high radical concentrations even more (Batch C), the enzyme consumption number for both enzymes increases to 4 and 4.6 U mM^{-1}. The higher enzyme consumption is also observed for GDH and indicates that the deactivation caused

by acetosyringone phenoxy radicals is concentration dependent and not connected with substrate turnover. Even more interesting results were found under a limiting redox mediator concentration in Batch D. After a short time a color change in the reaction vessel was observed, which indicates a degradation of the redox mediator acetosyringone. The most probable cause is a dimerization/polymerization by coupling of the formed phenoxy radicals or coupling to proteins. The low concentration of the redox mediator made this process obvious. A fast reduction of the acetosyringon phenoxy radical by NADH should stabilize the redox mediator in the process. Together with the destruction of the redox mediator also a deactivation of laccase slows down after the first hours and follows similar to GDH a time-dependent process.

In conclusion, an optimized process using the laccase/mediator system should fulfill the following criteria: i) A high concentration of NAD^+ to achieve a high specific GDH productivity. The actual NAD^+ concentration depends mostly on the initial coenzyme concentration and laccase activity; ii) A two-fold excess of laccase activity over GDH activity to ensure efficient regeneration of the coenzyme and high specific productivities of regenerating and synthetic enzymes. When the ratio drops, the NAD^+ concentration is low regardless of the added coenzyme concentration. iii) The enzyme activity ratio also affects the concentration of the oxidized redox mediator. High concentrations should be avoided to reduce enzyme deactivation. Also the redox mediator itself is susceptible to degradation and more stable in the reduced form. If necessary, additional redox mediator has to be added during the reaction. iv) The enzyme activities can be increased to increase STY until the oxygen concentration becomes a limiting factor. v) Modeling can be elegantly used to optimize the enzymatic activities as well as redox mediator and coenzyme concentrations to improve the productivity, stability and economics of the reaction.

When comparing the excellent obtained productivity and enzyme total turnover numbers of the optimized laccase/mediator regeneration system with recently reported requirements

for feasible industrial biocatalytic processes [17,25] the investigated regeneration reaction appears to be very useful to regenerate NAD(P)$^+$-dependent reactions.

Acknowledgements

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Nomenclature

ε	molar absorption coefficient (M^{-1} cm^{-1})
eff	efficiency
K_I	inhibition constant (µM)
K_M	Michaelis-Menten constant (µM)
r_1	rate of the regenerating (laccase) reaction (M s^{-1})
r_2	second order rate constant (M^{-1} s^{-1})
r_3	rate of the synthetic (GDH) reaction (M s^{-1})
r_{hyd}	rate of D-glucono-1,5-lactone hydrolysis (s^{-1})
RM	redox mediator
t	time (h)
V_{max}	maximum enzymatic turnover rate at infinite substrate concentration (M s^{-1}, U L^{-1})

Abbreviations

GLC	D-glucose
GL	D-glucono-1,5-lactone
GA	gluconic acid
NAD(P)H	nicotinamide adenine dinucleotide (phosphate)
O_2	molecular oxygen
STY	space-time yield (mM h^{-1})

References

[1] F. Hollmann, I.W.C.E. Arends, K. Buehler, A. Schallmey, B. Bühler, Green Chem. 13 (2011) 226.

[2] E.E. Ferrandi, D. Monti, I. Patel, R. Kittl, D. Haltrich, S. Riva, R. Ludwig, Adv. Synth. Catal. 354 (2012) 2821.

[3] D. Monti, G. Ottolina, G. Carrea, S. Riva, Chem. Rev. 111 (2011) 4111.

[4] R. Wichmann, D. Vasic-Racki, in:, U. Kragl (Ed.), Technol. Transf. Biotechnol. SE - 9, Springer Berlin Heidelberg, 2005, pp. 225–260.

[5] S. Kochius, A.O. Magnusson, F. Hollmann, J. Schrader, D. Holtmann, Appl. Microbiol. Biotechnol. 93 (2012) 2251.

[6] M.D. Leonida, Curr. Med. Chem. 8 (2001) 345.

[7] W. a van der Donk, H. Zhao, Curr. Opin. Biotechnol. 14 (2003) 421.

[8] H.K. Chenault, G. Whitesides, Appl. Biochem. Biotechnol. 14 (1987) 147.

[9] R. Lunzer, I. Ortner, D. Haltrich, K.D. Kulbe, B. Nidetzky, Biocatal. Biotransformation 16 (1998) 333.

[10] H. Keith Chenault, G.M. Whitesides, Bioorg. Chem. 17 (1989) 400.

[11] A. Martorana, R. Vazquez-Duhalt, S. a. Aguila, R. Basosi, M.C. Baratto, J. Mol. Catal. B Enzym. 107 (2014) 100.

[12] K. Li, F. Xu, K. Eriksson, Appl. Environ. Microbiol. 65 (1999) 2654.

[13] O. V Morozova, G.P. Shumakovich, M. a Gorbacheva, S. V Shleev, a I. Yaropolov, Biochem. Biokhimiia 72 (2007) 1136.

[14] S. Aksu, I.W.C.E. Arends, F. Hollmann, Adv. Synth. Catal. 351 (2009) 1211.

[15] P. Könst, S. Kara, S. Kochius, D. Holtmann, I.W.C.E. Arends, R. Ludwig, F. Hollmann, ChemCatChem 5 (2013) 3027.

[16] L.P. Christopher, B. Yao, Y. Ji, Front. Energy Res. 2 (2014) 1.

[17] G.W. Huisman, J. Liang, A. Krebber, Curr. Opin. Chem. Biol. 14 (2010) 122.

[18] H. Gröger, F. Chamouleau, N. Orologas, C. Rollmann, K. Drauz, W. Hummel, A. Weckbecker, O. May, Angew. Chem. Int. Ed. Engl. 45 (2006) 5677.

[19] V. Kaswurm, W. van Hecke, K.D. Kulbe, R. Ludwig, Biochem. Eng. J. 79 (2013) 104.

[20] W. Van Hecke, D. Haltrich, B. Frahm, H. Brod, J. Dewulf, H. Van Langenhove, R. Ludwig, J. Mol. Catal. B Enzym. 68 (2011) 154.

[21] C. Galhaup, S. Goller, C.K. Peterbauer, J. Strauss, D. Haltrich, Microbiology 148 (2002) 2159.

[22] C. Galhaup, D. Haltrich, Appl. Microbiol. Biotechnol. 56 (2001) 225.

[23] L.C. Petersen, H. Degn, Biochim. Biophys. Acta - Enzymol. 526 (1978) 85.

[24] V. Kaswurm, W. Van Hecke, K.D. Kulbe, R. Ludwig, Adv. Synth. Catal. 355 (2013) 1709.

[25] P. Tufvesson, J. Lima-Ramos, M. Nordblad, J.M. Woodley, Org. Process Res. Dev. 15 (2011) 266.

Table 1: Catalytic constants of laccase and GDH for their substrates and co-substrates. These constants were measured at 30°C in air-saturated, 100 mM sodium-citrate buffer, pH 5.0.

Enzyme/Substrate	*Wavelength monitored*	*Extinction coefficient*	V_{max}	K_M	k_{cat}	k_{cat}/K_M
Laccase	*(nm)*	*(mM^{-1} cm^{-1})*	*(U mg^{-1})*	*(µM)*	*(s^{-1})*	*(M^{-1} s^{-1})*
Acetosyringone	400	1.7	289 ± 5	161 ± 10	289 ± 5	1.78 x 10^{-6}
Syringaldehyde	380	1.6	113 ± 2	85 ± 5	113 ± 2	1.34 x 10^{-6}
Oxygen*	420	36	2900	410	2900	7.0 x 10^{6}
GDH	*(nm)*	*(mM^{-1} cm^{-1})*	*(U mg^{-1})*	*(mM)*	*(s^{-1})*	*(M^{-1} s^{-1})*
Glucose	340	6.22	21.6 ± 0.2	16.7 ± 0.6	37.7± 0.3	2.26 x 10^{-3}
NAD^+	340	6.22	28.9 ± 0.4	0.26 ± 0.01	50.5 ± 0.8	194 x 10^{-3}

*) From [21], were determined at 25 ^{0}C using 1 mM ABTS as the electron donor

Table 2. Batch conversion processes employing the system laccase/GDH/NAD(H)/acetosyringone. The reactions were performed with a working volume of 300 mL and an initial glucose concentration of 200 mM in 100 mM sodium-citrate buffer, pH 5.0. The measured and modeled data are taken after 10 h of reaction. Space-time yield (STY) and specific productivity are calculated for 10 h of reaction. Turnover numbers of enzymes, redox mediator and coenzyme were calculated by dividing the STY_{10} by the respective molar concentration.

Batch		A	B	C	D
Initial values					
Acetosyringone (µM)		500	500	500	100
NAD^+ (µM)		500	500	100	500
Laccase (U L^{-1})		900	380	900	900
GDH (U L^{-1})		220	480	300	500
Measured and modeled data					
Time to reach a conversion of 99% [h]	*modeled*	37.5	28.1	66.5	23.4
Conversion after 10 h [%]	*experiment*	30.2	37.7	17.7	13.0
	modeled	35.7	37.7	19.9	49.9
STY_{10} [mM h^{-1}]	*experiment*	6.04	7.69	3.61	2.65
	modeled	7.14	7.69	4.06	10.2
Specific $productivity_{LAC}$ [mmol kU^{-1} h^{-1}]	*experiment*	6.76	20.3	4.04	2.97
	modeled	7.98	20.2	4.54	11.4
Specific $productivity_{GDH}$ [mmol kU^{-1} h^{-1}]	*experiment*	27.3	16.0	11.9	5.29
	modeled	32.3	16.0	13.4	20.3
TN_{LAC} [h^{-1}]	*experiment*	6.65×10^5	1.99×10^6	3.97×10^5	2.92×10^5
	modeled	7.85×10^5	1.99×10^6	4.47×10^5	1.12×10^6
TN_{GDH} [h^{-1}]	*experiment*	2.03×10^5	1.19×10^5	8.86×10^4	3.93×10^4
	modeled	2.40×10^5	1.19×10^5	9.96×10^4	1.50×10^5
TN_{NAD^+} [h^{-1}]	*experiment*	12.1	15.4	36.1	5.31
	modeled	14.3	15.4	40.6	20.3
TN_{RM} [h^{-1}]	*experiment*	12.1	15.4	7.22	26.5
	modeled	14.3	15.4	8.12	102

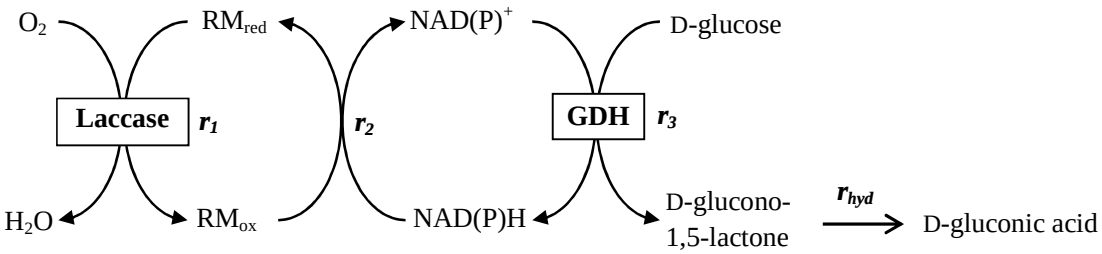

Figure 1. Reaction scheme for the bi-enzymatic system employing laccase as regenerating enzyme and glucose dehydrogenase (GDH) as synthetic enzyme. The redox mediator in its reduced form RM_{red} is oxidized by laccase to RM_{ox} with the rate r_1. The bimolecular rate observed for the reaction between RM_{ox} and the reduced form of the coenzyme NAD(P)H is given as r_2. NAD(P)H is reduced by GDH with the rate r_3. The concomitantly formed product is gluconolactone, an inhibitor of GDH. Its hydrolyzation rate to the non-inhibiting final product is r_{hyd}.

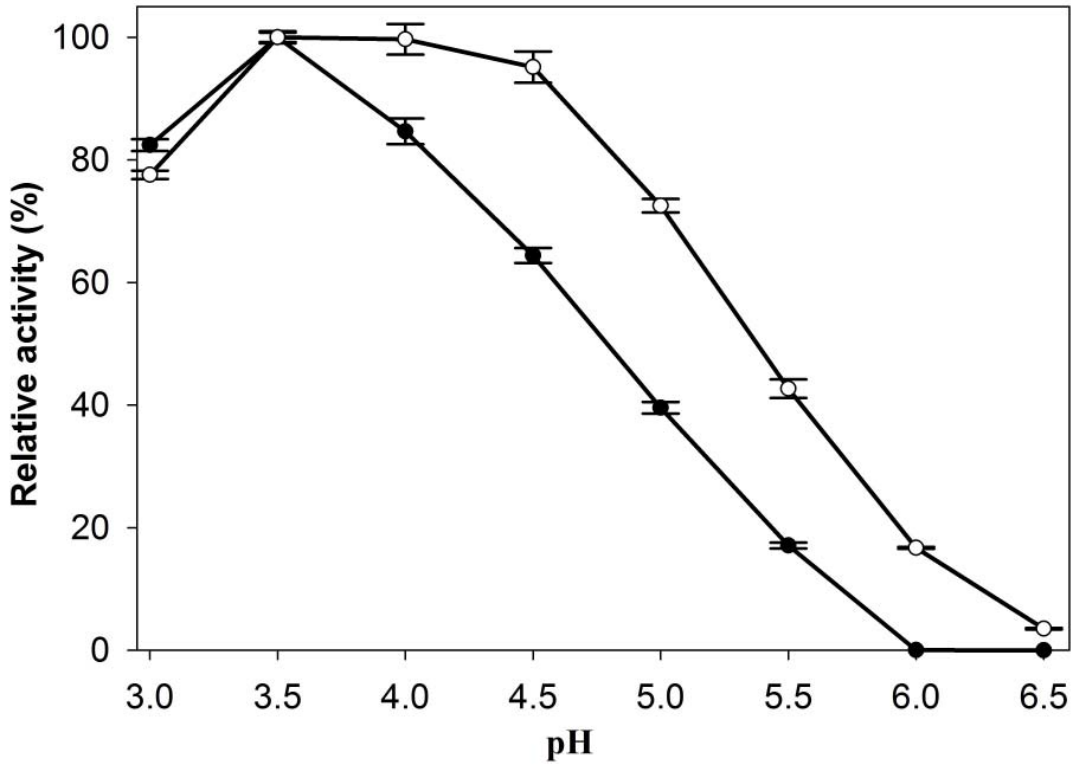

Figure 2. pH profile of laccase activity with either acetosyringone (○) or syringaldehyde as substrate (●). Relative activities are shown for better comparison of the shape of the profile. At pH 3.5 the specific activitiy of laccase for acetosyringone and syringaldehyde were 334 and 318 U/mg, respectively.

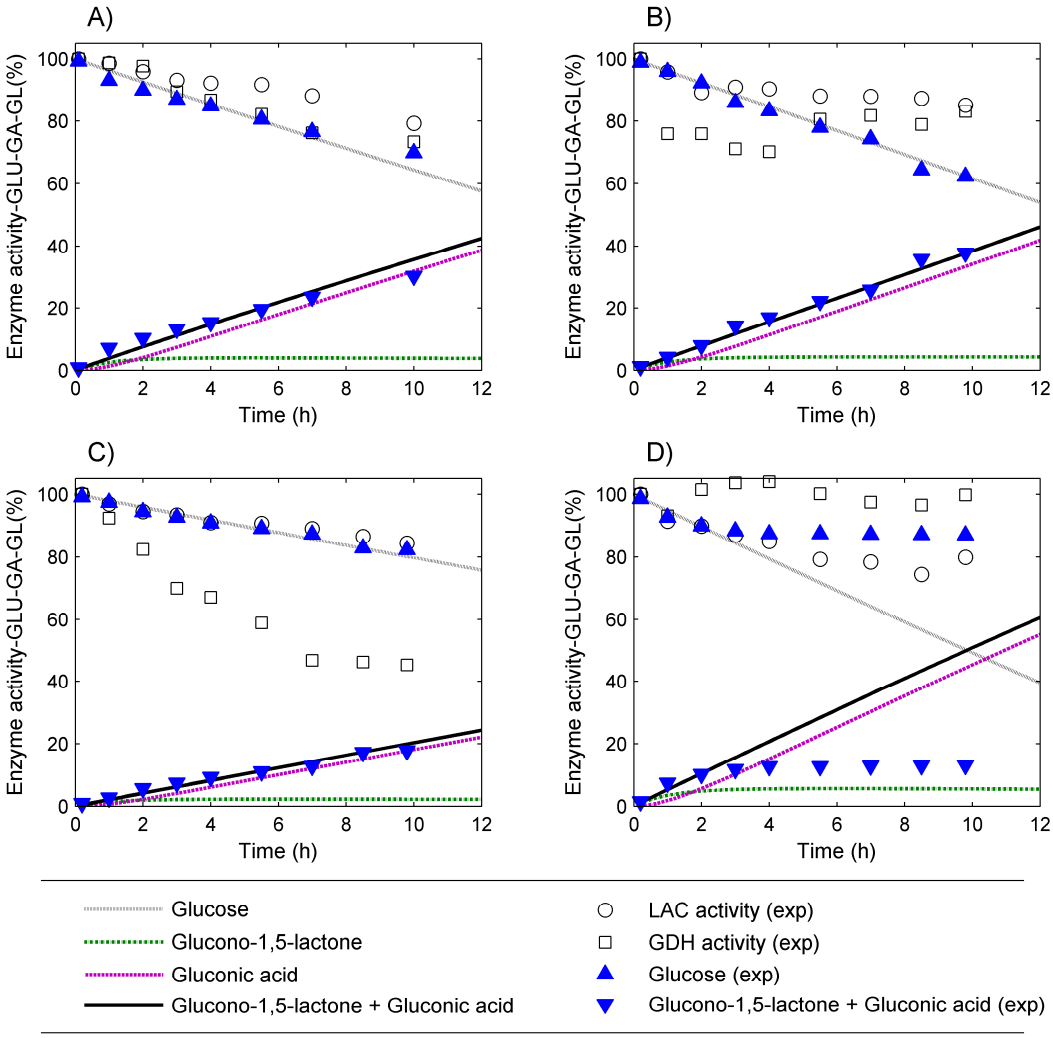

Figure 3. Batch conversions A–D. Measurements were stopped after 10 h of conversion. Measured values are indicated by data points, calculated values are indicated by lines. The Y-axis gives concentrations and activities as a percentage of their initial values to avoid multiple axes. Initial concentrations, activities and calculated performance numbers are given in Table 2.

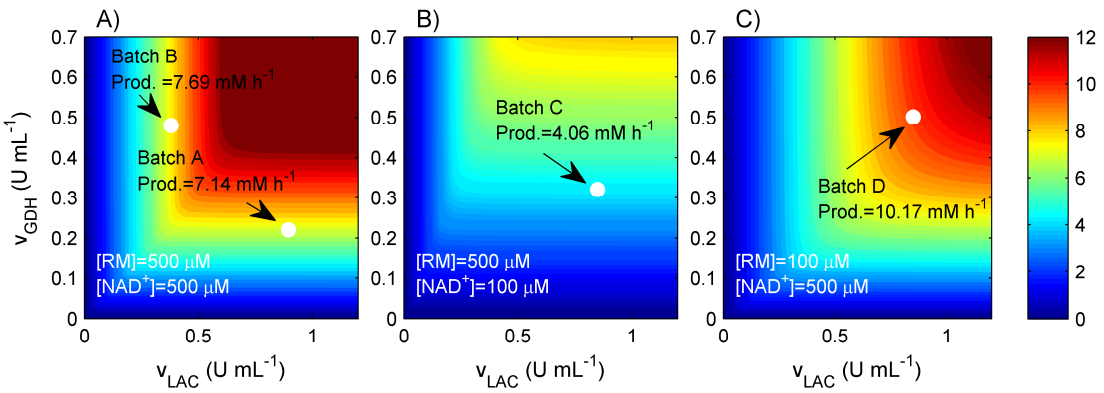

Figure 4. Isoproductivity charts calculated from space-time yields after 10 h of reaction. The simulation was based on the following initial concentrations: 200 mM glucose and (A) 500 µM redox mediator and 500 µM coenzyme (NAD^+), (B) 500 µM redox mediator and 100 µM coenzyme (NAD^+), (C) 100 µM redox mediator and 500 µM coenzyme (NAD^+).

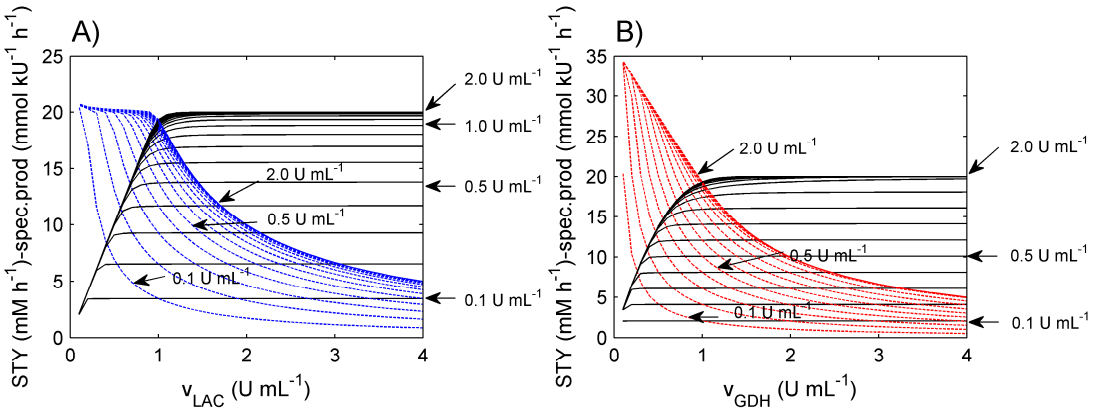

Figure 5. Isoproductivity plots based on the specific productivity after 10 h of reaction in regard to the laccase activity (A) and GDH (B) activity, simulated for the conditions in Batch A and B (500 µM redox mediator and 500 µM coenzyme). The activities indicated in the diagrams are the opposite enzyme as given on the X-axis. STY (straight lines), specific productivity (dashed lines).

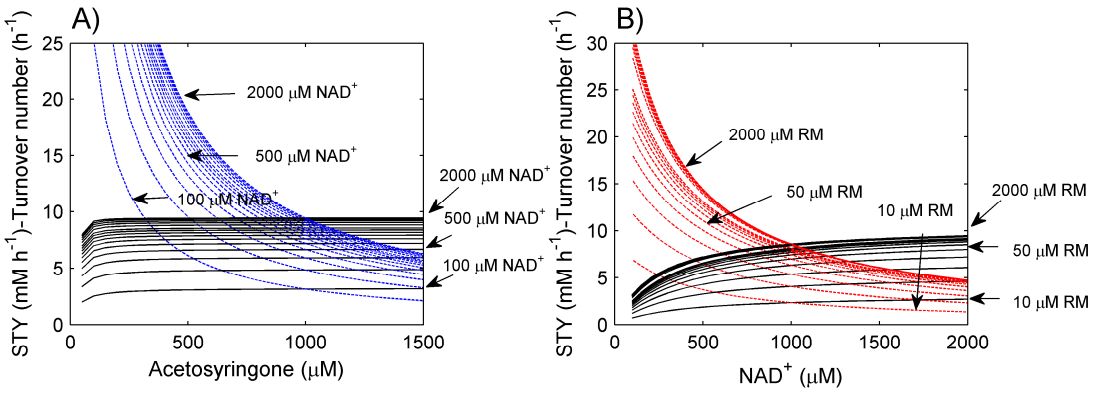

Figure 6. Effect of the initial redox mediator concentration (A) and coenzyme concentration (B) on STY and turnover numbers for the coenzyme and redox mediator after 10 h of reaction. The simulated laccase:GDH activity ratio corresponds to that used in Batch A (900 U L^{-1} laccase and 220 U L^{-1} GDH). STY (straight lines), turnover numbers (dashed lines), NAD^+ stands for the total of applied coenzyme which was in the oxidized form.

Supplementary Material

Engineering an Enzymatic Regeneration System for NAD(P)H Oxidation

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Table S1: Predicted NAD^+ concentration (µM) in the modeled batch conversion experiments. The initial coenzyme concentration is given at time 0. The coenzyme was added to the reactions in the form of NAD^+.

Time (h)	Batch			
	A	B	C	D
0	500.0	500.0	100.0	500.0
0.1	484.0	167.4	91.7	224.2
0.2	484.2	156.7	91.7	221.1
0.5	484.5	159.1	91.8	226.1
1	484.8	162.6	91.9	232.3
2	485.2	167.0	92.0	240.3
3	485.4	169.8	92.0	245.3
4	485.5	172.0	92.1	249.4
5	485.6	174.0	92.1	253.2
6	485.7	176.0	92.1	257.3
7	485.9	178.2	92.2	261.8
8	486.0	180.7	92.2	266.8
9	486.1	183.4	92.2	272.7
10	486.3	186.4	92.2	279.4

Table S2: Predicted acetosyringone concentration (µM) in the modeled batch conversion experiments. The initial redox mediator concentration is given at time 0. The redox mediator was added to the reactions in its reduced form, the concentration of the oxidized form, the acetosyringone phenoxy radical, can be calculated by subtracting the indicated acetosyringone concentration from the applied concentration.

Time (h)	Batch			
	A	B	C	D
0	500.0	500.0	500.0	100.0
0.1	38.3	478.1	16.2	64.2
0.2	37.7	478.9	16.1	64.6
0.5	36.7	478.8	15.9	64.1
1	35.5	478.5	15.7	63.4
2	34.3	478.3	15.5	62.6
3	33.7	478.1	15.4	62.0
4	33.2	477.9	15.3	61.5
5	32.8	477.8	15.2	61.1
6	32.5	477.6	15.1	60.6
7	32.1	477.5	15.1	60.0
8	31.7	477.3	15.0	59.4
9	31.3	477.1	15.0	58.6
10	30.9	476.9	14.9	57.7

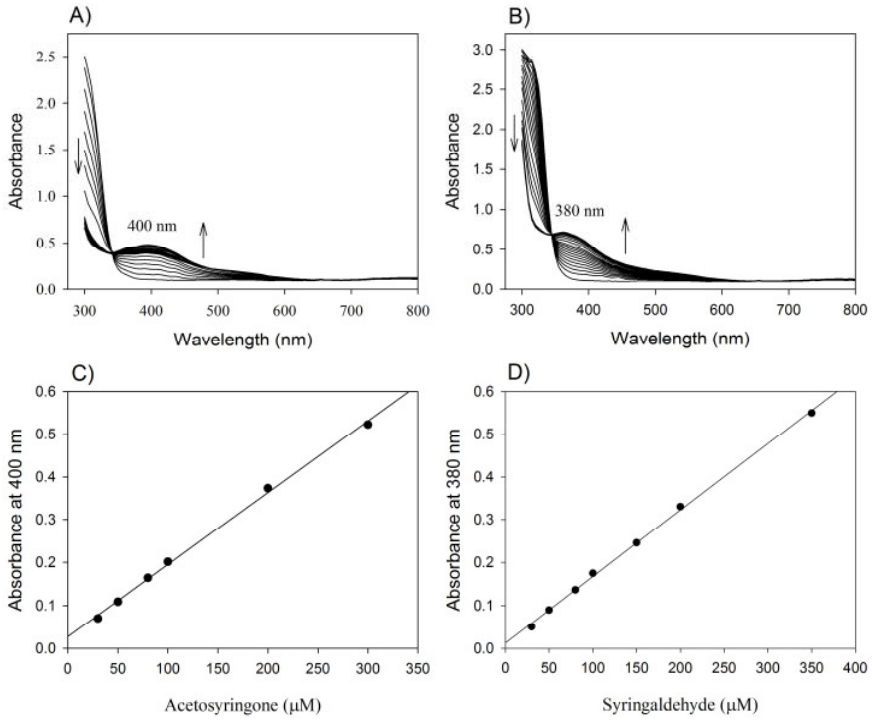

Figure S1: Determination of the peak wavelength and molar absorption coefficients of acetosyringone and syringaldehyde. Absorption spectra were recorded from a 200 µM solution of acetosyringone (A) and syringaldehyde (B) in 100 mM sodium-citrate buffer, pH 5.0. After addition of laccase the absorbance increases at the indicated wavelengths. The linear relationship between concentration of the oxidized redox mediator and absorbance is shown for acetosyringone (C) and syringaldehyde (D).

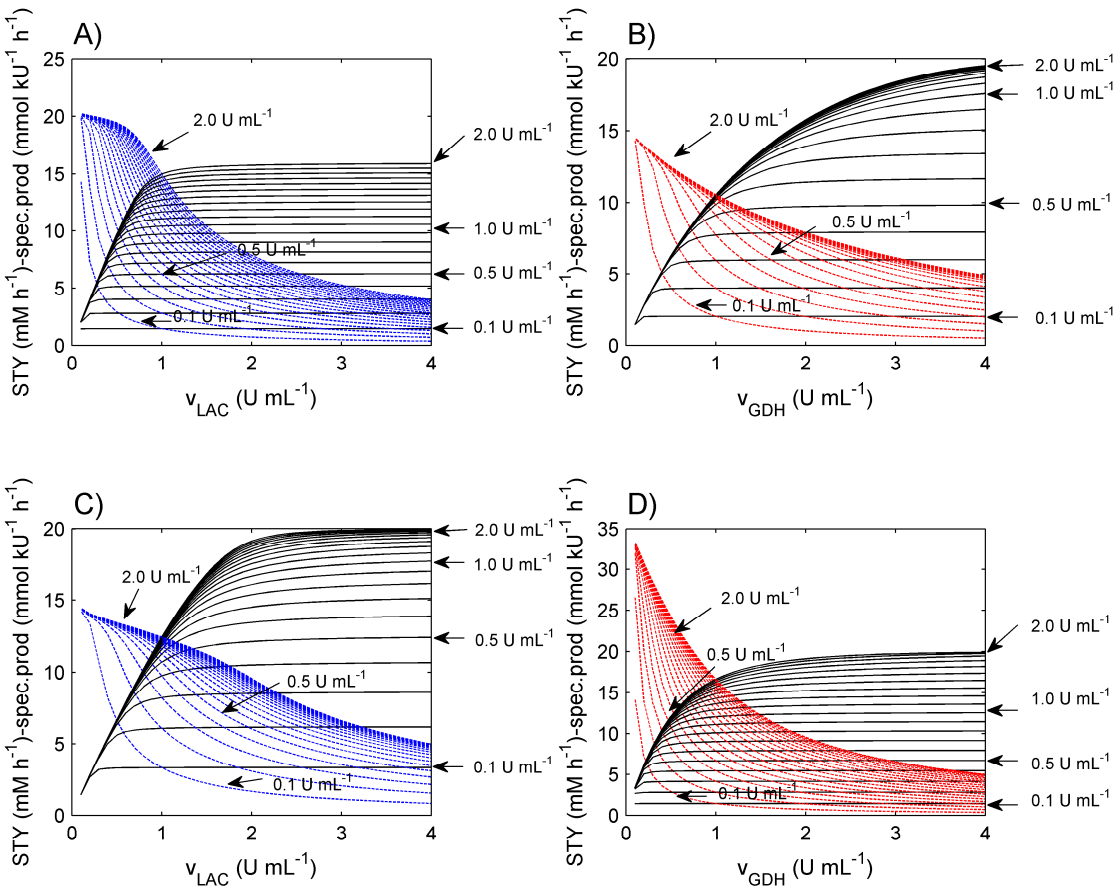

Figure S2. Isoproductivity plots based on the specific productivity after 10 h of reaction in regard to laccase (A) and GDH (B) activity, simulated for the conditions in Batch C (500 µM redox mediator, 100 µM coenzyme). The conditions in Batch D (100 µM redox mediator and 500 µM coenzyme) on the laccase and GDH are shown in C and D, respectively. The activities indicated in the diagrams are the opposite enzyme as given on the X-axis. STY (straight lines), specific productivity (dashed lines).

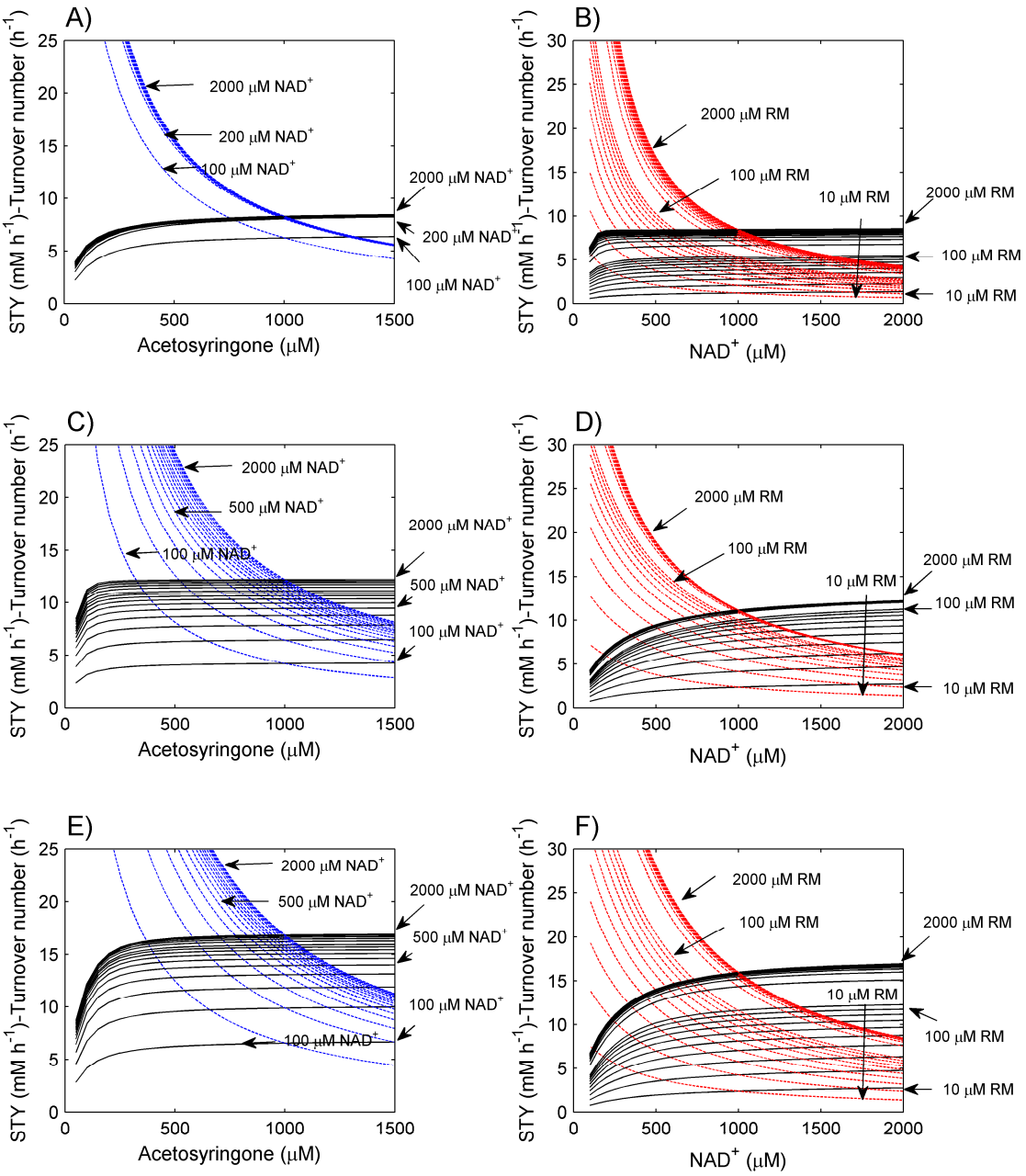

Figure S3. Effect of the initial redox mediator concentration (A, C, E) and coenzyme concentration (B, D, F) on STY and turnover numbers for the coenzyme and redox mediator after 10 h of reaction. The simulated laccase:GDH activity ratios correspond to those used in Batch B (380 U L^{-1} laccase, 480 U L^{-1} GDH, A, B); Batch C (900 U L^{-1} laccase, 300 U L^{-1} GDH, C, D); Batch D (900 U L^{-1} laccase, 500 U L^{-1} GDH, E, F). STY (straight lines), turnover numbers (dashed lines), NAD^+ stands for the total of applied coenzyme which was in the oxidized form.

APPENDIX B

Nature and biosynthesis of galacto-oligosaccharides related to oligosaccharides in human breast milk.

Intanon, M., Arreola, S. L., **Pham, N. H.,** Kneifel, W., Haltrich, D., and Nguyen, T.-H.

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MINIREVIEW

Nature and biosynthesis of galacto-oligosaccharides related to oligosaccharides in human breast milk

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β-galactosidases; transgalactosylation; galacto-oligosaccharides; human milk oligosaccharides; hetero-oligosaccharides.

Abstract

Human milk oligosaccharides (HMO) are prominent among the functional components of human breast milk. While HMO have potential applications in both infants and adults, this potential is limited by the difficulties in manufacturing these complex structures. Consequently, functional alternatives such as galacto-oligosaccharides are under investigation, and nowadays, infant formulae are supplemented with galacto-oligosaccharides to mimic the biological effects of HMO. Recently, approaches toward the production of defined human milk oligosaccharide structures using microbial, fermentative methods employing single, appropriately engineered microorganisms were introduced. Furthermore, galactose-containing hetero-oligosaccharides have attracted an increasing amount of attention because they are structurally more closely related to HMO. The synthesis of these novel oligosaccharides, which resemble the core of HMO, is of great interest for applications in the food industry.

Introduction

Certain oligosaccharides are considered to be beneficial for human and animal hosts due to their ability to stimulate selectively growth and/or activity of one or a limited number of bacteria in the colon. They are classified as 'prebiotics', new functional food ingredients that are of considerable interest. The prebiotic compounds are typically oligosaccharides of various compositions, and galacto-oligosaccharides (GOS), the products of transgalactosylation reactions catalyzed by β-galactosidases when using lactose as the substrate, are nondigestible carbohydrates meeting the criteria of 'prebiotics' (Roberfroid *et al.*, 2010). GOS are of special interest to human nutrition because of the presence of structurally related oligosaccharides together with different complex structures in human breast milk (Sangwan *et al.*, 2011). Several different functions are attributed to these human milk oligosaccharides (HMO). With respect to the influence on the intestinal microbiota, the neutral fraction of HMO seems to be a key factor for the development of the intestinal microbiota typical for breastfed infants and hence for the prebiotic effect. GOS together with inulin/fructo-oligosaccharides (FOS) and lactulose are among the most important and best-studied groups of prebiotic oligosaccharides. At present, these commercially important oligosaccharides with prebiotic status are available mainly in the Japanese, European and USA markets. A mixture of GOS and long-chain FOS was introduced in the market especially for the use in infant formula. This mixture can mimic HMO to some extent and shows a pronounced prebiotic effect; in that it stimulated the development of intestinal microbiota comparable with those found in breastfed infants (Boehm *et al.*, 2008). Hence, biocatalytically produced GOS can be of significant interest for the nutrition of infants. β-galactosidases have also been used to produce hetero-oligosaccharides (HOS) with potentially extended functionality in addition to GOS. Mannose, fructose, *N*-acetylneuraminic acid, glucuronic

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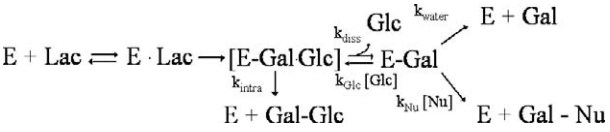
acid and a number of aromatic compounds have been shown to act as galactosyl acceptor for β-galactosidases (Gänzle, 2012). The choice of suitable acceptor and enzyme allows the formation of 'tailor-made' HOS of high interest for applications in the food industry. This article highlights the recent progress on research in microbial production of GOS. The emerging trends in the biosynthesis of the novel oligosaccharides, which are structurally more closely related to HMO, will be reviewed as well.

Microbial production of GOS

Transgalactosylation of lactose using β-galactosidases

β-galactosidases (β-gal; EC 3.2.1.23) catalyze the hydrolysis and transgalactosylation of β-D-galactopyranosides (such as lactose). GOS are the products of transgalactosylation reactions catalyzed by β-galactosidases when using lactose or other structurally related galactosides as the substrate. β-galactosidases undergo a two-step mechanism of catalysis. First, this mechanism involves the formation of a covalently linked galactosyl-enzyme intermediate. Subsequently, the galactosyl moiety linked to the nucleophile in the active site is transferred to a nucleophilic acceptor. Water, as well as all sugar species present in the reaction mixture, can serve as a galactosyl acceptor. Hence, the resulting final mixture contains hydrolysis products of lactose, which are glucose and galactose, unconverted lactose as well as di-, tri- and higher oligosaccharides. Scheme 1 illustrates possible lactose conversion reactions catalyzed by β-galactosidases, and structures of some GOS are given in Fig. 1.

β-galactosidases can be obtained from different sources including microorganisms, plants and animals. Microbial β-galactosidases have been isolated and characterized from yeasts, fungi and bacteria. The major industrial enzymes are obtained from *Aspergillus* spp. and *Kluyveromyces* spp. where *Kluyveromyces lactis* is probably the most widely used source (Kim *et al.*, 2004). Microbial sources of β-galactosidase are of great biotechnological interest because of easier handling, higher multiplication rates and production yield. Table 1 presents some of the commercially available bacterial, fungal and yeast



Scheme 1. Hydrolysis and galactosyl transfer reactions, both intra- and intermolecular, during the conversion of lactose catalyzed by β-galactosidases. E, enzyme; Lac, lactose; Gal, galactose; Glc, glucose; Nu, nucleophile.

β-galactosidases. An extensive list of bacterial and fungal sources of β-galactosidases, as well as the lactose conversion reaction conditions and GOS yields, are given in the review by Torres *et al.* (2010).

β-galactosidases from different species possess very different specificities for building glycosidic linkages and therefore produce different GOS mixtures. For example, the β-galactosidase from *K. lactis* produced predominantly β-(1→6)-linked GOS, the β-galactosidase from *Aspergillus oryzae* produced mainly β-(1→3) and β-(1→6) linkages, *Bacillus circulans* β-galactosidase forms mainly β-(1→4)-linked GOS (Rodriguez-Colinas *et al.*, 2014), whereas β-galactosidases from *Lactobacillus* spp. showed preference to form β-(1→6) as well as β-(1→3) linkages in transgalactosylation mode (Splechtna *et al.*, 2006; Nguyen *et al.*, 2012).

Production of GOS

Microbial sources of β-galactosidases for GOS production include crude enzymes, purified enzymes, recombinant enzymes, immobilized enzymes, whole-cell biotransformations, toluene-treated cells and immobilized cells. The enzyme sources, the process parameters as well as the yield and the productivity of these processes for GOS production are summarized in detail in recent reviews (Torres *et al.*, 2010; Sangwan *et al.*, 2011). The highest GOS productivity, 106 g L^{-1} h^{-1}, was observed when β-galactosidase from *A. oryzae* immobilized on cotton cloth was used for GOS production in a packed-bed reactor (Albayrak & Yang, 2002).

Bifidobacteria and lactobacilli have been studied intensively with respect to their enzymes for various different reasons, one of which is their 'generally recognized as safe' status and their safe use in food applications. It is anticipated that GOS produced by these β-galactosidases will have better selectivity for growth and metabolic activity of these bacterial genera in the gut and thus will lead to improved prebiotic effects. A number of studies report the presence of multiple β-galactosidases, for example, in *Bifidobacterium infantis*, *Bifidobacterium adolescentis* or *Bifidobacterium bifidum* (Hung & Lee, 2002; Hinz *et al.*, 2004; Goulas *et al.*, 2009). It was shown that these enzymes are very different with respect to substrate specificity and regulation of gene expression. Furthermore, these reports described the cloning and characterization of these enzymes and studied their transgalactosylation activity in detail; for example, β-galactosidase BgbII from *B. adolescentis* showed high preference toward the formation of β-(1→4) linkages, while no β-(1→6) linkages were formed (Hinz *et al.*, 2004). In contrast, the β-galactosidase BgbII from *B. bifidum* showed a clear preference for the synthesis of β-(1→6) linkages over β-(1→4)

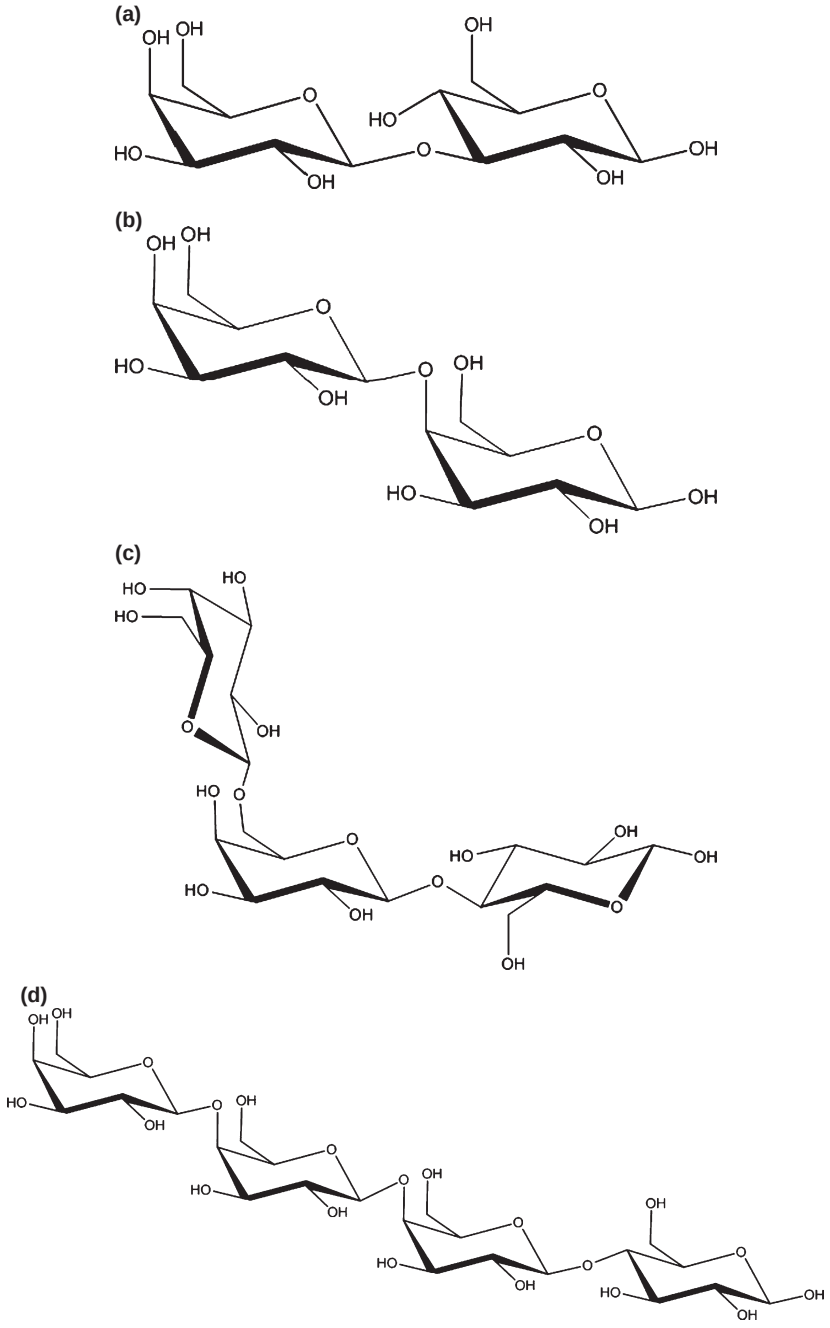


Fig. 1. Structures of some GOS: β-D-Gal*p*-(1→3)-D-Glc (a), β-D-Gal*p*-(1→4)-D-Gal (b), β-D-Gal*p*-(1→6)-Lac (c), β-D-Gal*p*-(1→4)-D-Gal*p*-(1→4)-Lac (d).

Table 1. Commercial β-galactosidases

Name	Manufacturer	Microorganism
BioLactase NTL-CONC	Biocon	*Bacillus circulans* (Rodriguez-Colinas *et al.*, 2014)
Lactozym pure 6500 L	Novozymes	*Kluyveromyces lactis* (Rodriguez-Colinas *et al.*, 2014)
Lactase F 'Amano'	Amano Enzyme Inc	*Aspergillus oryzae* (Rodriguez-Colinas *et al.*, 2014)
Biolacta FN5	Daiwa Fine Chemicals Co., Ltd	*Bacillus circulans*
LACTOLES L3	Biocon Ltd, Japan	*Bacillus circulans*
Maxilact	DSM Food Specialties	*Kluyveromyces lactis*
Tolerase	DSM Food Specialties	*Aspergillus oryzae*

linkages (Goulas *et al.*, 2009). A recombinant β-galactosidase from *B. infantis* (Hung & Lee, 2002) is an excellent biocatalyst for GOS production giving the highest GOS yield of 63% (mass of GOS of the total sugars in the reaction mixture). β-galactosidases of lactobacilli play an important role in a number of commercial processes, for example, milk processing or cheese making. Recent studies of β-galactosidases, especially with respect to their enzymatic and molecular properties, from *Lactobacillus reuteri* or *Lactobacillus bulgaricus* showed that these enzymes are very well suited for the production of GOS (Splechtna *et al.*, 2006; Nguyen *et al.*, 2012). Maximum GOS yields at 30 °C were *c.* 40% when using purified β-galactosidases from *L. reuteri* with initial lactose concentration of 205 g L^{-1} and at *c.* 80% lactose conversion (Splechtna *et al.*, 2006). Purified β-galactosidase from *L. bulgaricus* gave the highest yield of 50% for the lactobacillal enzymes at 90% lactose conversion (Nguyen *et al.*, 2012). To reduce enzyme costs, a crude β-galactosidase extract from *Lactobacillus* sp. directly obtained after cell disruption and separation of cell debris by centrifugation was used in lactose conversion for GOS production (Splechtna *et al.*, 2007b).

Choice of process technology

The choice of process technology either for lactose hydrolysis or GOS production depends on the nature of the substrate and the characteristics of the enzyme. The primary characteristic, which determines the choice and application of a given enzyme, is the operational pH range. Acid pH enzymes, which are mainly from fungi, are suitable for processing of acid whey and whey permeate, while the neutral pH enzymes from yeasts and bacteria are suitable for processing milk and sweet whey. Depending on the enzyme source, the pH value of the reaction mixture can be acidic when using, for example, the β-galactosidase from *A. oryzae* with an optimum GOS yield at pH 4.5 (Iwasaki *et al.*, 1996). The β-galactosidase from an acidophilic fungus, *Teratosphaeria acidotherma* AIU BGA-1, is stable over the pH range of 1.5–7.0 with optimal activity at pH 2.5–4.0 and 70 °C (Isobe *et al.*, 2013). In contrast, the maximum yield of GOS was observed at neutral pH for most bacterial and fungal β-galactosidases. The highest GOS yields are generally observed when the reaction proceeds to 45–90% lactose conversion (Torres *et al.*, 2010).

Studies of thermostable glycoside hydrolases have been conducted in pursuit of GOS production at high temperatures. These include β-glycosidases from *Alicyclobacillus acidocaldarius*, *Thermus thermophilus* KNOUC202 or *L. bulgaricus*, to name a few (Di Lauro *et al.*, 2008; Nam *et al.*, 2010; Nguyen *et al.*, 2012). Cold-active β-galactosidases have also attracted attention because their applications in the industrial processes of lactose hydrolysis and oligosaccharides synthesis can lower the risk of mesophiles contamination. Cold-active β-galactosidases were isolated from different sources such as *Paracoccus* sp. 32d, *Halorubrum lacusprofundi* and *Thalassospira frigidphilosprofundus* (Wierzbicka-Woś *et al.*, 2011; Karan *et al.*, 2013; Pulicherla *et al.*, 2013). Soluble cold-active β-galactosidase from *Paracoccus* sp. 32d was found to efficiently hydrolyze lactose in milk at 10 °C (Wierzbicka-Woś *et al.*, 2011). There has been relatively little research on GOS synthesis at low temperatures by these psychrophilic enzymes.

Reactor set-up is an important factor that can influence both the yield and the composition of the GOS mixtures formed. Continuous GOS production using a continuous stirred tank reactor (CSTR) with an external cross-flow membrane was compared with the batch-wise mode of conversion using β-galactosidase from *L. reuteri*. Marked differences were detected for the two reactor setups. Above 65% lactose conversion, the GOS yield was lower for the CSTR due to a lower content of tri- and tetrasaccharides in the reaction mixture. In the CSTR, β-gal from *L. reuteri* showed up to twofold higher specificity toward the formation of β-(1→6) linked GOS with β-D-Gal*p*-(1→6)-D-Glc and β-D-Gal*p*-(1→6)-D-Gal being the main GOS components formed under these conditions (Splechtna *et al.*, 2007a). A rotating disk membrane bioreactor was compared over batch mode to obtain purified GOS with high yield. It was found that GOS yield and purity were 32.4% and 77%, respectively, in batch mode followed by diafiltration-assisted nanofiltration, while in the immobilized state, they were 67.4% and 80.2% at 105 rad s^{-1} membrane speed. Retention of the monosaccharides that inhibit the enzyme in the reaction volume of batch mode reduced the yield of GOS. On the contrary, simultaneous production and purification of GOS in the rotating disk membrane bioreactor led to a high yield of GOS (Sen *et al.*, 2012).

Compared with soluble β-galactosidases, immobilized β-galactosidases may provide advantages such as high enzyme reusability, higher cell densities in bioreactors, improved enzyme stability, reutilization and continuous operation, and easier separation of the products (Verma *et al.*, 2012). Higher activity but lower thermostability was reported for β-galactosidase immobilized on chitosan nanoparticles than that bound onto macroparticles (Klein *et al.*, 2012). β-galactosidase immobilized in polyvinyl alcohol lenses was more stable and converted more lactose than when immobilized in solgel carriers (Jovanovic-Malinovska *et al.*, 2012). Compared with the corresponding free enzyme systems, immobilization resulted in less product inhibition by glucose and a higher stability at denaturing temperatures (Klein *et al.*, 2012; Verma *et al.*, 2012).

Prediction of GOS production by modeling techniques

Several kinetic mechanisms, either mechanistic, empirical or a combination of both, have been proposed to account for the transgalactosylation reactions and to subsequently define strategies to optimize GOS production. A six-parameter model was developed to describe oligosaccharide production from lactose hydrolysis by β-galactosidase from *B. circulans*; the model considered glucose inhibition, but ignored the formation of tetra- and higher oligosaccharides (Boon *et al.*, 2000). A model of *K. lactis* β-galactosidase describing both hydrolysis and transgalactosylation reactions with glucose and lactose as acceptors fitted well to the experimental data of the time course reactions at various concentrations of lactose (Kim *et al.*, 2004). A pseudo steady-state model for the kinetically controlled synthesis of GOS with *A. oryzae* β-galactosidase was presented by Vera *et al.* (2011). This model predicted substrate and product profiles during GOS synthesis in the temperature range between 40 and 55 °C, providing a useful tool for process scale-up and optimization. The model accounts for the total GOS production and its composition, which is a definite advantage over previously existing models. However, the model tends to underestimate disaccharides consumption and penta-GOS (GOS-5) formation and to overestimate glucose production.

Leveraging the power of protein engineering for GOS production

Protein engineering is a powerful approach to favor transgalactosylation over hydrolysis and hence to improve transgalactosylation yields. A truncated β-galactosidase from *B. bifidum* enhanced the transgalactosylation activity of the enzyme toward lactose, and as a result, a normal, hydrolytic β-galactosidase was converted to a highly efficient transgalactosylating enzyme (Jørgensen *et al.*, 2001). A mutagenesis approach was applied to the galactosidase BgaB of *Geobacillus stearothermophilus* KVE39 to improve its enzymatic transglycosylation of lactose into oligosaccharides. Exchange of one single amino acid, arginine Arg109, in β-galactosidase BgaB to either lysine, valine or tryptophan improved significantly the formation of the main trisaccharide, that is, 3′-galactosyl-lactose. The yield of this trisaccharide increased from 2% to 12%, 21% and 23%, respectively, for these different variants compared with that of the native enzyme (Placier *et al.*, 2009). Enhancement of the production of GOS was also achieved by mutagenesis of *Sulfolobus solfataricus* β-galactosidase, LacS. The GOS yield obtained from two mutants of LacS, F441Y and F359Q, was increased by 10.8% and 7.4%, respectively (Wu *et al.*, 2013). Although protein engineering strategies were successfully applied to enhance transgalactosylation activities of different β-galactosidases, this approach has not yet been reported to alter the linkage type of the GOS products as well.

Biosynthesis of oligosaccharides structurally related to those found in human milk

Mature human milk contains *c.* 7–12 g L^{-1} of free oligosaccharides in addition to lactose, which typically is present in concentrations of 55–70 g L^{-1} (Wu *et al.*, 2011). Currently, up to 200 unique oligosaccharide structures varying from 3 to 22 sugar units have been identified (Kobata, 2010). HMO are composed of the five monosaccharide building blocks D-glucose (Glc), D-galactose (Gal), *N*-acetylglucosamine (GlcNAc), L-fucose (Fuc) and sialic acid (*N*-acetylneuraminic acid). They can be grouped into neutral and charged oligosaccharides, the latter being sialylated and comprising *c.* 20% of all HMO (Wu *et al.*, 2011). The structures of HMO show typical patterns. Lactose (Gal-β-1,4-Glc) is found at the reducing end of HMO. This terminal lactose is typically elongated by lacto-*N*-biose units (LNB; Gal-β-1,3-GlcNAc) in type I or *N*-acetyl-lactosamine units (LacNAc; Gal-β-1,4-GlcNAc) in the rarer type II structures. Both LNB and LacNAc are attached via a β-1,3-linkage to the galactosyl moiety of the terminal lactose, with an additional β-1,6-linkage in branched HMO.

These LNB and LacNAc units can be repeated up to 25 times in larger HMO, forming the core region of these oligosaccharides. A further variation results from the attachment of fucosyl and sialic acid residues (Fig. 2). Thus, the simplest structures following this general scheme (apart from certain trisaccharides such as galactosyl-lactose, fucosyl-lactose and sialyl-lactose) are the tetrasaccharides lacto-*N*-tetraose, Gal-β-1,3-GlcNAc-β-1,3-Gal-β-1,4-Glc (type I), and lacto-*N*-*neo*-tetraose, Gal-β-1,4-GlcNAc-β-1,3-Gal-β-1,4-Glc (type II; Fig. 3). Urashima listed an HMO classification based on their 13 core structures; of which, the most abundant components are 2′-fucosyllactose (Fuc-α-1,2-Gal-β-1,4-Glc), lacto-*N*-tetraose (Gal-β-1,3-GlcNAc-β-1,3-Gal-β-1,4-Glc) and lacto-*N*-fucopentaose (Fuc-α-1,2-Gal-β-1,3-GlcNac-β-1,3-Gal-β-1,4-Glc) (Urashima *et al.*, 2013). Despite recent modern analytical techniques, HMO identification remains a challenge for researchers.

The composition and content of HMO can vary significantly between different mothers. It varies depending on both their blood group type and the time/length of lactation (Totten *et al.*, 2012). Nevertheless, mono- and difucosyl-lactose, lacto-*N*-tetraose and its fucosylated

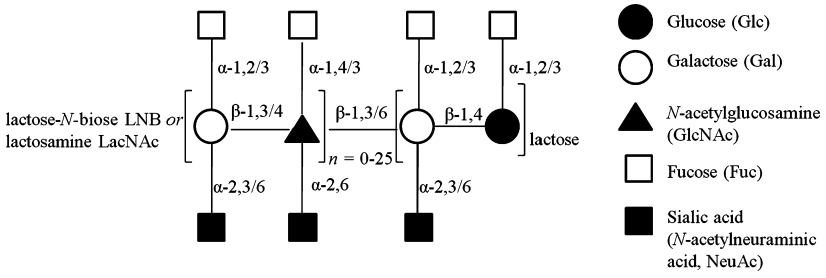


Fig. 2. Schematic structure of HMO.

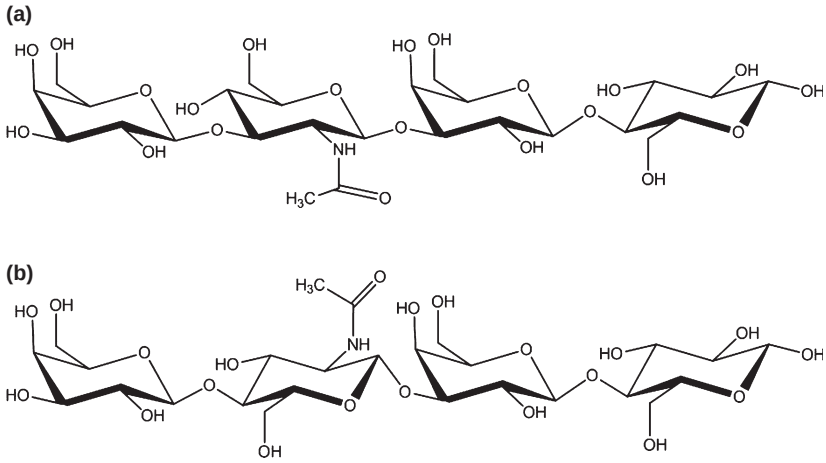


Fig. 3. Some simple structures of HMO: tetrasaccharides lacto-*N*-tetraose, Gal-β-1,3-GlcNAc-β-1,3-Gal-β-1,4-Glc (a), and lacto-*N*-*neo*-tetraose, Gal-β-1,4-GlcNAc-β-1,3-Gal-β-1,4-Glc (b).

derivatives as well as sialyl-lactose and the sialylated forms of lacto-*N*-tetraose are major HMO in human milk. The presence and absence of α-(1,3/4)- and α-(1,2)-fucosylated oligosaccharides indicate the activity of the Lewis (*Le*)- and secretor (*Se*) gene of the mother, with the latter being responsible for the expression of α-(1,2)-fucosyl transferase. Totten *et al.* (2012) described the four possible phenotypes expressed in HMOs and reported that West African populations have higher abundance of Lewis negative and nonsecretors than in European and American populations.

Approaches to biosynthesis of related HMO structures

Considerable interest exists in the efficient production of HMO, which is albeit hampered by the structural complexity as well as the complex mixture of HMO. Many manufacturers are trying to emulate HMO; however, essential ingredients are mostly absent from infant formula due to the lack of industrial production methods. Most often, GOS and/or FOS are added to infant formulas to mimic the effect of HMOs.

The most promising approach toward the production of defined HMO structures seems to be microbial, fermentative methods employing single, appropriately engineered microorganisms. This *in vivo* approach was introduced by the group of Eric Samain. They used a β-galactosidase-negative *Escherichia coli* strain over-expressing a β-1,3-*N*-acetylglucosaminyl-transferase gene from *Neisseria meningitidis*. When feeding lactose to this engineered strain, this disaccharide was taken up by the indigenous β-galactoside permease of *E. coli*. The recombinantly synthesized β-1,3-*N*-acetylglucosaminyl transferase then utilized the intracellular pool of UDP-GlcNAc to transfer GlcNAc residues regiospecifically to lactose, resulting in the formation of the trisaccharides GlcNac-β-1,3-Gal-β-1,4-Glc. This compound was released into the extracellular medium in yields of 6 g L^{-1} (Priem *et al.*, 2002). In a similar manner, an engineered *E. coli* strain, over-expressing an α-1,3-fucosyltransferase from *Helicobacter pylori* and genetically engineered to provide sufficient GDP fucose as the intracellular substrate for the glycosyltransferase, was used to produce various fucosylated HMO from lactose added to the medium (Dumon *et al.*, 2001). An engineered *E. coli* strain over-expressing the α-2,3-sialyltransferase gene from *N. meningitidis*, together with an engineered pathway to provide the activated sialic acid donor CMP-Neu5Ac as the substrate for the glycosyltransferase, produced 3′-sialyl-lactose in concentrations of up to 25 g L^{-1} in high cell density cultivations with continuous lactose feed (Fierfort & Samain, 2008). 6′-sialyl-lactose was efficiently produced when employing α-2,6-sialyltransferase from *Photobacterium* sp.

again in metabolically engineered *E. coli* (Drouillard *et al.*, 2010). In a very recent study, a recombinant *Pasteurella multocida* sialyltransferase exhibiting dual trans-sialidase activities catalyzed trans-sialylation using either 2-*O*-(*p*-nitrophenyl)-α-D-*N*-acetylneuraminic acid or casein glycomacropeptide (whey protein) as the sialyl donor and lactose as the acceptor, resulting in production of both 3′-sialyl-lactose and 6′-sialyl-lactose. The enzyme was capable of catalyzing the synthesis of both 3′- and 6′-sialylated GOS when GOS served as acceptors (Guo *et al.*, 2014). A mutant of the sialidase from the nonpathogenic *Trypanosoma rangeli* expressed in *Pichia pastoris* after codon optimization has been reported to exhibit trans-sialidase activity. The enzyme catalyzed the transfer of sialic acid from cGMP (casein glycomacropeptide) to lactose at high efficiency, giving a yield at the 5 L scale of 3.6 g 3′-sialyl-lactose. The estimated molar trans-sialylation yield was 50% for the 3′-sialyl residues in cGMP without substantial hydrolysis of 3′-sialyl-lactose. Lacto-*N*-tetraose and lacto-*N*-fucopentaoses also functioned as acceptor molecules demonstrating the versatility of this trans-sialidase for catalyzing sialyl-transfer toward different HMO (Michalak *et al.*, 2014).

LNB is a key disaccharide component of HMO such as lacto-*N*-tetraose and lacto-*N*-fucopentaose. LNB can be produced in a purely enzymatic approach, making use of the synthetic capacity of sugar phosphorylases. LNB phosphorylase, together with sucrose phosphorylase, UDP-glucose-hexose-1-phosphate uridylyltransferase and UDP-glucose 4-epimerase produced LNB from sucrose and GlcNAc in the presence of phosphate and catalytic amounts of UDP-Glc in yields of 85% (Nishimoto & Kitaoka, 2007). Recent development to enhance thermostability of galacto-*N*-biose/lacto-*N*-biose phosphorylase by directed evolution yielded a mutant that exhibited 20 °C higher thermostability than the wild type, which is suitable for industrial production of LNB at temperatures higher than 50 °C for faster reaction and prevention of microbial contamination (Koyama *et al.*, 2013).

Galactose-containing HOS

An approach that has received some interest is the synthesis of HOS; of which, some are expected to resemble HMO-like structures, using β-galactosidases. This approach is based on β-galactosidase-catalyzed transglycosylation with lactose as donor (thus transferring galactose onto suitable acceptors) and GlcNAc as acceptor, thus obtaining *N*-acetyl-lactosamine (LacNAc) and its regioisomers. Using this approach and a hyperthermophilic β-galactosidase from *S. solfataricus*, Gal-β-1,6-GlcNAc together with an unidentified sugar were the main products starting from a mixture of 1 M lactose and 1 M GlcNAc, while LacNAc and Gal-β-1,3-GlcNAc were formed as well, yet in lower concentrations (Reuter *et al.*, 1999). This reaction was also optimized for using β-galactosidase from *B. circulans* as the biocatalyst. This enzyme is known for its propensity to synthesize β-1,4-linkages in its transgalactosylation mode, and hence the main reaction product here was LacNAc together with smaller amounts of GlcNAc-containing higher oligosaccharides (one tri- and one tetrasaccharides) and Gal-β-1,6-GlcNAc. The total yield was 40% for these GlcNAc-containing oligosaccharides when starting from 0.5 M lactose and GlcNAc each (Li *et al.*, 2010). Crude cellular extracts of *L. bulgaricus* and *Lactococcus lactis* MG1363 expressing LacLM of *Lactobacillus plantarum* were used as sources of β-galactosidases for the formation of HOS by galactosylation of *N*-acetylglucosamine (GlcNAc) and fucose with the main products identified as Gal-β-(1→4)-GlcNAc, Gal-β-(1→6)-GlcNAc, Gal-β-(1→6)-Gal-β-(1→4)-GlcNAc and Gal-β-(1→6)-Gal-β-(1→6)-GlcNAc (Gänzle, 2012).

Conclusion

HMO yet cannot be commercially produced due to their structural and compositional complexity; however, increased biochemical knowledge on suitable glycosyltransferases may pave the road to microbial, fermentative methods employing single, appropriately engineered microorganisms. The presence of structurally related oligosaccharides together with different complex structures in human breast milk makes GOS attract increasing interests from researchers and manufacturers. The insights into the structures and the production of GOS together with advancement in the area of biotechnology will certainly result in the enhancement of the production of GOS in the future. The use of lactic acid bacteria and *Bifidobacteria* as the sources of β-galactosidases offers substantial potential for the production of GOS and is an interesting approach for the production of new carbohydrate-based functional food ingredients that are of interest in applications such as infant formula. Nowadays, infant formulae are supplemented with GOS to mimic the biological effects of HMO. Some structures of novel galactose-containing HOS resemble the core of HMO, and hence, these novel functionally enhanced, prebiotic oligosaccharides could be of interest for a wide range of applications.

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References

Albayrak N & Yang ST (2002) Production of galacto-oligosaccharides from lactose by *Aspergillus oryzae* β-galactosidase immobilized on cotton cloth. *Biotechnol Bioeng* **77**: 8–19.

Boehm G, Stahl B, Knol J & Garssen J (2008) Carbohydrates in human milk and infant formulas. *Carbohydrate Chemistry, Biology and Medical Applications* (Garg HG, Cowman MK & Hales CA, eds), pp. 275–291. Elsevier Ltd, Amsterdam, the Netherlands.

Boon MA, Janssen AEM & van't Riet K (2000) Effect of temperature and enzyme origin on the enzymatic synthesis of oligosaccharides. *Enzyme Microb Technol* **26**: 271–281.

Di Lauro B, Strazzulli A, Perugino G, La Cara F, Bedini E, Corsaro MM, Rossi M & Moracci M (2008) Isolation and characterization of a new family 42 β-galactosidase from the thermoacidophilic bacterium *Alicyclobacillus acidocaldarius:* identification of the active site residues. *Biochim Biophys Acta* **1784**: 292–301.

Drouillard S, Mine T, Kajiwara H, Yamamoto T & Samain E (2010) Efficient synthesis of 6′-sialyllactose, 6,6′-disialyllactose, and 6′-KDO-lactose by metabolically engineered *E. coli* expressing a multifunctional sialyltransferase from the *Photobacterium* sp. JT-ISH-224. *Carbohydr Res* **345**: 1394–1399.

Dumon C, Priem B, Martin SL, Heyraud A, Bosso C & Samain E (2001) *In vivo* fucosylation of lacto-*N*-neotetraose and lacto-*N*-neohexaose by heterologous expression of *Helicobacter pylori* α-1,3 fucosyltransferase in engineered *Escherichia coli*. *Glycoconj J* **18**: 465–474.

Fierfort N & Samain E (2008) Genetic engineering of *Escherichia coli* for the economical production of sialylated oligosaccharides. *J Biotechnol* **134**: 261–265.

Gänzle MG (2012) Enzymatic synthesis of galacto-oligosaccharides and other lactose derivatives (hetero-oligosaccharides) from lactose. *Int Dairy J* **22**: 116–122.

Goulas T, Goulas A, Tzortzis G & Gibson GR (2009) Comparative analysis of four β-galactosidases from *Bifidobacterium bifidum* NCIMB41171: purification and biochemical characterisation. *Appl Microbiol Biotechnol* **82**: 1079–1088.

Guo Y, Jers C, Meyer AS, Arnous A, Li H, Kirpekar F & Mikkelsen JD (2014) A *Pasteurella multocida* sialyltransferase displaying dual trans-sialidase activities for production of 3′-sialyl and 6′-sialyl glycans. *J Biotechnol* **170**: 60–67.

Hinz SWA, Van Den Broek LAM, Beldman G, Vincken JP & Voragen AGJ (2004) β-Galactosidase from *Bifidobacterium adolescentis* DSM20083 prefers β(1,4)-galactosides over lactose. *Appl Microbiol Biotechnol* **66**: 276–284.

Hung MN & Lee BH (2002) Purification and characterization of a recombinant b-galactosidase with transgalactosylation activity from *Bifidobacterium infantis* HL96. *Appl Microbiol Biotechnol* **58**: 439–445.

Isobe K, Yamashita M, Chiba S, Takahashi N & Koyama T (2013) Characterization of new β-galactosidase from acidophilic fungus, *Teratosphaeria acidotherma* AIU BGA-1. *J Biosci Bioeng* **116**: 293–297.

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

Jørgensen F, Hansen OC & Stougaard P (2001) High-efficiency synthesis of oligosaccharides with a truncated β-galactosidase from *Bifidobacterium bifidum*. *Appl Microbiol Biotechnol* **57**: 647–652.

Jovanovic-Malinovska R, Fernandes P, Winkelhausen E & Fonseca L (2012) Galacto-oligosaccharides synthesis from lactose and whey by β-galactosidase immobilized in PVA. *Appl Biochem Biotechnol* **168**: 1197–1211.

Karan R, Capes MD, DasSarma P & DasSarma S (2013) Cloning, overexpression, purification, and characterization of a polyextremophilic β-galactosidase from the Antarctic haloarchaeon *Halorubrum lacusprofundi*. *BMC Biotechnol* **13**: 3.

Kim CS, Ji E-S & Oh D-K (2004) A new kinetic model of recombinant b-galactosidase from *Kluyveromyces lactis* for both hydrolysis and transgalactosylation reactions. *Biochem Biophys Res Commun* **316**: 738–743.

Klein MP, Nunes MR, Rodrigues RC, Benvenutti EV, Costa TMH, Hertz PF & Ninow JL (2012) Effect of the support size on the properties of β-galactosidase immobilized on chitosan: advantages and disadvantages of macro and nanoparticles. *Biomacromolecules* **13**: 2456–2464.

Kobata A (2010) Structures and application of oligosaccharides in human milk. *Proc Jpn Acad Ser B Phys Biol Sci* **86**: 731–747.

Koyama Y, Hidaka M, Nishimoto M & Kitaoka M (2013) Directed evolution to enhance thermostability of galacto-*N*-biose/lacto-*N*- biose I phosphorylase. *Protein Eng Des Sel* **26**: 755–761.

Li W, Sun Y, Ye H & Zeng X (2010) Synthesis of oligosaccharides with lactose and *N*-acetylglucosamine as substrates by using β-D-galactosidase from *Bacillus circulans*. *Eur Food Res Technol* **231**: 55–63.

Michalak M, Larsen DM, Jers C *et al.* (2014) Biocatalytic production of 3′-sialyllactose by use of a modified sialidase with superior trans-sialidase activity. *Process Biochem* **49**: 265–270.

Nam ES, Kim MS, Lee HB & Ahn JK (2010) β-Glycosidase of *Thermus thermophilus* KNOUC202: gene and biochemical properties of the enzyme expressed in *Escherichia coli*. *Prikl Biokhim Mikrobiol* **46**: 562–571.

Nguyen TT, Nguyen HA, Arreola SL, Mlynek G, Djinović-Carugo K, Mathiesen G, Nguyen TH & Haltrich D

(2012) Homodimeric β-galactosidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 20081: expression in *Lactobacillus plantarum* and biochemical characterization. *J Agric Food Chem* **60**: 1713–1721.

Nishimoto M & Kitaoka M (2007) Identification of *N*-acetylhexosamine 1-kinase in the complete lacto-*N*-biose I/galacto-*N*-biose metabolic pathway in *Bifidobacterium longum*. *Appl Environ Microbiol* **73**: 6444–6449.

Placier G, Watzlawick H, Rabiller C & Mattes R (2009) Evolved β-galactosidases from *Geobacillus stearothermophilus* with improved transgalactosylation yield for galacto-oligosaccharide production. *Appl Environ Microbiol* **75**: 6312–6321.

Priem B, Gilbert M, Wakarchuk WW, Heyraud A & Samain E (2002) A new fermentation process allows large-scale production of human milk oligosaccharides by metabolically engineered bacteria. *Glycobiology* **12**: 235–240.

Pulicherla KK, Kumar PS, Manideep K, Rekha VPB, Ghosh M & Sambasiva Rao KRS (2013) Statistical approach for the enhanced production of cold-active β-galactosidase from *Thalassospira frigidphilosprofundus*: a novel marine psychrophile from deep waters of Bay of Bengal. *Prep Biochem Biotechnol* **43**: 766–780.

Reuter S, Rusborg Nygaard A & Zimmermann W (1999) β-Galactooligosaccharide synthesis with β-galactosidases from *Sulfolobus solfataricus*, *Aspergillus oryzae*, and *Escherichia coli*. *Enzyme Microb Technol* **25**: 509–516.

Roberfroid M, Gibson GR, Hoyles L *et al.* (2010) Prebiotic effects: metabolic and health benefits. *Br J Nutr* **104**: S1–S63.

Rodriguez-Colinas B, Fernandez-Arrojo L, Ballesteros AO & Plou FJ (2014) Galactooligosaccharides formation during enzymatic hydrolysis of lactose: towards a prebiotic-enriched milk. *Food Chem* **145**: 388–394.

Sangwan V, Tomar SK, Singh RRB, Singh AK & Ali B (2011) Galactooligosaccharides: novel components of designer foods. *J Food Sci* **76**: R103–R111.

Sen D, Sarkar A, Das S, Chowdhury R & Bhattacharjee C (2012) Batch hydrolysis and rotating disk membrane bioreactor for the production of galacto-oligosaccharides: a comparative study. *Ind Eng Chem Res* **51**: 10671–10681.

Splechtna B, Nguyen TH, Steinböck M, Kulbe KD, Lorenz W & Haltrich D (2006) Production of prebiotic galacto-oligosaccharides from lactose using β-galactosidases from *Lactobacillus reuteri*. *J Agric Food Chem* **54**: 4999–5006.

Splechtna B, Nguyen TH & Haltrich D (2007a) Comparison between discontinuous and continuous lactose conversion processes for the production of prebiotic galacto-oligosaccharides using β-galactosidase from *Lactobacilius reuteri*. *J Agric Food Chem* **55**: 6772–6777.

Splechtna B, Nguyen TH, Zehetner R, Lettner HP, Lorenz W & Haltrich D (2007b) Process development for the production of prebiotic galacto-oligosaccharides from lactose using β-galactosidase from *Lactobacillus* sp. *Biotechnol J* **2**: 480–485.

Torres DPM, Gonçalves MDPF, Teixeira JA & Rodrigues LR (2010) Galacto-oligosaccharides: production, properties, applications, and significance as prebiotics. *Compr Rev Food Sci Food Saf* **9**: 438–454.

Totten SM, Zivkovic AM, Wu S, Ngyuen U, Freeman SL, Ruhaak LR, Darboe MK, German JB, Prentice AM & Lebrilla CB (2012) Comprehensive profiles of human milk oligosaccharides yield highly sensitive and specific markers for determining secretor status in lactating mothers. *J Proteome Res* **11**: 6124–6133.

Urashima T, Taufik E, Fukuda K & Asakuma S (2013) Recent advances in studies on milk oligosaccharides of cows and other domestic farm animals. *Biosci Biotechnol Biochem* **77**: 455–466.

Vera C, Guerrero C, Illanes A & Conejeros R (2011) A pseudo steady-state model for galacto-oligosaccharides synthesis with β-galactosidase from *Aspergillus oryzae*. *Biotechnol Bioeng* **108**: 2270–2279.

Verma ML, Barrow CJ, Kennedy JF & Puri M (2012) Immobilization of β-D-galactosidase from *Kluyveromyces lactis* on functionalized silicon dioxide nanoparticles: characterization and lactose hydrolysis. *Int J Biol Macromol* **50**: 432–437.

Wierzbicka-Woś A, Cieśliński H, Wanarska M, Kozłowska-Tylingo K, Hildebrandt P & Kur J (2011) A novel cold-active β-D-galactosidase from the *Paracoccus* sp. 32d–gene cloning, purification and characterization. *Microb Cell Fact* **10**: 108.

Wu S, Grimm R, German JB & Lebrilla CB (2011) Annotation and structural analysis of sialylated human milk oligosaccharides. *J Proteome Res* **10**: 856–868.

Wu Y, Yuan S, Chen S, Wu D, Chen J & Wu J (2013) Enhancing the production of galacto-oligosaccharides by mutagenesis of *Sulfolobus solfataricus* β-galactosidase. *Food Chem* **138**: 1588–1595.

APPENDIX C

Two β-Galactosidases from the Human Isolate *Bifidobacterium breve* DSM 20213: Molecular Cloning and Expression, Biochemical Characterization and Synthesis of Galacto-Oligosaccharides.

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Two β-Galactosidases from the Human Isolate *Bifidobacterium breve* DSM 20213: Molecular Cloning and Expression, Biochemical Characterization and Synthesis of Galacto-Oligosaccharides

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Abstract

Two β-galactosidases, β-gal I and β-gal II, from *Bifidobacterium breve* DSM 20213, which was isolated from the intestine of an infant, were overexpressed in *Escherichia coli* with co-expression of the chaperones GroEL/GroES, purified to electrophoretic homogeneity and biochemically characterized. Both β-gal I and β-gal II belong to glycoside hydrolase family 2 and are homodimers with native molecular masses of 220 and 211 kDa, respectively. The optimum pH and temperature for hydrolysis of the two substrates *o*-nitrophenyl-β-D-galactopyranoside (oNPG) and lactose were determined at pH 7.0 and 50°C for β-gal I, and at pH 6.5 and 55°C for β-gal II, respectively. The k_{cat}/K_m values for oNPG and lactose hydrolysis are 722 and 7.4 $mM^{-1}s^{-1}$ for β-gal I, and 543 and 25 $mM^{-1}s^{-1}$ for β-gal II. Both β-gal I and β-gal II are only moderately inhibited by their reaction products D-galactose and D-glucose. Both enzymes were found to be very well suited for the production of galacto-oligosaccharides with total GOS yields of 33% and 44% of total sugars obtained with β-gal I and β-gal II, respectively. The predominant transgalactosylation products are β-D-Gal*p*-(1→6)-D-Glc (allolactose) and β-D-Gal*p*-(1→3)-D-Lac, accounting together for more than 75% and 65% of the GOS formed by transgalactosylation by β-gal I and β-gal II, respectively, indicating that both enzymes have a propensity to synthesize β-(1→6) and β-(1→3)-linked GOS. The resulting GOS mixtures contained relatively high fractions of allolactose, which results from the fact that glucose is a far better acceptor for galactosyl transfer than galactose and lactose, and intramolecular transgalactosylation contributes significantly to the formation of this disaccharide.

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Introduction

The colonic microbiota is composed of more than 400 different species, some of which have been related to health and well-being of the host [1]. In practice, the beneficial bacteria that serve as main targets to be increased in number and/or activity by different approaches are bifidobacteria and lactobacilli [2]. Members of the genus *Bifidobacterium* are one of the most common organisms found in the human gastro-intestinal tract [3,4]. These species are considered to be important in maintaining human health as they contribute to carbohydrate fermentations in the colon, and their diversity and number provide a marker for the stability of the human intestinal microflora [5]. Bifidobacteria thus play an important role in the eco-physiology of the colonic microbiota, although their population sizes and species composition vary among different groups of human population. The major *Bifidobacterium* species found in the adult microflora are *Bifidobacterium adolescentis* and *B. longum* while *B. infantis* and *B. breve* are the predominant bifidobacteria in infant intestinal tracts [4,6]. Activity of these bacteria has been linked to health effects such as increased resistance to infection, stimulation of the immune system activity, protection against cancer, or other prophylactic and therapeutic benefits. Bifidobacteria are also known to excrete a range of water-soluble vitamins such as folate, nicotinic acid, thiamine, pyridoxine, and vitamin B12 [7].

Table 1. β-Galactosidase activities in cell-free extracts of recombinant *E. coli* expressing *B. breve* β-gal I or β-gal II with and without coexpression of chaperones[a].

Enzyme	Volumetric activity (kU L^{-1} fermentation broth)[b]		Specific activity (U mg^{-1} protein)		Expression factor[c] (fold)
		with chaperones		with chaperones	
β-gal I	6.4	193.2	1.8	159.0	30.2
β-gal II	2.6	36.5	2.5	31.4	14.0

[a]Values are the mean of two cultivations.
[b]*o*NPG was used to determine enzyme activity.
[c]The expression factors are calculated as the ratios of the volumetric β-galactosidase activities obtained from the expressions with chaperones and without chaperones.
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Prebiotic oligosaccharides can serve as fermentable substrates for certain members of the gut microbiota and have been found to modulate the colonic flora by selective stimulation of the beneficial bacteria as well as inhibition of 'undesirable' bacteria [7–9]. Galacto-oligosaccharides (GOS) are the products of transgalactosylation reactions catalyzed by β-galactosidases when using lactose as the substrate and can include various trisaccharides, higher oligosaccharides as well as non-lactose disaccharides. GOS are non-digestible carbohydrates meeting the criteria of 'prebiotics' [10], and have attracted increasing attention because of the presence of structurally related oligosaccharides together with different complex structures in human breast milk. Therefore the use of GOS in infant formula is nowadays of great interest [11–13].

β-Galactosidases (β-gal; EC 3.2.1.23) catalyze the hydrolysis and transgalactosylation of β-D-galactopyranosides (such as lactose) [14–16] and are found widespread in nature. They catalyze the cleavage of lactose (or related compounds) in hydrolysis mode. An attractive biocatalytic application is found in the transgalactosylation potential of these enzymes, which is based on their catalytic mechanism [14,17]. The use of lactic acid bacteria (LAB) and bifidobacteria as sources of β-galactosidases may offer substantial potential for the production of GOS [18], and recent years have seen a significant increase in studies dealing with their biochemical properties or their ability to produce GOS in biocatalytic processes [19–34]. *Bifidobacterium breve* DSM 20213 is an isolate from the infant gut. The possibility of rationally targeting prebiotics to specific groups of bacteria such as certain known and approved probiotic strains is a promising prospect. One potential approach to this end is the use of enzymes, such as a β-galactosidase obtained from a probiotic strain, for the synthesis of oligosaccharides [35]. The two β-galactosidases from *B. breve* DSM 20213 selected for this work, β-gal I and β-gal II, are encoded by the corresponding *lacZ* genes (NCBI Reference No. EFE90149.1; EFE88654.1) and belong to glycoside hydrolase family 2 (GH2 family). β-Galactosidases of the GH2 family generally receive more attention in terms of transgalactosylation activity since they may have a high propensity to catalyze this reaction.

In this paper, we describe the cloning of two β-galactosidases from *Bifidobacterium breve* DSM 20213 and their expression in *Escherichia coli*. Furthermore, biochemical properties of these enzymes and their potential to produce GOS are also presented.

Materials and Methods

Chemicals and vectors

All chemicals and enzymes were purchased from Sigma (St. Louis, MO, USA) unless stated otherwise and were of the highest quality available. The test kit for the determination of D-glucose was obtained from Megazyme (Wicklow, Ireland). All restriction enzymes, T4 DNA ligase, and corresponding buffers were from Fermentas (Vilnius, Lithunia). The plasmid pET-21a (+) was from Novagen (Darmstadt, Germany) and the plasmid pGRO7

Table 2. Kinetic parameters of two recombinant β-galactosidases (β-gal I and β-gal II) from *B. breve* for the hydrolysis of lactose and *o*-nitrophenyl β-D-galactopyranoside (*o*NPG).

Substrate	Method for determination of enzyme activity	Kinetic parameter	β-gal I	β-gal II
Lactose	release of D-Glc	$v_{max,Lac}$ (μmol min^{-1} mg^{-1})	59±2	97±5
		$K_{m,Lac}$	15.3±3.2	7.5±0.9
		k_{cat} (s^{-1})	114±4	188±10
		k_{cat}/K_m (mM^{-1} s^{-1})	7.4±1.9	25±4
		$K_{i,Gal}$	28±9	27±6
*o*NPG	release of *o*NP	$v_{max,oNP}$ (µmol min^{-1} mg^{-1})	486±9	188±3
		$K_{m,oNP}$	1.3±0.1	0.67±0.07
		k_{cat} (s^{-1})	939±7	364±6
		k_{cat}/K_m (mM^{-1} s^{-1})	722±66	543±65
		$K_{i,Gal}$	15±3	34±5
		$K_{i,Glc}$	120±31	37±4

Data given are the mean of two independent experiments ± standard error.
doi:10.1371/journal.pone.0104056.t002

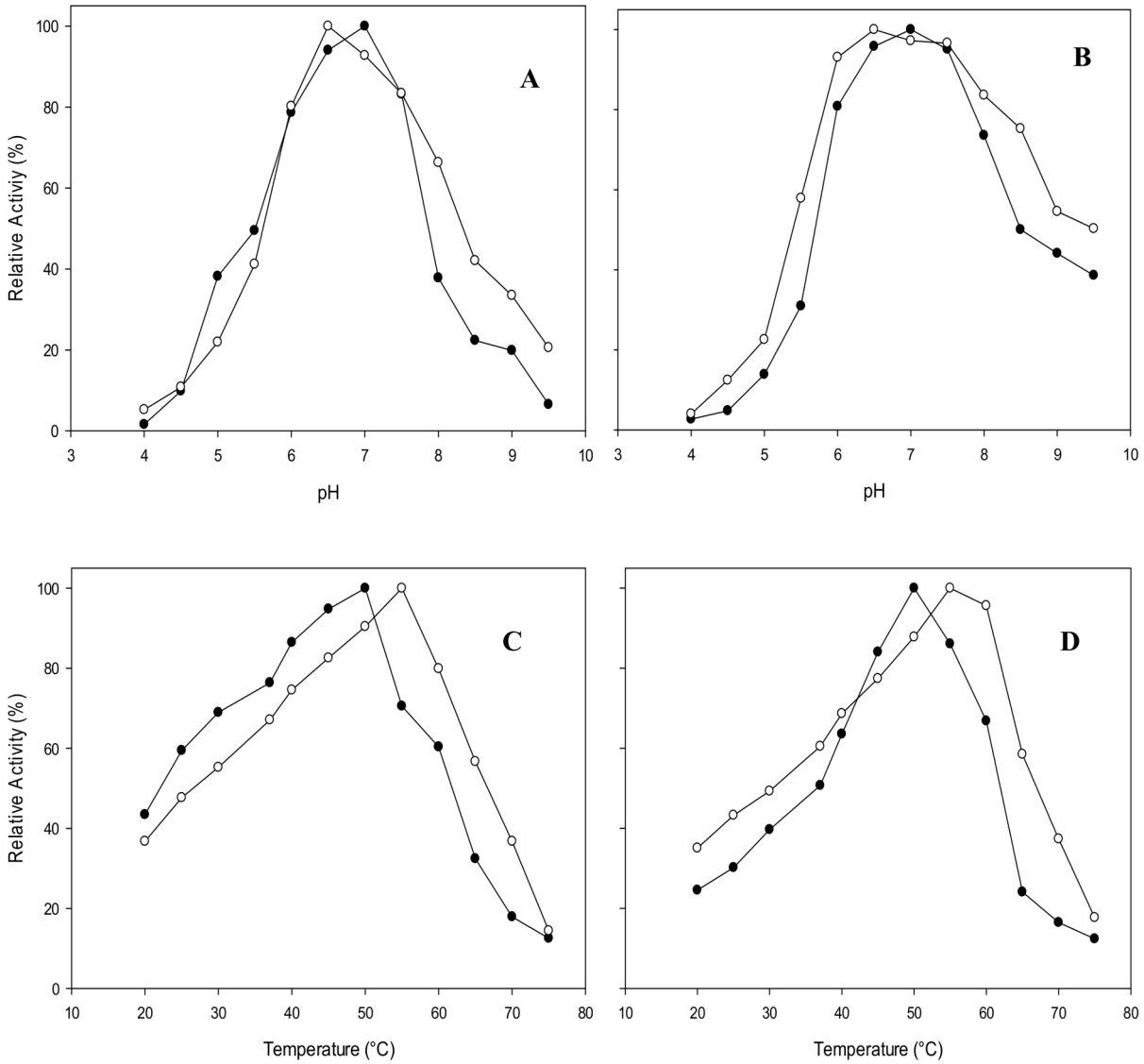

Figure 1. pH (A and B) and temperature (C and D) optimum of β-galactosidase activity for *B. breve* β-gal I (•) and β-gal II (○) using *o*NPG (A and C) and lactose (B and D) as substrate. Values are the mean of two independent experiments and the standard deviation was always less than 5%.
doi:10.1371/journal.pone.0104056.g001

encoding the chaperones GroEL and GroES was from TAKARA (Shiga, Japan). Galacto-oligosaccharide standards of β-D-Gal*p*-(1→3)-D-Glc, β-D-Gal*p*-(1→6)-D-Glc, β-D-Gal*p*-(1→3)-D-Gal, β-D-Gal*p*-(1→4)-D-Gal, β-D-Gal*p*-(1→6)-D-Gal, β-D-Gal*p*-(1→3)-D-Lac, β-D-Gal*p*-(1→4)-D-Lac, β-D-Gal*p*-(1→6)-D-Lac were purchased from Carbosynth (Berkshire, UK).

Bacterial strains and culture conditions

B. breve DSM 20213, an infant isolate, was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The strain was grown anaerobically at 37°C in MRS medium [36]. *Escherichia coli* DH5α (New England Biolabs, Frankfurt am Main, Germany) was used in the transformation experiments involving the subcloning of the DNA fragments. *Escherichia coli* T7 express (Novagen) was used as expression host for the vectors carrying the target DNA fragment encoding β-galactosidases.

Construction of β-galactosidase expression vectors

The β-gal I (NCBI Reference No. EFE90149.1) and β-gal II gene (NCBI Reference No. EFE88654.1) were amplified using proof-reading Phusion polymerase with the primer pairs 5BbBG1Nde1 (5′-AATACATATGCAAGGAAAGGCGAAAACC-3′), 3BbBG1Not1 (5′-ATAGCGGCCGCGATTAGTTCGAGTGTCACATCC-3′) and 5BbBG2Nde1 (5′-AATACATATGAACACAACCGACGATCAG-3′), 3BbBG2Not1 (5′-ATAGCGGCCGCGATGAGTTCGAGGTTCACGTC-3′), respectively. The forward primers contain *Nde*I and the reverse primers include *Not*I recognition sites (underlined). The template for the PCR reaction was obtained from cells scratched from an MRS agar plate and suspended in the PCR mix. The initial denaturation step at 98°C for 3 min was follow by 30 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 2 min, followed by a final extension step at 72°C for 5 min. The amplified genes were digested with the corresponding restriction enzymes. Subsequently, the gene frag-

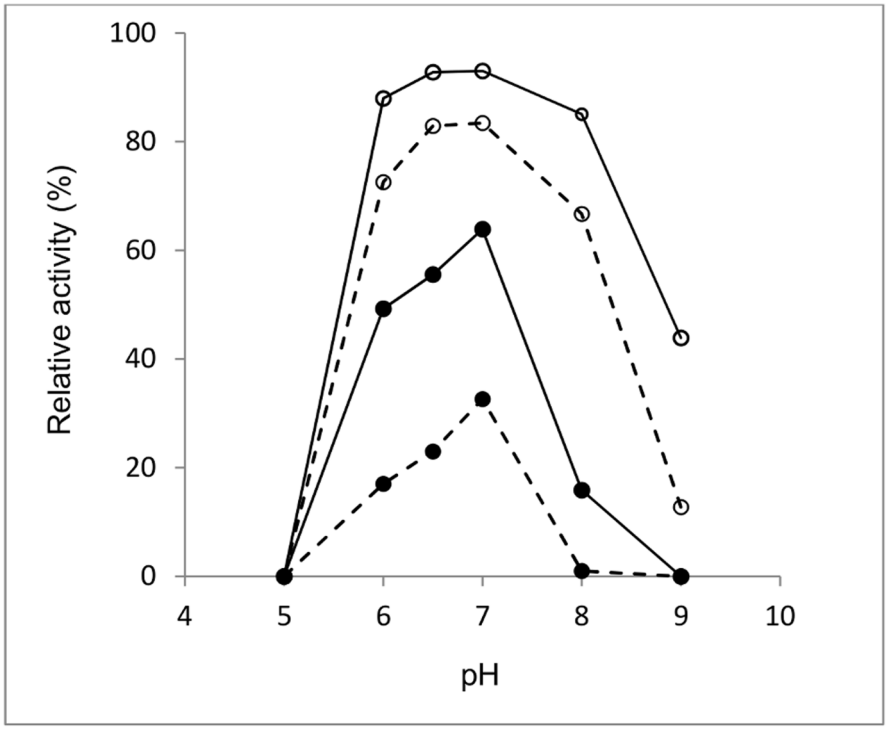

Figure 2. pH stability of the β-galactosidases from *B. breve* β-gal I (•) and β-gal II (○) incubated at 37°C in Britton-Robinson buffer over a pH range of pH 5.0–9.0 for 4 h (solid lines) and 10 h (dashed lines). The residual activity was measured after 4 h and 10 h (B) and *o*NPG was used as substrate for the enzyme assay. Values are the mean of two independent experiments and the standard deviation was always less than 5%.
doi:10.1371/journal.pone.0104056.g002

Table 3. Stability of β-galactosidases from *B. breve* at different temperatures in the absence of $MgCl_2$ as well as in the presence of 1 and 10 mM $MgCl_2$.

(A) β-gal I

Temperature (°C)	Sodium phosphate buffer, pH 7		Sodium phosphate buffer, pH 7 +1 mM Mg^{2+}		Sodium phosphate buffer, pH 7 +10 mM Mg^{2+}	
	k_{in} (h^{-1})	$\tau_{1/2}$ (h)	k_{in} (h^{-1})	$\tau_{1/2}$ (h)	k_{in} (h^{-1})	$\tau_{1/2}$ (h)
30	1.00 ($\pm$0.00)$\times10^{-2}$	73	2.00 ($\pm$0.01)$\times10^{-3}$	428	3.00 ($\pm$0.00)$\times10^{-3}$	235
37	0.32$\pm$0.03	2	2.00 ($\pm$0.00)$\times10^{-2}$	37	2.30 ($\pm$0.00)$\times10^{-2}$	28
45	9.56$\pm$0.26	0.07	0.96$\pm$0.04	0.72	1.18$\pm$0.06	0.59
50	36.7$\pm$1.2	0.02	9.00$\pm$0.39	0.08	10.80$\pm$0.37	0.06

(B) β-gal II

Temperature (°C)	Sodium phosphate buffer, pH 6.5		Sodium phosphate buffer, pH 6.5 +1 mM Mg^{2+}		Sodium phosphate buffer, pH 6.5+10 mM Mg^{2+}	
	k_{in} (h^{-1})	$\tau_{1/2}$ (h)	k_{in} (h^{-1})	$\tau_{1/2}$ (h)	k_{in} (h^{-1})	$\tau_{1/2}$ (h)
30	4.67 ($\pm$0.06)$\times10^{-3}$	109	2.58 ($\pm$0.08)$\times10^{-3}$	268	2.34 ($\pm$0.16)$\times10^{-3}$	297
37	2.07 ($\pm$0.07)$\times10^{-2}$	33	3.79 ($\pm$0.01)$\times10^{-3}$	183	3.78 ($\pm$0.07)$\times10^{-3}$	183
50	5.70$\pm$0.04	0.12	0.55$\pm$0.01	1.25	0.19$\pm$0.0.01	3.7

Data given are the inactivation constants k_{in} and the half-life times of activity $\tau_{1/2}$. Values are the mean of two independent experiments and the standard deviation was always less than 5%.
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Table 4. Relative hydrolytic activities of *B. breve* β-galactosidases for individual galactosides.

Substrate	% Conversion			
	β-gal I		β-gal II	
	30 min	60 min	30 min	60 min
Lactose	>99	>99	86.3	>99
β-D-Gal*p*-(1→6)-D-Glc	>99	>99	98.3	>99
β-D-Gal*p*-(1→3)-D-Glc	97.7	>99	61.7	88.6
β-D-Gal*p*-(1→3)-D-Gal	98.7	>99	73.1	90.9
β-D-Gal*p*-(1→4)-D-Gal	11.6	12.4	4.7	11.6
β-D-Gal*p*-(1→6)-D-Gal	52.1	79.0	48.2	80.4
β-D-Gal*p*-(1→3)-D-Lac	>99	>99	85.7	97.6
β-D-Gal*p*-(1→4)-D-Lac	22.1	29.1	21.8	26.2
β-D-Gal*p*-(1→6)-D-Lac	10.3	30.4	5.9	14.9

Results are expressed as a percentage of hydrolysis (or conversion) of each substrate after 30 and 60 min.
doi:10.1371/journal.pone.0104056.t004

ments were ligated into the pET-21a(+) vector without the natural stop codon and in frame with the C-terminal His_6-tag sequence on the vector, and transformed into *E. coli* DH5α cells. The resulting expression vectors β-gal I and β-gal II were transformed into two different hosts, *E. coli* T7 Express and *E. coli* T7 Express carrying the plasmid pGRO7 (*E. coli* T7 Express GRO), for comparison of the expression levels. The correct nucleotide sequences were confirmed by sequencing (VBC-Biotech, Vienna, Austria). The basis local alignment search tool (BLAST) from the National Center for Biotechnology Information BLAST website was used for database searches. The comparison of β-galactosidases from *B. breve* with homologous proteins was carried out using the program ClustalW2 (version 2.0) [37].

Heterologous expression of β-galactosidases

The expression levels of β-gal I and β-gal II with and without co-expression of the chaperones GroEL and GroES were compared. To this end, all cultures were grown at 37°C in 250 mL of MagicMedia (Invitrogen, Carlsbad, CA, USA) until an optical density at $OD_{600\ nm}$ of 0.6 was reached. MagicMedia promotes high expression levels of T7-regulated genes without the need of adding inducing reagent such as IPTG. The co-expression of the chaperons was induced with 1 mg mL^{-1} L-arabinose. The cultures were incubated further at 20°C overnight. The cells were harvested by centrifugation (6,000×*g*, 30 min, 4°C), washed twice with 50 mM sodium phosphate buffer, pH 6.5, and disrupted using a French press (AMINCO, Silver Spring, MD). The resulting homogenate was centrifuged at 25,000×*g* for 30 min at 4°C to remove the cell debris. The crude extracts were tested for protein concentration and β-galactosidase activity using the standard assay.

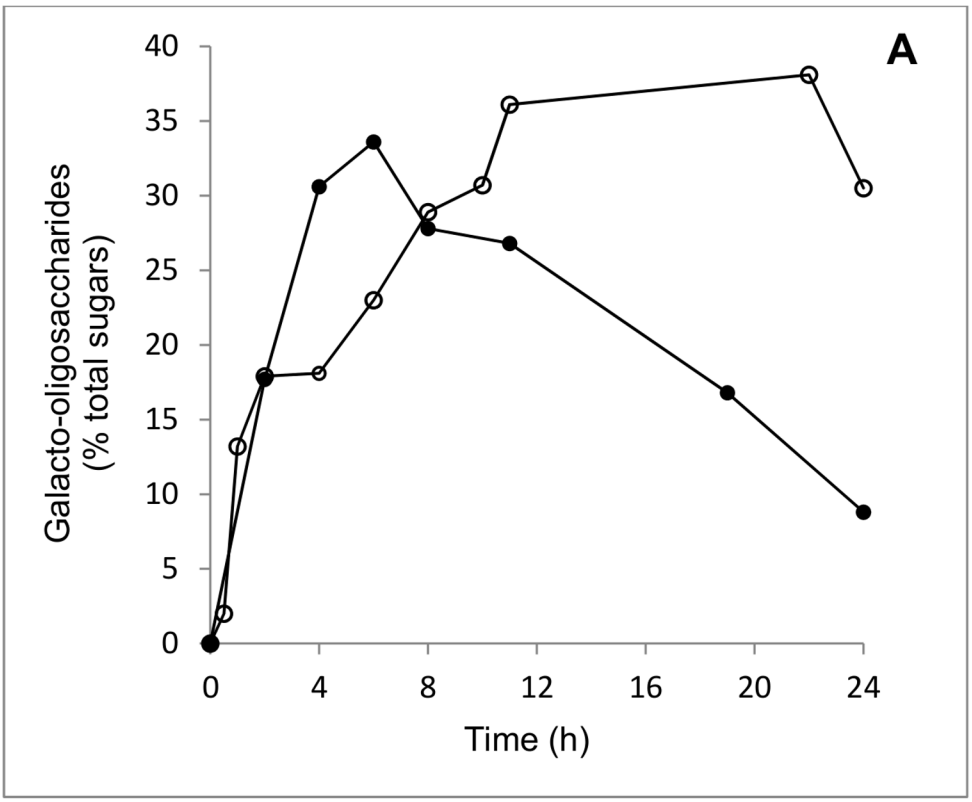
Subsequently, the expression of β-gal I and β-gal II with co-expression of the chaperones was studied further. In this investigation, LB medium was used instead of MagicMedia. Different induction conditions were compared by varying the concentrations of isopropyl-β-D-thiogalactopyranoside (IPTG) in LB medium. *E. coli* T7 express GRO cells carrying β-gal I and β-gal II plasmids, respectively, were grown at 37°C in 100 mL of LB medium containing 100 μg mL^{-1} ampicillin and 1 mg mL^{-1} L-arabinose for chaperone induction until an optical density at $OD_{600\ nm}$ of ~0.8 was reached. IPTG was added to the culture medium in various final concentrations (0.1, 0.5, 1.0 mM), and the cultures were incubated at 18°C for 16 h. The cultures were harvested, washed twice and resuspended in 1 mL of 50 mM sodium phosphate buffer, pH 6.5. Cells were disrupted in a bead beating homogenizer using 0.5 g of glass bead (Precellys 24 Technology; PEQLAB, Germany), which is practical for small sample volumes used here. The crude extracts obtained after centrifugation (16,000×*g* for 20 min at 4°C) were tested for β-galactosidase activity using the standard enzyme assay and protein concentrations.

Cultivation and purification of recombinant β-galactosidases

E. coli T7 express GRO cells carrying the plasmids β-gal I and β-gal II, respectively, were grown at 37°C in 1 L LB medium containing 100 μg mL^{-1} ampicillin, 20 μg mL^{-1} chloramphenicol and 1 mg mL^{-1} L-arabinose until $OD_{600\ nm}$ of 0.8 was reached. IPTG (0.5 mM for β-gal I and 1 mM for β-gal II) was added to the medium and the cultures were incubated further at 18°C for 16 h. The cultures were then harvested, washed twice with 50 mM sodium phosphate buffer, pH 6.5 and disrupted by using a French press. Cell debris was removed by centrifugation (25,000×*g*, 30 min, 4°C) and the lysate (crude extract) was loaded on a 15 mL Ni-immobilized metal ion affinity chromatography (IMAC) column (GE Healthcare, Uppsala, Sweden) that was pre-equilibrated with buffer A (20 mM phosphate buffer, 20 mM imidazole, 500 mM NaCl, pH 6.5). The His-tagged protein was eluted at a rate of 1 mL min^{-1} with a 150 mL linear gradient from 0 to 100% buffer B (20 mM sodium phosphate buffer, 500 mM imidazole, 500 mM NaCl, pH 6.5). Active fractions were pooled, desalted and concentrated by ultrafiltration using an Amicon Ultra centrifugal filter unit (Millipore, MA, USA) with a 30 kDa cut-off membrane. Purified enzymes were stored in 50 mM sodium phosphate buffer, pH 6.5 at 4°C for further analysis.

Gel electrophoresis analysis

The purity and the molecular mass of β-galactosidases were determined by SDS-PAGE. The enzymes were diluted to 1 mg protein mL^{-1} and incubated with 2×Laemmli buffer at 90°C for 5 min. Protein bands were visualized by staining with Bio-safe Coomassie (Bio-Rad). Unstained Precision plus Protein Standard (Bio-Rad) was used for mass determination.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Galacto-oligosaccharides (% total sugars)
Time (h)
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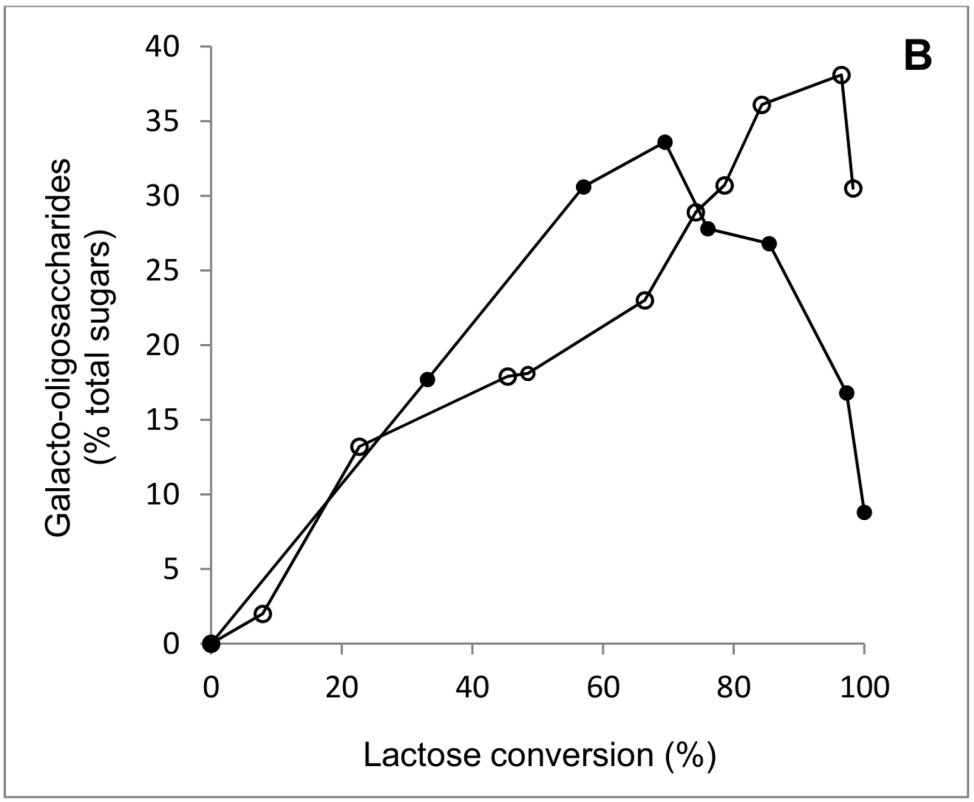
B
Galacto-oligosaccharides (% total sugars)
Lactose conversion (%)
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100

Figure 3. Time course of GOS formation (A) and formation and degradation of GOS during lactose conversion (B) catalyzed by *B. breve* β-gal I (•) and β-gal II (○). The reaction was performed at 30°C at an initial lactose concentration of 200 g L^{-1} in sodium phosphate buffer (pH 6.5) and 1 mM $MgCl_2$ using 1.5 U_{Lac} mL^{-1}. Values are the mean of two independent experiments and the standard deviation was always less than 5%.
doi:10.1371/journal.pone.0104056.g003

Size exclusion chromatography - Multi-angle laser light scattering analysis

Size exclusion chromatography (SEC) was performed with a Superdex S200 10/300 GL column (GE Healthcare) equilibrated in a buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM β-mercaptoethanol and 5 mM EDTA. The sample separations were performed at room temperature with a flow rate of 0.5 mL min^{-1}. The samples (50 µL) were injected as indicated at a concentration of 2.5 mg mL^{-1}. On-line multi-angle laser light scattering analysis (MALLS) detection was performed with a miniDawn Treos detector (Wyatt Technology, Santa Barbara, CA) using a laser emitting at 690 nm. The protein concentration was measured on-line by refractive index measurement using a Shodex RI-101 instrument (Showa Denko, Munich, Germany). Analysis of the data was performed with the ASTRA software (Wyatt Technology).

β-Galactosidase assays

The measurement of β-galactosidase activity using *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG) or lactose as the substrates was carried out as previously described [26]. Reactions were performed in 50 mM sodium phosphate buffer (pH 6.5) at 30°C, and the release of *o*-nitrophenol (*o*NP) was measured by determining the absorbance at 420 nm. One unit of *o*NPG activity was defined as the amount of enzyme releasing 1 µmol of *o*NP per minute under the described conditions. When lactose was used as the substrate, reactions were performed in 50 mM sodium phosphate buffer, pH 6.5, at 30°C. After stopping the reaction, the release of D-glucose was determined using the test kit from Megazyme, in which D-glucose was assessed colorimetrically using the GOD/POD (glucose oxidase/peroxidase) assay. One unit of lactase activity was defined as the amount of enzyme releasing 1 µmol of D-glucose per minute under the given conditions. Protein concentrations were determined by the method of Bradford [38] using bovine serum albumin as the standard.

Steady-state kinetic measurement

All steady-state kinetic measurements were obtained at 30°C using *o*NPG and lactose as the substrates in 50 mM sodium phosphate buffer, pH 6.5, with concentrations ranging from 0.5 to 22 mM for *o*NPG and from 1 to 600 mM for lactose, respectively. The inhibition of *o*NPG hydrolysis by D-galactose and D-glucose as well as that of lactose hydrolysis by D-galactose using various concentrations of the inhibitors (ranging from 0 to 200 mM) was investigated as well. The kinetic parameters and inhibition constants were calculated by nonlinear regression, and the observed data were fit to the Henri-Michaelis-Menten equation (SigmaPlot, SPSS Inc., Chicago, IL).

pH and temperature dependency of activity and stability

The pH dependency of the recombinant enzymes was evaluated by the standard assay with 22 mM *o*NPG in the pH range of 3–10 using Briton-Robinson buffer (20 mM acetic acid, 20 mM phosphoric acid, and 20 mM boric acid titrated with 1 M NaOH to the desired pH). To evaluate the pH stability of β-gal I and β-gal II, the enzyme samples were incubated at various pH values using Britton-Robinson buffers at 37°C and the remaining activity was measured at time intervals with *o*NPG as substrate. The temperature optima for hydrolytic activity of β-gal I and β-gal II with both substrates lactose and *o*NPG were determined at 20–90°C and measured at optimum pH of each enzyme. The thermostability was evaluated by incubating the pure enzyme in 50 mM sodium phosphate buffer (pH 6.5) at several temperatures. The residual activities were measured regularly with *o*NPG as substrate. Inactivation constants (k_{in}) were calculated using first-order rate equations and half-life time of activities ($\tau_{1/2}$) were calculated based on these k_{in} values.

Differential Scanning Calorimetery (DSC)

DSC measurements were performed using a MicroCal VP-DSC System (GE Healthcare) controlled by the VP-viewer program and equipped with a 0.137-mL cell. Studies were made with 1 mg mL^{-1} protein samples in 50 mM phosphate buffer (pH 6.5). Samples were analyzed using a programmed heating scan rate of 60°C h^{-1} in the range of 33–80°C. For baseline correction, a buffer blank was scanned in the second chamber and subtracted. Data analysis was performed with the MicroCal Origin software (GE Healthcare) and experimental data points were fitted to an MN2-State Model.

Substrate specificity

Substrate specificity of the recombinant enzymes was determined using various structurally related chromogenic substrates under standard assay conditions as described for *o*NPG. The chromogenic substrates tested were 2-nitrophenyl-β-D-glucopyranoside, 4-nitrophenyl-β-D-mannopyranoside, 4-nitrophenyl-α-D-galactopyranoside, and 4-nitrophenyl-α-D-glucopyranoside at substrate concentration of 22 mM.

Substrate affinities of the recombinant enzymes towards some galactosides were also evaluated. An appropriate amount of each enzyme was incubated with ~3 mM of each galactoside (lactose, β-D-Gal*p*-(1→3)-D-Glc, β-D-Gal*p*-(1→6)-D-Glc, β-D-Gal*p*-(1→3)-D-Gal, β-D-Gal*p*-(1→4)-D-Gal, β-D-Gal*p*-(1→6)-D-Gal, β-D-Gal*p*-(1→3)-D-Lac, β-D-Gal*p*-(1→4)-D-Lac, β-D-Gal*p*-(1→6)-D-Lac) at 30°C in 50 mM sodium phosphate buffer, pH 6.5. Samples were taken after 30 and 60 min and reactions were stopped by incubation at 95°C for 5 min. The relative activities of the recombinant enzymes towards each galactoside were determined considering the percentage of the hydrolysis (or conversion) of each galactoside under similar reaction conditions.

Galacto-oligosaccharides synthesis and analysis

Discontinuous conversion reactions were carried out at 30°C to determine the transgalactosylation reaction of the recombinant β-galactosidases from *B. breve*. The substrate lactose solution (200 g L^{-1}) was prepared in 50 mM sodium phosphate buffer containing 1 mM Mg^{2+}. Agitation was applied at 300 rpm with a thermomixer (Eppendorf, Hamburg, Germany). Samples were taken at certain time intervals to determine the residual activities and the carbohydrate contents in the reaction mixtures by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). HPAEC−PAD analysis was carried out on a Dionex DX-500 system as previously described in detail [27].

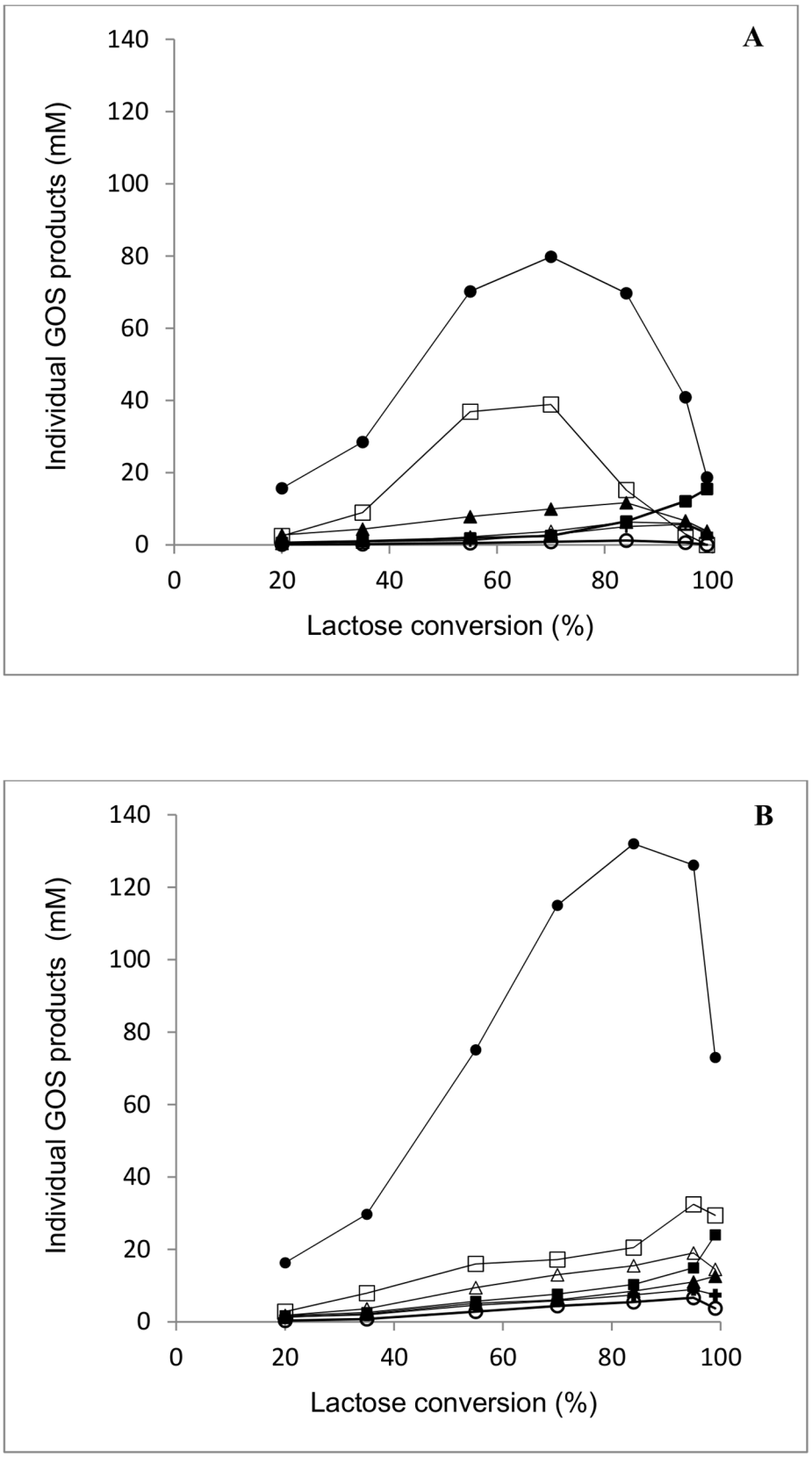
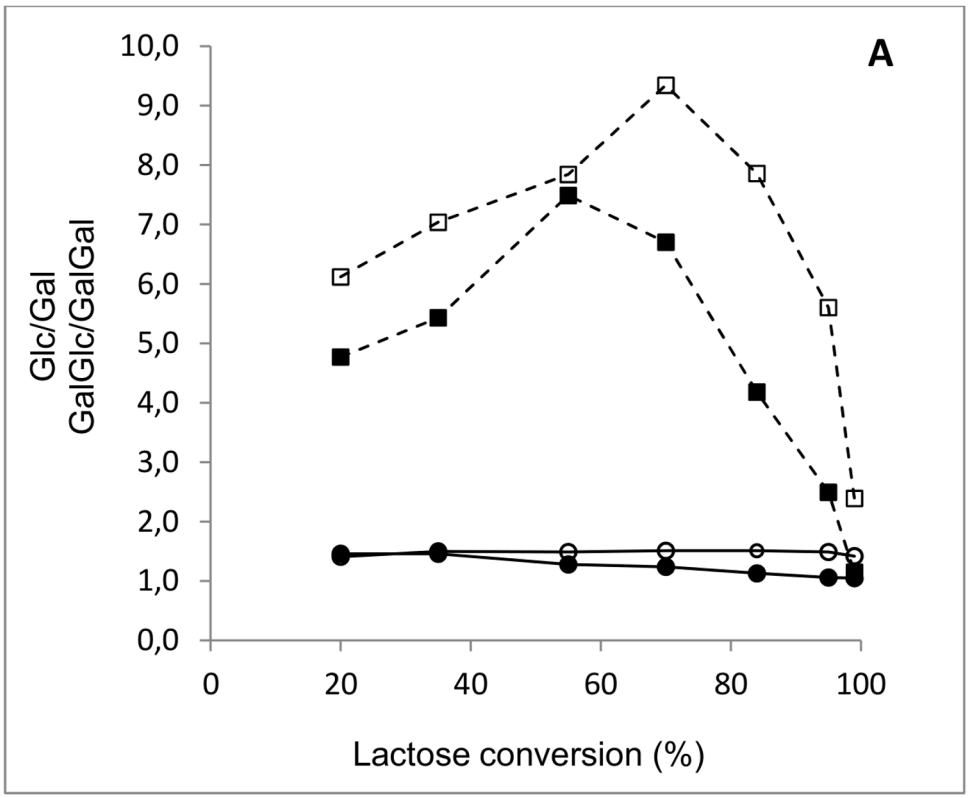


Figure 4. Formation and degradation of individual GOS formed by *B. breve* β-gal I (A) and β-gal II (B) during lactose conversion. Reaction conditions: initial lactose concentration of 200 g L^{-1} in 50 mM sodium phosphate buffer (pH 6.5) with 1 mM Mg^{2+} and 30°C and 1.0 U_{Lac} mL^{-1} β-gal I or 2.5 U_{Lac} mL^{-1} β-gal II. Symbols: (●) D-Gal*p*-(1→6)-D-Glc; (■) D-Gal*p*-(1→6)-D-Gal; (▲) Gal*p*-(1→3)-D-Gal; (Δ) D-Gal*p*-(1→3)-D-Glc; (□) D-Gal*p*-(1→3)-D-Lac; (○) D-Gal*p*-(1→4)-Lac, (+) D-Gal*p*-(1→6)-D-Lac.
doi:10.1371/journal.pone.0104056.g004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4,0
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Glc/Gal
GalGlc/GalGal
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40
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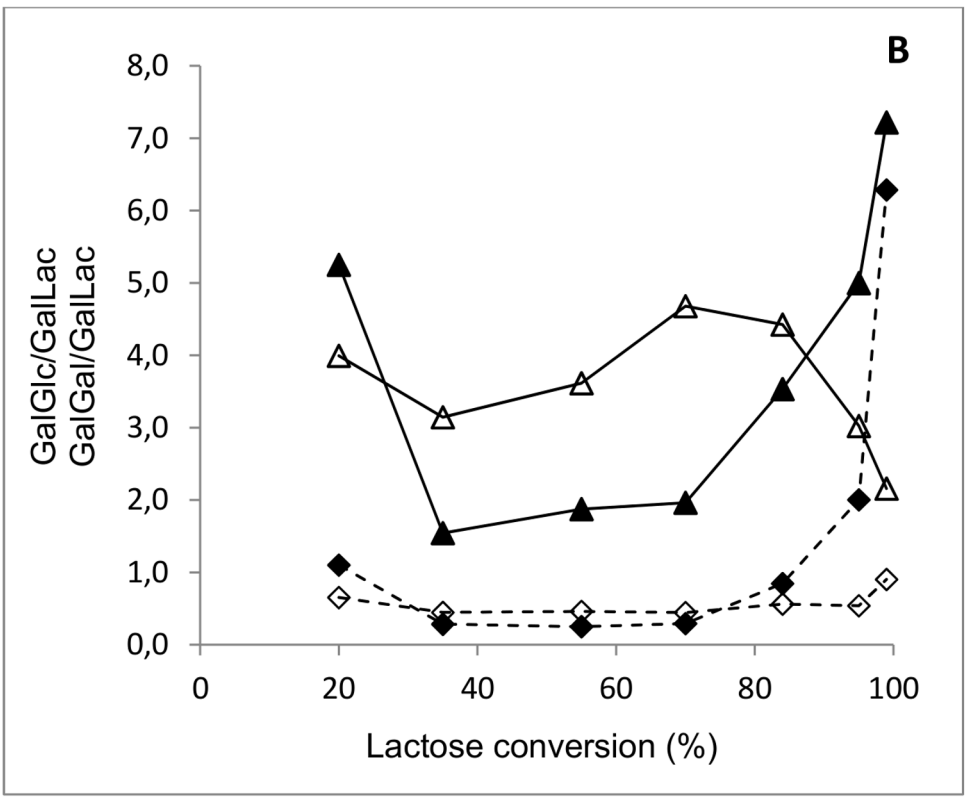
Lactose conversion (%)


B
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GalGlc/GalLac
GalGal/GalLac
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40
60
80
100
Lactose conversion (%)

Figure 5. D-Glucose/D-Galactose (solid lines) and GalGlc/GalGal (dashed lines) ratios (A); GalGlc/GalLac (solid lines) and GalGal/GalLac (dashed lines) ratios (B) during lactose conversion by *B. breve* β-gal I (close symbol) and β-gal II (open symbol). The reactions were performed at 30°C at an initial lactose concentration of 200 g L^{-1} in sodium phosphate buffer (pH 6.5) and 1 mM $MgCl_2$ with 1.0 U_{Lac} mL^{-1} β-gal I or 2.5 U_{Lac} mL^{-1} β-gal II.
doi:10.1371/journal.pone.0104056.g005

Statistical Analysis

All experiments and measurements were performed at least in duplicate, and the data are given as the mean±standard deviation when appropriate. The standard deviation was always less than 5%.

Results

Expression and purification of recombinant β-galactosidases from B. breve

The β-gal I and β-gal II genes were cloned into pET-21a (+). Comparison of amino acid sequences deduced from these two *lacZ* genes revealed 57% of sequence homology. The resulting expression vectors were then transformed into *E. coli* T7 express cells and T7 express cells carrying the plasmid pGRO7. The resulting clones were cultivated under inducing conditions in MagicMedia to compare the expression yields with and without chaperone co-expression. β-Gal I and β-gal II expressed in the strains with chaperones showed a 30- and 14-fold increase in activity compared to the activity obtained from the strains without chaperones, respectively (Table 1). When using these conditions, 193 kU per liter of fermentation broth with a specific activity of 159 U mg^{-1} of β-gal I and 36 kU per liter of fermentation broth with a specific activity of 31 U mg^{-1} of β-gal II were obtained.

The expression levels of both enzymes increased even further when LB medium was used instead of MagicMedia and gene expression was induced using IPTG. The highest yields were obtained when 0.5 mM IPTG was used for induction of β-gal I (683 kU per L of medium with a specific activity of 142 U mg^{-1}), and when using 1.0 mM IPTG for β-gal II expression (169 kU per L of medium with a specific activity of 34 U mg^{-1}). Both enzymes were subsequently purified with a single-step procedure using an IMAC column. The specific activities of the purified enzymes were 461 U mg^{-1} of protein for β-gal I and 196 U mg^{-1} of protein for β-gal II when using the standard *o*NPG assay. The purification procedure yielded a homogenous β-gal I and β-gal II preparation as judged by SDS-PAGE gel (Figure S1).

Gel electrophoresis analysis

Both recombinant β-galactosidases from *B. breve* showed molecular masses of approximately 120 kDa as judged by SDS-PAGE in comparison with reference proteins (Figure S1). Molecular masses of 116,127 and 116,594 Da were calculated for β-gal I and β-gal II, respectively, based on their DNA sequences. Size exclusion chromatography in combination with online multi-angle laser light scattering (SEC-MALLS) analysis revealed that the native molecular masses of β-gal I and β-gal II are 220 and 211 kDa, respectively. Therefore, it can be concluded that both enzymes are dimers and it is likely that they are homodimers consisting of two identical subunits as has been shown for other oligomeric β-galactosidases of the LacZ type [39,40].

Kinetic parameters

The steady-state kinetic constants and the inhibition constants of *B. breve* β-galactosidases determined for the hydrolysis of lactose and *o*NPG are summarized in Table 2. The k_{cat} values were calculated on the basis of the theoretical v_{max} values experimentally determined by nonlinear regression and using a molecular mass of 116 kDa for the catalytically active subunit. β-Gal I and β-gal II are not inhibited by their substrates, which are *o*NPG in concentrations of up to 25 mM or lactose in concentrations of up to 600 mM, as it is evident from the Michaelis-Menten plots (not shown). The end product D-galactose was found to competitively inhibit the hydrolysis of lactose by both enzymes. This inhibition, however, is only moderate as is obvious from the ratio of the Michaelis constant for lactose and the inhibition constant for D-galactose, which were calculated for both enzymes (β-gal I, $K_{i,Gal}/K_{m,Lac} = 1.8$; β-gal II, $K_{i,Gal}/K_{m,Lac} = 3.6$). D-Galactose was also found to be a competitive inhibitor against *o*NPG with inhibition constants of 15 mM for β-gal I and 34 mM for β-gal II. Based on the ratio of K_i to K_m this inhibition is even less pronounced (β-gal I, $K_{i,Gal}/K_{m,oNPG} = 11.5$; β-gal II, $K_{i,Gal}/K_{m,oNPG} = 50.7$). *o*NPG was also used as the substrate for studying inhibition by the second end product, D-glucose. Again, glucose is a competitive inhibitor of both enzymes, but this inhibiting effect is only moderate (β-gal I, $K_{i,Glc}/K_{m,oNPG} = 92$; β-gal II, $K_{i,Glc}/K_{m,oNPG} = 55$).

Effects of temperature and pH on enzyme activity and stability

Both *o*NPG and lactose were used as substrates to determine the temperature and pH optimum of β-gal I and β-gal II activity. The pH optimum of β-gal I is pH 7.0 for both *o*NPG and lactose hydrolysis (Figure 1A, B). This enzyme is also most stable at pH 7.0, retaining 60% and approximately 30% of its activity when incubated at pH 7.0 and 37°C for 4 and 10 h, respectively (Figure 2). β-Gal I has a half-life time of activity ($\tau_{1/2}$) of 5 h when incubated at pH 7.0 and 37°C. The pH optimum of β-gal II is pH 6.5 for both *o*NPG and lactose hydrolysis (Figure 1A, B), and the enzyme is most stable at pH 6.0–7.0. The residual activities of this enzyme after 10 h of incubation at pH 6.0, 6.5, and 7.0 at 37°C were 72%, 82%, and 83%, respectively (Figure 2). The stability of both enzymes rapidly dropped at pH values below 6.0 or above 8.0.

The optimum temperature of β-gal I activity was 50°C when using both *o*NPG and lactose as substrates under standard assay conditions. In comparison to β-gal I, β-gal II had higher optima, which were at 55°C for both substrates (Figure 1C, D). Both recombinant enzymes showed a half-life time of activity ($\tau_{1/2}$) of ~5 months at 4°C when stored in sodium phosphate buffer, pH 6.5. Both enzymes also showed their stability at 30°C with half-life time of activities ($\tau_{1/2}$) of 73 and 109 h for β-gal I and β-gal II, respectively (Table 3A, B).

Thermal stability of both β-galactosidases I and II was significantly improved in the presence of $MgCl_2$. Table 3 (A, B) shows the effect of 1 and 10 mM of $MgCl_2$ on the thermostability of β-gal I and β-gal II at 37°C and higher. In the presence of 1 mM $MgCl_2$ β-gal I showed 19-, 10- and 4-fold increase in $\tau_{1/2}$ at 37°C, 45°C and 50°C, respectively. A further increase of the Mg^{2+} concentration to 10 mM was less effective with respect to stabilization of this enzyme. At all conditions tested, β-gal II was found to be more stable than β-gal I. In the presence of 1 mM $MgCl_2$ $\tau_{1/2}$ of β-gal II activity at 50°C was increased to 1.25 h, compared to 0.12 h without Mg^{2+}. A further increase of the Mg^{2+} concentration to 10 mM was more effective in improving thermostability of β-gal II activity. It should be noted that, while

Mg^{2+} increased stability considerably, it had no effect on activity of both β-gal I and β-gal II. Stability was further studied by differential scanning calorimetry (DSC). Both enzymes showed a single endothermic peak in the DSC scans which fitted very well on the basis of a two-state transition model (data not shown). The observed melting temperatures T_m, 49.97 and 55.58°C for β-gal I and β-gal II, B, respectively, are in excellent agreement with the optimum temperatures of these two enzymes as shown in Figure 1C, D.

Substrate specificity

The two β-galactosidases from *B. breve* displayed a narrow substrate range when using chromogenic substances. Both enzymes showed 1% activity (relative to *o*NPG) when using 2-nitrophenyl-β-D-glucopyranoside while no activity (<0.05%) was observed when 4-nitrophenyl-β-D-mannopyranoside, 4-nitrophenyl-α-D-galactopyranoside, or 4-nitrophenyl-α-D-glucopyranoside were used as substrates.

Activities of *B. breve* β-galactosidases with individual galactosides are expressed as relative hydrolysis (or conversion) of each substrate after 30 and 60 min (Table 4). β-Gal I shows high hydrolytic activity towards lactose with more than 99% hydrolysis after 30 minutes of hydrolytic reaction. β-D-Gal*p*-(1→6)-D-Glc (allolactose), β-D-Gal*p*-(1→3)-D-Lac, β-D-Gal*p*-(1→3)-D-Glc and β-D-Gal*p*-(1→3)-D-Gal were hydrolyzes at comparable rates (Table 4). β-Gal II also showed high activities with lactose, β-D-Gal*p*-(1→6)-D-Glc (allolactose) and β-D-Gal*p*-(1→3)-D-Lac but these substrates were hydrolyzed at slightly lower rates than that by β-gal I. Both enzymes show low activity with the disaccharide β-D-Gal*p*-(1→4)-D-Gal and the trisaccharides β-D-Gal*p*-(1→4)-D-Lac and β-D-Gal*p*-(1→6)-D-Lac, which is evident from the slow hydrolysis rates of these substrates.

GOS synthesis

Figure 3 shows GOS (including non-lactose disaccharides) formation of a typical discontinuous conversion reaction at 30°C with an initial lactose concentration of 200 g L^{-1} in sodium phosphate buffer (pH 6.5) and 1 mM $MgCl_2$ using 1.5 U_{Lac} mL^{-1} of enzyme. Under these conditions, maximum GOS yields of 33% of total sugars (after 6 h of reaction and when 70% of the initial lactose were converted), and 38% of total sugars (after 22 h of reaction at 96% lactose conversion) were obtained with β-gal I and β-gal II, respectively. The amount of GOS expressed as percentage of total sugars is constantly rising from 0 to 33% and from 0 to 38%, when lactose conversion increased up to ~70% and ~90% using β-gal I and β-gal II, respectively. After these points, at which maximum GOS yields were obtained, the concentration of GOS decreased because they are also subjected to hydrolysis by the β-galactosidases. This is particularly pronounced for β-gal I. When the concentration of β-gal I in the conversion reaction was reduced to 1.0 U_{Lac} mL^{-1}, a slight difference on the maximum GOS yield, which was 30% of total sugars at 70% lactose conversion, was observed. Interestingly, when the concentration of β-gal II in the conversion reaction was increased to 2.5 U_{Lac} mL^{-1}, maximum GOS yield increased from 38% to 44% of total sugars, which was obtained at 84% lactose conversion, and also the time needed to obtain this maximum GOS yield was reduced to 6 h (data not shown).

Individual GOS can be separated effectively using a Carbopac PA1 column for HPAEC with pulsed amperometric detection (Figure S2A, B). It was possible to identify the main products of transgalactosylation by both β-gal I and β-gal II. These main transferase products formed and degraded at different lactose conversion are presented in Table S1 and Figure 4A, B. The predominant oligosaccharide product was identified as β-D-Gal*p*-(1→6)-D-Glc (allolactose), accounting for approximately 45% and 50% of the GOS formed by transgalactosylation by β-gal I and β-gal II, respectively, at maximum total GOS yield. β-D-Gal*p*-(1→3)-D-Lac was identified as the second important transferase product at the maximum total GOS yield point, contributing approximately 32% and 16% of the total GOS formed by β-gal I and β-gal II, respectively. Other identified products, including β-D-Gal*p*-(1→3)-Glc, β-D-Gal*p*-(1→3)-Gal, β-D-Gal*p*-(1→6)-Gal, β-D-Gal*p*-(1→6)-Lac and β-D-Gal*p*-(1→4)-Lac, make up approximately 12% and 20% of total GOS (at total GOS maximum yield) formed using β-gal I and β-gal II, respectively. 4′-Galactobiose was not detected at all during the course of lactose conversion. It should be noted that the unidentified peaks 8 and 14 were present in detectable concentrations (Figure S2A, B). However, the structure of these components has yet to be determined.

Discussion

In this study, two GH2 β-galactosidases, β-gal I and β-gal II, of the LacZ type of *B. breve* DSM 20213 were cloned, heterologously expressed in *E. coli* and biochemically characterized. The activity yields of 683 and 169 kU L^{-1} obtained in simple shaken flask cultures for β-gal I and β-gal II, respectively, correspond to values of ~1.5 and 0.86 g of recombinant protein produced per L of medium. These yields are significantly higher than the recently reported maximum yield of a β-galactosidase (BbgIV) from *Bifidobacterium bifidum* NCIMB 41171 expressed in *E. coli* DH5α, which was 0.4–0.5 g BbgIV per L of culture medium [41]. Furthermore, ~31% and 18% of the total soluble protein in the cellular extracts of *E. coli* overexpressing the genes encoding β-gal I and β-gal II, respectively, can be attributed to the recombinant proteins as judged by the specific activities. Co-expression of the chaperones GroEL/GroES significantly boosted expression levels of both β-galactosidases.

Kinetic constants were determined for the two substrates lactose and *o*NPG. The K_m values determined for lactose, 15.3 and 7.5 mM for β-gal I and β-gal II, respectively, are lower compared to the values reported for other β-galactosidases from *Bifidobacterium* spp. including *B. adolescentis* β-gal II (60 mM) [22], *B. breve* B24 (95.6 mM) [34], *B. bifidum* β-gal I (29.9 mM) and β-gal II (47.1 mM) [25], as well as fungal and yeast β-galactosidases that are commonly employed in technological applications, for example *A. oryzae* (36–180 mM), *A. niger* (54–99 mM), *K. fragilis* (15–52 mM) [42], *K. lactis* (35 mM) [43]. These K_m values of *B. breve* β-galactosidases compare favorably with the values reported for β-galactosidases from *B. bifidum* β-gal III (9.56 mM) [25], *L. reuteri* (13 mM) [26], and *L. crispatus* (14 mM) [44]. These relatively low K_m values of the *B. breve* β-galactosidases can be an advantage, e.g. when the complete hydrolysis of lactose is desired.

The inhibition by the end product galactose is moderate as is evident from the ratio of K_i to K_m calculated for this competitive inhibitor. This ratio of K_i to K_m represents a specificity constant, which determines preferential binding of the substrate lactose versus that of the monosaccharide end product, hence a high value for this ratio is desirable for efficient hydrolysis of lactose. The *B. breve* β-galactosidases display values for the $K_{i,Gal}/K_{m,Lac}$ ratio of 1.8 and 3.6 for β-gal I and β-gal II, respectively, indicating low inhibition. Values for the $K_{i,Gal}/K_{m,Lac}$ ratio reported for example for *B. licheniformis*, *A. oryzae, A. niger* and *K. fragilis* β-galactosidases are as low as 0.0055, 0.01, 0.006 and 0.84, respectively, indicating severe inhibition by the end product galactose [45,46].

Recombinant GH2 β-galactosidases from the infant isolate *B. breve* were found to be suitable for the production of GOS via transgalactosylation. Maximum total GOS yields of 33% and 44% were obtained when β-gal I and β-gal II were used in discontinuous conversion reactions with an initial lactose concentration of 200 g L^{-1}. The conversions were performed with this initial lactose concentration based on the solubility of lactose at ambient temperature. An increase in reaction temperature would help to increase the solubility of lactose, however this was not possible since both enzymes lack sufficient stability above 30°C. The maximum GOS yield obtained with β-gal II is comparable to the reported yields obtained with other β-galactosidases from *Bifidobacterium* spp., for example *B. angulatum* (43.8%), *B. bifidum* BB-12 (37.6%), *B. adolescentis* (43.1%) [47], and *B. breve* B24 (42%) [34], however the lactose conversions for GOS synthesis using these β-galactosidases were performed with initial lactose concentration of 30% (w/w). Additionally, Goulas et al. [48] reported a yield of 47% of GOS using BbgIV from *B. bifidum* NCIMB41171 at 40°C and 40% (w/w) initial lactose concentration, while Osman et al. [49] obtained a yield of 55% at 65°C and 43% (w/w) initial lactose concentration using the same enzyme. It was found by many authors that the initial lactose concentration has a significant impact on GOS yields [16,27,50].

Both enzymes show highest hydrolytic affinities towards lactose, allolactose and β-D-Gal*p*-(1→3)-D-Lac among the substrates tested. It is conceivable that the 'probiotic' β-galactosidases, which rapidly *hydrolyze* certain galacto-oligosaccharide structures, can preferentially *form* these glycosidic linkages as well when acting in transgalactosylation mode, and this is again confirmed in this study. The predominant transgalactosylation products were identified as β-D-Gal*p*-(1→6)-D-Glc (allolactose) and β-D-Gal*p*-(1→3)-D-Lac, together accounting for more than 75% and 65% of the GOS formed by transgalactosylation by β-gal I and β-gal II, respectively. Both enzymes show very low activity towards β-D-Gal*p*-(1→4)-D-Gal, and interestingly, this disaccharide was not detected and hence formed at all during lactose conversion by β-gal I and β-gal II.

Transgalactosylation is described to involve intermolecular as well as intramolecular reactions. Intramolecular or direct galactosyl transfer to D-glucose yields regio-isomers of lactose, and disaccharides are formed right from the beginning of the reaction even when hardly any monosaccharide galactosyl acceptors are available. In this reaction pathway the noncovalently enzyme-bound glucose is not released from the active site but linked immediately to the galactosyl enzyme intermediate. Different transfer rates for different acceptors are to some extent responsible for these phenomena. Figure 5A reveals the ratio between GalGlc and GalGal disaccharides at all lactose conversion levels formed during transgalactosylation using β-gal I and β-gal II. This ratio was as high as ≈5 (for β-gal I) or ≈6 (β-gal II) during the initial phase of the reaction (at 20% lactose conversion), at which the concentration of the main hydrolysis products D-Glc and D-Gal are relatively low. This indicates that D-glucose is an excellent galactosyl acceptor, in fact it is a far better acceptor than D-galactose for galactosyl transfer by both of these two enzymes. Figure 5B shows that D-glucose is also a better galactosyl acceptor than D-lactose when looking at the ratio between GalGlc disaccharides and GalLac trisaccharides. Especially at the beginning of the reaction this ratio was ≈5 (for β-gal I) or ≈4 (β-gal II) at 20% lactose conversion. This indicates that at least D-Gal*p*-(1→6)-D-Glc is formed by intramolecular transgalactosylation, that is, the D-Gal moiety is transferred onto D-Glc before it can leave the active site of β-galactosidase and another acceptor molecule or water can enter the active site. Since both enzymes form β-D-Gal*p*-(1→4)-Lac, it is conceivable that the galactosyl moiety can also be transferred onto glucose to form lactose (β-D-Gal*p*-(1→4)-Glc) as an intramolecular transgalactosylation product; however, this cannot be distinguished experimentally from lactose provided as a substrate. As β-D-Gal*p*-(1→3)-D-Lac is the second main product during transgalactosylation after D-Gal*p*-(1→6)-D-Glc and when looking at the ratio between GalGal disaccharides and GalLac trisaccharides (figure 5B), it can be concluded that D-lactose is preferred to D-galactose as galactosyl acceptor during intermolecular transgalactosylation.

In conclusion, two GH2 β-galactosidases from *B. breve* DSM 20213, β-gal I and β-gal II, were studied in detail regarding their biochemical properties, distribution of oligosaccharides formed, and linkages preferentially synthesized in transgalactosylation mode. Both enzymes were found to be very well suited for the production of galacto-oligosaccharides, components that are of great interest because of their use in functional food. The resulting GOS mixtures contained relatively high fractions of allolactose, which results from the fact that glucose is a far better acceptor for galactosyl transfer than galactose and lactose, and intramolecular transgalactosylation contributes significantly to the formation of this disaccharide. 3′-Galactosyl-lactose was found to be the major trisaccharide in the GOS mixtures. The β-galactosidases from *B. breve* DSM 20213 should be of considerable interest for the production of prebiotic GOS.

Supporting Information

Figure S1 SDS-PAGE analysis of recombinant β-galactosidases from *B. breve* stained with Coomassie blue. Lanes 1 and 4 shows the molecular mass marker (Amersham); lanes 2 and 5 are the crude extracts of β-gal I and β-gal II, lanes 3 and 6 are the purified enzymes of β-gal I and β-gal II.
(DOCX)

Figure S2 Separation and quantification by HPAEC-PAD of individual GOS produced during lactose conversion catalyzed by *B. breve* β-gal I (A), and *B. breve* β-gal II (B). The identified compounds are (1) D-galactose, (2) D-glucose, (3) D-Galp-(1→6)-D-Gal, (4) D-Galp-(1→6)-D-Glc (allolactose), (5) D-Galp-(1→4)-D-Glc (lactose), (6) D-Galp-(1→3)-D-Gal, (7) D-Galp-(1→6)-Lac, (9) D-Galp-(1→3)-D-Glc, (13) D-Galp-(1→4)-Lac and (15) D-Galp-(1→3)-Lac. Peaks 8, 10–12, 14, and 16–20 were not identified.
(DOCX)

Table S1 Individual GOS components produced by the transgalactosylation reaction of β-gal I (I) and β-gal II (II) from *Bifidobacterium breve* DSM 20031 using lactose as substrate. The reaction was performed at 30°C at an initial lactose concentration of 200 g L^{-1} in sodium phosphate buffer (pH 6.5) and 1 mM $MgCl_2$ using 1.0 U_{Lac} mL^{-1} (β-gal I) or 2.5 U_{Lac} mL^{-1} (β-gal II).
(DOCX)

Author Contributions

Conceived and designed the experiments: RK DH THN. Performed the experiments: SLA MI JS NHP. Analyzed the data: SLA MI PK DH THN. Contributed to the writing of the manuscript: SLA MI DH THN.

References

1. Gibson GR, Roberfroid MB (1995) Dietary modulation of the human colonic microbiota: Introducing the concept of prebiotics. J Nutr 125: 1401–1412.
2. Bouhnik Y, Raskine L, Simoneau G, Vicaut E, Neut C, et al. (2004) The capacity of nondigestible carbohydrates to stimulate fecal bifidobacteria in healthy humans: A double-blind, randomized, placebo-controlled, parallel-group, dose-response relation study. Am J ClinNutr 80: 1658–1664.
3. Matsuki T, Watanabe K, Tanaka R, Fukuda M, Oyaizu H (1999) Distribution of bifidobacterial species in human intestinal microflora examined with 16S rRNA-gene-targeted species-specific primers. Appl Environ Microbiol 65: 4506–4512.
4. Biavati B, Mattarelli P (2006) The family Bifidobacteriaceae. In: Dworkin M, Falkow S, Rosenberg E, Schleifer KH, Stackebrandt E, editors. The prokaryotes. 3rd ed. New York: Springer. pp. 322–382.
5. Tanaka R (1995) Clinical effects of bifidobacteria and lactobacilli. In: Fuller R, Heidt PJ, Rusch V, Waaij DVD, editors. Probiotics: prospects of use in opportunistic infections Old Herborn University seminar monograph 8: Institute for Microbiology and Biochemistry, Herborn-Dill, Germany. pp. 141–157.
6. Biavati B, Castagnoli P, Trovatelli LD (1986) Species of the genus *Bifidobacterium* in the feces of human adults. Microbiologica 9: 39–45.
7. Macfarlane GT, Steed H, Macfarlane S (2008) Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. J Appl Microbiol 104: 305–344.
8. Holzapfel WH, Schillinger U (2002) Introduction to pre- and probiotics. Food Res Int 35: 109–116.
9. Rastall RA, Gibson GR, Gill HS, Guarner F, Klaenhammer TR, et al. (2005) Modulation of the microbial ecology of the human colon by probiotics, prebiotics and synbiotics to enhance human health: An overview of enabling science and potential applications. FEMS Microbiol Ecol 52: 145–152.
10. Gibson GR, Probert HM, Van Loo J, Rastall RA, Roberfroid MB (2004) Dietary modulation of the human colonic microbiota: Updating the concept of prebiotics. Nutrition Research Reviews 17: 259–275.
11. Crittenden RG, Playne MJ (1996) Production, properties and applications of food-grade oligosaccharides. Trends Food Sci Tech 7: 353–361.
12. Gopal PK, Sullivan PA, Smart JB (2001) Utilisation of galacto-oligosaccharides as selective substrates for growth by lactic acid bateria including *Bifidobacterium lactis* DR10 and *Lactobacillus rhamnous* DR20. Int Dairy J 11: 19–25.
13. Sangwan V, Tomar SK, Singh RRB, Singh AK, Ali B (2011) Galactooligosaccharides: novel components of designer foods. J Food Sci 76: R103–R111.
14. Nakayama T, Amachi T (1999) β-Galactosidase, enzymology. In: Flickinger MC, Drew SW, editors. Encyclopedia of bioprocess technology: fermentation, biocatalysis, and bioseparation. New York: John Willey and Sons. pp. 1291–1305.
15. Pivarnik LF, Senegal AG, Rand AG (1995) Hydrolytic and transgalactosylic activities of commercial β-galactosidase (lactase) in food processing. In: Kinsella JE, Taylor SL, editors. Advances in Food and Nutrition research: Academic Press. pp. 1–102.
16. Prenosil JE, Stuker E, Bourne JR (1987) Formation of oligosaccharises during enzymatic lactose hydrolysis: Part I: State of art. Biotechnol Bioeng 30: 1019–1025.
17. Petzelbauer I, Zeleny R, Reiter A, Kulbe KD, Nidetzky B (2000) Development of an ultra-high-temperature process for the enzymatic hydrolysis of lactose: II. Oligosaccharide formation by two thermostable β-glycosidases. Biotechnol Bioeng 69: 140–149.
18. Gänzle MG, Haase G, Jelen P (2008) Lactose: Crystallization, hydrolysis and value-added derivatives. International Dairy Journal 18: 685–694.
19. Hung MN, Xia Z, Hu NT, Lee BH (2001) Molecular and biochemical analysis of two β-galactosidases from *Bifidobacterium infantis* HL96. Appl Environ Microbiol 67: 4256–4263.
20. Hung MN, Lee BH (2002) Purification and characterization of a recombinant β-galactosidase with transgalactosylation activity from *Bifidobacterium infantis* HL96. Appl Microbiol Biotechnol 58: 439–445.
21. Moller PL, Jorgensen F, Hansen OC, Madsen SM, Stougaard P (2001) Intra- and extracellular β-galactosidases from *Bifidobacterium bifidum* and *B. infantis*: molecular cloning, heterologous expression, and comparative characterization. Appl Environ Microbiol 67: 2276–2283.
22. Van Laere KMJ, Abee T, Schols HA, Beldman G, Voragen AGJ (2000) Characterization of a novel β-galactosidase from *Bifidobacterium adolescentis* DSM 20083 active towards transgalactooligosaccharides. Appl Environ Microbiol 66: 1379–1384.
23. Hinz SWA, Van Den Broek LAM, Beldman G, Vincken JP, Voragen AGJ (2004) β-Galactosidase from *Bifidobacterium adolescentis* DSM20083 prefers β(1,4)-galactosides over lactose. Appl Microbiol Biotechnol 66: 276–284.
24. Goulas TK, Goulas AK, Tzortzis G, Gibson GR (2007) Molecular cloning and comparative analysis of four β-galactosidase genes from *Bifidobacterium bifidum* NCIMB41171. Appl Microbiol Biotechnol 76: 1365–1372.
25. Goulas T, Goulas A, Tzortzis G, Gibson GR (2009) Comparative analysis of four β-galactosidases from *Bifidobacterium bifidum* NCIMB41171: Purification and biochemical characterisation. Appl Microbiol Biotechnol 82: 1079–1088.
26. Nguyen TH, Splechtna B, Steinböck M, Kneifel W, Lettner HP, et al. (2006) Purification and characterization of two novel β-galactosidases from *Lactobacillus reuteri*. J Agric Food Chem 54: 4989–4998.
27. Splechtna B, Nguyen TH, Steinböck M, Kulbe KD, Lorenz W, et al. (2006) Production of prebiotic galacto-oligosaccharides from lactose using β-galactosidases from *Lactobacillus reuteri*. J Agric Food Chem 54: 4999–5006.
28. Nguyen TH, Splechtna B, Krasteva S, Kneifel W, Kulbe KD, et al. (2007) Characterization and molecular cloning of a heterodimeric β-galactosidase from the probiotic strain *Lactobacillus acidophilus* R22. FEMS Microbiol Lett 269: 136–144.
29. Splechtna B, Nguyen TH, Haltrich D (2007) Comparison between discontinuous and continuous lactose conversion processes for the production of prebiotic galacto-oligosaccharides using β-galactosidase from *Lactobacilius reuteri*. J Agric Food Chem 55: 6772–6777.
30. Splechtna B, Nguyen TH, Zehetner R, Lettner HP, Lorenz W, et al. (2007) Process development for the production of prebiotic galacto-oligosaccharides from lactose using β-galactosidase from *Lactobacillus* sp. Biotechnol J 2: 480–485.
31. Iqbal S, Nguyen TH, Nguyen TT, Maischberger T, Haltrich D (2010) β-galactosidase from *Lactobacillus plantarum* WCFS1: Biochemical characterization and formation of prebiotic galacto-oligosaccharides. Carbohydr Res 345: 1408–1416.
32. Iqbal S, Nguyen TH, Nguyen HA, Nguyen TT, Maischberger T, et al. (2011) Characterization of a heterodimeric GH2 β-galactosidase from *Lactobacillus sakei* Lb790 and formation of prebiotic galacto-oligosaccharides. J Agric Food Chem 59: 3803–3811.
33. Maischberger T, Leitner E, Nitisinprasert S, Juajun O, Yamabhai M, et al. (2010) β-galactosidase from *Lactobacillus pentosus*: Purification, characterization and formation of galacto-oligosaccharides. Biotechnol J 5: 838–847.
34. Yi SH, Alli I, Park KH, Lee B (2011) Overexpression and characterization of a novel transgalactosylic and hydrolytic β-galactosidase from a human isolate *Bifidobacterium breve* B24. New Biotechnol 28: 806–813.
35. Rastall RA, Maitin V (2002) Prebiotics and synbiotics: towards the next generation. Curr Opin Biotechnol 13: 490–496.
36. de Man JD, Rogosa M, Sharpe ME (1960) A medium for the cultivation of *Lactobacilli*. J Appl Bacteriol 23: 130–135.
37. Larkin MA, Blackshields G, Brown NP, Chenna E, McGettigan PA, et al. (2007) Clustal W and Clustal X version 2.0. Bioinforma Oxf Engl 23: 2947–2948.
38. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.
39. Nguyen TT, Nguyen HA, Arreola SL, Mlynek G, Djinović-Carugo K, et al. (2012) Homodimeric β-galactosidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 20081: Expression in *Lactobacillus plantarum* and biochemical characterization. J Agric Food Chem 60: 1713–1721.
40. Juers DH, Matthews BW, Huber RE (2012) *LacZ* β-galactosidase: Structure and function of an enzyme of historical and molecular biological importance. Protein Sci 21: 1792–1807.
41. Osman A, Tzortzis G, Rastall RA, Charalampopoulos D (2013) High yield production of a soluble bifidobacterial β-galactosidase (BbgIV) in *E. coli* DH5α with improved catalytic efficiency for the synthesis of prebiotic galactooligosaccharides. J Agric Food Chem 61: 2213–2223.
42. de Roos A (2004) Industrial enzymes: enzymes in dairy applications. In: Aehle W, editor. Enzymes in Industry. 2nd ed: Wiley-VCH, Weinheim. pp. 144.
43. Kim CS, Ji ES, Oh DK (2003) Expression and characterization of *Kluyveromyces lactis* β-galactosidase in *Escherichia coli*. Biotechnol Lett 25: 1769–1774.
44. Kim JW, Rajagopal SN (2000) Isolation and characterization of β-galactosidase from *Lactobacillus crispatus*. Folia Microbiol 45: 29–34.
45. Juajun O, Nguyen TH, Maischberger T, Iqbal S, Haltrich D, et al. (2011) Cloning, purification, and characterization of β-galactosidase from *Bacillus licheniformis* DSM 13. Appl Microbiol Biotechnol 89: 645–654.
46. Jurado E, Camacho F, Luzón G, Vicaria JM (2002) A new kinetic model proposed for enzymatic hydrolysis of lactose by a β-galactosidase from *Kluyveromyces fragilis*. Enzyme Microb Technol 31: 300–309.
47. Rabiu BA, Jay AJ, Gibson GR, Rastall RA (2001) Synthesis and fermentation properties of novel galacto-oligosaccharides by β-galactosidases from *Bifidobacterium* species. Appl Environ Microbiol 67: 2526–2530.
48. Goulas T, Goulas A, Tzortzis G, Gibson GR (2009) Expression of four β-galactosidases from *Bifidobacterium bifidum* NCIMB41171 and their contribution on the hydrolysis and synthesis of galactooligosaccharides. Appl Microbiol Biotechnol 84: 899–907.
49. Osman A, Tzortzis G, Rastall RA, Charalampopoulos D (2012) BbgIV is an important *Bifidobacterium* β-galactosidase for the synthesis of prebiotic galactooligosaccharides at high temperatures. J Agric Food Chem 60: 740–748.
50. Mahoney RR (1998) Galactosyl-oligosaccharide formation during lactose hydrolysis: A review. Food Chem 63: 147–154.

APPENDIX D

Galacto-Oligosaccharides: Recent Progress on Research and Application As Prebiotics

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Book chapter

Galactose: Structure and Function in Biology and Medicine

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Chapter 4

GALACTO-OLIGOSACCHARIDES
RECENT PROGRESS ON RESEARCH AND APPLICATION AS PREBIOTICS

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ABSTRACT

Prebiotic oligosaccharides have attracted an increasing amount of attention because of their physiological importance and functional effects on human health, as well as their physico-chemical properties, which are of interest for various applications in the food industries. Galacto-oligosaccharides (GOS), one of the major groups of prebiotic oligosaccharides, are formed via the transgalactosylation reaction from lactose. This reaction is catalyzed by a number of β-galactosidases (lactases) in addition to their hydrolytic activity. GOS are complex mixtures of different oligosaccharides, and the spectrum of the oligosaccharides making up these mixtures strongly depends on the source of the enzyme used for the biocatalytic reaction as well as on the conversion conditions used in their production. These oligosaccharides are of great interest because of their proven prebiotic (bifidogenic) characteristics. A plethora of GOS is also found in human milk, and these differently substituted oligosaccharides are associated with a number of beneficial effects for the breast-fed infant. This chapter reviews the production, the properties, the biological effects as well as the applications of

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galacto-oligosaccharides as prebiotics. The chapter also includes emerging trends in the production of novel, galactose-containing hetero-oligosaccharides, which are structurally more closely related to human milk oligosaccharides.

INTRODUCTION

The concept of 'probiotics' with the emphasis on the human host was described as 'a mono- or mixed-culture of live microorganisms which when applied to man or animal affects beneficially the host by improving the properties of the indigenous microflora' [1, 2]. Presently, there is a general acceptance that 'probiotics' refer to viable microorganisms which promote or stimulate beneficially the microbial population of the gastro-intestinal tract (GIT) [3, 4]. It was stated in different reviews that such microorganisms may not necessarily be constant inhabitants of the GIT but they should beneficially affect the health of man and animal [4-7]. Lactic acid bacteria (LAB) and bifidobacteria are the major representatives of probiotic microorganisms [4, 8]. LAB and bifidobacteria have long been used in the production of a wide range of foods without adverse effects on humans [8]. *Bifidobacterium* and *Lactobacillus* species, among LAB, receive special attention in the applications of probiotic products because of their GRAS (generally recognized as safe) status and beneficial effects on human health. Their beneficial roles on the host were summarized by Klaenhammer (1998) [9] including maintenance of the normal microflora, pathogen interference, exclusion and antagonism, immuno-stimulation and immuno-modulation, anti-carcinogenic and anti-mutagenic activities, deconjugation of bile acids, and lactase presentation *in vivo*. A number of *Lactobacillus* and *Bifidobacterium* strains are used for applications in probiotic products and these include for example *L. acidophilus*, *L. amylovorus*, *L. casei*, *L. crispatus*, *L. gasseri*, *L. johnsonii*, *L. paracasei*, *L. plantarum*, *L. reuteri*, *L. rhamnosus, B. bifidum, B. longum, B. infantis, B. breve*, or *B. adolescentis* [8, 10-13].

Since its first introduction [14], the concept of prebiotics has attracted an increasing amount of attention and stimulated both scientific and industrial interests. This concept was later revised [15, 16], and according to an updated definition of the prebiotic concept, 'a dietary prebiotic is a selectively fermented ingredient that results in specific changes, in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health' [16]. Based on the criteria [15, 17] (i) resistance to gastric acidity, to hydrolysis by mammalian enzymes, and to gastrointestinal absorption; (ii) fermentation by intestinal microflora; and (iii) selective stimulation of growth and/or activity of intestinal

bacteria associated with health/well-being, only inulin/fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS) and lactulose are fulfilling these requirements for prebiotics as documented and proven in several studies, although promise exists for several other dietary oligosaccharides [15, 17-20].

Prebiotic oligosaccharides can serve as fermentable substrates for certain members of the gut microbiota, and have been found to modulate the colonic flora by selective stimulation of beneficial bacteria such as bifidobacteria and lactobacilli as well as inhibition of 'undesirable' bacteria [4, 21, 22]. Galacto-oligosaccharides (GOS), the products of transgalactosylation reactions catalyzed by β-galactosidases when using lactose as the substrate, are non-digestible carbohydrates meeting the criteria of 'prebiotics'. GOS are of special interest to human nutrition because of the presence of structurally related oligosaccharides together with different complex structures in human breast milk [23-25].

PRODUCTION OF GALACTO-OLIGOSACCHARIDES

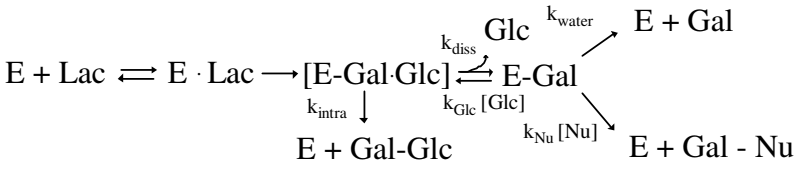
β-Galactosidases and Transgalactosylation of Lactose

Production of GOS (or sometimes referred to as TOS, transgalactosylated oligosaccharides) typically employs lactose as galactosyl donor and the transfer of the galactosyl moiety of lactose to suitable acceptor carbohydrates or nucleophiles using either glycoside hydrolases (EC 3.2.1.) or glycosyltransferases (EC 2.4.) [26-28]. Glycosyltransferases catalyze glycosidic bond formation employing sugar donors containing a nucleoside phosphate or a lipid phosphate leaving group, and are quite efficient as well as regio-and stereo-selective compared to glycoside hydrolases. However, due to limited supply, high price and necessity of specific sugar nucleotide as substrate of glycoside hydrolases, industrial GOS production favors the use of glycoside hydrolases [26].

Glycoside hydrolases (GH) are classified based on the stereochemical outcome of the hydrolysis reaction; they can be either *retaining* or *inverting* enzymes. Amino acid sequence similarities, hydrophobic cluster analysis, reaction mechanisms and the conservation of catalytic residues allow classification of β-galactosidases (β-gal; β-D-galactoside galactohydrolase E.C.3.2.1.23; lactase) in the GH families GH1, GH2, GH35, and GH42, indicating their structural diversity [28]. GH1 β-glycosidases are retaining enzymes of which the most commonly known enzymatic activities are myrosinases (thio-β-glucosidases),

β-mannosidases, β-galactosidases, phospho-β-glucosidases and phospho-β-galactosidases. GH1 β-galactosidases are predominant in the plant kingdom and have been explored for their 3D conformation, active site residues, and mechanism of action with few reports from Archaea and recently also from the Eubacteria *Meiothermus ruber* DSM1279 [29]. The GH2 family, to which most of the β-galactosidases belong, comprises the LacZ and LacLM β-galactosidases as isolated and described from *E. coli*, lactic acid bacteria and bifidobacteria. GH1 and GH2 β-galactosidases use only lactose, β-(1→3) and β-(1→6) linked galactosides as their substrates while those belonging to families GH35 and GH42 act on different galactose-containing glycosides including higher oligosaccharides and polysaccharides [28]. Owing to the different substrate specificities, β-galactosidases of GH2 and GH42 are often found in the same organism [30-32].

β-Galactosidases catalyze the hydrolysis and transgalactosylation of β-D-galactopyranosides (such as lactose) [33-35] and are found widespread in nature. They catalyze the cleavage of lactose (or related compounds) in their hydrolysis mode, and are thus used in the dairy industry to remove lactose from various products. An attractive biocatalytic application is found in the transgalactosylation potential of these enzymes, which is based on their catalytic mechanism [33, 36]. Retaining β-galactosidases undergo a two-step mechanism of catalysis. First, this mechanism involves the formation of a covalently linked galactosyl-enzyme intermediate. Subsequently, the galactosyl moiety linked to the nucleophile is transferred to a nucleophilic acceptor. Water, as well as all sugar species present in the reaction mixture, can serve as a galactosyl acceptor. Hence, the resulting final mixture contains hydrolysis products of lactose, which are glucose and galactose, unconverted lactose as well as di-, tri- and higher oligosaccharides [37, 38]. Scheme 1 illustrates the possible lactose conversion reactions catalyzed by β-galactosidases, and structures of some galacto-oligosaccharides are given in Figure 1.



Scheme 1. Hydrolysis and galactosyl transfer reactions, both intra- and intermolecular, during the conversion of lactose catalyzed by β-galactosidases. E, Enzyme; Lac, lactose; Gal, galactose; Glc, glucose; Nu, nucleophile.

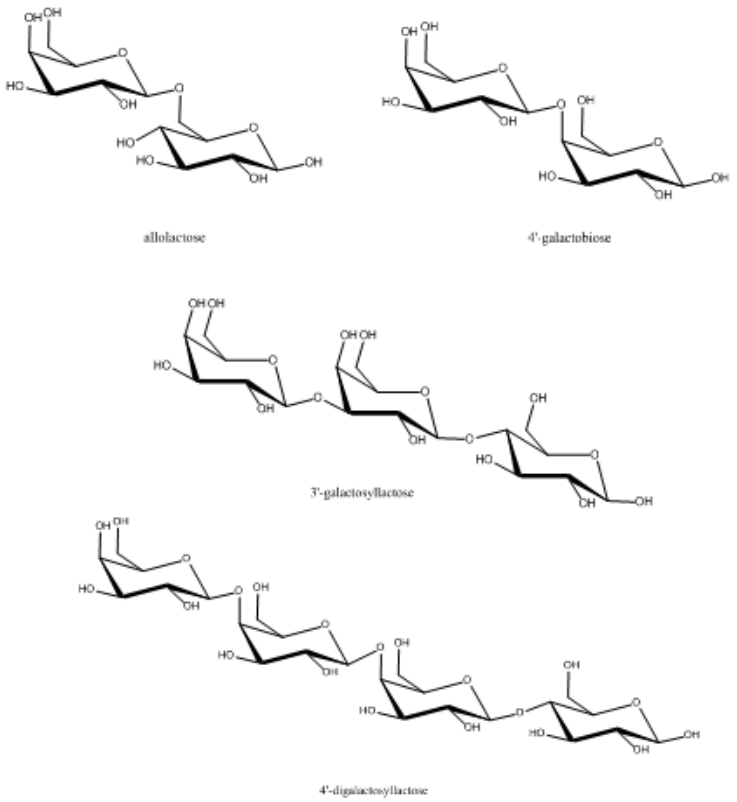


Figure 1. Structures of some galacto-oligosaccharides.

Transgalactosylation is described to involve intermolecular as well as intramolecular reactions. Intramolecular or direct galactosyl transfer to D-glucose yields regio-isomers of lactose. The glycosidic bond of lactose (β-D-Gal*p*-(1→4)-D-Glc) is cleaved and immediately formed again at a different position of the glucose molecule before it diffuses out of the active site. This is how allolactose (β-D-Gal*p*-(1→6)-D-Glc), the presumed natural inducer of β-galactosidases in certain microorganisms, can be formed even in the absence of significant amounts of free D-glucose [37, 39]. By intermolecular transgalactosylation, various di-, tri-, tetrasaccharides and eventually higher oligosaccharides are produced. Any sugar molecule in the reaction mixture can be the nucleophile accepting the galactosyl moiety from the galactosyl-enzyme complex, which is formed as an intermediate in the reaction. The GOS produced are not the product of an equilibrium reaction, but must be regarded as kinetic intermediates as they are also substrates for hydrolysis, and hence transgalactosylation reactions are kinetically controlled [39, 40]. For these

reasons, GOS yield and composition change dramatically with reaction time, and the GOS mixtures thus obtained are very complex and can hardly be predicted.

Enzyme Sources

β-Galactosidases can be obtained from different sources including microorganisms, plants and animals. Microbial sources of β-galactosidase are of great biotechnological interest because of easier handling, higher multiplication rates, and production yield.

Table 1 presents some of the commercially available bacterial, fungal and yeast β-galactosidases. Recently, a number of studies have focused on the use of the genera *Bifidobacterium* and *Lactobacillus* for the production and characterization of β-galactosidases, including the enzymes from *L. reuteri, L. acidophilus, L. plantarum, L. sakei, L. pentosus, L. bulgaricus, L. fermentum, L. crispatus*, *B. infantis, B. bifidum, B. angulatum, B. adolescentis, and B. pseudolongum and B. breve* [41-55]. Bifidobacteria and lactobacilli have been studied intensively with respect to their enzymes for various different reasons, one of which is their 'generally recognized as safe' (GRAS) status and their safe use in food applications. It is anticipated that GOS produced by these β-galactosidases will have better selectivity for growth and metabolic activity of these bacterial genera in the gut, and thus will lead to improved prebiotic effects [56].

Table 1. Commercial β-galactosidases

Name	Manufacturer	Microorganism
BioLactase NTL-CONC	Biocon Ltd.,	*Bacillus circulans* [153, 154]
Lactozym pure 6500 L	Novozymes	*Kluyveromyces lactis* [154]
Lactase F "Amano"	Amano Enzyme Inc.	*Aspergillus oryzae* [154, 155]
Biolacta FN5	Daiwa Fine Chemicals Co., Ltd.	*Bacillus circulans* [155, 156]
Lactoles® L3	Biocon Ltd.,	*Bacillus circulans* [155]
Maxilact	DSM Food Specialties	*Kluyveromyces lactis* [157, 158]
Tolerase	DSM Food Specialties	*Aspergillus oryzae* [157, 158]

An extensive list of bacterial and fungal sources of β-galactosidases, as well as the lactose conversion reaction conditions and GOS yields, is given in [57].

Studies of thermostable glycosyl hydrolases have been conducted in pursuit of GOS production at high temperatures. These include β-glycosidases from *Pyrococcus furiosus* (F426Y), *Thermotoga maritima*, *Penicillum simplicissimum*, *Saccharopolyspora rectivirgula, Aspergillus niger*, *Bifidobacterium bifidum*, *Bacillus stearothermophilus*, *Alicyclobacillus acidocaldarius*, *Thermus aquaticus* YT-1, *Thermus thermophilus* KNOUC202, and *L. bulgaricus,* to name a few [47, 58-63]. The GOS yields as influenced by transgalactosylation of lactose at different temperatures were given in recent reviews [57, 64]. Cold-active β-galactosidases have also attracted attention because their applications in the industrial processes of lactose hydrolysis and oligosaccharides synthesis can lower the risk of mesophiles contamination. Cold-active β-galactosidases were isolated from *P. haloplanktis* TAE 79, *Planococcus* sp., *Arthrobacter psychrolactophilus*, *Arthrobacter* sp. 32c, *Alkalilactibacillus ikkense*, *Paracoccus* sp. 32d, *Halorubrum lacusprofundi,* and *Thalassospira frigidphilosprofundus* [65-72] and have been reported for their potential use to hydrolyze lactose in dairy products processed at low temperatures. Soluble cold-active β-galactosidases from *Paracoccus* sp. 32d and *Lactococcus lactis* IL 1403 were found to efficiently hydrolyze lactose in milk at 10°C [71, 73].

It is a well-known fact that β-galactosidases from different species possess very different specificities for building glycosidic linkages, and therefore produce different GOS mixtures. For example, the β-galactosidase from *Kluyveromyces lactis* produced predominantly β-(1→6)-linked GOS [74], the β-galactosidase from *Aspergillus oryzae* produced mainly β-(1→3) and β-(1→6) linkages in their GOS [75], *Bacillus circulans* β-galactosidase forms β-(1→2), β-(1→3), β-(1→4), β-(1→6) linked GOS [76], whereas β-galactosidases from *Lactobacillus* spp. showed preference to form β-(1→3) and β-(1→6) linkages in transgalactosylation mode [39, 43, 45, 47].

Microbial Production of GOS

GOS are produced from lactose by microbial β-galactosidases employing different enzyme sources and preparations including crude enzymes, purified enzymes, recombinant enzymes, immobilized enzymes, whole-cell biotransformations, toluene-treated cells, and immobilized cells. The enzyme sources, the process parameters as well as the yield and

the productivity of these processes for GOS production are summarized in detail in recent reviews [25, 57, 64, 77]. The highest GOS productivity, 106 g L^{-1} h^{-1}, was observed when β-galactosidase from *Aspergillus oryzae* immobilized on cotton cloth was used for GOS production in a packed-bed reactor [78].

The choice of process technology either for lactose hydrolysis or GOS production depends on the nature of the substrate and the characteristics of the enzyme. The primary characteristic, which determines the choice and application of a given enzyme, is the operational pH range. Acid-pH enzymes, which are mainly from fungi, are suitable for processing of acid whey and whey permeate, while the neutral-pH enzymes from yeasts and bacteria are suitable for processing milk and sweet whey. Depending on the enzyme source, the pH value of the reaction mixture can be very acidic when using β-galactosidases from *A. oryzae* and *Bullera singularis* with optimum GOS yields at pH 4.5 and 3.7, respectively [79, 80]. Isobe and others studied the β-galactosidase from an acidophilic fungus, *Teratosphaeria acidotherma* AIU BGA-1, which was stable over the pH range of 1.5 to 7.0 and exhibited optimal activity at pH 2.5-4.0 and 70°C [81]. The maximum yield of GOS was observed at neutral pH for most bacteria and fungi though [44]. The time required to get maximum GOS depends inversely on the amount of enzyme. The highest GOS yields are generally observed when the reaction proceeds to 45 - 90% lactose conversion [57].

Despite the importance of LAB, and in particular *Lactobacillus* spp. and bifidobacteria, for food technology and dairy applications, and despite numerous studies on the gene clusters involved in lactose utilization by these bacteria, β-galactosidases from *Lactobacillus* and *Bifidobacterium* spp. have been characterized in detail pertaining to their biochemical properties or investigated for their ability to produce GOS in biocatalytic processes only recently. Previous studies reported the presence of multiple β-galactosidases in *B. infantis*, *B. adolescentis*, *B. bifidum,* as well as *B. breve* [51, 52, 55, 82-86], and revealed that these enzymes are very different with respect to substrate specificity and regulation of gene expression. Furthermore, these reports described the cloning and characterization of these enzymes and studied their transgalactosylation activity in detail, for example β-galactosidase BgbII from *B. adolescentis* showed high preference towards the formation of β-(1→4) linkages while no β-(1→6) linkages were formed [85]. In contrast, the β-galactosidase BgbII from *B. bifidum* showed a clear preference for the synthesis of β-(1→6) linkages over β-(1→4) linkages [32]. A recombinant β-galactosidase from *B. infantis* was found to be an excellent biocatalyst for

GOS production giving the highest GOS yield of 63% (mass of GOS of the total sugars in the reaction mixture) [51].

β-Galactosidases of lactobacilli play an important role in a number of commercial processes, e.g., milk processing or cheese making [87, 88]. Recent studies of β-galactosidases, especially with respect to their enzymatic and molecular properties, from *L. reuteri*, *L. acidophilus*, *L. plantarum*, *L. sakei*, *L. pentosus*, and *L. bulgaricus* [39, 41-43, 45-47, 89, 90] revealed that these enzymes were found to be very well suited for the production of galacto-oligosaccharides. Maximum GOS yields at 30°C were 38% when using purified β-galactosidases from *L. reuteri* and *L. acidophilus* with initial lactose concentration of 205 g/L and at ~80% lactose conversion [39, 42]. When using purified β-galactosidases from *L. plantarum* and *L. sakei*, the yields at 30°C were 41% with similar initial lactose concentrations and at 77% and 85% lactose conversion, respectively [43, 45]. Purified β-galactosidase from *L. bulgaricus* gave the highest yield of 50% at 90% lactose conversion [47], and on the other hand, purified β-galactosidase from *L. pentosus* gave the lowest yields, 31%, compared to the above-mentioned β-galactosidases from lactobacilli [46]. In order to reduce enzyme costs by avoiding laborious and expensive chromatographic steps for the purification of the biocatalyst, crude β-galactosidase extract from *Lactobacillus* sp. directly obtained after cell disruption and separation of cell debris by centrifugation was used in lactose conversion for GOS production [90]. It was reported that there was no obvious difference in the obtained GOS yields using either purified or crude β-galactosidase at 37°C, and in addition, the crude enzyme was found to be equally stable as the purified one. Therefore, crude β-galactosidase extracts are suitable for a convenient and simple process of GOS production. Because of the GRAS status of most *Lactobacillus* spp., it is also safe to use these crude extracts in food and feed applications. The reduction of reaction temperature to 17°C to limit microbial growth and the use of a cheap lactose source such as whey permeate powder did not have significant adverse effects on the GOS yield [90].

Protein engineering is a powerful approach to favor transgalactosylation over hydrolysis, and hence improves transgalactosylation yields. A truncated β-galactosidase from *B. bifidum* enhanced the transgalactosylation activity of the enzyme towards lactose and as a result a normal, hydrolytic β-galactosidase was converted to a highly efficient transgalactosylating enzyme [91]. A mutagenesis approach was applied to the galactosidase BgaB of *Geobacillus stearothermophilus* KVE39 in order to improve its enzymatic transglycosylation of lactose into oligosaccharides. Exchange of one amino acid, arginine Arg109, in β-galactosidase BgaB to either lysine, valine or tryptophan improved significantly the formation of the main trisaccharide, i.e. 3'-galactosyl

lactose. The yield of this trisaccharide increased from 2% to 12%, 21% and 23%, respectively, for these different variants compared to that of the native enzyme [92]. Enhancement of the production of GOS was achieved by mutagenesis of *Sulfolobus solfataricus* β-galactosidase LacS. Site-directed mutagenesis was performed to obtain two mutants of LacS, F441Y and F359Q, and the GOS yield was increased by 10.8 and 7.4%, respectively [93]. Although protein engineering strategies were successful to enhance transgalactosylation activities of different β-galactosidases, it has not been described for β-galactosidases yet that this approach was successful to alter also the linkage type of the GOS products [94].

Manufacturers of GOS

GOS are manufactured and commercialized mainly in Japan, the United States and Europe. The major manufacturers are Yakult Honsha (Japan) with their product Oligomate, Nissin Sugar Manufacturing (Japan) with Cup-Oligo, Snow Brand Milk Products (Japan) with P7L, GTC Nutrition (United States) with Purimune, Friesland Foods Domo (the Netherlands) with Vivinal® GOS, Clasado Ltd. (UK) with Bimuno, and Dairy Food Ingredients (Ireland) with Dairygold GOS [23, 25, 57, 95-97].

Commercial GOS preparations typically are transparent syrups or white powders containing oligosaccharides of different DP, non-converted lactose and the monosaccharides glucose and galactose. They differ in purity of the GOS products and also in the linkages of the oligosaccharide chains, which depend on the enzymes used for the GOS productions. Oligomate contains mainly β-(1→6) linked GOS, Vivinal® GOS, Cup-Oligo and Purimune contain mainly β-(1→4) linkages, whilst Bimuno contains mainly β-(1→3) linked GOS [57]. Table 2 presents some commercial GOS and the enzymes used in their productions.

PROPERTIES AND BIOLOGICAL EFFECTS OF GALACTO-OLIGOSACCHARIDES

Properties of GOS

The physico-chemical properties of galacto-oligosaccharides are of significant interest for their application in the food industries. Generally, GOS are transparent/colorless water-soluble products (80% w/w solubility), and more viscous than high-fructose corn syrup [22, 57]. GOS are stable during treatment at elevated temperature of up to 160°C and as

low as pH 2. They are also stable during long-term storage at room temperature under acidic conditions [38].The caloric value of GOS was estimated to be 1.7 kcal g^{-1}, this is approximately 30-50% of those of digestible carbohydrates such as sucrose [38]. GOS are low-calorie sweeteners since they pass through the human small intestine without being digested. GOS are undigested by pancreatic enzymes and gastric juice while passing the small intestine, which makes them suitable for low-calorie diets and for consumption by individuals with diabetes [57, 97]. They can be used as humectants because of their high moisture-retaining capacity to prevent excessive drying, hence to keep the foodstuff moist. They can alter the freezing temperature of frozen foods and reduce the amount of colouring due to Maillard reactions in heat-processed foods as relatively fewer reducing moieties are available [57, 97, 98]. These properties enable GOS to be applied in a wide variety of food products.

Table 2. Commercial galacto-oligosaccharides (GOS) in the market

Product	Company	Total GOS (% w/w)	Enzyme Source
Oligomate 55	Yakult Pharmaceutical Industry Co., Ltd. Tokyo, Japan	>55	*Sporobolomyces singularis*, *Kluyveromyces lactis* [159, 160]
Vivinal® GOS	Friesland Foods Domo, Amersfoort, The Netherlands	~60	*Bacillus circulans* [161, 162]
Purimune™	GTC Nutrition, Colorado, USA	≥ 90	*Bacillus circulans* [163, 164]
Bimuno® GOS	Clasado Ltd., Milton Keynes, England, UK	48-55	*Bifidobacterium bifidum* [165, 166]
Cup-oligo	Kowa Company Ltd., Tokyo, Japan	70	*Cryptococcus laurentii* [57, 167]

Apart from being used as sweeteners, GOS are nowadays incorporated in a wide range of products such as fermented milk products, breads, jams, snack bars, confectionery, beverages, infant milk formulas, and as sugar replacements [22, 23, 38].

Biological Effects

Prebiotic galacto-oligosaccharides can serve as fermentable substrates for certain members of the gut microbiota, and have been found to modulate the colonic flora [4, 21, 22]. The physiological importance and health benefits of prebiotic GOS have been reported extensively in several recent reviews on prebiotics and functional oligosaccharides [25, 99, 100]. The biological effects of GOS on human health are discovered in many different dimensions.

Positive Impact on the Intestinal Bifidobacteria and Lactobacilli Population

Galacto-oligosaccharides, like other prebiotics, are metabolized selectively in the gastrointestinal tract by beneficial bacteria associated with health benefits and well-being. These carbohydrates can thus positively modulate the colonic microbiota, which exerts an important influence on host health [15, 21, 22]. Bifidobacteria and lactobacilli play an important role in the eco-physiology of the colonic microbiota, although their population sizes and species composition vary among different groups of human population. The growth of these bacteria has been linked to beneficial health effects such as increased resistance to infection, stimulation of the immune system activity, protection against cancer, prophylactic and therapeutic benefits. Bifidobacteria are also known to excrete a range of water soluble vitamins such as folate, nicotinic acid, thiamine, pyridoxine, and vitamin B12 [22].

Different methods such as pure culture fermentations of single, selected strains [101, 102] and *in vitro* fermentations of mixed bacterial populations, particularly fecal bacteria, have been used as preliminary screening tools for prebiotic activities [103-105] whereas *in vivo* fermentations of non-digestible carbohydrates in animals and human subjects have been reported for evaluating the prebiotic effects of different oligosaccharide mixtures [15]. Pure culture fermentations are performed in appropriate basal media supplemented with the respective prebiotics, and the increase in cell numbers is quantified by turbidimetry of the cultures or

by viable cell count. pH-controlled batch cultures are, however, better models to investigate the interactions between the gut populations in response to certain carbohydrates. Here, fermentation is again based on basal media, with the test carbohydrate being the sole fermentable substrate present, but the use of fecal bacterial populations allows for an investigation of the interactions, competition and cross-feeding during growth on the selected substrate. Changes in fecal bacteria concentrations are monitored using molecular techniques such as fluorescent *in situ* hybridization (FISH) or real-time PCR [106, 107]. Alternatively, *in vitro* colonic models and ^{13}C labelling of substrates can be used to study the prebiotic activity [108]. The ability of galacto-oligosaccharide uptake generally seems to vary within the genus of *Lactobacillus* and *Bifidobacterium*, and hence different growth rates on various oligosaccharides can be observed. In a recent study, growth of single strains of *Bifidobacterium*, *Lactobacillus* and *Streptococcus* on various trisaccharides including 4´-galactosyl-lactose and 6´-galactosyl-lactose was evaluated, and in general, these strains grew faster on the trisaccharides with a β-(1→6)-galactosyl moiety [109]. A plethora of GOS is also found in human milk, and these differently substituted oligosaccharides are associated with a number of beneficial effects for the breast-fed infant. Because of this, GOS are incorporated in infant formula to achieve a bifidogenic effect and to imply a "breast-fed-like" flora [110]. A recent study demonstrated the prebiotic attributes of a purified Vivinal® GOS formulation in an *in-vitro* colon model. The authors observed an increase in numbers of lactobacilli and bifidobacteria as well other beneficial bacteria, with a concomitant decrease in numbers of *Bacteroides species, Eubacterium halii, Prevotella,* and *Lactococcus* [108].

Prebiotic effects of GOS depend significantly on the degree polymerization, the linkage types as well as the composition of the GOS mixtures, and also vary between individuals. These differences are known to be important when it comes to GOS assimilation by beneficial bacteria in the colon. It was reported that the administration of a GOS mixture containing β-(1→3) as well as β-(1→4) and β-(1→6) linkages proved to have a better bifidogenic effect than a mixture containing GOS with β-(1→4) and β-(1→6) linkages [56]. Furthermore, bifidogenic properties of GOS are dose dependent. It is known that bifidobacteria populations generally increase as the GOS dosage and purity increase. However, it has been shown that even when GOS were administered for many weeks and at high doses, there were still some individuals for whom a bifidogenic response did not occur [111].

Protective Effect against Infections and Intoxications

The use of prebiotics to prevent non-antibiotic associated gut diseases is promising. GOS are dietary prebiotic oligosaccharides and increasingly used as new food ingredient, especially in infant formula. Infants are more susceptible to gastrointestinal pathogens than adults. It is well known that a higher number of bifidobacteria in the intestine of breast-fed infants has been associated with a better health compared with formula-fed infants. It was suggested that GOS administration reduced intestinal infections and lowered the incidence of gastroenteritis in healthy infants during the first year of age [112]. GOS are not only effective against enteric infections in infants but also against respiratory infections. A study, in which new-born infants were fed a mixture of 4 probiotic bacterial strains along with galacto-oligosaccharides, revealed no effect on the incidence of any allergic diseases and it seemed to increase resistance to respiratory infections during the first two years of life [113]. A similar result was found in another study of preterm infants in Finland, which concluded that early supplementation of prebiotics (GOS and polydextrose mixture) or probiotics reduce the risk of virus-associated respiratory tract infections during the first year of life [114].

The use of GOS as functional mimics for a cell-surface toxin receptor is continuing to be developed for treatment or prevention of an acute or chronic disease associated with the adhesion or uptake of a cholera toxin (Ctx), which is a significant cause of gastrointestinal disease globally. Ctx antiadhesive activity of GOS was shown in a study [115], in which GOS fractions containing more than 5% hexasaccharides (DP6) exhibited more than 90% binding to the cell-surface toxin receptor (GM1) and its competitive inhibition was dose dependent. Supplementation of the prebiotic GOS in enteral nutrition was also found to significantly improve the intestinal barrier function in secondary infectious complications associated with severe acute pancreatitis rats, which might be partly attributed to an increase in the population of probiotic bifidobacteria, stimulation of the production of sIgA in the intestinal mucus, decrease in the apoptosis of intestinal epithelial cells, and regulation of the expression of the tight junction protein occluding [116].

Protective mechanisms of GOS against enteric infections and intoxications are associated with the potential to inhibit pathogen infections by blocking or competing for bacterial adhesion sites as well as competitive inhibition of adhesion or uptake of bacterial toxin in intestinal cells. GOS showed the best adherence inhibition of *Escherichia coli* strain E2348/69 on HEp-2 and Caco-2 cells with the depletion in adherence on both HEp-2 and Caco-2 cells by 65 and 70%, respectively, when compares with FOS, inulin, lactulose, and raffinose [117]. A protective mechanism

of GOS against other enteric pathogens such as *Salmonella* and *Listeria* was also reported. Purified GOS, derived from a mixture produced by the enzymatic activity of *Bifidobacterium bifidum*, were reported to reduce *Salmonella enterica* serovar Typhimurium adhesion and invasion both *in vitro* and *in vivo* [118]. It was demonstrated that ~2.5 mg GOS mL^{-1} significantly reduced the invasion of *S.* Typhimurium. The presence of GOS also prevented the adherence or invasion of *S.* Typhimurium to enterocytes, and thus reduced its associated pathology. It was also suggested that this protection appeared to correlate with significant reductions in the neutral and acidic mucins detected in goblet cells, possibly as a consequence of stimulating the cells to secrete the mucin into the lumen [118]. In a recent study, it was demonstrated that galacto-oligosaccharides, obtained from transgalactosylation of lactose (GOS-La) or lactulose (GOS-Lu), and their derivatives caseinomacropeptide hydrolysates (hCMP:GOS-La and hCMP:GOS-Lu) significantly reduced adhesion *of Salmonella enterica* CECT 443 and *Listeria monocytogenes* CECT 935. GOS-Lu and hCMP:GOS-Lu also inhibited the production of IL-1β, inflammatory cytokines, by intestinal cells stimulated by the pathogens tested [119].

Immunomodulation for the Prevention of Allergies and Gut Inflammatory Conditions

Currently, there is increasing interest in the utilization of prebiotics to modulate the immune system and attenuate inflammations in the colon. Most of these data originate from animal models and were obtained in relation to FOS and its prebiotic effect. Nevertheless, there are some studies that either suggest or prove an effect of GOS on the immune system, suggesting either a direct or indirect modulatory effect [97].

Immunity consists of the innate and adaptive immune system. The innate immune system is the first line of defense for the body and it comprises a physical barrier such as the skin, phagocytic, inflammatory, dendritic and natural killer (NK) cell, as well as other soluble components such as cytokines and complement proteins. The adaptive immune system response occurs after activation of the innate system. It is more antigen-specific and it involves the T- and B-lymphocytes. Lymphocytes play an important role in this component of the immune system, either by modulating the function of other immune cells, or by directly destroying infected cells [22]. Several animal model studies on the immunomodulatory effects of GOS were reported. A study in mice revealed that $CD25^+$ regulatory T-cells have an important role in modulated Flu-vaccine responses induced by orally supplied prebiotic

oligosaccharides containing GOS [120]. A recent study investigated the effect of GOS on colitis development and on immune variables in *Smad*3-deficient mice treated with the pathogen *Helicobacter hepaticus*. The results showed that GOS significantly reduced colitis severity in response to *H. hepaticus,* and it was suggested that GOS reduces colitis by modulating the function and trafficking of NK cells and may provide a novel therapeutic strategy for individuals with inflammatory bowel disease [121]. It is known that supplementation of non-digestible oligosaccharides during pregnancy has positive effects on hypertension as well as the metabolism, and may be used to ameliorate pregnancy-related metabolic disturbances. The effects of non-digestible oligosaccharides on the immune system during the pregnancy of mice supplemented with a specific mixture of short-chain galacto- and long-chain fructo-oligosaccharides (ratio of 9:1) was found to elicit a more tolerogenic immune reaction in pregnant mice [122].

In human studies, it has been suggested that GOS play a role in the development of the immune system in infants, and may consequently inhibit the onset of allergy. A specific prebiotic mixture of short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides (ratio of 9:1) has shown to reduce the incidence of atopic dermatitis at 6 months of age in infants at risk for allergy [123]. The synbiotic effect of several probiotic strains and GOS in preventing allergic diseases was demonstrated as well. High-risk pregnant women received probiotics for 2-4 weeks before delivery and their infants received a probiotic formulation with GOS for 6 months. It was shown that probiotics when taken together with GOS showed no effect on the incidence of all allergic diseases in high-risk children from birth to the age of two but significantly prevented eczema and especially atopic eczema [113].

Trophic Effects of Short-Chain Fatty Acids (SCFAs) on the Colonic Epithelium

Short-chain fatty acids (SCFAs), which are the end products of saccharolytic fermentations in the gut, are of interest as they are claimed to promote human health or may be antagonistic to intestinal competitors [15, 124]. The principal SCFAs that result from carbohydrate fermentations are acetate, propionate and butyrate. SCFAs stimulate colonic blood flow as well as fluid and electrolyte uptake [125], and inhibit proliferation and induce apoptosis in colon cancer cells [126]. SCFAs affect the synthesis of vitamins and essential amino acids, interaction with training of gut-associated lymphoid tissue; and resistance against colonization by enteric pathogens. Moreover, SCFAs affect colonic epithelial cell transport,

energy transduction in colonocytes, growth and cellular differentiation [22, 127]. These trophic properties have important physiological implications, in addition to maintaining the mucosal defense barrier against invading organism. *In vitro* studies involving fecal microbiota are useful models for studying fermentation activity of oligosaccharides. FOS and GOS (at 10 g L^{-1}) were shown to increase acetate and butyrate formation, with transient accumulation of lactate and succinate [128]. It was also demonstrated that a GOS mixture derived from lactose with 6'-galactosyl-lactose and 4'-galactosyl-lactose as the main components exhibited a bifidogenic effect in fecal slurries similar or slightly higher than the commercial Vivinal® GOS, and enhanced the production of acetic acid and SCFAs [129].

Mineral Absorption

Consumption of GOS significantly increases calcium absorption in humans and GOS play a role in increasing bioavailability of calcium, which is a key to bone mass density [22]. Different studies in rats and human trials have shown positive effects of GOS in promoting calcium absorption. In a study on the dose-response effect of GOS supplementation on calcium and magnesium absorption, mineral retention, bone properties and gut microbiota in growing rats, beneficial effects on calcium and magnesium absorption and retention, femur calcium uptake, bone strength, and bone mineral density were observed. These effects either directly or indirectly were attributed to a decrease in cecal pH, an increase in cecal wall and content weight, and an increase in number of bifidobacteria [130]. A similar study reported that a diet containing GOS stimulated calcium absorption and the ingestion of GOS prevents osteopenia in partially gastrectomized rats [131]. Recently, the dose-response relationship of GOS supplementation on calcium absorption in adolescent girls was investigated. Significant improvement in calcium absorption with both low and high doses of GOS was observed, but it was not a dose-response relationship [132]. These observations may have particular importance because bone mass accretion is maximal during adolescence [133].

Other Health Benefits of GOS

Other additional health benefits of GOS such as effects on serum cholesterol and high-density lipoprotein (HDL) cholesterol levels, improving brain functions, maintenance of brain health, and adjunctive treatment of neuropsychiatric disorders were reported. It is believed that

prebiotics can decrease serum cholesterol levels and increase HDL cholesterol levels when used in subjects with initial elevated serum cholesterol levels. However, the role of GOS regarding this health effect is still in question since it was demonstrated that 5.5 g of a B-GOS mixture administration to healthy elderly had no effect upon total serum cholesterol and HDL cholesterol levels [134]. In addition, it was shown that no differences in total cholesterol and low-density lipoprotein (LDL) cholesterol levels exist in infants receiving an infant formula supplemented with GOS and long-chain FOS in comparison with infants receiving a control infant formula [135]. More studies are required in order to demonstrate the underlying mechanisms of GOS in this respect.

Recently, evidence was presented that suggests effects of GOS in improving brain function with possible involvement of gut hormones. A study in rat reported that the effect of GOS on components of central *N*-methyl-D-aspartate receptor (NMDAR) signaling was greater than FOS, and it may reflect the proliferative potency of GOS on microbiota and increased brain derived neurotropic factor (BDNF) expression. This evidence suggested further investigations on the utility of prebiotics in the maintenance of brain health and adjunctive treatment of neuropsychiatric disorders [136]. In another recent study, it was reported that GOS or GOS-rich prebiotic yogurt could delay the onset of disease and prolong the lifespan in mice. They can attenuate motor neuron loss as well as muscle atrophy and dysfunction. Furthermore, they possess anti-inflammatory and anti-apoptotic effects through the regulation of related molecules. Altogether, GOS may have therapeutic potential for amyotrophic lateral sclerosis (ALS), and prebiotic yogurt may be considered as a nutritional therapy for this intractable disease [137].

EMERGING TRENDS IN THE PRODUCTION OF NOVEL HETERO-OLIGOSACCHARIDES

Human milk is known as the sole source of nourishment for breast-fed infants and for its promotion of a healthy development of newborns. Human milk is comprised of a complex mixture of oligosaccharides (5-10 g L^{-1} in addition to lactose) that are different in size, linkage and charge [138]. Human milk oligosaccharides (HMO) are a heterogenic group of about 200 molecular species consisting of mostly neutral and fucosylated oligosaccharides [139]. The potential health benefits of HMO have been studied with a special emphasis on prebiotic effects [138]. Recent studies have reported on the ability of HMO to selectively support the growth of specific strains of bifidobacteria thus providing insight on how HMO

modulate the infant intestinal microbiota [140-142]. These results suggest that the prebiotic, bifidogenic effects of HMO are structure-specific and may vary depending on the HMO composition in milk [138, 140-142]. In addition, HMO are also considered as a mechanism to protect infants against exogenous infections [140, 143]. *In vitro* studies have shown that HMO bind and block the infection of pathogenic bacteria to animal cells by acting as receptor analogues to the intestinal cell glycans [140, 144, 145]. Because of these benefits, prebiotic HMO are of great interest for human nutrition.

HMO are composed of both neutral and anionic species with building blocks of 5 monosaccharides: D-glucose, D-galactose, *N*-acetyl-D-glucosamine, L-fucose, and *N*-acetylneuraminic acid (sialic acid). The basic structure of HMO includes a lactose core at the reducing end which is elongated by *N*-acetyl-lactosamine units with at least 12 different types of glycosidic bonds, wherein fucose and sialic acid residues are added to terminal positions [140, 143]. The terminal lactose is typically elongated by lacto-*N*-biose units (LNB; Gal-β-1,3-GlcNAc) in type I or *N*-acetyl-lactosamine units (LacNAc; Gal-β-1,4-GlcNAc) in the rarer type II structures. Both LNB and LacNAc are attached via a β-1,3-linkage to the galactosyl moiety of the terminal lactose, with an additional β-1,6-linkage in branched HMO. These LNB and LacNAc units can be repeated up to 25 times in larger HMO, forming the core region of these oligosaccharides. A further variation results from the attachment of fucosyl and sialic acid residues. Thus, the simplest structures following this general scheme (apart from certain trisaccharides such as galactosyl-lactose, fucosyl-lactose and sialyl-lactose) are the tetrasaccharides lacto-*N*-tetraose, Gal-β-1,3-GlcNAc-β-1,3-Gal-β-1,4-Glc (type I), and lacto-*N*-*neo*-tetraose, Gal-β-1,4-GlcNAc-β-1,3-Gal-β-1,4-Glc (type II) (Figure 2) [146-149]. Mono- and difucosyl-lactose, lacto-*N*-tetraose and its fucosylated derivatives, as well as sialyl-lactose and the sialylated forms of lacto-*N*-tetraose, are major oligosaccharides in human milk.

Some of the above-mentioned HMO structures or structurally related compounds can be accessed through different approaches. One such approach that has received some interest is based on β-galactosidase-catalyzed transglycosylation with lactose as donor (thus transferring galactose onto suitable acceptors) and GlcNAc as acceptor, thus obtaining *N*-acetyl-lactosamine (LacNAc) and its regioisomers. Using this approach and a hyperthermophilic β-galactosidase from *Sulfolobus solfataricus*, Gal-β-1,6-GlcNAc together with an unidentified sugar were the main products starting from a mixture of 1 M lactose and 1 M GlcNAc, while LacNAc and Gal-β-1,3-GlcNAc were formed as well, yet in lower concentrations [150]. This reaction was also optimized using β-

galactosidase from *Bacillus circulans* as the biocatalyst. This enzyme is known for its propensity to synthesize β-1,4-linkages in its transgalactosylation mode, and hence the main reaction product here was LacNAc together with smaller amounts of GlcNAc-containing higher oligosaccharides (one tri- and one tetrasaccharides) and Gal-β-1,6-GlcNAc. The total yield was 40% for these GlcNAc-containing oligosaccharides when starting from 0.5 M lactose and GlcNAc each [151]. This reaction and the β-galactosidase from *B. circulans* were also compared to the enzyme from *Kluyveromyces lactis* and the reaction conditions were optimized. The latter enzyme was shown to form predominately Gal-β-1,6-GlcNAc. Again, both enzymes formed a mixture of various di- to tetra-saccharides [152]. Since these structures resemble the core of HMO, they could be of interest as prebiotic compounds to be added to food.

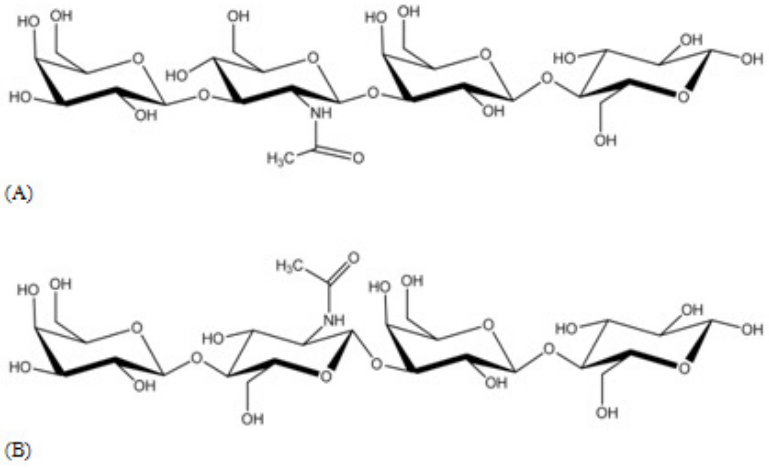


Figure 2. Some simple structures of human milk oligosaccharides: tetrasaccharides lacto-*N*-tetraose, Gal-β-1,3-GlcNAc-β-1,3-Gal-β-1,4-Glc (A), and lacto-*N*-*neo*-tetraose, Gal-β-1,4-GlcNAc-β-1,3-Gal-β-1,4-Glc (B).

CONCLUSION

The understanding of the relationship between certain oligosaccharide structures and prebiotic function already had an impact on novel prebiotic products that were brought to the market, and will have a more important impact in the future. The insights into the structures, production and biological effects of prebiotic galacto-oligosaccharides together with

advancement in the area of biotechnology will certainly result in the enhancement of the production of GOS. The presence of structurally related oligosaccharides together with different complex structures in human breast milk makes GOS attract increasing interests from researchers and manufacturers. Since the structures of GOS or novel hetero-oligosaccharides resemble the core of human milk oligosaccharides, they could be of interest as prebiotic compounds to be added to food, and intensified research in this field is needed to open the door for commercial production of these novel functionally enhanced, prebiotic oligosaccharides.

REFERENCES

[1] J. H. J. Huis in't Veld and R. Havenaar, *Eur. J. Clin. Nutr.,* 45, 29-31 (1991).

[2] M. E. Sanders, *Int. Dairy J.,* 8, 341-347 (1998).

[3] W. H. Holzapfel, P. Haberer, R. Geisen, J. Bjorkroth, and U. Schillinger, *Am. J. Clin. Nutri.,* 73, 365s-373s (2001).

[4] W. H. Holzapfel and U. Schillinger, *Food Res. Int.,* 35, 109-116 (2002).

[5] R. Fuller, *J. Appl. Bacteriol.,* 66, 365-378 (1989).

[6] R. Havenaar, B. Ten Brink, and J. H. J. Huis in't Veld, in R. Fuller (ed.), Probiotics, the scientific basis, Chapman & Hall, London, pp. 209-221 (1992).

[7] S. Salminen, M. A. Deighton, Y. Benno, and S. L. Gorbach, in S. Salminen and A. Von Wright (eds.), Lactic acid bacteria: Microbiology and Functional aspects, Marcel Dekker Inc., New York, pp. 211-254 (1998).

[8] J. K. Collins, G. Thornton, and G. O. Sullivan, *Int. Dairy J.,* 8, 487-490 (1998).

[9] T. R. Klaenhammer, *Int. Dairy J.,* 8, 497-505 (1998).

[10] W. H. Holzapfel, P. Haberer, J. Snel, U. Schillinger, and J. H. J. Huis in't Veld, *Int. J. Food Microbiol.,* 41, 85-101 (1998).

[11] L. J. Fooks, R. Fuller, and G. R. Gibson, *Int. Dairy J.,* 9, 53-61 (1999).

[12] C. Shortt, *Trends Food Sci. Technol.,* 10, 411-417 (1999).

[13] G. W. Tannock, *Curr. Issues Mol. Biol.,* 1, 53-64 (1999).

[14] G. R. Gibson and M. B. Roberfroid, *J. Nutr.,* 125, 1401-1412 (1995).

[15] G. R. Gibson, H. M. Probert, J. Van Loo, R. A. Rastall, and M. B. Roberfroid, *Nutr. Res. Rev.,* 17, 259-275 (2004).

[16] ISAPP, 6th Meeting of the International Scientific Association of Probiotics and Prebiotics, London, Ontario (2008).
[17] M. Roberfroid, *J. Nutr.,* 137, 830S-837S (2007).
[18] M. Roberfroid, G. R. Gibson, L. Hoyles, A. L. McCartney, R. Rastall, I. Rowland, D. Wolvers, B. Watzl, H. Szajewska, B. Stahl, F. Guarner, F. Respondek, K. Whelan, V. Coxam, M. J. Davicco, L. Léotoing, Y. Wittrant, N. M. Delzenne, P. D. Cani, A. M. Neyrinck, and A. Meheust, *Br. J. Nutr.,* 104, S1-S63 (2010).
[19] H. Barreteau, C. Delattre, and P. Michaud, *Food Technol. Biotechnol.,* 44, 323-333 (2006).
[20] T. Barclay, M. Ginic-Markovic, P. Cooper, and N. Petrovsky, *J. Exci. Food Chem.,* 1, 27-50 (2010).
[21] R. A. Rastall, G. R. Gibson, H. S. Gill, F. Guarner, T. R. Klaenhammer, B. Pot, G. Reid, I. R. Rowland, and M. E. Sanders, *FEMS Microbiol. Ecol.,* 52, 145-152 (2005).
[22] G. T. Macfarlane, H. Steed, and S. Macfarlane, *J. Appl. Microbiol.,* 104, 305-44 (2008).
[23] R. G. Crittenden and M. J. Playne, *Trends Food Sci. Tech.,* 7, 353-361 (1996).
[24] P. K. Gopal, P. A. Sullivan, and J. B. Smart, *Int. Dairy J.,* 11, 19-25 (2001).
[25] V. Sangwan, S. K. Tomar, R. R. B. Singh, A. K. Singh, and B. Ali, *J. Food Sci.,* 76, R103-R111 (2011).
[26] B. M. de Roode, M. C. Franssen, A. van der Padt, and R. M. Boom, *Biotechnol. Prog.,* 19, 1391-1402 (2003).
[27] H. D. Ly and S. G. Withers, *Annu. Rev. Biochem.,* 68, 487-522 (1999).
[28] B. L. Cantarel, P. M. Coutinho, C. Rancurel, T. Bernard, V. Lombard, and B. Henrissat, *Nucleic Acids Res.,* 37, D233-238 (2009).
[29] R. Gupta, T. Govil, N. Capalash, and P. Sharma, *Appl. Biochem. Biotechnol.,* 168, 1681-1693 (2012).
[30] E. Yoshida, H. Sakurama, M. Kiyohara, M. Nakajima, M. Kitaoka, H. Ashida, J. Hirose, T. Katayama, K. Yamamoto, and H. Kumagai, *Glycobiology,* 22, 361-368 (2012).
[31] C. Schwab, K. I. Sørensen, and M. G. Gänzle, *Syst. Appl. Microbiol.,* 33, 300-307 (2010).
[32] T. Goulas, A. Goulas, G. Tzortzis, and G. R. Gibson, *Appl. Microbiol. Biotechnol.,* 84, 899-907 (2009).
[33] T. Nakayama and T. Amachi, in M. C. Flickinger and S. W. Drew (eds.), Encyclopedia of bioprocess technology: fermentation, biocatalysis, and bioseparation, John Willey and Sons, New York, pp. 1291-1305 (1999).

[34] L. F. Pivarnik, A. G. Senegal, and A. G. Rand, in J. E. Kinsella and S. L. Taylor (eds.), *Adv. Food Nutr. Res.*, Academic Press, pp. 33-41 (1995).
[35] J. E. Prenosil, E. Stuker, and J. R. Bourne, *Biotechnol. Bioeng.,* 30, 1019-1025 (1987).
[36] I. Petzelbauer, R. Zeleny, A. Reiter, K. D. Kulbe, and B. Nidetzky, *Biotechnol. Bioeng.,* 69, 140-149. (2000).
[37] R. R. Mahoney, *Food Chem.,* 63, 147-154 (1998).
[38] T. Sako, K. Matsumoto, and R. Tanaka, *Int. Dairy J.,* 9, 69-80 (1999).
[39] B. Splechtna, T. H. Nguyen, M. Steinböck, K. D. Kulbe, W. Lorenz, and D. Haltrich, *J. Agric. Food Chem.,* 54, 4999-5006 (2006).
[40] M. A. Boon, A. E. M. Janssen, and K. van `t Riet, *Enzyme Microb. Technol.,* 26, 271-281 (2000).
[41] T. H. Nguyen, B. Splechtna, M. Steinböck, W. Kneifel, H. P. Lettner, K. D. Kulbe, and D. Haltrich, *J. Agric. Food Chem.,* 54, 4989-4998 (2006).
[42] T. H. Nguyen, B. Splechtna, S. Krasteva, W. Kneifel, K. D. Kulbe, C. Divne, and D. Haltrich, *FEMS Microbiol. Lett.,* 269, 136-144 (2007).
[43] S. Iqbal, T. H. Nguyen, T. T. Nguyen, T. Maischberger, and D. Haltrich, *Carbohydr. Res.,* 345, 1408-1416 (2010).
[44] D. Gobinath and S. G. Prapulla, *Biotechnol. Lett.,* (2013) doi: 10.1007/s10529-013-1345-9.
[45] S. Iqbal, T. H. Nguyen, H. A. Nguyen, T. T. Nguyen, T. Maischberger, R. Kittl, and D. Haltrich, *J. Agric. Food Chem.,* 59, 3803-3811 (2011).
[46] T. Maischberger, E. Leitner, S. Nitisinprasert, O. Juajun, M. Yamabhai, T. H. Nguyen, and D. Haltrich, *Biotechnol. J.,* 5, 838-847 (2010).
[47] T. T. Nguyen, H. A. Nguyen, S. L. Arreola, G. Mlynek, K. Djinović-Carugo, G. Mathiesen, T. H. Nguyen, and D. Haltrich, *J. Agric. Food Chem.,* 60, 1713-1721 (2012).
[48] B. A. Black, V. S. Y. Lee, Y. Y. Zhao, Y. Hu, J. M. Curtis, and M. G. Ganzle, *J. Agric. Food Chem.,* 60, 4886-4894 (2012).
[49] G. X. Liu, J. Kong, W. W. Lu, W. T. Kong, H. Tian, X. Y. Tian, and G. C. Huo, *J. Dairy Sci.,* 94, 5811-5820 (2011).
[50] C. Nie, B. Liu, Y. Zhang, G. Zhao, X. Fan, X. Ning, and W. Zhang, *Protein Expr. Purif.,* 92, 88-93 (2013).
[51] M. N. Hung, Z. Xia, N. T. Hu, and B. H. Lee, *Appl. Environ. Microbiol.,* 67, 4256-4263 (2001).
[52] T. Goulas, A. Goulas, G. Tzortzis, and G. R. Gibson, *Appl. Microbiol. Biotechnol.,* 82, 1079-1088 (2009).

[53] K. Kawabata, Y. Sugiyama, T. Sakano, and H. Ohigashi, *Biofactors,* 39, 422-429 (2013).
[54] B. A. Rabiu, A. J. Jay, G. R. Gibson, and R. A. Rastall, *Appl. Environ. Microbiol.,* 67, 2526-2530 (2001).
[55] S. H. Yi, I. Alli, K. H. Park, and B. Lee, *New Biotechnol.,* 28, 806-813 (2011).
[56] F. Depeint, G. Tzortzis, J. Vulevic, K. I'Anson, and G. R. Gibson, *Am. J. Clin. Nutr.,* 87, 785-791 (2008).
[57] D. P. M. Torres, M. d. P. F. Gonçalves, J. A. Teixeira, and L. R. Rodrigues, *Compr. Rev. Food Sci. Food Safety,* 9, 438-454 (2010).
[58] T. Hansson, T. Kaper, J. D. Van Oost, W. M. De Vos, and P. Adlercreutz, *Biotechnol. Bioeng.,* 73, 203-210 (2001).
[59] J. B. Moore, P. Markiewicz, and J. H. Miller, *Gene,* 147, 101-106 (1994).
[60] W. Chen, H. Chen, Y. Xia, J. Zhao, F. Tian, and H. Zhang, *J. Dairy Sci.,* 91, 1751-1758 (2008).
[61] B. Di Lauro, A. Strazzulli, G. Perugino, F. La Cara, E. Bedini, M. M. Corsaro, M. Rossi, and M. Moracci, *Biochim. Biophys. Acta,* 1784, 292-301 (2008).
[62] E. S. Nam, M. S. Kim, H. B. Lee, and J. K. Ahn, *Prikl. Biokhim. Mikrobiol.,* 46, 562-571 (2010).
[63] J. Marín-Navarro, D. Talens-Perales, A. Oude-Vrielink, F. J. Cañada, and J. Polaina, *World J. Microbiol. Biotechnol.,* (2013), doi 10.1007/s11274-013-1517-8.
[64] D. O. Otieno, *Compr. Rev. Food Sci. Food Safety,* 9, 471-482 (2010).
[65] K. K. Pulicherla, P. S. Kumar, K. Manideep, V. P. B. Rekha, M. Ghosh, and K. R. S. Sambasiva Rao, *Prep. Biochem. Biotechnol.,* 43, 766-780 (2013).
[66] A. Hoyoux, I. Jennes, P. Dubois, S. Genicot, F. Dubail, J. M. François, E. Baise, G. Feller, and C. Gerday, *Appl. Environ. Microbiol.,* 67, 1529-1535 (2001).
[67] J. M. Hu, H. Li, L. X. Cao, P. C. Wu, C. T. Zhang, S. L. Sang, X. Y. Zhang, M. J. Chen, J. Q. Lu, and Y. H. Liu, *J. Agric. Food Chem.,* 55, 2217-2224 (2007).
[68] T. Nakagawa, R. Ikehata, T. Myoda, T. Miyaji, and N. Tomizuka, *Protein Expr. Purif.,* 54, 295-299 (2007).
[69] P. Hildebrandt, M. Wanarska, and J. Kur, *BMC Microbiol.,* 9, 151 (2009).
[70] M. Schmidt and P. Stougaard, *Environ. Technol.,* 31, 1107-1114 (2010).

[71] A. Wierzbicka-Woś, H. Cieśliński, M. Wanarska, K. Kozłowska-Tylingo, P. Hildebrandt, and J. Kur, *Microbial cell factories,* 10, 108 (2011).
[72] R. Karan, M. D. Capes, P. DasSarma, and S. DasSarma, *BMC Biotechnol.,* 13, 3 (2013).
[73] V. Vincent, N. Aghajari, N. Pollet, A. Boisson, S. Boudebbouze, R. Haser, E. Maguin, and M. Rhimi, *Antonie van Leeuwenhoek,* 103, 701-712 (2012).
[74] N. G. Asp, A. Burvall, and A. Dahlqvist, *Food Chem.,* 5, 147-153 (1980).
[75] T. Toba, A. Yokota, and S. Adachi, *Food Chem.,* 16, 147-162 (1985).
[76] S. Yanahira, T. Kobayashi, T. Suguri, M. Nakakoshi, S. Miura, H. Ishikawa, and I. Nakajima, *Biosci. Biotechnol. Biochem.,* 59, 1021-1026. (1995).
[77] A. R. Park and D. K. Oh, *Appl. Microbiol. Biotechnol.,* 85, 1279-1286 (2010).
[78] N. Albayrak and S. T. Yang, *Biotechnol. Bioeng.,* 77, 8-19. (2002).
[79] K.-i. Iwasaki, M. Nakajima, and S.-i. Nakao, *Process Biochem.,* 31, 69-76 (1996).
[80] H. J. Shin, J. M. Park, and J. W. Yang, *Process Biochem.,* 33, 787-792 (1998).
[81] K. Isobe, M. Yamashita, S. Chiba, N. Takahashi, and T. Koyama, *J. Biosci. Bioeng.,* 116, 293-297 (2013).
[82] M.-N. Hung and B. H. Lee, *Appl. Microbiol. Biotechnol.,* 58, 439-445 (2002).
[83] P. L. Moller, F. Jorgensen, O. C. Hansen, S. M. Madsen, and P. Stougaard, *Appl. Environ. Microbiol.,* 67, 2276-2283. (2001).
[84] K. M. J. Van Laere, T. Abee, H. A. Schols, G. Beldman, and A. G. J. Voragen, *Appl. Environ. Microbiol.,* 66, 1379-1384 (2000).
[85] S. W. A. Hinz, L. A. M. Van Den Broek, G. Beldman, J. P. Vincken, and A. G. J. Voragen, *Appl. Microbiol. Biotechnol.,* 66, 276-284 (2004).
[86] T. K. Goulas, A. K. Goulas, G. Tzortzis, and G. R. Gibson, *Appl. Microbiol. Biotechnol.,* 76, 1365-1372 (2007).
[87] E. M. Hébert, R. R. Raya, P. Tailliez, and G. S. De Giori, *Int. J. Food Microbiol.,* 59, 19-27 (2000).
[88] A. C. Adam, M. Rubio-Texeira, and J. Polaina, *Crit. Rev. Food Sci. Nutr.,* 44, 553-557 (2004).
[89] B. Splechtna, T. H. Nguyen, and D. Haltrich, *J. Agric. Food Chem.,* 55, 6772-6777 (2007).
[90] B. Splechtna, T. H. Nguyen, R. Zehetner, H. P. Lettner, W. Lorenz, and D. Haltrich, *Biotechnol. J.,* 2, 480-485 (2007).

[91] F. Jørgensen, O. C. Hansen, and P. Stougaard, *Appl. Microbiol. Biotechnol.,* 57, 647-652 (2001).
[92] G. Placier, H. Watzlawick, C. Rabiller, and R. Mattes, *Appl. Environ. Microbiol.,* 75, 6312-6321 (2009).
[93] Y. Wu, S. Yuan, S. Chen, D. Wu, J. Chen, and J. Wu, *Food Chem.,* 138, 1588-1595 (2013).
[94] J. Seibel and K. Buchholz, in H. Derek (ed.), *Advances in Carbohydrate Chemistry and Biochemistry*, Academic Press, pp. 101-138 (2010).
[95] L. Coulier, J. Timmermans, B. Richard, R. Van Den Dool, I. Haaksman, B. Klarenbeek, T. Slaghek, and W. Van Dongen, *J. Agric. Food Chem.,* 57, 8488-8495 (2009).
[96] M. J. Playne, Crittenden, R. G., in P. L. H. McSweeney, Fox, P. F. (eds.), Lactose, water, salts and minor constitutents, Springer, New York, pp. 121-201 (2009).
[97] G. Tzortzis and J. Vulevic, in D. Charalampopoulos and R. Rastall (eds.), Prebiotics and Probiotics Science and Technology, Springer New York, pp. 207-244 (2009).
[98] B. P. Lamsal, *J. Sci. Food. Agric.,* 92, 2020-2028 (2012).
[99] S. Patel and A. Goyal, *World J. Microbiol. Biotechnol.,* 27, 1119-1128 (2011).
[100] X. Qiang, C. YongLie, and W. QianBing, *Carbohydr. Polym.,* 77, 435-441 (2009).
[101] H. Kaplan and R. W. Hutkins, *Appl. Environ. Microbiol.,* 66, 2682-2684 (2000).
[102] J. Huebner, R. L. Wehling, and R. W. Hutkins, *Int. Dairy J.,* 17, 770-775 (2007).
[103] R. Palframan, G. R. Gibson, and R. A. Rastall, *Lett. Appl. Microbiol.,* 37, 281-2814 (2003).
[104] C. E. Rycroft, M. R. Jones, G. R. Gibson, and R. A. Rastall, *J. Appl. Microbiol.,* 91, 878-887. (2001).
[105] J. Vulevic, R. A. Rastall, and G. R. Gibson, *FEMS Microbiol. Lett.,* 236, 153-159 (2004).
[106] A. Cardelle-Cobas, M. Fernández, N. Salazar, C. Martínez-Villaluenga, M. Villamiel, P. Ruas-Madiedo, and C. G. De Los Reyes-Gavilán, *J. Dairy Res.,* 76, 317-325 (2009).
[107] A. Cardelle-Cobas, A. Olano, N. Corzo, M. Villamiel, M. Collins, S. Kolida, and R. A. Rastall, *J. Agric. Food Chem.,* 60, 2024-2032 (2012).
[108] A. J. H. Maathuis, E. G. van den Heuvel, M. H. C. Schoterman, and K. Venema, *J. Nutr.,* 142, 1205-1212 (2012).
[109] A. Cardelle-Cobas, N. Corzo, A. Olano, C. Peláez, T. Requena, and M. Ávila, *Int. J. Food Microbiol.,* 149, 81-87 (2011).

[110] P. M. Sherman, M. Cabana, G. R. Gibson, B. V. Koletzko, J. Neu, G. Veereman-Wauters, E. E. Ziegler, and W. A. Walker, *J. Pediatr.,* 155, S61-70 (2009).
[111] L. M. G. Davis, I. Martínez, J. Walter, and R. Hutkins, *Int. J. Food Microbiol.,* 144, 285-292 (2010).
[112] E. Bruzzese, M. Volpicelli, V. Squeglia, D. Bruzzese, F. Salvini, M. Bisceglia, P. Lionetti, M. Cinquetti, G. Iacono, S. Amarri, and A. Guarino, *Clin. Nutr.,* 28, 156-161 (2009).
[113] K. Kukkonen, E. Savilahti, T. Haahtela, K. Juntunen-Backman, R. Korpela, T. Poussa, T. Tuure, and M. Kuitunen, *J. Allergy Clin. Immunol.,* 119, 192-198 (2007).
[114] R. Luoto, O. Ruuskanen, M. Waris, M. Kalliomaki, S. Salminen, and E. Isolauri, *J. Allergy Clin. Immunol.,* (2013) doi:10.1016/j.jaci.2013. 08.020.
[115] H. R. Sinclair, J. de Slegte, G. R. Gibson, and R. A. Rastall, *J. Agric. Food Chem.,* 57, 3113-3119 (2009).
[116] Y. Zhong, D. Cai, W. Cai, S. Geng, L. Chen, and T. Han, *Clin. Nutr.,* 28, 575-580 (2009).
[117] K. Shoaf, G. L. Mulvey, G. D. Armstrong, and R. W. Hutkins, *Infect. Immun.,* 74, 6920-6928 (2006).
[118] L. E. Searle, W. A. Cooley, G. Jones, A. Nunez, B. Crudgington, U. Weyer, A. H. Dugdale, G. Tzortzis, J. W. Collins, M. J. Woodward, and R. M. La Ragione, *J. Med. Microbiol.,* 59, 1428-1439 (2010).
[119] J. M. Laparra, O. Hernandez-Hernandez, F. J. Moreno, and Y. Sanz, *Food Res. Int.,* 54, 1096-1102 (2013).
[120] B. van't Land, M. Schijf, B. C. van Esch, J. van Bergenhenegouwen, J. Bastiaans, B. Schouten, L. Boon, and J. Garssen, *Vaccine,* 28, 5711-5717 (2010).
[121] A. Gopalakrishnan, J. F. Clinthorne, E. A. Rondini, S. J. McCaskey, E. A. Gurzell, I. M. Langohr, E. M. Gardner, and J. I. Fenton, *J. Nutr.,* 142, 1336-1342 (2012).
[122] N. van Vlies, A. Hogenkamp, S. Thijssen, G. M. Dingjan, K. Knipping, J. Garssen, and L. M. J. Knippels, *J. Reprod. Immunol.,* 94, 161-168 (2012).
[123] E. van Hoffen, B. Ruiter, J. Faber, L. M'Rabet, E. F. Knol, B. Stahl, S. Arslanoglu, G. Moro, G. Boehm, and J. Garssen, *Allergy,* 64, 484-487 (2009).
[124] J. H. Cummings, G. T. Macfarlane, and H. N. Englyst, *Am. J. Clin. Nutr.,* 73, 415S-420S (2001).
[125] D. L. Topping and P. M. Clifton, *Physiol. Rev.,* 81, 1031-1064 (2001).
[126] Y. Tang, Y. Chen, H. Jiang, and D. Nie, *Cell Death Differ.,* 18, 602-618 (2011).

[127] M. Blaut and T. Clavel, *J. Nutr.,* 137, 751S-755S (2007).
[128] M. J. Hopkins and G. T. Macfarlane, *Appl. Environ. Microbiol.,* 69, 1920-1927 (2003).
[129] A. Cardelle-Cobas, M. Fernandez, N. Salazar, C. Martinez-Villaluenga, M. Villamiel, P. Ruas-Madiedo, and C. G. de los Reyes-Gavilan, *J. Dairy Res.,* 76, 317-325 (2009).
[130] C. M. Weaver, B. R. Martin, C. H. Nakatsu, A. P. Armstrong, A. Clavijo, L. D. McCabe, G. P. McCabe, S. Duignan, M. H. C. Schoterman, and E. G. H. M. van den Heuvel, *J. Agric. Food Chem.,* 59, 6501-6510 (2011).
[131] E. F. dos Santos, K. H. Tsuboi, M. R. Araujo, N. A. Andreollo, and C. K. Miyasaka, *Rev. Col. Bras. Cir.,* 38, 186-191 (2011).
[132] C. M. Whisner, B. R. Martin, M. H. C. Schoterman, C. H. Nakatsu, L. D. McCabe, G. P. McCabe, M. E. Wastney, E. G. H. M. van den Heuvel, and C. M. Weaver, *Br. J. Nutr.,* 110, 1292-1303 (2013).
[133] L. Esterle, J. P. Sabatier, F. Guillon-Metz, O. Walrant-Debray, G. Guaydier-Souquieres, F. Jehan, and M. Garabedian, *Osteoporos. Int.,* 20, 567-575 (2009).
[134] J. Vulevic, A. Drakoularakou, P. Yaqoob, G. Tzortzis, and G. R. Gibson, *Am. J. Clin. Nutr.,* 88, 1438-1446 (2008).
[135] P. Alliet, P. Scholtens, M. Raes, K. Hensen, H. Jongen, J.-L. Rummens, G. Boehm, and Y. Vandenplas, *Nutrition,* 23, 719-723 (2007).
[136] H. M. Savignac, G. Corona, H. Mills, L. Chen, J. P. E. Spencer, G. Tzortzis, and P. W. J. Burnet, *Neurochem. Int.,* 63, 756-764 (2013).
[137] L. Song, Y. Gao, X. Zhang, and W. Le, *Neuroscience,* 246, 281-290 (2013).
[138] L. Bode, *Nutr. Rev.,* 67, S183-191 (2009).
[139] M. R. Ninonuevo, Y. Park, H. Yin, J. Zhang, R. E. Ward, B. H. Clowers, J. B. German, S. L. Freeman, K. Killeen, R. Grimm, and C. B. Lebrilla, *J. Agric. Food Chem.,* 54, 7471-7480 (2006).
[140] A. Marcobal, M. Barboza, J. W. Froehlich, D. E. Block, J. B. German, C. B. Lebrilla, and D. A. Mills, *J. Agric. Food Chem.,* 58, 5334-5340 (2010).
[141] R. G. LoCascio, M. R. Ninonuevo, S. L. Freeman, D. A. Sela, R. Grimm, C. B. Lebrilla, D. A. Mills, and J. B. German, *J. Agric. Food Chem.,* 55, 8914-8919 (2007).
[142] R. E. Ward, M. Niñonuevo, D. A. Mills, C. B. Lebrilla, and J. B. German, *Mol. Nutr. Food Res.,* 51, 1398-1405 (2007).
[143] D. S. Newburg, G. M. Ruiz-Palacios, and A. L. Morrow, *Annu. Rev. Nutr.,* 25, 37-58 (2005).

[144] G. M. Ruiz-Palacios, L. E. Cervantes, P. Ramos, B. Chavez-Munguia, and D. S. Newburg, *J. Biol. Chem.,* 278, 14112-14120 (2003).
[145] A. Cravioto, A. Tello, H. Villafan, J. Ruiz, S. Del Vedovo, and J. R. Neeser, *J. Infect. Dis.,* 163, 1247-1255 (1991).
[146] A. Kobata, *Proc. Jpn. Acad. Ser. B. Phys. Biol. Sci.,* 86, 731-747 (2010).
[147] C. Kunz, S. Rudloff, W. Baier, N. Klein, and S. Strobel, *Annu. Rev. Nutr.,* 20, 699-722 (2000).
[148] S. Wu, R. Grimm, J. B. German, and C. B. Lebrilla, *J. Proteome Res.,* 10, 856-868 (2011).
[149] S. Wu, N. Tao, J. B. German, R. Grimm, and C. B. Lebrilla, *J. Proteome Res.,* 9, 4138-4151 (2010).
[150] S. Reuter, A. Rusborg Nygaard, and W. Zimmermann, *Enzyme Microb. Technol.,* 25, 509-516 (1999).
[151] W. Li, Y. Sun, H. Ye, and X. Zeng, *Eur. Food Res. Technol.,* 231, 55-63 (2010).
[152] N. Bridiau and T. Maugard, *Biotechnol. Prog.,* 27, 386-394 (2011).
[153] S. A. Biocon Española, 2013 at http://www.biocon.es/en/productos-documentos.php?id=36.
[154] B. Rodriguez-Colinas, L. Fernandez-Arrojo, A. O. Ballesteros, and F. J. Plou, *Food Chem.,* 145, 388-394 (2014).
[155] Amano Enzyme Japan, 2007 at http://www.amano-enzyme.co.jp/eng/enzyme/23.html
[156] T. Palai, S. Mitra, and P. K. Bhattacharya, *J. Biosci. Bioeng.,* 114, 418-423 (2012).
[157] DSM Food Specialties, 2012 at http://www.dsm.com/le/en_US/maxilact/html/lactase.htm
[158] P. S. Panesar, S. Kumari, and R. Panesar, *Enzyme Res.,* Article ID 473137 (2010).
[159] Yakult Pharmaceutical Industry, at http://www.yakult.co.jp/ypi/en/product/ori.html
[160] US Food and Drug Administration, 2010 at http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=334
[161] Friesland Foods Domo, at http://vivinalgos.com/index.php?id=1
[162] US Food and Drug Administration, 2007 at http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=236
[163] GTC Nutrition, at http://www.innovadex.com/Food/Detail/1698/101070/PURIMUNE

[164] US Food and Drug Administration, 2009 at http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=286

[165] Clasado, at http://www.bimuno.com/

[166] EFSA Panel on Dietetic Products Nutrition and Allergies (NDA), *EFSA J.,* 11, 3259 (2013).

[167] R. Hartemink, K. M. Van Laere, C. C. Peeters, M. J. Nout, and F. M. Rombouts, *Lett. Appl. Microbiol.,* 25, 38-42 (1997).