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Production of Prebiotic Galacto-Oligosaccharides from Acid Whey Catalyzed by a Novel β-Galactosidase from *Thermothielavioides terrestris* and Commercial Lactases: A Comparative Study

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Abstract: The steadily increasing global popularity of Greek strained yoghurt has necessitated alternative valorization approaches for acid whey, the major straining process effluent. In this context, prebiotic galacto-oligosaccharides can be enzymatically synthesized from acid whey lactose, via either commercial or novel β-galactosidases. A comparative study of galacto-oligosaccharide production from acid whey was carried out, employing two commercial β-galactosidases (from *Kluyveromyces lactis* and *Aspergillus oryzae*) and one novel, in-house produced (from *Thermothielavioides terrestris*), as a function of the initial lactose content and enzyme load. Selected reaction conditions for β-galactosidases from *K. lactis*, *A. oryzae*, and *T. terrestris* were 35 °C at pH 7.2, 45 °C at pH 4.5, and 50 °C at pH 4.0, respectively. Maximum galacto-oligosaccharide yields equal to 23.7, 23.4, and 25.7% were achieved with, respectively, 0.13 U/mL of *K. lactis* β-galactosidase in non-concentrated acid whey, 4 U/mL of *A. oryzae* β-galactosidase, and 8 U/mL of *T. terrestris* β-galactosidase in acid whey concentrated to 20% *w*/*v* initial lactose content. The increased galacto-oligosaccharide productivity of the thermophilic β-galactosidase from *T. terrestris* can be a determining asset in a combined concentration and oligomerization industrial process. This will allow for high galacto-oligosaccharide yields for efficient, cost-effective production of valuable prebiotics from acid whey.

Keywords: whey lactose; biocatalysts; transgalactosylation; yoghurt by-products; valorization

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1. Introduction

Greek strained yoghurt has gained great popularity worldwide over the past two decades, becoming an exemplary success story in the global dairy market [1]. Acid whey is the liquid by-product separated during the straining process of Greek yoghurt production. It is a yellowish liquid containing mainly lactose (3.3–3.5% *w*/*v*), galactose (0.56–0.65% *w*/*v*), lactic acid (0.42–0.53% *w*/*v*), whey proteins (1.71–3.71 mg/g), minerals (0.67–0.75% *w*/*w*), and lipids depending on the fat content of the milk used for the yoghurt production [2]. For each kg of Greek yoghurt produced, 2 kg of acid whey are discharged [1]. Due to the rapid growth of the global Greek yoghurt market over the past decades, acid whey management problems have emerged. With 771,000 tn of Greek yoghurt produced in the USA in 2015, eliminating over 2,000,000 tn of acid whey [3] and 8.1% compound annual

growth rate of the global Greek yoghurt market between 2017 and 2021, estimated to reach 11.1% by 2032 [4], the prodigious amounts of acid whey discharged annually from dairy industries require special treatment in order to be discarded in the environment or incorporated in the food or feed chain. Several methods have been proposed for acid whey utilization, but all exhibit problems in their implementation. Spraying of acid whey over fields as a fertilizer causes emission of intense odors and a high biological load related to eutrophication [5]. Incorporation of acid whey in dairy animal feed is ineffective due to the inability of adult animals to metabolize lactose. Finally, the conversion of acid whey into biofuels is restricted by the high cost of the required processes compared to the value of the final product [1]. Additionally, the high amounts of biochemical oxygen demand (52,400–64,400 ppm) and chemical oxygen demand (45,800–50,500 ppm) of acid whey, in combination with its low pH value (4.21–4.48) due to the high concentration of lactic acid [2], prohibit its discharge into the environment without any pretreatment. Its management in conventional biological treatment plants is a poor option because it results in the overload of such systems [6]. Consequently, the quest for alternative methods of acid whey exploitation that are sustainable and bring commercial potential to this problematic yet valuable by-product is essential for the dairy industry.

The production of prebiotic galacto-oligosaccharides (GOSs) via the biocatalytic oligomerization of lactose has gained great scientific interest recently [7]. Although GOSs can be chemically synthesized from monosaccharides via a reaction called "reversion", the preferable method for GOS production is enzymatic synthesis with the application of a β-galactosidase, due to its higher yields and product specificity [8]. The β-galactosidase is a well-established enzyme in the dairy industry, utilized to hydrolyze milk lactose into its monomers, glucose and galactose, to produce low-lactose or lactose-free dairy products [9]. The GOS synthesis is a side reaction of lactose hydrolysis, called transgalactosylation. The operation mechanism of β-galactosidase consists of two steps. In the first step, the β-galactosidase attacks the anomeric center of the galactose monomer of the lactose molecule and creates a galactosyl–enzyme complex, while releasing the glucose monomer. The second step relies on the acceptor substrate. If the acceptor is water, then the galactosyl–enzyme complex releases the galactose monomer, resulting in a hydrolysis reaction. If the acceptor is lactose, a monosaccharide, or an oligosaccharide, then a galacto-oligosaccharide is formed as a result of the transgalactosylation reaction. Subsequently, the GOS molecule can serve either as an acceptor substrate to produce a GOS with a higher degree of polymerization, or as a donor, resulting in the degradation of the oligosaccharide. The type of bonds between the monomers and the degree of polymerization of GOS molecules formed via the transgalactosylation reaction are related to the specific enzyme and the reaction conditions, mainly the initial lactose content and enzyme source. On the onset of the catalysis, the high lactose content shifts the reaction towards transgalactosylation. With lactose depletion, the hydrolysis reaction is favored and the formed GOSs tend to disintegrate [7,10–12]. Hence, the enzymatic production of GOS from lactose is a kinetically controlled reaction, comprising the competitive reactions of hydrolysis and transgalactosylation. As such, having a grasp of how the reaction unfolds over time becomes crucial for pinpointing the optimal juncture of highest GOS yield.

Galacto-oligosaccharides have significant prebiotic properties [13]. They improve gut health, promoting the growth of the probiotic intestinal bacteria population [11]. Specifically, GOSs assist bifidobacteria and lactobacilli proliferation, contributing to several health benefits, such as an increase in mineral absorption, the inhibition of pathogens and the enhancement of the immune system [14]. The enzymatic production of GOSs directly from acid whey can contribute to an increase in acid whey's value, with a significant environmental impact. If such a process is applied in a dairy industry producing Greek strained yoghurt, it can contribute to a significant reduction in the amount of acid whey being discharged and to the production of another product with high nutritional value that can either be incorporated into current dairy products of the producing industry to boost their nutritional appeal or be sold as a food ingredient. Additionally, this process

comes with practically no side streams because acid whey is enzymatically treated with β-galactosidases and the whole amount of the reaction mixture, after inactivation of the enzyme, is further concentrated into a high GOS syrup or dried to become a food ingredient. The conversion of a by-product of minimal value into a product rich in GOSs with high nutritional and commercial value via a process with zero effluents is compatible with the circular economy drive.

Until recently, transgalactosylation reaction has been studied using pure lactose as a substrate and commercial β-galactosidases from various sources, like *Aspergillus oryzae* [15], *Kluyveromyces marxianus* [16], *Bacillus circulans*, and *Kluyveromyces lactis* [11]. Others have studied GOS production from pure lactose using recombinant β-galactosidases with desired properties, such as the thermophilic lactases from *Pantoea anthophila* [17], *Thermus thermophilus*, and *Pyrococcus furiosus* [18]. Little research has been published so far regarding the valorization of acid whey towards GOS production. Fischer and Kleinschmidt (2015) studied the application of two commercial enzymes from *Aspergillus oryzae* and *Kluyveromyces lactis* in sweet and acid whey and found significant potential for GOS production, with GOS yields up to 33.45% of total sugars [19]. Additionally, Fischer and Kleinschmidt (2021) investigated GOS synthesis from acid whey via the use of whole cells from *Cryptococcus laurentii* and demonstrated GOS yields up to 34.6% [20]. Finally, in our previous research we studied a novel thermophile β-galactosidase from *Thermothielavioides terrestris* exhibiting optimum activity at 50 °C and pH value of 4.0 and demonstrated the potential of GOS production from pure lactose and acid whey [21].

The aim of this research was the comparative investigation of GOS production from Greek strained yoghurt acid whey via the application of one novel, in-house produced thermophilic β-galactosidase from *Thermothielavioides terrestris* and two industrially applied β-galactosidases from *Aspergillus oryzae* and *Kluyveromyces lactis* in relation to the initial lactose content of acid whey and the enzyme load, at the optimum temperature and pH value for each biocatalyst, to determine the conditions that maximize GOS yield.

2. Results and Discussion

2.1. Acid Whey Characterization

The composition of acid whey utilized in the present research is presented in Table 1. The average concentration of total sugars in acid whey was 38.72 ± 2.81 g/L, consisting of 31.18 ± 2.34 g/L lactose, 7.42 ± 1.56 g/L galactose, and 0.12 ± 0.09 g/L glucose. These results are typical for liquid acid whey and are within the sugar concentration range mentioned in the literature [2,19,20]. Total protein concentration of acid whey was equal to 2.752 ± 0.421 g/L, as determined by the Kjeldahl method. Acid whey typically contains low amounts of whey proteins because most of the proteins remain in the final product during the production process of Greek strained yoghurt [2,22]. The acid whey used in the present study was discharged during the production of defatted Greek strained yoghurt; thus, its fat content was practically zero. Lactic acid concentration in acid whey was equal to 1.37 ± 0.05 g/L, giving it a pH value equal to 4.4 ± 0.2. Moreover, acid whey contained a wide range of metal cations, mainly K^+ (1131 ± 38 mg/L), Na^+ (343.2 ± 50.4 mg/L), Ca^{2+} (1175 ± 28 mg/L), and Mg^{2+} (29.26 ± 6.20 mg/L). Knowledge of the exact composition of acid whey is important when studying the enzymatic conversion of acid whey lactose into GOSs, since the activity of some enzymes catalyzing the transgalactosylation reaction is affected by acid whey components, mainly cations [19].

2.2. Characterization of Studied Biocatalysts

The relative enzyme activity of β-galactosidases from *K. lactis*, *A. oryzae*, and *T. terrestris* over pH and temperature is presented in Figure 1. Optimum enzyme activity of β-galactosidase from *K. lactis* was observed at a pH value of 7.2. As for β-galactosidase from *A. oryzae*, optimum enzyme activity was measured at a pH value of 4.5. Enzyme activity at a pH value equal to 5.0 was statistically not different ($p > 0.05$), but a pH value of 4.5 was considered as optimum because it coincides with the pH value of acid whey. Additionally,

according to our previous study [21], the optimum pH value of β-galactosidase from *T. terrestris* is 4.0. The optimum enzyme activity of β-galactosidase from *K. lactis* was measured at 35 °C. At this temperature, more that 96% of the enzyme activity remained after 10 h of incubation. The enzyme activity of β-galactosidase from *A. oryzae* was constantly increasing with the temperature, up to 55 °C. However, the optimum temperature for this biocatalyst was 45 °C because at higher temperatures the enzyme was not stable during the 10-h incubation, losing 20.3% and 42.8% of its activity after 10 h at 50 °C and 1 h at 55 °C, respectively (Figure 2). Moreover, in our previous study we observed optimum enzyme activity of β-galactosidase from *T. terrestris* at 50 °C [21].

Table 1. Composition of acid whey.

Component	Concentration (g/L)	Component	Concentration (mg/L)
Total sugars	38.72 ± 2.81	K^+	1131 ± 38
Lactose	31.18 ± 2.34	Na^+	343.2 ± 50.4
Galactose	7.42 ± 1.56	Ca^{2+}	1175 ± 28
Glucose	0.12 ± 0.09	Mg^{2+}	29.26 ± 6.20
Total proteins	2.752 ± 0.421	Mn^{2+}	0.019 ± 0.003
Fat	0.719 ± 0.120	Zn^{2+}	1.746 ± 0.121
Lactic acid	1.37 ± 0.05	Cu^{2+}	<0.01
Solid residue	53.75 ± 2.50	SO_4^{2-}	65.4 ± 0.6
Ash	8.964 ± 0.198		

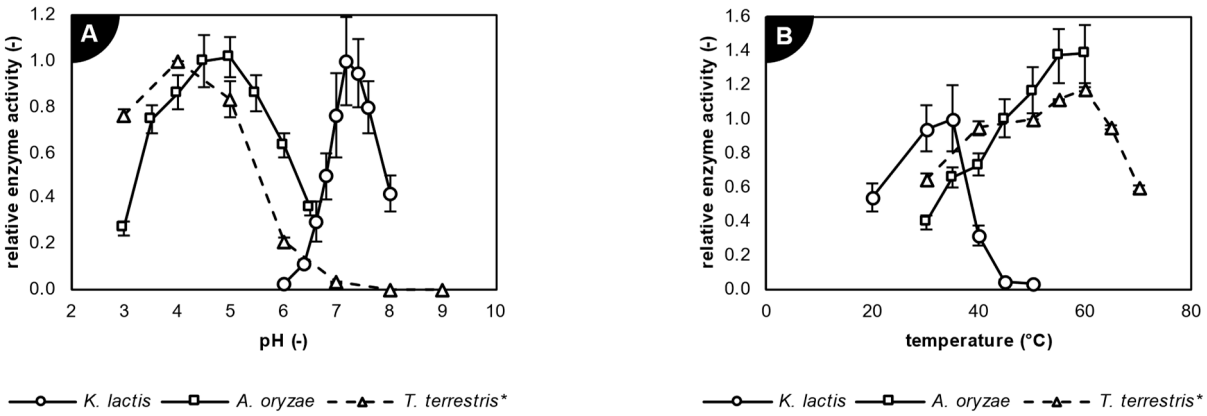

Figure 1. Effect of (**A**) pH and (**B**) temperature on enzyme activity of β-galactosidases from *Kluyveromyces lactis*, *Aspergillus oryzae*, and *Thermothielavioides terrestris*. * Data from our previous study [21] for comparative purposes.

The optimum activity conditions of β-galactosidases from *A. oryzae* and *K. lactis* are important for any bioprocess involving lactases, such as the production of GOSs from lactose, and have been reported by other researchers. Tanaka et al. (1975) found that β-galactosidase from *A. oryzae* acts optimally at 46 °C and at a pH value equal to 4.5 [23], values that are within the range mentioned by Tanriseven and Dogan (2002) [24] and this study. In contrast, Fischer and Kleinschmidt (2015) and Vera et al. (2011) reported higher temperatures (55–60 °C) as optimal for β-galactosidase from *A. oryzae* [19,25]. Fujimura et al. (2003) and Dickson et al. (1979) reported that the optimum conditions of β-galactosidase from *K. lactis* were measured at 35 °C and at a pH value equal to 7.0 [26,27], which are in accordance with the findings of this study.

The effect of the studied metal cations on the enzyme activity of β-galactosidases from *K. lactis* and *A. oryzae* is presented in Table 2. Enzyme activity of β-galactosidase from *K. lactis* is strongly enhanced by the presence of K^+, Na^+, and Ca^{2+}, which are the main metal cations found in acid whey, but inhibited by Mg^{2+}, Mn^{2+}, and Zn^{2+}, even at low concentrations (1 mM). As for β-galactosidase from *A. oryzae*, none of the studied

cations exhibited any significant effect on enzyme activity (remaining enzyme activity over 90%), at the optimum reaction conditions for this biocatalyst. The enzymatic activity of β-galactosidase from *T. terrestris* is not affected by any metal cation [21].

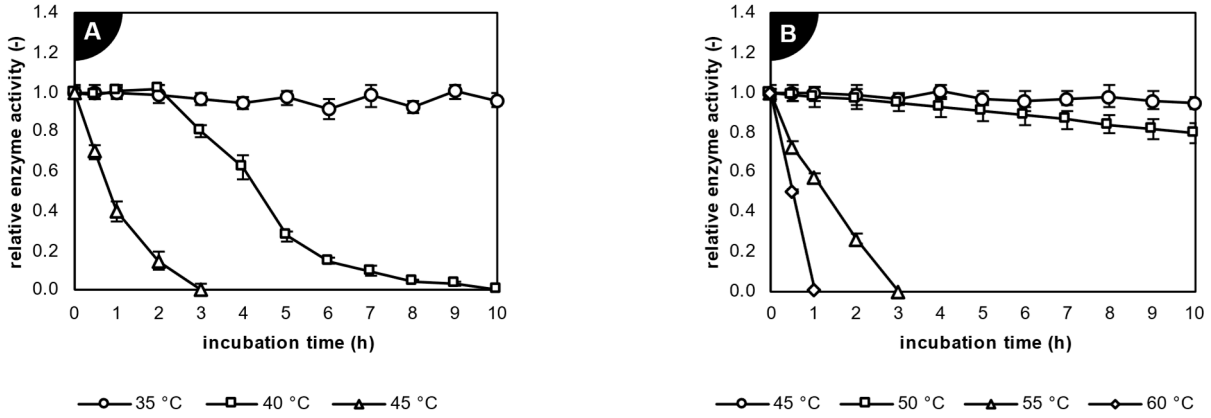

Figure 2. Remaining relative enzyme activity of β-galactosidases from (**A**) *Kluyveromyces lactis* and (**B**) *Aspergillus oryzae* over incubation time at different temperatures.

Table 2. Effect of metal cations on enzyme activity of β-galactosidases from *Kluyveromyces lactis* and *Aspergillus oryzae*.

Enzyme Source	Concentration (mM)	K^+	Na^+	Ca^{2+}	Mg^{2+}	Mn^{2+}	Zn^{2+}	Cu^{2+}
Kluyveromyces lactis	0				100 ± 9			
	1	231 ± 4	118 ± 3	183 ± 16	29 ± 3	25 ± 2	1.4 ± 0.3	n.d.
	10	393 ± 6	195 ± 9	256 ± 12	38 ± 5	n.d.	n.d.	n.d.
	100	651 ± 9	389 ± 7	n.d.	n.d.	n.d.	n.d.	n.d.
Aspergillus oryzae	0				100 ± 2			
	1	92 ± 2	99 ± 2	97 ± 3	97 ± 4	97 ± 3	104 ± 3	108 ± 2
	10	98 ± 3	98 ± 3	104 ± 7	100 ± 3	90 ± 6	103 ± 2	104 ± 3
	100	99 ± 2	101 ± 2	n.d.	101 ± 3	98 ± 10	n.d.	n.d.

n.d.: not determined.

2.3. Enzymatic Transgalactosylation of Acid Whey Lactose

2.3.1. Application of β-Galactosidase from *Kluyveromyces lactis*

Galacto-oligosaccharide yields (y_{GOS}) achieved via the application of β-galactosidase from *K. lactis* in acid whey over the reaction time, using enzyme loads of 0.06, 0.13, and 0.26 U/mL and initial lactose contents of 3.1, 10, 14, and 18% *w*/*v*, are presented in Figure 3. In all cases, GOS yields increased at the beginning of the enzymatic reactions, reached a maximum value, and consequently decreased, indicating the hydrolysis of the formed GOSs. This can be explained by the reaction mechanism described previously. Because transgalactosylation is a kinetically controlled reaction and GOSs serve both as reaction products and substrates at the two stages of the catalysis, at early reaction times GOSs are produced faster than hydrolyzed, due to the abundance of lactose serving as a substrate. As the lactose is consumed, the GOS production rate decreases and hydrolysis rate increases. At the time of the achievement of maximum GOS yield, the two rates are equalized. When the lactose is depleted, it becomes more likely that the enzyme will catalyze the hydrolysis of the glycosidic bond of a GOS molecule, rather than of the lactose; thus, the GOS concentration decreases [12].

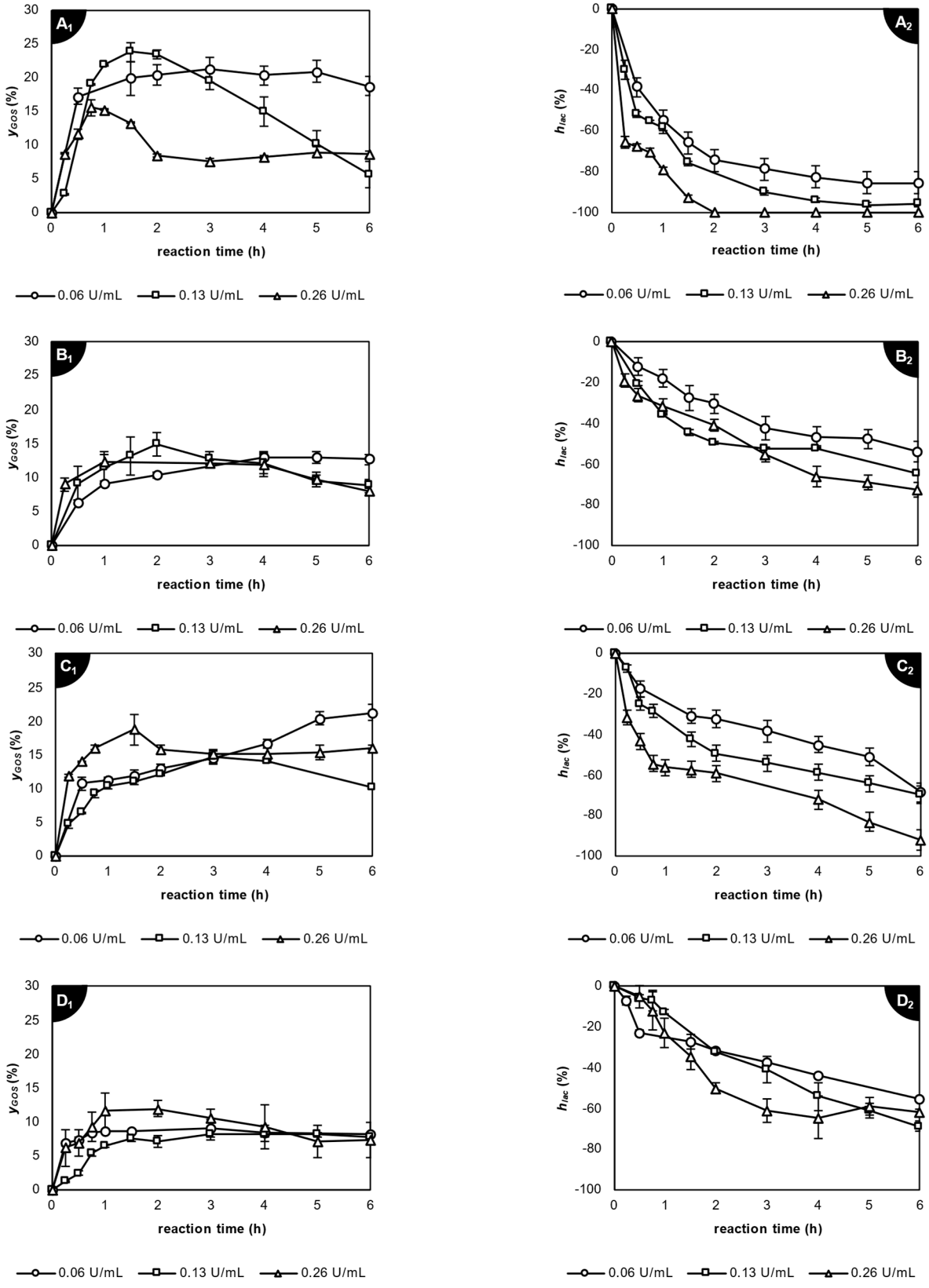

Figure 3. Galacto-oligosaccharide yields (y_{GOS}) and lactose hydrolysis degrees (h_{lac}) over time for enzymatic reactions with β-galactosidase from *Kluyveromyces lactis* in acid whey with initial lactose content of 3.1% *w*/*v* (**A**$_{\mathbf{1}}$ and **A**$_{\mathbf{2}}$, respectively), 10% *w*/*v* (**B**$_{\mathbf{1}}$ and **B**$_{\mathbf{2}}$, respectively), 14% *w*/*v* (**C**$_{\mathbf{1}}$ and **C**$_{\mathbf{2}}$, respectively), and 18% *w*/*v* (**D**$_{\mathbf{1}}$ and **D**$_{\mathbf{2}}$, respectively).

The application of β-galactosidase from *K. lactis* in non-concentrated acid whey resulted in higher GOS yields than in concentrated acid whey. Maximum GOS yield equal to 23.7 ± 1.4% was achieved with an enzyme load equal to 0.13 U/mL in non-concentrated acid whey with an initial lactose content equal to 3.1% *w*/*v*, after 1.5 h of enzymatic reaction, with an accompanying lactose hydrolysis degree of 76.0%. Higher initial lactose contents in acid whey did not result in the achievement of higher GOS yields with the application of β-galactosidase from *K. lactis*. This was not expected but can be explained by the strong effect of some cations present in acid whey, such as K^+ and Na^+, that result in an increase in enzyme activity, as described in Section 2.2. Concentrated acid whey has a higher concentration of metal ions that further increases the enzymatic activity of β-galactosidase from *K. lactis* possibly in favor of lactose hydrolysis, resulting in lower GOS yields.

From the analysis of the reaction products with high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), 14–18 carbohydrate products were identified in all enzymatic reactions with β-galactosidase from *K. lactis* in acid whey, including lactose, galactose, and glucose. The most dominant oligosaccharide produced was eluted at 35.6 min and is presumed to be 6′-*O*-β-galactosyl-lactose, according to other studies following a similar methodology for GOS identification [11,15]. The second most abundant oligosaccharide was 6-galactobiose. This reaction product was identified using a separate standard and was not included in the GOS concentration used to calculate the GOS yield because 6-galactobiose concentration changes differently over time in comparison to all other oligosaccharides. Specifically, the 6-galactobiose concentration was also increased at the early stages of the enzymatic reaction, reached a maximum value and then was hydrolyzed, especially when high enzyme loads and low initial lactose contents were used. However, in all cases, maximum 6-galactobiose concentration was reached later than all other GOSs. Consequently, 6-galactobiose yield ($y_{gal\text{-}gal}$) was selected to be presented separately. At the maximum GOS yield point (3.1% *w*/*v* initial lactose content, 0.13 U/mL enzyme load, 1.5 h of enzymatic reaction), the concentration of 6-galactobiose was equal to 0.110% *w*/*v* (3.72% of the initial lactose content). If this 6-galactobiose concentration was included in the maximum GOS yield, a y_{GOS} equal to 27.4 ± 1.5% was achieved, with 6-galactobiose constituting 13.6% of total oligosaccharides. The percentage of 6′-*O*-β-galactosyl-lactose at this reaction point was 31.5% of total oligosaccharides.

González-Delgado et al. (2016) studied the application of β-galactosidase from *K. lactis* in pure lactose for GOS production and found a maximum GOS yield equal to 12.18%, using an enzyme load of 5 U/mL at 40 °C and a pH value equal to 7, with an initial lactose content of 25% *w*/*v*, after 3 h of enzymatic reaction [28]. These results are not similar to those of the present research since the application of a much lower enzyme load (0.13 U/mL) in non-concentrated acid whey resulted in a much higher GOS yield, probably due to the effect of other components of acid whey in β-galactosidase from *K. lactis*, especially cations that make this particular lactase more active in transgalactosylation and hydrolysis reactions, as shown in the present research and as is in accordance with similar results found in the literature [19]. Yin et al. (2017) also studied the application of β-galactosidase from *K. lactis* in 30% *w*/*w* pure lactose solutions using an enzyme load of 3.75 U/g lactose (1.125 U/mL reaction mixture) and found a maximum GOS yield equal to 34.9 ± 1.8% after 6 h of enzymatic reaction at 40 °C and a pH value of 7.0, with 91.8 ± 0.8% lactose hydrolysis degree [11]. These results are in agreement with the present study. Galacto-oligosaccharide yields presented by Yin et al. (2017) are slightly higher than those achieved in the present study but were achieved in reaction solutions of pure lactose with an initial content of 30% *w*/*v*, which is 8.6 times the lactose content of non-concentrated acid whey. Moreover, they used an enzyme load of 1.125 U/mL, which is 8.6 times the enzyme load that resulted in the maximum GOS yield in the present study. Thus, the enzyme to lactose ratio is the same in both studies.

Mano et al. (2019) studied the application of β-galactosidase from *K. lactis* in whey permeate comparatively with other commercial lactases and found a maximum GOS yield equal to 25% using an enzyme load of 50 U/g in concentrated whey with an initial lactose content of 30% *w*/*v*, after 2 h of enzymatic reaction at 35 °C and a pH value equal to 7 [29]. These results are not in accordance with the present study because a similar maximum GOS yield (27.2 ± 1.5%) was achieved but in non-concentrated acid whey (3.1% *w*/*v* initial lactose content), while in concentrated acid whey much lower GOS yields were achieved (11.9 ± 1.2% for initial lactose content of 18% *w*/*v* and enzyme load of 0.26 U/mL), in the same reaction conditions (35 °C, pH = 7.2).

2.3.2. Application of β-Galactosidase from *Aspergillus oryzae*

In Figure 4, GOS yields (y_{GOS}) achieved via the application of β-galactosidase from *A. oryzae* in acid whey are shown over the reaction time, using enzyme loads of 0.5–8.0 U/mL and initial lactose contents of 3.1, 10, 15, and 20% *w*/*v*. As with β-galactosidase from *K. lactis*, the GOS yield initially increased, reached a maximum value, and consequently decreased. The GOS hydrolysis was more intense with lower initial lactose contents and higher enzyme loads, which was expected since the enzyme is more plentiful in the substrate. With a high initial lactose content, the lactose remains in abundance longer and allows for the enzyme to synthesize more GOSs, before hydrolyzing the reaction products. In non-concentrated acid whey, relatively low and statistically equal ($p > 0.05$) maximum GOS yields were achieved with all studied enzyme loads, up to 9.59 ± 0.56%. In concentrated acid whey, higher GOS yields were achieved. In acid whey with an initial lactose content of 10% *w*/*v*, statistically similar ($p > 0.05$) maximum GOS yields up to 18.5 ± 0.4% were achieved with enzyme loads of 1–4 U/mL, while a higher enzyme load of 8 U/mL resulted in a significantly lower ($p < 0.05$) maximum GOS yield (13.9 ± 0.7%). In more concentrated acid whey with an initial lactose content of 15% *w*/*v*, even higher GOS yields were achieved, with a maximum value amounting to 19.8 ± 0.9%, after 4 h of enzymatic reaction with an enzyme load of 4 U/mL. However, for higher initial lactose contents, longer reaction times were needed to achieve maximum GOS yields since the reaction is dependent on the substrate concentration.

The application of β-galactosidase from *A. oryzae* in acid whey resulted in the production of 18–21 carbohydrates, including galactose, glucose, and lactose, as identified from the HPAEC-PAD analysis. A maximum GOS yield of 23.4 ± 0.9% was achieved with an enzyme load of 4.0 U/mL in concentrated acid whey with an initial lactose content of 20% *w*/*v*, after 7 h of enzymatic reaction, with an accompanying lactose hydrolysis degree of 51.9%. The maximum GOS yield achieved with the application of β-galactosidase from *A. oryzae* in concentrated acid whey is statistically similar ($p > 0.05$) to that achieved with β-galactosidase from *K. lactis*. However, in the case of β-galactosidase from *K. lactis*, lactose hydrolysis at the time of maximum GOS yield achievement is much higher than in the case of β-galactosidase from *A. oryzae*. Nevertheless, the achievement of a similar GOS yield in pre-concentrated acid whey is advantageous for an industrial application since more GOSs are produced in less volume of acid whey. The most dominant oligosaccharide was eluted at 34.9 min and similarly to the case of *K. lactis* is presumed to be 6′-*O*-β-galactosyl-lactose, followed by 6-galactobiose eluted at 23.5 min. At the maximum GOS yield point (20% *w*/*v* initial lactose content, 4 U/mL, 7 h), the concentration of 6-galactobiose was equal to 0.966% *w*/*v* (4.78% of initial lactose content). If this 6-galactobiose concentration was included in the maximum GOS yield, a maximum y_{GOS} equal to 28.2 ± 1.1% is achieved, with 6-galactobiose constituting 17.0% of total oligosaccharides. The percentage of 6′-*O*-β-galactosyl-lactose at this reaction point was 41.9% of total oligosaccharides.

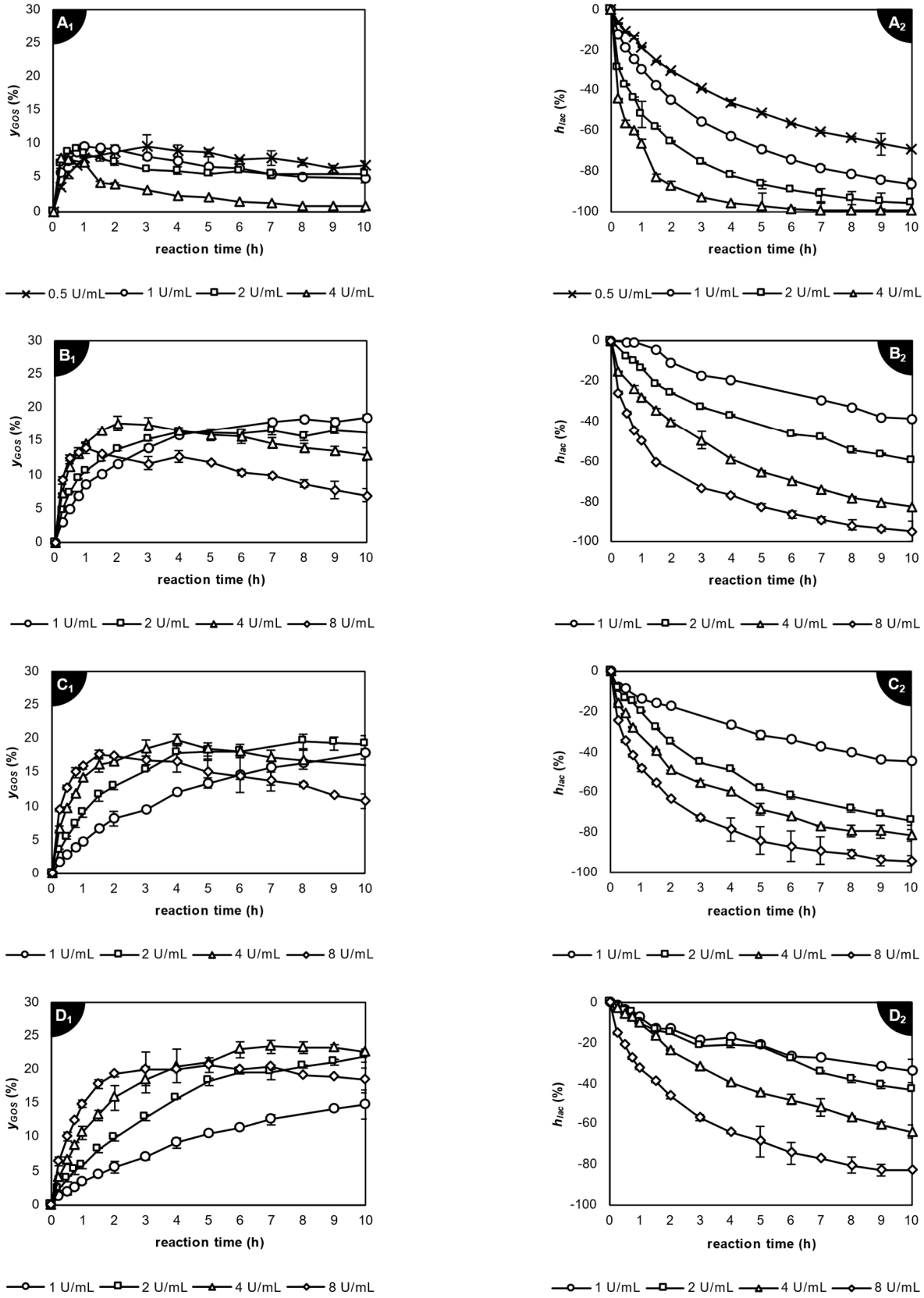

Figure 4. Galacto-oligosaccharide yields (y_{GOS}) and lactose hydrolysis degrees (h_{lac}) over time for enzymatic reactions with β-galactosidase from *Aspergillus oryzae* in acid whey with initial lactose content of 3.1% *w*/*v* (**A**$_{\mathbf{1}}$ and **A**$_{\mathbf{2}}$, respectively), 10% *w*/*v* (**B**$_{\mathbf{1}}$ and **B**$_{\mathbf{2}}$, respectively), 15% *w*/*v* (**C**$_{\mathbf{1}}$ and **C**$_{\mathbf{2}}$, respectively), and 20% *w*/*v* (**D**$_{\mathbf{1}}$ and **D**$_{\mathbf{2}}$, respectively).

Urrutia et al. (2013) applied β-galactosidase from *A. oryzae* in pure lactose solutions with 40% *w*/*v* initial lactose content using an enzyme load of 15 U/mL and found a maximum GOS yield of 26.8% *w*/*w*, with an accompanying lactose hydrolysis degree of about 70% [15]. Similarly to the present study, they identified more than 17 carbohydrates, with the predominant transgalactosylation product being 6′-*O*-β-galactosyl-lactose, amounting to almost a third of total oligosaccharides. Jenab et al. (2018) also used β-galactosidase from *A. oryzae* in pure lactose solutions and found a maximum GOS yield equal to 23% (initial lactose content 40% *w*/*v*, 50 °C, pH = 4.5) [30]. These results agree with the present study and confirm the feasibility of the application of a commercial lactase directly in acid whey for GOS production with high yields. Fischer and Kleinschmidt (2015) studied the application of β-galactosidase from *A. oryzae* comparatively in sweet and acid whey and reported a maximum GOS yield of 11.32 ± 0.59% in non-concentrated acid whey (initial lactose content 3.8% *w*/*v*, 1.9 U/mL) and 24.45 ± 0.33% in concentrated acid whey (initial lactose content 20% *w*/*v*, 10 U/mL) [19]. These results are also in accordance with the present research because similar GOS yields were achieved in both non-concentrated and concentrated acid whey, though the enzyme loads used were lower in this study.

2.3.3. Application of β-Galactosidase from *Thermothielavioides terrestris*

Galacto-oligosaccharide yields (y_{GOS}) achieved by applying the β-galactosidase from *T. terrestris* in acid whey over a reaction time, using enzyme loads of 1–16 U/mL and initial lactose contents of 3.1, 10, 15, and 20% *w*/*v*, are presented in Figure 5. Similarly to the use of the two industrial biocatalysts, the application of high enzyme loads of β-galactosidase from *T. terrestris* in acid whey with a low initial lactose content resulted in the achievement of a maximum GOS yield in the early stages of the enzymatic reaction that were quickly hydrolyzed. In the case of higher initial lactose contents, maximum GOS yields were higher and achieved at longer reaction times. Specifically, in non-concentrated acid whey (3.1% *w*/*v* initial lactose content), statistically similar ($p > 0.05$) maximum GOS yields up to 12.9 ± 0.6% were achieved for all the enzyme loads studied (1–4 U/mL), though the higher the enzyme load was, the faster the maximum GOS yield was achieved (from 4 to 1 h). The increase in initial lactose content to 10% *w*/*v* through the condensation of acid whey under vacuum prior to the enzymatic reaction resulted in an increase in the GOS yields achieved, rising up to 20.2 ± 2.4% after 3 h of enzymatic reaction, though a higher enzyme load equal to 8.0 U/mL was required. A further increase in the initial lactose content of acid whey to 15% *w*/*v* led to even higher GOS yields, up to 23.7 ± 0.4%, after 9 h of enzymatic reaction using an enzyme load of 8.0 U/mL. A maximum GOS yield of 25.7 ± 0.3% was achieved with an enzyme load equal to 8.0 U/mL in the most concentrated acid whey (20% *w*/*v* initial lactose content), after 7 h of enzymatic reaction, with an accompanying lactose hydrolysis degree of 48.8%. This yield was higher than the corresponding achieved with β-galactosidases from *K. lactis* and *A. oryzae*, under the optimum reaction conditions in terms of temperature, pH value, initial lactose content, enzyme load, and reaction time (Table 3). From the analysis of the reaction products with HPAEC-PAD, 17–22 carbohydrates were produced via the application of β-galactosidase from *T. terrestris* in acid whey, including galactose, glucose, and lactose. Again, the most dominant oligosaccharide was 6′-*O*-β-galactosyl-lactose, followed by 6-galactobiose. At the maximum GOS yield point (20% *w*/*v* initial lactose content, 8 U/mL, 7 h), the concentration of 6-galactobiose was equal to 1.13% *w*/*v* (6.07% of initial lactose content). If this 6-galactobiose concentration was included in the maximum GOS yield, a maximum y_{GOS} equal to 31.8 ± 0.4% was achieved, with 6-galactobiose constituting 19.1% of total oligosaccharides. The percentage of 6′-*O*-β-galactosyl-lactose at this reaction point was 45.6% of total oligosaccharides.

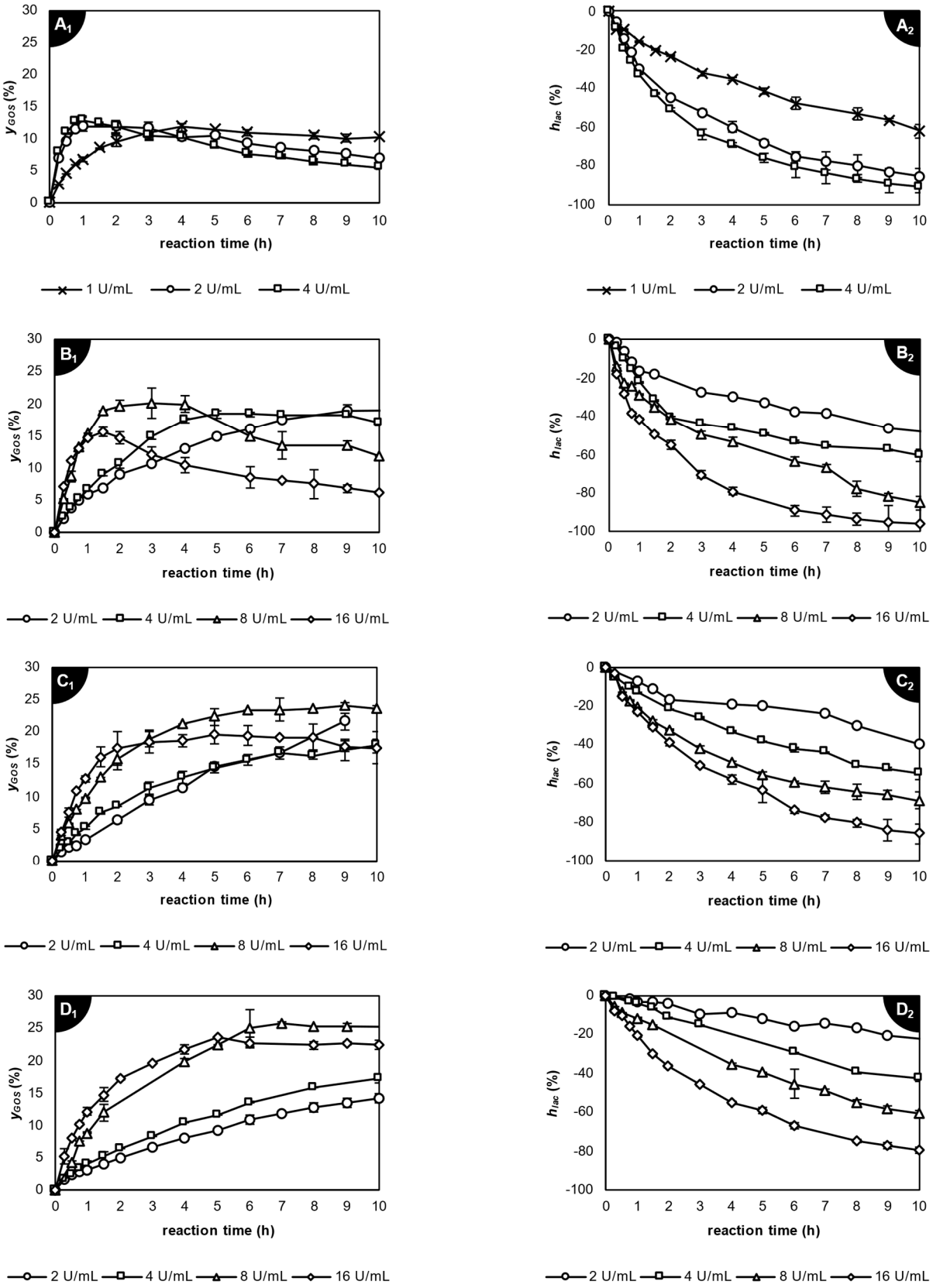

Figure 5. Galacto-oligosaccharide yields (y_{GOS}) and lactose hydrolysis degrees (h_{lac}) over time for enzymatic reactions with β-galactosidase from *Thermothielavioides terrestris* in acid whey with initial lactose content of 3.1% w/v (**A$_1$** and **A$_2$**, respectively), 10% w/v (**B$_1$** and **B$_2$**, respectively), 15% w/v (**C$_1$** and **C$_2$**, respectively), and 20% w/v (**D$_1$** and **D$_2$**, respectively).

Table 3. Maximum galacto-oligosaccharide yields ($y_{GOS(max)}$), time to achieve $y_{GOS(max)}$ ($t_{GOS(max)}$), lactose hydrolysis degrees (h_{lac}), and galactobiose yields ($y_{gal\text{-}gal}$) for all studied systems of galacto-oligosaccharide production from acid whey.

Enzyme Source	T (°C)	pH	$c_{lac(0)}$ (% *w*/*v*)	A_E (U/mL)	$y_{GOS(max)}$ (%)	$t_{GOS(max)}$ (h)	h_{lac} (%)	$y_{gal\text{-}gal}$ (%)
Kluyveromyces lactis	35	7.2	**3.1**	0.06	21.2 ± 1.8 ^{e}	3	−78.9	3.70 ± 0.01
				0.13	**23.7 ± 1.4 ^{g}**	**1.5**	**−76.0**	**3.72 ± 0.29**
				0.26	15.6 ± 1.2 ^{b}	0.75	−70.6	3.62 ± 0.17
			10	0.06	13.0 ± 0.6 ^{a,b,c}	4	−46.3	6.56 ± 0.11
				0.13	14.9 ± 1.8 ^{a,b}	2	−49.3	5.72 ± 0.09
				0.26	12.4 ± 1.0 ^{a,b,c}	1	−31.2	6.25 ± 0.17
			14	0.06	21.3 ± 1.1 ^{e}	6	−68.3	6.40 ± 0.29
				0.13	14.8 ± 1.1 ^{a,b}	3	−54.0	4.61 ± 0.11
				0.26	18.8 ± 2.3 ^{f}	1.5	−57.0	4.34 ± 0.01
			18	0.06	9.11 ± 1.39 ^{d}	3	−37.4	7.19 ± 0.01
				0.13	8.33 ± 1.24 ^{d}	4	−53.9	7.44 ± 0.34
				0.26	11.9 ± 1.2 ^{c}	2	−49.8	6.63 ± 0.21
Aspergillus oryzae	45	4.5	3.1	0.5	9.57 ± 1.81 ^{a}	3	−38.8	1.84 ± 0.01
				1	9.59 ± 0.56 ^{a}	1	−29.6	1.66 ± 0.02
				2	9.14 ± 0.53 ^{a}	0.75	−44.1	1.95 ± 0.02
				4	7.99 ± 0.45 ^{a}	0.25	−43.8	1.21 ± 0.01
			10	1	18.5 ± 0.4 ^{c,d}	10	−38.5	5.32 ± 0.01
				2	16.7 ± 0.7 ^{c}	7	−48.2	8.97 ± 0.09
				4	17.7 ± 1.0 ^{c}	2	−40.0	6.38 ± 0.10
				8	13.9 ± 0.7 ^{b}	1	−49.6	7.86 ± 0.02
			15	1	17.8 ± 0.7 ^{c}	10	−44.7	8.25 ± 0.18
				2	19.7 ± 1.1 ^{d,e}	8	−68.6	4.71 ± 0.10
				4	19.8 ± 0.9 ^{d,e}	4	−59.9	5.97 ± 0.08
				8	17.7 ± 0.6 ^{c}	1.5	−55.6	6.89 ± 0.09
			20	1	14.8 ± 2.1 ^{b}	10	−33.7	4.19 ± 0.06
				2	22.0 ± 0.9 ^{f,g}	10	−43.4	5.60 ± 0.38
				4	**23.4 ± 0.9 ^{g}**	**7**	**−51.9**	**4.78 ± 0.40**
				8	20.7 ± 1.0 ^{e,f}	5	−68.7	4.93 ± 0.54
Thermothielavioides terrestris	50	4.0	3.1	1	11.8 ± 0.6 ^{a}	4	−35.2	2.99 ± 0.06
				2	12.0 ± 0.6 ^{a}	2	−44.5	3.39 ± 0.05
				4	12.9 ± 0.6 ^{a,b}	1	−32.4	3.03 ± 0.05
			10	2	19.0 ± 1.0 ^{f,g,h}	9	−45.9	5.71 ± 0.29
				4	18.4 ± 0.6 ^{e,f,g}	5	−49.3	4.65 ± 0.04
				8	20.2 ± 2.4 ^{h}	3	−49.2	5.34 ± 0.15
				16	15.6 ± 0.8 ^{c,d}	1.5	−49.0	3.95 ± 0.06
			15	2	16.7 ± 0.8 ^{d,e}	9	−39.9	3.86 ± 0.01
				4	17.8 ± 1.0 ^{e,f}	10	−55.1	4.16 ± 0.23
				8	23.7 ± 0.4 ^{i}	9	−65.6	6.88 ± 0.26
				16	19.6 ± 1.5 ^{g,h}	5	−63.8	5.83 ± 0.55
			20	2	14.3 ± 0.7 ^{b,c}	10	−20.4	2.94 ± 0.01
				4	17.2 ± 0.6 ^{d,e}	10	−42.3	4.09 ± 0.16
				8	**25.7 ± 0.3 ^{j}**	**7**	**−48.8**	**6.07 ± 0.07**
				16	23.6 ± 0.2 ^{i}	5	−59.1	5.91 ± 0.18

$c_{lac(0)}$: initial lactose content, A_E: enzyme load. Different letters indicate significant differences between means (± standard deviation) of $y_{GOS(max)}$ according to Duncan's mean values post hoc comparison test for a significance level of $p = 0.05$.

Zeuner et al. (2016) studied the application of three thermophilic β-galactosidases from *Bacilus circulans*, *Thermus thermophilus*, and *Pyrococcus furiosus* in solutions containing lactose and N-acetylglucosamine, for the production of prebiotic N-acetyllactosamine [18]. The enzyme from *B. circulans* showed optimum activity and thermostability at 50 °C, similarly to β-galactosidase from *T. terrestris*, but had a higher optimum pH value (6.0). When β-galactosidase from *B. circulans* was applied in 27 mM phosphate-citrate buffer (pH 6.0) containing 50 mM lactose and 500 mM N-acetylglucosamine, they found that the maximum yield of N-acetyllactosamine produced reached 32% after 30 min of enzymatic reaction,

which is in accordance with our findings for the β-galactosidase from *T. terrestris*. The more thermostable β-galactosidases from *T. thermophilus* (optimum temperature 65 °C) and *P. furiosus* (optimum temperature 90 °C) exhibited significantly lower N-acetyllactosamine yields, reaching only up to 16% and 5.4%, respectively. Additionally, Yadav and Kayastha (2020) studied the transgalactosylation activity of β-galactosidase from *Lens culinaris*, using pure lactose solutions (200 g/L) as a substrate and reported a maximum GOS concentration of 68 g/L, after 16 h of enzymatic reaction at 55 °C and pH = 4.5, using 12 U/mL enzyme load [31]. This GOS concentration corresponds to a 34% GOS yield, which is slightly higher than the 31.3 ± 0.4% maximum GOS yield achieved in the present study using β-galactosidase from *T. terrestris* in acid whey, under almost identical reaction conditions. However, the maximum GOS yield with β-galactosidase from *L. culinaris* was achieved after a longer reaction time (16 h vs. 7 h with β-galactosidase from *T. terrestris*), using higher enzyme loads (16 U/mL vs. 8 U/mL with β-galactosidase from *T. terrestris*).

2.4. Comparison of Galacto-Oligosaccharide Profile Produced by the Studied Lactases

Figure 6 depicts the HPAEC-PAD chromatograms of the products from the enzymatic reactions via the application of the three studied β-galactosidases in acid whey, under the optimum reaction conditions in terms of temperature, pH value, initial lactose content, enzyme load, and reaction time, shown in bold in Table 3. The application of lactase from *K. lactis* in acid whey results in a different profile of GOS products (Figure 6A), compared to the application of the β-galactosidases from *A. oryzae* (Figure 6B) and *T. terrestris* (Figure 6C). The enzyme from *K. lactis* hydrolyses the lactose faster, producing higher concentrations of allolactose and lower concentrations of 6′-*O*-β-galactosyl-lactose, whereas β-galactosidase from *A. oryzae* and *T. terrestris* produces more 6′-*O*-β-galactosyl-lactose and less allolactose. If we compare the two acidophilic lactases from *A. oryzae* and *T. terrestris*, we see that the latter produces higher concentrations of 6′-*O*-β-galactosyl-lactose and lower concentrations of allolactose, compared to the former one. Specifically, the enzymatic conversion of acid whey lactose into GOSs using β-galactosidase from *K. lactis* under the optimum reaction conditions (3.1% *w*/*v* initial lactose content, 0.13 U/mL enzyme load, 1.5 h of enzymatic reaction, 35 °C, pH = 7.2) resulted in the production of a GOS mixture comprised of 7.13% allolactose and 31.5% 6′-*O*-β-galactosyl-lactose, on the basis of total oligosaccharides. The corresponding fractions in GOS mixtures produced under the optimum reaction conditions using β-galactosidase from *A. oryzae* (20% *w*/*v* initial lactose content, 4 U/mL, 7 h, 45 °C, pH = 4.5) and *T. terrestris* (20% *w*/*v* initial lactose content, 8 U/mL, 7 h, 50 °C, pH = 4.0) were 5.72% and 3.97% for allolactose and 41.9% and 45.6% for 6′-*O*-β-galactosyl-lactose, respectively. Regarding 6-galactobiose, a maximum fraction equal to 19.1% of total oligosaccharides was measured with the application of β-galactosidase from *T. terrestris*, followed by *A. oryzae* (17.0%) and *K. lactis* (13.6%), under the aforementioned optimum reaction conditions.

Rico-Rodríguez et al. (2021) studied the application of both β-galactosidases from *A. oryzae* and *K. lactis* separately and simultaneously in pure lactose and cheese whey with an initial lactose content equal to 40% *w*/*w* and found that in reactions with pure lactose maximum GOS yields equal to 32.7 and 41.7% of total carbohydrates were achieved via the application of β-galactosidases from *A. oryzae* and *K. lactis*, respectively. When cheese whey was used, respective maximum GOS yields were 35.6 and 29.6% of total carbohydrates. Additionally, they reported that although the combination of the two enzymes resulted in similar GOS yields to those obtained when the two β-galactosidases were studied separately, there were significant differences in the profile of the GOS products regarding their degree of polymerization [32].

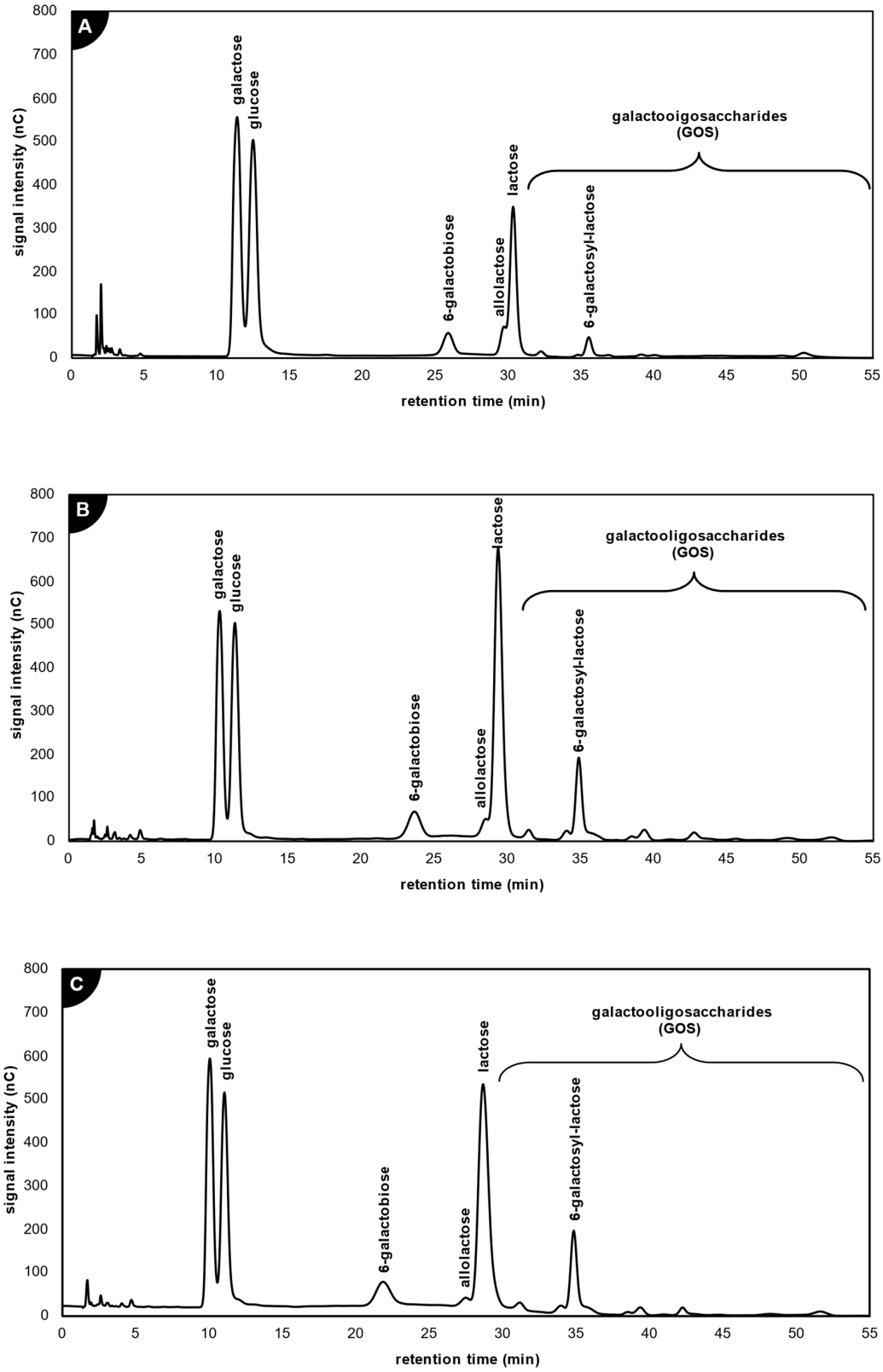

Figure 6. Chromatograms from high-performance anion exchange chromatography with pulsed amperometric detection of the enzymatic reaction products via the application of β-galactosidases from (**A**) *Kluyveromyces lactis* (sample dilution 1:200), (**B**) *Aspergillus oryzae* (sample dilution 1:1000), and (**C**) *Thermothielavioides terrestris* (sample dilution 1:1000) in acid whey, under the optimum reaction conditions. The peaks of allolactose and 6′-*O*-β-galactosyl-lactose were identified according to Urrutia et al. (2013) following a similar methodology for GOS identification [15].

3. Materials and Methods

3.1. Materials

Pasteurized acid whey from non-fat Greek strained yoghurt was provided by a leading Greek dairy industry and was kept frozen (−25 °C) until use. Two industrially applied β-galactosidases were used; one from yeast *Kluyveromyces lactis* acquired from Kerry Group plc (Tralee, Kerry, Ireland) and one from fungus *Aspergillus oryzae* acquired from Sigma-Aldrich (St. Louis, MO, USA). The novel, thermophile β-galactosidase from *Thermothielavioides terrestris*, heterologously expressed in *Pichia pastoris*, was produced and purified in-house, as described in our previous study [21]. All standards for chemical analyses were acquired from Sigma-Aldrich (St. Louis, MO, USA).

3.2. Characterization and Pretreatment of Acid Whey

A series of chemical analyses was performed for the full characterization of acid whey. Total sugar, lactose, glucose, and galactose concentrations were determined via the high-performance anion exchange chromatography with pulsed amperometric detection method described in Section 3.5. Determination of total nitrogen was carried out via the Kjeldahl method [33]. For calculation of the total protein content, a factor of 6.28 was used [34]. Fat content was measured via the Röse−Gottlieb method [35]. Lactic acid concentration was determined according to Borshchevskaya et al. (2016) [36]. Solid residue was determined gravimetrically, by drying liquid acid whey at 105 ± 3 °C for 24 h. Ash from the acid whey was measured gravimetrically, by incinerating the previously dried acid whey in a muffle furnace, at 500–550 °C for 16 h. Concentrations of K^+ and Na^+ were determined via atomic absorption spectrometry (AAS). Concentrations of Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+} were determined via inductively coupled plasma atomic emission spectroscopy (ICP-AES). The sulphate (SO_4^{2-}) concentration was determined turbidimetrically, via the sulphate turbidimetric method 9038 (1986) of the United States Environmental Protection Agency (EPA) [37]. The pH value of the acid whey was measured using an Amel 338 pH meter (AMEL Instruments, Milan, Italy) and a Hanna HI1131B 12 mm glass electrode (Hanna Instruments, Winsocket, RI, USA). All measurements were carried out in triplicate.

For the investigation of the effect of acid whey's initial lactose concentration on the production of GOS via the application of the aforementioned β-galactosidases, acid whey was concentrated using a Büchi RE-111 Rotavapor (Büchi Labortechnik AG, Flawil, Switzerland). The lactose content of concentrated acid whey was up to 20% w/v, which is the limit of lactose solubility at an ambient temperature [38].

3.3. Characterization of Biocatalysts

Enzyme activity of the studied β-galactosidases was measured via the o-nitrophenyl-β-D-galactopyranose (o-NPG) method. In glass test tubes, 2.5 mL of 0.3 mM of o-NPG in a suitable buffer solution with the desired pH value and 200 μL of appropriately diluted enzyme solution were added and incubated in a water bath at a constant temperature for 10 min. Dilution of the enzyme solution was performed in order for the substrate of the enzymatic reaction (o-NPG) to be in excess during the whole 10-min incubation. Immediately after incubation, 500 μL of 30% w/v Na_2CO_3 solution was added to alkalize the solution and thus terminate the reaction and turn the color of the produced o-nitrophenol (o-NP) to yellow. Subsequently, the absorbance of the solution at 410 nm was measured using a U-2900 spectrophotometer (Hitachi, Tokyo, Japan) and absorbance was converted to o-NP concentration via the appropriate calibration curve. Enzyme activity (A_E) was expressed in units and one unit was defined as the amount of β-galactosidase producing 1 μmol of o-NP per min of enzymatic reaction in substrate (o-NPG) excess, under corresponding studied conditions of temperature and pH.

For the determination of optimum catalysis conditions of studied β-galactosidases, enzyme activities were measured in pH and temperature ranges presented in Table 4, using the mentioned buffer solutions. The thermostability of each biocatalyst at the temperature range also mentioned in Table 4 was measured at the optimum pH value by incubating the

enzyme solution in a water bath for up to 10 h. At specified time intervals, 200 μL aliquots of the enzyme solution were removed and the remaining enzyme activity was measured via the aforementioned o-NPG protocol at the same temperature. Additionally, the effect of metal ions present in the acid whey on the enzyme activity of studied β-galactosidases was studied, under the optimum pH and temperature conditions of each biocatalyst. The metal ions were K^+, Na^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, and Cu^{2+} and were added to the buffer solution in a concentration of 1–100 mM. All measurements were carried out in triplicate.

Table 4. Buffer solutions and ranges of pH and temperature for the determination of enzyme activity and thermostability of β-galactosidases from *Kluyveromyces lactis*, *Aspergillus oryzae*, and *Thermothielavioides terrestris*.

β-Galactosidase Source	Buffer Solution	pH Range	Temperature Range (°C)
Kluyveromyces lactis	Na_2HPO_4—NaH_2PO_4	6.0–8.0	20–50
Aspergillus oryzae	citric acid—NaOH	3.0–6.5	30–60
Thermothielavioides terrestris *	citrate, phosphate, Tris—HCl	3.0–9.0	30–70

* Data from Zerva et al. (2021) for the purpose of comparative study [21].

3.4. Enzymatic Reaction for GOS Production

Enzymatic reactions for GOS production via the application of the studied β-galactosidases in non-concentrated acid whey with initial lactose content 3.1% *w*/*v* and concentrated acid whey with initial lactose content up to 20% *w*/*v* were performed in duplicate, under the optimum reaction conditions for each β-galactosidase studied, as determined by the analysis described in Section 3.3. Inside a 100 mL glass Erlenmeyer flask with a stopper, 90 mL of acid whey was transferred, and an appropriate volume of enzymatic solution was added to achieve the desired enzyme load. The flasks with the reaction mixture were incubated inside a Grant GLS400 linear shaking (160 rpm) water bath (Grant Instruments Ltd., Cambridge, United Kingdom). The range of enzyme loads studied was 0.06–0.26 U/mL for β-galactosidase from *K. lactis*, 0.5–8.0 U/mL for β-galactosidase from *A. oryzae*, and 1.0–16 U/mL for β-galactosidase from *T. terrestris*. At specified time intervals, 3 mL aliquots were transferred in glass tubes with a screw cap, incubated at 100 °C for 10 min inside a water bath (Memmert GmbH + Co. KG, Schwabach, Germany) for enzyme inactivation and kept frozen (−25 °C) until their analysis.

The selection of the enzyme loads studied was based on preliminary experiments and was not identical for all lactases. The enzymatic activity of the three β-galactosidases was determined in standard buffered o-NPG solutions, as described in Section 3.3, but it was applied in acid whey, containing other components, mainly metal cations, that affect the enzymatic activity of each biocatalyst differently, as described in Section 2.2. For this reason, the term "enzyme load" was used instead of "enzymatic activity" to describe the amount of the enzyme added to the reaction mixture to perform the bioconversion of acid whey lactose into galacto-oligosaccharides.

3.5. Analysis of Enzymatic Reaction Products

The products of the enzymatic reactions for GOS production via the application of the studied β-galactosidases in non-concentrated and concentrated acid whey comprised monosaccharides (galactose, glucose), disaccharides (lactose and GOS with a degree of polymerization equal to 2), and GOS with a degree of polymerization greater than 2. These products were analyzed via high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), using an ICS 6000 System (Thermo Scientific, Waltham, MA, USA). To separate enzymatic reaction products, a Dionex CarboPac PA1 analytical column (4 × 250 mm) with a Dionex CarboPac PA1 (4 × 50 mm) guard column (Thermo Scientific, Waltham, MA, USA) was used and a modified AOAC method 2001.02 was applied [39]. The eluents used were 12.5 mM NaOH (A), 125 mM NaOH (B), and 125 mM NaOH/500 mM CH_3COONa (C). The elution gradient was 0–20 min 95% A and 5%

B, 20–35 min gradient up to 100% B, 35–55 min 100% B, 55–65 min 100% C, 65–80 min 95% A and 5% B. The flow rate was set to 1 mL/min. System control and data acquisition were performed using Chromeleon 7 software (Thermo Scientific, Waltham, MA, USA). For the identification and quantification of the enzymatic products, five standard sugars (galactose, glucose, 6-galactobiose, lactose, raffinose) were used and corresponding standard curves were made. Galacto-oligosaccharides with a degree of polymerization 2 or greater, other than lactose and 6-galactobiose, were quantified as raffinose. Galacto-oligosaccharide yield (y_{GOS}), lactose hydrolysis degree (h_{lac}), and 6-galactobiose yield ($y_{gal\text{-}gal}$) were calculated according to Equation (1), Equation (2), and Equation (3), respectively.

$$y_{GOS} = \frac{c_{GOS}}{c_{lac(0)}} \cdot 100\ \% \quad (1)$$

$$h_{lac} = \frac{c_{lac} - c_{lac(0)}}{c_{lac(0)}} \cdot 100\ \% \quad (2)$$

$$y_{gal-gal} = \frac{c_{gal-gal}}{c_{lac(0)}} \cdot 100\ \% \quad (3)$$

where c_{lac} the lactose content each time (% w/v), $c_{lac(0)}$ the initial lactose content (% w/v), c_{GOS} the galacto-oligosaccharide content each time (% w/v), and $c_{gal\text{-}gal}$ the 6-galactobiose content each time (% w/v). The products of each enzymatic reaction were analyzed in duplicate.

4. Conclusions

The present comparative study of the enzymatic production of prebiotic galacto-oligosaccharides (GOS) from acid whey, via the applications of three β-galactosidases from *Kluyveromyces lactis*, *Aspergillus oryzae*, and *Thermothielavioides terrestris* demonstrates the feasibility of acid whey valorization via its conversion from a by-product to a high-nutritional and added-value product. The optimum reaction conditions for the three studied biocatalysts were determined at 35 °C and a pH equal to 7.2 for β-galactosidase from *K. lactis* and at 45 °C and a pH equal to 4.5 for β-galactosidase from *A. oryzae*, while for β-galactosidase from *T. terrestris* the optimum reaction conditions were specified at 50 °C and a pH equal to 4.0 from our previous study [21]. Under these reaction conditions, a maximum GOS yield equal to 25.7 ± 0.3% was achieved via the application of the thermophilic β-galactosidase from *T. terrestris* in concentrated acid whey with an initial lactose content equal to 20% w/v, with an enzyme load of 8 U/mL, after 7 h of enzymatic reaction. The corresponding maximum GOS yields achieved by the two commercial lactases from *K. lactis* and *A. oryzae* were lower, equal to 23.7% (3.1% w/v initial lactose content, 0.13 U/mL, 1.5 h) and 23.4% (20% w/v initial lactose content, 4 U/mL, 7 h), respectively. Regarding the profile of the produced GOS from each biocatalyst, the most dominant oligosaccharides produced were 6′-*O*-β-galactosyl-lactose, 6-galactobiose, and allolactose. On the basis of total oligosaccharides, the novel β-galactosidase from *T. terrestris* outmatched the other two commercial lactases from *K. lactis* and *A. oryzae* in the production of the trisaccharide 6′-*O*-β-galactosyl-lactose (45.6% over 31.5% and 41.9%, respectively) and the disaccharide 6-galactobiose (19.1% over 13.6% and 17.0%) but resulted in the production of less allolactose (3.97% over 7.13% and 5.72%, respectively). The increased production of GOSs by the thermophilic β-galactosidase derived from *T. terrestris* can play a pivotal role in a unified industrial process involving acid whey concentration and lactose oligomerization. This process could enable the generation of substantial GOS yields, facilitating the efficient and economical production of valuable prebiotics from acid whey that can be further incorporated into conventional products to increase their nutritional value and biofunctionality, as a viable alternative for acid whey management from dairy industries.

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References

1. Rocha-Mendoza, D.; Kosmerl, E.; Krentz, A.; Zhang, L.; Badiger, S.; Miyagusuku-Cruzado, G.; Mayta-Apaza, A.; Giusti, M.; Jiménez-Flores, R.; García-Cano, I. Invited Review: Acid Whey Trends and Health Benefits. *J. Dairy Sci.* **2021**, *104*, 1262–1275. [CrossRef] [PubMed]
2. Menchik, P.; Zuber, T.; Zuber, A.; Moraru, C.I. Short Communication: Composition of Coproduct Streams from Dairy Processing: Acid Whey and Milk Permeate. *J. Dairy Sci.* **2019**, *102*, 3978–3984. [CrossRef]
3. Erickson, B.E. Acid Whey: Is the Waste Product an Untapped Goldmine? Companies, Food Scientists Develop Innovative Solutions to Handle Tons of Greek Yogurt Byproduct. *Chem. Eng. News* **2017**, *95*, 26–30.
4. Greek Yogurt Market. 2022. Available online: https://www.factmr.com/report/269/greek-yogurt-market (accessed on 25 July 2023).
5. Ketterings, Q.; Czymmek, K.; Gami, S.; Godwin, G.; Ganoe, K. Guidelines for Land Application of Acid Whey. Cornell University Department of Animal Science Publication Series No. 247. 2017. Available online: http://nmsp.cals.cornell.edu/publications/extension/AcidWheyGuidelines2017.pdf (accessed on 25 July 2023).
6. Smithers, G.W. Whey and Whey Proteins-From "Gutter-to-Gold". *Int. Dairy J.* **2008**, *18*, 695–704. [CrossRef]
7. Fischer, C.; Kleinschmidt, T. Synthesis of Galactooligosaccharides in Milk and Whey: A Review. *Compr. Rev. Food Sci. Food Saf.* **2018**, *17*, 678–697. [CrossRef]
8. Torres, D.P.M.; Gonçalves, M.d.P.F.; Teixeira, J.A.; Rodrigues, L.R. Galacto-Oligosaccharides: Production, Properties, Applications, and Significance as Prebiotics. *Compr. Rev. Food Sci. Food Saf.* **2010**, *9*, 438–454. [CrossRef]
9. Erich, S.; Kuschel, B.; Schwarz, T.; Ewert, J.; Böhmer, N.; Niehaus, F.; Eck, J.; Lutz-Wahl, S.; Stressler, T.; Fischer, L. Novel High-Performance Metagenome β-Galactosidases for Lactose Hydrolysis in the Dairy Industry. *J. Biotechnol.* **2015**, *210*, 27–37. [CrossRef]
10. Gosling, A.; Stevens, G.W.; Barber, A.R.; Kentish, S.E.; Gras, S.L. Recent Advances Refining Galactooligosaccharide Production from Lactose. *Food Chem.* **2010**, *121*, 307–318. [CrossRef]
11. Yin, H.; Bultema, J.B.; Dijkhuizen, L.; van Leeuwen, S.S. Reaction Kinetics and Galactooligosaccharide Product Profiles of the β-Galactosidases from *Bacillus circulans*, *Kluyveromyces lactis* and *Aspergillus oryzae*. *Food Chem.* **2017**, *225*, 230–238. [CrossRef]
12. Vera, C.; Córdova, A.; Aburto, C.; Guerrero, C.; Suárez, S.; Illanes, A. Synthesis and Purification of Galacto-Oligosaccharides: State of the Art. *World J. Microbiol. Biotechnol.* **2016**, *32*, 197. [CrossRef] [PubMed]
13. Barile, D.; Rastall, R.A. Human Milk and Related Oligosaccharides as Prebiotics. *Curr. Opin. Biotechnol.* **2013**, *24*, 214–219. [CrossRef]
14. Rodriguez-Colinas, B.; Fernandez-Arrojo, L.; Ballesteros, A.O.; Plou, F.J. Galactooligosaccharides Formation during Enzymatic Hydrolysis of Lactose: Towards a Prebiotic-Enriched Milk. *Food Chem.* **2014**, *145*, 388–394. [CrossRef]
15. Urrutia, P.; Rodriguez-Colinas, B.; Fernandez-Arrojo, L.; Ballesteros, A.O.; Wilson, L.; Illanes, A.; Plou, F.J. Detailed Analysis of Galactooligosaccharides Synthesis with β-Galactosidase from *Aspergillus oryzae*. *J. Agric. Food Chem.* **2013**, *61*, 1081–1087. [CrossRef]
16. Srivastava, A.; Mishra, S.; Chand, S. Transgalactosylation of Lactose for Synthesis of Galacto-oligosaccharides Using *Kluyveromyces marxianus* NCIM 3551. *New Biotechnol.* **2015**, *32*, 412–418. [CrossRef]
17. Yañez-Ñeco, C.V.; Cervantes, F.V.; Amaya-Delgado, L.; Ballesteros, A.O.; Plou, F.J.; Arrizon, J. Synthesis of β(1→3) and β(1→6) Galactooligosaccharides from Lactose and Whey Using a Recombinant β-Galactosidase from *Pantoea anthophila*. *Electron. J. Biotechnol.* **2021**, *49*, 14–21. [CrossRef]

18. Zeuner, B.; Nyffenegger, C.; Mikkelsen, J.D.; Meyer, A.S. Thermostable β-Galactosidases for the Synthesis of Human Milk Oligosaccharides. *New Biotechnol.* **2016**, *33*, 355–360. [CrossRef]
19. Fischer, C.; Kleinschmidt, T. Synthesis of Galactooligosaccharides Using Sweet and Acid Whey as a Substrate. *Int. Dairy J.* **2015**, *48*, 15–22. [CrossRef]
20. Fischer, C.; Kleinschmidt, T. Synthesis of Galactooligosaccharides by *Cryptococcus laurentii* and *Aspergillus oryzae* Using Different Kinds of Acid Whey. *Int. Dairy J.* **2021**, *112*, 104867. [CrossRef]
21. Zerva, A.; Limnaios, A.; Kritikou, A.S.; Thomaidis, N.S.; Taoukis, P.; Topakas, E. A Novel Thermophile β-Galactosidase from *Thermothielavioides terrestris* Producing Galactooligosaccharides from Acid Whey. *New Biotechnol.* **2021**, *63*, 45–53. [CrossRef]
22. Jelen, P. WHEY PROCESSING | Utilization and Products. *Encycl. Dairy Sci.* **2011**, 731–737. [CrossRef]
23. Tanaka, Y.; Kagamiishi, A.; Kiuchi, A.; Horiuchi, T. Purification and Properties of β-Galactosidase from *Aspergillus oryzae*. *J. Biochem.* **1975**, *77*, 241–247.
24. Tanriseven, A.; Dogan, S. A Novel Method for the Immobilization of β-Galactosidase. *Process Biochem.* **2002**, *38*, 27–30. [CrossRef]
25. Vera, C.; Guerrero, C.; Illanes, A. Determination of the Transgalactosylation Activity of *Aspergillus oryzae* β-Galactosidase: Effect of pH, Temperature, and Galactose and Glucose Concentrations. *Carbohydr. Res.* **2011**, *346*, 745–752. [CrossRef]
26. Fujimura, Y.; Rokushika, S.; Ohnishi, M. Purification and Molecular Characterization of β-Galactosidase from Yeast *Kluyveromyces lactis*. *Int. J. Biol. Macromol.* **2003**, *3*, 97–103.
27. Dickson, R.C.; Dickson, L.R.; Markin, J.S. Purification and Properties of an Inducible β-Galactosidase Isolated from the Yeast *Kluyveromyces lactis*. *J. Bacteriol.* **1979**, *137*, 51–61. [CrossRef]
28. González-Delgado, I.; López-Muñoz, M.J.; Morales, G.; Segura, Y. Optimisation of the Synthesis of High Galacto-Oligosaccharides (GOS) from Lactose with β-Galactosidase from *Kluyveromyces lactis*. *Int. Dairy J.* **2016**, *61*, 211–219. [CrossRef]
29. Mano, M.C.R.; Paulino, B.N.; Pastore, G.M. Whey Permeate as the Raw Material in Galacto-oligosaccharide Synthesis Using Commercial Enzymes. *Food Res. Int.* **2019**, *124*, 78–85. [CrossRef]
30. Jenab, E.; Omidghane, M.; Mussone, P.; Armada, D.H.; Cartmell, J.; Montemagno, C. Enzymatic Conversion of Lactose into Galacto-oligosaccharides: The Effect of Process Parameters, Kinetics, Foam Architecture, and Product Characterization. *J. Food Eng.* **2018**, *222*, 63–72. [CrossRef]
31. Yadav, A.; Kayastha, A.M. *Lens culinaris* β-Galactosidase (*Lsbgal*): Insights into Its Purification, Biochemical Characterization and Trisaccharides Synthesis. *Bioorg. Chem.* **2020**, *95*, 103543. [CrossRef]
32. Rico-Rodríguez, F.; Noriega, M.A.; Lancheros, R.; Serrato-Bermúdez, J.C. Kinetics of Galactooligosaccharide (GOS) Production with Two β-Galactosidases Combined: Mathematical Model and Raw Material Effects. *Int. Dairy J.* **2021**, *118*, 105015. [CrossRef]
33. Kjeldahl, J. Neue Methode zur Bestimmung des Stickstoffs in Organischen Körpern. *Z. Anal. Chem.* **1883**, *22*, 366–382. [CrossRef]
34. van Boekel, M.A.J.S.; Ribadeau Dumas, B. Addendum to the Evaluation of the Kjeldahl Factor for Conversion of the Nitrogen Content of Milk and Milk Products to Protein Content. *Neth. Milk Dairy J.* **1987**, *41*, 281–284.
35. O'Connor, T.P.; O'Brien, N.M. LIPIDS | Analytical Methods. *Encycl. Dairy Sci.* **2002**, 1583–1587. [CrossRef]
36. Borshchevskaya, L.N.; Gordeeva, T.L.; Kalinina, A.N.; Sineokii, S.P. Spectrophotometric Determination of Lactic Acid. *J. Anal. Chem.* **2016**, *71*, 755–758. [CrossRef]
37. *Sulphate Turbidimetric Method 9038*; United States Environmental Protection Agency (EPA): Washington, DC, USA, 1986.
38. Yalkowsky, S.H.; He, Y.; Jain, P. *Handbook of Aqueous Solubility Data*, 2nd ed.; CRC Press: Boca Raton, FL, USA, 2010, ISBN 13: 978-1-4398-0246-5.
39. DIONEX Application Note 155 Determination of Trans-Galactooligosaccharides in Foods by AOAC Method 2001.02. 2016. Available online: https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/AN-155-IC-Trans-Galactooligosaccharides-Foods-LPN1547-EN.pdf (accessed on 25 July 2023).