WHEY VALORIZATION USING TRANSGALACTOSYLATION ACTIVITY OF *β*-GALACTOSIDASE

Ana Milivojević[1]*, Milica Carević[2], Marija Ćorović[2], Katarina Banjanac[1,3], Dejan Bezbradica[2]

[1] Innovation Center, Faculty of Technology and Metallurgy, University of Belgrade, Karnegijeva 4, 11000 Beograd, Serbia
[2] Faculty of Technology and Metallurgy, University of Belgrade, Karnegijeva 4, 11000 Beograd, Serbia
[3] Group for Metrology in Chemistry, Directorate of Measures and Precious Metals, Mike Alasa 14, 11000 Beograd, Serbia

*Corresponding author:
E-mail address: amilivojevic@tmf.bg.ac.rs

ABSTRACT

Whey is the most significant by-product from the dairy industry that can cause serious environmental pollution problems. Namely, one of the main whey constituents is lactose, which is responsible for high biological oxygen demand (BOD) and chemical oxygen demand (COD) values. Hence, owing to the abundant amount of lactose in whey, removal or conversion of lactose proved to be the promising approach for the whey valorization.
Hereby, we investigated a possibility of lactose bioconversion into wide range of valuable prebiotic compounds named galacto-oligosaccharides (GOS) using transgalactosylation activity of commercial *β*-galactosidase from *Aspergillus orzyae*. Obtained results showed that an ussual substrate for the GOS synthesis - lactose could be successfully replaced by much cheaper substrate - whey powder, since the quite similar composition of products (tri- and tetra- saccharides with dominant *β*-1,4 and *β*-1,6 linkages), as well as equal product yields have been achieved using both substrates. Therefore, in terms of yielding the high GOS yields, thorough optimization of the most important process parameters (enzyme concentration, temperature, pH, whey solution concentration and time) was performed. Finally, the highest obtained GOS concentration (60 g/l) was achieved using 40 % (w/v) whey solution (the highest examined concentration due to the low whey solubility) under the optimized conditions (50 ^{0} C, pH 4.5) after 10h.
Therefore, the findings of this study could provide a valuable contribution to the efficient and cost-effective production of physiologically active GOS, and simultaneously solve the considerable problem of whey utilization and its appropriate disposal.
***Keywords**: β-galactosidase, galacto-oligosaccharides, transgalactosylation, prebiotics, whey*

INTRODUCTION

Whey represents the most important cheese and casein industry by-product, manufactured in large quantities worldwide. It contains various valuable substances, including proteins, lactose, vitamins, minerals, essential amino acids, lactic acid, and various enzymes (Song *et al.*, 2013), yet its nutritional benefits are not fully exploited. With the genuine progress of biotechnology, whey is becoming a substrate of choice for diverse food and pharmaceutical industries (Lata *et al.*, 2018, Chandra *et al.,* 2018). However, significant amounts of whey are still regarded as a waste, causing severe environmental problems upon its disposal. The major factor responsible for high biological (BOD) and chemical oxygen demand (COD) values of whey is lactose, one of the major organic load fractions present in whey (Illanés 2011). Therefore, the great amount of effort is currently being invested in development of efficient lactose conversion processes that can tune whey into products with added value, and simultaneously solve the environmental issues.
Traditionally, lactose removal is performed by virtue of *β*-galactosidase hydrolytic activity, yielding products of enhanced sensorial and physicochemical properties that find application mainly in the food industry (Illanés 2011). Nevertheless, with the increased interest in functional foods and prebiotics, considerably more attention is nowadays drawn to lactose bioconversion using the transgalactosylic activity of the same enzyme under slightly changed reaction parameters (Lamsal 2012, Torres *et al.*, 2010). Namely, wide range of

physiologically active compounds collectively named galacto-oligosaccharides (GOS) could be produced by *β*-galactosidase mediated transfer of galactosyl moiety onto other saccharide molecules, thus resulting in the formation of a complex mixture of carbohydrates notably varying in polymerization degree, type of linkages, and their properties (Urrutia *et al.*, 2013, Lamsal 2012). These compounds exhibit remarkable prebiotic potential and their consumption ensures hosts' overall wellbeing since they are believed to support an improvement of lactose digestion and mineral absorption, reduction of serum cholesterol level, diminishing the risk of cancer, and enhancement of the host's immune system (Lamsal 2012, Torres *et al.*, 2010). Moreover, GOS are distinguished by high thermal and acid stability, excellent taste quality, low sweetness and caloric value that secure its wide application in the food industry (Lamsal 2012, Torres *et al.*, 2010).
Until recently, the vast majority of the galacto-oligosaccharides synthesis related research was focused on using pure lactose solutions as a transgalactosylation substrate (Lamsal 2012, Torres *et al.*, 2010). However, with arousing awareness on lactose abundance in dairy effluents, cheaper and more versatile substrates, such as whey and whey permeate, or even milk, have been taken into account (Nath *et al.*, 2015, Fischer and Kleinschmidt 2018). Therefore, the aim of this work was to examine the possibility of GOS synthesis from the commercial whey powder (containing minimum 70% of lactose) using the previously well-established transgalactosylating enzyme.

MATERIAL AND METHODS

Materials

Enzyme, namely *β*-galactosidase from *Aspergillus oryzae* (≥8 IU/mg) used throughout the study was purchased from Sigma Chemical Co (St Louis, USA). The substrates used for galacto-oligosaccharide (GOS) production were lactose and whey powder, obtained from Sigma Chemical Co and Dukat d.d. (Zagreb, Croatia), respectively. Identification and quantitative analysis of obtained products were performed by high-performance liquid chromatography (HPLC) using HPLC grade water obtained from Merck (Darmstadt, Germany) as sole mobile phase.

Enzymatic synthesis of galacto-oligosaccharides

All trensgalatosylation reactions were performed on a rotary shaker (IKA KS 4000i control, Werke GmbH and Co. KG, Staufen, Germany) at 50^0 C. Reaction mixtures were comprised of lactose or whey dissolved in 0.1 M acetate buffer (pH 4.5) with or without enzyme, while the concentrations were specified for each experiment separately. Reaction mixture component concentrations were in the following ranges: 7-28 % (w/w) for lactose, 10-40% (w/w) for whey powder, and 5-20 mg for *β*-galactosidase. After the predefined reaction time, the samples were taken and the reaction was stopped by heating samples at 100 ^{0} C for 10 min in order to inactivate the enzyme. Samples were diluted with HPLC grade water, deproteinized centrifuged and then analyzed using HPLC. Experiments without enzyme were performed, and obtained control samples were subjected to the same temperature and deproteinization treatment (the products were not detected in them). All experiments were carried out in duplicate and average values of the product concentrations obtained are presented throughout the manuscript.

HPLC analysis

Quantitative analysis of the previously prepared samples was performed using Dionex Ultimate 3000 Thermo Scientific HPLC system (Waltham, USA). Different saccharides were separated using the carbohydrate column (Hyper REZ XP Carbohydrate Ca^{2+}, 300 mm×7.7 mm, 8 µm) operating on 80 °C. Deionized water was used as the mobile phase with an elution rate 0.6 ml/min during the analysis. Detection of different saccharides was performed with RI detector RefractoMax 520, (ERC, Germany), and all data acquisition and processing was done using Chromeleon Software.

RESULTS AND DISCUSSION

Throughout this study, the possibility of whey powder utilization for GOS synthesis using enzyme *β*-galactosidase from *A. oryzae* was examined. This enzyme was chosen on the basis of its easy availability, relatively low prices, and more importantly, our previous findings claiming its outstanding transgalactosylytic activity (Carević *et al.,* 2016, Carević *et al*., 2017). Bearing in mind that this enzyme was successfully employed in our previous studies using concentrated lactose solutions as substrate, in the initial stage of research, several concentrated whey powder solutions (10-40 % w/v) were tested as GOS synthesis substrate, under the previously determined optimal reaction conditions (temperature 50^0 C and sodium acetate buffer, pH 4.5 as solvent).

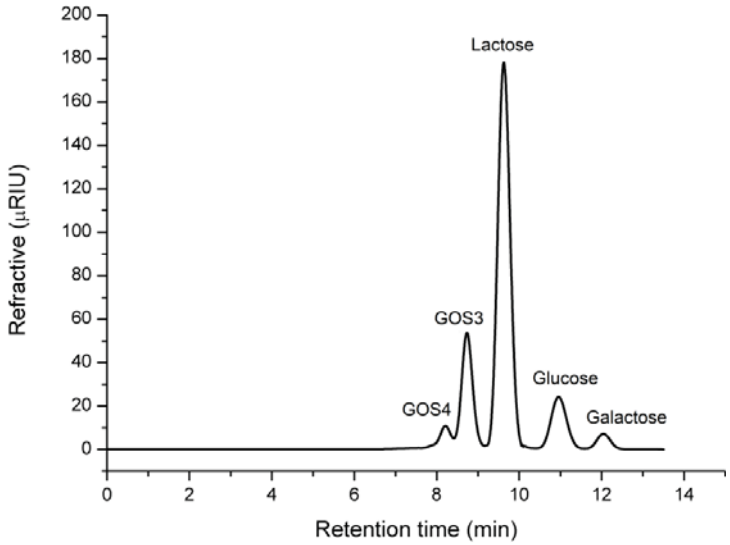

Figure 1. The characteristic HPLC reaction mixture chromatogram

The reaction course was monitored using HPLC, and the characteristic HPLC chromatogram featuring all reaction mixture constituents, namely galacto-oligosaccharides (tri-saccharides - GOS3 and tetra-saccharides - GOS4), lactose, glucose and galactose is presented in the Figure 1. Such composition was rather expected, bearing in mind that *β*-galactosidase from *A. oryzae* exhibits a low tendency towards creating other disaccharides, as well as the higher oligosaccharides (Carević *et al*., 2016).

In order to better understand the complex nature of transgalactosylation reaction, and gain better insight into the behavior of all reactants, reaction profiles of all present saccharides are depicted in Figure 2. In the first stage of the reaction, a significant decline in lactose concentration is observed, since it is simultaneously spent on the synthesis of GOS and in a small part on hydrolysis (glucose and galactose formation). During this stage, GOS3, GOS4 and glucose concentrations are rapidly increasing in all examined experiments, since lactose is hydrolyzed and galactose are incorporated into the newly formed oligosaccharide molecules. In this phase reaction of transgalactosylation is dominant, and this phase lasts until the lactose concentration becomes rather low. Accordingly, this phase is longer and the GOS yields are greater when initial concentrations of lactose are higher, because the availability of potential acceptor for the transgalactosylation reaction is higher, and the likelihood of galactose molecule transfer onto the molecule lactose instead of water is greater.

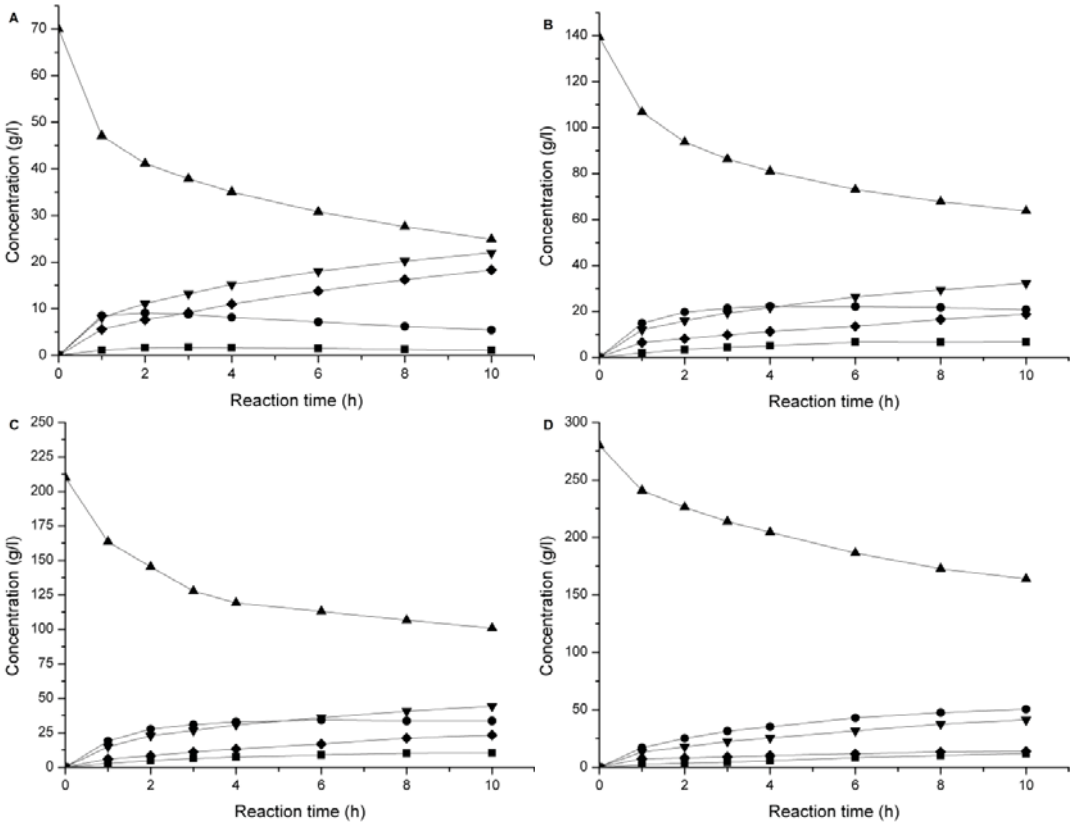

Figure 2. Transgalactosylation reaction kinetics: 10% (w/v) whey powder (A), 20% (w/v) whey powder (B), 30% (w/v) whey powder (C), 40% (w/v) whey powder solution (D) as substrate. Reaction mixture constituents: GOS3 (■), GOS4 (●), lactose (▲), glucose (▼) and galactose (♦). Reaction was conducted in orbital shaker (200 rpm) at 50 ^{0}C and pH 4.5.

Moreover, it can be seen that this difference between glucose and galactose concentrations is more pronounced in this first stage of the reaction, when the GOS synthesis prevails. Concentrations of glucose and galactose became similar as the reaction proceeds, due to the fact that the reaction of hydrolysis takes precedence in this stage. At the same time, GOS concentrations start to decrease, since they are prone to hydrolysis as well. Therefore, it is of great importance to correctly determine the optimum reaction time, in order to achieve the highest possible GOS yields. It can be clearly seen that the growth of whey solution concentration (constrained by low whey powder solubility) leads to an increase in the concentration of total synthesized GOS throughout the whole analyzed range. Also, it can be noted that as the concentration of whey increases, the time of reaching the maximum concentration of prebiotics is prolonged. Finally, it can be concluded that the highest concentration of GOS (62 g/l) was achieved with 40% whey in the reaction mixture in 10 hours (Figure 3).

After we adopted the most favorable conditions for achieving high GOS yields, the reasoning of the whey usage should be examined. Hence, in the subsequent experiment, GOS synthesis was performed using the corresponding lactose solutions (7-28 % w/v) in terms of fair comparison. Values of total GOS yields in both experiments (when using lactose and raw material containing lactose (whey powder) as substrate) are shown in Figure 3. Interestingly, the obtained results using both substrates are quite similar. Consequently, it can be concluded that the complex composition of the whey was not hampering lactose accessibility towards enzyme, and more importantly, did not impose presumed enzyme inactivation, thus could be successfully used as a reasonable substitution of lactose.

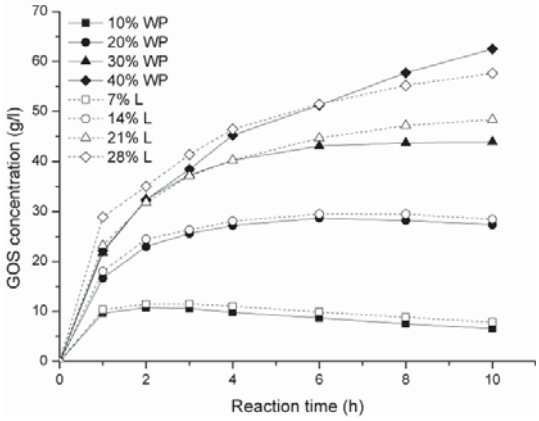

Figure 3. Transgalactosylation reaction kinetics with different concentrations of whey powder and lactose as substrates. Reactions were conducted in orbital shaker (200 rpm) at 50 ^{0}C and pH 4.5.

In terms of developing the economic enzymatic processes, especially when using waste materials as substrates, special attention should be paid to the employed enzyme concentration considering the high enzyme prices. Therefore, in the next set of experiments, the effect of different enzyme loadings in the GOS synthesis reaction is examined.

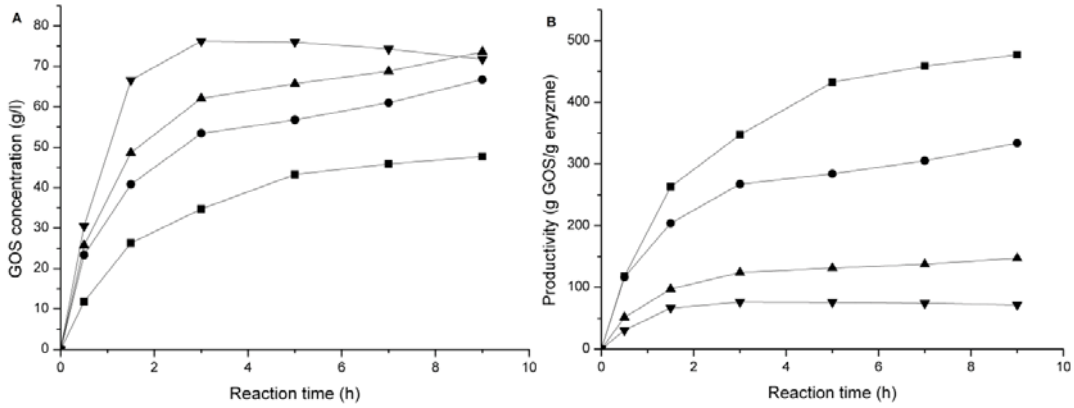

Figure 4. Influence of β-galactosidase concentration on GOS synthesis: achieved GOS concentration (A) and GOS synthesis productivity (B). Examined enzyme concentrations were: 0.1 mg/ml (■), 0.2 mg/ml (●), 0.5 mg/ml (▲) and 1 mg/ml (▼)

When considering both examined outputs (GOS concentration and productivity), although excerting conflicting results, it can be concluded that the most suitable enzyme concentration for further applications is 0.2 mg/ml. At this enzyme concentration, the maximum GOS concentration (Figure 4.) is attained after approximately 10h, and at the same time the reaction productivity is rather satisfactory (330 g GOS/g enzyme). The obtained results proved to be comparable with ones previously reported in the literature in terms of achieved yields (40-67 g/l), however, enzyme concentrations in the present study were at least ten fold lower (Fischer and Kleinschmidt 2018).

CONCLUSIONS

Whey is an abundant waste material whose valorization may be carried out using an enzymatic approach to produce galacto-oligosaccharides (GOS). In this study, the highest obtained GOS concentration (62.2 g/l) was achieved by conversion of 40 % (w/v) commercial sweet whey powder solution under the optimized conditions (50℃, pH 4.5) after 10h using *β*-

galactosidase from *A. oryzae* (0.2 mg/ml). Therefore, it can be concluded that lactose could be adequately replaced with cheaper substrate, enabling the establishment of cost-effective production of physiologically active GOS while solving the considerable problem of whey utilization at the same time.

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