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A Three-Step Process for the Bioconversion of Whey Permeate into a Glucose-Free D-Tagatose Syrup

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Abstract: We have developed a sustainable three-stage process for the revaluation of cheese whey permeate into D-tagatose, a rare sugar with functional properties used as sweetener. The experimental conditions (pH, temperature, cofactors, etc.) for each step were independently optimized. In the first step, concentrated whey containing 180–200 g/L of lactose was fully hydrolyzed by β-galactosidase from *Bifidobacterium bifidum* (Saphera®) in 3 h at 45 °C. Secondly, glucose was selectively removed by treatment with *Pichia pastoris* cells for 3 h at 30 °C. The best results were obtained with 350 mg of cells (previously grown for 16 h) per mL of solution. Finally, L-arabinose isomerase US100 from *Bacillus stearothermophilus* was employed to isomerize D-galactose into D-tagatose at pH 7.5 and 65 °C, in presence of 0.5 mM $MnSO_4$. After 7 h, the concentration of D-tagatose was approximately 30 g/L (33.3% yield, referred to the initial D-galactose present in whey). The proposed integrated process takes place under mild conditions (neutral pH, moderate temperatures) in a short time (13 h), yielding a glucose-free syrup containing D-tagatose and galactose in a ratio 1:2 (*w*/*w*).

Keywords: biocatalysis; glycosidases; isomerases; *Pichia pastoris*; sweeteners; rare sugars; cheese whey; sustainable chemistry

1. Introduction

D-Tagatose is one of the most promising low-calorie functional sweeteners [1]. It is a ketohexose, namely a C4 epimer of D-fructose. It is a rare sugar only found at small quantities in the gum exudates of the tropical tree *Sterculia setigera* and in several dairy products, e.g., in Ultra-High-Temperature (UHT) sterilized cow's milk [2]. It is heat tolerant, very stable at pH values between 2.0 to 7.0, and highly soluble in water (58% *w*/*w* at room temperature). It possesses a sucrose-like taste with 92% of its sweetness but contributing only 1.5 kcal/g (38% compared to sucrose) [3], with no cooling effect or aftertaste [4]. D-Tagatose was approved as a novel food in the European Union [3] and has obtained GRAS status by FDA in USA [5]. It is also approved in many other countries and is being widely used in foods, beverages, and dietary supplements [6,7].

Only 20% of the ingested D-tagatose is absorbed in the small intestine [8]. Since the remaining 80% seems to favourably modulate the composition of the gut microbiota [9], this sugar has been proposed as a potential prebiotic. In addition, D-tagatose is able to modulate lipid metabolism, minimize tooth

decay, promote blood health, and reduce the symptoms associated with type 2 diabetes, hyperglycemia, anemia, and haemophilia [10–12].

Several chemical [13,14] and chemoenzymatic [15,16] methods have been described for D-tagatose synthesis. In this context, the chemical manufacture of D-tagatose involves the use of metal hydroxides and calcium chloride, the neutralization with mineral acids and the implementation of complex purification steps due to by-product generation [6]. Therefore, enzymatic methods are the most appropriate for D-tagatose production in terms of selectivity, efficiency and environmental impact [17]. Several enzymes have been investigated for D-tagatose synthesis, e.g. phosphoglucose isomerase [18], galactitol 2-dehydrogenase [19] and L-arabinose isomerase (L-AI) [20]. The main reaction catalyzed by L-AI is the bioconversion of L-arabinose into L-ribulose, however, it also promotes efficiently the isomerization of D-galactose to D-tagatose [21]. In fact, most of the publications on the enzymatic synthesis of D-tagatose use L-AI as biocatalyst [22–24].

D-Tagatose production typically begins with D-galactose [25–28], which is quite expensive in its pure form. The valorization of galactose-rich byproducts such as agar [29] or whey [30] is much more attractive from the economic and environmental points of view. Moreover, cheese whey generates significant environmental and health issues due to its large volume production and high organic content [31]. With this aim in mind, several groups have reported the production of D-tagatose from cheese whey in liquid or powdered form [30,32–36], yielding a mixture of D-tagatose, D-galactose and D-glucose. Since the physicochemical properties of the three monosaccharides are quite similar, the isolation of pure D-tagatose is a difficult task [32].

In order to increase the efficiency of the process, several groups have co-expressed β-galactosidase and L-AI in *Pichia pastoris* [37], *Corynebacterium glutamicum* [33] or *Escherichia coli* [30,32,38] for the simultaneous lactose hydrolysis and D-galactose isomerization. The main drawback of this approach is that the optimum pH and temperature of L-AI and β-galactosidase are substantially different, thus limiting the efficiency of the whole process.

The presence of D-glucose in D-tagatose syrups is clearly undesirable, especially because one of the main properties of D-tagatose is its antidiabetic effect [6]. In order to eliminate the glucose released during lactose hydrolysis, Wanarska et al. took advantage of the presence of glucose to cultivate *Pichia pastoris* (which co-expressed L-AI and β-galactosidase) [37]. Another approach addressed by Zhan et al. [39] and Zheng et al. [32] was the implementation of a second step of D-glucose (and eventually D-galactose) fermentation by *Saccharomyces cerevisiae* which may render both D-tagatose and bioethanol.

In this work, we report a three-step process for the bioconversion of concentrated whey permeate into D-tagatose using free enzymes and whole cells. In the first stage, β-galactosidase hydrolyzed lactose. This was followed by treatment with *Pichia pastoris* cells to remove the released D-glucose. Finally, L-AI isomerized D-galactose into D-tagatose. The three stages were independently optimized to obtain the highest efficiency.

2. Results and Discussion

2.1. Chemical Composition of Whey Permeate

It is well reported the effect of metal ions on the activity and stability of many enzymes, including those involved in the transformation of carbohydrates, and in particular in the two enzymes involved in the present work: β-galactosidase [40] and L-arabinose isomerase [41]. In particular, several members of these two families are activated by divalent cations such as Co^{2+}, Mn^{2+} and Mg^{2+} [21,42]. For that reason, we measured by semiquantitative ICP-MS the composition of the concentrated whey permeate employed in this work. Table 1 summarizes the concentration of metal ions present at higher amounts than 0.1 ppm. As shown, the concentration of Mg^{2+} was significant (98 mg/L) but the presence of Co^{2+} and Mn^{2+} was negligible.

Table 1. Metal ions present in whey permeate at concentrations higher than 0.1 ppm, determined by semiquantitative ICP-MS.

Analyte	Concentration (ppm)
Na	390
Mg	98
K	1492
Ca	829
Ti	0.35
Fe	0.18
Zn	1.1
Rb	1.6
Sr	0.45
Mo	0.1

2.2. Hydrolysis of Whey Permeate by β-Galactosidase from *Bifidobacterium bifidus*

The concentration of lactose in the cheese whey permeates employed in this work varied between 180–200 g/L depending on the batch, as determined by HPAEC-PAD. The pH of whey was adjusted to 6.8. We selected *Bifidobacterium bifidum* β-galactosidase (Saphera®, Novozymes, Bagsværd, Denmark) for the hydrolysis of lactose in whey because we recently observed that at lactose concentrations lower than 200 g/L the main reaction catalyzed by this enzyme is the hydrolysis with negligible transgalactosylation [43]. In order to standardize the dose of enzyme, we measured the activity of Saphera with *o*-nitrophenyl-β-D-galactopyranoside (ONPG). The activity was 1506 ± 0.1 U/mL at 40 °C and pH 6.8, which are the typical preferred conditions for several β-galactosidases from *Bifidobacteria* [44,45].

First, we analyzed the effect of temperature on lactose hydrolysis in whey permeate. We assayed three different temperatures (30, 40 and 45 °C) using 2.5 μL (3.75 ONPG units) per mL of whey. HPAEC-PAD showed that the fastest reaction was the one performed at 45 °C (Table 2). Under these conditions, all the lactose is hydrolyzed in 3 h. We further increased the enzyme concentration up to 7.50 ONPG units per mL but the improvement in the hydrolysis process was not substantial. In order to minimize the cost for the process, we selected the lowest concentration of this enzyme.

Table 2. Effect of temperature on the progress of lactose hydrolysis in whey permeate by Saphera, using 2.5 μL of enzyme (3.75 ONPG units) per mL of whey permeate.

Temperature (°C)	Residual lactose (%) [a]					
	30 min	60 min	90 min	120 min	150 min	180 min
30	89.3	81.5	76.6	65.5	57.1	24.7
40	73.4	45.6	39.7	36.9	21.3	18.7
45	76.3	31.7	29.8	26.1	10.7	0

[a] Measured by HPAEC-PAD.

2.3. Study of the Growth Time and Concentration of *Pichia pastoris* for Elimination of D-Glucose

Pichia pastoris is capable of using D-glucose, glycerol and methanol as carbon sources but not D-galactose [46]. In this context, Avila-Fernandez et al. successfully employed *P. pastoris* cells to selectively eliminate glucose and fructose in a syrup of fructooligosaccharides obtained by agave fructan hydrolysis [47]. Based on such background, the elimination of D-glucose using *P. pastoris* cells was postulated for the second step of the reaction.

We first developed a simple and fast test to evaluate the effect of *P. pastoris* growing time on the further consumption of glucose. Thus, samples from a *P. pastoris* culture were taken every two hours (after an initial lag phase of 6 h), and the absorbance at 600 nm (as turbidity measurement) of all samples was adjusted to the absorbance at 6 h, to have the same concentration of cells but with

different growing times. The *P. pastoris* cells were then mixed with a solution containing 1 g/L of both D-glucose and D-galactose, and the mixture was incubated for 10 min at 30 °C. The cells were separated by centrifugation and the concentration of both sugars was analyzed by HPAEC-PAD (Figure 1). Our results showed that the most voracious cells were those corresponding to 12–16 h growing time. It is well reported that these times correspond the exponential phase of growth of *P. pastoris* [48].

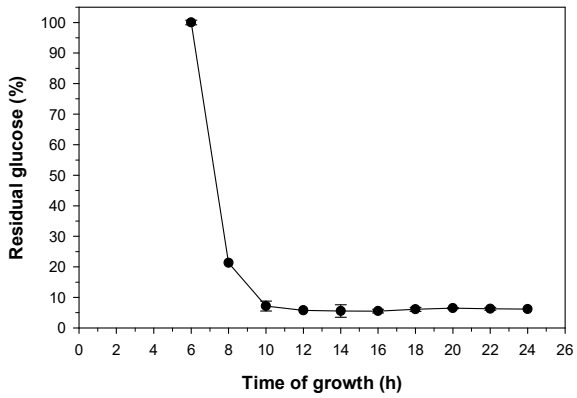

Figure 1. Effect of the growth time of *Pichia pastoris* on the elimination of D-glucose. Experimental conditions: 1 g/L D-glucose, 1 g/L D-galactose, 10 min, 30 °C.

The next step was to determine the amount of cells (grown for 16 h) required to consume the glucose (90–100 g/L) released after lactose hydrolysis in whey permeate. Different amounts of *Pichia pastoris* cells (100–600 mg of wet weight) were added to 1 mL of a solution containing 100 g/L of D-glucose and 100 g/L D-galactose. The residual glucose was analyzed at 1, 2 and 3 h by HPAEC-PAD (Figure 2). As illustrated, the glucose disappeared in one hour using 600 mg of *P. pastoris* cells per mL. However, adding 350 mg of cells per mL, the total disappearance of glucose took place in two hours, and the mass transfer limitations were less significant than with 600 mg cells per mL. On this basis, we selected 350 mg of cells per mL (3 h incubation) to assure the elimination of glucose in the integrated 3-step process for D-tagatose synthesis.

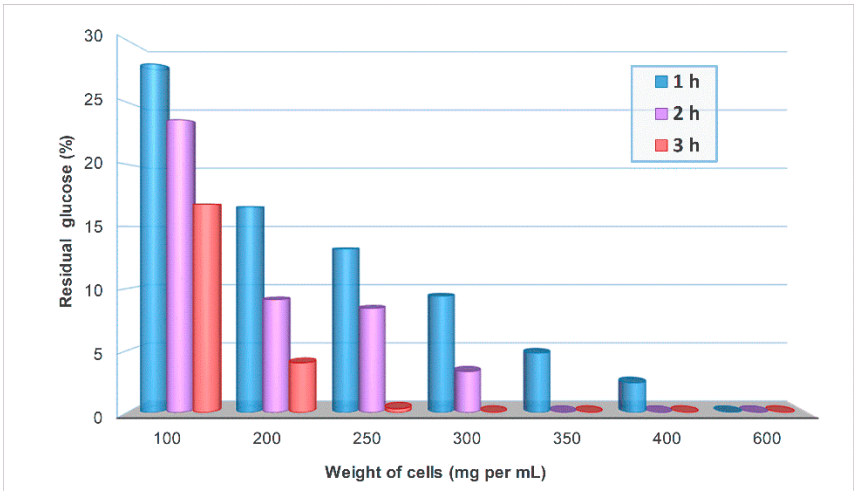

Figure 2. Effect of the amount of cells (wet weight) of *Pichia pastoris* on the elimination at 30 °C of D-glucose in a solution containing 100 g/L of both D-glucose and D-galactose.

2.4. *Effect of pH and Temperature on the Stability of L-Arabinose Isomerase*

The L-arabinose isomerase (L-AI US100) from *Bacillus stearothermophilus* is a multimeric enzyme formed by four 56 kDa monomers [49]. Compared with other L-arabinose isomerases, L-AI US100 is

relatively active at neutral and slightly acidic pH, and at moderate temperatures [50]. These properties are very valuable because at the typical conditions of alkaline pH and high temperature the formation of undesired byproducts in bioconversions of sugars is quite common [51]. For these reasons, L-AI US100 was selected for the transformation of whey permeate into D-tagatose.

In order to establish the optimal conditions for the biotransformation, the stability of L-AI US100 towards pH was evaluated by pre-incubation (at room temperature) of the enzyme for 1 h at pH values between 3.0 and 10.0 employing 0.1 M Britton & Robinson buffer. After that, the L-arabinose isomerase activity was measured following the standard activity assay (at 65 °C and pH 7.5, see Experimental Section). The results shown in Figure 3A represent the relative activity of pre-treated L-AI compared with the non-incubated enzyme. As illustrated, the enzyme is very unstable at pH values below 6.0 and is notably stable at neutral and moderately alkaline pH values.

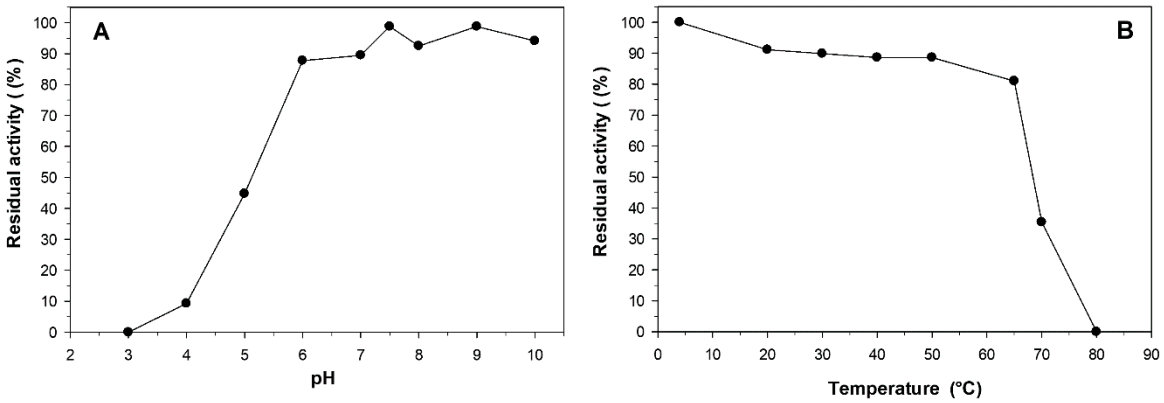

Figure 3. Stability of L-arabinose isomerase US100 from *Bacillus stearothermophilus* towards: (**A**) pH; (**B**) temperature. The pre-incubation time in both cases was 1 h.

The thermal stability of the enzyme was determined by pre-incubating the L-AI US100 for 1 h at temperatures between 4 and 80 °C in 100 mM MOPS buffer (pH 7.5). Following this incubation, the L-AI activity was measured by the standard activity assay (at 65 °C and pH 7.5). As shown in Figure 3B, the enzyme is very stable at temperatures up to 65 °C, and was fast inactivated at temperatures higher than 70 °C.

2.5. Effect of Cofactors, pH and Temperature on the Activity of L-Arabinose Isomerase

The effect of metal cofactors on the activity of L-arabinose isomerase (L-AI US100) from *B. stearothermophilus* at 65 °C and neutral pH was assessed. Four different reactions were performed in absence or presence of 0.5 mM $MnSO_4$ and/or 0.1 mM $CoCl_2$. The reactions were carried out with 20 g/L galactose in 100 mM MOPS buffer (pH 7.5) at 65 °C for 4 h. Results are displayed in Figure 4.

As shown, $CoCl_2$ had a negligible effect on the yield of D-tagatose under these conditions. However, the production of D-tagatose in the presence of 0.5 mM $MnSO_4$ was approximately 40% higher compared with the control.

These results correlate well with previous findings on the effect of metallic ions on the L-AI US100 activity and thermostability. The effect of divalent cations on L-AI US100 must be particularly considered at temperatures higher or equal to 65 °C [49]. In fact, at temperatures above 65 °C both Mn^{2+} and Co^{2+} exert a significant influence on the thermostability of L-AI US100. Since a temperature of 65 °C was selected for the isomerization reaction, and considering that the conversion from D-galactose to D-tagatose was higher in presence of $MnSO_4$ in the reaction medium, we decided to maintain $MnSO_4$ but not $CoCl_2$ in the integrated process from whey to D-tagatose.

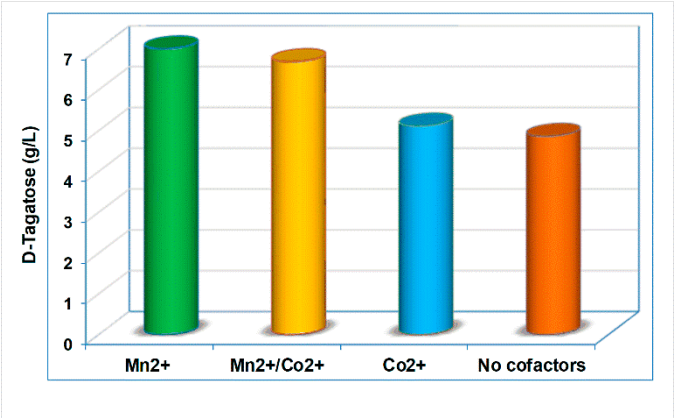

Figure 4. Effect of metal ions on the activity of L-arabinose isomerase US100 from *B. stearothermophilus*, in absence or presence of 0.5 mM $MnSO_4$ and/or 0.1 mM $CoCl_2$. Experimental conditions: 20 g/L D-galactose, 100 mM MOPS (pH 7.5), 0.65 U/mL L-AI, 65 °C. The concentration of D-tagatose was measured after 4 h by HPAEC-PAD.

Furthermore, we analysed the effect of pH and temperature on the production of D-tagatose. We selected the range of pH (7.0–9.5) and temperature (40–65 °C) at which the L-arabinose isomerase from *B. stearothermophilus* was stable (see Figure 3). The reactions were carried out with 20 g/L galactose in 100 mM MOPS buffer for 4 h, in presence of 0.5 mM $MnSO_4$. Results are shown in Figure 5. It remains clear that temperature substantially affects the reaction course. The highest production of D-tagatose in 4 h (7–8 g/L, 35–40% yield) was achieved at 65 °C. The reaction was 6–7 fold faster than at 40 °C. Regarding the pH, its effect was very much lower than that of temperature (Figure 5).

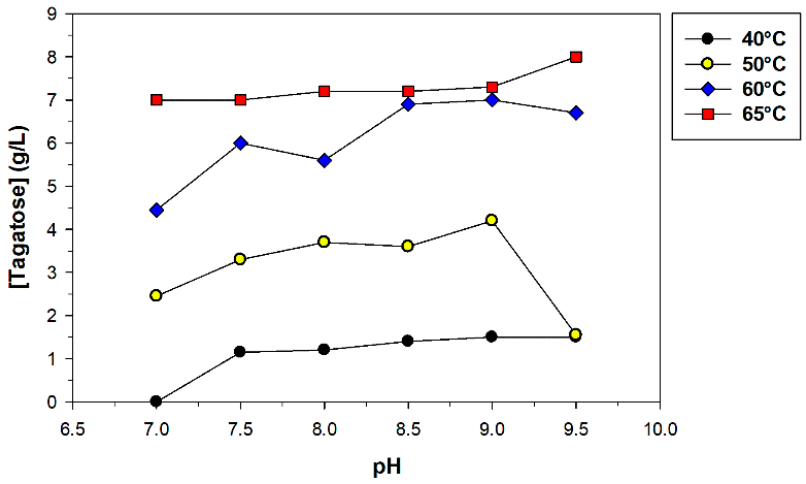

Figure 5. Effect of temperature and pH on the production of D-tagatose by L-arabinose isomerase US100 from *B. stearothermophilus*. Experimental conditions: 20 g/L D-galactose in buffer 100 mM MOPS containing 0.5 mM $MnSO_4$, 0.65 U/mL L-AI. The concentration of D-tagatose was measured after 4 h by HPAEC-PAD.

Considering the results on the effect of pH, temperature and metal cofactors on the stability and activity of L-AI US100, we selected pH 7.5, 65 °C and 0.5 mM $MnSO_4$ as the optimal experimental conditions for the integrated process. These results represent a compromise between activity and stability of the enzyme and correlate well with previous studies reported with this enzyme by using fast spectrophotometric assays [49,50,52] rather than chromatographic analysis by HPAEC-PAD performed in this work.

2.6. Sequential Biotransformation of Whey Permeate into D-Tagatose

We propose a three-step process for D-tagatose synthesis from whey permeate based on the experimental conditions that were previously optimized. In the first stage, the lactose (180–200 g/L) contained in whey is hydrolyzed by β-galactosidase from *Bifidobacterium bifidum* (7.5 U/mL). Total hydrolysis is achieved in 3 h at 45 °C. Figure 6 shows a HPAEC-PAD chromatogram illustrating the complete disappearance of lactose in the reaction medium.

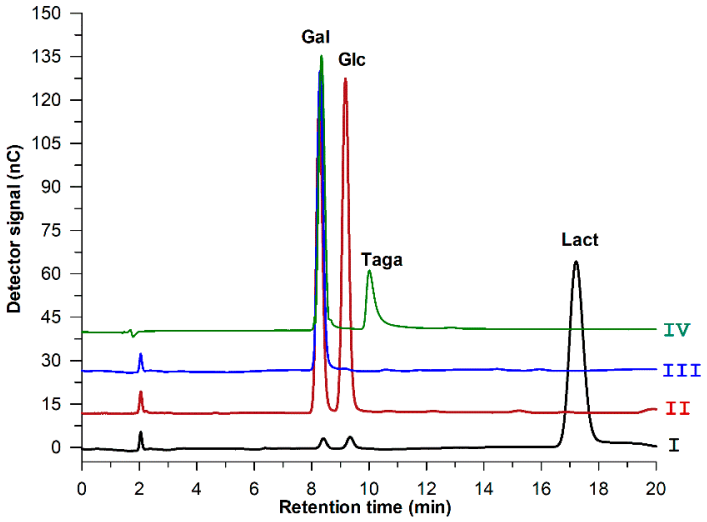

Figure 6. HPAEC-PAD chromatograms showing the bioconversion of whey permeate into D-tagatose syrup. (I) Original whey permeate; (II) After treatment with β-galactosidase from *B. bifidum*; (III) After removal of glucose with *P. pastoris* cells; (IV) After isomerization with of L-arabinose isomerase US100. Gal: D-Galactose; Glc: D-glucose; Taga: D-tagatose; Lact: D-Lactose.

In the second step, the hydrolyzate is treated with *Pichia pastoris* cells (350 mg of cells per mL, obtained as described elsewhere) during 3 h at 30 °C. As shown in Figure 6, all the glucose is metabolized by the yeast and the amount of D-galactose remains intact. After centrifugation to remove the cells, the pH of supernatant is adjusted to 7.5 (because the medium is acidified during the treatment with *P. pastoris*) and $MnSO_4$ is added to reach a final concentration of 0.5 mM. These adjustments prepare the mixture for the third step.

Finally, L-arabinose isomerase L-AI US100 is added (0.65 U per mL in the mixture, measured by the standard assay). The reaction is incubated at 65 °C and different aliquots are taken to follow the progress of the reaction. As depicted in Figure 6, D-tagatose is formed and the final product contains a mixture of D-tagatose and D-galactose.

Figure 7 shows the progress of D-tagatose formation over time. The concentration of D-tagatose increases rapidly during the first four hours and then tends to stabilize in approximately 30 g/L. Starting of a whey permeate with 180 g/L lactose, the yield of D-tagatose referred to lactose was 16.7%, and 33.3% referred to the D-galactose present in whey. We believe that this yield could be improved by using a higher concentration of L-AI thus minimizing the enzyme inactivation effects (see Figure 3).

Wanarska et al. reported a 30% yield of D-tagatose (referred to D-galactose) using a strain of *Pichia pastoris* that co-expressed L-AI and β-galactosidase, starting of a whey permeate containing 110 g/L lactose [37]. Zheng et al. employed a whey with 110 g/L lactose to get 43.6% yield of D-tagatose (referred to D-galactose) using a genetically engineered strain of *Escherichia coli* that expressed L-AI [32]. Similar results (40.4% yield) were reported by Xu et al. employing *E. coli* that co-expressed a β-galactosidase from *Thermus thermophilus* and L-arabinose isomerase from *Lactobacillus fermentum* [38,39]. Jayamuthunagai et al. reported 38% yield of D-tagatose (referred to D-galactose) from a hydrolyzed whey permeate containing 300 g/L D-galactose, using permeabilized

and alginate-entrapped cells of *Lactobacillus plantarum* [36]. Shen et al. expressed xylose isomerase and L-AI in a strain of *Corynebacterium glutamicum* able to metabolize lactose. Starting from whey containing 98 g/L lactose, they obtained 20.4 g/L D-tagatose (44% yield referred to D-galactose) [33].

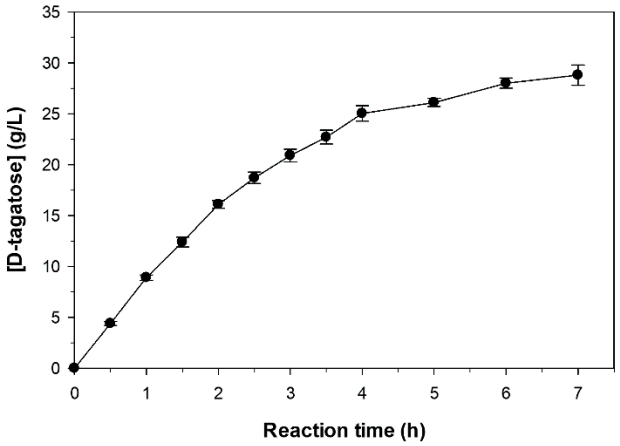

Figure 7. Progress of formation of D-tagatose from D-galactose syrup obtained by hydrolysis of whey permeate. Experimental conditions: 90 g/L D-galactose (coming from 180 g/L lactose in whey permeate), 0.65 U/mL L-arabinose isomerase US100, 0.5 mM $MnSO_4$, pH 7.5, 65 °C.

A scheme summarizing the different steps of the integrated process is presented in Figure 8.

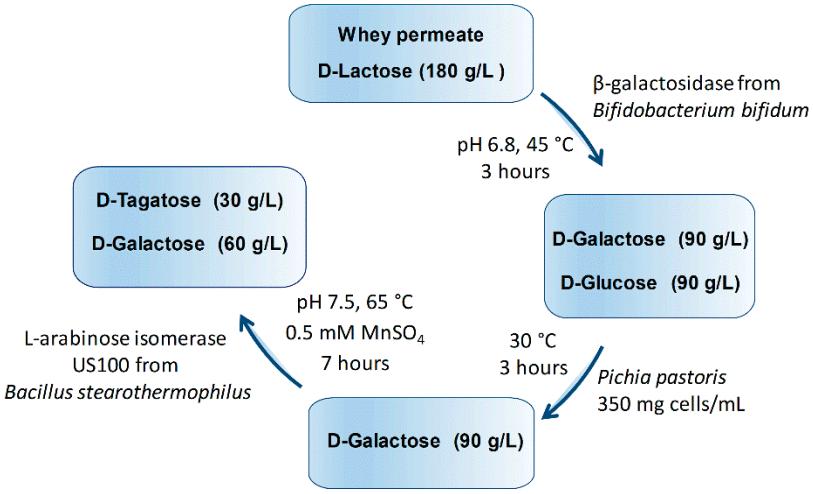

Figure 8. Scheme of the integrated process for bioconversion of whey permeate into D-tagatose syrup.

3. Materials and Methods

3.1. Enzymes and Reagents

L-Arabinose isomerase (L-AI) from *Bacillus staerothermophilus* US100 (L-AI US 100) was recombinantly produced in *E. coli* as described in previous publications [50,52,53]. The β-galactosidase from *Bifidobacterium bifidus* (Saphera®, Novozym 46091) was gently donated by Novozymes A/S (Bagsværd, Denmark). *Pichia pastoris* GS115 (*his4*) was obtained from Invitrogen (Carlsbad, CA, USA). Concentrated whey permeate (180–200 g/L) was kindly donated by Innolact (Castro de Rei, Lugo, Spain). D-Galactose and D-glucose were purchased from Sigma-Aldrich (Madrid, Spain). D-Tagatose was acquired from Tokyo Chemical Industry Co. (Tokyo, Japan). Lactose and sodium acetate trihydrate were from Fisher Chemical (Madrid, Spain). NaOH 50% (*v*/*v*) was from Acros Organics (Geel, Belgium). All other reagents and solvents were of the highest purity grade available.

3.2. Elemental Analysis of Whey Permeate using Inductive Coupled Plasma Mass Spectrometry

Semiquantitative analysis of metals in whey permeate was performed on a NexION 300X Inductively Coupled Plasma-Mass Spectrometer (ICP-MS, PerkinElmer, Waltham, MA, USA) equipped with Universal Cell Technology, as previously described [54].

3.3. β-Galactosidase Activity Assay

The assay of β-galactosidase activity was performed using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate. The activity was measured at 40 °C following *o*-nitrophenol (ONP) release at 405 nm using a microplate reader (Versamax, Molecular Devices, San Jose, CA, USA). The reaction was started by adding 10 μL of the enzyme solution (properly diluted) to 190 μL of 15 mM ONPG in 0.1 M sodium phosphate buffer (pH 6.8). The increase of absorbance at 405 nm was followed continuously at 40 °C during 5 min. The molar extinction coefficient of *o*-nitrophenol at pH 6.8 was 1627 M^{-1} cm^{-1}. One unit of activity (*U*) was defined as the corresponding to the hydrolysis of 1 μmol of ONPG per min under the above specified conditions.

3.4. Lactose Hydrolysis in Whey by Bifidobacterium Bifidum β-Galactosidase

Concentrated whey permeate (2 mL, containing 180–200 g/L lactose) was mixed with β-galactosidase from *Bifidobacterium bifidum* (Saphera®, 5–10 μL, 3.75–7.5 ONPG units per mL). The mixture was incubated at 30, 40 or 45 °C in an orbital shaker (Vortemp 1550, Labnet International, Big Flats, NY, USA) at 200 rpm. 200 μL aliquots were taken from the reaction vial each 30 minutes until 3 hours. The enzyme was then inactivated by incubating the samples in a Thermomixer (Eppendorf, Hamburg, Germany) for 10 min at 95 °C. Samples were then filtered using micro-centrifuge filter tubes, with 0.45 μm cellulose acetate filters (National Scientific, Claremont, CA, USA) at 6000 rpm for 5 min. The samples were diluted with water (1:400 and 1:4000) and then analysed using HPAEC-PAD.

3.5. Effect of Growth Time and Concentration of Pichia pastoris on the Elimination of D-Glucose

Pichia pastoris preculture was grown in 5 mL of 1% yeast extract, 1% peptone and 2% dextrose (all *w/v*) liquid medium (YEPD) at 30 °C and 1200 rpm for 24 h. After this time, the absorbance was measured, and 1 mL of preculture was added to a 250 mL flask containing 25 mL of YEPD, which was left growing at 30 °C and 1200 rpm. The first sample was taken at 6 h. The optical density (OD) was measured at 600 nm in a UV-1800 spectrophotometer (Shimadzu, Kioto, Japan) taking this OD as the reference value. The cells were centrifuged and washed three times with distilled water to remove the remaining medium from the cells. Samples were taken every 2 h until 24 h. Before centrifugation, all the samples were diluted to reach the reference value of absorbance, with the aim of having the same amount of cells in the experiments but with different growing times. Reactions with *P. pastoris* cells were performed adding 500 μL of a solution containing 1 g/L D-glucose and 1 g/L D-galactose to the cells. Reactions were incubated in a tube rotator (Argos Technologies Inc., Vernon Hills, IL, USA) for 10 min at 30 °C, then samples were centrifuged, the supernatant was inactivated at 95 °C for 10 min and analyzed by HPAEC-PAD. To determine the amount of cells required to consume the glucose expected in the sequential process for production of D-tagatose, different amounts of *P. pastoris* cells (300, 400, 500 and 600 mg of wet weight) were added to 1 mL of a solution containing 100 g/L D-glucose and 100 g/L D-galactose. Aliquots were withdrawn at 1, 2 and 3 h. After each extraction, the samples were centrifuged at 10,000 rpm for 3 min and the concentration of glucose and galactose in the supernatant was analyzed by HPAEC-PAD.

3.6. L-Arabinose Isomerase Activity Assay

The L-arabinose isomerase (L-AI) activity was determined adding the enzyme to a solution containing 20 g/L of D-galactose in 100 mM MOPS buffer (pH 7.5) containing 0.5 mM $MnSO_4$ and 0.1 mM $CoCl_2$. The mixture was incubated at 65 °C for 4 h, and the reaction stopped in a water bath at

90 °C for 10 min. The concentration of D-tagatose was measured by HPAEC-PAD. One unit of L-AI activity (U) was defined as the corresponding to the formation of 1 μmol of D-tagatose per minute, under the conditions specified above.

3.7. Stability of L-Arabinose Isomerase

The stability of L-arabinose isomerase (L-AI US 100) from *B. stearothermophilus* towards pH was assessed pre-incubating the enzyme at different pH values (3.0 to 10.0) in 0.1 M Britton & Robinson buffer [55] at room temperature for 1 h. The thermal stability of the enzyme was determined by pre-incubating the L-AI at 4, 20, 30, 40, 50, 65, 70 and 80 °C in 100 mM MOPS buffer (pH 7.5) for 1 h. After incubation, the remaining L-arabinose isomerase activity was measured following the standard activity assay (Section 3.6). The activity of pre-treated L-AI US100 was compared with the activity of non-pre-incubated enzyme that was taken as control (100%).

3.8. Effect of Metals, pH and Temperature on the Activity of L-Arabinose Isomerase

To analyze the effect of metal cofactors on the activity of L-arabinose isomerase (L-AI US 100), different reactions were carried out in presence of 0.5 mM $MnSO_4$ and/or 0.1 mM $CoCl_2$. The reactions were carried out with 20 g/L galactose in 100 mM MOPS buffer (pH 7.5) at 65 °C for 4 h, using 0.65 U/mL L-AI, and the concentration of D-tagatose was determined by HPAEC-PAD. A control reaction without cofactors was also performed. To determine the best reaction conditions for the enzyme, different values of pH (7, 7.5, 8, 8.5, 9, 9.5) and temperature (40, 50, 60 and 65 °C) were tested, in a reaction containing 20 g/L of D-galactose, 0.5 mM of $MnSO_4$ in 100 mM MOPS buffer (pH 7.5) and 0.65 U/mL L-AI. After 4 h, the reaction mixtures were analyzed by HPAEC-PAD.

3.9. Sequential Biotransformation of Whey Permeate into D-Tagatose

The biosynthesis of D-tagatose syrup from whey permeate was performed in three steps. Initially, the pH of whey was adjusted to 6.8. In the first stage, β-galactosidase from *Bifidobacterium bifidum* (5 μL, 7.5 U/mL measured with ONPG) was added to 2 mL of concentrated whey permeate (containing 180–200 g/L lactose, depending on the batch). The mixture was incubated for 3 h in an Envirogenie orbital stirrer (Scientific Industries Inc., Bohemia, NY, USA) at 45 °C. The second step involved the treatment of the hydrolyzate with 700 mg (wet weight) of *Pichia pastoris* cells (previously grown for 16 h) and the mixture was incubated in an orbital shaker (Orbitron, Infors HT, Surrey, UK) for 3 h at 30 °C. Then, the cells were removed by centrifugation (model 5810, Eppendorf) at 5000 rpm for 20 min at 4 °C. The supernatant was then separated from the cells and inactivated in a water bath at 90 °C for 10 min. In the third stage, the pH of supernatant was adjusted to 7.5 and $MnSO_4$ was added up to a final concentration of 0.5 mM. Then, 1.3 units of L-arabinose isomerase L-AI US100 were added (0.65 U/mL in the mixture). The reaction was incubated in a thermoshaker (model TS-100, Biosan, Riga, Latvia) at 65 °C and 1500 rpm. Aliquots were taken every 30 min, filtered on UltraFree centrifugal filters (0.45 μm, Millipore, Burlington, MA, USA) and analysed by HPAEC-PAD.

3.10. HPAEC-PAD Analysis

Sugar (D-galactose, D-glucose, D-tagatose, D-fructose and D-lactose) analysis was carried out by high performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) on an ICS3000 system (Dionex, Thermo Fischer Scientific Inc., Waltham, MA, USA) consisting of a SP gradient pump, an electrochemical detector with a gold working electrode and Ag/AgCl as reference electrode, and an autosampler (model AS-HV). All eluents were degassed by flushing with helium. A pellicular anion-exchange 4 × 250 mm Carbo-Pack PA-1 column (Dionex) connected to a 4 × 50 mm CarboPac PA-1 guard column was used at 30 °C. Eluent preparation was performed with MilliQ water, 50% (*w*/*v*) NaOH and sodium acetate trihydrate. The compounds were eluted by an isocratic method in which the mobile phase contained 10 mM NaOH and 2 mM sodium acetate, using a flow rate of 1 mL/min for 25 min. The peaks were analyzed using the Chromeleon

software. Identification of the different carbohydrates was carried out employing commercially available standards.

4. Conclusions

The present work describes an environmentally friendly process for the bioconversion of cheese whey, a lactose-rich byproduct of the food industry, into D-tagatose, a rare sugar that has become one of the most promising low-calorie sweeteners in the market due to its functional properties. The methodology is based in the complete hydrolysis of lactose by a bifidobacterial β-galactosidase, followed by the selective removal of glucose with *Pichia pastoris* cells, and finally the isomerization of the remaining D-galactose into D-tagatose by L-arabinose isomerase from *Bacillus stearothermophilus*. The three steps were optimized independently in such a way that the integrated process is carried out in a short time (13 h) yielding 33.3% of D-tagatose (referred to the initial D-galactose). The total time could be even reduced by using a higher concentration of L-AI in the third step. One of the main advantages of the proposed project is its sustainability, as the three steps take place under mild conditions of pH (6.8–7.5), moderate temperatures (30–65 °C) and with completely biodegradable catalysts. The final syrup is free of glucose, and contains D-tagatose and D-galactose in a ratio 1:2 (*w*/*w*). This work opens new possibilities for the synthesis of D-tagatose and for the development of different reaction engineering strategies including enzyme immobilization.

Author Contributions: F.J.P., S.B. and A.O.B. conceived and designed the experiments; F.V.C. carried out most of the experiments; S.N. and S.B. produced and characterized the L-AI US100; Z.M., J.V.-G. and M.F.-L. were in charge of the microbiological issues in this work; L.F.-A. contributed to the chromatographic analysis of carbohydrates; F.J.P. wrote the paper, which was improved by the rest of authors. All authors have read and agreed to the published version of the manuscript.

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