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Optimization of reaction conditions and the donor substrate in the synthesis of hexyl-β-D-galactoside

Carlos Vera*, Cecilia Guerrero, Lorena Wilson, Andrés Illanes

School of Biochemical Engineering, Pontificia Universidad Católica de Valparaíso, Avenida Brasil 2085, Valparaíso, Chile

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ABSTRACT

Reaction conditions were optimized and the donor substrate selected for maximizing the reaction yield and productivity of the enzymatic synthesis of hexyl-β-galactoside with β-galactosidase from *Aspergillus oryzae*. We independently studied the effect of water content, type of cosolvent, temperature, donor substrate concentration, and leaving group of the donor substrate on the yield and productivity of hexyl-β-galactoside synthesis. Reaction yield was maximum in the medium with 70% water content and acetone as cosolvent, corresponding to a water activity of 0.94. Temperature and donor substrate concentration had very little effect on the yield. The leaving group of the donor substrate was the most relevant variable. Lactose, lactulose, o-nitrophenyl-β-D-galactopyranoside, and propyl- and butyl-β-D-galactoside (the last two are enzymatically synthesized from lactose) were evaluated as donor substrates. Use of propyl- and butyl-β-galactoside as donor substrates allowed us to increase the product yield by 683% and 716% [vs. lactose (0.06 mol/mol)]. Because propyl- and butyl-β-galactosides can be synthesized at low cost from lactose, using them in a two-step process could be much better alternative than a one-step process with lactose; leading to a theoretical global molar yield of 0.41 and 0.37 mol/mol from propyl- and butyl-β-galactosides, respectively.

1. Introduction

Alkyl-glycosides (AGs) are nontoxic, hypoallergenic, and biodegradable nonionic surfactants [1,2]. Therefore, they are considered a sound alternative to conventional petroleum-derived surfactants such phenol ethoxylates that are suspected to harm the endocrine system [3,4]. Although AGs are considered ecofriendly, their production by chemical synthesis requires the use of high temperatures and pressures, obnoxious catalysts, and several protection/deprotection and activation steps that are environmentally threatening. Therefore, their enzymatic synthesis appears as a technology that is more in synchrony with the principles of sustainable chemistry [5,6]. In addition, the enzymatic synthesis of AGs has the advantage of a high regio- and enantioselectivity, producing anomerically pure products, which is not the case in chemical synthesis because of the presence of multiple equally reactive hydroxyl groups [5,7,8]. However, enzymatic synthesis has some drawbacks and remains technologically challenging as explained below. AGs are produced by a condensation reaction between a fatty alcohol and a carbohydrate [8]. The difference in solubility and polarity of the substrates is the major process limitation that leads to low yield and productivity of synthesis [8]. In the chemical process of synthesis, temperatures higher than 100 °C are used [5,8] to overcome this limitation, but this is not an option in the case of using enzyme catalysts. Therefore, enzymatic synthesis is usually done in the presence of an organic cosolvent to increase the solubility of the substrates [9,10]. The presence of the cosolvent also helps in reducing the water activity (a_W) of the reaction medium, thus depressing the undesirable reactions of hydrolysis [11]. However, the cosolvent may reduce the enzyme operational stability to a considerable extent [12], which is a key issue when evaluating the process economics [13]. Glycosidases have been extensively studied as catalysts for the synthesis of AGs because they are poorly selective, being capable of transferring glycosidic residues to a variety of hydroxyl-containing acceptors such as fatty alcohols [7,14]. Because values of a_W higher than 0.6 are required for glycosidase activity [5], the a_W resulting from the use of a cosolvent has to be carefully controlled. Other key variables in the enzymatic synthesis of AGs are the temperature (which affects the solubility of substrates), the reaction rate, and the enzyme stability, all of them affecting the economics of the process [15]. Synthesis of AGs with glycosidases can be conducted under thermodynamic control (reverse hydrolysis) or kinetic control. Different from lipases, glycosidases are poorly active at low a_W as this condition is inadequate for synthesis under thermodynamic control. Therefore, synthesis is usually conducted under kinetic control, where product composition is dictated

* Corresponding author.
E-mail address: carlos.vera@pucv.cl (C. Vera).

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by the enzyme kinetics, the concentrations of the substrates, their molar ratio [5,7,16], and the characteristics of the donor substrate. It has been reported that the yield and productivity of the enzymatic synthesis of transgalactosylated compounds are strongly dependent on the substrate donor, better results being obtained with non-natural donor substrates [11,17,18].

On the basis of this background information, the present work aimed to evaluate the effect of selected variables on the synthesis of alkyl-β-galactoside in order to design a suitable strategy of synthesis from lactose, within the framework of developing an attractive option for lactose upgrading. Lactose is a low-value and abundant by-product obtained from cheese whey, and its improper disposal may lead to severe environmental damages, so its upgrading is relevant for the cheese industry [19]. Enzymatic synthesis of hexyl-β-galactoside with *Aspergillus oryzae* is employed as a model reaction in this work, and the effect of the main reaction conditions are studied to maximize the product yield and productivity of synthesis. *A. oryzae* β-galactosidase was selected as the catalyst as it is commercially available at a reasonable price and has a high operational stability and transgalactosylation activity [18,20]. This enzyme has been used for decades in the food and pharmaceutical industry, having a Generally Recognized as Safe (GRAS) status given by the Food and Drug Administration of the United States of America. The effects of the type of cosolvent, water content in the reaction medium, concentration of the donor substrate, and nature of the leaving group on the yield and productivity of hexyl-β-galactoside synthesis were evaluated independently. The effect of the donor substrate was studied in detail. Five donor substrates (lactose, lactulose, 2-nitrophenyl-β-D-galactopyranoside (*o*-NPG), propyl-, and butyl-β-D-galactoside) were evaluated, and the product yield and productivity obtained for hexyl-β-galactoside synthesis was correlated with the values of the kinetic parameters (Michaelis constant and maximum initial hydrolysis rate) of the enzyme in the corresponding substrates.

2. Materials and methods

2.1. Chemical reagents and enzyme

β-Galactosidase from *A. oryzae* (Enzeco® Fungal Lactase) was kindly donated by Enzyme Development Corporation (EDC, New York, NY). The enzyme preparation was stored at 4 °C, remaining fully active during the experimental work. Monosaccharides, HPLC standards, α-lactose monohydrate, and hexyl-β-glucoside were purchased from Sigma (St. Louis, MO). *o*-NPG was obtained from Carbosynth Limited (Berkshire, UK). Lactulose (4-*O*-β-D-galactopyranosyl-D-fructose) was provided by Discovery Fine Chemicals (Wimborne, UK). All other reagents were of analytical or superior grade and purchased from Merck (Darmstadt, Germany).

2.2. Definition of β-galactosidase activity

One international unit of β-galactosidase activity (IU) was defined as the amount of enzyme that hydrolyzes 1 μmole of *o*-NPG per minute at 45 mM initial *o*-NPG concentration at 40 °C and pH 4.5. *o*-NPG hydrolysis was quantified following the release of *ortho*-nitrophenol (*o*-NP) at 420 nm using a Jenway 6715 spectrophotometer equipped with magnetically stirred cells and temperature control. The molar extinction coefficient of *o*-NP under the assay conditions was 248 M^{-1} cm^{-1}.

2.3. Quantification of substrates and products by HPLC

Donor substrates (lactose, lactulose, *o*-NPG, propyl-, and butyl-β-galactoside) and reaction products (fructose, glucose, galactose, and hexyl-β-galactoside) were quantified by high-performance liquid chromatography (HPLC) using a Jasco (Japan) delivery system equipped with a refractive index detector (RI-2031), an isocratic pump (PU-2080), an autosampler (AS-2055), and a column oven (CO-2065). Chromatograms were integrated using the ChromPass software provided by Jasco (Japan). The unreacted 1-hexanol and cosolvent in the reaction samples were removed under vacuum using a centrifugal concentrator SpeedVac SPD121P at 45 °C overnight. The resulting solid was dissolved in MilliQ water and passed through a 0.22-μm syringe filter. Samples were eluted through an Aminex® HPX-87H (300 × 7.8 mm) column using 0.005 N sulfuric acid as mobile phase at a flow rate of 0.6 mL min^{-1}. The column and detector were maintained at constant temperatures of 45 °C and 40 °C, respectively. The retention times for lactose, lactulose, glucose, fructose galactose, propyl-β-galactoside, butyl-β-galactoside, *o*-NPG, and hexyl-β-glucoside were 7.3, 7.6, 8.5, 8.9, 9.6, 11.2, 13.1, 21.0, and 23.7 min, respectively. Hexyl-β-glucoside was used as the standard for hexyl-β-galactoside as the standard for the latter was not available. Propyl- and butyl-β-galactosides were produced and purified as described by Vera et al. [21,22].

2.4. Effect of the water content and cosolvent type on the synthesis of hexyl-β-galactoside

Synthesis of hexyl-β-galactoside with *A. oryzae* β-galactosidase was conducted in 50 mL Schott bottles magnetically stirred at 900 rpm in a thermoregulated water bath at 35 ± 1 °C. All reactions had a reaction volume of 40 mL with a substrate donor (*o*-NPG or lactose) concentration of 16.6 mM and an acceptor substrate (1-hexanol) concentration of 166 mM (0.84 mL). The reactions were conducted at water contents of 10%, 20%, 50%, 60%, 70%, 80%, and 90% v/v (100 mM McIlvaine buffer pH 4.5); the remaining volume was occupied by the cosolvent (acetone or 1,4-dioxane). Reactions were started by adding 610 IU of β-galactosidase per mmol of donor substrate. Samples of 0.5 mL were removed at regular time intervals and added with an equal volume of 150 mM carbonate solution for stopping the reaction. Samples were analyzed and processed as described in Section 2.3. All assays were performed in triplicate. The water content and cosolvent producing the highest hexyl-β-galactoside yield were selected at this stage (see Section 3.1) to be used in the following experimental stages.

Reactions of synthesis were characterized in terms of the following parameters: yield (Y), specific productivity (π), and conversion (X). Because the synthesis of hexyl-β-galactoside is a kinetically controlled reaction, the above parameters were determined at the point of maximum product concentration and defined as follows:

-Yield (Y): corresponds to the moles of hexyl-β-galactoside (HG) produced per mole of donor substrate added (SD_0):

$$Y_i = \frac{HG}{SD_0} \quad (1)$$

where subscript i denotes the donor substrate.

-Specific productivity (π): corresponds to the moles of hexyl-β-galactoside (HG) produced per unit mass of enzyme preparation (M_E) and unit of reaction time (t):

$$\pi_i = \frac{HG}{M_E \cdot t} \quad (2)$$

-Conversion (X): corresponds to the percentage of donor substrate reacted during synthesis:

$$X_i = \left(\frac{SD_0 - SD}{SD_0}\right) \cdot 100 \quad (3)$$

where SD represents the nonreacted moles of donor substrate.

The incidence of secondary hydrolysis of hexyl-β-galactoside was evaluated by expanding the conceptualization from van Rantwijk et al. [7] for the efficiency of synthesis (η). This parameter is calculated using the ratio between the apparent initial rate of hexyl-β-galactoside synthesis ($r_{s,a}$) and the apparent initial rate of donor substrate hydro-