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Enzymatic Glycosidations in Dry Media on Mineral Supports

Mirjana Gelo-Pujic*, Eryka Guibé-Jampel, André Loupy

Laboratoire des Réactions Sélectives sur Supports, Institut de Chimie Moléculaire d'Orsay, Université de Paris-Sud, URA CNRS 478, Bt. 410, 91405 Orsay Cédex, France

Abstract: Preparation of 4-hydroxybutyl-β-D-glucoside was achieved by reversed hydrolysis from glucose using almond-β-glucosidase impregnated on mineral supports, while 4-hydroxybutyl-α-D-glucoside was obtained from glucose or by one-pot starch hydrolysis followed by glucosidation catalysed by γ-amylase (amyloglucosidase) from *Rhizopus* mold on celites. The influence of the support nature and of water activity were investigated in order to shift the equilibrium towards glycosidation.

Introduction

Glycohydrolases [3.2.1.x] mediated regio- and stereoselective syntheses of glycosides by transglycosidation or reversed hydrolysis are becoming widely used in the functionalisation of saccharides. The potential commercial importance of these biocatalysts is high in the field of production of different chemicals not easily synthesized by chemical means. These thermodynamically controlled glycosidations are strongly dependent upon the reaction conditions. In aqueous solution, hydrolysis is favoured by ca. 4 kcal / mol, while in anhydrous organic solvents the enzyme is inactivated and the solubility of the carbohydrates decreases. Two-phase reaction mixtures[1], addition of a water-miscible organic cosolvents[2,3], use of the acceptor molecules as the solvent with 10-40 % of water[4-8], enzymes crosslinked on duolite[9-11] and lipid coated enzymes[12-15] have been proposed to overcome the hydrolysis of the donors and the products as well as the enzyme denaturation in the presence of the acceptors, which are the major drawbacks of these reactions.

With the aim of increasing the yield in glycosidation and of limiting the excess of acceptor, we have investigated the reaction in "dry media" on mineral supports. Substrates and enzymes co-impregnated on the solid supports allow an homogenous reaction medium with products of different polarities, and at the same time slow down enzyme inactivation.

* fax: (33-1)69154679; E-mail: eriguibe@icmo.u-psud.fr

Results and Discussion

The well controlled heating of dry reaction mixture improves the kinetics of glycosidation. Higher temperatures increase the solubility of many compounds as well as the diffusion rate and allow the removal of volatile products, particularly in the absence of organic solvents.

At the same water concentration, the water activity is lower on the mineral support than in an organic solvent. The glycosidations can be performed at water activities a_w = 0.6-0.9[16].

Almond-β-glucosidase catalysed reversed hydrolysis

The reversed hydrolysis catalysed by almond-β-glucosidase is well documented[5-7]. These generally slow reactions can be considerably accelerated by working under dry conditions and at high temperatures. Glucosidations (Scheme 1) with 1,4-butanediol catalysed by almond-β-glucosidase were studied on different supports (Table 1 and Fig. 1).

Scheme 1

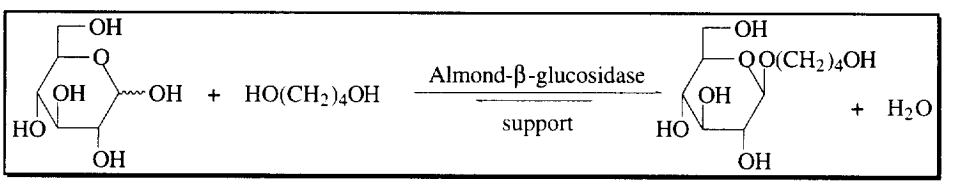


Table 1: Almond-β-glucosidase catalysed glycosidations with 1,4-butanediol (80°C, 1h).

Entry No.	Diol : Glucose (mmol)	Enzyme units / mg of substrate	Support [e] (1 g)	% Conv.	a_w [f] (starting)
1	3.8 : 1	2.5	Celite 545	36	0.8
2	4 : 1	2.3	Celite 545 AW	31	0.9
3	4 : 1	3.9	Celite 545 AW	53	0.9
4	3.7 : 1	2.5	Celite R-640	42	0.9
5	3.8 : 1	2.5	Neutral Al_2O_3	32	0.6
6	3.7 : 1[a]	2.5	Neutral Al_2O_3	55	0.6
7	4 : 1[b]	3.9	Celite 545AW	15	0.8
8	3.4 : 1[c]	2.4	Celite 545	28	0.9
9	3.6 : 1[d]	2.5	Celite 545	31	0.9

[a] surface covered with a filter paper
[b] reaction under microwaves
[c] combined conditions (Δ-0.5 h, then MW-0.5 h)
[d] 1,3-butanediol as acceptor
[e] specifications of supports: see ref. 17
[f] a_w of mixture enzyme/support/donor

Table 2: Glycosidations using *Rhizopus* mold γ-amylase on different supports (40 °C, a_w=1.0, acceptor 10 eq).

Entry No.	Support [a] (1 g)	t (days)	Conversion (%)
10	Celite 545 / Na_2SO_4	1.7	44
11	Celite 545	1.7	34
12	Celite 545 AW	2.7	28
13	Celite R-630	1.7	44
14	Celite R-640	1.7	58
15	Celite R-650	1.7	36
16	Neutral Al_2O_3	2.7	13
17[b]	Celite 545	1.7	47

[a] specifications of supports: see ref. 17
[b] 1,3-butanediol as acceptor

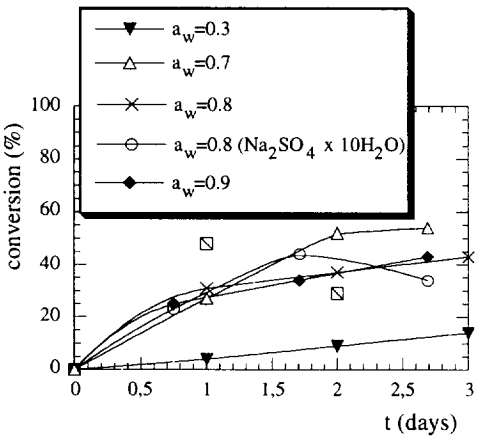


Figure 3: γ-Amylase glycosidations on celite 545 of different a_w.

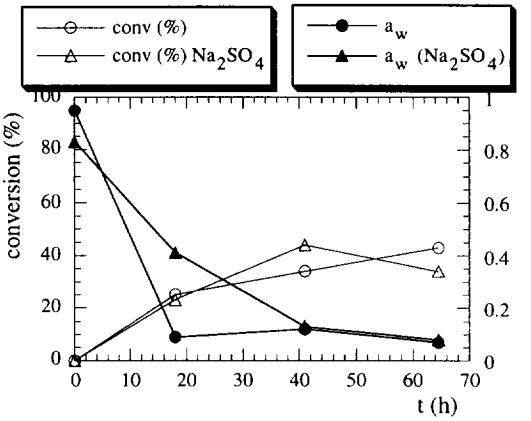


Figure 4: Conversions and a_w in the presence of an hydrated salt (Na_2SO_4 ,$10H_2O$).

Amyloglucosidase from *Rhizopus* mold is responsible for *in vivo* starch digestion which results in the breaking of every glucosidic bound with release of D-glucose. Recently, microwave irradiation has been found to facilitate acid catalysed starch hydrolysis[20]. Our previous work[21] on thermophilic *Sulfolobus solfataricus* glycosidase showed that mineral supports in the absence of organic solvent were by far the best conditions for microwave irradiation and an important enhancement in reaction kinetics was observed. We thus tested the starch hydrolysis on a mineral support and with minimal amounts of water (Scheme 2).

The excess of acceptor (ca. 300 fold), which was a solvent under "usual" conditions described in literature[5], was lowered to 3-4 equivalents and became only a reactant under solvent-free conditions. The best results (55 % conversions, entries 3 and 6) were obtained on celite 545 AW or neutral Al_2O_3 with a water activity a_w=0.9 and a_w=0.6 respectively. When neutral Al_2O_3 was used as the support, the surface was covered with a filter paper in order to avoid a rapid loss of water. Indeed, if the heating is carried out in an open system the a_w decreases rapidly (Fig 2). The glycosidations using almond-β-glucosidase stop to progress after 0.5-1 hour when a_w decreases to 0.1. This dramatic change in a_w cause the irreversible loss of enzymatic activity. In the case of almond-β-glucosidase, microwave has a significant negative effect on yield (entry 7), probably due the too fast and irreversible loss of water as microwave is more prone to water vaporization when compared to classical heating[18,19].

Almond-β-glucosidase catalysed synthesis of 4-hydroxybutyl-β-D-glucoside

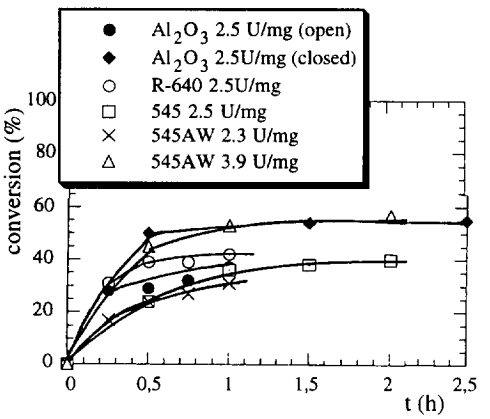


Figure 1: The influence of different supports.

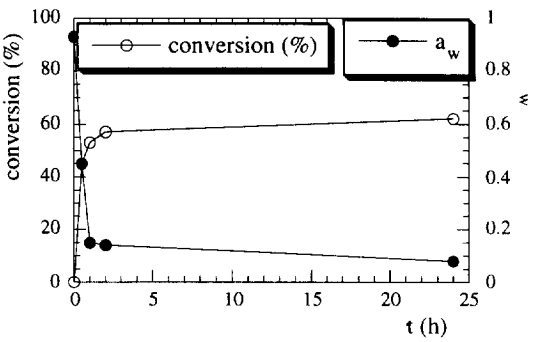


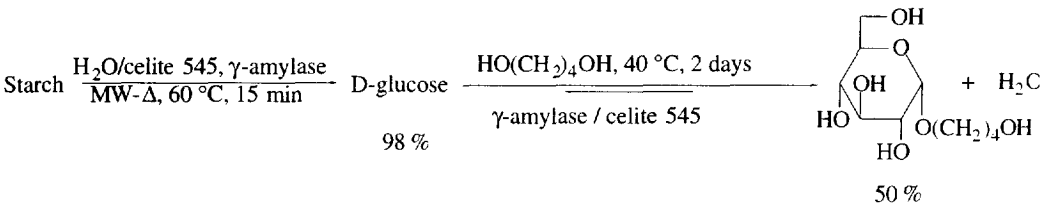
Figure 2: Conversion and a_w during the reaction.

Amyloglucosidase catalysed hydrolysis and glucosidations of starch

With the aim of comparing our glycosidation conditions with those previously described in water-organic solvent mixtures, we chose the γ-amylase (amyloglucosidase) from *Rhizopus* mold. Willemot and co-workers[5] have described the preparation of 4-hydroxybutyl-α-D-glucoside in the acceptor as a solvent (300 fold of molar excess) with 15 % of water. The equilibrium yield (25%) was obtained within 3 days.

We have investigated the same reaction under dry conditions and at the same temperature (40 °C). Under our conditions, the equilibrium is shifted towards product (yields 40-58 % obtained within 1-2 days, versus 25% in literature[5], Table 2 and Fig. 3). The best support for glycosidation in dry medium was celite R-640. The optimal a_w was found to be 0.7; at a_w inferior to 0.4 glycosidations become very slow. Water activity decreases slower in the presence of hydrated salts (Fig 4) but the conversions remain unchanged when compared with those obtained in the absence of salts.

Scheme 2



Amounts of 30-35 equivalents of water were found to be necessary for a rapid hydrolysis. The hydrolysis performed in an oil bath at 60 °C gave 30% of glucose within 5 min or 55 % within 15 min and could be accelerated under microwave irradiation, under which conditions 75 % of glucose was obtained within 5 min or 98 % within 15 min. The temperature under microwaves was controlled by modulation of power between 15 and 0 W. The mixture, thus obtained, was used directly, after addition of fresh enzyme and acceptor, in the glycosidation step, at 40 °C and under previously described conditions. The best result obtained was a conversion of 50 % after 2 days.

Experimental part

Materials and methods. Almond-β-glucosidase (activity 5 U/mg solid), amyloglucosidase from *Rhizopus* mold (activity 20 U/mg solid) and soluble starch were purchased from Sigma. Glucose, celite 545 AW, celite R-630, celite R-640 and celite R-650 were supplied from Fluka, while celite 545 and neutral Al_2O_3 (pH 7.5) from Prolabo. Supports (celite R-630, celite R-640 and celite R-650) were powdered before use. 1,3-Butanediol and 1,4-butanediol were products from Aldrich. Commercial solvents were used as such, without any further purification.

Microwave equipment. Reactions were performed in a monomode microwave reactor with focused electromagnetic field (Synthewave 402, Prolabo), fitted with a stirring system and an IR pyrometer[22].

Analytical methods. NMR spectra were recorded on Bruker instruments at 250 MHz in [D_5]Pyridine with TMS as an internal standard. Reactions were followed by GC after silylation[23] on a 6000 Vega Series with FID detector, Spectra-Physics SP 4290 integrator and a OV1 column (12 m); detector 300 °C, injector 290 °C and column temperature in the range 150-280 °C (10 °C/min). Water activity measurements were performed on a Novasina Humidat IC-3 instrument (Switzerland).

Glycoside synthesis using almond-β-glucosidase. Glucose (180-200 mg; 1-1.1 mmol) and almond-β-glucosidase (90-100 mg) dissolved in water (0.75-1 ml) were impregnated on different supports (1 g) and dried in a desiccator under vacuum (5 mmHg) till a_w values indicated in Tables and Figures. In case of Al_2O_3 lyophilisation was used to reach the a_w=0.6. The acceptor (360-400 mg; 3.4-4 mmol) was then added, and the reaction mixtures were incubated at 80 °C during the time periods indicated on Figure 1. After cooling, the mixture was washed with methanol; the products were analyzed by GC after silylation and compared with authentic samples. The product was purified on a silica gel column with $CHCl_3$-CH_3OH-H_2O 4-1-0.1 solvent mixture. Isolated yield was 5% lower than the GC yield. ^{1}H-NMR (250 MHz, [D_5]Pyr): δ (ppm) 5.29 (d, H-1, $J_{1,2}$ = 3.5 Hz), 3.85-4.65 (m, 8H: H-2, H-3, H-4, H-6a, H-6b, H-1'a, H-1'b), 3.78 (t, 2H: H-4'a, H-4'b),

3.58 (m, 1H: H-5), 1.65-1.98 (m, 4H: CH_2-CH_2 en 2' et 3'), ^{13}C-NMR: 103.2 (C-1), 71.4 (C-2), 73.7 (C-3), 70.0 (C-4), 76.5 (C-5), 62.2 (C-6), 70.8 (C-1'), 26.2 (C-2'), 29.0 (C-3'), 62.1 (C-4').

Glycoside synthesis using γ-amylase. Glucose (180-200 mg; 1-1.1 mmol) and γ-amylase (250 mg) dissolved in water (1-1.5 ml) were impregnated on different supports (1 g), and the acceptor (0.9 g; 10 mmol) was then added. The impregnation technic was the same as described above. Reaction mixtures were incubated at 40 °C during the time periods as indicated on Figure 3. After cooling to room temperature, the mixture was analyzed as described above.

Hydrolysis of starch. Celite 545 (1 g) was added to a suspension of starch (0.5-1 g) and γ-amylase (0.25-0.5 g) in water (2 ml). The mixture was irradiated under microwaves at 60 °C during 15 min with 15-0 W. Yield: 90-98% of glucose. The mixture was cooled to room temperature and fresh amyloglucosidase (0.5 g) on celite (1 g) was added, followed by addition of the acceptor (0.9 g, 10 mmol). The final mixture was incubated in an oil bath at 40 °C during 2 days. The mixture was analyzed and purified as described above. Yield: 50% of glucoside.

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16. For instance, 75 % water on celite (w/w) corresponds to $a_w = 0.9$.
17. Specifications of supports (Fluka): celite 545 particle size 20-45 μm; celite 545AW acid washed; celite R-630 pore volume 1.7 cm^3/g, surface area 3 m^2/g; celite R-640 pore volume 0.5 cm^3/g, surface area 65 m^2/g; celite R-650 pore volume 0.4 cm^3/g, surface area 60 m^2/g.
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