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Transglycosylation reactions using glycosyl hydrolases from *Thermotoga neapolitana*, a marine hydrogen-producing bacterium

Annabella Tramice, Edoardo Pagnotta, Ida Romano, Agata Gambacorta, Antonio Trincone *

Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, Via Campi Flegrei 34, 80078 Pozzuoli, Naples, Italy

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Abstract

The use of the extremophilic marine bacterium *Thermotoga neapolitana* (DSM 4359) for the bioproduction of hydrogen using complex carbohydrates as feedstock has been recently described in a patent. In this growth condition glycosyl hydrolases can be well expressed. An important issue to plan economical feasibility of biological hydrogen production comprises techniques for recovering useful materials such as physiologically active biomolecules from biomasses grown in large quantities.

The present paper describes a series of enzymatic transglycosylation reactions performed using the crude homogenate of *T. neapolitana*. The study is focused on synthetic features of the transglycosylating enzymes. Xylosidase/xylanase activity seems to be the most abundant leading to convenient syntheses of interesting series of pure (β-1,4)-xylooligosaccharides of different aglycones such as 1-hexanol (producing promising candidates for new surfactants), 9-fluorene methanol (obtaining anti-HSV agents), 1,4-butanediol (for the synthesis of new glycolipids), and geraniol (producing aroma compounds). Furthermore, the regioselectivity during galactose, fucose, glucose, and mannose enzymatic transfers is also investigated. The knowledge of synthetic characteristics of all these enzymes will be useful in the feasibility evaluation of large-scale processes of the biohydrogen production based on *T. neapolitana*.

Keywords: Glycosyl hydrolases; Xylosides; *Thermotoga*; Biocatalysis; Biohydrogen

1. Introduction

Marine ecosystem is considered a quite unexplored source of biological material and can be also a surprising font of enzymes endowed with new and interesting catalytic activities for applications in biocatalysis [1]. Marine sources are represented by marine microorganisms comprising bacterial extremophiles, and plants or animals.

Thermotoga neapolitana (DSM 4359) is an extremophilic marine bacterium belonging to the *Thermotogales* order. The ability of this microorganism to produce hydrogen has been described in a patent specifically in an environmental-friendly process using complex carbohydrates as feedstock [2]. Members of this order possess an array of important hyperthermophilic glycosyl hydrolases [3] that in the conditions adopted for hydrogen production can be well expressed.

In the last few years, glycosyl hydrolases gained interest as applicative enzymes for the convenient synthesis of biological relevant glycosidic linkages in the field of production of oligosaccharides [4,5]. They belong to inverting or retaining class on the basis of their mechanism of action. In the inverting mechanism, the hydrolysis occurs through a single displacement involving an oxocarbenium ion-like transition state. The retaining reaction mechanism involves a nucleophile group and an acid/base catalyst. This reaction proceeds by a double-displacement in which a covalent glycosyl-enzyme intermediate is firstly formed (glycosylation step) and then hydrolysed (deglycosylation step) in a general acid/base-catalysed process. Therefore, retaining glycosyl hydrolases can transglycosylate by

* Corresponding author. Tel.: +39 081 8675095; fax: +39 081 8041770.
E-mail address: antonio.trincone@icb.cnr.it (A. Trincone).

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transferring the glycone of the glycosyl enzyme to an acceptor rather than to water.

The knowledge of synthetic assets (stereoselectivity, yields, conversion of substrates, feasibility of reaction) of a new biocatalyst is a key point to plan biotechnological improvements of available enzymes [5]. Concerning glycosyl hydrolases as an example, the production of different mutants (e.g. glycosynthases, possessing further benefits in the synthetic applications [6]) was planned considering the useful features in the synthetic domain possessed by native enzymes.

An important issue in biological production of hydrogen consists of techniques for recovering useful materials including physiologically active biomolecules [7] from biomasses produced in large-scale cultivation plants. In the framework of our involvement in a research project based on biohydrogen production, we have focused on valuable catalytic activities of value in the synthesis of glycosidic linkages from *T. neapolitana*. Particular emphasis has been devoted to the use of the whole crude homogenate easily produced from the biomass. Together with the hydrolytic action, we investigated synthetic aspects of the enzymatic reactions, establishing a simple strategy for synthesis of xylooligosaccharides array with potential pharmacological interest. The knowledge of synthetic characteristics of the enzymes thus acquired is important for evaluating the economical feasibility of large-scale processes for the biohydrogen production.

2. Material and methods

2.1. Instruments and general methodology

Nitrophenyl glycosides and other substrates were obtained from Sigma–Aldrich Fluka. Reverse-phase silica gel and TLC silica gel plates were purchased from E. Merck (Darmstadt, Germany). All other chemicals were of analytical grade. Agilent UV–vis Spectroscopy System was used for analytical UV measurements. NMR spectra were recorded on Bruker instruments operating at 600, 400, and/or 300 MHz. Samples for NMR analysis were dissolved in the appropriate solvent ($CDCl_3$ for acetylated derivatives and CD_3OD for native compounds) and the downfield shift of the signal of the solvent was used as internal standard. Acetylation of compounds was performed with pyridine/Ac_2O at room temperature. The solvents were removed by a N_2 stream, and the reaction mixture was purified by silica gel chromatography or preparative TLC.

Protein concentrations were routinely estimated using the Bio-Rad Protein System, with the bovine serum albumin as the standard [8].

ESI-MS spectra were obtained on a Q-Tof mass spectrometer, Micro (Micromass).

2.2. Bacterial strain, culture conditions and preparation of crude extract

T. neapolitana DSM 4359 was obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen). Cells were grown in a 40-L fermentor (B. Brown Biotech International Micro DCU400) in TN medium (NaCl 10 g/L, KCl 0.1 g/L, $MgCl_2{\cdot}6H_2O$ 0.2 g/L, NH_4Cl 1 g/L, $CaCl_2{\cdot}2H_2O$ 0.1 g/L, K_2HPO_4 0.3 g/L, KH_2PO_4 0.3 g/L, cysteine·HCl 0.5 g/L, yeast extract 2 g/L, tryptone 2 g/L, and glucose 5 g/L). Trace metal solution (1 mL/L), and vitamin solution (1 mL/L) were prepared as described for medium 141 in the DSMZ catalogue. Anaerobic conditions were established and maintained by a continuous flow of nitrogen and pH was automatically adjusted during the growth, while temperature was controlled at 77 °C. Growth was monitored turbidimetrically at 540 nm and cells were harvested at OD 1390 and pH 5.48. The cells were collected by continuous centrifugation using an Alpha-Laval centrifuge and then pelleted by centrifugation at 10,000 rpm for 30 min and frozen. Cells (21 g, wet weight) are resuspended in 28 mL of 100 mM sodium phosphate buffer, pH 7.5, and disrupted by 2 passages through a French press cell at 1200–1300 PSI. Cell debris was removed by centrifugation at 10,000 rpm for 50 min at 4 °C, and the soluble fraction used as the crude enzyme preparation (28 mL, 1.8 mg/mL of total protein).

2.3. Enzyme assay

The presence of enzymatic activities in the crude homogenate was assayed under standard conditions: 70 °C in 0.5 mL of 100 mM Na-phosphate, pH 7.5, containing 5 mM aryl substrates (PNP-α-D-Gal, PNP-β-D-Man, PNP-α-L-Fuc, PNP-β-D-Glc, PNP-α-D-GlcNAc, PNP-α-D-Man, PNP-β-D-Gal, PNP-α-D-Glc, PNP-α-D-Xyl, PNP-β-D-Xyl, PNP-α-D-GalNAc, PNP-β-D-GalNAc, PNP-α-D-Rha, and PNP-β-D-Fuc). Aliquots (0.05 mL) were withdrawn at time intervals and the reaction was stopped with the addition of Na_2CO_3 1 M (0.45 mL) measuring the increase in absorbance at 405 nm due to *p*-nitrophenol liberated. One unit of enzyme activity was defined as the amount of protein required to catalyse the release of 1.0 μmol of *p*-nitrophenol per min from the substrates.

2.4. Enzyme reactions

The formation of transglycosylation products using PNP-β-D-Glc, PNP-β-D-Xyl, PNP-β-D-Gal, PNP-β-D-Man, PNP-β-D-Fuc was monitored at 70 °C in 100 mM Na-phosphate pH 7.5 using three different substrate concentration (15, 30, and 60 mM) and 126 μg/mL of crude protein. The same reaction was also performed on a series of di-, oligo- and poly-saccharides (5 mg substrate/mL, 90 μg/mL of crude protein). The products were analyzed by TLC (see Table 1).

Preparative reactions to study the regioselectivity in the transfer of galactose, mannose, glucose and fucose were conducted at 70 °C using the corresponding nitrophenyl β-D-glycopyranosides functioning as donor and acceptor at 300–500 mM in 100 mM Na-phosphate, pH 7.5 using ca. 950–1800 μg/mL of crude protein (galactose, 1260 μg/mL; mannose, 1800 μg/mL; glucose, 969 μg/mL; fucose, 1800 μg/mL). Almost total substrate conversion was obtained in each case after different reaction times. Chromatographic purification of the reaction mixture was based on reverse-phase chromatography (Lobar RP-18) eluting

Table 1
Hydrolysis and transglycosylation reactions performed by *Thermotoga neapolitana* crude homogenate

Substrate	Hydrolysis[a]	Transglycosylation[b]
PNP-β-D-Glc	+	*
PNP-β-D-Gal	+	*
PNP-β-D-Xyl	+	*
PNP-β-D-Fuc	+	*
PNP-β-D-Man	+	*
Maltose	−	−
Lactose	+/−	−
Saccharose	+/−	−
α-α-Trehalose	−	−
Maltoheptaose	+	ND
Pullulan	+/−	ND
Starch	+	ND
Curdlan	+	**
Xylan from birchwood	+	**
Laminarin	+	**

Hydrolysis products are clearly (+) or slightly (+/−) visible on TLC. ND: hydrolysis products are not detectable in the condition adopted. Products due to selftransglycosylation (*) or transglycosylation using different acceptors (**) are clearly visible on TLC.

[a] The hydrolysis products is analyzed by comparing the Rf with authentic sugar in different TLC solvent system.

[b] The transglycosylation products show positive UV absorbance and lower Rf than corresponding aryl substrates in EtOAc:CH_3OH:H_2O, 70:20:10 by vol or $CHCl_3$:CH_3OH: H_2O, 65:25:4 by vol TLC systems and show α-naphthol positive test and lower Rf than corresponding carbohydrate substrate in EtOAc:CH_3COOH:2-propanol:HCOOH:H_2O, 25:10:5:1:15 by vol TLC solvent.

the column in water, thus efficiently separating the chromophoric products from free saccharides in the mixture. Single chromophoric regioisomers were purified by silica gel chromatography (preparative TLC, solvent EtOAc/MeOH/H_2O 70:20:10).

2.4.1. Xylooligosaccharides of n-hexanol

The reaction for studying xylose transfer was performed at 70 °C using xylan from birchwood (20 g/L) in 100 mM Na-phosphate pH 7.5, at 1.5 M *n*-hexanol and using 540 μg/mL of crude protein. Aliquots of reaction mixture (3 mL) were withdrawn at 3, 7, 23, and 48 h and the products were initially purified by reverse phase chromatography (Lobar RP-18) eluting in H_2O with methanol increasing gradient. Silica gel chromatography was performed to obtain purified compounds which were peracetylated and subjected to NMR spectroscopy. Concentrations (mM) of major hexyl oligoxylosides obtained were determined gravimetrically.

2.4.2. 1,4-Butanediol β-D-xyloside

The reaction was conducted in 100 mM Na-phosphate, pH 7.5 containing 540 μg/mL of crude protein, 20 g/L xylan from birchwood, and 2.2 M 1,4-butanediol. After one night at 70 °C under agitation, the reaction mixture was purified on reverse-phase column chromatography (Lobar RP-18) eluting in H_2O/MeOH 7:3.

2.4.3. Geranyl β-D-xylopyranoside

The reaction was conducted in 100 mM Na-phosphate pH 7.5 containing 540 μg/mL of crude protein, 20 g/L xylan from birchwood, and 1.1 M geraniol. After one night at 70 °C under agitation, the reaction mixture was subjected to reverse-phase column chromatography (Lobar RP-18) eluting in water, thus efficiently separating the xylosylated product from free saccharides. The product was further subjected to silica gel column chromatography and preparative TLC (solvent EtOAc/MeOH/H_2O 70:20:10) and peracetylated for NMR spectroscopy.

2.4.4. β-D-Xylooligosaccharides of 9-fluorene methanol

The reaction was conducted in CH_3CN/100 mM Na-phosphate pH 7.5 (13:87 v/v) containing 257 μg/mL of crude protein, 20 g/L xylan from birchwood and 0.11 M 9-fluorene methanol. After 6.5 h at 70 °C under agitation, the reaction mixture was subjected to repeated reverse-phase column chromatography (Lobar RP-18) eluting in water thus efficiently separating the chromophoric xylosylated product from free saccharides. Single products were further purified by preparative TLC (solvent EtOAc/MeOH/H_2O 70:20:10).

2.5. Structural determination

After chromatographic purification and usual acetylation procedure, ^{1}H NMR spectra and 2D COSY and TOCSY experiments allowed chemical shifts assignment of the products and the unambiguous structure determination. In the COSY spectrum, starting from the anomeric proton signal of sugars and following the correlations through pyranosidic protons, it is easy to detect the glycosylated position as indicated by upfield shift of the signal due to the absence of acetyl group and the downfield shift of the corresponding carbon signal. β-Configuration of mannosides was established by the 160 Hz value of coupling constant ($J_{(C,H)}$), which was measured for the C-1 of the added mannose residue in the disaccharides formed [9].

Mass spectra of oligoxylosides were performed before acetylation to assess molecular weights of native oligosaccharides.

The assignments of diagnostic signals for the compounds synthesized are reported below.

Peracetyl derivative of hexyl β-xyloside **1** (Fig. 1): xylose moiety, 4.46 H1 (J = 7.8 Hz), 4.90 H2, 5.15 H3, 4.94 H4, 4.11–3.34 H5; aglycon moiety, 3.79–3.43 H1′, 1.55 H2′, 1.28 H3′–H5′, 0.88 H6′.

Peracetyl derivative of hexyl β-xylobioside **2** (Fig. 1): internal alkyl linked xylose moiety, 4.55 H1 (J = 7.8 Hz), 4.78 H2, 5.10 H3, 3.83 H4, 3.97–3.29 H5; external xylose moiety, 4.40 H1 (J = 7.9 Hz), 4.83 H2, 5.08 H3, 4.87 H4, 4.08–3.38 H5; aglycon moiety, 3.78–3.46 H1′, 1.56 H2′, 1.26 H3′–H5′, 0.87 H6′.

Peracetyl derivative of hexyl β-xylotrioside **3** (Fig. 1): internal xylose moieties (signals are interchangeables), 4.46, 4.39 H1 (J = 7.8 Hz), 4.82, 4.84 H2, 5.10 H3, 3.80 H4, 3.92–3.33 H5; external xylose moiety, 4.54 H1 (J = 7.9 Hz), 4.83 H2, 5.07 H3, 4.87 H4, 4.06–3.35 H5; aglycon moiety, 3.80–3.39 H1′, 1.55 H2′, 1.28 H3′–H5′, 0.89 H6′.

1,4-Butanediol β-D-xyloside **4** (Fig. 1): xylose moiety, 4.22 (H1, J = 7.8 Hz), 4.0–3.1 (H2–H5 xylose, H1′ and H4′ butanediol moiety), 1.7 (H2′–H3′ butanediol moiety). ^{13}C NMR: 105.1, 74.9, 71.2, 70.5, 66.9, 62.7, 30.2, 27.2.

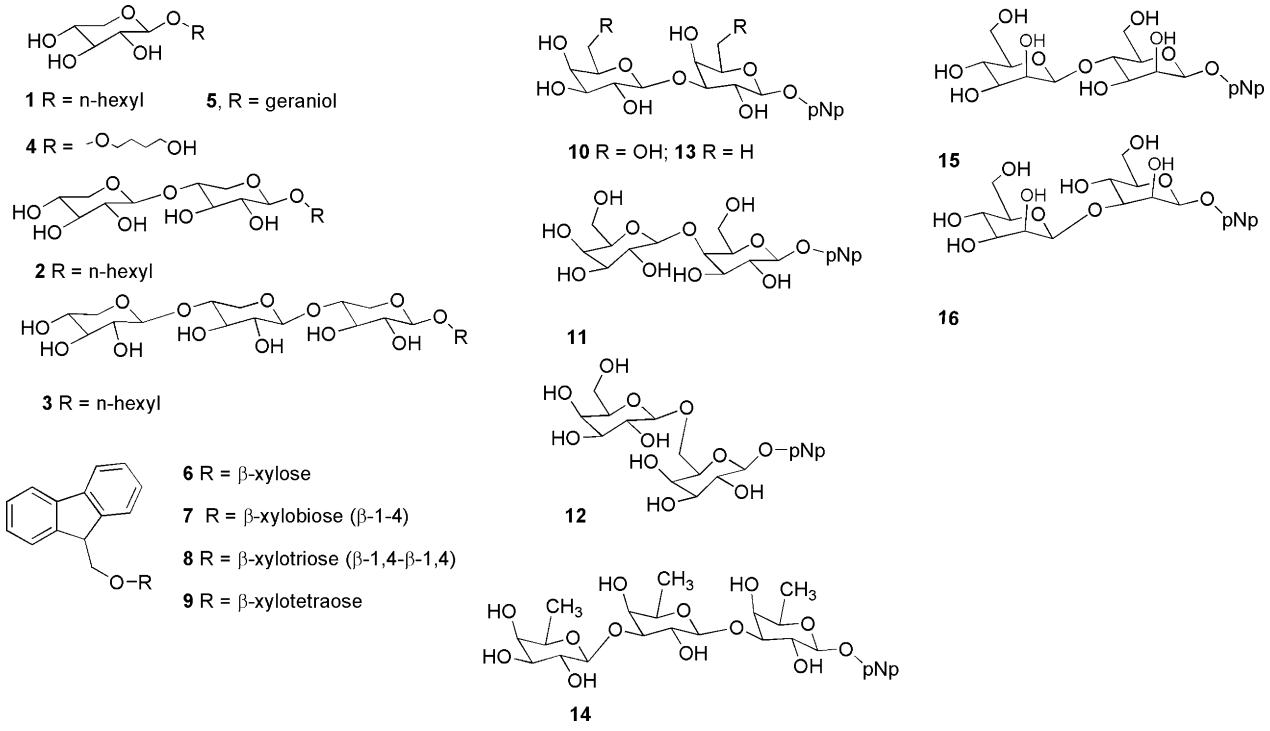

Fig. 1. Compounds synthesized by glycosyl hydrolases present in the crude homogenate of *Thermotoga neapolitana*.

*Peracetyl derivative of geranyl β-*D*-xylopyranoside* **5** (Fig. 1): xylose moiety, 4.49/99.2 H1 (J = 7.9 Hz), 4.91/70.9 H2, 5.17/71.7 H3, 4.97/69.1 H4, 4.13–3.48/62.1 H5; aglycon moiety, 4.28–4.16 H1′, 5.27 H2′, 2.06 H4′, 2.05–2.03 H5′, 5.10 H6′, 1.61 H8′, 1.69 H9′, 1.65 H10′.

*Peracetyl derivative of 9-fluorene methyl β-*D*-xylopyranoside* **6** (Fig. 1): xylose moiety, 4.55/100.5 H1 (J = 7.8 Hz), 5.06/70.6 H2, 5.17/71.3 H3, 4.98/68.7 H4, 4.16–3.39/61.9 H5; aglycon moiety, 4.24–3.58/71.9 Ar-CH_2-O-β-Xyl, 4.18/47.8 Ar-CH-CH_2-O-β-Xyl, 7.76, 7.60, 7.38, 7.29 aromatic ring protons.

*Peracetyl derivative of 9-fluorene methyl β-*D*-xylobioside* **7** (Fig. 1): internal xylose moiety, 4.49 H1 (J = 7.8 Hz), 4.99 H2, 5.12 H3, 3.86 H4, 3.98–3.29 H5; external xylose moiety, 4.56 H1 (J = 7.8 Hz), 4.79 H2, 5.09 H3, 4.87 H4, 4.10–3.39 H5; aglycon moiety, 4.26–3.54 Ar-CH_2-O-β-Xyl, 4.15 Ar-CH-CH_2-O-β-Xyl, 7.76, 7.58, 7.39, 7.30 aromatic ring protons.

*Peracetyl derivative of 9-fluorene methyl β-*D*-xylotrioside* **8** (Fig. 1): internal xylose moiety, 4.49/100.4 H1 (J = 7.8 Hz), 4.75/70.2 H2, 5.06 H3, 3.81 H4, 4.05–3.25 H5; middle xylose moiety, 4.55/99.4 H1 (J = 7.8 Hz), 4.79/70.3 H2, 5.09 H3, 3.84 H4, 4.05–3.25 H5; external xylose moiety, 4.47/100.7 H1 (J = 7.8 Hz), 4.89/71.03 H2, 5.11 H3, 4.87/68.2 H4, 4.09–3.45/61.4 H5; aglycon moiety, 4.25–3.50/70.9 Ar-CH_2-O-β-Xyl, 4.17/47.5 Ar-CH-CH_2-O-β-Xyl, 7.76/119.9, 7.56/124.9, 7.73/127.5, 7.28/126.9 aromatic ring protons. C3 of all xylose are at 71.8 × 2 and 72.2; C5 of internal and middle xylose were found at 62.4 and 62.6; C4 of internal and middle xylose are at 74.1 and 75.3.

*Peracetyl derivative of 9-fluorene methyl β-*D*-xylotetraoside* **9** (Fig. 1): 4.56, 4.50–4.46 four H1 signals, 4.96, 4.80–4.70 H2 signals, 5.15–5.02 H3 signals, 4.87, 3.72–3.85 H4 signals, 4.06–3.38 H5 external xylose, 3.90–4.00 and 3.25–3.35 other three set of H5 signals; aglycon moiety, 4.26–3.56 Ar-CH_2-O-β-Xyl, 4.17 Ar-CH-CH_2-O-β-Xyl, 7.76, 7.58, 7.39, 7.30 aromatic ring protons.

Peracetyl derivative of p-nitrophenyl digalactopyranoside (β-1-3) **10** (Fig. 1): internal galactose, 5.06 (H1), 5.49 (H2), 3.99 (H3), 5.46 (H4), 4.03 (H5), 4.17–4.06 (H6); external galactose 4.60 (H1), 5.11 (H2), 4.96 (H3), 5.35 (H4), 3.85 (H5), 4.17–4.06 (H6).

Peracetyl derivative of p-nitrophenyl digalactopyranoside (β-1-4) **11** (Fig. 1): internal galactose, 5.10 (H1), 5.40 (H2), 5.00 (H3), 4.19 (H4), 3.87 (H5), 3.87–4.12 (H6); external galactose 4.46 (H1), 5.26 (H2), 4.99 (H3), 5.37 (H4), 3.95 (H5), 4.38–4.28 (H6).

Peracetyl derivative of p-nitrophenyl digalactopyranoside (β-1-6) **12** (Fig. 1): internal galactose, 5.13 (H1), 5.51 (H2), 5.12 (H3), 5.42 (H4), 3.81 (H5), 3.75–3.88 (H6); external galactose 4.48 (H1), 5.23 (H2), 4.98 (H3), 5.39 (H4), 4.25 (H5), 4.35–4.23 (H6).

Peracetyl derivative of p-nitrophenyl difucopyranoside (β-1-3) **13** (Fig. 1): internal fucose, 5.06 (H1), 5.49 (H2), 3.97 (H3), 5.35 (H4), 3.96 (H5), 1.29 (H6); external fucose 4.54 (H1), 5.12 (H2), 4.94 (H3), 5.20 (H4), 3.75 (H5), 1.23 (H6).

Peracetyl derivative of p-nitrophenyl trifucopyranoside (β-1-3, β-1-4) **14** (Fig. 1): internal fucose, 5.11 (H1), 5.45 (H2), 4.01 (H3), 5.26 (H4), 4.01 (H5), 1.20 (H6); middle fucose 4.54 (H1), 5.01 (H2), 4.83 (H3), 3.83 (H4), 3.61 (H5), 1.30 (H6); external fucose 4.39 (H1), 5.20 (H2), 5.01 (H3), 5.20 (H4), 3.68 (H5), 1.14 (H6). Mass spectrum indicates m.w. 894 ($M + Na^+$).

Peracetyl derivative of p-nitrophenyl dimannopyranoside (β-1-3) **15** (Fig. 1): internal mannose, 5.30 (H1), 5.62 (H2), 4.05

(H3), 5.23 (H4), 3.88 (H5), 4.30 (H6); external mannose 4.74 (H1), 5.32 (H2), 5.12 (H3), 5.22 (H4), 3.64 (H5), 4.25–4.13 (H6).

Peracetyl derivative of p-nitrophenyl dimannopyranoside (β-1-4) **16** (Fig. 1): internal mannose, 5.32 (H1), 5.69 (H2), 5.14 (H3), 3.85 (H4), 3.70 (H5), 3.98–3.69 (H6); external mannose 4.63 (H1), 5.42 (H2), 4.85 (H3), 5.25 (H4), 3.58 (H5), 4.32–4.10 (H6).

3. Results and discussion

Despite of the huge amount of literature that can be found on glycosyl hydrolases from *Thermotogales*, there are few articles dealing with synthetic characteristics of such interesting enzymes. A survey of hydrolysis and transglycosylation capabilities of glycosyl hydrolases in the crude extract of *T. neapolitana*, and a thorough analysis of properties for the most abundant enzymes found, are discussed below.

The enzymatic action of retaining glycosyl hydrolases is based on the formation of a glycosyl ester of enzyme which soon reacts with a nucleophile present in reaction mixture to form the products. If the nucleophile is a water molecule, then the hydrolysis reaction takes place. Transglycosylation product(s) formation is observed if other nucleophiles are present in the reaction mixture. The formation of self-transglycosylation products usually occurs when the glycone moiety of enzyme glycosyl ester is transferred to another molecule of the substrate itself.

3.1. Monitoring the synthetic and hydrolytic potential of T. neapolitana crude extract

The crude homogenate of *T. neapolitana* (DSM 4359) shows β-D-gluco-(2 U/mg of crude protein), β-D-galacto-(1.3), β-D-fuco-(3.0), β-D-manno-(1.4), and β-D-xylosidase-(5.5) activities at a good extent. Different enzymatic activities are expressed at low level below 1 U/mg of crude protein (α-D-galacto and α-D-xylosidase at 0.3 and 0.2 U/mg of crude protein, respectively) or much less.

In Table 1, the results of a general investigation of the enzymatic reactions are reported for the hydrolysis and transglycosylation using different substrates (aryl glycosides and di-, oligo- and poly-saccharides of different nature). All aryl glycosides tested show the formation of dimers due to the self-transglycosylation reaction. The reaction is fast at all concentrations analyzed indicating the high activity of the enzyme used; in fact in the adopted conditions the substrate is generally consumed within 3–4 h and complete hydrolysis of products occurred after one night. Maltose and α,α-trehalose are not substrates. Interestingly oligosaccharides such as maltoheptaose and α-(starch, pullulan), and β-(laminarin, curdlan, xylan) polymers, are all positive substrates generating products of partial and total hydrolysis (Table 1). Synthetic reactions are possible using laminarin, curdlan and xylan using different acceptors, with the last one being the most interesting donor for synthetic purposes.

3.2. β-Xylosidase/β-xylanase activity

The crude homogenate of *T. neapolitana* hydrolyzes xylan from birchwood (90% xylose residue) to xylose through the formation of a series of xylooligosaccharides. The biotechnological importance of enzymatic reactions based on naturally occurring donors such as xylan for the production of valuable products such as oligoxylosides is a known issue [11]. This reaction, conducted using different acceptors (1-butanol, 1-hexanol, 1-octanol, 1-decanol, geraniol, 1,4-butanediol) shows the formation of important transxylosylation products. Among those used, 1,4-butanediol and 1-hexanol give excellent results; the last one soon forms β-xyloside, β-xylobioside (β-1-4), and β-xylotrioside (β-1-4-β-1-4).

Time course of 1-hexanol transxylosylation is studied in detail (Table 2), as long chain oligoxylosides are valuable new surfactants [11]. Using a 20 g/L solution of xylan from birchwood in the presence of 1.5 M 1-hexanol, after 3 h, 13.2, 8.7, and 2.8 mM 1-hexyl β-xyloside (**1**, Fig. 1), hexyl β-xylobioside (**2**, Fig. 1), and hexyl β-xylotrioside (**3**, Fig. 1) can be produced, respectively. Traces of a more polar 1-hexyl based tetrasaccharide are also visible in the first aliquot (3 h) as indicated by ^{1}H NMR spectrum of a minor product after purification. Either this product and the trisaccharide **3** (Fig. 1), soon disappear from reaction mixture indicating an hydrolytic action of a xylanase/xylosidase activities on the products formed. This is further confirmed by the molar ratio of monosaccharide/disaccharide which increases from 1.5 to 11.4 ranging from 3 to 48 h (Table 2). Shuffling of the interglycosidic linkage in the hexyl β-xylobioside (**2**, Fig. 1) is not observed as indicated by close inspection of the two ^{1}H NMR spectra of this peracetylated disaccharide isolated at 3 and 48 h from reaction mixture thus securing on the purity of material at level of interglycosidic linkages. Hexyl β-xyloside represents the kinetically favoured product obtained in a final concentration of 19.5 mM at 48 h, corresponding to a molar yield of ca. 17% with respect to xylose available (average m.w. xylan 25.000). This high yield, corresponding to a concentration of ca. 5 g/L (228 mg/g of xylan) is an order of magnitude higher than that obtained using *Trichoderma longibrachiatum* xylanase (0.4 g/L) [12] and fourfold higher (54 mg/g of xylan) than that obtained using *Aureobasidium pullulans* [11] in the synthesis of octyl β-xyloside, a very similar compound.

Good yield of monoxyloside product (**4**, Fig. 1) is also obtained using as acceptor the bifunctional 1,4-butanediol. The

Table 2
Concentrations (mM) of various hexyl oligoxylosides obtained in the reaction with crude homogenate of *Thermotoga neapolitana* in the condition described in Section 2

Products	Reaction time (h)			
	3	7	23	48
Hexyl β-xyloside	13.2	13.0	15.3	19.5
Hexyl β-xylobioside	8.7	6.1	5.0	1.7
Hexyl β-xylotrioside	2.8	–	–	–
Hexyl β-xylotetraoside	Trace	–	–	–

product is formed at a concentration of 32 mM (360 mg/g of xylan) after one night. Such a compound can be useful as a reagent in the conjugation with phosphatidylcholine, mediated by phospholipase D, for the biocatalyzed synthesis of neoglycolipids [13]. Yields are worst using 10 carbon aglycones: geraniol is xylosylated obtaining geranyl β-xyloside (**5**, Fig. 1) after one night at 90 mg/g xylan.

9-Fluorene methanol is an interesting aglycone listed among IFN-inducing (interferon inducing) and anti-HSV (Herpes Simplex Virus) active compounds. The β-glucoside of 9-fluorene methanol, previously obtained by us using a biocatalyzed process, has been reported to be an active anti-viral agent and glycosylation judged as a direct element to improve its pharmacological profile [14]. 9-Fluorene methanol is used as an acceptor for the synthesis of xylooligosides by *T. neapolitana* xylanase activity, exploiting the usual resistance of thermophilic enzyme in the presence of high concentration of organics (miscible solvent and acceptor itself). In fact, the reaction is conducted in 13% acetonitrile necessary to dissolve 0.1 M aglycone, and stopped after 6.5 h to achieve sufficient amounts of longer oligosaccharides (**6–9**, Fig. 1, total amount 150 mg/g xylan) before hydrolysis to monoxyloside. Prior to acetylation, used to establish the interglycosidic linkages via NMR spectroscopy, mass spectra indicate molecular weights of native oligoxylosides thus ruling out the presence of acetyl groups found in the natural xylan blocks enzymatically transferred [10]: **6**, **7**, **8**, and **9** (Fig. 1), 351, 483, 615, and 724 $M+Na^+$, respectively. 9-Fluorene methyl β-D-xylopyranoside (**6**, Fig. 1) reach 1.5 mM while disaccharide **7** (Fig. 1) is at 0.35 mM, trisaccharide **8** (Fig. 1) at 0.5 mM and the tetrasaccharide **9** (Fig. 1) at 0.15 mM. Additionally three minor products are detected at lower Rf in TLC, amounting to 13.2 mg/g xylan; they contain sugars different from xylose, as indicated by inspection of ^1H NMR spectra.

The above results demonstrated that *T. neapolitana* crude homogenate is able to transfer xylan blocks up to tetra- and penta-saccharides to different acceptors such as 10 carbon atom chain or to 9-fluorene methanol, an aromatic acceptor never tested before for the formation of xylosides. The unvarying purity of the products at level of interglycosidic linkage (β-1,4 linear products) during reaction time is interesting from a synthetic point of view as well as the formation of higher saccharides containing acidic sugars [10]. This could indicate interesting ability of the enzyme(s) active site responsible for sugar accommodation in the transfer. The limit of four carbon atoms for the acceptor, which was not further investigated for *T. maritima* enzyme [15], is extended here for *T. neapolitana* catalyst(s) confirming the biotechnological interest for this biomass.

3.3. β-Galactosidase, β-glucosidase and β-fucosidase activities

It has been recently reported [16] that a recombinant β-glucosidase from *T. neapolitana* transfers glucose to arbutin in different positions while the transfer of galactose to cellobiose (or lactose) in two different positions (β-1-6, β-1-3) has been only predicted. So it has been considered interesting to evaluate experimentally the regioselectivity of galactose transfer in the formation of dimers and trimers during the reaction of PNP-β-D-Gal with crude homogenate from *T. neapolitana*. The reaction can be conveniently conducted at 400 mM initial concentration of substrate exploiting the known resistance of hyperthermophilic biocatalysts to high substrate concentration and common resistance to substrate/product inhibition [17]. Almost total conversion (90%) of PNP-β-D-Gal quickly takes place (2.5 h) recovering at this stage ca. 58% of free galactose due to the hydrolysis reaction. Transfer products (42% galactose equivalent) due to transgalactosylation consist mainly of three galactosyl disaccharides of *p*-nitrophenol (**10–12**, Fig. 1; **10** β-1-3 66%, **11** β-1-4 18%, **12** β-1-6 15%) and a minor amount (ca. 1.0%) of trisaccharide(s).

Regioselectivity in the transfer of glucose is investigated by the *o*-nitrophenyl β-glucopyranoside self-condensation reaction. The products are identified by comparison with authentic standards previously obtained by us in the study of glycosynthase reaction using *S. solfataricus* enzyme [17]. Results are shown in Fig. 2: the rapid formation of *o*-nitrophenyl β-laminaribioside (β-1-3), the most abundant produced disaccharide, is initially detected but this compound is also actively enzymatically hydrolyzed and soon disappeared. The worst disaccharidic substrate is the *o*-nitrophenyl β-gentobioside (β-1-6), which still remain in the reaction mixture after 4 h. The formation of a very scarce amount of trisaccharides of *o*-nitrophenol and transfer of glucose to free glucose molecules are also observed in the reaction conditions adopted (Fig. 2).

The reaction of fucose transfer can be conducted at 400 mM initial concentration of substrate. Almost total conversion (87%) of PNP-β-D-Fuc occurs in 6.5 h recovering 71% of free fucose. Transfer products (29% fucose equivalent) due to transfucosylation consist mainly of a disaccharide (95%, β-1-3) and a major

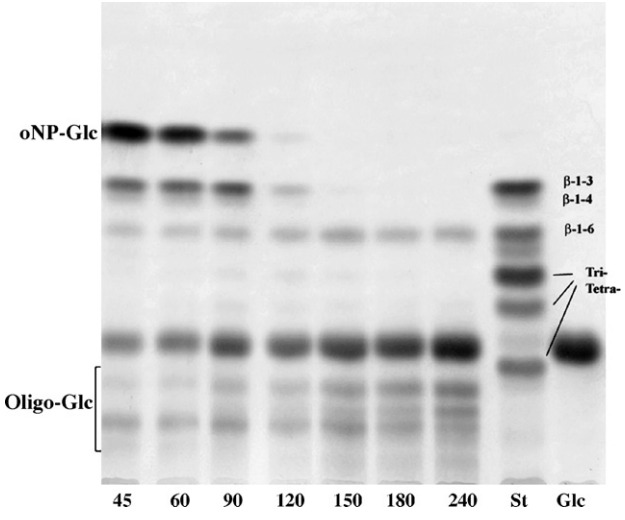

Fig. 2. TLC of reaction mixture at different time intervals (45–240 min) showing the enzymatic formation and hydrolysis of *o*-nitrophenyl disaccharides as catalyzed by *T. neapolitana* crude homogenate. St: reaction mixture obtained using *S. solfataricus* β-glycosynthase—β-1-4, β-1-3, β-1-6 are the corresponding disaccharides; tri- and tetra- correspond to oligoglucosides of *o*-nitrophenol of known structure as reported in reference 17; Oligo-Glc: non-chromophoric oligoglucosides.

trisaccharide (ca. 4%); additional trace amount of a different trisaccharide is also present. Mass spectrum of peracetylated major trisaccharide (**14**, Fig. 1) indicates the molecular weight 894 ($M + Na^+$). The exact assignment of chemical shifts of signals and the unambiguous structure for this compound are established starting from signals of anomeric protons of fucose attached to aryl group (5.11 ppm internal fucose) with those of middle (4.54 ppm) and external (4.39 ppm) fucose units and following all correlations through pyranosidic rings in the COSY spectrum.

The results observed for the formation of different dimers and trimers of aryl β-glycosides fully confirm the characteristics of the previously reported recombinant β-glucosidase from *T. neapolitana* [18]. It is interesting the possible transfer of β-D-fucose other than glucose and galactose and the formation of trifucosyl derivative never reported before.

The ability of the above biocatalyst(s) to form novel compounds such as oligosaccharides might prove usefulness in biotechnological applications.

3.4. β-Mannosidase activity

Reports dealing with β-mannosidase activity in the synthesis are not frequent reflecting the less abundancy of catalytic activities of this type. However, a β-mannosidase has been reported for both *T. maritima* and for *T. neapolitana* [18] and a β-mannanase from the last one was used for the hydrolysis of guar gum [19]. However, the study of transglycosylation properties has not been performed up until now using these enzymes which are considered the most thermostable and thermoactive versions of these biocatalysts.

The formation of chromophoric mannodisaccharides by self-condensation reaction using PNP-β-D-mannopyranoside as donor and acceptor, is conducted at 500 mM initial concentration of substrate; ca. 80% of substrate conversion is obtained after 6 h. The total molar yield of two disaccharides (β-1-4/β-1-3, 1:1 ratio) is 22%. This is an interesting result in the light of usually reported low yields for β-mannosylation reactions [20]. A further useful element is the possibility of working at high concentration of substrate thus increasing the efficiency of each reaction batch.

The marine source confirmed to be important for β-mannosidase discovery: similar yield of products, but different regioselectivity, was reported for the enzyme found in *Aplysia fasciata*, a marine mollusc [21].

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