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Screening of strains and recombinant enzymes from *Thermus thermophilus* for their use in disaccharide synthesis

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ABSTRACT

Cell extracts from ten different strains of *Thermus thermophilus* have been screened for glycosidase activity. Among these, the sequenced strain HB27 hydrolyzed a wide variety of glycosides and increased six fold its β-glycosidase activity when grown with cellobiose in nutrient-limited media. We selected five genes encoding (putative) glycosidases (TTP0042, TTP0072, TTP0220, TTC0107 and TTP0222) from the genome of this strain, and the corresponding recombinant enzymes were overexpressed and purified. Several transglycosylation reactions using cellobiose-induced HB27 cell extracts and the purified recombinant enzymes were assayed. Biochemical properties and biosynthetic capabilities of the HB27 cell extracts and the TTP0042 enzyme were very similar, suggesting that this enzyme was responsible for most of the β-glycosidase activity detected in the HB27 strain. This was confirmed through the isolation and analysis of a null mutant of its encoding gene. With both, HB27 cell extracts and purified TTP0042 recombinant enzyme, we finally achieved high yields conditions for disaccharide production by transglycosylation with low amounts of self-condensed donor when high concentrations of a 1:5 donor:acceptor molar ratio was used.

1. Introduction

Oligosaccharides of glycoproteins and glycolipids play an important role in molecular interactions between cell membranes that are relevant for process such as embryogenesis, neuronal proliferation and apoptosis [1,2,3,4,5]. Thus, the synthesis of oligosaccharides requires a special control of the regio and stereoselectivity of the reaction that is difficult to achieve by chemical synthesis. For that reason the synthesis of oligosaccharides and glycoconjugates is often achieved by enzymatic methods, with glycosyltransferases, glycosidases and glycosynthases as biocatalysts [6–12]. Glycosidases (E.C. 3.2.1.) are enzymes whose natural function is the hydrolysis of glycosides. Reaction equilibrium of glycosidases can be controlled by competition of a glycoside acceptor in the active site of the enzyme against water molecules in order to develop synthesis of oligosaccharides and glycoconjugates. This type of reaction is known as transglycosylation, and it is used for oligosaccharide synthesis [13–15] through the appropriate control of the reaction conditions by the use of low water environment or activated glycosyl donors. An example for an important disaccharide in intercellular interactions is Gal-β(1→4)GlcNAc that constitutes the core of H2 Lewis antigen [16] and it is also well known as an specific substrate for *Streptococcus pneumoniae* β-galactosidase. Such enzyme could be related to the infection of human tissues by this bacterium because of its ability to deglycosylate *N*-linked glycans present on the surface of the cytoplasmic membrane [17–19].

The species *Thermus thermophilus* includes several strains of extreme thermophilic bacteria that constitute a potential source of thermostable and solvent-resistant enzymes, properties that could be required for the efficient synthesis of disaccharides if an appropriate glycosidase could be identified.

One of the main problems in enzymatic synthesis of disaccharide using β-glycosidases from *Thermus thermophilus* has been the self-condensation of the nitrophenyl donor used [20–24]. This have generated different strategies to improve disaccharide synthesis, including: directed evolution [25], glycosynthases design [26], acceptors engineering [27] and chemoenzymatic synthesis [28] to achieve specific sugars.

In this study we screened several strains of *Thermus thermophilus* for glycosidase activity against different substrates and characterized the biochemical properties from the best ones. We

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Table 1
Oligonucleotides used in this work. Letters in bold indicate restriction sites used for cloning of the amplified genes.

Name	Sequence (5′ > 3′)	Purpose
oTTP0042NdeI	AAA**CATATG**ACCGAGAACGCCGA	Forward, cloning TTP0042
oTTP0042HindIII	AA**AAGCTT**AGGTCTGGGCCCGC	Reverse, cloning TTP0042
oTTP0072NdeI	AAAA**CATATG**AGGCTGAACCTAGGA	Forward, cloning TTP0072
oTTP0072HindIII	AA**AAGCTT**ATAGAAGGGGGGCA	Reverse, cloning TTP0072
oTTP0220NdeI	AAAA**CATATG**CGGCTGGACCCCA	Forward, cloning TTP0220
oTTP0220HindIII	AA**AAGCTT**CTACTCCGCGAGAAGC	Reverse, cloning TTP0220
oTTP0222NdeI	AAAA**CATATG**AGGGTGGAGAAGGC	Forward, cloning TTP0222
oTTP0222HindIII	AA**AAGCTT**CACCAGGCCACCCT	Reverse, cloning TTP0222
oTTC0107NdeI	AAAA**CATATG**TGGTGGAAAGAGGCG	Forward, cloning TTC0107
oTTC0107HindIII	AA**AAGCTT**CTAGTCTAGCCGCACC	Reverse, cloning TTC0107
oMUT42EcoRIdir	AAAA**GAATTC**AAGGGCCTCGCCTTCT	Forward, mutant TTC0042
oMUT42HindIIIrev	AAAA**AAGCTT**CCCGTTTTCCGTGACG	Reverse, mutant TTC004

studied different strategies to improve their activities and evaluated their use as biocatalyst in glycoconjugate synthesis. Finally we cloned, expressed in *Escherichia coli* and in *Thermus thermophilus* HB27, and purified recombinant glycosidases from the strain HB27. The best glycosidase activities were analysed for their use in transglycosylation reactions.

2. Materials and methods

2.1. General

p-nitrophenol (*p*NP), *p*-nitrophenyl glycosides and bovine serum albumin (BSA), analytical standards of monosaccharides and disaccharides (D-(+)-galactose, D-(+)-fucose, D-(+)-mannose, D-(+)-glucose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, Gal-(β1→4)-GlcNAc and Gal-(β1→6)-GlcNAc) for HPLC were purchased from Sigma–Aldrich; dye reagent for protein determination was purchased from Bio Rad. All other chemicals were from analytical grade. UV–visible spectra were recorded on a UV-2401 PC Shimadzu. HPLC Agilent 1100 with UV-vis detector using Mediterraneasea18 15 cm × 0.46 5 mm column (Teknokroma) with water: acetonitrile (75:25) as a mobile phase at a flow of 0.7 mL/min. HPLC Jasco with evaporative light scattering (ELSD), detector using NH2P50-4E amino column (Asahipak, Japan) with acetonitrile:water (80:20) as a mobile phase at a flow of 0.8 mL/min. NMR spectra were recorded on Bruker Avance 500 MHz spectrometer.

2.2. Bacterial strains, enzymes and growth conditions

Thermus thermophilus strains CC16, NR17, PRQ16, VG7, B, RQ1, PRQ25 and HN1.11 were a gift from Professor Milton da Costa. HB27 strain was a gift from Prof. Y. Koyama, and NAR1 strain was previously described [29]. *Thermus thermophilus* HB27nar is a derivative of HB27 that can grow anaerobically with nitrate as electrons acceptor [30]. *Escherichia coli* strains DH5α and BL21DE3 were used for gene cloning and protein overexpression, respectively. *Thermus thermophilus* strains were routinely grown in TB media [31], and *E. coli* was grown in LB. For plates, agar (1.5%, w/v) was added to these media. Ampicillin (100 mg/L) or kanamycin (30 mg/L) was added when required for plasmid selection. Transformation of *Escherichia coli* was carried out by standard methods and transformation of *Thermus thermophilus* was carried out by natural competence.

2.3. Screening of glycosidase activities in Thermus thermophilus strains

The above *Thermus thermophilus* strains were grown in TB media [31] under aerobic conditions for 16 h at 65 °C. Cells were harvested by centrifugation at 5000 × *g* for 10 min and suspended in phosphate buffer 30 mM pH 7.0. Cell concentration was analyzed with a Neubauer counting chamber. Glycosidase activity was determined using a continuous method as described below.

2.4. Effect of inducers in culture media over the glycosidase activity of Thermus thermophilus

To avoid putative repression by preferential catabolites, we used a nutrient-limited ¼ diluted TB medium to which we added 0.2% of saccharose, lactose, mannose, cellobiose, or melibiose. As reference control we used a parallel culture with similar concentration of glucose, which acts as preferential energy and carbon source in many bacteria, inhibiting the use of alternative sugars. Cells grown at 65 °C were harvested by centrifugation at 5000 × *g* for 10 min. Cells pellet was resuspended in phosphate buffer 50 mM, NaCl 50 mM, pH 7.30 and disrupted by sonication (three 30 s pulses, 300 MHz). Unbroken cells and insoluble debris were eliminated by centrifugation (14,000 × *g* for 15 min at 4 °C). Glycosidase activity was determined using a discontinuous method (see below) with identical amount of cell extract protein.

2.5. Enzyme assays and protein determination

Hydrolytic activities were determined by quantification of *p*NP liberated from the corresponding *p*NP-Glycosides. Reactions were carried out in a sodium phosphate buffer 50 mM pH 7.3 using either a continuous or a discontinuous method. Continuous method: increase in absorbance at 410 nm during 2.5 min at 65 °C in a 100 μL cell with 5 mM of the *p*NP-Glycoside. Discontinuous method: 200 μL reaction with 1.0 mM of the *p*NP-Glycoside at 80 °C for 10 min and stopping the reaction with1.00 mL of Na_2CO_3 0.20 M [20,32,33]. Protein concentration was determined by the Bradford method [34] with BSA (bovine serum albumin) as standard. One enzyme unit was defined as the amount of protein that hydrolyses 1 μmol of *p*NP-glycoside per minute under the conditions described above.

2.6. Cloning and overexpression of putative glycosydases from Thermus thermopohilus HB27

A bioinformatic search for putative glycosidases was carried out on the genome of *Thermus thermophilus* HB27 leading to the identification of five genes of codes TTP0042, TTP0072, TTP0220, TTP0222, and TTC0107. (http://wishart.biology.ualberta.ca/BacMap/). Every gene was amplified by PCR (Polymerase Chain Reaction) with the corresponding pair of plasmids (Table 1) that included sites for the enzyme of restriction NdeI at the 5′ end and HindIII at the 3′ end. The amplified genes were inserted into the equivalent restriction sites of plasmid pET28b+ (Novagen) from which they can be produced as N-terminal six-histidines tagged proteins. For overexpression of the corresponding proteins, cells of *E. coli*

BL21DE3 transformed with the appropriate plasmids were grown at 37 °C in LB with kanamycin for plasmid selection up to an OD550 nm of 0.6 before the addition of 0,5 mM of IPTG (isopropyl-β-D-thiogalactopyranoside), keeping the incubation for 12 h. To overexpress the TTP0042 protein in *Thermus thermophilus* we used a derivative of plasmid pMKE2 [35]. The plasmid pMKE2-TTP042 expresses a six-histidine tagged (His_6tag) TTP0042 protein under the control of the promoter of the respiratory nitrate reductase (Pnar) in the facultative strain *Thermus thermophilus* HB27nar [30]. HB27nar transformants were grown aerobically at 70 °C in TB with kanamicin up to an OD550 of 0.4. Then, potassium nitrate (40 mM) was added, and the culture shaking was stopped for 12 h at 70 °C. The lack of shaking combined with the high temperature makes this culture almost anaerobic, allowing the expression of Pnar promoter when nitrate is present.

2.7. Isolation of TTP0042 mutants

The isolation of a mutant in the TTP0042 gene was achieved by using the suicide vector pK18 [36]. For this an internal fragment of the gene was amplified by PCR with the oligonucleotides oMUT42EcoRIdir and oMUT42HindIIIrev (Table 1), and the product was cloned into the pK18 plasmid using the EcoRI and HindIII restriction sites included in the sequence of the oligonucleotides. The plasmid obtained was used to transform *Thermus thermophilus* HB27 and the kanamycin resistant colonies obtained were checked by PCR for the presence of the mutation. A colony of this T42kat mutant was selected for further studies.

2.8. Purification and screening of the recombinant glycosidases

The proteins encoded by the TTP0042, TTP0072, TTP0220, TTP0222 and TTC0107 genes were overexpressed in *E. coli* as described above. In order to obtain cell lysates, overproducing cells were harvested and disrupted (as mentioned above). The supernatant was incubated at 70 °C for 40 min to denature most of *E. coli* proteins that were further removed by centrifugation (14,000 × *g*, 20 min, 4 °C). The clarified solution obtained was passed through a Ni^{2+}-agarose column (3 mL), where the His_6tagged proteins were bound. The enzymes were further eluted from the column with imidazole according to manufacturer's protocol (ABT beads). Fractions were monitored by absorbance at 280 nm, pooled, and concentrated and desalted in an Amicon ultra centrifuge filter (Millipore). The His_6tagged TTP0042 protein was also overexpressed in *Thermus thermophilus* HB27nar as described above and the protein was purified from the soluble cell extract by affinity chromatography on Ni^{2+}-agarose as above. Glycosidase activities of the recombinant enzymes were tested using the continuous method.

2.9. Comparison between native cell extracts and the recombinant TTP0042 His_6tagged enzyme

According to the results of the screening, we decided to develop a comparison of the β-galactosidase activity between cell extracts of HB27 (induced with cellobiose) and the recombinant TTP0042 His_6tagged enzyme using optimal pH, optimal buffer and optimal temperature as comparison parameters. *p*NP-β-Gal was used as substrate for β-glycosidase activity. The effect of pH on the enzymatic activity was tested by discontinuous method. Buffers employed were 50 mM citrate/phosphate pH range of 4.0–5.50 and 50 mM phosphate buffer range of pH 5.5–8.0. The pH values of each buffer were measured at 25 °C. Once the pH optimum defined, we assayed the effect of the buffer type using 50 mM of the following buffers: sodium citrate/phosphate (50% molar of each compound), sodium phosphate, sodium acetate, 2-(*N*-morpholino) ethanesulfonic acid (MES), sodium citrate and sodium acetate. The effect of temperature on the enzyme activities was investigated by measure hydrolysis at temperatures 60, 65, 70, 75, 80, 85 and 90 °C in optimal pH and buffer conditions.

2.10. General procedure for tranglycosylation reactions

A 1:5 molar ratio donor:acceptor was used, using 42.5 mM of the donor and 213 mM of the acceptor. Donor employed was *p*-nitrophenyl-β-D-galactopyranoside (*p*NP-β-Gal), and acceptors used were: *p*NP-β-Gal (autocondensation reaction) D-(+)-galactose (Gal), D-(+)-mannose (Man), D-(+)-fucose (Fuc), D-(+)-glucose (Glc), *N*-acetyl-D-glucosamine (GlcNAc), *N*-acetyl-D-galactosamine (GalNAc). Final volume of reactions was set on 1.00 mL. Reagents were pre-warmed at appropriate temperatures according to the enzyme assayed. Reaction started by addition of the respective enzyme. 50 μL samples were taken at different times from the reaction media, and 450 μL of pure methanol was added in order to stop the reactions, which were immediately transferred and kept at −20 °C. The progress of the reactions was monitored by HPLC for studying the proportions of products in reaction mixtures. Periodically samples of reaction were removed (each 30 min) and analyzed by HPLC, after 3 h the reaction was stopped. For determination of products by HPLC we used several standards (p-nitrophenyl-β-D-galactopyranoside, D-(+)-galactose, D-(+)-fucose, D-(+)-mannose, D-(+)-glucose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, Gal-(β1→4)-GlcNAc and Gal-(β1→6)-GlcNAc). Isolation of new compounds was done by column chromatography using activated carbon (50% m/m) and celite (50% m/m), column was eluted with milliQ water, 5% and 15% ethanol (in water). The structure of the enzymatically synthesized disaccharides were assigned by NMR, spectra were consistent with reference literature [37]: Gal-(β1→4)-GlcNAc (*N*-acetyl-D-lactosamine): ^{1}H NMR (700 MHz, D_2O): 1.93 (s, 3H, Ac), 4.37 (d, 1H, H1′, $J_{1',2'}$ = 7.8 Hz), 4.62 (d, 0.4H, $H1_\beta$, $J_{1\beta,2}$ = 8.1 Hz) 5.10 (d, 0.6H, $H1_\alpha$, $J_{1\alpha,2}$ = 2.2 Hz). ^{13}C NMR: 21.80 (Me, α), 22.10 (Me, β), 53.65 (C-2α), 56.13 (C-2β), 59.86 (C-6α), 59.99 (C-6β), 60.96 (C-6′), 68.48 (C-4′), 69.21 (C-3α), 70.20 (C-5α), 70.90 (C-2′), 72.43 (C-3β, C-3′), 74.79 (C-5β), 75.29 (C-5′), 78.24 (C-4β), 78.68 (C-4α), 90.46 (C-1α), 94.80 (C-1β), 102.80 (C-1′β), 102.86 (C-1′α), 174.40 (C═O, α), 174.66 (C═O, β).

2.10.1. Transglycosylation reactions using cell extracts

10 units (measured by discontinuous method) of lyophilized powder from induced HB27 cell extracts were dissolved in 1.0 mL sodium phosphate buffer pH 5.50 (25 mM) at 80 °C. In order to check the putative relationship between the observed synthetic activity in HB27 cell extracts and the TTP0042 enzyme, we performed five reactions using lyophilized powder from cell extracts of the T42kat mutant (grown in enriched cellobiose medium). Enzymatic activity from T42Kat was so low that we used 43.3 mg of proteins. Reactions with T42Kat extracts were performed using identical conditions as with the HB27 parental.

2.10.2. Transglycosidation reactions with recombinant enzymes

Transglycosylation reactions with pure enzymes were done for 3 h at 65 °C, in sodium phosphate buffer 50 mM pH 6.0 and 0.1 units/mL of enzyme (measured by discontinuous method) expressed in *E. coli* and in *T. thermophilus* HB27nar.

2.11. Study of reaction conditions for transglycosylation

In order to improve the yields of the most relevant transglycosylation found for TTP0042 we performed the reaction on a four-folds concentrated media (0.17 M *p*NP-β-Gal as donor and 0.85 M GlcNAc as acceptor) and 1.8 units/mL of enzyme (determined by discontinuous method).

3. Results and discussion

3.1. Screening of Thermus thermophilus strains

Ten strains (Table 2) were screened for the presence of glycosidase activities within the cells. All the strains assayed showed β-galactosidase, α-glucosidase and β-fucosidase activities. In addition, NR17, PRQ16, B, NAR1, HB27 and HN1.11 also exhibited α-galactosidase activity. We also assayed the supernatant of the growth medium to check for putative secreted enzymes, but only low activity for a few reactions was detected (data not shown). For this reason, we conclude that these strains have only intracellular glycosidases activities, being the activities found in the medium the likely consequence of cell lysis.

As result of this screening we found many strains that presented very high hydrolitic activities against some of the substrates tested: NR17 presented very high β-fucosidase, β-galactosidase and α-glucosidase activities, PRQ16 showed β-fucosidase and α-glucosidase activities, and VG7 exhibit high α-glucosidase activity. Others strains, like HB27, presented a great diversity of activities.

3.2. Effect of inducers on glycosidase activities in T. thermophilus cell extracts

The results above showed that the strain with higher glycosidase activities were NR17, PRQ16, VG7 and HB27. As only the sequence of the HB27 strain is known [38], we decided to focus on the genes encoding these putative activities in this strain in order to define the relationship between the activities observed on different substrates and specific enzymes. An additional advantage of this strain is that it can be manipulated genetically.

In Fig. 1 we show the effects of the presence of disaccharides as putative inducers of the expression of β-fucosidase, β-galactosidase, and α-glucosidase activities in the HB27 strain, while a culture with glucose was used for uninduced reference as a likely catabolite repressor. As it can be seen, only cellobiose produced a significant increase in both the β-fucosidase and the β-galactosidase activities, whereas the α-glucosidase was slightly increased in the presence of saccharose and maltose. In previous works, cellobiose was reported as an inducer of the β-glucosidase activity by 4 fold in the genus *Thermus* [39], and also acts as inducer in different mesophiles [40–43]. In our experiments a 6-fold increase in β-galactosidase and β-fucosidase activities was detected, suggesting either that two specific enzymes were being induced or that a single enzyme with both activities was induced by this disaccharide. Noteworthy, none of the other β-disaccharides used produced any significant increase in these activities, supporting the existence of a quite specific signalling system for cellobiose.

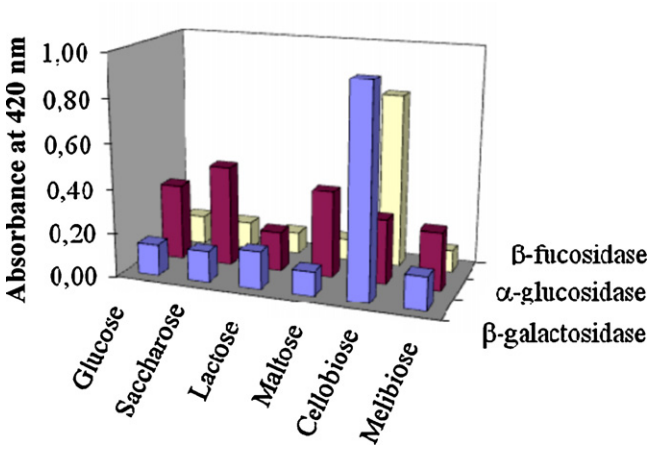


Fig. 1. Effects of disaccharides on the glycosidase activities of *Thermus thermophilus* HB27. Cells were grown in ¼ TB medium containing 0.2% of the indicated disaccharides, or glucose, used as reference control. The β-fucosidase, β-galactosidase, and α-glucosidase activities were measured through the absorbance at 420 nm corresponding to the liberation of *p*NP from *p*NP-β-Fuc, *p*NP-β-Gal, and *p*NP-α-Glc, respectively.

3.3. Glycosidases activity screening of recombinant enzymes from HB27

In an attempt to identify the putative enzyme(s) responsible for the two activities observed after induction with cellobiose, we searched the genome of the HB27 strain and selected five genes that were predicted to encode putative glycosidases. The five genes were cloned, overexpressed, and their recombinant protein products purified as derivatives carrying a N-terminal 6-histidine tag (His_6tag-enzymes). The purified enzymes were subjected assayed with a set of substrates.

As shown in Table 3, only the protein of code TTC107 showed a significant α-glucosidase activity (180 U/mg prot). It has been described recently that this enzyme uses the osmolyte trehalose as its main substrate, playing an important role in its metabolism [44]. Regarding the β-galactosidase and the β-fucosidase activities, both were basically found associated to the protein TTP0042, which also presented a relevant β-glucosidase and β-manosidase activities. A relatively low β-galactosidase activity was found associated to TTP0222. In consequence, the β-galactosidase and the β-fucosidase activities induced by cellobiose in HB27 are most likely due to the expression of a single enzyme, the TTP0042 protein.

3.4. Identification of TTP0042 as the main glycosidase of HB27

To confirm the nature of TTP0042 as the main β-glycosidase in HB27, we isolated a mutant (T42kat) in which is coding gene was disrupted by insertional-mutagenesis with a gene cassette

Table 2
Screening of intracellular glycosidase activity from cell extracts of *Thermus thermophilus* strains (± 5%, enzymatic units per million of cells).

	CC16	NR17	PRQ16	VG7	B	NAR1	HB27	RQ1	PRQ25	HN1.11
*p*NP-α-Gal	–	191	140	–	123	131	174	–	–	101
*p*NP-β-Gal	104	494	286	252	225	103	146	86	216	60
*p*NP-α-Glc	161	513	948	618	590	489	422	444	337	560
*p*NP-β-Glc	105	–	343	269	172	66	130	41	215	–
*p*NP-α-Man	–	–	–	–	–	–	–	86	–	–
*p*NP-β-Man	–	–	–	–	–	–	–	–	–	–
*p*NP-α-Fuc	–	97	–	–	–	–	–	–	–	–
*p*NP-β-Fuc	180	673	531	326	410	134	293	130	236	103
*p*NP-α-GalNAc	–	259	–	–	–	–	–	–	–	–
*p*NP-β-GalNAc	–	–	–	–	–	–	–	–	–	–
*p*NP-α-GlcNAc	–	–	–	–	–	–	–	–	–	–
*p*NP-β-GlcNAc	–	–	–	–	–	–	107	70	–	–

Table 3
Glycosidases activities (±5%) of recombinant His_6tag enzymes determined at 5 mM substrate concentration and expressed as U/mg prot.

Substrates	TTP 0042	TTP 0072	TTC 00107	TTP 0220	TTP 0222
*p*NP-α-Glc	–	0.16	180	0.0057	–
*p*NP-β-Glc	23.2	–	–	–	0.82
*p*NP-α-Gal	0.0011	2.19	–	–	–
*p*NP-β-Gal	36.3	–	–	–	1.15
*p*NP-α-Fuc	0.0006	–	0.051	–	–
*p*NP-β-Fuc	47.9	–	–	–	–
*p*NP-α-Man	0.0007	–	0.23	–	0.44
*p*NP-β-Man	3.93	–	–	–	–
*p*NP-α-GlcNAc	0.0008	–	–	–	–
*p*NP-β-GlcNAc	0.0030	0.44	–	–	1.34
*p*NP-α-GalNAc	–	–	–	–	–
*p*NP-β-GalNAc	0.0037	0.69	–	–	–

encoding a thermostable resistance to kanamycin. The analysis of the cell extracts from cultures of this mutant grown under inducing conditions with cellobiose revealed almost negligible β-glycosidase activity (8% hydrolysis of *p*NP-β-Gal with respect to HB27). Therefore we concluded without any doubt that TTP0042 is the enzyme responsible for the mayor β-glycosidase activity of HB27.

3.5. Comparison between HB27 cell extracts and TTP0042 His_6tag enzyme

In order to determine similarities in the biochemical behaviour between HB27 cell extracts (induced) and the recombinant TTP0042 His_6tagged protein, we analyzed the optimal pH and temperature for both biocatalysts. Optimal pH was determined with *p*NP-β-Gal at 80 °C in a range of pH from 4.0 to 8.0 with both, citrate/phosphate and phosphate buffers (Fig. 2). The HB27 cell extracts displayed a maximum activity at pH 5.5–6.5, while TTP0042 His_6tag displayed a wider range for its maximum (5.0–7.0). Such range is usual for many glycosidases from the genus *Thermus* [20,33,45–47].

We also compared the activity on different buffers at pH 5.5 and 6.0, of the cell extract and the recombinant protein, respectively, leading to select in both cases a sodium phosphate buffer (Table 4) for the analysis of the optimum temperature. Under these conditions, the maximum activity was found at 90 °C for the HB27 cell extracts, while TTP0042 showed its maximum at 80 °C (Fig. 3). Such difference between the properties of the activity found in cell extracts and that of the recombinant protein could be related to the presence of a His_6tag at the *N*-terminus of the latter.

The results presented so far point to TTP0042 as the main and quite promiscuous β-glycosidase in HB27. Other strains from *Thermus thermophilus* present one or more β-glycosidases with high similarities in amino acid sequences to TTP0042. As examples the enzyme from *Thermus flavus* AT-62 [45] has 98% of identity, and another from *T. thermophilus* HJ6 shows 95,6% of identity [24]. Moreover, *Thermus sp*. IB-21 encodes two lactose hydrolases, BglA and BglB, that show sequence identities of 94% and 98%, respectively to TTP0042 [33]. These data and the fact that the TTP0042 is encoded within a variable region of a megaplasmid in HB27

Table 4
Optimal buffer for HB27 cell extracts (induced with cellobiose) and TTP0042 His_6tag.

Buffer	HB27 (pH 5.50)	TTP0042 His_6tag (pH 6.00)
Citric acid/sodium citrate	(97 ± 3)%	(95 ± 3)%
Sodium citrate/phosphate	(91 ± 3)%	(91 ± 3)%
Sodium phosphate	(100 ± 3)%	(100 ± 3)%
Acetic acid/sodium acetate	(91 ± 3)%	(93 ± 3)%
MES	(84 ± 3)%	(93 ± 3)%

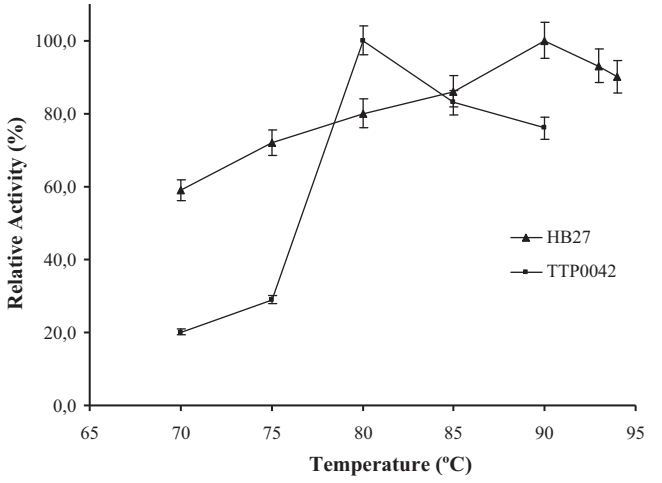


Fig. 3. Optimal temperature for HB27 cell extracts (induced with cellobiose) and TTP0042 His_6tag.

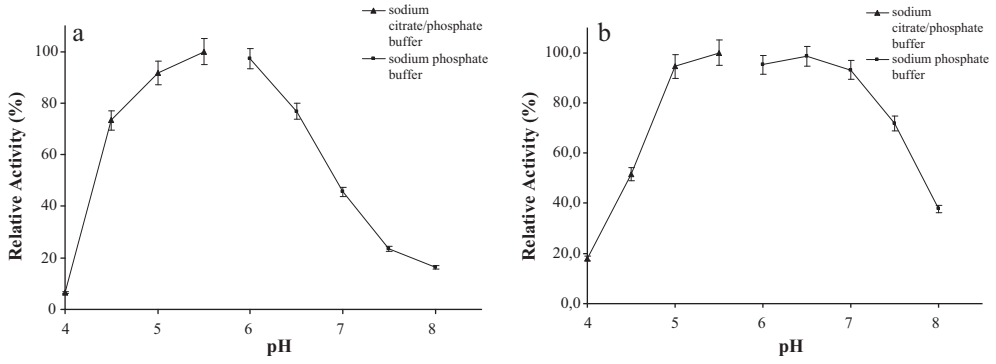
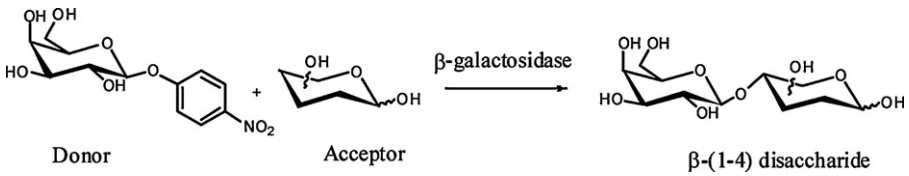


Fig. 2. Optimal pH for (a) HB27 cell extracts (induced with cellobiose) and (b) recombinant TTP0042 His_6tag.



Scheme 1. Scheme of transglycosylation reaction.

Table 5
Transglycosylation reactions (% yields) using cell extracts from HB27 and T42Kat as catalyst and *p*NP-β-Gal as donor.

Catalyst	Acceptor	Autocondensation β(1→3)	Autocondensation β(1→6)	Hydrolysis	Disaccharide β(1→4)	Disaccharide β(1→6)
HB 27	*p*NP-β-Gal	1.2	95.8	–	–	–
HB 27	Gal	–	–	100	–	–
HB 27	Man	–	–	100	–	–
HB 27	Fuc	37.1	–	62.9	–	–
HB 27	Glc	–	–	100	–	–
HB 27	GlcNAc	40	17	0	41	2
HB 27	GalNAc	–	–	100	–	–
T42Kat	*p*NP-β-Gal	20.9	32.6	–	–	–
T42Kat	GlcNAc	2.5	58.2	–	–	–
T42Kat	GalNAc	77.7	–	22.3	–	–

suggest that these enzymes are likely the subject of frequent events of Lateral Gene Transfer (LGT) among different strains of *Thermus* sp.

3.6. Transglycosylation reactions

Several articles report the use of recombinant TTP0042 enzyme from HB27 strain of *Thermus thermopilus* in transglycosylation reactions with nitrophenyl-β-glycosides as donors leading to self condensation products, with high preference toward β(1→3) linkages as undesired products [20–22]. Nitrophenyl-β-glycosides were also used both as donor and acceptor with this enzyme and with an mutant of this protein, leading to obtain a nitrophenyl tag attached to the final disaccharide as the main reaction product [48]. In our case, we used *p*-nitrophenyl-β-glycosides as donors and different monosaccharides as acceptors in order to synthesize a non tagged disaccharide (Scheme 1). First, we investigated transglycosylation reaction in sodium phosphate buffer 50 mM pH 5.5 at 80 °C catalyzed by cellobiose-induced cell extracts from the wild type HB27 strain and from its T42kat mutant using (42.5 mM) *p*NP-β-Gal as donor and different acceptors (212.5 mM): *p*NP-β-Gal, Gal, Man, Fuc, Glc, GlcNAc, GalNAc.

As shown in Table 5, wild type HB27 cell extracts presented a hydrolytic activity of the donor substrate under these conditions that was reduced in the T42kat mutant, which by contrast was able to show relevant autocondensing activity of the donor molecules (Gal-β(1→3)-Gal-β-*p*NP and Gal-β(1→6)-Gal-β-*p*NP). Noteworthy, HB27 cell extracts produce disaccharides with GlcNAc as acceptor, which were not produced by the extracts of the T42kat mutant. To confirm this, we also carried out parallel reactions under similar conditions with the recombinant TTP0042 protein overexpressed and purified from both *E. coli* and *Thermus thermophilus* (Table 6). An important issue regarded with these results is the presence of GlcNAc as acceptor affecting the hydrolysis rates on transglycosylation with cells extracts. The effects of an *N*-acetylated glycoside in the active centre of TTP0042 affecting regioselectivity of this biocatalyst in transglycosylation reaction have been previously reported [48], assuming this enzyme as the main glycosidase on HB27 cell extracts, we point out that GlcNAc could be the responsible for the change on the traditional hydrolysis behaviour observed in the acceptor screening (Table 5).

Results with TTP0042 His_6tag expressed in *E. coli* or in HB27nar (Table 6) show a high tendency toward autocondensation through β(1→3) bonds but hydrolysis of substrate was negligible in most cases. On the other hand, cell extracts display a mostly hydrolytic behaviour with low biosynthetic activity that produces β(1→6) self-condensates. This difference between to the results obtained with cell extracts of HB27, wild type with preference for hydrolysis (Table 5), and those obtained with His_6tag-TTP0042, with preference for auto-condensation (Table 6), could be related to the presence of additional activities in the cell extracts that could hydrolyze the β(1→6) auto-condensates. Reactions followed by HPLC confirm this hypothesis. At the beginning of the reaction (30 min) with the cell extracts small amounts of auto-condensation

Table 6
Transglycosylation reactions (% yields) using recombinant enzymes (His_6tag) from HB27 (expressed in *E. coli* and HB27nar) as catalyst and *p*NP-β-Gal as donor.

Catalyst (expression)	Acceptor	Autocondensation β(1→3)	Autocondensation β(1→6)	Hydrolysis	Disaccharide β(1→4)	Disaccharide β(1→6)
TTP0042 (*E. coli*)	*p*NP-β-Gal	78.3	21.7	–	–	–
TTP0042 (*E. coli*)	Gal	90.6	9.4	–	–	–
TTP0042 (*E. coli*)	Man	90.6	9.4	–	–	–
TTP0042 (*E. coli*)	Fuc	72.6	27.4	–	–	–
TTP0042 (*E. coli*)	Glc	73.6	7.1	–	–	19.3
TTP0042 (*E. coli*)	GlcNAc	80.0	7.7	–	12.3	–
TTP0042 (*E. coli*)	GalNAc	77.2	22.8	–	–	–
TTP0042 (HB27)	*p*NP-β-Gal	88.1	11.9	–	–	–
TTP0042 (HB27)	GlcNAc	79.5	3.6	–	16.9	–
TTP0042 (HB27)	GalNAc	77.5	21.5	–	–	–

Table 7
Effect of substrates concentration over reaction yields (%).

Donor (mM) *p*NP-β-Gal	Acceptor (mM) GlcNAc	Autocondensation β(1→3)	Autocondensation β(1→6)	Hydrolysis	Disaccharide β(1→4)	Disaccharide β(1→6)
43 mM	213 mM	80	8	–	12	–
170 mM	850 mM	38	16	–	46	–

product were detected, whereas 3 h later the small or negligible amounts of were found. This result strongly support that others enzymes in cell extract hydrolyze the auto-condensation product.

Recently, a mutant β-glycosidase was designed starting from native TTP0042 and both enzymes in presence of *o*NP-β-GlcNAc changed their regioselectivity from β(1→3) to β(1→4) achieving Gal-β(1→4)-GlcNac-*o*NP. However, auto-condensation β(1→3) product still remained as important product of the reaction [48]. In this study we found that GlcNAc can act as a non-activated acceptor producing Gal-β(1→4)-GlcNAc as the main final product of reaction. Therefore, the most biotechnologically relevant product detected was Gal-β(1→4)-GlcNAc, with yields around 17% with the enzyme produced in HB27. This disaccharide has a high pharmacological interest as precursor of H2 antigen [49].

3.7. Transglycosylation reactions with increased concentrations of the substrate

The results of Table 6 supported that TTP0042 could be used for the synthesis of disaccharide if higher product yields could be obtained. In our case, we modified the reaction conditions using the same donor:acceptor molar ratio at 65 °C, but increasing four times their concentration (0.17 M donor and 0.85 M acceptor) and also that of the enzyme (1.8 units/mL, approximately 0.22 mg of purified enzyme). Under these conditions the yield for synthesis of Gal-β(1→4)-GlcNAc was improved up to a 46–50% d (Table 7). This phenomenon could be explained by a deep modification of the chemical environment around the enzyme. The huge amount of donor and acceptor molecules could increase effective collisions in the active site of the enzyme and favour a new trend in the regioselectivty of the reaction.

4. Conclusions

In this work we demonstrate that among the strains tested, *Thermus thermophilus* HB27 presents a very interesting β-glycosidase activity. As it could be expected for a catabolic enzyme, this activity was induced in a nutrient-limited media containing a disaccharide with a β(1→4) bond like cellobiose (0.1–0.2%). Other disaccharides of similar structure like lactose were not inducers, supporting the existence of a highly specific detection system that discriminates them. To identify the enzyme responsible for this activity, we produced in *E. coli* and assayed the properties up to five protein candidates encoded within the genome of the HB27 strain, leading us to conclude that TTP0042 presents the highest similarity regarding biochemical and synthetic capabilities to the activity measured in the cell extracts. Actually, the knockout of the corresponding gene in the T42kat mutant confirmed this hypothesis and probed that this enzyme is the responsible for the main β-galactosidase activity in cell extracts of HB27. However, crude cell extracts of HB27 present more hydrolytic behaviour in transglycosylation reactions than pure TTP0042, a property most likely related to the presence of other enzymatic activity that hydrolyzes condensation products synthesized in the same reaction. The main problem for the use of TTP0042 in the synthesis of disaccharide is the auto-condensation of the *p*-nitrophenyl donor. This problem appears in reactions performed with cellobiose-induced cell extracts, and also with the purified enzyme. However, it was possible to achieve much higher yields in the desired disaccharide synthesis using a more concentrated media, a 1:5 donor:acceptor molar ratio, 1.8 units/mL of TTP0042 and 1 h of reaction. In this sense, the most relevant disaccharide obtained is the Gal-β(1→4)-GlcNAc.

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