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Trichoderma reesei acetyl esterase catalyzes transesterification in water

Lubomír Kremnický [a], Vladimír Mastihuba [b], Gregory L. Côté [c,*]

[a] *Institute of Chemistry, Slovak Academy of Sciences, Dúbravská cesta 9, 845 38 Bratislava, Slovak Republic*
[b] *Department of Food Science and Technology, Faculty of Chemical and Food Technology, Slovak University of Technology, Radlinského 9, 812 37 Bratislava, Slovak Republic*
[c] *NCAUR, ARS, USDA, 1815 North University Street, Peoria, IL 61604, USA*

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Abstract

Partially purified *Trichoderma reesei* RUT-C30 acetyl esterase preparation was found to catalyze acyl transfer reactions in organic solvents, mixtures of organic solvents with water and even in water. Using different acyl donors, the best results for acetyl transfer in water were obtained using vinyl acetate. As acetyl acceptors, a variety of hydroxyl bearing compounds in aqueous solutions were used. Degree of conversion and the number of newly formed acetates varied according to the acceptor used. Conversions over 50% were observed for the majority of several common monosaccharides, their methyl and deoxy derivatives and oligosaccharides. In several cases, the transesterification reaction exhibited strict regioselectivity, leading to only one acetyl derivative. Preparative potential of the transesterification in water was demonstrated by acetylation of methyl β-D-glucopyranoside, 4-nitrophenyl β-D-glucopyranoside and kojic acid, yielding 56.4% of methyl 3-*O*-acetyl β-D-glucopyranoside, 70.2% of 4-nitrophenyl 3-*O*-acetyl β-D-glucopyranoside and 30.9% of 7-*O*-acetyl-kojic acid as the only reaction products.

This enzymatically catalyzed transacetylation in water, which is applied to transformation of saccharides for the first time, opens a new area in chemoenzymatic synthesis. Its major advantages are simplicity, highly regioselective esterification of polar compounds, high yields, low enzyme consumption and elimination of the need to use toxic organic solvents.

Keywords: Acetyl esterase; Transesterification; Acylation; *Trichoderma reesei*

1. Introduction

The use of lipases and proteases to catalyze ester synthesis in non-aqueous solvents has been known and well recognized for nearly 20 years. Enzymes are often more thermostable in non-aqueous, organic environments [1], although the apparent activity in many such solvents can be lower by orders of magnitude. This could be an ideal situation for reactions where ester bonds are created, although water complicates the process by inducing hydrolytic side-reactions. The esters thus formed and often the acyl donor as well are usually soluble in the organic solvent, whereas the typically insoluble enzymes can be easily separated by filtration. The enzyme is usually stable enough to be reused several times, drastically reducing cost of the reaction [2].

The situation changes, however, when saccharides are to be esterified. Due to their low solubility in most non-polar organic liquids, potentially toxic or environmentally unfriendly solvents such as pyridine [3], *N*,*N*-dimethylformamide [4], tertiary butanol [5], dimethyl sulfoxide [6], 2-pyrrolidone [7], acetonitrile [8], etc. are usually employed, eliminating one of the main advantage of biotransformation techniques—reduction in the use of harmful reagents.

Derivatization of sugars is sometimes performed to improve their solubility in organic solvents. Glycosides [9], sugar acetals [10], complexes of saccharides with organoborates [5], etc. have been successfully esterified using enzymes. Attempts to dissolve the enzyme instead of substrate in order to attain contact of reactants with the catalyst have been accomplished through hydrophobization of the enzyme surface by various techniques, such as hydrophobic ion pairing [11] or lipid-coating [12–14]. Derivatization of either the reactants or the catalyst can negatively affect both cost of the process and purity of the product.

* Corresponding author. Tel.: +1-309-681-6319; fax: +1-309-681-6427.
E-mail address: COTEGL@ncaur.usda.gov (G.L. Côté).

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Since the early experiments of Klibanov [15,16], it has been generally accepted that the content of water in non-aqueous reaction systems has a great impact on behavior of the biocatalyst. This impact depends on the nature of enzyme and especially on the polarity of the solvent. The theory generally states that in non-polar solvents only a minimal amount of water is required to "polarize" reactive groups in the active site and on the surface of the enzyme. There is typically an optimal water content for every combination of enzyme, solvent, substrate, and reaction type. This water is kept in contact with the enzyme molecule as a "water shell" by hydrophobicity of the environment. Polar, water-miscible solvents can strip the water off the enzyme surface, thus causing its denaturation or at least decreasing its activity and stability. The requirement for water for maintenance of enzyme activity differs for various enzyme classes and each individual enzyme. Lipases usually require low water content (or more accurately, water activity) which influences not only overall enzyme activity but especially reactivity in acyl transfer reactions. However, glycosidases may have maximal transglycosidation activity at water activity as high as 0.9 [17].

Introducing detergent allows for one to increase the water content to a higher level, thus creating microemulsions (reverse micelles) where reactants are partitioned according to their relative solubility in water and in the organic phase. In these microemulsions, the enzyme occurs in the water pool or as deposits on the phase interface. Since lipases are activated at water/oil interfaces, microemulsions seem to be especially useful for esterifications catalyzed by this group of ester hydrolases. It is even possible to perform enzymatic esterifications in two-phase systems where both phases occur as separate pools without emulsification. This is possible when the product ester is so hydrophobic that it rapidly leaves the water phase where the enzyme is dissolved, thus preventing its hydrolysis. This is not the case when the product is a partially esterified carbohydrate, especially when short aliphatic acids are involved.

Our aim was to use a class of enzymes other than lipases or proteases for the esterification of saccharides in non-aqueous media. We selected acetyl esterases, a group of enzymes which have evolved in various microbes for the purpose of removing acetyl groups from native hemicellulose in plant cell walls. These enzymes are part of a complex that degrades plant matter for microbial catabolism. Examples include acetylxylan esterase and acetylmannan esterase [18–20]. Our strategy was based on the assumption that enzymes which naturally act on poly-, oligo- or saccharide esters would be more likely to catalyze their esterification. A similar approach has recently been described by our colleagues [21] using a related acetylxylan esterase in microemulsions in the presence of non-aqueous solvents.

We chose esterases present in the cellulolytic/hemicellulolytic enzyme system of the wood-rot fungus *Trichoderma reesei* [22,23]. Using crude media lyophilizate for transesterification of methyl β-D-xylopyranoside in 2-propanol, we have found that the reaction proceeds much better when water is present in reaction mixtures. Various proportions of water were added to non-aqueous solvents in an attempt to determine how much water was necessary to solubilize the substrates and enzymes without compromising the ability of the enzyme to catalyze the synthesis rather than the hydrolysis of ester bonds. It was presumed that some hydrolysis would invariably occur as soon as a significant level of water was present. Surprisingly, it was discovered that water could be added to the acylation reactions at levels far beyond what one would expect. In fact, acylations may be carried out in a purely aqueous environment, provided that an activated acyl donor is used. This very interesting observation led us to study the acyl tranferase activity of *T. reesei* enzyme preparations in water and to demonstrate its preparative versatility.

2. Materials and methods

2.1. Chemicals

Methyl 2,3,4-tri-*O*-acetyl-β-D-xylopyranoside was prepared by acetylation of commercially available methyl β-D-xylopyranoside by refluxing it with a mixture of sodium acetate in acetic anhydride [24] and recrystallized from ethanol.

Cyclic alternan tetrasaccharide was prepared from alternanase-hydrolyzed alternan [25]. Native, high-molecular weight alternan and limit branched dextran from *Leuconostoc mesenteroides* NRRL B-742 treated with endodextranase were both prepared as described by Côté et al. [26]. The limit branched dextran was purified by gel-filtration chromatography over Bio-Gel P-2 prior to use. Dextran T2000, larch xylan, pullulan, and all other acyl acceptors were commercial preparations.

2.2. Trichoderma reesei RUT-C30 cultivation and enzyme preparation

T. reesei RUT-C30 (NRRL 11460, ATCC 56765) was cultivated according to Biely et al. [27] on cellulose containing media. After 5 days, the culture was filtered on cheesecloth. Cell free medium was desalted and concentrated by ultrafiltration on Pellicon ultrafiltration unit equipped with Millipore PTGC 10,000 MW cut-off membrane against water. When not used immediately, desalted enzyme preparation was either lyophilized or frozen and stored in refrigerator or freezer till further use. Melted or solubilized enzyme preparations were clarified by centrifugation before use.

Acetyl esterase was partially purified by modified procedure according to Poutanen and Sundberg [22]. Desalted cell-free medium (acetyl esterase activity 883.6 U, proteins 0.5 g) in 0.01 M citrate buffer pH 4.0 was applied to a CM-Sepharose column (5 cm × 15 cm). The column was first washed with 0.01 M citrate buffer pH 4.0 (1000 ml)

and then with 0.22 M NaCl in the same buffer (770 ml). After applying a linear NaCl gradient (0.22–0.35 M NaCl in 0.01 M citrate buffer pH 4.0), acetyl esterase activity was eluted at about 0.30 M NaCl. Fractions containing over 30% of maximal acetyl esterase activity in its peak were pooled (660 ml), desalted against deionized water and concentrated (acetyl esterase activity 607.9 U, proteins 33.3 mg).

Acetylxylan esterase fractions separated by CM Sepharose according to Sundberg and Poutanen [23] did not show acetyl transfer activity in water, so enzyme content was not quantified.

2.3. Acetyl transfer reactions—preliminary experiments

The reaction mixture contained methyl β−D−xylopyranoside (20 mg), 7 mg desalted crude media lyophilizate of *T. reesei* RUT-C30 and 0.5 ml of organic solvent. Altering compounds of the reaction mixtures were vinyl acetate (0.1 ml), water (1.5–5 μl), sodium sulphate (50 mg).

2.4. Acyl transfer and deacetylation reactions

A typical reaction mixture contained the compound to be tested (20 mg) and desalted crude medium of *T. reesei* RUT-C30 (0.212 mg of proteins, 0.375 U of acetyl esterase activity) or partially purified acetyl esterase (0.026 mg of proteins, 0.411 U acetyl esterase activity) in 0.5 ml water or mixture of water/2-propanol or water/acetonitrile.

Acylation was initiated by addition of acyl donor (0.1 ml of liquid or 0.1 g of solid), and deacetylation by addition of an aqueous solution of the enzyme preparation. The mixtures were rocked (1 cycle per second) at room temperature and analyzed by TLC. Control samples lacking enzyme preparation or acyl donor were run simultaneously.

Reaction mixtures for following the kinetics of acetylation of methyl β-D-glucopyranoside and 4-nitrophenyl β-D-glucopyranoside contained 0.5 ml water solution of glucoside (10 mg), partially purified acetyl esterase (6.7×10^{-6} mg of proteins, 0.019 U acetyl esterase activity) and 0.1 ml of vinyl acetate.

3. Preparative synthesis

3.1. 4-Nitrophenyl 3-O-acetyl-β-D-glucopyranoside

The reaction mixture contained 4-nitrophenyl β-D-glucopyranoside (0.2 g), partially purified acetyl esterase from *T. reesei* RUT-C30 (1.52 U) in 10 ml of water and 3 ml of vinyl acetate. The mixture was gently stirred on magnetic stirrer at room temperature for 3 days. Reaction was stopped by freezing the bottom water phase, the liquid phase on the top (vinyl acetate) was removed and the water phase was then lyophilized.

The lyophilized mixture was solubilized in ethyl acetate, applied to a silica gel column and eluted with ethyl acetate. Fractions containing the only product of enzymatic transacetylation were pooled and the solvent was removed by evaporation. The product was then redissolved in chloroform and evaporated again to remove traces of ethyl acetate. The dry product was analyzed by NMR. The yield was 0.160 g of 4-nitrophenyl 3-*O*-acetyl-β-D-glucopyranoside, molar yield 70.2%.

3.2. Methyl 3-O-acetyl-β-D-glucopyranoside

The reaction mixture contained methyl β-D-glucopyranoside (0.3 g) and 0.1 ml of partially purified acetyl esterase from *T. reesei* RUT C-30 (1.52 U) in 6.0 ml of water. After addition of vinyl acetate (3 ml), the reaction mixture was gently stirred on a magnetic stirrer at room temperature for 3 days. Reaction was stopped by freezing the bottom water phase. The liquid phase (vinyl acetate) on the top was removed, and the water phase was lyophilized. The lyophilized mixture was resolubilized in ethyl acetate and applied to a silica gel column and eluted with ethyl acetate/toluene/ethanol (10:5:6). Fractions containing the only product of enzymatic transacetylation reaction were pooled and the solvent was removed by evaporation. Chloroform was then added to the product and evaporated to remove traces of ethyl acetate. The dried product was then analyzed by NMR. The yield was 0.206 g methyl 3-*O*-acetyl-β-D-glucopyranoside, molar yield 56.4%.

3.3. 7-O-Acetyl kojic acid (2-O-acetyloxymethyl-5-hydroxy-4H-pyran-4-one)

The reaction mixture contained kojic acid (0.2 g) and partially purified acetyl esterase of *T. reesei* RUT-C30 (1.52 U) in 10.0 ml of water. After addition of vinyl acetate (3 ml) the reaction mixture was gently stirred on a magnetic stirrer at room temperature for 3 days. The reaction was stopped by freezing the bottom water phase. The liquid phase (vinyl acetate) on the top was removed and the water phase was lyophilized.

The lyophilized mixture was solubilized in ethyl acetate, applied to a silica gel column and eluted with ethyl acetate. Fractions containing the only product of enzymatic transacetylation reaction were pooled, solvent evaporated. Product was then redissolved in chloroform, followed by its evaporation to remove traces of ethyl acetate. The residue was then analyzed by NMR. Yield 0.080 g of 7-*O*-acetyl kojic acid (2-*O*-acetyloxymethyl-5-hydroxy-4H-pyran-4-one), molar yield 30.9%.

3.4. Acetylation of polysaccharides

Reaction mixtures consisting of an aqueous solution of polysaccharide (0.312 g/6 ml), partially purified *T. reesei* acetyl esterase (8.2 U) and 1.2 ml of vinyl acetate were

rocked at room temperature (25 °C) at 1 cycle per second for 23 days. Blanks without vinyl acetate and/or enzyme were run in parallel. Sodium azide (0.02% (w/v)) was added to samples without vinyl acetate as a protection against microbial contamination. Enzymatic reaction was stopped by freezing the aqueous phase. The liquid layer of vinyl acetate on the top was removed and samples were lyophilized. Limit branched dextran was treated as described above, except the incubation was shortened to 4 days and the lyophilized sample was washed several times with acetone prior to homogenization. Samples were analyzed by FTIR spectroscopy.

4. Analytical methods

4.1. Thin layer chromatography

Products of enzymic reactions were followed by TLC using Silica gel 60 or Silica gel 60 F_{254} (Merck). Methyl β-D-xylopyranoside and its derivatives were separated in solvent systems consisting of either ethyl acetate/toluene/2-propanol or ethyl acetate/benzene/2-propanol (2:1:0.1 (v/v)). The system used for cyclic oligosaccharides and their derivatives consisted of nitroethane/acetonitrile/ethanol/H_2O (1:4:3:2 (v/v)). The systems for following the kinetics of acetylation of methyl β-D-glucopyranoside and 4-nitrophenyl β-D-glucopyranoside consisted of ethyl acetate/toluene/ethanol (2:1:1.2 (v/v) and 2:1:0.4 (v/v), respectively). All other compounds were separated in acetonitrile/H_2O (9:1 (v/v)). Separated compounds were detected with *N*-(1-naphthyl)ethylenediamine dihydrochloride reagent [28], or $KMnO_4$ (20 g $KMnO_4$ and 40 g K_2CO_3 per 1 l of H_2O) or by UV_{254} quenching. Amino acids were detected with ninhydrin spray.

4.2. NMR

^1H and ^{13}C NMR spectra (internal standard Me_4Si) were performed with a Bruker AM 300 spectrometer equipped with 5 mm probe at 300.13 MHz. Structures of isolated products were deduced from 2D ^1H–^1H COSY and HSQC spectra.

4.3. Molecular mass determination

Molecular mass was determined on MALDI IV (Shimadzu, Kratos Analytical, Japan). 2,5-Dihydroxybenzoic acid was used as a matrix, ion acceleration voltage was 5 kV. Samples were irradiated by 337 nm photons from nitrogen laser and typically 100 shots were summed into a single mass spectrum.

4.4. FTIR spectroscopy

Dry lyophilizates were pulverized with KBr and pressed into transparent disks for analysis by FTIR spectrometry. A typical test sample (2.0 mg) was pulverized at liquid nitrogen temperature in a sealed stainless steel vial containing a stainless steel ball bearing for 30 s on a Wig-L-Bug amalgamator (Crescent Dental Manufacturing, Lyons, IL). The vial was allowed to warm to room temperature before KBr (698 mg) was added. The KBr/sample mixture was pulverized at liquid nitrogen temperature in the vial with the ball bearing on the amalgamator for 5 s. After warming to room temperature, 300 mg of the 700 mg KBr/sample mixture was transferred to a KBr die (Perkin-Elmer, Norwalk, CT) and pressed under vacuum at 110 MPa on a laboratory press (Fred S. Carver, Menominee Falls, WI). FTIR spectra were measured on an FTS 6000 spectrometer (Digilab, Cambridge, CT) equipped with a DTGS detector. Absorbance spectra were acquired at 4 cm^{-1} resolution and signal-averaged over 32 scans. Spectra were baseline corrected and scaled to adjust for small differences in sample weights.

4.5. Other methods

Acetyl esterase activity was assayed according to Biely et al. [29], except potassium phosphate buffer was used instead of sodium phosphate. One unit of esterase activity is defined as 1 μmol of 4-nitrophenol released from 4-nitrophenyl acetate in 1 min at 25 °C.

Protein concentrations were determined using the Bio-Rad assay of Bradford [30].

5. Results and discussion

In our initial experiments, we found that the enzyme system secreted into the cultivation media by *T. reesei* during its growth on cellulose is capable of acetylating methyl β-D-xylopyranoside in 2-propanol, using vinyl acetate or ethyl acetate as acyl donors. We also observed that this ability varied considerably among otherwise comparable *T. reesei* preparations.

The enzyme systems were used in reactions as freeze-dried preparations. While searching for the possible cause of such irregularities in our results, we noticed that one of the factors influencing the acetylation was the presence of small amounts of moisture in some freeze-dried enzyme samples.

To investigate this observation we added very small amounts of water (0.3–1%) to reaction mixtures. Besides the enzyme preparation and 2-propanol, reaction mixtures contained methyl β-D-xylopyranoside and vinyl acetate. In samples where water was not added, acetylation proceeded weakly, but good acetylation occurred in samples with water added. The significance of water was confirmed in a parallel experiment when addition of sodium sulfate (a water-binding agent) to the reaction mixture resulted in complete inhibition of acetylation.

An experiment was designed to determine the critical concentration of water necessary to cause a shift from transacetylation to deacetylation, since the latter process is

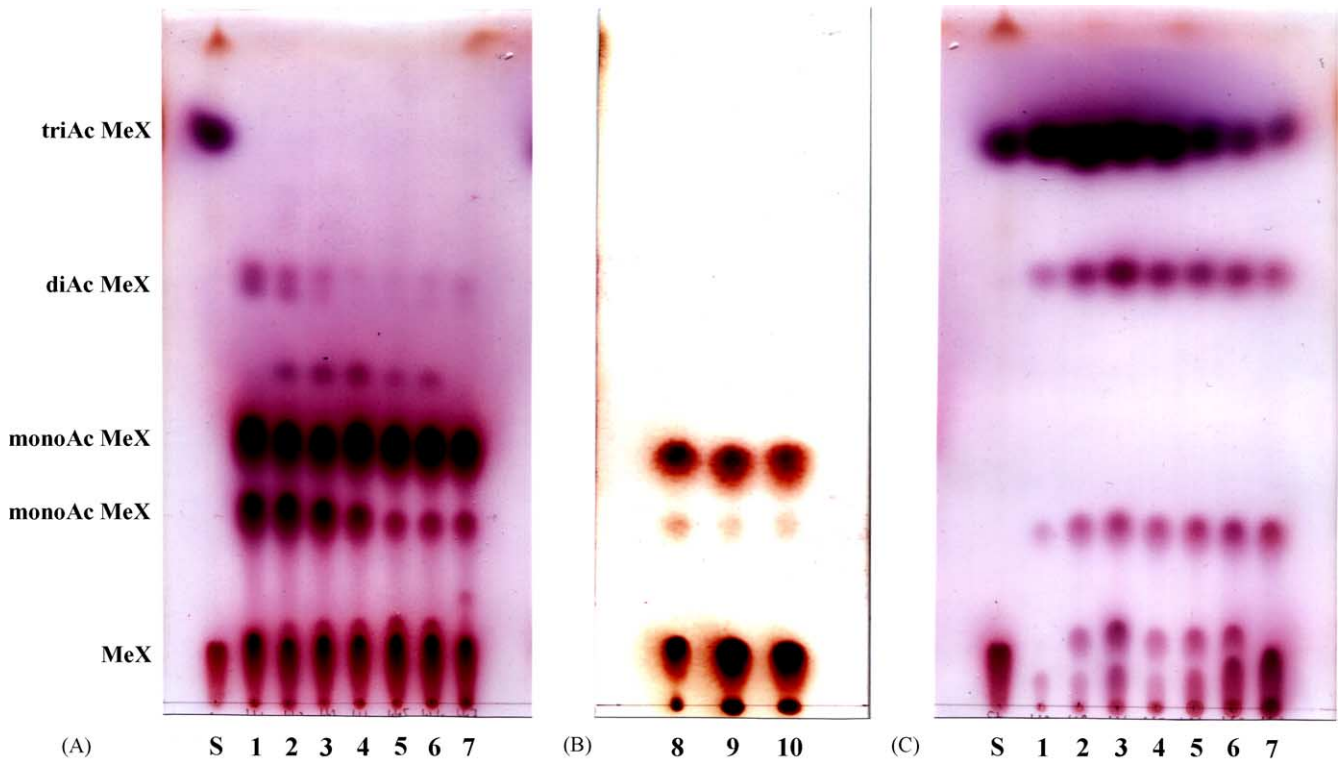

Fig. 1. *Trichoderma reesei* media preparation catalyzed acetylation of methyl β−D-xylopyranoside in (A), 2−propanol/water and in (B), acetonitrile/water using vinyl acetate as acyl donor and (C), deacetylation of methyl 2,3,4-tri-*O*-acetyl-β−D-xylopyranoside in 2-propanol/water. Water content in mixtures of water/2−propanol: (1) 3.2; (2) 17.7; (3) 32.2; (4) 51.6; (5) 71.0; (6) 85.5; and (7) 100%, in mixtures of water/acetonitrile: (8) 32.2; (9) 51.6; and (10) 71.0%. Twenty-second hour of reaction. MeX: methyl β−D-xylopyranoside; triAc MeX: methyl 2,3,4-tri-*O*-acetyl-β−D-xylopyranoside; diAc MeX: methyl di−*O*−acetyl-β−D-xylopyranoside; monoAc MeX, methyl *O*−acetyl-β−D-xylopyranoside, S, MeX and triAc MeX.

considered to be the natural function of the enzyme. Using mixtures of 2-propanol and water in which the 2-propanol was gradually substituted with water, we discovered that acetylation of methyl β-D-xylopyranoside with vinyl acetate is possible even in pure water, i.e. in the complete absence of 2-propanol (Fig. 1A). Moreover, with increased concentrations of water we observed higher regioselectivity of transacetylation. While in a predominantly organic environment we could observe equal formation of two monoacetates, a higher water content in reaction mixtures resulted in the production of a single monoacetate. Formation of diacetates seemed to be more pronounced in less polar mixtures. Reaction proceeded also in the acetonitrile/water mixtures, and the higher regioselectivity in such cases resulted in the formation of monoacetates (Fig. 1B).

Transacetylation was compared with deacetylation of fully acetylated methyl β-D-xylopyranoside in the same 2-propanol–water mixtures in the absence of vinyl acetate (Fig. 1C). Only one monoacetate of methyl β-D-xylopyranoside was observed on TLC during hydrolysis of methyl 2,3,4-tri-*O*-acetyl-β-D-xylopyranoside, and it was different from the major product synthesized by acetylation in water. The highest rate of hydrolysis was in polar solutions.

Parallel control experiments for both transacetylation and deacetylation in the absence of enzymes or vinyl acetate confirmed the fact that transacetylation in water required the presence of *T. reesei* enzyme preparation, which suggests controlled enzymic reaction.

Reaction mixtures presented in Fig. 1A and C (1 day of reaction) were also analyzed on the seventh day of incubation (not shown). In all cases, compositions of reaction mixtures analyzed after 7 days of incubation were practically identical to those analyzed after 1 day. Starting compounds were present in all reaction mixtures and neither complete deacetylation of methyl 2,3,4-tri-*O*-acetyl-β-D-xylopyranoside nor complete acetylation of methyl β-D-xylopyranoside were observed, indicating that the mixtures represent reaction states close to equilibrium.

Deacetylation of methyl 2,3,4-tri-*O*-acetyl-β-D-xylopyranoside in water and acetylation of methyl β-D-xylopyranoside in water/vinyl acetate within first 22 h of incubation is documented on thin layer plates presented in Fig. 2. Although formation of new products is seen since the very beginning of both reactions (0.3 h), transacetylation appears to be significantly faster than deacetylation. Similar kinetics were observed also in water/2-propanol reaction mixtures (22.6/77.4 (v/v) and 67.7/32.3 (v/v), results not shown).

Similarly to results presented on Fig. 1, the only monoacetate formed by hydrolysis differed from major product formed by transacetylation. According to their substrate specificity, esterases and lipases have preferences for re-

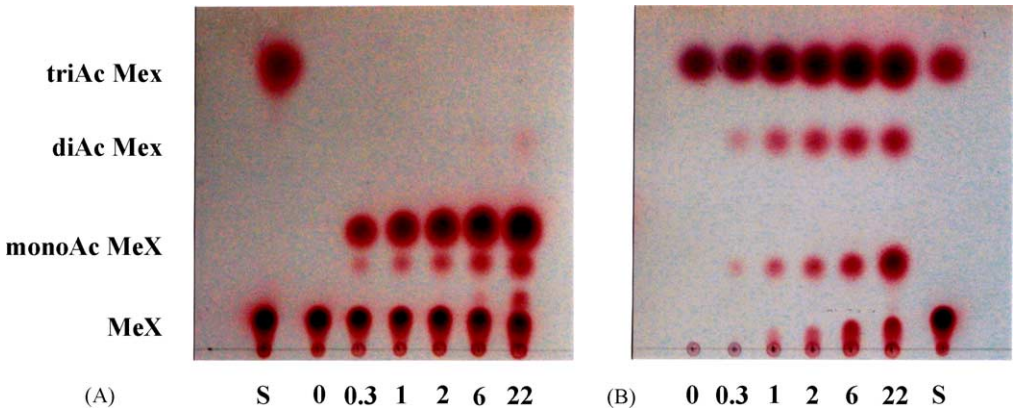

Fig. 2. Kinetics of *Trichoderma reesei* media preparation catalyzed A, acetylation of methyl β−D-xylopyranoside in water using vinyl acetate as acyl donor and B, deacetylation of methyl 2,3,4-tri-*O*-acetyl-β−D-xylopyranoside in water. 0, 0.3, 1, 2, 6 and 22 h of reaction. MeX, methyl β−D-xylopyranoside; triAc MeX methyl 2,3,4-tri-*O*-acetyl-β−D-xylopyranoside; monoAc MeX, methyl *O*−acetyl-β−D-xylopyranosides, S, MeX and triAc MeX.

leasing acetyl group in certain position of acetylated compounds, including acetyl groups present on acetylated carbohydrates [29,31–33]. The fact, that monoacetates formed by transacetylation differ from those formed by deacetylation can be explained by assumption, that in the first step enzyme(s) catalyzing process preferably acetylate(s) and deacetylate(s) the same position on the xyloside molecule.

In both transacetylation and deacetylation mixtures which contained 17.7–85.5% of H_2O in 2-propanol we observed the formation of a compound with chromatographic mobility slightly higher than methyl β-D-xylopyranoside (Fig. 1A,C). Since this product was present also in control experiments containing enzyme preparation but lacking vinyl acetate, we assume that this compound is 2-propyl xylopyranoside formed by transglycosylation catalyzed by β−xylosidase, present in the enzyme mixture [34,35]. We also assume, that the compound with TLC mobility between mono- and diacetates of methyl β-D-xylopyranoside, present in the same mixtures as 2−propyl xylopyranoside is the monoacetate of 2−propyl xylopyranoside. Putative 2−propyl xylopyranoside and its monoacetate were absent in reaction mixtures containing minimal (3.2%) and maximal (100%) amounts of water.

Formation of these products was not observed when acetonitrile was used instead of 2−propanol (Fig. 1B).

Despite recent publications [36–38] transacetylation in water is still a new phenomenon and the objection may be raised that this reaction can be caused by any protein, or some other component of the culture medium. To prove that the reaction is enzymatically catalyzed, we boiled or autoclaved several different *T. reesei* enzyme preparations to inactivate any enzymes present and used the boiled sample to study acetylation of methyl β−D−xylopyranoside and deacetylation of methyl 2,3,4-tri-*O*-acetyl-β-D-xylopyranoside. Transacetylation was attempted in acetonitrile/water mixture containing less than 25% of water and in water; deacetylation was attempted in water. None of the heated samples exhibited deacetylation or transacetylation activity. All unheated controls were active under the same reaction conditions. This supported the idea that these reactions do not result from the non-specific action of media components, but from enzymes in their native, active state.

Use of vinyl esters as acylating agents is a standard procedure for chemoenzymic esterifications in organic solvents. One advantage lies in quickly achieving of high yields of products, since the esterification process is kinetically controlled owing to oxo-enol tautomerisation of reaction side product—vinyl alcohol—to acetaldehyde [39], thus rendering the acyl transfer irreversible.

Taking into consideration that enzymes catalyzing hydrolysis can, under specific conditions, catalyze reverse reactions, the enzymes most likely to be responsible for acetylation are acetyl esterases. While growing on cellulose, *T. reesei* produces two enzymes capable of hydrolyzing acetyl bonds—acetylesterase and acetylxylan esterase [22,23].

CM-Sepharose fractions obtained by following the first steps of the procedures for acetylxylan esterase and acetyl esterase purification ([22,23], Materials and Methods 2.2) were analyzed for acetyl transfer activity using vinyl acetate as acetyl donor and methyl β-D-xylopyranoside solution in water as acceptor. Fractions capable of acetyl transfer reactions in water were analyzed also for acetyl esterase activity towards 4-nitrophenyl acetate. Analysis showed that transacetylation activity in water corresponded to fractions containing acetyl esterase, whereas acetylxylan esterase fractions appeared to be inactive.

Partially purified acetyl esterase showed similar patterns for acetylation of methyl β-D-xylopyranoside and deacetylation of methyl 2,3,4-tri-*O*-acetyl-β-D-xylopyranoside in water or in 2-propanol/water mixtures. The acetyl esterase preparation was free of β−xylosidase activity and no other products than methyl β−D−xylopyranoside and its monoacetates were detected by transesterification in reaction mixtures. This also supported our hypothesis that

β−xylosidase transglycosidation activity was involved in experiments where crude media preparations were used. Acetyl esterase was considered pure enough to be used in further experiments.

Reaction mixtures containing higher concentrations of water did not mix with vinyl acetate, but formed biphasic systems. Enzymic reactions carried out in water solutions of methyl β-D-xylopyranoside saturated with vinyl acetate showed that transacetylation could be accomplished in monophasic systems, taking place in the aqueous phase. However, the excess of vinyl acetate in biphasic systems resulted in higher conversion to acetates, especially upon prolonged incubation or at higher concentration of methyl β−D-xylopyranoside. The vinyl acetate layer served only as a means to ensure saturation of the aqueous phase with vinyl acetate, which has only limited solubility in water. Although vinyl acetate can be hydrolyzed by the enzyme or spontaneously, its top layer was always present throughout the experiments. The excess used in our experiments was thus considered sufficient and was not further optimized.

All of the acetylation experiments were performed with methyl β−D-xylopyranoside as a model molecule. To extend our knowledge about other possible acetyl acceptors, we have used the partially purified acetylesterase of *T. reesei* to acetylate a wide spectrum of mono- and oligosaccharides and their derivatives in water. Vinyl acetate served as an acetyl donor.

Results presented in Table 1 indicate that acetylation occurred for nearly every compound tested, from glycerol to cyclic alternan tetrasaccharide. The only exception was cyclodextrin, which was unreactive. The degree and number of newly formed acetyl derivatives varied. Whereas in some cases the regioselectivity led to formation of only one compound, in other instances, we could detect the formation of at least three compounds. For example, although glucopyranoside and xylopyranoside have similar structures, acetylation of the latter is less selective, giving two products. This can be explained either by higher specificity of the enzyme towards glucopyranoside or by a high rate of acetyl migration along the more flexible xylopyranoside ring [40]. In mixtures with several products, usually one or two of the products were in significantly higher concentration than the other(s). Data presented in Table 1 are informative mainly for their qualitative value, since they were obtained using thin layer chromatography and may not be able to distinguish the exact number of newly formed products. The number of products detected can be influenced by spontaneous acetyl migration [40–43] or by failure to resolve products with similar mobility by TLC.

Such a wide range of acetyl acceptor molecules inspired us to try to acetylate hydroxyl amino acids and peptides. Although we did not use the most suitable system for resolution by TLC, we observed formation of new products in reaction mixtures containing L-serine, L-threonine, BOC-serine and trans-4-hydroxy L-proline. Reaction mixtures were compared against controls without enzyme. These results complement those recently described by Guranda et al. [44].

Besides different acetyl acceptors, we studied the ability of a variety of vinyl esters to serve as acyl donors in aqueous solutions. D−xylose, methyl α−D−mannopyranoside and methyl β−D−glucopyranoside were used as model acyl acceptors. Using partially purified *T. reesei* acetyl esterase and vinyl esters with increasing chain length we found that the best acyl donor was vinyl acetate. Vinyl propionate was somewhat less effective, and vinyl esters of acids with acyl chains longer than four carbons were ineffective, which is in agreement with the natural preference of acetyl esterases towards acetyl esters.

T. reesei acetyl esterase also catalyzed acylation in water with 2,2,2-trichloroethyl acetate and 2,2,2-trifluoroethyl butyrate as acyl donors. Rates of these reactions were much slower than those observed for corresponding vinyl esters. Ethyl acetate, a good acetyl donor in non-aqueous organic solutions, appeared to be relatively ineffective for acylations in water. These results confirmed our hypothesis that activated acyl donors are required for successful acylations in water.

Most of the reactions described above were carried out in unbuffered mixtures using deionized water and desalted enzyme preparations. Reactions performed in sodium citrate buffer (17 mM, pH 5.4) and/or in the presence of a low concentration of NaCl (20 mM) showed no significant difference in comparison to controls in water. By way of contrast, 20 mM NaCl inhibits acetylation in predominantly organic solvents.

6. Preparative synthesis

Results of screening for different acetyl acceptors showed that enzymatic acetylation of methyl β-D-glucopyranoside and 4-nitrophenyl β-D-glucopyranoside leads to formation of sole products (Table 1). Monitoring the formation of corresponding monoacetates within the first 22 h of reaction (Fig. 3) confirmed those results.

Methyl β-D-glucopyranoside and 4-nitrophenyl β-D-glucopyranoside were chosen to prove the effectiveness of preparative acetylation in water, using vinyl acetate as acetyl donor. A nonsaccharidic compound, kojic acid, which also gave only one acetylated product in the screening (Table 1) was included in this preparative experiment as well.

The sole products of transesterification catalyzed by *T. reesei* acetyl esterase were isolated and characterized. ^{1}H NMR spectra for methyl β-D-glucopyranoside and acetyl kojic acid monoacetates (Tables 2 and 3) were in agreement with published data for 3-*O*-acetyl methyl β-D-glucopyranoside [45] and 7-*O*-acetyl kojic acid (2-*O*-acetyloxymetyl-5-hydroxy-4H-pyran-4-on) [46], showing acetylation of methyl β-D-glucopyranoside in the C3 position and acetylation of the primary hydroxy group of kojic acid. The third compound, a monoacetate of

Table 1
Examples of mono- and oligosaccharides and their derivatives acetylated by *T. reesei* acetylesterase in 1 or 2 days

Compound	Degree of acetylation	Number of products	Number of major + minor products
Glycerol	+++	2	1 + 1
L-Arabinose	++	3	2 + 1
D-Ribose	+++	2	1 + 1
D−Xylose	+++	2	2
D-Fructose	++	4	1 + 3
D-Galactose	+++	3	1 + 2
D-Glucose	+++	3	1 + 2
D-Mannose	+++	2	1 + 1
L-Rhamnose	+	1	1
L-Sorbose	+++	2	1 + 1
D-Glucoheptose	+	2	1 + 1
Cellobiose	+++	3	1 + 2
3-*O*-β-D-Galactopyranosyl-D-arabinose	+++	2	1 + 1
Lactose	+++	2	2
Maltose	++	2	1 + 1
Melibiose	+++	2	1 + 1
Palatinose	+++	3	2 + 1
Sucrose	+	4	2 + 2
Trehalose	+++	3	1 + 2
Raffinose	+++	2	1 + 1
Maltotriose	+	1	1
Isomaltose	++	2	1 + 1
Cyclic alternan tetrasaccharide	+++	2	1 + 1
Cyclodextrin	–	–	–
Maltitol	+	1	1
Mannitol	+	1	1
Myo-inositol	+++	3	2 + 1
D-Sorbitol	+	2	2
Xylitol	++	2	1 + 1
6-Deoxy-L-galactose (L-fucose)	+++	2	1 + 1
2-Deoxy-D-galactose	+++	2	2
2-Deoxy-D-glucose	+++	2	1 + 1
2-Deoxy-D-ribose	+	1	1
Methyl α-D-galactopyranoside	+++	2	1 + 1
Methyl β-D-galactopyranoside	+++	1	1
Methyl α-D-glucopyranoside	+++	2	1 + 1
Methyl β-D-glucopyranoside	+++	1	1
4-Nitrophenyl β-D-glucopyranoside	+++	1	1
n-Octyl β-D-glucopyranoside	+++	1	1
1,2-Isopropyl-glucofuranoside	++	1	1
Methyl α-D-mannopyranoside	+++	1	1
Methyl β-D-mannopyranoside	+++	1	1
Methyl α-D−xylopyranoside	+++	3	1 + 2
Methyl β-D−xylopyranoside	+++	2	1 + 1
α-Glucoheptonic lactone	+	1	1
D-Gulono-γ-lactone	+	1	1
Glucuronoamide Glc-NH_2·HCl	++	1	1
D-Galacturonic acid	+	1	1
D-Glucuronic acid	+	1	1
D-Glucose-6-phosphate	+	1	1
Ascorbic acid	+	1	1
Kojic acid	++	1	1

Degree of acetylation estimated from intensity of spots after TLC: + traces up to 24%, ++ 25–49%, +++ 50–99%, ++++ 100%.

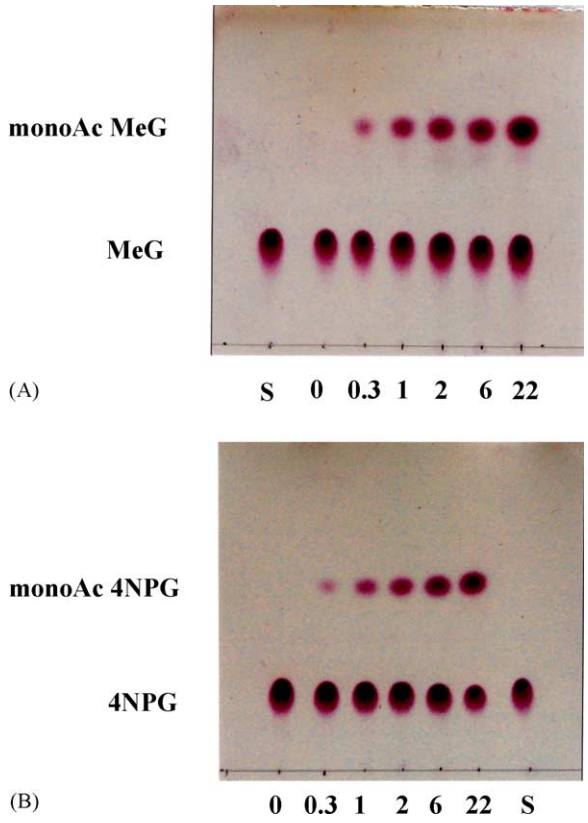

Fig. 3. Kinetics of *Trichoderma reesei* partially purified acetyl esterase catalyzed acetylation of A, methyl β−D-glucopyranoside and B, 4-nitrophenyl β−D-glucopyranoside in water using vinyl acetate as acyl donor. 0, 0.3, 1, 2, 6 and 22 h of reaction. (A) MeG: methyl β−D-glucopyranoside; monoAc MeG: methyl *O*−acetyl-β−D-glucopyranoside, S, methyl β−D-glucopyranoside. (B) 4NPG: 4-nitrophenyl β−D-glucopyranoside; monoAc 4NPG, 4-nitrophenyl *O*−acetyl-β−D-glucopyranoside, S, 4-nitrophenyl β−D-glucopyranoside.

Table 2
^{1}H NMR data of 7-*O*-acetyl kojic acid ($CDCl_3$, 300 MHz)

	^{1}H Chemical shifts (ppm)[a]			
H	CH_2	3	6	$COCH_3$
^{1}H	4.93	6.49	7.84	2.16

[a] Relative to TMS at 0 ppm.

4-nitrophenyl β-D-glucopyranoside, was identified by ^{1}H NMR, ^{13}C NMR and 2D NMR methods (H–H COSY and HSQC) as 4-nitrophenyl 3-*O*-acetyl-β-D-glucopyranoside. Similarly to methyl 3-*O*-acetyl-β-D-glucopyranoside, in the case of 4-nitrophenyl β-D-glucopyranoside the introduction of an *O*-acetyl group in position 3 of β-D-glucopyranoside unit causes deshielding of C-3 (1.88 ppm) and H-3 (1.38 ppm) (Table 4). Molecular mass (*M*) of 343.30 g/mol was confirmed by MALDI-MS, where mass/charge ratios were (Na + *M*) = 366.55 and (K + *M*) = 382.30 (366.29 and 382.40 calculated). Optical rotation was $[\alpha]_D^{20} = -60.0^\circ$ (methanol, $c = 1.0$).

These results confirm enzyme preference for acetylation of C3 position of glucopyranoside unit and they are also in agreement with acetyl esterase specificity for deacetylation of C-3′ position of acetylated xylobiose [47].

Transacetylation reactions yielded 56.4% (mol) of methyl 4-nitrophenyl 3-*O*-acetyl-β-D-glucopyranoside, 70.2% (mol) of 3-*O*-acetyl 4-nitrophenyl β-D-glucopyranoside and 30.9% (mol) of 7-*O*-acetyl kojic acid (2-*O*-acetyloxymetyl-5-hydroxy-4H-pyran-4-one). Raku and Tokiwa [48] recently described the use of protease for the regioselective acylation of kojic acid at the primary hydroxyl position. The reaction presented here thus complements their procedure.

Although transacetylation reactions were not quantitative, they were not optimized and yields are comparable with those reported for lipases catalyzed transacylation in organic solvents. All three isolated derivatives were deacetylated to the original compounds in aqueous solutions by the same *T. reesei* acetyl esterase preparation which catalyzed transacetylation.

In the screening for suitable saccharides as acyl acceptors, we showed that practically any saccharide or derivative containing a hydroxyl group can by acetylated. Successful acetylation of cyclic tetraoligosaccharide indicated that acetylation/acylation can also be done on higher saccharides.

We tried partially purified *T. reesei* acetylesterase, free of polysaccharide-hydrolyzing activities to acetylate several polysaccharides, namely alternan, dextran T-2000, inulin, levan, pullulan, soluble starch and xylan, in water solutions using vinyl acetate as the acetyl donor. Infrared spectra showed a slight increase of acetyl content in alternan, dextran, pullulan and xylan samples. Unfortunately, all polysaccharides contained small amount of oligosaccharides. Because even traces of oligosaccharides could influence the results in favor of acetylation, in subsequent experiment we used an ultrafiltered, high molecular weight fraction of limit branched dextran. Infrared spectra showed tiny increase of absorbance in acetyl region of enzyme treated sample. According to the literature, acetylesterase of *T. reesei* is inactive towards acetyl groups on polysaccharides and

Table 3
^{1}H NMR data of methyl 3-*O*-acetyl-β-D-glucopyranoside ($CDCl_3$, 300 MHz)

	^{1}H Chemical shifts (ppm)[a] and coupling constants (Hz)								
H	1	2	3	4	5	6	6′	OCH_3	$COCH_3$
^{1}H	4.31	3.48	4.92	3.69	3.42	3.84	3.94	3.58	2.18
$^3J_{n,n+1}$	$7.8_{1,2}$	$9.5_{2,3}$	$9.4_{3,4}$	$9.6_{4,5}$	$4.4_{5,6'}$	$3.4_{5,6}$	$11.9_{6,6'}$		

[a] Relative to TMS at 0 ppm.

Table 4
^{1}H and ^{13}C NMR data of 4-nitrophenyl β-D-glucopyranoside and 4-nitrophenyl 3-*O*-acetyl-β-D-glucopyranoside (CD_3OD, 300 MHz)

	^{1}H, ^{13}C chemical shifts (ppm)[a] and coupling constants (Hz)									
	H, C	1	2	3	4	5	6	6′	4-NPh	$COCH_3$
4-NPh Glc*p*	^{1}H	5.06	3.49	3.49	3.41	3.53	3.70	3.90	2 × 7.24; 2 × 8.21	
4-NPh 3-Ac Glc*p*	^{1}H	5.17	3.63	4.87	3.57	3.60	3.72	3.90	2 × 7.24; 2 × 8.22	2.14
		(0.11)	(0.14)	(1.38)	(0.16)	(0.07)	(0.02)	(0)		
4-NPh Glc*p*	$^{3}J_{n,n+1}$	$7.4_{1,2}$		$7.6_{3,4}$		$5.6_{5,6'}$	$2.1_{5,6}$	$12.1_{6,6'}$	$9.3_{o,m}$	
4-NPh 3-Ac Glc*p*	$^{3}J_{n,n+1}$	$7.8_{1,2}$	$9.6_{2,3}$	$7.8_{3,4}$	$8.1_{4,5}$	$4.7_{5,6'}$	$1.7_{5,6}$	$11.9_{6,6'}$	$9.3_{o,m}$	
4-NPh Glc*p*	^{13}C	101.76	74.77	77.92	71.26	78.47	62.47		163.97; 2 × 126.66 2 × 143.96; 117.80	
4-NPh 3-Ac Glc*p*	^{13}C	101.49	73.03	78.75	69.34	78.20	62.09		163.76; 2 × 126.68 2 × 144.05; 117.81	172.65; 21.13
		(−0.26)	(−1.77)	(+1.88)	(−1.96)	(−0.27)	(−0.37)			

The values in parentheses are the chemical shifts differences to the parent 4-nitrophenyl β-D-glucopyranoside. Positive differences indicate downfield shifts. 4-NPh Glc*p*: 4-nitrophenyl β-D-glucopyranoside, 4-NPh 3-Ac Glc*p*: 4-nitrophenyl 3-O-acetyl-β-D-glucopyranoside.
[a] Relative to TMS at 0 ppm.

preferably deacetylates short oligomeric and monomeric acetates. This indicates that the enzyme may be unable to acetylate polysaccharides. Further studies are planned to find out if these phenomena are unique to *T. reesei* acetyl esterase.

7. Conclusions

Partially purified *T. reesei* acetyl esterase is capable of catalyzing the acylation of compounds containing one or more hydroxyl groups in water or aqueous environments in the presence of activated esters of organic acids serving as acyl donors. This enzyme catalyzed transacylation reaction is very specific and can lead to enzymatic/chemoenzymatic synthesis of new compounds or to more economic production of compounds already known. Although enzymatic acylation of saccharides has been known for a long time, previous reactions were carried out in organic solutions, which can be limited by the solubility of the hydroxyl containing compounds, solubility of the enzymes, or economic factors such as price of the solvent and cost of recovery or disposal. The enzymatic process described here introduces an attractive alternative to the chemical preparation of specifically acylated hydroxyl-containing compounds.

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