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Transglycosylation reaction of endoxylanase from *Trichoderma longibrachiatum*

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Abstract

The study of hydrolytic activities of several enzymatic preparations showed that the Glucanase GL-200 and Xylanase XL-200 enzymatic preparations from *Trichoderma longibrachiatum* and the Xylanase from *Trichoderma viride*, possessed an endoxylanase activity useful for the transfer reaction. The enzymatic synthesis of hexylxyloside and hexylxylobioside were achieved by xylose transfer, catalysed by *T. longibrachiatum* XL-200 xylanase crude preparation, from xylan (donor) to hexanol, with (50%, v:v) or without *n*-hexane used as co-solvent. Benzyl alcohol was also used as acceptor for the synthesis reaction of benzylxyloside, benzylxylobioside, and benzylxylotrioside, with the *T. longibrachiatum* XL-200 xylanase crude preparation and partially pure *T. longibrachiatum* endoxylanase. The transfer reaction due to the *T. longibrachiatum* endoxylanase was confirmed by the enzymatic synthesis, catalysed by *T. longibrachiatum* partially pure endoxylanase, of phenyl primeveroside performed from phenyl glucoside and xylan, in the presence of *n*-hexane. We showed that the *T. longibrachiatum* endoxylanase was a good tool for the synthesis of xylosyl derivatives (homo- and hetero-xylosides), by transfer reaction.
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Keywords: Hydrolytic activities; Transfer reaction; Endoxylanase; Enzymatic synthesis

1. Introduction

During the last twenty years chemical and enzymatic synthesis of alkyl glycosides, among them alkyl xylosides, have attracted considerable interest. These compounds, which possess surface and antimicrobial activities and are biodegradables, constituted a new class of non-ionic surfactants which have numerous applications in chemistry, foods, cosmetics, pharmaceutics and medicine (Matsumura, Ando, Toshima, & Kawada, 1998). Their chemical regioselective synthesis is complicated by the requirement of protection and deprotection steps and the use of heavy metal salts as catalysts. The enzymatic synthesis provides convenience because of the synthesis of a regio- and stereoselective product commonly in one-step reaction, without environmental injuries. The use of plant or microbial enzymes for the synthesis of alkyl xylosides with surfactant properties has been extensively studied (Drouet, Zhang, & Legoy, 1994; Matsumura, Kinta, Sakiyama, & Toshima, 1996; Matsumura, Takahashi, Nishikitami, Kubota, & Kobayashi, 1997; Matsumura et al., 1998; Matsumura, Sakiyama, & Toshima, 1999; Nakamura, Toshima, & Matsumura, 2000; Yu, Xu, Lu, & Lin, 2007). Reports on the enzymatic synthesis of xylosides consisting of aroma compounds are more scarce (Kadi, Belloy, Chalier, & Crouzet, 2002; Shinoyama, Ando, Fujii, & Yasui, 1991).

Primeverosides or 6-*O*-β-xylopyranosyl-β-D-glucopyranosides are more generally present in leaves of plant belonging to the Camellia family. Some of them are known as the main precursors of floral aroma of tea (*Camellia sinensis*) developed during processing (Wang, Kurasawa, Yamaguchi, Kubota, & Kobayashi, 2001). Primeverosides were also identified in fruits, leaves, roots and flowers

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(Kijima et al., 1997; Schwab & Schreier, 1990; Watanabe et al., 1993).

β-Xylosidase from *Aspergillus pulverulens* induced a β-xylosyl transfer reaction from xylobiose to *p*-nitrophenyl β-D-glucopyranoside; *p*-nitrophenyl β-primeveroside was the dominant synthesized product relatively to its isomers (Murata, Shimida, Watanabe, Sakata, & Usui, 1999). More recently, enzymatic synthesis of a series of β-primeverosides as aroma precursors, via β-primeverosyl transfer reaction, was reported by Tsuruhami, Mori, Sakata, Amarume, and Saruwatari (2005).

The enzyme-catalysed synthesis of glycosides can be achieved through two ways, reverse hydrolysis (thermodynamic control) or transglycosylation (kinetic control), from renewable and easily available raw materials such corn starch, lactose or xylan and relatively inexpensive industrial enzymes. The transglycosylation which involves one or more saccharidic unit transfer from a donor to an acceptor is faster than reverse hydrolysis. Transglycosylation reactions generally take a few hours rather than days. Two kinds of enzyme are involved in xylosylation reaction, endo-β-1,4-xylanase or endoxylanase or EX for transxylosylation (Eneyskaya et al., 2003; Jiang et al., 2004) and exo-β-1,4-xylanase (or β-xylosidase) for reverse hydrolysis (Biely, 2003).

So, the aim of this work was the screening of various enzymatic preparations to find an endoxylanase activity to synthesize xylosyl derivatives, by transglycosylation.

2. Materials and methods

2.1. Enzymes

Xylanase XL-200 and Glucanase GL-200 from *Trichoderma longibrachiatum* were obtained as a gift from Saf-Isis (Soustons, France), Xylanase from *Trichoderma viride* and Pectinase from *Aspergillus niger* were furnished by Sigma (St Quentin Fallavier, France), hemicellulase REG2 from *A. niger* was a Gist Brocades (Seclin, France) preparation.

2.2. Products

n-Hexane (95% purity) and ethyl acetate (99% purity) were from Prolabo and acetonitrile (99.8% purity) was from SDS (Peypin, France).

Water, conductivity 18.2 MΩ cm, was furnished by a Purelab Plus system (US filter).

Aroma compounds (hexanol, benzyl alcohol) used in the present work (purity 97–100%) were from Sigma-Aldrich.

Xylan, from birch wood, D-(+)-xylose and D-(+)-xylobiose (purity more than 97%) and [*N*-methyl-bis(trifluoroacetamide)] reagent (TFA) were obtained from Sigma.

Phenyl-, *n*-hexyl-, and heptyl-glucosides (purity 98%) were purchased at Sigma.

Xylobiose was prepared by enzymatic hydrolysis of xylan. 300 mL of a xylan solution (10 g/L) in sodium acetate buffer, 0.1 M, pH 5, were incubated during 14 h at 40 °C under stirring in the presence of 1.2 mL of a crude enzymatic preparation of xylanase XL-200 (14×10^3 UI/mL). After elimination of the excess of xylan by centrifugation, the supernatant was concentrated to 20 mL. Then the addition of fresh ethanol (40 mL) and further incubation for 5 h at −18 °C allowed the precipitation of the residual xylan polymer. A last centrifugation of the mixture for 45 min, at 4 °C and 17,700*g* was carried out, following by concentration of the supernatant to 5 mL, by rotary evaporator under reduced pressure. Xylobiose was separated by gel filtration on Bio-Gel P-2 (Bio-Rad, Hercules, CA). The various fractions with xylobiose and xylotriose were then collected, pooled, and dried-frozen. The quantification of xylobiose was performed by HPLC on a Spheri-5 amino column (22 cm × 4.6 mm i.d.) from Touzart & Matignon, by comparison with a pure (98%) sample of xylobiose.

2.3. Protein determination

Proteins present in enzymatic preparations were determined according to the method of Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin as standard.

2.4. Xylan (or xylobiose) hydrolysis by various enzyme preparations

Hydrolysis of 6 mL of xylan (10 g/L) or xylobiose (50 mM) by several enzyme preparations (300 μL) was performed at 40 °C for 4 h, under stirring (400 rpm), in sodium acetate buffer (0.1 M, pH 5). After enzyme inactivation (10 min at 100 °C) and centrifugation (15 min at 25,000*g*) for elimination of xylan excess, the xylose oligomeres with different condensation degrees were separated and quantified by HPLC on a spheri-5 amino column (22 cm × 4.6 mm i.d.) from Touzart & Matignon.

2.5. Endoxylanase activity measurements

Hydrolysis was carried out by incubation of 250 μL of xylan solution (10 g/L) and 250 μL of diluted enzyme preparation for a total volume of 0.5 mL, in sodium acetate buffer (0.1 M, pH 5), at 40 °C during 10 min. The liberated reducing sugars were determined by Somogyi–Nelson (Somogyi, 1952) method with xylose as standard. The activity was expressed in International Unit (IU). One International Unit is defined as the enzyme amount catalyzing the release of one μmol of equivalent xylose per minute.

2.6. Xylosidase activity measurements

A 4 mM solution (200 μL) of *p*-NP xyloside in 100 mM sodium acetate buffer (pH 5.0) and 200 μL of enzyme preparation, suitable diluted, were incubated at 40 °C for 10 min. The reaction was stopped by adding 1.2 mL of 1 M sodium carbonate. The resulting yellow color was

measured at 400 nm by a spectrophotometer and the amount of released *p*-nitrophenol determined using a calibration curve with *p*-nitrophenol as standard. The results were expressed as μmole of *p*-nitrophenol released per min and per proteins mg (IU/mg).

2.7. Determination of the effects of organic compounds on endoxylanase activity

The determination of the endoxylanase activity after incubation in various organic solvents constitutes a simple measure of the inhibition degree of these compounds (Klibanov, 1989). The reaction was carried out in a total volume of 2 mL with a 1:1 (v/v) proportion of aqueous/organic media. The partially pure endoxylanase (100 μL) was incubated 30 min, at 40 °C, under stirring, in 0.9 mL of acetate buffer (0.1 M, pH 5) and 1 mL of various organic solvents, or organic compounds, potential donors in transfer reaction.

The reaction was stopped by incubation of each tube in ice. The aqueous phase (1 mL) was collected and the xylanase activity was evaluated according to the Somogyi–Nelson method (Somogyi, 1952).

The control was constituted of the partially pure endoxylanase (100 μL), and 1.9 mL of acetate buffer (0.1 M, pH 5) and was incubated 30 min, at 40 °C, under stirring. Subsequently, it was treated as the samples.

2.8. Partial purification of endoxylanase

The industrial XL-200 enzyme preparation (10 mL) was treated by ultrafiltration using an Amicon model 52 cell fitted with PM 10 membrane, at 4 °C under 2×10^5 Pa nitrogen pressure. The preparation was washed with 40 mL of sodium acetate buffer, 20 mM, pH 5 and finally adjusted to 10 mL with the same buffer.

This crude enzyme was partially purified by FPLC using an AKTA basic 900 unit, (Amersham Pharmacia Biotech, Uppsala, Sweden), with a 20 cm × 5 cm i.d. XK 50/20 Pharmacia Biotech column filled with CM Sepharose LC-6B (weak cations exchanger) equilibrated with a sodium acetate buffer, 20 mM, pH 5. The protein elution was followed by spectrophotometry at 260 nm. Crude enzyme preparation (10 mL) was loaded on the column and elution was performed with sodium acetate buffer at 2 mL/min. The elution was performed with a gradient 0–40% of NaCl 0.5 M during 30 min and by a second 40–100% (v/v) NaCl gradient. Twenty milliliter fractions were collected, the fractions containing an endoxylanase activity were pooled and concentrated by ultrafiltration to a final volume of 10 mL of sodium acetate buffer (0.1 M, pH 5).

A further purification was carried out on a gel filtration column (Superdex 200, Amersham Pharmacia), with the sodium acetate (20 mM, pH 5) and NaCl (15 mM), as elution buffer. The spectrophotometer detection was 260 nm, and the fractions with the endoxylanase activity were pooled and concentrated by ultrafiltration.

2.9. Purity of the purified endoxylanase

The purity of the transfer activity endoxylanase, purified on weak cations exchange column, was evaluated by SDS-polyacrylamide gel 10%. The migration was carried out at 40 mA during 2 h. The gel was washed in 12% trichloroacetate solution followed by staining in coomassie blue solution for 2 h. The destain buffer consisted of methanol, acetic acid and water.

The relative ratio of proteins was performed by densitometry with the Clara Vision pictures programme (Gel Grab, USA).

2.10. Xylosides biosynthesis

2.10.1. Organic phase as only acceptor

In a typical experiment, a mixture containing: 50 mg xylan, 9 mL of aroma compound (hexyl alcohol or benzyl alcohol), and 1 mL of xylanase XL-200 enzymatic preparation were stirred under magnetic agitation (400 rpm) at 50 °C, for 3 h, in a tightly closed flask. The reaction was stopped by heating in boiling water for 10 min. The excess of aroma compound was then distilled under vacuum and the residue dissolved in 10 mL of water. The mixture was flash purified on a RP18 cartridge after 3 × 2 mL of water used for washing and the elution was performed using 3 × 2 mL of methanol. To prepare derivatives for GC-MS, an aliquot of the methanolic solution, obtained after elution of the RP 18 column, was concentrated to dryness in a screw-capped vial at 60 °C under a stream of nitrogen. Anhydrous pyridine (20 μL) and 20 μL of trifluoroacetylating (TFA) reagent: [*N*-methyl-bis (trifluoroacetamide)] were added. The tightly closed vial was heated at 60 °C for 20 min under stirring, and then chilled to room temperature (Chassagne, Crouzet, Baumes, Lepoutre, & Bayonove, 1995).

2.10.2. In the presence of co-solvent

For a constant total volume of 10 mL with 1 mL of aqueous phase (enzyme), 5 mg of xylan (donor) was added to 4 mL of benzyl alcohol or hexanol (substrate) and 5 mL of *n*-hexane or ethyl acetate or acetonitrile. The mixtures were incubated for 3 h, at 50 °C, under stirring. The subsequent treatments of the mixtures were the same than before.

2.11. Phenyl primeveroside biosynthesis

The mixture contained: 20 mg xylan, 10 mM of phenylglucoside, 9 mL of *n*-hexane (solvent) and 1 mL of partially pure endoxylanase from XL-200 enzymatic preparation. The mixture was stirred under magnetic agitation (400 rpm) at 50 °C for 3 h in a tightly closed flask. The reaction was stopped by heating in boiling water for 10 min. The hexane was then evaporated and the remaining residue dissolved in 10 mL of water. The mixture was flash purified on a RP18 cartridge, 3 × 2 mL of water were used for washing and the elution was performed using 3 × 2 mL

of methanol. TFA derivatives for the GC-MS studies were prepared as before.

2.12. Gas chromatography analysis

A DB-5 MS fused silica capillary column, 30 m × 0.25 mm i.d., 0.25 μm (J&W Scientific, Folson, CA) was used for GC. The column temperature was isothermal at 125 °C during 5 min then raised from 125 °C to 220 °C at 3 °C/min, and increased to 280 °C at 6 °C/min. Injector and detector temperatures were 280 °C and 300 °C, respectively. The flow rate for the carrier gas, hydrogen, was 1.8 mL/min. Split mode injection with a 1/10 ratio, and a makeup of 30 mL/min of nitrogen were used. Heptyl, hexyl or phenyl glucosides were used as internal standards.

2.13. Electron impact mass spectrometry (EI-MS)

EI-MS spectra were recorded by coupling a Varian 3400, (Walnut Creek, CA) gas chromatograph equipped with a DB-5 MS fused silica capillary column (30 m × 0.25 mm i.d., 0.25 μm bonded phase), to a Automass 020 (Unicam, Argenteuil, France) mass spectrometer. Injections were about 1 μL. The transfer line was maintained at 290 °C and the injector temperature was 280 °C. The column temperature programmation was the same as that used in GC experiments. Helium at 1.2 mL/min was the carrier gas. Source temperature was 200 °C, mass spectra were scanned at 70 eV in the m/z range 60–600 mass units.

2.14. Negative chemical ionisation (NCI)

NCI-MS spectra were recorded for the TFA derivatives by coupling a Helwett-Packard (HP) 5890 gas chromatograph fitted with a DB-5 M fused silica capillary column (30 m × 0.32 mm i.d.; 0.25 μm bonded phase), to a HP 5889 A mass spectrometer. The transfer line was maintained at 290 °C. Injections about 1 μL were on column; the injector temperature was programmed at 60 °C/min from 110 °C to 260 °C, then held at this temperature for 55 min. The column temperature was programmed at 3 °C/min from 125 to 290 °C with helium as carrier gas at 1.1 mL/min.

The operating conditions were as follows: emission current: 350 μA; energy of the electrons: 200 eV ; the temperature of the source and quadrupole were 200 and 120 °C, respectively ; methane at 80 Pa was the reactant gas. The ion source tuning was carried out in positive mode by using perfluorotributylamine. Mass spectra were scanned in the range 100–1400 at 500 ms intervals with a repeller potential of 7 V. The mass spectra were recorded when the abundance of pseudo-molecular ions maximized.

2.15. Positive fast atom bombardement (FAB)

Positive FAB spectra were obtained using mass spectrometer Jeol DX 300 (Laboratoire de measures physiques, Université de Montpellier 2 France). Xenon was used as inert gas and nitrobenzyl alcohol as matrix.

3. Results and discussions

Because hydrolytic activity generally has close relationship with transglycosylation activity, a first part of this work was about the study of hydrolytic activities from various commercial enzymatic preparations. On the second part, we used the data obtained for the hydrolytic study to find the best conditions to synthesize mono- and hetero-glycosides.

3.1. Xylan and xylobiose hydrolysis

To find an endoxylanase (EX) activity for the transfer of one (or more) xylose unit(s), the xylan and xylobiose hydrolysis by various enzymatic preparations were performed in aqueous media.

The enzymatic preparations were chosen according to their easily availability, as well as this one of the potential substrates. So, the commercial enzymes used were: xylanase XL-200 and glucanase GL-200 from *T. longibrachiatum, T. viride* xylanase, *A. niger* pectinase and *A. niger* hemicellulase REG2. The two last preparations were known to develop "glycosidase activities" (Sarry & Günata, 2004; Winterhalter & Skouroumounis, 1997) and were used for glycoside hydrolysis (Günata, Bayonove, Tapiero, & Cordonnier, 1990; Sakho, Chassagne, & Crouzet, 1997) or for enzymatic synthesis of glycosides (Günata, Vallier, Sapis, Baumes, & Bayonove, 1994).

The relative percent of xylose, xylobiose, xylotriose, xylotetraose and xylopentaose released by the action of these enzymes from the xylan, were given Fig. 1. Only xylose was released by the action of *A. niger* pectinase and hemicellulase REG2 preparations indicating that only β-xylosidase activities were present. This finding was supported by the results of hydrolysis activity toward *p*-NP-xyloside, used for the exoxylosidase activity assessement. The measurements were 0.51 (from *A. niger* pectinase) and 0.43 IU/mg of proteins (for Hemicellulase REG2) (Table 1).

Xylose oligomers with DP $\geqslant$ 2 were the major products released when the two Xylanases and Glucanase were used. Only traces of xylose were detected among the hydrolysis products resulting from Xylanase XL-200 and Glucanase GL-200 action. The hydrolysis activity toward *p*-NP-xyloside was found to be less than 0.060 and 0.020 IU/mg of proteins for *T. longibrachiatum* XL-200 and GL-200 preparations, respectively, and less than 0.001 IU/mg of proteins for *T. viride* xylanase, in agreement with xylan hydrolysis data (Table 1).

To confirm the weak exoxylosidase activities of the enzymatic preparations of *T. longibrachiatum* xylanase XL-200 and glucanase GL-200, and *T. viride* xylanase, the study of the xylobiose hydrolysis was performed. After 4 h, no release of a xylose unit was noticed for these three

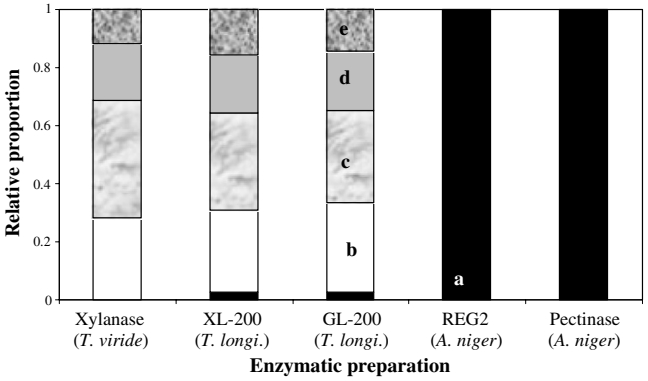

Fig. 1. Relative proportion of xylose (a), xylobiose (b), xylotriose (c), xylotetraose (d) and xylopentaose (e), released by the action of various enzymatic preparations, from xylan (10 g/L), in the sodium acetate buffer (0.1 M, pH 5), at 40 °C, 4 h.

enzymatic preparations. The traces of xylose observed for the *T. longibrachiatum* xylanase and glucanase preparations during the xylan hydrolysis study, were probably due to the parasites activities present in these crude preparations.

So, the main xylosidase activity developed by these three preparations was an endoxylanase activity, as indicated by the results reported Table 1. The most important specific activity was found in *T. viride* Xylanase. However *T. longibrachiatum* Xylanase XL-200 was cheaper than *T. viride* xylanase. This industrial enzyme, available in large amount, was kept for following experiments.

3.2. Trichoderma longibrachiatum endoxylanase purification

By FPLC ion exchange chromatography on CM Sepharose, a strong peak of activity was detected for a 434 mL retention volume after loading of *T. longibrachiatum* ultrafiltred xylanase XL-200 preparation, as well as a weak activity was present for a 126 mL retention volume.

The peak isolated between 400 and 460 mL retention time and analyzed by SDS-PAGE gel electrophoresis showed the presence of three protein strips possessing the following molecular mass and ratio: 21.3 kD (79.2%), 33.0 kD (20%) and 49.3 kD (0.8%). As previously reported by Royer and Nakas (1991) and, Chen, Chen, and Lin (1997) using different *T. longibrachiatum* strains, the main strip (21.3 kD) contained the endoxylanase activity whereas the strip at 33. kD correspond to β-exoxylosidase activity.

The purity of the endoxylanase was increased 15 fold after ion exchange column, and 60% of the specific activity is conserved after this first step of purification (Table 2).

A further purification of the main peak obtained after ion exchange chromatography and possessing the endoxylanase activity was carried out on gel filtration column. The endoxylanase was then totally pure, but 80% of the total activity was lost (Table 2).

Our aim was the reduction of the parasites activities of the crude *T. longibrachiatum* xylanase preparation and the preservation of the maximum of activity. So, only the partial purification step after ion exchange column was kept without more purification.

3.3. Influence of organic compounds on partially pure enzyme stability

The results obtained at pH 5, in the presence of 50% (v:v) of organic compounds, expressed in percent relatively to those obtained in the absence of these compounds (Fig. 2) showed that the xylan hydrolysis by the enzyme was completely inhibited by acetonitrile, *n*-butanol and ethyl acetate. Moreover, in the experimental conditions used, 70% of the activity was preserved in presence of hexanol, more than 25% in the presence of *n*-hexane and less than 5% of the activity was recovered after incubation in benzyl alcohol. It would seem that in a biphasic medium, the enzyme would remain in the active form despite the loss of water (Van Rantwijk, Woudenberg Van Oosterom, & Sheldon, 1999).

Moreover it was known that a protective effect against enzyme deactivation is afforded in the presence of an excess of substrate. Consequently, glycosidically bound aroma compounds synthesis were carried out in two conditions: by using of system where the organic phase consisted only of the acceptor of the reaction (hexanol or benzyl alcohol), or by using of a co-solvent (50%, v:v).

3.4. Glycosidically bound aroma compounds synthesis assay

3.4.1. Organic phase as only acceptor

After 3 h of incubation of hexanol (9 mL), xylan (5 g/L) and 1 mL of crude XL-200 xylanase preparation from *T. longibrachiatum*, two peaks eluting at 9.2 min and

Table 1
β-Xylosidase and endoxylanase activities of several enzymatic preparations

Enzymatic preparation activity	Proteins (mg/mL)	β-Xylosidase activity (IU)[a]	Endoxylanase activity (IU)[b]
Hemicellulase REG2 (*A. niger*)	0.29	0.432	nd
Pectinase (*A. niger*)	22.14	0.510	nd
GL-200 (*T. longibrachiatum*)	90.09	<0.020	101
XL-200 (*T. longibrachiatum*)	56.36	<0.060	852
Xylanase (*T. viride*)	0.36	<0.001	42×10^3

[a] One International Unit (IU) is defined as the enzyme amount catalyzing the release of one μmol of *p*nitrophenol per minute and per mg of proteins.
[b] One International Unit (IU) is defined as the enzyme amount catalyzing the release of one μmol of xylose equivalent per minute and per mg of proteins.
nd: Not determined.

Table 2
Activities of the endoxylanase from the *Trichoderma longibrachiatum* XL-200 preparation, in each steps of the purification

	Molecular weight (Da)	Proportion (%)	Proteins (mg/mL)	Total activity (IU[a]/mL)	Specific activity (IU[a]/mg)	Yield (%)	Purity
XL-200 crude preparation	21,300	13	56.36	48×10^3	852	100	1
	33,000	11					
	>49,000	76					
After ion exchange	21,300	79.2	2.43	32×10^3	13169	66.3	15.4
	33,000	20					
	>49,000	0.8					
After gel filtration	21,300	0	1.51	9.5×10^3	6291	19.8	0.5
	33,000	100					

[a] One International Unit (IU) is defined as enzyme amount catalyzing the release of one μmol of xylose equivalent per minute.

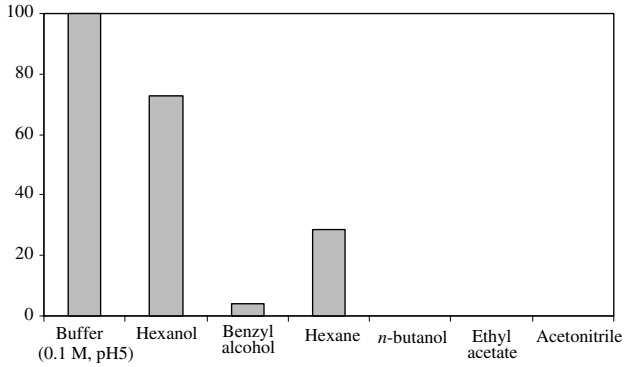

Fig. 2. Endoxylanase activity of the XL-200 Xylanase preparation from *T. longibrachiatum* diluted in sodium acetate buffer (0.1 M, pH 5), mixed with several organic compounds (1:1), at 40 °C, 30 min. The results are expressed in percent relatively to activity in buffer only.

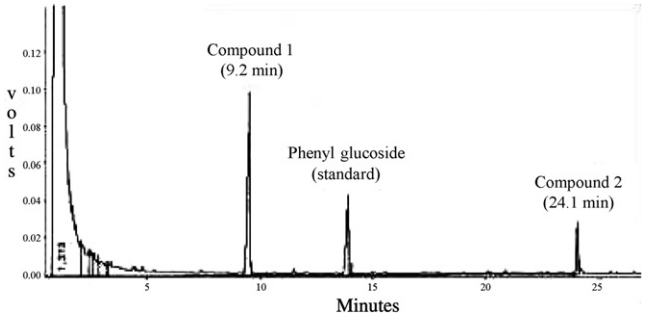

Fig. 3. Gas chromatogram of TFA derivatives of the compounds separated from the reaction medium: 9 mL of hexanol and 1 mL of *Trichoderma longibrachiatum* xylanase XL-200 enzymatic preparation containing xylan (5 g/L), after 3 h, at 40 °C, and with the phenyl glucoside as internal standard.

24.1 min and that of the internal standard (TFA phenylglucoside), were detected by GC of the TFA derivative obtained from the reaction medium (Fig. 3).

These two compounds separated by preparative layer chromatography and derivatized as TFA compounds, were analyzed by electron impact (Voirin, Baumes, Sapis, & Bayonove, 1992) and negative chemical ionisation GC-MS (Chassagne et al., 1995). Moreover the results obtained by these two methods were confirmed by FAB^+-MS (Salles, Essaied, Chalier, Jallageas, & Crouzet, 1988) directly from the mixture, without preliminary separation and derivatization (Table 3).

The EI mass spectra were similar for the two derivatives separated by thin layer chromatography with peaks at *m/z* 57, 69, 85 for the aglycone moiety (hexyl) and peaks at *m/z* 165, 193, 265, 278, 307 and 421 characteristic of the xylose moiety (Wang, Yoshimura, Kubota, & Kobayashi, 2000).

The NCI mass spectrum of the compound having a 9.2 min retention time was characterized by the presence of fragment ions at *m/z* 409, 522 and 635, whereas fragment ions at *m/z* 733, 846 and 959 were detected in the mass spectrum of the second compound having a 24.1 min retention time. These data were in agreement with fragments at $[M-113]^-$, M^- and $[M+113]^-$ of hexylxyloside and hexylxylobioside, respectively (Table 3).

The FAB^+ spectrum of the TFA xylosides mixture showed characteristic pseudo-molecular ions at *m/z* 235 $[M-1]^+$and *m/z* 257 $[M+23]^+$ for one of them, and at *m/z* 367 $[M+1]^+$ and *m/z* 389 $[M+23]^+$, for the other one (Adinolfi, Mangoni, Marino, Parrilli, & Self, 1984; Salles et al., 1988). These results were in agreement with the identification of hexylxyloside ($M=234$) and hexylxylobioside ($M=366$) (Table 3).

Moreover, when the xylobiose is used instead of the xylan as donor of the synthesis reaction, only the hexylxyloside was synthesized. And when the xylose is used instead of the xylan as donor, no hexylxylosides were synthesized.

Similar results were obtained when the synthesis was performed with benzyl alcohol as acceptor of the reaction. Benzylxyloside, benzylxylobioside and benzylxylotrioside were obtained (Kadi et al., 2002), only when xylan was used as donor of the reaction.

All these results indicated that a transfer of a xylose unit, by the endoxylanase from *T. longibrachiatum* XL-200 preparation, was probably involved by transfer reaction instead of reverse hydrolysis reaction. These results were consistent with those previously reported by Jiang et al. (2004) showing that xylanase B of *Thermotoga maritima*, showed a great ability to transfer xylose and xylobiose to alcohols from xylan. In the contrary at least

Table 3
Identification of hexyl xyloside, hexyl xylobioside and phenyl primeveroside by coupled gas chromatography-electron impact mass spectrometry (EI-GC-MS), coupled chromatography-negative chemical mass spectrometry (NCI-GC-MS) of their trifluoroacetyl derivatives and positive fast bombardment spectrometry (FAB^+)

Compound	Rt (min)	EI-GC-MS		NCI-GC-MS			FAB^+	
		Aglycon moiety	Saccharidic moiety	*M*	*M* + 113	*M* − 113	*M* + 1	*M* + 23
Hexyl xyloside	9.2	50(93), 51(63), 57(65), 69(97), 85(100)	165(4), 177(7), 193(73), 265(6), 278(7), 307(7), 319(15), 421(4)	522	635	409	235	257
Hexyl xylobioside	24.1	50(41), 91(100), 92(16), 107(10),	177(3), 193(24), 265(2), 278(1), 310(2), 319(5), 421(1)	846	959	733	367	389
Phenyl primeveroside	30.6	69(39), 91(12), 94(88)	177(3), 193(15), 265(2), 319(52), 193(15), 278(1), 279(3), 307(1), 421(3)	nd	nd	nd	389	411

Rt: Retention time.
M: Molecular weight.
nd: Not determined.

a xylotriose unit was required for the synthesis of alkyl xylosides catalysed by *Aureobasidium pullulans* xylanase (Matsumura et al., 1999).

Moreover, the use of the partially purified endoxylanase instead of the crude XL-200 xylanase preparation gave similar results and the same benzylxylosides products, confirming that the transfer reaction is due to the endoxylanase present in the crude preparation XL-200.

3.4.2. In the presence of co-solvent

The synthesis reactions were carried out with xylan (5 g/L) as donor, benzyl alcohol (4 mL) as acceptor, 5 mL of a co-solvent (*n*-hexane, ethyl acetate or acetonitrile) and 1 mL of xylanase XL-200 crude preparation, at 50 °C, under stirring (400 rpm) during 3 h.

The results reported Table 4, shows that benzyl xylosides were formed in higher amount when *n*-hexane was the co-solvent, relatively to the results obtained in the presence of ethyl acetate or acetonitrile. We noticed that the production of xylosides increased with the hydrophobicity of the co-solvent. These results were consistent with previously reported data (Laroute & Villemot, 1992) showing that enzymes were often more denatured by water miscible compounds than by compounds producing biphasic media. However they were in contradiction with most of the results reported in the literature indicating an increased synthesis in the presence of water miscible solvents relatively to the synthesis in the presence of hydrophobic solvents, for instance the synthesis of alkylglycosides was increased by amphiphilic phase enzyme reaction (Park, Kim, Jung, Haam, & Kim, 2000). These results were probably dependent of several parameters such as operative conditions for the reaction, pH, nature of the enzyme and quantity of water used.

Moreover, the synthesis of hexylxylosides was attempted in 50% of *n*-hexane in the reaction medium with hexanol as acceptor instead of benzyl alcohol. We noticed that only the hexylxyloside (118.8 mg/L) was synthesized under these conditions, instead of the hexylxyloside (382.1 mg/L) and hexylxylobioside (80.4 mg/L) mixture when there was no co-solvent in the medium (Table 5).

Consequently, these results showed that the nature of the acceptor is important for the course of the transfer reaction of one, two or three xylose units. And, the use of hexane as co-solvent allowed the synthesis of hexyl- and benzyl-xylosides, but led to a shift to the simplest oligoxylosides synthesized.

Table 4
Enzymatic synthesis by the XL-200 xylanase preparation from *Trichoderma longibrachiatum* of benzylxyloside, benzylxylobioside and benzylxylotrioside (in mg per liter), in absence or presence (50%, v:v) of co-solvent

Co-solvent	Benzyl xyloside	Benzyl xylobioside	Benzyl xylotrioside
Without	488.6	204.5	56.8
n-Hexane (50%)	1113.6	113.6	ns
Ethyl acetate (50%)	754.1	ns	ns
Acetonitrile (50%)	112.9	ns	ns

ns: Not synthesized.

Table 5
Enzymatic synthesis by the XL-200 xylanase preparation from *Trichoderma longibrachiatum* of hexylxyloside and hexylxylobioside (in mg per liter), in absence or presence (50%, v:v) of *n*-hexane

Co-solvent	Hexyl xyloside	Hexyl xylobioside
Without	382.1	80.4
n-Hexane (50%)	118.8	ns

ns: Not synthesized.

These results were confirmed by the synthesis of hetero-glycoside (primeveroside). Indeed, the enzymatic transfer of one xylose unit from xylan (donor) to phenylglucoside (acceptor) by partially pure *T. longibrachiatum* xylanase (10%, v:v) was undertaken in the presence of *n*-hexane (90%, v:v). After 1 h of reaction, the TFA derivative of the synthesized compound (51.8 mg/L) with the same retention time (30.6 min) as that of an authentic standard (obtained by chemical synthesis by Pr. Kobayashi), was detected by GC. The fragment ions obtained by EI-MS, characteristic of the aglycone moiety (phenyl) at *m*/*z* 50, 91, 92, 107, 108 and of the glucose unit *m*/*z* 177, 193, 265, 319 and of the xylose unit *m*/*z* 193, 278, 307, 421, were present in the spectra of the enzymatically synthesized compound (Table 3) which consisted of a phenyl, xylose and glucose moieties (Kadi & Crouzet, 2006).

4. Conclusions

By screening of various enzymatic preparations, we found that XL-200 xylanase preparation from *T. longibrachiatum* was able to achieve the transfer of one xylose unit from xylan by endoxylanase activity present in this preparation, with or without *n*-hexane used as co-solvent. The nature of the donor of the synthesis reactions was determinent, since the same result could be carried out, in different conditions, when the donor was xylobiose instead of xylan. We also showed that the nature of the acceptor is important for the course of the reaction and the nature of the synthesized compounds, and must deeply studied.

We can also conclude that the study of hydrolytic activities of hydrolases can be relevant, in the first place, to find the activities useful for the transfer reaction.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2007.05.089.

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