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# Hemicellulose extract from *Cynara cardunculus*: a source of value-added biomolecules produced by xylanolytic thermozymes

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**Abstract:**

*Cynara cardunculus* hemicellulose fraction was recovered from its stems and leaves biomass and converted in valuable molecules by exploiting the extracellular xylanase and β-xylosidase activities produced by the thermophilic bacterium *Geobacillus thermantarcticus*. Several degradation procedures of the arabinoglucuronoxylan extract were proposed by using efficient and different enzymatic preparations, containing both or only one xylanolytic activity of *G. thermantarcticus.* In particular, when the xylanase and β-xylosidase activities were used separately into hydrolysis reactions, xyloglucurono-oligosaccharides or xylose were obtained with a yield of 32% and 62.6 % respectively, with reference to hemicellulosic extract. Furthermore, the synergic action of β-xylosidase/xylanase activities were exploited in transglycosyaltion processes for the production of xylo-conjugated; xylosides of primary alcohols with increasing carbon chains and aromatic alcohols were produced starting from the *C. cardunculus* hemicellulose, which was selected as cheap donor. When 2-phenoxyethanol was selected as acceptor, 2-phenoxyethyl β-xyloside, xylobioside and xylotrioside were prepared with a yield of 38.5 % with respect to hemicellulosic extract and spectroscopically characterized.

**1. Introduction**

Biorefinery goals are closely related to two main subjects: value-added bioproducts (chemical building blocks and materials) and bioenergy (biofuels, power and heat) from biomass.

Recently, particular attention has been devoted to biorefinery of lignocellulosic biomass, in the form of agro-residues and forestry biomass, as a more different and valuable source of chemicals and second-generation biofuels[1]. Annual crops and perennial herbaceous species (grasses), in particular, represent abundant and low cost feedstock for lignocellulosic biorefinery. Nevertheless, the competition for land between food and non-food crops must be avoided, because the diffusion of energy crops could have negative effects on agricultural markets, increasing food prices and consequently reducing the food availability[2], therefore causing not sustainable social impacts[3]. For these reasons, different researches have been addressed to identify cropland not suitable for traditional food crops, and so useable for energy crops, such as polluted soils, marginal lands subjected to salinization or accelerated erosion[4].

However, the structural heterogeneity of lignocelluloses in terms of proportion of their three components cellulose, hemicellulose and lignin, as well as the spatial distribution of the constituent molecules, is perhaps one of the major hindrances in developing as much as possible universal multi-step procedures, which include mechanical, chemical pre-treatments and enzyme-based

bioconversion processes. Their focus is the fermentation of sugars extracted from feedstock biomass to produce ethanol and/or other building block chemicals[1,5].

According to its chemical composition and the huge biomass productivity, *Cynara cardunculus* L., also known as wild cardoon or Castilian thistle, represents a very attractive lignocellulosic feedstock in biorefinery schemes. *Cynara cardunculus* is a perennial herbaceous species (grasses) with annual growth cycle, making it appropriate for mediterranean climates where it is traditionally grown for horticultural purposes. It produces great amount of lignocellulosic biomass and also an interesting amount of oilseeds. In fact, it is well known as a crop adaptable to low fertility soils and resistant to the environmental stresses typical of marginal lands, therefore minimizing the social impact of biomass crops[6]. It is present in three different varieties, namely, var. *sylvestris* (Lamk) Fiori (wild cardoon), var. *scolymus* (L.) (globe artichoke), and var. *altilis* (DC) (cultivated cardoon)[7]. For biorefinery applications, *C. cardunculus* is grown as a perennial field crop in dry farming[8].

The cardoon hemicellulose fraction represents the second largest constituent (20-30%) of its stalk-wall biomass[9], therefore being an important source of β-1,4-xylan.

Cardoon arabinoglucuronoxylan can be recovered and further valued by coupling proper chemical pre-treatments to degrading processes carried out by using xylanolytic enzymes. In a multi-step procedure, it is possible to obtain efficiently bio-molecules, such as xylo-oligosaccharides (XOs), D-xylose and to a lesser extent L-arabinose.

Besides, xylose represents the 2$^{nd}$ most abundant sugar present in lignocellulosic biomass after glucose. Given this high abundance in the context of lignocellulosic biorefinering, the processes for conversion of D-xylose into ethanol, or other useful chemicals, is of considerable economic importance and several efforts are doing to get an efficient xylose fermentable microorganism for a commercially viable ethanol production process[10]. On the other hand, widening the use of xylose and arabinose as commodity chemicals and platform intermediates with respect to current ones, which is limited by the scarce quality of these sugar preparations, would increase the sustainability of lignocellulosic biomass[11].

It is known that the main enzymes involved in the xylan backbone hydrolysis are the endo-1,4- β -xylanase (1,4- β -D-xylan xylanohydrolase; EC 3.2.1.8) and the β -xylosidase (1,4- β -D-xylan xylohydrolase; EC 3.2.1.37), although the activities of several accessory enzymes are also essential for the complete hydrolysis of xylan backbone into its constituent sugars, useful for the subsequent biofuel hemicellulose fermentations[12]. Indeed, xylanolytic enzyme systems, have been found to be

quite widespread among microorganisms (fungi, actinomycetes and bacteria) belonging to several ecological niches[13].

However, xylanases and β-xylosidases from thermophilic strains of bacteria and fungi are important from biotechnological viewpoint, owing to their higher thermostability, and other biochemical properties, which are required for industrial applications[5,14]. Moreover, in order to make the enzymatic applications more economical at the industrial level, the enzymatic production using low-cost substrates such as agro-residues has been also recommended[15].

Important examples are reported in literature of thermophilic microorganisms owing both xylanase and β-xylosidase activities. In a recent paper, *G. thermodenitrificans* TSAA1 resulted an interesting producer of extracellular and synergistic acting endoxylanase and β-xylosidase[16]. It should be also cited the case of a trifunctional enzyme constructed by associating the *Thermoanaerobacter ethanolicus* xylosidase and arabinosidase with the *Thermomyces lanuginosus* xylanase activity and producing them in *E. coli* with the aim to study the effect of this physical and enzymatic association in arabinoxylan hydrolytic processes[17]. More recently hemicellulase and cellulase from the extremely thermophilic bacterium *Caldicellulosiruptor owensensis*[18], a thermostable xylanase from *Geobacillus* sp. strain WSUCF1[19] and hemicellulolytic enzymes from *Thermobacillus xylanilyticus*[20] were exploited for the efficient hydrolyses of several lignocellulosic biomass.

It is worth mentioning that some bacterial thermophilic xylanases and β-xylosidases were employed not only in xylan and xylo-oligosaccharide hydrolyses but also in transglycosylation processes for the production preferentially of alkylxylosides as valuable biosurfactants with interesting foaming, antimicrobial properties and biodegradability[21]. In this sense, efficient transglycosylation reactions were performed with a xylanase from *Thermobacillus xylanilyticus* (Tx-xylanase) or a commercial xylanase (Novozymes NS-50030); in optimized reaction conditions, xylooligosaccharides generated from hydrothermally pretreated and destarched wheat bran were used as possible donors and *n*-pentanol or *n*-octanol as acceptors[22]. Similarly, a mixture of XOs, which were enzymatically produced from a de-starched wheat bran extract, was employed as donors in xylosylation processes of aliphatic alcohols with short chains performed by using a recombinant β-xylosidase from *Geobacillus thermodenitrificans* TSAA1[14]. Recently, an overview on the possibility to synthesize 9-fluorene methanol xylosides in transglycosylation processes, in which an hemicellulolose extract from *Arundo donax* rhizomes was exploited as donor, and by using *Thermotoga maritima* xylanase and xylanase/β-xylosidase activities from *T. neapolitana*, *T. thermostercoris* and *Geobacillus thermantarcticus* crude enzyme solutions, was described by our research group[23].

In this paper, enzymatic procedures, involving xylanase and β-xylosidase activities from *Geobacillus thermantarcticus*, for the conversion of hemicellulose extract recovered from waste stems and leaves biomass of *C. cardunculus* into saleable, eco-friendly and value-added products were proposed.

*G. thermantarcticus* is an aerobic, Gram-positive, thermophilic and highly xylanolytic bacterium[24]. Two extracellular thermostable activities were previously purified and characterized from this microorganism: a xylanase (1,4-beta-D-xylan xylanohydrolase; E.C. 3.2.1.8) and β-xylosidase (1,4-beta-D-xylan xylohydrolase; E.C. 3.2.1.37)[25].

*G. thermantarcticus* xylanolytic activities were here investigated for their ability to degrade the *Cynara cardunculus* hemicellulolytic fraction with/without production of small useful oligosaccharides, and for the possibility to set-up efficient transglycosylation reactions. In these reactions, a *Cynara cardunculus* hemicellulolytic extract was used as donor. Primary alcohols with increasing carbon chains were selected as possible acceptors according to the non-ionic surfactant properties, the alkaline pH stability and biodegradability of alkyl xylosides, which make these molecules usable into the formulation of foods, hygiene, pharmaceuticals and cosmetics industries and for biological/biomedical applications[26,27]. Furthermore, considering that, few examples are reported in literature about the use of xylanase or β-xylosidase enzymes for the synthesis of lignin model aromatic compounds xylosides[28-31], phenol, polyphenols, aromatic alcohols were also investigated as possible acceptors. Particular attention was devoted to 2-phenoxyethanol xylosylation; in fact, 2-phenoxyethanol together with benzyl alcohol are among the most commonly used antimicrobial preservatives in licensed parenteral products like peptide and protein based drugs, vaccines and pharmaceutical products[32].

## 2. Results and discussion

### 2.1. *Cynara cardunculus* biomass yield

*Cynara cardunculus* represents an interesting crop for its double attitude to produce both biomass from the whole plant, and oil from the seeds previously removed from the plants[33]. Cardoon biomass yield after irrigated and not irrigated treatments (see section 3.2), resulted not significantly different in all the 3 years of monitoring, thus confirming the resistance of this species to arid conditions[34] and to low input cropping systems[6]. In fact, biomass yield decreased during the 3 year from 20 to 16 t $ha^{-1}$ of dry matter, on the average.

### 2.2 Isolation and characterization of hemicellulosic extract from stems and leaves of *Cynara cardunculus*

Arabinoglucuronoxylans are typically found in the lignocellulose isolated from grasses and these polysaccharides possess the composition of biomass currently targeted for biofuel production, having arabinose, methyl glucuronic acid, and acetyl side chains linked to the xylose backbone[35].

Aiming to extract the hemicellulosic fraction from stems and leaves of *Cynara cardunculus*, an alkaline treatment, previously adopted by our research group for the isolation of xylan from *Arundo donax* rhizomes[23],was considered the most appropriate in comparison with other methods for the extraction of hemicellulose from agro-residues[36,37].

In fact, under solubilisation with 2N KOH for 72 h and at room temperature, the extracted hemicellulose fraction of *Cynara cardunculus* (CK72h), represented the 17% (w/w) of the dried biomass. Using this alkali treatment, the yields of recovered cellulose and lignin were, respectively, the 42 % and the 12% of starting biomass.

Furthermore the monosaccharidic composition of hemicellulose extract (CK72h) was determined, as described in section 3.1, and results were reported in Table 1. Arabinose to xylose ratio (Ara/Xyl) resulted indicative of the degree of linearity of hemicellulose[36]. In the case of polysaccharide extracted from stems and leaves of *Cynara cardunculus*, the arabinose/xylose ratio of was 0.020 (Table 1), thus suggesting that the obtained *C. cardunculus* hemicellulose occurred in an essentially de-branched form. This information was also supported by $^{13}C$ NMR spectrum of CK72h saturated solution in *d*-DMSO at 40°C: intense signals were recorded, due to xylan main chain, at 101.7, 75.4, 73.9, 72.5 and 63.2 δ, with typical values of C1 (101.7) and C4 (75.3) linked β-xyloses; others less intense signals got confused in the noise spectrum.

### 2.3 Recovery and separation procedures of xylanase and β-xylosidase activities from *Geobacillus thermantarcticus*

The crude enzymatic solution **ES-GTM**, prepared as described in section 3.4, was processed for the separation of xylanase and β-xylosidase activities. Two methods were employed to set-up an effortless procedure able to split the xylanase and β-xylosidase activities. The first procedure exploited the different thermal stability of the two proteins[25]; in fact, the β-xylosidase activity could be simply eliminated by heat treatment at 80°C for 20 min (section 3.5), starting from **ES-GTM**. These conditions were found to be the most convenient for the recovery of the xylanase solution (0.46 mg/ml of total protein) named "**ES-GTM endo-xyl"**; it represented the best compromise between the almost complete loss of β-xylosidase (95%) and the preservation of endo-xylanase (100%) activities, with a residual total protein content of 34%.

The second method for separating the xylanase and β-xylosidase activities was based on the ability of many xylanase enzymes to absorb on xylan[38] through a simple method, as described in section 3.5. From the extracellular enzymatic pool of *Geobacillus thermantarcticus,* were obtained a xylan-bound fraction that contained the 100 % of inactive xylanase, and an unbound fraction that contained the 100% of active β-xylosidase, named "**ES-GTM β-xyl"** solution (0.9 mg/ml of total protein) with a residual total protein content of 67%.

**2.4 Digestion of *Cynara cardunculus* hemicellulolytic extract and selective production of XOs or xylose**

The enzymatic bioconversion of the hemicellulosic material CK72h extracted from *C. cardunculus* (CK72h) for the production of xylo-oligosaccharides, xylo-conjugated and xylose was resumed in Figure 1 and the results were reported in Table 2.

In particular, at the beginning, the enzymatic digestion was conducted through the simultaneous use of endoxylanase and β-xylosidase activities of *G. thermantarcticus* (**ES-GTM** enzymatic solution, Figure 1A and section 3.6); it furnished a bioconversion yield of 62% and 44%, in terms of reducing sugars and xylose releases, respectively (line 1 of Table 2).

At a later stage, the xylanase and β-xylosidase activities, separated through the effortless procedure, as mentioned in section 2.3, were used consecutively into cascade hydrolysis reactions aiming to accumulate xylose or xylo-derivatives selectively into the reactions medium, as illustrated in Figure 1B and 1C. Indeed, the polysaccharide bioconversion after the **ES-GTM endo-xyl** attach (Figure 1B and line 2 of Table 2,) reached the 43.7% of reducing sugars with only 6.8% of xylose content; moreover the hydrolysis products were essentially composed of xylobiose (26.5%), xylotriose (5.5%), and oligomers (DP > 3), as revealed by HPAE-PAD chromatogram profile (black trace, Figure 2) and TLC analysis (Figure 1S of supplementary materials). $ESI^+$-MS spectrum of reaction mixture after 24 h confirmed the presence of xylobiose as base peak at *m/z* 305 [$M+Na^+$], xylotriose at *m/z* 437 [$M+Na^+$], and xylose at *m/z* 173 [$M+Na^+$]; the composition of products in the reaction above described was in agreement with the β-xylosidase activity absence.

However, in the second step of an enzymatic cascade-reaction (Figure 1C and line 3 of Table 2) XOs produced from reaction depicted in Figure 1B (line 1 of Table 2) were degraded by the **ES-GTM β-xyl**, obtaining 73% of reducing sugars with a xylose yield of 62% into the reaction mixture; the hydrolysis products were essentially xylose as revealed by the HPAE-PAD chromatogram profile (violet trace, Figure 2) and TLC analyses (Figure 1S of supplementary materials) as also

confirmed by MS analyses in which a base peak signal at *m/z* 173 [M+$Na^+$] was recorded, corresponding to xylose.

Most of microbial investigated β-xylosidases exhibited a strong xylose inhibition[39]. However, *G. thermantarcticus* β-xylosidase produced a large amount of xylose (62.6 %, line 3 of Table 2), showing a scarce substrate inhibition in agreement with the few recently discovered β-xylosidases behaviour[40] and it suggested a possible use of *G. thermantarcticus* β-xylosidase for industrial purposes.

Exploiting the different optimal temperatures and thermostabilities of each enzyme[25], a more efficient hydrolysis was obtained (increase of 10% of reducing sugars and about 20% of xylose) through a sequential addition of two activities as reported in Table 2, line 3 (Figure 1C). In enzymatic cascade-attach represented in Figure 1C, reactions were carried out at 70 °C for the step 1 (xylanase activity), and at 60 °C for step 2 (β-xylosidase activity), respect to reaction conditions described in Figure 1A (70°C), when the concomitant action of two enzymes was evaluated. These results encourage the application of these enzymatic separation techniques to industrial scale for β-xylosidase and xylanase production from *G. thermantarcticus*, suggesting their efficient use into bioconversion of agro-residues for obtaining both xylo-oligomers and fermentable sugars. In particular, with the separation of the two activities, we are able to produce XOs (line 2 of Table 2,) for the 32% or xylose for the 62.6 % (line 3 of Table 2,). Our results encouraged the sequential use of these activities in industrial production processes. In addiction, an overview on the most recent enzymatic strategies involving thermophilic hemicellulase activities for the bioconversion of lignocellulose biomass from several agro-residues, indicated that the hydrolyses yields here presented had similar values to these reported in literature[18-20] and due to the possibility to easily select the β-xylosidase and xylanase from *G. thermantarcticus,* it suggested the application of these procedures to other agricultural feedstock biomass.

These results acquired a great value considering that XOs are used in both food and no-food applications: they find in fact application for example as prebiotics, symbiotic and antiobesity food or in pharmaceutical field as active agent against osteoporosis, pruritus cutaneous, otitis, skin and hair disorders. In agricultural sector, the XOs can be used as growth stimulator and accelerator, yield enhancer and ripening agent; finally they are involved in feed for domestic animals and fishes[41,42].

### 2.5 Isolation and characterization of oligosaccharides produced by hydrolysis of *Cynara cardunculus* hemicellulosic extract

The chromatographic investigation of hydrolysis products by HPAE-PAD analysis (Figure 2) and TLC (Figure 1S of supplementary materials) of reactions reported in Table 2 at lines 2 and 3, showed the presence of the two unknown oligosaccharides. In order to isolate and characterize them, they were produced by enzymatic digestion of CK72 hemicellulosic extract, which was carried out with **ES-GTM endo-xyl** solution (Figure 1B), as reported in section 3.7.

When the reaction was stopped after 24 h, 39 % of reducing sugars were obtained, of which 7.6% was free xylose (line 4 of Table 2,). After biogel purification of reaction mixture, ESI$^-$-MS analyses of the two oligosaccharidic fractions revealed the presence of two peaks at *m/z:* 605 and 591 [M+H$^-$ ] respectively; these values resulted in agreement with tetrasaccharidic chains constituted of three xylose and an uronic acid unit, with or without methoxyl functionalization. 22% of molecule with *m/z* 605 and 13% of molecule with *m/z* 590 were recovered after purification.

Their structures were identified and chemical shifts totally assigned by NMR spectroscopy and they corresponded to 2'-*O*-(4-O-methyl-α-D-glucuronosyl) xylotriose (compound **1**, α-D-4OMeGlcA-(1-2)-β-D-Xyl-(1-4)-β-D-Xyl-(1-4)-β-D-Xyl) and 2'-*O*-α-D-glucuronosyl xylotriose (compound **2**, α-D-GlcA-(1-2)-β-D-Xyl-(1-4)-β-D-Xyl-(1-4)-β-D-Xyl), respectively. $^1$H and $^{13}$C NMR chemical shift values were reported in section 3.7 and in Figure 3, $^{13}$C NMR spectra were reported of both oligosaccharides. Collected data secured that these compounds were constituted by three units of β-xylose 1,4 linked ($Xyl_1$ to $Xyl_3$ starting from reducing end) and one unit of 4*O*-methyl glucuronic acid or glucuronic acid respectively (Figure 3).

In compound **1**, positioning of MeGlcA was confirmed by Noesy experiments; dipolar coupling were recorded between anomeric proton of MeGlcA (5.22 δ) and proton in position 2 of $Xyl_3$ (3.32 δ) as reported in Figure 2S-A of supplementary materials. In HMBC experiment, long-range correlation between carboxyl carbon at 177.6 δ and the proton at position 5 of MeGlcA at 4.20 δ and also the scalar contact between carbon of methoxyl group at 60.87 δ and proton at position 4 of MeGlcA at 3.14 δ secured the sites of substitution of monosaccharidic unit (Figure 2S-B and 2S-C of supplementary materials, respectively). Furthermore the presence of a signal at 4.21 δ (H5 $^{MeGlcA}$) with a coupling constant of 9.71 Hz confirmed the gluco-configuration (1,3 trans diaxial H4-H5 coupling) of this monosaccharidic unit[43].

Similarly, in compound **2** NOE-effect between MeGlcA H1(5.25 δ) and H2 (3.39 δ) of $Xyl_3$, between H1 of $Xyl_3$ (4.54 δ) and H4 (3.71 δ) $Xyl_2$ and also long-range correlation between H1 of

$Xyl_2$ (4.39 δ) and C4 (77.47 δ) $Xyl_1$ secured the intra-residues connections (Figure 3S-A and 3S-B of supplementary materials). In literature, it is reported that acidic oligosaccharides of specific length are produced by endoxylanases belonging to family 10 and 11. Compound **1** was previously isolated and only partially characterized by NMR investigation. Spectroscopic data of compound **1** reported in this paper confirmed and complete the previous structural information[44].

### 2.6 *Cynara cardunculus* hemicellulose extract in transglycosylation reactions

#### 2.6.1 Analytical scale transglycosylation reactions

The substrate specificity in transglycosylation processes of both xylanase/β-xylosidase activities from the *G. thermantarcticus* (**ES-GTM** enzymatic solution) was investigated by using several acceptors with alcoholic and/or phenolic hydroxyl groups in their aliphatic or aromatic molecular skeletons (section 3.8.1) and the regio- and the chemo-selectivity in their transglycosylation processes were examined.

Results of each reaction, which were schematically represented in Figure 1D, were reported in Table 3. Each process was monitored by TLC and ESI-MS analyses and taking into account both the production of xylo-conjugated and the accumulation of mono- and oligo-saccharides from hemicellulose hydrolysis. In all cases, MS spectra of reactions after 24 h evidenced the presence of xylobiose, xylotriose, xylotetraose and in most of cases, of the compound **1** previously described.

Among primary alcohols (lines 1-4), hexanol resulted the best acceptor (line 3); the hexyl β-xyloside intense spot was detected by TLC analyses at rf 0.75 and a signal at *m/z* 257 [$M+Na^+$] in MS spectrum of reaction at 24h confirmed its presence. Ethanol and *n*-octanol (lines 2 and 4, respectively) resulted also possible acceptors. After 24h, the ethyl β-xyloside presence was confirmed by a signal at *m/z* 233 [$M+Na^+$] in MS spectrum of reaction; similarly traces of *n*-octyl β-xyloside were also detected at 4 and 24 h by TLC (rf 0.73) and MS analyses with a signal at *m/z* 285 [$M+Na^+$]. Phenol (line 5) resulted a poor acceptor, although CK72h hemicellulose hydrolysis was not influenced by its presence and accumulation of oligosaccharides was observed into the reaction medium. 4-(2-methoxyethyl)phenol (line 15) was on the contrary xylosylated and the 4-(2-methoxyethyl)phenyl β-xyloside and β-xylobioside peaks were detected in MS spectrum of reaction after 4 h at *m/z* 307 and 439 [$M+Na^+$] respectively and as well as on TLC plates, two spots at rf 0.76 and 0.65 were recorded, respectively. Resorcinol β-xylobioside (line 6) presence was confirmed by a TLC spot at rf 0.66 and by a signal in MS spectrum of reaction at *m/z* 398 [$M+H^+$]. It was accumulated over the reaction time and partially hydrolysed after 24h giving the

corresponding resorcinol β-xyloside (*m/z* 306 [M+$Na^+$+$H_2O$]). Hydroquinone resulted a good acceptor (line 7). It was observed by TLC analyses that the hydroquinone β-xylobioside at rf 0.61 (signal at *m/z* 397 [M+$Na^+$] in MS spectrum) was preferentially accumulated into the reaction medium together with traces of hydroquinone β-xyloside at rf 0.62 (*m/z* 319 [M+$Na^+$+3*$H_2O$] in MS spectrum). Avarol (line 14), in which the hydroquinone ring resulted functionalized with a sesquiterpenyl substituent, was not xylosylated, as a consequence probably of the terpene portion hindrance, even though CK72h hemicellulose hydrolysis occurred. (±)Catechin (line 17) could represent a potential acceptor but it was weakly xylosilated; in the reaction conditions, the molecule resulted unstable as observed by TLC monitoring of reaction[45]. Caffeic and gallic acids (lines 12 and 13), vanillin (line 16), with a catechol like molecular skeletons differently functionalized were not xylosylated; these results suggested some accessibility difficulties to phenol sites of glycosylation for the presence of *ortho*-substituents. 2-hydroxymethyl-2,5,7,8-tetramethylchroman-6-ol (line 10) was a poor acceptor also after 24 h and in addiction hemicellulose hydrolysis did not occur. MS investigation after 24 h of the tyrosol (2-(4-hydroxyphenyl)ethanol) (line 9) transglycosylation reaction showed the presence of mono-, di-, tri-xylosides derivatives of tyrosol at *m/z* 293, 425, 557 [M+$Na^+$] values, respectively. Furthermore, TLC analysis of reaction after 5h and 24h revealed the contemporary presence of mono- and poli- glycosylation products to aromatic and alcoholic sites (data not shown)[46]. Benzyl alcohol (line11) was mono-, di- and tri- xylosylated; signals in MS spectrum at m/z: 263, 395, 527 [M+$Na^+$] respectively confirmed their presence; a TLC monitoring of reaction after 5h, revealed the presence of the most abundant benzyl β-xylobioside (rf 0.65), traces benzyl β-xylotrioside (rf 0.52) after 5h and the absence of benzyl β-xyloside (rf 0.76), which appeared after 24h. However, interesting results were recorded for 2-phenoxyethanol transglycosylation (line 8, Figure 1D). After 5h, MS analyses secured the presence of 2-phenoxyethyl β-xyloside, xylobioside and xylotrioside (*m/z* 293, 425, 557 [M+$Na^+$], respectively). Spots on TLC plates were recorded at rf 0.71, 0.62, and 0.49, respectively.

This last process was investigated in more detail aiming to improve its yield; therefore, 2-phenoxyethyl β-xyloside, xylobioside, xylotrioside were preliminary and in not optimized conditions produced (4% of yield) and then purified (see section 3.8.2). In particular, 2-phenoxyethanol mono- di- and tri-saccharidic derivatives (compound **3**, **4**, **5** in Figure 4) were isolated in a molar ratio of 1:0.4:0.6 respectively and their molecular structure was confirmed by 1D and 2D NMR spectra, as reported in 3.8.3 section.

**2.6.3 Optimization of 2-phenoxyethanol xylosylation**

Aiming to identify the best conditions for 2-phenoxyethanol xylosylation, a first set of reactions were conducted at different concentrations of 2-phenoxyethanol (50, 70, 100, 200 mM) and taking constant the CK72h hemicellulosic extract amount (4mg/ml) into reaction medium (see 3.8.4 section). In these reactions it was evaluated the possibility to use not only the **ES-GTM** enzymatic solution, but also the endoxylanase activity recovered after heat treatment (**ES-GTM endo-xyl**), as described in 3.5 section. Aliquots of each reaction were collected over time and analysed by TLC and HPLC; the reaction yields were calculated by evaluating the percent ratio of produced 2-phenoxyethyl β-xyloside, xylobioside, xylotrioside (compound **3**, **4**, **5** in Figure 4), according to calibration curve as reported in section 3.1, and the initial acceptor concentration.

Results of the first set of experiments, in which both endoxylanase/β-xylosidase were active (**ES-GTM**) into the reactions medium, suggested that the reactions were considered finished after 24 h; in fact at 48h for each process no increase of xylosylation yield was recorded; however, at 24 h in the reaction at 70 mM of acceptor concentration, it was possible produce 385 mg of xylosylated products per gram of polysaccharidic extract CK72h (5.4% of yield according to initial 2-phenoxyethanol concentration). In this process, molar ratios of mono-,di-, and tri-xylosylated products were 1:0.5:1.14 (line 6 of Table 2). Similar results were recorded also for processes carried out in presence of **ES-GTM endo-xyl**; in particular in the best reaction at 70 mM of 2-phenoxyethanol (line 5 of Table 2,), after 24 h, molar ratios of mono-,di-, and tri-xylosylated products resulted 1:0.5:0.8 and 205 mg of xylosylated products per gram of polysaccharidic extract CK72h were prepared (3.1 % of yield according to initial 2-phenoxyethanol concentration). These data suggested that in transglycosylation processes, the synergic action of the β-xylosidase/xylanase activities resulted more efficient than the single xylanase activity of *G. thermantarcticus*. The different distribution of products resulted interesting: if in the presence of xylanase activity, mono-xylosylated product was the most abundant ones, when both β-xylosidase/xylanase activities worked, the tri-xylosylated product was mainly produced, indicating a strong participation of β-xylosidase activity in the formation of oligo-xyloside compounds.

Differently, in a second set of experiments the concentration of CK72h donor was increased (4, 8, 16 mg/ml) and the 2-phenoxyethanol concentration was fixed at 50 mM; aliquots of each reaction were collected over time and analysed as previously described.

After 24 h, the reactions yields increased from 3.4 % (166 mg of products /g of CK72h extract) to 9.7 % (228 mg of products /g of CK72h extract) and up to 19.3 % (221 mg of products /g of CK72h extract).

Combining the results of these two set of experiments, a reaction at 70 mM of acceptor and 16 mg/ml of CK72h extract was carried out and monitored by HPLC over the time (see section 3.8.4). Between 24 h and 29 h, it was possible to record a conversion of about 18-19 % with a production of 324-347 mg of xylosyl derivatives per gram of CK72h extract. In these experimental conditions, 2-phenoxyethyl oligoxylosides were produced preferentially; at 29 h, molar ratios of mono-, di-, and tri-xylosylated products were of 1:0.35:1.12 (line 7 of Table 2). These yields resulted mainly interesting if compared to those reported in literature about an enzymatic synthesis of alkyl β-xylosides from the hemicellulose extract of hydrothermally treated wheat bran[22]. In this case, using a xylanase from *Thermobacillus xylanolyticus*, xylooligosaccharides generated from hydrothermally pretreated and destarched wheat bran, as donor and 20% of *n*-octanol as acceptor, 222.2 mg of octyl xylosides per gram of arabinoxylan equivalent were produced. Furthermore, in different experimental conditions by using lactose as donor, the 2-phenoxyethanol galactoside was produced with higher yield (50% of conversion starting from 80 mM of acceptor after 24 h)[47] . Furthermore, authors reported that galactosylation of 2-phenoxyethanol reduced its cytotoxicity without influence on antibacterial activity. In fact, as 2-phenoxyethanol, which is moderately soluble in water (20g/L)[48] , is effective against gram negative organisms such as *P. aeruginosa*, it is an ingredient used in many hygiene and cosmetics industries as a single agent or in combination with other preservatives, up to a maximum allowed concentration of 1%[49]. Although it has one of the best toxicological profile among the antimicrobial additives, several animal studies demonstrate that it is toxic[50] with effects on the brain and the nervous system[51]. In this sense, its mono- and poli-xylosides products might decrease the drawbacks of solubility, modulate the toxicity and then allow the extension of the usefulness of this compound into broader fields.

## 3. Experimental

### 3.1 General

Chemicals were purchased from Sigma (St. Louis, MO). Silica gel and reverse-phase silica gel and TLC silica gel plates were from E. Merck (Darmstadt, Germany). Compounds on TLC plates were visualized under UV light or charring with α-naphthol reagent.

Chemical determination of reducing sugar concentrations was determined by Bernfeld method[52] using a xylose-based calibration curve. Protein concentration was determined by the method of Bradford[53] using bovine serum albumin as standard. Dialyses were performed by using Spectrapore dialysis tubes (12,000-14,000 MW cut-off). Compounds were weighed using a Mettler Toledo analytical balance. $^{1}H$ and $^{13}C$ NMR spectra were recorded at 600.13 MHz on a Bruker DRX-600 spectrometer, equipped with a TCI Cryo Probe TM, fitted with a gradient along the Zaxis and on

Bruker instruments at 400 MHz. Samples for NMR analysis were dissolved in the appropriate solvent; spectra in $D_2O$ were referenced to internal sodium 3-(trimethyl-silyl)-(2,2,3,3-2H4) propionate (Aldrich, Milwaukee, WI); for other solvents downfield shift of the signal of the solvent was used as internal standard.$^{13}$C NMR, COSY, TOCSY, HSQC, HSQC-EDITED, HMBC ($^3J$: 7 and 10 Hz), NOESY (mixing time at 100, 200, 300 msec) experiments were used for structural determinations.

Mass spectra were acquired on a microQ-Tof mass spectrometer coupled with an Alliance HPLC (Waters, Milford, MA) equipped with an ESI positive and negative source.

TLC solvent systems: (A): *n*-BuOH/AcOH/$H_2O$ 6:2:2 by vol., (B): EtOAc/AcOH/2-propanol/HCOOH/$H_2O$, 25:10:5:1:15 by vol, (C): EtOAc/MeOH/$H_2O$, 7:2:1 by vol., (D): $CH_3CN$/$H_2O$ 8:2, by vol.

HPLC analyses were performed on Shimadzu LC10Ai instrument equipped with a UV/VIS photodiode array detector SPD-M20A prominence; column: Phenomenex Kinetex 5 μm C18 100 A, 250 x 4.60 mm. Elution gradient condition: solvent **A**: $H_2O$ + 0.1% of TFA; solvent **B**: $CH_3CN$ + 0.1% of TFA; at t0 **A**:**B** ratio 95:5; t0–t1=30 min from 5% to 100% of **B** , t1–t2=35 min 100% of **B** ; flow 0.9 mL/min; detection at λ 220 nm. In these elution conditions, calibration curve of 2-phenoxyethanol (rt: 12.95 min), 2-phenoxyethanoyl xyloside (rt: 11.7 min), 2-phenoxyethanoyl xylobioside (rt: 11.1 min), 2-phenoxyethanoyl xylotrioside (rt: 10.5 min) were elaborated by using pure solutions of enzymatically prepared compounds (section 3.8.2) in the interval of concentration of 0.025:1.25 mM and 0.03125:0.5 mg/ml for 2-phenoxyethanol and its xyloside derivatives, respectively. All experimental data of absorption showed a linear correlation to the concentration of the analysed compounds (straight line resulting from the fit by linear regression, r2 0.99–0.96); each point was the average of three determinations (SD < 0.05).

The reaction products were analyzed by HPLC as described below to determine the degree of polymerization (DP) of the XOs. Apart of identification by TLC (system solvents A and B), both acid and enzymatic hydrolyses of *Cynara cardunculus* hemicellulosic extract were analysed by high-performance anion-exchange chromatography (HPAE-PAD Dionex *ICS 5000$^+$ DC*) with a CARBOPAC PA1 column at a solvent flow of 0.25 ml/min. The samples were centrifuged at 16,000 x g for 10 min properly diluted with water before each injection.

Monosaccharides composition of hemicellulosic extract was determined by acid hydrolysis with 2N trifluoroacetic acid (TFA) at 120 °C for 2 h and the percentage of each monosaccharide was determined as ratio of each peak area with respect to total area of the chromatogram (Table 1) (HPAE-PAD Dionex).

The carbohydrate standards were obtained from Sigma-Aldrich and analysed in proper Dionex chromatographic conditions. Xylose and xylo-oligosaccharides calibration curves were constructed according to the concentration interval ranging from 0.16 mg/ml to 0.0016 mg/ml.

The sugars separation of acid hydrolysate of *C. cardunculus* hemicellulosic extract was obtained with a following linear gradient elution program: eluent A was 18mM NaOH; eluent B was 500mM NaOAc - 100mM NaOH; eluent C was 500mM NaOH and eluent D was $H_2O$; 0-20 min, eluent A 100%; 20.1-30 min eluent B 10%, eluent C 36%, eluent D 54%; 30-30.1 min eluent B 40%, eluent C 24%, eluent D 36%; 30-40 min eluent B 100%; 40-50 min eluent A 100%. Retention times, determined at 25°C, of rhamnose, arabinose, galactose, glucose, xylose, galacturonic acid and glucuronic acid solution standard resulted (min): 5.27, 5.71, 6.89, 7.46, 8.42, 28.32, 28.48, respectively.

The separation of saccharides obtained from enzymatic digestions was achieved with 333 mM NaOH as the isocratic elution system. Standards retention times for xylose, xylobiose, xylotriose, xylotetraose, xylopentaose and xyloexaose at 25°C were (min) 2.59, 3.61, 5.5, 9.17, 16.15 and 29.66, respectively.

**3.2 Lignocellulosic biomass production**

*Cynara cardunculus* plants were harvested at the experimental farm of the University of Naples "Federico II" and were obtained from an experimental plantation located in the Sele river plain of Campania Region (Southern Italy, 40°61'N, 14°92'E, 30 m a.s.l.) where they were grown under mediterranean climatic conditions with low fertilization doses (100 kg $ha^{-1}$ of N per year). Sowing was made on spring 2009, harvests were made on August 2010, 2011, 2012. In a randomized block design with three replications, two water regimes were compared (irrigated *vs*. not irrigated). Aerial biomass of the last harvest, represented by crop residues after seeds separation, was collected in a sample area, air-dried until constant weight and grinded at 4 mm. Successively, the material was further grinded in order to obtain a homogenous fine powder (particle size of about 1 mm) by a kitchen mixer (Waring).

**3.3 Preparation of hemicellulose extract from stems and leaves of *Cynara cardunculus***

1 g of grinded dry steam and leaves from *Cynara cardunculus* (CC) was suspended in alkali medium (50 mL of 2N KOH) by continuous magnetic stirring at room temperature for 72 h. Later the suspension was processed as previously reported for the hemicellulose recovery from *Arundo donax* rhizomes[23]. The yield of lyophilized material (CK72h) was expressed in weight percentage with respect to the initial dry biomass (%, w/w).

### 3.4 Microorganism, xylanase and β-xylosidase activity assays

*Geobacillus thermantarcticus* (DSM 9572$^T$) isolated from Antarctic geothermal soil was grown in a flask at 60°C on a complex medium **YN** containing (w/v): 0.6% yeast extract, 0.3% NaCl at pH 6.0. Growth was followed for 24 h by measuring the optical density at λ 540 nm. A crude extracellular enzymatic solution, containing both xylanase and β-xylosidase activities, named "**ES-GTM**" (1.34 mg/ml of proteins) was produced, partially purified with ammonium sulphate and assayed using a previously established protocol[25]. In particular **ES-GTM**, appropriately diluted, was assayed for xylanase and β-xylosidase activities, by using 1% (w/v) of birchwood xylan (Sigma) as substrate at 80°C for 10 min and 1mM of *p*-nitrophenyl-β-D-xylopyranoside (Sigma) at 70°C for 10 min, respectively.

**ES-GTM** was used in all hemicellulose hydrolyses and transglycosylation processes reported below.

### 3.5 Separation procedures of xylanase and β-xylosidase enzymes from *Geobacillus thermantarcticus*

The extracellular suspension **ES-GTM** was processed by following two diverse protocols for separating quickly the two xylanolytic activities. Exploiting the different thermal stability of the two investigated activities, **ES-GTM** enzymatic solution was subjected to a thermal treatment at 80°C for an interval time of 10, 20 and 30 minutes for the β-xylosidase denaturation and the xylanase enzyme recovery. After each interval time with thermal treatment, the ES-GTM protein content, xylanase and β-xylosidase activities were assayed according to Lama et al.[25].

**ES-GTM** enzymatic solution which was thermally treated at 80°C for 20 min (**ES-GTM endo-xyl** 0.46 mg/ml of protein) was used in the processes later described.

Instead, to obtain **ES-GTM** containing essentially β-xylosidase, xylanase-free, the ability of adsorption of xylanase on xylan was exploited. Therefore, ES-GTM enzymatic solution was mixed with 2% xylan from birchwood in 50mM acetate buffer pH 5.6. After gentle shaking for 1 h at room temperature, the sample was centrifuged at 7000 g for 10 min and the precipitate washed three times with the same buffer. After xylan-affinity based treatment, the un-bound enzymatic fraction was assayed for protein content, β-xylosidase and xylanase activities, according to Lama et al.[25]. This xylan-unbound enzymatic solution, containing β-xylosidase activity (**ES-GTM β-xyl,** 0.9 mg/ml of protein) was used in the processes later described.

### 3.6 Enzymatic degradation of *Cynara cardunculus* hemicellulolytic extract and selective production of XOs or xylose

Enzymatic digestion (2 mL) of hemicellulosic material extracted from *C. cardunculus* (CK72h) (8 mg), solubilised in 50 mM sodium acetate buffer (pH 5.6), was carried out by incubating **ES-GTM** solution (2.23 mg of total proteins) for 24 h at 70 °C (Figure 1A). For selective production of xylo-derivatives, 1 ml of xylanase enzyme, (**ES-GTM endo-xyl** enzymatic solution), was mixed with 8 mg of the hemicellulosic extract CK72h at 70°C for 24 hours in 2 mL as final volume (Figure 1B). For higher xylose recovery an enzymatic cascade-reaction was set up; the xylanase hydrolysis products were then subjected to further hydrolysis, by 1 ml of β-xylosidase contained in the xylan-unbound fraction (**ES-GTM β-xyl** enzymatic solution), at 60°C for 24 hours (Figure 1C). All enzymatic reactions were performed in sealed glass vessels, under magnetic stirring and stopped by cooling in an ice-bath. The results of hydrolysis reactions were expressed in terms of reducing sugars and xylose (% w/w, respect to initial substrate amount). The reaction products were further analysed by TLC and HPAE-PAD Dionex, to determine the mono- and oligo-saccharidic composition of products.

### 3.7 Semi-preparative scale production of XOs

10 ml of **ES-GTM endo-xyl** enzymatic solution were reacted with 40 mg of CK72h at 70°C for 24h, under magnetic stirring. Hydrolysis reaction mixture was stopped by cooling in ice-bath for 4 minutes and then concentrated under vacuum. The percentages of total reducing sugars and xylose were evaluated. The dry sample was re-suspended in 5ml of distilled water and loaded onto a Biogel P-2 column (1 × 47 cm) equilibrated and eluted at 0.3 ml/min with water. The fractions containing the carbohydrates were pooled and freeze-dried. Purified compounds were subjected to extensive MS and NMR spectroscopic investigation.

**Compound 1**: α-D-4OMeGLcA-(1-2)-β-D-Xyl-(1-4)-β-D-Xyl-(1-4)-β-D-Xyl; δ $^{1}$H ( δ $^{13}$C): MeGlcA-Xyl$_3$-Xyl$_2$-**Xyl$_1$,** H1 α 5.09, β 4.49 (*8.29 Hz*) (α 93.01, β 97.51 ) H2 3.16 (73.64), H3 3.46 (72.36), H4 3.68 (77.6), H5 3.29, 3.96 (63.95); MeGlcA-Xyl$_3$-**Xyl$_2$**-Xyl$_1$**,** H1 4.38 (*7.79 Hz*) (102.65 ) H2 3.19 (73.66), H3 3.47 (74.91-74.95), H4 3.70 (77.4), H5 3.35, 4.06 (63.86); MeGlcA-**Xyl$_3$**-Xyl$_2$-Xyl$_1$**,** H1 4.52 (*7.57 Hz*) (102.61 ) H2 3.32 (77.07) H3 3.38 (75.40) H4 3.55 (70.42), H5 3.21, 3.89 (65.91); **MeGlcA**-Xyl$_3$-Xyl$_2$-Xyl$_1$, H1 5.22 (*4.16 Hz*) (98.45) H2 3.49 (74.95) H3 3.67 (73.24), H4 3.14 (83.46), H5 4.21 (*10.2 Hz*) (73.17), -COOH (177.6), -OMe 3.37 (60.87). ESI-MS *m/z:* 605 [M+H$^-$]

**Compound 2** : α-D-GLcA-(1-2)-β-D-Xyl-(1-4)-β-D-Xyl-(1-4)-β-D-Xyl; δ $^{1}$H ( δ $^{13}$C): GlcA-Xyl$_3$-Xyl$_2$-**Xyl$_1$,** H1 α 5.11, β 4.51 (*7.90 Hz*) (α 93.03, β 97.55 ) H2 3.17 (75.02), H3 3.48 (74.97), H4 3.70 (77.4), H5 3.31, 3.96 (63.99); GlcA-Xyl$_3$-**Xyl$_2$**-Xyl$_1$**,** H1 4.39 (*7.84 Hz*) (102.68)

H2 3.21 (73.67), H3 3.50 (74.76), H4 3.72 (77.53), H5 3.37, 4.09 (63.91); MeGlcA-**$Xyl_3$**-$Xyl_2$-$Xyl_1$, H1 4.54 (*7.58 Hz*) (102.68 ) H2 3.39 (77.15) H3 3.57 (75.47) H4 3.58 (70.45), H5 3.23, 3.92 (65.98); **GlcA**-$Xyl_3$-$Xyl_2$-$Xyl_1$, H1 5.25 (*3.93 Hz*) (98.56) H2 3.47 (72.20) H3 3.66 (73.10), H4 3.40 (73.65), H5 4.26 (*10.7 Hz*) (73.09), -COOH (178.70). ESI-MS *m/z:* 591 [$M+H^-$].

### 3.8. Transglycosylation reactions

#### 3.8.1 Analytical scale reactions

Methanol, ethanol, hexanol, *n*-octanol, phenol, resorcinol, 2-phenoxyethanol, benzyl alcohol, hydroquinone, caffeic acid, gallic acid, tyrosol, vanillin, (±)catechin, 4-(2-Methoxyethyl)phenol, 2-hydroxymethyl-2,5,7,8-tetramethylchroman-6-ol and avarol were tested as possible acceptors in analytical transglycosylation reactions. 0.5 ml of a CK72 solution at a concentration of 4mg/ml containing 50 mM of each acceptor in 50 mM Na-acetate buffer at pH 5.6 and in presence 0.1 ml of enzymatic solution (**ES-GTM**) were put at 70°C under magnetic stirring for 24 h. The reaction were monitored by TLC analysis over the time (TLC system solvent C and D); reactions with 2-hydroxymethyl-2,5,7,8-tetramethylchroman-6-ol and avarol were performed by using 10% of DMSO as organic co-solvent. The reactions were carried out in tight screw vials and they were stopped by cooling in ice-bath for 4 minutes; after 24 h, aliquots of reactions were collected and analysed by ESI-MS

#### 3.8.2 Production of 2-phenoxyethanol xylosides

20 mg of CK72h polysaccharidic extract were dissolved at 70°C in 5 ml in Na-acetate buffer 50 mM, pH 5.6) in presence of 48.3 mg of 2-phenoxyethanol (70 mM) and 1 ml (1.34 mg) of **ES-GTM** enzymatic solution from *G. thermantarcticus*. The reaction was monitored by TLC analyses over the time (system solvent C); after 48h it was stopped by cooling in ice-bath for 4 minutes and immediately subjected to reverse-phase C-18 column chromatography eluting with water, and methanol, thus efficiently recovering in methanol fractions total chromophoric xylosylated products and unreacted acceptor. The methanol fractions were collected (23 mg) and further purified on silica gel column by eluting with a gradient of methanol in ethyl acetate. The structures of purified 2-phenoxyethanoyl xyloside, xylobioside, xylotrioside were established by ESI-MS and 1D and 2D NMR analyses. Pure molecules were then used for the construction of calibration curve by HPLC as described in 2.1 section. The products mixture composition was established by HPLC.

#### 3.8.3 NMR characterization of 2-phenoxyethyl β-xylosides

**2-phenoxyethyl β-xyloside.** *Saccharidic moiety* δ $^1H$ ($^{13}C$): H1 4.36 ( *d*, J=7.8 Hz) (104.7), H2 3.24 (74.72), H3 3.62 (77.31), H4 3.52 (70.80), H5-H5' 3.91-3.25(66.71). *Aglycone moiety* δ $^1H$

($^{13}$C): Φ–O-$\underline{CH_2}$–$CH_2$–O–β-xyl: 4.19 (68.28), Φ–$CH_2$–$\underline{CH_2}$–O–β–xyl 4.15-3.95 (68.86), aromatic signals (from 1' to 4' positions): (159.5), 6.96 (115.32), 7.29 ( *dd*, J= 8.03, 7.93 Hz) (130.11), 6.95 (121.81).

**2-phenoxyethyl β-xylobioside:** *Saccharidic moiety* δ $^1$H ($^{13}$C): O–β–**xyl**–(1,4)–xyl: H1 4.37 ( *d*, J=7.4 Hz) (105.11), H2 3.30 (74.72), H3 3.68 (78.27), H4 3.50 (**75.86**), H5-H5' 4.06-3.36 (64.48); O–β–xyl–(1,4)–**xyl**: H1 4.35 ( *d*, J=7.61 Hz) (104.02), H2 3.26 (74.39), H3 3.35 (77.64), H4 3.54 (**71.19**), H5-H5' 3.94-3.27 (67.13). *Aglycone moiety* δ $^1$H ($^{13}$C): Φ–O-$\underline{CH_2}$–$CH_2$–O–β-$xyl_2$: 4.21 (68.57), Φ–$CH_2$–$\underline{CH_2}$–O–β–$xyl_2$ 4.15-3.95 (69.23), aromatic signals (from 1' to 4' positions): (160.40), 6.97 (115.66), 7.29 (*dd*, J=8.10, 7.99 Hz) (130.46), 6.95 (121.95).

Long range correlations between signal at 4.15 δ of methylene protons in position 2 of aglycone and anomeric carbon at 105.3 δ and anomeric proton at 4.35 δ in reducing xylose unit with the carbon in position 4 of non-reducing xylose secured the connection between the different portions of molecule.

**2-phenoxyethyl β-xylotrioside.** *Saccharidic moiety* δ $^1$H ($^{13}$C): O–β–**xyl**–(1,4)–xyl–β–(1,4)– xyl: H1 4.38 (105.12), H2 3.29 (74.65), H3 3.69 (78.11), H4 3.49 (**75.71,75.78**), H5-H5' 4.09-3.38 (64.70); O–β–xyl–(1,4)–**xyl**–β–(1,4)– xyl: H1 4.37 (104.04), H2 3.25 (74.35), H3 3.71 (77.65), H4 3.51 (**75.71,75.78**), H5-H5' 4.06-3.36 (64.49); O–β–xyl–(1,4)–xyl–β–(1,4)–**xyl**: H1 4.36 Hz (103.71), H2 3.22 (74.14), H3 3.35 (77.64), H4 3.53 (**71.06**), H5-H5' 3.93-3.27 (67.11); *Aglycone moiety* δ $^1$H ($^{13}$C): Φ–O-$\underline{CH_2}$–$CH_2$–O–β-$xyl_2$: 4.21 (68.46), Φ–$CH_2$– $\underline{CH_2}$–O–β–$xyl_2$ 4.15-3.96 (69.17), aromatic signals (from 1' to 4' positions): (160.10), 6.96 (J=) (115.67), 7.29 (*dd*, J=8.11, 7.98 Hz) (130.44), 6.94 (121.94) . The long-range correlation between signal at 69.17 δ of methylene carbon in position 2 of aglycone and anomeric proton at 4.38 δ and the chemical shift values of carbon in position 4 of each xylose residue confirmed the molecular structure.

### 3.8.4 Optimization of 2-phenoxyethanol xylosylation

Glycosylation reactions were performed at different acceptor concentrations and at a fixed amount of polysaccharidic CK72h extract or differently by varying the donor amount and at a selected acceptor concentration. In all cases, the ratio between the mg of CK72h xylan and the ml of total protein of used enzymatic solution was fixed. Reactions were carried out on analytical scale (total volume of 0.5 ml) in Na-acetate buffer (50 mM, pH 5.6), at 70°C under magnetic stirring, for 48 h and in presence of 50 μl of β-xylosidase/xylanase enzymatic solution (67 μg) from *G. thermantarcticus* (**ES-GTM**) or 150 μl of xylanase (69 μg) recovered after thermal treatment at 80°C for 20 min (**ES-GTM endo-xyl**) per each mg of CK72h polysaccharidic extract employed.

The reactions were monitored by TLC analysis (system solvent C), as above described. After 24 and 48 h, aliquots of 10 or 20 µl of each reactions were collected and diluted 1:20, 1:40 1:80 with $H_2O$ with respect to initial concentration of acceptor and donor; they were stopped by cooling, centrifuged for 20 minutes at 13500 rpm and analysed by HPLC, according to method reported in section 2.1. The identification of reaction products was possible by comparison with the retention time of pure compounds previously produced, isolated and characterized.

In a first set of reactions, 4 mg/ml of CK72h polysaccharide extract was put in presence of increasing concentration of 2-phenoxyethanol (50, 70, 100, 200 mM); in these reactions 100 µl of **ES-GTM** enzymatic solution (134 µg) or 300 µl of **ES-GTM endo-xyl** enzymatic solution (138 µg) were used.

In a second set of reactions, the acceptor concentration was fixed at 50 mM and 4, 8, 16 mg/ml of CK72h polysaccharide extract were digested in presence of 100, 200 and 400 µl of **ES-GTM** enzymatic solution (134, 268, 536 µg) respectively.

A further reaction was performed at 70 mM of 2-phenoxyethanol and 16 mg/ml of CK72 polysaccharide extract by using 400 µl of **ES-GTM** enzymatic solution (536 µg) in 500 µl of buffer as previously described. Time course of reaction was made by monitoring the process over the time.

## 4. Conclusions

Arabinoglucuronoxylan extracted from stems and leaves of *Cynara cardunculus* not only reflect the composition of biomass currently targeted for biofuel production but also represents a valuable source of biomolecules usable in different fields. In this work, we presented several processes involving *C. cardunculus* hemicellulose and extracellular β-xylosidase/xylanase activities from thermophilic *G. thermantarcticus*. In all cases enzymatic solutions of single or coupled β-xylosidase and xylanase activities from *G. thermantarcticus* extracellular were easily recovered with simple procedures and exploited for the production of (i) fermentable sugars when the xylanase and the β-xylosidase activities from *G. thermantarcticus* were consecutively used for a cascade enzymatic attach; (ii) xylooligosaccharides and in particular glucuronoxylo-oligosaccharides (compounds **1** and **2**) when the *G. thermantarcticus* xylanase activity digested the *C. cardunculus* hemicellulose extract; (iii) oligoxylosides of 2-phenoxyethanol, by transglicosylation processes in which xylanolitic fraction of *C. cardunculus* agroresidue was used as cheap donor and xylanase and β-xylosidase activities of *G. thermantarcticus* were synergically employed.

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**Table 1.** Monosaccharide composition of *Cynara cardunculus* hemicellulose fraction (CK72h), extracted with KOH 2 N at 72 h.

| Monosaccharide | (%) |
|---|---|
| **Xyl** | 89.8 |
| **Glc** | 3.8 |
| **Ara** | 1.8 |
| **Man** | 0 |
| **Gal** | 2.51 |
| **Rha** | 0.4 |
| **GalA** | 0.63 |
| **GlcA** | 1.18 |

**Xyl:** Xylose**; Glc:** Glucose; **Gal:** Galactose; **Ara:** Arabinose; **Rha:** Rhamnose; **Man:** Mannose; **GlcA:** Glucuronic acid; **GalA,** Galacturonic acid.

**Table 2**

Enzymatic processes (hydrolyses **1-4** and transglycolylations **5-7**) performed by using extracellular xylanase and β-xylosidase from *G. thermantarcticus* for the production of fermenTable sugars, oligosaccharides and xylo-conjugated molecules

| | Biocatalyst | | substrates | | | | | | Useful results |
|---|---|---|---|---|---|---|---|---|---|
| | | B/S[1] | CK77h mg/ml | acceptor mM | RS[2] % | Xyl % | $Xyl_2$ % | $Xyl_3$ % | |
| **1** | **ES-GTM** | 278 | 4 | -- | 62 | 44 | | | Presence of $Xyl_3$, $Xyl_4$* |
| **2** | **ES-GTM endo-xyl** | 57 | 4 | -- | 43.7 | 6.8 | 26.5 | 5.5 | Traces of oligosaccharides >3 units: compounds **1** and **2**** |
| **3** | **ES-GTM endo-xyl + ES-GTM β-xyl** | 57+112 | *** | -- | 73.6 | 62.6 | | | Traces of oligosaccharides >3 units# |
| **4** | **ES-GTM endo-xyl** | 57 | 4 | -- | 39 | 7.6 | nd[5] | nd[5] | compounds **1** 22 % and **2** 13%## |
| | | | | | **Yield[3] %** | **Yield[4] %** | **Molar ratio Compound 3:4:5** | | |
| **5** | **ES-GTM endo-xyl** | 69 | 4 | 70 | 3.1 | 20.5 | 1:0.5:0.8 | | |
| **6** | **ES-GTM** | 67 | 4 | 70 | 5.4 | 38.5 | 1:0.5:1.14 | | |
| **7** | **ES-GTM** | 67 | 16 | 70 | 19.3 | 34.7 | 1:0.35:1.12 | | |

[1]B/S: μg of enzymatic solutions protein /mg of CK72h hemicellulose extract

[2]RS = Reducing sugars yield %.

[3] yields were calculated by evaluating the percent ratio of produced 2-phenoxyethyl β-xyloside, xylobioside, xylotrioside (compound **3**, **4**, **5**, in Figure 4) and the initial acceptor concentrations.

[4] yields were calculated as percentage ratio of total g of xylosilated products and g of CK72h hemicelulosic extract.

[5] nd: not detected.

* MS analysis of reaction after 24 h indicated the presence into reaction mixture of xylose ( m/z:173 [M+Na+]), xylotriose (m/z: 437 [M+Na+]), xylotetraose (m/z: 569 [M+Na+]) and also of a disaccharide constituted of xylose and o-methyl glucuronic acid (m/z: 341 [M+H+]) and a disaccharide constituted of xylose and glucuronic acid (m/z: 327 [M+H+]).

** Results were obtained by HPAE-PAD investigations (black trace, Figure 2) and TLC analyses

(Figure 1S of supplementary materials).

*** Substrate of this reaction were the hydrolysis products of reaction 2

[#] Results were obtained by HPAE-PAD investigations (violet trace, Figure 2) and TLC analyses (Figure 1S of supplementary materials).

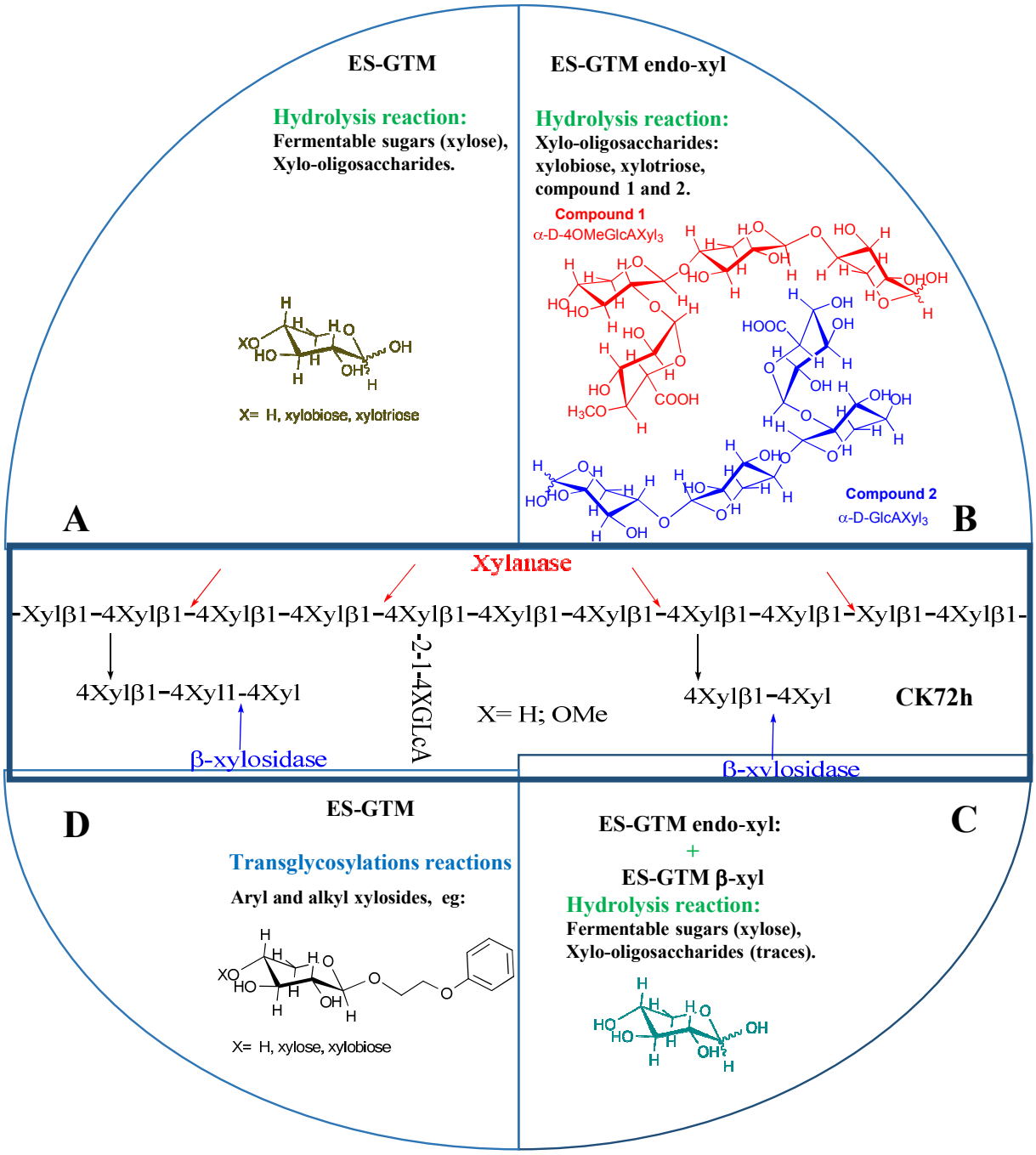
[##] Results of semi-preparative scale reaction reported in section 3.7.

**Table 3**: Qualitative evaluation of xylosylation of different acceptors by xylanase/β-xylosidase activities from the *G. thermantarcticus* **ES-GTM** enzymatic solution; concurrent hydrolyses of CK72h hemicellulosic extract were analysed.

| | Acceptors | Reactivity* | | Hydrolysis* |
|---|---|---|---|---|
| | | 5 h | 24h | 24h |
| **1** | Methanol | - | - | - |
| **2** | Ethanol | + | + | + |
| **3** | Hexanol | ++ | + | ++ |
| **4** | *n*-Octanol | + | +/- | ++ |
| **5** | Phenol | +/- | - | +++ |
| **6** | Resorcinol | + | ++ | +++ |
| **7** | Hydroquinone | + | ++ | ++ |
| **8** | 2-phenoxyethanol | ++ | ++ | +++ |
| **9** | Tyrosol[a] | ++ | ++ | +++ |
| **10** | 2-Hydroxymethyl 2,5,7,8 tetramethylchroman-6-ol | - | - | - |
| **11** | Benzyl alcohol | + | ++ | +++ |
| **12** | Caffeic acid | - | - | - |
| **13** | Gallic acid | - | - | - |
| **14** | Avarol | - | - | ++ |
| **15** | 4-(2Methoxyethyl)phenol | +/- | + | + |
| **16** | Vanillin | - | - | ++ |
| **17** | (±)Catechin | + | + | + |

*Notes :-/+ percentage of products below 10%; + 10-15% of products; ++ 15-30% of products;+++: percentage of products more than 30%. In general, the adopted chromatographic conditions (TLC system solvent C) were suitable for the complete separation of mono- and oligo xylosides of acceptors and hydrolysis products (xylose, xylobiose, xylo-oligosaccharides)
[a]: TLC system solvent D

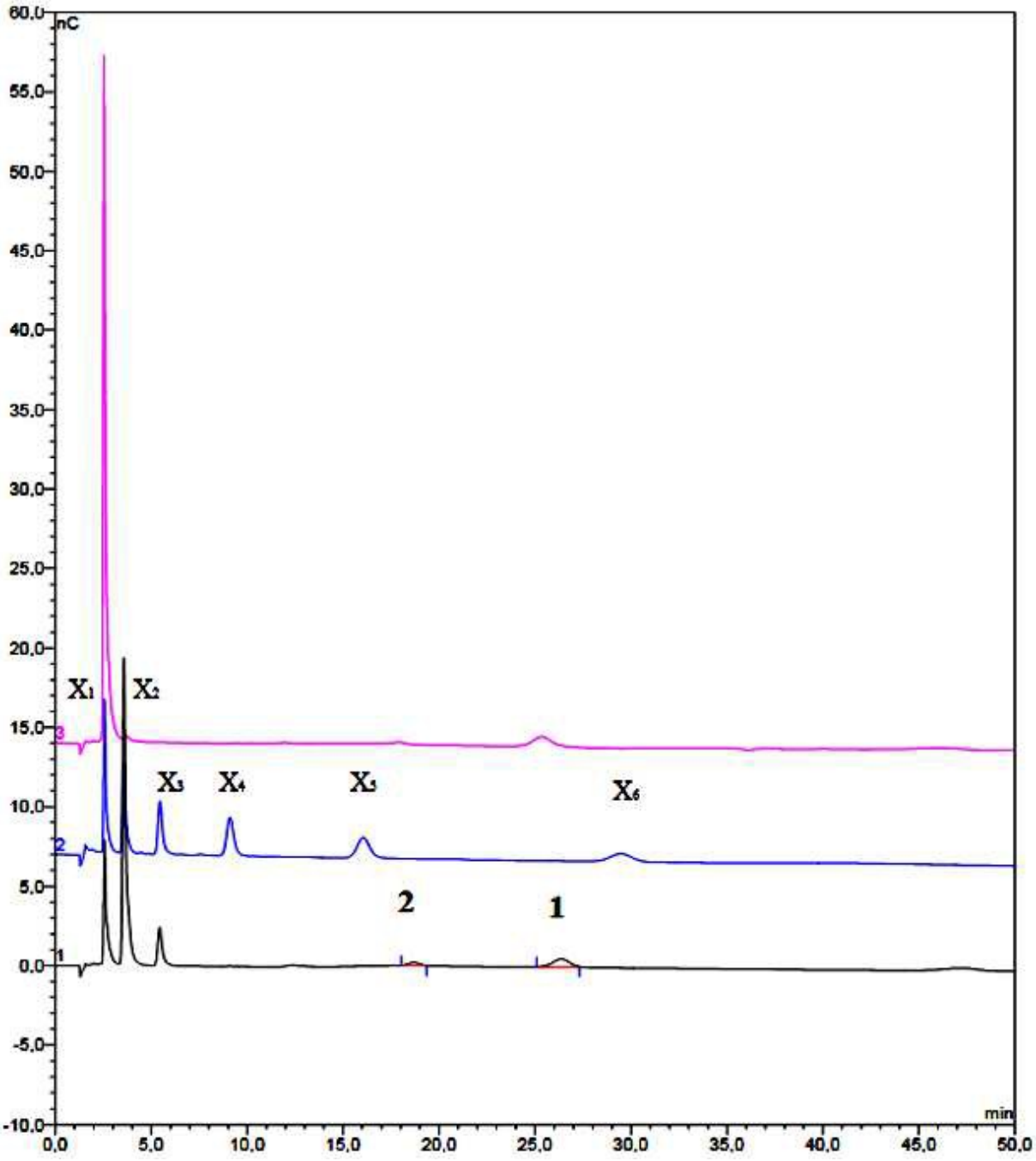
**Figure 1**: Exploitation of xylanolitc activities from *G. thermantarcticus* for high value-added molecules production from *Cynara cardunculus* hemicellulose extract



**CK72h**: Schematic representation of xylan backbone of hemicellulosic extract from *Cynara cardunculus* (central box); arrows in red and blue indicated the possible break-sites by xylanase and β-xylosidase activities, respectively.

**1A**:**1C**: hydrolysis reactions of hemicellulose extract CK72h by *G. thermantarcticus* xylanolitic activities; **1D**: transglycosylation reactions with selected aryl and alkyl alcohols as acceptors (Table 3) and CK72h extract as donor by *G. thermantarcticus* xylanolitic activities; **ES-GTM**: enzymatic solution containing both xylanase and β-xylosidase activities; **ES-GTM endo-xyl:** enzymatic solution containing xylanase activity**; ES-GTM β-xyl**: enzymatic solution containing β-xylosidase activity.

**Figure 2:** HPAE-PAD analyses of hydrolysis reactions of *C. cardunculus* hemicellulose extract (CK72h) by using **ES-GTM endo-xyl** (Figure 1B) and by the combined enzymatic attach with **ES-GTM endo-xyl** and **ES-GTM β-xyl** (Figure 1C)




Notes: **Trace black**: chromatographic profile of CK72h hydrolysis by **ES-GTM endo-xyl** (Figure 1B and line 2 of Table 2) **1**: compound **1** at rt 26.36 min, **2:** compound 2 at rt 18.67 min (Figure 3);

**Trace blue**: chromatographic profile of xylo-oligosaccharides standards (X1: xylose, X2: xylobiose, X3: xylotriose, X4: xylotetraose, X5: xylopentaose, X6: xyloexaose; **Trace violet**: chromatographic profile of enzymatic cascade-reaction: products of CK72h digestion by **ES-GTM endo-xyl** (Figure 1B and line 2 of Table 2) were further degraded by the **ES-GTM β-xyl** activity (Figure 1C and line 3 of Table 2) .

**Figure 3**: $^{13}C$ NMR spectra of compounds **1** and **2** produced from CK72h hemicellulose extract by **ES-GTM endo-xyl** enzymatic solution recovered after thermal treatment.

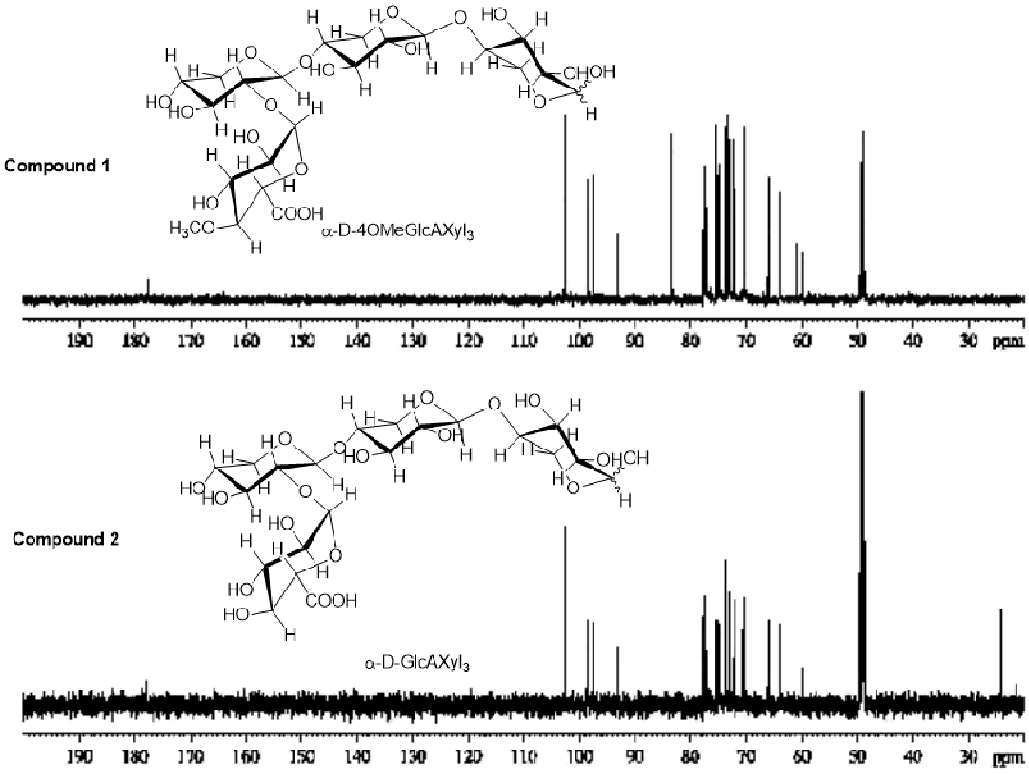
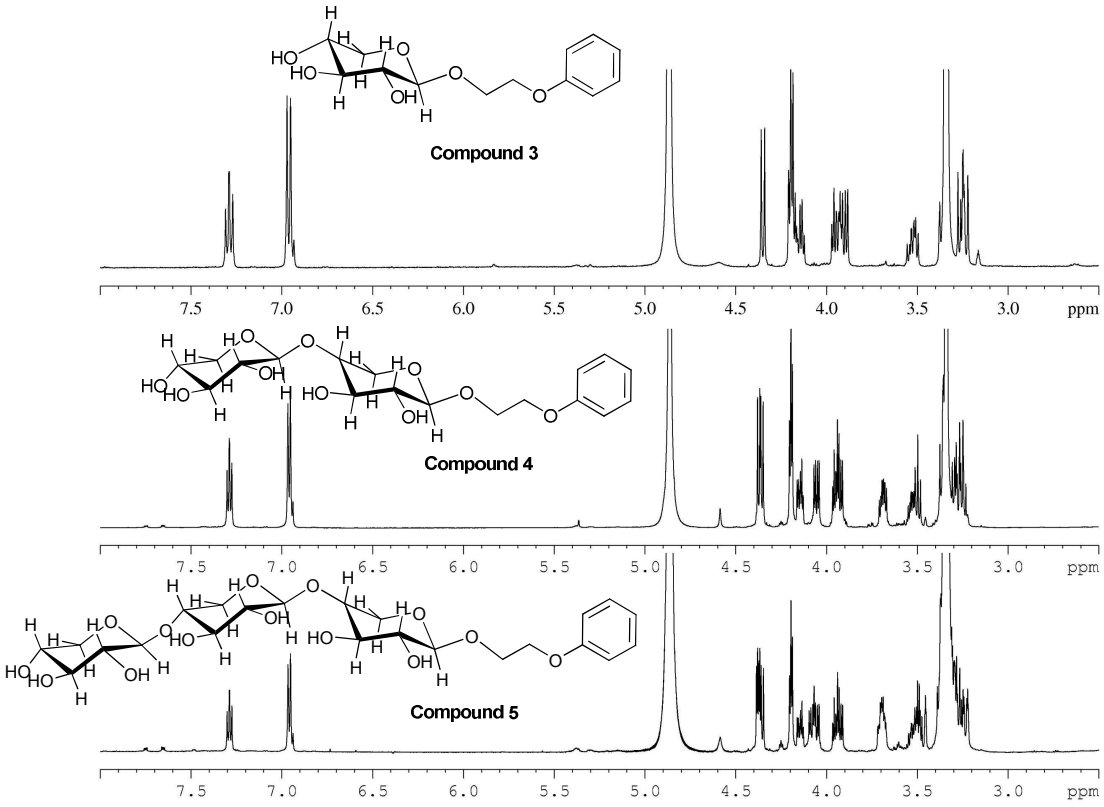
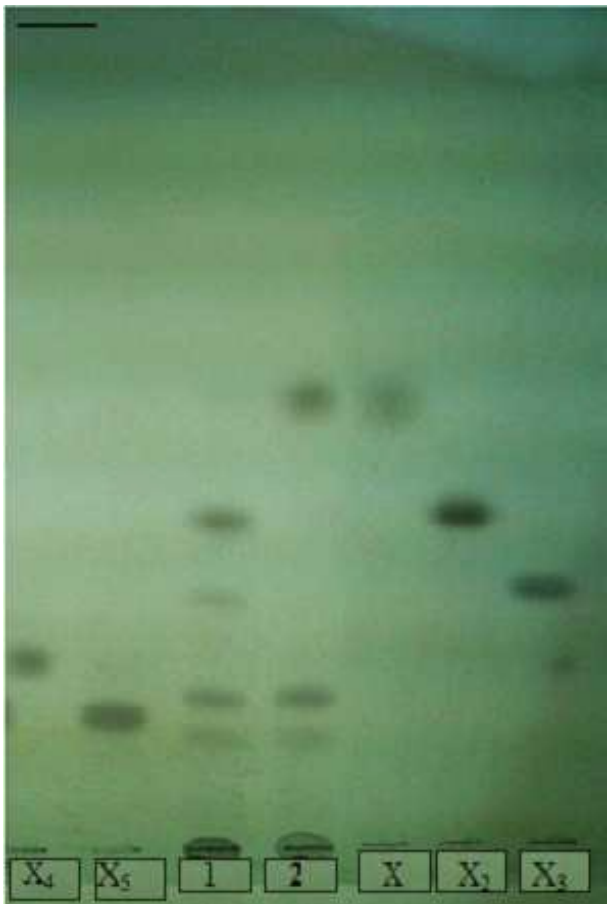


**Figure 4**: $^{1}$H NMR spectra of compounds **3**, **4** and **5** produced in transglycosylation process by **ES-GTM** enzymatic solution, CK72h hemicellulose extract as donor and 2-phenoxyethanol as acceptor



**Supplementary materials**

**Figure 1S**. TLC analyses of hydrolysis reactions of *C. cardunculus* hemicellulose extract (CK72h) by using **ES-GTM endo-xyl** (lane 1) and by the combined enzymatic attach with **ES-GTM endo-xyl** and **ES-GTM β-xyl** (lane 2).




Notes: **X**: xylose; **$X_2$-$X_5$** = β-1,4-xylooligomers. TLC System solvent A.

**Figure 2S:** Partial plot of Noesy (2S-A) an HMBC experiments (2S-B and C) of compound **1**

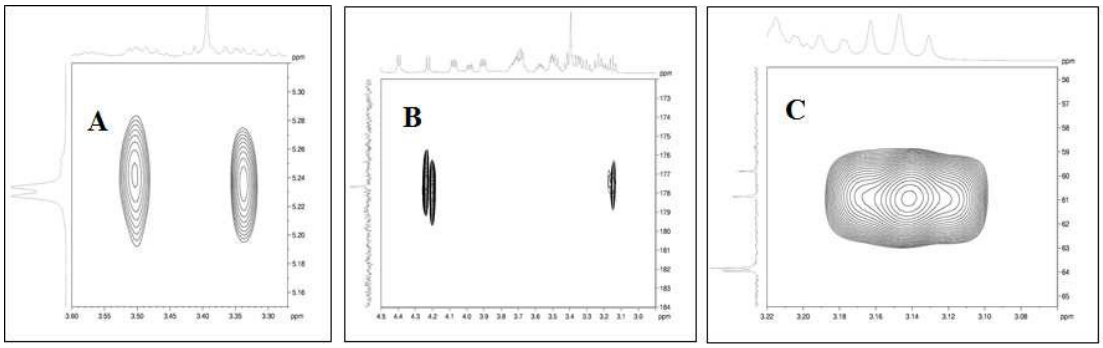
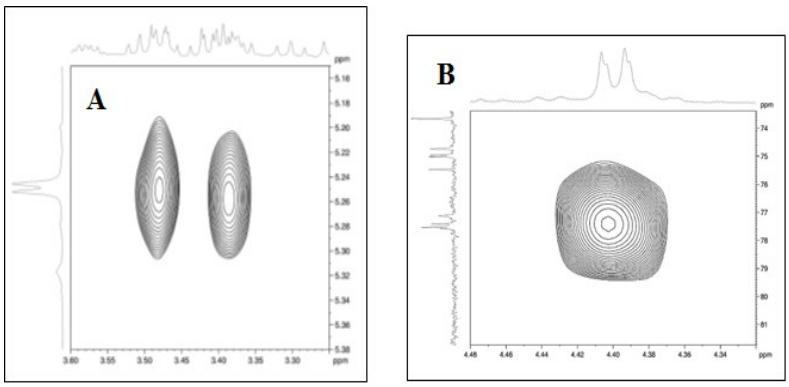


**Figure 3S:** Partial plot of Noesy (3S-A) an HMBC experiments (3S-B ) of compound **2**



Hemicellulose fraction from *Cynara cardunculus* was efficiently degraded and converted in xylo-conjugates by the thermophilic xylanolytic enzymatic systems of *Geobacillus thermantarcticus.*