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Innovative enzymes for bioethanol production from lignocellulosic materials

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Abbreviations

AAS	aqueous ammonia soaking
BSG	brewery's spent grains
EDTA	ethylenediaminetetraacetic acid
LCMs	lignocellulosic materials
PMSF	phenylmethylsulfonyl fluoride
pNPAF	p-nitrophenyl (pNP)-α-L-arabinofuranoside
pNPXP	p-nitrophenyl-β-Dxylopyranoside
XOS	xylooligosaccharides

1. Introduction

Increasing demand and uncertain availability of fossil fuels urge us to find alternative resources available in large quantities. It can no longer come as a surprise that the ecological impact of human beings is having a significantly adverse effect on the global environment. Along with increasing energy consumption, environmental aspects such as green house gas emission should be considered to obtain a sustainable environment (Solomon, 2010). Depletion of fossil fuels has increased the carbon dioxide content of the atmosphere, causing a steady increase in global temperatures (Kerr, 2007). Renewable energy sources such as solar energy, wind and water power, or biomass, when exploited in combination, have a good potential to complement fossil fuels in the growing energy market (de Vries et al, 2007) and to fulfill the requirements to reduce green house gas emissions set by the Kyoto protocol in 1998. At present, bioethanol and biodiesel are produced and blended with gasoline and diesel worldwide: bioethanol is produced in North America mainly from cornstarch and in Brazil from sugarcane, while biodiesel is produced in Europe from rapeseed oil (the so call "first generation" biofuel). However, fuels produced from these materials are not considered sustainable for several reasons: because starch, sugar and vegetable oils can be consumed as food or feed (the "food versus fuel" debate), and because the net green house gas and energy savings of these fuels are modest. Thus, recent efforts have been directed towards the use of sustainable raw materials, such as residues from forestry and agriculture as well as municipal wastes (EPA, 2009; Solomon, 2010; Bonin and Lal, 2012)

Often being touted as the world's most abundant polymers, (hemi)cellulose and other plant carbohydrates are believed to be the renewable energy source that can provide liquid fuels and chemicals on a sustainable basis (Gomez et al, 2008; Kamm and Kamm, 2008; Ragauskas et al, 2006). It can be produced from all kinds of plant materials, ranging from corn stover and wheat straw to forest and agricultural residues. Cellulosic bioethanol, also known as second-generation bioethanol, is seen as a more attractive alternative (Service, 2007); however, the cost of production is a major deterrent to its commercialization and the process optimization and economics need to be improved in order for bioethanol to gain wide acceptance (Binder and Raines, 2010).

The conversion of biomass to fermentable sugars (Fig.1) is hindered primarily by the complexity of lignocellulosic substrates as well as by the performance of the hydrolytic enzymes, the major cost factors in the biomass conversion process are due to the feedstock, pretreatment and the enzymes (Humbird et al, 2011). Processes under discussion will, in principle, focus on the careful degradation of the lignocellulose complex to its building compounds and subsequent chemical and/or biological processing of the different subunits to various value-added products (McKendry, 2002; Aristidou and Penttila, 2000). The term "careful degradation" means that the major substructures: cellulose, hemicellulose and lignin are first separated from each other by a physical, chemical, or physico-chemical treatment, and thereafter processed individually by the most suitable often enzymatic method. For the degradation of the polymers under discussion enzymatic methods are superior to chemical techniques to many aspects and consequently are in the focus of current lignocellulose research (Nguyen and Saddler, 1991; Jeffries, 1990).

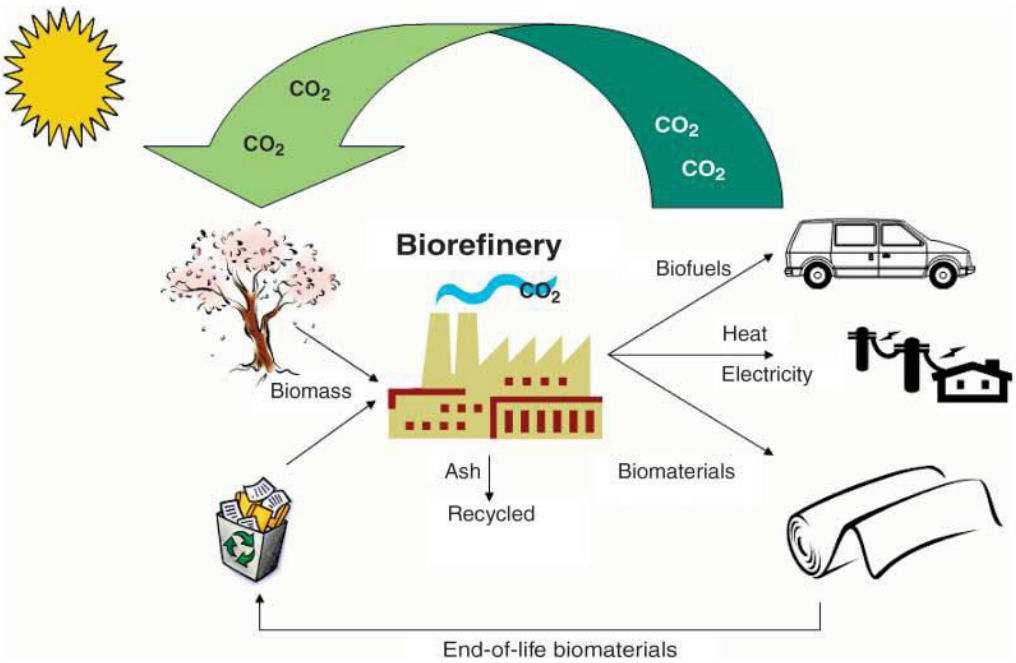

Fig. 1: The fully integrated agro-biofuel-biomaterial-biopower cycle for sustainable technology

1.1 Lignocellulosic biomass, a renewable raw material

Lignocellulosic biomass is the most abundant material on Earth. Its sources, including raw materials like agricultural residues (e.g. corn stover and wheat straw), forestry residues (e.g. sawdust and mill wastes), portions of municipal solid waste (e.g. waste, paper) and various industrial wastes have a great potential to be used in the industrial processes.

A defining feature of terrestrial plants is the highly developed cell walls. The plant cell wall is described as a dynamic structure and is a complex composite of polysaccharides, aromatic compounds and proteins. In general, the three major constituents of secondary cell walls are cellulose, hemicelluloses and lignin, from which the generic term *lignocellulose*. The composition of lignocellulosic biomass varies significantly between plant species but contains approximately 30-50% cellulose, 20-30% hemicellulose and 10-25% lignin (Sanchez, 2009), which are strongly bonded by non-covalent cross linkages. Various biomasses may also contain small amounts of pectin, starch, minerals (ash) and various extractives.

The plant species differ in the relative amounts (Table 1) (Deobald et al, 1997), as well as in the chemical structures of these main polymers.

Variation also occurs between plant tissues. Furthermore, different layers of plant cell walls, such as primary and secondary cell walls in wood, differ in the relative amounts of the cell wall components. Cell walls are built up from several layers: the middle lamella, the primary and secondary cell walls, and the warty layer (Sjöström, 1993). The middle lamella, which is highly lignified, is located between cells and binds the cells together. The outmost layer of the cell, the primary cell wall, consists of an irregular network of cellulose microfibrils combined with hemicellulose, lignin, pectin and proteins. The primary cell wall is supported by a thicker secondary cell wall, which can be divided into three distinct sublayers consisting of highly organised cellulosic microfibrils with various orientations. The middle layer of the secondary cell wall comprises most of the lignin in the cell wall because of its thickness, even though the concentration of lignin is higher in the middle lamella and the primary wall. In the cell wall, hemicelluloses and cellulose have been reported to be associated to each other and cross-linked with lignin (Tenkanen et al, 1999).

Table 1. Lignocellulose composition of several agricultural waste.

Lignocellulosic materials	**Cellulose** (%)	**Hemicellulose** (%)	**Lignin** (%)
Hardwood	40-55	24-40	18-25
Softwood	45-50	25-35	25-35
Nut shell	25-30	25-30	30-40
Chestnut shell	27.4	10	44.6
Grape stalk	38	15	33
Corn stover	36.7	13.33	33
Wheat straw	30	50	15
Rice straw	32.1	24	18
Brewer's spent grain	16.8	28.4	27.8
Paper	85-99	0	0-15
Leaves	15-20	80-85	0
Cotton seeds hairs	80-95	5-20	0
Newspaper	40-55	25-40	18-30
Waste paper from chemical pulps	60-70	10-20	5-10
Switch grass	45	31.4	12.0

Modified from (Jorgensen et al, 2007)

The structure of lignocellulose is therefore strongly dependent on its origin. A general presentation of polymer deposition in a lignified secondary cell wall is shown in Fig. 2.

In plants, linear cellulose chains contribute to tensile strength, while hydrophobic amorphous lignin is responsible for chemical resistance, in particular protection against water. Hemicellulose provides bonding between cellulose and lignin.

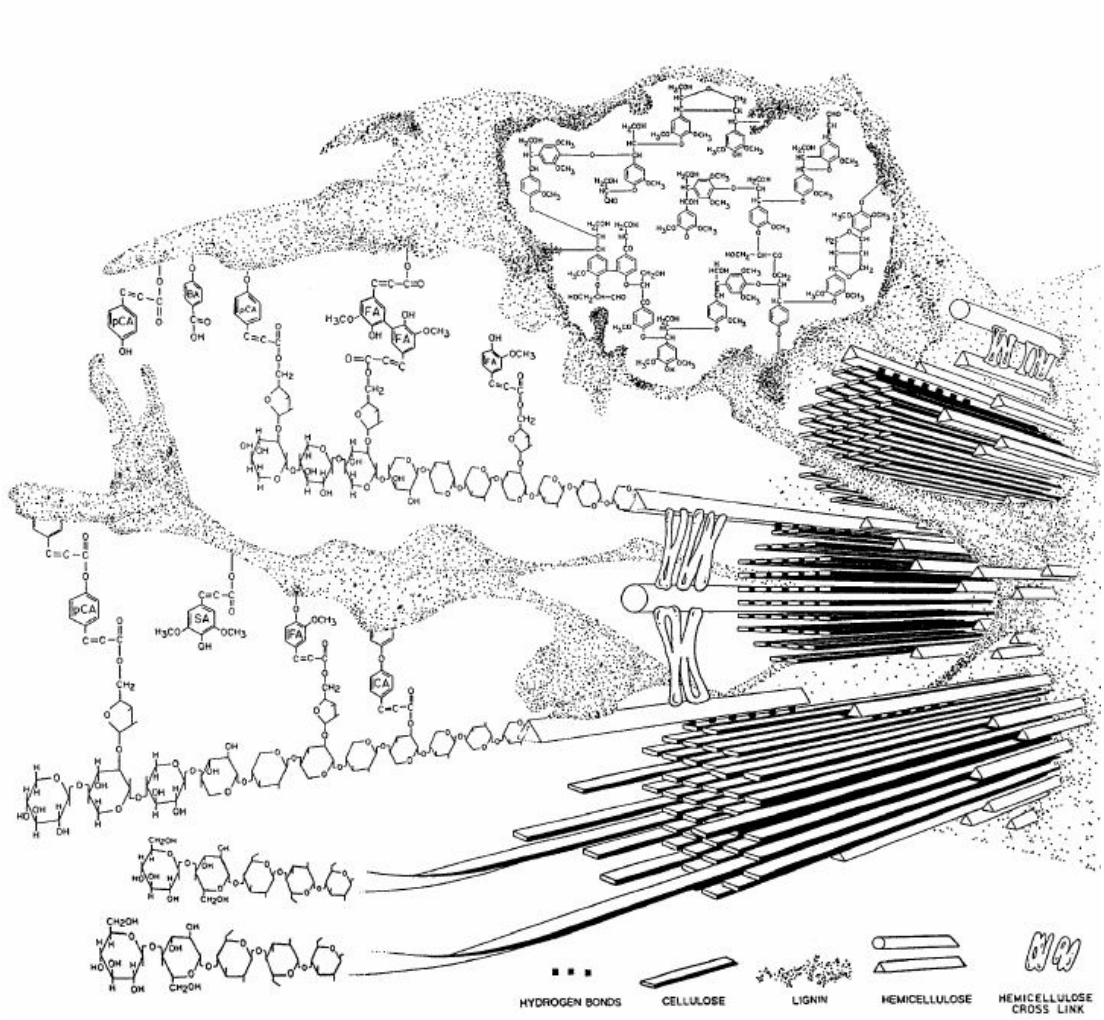

Figure 2: A model of the molecular structure of the main constituents of lignocellulosic material (secondary plant cell wall). Components are arranged so that the cellulose and hemicellulosic chains are embedded in lignin. Parallel cellulose chains bound tightly together by hydrogen bonds provide rigidity to the plant material, while surrounding hydrophobic lignin "glues" the components together and acts as a physical barrier for microorganisms and water. Hemicellulose connects cellulose and lignin through a network of cross-linked fibres (Bidlack et al, 1992).

Thus, two major obstacles hinder the hydrolysis of cellulose in lignocellulosic material. They are the recalcitrance of crystalline cellulose itself (emerging from the linear cellulose chain structure tightly bound in microfibrils), and the highly protective lignin surrounding it, and acting as a physical barrier for microorganisms (i.e. enzymatic attack) (Carpita and Gibeaut, 1993). The main polysaccharides that the walls of plant are composed of, are show in Table 2

Table 2. Main polysaccharides of the wall cell plants

Polysaccharides	**Main Chain Residue**	**Branch Residue**	**Enzymes**
Mannans	Mannose	Sometimes galactose	Endo-β-mannanase
Glucomannans	Mannose Glucose	Galactose	Endo-β-mannanase Endo-β-glucanase α-galactosidase
Galactomannas	Mannose	Galactose	Endo-β-mannanase Exo-β- glucanase α-galactosidase
Xyloglucan	Glucose	Xylose Galactose Fucose Arabinose	Xyloglucan-endo-β-transglucosidase Xyloglucan-endo-β-glucanase β-galactosidase α-xylosidase β-glucosidase α-fucosidase
Galactans	Galactose	Arabinose	Exo-galactanase α-arabinosidase
Arabinoxylans	Xylose	Arabinose Glucuronic Acid	β-xylosidase endo-β-xylanase α-arabinosidase α-glucuronodase
β-Glucan	Glucose	None	Lichenase β-(1,3)-(1,4)-glucosidase
Callose	Glucose	None	β-(1,3)-glucanase
Cellulose	Glucose	None	Cellulase endo-β-(1,4)-glucanase

1.2 Lignocellulose Chemistry

1.2.1 Cellulose

Polysaccharides are synthesized by plants, animals and humans to be stored for food, structural support, or metabolized for energy. Anselme Payen (1795-1871), a pioneer in wood chemistry, first isolated a fibrous substance commonly found in wood, cotton, and other plants: the cellulose, as he coined and introduced it into the scientific literature in 1839, (Payen 1938; Guo et al, 2008). Cellulose constitutes the most abundant and renewable organic polymer resource available on Earth (Zhu et al, 2006; Habibi, 2010).

It is an insoluble crystalline substrate, flavorless, odorless, hydrophilic, insoluble in water and in most organic solvents, chiral and with a wide chemical variability. It is a structural component of the cell wall of green plants accounting for almost 35–50% of the the total biomass. It is also biosynthesized in other living systems such as Bacteria and Algae.

Cellulose produced by plants usually exists within a matrix of other polymers primarily hemicellulose, lignin, pectin and other substances, forming the so-called lignocellulosic biomass, while microbial cellulose is quite pure, has a higher water content and consists of long chains. It is a carbohydrate polymer with formula $(C_6H_{10}O_5)_n$, where n is the degrees of polymerization (DP) of the polymer and gives an indication of the number of anhydroglucose units joined by glycosidic bonds in the cellulose chain; this number can vary dramatically depending on the source. Cellulose consists of a linear chain of several hundred to over ten thousand 1,4-β-D-glucose units (Timell, 1967) linked through acetal functions between the equatorial -OH group of C4 and the C1 carbon atom (Jagtap and Rao, 2005; Morana et al, 2011). The high stability of this conformation leads to a reduced flexibility of the polymer, so this is usually described as a real tape. The structure of cellulose is showed in Figure 3.

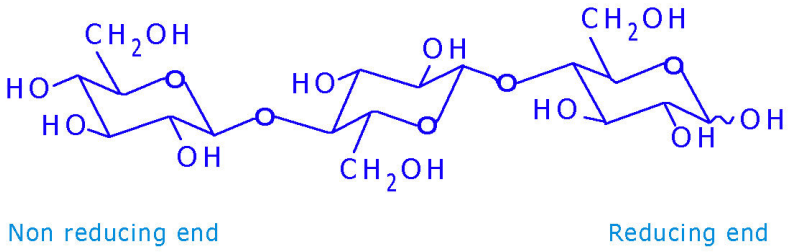

Figure 3: Structure of cellulose

The intramolecular bonds hold glucose residues together to form a long cellulose chain, while intermolecular bonds (van der Waals forces and hydrogen bonds) form between roughly 40 cellulose chains to create a microfibril, which is a packed network of cellulose with a sheet-like formation giving a stiff and rigid structure (Bidlack et al, 1992), containing both ordered (crystalline) and less ordered (amorphous) regions (Ramos, 2003). The organization of cellulose microfibrils, and hence the crystallinity, changes from layer to layer in the primary and secondary cell walls along with the composition of the layers (Sjöström, 1993). Cellulose crystals vary in size depending on their origin, the crystal width (cross-section) being 3–4 nm in wood (Hult et al, 2001; Andersson et al, 2004). The microfibrils prevent cellulases from breaking down this resistant structure and also prevent water molecules from penetrating into the structure.

Larsson et al (1997), investigated molecular ordering of cellulose and reported that most of the amorphous regions correspond to the chains located at the surface, whereas crystalline components occupy the core of the microfibril. A different molecular architecture of crystalline and amorphous cellulose is suggested by Moiser et al (1999) and Tenkanen et al (2003). They describe cellulose as being semi-crystalline, with regions of high crystallinity averaging approximately 200 glucose residues in length separated by amorphous regions.

Cellulose exists in seven crystal structures (polymorphs) designated as celluloses Iα, Iβ, II, IIII, IIIII, IVI, and IVII (O'Sullivan, 1997). Initially, crystalline structure of native cellulose (cellulose I) has been studied by X-ray diffraction and has been defined as monoclinic unit cells with two cellulose chains with a twofold screw axis in a parallel orientation forming slight crystalline microfibrils (Gardner and Blackwell, 2004; Klemm et al, 2005). Afterwards, it was discovered by using 3C-

CP/MAS NMR spectroscopy, that the native cellulose was present in two crystalline forms obtained by modifications of cellulose I: Iα with triclinic unit cells and Iß with monoclinic unit cells. The ratio between Iα and Iß changes in relation to the source of the cellulose (Atalla and Vanderhart, 1984).

Cellulose II can be prepared by regeneration, dissolving cellulose into an aqueous medium and then recrystallization, or exposing native cellulose to the process of mercerization. The conversion of cellulose I into cellulose II is an irreversible process. The cellulose I, result the less stable thermodynamically, while the cellulose II is the most stable structure (Klemm et al, 2005; Fengel and Wegener, 1984). The cellulose I can turn into other forms using different treatments; for example, by mercerization, using aqueous sodium hydroxide or dissolution followed by precipitation and regeneration (formation of fiber and film) (O'Sullivan, 1997; Nishiyama et al, 2002).

However, additional information on the structure of noncrystalline random cellulose chain segments are needed because it is very important for the accessibility and reactivity of the polymer and the characteristics of cellulose fibers (Paakkari et al, 1989).

1.2.2 Hemicellulose

Hemicelluloses is the second most abundant renewable organic material on the Earth, after cellulose. In the conversion of lignocellulose to fuels and chemicals, utilization of hemicellulose is essential to make feasible overall economics of wood processing into chemicals.

In the past hemicelluloses were believed to be intermediates in the biological synthesis of cellulose. Today it is known that hemicelluloses are formed through entirely different biosynthetic pathways and in contrast to cellulose, are heteropolysaccharides (Sjöström, 1993). Hemicelluloses are solid materials, white, that are rarely crystalline or fibrous in nature, but fill out the flesh of the wood fibre.

Hemicellulose forms a physical barrier which surrounds cellulose. In the primary cell wall, hemicellulose interacts with other polymers to maintain the physical properties of the wall. In many types of plants, hemicellulose strengthens the

secondary cell wall, facilitates transport of water to the plant and can also act as a seed storage carbohydrate (Dashtban et al, 2009; Scheller and Ulvskov, 2010).

Table 3. The major hemicellulose components (Eriksson et al, 1990).

Hemicellulose type	Occurance	Ammount (% of wood)	Composition			DP
			Units	Molar ratios	Linkage	
Galactoglucomannan	Softwood	5.0-8.0	β-D-Manp β-DGlcp α-D-Galp Acetyl	3 1 1 1	1,4 1,4 1,6	~200
Galactoglucomannan	Softwood	10.-15.0	β-D-Manp β-DGlcp α-D-Galp Acetyl	4 1 0.4 1	1,4 1,4 1,6	~200
Arabinoglucuronoxylan	Softwood	7.0-10.0	β-D-Xylp 4-O-Me-α-D-GlcpA α-L-Ara	10 2 1.3	1,4 1,2 1,3	~200
Arabinogalactan	Larchwood	5.0-35.0	β-D-Galp α-L-Araf β-D-Arap β-D-GlcpA	6 ~2/3 ~1/3 Little	1,3 1,6 1,3 1,6	350
Glucuronoxylan	Hardwood	15.0-30.0	β-D-Xylp 4-O-Me-α-D-GlcpA Acetyl	10 1 7	1,4 1,2	200
Glucomannan	Hardwood	2.0-5.0	β-D-Manp β-D-Glcp	~1-2 1	1,4 1,4	200

DP: Degree of polymerization; β-D-Manp: β-D-Mannopyranose; β-D-Glcp: β-D-Glucopyranose; α-DGalp: α-D-Galactopyranose; β-D-Xylp: β-D-Xylopyranose; 4-O-Me-α-D-GlcpA: 4-O-Mehyl-α-DGlucuronic acid; α-L-Araf: α-L-Arabinopyranose; β-D-Galp: β-D-Galactopyranose; β-L-Arap: β-LArabinofuranose; β-D-GlcpA: β-D-Glycopyranosyl-uronic acid.

The heteropolysaccharides together with lignin form a matrix surrounding the network of cellulosic fibrils. Both glycosidic and ester linkages are present in hemicelluloses that are branched heterogeneous polymers consisting of many different sugar monomers (Table 3) such as: D-xylose, L-arabinose (pentoses), D-mannose, D-glucose, D-galactose, L-rhamnose (hexoses), 4-O-methyl-D-glucuronic acid, D-glucuronic acid and D-galacturonic acid (uronic acids) (Hon and Shiraishi, 1991). They have substantially different DP (70-200) depending on the wood species (Fengel and Wegener, 1983; Sjöström, 1993; Gong et al, 1981).

Due to their branched structure, hemicelluloses are more soluble than cellulose. The type of hemicellulose varies depending on monomer composition and account for 20 – 30 % of dry weight in wood and 20–40 % and of dry weight in herbaceous crops (Buranov and Mazza 2008; Sjöström, 1993).

Xylans consist of a homopolymer backbone of β-D-xylopyranose units with β-1,4 linkages (Biely, 1993) as shown in Fig. 4. They account for up to 30% of the cell wall material of annual plants, 15-30 % in hardwood and 7-10 % in softwood (Subramaniyan and Prema, 2003). Typically, hardwood from temperate zones contain more xylan and less glucomannan and lignin than hardwoods from tropical climates (Timell, 1964). The structure and composition of xylans differ depending on the biomass source. The biggest differences are seen in the xylans from hardwoods, softwoods and cereals.

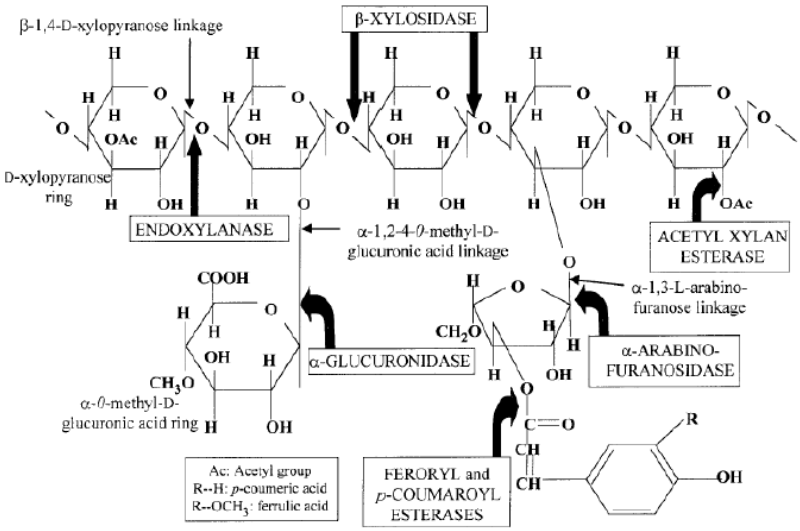

Figure 4: A hypothetical plant xylan structure showing different substituent groups with sites of attack by microbial xylanases (Beng et al, 2001).

In softwoods, galactoglucomannan is the most abundant type of hemicellulose (11–17 wt %) and lower quantities of xylans (6–8 wt %) are also present (Willför et al, 2005a). Xylan-type polysaccharides constitute about 20–30% of the biomass of dicotyl plants (hardwoods and herbaceous plants) and 50% of monocotyl plants (grasses and cereals), while only 10–15% of softwood cell walls (Timell, 1967). In hardwoods, xylans account typically for 15–25 wt % (Willför et al, 2005b) although glucomannans are also present to a lower degree. O-acetyl-4-O-

methylglucuronoxylan is the most representative hemicelluloses in hardwood (15-30% of the dry mass). The backbone consists of 1,4 linked β-D-xylopyranose units with an α-1,2 linked 4-O-methylglucuronic acid on about every tenth xylose unit. The DP of the hardwoods glucuronoxylan is 15 – 200 (Coughlan et al, 1993). The 4-O-methylglucuronic acid link is the strongest link in the polymer structure and is belived to act as a barrier against wood degradation (Subramaniyan and Prema, 2003).

In softwood and agricultural plants, xylans are not acetylated, but contain L-arabinofuranose, attached to about every nine xylose unit (Puls and Schuseil, 1993).

Xylose units can also be acetylated. Unlike xylans in hardwood or annual plants, softwood xylans are not esterified by acetyl groups. In softwood and agricultural plants, β-D-xylopyranose units may be substituted at the positions C-2 and C-3 with α-L-arabinofuranose units. Sometimes the L-arabinofuranose side chains are esterified with ferulic and *p*-coumaric acid in annual plants (Iiyama et al, 1994; Hatfield et al, 1999).

In cereals the main portion of hemicelluloses consist of arabinoxylans (7-8% dry weight) (Montgomery and Smith, 1956). The xylan backbone is made up of 1,4 linked xylose units where 20% of the xylose residues are substituted at C-2 with (4-O-methyl-)α-D-glucopyranuronic acid residues and 10-15 % with arabinose residues, which can be esterified in hardwood or agricultural plants by an *O*-acetyl group at C-2 or more frequently at C-3 (Timell, 1967; Kabel et al, 2002; Willför et al, 2005a; Willför et al, 2005b; Ebringerová and Heinze, 2000). Xyloglucans in the primary cell walls of the plants acts as interface between cellulose and other polysaccharides (Coughlan et al, 1993). It consists of a 1,4 linked backbone of β-glucose residues with side chain of 1,6-α-xylose residues. Arabinogalactan is known to occur to a lesser degree in softwood and hardwood, but in larchwood the quantity amount to 10-20 % of the dry weight. It consist of a β-1,3 linked galactose backbone of which each residues is substituted with a β-1,6 linked galactose side chain. Arabinan is the polymer of α-1,5-linked L-arabinofuranose residues. It can be extracted from beet pulp which contains 20-25% of the

polysaccharide (Coughlan et al, 1993). Single unit side chain of arabinose are sometimes attached to the backbone.

Pectins, composed mainly of acidic sugars (galacturonic acids) are present in wood primary cell walls and middle lamella in low quantities (1.5–3 wt %) (Willför et al, 2005a; Willför et al, 2005b).

1.2.3 Lignin

Lignin is the third most abundant polymer in plants due to which plants are rigid, impermeable, resistant to microbial attack and oxidative stress (Weng & Chapple, 2010). It is an aromatic cell wall polymer accounting typically for 26–32 % and 20–25 % of total mass in softwoods and hardwoods, respectively (Sjöström, 1993). The lignin content varies in different plants depending on the species, cell type and stage of tissue development.

Lignin is an amorphous heteropolymer network composed of *p*-hydroxyphenoyl, guaiacyl and/or syringyl monomers linked in three dimensions of phenyl propane units (Douglas, 1996), having molecular weight of more than 100 KD.

The polymeric structure of lignin is complex because of the great number of possible linkage types between the aromatic units and the random occurrence of the different linkage types. Traditional lignin models describe a cross-linked or branched polymer (Fengel & Wegener, 1984), although a recent study by Crestini et al, (2011) demonstrated that isolated softwood lignin consists mainly of 6–12 phenolic units long linear oligomers.

The exact structure of native lignin remains an open question because lignin characterisation *in situ* lacks powerful analytical techniques, and lignin isolation prior to chemical characterisation always alters the polymeric structure. In general, softwood lignin contains predominantly guaiacyl units with a minor proportion of unmethoxylated *p*-hydroxyphenyl units (Obst and Landucci, 1986); hardwood lignin is richer in syringyl units, however, with guaiacyl units dominating (Fergus and Goring, 1970); and annual gramineous plants contain all three units in fairly equal amounts (Buranov and Mazza, 2008). In addition, lignins in annual plants are also etherified and esterified by phenolic acids, *i.e.* by ferulic and *p*-coumaric acids (Scalbert et al, 1985; Lam et al, 2001; Buranov and

Mazza, 2008). In grass arabinoxylans, phenolic acids have been found to esterify hydroxyl groups in α-L-arabinofuranosyl residues, mostly at the position C-5 (Iiyama et al, 1994; Hatfield et al, 1999; Vogel, 2008). Ferulic acids can form various dimers with free-radical condensation reactions, similar to the polymerisation reactions of polysaccharides with lignin (Ralph et al, 1995).

In the process of enzymatic hydrolysis of lignocellulose (Vinzant et al, 1997; Mooney et al, 1998) lignin is an undesirable component and it is generally necessary to remove it by chemical treatments. In fact covalent linkages between hemicellulose and lignin by ferulic acid ester linkages (Gray et al, 2006) have been reported in wood and other plant materials (Iiyama et al, 1994; Vogel, 2008), consequently influencing the amount of enzyme needed for the hydrolysis, but also hindering the recovery of the enzymes after the hydrolysis (Lu et al, 2002).

1.2.4 Extractive and Ash

This group represent a minor fraction (1-8 %) characterized by wood compounds soluble in water and in neutral organic solvents (Biermann,1996). Terpens are class of compounds that are present in softwood in large quantities.

A small quantity of ash (about 0.5 %) is found in wood. It is composed by metallic ions and its composition is determined by the controlled combustion of the wood (Biermann, 1996).

1.3 Bioconversion of Lignocellulose

In economically feasible industrial processes for conversion of lignocellulosic materials into energy carriers in liquid or gaseous forms, for the production of biofuels, chemicals, heat or electric power, both cellulose and hemicellulose needs to be hydrolyzed to sugars that in proceeding steps can be further converted (Wyman, 2003).

The conversion of lignocellulose into other products involve basically three steps: the pretreatment, that consists into the delignification to liberate the cellulose and hemicelluloses fibres from their complex with the lignin. The second step is the hydrolysis with the depolymerisation of the carbohydrates to obtain free sugar

residuals. The fermentation is the last step, with a microbial conversion of the resulting sugars mixture of hexose and pentose to produce added value products.

1.4 Pretreatment

The main goal of pretreatment is to remove the lignin, that is the hardest part of the lignocellulosic biomasses, decrease the crystallinity and increase of surface area of cellulose, expose the cellulose from hemicellulose, and this process can be done by various methods which are categorized into three main groups (Table 4) as chemical, physical or physic-chemical and biological (Taherzadeh and Karimi, 2008).

The drawback of the pretreatment processes, besides the obvious economical impact, is the generation of inhibitory compounds that can negatively influence the action of enzymes and microorganisms (Chandra et al, 2007). Inhibitors are produced as a consequence of extreme pH and high temperature treatment of lignocellulosic materials (Olsson et al, 2004).

Physical pretreatment methods break down the lignocellulose and reduce the particle size, which increases the surface area for acid or enzymatic attack (Fan et al, 1982). They can be classified into two general categories, mechanical and non-mechanical pretreatments (irradiation, high pressure steaming and pyrolysis). Steam with or without explosion is a popular physical pretreatment. It removes the major part of hemicelluloses, that is predominantly solubilised, from the material and makes cellulose more susceptible to chemical or enzymatic digestion. The biomass is treated with high pressure steam. Steam explosion is initiated at a temperature of 160-206 °C from several seconds to few minutes before the material is exposed to atmospheric pressure (Galbe and Zacchi, 2002; Ruiz et al, 2006). Steam explosion is a basic pretreatment method because it has a relatively moderate energy cost on commercial scale. It has been shown that the effect of steam pretreatment, which renders a material suitable for enzymatic hydrolysis, is more likely due to acid hydrolysis of the hemicellulose than the "explosive" action when the pressure is released (Galbe and Zacchi, 2007). Drawbacks of steam pretreatment are partial degradation of hemicellulose,

production of enzymes and fermentation inhibitors, and incomplete separation of lignin and cellulose (Hendriks and Zeeman, 2009, Sun and Cheng, 2002).

Chemical pretreatment methods alter the crystal structure of glucose and hemicellulose. Common chemical pretreatments use sodium hydroxide, ammonia, lime, ammonia fibre explosion, concentrated acid and dilute acid.

Alkaline pretreatment refers to the material soaking in an alkaline solution, such as NaOH, and then heating it for a certain time. The swelling increases the internal surface area and decreases the degree of polymerisation and crystallinity. Usually the major fraction of lignin is solubilised together with part of the hemicellulose that is recovered mainly as oligomer. Alkaline pretreatment proved to be more effective on agricultural residues and herbaceous crops than on woody materials (Galbe and Zacchi, 2007).

Dilute acid pretreatment is performed by soaking the material in dilute acid solution, usually below 4%, and then heating to temperatures between 120 and 200°C. The residence time varies from several minutes up to few hours. The most widely investigated approaches are based on dilute sulphuric acid (Lloyd and Wyman, 2005, Torget et al, 1992), since sulphuric acid is inexpensive and effective. The hemicellulose is hydrolysed and the main part is usually obtained as monomer sugars. It has been shown that materials that have been subjected to dilute acid pretreatment may be harder to ferment because of the inhibitors formed during the treatment (Larsson et al, 1999).

The main advantages of these methods are high effectiveness (high glucose yield) and minimal formation of inhibitors (in particular with the concentrated acid treatment). On the other side, the need of specialized corrosion resistant equipment, and necessity of subsequent extensive washing, together with the disposal of chemical waste should be stated as the main disadvantages of these processes.

The use of ionic liquids (e.g. 3-methyl-N-butylpyridinium chloride, 1-*n*-butyl-3-methylimidazolium chloride and 1-allyl-3-methylimidazolium chloride) for pretreatment has recently received attention as green solvents. Ionic liquids are salts with very low volatility and high thermal stability (up to 300°C), and can dissolve carbohydrates and lignin simultaneously forming hydrogen bonds

between the non-hydrated chloride ions and the hydrogen atoms of the hydroxyl groups, while minimizing formation of degradation products (Dadi et al, 2006). However, it is still uncertain whether ionic liquids can be a feasible option in full-scale pretreatment of lignocelluloses (Yang and Wyman, 2008).

Table 4. Methods used for the pretreatment of lignocellulosic materials

Physical[a]	Chemical[a]	Biological[b]
Ball-miling	Alkali: Sodium hydroxide	Fungi: Brown rots (attack cellulose)
Two roll milling	Ammonia	*Piptropus betulinus*
Hammer milling	Ammonium sulfite	*Laetiporus sulphureus*
Colloid milling	Acid: Sulfuric acid	*Trametes quercina*
Vibro energy milling	Hydrochloric acid	*Fomitopsis pinicola*
High pressure steaming	Phosphoric acid	*Gloephilium saepiarum*
Extrusion	Gas: Chlorine dioxide	White rots (attack both lignin and cellulose)
Expansion	Nitrogen dioxide	*Formes fomentarius*
Pyrolysis	Sulfur dioxide	*Phelliunus igniarus*
High energy radiation	Oxidizing agents: Hydrogen peroxide Ozone	*Ganoderma appalanatum* *Amilaria mellea*
	Cellulose solvents:	*Pleurotus ostreatus*
	Cadoxen (ethylene diamine and water) CMCS	Red rot (attack both lignin and cellulose) *Formitopsis annos*
	Solvent extraction of lignin: Ethanol-water extraction	Bacteria
	Benzene-ethanol extraction Ethylene- glycol extraction Butanol-water extraction	
	Swellin agents	

[a]From Fan et al., 1982; [b] From Schurz, 1978; CMCS: is composed of sodium tartarate, ferric chloride, sodium sulfite, and sodium hydroxide solution.

Biological pretreatment utilizes wood attacking microorganisms that can degrade lignin. White rot fungi have been identified as the most promising group for the lignocellulose pretreatment (Ander and Eriksson, 1979). and are considered

environmentally friendly methods. Generally, soft and brown rot fungi primarily degrade the hemicellulose, while white-rot fungi can attack the lignin fraction (Ander and Eriksson, 1977). Major disadvantages of biological pretreatments are the long residence time (10–14 days), thus large space requirement, and the need for careful control of growth conditions (Wyman, 1996).

Cellulose, hemicellulose and lignin each have different reactivities towards these pretreatment technologies, making the process very difficult to generalize. The type of pretreatment method chosen ultimately depends on the type of lignocellulose (Taherzdeh and Karimi, 2008; Mosier et al, 2005; Kumar et al, 2009).

1.5 Enzymes for (hemi)cellulose degradation

After pretreatment, the biomass should be much more susceptible to enzymatic attack. Enzymes are added to the biomass, acting as catalysts to break down the glycosidic bonds of the polysaccharides. The mechanism involved in the enzymatic degradation of lignocellulose depends on the chemical nature and physical structure of the substrate (Taherzdeh and Karimi, 2008). Several enzymes are needed to work together synergistically to break down the various polysaccharides. On a commercial scale, the cost of enzymes needs to be lowered since it is estimated to contribute to 10-20% of the total cost of bioethanol production (Barta et al, 2010). This would require efficient enzymes in order to minimize the biocatalyst amount needed and keep the cost at a minimum.

1.5.1 Cellulases

Cellulases belong to a class of enzymes that catalyze the hydrolysis of cellulose and are chiefly produced by Fungi, Bacteria, and Protozoa, as well as other organisms like plants and animals. The cellulolytic enzymes are inducible since they are synthesized by microorganisms during their growth on cellulosic materials (Lee and Koo, 2001).

A variety of different kinds of cellulose-degrading enzymes are known, with different structure and mechanism of action. The hydrolytic enzymes were first classified based on enzymatic activities (EC classification). The classification of

glycoside hydrolases (GHs) (http://www.cazy.org/) into families is based on their amino acid sequence and their three-dimensional structure organization (Henrissat, 1991; Henrissat and Davies, 1997; Cantarel et al, 2009).

Enzymes classified into a family have been known to exhibit similar catalytic properties, because enzyme fold and morphology of active site are closely related to determine the catalytic behavior of the protein. Enzymes that showed similar physic-chemical properties are grouped into the same families.

In general, the "cellulolytic enzyme complex" breaks down cellulose to β-glucose and involves the following types of enzymes: endoglucanases, exoglucanases, cellobiohydrolases, and β-glucosidases (Li et al, 2010).

Endoglucanases (EC 3.2.1.4) hydrolyze randomly internal glycosidic linkages in soluble and amorphous regions of the cellulose and produce new ends by cutting into long cellulose strands. This action results in a rapid decrease of the polymer length and in a gradual increase of reducing sugars concentration. Exoglucanases (EC 3.2.1.74) hydrolyze cellulose chain and oligosaccharides with high DP removing successive β-glucose units. Cellobiohydrolases (EC 3.2.1.91) hydrolyze cellulose chains by removing processively 2 units (cellobiose) either from the non-reducing and reducing ends. This action results in rapid release of reducing sugars but little changes in polymer length occurr. Cellobiohydrolases with specificity for the reducing and the non-reducing end have to work together. β-Glucosidases (EC 3.2.1.21) convert the resulting oligosaccharide products to glucose (Bhat, 2000).

The cellulolytic enzymes are inhibited by their products, cellobiose and glucose, disrupting the cellulase system. Cellobiose is an inhibitor of endoglucanases and cellobiohydrolases (Zhao et al, 2004), therefore, cellobiose accumulation will impede the release of glucose during enzymatic hydrolysis. In order to overcome this inhibition, cellobiose needs to be broken down by β-glucosidase.

Only a high level of synergism between such enzymes can guarantee the complete degradation of cellulosic materials (Wood and McCrae, 1979; Henrissat et al, 1985).

Cellulolytic systems seem to differ between aerobic and anaerobic microorganisms (Lynd et al, 2002). In aerobes such as saprophytic fungi, various

cellulases and other plant cell wall degrading enzymes are mostly secreted extracellularly and they exhibit extensive biochemical synergy. In contrast, the hydrolytic enzymes of most anaerobic microorganisms such as cellulose-degrading rumen bacteria are associated to an integrating subunit (scaffoldin) which contains a carbohydrate binding module (CBM), forming a supramolecular complex called cellulosome (Gilbert, 2007) to facilitate sufficient contact and orientation between the catalytic domain (CD) and the substrate. All known cellulosomes contain a large polypeptide (cohesin) which serves as an anchoring protein for the catalytic domains of cellulases and hemicellulases. The cellulosome of the anaerobic bacterium *Clostridium thermocellum* is the most studied complexed cellulase system (Bayer et al, 2004).

The cellulosome enzymatic components contain not only cellulases but also a large array of hydrolytic activities such as hemicellulases (Kosugi et al, 2002), pectinases (Tamaru and Doi, 2001), chitinases, lichenases, mannanases and esterases. This extraordinary enzymes diversity reflect the chemical and structural complexity of the substrate, the plant cell wall, that can be efficiently attacked and degraded only by the concerted action of different enzymatic activities (Fontes and Gilbert, 2010).

Due to its excellent ability to produce and secrete a complete set of cellulose degrading enzymes in such amounts, that makes their production viable at industrial scale, the soft rot fungus *Trichoderma reesei* has been in the focus of cellulase research for decades (Persson et al, 1991). The cellulolytic enzyme system of *T. reesei* has served as a model for basic cellulose degradation studies, and strains have been tailored to produce specific mixtures of these enzymes for various industrial applications. In addition, *T. reesei* strains serve as production hosts for efficient heterologuous expression of proteins of other organisms. Surprisingly, the genome of the well-studied fungus *T. reesei* carries about only 200 glycoside hydrolase (GH) genes encoding plant cell wall polysaccharide degrading enzymes (Martinez et al, 2008). Despite its high hydrolytic efficiency, the genome of *T. reesei* encodes only 10 cellulolytic and 16 hemicellulolytic enzymes, which is actually less than in many other sequenced fungi. The

cellulolytic enzyme system of *T. reesei* strains have been tailored to produce specific mixtures of these enzymes for various industrial applications.

1.5.2 Hemicellulases

Hemicellulases are a broad group of enzymes that degrade the complex structures of different hemicellulosic polymers composed of sugars, sugar acids and esterified acids. Because of the complex structures composed of versatile building blocks, a vast array of enzymes is needed for the complete depolymerisation of hemicelluloses (Table 5) (Beg et al, 2001; Saha, 2003).

Table 5. Enzymes associated with hemicelluloses degradation and their mode of action

Enzyme	Mode of action	Catalytic classification
Exo-xylanase	Hydrolyses β-1,4-xylose linkages releasing xylobiose	GH
Endo-xylanase	Hydrolyses mainly interior β-1,4-xylose linkages of the xylan backbone	GH
β-xylosidase	Releases xylose from xylobiose and short chain xyloologosaccharides	GH
α-Arabinofuranosidase	Hydrolyses terminal non reducing α-arabinofuranose from arabinoxylan	GH
α-Glucuronidase	Releases glucuronic acid from glucuronoxylan	GH
Acetyl xylan esterase	Hydrolyses acetylester bonds in acetyl xylans	CE
Ferulic acid esterase	Hydrolyses feruloyester bonds in xylan	GH
p-Coumaric acid esterase	Hydrolyses *p*-Coumaryl ester bonds in xylans	GH
Endo-β-1,4-mannanase	Hydrolyse mannan-based hemicelluloses, liberate β-1,4-mannooligomers	GH
Endo- β-1,4--mannosidase	Hydrolyse β-1,4-mannooligomers to mannose	GH
Endo-galactanase	Hydrolyses β-1,4- galactan	GH
Acetyl mannan esterase	2- or 3-O-acetyl xylan	CE

GH: glycoside hydrolysis
CE: carbohydrate esterases

Hemicellulases may be classified into depolymerising enzymes that act on the hemicellulose backbone and into debranching enzymes (also referred to as accessory enzymes) that act on the polymer branches. Backbone-depolymerising hemicellulases are hydrolases with different specificities for the various hemicellulose structures. They include xylanases, mannanases, β-glucanases and

xyloglucanases (Decker et al, 2008). Analogously to cellulases, the depolymerising hemicellulases may also be considered as endo- or exo-acting depending on the site of attack in the hemicelluloses backbone, although many enzymes have been shown to act in both modes. Certain hemicellulases degrade only the small oligomeric fragments arising from degradation of the polymeric molecules (Decker et al, 2008). In general, depolymerising hemicellulases may be highly site- and conformation-specific or they may possess a broader spectrum potential of substrates.

A description of these enzymes is given below.

Endo and Exo-1,4-β xylanase

Endo-1,4-β xylanase (1,4-β-D-xylanxylnanohydrolase, EC 3.2.1.8) cleaves the glycosidic bond in the xylan structure, reducing the degree of polymerization of the substrate and the required chain length depends on the family of endo-xylanase, but is usually more than eight. Since branching and side chains cause steric hindrance to the action of the enzyme (Coughlan et al, 1993) the endo-xylanase enzymes require a long chain of consecutively unsubstituted xylopyranose units. Most xylanases with endo-1,4-β xylanase activity (EC 3.2.1.8), capable of hydrolysing xylan backbone, fall into the GH families 10 and 11 in the CAZy database (Kolenová et al, 2006), although interesting enzymes with distinctive substrate specificities are also found from other GH families, such as GH30 (St John et al, 2010), GH8 (Pollet et al, 2010), GH5 and GH43. Family 10 contains high molecular mass molecules with low isoelectric point values, while low molecular mass molecules with high pI values belong to family 11. Moreover there are differences in catalytic activities. The enzymes of family 10 show less substrate specifity and the endo- xylanase are capable of attacking the glucosidic linkage next to branch points and towards the non-reducing end, whereas the endo- xylanase of family 11 are not (Subramaniyan and Prema, 2003). Also, the endo-xylanase of family 10 require two unsubstituted xylopyranosyl residues between branches, whereas the same enzymes belonging to the family 11 require three consecutively unsubstituted xylopyranosyl residues.

Two mechanism of hydrolysis are observed in the action of xylanases. The one is a single displacement mechanism, where the configuration about the anomeric

center is inversed, and the other is a double displacement mechanism, where the configuration is retained. Enzymes belonging to the families 10 and 11 exhibit the double displacement mechanism (Biely et al, 1994). Exoxylohydrolase has been shown to preferentially remove xylose from the non reducing ends of xylans with inversion of configuration (single displacement mechanism) (Coughlan et al, 1993).

β-xylosidase

1,4-β-xylosidase (1,4-β-D-xylan xylohydrolase EC 3.2.1.37) hydrolyse short xylo-oligomers chains into the monosugar, xylose. The action of the enzyme is preferential to removal the monomers from the non-reducing end of the xylooligomer with affinity increasing with decreasing DP (Coughlan et al, 1993). The best substrate for β-xylosidase is xylobiose and their affinity for xylo-oligosaccharides is inversely proportional to its degree of polymerization. The main role of this enzyme is to act after the xylan has suffered several hydrolysis by xylanase. Due to this reaction the accumulation of short oligomers can inhibits the endoxylanase, and the β-xylosidase hydrolyzes these products, increasing xylan hydrolysis. An important consideration in the use of the β-xylosidase is also its susceptibility by xylose end-product (Poutanen et al, 1991).

β-xylosidase are found in GH families 3, 39, 43, 52 and 54 based on their amino acid sequence similarities (Shallom and Shoham, 2003). In general, all the families have common reaction mechanism (except for GH43) with double displacement (retaining) mechanism. The substrate (glycone) forms a covalent glycosyl-enzyme intermediate, which is attacked by water molecule in hydrolysis and by a sugar residue leading to the formation of a new glycosidic linkage in transglycosylation (Knob et al, 2010). The family GH52 β-xylosidases use p-nitrophenyl β-D-xylopyranoside (pNPX) or β-xylo-oligosaccharides (XOs) of the degree of polymerization 2 and 3 in transxylosylations, resulting in the formation of pNP-β-xylo-oligosaccharides or longer β-xylo-oligosaccharides with mainly β-1,4 linkages (Henrissat and Davies, 1997). There is no report on GH52 β-xylosidase transxylosylation with acceptors other than xylose or β-xylo-oligosaccharides (Bravman et al, 2003). Although β-xylosidases from other families have broad acceptor specificity, the transglycosylation capacity of GH52

was previously tested only with a few carbohydrates as acceptors (Bravman et al, 2001; Bravman et al, 2003).

α -arabinofuranosidases

α-L-arabinofuranosidase (EC 3.2.1.55) are reported to liberate arabinose from arabinans, arabinoxylans and arabinogalactans. When the arabinose is a substituent on xylan, the enzymes shows preference to the arabinose linked oligosaccharides, rather than the xylan itself (Coughlan et al, 1993; Subramaniyan and Prema, 2003).

α-glucuronidases

α-glucuronidases (EC 3.2.1.139) hydrolyzes the α-1,2 bonds between glucuronic acid residues and β-D-xylopyranosyl backbone units found in glucuronoxylan. Acetyl groups close to the glucoronosyl substituents can partially hinder the α-glucuronidase activity (Polizeli et al, 2005).

Esterases

Esterases remove substituent bound by ester linkage. The O-acetyl group found in hardwood and ceral xylan is liberated by acetyl xylan esterases. The esterases have been found to act on both the xylan polymer and xyloligomers. For example ferulolyl esterase remove ferulic acid bound to the arabinoxylans of certain cereal xylans

Mannanases

The hydrolysis of mannan containing hemicelluloses also require a wide array of enzymes (Stålbrand, 2003; Moreira and Filho, 2008). Endo-1,4-β-mannanase (EC.3.2.1.78) cleaves the (galacto)glucomannan main chain to oligosaccharides leaving a mannose residue on the reducing end (Tenkanen et al, 1997).

The formed oligosaccharides are further hydrolysed by β-mannosidase (EC 3.2.1.25) and β-glucosidase (EC 3.2.1.21), liberating mannose and glucose residues at the non-reducing-end respectively. Analogously to endo-1,4-β-xylanases, the action of endo-1,4-β-mannanases is restricted by the substitution groups on the backbone, and the galactosyl side groups can be cleaved off by α-galactosidase (EC 3.2.1.22) (McCleary and Matheson, 1983; Tenkanen et al, 1997). Acetylmannan esterase (EC 3.1.1.6) catalyses the deacetylation of the glucomannan backbone in softwood (Tenkanen et al, 1995).

Synergistic action of multiple hemicellulolytic enzymes is required for complete degradation of hemicellulose. The side chain cleaving enzymes improve accessibility to the backbone, infact it has been proven that the endo-type enzymes shows shows low or no activity toward substituted xylose units. The reason is suspected to be the complex nature of a lot of substrates and the interference of the side chains (Coughlan, 1992).

Their action is dependent on the complete removal of the side chains from the backbone carried out by accessory enzyme. Moreover several accessory enzymes show preference to remove substituents on the oligomers and not the polymer (Saha, 2003). The highest yield have been observed when different enzymes are used simultaneously (Poutanen et al, 1991) and xylan degrading microorganism have been found to produce a whole range of xylan hydrolyses.

The synthesis of xylanolitic enzymes in microorganisms are controlled by mechanisms of induction or repression and the constitutive expressions are rare (Srivastava et al, 1993). The production of the enzyme takes place in the presence of metabolizable carbon sources such as xylan and rarely also cellulose (Kulkarni et al, 1999), whereas it is inhibited by glucose and xylose. In *Streptomyces*, for example, the endoxylanase activity seems to increase with increasing of the crystallinity of cellulose substrate (Morosoli et al, 1986), while cellulosic and hemicellulosic substrates are inducers of xylanase and β-xylosidase in *Cellulomonas flavigena* (Perez-Avalos et al, 1996). It is still unclear, however, if the inducing effect of cellulose may be related to the presence of xylan contaminant. The xylan, being a polymer of high molecular weight, can not cross the cell membrane, and then the signal for the synthesis is represented by fragments of lower molecular weight, such as xylobiose, xylotriose and other xyloligosaccharides. These are formed through the hydrolysis of the xylan by the small amount of constitutively enzyme produced.

Typically the induction of xylanase is a complex phenomenon (Fig.5) and the degradation products of xylan, which often act as inducers of production of xylanase, at higher concentrations act as repressors. Furthermore, the levels of response to an inducer vary with the species, for example an inductor that stimulates the xylanase activity in a species can inhibit in another.

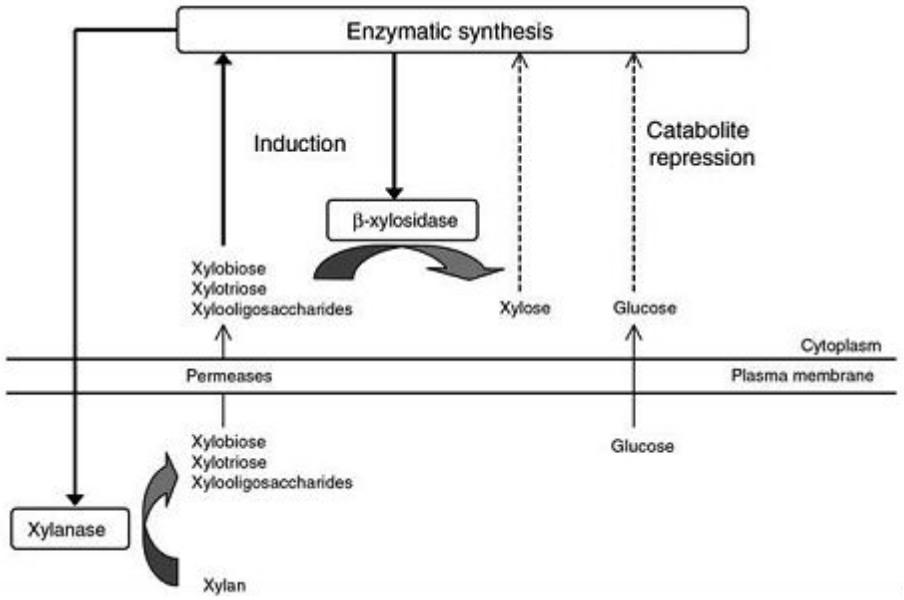

Figure 5: Regolation of the enzymatic syntesis

1.6 (Hemi)cellulolytic activities from microorganism

The search of potential microbial strains of (hemi)cellulolytic enzymes is continuing in the interest of cellulose biotechnology. There are many microorganisms (Bacteria, Archea and Fungi) that play a significant role in the carbon cycle on Earth, since they have a key function in the conversion of plant biomass into useful products. The ability to digest cellulose and hemicellulose is widely distributed among Bacteria and Fungi.

The different strategy of degradation between the anaerobic and aerobic groups resides in the production of complex systems, exemplified by the well-characterized cellulosome from the *Clostridium* genus (Beguin and Lemaire, 1996; Schwarz, 2001), or the extracellular cellulases freely released in the culture supernatant, respectively (Wachinger et al, 1989).

In the last years, a great deal of attention has been focused on enzymes capable of degrading biomass for a number of applications and on their potential to be produced industrially. However, the cost of producing sugars from lignocellulosic waste for fermentation into bioethanol is still high to attract industrial attention, mainly due to low enzyme yields from microorganisms. Cellulases produced by Fungi such as the *Aspergillus* and *Penicillium* species have been widely studied

by numerous researchers, in addition to cellulases from *T. reesei* (van Peij et al, 1998; Jun et al, 1992).

Fungal cellulases are well-studied enzymes used in various industrial processes (Bhat, 2000). A variety of aerobic and anaerobic Fungi are producers of cellulose-degrading enzymes. The aerobic Fungi play a major role in the degradation of plant materials and are found on the decomposing wood and plants, in the soil, and on the agricultural residues. The cellulase systems of a lot of aerobic Fungi (*Trichoderma reesei*, *Penicillium pinophilum*, *Fusarium solani* and *Rhizopus oryzae*) are well characterized (Bhat and Bhat, 1997). Much of the knowledge on enzymatic depolymerization of cellulosic material has come from *Trichoderma* cellulase system. In particular, the cellulase system of *T. reesei* has been the focus of research for 60 years (Reese et al, 1959; Reese and Mandels, 1971). *T. reseei* produces readily, and in large quantities, a complete set of extracellular cellulases, wich also includes endoglucanases, and consequently it has a high commercial value (Miettinen-Oinonen and Suominen, 2002).

The optimal temperature and pH at which the enzymes produced from *T. reseei* work are 30°C and 5.0, respectively (Biely et al, 1991). In general enzymes purified from Fungi are acidophilic and work at mesophilic temperatures (20-40°C).

The Fungus *Acremonium cellulolyticus*, isolated in 1987, is known to be a potent producer of cellulases as *T. reesei*, even if many cellulases and β-glucosidases have not been as well characterized as those produced by *T. reesei* (Yamanobe et al, 1987; Ikeda et al, 2007). Since enzymatic saccharification using cellulases has proven to be a powerful method in the production of bioethanol, a comparison between cellulase activity from the two fungi against three lignocellulosic materials (eucalyptus, Douglas fir wood chip and rice straw) has been performed by Fujii et al (2009). Saccharification efficiency of both culture supernatants and commercial preparations (AC derived from *A. cellulolyticus* and Accellerase 1000 derived from *T. reesei*) was investigated. The culture supernatant from *A. cellulolyticus* produced higher glucose yield from lignocellulosic materials than the *T. reesei* supernatant. In the same way, AC produced a greater amount of glucose from lignocellulosic materials than Accellerase 1000.

Identification, purification and characterization of (hemi)cellulases are continuously increasing and always in progress, with incessant research and isolation of new microorganisms able to produce novel (hemi)cellulolytic activities. Some of the bacterial strain producing (hemi)cellulases are *Cellulomonas* species, *Clostridium stercorarium*, *Bacillus* species, *Pyrococcus furiosus*, *Saccharofagus dregradans* (Hutnan et al, 2000; Taylor et al, 200; Das et al, 2007).

Bacterial cellulases exist as discrete multi-enzyme complexes, the cellulosome, that consist of multiple subunits that, interacting each other synergistically, efficiently degrade cellulosic substrate (Bayer et al, 2004). The cellulosome is believed to allow concerted enzyme activity in close proximity to the bacterial cells, minimizing the distance over which cellulose hydrolysis products must diffuse, allowing efficient uptake of these oligosaccharides by the hoist cell (Schwarz, 2001).

Among the anaerobic cellulase-producing bacteria, the *Clostridium* genus is without doubt the most studied. It numbers mesophilic and thermophilic representatives and multi-enzyme complexes having high activity against crystalline cellulose, have been identified and characterized in many of these Bacteria.

Many microorganisms belonging to *Bacillus* sp. are producers of cellulases and hemicellulase, in particular alkaline enzymes, even if other microorganisms also possess cellulases active at high pH value. Since the discovery of an alkaline cellulase by Horikoshi et al (1984), many other alkaline cellulases from alkaliphilic *Bacillus* strains have been identified. Basing on the characteristics of this novel strain of *Bacillus*, Lee et al (2008) aimed to develop an economical process for production of cellulases by using cellulosic waste as inexpensive and widely distributed carbon source.

As already mentioned, microorganisms different from *Bacillus* are also capable of producing alkaline cellulases. *Marinobacter* sp. (MSI032), isolated from the marine sponge *Dendrilla nigra*, produces an extracellular alkaline cellulase at 27°C and pH 9.0 (Shanmughapriya et al, 2009).

Most of the information regarding the biochemical characteristics of hemicellulases refer to those isolated from either bacteria that by fungi. The microbial xylanase are monomeric proteins with molecular weights ranging between 8 and 145 kDa (Sunna and Antranikian, 1997). Generally, the bacteria are capable of producing two types of xylanase, respectively higher molecular weight (> 30kDa) or lower (<30kDa); in fungi, however, most frequently low molecular weight xylanase are found.

The fungal xylanase show an optimum pH around 5 and their activities normally remains stable for pH values comprised between 2 and 9; the microbial ones usually are stable in a wide range of pH values, ranging from 3 to 10, showing best activity at the higher pH between 4 and 7; in particular the alcalophililic bacilli and actinomycetes produce xylanase active at alkaline pH-values.

Most of the fungi and bacteria produces xylanase with optimal temperatures for the activity between 40 and 60°C, however, the fungal xylanase appear to be less thermostable than the bacterial.

A common post-translational modification of these carbohydrates active proteins is the glycosilation. The sugars can be covalently bound to the protein or be present as complexes removable by the enzyme (Kulkarni et al, 1999; Wong et al, 1988) and the percentage of residues varies between different species. For example endoxylanases Xl, X-II A and II-B X from *Streptomyces* sp. contain less than 1% sugar (Marui et al, 1985), while the three xylanase of *Talaromyces byssochlamydoides* YH-50 containing 36.5, 31.5 and 14.2% of the sugar residues consisting of polymers of glucose, mannose and fructose (Yoshioka et al, 1981)

In the last years thermophilic bacteria have received considerable attention as sources of highly active and thermostable cellulolytic and xylanolytic enzymes. In fact one of major drawbacks in industrial terms is that most cellulases and hemicellulases apparently lack thermostability. Currently, considerable interest is focused on the use of hemicellulases in the processing of paper pulp. Use of xylanases in bleaching step has been shown to reduce the amount of chlorine required to achieve comparable levels of paper brightness. However, the mesophilic enzymes currently in use have limitations due to high temperature

used in industrial processes (Gibbs et al, 1995; Morris et al, 1995; Morris et al, 1998; Sunna et al, 2000, Viikari et al, 1994).

Depending on their optimal growth temperature, thermophilic microorganisms are grouped in thermophiles (45–80°C) and hyperthermophiles (80–122°C). The latter are dominated by the Archaea microorganism even if some Bacteria, such as Thermotoga and Aquifex, tolerate temperatures around 100 °C. Degradation of cellulosic and hemicellulosic substrates among thermophiles is mostly due to Bacteria species with few Archaea species (Cobucci-Ponzano et al, 2013).

Thermophiles and hyperthermophiles are therefore considered to be organisms whose optimal growth temperatures are above the normal range of the environment. These kinds of environments are rare in nature and occur only under special conditions, such as in compost piles or in geothermal areas that are the main "permanently hot" places on this planet. Such areas, for example, hot soil and hot spring are the main natural habitats where thermophiles and hyperthermophiles have been isolated. Moreover, they can also be found in artificial environments such as the boiling outflows of geothermal power plants (Kristjansson and Hreggvidsson, 1995).

There are thermostable cellulolytic enzymes characterized from thermophilic cellulolytic bacteria such as *Clostridium thermocellum, Caldocellum saccharolyticum* and *Acidothermus cellulolyticus* (Gibbs et al, 1995; Te'o et al, 1995; Bergquist et al, 1999). The most stable of these, from *Acidothermus*, has a half-life of less than 20 min at 85°C (Sakon, et al, 1996). Recently, hyperthermal cellulases and hemicellulases have been isolated and some of them have been purified and characterized. Most of the enzymes isolated from hyperthermophilic genus *Thermotoga* have temperature optima at 90 °C or higher. The unusual properties of these enzymes have resulted in the development of the novel biotechnology processes that can be accomplished within a wide range of conditions, including the reactions that are beyond the capability of biologically based systems (Bronnenmeier et al, 1995; Leuschner and Antranikian, 1995; Ruttersmith and Daniel, 1991; Saul et al, 1995; Winterhalter and Liebl, 1995).

The order Thermoanaerobacteriales includes several species that utilize (hemi)cellulose as growth substrates. The most thermophilic species belong to the

genus *Caldicellulosiruptor*, but, while all species hydrolyze hemicellulose, not all degrade crystalline cellulose. *C. kristjanssonii* (78°C) and *C. bescii* (80°C), which is the most thermophilic cellulolytic bacterium characterized to date, can grow on crystalline cellulose and unprocessed plant biomasses (Bredholt et al, 1999; Yang et al, 2009; 2010). Recently, a novel cellulolytic bacterium has been isolated from Obsidian Pool in Yellowstone National Park, *C. obsidiansis* sp. nov. (78°C), which exhibits fermentative growth on arabinogalactan, xylan, Avicel, filter paper, dilute acid-pretreated switchgrass, and poplar, whereas is unable to grow on lignin (Hamilton-Brehm et al, 2010).

The number of characterized thermophilic microorganisms that utilize xylan exceeds the number of cellulose-degrading ones. Xylan-utilizing microorganisms (Tab. 6) are widely distributed within the order Thermoanaerobacteriales. *T. zeae* (68°C), isolated from industrial environments, utilizes cracked corn and xylan, but not cellulose, and produces ethanol as the main product after sugars fermentation (Cann et al, 2001). Xylan-degrading bacteria have also been found in the genera Thermotoga. *T. hypogea* sp. nov. (70°C) produces trace amounts of ethanol during xylan fermentation (Fardeau et al, 1997).

In addition to traditional bioethanol production processes, consolidated bioprocessing (CBP), which combines in one step saccharification with fermentation using a whole cells-based approach, represents an alternative method with outstanding potential for low-cost processing of lignocellulosic biomass (Lynd et al, 2005). No ideal CBP microbe able to degrade efficiently lignocellulose and, at the same time, to utilize the released sugars to produce ethanol is currently available. A newly discovered thermophilic microorganism, *Geobacillus* sp. R7 (60 °C), is a facultative anaerobic bacterium isolated from soil samples (Homestake gold mine, South Dakota). It produces a thermostable cellulase when grown on extrusion-pretreated agricultural residues such corn stover and prairie cord grass, and ferments lignocellulosic substrates to ethanol in a single step (Zambare et al, 2011).

In comparison with the anaerobic thermophilic bacteria few aerobic have been described to produce cellulases and xylanases. The aerobic thermophiles *R. marinus* produces a highly thermostable cellulase (Cel12A) and three glycoside

hydrolases belonging to family GH10 of the CAZy database (Alfredsson et al, 1988; Cantarel et al, 2009). *Aquifex aeolicus*, isolated in the Aeolic Islands in Sicily (Italy), represents one of the most thermophilic bacteria since its growth temperature can reach 95°C (Deckert et al, 1998).

Table 6. Thermophilic (hemi)cellulolytic enzymes (Cobucci-Ponzano et al, 2013)

Organism	Enzyme	Temp opt (°C)	pH opt	References
Bacillus sp. AR-009	Xylanase	70–75	9.0–10.0	Gessesse (1998)
C. cellulovorans	Xylanase	70	6.0	Sunna et al. (2000)
G. stearothermophilus	Xylan 1,4-β-D-	70	6.0	Nanmori et al. (1990)
	xylosidase	75	6.5	Khasin et al. (1993)
G. stearothermophilus	Xylanase	65	5.5	Zaide et al. (2001)
T-6	α-D-glucuronidase	95	4.0	Cannio et al. (2004)
S. solfataricus	Xylanase	80	6.5	Morana et al. (2007)
T. ethanolicus	β-D-xylosidase/ α-L-arabinosidase	93	5.8-6.0	Shao and Wiegel (1992)
	β-D-xylosidase/ α-L-arabinosidase	105	6.2	Simpson et al. (1991)
		95	6.1	Xue and Shao (2004)
T.maritima	Xylanase	85	6.3	Ruile et al. (1997)
	Xylan 1,4-β-D-xylosidase α-D-glucuronidase			

Among Archaea, the genus Pyrococcus and Sulfolobus have been found to produce cellulases and xylanases (Maurelli et al, 2008). Within the order Sulfolobales, *Sulfolobus* species are commonly isolated from acidic thermal pools (T up to 90 °C). A specific strain *S. solfataricus* (Oα) can grow on xylan as sole carbon source (Cannio et al, 2004), but crystalline cellulose is not a growth substrate for any species of *Sulfolobus* so far reported.

Most of the studied thermophilic xylanase belong to GH 10 and GH 11 families. The xylanase belonging to the GH10 family have been isolated from thermophilic and hyperthermophilic microorganisms such as *Thermotoga* sp. (Winterhalter et al, 1995), *Rhodothermus marinus* (Abou-Hachem et al, 2003), *Bacillus stearothermophilus* (Khasin et al, 1993) and *Clostridium thermocellum* (Lo Leggio et al, 1999). An example is the xylanase from *Thermotoga maritima* MSB, called XynA, which showed the optimal values of activity at a temperature of 105

°C and a half life of 90 minutes at 95 °C (Simpson et al, 1991). The thermophilic xylanase belonging to family 11 have been isolated from *Thermomyces lanuginosus* (Singh et al, 2003;), *Dictyoglomus thermophilum* (McCarthy et al, 2000) and *Bacillu*s D3 (Harris et al, 1997).

Studies on the structure and the sequences of the xylanase belonging to the families GH 10 and GH 11, together with mutagenesis experiments, have allowed to discover the strategies for the adaptation and the mode of action of these molecules at high temperatures. The thermophilic xylanase, compared to mesophilic, present a series of structural changes that make them more rigid and stable, consequently to an increase in the number of salt bridges and hydrogen bonds, a better packaging in the internal regions of the structure (Hakulinen et al, 2003), a greater number of charged amino acids on the surface (Turune et al, 2002), the presence of structural domains often repeated in tandem (Winterhalter et al, 1995; Zverlov et al, 1996 Fontes et al, 1995) as well as a greater number of disulfide bridges in the N or C-terminal regions of alpha helics (Kumar et al, 2000; Turunen et al, 2001).

1.7 *Geobacillus thermodenitrificans*

Bacillus species have always been the major workhorse industrial microorganisms, being ubiquitous, non pathogenic bacteria that can be isolated from various environments also at temperature higher than 60°C. They can be easily cultivated in laboratory at high growth rates and are able to produce huge amounts of a great variety of extracellular enzymes. Among them, xylanases are of particular biotechnological relevance, owing to industrial interest in *Geobacillus* species has arisen from their potential applications in biotechnological processes, for example as sources of thermostable enzymes (de Champdore´ et al, 2007), as platforms for biofuel production (Cripps et al, 2009; Taylor et al, 2009) and as potential components of bioremediation strategies (Obojska et al, 2002; Perfumo et al, 2007), since they are equipped with numerous thermophilic enzymatic activities involved in the organic carbon recycling in hot natural and artificial environments where they thrive.

The genus *Bacillus* include a large and diverse collection of aerobic and facultatively anaerobic, rod-shaped, Gram-positive (to Gram-variable), endospore-forming bacteria. Whereas *Bacillus* and related genera consists of psychrophiles, acidophiles, alkalophiles and halophilic bacteria, able to utilize a wide range of carbon sources for heterotrophic or autotrophic growth, it is a specific group of thermophiles, the genus *Geobacillus* (Nazina et al, 2001).

16S rRNA gene sequence analysis has revealed high phylogenetic heterogeneity in the genus *Bacillus*, and the majority of the thermophilic species described belong to the genus *Bacillus* genetic groups 1 and 5 (Ash et al, 1991; Rainey et al, 1994). Depending on the strain, the temperature range for growth can extend as low as 35°C or as high as 80°C (Zeigler, 2014). But for most isolates, temperatures between 45 and 70°C are required for thermophilic aerobic spore-forming bacteria, that are classified into the genera *Bacillus*, *Alicyclobacillus*, *Brevibacillus*, *Aneurinibacillus*, *Sulfobacillus*, *Thermoactinomyces* and *Thermobacillus* (Sneath, 1986; Wisotzkey et al, 1992; Dufresne et al, 1996; Heyndrickx et al, 1997; Touzel et al, 2000). Subsequently group 5 isolates were found to be a phenotypically and phylogenetically coherent group of thermophilic bacilli (Fig. 5) with a high 16S rRNA sequence similarity (98.5–99.2%). As a consequence, in 2001 the thermophilic bacteria belonging to *Bacillus* genetic group 5 were reclassified as being members of *Geobacillus* gen. nov., meaning earth or soil *Bacillus*, with the well-known *Geobacillus* (*Bacillus*) *stearothermophilus* being assigned as the type strain (Nazina et al, 2001).

Thermophilic bacilli, including *Geobacillus*, are widely distributed and have been isolated from all continents where geothermal areas occur (Sharp et al, 1992). Geobacilli are also isolated from shallow marine hot springs and from deep-sea hydrothermal vents (Maugeri et al, 2002). High-temperature oilfields have also yielded strains of *Geobacillus* (Nazina et al, 2001) reporting two novel species *G. subterraneus* and *G. uzenensis*, isolated from the Uzen oilfield in Kazakhstan.

In addition *Geobacillus* species have also been recovered from artificial hot environments such as hot water pipelines, heat exchangers, waste treatment plants, bioremediation biopiles and from temperate soil environments (Marchant et al, 2002; Maugeri et al, 2002; Obojska et al, 2002).

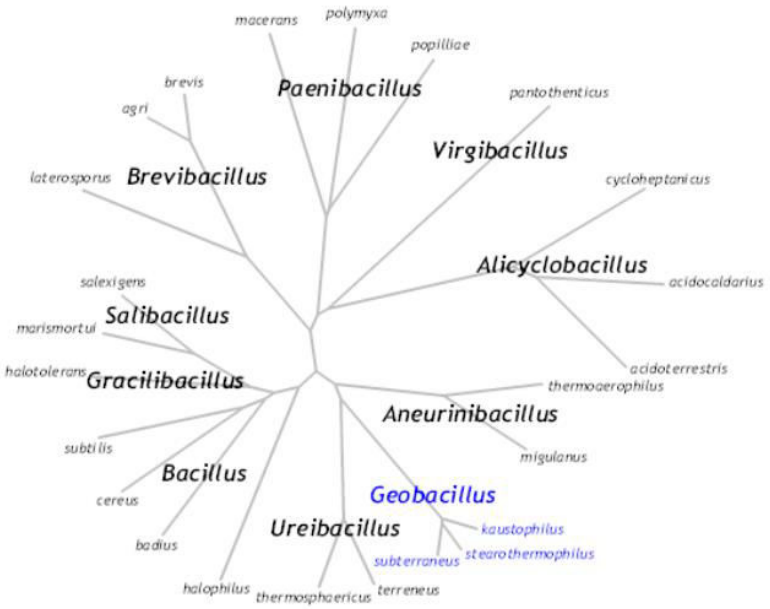

Figure 6: Phylogenetic tree based on 16S rRNA gene alignments.

Initial studies showed that thermophilic aerobic bacilli could be isolated in large numbers from a range of soils from geographically dispersed temperate and cool environments (McMullan et al, 2004), demonstrating great capabilities for adaptation to a wide variety of environmental niches. Obligate thermophiles, however, face a significant challenge outside their environment: they must survive on a planet with an average annual land surface temperature that has only ranged between 7 and 10 °C over the past three centuries (Rohde et al, 2013).

Industrial interest in *Geobacillus* species has arisen from their potential applications in biotechnological processes, for example as sources of thermostable enzymes (de Champdore´ et al, 2007), as platforms for biofuel production (Cripps et al, 2009; Taylor et al, 2009) and as potential components of bioremediation strategies (Obojska et al, 2002; Perfumo et al, 2007), since they are equipped with numerous thermophilic enzymatic activities involved in the organic carbon recycling in hot natural and artificial environments where they thrive.

Geobacillus thermodenitrificans is a Gram-positive, aerobic optional and facultative soil bacteria, ubiquitous and non pathogenic, with distinctive property of denitrification (Manachini et al, 2000). Different strains were isolated from different environments such as geothermal areas in terrestrial and marine

environments (Canakci et al, 2007) and biomass subjected to composting process (Gerasimova and Kuisiene, 2012).

They can be easily cultivated in laboratory at high growth rates and are able to produce huge amounts of a great variety of extracellular enzymes. Among them, xylanases are of particular biotechnological relevance owing to their good level of thermophilicity and thermostability and the ability to be highly active in alkaline conditions (Satyanarayana et al, 2012; Subramaniyan and Prema, 2000). *G. thermodenitrificans* is distinguished by its ability to metabolize a number of carbon sources larger than those utilized by other species of the genus and it is endowed with genes encoding all the glycolytic activities necessary to utilize xylans as the sole carbon sources (Feng et al, 2007). It shows the ability to grow between 45 and 73°C with an optimum at 65°C and use different substrates as a carbon source (Feng et al, 2007).

These characteristics appear to be advantageous for various industrial applications. An example is represented by *Anoxybacillus flavithermus* TWXYL, whose extracellular xylanase is active and stable at high temperatures required in the paper industry (Ellis and Magnuson, 2012).

Bacillus stearotermophilus T6 is known to synthesize an extracellular xylanase, which is also used in the paper industry to facilitate the lignin removal from the cellulose pulp during the paper bleaching (Khasin et al, 1993). *Bacillus* SSP-34 is able to sensitize xylanase maximally active at pH between 6 and 8 with optimum temperature of 50 °C. These xylanases are stable for up to two hours at a pH of from 4 to 9 and at temperatures between 30 and 45°C while at 50°C are stable for 20 minutes (Subramaniyan et al, 1997). Another interesting example is *Bacillus* D3, whose thermophilic xylanase has optimal activity at 75 °C and is used in the paper industry, in which are frequent high temperatures and alkaline conditions. Its optimum pH is 6, but the enzyme can be active in a broad pH range, especially in extreme conditions typical of industrial processes (Harris et al, 1997).

In this thesis is reported the isolation, purification and characterization of an endoxylanase from the *G. thermodenitrificans* A333, microorganism firstly isolated by Coleri et al, 2009.

1.8 *Anoxybacillus*

The Bacillaceae are a family of Gram-positive rod- or coccus shaped bacteria that for the most part produce endospores, as previously described. Based on the UniProt Taxonomy (2012), the family consists of a total of more than 19,000 described species, subspecies, strains pending proper descriptions, and strains identified directly from environmental samples.

Among the genera of Bacilli, *Bacillus* remains the largest and best-studied genus in terms of the total number of isolates, studies conducted, and published reports (De Boer et al, 1994; Kolstø et al, 2009).

A new anaerobic strain K1T from animal manure (Pikuta et al, 2000) was isolated. Based on the 16S rRNA, DNA‑DNA hybridization analyses and the phenotypic features of strain K1T, *Anoxybacillus* was proposed as a new genus of the Bacillaceae. Strain K1T was thus named *Anoxybacillus pushchinensis*. A number of *Anoxybacillus spp*. have been isolated around the world since the first introduction of the genus in 2000. To date, a total of 22 species and two subspecies of *Anoxybacillus* are described (Goh et al, 2014; Cihan et al, 2013; Deep et al, 2013). As *A. pushchinensis* K1T and *B. flavithermus* are phylogenetically clustered together and are distinct from Bacillus, *B. flavithermus* (Heinen et al, 1982), was reclassified as *Anoxybacillus flavithermus* (Pikuta et al, 2000).

The similarity of the 16S rRNA sequences throughout the genera is generally higher than 80 %. The *Anoxybacillus* species clustered together (92–99% similarity) and their nearest neighbors are *Geobacillus*, *Saccharococcus*, and *Aeribacillus* in the range of 90–95% similarity, whereas *Caldalkalibacillus* and *Calditerricola* shows lightly lower similarity to *Anoxybacillus* (85–88%) (Goh et al, 2014).

Anoxybacillus is a small, rodshaped (bacilli) microorganism, straight or slightly curved, often present in pairs or short chains, and it forms endospores. Interestingly, *Anoxybacillus* spp. can be either alkaliphilic or alkalitolerant and most of them are able to grow well at neutral pH. *Anoxybacillus spp*. are moderately thermophilic (50-62°C) with a slightly lower growth temperatures than *Geobacillus spp*. (55-65°C). The *Anoxybacillus spp*. are either aerobes or

facultative anaerobes (Pikuta et al, 2003; Pikuta et al, 2009). Among the *Anoxybacillus spp*., the genome of *A. flavithermus* WK1 remains the only completely sequenced genome (Saw et al, 2008). As previously described for *Geobacillus* species, Anoxybacilli are also isolated from different environments, such as geothermal springs (Atanassova et al, 2008), hot spring (Dülger et al, 2004; Kevbrin et al, 2005; Derekova et al, 2007), well-pipeline sediment (Cinhan et al, 2011) showing a high degree of adaptability to different environmental conditions.

Industrial interest in *Anoxybacillus* species has arisen from their potential applications in biotechnological processes such as biobleaching of paper pulp, the degradation of lignocellulosic material for production of bioethanol and the synthesis of oligosaccharide for the food industry. In general, the enzymes from the microorganism belonging to this species are heat tolerant and stable in alkaline conditions. Most reported protein sequences are distinct from their *Bacillus* counterparts but are similar to those from *Geobacillus* sources.

Studies of lignocellulolytic enzymes produced by *Anoxybacillus* are relatively new and limited compared to those of fungi such as *T. reesei* (Couturier et al, 2012). As previously mentioned, such applications require thermostable (hemi)cellulases due to their structural features which confer thermostability as well as for potential advantages in industrial processes, that requires particular harsh condition such as high temperature, extremes pH, presence of metal ions.

Xylanolytic activity was observed in *A. flavithermus* BC (Kambourova et al, 2007), *A. pushchinoensis* A8 (Kacagan et al, 2008), *Anoxybacillus sp.* E2 (Wang et al, 2010), and several strains isolated from Turkish hot springs (İnan et al, 2011). The xylanase purified from these sources exhibited different biochemical characteristics.

For example the xylanase from *Anoxybacillus sp.* A8 worked optimally at the acidic pH of 6.5 (Kacagan et al, 2008). As another example, the xylanase from E2 was thermostable at 60°C, exhibited activity in a broad pH range of 4.6‑12.0 and had no detectable cellulase activity (Wang et al., 2010). The presence of an arabinofuranosidase could facilitate the hydrolysis of the α-1,2- and α-1,3-arabinofuranosidic bonds in hemicelluloses (Saha, 2003). The first α-L-

arabinofuranosidase gene from *Anoxybacillus kestanbolensis* was cloned and expressed in *Escherichia coli* BL21 (Çanakçi et al, 2008), with an optimal pH of 5.5 and an optimal temperature of 65 °C. A study conducted by Thitikorn-Amorn et al (2012) elucidated the presence of xylanase, β-xylosidase, α-L-arabinofuranosidase and acetyl esterase activities in cultures of *Anoxybacillus sp*. JT-12. Xylotriose was found to be the predominant xylooligosaccharide in the supernatant of cells grown in minimal salt medium containing birchwood xylan at 55°C and pH 7.0 for at least 42 h.

Indeed the complete degradation of xylan requires several xylanolytic enzymes, and among them, endoxylanases and β-xylosidases are prominent in xylan hydrolysis. As described, the β-xylosidases catalyze the hydrolysis of xylo-oligosaccharides to release xylose or other oligosaccharides from the non-reducing end (Guerfali et al, 2009; Shi et al, 2013).

Although many β-xylosidases and their coding genes have been manipulated and characterized in plant, fungi, bacteria as well as archaea, few β-xylosidases have been reported from thermophilic Bacteria and Archaea (Cantarel et al, 2009). A β-xylosidases activity was characterized from aerobic bacteria such as *Geobacillus pallidus*. The hydrolytic enzyme that belong to GH52 family, showed its optimum activity at 70 °C and pH close to 8.0 and was fully stable at 55°C for 72 h (Quintero et al, 2007). A β-xylosidase gene (XsidB) (GH52) of the thermophile *G. thermodenitrificans* TSAA1 has been cloned and expressed in *E. coli*. The purified β-xylosidase is alkalitolerant (working pH range between 5.0 and 8.0) as well as thermostable (activity range 40-70 °C, with optimum at 60 °C), and it has the required characteristics for a biocatalyst exploitable in the production fermentable sugars from agro-residues and in the biosynthesis of methylxylosides (Jain et al, 2014).

In this thesis is reported the purification and characterization of a β-xylosidase from the *Anoxybacillus sp.* strain 3M, that was isolated by Marques et al (1998) from terrestrial hot springs (90°C) in Sao Miguel, Azores.

Anoxybacillus sp. 3M exhibited hemicellulolitic activities in the crude extract and an extracellular xylanase activity was identified and partially characterized (Marquez et al, 1998). The study showed that the xylanase activity produced by

Bacillus sp 3M was stable at 55°C for 3 days, which could be useful for industrial processes.

1.9 Applications of cellulases and hemicellulases

Cellulases and hemicellulases were initially investigated several decades back for the bioconversion of biomass which gave way to research in the industrial applications of the enzymes in animal feed, food, textiles and detergents and in the paper industry. The technologies and products using (hemi)cellulases have reached the stage where these enzymes have become indispensable.

1.9.1 Textile industry

Cellulases have become the third largest group of enzymes used in the industry since their introduction only since a decade. They are used in the bio- stoning of garments. For example *Humicola insolens* cellulase is most commonly employed in combination with the equally good cellulases mixtures and are utilized for digesting off the small fiber ends protruding from the fabric softening and defibrillation , and in processes for providing localized variation in the color density of fibers (Schülein, 1997).

Cellulases are commonly used in detergents for cleaning textiles and are commonly added in washing powders, and in detergents.

1.9.2 Food and animal feed

In food industry, cellulases are used in extraction and clarification of fruit and vegetable juices, and in the extraction of olive oil. Glucanases are added to improve the malting of barley in beer manufacturing and in wine industry, better maceration and color extraction is achieved by use of exogenous hemicellulases and glucanases. Cellulases are also used in carotenoid extraction in the production of food coloring agents (Kulkarni et al, 1999).

Enzyme preparations containing hemicellulase and pectinase in addition to cellulases are used to improve the nutritive quality of forages. Several reports can be cited on the use of *Trichoderma* cellulases as feed additive for improving the feed conversion ratio and increasing the digestibility of a cereal-based feed (Nidetzky et al, 1994).

1.9.3 Pulp and paper industry

In the pulp and paper industry, cellulases and hemicellulases have been employed for biomechanical pulping, for modification of the coarse mechanical pulp and hand sheet strength properties, de-inking of recycled fibers and for improving drainage and run ability of paper mills (Viikari et al., 1994). Cellulases are also used in preparation of easily biodegradable cardboard. The enzyme is employed in the manufacture of soft paper including paper towels and sanitary paper and preparations containing cellulases are used to remove adhered paper.

1.9.4 Biofuel

Perhaps the most important application currently being investigated is in the utilization of lignocellulosic wastes for the production of biofuel. The application of cellulose and hemicellulase is the conversion of cellulosic materials into fermentable sugars, which in turn can be, used as microbial substrates for the production of ethanol (Gessesse, 1998). The use of pure enzymes in the conversion of biomass to ethanol or to fermentation products is currently uneconomical due to the high cost of commercial (hemi)cellulases.

Effective strategies are yet under investigation to solve this issue and active research has to be taken up in this direction. Overall, cellulosic biomass is an attractive resource that can serve as substrate for the production of value added metabolites and cellulases as such.

1.10 Aim of the study

The general aim of this work was to add new knowledge on novel hemicellulolytic enzymes involved in the hydrolysis of lignocellulosic materials, considered as a key process for the bioethanol production.

Therefore, it is not only focused on (hemi)cellulolytic enzymes from mesophilic fungi and bacteria but also on newly isolated and characterized xylanase and β-xylosidase from the thermophilic bacteria *Geobacillus thermodenitrificans* A333 and *Anoxybacillus* sp. 3M, respectively.

The covered fields involved the production and purification of the novel thermo-alkali tolerant hemicellulases and the characterization of these enzymes by their activity, substrate specificity and hydrolytic potential. The present work was also focused on the definition of enzymatic cocktails through the identification of the enzyme components of bacterial and fungal origin most useful for improving the complete hydrolysis of lignocelluloses and the exploration of the possible synergies among them.

In order to convert lignocellulosic materials from agro-residues into reducing sugars, pretreatments and enzymatic hydrolysis of the biomasses has been performed. Different enzyme cocktails composition were defined by using various commercial and in house developed cellulases, xylanases, β-xylosidases and arabinosidases, to obtain the efficient hydrolysis of *Arundo donax*, corn cobs and BSG. Since each biomass responds differently to pretreatment methods, an appropriate chemical pretreatment method was used for BSG and two different pretreatments involving chemical and physical methods were tested for their efficiency on *A. donax*.

Though research about the conversion of biomass to ethanol is vast and this productive process is since long time known, its commercialization is restricted by the elevated costs and the limited efficiency. This research intends to demonstrate that using agro-residues as substrates, high yields of reducing sugars are possible, using the appropriate pretreatments and enzyme cocktails. The removal of the limitation imposed by poor yields after the enzymatic hydrolysis, would result in an increased efficiency for ethanol production, which is a key stepping stone to its

commercialization. Also, increasing the recovery of the co-product of (hemi)celluloses hydrolysis, as xylose and arabinose, would also allow an effective utilization of this resource.

2. Materials and Methods

Part I

Isolation, purification and characterization of novel hemicellulolytic activities

2.1 *Geobacillus thermodenitrificans* A333 endo-xylanase purification, characterization and cloning

2.1.1 Substrates and reagents

Meat extract and Peptone for the nutrient broth (NB) preparation were purchased from Oxoid Ltd (Oxford, UK). Xylans from beechwood, oat spelt and birchwood respectively, carboxymethyl cellulose (CMC), Avicel, laminarin, lichenan, gelrite (Gellan gum), p-nitrophenyl (*p*NP) glycoside substrates, Congo Red, sodium azide (NaN_3), phenylmethylsulfonyl fluoride (PMSF), Coomassie Brilliant Blue G-250, β-mercaptoethanol (2-ME), 3-[(3-cholamidopropyl)dimethylammonium]-1-propanesulfonate (CHAPS), ethylenediaminetetraacetic acid (EDTA), lysozyme, urea, Triton X-100, Tween 20 and 80, SDS, Cetyltrimethylammonium bromide (CTAB), PEG 8000, octylphenoxypolyethoxyethanol (Nonidet), proteinase K (from *Tritirachium album*), trypsin and α-chymotrypsin (from bovine pancreas), subtilisin A (from *Bacillus licheniformis*), collagenase (from *Clostridium histolyticum*), xylose and glucose were obtained from Sigma-Aldrich Co. (St. Louis MO).

2% 4-0-methyl-D-glucurono-D-xylan-Remazol Brilliant Blue R (Azo-Oat Spelt Xylan), xylobiose, xylotriose, xylotetraose and xylopentaose (X_2-X_5) were from Megazyme Co. (Bray, Ireland).

The GeneElute Bacterial Genomic DNA Kit and the pGEM T-easy plasmid were from Sigma-Aldrich Co. and Promega Co. (Madison, USA) respectively. The Phusion High Fidelity DNA polymerase and dNTP, were obtained from NEB (Ipswich MA, USA). All chemicals were of reagent grade, and all solutions were made with distilled and deionized water.

2.1.2 Media and growth conditions

Geobacillus thermodenitrificans A333, (catalog number DSM22625), was supplied by Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). The microorganism, isolated by Coleri et al (2009), was grown aerobically at 60°C and pH 7.0 in the basal medium NB, having the following composition (per liter of distilled water): 5 g peptone, 3 g meat extract. *G. thermodenitrificans* was gradually adapted to utilize xylan as a carbon source by spotting aliquots of 10^6 cells onto solid media containing NB, increasing amounts of beechwood xylan up 0.15% (w/v) and 0.8% (w/v) gelrite (Gellan gum).

Single colonies obtained onto the plates, incubated at 60°C for 16 h were tested for xylanase production by staining with a Congo Red (Sigma-Aldrich Co., St. Louis MO) aqueous solution (1.0% w/v) and destaining with 1.0 M NaCl. Isolates surrounded by clear zone were considered as xylanase positive. The enzyme production was obtained in liquid culture by growing the microorganism in NB medium supplemented with 0.15% (w/v) beechwood xylan as carbon source and buffered at pH 7.0. A 250 mL Erlenmeyer flask, containing 50 mL of liquid medium, was inoculated with a single xylanase positive colony and incubated at 60°C for 16 h in a rotatory shaker (New Brunswick Scientific Co., USA) at 150 rpm. Then, the culture was scaled up to 2 L and the biocatalyst was produced into four 2 L Erlenmeyer flasks. After 16 h of incubation, the bacteria were harvested by centrifugation at 10,800 x *g* for 20 min at 4°C. The cell-free supernatant was used as crude enzyme solution and was stored at 4°C after the addition of NaN_3 and PMSF at a final concentration of 0.02% and 0.2 mM, respectively.

2.1.3 Enzyme assays

Xylanase activity, in the culture supernatant and during the purification procedure, was estimated spectrophotometrically using a soluble chromogenic xylan as the substrate (Biely et al, 1985). The activity was measured by adding 150 µL of 2% 4-0-methyl-D-glucurono-D-xylan-Remazol Brilliant Blue R (Azo-Oat Spelt Xylan) in 100 mM sodium phosphate buffer pH 7.5 to 150 µL of properly diluted enzyme solution, and incubating at 50°C for 30 min. The reaction was stopped

with the addition of 750 µL of 97% ethanol, followed by incubation at room temperature for 5 min and centrifugation at 9,000 x *g* for 10 min. The supernatant absorbance was measured at 590 nm. One unit of xylanase activity was defined as the amount of enzyme required to increase the absorbance at 590 nm of 1 A min^{-1} under the experimental conditions.

The purified xylanase activity was assayed by incubating 50 µL of 1% beechwood xylan in 100 mM sodium phosphate buffer pH 7.5 with 50 µL of appropriately diluted enzyme, in the same buffer; boiled enzyme samples were used in the control. After incubation at 50°C for 10 min, the released reducing sugars were estimated with the Somogyi-Nelson method (Nelson 1944). One unit of xylanase activity was defined as the amount of enzyme required to release 1 µmol of xylose-equivalent reducing groups per min under the described conditions.

All the enzymatic measurements were performed in triplicate.

2.1.4 Localization of xylanase

The microorganism was grown, as previously described, in 50 mL of liquid medium, and when the culture reached the late exponential phase, it was centrifuged at 10,800 x *g* for 5 min at 4°C. The presence of the extracellular activity was evaluated on cell-free supernatant. For cell-wall bound and intracellular activity, the cells, washed with 100 mM sodium phosphate buffer pH 7.5 and re-suspended in 1 mL of the same buffer containing 5.0 mM EDTA and lysozyme (1 mg mL^{-1}), were incubated for 30 min at 30°C. Subsequently, the bacterial cells were broken by sonication with the ultrasonic cell disruptor (Soniprep Braun Labsonic) according to the following procedure: ten cycles of 1 min sonication on ice with 1 min pause. Supernatant was separated from cell debris by centrifugation (20,000 x *g*, 30 min and 4 °C). Cell debris was re-suspended in 200 µL of 100 mM sodium phosphate buffer pH 7.5 and tested in parallel with the supernatant, for the presence of xylanase activity utilizing the azo-xylan as the substrate.

2.1.5 Induction of xylanase expression

The xylanase induction was evaluated by growing *G. thermodenitrificans* A333, under the previously specified culture conditions, in different media obtained by replacing the beechwood xylan with monosaccharides such as xylose and glucose at 0.1% (w/v) final concentration. The effect of other xylan types, such as oat spelt xylan and birchwood xylan at 0.15% (w/v) final concentration, on GtXyn production was also evaluated. The growth and the extracellular xylanase production were checked analyzing culture samples withdrawn at various intervals of time. Samples from each culture broth were centrifuged at 10,800 x *g* and 4ºC for 15 min to remove the bacterial cells and the supernatants were used as enzyme extracts for the activity assays.

The effect of xylose addition at different concentrations on GtXyn production was also investigated. The microorganism was grown in basal medium supplemented with 0.1, 0.25, 0.5 and 1% (w/v) sugar and the extracellular xylanase production was tested along the growth, as above described.

Furthermore, to verify a potential catabolite repression effect of ready metabolizable carbon sources, xylose at a 1% (w/v) final concentration was added at mid-exponential phase of growth to a culture obtained in basal medium containing 0.15% (w/v) beechwood xylan.

2.1.6 Xylanase production from agro-industrial raw materials

Different agro-industrial raw materials including brewery's spent grains (BSG), corn cobs, grape cane and wheat straw were used as carbon sources for xylanase production by *G. thermodenitrificans* A333. BSG were provided by Maltovivo micro-brewery, Avellino, Italy. This biomass was dried at 50°C, milled by an homogenizer to obtain a fine powder, and stored in sealed vials at room temperature until use. The processed lot contained (w/w, oven dry basis) 26.3% cellulose, 24.1% hemicelluloses, 9.8% Klason lignin and 27.1% protein. Wheat straw supplied by Estação Nacional de Melhoramento de Plantas, Elvas, Portugal, was dried and grounded to particles of about 0.5 mm thickness. The processed lot was composed of 38.8% cellulose, 27.6% hemicelluloses and 16% Klason lignin. Grape cane was collected from a farm at Oleiros, Portugal. This raw material was

milled to attain 0.5 mm of particle size. The processed lot was composed of 39.1% cellulose, 17.9% hemicelluloses and 24.7% Klason lignin. Corn cobs were obtained from Companhia das Lezírias (Samora Correia, Portugal), dried up to a moisture content less than 10% (w/w), and milled to particles smaller than 3 mm. The average composition of this feedstock was 38% cellulose, 35% hemicelluloses and 19% Klason lignin.

For the xylanase production, *G. thermodenitrificans* A333 cultivation started from the inoculum obtained transferring the microorganism from a fresh slant (less than 24 h old) to a 500 mL Erlenmeyer flask containing 100 mL of NB basal medium, supplemented with 0.15% (w/v) beechwood xylan pH 7.0 and incubated at 60°C for 16 h under shaking conditions (150 rpm). Subsequently, 10% of this culture was used to inoculate a 500 mL Erlenmeyer flasks containing 100 mL of basal medium supplemented with 1.0% (w/v) of each carbon source listed above as inducer substrates. The basal medium and the C-sources were previously sterilized by autoclave (121°C, 1 atm for 15 min). The cultures were grown by shaking (150 rpm) at 60ºC for 2-6 days. The samples from each culture broth, withdrawn at different intervals of time, were checked for xylanase activity.

2.1.7 Xylanase purification procedures

The culture supernatant was concentrated 20-fold by ultrafiltration through a YM10 membrane (cut-off 10 kDa) in an Amicon cell (Millipore Merck KGaA, Darmstadt, Germany) and dialyzed against 20 mM sodium acetate buffer pH 5.0 at 4°C.

The dialyzed enzyme solution was applied, at a flow rate of 0.5 mL min^{-1}, to a SP-Sepharose fast flow column (2.6 x 10 cm) (GE Healthcare, Uppsala, Sweden) previously equilibrated with 20 mM sodium acetate pH 5.0 (buffer A). After washing with buffer A to remove the unbound proteins, the elution was performed with a linear salt gradient from 0 to 1.0 M NaCl in buffer A, at a flow rate of 2 mL min^{-1}. The eluted fractions, dialyzed against buffer A at 4°C for 16 h, were checked for the enzymatic activity.

The pool of xylanase positive fractions were filtered using a 0.22 µm filter (Millipore Merck KGaA, Darmstadt, Germany) and applied, at a flow rate of 0.5

mL min^{-1}, onto a Resource S column (GE Healthcare, Uppsala, Sweden) (6.4 x 30 mm) pre-equilibrated with buffer A. After washing with the same buffer, the elution was carried out at a flow rate of 1 mL min^{-1} with a step gradient 0-1.0 M NaCl in buffer A, with the following profile: from 0 to 0.43 M NaCl in 2 min, from 0.43 to 0.60 M NaCl in 15 min, from 0.60 to 1.0 M NaCl in 3 min. The active fractions pooled were dialyzed against 50 mM sodium phosphate buffer pH 7.5 at 4°C for 16 h and concentrated using a Centricon system (Millipore Merck KGaA, Darmstadt, Germany) of 10 kDa cut-off. The concentrated enzyme solution (150 µL) was loaded onto a gel filtration column Superdex 75 HR (1.0 x 30 cm) (Amersham Pharmacia Biotech, Uppsala, Sweden) and the enzyme elution was carried out with 50 mM sodium phosphate buffer pH 7.5 containing 150 mM NaCl at a flow rate of 1 mL min^{-1}. The pool of active fractions were used as purified enzyme for subsequent analysis.

2.1.8 Protein determination

Protein concentration was determined as described by Bradford, using the BioRad protein staining assay and bovine serum albumine as the standard (Bradford, 1976).

2.1.9 Electrophoresis and activity staining

The purification levels of the enzymatic protein after each step were analyzed by sodium dodecyl sulfate polyacrilammide gel electrophoresis (SDS-PAGE). Enzyme samples were loaded onto a SDS-PAGE 10% containing 0.1% (w/v) beechwood xylan. The SDS-PAGE was performed according to the discontinuous Laemmli method (1970). Proteins were visualized by staining with Coomassie Brilliant Blue G-250. Zymogram analysis was performed as described by Tseng et al. (2002). After the electrophoretic run, the gel was washed twice for 20 min at room temperature in 50 mM sodium phosphate buffer (pH 7.5) containing 25% (v/v) 2-propanol, then rinsed in 50 mM sodium phosphate buffer (pH 7.5) and incubated at 60°C for 16 h in the same buffer. The gel was flooded with 0.1% (w/v) Congo Red dye aqueous solution for 30 min and repeatedly washed with 1.0 M NaCl solution.

2.1.10 NH_2-terminal sequence

The NH_2-terminal amino acid sequence from the pure protein band electroblotted onto polyvinylidene difluoride membrane (PVDF) according to Matsudaira (1987) was performed by automated Edman degradation utilizing an Applied Biosystems 477 A Protein Sequencer, equipped on line with a phenylthiohydantoin analyzer (model 120A). Degradation proceeded for 25 cycles according to the manufacturer's instructions, and produced identifiable amino acid residues.

2.1.11 Gene isolation and sequencing

Genomic DNA from *G. thermodenitrificans* A333 was extracted using the GeneElute Bacterial Genomic DNA Kit, following the manufacturer's instructions. The complete coding sequence for GtXyn was amplified by PCR using synthetic primers (PRIMM s.r.l, Milano, Italy) designed on the basis of the end regions of the extracellular xylanase gene (NC0093281*)* from *G. thermodenitrificans* NG80-2 whose genome is available on line. The primers sequences were the following:

5'A333: -5′-ATGTTGAAAAGATCGCGAAAA-3′

3'A333: -5′-TCACTTATGATCGATAATAGC-3'

The PCR reaction was performed by using 50 ng of genomic DNA, the Phusion High Fidelity DNA polymerase and the following program: 1 cycle 95°C 10 min; 40 cycles 95°C 1 min, 65°C 1 min, 72°C 1 min and 1 cycle 72°C 10 min. The PCR product was first cloned into the pGEM T-easy plasmid and sequenced by using an automatic sequencer.

2.1.12 Effect of pH and temperature on xylanase activity and stability

The influence of pH was determined at 50°C by the xylanase standard assay in the range 5.0-11.0 using the following buffers (100 mM): sodium acetate buffer (pH 5.0, 5.5, 6.0), sodium phosphate buffer (pH 6.0, 6.5, 7.0,7.5, 8.0), borate-borax buffer (pH 8.0, 8.5, 9.0), and borax-NaOH buffer (pH 9.0, 9.5, 10.0, 11.0). The pH stability was evaluated by pre-incubation of the enzyme (0.1 mg mL^{-1}) at 50°C at pH 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 for 24 h followed by the residual xylanase

activity measurement. The activity obtained without pre-incubation was expressed as 100%.

The effect of temperature was investigated at different values ranging from 20 to 90°C at pH 7.5 by the xylanase standard assay. To determine the thermal stability, the purified GtXyn (0.1 mg mL^{-1}) was pre-incubated in 100 mM sodium phosphate buffer pH 7.5 at defined temperatures (58, 60, 62, 65, 70°C) for 60 min without substrate and the residual activity was determined at standard assay conditions. The activity obtained without pre-incubation was expressed as 100%. The time dependent profile of heat inactivation was studied by measuring the residual activity after pre-incubating the enzyme (0.1 mg mL^{-1}) in 100 mM sodium phosphate buffer pH 7.5 at different temperatures (58, 60, 62, 65 and 70°C) for 1, 2, 5, 10 and 24 h.

The kinetic parameters for thermal inactivation were obtained utilizing the experimental data in the equation reported below:

$\ln A/A_0 = K_d t$

where A_0 is the initial activity obtained without pre-incubation, A is the residual activity after heat treatment, K_d is thermal inactivation rate constant (h^{-1}) and t is the exposure time (h).

The half-life of the xylanase ($t_{1/2}$, h) was determined from the relationship:

$t_{1/2} = \ln 2/K_d$.

2.1.13 Effects of metal ions, detergents and other chemical reagents

The effect of metal ions and chemical reagents was examined by pre-incubating the xylanase (0.1 mg mL^{-1}) in 100 mM sodium phosphate buffer pH 7.5, at 60°C for 1 h, in presence of different mono-, di- and trivalent cations either as sulphate and chloride salts, EDTA, PMSF and β-mercaptoethanol (2-ME), at final concentrations of 1.0 and 10 mM. Moreover, the influence of urea at 1% (w/v) final concentration was evaluated. The salt stability was also investigated by pre-incubating the enzyme with 1.0, 2.0 and 3.0 M NaCl at 60°C for 1 h.

The GtXyn resistance to detergents was determined by pre-incubating the enzyme at 60°C for 1 h, under constant agitation, with Triton X-100, Tween 20 and 80, SDS, Cetyltrimethylammonium bromide (CTAB), PEG 8000,

octylphenoxypolyethoxyethanol (Nonidet) and 3-[(3-cholamidopropyl)dimethylammonium]-1-propanesulfonate (CHAPS) at a final concentration of 1% with the sole exception of SDS that was tested at 1.0 and 10 mM. Residual activity values were determined under the Somogyi-Nelson method, and expressed as percentage of a control sample incubated in absence of any additive.

2.1.14 Effect of organic solvents

The effect of 15% (v/v) organic solvents was investigated by pre-incubating the enzyme in presence of different solvents at 25°C for 30, 60 and 120 min, under constant agitation. The xylanase activity towards beechwood xylan was determined with the standard method and expressed as percentage of a control pre-incubated with distilled water instead of solvents. Furthermore, the effect of various ethanol amounts was investigated by GtXyn pre-incubation at 25°C for 30 min in presence of concentrations ranging from 10 to 80% (v/v).

The time-resistance in presence of ethanol was also evaluated by pre-incubating the xylanase at 25°C in presence of 40% (v/v) ethanol for different time intervals (1, 2, 5 and 24 h). The residual activity was measured by the standard assay, previously mentioned.

2.1.15 Effect of proteases on enzyme stability

The resistance to different proteases was tested in presence of 10 μg mL^{-1} of proteinase K (from *Tritirachium album*), trypsin and α-chymotrypsin (from bovine pancreas), subtilisin A (from *Bacillus licheniformis*) and collagenase (from *Clostridium histolyticum*). For this purpose, the purified xylanase (150 μg mL^{-1}) was pre-incubated in presence of the different proteolitic activities in 100 mM sodium phosphate buffer at pH 7.5, and 37°C for 1, 2 and 3 h. The enzyme, pre-incubated under the same conditions in the absence of proteases, was used as control. The residual xylanase activity was measured with the standard assay.

2.1.16 Substrate specificity

The specificity of the purified GtXyn towards different polysaccharides was determined by the Somogyi–Nelson standard assay, using the following substrates: beechwood xylan, birchwood xylan, oat spelt xylan, carboxymethyl cellulose, Avicel, laminarin, lichenan, tamarind xyloglucan, and locust bean gum. β-Xylosidase activity was determined, according to Biely et al. (2000), by utilizing the soluble chromogenic substrate *p*-nitrophenyl-β-D-xylopyranoside (*p*NPXP). The assay was started by adding 50 µl of appropriately diluted enzyme in 50 mM sodium phosphate buffer pH 7.5, to 450 µL of 1.5 mM *p*NPXP, in the same buffer. After heating the assay mixture at 50°C for 10 min, the reaction was stopped by adding 1 mL of 1.0 M sodium carbonate and the release of *p*-nitrophenol was detected by measuring the absorbance at 405 nm. One unit of β-xylosidase was defined as the amount of enzyme required to release 1 µmol of *p*-nitrophenol per min under the assay conditions. α-Arabinofuranosidase, α-glucosidase and β-glucosidase activities were evaluated by the same assay method utilizing *p*-nitrophenyl (*p*NP)-α-L-arabinofuranoside, *p*NP-α-D-glucopyranoside, *p*NP-β-D-glucopyranoside as substrates, respectively. All the enzymatic measurements were performed in triplicate.

2.1.17 Analysis of hydrolysis products

To determine the mode of action of the enzyme, 1.5 U of purified GtXyn were incubated at 50°C in 50 mM sodium phosphate buffer (pH 7.5) in presence of 25 mM xylobiose, xylotriose, xylotetraose and xylopentaose (X_2-X_5) up to 24 h, and 5% (w/v) beechwood xylan up to 72 h. The determination of the hydrolysis products was performed withdrawing samples at different time intervals and analyzing, on silica plate by thin layer chromatography (TLC), the released sugars. The plates (20 x 20 cm) (Merck Millipore KGaA, Darmstadt, Germany), developed with one run of butanol-acetic acid-water (2:1:1 vol.), were then sprayed with a methanol-sulfuric acid mixture (95:5 v/v) and heated for few min at 130°C. The sugar hydrolysis products were identified by comparing the Rf values of the samples with those of the above cited xylooligosaccharides and xylohexaose (X_6), utilized as standards.

2.1.18 Saccharification of agro-derived materials

Saccharification experiments of various lignocellulosic materials (LCMs) such as BSG, corn cobs and biomass from giant reed (*Arundo donax*), pretreated with an aqueous ammonia solution as described by Maurelli et al (2013), were carried out. The raw materials, dried at 50°C and milled by an homogenizer to obtain a fine powder, were soaked in 10% (v/v) aqueous ammonia solution at a solid loading of 5% at 70°C for 22 h in screw-capped 25 mL bottles to reduce the evaporation. The alkaline mixtures were centrifuged at 800 x *g* and the residues were extensively washed with 50 mM sodium phosphate buffer until obtaining pH 7.5. The hydrolysis of the xylan fractions was carried out at 50°C for 72 h in a total volume of 5 mL (50 mM sodium phosphate buffer pH 7.5 plus 1.5 U of enzyme), at a solid loading of 5%. Samples were withdrawn at different time intervals and the released sugars were analyzed by TLC as described above. The amounts of reducing sugars liberated by the enzyme action were quantified with the Somogyi-Nelson method.

2.1.19 Cloning, expression and purification of the recombinant xylanase

The coding sequence for an N-terminally truncated GtXyn version, namely a protein lacking the signal peptide region (amino acids 1-27) was cloned as follows: a PCR fragment was obtained with the synthetic primers 1761NheI (**GCTAGC**AAAACTGAACAATCATACGCT) and 1761BamHI (**GGATCC**TCACTTATGATCGATAATAGC) (PRIMM s.r.l, Milano, Italy). The primers were designed on the basis of the end regions of the extracellular xylanase gene (NC0093281) from *G. thermodenitrificans* NG80-2, whose genome is available on line. The primers sequences contained the NheI and BamHI restriction sites (bold letters) at the start and at the end of the truncated GtXyn coding sequence. The *G. thermodenitrificans* A333 genomic DNA, used as the template for amplifications, was extracted using the GeneElute Bacterial Genomic DNA Kit (SIGMA) following the manifacturer's instructions. The PCR product was first cloned into the pGEM T-easy plasmid (Promega) and sequenced to confirm the identity of the GtXyn gene. After partial digestion of the recombinant plasmid with NheI and BamHI, a fragment of 1.149 kb, corresponding to the

coding gene, was cloned into the similarly digested pET-28c expression vector (Novagen). The pET-28/GtXyn expression construct was used to transform *E. coli* BL21(DE3) cells and to produce a recombinant GtXyn, containing a N-terminal histidine tag.

For the expression, cells were grown overnight in 10 ml of LB medium with 25 μg/ml kanamycin at 22°C for 16 h. After scale up to 1 L culture, the growth was continued up to an A_{600} of about 0.8-1.0, and IPTG was added to a final concentration of 0.4 mM. The BL21(DE3) cells were incubated for further 3 h at 22°C. Cells were harvested by centrifugation, suspended in 20 ml of buffer 50 mM sodium phosphate, pH 7.5, 2 mM 2-mercaptoethanol, 0.7 mM PMSF, and disrupted by sonication for 5 cycles of 1 min each (Soniprep Braun Labsonic sonicator) and cell debris was removed by centrifugation. The supernatant was incubated at 55°C for 10 min, and after 30 minutes in ice, at 60°C for 10 min again. The precipitate was removed by centrifugation and the supernatant was dialysed, against 50 mM Tris-HCl pH 7.5, at 4 °C for 16 h. The dialyzed supernatant was applied to an affinity chromatography using a PolyPrep Chromatography column (Bio-Rad) packed with 1 ml His Select™ HF Nickel Affinity Gel (Sigma), equilibrated in 50 mM Tris-HCl pH 7.5. The recombinant proteins were eluted with the same buffer containing increasing Imidazole concentrations (5 mM, 20 mM, 50 mM, 200 mM, 500 mM). The cromathografic fractions were dialyzed against 50mM Tris-HCl pH 7.5 and analyzed by a 10% SDS-Page.

2.2 Isolation, purification and characterization of a β-xylosidase from *Anoxybacillus* sp. 3M

2.2.1 Substrates and reagents

Yaest extract and Peptone were purchased from Oxoid Ltd (Oxford, UK). Xylan from beechwood, carboxymethyl cellulose (CMC), Congo Red, Coomassie Brilliant Blue G-250, β-mercaptoethanol (2-ME), sodium azide (NaN_3), PMSF, EDTA, all the salts and the reagents were obtained from Sigma-Aldrich Co. (St. Louis MO).

2% 4-0-methyl-D-glucurono-D-xylan-Remazol Brilliant Blue R (Azo-Oat Spelt Xylan) was from Megazyme Co. (Bray, Ireland).

All chemicals were of reagent grade, and all solutions were made with distilled and deionized water.

2.2.2 Media and growth conditions

Anoxybacillus sp. 3M was provided by Dott. Luis Alves, National Laboratory on Energy and Geology (Portugal). The microorganism was aerobically grown at 55°C and pH 7.0 in the medium, called AM, having the following composition (per liter of distilled water): 1.5 g $(NH_4)_2SO_4$, 1.7 g KH_2PO_4, 1.7 g $Na_2HPO_4x2H_20$, 0.2 g $MgSO_4x7H_20$, 0.5 mL mineral solution (Vishniac and Santer, 1957), 1 g yeast extract and 1 g peptone. The microorganism was gradually adapted to utilize the xylan as a carbon source by spotting aliquots of 10^6 cells onto solid media containing the AM medium and increasing amounts of beechwood xylan up 1% (w/v) and 0.8% (w/v) gelrite (Gellan gum).

The colonies obtained onto the plates, incubated at 55°C for 18 h, were tested for hemicellulase production by staining with a Congo Red aqueous solution (1.0% w/v) and destaining with 1.0 M NaCl. Isolates surrounded by clear zone were considered as xylanase positive. The enzyme production was obtained in liquid culture by growing the microorganism in the AM medium supplemented with 1% (w/v) beechwood xylan as carbon source and buffered at pH 7.0. A single xylanase positive colony was inoculated in 50 mL of liquid medium and incubated at 55°C for 18 h in a rotatory shaker (New Brunswick Scientific Co., USA) at 150 rpm. The culture was scaled up to 1 L and the biocatalyst was produced into four 1 L Erlenmeyer flasks. After 18 h of incubation, the bacteria were harvested by centrifugation at 10,800 x *g* for 30 min at 4°C. The cell-free supernatant was used as crude enzyme solution and was stored at 4°C after the addition of NaN_3 and PMSF at a final concentration of 0.02% and 0.2 mM, respectively.

2.2.3 Protein determination, electrophoresis and activity staining

Protein concentrations were determined by the Bradford method using the Bio-Rad protein staining assay, as previously described.

The purification levels of the enzymatic protein after each step were analyzed by SDS-PAGE. Enzyme samples were loaded onto a SDSPAGE 8% containing 0.1% (w/v) beechwood xylan. The SDS-PAGE was performed as described in the section electrophoresis and activity staining of *G. thermodenitrificans*.

2.2.4 One-dimensional SDS-PAGE Separation and nano-HPLC-ESI-MS/MS analysis

Enzyme samples (30 µg) were loaded onto a SDS-PAGE 8 %, as previously described. The gel was fixed and stained with Coomassie Brilliant Blue G-250. The resulting gel lanes were sequentially cut into slices (3 mm each) and protein in-gel digestion (Shevchenko et al, 1997) by using 6 ng/µL trypsin or 12 ng/µL chymotrypsin in 50mM NH_4HCO_3 10% acenotrile (ACN) overnight. Reduction and alkylation steps were not performed. Peptides were extracted sequentially using 100 µl 30% ACN 3.5% formic acid (HCOOH), 100 µl 50% ACN 5% HCOOH and 100 µl ACN. The fractions were combined, vacuum dried and analyzed by nanoflow-reversed phase (RP)-highpreformance liquid chromatography (HPLC)-electrospray ionization (ESI)-MS/MS (HPLC-ESI-MS/MS) using the QStar Elite (Applied Biosystems, Foster City, CA, USA) equipped with a nanoflow electrospray ion source and interfaced with an Ultimate 3000 (LCPackings, Sunnyvale, CA, USA) HPLC system. Twenty microliter samples were loaded, purified and concentrated on a RP monolithic precolumn, 200 µm ID × 5mm length (LCPackings, Sunnyvale, CA, USA) at 25 µl/min flow rate. Peptides were separated at a flow rate of 300 µl/min on a PepSwift Monolithic column, 100 µm ID × 5 cm length (LCPackings, Sunnyvale, CA, USA) using the following gradient: solvent A: 2% acetonitrile, 0.1% formic acid solvent B: 98% acetonitrile, 0.1% (v/v) formic acid 5-50% B in 30 min, 50-98% B in 6 s for 10 min. Eluted peptides were analyzed in information dependent acquisition mode using the QSTAR Elite (Applied Biosystems, Foster City, CA, USA) equipped with a nanoflow electrospray ion source. Analyst QS 2.0 software (Applied Biosystems, Foster City, CA, USA) was used with default parameters to generate and to analyze peak lists extracted from information dependent acquisition mass spectra. Mascot v.2.2 (Applied Biosystems, Foster City, CA,

USA) was used for protein identification in the unreviewed set of protein entries that are present in the NCBInr database for all bacteria.

2.2.5 β-Xylosidase purification

The culture supernatant was concentrated 20-fold by ultrafiltration through a YM10 membrane (cut-off 10 kDa) in an Amicon cell (Millipore Merck KGaA, Darmstadt, Germany) and dialyzed against 20 mM sodium phosphate buffer pH 7.5, at 4°C.

The dialyzed enzyme solution was applied, at a flow rate of 0.5 mL min^{-1}, to a Q-Sepharose fast flow column (2.6 x 10 cm) (GE Healthcare, Uppsala, Sweden) previously equilibrated with 20 mM sodium phosphate pH 7.5 (buffer A). The unbound proteins were removed washing the column with the buffer A, and the elution was performed with a linear salt gradient from 0 to 1.0 M NaCl in buffer A, at a flow rate of 1 mL min^{-1}. The eluted fractions, dialyzed against buffer A at 4°C, were checked for the enzymatic activity.

The pool of hemicellulase positive fractions were filtered using a 0.22 µm filter (Millipore Merck KGaA, Darmstadt, Germany). At a flow rate of 0.5 mL min^{-1}, the pool was applied onto a MonoQ HR 5/5 (1 ml) high resolution column (GE Healthcare, Uppsala, Sweden) pre-equilibrated with buffer A. The elution was carried out at a flow rate of 1 mL min^{-1} with a gradient 0-1.0 M NaCl in buffer A in 15 min. The active fractions were pooled, dialyzed against buffer A at 4°C for 16 h. The enzyme solution (200 µL) was loaded onto a gel filtration column Superdex 200 HR 10/30 (Amersham Pharmacia Biotech, Uppsala, Sweden) and the elution was carried out with 20 mM sodium phosphate buffer pH 7.5 containing 150 mM NaCl, at a flow rate of 1 mL min^{-1}. The pool of active fractions represented the purified enzyme.

2.2.6 Enzyme assays

β-Xylosidase activity in the culture supernatant and during the purification procedure was determined, according to Biely et al (2000), using the soluble substrate *p*-nitrophenyl-β-D-xylopyranoside (*p*NPXP). 50 µl of appropriately diluted enzyme were added in 50 mM sodium citrate buffer pH 5.5, to 450 µL of

1.5 mM *p*NPXP, in the same buffer. After heating the assay mixture at 60°C for 20 min, the reaction was stopped by adding 1 mL of 1.0 M sodium carbonate and the release of *p*-nitrophenol was detected by measuring the absorbance at 405 nm. One unit of β-xylosidase was defined as the amount of enzyme required to release 1 µmol of *p*-nitrophenol per min under the assay conditions.

2.2.7 Effect of pH and temperature on xylanase activity and stability

The influence of pH was determined at 60°C by the β-xylosidase standard assay in the range 3.0-9.0 using the following buffers (50 mM): sodium citrate buffer (pH 3.0, 4.0, 4.5, 5.0, 5.5, 6.0), sodium phosphate buffer (pH 6.0, 6.5, 7.0, 7.5, 8.0) and borate-borax buffer (pH 8.0, 8.5, 9.0). The pH stability was evaluated by pre-incubation of the purified enzyme (0.1 mg mL^{-1}) at 40°C at pH 4.0, 5.0, 6.0, 7.0 and 8.0 for 24 h, followed by the residual β-xylosidase activity measurement. The activity obtained without pre-incubation was expressed as 100%.

The effect of temperature was investigated at different values ranging from 20 to 90°C by the standard assay. To determine the thermal stability, the purified β-xylosidase (0.1 mg mL^{-1}) was pre-incubated in 50 mM sodium citrate buffer pH 5.5 at defined temperatures (40, 50, 55, 58, 60, 62, 65, 70°C) for 1, 2, 5, 10 and 24 h. The incubation was followed by the residual β-xylosidase activity assay, above mentioned. The activity obtained without pre-incubation was expressed as 100%.

2.2.8 Effects of metal ions, organic solvents and other chemical reagents

The effect of metal ions and chemical reagents was examined by pre-incubating the xylanase (0.1 mg mL^{-1}) in 50 mM sodium citrate buffer pH 5.5, at 40°C for 1 h, in presence of different mono-, di- and trivalent cations, EDTA, PMSF, SDS and β-mercaptoethanol (2-ME), at the concentrations of 1.0 and 10 mM. Residual activity values were determined using the pNPX method, and expressed as percentage of a control sample incubated in absence of any additive.

The effect of 15% (v/v) organic solvents was investigated by pre-incubating the enzyme, under constant agitation, in presence of different solvents at 25°C for 30, 60 and 120 min. The β-xylosidase activity was determined with the standard assay

method and expressed as percentage of a control pre-incubated without the solvents.

2.2.9 Substrate specificity

The same assay method exploited for β-Xylosidase activity was also used to evaluate α-arabinofuranosidase, α-glucosidase and β-glucosidase activities, with the use *p*-nitrophenyl (*p*NP)-α-L-arabinofuranoside, *p*NP-α-D-glucopyranoside, *p*NP-β-D-glucopyranoside as substrates, respectively. The enzymatic measurements were performed in triplicate.

The presence of xylanase activity in the culture supernatant and during the purification procedure was estimated using a soluble chromogenic xylan as the substrate (Biely et al, 1985). The activity was measured using 2% 4-0-methyl-D-glucurono-D-xylan-Remazol Brilliant Blue R (Azo-Oat Spelt Xylan) as substrate, utilizing of the method described under the section material and methods "enzyme assays of *G. thermodenitrificans*" and incubating substrate and enzyme for 2 h at a temperature of 55°C.

The supernatant and the purified enzyme were checked also for the cellulase activity. The chromogenic cellulase used as substrate was carboxymethyl cellulose-Remazol Brilliant Blue R (Azo-CM-Cellulose). The cellulase activity was measured by adding 150 µL of 2% substrate in 100 mM sodium phosphate buffer pH 7.5 to 150 µL of properly diluted enzyme solution, and incubating at 55°C for 2 h. The reaction was stopped with the addition of 750 µL of 97% precipitant solution, followed by incubation at room temperature for 10 min and centrifugation at 9,000 x *g* for 10 min. The absorbance of the supernatant was read at 590 nm, and the enzyme units were calculated from a standard curve constructed with known amounts of cellulase from *T. viride*.

Part II

2.3 Enzymatic hydrolysis of Ligno Cellulosic Materials

2.3.1 Raw materials

Different agro-industrial raw materials including giant reed (*A. donax*), brewery's spent grains (BSG) and corn cobs were utilized for the saccharification experiments. Untreated *A. donax* biomass was kindly provided by Enea Trisaia Research Center (Italy) and consisted of chips of approximately 3 x 2 x 0.5 cm of size obtained from whole plants, including leaves. The biomass was then dried at 50°C, milled by an homogenizer to obtain a fine powder, and stored in sealed vials at room temperature until use. BSG were provided by Maltovivo micro-brewery, Avellino, Italy and corn cobs were obtained from Companhia das Lezírias (Samora Correia, Portugal). Both materials were dried up at 50°C to a moisture content less than 10% (w/w), milled to particles smaller than 3 mm and stored in sealed vials at room temperature until use.

2.3.2 Enzymatic activity for hydrolysis with commercial enzymes, CelStrep and rPoAbf

The following commercial enzymatic preparations: Accellerase 1500 (cellulase enzyme complex), Accellerase XY (xylanase enzyme complex), and Accellerase BG (β-glucosidase) were kindly provided by Danisco US Inc., Genencor Division (Palo Alto, CA, USA). Cellulase from *Trichoderma reesei* ATCC26921, β-glucosidase from *Aspergillus niger*, Cellobiase from *A. niger*, xylanase from *Trichoderma viride* and thermostable β-xylosidase were purchased from Sigma (St. Louis, MO). The cellulase CelStrep from *Streptomyces sp*. G12 recombinantly expressed in *E. coli* (Amore et al, 2012b), the α-L-arabinofuranosidase from *P. ostreatus* recombinantly expressed in *P. pastoris* (rPoAbf) (Amore et al, 2012a) and a mutant of rPoAbf indicated as rPoAbf F435Y/Y446F (Giacobbe et al, 2014) were utilized in combination with the commercial enzymes.

The commercial enzymatic activities and the CelStrep activity were measured at 50°C in 50 mM sodium acetate buffer (pH 5.0). The cellulase activity was

determined utilizing the soluble chromogenic substrate AZOCM-Cellulose (Megazyme Co., Bray Ireland), as described. The xylanases activities were evaluated by the same assay method utilizing methyl-D-glucurono-D-xylan-Remazol Brilliant Blue R, previously mentioned. The activities of β-xylosidase and β-glucosidase were evaluated by the standard assay using the soluble chromogenic substrate p-nitrophenyl-β-Dxylopyranoside (pNPXP) and pNP-β-D-glucopyranoside, respectively, using the experimental procedure described in the previous sections. The α-L-arabinosidase activity of rPoAbf and rPoAbf F435Y/Y446F was measured as described for the other arabinosidase activities, using pNPAF as substrate in 50 mM sodium acetate buffer pH 5.0, at 50°C for 10 min. All the enzymatic measurements were performed in triplicates.

2.3.3 Pretreatments and determination of chemical composition

The different raw materials, including *A. donax*, BSG and corn cobs, were subjected to a pretreatment with an aqueous ammonia solution (AAS) on a lab-scale as described previously in **"**Saccharification of agro-derived materials" section. The residues were extensively washed with 50 mM sodium acetate buffer or 50 mM sodium phosphate buffer, until obtaining the required pH for the subsequent enzymatic saccharification process (pH 5.0, 6.0 and 7.0, respectively). The biomass from *A. donax* pretreated by steam explosion (Di Pasqua et al., 2014) was also analyzed.

Carbohydrate compositions of the biomass samples untreated and pretreated were determined according to the method of Davis (1998). This procedure involved an acid hydrolysis carried out in two step which fractionate the polysaccharides into their corresponding monomers. First, the samples were soaked in 72% v/v H_2SO_4, at a solid loading of 10%, at 30°C, for 1 h; then the mixtures were diluted to 4% (v/v) H_2SO_4 with distilled water, fucose was added as an internal standard, and the secondary hydrolysis was performed for 1 h at 120°C. After filtration through 0.45 µm Teflon syringe filters (National Scientific, Lawrenceville, GA), the hydrolysis products were analyzed via HPLC as described below. The acid insoluble lignin (Klason lignin) was determined by weighting the dried residue after total removal of the sugars.

2.3.4 Saccharification experiments with commercial enzymes, CelStrep, rPoAbf and its variant

The saccharification experiments were carried out at 50°C for 72 h in a total volume of 5 mL (50 mM sodium acetate buffer pH 5.0 with the addition of the enzyme cocktail), at a solid loading of 5%. The hydrolysis of pretreated BSG, corn cobs and *A. donax* were carried out with enzyme cocktails prepared with the following commercial products at the indicated amounts expressed as units per grams of pretreated biomass: 5.4 U/ gds of Accellerase 1500, 4000 U/ gds of Accellerase XY and 145 U/gds of Accellerase BG (mix 1) or 5.4 U/gds Cellulase from *T. reesei* ATCC26921, 145 U/gds of Cellobiase from *A. niger*, 80U/gds of Xylanase from *T. viride*, 8U/gds of thermostable β-xylosidase (mix 2). Because of its high glucose content, Accellerase BG was dialyzed against 50 mM sodium acetate pH 5.0, before addition to the pretreated biomass.

Subsequent experiments were performed utilizing cellulase CelStrep in each commercial cocktail, mix 1 or mix 2, in substitution of the cellulolytic component and the mixtures then named mix 1CS and mix 2CS, respectively.

The action of rPoAbf and rPoAbfF 435Y/Y446F mutant was evaluated in combination with mix1, mix 2, mix 1CS and mix 2CS. The saccharification mixtures together with blanks (pretreated lignocellulosic materials without enzyme cocktail) were incubated in a rotary thermo-block (Themo Mixer C, from Eppendorf) at 50 °C and 600 rpm, for 72 h. Samples were withdrawn at different time intervals, chilled on ice and centrifuged at 16,500 x g for 30 min at 4°C. The supernatants were

analyzed to quantify the amount of sugars released as described below. The saccharification yield was expressed as percentage of sugar production calculated respect to the amount of sugars present in the dry weight pretreated materials before the hydrolysis processes. The saccharification experiments were run in triplicate.

2.3.5 Saccharification experiments of pre-treated BSG with xylanase from *Bacillus amyloliquefaciens* XR44A

The xylanase from *Bacillus amyloliquefaciens* XR44A (Amore et al, 2015) was provided by Prof. Faraco V. (Department of Chemical Science, University of Naples Federico II). Xylanase assay was performed were evaluated by the same method previously described utilizing methyl-D-glucurono-D-xylan-Remazol Brilliant Blue R, using sodium acetate buffer (50 mM, pH 5.0). Substrate and enzyme were incubated at 40 °C for 5min. The commercial enzymatic activities were assayed as before mentioned. All the enzymatic measurements were performed in triplicates.

The saccharification experiments were carried out at 30°C for 72 h in a total volume of 5mL (50 mM sodium acetate buffer pH 5.0 plus enzyme cocktail), at a solid loading of 5%. The hydrolysis of pretreated BSG was carried out with enzyme cocktails prepared with the following commercial and non commercial products at the indicated amounts expressed as units per gram of pretreated biomass: 5.4 U g^{-1} of cellulase from *Trichoderma reesei* ATCC26921 (Sigma), 145 U g^{-1} of cellobiase from *Aspergillus niger* (Sigma), 1.5 U g^{-1} of xylanase from *Trichoderma viride* (Sigma) or 1.5 U g^{-1} of xylanase from *Bacillus amyloliquefaciens* XR44A, 0.5 U g^{-1} of thermostable β-xylosidase (Sigma), and 145 U g^{-1} of cellobiase from *Aspergillus niger* (Sigma). The saccharification mixtures together with blanks (pretreated lignocellulosic materials without enzyme cocktail) were incubated in a shaking Thermo Mixer C (Eppendorf) at 30°C and 800 rpm, for 72 h. Samples were withdrawn at different times, chilled on ice and centrifuged at 16,500 ×g for 30 min at 4°C. The supernatants were analyzed to quantify the amount of sugars released as described below. The saccharification yield was expressed as percentage of sugar production calculated with respect to the amount of sugars present in the dry weight pretreated materials before the hydrolysis processes. Each saccharification experiment was run in triplicate.

2.3.6 Saccharification experiments of pre-treated BSG with GtXyn from *G. thermodenitrificans*, β-xilosidase from *Anoxybacillus* sp. 3M and arabinosidase from *S. solfataricus*

The xylanase used in the enzymatic hydrolysis, in combination with the commercial enzymes, was GtXyn from *G. thermodenitrificans,* above described; the β-xilosidase (AbXyl) was the purified enzyme from *Anoxybacillus sp.* 3M; the bi-functional β-D-xylosidase/α-L-arabinosidase (SsAra/Xyl) from *S. solfataricus*, recombinantly expressed in *Escherichia coli*, was kindly provided by Dott. Morana A. (Institute of Bioscience and BioResorces, CNR, Napoli). The purification of the bi-functional enzyme was carried out as described by Morana et al, (2007).

The activity of the bi-functional β-D-xylosidase/α-L-arabinosidase from *S. solfataricus* were evaluated utilizing p-nitrophenyl (pNP)-α-L-arabinofuranoside (pNPAF) and pNPXP as the substrates. 50 µl of appropriately diluted enzyme were added in 50 mM sodium phosphate buffer pH 6.5, to 450 µL of 1.5 mM *p*NPXP, or 2.0 mM pNPAF, in the same buffer. The assay mixture was heated at 75°C for 2 min, the reaction was stopped by adding 1 mL of 1.0 M sodium carbonate and the release of *p*-nitrophenol was detected by measuring the absorbance at 405 nm, as above described. The commercial enzymatic activities were assayed above mentioned. All the enzymatic measurements were performed in triplicates.

A typical hydrolysis mixture consisted of 125 mg of BSG, subjected to pretreatment with AAS, in a total volume of 2.5 ml of 50 mM sodium phosphate buffer pH 6.0 plus enzyme cocktail, at a solid loading of 5%. The cocktail enzyme was composed as follow: 5.4 U/gram dry of dry substrate (gds) of Cellulase from *T. reesei* ATCC26921, 145 U/gds of β-glucosidase from *A. niger*, 40 U/gds of xylanase from *G. thermodenitrificans*, 4 U/gds of β-xylosidase from *Anoxybacillus sp*. 3M and 13.6 U/gds of α-L-arabinosidase from *Sulfolobus solfataricus*, supplemented with antibiotics tetracycline (40 µg/ml) and cycloheximide (30 µlg/ml) to prevent any microbial contamination.

The mixture was prepared in a caped tube and was incubated together with blank (pretreated lignocellulosic material without enzyme cocktail) on a rotary thermo-

block (Themo Mixer C, from Eppendorf) at 600 rpm, at temperature and time conditions specified in the following paragraphs Plackett-Burman (PB) design. Samples were taken from the reaction mixture at different time intervals (24, 48 and 72 h) chilled on ice and centrifuged at 16,500 x g for 30 min at 4°C. The supernatant was used for the reducing sugar analysis.

2.3.7 Plackett-Burman (PB) design

A total of 11 (N) variables (columns) including temperature (°C), amount of biomass (%, w/v), pH, concentration of xylanase from *Geobacillus thermodenitrificans*, xylosidase from *Anoxybacillus* and arabinofuranosidase/xylosidase from *Sulfolobus sofataricus*, and 5 unassigned variables (dummy) were studied in 12 (N+1) experiments (rows). Each variable was examined at two levels, high and low denoted by (+1) and (−1) signs, respectively (Table 7). Also 3 centre points were included, in which the medium level with the code (0) was considered. The values for the variables were chosen taking into account the results of preliminary assays and the possibility of enzyme inactivation or denaturation at some experimental conditions.

The main effect of each variable was determined using to the following equation:

$E_{xi} = (\Sigma M_{i+} - \Sigma M_{i-}) / n$

where E_{xi} is the variable main effect, M_{i+} and M_{i-} are the sum of the responses in runs, in which the independent variable (xi) was present in high and low levels, respectively, while n is the half number of runs considered Stowe and Mayer (1966). If the main effect of the tested variable is positive, it means that the influence of the concerning variable is greater at the high level tested, and when it is negative, the influence of the given variable is greater at the low level. The significance of each variable was determined by applying the Student t-test. The regression coefficients, analysis of variance (ANOVA), *p* and F values were used to estimate the statistical parameters employed by the same software. ANOVA table consists of calculations that provide information about levels of variability within a regression model and form a basis for tests of significance.

Table 7. Levels decoded of the tested parameters in the Plackett-Burman Screening Design.

	Levels		
Variables	**-1**	**0**	**+1**
Temperature (°C)	50	55	60
% Biomass (w/v)	3	5	7
pH	5	6	7
GtXyn (U/gds)	0	40	80
AbXyl (U/gds)	0	4	8
SsAra/Xyl (U/gds)	0	13.6	27.2

2.3.8 Determination of sugar content

For estimation of the sugars released from untreated and pretreated biomasses, obtained as described in "Pretreatments and determination of chemical composition" and enzymatic hydrolysates prepared as previously described, the cleared supernatants were opportunely diluted, and analyzed by a high-performance liquid chromatographic (HPLC) system (Dionex, Sunnyvale, CA, USA), equipped with an anionic exchange column (Carbopac PA-100) and a pulsed electrochemical detector.

Glucose, xylose and arabinose were separated with 16 mM sodium hydroxide at a flow rate of 0.25 mL/min, and identified by the respective standards. Fucose was used as internal standard.

3. Results and Discussion

Part I

Isolation, purification and characterization of novel hemicellulolytic activities.

3.1 *Geobacillus thermodenitrificans* A333 endo-xylanase purification characterization and cloning

3.1.1 Identification, production and localization of the xylanase activity from *Geobacillus thermodenitrificans*

Several extracellular xylanases from bacteria of the genus *Geobacillus* have been studied and in particular three strains identified as *Geobacillus thermodenitrificans* were reported as extracellular xylanase producers (Anand et al, 2013; Canakci et al, 2007; Gerasimova and Kuisiene, 2012). The detailed characterization of the native *Geobacillus thermodenitrificans* A333 xylanase, called GtXyn, reported in this study, allows to appreciate the relevant biotechnological characteristics of such a biocatalyst. The data reported in this thesis also highlight that the catalytic properties of GtXyn are quite different from those of the recombinant endo-xylanase studied by Gerasimova and Kuisiene (2012), whose primary sequence differed in only two amino acids from that of the enzyme produced by the wild type strain A333 of *G. thermodenitrificans.*

The identification of the xylanase activity started with the growth of the selected microorganism *G. thermodenitrificans* A333, that was gradually adapted to utilize the xylan as a carbon source onto solid media. On plates with 0.15% (w/v) xylan, 37 - 42 colonies were obtained by streaking and, after staining with Congo Red, 15-20 colonies surrounded by clear halos of 5 to 15 mm diameter were detected confirming the microorganism capability to hydrolyze xylan. The xylanase production was also investigated by growing *G. thermodenitrificans* A333 in liquid basal medium supplemented with 0.15% (w/v) beechwood xylan. The analysis of the time-course profile revealed that the extracellular xylanase production was not related to the growth and that the enzyme expression started in the exponential phase, reaching the highest level in the late stationary phase, when

the microorganism utilized the xylan as carbon source only after the depletion of the primary sources (Figure 7).

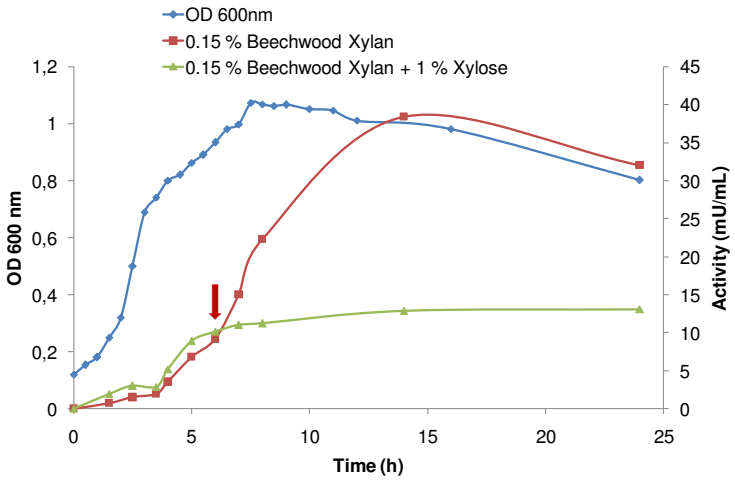

Fig. 7: Xylanase production. Time-course of *G. thermodenitrificans* A333 grown in NB liquid medium supplemented with 0.15% beechwood xylan at 60°C. Red arrow indicates the time of xylose addition to a culture of *G. thermodenitrificans* A333 grown in presence of 0.15% beechwood xylan

The localization of xylanase was investigated in cultures of *G. thermodenitrificans* A333, grown on beechwood xylan by measuring the activity in the cell-free supernatant, crude extract and membrane fragments. The activity was retrieved almost exclusively in the cell-free supernatant (94.2%), while the intracellular and cell bound levels were 4.2 and 1.6%, respectively. This result is in complete agreement with Signal IP analysis of the protein primary sequence (Nielsen et al, 1999) that indicated the presence of a signal peptide for the amino acid positions 1–28, thus attesting that the xylanase is a secreted enzyme, as many microorganisms with (hemi)cellulolitic activities (Biely, 1985).

The induction is a complex mechanism, especially when the substrate acts also as the inductor. The identification of the inductor which allows to obtain the higher levels of enzyme production is not always immediate; often the molecules derived from the substrate or the end products of its hydrolysis can act as inducers or repressors, also depending from the different concentrations. In several bacteria and yeasts, xylanolytic enzymes appear to be inducible: xylanase and xylosidase are produced in high amounts during growth on xylan and synthesis of the

enzyme is subject to catabolite repression by well metabolite such as glucose or xylose. Xylan cannot cross the cells, so that the signal for accelerated synthesis of xylanolytic enzymes must involve lower molecular weight fragments. The oligosaccharides are formed by hydrolysis of xylan in the medium by tiny amounts of enzymes (Biely, 1985).

In this study the utilization of monosaccharides such as 0.1% (w/v) xylose, as supplement to basal medium, increased by 3.5-fold the enzymatic activity production that was further enhanced by xylan (Fig. 8). In particular, the effect of the three different xylan types was tested and the best result was reached with the use of oat spelt xylan, that yielded a level of xylanase expression 6-fold higher than the basal one (7.24 mU/mL and 43.57 mU/mL were the maximum activities with NB and oat spelt xylan, respectively). These findings clearly indicate that the xylanase is produced at significant amounts only in presence of inducers. Owing to the commercially unavailability of the oat spelt xylan, we were forced to utilize beechwood xylan for GtXyn production. Xylose gradually lost its function of inducer at increasing concentrations and only a basal xylanase production was achieved at 1% (w/v) (data not shown). Furthermore, the addition of 1% xylose, during the exponential phase of *G. thermodenitrificans* A333 growth carried out in presence of 0.15% beechwood xylan, blocked the xylanase production (Fig. 7). Glucose demonstrated to repress the xylanase expression (data not shown).

The investigation on the GtXyn production highlighted the microorganism capability to utilize several cheap and widely available carbon sources, such as brewery's spent grains, corn cobs, wheat straw and grape cane for the bacterial growth and enzyme secretion. Xylanase production, in presence of the before mentioned agricultural wastes, showed an enhanced activity concentrations respect to those reached with the commercial xylan preparations (239, 171, 258 and 351 mU/mL obtained in presence of BSG, grape cane, corn cobs and wheat straw, respectively). This outcome would allow to exploit these raw materials in a suitable fermentation processes, instead of the commercially available xylans, to reduce the costs of the biocatalyst hyper-production.

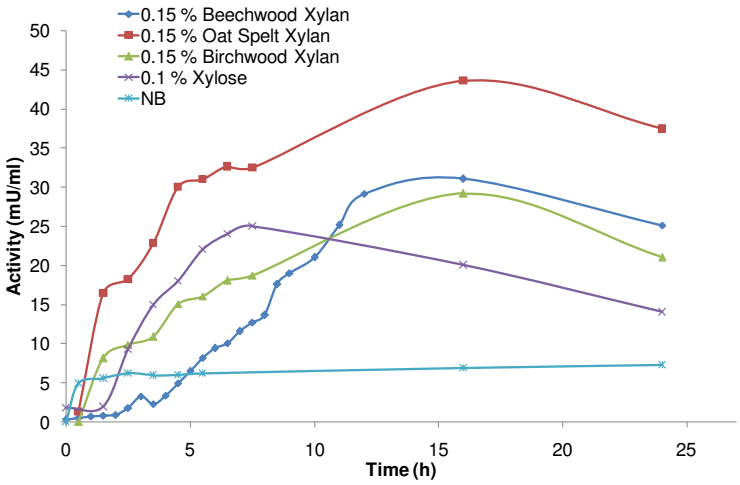

Fig. 8: Effect of different carbon sources on GtXyn production

3.1.2 Purification of the *G. thermodenitrificans* A333 xylanase

A high enzyme purity, even if it is generally not important for industrial applications due to economic reasons, is relevant to investigate on the enzyme properties.

The purification procedure of the xylanase is summarized in Table 8. The cell-free supernatant from 2 L culture, 20-fold concentrated and dialyzed, was tentatively absorbed onto different resins but successful results were only obtained with the strong cation exchange matrix.

Table 8. Purification procedure of the xylanase from *G. thermodenitrificans* A333

Purification steps[a]	Volume (mL)	Total Units (U)[b]	Proteins (mg)[c]	Specific Activity (U/mg)	Yield (%)	Purification fold
Crude broth	2000	37.83	333.4	0.114	100	1
Ultrafiltration	100	29.5	238	0.124	78	1.1
SP-Sepharose	20	17.14	75.17	0.23	45.3	2.01
Resource S	1	7.39	2.78	2.66	20	23.3
Superdex 75	1	5.05	0.65	7.78	13.3	68.2

[a]Purification was performed utilizing the cell-free supernatant from 2 L culture of *G. thermodenitrificans* A333 obtained in liquid basal medium supplemented with 0.15 % (w/v) beechwood xylan

[b]Xylanase activity was measured as described in Materials and Methods.

[c]Measured by Bradford method using bovine serum albumine as a standard (Bradford, 1976).

Therefore, the first two separation steps were performed on SP-Sepharose and Resource S columns, the latter characterized by a higher resolution capacity, allowed to increase of 11-folds the purification factor being. The last chromatographic step, carried out by a molecular exclusion chromatography on Superdex 75 HR10/30, allowed to obtain a specific activity of 7.78 U/mg, with a purification factor of 68.2.

As judged by SDS-PAGE and zymographic analyses, the xylanase showed only a single band, confirming its homogeneity (Fig. 9). A molecular mass of about 44 kDa was calculated. Among the xylanses of *G. thermodenitrificans* previously cited, GtXyn differs only from the molecular weight of the partially characterized enzyme by Canakci et al. (2007), that was 30 kDa in size. Moreover, the xylanase was active as confirmed by the clear band on the zymogram gel after the Congo red staining. The molecular mass of 44 KDa was also confirmed by gel filtration chromatography, allowing to determine a monomeric structure for the enzyme.

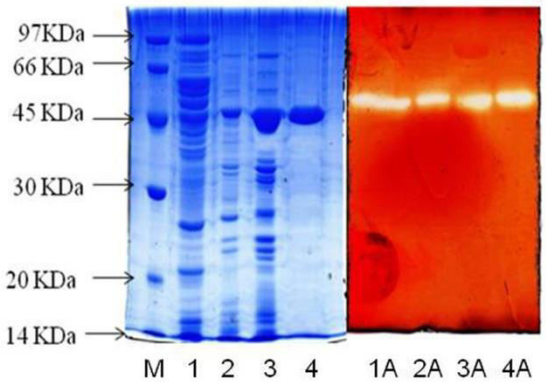

Fig. 9: 10% SDS-PAGE followed by Coomassie Blue staining and zymogram. *Lanes*: protein markers **(M)**; cell-free supernatant **(1, 1A)**; pooled fractions after SP-Sepharose **(2, 2A)**; pooled fractions after Resource S **(3, 3A)**; pooled fractions after Superdex 75 **(4, 4A)**.

3.1.3 Primary sequence analysis

The identified 25 residues of the GtXyn N-terminal sequence showed a perfect match to the amino acid sequence of the extracellular endo-xylanase from *Geobacillus thermodenitrificans* NG80-2. Consequently, the gene sequence encoding this protein (NC009328) was utilized for the construction of the primers to amplify the full-length xylanase gene from *G. thermodenitrificans* A333. The

complete recombinant gene of 1224 bp size, starting with ATG and with TAG as stop codon, was obtained. It encoded a 407 amino acid residue protein, whose deduced sequence revealed the highest homology with xylanases from *Geobacillus* strains belonging to the GH10 (www.cazy.org, Cantarel et al, 2009), as almost known xylanase from geobacilli, such as *Geobacillus thermodenitrificans* JK1 (GenBank Accession No AEP39603, identity 99%), *Geobacillus thermodenitrificans* NG80-2 (GenBank Accession No YP001125870, identity 99%), *Geobacillus* sp. TC-W7 (GenBank Accession No ACX42569, identity 99%), *Geobacillus* sp. 71 (GenBank Accession No AEO96821, 94% identity) *Geobacillus kaustophilus* (GenBank Accession No WP02027929, identity 94%), *Geobacillus* sp. C56-T3 (GenBank Accession No YP003671234, identity 91%), and *Geobacillus stearothermophilus* (GenBank Accession No ABI49951, identity 86%).

The analysis with Pfam program confirmed for GtXyn the presence of the family GH10 catalytic domain, containing at the positions 230 and 293 two glutamic acids as catalytic residues. Furthermore, it was evidenced that GtXyn lacks of a substrate binding domain and bear a N-terminal stretch of 28 residues recognized as a signal peptide, in full agreement with the outcome from SignalIP. The theoretical protein molecular weight of 47.3 kDa with the signal sequence and 44.2 kDa without it, was in accordance with the previously analysis of the purified xylanase.

3.1.4 Influence of pH and temperature on enzyme activity and stability

The xylanase purified from *G. thermodenitrificans* A333 presents similar optimal pH and temperature of the xylanase from geobacilli, belonging to the family GH10 (Canakci et al, 2007; Canakci et al, 2012; Khasin et al, 1993; Liu et al, 2012 ; Wu et al, 2006). In details, the pH optimum for the xylanase was found between 7.0 and 7.5 with an appreciable level of stability in wide intervals around these values, even if GtXyn was active over the whole pH range 5.0-11.0 (Fig. 10). In particular, the enzyme was more tolerant to alkaline conditions than to the acidic ones, showing 75.6% of the maximal activity at pH 10.0 and 23% at pH 6.0. The stability was also pronounced at alkaline pHs as demonstrated by residual

activity values of 97, 95 and 75% at pH 8.0, 9.0 and 10.0, respectively after 24 h of incubation at 50°C.

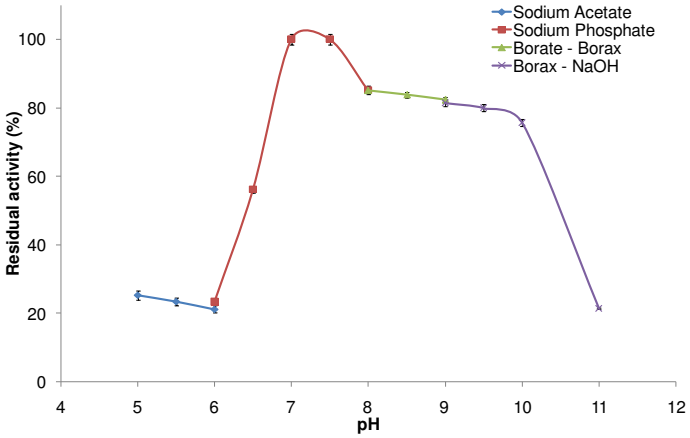

Fig. 10: Effect of pH on purified GtXyn activity. All the activity values, measured at 50°C, are means of three replicates ±SD

The enzymatic activity was detected over a broad range of temperature (20-90°C) and showed its maximum at 70°C. Moreover, high activity levels were observed between 60 and 80°C, with values comprised between 80 and 50% of the maximal activity at the extremes of this temperature range (Fig. 11). Thermostability was also evaluated by measuring the residual activity after 60 min of incubation at increasing temperatures, as described under "Materials and methods". The temperature at which 50% of the enzyme was inactivated was 70°C, while the residual activity values at 58, 60, 62 and 65°C were 98, 95, 88 and 83% respectively.

The relevant GtXyn resistance to thermo-alkaline conditions is comparable to that reported for other xylanases (Canakci et al, 2012; Gerasimova and Kuisiene 2012; Kumar and Satyanarayana 2011; Liu et al, 2012; Verma and Satyanarayana 2012; Wu et al, 2006). This is a crucial characteristic that makes the enzyme suitable for several industrial applications.

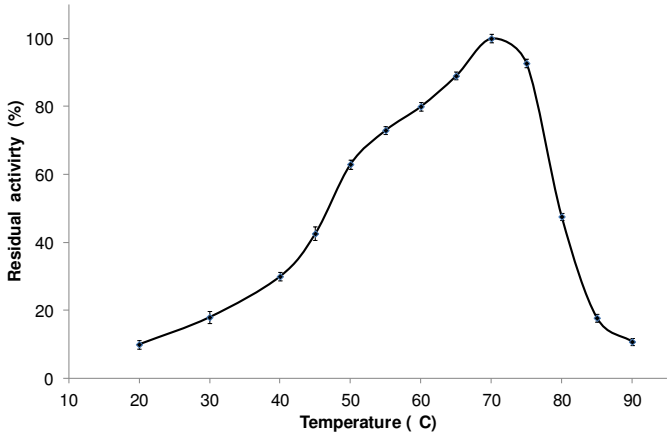

Fig. 11: Effect of temperature on purified GtXyn. Thermophilicity of the purified enzyme was estimated in presence of the substrate. All the activity values, measured at pH 7.5, are means of three replicates ±SD

Kinetic parameters of GtXyn thermal inactivation were determined by subjecting the enzyme to incubations at different temperatures in the range 58-70°C, and measuring the residual activity under standard conditions. As observed in Fig. 12, the plots of the GtXyn residual activity versus the incubation time were linear, allowing the expression of the thermal inactivation with a first order kinetic.

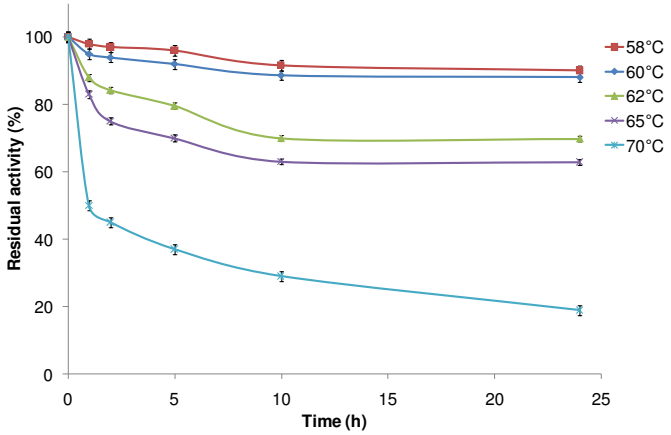

Fig. 12: Effect of temperature on purified GtXyn. The percent residual activity values were plotted against the corresponding incubation times. All the activity values, measured at pH 7.5, are means of three replicates ±SD

The results concerning the inactivation rate constants and the xylanase half-lives at different temperatures are presented in Table 9. In overall, it can be observed that GtXyn is characterized by a good thermal stability showing at 58 and 60°C a

residual activity of 90 and 88% after 24 h of incubation and half-life of 94.6 and 73 h, respectively.

Table 9. Inactivation kinetic parameters of purified GtXyn

Temperature (°C)	K_d (h^{-1})	$t_{1/2}$ (h)
58	0.0074	94.6
60	0.0096	72.92
62	0.0333	21.02
65	0.0474	14.77
70	0.184	3.76

K_d = Thermal inactivation rate constant
$t_{1/2}$ = Half-life

3.1.5 Effect of metal ions and chemical reagents on xylanase

Impurities such as metal ions, which are present in the agro-industrial wastes, can potentially inhibit the activity of xylanases. The influence of metal ions either as chloride or sulfate salts and of the metal chelator EDTA were investigated at final concentrations of 1.0 and 10 mM. As reported in Table 10, monovalent cations, such as Na^+ and Li^+, did not significantly affect the enzymatic activity. On the contrary, K^+ exhibited a marked stimulating effect, at both 1.0 and 10 mM concentrations, enhancing the activity, as chloride salt, by 42 and 45%, respectively. Bivalent cations such as Ba^{2+} did not substantially influence the enzyme, while an activity decrease up to 29 and 36% with Ca^{2+} and Mg^{2+}, respectively was observed. Transition metals such as Ag^+, Cu^{2+}, Ni^{2+}, Zn^{2+}, Mn^{2+}, Cd^{2+}, Al^{3+} slightly affected the enzyme leading to modifications of the activity within 25%, while Fe^{2+}, Fe^{3+}, Hg^{2+} and Co^{2+} had a marked impact on the activity. In particular, Fe ions caused an almost complete inhibition at 10 mM. Conversely, Hg^{2+} and Co^{2+} highly lowered the activity at both concentrations, with Hg^{2+} which exhibited a total inhibition effect and Co^{2+} that caused an activity reduction up to 91%. EDTA did not affected enzyme activity, indicating that metal ions neither are required nor substantially influence the xylanase activity. Although the activity of GtXyn was not significantly influenced in the presence of EDTA and several metal ions, K^+, Hg^{2+}, Co^{2+}, Fe^{2+} and Fe^{3+} highly affected its activity. In particular, K^+ markedly stimulated the enzyme, probably due to the role of this

metal ion as cofactor for enzyme-substrate reactions or its stabilizing effect on the protein molecular structure. On the other hand, GtXyn was strongly inhibited by Hg^{2+}, Co^{2+}, Fe^{2+} and Fe^{3+}, probably for the oxidizing nature of these metal ions (Vieille and Zeikus, 2001).

Table 10. Effect of metal ions and various reagents on the purified GtXyn

Metal ions or chemical agents	Residual activity (%) 1 mM	10 mM
EDTA	121	104
NaCl	122	106
Na_2SO_4	107	102
KCl	142	145
K_2SO_4	132	127
LiCl	93	99
$CaCl_2$	90	90
$CaSO_4$	97	71
$MgCl_2$	72	70
$MgSO_4$	67	64
$BaCl_2$	116	92
$HgCl_2$	0	0
$CoCl_2$	42	9
$CoSO_4$	30	15
$FeCl_3$	91	7.6
$FeSO_4$	80	3
$CuCl_2$	75	81
$CuSO_4$	79	76
$NiCl_2$	99	94
$NiSO_4$	91	101
$ZnSO_4$	80	80
$AlCl_3$	125	86
$CdCl_2$	96	81
$MnCl_2$	90	89
$AgNO_3$	105	89
PMSF	89	92
2-ME	105	113
Urea[a]	56	

The effect of different reagent was determined by pre-incubating the purified GtXyn in100 mM phosphate buffer at 60 °C and pH 7.5 in presence of the listed substances for 1 h. The activity of GtXyn, measured in the absence of any substance, was set as 100 %.Values are means of three experiments.

[a] Urea was tested at concentration of 1%.

The effect of potential inhibitors or activators such as PMSF, 2-ME and urea was also examined. PMSF and 2-ME did not substantially affect the enzymatic activity, while urea inhibited GtXyn by 44%.

The xylanase halo-tolerance was evaluated revealing that, after 1 h incubation at 60°C in presence of increasing NaCl concentrations (1.0-3.0 M), the activity underwent a 30% reduction (data not shown).

The influence of various detergents at 1% concentration on the GtXyn activity was investigated (Table 11). The xylanase in the presence of non-ionic surfactants was highly activated. Upon the addition of Triton X-100, Tween 20 and 80 and PEG 8000, an activity increase up to 54% was observed. The sole exception was represented by Nonidet P40 that showed a slight inhibition effect, reducing the activity by 25%. In contrast, considerable activity losses with ionic surfactants were detected: in presence of CTAB, an activity decrease of 86% was observed while SDS, tested at 1.0 and 10 mM final concentrations, gave activity reductions of 13 and 79%, respectively. Finally, the zwitterionic detergent CHAPS did not affect the activity.

Table 11. GtXyn stability in surfactants

Surfactants (1%)	**Residual activity (%)**
Tween 20	132
Tween 80	152
Triton x100	154
PEG 8000	137
Nonidet P40	75
CHAPS	106
CTAB	14
SDS (1-10 mM)	87 - 21

The purified enzyme was treated with the indicated concentrations of the effectors for 1 h at 60°C.
The activity of GtXyn, measured in the absence of any substance, was set as 100 %.
Values aremeans of three experiments

GtXyn was among the few reported xylanases endowed with the capability to tolerate high salinity levels, solvents and surfactants (Woldesenbet et al, 2012; Zhou et al, 2012). GtXyn was able to retain 70% of its activity for 1 h on presence of 3.0 M NaCl, showing a salt tolerance comparable to that of xylanases isolated from microorganisms harbored in marine or saline habitats (Guo et al, 2009; Khandeparker et al, 2011; Menon et al, 2010). For this reason the enzyme could be utilized in the processing of sea and saline food and in the production of

bioethanol from seaweeds. The marked halostability also accounts for the GtXyn resistance to the organic solvents (Sellek and Chaudhuri, 1999).

The time resistance of GtXyn to organic solvents with different log P values, at 15% (v/v) final concentration, was evaluated (Table 12). The enzyme was not substantially influenced by most of the tested solvents even after 2 h of incubation. GtXyn activity reductions only of 15, 13 and 8%, were detected in presence of DMSO, acetonitrile and 2-propanol, respectively, while *n*-butanol enhanced the activity by 14%. Formaldehyde and glycerol markedly inhibited the enzyme; after 2 h of incubation an activity reductions of 71% and 62%, respectively, were detected. The data also demonstrated that GtXyn stability in organic solvents did not follow log P trend.

Table 12. GtXyn stability in organic solvents

Solvents (15% v/v)	**logP**	**Residual activity (%)**		
		30 min	**60 min**	**120 min**
Ethanol	-0.3	100	94	96
Methanol	-0.76	100	94	99
Acetone	-0.24	92	97	97
Acetonitrile	-0.34	98	92	87
Ethyl acetate	0.71	100	98	98
2-propanol	0.07	99	95	92
DMSO	-1.35	96	88	85
DMF	-1.04	100	99	99
n-Butanol	0.88	100	100	114
Formaldehyde	0.35	60	42	29
Glycerol	-2.55	58	46	38

The purified enzyme was treated with various solvents for different intervals of time. The activity of GtXyn, measured in the absence of any substance, was set as 100 %.
Values are means of three experiments

The effect of the addition of different ethanol amounts was investigated, indicating that the enzyme was marginally influenced by the exposition, for 30 min, to increasing concentrations of this organic solvent (Fig. 13). In fact, GtXyn, underwent a weak activation up to 20% ethanol and then a gradual slight inhibition that led to a residual activity of 70% in presence of 80% ethanol. GtXyn activation was also evident upon prolonged times of incubation (1, 2, 5 and 24 h) in presence of 40% ethanol, reaching the highest levels after 1 and 2 h, with values of residual activity around 120 and 118%, respectively (data not shown).

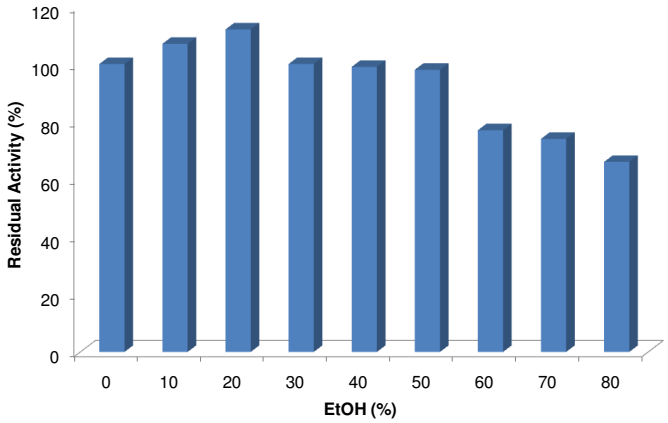

Fig. 13: Effect on GtXyn activity of different ethanol concentrations. Each value represents the mean of triplicate measurements.

The marked resistance to ethanol makes GtXyn suitable for different applications, included those for production of the alcoholic beverages. As reported by Sato et al. (2010), the addition of xylan-degrading enzymes during the simultaneous hydrolysis and fermentation process of rice starch, significantly enhanced the alcohol yield in the sake preparation. GtXyn can also be regarded as a promising candidate for bioethanol production in consolidated bioprocessing due to the enzyme capability to remain fully active at ethanol concentrations of 23–63 g/L, that are normally reached in these industrial processes (Morrison et al, 2011). Moreover, the positive effect of non ionic detergents on its activity could also allows to improve the rate and extent of lignocellulosic substrate hydrolysis (Kaya et al, 1995).

3.1.6 Protease resistance

The purified GtXyn exhibited good resistance to neutral and alkaline proteases as demonstrated by the residual activity values detected upon incubation up to 3 h with 10 µg/mL of trypsin, chymotrypsin, collagenase, subtilisin A and proteinase K. In particular, GtXyn activity remained almost unchanged after pre-incubation for 2 h with collagenase and for 1 h with trypsin, while, after 1 h, the residual activity was 90 % with subtilisin A and proteinase K, and 80 % with chymotrypsin. A residual activity of 80 % was also registered after 3 h in presence

of all the tested proteases with the sole exception of chymotrypsin that gave a reduction of 38 %.

The protease-resistance showed by GtXyn could be explained with the enzyme extracellular localization and thermostability (Fontes et al, 1995), even if the mechanism underlying this behavior is not completely clear. This relevant characteristic makes GtXyn useful for potential applications in textile and food industry for cotton scouring and improvement of animal feed (Li et al, 2008).

3.1.7 Substrate specificity

The substrate specificity of the purified xylanase using various natural and synthetic substrates was tested. GtXyn acted only on xylans and lacked of activity towards both amorphous and crystalline cellulose represented by carboxymethylcellulose and Avicel, respectively, suggesting that the enzyme is a cellulase-free xylanase. The enzyme failed also to hydrolyze the natural glucose formed polysaccharides lichenan and laminarin, and was unable to breakdown cell wall components such as mannan, xyloglucan and arabinan. Respect to the xylan hydrolysis, GtXyn did not show a marked specificity towards one of the three natural xylans tested, but only a slight preference towards beechwood xylan was evidenced as demonstrated by the activity values of 6.9, 7.10 and 8.76 U/mg in presence of birchwood, oat spelt and beechwood xylan, respectively. Moreover, no detectable α-arabinofuranosidase, β-xylosidase, α- and β-glucosidase activities were observed, since GtXyn was unable to hydrolyze the pNP-glycosides utilized as respective substrates (data not shown). These results clearly highlight the strict specificity of GtXyn toward polysaccharides containing 1,4 β-xylan bonds.

These characteristics, in addition with others previously described, make relevant GtXyn applicability in the pulp and paper industry. Due to the resistance to alkaline pHs and the cellulase-free activity, the biocatalyst utilization before the bleaching kraft pulp process would reduce the chlorine consumption up to 25–35 % and avoid time/cost consuming pH adjustments (Archana and Satyanarayana, 1997; Sharma et al, 2007) and the cellulose degradation. The possibility to utilize the xylanase in presence of detergents could also allow to

obtain better results than those reached with the traditional chemical methods in the process of paper pulping and deinking of recovered paper (Kaya et al, 1995).

3.1.8 Analysis of hydrolysis products and saccharification of agro-derived biomass

The mode of action of the purified GtXyn was determined using xylooligosaccharides (X_2–X_5) as test substrates and analyzing the hydrolysis products by TLC (Fig. 12a). X_2 was not hydrolyzed even after 48 h of incubation, X_3 was slowly transformed, whereas X_4 and X_5 were rapidly degraded. Most of the X_3 was converted to X_2 upon 24 h of incubation and, after 48 h, detectable quantities of X_3 in conjunction with negligible amounts of xylose were still observed. X_4 was already hydrolyzed after 30 min of incubation and the prevalent product, X_2, was accompanied by low amounts of xylose. X_5 disappeared after 30 min of incubation producing X_2 and X_3. The disappearance of the X_3, simultaneously to the presence in trace of xylose, was observed after 48 h of incubation. The xylanase preferentially cleaves the internal glycoside bonds of the oligosaccharides, as suggested by the appearance of relatively high concentrations of X_2 and X_3, in the X_3, X_4 and X_5 reaction mixtures. It can, therefore, be concluded that GtXyn is a typical endo-β-1,4-xylanase.

Beechwood xylan was treated with the purified GtXyn and incubated up to 72 h (Fig. 12b). The main reaction products were X_5, X_3 and X_2, with a prevalence of X_2 upon long time incubation. After few seconds GtXyn was already able to produce xylooligosaccharides with DP comprised between 6 and 2 but X_2, X_3 and X_5 were the main products observed after 2 h. X_3 completely disappeared at 48 h and only X_2 and X_5 were detected together with minimal amounts of xylose; this situation remained unaltered up to 72 h. The only difference with the results obtained from the hydrolysis of xylooligosaccharides, is represented by the presence of X_5 even after 72 h of incubation. It could be due to substituents moyeties on the xylan backbone, that would hinder the GtXyn attack.

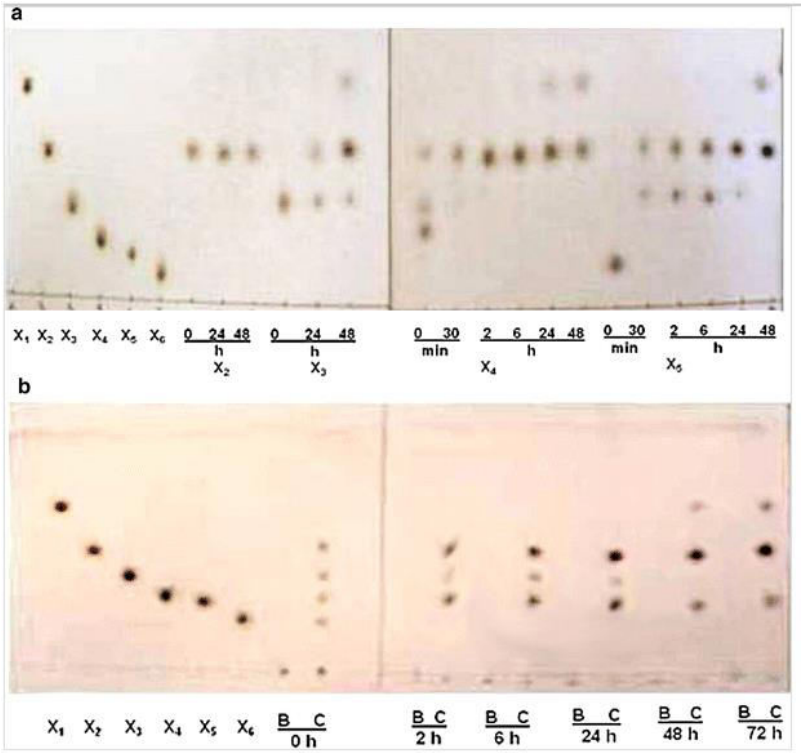

Fig. 12: Time course of xylooligosaccharides (a) and beechwood xylan (b) hydrolysis. Standards: xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5) and xylohesaose (X6). In b B (beechwood xylan incubated without GtXyn), C (beechwood xylan incubated with GtXyn).

The saccharification of agro-derived biomasses (BSG, corn cobs and *Arundo donax*), pretreated by soaking in aqueous ammonia solution, was carried with the purified GtXyn. The end-products profiles, analyzed by TLC, were identical to that obtained with the beechwood xylan (data not shown). On the contrary, the evaluation of the reducing sugars released revealed that the three tested materials reached different degrees of xylan hydrolysis (Table 13). Comparable levels of xylan conversion were observed with BSG and corn cobs after 6 and 24 h of reaction, but upon prolonged incubation times different results were achieved. Xylan hydrolysis percentage from BSG did not exceeded the 32 %, reached after 24 h of incubation. Conversely, the percentage of xylan degradation with corn cobs reached the 68.6 % after 72 h and was double respect to that obtained after 24 h. The percentage of xylan degradation with *A. donax*, after 72 h incubation, was very low.

Table 13. Hydrolysis of agro-derived materials by purified GtXyn

	Incubation time (h)	Reducing sugar yield[a] (mg xylose equivalent mL^{-1})	Xylan hydrolysis (%)
Arundo donax	0	0.17	1.85
	6	0.24	2.61
	24	0.52	5.65
	48	0.46	5.00
	72	0.66	7.17
BSG	0	0.02	0.16
	6	2.61	21.7
	24	3.83	31.9
	48	4.06	33.8
	72	3.8	31.6
Corn Cobs	0	0.09	0.51
	6	4.8	27.4
	24	6.2	35.4
	48	10.1	57.7
	72	12.0	68.6

[a]Reducing sugars released in the incubation mixtures were estimated by the Somogyi-Nelson assay.

The use of agricultural residues and non-food crops as alternative cheap substrates has considerable potentialities for the cost-effective XOS synthesis. For this purpose, corn cobs, BSG and the biomass from *A. donax* were utilized for saccharification experiments with GtXyn. The first two materials are agro-industrial by-products characterized by a high content of xylan, that accounts for the 35 and 24.1 % of their dry weights, respectively. *A. donax* crop is one of the highest yielding species for biomass production in southern Europe, with a xylan content of 20 % of its dry weight (Scordia et al, 2011). It has also been recognized as very robust species, able to grow well on marginal, polluted and non-agricultural soils, thus avoiding the competition with food crops for lands (Krasuska et al, 2010). As frequently reported in literature, in order to improve the xylan digestibility, the raw materials are subjected to a pretreatment to remove the lignin fraction that shields the cell wall polysaccharides from the enzymatic attack. In this work the pretreatment was realized by soaking the LCMs in aqueous ammonia solution, due to the high efficacy of this method in breaking the ester bonds between lignin, hemicellulose and cellulose without degrading the

hemicellulose polymers, as confirmed by Maurelli et al. (2013). Among the three tested materials, corn cobs, that is characterized by the most consistent xylan content, reached the highest percentage of xylan hydrolysis and the best yield of reducing sugar released after 72 h. On the other hand, for BSG, the polysaccharide conversion process was completed in the shortest reaction time, namely 24 h, probably due to its lowest lignin content.

In conclusion, GtXyn, owing to its mechanism of action and alkali-thermostable nature, is suitable for the production of XOS, known to posses many beneficial biomedical and health effects, such as the stimulation of the human intestinal Bifidobacteria and Lactobacilli growth (Moure et al, 2006; Vazquez et al, 2000; Yang et al, 2005). The analysis of the hydrolysis end products, obtained from the saccharification of beechwood xylan and the three agro-derived feedstocks, revealed the presence of XOS with a DP comprised between 6 and 2, with a prevalence of xylobiose upon long times of reaction. The presence of only minimal amount of xylose suggests a profitable GtXyn applicability in the bioconversion of LCMs into XOS. Currently, XOS are mainly obtained by enzymatic hydrolysis due to the high specificity and negligible side product generation (Tan et al, 2008); however, the high cost of production requires further developments in the production processes. XOS preparation is generally performed using neutral xylanases but the employment of alkaline enzymes, such as GtXyn, may improve the yield due to the higher solubility of xylan in alkaline solutions (Mamo et al, 2007).

3.1.9 Cloning and expression of recombinant xylanase

To exploit bacterial thermostable (hemi)cellulases for industrial applications, over-expression of these enzymes is desirable. There are several published reports where *E. coli* has been used as heterologous host. For example, thermostable endo-xylanases from *Geobacillus sp*. MT-1 (Wu et al, 2006), *Geobacillus sp*. TC-W7 (Liu et al, 2012) and *Geobacillus thermoleovorans* (Verma and Satyanarayana, 2012) have successfully been over expressed in *E. coli*.

Therefore, in this study, the *G. thermodenitrificans* A333 endo-xylanase encoding gene (1149 bp) was cloned into pET 28c(+) and successfully over-expressed in *E.*

coli BL21 (DE3), obtaining a 73-fold increase in activity (369.45 and 5.05.U/mL for the recombinant and the native protein, respectively).

After induction with IPTG, cell homogenate was centrifuged and the supernatant was heat precipitated and applied to a nickel affinity resin (His Trap). The His-tagged xylanase was eluted at 200 mM Imidazole. A single band of about 45 kDa was observed for the purified endo-xylanase on SDS–PAGE, after staining with Coomassie Blue which corresponded to a clear band obtained by zymogram analysis (data not shown). The partial and in progress characterization of the cloned enzyme, with the same methods and conditions utilizing for the native GtXyn, revealed no significant differences from the native purified protein.

3.2 Isolation,purification and characterization of a β-xylosidase from *Anoxybacillus* sp. 3M

3.2.1 Identification and production of the β-xylosidase activity from *Anoxybacillus* sp. 3M

In the last years numeros hemicellulolytic activities, belonging to Bacillus species, have been studied. Investigation on lignocellulolytic enzymes produced by *Anoxybacillus* sp. are relatively new and limited compared to those on similar biocatalysts from fungi and other bacteria. Xylanolytic activity was observed in *A. flavithermus* BC (Kambourova et al, 2007), *A. pushchinoensis* A8 (Kacagan et al, 2008), *Anoxybacillus sp*. E2 (Wang et al, 2010), and several strains isolated from Turkish hot springs (İnan et al, 2011).

There are only few identified and characterized β-xylosidase from Anoxybacillus species. *A. flavithermus* xylanolytic enzymes had hydrolytic activity on xylan that was at least three folds higher than that of the *Sulfolobus solfataricus* β-xylosidase/α-arabinosidase. A combination of enzymes from the two thermophiles was able to hydrolyze beechwood xylan, performing better than *Bacillus thermoantarticus* xylanolytic activities under the same reaction conditions (Kambourova et al, 2007).

Moreover the presence of xylanase, β-xylosidase, α-L-arabinofuranosidase and acetyl esterase activities in cultures of *Anoxybacillus sp*. JT-12, was recently discoverd (Thitikorn-Amorn et al, 2012).

The existence of hemicellulolitic activities in *Anoxybacillus sp.* 3M was firstly identified by Marques et al, (1998), that isolated the microorganism from terrestrial hot springs (T 90°C) in Sao Miguel, Azores.

The identification of the hemicellulolytic activities started with the growth of *Anoxybacillus sp.* 3M, that was adapted to utilize the xylan as a carbon source on solid media. Onto plates with 1% (w/v) xylan, several colonies were obtained by streaking and, after staining with Congo Red, colonies surrounded by clear halos of 5 to 15 mm diameter were detected confirming the microorganism ability to hydrolyze the xylan. The xylanolytic activity was also investigated by growing the microorganism in liquid basal medium supplemented with 1% (w/v) beechwood xylan. The analysis of the enzyme activity during the growth revealed that the extracellular xylanolitic production was not growth-linked and the expression of different hemicellulolitic activities reached the highest level in the late stationary phase (data not shown).

3.2.2 Purification of the *Anoxybacillus* sp. 3M β-xylosidase

The purification of a new identified enzyme is very important to understand the catalytic characteristics of a microorganism and in particular the enzyme properties.

Different chromatographic resins were used to bind the extracellular enzyme endowed with β-xylosidase activity present in the cell free supernatant from 1 L culture that was previously 20 fold concentrated and dialyzed. The use of a strong anion exchange matrix allowed the enzyme binding and two sequential separation steps were performed on Q-Sepharose and MonoQ columns, with an increase of 13 folds of the purification factor. The purification procedure of the β-xylosidase is summarized in Table 14. Finally a molecular exclusion chromatography on Superdex 200 HR 10/30, allowed to obtain a specific activity of 7.2 U/mg, with a purification factor of 74.14.

Table 14. Purification procedure of the β-xylosidase from *Anoxybacillus sp.*3M

Purification steps[a]	Volume (mL)	Total Units (U)[b]	Proteins (mg)[c]	Specific Activity (U/mg)	Yield (%)	Purification fold
Crude broth	1000	52.7	550	0.0952	100	1
Ultrafiltration	50	41.5	345	0.120	79	1.26
Q-Sepharose	10	8.3	52.6	0.160	15.7	1.68
MonoQ	1	7.6	3.5	2.17	14.4	22.8
Superdex 200	1	7.2	1.02	7.06	13.7	74.14

[a] Purification was performed utilizing the cell-free supernatant from 1 L culture of *Anoxybacillus sp.* 3M obtained in liquid basal medium supplemented with 1 % (w/v) beechwood xylan
[b] β-xylosidase activity was measured as described in Materials and Methods.
[c]Measured by Bradford method using bovine serum albumine as a standard (Bradford, 1976).

The β-xylosidase, (AbXyl) showed a single band on SDS-PAGE confirming its homogeneity (Figure 13). The molecular mass of the enzyme was determined to be of about 70 kDa under SDS-PAGE denaturing conditions, whilst the gel filtration chromatographic analysis revealed that in the native state the molecular mass of the β-xylosidase was about 140 kDa. This outcome indicated for AbXyl a homodimeric structure composed by two subunits was of 70 kDa (data not included). Generally β-xylosidases reported from bacteria are dimeric in nature except that of *Geobacillus. pallidus* which is a trimer (Quintero et al, 2007).

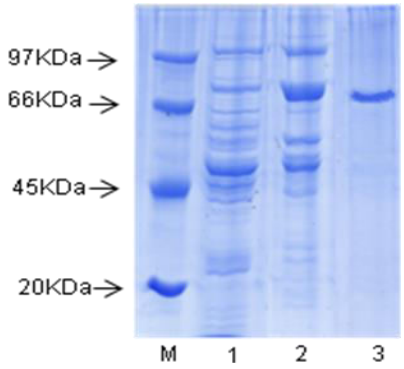

Fig. 13: 8% SDS-PAGE followed by Coomassie Blue staining: protein markers (M); pooled fractions after Q-Sepharose **(1)**; pooled fractions after MonoQ **(2)**; pooled fractions after Superdex 200 **(3)**.

The fractions from the different chromatographic steps were analyzed, using the assay described in Material and Methods, for the detection of β-xylosidase

activity. The pooled fraction, showing the enzymatic activity, were loaded on SDS-PAGE. After the last step of purification, the single band obtained on the Comassie blue stained SDS-PAGE gel was excised and the peptide mixtures was subjected to protein identification by *in situ* digestion and LC-MS/MS analysis. The acquired MS/MS spectra were transformed in *Mascot generic* format (.mgf) and used for protein identification with the 2.4.0 licensed version of MASCOT software (www.matrixscience.com) against the whole unreviewed set of protein entries of the NCBInr database for all bacteria. It is worth noting that a β-xylosidase (NCBInr entry: gi|516003689) attributed to *Geobacillus caldoxyloliticus* (five peptides, 27% sequence coverage) and a β-xylosidase (NCBInr entry: gi|13889531) attributed to *Geobacillus thermodenitrificans* NG80-2 (five peptides, 20% sequence coverage), both belonging to the GH52 family (CAZy database: http://www.cazy. org/), were identified. So the enzyme responsible for the activity produced by *Anoxybacillus sp.* 3M was recognized as a β-xylosidase, using an approach based on a combination of enzymatic assays and mass spectrometry analysis, presumably belonging to the GH52 family.

Compared to GH39 and GH43 xylosidase, only a limited number of GH52 enzymes have been discovered, purified, and biochemically characterized. However, few β-xylosidases have been reported from thermophilic Bacteria and Archaea (Cantarel et al, 2009). Some of the β-xylosidases characterized from aerobic bacteria are those from *Deinococcus geothermalis* (Cournoyer and Faure, 2003), *Rhodothermus marinus* (Manelius et al, 1994) and *Geobacillus stearothermophilus* (Brux et al, 2006).

Moreover, the molecular masses of other GH 52 belonging to the Bacillus species, such as the β-xylosidases from *B. stearothermophilus* T-6 and *G. thermodenitrificans* (Bravman et al, 2001) are close to that of identified enzyme of *Anoxybacillus sp.* 3M.

Since there isn't any report on the characterization of β-xylosidase from the bacterium *Anoxybacillus sp.* 3M, the enzyme can be considered as novel.

3.2.3 Influence of pH and temperature on enzyme activity and stability

The β-xylosidase purified from *Anoxybacillus sp*. 3M was active in the pH range between 5.0 and 7.0, with optimum at pH 5.5, as shown in Figure 14.

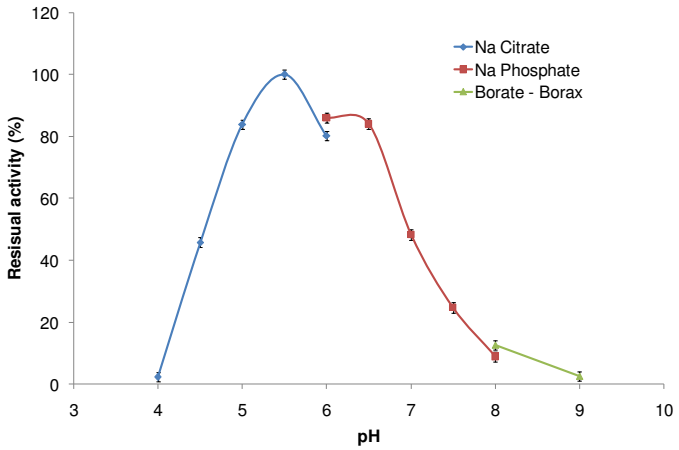

Fig.14: Effect of pH on purified β-xylosidase activity. All the activity values, measured at 60°C, are means of three replicates ±SD.

It was noteworthy that the purified enzyme was tolerant to a range of pH between 5.0 and 9.0. The β-xylosidase retained 99% of its activity at pH 5.0 after 24 h of incubation at 40°C, and it lost only around 12% of its initial activity at pH 6.0, 7.0, 8.0.

In particular, the enzyme was very tolerant to alkaline conditions, showing 74.8% of the maximal activity at pH 9.0 also after 24 h of incubation at 40°C, whilst activity decreased at pH 4.0, where the residual activity was around 21% after 5 h, in the previously mentioned conditions (Figure 15).

The range of pH 5.0-7.0 that was found for β-xylosidase activity is in agreement with that found for other similar bacterial enzymes, but only few are known to exhibit activity in the alkaline pH range (Wagschal et al, 2009; Shi et al, 2013). The ability of enzyme to be active at alkaline pH suggests its applicability in generating fermentable sugars from agro-residues (Kumar and Satyanarayana, 2013).

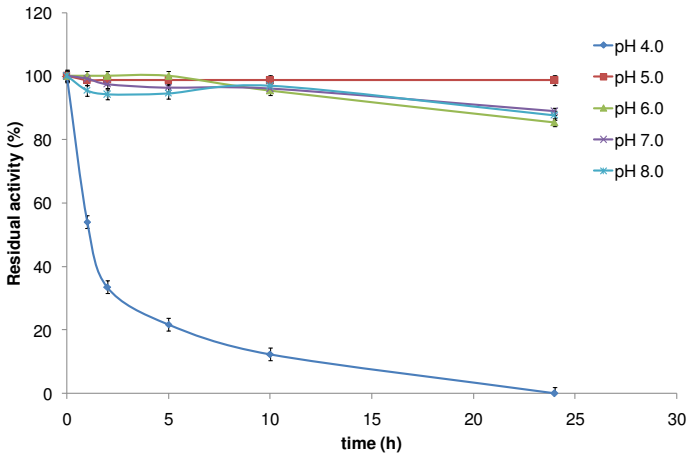

Fig.15: Effect of pH on purified β-xylosidase. The percent residual activity values were plotted against the corresponding incubation times. All the activity values are means of three replicates ±SD.

The enzymatic activity was detected over a broad range of temperature (20-90°C) and the enzyme showed its maximum at 60°C. Moreover, high activity levels were also observed at 55 and 65°C, with values of 83 and 98.9% of the maximal activity, respectively (Fig. 16). Interestingly, the enzyme at 70°C showed even 41% of the maximum activity.

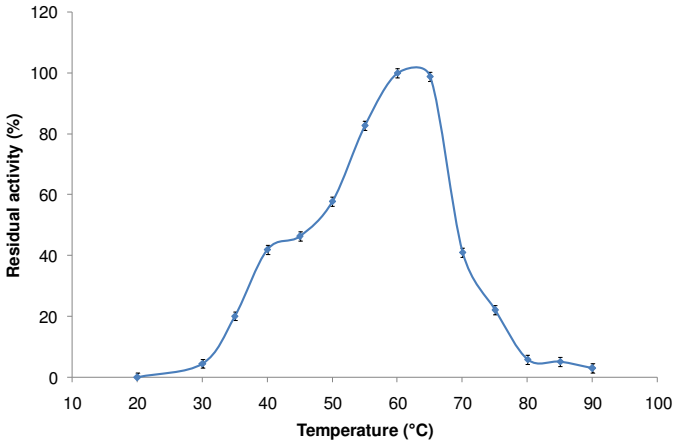

Fig.16: Effect of temperature on purified β-xilosidase. Thermophilicity of the purified enzyme was estimated in presence of the substrate. All the activity values are means of three replicates ± SD.

Thermostability was also evaluated by measuring the residual activity after several interval of time (1, 2, 5, 10 and 24 h) of incubation at increasing temperatures, as described in the section Material and Methods. The temperature

at which 50% of the enzyme was inactivated was 60°C after 10 h of incubation, while the residual activity values at 40, 50, 55 and 58°C were 93.8, 90.2, 90.4 and 67.7% respectively (Figure 17).

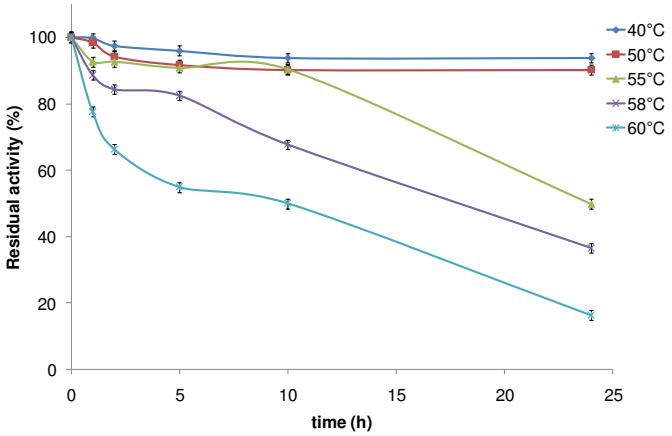

Fig.17: Effect of temperature on purified AbXyl. The percent residual activity values were plotted against the corresponding incubation times. All the activity values are means of three replicates ± SD

The xylosidase exhibited its maximal activity at 60°C , similarly to the xylosidase from *Bacillus stearothermophilus* T-6 (65 °C) (Bravman et al, 2001). Several xylosidases have been found to show their optimal activity at or above mesophilic temperatures. XylC from *Thermoanaerobacterium saccharolyticum* JW/SL-YS485 exhibited the maximum activity at 65°C, pH 6.0 (Shao et al, 2011). Xylosidase from *Geobacillus pallidus*, *Geobacillus thermantarcticus*, and *G. stearothermophilus* exhibited maximum activity at 70 °C (Lama et al, 2004; Nanmori et al, 1990; Quintero et al, 2007). A thermostable xylosidase from *Thermotoga maritime* exhibited the maximum activity at 90 °C, pH 6.1, which had an half-life of over 33 min at 95 °C, and retained over 57% of its activity at a pH value from 5.4 to 8.5 at 80 °C for 1 h (Xue and Shao, 2004). The β-xylosidase from *Anoxybacillus sp*. 3M exhibited a similar thermal stability to its aforementioned counterparts except for the xylosidase from *Thermotoga maritima*. Thermal stability of enzyme is a key factor limiting its application value in industry, and thus the enzymatic activity reported here can be used for several industrial application. In future studies the enzyme activity can be further

improved in this respect by genetic engineering technology, for a better application in industry.

3.2.4 Effect of metal ions and chemical reagents on β-xylosidase

The metal ions are known for their involvement in enzyme catalysis in a variety of ways: acceptor or donor of electrons, as coordinating group between enzyme and substrate, or they may simply stabilize a catalytically active conformation of the enzyme (Mohana et al, 2008). The β-xylosidase activity from *Anoxybacillus sp*. 3M was not affected by the presence of most of the metal ions tested (Table 15).

Table 15. Effect of metal ions and reagents on the purified β-xylosidase

Metal ions or chemical agents	Residual activity (%)	
	1 mM	10 mM
EDTA	97.6	97.3
Na^{+}	92.6	91.3
K^{+}	99.4	96.2
Ca^{2+}	88.4	81.9
Mg^{2+}	96.3	95.8
Hg^{2+}	0	0
Co^{2+}	101.7	99.8
Fe^{2+}	93.8	52.5
Cu^{2+}	62.5	30
Ni^{2+}	91	85
Zn^{2+}	91.8	93.9
Al^{3+}	93.7	9.5
Mn^{2+}	90.4	88.3
Ag^{+}	1.7	1.6
2-ME	99.2	101.6
SDS	111	133.6

The effect of different reagent was determined by pre-incubating the purified AbXyl in100 mM citrate buffer at 40°C and pH 5.5 in presence of the listed substances for 1 h. The activity of the β-xylosidase, measured in the absence of any substance, was set as 100 %.Values are means of three experiments.

It was, however, strongly inhibited by high concentrations (10 mM) of Fe^{2+}, Cu^{2+}, and Al^{3+} probably for the nature of oxidant of such metal ions (Vieille and Zeikus, 2001). The enzyme was almost completely inhibited at the lower concentrations (1.0 mM) of Ag^{+}, and inactivated by Hg^{2+}. The strong inhibition even at lower

concentration (1.0 mM) of Hg^{2+} suggests the possible presence of tryptophan residue in the activity site of the enzyme. The presence of this residues and their role in activity is well known in xylanases (Kumar and Satyanarayana, 2011). The effect of potential inhibitors or activators such as SDS and 2-ME was also examined and they did not substantially affect the enzymatic activity, probably because of stability of the tertiary structure of the enzyme, and no cysteines or disulfide bonds are involved in the catalytic site of the enzyme.

The enzyme activity was almost completely unaffected by the exposure to organic solvents, even after 120 min of exposure. β-xylosidase retained between 96.7 and 68% of residual activity in the presence of the solvents tested (15% v/v) after 120 min of incubation (Table 16), showing a slight increase when it was incubated with ethanol, methanol and *n*-butanol for 30 min. In general, thermostable enzymes are known to be tolerant to organic solvents and this property can be attributed to the presence, , of a relatively higher number of hydrophobic amino acids residues, in their primary sequence, than the mesophilic counterparts. (Klibanov, 2001).

Table 16. β-xylosidase stability in organic solvents

Solvents (15% v/v)	**logP**	**Residual activity (%)**		
		30 min	**60 min**	**120 min**
Ethanol	-0.24	105.5	103.4	93.1
Methanol	-0.76	102.8	102.5	96.7
2-propanol	0.07	90	81.1	68.2
n-Butanol	0.88	105.4	96.1	95.7

The purified enzyme was treated with various solvents for different intervals of time. The activity of the enzyme, measured in the absence of any substance, was set as 100 %.
Values are means of three experiments

The high resistance to organic solvents could make the β-xylosidase from *Anoxybacillu sp.* 3M a good candidate in many industrial processes, that request solvents tolerant enzymes applied for bioremediation of solvent-contaminated industrial wastewaters, in the deiking of recycled paper and solvent tolerance facilitates the selective precipitation, recovery and reuse of enzymes.

Moreover, the resistance in presence of several metal ions could make the studied enzyme suitable for industrial applications, in particular for the improvement of lignocellulosic hydrolysis (Woldesenbet et al, 2012).

3.2.5 Substrate specificity

The substrate specificity of the purified β-xylosidase using various substrates was tested. No detectable α-arabinofuranosidase, α- and β-glucosidase activities were observed, as it can be deduced from the purified AbXyl unability to hydrolyze the pNP-glycosides utilized as analogous substrates (data not shown). This was in complete agreement with the putative attribution of this activity to the GH52, that it's a family of glycosil hydrolases that include only β-xylosidase activities. The substrate spectrum analysis suggested p-NPX as the preferred substrate and this characteristic is similar to that of other GH52 β-xylosidases also of bacterial origin. Although β-xylosidase is a critical component of microbial xylanolytic systems, there are very few reports on their characterization (Bravman et al, 2003; Shallom and Shoham, 2003).

3.2.6 Conclusions

Enzymes with hydrolytic activities are major the biocatalysts used in industry representing about 75% of the global market in this sector. In particular glycosidases represent the largest group of enzymes with biotechnological relevance after proteases and among them hemicellulases have long attracted considerable interest for the large number of industrial applications in which they are exploited.

A cocktail of enzymes containing mainly glycoside hydrolases activities such as cellulases, and hemicellulases produced by microorganisms are indispensable for the complete degradation of plant biomass. When bioethanol is to be produced, hemicelluloses are the potential bottleneck as the industrial fermentation of pentoses has not yet been achieved in a cost effective manner. The development of integrated biorefineries requires the utilization of the entire plant biomass, and in this context, new opportunities emerge from the conversion of hemicellulose component of plant cell walls for producing high value molecules and bioethanol.

The abundantly available lignocellulosic materials are soluble at high pH and elevated temperatures, and therefore, the use of thermostable and alkalistable hemicellulases is desirable. Based on these considerations, the present study has been directed towards the individuation of enzymes with xylanolytic activities with biotechnologically advantageous features such as good levels of activity and stability over a wide range of pH, in particular alkaline pHs, and at high temperature. For this purpose, the interest of the research has been focused on the study of two novel hemicellulolytic enzymes: the endoxylanasic activity belonging to the thermophilic bacterium *Geobacillus thermodenitrificans* A333 and the β-xylosidase activity from the bacterium *Anoxybacillus sp*. 3M. These bacteria are characterized by high levels of production of enzymes with xylanasic activities.

The purified and characterized enzymes were capable to work at high pH and temperature and the presence of most of the metallic ions, usually present in the industrial processes, and of organic solvents do not negatively affect their activity. Moreover the endo-xylanase GtXyn was able to remain active also at high concentrations of NaCl. These characteristics make them valuable candidate for possible applications in consolidated bioprocesses for the transformation of agro-industrial waste into bioethanol. Another important application would be the fermentation processes for the production of alcoholic beverages where the xylanolitic activities, hydrolysing the hemicellulose present in the cell wall of the endosperm containing starch, makes it more easily accessible to hydrolysis by the enzymes responsible for its saccharification.

Furthermore, the ability of the xylanase to be active at alkaline pH, the absence of cellulase activity and the strong resistance in presence of detergents makes advantageous its use in several processes for the production and the recovery of the recycled paper, where the use of detergents in combination with enzymatic activities can allow improved results than those achieved with traditional chemical methods. Moreover, the end products of the xylan hydrolysis by the studied xylanase from *G. thermodenitrificans* consist of oligosaccharides with degree of polymerization greater than two. This makes GtXyn an important candidate for

the production of xyloolisaccharides, regarded as high added value products as with important applications in the pharmaceutical and food industries.

In conclusion, the thermo-alkali-stable and cellulase-free endo-β-1,4-xylanase from *G. thermodenitrificans* A333 can be certainly considered one of the xylanases that, owing to its stability at extreme process conditions, could be efficaciously used in several industrial applications included the XOS production from lignocellulosic biomasses and agricultural residues.

Moreover, is noteworthy the identification and purification of a novel hemicellulolytic enzyme from *Anoxybacillus* since xylosidase activities from Bacilli spp are relatively new and in limited number compared to those isolated from fungi and other bacteria. The novel β-xylosidase from the thermophile *Anoxybacillus sp*. 3M has been purified and characterized revealing an alkali tolerant as well as thermostable activity. These are valuable characteristics for AbXyl exploitation as a biocatalyst for the production of fermentable sugars, in particular from agro-residues.

Part II

Enzymatic Hydrolysis Of Ligno Cellulosic Materials

3.3 Saccharification experiments with commercial and innovative enzymes.

3.3.1 Effect of rPoAbf and its variant in *Arundo donax,* pretreated by steam explosion, bioconversion

The use of agricultural residues and non-food crops such as *A. donax* as alternative cheap substrates has considerable potentialities for the cost-effective biofuel production. *A. donax* crop is considered a valuable feedstock, being one of the highest yielding species for biomass production in southern Europe, with a high xylan content representing the 20% of its dry weight (Scordia et al, 2011). It has also been recognized as a robust species with the characteristic to avoid competition with food crops for lands, due to its ability to grow on marginal and non agricultural soils (Krasuska et al, 2010). *A. donax* pretreated by steam explosion (Di Pasqua et al, 2014) was subjected to saccharification using cocktails of commercial enzymes exclusively of fungal origin, with the addition of the *P. ostreatus* arabinofuranosidase recombinantly expressed in *P. pastoris* rPoAbf (Amore et al, 2012a) or its evolved variant rPoAbf F435Y/Y446F (Giacobbe et al, 2014). These arabinofuranosidases were also tested in combination with the recently investigated cellulase from *Streptomyces sp.* G12 CelStrep recombinantly expressed in *E. coli* (Amore et al, 2012b) to verify a possible synergism with a bacterial cellulase. The conditions of pH and temperature adopted in saccharification experiments, at which all the tested enzymes retained 80% of their initial activities, were those described in "Enzymatic hydrolysis with commercial enzymes, CelStrep and rPoAbf" under the section Material and Methods. The following main effects were revealed in the different tested conditions.

3.3.2 Effect of addition of rPoAbf or its variant to the commercial enzymatic mix 1

The use of wild-type rPoAbf in combination with the enzymatic mix 1 from Genencor (Fig. 18) improved the xylose yield and speeded up, in the conducted experiments, the xylan degradation. The use of the evolved mutant of rPoAbf allowed further increasing of the xylose recovery and achieving the highest monosaccharide yield. This can be due to the increase of the xylan accessibility and hydrolysis caused by removal of the arabinosyl side chains from hemicelluloses catalyzed by the arabinofuranosidases. Conversely, the glucose yield, conversely, was not affected by the addition of the arabinofuranosidases (data not shown).

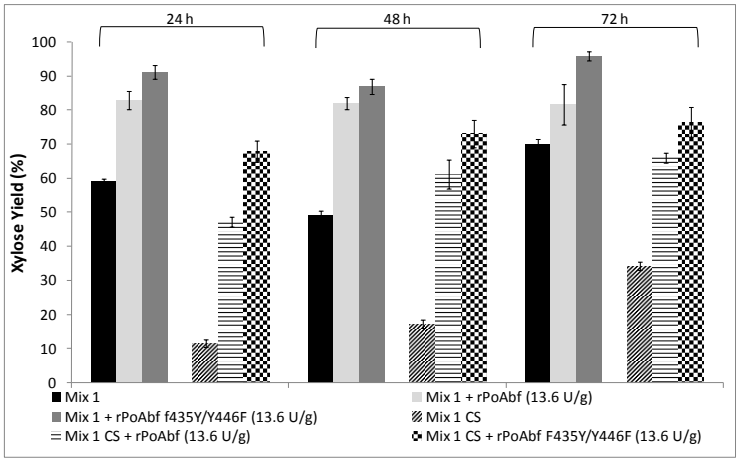

Fig. 18: Xylose yield of saccharification of *A. donax* pretreated by steam explosion obtained by adopting the mix 1 (5.4 U g^{-1} of Accellerase 1500, 4000 U g^{-1} of Accellerase XY and 145 U g^{-1} of Accellerase BG) or mix 1CS (mix 1 containing the recombinant enzyme CelStrep instead of Accellerase 1500) with or without addition of rPoAbf or rPoAbfF 435Y/Y446F mutant.

3.3.3 Effect of rPoAbf and its variant addition to the commercial enzymatic mix1 containing CelStrep instead of the commercial cellulase

When the recombinant enzyme CelStrep was used in the mix 1 instead of Accellerase 1500 (Fig. 18), the ability of rPoAbf and its evolved variant to improve the xylan degradation became more evident. As reported by Gao et al. (2011), for evaluating the role of individual biocatalysts in biomass hydrolysis, the employment of a characterized enzyme, such as the recombinant CelStrep, is

more advantageous than the utilization of enzymatic preparations lacking a defined composition. Using CelStrep, the xylose yield was highly reduced due to the absence of the hemicellulolytic activities reported for the Accellerase 1500 preparation. The addition of wild-type rPoAbf caused a marked increase in the xylose recovery that became closer to that obtained with Accellerase 1500. In particular, the xylose recovery after 48 h was 12.1% higher than that obtained with the mix 1. The evolved variant of rPoAbf was able to improve the xylose yield and it is worth noting that the substitution of Accellerase 1500 with CelStrep plus the mutated rPoAbf enhanced the xylose yield up to 24% (after 48 h of conversion). The reported data clearly highlight that the limitation of the commercial preparation in xylan hydrolysis derives from the prevalence in their composition of backbone-acting xylanases at the expense of the auxiliary hemicellulolytic enzymes active on the side groups. Hence, the removal of side chains which impede endoxylanase activity are crucial to obtain high xylose yields (Banerjee et al, 2010). As concerning the glucose recovery, it was reduced in presence of CelStrep and was not affected by the addition of the arabinofuranosidases (data not shown).

3.3.4 Effect of rPoAbf or its variant addition to the commercial enzymatic mix 2

In order to identify enzyme combinations that could improve the hydrolysis yield, further trials were performed using a cocktail of commercial enzymes from Sigma (mix 2) including cellulolytic and xylanolytic activities (Table 17). The result was 96.4% xylose recovery after 72 h. The addition of rPoAbf did not substantially influence the xylose recovery whilst the utilization of rPoAbf F435Y/Y446F speeded up the xylan degradation without modifying the maximum xylose yield.

Table 17. *Arundo donax* pretreated by steam explosion (Di Pasqua et al, 2014).

	Composition	Xylose Yield (%)		
		24 h	48 h	72 h
	Mix 2	50.1 ± 1.9	64.7 ± 2.6	96.4 ± 0.6
rPoAbf (13.6 U/g)	Mix 2	44.5 ± 5.4	59.9 ± 4.5	93.2 ± 3.7
rPoAbf F 435Y/Y446F (13.6 U/g)	Mix 2	48.5 ± 6.1	77.7 ± 3.0	98.2 ± 1.3
	Mix 2 CS1	49.0 ± 4.0	51.0 ± 4.3	65.6 ± 3.2
rPoAbf (13.6 U/g)	Mix 2 CS1	48.6 ± 5.6	52.6 ± 4.2	60.8 ± 4.4
rPoAbf F 435Y/Y446F (13.6 U/g)	Mix 2 CS1	64.2 ± 5.4	65.3 ± 3.2	68.4 ± 5.2
rPoAbf F 435Y/Y446F (27.2 U/g)	Mix 2 CS1	44.6 ± 2.8	45.5 ± 2.6	90.0 ± 4.0
rPoAbf F 435Y/Y446F (13.6 U/g)	Mix 2 CS2	53.0 ± 3.3	57.9 ± 5.8	63.8 ± 5.1
rPoAbf F 435Y/Y446F (27.2 U/g)	Mix 2 CS2	50.6 ± 3.7	67.1 ± 5.5	90.5 ± 4.6

Biotransformations in 50 mM buffer acetate pH 5.0 T = 50 °C, for 24, 48 and 72 h.
Mix 2: C2730 cellulase from *T. reesei* ATCC 26921 (5.4 U g^{-1} of pretreated biomass); C6105 Cellobiase from *A. niger* (145 U g^{-1}); X3876 xylanase from *T. viride* (80 U g^{-1}); X3504 β-xylosidase, thermostable (8 U g^{-1}).
Mix 2 CS1 or Mix 2 CS2: Mix 2 containing CelStrep, cellulase from *Streptomyces sp*., (5.4 or 10.8 U g^{-1}) instead of C2730, cellulase from *T. reesei* ATCC 26921.

3.3.5 Effect of rPoAbf addition to the commercial enzymatic mix 2 containing CelStrep instead of the commercial cellulase

The use of CelStrep in the enzyme mix 2 instead of the commercial cellulase reduced the glucose yield and the most evident effect was represented by halving of the maximum value (64%) at 72 h (data not shown). Also the recovery of xylose decreased (Table 17), that can be explained by the lack, for CelStrep, of xylanolytic side activities present in the commercial cellulase. Differently from the results obtained with CelStrep in combination with Genencor enzymes (mix 1

CS), this situation was not modified by the addition of wild-type rPoAbf. These data indicate that, in presence of CelStrep, rPoAbf works better in combination with the Genencor commercial cocktail than with the Sigma preparation. Probably, the different specificities of the biocatalysts involved in the enzymatic cocktails towards the same substrate was at the basis of the conversion yields reported.

3.3.6 Effect of rPoAbf variant addition to the commercial enzymatic mix 2 containing CelStrep instead of the commercial cellulase

The use of the evolved mutant rPoAbf increased the xylose recovery and allowed to achieve, after 24 h, a yield of xylose by 14.1% higher than that obtained with the commercial enzymes mix 2 (Table 17). When the units of the evolved mutant of rPoAbf were doubled, the recovery of xylose was 90% after 72 h. This value was closer to that of 98.2% obtained with the mix 2, in presence of the evolved mutant. Doubling CelStrep resulted in an improvement of cellulose hydrolysis (data not shown). Moreover, when double amount of CelStrep was used in combination with the evolved rPoAbf mutant, an acceleration of xylose recovery was also observed. In particular, a 21.6% increase in xylose yield was observed after 48 h compared to the results obtained when only a double amount of rPoAbf mutant was utilized. A possible synergism between the action of CelStrep and the evolved rPoAbf mutant can be at the basis of the acceleration of the detected xylose recovery, when the amounts of the cellulase and the rPoAbf mutant were increased at the same time. These findings highlighted that, besides the frequently cited effect of improvement in glucan conversion following the xylan removal (Polizeli et al, 2005), also the progress of the cellulose hydrolysis can facilitate the breakdown of the xylan fraction which is intimately adsorbed onto the cellulose microfibrils (Dammstrom et al, 2009).

3.3.7 Effect of PoAbf and its variant in bioconversion of *A. donax* and other biomasses pretreated by AAS

In addition to *A. donax* others lignocellulosic biomasses, namely BSG and corn cobs, were also tested in the saccharification experiments. The former two

materials are agro-industrial by-products characterized by a high xylan content, that accounts for the 35% and 24.1% of their dry weights, respectively. In these trials, wild type rPoAbf or its evolved variant were used in combination with two kinds of enzymatic cocktails: the mix 2 and this cocktail containing CelStrep in substitution of the commercial cellulase. In all the experiments, the maximum amounts for both CelStrep and rPoAbf previously utilized were tested. In order to improve the xylan digestibility the BSG, corn cobs and *A. donax* were then subjected to pretreatment with aqueous ammonia soaking (AAS). Beacuse this pretreatment modifies the structure and composition of lignocelluloses leaving behind a substantial amount of hemicellulose, it is conceivable that the effectiveness of side-chain acting hemicellulases is better highlighted on alkaline-treated materials. In Table 18, the macromolecular composition of all the materials before and after the pretreatments, in terms of percentage of glucans, xylans and Klason lignin, are reported. It is possible to see that the pretreatment with AAS mainly caused the removal of lignin with a consequent enrichment of glucans and xylans. For *A. donax*, pretreated also by steam explosion, it was possible to compare the effects of the two pretreatment methods highlighting that steam explosion mainly provoked the removal of a part of the xylan.

Table 18. Macromolecular composition of untreated and pretreated biomasses.

Chemical Composition (% total dry weight)							
Polysaccharides	**Corn cobs (untreated)**	**Corn cobs (after AAS)**	**BSG (untreated)**	**BSG (after AAS)**	***Arundo donax* (untreated)**	***Arundo donax* (after AAS)**	***Arundo donax* (after steam explosion)**
Glucan	38.0 ± 0.4	51.2 ± 1.2	26.3 ± 1.6	49.9 ± 0.3	37.8 ± 2.0	63.2 ± 1.5	38.2 ± 1.2
Xylan	35.0 ± 0.7	43.3 ± 0.8	24.1 ± 1.2	40.7 ± 0.6	19.3 ± 0.9	26.0 ± 0.7	5.7 ± 0.9
Klason lignin	19.1 ± 0.5	5.4 ± 0.7	9.8 ± 0.4	5.2 ± 0.3	24.9 ± 1.3	10.7 ± 1.1	36.1 ± 0.6

3.3.8 Effect of PoAbf and its variant in *A. donax* bioconversion

Analyzing the results of conversion of *A. donax* pretreated with AAS using the enzyme cocktail 2 in combination with wild-type rPoAbf or its evolved variant, it was noticed that the yield of glucose remained unchanged (data not shown) whilst a higher yield in xylose was obtained with the evolved variant of rPoAbf (Table 19).

Table 19. *A. donax* 5% w/v, pretreated with NH4OH. Biotransformation in 50 mM buffer acetate pH 5.0, T = 50 °C, for 24, 48 and 72 h incubation.

	Composition	**Xylose Yield (%)**		
		24 h	**48 h**	**72 h**
rPoAbf (27.2 U/g)	Mix 2	41.0 ± 2.2	49.9 ± 1.8	72.1 ± 2.0
rPoAbf F 435Y/Y446F (27.2 U/g)	Mix 2	42.8 ± 3.5	57.7 ± 2.2	82.5 ± 2.4
rPoAbf (27.2 U/g)	Mix 2 CS 2	29.8 ± 2.9	33.6 ± 3.7	64.9 ± 4.1
rPoAbf F 435Y/Y446F (27.2 U/g)	Mix 2 CS 2	33.7 ± 3.5	47.5 ± 4.2	62.9 ± 4.3

Mix 2: C2730 cellulase from *Trichoderma reesei* ATCC 26921 (5.4 U g^{-1} of pretreated biomass); C6105 Cellobiase from *Aspergillus niger* (145 U g^{-1}); X3876 xylanase from *Trichoderma viride* (80 U g^{-1}); X3504 β-xylosidase, thermostable (8 U g^{-1}).
Mix 2 CS2: Mix 2 containing cellulase CelStrep (10.8 U g^{-1}) from *Streptomyces sp*. instead of C2730 cellulase from *Trichoderma reesei* ATCC 26921.

The addition of the evolved variant of rPoAbf to the cocktail containing CelStrep allowed to increase the yield in xylose up to 14% respect to that obtained with the wild type. However, this effect was evident only after 48 h whilst the yield after 72 h remained unchanged (Table 19). Comparing the results of conversion of *A. donax* pretreated with AAS and by steam explosion it is noticed that the saccharification of the biomass pretreated with AAS allowed a lower yield of xylose expressed as percentage on the amount of sugars present before the hydrolysis (i.e., 90.5% instead of 62.9%, after 72 h of conversion with the mixture containing 27.2 U/g of mutated rPoAbf and Mix 2 CS2). This behavior can be explained considering that the pretreatment with AAS causes the removal of a larger fraction of lignin, leading to an enrichment of glucans and xylans in the

pretreated biomass, that leads to a lower fraction of xylose expressed in percentage on the total amount of these polysaccharides. On the other hand, if the absolute amounts of xylose are compared, the values after AAS pretreatment were higher than those achieved by steam explosion (i.e., 8.12 instead of 3.46 g L^{-1} of xylose, recovered after 72 h of conversion with the mixture containing CelStrep and mutated rPoAbf) (data not shown). This can be explained considering that the pretreatment with AAS reduce the content of lignin and the absorption of hydrolytic enzymes on it, thus facilitating the biodegradation of polysaccharides (Maurelli et al, 2013).

3.3.9 Effect of PoAbf and its variant in BSG and corn cobs bioconversion

Analyzing the results of saccharification of BSG and corn cobs, it is possible to see that the addition of the evolved variant of PoAbf in comparison with the wild-type led to an improvement of the saccharification yield in terms of xylose. The best results were obtained when the enzyme was used in addition to the mix 2 (Tables 20 and 21).

Table 20. BSG 5% w/v, pretreated with NH_4OH. Biotransformation in 50 mM buffer acetate pH 5.0, T = 50°C, for 24, 48 and 72 h incubation.

	Composition	**Xylose Yield (%)**		
		24 h	**48 h**	**72 h**
rPoAbf (27.2 U/g)	Mix 2	57.6 ± 3.8	63.5 ± 3.0	73.1 ± 4.2
rPoAbf F 435Y/Y446F (27.2 U/g)	Mix 2	64.4 ± 1.5	72.7 ± 2.9	85.8 ± 3.6
rPoAbf (27.2 U/g)	Mix 2 CS 2	53.4 ± 2.3	58.4 ± 2.8	63.3 ± 4.2
rPoAbf F 435Y/Y446F (27.2 U/g)	Mix 2 CS 2	58.5 ± 2.9	64.1 ± 2.4	66.4 ± 4.1

Mix 2: C2730 cellulase from *Trichoderma reesei* ATCC 26921 (5.4 U g^{-1} of pretreated biomass); C6105 Cellobiase from *Aspergillus niger* (145 U g^{-1}); X3876 xylanase from Trichoderma viride (80 U g^{-1}); X3504 β-xylosidase, thermostable (8 U g^{-1}).
Mix 2 CS2: Mix 2 containing cellulase CelStrep (10.8 U g^{-1}) from *Streptomyces sp*. instead of C2730, cellulase from *Trichoderma reesei* ATCC 26921.

Table 21. Corn cobs 5% w/v, pretreated with NH4OH. Biotransformation in 50 mM buffer acetate pH 5.0, T = 50 °C, for 24, 48 and 72 h incubation.

	Composition	Xylose Yield (%)		
		24 h	48 h	72 h
rPoAbf (27.2 U/g)	Mix 2	53.7 ± 1.0	64.5 ± 3.1	69.4 ± 5.0
rPoAbf F 435Y/Y446F (27.2 U/g)	Mix 2	58.8 ± 2.0	64.0 ± 3.4	85.5 ± 4.2
rPoAbf (27.2 U/g)	Mix 2 CS 2	42.6 ± 3.0	47.9 ± 5.1	56.1 ± 4.5
rPoAbf F 435Y/Y446F (27.2 U/g)	Mix 2 CS 2	50.6 ± 2.8	57.5 ± 4.3	64.2 ± 6.8

Mix 2: C2730 cellulase from *Trichoderma reesei* ATCC 26921 (5.4 U g^{-1} of pretreated biomass); C6105 Cellobiase from *Aspergillus niger* (145 U g^{-1}); X3876 xylanase from *Trichoderma viride* (80 U g^{-1}); X3504 β-xylosidase, thermostable (8 U g^{-1}).
Mix 2 CS2: Mix 2 containing cellulase CelStrep (10.8 U g^{-1}) from *Streptomyces sp*. instead of C2730 cellulase from *Trichoderma reesei* ATCC 26921.

This outcome was more evident after 72 h of incubation when increases of 14.5% and 16.1% were observed for BSG and corn cobs, respectively. As concerning the glucose recovery, it was reduced in presence of CelStrep and unaltered after the addition of the arabinofuranosidases (data not shown). Moreover, even if the yields of xylose after 72 h were similar for the two tested materials with the mix 2 in combination with the evolved variant of rPoAbf (82–85%), the hydrolysis of BSG appeared to be the fastest. In fact, after 24 e 48 h of BSG saccharification, the highest xylose yields, corresponding to 64.4% and 72.7% respectively, were obtained. The explanation could be that the lower lignin content of this biomass can be at the basis of the higher biodegradability of its xylan fraction. This can also justify the similar absolute amounts of xylose obtained for BSG and corn cobs, despite the higher xylan content of corn cobs (at maximum 17.32 and 18.46 g L^{-1} of xylose obtained from BSG and corn cobs, respectively, after 72 h incubation with commercial enzymes and mutated rPoAbf) (data not shown). A similar behaviour was verified analyzing the amounts (g L^{-1}) of arabinose obtained from the hydrolysis of both BSG and corn cobs (data not shown). The highest arabinose recovery was obtained for BSG after 72 h with the evolved

rPoAbf mutant that gave a two folds higher yield respect to those obtained with the wild type. The arabinofuranosidase showed a better results in combination with the commercial cocktail (1.73 g L^{-1}) than in presence of CelStrep (1.65 g L^{-1}). Interestingly, it must be underlined that the evolved rPoAbf variant led to the best yields for arabinose and the xylose at the same time. These findings highlight the synergistic action of rPoAbf with the xylanolytic components of the enzymatic cocktails. The arabinose recovery from corn cobs was undoubtedly lower with all the enzymatic cocktails tested (around 1–1.2 g L^{-1}) and the worst results were obtained with wild type rPoAbf in combination with the mixture containing CelStrep (data not shown). The higher arabinose content for BSG (20–22%) in comparison with that of corn cobs (5.9%) and its lower lignin content (Musatto et al, 2006) can account for these data.

3.4 Saccharification of pre-treated BSG using a new xylanase from *Bacillus amyloliquefaciens* XR44A

3.4.1 Substrate chemical composition

BSG represents the major by-product of the brewing industry accounting for about 85% of the total residues generated after mashing and lautering processes (Mussatto, 2014) and is available in large quantities throughout the year. This residue has an elevated potential as feedstock for the production of ethanol, since it is characterized by a high (hemi)cellulose content. The chemical composition of BSG varies according to several factors such as the barley variety, the harvest time, the malting and mashing conditions, and also the quality and type of additives added in the brewing process (Robertson et al, 2010; Santos et al, 2003). It generally consists of about 16.8‑25.4% cellulose, 21.8‑28.4% hemicellulose (mostly arabinoxylans) and 11.9‑27.8% lignin (Aliyu and Bala, 2011; Sežun et al, 2011). The composition of the raw material utilized showed high amounts of cellulose and xylan (in the form of arabinoxylan), and low lignin content, in comparison to the composition of other types of brewer's spent grain reported in the literature. In Table 18 the composition of the material after pretreatment was reported.

Pretreatment is a key step for effective enzymatic lignocelluloses hydrolysis, having the important function of removing the lignin fraction that shields the cell wall polysaccharides from enzymatic attack (Agbor et al, 2011). In this experiment the pretreatment by aqueous ammonia soaking was set up for BSG adapting the conditions reported by Kim et al. (2008) and Maurelli et al. (2013) for barley hull and chestnut shell, respectively. This method was chosen due to its high efficacy in breaking the ester bonds between lignin, hemicellulose and cellulose without degrading the hemicellulose polymers (Chang and Holtzapple, 2000). As shown in Table 18, the delignification percentage was 78% after 22 h incubation, with an enrichment of glucans and xylans fractions. Furthermore, it was shown that by prolonging the pretreatment time no significant improvements were achieved (data not shown).

3.4.2 Effect of *B. amyloliquefaciens* XR44A xylanase in bioconversion of BSG

The culture supernatant of *B. amyloliquefaciens* XR44A with xylanase activity was utilized after ammonium sulfate precipitation and dialysis (Amore et al, 2015) for the saccharification of brewer's spent grains (BSG), which is considered a valuable low-cost feedstock, with an increasing attractiveness for energy production. The pretreatment by aqueous ammonia soaking was set up for BSG as previously described. The results regarding the saccharification of the pretreated BSG with *B. amyloliquefaciens* XR44A protein preparation endowed with xylanase activity (1.5 U g^{-1} pretreated biomass) were reported in Table 22.

Table 22. BSG 5% w/v, pretreated with NH_4OH. Biotransformation in 50 mM buffer acetate pH 5.0, T = 30°C, for 24, 48 and 72 h incubation

Composition	Glucose Yield (%)			Xylose Yield (%)			Arabinose Yield (%)		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Mix 1	35.3	47.9	51.5	30.5	40.0	43.8	52.4	56.1	53.4
Mix 2	35.1	74.3	79.8	24.2	48.2	43.7	48.1	70.0	92.3

Mix 1: cellulase from *T. reesei* ATCC 26921 (5.4 U g^{-1} of pretreated biomass); C6105 Cellobiase from *A. niger* (145 U g^{-1}); X3876 xylanase from *T. viride* (1.5 U g^{-1}); X3504 β-xylosidase, thermostable (8 U g^{-1}).
Mix 2: Mix 1 containing xylanase from *Bacillus amyloliquefaciens* XR44A (1.58 U g^{-1}) instead of X3876 xylanase from *T. viride*.

The new enzymatic activity developed, or alternatively, the same amount of a commercial xylanase from *Trichoderma viride* (Sigma), were used in combination with different commercial enzymes (Sigma): cellulase from *T. reesei* ATCC 26921 (5.4 U g^{-1}), cellobiase from *A. niger* (145 U g^{-1}) and β-xylosidase (0.5 U g^{-1}). It is worth noting that the utilization of the enzyme preparation from *B. amyloliquefaciens* XR44A led to results similar to those obtained with the commercial xylanase from *T. viride* (Sigma). In both cases, the maximum xylose recovery was around 43%, after 48 h of hydrolysis reaction, and it did not change over prolonged incubation times. Interestingly, the utilization of *B. amyloliquefaciens* XR44A xylanase preparation improved the glucan fraction degradation, which was enhanced from 51.5 to 79.8% after 72 h incubation. However, also in this case most of the cellulose conversion was completed in 48 h. A potentially better synergism between the xylanase activity from *B. amyloliquefaciens* XR44A and the commercial cellulase could be at the basis of an easier cellulose deconstruction. As regards the arabinose, due to the lack of arabinofuranosidase activity in both the *B. amyloliquefaciens* XR44A enzyme preparation and the commercial xylanase (as demonstrated by assaying it towards the substrate p-nitrophenyl α-L-arabinofuranoside), the release of this monosaccharide must be ascribed to the presence of arabinofuranosidase activities in the other commercial enzymes. As a matter of fact, values of 0.11, 1.22 and 0.69 U mL^{-1} of arabinofuranosidase activity were detected for arabinofuranosidase activity in the commercial cellulase, cellobiase and xylosidase, respectively. Moreover, a better arabinose yield, that exceeded by up to 38.9% the result reached with the commercial xylanase (56.4% after 72 h), was obtained in the presence of *B. amyloliquefaciens* XR44A enzyme preparation. This revealed that the arabinosidases worked better in combination with the new xylanase than in the presence of its commercial counterpart. As also reported in this thesis the diverse values of arabinose recovery in the presence of the two different xylanase activities can be explained by their specificities towards the same substrate, which account also for the synergism reached with the xylan debranching enzymes. These results, arising from the synergistic behavior of the various enzymes involved in the hydrolysis of (hemi)cellulosic substrates, are in

agreement with other investigation described in the literature (Hashimoto and Nakata, 2003; Gao et al, 2011).

3.5 Saccharification experiments with endo-xylanase (GtXyn) from *G. thermodenitrificans*, β-xilosidase (AbXyl) from *Anoxybacillus* sp. and arabinosidase (SsAra/Xyl) from *S. solfataricus*

3.5.1 Enzymes and experimental conditions

Among the bio-processes for obtaining ethanol and other added value products from lignocellulosic biomass in a biorefining perspective, the breakdown of cell wall polysaccharides into fermentable sugars by enzymatic hydrolysis has an essential role.

Cellulase from *Trichoderma reesei* ATCC26921, β-glucosidase from *Aspergillus niger*, purchased from Sigma, were the cellulolytic enzymes used in these bioconversions experiments.

The hemicellulolytic enzymes for xylan degradation were the following purified biocatalysts: the xylanase (GtXyn) from *Geobacillus thermodenitrificans* A333 recombinantly expressed in *E. coli*; the β-xylosidase (AbXyl) from *Anoxybacillus sp.* 3M and the bi-functional β-D-xylosidase/α-L-arabinosidase from *Sulfolobus solfataricus* (SsAra/Xyl) recombinantly expressed in *E. coli*. The comprehensive characterization of these novel enzymes was previously reported in this thesis and by Morana et al. (2007), showing protocols for production and purification.

The enzymes were tested for their activity on different substrates at pH 5.0 and 55°C, conditions used for the enzymatic hydrolysis (PB experimental design) where these biocatalysts were included among the variables. Despite the values of pH and temperature were not ideal for each of the activities studied, they represent a good compromise in the operating conditions of the enzymatic mix utilized in the hydrolysis experiments aimed to understand the interaction among the biocatalysts, and the other parameters, namely temperature, pH and biomass.

While the xylanase from *G. thermodenitrificans* and the β-xylosidase from *Anoxybacillus sp*. 3M were devoid of side activities, the other components of the enzymatic cocktail were active on more than one of the tested substrates, as

shown in Table 23. Besides the arabinofuranosidase from *S. solfataricus,* that was already described as presenting a bifunctional activity, the cellulase from *T.reesei* and β-glucosidase from *A. niger* worked also on xylose containing substrates. Nevertheless, the xylanase and xylosidase activity revealed by the commercial cellulolytic biocatalysts were residual compared to the corresponding ones showed by the in house developed enzymes.

Table 23. Activity values for the (hemi)cellulolytic enzymes on different substrates

	Specific activity (U/mg)					
Enzymes	CMC[a]	Beechwood xylan	Avicel	*p*NPG[b]	*p*NPAf[c]	*p*NPXP[d]
GtXyn	-	8.76	-	-	-	-
AbXyl	-	-	-	-	-	7.06
SsAra/Xyl	-	-	-	-	17.16	2.45
Cellulase from *T. reesei* ATCC26921	0.7	0.093	-	-	-	0.019
β-glucosidase from *A. niger*	-	-	-	1.2	-	0.08

[a]CMC (carboxymethyl cellulose), [b]*p*NPG (*p*NP-β-D-glucopyranoside), [c]*p*NPAf (*p*-nitrophenyl-α-L-arabinopyranoside), [d]*p*NPXP (*p*-nitrophenyl-β-D-xylopyranoside).

3.5.2 Screening of parameters affecting the sugars released during the enzymatic hydrolysis of BSG by PB design

Several factors affect the enzymatic hydrolysis of (hemi)cellulose including substrate loading, enzymes concentration, and reaction conditions (temperature and pH). A statistical experimental design is a widely applied method to improve yield and rate of the enzymatic hydrolysis and represent an alternative to the traditional time and cost consuming approach, which involves the change of one independent variable, holding all others constant. The use of the Plackett- Burman design is an efficient way to identify, among a large number of variables, which are those that affect the process and determine their main effects (Plackett and Burman, 1946). The PB design is based on a two-level multifactorial matrix composed of balanced incomplete blocks (Stanbury et al, 1986) that allow the study of up to N-1 components in N assemblies, where N must be a multiple of 4.

In this biotransformation experiments the effect of six variables - the concentration (units/g dry substrate) of three in house developed hemicellulases such as GtXyn, AbXyl and SsAra/Xyl temperature (°C), biomass loading (% w/v) and pH - on the sugars released during the enzymatic hydrolysis of BSG biomass pretreated by AAS, was analyzed through the PB screening design. To determine the protein concentrations to be used in the experimental design, preliminary tests were performed and kinetic curves were generated to verify the minimum protein concentration to be employed in the experiments. Thus, the concentrations were set at GtXyn 40 U/g dry substrate (gds), AbXyl 4 U/gds, SsAra/Xyl 13.6 U/gds.

The addition of the commercial enzymatic mix C (5.4 U/gds of cellulase from *T. reesei* ATCC26921 and 145 U/ gds β-glucosidase from *A. niger*) in the hydrolysis reaction was necessary to obtain the complete carbohydrates hydrolysis into the respective monomers, since the cellulase and β-glucosidase activities were not detected for the novel xylanolitic enzymes utilized.

Based on Table 24, which shows the distribution of factors according to the design matrix and the results obtained in this study, the highest and the lowest xylan conversion yields after 72 h of biotransformation were found to be 4.8 and 37.1 % (v/v) as observed in runs 9 and 4, respectively. As showed by the ANOVA analysis (data not shown) carried out on the data obtained after 72 h of biotransformation, the most significant influence on the xylan hydrolysis was exercised by the following factors: concentrations of the three in house developed enzymes and two operation parameters (pH and temperature). In Figure 21 the Pareto Chart (R^2 = 0.96 and R^2 adj. = 0.93), showing the five significant factors at 95% of confidence level ($p < 0.05$), was reported. In detail, the concentrations of GtXyn ($p< 0.0001$), SsAra/Xyl ($p=0.0002$) and AbXyl ($p=0.0026$) were among the most important factors for the xylose recovery, exercising positive effects of 10.04, 9.20 and 5.92, respectively. As showed by the Pareto chart plots obtained with the results achieved after 24 and 48 h, the contribution of the three enzymes to the xylan hydrolysis increased with the time (Fig. 19 and 20). The effect of GtXyn after 24 and 48 h was of 6.66 and 7.39 respectively and similar values were measured for SsAra/Xyl too (6.45 and 7.98). The AbXyl influence, instead, became significant only after 48 h ($p=0.0169$) producing a positive effect of 3.00,

that was far less important than those of the other biocatalysts and remained the lowest also after 72 h. As concerning pH and temperature they exercised a negative effect on xylose recovery that was more pronounced for the first parameter. Whilst for the pH estimated effects of -4.03, -4.66, -7.51 were detected after 24, 48 and 72 h respectively, the temperature became significant only after 72 h ($p=0.029$), and showed an effect of -5.80.

Table 24: Plackett-Burman 11/12 Screening design - Two levels, 6 factors, 3 central points and 5 dummies factors (D1, D2, D3, D4 and D5).

Run	T (°C)	Biomass % (w/v)	pH	GtXyn (U/gds)	AbXyl (U/gds)	Ss Ara/Xyl (U/gds)	D 1	D 2	D 3	D 4	D 5	Xylose Yield % (v/v) 24 h	48 h	72 h	Glucose Yield % (v/v) 72 h
1	60	7	7	80	8	0	-1	-1	-1	1	1	6.63	9.16	9.87	7.41
2	50	3	7	80	0	0	1	1	-1	1	1	3.20	3.72	6.81	1.6
3	50	7	7	0	8	27.2	1	-1	-1	-1	1	8.67	11.44	13.86	10.43
4	50	3	5	80	8	27.2	1	-1	1	1	-1	18.60	26.96	37.10	32.5
5	55	5	6	40	4	13.6	0	0	0	0	0	10.40	13.32	17.10	14.68
6	55	5	6	40	4	13.6	0	0	0	0	0	7.59	12.09	16.1	19.89
7	50	7	5	80	8	0	-1	1	1	-1	1	17.66	17.79	29.64	50.33
8	50	7	7	0	0	27.2	-1	1	1	1	-1	9.92	10.97	15.94	4.68
9	60	7	5	0	0	0	1	-1	1	1	1	3.45	4.11	4.80	6.28
10	60	3	7	0	8	0	1	1	1	-1	-1	4.69	7.04	7.21	4.22
11	60	3	7	80	0	27.2	-1	-1	1	-1	1	15.57	17.11	17.74	17.74
12	60	3	5	0	8	27.2	-1	1	-1	1	1	8.30	10.01	14.02	10.86
13	60	7	5	80	0	27.28	1	1	-1	-1	-1	19.08	20.85	22.9	22.13
14	55	5	6	40	4	13.6	0	0	0	0	0	9.37	14.83	19.26	15.33
15	50	3	5	0	0	0	-1	-1	-1	-1	-1	5.78	7.67	8.010	12.12

More details can be obtained from the results of the different runs reported in Table 24. By the comparison between the runs 2 and 3, after 72 h, GtXyn in absence of AbXyl and SsAra/Xyl reduced of 50.8 % the xylose recovery respect to that achieved when xylosidase and arabinofuranosidase activities were utilized without the xylanase GtXyn. This finding suggested that SsAra/Xyl and AbXyl worked better in combination with the xylanolytic activities present in the commercial enzymatic mix C than with GtXyn, at 50°C and pH 7.0. Moreover the runs 1 and 11 evidenced that SsAra/Xyl, when used instead of AbXyl in combination with GtXyn, increased by 40% of the xylose recovery after 72 of incubation, at 60°C and pH 7.0. A temperature decrease reduced this effect and, at 50°C, the absence of SsAra/Xyl reduced the xylose yield only by 21% after 72 h of biotransformation (run 4 and 7). This outcome supports the higher significance level of SsAra/Xyl respect to AbXyl, as evidenced by the Pareto chart analysis. As regards the glucose recovery, the crucial factors after 72 h of incubation were the pH ($p<0.0001$), the biomass amount ($p=0.025$) and the xylanase concentration ($p=0.026$) with estimated effects that were identical for the two latter variables (0.1) and negative for the pH (-5.6) (data not shown). The best result for glucose yield (50.3 %) was observed in correspondence to one of the highest xylose yields (29.64 %, achieved in run 7) after 72 h at pH 5 and in presence of the maximal biomass loading (7%) and xylanase concentration (80 U/gds). Varying the actual level of both factors, pH (from 5 to7) and biomass loading (from 7 to 3 %), also in presence of the highest xylanase concentration, the glucose yield reached its minimum value (1.6%) after 72 h. If only the biomass loading was reduced (3%), the glucose yield underwent a slighter reduction that went from 50.3 to 32.5%, after 72 h (Table 24) thus confirming the more relevant effect of the pH than the biomass loading on the monosaccharide recovery.

It was evident from the obtained data that the xylanase action was crucial to achieve the best level of glucose recovery that were reached in correspondence to one of the highest xylose yields (run 7). These findings were in complete agreement with the assumption that hydrolyzing xylan enhances the accessibility of the cellulases to the residual cellulose microfibrils; thereby, the higher glucan conversions can be reached in the presence of suitable hemicellulases (Gao et al,

2010). Finally, also the best result for total reducing sugars yield (40.45%) was obtained after 72 h of biotransformation, at the condition above specified for the run 7, and was comparable to those reported by several authors for BSG enzymatic hydrolysis (Forssell et al, 2008; Xiros et al, 2008a; Xiros et al, 2008b).

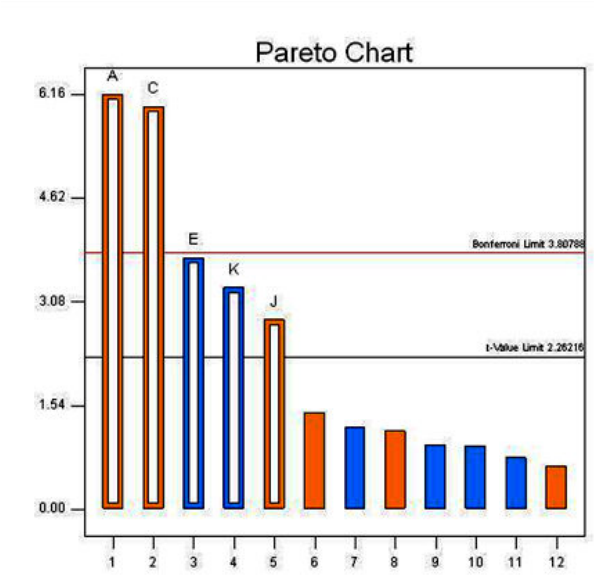

Figure 19: Pareto Chart of xylose released during the enzymatic hydrolysis of BSG at 24 h (R^2 = 0.92 and R^2 adj. = 0.88), showing the significant factors at 95% of confidence level ($p < 0.05$). Significant factor were GtXyn concentration (p=0.003), SsAra/Xyl concentration (p=0.004) and pH (p=0.066), with an effect of +6.66, +6.45 and -4.3, respectively. The empty columns represent significant factors, those in orange indicated positive effects while the blue coloured negative effects.

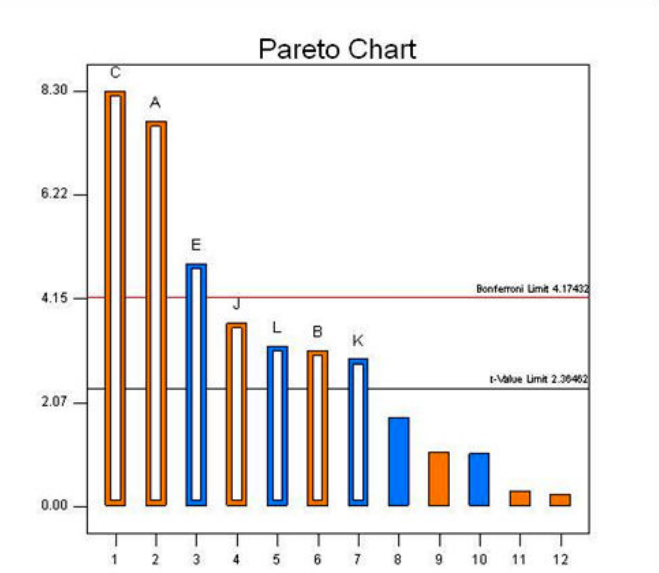

Figure 20: Pareto Chart of xylose released during the enzymatic hydrolysis of BSG at 48 h (R^2 = 0.96 and R^2 adj. = 0.93), showing the significant factors at 95% of confidence level ($p < 0.05$).). Significant factor were SsAra/Xyl concentration ($p< 0.0001$), GtXyn concentration (p=0.0001), pH (p=0.0019), AbXyl concentration (p=0.0169) and temperature (p=0.0015) and with an effect of +7.98, +7.38, -4.66, +3.0 and -4.66, respectively. The empty columns represent significant factors, those in orange indicated positive effects while the blue coloured negative effects.

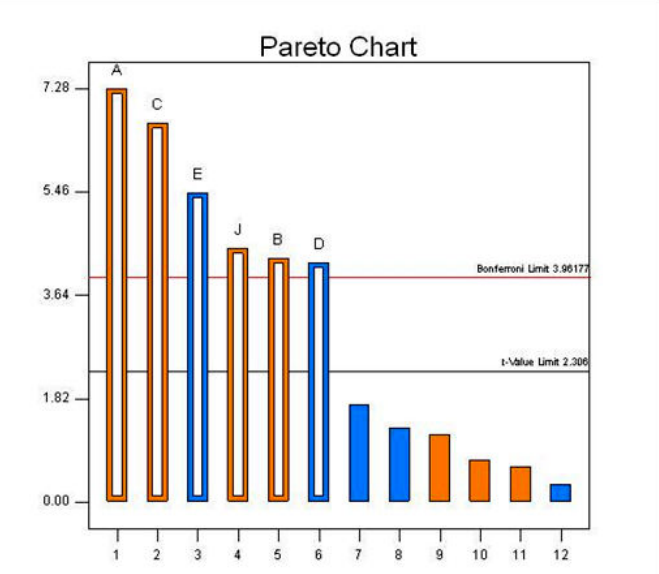

Figure 21: Pareto Chart of xylose released during the enzymatic hydrolysis of BSG at 72 h (R^2 = 0.96) and R^2 adj. =0.93), showing the significant factors at 95% of confidence level ($p < 0.05$). Significant factor were GtXyn concentration ($p < 0.0001$), SsAra/Xyl concentration(p=0.0002), pH (p=0.0006), AbXyl concentration (p=0.0026) and temperature (p=0.0029) and with an effect of +10.04, +9.2, -7.51, +5.92 and -5.8, respectively. The empty columns represent significant factors, those in orange indicated positive effects while the blue coloured negative effects

3.6 Conclusions

The study conducted on several biomasses, processed at different pretreatment, with the combined use of novel and commercial enzymes, highlights the relevance of the white rot fungus *P. ostreatus* as source of side-chain acting hemicellulases and arabinosidases, which play a crucial role in complete xylan hydrolysis via their cooperative action with the hydrolytic activities targeted to polysaccharides backbone. The results obtained showed that the addition of wild-type or directed evolved variant of the arabinosidase from *P. ostreatus* PoAbf improved the hydrolytic efficiency of the commercial enzymatic cocktails on *A. donax* pretreated by steam explosion and aqueous ammonia soaking and on Brewers's spent grain (BSG) and corn cobs pretreated by aqueous ammonia soaking. It is conceivable that, due to the low content of auxiliary hemicellulolytic enzymes in the commercial preparation, the supplementation with enzymes, that removes side chain on xylan backbone, such as the arabinofuranosidases, are required to develop enzyme mixtures better adapted for lignocellulose hydrolysis.

Interestingly, it was determined that the biomasses pretreated by aqueous ammonia soaking showed an higher hydrolizability by the (hemi)cellulolitic enzymes than that treated with the steam explosion method. For this reason the AAS method was used, due to its high efficacy in breaking the ester bonds between lignin, hemicellulose and cellulose without degrading the hemicellulose polymers, for the BSG pretreatment. BSG was the biomass chosen for the biotransformation using different novel enzymes. This biomass is available at low or no cost, and is produced in big quantities not only by large, but also small breweries. Despite the large amounts that are generated throughout the year, BSG has received little attention as a low-cost raw material and its use is still limited.

With the utilization of BSG as raw material, the xylanolytic activity of *B. amyloliquefaciens* XR44A showed performances similar to a commercial xylanase from *Trichoderma viride*, with a relevant xylose yield. Moreover, the presence of the xylanase in the commercial enzymatic cocktail favoured an increase of arabinose recovery due to better synergism than the commercial counterpart, established in association with the arabinofuranosidases activities present in the commercial preparations.

Thermostable enzymes offer several potential advantages in the hydrolysis of lignocellulosic materials owing to their stability also at harsh industrial conditions. In this perspective, the thermo-alkali enzymes, purified and characterized in this study, were used for the hydrolysis of BSG, using an experimental design in order to optimize the degrading process. The experimental design was of great importance for understanding the action of enzymes when combined and to optimize the enzymes necessary for elaboration of less costly enzymatic cocktails, thus enabling the development of target biotechnological applications. Collectively, the findings indicate the best way to direct xylose and arabinose production. The results obtained allowed to elucidated the effect of several variables, that include different hemicelullolytic enzymes tested in the Placket-Burman experimental design, in combination with other parameters, namely pH, temperature and biomass loading.

Knowledge of how the novel enzymes used in the hydrolytic processes (xylanase from *G. thermodenitrificans*, β-xylosidase from *Anoxybacillus sp.* 3M and β-D-xylosidase/α-L-arabinosidase from *S. solfataricus*) were able to degrade complex xylans contained in the BSG biomass will underpin their industrial exploitation, particularly in the continued quest to convert lignocellulosic material into biofuels. The results obtained by the biotransformation experiments clearly demonstrated the potential application of hemicelulolytic enzymes, for biofuel production, as well as for XOS production according to the chosen strategy.

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ORIGINAL PAPER

Properties of an alkali-thermo stable xylanase from *Geobacillus thermodenitrificans* A333 and applicability in xylooligosaccharides generation

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Abstract An extracellular thermo-alkali-stable and cellulase-free xylanase from *Geobacillus thermodenitrificans* A333 was purified to homogeneity by ion exchange and size exclusion chromatography. Its molecular mass was 44 kDa as estimated in native and denaturing conditions by gel filtration and SDS-PAGE analysis, respectively. The xylanase (GtXyn) exhibited maximum activity at 70 °C and pH 7.5. It was stable over broad ranges of temperature and pH retaining 88 % of activity at 60 °C and up to 97 % in the pH range 7.5–10.0 after 24 h. Moreover, the enzyme was active up to 3.0 M sodium chloride concentration, exhibiting at that value 70 % residual activity after 1 h. The presence of other metal ions did not affect the activity with the sole exceptions of K^+ that showed a stimulating effect, and Fe^{2+}, Co^{2+} and Hg^{2+}, which inhibited the enzyme. The xylanase was activated by non-ionic surfactants and was stable in organic solvents remaining fully active over 24 h of incubation in 40 % ethanol at 25 °C. Furthermore, the enzyme was resistant to most of the neutral and alkaline proteases tested. The enzyme was active only on xylan, showing no marked preference towards xylans from different origins. The hydrolysis of beechwood xylan and agriculture-based biomass materials yielded xylooligosaccharides with a polymerization degree ranging from 2 to 6 units and xylobiose and xylotriose as main products. These properties indicate *G. thermodenitrificans* A333 xylanase as a promising candidate for several biotechnological applications, such as xylooligosaccharides preparation.

Keywords Thermo-alkali stable xylanase · *Geobacillus thermodenitrificans* A333 · Agro-derived lignocellulosics · Xylooligosaccharides

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Introduction

Worldwide there is a growing interest in the utilization, for bioenergetic purposes, of lignocellulosic materials (LCMs), the most abundant and renewable resources on Earth that constitute a large component of the wastes originated from municipal, agricultural, forestry and some industrial sources. In this scenario, xylanolytic enzymes of microbial origin play an important role in the bioconversion of LCMs into high value products as they act on the xylan, the main component of hemicellulose, which is the most represented polysaccharide in plant biomass after cellulose (Bastawde 1992; Timell 1967). Xylan is a heteropolysaccharide consisting of a linear backbone of 1,4-β-linked D-xylose units, substituted with various side groups such as 4-O-methyl-D-glucuronosyl, α-arabinofuranosyl residues and others. Due to its heterogeneity, the complete hydrolysis of xylan requires a system of enzymes including endo-β-D-xylanases, β-D-xylosidases, α-L-arabinofuranosidases, α-D-glucuronidases, acetylxylan esterases, ferulic and *p*-coumaric acid esterases that synergically act on the linear chain as well as on the side chains (Juturu and Wu 2012). Among them, the xylanases, whose function is to randomly cleave the linear chain internal linkages releasing xylooligosaccharides (XOS), are catalysts of significant industrial importance, due to their high

specificity, mild reaction conditions, negligible substrate loss and side products generation (Kulkarni et al. 1999). The enzyme stability at the industrial process conditions is the main requirement for convenient commercial applications. Differently from most of the xylanases described so far, active at neutral or acidic pH and below 45 °C (Collins et al. 2005), xylanases working at higher temperatures and pHs are of great interest for several biotechnological applications such as those in paper and feed industries, textile processes, enzymatic saccharification of LCMs, and waste treatments. In the pulp and paper industry (Viikari et al. 1994), for example, where xylan hydrolysis facilitates the release of lignin from wood pulp, the use of thermo-alkaline xylanases allows direct enzymatic treatments of the hot and alkaline incoming pulp, avoiding the cost and time consuming steps of temperature and pH readjustments. Moreover, due to the better solubility of xylan at alkaline conditions, xylanases active at these pH values may also find potential applications in waste management programs to hydrolyze the xylan in industrial, agricultural and municipal residues (Gessesse 1998). On this basis, xylanases obtained from extremophilic microorganisms are of special interest (Collins et al. 2005) and, in particular, those from Bacteria of the genus *Bacillus* "latu sensu" attract considerable attention. *Bacillus* species have always been the major workhorse industrial microorganisms, being ubiquitous, non pathogenic bacteria that can be isolated from various environments also at temperature higher than 60 °C. They can be easily cultivated in laboratory at high growth rates and are able to produce huge amounts of a great variety of extracellular enzymes. Among them, xylanases are of particular biotechnological relevance owing to their good level of thermophilicity and thermostability and the ability to be highly active in alkaline conditions (Satyanarayana et al. 2012; Subramaniyan and Prema 2000). Among the *Bacillus* species, those belonging to the genus *Geobacillus* are regarded with special interest since they are equipped with numerous thermophilic enzymatic activities involved in the organic carbon recycling in hot natural and artificial environments where they grow. Specifically, *Geobacillus thermodenitrificans* is distinguished by its ability to metabolize a larger number of carbon sources larger than the other species of the genus and it is endowed with genes encoding all the glycolytic activities needed to utilize xylans as the sole carbon sources (Feng et al. 2007). In this paper we report on the isolation, purification, and characterization of an endo-xylanase from the thermophilic *G. thermodenitrificans* strain A333. Our efforts were also aimed to the exploitation of cheap and widely available substrates such as lignocellulosic residues for the enzyme production to reduce the high costs required for enzyme industrial application. Finally, in a biorefining perspective, we also investigated on the possibility of xylanase utilization for the bioconversion of xylan into xylooligosaccharides, useful high value end products, endowed with beneficial documented biomedical and health effects (Vazquez et al. 2000).

Materials and methods

Substrates and reagents

Nutrient broth (NB) was purchased.from Oxoid Ltd (Oxford, UK). Xylans from beechwood, oat spelt and birchwood, carboxymethyl cellulose (CMC), Avicel, laminarin, lichenan, gelrite (Gellan gum), *p*-nitrophenyl (*p*NP) glycoside substrates, Congo Red, sodium azide (NaN_3), phenylmethylsulfonyl fluoride (PMSF), EDTA Coomassie Brilliant Blue G-250, lysozyme, β-mercaptoethanol (2-ME), urea, 3-[(3-cholamidopropyl)dimethylammonium]-1-propanesulfonate (CHAPS), Triton X-100, Tween 20 and 80, SDS, Cetyltrimethylammonium bromide (CTAB), PEG 8000, octylphenoxypolyethoxyethanol (Nonidet), proteinase K (from *Tritirachium album*), trypsin and α-chymotrypsin (from bovine pancreas), subtilisin A (from *Bacillus licheniformis*), collagenase (from *Clostridium histolyticum*), xylose and glucose were obtained from Sigma-Aldrich Co. (St. Louis MO).

2 % 4-0-methyl-D-glucurono-D-xylan-Remazol Brilliant Blue R (Azo-Oat Spelt Xylan), xylobiose, xylotriose, xylotetraose and xylopentaose (X_2–X_5) were from Megazyme Co. (Bray, Ireland).

The GeneElute Bacterial Genomic DNA Kit and the pGEM T-easy plasmid were from Sigma-Aldrich Co.and Promega Co. (Madison, USA), respectively. The Phusion High Fidelity DNA polymerase and dNTP, were obtained from NEB (Ipswich MA USA). All chemicals were of reagent grade, and all solutions were made with distilled and deionized water.

Media and growth conditions

Geobacillus thermodenitrificans A333, (catalog number DSM22625), was supplied by Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). The microorganism, isolated by Coleri et al. (2009), was grown aerobically at 60 °C and pH 7.0 in the basal medium nutrient broth (NB) and gradually adapted to utilize xylan as a carbon source by spotting aliquots of 10^6 cells onto solid media containing NB, increasing concentration of beechwood xylan up 0.15 % (w/v) and 0.8 % (w/v) gelrite (Gellan gum).

Single colonies obtained onto the plates, incubated at 60 °C for 16 h were tested for xylanase production by staining with a 1.0 % (w/v) Congo Red aqueous solution and destaining with 1.0 M NaCl. Isolates surrounded by

clear zone were considered as xylanase positive. The enzyme production was obtained in liquid culture by growing the microorganism in NB medium supplemented with 0.15 % (w/v) beechwood xylan as carbon source and buffered at pH 7.0. A 250 mL Erlenmeyer flask, containing 50 mL of liquid medium, was inoculated with a single xylanase positive colony and incubated at 60 °C for 16 h in a rotatory shaker (New Brunswick Scientific Co., USA) at 150 rpm. Finally, the culture was scaled up to 2 L and the biocatalyst was produced into four 2 L Erlenmeyer flasks. After 16 h of incubation, the bacterial cells were harvested by centrifugation at 10,800×*g* for 20 min at 4 °C. The cell-free supernatant was used as crude enzyme preparation and was stored at 4 °C after the addition of sodium azide (NaN_3) and phenylmethylsulfonyl fluoride (PMSF) at a final concentrations of 0.02 % and 0.2 mM, respectively.

Enzyme assays

Xylanase activity in the crude enzyme preparation and during the purification procedure was estimated spectrophotometrically using a soluble chromogenic xylan as the substrate (Biely et al. 1985). The activity was measured by adding 150 µL of 2 % 4-0-methyl-D-glucurono-D-xylan-Remazol Brilliant Blue R (Azo-Oat Spelt Xylan) in 100 mM sodium phosphate buffer pH 7.5–150 µL of properly diluted enzyme solution, and incubating at 50 °C for 30 min. The reaction was stopped with the addition of 750 µL of 97 % ethanol, followed by incubation at room temperature for 5 min and centrifugation at 9,000×*g* for 10 min. The supernatant absorbance was measured at 590 nm. One unit of xylanase activity was defined as the amount of enzyme required to increase the absorbance at 590 nm of 1 A/min under the experimental conditions.

The purified xylanase activity was assayed by incubating 50 µL of 1 % beechwood xylan in 100 mM sodium phosphate buffer pH 7.5 with 50 µL of appropriately diluted enzyme, in the same buffer; boiled enzyme samples were used in the control. After incubation at 50 °C for 10 min, the released reducing sugars were estimated with the Somogyi–Nelson method (Nelson 1944). One unit of xylanase activity was defined as the amount of enzyme required to release 1 µmol of xylose-equivalent reducing groups per min under the described conditions.

All the enzymatic measurements were performed in triplicate.

Localization of xylanase

The microorganism was grown, as previously described for the enzyme production, in 50 mL of liquid medium, and when the culture reached the late exponential phase, it was centrifuged at 10,800×*g* for 5 min at 4 °C. Cell-free supernatant was used for evaluating the presence of the extracellular activity. For cell-wall bound and intracellular activity, the cells, washed with 100 mM sodium phosphate buffer pH 7.5 and re-suspended in 1 mL of the same buffer containing 5.0 mM EDTA and lysozyme (1 mg/mL), were incubated for 30 min at 30 °C. Subsequently, the bacterial cells were broken by sonication with the ultrasonic cell disruptor (Soniprep Braun Labsonic) according to the following procedure: ten cycles of 1 min sonication on ice with 1 min pause after each cycle. Supernatant was separated from cell debris by centrifugation (20,000×*g*, 30 min and 4 °C). Cell debris was re-suspended in 200 µL of 100 mM sodium phosphate buffer pH 7.5 and tested in parallel with the supernatant, for the presence of xylanase activity.

Induction of xylanase expression

The xylanase induction was evaluated by growing *G. thermodenitrificans* A333, under the previously specified culture conditions, in different media obtained by replacing the beechwood xylan with monosaccharides such as xylose and glucose at 0.1 % (w/v) final concentration. The effect of other xylan types such as oat spelt xylan and birchwood xylan at 0.15 % (w/v) final concentration on GtXyn production was also evaluated. The growth and the extracellular xylanase production were checked analyzing culture samples withdrawn at various intervals of time. Samples from each culture broth were centrifuged at 10,800×*g* and 4 °C for 15 min to remove the bacterial cells and the supernatants were used for the activity assays.

The effect of xylose addition at different concentrations on GtXyn production was also investigated. The microorganism was grown in basal medium supplemented with 0.1, 0.25, 0.5 and 1 % (w/v) sugar and the extracellular xylanase production was tested along the growth, as above described.

Furthermore, to verify a potential catabolite repression effect by ready metabolizable carbon sources, xylose at a 1 % (w/v) final concentration was added during the mid-exponential phase of growth to a culture obtained in basal medium containing 0.15 % (w/v) beechwood xylan.

Xylanase production from agro-industrial raw materials

Different agro-industrial raw materials including brewery's spent grains (BSG), corn cobs, grape cane and wheat straw were used as carbon sources for xylanase production by *G. thermodenitrificans* A333. BSG were provided by Maltovivo micro-brewery, Avellino, Italy. This biomass was dried at 50 °C, milled by an homogenizer to obtain a fine powder, and stored in sealed vials at room temperature until use. The processed lot contained (w/w, oven dry

basis) 26.3 % cellulose, 24.1 % hemicelluloses, 9.8 % Klason lignin and 27.1 % protein. Wheat straw supplied by Estação Nacional de Melhoramento de Plantas, Elvas, Portugal, was dried and grounded to particles of about 0.5 mm thickness. The processed lot was composed of 38.8 % cellulose, 27.6 % hemicelluloses and 16 % Klason lignin. Grape cane was collected from a farm at Oleiros, Portugal. This raw material was milled to attain 0.5 mm of particle size. The processed lot was composed of 39.1 % cellulose, 17.9 % hemicelluloses and 24.7 % Klason lignin. Corn cobs were obtained from Companhia das Lezírias (Samora Correia, Portugal), dried up to a moisture content less than 10 % (w/w), and milled to particles smaller than 3 mm. The average composition of this feedstock was 38 % cellulose, 35 % hemicelluloses and 19 % Klason lignin.

For xylanase production, *G. thermodenitrificans* A333 cultivation started from the inoculum obtained transferring the microorganism from a fresh slant (less than 24 h old) to a 500 mL Erlenmeyer flask containing 100 mL of NB basal medium, supplemented with 0.15 % (w/v) beechwood xylan pH 7.0 and incubated at 60 °C for 16 h under shaking conditions (150 rpm). Subsequently, 10 % of this culture was used to inoculate a 500 mL Erlenmeyer flasks containing 100 mL of basal medium supplemented with 1.0 % (w/v) of each carbon source listed above as inducer substrate. The basal medium and the C-sources were previously sterilized by autoclave (121 °C, 1 atm for 15 min). The cultures were grown by shaking (150 rpm) at 60 °C for 2–6 days. The samples from each culture broth, withdrawn at different intervals of time, were checked for xylanase activity.

Xylanase purification procedures

The culture supernatant was concentrated 20-fold by ultrafiltration through a YM10 membrane (cut-off 10 kDa) in an Amicon cell (Millipore Merck KGaA, Darmstadt, Germany) and dialyzed against 20 mM sodium acetate buffer pH 5.0 at 4 °C.

The dialyzed enzyme solution was applied, at a flow rate of 0.5 mL/min, to a SP-Sepharose fast flow column (2.6 × 10 cm) (GE Healthcare, Uppsala, Sweden) previously equilibrated with 20 mM sodium acetate pH 5.0 (buffer A). After washing with five column volumes of buffer A to remove the unbound proteins, the elution was performed with a linear salt gradient from 0 to 1.0 M NaCl in buffer A, at a flow rate of 2 mL/min. The eluted fractions, dialyzed against buffer A at 4 °C for 16 h, were checked for the enzymatic activity.

The xylanase positive fractions were pooled, filtered using a 0.22 μm filter (Millipore Merck KGaA, Darmstadt, Germany) and applied, at a flow rate of 0.5 mL/min, onto a Resource S column (GE Healthcare, Uppsala, Sweden) (6.4 × 30 mm) pre-equilibrated with buffer A. After washing with the same buffer, the elution was carried out at a flow rate of 1 mL/min with a step gradient 0–1.0 M NaCl in buffer A with the following profile: from 0 to 0.43 M NaCl in 2 min, from 0.43 to 0.60 M NaCl in 15 min, from 0.60 to 1.0 M NaCl in 3 min. The active fractions were pooled, dialyzed against 50 mM sodium phosphate buffer pH 7.5 at 4 °C for 16 h and concentrated using a Centricon system (Millipore Merck KGaA, Darmstadt, Germany) of 10 kDa cut-off. The concentrated enzyme solution (150 μL) was loaded onto a gel filtration column Superdex 75 HR (1.0 × 30 cm) (Amersham Pharmacia Biotech, Uppsala, Sweden) and the enzyme elution was carried out with 50 mM sodium phosphate buffer pH 7.5 containing 150 mM NaCl at a flow rate of 1 mL/min. The active fractions were pooled and used as purified enzyme for subsequent analysis.

Protein determination

Protein concentration was determined as described by Bradford using the BioRad protein staining assay and bovine serum albumine as the standard (Bradford 1976).

Electrophoresis and activity staining

The purification grade of the xylanase after each step was checked by sodium dodecyl sulfate polyacrilammide gel electrophoresis (SDS-PAGE). Enzyme samples were loaded onto a SDS-10 % polyacrylamide gel containing 0.1 % (w/v) beechwood xylan. The SDS-PAGE was performed according to the discontinuous Laemmli method (1970). Proteins were visualized by staining with Coomassie Brilliant Blue G-250. Zymogram analysis was performed as described by Tseng et al. (2002). After the electrophoretic run, the gel was washed twice for 20 min at room temperature in 50 mM sodium phosphate buffer (pH 7.5) containing 25 % (v/v) 2-propanol, then rinsed in 50 mM sodium phosphate buffer (pH 7.5) and incubated at 60 °C for 16 h in the same buffer. The gel was flooded with 0.1 % (w/v) Congo Red dye aqueous solution for 30 min and repeatedly washed with 1.0 M NaCl solution.

NH_2-terminal sequence

The NH_2-terminal amino acid sequence from the pure protein band electroblotted onto polyvinylidene difluoride membrane (PVDF) according to Matsudaira (1987) was determined by automated Edman degradation utilizing an Applied Biosystems 477 A Protein Sequencer, equipped on line with a phenylthiohydantoin analyzer (model 120A). Degradation proceeded for 25 cycles according to the

manufacturer's instructions, and produced identifiable amino acid residues.

Gene isolation and sequencing

Genomic DNA from *G. thermodenitrificans* A333 was extracted using the GeneElute Bacterial Genomic DNA Kit, following the manufacturer's instructions. The complete coding sequence for GtXyn was amplified by PCR using synthetic primers (PRIMM s.r.l, Milano, Italy) designed on the basis of the end regions of the extracellular xylanase gene (NC0093281*)* from *G. thermodenitrificans* NG80-2 whose genome is available on line. The primers sequences were the following:

5′A333: -5′-ATGTTGAAAAGATCGCGAAAA-3′
3′A333: -5′-TCACTTATGATCGATAATAGC-3′

The PCR reaction was performed by using 50 ng of genomic DNA, the Phusion High Fidelity DNA polymerase and the following program: 1 cycle 95 °C 10 min; 40 cycles 95 °C 1 min, 65 °C 1 min, 72 °C 1 min and 1 cycle 72 °C 10 min. The PCR product was first cloned into the pGEM T-easy plasmid and sequenced by using an automatic sequencer.

Effect of pH and temperature on xylanase activity and stability

The influence of pH was determined at 50 °C through the xylanase standard assay in the range 5.0–11.0 using the following buffers (100 mM): sodium acetate buffer (pH 5.0, 5.5, 6.0), sodium phosphate buffer (pH 6.0, 6.5, 7.0,7.5, 8.0), borate-borax buffer (pH 8.0, 8.5, 9.0), and borax-NaOH buffer (pH 9.0, 9.5, 10.0, 11.0). The pH stability was evaluated by pre-incubation of the enzyme (0.1 mg/mL) at 50 °C at pH 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 for 24 h followed by the residual xylanase activity measurement. The activity obtained without pre-incubation was expressed as 100 %.

The effect of temperature was investigated at different values ranging from 20 to 90 °C at pH 7.5 by the xylanase standard assay. To determine the thermal stability, the purified GtXyn (0.1 mg/mL) was pre-incubated in 100 mM sodium phosphate buffer pH 7.5 at defined temperatures (58, 60, 62, 65, 70 °C) for 60 min without substrate and the residual activity was determined at standard assay conditions. The activity obtained without pre-incubation was expressed as 100 %. The time dependent profile of heat inactivation was studied by measuring the residual activity after pre-incubating the enzyme (0.1 mg/mL) in 100 mM sodium phosphate buffer pH 7.5 at different temperatures (58, 60, 62, 65 and 70 °C) for 1, 2, 5, 10 and 24 h.

The kinetic parameters for thermal inactivation were obtained utilizing the experimental data in the equation reported below:

$$\ln A/A_0 = K_d t$$

where A_0 is the initial activity obtained without pre-incubation, A is the residual activity after heat treatment, K_d is thermal inactivation rate constant (h^{-1}) and t is the exposure time (h).

The half-life of the xylanase ($t_{1/2}$, h) was determined from the relationship:

$$t_{1/2} = \ln 2/K_d.$$

Effects of metal ions, detergents and other chemical reagents

The effect of metal ions and chemical reagents was examined by pre-incubating the xylanase (0.1 mg/mL) in 100 mM sodium phosphate buffer pH 7.5, at 60 °C for 1 h, in presence of different mono-, di- and trivalent cations either as sulphate and chloride salts, ethylenediaminetetraacetic acid (EDTA), PMSF and β-mercaptoethanol (2-ME), at final concentrations of 1.0 and 10 mM. Moreover, the influence of urea at 1 % (w/v) final concentration was evaluated. The salt stability was also investigated by pre-incubating the enzyme with 1.0, 2.0 and 3.0 M NaCl at 60 °C for 1 h.

The GtXyn resistance to detergents was determined by pre-incubating the enzyme at 60 °C for 1 h, under constant agitation, with Triton X-100, Tween 20 and 80, SDS, Cetyltrimethylammonium bromide (CTAB), PEG 8000, octylphenoxypolyethoxyethanol (Nonidet) and 3-[(3-cholamidopropyl)dimethylammonium]-1-propanesulfonate (CHAPS) at a final concentration of 1 % with the sole exception of SDS that was tested at 1.0 and 10 mM. Residual activity values were determined under standard assay condition, and expressed as percentage of a control sample incubated in absence of any additive.

Effect of organic solvents

The effect of 15 % (v/v) organic solvents was investigated by pre-incubating the enzyme in presence of different solvents at 25 °C for 30, 60 and 120 min, under constant agitation. The xylanase activity towards beechwood xylan was assessed utilizing aliquots of the incubated enzyme in a reaction mixture with a solvent concentration less than 1 % (v/v). Residual activity values were expressed as percentage of a control pre-incubated with distilled water instead of solvents. Furthermore, the effect of various ethanol amounts was investigated by GtXyn pre-incubation

at 25 °C for 30 min in presence of concentrations ranging from 10 to 80 % (v/v).

The time-resistance in presence of ethanol was also evaluated by pre-incubating the xylanase at 25 °C in presence of 40 % (v/v) ethanol for different time intervals (1, 2, 5 and 24 h). The residual activity was measured under the assay conditions previously mentioned.

Effect of proteases on enzyme stability

The resistance to different proteases was tested in presence of 10 µg/mL of proteinase K (from *Tritirachium album*), trypsin and α-chymotrypsin (from bovine pancreas), subtilisin A (from *Bacillus licheniformis*) and collagenase (from *Clostridium histolyticum*). For this purpose, the purified xylanase (150 µg/mL) was pre-incubated in presence of the different proteolytic activities in 100 mM sodium phosphate buffer at pH 7.5, and 37 °C for 1, 2 and 3 h. The residual xylanase activity was measured at the standard conditions. The enzyme, pre-incubated under the same conditions in the absence of proteases, was used as control.

Substrate specificity

The specificity of the purified GtXyn towards different polysaccharides was determined, under standard conditions, using the following 1.0 % (w/v) substrates: beechwood xylan, birchwood xylan, oat spelt xylan, carboxymethyl cellulose, Avicel, laminarin, lichenan, tamarind xyloglucan, and locust bean gum.

β-Xylosidase activity was determined, according to Biely et al. (2000), by utilizing the soluble chromogenic substrate *p*-nitrophenyl-β-D-xylopyranoside (*p*NPXP). Briefly, the assay was started by adding 50 µl of appropriately diluted enzyme in 50 mM sodium phosphate buffer pH 7.5, to 450 µL of 2 mM *p*NPXP, in the same buffer. After heating the assay mixture at 50 °C for 10 min, the reaction was stopped by adding 1 mL of 1.0 M sodium carbonate and the release of *p*-nitrophenol was detected by measuring the absorbance at 405 nm. One unit of β-xylosidase was defined as the amount of enzyme required to release 1 µmol of *p*-nitrophenol per min under the assay conditions. α-Arabinofuranosidase, α-glucosidase and β-glucosidase activities were evaluated by the same assay method utilizing *p*-nitrophenyl (*p*NP)-α-L-arabinofuranoside, *p*NP-α-D-glucopyranoside, *p*NP-β-D-glucopyranoside as substrates, respectively. All the enzymatic measurements were performed in triplicate.

Analysis of hydrolysis products

To determine the mode of action of the enzyme, 1.5 U of purified GtXyn were incubated at 50 °C in 50 mM sodium phosphate buffer (pH 7.5) in presence of 25 mM xylobiose, xylotriose, xylotetraose and xylopentaose (X_2–X_5) up to 24 h, and 5 % (w/v) beechwood xylan up to 72 h. The determination of the hydrolysis products was performed withdrawing samples at different time intervals and analyzing, by thin layer chromatography (TLC), the released sugars. The silica gel plates (20 × 20 cm) (Merck Millipore KGaA, Darmstadt, Germany), developed with one run of butanol-acetic acid–water (2:1:1 by vol.), were then sprayed with a methanol-sulfuric acid mixture (95:5 v/v) and heated for few min at 130 °C. The sugar hydrolysis products were identified by comparing the Rf values of the samples with those of the above cited xylooligosaccharides and xylohexaose (X_6), utilized as standards.

Saccharification of agro-derived materials

Saccharification experiments of various LCMs such as BSG, corn cobs and biomass from giant reed (*Arundo donax*), pretreated with an aqueous ammonia solution as described by Maurelli et al. (2013), were also carried out. The raw materials, dried at 50 °C and milled by an homogenizer to obtain a fine powder, were soaked in 10 % (v/v) aqueous ammonia solution at a solid loading of 5 % at 70 °C for 22 h in screw-capped 25 mL bottles to reduce the evaporation. The alkaline mixtures were centrifuged at 800×*g*, and the residues were extensively washed with 50 mM sodium phosphate buffer until obtaining pH 7.5. The hydrolysis of the xylan fractions was carried out at 50 °C for 72 h in a total volume of 5 mL (50 mM sodium phosphate buffer pH 7.5 plus 1.5 U of enzyme), at a solid loading of 5 %. Samples were withdrawn at different time intervals and the released sugars were analyzed by TLC as described above. The amounts of reducing sugars liberated by the enzyme action were quantified with the Somogyi–Nelson method.

Results

Identification, production and localization of the xylanase activity

Geobacillus thermodenitrificans A333 was gradually adapted to utilize the xylan as a carbon source onto solid media. On plates with 0.15 % (w/v) xylan, 37–42 colonies were obtained by streaking and, after staining with Congo Red, 15–20 colonies surrounded by clear halos of 5–15 mm diameter were detected confirming the microorganism ability to hydrolyze the xylan. The xylanase production was also investigated by growing *G. thermodenitrificans* A333 in liquid basal medium supplemented with 0.15 % (w/v) beechwood xylan. The analysis of the time-course profile revealed that the extracellular xylanase production was not

growth-linked as the enzyme expression started in the exponential phase and reached the highest level in the late stationary phase (Fig. 1a).

Since the bacterial growth in NB basal medium without any carbon supplement yielded low enzyme concentrations, the regulation of xylanase expression was investigated. The utilization of monosaccharides such as 0.1 % (w/v) xylose, as supplement to basal medium, increased by 3.5-fold the enzymatic activity production that was further enhanced by xylan (Fig. 1b). In particular, testing the effect of the three different xylan types, the best result was reached with oat spelt xylan that yielded a level of xylanase expression sixfold higher than the basal one (7.24 and 43.57 mU/mL maximum activities with NB and oat spelt xylan, respectively). These findings clearly indicate that the xylanase is produced at significant amounts only in presence of inducers. Owing to the commercially unavailability of the oat spelt xylan, we were forced to utilize beechwood xylan for GtXyn production. Xylose gradually lost its function of inducer at increasing concentrations, and only a basal xylanase production was achieved at 1 % (w/v) (data not shown). Furthermore, the addition of 1 % xylose, during the exponential phase of *G. thermodenitrificans* A333 growth carried out in presence of 0.15 % beechwood xylan, stopped the xylanase production (Fig. 1a). Glucose had the function to block the xylanase expression (data not shown).

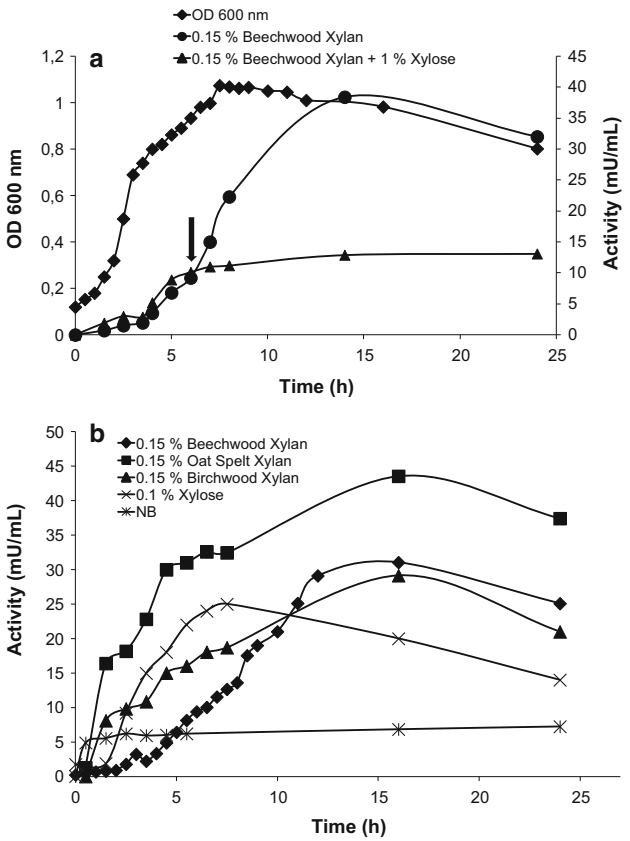

Fig. 1 Xylanase production by *G. thermodenitrificans* A333. Time-course of *G. thermodenitrificans* A333 grown in NB basal liquid medium supplemented with 0.15 % beechwood xylan at 60 °C. Arrow indicates the time of xylose addition to a culture of *G. thermodenitrificans* A333 grown in presence of 0.15 % beechwood xylan (**a**). Effect of different carbon sources (**b**)

G thermodenitrificans A333 growth in presence of agricultural wastes such as BSG, corn cobs, grape cane and wheat straw gave enhanced enzyme amounts respect to those reached with the commercial xylan preparations (239, 171, 258 and 351 mU/mL obtained in presence of BSG, grape cane, corn cobs and wheat straw, respectively).

The localization of xylanase was investigated in cultures of *G. thermodenitrificans* A333, grown on beechwood xylan by measuring the activity in the cell-free supernatant, crude extract and membrane fragments. The activity was retrieved almost exclusively in the cell-free supernatant (94.2 %), while the intracellular and cell bound levels were 4.2 and 1.6 %, respectively. This result is in complete agreement with Signal IP analysis of the protein primary sequence (Nielsen et al. 1999) that indicated the presence of a signal peptide for the amino acid positions 1–28, thus attesting that the xylanase is a secreted enzyme.

Purification of the *G. thermodenitrificans* A333 xylanase

The purification procedure of the xylanase is summarized in Table 1. The cell-free supernatant from 2 L culture, 20 fold concentrated and dialyzed, was tentatively absorbed onto different resins but successful results were only obtained with the strong cation exchange matrix. Therefore, the first two separation steps were performed on SP-Sepharose and Resource S columns, the latter allowing 11 folds increase of the purification factor being characterized by a higher resolution capacity.

The last chromatographic step, carried out by a molecular exclusion chromatography on Superdex 75 HR10/30, allowed to obtain a specific activity of 7.78 U/mg, with a purification factor of 68.2. As judged by SDS-PAGE and zymographic analyses, the xylanase migrates as a single band with a molecular mass of approximately 44 KDa, confirming its homogeneity (Fig. 2). Moreover, the xylanase has been shown to be active giving a clear band on the zymogram gel after the Congo red staining. The molecular mass of 44 KDa was also confirmed by gel filtration chromatography, allowing to ascertain a monomeric structure for the enzyme.

Primary sequence analysis

The identified 25 residues of the GtXyn N-terminal sequence showed a perfect match to the amino acid sequence

Table 1 Purification procedure of the xylanase from *G. thermodenitrificans* A333

Purification steps[a]	Volume (mL)	Total Units (U)[b]	Proteins (mg)[c]	Specific Activity (U/mg)	Yield (%)	Purification fold
Crude broth	2,000	37.83	333.4	0.114	100	1
Ultrafiltration	100	29.5	238	0.124	78	1.1
SP-Sepharose	20	17.14	75.17	0.23	45.3	2.01
Resource S	1	7.39	2.78	2.66	20	23.3
Superdex 75	1	5.05	0.65	7.78	13.3	68.2

[a] Purification was performed utilizing the cell-free supernatant from 2 L culture of *G. thermodenitrificans* A333 obtained in liquid basal medium supplemented with 0.15 % (w/v) beechwood xylan

[b] Xylanase activity was measured in 50 mM sodium phosphate buffer pH 7.5 at 50 °C for 30 min using 1 % (w/v) AZO-xylan as substrate

[c] Measured by Bradford method using bovine serum albumine as a standard (Bradford 1976)

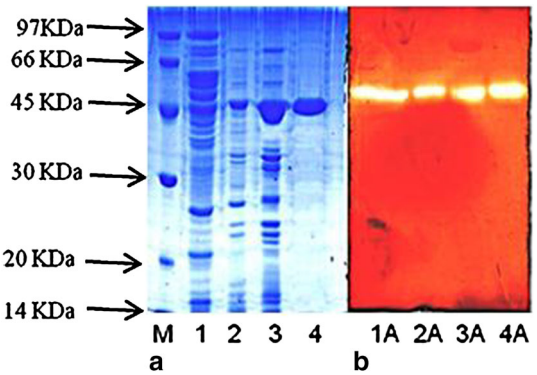

Fig. 2 10 % SDS-PAGE followed by Coomassie Blue staining (**a**) and zymogram (**b**). *Lanes* protein markers (*M*); cell-free supernatant (*1*, *1A*); pooled fractions after SP-Sepharose (*2*, *2A*); pooled fractions after Resource S (*3*, *3A*); pooled fractions after Superdex 75 (*4*, *4A*)

of the extracellular endo-xylanase from *G. thermodenitrificans* NG80-2. Consequently, the gene sequence encoding this protein (NC009328) was utilized for the construction of the primers to amplify the full-length xylanase gene from *G. thermodenitrificans* A333. The complete recombinant gene of 1,224 bp size, starting with ATG as initiation and ending in TAG as stop codon was obtained. It encoded a 407 amino acid residue protein, whose deduced sequence revealed the highest homology with xylanases from *Geobacillus* strains belonging to the GH10 such as *G. thermodenitrificans JK1* (GenBank Accession No. AEP39603, identity 99 %), *G. thermodenitrificans* NG80-2 (GenBank Accession No. YP001125870, identity 99 %), *Geobacillus* sp. TC-W7 (GenBank Accession No. ACX42569, identity 99 %), *Geobacillus* sp. 71 (GenBank Accession No. AEO96821, 94 % identity) *Geobacillus kaustophilus* (GenBank Accession No. WP02027929, identity 94 %), *Geobacillus* sp. C56-T3 (GenBank Accession No. YP_003671234, identity 91 %), and *Geobacillus stearothermophilus* (GenBank Accession No. ABI49951, identity 86 %).

The analysis with Pfam program confirmed for GtXyn the presence of the family GH10 catalytic domain, containing at the positions 230 and 293 two glutamic acids as catalytic residues. Furthermore, it was evidenced that GtXyn lacks of a substrate binding domain and bear a N-terminal stretch of 28 residues recognized as a signal peptide, in perfect agreement with the outcome from SignalIP. The theoretical protein molecular weight of 47.3 kDa with the signal sequence and 44.2 kDa without it, was in accordance with the zymographic analysis of the purified xylanase.

Influence of pH and temperature on enzyme activity and stability

The pH optimum for the xylanase was found between 7.0 and 7.5, even if GtXyn was active over the whole pH range 5.0–11.0 (Fig. 3). In particular, the enzyme was more tolerant to alkaline conditions than to the acidic ones, showing 75.6 % of the maximal activity at pH 10.0 and a sharp activity decrease at pH 6.0, where the residual activity was around 23 %. The stability was also pronounced at alkaline pHs as demonstrated by residual activity of 97, 95 and 75 % at pH 8.0, 9.0 and 10.0, respectively after 24 h of incubation at 50 °C (data not shown).

The enzymatic activity was detected over a broad range of temperature (20–90 °C) and showed its maximum at 70 °C. Moreover, high activity levels were observed between 60 and 80 °C, with values comprised between 80 and 50 % of the maximal activity at the extremes of this temperature range (Fig. 4a). Heating resistance was also evaluated by measuring the residual activity after 60 min of incubation at increasing temperatures, as described under "Materials and methods". The temperature at which 50 % of the enzyme was inactivated was 70 °C, while the residual activity values at 58, 60, 62 and 65 °C were 98, 95, 88 and 83 % respectively (data not shown).

Kinetic parameters of GtXyn thermal inactivation were determined by subjecting the enzyme to prolonged

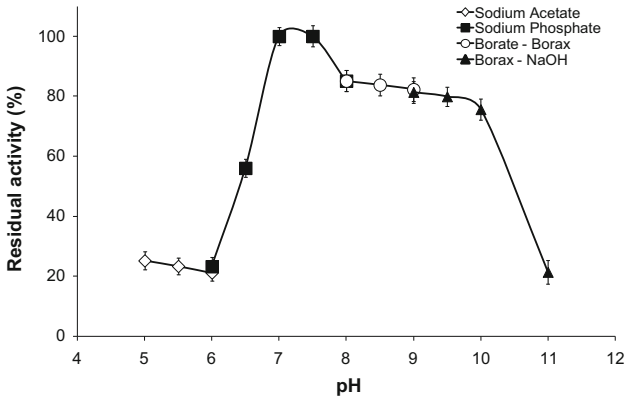

Fig. 3 Effect of pH on purified GtXyn activity. All the activity values, measured at 50 °C, are means of three replicates ± SD

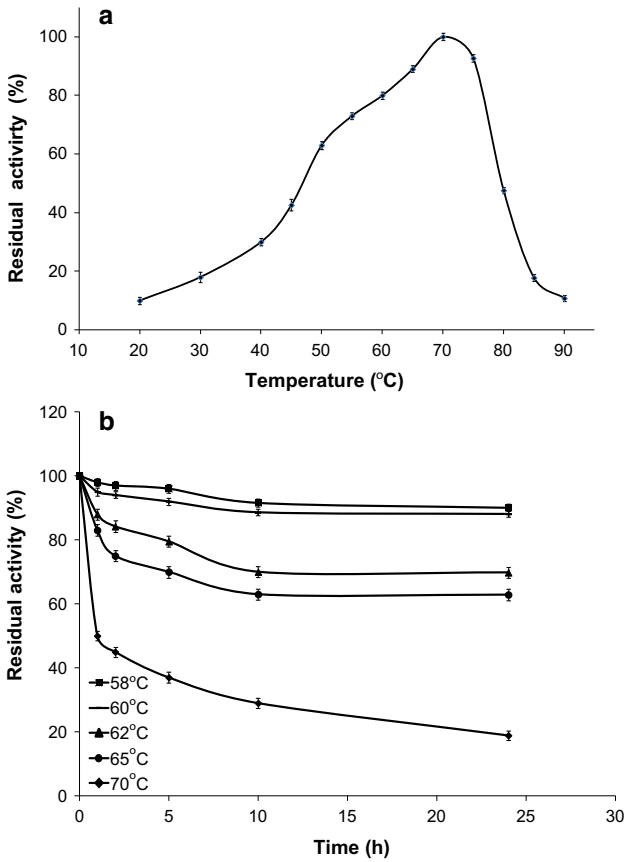

Fig. 4 Effect of temperature on purified GtXyn. Thermophilicity of the purified enzyme was estimated in presence of the substrate (**a**). Thermostability was investigated by incubating the enzyme without substrate. After the indicated intervals of time, aliquots were withdrawn and assayed for the activity as previously described. The percent residual activity values were plotted against the corresponding incubation times (**b**). All the activity values, measured at pH 7.5, are means of three replicates ± SD

incubations at different temperatures in the range 58–70 °C, and measuring the residual activity under standard conditions. As observed in Fig. 4b, the plots of the

Table 2 Inactivation kinetic parameters of purified GtXyn

Temperature (°C)	K_d (h^{-1})	$t_{1/2}$ (h)
58	0.0074	94.6
60	0.0096	72.92
62	0.0333	21.02
65	0.0474	14.77
70	0.184	3.76

K_d = Thermal inactivation rate constant

$t_{1/2}$ = Half-life

GtXyn residual activity versus the incubation time were linear, allowing the expression of the thermal inactivation with a first order kinetic. The results concerning the inactivation rate constants and the xylanase half-lives at different temperatures are presented in Table 2. In overall, it can be observed that GtXyn is characterized by a good thermal stability showing at 58 and 60 °C a residual activity of 90 and 88 % after 24 h of incubation and half-lives of 94.6 and 73 h respectively.

Effect of metal ions and chemical reagents on xylanase

The influence of metal ions either as chloride or sulfate salts and of the metal chelator EDTA were investigated at final concentrations of 1.0 and 10 mM. As reported in Table 3, monovalent cations, such as Na^+ and Li^+, did not significantly affect the enzymatic activity. On the contrary, K^+ exhibited a marked stimulating effect, at both 1.0 and 10 mM concentrations, enhancing the activity, as chloride salt, by 42 and 45 %, respectively. Bivalent cations such as Ba^{2+} did not substantially influence the enzyme, while an activity decrease up to 29 and 36 % with Ca^{2+} and Mg^{2+}, respectively was observed. Transition metals such as Ag^+, Cu^{2+}, Ni^{2+}, Zn^{2+}, Mn^{2+}, Cd^{2+}, Al^{3+} slightly affected the enzyme leading to modifications of the activity within 25 %, while Fe^{2+}, Fe^{3+}, Hg^{2+} and Co^{2+} had a marked impact on the activity. In particular, Fe ions caused an almost complete inhibition at 10 mM. Conversely, Hg^{2+} and Co^{2+} highly lowered the activity at both concentrations, with Hg^{2+} which exhibited a total inhibition effect, and Co^{2+} that caused an activity reduction up to 91 %. EDTA did not fundamentally modify the activity indicating that metal ions neither are required nor substantially influence the xylanase activity.

The effect of potential inhibitors or activators such as PMSF, 2-ME and urea was also examined. PMSF and 2-ME did not substantially affect the enzymatic activity, while urea inhibited GtXyn by 44 %.

The xylanase halo-tolerance was evaluated revealing that, after incubation for 1 h at 60 °C in presence of increasing NaCl concentrations (1.0–3.0 M), the activity underwent slight reduction up to 30 % (data not shown).

Table 3 Effect of metal ions and substances on the purified GtXyn

Metal ions or chemical agents	Residual activity (%)	
	1 mM	10 mM
EDTA	121	104
NaCl	122	106
Na_2SO_4	107	102
KCl	142	145
K_2SO_4	132	127
LiCl	93	99
$CaCl_2$	90	90
$CaSO_4$	97	71
$MgCl_2$	72	70
$MgSO_4$	67	64
$BaCl_2$	116	92
$HgCl_2$	0	0
$CoCl_2$	42	9
$CoSO_4$	30	15
$FeCl_3$	91	7.6
$FeSO_4$	80	3
$CuCl_2$	75	81
$CuSO_4$	79	76
$NiCl_2$	99	94
$NiSO_4$	91	101
$ZnSO_4$	80	80
$AlCl_3$	125	86
$CdCl_2$	96	81
$MnCl_2$	90	89
$AgNO_3$	105	89
PMSF	89	92
2-ME	105	113
Urea[a]	56	

The effect of different reagents was determined by pre-incubating the purified GtXyn in100 mM phosphate buffer at 60 °C and pH 7.5 in presence of the listed substances for 1 h. The activity of GtXyn, measured in the absence of any substance, was set as 100 %. Values are means of three experiments

[a] Urea was tested at concentration of 1 %

The influence of various detergents at 1 % concentration on the GtXyn activity was investigated (Table 4). The xylanase in the presence of non-ionic surfactants was highly activated. Upon the addition of Triton X-100, Tween 20 and 80 and PEG 8000, an activity increase up to 54 % was observed. The sole exception was represented by Nonidet P40 that showed a slight inhibition effect, reducing the activity by 25 %. In contrast, considerable activity losses with ionic surfactants were detected: in presence of CTAB, an activity decrease of 86 % was observed while SDS, tested at 1.0 and 10 mM final concentrations, gave activity reductions of 13 and 79 %, respectively. Finally, the zwitterionic detergent CHAPS did not affect the activity.

Table 4 GtXyn stability in surfactants

Surfactants (1 %)	Residual activity (%)
Tween 20	132
Tween 80	152
Triton ×100	154
PEG 8000	137
Nonidet P40	75
CHAPS	106
CTAB	14
SDS (1–10 mM)	87–21

The purified enzyme was treated with the indicated concentration of the effectors for 1 h at 60 °C. The activity of GtXyn, measured in the absence of any substance, was set as 100 %. Values are means of three experiments

The time resistance of GtXyn to organic solvents with different log *P* values, at 15 % (v/v) final concentration, was evaluated (Table 5). The enzyme was not substantially influenced by most of the tested substances even after 2 h of incubation. Slight effects on GtXyn consisting in activity reductions by 15, 13 and 8 %, were detected in presence of DMSO, acetonitrile and 2-propanol, respectively, while *n*-butanol enhanced the activity by 14 %. Formaldehyde and glycerol markedly inhibited the enzyme; after 2 h of incubation activity reduction of 71 and 62 %, in presence of formaldehyde and glycerol respectively, were detected. The data also demonstrated that GtXyn stability in organic solvents did not follow log *P* trend.

The effect of the addition of different ethanol amounts was investigated, indicating that the enzyme was marginally influenced by the exposition, for 30 min, to increasing concentrations of this organic solvent (Fig. 5). In fact, GtXyn, underwent a weak activation up to 20 % ethanol and then a gradual slight inhibition that led to a residual activity of 70 % in presence of 80 % ethanol. GtXyn activation was also evident upon prolonged incubation times (1, 2, 5 and 24 h) in presence of 40 % ethanol, reaching the highest levels after 1 and 2 h, with values of residual activity around 120 and 118 %, respectively (data not shown).

Protease resistance

The purified GtXyn exhibited good resistance to neutral and alkaline proteases as demonstrated by the residual activity values detected upon incubation up to 3 h with 10 μg/mL of trypsin, chymotrypsin, collagenase, subtilisin A and proteinase K. In particular, GtXyn activity remained almost unchanged after pre-incubation for 2 h with collagenase and for 1 h with trypsin, while, after 1 h, the residual activity was 90 % with subtilisin A and proteinase K,

Table 5 GtXyn stability in organic solvents

Solvents (15 % v/v)	logP	Residual activity (%)		
		30 min	60 min	120 min
Ethanol	−0.3	100	94	96
Methanol	−0.76	100	94	99
Acetone	−0.24	92	97	97
Acetonitrile	−0.34	98	92	87
Ethyl acetate	0.71	100	98	98
2-Propanol	0.07	99	95	92
DMSO	−1.35	96	88	85
DMF	−1.04	100	99	99
n-Butanol	0.88	100	100	114
Formaldehyde	0.35	60	42	29
Glycerol	−2.55	58	46	38

The purified enzyme was treated with various solvents for different intervals of time. The activity of GtXyn, measured in the absence of any substance, was set as 100 %. Values are means of three experiments

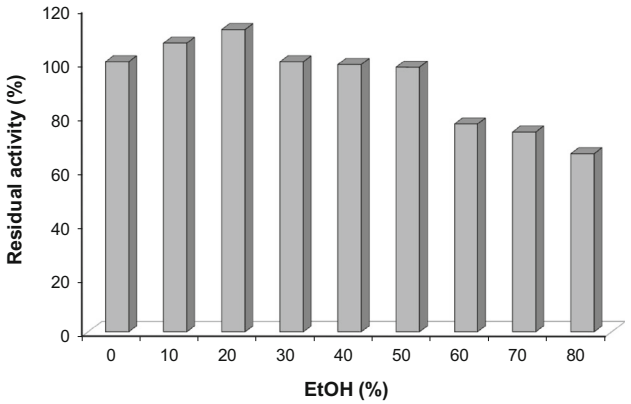

Fig. 5 Effect on GtXyn activity of different ethanol concentrations. The enzyme was incubated with the indicated concentrations of ethanol for 30 min at 25 °C. Each value represents the mean of triplicate measurements

and 80 % with chymotrypsin. A residual activity of 80 % was also registered after 3 h in presence of all the tested proteases with the sole exception of chymotrypsin that gave a reduction of 38 %.

Substrate specificity

The substrate specificity of the purified xylanase using various natural and synthetic substrates was tested.

GtXyn acted only on xylans and lacked of activity towards both amorphous and crystalline cellulose represented by carboxymethylcellulose and Avicel, respectively, suggesting that the enzyme is a cellulase-free xylanase. The enzyme failed also to hydrolyze the natural glucose formed polysaccharides lichenan and laminarin, and was unable to breakdown cell wall components such as mannan, xyloglucan and arabinan. Respect to the xylan hydrolysis, GtXyn did not show a marked specificity towards one of the three natural xylans tested but only a slight preference towards beechwood xylan was evidenced as demonstrated by the activity values of 6.9, 7.10 and 8.76 U/mg in presence of birchwood, oat spelt and beechwood xylan, respectively. Moreover, no detectable α-arabinofuranosidase, β-xylosidase, α- and β-glucosidase activities were observed, since GtXyn was unable to hydrolyze the *p*NP-glycosides utilized as respective substrates (data not shown). These results clearly highlight the strict specificity of GtXyn toward polysaccharides containing 1,4 β-xylan bonds.

Analysis of hydrolysis products and saccharification of agro-derived biomass

The mode of action of the purified GtXyn was determined using xylooligosaccharides (X_2–X_5) as test substrates and analyzing the hydrolysis products by TLC (Fig. 6a). X_2 was not hydrolyzed even after 48 h of incubation, and X_3 was slowly transformed, differently from X_4 and X_5, that were rapidly degraded. Most of the X_3 was converted to X_2 upon 24 h of incubation, and after 48 h detectable quantities of X_3 in conjunction with negligible amounts of xylose were still observed. X_4 was already hydrolyzed after 30 min of incubation, and the prevalent product, X_2, was accompanied by irrelevant amounts of xylose. X_5 disappeared after 30 min of incubation producing X_2 and X_3. The disappearance of the X_3, simultaneously to the appearance of xylose trace, was observed after 48 h of incubation. The presence of relatively high concentrations of X_2 and X_3, in the X_3, X_4 and X_5 reaction mixtures, suggests that the xylanase preferentially cleaves the internal glycoside bonds of the oligosaccharides. It can, therefore, be concluded that GtXyn is a typical endo-β-1,4-xylanase. Beechwood xylan was treated with the purified GtXyn and incubated up to 72 h (Fig. 6b). The main reaction products were X_5, X_3 and X_2, with a prevalence of X_2 upon long time incubation. After few seconds GtXyn was already able to produce xylooligosaccharides with DP comprised between 6 and 2 but X_2, X_3 and X_5 were the main products observed after 2 h. X_3 completely disappeared at 48 h and only X_2 and X_5 were detected together with minimal amounts of xylose; this situation remained unaltered up to 72 h. The only difference with the results obtained from the hydrolysis of xylooligosaccharides, is represented by the presence of X_5 even after 72 h of incubation. It could be due to the substituents, present on the xylan backbone, that would hinder and slow down the GtXyn attack.

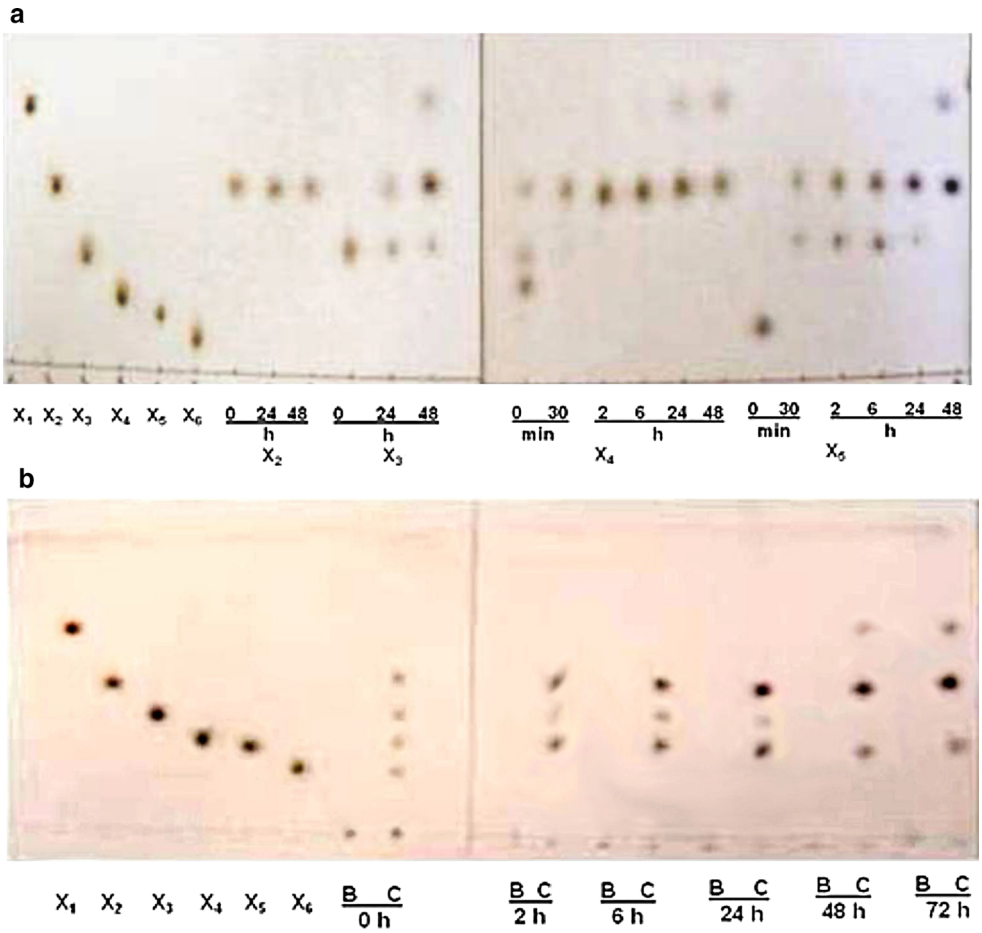

Fig. 6 Time course of xylooligosaccharides (**a**) and beechwood xylan (**b**) hydrolysis. Standards: xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5) and xylohesaose (X6). In **b** B (beechwood xylan incubated without GtXyn), C (beechwood xylan incubated with GtXyn)

The saccharification of agro-derived biomasses (BSG, corn cobs and *A. donax* biomass), pretreated by soaking in aqueous ammonia solution, was carried with the purified GtXyn. The end-products profiles, analyzed by TLC, were identical to that obtained with the beechwood xylan (data not shown). On the contrary, the evaluation of the reducing sugars released revealed that the three tested materials reached different degrees of xylan hydrolysis (Table 6). Comparable levels of xylan conversion were observed with BSG and corn cobs after 6 and 24 h of reaction, but upon prolonged incubation times different results were achieved. Xylan hydrolysis percentage from BSG did not exceeded the 32 %, reached after 24 h of incubation. Conversely, the percentage of xylan degradation with corn cobs reached the 68.6 % after 72 h and was double respect to that obtained after 24 h. The percentage of xylan degradation with *A. donax*, was very low, reaching 7.17 % after 72 h.

Table 6 Hydrolysis of agro-derived materials by purified GtXyn

	Incubation time (h)	Reducing sugar yield (mg xylose equivalent mL^{-1})[a]	Xylan hydrolysis (%)
Arundo donax	0	0.17	1.85
	6	0.24	2.61
	24	0.52	5.65
	48	0.46	5.00
	72	0.66	7.17
BSG	0	0.02	0.16
	6	2.61	21.7
	24	3.83	31.9
	48	4.06	33.8
	72	3.8	31.6
Corn Cobs	0	0.09	0.51
	6	4.8	27.4
	24	6.2	35.4
	48	10.1	57.7
	72	12.0	68.6

[a] Reducing sugars released in the incubation mixtures were estimated by the Somogyi–Nelson assay

Discussion

Several extracellular xylanases from bacteria of the genus *Geobacillus* have been studied and in particular three

strains identified as *G. thermodenitrificans* were reported as extracellular xylanase producers (Anand et al. 2013; Canakci et al. 2007; Gerasimova and Kuisiene 2012). The detailed characterization of the native *G. thermodenitrificans* A333 xylanase allows to appreciate the relevant biotechnological characteristics of such a biocatalyst. The data reported in the present work also highlight that the catalytic properties of GtXyn are quite different from those of the recombinant endo-xylanase studied by Gerasimova and Kuisiene (2012), whose primary sequence differed in only two amino acids from that of the enzyme produced by the wild type strain A333 of *G. thermodenitrificans.* The investigation on GtXyn started from its production, highlighting the microorganism capability to utilize several cheap and widely available carbon sources, such as brewery's spent grains, corn cobs, wheat straw and grape cane for the bacterial growth and enzyme secretion. This outcome would allow to exploit these raw materials in properly developed fermentation processes, instead of the commercially available xylans, to reduce the costs of the biocatalyst hyper-production. A high enzyme purity, even if it is generally not important for industrial applications due to economic reasons, is relevant to investigate on the enzyme properties. On SDS-PAGE, GtXyn resulted purified to homogeneity and monomeric. It showed a molecular mass of about 44 kDa which, among those previously cited, differs only from the molecular weight of the partially characterized enzyme by Canakci et al. (2007), that was 30 kDa in size. As almost all known xylanases from geobacilli, also GtXyn belongs to the GH10 family (www.cazy.org, Cantarel et al. 2009) and presents similar optimal pH and temperature (Canakci et al. 2007, 2012; Khasin et al. 1993; Liu et al.2012; Wu et al. 2006). In details, GtXyn showed the highest activity at 70 °C and pH 7.0–7.5, and an appreciable level of stability in wide intervals around these values. A good thermal stability was demonstrated by residual activities of 90 and 88 %, after 24 h of incubation at 58 and 60 °C, respectively, along with half-lives of 94.6 and 73 h. Noteworthy was also the enzyme stability at alkaline pHs, as showed by activity values almost unaltered for 24 h at pH 8.0 and 9.0, and by a residual activity of 75 %, at pH 10.0 after 24 h. The relevant GtXyn resistance to thermo-alkaline conditions is comparable to that reported for other xylanases (Canakci et al. 2012; Gerasimova and Kuisiene 2012; Kumar and Satyanarayana 2011; Liu et al. 2012; Verma and Satyanarayana 2012; Wu et al. 2006). This is a crucial factor that makes the enzyme suitable for several industrial applications.

Impurities such as metal ions, which are present in the agro-industrial wastes, can potentially inhibit the activity of xylanases. Although the activity of GtXyn was not significantly influenced in the presence of EDTA and several metal ions, K^+, Hg^{2+}, Co^{2+}, Fe^{2+} and Fe^{3+} highly affected its activity. In particular, K^+ markedly stimulated the enzyme, probably due to the role of this metal ion as cofactor for enzyme-substrate reactions or its stabilizing effect on the protein molecular structure. On the other hand, GtXyn was strongly inhibited by Hg^{2+}, Co^{2+}, Fe^{2+} and Fe^{3+}, probably for the oxidizing nature of these metal ions (Vieille and Zeikus 2001). In addition, GtXyn was among the few reported xylanases endowed with the capability to tolerate high salinity levels, solvents and surfactants (Woldesenbet et al. 2012; Zhou et al. 2012). GtXyn, was able to retain 70 % of its activity for 1 h at 3.0 M NaCl, showing a salt tolerance comparable to that of xylanases isolated from microorganisms harbored in marine or saline habitats (Guo et al. 2009; Khandeparker et al. 2011; Menon et al. 2010). For this reason the enzyme can find applications in the processing of sea and saline food and in the production of bioethanol from seaweeds. The marked halostability also accounts for the GtXyn resistance to the organic solvents (Sellek and Chaudhuri 1999). The high resistance, in particular to ethanol, makes GtXyn suitable for different applications, included those for production of the alcoholic beverages. As reported by Sato et al. (2010), the addition of xylan-degrading enzymes during the simultaneous hydrolysis and fermentation process of rice starch, significantly enhanced the alcohol yield in the sake preparation. GtXyn can also be regarded as a promising candidate for bioethanol production in consolidated bioprocessing due to the enzyme capability to remain fully active at ethanol concentrations of 23–63 g/L, that are normally reached in these types of industrial procedures (Morrison et al. 2011). Moreover, the positive effect of non ionic detergents on its activity could also allow to improve the rate and extent of lignocellulosic substrate hydrolysis (Kaya et al. 1995).

GtXyn applicability in the pulp and paper industry would also be relevant. Due to the resistance to alkaline pHs and the cellulase-free activity, the biocatalyst utilization before the bleaching kraft pulp process would reduce the chlorine consumption up to 25–35 % and avoid time and cost consuming pH adjustments (Archana and Satyanarayana 1997; Sharma et al. 2007) and the cellulose degradation. The possibility to utilize the xylanase in conjunction with detergents could also allow to obtain better results than those reached with the traditional chemical methods in the process of paper pulping and deinking of recovered paper (Kaya et al. 1995). GtXyn was also found to be protease-resistant and, even if the mechanism underlying this behavior is not completely clear, the insensitivity to proteolytic attack could be explained with the enzyme extracellular localization and thermostability (Fontes et al. 1995).This relevant characteristic makes GtXyn useful for potential applications in textile and food

industry for cotton scouring and improvement of animal feed, respectively (Li et al. 2008).

Finally GtXyn, owing to its mechanism of action and alkali-thermostable nature, is suitable for the production of XOS, known to posses many beneficial biomedical and health effects, such as the stimulation of the human intestinal *Bifidobacteria* and *Lactobacilli* growth (Moure et al.2006; Vazquez et al. 2000; Yang et al. 2005). Currently, XOS are mainly obtained by enzymatic hydrolysis due to the high specificity and negligible side product generation (Tan et al. 2008); however, the high cost of production requires further developments in the production processes. XOS preparation is generally performed using neutral xylanases but the employment of alkaline enzymes, such as GtXyn, may improve the yield due to the higher solubility of xylan in alkaline solutions (Mamo et al. 2007). Moreover, the use of agricultural residues and non-food crops as alternative cheap substrates has considerable potentialities for the cost-effective XOS synthesis. For this purpose, corn cobs, BSG and the biomass from *A. donax* were utilized for saccharification experiments with GtXyn. The first two materials are agro-industrial by-products characterized by a high content of xylan, that accounts for the 35 and 24.1 % of their dry weights, respectively. *A. donax* crop is one of the highest yielding species for biomass production in southern Europe, with a high xylan content (20 % of its dry weight) (Scordia et al. 2011). It has also been recognized as very robust species, able to grow well on marginal and non-agricultural soils, thus avoiding the competition with food crops for lands (Krasuska et al. 2010). As frequently reported in literature, in order to improve the xylan digestibility, the raw materials are subjected to a pretreatment to remove the lignin fraction that shields the cell wall polysaccharides from the enzymatic attack. In the present paper, the pretreatment was realized by soaking the LCMs in aqueous ammonia solution, due to the high efficacy of this method in breaking the ester bonds between lignin, hemicellulose and cellulose without degrading the hemicellulose polymers, as recently confirmed by Maurelli et al. (2013). Among the three tested materials, corn cobs, that is characterized by the most consistent xylan content, reached the highest percentage of xylan hydrolysis and the best yield of reducing sugar released after 72 h. On the other hand, for BSG, the polysaccharide conversion process was completed in the shortest reaction time, namely 24 h, probably due to its lowest lignin content. The profile of the hydrolysis end products, obtained from the saccharification of beechwood xylan and the three agro-derived feedstocks, revealed the presence of XOS with a DP comprised between 6 and 2, with a prevalence of xylobiose upon long times of reaction. The presence of only minimal amount of xylose suggests a profitable GtXyn applicability in the bioconversion of LCMs into XOS.

In conclusion, the thermo-alkali-stable and cellulase-free endo-β-1,4-xylanase from *G. thermodenitrificans* A333 can be certainly regarded as one of the xylanases that, owing to its stability at extreme process conditions, could be efficaciously used in several industrial applications included the XOS production from lignocellulosic biomasses and agricultural residues. Further work is underway to clone and express GtXyn in an suitable host to enhance the production of the enzyme with unchanged biotechnological relevant properties of the native protein.

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The effect of *Pleurotus ostreatus* arabinofuranosidase and its evolved variant in lignocellulosic biomasses conversion

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ABSTRACT

The fungal arabinofuranosidase from *Pleurotus ostreatus* PoAbf recombinantly expressed in *Pichia pastoris* rPoAbf and its evolved variant rPoAbf F435Y/Y446F were tested for their effectiveness to enhance the enzymatic saccharification of three lignocellulosic biomasses, namely *Arundo donax*, corn cobs and brewer's spent grains (BSG), after chemical or chemical–physical pretreatment. All the raw materials were subjected to an alkaline pretreatment by soaking in aqueous ammonia solution whilst the biomass from *A. donax* was also pretreated by steam explosion. The capability of the wild-type and mutant rPoAbf to increase the fermentable sugars recovery was assessed by using these enzymes in combination with different (hemi)cellulolytic activities. These enzymatic mixtures were either entirely of commercial origin or contained the cellulase from *Streptomyces* sp. G12 CelStrep recombinantly expressed in *Escherichia coli* in substitution to the commercial counterparts. The addition of the arabinofuranosidases from *P. ostreatus* improved the hydrolytic efficiency of the commercial enzymatic cocktails on all the pretreated biomasses. The best results were obtained using the rPoAbf evolved variant and are represented by increases of the xylose recovery up to 56.4%. These data clearly highlight the important role of the accessory hemicellulolytic activities to optimize the xylan bioconversion yields.

1. Introduction

Lignocellulose represents a favoured feedstock for development of a 'no oil' bioeconomy since it is the most abundant biomass, representing near 70% of the total plant biomass, and its use does not provide competition with food industries. The efficient enzymatic hydrolysis of pretreated biomass to achieve both high glucose and xylose yields requires finely tuned mixtures of enzymes acting on the complex carbohydrate matrix. Cellulases, hemicellulases (xylanases) and accessory enzymes (e.g., arabinofuranosidases, pectinases, mannanases) are needed for conversion of pretreated lignocellulose into fermentable sugars. Hemicellulose represents about 20–35% of lignocellulosic biomass and it is a very complex polysaccharide composed of a wide variety of sugars including xylose, arabinose, mannose, and galactose (Saha, 2003). The importance of side-chain acting hemicellulases, such as α-L-arabinofuranosidase (EC 3.2.1.55), feruloyl esterase (EC 3.1.1.73), α-glucuronidase (EC 3.2.1.139), and acetyl xylan esterase (EC 3.1.1.72), in lignocellulosic biomass degradation has been clearly evidenced in literature demonstrating marked increases of the efficiency of lignocellulosic hydrolysis in presence of these auxiliary enzymes (Gao et al., 2011; Ravalason et al., 2012).

The fungi that obtain their nutrition from the breakdown of plant biomass are interesting producers of cell wall-degrading enzymes including side-chain acting hemicellulases (Van den Brink and de Vries, 2011). Several fungi such as *Aspergillus terreus* (Le Clinche et al., 1997), *Aspergillus awamori* (Kaneko et al., 1998), *Penicillium purpurogenum* (De Ioannes et al., 2000), *Aspergillus niger* (vd Veen et al., 1991) and *Aspergillus nidulans* (Ramón et al., 1993) have been reported to be able to produce α-L-arabinofuranosidases.

The white rot fungus *Pleurotus ostreatus* has been recently investigated for its hemicellulolytic capability (Iandolo et al., 2011) and an α-L-arabinofuranosidase, named PoAbf, was identified during its solid state fermentation on tomato pomace (Amore et al., 2012a). The enzyme PoAbf recombinantly expressed in *Pichia pastoris*, rPoAbf, was characterized and further

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experiments of its directed evolution allowed developing a variant, rPoAbf F435Y/Y446F, with improved catalytic properties (Giacobbe et al., 2014). As a matter of fact, this variant when assayed with p-nitrophenyl α-L-arabinofuranoside showed a k_{cat} about 3-fold higher and a K_M 30% lower than that of the wild type, and an improved ability to hydrolyze insoluble substrates such as larch arabinogalactan, arabinoxylan and arabinan, suggesting its improved potential for hemicellulose conversion.

Our work was aimed at assessing the ability of the α -L-arabinofuranosidase rPoAbf and its directed evolved variant rPoAbf F435Y/Y446F to improve the biodegradation of three different lignocellulosic biomasses, namely *Arundo donax*, corn cobs and brewer's spent grain. Since the commercially available hemicellulases are typically dominated by backbone-acting xylanases and β-xylosidases with low side-chain acting hemicellulases, the effect of the addition of PoAbf and its directed evolved variant to commercial cellulases, xylanases, β-xylosidase and β-glucosidase was analyzed. These enzymes were also tested in a cocktail containing the cellulase CelStrep from *Streptomyces* sp. G12 recombinantly expressed in *Escherichia coli* (Amore et al., 2012b) in substitution to the commercial cellulase.

2. Material and methods

2.1. Raw materials

Different agro-industrial raw materials including giant reed (*A. donax*), brewery's spent grains (BSG) and corn cobs were utilized for the saccharification experiments. Untreated *A. donax* biomass was kindly provided by Enea Trisaia Research Center – Italy and consisted of chips of approximately 3 × 2 × 0.5 cm of size obtained from whole plants, including leaves. The biomass was then dried at 50 °C, milled by an homogenizer to obtain a fine powder, and stored in sealed vials at room temperature until use. BSG were provided by Maltovivo micro-brewery, Avellino, Italy and corn cobs were obtained from Companhia das Lezírias (Samora Correia, Portugal). Both materials were dried up at 50 °C to a moisture content less than 10% (w/w), milled to particles smaller than 3 mm and stored in sealed vials at room temperature until use.

2.2. Pretreatments and determination of chemical composition

The different raw materials were subjected to a pretreatment with an aqueous ammonia solution on a lab-scale as described by Maurelli et al. (2013). The biomasses reduced to a fine powder, were soaked in 10% (v/v) aqueous ammonia solution at a solid loading of 5% at 70 °C for 22 h in screw-capped 25 mL bottles to reduce the evaporation. The alkaline mixtures were centrifuged at 800 × *g*, and the residues were extensively washed with 50 mM sodium acetate buffer until obtaining the required pH for the subsequent enzymatic saccharification process (pH 5.0).

The biomass from *A. donax* pretreated by steam explosion (Di Pasqua et al., 2014) was also analyzed.

Carbohydrate compositions of the biomass samples untreated and pretreated were determined according to the method of Davis (1998). This procedure involved an acid hydrolysis carried out in two step which fractionate the polysaccharides into their corresponding monomers. First, the samples were soaked in 72% v/v H_2SO_4, at a solid loading of 10%, at 30 °C, for 1 h; then the mixtures were diluted to 4% (v/v) H_2SO_4 with distilled water, fucose was added as an internal standard, and the secondary hydrolysis was performed for 1 h at 120 °C. After filtration through 0.45 μm Teflon syringe filters (National Scientific, Lawrenceville, GA), the hydrolysis products were analyzed via HPLC as described below. The acid insoluble lignin (Klason lignin) was determined by weighting the dried residue after total removal of the sugars.

2.3. Enzymatic activities

The following commercial enzymatic preparations: Accellerase 1500 (cellulase enzyme complex), Accellerase XY (xylanase enzyme complex), and Accellerase BG (β-glucosidase) were kindly provided by Danisco US Inc., Genencor Division (Palo Alto, CA, USA). Cellulase from *Trichoderma reesei* ATCC26921, Cellobiase from *A. niger*, Xylanase from *Trichoderma viride* and thermostable β-xylosidase were purchased from Sigma (St. Louis, MO). The cellulase CelStrep from *Streptomyces* sp. G12 recombinantly expressed in *E. coli* (Amore et al., 2012b), the α-L-arabinofuranosidase from *P. ostreatus* recombinantly expressed in *P. pastoris* (rPoAbf) (Amore et al., 2012a) and a mutant of rPoAbf indicated as rPoAbf F435Y/Y446F (Giacobbe et al., 2014) were utilized in combination with the commercial enzymatic preparations.

The enzymatic activities were measured at 50 °C in 50 mM sodium acetate buffer (pH 5.0). Cellulase activity was determined spectrophotometrically utilizing the soluble chromogenic substrate carboxymethyl cellulose-Remazol Brilliant Blue R (AZO-CM-Cellulose) (Megazyme Co., Bray Ireland). The standard assay mixture, containing 150 μL of 0.2% (w/v) substrate in 50 mM sodium acetate buffer, and 150 μL of properly diluted enzyme solution, was incubated for 30 min. The reaction was stopped by adding 750 μL of 96% ethanol to the mixture, followed by incubation at room temperature for 10 min and centrifugation at 9000 × *g* for 10 min. The absorbance of the supernatant was read at 590 nm, and the enzyme units were calculated from a standard curve constructed with known amounts of cellulase from *T. viride*. The xylanase activity was evaluated by the same assay method utilizing methyl-D-glucurono-D-xylan-Remazol Brilliant Blue R (Azo-Oat Spelt Xylan) (Megazyme Co., Bray Ireland), as the substrate (Biely et al., 1985). One unit of xylanase activity was defined as the amount of enzyme required to increase the absorbance at 590 nm of 1 A min^{-1} under the experimental conditions. β-xylosidase activity was determined, according to Biely et al. (2000), utilizing the soluble chromogenic substrate *p*-nitrophenyl-β-D-xylopyranoside (*p*NPXP) (Sigma, St. Louis, MO). The assay was started by adding 50 μl of appropriately diluted enzyme in 50 mM sodium acetate buffer pH 5.0 to 450 μL of 2 mM *p*NPXP, in the same buffer. After heating the assay mixture at 50 °C for 10 min, the reaction was stopped by adding 1 mL of 1.0 M sodium carbonate and the release of *p*-nitrophenol was detected by measuring the absorbance at 405 nm. One unit of β-xylosidase was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol per min under the assay conditions. α-Arabinofuranosidase and β-glucosidase activities were evaluated by the same assay method utilizing *p*-nitrophenyl (*p*NP)-α-L-arabinofuranoside and *p*NP-β-D-glucopyranoside (Sigma, St. Louis, MO) as the substrates, respectively. All the enzymatic measurements were performed in triplicates.

2.4. Enzymatic hydrolysis

The saccharification experiments were carried out at 50 °C for 72 h in a total volume of 5 mL (50 mM sodium acetate buffer pH 5.0 plus enzyme cocktail), at a solid loading of 5%. The hydrolysis of pretreated lignocellulosic materials was carried out with enzyme cocktails prepared with the following commercial products at the indicated amounts expressed as units per grams of pretreated biomass: 5.4 U g^{-1} of Accellerase 1500, 4000 U g^{-1} of Accellerase XY and 145 U g^{-1} of Accellerase BG (mix 1) or 5.4 U g^{-1} Cellulase from *T. reesei* ATCC26921, 145 U g^{-1} of Cellobiase from *A. niger*, 80 U g^{-1} of Xylanase from *T. viride*, 8 U g^{-1} of thermostable β-xylosidase (mix 2). Because of its high glucose content, Accellerase BG was dialyzed against 50 mM sodium acetate before addition to the pretreated biomass.

Subsequent experiments were performed utilizing cellulase CelStrep in each commercial cocktail, mix 1 or mix 2, in substitution of the cellulolytic component and the mixtures then named mix 1CS and mix 2CS, respectively.

The action of rPoAbf and rPoAbfF 435Y/Y446F mutant was evaluated in combination with mix1, mix 2, mix 1CS and mix 2CS.

The saccharification mixtures together with blanks (pretreated lignocellulosic materials without enzyme cocktail) were incubated in a shaking water bath at 50 °C and 140 rpm for 72 h. Samples were withdrawn at different time intervals, chilled on ice and centrifuged at 16,500 × *g* for 30 min at 4 °C. The supernatants were analyzed to quantify the amount of sugars released as described below. The saccharification yield was expressed as percentage of sugar production calculated respect to the amount of sugars present in the dry weight pretreated materials before the hydrolysis processes. Each saccharification experiment was run in triplicate.

2.5. Determination of sugar content

For estimation of the sugars released from untreated and pretreated biomasses prepared as described in Section 2.2 and enzymatic hydrolysates prepared as described in Section 2.4, the cleared supernatants were opportunely diluted, and analyzed by a high-performance liquid chromatographic (HPLC) system (Dionex, Sunnyvale, CA, USA), equipped with an anionic exchange column (Carbopac PA-100) and a pulsed electrochemical detector. Glucose and xylose were separated with 16 mM sodium hydroxide at a flow rate of 0.25 mL/min, and identified by the respective standards. Fucose was used as internal standard.

3. Results and discussion

3.1. Effect of rPoAbf and its variant in bioconversion of A. donax pretreated by steam explosion

The use of agricultural residues and non-food crops such as *A. donax* as alternative cheap substrates has considerable potentialities for the cost-effective biofuel production. *A. donax* crop is considered a valuable feedstock, being one of the highest yielding species for biomass production in southern Europe, with a high xylan content representing the 20% of its dry weight (Scordia et al., 2011). It has also been recognized as a very robust species with the characteristic to avoid competition with food crops for lands, due to its ability to grow well on marginal and non-agricultural soils (Krasuska et al., 2010).

A. donax pretreated by steam explosion (Di Pasqua et al., 2014) was subjected to saccharification using cocktails of commercial enzymes exclusively of fungal origin, with the addition of the *P. ostreatus* arabinofuranosidase recombinantly expressed in *P. pastoris* rPoAbf or its evolved variant rPoAbf F435Y/Y446F. These arabinofuranosidases were also tested in combination with the recently investigated cellulase from *Streptomyces* sp. G12 CelStrep recombinantly expressed in *E. coli* to verify a possible synergism with a bacterial cellulase. The conditions of pH and temperature adopted in saccharification experiments were those described in the Section 2.4 at which all the tested enzymes retained 80% of their initial activities. The following main effects were revealed in the different tested conditions.

3.1.1. Effect of addition of rPoAbf or its variant to the commercial enzymatic mix 1

It was shown that the use of wild-type rPoAbf in combination with the mix 1 from Genencor (Fig. 1) improved the xylose yield and speeded up xylan degradation. The use of the evolved mutant of rPoAbf allowed further increasing the xylose recovery and achieving the highest monosaccharide yield. This can be due to the increase of the xylan accessibility and hydrolysis caused by removal of the arabinosyl side chains from hemicellulose catalyzed by the arabinofuranosidases. The glucose yield, conversely, was not affected by the addition of the arabinofuranosidases (Supplementary Table 1).

3.1.2. Effect of addition of rPoAbf and its variant to the commercial enzymatic mix1 containing CelStrep instead of the commercial cellulase

When the recombinant enzyme CelStrep was used in the mix 1 instead of Accellerase 1500 (Fig. 1), the ability of rPoAbf and its evolved variant to improve the xylan degradation became even more evident. As reported by Gao et al. (2011), for evaluating the role of individual biocatalysts in biomass hydrolysis, the employment of a characterized enzyme, such as the recombinant CelStrep, is more advantageous than the utilization of enzymatic preparations lacking a defined composition. Using CelStrep, the xylose yield was highly reduced due to the absence of the hemicellulolytic activities reported for the Accellerase 1500 preparation. The addition of wild-type rPoAbf caused a marked increase in the xylose recovery that became closer to that obtained with Accellerase 1500. In particular, the xylose recovery after 48 h was 12.1% higher than that obtained with the mix 1. The evolved variant of rPoAbf was able to further improve the xylose yield and it is worth noting that the substitution of Accellerase 1500 with CelStrep plus the mutated rPoAbf enhanced the xylose yield up to 24% (after 48 h of conversion). The reported data clearly highlight that the limitation of the commercial preparation in xylan hydrolysis derives from the prevalence in their composition of backbone-acting xylanases at the expense of the auxiliary hemicellulolytic enzymes active on the side groups. Hence, the removal of side chains which impede endoxylanase activity are crucial to obtain high xylose yields (Banerjee et al., 2010). As concerning the glucose recovery, it was reduced in presence of CelStrep and was not affected by the addition of the arabinofuranosidases (Supplementary Table 1).

3.1.3. Effect of addition of rPoAbf or its variant to the commercial enzymatic mix 2

In order to identify enzyme combinations that could improve the hydrolysis yield, further trials were performed using a cocktail of commercial enzymes from Sigma (mix 2) including cellulolytic and xylanolytic activities (Table 1). This gave a 96.4% xylose recovery after 72 h. The addition of rPoAbf did not substantially influence the xylose recovery whilst the utilization of rPoAbf F435Y/Y446F speeded up the xylan degradation without modifying the maximum xylose yield.

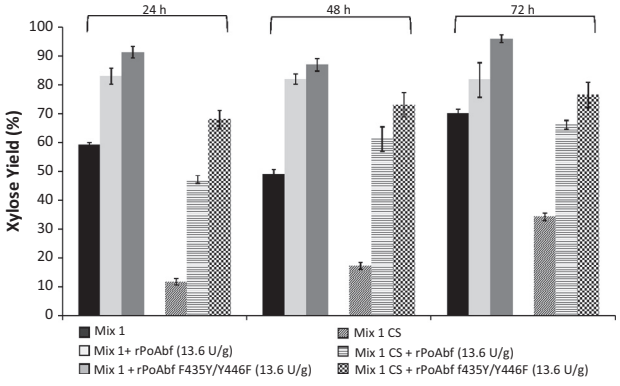

Fig. 1. Xylose yield of saccharification of *Arundo donax* pretreated by steam explosion obtained by adopting the mix 1 (5.4 U g^{-1} of Accellerase 1500, 4000 U g^{-1} of Accellerase XY and 145 U g^{-1} of Accellerase BG) or mix 1CS (mix 1 containing the recombinant enzyme CelStrep instead of Accellerase 1500) with or without addition of rPoAbf or rPoAbfF 435Y/Y446F mutant.

3.1.4. Effect of addition of rPoAbf to the commercial enzymatic mix 2 containing CelStrep instead of the commercial cellulase

The use of CelStrep in the enzyme mix 2 instead of the commercial cellulase reduced the glucose yield and the most evident effect was represented by halving of the maximum value (64%) at 72 h (Supplementary Table 2). Also the recovery of xylose decreased (Table 1), that can be explained by the lack, for CelStrep, of side xylanolytic activities present in the commercial cellulase. Differently from the results obtained with CelStrep in combination with Genencor enzymes (mix 1 CS), this situation was not modified by the addition of wild-type rPoAbf. These data indicate that, in presence of CelStrep, rPoAbf works better in combination with the Genencor commercial cocktail than with the Sigma preparation. Probably, the diverse specificities of the biocatalysts involved in the enzymatic cocktails towards the same substrate was at the basis of the different conversion yields reported.

3.1.5. Effect of addition of rPoAbf variant to the commercial enzymatic mix 2 containing CelStrep instead of the commercial cellulase

The use of the evolved mutant rPoAbf increased the xylose recovery and allowed to achieve, after 24 h, a yield of xylose by 14.1% higher than that obtained with the commercial enzymes mix 2 (Table 1). When the units of the evolved mutant of rPoAbf were doubled, the recovery of xylose achieved 90% after 72 h. This value was closer to that of 98.2% obtained with the mix 2, in presence of the evolved mutant. Doubling CelStrep gave an improvement in cellulose hydrolysis (Supplementary Table 2). Moreover, when double amount of CelStrep was used in combination with the evolved rPoAbf mutant, an acceleration of xylose recovery was also observed. In particular, a 21.6% increase in xylose yield was observed after 48 h compared to the results obtained when only a double amount of rPoAbf mutant was utilized. A possible synergism between the actions of CelStrep and the evolved rPoAbf mutant can be at the basis of the acceleration of the xylose recovery detected when the amounts of the cellulase and the rPoAbf mutant were increased at the same time. These findings highlighted that, besides the frequently cited effect of improvement in glucan conversion following the xylan removal (Polizeli et al., 2005), also the progress of the cellulose hydrolysis can facilitate the breakdown of the xylan fraction which is intimately adsorbed onto the cellulose microfibrils (Dammstrom et al., 2009).

3.2. Effect of PoAbf and its variant in bioconversion of A. donax and other biomasses pretreated by AAS

Different lignocellulosic biomasses, namely BSG and corn cobs, were also tested in the saccharification experiments in addition to *A. donax*. The former two materials are agro-industrial by-products characterized by a high xylan content of, that accounts for the 35% and 24.1% of their dry weights, respectively. In these trials, wild type rPoAbf or its evolved variant were used in combination with two kinds of enzymatic cocktails: the mix 2 and this cocktail containing CelStrep in substitution of the commercial cellulase. In all the experiments, the maximum amounts for both CelStrep and rPoAbf previously adopted were tested. In order to improve the xylan digestibility, the three biomasses BSG, corn cobs and *A. donax* were then subjected to pretreatment with aqueous ammonia soaking (AAS). As this kind of pretreatment modifies the structure and composition of lignocelluloses leaving behind a substantial amount of hemicellulose, it is conceivable that the effectiveness of side-chain acting hemicellulases is better highlighted on alkaline-treated materials. In Table 2, the macromolecular composition of all the materials before and after the pretreatments, in terms of percentage of glucans, xylans and Klason lignin, is reported. As it is possible to see, the pretreatment with AAS mainly caused the removal of lignin with a consequent enrichment of glucans and xylans. For *A. donax*, pretreated also by steam explosion, it was possible to compare the effects of the two pretreatment methods highlighting that steam explosion mainly provoked the removal of a part of the xylan.

3.2.1. Effect of PoAbf and its variant in bioconversion of A. donax

Analyzing the results of conversion of *A. donax* pretreated with AAS using the enzyme cocktail 2 in combination with wild-type rPoAbf or its evolved variant, it is possible to see that the yield of glucose remained unchanged (Supplementary Table 3) whilst a higher yield in xylose was obtained with the evolved variant of rPoAbf (Table 3).

The addition of the evolved variant of rPoAbf to the cocktail containing CelStrep allowed increasing the yield in xylose up to 14% respect to that obtained with the wild type. However, this effect was evident only after 48 h whilst the yield after 72 h remained unchanged (Table 3).

Comparing the results of conversion of *A. donax* pretreated with AAS and by steam explosion it is noticed that the saccharification of the biomass pretreated with AAS led to a lower yield of xylose expressed as percentage on the amount of sugars present before the hydrolysis (i.e., 90.5% instead of 62.9%, after 72 h of conversion with the mixture containing 27.2 U/g of mutated rPoAbf and Mix 2 CS2). This can be explained considering that the pretreatment with AAS causes the removal of a larger fraction of lignin, leading to an enrichment of glucans and xylans in the pretreated biomass, that leads to a lower fraction of xylose expressed in percentage on the total amount of these polysaccharides. On the other hand, if the absolute amounts of xylose are compared, the values after AAS pretreatment were higher than those achieved by steam explosion (i.e., 8.12 instead of 3.46 g $L^{-1}$of xylose, recovered after 72 h of conversion with the mixture containing CelStrep and

Table 1
Arundo donax pretreated by steam explosion (Di Pasqua et al., 2014).

	Composition	Xylose yield (%)		
		24 h	48 h	72 h
	Mix 2	50.1 ± 1.9	64.7 ± 2.6	96.4 ± 0.6
rPoAbf (13.6 U/g)	Mix 2	44.5 ± 5.4	59.9 ± 4.5	93.2 ± 3.7
rPoAbf F 435Y/Y446F (13.6 U/g)	Mix 2	48.5 ± 6.1	77.7 ± 3.0	98.2 ± 1.3
	Mix 2 CS1	49.0 ± 4.0	51.0 ± 4.3	65.6 ± 3.2
rPoAbf (13.6 U/g)	Mix 2 CS1	48.6 ± 5.6	52.6 ± 4.2	60.8 ± 4.4
rPoAbf F 435Y/Y446F (13.6 U/g)	Mix 2 CS1	64.2 ± 5.4	65.3 ± 3.2	68.4 ± 5.2
rPoAbf F 435Y/Y446F (27.2 U/g)	Mix 2 CS1	44.6 ± 2.8	45.5 ± 2.6	90.0 ± 4.0
rPoAbf F 435Y/Y446F (13.6 U/g)	Mix 2 CS2	53.0 ± 3.3	57.9 ± 5.8	63.8 ± 5.1
rPoAbf F 435Y/Y446F (27.2 U/g)	Mix 2 CS2	50.6 ± 3.7	67.1 ± 5.5	90.5 ± 4.6

Biotransformations in 50 mM buffer acetate pH 5.0 $T = 50$ °C, for 24, 48 and 72 h.
Mix 2: C2730 cellulase from *Trichoderma reesei* ATCC 26921 (5.4 U g^{-1} of pretreated biomass); C6105 Cellobiase from *Aspergillus niger* (145 U g^{-1}); X3876 xylanase from *Trichoderma viride* (80 U g^{-1}); X3504 β-xylosidase, thermostable (8 U g^{-1}).
Mix 2 CS1 or Mix 2 CS2: Mix 2 containing CelStrep, cellulase from *Streptomyces* sp., (5.4 or 10.8 U g^{-1}) instead of C2730, cellulase from *Trichoderma reesei* ATCC 26921.

Table 2
Macromolecular composition of untreated and pretreated biomasses.

Chemical composition (% total dry weight)							
Polysaccharides	Corn cobs (untreated)	Corn cobs (after AAS)	BSG (untreated)	BSG (after AAS)	*Arundo donax* (untreated)	*Arundo donax* (after AAS)	*Arundo donax* (after steam explosion)
Glucan	38.0 ± 0.4	51.2 ± 1.2	26.3 ± 1.6	49.9 ± 0.3	37.8 ± 2.0	63.2 ± 1.5	38.2 ± 1.2
Xylan	35.0 ± 0.7	43.3 ± 0.8	24.1 ± 1.2	40.7 ± 0.6	19.3 ± 0.9	26.0 ± 0.7	5.7 ± 0.9
Klason lignin	19.1 ± 0.5	5.4 ± 0.7	9.8 ± 0.4	5.2 ± 0.3	24.9 ± 1.3	10.7 ± 1.1	36.1 ± 0.6

Table 3
A. donax 5% w/v, pretreated with NH_4OH. Biotransformation in 50 mM buffer acetate pH 5.0 T = 50 °C, for 24, 48 and 72 h incubation.

		Composition	Xylose yield (%)		
			24 h	48 h	72 h
Arundo donax	rPoAbf (27.2 U/g)	Mix 2	41.0 ± 2.2	49.9 ± 1.8	72.1 ± 2.0
	rPoAbf F 435Y/Y446F (27.2 U/g)	Mix 2	42.8 ± 3.5	57.7 ± 2.2	82.5 ± 2.4
	rPoAbf (27.2 U/g)	Mix 2 CS2	29.8 ± 2.9	33.6 ± 3.7	64.9 ± 4.1
	rPoAbf F 435Y/Y446F (27.2 U/g)	Mix 2 CS2	33.7 ± 3.5	47.5 ± 4.2	62.9 ± 4.3

Mix 2: C2730 cellulase from *Trichoderma reesei* ATCC 26921 (5.4 U g^{-1} of pretreated biomass); C6105 Cellobiase from *Aspergillus niger* (145 U g^{-1}); X3876 xylanase from *Trichoderma viride* (80 U g^{-1}); X3504 β-xylosidase, thermostable (8 U g^{-1}).
Mix 2 CS2: Mix 2 containing cellulase CelStrep (10.8 U g^{-1}) from *Streptomyces* sp. instead of C2730 cellulase from *Trichoderma reesei* ATCC 26921.

Table 4
BSG 5% w/v, pretreated with NH_4OH. Biotransformation in 50 mM buffer acetate pH 5.0 T = 50 °C, for 24, 48 and 72 h incubation.

		Composition	Xylose yield (%)		
			24 h	48 h	72 h
BSG	rPoAbf (27.2 U/g)	Mix 2	57.6 ± 3.8	63.5 ± 3.0	73.1 ± 4.2
	rPoAbf F 435Y/Y446F (27.2 U/g)	Mix 2	64.4 ± 1.5	72.7 ± 2.9	85.8 ± 3.6
	rPoAbf (27.2 U/g)	Mix 2 CS2	53.4 ± 2.3	58.4 ± 2.8	63.3 ± 4.2
	rPoAbf F 435Y/Y446F (27.2 U/g)	Mix 2 CS2	58.5 ± 2.9	64.1 ± 2.4	66.4 ± 4.1

Mix 2: C2730 cellulase from *Trichoderma reesei* ATCC 26921 (5.4 U g^{-1} of pretreated biomass); C6105 Cellobiase from *Aspergillus niger* (145 U g^{-1}); X3876 xylanase from *Trichoderma viride* (80 U g^{-1}); X3504 β-xylosidase, thermostable (8 U g^{-1}).
Mix 2 CS2: Mix 2 containing cellulase CelStrep (10.8 U g^{-1}) from *Streptomyces* sp. instead of C2730, cellulase from *Trichoderma reesei* ATCC 26921.

Table 5
Corn cobs 5% w/v, pretreated with NH_4OH. Biotransformation in 50 mM buffer acetate pH 5.0 T = 50 °C, for 24, 48 and 72 h incubation.

		Composition	Xylose yield (%)		
			24 h	48 h	72 h
Corn cobs	rPoAbf (27.2 U/g)	Mix 2	53.7 ± 1.0	64.5 ± 3.1	69.4 ± 5.0
	rPoAbf F 435Y/Y446F (27.2 U/g)	Mix 2	58.8 ± 2.0	64.0 ± 3.4	85.5 ± 4.2
	rPoAbf (27.2 U/g)	Mix 2 CS2	42.6 ± 3.0	47.9 ± 5.1	56.1 ± 4.5
	rPoAbf F 435Y/Y446F (27.2 U/g)	Mix 2 CS2	50.6 ± 2.8	57.5 ± 4.3	64.2 ± 6.8

Mix 2: C2730 cellulase from *Trichoderma reesei* ATCC 26921 (5.4 U g^{-1} of pretreated biomass); C6105 Cellobiase from *Aspergillus niger* (145 U g^{-1}); X3876 xylanase from *Trichoderma viride* (80 U g^{-1}); X3504 β-xylosidase, thermostable (8 U g^{-1}).
Mix 2 CS2: Mix 2 containing cellulase CelStrep (10.8 U g^{-1}) from *Streptomyces* sp. instead of C2730 cellulase from *Trichoderma reesei* ATCC 26921.

mutated rPoAbf) (data not shown). This can be explained considering that the pretreatment with AAS reduce the content of lignin and the absorption of hydrolytic enzymes on it, thus facilitating the biodegradation of polysaccharides (Maurelli et al., 2013).

3.2.2. Effect of PoAbf and its variant in bioconversion of BSG and corn cobs

Analyzing the results of saccharification of BSG and corn cobs, it is possible to see that the addition of the evolved variant of PoAbf in comparison with the wild-type led to an improvement of the saccharification yield in terms of xylose and the best results were obtained when the enzyme was used in addition to the mix 2 (Tables 4 and 5). This outcome was more evident after 72 h of incubation when increases of 14.5% and 16.1% were observed for BSG and corn cobs, respectively. As concerning the glucose recovery, it was reduced in presence of CelStrep and unaltered after the addition of the arabinofuranosidases (Supplementary Tables 4 and 5). Moreover, even if the yields of xylose after 72 h were similar for the two tested materials with the mix 2 in combination with the evolved variant of rPoAbf (82–85%), the hydrolysis of BSG appeared to be the fastest. In fact, after 24 e 48 h of BSG saccharification, the highest xylose yields, corresponding to 64.4% and 72.7% respectively, were obtained. The explanation could be that the lower lignin content of this biomass can be at the basis of the higher biodegradability of its xylan fraction. This can also justify the similar absolute amounts of xylose obtained for BSG and corn cobs, despite the higher xylan content of corn cobs (at maximum 17.32 and 18.46 g L^{-1} of xylose obtained from BSG and corn cobs, respectively, after 72 h incubation with commercial enzymes and mutated rPoAbf) (data not shown). A similar situation was verified also analyzing the amounts (g L^{-1}) of arabinose obtained from the hydrolysis of both BSG and corn cobs (data not shown). The

highest arabinose recovery was obtained for BSG after 72 h with the evolved rPoAbf mutant that gave a two folds higher yield respect to those obtained with the wild type. The arabinofuranosidase gave better results in combination with the commercial cocktail (1.73 g L^{-1}) than in presence of CelStrep (1.65 g L^{-1}). Interestingly, it must be underlined that the evolved rPoAbf variant led to the best yields for arabinose and the xylose at the same time. These findings highlight the synergistic action of rPoAbf with the xylanolytic components of the enzymatic cocktails. The arabinose recovery from corn cobs was undoubtedly lower with all the enzymatic cocktails tested (around 1–1.2 g L^{-1}) and the worst results were obtained with wild type rPoAbf in combination with the mixture containing CelStrep (data not shown). The higher arabinose content for BSG (20–22%) in comparison with that of corn cobs (5.9%) and its lower lignin content (Musatto et al., 2006) can account for these data.

4. Conclusions

This study highlights the relevance of the white rot fungus *P. ostreatus* as source of side-chain acting hemicellulases and arabinofuranosidases, which play a crucial role in complete xylan hydrolysis via their cooperative action with the hydrolytic activities targeted to polysaccharides backbone.

The results obtained showed that the addition of wild-type or directed evolved variant of the arabinofuranosidase from *P. ostreatus* PoAbf improved the hydrolytic efficiency of the commercial enzymatic cocktails on *A. donax* pretreated by steam explosion and aqueous ammonia soaking and on BSG and corn cobs pretreated by aqueous ammonia soaking. It is conceivable that, due to the low content of auxiliary hemicellulolytic enzymes in the commercial preparation, the supplementation with enzymes, that removes side chain on xylan backbone, such as the arabinofuranosidases, are required to develop enzyme mixtures better adapted for lignocellulose hydrolysis.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2014.07.003.

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Research Article

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Application of a new xylanase activity from *Bacillus amyloliquefaciens* XR44A in brewer's spent grain saccharification

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Abstract

BACKGROUND: Cellulases and xylanases are the key enzymes involved in the conversion of lignocelluloses into fermentable sugars. Western Ghat region (India) has been recognized as an active hot spot for the isolation of new microorganisms. The aim of this work was to isolate new microorganisms producing cellulases and xylanases to be applied in brewer's spent grain saccharification.

RESULTS: 93 microorganisms were isolated from Western Ghat and screened for the production of cellulase and xylanase activities. Fourteen cellulolytic and seven xylanolytic microorganisms were further screened in liquid culture. Particular attention was focused on the new isolate *Bacillus amyloliquefaciens* XR44A, producing xylanase activity up to 10.5 U mL^{-1}. A novel endo-1,4-beta xylanase was identified combining zymography and proteomics and recognized as the main enzyme responsible for *B. amyloliquefaciens* XR44A xylanase activity. The new xylanase activity was partially characterized and its application in saccharification of brewer's spent grain, pretreated by aqueous ammonia soaking, was investigated.

CONCLUSION: The culture supernatant of *B. amyloliquefaciens* XR44A with xylanase activity allowed a recovery of around 43% xylose during brewer's spent grain saccharification, similar to the value obtained with a commercial xylanase from *Trichoderma viride*, and a maximum arabinose yield of 92%, around 2-fold higher than that achieved with the commercial xylanase.

Keywords: *Bacillus*; xylanase; brewer's spent grain; saccharification

INTRODUCTION

The conversion of lignocelluloses into fermentable sugars for fuel production is preferentially performed by enzymatic hydrolysis of polysaccharides, resulting from biomass pretreatment, with cellulases and hemicellulases.[1] Filamentous fungi are the major source of cellulases and hemicellulases.[2] However, bacterial cellulases and hemicellulases are more often investigated due to the advantages presented by bacteria as a source of enzymes in comparison to fungi, such as higher growth rate, the possibility to be easily engineered, and the facility to be isolated from niches naturally driving them to produce enzymes resistant to environmental stresses.[3]

Cellulolytic and hemicellulolytic bacteria have been isolated from a wide diversity of composts, soils and habitats,[4–16] and they represent one of the main enzyme sources currently exploited for the development of lignocellulose biorefinery.[3,17]

Huge amounts of lignocellulosic wastes are produced annually all around the world. They include agricultural residues, food farming wastes, green-grocer's wastes, tree pruning residues, and organic and paper fractions of urban solid wastes. A wide range of high added value products, such as biofuels, organic acids, biopolymers, bioelectricity and molecules for food and pharmaceutical industries,[18] can be obtained by upgrading solid wastes using biotechnological processes.

Among the lignocellulosic wastes, brewer's spent grain (BSG), the most abundant by-product generated during the beer-brewing process, has generated scientific interest for its application in energy production, due to its composition rich in cellulose and xylan.[19]

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BSG has been exploited for ethanol production in simultaneous saccharification and fermentation using commercial enzymes belonging to the classes glucoamylase, cellulase and hemicellulase,[20] and in consolidate bioprocessing, exploiting the ability of the fungus *Fusarium oxysporium* to concomitantly produce hydrolytic enzymes and ferment sugars into ethanol.[21]

In this study, the Western Ghat region of Kerala was explored for the isolation of new microorganisms with cellulolytic and xylanolytic abilities. An endo-1,4-beta xylanase was identified from the new isolate *Bacillus amyloliquefaciens* XR44A and recognized as the main enzyme responsible for xylanase activity. The xylanase activity produced by *B. amyloliquefaciens* XR44A was investigated for its ability to hydrolyse brewer's spent grain pre-treated by aqueous ammonia soaking. The new xylanase activity was shown able to yield an amount of xylose comparable with the level obtained using a commercial enzyme applied to the pre-treated BSG, in the same reaction conditions. Interestingly, application of the new xylanase activity gave a maximum arabinose yield around 2-fold higher than that achieved with the commercial enzyme.

MATERIALS AND METHODS

Isolation of samples

Samples used in this study were obtained from three areas of Western Ghat region (Arikan para, Puchi, Neelikal), Kerala, India. Samples, mainly collected from humic soil, the inner part of rock, rock surface and grass land, were aseptically stored in sterilized plastic bags at 4 °C.

Isolation of bacteria

Serial dilutions (10^{-1}–10^{-5}) of the samples were used to inoculate Petri dishes containing LB medium (10 g L^{-1} bactotryptone, 10 g L^{-1} NaCl, and 5 g L^{-1} yeast extract). Plates were incubated at 37 °C for a period varying from 2 to 4 days, depending on the speed of growth of the isolates. The isolates were purified, grown in liquid media and preserved in a 20% glycerol solution at −80 °C.

Screening of bacterial isolates for cellulolytic/xylanolytic activity production on solid and liquid media

In order to determine the production of cellulases and xylanases by the isolates, pure cultures of microorganisms were individually transferred onto solid medium ($NaNO_3$ 1 g L^{-1}, K_2HPO_4 1 g L^{-1}, KCl 1 g L^{-1}, $MgSO_4$ 0.5 g L^{-1}, yeast extract 0.5 g L^{-1}, glucose 1 g L^{-1}, agar 17 g L^{-1}) containing 5 g L^{-1} of carboxymethylcellulose (CMC, Sigma) or Xylan (Sigma), respectively. After incubation for 2–4 days, depending on the speed of growth of the isolates, the cellulolytic/xylanolytic strains were assayed for their ability to degrade CMC/xylan by incubation with 0.1% Congo Red solution for 30 min, followed by washing with 5 mol L^{-1} NaCl as reported in Amore *et al.*[6] All the strains with a clear halo around the colonies were chosen as positive.

The liquid medium adopted for analysis of cellulase and xylanase production levels contained 1% CMC or xylan, respectively, 0.7% yeast extract, 4 g L^{-1} KH_2PO_4, 4 g L^{-1} Na_2HPO_4, 0.2 g L^{-1} $MgSO_4.7H_2O$, 0.001 g L^{-1} $CaCl_2.2H_2O$, 0.004 g L^{-1} $FeSO_4.7H_2O$.[13]

Phenotypic characterization of microbial isolates

Morphological analysis of the colony of each bacterial strain was carried out observing shape (regular/irregular/rhizoid/punctiform/filamentous), edge (entire/undulate), surface (dry/viscid/powdery), elevation (flat/raised) and color of colony.

Inoculum preparation and incubation

The bacterial strains were pre-inoculated dissolving a single colony in 3 mL of liquid medium having the composition described in the paragraph 'Screening of bacterial isolates for cellulolytic/xylanolytic activity production on solid and liquid media', and incubated over night at 37 °C. Fermentation was carried out in 100 mL plugged Erlenmeyer flasks, each containing 20 mL of medium, and inoculated with volumes of pre-inoculum corresponding to 0.1 OD. Fermentations were incubated at 37 °C on a rotary shaker at 125 rpm. At the 6th or 24th hour, samples of liquid cultures were withdrawn and used to measure optical density (OD_{600nm}) and extracellular cellulase and xylanase activity. The results of the analytical determinations reported in the figures correspond to mean values of three independent experiments, each one performed in three replicates.

Azo-CMCase assay

endo-1,4-ß-glucanase activity produced in liquid or submerged culture was assayed using Azo-CMC (Megazyme, Ireland) as substrate, following supplier's instructions, and determined by referring to a standard curve.

Xylanase assay

Xylanase assay was performed according to Bailey *et al.*[22] The reaction mixture consisting of 1.8 mL of a 1.0% (w/v) suspension of birch-wood xylan in 50 mmol L^{-1} Na citrate at pH 5.3 and 0.2 mL of enzyme dilution (in 50 mmol L^{-1} sodium citrate at pH 5.3) was incubated at 50 °C for 5 min. Released reducing sugars were determined by adding 3 mL of 3,5-dinitrosalicylic acid solution and then incubating the mixture at 95 °C for 5 min. Absorbance was measured at 540 nm. One unit of enzyme is defined as the amount of enzyme catalyzing the release of 1 µmol of xylose equivalent per min.

Arabinofuranosidase activity assay

α-L-arabinofuranosidase activity was measured spectrophotometrically with p-nitrophenyl α-L-arabinofuranoside (pNPA) (Gold Biotechnology, St Louis, MO, USA) as substrate as described in Amore *et al.*[23] One unit of α-L-arabinofuranosidase activity was defined as the amount of enzyme releasing 1 µmol of p-nitrophenol per minute in the reaction mixture under these assay conditions.

16S rRNA gene partial sequence

Genomic DNA extraction, 16S rRNA gene amplification and sequencing of PCR amplification fragments were performed according to Rameshkumar and Nair.[24] The purified PCR product was used directly for DNA sequencing, using the dideoxy chain termination method with Big Dye Terminator kit (Applied Biosystems). The reaction products were analysed using capillary electrophoresis on an ABI 310 Genetic Analyser (Applied Biosystems).

The sequences were compared with the GenBank nucleotide data library using the Blast software at the National Centre of Biotechnology Information website (http://www.ncbi.nlm.nih.gov), in order to determine their closest phylogenetic relatives.

The partial 16S rDNA sequences of the isolates selected for cellulase and xylanase activity have been submitted to EMBL, and the accession numbers are reported in Table 1.

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Table 1. Similarity analyses and accession number to EMBL nucleotide sequence database of the new isolates by BLAST analyses

Microorganism code	Percentage identity with other sequences	Accession number to EMBL nucleotide sequence database
Cellulolytic microorganisms		
R7A	*Streptomyces sp.* (100%)	HG975526
R10B	*Bacillus* sp. (100%)	HG975527
R13A	*Bacillus* sp. (100%)	HG975528
R13B	*Bacillus* sp. (100%)	HG975529
R14B	*Bacillus* sp. (100%)	HG975530
R21A	*Bacillus* sp. (100%)	HG975531
R43B	*Bacillus* sp. (100%)	HG975532
R77A	*Bacillus* sp. (100%)	HG975533
R79A	*Streptomyces griseoruber* (100%)	HG975534
R79C	*Streptomyces costaricanus* (100%)	HG975535
R79A	*Bacillus* sp. (100%)	HG975536
R103A	*Bacillus* sp. (100%)	HG975537
R5A	*Bacillus* sp. (100%)	HG975538
R55B	*Paenibacillus* sp. (100%)	HG975539
Xylanolytic microorganisms		
XR13A	*Bacillus* sp. (100%)	HG975540
XR18B	*Bacillus* sp. (100%)	HG975541
XR24A	*Bacillus* sp. (100%)	HG975542
XR44A	*Bacillus* sp. (100%)	HG975543
XR84A	*Lysinibacillus xylanilyticus* (100%)	HG975544
XR84A	*Bacillus* sp. (100%)	HG975545
XR80B	*Streptomyces olivochromogenes* (100%)	HG975546

Strain identification and deposition in culture collection

The major strain XR44A used in this study was identified at the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, GERMANY).

Determination of protein concentration

Protein concentration of crude enzyme preparation was determined by Bradford method using Biorad reactive (München, Germany) and following the procedure suggested by the supplier. Bovin serum albumin (BSA) was used to set up the standard curve.

Protein fractionation

Secreted proteins produced by the strains XR18B and XR44A were precipitated from the cultures corresponding to the maximum xylanase production by the addition of ammonium sulphate up to 80% saturation, after removing cells by centrifugation. Precipitated proteins were recovered by centrifugation at 7500 rpm for 45 min at 4 °C and brought in 20 mmol L^{-1} Tris-HCl pH 7, by extensive dialysis.

Zymogram analyses

Semi-denaturing gel electrophoresis was carried out loading non-denatured and not-reduced samples on a SDS polyacrylamide gel, performed as described by Laemmli.[25] Proteins showing xilanolytic activity were visualized following a modified version of the method reported by Béguin.[26] After electrophoresis, the gel was soaked in the same buffer used for dissolving proteins and gently shaken to remove SDS and renature the proteins in the gel. The gel was then laid on the top of a thin sheet of 1.5% agar containing 1% xylan. After 1 h incubation at 40 °C, zones of xylan hydrolysis were revealed by staining the agar replica with 0.1% Congo red.

Protein identification by mass spectrometry

SDS polyacrylamide gel (SDS-PAGE) and zymogram analyses were aligned to select bands of interest, which were then cut from the SDS-PAGE, destained by washes with 0.1 mol L^{-1} NH_4HCO_3 pH 7.5 and acetonitrile, reduced for 45 min in 100 µL of 10 mmol L^{-1} dithiothreitol, 0.1 mol L^{-1} NH_4HCO_3, pH 7.5, and carboxyamidomethylated for 30 min in the dark by the addition of 100 µL of 55 mmol L^{-1} iodoacetamide dissolved in the same buffer. Enzymatic digestion was performed by adding to each slice 100 ng of proteomic-grade trypsin (SIGMA) in 10 µL of 10 mmol L^{-1} NH_4HCO_3 pH 7.5 for 2 h at 4 °C. The buffer solution was then removed and 50 µL of 10 mmol L^{-1} NH_4HCO_3 pH 7.5 were added and incubated for 18 h at 37 °C. Peptides were extracted with 20 µL of 10 mmol L^{-1} NH_4HCO_3, 1% formic acid, 50% acetonitrile at room temperature.

Peptide mixtures were filtered on 0.22 µm cellulose acetate spin filter membranes (Agilent Technologies) and analysed by LC-MSMS on a 6520 Accurate-Mass Q-TOF LC/MS System (Agilent Technologies, Palo Alto, CA) equipped with a 1200 HPLC system and a chip cube (Agilent Technologies). After loading, the peptide mixture was first concentrated and washed in 40 nL enrichment column (Agilent Technologies chip), with 0.1% formic acid in 2% acetonitrile as the eluent. The sample was then fractionated on a C18 reverse-phase capillary column (Agilent Technologies chip) at a flow rate of 400 nL min^{-1}, with a linear gradient of eluent B (0.1% formic acid in 95% acetonitrile) in A (0.1% formic acid in 2% acetonitrile) from 7 to 80% in 50 min. Peptides analysis was performed using data-dependent acquisition of one MS scan (mass range from 300 to 1800 m/z) followed by MS/MS scans of the five most abundant ions in each MS scan. MS/MS spectra were measured automatically when the MS signal surpassed the threshold of 50 000 counts. Double and triple charged ions were preferably isolated and fragmented. The acquired MS/MS spectra were transformed in *Mascot generic* format (.mgf) and used for protein identification in the unreviewed set of protein entries that are present in the NCBInr database for all bacteria, with a licensed version of MASCOT software (www.matrixscience.com) version 2.4.0.

Additional MASCOT search parameters were: peptide mass tolerance 10 ppm, fragment mass tolerance 0.6 Da, allowed missed cleavages up to 3, carbamidomethylation of cysteines as fixed modification, oxidation of methionine, pyro-Glu N-term Q, as variable modifications. Only doubly and triply charge ions were considered. Ions score was −10 log(P), where P is the probability that the observed match is a random event. Individual ion scores >45 indicated identity or extensive homology ($P < 0.05$). Protein scores were derived from ion scores as a nonprobabilistic basis for ranking protein hits (http://www.matrixscience.com/help/interpretation_help.html).

Acrylamide/bis-acrylamide, ammonium persulfate, trypsin, dithiothreitol, iodoacetamide, spin filters and NH_4HCO_3 were purchased from SIGMA; Temed was purchased from AllChem. Trifluoroacetic acid (TFA)-HPLC grade was from Carlo Erba (Milan, Italy). All other reagents and solvents were of the highest purity available from Baker.

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Optimum temperature and thermo-resistance

Supernatant of the strain XR44A containing xylanase activity after 24 h incubation was assayed in order to study the optimum temperature and thermo-resistance.

To determine the optimum temperature, the substrate of the activity assay (birch-wood xylan) was dissolved in 50 mmol L^{-1} Na citrate at pH 5.3 and the incubation (5 min) was performed at 40 °C, 50 °C, 60 °C and 70 °C. The thermo-resistance of the xylanase activity was studied by incubating the supernatant of the strain XR44A containing xylanase activity in 50 mmol L^{-1}, Na citrate at pH 5.3, at 30 °C, 40 °C, 50 °C, 60 °C and 70 °C. The samples withdrawn were assayed for residual xylanase activity as described above.

The reported results are mean values of the three independent experiments, each one performed in three replicates.

Optimum pH and pH resistance

To determine the optimum pH of XR44A xylanase activity, xylanase activity assay was performed using the substrate birch-wood xylan dissolved in 50 mmol L^{-1} citrate phosphate buffers,[27] with pH values between 3.0 and 9.0 and performing the incubation (5 min) at 50 °C.

The pH resistance of the supernatant of the strain XR44A containing xylanase activity was studied by diluting it in citrate phosphate buffers, pH 3–9 and incubating at 25 °C. From time to time, samples were withdrawn and immediately assayed for residual xylanase activity as described above.

The reported results correspond to mean values of the three independent experiments, each one performed in three replicates.

Pretreatments and determination of chemical composition

The brewer's spent grain (BSG) provided by the micro-brewery Maneba (Striano, Naples, Italy) was subjected to pretreatment with an aqueous ammonia solution at lab-scale, following conditions described by Maurelli *et al.* for chestnut shell.[28] The biomass reduced to a fine powder, was soaked in 5% (v/v) aqueous ammonia solution at a solid loading of 10%, then incubated at 70 °C for 22 h in screw-capped 25 mL bottles to reduce evaporation. The alkaline mixtures were centrifuged at 800 × g, and the residues were washed extensively with 50 mmol L^{-1} sodium acetate buffer until obtaining the requested pH value for the subsequent enzymatic saccharification process (pH 5.0).

Carbohydrate compositions of the biomass samples untreated and pretreated were determined according to the method of Davis.[29] This procedure involved an acid hydrolysis carried out in two steps to fractionate the polysaccharides into their corresponding monomers. First, the samples were soaked in 72% v/v H_2SO_4, at a solid loading of 10%, at 30 °C, for 1 h; then the mixtures were diluted to 4% (v/v) H_2SO_4 with distilled water, and the secondary hydrolysis was performed for 1 h at 120 °C, after adding fucose as an internal standard. After filtration through 0.45 µm Teflon syringe filters (National Scientific, Lawrenceville, GA), the hydrolysis products were analysed via HPLC as described below. The acid insoluble lignin (Klason lignin) was determined by weighing the dried residue after total removal of the sugars.

Enzymatic hydrolysis

The saccharification experiments were carried out at 30 °C for 72 h in a total volume of 5 mL (50 mmol L^{-1} sodium acetate buffer pH 5.0 plus enzyme cocktail), at a solid loading of 5%. The hydrolysis of pretreated lignocellulosic materials was carried out with enzyme cocktails prepared with the following commercial and non commercial products at the indicated amounts expressed as units per gram of pretreated biomass: 5.4 U g^{-1} of cellulase from *Trichoderma reesei* ATCC26921 (Sigma), 145 U g^{-1} of cellobiase from *Aspergillus niger* (Sigma), 1.5 U g^{-1} of xylanase from *Trichoderma viride* (Sigma) or 1.5 U g^{-1} of xylanase from the strain XR44A isolated in this study, 0.5 U g^{-1} of thermostable β-xylosidase (Sigma), and 145 U g^{-1} of cellobiase from *Aspergillus niger* (Sigma). The saccharification mixtures together with blanks (pretreated lignocellulosic materials without enzyme cocktail) were incubated in a shaking ThermoMixer C (Eppendorf) at 30 °C and 800 rpm, for 72 h. Samples were withdrawn at different times, chilled on ice and centrifuged at 16 500 × g for 30 min at 4 °C. The supernatants were analysed to quantify the amount of sugars released as described below. The saccharification yield was expressed as percentage of sugar production calculated with respect to the amount of sugars present in the dry weight pretreated materials before the hydrolysis processes. Each saccharification experiment was run in triplicate.

Determination of sugar content

For estimation of the sugars released from pretreated biomasses, the cleared supernatants were opportunely diluted, and analysed by high-performance liquid chromatography (HPLC; Dionex, Sunnyvale, CA, USA) equipped with an anionic exchange column (Carbopac PA-100) and a pulsed electrochemical detector. Glucose and xylose were separated with 16 mmol L^{-1} sodium hydroxide at a flow rate of 0.25 mL min^{-1}, and identified by the respective standards. Fucose was used as internal standard.

RESULTS AND DISCUSSION

Isolation and screening of xylanolytic and cellulolytic microorganisms from Western Ghat region

The biodiversity of the Western Ghat regions of Kerala has been exploited for the isolation of new microorganisms able to produce cellulolytic and xylanolytic activities. Western Ghats region (India) has been so far described as an active biodiversity hot spot.[30]

Three areas of Western Ghat Region, namely Arikan Para, Puchi Para, Neelikal, were explored and 50 samples of different nature, such as humic soil, grass land, inner part of rock, rock surface, excrement, laterite, were used for isolation of bacteria and actinomycetes. 93 purified microorganisms were screened for xylanase and cellulase activity production by Congo Red assay on xylan- and CMC-containing plates, respectively. The screening led to the selection of seven xylanolytic microorganisms and 14 cellulolytic microorganisms showing activity halos on xylan-and CMC-containing plates, with a diameter greater than 5 mm. Based on morphological analyses, these microorganisms were divided into bacteria – mainly *Bacillus* spp. – and actinomycetes.

Sequencing of 16S rRNA genes of xylanolytic and cellulolytic isolates

The 21 microorganisms isolated and selected on solid medium were identified by 16S rRNA sequencing. Data derived from BLAST showed that the new isolated strains have high sequence similarities with different species (Table 1): 15 belong to the genus *Bacillus,* four to the genus *Streptomyces,* one to the genus *Lysinobacillus,* and one to the genus *Paenibacillus*.

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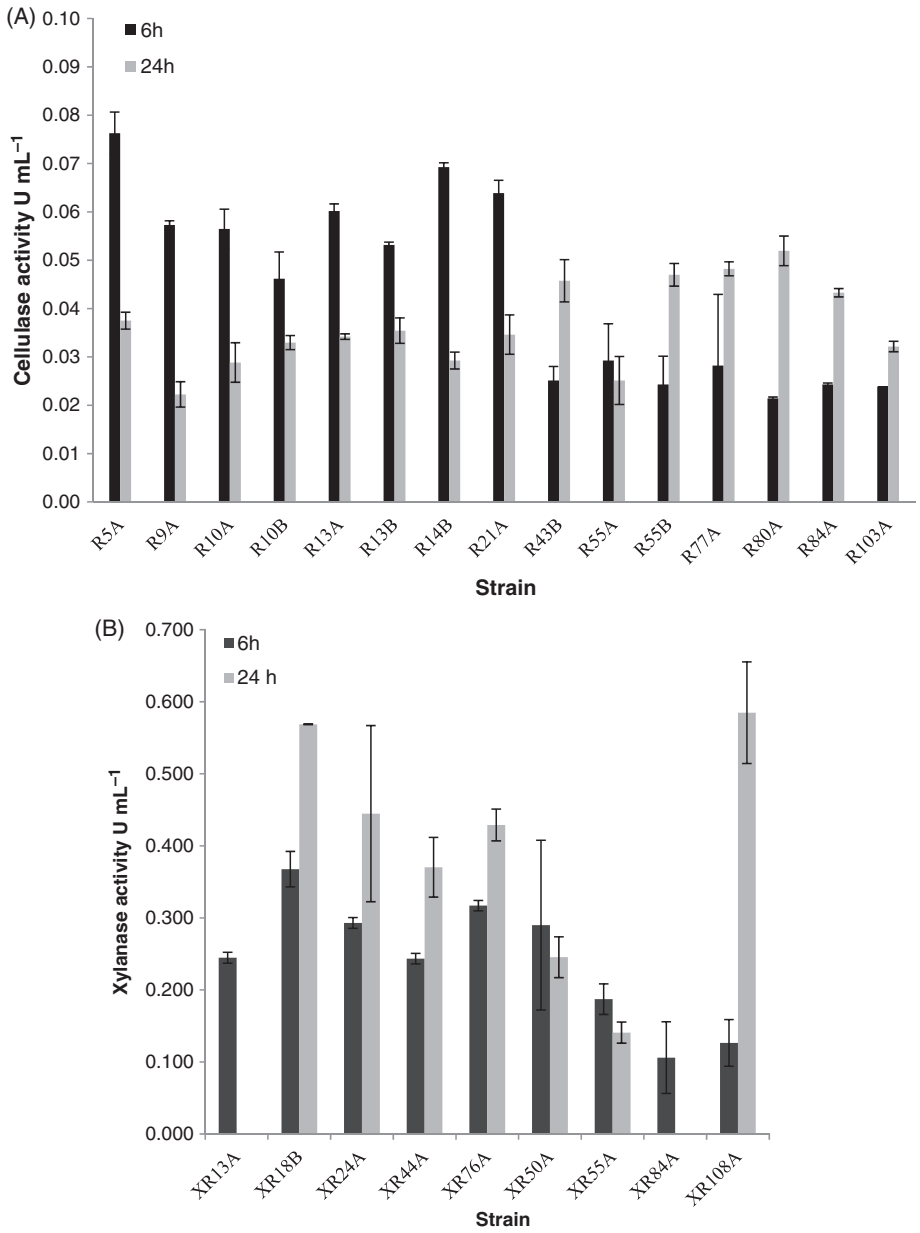

Figure 1. Screening of cellulolytic (A) and xylanolytic (B) strains in liquid medium.

Screening of the isolates in liquid medium

A further screening of the seven xylanolytic and fourteen cellulolytic strains was carried out in liquid culture, using a 1% xylan- and 1% CMC-containing medium, respectively. Xylanase (Fig. 1(A)) and CMCase activity (Fig. 1(B)) were assayed after 6 and 24 h of incubation. Eight microorganisms (R5A, R9A, R10A, R10B, R13A, R13B, R14B, R21A) were identified as the best producers of the cellulase activity with values of AZO-CMCase activity ranging from 0.045 up to 0.075 U mL^{-1}, after 6 h incubation. XR18B, XR44A, XR76A and XR108A produced the highest levels of xylanase activity, ranging from 0.4 to 0.6 UmL^{-1}, similarly to other xylanase-producing *Bacillus* strains.[10,13]

In order to increase the level of activity production, the volume culture of the selected strains was increased to 50 mL, and the temperature and shaking rotation adjusted to 40 °C and 250 rpm, respectively. Interestingly, these conditions increased xylanase activity production to 9.4 and 10.5 U mL^{-1}, for XR18B and XR44A, respectively.

Zymogram analyses of xylanase in different selected isolates

Proteins responsible for xylanase activity of the most productive xylanolytic strains XR18B and XR44A were analysed by fractionation on a semi-denaturing SDS-PAGE. Samples from the supernatant of the cell cultures after 6 h were loaded on SDS-PAGE without boiling them and treating with reducing agents. The resulting gel was laid over another thin gel containing 1% xylan as substrate for xylanase activity detection. SDS-PAGE and zymogram were aligned to select bands of interest. An activity protein band, detected by Congo red staining as described in the section Materials and methods, was visualized only for the strain XR44A (Fig. 2, lane A), and the corresponding band in the colloidal Comassie blue stained SDS-PAGE gel (Fig. 2, lane B) was excised and subjected

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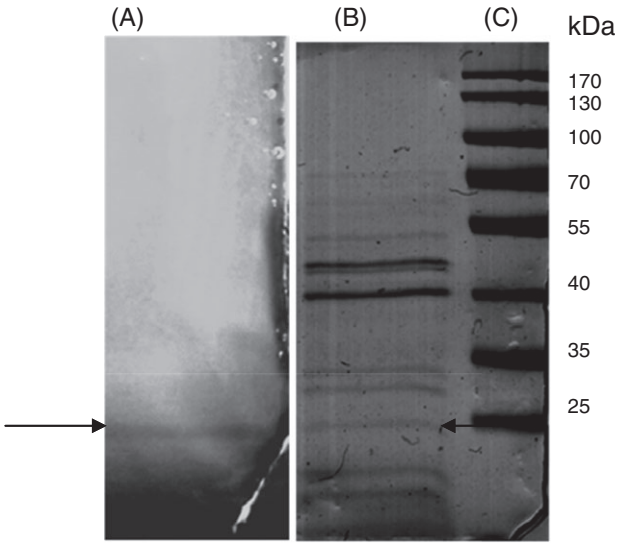

Figure 2. Detection of xylanase activity produced by XR44A after SDS-PAGE. Lane A: XR44A (0.25 U total xylanase activity); Lane B: XR44A (10 γ of total protein loaded); Lane C: Markers.

to protein identification by *in situ* digestion and LC-MS/MS analysis of the peptide mixtures. The acquired MS/MS spectra were transformed in *Mascot generic* (.mgf) format and used for protein identification with a licensed version of MASCOT software (www.matrixscience.com) version 2.4.0. against the whole unreviewed set of protein entries that are present in the NCBInr database for all bacteria. Protein identifications in the selected band are reported in Table 2.

It is worth noting that among the identified proteins, an endo-1,4-beta-xylanase (NCBInr entry: gi70998142) attributed to *Paenibacillus macerans* was identified (three peptides, 20% sequence coverage).

Strain identification

XR44A was identified as a *Bacillus amyloliquefaciens* by means of cellular fatty acids analysis, physiological study and partial sequencing of 16SrDNA.

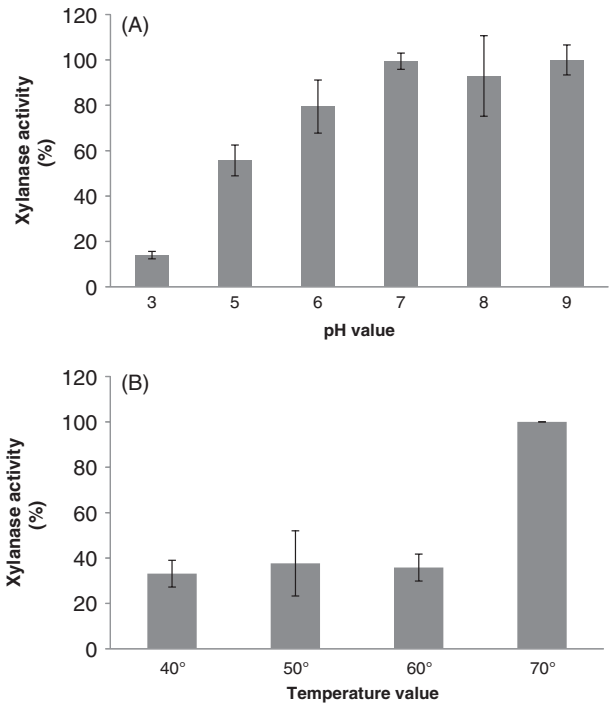

Figure 3. Optimum pH (A) and temperature (B) of xylanase activity produced by *B. amyloliquefaciens* XR44A.

Partial characterization of the xylanase activity to define the enzymatic properties useful for its application

The optimum pH of *B. amyloliquefaciens* XR44A xylanase activity (assayed in the range 3–9) is in the range 7–9 (Fig. 3(A)). Other xylanases from *Bacillus* strains so far characterized generally show wide differences in their optimal pH, going from acidic values, such as 4 for the glycosyl hydrolase family 11 xylanase from *B. amyloliquefaciens* CH51 strain,[32] 5 for the xylanase activity produced by *Bacillus subtilis* GN156,[33] 5.8 for the xylanase from *B. subtilis* str. CXJZ,[34] up to 9 as in the case of the endoxylanase activity from *B. halodurans* TSEV1.[31]

The optimal temperature of *B. amyloliquefaciens* XR44A xylanase activity (assayed in the range 40–70 °C) is 70 °C (Fig. 3(b)), near to that of the xylanases from *Bacillus halodurans* TSEV1 (80 °C)[31] and *B. subtilis* str. CXJZ isolated from the degumming line (60 °C),[34]

Table 2. Mascot search results of LC-MS/MS data against NCBInr database showing identified proteins' nominal mass, peptide ion score, peptide sequence and protein sequence coverage

			Mascot		
NCBInr ID	Protein	nominal mass (Mr)	Score	Peptide	Sequence coverage (%)
gi|70998142	endo-1,4-beta-xylanase [Paenibacillus macerans]	23264	24	GWTTGSPFR	20
			65	SDGGTYDIYTTTR	
			61	YNAPSIDGDNTTFTQYWSVR	
gi|14278871	flagellin [Bacillus subtilis]	35615	25	LGAVQNR	14
			27	QLNAGSNSAAK	
			39	TAIDTVSSER	
			41	LEHTINNLGTSSENLTSAESR	
gi|154686187	chitin binding protein [Bacillus amyloliquefaciens FZB42]	22437	31	ADTNLTHK	22
			31	YGSVIDNPQSVEGPK	
			28	DEFELIGTVNHDGSK	

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but distant from that of the xylanases produced by *Bacillus subtilis* GN156 (40 °C)[33] and *B. amyloliquefaciens* CH51 strain(25 °C).[32]

The novel *B. amyloliquefaciens* XR44A xylanase activity shows a half life of 5 min at 70 °C, 15 min at both 50 °C and 60 °C, and 2 h at 40 °C. Interestingly, it retains 90% of activity for at least 2 days at 30 °C, with a half-life of 7 days. The enzyme immediately loses activity at temperatures higher than 70 °C, differently from the xylanase produced by *Bacillus aerophilus* KGJ2, which retained more than 90% activity after incubation at 80–90 °C for 60 min.[35]

Bacillus amyloliquefaciens XR44A xylanase activity shows very high stability over a broad range of pHs, with a half-life of 7 days at pH3, pH 5, pH 6, pH 7, pH 8 and pH 9.

Table 3. Macromolecular composition of BSG before and after aqueous ammonia soaking (AAS) pretreatment

		Solid composition[b]			
	SR[a] (%)	Lignin (%)	Glucan (%)	Xylan (%)	Arabinan (%)
BSG untreated	-	12.8	27.5	28.8	4.32
BSG AAS	59.96	2.09	25.9	23.3	3.26

[a] Solid remaining after reaction.
[b] Solid percentage composition based on oven dry untreated biomass.

Saccharification of pre-treated brewer's spent grain using new xylanase

The culture supernatant of *B. amyloliquefaciens* XR44A with xylanase activity was utilized after ammonium sulfate precipitation and dialysis for the saccharification of brewer's spent grains (BSG), which is considered a valuable low-cost feedstock, with an increasing attractiveness for energy production. BSG represents the major by-product of the brewing industry accounting for about 85% of the total residues generated after mashing and lautering processes[19] and is available in large quantities throughout the year. This lignocellulosic residue has an elevated potential as feedstock for the production of ethanol, since it is characterized by a high (hemi)cellulose content. The chemical composition of BSG varies according to several factors such as the barley variety, the harvest time, the malting and mashing conditions, and also the quality and type of additives added in the brewing process.[36,37] It generally consists of about 16.8–25.4% cellulose, 21.8–28.4% hemicellulose (mostly arabinoxylans) and 11.9–27.8% lignin.[38,39] The composition of the raw material utilized in this work (Table 3) shows very high amounts of cellulose and xylan (in the form of arabinoxylan), and very low lignin content, in comparison to the composition of other types of brewer's spent grain reported in the literature. In Table 3 the composition of the material after pretreatment is also reported. As witnessed by a huge amount of scientific literature, pretreatment is a key step for effective enzymatic lignocellulose hydrolysis, having the important function of removing the lignin fraction that shields the cell wall polysaccharides from enzymatic attack.[40] In this work, pretreatment by aqueous ammonia soaking was set up for BSG adapting the conditions reported by Kim *et al.*[41] and Maurelli *et al.*[28] for barley hull and chestnut shell, respectively. This method was chosen due to its high efficacy in breaking the ester bonds between lignin, hemicellulose and cellulose without degrading the hemicellulose polymers.[42] As shown in Table 3, the delignification percentage was 78% after 22 h incubation, and the glucan and xylan contents were only marginally modified (1.63 and 5.57% of glucan and xylan dissolution, respectively, were reported). Furthermore, it was shown that by prolonging the pretreatment time no significant improvements were achieved (data not shown).

Table 4 reports the results regarding the saccharification of the pretreated BSG with *B. amyloliquefaciens* XR44A protein preparation endowed with xylanase activity (1.5 U g^{-1} pretreated biomass). The new enzymatic activity developed in this work, or alternatively, the same dosage of a commercial xylanase from *Trichoderma viride* (Sigma), were used in combination with different commercial enzymes (Sigma) – cellulase from *Trichoderma reesei* ATCC 26921 (5.4 U g^{-1}), cellobiase from *Aspergillus niger* (145 U g^{-1}) and β – xylosidase (0.5 U g^{-1}). It is worth noting that the utilization of the enzyme preparation from *B. amyloliquefaciens* XR44A led to results similar to those obtained with the commercial xylanase from *Trichoderma viride* (Sigma). In both cases, the maximum xylose recovery was around 43%, after 48 h of hydrolysis reaction, and it did not change over prolonged incubation times. Interestingly, the utilization of *B. amyloliquefaciens* XR44A xylanase preparation improved the glucan fraction degradation, which was enhanced from 51.5 to 79.8% after 72 h incubation. However, also in this case most of the cellulose conversion was completed in 48 h.

A potentially better synergism between the xylanase activity from *B. amyloliquefaciens* XR44A and the commercial cellulase could be at the basis of the easier cellulose deconstruction.

As regards the arabinose liberation, due to the lack of arabinofuranosidase activity in both the *B. amyloliquefaciens* XR44A

Table 4. Results of saccharification with sigma enzymes – cellulase from *Trichoderma reesei* ATCC 26921 (5.4 U g^{-1} of pretreated biomass); cellobiase from *Aspergillus niger* (145 U g^{-1} of pretreated biomass); xylanase from *Trichoderma viride* (1.5 U g^{-1} of pretreated biomass); β-xylosidase, thermostable (0.5 U g^{-1} of pretreated biomass) – and xylanase from *Bacillus amyloliquefaciens* XR44A (1.5 U g^{-1} of pretreated biomass)

	Glucose yield (%)			Xylose yield (%)			Arabinose yield (%)		
Composition	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Cellulase Cellobiase Xylanase β-Xylosidase	35.3	47.9	51.5	30.5	40.0	43.8	52.4	56.1	56.4
Cellulase Cellobiase Xylanase (*Bacillus amyloliquefaciens* XR44A) β-Xylosidase	35.1	74.3	79.8	24.2	43.2	43.7	48.1	70.0	92.3

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enzyme preparation and the commercial xylanase (as demonstrated by assaying it towards the substrate p-nitrophenyl α – L-arabinofuranoside), the release of this monosaccharide must be ascribed to the presence of arabinofuranosidase activities in the other commercial enzymes. As a matter of fact, values of 0.11, 1.22 and 0.69 Um L^{-1} of arabinofuranosidase activity were detected for arabinofuranosidase activity in the commercial cellulase, cellobiase and xylosidase, respectively.

Moreover, a better arabinose yield, that exceeded by up to 38.9% the result reached with the commercial xylanase (56.4% after 72 h), was obtained in the presence of *B. amyloliquefaciens* XR44A enzyme preparation. This revealed that the arabinofuranosidases worked better in combination with the new xylanase than in the presence of its commercial counterpart. As already reported by Marcolongo *et al.*,[43] the diverse values of arabinose recovery in the presence of the two different xylanase activities can be explained by their different specificities towards the same substrate, which account also for their different level of synergism reached with the xylan debranching enzymes.

These results, arising from the synergistic behavior of the various enzymes involved in the hydrolysis of (hemi)cellulosic substrates, are in agreement with other works described in the literature.[44,45]

CONCLUSION

In this work, 93 microorganisms were isolated from Western Ghat region (Kerale, Indi) and screened for cellulase and xylanase activity. 21 microorganisms were selected by screening on solid medium, and further analysed for the production of enzymes of interest in liquid medium. The xylanolytic strain *Bacillus amyloliquefaciens* XR44A was selected as the best producer of xylanase activity, with a value up to 10.5 U mL^{-1} under optimized growth conditions. The enzyme responsible for the xylanase activity produced by *Bacillus amyloliquefaciens* XR44A was identified as an endo-1,4-beta-xylanase using an approach based on a combination of zymography and mass spectrometral analyses. The novel enzyme in the crude supernatant was partially characterized in order to define the properties useful for its application, and it was tested in brewer's spent grain saccharification, this being recognized as an attractive lignocellulose feedstock for fermentable sugars production. Interestingly, the novel xylanolytic activity showed performances similar to a commercial xylanase from *Trichoderma viride*, with a xylose yield of 43%. Moreover, the presence of the new xylanase in the commercial enzymatic cocktail favoured an increase of arabinose recovery by up to 38% due to better synergism than the commercial counterpart, established in association with the arabinofuranosidases activities present in the commercial preparations.

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