Chapter 19
Technical Developments for Vegetable Waste Biomass Degradation by Thermophiles

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1 World Vegetable Waste Biomass Production: Overview of Main Lignocellulosic Residues Production

Biomass is the main renewable feedstock that can be used for the sustainable generation of energy and useful molecules. Biomass exploitation for the production of bioenergy and platform chemicals could afford great reduction of greenhouse gas emissions, thus it represents a valuable alternative to the present fossil fuels-based economy. In this line, exploitation of biomass could represent a sustainable environmental practice for a biobased economy in which no resource (arable land, crops) is diverted from food chain to biofuels production. In this frame green chemistry is presently the most promising approach to convert biomass into bioenergy (biofuels and biopower) and biochemicals, according to the integrated biorefinery approach. Indeed, by means of a combination of chemical, physical and biochemical technologies, different kinds of biomass are used as starting materials to produce chemicals, biopolymers, biotechnologically useful enzymes/microorganisms and energy. The generation of energy and chemicals typically based on non-renewable fossil resources, shifts to a sustainable and low environmentally impacting system.

A big portion of biomass is represented by vegetable wastes and residues that are generated in huge amounts from plants and crops, during all the phases of cultivation

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and industrial processing. These residues can be defined as: primary wastes, i.e. harvesting residues like for example crop residues (stover, stalks, bagasse) and forestry residues; secondary wastes, that are generated by agro-industries i.e. industries that use agriculture products (husks, exhausted pulps, peels and seeds) or forestry products (wood chips).

A main fraction of these wastes is represented by lignocellulosic residues, that are currently used for several purposes like for example biofuel production or extraction of value added compounds (natural antioxidants, lipids, oligosaccharides, fibres and proteins).

In particular, lignocellulose residues are constituted of cellulose, hemicellulose and lignin that, in natural sources, are found as interconnected macromolecular structures, i.e. fibrils. Their heterogeneous structure due to the variable proportion of cellulose, hemicellulose and lignin in diverse plant sources, is perhaps one of the major hindrances in developing as much as possible universal multi-step procedures, which include mechanical and chemical pre-treatments and also enzyme-based bioconversion processes. Their focus is to ferment the sugars, obtained from biomass feedstocks, to generate ethanol besides other useful molecules (FitzPatrick et al. 2010).

Cellulose, as important renewable source of bioenergy, is involved in several treatments useful for its conversion to fermentable glucose (Bhalla et al. 2013). Differently, the hemicellulose for its intrinsic structure, can furnish also oligosaccharides extremely interesting in the field of nutrition as prebiotics and then used for several purposes, like for example nutrition research and applications (Azevedo Carvalho et al. 2013).

Nevertheless these residues are still an under-utilised feedstock that, by means of bioprocesses, could be used for the production of several bioproducts like for example biotechnologically useful extremophilic microorganisms and enzymes. Indeed, thanks to their complex chemical composition, these residues could be exploited as sole carbon source to promote and sustain microbial growth. Therefore, waste biomass could afford a cheap and environmentally friendly way to produce useful microorganisms (like for example extremophiles) and their related enzymes. The latter are the object of increasing interest since they find manifold applications in the production of second generation biofuels or value added compounds like for example biologically active oligosaccharides that could be used for the production of other useful chemicals.

Every year, million tonnes of these kinds of wastes are produced and therefore high amounts of cellulose and hemicellulose are lost. As reported in Table 19.1, significantly huge amounts of this cheap and renewable feedstock are available. Food wastes that are rich sources of cellulose and hemicellulose comprise fruits, vegetables and cereal residues that are produced by agro-industries like canning or packing industries.

Cellulose and hemicellulose are the main constituents of pomace, the waste of processing of fruits like apples, black currant, cherry, chokeberry and pears that are usually employed for juice or jams production. Apple pomace is one of the most abundant pomace residue and is a very rich source of cellulose and hemicellulose that account for 43.6 % and 24.4 % of the dry waste weight, respectively. About 0.5

Table 19.1 Annual production of main vegetable waste biomass that are renewable sources of cellulose and hemicellulose

Category	Waste type	Quantity/year	Reference
Food wastes	Apple pomace	3.42 Mton	Nawirska and Kwasniewska (2005)
	Black currant, Cherry, Chokeberry, Pear pomace	0.5 Mton	Nawirska and Kwasniewska (2005)
	Orange peels	7.4 Mton	Sanchez-Vazquez et al. (2013)
	Mandarin peels	3.1 Mton	
	Carrot residues	≈11 Mton	Nawirska and Kwasniewska (2005), Sanchez-Vazquez et al. (2013)
	Corn bran	7.8 Mton	Rose et al. (2010)
	Tomato	11 Mton	Tommonaro et al. (2008), Das and Sing (2004)
Crop residues	Cassava bagasse	≈200 Mton	Das and Sing (2004)
	Sugar cane bagasse	300 Mton	Cardona et al. (2010)
	Corn stover	696 Mton	Sanchez-Vazquez et al. (2013)
	Barley straw	≈5.6–9.8 Mton	Das and Sing (2004)
	Oat straw	≈10.5–15.7 Mton	Das and Sing (2004)
	Rice straw	731 Mton	Das and Sing (2004), Binod et al. (2010)
	Sorghum straw	≈28–43 Mton	Das and Sing (2004)
	Wheat straw	550 Mton	Kuan and Liong (2008), Das and Sing (2004)
Forestry/ wood residues	Wood residues	210 Mm^3	FAO data (http://faostat3.fao.org/home/E)
	Wood pellets	22 Mton	
	Chips and particles	250 Mm^3	

million tonnes of pomace are produced from the other above listed fruits: complex residues from processing of black currant, cherry, chokeberry and pears are on average made up on a weight basis of 34.6 % cellulose and 33.5 % hemicellulose (Nawirska and Kwasniewska 2005). Citrus fruits also generate abundant wastes that are cheap sources of cellulose and hemicellulose: on a weight basis in the case of orange pomace they represent 37.1 % and 11.0 % respectively of the dry weight, while for mandarin they account for 22.6 % and 6.0 % respectively (Sanchez-Vazquez et al. 2013). High cellulose contents can be found also in carrot and bran wastes: residues of carrot selection and processing for juice production are composed for 51.6 % w/w and 12.3 % w/w respectively by cellulose and hemicellulose; corn bran, that is generated by dry-milling of grains, accounts for about 7.8 Mtons that are composed by 70.0 % w/w of cellulose and by 28.0 % w/w of hemicellulose. Finally, canning residues from tomato processing can also be a valuable source of hemicellulose and cellulose that represent 7.5–11 % and 9.1 % respectively of the weight of these residues, mainly composed by peels and seeds.

Crop residues comprise the vegetable material left on the ground after harvesting i.e. stalks, leaves, straw (that on average represent more than 50 % of the crop), stover or bagasse. These wastes contain high amounts of cellulose and hemicellulose, therefore they are at world level the main renewable source lignocellulose (Sanchez-Vazquez et al. 2013). The main waste sources of lignocellulose are: corn stover (comprising leaves, shells, and stalks) that contains about 33.0 % w/w cellulose and 26.0 % w/w hemicellulose (Sanchez-Vazquez et al. 2013); rice straw that contains about 27.8 % w/w cellulose and 32.0 % w/w hemicellulose (Das and Sing 2004; Binod et al. 2010); wheat straw that is composed by both cellulose and hemicellulose that respectively account for 39.0 % and 36.0 % of dry biomass weight (Kuan and Liong 2008; Das and Sing 2004). Barley, oat and sorghum straw are produced in lesser amounts, nevertheless they also are very rich in cellulose and hemicellulose that on average respectively represent 38.6 % and 24.3 % of dry waste biomass weight (Das and Sing 2004).

Forestry residues are almost exclusively lignocellulosic materials and in general they comprise the biomass remaining in forests after harvesting and all the kinds of residues produced by wood processing industries (pellets; chips and particles). According to FAOSTAT database, the total world solid volume of forest wood residues produced in 2013 was about 210 million cubic meters: such biomass includes logging residues, excess small trees, rough or rotten dead wood. Usually these residues are either left in the forest or burned. With regard to wood processing wastes, about 22 million tonnes of wood pellets were produced in 2013: usually they are exploited as pellet fuel and are produced by using sawdust, residues of lumber's milling or wastes of manufacture of wood, furniture and construction. Finally the other main woody residues that could represent a cheap lignocellulose reserve is represented by chips and particles i.e. wood that has been reduced to small pieces and is suitable for pulping, fibreboard production. These wastes too, thank to their high cellulose content, could be used as cheap fermentation media for microorganisms and enzyme's production.

1.1 Main Current Technologies for Pre-treatment of Lignocellulose Waste Biomass

Food wastes, crop and forestry/wood residues are all polysaccharide rich materials that, as previously mentioned, could serve for several biotechnological applications. Indeed, thanks to their rich chemical composition, they can be used as growth media to produce useful microorganisms (like for example extremophiles) and their related enzymes: with this regard, several examples of wastes to be used as fermentation media are available in literature (Das and Sing 2004; Di Donato et al. 2011). However lignocellulosic residues are a recalcitrant biomass since plant cell wall's cellulose is deeply inset in a network composed of hemicellulose and lignin that hamper microorganisms or enzyme's access to the polysaccharide matrix. Therefore a kind of pre-treatment is necessary to open the lignin sheath in order to make

Table 19.2 Main techniques for waste lignocellulosic biomass pre-treatments

Pre-treatment type	Method	Biomass	Reference
Physical	Comminution	Crop residues (wheat straw, rice straw, bagasse); forestry residues	Zheng et al. (2014)
	Extrusion	Crop residues (barley straw, maize)	
	Liquid hot water	Crop residues (wheat and rice straw; sugarcane bagasse)	
Chemical	Acid hydrolysis	Crop residues (straws; sugarcane bagasse)	Sun and Cheng (2002)
	Alkaline hydrolysis	Food wastes (pomace); crop residues (wheat and rice straw, corn stover, sugarcane bagasse); forestry residues (leaves, wood residues)	Chang and Holtzapple (2000), Zhang et al. (2007), Kim et al. (2003)
	Organosolv	Crop residues (wheat and rice straw, sugarcane bagasse); forestry residues (wood residues)	Sun and Cheng (2002)
Combined physico-chemical	Afex	Crop residues (wheat and rice straw, corn stover); forestry residues (wood chips)	Sun and Cheng (2002)
	Steam explosion	Crop residues (wheat straw, corn stover); forestry residues (wood chips)	
Biological	Fungi digestion	Crop residues (wheat straw); forestry residues (wood chips)	Sun and Cheng (2002)

cellulose accessible for further processes. Different pre-treatment techniques are described in literature and they include physical, chemical, biological or combined treatments (Table 19.2): depending on the process applied, partial hydrolysis of hemicellulose as well as degradation of the lignin matrix may occur. The choice of the most appropriate pre-treatment is determined by the nature of lignocellulosic biomass and by the wanted products, therefore it is not possible to identify the best technique. In Table 19.2 are listed some of the most representative technologies that are currently used for different biomass pre-treatment, either at laboratory or at industrial scale.

Comminution is a physical pre-treatment that is implemented by means of milling or grinding machines and is used to reduce biomass particle size. Although this technique can affect the ultrastructure of cellulose, it is useful to increase the accessible surface area of the polymer; moreover it allows to reduce both the extent of crystallinity and the polymerization of cellulose thus improving its enzyme digestion. Extruders are schematically constituted by a barrel inside which a driving screw is allowed to move along the barrel: by means of this technique different mechanical treatments are applied to the biomass i.e. friction, mixing and shearing forces. Extrusion allows more accessibility of cellulose and in some cases it can also cause partial depolymerization of cellulose, besides degradation of both hemicellulose and lignin matrices. In liquid hot water (LHW) technique, biomass is treated

at high pressures that allow to hot liquid water to penetrate plant cell walls thus hydrating cellulose: in such a way hemicellulose can be solubilised while lignin is partially removed. Therefore by increasing the accessible surface area of cellulose, LHW can improve subsequent cellulose degradation by microorganisms and enzymes (Zheng et al. 2014).

Acid hydrolysis is a chemical method that can be implemented by using either diluted or concentrated acids. In diluted acids pre-treatment, biomass is sprayed with sulphuric, maleic or fumaric acid and then it is heated up to 160–220 °C for a small number of minutes. Such a treatment usually causes hemicellulose removal and increase in porosity: in this way enzyme hydrolysis affords oligosaccharides or monomer sugars for further applications (for example fermentation to ethanol). In concentrated acids pre- treatment, sulphuric or hydrochloric are the most widely used acids: although this technique affords high yields for cellulose depolymerisation (that doesn't require further enzyme hydrolysis) it is more costly due to the corrosion of apparatuses and to the need of recycling of reagents (Sun and Cheng 2002).

Alkaline hydrolysis can be carried out by using calcium or sodium hydroxides or aqueous ammonia either at low or at higher temperatures (Kim et al. 2003): the main effect of these treatments, that require long reaction times, is the solubilisation of lignin (up to 70–80 %) and the very limited degradation of sugars (Chang and Holtzapple 2000). Finally, organosolv processes allow to remove lignin by treating biomass with pure organic solvents (ethanol, methanol, acetone, ethylene glycol) or their mixtures: the treatment is carried out either at low or at high temperatures depending on the biomass type. The main advantage is represented by solubilisation of lignin and hemicellulose depolymerisation, that consequently improve cellulose purity and accessibility to enzymes or microorganisms (Sun and Cheng 2002).

The most known combined physico-chemical treatments are ammonia fiber explosion (AFEX) and catalysed steam explosion. AFEX technique is based on liquid ammonia treatment that is conducted at high values of temperature and pressure (Bals et al. 2010) for about 30 min. This method affords minor hemicellulose depolymerization but efficient removal of lignin, although the cost of ammonia strongly limits its use. On the other hand steam explosion is more convenient for both economical and environmental aspects: indeed it requires minor quantities of chemicals and a lower energy input, therefore being a less environmentally impacting technique. Typically, biomass is treated with saturated steam at high-pressures and in the presence of 1 % H_2SO_4 as catalyst: the process lasts for few minutes at 160–260 °C and 0.69–4.83 MPa, then the material is returned to atmospheric pressure. By means of this process, hemicellulose degradation and lignin solubilisation are quite complete, and the potential of cellulose enzymatic hydrolysis is significantly increased (Sun and Cheng 2002).

Biological pre-treatments exploit white or brown or soft rot-fungi, that act by degrading hemicellulose and lignin matrices. These methods are very low-impacting since they require mild treatment conditions and very low energy inputs, nevertheless their application is still limited to lab-scale processes due to the low rate of biological

degradation of lignin polymer and of hemicellulose whose sugars are also assimilated by fungi (Sun and Cheng 2002). Presently the only cost-effective methods are steam explosion besides liquid hot water or acid hydrolysis pre-treatments.

1.2 Extraction Procedures of Xylan from Vegetable Biomass

One of the conditions of an efficient use of biomass such as cereal straws and grasses is the separation of the main components, polysaccharides and lignin, by a relatively mild processes, able to guarantee their minimum physical and chemical modifications as well as an efficient extraction procedure. Various processes have been described for the exhaustive extraction of hemicellulose to obtain xylan useful for enzymatic applications (Aachary and Prapulla 2009).

However, before starting any pre-treatment, the raw material should be prepared by washing with organic solvents (ethanol or ethyl acetate) to separate impurities and other molecules (e.g., waxes and pectin) and make the following processes (like xylo-oligosaccharides steps) more simple (Brienzo et al. 2009). In some cases, alcohols and ketones could be exploited in the collection of soluble xylan or to concentrate the released XOS (xylo-oligosaccharides) after enzymatic or chemical reactions (Akpinar et al. 2009).

Dilute acid treatment, dilute alkali extraction, autohydrolysis, enzymatic hydrolyses represent the current pre-treatments employed to isolate hemicellulosic polymers from agro-residues (Fig. 19.1) (Azevedo Carvalho et al. 2013; Chapla et al. 2012; Aachary and Prapulla 2009; Akpinar et al. 2010). In some cases, organic solvent are used as extraction co-solvents, but these procedures, although efficient, are not totally eco-friendly and economical. Each of these procedures has its advantages and its drawbacks.

Autohydrolysis is the deacetylation of xylan by the thermal hydrolysis of hemicellulose to generate acetic acid. It is essential to operate with specialized equipment for reaching high temperatures and pressures (Kabel et al. 2002).

In recent cases, hydrothermal methods consider the combined use of microwave heating or steam explosion procedures. According to the temperature and time of working, it is possible to modulate the degree of de-polymerization and then the molecular weight interval of xylan/xylo-oligosaccharides obtained (Aguedo et al. 2014). Hemicellulose material is usually recovered in a liquid phase, whereas the precipitate solids are rich of cellulose, which is available for other purposes (Azevedo Carvalho et al. 2013). Unfortunately, the products obtained from lignocellulosic materials by this procedure were contaminated with several undesirable

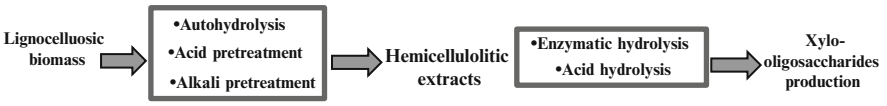

Fig. 19.1 Pre-treatments for xylan extraction and procedures for XOS production

components such as lignin, furfural, and others, thereby demanding further purification (Zhu et al. 2006).

Acid pretreatments consist of the exposure of hemicelluloses to acid solutions. Regardless of the vegetable initial biomass, these extractions are carried out in acidic mild conditions (generally from 0.01 to 0.5 M of H_2SO_4) to limit the amount of xylose released and avoid or decrease the resulting corrosion. However, acid methods are always coupled with the steaming of lignocellulosic material (>120 °C) allowing the removal of lignin: in this case the time of process is important for modulating the degradation of xylan to xylose and for avoiding its complete de-polymerization and the undesirable formation of toxic furfural and hydroxymethylfurfural (HMF) (Akpinar et al. 2009). Indeed, the production of monosaccharides and these toxic components represent the major impediment of the acid treatment that requires a greater degree of purification also for these extraction procedures.

Alkali treatments have been widely accepted because they destroy the cell wall structure and cleave the hydrogen bonds, the ester linkages with acetyl units and hydroxycinnamic acids and covalent bonds with lignin (mainly α-benzyl ether linkages) in the cell wall matrix, liberating hemicelluloses polymers in the aqueous media. The limitation is that the alkaline pre-hydrolysis does not preserve the acetyl groups on the xylan chains (Lama et al. 2014; Bian et al. 2012). Aqueous solutions of potassium, sodium, barium, calcium and lithium hydroxide have been widely used to recover hemicelluloses at different temperatures, in particular from 4 °C to 40 °C (Bian et al. 2012). Nevertheless, the favorite alkali is the potassium hydroxide because the potassium acetate produced during the neutralization of the alkali extract is more soluble in alcohol used for precipitation than other acetate (Lawther et al. 1996). According to nature of lignocelluosic material, the percentage of alkali used could be different. Moreover, sometimes the presence of co-solvents (e.g. ethanol) could be employed (Li et al. 2015). A good procedure of xylan recovery involves both alkali at higher concentration and thermal hydrolysis: it was described for corncob biomass (Samanta et al. 2012).

Akaline pretreatments with hydrogen peroxide (2–4 %) at lower temperature (20–60 °C) furnish good results in delignification and hemicellulose solubilization from straws and grasses, preserving its chemical structure, as reported for sugarcane bagasse hemicellulose extraction (Brienzo et al. 2009).

The microwave irradiation coupled with alkali solutions may be an alternative pretreatment approach for lignocellulosic materials at lower temperature to overcome the problems related to the production at high temperature (autohydrolysis) of undesired and harmful compounds for the following enzymatic procedures (Zhu et al. 2006).

Pre-treatments with selected enzymes could be more attractive because it does not generate undesirable by-products or high amounts of monosaccharides and does not require special instruments, which work at high temperatures, for example. Unfortunately, they are useful only for susceptible materials such as citrus peels to produce XOS with direct enzymatic treatment of xylan-containing materials (Aachary and Prapulla 2011).

Microwave-assisted enzymatic hydrolysis has recently considered as a possible method to produce xylo-oligosaccharides by reducing the reaction time and avoiding undesirable products; it was described for the production of prebiotics from wheat bran by using commercial xylanases (Wang and Lu 2013).

The extracted xylan, obtained from the pretreatment of the lignocellulosic material, could be converted to monosaccharides by strong acidic reactions or treated by enzymatic hydrolysis (Azevedo Carvalho et al. 2013) if the aim is the preparation of oligosaccharides. In fact enzymatic hydrolysis is evaluated as the best option to produce xylo-oligosaccharides (XOS) for the food industry.

Preparation of XOS is performed by an endo-xylanase, which hydrolyses β-1,4 bonds in the main chain of xylan. If enzymatic complex are used for this aim, they must have a low activity of exoxylanases to avoid or reduce the production of xylose, which inhibits the XOS formation (Fig. 19.1) (Vázquez et al. 2005).

2 Waste Biomass Development as Alternative Carbon Source for Extremophilic Microbial Growth

A still not completely explored opportunity in waste biomass improvement is its use as alternative source for supporting microbial growth. The microbes can be considered as an inestimable factory of molecules with numerous uses in several fields and for different industrial processes. For this reason, the reduction of costs due to the needed chemicals for microbial fermentation even more so at industrial scale, represents an attracting prospective. On the other hand, this change permits to restore a commercial value to the waste so extending the duration life cycle of starting material. In addition, this could offer a manner to amortise the disposal waste costs (Di Donato et al. 2011). The above mentioned concepts are more amplified when the factories are represented by the extremophilic microorganisms, so called because they prefer environmental niches characterized by one or more stress-factors, such as high or low temperature, pressure and pH, absence of oxygen, presence of radiations, high saline and metal concentration. Therefore, they are able to thrive under stressful conditions and so their use result more advantageous than the mesophilic microorganisms.

Knowing of these organisms allows us to better understand how life originated and developed on Earth but also allows us to understand how we can improve some processes or obtain better results from already known processes (Mastascusa et al. 2014). These particular microorganisms, in fact, to survive and thrive in such adverse conditions, have developed special properties such as chemical structures and components specific for cell membranes, incredible metabolic processes, mechanisms of energy transformation and regulation of the intracellular environment (Antranikian and Egorova 2007). Thermophilic bacteria are currently used in the production of alcohols and other biologically active compounds (carotenoids, amino acids, antibiotics), in the removal of metal ions and organic compounds from waste solids or aqueous. Thermozymes find application in food industry for the syrups

production with a high content of sugar (amylase, xylose isomerase, pullulanase) and to improve the organoleptic properties (pectinase) or the digestibility of certain foods (beta galactosidase); several thermoactive protease and lipase are used in the detergent industry and in the process of baking; the protease thermolysin from *Bacillus thermoproteolyticus* is used in the synthesis of the dipeptide aspartame, a low calorie sweetener (Ogino et al. 1995). Thermophilic enzymes are also used in the medical and biological precursors of such drugs, or, as the DNA polymerase and DNA ligase thermophilic, used in the PCR (Polymerase Chain Reaction) used in diagnostic medicine, molecular biology and taxonomy.

The adapting strategies of extreme microbes allowed them to carry out all metabolic processes even if in the presence of chemico-physical stress factors. These strategies come in handy in biotechnology researches, by means of pharmaceutical, food, genetic, feed, environmental, etc. Indeed, various industrial production processes are based on the exploitation of the extremophile potentialities together with their bio-molecules (enzymes, polymers, lipids, etc.) (Elleuche et al. 2014). For all those motivations, it's clear how convenient could be to search for a cheaper manner to growth extremophilic microorganisms. Serious attention is being paid in the research to this subject matter and several efforts are already reported into the literature.

An example of interesting molecules are the polyhydroxyalcanoates, PHAs; they are biodegradable polymers produced essentially by halophilic bacteria, microorganisms growing in presence of high salt concentrations. The PHAs are accumulated into the microbial cells in granules as reserve energy and as strategy to contrast the unbalance growing conditions. The bio-compatibility of PHAs has allowed its utilization up to industrial level in several fields (packaging, medicine, agriculture, drug delivery etc.) (Shrivastav et al. 2013).

Danis et al. (2015) studied the production of polyhydroxyalcanoates by the halophilic archaea *Natrinema* strain 1KYS1 in a cheaper manner using wastes as sole carbon and energy sources such as corn starch, sucrose, whey, melon, apple and tomato wastes. Among the waste investigated, the media containing corn starch resulted the most suitable for the PHA cell accumulation, with a yield of 53.14 % of dry cell weight (Danis et al. 2015). Also the haloarchaea *Haloterigena hispanica* was tested for its capability to utilize tomato and carrot wastes for growth and polyhydroxybutyric acid (PHB) production. The best substrate was the carrot that gave a PHB yield comparable to that obtained with standard complex broth, 1.25 ± 0.05 mg/g^{-1} of dry cell and 1.35 ± 0.06 mg/g^{-1} of dry cell, respectively (Di Donato et al. 2014). Huang et al. (2006) investigated the chance to increase the (PHA)s accumulation into the cells of another extremely halophilic archaea *Haloferax mediterranei* by using as carbon source pre-treated rice bran and starch, coupled to a repeated batch fermentation for growth. The highest PHA yield reached was of 55.6 % of dry cell weight.

In field of polymers, many attempts are doing continuously mainly for microbial exopolysaccharides (EPS), of which the biotechnological applications are already known and they extend from food to medicine, pharmacology, nutraceutical, cosmetic, herbicides and insecticides (Nwodo et al. 2012). There are several kinds of EPS produced by alternative carbon sources, but in the field of extremophilic

microorganisms the levan can be cited; its an homopolymer of fructose, usable in food, medical, pharmaceutical and agricultural applications (Rhee et al. 2005). Speaking of which, an example of fermentation on substrates deriving from exhausted productive processes for extracellular levan production is the study of the halophilic microorganism *Halomonas smyrniensis* AAD6. The microbial growths were set up by using starch molasses and sugar beet molasses, after diverse pretreatments, as alternative sources to sucrose; beet molasses resulted to be better utilized from the microorganism for the EPS release (12.4 g/L^{-1}) (Küçükaşik et al. 2011).

Among the possible molecules of microbial origin, the enzymes represent probably the most exploited ones in productive industrial processes. Especially the thermophilic enzymes, for their thermostability, tolerance to organic solvents and metals, always find purpose in several fields. Deeply investigated are the amylase, cellulase and xylanase activities, so called for their ability to hydrolyse the starch, cellulose and xylan polymers, respectively. The polysaccharides are the most common in nature and represent an inestimable source of by-products (mono and oligosaccharides) with high economical potential. The amylase commercial applications are ascribable to pharmaceutical, paper, food, textile, fuel, detergent and starch industries (de Souza and Magalhães 2010). Therefore, the thermophilic *Anoxybacillus amylolyticus* was grown in submerged and solid state fermentation on diverse carbon sources, comprised agro-wastes, to investigate the microorganism capability to utilize them for growing and producing α-amylase. The waste sources tested and added to minimal medium (1 %, w/v) were rhizomes from *Arundo donax* L., steam and leaf from *Cynara cardunculus* and potato peel wastes. The growth and enzymatic yields were compared to those obtained from conventional chemicals such as yeast extract and soluble starch. The rhizomes from *Arundo donax* L., in submerged fermentation conditions, showed to be the most suitable inducer for the recovery of amylase from *A. amylolyticus*, exactly 2.2-fold higher respect to that observed when the microorganism was grown on yeast extract (Finore et al. 2014). Another thermophilic microorganisms tested for its ability to utilize waste sources was *Geobacillus thermoleovorans* subsp. s*tromboliensis*; it produced an extracellular α-amylase able to hydrolyse raw starches also in presence of high ethanol concentration; it makes this enzyme a suitable candidate for a cloning procedure in heterologous host such as *Saccharomyces cerevisiae* for a simultaneous saccharification and fermentation in order to obtain ethanol directly from un-treated starch (Finore et al. 2011; Kasavi et al. 2012). Therefore, *G. thermoleovorans* was grown both in batch and dialysis fermentation in presence of the following waste biomass: lemon, tomato, fennel, carrot and rhizomes from *Arundo donax* L. The investigated amylase activity reached the highest value in presence of the rhizomes from *Arundo donax* L., its specific activity was 110 % higher than that collected under standard complex medium; while lemon wastes gave only 39 % of specific activity compared to the complex medium (Di Donato et al. 2014). The industrial processes involving xylanase enzymes are numerous and concern the animal feed, paper industry, cellulose bleaching, etc. (Polizeli et al. 2005). *Thermobacillus xylanilyticus* is a thermophilic bacterium with complete stable hemicellulotic enzymes. Its growth was tested on wheat bran and straw and compared to glucose and xylans. The experiments

showed that the microorganism utilized all tested carbon sources and in any case, the most abundant activity produced was the xylanase, while the medium composition affected the relative presence of the debranching enzymes; more in detail, the esterase activity was more produced in presence of wheat straw and the arabinofuranosidase activity resulted more abundant when the bacterium grew on straw (Rakotoarivonina et al. 2012). Also *Geobacillus thermantarcticus* is a thermophilic microorganism producing an extracellular thermophilic xylanase and a β-xylosidase (Lama et al. 2004). For this bacterium the cheaper medium containing steam and leafs of *Cynara cardunculus* increased the xylanase release up to the 160 % respect to that measured in standard growth conditions (Di Donato et al. 2014).

Fennel, carrot and tomato wastes were evaluated as possible substrates for the growth of the halophilic bacterium *Halobacillus alkaliphilus* and the intracellular α-glucosidase production was investigated. Waste biomass was added at 1 % (w/v) in minimal medium both in dialysis and in batch fermentation. All vegetable wastes supported the growth of microorganism and in the presence of fennel waste the enzyme yield was found comparable with the respect to the activity measured in standard growth conditions (Di Donato et al. 2014).

3 Extremophilic Enzymes Useful in Biomass Conversion to Obtain Biofuel

Through acid or enzymatic treatments, the cellulose and hemicelluloses are converted into hexose and pentose sugars. Enzymatic hydrolysis by (hemi)cellulases is the better method, indeed it allows to reach higher conversions and, at the same time, results more eco-friendly because less toxic respect to the acid hydrolysis. Fermentation of all free sugars into ethanol is obtained by yeasts or bacteria. Nowadays, the use of commercial enzymes for hemicelluloses and celluloses conversion, during the ethanol production process, results the most expensive aspect in the production of bioethanol (Elleuche et al. 2014). In fact the available enzymes have been produced by mesophilic organisms and the enzymatic reactions are performed at ≤50 °C. This caused slow and, sometimes, incomplete enzymatic hydrolysis with low yields of bioconversion of lignocellulose biomass; furthermore high amount of enzyme is necessary and there is also an high contamination risk (Bhalla et al. 2013).

To solve these problems, many efforts have been made by the research for improving the hydrolysis procedures, increasing the cellulase activities, optimizing the reaction parameters, such as appropriate enzyme and substrate combination, cascade-reactions, enzyme reusing.

During last studies aiming to optimize the lignocellulosic biomass hydrolysis, statistical approach coupled with factorial design was applied; therefore enzymes of various origin were combined and mixed in an appropriate amounts (Zhou et al. 2009). A considerable decrease in the proteins content used has been reached (twofold) to obtain glucose from glucan and xylose from xylan, 99 % and 88 % conversion

yields, respectively, so validating the statistical approach (Berlin et al. 2005). Several efforts are underway to reduce the cost and maximize enzyme production.

Some of the strategies include enhancing the enzymatic catalytic abilities by increasing the specific activity (by directed evolution and site directed mutagenesis) and thereby minimizing enzyme dosage or reduce the cost of enzyme production by improving cellulase titers during fermentation (through process engineering approaches by using cheap substrates including biomass, producing enzymes near biorefinery, or expression of enzyme in plants). An optimal enzymatic system in biorefinery should be engendered in situ and usable in continuous culture, having elevate activity, being stable under process conditions such as high temperature and inhibitory compounds presence (aldehydes), and possessing a considerable half-life.

3.1 Thermophilic Bacteria and Thermostable Enzymes

Several problems connected to the conversion of biomass into biofuels could be solved by utilizing extremophilic microorganisms and their thermostable enzymes.

Extremophiles have always found applications in the frame of bioenergies; furthermore recently, new attention has been pointed out on to these particular forms of life. Indeed extremophilic microorganisms are able to survive under unique environmental conditions, by means of low and high pH, temperature, elevate salinity or pressure (Bhalla et al. 2013). In addition, these microorganisms thrive in similar niches thanks to metabolic and physiological strategic adaptations. Extremozymes show kinetics that enable integration into processes carried out under stressful conditions.

In going over the knowledge concerning the applications of extremophilic microorganisms in biofuel process production processes, it is evident that most of microbial sources are essentially thermophilic. This has not astound, because thermophiles are able to put up with pH, temperature and environmental variations, proprieties which make them interesting for several applications into commercially valuable fuel production processes (Bhalla et al. 2013).

The employ of extremophilic, in particularly of their resistant thermoenzymes, represents a great potential in the bioconversion of biomass, crucial step for the generation of a commercial process. It's widely reported in the literature that extremozymes in biofuel production are mainly collocate in the hydrolysis of polymers from the various feedstocks.

Biofuel industries need enzymatic activities highly substrate-specific, not inhibited by the end-products accumulation and stable even during the process parameters variation. Enzymes produced by acidophilic and thermophilic microorganisms are obtaining always more interest because of their characteristic properties.

Indeed, to utilize thermostable enzymes implies numerous advantages during the processes of lignocellulosic biomass hydrolysis; first of all, the high temperature guarantees a major solubility of both reactants and products, so resulting in a more efficient process, mainly in terms of reaction velocity and therefore in quantity of

request enzyme (Zhang et al. 2011); a lower contamination risk and, in this manner an improved productivity; an easier recovery of volatile released compounds (e.g. ethanol); reduced energy costs associated to the cooling after the thermal treatment; an higher stability that permits a longer time of hydrolysis and a potentially more flexible production process.

It is commonly known that the bacteria producing thermostable cellulases and xylanases are the most able microbes in the depolymerization of lignocellulose (Liang et al. 2011).

Research is also interested on activities able to put up with acid and heat which both may improve the lignocellulose processing. These enzymatic activities could be derived by iper-thermophilic microbes/extremophiles (Bhalla et al. 2013). The applicative potential of such microorganisms together with their enzymes could increase, mainly, in the biofuel industry.

3.2 Cellulose Deconstruction

Cellulose represent the most abundant polymer of all plants and the most copious organic molecule on globe. Cellulose is a glucan homopolysaccharide comprised of β-D-glucopyranose units linked together by β-1,4-glycosidic bonds. Cellulases hydrolyse the glycosidic bonds of both crystalline and amorphous cellulose (Mischnick and Momcilovic 2010), and according to the cellulase, different portions and chains of cellulose are targeted.

The conversion of cellulose to monomer of glucose entails the interventation of endocellulases (Enzyme Commission EC 3.2.1.4), exocellulases (cellobiohydrolases, CBH, EC 3.2.1.91; glucanohydrolases, EC 3.2.1.74), and beta-glucosidases (EC 3.2.1.21). The endocellulases act randomly on internal glycosidic bonds; it leads to a fast reduction of polymer extension and a progressive enhancement of reducing sugars. Exocellulase activities cut cellulose by removing essentially cellobiose starting from both the reducing and the not-reducing ends. In this manner, it's obtained a rapid increase in reducing sugars concentration but small variation in terms of polymer length. The hydrolytic action of endocellulase and exocellulase enzymes is synergic and results in cello-oligosaccharides and cellobiose release, which are then converted into glucose by beta-glucosidase (Kumar et al. 2008).

However, the conversion procedures of lignocellulose biomass are crucial; it's necessary to search for new and more efficient enzymes. The most problematic and slow step in biofuel production from cellulose is represented by the initial enzymatic attack on the ordered and unsoluble structure of crystalline cellulose.

A diverse combination of hydrolytic activities is necessary for a total degradation of hemicellulose of various origin. Even though cellulases hydrolyze one type of bond, crystalline substrates because of their of wide pattern of bonding require the participation of a set of enzymes or multi-component systems named cellulosomes (Kumar et al. 2008). They consist of a multi-domain scaffoldin that carries at least one Carbohydrate Binding Module (CBM) and various cohesion modules.

Essentially the carbohydrate-binding module CMB are non-catalytic modules, that facilitates the targeting of the enzymes to the insoluble polymers, and the dockerin module that mediates the binding of the catalytic module via cohesion-dockerin interactions, improving the degradation efficiency, mainly on raw lignocellulose biomass. A more efficient degradation of cellulose, in terms of speed and costs would produce both environmental and economic advantages, motivating trials using enzyme mixtures, as well as engineered cells, and is still a stimulus for the researchers.

The main properties of thermostable endoglucanases from many thermophilic and hyperthermophilic bacteria are reported in Table 19.3. It's reported that a great number of bacteria and fungi are source of thermostable cellulases. Thermophilic and mesophilic fungal genera belonging to the *Rhizopus, Trichoderma*, *Aspergillus* and *Sclerotium, Thermoascus thermophile* var. *coprophile, Chaetomium thermophile*, *Sporotrichum thermophile*, *Coniochaeta ligniaria* (Barnard et al. 2010) presented cellulases, but the hydrolysis of cellulose was often not complete. As an instance, one of the major bad aspect of *Trichoderma* is that it possesses a low amount of beta-glucosidase enzyme (Rahman et al. 2009). The union of cellulases, cellobiose dehydrogenase and glycoside hydrolase 61 (GH61) family of proteins, found in many species of thermophilic fungi (Dimarogona et al. 2012) have been reported to drive to an interesting increase in to lignocellulose hydrolysis (Horn et al. 2012).

Pyrococcus (Kim and Ishikawa 2010), *Sulfolobus* (Girfoglio et al. 2012) *Thermotoga* (Hong et al. 2007), *Geobacillus* (Rastogi et al. 2011) and *Thermus* (Antranikian and Egorova 2007) represent some Archaea and Bacteria examples in which thermostable endoglucanases have been found. Moreover, in *Pyrococcus* and *Thermus* spp. (Chang et al. 2001; Xiangyuan et al. 2001) exocellulases and glucosidases have been described. The improvements in terms of enzyme specificity and activity have been achieved by molecular biology (Table 19.3). Since the degradation for example of lignocellulosic materials requires a huge amount of enzymes (Lynd et al. 2005), the cellulases employed came from recombinant strains of aerobic fungi, such as *Trichoderma reesei* (syn. *Hypocrea jecorina*) and *Humicola insolens* (Karlsson et al. 2002).

The absence of CBM is responsible for unable of several hyperthermophilic microorganisms to decompose crystalline cellulose efficiently at temperatures above 75 °C, even if the employment of a multidomain hyperthermophilic cellulase could be responsible of a good degradation of lignocellulose at temperature above 90 °C (Graham et al. 2011).

Thermostable endoglucanases could operate at different optimum pH values. *Acidothermus cellulolyticus* presents an endoglucanase that works optimally at pH of 5.0 and a temperature of 80 °C (Lindenmuth and McDonald 2011). The thermoacidophilic *Alicyclobacillus* sp. A4 presents an extremely acidic β-1,4-glucanase (optimum pH 2.6 and 65 °C) (Bai et al. 2010a). *Bacillus* KSM-S237 shows a thermostable alkaline endoglucanase (optimum pH 8.6–9.0), and stored more than 30 % of the original activity after exposure at temperature of 100 °C, at pH 9.0 for 0.17 h (Hakamada et al. 1997). The use of these thermoacidophilic and thermoalkaliphilic enzymes are very widespread for several reason. For example the ligno-

Table 19.3 Characteristics of thermostable endoglucanases from various thermophilic and hyperthermophilic bacteria

Organisms	Enzymes	pH optimum	Temperature optimum (°C)	Stability	References
Thermotoga sp.	Exoglucanase	6.8–7.8	100–105	Half-life 70 min at 108 °C	
T. neapolitana	Endoglucanase Cel B	6.0	95	Half-life of 2.16 h at 106	Bok et al. (1998)
T. neapolitana	Endoglucanase Cel A	6.0–6.6	100	Half-life of 0.43 h at 110	Bok et al. (1998)
Rhodothermus marinus	Endoglucanase	7.0	95	Retained 50 % of its activity after 3.5 h at 100 °C and 80 % after 16 h at 90 °C	Hreggvidsson et al. (1996)
Alicyclobacillus acidocaldarius ATCC27009	Endoglucanase	4.0	80	Retained 60 % of activity after incubation for 1 h at 80 °C	Eckert and Schneider (2003)
Caldibacillus cellulovorans	Endoglucanase	6.5–7.0	80	Half-lifeof 0.53 h at 80 °C, and 0.03 h at 85 °C, and retained 83 % activity after 3 h at 70 °C	Huang and Monk (2004)
Geobacillus thermoleovorans T4	Endoglucanase	7.0	70	Retained more than 10 % of the original activity for 1 h at 90 °C and 100 °C	Tai et al. (2004)
Geobacillus sp. DUSEL R7 NA	Endoglucanase	5.0	75	Retained 26 % activity at 60 °C up to a period of 300 h	Rastogi et al. (2009)
Alicyclobacillus sp. A4	Endoglucanase	2.6	65	Retained 90 % activity after 1 h at 60 °C	Bai et al. (2010a)
Clostridium thermocellum	Endoglucanase	7.0	70	50 % of activity remained after 48 h at 60 °C	Romaniec et al. (1992)
Clostridium stercorarium	Endoglucanase	6.0–6.5	90	Stable for several days	Bronnenmeier and Staudenbauer (1990)
Rhodothermus marinus	Endoglucanase	7.0	95	50 % of activity remained after 3.5 h at 100 °C, 80 % after 16 h at 90 °C	Hreggvidsson et al. (1996)
Acidothermus cellulolyticus	Endoglucanase	5.0	83	Inactivated at 110 °C	Himmel et al. (1994)
Thermosipho sp. strain 3	Endoglucanase	5.6	70	Retained 50 % after 90 h at 70 °C in the presence of Ca2	Dipasquale et al. (2014)
E. coli expressing gene from *Thermoanaerobacter tengcongensis* MB4	Endoglucanase	6.0–6.5	75–80	Half-life of ½h at 82 °C	Liang et al. (2011)
E. coli expressing gene from *Clostridium thermocellum*	Endoglucanase	6.4	80	Cumulative activity after 1/2 h at 90 °C was 15 % of that at 80 °C	Zverlov et al. (2005)

cellulosic biomass have to be treated by acids or alkali, followed by neutralization step, before the action of degrading enzymes (Zambare et al. 2011). On the other hand when thermoacidophilic and thermoalkaliphilic enzymes are used for the hydrolysis of lignocellolosic biomass, the neutralization step could be eliminated (Zambare et al. 2011).

3.3 Hemicellulose Deconstruction

Hemicellulose is a complex polymer that could be homo- and hetero-polymer and contains units of xylo- manno-, gluco- and galacto-pyranose that constitute the main chain and is responsible for 25–35 % of lignocellulosic biomass (Jain et al. 2014). Pentoses such as D-xylose, D-arabinose, hexoses such as D-mannose, D-glucose, D-galactose and sugar acids, in addition to other substituents in the branching, make this heteropolymer very complex and unique for each plant source. Xylan represents the major component of hemicelluloses and also the second polymer for its abundance representing about one-third of the renewable biomass available on our planet (Dhiman et al. 2008). From structural point of view xylan is a heteropolymer in which the repeating unit is formed by beta-1,4-linked xylose (main chain) that appears decorated with 4-O-methylglucuronopyranosyl, alpha-L arabinofuranosyl, alpha-D-glucuronyl residues, acetyl, feruloyl, and/or *p*-coumaroyl units (Sun et al. 2005).

Hardwoods are the natural source of xylan as O-acetyl-4-O-methylglucuronoxylan, instead softwoods represented the source of arabino-4-O-methylglucuronoxylans: they have next to the 4-O-methylglucuronic acid a substitution with α-(1,3)-L-arabinofuranosyl residues. In the endosperm with high content of starch and the external parts of cereals, it is possible to recover arabinoxylans with a substitution of the β-(1,4)-D-xylopyranose backbone at position C2 or C3 with α-L-arabinofuranose, which can be link by esterification with phenolic acids, and/or 4-O-methyl-D-glucuronic acid. In woody part of grasses and cereals are isolated several polymers such as (glucurono)arabinoxylans linked by acetylation and esterification with ferulic acid. Differently, in the case of cereal stalks, seeds and gums, these heteropolymers are more complex thank to many substitutions of monosaccharides or oligosaccharides (Sedlmeyer 2011). Hardwood and softwood beside the presence of xylan and glucomannan respectively, are also the source of xyloglucan, glucomannan, galactoglucomannan and arabinogalactan.

Several enzymes are necessary in order to hydrolyse completely the complex structure of hemicellulose into fermentable sugars such as pentoses (D-xylose and D-arabinose), hexoses (D-glucose, D-galactose and D-mannose) and sugar acids (Subramaniyan and Prema 2000, 2002). Glycoside hydrolases, polysaccharide lyases, carbohydrate esterases are some example of digesting enzyme for hemicellulose hydrolysis: these enzyme act together to break glycosidic and ester bonds, and in the removal substituents from chains (Sweeney and Xu 2012). The enzymes involved are mainly endo-beta-1,4 xylanases ([EC 3.2.1.8]), xylan 1,4-beta-xylosidases

([EC 3.2.1.37]), alpha-L-arabinofuranosidases ([EC 3.2.1.55]), alpha-glucuronidases ([EC 3.2.1.139]), acetylxylan esterases ([EC 3.1.1.72]), feruloyl esterases ([EC 3.1.1.73]), mannan endo-1,4-beta-mannanases ([EC 3.2.1.78]), beta-1,4-mannosidases ([EC 3.2.1.25]), and arabinan endo-1,5-alpha-L-arabinosidases ([EC 3.2.1.99]) (Collins et al. 2005).

In the hydrolysis of hemicelluloses, endoxylanases and exoxylanases are useful to start the break the cross-linked polymers, then β-xylosidases convert xylo-oligosaccharides to xylose with xylo-oligomers of various lengths; on the other hand α-arabinofuranosidase breaks arabinose units in both furanose and pyranose forms; methyl glucuronic acid substitutes are hydrolised by α-glucoronisidase while acetylxylan esterase and ferulic acid esterase hydrolyses acetyl substitutes, arbinose and ferulic acids. To be noted that several of these enzymes are able to hydrolise also other compounds: this aspect make hard to know their enzymatic activities when a lignocellulosic material is digested. Nowadays there are many examples of protein combinations with good activity and high resistance to inhibition that contain hydrolitic enzymes that came from fungi or bacteria.

In Table 19.4 are listed examples of endoxylanases isolated from thermophilic and hyperthermophilic microorganisms. Thermostable xylanases are mainly produced by bacteria and fungi (Collins et al. 2005). Examples of fungi producing thermostable xylanase are *Thermoascus aurantiacus* (Zhang et al. 2011), *Rhizomucor miehei* (Fawzi 2011), *Thermomyces lanuginosus* (Singh et al. 2003), *Nonomuraea flexuosa* (Zhang et al. 2011), *Laetiporus sulphureus* (Lee et al. 2009), *Talaromyces thermophiles (*Maalej et al. 2009). Since xylanases produced by bacteria possess a higher optimum temperature and higher thermostability are usually employed for lignocellulosic materials deconstruction with respect to xylanases from fungi (Bhalla et al. 2013). Bacteria belong to *Alicyclobacillus, Anoxybacillus, Paenibacillus, Thermoanaerobacterium, Actinomadura, Nesterenkonia, Enterobacter, Acidothermus, Cellulomonas, Bacillus, Geobacillus, Thermotoga* genera have been found to possess thermostable xylanases.

Geobacillus thermantarcticus, a thermophiles microorganism collected from Antarctica, produced extracellular xylanase and β-xylosidase. These activities show interesting properties for biotechnological applications, such as optimal pH and temperature activity, thermostability, the absence of cellulolytic activities and high portions of low-member xylo-oligomers. *G. thermantarcticus,* when use xylan as unique organic source, presents a characteristic xylan digestion system in two steps that is advantageous for recovery of hydrolysis products by modulating growth conditions and physico-chemical parameters (Lama et al. 2004).

Other sources of thermostable xylanases are represented by thermoalkaliphiles, thermoacidophiles, and thermohalophiles. *Thermoanaerobacterium saccharolyticum* NTOU1 is a marine halophilic bacterium whose xylanase stored 71 % activity for 24 h when incubated in the presence of 2 M NaCl (Hung et al. 2011). *Alicyclobacillus* sp. A4 possesses an interesting xylanase stables in a wide range of pH from 3.8 to 9.4: this enzyme, that has been cloned in *Escherichia coli,* stored 90 % enzyme activity for 1 h after incubation at 60 °C (Bai et al. 2010b). Other examples of thermostable xylanase has been found in *Enterobacter* sp. MTCC 5112 (Khandeparkar and Bhosle 2006).

Table 19.4 Characteristics of thermostable xylanase from various thermophilic and hyperthermophilic bacteria

Organisms	Enzymes	pH optimum	Temperature optimum (°C)	Stability	References
Thermotoga sp. strain FjSS3-B. 1	β-1,4-xylanase	5.5	80	Half-lives of 1.5, 0.13 and <0.03 h at 95 °C, 100 °C, and 105 °C, respectively	Simpson et al. (1991)
Bacillus stearothermophilus T-6	β -1,4-xylanase	6.5	75	Half-lives of about 14.5 and 0.33 h at 70 °C and 75 °C, respectively	Khasin et al. (1993)
B. flavothermus strain LB3A	β -1,4-xylanase	7.0	70	Half-life of 0.16 h at 80 °C	Sunna et al. (1997)
B. thermoleovorans strain K-3d	β -1,4-xylanase	7.0	70–80	Half-life of 0.3 h at 80 °C	Sunna et al. (1997)
Bacillus sp. strain SPS-0	β -1,4-xylanase	6.0	75	Retained 80 % activity for 4 h at 70 °C in presence of xylan, and 20 % without xylan	Bataillon et al. (2000)
B. licheniformis	β -1,4-xylanase I	7.0	70	Retained more than 90 % of activity for 1 h at 50 °C and 60 °C for X-I and X-II, respectively	Damiano et al. (2006)
	β -1,4-xylanase II	8.0–10.0	75		
B. subtilis	β -1,4-xylanase	8.0	60	Half-lives at 60 °C, 70 °C and 80 °C were 16.2, 9.6 and 2.8 h, respectively	Saleem et al. (2011)
Clostridium sp. TCW1	β -1,4-xylanase	6.0	75	NA	Lo et al. (2011)
Bacillus sp.	β -1,4-xylanase	6.5, 8.5 and 10.5	50	Retained full activity at 50 °C for more than 23 h	Sapre et al. (2005)
Enterobacter sp. MTCC	β -1,4-xylanase	9.0	100	Retained 85 % and 64 % of its activity for 18 h at 60 °C and 70 °C, respectively	Khandeparkar and Bhosle (2006)
Paenibacillus macerans IIPSP3	β -1,4-xylanase	4.5	60	Half-life of 6 h at 60 °C and 2 h at 90 °C	Dheeran et al. (2012)
E. coli expressing *Alicyclobacillus* sp. A4	β -1,4-xylanase	7.0	55	Half-life of 6.5, 0.28, and 0.05 h at 60 °C, 65 °C, and 70 °C, respectively	Bai et al. (2010b)
Geobacillus thermantarcticus	β -1,4-xylanase	5.6	80	Half life at 70 °C of 24 h and at 80 °C for 50 min	Lama et al. (2004)
	β-xylosidase	6.0	70	Retained full activity at 1 h at 60 °C	

3.4 Lignin Deconstruction

Lignin structure is a complex network originated by the oxidative coupling of three phenolic precursors i.e. coniferyl alcohol, sinapyl and *p*-coumaryl. Precursors form, respectively, guiacyl, syringyl and hydroxyphenyl phenylpropanoid subunits. Since lignin is a poor source of fermentable carbon, it has to be removed to allow efficient biomass processing as it effectively hinder the access to the cellulose and hemicellulose polymers. Several microorganisms have been studied that are able to depolymerize lignin by means of enzymes or chemical oxidative mechanisms. Fungi (e.g. basidiomycetes) could hydrolyse lignin by means of different kind of peroxidases including manganese (EC 1.11.1.13), lignin (EC 1.11.1.14), versatile (EC 1.11.1.16) and phenol oxidases (laccases) (EC 1.10.3.2). Also other enzymes are required for the oxidative degradation of lignin such as cellobiose dehydrogenase (EC 1.1.99.18), glyoxal oxidase (EC 1.2.3.5), aryl alcohol oxidase (EC 1.1.3.7) and cellobiose/quinone oxidoreductase (EC 1.1.5.1). These enzymes are able of producing H_2O_2 that destroy the lignin polymer in a non-specifical manner (Wong 2009).

3.5 Thermostable Enzymes: Their Overexpression for Lignocellulose Degradation

The majority of thermophilic bacteria, even under optimal growth conditions, do not generate significant amounts of enzymes: as an example at 60° *Geobacillus* spp. produce endoglucanase activities from 0.0113 U/ml C (Tai et al. 2004) up to 0.058 U/ml (Rastogi et al. 2009). Similarly at 55 °C *Geobacillus* sp. has been reported to produce 0.064 U/ml endoglucanase (Abdel-Fattah et al. 2007). Therefore in order to use such thermostable enzymes at industrial scale, it is necessary to over produce them by overexpression in suitable hosts, usually *E. coli*, but also species that belong to *Bacillus* genus such as *B. subtilis* and *B. megaterium*. In addition, *Pichia pastoris* has been described as good producer of recombinant cellulase and xylanase (Sriyapai et al. 2011; Lindenmuth and McDonald 2011). An endoglucanase produced by the thermophile *Geobacillus* sp. 70PC53 showing optimum activity at 65 °C, was efficiently expressed in *E. coli* (Ng et al. 2009). *E. coli* was also exploited for the expression of a xylanase gene from a novel thermophilic strain *Geobacillus* sp. MT-1: both the recombinant and wild-type xylanases showed their optimal activity at 70 °C, besides similar activity profiles in the temperature range from 20 °C to 90 °C (Wu et al. 2006). Interestingly also other endoglucanases like those from *Bacillus subtilis* strain I15 (Yang et al. 2010), *Bacillus* sp. (Afzal et al. 2010), *B. subtilis* (Li et al. 2000, 2009), *Thermoanaerobacter tengcongensis* MB4 (Liang et al. 2011) and finally *Fervidobacterium nodosum* Rt17-B1 (Wang et al. 2010) were successfully expressed in *E. coli*. The production of recombinant cellulases and xylanases has been implemented also by means of other hosts like *Bacillus megaterium*, *B. subtilis* and *Pichia pastoris* (Lindenmuth and McDonald 2011). The last host

showed to be more effective in the case of the thermostable xylanase from *Actinomadura* sp. S14: the recombinant enzyme obtained by expression in *P. pastoris* was more thermostable (50 % activity retained after 2 h of incubation at 80 °C) that obtained by expression in *E. coli* (30 % activity retained after 2 h of incubation at 80 °C) (Sriyapai et al. 2011). Such results suggested that expression in mesophilic systems could result in different post-translational modifications such as probably protein folding or glycosylation (Gao et al. 2012). Today, few data are still available regarding the expression of lignocellulose depolymerizing enzymes in thermophilic hosts.

3.6 Starch Hydrolysis by Extremophiles

Starch is the main carbohydrate energy reserve in many cereals like maize, wheat, rice, oat, potato, cassava, etc. that represent a major potential feedstock for the sustainable generation of energy (as gas or liquid biofuels) and chemicals (Kumar et al. 2007).

Starch degrading enzymes belong to the alpha-amylase superfamily: such group include numerous enzymes that show high similarity in primary sequence and act by means of a retaining catalytic mechanism (Sinnott 1990) thus releasing sugars in the alpha-configuration. The alpha-amylase superfamily is grouped in the glycoside hydrolase clan GH-H, and encompasses three sequence-related families of the GH13 family. The specificity variability of such enzymes is due to the specific consensus sequences, and to a variable number of domains, that in turn result in different hydrolytic or transferase activity, as well as in diverse substrate specificity.

The industrial process of starch's conversion to glucose is a two-step process, namely it requires a first energy-intensive step of liquefaction followed by the saccharification (Sivaramakrishnan et al. 2006). Liquefaction is carried out at high temperatures (above 100 °C) thus it requires the employ of highly thermostable enzymes. Thermophilic α-amylases produced by the hyperthermophilic archaea belonging to the genera *Methanocaldococcus*, *Pyrococcus, Sulfolobus* and *Thermococcus* (Kim and Peeples 2006; Van et al. 2007; Yang et al. 2004) have been widely investigated. These enzymes showed optimal activity typically around 90 °C besides possessing an impressive thermostability with activity being retained also after 4 h treatment at 120 °C. Most remarkable examples are the amylase activity produced by *Methanocaldococcus jannaschii* (T_{opt} 120 °C; T1/2 = 50 h at 100 °C), by *Pyrococcus furiosus* (T_{opt} 100 °C; T1/2 = 13 h at 98 °C) and by *Thermococcus kadakaraensis* (T_{opt} 90 °C; T1/2 = 24 h at 70 °C) (Antranikian and Egorova 2007). Also the moderately thermostable amylase produced by *Bacillus licheniformis* is extensively exploited by starch industry (Bravo Rodriguez et al. 2006). In addition, also glycoside hydrolases from halophilic species have been studied because of their tolerance to high salt and solvent concentrations (Antranikian and Egorova 2007). In particular also some haloarchaea's amylases have been described since they display significant activity in several solvents (like for example toluene,

benzene or chloroform), and in the presence of high salt concentrations (up to 4.5 M NaCl) or at high pH values (upto 10) (Antranikian and Egorova 2007). Also other hydrolytic enzymes are required to complete starch's degradation like for example glucoamylase (EC 3.2.1.3) and α-glucosidase (EC 3.2.1.20). Glucoamylase enzymes release α-1,4-linked D-glucose units from the non-reducing ends of the polymer: interesting thermostable and acidophilic glucoamylases are those produced by some archaeal species belonging to the genus *Sulfolobus* (Kim et al. 2004), to the genus *Picrophilus* (Topt 90 °C; T1/2 = 24 h at 90 °C; $pH_{opt} = 2$) and to the genus *Thermoplasma* (T_{opt} 75–90 °C; T1/2 = 24–40 h at 60–90 °C; $pH_{opt} = 2–5$) (Serour and Antranikian 2002). α-Glucosidase acts on α-1,4-bonds in dimers, trimers and tetramers of D-glucose: remarkable examples of such enzymes are those found in by species of the genera *Sulfolobus, Ferroplasma, Pyrococcus* and *Thermococcus* that produce highly thermotolerant enzymes (Chang et al. 2001; Piller et al. 1996; Schiraldi et al. 2000).

4 Advance Procedures for Vegetable Biomass Degradation

In the range of second-generation biofuels, also named advanced biofuels, the chemical characterization of de-starched polysaccharidic and oligosaccharidic materials, which are recovered from vegetable biomass feedstock, is essential for their exploitation in the production of value added-compounds. In particular, it is matter of lignocellulosic materials (as agro-residues and forestry biomass), which represent a precious low cost and abundant renewable biomass resource with considerable potential for the bioconversion to special bio-products (Kamm and Kamm 2004).

In this section, an overview on the chemical procedures for the characterization of compounds obtained from lignocellulosic materials will be presented. Particular attention will be devoted to the oligosaccharides and glyco-conjugated prepared by using extremophilic enzymes.

4.1 Monosaccharidic Composition of Hemicellulosic Extract

In order to know the nature of hemicelluloses extracted from vegetable biomass, it is essential to analyze their monomeric composition. Arabionose/xylose or glucuronic acids/xylose ratios are considered indicative of the degree of linearity o branching of hemicelluloses (Verbruggen et al. 1995).

The monomeric composition analysis of hemicelluloses extracted from vegetable biomass is essential to know their nature.

Usually, they are exhaustively hydrolyzed with sulfuric acid, in different experimental conditions. In the case of wheat bran extract, for example, hydrolysis was carried out in 1 M H_2SO_4 for 3 h at 100 °C; for eucalyptus cell wall extract and hemicellulose from barley straw for 2.5 h at 105 °C.

After neutralization, total monosaccharides (xylose, rhamnose, arabinose, mannose, glucose, galactose, glucuronic and galacturonic acids) were analyzed by HPAEC-PAD (High-performance anion-exchange chromatography with amperometric detection) systems in proper elution conditions (Aguedo et al. 2014; Lina et al. 2006).

In the case of sugarcane bagasse hemicelluloses, neutral monosaccharides were hydrolysed by 2 M trifluoroacetic acid (TFA) in 2 h at 120 °C and were converted in alditol peracetylated and analyzed by GC (Sun et al. 2004).

Uronic acids were analyzed in arabinoxylans from wheat bran by hydrolyzing with TFA 2 N for 4 h at 105 °C (Aguedo et al. 2014). Following the classical procedure for detecting the primary structure of saccharidic chains, monosaccharide composition is determined by GC-MS as paracetylated methyl glycosides; in the case of hemicellulose from rhizome of *Arundo donax*, methanolysis was performed at 1.25 M HCl/MeOH, 80 °C and overnight (Lama et al. 2014).

It is worth to note that sometimes, monosaccharidic composition of totally hydrolyzed xylan extracts could be qualitatively and quickly detected by Thin-layer chromatography (TLC) analyses in proper eluting conditions (*n*-BuOH/AcOH/H_2O 6:2:2 by vol., or EtOAc/ H_2O/ AcOH/2-propanol/HCOOH/, 25:15:10:5:1: by vol) (Lama et al. 2014).

4.2 Chromatographic Characterization of Oligosaccharides Produced from Hemicellulosic Agro-residues

High-Performance Anion-Exchange Chromatography (HPAEC) is the typical procedure employed for the chromatographic analyses of xylo-oligosaccharides obtained by enzymatic or chemical hydrolyses of xylan extracts. The identification of oligosaccharides and their concentrations values are recovered by using proper standards.

HPAEC-PAD analyses of arabinoxylo-oligosaccharides (AXOS) produced from wheat arabinoxylan (AX) by using Shearzyme (GH10 endo-1,4-β-D-xylanase) and two α-L-arabinofuranosidases (AXH-m and AXHd3).

HPAEC-PAD analysis of these reactions furnished chromatographic profiles in which not only the shortest xylo-oligosaccharides are eluted from the column in advance, but the corresponding elution order is followed within the collections of singly and doubly α-L-Arabinofuranosyl (Ara*f*) substituted arabinoxylan oligosaccharides (AXOS); furthermore the α-I-Ara*f* linkage position on the β-D-xylopyranosyl (Xyl*p*) unit also influences the elution order (Pastell et al. 2008).

Recently, an extracellular endoxylanase from *Bacillus halodurans* TSEV1, which resulted stable to heat and alkaline pHs, was cloned and expressed in *E. coli* and then exploited in the xylo-oligosaccharides production from several agro-residues. The monitoring of the saccharifications was performed by high pressure liquid chromatography (HPLC) equipped with a differential refractive index detector (Kumar and Satyanarayana 2014).

However, sometimes a qualitative monitoring by TLC analyses can result sufficient for the identification of xylo-oligosaccharides produced from agro-residues; the degradation study by TLC of the wheat bran hemicellulose (TLC system solvent: *n*-butanol:ethanol:water 5:3:2 by vol) for the oligosaccharides production by using a thermostable endoxylanase from the thermophilic bacterium *Geobacillus thermodenitrificans* TSAA1 is a useful example (Anand et al. 2013). Furthermore, a TLC investigation of enzymatic digestions using *A. donax* hemicelluloses extract and different thermopilic enzymatic preparations (cell-free extract of *T. neapolitana* and *Thermoanaeorbacterium thermostercoris*, extracellular suspension of *Geobacillus thermantarcticus*, commercial xylanases from *Thermomyces lanuginosus* and *Thermotoga maritima*) resulted fundamental for the individuation of unknown oligosaccharides into mixture reactions, which were later spectroscopically characterized (Lama et al. 2014).

4.3 Spectroscopic Investigation of Hemicellulosic Fractions Before and After Enzymatic Digestion: NMR, MS, FT-IR Analyses

1D and 2D NMR investigations of xylan polysaccharides from lignocellulosic sources are essential before any procedure designed to produce xylo-oligosaccharides or their value-added derivatives.

Nuclear magnetic resonance (NMR) analyses gives structural information about the nature, the configuration and relative content of monosaccharide essential to identifying the structure of several hemicellulosic extract. In most of cases, the homogeneity of polysaccharidic extracts corresponds to relatively simple spectra with well resolved signals.

In general, in protonic and carbon NMR spectra of arabino(glucurono)xylans and their oligosaccharidic derivatives it is possible to detect some diagnostic signals, of which intensity is variable, according to the branching of saccharidic chains (Jin et al. 2009).

In Table 19.5 we summarise peculiar values of chemical shifts belonging to hemicellulose structures.

In the case of hemicellulose isolated from rhizomes of *A. donax*, ^{13}C NMR spectrum in *d*-DMSO at 40 °C of hemicellulosic substrate showed intense and diagnostic signals attributed to xylan main chain as reported in Table 19.5. These signals represent, in general, the major signals of (1 → 4)-linked-β-xylan (Bendahou et al. 2007). Furthermore, in the ^{13}C NMR of *A. donax* hemicellulose extract, small signals were present corresponding to arabinose residues. These values confirmed the arabinoxylan structure of polysaccharidic extract and their intensity suggested a partially de-branched skeleton.

The analysis of ^{13}C and ^{1}H NMR spectra (in D_2O) of hemicellulose extracts at different temperature from sugarcane bagasse (Table 19.5) presented together with the signals of β-xylose chain and arabinose residues, also other signals of smaller intensity belonging to 4-O-methyl-α-D-glucuronic acid units (Bian et al. 2012).

Table 19.5 Diagnostic chemical shifts in ^{1}H and ^{13}C spectra of xylan from lignocellulosic sources

Compounds	Structural elements	NMR	
		^{1}H (ppm)	^{13}C (ppm)
(acetyl)arabinoglucoronoxylans (Hoffmann et al. 1992a, b)			
	Anomeric sites of β-D-xylose residues which are substituted at C-2 and C-3 (disubstituted), C-3 (monosubstituted), or unsubstituted	4.5:4.8	
	Anomeric sites of Ara*f* belonging to short side chains	~5.4	
	Sites 2:5 belonging to xylose and arabinose residues	3.1:4.3	
	Anomeric site of methyl glucuronic acid	5.3	
	Methyl signals of methyl glucuronic acid	1.8	
	Acetyl groups of xylan chain	2.6	
Arabinoxylan isolated from rhizomes of *A. donax* (Lama et al. 2014)			
	Anomeric site of xylan main chain		101.7
	Site 4 of 1,4-β-linked –xyloses		75.3
	Site 2,3,5 of xylose in 1,4 β-linked xyloses		73.9, 72.5, 63.2
	Anomeric site of arabinofuranose residues		107.1
	Site 2: 5 of α-L-Ara*f* linked to position 3 of xylose residue		87.3, 86.0, 80.2, 77.8, 61.8
Hemicellulose from sugarcane bagasse (Bian et al. 2012)			
	Site 2:5 of 1,4-β-linked xylose residues of main chain	4.34 (H-1), 3.96 (H-5 eq), 3.66 (H-4), 3.40 (H-3), 3.25 (H-5ax), 3.18 (H-2)	102.32, 75.89, 74.91, 73.28, 63.27
	Site 2:5 of α-L-Ara*f*		109.50, 86.46, 80.23, 78.37, 61.72
	Sites1: 6 and OCH_3-4 substitution of 4-O-methy-α-D-glucuronic acid residues		177.01 (C-6), 97.46 (C-1), 75.3 (C-2) 72.12 (C-3), 73.86 (C-5), 79.26 (C-4), 59.53 (OCH_3-4)
Hemicellulose from *Eucalyptus* cell walls (Li et al. 2015)	Sites 1,2,5 6 and OCH_3-4 substitution of 4-O-methy-α-D-glucuronic acid residues	5.14 (H-1), 4.15 (H-5), 3.49 (H-2), 3.35 (–OCH3)	

Analogously, in the protonic spectra of hemicellulose extract of *Eucalyptus* cell walls, recently investigated, although the signals of β-xylan backbone were predominant, minor signals were detected and attributed to 4-O-methyl-α-glucuronic acid residues (Li et al. 2015).

2D-NMR experiments, such as COSY (COrrelated SpectroscopY), TOCSY (TOtal Correlation SpectroscopY), HSQC (Heteronuclear Single Quantum Correlation), HSQC-EDITED (multiplicity-edited HSQC), HMBC (Heteronuclear Multiple-Bond Correlation Spectroscopy), NOESY (Nuclear Overhauser Spectroscopy) are necessary for the assignment of the protonic and carbon value signals of each position within each monosaccharidic residue; it allows to establish the primary structure of polysaccharidic chains and/or also the sequence of monosaccharide units into an oligosaccharide skeleton.

COSY, TOCSY and HSQC experiments are essential for the identification of spin systems; positioning of acidic residues or arabinoses, for example, along the xyloses backbone is possible by evaluating the long-range correlations C–H in HMBC experiments or dipolar coupling H–H in NOE experiments.

In fact, an exhaustive 2D-NMR spectroscopic investigation resulted essential to characterize the structure of a pentasaccharide (Fig. 19.2, compound 1) and a tetrasaccharide (Fig. 19.2, compound 2) obtained by enzymatic digestion of hemicellulose extract of *Arundo donax* rhizome and by using the commercial *Thermomyces lanuginosus* xylanase and the xylanase from cell free extract of *Thermoanaeorbacterium thermostercoris* (Lama et al. 2014).

The analyses of oligosaccharides released from xylans by mass spectrometry parallel and support the NMR investigations. Usually, Matrix-Assisted Laser Desorption/Ionisation Time-Of-Flight Mass Spectrometry (MALDI-TOF-MS) and

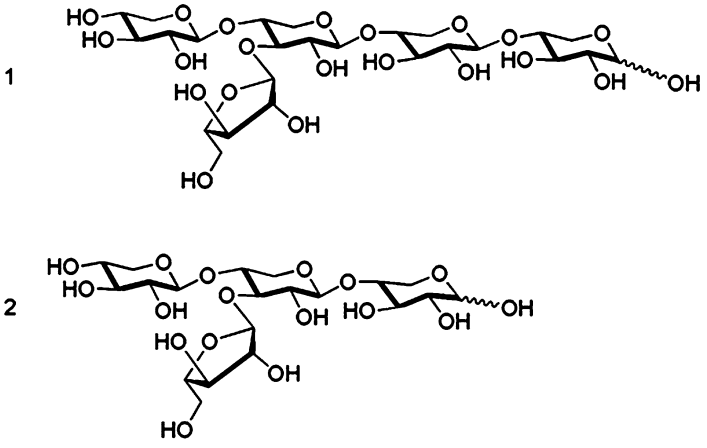

Fig. 19.2 The pentasaccharide β-D-Xylp-(1–4)-[α-L-Araf-(1–3)]-β- D-Xylp-(1–4)-β-D-Xylp-(1–4)-β-D-Xylp (1) from hydrolysis mixture using T. lanuginosus enzyme and β-D-Xylp-(1–4)-[α-L-Araf-(1–3)]- β-D-Xylp-(1–4)-D-Xylp (2) the tetrasaccharide, from the hydrolysis reaction mixture using crude extract of *T. thermostercoris*

electrospray mass spectrometry (ESI-MS) are the most common methods of analysis (Aachary and Prapulla 2011; Pastell et al. 2008).

In some cases, these analyses are performed in association with separation procedures such as size exclusion chromatography (SEC), high-performance anion-exchange chromatography (HPAEC) and reversed phase high-performance liquid chromatography (RPHPLC).

Lately, positive tandem mass spectrometry using ESI has been proposed for the characterization of underivatized or acetylated neutral and acidic XOS (Reis et al. 2005).

Furthermore, in the case of a mixture of xylo-oligosacchrides produced by enzymatic digestion for example, the MS studies furnish the xylo-oligosaccharide molecular weights, which in combination with information about the primary structure of initial xylan, allow possible structures to be proposed. On the other hand, certainty about the structures supposed can only be obtained by tandem mass spectrometry investigations (ESI-MS/MS) (Reis et al. 2005).

Infrared spectroscopy has been widely used for investigating the functional groups of polysaccharides (Li et al. 2015).

In IR spectra of hemicelluloses, stretching signal of C=O belonging to acetyl, uronic, and ferulic ester groups is recorded at about 1745 cm^{-1} together with a bending signal at 1249 cm^{-1} corresponding to –C–O– in ester groups.

Furthermore, in the carbonyl group stretching region, the signals at 1463, 1426, 1382, and 1318 cm^{-1} are attributed to –CH2 symmetric bending, CH and OH bending, OH in-plane bending, and –CH wagging, respectively (Sun et al. 1996). However, the presence of a band at 903 cm^{-1} is indicative of the dominant β-glycosidic linkages between the sugar units in the hemicellulosic extracts (Robert et al. 2005).

In the 1120–1000 cm^{-1} region, signals with multiple peaks is a distinctive characteristic of highly substituted arabinoxylans, and the arabinose substitution at C-3 site of xylose residues furnishes a band at 1173 cm^{-1} (Subba and Muralikrishna 2004). In general, the intensity of bands between 990 and 900 cm^{-1} is strongly dependent on the amount of Ara*f* units along the xylan backbone (Robert et al. 2005).

4.4 Transglycosylation Processes with Hemicelluloses Extracts

An alternative to exploit lignocellulosic biomass for the biofuel recovery, could be the syntheses of high value-added molecules. In this context, xylanases which naturally hydrolyze xylans into oligoxylosides and xylose monomers and xylosidases which hydrolyse oligoxylosides into xylose monosaccharides, result extremely useful in transglycosylation processes for the production xylo-conjugated.

As we know, alkyl substituted saccharides represent nonionic surfactants that are currently added in liquid and powder detergents, in pharmaceuticals preparations and in personal care commodities. Alkylxylosides, as example of the important group of the pentose-based surfactants, have very stimulating surfactant properties (Xu et al. 2012), and these molecules can be produced from lignocellulosic biomass

in order to decrease the cost of production by enzymatic synthesis in environment-friendly conditions.

In this context, a recombinant β-xylosidase from *Geobacillus thermodenidrificans* TSAA1 has been recently used for the production of alkyl xylosides, starting from aliphatic alcohols with short chains (C1-C5) as acceptors and a mixture of XOs, which were enzymatically produced from a de-starched wheat bran extract, as donors.

In proper reaction conditions and after 16 h of reaction between methanol and wheat bran hydrolysates, the corresponding band of methyl xyloside was well detected on TLC plate; furthermore in HPLC chromatogram, the presence of peaks corresponding to methyl xyololigosaccharides was recorded other than that of methyl xyloside (Jain et al. 2014).

More efficient transglycosylation reactions were carried out in presence of a xylanase from *Thermobacillus xylanilyticus* (Tx-xylanase) and a commercial xylanase (Novozymes NS-50030). In this case, birchwood or oat spelt xylans were used as donor or also alternatively xylo-oligosaccharides generated from hydrothermally pretreated and destarched wheat bran; the acceptors were aliphatic alcohols with growing chain length (from methanol to decanol) (Ochs et al. 2011).

Experiments with Tx-xylanase and NS-50030 xylanase were focused on the analysis of reaction parameters with the aim to induce their best transglycosylation capacity in presence of a partially water miscible alcohol (pentan-1-ol) and a non water-miscible alcohol (octan-1-ol) as acceptors.

Destarched wheat bran was subjected to autohydrolysis; after 1 h of reaction at 135 °C, the arabinoxylan present in the bran was solubilized. The oligosaccharidic solution after filtration and lyophilization was used as donor at a concentration of 2 % (w/v) of arabinoxylan equivalent. This donor was used in a 1 h reaction with octan-1-ol (20 %, v/v), tert-butanol (20 %, v/v) as co-solvent and Tx-xylanase (20 IU mL^{-1}).

Surprisingly, the highest total yield was recorded (222.2 mg g^{-1} arabinoxylan equivalent) when using the supernatant obtained from pretreated wheat bran, with respect to the process in which 2 % (w/v) birchwood xylan was used as substrate (146.6 mg g^{-1} arabinoxylan equivalent).

It was previously reported that 9-fluorenylmethyl glycosides showed interesting antiviral and antiproliferative on ovarian cancer cells activities (Tramice et al. 2008, 2009).

Recently, hemicellulolitic extract from *Arundo donax* rhizomes was employed as donor in transglycosylation processes with the aim to produce 9-fluorene methanol xylosides (Lama et al. 2014) by using *T. maritima* xylanase and xylanase/β-xylosidase activities from *T. neapolitana*, *T. thermostercoris* and *G. thermantarcticus* crude enzymatic homogenates. Methanol extracts of reactions performed with each enzymatic system were analyzed by MS spectroscopy, revealing the production of mono- and di-xylosylated derivatives of 9-fluorenyl methanol. Signals at *m/z* 351 and 483 $[M+Na]^+$ were recorded. Furthermore, in the reaction with *G. thermantarcticus* crude homogenate, the presence into the reaction medium of trixylosylated 9-fluorenyl methanol was secured by a signal in the reaction MS spectrum at m/z 651 $[M+Na]^+$.

5 Biotechnological Application of Extremophiles in the Valorization of Waste Biomass: Biofuels and Biohydrogen Production

One of the main applications of extremophilic microorganisms is their use for the conversion of waste derived from agriculture and forestry in order to obtain biofuels. In particular, lignocellulosic agricultural and forestry wastes, that represent the starting materials for second-generation biofuels, are no-food materials that aren't in competition with food, resulting irrelevant to the increase of the food prices. In view of this, the search of new thermophilic bacteria with interesting thermostable enzymes is a critical point to overlap the best utilization of these wastes biomass. In order to access to cellulose and hemicellulose, lignocellulosic start material needs of several pretreatments, usually a thermomechanically pretreatment to open the lignin sheath, following by chemical treatment as described in Table 19.2. After that, enzymatic hydrolysis are requested, usually cellulases, hemicellulases and xylanase in order to release fermentable sugars. These enzymatic hydrolysis are usually performed at <50 °C and in that condition the rate of hydrolysis appears slow, the enzyme concentration have to be high as well as the risks related to microbial contamination and in general the yields of fermentable sugars appear low. In this scenario the use of thermophilic microorganisms and their thermostable enzymes is very useful to overcome the limitations of lignocellulosic biomass conversion and to improve the whole process (Bhalla et al. 2013). In fact, the employ of these thermozymes, since they usually require working temperature ranging from 50 °C to 80 °C, implicates a shorter hydrolysis times, an increase of reagent and product solubility, a higher rate of hydrolysis, a lower microbial contamination and a facilitation in volatile product recovery (Zhang et al. 2011; Liang et al. 2011).

Since thermozymes possess a great adaptability to work in different pH values, this feature makes these enzymes ideal actors for lignocellulose conversion by extremophilic microorganisms, overall in the case in which acid or alkaline chemical pretreatments are required. The lignocellulosic biomass conversion could be achieved by using the thermozymes able to make an efficient lignocellulose deconstruction: the use of a mix of thermostable enzymes in combination with ethanologenic thermophiles, increase the yield of hydrolysis and in this condition the fermentation could be carried out at higher temperatures using just one fermentator (Thermophilic Simultaneous Saccharification and Fermentation -SSF) (Podkaminer et al. 2011; Shaw et al. 2008). This bioprocess was used for example by Shaw et al. (2008) that employed the thermophile *Thermoanaerobacterium saccharolyticum* strain ALK2 obtaining a 2.5-fold reduction in terms of quantity of enzymes required to achieve equivalent hydrolysis for SSF process at 50 °C compared to SSF performed by *S. cerevisiae* that worked at 37 °C. In literature there are several examples of fermentative thermophilic bacteria (fermentation temperature ranging from 45 °C to 70 °C) that are able to use xylose, glucose, cellobiose, galactose, mannose but also mixed sugars that derive from biological decomposition of lignocellulosic biomass such as corn stover, in order to obtain ethanol; in this case the ethanol production lies in the interval between 0.16 and 0.47 g of ethanol/g of saccharides

or cellulose reduced even if other products could be achieved such as acetate, lactate, pyruvate and succinate (Cai et al. 2011; Cripps et al. 2009; Georgieva et al. 2008). However, the best results have been obtained using Consolidated Bioprocessing (CBP) that could involve saccharolytic fermentative thermophiles using the pre-treated lignocellulose biomass (Olson et al. 2012). The most challenging task associated with CBP is the selection of appropriate microorganisms or a microbial community (*consortium*) that can digest the lignocellulosic materials producing ethanol. For this aim the raw material should not required any pre-treatments such as chemical, physical or enzymatic actions, and just a reduction of particle size should be enough (Paulová et al. 2014). In CBP the production of hydrolytic enzymes, the degradation of lignocellulosic waste and the use of the monosaccharides released by fermentation occurred efficiently in a single reactor, led up to a cost reduction of a fourfold in comparison with SSCF (simultaneous saccharification and co-fermentation) (Lynd et al. 2005). There are two categories of microorganisms used in the CBP: category I or cellulase producers and category II or ethanol producers. At first category belong for example *Clostridium thermocellum, Geobacillus thermoglucosidans, Thermoanaerobacter mathranii* and cellulolytic fungi (*Tricoderma reesei*, *Paecilomyces variotii*) (Paulová et al. 2014). Category II CBP producers include ethanol producers microorganisms conveniently engineered such as *Saccharomyces cerevisiae*, *Kluyveromyces marxianus* and *Zymomonas mobilis* (Paulová et al. 2014). These saccharolytic thermophiles have been improved by genetic engineering providing constructed microorganisms containing a set of interesting hydrolytic enzymes. This is the example of *Clostridium thermocellum*, able to convert plant biomass into ethanol by CBP. In order to increase ethanol tolerance and to obtain a high yield in ethanol production, it has been mutagenised at level of alcohol dehydrogenase as reported by Brown et al. (2011). The possibilities to use a microbial consortium combining both category CBP producers (I and II) have been also reported. Ethanol production has been also studied using a microbial community consisting of *S. cerevisiae*, *T. reesei* and *Scheffersomyces stipitis*. It was observed a maximum ethanol concentration of 9.8 g/l^{-1} using an acid pretreated wheat straw with a theoretical ethanol yield of 69 % (the theoretical ethanol yield of 0.51 g/g^{-1} is taken as 100 % and represent the yield achieved by *S. cerevisiae* during ethanol fermentation of glucose). Other approaches to decrease the costs linked to lignocellulose conversion to biofuels were the use of immobilized biocatalysts, such as cellulolytic enzymes and microorganisms. Apparently, the microbial cell immobilization gave better results compared with cellulase immobilization in that a decrease in enzymatic activities occurred after enzyme immobilization (Paulová et al. 2014).

Moreover, several efficient hydrogen cell factories have been developed using anaerobic thermophiles able also to produce thermostable cellulolytic and xylanolytic enzymes. *Caldicellulosiruptor saccharolyticus* represented a suitable candidate for hydrogen production using lignocellulosic biomass such as sugarcane bagasse and sweet sorghum (VanFossen et al. 2009). The efficiently of the whole process of bioconversion of lignocellulosic biomass into biofuels reached the maximum expression when *Caldicellulosiruptor bescii* DSM 6725 was used in a single

reactor. In fact, this microorganism, with an optimum growth temperature of 80 °C, was shown to convert untreated biomass such as bermuda grass and switch grass into hydrogen (Yang et al. 2009). In this case, the high growth temperature of *C. bescii* helped the recovery of volatile products (ethanol) from bioreactor (Chang and Yao 2011).

6 Conclusion

The active research of new thermophilic bacteria with interesting thermostable enzymes able to convert lignocellulosic materials could improve the productivity of this process and the energy consumption. In addition to classic approaches, the knowledge of draft genome sequence of thermophilic microorganisms able to degrade lignocellulosic substrates, revealing genes encoding cellulose or xylan-degrading enzymes, could help into exploration for the biofuel production processes. In fact, even using all the information concerning both the waste composition and the microorganism pathways, not all attempts in waste utilization for microbial growth result be successfully. Indeed, the last tendencies recognize the helpful contribute coming from the information contained into microbial genome. The knowledge of whole genome could permit a preliminary screening of the suitable extremophilic microorganisms to be used for the waste biomass utilization (Studholme 2015).

In the world energy scenario, the absolute certainty consists of the quickly fossil fuel depletion that, coupled with the boost population, has imposed the search, at every level, for an efficient and valuable alternative. Different renewable energy are currently being examined. In this chapter, the vegetable waste biomass are consider a source of added value products. As extensively argued, they are produced in huge amount and need to be correctly disposed of during expansive appropriate practices. The development of procedures aiming to reuse and valorize useful waste materials. Therefore, the innovative idea for all industrial and agricultural residues is to tend to a zero-waste emission. In the Fig. 19.3 is reported the degradation of waste biomass (rhizomes of *Arundo donax* L.), utilizing thermophilic microorganisms, to obtain polymeric components (starch, lignin, cellulose, xylan) and their monosaccharides and oligosaccharides useful for biofuel production. In addition, vegetable biomass could be exploited as organic and energy supporting materials for thermophiles growths and their enzyme hyper-production (Finore et al. 2014; Lama et al. 2014). In this integrated system the residues of each treatment represent the starting materials for a further transformation with the aim of zero-emission processes.

In this contest the emergent Synthetic Biology offers great potential to overcome the challenges associated with lignocellulose conversion. Synthetic biology is considered a recent discipline with the interesting properties to construct new biological systems (Keasling 2008) and in the field of renewable sources, it represents a cheaper way to get biofuels and/or chemicals (Nieves et al. 2015). This discipline is based on the design and the build of new biological components ranging from enzymes, metabolic pathways, genetic circuits up to the whole cells, in order to assembly an inte-

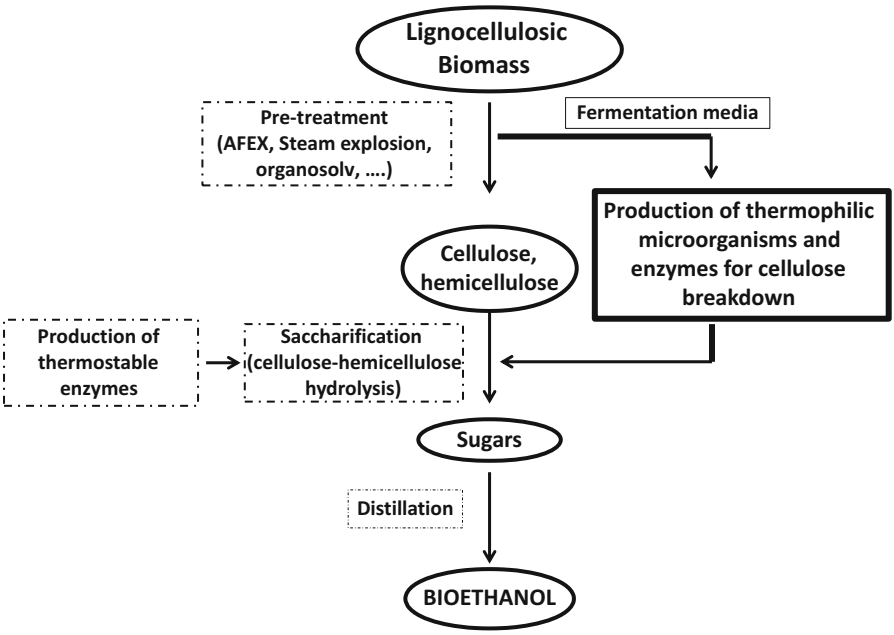

Fig. 19.3 Scheme of integrated treatment of lignocellulosic biomass from rhizomes of *Arundo donax* L

grated systems that can solve specific problem. In the case of lignocellulose conversion for example, the main goals for microbial catalysts are represented by a good utilization of xylose and resistance to furan aldehydes (Sandoval et al. 2012; Wang et al. 2013). Next step will be the construction of a cell able to provide a lignocellulosic conversion thank to the new developed genetic techniques taking advantages from the genome sequence of lignocellulose-degrading thermophiles.

Conflict of Interest Annarita Poli, Ilaria Finore, Annabella Tramice, Paola Di Donato, Barbara Nicolaus, and Licia Lama declare that they have no conflict of interest.

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