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"New multifunctional degrading microorganisms and optimization of renewable source conversion in biofuels of second and third generation"

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Chapter 1 Introduction

1.1 Bioenergy

Fossil fuels have remained the major energy source since last few centuries; however, the rapid depletion of their reservoirs has been brought under spotlight only recently (Khattak et al., 2014a). Besides energy concerns, serious environmental complications have originated from fossil fuel depletion (Khattak et al., 2014b). These concerns have been intensely debated on several platforms and demands have been put forward for strenuous efforts for an urgent resolution. In this scenario, renewable energy technology has been proposed as the only ultimate solution to overcome issues associated with fossil fuels (Johansson et al., 1993; Wang et al., 2013; Edwards and Doran-Peterson 2012). So, the need for climate change adaptation and the growing concerns over energy security, but also to encourage the diversification of the production of oil companies so to increase their profits, are the main drivers behind the policies of many countries of the Organization for Economic Cooperation and Development (OECD) and of the United States of America that encourage the growth of renewable energy. Today, renewable energy contributes 13% of the total global energy consumption, in which bioenergy accounts for approximately 10% (IEA, 2013). Bioenergy refers to the energy content in solid, liquid and gaseous products derived from biological raw materials (biomass) (IEA, 2010). Therefore bioenergy is the chemical energy stored in organic material, which can be directly converted into useful energy sources by biological, mechanical or thermochemical processes (Bessou et al., 2011). The term bioenergy refers to the renewable energy from biological sources that can be used for heating, electricity and fuel as well as their by-products. Regarding modern bioenergy, bioethanol, biodiesel and biogas are the three main bioenergetic products (Yuan et al., 2008). Bioenergy has been promoted as a fossil energy substitute to

reduce greenhouse gas (GHG) emissions and the dependence on energy imports (Haberl et al., 2010). The term "biofuel" is used to describe the intermediate products in the biomass energy chain that are obtained from raw materials, through their pretreatment or conditioning by physical, thermochemical, chemical or biological processes. Depending on their nature, it is worth distinguishing among solid, liquid and gaseous biofuels (López-Bellido et al., 2014). Generally, biofuels used in the transportation sector are currently represented mainly by liquid bioethanol and biodiesel. In contrast, biogas that is obtained by the anaerobic treatment of organic waste is currently the main gaseous biofuel (Antizar-Ladislao and Turrión-Gómez, 2008). Bioenergy offers the advantage of generally low-level investments. In addition, the diversity of raw materials and the transformation processes offer a broad range of possibilities that can be adapted to different geographical regions, environments and needs (Bessou et al., 2011). There are five crucial aspects of bioenergy production: (i)energy security, (ii) food security and rural development, (iii) environmental impact, (iv) technical innovation, and (v) political and institutional roles (Winslow and Ortiz, 2010). However, the main factor that will limit biofuel development will be land availability (Bessou et al., 2011) if we want to avoid the reduction of food production.

1.2 First-generation biofuels

Biofuel production has been increasing rapidly in the last decade and currently supplies 3.4% of global road transport fuel requirements, with a considerable share in Brazil (21%), and an increasing share in the United State (US; 4%) and the European Union (EU; 3%) (IEA, 2013). Around 40 million gross ha (2.5% of global cropland) (FAOSTAT, 2011) are used for bioenergy crops, mainly for biofuel production as bioethanol and biodiesel, and biogas. The traditional feedstocks for first generation biofuels can be categorized as starch and sugar crops (for bioethanol), and oil seeds (for biodiesel). In particular, the first

generation bioethanol is produced by fermentation of crops high in sugar (e.g. sugarcane, sugar beet, and sweet sorghum) or by a series of hydrolysis/fermentation steps for starchy crops (e.g. corn, wheat, and cassava). Biodiesel can be produced by combining oil extracted from seeds and oil-rich nuts with an alcohol through a chemical process known as transesterification (Balat and Balat, 2010). The most common oil crops are rapeseed, soybean, palm and coconut oil. Recent analyses of energy crops have shown that current practices to convert food-product carbohydrates or plant oils into ethanol and biodiesel (i.e. first generation bioenergy technologies) are inefficient and have only limited capabilities to reduce emissions (Bessou et al., 2011). This first generation of biofuel production also competes directly with food production for the most fertile lands (Jaradat, 2010; Markevi˘cius et al., 2010; Bessou et al., 2011; López-Bellido and López-Bellido, 2012). The cost and sustainability of crops to produce first generation biofuels, with the exception of sugarcane, have been criticized as expensive sources for satisfying environmental objectives and supplying alternative energy. Similarly, the production costs for biodiesel remain a major obstacle towards its commercial large-scale use, mainly due to the high costs of vegetable oils. Globally, the cost of the raw material represents the largest part of the final price of any first generation biofuel, where as processing and transportation costs represent most of the remainder (Jaradat, 2010). For ethanol production, the cost of the raw material comprises 50–70% of the production costs, where as for biodiesel, the raw materials can represent up to 60–80% of the final cost (Langeveldet et al., 2010). These limitations could be partially overcome by second-generation bioenergy technologies based on the conversion of lignocellulosic plant materials from fast growing tree and perennial grass species, as well as residues from agriculture and forestry (Jaradat, 2010) and industry.

1.3 Second-generation biofuels

It is expected that both second-generation biofuel production technology and energy crops used will be more efficient than first-generation biofuel and energy crops associated. These crops will supply energy derived from cellulose and non-oxygenated fuels from pure hydrocarbons, such as biomass-to-liquid fuels. Biofuels produced biochemical and thermochemical from lignocellulosic crops have higher energy content (GJ $ha^{-1}yr^{-1}$) than many energy crops used to produce first-generation biofuels (López-Bellido et al., 2014). These crops may avoid many environmental concerns and offer greater reductions in the potential costs in the long-term (Jaradat, 2010). Nonetheless, there are still technical limitations for crop growth and fuel production from second-generation technology. The net GHG emissions from cellulosic ethanol or biomass-to-liquid fuels are substantially lower than ethanol produced from crops used to produce first-generation biofuels, such as corn. The potential crops that could serve as a raw material source for cellulosic ethanol include the following: short rotation woody crops, rapidly growing trees and perennial herbaceous species. Because the entire crop will be used, an ideal plant species will rapidly produce large amounts of biomass. Perennial species can reduce production costs, carbon depletion in the soil and erosion while simultaneously increasing biomass performance. In addition, a number of plant species are adapted to poor and degraded soils, which in theory would provide opportunities for land rehabilitation and avoid competition for land with food crops (Payne, 2010). Included among the crops to produce second-generation biofuels are perennial grasses, such as miscanthus, switchgrass, reed canary, giant reed and alfalfa and short rotation wood crops such as species of poplar, willow and eucalyptus (Dang et al., 2014). Herbaceous oil seed crops, new woody plant species for oil seed production are under investigation to assess their suitability as biodiesel production crops. Jatropha has been seen as an ideal crop for cheap biodiesel

production. Some other species with biodiesel potential include pongamia, mahua, castor and linseed (Koh and Ghazi, 2011). The use non edible oil as raw materials to produce biodiesel by second-generation technology eliminates competition between foods for human consumption and fodder (Ahmad et al., 2011). Non edible oil conversion to biodiesel is comparable to the conversion of edible oils in terms of production and quality. Moreover, less land is required to cultivate these crops, and a mix of crops can be used to produce useful by products, which can be used in other chemical processes or can be burned for heating or energy. The second-generation technologies are based on the use of energy crops that are grown specifically using low external inputs and conversion methods that result in a high net energy efficiency (output/input). The research seeks to develop enzymatic processes for the cost-efficient decomposition of cellulose into its monosaccharide components, which can then be fermented to produce ethanol. The development of key enzymes, pretreatments and fermentation processes is needed to ensure more efficient (cost versus energy) processing (Antizar-Ladislao and Turrión-Gómez, 2008). The commercialization of second-generation biofuels will also require developing of new infrastructure to collect, transport, store and refine biomass. In summary, second-generation biofuels generated from lignocellulosic raw materials are unique in terms of their repercussions on agricultural and food security. Compared to the agricultural crops currently in use for first-generation technologies, second-generation biofuels use a larger variety of lignocellulosic raw materials originated from different perennial crops with, but also from agriculture and forestry residues, municipal and industrial wastes. The effect of this fuel on basic markets, land use and environmental changes as well as their influence on future production and conversion technologies will also vary (FAO, 2008).

Recently, the search for cheaper feedstocks for the production of biodiesel has turned attention to various forms of waste alternative raw materials, including waste animal fats, waste cooking oil and lipids in sewage sludge.

Sewage sludge is a waste formed during treatment of wastewater (Olkiewicza et al., 2012). Wastewater treatment plants produce each year higher quantities of sludge due to the increase of urbanization and industrialization, it can be considered as a continuous process in the future. It was estimated the total sludge generation in the EU in 2020 will be approximately 12.9 million tones of dry sludge per year, compared with 10 million tones in 2005, an overall increase by about 30% (European Comission, 2010). Additionally, the sludge could comprise up to 30 wt% of a lipid fraction (Dufreche et al., 2007), which could be converted into biodiesel.

1.4 Lignocellulosic biomass composition

Lignocellulose consists of lignin, carbohydrates such as cellulose and hemicellulose, pectin, proteins, ash, salt and minerals. (Van Dyk and Pletschke, 2012). Cellulose and hemicelluloses make up approximately 70% of the entire biomass and are tightly linked to the lignin component through covalent and hydrogenic bonds that make the structure highly robust and resistant to any treatment (Knauf and Moniruzzaman, 2004; Mielenz, 2001; Edye and Doherty 2008). A representative diagrammatic framework of lignocellulosic biomass is illustrated in Figure 1.

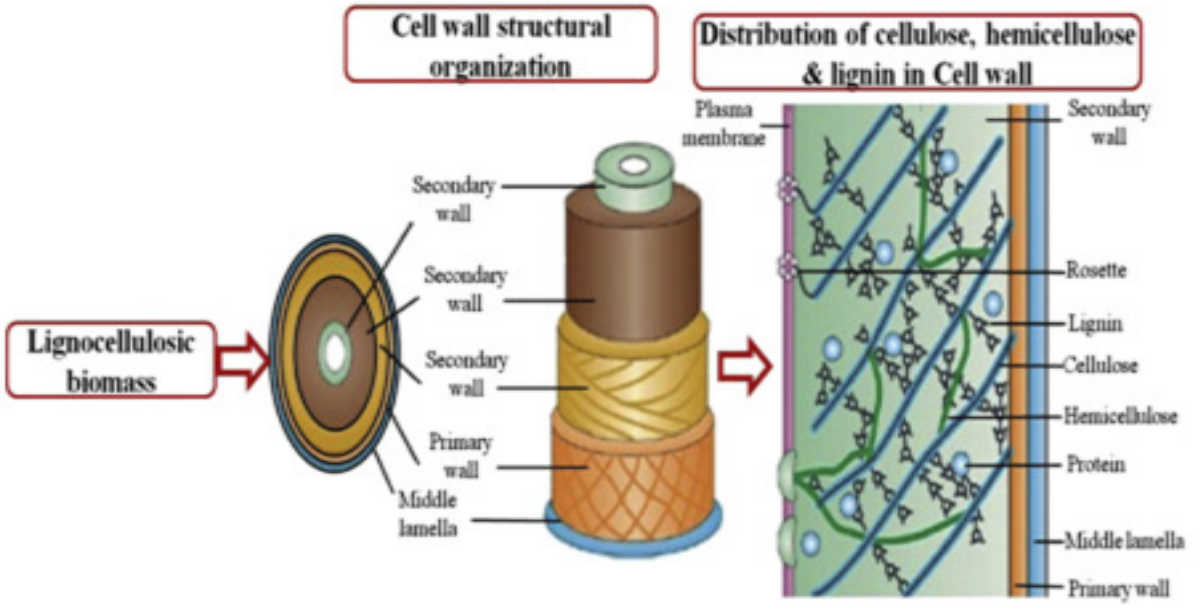

Figure 1. Diagrammatic illustration of the framework of lignocelluloses (Menon and Rao, 2012).

1.4.1 **Cellulose**

Cellulose is a structural linear component of a plant's cell wall consisting of a long-chain of glucose monomers linked β-(1-4)-glycosidic bonds that can reach several thousand glucose units in length. The extensive hydrogen linkages among molecules lead to a crystalline and strong matrix structure (Ebringerova et al., 2005). This cross-linkage of numerous hydroxyl groups constitutes the microfibrils that give the molecule more strength and compactness. The molecules of individual microfibrils in the crystalline cellulose are packed so tightly that not only enzymes but even small molecules like water cannot enter the complex framework. Some parts of the microfibrils have a less ordered. No crystalline structure referred to as amorphous region (Arantes and Saddler 2010). The high molecular weight and ordered tertiary structure make natural cellulose insoluble in water. The crystalline regions of cellulose are more resistant to biodegradation than the amorphous parts. The cellulose is further embedded in a matrix of hemicellulose, pectin and lignin (Eriksson and Bermek, 2009; Raven et al., 1999). Lignin and hemicellulose are found in the spaces

between cellulose microfibrils in primary and secondary cell walls, as well as the middle lamellae (Eriksson and Bermek, 2009).

1.4.2 **Hemicellulose**

Hemicellulose is an amorphous and variable structure formed of heteropolymers including hexoses (D-glucose, D-galactose and D-mannose) as well as pentose (D-xylose and L-arabinose) and may contain sugar acids (uronic acids) namely, D-glucuronic, D-galacturonic and methylgalacturonic acids. Its backbone chain is primarily composed of xylan β-(1-4)-linkages that include D-xylose (nearly 90%) and L-arabinose (approximately 10%) (Girio et al., 2010). Branch frequencies vary depending on the nature and the source of feedstocks. In plants, the xylan forms an overlying layer through hydrogen bonding with the cellulose, while covalently linked with lignin which forms an outside sheath to protect the plant (Beg et al., 2001). The hemicelluloses of softwood are typically glucomannans while hardwood hemicellulose is more frequently composed of xylans (McMillan JD, 1993). Although the most abundant component in hemicellulose, xylan composition still varies in each feedstock. Because of the diversity of its sugars, hemicellulose requires a wide range of enzymes to be completely hydrolyzed into free monomers.

1.4.3 **Lignin**

Lignin is an aromatic and rigid biopolymer with a molecular weight of 10,000 Da bonded via covalent bonds to xylans (hemicelluloses portion) conferring rigidity and high level of compactness to the plant cell wall (Mielenz, 2001). Lignin is composed of three phenolic monomers of phenyl propionic alcohol namely, coumaryl, coniferyl and sinapyl alcohol. Lignification is achieved by cross-linking reactions of a monomer with the growing polymer or by polymer–polymer coupling via radicals generated by oxidase enzymes (Wong, 2009). Lignin and other aromatics link with, and physically mask, the polysaccharides

present in lignocellulose, protecting them from hydrolytic activities. However, due to its chemical composition and complex structure, lignin is characterized by a high recalcitrance, thus requiring very harsh conditions such as chemical or chemical physical treatments. Previously, little interest has been given to lignin chemistry potential on hydrolysis. However, lignin components are gaining importance because of their dilution effect on the process once solids are added to a fed batch hydrolytic or fermentation bioreactor in addition to their structure and concentration effects that would affect potential hydrolysis (Ladisch, et al., 2010) For instance, the adsorption of lignin to cellulases requires a higher enzyme loading because this binding generates a non-productive enzyme attachment and limits the accessibility of cellulose to cellulase (Chen et al., 2006). Furthermore, phenolic groups are formed from the degradation of lignin. These components substantially deactivate cellulolytic enzymes and hence influence enzymatic hydrolysis. This negative impact caused by lignin has led to interest in lowering the lignin negative effect.

1.4.4 **Pectin**

Pectin is the versatile and structurally homo polysaccharide contained in the middle lamella and primary cell walls of terrestrial plants, (Gupta et al. 2008). Pectin, a hydrophilic polysaccharide, forms part of the intracellular network and imparts pliability to the cell through its association with water (Raven et al., 1999). Pectin containing long galacturonic acid chains with residues of carboxyl groups and with varying degree of methylesters (Voragen et al. 2009). Other components of pectin include rhamnose, arabinose, galactose, fucose and apiose, (Willats et al., 2006). Pectin has found widespread commercial use, especially in the textile industry and in the food industry as a thickener. Despite these applications, pectins are similar to cellulose and hemicelluloses, common waste materials that can be converted to soluble sugars, ethanol, and biogas (Doran JB, 2000).

1.5 Bioconversion of lignocellulosic biomasses

Bioconversion of lignocellulosic material to useful products normally requires multi-step processes that include: pretreatment, hydrolysis and fermentation. The enzymes play a key role in the bioconversion process. In particular, in pretreatment and hydrolysis phase. These enzymes are produced by several microorganisms, commonly by bacteria and fungi. These microorganisms can be aerobic or anaerobic, mesophilic or thermophilic (Balat, 2011). In the fermentation phase much important is to add microorganisms capable to ferment to the resulting monosaccharides generated during hydrolysis to bioethanol.

1.5.1 Enzymes required to degrade lignocellulose

A large variety of enzymes with different specificities are required to degrade all components of lignocellulose. Table 1 gives a brief overview of the types of enzymes that are required to degrade complex lignocellulose substrates. However, there are indications that many other proteins may contribute to lignocelluloses degradation in ways that are not yet clearly understood (Van Dyk and Pletschke, 2012).

Table 1. Some of the main enzymes required to degrade lignocellulose to monomers.

Substrate	Types of Enzyme
Cellulose	Cellobiohydrolase, endoglucanase, β-glucosidase
Hemicellulose	Endo-xylanase, acetyl xylan esterase, β-xylosidase, endomannanase, β-mannosidase, α-L-arabinofuranosidase, α-glucuronidase, ferulic acid esterase, α-galactosidase, p-coumaric acid esterase
Lignin	Laccase, Manganese peroxidase, Lignin peroxidase
Pectin	Pectin methyl esterase, pectate lyase, polygalacturonase, rhamnogalacturonan lyase

It is generally accepted that three types of enzymes are required to hydrolyse cellulose into glucose monomers, namely exo-1,4-β- glucanases, EC 3.2.1.91 and EC 3.2.1.176 (cellobiohydrolase), endo- 1,4-β-glucanases, EC 3.2.1.4 and β-

glucosidases, EC 3.2.1.21 (cellobiases) (Van Dyk and Pletschke, 2012). Cellobiohydrolases attack the ends of cellulose chains while endo-glucanases cleave cellulose chains in the middle and reduce the degree of polymerisation. Cellobiohydrolases may have a preference for attacking the reducing or non-reducing ends of cellulose chains (Teeri, 1997). Cellobiase hydrolyze cellobiose to glucose in order to eliminate cellobiose inhibition. Cellobiase complete the hydrolysis process by catalyzing the hydrolysis of cellobiose to glucose (Balat, 2011).

Hemicellulose has a more varied composition compared to cellulose and therefore requires a large number of enzymes to hydrolyze effectively. Enzymes degrading hemicellulose can be divided into depolymerising enzymes which cleave the backbone and enzymes that remove substituents which may pose steric hindrances to the depolymerising enzymes. The core enzymes for degradation of xylan to monomers are the endo-xylanases, which cleave the xylan backbone into shorter oligosaccharides and β-xylosidase, which cleaves short xylo-oligosaccharides into xylose. Similarly, the core enzymes for degradation of mannan are the endo-mannanase and β-mannosidase. However, xylans and mannans generally have several different substituents linked to the main backbone, such as arabinose, acetyl, galactose and glucose which require a host of ancillary enzymes to remove from the backbone to give access to the core enzymes to degrade the backbone. Some of these ancillary enzymes are α-L-arabinofuranosidases, α-glucuronidase, ferulic acid esterase, α-galactosidase, feruroyl esterase, acetyl xylan esterase and acetyl mannan esterase. Ferulic acid esterases specifically cleave the linkages between hemicellulose and lignin. α-L-arabinofuranosidases also have different specificities with some cleaving 1,2 linkages or 1,3 linkages, while others are able to cleave doubly substituted arabinose residues from arabinoxylan (Meyer et al., 2009).

Several enzymes are involved in degrading pectin. The pectin methyl esterases remove methyl groups from pectin to give access to depolymerising enzymes

such as polygalacturonase, pectin lyase or pectate lyase (Jayani et al., 2005). Additional rhamnogalacturonases and rhamnogalacturonan lyases are responsible for degradation of the "hairy regions" in the pectin (Michaud et al., 2003), while pectin acetylesterases remove acetyl groups from acetylated homogalacturonan and rhamnogalacturonan (Bonnin et al., 2008).

The major groups of ligninolytic enzymes include lignin peroxidases, manganese peroxidases, versatile peroxidases, and laccases. The peroxidases are heme-containing enzymes with catalytic cycles that involve the activation by H_2O_2 and substrate reduction of compound I and compound II intermediates. Lignin peroxidases have the unique ability to catalyze oxidative cleavage of C-C bonds and ether (C-O-C) bonds in non-phenolic aromatic substrates of high redox potential. Manganese peroxidases oxidize Mn(II) to Mn(III), which facilitates the degradation of phenolic compounds or, in turn, oxidizes a second mediator for the breakdown of non-phenolic compounds. Versatile peroxidases are hybrids of lignin peroxidase and manganese peroxidase with a bi-functional characteristic. Laccases are multi-copper-containing proteins that catalyze the oxidation of phenolic substrates with concomitant reduction of molecular oxygen to water (Wong, 2009).

1.5.2 **Microorganisms related to bioethanol fermentation**

Microorganisms for bioethanol fermentation can best be described in terms of their performance parameters and other requirements such as compatibility with existing products, processes and equipment. The performance parameters of fermentation are: temperature range, pH range, alcohol tolerance, growth rate, productivity, osmotic tolerance, specificity, yield, genetic stability, and inhibitor tolerance. The characteristics required for an industrially suitable microorganism are summarized in Table 2 (Dien et al., 2003).

Table 2. Important traits for bioethanol fermentation process.

Trait	**Requirement**
Bioethanol yield	>90% of theoretical*
Bioethanol tolerance	>40 g l^{-1}
Bioethanol productivity	>1 g l^{-1} h^{-1}
Robust grower and simple growth requirements	Inexpensive medium formulation
Able to grow in undiluted hydrolysates	Resistance to inhibitors
Culture growth conditions retard contaminants	Acidic pH or higher temperatures

*$\%\textbf{Theoretical ethanol yield} = \frac{[Ethanol1]-[Ethanol0]}{0.51\,(1.11\,f\,[BIOMASS]} * 100$ **[** ***Ethanol*1]** Ethanol concentration at the end of the fermentation (g L^{-1}) **[** ***Ethanol*0]** Ethanol concentration at the beginning of the fermentation (g L^{-1}) [***Biomass***] Initial solids concentration (g L^{-1}) f Cellulose fraction in dry biomass (g/g) **1.11** Conversion factor for glucan to glucose **0.51** Conversion factor for glucose to ethanol

Traditionally, *Saccharomyces* (S.) *cerevisiae* and *Zymomonas* (Z.) *mobilis* have been used for bioethanol fermentation. They are capable of efficiently fermenting glucose into bioethanol, but are unable to ferment xylose (Keshwani and Cheng 2009). However, some natural ethanologenic yeast species such as *Pichia* (P.) *stipitis, Pachysolen tannophilus, Kluyveromyces marxianus* and *Candida* (C.) *shehatae* appeared to have promise in replacing *S. cerevisiae* in lignocellulosic-based ethanol fermentation (Keshwani and Chen, 2009). Nevertheless, these wild yeasts still require further development to survive bioethanol fermentation conditions and yield an optimal ethanol concentration. The competitive exclusion as well as repression catabolism (competitive inhibition of hexose and pentose sugar transport) among these microorganisms in the bioethanol ecosystem makes addition of a selective agent to not be of particular value for improving yield performance (Olofsson et al, 2008). Natural xylose-fermenting yeasts, such as

P. stipitis, C. shehatae, and C. parapsilosis, can metabolize xylose via the action of xylose reductase (XR) to convert xylose to xylitol, and of xylitol dehydrogenase (XDH) to convert xylitol to xylulose. Therefore, bioethanol fermentation from xylose can be successfully performed by recombinant *S.*

cerevisiae carrying heterologous XR and XDH from *P. stipitis*, and xylulokinase (XK) from *S. cerevisiae* (Katakira et al., 2006).

In bacteria, a xylose isomerase (XI) converts xylose to xylulose, which after phosphorylation, is metabolized through the pentose phosphate pathway (PPP). The most frequently used microorganism for fermenting bioethanol in industrial processes is *S. cerevisiae*, which has proved to be very robust and well suited to the fermentation of lignocellulosic hydrolysates. *S. cerevisiae* can easily ferment hexoses, but hardly xylose in lignocellulose hydrolysates, because *S. cerevisiae* lacks enzymes that convert xylose to xylulose. However, this yeast can ferment xylulose. For xylose-using *S. cerevisiae*, high bioethanol yields from xylose also require metabolic engineering strategies to enhance the xylose flux (Balat, 2011).

Bacteria, such as *Z. mobilis, Escherichia coli* and *Klebsiella oxytoca*, have attracted particular interest, given their rapid fermentation, which can be minutes compared to hours for yeasts (Hayes, 2009). *Z. mobilis*, a Gram-negative bacterium, is well recognized for its ability to efficiently produce bioethanol at high rates from glucose, fructose, and sucrose. When *Z. mobilis* and *S. cerevisiae* were compared fortheir efficiency to produce bioethanol from glucose and starch hydrolysate, higher yield was observed for *Z. mobilis* (Balat, 2011). Comparative performance trials on glucose have shown that *Z. mobilis* can achieve 5% higher bioethanol yields and up to 5-fold higher bioethanol volumetric productivity compared to traditional *S. cerevisiae* yeast (Saez-Miranda et al., 2008). It has a theoretical yield of 97% (Abril and Abril, 2009). *Z. mobilis* efficiently produces bioethanol from the hexose sugars glucose and fructose but not from pentose sugars. However, remains deficient as optimal ethanol producer in comparison with *S. cerevisiae* in terms of resistance to high alcohol concentration and chemical inhibitors (Limayem and Ricke, 2012).

Thermophilic anaerobic bacteria have also been extensively examined for their potential as bioethanol producers. These bacteria include *Thermoanaerobacter*

ethanolicus, Clostridium thermohydrosulfuricum, Thermoanaerobacter mathranii, Thermoanaerobium brockii, Clostridium thermosaccharolyticum, etc. Thermophilic anaerobic bacteria have a distinct advantage over conventional yeasts for bioethanol production in their ability to use a variety of inexpensive biomass feedstocks and their ability to withstand temperature extremes (Balat, 2011). The low bioethanol tolerance of thermophilic anaerobic bacteria (<2%, v/v) is a major obstacle for their industrial exploitation for bioethanol production Georgieva et al., 2007).

1.6 Bioethanol from lignocellulosic materials via the biochemical pathway

Biochemical conversion of lignocellulosic materials through saccharification and fermentation is a major pathway for bioethanol production from biomass. Bioconversion of lignocellulosics to bioethanol is difficult due to (1) the resistant nature of biomass to breakdown (2) the variety of sugars which are released when the hemicellulose and cellulose polymers are broken and the need to find or genetically engineer organisms to efficiently ferment these sugars; (3) costs for collection and storage of low density lignocellosic materials..

The basic process steps in producing bioethanol from lignocellulosic materials are: pretreatment, hydrolysis, fermentation and product separation/distillation (Balat, 2011).

1.6.1 **Pretreatment categories of the biomasses**

The crucial step in the production of biofuels from lignocellulosic biomass is pretreatment. Pretreatment affords the solubilization or separation of the major components of biomass ie cellulose, hemicellulose and lignin and thus render the digestibility of lignocellulosic material. The choice of pretreatment should consider the overall compatibility of feedstocks, enzymes and organisms to be applied. Pretreatment is not only costly in its own right but has a pervasive impact on the cost of virtually all other biological processing operations,

including those preceding pretreatment, the handling of the liquid stream generated, the processing of the solids from pretreatment, waste treatment, and potential production of co-products. To implement successfully the bioethanol production process, the first impediment to be resolved is the efficient removal of lignin and hemicellulose through a cost effective pretreatment process. During the past few decades, several approaches have been used for developing low cost pretreatments for generating sugar syrups from cellulose and hemicellulose (Yang and Wyman 2008).

Pretreatment methods can be roughly divided into different categories: physical, physicochemical, chemical, biological, or a combination of these (Kumar et al., 2009).

The choice of the optimum pretreatment process depends on the feedstocks and its economic assessment and environmental impact. There are a number of reports on pretreatment options for various biomass types. However, none of those can be declared outstanding as each pretreatment has its intrinsic advantages and disadvantages. An effective pretreatment is characterized by several criteria: avoiding size reduction, preserving hemicellulose fractions, limiting formation of inhibitors due to degradation products, minimizing energy input, and being cost-effective. Except for these criteria, several other factors are also needed to be considered, including recovery of high value added co-products (e.g., lignin and protein), pretreatment catalyst, catalyst recycling, and waste treatment (Banerjee et al., 2010). When comparing various pretreatment options, all the above mentioned criteria should be comprehensively considered as a basis. At the moment efforts on the production of ethanol from lignocellulose is growing rapidly and by analyzing the industrial scenario in this field more knowledge can be gained on the applied pretreatment methods.

- ✓ *Physical pretreatment*

Most of the lignocellulosic biomass requires some of the mechanical processing for size reduction. Several pretreatment methods such as milling, irradiation

(using gamma rays, electron beam, microwave radiations etc) and extrusion are commonly used to improve the enzymatic hydrolysis of biodegradability of lignocellulosic materials. Improved hydrolysis results due to the reduction in crystallinity and improved mass transfer characteristics from reduction in particle size. The energy requirements for physical pretreatment are dependent on the final particle size and reduction in crystallinity of the lignocellulosic material. In most cases where the only option available for pretreatment is physical, the required energy is higher than the theoretical energy content available in the biomass. These methods are expensive and likely will not be used in a full-scale process (Menon and Rao, 2012).

- ✓ *Physico-chemical pretreatment*

Pretreatments that combine both chemical and physical processes are referred to as physicoechemical processes. The most important processes of this group includes: - steam explosion, catalyzed (SO2 or CO2) steam explosion, ammonia fiber explosion (AFEX), liquid hotwater, microwave-chemical pretreatment (Brodeur et al., 2011).

- ✓ *Chemical pretreatments*

Chemical pretreatments were originally developed and have been extensively used in the paper industry for delignification of cellulosic materials to produce high quality paper products. The possibility of developing effective and inexpensive pretreatment techniques by modifying the pulping processes has been considered. Chemical pretreatments that have been studied to date have had the primary goal of improving the biodegradability of cellulose by removing lignin and/or hemicellulose, and to a lesser degree decreasing the degree of polymerization (DP) and crystallinity of the cellulose component. Chemical pretreatment is the most studied pretreatment technique among pretreatment categories. The various commonly used chemical pretreatments includes: acid, alkali, organic acids, pH-controlled liquid hotwater and ionic liquids (Menon and Rao, 2012).

✓ *Biological pretreatment*

Biological pretreatment employs wood degrading microorganisms, including white-, brown-, soft-rot fungi, and bacteria to modify the chemical composition and/or structure of the lignocellulosic biomass so that the modified biomass is more amenable to enzyme digestion. Although it has so far attracted little attention due to many inherent limitations, biological pretreatment has multiple benefits. Increasing understanding of termites and fungal systems has provided insights for developing more effective pretreatment technologies to realize these benefits. Fungi have distinct degradation characteristics on lignocellulosic biomass. In general, brown and soft rots mainly attack cellulose while imparting minor modifications to lignin, and white-rot fungi are more actively degrade the lignin component (Sun and Cheng 2002) Present research is aimed towards finding those organisms which can degrade lignin more effectively and more specifically. White-rot fungi were considered the most promising basidiomycetes for biopretreatment of biomass and were the most studied biomass degrading microorganisms (Sánchez, 2009).

Several white-rot fungi such as *Phanerochaete* (P.) *chrysosporium*, *Ceriporia lacerata*, *Cyathus stercolerus*, *Ceriporiopsis subvermispora*, *Pycnoporus cinnarbarinus* and *Pleurotus ostreaus* have been examined on different lignocellulosic biomass showing high delignification efficiency. Although the microbial degradation of lignin has been most intensively studied in white-rot and brown-rot fungi, there are a number of reports of bacteria that can break down lignin. In the *α-Proteobacteria* class, the genera *Brucella*, *Ochrobactrum*, *Sphingomonas*, and *Sphingobium* contain numerous aromatic-degrading strains. Moreover, *γ-Proteobacteria* include ligninolytic bacteria of which *Pseudomonas* strains were among the first to be characterized. *Enterobacter*, another genus of *γ-Proteobacteria*, contains facultative anaerobic bacteria capable of lignin digestion. *Pandoraea* and *Burkholderia*, two genera of *β-Proteobacteria* contain lignin-degrading strains. Some *δ-Proteobacteria*, particularly *Geobacter*

metallireducens, are involved in the degradation of anaerobic aromatic substances in nature. *Actinobacteria* are known for their ability to produce a large range of catabolic enzymes such as peroxidases. *Streptomyces* genera contain some famous plant pathogens that are also well-known lignin-degrading bacteria. In addition, the *Mycobacteria* genus contains a number of catalase and/or peroxidase producing strains, indicating their ability to decompose aromatic compounds (Le Roes-Hill et al. 2011). Furthemore, the aromatic-degrading ability of some *Microbacteria* species in the *Actinomycetales* order was recently characterized. Most ligninolytic Firmicutes strains discovered to date belong to the *Bacilli* class. Production of key ligninolytic enzymes, especially laccases, has been reported for a number of Bacillus strains. Although little knowledge has been acquired on archaeal laccase, five genes encoding laccase-like enzymes have been identified in the *archaea* kingdom to date, three of which belong to *Halobacteriales* and one to *Thermoproteales.*

The biological pretreatment appears to be a promising technique and has very evident advantages, including no chemical requirement, low energy input, mild environmental conditions, and environmentally friendly working manner (Salvachúa et al., 2011). However, its disadvantages are as apparent as its advantages since biological pretreatment is very slow and requires careful control of growth conditions and large amount of space to perform treatment. In addition, most ligninolytic microorganisms solubilize/consume not only lignin but also hemicellulose and cellulose. Therefore, the biological pretreatment faces technoeconomic challenges and is less attractive commercially (Eggeman and Elander, 2005).

1.7 Hydrolysis techniques

The success of the hydrolysis step is essential to the effectiveness of a pretreatment operation (Gamage et al., 2010). During this reaction, the released polymer sugars, cellulose and hemicellulose are hydrolyzed into free monomer

molecules readily available for fermentation conversion to bioethanol (Chandel et al., 2007).

Various methods for the hydrolysis of lignocellulosic materials have recently been described. The most commonly applied methods can be classified in two groups: chemical hydrolysis (dilute and concentrated acid hydrolysis) and enzymatic hydrolysis. There are some other hydrolysis methods in which no chemicals or enzymes are applied. For instance, lignocellulose may be hydrolyzed by gamma-ray or electron-beam irradiation, or microwave irradiation. However, those processes are commercially unimportant.

Several products can result from hydrolysis of lignocellulosic material (Figure 2) (Demirbas, 2008).

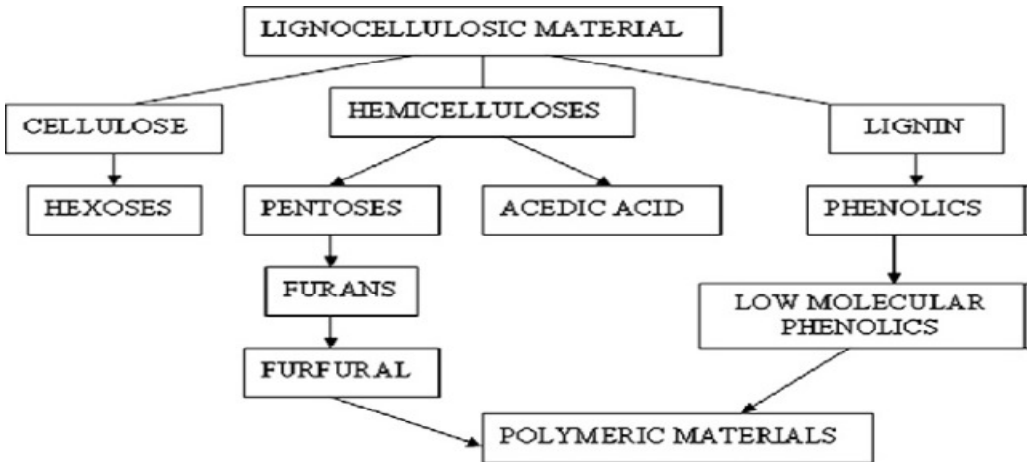

Figure 2. Degradation products occurring during hydrolysis of lignocellulosic material. (Balat, 2011).

When hemicelluloses are hydrolyzed to xylose, mannose, acetic acid, galactose, and glucose are liberated. The main application of xylose is its bioconversion to xylitol, a functional sweetener with important technological properties (Gamez et al., 2004), and bioethanol. Hemicellulosic hydrolysis can be generalized as:

Hemicelluloses ▸Xylan ▸Xylose ▸Furfural

Acetyl groups ▸Acetic acid

Cellulose is hydrolyzed to glucose. The following reaction is proposed for hydrolysis of cellulose:

Cellulose ➔Glucan ➔Glucose.

1.7.1 **Chemical hydrolysis**

Chemical hydrolysis involves exposure of lignocellulosic materials to a chemical for a period of time at a specific temperature, and results in sugar monomers from cellulose and emicellulose polymers. In the chemical hydrolysis, the pretreatment and the hydrolysis may be carried out in a single step. Acids are predominantly applied in chemical hydrolysis (Taherzadeh and Karimi, 2007). There are two basic types of acid hydrolysis processes: dilute acid and concentrated acid.

Dilute hydrolysis (1-3%) requires a high temperature of 200-240 °C to disrupt cellulose crystals (Xiang et al., 2004). It is followed by hexose and pentose degradation and formation of high concentrations of toxic compounds including HMF and phenolics detrimental to an effective saccharification (Sun and Cheng, 2002). The Madison wood-sugar process was developed in the 1940s to optimize alcohol yield and reduce inhibitory and toxic byproducts. This process uses sulfuric acid H_2SO_4 (0.5 wt%) that flows continuouslyto the biomass at a high temperature of 150-180 °C in a short period of time allowing for a greater sugar recovery. Concentrated acid hydrolysis, the more prevalent method, has been considered to be the most practical approach. Unlike dilute acid hydrolysis, concentrated acid hydrolysis is not followed by high concentrations of inhibitors and produces a high yield of free sugars (90%); however, it requires large quantities of acid as well as costly acid recycling, which makes it commercially less attractive (Hamelinck et al., 2005).

While acid pretreatment results in a formation of reactive substrates when acid is used as a catalyst, acid hydrolysis causes significant chemical dehydration of the monosaccharides formed such as aldehydes and other types of degradation

products are generated. This particular issue has driven development of research to improve cellulolytic-enzymes and enzymatic hydrolysis (Limayem and Ricke, 2012).

1.7.2 **Enzymatic hydrolysis**

Acid hydrolysis has a major disadvantage where the sugars are converted to degradation products like tars. This degradation can be prevented by using enzymes favoring 100% selective conversion of cellulose to glucose. When hydrolysis is catalyzed by such enzymes, the process is known as enzymatic hydrolysis (Balat, 2011) Enzymatic hydrolysis of natural lignocellulosic materials is a very slow process because cellulose hydrolysis is hindered by structural parameters of the substrate, such as lignin and hemicellulose content, surface area, and cellulose crystallinity (Pan et al., 2006). Utility cost of enzymatic hydrolysis is low compared to acid or alkaline hydrolysis because enzyme hydrolysis is usually conducted at mild conditions (pH 4.8) and temperature (45‑50 °C) and does not have a corrosion problem (Sun and Cheng, 2002). The enzymatic hydrolysis has currentlyhigh yields (75‑85%) and improvements are still projected (85‑95%), as the research field is only a decade young (Hamelinck et al., 2005).

Comparison of process conditions and performance of three cellulose hydrolysis processes is given in Table 3.

Table 3. Comparison of process conditions and performance of three hydrolysis processes.

	Consumables	**Temperature °C**	**Time**	**Glucose yield (%)**
Dilute acid	1-3% H_2SO_4	24-240	3 min	50
Concentrated acid	30‑70% H_2SO_4	150-180	2-6 h	70-90
Enzymatic	Cellulase	45-50	1-3 day	75-95

Enzymatic hydrolysis is an environmentally friendly alternative that involves using carbohydrate degrading enzymes (cellulases and hemicellulases) to hydrolyze lignocelluloses into fermentable sugars (Keshwani and Cheng, 2009): glucose and xilose.

Hemicellulases action is fundamental to facilitate cellulose hydrolysis by exposing the cellulose fibers, thus making them more accessible.

Cellulose is typically hydrolyzed by an enzyme called cellulase that is a group of enzymes that synergistically hydrolyzes cellulose.

These enzymes are produced by several microorganisms, commonly by bacteria and fungi. These microorganisms can be aerobic or anaerobic, mesophilic or thermophilic. Bacteria belonging to *Clostridium*, *Cellulomonas*, *Bacillus* (Ventorino et al., 2015), *Thermomonospora*, *Ruminococcus*, *Bacteriodes*, *Erwinia*, *Acetovibrio*, *Microbispora*, and *Streptomyces* can produce cellulases effectively (Sun and Cheng, 2002). Fungi such as *Sclerotium rolfsii*, *P. chrysosporium* and species of *Trichoderma*, *Aspergillus*, *Schizophyllum* and *Penicilium* are used to produce cellulases (Balat, 2011). Unlike cellulose, xylans are chemically quite complex, and their degradation requires multiple enzymes. Xylan-degrading enzymes are produced by a wide variety of fungi and bacteria such as *Trichodrema spp.*, *Talaromyces spp.*, (Balat, 2011), *Penicillium spp.* (Jorgensen et al., 2003), *Aspergillus spp.* (Dos Reis et al., 2005), and *Bacillus spp.* (Ventorino et al., 2015)

There are different factors that affect the enzymatic hydrolysis of cellulose, namely, substrates, cellulase activity, reaction conditions (temperature, pH as well as other parameters), and a strong product inhibition. To improve the yield and rate of enzymatic hydrolysis, research has been focused on optimizing the hydrolysis process and enhancing the cellulase activity problem (Sun and Cheng, 2002). The rate of enzymatic hydrolysis of cellulose is dependent upon its several structural features known to affect the rate of hydrolysis: (1) molecular structure of cellulose (2) crystallinity of cellulose, (3) surface area of

cellulose fiber, (4) degree of swelling of cellulose fiber, (5) degree of polymerization, and (6) associated lignin or other materials. A low substrate concentration gives low yield and rate, and a high cellulase dosage may increase the costs disproportional (Balat, 2011).

Most desired attributes of cellulases for lignocelluloses bioconversion are the complete hydrolytic machinery, high specific activity, high rate of turn over with native cellulose/biomass as substrate, thermal stability, decreased susceptibility to enzyme inhibition by cellobiose and glucose, selective adsorption on cellulose, synergism among the different enzymes and ability to withstand shear forces (Maki et al., 2009). Despite intensive research over the few past decades, the enzyme hydrolysis step remains as a major techno-economic bottleneck in lignocellulosic biomass-to ethanol bioconversion process (Menon and Rao, 2012).

1.8 Fermentation

Pretreatment and hydrolysis processes are designed to optimize the fermentation process (Gamage et al., 2010). This natural, biological pathway depending on the conditions and raw material used requires the presence of microorganisms to ferment sugar into alcohol (Limayem and Ricke, 2012).

The supernatant from enzymatic hydrolysis of lignocellulosic can contain both six-carbon (hexoses) and five-carbon (pentoses) sugars (if both cellulose and hemicellulose are hydrolyzed).

Depending on the lignocellulose source, the hydrolysate typically consists of glucose, xylose, arabinose, galactose, mannose, fucose, and rhamnose (Keshwani and Cheng 2009). One ton of glucan, galactan, or mannan yields 1.11 tons of six-carbon sugars and could be fermented theoretically into 172.0 gallons of bioethanol. One ton of arabinan or xylan yields 1.14 tons of five-carbon sugars and could be fermented theoretically into 176.0 gallons of bioethanol

(Szulczyk et al., 2010). Microorganisms can be used to ferment all lignocellulose-derived sugars to bioethanol.

Fermentation can be performed as a batch, fed-batch or continuous process. The choice of most suitable process will depend upon the kinetic properties of microorganisms and type of lignocellulosic hydrolysate in addition to process economics aspects (Chandel et al., 2007).

Batch culture can be considered as a closed culture system which contains an initial, limited amount of nutrient, which is inoculated with microorganisms to allow the fermentation (Abtahi, 2008). It is very simple method, during the fermentation nothing is added after inoculation except possibly acid or alkali for pH control or air for aerobic fermentation.

Fed-batch reactors are widely used in industrial applications because they combine the advantages from both batch and continuous processes. The major advantage of fed-batch, comparing to batch, is the ability to increase maximum viable cell concentration, prolong culture lifetime, and allow product accumulation to a higher concentration. This process allows for the maintenance of critical process variables (e.g. temperature, pH, and dissolved oxygen) at specific levels through feedback control.

In the continuous process, feed, which contains substrate, culture medium and other required nutrients, is pumped continuously into an agitated vessel where the microorganisms are active. The product, which is taken from the top of the bioreactor, contains bioethanol, cells, and residual sugar. One of the first approaches taken in improving the yeast bioethanol fermentation process involved operating the fermenters in a continuous mode rather than the more conventional batch mode and thereby increasing the productivity about threefold from about 2 to 6 g EtOH/l/h (Balat, 2011).

1.9 Processes configuration to comparison

The pretreated biomass can be processed using variety of process configurations such as separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), Prehydrolysis and simultaneous saccharification and fermentation (PSSF), simultaneous saccharification and co-fermentation (SSCF) and consolidated biomass processing (CBP) (Figure 3).

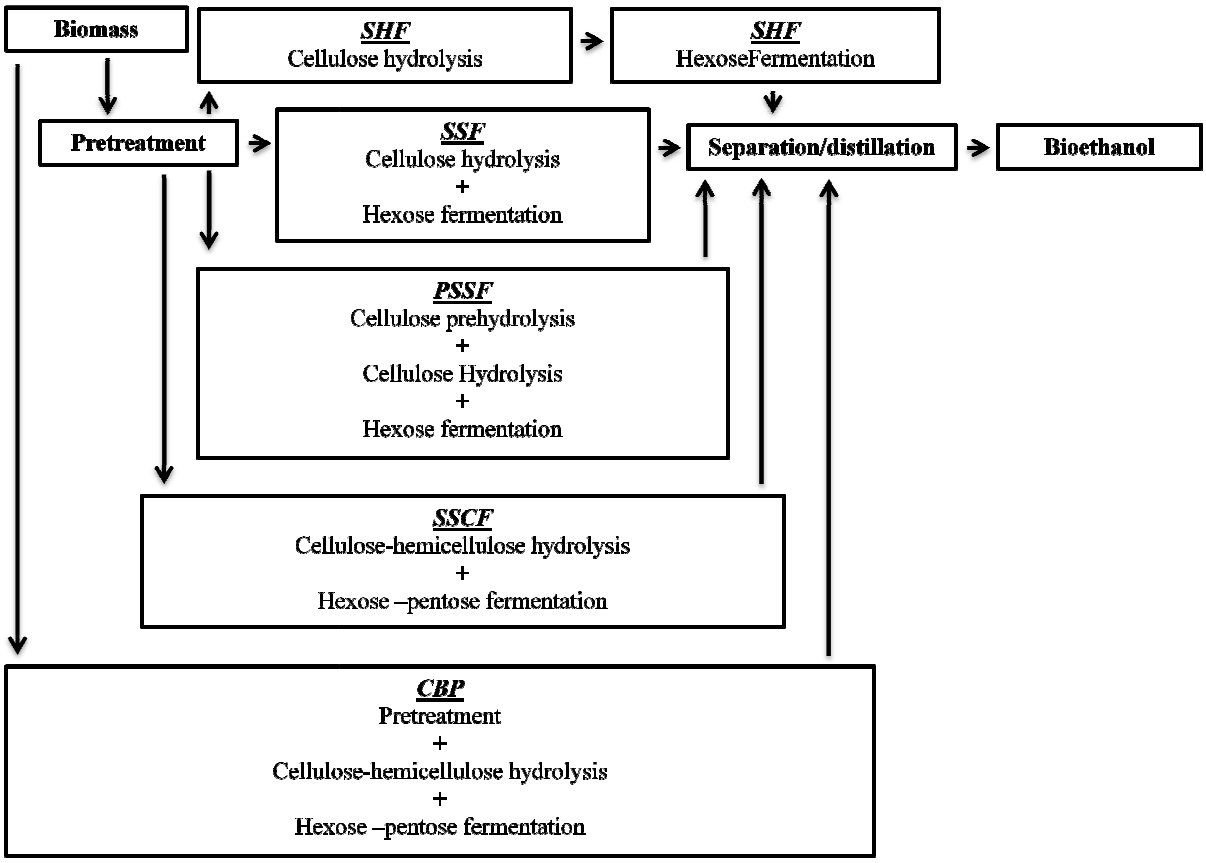

Figure 3. Fermentation strategies and consolidated biomass processing.

SHF is a conventional two step process where the lignocellulose is hydrolysed using the enzymes to form the reducing sugars in the first step and the sugars, thus formed, are fermented to ethanol in the second step using various yeasts such as *Saccharomyces*, *Kluyveromyces*, *Debaryomyces*, *Pichia* as well as their recombinants. In SHF was able to achieve 191.5 g of ethanol/Kg untreated biomass at ethanol concentration of 40 g/L (Menon and Rao, 2012). The process

is carried out without washing of pretreated biomass, detoxification and nutrient supplementation Lau and Dale, 2009). The saccharification of the lignocellulosic biomass by the enzymes and the subsequent fermentation of the sugars to ethanol by yeast take place in the same vessel in SSF. The compatibility of both saccharification and fermentation process with respect to various conditions such as pH, temperature, substrate concentration, enzymatic amount etc. is one of the most important factors governing the success of the SSF process (Doran-Peterson et al., 2010). The main advantage of using SSF for the ethanol bioconversion is enhanced rate of hydrolysis of lignocellulosic biomass (cellulose and hemicellulose) due to removal of end product inhibition. Another factor of great importance in the fermentative processes is the cultivation conditions, which, if inadequate, can stimulate the inhibitory action of the toxic compounds (Ballesteros et al., 2004). Thermotolerant yeast is an added advantage for SSF and thermotolerant yeast strains, to allow fermentation at temperatures closer to the optimal temperature for the enzymes. Running SSF in fed-batch mode does not necessarily give higher ethanol yields than running in batch mode, but when the enzyme adding method is suitable, similar or slightly higher ethanol yields could be obtained in fed batch SSF compared with batch mode.

A major SSF drawback is the mismatch between the optimum temperature of enzymes and microorganism and to reduce viscosity of the solid–liquid mixture prior to the addition of the microorganism. PSSF configuration could be conducted in an attempt to partially solve this disadvantage. PSSF configuration could be conducted in an attempt to partially solve this disadvantage. This consists of an enzymatic hydrolysis followed by an SSF process in one batch (Ohgren et al., 2007).

Simultaneous saccharification and co-fermentation (SSCF) has been recognized as a feasible option for ethanol production from xylose-rich lignocellulosic materials. Simultaneous saccharification of cellulose and hemicellulose and co-

fermentation of end products glucose and xylose is carried out by genetically engineered microbes or wild type. Another promising alternative for simultaneous utilization of pentoses and hexoses is the conversion of xylose into xylulose by xylose isomerase or oxidoreductase. The fermentation of xylulose begins by its intracellular phosphorylation to xylulose-5-phosphate, which enters the pentose phosphate pathway and eventually forms ethanol. Yeasts like *S. cerevisiae* and *Schizosaccharomyces pombe* are capable of fermenting xylulose to ethanol, along with glucose (Menon and Rao, 2012).

Lignocellulosic bioprospecting involving enzymes or microbial systems commonly includes three major biologically mediated processes: production of saccharolytic enzymes (cellulases and hemicellulases), hydrolysis of cellulose and hemicellulose to monomeric sugars, fermentation of hexose and pentose sugars. These three biotransformations occurring in a single process configuration is called as consolidated biomass processing (CBP). CBP offers the potential for lower cost and higher efficiency than processes featuring dedicated cellulase production. This result from avoided costs for capital, substrate and other raw materials, and utilities associated with cellulase production. In addition, several factors support the possibility of realizing higher hydrolysis rates, and hence reduced reactor volume and capital investment, using CBP. These include enzyme-microbe synergy as well as the use of thermophilic organisms and/or complexed cellulase systems. CBP requires a microbial culture that combines properties related to both substrate utilization and product formation. Desired substrate utilization properties include the production of a hydrolytic enzyme system allowing high rates of hydrolysis and utilization of resulting hydrolysis products under anaerobic conditions with a practical growth medium. Desired product formation properties include high product selectivity and concentrations. A cellulolytic culture with this combination of properties has not been described to date. Development of microorganisms for cellulose conversion via CBP can be pursued according to

two strategies. The native cellulolytic strategy involves naturally occurring cellulolytic microorganisms to improve product-related properties such as yield and tolerance. The recombinant cellulolytic strategy involves engineering non-cellulolytic microorganisms that exhibit high product yields and tolerance so that they become able to utilize cellulose as a result of a heterologous cellulase system (Carrad et al., 2000). Both CBP organism development strategies involve very large challenges that will probably require a substantial sustained effort to overcome. The feasibility of CBP will be fully established only when a microorganism or microbial consortium is developed that satisfies the requirements.

Process integration plays a major role in efficient process design. When several operations can be performed in a same single unit, the possibilities for improving the performance of the process are higher. Similarly, the integration of different chemical and biological processes for the complete utilization of the feedstocks should lead to the development of big "biorefineries" that allow the production of large amounts of fuel ethanol and many other valuable co-products at smaller volumes, improving the overall economical effectiveness of the conversion of a given raw material. Integration opportunities may provide the ways for a qualitative and quantitative improvement of the process to make it techno-economical and eco-friendly (Menon and Rao, 2012).

1.10 Separation/distillation

Bioethanol obtained from a fermentation conversion requires further separation and purification of ethanol from water through a distillation process. Fractional distillation is a process implemented to separate ethanol from water based on their different volatilities. This process consists simply of boiling the ethanol water mixture. Because the boiling point of water (100 °C) is higher than the ethanol-boiling point (78.3 °C), ethanol will be converted to steam before water. Thus, water can be separated via a condensation procedure and ethanol distillate

recaptured at a concentration of 95% (Cardona and Sanchez, 2007) Typically, most large scale industries and biorefineries use a continuous distillation column system with multiple effects. Liquid mixtures are heated and allowed to flow continuously all along the column. At the top of the column, volatiles are (Olkiewicza et al., 2012) separated as a distillate and residue is recovered at the bottom of the column (Limayem and Ricke, 2012).

1.11 Municipal sewage sludge

Sludge is characterized by an extremely liquid residue (over 95% water), its composition varies depending on the initial load wastewater pollution and technical characteristics of the wastewater treatment performed.

Treatment plant produces primary and secondary sludges, generally after primary and secondary treatment with significant differences between their compositions. The primary sludge is a combination of floating grease and solids collected at the bottom of the primary settler after screening and grit removal.

The secondary or activated sludge is composed mainly of microbial cells and suspended solids produced during the aerobic biological treatment and collected in the secondary settler (Olkiewicza et al., 2012).

Municipal sewage sludge contains significant concentrations of lipids derived from the direct adsorption of lipids onto the sludge. These energy-containing lipids include triglycerides, diglycerides, monoglycerides, phospholipids, and free fatty acids contained in the oils and fats. In addition, microorganisms used in the wastewater treatment process utilize organic and inorganic compounds in the wastewater as a source of energy, carbon, and nutrients. The cell membrane of these microorganisms is a major component of sewage sludge and is composed primarily of phospholipids. It is estimated at 24% to 25% of dry mass of the cell 4.5 and yields about 7% oil from the dried secondary sludge. Was demonstrated that up to 36.8 wt% of the dry sludge is comprised of fatty acids and steroids. With the fatty acids from sludge predominantly in the range

of C10 to C18, these are excellent for the production of biodiesel (Kargbo, 2010).

The extraction and transformation of the lipids could yield an unexploited source of cheap and readily available feedstock for biodiesel production. Furthermore, it is one possible alternative to take advantage of the excess of sludge, reusing it as a source of lipid for the production of biodiesel (Olkiewicza et. al., 2012).

1.12 The biodiesel production methods from wastewater sludge

There are four primary techniques for biodiesel production – direct use and blending of raw oils, micro-emulsions, thermal cracking and transesterification. The most commonly used method for biodiesel production is transterification (also known as alcoholysis) reaction in presence of a catalyst. Transesterification is the process of exchanging the alkoxy group of an ester compound with another alcohol (Figure 4).

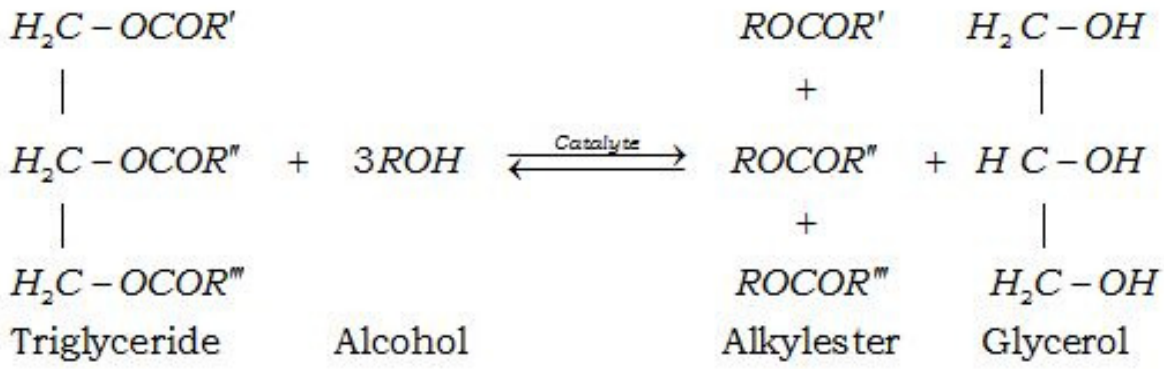

Figure 4. The general form of transesterification reaction (Siddiquee and Rohani, 2011).

The R', R'', and R''' are long hydrocarbon chains, called fatty acid chains. This transesterification reaction consists of a series of consecutive, reversible reactions. The triglyceride is converted stepwise by reacting with primary alcohol to diglyceride, monoglyceride and finally glycerol.

Triglyceride + ROH ↔ Diglyceride + RCOOR'

Diglyceride + ROH ↔ Monoglyceride + RCOOR''

Monoglyceride + ROH ↔ Glycerine + RCOOR'''

The primary alcohol used to form the ester is a major feedstock in biodiesel production process. Methanol is widely used as the alcohol for producing biodiesel because it is the least expensive alcohol. Besides it has some advantages – (i) its reactivity is high, (ii) it does not absorb water that interferes with transesterification reaction, (iii) prevents soap formation and (iv) its recovery is easier, as it does not form azeotrope like ethanol. Excess methanol (60–100% more methanol than required) is added in order to ensure total conversion of the vegetable oil or animal fat to its esters.

The yield of transesterification depends on several factors including the type of catalyst (base, acid, enzyme or heterogeneous), alcohol/vegetable oil molar ratio, temperature, and duration of reaction, water content and free fatty acid content. Water can consume the catalyst and reduce catalyst efficiency.

Base catalyst transesterification is widely used commercially due to very fast reaction rate compared to other catalysts. Base catalyzed process is very sensitive to water and free fatty acids (FFA) present in lipid sources due to soap formation. It leads to catalyst consumption and soap formation that inhibits separation of the glycerol from the methyl esters and contributes to emulsion formation during the water wash.

Fatty acid (R1COOH) + NaOH ↔ Soap (R1COONa) + Water (H2O)

On the other hand, acid catalyzed transterification is 4000 times slower than base catalyzed transesterification and requires high triglyceride to alcohol ratio.

But acid catalyst is able to catalyze both the esterification and transesterification and more biodiesel is produced. If water accumulates, it can stop the reaction. So, this approach requires a water management technique.

Fatty acid (R1COOH) + Alcohol (ROH)
↔ Mono-alkyl-ester (R1COOR) + Water (H2O)

The use of heterogeneous catalysts has shown greater promise toward transesterification to obtain biodiesel. The commonly used heterogeneous catalysts are Mg/La mixed oxide, S-ZrO_2 sulfated zirconia, KOH/Nax zeolite, Li/CaO, CaO, KI/Al_2O_3, (ZS/Si) zinc stearate immobilized on silicagel, KNO_3/Al_2O_3, $SO_4^{2-}/TiO_2–SiO_2$, etc. Heterogeneous catalysts can be recovered conveniently fromreaction products. The undesired saponification reactions can be avoided by using heterogeneous acid catalysts. This catalyst enables the transesterification of vegetable oils or animal fats with high contents of FFA. Solid catalyst can be reused and provides the possibility for carrying out both transesterification and esterification reactions simultaneously. However, the cost of solid catalyst is very high (Siddiquee and Rohani, 2011).

For the lipid feedstocks in sewage sludges containing >1% FFA, acid catalysis followed by base catalysis is recommended. Here, FFAs are first converted to methyl esters until FFA< 0.5%, when additional methanol is added followed by the addition of a base catalyst to transesterify the triglycerides to biodiesel (Kargbo, 2010). However, enzymatic transesterification especially those using lipase has drawn researcher's attention in last ten years due to the downstream processing problem posed by chemical transesterification. Huge amount of wastewater generation and difficulty in glycerol recovery are among problems that eventually increase the overall biodiesel production cost and being not environmental benign. In contrast, enzyme catalysis proceeds without the

generation of by-products, easy recovery of product, mild reaction condition, insensitive to high FFA oil and catalyst can be reuse (Kulkarni and Dalai, 2006). These advantages prove that enzyme catalyzed biodiesel production has high potential to be an eco-friendly process and a promising alternative to the chemical process. However, it still has its fair share of constraints especially when implemented in industrial scale such as high cost of enzyme, slow reaction rate and enzyme deactivation (Bajaj et al., 2010).

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Chapter 2 Aims of the research

This research was focused on the improvement and development of current techniques for biofuels production to latest generation from renewable sources. This aims has been achieved investigating in three different ways:

- Isolation and characterization of microorganisms of biotechnological interest for the application in bioenergetic systems. In particular, this objective was reached with isolation, biotechnological characterization and molecular identification of microorganisms able to degrade cellulose, hemi-cellulose, pectin and lignin, and microorganisms able to ferment monosaccharides (hexose and pentose) into bioethanol from *Arundo donax*, *Populus nigra* and *Eucalyptus camaldulensis* chipped.

- Evaluation and optimization to parameters that usually influence the process bioethanol production: enzyme concentration, biomass pretreatment amount and temperature. Moreover, was selected the best configuration process for bioethanol production, comparing between Prehydrolysis and Simultaneous Saccharification and Fermentation (PSSF), Simultaneous Saccharification and Fermentation (SSF) and Simultaneous Saccharification and Co-Fermentation (SSCF).

- Identify the best point of equilibrium of the acid and enzymatic esterification reactions for Free Fatty Acid conversion present in the sewage sludge. Moreover, evaluation of biodiesel production with acid esterification followed by basic transesterification.

Chapter 3 New microorganisms able to bioconvert the lignocellulose biomass

3.1 Introduction

Lignocellulosic biomass includes dedicated energy crops, such as *Miscanthus, switchgrass, Arundo donax, Populus nigra* and *Eucalyptus camaldulensis*. These plants can easily grow in farmland not suitable for food crops or in soils subjected to accelerated erosion, which affects most of the Mediterranean hilly croplands (Diodato et al., 2009; Fiorentino et al., 2013; Ventorino et al., 2015).
Vegetable biomasses represent an inexpensive alternative to fossil sources of fermentable sugars that can be utilised in several industrial applications, including last generation biofuels and biopolymer production. The enzymatic hydrolysis of plant carbohydrates has emerged as the most prominent eco-technology for the degradation of such biomasses. By a microbiological point of view, lignocellulosic biomass represents a complex ecosystem in which environmental conditions influence living organisms. In particular, geochemical (pH and salinity) and physical (temperature, pressure and radiation) factors can have a selective pressure on the biodiversity of microorganisms (Van Den Burg, 2003). As a consequence, autochthonous microbial communities may prevail over other microorganisms because they possess enzymes able to degrade complex molecules such as cellulose, hemicellulose, lignin and pectin. Therefore, the monosaccharides derived by these enzymatic activities, can be fermented by others autochthonous microorganisms.
Generally, on industrial level, the bioconversion of pretreated lignocellulose-based materials into ethanol is performed by using a reaction mixture. This mixture is composed by multiple enzymes, for complete hydrolysis, and yeasts for fermentation. However, the biorefining process is still economically unfeasible due to the operative steps of cooling, oxygen pumping, stirring and

neutralisation, as well as the intrinsically high cost of hydrolytic enzyme and efficiency of fermentation. New biocatalysts bacteria and yeasts could help to overcome these costly hurdles (Maki et al., 2009).

3.2 Materials and method

3.2.1 **Lignocellulosic Biomasses and Sampling**

Chipped wood from *A. donax*, *E. camaldulensis* and *P. nigra* has been processed for biodegradation under natural conditions for 90 days. The chipped wood was used to form two piles of approximately 30 kg (length 0.85 m, width 0.85 m and height 0.70 m) for each plant species. In order to increase microbial biodiversity, the piles were placed under two different environmental conditions: open field and underwood conditions. In open field, the biomass piles were placed on an agronomic soil without any coverage shady trees, inside the experimental station of Department of Agricultural Sciences (Naples, Italy; 40°48'50.1''N, 14°20'48.2''E). The underwood conditions were performed reproducing the natural environments that usually arise in a forest, the piles were placed under oak trees at the Department of Agricultural Sciences woodland (Naples, Italy; 40°48'47.8''N, 14°20'50.4''E). Samples of 0.5 kg were collected from the external part (right and left side of the pile) and the internal central part of the biomass in three different times: immediately after preparation (T0), after 45 days and after 90 days (T1 and T2 respectively) of biodegradation. During the process, temperature (°C) was measured by using specific temperature sensors (VWR International PBI, Milan, Italy) placed directly in the core of the piles at a depth of 30–40 cm. The water activity (a_w, water readily available for microbial metabolic activities) was also monitored using a HygroPalm23-AW (Rotronic AG, Basserdorf, Germany).

3.2.2 **Enumeration and isolation of microbial functional groups and yeast in the lignocellulosic biomasses**

Different samples were collected from the chipped vegetable biomass piles of *A. donax*, *E. camaldulensis* and *P. nigra* for the isolation yeasts content as well as different eco-physiological microbial groups involved in the carbon cycle, such as aerobic and anaerobic microorganisms cellulolytic, pectinolytic, hemicellulolytic and ligninolytic. Serial dilutions (0.1 mL) of the samples were spread on the surface of plates containing differential selective solid substrates.
Basal medium (1 g L^{-1} of $(NH_4)NO_3$, 1 g L^{-1} of yeast extract, 50 mL L^{-1} of standard salt solution [5.00 g L^{-1} of K_2HPO_4, 2.50 g L^{-1} of $MgSO_4$, 2,50 g L^{-1} of NaCl, 0,05 g L^{-1} of $Fe_2(SO_4)_3$, 0,05 g L^{-1} of $MnSO_4$], 1 mL L^{-1} of trace elements solution [0.05 g L^{-1} of K_2MoO_4*5 H_2O, 0.05 g L^{-1} of $Na_2B_4O_7$*10 H_2O, 0.05 g L^{-1} of $Co(NO_3)_2$*6 H_2O, 0.05 g L^{-1} of $MnSO_4$, 0.05 g L^{-1} of $CdSO_4$, 0.05 g L^{-1} of $ZnSO_4$*7 H_2O, 0.05 g L^{-1} of $CuSO_4$*H_2O, 0.10 g L^{-1} of $FeCl_3$], 15 g L^{-1} of Agar bacteriological [Oxoid, Milan, Italy] pH 7.0) with 0.1 % of Congo red (Sigma-Aldrich) and 5 g L^{-1} of Avicel (Sigma-Aldrich, Milan, Italy) or 5 g L^{-1} of carboxymethylcellulose (CMC), was used to count aerobic and anaerobic exo-cellulolytic, endo-cellulolytic, respectively. Anaerobic conditions were obtained by using Anaerogen™ System (Oxoid, Milan, Italy). For hemicellulolytic enumeration 5 g L^{-1} of Xilan (Sigma-Aldrich) were added to basal medium. After 4 days of incubation at 28ºC, exo-cellulolytic colonies appeared surrounding by a clear halo on CMC. The colonies of putative exo-cellulolytic and hemicellulolytic microorganisms able to grow on the Avicel and Xylan substrates, as the only carbon source, were counted (Ventorino et al, 2015).
Pectinolytic microrganisms were counted using the following substrate: basal medium (1 g L^{-1} of yeast extract, 50 mL L^{-1} of standard salt solution, 1 mL L^{-1} of trace elements solution, 15 g L^{-1} of Agar bacteriological, pH 6.8) with 5 g L^{-1}

Pectin (Sigma-Aldrich). After 5 days of incubation at 28ºC, the colonies grown were enumerated. (Pepe et al., 2013).

Lignin agar substrate was used to ligninolytic microorganisms. This medium was composed by Liquid Basal Medium (LBM) (1 g L^{-1} of KH_2P0_4, 0.01 g L^{-1} of Yeast Extract, 0.001 g L^{-1} of $CuS0_4$*5 H_20, 0.5 g L^{-1} of $C_4H_{12}N_20_6$, 0.5 g L^{-1} of $MgS0_4$*7 H_20, 0.001 g L^{-1} of Fe_2 $(S0_4)_3$, 0.01 g L^{-1} of $CaCl_2$*2 H_20) supplemented with 2.5 g L^{-1} of Lignin (Sigma-Aldrich) and 16 g L^{-1} of Agar bacteriological. The colonies were counted after 5 days of incubation at 25°C in darkness (Pointing, 1999).

Yeasts were counted using YPD Agar medium (20 g L^{-1} of Glucose, 20 g L^{-1} of Peptone, 10 g L^{-1} of Yeast Extract, 18 g L^{-1} of Agar bacteriological) added with 100 ppm of chloramphenicol (Sigma-Aldrich) to inhibit the growth of prokaryotic microorganisms. The plates were incubated at 28°C for 4 days.

Morphologically different colonies, were isolated by each substrate of count, purified on the same medium and stored at 4°C until their characterization.

3.2.3 **Screening for functional activities on solid media**

Biotechnological characterization of all isolates was performed. In particular, qualitative and semi-quantitative tests were carried out evaluating the following enzymatic activities: endo-cellulolase, exo-cellulase, cellobiase, xylanase, pectinase, ligninase, peoxidase and laccase. The isolates, grown at 28°C for 24 h in the specific liquid substrate, were spotted (10µL broth) on the solid medium in triplicate.

In particular, the endo-cellulolase activity was measured on CMC agar (1 g L^{-1} of $(NH_4)NO_3$, 1 g L^{-1} of yeast extract, 50 mL L^{-1} of standard salt solution, 1 mL L^{-1} of trace elements solution, 15 g L^{-1} of agar bacteriological, 5 g L^{-1} carboxymethylcellulose, 18 g L^{-1} Agar bacteriological, pH 7.0). After 4 days incubation at 28°C, the plates were stained with 0.1% Congo red solution for 30 min followed by washing with 5 M NaCl (Ventorino et al, 2015). Cellulase

activities were recorded as the ‘‘Indices of Relative Enzyme Activity, I_{CMC} = diameter of clearing or halo zone/colony diameter’’ (Saini and Tewari, 2012). The exo-cellulase activity was estimated in a qualitative way by spotting all the isolates on the minimal medium agar (6.0 g L^{-1} of $NaNO_3$, 1.5 g L^{-1} of KH_2PO_4, 0.5 g L^{-1} of KCl, 0.5 g L^{-1} of $MgSO_4$, 0.01 g L^{-1} of $FeSO_4$, 0.01 g L^{-1} of $ZnSO_4$ and 18 g L^{-1} of Agar bacteriological) with 10 g L^{-1} of Avicel and observing the development of microbial colonies after incubation at 28°C for 10 days (Soares et al., 2008).

Cellobiase activity was assessed with cellobiose agar spotting (10 g L^{-1} bacteriological peptone, 15 g L^{-1} Agar bacteriological, 7.5 g L^{-1} cellobiose and 0.06 g L^{-1} bromothymol blue [Sigma-Aldrich]). After incubation at 28°C for 3 days, the cellobiase activity was detected by observing yellow haloes around the colonies (Ventorino et al., 2015). Xylanase activity was monitored spotting microorganisms on LB agar medium (10 g L^{-1} of Peptone, 5 g L^{-1} of Yeast Extract, 5 g L^{-1} of Na Cl, 20 g L^{-1} of Agar bacteriological, pH=7) added of 0.05% Remazol brilliant blue-R and 0.5% of sonicated (45 min) Xylan solution (FALC Instruments, HK3300). After 3–5 days of incubation at 28°C, the hydrolysis of Xylan was detected by observing a clear zone around the colonies (Ventorino et al., 2015). Pectinase activity was considered using basal medium (1 g L^{-1} of yeast extract, 50 mL L^{-1} of standard salt solution, 1 mL L^{-1} of trace elements solution, 15 g L^{-1} of Agar bacteriological, pH=6.8) with 5 g L^{-1} of Pectin. After incubation at 28°C for 4 days, the plates were flooded with 1% of hexadecyltrimethylammonium bromide (Sigma-Aldrich) solution that plummeted the intact pectin. After 20-30 minutes, pectinase activity was detected by developing hydrolysis halo around the colony (Pepe et al., 2013). Pectinase activity was recorded as the ‘‘Indices of Relative Enzyme Activity, (I_{PEC}) = diameter of clearing or halo zone/colony diameter’’.

Ligninase activity was detected by using the CGA culture medium (2 g L^{-1} of sugarcane bagasse, 0.1 g L^{-1} of Guaiacol [Sigma-Aldrich], 16 g L^{-1} of Agar

bacteriological) (Okino et al., 2000) modified by replacing the lignin with vegetable biomass powder from *A. donax* or 2 g L^{-1} of lignin alkali. A reddish brown halo around a colony indicates the successful oxidation of guaiacol by ligninolytic enzymes (Okino et al., 2000). Laccase activity was determined for all the isolated strains by growth on agar medium composed of (LBM) supplemented with 1 g L^{-1} of 2.29-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid [ABTS], Sigma-Aldrich) and 16 g L^{-1} of Agar bacteriological. After 10 days of incubation at 25°C in darkness, a green colour in the medium indicated the presence of laccase activity (Ventorino et al., 2015). Azure-B agar (LBM, 0.1 g L^{-1} of Azure-B [Sigma-Aldrich] and 16 g L^{-1} Agar bacteriological) was used to evaluate the production of lignin peroxidase and manganese-dependent peroxidase by the formation of a clear halo around a colony after 10–30 days of incubation at 25°C in darkness (Ventorino et al., 2015).

3.2.4 **Resistance to thermal stress in *S. cerevisiae***

The growth of nineteen strains of *S. cerevisiae* (Yeasts Collection of Department of Agricultural Sciences – Division of Microbiology, Naples, Italy) with high alcohol tolerance and an elevated fermentative power was monitored at different temperatures (30°C, 37°C, 40°C and 45°C). The tests were carried out inoculating $1*10^6$ CFU mL^{-1} in 10 mL of YPD for 48 h. After, 220 µL of broth were dispensed into a microtiter plate. The wells were covered with two drops of sterile paraffin oil. The growth curves were obtained by monitoring the Optical Density [OD 600 nm] with Microtiter Instrument (Bio-Tek ELx808) every 30 minutes for 48 h.

3.2.5 **Fermentation of pentose and hexose by non-*Saccharomyces* yeasts**

Fermentation pentose was evaluated of all wild type isolated yeasts and 121 non-*Saccharomyces* yeasts (Yeasts Collection of Department of Agricultural Sciences – Division of Microbiology, Naples, Italy). Two strains, *Candida*

shehatae (CBS5853, CBS, Utrecht, Netherlands) and *Spathaspora passalidarum* (CBS10155, CBS, Utrecht, Netherlands) were used as positive control. The strains were inoculated in 10 mL of liquid substrate with 0.67 g L^{-1} of Yeasts Nitrogen Base (YNB, Sigma-Aldrich) and 5 g L^{-1} of Xylose (Sigma-Aldrich) as only carbon source (YX medium) (Cadete et al., 2012). The strains, able to grow in YX medium, were tested to evaluate their ability to ferment Xylose. The strains were inoculated in 20 mL of YPX (10 g L^{-1} of Yeast Extract, 20 g L^{-1} of Peptone, 20 g L^{-1} of Xylose) and incubated at 30°C shaking at 120 rpm. After 24 h, the broth was centrifuged (6500 rpm for 10 min) and pellet was used to inoculate ($1*10^7$ CFU mL^{-1}) 50 mL of substrate (0.67 g L^{-1} of YNB, 50 g L^{-1} of Xylose, diluted in Na-Acetate buffer 0.05 N, pH=5). The fermentation test was performed at 30°C shaking at 120 rpm. After 96 h, the ethanol produced was quantified by HPLC (Refractive index detector 133; Gilson; pump 307, column Metacarb 67h [Varian] isocratic flow 0,4 mL min^{-1}, mobil phase H_2SO_4 0.01 N, temperature 65°C). Similarly tests were carried out to evaluate the ability to ferment glucose of all strains wild type and *Pichia* (P.) *caribbica* NS117 (Yeasts Collection of Department of Agricultural Sciences – Division of Microbiology, Naples, Italy). The high alcohol tolerance and an elevated fermentative power strain *S. cerevisiae* NA227 (Yeasts Collection of Department of Agricultural Sciences – Division of Microbiology, Naples, Italy) was used as positive control. The strains were inoculated in 10 mL of YPD and incubated at 30°C shaking at 120 rpm. After 24 h, the broth was centrifuged (6500 rpm for 10 min) and pellet inoculated ($1*10^7$ CFU mL^{-1}) in 50 mL of substrate to fermentation (0.67 g L^{-1} of YNB, 30 g L^{-1} of Glucose [Sigma-Aldrich], diluted in Na-Acetate buffer 0.05 N, pH=5). The test was carried out at 30°C under shaking at 120 rpm. After 24 h, the ethanol produced was quantified by HPLC analysis such as described above.

3.2.6 **Identification and phylogenetic analysis of bacterial strains with multi-enzymatic activities**

Bacterial isolates were identified by molecular method (16S rRNA gene sequencing). Total genomic DNA was extracted and purified by InstaGeneTM Matrix (Bio-Rad Laboratories, Hercules, CA, USA) according to the supplier's recommendations.

Approximately 50 ng of DNA was used as the template for PCR assays. Synthetic oligonucleotide primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGGAGGTGATCCAGCC-3') were used to amplify the 16S rRNA gene. The PCR mixture was prepared as follows: 5 uL of DNA teplate, 36.3 µL of bi-distilled water, 5 µL of Taq Polymerase Buffer (1x), 2.5 µL of $MgCl_2$ (2.5 mM), 0.5 µL of Mix dNTP (250 µM), 0.5 µL Taq Polymerase (2.5 U/50 µL), 0.1 µL of Primer 1 (0.2 µM), 0.1 µL of Primer 2 (0.2 µM) (Alfonzo et al., 2012).

The amplification program was conducted using a thermacycler (PTC-100 MJ-Research Inc., Watetrtown, MA, USA). The program included one initial denaturation step of 95°C for 3 minutes, 30 cycles of amplification (94°C for 1 minute, 54°C for 45 seconds, 72°C for 2 minutes) and a final extension step of 7 minutes at 72°C (Pepe et al., 2013).

The UV visualization was conducted by agarose (1.5% wt/vol) gel electrophoresis at 100 V for 1 h in TBE 1X (Trizma base, boric Acid, EDTA 0.5M, pH = 8), using such marker l'1 Kb DNA ladder plus (Invitrogen), The PCR products were purified by using a QIAquick gel extraction kit (Qiagen S.p.A., Milan, Italy) and sequenced. The DNA sequences were analysed with the software FinchTV version 1.4 (Geospiza) and compared with the GenBank nucleotide data library using Blast software at the National Centre of Biotechnology Information website (http://www.ncbi.nlm.nih.gov/Blast.cgi).

Multiple nucleotide alignments of nearly full-length 16S rRNA sequences of isolated strains and type strains within each of the defined species were performed using the ClustalWprogram (Ventorino et al., 2015) from MEGA version 4.067. The nucleotide sequences of the type strains were retrieved from the Ribosomal Database Project (RDP - http://rdp.cme.msu.edu/). The phylogenetic tree was inferred by using the Neighbour-Joining method with the Maximum Composite Likelihood model in MEGA4 program with bootstrap values based on 1,000 replications.

3.2.7 **Identification and phylogenetic analysis of yeasts strains**

Yeasts Yeast wild type isolates were identified by molecular method (ITS1-5.8S-ITS2 rDNA region sequencing). Genomic DNA was extracted and purified by InstaGeneTM Matrix (Bio-Rad Laboratories, Hercules, CA, USA) according to the supplier's recommendations.

Approximately 50 ng of DNA was used as the template for PCR assays. Synthetic oligonucleotide primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used to amplify the ITS1, ITS2 and 5.8S region rDNA (White et al., 1990).

The PCR mixture was prepared as follows: 2 uL of DNA teplate, 39.5 µL of bi-distilled water, 5 µL of Taq Polymerase Buffer (1x), 2.5 µL of $MgCl_2$ (50 mM), 0.5 µL of Mix dNTP (250 µM) , 0.25 µL Taq Polymerase (5 U μL^{-1}), 0.125 µL of Primer 1 (0.1 mM), 0.125 µL of Primer 2 (0.1 mM) (White et al., 1990).

The amplification program was conducted using a thermacycler (PTC-100 MJ-Research Inc., Watetrtown, MA, USA). The program included one initial denaturation step of 95°C for 3 minutes, 40 cycles of amplification (94°C for 1 minute, 56°C for 2 minutes, 72°C for 2 minutes) and a final extension step of 10 minutes at 72°C (White et al., 1990).

The UV visualization was conducted by agarose (1.5% wt/vol) gel electrophoresis at 100 V for 1 h in TBE 1X (Trizma base, boric Acid, EDTA

0.5M, pH = 8), using such marker l'1 Kb DNA ladder plus (Invitrogen), The PCR products were purified by using a QIAquick gel extraction kit (Qiagen S.p.A., Milan, Italy) and sequenced. The DNA sequences were analysed with the software FinchTV version 1.4 (Geospiza) and compared with the GenBank nucleotide data library using Blast software at the National Centre of Biotechnology Information website (http://www.ncbi.nlm.nih.gov/Blast.cgi). Multiple nucleotide alignments of nearly full-length ITS1-5.8S-ITS2 region rDNA sequences of isolated strains and type strains within each of the defined species were performed using the ClustalWprogram (Ventorino et al., 2015) from MEGA version 4.067. The nucleotide sequences of the type strains were retrieved from the Ribosomal Database Project (RDP - http://rdp.cme.msu.edu/). The phylogenetic tree was inferred by using the Neighbour-Joining method with the Maximum Composite Likelihood model in MEGA4 program with bootstrap values based on 1.000 replications.

3.2.8 **Statistical Analyses**

One-way ANOVA followed by Tukey's HSD post hoc for pairwise comparison of means (at P <0.05) was used to assess the difference in the enzymatic activities of isolated strains such as I_{CMC}, I_{PEC} and Azo-CMCase. Statistical analyses were performed using SPSS 13.0 statistical software package (SPSS Inc., Cary, NC, USA).

3.3 Results

3.3.1 **Physicochemical measurement**

The temperature values observed in the piles were approximately 24°C during the first 45 days. This value increased up to about 29°C after 90 days of biodegradation. The values of a_w ranged from to 0.91 to 0.99.

3.3.2 **Microbial count and isolation**

The media used in this work allowed differentially to enumerate and isolate microorganisms involved in the biomasses conversion.

At the beginning, the aerobic microorganisms involved in the carbon cycle showed a microbial concentration comprised between 6.86±0.09 and 8.91±0.22 log CFU g^{-1}, while the anaerobic populations were comprised between 6.90±0.14 and 7.74±0.10 log CFU g^{-1}. However, microbial groups in *E. camaldulensis* were 1 log CFU g^{-1} smaller than those found in *A. donax* and *P. nigra* biomasses, except for the Endo-cellulolytic. Yeasts and moulds population reached concentrations between 4.48±0.18 and 7.3±0.12 log CFU g^{-1} (Table 4).

Table 4. Enumeration (Log CFU g^{-1}) of microbial groups immediately after the preparation of different lignocellulosic biomasses piles.

	T0		
	Arundo donax	***Populus nigra***	***Eucalyptus camaldulensis***
Hemicellulolytic	8.32±0.10	8.65±0.30	7.18±0.06
Pectinolytic	8.53±0.12	8.46±0.10	6,86±0.09
Aerobic Endo-cellulolytic	8.40±0.08	8.86±0.15	8,91±0.22
Aerobic Exo-cellulolytic	8.38±0.11	8.57±0.06	7.15±0.04
Anaerobic Endo-cellulolytic	7.40±0.10	7.74±0.10	6.90±0.14
Anaerobic Exo-cellulolytic	7,28±0.03	7.54±0.10	6.90±0.03
Ligninolytic	7.53±0.01	7.41±0.24	7.23±0.55
Yeast	6.00±0.20	7.3±0.12	6.00±0.66
Mould	6.30±0.08	4.48±0.18	5.28±0.02

Values are means ± Standard Deviation of three replicate counts.

At T1 in underwood condition, aerobic microbial concentration of piles was between 7.78±0.63 and 9.93±0.56 log CFU g^{-1}, while in open field was between 7.40±0.42 and 9.48±0.04 log CFU g^{-1}, with 1 log higher in *A. donax* compared to other biomasses. Anaerobic microbial concentration was between 7.07±0.30 and 8.81±0.21 log CFU g^{-1}, in each condition the best results were observed in *A.*

donax. Yeasts and moulds population reached concentrations between 5.00±0.26 and 8.70±0.18 log CFU g^{-1}. In particular, yeasts grew only on *P. nigra* (Table 5).

Table 5. Enumeration (Log CFU g^{-1}) of microbial groups after 45 days of natural biodegradation of different lignocellulosic biomasses.

	T1C*			**T1SB+**		
	A. donax	***P.nigra***	***E. camaldulensis***	***A. donax***	***P.nigra***	***E. camaldulensis***
Hemicellulolytic	9.30±0.20	8.46±0.33	8.18±0.05	9.93±0.56	8.00±0.46	8.46±0.20
Pectinolytic	8.99±0.26	8.23±0.85	8.11±0.29	8.73±0.25	8.15±0.11	8.70±0.20
Aerobic Endo-cellulolytic	9.48±0.04	8.23±0.20	8.28±0.36	8.85±0.09	8.08±0.20	18.20±0.27
Aerobic Exo-cellulolytic	9.32±0.11	8.34±0.20	8.52±0.22	8.81±0.13	8.83±0.26	8.30±0.58
Anaerobic Endo-cellulolytic	8.74±0.55	7.40±0.78	7.93±0.20	8.95±0.45	7.39±0.29	7.55±0.16
Anaerobic Exo-cellulolytic	8.81±0.21	7.30±0.11	7.78±0.20	7.30±0.07	7.07±0.30	7.47±0.13
Ligninolytic	8.48±0.03	7.40±0.42	8.00±0.10	8.00±0.66	7.78±0.63	9.36±0.85
Yeast	-	5.00±0.26	-	-	5.00±0.19	-
Mould	8.70±0.18	6.68±0.01	7.08±0.63	7.95±0.48	7.78±0.78	7.10±0.33

*Open field (the biomass piles were placed on an agronomic soil without any coverage shady trees) +Underwood (the piles were placed under oak trees). Values are means ± Standard Deviation of three replicate counts.

After 90 days of biodegradation in both condition aerobic microbial concentration was between 7 and 9 log CFU g^{-1}, higher than anaerobic concentration (6 and 7 log CFU g^{-1}). Moulds concentration was between 7 and 8 log CFU g^{-1}. Yeasts were not detected (Table 6).

Table 6. Enumeration (Log CFU g^{-1}) of microbial groups after 90 days of natural biodegradation of different lignocellulosic biomasses.

	T2C*			**T2SB⁺**		
	A. donax	***P.nigra***	***E. camaldulensis***	***A. donax***	***P.nigra***	***E. camaldulensis***
Hemicellulolytic	8.67±0.11	7.92±0.74	7.88±0.06	8.83±0.05	8.30±0.25	8.93±0.92
Pectinolytic	8.54±0.09	8.60±0.36	7.70±0.69	8.60±0.02	7.85±0.11	8.20±0.55
Aerobic Endo-cellulolytic	8.90±0.22	8.46±0.33	8.26±0.58	9.60±0.05	8.51±0.10	8.40±0.08
Aerobic Exo-cellulolytic	8.70±0.96	8.00±0.19	8.30±0.04	8.64±0.15	8.30±0.10	8.30±0.23
Anaerobic Endo-cellulolytic	7.48±0.05	6.85±0.03	6.28±0.11	7.30±0.29	6.97±0.65	7.34±0.20
Anaerobic Exo-cellulolytic	6.95±0.52	6.70±0.54	6.11±0.22	6.75±0.38	6.54±0.73	6.32±0.14
Ligninolytic	8.04±0.21	7.54±0.34	7.34±0.22	8.38±0.08	7.18±0.11	7.34±0.22
Yeast	-	-	-	-	-	-
Mould	8.00±0.32	7.18±0.27	6.90±0.20	7.70±0.66	8.30±0.33	7.40±0.84

*Open field (the biomass piles were placed on an agronomic soil without any coverage shady trees) ⁺Underwood (the piles were placed under oak trees). Values are means ± Standard Deviation of three replicate counts.

We proceeded to the isolation and purification of 1291 of eukaryotes (36.95%) and prokaryotes (63.05%) microorganisms. In particular, 206 microorganisms were isolated as putative Hemicellulolytic, 169 as putative Pectinolytic, 231 as putative aerobic Endo-cellulolytic, 200 as putative aerobic Exo-cellulolytic, 118 as putative anaerobic Endo-cellulolytic, 99 as putative anaerobic Exo-cellulolytic and 134 as putative Ligninolytic. 23 yeasts and 112 moulds were isolated on YPD with chloramphenicol. Prokaryotic microorganisms were in higher concentration in all microbial groups and in all biomasses. Moreover, more than 400 isolates were obtained from each pile, although the *A. donax* biomass allowed obtaining the highest number of isolates (448) (table 7).

Table 7. Number and percentage of prokaryotes and eukaryotes isolated from lignocellulosic biomasses.

	A. donax			*P. nigra*			*E. camaldulensis*			Total	%Total	%Total
	Isolates	%Eukaryote	%Prokaryote	Isolates	%Eukaryotes	%Prokaryotes	Isolates	%Eukaryotes	%Prokaryotes	Isolates	Eukaryotes	Prokaryotes
Hemicellulolytic	**73**	**38.67**	**61.33**	**71**	**32.26**	**67.74**	**62**	**43.90**	**56.10**	**206**	**37.86**	**62.14**
Pectinolytic	**52**	**44.23**	**55.77**	**45**	**28.89**	**71.11**	**72**	**41.67**	**58.33**	**169**	**44.97**	**55.03**
Aerobic Endo-cellulolytic	**84**	**39.28**	**60.72**	**73**	**19.18**	**80.82**	**74**	**39.19**	**60.81**	**231**	**32.90**	**67.10**
Aerobic Exo-cellulolytic	**71**	**43.66**	**56.34**	**66**	**12.12**	**87.88**	**63**	**41.27**	**58.73**	**200**	**32.50**	**67.50**
Anaerobic Endo-cellulolytic	**49**	**0**	**100.00**	**36**	**0**	**100.00**	**33**	**0**	**100.00**	**118**	**0**	**100.00**
Anaerobic Exo-cellulolytic	**26**	**0**	**100.00**	**43**	**0**	**100.00**	**30**	**0**	**100.00**	**99**	**0**	**100.00**
Ligninolytic	**46**	**69.56**	**30.44**	**48**	**62.50**	**37.50**	**40**	**55.00**	**45.00**	**134**	**62.68**	**37.32**
Yeast	**9**	**100.00**	**0**	**10**	**100**	**0**	**3**	**100.00**	**0**	**22**	**100.00**	**0**
Mould	**38**	**100.00**	**0**	**31**	**100.00**	**0**	**43**	**100.00**	**0**	**112**	**100.00**	**0**
Total	**448**	**43.30**	**56.70**	**423**	**30.50**	**69.50**	**420**	**36.67**	**63.33**	**1291**	**36.95**	**63.05**

3.3.1 **Characterization of microorganisms for enzymatic activities**

To establish the number of putative multifunctional-degrading microorganisms, all isolates obtained from different selective and differential were assayed for different enzymatic activities using the methods described above.

About 90% of the microorganisms isolated as potential hemicellulolytic showed xilanase activity (Figure 5). Moreover, most percentage of isolates with xilanase activity was obtained from *A. donax* and *P. nigra*. A lot of the isolates showed endo-cellulase, exo-cellulase, cellobiase, xylanase, peroxidase and pectinase activities, while few demonstrated ligninase and laccase activities (Table 8).

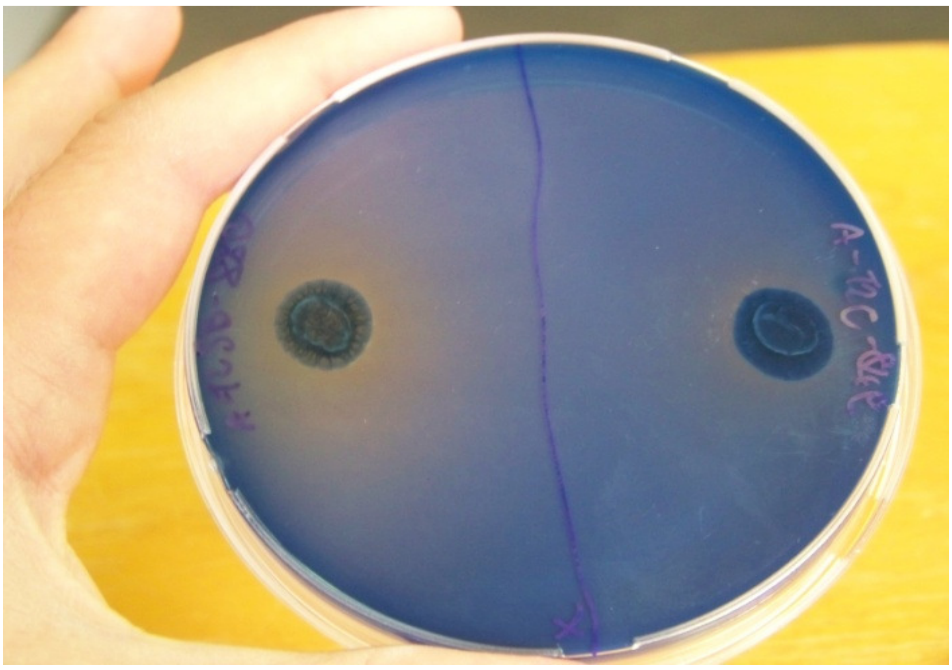

Figure 5. Hemicellulase activity on LB agar medium added of 0.05% Remazol brilliant blue-R and 0.5% of Xylan solution. The hydrolysis of Xylan was detected by observing a clear zone around the colonies.

Table 8. Enzymatic activities of putative hemicellulolytic isolates.

Enzymatic Activity	*A. donax*		*P. nigra*		*E. camaldulensis*		Total isolates	%Total Positive
	Isolates	%Positive	Isolates	%Positive	Isolates	%Positive		
Endo-cellulase	73	79.45	71	64.79	62	51.61	206	66.02
Exo-cellulase		56.16		78.87		69.35		67.96
Cellobiase		41.10		33.80		51.61		41.75
Xilanase		94.52		91.55		82.26		89.81
Pectinase		68.49		52.11		37.10		53.40
Ligninase (*A. donax* + Guaiacol)		5.48		2.82		0		2.91
Ligninase (Lignin+ Guaiacol)		0		0		1.61		0.49
Peroxidase		57.53		39.44		20.97		40.29
Laccase		5.48		8.45		1.61		5.34

40% of microorganisms, isolated as putative pectinolytic, showed enzymatic activity (Figure 6), with similar percentages recorded between the different biomasses. Many isolates proved endo-cellulase, exo-cellulase, cellobiase, xilanase activities, while the lignin degrades ability was expressed from a few isolates (Table 9).

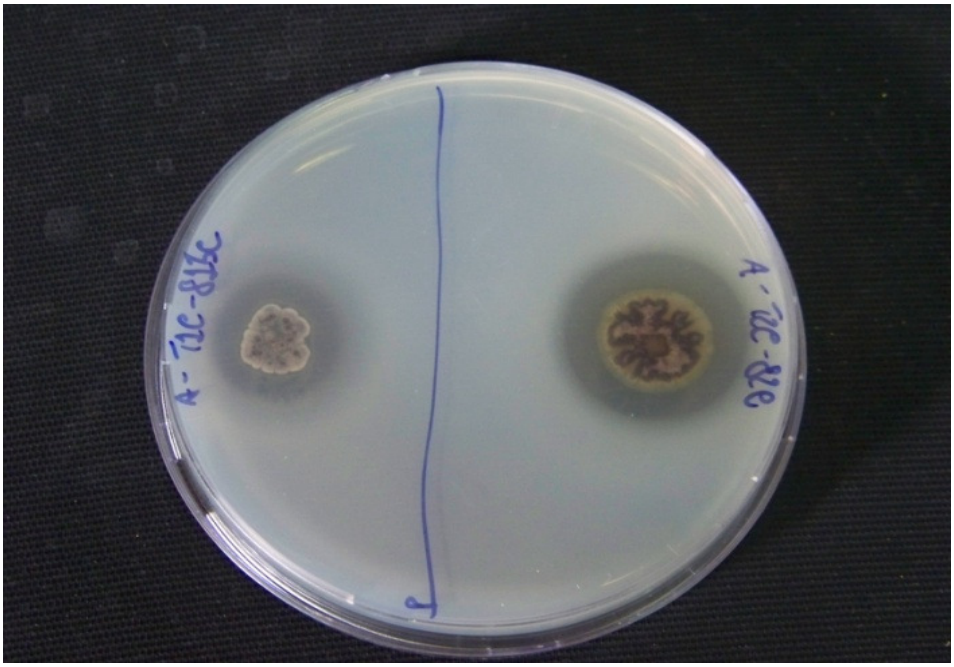

Figure 6. Pectinase activity on basal medium added 5 g L^{-1} of Pectin. After incubation, the plates were flooded with 1% of hexadecyltrimethylammonium bromide solution that plummeted the intact pectin. Pectinase activity was detected by developing hydrolysis halo around the colonies.

Table 9. Enzymatic activities of putative pectinolytic isolates.

Enzymatic Activity	*A. donax*		*P. nigra*		*E. camaldulensis*		Total isolates	%Total Positive
	isolates	%Positive	isolates	%Positive	isolates	%Positive		
Endo-cellulase		67.31		48.89		56.94		57.99
Exo-cellulase		80.77		80.00		88.89		84.02
Cellobiase		63.46		33.33		54.17		51.48
Xilanase		94.23		80.00		80.95		84.61
Pectinase		48.08		37.80		36.11		40.23
Ligninase (*A. donax* + Guaiacol)	52	9.61	45	0.00	72	1.34	169	3.55
Ligninase (Lignin+ Guaiacol)		0		0		2.77		1.18
Peroxidase		0		0		0		0
Laccase		7.69		6.67		12.50		9.47

53.38% of 231 isolates on CMC Agar showed endo-cellulase activity (Figure 7). Most of isolates with endo-cellulase activity were obtained from *A. donax.* A lot of the isolates showed also exo-cellulase, cellobiase, xylanase and pectinase activities, while few demonstrated ligninase, peroxidase and laccase activities (Table 10).

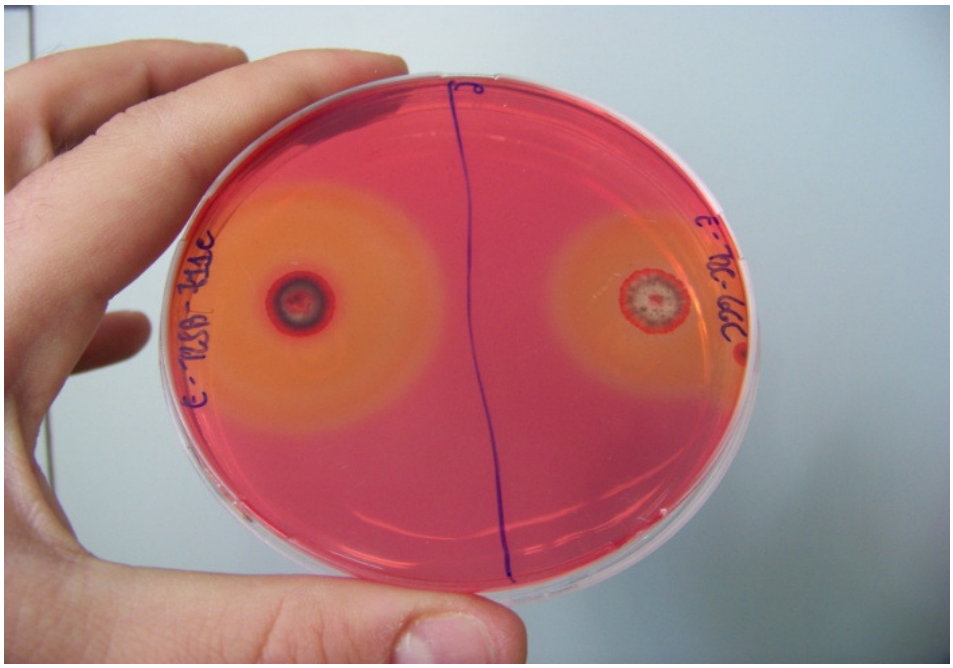

Figure 7. Endo-celluase activity on CMC agar. After incubation, the plates were stained with 0.1% Congo red solution followed by washing with 5 M NaCl. The hydrolysis of CMC was detected by observing halo around the colonies.

Table 10. Enzymatic activities of putative endo-cellulolytic isolates.

Enzymatic Activity	***A. donax***		***P. nigra***		***E. camaldulensis***		**Total Isolates**	**%Total Positive**
	Isolates	**%Positive**	**Isolates**	**%Positive**	**Isolates**	**%Positive**		
Endo-cellulase		71.43		31.51		55.41		53.68
Exo-cellulase		61.64		38.09		45.94		48.05
Cellobiase		61.90		54.80		43.24		53.68
Xilanase		75.00		41.10		51.36		56,71
Pectinase		77.38		31.51		44.59		51.95
Ligninase (*A. donax* + Guaiacol)	84	1.19	73	0	74	0	231	0.43
Ligninase (Lignin+ Guaiacol)		1.19		0		0		0,43
Peroxidase		11.90		6.84		6.75		8.65
Laccase		3.57		4.11		4.05		3.89

The biotechnological characterization of 200 putative exo-cellulolytic isolates was carried out. 93.50% of the isolates proved exo-cellulase activity (Figure 8). Several isolates showed also endo-cellulase, xilanase, pectinase and cellobiase activities. All ligninase activities were detected in *E. camaldulensis* isolates indeed only peroxidase or laccase activity was detected in *P. nigra* or *A. donax* isolates, respectively (Table 11).

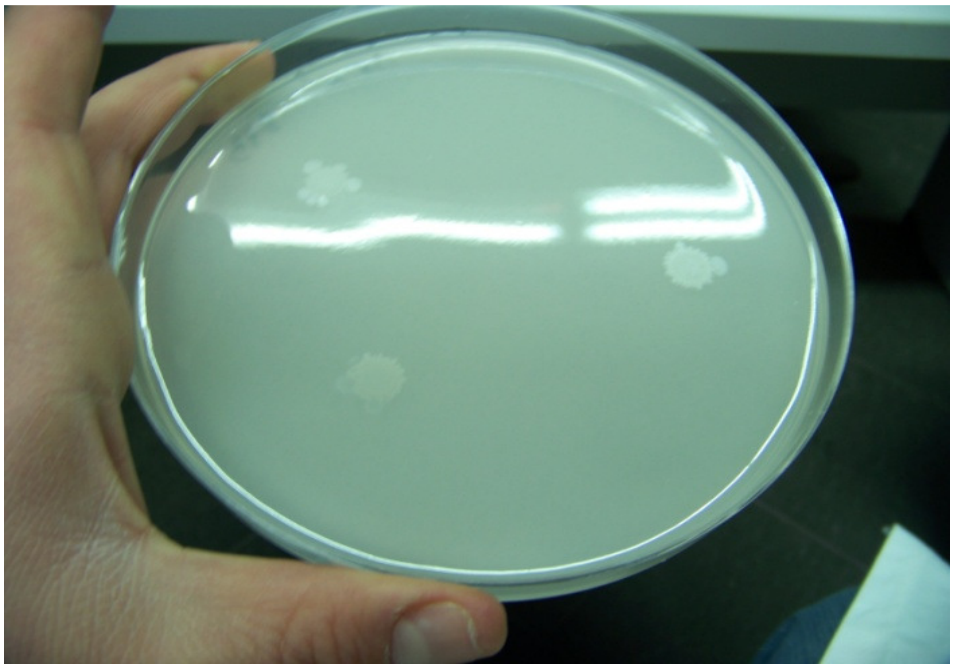

Figure 8. Exo-celluase activity was estimated on minimal medium agar added 10 g L^{-1} of Avicel. The hydrolysis of medium was detected by development of microbial colonies.

Table 11. Enzymatic activities of putative exo-cellulolytic isolates.

Enzymatic Activity	***A. donax***		***P. nigra***		***E. camaldulensis***		**Total Isolates**	**%Total Positive**
	Isolates	**%Positive**	**Isolates**	**%Positive**	**Isolates**	**%Positive**		
Endo-cellulase		54.93		53.03		58.73		55.50
Exo-cellulase		95.77		96.97		87.30		93.50
Cellobiase		53.52		65.15		66.67		61.50
Xilanase		94.36		90.91		84.13		88.50
Pectinase		35.21		25.76		23.81		28.50
Ligninase (*A. donax* + Guaiacol)	71	0	66	0	63	4.76	200	1.50
Ligninase (Lignin+ Guaiacol)		0		0		3.17		1.00
Peroxidase		0		10.61		3.17		4.50
Laccase		12.67		0		12.70		8.50

Some of the 134 isolates as potential ligninolytics showed ligninase (Figure 9), peroxidase (Figure 10) and laccase (Figure 11) activities. Laccase activity detected in isolates of all vegetable biomasses, whereas peroxidase and ligninase activities were showed only for microorganisms isolated from *P. nigra* and *E. camaldulensis*. Furthermore, most isolates proved also endo-cellulase, exo-cellulase, cellobiase, xilanase and pectinase activities (Table 12).

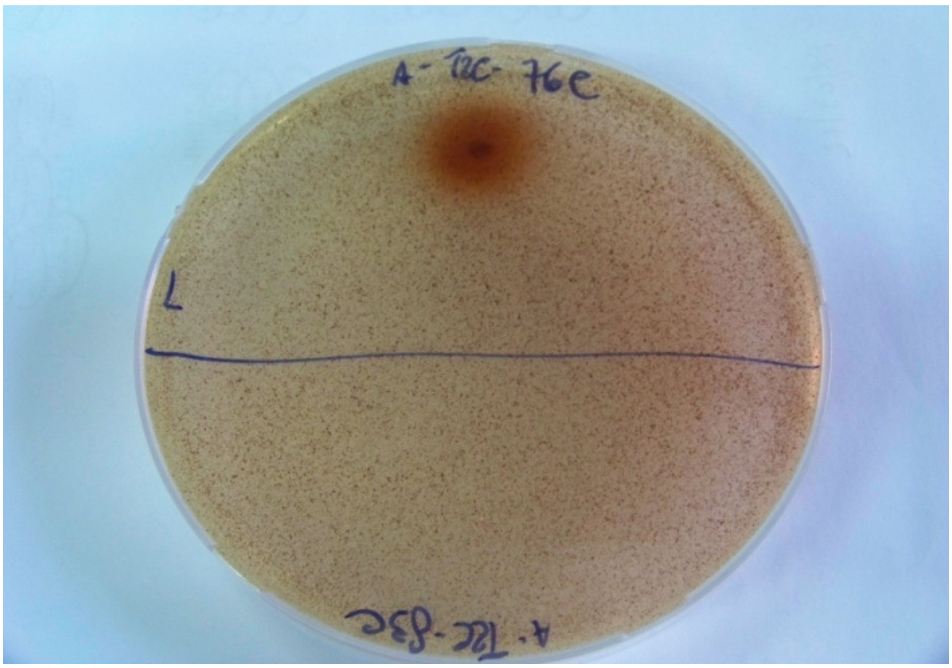

Figure 9. Ligninase activity was detected on CGA culture medium added powder from *A.* donax or lignin alkali. A reddish brown halo around a colony indicates the successful oxidation of guaiacol by ligninolytic enzymes.

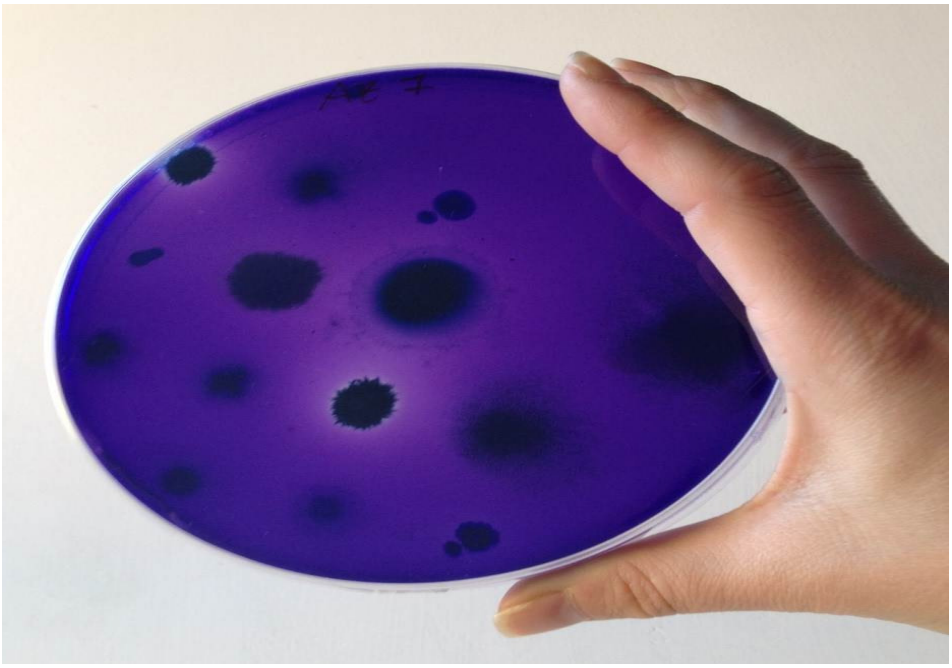

Figure 10. Peroxidase activity on Azure-B agar. Peroxidase activity was detected by developing oxidation halo around the colonies.

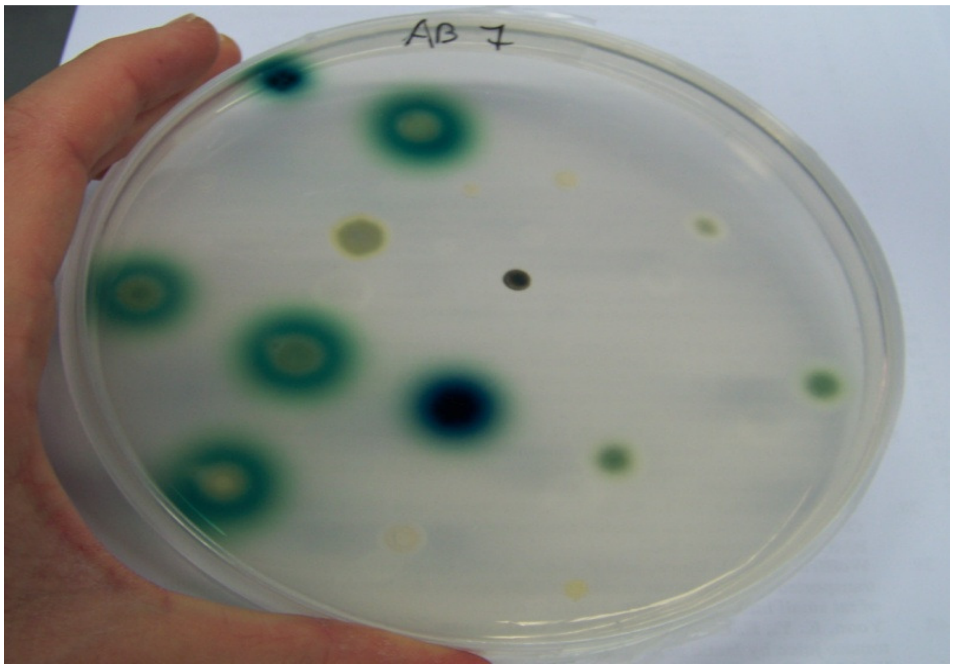

Figure 11. Laccase activity on LBM agar medium added ABTS. A green colour in the medium indicated the presence of laccase activity.

Table 11. Enzymatic activities of putative ligninolytic isolates.

Enzymatic Activity	***A. donax***		***P. nigra***		***E. camaldulensis***		**Total Isolates**	**%Total Positive**
	Isolates	**%Positive**	**Isolates**	**%Positive**	**Isolates**	**%Positive**		
Endo-cellulase	46	45.65	48	54.17	40	60.00	134	52.98
Exo-cellulase		76.08		64.58		80.00		73.13
Cellobiase		34.78		20.83		20.00		25.37
Xilanase		60.87		37.50		67.50		54.48
Pectinase		17.39		27.08		22.50		22.39
Ligninase (*A. donax* + Guaiacol)		0		16.67		10.00		8.95
Ligninase (Lignin+ Guaiacol)		0		0		0		0
Peroxidase		0		4.16		2.50		2.23
Laccase		28.26		33.34		42.50		34.33

Due to the complexity in using anaerobic microorganisms in industry, they are not characterized at the moment but they represent an interesting biological source that should be used in specific biotechnological process.

At the end of this part of research were isolated 540 endo-cellulolytic, 678 exo-cellulolytic, 709 hemicellulolytic, 385 pectinolytic and 248 ligninolytic microorganisms (total 1291 isolates) of which 753 showed multi-enzymatic

activities. Some isolates were able to convert all the principal components of vegetable biomasses such as cellulose, hemicelluloses, lignin and pectin.

3.3.2 **Characterization of yeasts for the hexose and pentose sugars fermentation**

19 strains of *S. cerevisiae* tested showed different resistance to thermal stress and growth ability. At 45°C only *S. cerevisiae* NA 231 showed higher resistance with a value of 0.97 O.D. after 3 hours of incubation in which the stationary phase was reached (Figure 12).

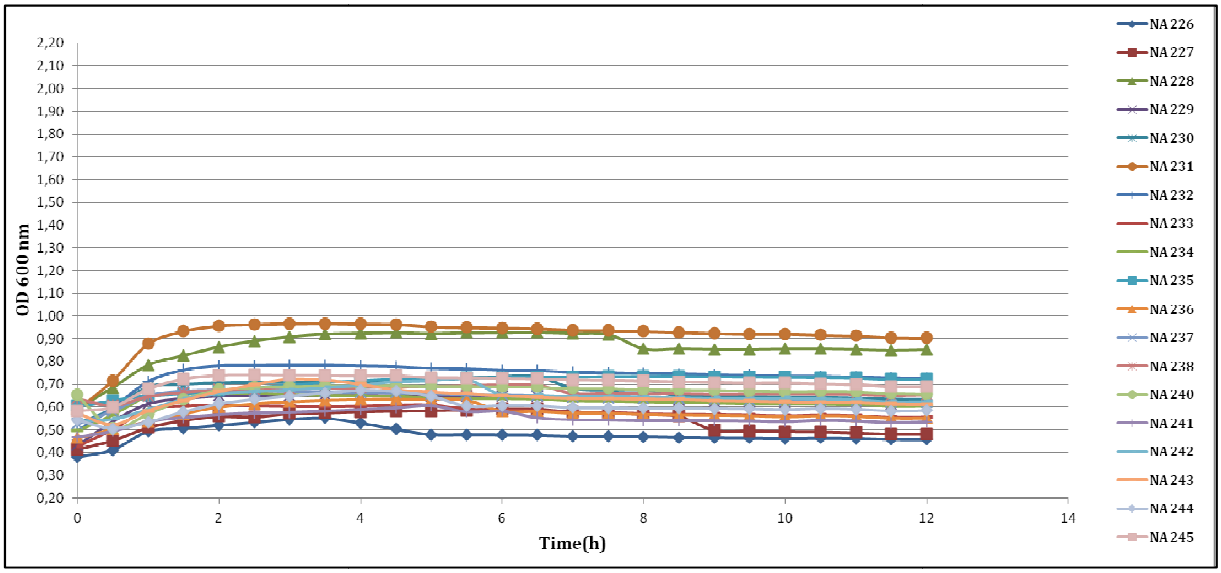

Figure 12. Growth curves at 45°C of *S. cerevisiae* strains (Yeasts Collection of Department of Agricultural Sciences – Division of Microbiology, Naples, Italy).

At 40°C, 15 yeasts strains showed a good resistance to thermal stress. The best performance was shown by *S. cerevisiae* NA227 strain that reached 1.80 O.D. after 10 h of incubation (Figure 13).

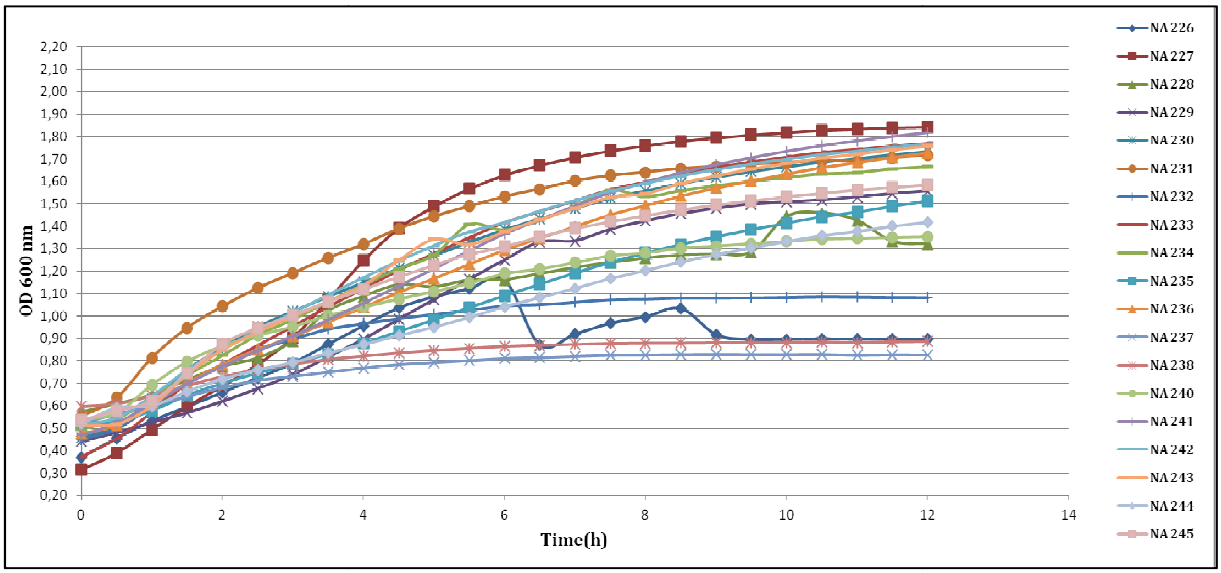

Figure 13. Growth curves at 40°C of *S. cerevisiae* strains (Yeasts Collection of Department of Agricultural Sciences – Division of Microbiology, Naples, Italy).

When the temperature was lower all yeast strains grew rapidly reaching O.D. values that ranged from about 1.6 and 2.1 after 12 h of incubation at 37 and 30 °C when the yeast strains proved the end of exponential phase (Figure 14 and 15).

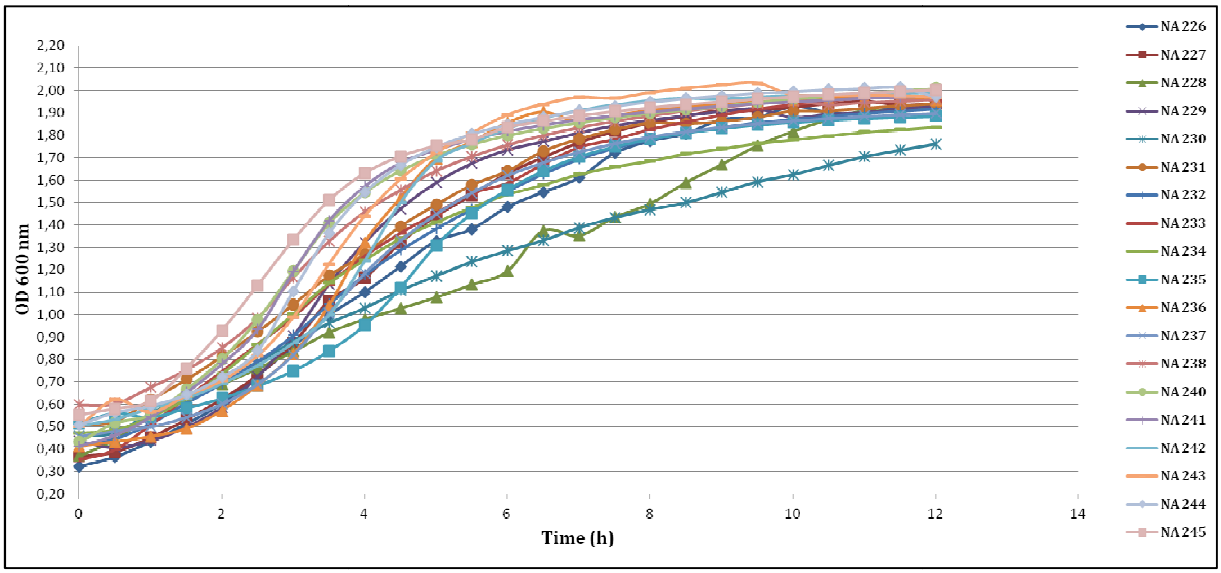

Figure 14. Growth curves at 37°C of *S. cerevisiae* strains (Yeasts Collection of Department of Agricultural Sciences – Division of Microbiology, Naples, Italy).

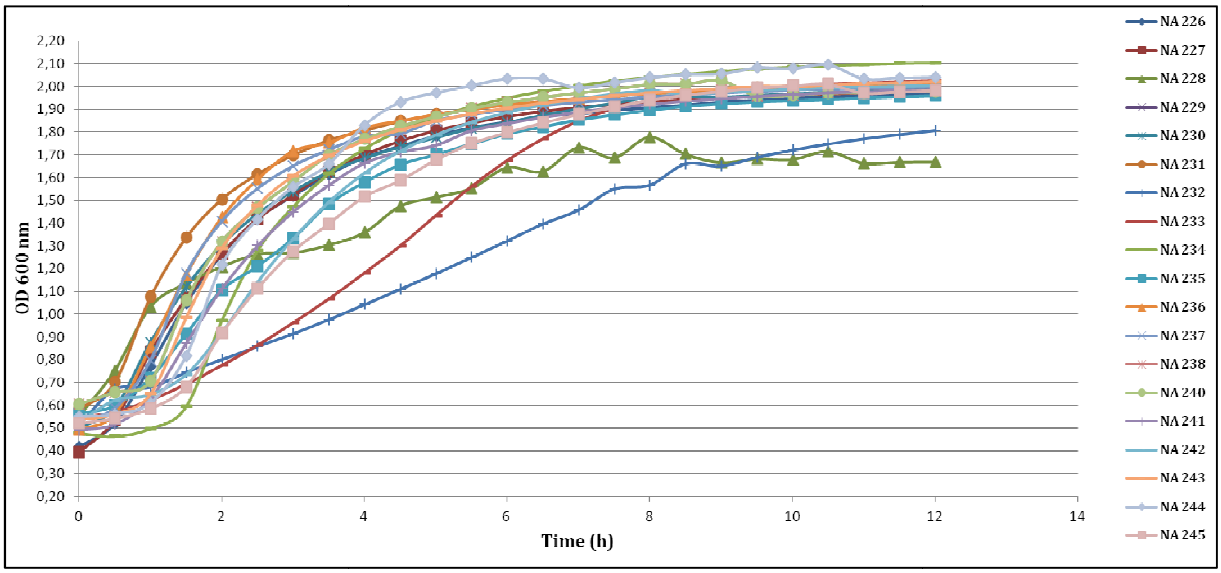

Figure 15. Growth curves at 30°C of *S. cerevisiae* strains (Yeasts Collection of Department of Agricultural Sciences – Division of Microbiology, Naples, Italy).

S. cerevisiae NA227 showed a great resistance to thermal stress and a high ability to grow at different temperatures, thus, it was selected for the fermentation tests using hydrolyzed pretreated of lignocellulosic biomasses.

Seventy-six non-*Saccharomyces* strains and eighteen wild type isolates were capable to grow in minimal medium with xylose as sole carbon source.

Only *Cyberlindnera* (C.) *maclurae* E41L wild type, isolated from *Eucalyptus camaldulensis* chipped, and *P. caribbica* NS117 of the Yeasts Collection of Department of Agricultural Sciences – Division of Microbiology, Naples, Italy, showed the ability to ferment xylose in ethanol. In particular, *C. maclurae* E41L and *P. caribbica* NS117 produced higher amount of ethanol than *Candida shehatae* (CBS5853, CBS, Utrecht, Netherlands) and *Spathaspora passalidarum* (CBS10155, CBS, Utrecht, Netherlands) used as positive control (figure 16).

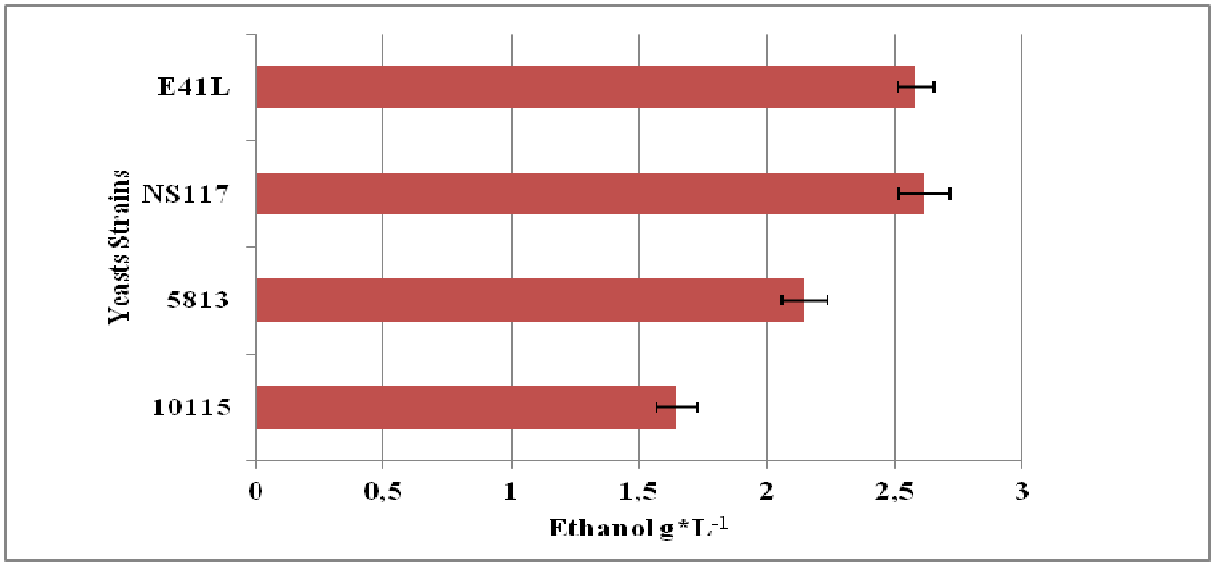

Figure 16. Ethanol produced to xylose fermentation in 96 h by *C. maclurae* E-T5SB-41L, *P. caribbica* NS117, *Candida shehatae* (CBS5853, positive control) and *Spathaspora passalidarum* (CBS10155, positive control) (error bars = ± Standard Deviation of triplicate fermentation).

15 wild type isolated from *P. nigra* (P51, P52L, P53L, P54L, P61L, P62L, P63L, PC41L, PC51L, PSB51L, PSB52L, PC53L) and from *E. camaldulensis* (E41L, E42L, E43L) and *P. caribbica* NS117 were able to ferment glucose in ethanol. In particular, *Cryptococcus* (C.) *flavescens* P51L and *P. caribbica* NS117 produced high amounts of ethanol. The yeasts strains isolated from *A. donax* (A51L, A52L, A53L, A54L, A55L, A56L, A57L, A58L, A61L) were not able to ferment glucose (Figure 17).

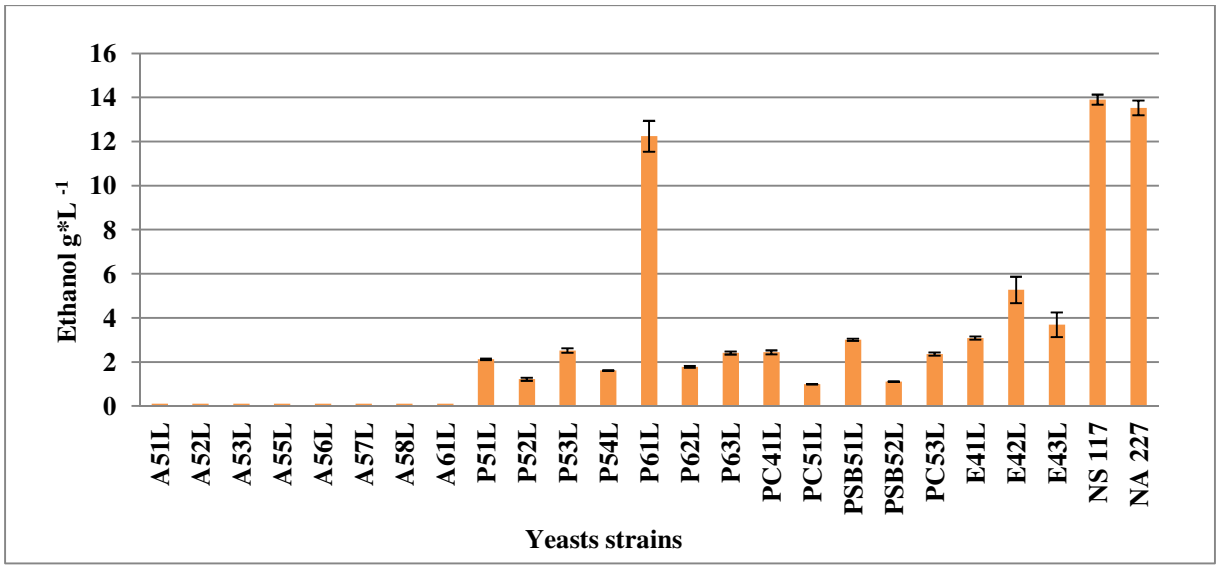

Figure 17. Ethanol produced to glucose fermentation in 24 h by wild type yeasts strains, *P. caribbica* NS117 and *S. cerevisiae* NA227 (positive control) (error bars = ± Standard Deviation of triplicate fermentation).

C. maclurae E41L and *P. caribbica* NS117 were able to ferment glucose and xylose and were used in the tests of co-fermentation.

3.3.3 **Molecular identification and biotechnological characterization of selected bacteria**

Forty aerobic bacterial strains that showed higher cellulase activity (I_{CMC} values >10) and other enzymatic activities were chosen for molecular identification. The results showed a great biodiversity, in fact, 20 genera and 27 different species were found (Table 12). *Curtobacterium spp.* and *Bacillus spp.* were the most representative genera with the species *Curtobacterium citreum* as well as *Bacillus amyloliquefaciens*, *Bacillus licheniformis* and *Bacillus subtilis*, respectively. The bacterial strains showed for 3 to 6 different enzymatic activities. All strains showed endo- and exo-cellulolytic activities and, at same time, expressed cellobiase, xylanase and pectinase (Table 12). The highest endo-cellulase activity was observed in the CE86 strain, identified as *Promicromonospora sukumoe* (I_{CMC} =34, P <0.05), in *Isoptericola variabilis*

CA84b (I_{CMC} =32, *P* <0.05) and *Staphylococcus warneri* CE83 (I_{CMC} =32, *P* <0.05). The first two strains were also able to degrade exo-cellulose, cellobiose and xylan. Moreover, *Isoptericola variabilis* CA84b produced the highest pectinase activity (I_{PEC} =22, P <0.05). *Curtobacterium citreum* CE711 and *Bacillus amyloliquefaciens* CA81 represented the most versatile strains because they possessed six different enzymatic activities. 22 bacterial strains showed, in addition to endo- and exo-cellulases, cellobiase and xylanase activities that were missed in only 10% of the screened strains (*Staphylococcus warneri* CE83, *Lysobacter enzymogenes* CE710, *Lysobacter gummosus* CP72, *Mycobacterium frederiksbergense* CP710b, *Promicromonospora citrea* CE73, *Novosphingobium resinovorum* CE77, *Bacillus subtilis subsp. subtilis* CA816 and *Aurantimonas altamirensis* SBP73). All the strains belonging to the different *Bacillus spp.* and *Curtobacterium spp.* had the highest cellobiase activity. In particular, the genus *Curtobacterium*, represented by the species *Curtobacterium citreum* and *Curtobacterium flaccumfaciens*, was able to grow at least on 4-5 different substrates and, in one case, also showed peroxidase activity. In total, 87.5% of the bacterial strains produced xylanase (Table 12). Similar behavior was observed for pectinase activity as only 15% of the strains possessed an I_{PEC} from 12 to 22. Laccase and peroxidase activities were detected in 5 strains belonging to the species *Curtobacterium citreum* CE711, *Novosphingobium resinovorum* CE77 and CE84, *Lysobacter enzymogenes* CE710 and *Bacillus amyloliquefaciens* CA81. Lignin hydrolysis was not exhibited by any of the strains identified.

Table 12. Identification and enzymatic activities of bacterial strains from different lignicellulosic biomasses.

Strain	Source	C[a]	A[b]	CE[c]	X[c]	P[a]	AZ[c]	AB[c]	L[c]	AD[c]	Identification (% identity)	Accession Number
CP81	*P. nigra*	36	+	+	+	8	-	-	-	-	*Xanthomonas campestris* (100%)	KF040971
CE86	*E. camaldulensis*	34	+	++	+	-	-	-	-	-	*Promicromonospora sukumoe* (99%)	KF057947
CA84b	*A. donax*	32	+	++	+	22	-	-	-	-	*Isoptericola variabilis* (98%)	KF057948
CE83	*E. camaldulensis*	32	+	+	-	-	-	-	-	-	*Staphylococcus warneri* (100%)	KF057949
CP72	*P. nigra*	28	+	-	++	-	-	-	-	-	*Lysobacter gummosus* (99%)	KF040972
CP710b	*P. nigra*	28	+	-	+	12	-	-	-	-	*Mycobacterium frederiksbergense* (99%)	KF057950
CA817	*A. donax*	27	+	+	+	2	-	-	-	-	*Cellulosimicrobium cellulans* (99%)	KF040973
CA812	*A. donax*	27	+	+++	+++	-	-	-	-	-	*Bacillus amyloliquefaciens* (99%)	KF040974
SBP79	*P. nigra*	26	+	++	+	12	-	-	-	-	*Promicromonospora citrea* (99%)	KF057951
SBA88	*A. donax*	26	+	+	+	-	-	-	-	-	*Microbacterium lacus* (99%)	KF057952
SBE74	*E. camaldulensis*	24	+	±	+	-	-	-	-	-	*Enterobacter aerogenes/Kluyvera cryocrescens* (98%)	KF057960
CE77	*E. camaldulensis*	22	+	-	-	-	++	-	-	-	*Novosphingobium resinovorum* (100%)	KF040976
CP77	*P. nigra*	22	+	+	+	4	-	-	-	-	*Xanthomonas orizae* (98%)	KF040975
CE75b	*E. camaldulensis*	22	+	++	+	-	-	-	-	-	*Pediococcus acidilactici* (99%)	KF057953
CP77b	*P. nigra*	22	+	++	±	-	-	-	-	-	*Curtobacterium flaccumfaciens* (99%)	KF057954
SBA76	*A. donax*	22	+	+	+	-	-	-	-	-	*Pantoea ananatis* (99%)	KF057955
SBP71	*P. nigra*	22	+	+	+	-	-	-	-	-	*Schumannella luteola* (97%)	KF057956
CP78b	*P. nigra*	22	+	±	+	12	-	-	-	-	*Mycobacterium frederiksbergense* (99%)	KF057957
CA816	*A. donax*	21	+	+++	-	20	-	-	-	-	*Bacillus subtilis subsp. subtilis* (99%)	KF040977
CA81	*A. donax*	20	+	+++	+++	-	+	+	-	-	*Bacillus amyloliquefaciens* (99%)	KF040978
CA82	*A. donax*	20	+	+++	++	4	-	-	-	-	*Bacillus licheniformis* (99%)	KF040979
CE84	*E. camaldulensis*	20	+	+	+++	-	++	-	-	-	*Novosphingobium resinovorum* (99%)	KF040980
CA81b	*A. donax*	20	+	+++	++	4	-	-	-	-	*Bacillus licheniformis* (99%)	KF040981
CE73b	*E. camaldulensis*	20	+	++	+	-	-	-	-	-	*Pediococcus acidilactici* (99%)	KF057958
SBP73	*P. nigra*	20	+	-	±	-	-	-	-	-	*Aurantimonas altamirensis* (99%)	KF057959
CE85	*E. camaldulensis*	18	+	++	++	2	-	-	-	-	*Isoptericola variabilis* (98%)	KF040982
CE710	*E. camaldulensis*	18	+	-	++	2	-	++	-	-	*Lysobacter enzymogenes* (99%)	KF040983
CP81b	*P. nigra*	18	+	++	++	-	-	-	-	-	*Promicromonospora sukumoe* (99%)	KF040984
CA83	*A. donax*	17	+	++	+	2	-	-	-	-	*Curtobacterium flaccumfaciens* (99%)	KF040985
CA77	*A. donax*	16	+	+	+++	2	-	-	-	-	*Sphingobacterium multivorum* (99%)	KF040986
CE75	*E. camaldulensis*	16	+	++	+	2	-	-	-	-	*Curtobacterium flaccumfaciens* (99%)	KF040987
CE73	*E. camaldulensis*	16	+	+	-	-	-	-	-	-	*Promicromonospora citrea* (99%)	KF040988
CA83b	*A. donax*	15	+	++	++	-	-	-	-	-	*Curtobacterium flaccumfaciens* (99%)	KF040989
CA84	*A. donax*	14	+	+++	+	-	-	-	-	-	*Curtobacterium citreum* (99%)	KF040990
CP713	*P. nigra*	14	+	+++	++	20	-	-	-	-	*Cellulomonas flavigena* (98%)	KF040991
CA818	*A. donax*	13	+	++	+	2	-	-	-	-	*Microbacterium testaceum* (99%)	KF040992
CE78	*E. camaldulensis*	12	+	++	+	2	-	-	-	-	*Curtobacterium citreum* (99%)	KF040993
CP710	*P. nigra*	12	+	+	+	-	-	-	-	-	*Labedella gwakjiensis* (99%)	KF040994
CE711	*E. camaldulensis*	10	+	++	+	2	+	-	-	-	*Curtobacterium citreum* (99%)	KF040995
CP78	*P. nigra*	10	+	+++	+	-	-	-	-	-	*Raoultella terrigena* (99%)	KF040996

Enzymatic activities: C=Endo-cellulase; A=Eso-cellulase; CE=Cellobiase; X=Xylanase; P=Pectinase; AZ=Peroxidase; AB=Laccase; L=Ligninase with Guaiacol and lignin alkal AD=Ligninase with Guaiacol and *Arundo Donax*; [a]$I_{CMC=\text{ diameter of clearing or halo zone/colony diameter}}$ o $I_{PEC=\text{ diameter of clearing or halo zone/colony diameter}}$; [b]growth; [c] – negative; + low intensity; ++ middle intensity; +++ high intensity.

3.3.4 **Phylogenetic analysis of selected bacteria**

16S rRNA gene sequences of the forty cellulolytic bacteria identified as described below were grouped by phylogenetic analysis into five different clusters, generating a *consensus tree*. The clusters were *Actinobacteria* (n=21; 52.5%), *Bacilli* (n=8; 20.0%), *α-Proteobacteria* (n=3; 7.5%), *γ-Proteobacteria* (n=7; 17.5%), *Sphingobacteria* (n=1; 4%) (Figure 18). A phylogenetic tree was generated from the distance data using the Neighbour-Joining method with the Maximum Composite Likelihood model in a MEGA4 Program (Figure 19). The nucleotide sequences of related type strains of different genera were included in the data set. High bootstrap values were observed and indicated significant branching points in the phylogenetic tree. Strains representative of the dominant class of *Actinobacteria* were placed in a cluster with bootstrap values higher than 52% and could be differentiated into nine subclusters of different genera. The bacterial strains primarily belonged to *Curtobacterium* (33.3%) and *Promicromonospora* (19.0%), whereas other genera (*Schumannella*, *Labedella*, *Cellulomonas*, *Cellulosimicrobium*, *Microbacterium*, *Isoptericola*, and *Mycobacterium*) were represented by one or two strains. The strains *Curtobacterium citreum* CE711, *Curtobacterium citreum* CA84 and *Curtobacterium citreum* CE78 showed a sequence similarity of 100%. Representatives of the Bacilli class were placed in three clusters of the genera *Bacillus* (62.5%), *Staphylococcus* (12.5%) and *Pediococcus* (25.0%). The strains belonging to *Bacillus licheniformis* CA82 and CA81b showed a similarity level of 100%, as did *Bacillus amyloliquefaciens* CA812 and CA81 and *Pediococcus acidilactici* CE75b and CE73b (Figure 19). The *α-Proteobacteria* strains were placed in 2 different genera, in which 2 strains of *Novosphingobium resivorum* (CE77 and CE84) exhibited a sequence similarity of 100% with 100% of the bootstrap analysis. The other strain, belonging to *Aurantimonas altamirensis* (SBP73), formed a phylogenetically distinct cluster

with a low 16S rRNA gene sequence similarity (60%) (Figure 19). The representative strains of the *γ-Proteobacteria* cluster belonged to the genera *Xanthomonas* and *Lysobacter* and included the species *Xanthomonas campestris*, *Xanthomonas oryzae*, *Lysobacter gummosus*, and *Lysobacter enzymogenes*. The other genera, identified as the *Enterobacter aerogenes/Klebsiella cryocrescens* group, *Raoultella* and *Pantoea*, were represented by 1 strain. The strain *Sphingobacterium multivorum* CA77 formed a phylogenetically distinct cluster with the type strain of the respective species with 100% of the bootstrap analysis (Figure 19).

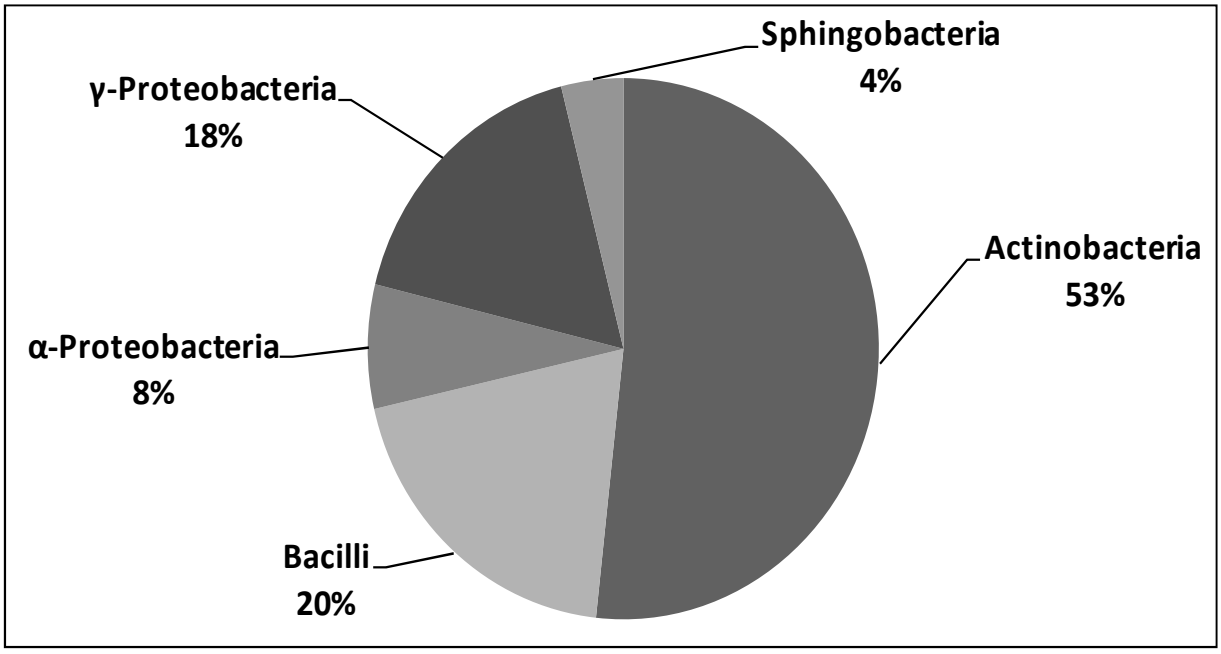

Figure 18. Percentage composition of different phyla of eso- and endo-cellulolytic bacteria isolated from lignocellulosic biomasses on the basis of 16S rRNA gene sequence similarity.

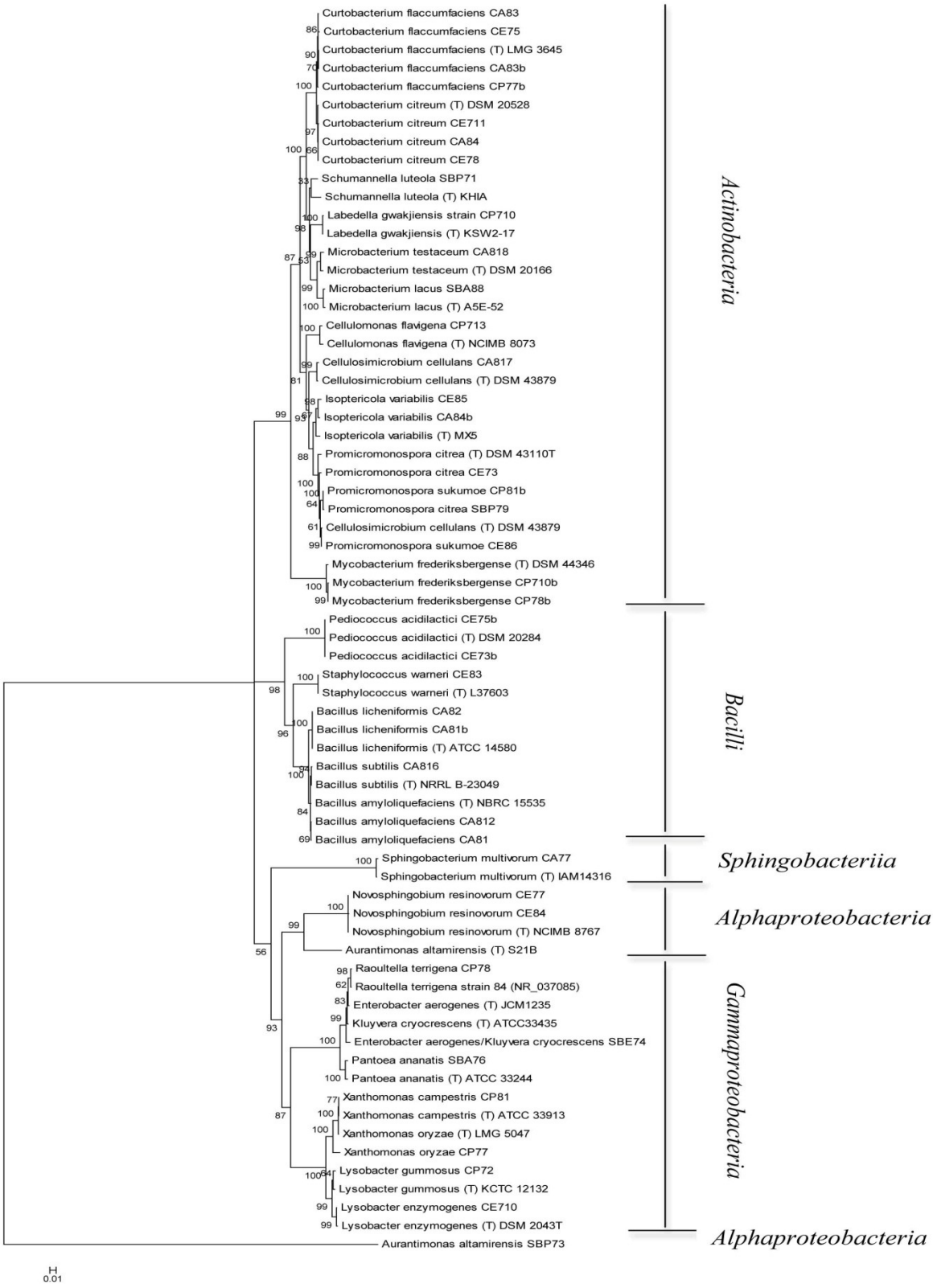

Figure 19. Neighbour-Joining tree based on the comparison of 16S rRNA gene sequences showing the relationships among cellulolytic strains. Bootstrap values (expressed as percentages of 1,000 replications) greater than 50% are given at the nodes. Strains marked with ‘‘(T)’’ represent type strains. The scale bar estimates the number of substitutions per site.

3.3.5 **Molecular identification and phylogenetic analysis of wild type yeasts**

23 Twenty-three yeasts isolated from lignocellulosic biomasses were identified. *Komagataella* (*K.*) *pseudopastoris* isolated from *P. nigra* chipped was the most representative species, one strain, isolated from *P. nigra* chipped, was identified as *Cryptococcus flavescens*. Two strains identified as *C. flavescens*, three as *Rhodotorula* (R.) *sloofiae* and two as *Pseudozyma* (P.) *graminicola* were isolated from *A. donax*. Two *C. maclurae* and 1 of *Williopsis californica* were recovered from woodchips of *E. camaldulensis* (Table 13).

The greater biodiversity was observed in *A. donax* chipped with 3 different species.

Table 13. Identification wild type yeasts isolates from *A. donax*, *P. nigra* and *E. camaldulensis* chipped.

Strain	Source	Identification (% identity)
A51L	*A. donax*	***Rhodotorula slooffiae* (100%)**
A52L	*A. donax*	***Rhodotorula slooffiae* (99%)**
A53L	*A. donax*	***Cryptococcus flavescens* (99%)**
A55L	*A. donax*	***Cryptococcus flavescens* (99%)**
A56L	*A. donax*	***Pseudozyma graminicola* (99%)**
A57L	*A. donax*	***Pseudozyma graminicola* (99%)**
A58L	*A. donax*	***Cryptococcus flavescens* (99%)**
A61L	*A. donax*	***Rhodotorula slooffiae* (100%)**
P52L	*P. nigra*	***Komagataella pseudopastoris* (97%)**
P54L	*P. nigra*	***Komagataella pseudopastoris* (97%)**
P51L	*P. nigra*	***Komagataella pseudopastoris* (97%)**
P53L	*P. nigra*	***Komagataella pseudopastoris* (97%)**
P61L	*P. nigra*	***Cryptococcus flavescens* (97%)**
P 62L	*P. nigra*	***Komagataella pseudopastoris* (97%)**
P-63L	*P. nigra*	***Komagataella pseudopastoris* (98%)**
PC41L	*P. nigra*	***Komagataella pseudopastoris* (98%)**
PC51L	*P. nigra*	***Komagataella pseudopastoris* (97%)**
PSB51L	*P. nigra*	***Komagataella pseudopastoris* (97%)**
PSB52L	*P. nigra*	***Komagataella pseudopastoris* (98%)**
PSB53L	*P. nigra*	***Komagataella pseudopastoris* (98%)**
E41L	*E. camaldulensis*	***Cyberlindnera maclurae* (100%)**
E42L	*E. camaldulensis*	***Williopsis californica* (99%)**
E43L	*E. camaldulensis*	***Cyberlindnera maclurae* (99%)**

The ITS1-5.8S-ITS2 rDNA region sequences of the 23 yeast strains identified as described above were grouped by phylogenetic analysis generating a *consensus tree*. The phylogenetic tree was generated from the distance data using the

Neighbour-Joining method with the Maximum Composite Likelihood model in a MEGA4 Program (Figure 20). The nucleotide sequences of related type strains of different species were included in the data set. Isolates could be grouped into 4 different clusters at a taxonomic level of class, and in particular *Saccharomycetes* (n=14; 60.9%), *Tremellomycetes* (n=4; 17.4%), *Cystobasidiomycetes* (n=3; 13.0%), *Ustilaginomycetes* (n=2; 8.7%) (Figure 20). Strains representative of the dominant class of *Saccharomycetes* could be differentiated into three subclusters of different genera. In particular, the yeast strains primarily belonged to *Komagatella* (47.8%), whereas the genera *Cyberlindnera* and *Williopsis* were represented by 1 or 2 strains, respectively.

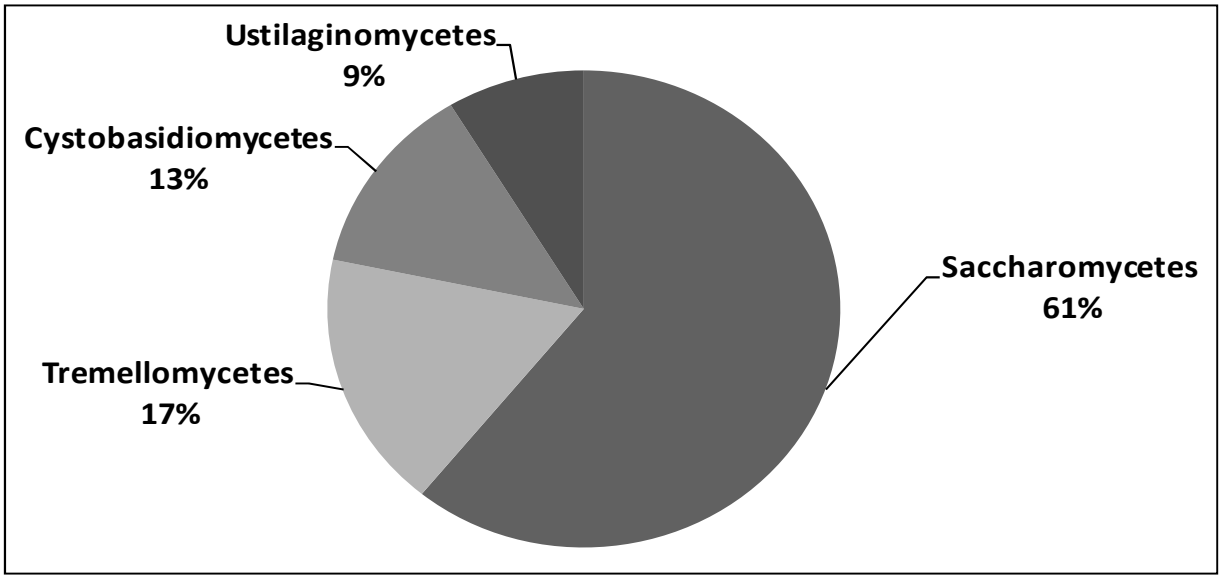

Figure 20. Percentage composition of different classes of yeasts isolated from lignocellulosic biomasses on the basis of ITS1-5.8S-ITS2 rDNA sequences similarity.

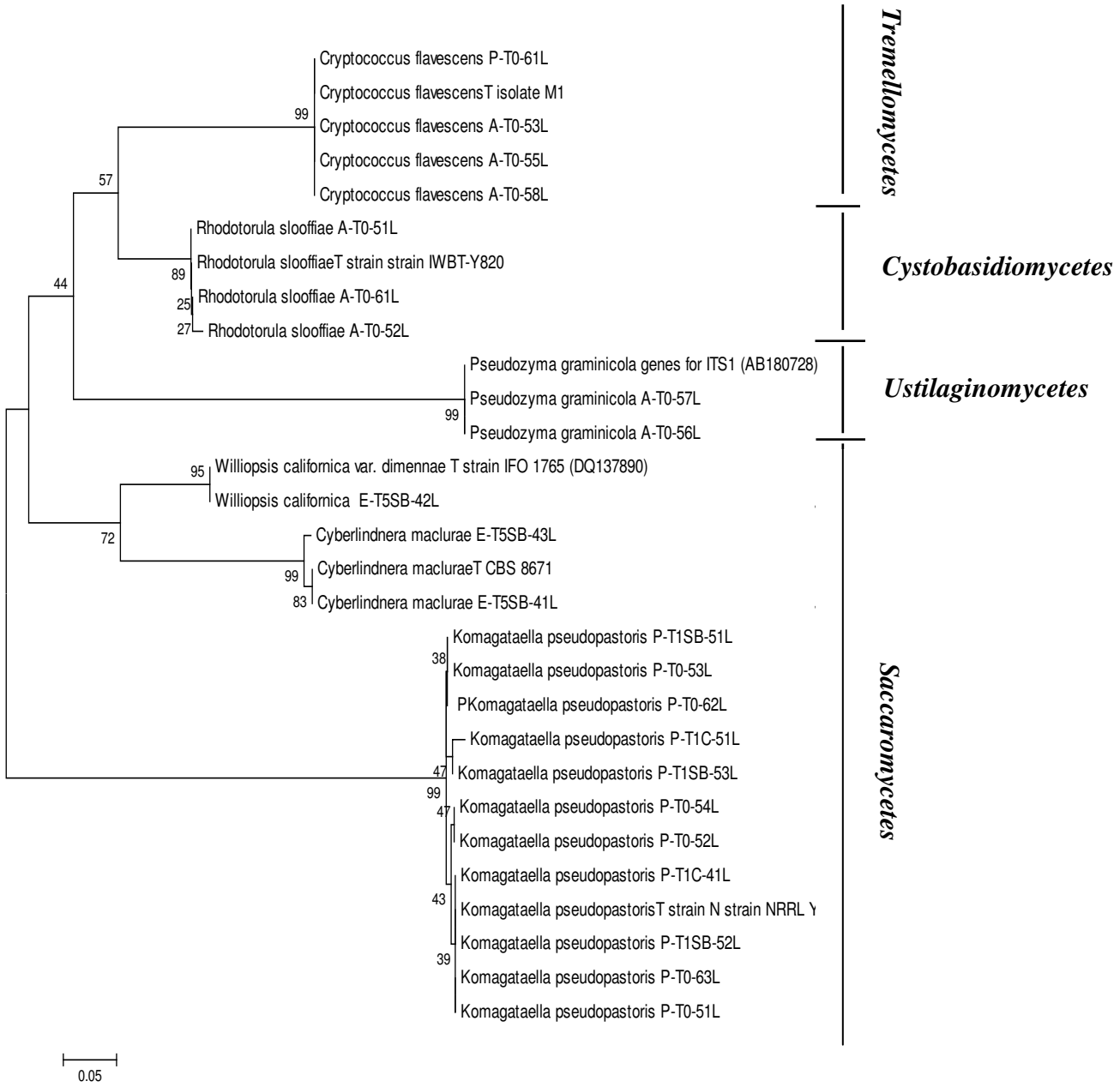

Figure 21. Neighbour-Joining tree based on the comparison of ITS1-5.8S rDNA-ITS2 sequences showing the relationships among yeasts strains. Bootstrap values (expressed as percentages of 1,000 replications) greater than 50% are given at the nodes. Strains marked with ‘‘(T)’’ represent type strains. The scale bar estimates the number of substitutions per site.

3.4 Discussion

In recent years, the competitive production of alternative renewable biofuels has stimulated research into new microrganisms as a source of enzymes for lignocellulosic material conversion. They exhibit several advantages such as a fast growth rate, production of enzymes that are often more effective catalysts

due to less feedback inhibition, and secretion of a complete multi-enzyme system for an efficient conversion of lignocelluloses into fermentable sugars (Amore et al. 2012; Saini and Tewari 2012), that can be metabolized by microorganisms and converted to desired chemical products, including alcohols, fatty acids, organic acids and amino acids in microbial fermentation (Hasunuma et al., 2013). In nature, several hydrolytic enzymes produced by microorganisms, operate in synergy to degrade lignocelluloses (Adsul et al., 2011).

In this context, particular attention must be given to exploring the biodiversity of natural niches so that multifunctional degradative and fermentative migroorganisms can be isolated and characterised.

For these reasons, in this work, the microbial diversity of natural ecosystems, represented by lignocellulosic biomasses of *A. donax*, *E. camaldulensis* and *P. nigra*, was evaluated by culture-independent and culture-dependent approaches. These vegetable species were selected because of their tolerance to environmental conditions of areas not suitable for food crops (Diodato et al., 2009; Fiorentino et al., 2010).

The culture-dependent methodology used here, which was based on a functional approach of detection and isolation to find new lignocellulose-degrading microorganisms strains, provides us with key insight. Special attention to the methods was required to determine the optimal culture and assay conditions. A comparison with another studies (Saini and Tewari 2012; Pepe et al., 2013; Ventorino et al., 2015) revealed that differential substrates containing CMC, Avicel, Xilan and Pectin are effective for the enumeration and isolation of colonies of Endo- and Exo- cellulolytic, Hemicellulolytic and Pectinolytic microrganisms, respectively. Few ligninolytic isolates have been found since a specific pool of enzymes is required to specifically degrade lignin that is characterised by a variable and very resistant composition (Hendriks and Zeeman, 2009). A lot of isolates from the biomasses showed multienzymatic

activities useful to perform the hydrolysis of a complex substrate such as lignocellulose, an important initial step in many technological applications (Van Dyk and Pletschke, 2012) that require the action of different specific enzymes. Moreover, microorganisms with enzymatic multi-activity are very important for commercial enzymatic preparations (Adsul et al., 2011) used in lignocellulosic material conversion.

Since bacterial enzymes are more thermo-stable than those produced by fungi and are known to perform also under alkaline conditions (Bon and Picataggio, 2002; Ferreira-Leitao et al., 2003), 40 prokaryotes were identified. These strains showed both endo- and exo-glucanase activities, confirming that these enzymes synergistically act during the cellulose saccharification. These observations were reinforced by the fact that many of these bacterial strains also possessed cellobiase as well as cellulolytic activities. Xylanase activity was also commonly observed, which is unsurprising because a close correlation between cellulase and xylanase activities has been demonstrated and is due to their coexpression in the same operon (Ventorino et al., 2015).

The Neighbour-Joining phylogenetic method generated a consensus tree that grouped all 16S rRNA gene sequences of the isolated strains into five different clusters at the class level. Culture-dependent data showed similar predominant bacterial classes detected by high-throughput sequencing in the lignocellulosic biomasses, with an abundance of *Actinobacteria*, *Bacilli*, *α-Proteobacteria*, *γ-Proteobacteria* and *Sphingobacteria*. Moreover, the bacteria isolated are dominant players since these microbial strains were isolated from a high serial decimal dilution (10^{-6}-10^{-7}). According to phylogenetic research on cellulose-decomposing bacteria isolated from soil carried out by Ulrich et al., 2008, *Actinobacteria* are the most prevalent bacterial group based on 16S rRNA gene sequences. This cluster included the species *Curtobacterium citreum*, which is able to use up to six different lignocellulose components and that is phylogenetically related to *Microbacterium testaceum* and *Microbacterium*

lacus. In particular, previous studies reported the production of enzymes involved in cellulose and xylan degradation by *Microbacterium* species (Okeke and Lu, 2011). The *Actinobacteria* cluster included other genera involved in lignocellulosic biomass degradation, such as *Cellulomonas*. Akasaka et al., 2003, reported that more than 60% of isolates from rice plant residues was closely related to *Cellulomonas* and involved in their degradation. Our study revealed the production of cellobiase and pectinase activities in *Actinobacteria* members, such as *Cellulosimicrobium cellulans*, *Isoptericola variabilis*, *Promicromonospora sukumoe* and *Promicromonospora citrea*, which belong to the suborder *Micrococcineae*. Many of the representatives of the *Promicrosporaceae* and *Corinebacteriaceae* families can degrade polysaccharides such as cellulose and xylan (Lo et al., 2009; Song and Wei, 2010). The *Bacilli* cluster was primarily represented by the *Pediococcus* and *Bacillus* genera on the basis of 16S rRNA gene sequence analysis. Interesting, Zhao and co-workers (2013), reported the use of a strain of *Pediococcus acidilactici* with high tolerance to temperature and a lignocellulose-derived inhibitor in simultaneous saccharification and fermentation (SSF) for high lignocellulosic lactic acid production. The strains CA812, CA81, CA816, CA82 and CA81b, isolated from the *A. donax* biomass and phylogenetically correlated to the *Bacillus* genus, showed high endo-cellulolytic and multi-enzymatic activities. The capacity of *Bacillus* strains to produce large quantities of extracellular enzymes has placed them among the most important industrial enzyme producers isolated from compost, soil and several other natural habitats (Amore et al., 2013a). In particular, *Bacillus amyloliquefaciens*, *Bacillus subtilis* and *Bacillus licheniformis*, isolated from soil and compost, are able to hydrolyse cellulosic waste-material (Amore et al., 2013b) by both cellulolytic activities (Kim et al., 2012) and multi-enzyme complexes (Jones et al., 2012). The selected bacterial strains could have cross-specificity facilitated by specific or non-specific active sites and also distinct catalytic domains binding to different

substrates (Van Dyk and Pletschke, 2012). *Xanthomonas campestris*, known as a phytopathogeni bacterium, and *Lysobacter gummosus* and *Lysobacter enzymogene*s, potent biocontrol agents that release cellulolytic enzymes such as glucanase (Palumbo et al., 2005), were identified in the phylogenetic group of γ-*Proteobacteria*. Other strains of this class, such as *Enterobacter sp.* and *Pantoea sp.*, although are known as insect-associated bacteria that are able to produce bioactive compounds and digestive enzymes that are responsible for lignocellulose degradation (Anand et al., 2010), they are less attractive for possible biotechnological application since human disease has been reported to be caused by these bacteria as well as *Raoultella terrigena* and *Sphingobacterium multivorum*. The two strains CE77 and CE84, included in the *α-Proteobacteria* cluster and characterised for their peroxidase activity, were closely related to the species *Novosphingobium resinovorum*, previously isolated from soil and studied for its capacity to degrade oil resins (Lim et al., 2005). Interestingly, the strain *Aurantimonas altamirensis* SBP73 showed multienzymatic activity. To our knowledge, this study is the first to report the multienzymatic activity of this specie.

The results demonstrated that the bacterial strains isolated and characterized in this study could represent a very interesting biological source for the conversion of lignocellulose material into products of commercial significance. Moreover, their biotechnological performance could be enhanced by modifying the biotechnological parameters of the fermentation process such as the temperature, pH and solid loading (Van Dyk and Pletschke, 2012).

Another important topic for bioconversion lignocellulosic biomass is the selection of strains able to ferment glucose (C6) and xylose (C5). In fact, the supernatant from enzymatic hydrolysis of lignocelluloses contains both six-carbon (hexoses) and five-carbon (pentoses) sugars (Balat, 2011). The general requirement of an organism to be used for ethanol production is that it should provide high ethanol yield. It should be able to withstand high ethanol

concentrations in order to keep distillation cost low (vonSivers and Zacchi, 1996). In addition, tolerance to high temperature as well as chemical inhibitors and the ability to utilize multiple sugars are essential for SSF applications. Moreover, tolerance towards low pH-values also minimizes the risk of contamination (Olofsson et al., 2008).

The yeast *S. cerevisiae* is one of the well-studied microorganisms for ethanol fermentation (Hasunuma et al., 2013). *S. cerevisiae* displays several advantageous characteristics for use in industrial applications, including its inherent resistance to low pH and various chemical inhibitors (Hasunuma and Kondo, 2011) and hence it is suitable for fermentation of lignocellulosic materials (Olofsson et al., 2008). This yeast specie produces ethanol at a high yield (higher than 0.45 g g^{-1} at optimal conditions) and a high specific rate (up to 1.3 g g^{-1} cell mass h^{-1}) (Verduyn et al., 1990). Moreover, *S. cerevisiae* has a very high ethanol tolerance and some stains are tolerant to a concentration over 100 g L^{-1} in specific fermentation conditions. (Casey and Ingledew, 1986). Furthermore, thermotolerance is a clearly important topic for bioethanol production by *S. cerevisiae* (Olofsson et al, 2008). In this study, since *S. cerevisiae* strains tested showed an elevated fermentative power and a high alcohol tolerance, their resistance to thermal stress was also evaluated. At 30°C and 37°C all *S. cerevisiae* strains tested showed high growth ability, while, at 40°C and 45°C several strains not were able to grow. Even if, *S. cerevisiae* has an optimal growth temperature around 30°C (Limayem and Ricke, 2012), bioethanol production experiments are often performed at a temperature of 37°C (Olofsson et al., 2008) since this is considered the highest temperature that *S. cerevisiae* can tolerate (Sassner et al., 2006). In addition, the temperature of 37°C can be a perfect compromise between the optimal temperatures for the cellulolytic enzymes and the yeast alcoholic fermentation (Rudolf et al., 2005). *S. cerevisiae* specie is capable of efficiently fermenting glucose into bioethanol, but is unable to ferment xylose (Adsul et al., 2011). However, D-xylose is the

second most abundant sugar in lignocellulose. Therefore, the selected strain *S. cerevisiae* NA227 was used in bioethanol production experiment since it showed highest termotolerance among tested strains. The efficient use of lignocellulosic biomass as a substrate for ethanol production requires effective utilization of also D-xylose (Nakamura et al., 2008). Yeasts that produce ethanol from D-xylose have been isolated from various locations, including tree exudates, wood-boring insects, decaying wood, rotten fruit and tree bark (Cadete et al., 2012). The non-*Saccharomyces* yeast strains used in this work showed different metabolic characteristics in sugar fermentation. According to Saucedo-Luna et al. (2011), the strain *P. caribbica* NS117, isolated from Aglianico grape of Montoro Superiore (Avellino, Italy), was able to ferment xylose and glucose to ethanol by fermentation. Interestingly, Duarte and co-workers (2013) reported this specie also as cellobiose degrader that could avoid the inhibition by end-product during hydrolysis and fermentation of the lignocellulosic material (Van Dyk and Pletschke, 2012). Moreover, *P. caribbica* is also reported capable to convert pure xylose into xylitol, commonly used in chemical and pharmaceutical industries (Mukherji et al., 2013), and to synthesize xylolipid biosurfactants that are advantageous over currently used petrochemical-based products (Joshi-Navarea et al., 2014).

All strains isolated in this work from lignocellulosic biomasses were identified as non-*Saccharomyces* yeast. These strains showed different ability in sugar (C5 and C6) fermentation. In particular, the three strains *C. maclurae* E41L, *C. maclurae* E43L and *W. californica* E42L, isolated from *E. camaldulensis* chipped, were able to ferment glucose into ethanol. These results are in according with previously works that reported the ability of these species to ferment C6 sugars in ethanol (Kurtzman, 2000; Ni et al., 2013). Moreover, interestingly, although Minter (2009) reported that the specie *C. maclurae* not able to ferment xylose into ethanol, the strain *C. maclurae* E41L showed also this ability. This result suggests that the xylose fermentative capacity could be a

strain-dependent specific metabolic characteristic. In addition, *W. californica* E42L could be particularly interesting, since other strains of this species are reported as lipase (Ciafardini et al., 2006) and nariginase (Ni et al., 2013) producer. Lipases are extremely versatile because they catalyze numerous different reactions, being widely used in industrial applications, such as in dairy and food manufacture, leather and detergent industry, production of cosmetics and pharmaceuticals (Sharma et al., 2001; Gupta et al., 2002) and biodiesel production (Olkiewicza et al., 2012). Naringinase enzyme has many significant commercial applications in food and pharmaceutical industry (Puri and Banerjee, 2000).

The wild type strains P51L, P52L, P53L, P54L, P62L, P63L, PC41L, PC51L, PSB51L, PSB52L and PC53L, isolated from *P. nigra* chipped, were identified as *K. pseudopastoris*. According to Dlauchy et al. (2003), strains belonged to this specie showed ability to ferment glucose in ethanol, while they did not able to ferment xylose. Furthermore, *K. pseudopastoris* is widely used as a host system for heterologous protein expression in both academia and industry (Hesketh et al., 2013). This characteristic could be used to design recombinant xylose fermenting strains of this yeast specie.

The other specie isolated from *P. nigra* chipped, *C. flavescens* strain P61L ferment glucose but, the two strains *C. flavescens* A53L and A58L, isolated from *A. donax* chipped, were not able to ferment C6 and C5 monosaccharides. Takashima et al. (2003) reported that this specie is unable to ferment sugars. Therefore, the results obtained in this study demonstrate that the ability of *C. flavescens* specie could be strain-dependent specific trait. However, this specie could have other interesting properties since was also reported as producer of lipase enzyme (Mase et al., 2013).

Acording to Fell and co-workers (2000) and Golubev et al. (2007), the strains A51L, A52L and A61L belonged to *R. slooffiae* specie as well as the strains A56L and A57L belonged to *P. graminicola* specie, isolated from *A. donax*

biomass, were able to ferment neither glucose nor xylose. Moreover, *R. slooffiae* specie showed peroxidase (Yang et al., 2013) and pectinase (Minegishi et al., 2006) activities as well as ability to synthesize lipases (Yalçın et al., 2013). These traits are very important in lignocellulose bioconversion (Ventorino et al., 2015) and in biodiesel production (Olkiewicza et al., 2012). In addition, *P. graminicola* specie is likely to have great potential for use in oil-in-water type emulsifiers and/or washing detergents, and would thus facilitate a broad range of applications for the promising yeast biosurfactants (Morita et al., 2008). Moreover, *P. graminicola* strain isolated from plants inhibited the growth of *Ascomycetes* and *Basidiomycetes* (over 270 species of ca. 100 genera) including pathogenic species secreting fungicidal agent (Golubev et al., 2008).

Overall results indicate that the yeasts selected in this work are a potential biological source for the production of second generation biofuels as well as for application in many eco-friendly technologies in food, agricultural and biochemical industries.

3.5 Reference

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Chapter 4 Optimization of the process of second generation bioethanol production from pretreated *A. donax* biomass

4.1 Introduction

In In recent years, climate changes and energy issues have become prominent in public life. In general, almost all economically viable commercial bio-ethanol production relies on the fermentation of sucrose from sugarcane and molasses or glucose derived from starch-based crops such as corn, wheat, and cassava. On the other hand, lignocelluloses are the most abundant and lowest-cost biomass in the world and, thus, are currently being considered as one of the best alternative raw materials for the production of fuel ethanol (Peng and Chen, 2011).

Bioethanol derived from lignocellulosic biomass is a renewable energy source that is being rapidly developed and commercialized as a substitute for fossil fuels in road transport in many countries (Wang et al., 2012). Moreover, lignocellulosics biomasses for ethanol production are a promising way to decrease greenhouse gas emissions (Chu et al., 2013). Sources of lignocellulose include agricultural waste such as corn stover, bagasse, wood, grass, municipal waste and dedicated energy crops such as miscanthus, switchgrass (Gomez et al, 2008), *A. donax* (Ask et al., 2012) *P. nigra* (Guerra et al., 2013) and *Eucalyptus* (McIntosh et al., 2012). *A. donax*, also known as giant reed, is a perennial herbaceous crop native to East Asia that has shown to thrive in Southern Europe. Perennial herbaceous energy crops like *A. donax* have the advantage of not requiring annual reseeding, need less energy input than annual crop land and reduce soil erosion. Although *A. donax* has been discussed as energy crop for some time, there are few studies reporting assessment of the material for lignocellulosic ethanol production (Ask et al., 2012).

The biological degradation of the carbohydrates within the biomass is achieved using multiple enzymes in defined ratios to convert the carbohydrates to their

monomer sugars. This is followed by the fermentation of these sugars into bio-ethanol using different microorganisms such as *S. cerevisiae* and *Zymomonas mobilis* (Balat, 2011).

The pretreated biomass can be processed using a variety of process configurations: Prehydrolysis and Simultaneous Saccharification and Fermentation (PSSF), Simultaneous Saccharification and Fermentation (SSF), Simultaneous Saccharification and Co-Fermentation (SSCF) (Menon and Rao, 2012). There are several advantages for bioethanol production: produced from a variety of raw materials; it is non-toxic; easily introduced into the existing infrastructure (Olkiewicza et al., 2012). However, ethanol production from lignocellulosics is still not widely and commercially viable in terms of high operating cost. The ethanol yield, concentration and production rate are highly affected by the operating parameters, including solid biomass concentration, enzyme loading, temperature (Wang et al, 2012) and processes configuration.

4.2 Materials and method

4.2.1 **Biomass and pretreated**

Lignocellulosic pretreated *A. donax* biomass was produced by Biochemtex (Strada Ribrocca, 11 15057, Tortona [AL], Italy) obtained by innovative process which use only steam and water without the requirement of additional chemical products. The pretreated material moisture content it was of the 55%. The material was composed from an soluble fraction (0.01% of Glucose, 0.26% Xylose, 0.17% Acetic acid, 0.04% of 5-(Hydroxymethyl) furfural, 0.35% of Glucolygomers, 2.89% of Xylolygimers, 0.21 of Arabinan, 0.37 of Acetyls and 3.82% of other [% weigth of dry basis]) and an insoluble fraction (38.18% of Glucans, 5.74% of Xylans, 0.93% Acetyls, 36.13% of Klason lignin and 10.88% of other [% weight of dry basis]). Pretreated *A. donax* was dried for 24 h at 60°C

in a muffle and grinded using a mixer (HMHF Turbo Homogenizer, PBI International, Milan, Italy).

4.2.2 **Enzyme activities**

The commercial cellulase (Cellic CTec2, Novozymes, Bagsværd, Denmark) and β-glucosidase (Cellobiase from *Aspergillus niger*, Sigma-Aldrich) was used in enzymatic hydrolysis process.

Filter paper unit activity of Cellic CTec2 was measured in terms of Filter Paper Unit (FPU) mL^{-1}. Enzyme (Cellic CTec2) was diluted in citrate buffer (1:100, 1:500, 1:750 and 1:1000). 0.5 mL of enzyme diluted was added in 1.0 mL of 0.05 M sodium citrate buffer, pH=4.8. After, one filter paper strip (Whatman No. 1 [= 50mg]) was added and mixed. The samples were incubated 50°C for 60 min. 3.0 mL of DNS (10 g L^{-1} of Dinitrosalicylic acid, 2 g L^{-1} of phenol, 0.5 g L^{-1} of Sodium sulfite and 10 g L^{-1} of Sodium hydroxide) was added, mixed and boiled to 5 min.

After, 20 mL of deionized water was added and mixed by completely inverting the tube several times. After at least 20 min, the color formed was measured against the spectro zero (negative control) at 540 nm (O.D.). The glucose, produced in the experiments, was calculated from a standard curve constructed with known amounts of glucose.

The dilution values were translated into enzyme concentration such as: volume of enzyme diluition/total volume of diluition. The concentration of enzyme was estimated as released of 2 mg of glucose by plotting glucose liberated against enzyme concentration on semilogarithmic graph. FPU was calculated such as 0.37/enzyme concentration to release 2.0 mg of glucose (Ghose, 1987).

Beta-glucosidase activity was measured by using p-nitrophenyl-beta-D-glucoside (p-NPG) as the substrate. Cellobiase was diluited 1:1000 with Sodium Acetate buffer (0.05 M, pH=5). 0.5 mL of enzyme plus buffer was added to 450

µL 2 mM p-NPG in 0.05 M sodium acetate buffer. The reaction mixture was incubated for 2 min and stopped by addition of 1 M Na_2CO_3 (1 mL). The color formed was measured against the spectro zero (negative control) at 405 nm (O.D.). The amount of released p-nitrophenol was measured at 405 nm. One enzyme unit was defined as the amount of enzyme releasing 1 µmol of p-nitrophenol per minute under the described conditions (Maurelli et al., 2013).

4.2.3 **Microorganism and inoculum preparation**

The The selected yeasts *S. cerevisiae* NA227, *Pichia* (P.) *caribbica* NS117 and *Cyberlindnera* (*C.*) *maclurae* E41L stored a 4°C in YPD agar medium, were revitalizing.

To standardize the yeasts concentration, a loopful of yeasts was inoculated in 10 mL of YPD broth and incubated at 30°C. After 24 h, 0.1 mL of brothculture (1%) were transferred in 10 mL of the same medium and incubated at 30°C After 48 h, serial dilutions (0.1 mL) of the samples were spread on the surface of plates containing YPD agar were incubated at 28°C for 48 h. Yeast concentration was $3*10^8$ CFU mL^{-1} for *S. cerevisiae* NA227 and $2*10^8$ CFU mL^{-1} for *P. caribbica* NS117 and *C. maclurae* E41L.

The inoculum of yeasts was performed by adding 0.3 mL of broth (1%) in 30 mL of YPD broth incubating at 30°C. After 48 h, the cells were harvested by centrifugation (6500 rpm for 10 min), washing and centrifugation three times to remove residual sugar and ethanol, and the pellet was inoculated (about 10^8 CFU mL^{-1} for each yeast) in the fermentation media.

4.2.4 **Influence Influence of enzymatic concentration and solid amounts of pretreated *A. donax* in PSSF and SSF processes at 37°C**

The evaluation of bioethanol process was performed investigating the effects of different cellulase concentrations and the influence of pretreated *A. donax*

biomass loading in two configuration process (PSSF and SSF). SSF process allowed the cellulose hydrolysis and glucose fermentation in a single step, in the fairly viscous slurry of diluted pretreated biomass. The viscosity of the slurry was due to the increase of the dry matter, which leads to severe mixing problems. However, the fermentation medium in SSF offering the advantage of preventing the end-product inhibition produced by enzymatic saccharification, PSSF configuration could partially solve this disadvantage because it consists in the preliminary enzymatic hydrolysis that decrease the viscosity, followed by an SSF process.

In PSSF, enzymatic hydrolysis experiments were conducted with substrate loadings of 5.0%, 7.5% and 10.0% (w/v) based on dried material of pretreated *A. donax* biomass (*A. donax* broth), seven different cellulase concentrations (1.09, 2.18, 4.35, 8.07, 17.41, 34.81 and 69.63 FPU g^{-1}) and supplemented with 36.25 International Unit (IU) ß-glucosidase g^{-1} of cellulose.

PSSF with each loading (enzyme and pretreated biomass) was performed in triplicate in 250 mL Erlenmeyer flasks sealed with a rubber stopper fitted with a one way air valve (Check valve, Fisher Scientific, Pittsburgh, PA) to maintain an anaerobic environment Each flask contained 100 mL of sodium acetate buffer (0.05 M, pH=5) (Shen et al., 2012). *A. donax* broth was autoclaved at 121°C for 15 min and then cooled to the room temperature (Wang et al., 2013) to add the enzymes amounts. All flasks were incubated at 37°C for 72 h in an orbital shaker (ES-80 Shaker-incubator, Grant Bio, Cambrige, UK,) at 120 rpm. After hydrolysis, *S. cerevisiae* strain NA227 (about 10^8 CFU mL^{-1}) was added to start the fermentation step that was conducted at 37°C for 96 h.

On the basis of the results obtained in the PSSF test, the SSF process was carried by using two Cellic Ctec2 concentrations (34.81 FPU g^{-1} of cellulose and 69.63 FPU g^{-1} of cellulose). The SSF was started by adding simultaneously enzymes and *S. cerevisiae* NA227 (about 10^8 CFU mL^{-1}). The tests were performed in

100 mL of sodium acetate buffer (0.05 M, pH=5) at 37°C for 168 h, shaking at 120 rpm (Dowe and McMillan, 2008).

4.2.5 **Influence of enzymatic concentration and solid amounts of pretreated *A. donax* in PSSF process at 50°C in prehydrolysis step**

PSSF consists in enzymatic hydrolysis followed by an SSF process in which hydrolysis and fermentation carried out in presence of *S. cerevisiae*. Prehydrolysis was performed at 50°C, which is more suitable for the enzymes, to evaluate the influence of the temperature on the yield of glucose and ethanol. Three different concentrations of pretreated biomass, 5%, 7.5% and 10% (w/v) based on dried material supplemented with 36.25 International Unit (IU) ß-glucosidase g^{-1} of cellulose, were used. According to the results obtained by the previous tests only two concentrations of cellulase (34.81 FPU g^{-1} of cellulose and 69.63 FPU g^{-1} of cellulose) were used. The hydrolysis step was carried out at 50°C for 72 h. The following SSF step was performed at 37°C for 96 h.

4.2.6 **Effect of high solid loading in SSF process**

Another set of SSF experiments was performed in order to determine the effect of increased solid loading. SSF experiments were conducted with dry substrate loading of 10%, 15%, 20%, 25%, 30% and 40% (w/v). On the base of results 1 concentration of cellulose (69.63 FPU g^{-1} of cellulose) was used supplemented with 36.25 International Unit (IU) ß-glucosidase g^{-1} of cellulose. The tests were performed in the same operating condition of the SSF described above.

4.2.7 **Simultaneous saccharification and co-fermentation (SSCF) experiments**

The The SSCF process consists in performing hydrolysis and glucose and xylose fermentation in a single step. The tests were carried out using a cellulase

concentration of 69.63 FPU g^{-1} of cellulose, 36.25 International Unit (IU) of ß-glucosidase g^{-1} of cellulose and 15% of pretreated biomass. The tests were performed in the same condition described above. *S. cerevisiae* NA227 and *P. caribbica* NS117 or *C. maclurae* E41L were co-inoculated (about 10^8 CFU mL^{-1} for each strains) in the substrate of fermentation at the beginning of the experiment. In a second test, *S. cerevisiae* NA227 was inoculated (about 10^8 CFU mL^{-1}) at the beginning of the experiment, while *P. caribbica* NS117 or *C. maclurae* E41L were added (about 10^8 CFU mL^{-1}) after 96 h.

4.2.8 **Analysis methods for glucose and ethanol quantification**

In all experiments, aliquots of sample (1 mL) taken at 24 h, 48 h, 72 h, 96 h and 168 h were centrifuged for 5 min at 14000 rpm. The supernatants were subsequently filtered through a 0.22 mL syringe filter (Minisart NML 0.2 um, Sartorius AG, Goettingen, Germany). The filtrate was stored at -20°C freezer till for the sugar and ethanol analysis.

The concentrations of ethanol and glucose in the samples were analyzed by the HPLC (Gilson, Refractive Index Detector 133, Pump 307) equipped with an Varian METACARB 67H column (Varian, Palo Alto, CA, USA). The column was used at 65°C with 0.01 N H_2SO_4 as eluent flowing at 0.4 mL min^{-1}. All analytical determinations were performed in triplicate and the average results are shown.

The glucose yield was calculated as % of the theoretical yield (% Hydrolysis) by using the following formula (Dowe and McMillan, 2008):

$$\%\mathbf{Hydrolysis} = \frac{[\boldsymbol{Glucose}]}{\mathbf{1.11}\, \boldsymbol{f}\, [\boldsymbol{BIOMASS}]} * \mathbf{100}$$

[***Glucose***] Glucose concentration at end of hydrolysis (g L^{-1})

[***Biomass***] Initial solids concentration (g L^{-1})

f Cellulose fraction in dry biomass (g g^{-1})

1.11 Conversion factor for glucan to glucose

The % theoretical ethanol yield or % cellulose conversion was calculate by using the following formula (Dowe and McMillan, 2008):

$$\%\mathbf{Cellulose\ Conversion} = \frac{[\boldsymbol{Ethanol}\ \mathbf{1}] - [\boldsymbol{Ethanol}\ \mathbf{0}]}{\mathbf{0.51}\ (\mathbf{1.11}\, \boldsymbol{f}\, [\boldsymbol{BIOMASS}]} * \mathbf{100}$$

[*Ethanol* 1] Ethanol concentration at the end of the fermentation (g L^{-1})

[*Ethanol* 0] Ethanol concentration at the beginning of the fermentation (g L^{-1})

[***Biomass***] Initial solids concentration (g L^{-1})

f Cellulose fraction in dry biomass (g g^{-1})

1.11 Conversion factor for glucan to glucose

0.51 Conversion factor for glucose to ethanol

The concentration of sugar and ethanol was determined from the standard curve correlating peak area to concentration.

4.2.9 **Statistical Analyses**

Coupled T-test (at P <0.01) were performed to assess the difference amongst the different runs. Statistical analyses were performed using SPSS 13.0 statistical software package (SPSS Inc., Cary, NC, USA).

4.3 Results

4.3.1 Enzymatic activity of the commercial preparation

Cellic Ctec2 had expressed filter paper activities 162 FPU mL^{-1}. Novozyme 188 had expressed β-glucosidase activity of 670 IU ß-glucosidase mL^{-1}.

4.3.2 Effect of enzyme loading and solid amounts in PSSF and SSF processes at 37°C

In In PSSF process, that consists in enzymatic hydrolysis followed by a fermentation step, at 37°C, a positive correlation was found between enzyme concentration and hydrolysis percentage since increasing the Cellic CTec2 loading from 1.09 to 69.63 FPU g^{-1} of cellulose a significant increase in glucan hydrolysis was detected (P <0.01). In particular, using 5% of solid loading, the percentage of hydrolysis increased from 12.44±0.25% (1.09 FPU g^{-1} of cellulose) to 94.06±0.47% (69.63 FPU g^{-1} of cellulose) after 72 h (Figure 22). Moreover, at 7.5% of solid amount, the percentage of hydrolysis ranged from 12.06±0.66% (1.09 FPU g^{-1} of cellulose) to 85.72±1.32% (69.63 FPU g^{-1} of cellulose) after 72 h (Figure 23). Finally, at 10% of solid, the percentage of hydrolysis increased from 11.75±0.23% (1.09 FPU g^{-1} of cellulose) to 81.05±0.55% (69.63 FPU g^{-1} of cellulose) during 72 h (Figure 24). At 5% (Figure 25), 7.5% (Figure 26) and 10% (Figure 27), significant higher amounts of glucose (at P <0.01) were produced using 69.63 FPU g^{-1} of cellulose.
The glucose produced at 72 h with substrate loading of 5.0%, 7.5% and 10.0% was 17.92±0.09 g L^{-1}, 24.52±0.38 g L^{-1}, 30.88±0.21 g L^{-1}, respectively.

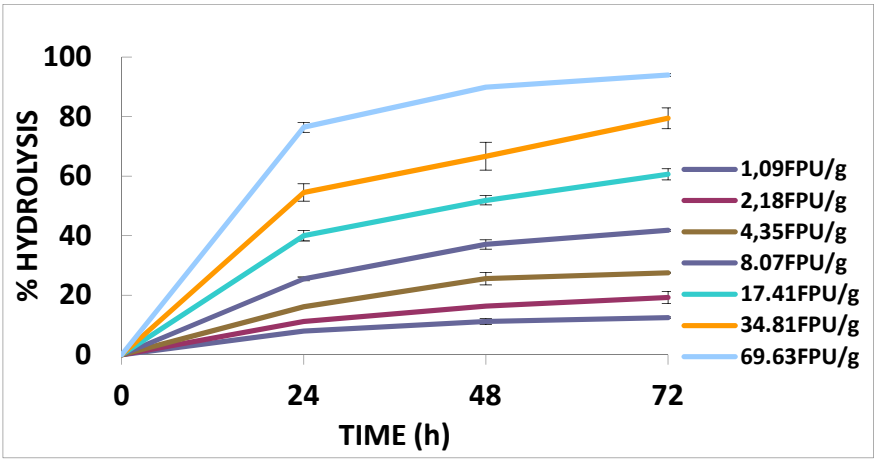

Figure 22. Percentage of hydrolysis, with 5% of pretreated *A. donax* biomass, using different enzyme concentrations.

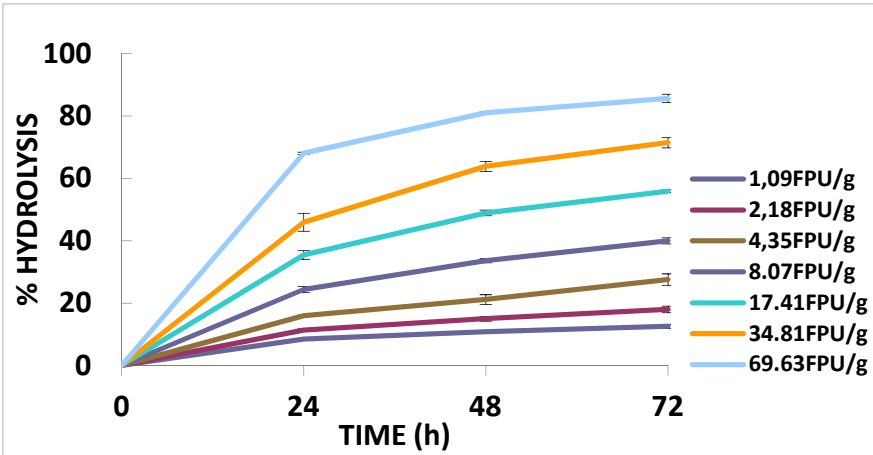

Figure 23. Percentage of hydrolysis, with 7.5% of pretreated *A. donax* biomass, using different enzyme concentrations.

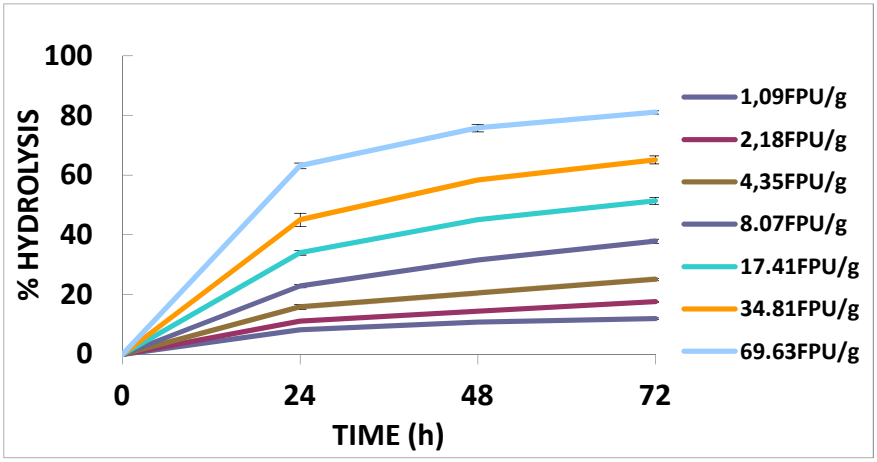

Figure 24. Percentage of hydrolysis, with 10% of pretreated *A. donax* biomass, using different enzyme concentrations.

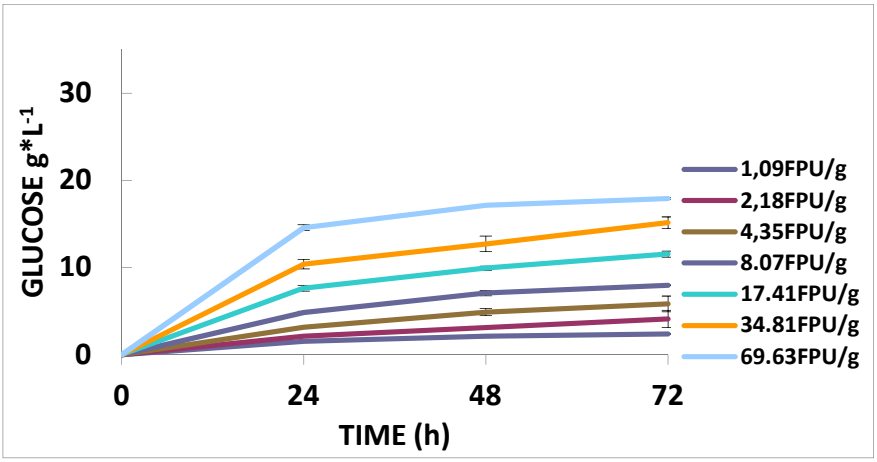

Figure 25. Glucose yield (g L^{-1}), with 5% of pretreated *A. donax* biomass, using different enzyme concentrations.

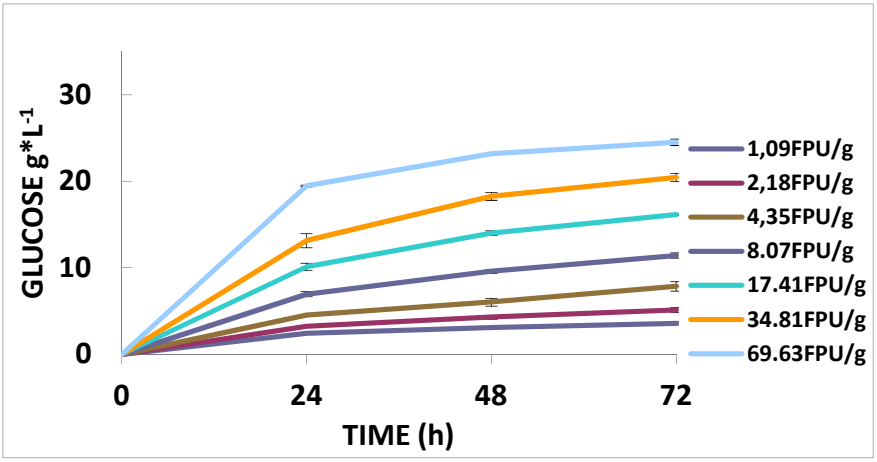

Figure 26. Glucose yield (g L^{-1}), with 7.5% of pretreated *A. donax* biomass, using different enzyme concentrations.

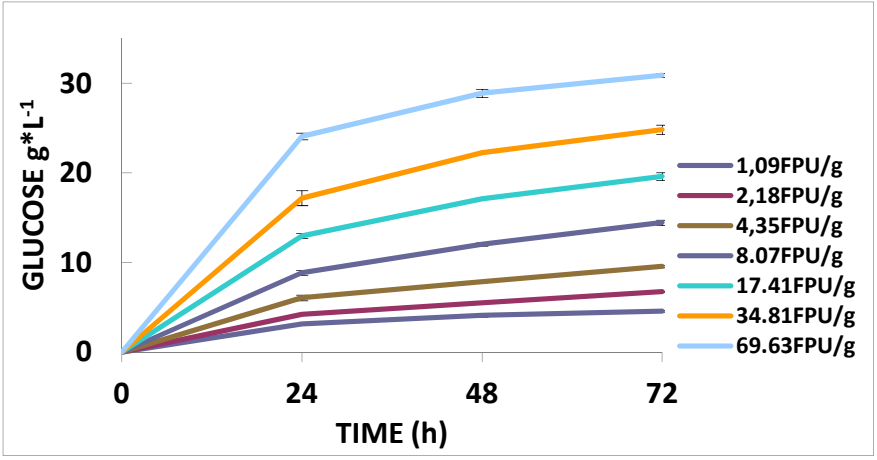

Figure 27. Glucose yield (g L^{-1}), with 10% of pretreated *A. donax* biomass, using different enzyme concentrations.

At 69.63 FPU g^{-1}, the increase of solid loading from 5% to 10% led to a significant decrease (at P <0.01) of the percentage of hydrolysis from 94.06±0.47% to 81.05±0.55% (Figure 28). However, amount of glucose increased from 17.92±0.09 g L^{-1} to 30.88±0.21 g L^{-1} (P <0.01) when the concentration of solid ranged from 5% to 10% (Figure 29).

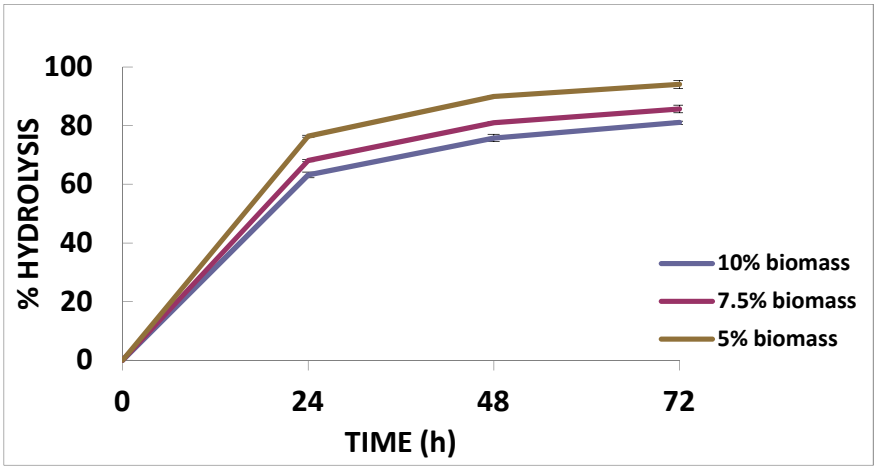

Figure 28. Percentage of hydrolysis, with 5%, 7.5% and 10% of pretreated *A. donax*, using 69.63 FPU g^{-1} of cellulose as enzymatic concentration.

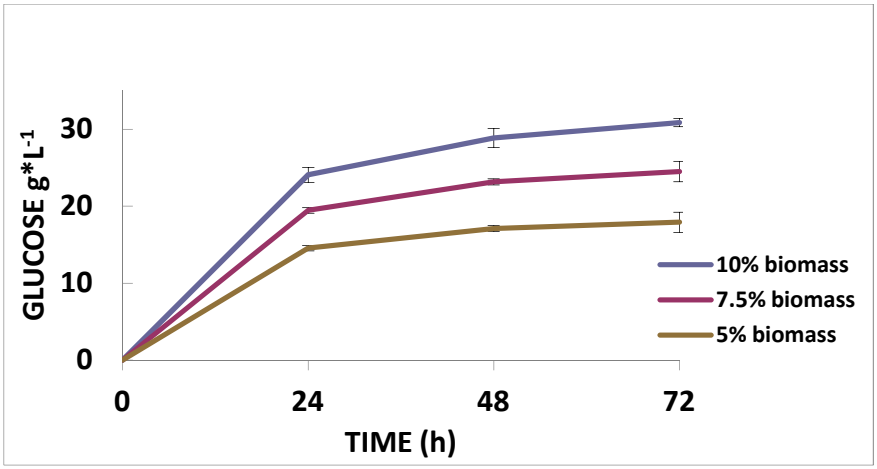

Figure 29. Glucose yield (g L^{-1}), with 5%, 7.5% and 10% of pretreated *A. donax*, using 69.63 FPU g^{-1} of cellulose as enzymatic concentration.

Higher enzymatic concentration and the substrate amounts, determined higher ethanol production (P <0.01) in the fermentation step. In particular, at 5% of solid loading, the ethanol amount increased from 1.50±0.03 g L^{-1} (1.09 FPU g^{-1} of cellulose) to 9.94±0.09 g L^{-1} (69.63 FPU g^{-1} of cellulose) (Figure 30). In addition, using 7.5% of solid amount, the ethanol produced increased from 2.57±0.05 g L^{-1} (1.09 FPU g^{-1} of cellulose) to 14.50±0.05 g L^{-1} (69.63 FPU g^{-1} of cellulose) (Figure 31). The ethanol increased from 3.65±0.32 g L^{-1} (1.09 FPU g^{-1} of cellulose) to 18.78±0.05 g L^{-1} (69.63 FPU g^{-1} of cellulose) using 10% of solid, (Figure 32). At 5% (Figure 33), 7.5% (Figure 34) and 10% (Figure 35) of pretreated *A. donax*, the percentage of cellulose conversion (P <0.01) were obtained using 69.63 FPU g^{-1} of cellulose. In particular, percentage cellulose conversion was 91.91±0.85%, 89.37±0.54% and 86.86±0.21% with 5%, 7.5% and 10% of solid loading, respectively.

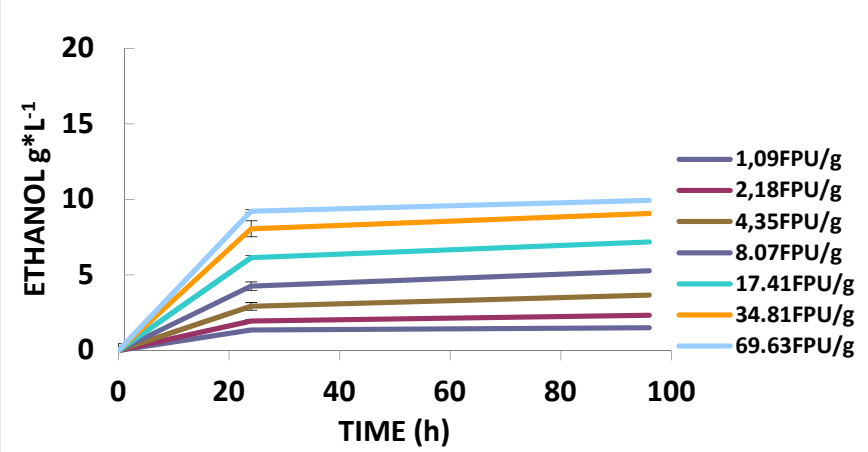

Figure 30. Ethanol yield (g L^{-1}), with 5% of pretreated *A. donax* biomass, using different enzyme concentrations.

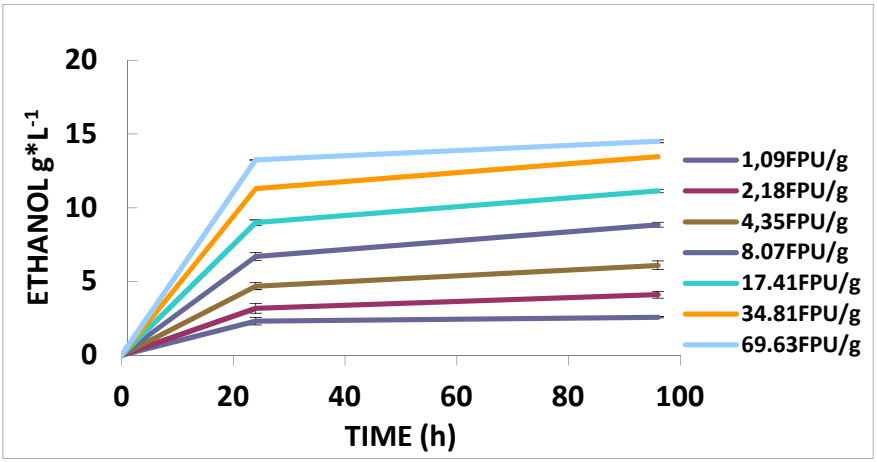

Figure 31. Ethanol yield (g L^{-1}), with 7.5% of pretreated *A. donax* biomass, using different enzyme concentrations.

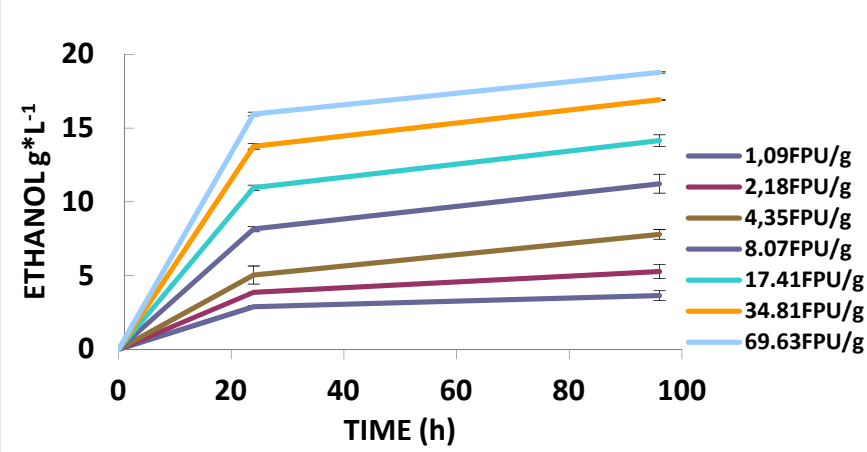

Figure 32. Ethanol yield (g L^{-1}), with 10% of pretreated *A. donax* biomass, using different enzyme concentrations.

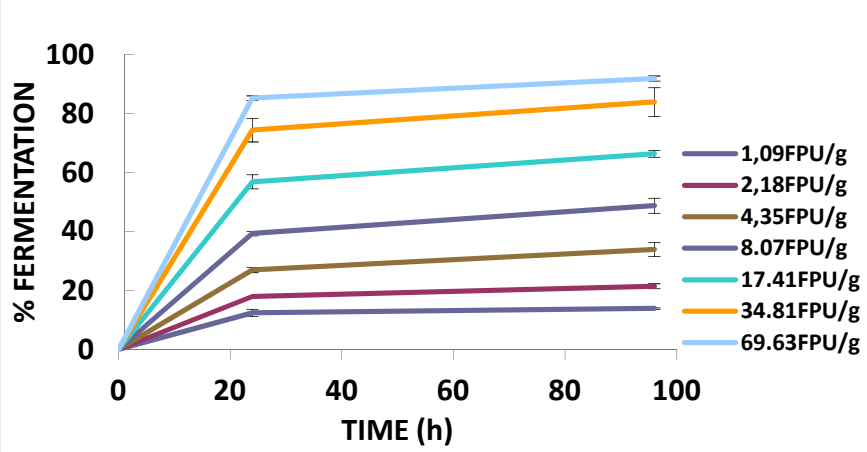

Figure 33. Percentage of cellulose conversion, with 5% of pretreated *A. donax* biomass, using different enzyme concentrations.

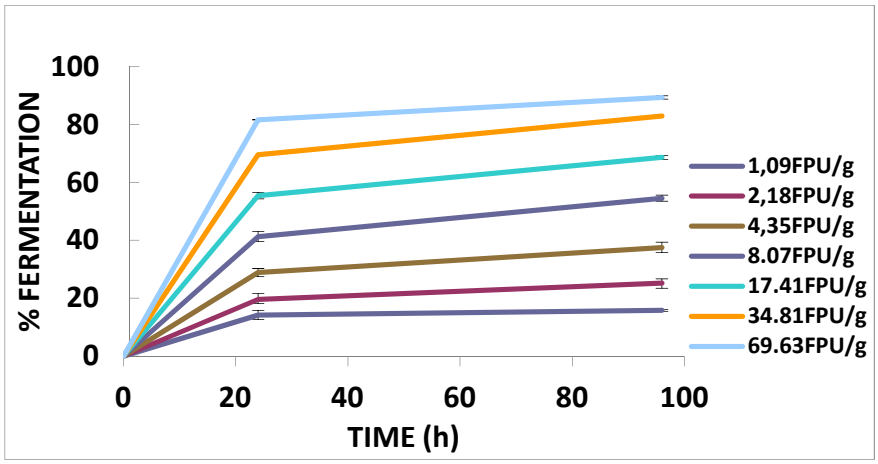

Figure 34. Percentage of cellulose conversion, with 7.5% of pretreated *A. donax* biomass, using different enzyme concentrations.

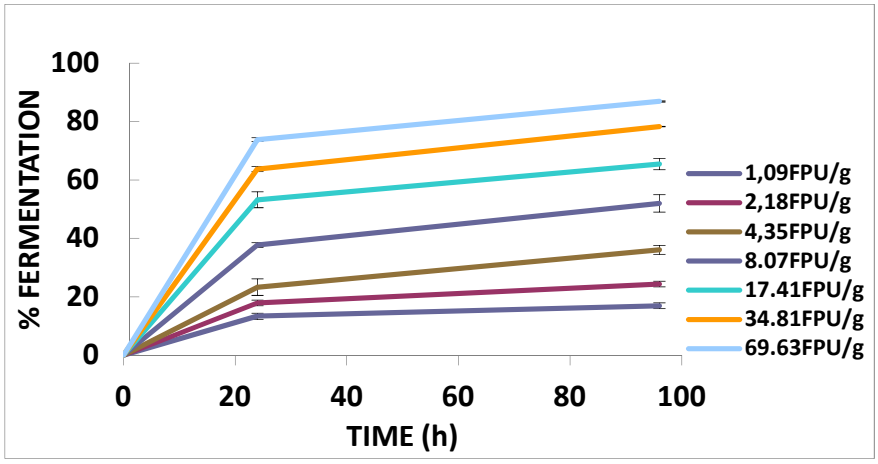

Figure 35. Percentage of cellulose conversion, with 10% of pretreated *A. donax* biomass, using different enzyme concentrations.

Moreover, using 69.63 FPU g^{-1} of cellulose and increasing percent solid loading from 5% to 10%, a decrease (P <0.01) of cellulose conversion from 91.91±0.85% to 86.86±0.21%, was observed (Figure 36). However, the amount of ethanol increased (P <0.01) from 9.94±0.09 g L^{-1} to 18.78±0.05 g L^{-1} (Figure 37).

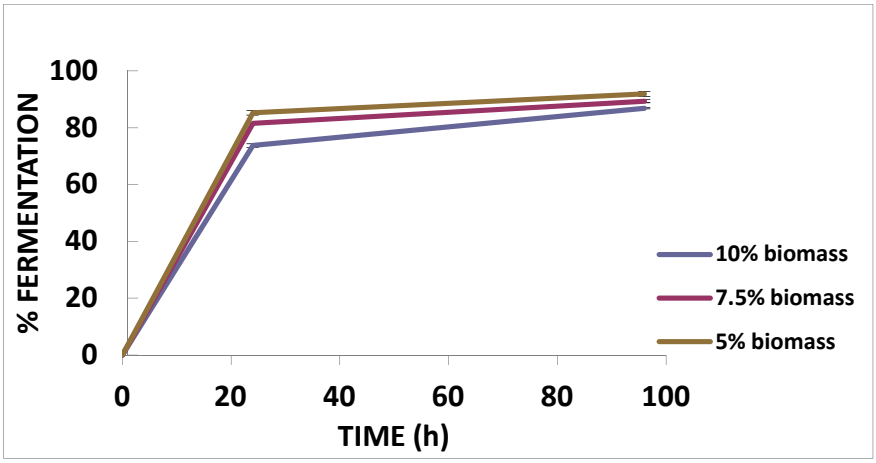

Figure 36. Percentage of cellulose conversion, with 5%, 7.5% and 10% of pretreated *A. donax* biomass, using 69.63 FPU g^{-1} of cellulose as enzymatic concentration.

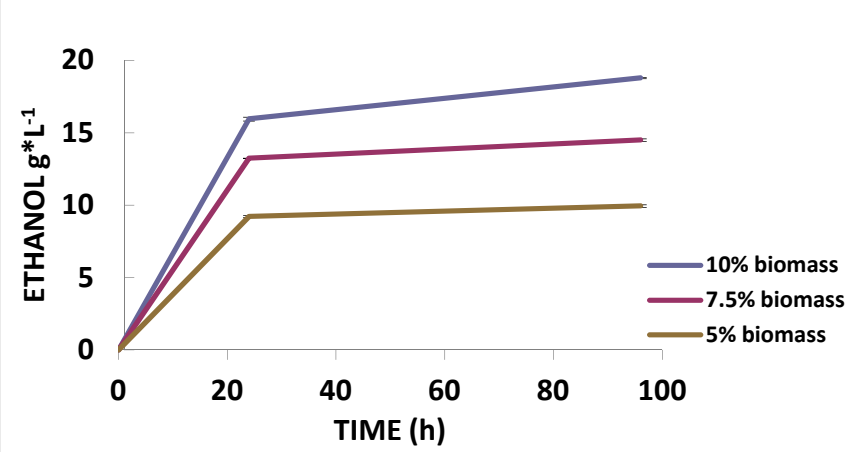

Figure 37. Ethanol yield (g L^{-1}), with 5%, 7.5% and 10% of pretreated *A. donax* biomass, using 69.63 FPU g^{-1} of cellulose as enzymatic concentration.

In PSSF, performed at 37°C, best results (30.88±0.21 g L^{-1} of glucose and 18.78±0.05 g L^{-1} of ethanol, at P <0.01) were obtained using an enzymatic concentration of 69.63 FPU g^{-1} of cellulose and 10% (w/v) of biomass pretreated.

Similar results were obtained in SSF process (hydrolysis and fermentation glucose in a single step were performed) with all substrate concentrations. In fact, high cellulose percentage conversion and ethanol amount (P <0.01) were obtained increasing the cellulase amount from 34.88 FPU g^{-1} to 69.76 FPU g^{-1} of

cellulose. In particular, using 5% of pretreated *A. donax*, the cellulose percentage conversion increased from 86.49±0.01% (34.81 FPU g^{-1} of cellulose) to 90.69±4.64% (69.63 FPU g^{-1} of cellulose) (Figure 38). Moreover, with 7.5% of solid amount, the percentage of cellulose conversion increased from 85.03±0.07% (34.81 FPU g^{-1} of cellulose) to 93.13±1.38% (69.63 FPU g^{-1} of cellulose) (Figure 39).

Finally, at 10% of solid amount, the cellulose percentage conversion incremented from 80.44±1.90% (34.81 FPU g^{-1} of cellulose) to 91.40±0.48% (69.63 FPU g^{-1} of cellulose) (Figure 40). At 5% (Figure 41), 7.5% (Figure 42) and 10% (Figure 43), the higher amounts of ethanol (at P <0.01) were produced using 69.63 FPU g^{-1} of cellulose. After 168 h of SSF process, the ethanol produced with 5%, 7.5% and 10% of pretreated biomass was 9.81±0.50 g L^{-1}, 15.11±0.22 g L^{-1} and 19.77±0.11 g L^{-1}, respectively.

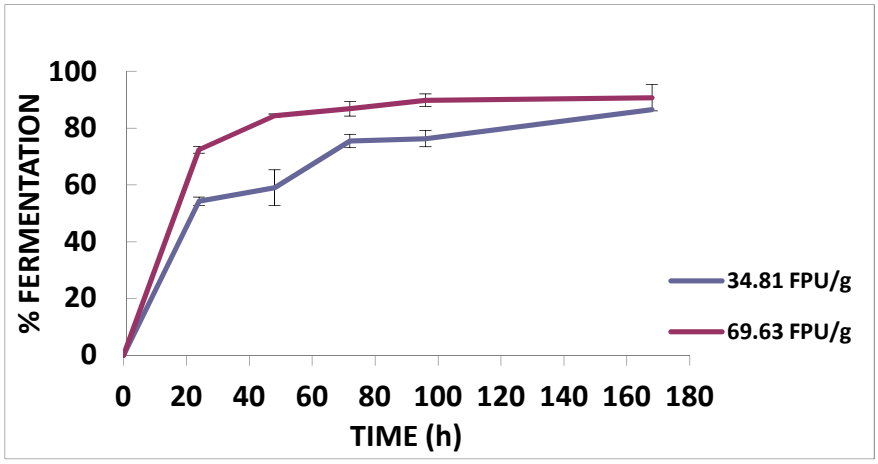

Figure 38. Percentage of cellulose conversion, with 5% of pretreated *A. donax* biomass, using different enzyme concentrations.

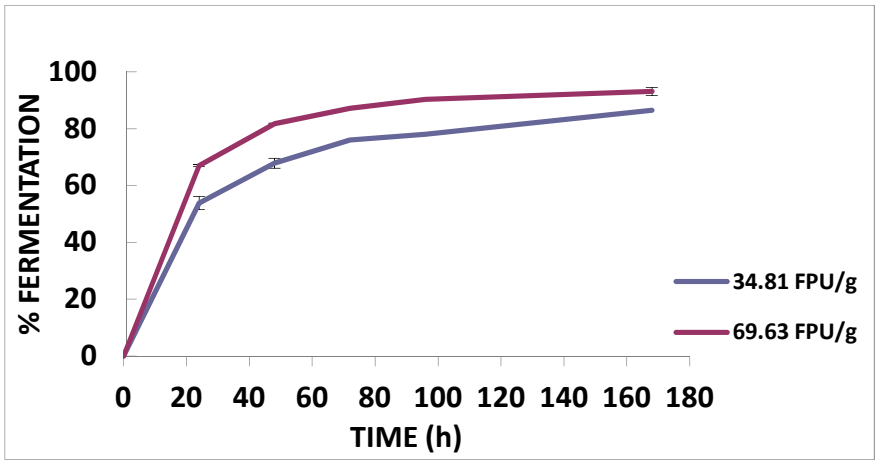

Figure 39. Percentage of cellulose conversion, with 7.5% of pretreated *A. donax* biomass, using different enzyme concentrations.

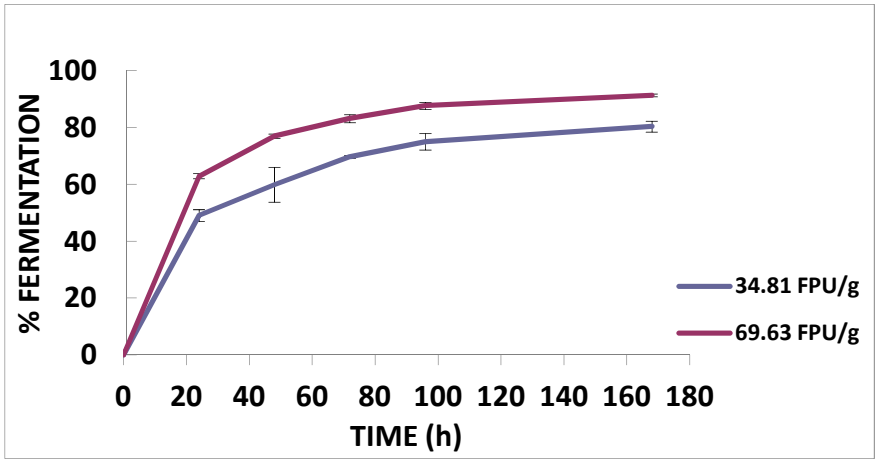

Figure 40. Percentage of cellulose conversion, with 10% of pretreated *A. donax* biomass, using different enzyme concentrations.

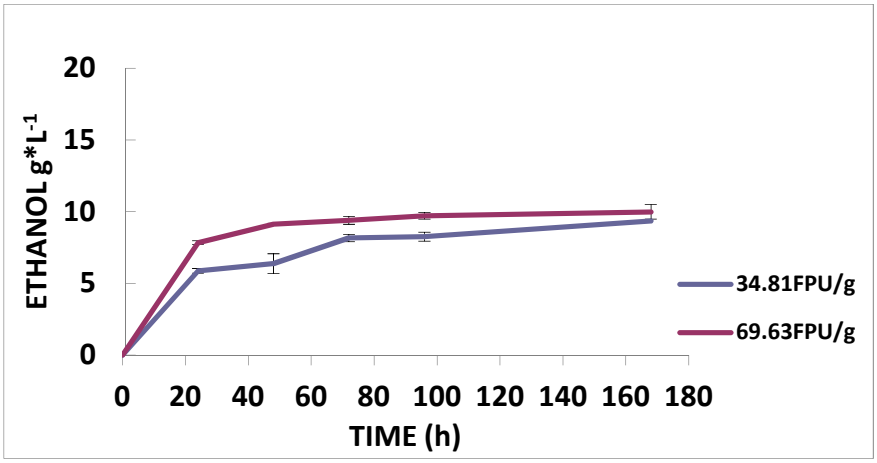

Figure 41. Ethanol yield (g L^{-1}), with 5% of pretreated *A. donax* biomass, using different enzyme concentrations.

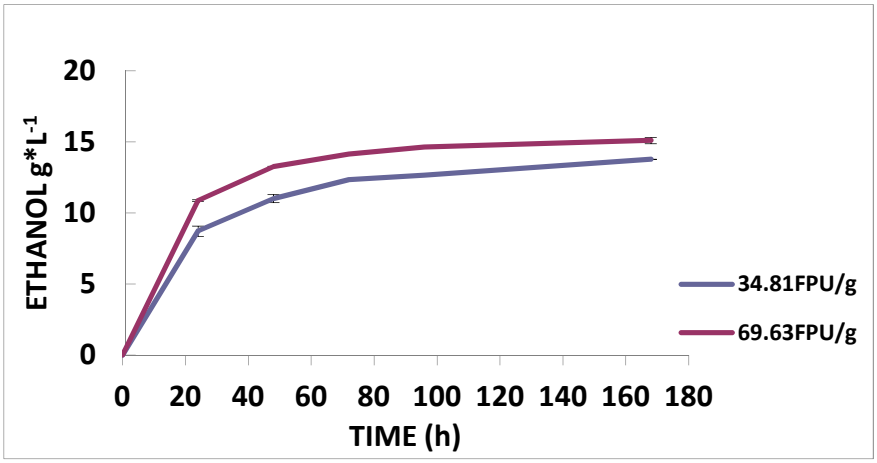

Figure 42. Ethanol yield (g L^{-1}), with 7.5% of pretreated *A. donax* biomass, using different enzyme concentrations.

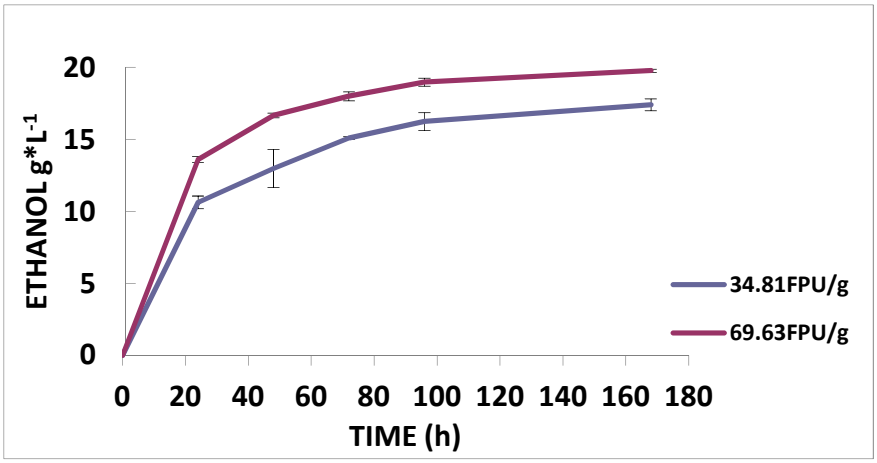

Figure 43. Ethanol yield (g L^{-1}), with 10% of pretreated *A. donax* biomass, using different enzyme concentrations.

In addition, with 69.63 FPU g^{-1} of cellulose, the ethanol amounts increased (P <0.01) from 9.81±0.50 g L^{-1} to 19.77±0.11 g L^{-1}, increasing solid from 5% to 10%, (Figure 44). However, not significant increases of cellulose conversion (P <0.01) were observed incrementing solid loading from 5% to 10% (90.69±4.64% and 91.40±0.48%, respectively) (Figure 45).

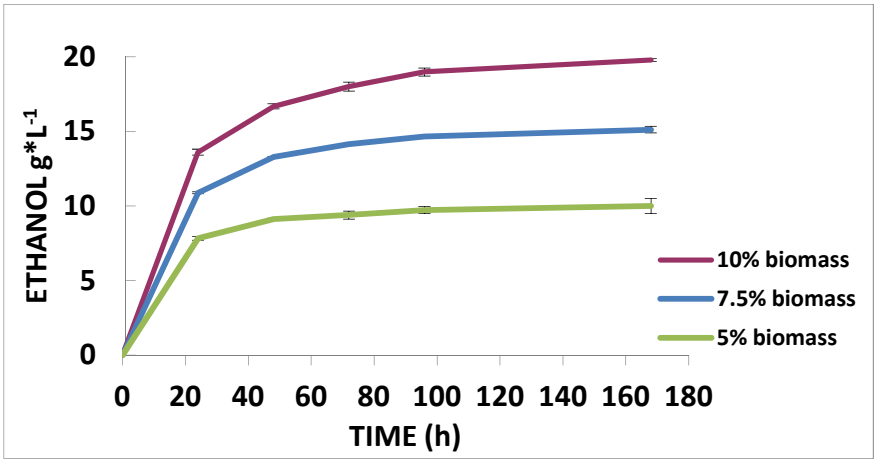

Figure 44. Ethanol yield (g L^{-1}), with 5%, 7.5% and 10% of pretreated *A. donax* biomass, using 69.63 FPU g^{-1} of cellulose as enzymatic concentration.

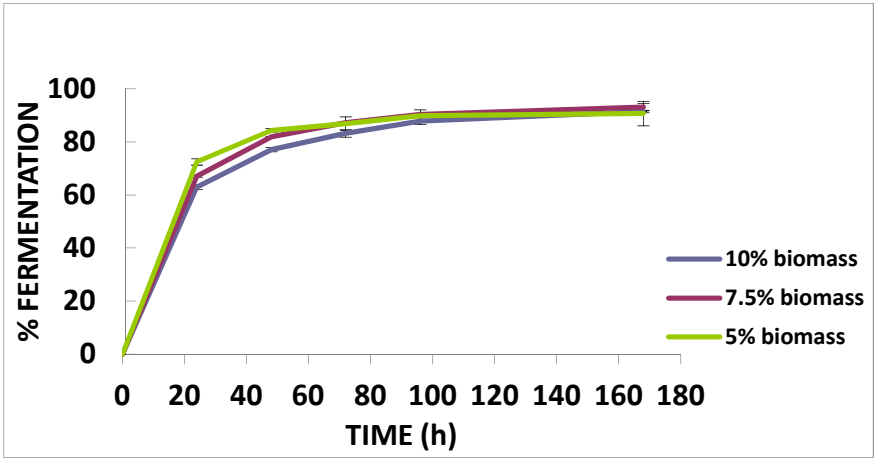

Figure 45. Percentage of cellulose conversion, with 5%, 7.5% and 10% of pretreated *A. donax* biomass, using 69.63 FPU g^{-1} of cellulose as enzymatic concentration.

In SSF, the best performance (19.77±0.11 g L^{-1} of ethanol, at P <0.01) were obtained using 10% of pretreated biomass and 69.63 FPU g^{-1} of cellulose.

At 7.5% and 10% of pretreated biomass (69.63 FPU g^{-1} of cellulose), SSF process allowed to achieved higher ethanol amount compared to PSSF process, while, with 5% of solid amount, not difference were observed (P <0.01). In particular, with 7.5% of pretreated *A. donax*, the ethanol amount was 15.11±0.22 g L^{-1} and 14.50±0.05 g L^{-1} in SSF process and PSSF process, respectively. With 10% of solid loading, the ethanol amount was 19.77±0.11 g L^{-1} and 18.78±0.05 g L^{-1} in SSF and PSSF, respectively. Finally, with 5% of pretreated *A. donax* biomass was 9.81±0.50 g L^{-1} and 9.94±0.09 g L^{-1} in SSF and PSSF, respectively.

4.3.3 **Effect of enzyme loading and solid amounts in PSSF process at 50°C in prehydrolysis step**

In PSSF, with prehydrolysis conducted at 50°C and fermentation step at 37°C, a positive correlation was found between enzyme concentration and hydrolysis percentage since increasing the Cellic CTec2 loading from 34.81 to 69.63 FPU g^{-1} of cellulose a significant increase in glucan hydrolysis was detected (P <0.01). In particular, using 5% of solid biomass loading, the percentage of hydrolysis increased from 81.80±1.99% (34.81 FPU g^{-1} of cellulose) to 95.80±1.53% (69.63 FPU g^{-1} of cellulose) after 72 h of fermentation (Figure 46). Moreover, at 7.5% of solid amount, the percentage of hydrolysis incremented from 75.57±0.54% (34.81 FPU g^{-1} of cellulose) to 89.26±0.59% (69.63 FPU g^{-1} of cellulose) after 72 h (Figure 47). Finally, at 10% of solid quantity, the percentage of hydrolysis increased from 68.68±0.67% (34.81 FPU g^{-1} of cellulose) to 84.92±0.35% (69.63 FPU g^{-1} of cellulose) with hydrolysis time to 72 h (Figure 48). At 5% (Figure 49), 7.5% (Figure 50) and 10% (Figure 51), the significantly higher amounts of glucose (at P <0.01) were produced using 69.63 FPU g^{-1} of cellulose.

The glucose produced at 72 h with substrate loading of 5.0%, 7.5% and 10.0% was 18.25±0.29 g L^{-1}, 25.53±0.17 g L^{-1}, 32.35±0.13 g L^{-1}, respectively. Moreover, at 7.5% and 10% of pretreated biomass (69.63 FPU g^{-1} of cellulose), prehydrolysis step performed at 50°C, allowed to achieved significantly higher glucose amount compared to prehydrolysis step carried out at 37°C, while, at 5% of solid amount, not difference were observed (at P <0.01). In particular, at 7.5% of pretreated *A. donax*, the glucose amount was 25.53±0.17 g L^{-1} and 24.52±0.38 g L^{-1} in prehydrolysis step conducted at 50°C) and prehydrolys step performed at 37°C, respectively. At 10% of solid loading, the glucose yield was, 32.35±0.13 g L^{-1} and 30.88±0.21 g L^{-1} in prehydrolysis step conducted 50°C and prehydrolysis step carried out at 37°C, respectively. Finally, at 5% of pretreated *A. donax*, the glucose released was 18.25±0.29 g L^{-1} and 17.92±0.09 g L^{-1} in prehydrolysis step carried out (50°C) and prehydrolysis step conducted (37°C), respectively.

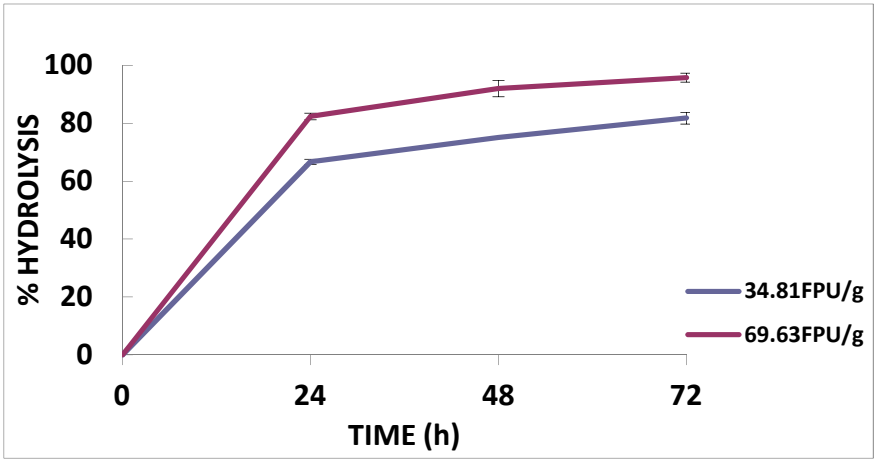

Figure 46. Percentage of hydrolysis, with 5% of pretreated *A. donax* biomass, using different enzyme concentrations.

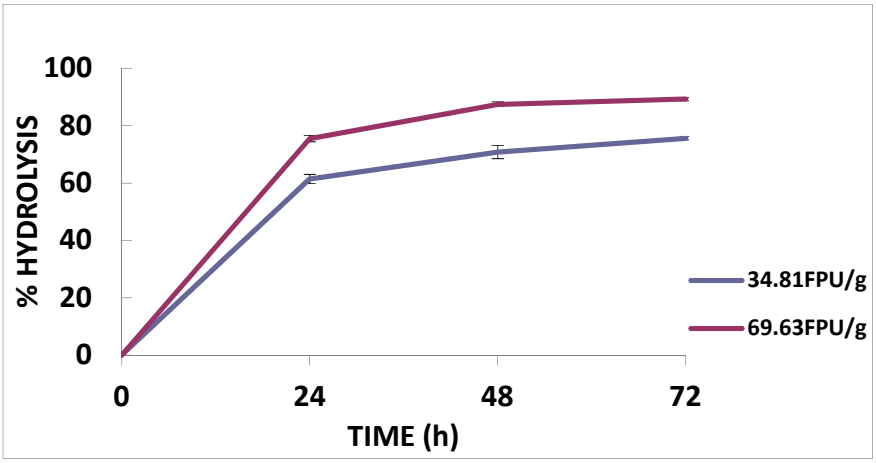

Figure 47. Percentage of hydrolysis, with 7.5% of pretreated *A. donax* biomass, using different enzyme concentrations.

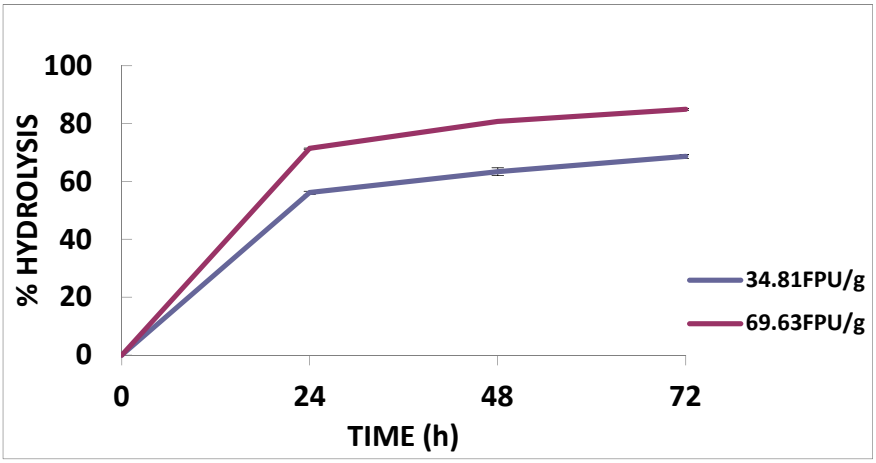

Figure 48. Percentage of hydrolysis, with 10% of pretreated *A. donax* biomass, using different enzyme concentrations.

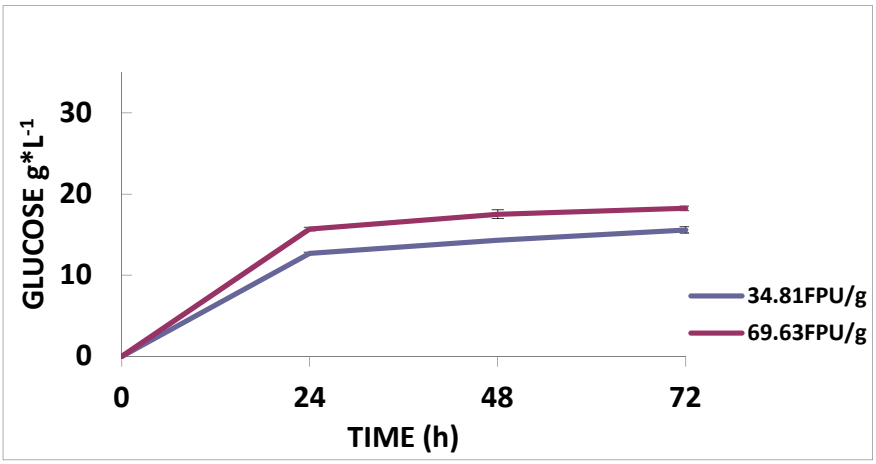

Figure 49. Glucose yield (g L^{-1}), with 10% of pretreated *A. donax* biomass, using different enzyme concentrations.

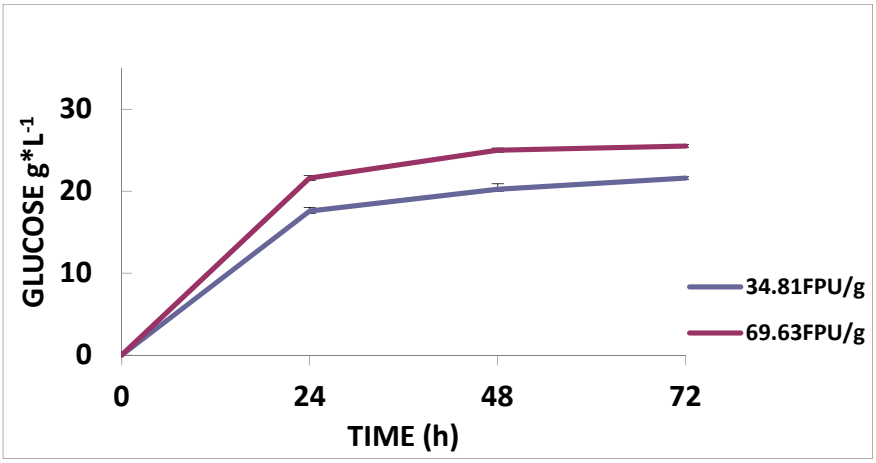

Figure 50. Glucose yield (g L^{-1}), with 7.5% of pretreated *A. donax* biomass, using different enzyme concentrations.

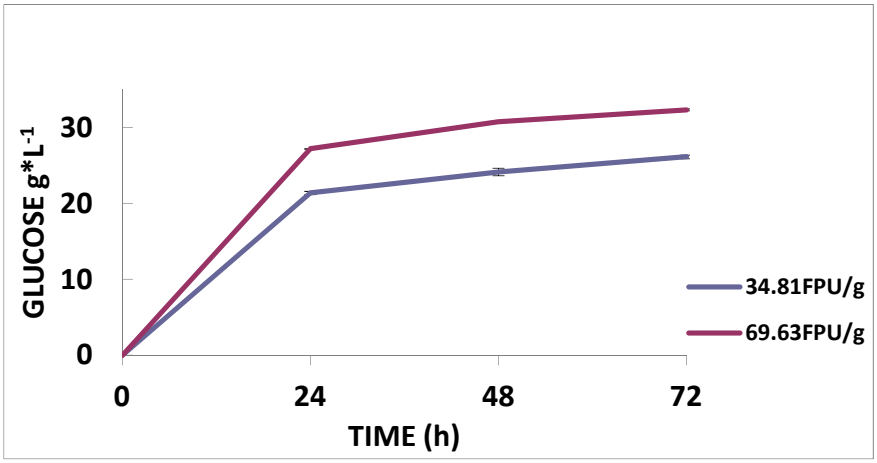

Figure 51. Glucose yield (g L^{-1}), with 10% of pretreated *A. donax* biomass, using different enzyme concentrations.

Higher enzymatic concentration and solid amounts, determined higher ethanol production (P <0.01) in the fermentation step. In particular, at 5% of solid loading, the ethanol amount increased from 8.14±0.10 g L^{-1} (34.81 FPU g^{-1} of cellulose) to 9.44±0.03 g L^{-1} (69.63 FPU g^{-1} of cellulose) (Figure 52). In addition, using 7.5% of solid amount, the ethanol produced increased from 11.82±0.16 g L^{-1} (34.81 FPU g^{-1} of cellulose) to 13.71±0.01 g L^{-1} (69.63 FPU g^{-1} of cellulose) (Figure 53). At last, using 10% of solid quantity, the ethanol produced increased from 14.60±0.07 g L^{-1} (34.81 FPU g^{-1} of cellulose) to 17.66±0.21 g L^{-1} (69.63 FPU g^{-1} of cellulose) (Figure 54). At 5% (Figure 55),

7.5% (Figure 56) and 10% (Figure 57) of pretreated *A. donax*, the significantly cellulose percentage conversion (P <0.01) were obtained using 69.63 FPU g^{-1} of cellulose. In particular, cellulose percentage conversion was 87.35±0.32%, 84.52±0.03% and 81.62±0.97% at 5%, 7.5% and 10% of solid loading, respectively.

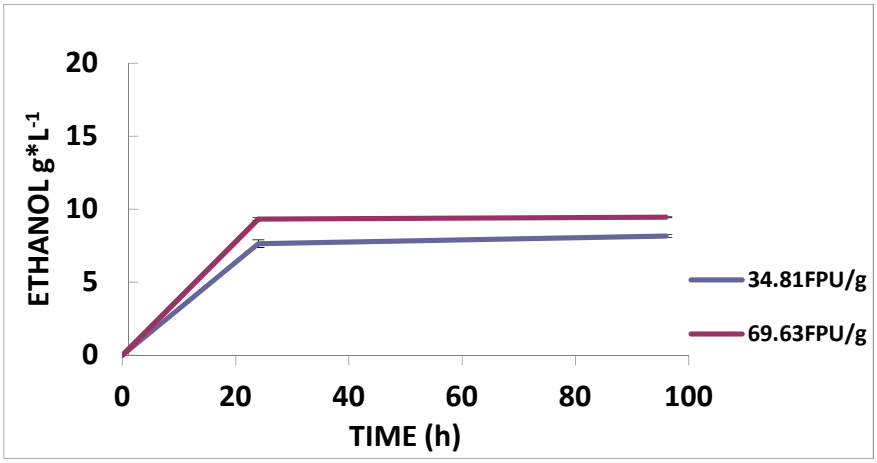

Figure 52. Ethanol yield (g L^{-1}), with 5% of pretreated *A. donax* biomass, using different enzyme concentrations.

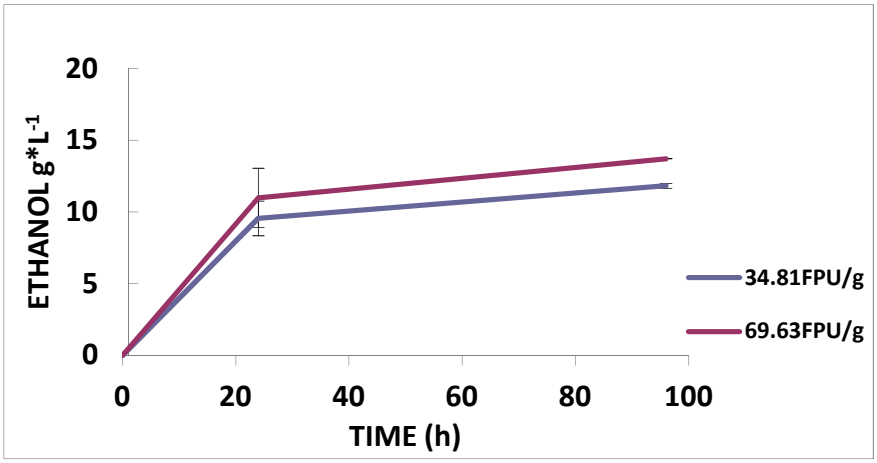

Figure 53. Ethanol yield (g L^{-1}), with 7.5% of pretreated *A. donax* biomass, using different enzyme concentrations.

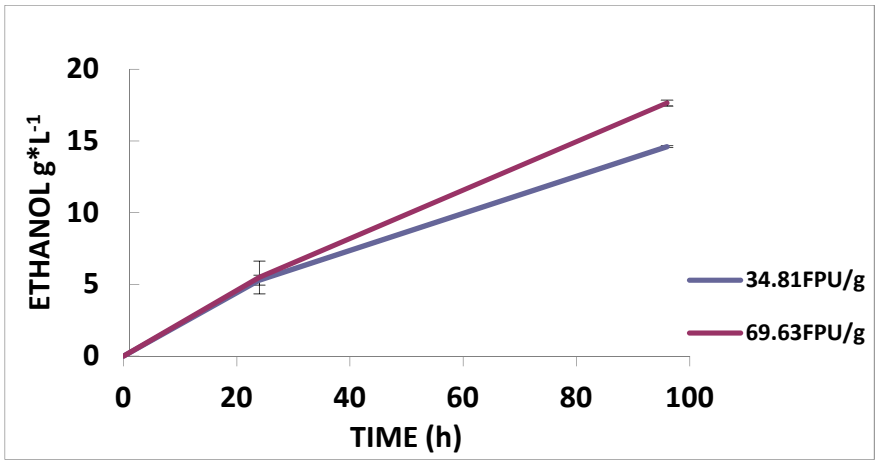

Figure 54. Ethanol yield (g L^{-1}), with 10% of pretreated *A. donax* biomass, using different enzyme concentrations.

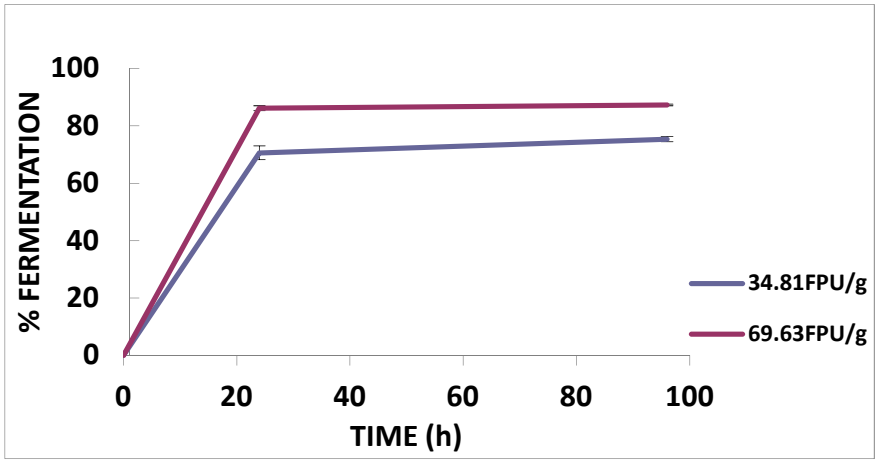

Figure 55. Percentage of cellulose conversion, with 5% of pretreated *A. donax* biomass, using different enzyme concentrations.

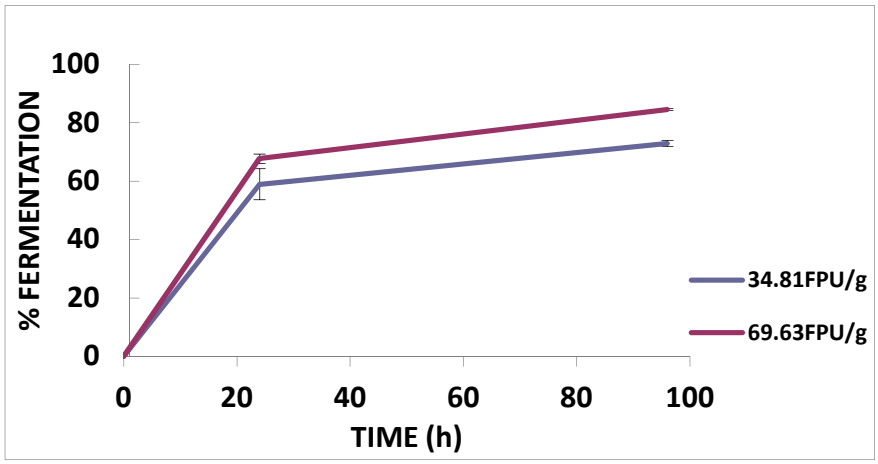

Figure 56. Percentage of cellulose conversion, with 7.5% of pretreated *A. donax* biomass, using different enzyme concentrations.

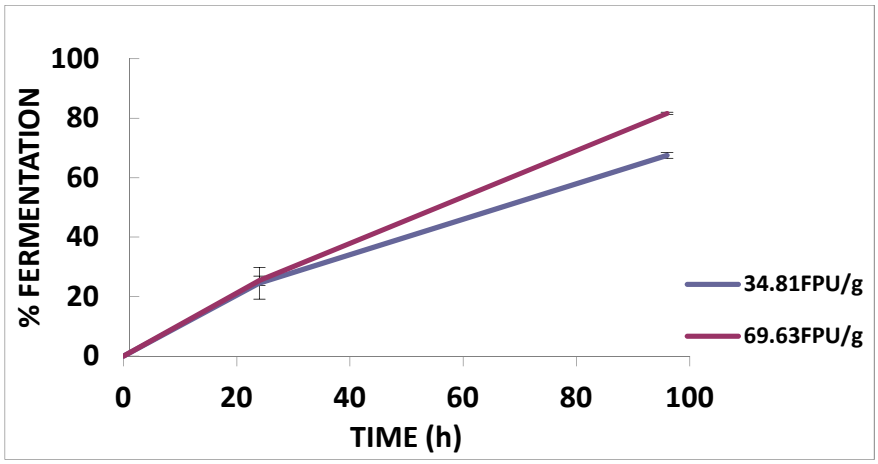

Figure 57. Percentage of cellulose conversion, with 10% of pretreated *A. donax* biomass, using different enzyme concentrations.

Moreover, using 69.63 FPU g^{-1} of cellulose and increasing percent solid loading from 5% to 10% led to an significant decrease (at P <0.01) of the percentage cellulose conversion, from 87.35±0.32% to 81.62±0.97% (Figure 58). However, using the same enzyme concentration, the amount of ethanol produced increased significantly (at P <0.01) with increasing solid from 5% to 10%, from 9.45±0.10 g L^{-1} to 17.68±0.21 g L^{-1} (Figure 59). Therefore, using the 10% of the biomass pretreated, very slow fermentation process was observed compared to 5% and to 7.5% of solids loading.

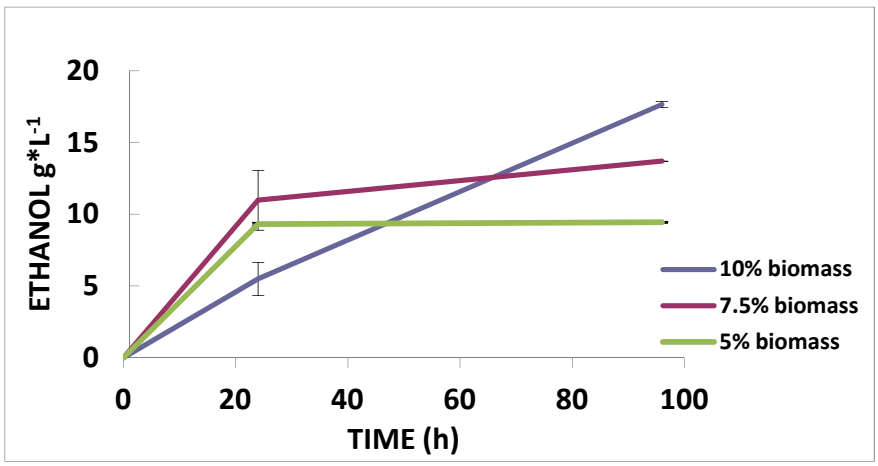

Figure 58. Ethanol yield (g L^{-1}), with 5%, 7.5% and 10% of pretreated *A. donax* biomass, using 69.63 FPU g^{-1} of cellulose as enzymatic concentration.

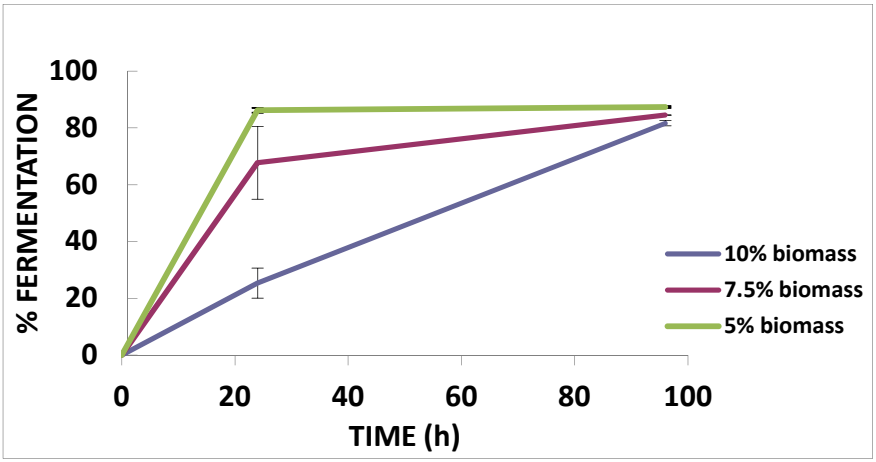

Figure 59. Percentage of cellulose conversion, with 5%, 7.5% and 10% of pretreated *A. donax* biomass, using 69.63 FPU g^{-1} of cellulose as enzymatic concentration.

In PSSF, with prehydrolysis step conducted at 50°C and fermentation step at 37°C, the best performance (32.35±0.13 g L^{-1} of glucose and 17.68±0.21 g L^{-1} of ethanol, at P <0.01) were obtained using 10% of pretreated biomass and 69.63 FPU g^{-1} of cellulose.

At 5%, 7.5% and 10% of pretreated biomass (69.63 FPU g^{-1} of cellulose), PSSF process, with prehydrolysis step performed at 50°C and fermentation step at 37°C, allowed to achieved significantly lower ethanol amount compared to SSF process. In particular, at 5% of pretreated *A. donax*, the ethanol amount was 9.45±0.10 g L^{-1} and 9,81±0.50 g L^{-1} in PSSF (prehydrolysis step performed at 50°C and fermentation step at 37°C) and SSF carried out at 37°C, respectively. Moreover, at 7.5% of solid loading, the ethanol yield was 13.71±0.01 g L^{-1} and 15.11±0.22 g L^{-1} in PSSF (prehydrolysis step conducted at 50°C and fermentation step at 37°C) and SSF performed at 37°C, respectively. Finally, at 10% of solid amount, the ethanol produced was 17.68±0.21 g L^{-1} and 19.77±0.11 g L^{-1} in PSSF (prehydrolysis step carried out at 50°C and fermentation step at 37°C) and SSF performed at 37°C, respectively.

4.3.4 **Effect of high solid loading in SSF process**

Considering the results obtained, tests of SSF (hydrolysis and fermentation glucose in a single step were conducted) were carried out, with a high concentration of solids (10%, 15%, 20%, 25%, 30% and 40%), to evaluate the increase in ethanol yield.

Cellic CTec2 69.63 FPU g^{-1} of cellulose was using in this tests. The effect of substrate loading on cellulose conversion is shown in Figure 60. The percentage of cellulose conversion at 168 h decreased from 90.69±4.64% to 0.65±0.05% with increasing the substrate loading from 10% to 40%,

In addition, the ethanol yield increased from 10% to 20%, while, decreased until 40% of pretreated *A. donax* biomass concentration (Figure 61). In particular, the ethanol yield increased ($P < 0.01$) from 10% to 15% of solid amount (19.77±0.11 g L^{-1} and 25.74±0.50 g L^{-1}, 10% and 15%, respectively) and from 10 to 20% of solid amount (19.77±0.11 g L^{-1} and 29.05±0.50 g L^{-1}, 10% and 20%, respectively). No difference (at $P < 0.01$) was monitored increasing from 15% to 20% of solid loading (25.74±0.50 g L^{-1} and 29.05±0.50 g L^{-1}, 15% and 20%, respectively) even if the last allowed very slow fermentation. Moreover, using 25%, 30% and 40% of the pretreated biomass, very low ethanol yield were observed (5.02, 1.24 and 0.56 g L^{-1}, respectively) (Figure 61).

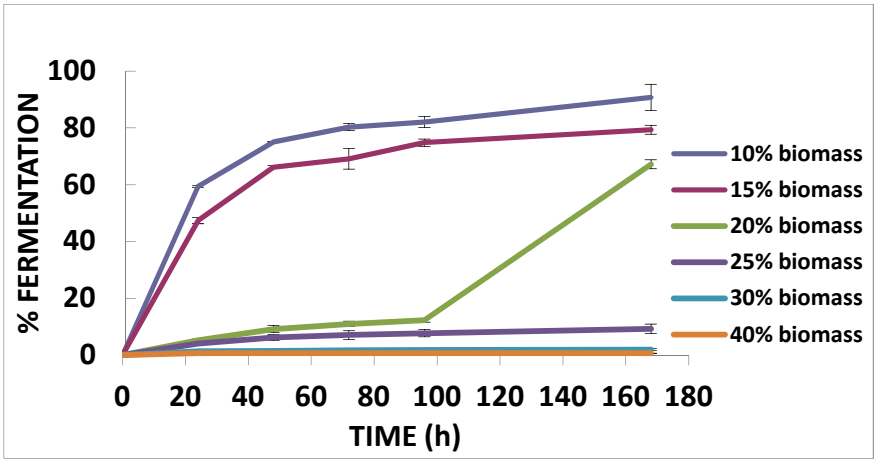

Figure 60. Percentage of cellulose conversion, with 10%, 15%, 20%, 25%, 30% and 40% of pretreated *A. donax* biomass, using 69.63 FPU g^{-1} of cellulose as enzymatic concentration.

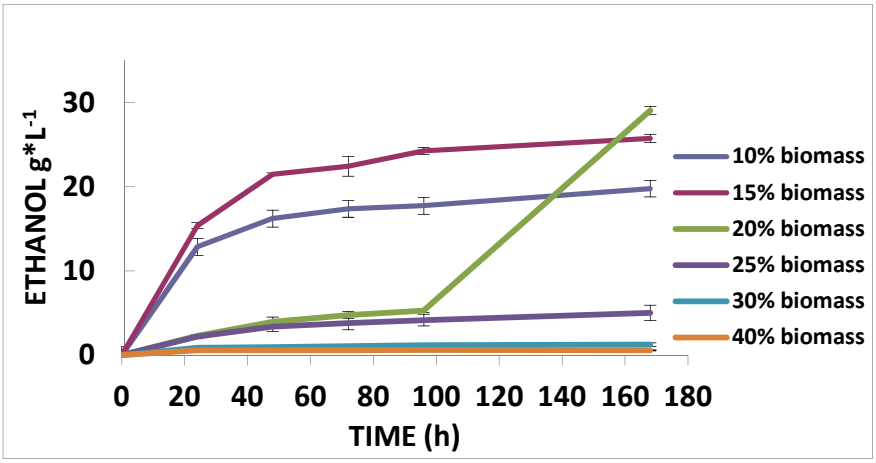

Figure 61. Ethanol yield (g L^{-1}), with 10%, 15%, 20%, 25%, 30% and 40% of pretreated *A. donax* biomass, using 69.63 FPU g^{-1} of cellulose as enzymatic concentration.

These results allowed identifying the optimal concentration of pretreated *A. donax* biomass equal to 15%. These finding allowed to choice 15% of pretreated biomass.

4.3.5 **Simultaneous saccharification and co-fermentation (SSCF) experiments**

In Among SSCF experiments (hydrolysis and fermentation glucose and xylose sugars in a single step were performed), the best performance (28.03±0.39 g L^{-1} of ethanol and 86.37±1.20% cellulose conversion (P <0.01) were obtained inoculating *S. cerevisiae* NA227 at the beginning of the experiment and adding *P. caribbica* NS117 after 96 h of fermentation (Figures 62 and 63). In these conditions was recorded higher ethanol yield (P <0.01) than SSF process and the other SSCF experiments performed using the conditions above reported (Table 14).

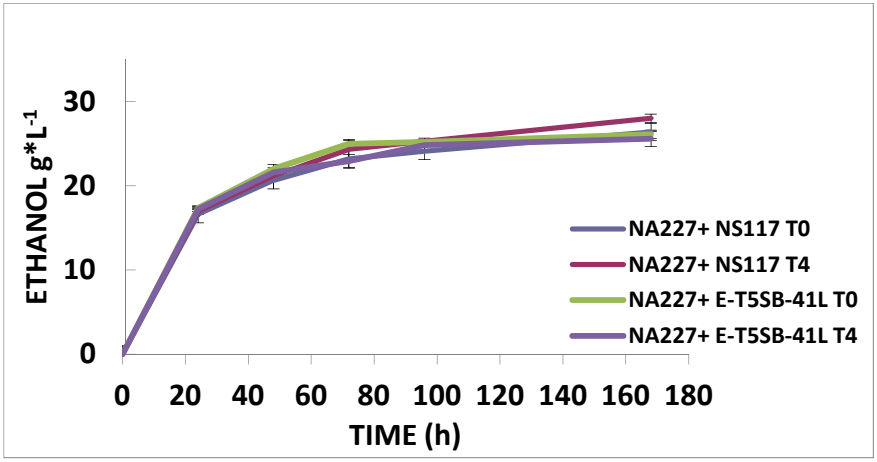

Figure 62. Ethanol yield (g L^{-1}), with 10% of pretreated *A. donax* biomass and 69.63 FPU g^{-1} of cellulose as enzymatic concentration, in different SSCF conditions.

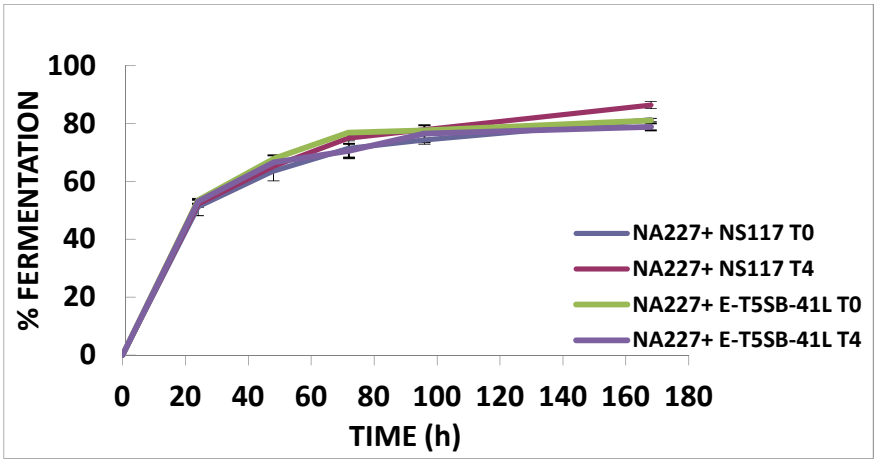

Figure 63. Percentage of cellulose conversion, with 10% of pretreated *A. donax* biomass and 69.63 FPU g^{-1} of cellulose as enzymatic concentration, in different SSCF conditions.

Table 14. Effect of the microbial inoculation on the ethanol yield by using Cellic Ctec2 69.63 FPU g^{-1} of cellulose and 15% of solid loading.

Strain T0	Strain T4	% cellulose conversion	Ethanol Yield g L^{-1}
NA227	NS117	86.37±1.20	28.03±0.39
NA227	E41L	78.86±1.24	25.59±0.40
NA227+ NS117	-	81.27±0.52	26.37±0.17
NA227+ E41L	-	81.03±0.18	26.13±0.29
NA227	-	79.34±1.54	25.74±0.50

T0= immediately after the microbial inoculation
T4= inoculation after 96 h of process
NA227: *S. cerevisiae*; E41L: *C. maclurae*; NS117: *P. caribbica*

In conclusion, the complex experiments of saccharification and fermentation carried out, allowed to define the best conditions for the second generation bioethanol production: 15% of pretreated *A. donax* biomass, 69.63 FPU g^{-1} of cellulose, temperature of 37°C, inocula separated of *S. cerevisiae* NA227 and *P. caribbica* NS117 (about 10^8 CFU mL^{-1} for each strain) and simultaneous saccharification and co-fermentation process (SSCF) as configuration process.

4.4 Discussion

Climate changes and shortage of fossil fuels have sparked a growing demand for liquid biofuels which in turn has increased the interest of research into the production of lignocellulose-derived bioethanol (Gray et al., 2009; Jørgensen et al., 2007). However, lignocellulosic materials pose several challenges in conversion to fermentable sugars and ethanol since they are an insoluble and highly heterogeneous substrate (Kristensen et al., 2009; Olofsson et al. 2008). In fact, hydrolysis and ethanol yields are highly affected by the operating parameters, including enzyme concentration, solid biomass loading and temperature (Wang et al, 2012). Moreover, the production of ethanol can be accomplished following several process strategies, and it is useful to compare them in order to select the best one (López-Linares et al., 2014). These strategies include prehydrolysis and simultaneous saccharification and fermentation (PSSF), simultaneous saccharification and fermentation (SSF) and simultaneous saccharification and co-fermentation (SSCF) (López-Linares et al., 2014; Pessani et al., 2011).

In the current study, *A. donax*, a perennial herbaceous plant that has shown to have great potential as energy crop in the Mediterranean region (Fiorentino et al., 2013), was assessed as a feedstock for bioethanol production investigating and optimizing different parameters and process strategies.

The first evaluated parameter was the selection of optimal enzymatic concentration to use in the bioethanol production. Although this is clearly important for the process economy, the economic sensitivity towards the enzyme loading is difficult to predict due to the large uncertainties of the cost of enzymes, and lack of sufficient experimental data on the effect of enzyme load (Olofsson et al., 2008). According to previous works (Wang et al., 2012; Van Dyk and Pletschke, 2012; Linde et al., 2007) the sugar yield and ethanol concentration increased in function of enzyme loading. In fact, in PSSF process

a positive correlation between enzyme concentration and hydrolysis and fermentation percentage was found. In particular, increasing the Cellic CTec2 loading a significant increase in glucan hydrolysis as well as ethanol yield was detected, when the prehydrolysis step carried out both at 37°C and 50°C. According to Eklund and Zacchi (1995) and Pessani et al. (2011), similar result was observed in SSF process. However, the minimum enzyme loading required to degrade a substrate depends on the specific substrate and its composition, as well as the type of pretreatment. In our work, in terms of economical optimization of the process, the best enzyme concentration was found to be 69.63 FPU g^{-1} cellulose. In fact, it allowed obtain about 90% in cellulose conversion of a pretreated biomass rich in lignin (36%) such as *A. donax*. Although, Sun and Cheng (2002) reported that cellulase concentration of 7-33 FPU g^{-1} substrate is generally used, Olsen and co-workers (2011) demonstrated that enzyme concentration of about 70 FPU g^{-1} cellulose is necessary to increase hydrolysis yield of corn stover biomass. Furthermore, Boussaid and Saddler (1999) reported that enzyme loading of 750 FPU g^{-1} of cellulose were not able to fully hydrolyze the pulp which contained 28% lignin. Moreover, substrates with a high lignin composition may require higher enzyme loadings due to non-productive adsorption of enzymes to the lignin portion (Van Dyk and Pletschke, 2012).

In order to achieve an economically feasible process, a high substrate loading, and thus a high water-insoluble substances (WIS) content, is required to obtain a high final ethanol concentration (Li et al., 2011), so as to minimize the energy demand in the downstream processes, for instance, distillation and evaporation (Wingren et al., 2003). Therefore, different substrate loadings (5%, 7.5% and 10% WIS) were evaluated in this study to optimize the bioethanol production. According to Hoyer et al. (2009) and López-Linares and co-workers (2013) that using different substrate loadings (from 5% to 10% WIS) in the SSF of spruce

and rapeseed straw pretreated, no significant reduction in cellulose conversion percentage were detected increasing solid amount (constant enzyme to substrate ratio). This result could be due to the fact that no end-product accumulation occurs during this process configuration since hydrolysis and fermentation steps took place simultaneously. Moreover, our results are very interesting because the use of higher substrate loading produced more concentrated ethanol solutions, and, at the same time, no losses in cellulose conversion percentage.

In PSSF process, the cellulose conversion percentage decreased increasing solid concentrations (constant enzyme to substrate ratio) as reported by Manzanares et al. 2011. In fact, during the PSSF process, several factors could affect the ability of enzymes to convert cellulose, including end-product inhibition, changes in substrate reactivity during enzymatic hydrolysis, mass transfer limitations or other effects related to the increased content of insoluble solids, such as non-productive adsorption of enzymes, deactivation of the enzyme and the intrinsic structural features of the substrate (Berlin et al., 2005; Lin and Tanaka, 2006; Rosgaard et al., 2007). Among these factors, the inhibition of enzyme adsorption by end-products has been suggested as the main cause of the decreasing yields increasing substrate concentrations of cellulosic biomass (Kristensen et al. 2009). Since, also the process configuration could affect the ethanol yields (Ask et al., 2012) it was reported, to our knowledge for the first time, the comparison between PSSF and SSF process configurations using pretreated *A. donax* biomass as substrate. The obtained glucose concentration was lower when the prehydrolysis was performed at 37°C than that at 50°C because, generally, the last one is an optimum of temperature for the enzymatic activity (Kumar et al., 2008). Nevertheless, the ethanol concentration was significantly lower in PSSF process conducted at a temperature of 50°C in prehydrolysis step than that obtained the same process performed at a constant temperature of 37°C. In fact, Tenborg and co-workers (2001) reported that high

temperature produced high degree of enzymatic deactivation in prehydrolysis step and thus lower hydrolysis and ethanol production in the following SSF phase.

In our study significantly lower ethanol amount was observed in PSSF process than that obtained in SSF process as also reported by Ohgren et al. (2008) and Hoyer et al. (2009) using steam pretreated corn stover and softwood, respectively. In the PSSF configuration, when the yeast is added to the prehydrolyzed slurry containing an excess of glucose, some sugar are transformed in by-products, thus lowering the final ethanol amount (Linde et al., 2007). Thus, according to Manzanares et al. (2011), the SSF process is a promising option since it is carried out in one vessel and end-product inhibition is minimized, allowing the use of higher solids levels. In addition, an important and interesting finding was that the cellulose conversion percentage obtained (91.4%) in SSF process is similar to the maximal theoretical yield assumed by Wyman et al. (1992) (about 95% from lignocellulose biomasses). In contrast, as reported by Olofsson et al. (2008), the main drawback of SSF is that it is usually conducted at temperatures lower than that optimal for the activity of cellulolytic enzymes. Several authors adviced PSSF process configuration as method to partially solve this problem, since it enables the use of higher temperature throught the initial prehydrolysis step, potentially increasing the enzymatic activity and, as consequence, the cellulose percentage conversion (Ohgren et al., 2008). Moreover, Ohgren et al. (2008), Olofsson et al. (2008) reported the PSSF process able to reduce the viscosity of the slurry making the WIS more soluble in order to facilitate the subsequent SSF step.

First results of this study showed that the SSF process was the most promising process configuration. Therefore, additional SSF experiments were performed using different high WIS amount (from 10% to 40%) to optimize the solid concentration (enzyme to substrate ratio = 69.63 FPU g^{-1} of cellulose pretreated)

in order to obtain the highest ethanol production. Ethanol concentration increased using up to 20% of pretreated *A. donax* biomass, while it decreased using a greater solid concentration. However, since in SSF experiments a high glucose yield was observed using up to 40% of pretreated *A. donax* biomass, no hight fermentation rates were observed. It can claim that the yeast viability and not the enzymatic hydrolysis limits SSF at high dry matter content (Manzanares et al., 2013).

Moreover, at 20% of solid loading a protracted lag phase (96 h) was observed in the SSF process. Linde et al. (2007) reported that a lag phase of 96 h occurred already using a concentration of solid amount equal to 10%. During fermentation a lag phase occurs when the yeast meets a new medium to which it must adapt (Russel, 2008) due to the presence of inhibition substances (Larsson et al., 1999). For example, fydroxymethylfurfural and furfural are two inhibitors formed when hexose and pentose sugars are degraded during the pretreatment step (Palmqvist et al., 1996). *S. cerevisiae* strains are able to metabolize the furfural and to convert it in furfuryl alcohol, reducing the ethanol yield determining also a lag phase during fermentation (Linde et al., 2007). Stenberg et al. (2000) and Hoyer and co-workers (2009) observed no fermentation products in SSF using a dry matter content of 10% and 12%, respectively. Nevertheless, since the selected strain *S. cerevisiae* NA227 used in this study was able to produce high ethanol yield using 15% and 20% of solid loading. Thus, it showed a higher resistance to inhibitors as well as a higher adapting capacity to high WIS concentration than others *S. cerevisiae* strains (Stenberg et al., 2000; Linde et. al., 2007; Hoyer et al., 2009).

Finally, SSCF process configuration, proposed as a promising strategy for maximizing ethanol production from lignocellulosic biomass hydrolysates with the co-fermentation of glucose and xylose (Koppram et al. 2013), was also tested in this work. This process has been performed using the optimal

parameters selected in previous process configuration experiments (enzyme concentration 69.63 FPU g^{-1} and 15% solid loading).

Although it is known that high solid loading reduced the cellulose conversion percentage, we obtained very interesting results in SSCF process since 86% of cellulose conversion percentage using 15% of pretreated *A. donax* biomass. Suriyachai et al. (2013) reported a cellulose conversion percentage of 88% and 86% using 8% and 10% of rice straw biomass concentration, respectively.

Moreover, a significant increase of cellulose conversion percentage and ethanol yield, compared to SSF process, were obtained inoculating *S. cerevisiae* NA227 at the beginning of the experiment and *P. caribbica* NS117, able to convert xylose in ethanol (Saucedo-Luna et al., 2011), after 96 h (T4) of process. This finding could be explained by the fact that *S. cerevisiae* does not ferment pentoses, and other yeast strains must be employed for fermentation of mixture containing arabinose and xylose (Brethauer and Wyman, 2010).

The time span between the two inocula (*S. cerevisiae* NA227 and *P. caribbica* NS117) is fundamental to reduce the glucose concentration in the batch by *S. cerevisiae* NA227 and thus promote the use of xylose by *P. caribbica* NS117. In fact, the growth of mixed cultures is diauxic, that is, when they grow in a mixture of sugars, the utilization of other sugars starts only after glucose depletion (glucose repression) (Zaldivar et al., 2001). The optimized SSCF process developed in this study allow an efficient conversion of pretreated *A. donax* biomass into bioethanol (86.37±1.20% cellulose conversion and 28.03±0.39 g L^{-1} of ethanol).

In conclusion, the results obtained in this work allowed the optimization of the process operational phases selecting the best enzyme concentration (69.63 FPU g^{-1} of cellulose) and solid loading (15%) as well as the selection of the best process configuration (SSCF) for bioethanol production from pretreated *A. donax* biomass.

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Chapter 5 Two step esterification-transesterification process of wet greasy sewage sluge for biodiesel production

5.1 Introduction

Biodiesel (fatty-acid alkyl esters) is a renewable and environmentally friendly energy source. It can be produced from plant oils, animal fats and nonedible feedstock. Since vegetable oils may also be used for human consumption, which can increases the cost of biodiesel production, alternative feedstock as; municipal sewage sludge containing high amounts of lipids have been gaining more attention nowadays in biodiesel production. In fact, Mondala et al. (2009) reported that biodiesel can be produced through the *in situ* transesterification of municipal primary and secondary sludges. Similarly, Revellame, et al. (2009) showed that activated sludge can be used as biodiesel feedstock. Currently, biodiesel productions from wet wastewater sludge to reduce the operational cost processes have been studied. In this sense, Choi et al. (2104) demonstrated that the transesterification method using xylene as a co-solvent can be applied effectively and economically for biodiesel recovery from wet wastewater sludge without drying process. In addition, greasy sludge (wet or dried) showed high potential to produced biodiesel by in situ transesterification using acid and enzymatic catalyst (Sangaletti et al., 2015).

The lipid fraction of primary and secondary municipal sludge contains around 65% of free fatty acids, 7% glycerides and 27% of unsaponifiable material. This means that 70% of sewage sludge lipids could be converted into biodiesel. Considering that sewage sludge may contain in average (for primary and secondary sludge) a lipid fraction of 6%. However, the high FFA content (up to 65%) and the low glyceride fatty acids content (up to 7%) in primary and secondary sewage sludge suggests the use of an esterification pre-treatment step. The high FFA content of sewage sludge can be explained based on the

conceptual model of lipids transformation, where FFA are a product obtained of the hydrolysis of lipids (glycerides) by enzymes (mainly lipases) of bacteria. In addition, lipids, especially FFA, are less responsive to biodegradation compared to other organic substrates such as sugars or amino acids and hence tend to accumulate during the biological treatment of wastewater (Navia and Mittelbach, 2015). In this sense, acid or enzymatic catalysis followed by base catalysis is recommended because of soap formation with alkali-catalyzed transesterification and high FFAs content (El-Mashad et al., 2008; Kargbo, 2010). Several researches have obtained yield above 98% of biodiesel produced from oil with high FFA contents, in a two-step process of acid esterification reaction and alkali transesterification reaction (Ghadge and Raheman, 2005; Tiwari et al., 2007; Marx and Venter, 2014).

5.2 Materilas and methods

5.2.1 **Chemicals**

Samples of sewage sludge were collected during October of 2014 from a municipal wastewater treatment plant (MWWTP) located in Traiguén city belonging to the Araucanía Region, Chile. Sulfuric acid (H_2SO_4) purchased from Sigma-Aldrich and Novozym®435 donated by Novozyme S/A (Bagsvaerd, Denmark) were use as catalysts. Methanol, hexane and all others reagents were of analytical grade and purchased from Merck KGaA (Darmstadt, Germany). Methyl heptadecanoate (Sigma-Aldrich, USA) was used as internal standard in gas chromatography assays to determine fatty acid methyl esters (FAME). N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA), glycerol, monoolein, diolein and triolein purchased from Sigma-Aldrich were used as standard in gas chromatography assays to determine mono-, di- and triacylglycerol.

5.2.2 **Sewage sludge and handling**

The MWWTP of Traiguén employs an activated sludge process, which produces three sludges types: (i) Greasy sludge from the oil, grease and lighter solids collected in the degreaser (11.4% solid content), (ii) a primary-settling sludge, including the heavy solids collected by sedimentation in a settler (2.0% solid content), and (iii) a secondary sludge produced from the biological treatments, which is subsequently pressed (13.0% solid content). In this studied, only the greasy and secondary sludges were evaluated due to their higher solids content compared to the primary-settling sludge.

The sludge samples were collected and transported in a refrigerated container to the laboratory. The fresh greasy sludge was ground in a blender until obtaining a homogeneous mass. The samples (greasy and secondary sludge) were stored in a freezer at -20°C for further assays.

5.2.3 **Characterization of sludge**

The greasy and secondary sludges were characterized in order to determine oil and fat content (%) according to Standard Methods 21th Edition 5520-E and total solid content (%) (ISO11465, 1994). The lipids fraction extracted from sludge was analyzed for unsaponifiable matter (%) (AOCS Ca 6b-53), saponification value Cd 3-25 (AOCS, 2003), acid value (mg KOH g^{-1}) and free fatty acids (% FFA) (Ca 5a-40 AOCS, 2003). The lipid fraction of each sludge sample was obtained using the methodology described by Bligh and Dyer (1979) with minor modifications according to Sangaletti-Gerhard et al. (2015). Table 1 showed the characteristics of greasy and secondary sludge.

Table 15. Characteristics of greasy and secondary sludge

Parameters	**Greasy sludge**	**Secondary sludge**
Solids content of sludge[a] (%)	11.4±0.2	13.0±0.1
Oil and fats content of sludge[a,b] (%)	41.3±1.7	7.5±0.1
Free fatty acid of lipid fraction[a] (% oleic acid)	65.4±4.5	43.6±0.5
Acid value[a] (mg KOH/g)	311.8±6.5	86.7±1.0
Saponification value[a] (mg KOH/g)	130.0±9.0	N.D
Unsaponifiable matter of lipid fraction[a] (%)	9.1±1.1	21.3±0.0

[a] Values are presented as mean ± standard deviation [b] Dry weight base. N.D Not determined

5.2.4 **In situ esterification of sludge**

Esterification reactions were conducted using methanol 99% as acyl acceptor and sulfuric acid 98% (H_2SO_4) and Novozym®435 lipase as catalysts. Reaction conditions were fixed according to the study carried out by Sangaletti-Gerhard et al. (2015) with minor modifications.

5.2.5 **In situ esterification of sludge using H_2SO_4 catalyst**

In a closed bottle Schott Duran type, 10 g sludge (wet weight base) were suspended with methanol and sulfuric acid catalyst previously prepared (methanol-acid solution). For the reaction, 200 mL of a mixture methanol-sulphuric acid (prepared by adding 1 mL H_2SO_4 in 999 mL methanol) was added into the bottle with greasy or secondary sludge preheated. The mixtures were incubated in an orbital shaker at 4.17 Hz at 60 °C.

For the kinetic assay the samples were collected at 0, 0.5, 1, 2, 4, 6, 8, 10, 14 and 19 h after the onset of the reaction. After reactions, each sample was cooling down to room temperature, and the lipid fraction extracted with hexane (30 mL) three times were collected mixed and washed with a saturated sodium chloride solution, with sodium bicarbonate (2%) and distilled water until water remained colorless upon phenolphthalein indicator. Finally, the organic phase was dried with anhydrous sodium sulfate (Na_2SO_4) and filtered. Hexane was removed under vacuum at 45 °C until complete evaporation. The remaining liquid product was dried at 105 °C for 15 min to remove moisture and residual solvent

presence. After cooling, the esterification product was weighed and the methyl esters (FAME) and glycerides (triglycerides, diglycerides and monoglycerides) were quantified. The tests were made in duplicate.

5.2.6 **In situ esterification of sludge using Novozym®435 catalyst**

In a closed bottle Schott Duran type, 1 g Novozym®435 lipase was added in 10 g sludge with 40 mL methanol homogenized and preheated. The mixture was incubated in an orbital shaker at 4.17 Hz at 45 °C.

For the kinetic assay the samples were collected at 0, 0.5, 1, 2, 4, 8, 16, 20, 24, 28, 32 and 38 h after the onset of the reaction. After reaction, the produced FAMEs where separated using the methodology described in Section 2.4.1. However, the hexane phase was only washed with distilled water instead saturated sodium chloride solution and sodium bicarbonate (2%). The tests were made in duplicate.

5.2.7 **Transesterification reaction**

The fraction obtained from the *in situ* reaction of sludge using acid catalyst (mixture of FFA esters and acyl-glycerides) named ester fraction, was subjected to alkaline transesterification reaction in order to increase the yield of methyl esters. The transesterification reaction was carried out using methanol as acyl acceptor and sodium hydroxide and potassium hydroxide as catalyst. The reaction conditions evaluated were four: (1) potassium hydroxide (KOH) 0.9% in mass of ester fraction, methanol:ester fraction molar ratio 6.5:1 at 60 °C for 90 min (Zhang and Jiang, 2008), (2) KOH 0.7% in mass of ester fraction, methanol:ester fraction molar ratio of 8:1 at 50 °C for 60 min (Sharma, 2010), (3) sodium hydroxide (NaOH) 0.5% in mass of ester fraction, methanol:ester fraction molar ratio of 12:1 at 60 °C for 60 min (adapted from Marx and Venter,

2014) and (4) NaOH 0.3% in mass of ester fraction, methanol:ester fraction molar ratio of 12:1 at 60 °C for 60 min (adapted from Marx and Venter, 2014).
All experiments were performed in closed bottles type Schott Duran in an orbital shaker (4.17 Hz) with temperature control. The excess methanol was removed in rotary evaporator under reduced pressure at 45°C and the glycerin phase was recovered after phase separation.
The methyl ester phase was washed times using deionized water (80 °C) and then dried in magnesium sulfate monohydrate. The compositions of the final product as the FAMEs and glycerides (triglycerides, diglycerides and monoglycerides) were quantified. The tests were made in duplicate.

5.2.8 **Methyl esters analysis**

Identification and quantification of fatty acid methyl esters (FAME) was carried out using a Clarus 600 chromatograph coupled to a flame ionization detector from Perkin Elmer (GC-FID) according to the method described by the Comité Européen de Normalisation (EN14103). An Elite-5MS capillary column with a length of 30 m, thickness of 0.1 μm and internal diameter of 0.25 mm was used. The vials were prepared by adding 10 μL of sample to 233 μL methyl heptadecanoate as an internal standard (initial concentration of 2060 mg L^{-1}). FAMEs yield (% based on lipid content) was calculated as the ratio between methyl ester mass (g) and lipid mass (g) multiplied by 100.

5.2.9 **Triglycerides, diglycerides and monoglycerides analysis**

Detection and quantification of triglycerides (TG), diglycerides (DG) and monoglycerides (MG) was carried out by gas chromatography in accordance with the American Standard ASTM D6584. A GC-FID was fitted with a MXT-Biodiesel TG capillary column (10 m x 0.32 mm ID with a 2 m x 0.53 mm ID). Esterification product of each sample was conducted dissolving 15 μL of

sample, 50 uL of N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) as silylation agent in 700 uL of heptane, and 1μL of this solution was injected in GC. Two internal standards were used, one for glycerol and other for glycerides. Monoglycerides (MG), diglycerides (DG) and triglycerides (TG) content were expressed as mass fraction on FAME (%). The instrument was calibrated using monoolein, diolein and triolein in solutions of *n*-heptane, in accordance with standard D6584, ASTM.

5.3 Results

5.3.1 In situ esterification of sludge using H_2SO_4 catalyst

The equilibrium of the esterification reaction, using the greasy sludge and H_2SO_4 as catalyst, was obtained after 8 h (63.53±1.2% FAME), while, using the secondary sludge, no equilibrium of the reaction was observed after 19 h (11.76±3.2% FAME). Moreover, the percentage of FAME obtained was higher using greasy slude than secondary sludge (Figure 64). Considering the results of esterification product by greasy sludge the FFA percentage and Tri- Di- Mono-glycerides were only determinate. The FFA percentage decreased from 65.40±4.5 to 1.67±0.12 and Tri- glycerides loading was reduced from 4.32±0.35 to 1.81±0.37, after 8 h. In addition, Di- Mono- glycerides were absent (Figure 65).

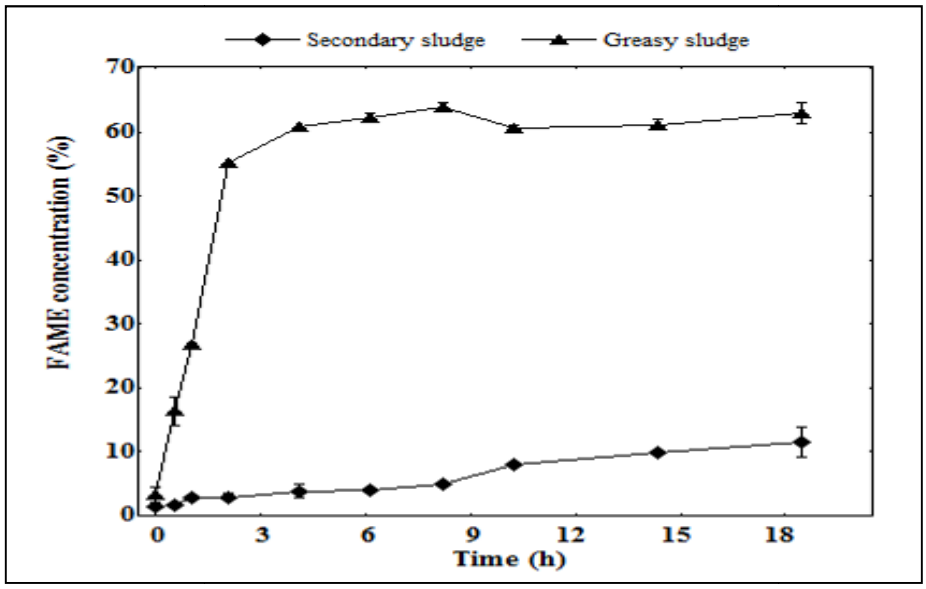

Figure 64. Fatty acid methyl esters (%) in the esterification product from secondary and greasy sludge using acid catalyst in the kinetic assay. Error bars indicate standard deviation of three independent experiments.

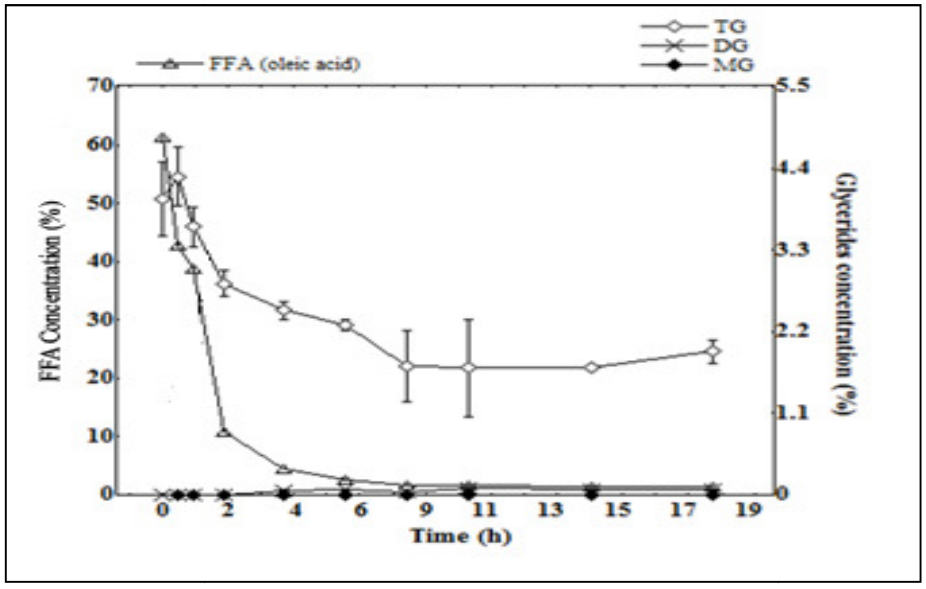

Figure 65. FFA and Glycerides concentration of greasy sludge after esterification process using acid catalyst during the kinetic assay. Error bars indicate standard deviation of three independent experiments.

5.3.2 **In situ esterification of sludge using Novozym®435 catalyst**

The equilibrium of the esterification reaction, using the greasy sludge and enzyme as catalyst, was obtained after 12 h (58.23±4.3% FAME), while, employing the secondary sludge, the equilibrium of the reaction was not detected after 38 h (16.47±3.2% FAME). Moreover, the percentage of FAME was higher using greasy slude than secondary sludge (Figure 66). In reason of the finding reported above, the FFA percentage and Tri- Di- Mono- glycerides were only analyzed. The FFA percentage decreased till 10.00±0.07% in 24 h, even if at equilibrium it was 13.47±0.10%. The tryglicerides fraction went to 0 in 8 h. Thus, the basic transesterification was not carried out.

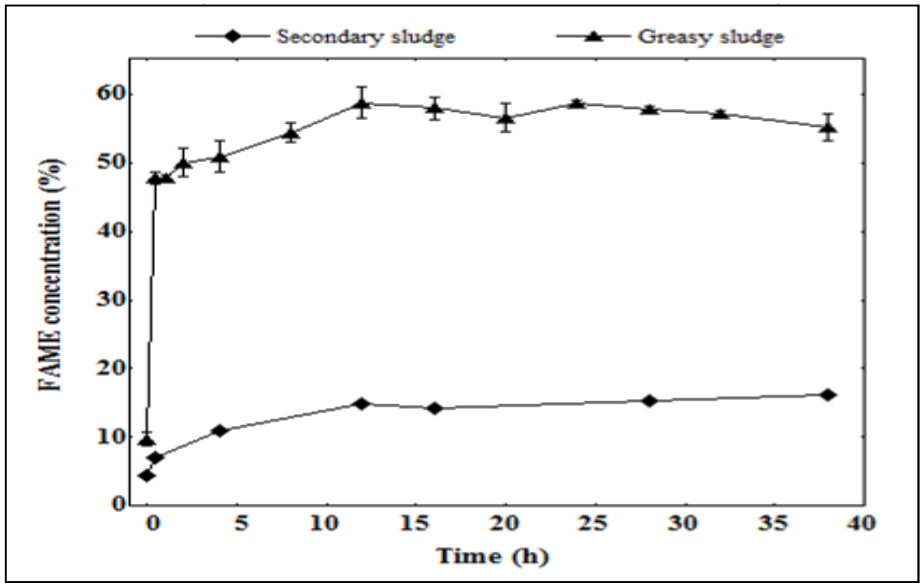

Figure 66. Fatty acid methyl esters (%) in the esterification product from secondary and greasy sludge using enzyme catalyst in the kinetic assay. Error bars indicate standard deviation of three independent experiments.

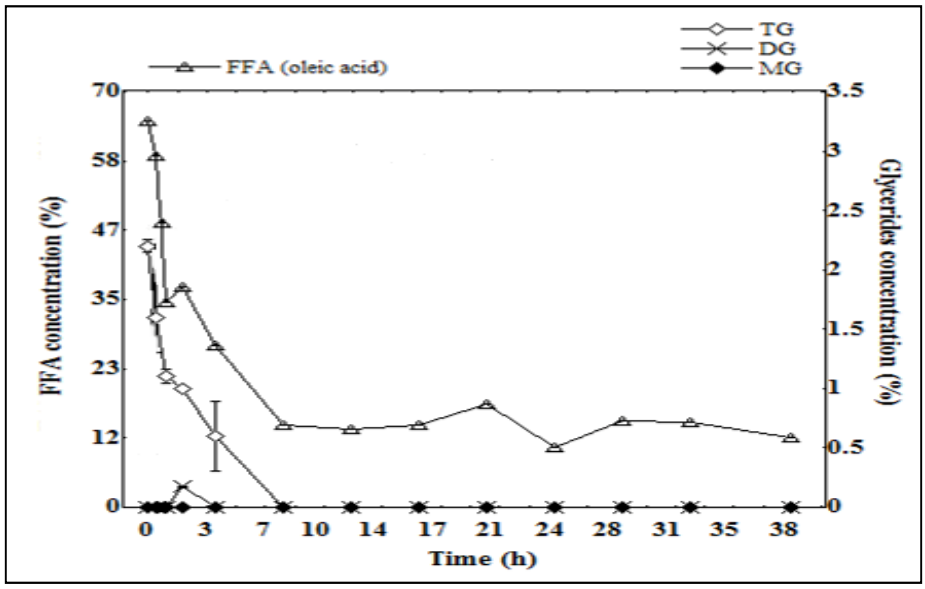

Figure 67. FFA and Glycerides concentration of greasy sludge after esterification process using enzymatic catalyst during the kinetic assay. Error bars indicate standard deviation of three independent experiments.

5.3.3 **Basic transesterification reaction using different catalysts**

The best performance, using acid esterification product, was obtained with NaOH 0.5%, methanol:ester fraction molar ratio of 12:1. The Fame percentage was 65.36±0.10 with residual FFA 1.38±0.00% (Table16).

Table 16. Alkaline transesterification reactions from of esterification product of greasy sludge as feedstock uder different operational conditions (section 2.5) and its effect on FFA and FAME yields

	Acid catalyst	
Reactions	FFA (% oleic acid)	FAME (%)
Esterification	1.67±0.12	63.53±1.20
Transesterification		
1	1.16±0.00	63.65±0.20
2	1.17±0.00	65.15±0.10
3	1.38±0.00	65.36±0.10
4	1.29±0.00	63.87±0.50

5.4 Discussion

According to Mandela et al. (2009), the maximum FAME percentage was obtained using greasy sludge than secondary sludge.

Moreover, the time necessary to obtain the equilibrium of the esterification reaction by using H_2SO_4 as catalyst was shorter compared to the enzymatic esterification. This could be due to the slower reaction rate as reported by Siddiquee and Rohani (2011).

In addition, the lower percentage of FAME obtained by using the enzymatic esterification product than acid esterification product could be imputed to the enzyme deactivation as reported by Bajaj et al. (2010). Moreove, the results demonstrate than, in the two-stage processes for biodiesel production, is more convenient first acid catalysis (esterification) and then alkaline transesterification as reported by Kargbo (2010).

5.5 Reference

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Chapter 6 Conclusion

The final outcomes of the Ph.D project are:

- Many procariotyc and eucariotic strains were characterised, identified and selected for their multi-enzymatic activities. They represent a precious biological and genetic source to upgrade the feasibility of lignocellulose conversion for the 'greener' technology of second-generation biofuel.

- The optimization of the bioethanol production required to find the best performances that were enhanced by modifying the biotechnological parameters of the hydrolysis and fermentation process such as the temperature, the enzymes concentration, the pretreated *Arundo donax* biomass loading, the process configuration as well as the selection of *Saccharomyces* and non-*Saccharomyces* yeast strains.

- Preliminary results in biodiesel production from sewage sludge could give an important indication about the performances obtained using different catalysts.

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Chapter 8 Appendix

8.1 Papers

Vincenza Faraco

Cellulolytic *Bacillus* strains from natural habitats - A review

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KEYWORDS

Bacillus; biodiversity; compost; soil.

ABSTRACT

Fossil fuel reserves depletion, global warming, costly and problematic waste recycling and population growth greatly induce to find renewable energy sources. Second generation bioethanol produced from lignocellulosic materials exhibits great potential as liquid biofuel to substitute gasoline. Production costs of enzymes involved in cellulose hydrolysis into fermentable sugars represent the main obstacle to achieve competitive production of cellulosic ethanol. Cheaper and more efficient biocatalysts for the saccharification step are, therefore, required for making the whole process more competitive. The biodiversity of natural niches has been so far exploited for the isolation of new cellulolytic microorganisms whose enzymes are naturally evolved for an efficient conversion of cellulose into fermentable sugars. This review discusses advances in isolation of bacteria, namely *Bacillus spp.*, from several natural habitats and their ability to produce cellulase activity.

INTRODUCTION

Second generation ethanol, produced from lignocellulosic materials, such as agricultural wastes, plant wastes and other industrial wastes, offers a more attractive greenhouse gas emissions profile than other biofuels, with a 86 % reduction of greenhouse gas emissions in comparison to gasoline (1). Moreover, use of lignocelluloses favors overcoming the so called *fuel versus food* conflict rising from the use of agricultural products (first generation bioethanol) normally addressed to human diet. The main challenges to improve the whole process associated with the conversion of cellulosic biomass to ethanol include increasing polysaccharides hydrolysis yield, decreasing loading of hydrolytic enzymes, optimizing or eliminating the pretreatment and developing a consolidated bioprocessing where the enzyme production, the hydrolysis step and fermentation of sugars into ethanol occur in a single process (2, 3).

An enormous range of different habitats such as soil, compost piles, decaying plant materials, rumens, sewage sludge, invertebrates like termites (gut), forest waste piles, wood processing plants, leaf litter, animal faces, paper mills and water, especially from hot springs have been so far investigated as sources of both aerobic and anaerobic bacteria producing several different enzymes of industrial interest. The aerobic microorganisms, especially fungi, play a major role in the degradation of plant materials and they are found on decomposing wood and plants, in the soil and in agricultural wastes (2). However, the isolation of bacteria as a source of novel enzymes for cellulose hydrolysis is now strongly investigated for the several advantages they exhibit in comparison to fungi. The high growth rates of bacteria, the possibility to easily engineer them, and, last but not least, the higher probability to isolate them from environmental niches that naturally drive them to the production of enzymes resistant to environmental stresses are some their main advantages. This review focuses on the isolation of *Bacillus* strains that have been so far recognized as good producers of cellulase enzymes, from compost, soil and other sources explored for their natural biodiversity (table 1).

COMPOST AS A SOURCE OF NEW CELLULOLYTIC *BACILLUS* STRAINS

Composting process mainly depends on the combined and sequential action of specific taxonomic and functional microbial groups, which produce a series of hydrolytic enzymes that contribute to the bioconversion of the organic materials. During composting, numerous types of microorganisms are involved in degradation of wastes and their diversity is influenced by biomass type. Since the organic substances used for this process are mostly derived from plant material, composting of this recalcitrant biomass is more likely to be led by a microbial community with high lignocellulolytic activity. Therefore, in the last decades, composting has been used as source of novel microorganisms and enzymes to be applied to the conversion of lignocellulose to biofuels (4, 5). Although fungi are the main cellulase-producing microorganisms and most cellulases currently used in industry are of fungal origin, bacteria and actinomycetes have also been reported to produce cellulases (6, 7) and play specific roles in the biodegradation of organic materials during composting.

Cellulolytic bacteria have been isolated from a wide diversity of composts. Bacteria species such as *Pseudomonas*, *Klebsiella* and *Bacillus* (*B.*) have been isolated from different compost environments. Strom (8) reported that bacteria that are the dominant in the thermophilic phase of the process include

Bacillus since the 87% of the randomly selected colonies during this phase belong to this genus. Similarly, Raut et al. (9) observed an increase in bacterial biomass during rapid composting due to dominance of actinomycetes and thermophilic bacteria, especially *Bacillus* spp. formed during the thermophilic phase. In particular, *B. subtilis*, *B. licheniformis* and *B. circulans* are reported as typical bacteria of the thermophilic phase of composting process (10).

Eida and co-workers (11) isolated cellulolytic bacteria using Dubos mineral salt agar containing azurine crosslinked (AZCL) HE-cellulose. Bacterial identification was performed based on the sequence analysis of 16S rRNA genes, and cellulase, xylanase, β-glucanase, mannanase, and protease activities were characterized using insoluble AZCL-linked substrates. Twenty-one isolates were obtained belonging to the genera *Streptomyces*, *Microbispora*, *Paenibacillus*, and *Cohnella,* all showing cellulase, xylanase, β-glucanase, and mannanase activities. Based on enzyme activities expressed as the ratio of hydrolysis zone diameter to colony diameter, it was suggested that some *Paenibacillus* strains (SDCB10, SDCB11) contribute to efficient cellulolytic and hemicellulolytic processes during composting, clarifying the role of these bacteria in the biodegradation of organic wastes.

In their study, Abu-Taleb and co-workers (12) isolated from compost the strain *B. amyloliquefaciens* C23 that showed a high ability to produce cellulase enzymes. Since cellulase yield depends on a complex relationship involving a variety of factors such as inoculum size, pH value, temperature, presence of inducers, medium additives, aeration and growth time, these authors investigated and optimized the nutritional and environmental parameters for improving cellulase production by this cellulolytic bacterial strain.

Recently, 90 bacteria were isolated from raw composting materials obtained from vegetable processing industry wastes using carboxymethylcellulose (CMC) as substrate (13). The bacteria producing the highest cellulolytic activity levels were identified by morphological, physiological, biochemical and molecular analyses as *B. licheniformis* strain 1, *B. subtilis* subsp. *subtilis* strain B7B, *B. subtilis* subsp. *spizizenii* strain 6, and *B. amyloliquefaciens* strain B31C. The strain *B. amyloliquefaciens* B31C was shown to produce the highest cellulase activity levels in comparison to the other isolated *Bacillus* strains. The cellulase purified from this strain showed a significant thermo-resistance and a range of optimum temperatures from 50 to 70°C, resulting an interesting candidate as biocatalyst in lignocelluloses conversion for second generation bioethanol production.

Thermophilic microorganisms from compost, identified as *Bacillus* strains by 16S rDNA sequence analysis, were isolated by Mayende et al. (14) using CMC as substrate. Authors screened the isolates for cellulase and polyphenol oxidase activity and performed temperature optimum and temperature stability studies on the enzymes. Their results showed that thermophilic microorganisms isolated from a composting environment are able to produce thermophilic cellulases which show activity at temperature up to 80° C.

Cellulolytic *Bacillus* strains were also isolated by Kim and co-workers (15) from different environments. Three *Bacillus* strains were selected for their highest cellulase activity and in particular, the strain C5-16, isolated from compost and identified as *B. subtilis*, produced different enzymatic activities, such as CMCase, avicelase, β-glucosidase, and xylanase.

SOIL AS A SOURCE OF NEW CELLULOLYTIC BACILLUS STRAINS

Among the different microorganisms inhabiting the soil, bacteria are the most abundant and predominant organisms, *Bacilli* being the most numerous, followed by *Cocci* and *Spirilla*.

As proposed in the Bergey's Manual of Systematic Bacteriology, most of the soil bacteria can be taxonomically divided in the three orders *Pseudomonadales*, *Eubacteriales* and *Actinomycetales*.

Soil bacteria can also be classified on the basis of their physiological activity and the way in which they obtain carbon, nitrogen, energy and other nutrient requirements (autotrophs or heterotrophs).

Soil bacteria are able to degrade all the components of lignocelluloses. Particularly, the most abundant bacteria of the genus *Bacillus* spp. are involved in cellulose, hemicelluloses and proteins degradation, producing a wide spectra of enzymes used for these reactions.

Microorganism	Source	Enzymatic activity	References
Paenibacillus strains (SDCB10, SDCB11)	Compost	Cellulase/ hemicellulase	[11]
Bacillus amyloliquefaciens C23	Compost	Cellulase	[12]
Bacillus amyloliquefaciens B31C	Compost from vegetable processing of industry wastes.	Cellulase	[13]
Bacillus strains	Compost	Cellulase	[14]
Bacillus spp.	Compost	CMCase, avicelase, β-glucosidase, xylanase	[15]
Bacillus strain DLG	Soil near Lake Mendota (Wisconsin.	Endoglucanase and exoglucanase	[16]
Bacillus strains	Amazonian environment	Cellulase	[17]
Bacillus alcalophilus S39	Soil	CMCase, filter paper activity, β-glucosidases	[12]
Bacillus amyloliquefaciens DL-3	Soil	CMCase	[18]
Bacillus subtilis S52-2	Soil	CMCase, avicelase, β-glucosidase, xylanase	[19]
Bacillus cereus MRLB1	Soil	Cellulase	[20]
Bacillus LFC15	Landfill ecosystem	CMCase	[21]
Bacillus sp.	Nagercoil, Kanyakumari (southwestern region of India)	CMCase	[22]
Bacillus subtilis SJ01	Soil samples in Grahamstown, South Africa	CMCase, avicelase and xylanase	[23]
Bacillus subtilis and *Bacillus circulans*	Benin city, nigeria.	Cellulase	[24]
Anoxybacillus flavithermus, *Geobacillus thermodenitrificans*, *Geobacillus stearothermophilus*	Hot spring, Hammam pharoan, in Sinai desert.	Cellulase	[28]
Bacillus subtilis	Cow dung	CMCase	[30]
Bacillus subtilis	Cow dung	Cellulase	[31]
Bacillus subtilis	Cow dung	CMCase	[32]
Bacillus thermoalcaliphus	Termite mound	Cellulase	[33]
Bacillus licheniformis SVD1	Biosulphidogenic bioreactor	Multy-enzyme complexes	[35]
Bacillus FME1strain *Bacillus* FME2 strain	Flour mill effluents around Tirupati, India	CMCase and FPactivity	[36]
Brevibacillus sp.	Degraded swine waste performing with an aerobic and thermophilic process (Southern Illinois University)	Cellulase	[37]
Bacillus strains *Bacillus* strains	Litter of swampy forest in Pesisir Selatan Mountain forest in Lembah Anai Tanah Datar	Exoglucanase, endoglucanase	[38]
Bacillus subtilis	Molasses obtained in Kom Ombo sugars factory	Cellulase	[39]
Cellulase-producing bacteria	Dry and aged waste products leftover from pulp and paper mill processing (Ontario, Canada); sludge material from the kraft processing of fine paper (Ontario, Canada); commercial fertilizer	Cellulase	[40]
Bacillus licheniformis MVS1 and *Bacillus* sp. MVS3	Water samples	Cellulase	[41]
Bacillus spp. CH43	Hot springs in Chiredzi (43°C, ph 8.5), Zimbabwe	Cellulase	[42]
Bacillus spp. HR68	Chimanimani (55°C, pH 8.5–9), Zimbabwe	Cellulase	[42]

Table 1. Various Bacillus sp. isolated from natural habitats and tested for cellulase activity.

A lot of cellulolytic bacteria belonging to *Bacillus* genus have been so far isolated from soil and characterized for the production of enzymes needed in lignocelluloses to ethanol process.

In 1984, Robson and Chambliss (16) reported isolation of a cellulolytic *Bacillus* strain, DLG, from soil near Lake Mendota (Wisconsin), representing one of the first examples of *Bacillus* sp. isolation from soil.

Heck et al. (17) demonstrated the potential of some *Bacillus* strains isolated from Amazonian

environment for production of cellulases. The isolates were proved to be excellent biological systems, showing some advantages over filamentous fungi, such as faster growth and lower probability of contaminations.
B. alcalophilus S39 was isolated from soil and it distinguished as a potential cellulase producer (12). Studying the effect of growth parameters on the enzyme production levels of this strain, it was shown that glucose represents the best carbon source for biomass accumulation, whilst CMC was the best substrate for CMCase, filter paper (FP) and b-glucosidases production. As far as nitrogen source is concerned, ammonium sulphate and peptone were shown to be the most suitable nitrogen source for cells growth and cellulase activity production, respectively, whilst pH 7 was optimum for enzyme production. The production of cellulase by the new isolate was demonstrated to be influenced even by physical parameters such as temperature and shaker rate, 30°C and 150-200 rpm being the best values, respectively.
B. amyloliquefaciens DL-3 was isolated from soil as a bacterium able to hydrolyze rice hull, a major cellulosic waste-material in Korea, consisting of lignin, cellulose and hemicelluloses (18). Even in this case, cellulase activity production was optimized by changing both carbon and nitrogen source, demonstrating that rice hull is a better carbon source than glucose, fructose, sucrose and maltose. Recently, Kim et al. (15) collected a total of 176 samples from soil, compost, and animal waste slurry on Jeju Island, South Korea. The isolates were screened for cellulolytic activity production and three clones showing relatively higher cellulolytic activity and broader pH optimum were selected. The selected isolate from the soil was designated as *B. subtilis* S52-2 and characterized from both morphological and physiological points of view. It was shown able to produce CMCase, avicelase, beta-glucosidase and even xylanase activities.
Afzal et al. (19) defined the best parameters for cellulase production by a *Bacillus* strain closely related to *B. cereus*, and named *B. cereus* MRLB1.
Total population of cellulose degrading bacteria was studied in a landfill ecosystem as a part of microbial diversity study (20). Samples were obtained from 3 and 5 feet depth of a local landfill being operated for past 10 years. Among many isolates, 22 bacterial strains were selected based on their capability to decompose CMC. All isolates were Gram positive, endospore forming and alkalophilic bacteria with optimum growth pH 9–10. They were grouped based on the phenotypic and chemotaxonomic characters and analysis of 16S rRNA gene indicated that these strains belong to different species of the genus *Bacillus*. Maximum CMCase activity of 4.8 U/ml at 50°C was obtained by strain *Bacillus* LFC15.
In their study, Vijayaraghavan et al. (21) isolated five cellulase-secreting bacterial strains from a paddy field located in Nagercoil, Kanyakumari (southwestern region of India). Among the five bacteria, the strain showing the highest cellulolytic activity on the CMC agar plate was identified as *Bacillus* sp., based on morphological and biochemical characteristics, according to Bergey's Manual of Systematic Bacteriology. Effect of pH on cellulase activity production was studied, showing that cellulase production is higher at pH 6.5, similarly to most of the *Bacillus* strains.
B. subtilis SJ01 was isolated from soil samples in Grahamstown, South Africa and studied for CMCase, avicelase and xylanase activity production. The enzymes have been purified and analysed by zymography, showing their ability to hydrolyze natural substrates like birchwood xylan, as a multi-enzyme complex (22). Similarly, Otajevwo et al. (23) selected two *Bacillus* strains, *B. subtilis* and *B. circulans* from a collection of microorganisms isolated from soil situated in three different locations of Benin City, in Nigeria. Both the isolates produce the highest cellulolytic activities level at around 35 °C, *B. subtilis* giving an optimal yield of residual fermentable sugars at pH 5.
Soil samples from agricultural fields, the premises of paper, cotton and wool industries and rich in rotting rice straw have also been shown a good source for cellulase-producing bacteria (24, 25, 26).
Soil samples from a hot spring, Hammam pharoan, in Sinai desert (in the east of Egypt, at the red sea beach), have been enriched with cellulose with the aim of isolating thermophilic cellullase producing bacteria (27). Isolation of cellulolytic microorganisms from soil samples enriched by incubation with cellulose for about 4 weeks and incubated at 70°C, resulted in 3 different microorganisms showing high similarity with *Anoxybacillus flavithermus*, *Geobacillus thermodenitrificans* and *Geobacillus stearothermophilus*.

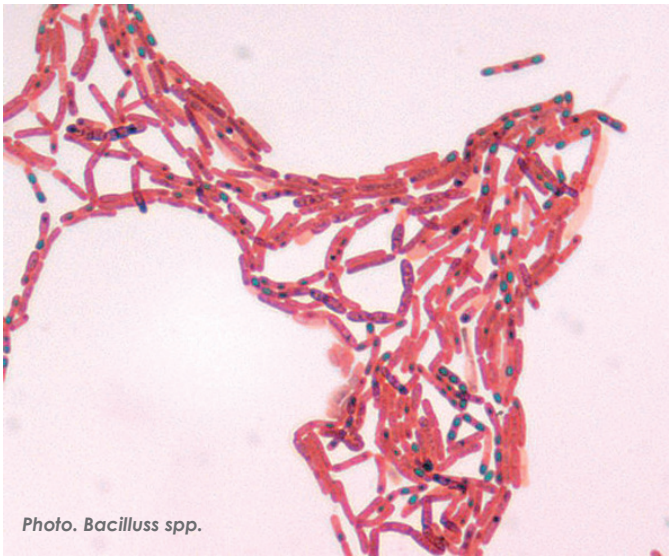

Photo. Bacilluss spp.

OTHER HABITATS FOR NEW CELLULOLYTIC *BACILLUS* STRAINS

Several other sources have been exploited for the isolation of cellulolytic bacteria belonging to *Bacillus* genus. Besides soil, decaying plants materials and compost piles, bacteria occur in the anaerobic rumen of various ruminants and the gut of termites, where they hydrolyze plant materials for the host organism's nutrition. The aerobic bacteria are also usually found in water, on plant materials, in humus, in animal faeces, in sugar cane fields and in leaf litter.
Several manuscripts show the potential of cow dung as a good source for bacteria involved in several physiological roles. Cow-dung is a mixture of dung and urine, generally in the ratio of 3:1, containing crude fibre, crude protein, cellulose, hemicellulose and several minerals such as N, K, S, traces of P, Fe, Co, Mg, P, Cl, Mn, etc. (28). As reported by Swain and Ray (29), *B. subtilis* strains isolated from cow-dung have several beneficial attributes, including biocontrol, plant growth promotion, sulphur oxidation, phosphorus solubilization and production of industrially important enzymes amylase and cellulase (1.5–1.8 mg of reducing sugar $24h^{-1}$ ml^{-1}). Cow dung has been shown an interesting source of cellulolytic bacteria even by Bai et al. (30) and Deka et al.(31) who both isolated *B. subtilis* strains.
Microbial system found in the gut of organisms thriving on cellulosic biomasses is one of the major sources for cellulolytic enzymes. In fact, invertebrates like termites (*Isopteran*), bookworm (*Lepidoptera*), snails have a syntrophic symbiotic microflora in their guts responsible for cellulosic feed digestion. Sarkar and Upadhyay (32) isolated a novel *B. thermoalcaliphus*

from a termite mound, producing a cellulase that is stable at elevated temperatures under alkaline conditions.
In their work, Huang et al. (33) isolated 270 aerobic and facultative anaerobic bacteria from the gut of *Holotrichia parallela* larvae. A *B. licheniformis* SVD1 strain was isolated from a biosulphidogenic bioreactor and studied for the presence of multy-enzyme complexes acting on several substrates such as cellulose, birchwood xylan and bagasse (34).
Bacterial cultures have been isolated also from the flour-mill effluents around Tirupati, India and among the isolates two *Bacillus* strains, FME 1 and FME 2, were identified. Both the isolates were shown able to produce CMCase and FP activities on cellulose powder, whatman filter paper and a lignocellulosic substrate, namely rice husk (35).
Liang et al. (36) used, as the original bacterial source, lyophilized samples from a pilot plant performing an aerobic and thermophilic process to degrade swine waste from a swine finishing building (Southern Illinois University). The new isolate *Brevibacillus* sp. was shown able to grow on different carbon sources, and, except in the presence of xylan and xylose, to release cellulases in the medium as revealed by the filter paper activity assay.
Litter of swampy forest in Pesisir Selatan and mountain forest in Lembah Anai Tanah Datar were shown suitable cellulolytic bacteria sources. The isolates were cultivated in selective media to obtain bacteria from the genus *Bacillus*; thus six *Bacillus* strains from swampy forest and three *Bacillus* strains from mountain forest were obtained (37).
A *B. subtilis* strain has been isolated from molasses obtained in Kom Ombo sugars factory. Factors affecting cellulase activity production have also been studied, showing that the strain is able to use as carbon source for cellulase production a wide spectrum of substrates such as cellulose, filter paper or starch (38).
Maki et al. (39) used different lignocellulosic samples for isolation of cellulase-producing bacteria. Dry and aged waste products leftover from pulp and paper mill processing were obtained from the area of Red Rock (Ontario, Canada) whilst a sludge material produced from the kraft processing of fine paper was obtained from a paper mill in Thunder Bay (Ontario, Canada). Moreover, a commercial fertilizer called Efficient Microorganism Dust was analyzed as bacteria source. Several of these isolates were analyzed for CMcellulase and Filter paper activities production, showing great potential for the application in lignocelluloses conversion.
Water samples are also of great interest for isolation of biotechnologically useful microbes, especially when originated from the so called "hot spring". For instance, Acharya and Chaudhary (40) isolated and identified two new *Bacillus* strains, as *B. licheniformis* MVS1 and *Bacillus* sp. MVS3. Effects on cellulase activity production of several parameters were studied, showing that rice straw and wheat bran can be used as a cheap carbon source which stimulates the production of thermophilic cellulases.
Bacillus spp. CH43 and HR68 were isolated from hot springs in Chiredzi (43°C, pH 8.5) and Chimanimani (55°C, pH 8.5–9), respectively (Zimbabwe), and their cellulases have been purified and characterized by Mawadza et al. (41).

CONCLUSIONS AND PERSPECTIVES

Second generation bioethanol represents a good alternative to the fossil fuels and moreover, its production can be achieved in frame of a biorefinery where the traditional chemical processes can be easily substituted with bioprocesses for the production of a wide spread of industrial green products.
Cellulases are needed in the hydrolysis step involved in the second generation ethanol for cellulose conversion into fermentable sugars but cost of their production are still high, thus efforts to improve the lignocellulose to ethanol conversion process are needed. For instance, research aims at cellulases having better catalytic performances thus requiring lower enzymes loading and reducing time of reaction in the lignocelluloses hydrolysis step. Habitats as soil, compost, decaying plant material, rumens, sewage sludge, invertebrates gut, forest waste piles, wood processing plants, leaf litter, animal faces, paper mills and water have been successfully explored as a source of novel cellulolytic microorganisms. The ability of the most of these microbes to produce cellulolytic enzymes resistant to the recalcitrant conditions typically founded in the process of second generation bioethanol production could help improving competitiveness of the whole process.

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Chestnut Biomass Biodegradation for Sustainable Agriculture

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Biodegradation of lignocellulosic waste from chestnut residues is an important biological process of added value for this sector according to the Chestnut National Plan for agriculture. Dynamic parameters during biodegradation under natural conditions of chestnut burr, leaf, and plant biomass litter are reported in this study. Microbiological and physical-chemical characterisation of chestnut wastes was carried out to monitor this specific biodegradation, to understand the progress and limits of the process and to analyse the compost-like organic substance obtained. Physical-chemical parameters, such as temperature, pH, and water activity, were influenced by the composition of the raw materials and by seasonal climatic conditions. Moreover, microbiological monitoring was assessed by culture-dependent and independent methods. Cellulolytic, hemicellulolytic, and ligninolytic populations were counted to determine different microbial activity during biodegradation process. The functional microbial groups analysed showed different trends, but all were found at high concentrations (7 to 9 log CFU/g). In addition, PCR-DGGE was performed for bacterial and fungal populations to evaluate the microbial diversity. The similarity level during the process was generally very high, both for bacterial and fungal populations. These data are the first on suitable natural degradation for chestnut forests.

Keywords: Chestnut composting; Microbiological monitoring; PCR-DGGE; Sustainable fertilisation

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INTRODUCTION

The Italian Chestnut National Plan in Italy supports the need to guide the chestnut population toward more sustainable forms of production, characterised by a higher ability to meet the needs and interests of consumers and producers by offering new economic opportunities in agriculture. These measures are justified by the need to sequester carbon, reducing greenhouse gas emissions into the atmosphere, recovering biomass for agronomic purposes, and increasing the sustainability of agricultural productions (Marmo 2007).

The use of biomass is interesting at both the EU and national level, especially after the agreements of the Kyoto Protocol (reduction of CO_2 emissions into the atmosphere). Recently, on-farm composting processes have been developed to transform biomass waste of agricultural origin (Tuomela *et al.* 2000). The use of lignocellulosic materials in composting has several important roles, such as structural, absorptive, and biochemical changes (Hubbe *et al.* 2010). An interesting example is the composting of lignocellulosic winery wastes (Paradelo *et al.* 2013*)*. The evaluation of compost made from chestnut residues obtained from cleaning and pruning can be a source of added value for the sector. Although composting procedures are well known, very few reports

exist on the biodegradation of chestnut wood remains and their use in agriculture, and these residues are often burned (Guerra-Rodrìguez *et al.* 2006). Chestnut residue composting, related practices, and their utilisation in agriculture would be possible approaches to a sustainable management of chestnut forests. Benefits of application of biodegraded matter are well recognised. The use of compost, for instance, contributes to the following: (1) improving the soil structural stability, increasing its fertility, and recycling organic matter (humus) and nutrients in a slow release; (2) restoring microbiological diversity in soil and limiting the development of plant pathogens and plant diseases; (3) increasing biological activity and root aeration; (4) reducing the environmental impact, contributing to the degradation of pollutants, and increasing crop protection and carbon dioxide storage in soils; and (5) reducing the use of chemicals and disposal costs.

Moreover, a further relevant aspect involves mechanisms by which compost's microflora are able to contain pathogens. This consists of competition between pathogens and antagonists, antibiosis, parasitism, and induction resistance in the host plant (Hoitink *et al*. 2001). Particularly interesting is the use of compost to suppress soil-borne pathogens. This aspect has been extensively studied by several authors (De Ceuster and Hoitink 1999; Pointing 1999; Ryckeboer *et al*. 2003). The studies of biotic and agronomic components represent key points to characterise the compost (Pepe *et al*. 2013a).

Many studies have been conducted in pilot-scale reactors that enable easier tracking of the biological process: field experiments do not allow monitoring developments in biodegradation because it depends on many environmental factors. Static aerated heaps have been developed for the purpose of natural degradation of lignocellulosic waste obtained from the cleaning of chestnut. Microbiological monitoring using culture-dependent and culture-independent methods of physical-chemical parameters such as temperature, pH, and water activity have been carried out during the process of biodegradation under natural conditions. In fact, the efficiency of process stages depends on a variety of parameters, including aeration, temperature, and content of moisture in the waste. The most important aspect, however, is represented by the microorganisms involved and their functional activity (Neklyudov *et al*. 2008; Pepe *et al*. 2013a).

The present study aims to create useful innovations to increase the sustainability and profitability of production, in accordance with the regulations of organic farming and environmentally-friendly criteria. Biotechnological and agronomic innovations will contribute to the well-being of those who work in remote areas, creating less impact on the environment and improving the quality of the product. On the basis of these considerations, microbiological and physical-chemical characterisation of chestnut biomass submitted to biodegradation was carried out to investigate the bio-oxidative processes at different times and analyse the compost-like organic substance obtained from a microbial and agronomic perspective to understand the progress and limits of the process.

EXPERIMENTAL

Characteristics of the Lignocellulosic Waste

The experimental field was in Roccamonfina (province of Caserta), an area in southern Italy particularly suitable for chestnut forests. The compost-like organic substance (CLOS) was obtained from a composting bin, and the resulting materials were placed in piles. The size of the bin was 1.50 m x 1.50 m x 1.80 m (length x width x height). Fagots were placed under the bin to avoid water retention. The structure was filled with the resulting material of chestnut cleaning (without shredding), such as fresh shoots, chipboard obtained from the cleaning of stakes, undergrowth vegetation (ferns, vetch, sainfoin, wild oats, horsetail grass, and calenzuola), old leaves, and curly. All components were added in equal amounts in terms of weight (about 60 kg) and submitted to natural degradation. Finally, the vegetable biomass was covered with 5 cm of soil taken from the chestnut forest to create a sort of natural lid, which can also enhance the biodegradation process.

The degradation of lignocellulosic wastes was carried out in natural conditions for 105 days. During the whole process, the pile was never turned. Moreover, aeration was also ensured by gaps formed by lateral and basal fagots.

Sampling

Since the process was submitted to natural degradation, it was assumed that it was slow and therefore the samples were collected every two weeks. Samples of 1 kg were collected from the external (right and left side of the pile) and from the internal central part of the biomass and mixed before analyses. Sampling was performed immediately after preparation (T0) and at 15, 30, 45, 60, 75, 90, and 105 days (T1, T2, T3, T4, T5, T6, and T7, respectively) of biodegradation.

Physical-chemical Monitoring and Microbiological Count

CLOS samples were monitored using physical-chemical and microbiological parameters at T1, T2, T5, T6, and T7.

Temperature (°C) was monitored using specific sensors (1500 mm) placed in the core of the pile. The pH was also measured by resuspending 25 g of sample in 250 mL Ringer's solution. Finally, water activity (a_w) was evaluated using a HygroPalm23-AW (Rotronic AG, Basserdorf, Germany). C/N ratio was determined based on the concentration of carbon (Nelson and Sommers 1996) and of nitrogen value (Stevenson 1996).

For microbiological counting, an initial suspension was prepared by the addition of 25 g (wt/vol) of the samples to 180 mL of quarter-strength Ringer's solution (Oxoid, Milan, Italy) in 225-mL Erlenmeyer flasks. After shaking, suitable dilutions were made to the sample solution, which was then used to inoculate different solid growth media. Specific functional groups of the soil microbial community involved in the carbon cycle, such as cellulolytic, hemicellulolytic, and ligninolytic populations, were detected at 28 °C using the surface spread plate count method. Cellulolytic microorganisms were counted using a minimal medium (1 g L^{-1} $(NH_4)NO_3$, 1 g L^{-1} yeast extract, 50 mL L^{-1} standard salt solution, 1 mL L^{-1} trace elements solution, and 15 g L^{-1} bacteriological agar, at pH 7.0) with carboxymethylcellulose (5 g L^{-1}) as the sole carbon source and Remazol Brilliant Blue R to show evidence of cellulolytic activities by developing clear haloes around the colonies (Ventorino *et al*. 2010). The medium used for hemicellulolytic population determination was the minimal medium described above with xylan (5 g L^{-1}) as the sole

carbon source. Finally, ligninolytic microorganisms were counted using LME basal medium (1 g L^{-1} KH_2PO_4, 0.5 g L^{-1}, $C_4H_{12}N_2O_6$, 0.5 g L^{-1} $MgSO_4$, 0.01 g L^{-1} $CaCl_2$, 0.01 g L^{-1} yeast extract, 0.001 g L^{-1} $CuSO_4$, 0.001 g L^{-1} $Fe_2(SO_4)_3$, 0.001 g L^{-1} $MnSO_4$, and 16 g L^{-1} bacteriological agar) supplemented with 0.25 % (wt/vol) lignin as the carbon source, as described by Pointing (1999).

DNA Extraction

Microbiological monitoring with a culture-independent method was performed for all samples (T0, T1, T2, T3, T4, T5, T6, and T7).

Microbial cells were desorbed from CLOS matrices, and pellets were obtained prior to community DNA extraction, as described by Rosewarne *et al*. (2011) with some modifications. First, 100 g of the samples was resuspended in 400 mL of acidic solution (pH 2.0), shaken at 240 rpm for 10 min, and filtered and centrifuged at 10,000 rpm for 20 min. Microbial cells were resuspended in 45 mL of sterile phosphate buffered saline (PBS), then recovered by centrifugation at 6500 rpm for 20 min. The DNA was extracted from compost samples using the FastDNA Spin Kit for Soil (MP Biomedicals) according to the supplier's recommendation.

PCR-DGGE

The primers V3f (5'-CCTACGGGAGGCAGCAG-3') and V3r (5'-ATTACC GCGGCTGCTGG -3') spanning the 200-bp V3 region of the 16S rDNA of *E. coli* were used for bacterial PCR-DGGE analysis. The PCR mixture and conditions were as previously described (Pepe *et al*. 2013b). To analyse the yeast population, the primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and LS2 (5'-ATTCCCAAACAA-CTCGACTC-3') corresponding to nucleotide positions 266 to 285 on the *S. cerevisiae* 26S rRNA gene were used. The PCR mixture and conditions were as previously described (Palomba *et al*. 2011). A GC-clamp was added to the forward primer in all PCR reactions, following Muyzer *et al*. (1993).

DGGE analyses were performed using a Bio-Rad DCode Universal Mutation System (Bio-Rad Laboratories). PCR products were loaded in a 0.8-mm polyacrylamide gel [8% (wt/vol) acrylamide-bisacrylamide (37:5:1)] using a denaturant gradient from 30 to 60% increasing in the direction of electrophoresis for both bacterial and fungal analysis. Electrophoresis was performed at 60 °C, initially at 50 V (5 min) and then at 200 V (240 min). The gels were stained in an ethidium bromide solution (5 min) and rinsed in distilled water (20 min).

Statistical analysis

Bands were automatically detected using the software Phoretix 1 advanced version 3.01 (Phoretix International Limited, Newcastle upon Tyne, England). The cluster analysis was performed by the program after band matching; the method described by Saitou and Nei (1987) was used to obtain the correlation matrix of the DGGE patterns. The resulting matrix was used in the average linkage method by the Cluster procedure of Systat 5.2.1 to estimate the percentage of similarity (S) of microbial populations in the composting process.

RESULTS AND DISCUSSION

The site used for setting up the experiment was chosen because its climatic characteristics (400 m above sea level and north-west sun exposure) are representative of a chestnut forest. The biodegradation bin was filled with the resulting material of the chestnut cleaning, as shown in Fig. 1.

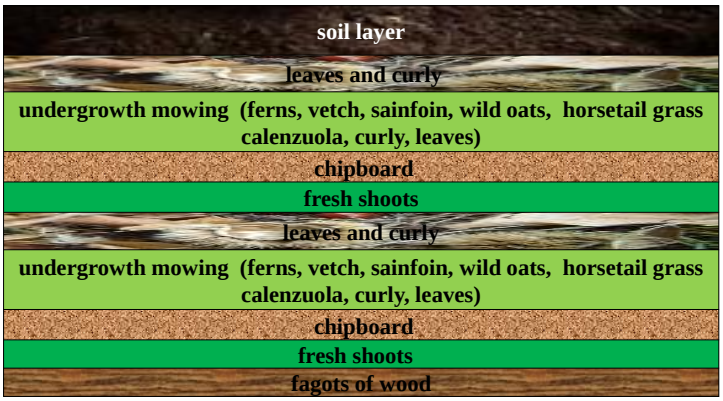

Fig. 1. Order and composition of layers in the biodegradation bin

The biochemical composition of the raw materials was: UR 40.2%, pH 4.54, organic carbon 28.9 % (dry weight), total nitrogen 0.5 % (dry weight), ammonia nitrogen 0.45 % (dry weight), C/N ratio 57.8.

The periodic measurement of the temperature during the biodegradation process is an important index to monitor the fermentation process because it is closely related to environmental conditions and to the metabolic activities of microorganisms. After 15 d, the temperature value observed in the pile was 27.1±0.06 °C (T1 sample). This value increased to 28.9±0.06 °C after 90 days of biodegradation (T6 sample). Since the biodegradation process was not carried out in controlled conditions but in a chestnut forest, it might be inferred that the temperature values in the internal part of the pile were strongly affected by the seasonal climatic conditions. In fact, as shown in Fig. 2, the temperature increased until September (28.9±0.06 °C) and underwent a sudden fall in October (26.6±0.07 °C). The detected environmental temperature showed a similar trend (Fig. 2). Although there are no common definitions of mesophilic and thermophilic phases during the composting process, mesophilic generally refers to temperatures up to approximately 40 °C, and thermophilic refers to temperatures from 45 to 70 °C (Miller 1996). The temperature values detected in this work suggest that the natural degradation process was in the mesophilic phase after 105 days.

Moreover, the detected carbon/nitrogen ratio of raw materials was very high (57.8) to reach a complete degradation. Guerra-Rodríguez *et al*. (2001a) reported that the composting of chestnut burr and leaf litter did not occur because the C/N ratio was too high and the compost never reached the thermophilic phase. In contrast, co-composting with solid poultry manure reached the temperature needed for the thermophilic phase of the process (about 60 °C) in 14 days (Guerra-Rodríguez *et al*. 2001a).

Moreover, measurements of other parameters that influenced microbial activity, such as pH and water activity (a_w), were monitored to understand the progress and limits of the process.

The pH values observed were quite stable, ranging from 6.75±0.03 to 6.8±0.02 at T1 and T7, respectively (Fig. 3). The biodegradation process should occur at a pH value from 3 to 11 (Guerra-Rodríguez *et al*. 2001b), but the best results are obtained in the range of 5 to 8 (Sundberg *et al*. 2004). Beck-Friis *et al*. (2001) reported that the change from the mesophilic to the thermophilic phase in composting corresponded to a change in pH from a range of 4.5 to 5.5 to a range of 8 to 9. In fact, in the first phase of the process, the acidic pH is due to the production of organic acid by microorganisms (Guerra-Rodríguez *et al*. 2001b), while the increase in pH up to 9 is due to ammonium production from protein degradation (Mahimaraja *et al*. 1994).

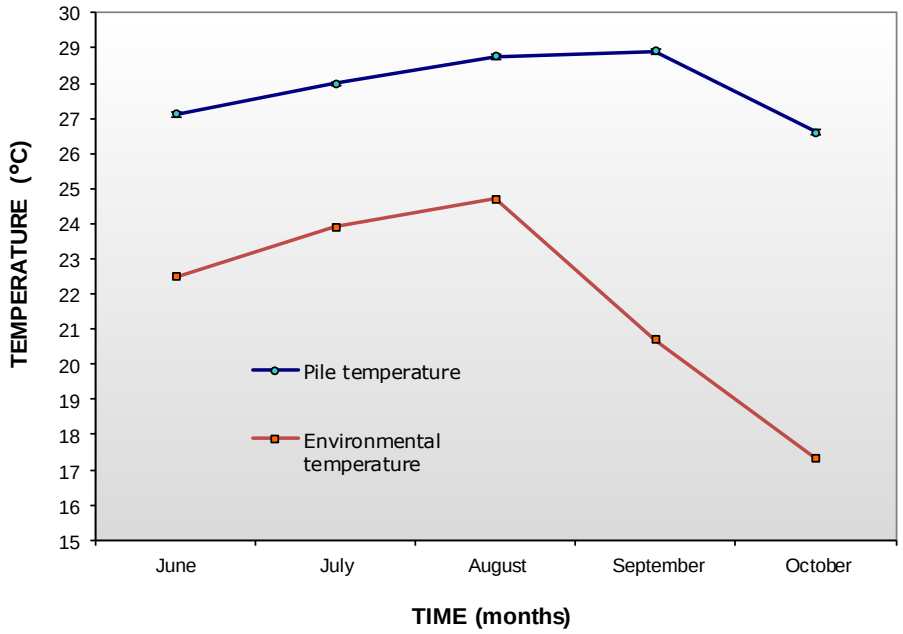

Fig. 2. Temperature values determined during the natural degradation process

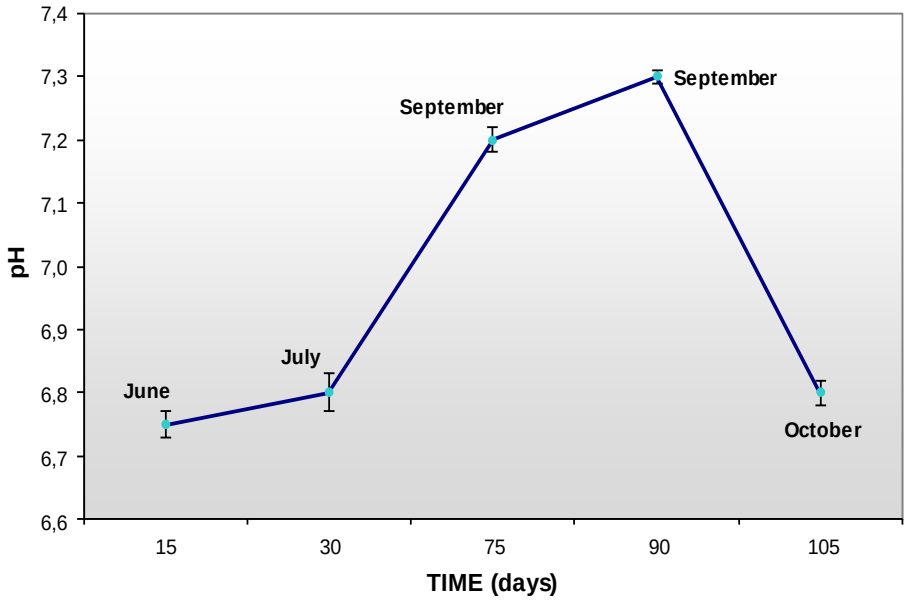

Fig. 3. pH values determined during the natural degradation process

Water activity (a_w) (water readily available for microbial metabolic activities) was also measured. The value of a_w was 0.94±0.003 after 15 d of experimentation and increased up to 0.98±0.001 after 105 days of the natural degradation process (Fig. 4).

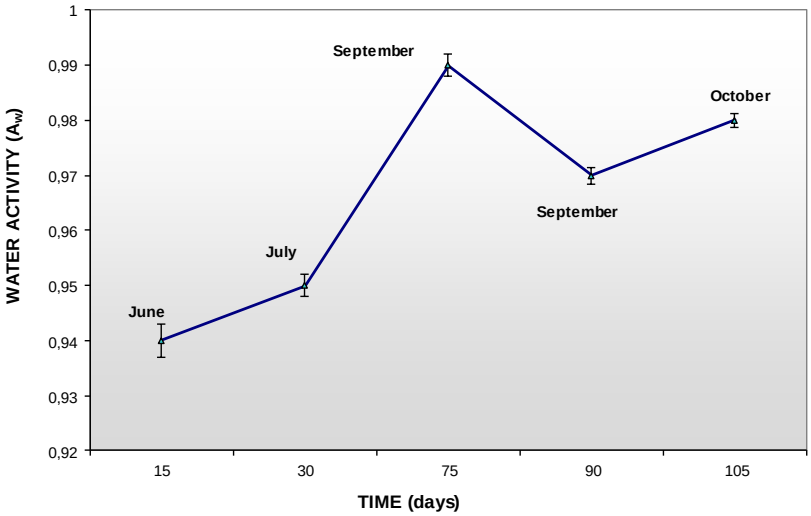

Fig. 4. Water activity values determined during the natural degradation process

Biodegradation of lignocellulosic materials is a result of the combined and sequential action of specific functional microbial groups, but little is known about the diversity of microorganisms in the carbon cycle that is specific and essential for vegetable biomass degradation. A large number of microorganisms have been studied for their potential ability of cellulose, hemicellulose, and lignin degradation in different experimental conditions (Huang *et al.* 2010a). The effectiveness of the process depends largely on the metabolic activities of microorganisms affecting the quality of the end product. For this reason, knowledge of the importance of specific functional groups can still be improved (Pepe *et al.* 2013a).

Because lignocellulosic materials are complex molecules of hemicellulose, cellulose, and lignin, three different functional microbial groups were analysed. The three functional groups involved in the carbon cycle showed different trends during biodegradation under natural conditions. Hemicellulolytic populations showed a value of about 9 log CFU g^{-1} of sample during the experimental process (Table 1). Indeed, after 105 days of biodegradation (T7), it was possible to detect an increase in cellulolytic populations, showing a concentration reaching to 10.18±0.04 log CFU g^{-1}. Observing the growth of aerobic cellulolytics, it was thought that the organic matrix possibly showed a good starting composition of cellulosic material. Pepe *et al.* (2013a) observed an increase in aerobic cellulolytics growth during composting of agro-industrial waste. The presence of hemicellulolytics and cellulolytics during the whole biodegradation process plays an important role with respect to cellulose, which represents the principal constituent of vegetable waste and whose degradation is restricted to a narrow range of microbial enzymes such as hemicellulase and cellulase (Herrmann and Shann 1997; Amore *et al.* 2013). Ligninolytic microorganisms showed an opposing trend. For this population, a significant decrease of about 1 log unit (from 8.48±0.00 to 7.59±0.16 log CFU g^{-1}) was detected (Table 1). It is probable that the decrease of temperature at T7 could negatively influence ligninolytic microbial activities, especially with regard to the fungal population.

In fact, microorganisms usually involved in the degradation of structural polymers (cellulose, hemicellulose, and lignin) such as fungi and actinomycetes (Huang *et al.* 2008; Amore *et al.* 2012) could be negatively affected by low temperature because their growth is favoured by higher temperatures (Ishii *et al.* 2000). Huang and co-workers (2010b) reported a continuous change in microbial population structure during composting of lignocellulosic waste. In particular after 3 days they detected a decrease of quinone species Q-9 associated with lignin degraders and an increase of Q-9(H2) and Q-10(H2) associated with cellulose-degrading ability.

Table 1. Microbial Contents (Log CFU g^{-1}) During Biodegradation Process

TIME SAMPLING (days)*	MICROBIAL FUNCTIONAL GROUP		
	Hemicellulolytic	*Cellulolytic*	*Ligninolytic*
15	9.04±0.06	9.00±0.06	8.48±0.00
75	9.36±0.08	9.65±0.07	9.25±0.07
90	8.58±0.03	8.64±0.19	7.34±0.19
105	8.97±0.21	10.18±0.04	7.59±0.16
* 15, T1; 75, T5; 90, T6; 105, T7.			

Microbiological monitoring with a culture-independent method was carried out during the natural degradation process. The results of the PCR-DGGE analysis performed on the samples during biodegradation are shown in Fig. 5. After 15 d, the bacterial and fungal populations showed very different profiles compared to the beginning of the process (T0). Instead, during natural degradation, the microbial community varied slightly, showing similar profiles both in bacterial and fungal populations. Ahmad *et al*. (2011) reported that the microbial community was influenced by environmental conditions and substrate characteristics, but did not change significantly during a composting process. In fact, they reported that the DGGE analysis showed similar profiles and the presence of same major bands.

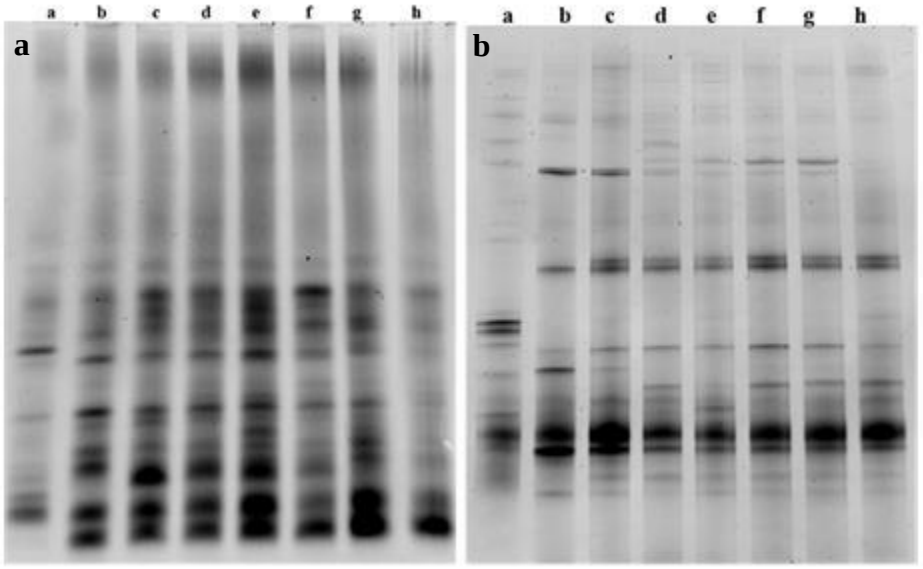

Fig. 5. DGGE profiles of bacterial (a) and fungal (b) populations during biodegradation process. Lanes: a, T0 (0 d); b, T1 (15 d); c, T2 (30 d); d, T3 (45 d); e, T4 (60 d); f, T5 (75 d); g, T6 (90 d); h, T7 (105 d)

The similarity level (S) in bacterial populations during chestnut biomass biodegradation was generally very high, showing values from 95% to 100%, except for at T0 (Fig. 6a). In fact, only at the beginning of the process were the bacterial populations very different, showing a similarity value of 60%, unlike the other sampling times. A similar trend was observed in fungal populations (Fig. 6b). In this case, the microbial diversity detected at T0 was the highest, showing a similarity value of 42% with the other times. Moreover, the levels of similarity among the fungal populations during the natural degradation varied more gradually than those detected among bacterial populations, as shown in Fig. 6.

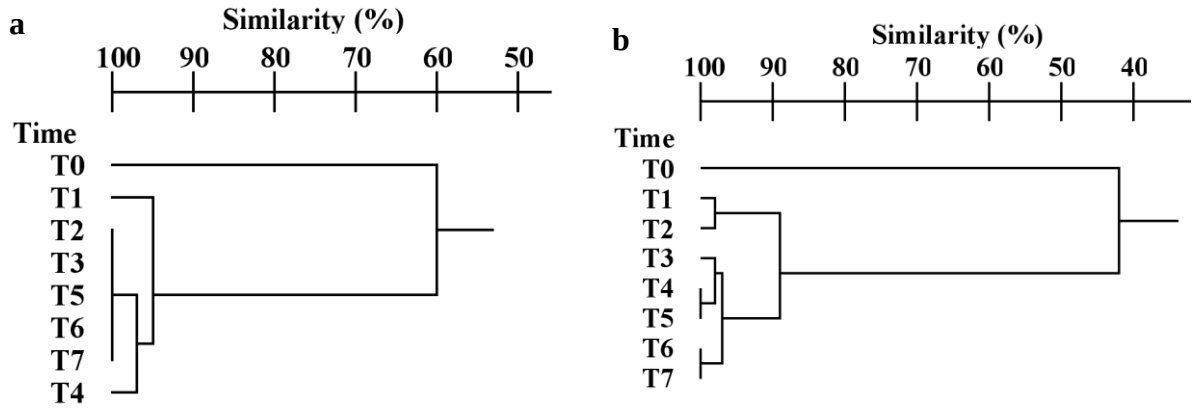

Fig. 6. Dendrogram showing the degree of similarity (%) of PCR-DGGE profiles of the bacterial (a) and fungal (b) populations during natural degradation of chestnut biomass

These results showed that the bacterial population was less influenced by the environmental conditions than the cellulolytic microbial functional group. In contrast, the fungal population suffered a greater variation during the biodegradation process, showing behaviour similar to a ligninolytic functional group. Because the ligninolytic activity of fungal populations is widely recognised (Feng *et al.* 2011), it is possible that the ligninolytic group was composed mainly of fungi and less so of bacteria, which exhibit more cellulolytic and hemicellulolytic activities. Despite recent findings, our results are encouraging because there have been few reports on biodegradation of chestnut biomass and there are no previous investigations about its microbiological characterisation.

CONCLUSIONS

1. The temperature values in the internal part of the pile were strongly affected by the seasonal climatic conditions.
2. Cellulolytics increased during biodegradation of chestnut biomass, while ligninolytics decreased as an effect of microbial competition and environmental conditions.
3. DGGE analysis showed similar profiles during the natural degradation process for both bacterial and fungal populations, except at the beginning of the process.
4. Because lignocellulosic biomass is also an attractive waste material for production of a wide range of high value-added products, cellulolytic, hemicellulolytic, and lignin-

lytic microorganisms should be isolated and selected for their biotechnological applications and used to improve the process.

5. To the authors' knowledge, there have been no previous investigations of the microbiological characterisation of chestnut biomass submitted to biodegradation under natural conditions. The first preliminary results are encouraging, but further tests must be carried out to improve the understanding of natural degradation of chestnut and the final product.

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Influence of Different Lignocellulose Sources on Endo-1,4-β-Glucanase Gene Expression and Enzymatic Activity of *Bacillus amyloliquefaciens* B31C

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Conversion of cellulose into fermentable sugars for ethanol production is currently performed by enzymatic hydrolysis catalyzed by cellulases. The cellulases are produced by a wide variety of microorganisms, playing a major role in the recycling of biomass. The endo-1,4-β-glucanase (CelB31C) from *Bacillus amyloliquefaciens* B31C, isolated from compost and previously selected on the basis of highest cellulase activity levels among *Bacillus* isolated, was characterized as being a potential candidate for a biocatalyst in lignocellulose conversion for second-generation bioethanol production. The aim of this work was to evaluate the changes in production of enzymatic activity of the endo-1,4-β-glucanase (CelB31C) and the expression of its gene (*bglC*) using a carboxymethylcellulase activity assay and qRT-PCR analysis, respectively, during growth of *B. amyloliquefaciens* B31C on different cellulose sources: carboxymethylcellulose (CMC), pure cellulose from *Arundo donax*, pretreated *Arundo donax* biomass (Chemtex), and microcrystalline cellulose (Avicel). The results showed that both the expression of *bglC* gene and the enzymatic activity production are related to the type of cellulose source. The strain showed a high enzymatic activity on lignocellulosic biomass and on microcrystalline cellulose. Furthermore, the highest gene expression occurred during the exponential phase of growth, except in the presence of Avicel.

Keywords: Bioethanol; Bacillus amyloliquefaciens; endo-1,4-β-glucanase; Arundo donax biomass; qRT-PCR; *bglC gene*

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INTRODUCTION

Lignocellulosic biomass is the most abundant renewable bioresource as a collectable, transportable, and storable chemical energy, and it is far from fully utilized. It is mainly composed of three major biopolymeric components: cellulose, hemicellulose, and lignin (Sathitsuksanoh *et al.* 2012). Strongly interwoven linkages among the biopolymers result in a naturally recalcitrant composite, and pretreatments are needed to make the cellulosic and hemicellulosic fractions accessible to enzymatic hydrolysis by opening the lignin sheath (Bhalla *et al.* 2013) and improving the enzymatic digestibility of pretreated lignocellulosic biomass.

In recent years, largely in response to an uncertain fuel supply and the need to reduce carbon dioxide emissions, bioethanol (along with biodiesel) has become one of the most promising biofuels today and is considered the only feasible alternative, in the

short and medium time frame, to fossil transport fuels in Europe and in the wider world (Onuki *et al.* 2008). To achieve energy and climate goals, the potential of bioenergy is a key issue (Ordóñez *et al*. 2013).

Bioethanol from traditional means, or first-generation bioethanol, is based on starch crops such as corn and wheat and on the bagasse byproducts from sugar crops such as sugar cane and sugar beet. Lignocellulose (excluding lignin) is an abundant carbohydrate source and has significant potential for conversion into liquid and gaseous biofuels (Bhalla *et al*. 2013).

In addition, the development of lignocellulosic technology has meant that not only high-energy content starch and sugar crops can be used, but also woody biomass or waste residues from forestry for 2nd generation biofuels.

The technology necessary to utilize the entire plants' biomass for ethanol production requires technologies that can break the cellulose into sugars and then ferment them to produce ethanol. Conversion of cellulose into fermentable sugars for ethanol production is currently performed by enzymatic hydrolysis catalyzed by cellulases, which are produced by a wide variety of microorganisms, depolymerizing raw materials and playing a major role in recycling of the biomass (Amore *et al*. 2012). Cellulases are needed in the hydrolysis step involved in second-generation ethanol for cellulose conversion into fermentable sugars, but costs for their production are still high; thus, efforts to improve the lignocellulose-to-ethanol conversion process are needed (Amore *et al*. 2013a). Despite its many advantages, cellulosic bioethanol is not yet industrially produced at a competitive level, mostly because of the high cost of cellulolytic enzymes. Because of this, more efficient and cheaper cellulolytic enzymes should be developed (Amore *et al*. 2013b).

Recently, 90 bacteria were isolated from raw composting materials obtained from vegetable processing industry wastes, using carboxymethylcellulose (CMC) as a carbon source (Ventorino *et al.* 2010; Pepe *et al*. 2013). A strain of *B. amyloliquefaciens* (B31C) was shown to produce the highest cellulase activity levels in comparison to the other isolates. The endo-1,4-β-glucanase CelB31C produced by *B. amyloliquefaciens* B31C was characterized as being a potential biocatalyst candidate in lignocellulose conversion for second-generation bioethanol production (Amore *et al*. 2013b).

The aim of this work was to evaluate the changes in cellulase activity and the expression of *bglC* during the three phases of growth of *B. amyloliquefaciens* B31C (lag, exponential, and early stationary) on different sources of cellulose. This is one of the most important factors affecting the production cost and yield of β-glucanase (Verma *et al.* 2013).

EXPERIMENTAL

Bacterial Strain, Media, and Growth Conditions

B. amyloliquefaciens strain B31C was used in this study; it was grown in liquid medium prepared as follows: 5 g L^{-1} CMC, 7 g L^{-1} yeast extract, 4 g L^{-1} KH_2PO_4, 4 g L^{-1} Na_2HPO_4, 0.2 g L^{-1} $MgSO_4.7H_2O$, 0.001 g L^{-1} $CaCl_2.2H_2O$, and 0.004 g L^{-1} $FeSO_4.7H_2O$ (Abou-Taleb *et al.* 2009). After an overnight incubation at 30 ºC, a suitable volume of the broth culture was used to inoculate 30 mL of the same medium, modified in the cellulose composition. The different pretreated lignocellulosic biomasses and the commercial celluloses added in the liquid medium (10 g L^{-1}) are listed in Table 1.

Pretreated *A. donax* was received from Chemtex Italia S.r.l. One batch of steam-exploded material was used, with 1% (w/w) water-insoluble solids (WIS) (Table 1). During incubation at 30 °C, liquid culture samples, monitored by measuring the optical density (OD at 600 nm) and viable counting, were withdrawn during different stages of growth.

The experiments were performed in triplicates.

Table 1. Lignocellulosic Biomasses and the Commercial Celluloses Added

Source/Manufacturer	Commercial name - Origin
Sigma-Aldrich, Germany	Carboxymethylcellulose CMC Sodium Salt
Sigma-Aldrich, Germany	Avicel - Microcrystalline Cellulose
Arundo donax	Pure cellulose
Chemtex-Italia (GM-Group)	Pretreated *Arundo donax* Lignocellulosic Biomass

qRT-PCR Analysis

Total RNA was isolated from the microbial cells collected at different stages of growth, as described above, using a RiboPure™-Bacteria RNA isolation kit (Ambion, Milano, Italy), according to the manufacturer's instructions. Twenty nanograms of RNA (DNA-free) were first reverse transcribed in cDNA using iScript™ cDNA Synthesis; then, the gene of interest and the housekeeping gene (16S rRNA has been used as reference gene) were amplified using the iQ™ SYBR® Green Supermix Kit according to the manufacturer's instructions in a Chrom4 System Thermocycler (the kits used for the retrotranscription and the amplification, and the thermocycler, were purchased from Bio-Rad Milano). Based on the genome sequence of *B. amyloliquefaciens* FZB42 (GenBank: CP000560.1 - GeneID: 5461442), primers were designed to amplify portions of *bglC* codifying for the endo-1,4-beta-glucanase enzyme. All primers (Table 2) were purchased from Primm (Milano, Italy). The qRT-PCR running protocol was performed according to the manufacturer's instructions. To confirm that there was no background contamination, a negative control was included for each run. For each target gene, PCR efficiency was determined. Melt curves were calculated to check the amplified products.

Table 2. Primers Used for qRT-PCR

Genes	*Primers*	*T°m*	Efficency %	Fragment length (bp)	Source
16S-rRNA	f: CAAGCGTTGTCCGGAATTAT r: CTCAAGTTCCCCAGTTTCCA	60	101	112	This study
bglC	f: TAAGCTGGCTGAACGGCTAT r: TCCTGATCCGTTTCAGATCC	60	98	90	This study

The PCR efficiency (E) for each primer set was determined by generating cDNA dilution curves obtained by plotting the threshold cycle (Ct) for each cDNA amount against the log of the cDNA concentration.

The relative expression ratio was calculated for each gene of interest by using a mathematical model described by Pfaffl (2001) as follows:

$$\text{Ratio} = (E_{\text{target}})^{\Delta Ct,\ \text{target (control-sample)}} / (E_{\text{reference}})^{\Delta Ct,\ \text{reference (control-sample)}}$$

All measurements of gene expression were conducted in triplicates, and the mean of these values was used for the analysis.

Azo-CMCase Assay

The cells for the RNA extraction were collected by centrifugation at 12,000⊆g for 10 min, and the supernatants were collected to be processed for extracellular endo-1,4-ß-glucanase activity by AZO-CMCase assay (Megazyme, Ireland), following the supplier's instructions. The analytical determinations correspond to the mean value of three replicates.

RESULTS AND DISCUSSION

The growth of *B. amyloliquefaciens* B31C was monitored during lag, exponential, and early-stationary phases. No differences were found during the growth on the different cellulosic media used in this study (Table 1). Table 3 shows the number of cells (CFU mL^{-1}) and the OD value (600 nm) during the different stages of growth of the bacterium in different cellulose sources.

Table 3. Growth Phase Values of B31C

		CMC	Avicel	*A. donax*	Chemtex
Growth Phase	**OD 600 nm**	**CFU mL^{-1}**			
Lag (1.5 h) *	0.18	$1.0x10^{6a}$	$3.0x10^{6a}$	$1.6x10^{6a}$	$1.5x10^{6a}$
Exponential (3.5 h)	0.50	$2.0x10^{7b}$	$4.0x10^{7b}$	$2.5x10^{7b}$	$2.0x10^{7b}$
Sub-stationary (5.5 h)	0.80	$2.0x10^{8c}$	$3.0x10^{8c}$	$2.8x10^{8c}$	$3.0x10^{8c}$

***** Time elapsed after inoculum. O.D. standard deviation < 0.002; CFU mL^{-1} <0.015; The letters in the columns indicate significant differences p ≤ 0.01 (t-test).

The expression of *bglC* was determined by qRT-PCR as described earlier. Results, reported in Fig. 1, show an increase in *bglC* expression during the exponential phase of growth and a reduction during the early stationary phase. Regarding the carbon source, it was observed that an overexpression occurred during the lag phase, except in the presence of Chemtex's pretreated cellulose. During the following phases of growth, the degree of expression of the gene, compared to the 16S rRNA (reference gene), was detected 5.15-fold in the presence of Avicel, up to 300-fold in the presence of *A. donax*, rising generally to a level of expression higher than 10-fold during the early stationary phase.

The highest enzymatic activity (Fig. 2) was detected in the presence of Avicel, while the strain showed the lowest enzymatic activity in the presence of CMC. Generally, a slight variation of the activity during the different growth phases was noticeable. However, during the early stationary phase, the activity, except in the presence of Avicel, appeared higher. The pattern of enzyme production in the presence of different carbon sources during the early-stationary phase was Avicel > Chemtex pretreated biomass > *A. donax* > CMC.

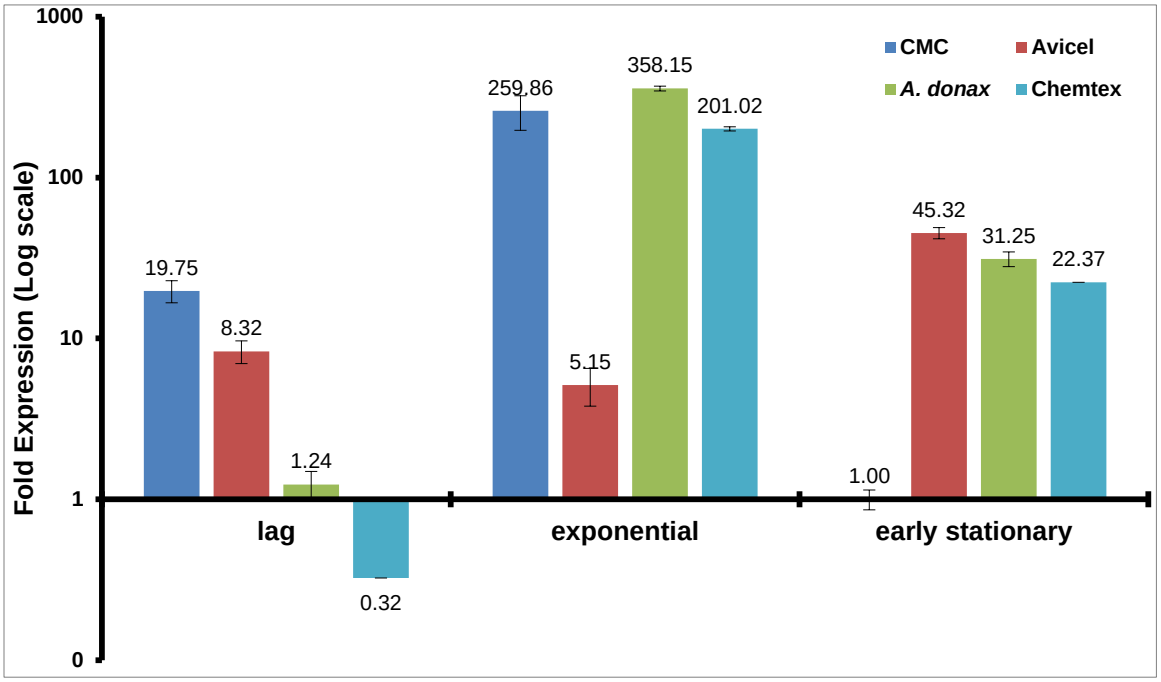

Fig. 1. Expression of *bglC* during different growth phases

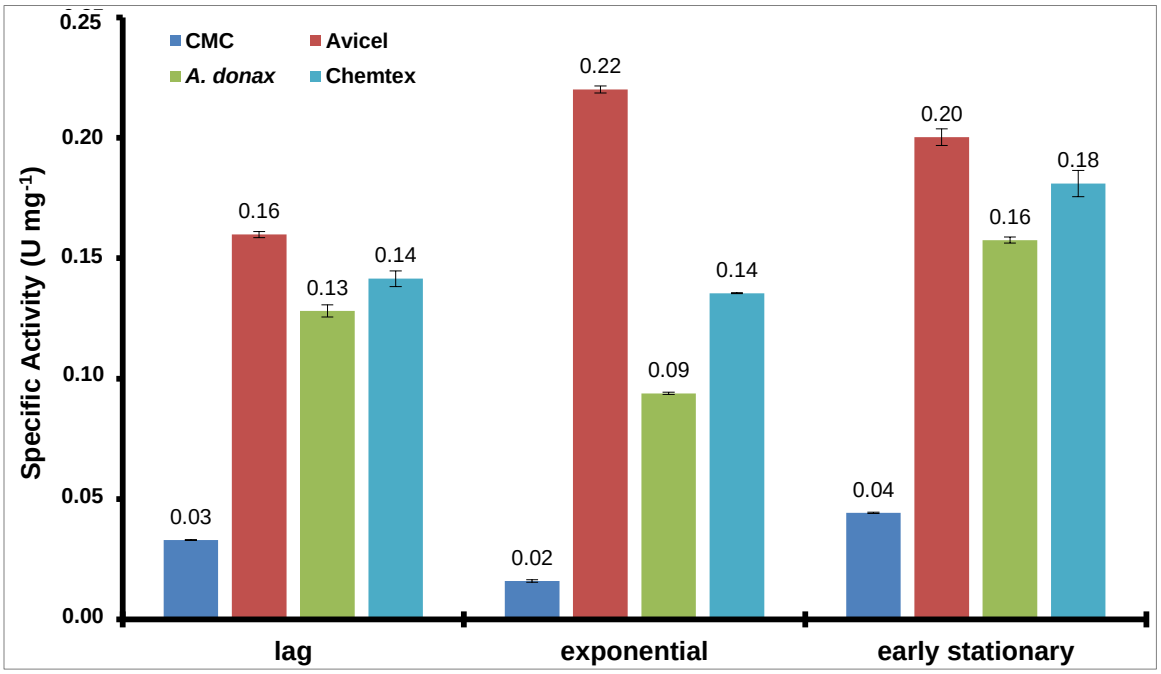

Fig. 2. Endo-glucanase activity with different cellulose sources (Azo-CMC assay)

In line with these results, Sethi *et al.* (2013) determined the effects of different agro-based waste source on the endo-cellulase activity of different bacteria isolated from soil, including a *Bacillus* strain. They underlined the involvement of the cellulose source on the enzymatic activity. As far as is known, this is the first report on the activity, as well as the gene expression, of the endo-1,4-β-glucanase of a *B. amyloliquefaciens* strain grown on different types of cellulose as carbon sources. A general overexpression of the investigated genes is evident from the results. On the other hand, this does not always

correspond to an increase of the enzymatic activity. It has been reported (Di Pasqua *et al.* 2013; De Filippis *et al.* 2013) that an increasing in gene expression does not always correspond to an increased protein regulation or high metabolites concentrations.

The gene expression increased in the presence of CMC more than in the presence of Avicel. This can be due to the microcrystalline structure of Avicel, the degradation of which requires a primary action of exo-cellulase enzymes (Soares *et al.* 2012). This could explain the lower expression of the gene compared to that in the presence of CMC during the lag and exponential growth phases. However, it is presumed that during the last phase of growth, in the presence of Avicel, the assumptive exo-cellulase enzymatic activity might had been replaced by endo-glucanase activity. This explains both the higher enzymatic activity and the high *bglC* expression. These findings are in line with those found by Wei *et al*. (2012). They reported that the expression pattern of cellulase activity takes place in a coordinated way that can enhance the overall efficiency of cellulose degradation.

An unexpected result was obtained in the presence of pure cellulose from *A. donax* and pretreated *A. donax* lignocellulose biomass because the abundance of lignin in the Chemtex biomass (data not shown) could reduce the enzymatic activity compared to that found in presence of pure cellulose from *A. donax*. A high induction of endo-1,4-β-glucanase, regardless of the concentration of lignin, has been reported by Bano *et al.* (2013). Recently, it has been proposed that lignin is melted and relocalized to the outer surface of the cell wall during high-temperature pretreatment, increasing the accessibility of the cellulose within, which might be a consequence of change in the S/G ratio of the lignin structure (S: syringyl-like lignin structures; G: guaiacyl-like lignin structures) (Li *et al*. 2010). S-rich lignin is more linear and often has a lower degree of polymerization.

It is tempting to speculate that the high-temperature pretreatment led to a relocalization and reorganization of the structure, increasing the S/G ratio, which in turn increased the enzymatic activity. Finally, the complex composition of *A. donax* lignocellulose biomass as multiple carbon source, could induce higher enzymatic activity (Xiong e*t al.* 2010).

CONCLUSIONS

1. This study showed that the enzymatic activity of *B. amyloliquefaciens* B31C strain is not related to the gene expression, representing a promising outcome for an application on a larger scale, to confirm the use of this enzyme as an interesting candidate for cellulose conversion in bioethanol production.
2. The growth of the *B. amyloliquefaciens* B31C strain was not affected by the different cellulose sources used in this study.
3. The pretreated lignocellulosic biomass (Chemtex) represents a good candidate for the industrial production of bioethanol.
4. Although the tests were done on a laboratory scale, the endo-glucanase activity of the *B.amyloliquefaciens* B31C strain was encouraging for defining the technical functions of the strain as promising for industry cellulose conversion in bioethanol.

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Exploring the microbiota dynamics related to vegetable biomasses degradation and study of lignocellulose-degrading bacteria for industrial biotechnological application

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The aims of this study were to evaluate the microbial diversity of different lignocellulosic biomasses during degradation under natural conditions and to isolate, select, characterise new well-adapted bacterial strains to detect potentially improved enzyme-producing bacteria. The microbiota of biomass piles of *Arundo donax*, *Eucalyptus camaldulensis* and *Populus nigra* were evaluated by high-throughput sequencing. A highly complex bacterial community was found, composed of ubiquitous bacteria, with the highest representation by the *Actinobacteria*, *Proteobacteria*, *Bacteroidetes* and *Firmicutes* phyla. The abundances of the major and minor taxa retrieved during the process were determined by the selective pressure produced by the lignocellulosic plant species and degradation conditions. Moreover, cellulolytic bacteria were isolated using differential substrates and screened for cellulase, cellobiase, xylanase, pectinase and ligninase activities. Forty strains that showed multienzymatic activity were selected and identified. The highest endo-cellulase activity was seen in *Promicromonospora sukumoe* CE86 and *Isoptericola variabilis* CA84, which were able to degrade cellulose, cellobiose and xylan. Sixty-two percent of bacterial strains tested exhibited high extracellular *endo*-1,4-ß-glucanase activity in liquid media. These approaches show that the microbiota of lignocellulosic biomasses can be considered an important source of bacterial strains to upgrade the feasibility of lignocellulose conversion for the 'greener' technology of second-generation biofuels.

Lignocellulosic biomass includes dedicated energy crops, such as miscanthus, switchgrass, *Arundo donax*, *Populus nigra* and *Eucalyptus camaldulensis*. These plants can easily grow in farmland not suitable for food crops or in soils subjected to accelerated erosion, which affects most of the Mediterranean hilly croplands[1–3]. Vegetable biomasses represent an inexpensive alternative to fossil sources of fermentable sugars that can be utilised in several industrial applications, including second-generation biofuels and biopolymer production[4].

The enzymatic hydrolysis of plant carbohydrates has emerged as the most prominent eco-technology for the degradation of such biomasses. From the microbiological point of view, lignocellulosic biomass represents a complex ecosystem in which environmental conditions influence living organisms. In particular, geochemical (pH and salinity) and physical (temperature, pressure and radiation) factors can have a selective pressure on the biodiversity of microorganisms[5]. As a consequence, autochthonous microbial communities may prevail over other microorganisms because they possess enzymes that are able to degrade complex molecules such as cellulose and hemicellulose, forming the lignocellulosic biomasses that are the most abundant renewable energy source on Earth[6].

Generally, at an industrial level, the bioconversion of pretreated cellulose-based materials into fermentable sugars is performed by using a reaction mixture composed of multiple enzymes for complete hydrolysis. However, because the biorefining process is still economically unfeasible, novel biocatalysts from bacteria could

help overcome costly hurdles due to the operative steps of cooling, oxygen pumping, stirring and neutralisation, as well as the intrinsically high cost of hydrolytic enzyme production[7]. Different microorganisms producing hemicellulolytic enzymes that are potentially usable as new biocatalysts for hemicellulose hydrolysis have been isolated from different natural environments such as compost[8,9]. They belong to specific groups of microorganisms that are able to synthesise cellulase, xylanases and other biocatalysts necessary to allow a complete hydrolysis of the recalcitrant components of the lignocellulosic biomass[10]. Cellulolytic microorganisms can synthesise distinct enzymes such as endoglucanases, exoglucanases, including d-cellodextrinases, cellobiohydrolases and ß-glycosidase, which cooperate in cellulose degradation. Indeed, the hemicellulolytic microorganisms produce xylanases for degrading xylan into xylose that include endo-β-1,4-xylanases and β-xylosidases as well as auxiliary enzymes such as α-glucuronidases, α-arabinofuranosidases, acetylesterases and acetyl xylan esterases[11].

The use of culture-independent high-throughput sequencing can potentially reveal uncultivable microbiota and enables the study of the microbial ecology and taxonomic diversity at a high resolution. A thorough determination of the microbial diversity in biomass degradation can be fundamental to evaluating potential sources of novel enzymes and activities[12,13].

In the present work, the changes in the microbiota during the natural biodegradation of lignocellulosic biomasses of *A. donax*, *E. camaldulensis* and *P. nigra* were studied. In addition, new well-adapted bacterial strains from the three lignocellulose biomasses were isolated, identified and characterised. This study shows that the microbiota of lignocellulosic biomasses can be considered an important source of bacterial strains to upgrade the feasibility of lignocellulose conversion for the 'greener' technology of second-generation biofuels.

Results

Physicochemical measurement. The temperature values observed in the piles were approximately 24°C during the first 45 days. This value increased up to about 29°C after 135 days of biodegradation, before declining up to 25°C at the end of the experiment (180 days). The values of a_w ranged from to 0.91 to 0.99. The environmental temperature increased from April (20.5°C on the average) to August (34.2°C on the average) and declined in September (29.2°C).

Microbial Diversity of Lignocellulosic Biomasses by High-Throughput Sequencing. The microbiota of three different lignocellulosic biomasses (*A. donax*, *E. camaldulensis* and *P. nigra*) was characterised by partial 16S rRNA gene sequencing obtained from DNA directly extracted from environmental samples. A total of 238,450 number of reads were obtained by high-throughput sequencing. However, following the removal of short, ambiguous and/or low-quality pyrotag reads, the final data set consisted of 138,336 high-quality reads with an average sequence length of 456 bp. The alpha-diversity was determined by calculating the Shannon diversity index and the Chao1 richness index based on OTUs of 97% identity (Table 1). The results showed that the highest diversity indices were observed in the *E. camaldulensis* and *P. nigra* biomasses after 180 days of biodegradation in the underwood condition ($P < 0.05$) but were quite variable in the *A. donax* biomass (Table 1). Good's coverage indicated that more than 90% of the microbial diversity was described in most of the samples.

The relative abundances of bacterial taxa were examined at the level of phyla and class to determine whether there were any significant shifts in the composition of the bacterial communities according to the plant species, degradation conditions and sampling time.

In total, twenty-six different phyla were detected in the biomass samples, but only *Actinobacteria*, *Proteobacteria*, *Bacteroidetes* and *Firmicutes* were detected in all samples (Fig. 1). These phyla together

Table 1 | Number of sequences analysed, observed diversity and estimated sample coverage for 16S rRNA amplification from DNA extracted from the chipped lingo-cellulosic biomasses

Sample	No. reads	No. OTUs	Chao1	Shannon index[a]	Good's coverage (%)
At0	12,021	322	432.68	4.28^{AB}	99.24
At1OF	6,235	477	682.92	6.59^{DE}	97.45
At1UW	9,713	506	713.56	5.51^{BC}	98.38
At2OF	4,158	592	955.73	7.09^{DEF}	93.70
At2UW	2,564	286	443.81	6.09^{C}	95.44
At3OF	1,699	404	618.70	7.33^{F}	88.99
At3UW	5,451	441	561.42	6.36^{CDE}	97.54
At4OF	5,160	620	1111.02	6.94^{DEF}	94.46
At4UW	4,293	501	671.50	6.61^{DE}	95.64
Et0	5,582	340	555.28	6.05^{C}	97.76
Et1OF	5,271	322	485.11	3.92^{A}	97.67
Et1UW	3,207	286	430.38	4.65^{B}	96.35
Et2OF	3,983	453	656.84	6.65^{DE}	95.81
Et2UW	1,069	228	405.60	6.52^{DE}	89.5
Et3OF	4,520	470	671.11	6.52^{DE}	96.00
Et3UW	7,091	623	978.51	6.46^{CDE}	96.63
Et4OF	4,271	571	882.78	7.49^{FG}	94.66
Et4UW	5,309	887	1367.25	8.37^{GH}	93.60
Pt0	10,116	471	623.72	5.94^{C}	98.65
Pt1OF	4,292	501	865.63	6.18^{CD}	94.43
Pt1UW	10,117	845	1128.45	8.24^{G}	97.68
Pt2OF	3,524	728	1097.07	7.89^{FG}	91.06
Pt2UW	1,226	367	727.57	7.58^{FG}	84.01
Pt3OF	5,031	743	1086.64	6.63^{DE}	93.76
Pt3UW	2,188	583	1169.58	8.08^{FG}	86.15
Pt4OF	2,576	654	975.19	7.82^{FG}	88.94
Pt4UW	7,669	1,629	3122.43	9.48^{H}	89.79

[a]Different letters after Shannon index values indicate significant differences ($P < 0.05$).
Abbreviations. A: *A. donax*; E: *E. camaldulensis*; P: *P. nigra*; T0: 0 days of degradation; t1 : 45 days of degradation; t2: 90 days of degradation; t3: 135 days of degradation; t4: 180 days of degradation; OF: open field degradation condition; UW: underwood degradation condition.

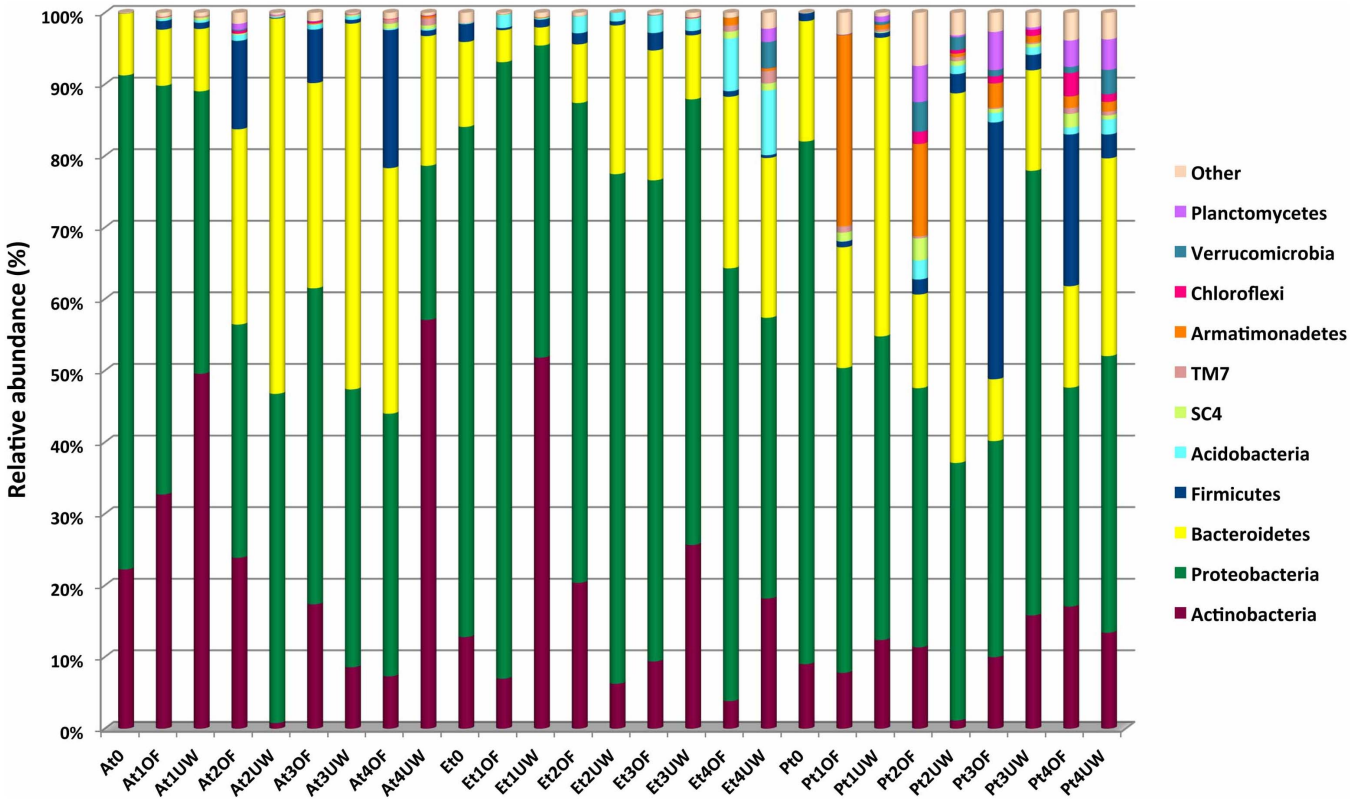

Figure 1 | Abundance of bacterial phyla in lignocellulosic biomasses during the biodegradation process. Only OTUs with an incidence >1% in at least two samples are shown. *Abbreviations.* A: *A. donax*; E: *E. camaldulensis*; P: *P. nigra*; T0: 0 days of degradation; t1: 45 days of degradation; t2: 90 days of degradation; t3: 135 days of degradation; t4: 180 days of degradation; OF: open field degradation condition; UW: underwood degradation condition.

accounted for approximately 98%, 99% and 91% of the total biodiversity in *A. donax*, *E. camaldulensis* and *P. nigra*, respectively.

The native composition of the microbial community in the *A. donax* biomass was strongly dominated by *Proteobacteria* (69.03%), followed by *Actinobacteria* (22.23%) and *Bacteroidetes* (8.61%). During the biodegradation process, the microbial composition remained the same in terms of diversity but varied in terms of abundance. This pattern was primarily observed in the piles processed under the open field condition (Fig. 1). Interestingly, in this environment, an inverse correlation was observed between *Actinobacteria* and *Bacteroidetes*. In particular, *Actinobacteria* decreased from 32.70% after 45 days of degradation under the open field condition to 7.31% after 180 days ($P < 0.05$), whereas *Bacteroidetes* increased from 7.84% after 45 days to 34.30% after 180 days ($P < 0.05$). *Firmicutes* was very low at the initial time (0.02%) and remained roughly constant in the underwood pile (0.77% after 180 days of biodegradation). By contrast, in the open field pile, the relative abundance of this taxon gradually increased from 1.20% after 45 days of biodegradation to 19.28% after 180 days ($P < 0.05$). Minor phyla included *Acidobacteria* and candidate phyla SC4 and TM7 were recovered in all *A. donax* biomass samples, although at an incidence ≤ 1% (Fig. 1).

As in the *A. donax* biomass, *Proteobacteria* was the taxa that heavily dominated the native microbial community in *E. camaldulensis* and *P. nigra* (71.26% and 73.01%, respectively) ($P < 0.05$), remaining high during all degradation processes, followed by *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Acidobacteria* (Fig. 1). In the *E. camaldulensis* pile, *Acidobacteria* increased to 7.35% and 9.02% after 180 days of degradation under the open field and underwood conditions, respectively, whereas a slight variation was detected in the *P. nigra* samples. By contrast, the abundance of *Firmicutes* increased in the *P. nigra* pile after 135 (35.88%, $P < 0.05$) and 180 days (21.20%, $P < 0.05$) of degradation under the open field condition and was relatively stable in the *E. camaldulensis* pile (Fig. 1). Other taxa, including *Armatimonadetes*, *Chloroflexi*, *Planctomycetes*, *Verrucomicrobia*, SC4 and TM7, were also detected. Interestingly, *Armatimonadetes* increased in the *P. nigra* pile after 45 days under the open field condition (26.70%, $P < 0.05$) and then gradually decreased during the degradation process to 1.63% (Fig. 1).

The microbial diversity was also analysed at a deeper taxonomic level. The identification of OTUs at the class level is reported in the heatmap shown in Fig. 2. *Actinobacteria*, *γ-Proteobacteria*, *β-Proteobacteria*, *α-Proteobacteria*, *Acidobacteria*, *Sphingobacteria*, *Flavobacteria* and *Bacilli* were recovered in all samples, with the exception of *Acidobacteria*, which was not detected in the *A. donax* biomass at the initial time. The abundance of *α-Proteobacteria* was high and exhibited the same variations during the biodegradation experiment in all three lignocellulosic biomasses. Interestingly, *γ-Proteobacteria* was the dominant taxa at the beginning of the experiment, with an abundance of 58.32%, 42.64% and 67.48% in the *A. donax*, *E. camaldulensis* and *P. nigra* samples, respectively; however, a dramatic reduction was recorded in all plant biomasses after 180 days (1.07%, 8.12% and 11.49%, respectively) (Fig. 2). A steady reduction in class *Actinobacteria* during degradation of the *A. donax* biomass under the open field condition was observed. By contrast, *Bacilli* showed an opposite trend, increasing from 0.02% at time zero to 19.26% after 180 days. Under the underwood condition, the middle phase of the biodegradation process was primarily dominated by *Sphingobacteria*, which showed an abundance of approximately 50%. Ever-increasing *Sphingobacteria* and *β-Proteobacteria* taxa abundance was recorded in the *E. camaldulensis* pile during the degradation process under the open field experiment. *Acidobacteria* showed a similar trend under both the open field and underwood conditions. The *P. nigra* biomass was characterised by an increase in the incidence of *Bacilli* and *β-Proteobacteria* during the biodegradation process under the open field conditions (Fig. 2). Other taxa were present at a very low incidence and with a great variability in the different

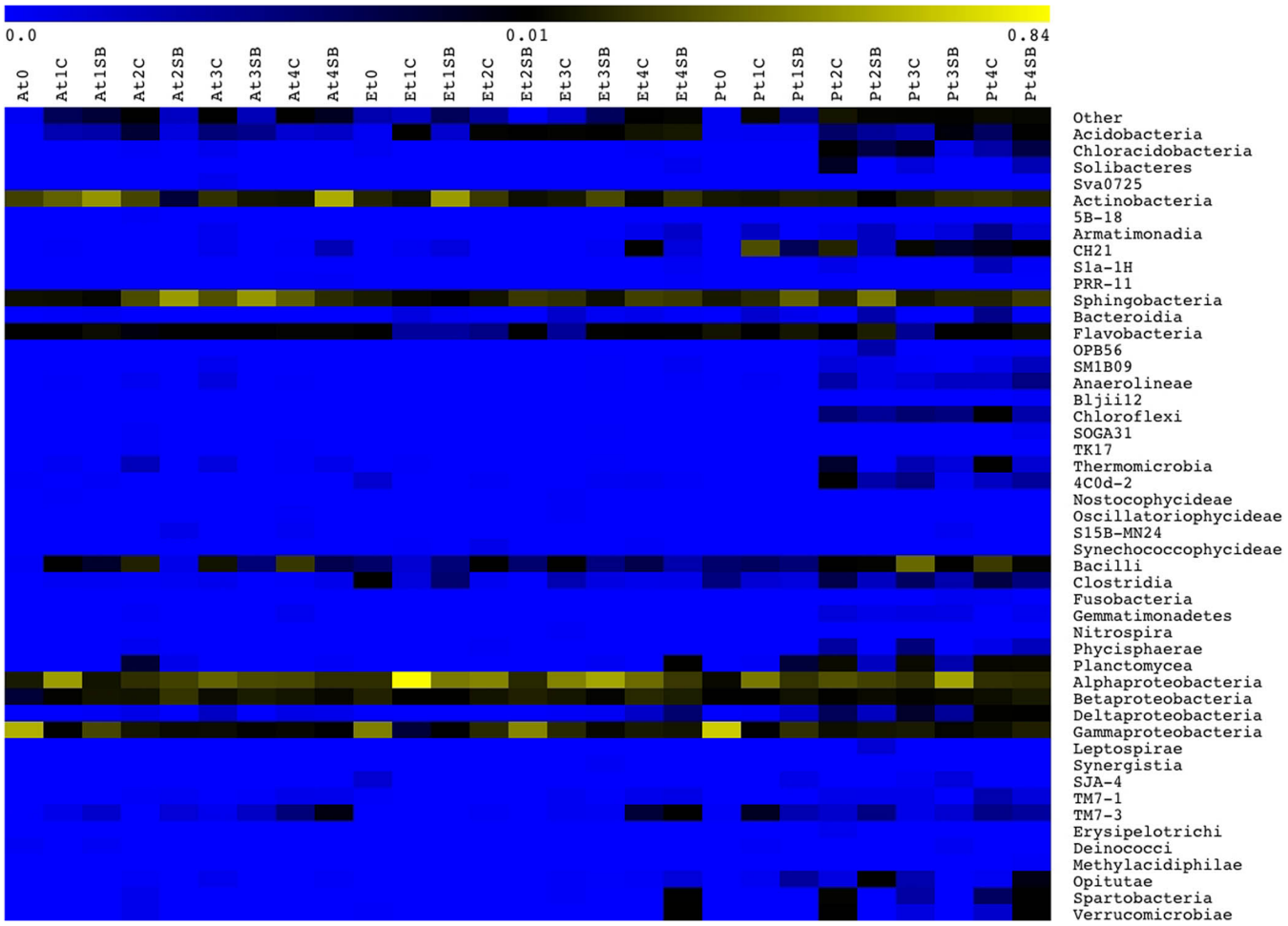

Figure 2 | Distribution of bacterial classes in lignocellulosic biomasses during the biodegradation process. Colour scale indicates the relative abundance of each OTU within the samples. *Abbreviations.* A: *A. donax*; E: *E. camaldulensis*; P: *P. nigra*; T0: 0 days of degradation; t1: 45 days of degradation; t2: 90 days of degradation; t3: 135 days of degradation; t4: 180 days of degradation; OF: open field degradation condition; UW: underwood degradation condition.

samples. However, as shown in Fig. 2, the highest bacterial diversity was recorded in the *P. nigra* biomass. An interesting finding was the high incidence of the uncultured bacterium CH21 (26.45%) in the *P. nigra* pile during the first phase (after 45 days) and its constant decrease during the other phases of the degradation process in the open field. *Chloroflexi* was detected only in the *P. nigra* biomass and from 90 days to 180 days of biodegradation, showing an abundance ranging from 0.33% to 1.55%.

However, the PCoA of the weighted UniFrac community distances showed a marked difference between the native microbiota of the lignocellulosic biomasses and the microbiota in the analysed samples during the biodegradation process. The three samples of the chipped biomasses of *A. donax*, *E. camaldulensis* and *P. nigra* at harvest time clustered separately (Fig. 3). However, the microbial diversity seemed to be influenced by a correlation between the degradation condition and the biomass type. In fact, no difference was observed among the samples during the biodegradation process under the underwood condition (Fig. 3 panel a). By contrast, under the open field condition, *P. nigra* samples at different time points clustered separately from the other biomasses (Fig. 3, panel b). A similar trend was observed in the *A. donax* biomass, except for the samples collected after 45 days of degradation (Fig. 3, panel b). Moreover, the statistical ADONIS test showed that the composition of bacterial community in the different piles during biodegradation process was significantly influenced by lignocellulosic plant species ($P < 0.001$), by the degradation conditions ($P < 0.05$) and by sampling time ($P < 0.01$). This difference increased combining the three factors. In fact, the analysis performed with ADONIS found significant difference combining *lignocellulosic plant species* x *sampling time* ($P < 0.001$), *lignocellulosic plant species* x *degradation condition* ($P < 0.001$) and *degradation condition* x *sampling time* ($P < 0.01$).

Multienzymatic Screening and Identification of Isolated Cellulolytic Bacterial Strains. A culture-dependent approach enables the isolation of putative cellulolytic strains. A total of 366 aerobic endo- and exo-cellulolytic bacteria were isolated from samples of the *A. donax*, *E. camaldulensis*, and *P. nigra* biomasses. In particular, more than one hundred isolates were obtained from each pile, although the *P. nigra* biomass produced the highest number (127 isolates). In total, 95.9% of the isolates that were obtained from Avicel agar plates, exhibited exo-cellulase activity, whereas isolates with endo-cellulase activity on the CMC agar plates were detected at values less than 50%. To establish the number of putative multifunctional-degrading bacteria, all isolates were assayed for different enzymatic activities using the methods described below. Forty aerobic endo- and exo-cellulolytic strains that showed higher I_{CMC} values (> 10) were chosen for further characterisation. The polyphasic approach of identification resulted in bacterial isolates with different shapes, dimensions and, in some cases, spore presence (data not shown) and great biodiversity, as twenty genera and twenty-seven different species were found (Table 2). *Curtobacterium* spp. and *Bacillus* spp. were the most representative genera with the species *Curtobacterium flaccumfaciens* and

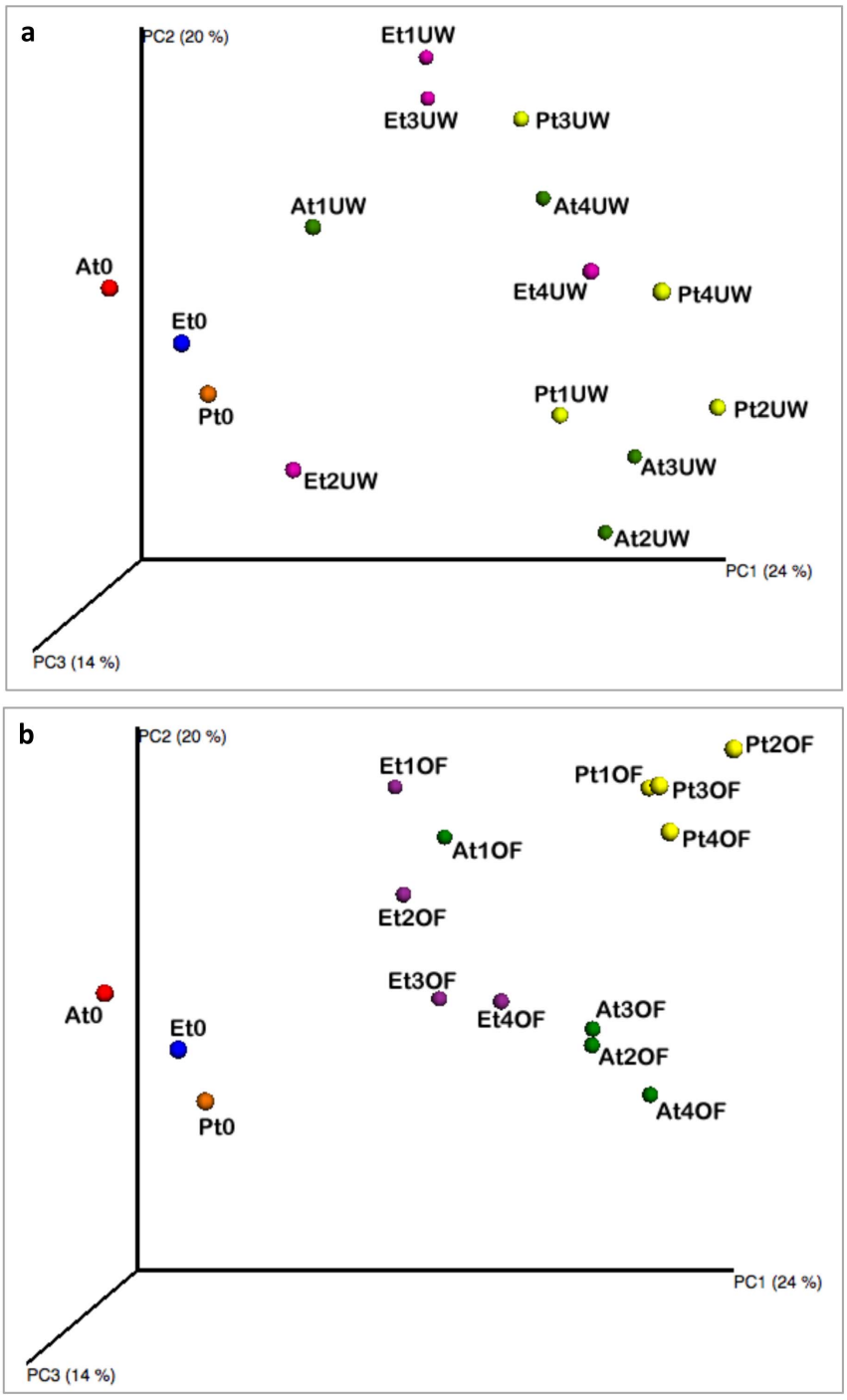

Figure 3 | Principal Coordinates Analysis of weighted UniFrac distances for 16S rRNA gene sequence data of lignocellulosic biomasses during the biodegradation process under the underwood (panel a) and open field (panel b) conditions. *Colour label.* red: *A. donax* at harvest time; blue: *E. camaldulensis* at harvest time; orange: *P. nigra* at harvest time; green: *A. donax*; violet: *E. camaldulensis*; yellow: *P. nigra*. *Abbreviations.* A: *A. donax*; E: *E. camaldulensis*; P: *P. nigra*; T0: 0 days of degradation; t1: 45 days of degradation; t2: 90 days of degradation; t3: 135 days of degradation; t4: 180 days of degradation; OF: open field degradation condition; UW: underwood degradation condition.

Curtobacterium citreum as well as *Bacillus amyloliquefaciens*, *Bacillus licheniformis* and *Bacillus subtilis*, respectively. The bacterial strains showed activities for three to six different enzymes. All strains showed endo- and exo-cellulolytic activities and, at same time, expressed cellobiase, xylanase and pectinase (Table 2). The highest endo-cellulase activity was observed in the CE86 strain, identified as *Promicromonospora sukumoe* (I_{CMC} = 34, $P < 0.05$), in *Isoptericola variabilis* CA84b (I_{CMC} = 32, $P < 0.05$) and *Staphylococcus warneri* CE83 (I_{CMC} = 32, $P < 0.05$). The first two strains were also able to degrade exo-cellulose, cellobiose and xylan. Moreover, *Isoptericola variabilis* CA84b produced the highest pectinase activity (I_{PEC} = 22, $P < 0.05$). *Curtobacterium citreum* CE711 and *Bacillus amyloliquefaciens* CA81 represented the most versatile strains because they possessed six different enzymatic activities. Thirty-two bacterial strains showed, in addition to endo- and exo-cellulases, cellobiase and xylanase activities that were missed in only 10% of the screened strains (*Staphylococcus warneri* CE83, *Lysobacter enzymogenes* CE710, *Lysobacter gummosus* CP72, *Mycobacterium frederiksbergense*

Table 2 | Identification and enzymatic activities of bacterial strains isolated from different lignocellulosic biomasses

Strain	Source	C[a]	A[b]	CE[c]	X[c]	P[a]	AZ[c]	AB[c]	L[c]	AD[c]	Identification (% identity)	Accession Number
CE86	*E. camaldulensis*	34 ± 1.0^{N}	+	++	+	0.0 ± 0.0^{A}	-	-	-	-	*Promicromonospora sukumoe* (99%)	KF057947
CA84b	*A. donax*	32 ± 0.0^{N}	+	++	+	22 ± 0.5^{G}	-	-	-	-	*Isoptericola variabilis* (98%)	KF057948
CE83	*E. camaldulensis*	32 ± 1.0^{N}	+	+	-	0.0 ± 0.0^{A}	-	-	-	-	*Staphylococcus warneri* (100%)	KF057949
CP72	*P. nigra*	28 ± 1.0^{M}	+	-	++	0.0 ± 0.0^{A}	-	-	-	-	*Lysobacter gummosus* (99%)	KF040972
CP710b	*P. nigra*	28 ± 0.5^{M}	+	-	+	12 ± 0.5^{E}	-	-	-	-	*Mycobacterium frederiksbergense* (99%)	KF057950
CA817	*A. donax*	27 ± 0.5^{M}	+	+	+	2 ± 0.2^{B}	-	-	-	-	*Cellulosimicrobium cellulans* (99%)	KF040973
CA812	*A. donax*	27 ± 1.0^{M}	+	+++	+++	0.0 ± 0.0^{A}	-	-	-	-	*Bacillus amyloliquefaciens* (99%)	KF040974
SBP79	*P. nigra*	26 ± 0.5^{LM}	+	++	+	12 ± 0.0^{E}	-	-	-	-	*Promicromonospora citrea* (99%)	KF057951
SBA88	*A. donax*	26 ± 0.3^{LM}	+	+	+	0.0 ± 0.0^{A}	-	-	-	-	*Microbacterium lacus* (99%)	KF057952
SBE74	*E. camaldulensis*	24 ± 1.0^{IL}	+	±	+	0.0 ± 0.0^{A}	-	-	-	-	*Enterobacter aerogenes/Kluyvera cryocrescens* (98%)	KF057960
CE77	*E. camaldulensis*	22 ± 1.0^{HI}	+	-	-	0.0 ± 0.0^{A}	++	-	-	-	*Novosphingobium resinovorum* (100%)	KF040976
CP81	*P. nigra*	22 ± 0.5^{HI}	+	+	+	3 ± 0.0^{C}	-	-	-	-	*Xanthomonas campestris* (100%)	KF040971
CP77	*P. nigra*	22 ± 0.9^{HI}	+	+	+	4 ± 0.0^{D}	-	-	-	-	*Xanthomonas orizae* (98%)	KF040975
CE75b	*E. camaldulensis*	22 ± 0.2^{HI}	+	++	+	0.0 ± 0.0^{A}	-	-	-	-	*Pediococcus acidilactici* (99%)	KF057953
CP77b	*P. nigra*	22 ± 0.5^{HI}	+	++	±	0.0 ± 0.0^{A}	-	-	-	-	*Curtobacterium flaccumfaciens* (99%)	KF057954
SBA76	*A. donax*	22 ± 1.0^{HI}	+	+	+	0.0 ± 0.0^{A}	-	-	-	-	*Pantoea ananatis* (99%)	KF057955
SBP71	*P. nigra*	22 ± 1.0^{HI}	+	+	+	0.0 ± 0.0^{A}	-	-	-	-	*Schumannella luteola* (97%)	KF057956
CP78b	*P. nigra*	22 ± 0.0^{HI}	+	±	+	12 ± 0.0^{E}	-	-	-	-	*Mycobacterium frederiksbergense* (99%)	KF057957
CA816	*A. donax*	21 ± 0.5^{H}	+	+++	-	20 ± 1.0^{F}	-	-	-	-	*Bacillus subtilis subsp. subtilis* (99%)	KF040977
CA81	*A. donax*	20 ± 1.0^{GH}	+	+++	+++	0.0 ± 0.0^{A}	+	+	-	-	*Bacillus amyloliquefaciens* (99%)	KF040978
CA82	*A. donax*	20 ± 0.0^{GH}	+	+++	++	4 ± 0.2^{D}	-	-	-	-	*Bacillus licheniformis* (99%)	KF040979
CE84	*E. camaldulensis*	20 ± 0.5^{GH}	+	+	+++	0.0 ± 0.0^{A}	++	-	-	-	*Novosphingobium resinovorum* (99%)	KF040980
CA81b	*A. donax*	20 ± 1.0^{GH}	+	+++	++	4 ± 0.1^{D}	-	-	-	-	*Bacillus licheniformis* (99%)	KF040981
CE73b	*E. camaldulensis*	20 ± 0.9^{GH}	+	++	+	0.0 ± 0.0^{A}	-	-	-	-	*Pediococcus acidilactici* (99%)	KF057958
SBP73	*P. nigra*	20 ± 0.0^{GH}	+	-	±	0.0 ± 0.0^{A}	-	-	-	-	*Aurantimonas altamirensis* (99%)	KF057959
CE85	*E. camaldulensis*	18 ± 1.0^{FG}	+	++	++	2 ± 0.0^{B}	-	-	-	-	*Isoptericola variabilis* (98%)	KF040982
CE710	*E. camaldulensis*	18 ± 0.5^{FG}	+	-	++	2 ± 0.1^{B}	-	++	-	-	*Lysobacter enzymogenes* (99%)	KF040983
CP81b	*P. nigra*	18 ± 1.0^{FG}	+	++	++	0.0 ± 0.0^{A}	-	-	-	-	*Promicromonospora sukumoe* (99%)	KF040984
CA83	*A. donax*	17 ± 1.0^{EF}	+	++	+	2 ± 0.1^{B}	-	-	-	-	*Curtobacterium flaccumfaciens* (99%)	KF040985
CA77	*A. donax*	16 ± 0.5^{DEF}	+	+	+++	2 ± 0.0^{B}	-	-	-	-	*Sphingobacterium multivorum* (99%)	KF040986
CE75	*E. camaldulensis*	16 ± 1.0^{DEF}	+	++	+	2 ± 0.1^{B}	-	-	-	-	*Curtobacterium flaccumfaciens* (99%)	KF040987
CE73	*E. camaldulensis*	16 ± 0.5^{DEF}	+	+	-	0.0 ± 0.0^{A}	-	-	-	-	*Promicromonospora citrea* (99%)	KF040988
CA83b	*A. donax*	15 ± 0.3^{CDE}	+	++	++	0.0 ± 0.0^{A}	-	-	-	-	*Curtobacterium flaccumfaciens* (99%)	KF040989
CA84	*A. donax*	14 ± 0.5^{BCD}	+	+++	+	0.0 ± 0.0^{A}	-	-	-	-	*Curtobacterium citreum* (99%)	KF040990
CP713	*P. nigra*	14 ± 0.3^{BCD}	+	+++	++	20 ± 0.3^{F}	-	-	-	-	*Cellulomonas flavigena* (98%)	KF040991
CA818	*A. donax*	13 ± 1.0^{BC}	+	++	+	2 ± 0.0^{B}	-	-	-	-	*Microbacterium testaceum* (99%)	KF040992
CE78	*E. camaldulensis*	12 ± 0.0^{AB}	+	++	+	2 ± 0.3^{B}	-	-	-	-	*Curtobacterium citreum* (99%)	KF040993
CP710	*P. nigra*	12 ± 0.5^{AB}	+	+	+	0.0 ± 0.0^{A}	-	-	-	-	*Labedella gwakjiensis* (99%)	KF040994
CE711	*E. camaldulensis*	10 ± 0.9^{A}	+	++	+	2 ± 0.0^{B}	+	-	-	-	*Curtobacterium citreum* (99%)	KF040995
CP78	*P. nigra*	10 ± 0.5^{A}	+	+++	+	0.0 ± 0.0^{A}	-	-	-	-	*Raoultella terrigena* (99%)	KF040996

Enzymatic activities: C = endo-cellulase; A = eso-cellulase; CE = cellobiase; X = xylanase; P = pectinase; AZ = peroxidase; AB = laccase; L = ligninase with guaiacol and lignin alkali; AD = ligninase with guaiacol and *Arundo donax*; [a]I_{CMC} or I_{PEC} index, values represent the means ± SD of three replicates. Different letters after values indicate significant differences ($P<0.05$); [b]growth; [c]– negative; + low intensity; ++ middle intensity; +++ high intensity.

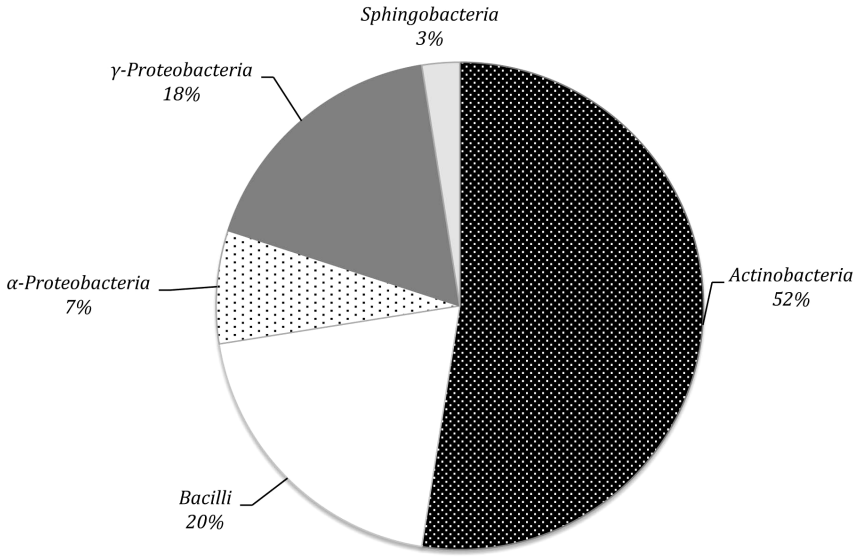

Figure 4 | Percentage composition of different phyla of eso- and endo-cellulolytic bacteria isolated from lignocellulosic biomasses on the basis of 16S rRNA gene sequence similarity.

CP710b, *Promicromonospora citrea* CE73, *Novosphingobium resinovorum* CE77, *Bacillus subtilis* subsp. *subtilis* CA816 and *Aurantimonas altamirensis* SBP73). All the strains belonging to the different *Bacillus* spp. and *Curtobacterium* spp. had the highest cellobiase activities. In particular, the genus *Curtobacterium,* represented by the species *Curtobacterium citreum* and *Curtobacterium flaccumfaciens,* was able to grow at least on four/five different substrates and, in one case, also showed peroxidase activity. In total, 87.5% of the bacterial strains produced xylanase (Table 2). Similar behaviour was observed for pectinase activity production as only 15% of the strains possessed an I_{PEC} from 12 to 22. Laccase and peroxidase activities were detected in five strains belonging to the species *Curtobacterium citreum* CE711, *Novosphingobium resinovorum* CE77 and CE84, *Lysobacter enzymogenes* CE710 and *Bacillus amyloliquefaciens* CA81. Lignin hydrolysis was not detected in any of the strains tested.

Phylogenetic Analysis of Selected Cellulolytic Bacteria. 16S rRNA gene sequences of the forty cellulolytic bacteria identified as described below were grouped by phylogenetic analysis into five different clusters, generating a *consensus* tree. The clusters were *Actinobacteria* (n=21; 52.5%), *Bacilli* (n=8; 20.0%), *α-Proteobacteria* (n=3; 7.5%), *γ-Proteobacteria* (n=7; 17.5%), *Sphingobacteria* (n=1; 4%) (Fig. 4). A phylogenetic tree was generated from the distance data using the Neighbour-Joining method with the Maximum Composite Likelihood model in a MEGA4 Program (Fig. 5). The nucleotide sequences of related type strains of different genera were included in the data set. High bootstrap values were observed and indicated significant branching points in the phylogenetic tree. Strains representative of the dominant class of *Actinobacteria* were placed in a cluster with bootstrap values higher than 52% and could be differentiated into nine subclusters of different genera. The bacterial strains primarily belonged to *Curtobacterium* (33.3%) and *Promicromonospora* (19.0%), whereas other genera (*Schumannella*, *Labedella*, *Cellulomonas*, *Cellulosimicrobium*, *Microbacterium*, *Isoptericola*, and *Mycobacterium*) were represented by one or two strains. The strains *Curtobacterium citreum* CE711, *Curtobacterium citreum* CA84 and *Curtobacterium citreum* CE78 showed a sequence similarity of 100%. Representatives of the *Bacilli* class were placed in three clusters of the genera *Bacillus* (62.5%), *Staphylococcus* (12.5%) and *Pediococcus* (25.0%). The strains belonging to *Bacillus licheniformis* CA82 and CA81b showed a similarity level of 100%, as did *Bacillus amyloliquefaciens* CA812 and CA81 and *Pediococcus acidilactici* CE75b and CE73b (Fig. 5). The *α-Proteobacteria* strains were placed in two different genera, in which two strains of *Novosphingobium resivorum* (CE77 and CE84) exhibited a sequence similarity of 100% with 100% of the bootstrap analysis. The other strain, belonging to *Aurantimonas altamirensis* (SBP73), formed a phylogenetically distinct cluster with a low 16S rRNA gene sequence similarity (60%) (Fig. 5). The representative strains of the *γ-Proteobacteria* cluster belonged to the genera *Xanthomonas* and *Lysobacter* and included the species *Xanthomonas campestris*, *Xanthomonas oryzae*, *Lysobacter gummosus*, and *Lysobacter enzymogenes*. The other genera, identified as the *Enterobacter aerogenes/Klebsiella cryocrescens* group, *Raoultella* and *Pantoea*, were represented by one strain. The strain *Sphingobacterium multivorum* CA77 formed a phylogenetically distinct cluster with the type strain of the respective species with 100% of the bootstrap analysis (Fig. 5).

Screening of Cellulolytic Microorganisms in Liquid Medium. Sixteen bacterial strains were selected based on at least one of these characteristics: higher halo dimension on CMC agar, multienzymatic activities, high pectinase activity or high peroxidase activity, and no pathogenicity for humans; these strains were submitted to a quantitative CMCase assay. All the analysed strains achieving the maximum AZO-CMCase level of production between the 8th and 26th hours, corresponding to the exponential and stationary phases of growth, respectively (data not shown). In Table 3, the values of the maximum AZO-CMCase activity measured for each strain and the corresponding time of production are reported. *Schumannella luteola* SBP71 showed the highest AZO-CMCase activity, at 0.33 ± 0.09 U mL^{-1}, although no significant differences were found with other strains. In particular, high activity levels ranging from 0.20 ± 0.06 to 0.32 ± 0.08 U mL^{-1} were also found for *Pediococcus acidilactici* CE75b, *Curtobacterium citreum* CA84, *Curtobacterium flaccumfaciens* CA83, *Cellulomonas flavigena* CP713 and *Pantoea ananatis* SBA76a (Table 3). *Mycobacterium frederiksbergense* CP78b, *Curtobacterium flaccumfaciens* CP77b and *Promicromonospora citrea* SBP79 (with a maximum value of 0.18 ± 0.02 U mL^{-1} and 0.16 ± 0.02 U mL^{-1}) exhibited less AZO-CMCase activity than the microorganisms mentioned above but more than *Cellulosimicrobium cellulans* CA817 (0.15 ± 0.01 U mL^{-1}) and *Isoptericola variabilis* CA84b (0.13 ± 0.01 U mL^{-1}). All other bacterial strains tested showed values lower than 0.10 U mL^{-1}.

Figure 5 | Neighbour-Joining tree based on the comparison of 16S rRNA gene sequences showing the relationships among cellulolytic strains. Bootstrap values (expressed as percentages of 1,000 replications) greater than 50% are given at the nodes. Strains marked with "(T)" represent type strains. The *scale bar* estimates the number of substitutions per site.

Table 3 | Maximum value of Azo-CMCase activity measured for each strain and the corresponding time of production

Bacterial strains	Maximum value of AZO-CMCase activity[a] (U mL^{-1})	Time (h)
Schumannella luteola SBP71	0.33 ± 0.09^{F}	12
Pediococcus acidilactici CE75b	0.32 ± 0.10^{EF}	12
Curtobacterium citreum CA84	$0.29 \pm 0.01^{D-F}$	17
Curtobacterium flaccumfaciens CA83	$0.24 \pm 0.03^{C-F}$	15
Cellulomonas flavigena CP713	$0.22 \pm 0.10^{B-F}$	20
Pantoea ananatis SBA76a	$0.20 \pm 0.06^{A-F}$	8
Mycobacterium frederiksbergense CP78b	$0.18 \pm 0.02^{A-E}$	26
Curtobacterium flaccumfaciens CP77b	$0.16 \pm 0.02^{A-D}$	20
Promicromonospora citrea SBP79	$0.16 \pm 0.02^{A-D}$	12
Cellulosimicrobium cellulans CA817	$0.15 \pm 0.01^{A-D}$	20
Isoptericola variabilis CA84b	$0.13 \pm 0.01^{A-C}$	12
Bacillus subtilis subsp. *subtilis* CA816	0.09 ± 0.00^{AB}	12
Microbacterium testaceum CA818	0.09 ± 0.01^{AB}	17
Curtobacterium flaccumfaciens CA83b	0.08 ± 0.03^{AB}	12
Promicromonospora sukumoe CE86	0.07 ± 0.02^{A}	14
Bacillus licheniformis CA82	0.07 ± 0.00^{A}	15

[a]The values represent the means ± SD of three replicates of three independent experiments. Different letters after the values indicate significant differences ($P < 0.05$).

Discussion

In recent years, the competitive production of alternative renewable biofuels has stimulated research into new bacteria as a source of highly active and specific cellulases. They exhibit several advantages such as a fast growth rate, production of enzymes that are often more effective catalysts due to less feedback inhibition, and secretion of a complete multi-enzyme system for an efficient conversion of lignocelluloses into fermentable sugars[11,14]. In this context, particular attention must be given to exploring the biodiversity of natural niches so that cellulase-producing bacteria can be isolated and characterised. For these reasons, in this work, the microbial diversity of natural ecosystems, represented by lignocellulosic biomasses of *A. donax*, *E. camaldulensis* and *P. nigra*, was evaluated by culture-independent and culture-dependent approaches.

A highly complex bacterial community was found, in which the most frequently occurring bacteria were those belonging to the *Actinobacteria*, *Proteobacteria*, *Bacteroidetes* and *Firmicutes* phyla. *Proteobacteria* was the most abundant taxa recovered in the *E. camaldulensis* and *P. nigra* piles, followed by *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Acidobacteria*. These taxa are related to microorganisms previously characterised as biomass degraders. The biodiversity of the microbial community in our study corresponded well with a previous study in which *Proteobacteria*, *Firmicutes* and *Bacteroidetes*, along with members of the class *Proteobacteria*, comprised 83% of the microbial richness and heavily dominated switchgrass-adapted communities[15]. Moreover, bacterial species belonging to *Proteobacteria* and *Acidobacteria*, together with *Firmicutes* (*Clostridium* and *Bacillus* genera) and followed by *Bacteroidetes*, *Chlamydiae/Verrucomicrobia* and *Actinobacteria* (mainly Streptomyces), are known as the major plant biomass-degrading microbes in peat swamp forests[16]. During the natural biodegradation process of plant substrates, the indigenous bacterial community would initially have grown by utilising the more accessible cellulose and hemicellulose and only later would use the more resilient lignin component[17]. With regard to lignin decomposition, *Actinobacteria, Firmicutes* and *Acidobacteria* are the major taxa involved in this process[18]. *Acidobacteria* was recovered in the late stage of our experiment and its abundance increased during the biodegradation process, especially in the *E. camaldulensis* piles. *Firmicutes* showed a similar trend in the *A. donax* and *P. nigra* biomasses, with the relative abundance of this taxon gradually increasing in the piles processed under the open field condition. Wu and He[19] reported that *Firmicutes* could be the main microbes for lignin depolymerisation since a dominance of this phylum was recovered in enriched microbial consortia using a medium with lignin. Moreover, this phylum is common in natural processes such as rice straw compost[20] and decaying wood[21], suggesting its importance in the degradation of lignocellulolytic materials.

Analysing the microbial diversity more deeply, *Actinobacteria, γ-Proteobacteria, β-Proteobacteria, α-Proteobacteria, Acidobacteria, Sphingobacteria, Flavobacteria, Bacilli* and *Acidobacteria* were recovered in all samples. In particular, *Actinobacteria, α-Proteobacteria, Sphingobacteria* and *β-Proteobacteria*, all potent plant polysaccharide-degrading microbes that play an important role in plant biomass degradation in the tropical peat swamp forest ecosystem[16], were the most abundant taxa during the biodegradation process in all lignocellulosic piles.

Different bacteria belonging to the *Actinobacteria* class are involved in complex glycoside degradation such as chitin and cellulose and are fundamental in lignin and polyphenol degradation[22]. Martins and coworkers[23] reported that biomass degradation in the composting process, including the deconstruction of recalcitrant lignocellulose, is fully performed by bacterial enzymes, most likely by members of the *Clostridiales* and *Actinomycetales* orders. *β-Proteobacteria* and *α-Proteobacteria* were also recovered in our study. According to Castillo et al.[22], the dominance of members of the phylum *Proteobacteria* such as *γ-Proteobacteria* is observed only at the beginning of the biodegradation process, and its strong reduction during the experiment could be due to its involvement in lignocellulosic waste declining during the early stages of the process. In the open field experiment, *Bacilli* increased in both the *A. donax* and *P. nigra* biomasses. Members belonging to this taxon are known to have specific genes encoding enzymes involved in cellulose and hemicellulose degradation[24,25].

The constant increase in the relative abundance of *Sphingobacteria* and *Acidobacteria* recorded in the *E. camaldulensis* pile during the degradation process in the open field experiment suggests that these species play a role in the decomposition of lignocellulosic material. Kanokratana et al.[26], using complementary shotgun pyrosequencing, identified different genes encoding glycosyl hydrolases targeting cellulose and hemicellulose degradation in a bagasse pile, most of which were found in orders *Clostridiales*, *Bacteroidales*, *Sphingobacteriales* and *Cytophagales*. Moreover, the *Acidobactria* taxon is able to a use a diversity of carbon sources, from simple sugars to complex plant biomass substrates[26].

Another adapted-lignocellulosic taxon was *β-Proteobacteria*, which increased in the *E. camaldulensis* and *P. nigra* piles. In recent work, Stursova et al.[27] identified *β-Proteobacteria, Bacteroidetes* and *Acidobacteria* as the primary cellulose decomposers in forest litter.

An interesting finding was the high incidence of the uncultured bacterium CH21 in the *P. nigra* pile during the first phase of the

degradation process in the open field. CH21 is a member of the phylum *Armatimonadetes*, formerly called candidate division OP10[28]. The phylum *Armatimonadetes* is very poorly studied and its phylogeny is still poorly defined[29]. Members of this phylum are detected in different ecosystems and they are phylogenetically different. This phylum includes species with a wide variety of metabolic potentials[30]. Wang and co-workers[31] reported that their prevalence in plant-fed anaerobic bioreactors indicates a role in degradation of plant material. In our research, CH21 is the only recovered member of this phylum. Since it's relative abundance increased in the *Populus nigra* pile after 45 days and decreased during the other phases of the degradation process in the open field, it is possible its involving in the biodegradation of this specific lignocellulosic biomass during the first phase of the process.

The differing trends observed in this study in terms of taxa abundance during the biodegradation process and between the vegetable species used demonstrated a local selective pressure in the lignocellulosic ecosystems.

The culture-dependent methodology used here, which was based on a functional approach of detection and isolation to find new lignocellulose-degrading bacterial strains, provides us with key insight. Special attention to the methodology was required to determine the optimal culture and assay conditions. A comparison with another study[11] revealed that differential substrates containing CMC and Avicel are effective for the enumeration and isolation of putative colonies of cellulolytic microorganisms[32]. According to Soares et al.[6], exo- and endo-cellulolytic bacterial isolates are found at different frequencies, and the number of microorganisms that were able to grow on Avicel as the sole carbon source was high. The cellulolytic strains isolated from the biomasses showed multienzymatic activities useful to perform the hydrolysis of a complex substrate such as lignocellulose, an important initial step in many technological applications[33] that require the action of different specific enzymes. All forty bacterial strains submitted to the multienzymatic screening showed both endo- and exo-glucanase activities, confirming that these enzymes act synergistically during the saccharification of celluloses. These observations were reinforced by the fact that many of these bacterial strains also possessed β-cellobiase as well as cellulolytic activity. Xylanase activity was also commonly observed, which is unsurprising because a close correlation between cellulase and xylanase activities has been demonstrated and is due to their coexpression in the same operon.

The Neighbour-Joining phylogenetic method generated a consensus tree that grouped all 16S rRNA gene sequences of the isolated strains into five different clusters at the class level. Culture-dependent data showed similar predominant bacterial classes detected by high-throughput sequencing in the lignocellulosic biomasses, with an abundance of *Actinobacteria*, *Bacilli*, *α-Proteobacteria, γ-Proteobacteria* and *Sphingobacteria*. Moreover, the bacteria isolated are dominant players since these microbial strains were isolated from a high serial decimal dilutions (10^{-6}–10^{-7}). According to phylogenetic research on cellulose-decomposing bacteria isolated from soil carried out by Ulrich et al.[34], *Actinobacteria* are the most prevalent bacterial group based on 16S rRNA gene sequences. This cluster included the species *Curtobacterium citreum,* which is able to use up to six different lignocellulose components and that is phylogenetically related to *Microbacterium testaceum* and *Microbacterium lacus.* In particular, previous studies reported the production of enzymes involved in cellulose and xylan degradation by *Microbacterium* species[35]. The *Actinobacteria* cluster included other genera involved in lignocellulosic biomass degradation, such as *Cellulomonas.* Akasaka et al.[36] reported that more than 60% of isolates from rice plant residues was closely related to *Cellulomonas* and involved in their degradation. Our study revealed the production of cellobiase and pectinase activities in *Actinobacteria* members, such as *Cellulosimicrobium cellulans*, *Isoptericola variabilis, Promicromonospora sukumoe* and *Promicromonospora citrea*, which belong to the suborder *Micrococcineae*. Many of the representatives of the *Promicrosporaceae* and *Corinebacteriaceae* families can degrade polysaccharides such as cellulose and xylan[37,38]. The *Bacilli* cluster was primarily represented by the *Pediococcus* and *Bacillus* genera on the basis of 16S rRNA gene sequence analysis. Interesting, Zhao and co-workers[39] reported the use of a strain of *Pediococcus acidilactici* with high tolerance to temperature and a lignocellulose-derived inhibitor in simultaneous saccharification and fermentation (SSF) for high lignocellulosic lactic acid production. The strains CA812, CA81, CA816, CA82 and CA81b, isolated from the *A. donax* biomass and phylogenetically correlated to the *Bacillus* genus, showed high endo-cellulolytic and multi-enzymatic activities. The capacity of *Bacillus* strains to produce large quantities of extracellular enzymes has placed them among the most important industrial enzyme producers isolated from compost, soil and several other natural habitats[40]. In particular, *Bacillus amyloliquefaciens, Bacillus subtilis* and *Bacillus licheniformis*, isolated from soil and compost, are able to hydrolyse cellulosic waste-material[8] by both cellulolytic activities[41] and multi-enzyme complexes[42]. The selected bacterial strains could have cross-specificity facilitated by specific or non-specific active sites and also distinct catalytic domains binding to different substrates[33]. *Xanthomonas campestris*, known as a phytopathogenic bacterium, and *Lysobacter gummosus* and *Lysobacter enzymogenes*, potent biocontrol agents that release cellulolytic enzymes such as glucanase[43], were identified in the phylogenetic group of *γ-Proteobacteria*. Other strains of this class, such as *Enterobacter* sp. and *Pantoea* sp., although are known as insect-associated bacteria that are able to produce bioactive compounds and digestive enzymes that are responsible for lignocellulose degradation[44], they are less attractive for possible biotechnological application since human disease has been reported to be caused by these bacteria as well as *Raoultella terrigena* and *Sphingobacterium multivorum*.

The two strains CE77 and CE84, included in the *α-Proteobacteria* cluster and characterised for their peroxidase activity, were closely related to the species *Novosphingobium resinovorum*, previously isolated from soil and studied for its capacity to degrade oil resins[45]. Interestingly, the strain *Aurantimonas altamirensis* SBP73 showed multienzymatic activity. To our knowledge, this study is the first to report the multienzymatic activity of this specie.

The bacterial strains screened in liquid medium showed enzymatic activity levels similar to the values reported in previous studies[46]. For example, Ekperigin[47] reported that the maximum activity of the cellulose-degrading enzyme determined for CMC in the culture supernatant of *Branhamella* spp. was 0.34 U mL^{-1}. Even though comparing cellulase production across studies is difficult because there are too many differences in the production of extracellular enzymes (e.g., media composition, fermentation conditions and raw materials), the results demonstrated that the bacterial strains isolated and characterised in this study could represent a very interesting biological source for the conversion of lignocellulose carbohydrates into products of commercial significance. Moreover, their biotechnological performance could be enhanced by modifying the biotechnological parameters of the fermentation process such as the temperature[33].

In conclusion, in this work, pyrosequencing-based technology increased our knowledge of lignocellulosic-adapted microbiota and the microbial dynamic during the degradation of three different biomasses under natural conditions. The dominant taxa found during the biodegradation process were members of classes *Actinobacteria*, *γ-Proteobacteria*, *α-Proteobacteria* and *Sphingobacteria*. However, the abundance of the major and minor taxa retrieved during the process was determined by selective pressure employed by the lignocellulosic plant species and the degradation conditions.

In addition, new multifunctional degrading bacteria have been selected and identified that are potential producers of multiple enzymes that have synergistic actions on cellulose and hemicellulose.

This step is fundamental for biotechnological applications of interest to industry because they constitute a microbial source of new multi-functional enzymes that can increase the efficiency of the hydrolysis of lignocellulosic biomass into fermentable sugars for biofuel eco-technology.

Methods

Lignocellulosic Biomasses and Sampling. Chipped wood from *A. donax*, *E. camaldulensis* and *P. nigra* was processed for biodegradation under natural conditions for 180 days. The chipped wood was used to form two piles of approximately 30 kg (length 0.85 m, width 0.85 m and height 0.70 m) for each plant species; on March 22, 2012, the piles were placed under two different environmental conditions in order to increase microbial biodiversity: open field conditions, in which the biomass piles were established on agronomic soil without any coverage shady trees, in the experimental station of Department of Agriculture (Naples, Italy; 40°48′50.1″N, 14°20′48.2″E); the underwood conditions performed reproducing the natural environment that usually arise in a forest since the piles were placed under oak trees in the woodland at the Department of Agriculture (Naples, Italy; 40°48′47.8″N, 14°20′50.4″E). Samples of 0.5 kg were collected from the external part (right and left side of the pile) and the internal central part of the biomass immediately after preparation (T0) and at 45, 90, 135 and 180 days (T1, T2, T3 and T4, respectively) of biodegradation. During the process, temperature (°C) was measured by using specific temperature sensors (VWR International PBI, Milan, Italy) placed directly in the core of the piles at a depth of 30–40 cm. The water activity (a_w, water readily available for microbial metabolic activities) was also monitored using a HygroPalm23-AW (Rotronic AG, Basserdorf, Germany).

Analysis of the Microbiota by High-Throughput Sequencing of the 16S rRNA Gene. Total DNA recovery from microorganisms adherent to the plant biomass was performed as previously described[48]. The bacterial diversity was evaluated by pyrosequencing using the primers Gray28F (5′-TTTGATCNTGGCTCAG-3′) and Gray519r (5′-GTNTTACNGCGGCKGCTG-3′) spanning the V1-V3 region of the 16S rDNA of *E. coli*[49]. 454-adaptors were included in the forward primer followed by a 10-bp sample-specific Multiplex Identifier (MID). The PCR mixture (50-µl total volume) included 20 ng of target DNA, 1 x *Taq* DNA polymerase buffer (Invitrogen, Milano, Italy), 2.5 mM $MgCl_2$, 0.5 mM of each dNTP, 0.4 µM of each primer and 2.5 U of *Taq* DNA polymerase (Invitrogen). PCR amplification was performed as previously reported[50]. After agarose gel electrophoresis, PCR products were purified twice by Agencourt AMPure kit (Beckman Coulter, Milano, Italy) and quantified using the QuantiFluor™ (Promega, Milano, Italy), and an equimolar pool was obtained prior to further processing. The amplicon pool was used for pyrosequencing on a GS Junior platform (454 Life Sciences, Roche, Italy) according to the manufacturer's instructions using Titanium chemistry.

Bioinformatics and Data Analysis. The pyrosequencing data were filtered using the following quality check parameters using the QIIME software package[51]: a minimum sequence length of 200 bp, maximum homopolymer number of 5, minimum quality score of 25, and maximum number of ambiguous bases and primer mismatches of 0. The QIIME software was also used to denoise, split sequences into the proper samples, and pick operational taxonomy units (OTUs) at 97% sequence identity using UCLUST[52]. The representative sequences were submitted to the RDPII classifier[53] to obtain a taxonomy assignment using the Greengenes 16S rRNA gene database[54].

Alpha diversity was evaluated by rarefaction curves, Good's coverage, Chao1 richness[55] and Shannon diversity indices[56]. To test for significant differences in alpha diversity, QIIME's compare_alpha_diversity.py script was used to run nonparametric two-sample *t*-tests. The default number of Monte Carlo permutations (999) was used to calculate *P*-values in the nonparametric *t*-tests, and a significance threshold of $P < 0.05$ was used. Statistical tests for calculating the influence of sample variables on microbial ecology were applied using QIIME's group_significance.py (ANOVA) and compare_categories.py (ADONIS) scripts. The OTU taxonomy tables generated by QIIME were used to produce heatmaps using the software TMeV v 4.8[57]. Beta diversity was also evaluated by UniFrac[58], and principal coordinate analysis (PCoA) was generated by QIIME. Unweighted UniFrac distances were also calculated, but we only report weighted distances here because sample clustering was more informative.

Isolation of Cellulolytic Functional Groups in the Lignocellulosic Biomasses. Different samples were collected from the chipped vegetable biomass piles of *A. donax*, *E. camaldulensis* and *P. nigra* for the isolation of aerobic cellulolytic bacteria. Serial dilutions (0.1 mL) of the samples were spread on the surface of plates containing basal medium (1 g L^{-1} $[NH_4]NO_3$, 1 g L^{-1} yeast extract, 50 mL L^{-1} standard salt solution, 1 mL L^{-1} trace element solution and 15 g L^{-1} agar bacteriological, pH 7.0) with 5 g L^{-1}Avicel (Sigma-Aldrich, Milan, Italy) or 5 g L^{-1} carboxymethylcellulose (CMC) with 0.1% Congo red (Sigma-Aldrich) for exo-cellulolytic or endo-cellulolytic differential isolation, respectively. After 4 days under aerobic conditions at 28°C, the endo-cellulolytic colonies were differentially visualised by a clear halo on CMC. All colonies grown on the substrate Avicel as only carbon source were considered as exo-cellulolytic bacteria. Morphologically different exo- and endo-cellulolytic colonies were isolated, purified again on the same isolation media and stored at 4°C until their characterisation.

Screening for Functional Activities on Solid Media. The endo-cellulolytic activity of all isolates was measured on CMC by a semi-quantitative agar spot method. After adjusting the turbidity of the bacterial suspensions (0.5 of McFarland Turbidity Standard corresponding to approximately 1.5×10^8 CFU mL^{-1}), the cells were spotted on CMC agar medium in triplicate. After a 4-day incubation at 28°C, the plates were stained with 0.1% Congo red solution for 30 min followed by washing with 5 M NaCl[8,14]. Cellulase activities on screening media were recorded as the "Indices of Relative Enzyme Activity, I_{CMC}= diameter of clearing or halo zone/colony diameter"[11]. The exo-cellulase activity was similarly estimated in a semi-quantitative way by inoculating all the isolates by spotting on the Avicel agar and observing the development of bacterial colonies after incubation at 28°C for 10 days.

All isolates were also assayed for cellobiase, xylanase, pectinase and ligninase activities. In particular, cellobiase activity was assessed with cellobiose agar (10 g L^{-1} bacteriological peptone, 15 g L^{-1} agar bacteriological [Oxoid, Milan, Italy], 7.5 g L^{-1} cellobiose and 0.06 g L^{-1} bromothymol blue [Sigma-Aldrich]). After incubation at 28°C for 3 days, the cellobiase activity was detected by observing yellow haloes around the colonies. For xylanase detection, the method described by Ko et al.[59] was applied, adding 0.05% Remazol brilliant blue-R and a 0.5% solution of sonicated (FALC Instruments, HK3300) xylan (Sigma-Aldrich) to the LB Agar medium (Oxoid). After 3–5 days of incubation at 28°C, the hydrolysis of xylan was detected by observing a clear zone around the colonies. Pectinase activity was measured following the method described by Pepe et al.[60] and the results were recorded as the "Indices of Relative Enzyme Activity, (I_{PEC}) = diameter of clearing or halo zone/colony diameter".

Ligninase activity was detected by using the CGA culture medium described by Okino et al.[61], modified by replacing the sugarcane bagasse lignin with vegetable biomass powder from *A. donax* or 2 g L^{-1} lignin alkali (Sigma-Aldrich). A reddish-brown halo around a colony indicates the successful oxidation of guaiacol by lignin-olytic enzymes. Laccase activity was determined for all the isolated strains by growth on agar medium composed of liquid basal medium (LBM) supplemented with 0.1% 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid [ABTS], Sigma-Aldrich) as described by Toutella et al.[62]. After 10 days of incubation at 25°C in darkness, a green colour in the medium indicated the presence of laccase activity. Azure-B (Sigma-Aldrich) agar was used to evaluate the production of lignin peroxidase and manganese-dependent peroxidase by the formation of a clear halo around a colony after 10–30 days of incubation at 25°C in darkness[63].

Identification and Phylogenetic Analysis of Cellulolytic Strains. Bacterial isolates were identified by molecular method (16S rRNA gene sequencing). Total genomic DNA was extracted and purified by InstaGene™ Matrix (Bio-Rad Laboratories, Hercules, CA, USA) according to the supplier's recommendations. Approximately 50 ng of DNA was used as the template for PCR assays. Synthetic oligonucleotide primers fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rD1 (5′-AAGGAGGTGATCCAGCC-3′) were used to amplify the 16S rRNA gene. The PCR mixture was prepared as reported by Alfonzo et al.[64]. The PCR conditions were performed as described by Pepe et al.[65]. The PCR products, after visualisation by agarose (1.5% wt/vol) gel electrophoresis at 100 V for 1 h, were purified by using a QIAquick gel extraction kit (Qiagen S.p.A., Milan, Italy) and sequenced. The DNA sequences were analysed as previously reported[61] and compared with the GenBank nucleotide data library using Blast software at the National Centre of Biotechnology Information website (http://www.ncbi.nlm.nih.gov/Blast.cgi).

Multiple nucleotide alignments of nearly full-length 16S rRNA sequences of isolated strains and type strains within each of the defined species were performed using the Clustal W program[66] from MEGA version 4.0[67]. The nucleotide sequences of the type strains were retrieved from the Ribosomal Database Project (RDP - http://rdp.cme.msu.edu/). The phylogenetic tree was inferred by using the Neighbour-Joining method with the Maximum Composite Likelihood model in MEGA4 program with bootstrap values based on 1,000 replications.

Screening of Cellulolytic Activity in Liquid Media. The bacterial strains were pre-inoculated by dissolving a single colony in 3 mL of liquid medium containing 0.5% CMC, 0.7% yeast extract, 4 g L^{-1} KH_2PO_4, 4 g L^{-1} Na_2HPO_4, 0.2 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.001 g L^{-1} $CaCl_2 \cdot 2H_2O$ and 0.004 g L^{-1} $FeSO_4 \cdot 7H_2O$. After overnight incubation at 37°C, a volume of the broth culture corresponding to 0.1 O.D. was used to inoculate 100-mL plugged Erlenmeyer flasks, each containing 20 mL of the same medium. During incubation at 37°C on a rotary shaker at 225 rpm, samples of the liquid culture were withdrawn and used to measure the optical density ($O.D._{600nm}$) and the extracellular *endo*-1,4-ß-glucanase activity by AZO-CMCase assay (Megazyme, Ireland), following the supplier's instructions. The analytical determinations correspond to the mean value of three replicates.

Accession Numbers. The 16S rRNA gene sequences obtained were deposited in the GenBank nucleotide database under accession numbers from KF040971 to KF040996 and from KF057947 to KF057960 (http://www.ncbi.nlm.nih.gov/Blast.cgi).

The pyrosequencing data are available in the Sequence Read Archive database of the National Center of Biotechnology Information (PRJNA248067).

Statistical Analyses. One-way ANOVA followed by Tukey's HSD *post hoc* for pair-wise comparison of means (at $P < 0.05$) was used to assess the difference in the

enzymatic activities of isolated strains such as I_{CMC}, I_{PEC} and Azo-CMCase. Statistical analyses were performed using SPSS 13.0 statistical software package (SPSS Inc., Cary, NC, USA).

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Author contributions

V.V. wrote the main manuscript text and, in particular, microbial diversity of lignocellulosic biomasses by High-Throughput Sequencing and Phylogenetic analysis of selected cellulolytic bacteria. She prepared Figure 1, 2, 3, 4, 5 and table 1. A.Aliberti and A.R. wrote the isolation and identification of cellulolytic bacterial strains by culture-dependent approach and selection by multienzymatic screening. They prepared Table 2. V.F., S.G. and A.Amore wrote the screening of cellulolytic microorganisms in liquid medium and prepared Table 3. D.E. wrote the microbial diversity of lignocellulosic biomasses by High-Throughput Sequencing. M.F. wrote lignocellulosic biomasses and sampling. O.P. is the corresponding author. All authors reviewed the manuscript.

Additional information

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