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Master Degree
"Microbial Biotechnology"

Microbial diversity studies in thermal spring fields with respect to plant biomass hydrolysis

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THESIS SUMMARY

The present thesis represents the first approach on the evaluation of the aerobic functional microbial diversity, in a unique Greek thermophilic environment near Edipsos area, Evia island, Greece. Degradation of lignocellulosic agro-industrial residues by means of complex microbial community is a promising approach providing efficient biomass decomposition for subsequent conversion to value-added products. In this study, an active thermophilic lignocellulose degrading microbial consortium was bred from high-temperature, lignocellulosic soil samples by successive subcultivation under aerobic conditions. The microbial consortium showed efficient degradation activity on potential biorefinery cellulosic substrates, including alkali pre-treated corn stover and phosphoric acid swollen cellulose. The consortium was structurally stable with the co-existence of two major microbes, comprising aerobic bacterial genera *Rhodothermus* and *Geobacillus*, along with anaerobic and uncultured bacteria. Majority of the lignocellulolytic activities including endo-glucanase, xylanase and -glucanase was present in the crude culture supernatant. This work presents the first report on analysis of the complex structurally stable lignocellulose degrading microbial consortium together with the characterization of its lignocellulolytic enzyme system applicable for biomass degradation and conversion in biotechnological industry.

Introduction

1. Introduction

1.1 Lignocellulosic biofuel as a promising alternative form of energy

Nowadays the growing demand of energy for transportation and industrial processes have increased substantially. Large quantities of fuel, especially petroleum-based liquid fuel, are constantly required to meet this demand. Nevertheless, this is a highly pollutant non-renewable fuel extracted from fossils. Hence, it is crucial to foster the quest for fuels obtained from renewable sources that do not contaminate the environment (Lynd et al. 2008; Ho, Ngo, and Guo 2014). There is a worldwide search for renewable forms of energy that are environmentally clean and economically efficient (Kotchoni, Gachomo, and Others 2008; Shao et al. 2010). Biofuel technology is now considered to be a promising interim, and possibly long-term technology to replace fossil fuels with liquid fuels produced from renewable sources such as cellulosic biomass (Somerville et al. 2010). Among various technical options, the fermentation of biomass hydrolysates is a favored approach, but there has been slow progress in this area due to lack of microorganisms that are capable of metabolizing all the sugars present in the hydrolysates and conversion into useful products (Cripps et al. 2009). Work has been done on engineering of microorganisms for production of biobutanol and biodiesel, but their productivity is low and the microorganisms being used have low tolerance to the solvent products. Sakuragi et al. suggested that current scientific studies should be focused on engineering of microorganisms for the degradation of cellulosic biomass and the production of biofuels such as biobutanol, biodiesel and bioethanol at high efficiency and low cost.
(Sakuragi, Kuroda, and Ueda 2011)

1.2 Lignocellulosic biomass

1.2.1 Structure of lignocellulose

Lignocellulosic biomass is mainly composed of three polymers; cellulose, hemicellulose and lignin together with small amounts of other components, like acetyl groups, minerals and phenolic substituents (Figure 1.1). Lignocellulose has evolved to resist degradation and this robustness or recalcitrance of lignocellulose stems from the crystallinity of cellulose, hydrophobicity of lignin, and encapsulation of cellulose by the lignin-hemicellulose matrix (Isikgor and Remzi Becer 2015).

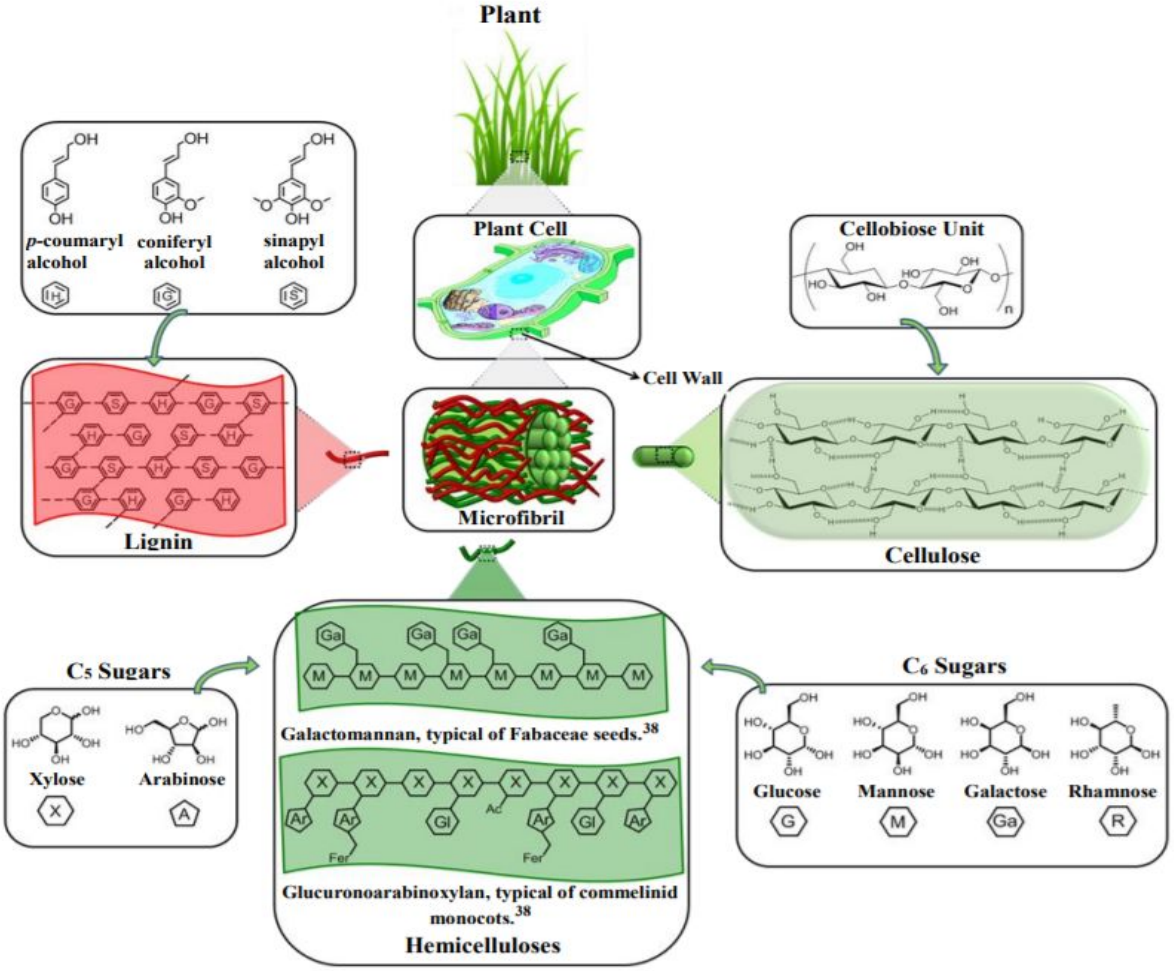


Figure 1.1: Structure of lignocellulose. Reproduced from (Isikgor and Remzi Becer 2015)

1.2.1.1 Cellulose

The major component of lignocellulosic biomass is cellulose. Cellulose is made up of glucose molecules linked by β-1,4 glycosidic bonds, forming long polymeric chains (Figure 1.2) (Volynets, Ein-Mozaffari, and Dahman 2017). The cellulose chains are joined together by hydrogen bonds, and form bundles of cellulose which are substantially crystalline (Gilbert 2010). The bundles of linear chains of cellulose are longitudinally arranged in the cell wall. Since about half of the organic carbon in the biosphere is present in the form of cellulose, the conversion of cellulose into fuels and valuable chemicals has a paramount importance (Himmel and Bayer 2009).

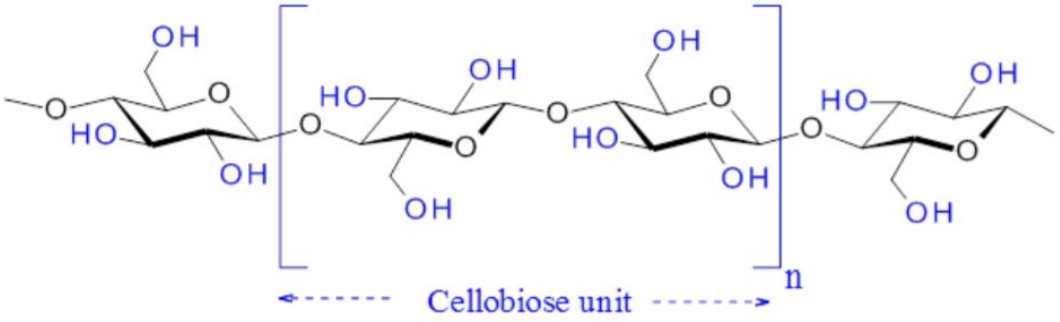


Figure 1.2: Schematic diagram of partial molecular structure of cellulose chain with numbering for carbon atoms and n = number of cellobiose repeating unit.

1.2.1.2 Hemicellulose

Hemicellulose is the second most abundant polymer. Unlike cellulose, hemicellulose has different monosaccharide units including pentoses, branched polymer chains and is lacking crystallinity. Sugar monomers in hemicellulose include xylose, mannose, galactose, rhamnose and arabinose and glucose (Figure 1.3). Xylan represents the most abundant hemicellulosic polysaccharide and is primarily composed of xylose, arabinose, and glucuronic acid (Feldman 1985). Hemicelluloses are imbedded in the plant cell walls to form a complex network of bonds that provide structural strength by linking cellulose fibres into microfibrils and cross-linking with lignin (Isikgor and Remzi Becer 2015).

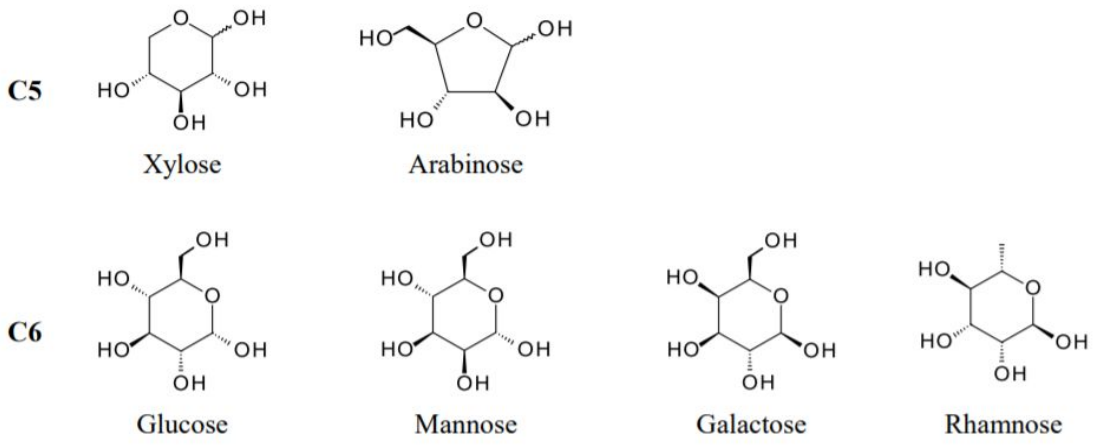


Figure 1.3: Common C5 and C6 sugars found in hemicellulose.

1.2.1.3 Lignin

Lignin is a three-dimensional polymer of phenylpropanoid units. It functions as the cellular glue which provides compressive strength to the plant tissue and the individual fibres, stiffness to the cell wall and resistance against insects and pathogens (Rubin 2008). The oxidative coupling of three different phenylpropane building blocks; monolignols: p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, forms the structure of lignin. The corresponding phenylpropanoid monomeric units in the lignin polymer are identified as p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, respectively (Figure 1.4) (Abdel-Hamid, Solbiati, and Cann 2013).

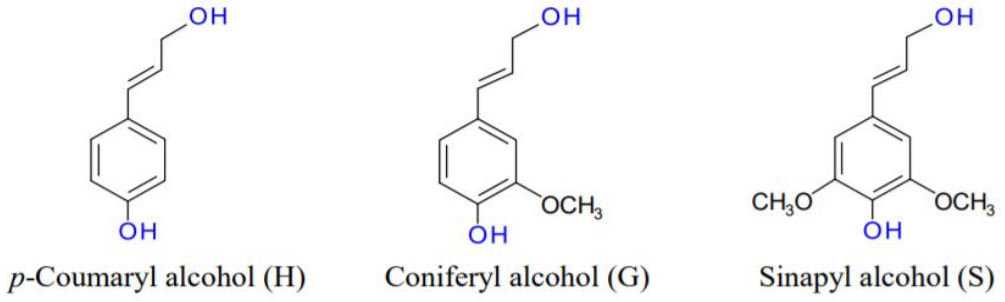


Figure 1.4: Chemical structure of lignin monomeric units.

1.3 Pretreatment of biomass material

Pretreatment is carried out with the purpose of modifying or altering the structural arrangements of the complex lignocellulosic materials, in order to improve the accessibility of the enzymes for cellulose digestion. The main purpose of the pretreatment is to remove the coverage of the cellulose, for example, lignin, and to break the crystalline structure, opening it up for hydrolysis. Various pretreatment methods for improving enzyme accessibility have been developed in the last decades. Nevertheless, pretreatment is still conceived as the second most expensive process unit on an industrial scale (Alvira et al. 2010). A global summary of the different pretreatment methods developed is presented in Table 1.

Table 1: The different categories of pretreatment processes, with relevant examples, effects on lignocellulose, and issues for biofuels adoption (Sun and Cheng 2002).

Process	Examples	Effects	Issues
Mechanical	Milling, steam, AFEX	Increases surface area	Energy cost
Thermal	Hot water	Increases surface area, removes hemicellulose	Byproduct formation
Chemical	Dilute sulfuric acid, concentrated acid, NaOH	Hydrolysis of bonds, hemicellulose and lignin modification/removal	Corrosive, byproduct formation
Biological	Enzymatic, Fungal	Degrades cell wall, delignification	Slow
Solvents	Ammonia, OrganoSolv, NMMO, ILs	Partial or full solubilization of the plant, decrystallization of cellulose	Recycle

1.4 (Hemi)cellulose degrading enzymes

1.4.1 Cellulases

Cellulosic biomass is the most abundant substrate for the economical and environmentally sustainable production of fuels, solvents and other building blocks (Mazzoli 2012). Cellulose is highly recalcitrant to biodegradation, and requires a mixture of enzymes, known collectively as cellulose, for complete hydrolysis (Mazzoli 2012; Olson et al. 2012). The costs of pretreatment and production of cellulases and ancillary enzymes are still the most difficult hurdles to overcome for commercial cellulosic biofuel production (Mohanram et al. 2013).

A classic cellulase enzyme system, in fungi and most bacteria, is made up of three types of enzymes, and these work synergistically during the hydrolysis of cellulose to glucose (Bayer et al. 2010). The three types of enzymes are:

• The "endo-1,4-β-glucanases" or 1,4-β-D-glucan 4-glucanohydrolases (EC 3.2.1.4), which act randomly on soluble and insoluble 1,4-β-glucan substrates and are commonly measured by detecting the reducing groups released from carboxymethylcellulose (CMC).

• The "exo-1,4-β-D-glucanases", including both the 1,4-β-D-glucan glucohydrolases (EC 3.2.1.74), which liberate D - glucose from 1,4-β-D-glucans and hydrolyze D - cellobiose slowly, and 1,4-β-D-glucan cellobiohydrolase (EC 3.2.1.91), which liberates D - cellobiose from 1,4-β-glucans.

• The "β-D-glucosidases" or β-D-glucoside glucohydrolases (EC 3.2.1.21), which act to release D-glucose units from cellobiose and soluble cellodextrins, as well as from an array of glycosides with low degree of polymerization.

Endoglucanases are involved in the random hydrolysis of the intramolecular β-1,4-glucosidic bonds of amorphous regions of cellulose chains resulting in production of new chain ends (Zheng, Pan, and Zhang 2009). The exoglucanases (cellobiohydrolases) attack the cellulose chain ends, to produce cellobiose, the repeating unit of cellulose, or glucose, while β-glucosidase hydrolyses cellobiose to glucose (Zheng, Pan, and Zhang 2009) (Fig. 1.5). Crystalline cellulose is degraded from the chain ends by a combination of exoglucanases and β-glucosidase.

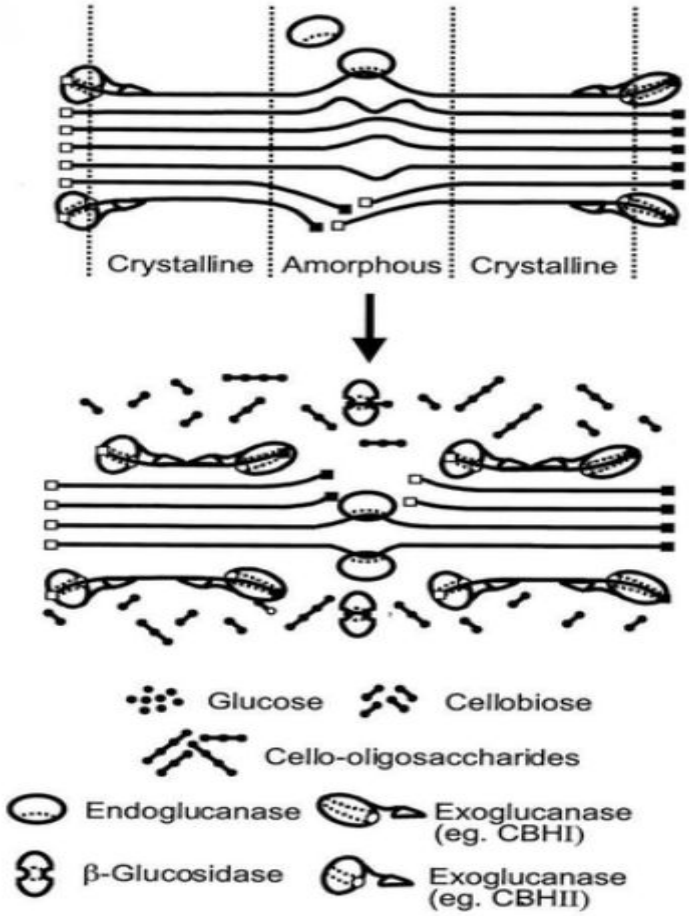


Figure 1.5: Schematic representation of the hydrolysis of amorphous and microcrystalline cellulose by noncomplexed cellulase system (Elnahrawy 2015).

1.4.2 Hemicellulases

The successful deconstruction and modification of hemicellulose requires a complex mixture of enzymes, collectively called hemicellulases (Karboune et al. 2009) (Figure 1.6). Enzyme degradation of hemicellulases involves mild conditions and does not result in the formation of toxic products such as those formed when acid hydrolysis is used (Juturu and Wu 2013).

The post translational modifications of hemicellulases result into production of many types of enzymes, enabling them to deconstruct the heterogeneous hemicellulose structure (Juturu and Wu 2013). Because of the structural complexity of hemicellulose these enzymes act synergistically to achieve full hemicellulose hydrolysis (Karboune et al.

2009; Juturu and Wu 2013). They include endoxylanase (endo-1,4-β-xylanase, E.C.3.2.1.8), β-xylosidase (xylan-1,4-β-xylosidase, E.C.3.2.1.37), arabinase (endo α-L-arabinase, E.C.3.2.1.99), α-arabinofuranosidase (α-L-arabinofuranosidase, E.C.3.2.1.55), feruloyl xylan esterase (E.C.3.2.1.73), α-glucuronidase (α-glucosiduronase, E.C.3.2.1.139) and acetyl xylan esterase (E.C.3.2.1.1.72) (Juturu and Wu 2013). Cellulases and hemicellulases act synergistically to achieve complete hydrolysis of the cellulose and hemicellulose components of the lignocellulosic biomass (Shallom and Shoham 2003).

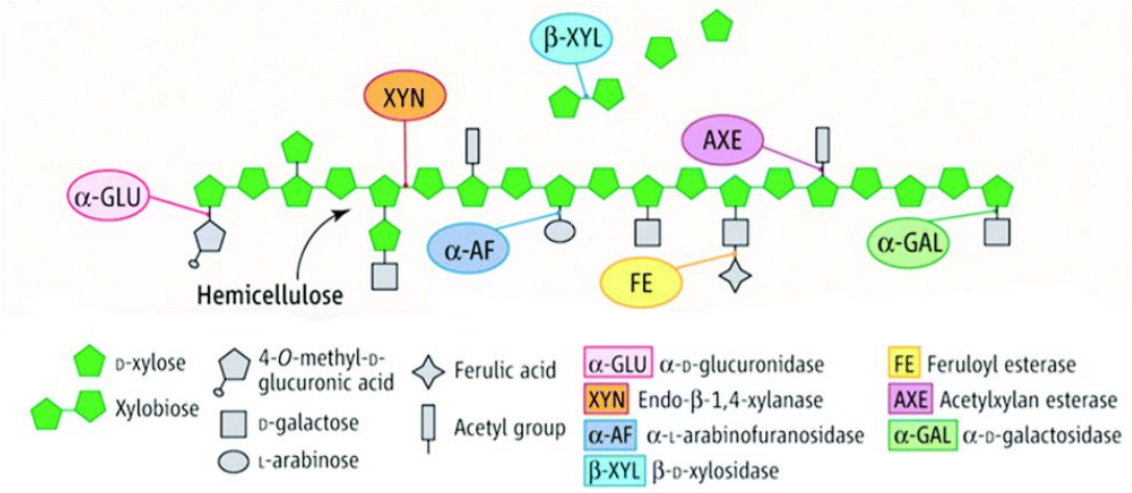


Figure 1.6: Schematic representation of the hydrolysis of hemicellulose by hemicellulase system (Adebayo and Martinez-Carrera 2015).

1.5 Glycoside hydrolases and carbohydrate binding modules

Glycoside hydrolases are the most diverse group of enzymes used by microbes to degrade biomass (Murphy et al. 2011). They are produced by a range of bacteria and fungi (McCartney et al. 2004), and they often contain multiple, structurally and functionally, diverse modules on the same polypeptide chain (Bayer et al. 1998). Glycoside hydrolases typically hydrolyse the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety (Henrissat and Davies 2000). The cellulases have two independent globular modules (Arai, Araki et al. 2003): a catalytic domain, responsible for the hydrolysis reaction itself, and a cellulose-binding module, without catalytic activity, that promotes the adsorption of the enzyme onto the substrate (Henrissat

and Davies 2000).

There is a wide stereochemical variation among carbohydrates, which is paralleled by the large multiplicity of enzymes involved in their metabolism. Since the process of hydrolysis is related to energy metabolism and storage, selective hydrolysis of the glycosidic bonds is therefore very important for energy uptake (Fleming and Pfaendtner 2013).

1.5.1 Classification of glycoside hydrolases

As mentioned above, cellulases and hemicellulases belong to a large group of enzymes called glycoside hydrolases, which hydrolyze the glycosidic bond between two or more carbohydrates or between a carbohydrate and a noncarbohydrate moiety. Previous classification schemes have been based usually on the substrate specificities of the enzyme. Such classification is largely inappropriate for the glycoside hydrolases, because single protein folds are known to harbor a diversity of substrate specificities. A better classification scheme has been instituted over two decades ago by Bernard Henrissat and colleagues (Henrissat and Davies 2000; Cantarel et al. 2009), which is based on the amino acid sequence and consequent fold of the protein. The various glycoside hydrolases are thus divided into families, which currently number 153. This scheme serves to provide comparative structural features of the enzymes within a family, their evolutionary relationships, and their mechanism of action. A compendium of the glycoside hydrolases and related carbohydrate - active enzymes (CAZymes) can be found on the CAZy website (http://www.cazy.org/). The members of most of the glycoside hydrolase families, exhibit multiple types of activities on either cellulosic and/or hemicellulosic substrates — independent of the fold, although some of the families are restricted to a certain type of activity. The specificity of these enzymes is thus a function of the architecture of the active site, the carbohydrate binding modules(s), and the linker peptide(s); not necessarily dictated by the overall structure of the enzyme. The enzymes of some families occur mainly or exclusively in fungi, for example, GH7, GH45, and GH61. Conversely, members of some other families occur mainly or exclusively in bacteria, for example, GH8, GH44, and GH48. The major glycoside hydrolases and their key substrate activities are listed in

Table 1.2.

Table 1.2: Major glycoside hydrolase families and their enzymatic activities. The glycoside hydrolase families (GHn) in which some members exhibit standard cellulase activities are shown in bold. GH families that include cellulases exclusively are followed by an asterisk (*) (Bayer et al. 2010).

GH Family	Enzymes
GH1	Numerous activities, including β-glucosidase, β-galactosidase, β-mannosidase, and β-glucuronidase; but *not* β-xylosidase activity
GH2	Numerous activities, including β-galactosidase, β-mannosidase, and β-glucuronidase; but *neither* β-glucosidase nor β-xylosidase activities
GH3	Numerous activities, notably not only β-glucosidase and β-xylosidase activities, but also glucan 1,3-β-glucosidase, glucan 1,4-β-glucosidase, and exo-1,3(4)-glucanase activities
GH5	Broad spectrum of cellulase and hemicellulase activities, including cellulase, xylanase, 1,3-β-mannanase; β-mannosidase, glucan 1,3-β-glucosidase, licheninase, glucan endo-1,6-β–glucosidase, mannan endo-1,4-β-mannosidase, endo-1,6-β-galactanase, and xyloglucan-specific endo-1,4-β-glucanase activities
GH6*	Cellulase activities in both aerobic bacteria and fungi (not found in archaea): both endo- and exo-glucanase (cellobiohydrolase) activities
GH7*	Cellulase activities *exclusive* to the fungi: both endo- and exo-glucanase (cellobiohydrolase) activities
GH8	Cellulase, lichenanase, xylanase activities; exclusive to bacteria
GH9*	Endo-, processive endo-, and exo-glucanase (cellobiohydrolase) activities in bacteria, plants, and fungi (but not in archaea)
GH10	Endo-1,4-β-xylanase and endo-1,3-β-xylanase activities in bacteria and fungi
GH11	Xylanase activities in bacteria and fungi
GH12	Endoglucanase, xyloglucanase, and 1,3(4)-β-glucanase in the three domains of life
GH16	Endo-1,3-β-glucanase, endo-1,3(4)-β-glucanase, lichenanase, and xyloglucanase activities
GH17	Glucan 1,3-β-glucosidase and lichenanase activities
GH18	Chitinases
GH19	Chitinases
GH26	β-Mannanase and 1,3-β-xylanase activities
GH30	1,6-β-Glucanase and β-xylosidase activities
GH39	β-Xylosidase activity
GH42	β-Galactosidase activity
GH43	Broad spectrum of hemicellulase activities, including xylanase, arabinanase, β-arabinofuranosidase, β-xylosidase, and galactan 1,3-β-galactosidase activities in bacteria and fungi
GH44	Endoglucanase and xyloglucanase activities, mainly in bacteria
GH45*	Endoglucanase activity, mainly in fungi (some bacteria)
GH47	α-Mannosidase activity, mainly in fungi
GH48*	Cellobiohydrolases and endo-processive cellulases; mainly in bacteria; an important enzyme in all cellulosomes and in some noncellulosomal systems
GH51	α-L-Arabinofuranosidase and endoglucanase activities
GH52	β-Xylosidase activity
GH53	Endo-1,4-β-galactanase activity
GH54	α-L-Arabinofuranosidase and β-xylosidase activities, mainly in fungi
GH55	Exo- and endo-1,3-glucanase activities, mainly in fungi
GH61	Exclusive to fungi. In some cases, annotated as endoglucanases, but probably disrupt cellulose structure rather than cleaving glucoside bonds.
GH62	α-L-Arabinofuranosidase activity
GH64	1,3-β-Glucanase activities; mainly in bacteria
GH67	α-Glucuronidase and xylan α-1,2-glucuronosidase activities
GH74	Xyloglucanase and endoglucanase activities
GH81	1,3-β-Glucanase activity

1.5.2 Glycoside hydrolases mechanism of action

Enzymatic hydrolysis of glycosidic bonds follows general acid catalysis that needs two critical residues: a proton donor and a nucleophile/base (Sinnott 1990). There are two major mechanisms for glycoside hydrolases, leading to overall retention or inversion of the stereochemistry at the cleavage point (Sinnott 1990), as shown in Fig.1.7.

Both mechanisms employ a pair of carboxylic acids in the active site. In retaining enzymes, one residue acts as a nucleophile and the other as a general acid/base catalyst and are only 5.5 Å apart (Fig. 1.7A). In inverting enzymes, on the other hand, one residue acts as a general acid and the other as a general base catalyst, and are suitably placed, about 10 Å apart, to allow both a substrate and a water molecule between them (Fig. 1.7B). The inverting mechanism proceeds via a single displacement involving an oxocarbenium ion-like transition state. By contrast, the reaction of retaining enzymes proceeds via a double displacement mechanism involving a covalent glycosyl-enzyme intermediate. First, one of the carboxylic residues functions as a general acid catalyst by protonation of the glycosidic oxygen. The other carboxylic residue acts as a nucleophile, by attacking the C1 atom of the glycosidic bond and forming a covalent glycosyl-enzyme intermediate. In the next step, the carboxyl group that first acted as an acid catalyst, now acts as a general base catalyst by de-protonating an incoming water molecule which attacks at the anomeric center, resulting in the release of the sugar molecule. For a chitinase it has been demonstrated that the acetamido group at C-2 of the substrate can take over the role of catalytic nucleophile, and thus shows substrate assisted catalysis (McCarter and Withers 1994).

One of the mechanisms has been found to use NAD as a cofactor. The glycoside hydrolases of family 4 (Rajan et al. 2004) and 109 use a mechanism that requires a NAD cofactor (Sulzenbacher et al. 2010). The NAD remains tightly bound throughout catalysis and the mechanism involves anionic transition states with elimination and redox steps (Rajan et al. 2004).

Some GH families are also able to employ novel mechanisms other than the typical carboxylate base/nucleophile. These include substrate assisted mechanisms, proton transferring networks, utilization of non-carboxylate residues and utilization of an

exogenous base/nucleophile (Vuong and Wilson 2010).

Glycoside hydrolases have also developed mechanisms to lower the energy barrier of the hydrolysis reaction by distorting their substrate into a sofa or half-chair conformation (Strynadka and James 1991; Kuroki, Weaver, and Matthews 1993). Protonation of the glycosidic bond is accompanied by a substantial lengthening of this bond (Sakon et al. 1997).

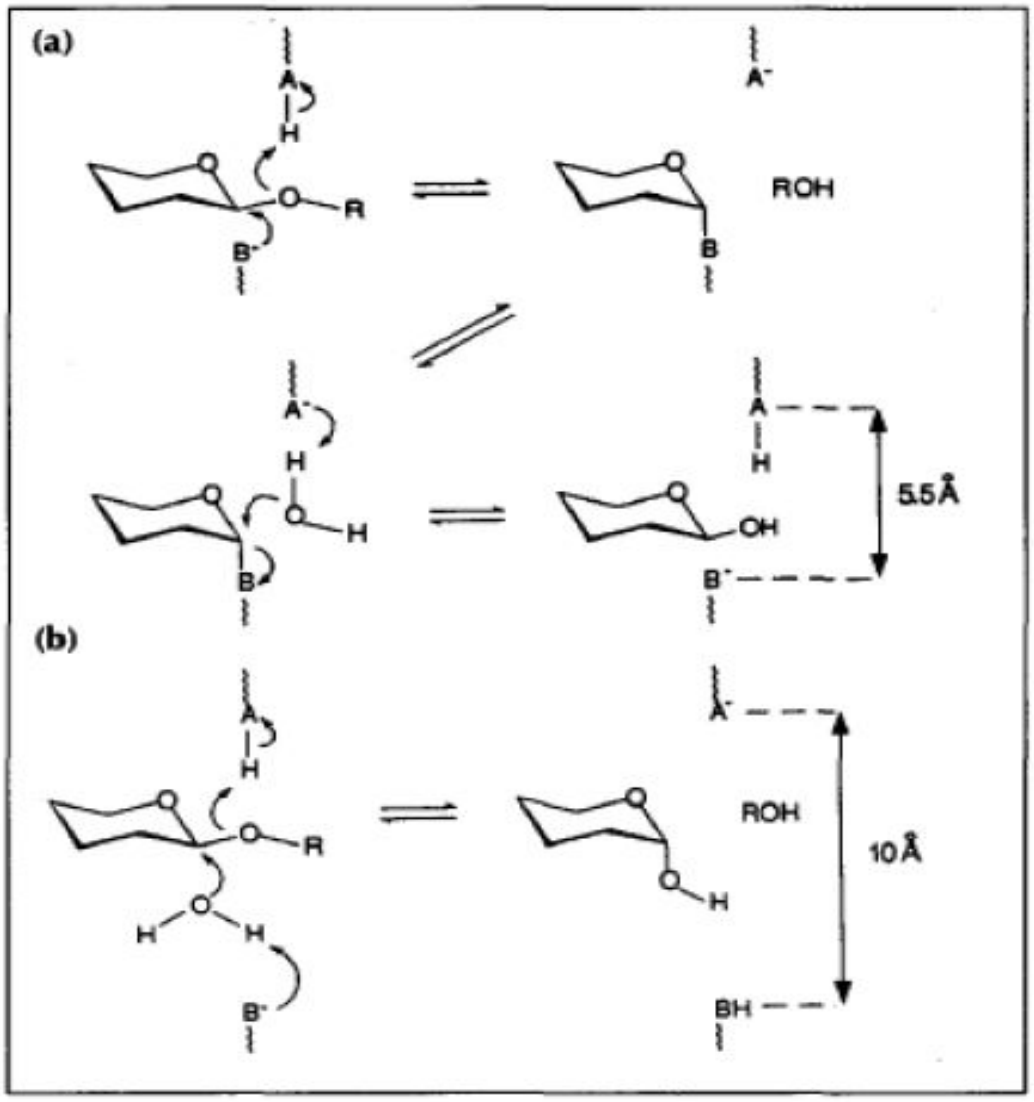


Figure 1.7: The two major mechanisms for glycoside hydrolases. The mechanisms were proposed by Koshland (1953). (a) The retaining mechanism, which involves the protonation of glycosidic oxygen by the acid catalyst (AH) and departure of the aglycon assisted by the nucleophile base (B-). The formed glycoside enzyme gets hydrolyzed by a water molecule and results into a product with the same stereochemistry as the substrate. (b) The inverting mechanism, in which the glycosidic oxygen gets protonated. A water molecule that is activated by the base residue (B-) attacks at the anomeric carbon as the aglycon departs. The resulting product has an opposite stereochemistry to the substrate. Copied from (Henrissat and Davies 2000).

1.5.3 Carbohydrate binding modules (CBMs)

The primary type of ancillary module, which is common to most glycoside hydrolases, is the CBM (Boraston et al. 2004). Many CBMs serve to target the parent glycoside hydrolase to the substrate. The first CBMs that have been described were initially termed CBDs (cellulose - binding domains), owing to their substrate specificities and binding to crystalline types of cellulose. The CBDs were thus divided into "types" on the basis of amino acid sequence, in a manner similar to the GH families. Further work, however, revealed that some of the CBD types were not specific for crystalline cellulose (such as type 4) or to cellulose at all (some members of type 2 bound to cellulose, whereas others bound to xylan). Moreover, some protein modules were found to exhibit binding specificity to non – cellulosic polysaccharides. Today, the different CBMs are now divided into over 84 different families showing broad specificity patterns, sometimes within a given family and even by a given module (CAZypedia Consortium 2018). The CBMs exhibit various functions, including targeting of the parent enzyme to the undigested substrate, targeting of given modules to portions (conformations) of the substrate during deconstruction, and attachment of the parent enzyme to the microbial surface.

1.6 Thermophiles and ethanol

Bacteria that grow optimally at temperatures above 55 °C are called thermophiles. These have been reported to be ideal microbes for improved production of bioethanol and other more advanced biofuels. Thermophilic bacteria such as *Clostridium thermocellum*, *Thermoanaerobacter thermosaccharolyticum*, *T. thermohydrosulfuricus*, *T. ethanolicus*, *T. brockii*, *T. saccharolyticum* and *Geobacillus* spp., have been evaluated for biofuel production and have been found to have a number of advantages over mesophilic bacteria (Sommer, Georgieva, and Ahring 2004; Shaw et al. 2008). Thermophilic bacteria are capable of producing robust enzymes suitable for use in industrial processes (Wiegel 1980; Rhee et al. 2000; Bhalla et al. 2013). Especially for 2nd generation biorefinery applications:
i. Many thermophiles readily utilize pentoses, glucose, and other complex carbohydrates, and therefore have a high potential for producing ethanol from lignocellulose.

ii. A number of thermophiles express cellulase systems.

iii. They generally display high maximum specific growth rates (μ_{max}) and have high maintenance energies resulting in low cell yields and conversely high substrate conversion to product.

iv. High temperatures result in an increased vapour pressure of ethanol, facilitating ethanol removal and recovery.

v. Substrates are more soluble at high temperatures, allowing increased concentrations of carbohydrate to be used during the process.

vi. Many glycolytic thermophiles are able to use polymeric or short oligomeric carbohydrates.

vii. Oxygen is less soluble at high temperatures, thus facilitating the maintenance of anaerobic conditions required for the fermentation process.

viii. High temperatures reduce the risk of contamination by non-thermophiles during the process.

ix. There is no requirement for cooling during fermentation with thermophiles.

An earlier study (Rastogi et al. 2010) indicated that bacteria belonging to the genera *Geobacillus*, *Thermobacillus*, *Cohnella* and *Thermus* are capable of degrading amorphous cellulose, carboxymethylcellulose (CMC), or ponderosa pine saw dust. Previous studies have reported isolation of cellulose-degrading bacteria from compost systems (Lu et al. 2005; Ng et al. 2009), soils (Lee et al. 2008), wastewaters (Tai et al. 2004), and from deep biosphere of gold mines (Rastogi et al. 2009). Cellulase enzymes from these bacteria can probably withstand extreme conditions such as temperature and toxic inhibitors (Rastogi et al. 2010). Efforts to produce ethanol from thermophiles have been hampered due to limited biochemical knowledge, limited genetic tools, poorly understood host transformation systems and scarcity of sequence data for these microorganisms.

1.7 Aim of the project

The main aim of this project was to investigate the possibility of isolating thermophilic biomass degrading bacteria from the thermal springs area of Edipsos in Evia island, Greece. The site has never been examined in the past with respect of its microbial diversity, under any respect. The temperature of the waters in the area are well above 60 ^{o}C and as a result, we expect the corresponding sediments to harbor thermophilic microorganisms with enzymes of high thermal stabilities and activity optima.

Our approach will involve the use of enrichment cultures, using specific biomass related carbon sources in order to isolate microorganisms with the corresponding biomass degrading abilities. For this reason, we have selected alkali pretreated corn stover (*Zea mays* ssp. *mays* L) as well as phosphoric acid swollen cellulose, in order to identify the enzymes, and the associated organisms involved in the process. This would act as a guide to important functions necessary for hemi (and cellulose) degradation and hence the proteins that would need to be isolated and purified.

Specific objectives.

- Use the pretreated corn stover and phosphoric acid swollen cellulose as substrates to isolate thermophilic bacteria which can degrade the complex oligomers and polymers present in the pre-treated material
- Characterize the resulting final lignocellulosic mixed cultures for enzymes that are responsible for producing enzymes that can hydrolyse (hemi)cellulose
- Identify the bacteria isolated from the mixed culture through analysis of their 16S RNA gene analysis
- Characterize the secreted (hemi)cellulases from the mixed cultures through zymogram analysis

Materials and Methods

2. Materials and Methods

2.1 Isolation of microorganisms with biomass degrading activities from Central Greece

2.1.1 Samples collection

Sediment and water samples were collected from a hydrothermal vent in Aidipsos, Greece, during October 2017. The water temperature at the sampling point was 64.5°C with a pH value of 6.95. Sediment cores (50 cm deep) and water samples were taken under aseptic conditions, using sterile equipment with water samples being collected in sterilized one-liter polypropylene bottles. Sterilized spatulas were used for transferring the sediment samples from different core depths into 50 mL polypropylene Falcon tubes, preventing air exposure as much as possible. Following sampling, the tubes were stored at 4-6 °C until further processing.

2.1.2 Preparation of isolation media

The isolation medium (modified from (Widdel and Pfennig 1981) contained (values in g/L): $NaH_2PO_4 \cdot H_2O$, 3.8; $Na_2HPO_4 \cdot 7H_2O$, 3; NH_4NO_3, 1; Na_2SO_4, 0.23; and NaCl, 8.5; KCl, 0.5. The following components were sequentially added, to the autoclaved cooled medium, from sterile stock solutions:

Solution A: (1 mL/L) - $MgCl_2 \cdot 6H_2O$ (324 g/L) and $CaCl_2 \cdot 2H_2O$ (29.4 g/L)

Solution B: (1 mL/L) - containing (per L) 6 mg $Na_2SeO_3 \cdot 5\ H_2O$, 8 mg $Na_2WO_4 \cdot 2H_2O$ and 0.4 g NaOH

Trace element stock solution: (1 mL/L)

Vitamins stock solution: (1 mL/L), containing (per L): 10 mg D(+) biotin, 40 mg 4-aminobenzoic acid, 100 mg nicotinic acid, 50 mg calcium D-(+)-pantothenate, 25 mg pyridoxamine-dihydrochloride (changed), 50 mg thiamin dihydrochloride/L (changed); vitamin B_{12} solution with 50 mg.

The carbon sources added in the isolation medium were 10 g/L of PASC (Phosphoric acid swollen cellulose) and 10 g/L of Alkali pre-treated corn stover. Agar powder was added at

a final concentration of 15 g/L in order to prepare solid agar plates with the same carbon sources. The pH of the medium (normally between 7 and 7.5) was adjusted at pH 7 with 2N HCl or NaOH before autoclaving.

2.1.3 Enrichment cultures

Enrichment cultures under aerobic conditions were performed with 1% w/v PASC (Phosphoric acid swollen cellulose) and 1% w/v APCS (alkali pre-treated corn stover) at an incubation temperature of 65 °C. Conical flasks with a volume of 100 mL were filled with 20 mL modified phosphate buffered minimal salt medium (Section 2.1.2). Enrichment cultures were inoculated with 5 grams (wet weight) of sediment sample and incubation took place for 7 days, before 2 mL of the respective enrichment culture was transferred into 18 mL fresh medium for a further 7 day incubation.

2.1.4 Isolation of cellulolytic/hemicellulolytic strains

Following 4 enrichment cycles, whole culture broth from the different enrichment cultures was serially diluted in sterile PBS. 0.1 mL from the 10^{-2}, 10^{-4} and 10^{-6} serial dilutions were spread on agar plates of medium with different carbon sources. The plates were prepared in duplicate and incubated at 65 °C. The NA plates were incubated overnight, while the PASC plates and the APCS plates were incubated for 3 days.

Developed colonies were purified by streaking each colony on a new plate of fresh medium, identical to the one used for the initial isolation. The purification was performed several times till pure colonies were obtained.

2.1.5 Extraction of genomic DNA and amplification of the 16s rRNA gene region

2.1.5.1 Agarose gel preparation

Agarose gels (0.8% w/v) were used to separate PCR products. Appropriate amounts of agarose were weighed and dissolved in 1x TAE prepared from 50x TAE buffer (242 g Tris Base, 57.1 mL Glacial acetic acid and 100 mL of 0.5 M EDTA, pH 8) followed by heating in a microwave oven (850 watts) until all agarose was dissolved. The solution was then cooled at room temperature and 3.5 µL of EtBr, per 100 ml gel, was safely added. The gel was poured into a mould and allowed to solidify.

2.1.5.2 Isolation of Genomic DNA

Bacterial genomic DNA was extracted using a modified PCR lysis buffer protocol[DGH8] . The PCR lysis buffer consisted of 100 mL 1x TE pH 8 and 0.1% w/v Triton – X100. In brief, microbial isolates were grown in Nutrient broth agar plates (values in g/L) (meat extract, 3; gelatin, 5; peptone[DGH9] ; agar, 1.5) at 65 ˚C for 24 hours. The collected colonies were boiled for 5 minutes in 15 µL of the PCR lysis buffer and spinned down (~13000 x g) for 10 minutes. 5 µL of the supernatant were used as template for the polymerase chain reaction (PCR).

2.1.5.3 Polymerase Chain Reaction (PCR)

Primer sequences used for the colony PCR are displayed in the table 2.1. Sterile 0.5 mL PCR tubes (Scientific Specialities Inc.) were used to carry out PCR reactions for bacterial 16S rRNA gene (primers 27F and 1492R), using a thermal cycler (Eppendorf Mastercycle gradient) and a total volume of 50 µL (made up of distilled water (ddH_2O), 20 µL of HF buffer, 2 µL of 10 mM dNTPs, 5 µL of forward primer (10 pmol/µL), 5 µL of reverse primer (10 pmol/µL), 5 µL of template and 1 µL Phusion hot start DNA polymerase (2 U/µL).

The cycling conditions for colony PCR were carried out with an initial denaturation for 3 minutes at 98°C followed by 35 cycles of denaturation at 98°C (10 s), annealing at 59-62°C (30 s), and final extension at 72°C (45s). After completion the reaction was held at 4-10°C.

Table 2.1 Sequences of primers used for PCR.

Primer	**Sequence** (5' ▬ 3')
27F	GAGAGTTTGATCCTGGCTCAG
1492R	GGTTACCTTGTTACGACTT

2.1.5.4 DNA fragment purification

After separating the DNA, the band of interest was excised from the agarose gel under ultra violet (UV) light (making sure that the DNA was not exposed to UV light for too long, to avoid DNA damage). The PCR products were purified with NucleoSpin Gel and PCR clean up Kit (MACHEREY-NAGEL) according to the manufacturer's instructions. The purified DNA was stored at -20°C.

2.1.5.5 DNA Quantification

DNA quantification was carried out by measuring the absorbance of samples at 260/280 nm. This was performed by diluting 2 µL of DNA sample with 18 µL of distilled water and loading the sample into a 384 UV/Vis Multiwell Plate (Corning). The absorbance was read at 260 nm and 280 nm on a Synergy HT multi-detection microplate reader.

2.2 Phosphoric acid swollen cellulose

Regenerated amorphous cellulose was prepared by adding approximately 0.2 g of microcrystalline cellulose to a 50 mL centrifuge tube, and dissolving it with 0.6 mL distilled water in order to form a uniform slurry. Ten mL of ice-cold 86.2% H_3PO_4 was slowly added to the slurry with vigorous stirring. The cellulose was mixed evenly before the addition of the last 2 mL acid. The cellulose mixture turned transparent within several minutes and was left on ice with occasional stirring for an hour.

Approximately 40 mL of ice-cold water were added in 10 ml batches, with vigorous stirring between additions, resulting in a white cloudy precipitate. The precipitated cellulose was centrifuged at 4000 x g for 20 min. The pellet was suspended by adding ice-cold water and re-centrifuged several times, to remove the acid. Approximately 0.5 mL of 2 M Na_2CO_3 was added to neutralize the residual phosphoric acid, and finally, 45 mL of ice-cold distilled water was used to suspend the cellulose pellet. After centrifugation, the pellet was suspended in distilled water and centrifuged until pH 7. The regenerated (homogeneous) cellulose could be kept at 4°C for a long time by adding a small amount of sodium azide .

2.3 Alkaline (NaOH) pretreatment of corn stover

Corn stover (Zea *mays ssp. mays L*) was obtained from a local farm in July 2017. Corn stover was washed with tap water to remove surface impurities, cut into 20-30 cm pieces, and then milled to a particle size of 1 mm. Then, it was air-dried at 100 °C in order to reduce moisture to less than 5% by weight and then milled again to a particle size of 0.7 mm. The milled corn stover was transferred to the laboratory and was stored in sealed plastic bags at room temperature until use.

Milled corn stover particles were pretreated with 0.13g NaOH/g dry biomass. Treatments were performed in a water batch at 92 °C, for 134 minutes. Pretreated corn stover particles were washed on a 0.125 mm sieve several times with tap and finally with distilled water. This washing step caused the removal of excess alkali as well as the re-deposition of lignin and other inhibitors. The composition of the material was changed and provided increased

access for enzymes. The dry mass of the pretreated corn stover was dried in an oven at 60 °C for 72 h and passed from a mill (0.7 mm) until storage at RT.

2.4 Protein methods and enzyme assay protocols

2.4.1 Preparation of 0.1% Congo Red

The 0.1% w/v Congo red solution was prepared by dissolving 0.5 g of Congo red powder (Sigma) into 500 ml of distilled water.

2.4.2 Qualitative assessment of cellulase activity on salts medium containing PASC and APCS

Three different compositions of agar plates were prepared as follows: 1. Minimal salt (MS) medium (control), 2. MS + 0.5% PASC, 3. MS + 0.5% APCS.
Bacterial strains were inoculated on the above agar medium plates that contained different carbon sources as well as on the control plate. The plates were incubated at 65°C incubator for 48 h, then flooded with Congo red solution and left to stand at room temperature for 1 h. The Congo red was poured off and the plates were subsequently flooded with 1 M NaCl. After 5 min this was poured off and then flooded again with 1 M NaCl (this step was repeated three times).

2.5 Determination of enzyme activities

2.5.1 Reagents

2.5.1.1 Preparation of 3,5-dinitrosalicylic acid (DNS) solution

The DNS solution was prepared by dissolving 10 g of 3,5-Dinitrosalicylic acid in 500 mL of dH_2O on a magnetic stirring platform at 40 (± 2) ˚C. Then, 300 g of Potassium Sodium (+) – tartarate was gradually added until all solids were dissolved. Finally, 16 g of NaOH were slowly added. The solution was made up to 1 L.

2.5.1.2 Preparation of 4% w/v Carboxymethyl cellulose (CMC)

Carboxymethyl cellulose (CMC) (Sigma-Aldrich, St Louis, MI, USA) was dissolved in a 4% w/v solution in MOPS-HCl buffer (pH = 7).

2.5.1.3 Preparation of 4% w/v Xylan from beechwood

Beechwood xylan (Sigma-Aldrich, St Louis, MI, USA) was dissolved in a 4% w/v solution in MOPS-HCl buffer (pH = 7).

2.5.1.4 Preparation of 1% v/v Phosphoric acid swollen cellulose (PASC)

Phosphoric acid swollen cellulose[DGH13] was dissolved in a 1% v/v solution in MOPS-HCl buffer (pH=7).

2.5.2 Enzyme assay

The assay mixture for detecting xylanase activity contained 100 µL of 4% beechwood xylan (Sigma-Aldrich, St Louis, MI, USA) in MOPS-HCl buffer pH 7, 50 µL MOPS-HCl buffer pH 7 and 50 µL of culture supernatant. A control sample was prepared without sample (substituted with 50 µL MOPS-HCl buffer) and incubated in the same way. Samples were incubated for 18 - 23 h at 60 °C in a thermoshaker (BOECO GmbH, Hamburg, Germany) at 1000 rpm. The reaction was terminated by immediately placing samples into an ice bath followed by the addition of 200 µL of dinitrosalycilic acid (DNS) solution. The tubes were placed in a boiling water bath for 5 min and centrifuged (10 min) at 11000 x g for the removal of the residual xylan solids. Supernatants (200 µL) from each tube were transferred to clean test tubes and 1600 µL distilled water were added, followed by brief vortexing. Aliquots (200 µL) were transferred to a 96-well microplate (Multiscan™ Go, Thermo Scientific), and the absorbance at 540 nm was measured. A xylose standard curve was used to determine the amount of xylooligosaccharides released. Endoglucanase activity was estimated in the same way but with carboxymethyl cellulose (CMC, Sigma-Aldrich, St Louis, MI, USA) and PASC at 2.0 % w/v (final conc.) and 0.5 % v/v (final conc.), respectively, as substrate, omission of the centrifugation step (CMC is soluble), and using glucose for the DNS reference curve.

All reactions were conducted at least in triplicate. Enzyme activities were expressed in Units (U), defined as the amount of enzyme necessary to catalyze the production of 1 μmol of product per minute under the above-described pH and temperature conditions.

2.6 Protein concentration by filtration and assay

The proteins in the culture supernatants of strains were concentrated using an Amicon® Ultra-2mL Centrifugal Filter Devices (MilliporeSigma) of 10 kDa MW cut-off.

2.7 Protein assay

The Bio-Rad DC protein assay method based on the Bradford method (Bradford, 1976) was used to determine protein concentrations in the culture supernatants as specified by the manufacturer's instructions. A standard curve was prepared using Bovine Serum Albumin (BSA) at concentrations 0, 0.125, 0.25, 0.5, 0.7, 1 and 1.25 mg/ml which was used for calculating the experimental protein concentrations.

2.8 SDS PAGE

2.8.1 Preparation of gels

The 10% lower gel (20 mL) was prepared by first mixing 8.1 mL ddH2O, 6.7 mL acryl amide/bisacrylamide stock solution (Applichem), 5 mL Tris-HCl (1.5 M, pH 8.8), followed by the addition of 0.2 mL freshly made 10% ammonium persulfate and 0.008 mL TEMED (Fisher Scientific) in that order. The mixture was promptly pipetted into the assembled gel plates (1.0 mm) evenly from side to side up to about 1 cm from the marked edge of the gel comb. The gel was immediately covered by a thin layer of isobutanol to even the surface, and allowed to dry (5-30 min). The isobutanol was poured-off from the resolving gel and the surface was blotted dry using paper roll.

The upper gel (5 ml) was prepared by mixing 3.45 mL ddH2O, 0.83 mL acryl amide/bisacrylamide stock solution, 0.63 mL Tris-HCl (1 M, pH 6.8), followed by the addition of 0.05 mL freshly made 10% ammonium persulfate and 0.005 mL TEMED

(Fisher Scientific) in that order. The mixed components of the upper gel were promptly pipetted into the assembled gel plates (1.0 mm) on top of the resolving gel evenly from side to side. The plates were filled with stacking gel such that it overflew upon addition of the comb. The comb (10 wells) was inserted between the gel plates carefully, to avoid bubbles from persisting. The stacking gel was allowed to solidify (5-30 min).

2.8.2 Preparation of Coomassie blue stain

The stain was prepared by mixing 1.25g Coomassie blue R250 (0.25% w/v final conc.), 200 mL methanol (40% v/v final conc.), 50 mL acetic acid (glacial, 10% v/v final conc.) and 250 mL dH_2O to obtain a final volume of 500 mL. The Coomassie stain was reused several times.

2.8.3 Coomassie blue destaining solution

The destain solution was prepared by mixing 100 mL of methanol (20% v/v final conc.), 25 mL of acetic acid (glacial, 5% v/v final conc.) and 375 mL of dH_2O to obtain a final volume of 500 mL.

2.8.4 Running buffer

A litre of 10x running buffer containing 30 g Tris-base, 144 g glycine and 10 g SDS.

2.8.5 Running SDS PAGE gels

For sample preparation, sample's supernatant were mixed with 4 x Laemmli buffer and heated at 95 °C for 5 min thus leading to the denaturation of proteins. When loading the gel, 7 µl of a protein molecular weight marker ["Prestained Protein Marker" (NEB, Schwalbach)] were applied to one of the gel wells in order to later serve as size standard to which sample bands could be compared to. Twenty µL of the respective samples were applied to the remaining gel pockets and the gel was run at 100 V power supply from a BioRad power supply by using BioRad Gel Electrophoresis tanks (BioRad, UK).

2.9 Zymogram analysis

Zymogram analysis for xylanase and endoglucanase activity was performed under denaturing conditions in a 10% SDS-PAGE gel containing either 0.5% w/v beechwood xylan (Sigma-Aldrich, St Louis, MI, USA) or 0.5% w/v carboxymethyl cellulose (CMC, Sigma-Aldrich, St Louis, MI, USA). Following electrophoresis, the gel was first soaked in 1% w/v Triton X-100 at room temperature for 1 h. It was then washed with Milli Q water, and incubated in 50 mM MOPS-HCl buffer pH 7 for 1 h at 65°C. In some cases, the incubation needed to last up to 48 h. The buffer was drained out and the gel was washed with deionized water for several times before stained with 0.1% Congo red stain for 2 h at room temperature. The gel was destained with 1 M NaCl until the development of visible hydrolysis bands, usually between 1 and 2 h, or sometimes in less than 1 h.

2.10 Frozen Glycerol Stocks

2.10.1 Preparation of 50% (v/v) glycerol

The 50% v/v glycerol was prepared by mixing 25 mL of sterile 99.5% glycerol and 25 mL sterile 1x phosphate buffered saline (PBS).

2.10.2 Preparation and storage of stocks

Stocks were prepared by mixing 5 mL of sterile 50% (v/v) glycerol (2.10.1) and 5 mL of the fresh culture and immediately stored in a -20°C freezer.

Results

3. Results

3.1 Sampling site and procedure

The aim of this study was to investigate the biomass degrading potential of the aerobic microbial population at the Saranta Platania hydrothermal vent in the Area of Edipsos, Evia island, Greece (Figure 3.1). The area is described as a deep hydrothermal field, with several karstic groundwater escapes that culminate in a central creek flowing parallel to a public road. As shown in Figure 3.1, one side of the creek is adjacent to heavy plant vegetation that in some points fell and degrade inside the creek.

Figure 3.1: Wide view of the sampling area (https://goo.gl/maps/Mg1zF3E8Xf52). The red circle surrounds the sampling points.

The temperature of the surface waters in the area is around 65 ºC, a value that remains stable throughout the year, supported by the continuous groundwater flow. Due to the geometry of the creek bed, degrading biomass mainly accumulates in the central areas of the bed and thus, we selected to obtain our sample core from the central area of the creek (Figure 3.2).

Based on that, core II (Figure 3.2, red circle) was selected for further analysis as we considered that the location from which it was harvested is where the decomposition of

plant biomass takes place at a higher rate and thus, is also the site where is most likely to harbor microbial consortia with a plant degradation capacity.

Figure 3.2: View of the sampling sites. The red circle indicates the sampling point from which the sample core was obtained.

Core II, of 50 cm length, was divided into five individual layers based on depth, three of which (I, III, V) (Figure 3.3) were selected as the starting inoculum for the enrichment cultures.

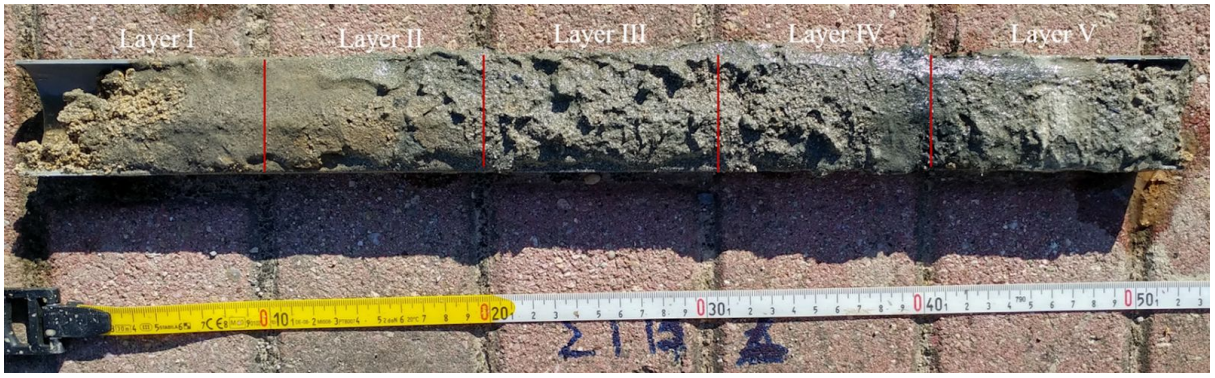


Figure 3.3: Picture of Core II, divided into five individual layers, three of which were selected as the starting inoculum for the enrichment cultures.

3.2 Glycoside hydrolase activities in enrichment cultures

Enrichment cultures were performed in order to increase the abundance of the target organisms (biomass degraders) in the sample inoculum. Two different substrates were used

as sole carbon and energy sources: Alkali pretreated corn stover (APCS) and Phosphoric acid swollen cellulose (PASC). APCS is an agricultural lignocellulosic residue rich in both cellulose and hemicelluloses. Alkali pretreatment removed most of the lignin and loosen the cellulose - hemicelluloses structure rendering it more accessible to hydrolytic enzymes. PASC on the other side is a pure cellulosic substrate based on crystalline cellulose, swollen by phosphoric acid in order to increase its susceptibility to enzymatic attack.

At the end of each weekly enrichment cycle, assays for endocellulases and xylanases were performed in order to confirm the success and the gradual enrichment progress (4 cycles). The observed enzyme activities for each substrate are recorded in Figure 3.4 and 3.5 for APCS and PASC, respectively.

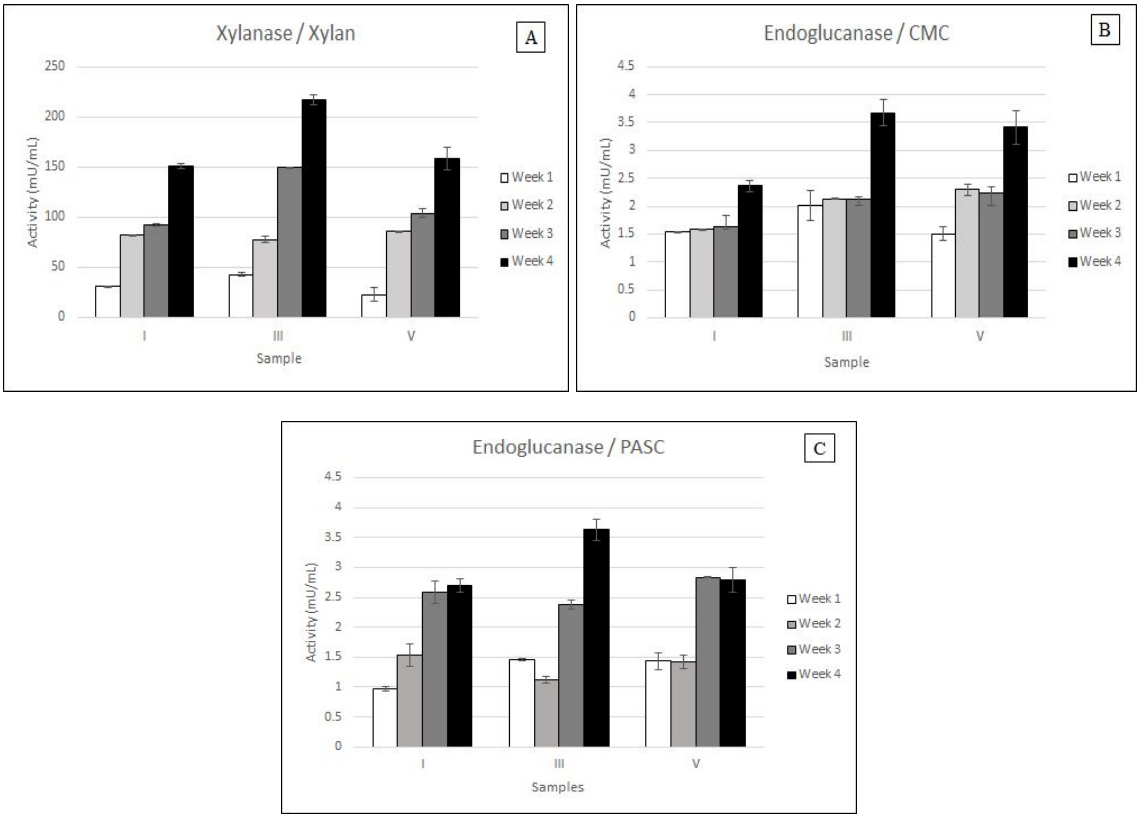


Figure 3.4(A-C): Enzyme activities in the supernatant of the APCS enrichment cultures at the end of each enrichment cycle. (A) Xylanase activity on xylan as substrate, (B) Endocellulase activity of CMC substrate, (C) Endocellulase activity on PASC as substrate. Each reaction was performed in triplicate, and standard deviations are indicated.

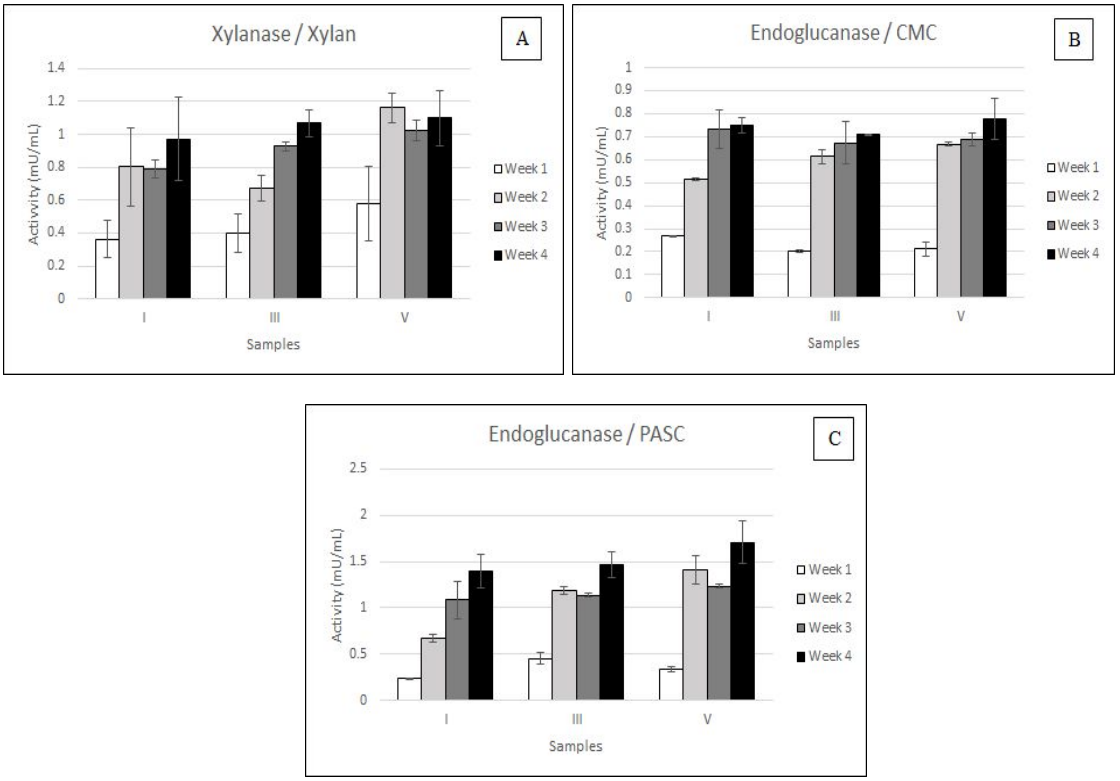


Figure 3.5(A-C): Enzyme activities in the supernatant of the enrichment cultures of PASC at the end of each enrichment cycle. (A) Xylanase activity on xylan as substrate, (B) Endocellulase activity of CMC substrate, (C) Endocellulase activity on PASC as substrate. Each reaction was performed in triplicate, and standard deviations are indicated.

Xylanase activity in the APCS enrichment cultures steadily increased with each enrichment cycle indicating a gradual adaptation of the microbial population towards more hemicellulolytic microorganisms. Xylanase activity levels were relatively high and reached over 200 mU/mL in the middle sample of the core. The cultures of this particular sample (III) had, in general, the highest xylanase activities, while there was no significant differentiation among the enrichment cultures of the other two cores. Cellulase activity in APCS enrichment cultures' supernatants was much lower compared to xylanase (for both substrates used). It is noteworthy that cellulase activity started to increase only after the 2nd or 3rd enrichment cycle. Since APCS is a substrate that contains both cellulose and hemicelluloses at almost equal quantities, this result probably reflects the fact hemicellulose adaptation of the microbial community is a faster (or easier) process. There were no marked differences in the final cellulase activity levels among the different core samples, with sample III, again showing a slightly higher cellulase concentration.

The enzyme activity profile in the PASC enrichment culture supernatants was quite different. Both xylanase and endocellulase activity levels were two and one order of magnitude lower than those of the corresponding APCS cultures representing a poor cellulolytic induction capacity for PASC. Interestingly though, the time pattern of cellulase activity practically leveled off, already from the first (samples II and III) or the second (sample I) enrichment cycle. This latter result reflects a low diversity of cellulose degraders among the sample's population, that is already established after one or two enrichment cycles.

3.3 SDS-PAGE gel analysis and zymogram of enrichment culture supernatants

Most probably the activities detected in the supernatant of the enrichment cultures are the cumulative activities of a number of enzymes. In order to verify the existence of hemicellulases and cellulases in the supernatants of the enrichment cultures, they were concentrated and run on SDS-PAGE gels supplemented with xylan (0.5%) and CMC (0.5%) substrates, respectively. Two gels were run for each protein sample; one was stained with Congo red stain to detect enzyme activity on the zymogram and identify the location of the corresponding bands. The second gel was stained with Coomassie brilliant blue stain and used to establish the relative abundance of the proteins that showed activity on the zymogram (Figures 3.6 and 3.7).

Xylanase activity was detected in all three APCS enrichment samples tested (Fig 3.6A). All of the samples (Fig. 3.6A, lanes E.C(I), E.C(III) and E.C(V)) showed clear zones, indicating the presence of xylan-degrading glycoside hydrolases of varying sizes ranging from ~37 to ~190 kDa. As shown in Fig. 3.6A, by comparing the molecular weights of the different bands, the conclusion that can be drawn is that all 3 layers include the same xylan degrading glycoside hydrolases. A probable difference is observed for sample E.C(III) that had an additional strong xylanase band at 37 kDa that was not present in the other two samples. PASC enrichment samples also showed significant xylanase activity in the corresponding zymograms (Figure 3.7A). A notable difference, in comparison with the APCS enrichment cultures, is the fact that the number of the xylanases detected in the PASC cultures was significantly lower, while their MWs were confined at values above 70 kDa.

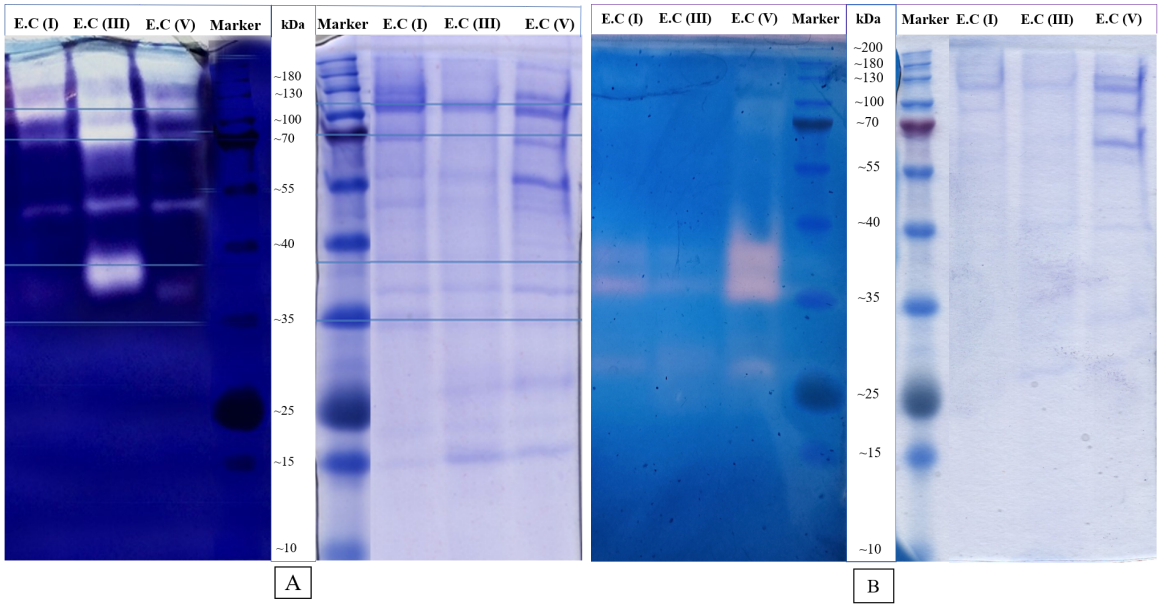


Figure 3.6: APCS enrichment culture's supernatant assayed for xylanase (A) and endocellulase (B) activity in 10% SDS-PAGE gels. The samples were prepared as described in section 2.8. All lanes were loaded with the same amount of protein.

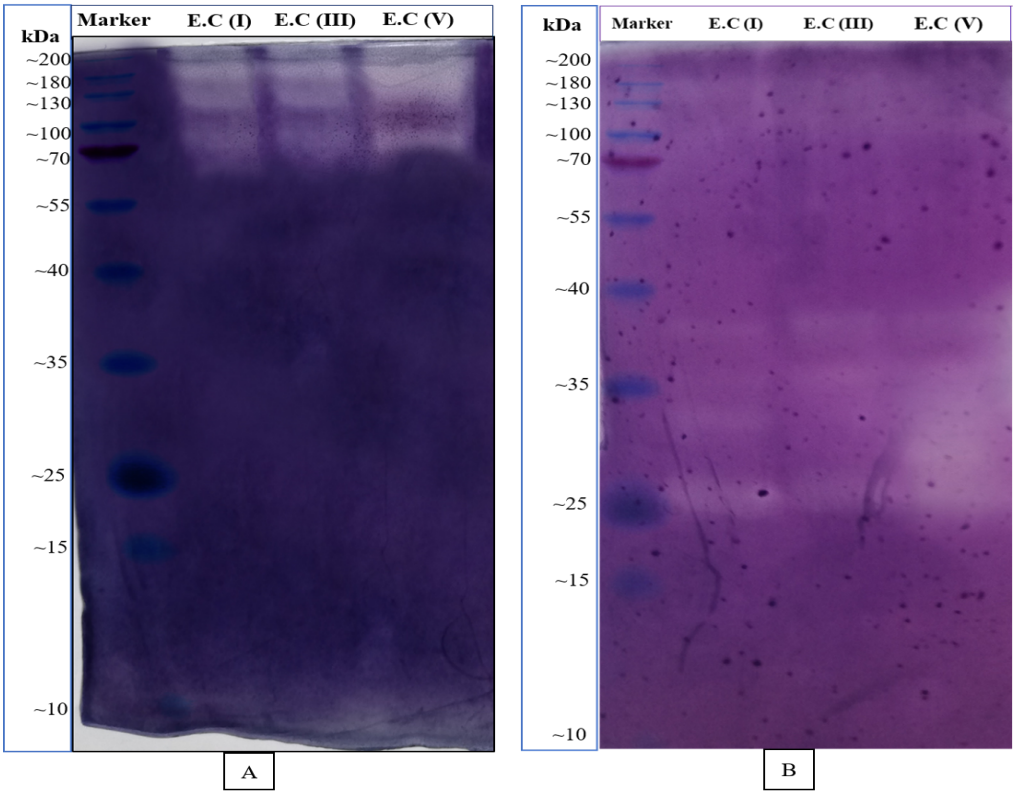


Figure 3.7: PASC enrichment culture's supernatant assayed for xylanase (A) and endocellulase (B) activity in 10% SDS-PAGE gels. The samples were prepared as described in section 2.8. All lanes were loaded with the same amount of protein.

Cellulase activity in APCS cultures was detected in all layer samples but with much lower diversity compared to the xylanases. Two bands at approx. 36 and 38 kDa were observed, but the corresponding CMC activity bands in sample E.C(III) were much more intense. In the latter sample, an additional band at 27 kDa was detected that was absent from the other two samples. A uniform CMCase activity pattern was observed in the samples of all PASC enrichment cultures' supernatants (Figure. 3.7B). A notable difference in comparison to the APCS cultures was the appearance of an additional clear CMC band at approx. 30 kDa.

3.4 Isolation of biomass-degrading strains

Whole broth from all final enrichment cultures was serially diluted in PBS up to the 10^{-6} and then 0.1 mL were spread on agar plates containing either APCS or PASC as a sole carbon and energy source. Figures 3.8 and 3.9 show the relative abundance (total cell number) of the aerobic bacterial population originated from the APCS and PASC enrichment cultures, respectively.

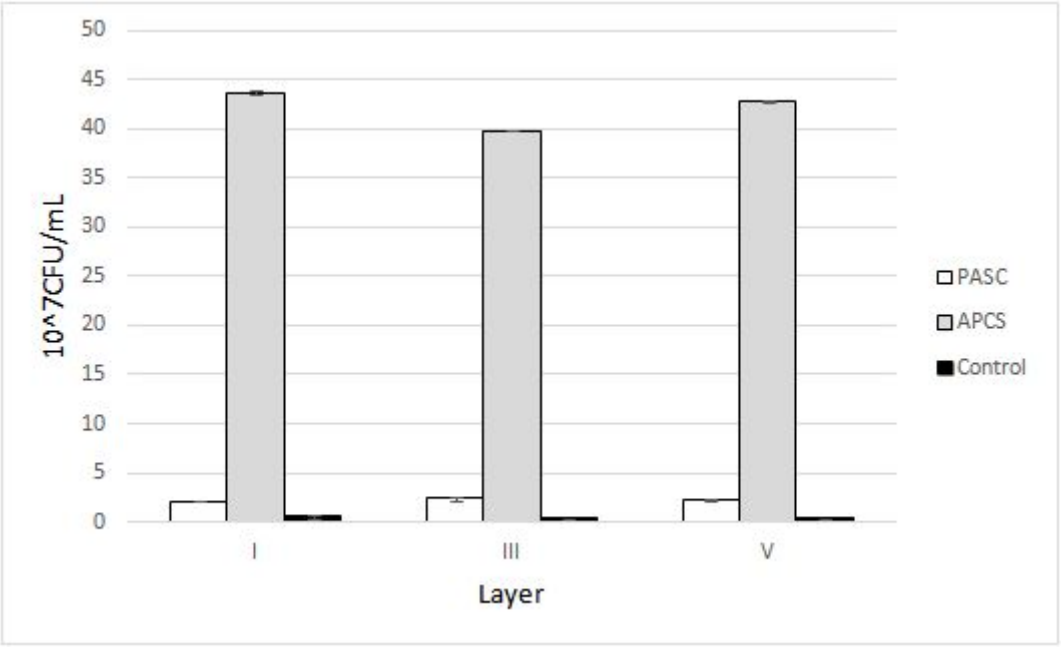


Figure 3.8: Relative abundance of isolates from the final APCS enrichments at the different core layers grown on plates with the two different carbon sources used (APCS and PASC). The control represents a plate without any carbon source added.

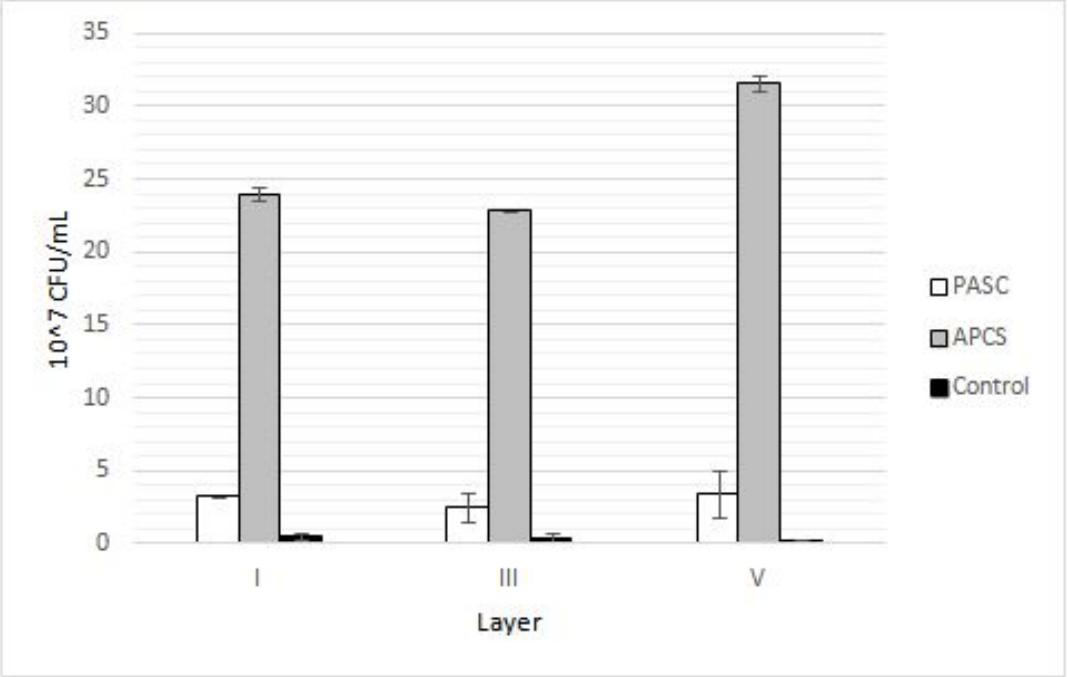


Figure 3.9: Relative abundance of isolates from the final PASC enrichments at the different core layers grown on plates with the two different carbon sources used (APCS and PASC). The control represents a plate without any carbon source added.

The microbial population grown in PASC plates was one order of magnitude lower than the corresponding population seen in APCS cultures, in all conditions. In line with the enzyme activity studies, the microbial population from APCS enrichment cultures was significantly higher than that of PASC enrichments when counted on APCS plates. When counted on PASC plates, there was not any detectable difference in the microbial population between the two enrichment populations. In addition, there were no significant variations among the aerobic microbial populations in the different core layers.

Based on the morphology of the colonies we isolated 23 distinct pure bacterial colonies for further investigation. Genomic DNA was extracted from all of them and was used as the template for PCR analysis the determination of the corresponding 16S rDNA. The PCR products (16S rRNA gene) were purified with NucleoSpin Gel and PCR Clean-up Kit (MACHEREY-NAGEL) according to the manufacturer's instructions. This step removes any possibility of the presence of compounds that could affect the purity of the 16S rRNA gene (e.g. proteins, extra primers, salts and dNTPs). The PCR products were sequenced by CeMIA SA (Larissa, Greece) by the SANGER method.

Analysis of all obtained PCR sequences revealed that they all group into only three different species namely ThP10, ThP11 and ThP12 (Figure 3.10).

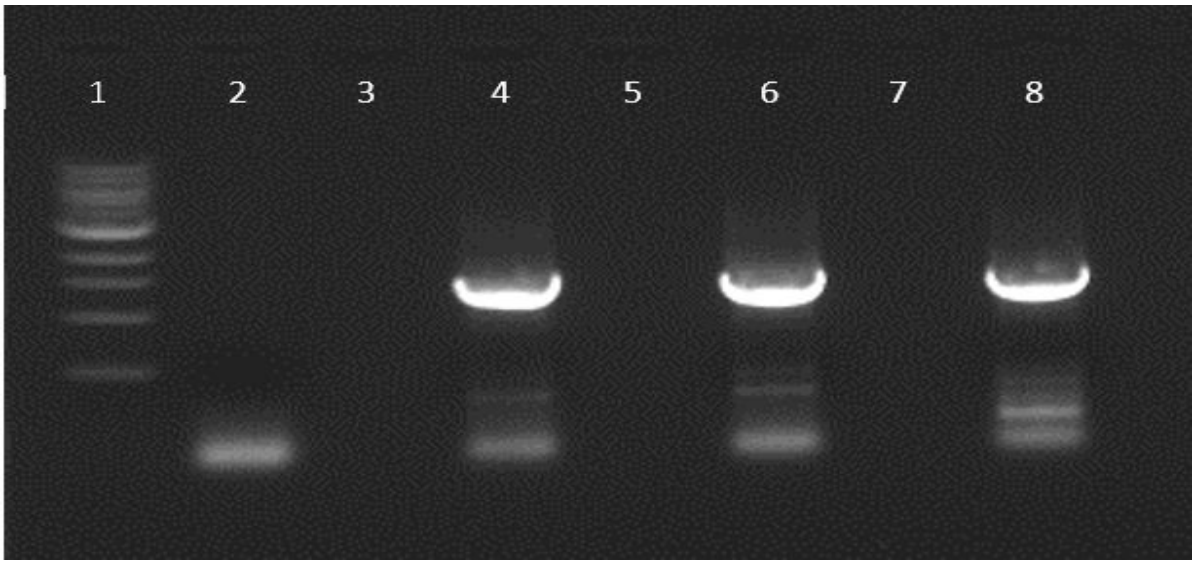
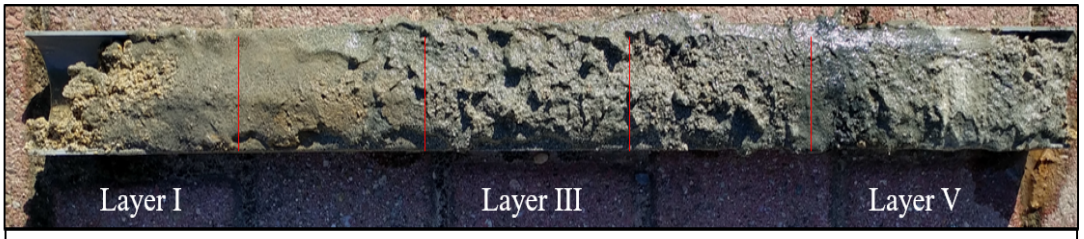


Figure 3.10: Agarose gel electrophoresis (1%) with ethidium bromide showing the amplification product of 16S rRNA gene with a size of 1.5 kb from the PCR involving the use of universal bacterial primers for the genomic DNA of ThP10 (lane 4), ThP11 (lanes 6) and ThP12 (lane 8). The ladder is lane 1 and the negative control is lane 2.

It is of great interest the fact that strains ThP10 and ThP11 are ubiquitous since they were isolated from both APCS and PASC enrichment cultures, and also from all three layers the sample core (Figure 3.11). Strain ThP12 though was also present in all layers but was isolated only from the enrichment cultures that contained APCS as carbon source.



	Layer I	Layer III	Layer V
PASC	ThP10 ThP11	ThP10 ThP11	ThP10 ThP11
APCS	ThP10 ThP11 ThP12	ThP10 ThP11 ThP12	ThP10 ThP11 ThP12

Figure 3.11: Relative localization of the three isolated strains with respect to the sample core layers and the carbon source used in the enrichment culture.

3.5 Identification and phylogenesis of bacterial isolates

The sequences of 16S rDNA of the three isolates were compared with the database at GenBank using BLAST-N search program in National Center Biotechnology Information (http://www.ncbi.nlm.nih.gov). The results are presented in Figures 3.12, 3.13 and 3.14, that follow.

GCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCCGCAAGACCGGGATAACTCCGGGAAAC
CGGAGCTAATACCGGATAACACCGAAGACCGCATGGTCTTCGGTTGAAAGGCGGCCTTTGGCTG
TCACTTGCGGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACG
ATGCGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTA
CGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCGACGCCGCGTGA
GCGAAGAAGGCCTTCGGGTCGTAAAGCTCTGTTGTGAGGGACGAAGGAGCGCCGTTTGAACAA
GGCGGCGCGGTGACGGTACCTCACGAGAAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTA
ATACGTAGGGGGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTCCTTAA
GTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGGACTTGAGTGC
AGGAGAGGAGAGCGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCA
GTGGCGAAGGCGGCTCTCTGGCCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACA
GGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGGTCACACC
CTTTAGTGCTGCAGCTAACGCGATAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACT
CAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGA
AGAACCTTACCAGGTCTTGACATCCCCTGACAACCCAAGAGATTGGGCGTTCCCCCTTCGGGGG
GACAGGGTGACAGGTGGTGCATGGTTGTCGTCAGCTcGTGTCGTGAGATGTTGGGTTAAGTCCC
GCAACGAGCGCAACCCTTGCCTCTAGTTGCCAGCATTCAGTTGGGCACTCTAGAGGGACTGCCG
GCGAAAAGTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTAC
ACACGTGCTACAATGGGCGGTACAAAGGGCTGCGAACCCGCGAGGGGGAGCG

Description	Max score	Total score	Query cover	E value	Ident	Accession
Geobacillus sp. strain Lhs-4 16S ribosomal RNA gene, partial sequence	2152	2152	100%	0.0	100%	MH368296.1
Geobacillus subterraneus strain KCTC 3922, complete genome	2152	19267	100%	0.0	100%	CP014342.1
Geobacillus sp. enrichment culture clone RA-555 16S ribosomal RNA gene, partial sequence	2152	2152	100%	0.0	100%	JQ083173.1
Geobacillus uzenensis partial 16S rRNA gene, strain LMG 24725	2152	2152	100%	0.0	100%	FN428634.1

Figure 3.12: 16S rRNA Sequence ThP10 isolate (length of sequence is 1193 bp) and 4 best hits from the Blast result.

GCCACGCCGCGTGGAGGATGAAGCCCTTCGGGGTGTAAACTCCTTTTCTGGGGgAAGAAATCCC
GACGCTGGTCGGGACTGACGGTACCCCAGGAATAAGCGCCGGCTAACTCCGTGCCAGCAGCCG
CGGTAATACGGAGGGCGCAAGCGTTGCCCGGAATCACTGGGTGTAAAGGGTGCGTAGGCGGGG
CTGTAAGTCAGAGGTGAAAGCCTCCGGCTCAACCGGAGAATTGCCTCTGATACTGCAGCTCTTG
AGTCCCGGAGAGGCCGCTGGAATTCCTGGTGTAGCGGTGAAATGCGTAGATATCAGGAGGAAC
ACCGGAGGCGAAGGCGGGCGGCTGGACGGGGACTGACGCTGAGGCACGAAAGCGTGGGGAGC
AAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGGATGCTCGGCGTTGCCGCCTTC
GGGCGGCAGTGCCTAAGCTAACGCGGTAAGCATCCCACCTGGGGAGTACGGCCGCAAGGTTGA
AACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGCTTAATTCGATGCTAC
GCGAGGAACCTTACCTGGGCTCGAACACCACCGGACAGCCCCCGAAAGGGGGTCTCCCGAAAG
GGCTGGTGGTGAGGTG

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhodothermus marinus gene for 16S ribosomal RNA, partial sequence, strain: CY14	1169	1169	100%	0.0	100%	AB862157.1
Rhodothermus marinus strain ir-1 16S ribosomal RNA gene, partial sequence	1169	1169	100%	0.0	100%	EU214606.1
Rhodothermus marinus strain WL 16S ribosomal RNA gene, partial sequence	1169	1169	100%	0.0	100%	DQ812981.1
Rhodothermus marinus strain mm-16 16S ribosomal RNA gene, partial sequence	1169	1169	100%	0.0	100%	EU652068.1

Figure 3.13: 16S rRNA Sequence ThP11 isolate (length of sequence is 648 bp) and 4 best hits from the Blast result.

GGGTGCGTAACGCGTAGGTAACCTGCCCTCGAGTGGGGGATAACTCCGGGAAACCGGGGCTAA
TACCGCATATTGTCCCGGGACCGCATGGCCCCGGGATGAAAGCCCTCTGGGCGCTCGAGGATGG
GCCTGCGTCGGATTAGCTAGTTGGTGGGGTAACGGCCCACCAAGGCGACGATCCGTAGCTGGTC
TGAGAGGACGACCAGCCACACTGGCACTGAGACACGGGCCAGACTACTACGGGAGGCAGCAGT
GGGGAATCTTGGGCAATGGGCGAAAGCCTGACCCAGCCACGCCGCGTGGAGGATGAAGCCCTT
CGGGGTGTAAACTCCTTTTCTGGGGGAAGAAATCCCGGCTATGGTCGGGACTGACGGTACCCCA
GGAATAAGCGCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGCGCAAGCGTTGCC
CGGAATCACTGGGTGTAAAGGGTGCGTAGGCGGGGCTGTAAGTCAGAGGTGAAAGCCTCCGGC
TCAACCGGAGAATTGCCTCTGATACTGCAGCTCTTGAGTCCCGGAGAGGCCGCTTCCCGGAGAG
GCCGCTGGAATTCCTGGTGTAGCGGTGAAATGCGTAGATATCAGGAGGAACACCGGAGGCGAA
GGCGGGCGGCTGGACGGGGACTGACGCTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAG
ATACCCTGGTAGTCCACGCCGTAAACGATGGATGCTCGGCGTTGCCGCCTTCGGGCGGCAGTGC
CTAAGCTAACGCGGTAAGCATCCCACCTGGGGAGTACGGCCGCAAGGTTGAAACTCAAAGGAA
TTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGCTTAATTCGATGCTACGCGAGGAACCTTA
CCTGGGCTCGAACACCACCGGACAGCCCCCGAAAGGGGGTTTCCCGAAAGGGCTGGTGGTGAG
GTGCTGCATGGCTGTCGTCAGCTCGTGCCGTGAGGTGTTGGGTTAAGTCCCGCAACGAGCGCAA
CCCCTATCGCCAGTTACCAGCGGGTAATGCCGGGGACTCTGGCGAGACTGCCTGCGCAAGCAGG
AGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGCCCAGGGCTGCACACGTGCTACA
ATGGCCGGTACAATGGGCAGCCACCCCGCGAGGGGGAGCGAATCCCTAAAGCCGGTCTCAGTT
CGGATTGGAGTCTGCAACTCGACTCCATGAAGCCGGAATCGCTAGTAATCGCGCATCAGCTACG
GCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCAACCCATGGGAGCCGGGGGCGC
CCGAAGTCCGTGACCT

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhodothermus obamensis strain JCM 9785T 16S ribosomal RNA gene, partial sequence	2362	2362	99%	0.0	99%	AF217493.1
Uncultured bacterium clone Z89 16S ribosomal RNA gene, partial sequence	2357	2357	99%	0.0	98%	JN091905.1
Rhodothermus marinus strain WL 16S ribosomal RNA gene, partial sequence	2357	2357	99%	0.0	98%	DQ812981.1
Rhodothermus marinus strain NR-29 16S ribosomal RNA gene, partial sequence	2357	2357	99%	0.0	98%	AF217498.1

Figure 3.14: 16S rRNA Sequence ThP12 isolate (length of sequence is 1348 bp) and 4 best hits from the Blast result.

On the basis of 16s rRNA gene sequencing, the isolates were identified as *Rhodothermus marinus* (ThP11 and ThP12) and *Geobacillus subterraneus* (ThP10). The evolutionary relationship between the organisms in the current study and other organisms is given in Fig. 3.15. As seen in Fig. 3.15 *Rhodothermus marinus* is clustered in a distinct and well-supported clade, together with other Bacteroidetes representatives. On the other

hand, *Geobacillus subterraneus* forms an external clade of the well supported Firmicutes cluster, exhibiting high affinities with *Bacillus*, *Lactobacillus*, as expected.

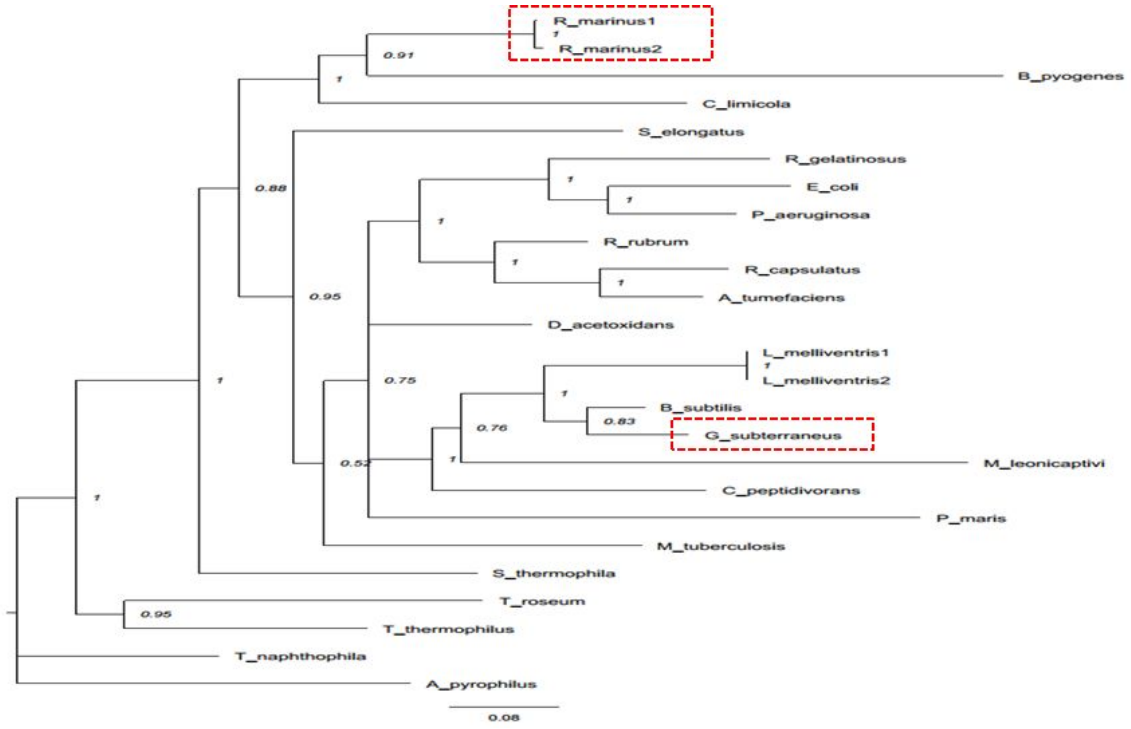


Figure 3.15: Bayesian phylogenetic tree (MCMC 10M generations) of the 16s rRNA gene of major bacterial phyla. Red arrows indicate the biomass degrading isolates. Numbers at nodes represent the posterior probability of each subclade and the scale bar corresponds to the number of substitutions per site

3.6 Glycoside hydrolase activities of the isolated strains

The cellulase and xylanase production capacity by the three isolates was examined by growing all three ThP strains on plates containing MS + 0.5% PASC, MS + 0.5% APCS, MS + 0.5% CMC and MS + 0.5% beechwood xylan, and staining the plates with Congo red (Figures 3.16 and 3.17). The presence of halos around the colonies after staining indicates zones of substrate clearance.

Bacterial colonies developed on all plates. ThP11 and ThP12 strains showed significant hydrolytic activity against all substrates (APCS, CMC, xylan and PASC), with both strains showing the largest clearance zone on APCS and the smallest on CMC. ThP10 strain did not show any apparent cellulolytic/hemicellulolytic activity. Halos were also

present on MS + 0.5% PASC, though they are barely visible in Fig. 3.17 due to the opaqueness of PASC (Figure 3.17).

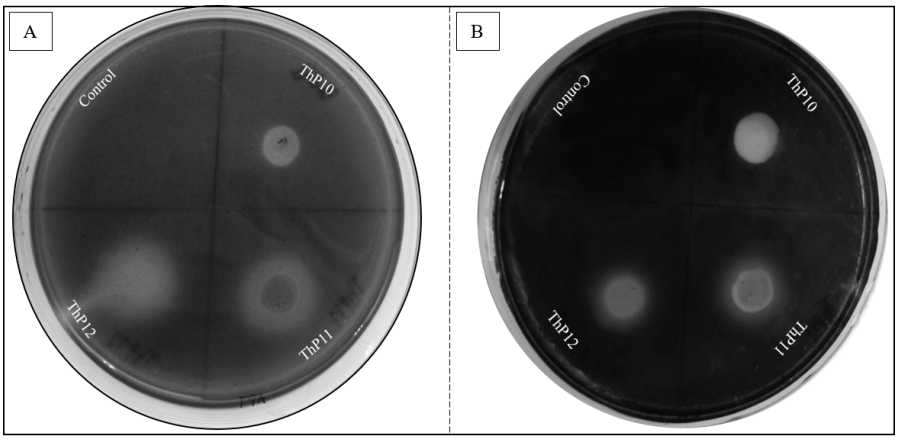


Figure 3.16: Congo red staining for hydrolytic activity in ThP strains plate cultures. The cultures were screened for xylanase activity after growth on A: MS + 0.5% APCS and B: MS + 0.5% beechwood xylan. Control indicated non inoculated areas.

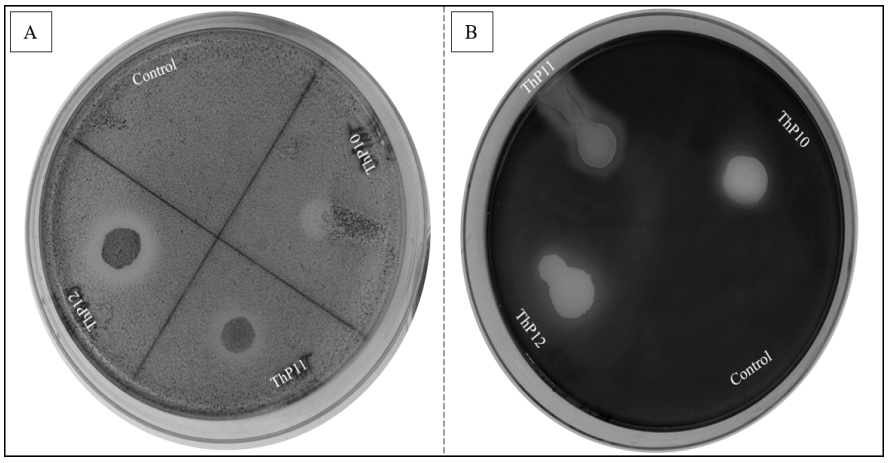


Figure 3.17: Congo red staining for hydrolytic activity in ThP strains plate cultures. The cultures were screened for xylanase activity after growth on A: MS + 0.5% APCS and B: MS + 0.5% beechwood xylan. Control indicated non inoculated areas.

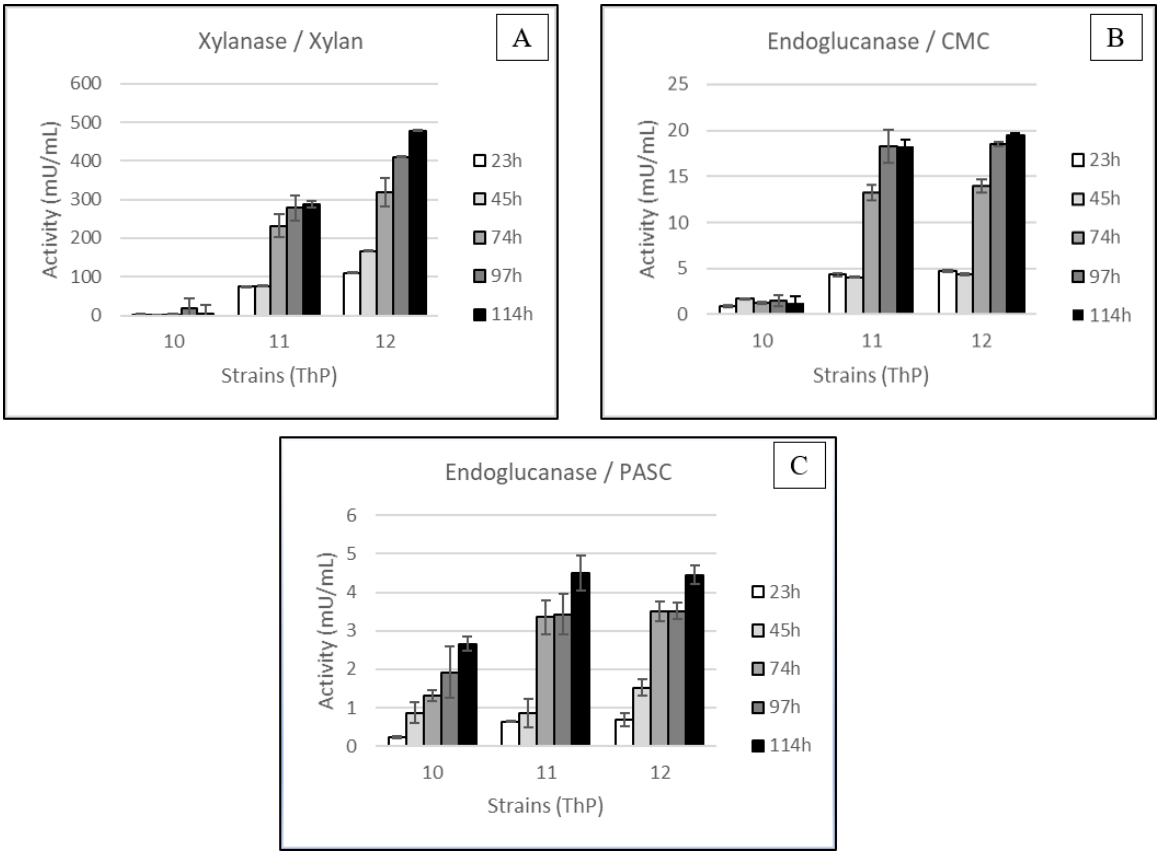


Figure 3.18: Enzyme activities in the supernatants of the liquid cultures with APCS as carbon source for the three strains (ThP10, 11, 12). All cultures were performed in triplicates and error bars represents the standard deviation.

The production of (hemi)cellulolytic activities of the three isolates was additionally examined in liquid cultures on MS medium supplemented with either 0.5% APCS or PASC. Endoglucanase (CMC and PASC as substrates) and xylanase (beechwood xylan as substrate) activity was determined in the corresponding culture supernatants at various time intervals (Figures 3.18 and 3.19).

Xylanase activity in APCS cultures was gradually increasing for strains ThP11 and ThP12, with the latter strain reaching the levels of 0.5 U/mL after 114 h of growth. ThP10 on the contrary, produced only traces of xylanase activity. The same pattern was observed for endocellulase activity when measured with CMC as a substrate. When endocellulase activity was measured with PASC (a more recalcitrant substrate) activity levels were lower, but detectable activity was also observed and for ThP10 strain (*G. subterraneus*).

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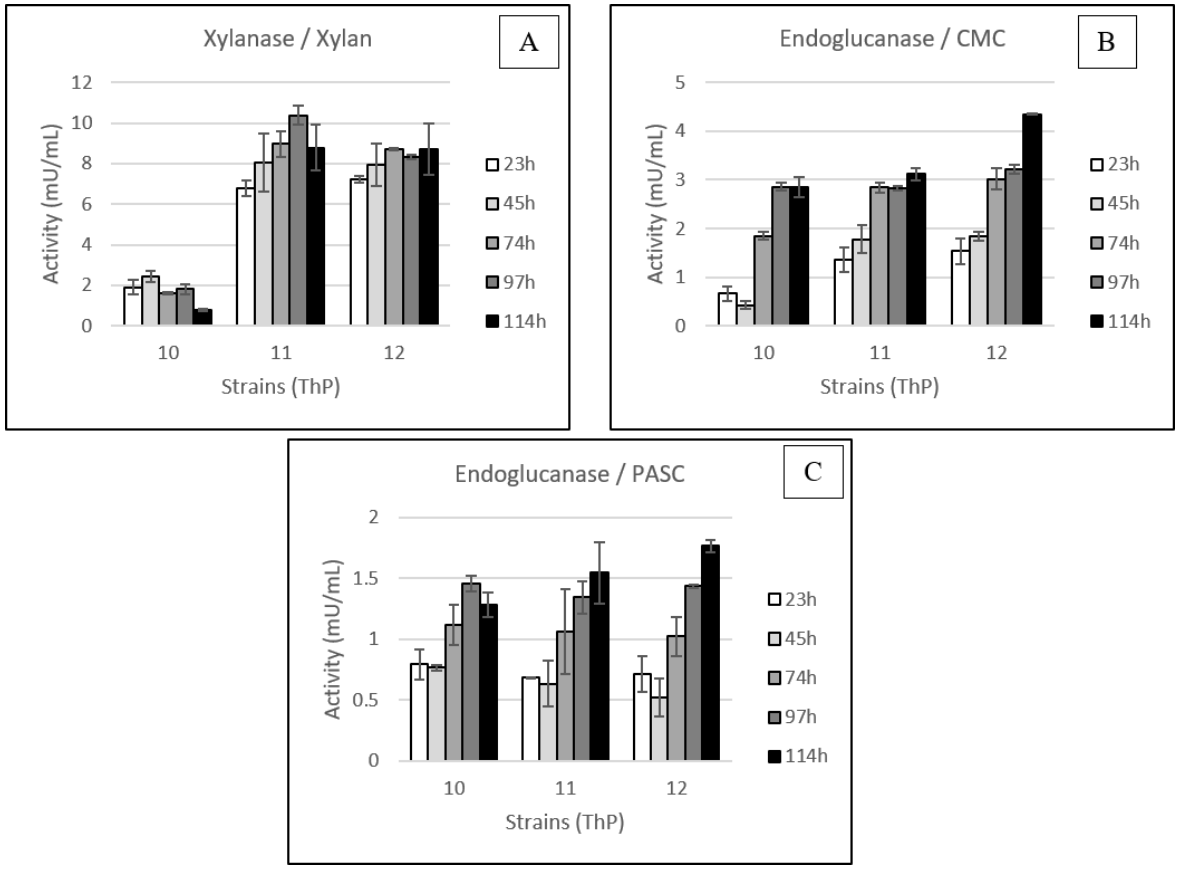

Figure 3.19: Enzyme activities in the supernatants of the liquid cultures with PASC as carbon source for the three strains (ThP10, 11, 12). All cultures were performed in triplicates and error bars represents the standard deviation.

Enzyme activities detected in the supernatants of the cultures with PASC as carbon source were significantly lower compared to the corresponding APCS cultures. Xylanase activities were two orders of magnitude lower, an expected result, since PASC is a pure cellulosic substrate. In this case though, all strains (including ThP10) produced almost equivalent endocellulase activities which were again higher, when determined with CMC as substrate.

The supernatants of 114 h of growth from all above cultures, were concentrated with ultrafiltration and were subjected to SDS-PAGE and zymogram analysis in 10% gels supplemented with 0.5% xylan or CMC. The corresponding zymograms are shown in Figures 3.20 and 3.21 for xylan and CMC, respectively.

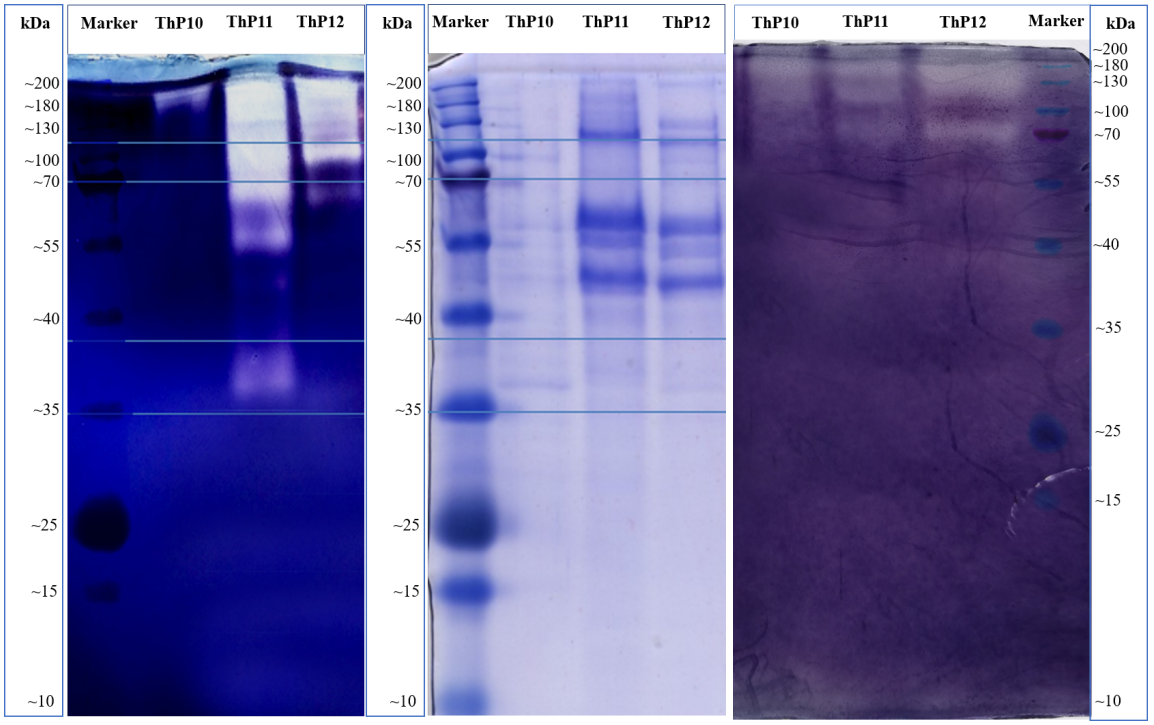

Figure 3.20: Zymogram analysis with xylan as substrate, of the culture supernatants after 114 h of growth for strains ThP10, 11 and 12, grown on xylan (left) and PASC (right) as sole carbon sources.

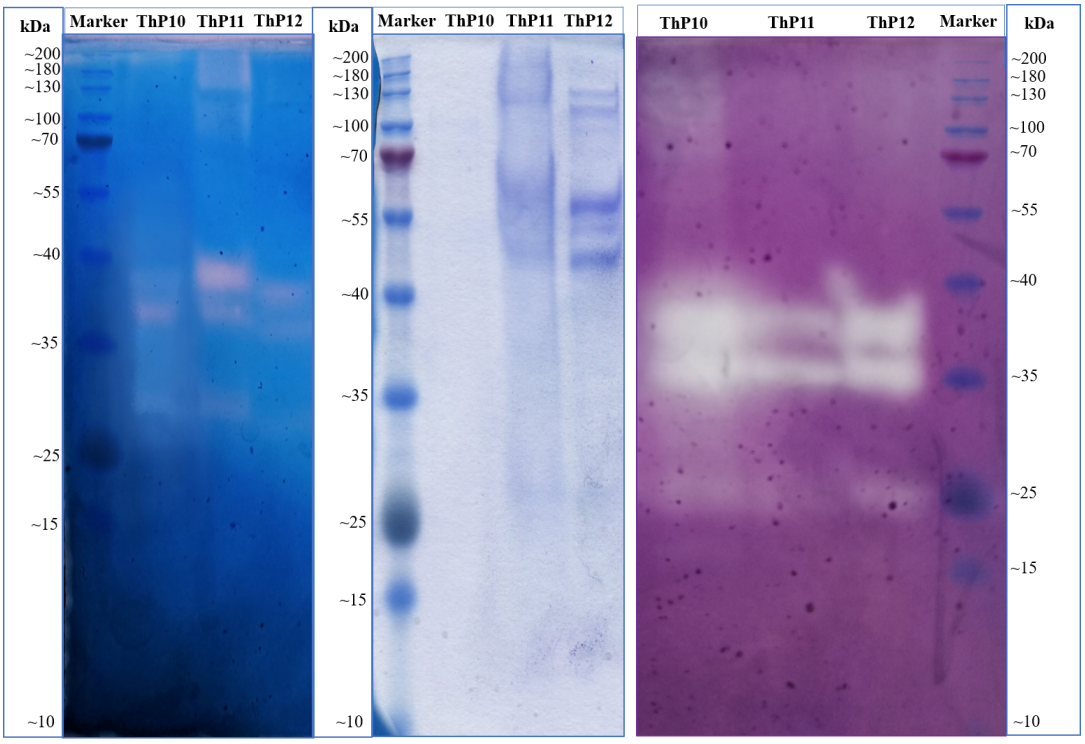

Figure 3.21: Zymogram analysis with CMC as substrate, of the culture supernatants for strains ThP10, 11 and 12, grown on xylan (left) and PASC (right) as sole carbon sources.

When grown on xylan, the three strains exhibited different xylanase activity patterns on the zymograms (Figure 3.20 - left). In accordance to the assayed extracellular activities, strain ThP10 (*G. subterraneus*) showed only a faint xylanase band over 150 kDa. The two *Rhodothermus marinus* strains (ThP11 and ThP12) on the contrary, had almost similar xylanase patterns which appeared more intense for ThP11. A unique xylanase activity band appeared only for ThP11 at approximately 37 kDa. All three strains showed also some residual endoglucanase zymogram activity when grow on xylan (Figure 3.21 - left). The corresponding activities where detected within a MW range between 28 and 39 kDa.

Upon growth on a pure cellulosic substrate, such as PASC (Figure 3.20 - right), a weak xylanase activity pattern was observed in the zymogram for all strains at the high molecular weight area. Compared to strain ThP10, strains ThP11 and ThP12 had an additional xylanase activity band at 70 kDa. Quite surprisingly, growth in PASC induced three distinct and intense endoglucanase activity bands at identical molecular weights, namely 25, 35 and 38 kDa.

Discussion

4. Discussion

Renewable energy has become a field of high interest over the past decade, and production of biofuels from lignocellulosic substrates has a particularly high potential as an alternative source of energy. Industrial deconstruction of biomass, however, is an onerous, exothermic process, the cost of which could be decreased significantly by the use of hyperthermophilic enzymes and microorganisms. Despite the numerous cellulases and hemicellulases that have been isolated and characterized by mesophilic and moderately thermophilic bacterial and fungal species, the number of such enzymes that are able to function at the demanding temperature range above 65 ^{o}C is very limited. Within this framework, the aim of this study was to isolate and evaluate novel thermophilic microorganisms with lignocellulose degrading capabilities from an environment where the temperatures remains above 60 ^{o}C all year round.

The area of Edipsos in Evia Island, Greece, satisfied the above thermal requirements since it is full of hot springs were the temperatures at their surface escapes ranges between 60 and 90 ^{o}C (reference). A second vital requirement for our specific site selection concerns the parallel long-term existence of degrading lignocellulosic biomass within the high temperature fields, preferably in the sediment of a creek formed by the outflowing waters of a thermal spring. Saranta Platania hydrothermal vent in the Edipsos area satisfies also this criterion. The area is described as a deep hydrothermal field, with several karstic groundwater escapes that culminate in a central creek flowing parallel to a public road. The north side of the creek is adjacent to heavy plant vegetation that in some points fell and degrade inside the creek, while the temperature of the waters at the date of sampling was recorded at 64.5 ^{o}C (October 20, 2017). The central area of the creek bed was chosen as the sampling point since, due to the semi-circular geometry of the bed, most of the sediment is being accumulated there.

The ~50 cm core that was collected was splitted into five parts in order to evaluate the microbial diversity as a function of depth. The top (I), middle (III) and bottom (V) section were used as inoculum for the enrichment cultures. Since we aimed to select and isolate for thermophilic microorganisms with ability ability to degrade lignocellulose, it was important to use (hemi)cellulose-rich substrates as sole carbon and energy sources, in

an effort to enrich the cultures with microorganism that have an efficient secretion system for the corresponding enzyme activities. A suitable substrate and correct pretreatment methods are required for efficient degradation (Chundawat et al. 2010), as different sets of enzymes are needed to degrade cellulosic materials to different extents (Sharma et al. 2016). A suitable pretreatment method should result in opening up the lignocellulose structure, to make it more accessible to enzymes (Ravindran and Jaiswal 2016; Zheng, Pan, and Zhang 2009). Since we wanted to evaluate both the cellulolytic as well as the hemicellulolytic microbial diversity of our original sediment samples, two different substrates were used as carbon and energy sources for the enrichments. The first was **Alkali Pretreated Corn Stover (APCS)**, an agricultural lignocellulosic residue rich in both cellulose and hemicelluloses (Kawaguchi et al. 2016; Chen et al. 2013). Alkali pretreatment has removed most of the lignin and loosen the cellulose - hemicelluloses structure, thus rendering both substrates more accessible to hydrolytic enzymes (Chen et al. 2013; Wang et al. 2014; Wilkinson, Smart, and Cook 2014). The second substrate was **Phosphoric Acid Swollen Cellulose (PASC)**, a pure cellulosic substrate based on crystalline cellulose (Ravindran and Jaiswal 2016), swollen by phosphoric acid in order to increase its susceptibility to enzymatic attack (Zhang et al. 2006).

Based on our results concerning the time profile of enzyme activities determined at the end of every enrichment culture, we may conclude that bacterial consortia, adapted to grow on one biomass feedstock, respond to perturbation with different biomass substrates by shifting its community composition and enzymatic activity profiles. The difference in the composition of the two carbon sources used in the enrichment cultures were clearly reflected in the final enzyme activities and the bacterial populations at the end of the four enrichment cycles. APCS is pretreated corn stover, where most of the lignin has been removed through the action of low NaOH concentrations, in the range of 100 mg per gram of untreated material (Chen et al. 2013; Kaar and Holtzapple 2000). As a result, hemicellulose and cellulose fibers remain exposed to the action of hemicellulases and cellulases produced by biomass degrading microorganisms in the samples. Due to the availability of both cellulose and hemicellulose in APCS, this substrate proved quite successful at least in promoting the growth of the microorganisms. This was also reflected in the corresponding enzyme activities as depicted in the zymograms. Specifically, APCS enrichment cultures showed very high xylanase activities that were gradually and

constantly increasing after each enrichment cycle. On the other hand, APCS failed to induce equally high cellulase activities despite the fact that several distinct cellulase specific bands appeared in the corresponding zymograms. This result clearly shows the establishment of a growing hemicellulolytic consortium induced by the presence of APCS.

The PASC enrichment results revealed a different phenotype. This substrate is much more difficult to digest since it is comprised of pure crystalline cellulose swelled by phosphoric acid in order to additionally acquire amorphous regions (Rastogi et al. 2009). As a result, we expect this substrate to sustain microbial communities able to digest only cellulose by excreting active cellulases. As expected, in PASC cultures, xylanase activities were very low compared to APCS cultures while significant cellulase activities were observed. One interesting result, is the fact that the cellulase activity levels in the supernatants practically leveled-off after the first enrichment, indicating a small proportion of cellulolytic microorganisms within the initial microbial populations.

In conclusion, the selected approach, of enriching our initial microbial communities by sequential growth on lignocellulosic substrates under thermophilic conditions, enabled to identify bacterial consortia that produced glycoside hydrolase enzymes. With appropriate selection of the lignocellulosic source this method is proves to be a useful tool for developing simplified biomass-degrading consortia tailored to deconstruct a designated feedstock under defined conditions, such as temperature or pH (Gladden et al. 2011).

A second important goal of our research was to isolate and the related biomass-deconstructing bacteria. Both enrichments converged to closely related consortia with similar microbial community compositions and levels of secreted glycoside hydrolase activities. Although we initially isolated over 20 pure individual bacterial colonies, 16S sequencing grouped them into only two microbial species. That of *Geobacillus subterraneous* and that of *Rhodothermus marinus*. Quite surprisingly, the *Rhodothermus marinus* 16S sequence was shared among two phenotypically different strains one of which lacked the characteristic pink *Rhodothermus* pigment color (Bjornsdottir et al. 2006) - (Figure 4.1). This result is very peculiar since color loss in *Rhodothermus marinus* strains has only been reported only after targeted deletion of the corresponding carotenoid genes (Bjornsdottir et al. 2011). Both of these groups have cultured relatives known to degrade biomass and are a source of many glycoside hydrolases. It was interesting that only this

two genera dominated the examined biomass degrading habitat. To the best of our knowledge, *Rhodothermus marinus* is being reported for the first time from a Greek habitat.

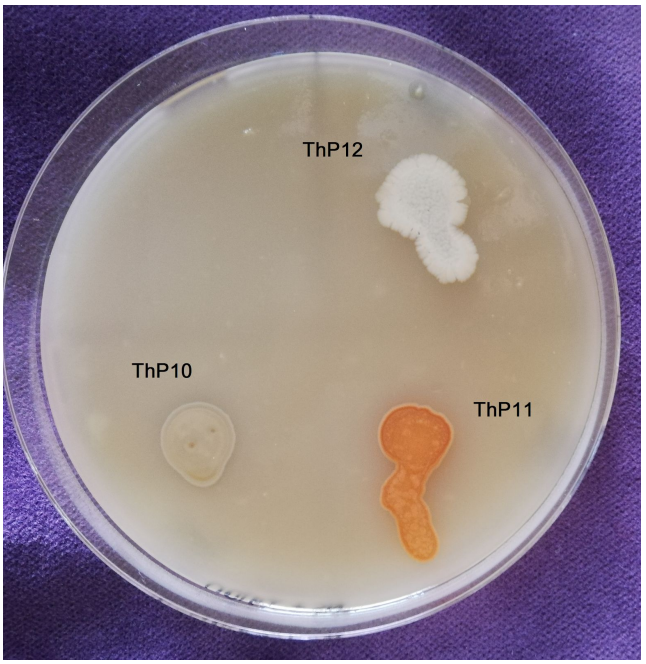

Figure 4.1: Colonies of the three isolates from Edipsos area. Based on their 16S gene sequence ThP10 is a *Geobacillus subterraneus* and ThP11 and ThP12 are *Rhodothermus marinus* strains.

Isolates of *R. marinus* have previously been demonstrated to secrete endoglucanases when grown in culture with carboxymethylcellulose, a derivatized form of cellulose that is soluble (Holst, Eggertsson, and Palsdottir 1996). However, there are no reports of isolated *R. marinus* growing on microcrystalline cellulose or other recalcitrant forms of cellulose (Bjornsdottir et al. 2006).

Regarding the *Geobacillus* isolates, in a recent analysis De Maayer, Brumm, Mead, and Cowan (2014) (De Maayer et al. 2014) have shown that most of the sequenced and partially sequenced strains of *Geobacillus* spp. have a range of hemicellulose utilization genes which makes this genus mainly hemicellulolytic. There is no evidence for true cellulolytic activity (ability to degrade crystalline cellulose) in *Geobacillus* spp., although extracellular enzymes showing endoglucanase activity (probably low specificity GH5 (Aspeborg et al. 2012) has been detected.

All three strains were able to grow and produce enzymes in liquid cultures with APCS or PASC as carbon sources. APCS again, was a particularly good substrate since it induced relatively high xylanase and cellulase activity in all strains, even though the

induction levels for the *Geobacillus* ThP10 were significantly lower than those of *Rhodothermus marinus* ThP11 and 12. In PASC, all strains produced very small extracellular xylanase activities and almost equal amounts of cellulolytic activity. These results indicate the existence of common induction pattern in all strains.

Of particular interest are the corresponding zymograms of the culture supernatants, that we present in parallel with the corresponding zymograms of the final enrichment culture supernatants in Figures 4.2 and 4.3, respectively.

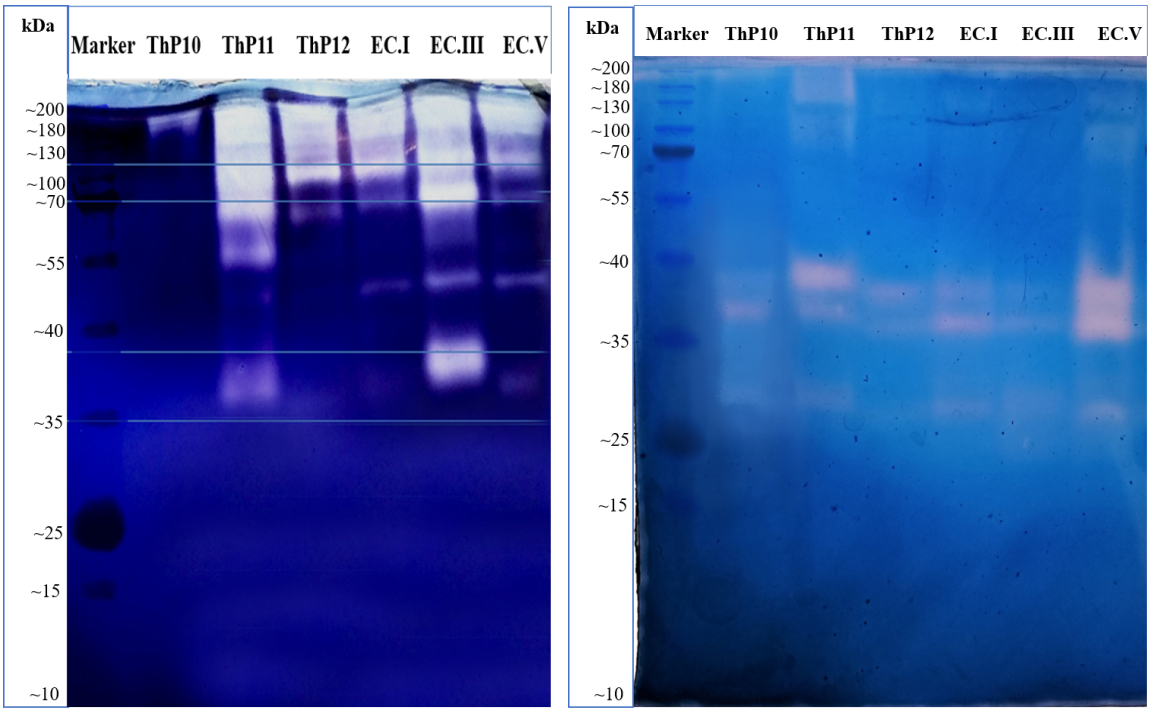

Figure 4.2: Comparative zymogram analysis of culture supernatants of the three strains grown in APCS liquid cultures (ThP10, 11, 12) with the culture supernatants of the APCS enrichments of the three samples (EC.I, EC.III, EC.V). Left: Xylan as substrate in the gel, Right: CMC as substrate in the gel.

Considering APCS as a global substrate for xylanase (Figure 4.2, left) and cellulase (Figure 4.2, right) production, we conclude that all xylanase activity bands in the final enrichment cultures seem to originate from the three isolates and especially the two *Rhodothermus marinus* strains (ThP11 and Thp12). One exception is a 55 kDa xylanase activity band in R. marinus ThP11 that did not apparently seemed to be produced during growth of the microorganism in the enrichment process. Quite surprisingly, the three cellulase activity bands are present in all APCS culture supernatants. Although such an observation is

expected for ThP11 and ThP12 (*R. marinus* strains) the existence of these same bands in ThP10 isolate (*G. subterraneus*) is somewhat unexpected, but may very well though reflect an incident of horizontal transfer among these two species that dominate this oligotrophic thermophilic environment.

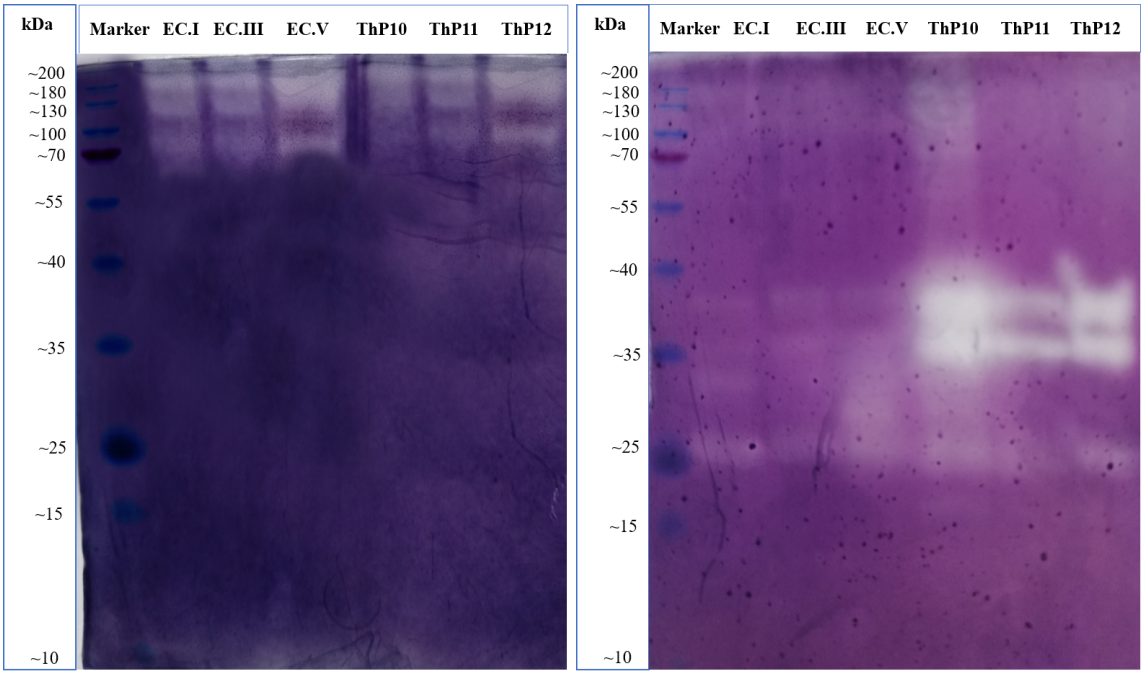

Figure 4.3: Comparative zymogram analysis of culture supernatants of the three strains grown in PASC liquid cultures (ThP10, 11, 12) with the culture supernatants of the PASC enrichments of the three samples (EC.I, EC.III, EC.V). Left: Xylan as substrate in the gel, Right: CMC as substrate in the gel.

Analogous results are obtained during the evaluation of all zymograms from PASC cultures (Figure 4.3). The same three cellulase bands appeared in both the zymograms of all three enrichments as well as in the zymograms of the PASC culture supernatants. A minor additional cellulase activity band at 33 kDa appeared in the zymograms of the enrichment culture supernatants. This band could probably have originated from an additional strain that was not able to be isolated.

In conclusion, our study was able to characterize the culturable aerobic thermophilic microbial diversity related to biomass degradation of the Saranta Platania thermal springs field. Using two rationally selected lignocellulosic substrates, microbial community analysis has demonstrated that the corresponding microbial consortia are composed of a few dominant phylotypes that consist of both well-studied and novel

biomass-deconstructing bacteria. These consortia are amenable to detailed genomic and proteomic investigations which will reveal the suite of bacterial glycoside hydrolases used to deconstruct complex biomass. This approach will allow the characterization of new bacterial glycoside hydrolases and accessory proteins from uncultivated organisms that will enhance biomass deconstruction.

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