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Ancestral sequence reconstruction for protein engineering: improving celulases for biomass hydrolysis

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- PhD Thesis -

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by

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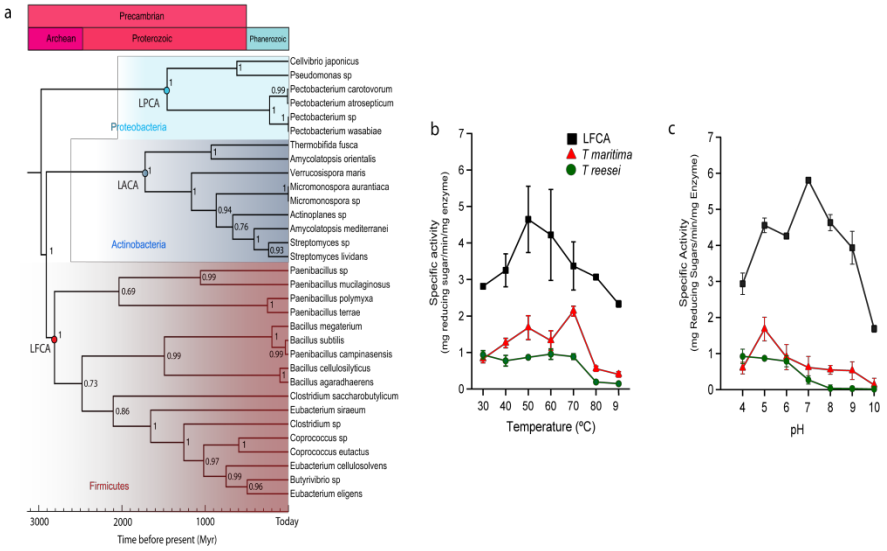
Laburpena

Aplikazio industrialetan entzimak erabiltzea beharrezkoa da proteina-ingeniaritzaren hainbat teknika hobetzeko. Beharrezkoa da baldintza industrial askotan entzimak desaktibatzen dituzten muturreko kondizioak erabiltzen direlako. Kasu askotan, muturreko baldintzak jasateko gai diren entzimak erabiliz emaitza onak lortzen badira ere, beste kasu batzuetan, horiek hobetzea ere ezinbesteko bihurtzen da. Azken urteotan, entzima horiek hobetzeko esfortzu ugari egin da. Zuzendutako in vitro eboluzioa izan da, muturreko baldintza horiek lortzea helburu, bioteknologiak gehien erabili duen metodoa. Protokolo hau, ordea, oso garestia da; izan ere, mutanteen liburutegi bat sortu behar da lehenbizi, horren ondorioz aldagai ugari sortzen dira, eta hau guztia, gainera, esperimentalki egin beharreko zerbait da. Are gehiago, sortutako mutante guztiak probatzeko ezintasuna dela eta, prozesu honek ez du bermatzen entzima onenak hautatzen direla. Beraz, prozesua neketsua eta garestia izateaz gain, ez da oso eraginkorra kasu gehienetan. Hau horrela, bada azken urteotan erabiltzen hasi den beste teknika bat: antzinako sekuentzien berreraikuntza.

Horretarako, antzinako proteinen berpizkundearen teknika berria erabiltzen da. Teknika horretan, organismo modernoen entzimen sekuentziak erabiltzen dira eta horien filogenia harremanak aztertzen dira. Zuhaitz filogenetiko bat lortzen den unean teknika estatistikoak aplikatu daitezke arbasoen sekuentzia lortzeko eta laborategian aztertzeko. Ikerketa gutxi egin dira, gaur egun arte, aipatutako teknikak izan ditzakeen aplikazio industriala eta biomedikoa aztertzeko. Berpiztutako proteinek egonkortasun termikoa, kimikoa edo zinetikoa duten propietate bereziak dituzte. Propietate horien hobekuntzak arrazoi bat dauka: gure planetak historian zehar izan dituen ingurumen-baldintza ezberdinak. Kondizio horietan bizi diren organismoen sekuentzia proteinak besterik ez dira aurkitu. Errendimendu handiak espero dira antzinako entzimak nahasiak direla erakusten baitute, hautakortasun txikiagoarekin lan egiten dute, eta beraz, substratu mota desberdinetarako eraginkorragoak dira.

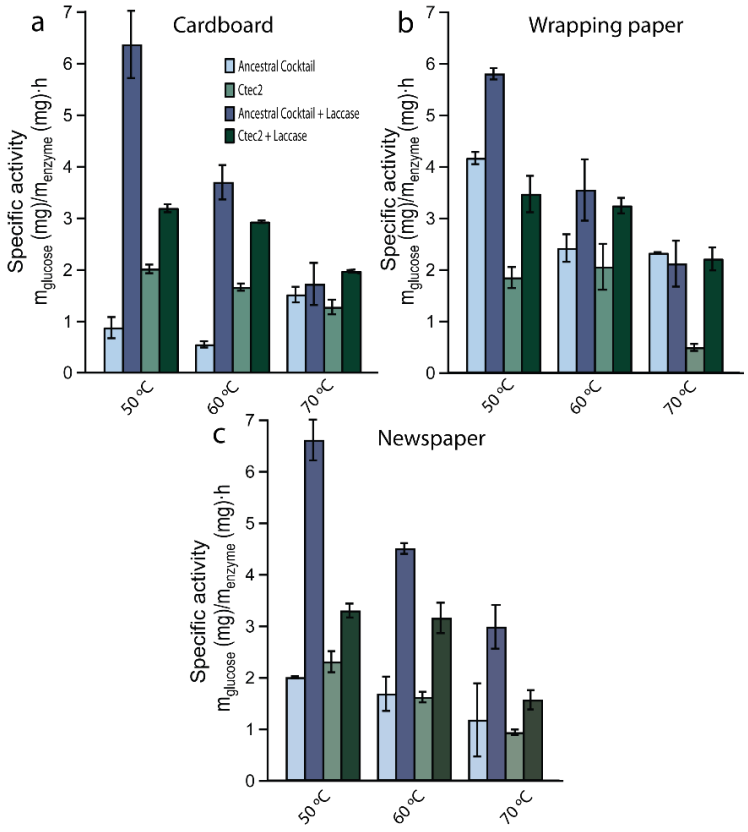
Zelulasen eraginkortasuna oso mugatua da 60 ° C-tik gorako tenperatura eta muturreko pH-etan. Hori dela eta, propietate horiek hobetzea da bioteknologia arloko ikerkuntzaren helburua. Zelulasak zelulosa hidrolizatzeko arduradun diren entzima talde baten izena da. Azaldutako teknika erabilita 3000 milioi urte arteko zelulasak berpiztu ditut. Egin ditudan azterketa guztietan antzinako zelulasen errendimendua neurtu ditut, industrian erabiltzen diren entzima komertzial batzuekin alderatuta. Neurtutako baldintza guztietan berpiztutako entzimen aktibitatea komertzialena baino handiagoa izan da, hala ere, tenperatura eta pH altuetan lortu ditut desberdintasun nabarienak. Hurrengo irudian (**1go irudia**) endozelulasa entzimarentzat egindako azterketa ikus daiteke.



1 irudia. Endozelulazarentzat egindako azterketa. *a) Endozelulasaren kronograma edo arbola filogenetikoa. Bakterioen hiru erreinutako (aktinobakteria, firmikutes eta proteobakteria) 32 sekuentzia erabilita egin da zuhaitza. Bertako arbaso bat hartuta egin da berpizketa, b) Tenperaturaren menpe (30-90) pH 4.8-an egindako aktibitate espezifikoaren azterketa c) pH-rekiko (4-10) menpekotasun diagrama 50ºC-tan eginikoa.*

Irudian ikus daitekeenez, baldintza guztietan emaitza hobeak lortu dira antzinako entzima erabilita. Horretaz gain, antzinako koktelaren jardueraren azterketak egin dira (hiru zelulasa: endozelulasa, exozelulasa eta beta-glukosidasa), hainbat tenperaturatan substratu lignozelulosikoen degradazioa egin dut.

Substratu idealak (CMC eta iragaz papera) erabiltzeaz gain, material lignozelulosiko desberdinekin azterketak egin ditut, hala nola, kartoia, batzeko papera eta egunkari-papera. Jarraian dagoen irudian (**2.irudia**) ikus daiteke.



2 irudia. Zelulasa koktelarentzat eginiko azterketa *Koktel zaharreko jarduerak (CKA), koktel komertziala (CKC), koktel zaharrean T. pubescens laccase (CKA + L) eta komertzial koktelaren presentzia T. pubescens laccase (CKC + L) ikusgai 50-70 ºC-ko tenperaturan eta pH 4.8. Azterketak hiru substratu desberdinetan burutu ziren: kartoia (a), egunkaria (b) eta paperean (c).*

Azterketa guztietan antzinako zelulazen errendimendu hobea neurtu da, bai tenperatura altuetan, bai eta pH ezberdinetan ere. Biokonbertsio prozesuen (pH azidoa eta tenperatura altua) muturreko baldintzei aurre egiteko gaitasun hobea dutenaren

hipotesia frogatu da, beraz. Horrela, prozesuaren kostua murriztu daiteke eta bere bideragarritasun industriala hobetuko litzateke. Baina ez hori bakarrik, gure antzinako koktelak material lignozelulosikoa degradatzeko erabiltzen diren beste entzima batzuekin daukan sinergia ere erakutsi dugu. Izan ere, material lignozelulosikoa zelulosaz gain, lignina eta hemizelulosaz osatua dago. Horiek hidrolizatzeko asmoz, lakasak eta xilanasak erabiltzen dira. Guk, azterketa honen bitartez, frogatu dugu gure entzimek beraien kabuz lan egiteaz gain, beste entzima horiekin lan egitean errendimendua asko hobetzen dela.

Zelulasa disolbagarriez gain, bakterioek sortzen duten zelulasa makromolekular konplexu batek ere industriaren interes potentziala izan lezake, konplexutasun horren egonkortasunagatik. Bakterioek naturalki sortzen dituzten konplexu horiek zelulosoma deitzen dira. Gaur egun, ordea, zelulosoma sintetikoak ere diseinatu daitezke, zelulasa desberdinen osagai indibidualak konbinatzen zelulosoma-itxurako konplexuak ekoiztuz. Gure zelulasak erabiliz antzinako zelulosoma (endozelulasa, exozelulasa eta beta-glukosidasa) berri bat diseinatu eta sortu nahi da, tenperatura altuko, estres mekanikoko eta pH azidoaren inguruko industria-inguruneetan funtziona dezan.

Material lignozelulosikoa lehengai ugaria da lurrean. Bioetanola ekoizteko erabiltzen da, baina bioetanolaren produkzioan entzimak muga dira oraindik. Zelulasa entzimek, euren gaitasunak mugatzen dituzten kondizio industrialetan, eraginkortasun handiko lanak behar dituzte. Bioetanolaren kasua da gure entzimak erabiliz hobetu litezkeen prozesuen adibideetako bat.

Summary

The use of enzymes in industrial applications requires an improvement thereof with various techniques of protein engineering. This is necessary since in many industrial conditions there are extreme conditions that inactivate enzymes. In many cases the use of enzymes from extremophiles produce satisfactory results, but there are situations in which even these extremophile enzymes should be improved. The method most used by biotech companies is directed evolution in vitro. However, this protocol is very expensive since the generation of mutant library and subsequent screening has to be done experimentally and often, thousands of variants are created. In addition, due to the inability to test all variants generated, this process does not guarantee that the best enzymes are selected. So there is a need of another technique for improving enzymes.

For this purpose the novel technique of the resurrection of ancient proteins is used. In this technique, sequences of enzymes from modern organisms are used and their phylogenetic relationship is studied. Once a phylogenetic tree is obtained, statistical techniques can be applied to obtain the sequence of ancestors and those can be studied in the laboratory. Nowadays, there are relatively few studies of resurrection of ancient proteins and possible industrial and biomedical applications. Ancestral proteins and enzymes often show exceptional properties related to its thermal stability, chemical or kinetic, in addition to its activity. This is due to the fact that our planet has been subjected to all

kinds of environmental conditions throughout history. Just the protein sequences of organisms that lived in those conditions have to be found. High activities are expected since it has been shown that ancestral enzymes are promiscuous, they are able to work with lower selectivity and therefore more effective with different types of substrates.

Cellulases enzymes are required to work with high efficiency under industrial conditions that limit their capabilities. Cellulase is the name of a group of enzymes responsible for the hydrolization of cellulose. We have brought back to life ancestral cellulases up to 3000 million years. In all the assays we have measured a better performance of the ancestral cellulases, both in high temperatures and in a range of pH. Cellulases are quite limited in their properties being difficult to use at temperatures above 60 ° C and extreme pH, improving these properties is a goal in biotechnology research.

Apart from soluble cellulases, it has been suggested that bacterial cellulases forming the macromolecular cellulosome complex may be of potential interest for industry due to the increased stability and cellulase activity of the complex. Efforts to develop cellulosomes for industrial applications have been focused on so-called Designer Cellulosomes, where individual components of different cellulosomes are combined to produce defined cellulosome-like complexes. Another intention is to redesign and create a new cellulosome composed of ancestral cellulases (endoglucanase, exoglucanase and beta-glucosidase) to function in the relevant industrial environment of high temperature, mechanical stress and acidic pH.

Chapter 1: Introduction

Enzymes are widely used in the chemical and biotechnological industry, being essential biocatalysts in diverse areas such as bioenergy, cosmetics, food industry, detergents and textile [1]. Natural enzymes are suited for their biological function, but when these enzymes are used as industrial catalysts they present significant limitations for the specific requirements for industrial application. In the past decades, research has been focused on the improvement of enzymes properties, paying special attention to the enhancement of the thermal stability, the increase of the specific activity, the improvement of their substrate promiscuity and the increase of the production rate [2-8]. In some cases the use of enzymes from extremophiles produce satisfactory results, but there are situations in which even these extremophile enzymes should be improved. Such non-natural conditions often result in poor enzyme activity, or complete deactivation due to denaturation or chemical modifications. Developments in protein engineering over the past ten years have enabled enzymes to be evolved in vitro for properties that favor the required process conditions, and also to obtain enzyme variants with altered substrate specificity or enantioselectivity [9, 10].

In order to obtain enhanced enzymes with improved efficiency several methods such as directed mutagenesis, DNA shuffling, error-prone PCR, directed evolution and rational design have been implemented [3, 5, 8, 11, 12]. The most classical method in enzyme engineering is rational design, which involves site directed mutagenesis introducing a specific amino acid into a target gene [13, 14]. The amino acid is chosen considering the structure and function of the enzyme. Its major drawback is that detailed structural knowledge of an enzyme is often unavailable. In addition, rational design does not allow the introduction of numerous mutations without perturbing the structure or function of the protein. DNA shuffling consists on the fragmentation of gene parents, followed by some PCR (polymerase chain reaction) cycles to obtain different mutants of a gene. Regarding error-prone PCR, this is a method by which random mutants maybe inserted into any piece of DNA. The technique is based on the well-founded PCR [15]. Nevertheless, the method that the biotechnological companies use the most is directed evolution in vitro [16, 17]. This is a method used in protein engineering that mimics the process of natural selection to evolve proteins or nucleic acids toward a user-defined condition. Using this method, thousands of variants of the enzyme of interest are created introducing random mutations, generating a library of mutants. These mutant enzymes are exposed to the desired conditions and the variants that best perform under these conditions are identified and selected for commercial exploitation. The likelihood of success in a directed evolution experiment is directly related to the total library size, as evaluating more mutants increases the chances of finding one with the desired properties [18]. However, this protocol is very expensive since the generation of a mutant library and subsequent screening has to be done experimentally and often, thousands of variants are created.

In addition, due to the inability to test all variants generated, this process does not guarantee that the best enzymes are selected [19].

Despite these advances, the limitation of these engineered enzymes is still a serious barrier in the chemical industry. Nowadays no methodology seems to be able to enhance, for instance, the temperature and pH operability, the expression level or the specific activity of enzymes, all at once. Developing a strategy capable of vastly improving the catalytic properties of enzymes in a cost efficient manner may revolutionize the biotechnology and chemical industries. In the past years, the so-called Ancestral Sequence Reconstruction technique (ASR) has been used to study the evolution of genes, proteins and enzymes [20-23]. Surprisingly, reconstructed ancestral enzymes displayed enhanced thermal stability, better pH response, improved activity and expression level, chemical promiscuity and in some cases, all of this at once [21, 22, 24-28] [29]. This technique is based on the evolution theory, which states that groups of organisms change over time so that descendants differ structurally and functionally from their ancestor. Today, combined with biophysical and biochemical state-of-the-art techniques, ancestral sequence reconstruction allows to study and compare features of extinct proteins and genes that are otherwise inaccessible [20-22, 30].

Phylogenetic methods applied to genomic information have made it possible to establish evolutionary relationships among different living organisms, including the possibility of inferring the putative sequences of the genes of their already extinct ancestors [31, 32]. Since Charles Robert Darwin (**Fig 1.1**) sketched an evolutionary tree in 1837 for the first time, to the current Time Tree of Life for all known living organisms a lot has been learnt in organismal and molecular evolution.

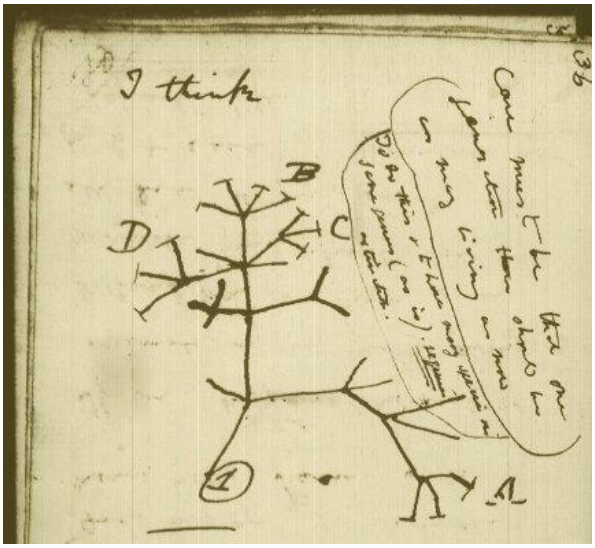


Figure 1.1. First sketch of an evolutionary tree in the notebook of Charles R. Darwin "Transmutation of Species B".

The two primary components of evolutionary history are the relationship of organisms (phylogeny) and their times of divergence which together form a phylogenetic tree scaled to time, i.e., a chronogram. Therefore, the two main goals of phylogenetic analysis are to reconstruct the correct genealogical relationships between organisms and estimate the time of divergence between organisms since they shared a common ancestor.

Ancestral Sequence Reconstruction allows determining the sequences of genes and proteins of the ancestor of modern species. The process comprises various steps (**Fig 1.2**). Protein or gene sequences of different species are acquired from the available databases. These sequences are then processed by bioinformatics tools to infer the phylogenetic tree. The genetic

code of the ancestor is obtained from the inferred tree and the protein can be resurrected in the laboratory using molecular cloning. This methodology is deeply explained in Chapter 3.

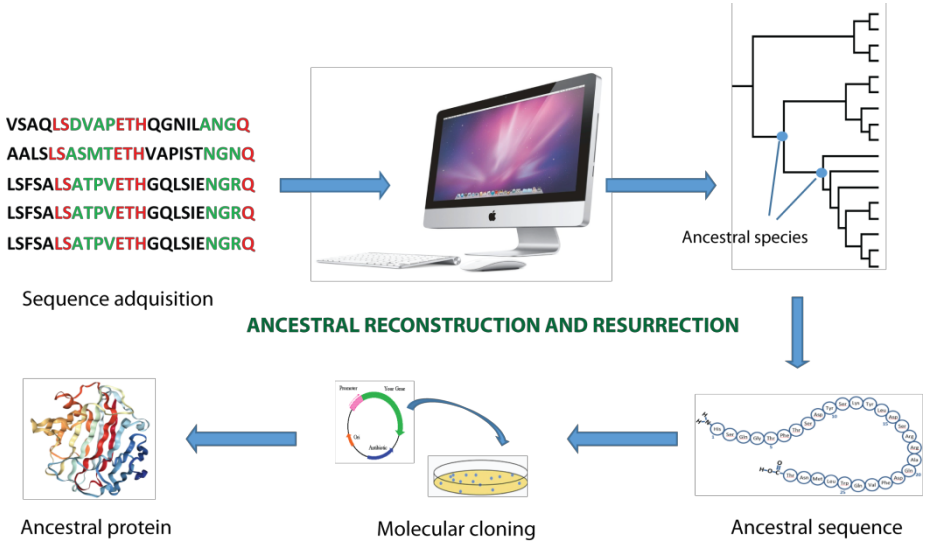


Figure 1.2. Schematic explanation of ancestral reconstruction and resurrection process. *First, the sequences are retrieved from online databases, and then the tree is inferred by bioinformatics tools. After that, the selected sequence is cloned in the lab and brought back to life by molecular biology techniques.*

The first reconstruction of an ancestral protein was carried in 1994 by Shindyalov et al [33]. They predicted the tertiary structure of….Since them numerous studies have been carried out using this technique, providing information not only related to physiological and metabolic features [34], but also information about the environmental conditions that hosted ancestral organisms [19, 20]. Precisely, the deduction of the environmental conditions of different geological eras is another important application of this technique. Several studies carried out with enzymes have reported the conditions of the earth at the

Precambrian era [19, 20, [35-37]. On od the first studies reporting environmental information was carried out by Gaucher and collaborators [21]. They reconstructed the translation elongation factors of species that lived in the range of 3.5-0.5 Gyr ago and calculated their melting temperature. According to this study, the temperature on earth cooled down over 30 ºC during that period, matching previous work related to the temperature of ancient oceans calculated from silicon isotopes [38]. Some years later, some new studies, such as, the one developed by Perez-Jimenez et al. studied the thermochemical evolution of thioredoxins from 4 to 1.4 Gyr with single-molecule force spectroscopy [22] confirming this cooling trend. The obtained results showed that ancestral enzymes have higher thermal stability than the extant ones.

Ancestral proteins and enzymes often show exceptional properties related to its thermal stability, chemical or kinetic, in addition to its activity. This is due to the fact that our planet has been subjected to extreme environmental conditions throughout history. Organisms and their molecules were adapted to these conditions. In addition, it has been shown that ancestral enzymes are promiscuous [24, 27], they are able to work with lower selectivity and therefore more effective with different types of substrates.

Nowadays, there are relatively few studies of resurrection of ancient enzymes with possible industrial and biomedical applications. These include most notably thioredoxin, or the lactamases [24]. These studies have shown that the study of ancestral enzymes not only has a great value from an evolutionary point of view, but can also have multiple applications in areas such as bioengineering and biomedicine.

Following the mentioned assumptions, we are going to explore the possible applications of ancestral enzymes for biotechnological and industrial applications. In this sense, we have decided to reconstruction ancestral variants of a group of enzymes widely used in industry, i.e., cellulases. These enzymes are among the most utilized enzymes in chemical industry [39] because of their ability to completely hydrolyze cellulose into glucose monomers (**Fig 1.3**).

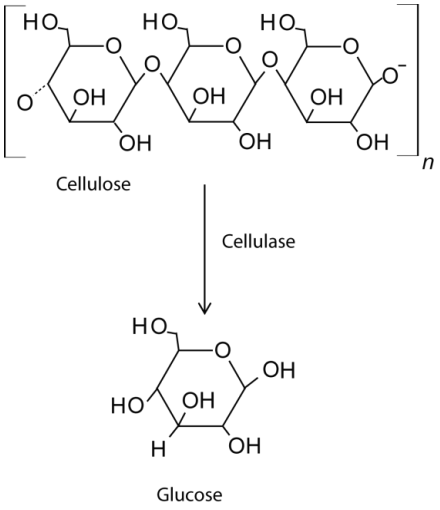


Figure 1.3. Hydrolysis of cellulose chain into glucose by cellulase enzymes.

Cellulases are quite limited in their properties being difficult to use at temperatures above 60 ° C and extreme pH, improving these properties is a goal in biotechnology research. Increasing

the thermal operability and activity of cellulases is perhaps the most investigated aspect for modifying them for industrial implementation [3, 11, 40-42] as well as other lignocellulosic enzymes. As I have explained before, several methods, such as directed mutagenesis, DNA shuffling, error-prone PCR, and directed evolution, have been implemented to obtain enhanced cellulases [3,[12, 40] that considerably improve the efficiency of cellulose bioconversion. Despite these improvements, the limitations of the enzymes is still a serious barrier in their industrial applications and we hypothesize that this methodology will help to enhance the temperature and pH resistance, the expression level or the specific activity of cellulases, hopefully all at once.

Cellulose is one of the major components in plant cell walls and is the most abundant organic polymer on the planet [43]. It is a homo-polysaccharide composed entirely of D-glucose monomers linked together by β-1,4-glucosidic bonds. There is an enormous variety of raw materials rich in cellulose, such as agricultural, industrial, and urban waste that can be used as sources for fermentable sugars [44].

Lignocellulose can be obtained from a variety of sources, such as agricultural residues, forestry residues, energy crops and bio-waste streams (**Table 1.1**).

Lignocellulosic materials	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwood stems	40-50	24-40	18-25
Softwood stems	45-50	25-35	25-35
Paper	85-99	0	0-15
Newspaper	40-55	25-40	18-30
Leaves	15-20	80-85	0
Switch grass	45	31.4	12
Corn cobs	45	35	15
Waste paper from chemical pulps	60-70	10-20	5-10
Primary wastewater solids	8-15	NA	24-29

Table 1.1. Lignocellulose contents of common agricultural residues and wastes [45] .

In lignocellulosic biomass, lignin and hemicellulose form an amorphous matrix in which, crystalline cellulose fibrils are embedded **(Fig 1.4)**. The combination of hemicellulose and lignin provides a protective sheath around cellulose and the crystalline structure of cellulose makes it highly resistant to attack. This arrangement makes lignocellulosic biomass recalcitrant and a complex substrate to convert into valuable products.

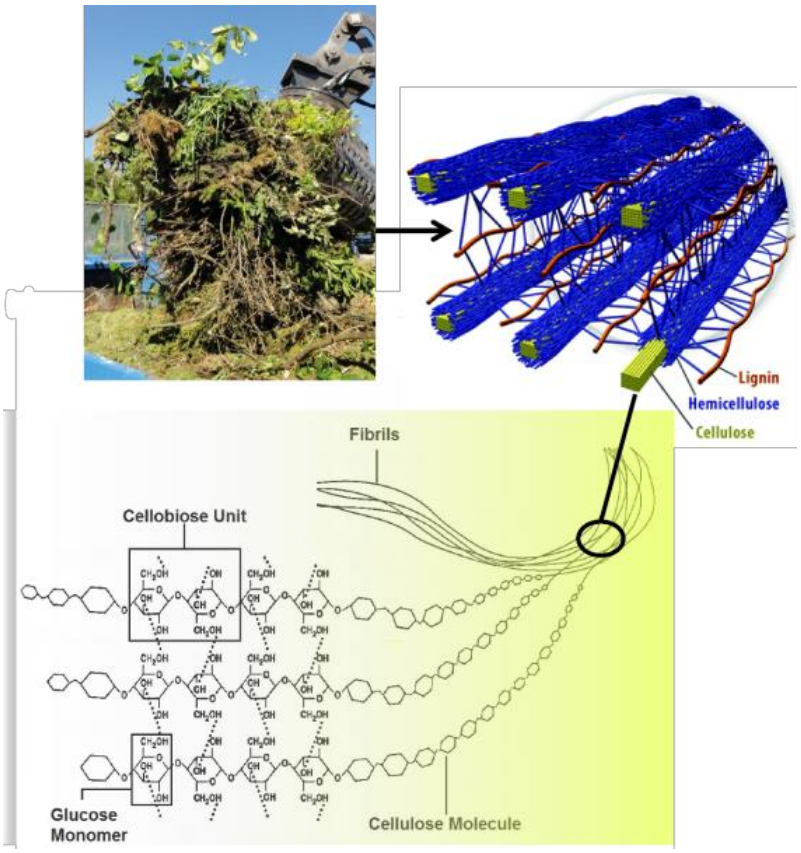


Figure 1.4. Schematic representation of the structure of lignocellulosic biomass. *The picture shows that lignocellulosic biomass (on top in the left) if composed many of cellulose. This cellulose is embedded in lignin and hemicellulose (top in the right). Above, the structure of cellulose is drawn. Glucose monomers form long polymer chains which form the microfibrils.*

Cellulose chains, with a degree of polymerization between 10.000 and 15.000, are linked by strong hydrogen bonds which form cellulose chains into microfibrils, making it crystalline in nature

and very recalcitrant to degradation (**Fig 1.4).** Three types of cellulases are involved in the complete hydrolysis of cellulose into sugar, and all of them cleave β-1,4-glycosidic bonds (**Fig 1.5**): endo-β-glucanases (EG) randomly break cellulose fibers; exo-β-glucanases (or cellobiohydrolases, CBH) cleave cellulose chains and release cellobiose; and cellobiose is further hydrolyzed to glucose by β-glucosidase.

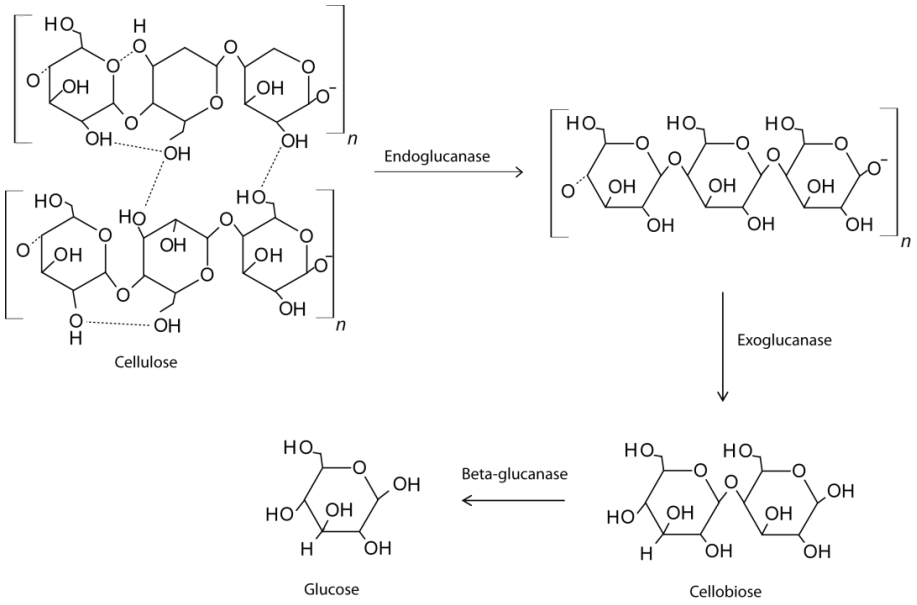


Figure 1.5. **Synergistic action of cellulases in cellulose degradation.** *Endo-1,4-β-glucanases break down randomly the internal β-1,4-glucosidic bonds of the cellulose chains, whereas exo-1,4-β-glucanases cleave off cellulobiose units from the end of the chains. These cellulobiose units are broken down by β-glucosidases into glucose monomers.*

Most endoglucanases and exoglucanases have a two-domain structure that contains a catalytic domain (CD) and a carbohydrate binding module (CBM) [46-48] which are connected by a peptide

linker that maintains the separation between the CD and the CBM (**Fig 1.6**). The CD contains the enzyme active site, responsible for cellulose hydrolysis. The CBM is a contiguous amino acid sequence that anchors the CD onto the surface of cellulose through hydrogen bonding and van der Waals interactions [49, 50]. According to sequence similarities within their CDs and CBMs, cellulases can be grouped into different families [51].

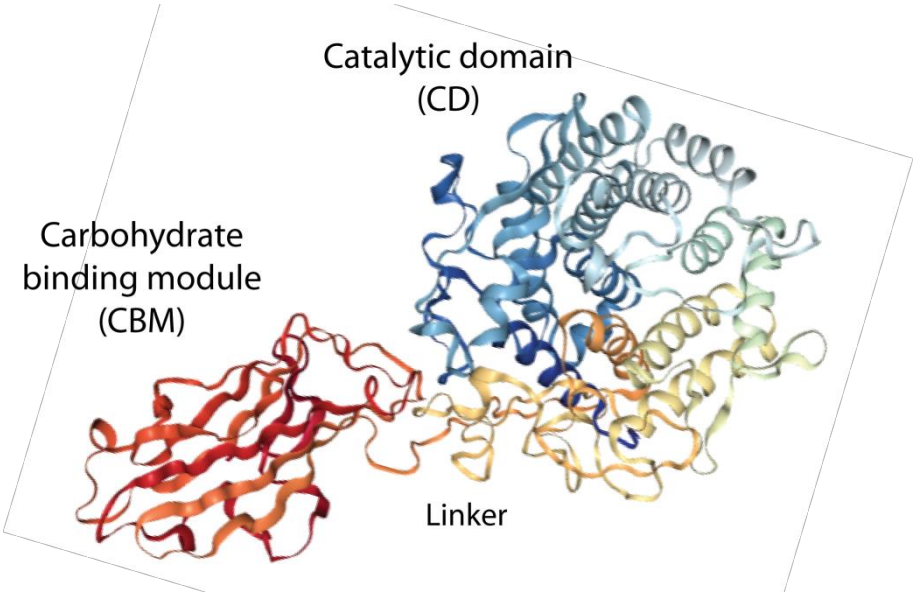


Figure 1.6. Schematic structure of the different domains of a typical endoglucanase. *It contains a catalytic domain (CD) and a carbohydrate binding module (CBM)). These two domains are connected by a peptide linker, which is known to maintain the separation between the CD and the CBM.*

In order to produce cellulases, fungi and bacteria have been heavily exploited, but the use of fungi has been more important because of their capability to produce high amounts of cellulases

and hemicellulases which are secreted to the medium for easy extraction and purification. In addition, fungal enzymes are often less complex than bacterial cellulases and can be more rapidly cloned and produced via recombination in a rapidly growing bacterial host, for example E.coli. However, for several reasons the use of bacterial cellulases is becoming widely exploited. On the one hand, bacteria often have a higher growth rate that fungi, which allows a higher recombinant production of enzymes. On the other hand, bacterial cellulases are more complex, therefore they are often expressed in multi-enzyme complexes. In this way, their function and synergy increase. Although those two reasons are important, there is a more important one; bacteria inhabit a wide variety of environmental and industrial niches, it is for that reason that the cellulolytic strains produced are extremely resistant to environmental stress. These include strains that are: thermophilic, psychrophilic, alkaliphilic, acidiophilic or halophilic. Thus, besides surviving the harsh conditions found in the bioconversion process, they often produce enzymes that are stable under extreme conditions present in bioconversion process. Those enzymes should be suitable as they may increase rates of enzymatic hydrolysis, fermentation and product recovery [12, 52]. Thus, using cellulases from bacteria has a greater potential for improvement than those from fungi. Fungal cellulases are produced in large quantities, which is the basis for their use in industrial processes so far. However, some bacterial cellulases present much higher specific activities and therefore have the potential to be excellent components for a more efficient saccharification processes [52].

Lignocellulosic substrates present a high degree of chemical and structural diversity (comprising mainly cellulose, hemicellulose, and pectin, as well as the non-carbohydrate lignin polymer, all in varying proportions), which makes it extremely recalcitrant such

that only some microorganisms are able to degrade it. But due to the different components that form lignocellulosic biomass, multiple enzymes that cooperate synergistically are used in the hydrolysis step. The core enzymes for cellulose hydrolysis are cellulases and the reaction can be favored by other enzymes that break down the structure of lignin and hemicellulose increasing cellulose accessibility (**Table 1.2**).

Component	Enzyme
Cellulose	Endo-1,4-β-glucanase, exo-1,4-β-glucanase, β-glucosidase
Hemicellulose	Endo-xylanase, β-xylosidase, acetyl xylan esterase, endo-mannanase, β-mannosidase, α-glucuronidase, ferulic acid esterase, α-galactosidase, p-coumaric acid esterase
Lignin	Laccase, Lignin peroxidase, Manganese peroxidase
Pectin	Pectin methyl esterase, pectate lyase, polygalacturonase, rhamnogalacturonan lyase

Table 1.2. Some of the main enzymes required to degrade lignocellulose to monomers[53]*. The column in the left shows the different components of the lignocellulosic material. The column in the right shows the enzymes that are able to hydrolase the components of the lignocellulosic material.*

Some parts of the cellulose structure may be amorphous in nature, which are easier to degrade. In regard to hemicelluloses, they consist of short highly branched chains of various sugars: mainly

xylose, and further arabinose, galactose, glucose and mannose [54]. They are classified according to the main sugar in the backbone. Due to the branches, hemicelluloses are amorphous in structure and relatively easier than cellulose to degrade. Concerning lignin, it is a complex three-dimensional network formed by the polymerization of phenyl propane units and forms a protective seal around the other two components. It is the most abundant natural phenolic polymer (**Fig 1.7**). It is formed mainly by three monomers: p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. These monomers are linked together by alkyl-aryl, alkyl-alkyl and aryl-aryl ether bonds (**Fig 1.7**). It makes the cell wall impermeable and resistant against microbial and oxidative attack[55].

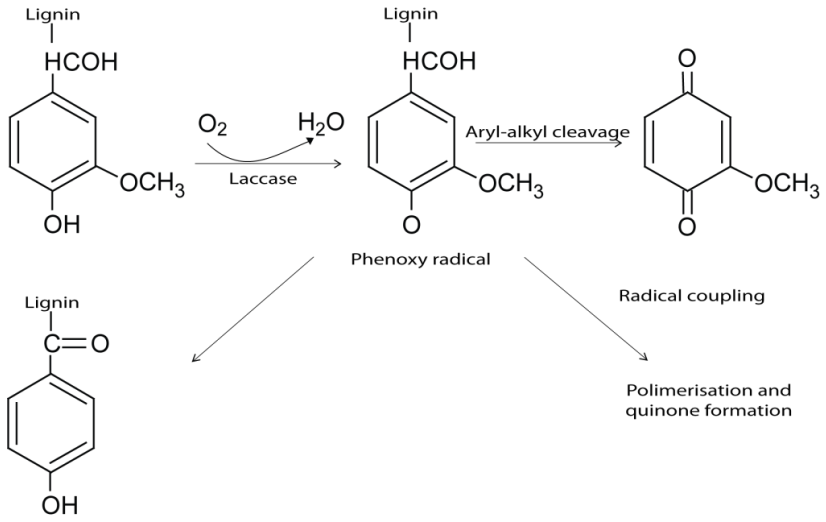


Figure 1.7. Diagram of Laccase enzymatic activity. *Oxidation of phenolic subunits of lignin by laccase, Laccase functions via the catalyzation of one-electron substrate oxidations with a concurrent four-electron reduction of molecular oxygen to water.*

In order to hydrolyze lignocellulosic material, we can use free enzymes. However, one efficient approach for degradation of lignocellulosic material in nature is the integration of cellulases and other associated enzymes into multi-enzymatic complex named the cellulosome [56]. Some anaerobic bacteria have evolved this specialized structure, a self-assembled nanomachine, the so-called cellulosome, that is highly efficient in this process. This molecular machinery is extremely complex and varied and seems to reflect the adaptation of the bacteria to the complexity of the plant cell wall. The cellulosome is composed of a scaffoldin (non-catalytic) subunit, two dockerins (recognition modules) and a cohesin, that are able to integrate various enzymes into the complex [57, 58]. *Clostridium thermocellum* is the most studied cellulosome producer. Its scaffoldin subunit comprises a series of 9 repeating cohesin modules, a single carbohydrate-binding module (CBM) and an X-dockerin that interacts with an attaching scaffoldin at the cell surface[59]. Cellulosomal enzymes contain a catalytic module and a specific dockerin module, which binds to the cohesins of the primary scaffoldin (**Fig1.8**).

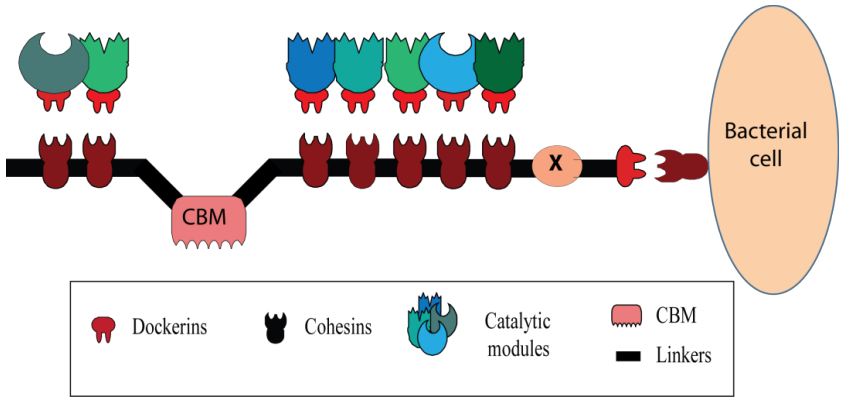


Figure 1.8. Schematic representation of a cellulosome. *In the legend the different components of the cellulosome are described. Each cohesin is selective for each dockerin, so each catalytic module has its own place in the designer cellulosome.*

We are able to mimic this natural machinery creating a designer cellulosome for promoting synergistic action among enzyme components [60, 61]. This artificial complex uses recombinant chimaeric scaffoldins composed of cohesin modules originating from different bacterial species. Each cohesin binds specifically to the matching dockerin of the same species, containing the different enzymes. These nanomachines allow controlling the composition and architecture of the cellulosome assembly and have demonstrated to be efficient in the degradation of lignocellulosic materials [62-64].

The improvement of cellulases may be of great interest for different industries such as textile, food industry, but especially for the bioenergy industry because these enzymes are necessary in the bioconversion of biomass to bioethanol. The enzymes that we selected for improvement in this thesis are have been targeted thinking of their possible implementation in the production of bioethanol.

The degradation of linognocellulosic materials can produce large amounts of value-added products that can be obtained by different thermo-chemical and biochemical processes. Lignocellulosic materials, consisting of approximately 75% polysaccharide sugars, can be converted to bioethanol and fine chemicals (**Fig 1.9**). In the industrial process, a thermochemical pretreatment is required to liberate cellulose from hemicellulose and lignin [65-67].

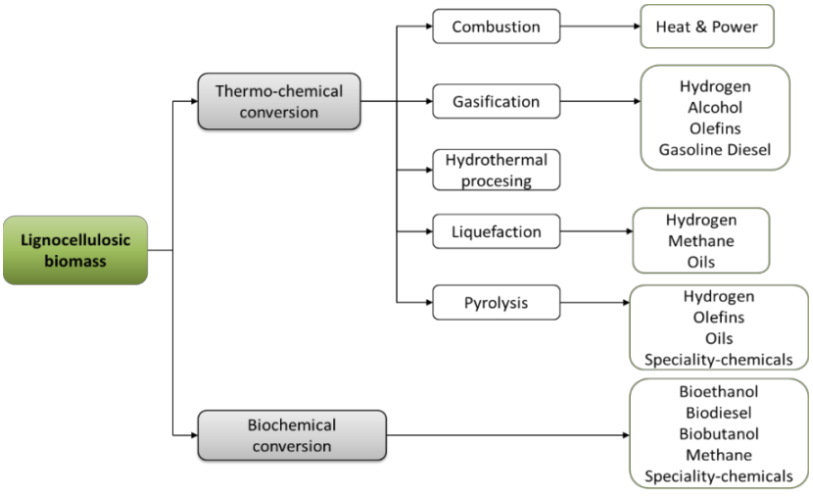


Figure 1.9. Thermochemical and biochemical processing of lignocellulosic biomass [68].

Global climate change due to excessive carbon emissions, as well as the uncertainty and price instability of petroleum resources, have encouraged the development of new sources of energy that are sustainable and environmentally friendly, and can reduce the dependence on fossil fuels. In the past decade, numerous efforts have been made to implement bioethanol as a semi-renewable fuel, as it is capable of alleviating some of the issues associated with fossil fuels, especially those related to the environment [69].

New mandates have compelled the rapid implementation of bioethanol [44, 70], and it is currently used in fuel at a concentration of between 5 and 25%, depending on the country.

Plant cell wall cellulosic biomass is the most abundant source of energy-rich carbon in the biosphere [71]. This source of energy, gives several advantages compared with oil. Bioethanol can be produced virtually in any country, thus reducing oil dependence. Also, it reduces CO_2 emission palliating part of the environmental problem related to fossil fuels. In addition, the use of bioethanol is less toxic to humans as it does not contain sulfur and produces a lower level of particulates and toxic emissions such as sulfur dioxide. Besides, its volatility is lower and hence the smog formation is reduced [72]. Natural resources are nowadays used for the production of bioethanol because of their low cost and abundant supply. These sources include city and agricultural waste, giving the opportunity to generate value for waste product. Bioethanol generated from lignocellulosic material s nowadays called second-generation bioethanol as opposed to first-generation ethanol that uses food crops. However, generating bioethanol from cellulose is more complex and expensive than in the case of first-generation ethanol. The conversion of lignocellulosic bio-wastes into products of interest requires several steps (**Fig. 1.10**), which include the pre-treatment of the substrate, its saccharification to obtain fermentable sugars, and finally fermentation using microbes to produce the final chemical products of interest.

Once that lignocellulose is pretreated, enzymatic hydrolysis also known as enzymatic saccharification, is carried out at 50 ºC. The biological degradation of cellulose into glucose and pentose monomers is achieved using multiple enzymes in defined ratios that cooperate synergistically. Then, sugars can be fermented at 50 ºC to obtain ethanol and other chemicals by different

organisms. These two steps can be done separately (separate hydrolysis and fermentation, SHF) or simultaneously (simultaneous saccharification and fermentation, SSF) [73].

For successful cellulose degradation to sugar, enzymes must withstand the conditions of the industrial bioconversion process, such as high temperature, generally above 50 °C, and low or high pH [11, 67]. The lower efficiency of the enzymes under these conditions, make the saccharification process a critical bottleneck in the bioconversion of cellulose.

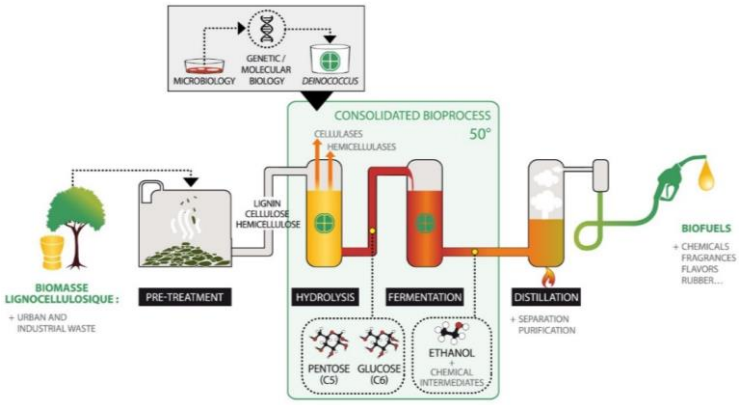


Figure 1.10. ***Enzymatic lignocellulose conversion process into valuable products such as bioethanol.***

In this thesis we have improved a set of enzymes that can produce sugar from lignocellulosic material in a wide range of conditions. The activity of the reconstructed cellulase enzymes was compared to that of modern enzymes. In particular, we used a bacterial endoglucanase from *Thermotoga maritima, T.reesei* enzyme preparation and Ctec2 enzymes cocktail. These enzymes are commonly used for the bioconversion of cellulose. The ancestral cellulases showed considerably higher specific activities than those of modern ones under a broad range of temperatures and pH

values with various substrates. We observed that an efficient bioconversion can be achieved by reconstructing a set of three enzymes as compared to other methodologies were hundreds of variants need to be tested. The reconstructed endoglucanase enzyme also displayed higher efficiency when integrated in a bacterial cellulosome, a macromolecular machine for cellulose degradation [59, 74], that has been also proposed for industrial implementation [12, 75]. The intention is to integrate the other two enzymes in the same cellulosome.

Our ancestral enzymes also showed very good synergy with other lignocellulosic enzymes such as laccase and xylanase, as well as incorporated into a bacterial cellulosome. We anticipate that the incorporation of additional enzymes with complementary activities towards cellulosic biomass degradation may result in even higher synergies and overall activities. Our resurrected enzyme targets a critical step of the process and is expected to result in an important reduction of the enzyme cost in industrial biomass degradation. Finally, the ancestral enzymes could be combined in the future with other reconstructed lignocellulosic enzymes to generate highly efficient cocktails providing the long-awaited improvement of the saccharification of cellulosic substrates.

Chapter 2: Methods for phylogenetic analysis

This chapter of this thesis, explains the methodology used in phylogeny in order to create phylogenetic trees and reconstruct ancestral sequences.

2.1. Introduction

The common name used for the mathematical and statistical methods used to infer ancient information (in the form of strings of characters) from current data is **ancestral reconstruction**. The main use of this technique, although it has some non-biological applications such as the phylogenies of the phonemes and vocabulary of ancient languages [76], oral traditions of extinct cultures [77] or ancestral marriage practices [78], is in the field of phylogenetics. That is why, the most common used characters are either protein or nucleic acid sequences. and the current data comes from the extant species that have been sequenced.

Phylogenetics is the study and correlation of the evolutionary relationships between extant individuals, species and populations and their corresponding ancestors [79]. Nowadays, by means of the so-called **ancestral sequence reconstraction**, it is possible to reconstruct ancestral biological macromolecules; polynucleotide sequences of DNA and distinct types of RNA, or amino acid sequences of proteins.

This technique is based on a sufficiently realistic statistical model of evolution to accurately recover ancestral states. In order to determine the route of the evolution [80] the genetic information already obtained through methods such as phylogenetics is used.

One of the precursors of the modern phyligenetics is cladistics, in fact, the idea of reconstructing ancestors from measurable biological characteristics, comes from cladistics. In cladistics, the organisms are classified based on the common characteristics that they share. Cladistics appeared as early as 1901 and infer the evolutionary relationships of species on the basis of the distribution of shared characteristics, of which some are inferred to be descended from common ancestors. The first person who is known to have carried out a cladistics analysis for birds is Peter Chalmers Mitchell [81, 82], followed by the works of Robert John Tillyard for insects (1921) [83] and Walter Max Zimmermann for plants (1943).

Emile Zuckerkandl and Linus Pauling in 1963 were the first persons that carried out works in ancestral sequence reconstruction. In 1955, Frederick Sangerce started developing techniques for sequencing the primary structure of proteins. This helped, Zuckerkandl and Pauling to propose that, based on the amino acid sequence of extant proteins, it is possible to infer the phylogeny of that protein and the sequences of all the common ancestors, including the earliest point of the tree [84, 85]. Beside

those pioneers works, it was in 1971 when Walter M. Fitch developed the first algorithm for ancestral sequence reconstruction using the principles of maximum parsimony [86].

2.2. Theory

Every effort on reconstructing ancestors starts with a phylogeny, a hypothetical tree that includes the order in how species are correlated between each other by descent from common ancestors, starting with the last universal common ancestor (LUCA). In a phylogenetic tree, terminal nodes correspond to the extant species. These nodes are successively connected to their common ancestors by branches. The common ancestors are the inner nodes. At the end, all the species and, therefore, all the evolutionary lines converge in the LUCA (**Fig. 2.1**).

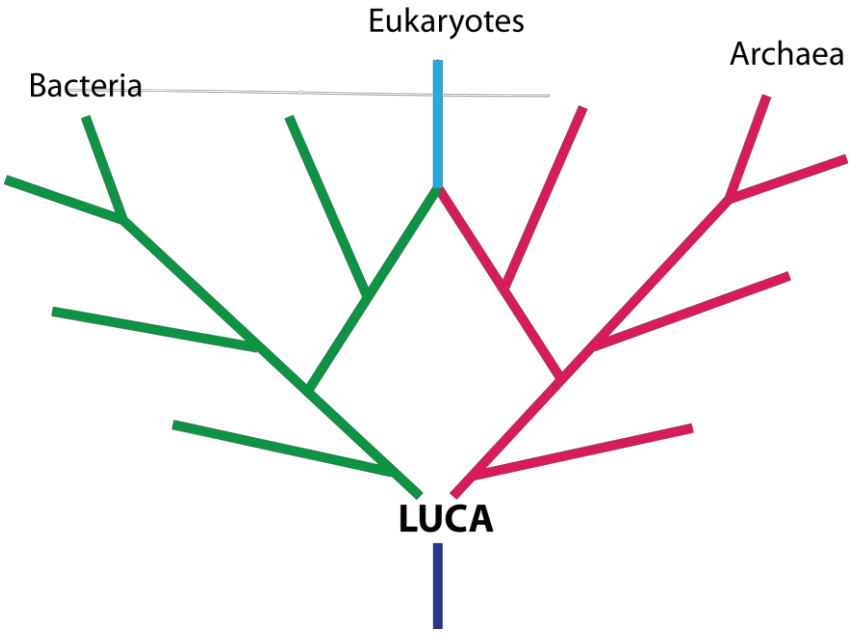


Figure 2.1. Schematic representation of a phylogenetic tree. *The three kingdoms are represented, Bacteria in green, Archea in re and Eukaryotes in blue. The last node (root) represents LUCA: Last Universal Common Ancestral. LUCA is the common ancestor of the three kingdoms.*

2.2.1. Methods

The maximum parsimony method is a non-parametric statistical method. In order to infer the tree, it uses a set of extant sequences minimizing the amount of mutations that are necessary to match the available data. Some years later, in 1975, David Sankoff optimized this algorithm adding a cost to the mutations [87]. Because of the development of this work David.L.Swofford developed the first phylogenetics program [88] in 1989, called PAUP. It soon became very popular in the phylogenetics community.

At the same time, the exponential increase of the computing power made the implementation of much more complex algorithms possible: maximum likelihood approaches [89-91] or Bayesian methods [92-96].

The maximum likelihood algorithm searches for the most probable tree when the phylogenetics model and the extant sequences are estimated. In the Bayesian approach, the computer program searches for the highest posterior probability, which is determined on the one hand by the likelihood of the data under a certain evolutionary model and on the other hand by a set of prior probabilities set for the trees. Nowadays most of the procedures for ancestral sequence reconstruction are based in maximum parsimony, maximum likelihood and Bayesian inference.

In this thesis, Bayesian inference has been used for computing the phylogenetic tree, whereas maximum likelihood has been chosen to infer the extant sequences.

2.2.1.1 Parsimony

Parsimony refers to the principle of selecting the simplest of competing hypotheses. In the context of ancestral reconstruction, parsimony endeavours to find the distribution of ancestral states within a given tree which minimizes the total number of character state changes that would be necessary to explain the states observed at the tips of the tree.

One of the earliest examples of maximum parsimony implementation is Fitch's method [86], which assigns ancestral character states by parsimony via two traversals of a rooted binary tree. In spite of being really used, it has some evident limitations, Fitch's approach overestimates the amount of rare changes [97].

Maximum parsimony is very useful due to its low computational costs and high efficiency for huge datasets and when ab initio phylogenies are needed [98] to optimize more complex algorithms. They are still used in some cases to seed maximum likelihood optimization algorithms with an initial phylogeny. However, the underlying assumption that evolution attained a certain end result as fast as possible is inaccurate. Parsimony methods impose five general assumptions that are not valid most of the times:

1. *Variation in rates of evolution.* Fitch's method assumes that changes between all character states are equally likely to occur; thus, any change incurs the same cost for a given tree. This assumption is often unrealistic and can limit the accuracy of such methods [99].
2. *Rapid evolution.* It assumes that mutations are rare. This assumption is not correct in cases of rapid evolution, such as some retroviruses [100-102].
3. *Changes in time among lineages.* Those methods accept that the same amount of evolutionary time has passed along every branch of the tree without taking into account the variation in branch lengths in the tree. They are often used to quantify the passage of evolutionary or chronological time. This limitation makes the technique responsible to infer that one change occurred on a very short branch rather than multiple changes occurring on a very long branch, for example [103]. In addition, it is possible that some branches of the tree could be experiencing higher selection and change rates than others, some periods of time may represent more rapid evolution than others, when this happens parsimony becomes inaccurate [104].

4. *Statistical justification.* Without a statistical model underlying the method, its estimates do not have well-defined uncertainties [101, 103, 105].
5. *Convergent evolution.* When considering a single character state, parsimony will automatically assume that two organisms that share that characteristic will be more closely related than those who don't.

2.2.1.2 Maximum likelihood

This method assumes that the ancestral states are those which are statistically most likely, based on the observed phenotypes. The first works developed using an approach of this method was developed in the context of genetic sequence evolution [89, 90, 106]; similar models were also developed for the analogous case of discrete character evolution.

Using a model of evolution needs to take into account the fact that not all events are equally likely to occur. But it does not mean that they need to happen just because they are more likely to take place. Sometimes, the one with the less probability occurs, and in those cases, maximum parsimony may actually be more accurate because it is more willing to make large, unlikely leaps than maximum likelihood. Maximum likelihood is really reliable in reconstructing character states. However it not so good in giving accurate estimations of the stability of proteins as overestimates, since it assumes that the proteins that were made and used were the most stable and optimal [107].

In maximum likelihood Markov process models the evolution of the sequence, assuming that all the mutations are independent [108]. The likelihood of the phylogeny is calculated from a sum of intermediate probabilities of the nodes for the proposed tree.

This basic model is frequently extended to allow different rates on each branch of the tree. In reality, mutation rates may also vary over time (due, for example, to environmental changes). This can be modelled by allowing the rate parameters to evolve along the tree, at the expense of having an increased number of parameters. A model defines transition probabilities from states *i* to *j* along a branch of length *t* (in units of evolutionary time). At each node, the likelihood of its descendants is summed over all possible ancestral character states at that node:

$$Lx = \sum_{Sx\in\Omega} P(Sx) \left(\sum_{Sy\in\Omega} P(Sy|Sx, txy) Ly \sum_{Sz\in\Omega} P(Sz|Sx, txz) Lz\right), \quad (2.1)$$

where the node x is the ancestor of y and z. Sx represents the sequence of the i-th node, tij refers to the branch length from i to j.

Ω is the set of all the possible combinations (the four nucleotides or the 20 basic amino acids).

Thus, the objective of ancestral reconstruction is to find the assignment for all *x* internal nodes that maximizes the likelihood of the observed data for a given tree.

Marginal and joint likelihood

The problem for ancestral reconstruction is to find the combination of character states at each ancestral node with the highest marginal maximum likelihood. In order to find the most probable evolutionary lineage to the common ancestor, two different conventions have been proposed. First, one can consider the probabilities of all the descendants for a certain ancestor and

calculate the joint combination with the maximum likelihood. This approach is called joint reconstruction. And second, instead of calculating the global likelihood, one can successively select the most likely ancestor for every node. This procedure is referred to marginal reconstruction. Joint reconstruction is more computationally complex than marginal reconstruction. Nevertheless, efficient algorithms for joint reconstruction have been developed with a time complexity that is generally linear with the number of observed taxa or sequences [91].

2.2.1.3 Bayesian inference

Bayesian inference employs both the likelihood of the experimental data, described before, and a prior knowledge about the possible solutions. Thus, the aim in ancestral sequence reconstruction is to obtain the posterior probabilities for every internal node of a known tree. Furthermore, the posterior probabilities can be combined with the posterior distributions over the parameters for a given evolutionary model and the structure of all possible trees. This results in the following applications of Bayes' theorem:

$$P(S \mid D, \theta) = \frac{P(D \mid S, \theta)\ P(S \mid \theta)}{P(D \mid \theta)} \tag{2.2}$$

$$\alpha\ P(D|S, \theta)P(S|\theta)P(\theta), \tag{2.3}$$

where D is the experimental data, S corresponds to the ancestral states and θ represents the phylogenetic tree and the evolutionary

model. Equation (2.2), $(D \mid S,)$ represents the likelihood of the experimental data that could be computed, $(S \mid \theta)$ refers to the prior probability of an ancestral node for a known tree and model and $(D \mid \theta)$ corresponds to the probability of the data for a known tree and model, integrated for all possible ancestral states.

Note that two different formulations have been given (2.2) and (2.3), one for each of the applications of Bayesian inference, the empirical and the hierarchical Bayes. Empirical Bayes approach estimates the probabilities of several ancestral nodes for a given tree and model of evolution. On the other hand, hierarchical Bayes approach calculates these probabilities over all possible trees and model of evolution, comparing how likely they are, with a given experimental data [109].

2.3. Methodology

In order to construct the ancestral trees of cellulases, bioinformatics tools have been used. The use of different software allows the whole process of constructing those trees. These different programs are described below.

First of all, a query was selected and making a blast of it in the protein databank, the amino acid sequences were obtained. It is a comprehensive resource for protein sequence and annotation data that includes different databanks. After downloading these sequences, they have to be aligned, cleaned and finally the tree has to be constructed, the following programs were the ones used. The scheme of the used methodology and software is shown in **Figure 2.1**.

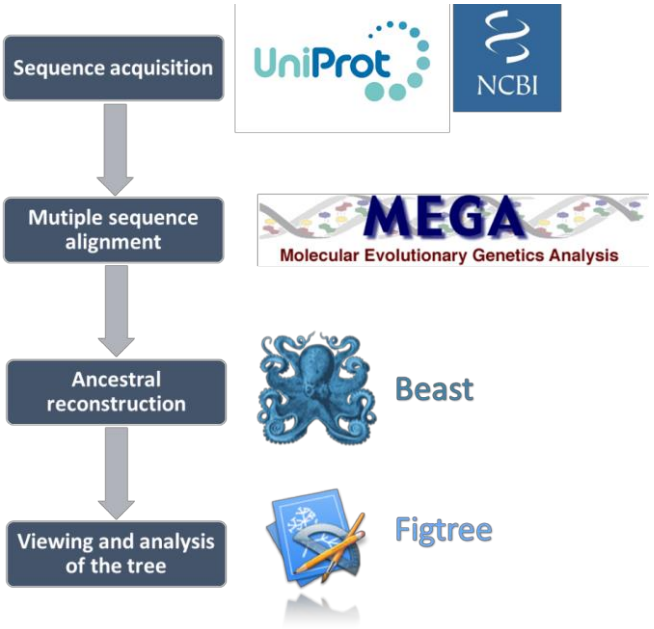


Figure 2.1. Methodology used for the reconstruction of ancestral sequences consisting in four steps: (1) Selection of extant sequences, (2) creation of a multiple alignment, (3) construction of a phylogeny, and (4) reconstruction of ancestral sequence*. Close to each step the icon of the program that has been used for each step is shown.*

2.3.1. Selection of extant sequences: Uniprot

In order to reconstruct a phylogenetic tree first, homologous sequences of the protein of interest need to be found. This means that, sequences of the different species chosen descend from the same common ancestor. In this way, those residues that are exactly the same at the same position are identical by character state in the particular sequences. Homologue sequences in this work, were retrieved from UniProtKB [110] online database using BLAST (Basic Local Alignment Search Tool) [111]. By using

this tool, regions of local similarity were searched between sequences, which gave later the possibility to infer evolutionary relationships. UniProtKB (Universal Protein Resource Knowledge Base) [39] is a catalog of information on proteins. To find the protein of interest (for now on, query), one can either use the search tool (**Fig. 2.2**) or directly enter the protein sequence, or its UniProt identifier

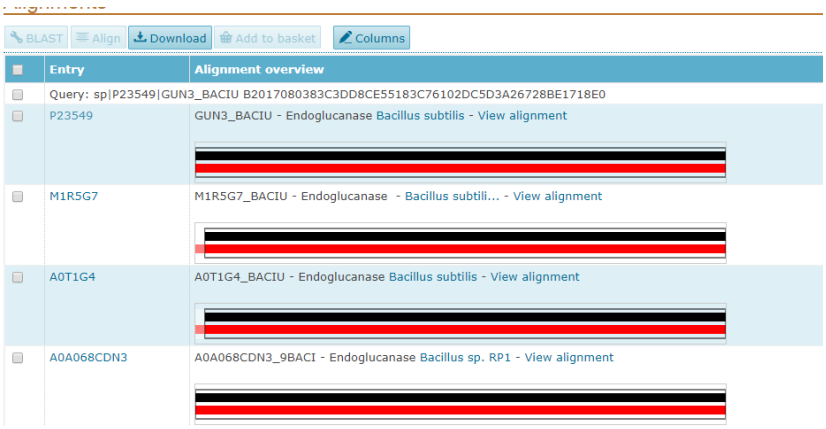


Figure 2.2. Search tool of the Uniprot Database. *The search of the sequences was made by using a query. This query is the sequence of the specie used for the search of the homologous sequences.*

The first step in this database was the selection of the query of the desired protein. This is the sequence from which the database will select the rest of the homologous proteins. Once the selection of the query was done, the BLAST tool was used to find the homologous sequences. Some parameters were set (those parameters are described below **table 2.1**):

Parameter	Meaning
Target Database	The search is performed against this database. Different phyla can be chosen for search.
E-Threshold	Statistical measure of the number of expected matches in a database. The bigger this value is, the more unlikely to be significant a match.
Matrix	It gives a probability score for the position of each aminoacid in an alignment. For this probability, the BLOSUM [112] matrix is based on the frequency with which that substitution is known to occur among consensus blocks within related proteins.
Filtering	The filtering can be made by masking the lookup table or by lower complexity regions.
Gapped	Gaps can be introduced after having selected the sequences.
Hits	The number of hits in a search can be chosen.

Table 2.1. Description of the parameters that can be changed in Uniprot Search tool. *The search of the homologous sequences was done by changing these parameters.*

The values used for each parameter in this work were the ones show in **Figure 2.3**.

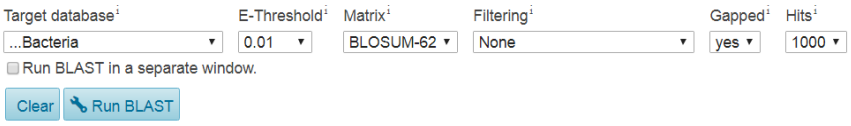


Figure 2.3. Uniprot search tool. *According to the description of the parameters done in* ***Table 2.1****, these were the selected parameters in the search of our sequences.*

After selecting all the parameters as shown in **Figure 2.3** "Run BLAST" was pressed in order to obtain the homologous sequences of the previously selected query. The process can take several minutes depending on different reasons, such as complexity of the query sequence, length of the sequence or the applied parameters. Once the process was over, homologous sequences were shown.

The next step consisted on selecting the sequences of interest. To do this, the identity between the regions of the different sequences with the query was taken into account. Finally, the selected sequences were download in the most appropriate format for the following steps: FASTA format, which is a text file that is commonly used for the aminoacid sequences. This format is shown in **Figure 2.4**.

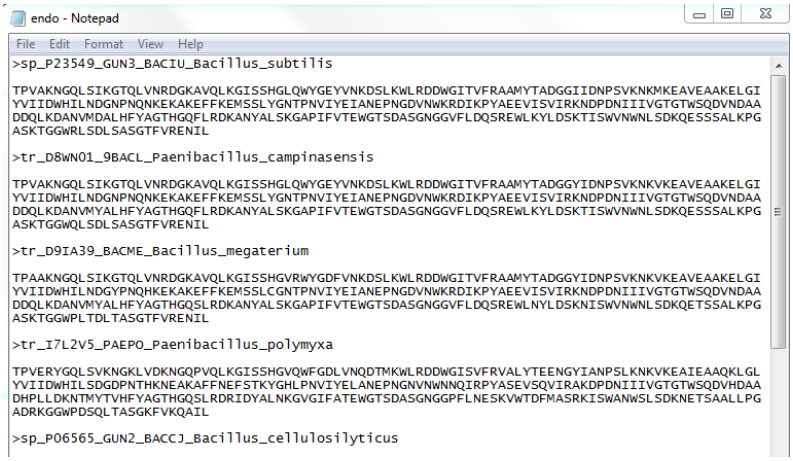


Figure 2.4. Fasta format file. *Example of sequences in fasta format, this type of files can be opened in text format.*

2.3.2. Creation of a multiple alignment: MUSCLE

Following the methodology to construct ancestral trees, the next step was the multiple alignment of the sequences selected in the previous step. MEGA is a very versatile tool for phylogenetic and molecular evolution analysis. This program is a package that is useful for: aligning sequences by ClustalW and MUSCLE, estimating phylogenetic trees by a variety of methods (Neighbor Joining, Maximum Parsimony and Maximum Likelihood), estimating rates of molecular evolution, inferring ancestral sequences and drawing those trees in different ways. Besides it´s multiple applications, in this work, it was used for two main purpose, to do the alignment and to select the best model [113]. Regarding to the alignment, over the last years, many algorithms were developed for this this purpose, with Clustal [114] and MUSCLE [115, 116] being the most popular in the phylogenetic community. In this work, the MUSCLE algorithm was used for all the multiple sequence alignments. In order to make the sequence alignment of the selected sequences, the FASTA file downloaded from the UniProtKB was loaded in MEGA [117-119]. The sequences appeared unaligned as it is shown in **Figure 2.5**.

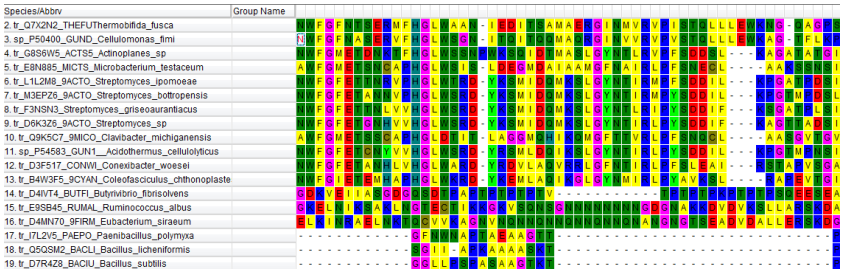


Figure 2.5. Sequence alignment before aligning. *This figure shows the sequences before aligning and editing the alignment.*

Once this appeared, MUSCLE was run by pressing "align with MUSCLE". Another window appeared the showing the different parameters that can be changed before running the algorithm (**Fig. 2.6**). The parameters are shown in **Table 2.2**.

Parameter	Meaning
Gap Opening Penalty	Increasing or decreasing this value gaps become more or less frequent in the alignment
Gap Extension Penalty	The bigger this value is, the shorter the gaps are in the alignment. Terminal gaps do not penalize
Max Memory in MB	The upper limit of the memory used by the algorithm in the computer. By living it default, the use of all the computer resources is avoid
Max Iterations	Maximum number of allowed iterations
Clustering Method (1,2 iterations)	The clustering method used for the first two iterations
Cluster Method	The clustering method use for the rest of iterations
Max Diagonal Length	Maximum length of the diagonal of the matrix made by aligning the sequences

Table 2.2. Description of parameters that can be changed in MEGA software in order to align the protein sequences. *By changing these parameters the alignment was done.*

In this work, the following parameters were used for the aligning (**Fig 2.6**).

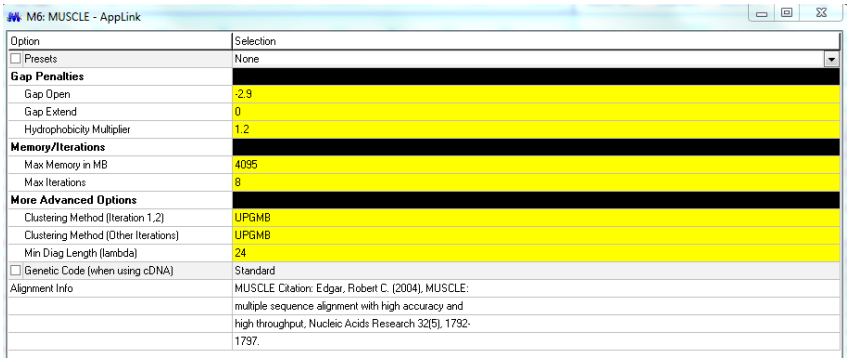


Figure 2.6. Screenshot of the window with the parameters that can be changed for the alignment of the sequences. *According to the description of the parameters done in* ***Table 2.1****, these were the selected parameters in the search of our sequences.*

In order to run the algorithm "Compute" was selected. Depending on how many iterations have been selected the process will last longer or shorter. Once all the iterations were finished, the alignment of all the sequences was obtained, as shown in the **Figure 2.7**.

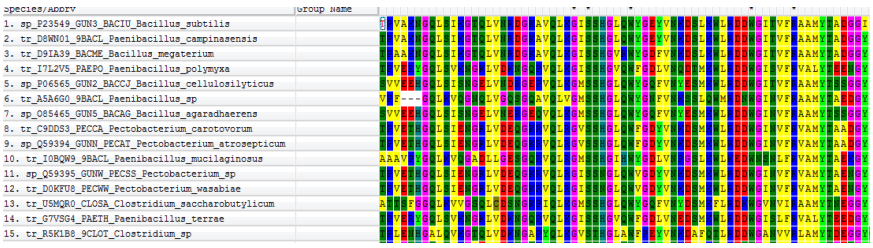
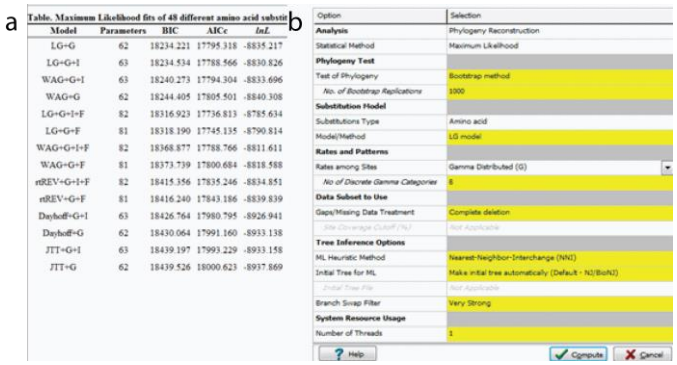


Figure 2.7. Image of the protein sequences once the alignment was done and it was edited. *The edition of the alignment was done manually and after it an almost no-gap matrix was obtained.*

Analyzing the alignment some asterisk can be seen in the top of the alignment, which means that the residue below the asterisk is conserved in all the species. The colors, they are related to the biochemical properties of the aminoacids. So as to remove ambiguously aligned regions, GBLOCKS [120] can be used or there is a possibility of doing it manually. The other tool of MEGA used in this work was the choosing of the best model (**Fig 2.8**).


a
Table. Maximum Likelihood fits of 48 different amino acid substit

Model	Parameters	BIC	AICc	lnL
LG+G	62	18234.221	17795.318	-8835.217
LG+G+I	63	18234.534	17788.566	-8830.826
WAG+G+I	63	18240.273	17794.304	-8833.696
WAG+G	62	18244.405	17805.501	-8840.308
LG+G+I+F	82	18316.923	17736.813	-8785.634
LG+G+F	81	18318.190	17745.135	-8790.814
WAG+G+I+F	82	18368.877	17788.766	-8811.611
WAG+G+F	81	18373.739	17800.684	-8818.588
mREV+G+I+F	82	18415.356	17835.246	-8834.851
mREV+G+F	81	18416.240	17843.186	-8839.839
Dayhoff+G+I	63	18426.764	17980.795	-8926.941
Dayhoff+G	62	18430.064	17991.160	-8933.138
JTT+G+I	63	18439.197	17993.229	-8933.158
JTT+G	62	18439.526	18000.623	-8937.869

b

Option	Selection
Analysis	Phylogeny Reconstruction
Statistical Method	Maximum Likelihood
Phylogeny Test	
Test of Phylogeny	Bootstrap method
No. of Bootstrap Replications	1000
Substitution Model	
Substitutions Type	Amino acid
Model/Method	LG model
Rates and Patterns	
Rates among Sites	Gamma Distributed (G)
No of Discrete Gamma Categories	5
Data Subset to Use	
Gaps/Missing Data Treatment	Complete deletion
Site Coverage Cutoff (%)	*Not Applicable*
Tree Inference Options	
ML Heuristic Method	Nearest-Neighbor-Interchange (NNI)
Initial Tree for ML	Make initial tree automatically (Default - NJ/BioNJ)
Initial Tree File	*Not Applicable*
Branch Swap Filter	Very Strong
System Resource Usage	
Number of Threads	1

Help Compute Cancel

Figure 2.8. Screenshot of the parameters available to modify in order to select the best model for the construction of the tree. *a) Best model selection in MEGA, b) Parameters used for the tree construction available in MEGA.*

2.3.3. Computing a phylogenetic tree

The way the phylogenetic trees were made in this work was by using BEAST (Bayesian Evolutionary Analysis Sampling Trees) [121, 122]. This a package of programs for Bayesian analysis of molecular sequences using MCMC (Markov chain Monte Carlo), a class of algorithm for sampling the probability distribution based on constructing a Markov chain. BEAST can be used for reconstructing phylogenies using MCMC to average over tree space. In this way, each tree is weighted proportional to its

posterior probability. It is a cross-platform program for Bayesian MCMC analysis of molecular sequences. It is entirely orientated towards rooted, time-measured phylogenies inferred using strict or relaxed molecular clock models. It can be used as a method of reconstructing phylogenies but is also a framework for testing evolutionary hypotheses without conditioning on a single tree topology. MCMC is used to average over tree space, so that each tree is weighted proportional to its posterior probability. It includes a graphical user-interface for setting up standard analyses and a suit of programs for analyzing the results. It is a software package that allows a phylogeny analysis [122].

2.3.3.1. Beauti

By using Bayesian Evolutionary Analysis Utility (BEAUti) a input file (.xml) was created in order to run BEAST. It is a graphical user interface that allows to set the evolutionary model and options for the MCMC Once the aligned sequences are imported (through a NEXUS file), the interface with several tabs permits modifying many parameters as it is shown in **Figure 2.9** and explained in **Table 2.3**.

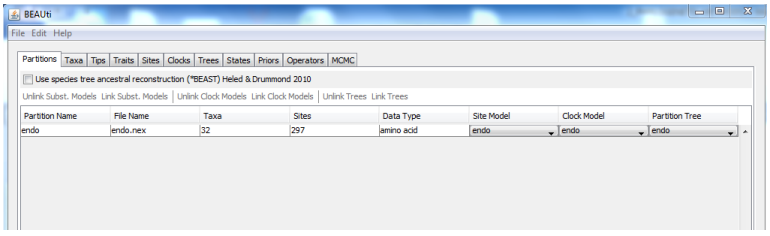


Figure 2.9. Graphical user-interface (GUI) application of BEAUti. *The figure shows the parameters that can be set to generate the file to construct the tree. The description of each parameter is shown in* ***Table 2.3****.*

Parameter	Meaning
Partitions	Makes possible to load sequences that were not included initially and making partitions
Taxa	Gives the possibility of making subgroups with the taxa. Also, this subgroups can be force to be monophyletic
Tips	Allows data selection of individual taxa
Traits	Phenotypic trait analysis can be set
Sites	Selection of the substitution model is allowed and the site heterogeneity model
Clocks	Gives the chance to choose the clock model. The mutation rate of biomolecules is used in those models to estimate when they diverged
Trees	The tree prior is set
States	Permits to reconstruct the states of all the ancestors or only choosing some subgroups
Priors	Sets the prior distribution for the subgroups
Operators	Allows to use or not some of the parameters in other tags
MCMC	MCMC value for the computing of the tree can be choose

Table 2.3. Description of the parameters available to adjust in order to make the tree. *By changing these parameters the correct file is generated in order to run the tree (explained in section 2.3.3.2).*

As it was explained before, this programs, has a tool that gives the opportunity of forcing phylogenetic groups. In this work this tool was used and it can be seen in the **Figure 2.10**.

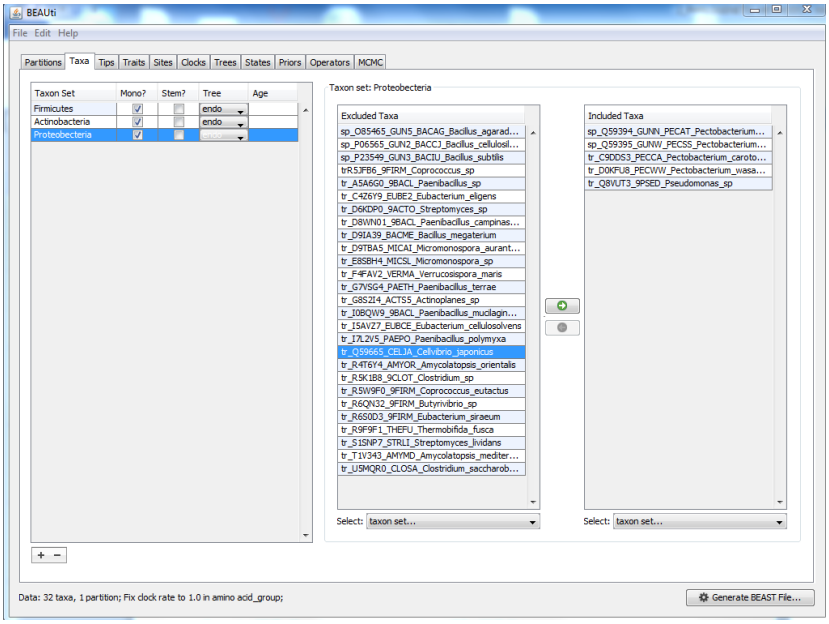


Figure 2.10. Description of the way the interface allows to make different groups in a set of sequences.

"Generate BEAST File…" was the next step to obtain the XML file for a further phylogeny computing using BEAST.

2.3.3.2. Beast

In order to run BEAST the previously generated ,xml file was used, The output was a .log file. The log file records a sample of the states that the Markov chain found. In order to compute the phylogeny, BEAST graphical interface was opened first (**Fig. 2.11**). Here, the XML file was opened. The application also has the option to activate the BEAGLE library [123]. BEAGLE is a high-performance library that takes advantage of the parallel

processors available in most of current PCs. Using this option is highly advisable to improve the performance of the program.

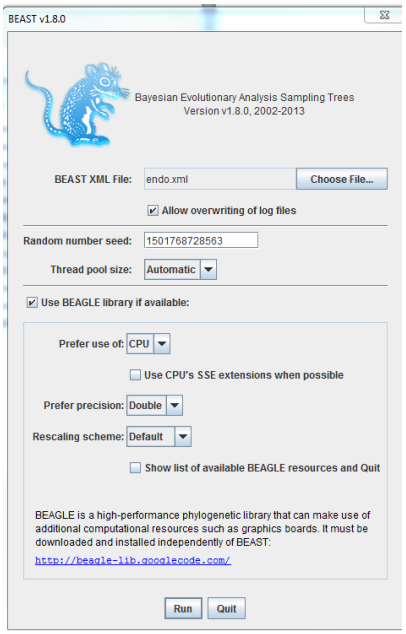


Figure 2.11. Graphical user-interface (GUI) application of BEAST. *The file generated by Beauti (xml file) can be run to construct the tree.*

Once the XML file of interest was selected and BEAGLE library option chosen, the program was run and the phylogeny started computing (**Fig. 2.12**).

Once the process was over, the program generated the log files that contain in the information of the process. An examination of this output was needed to determine whether the Markov chain was run for long enough to obtain accurate estimates of the parameters. Another application, Tracer, was used for this analysis (**Fig 2.13**).

2.3.3.3. Treeanotator

By using TreeAnotator the sample of trees generated by BEAST was summarize in a single consensus tree. The obtained tree contains information about the posterior probabilities of the nodes in the consensus tree, the posterior estimates and the rates. In the following figure (**Fig 2.12**) the different parameters are described.

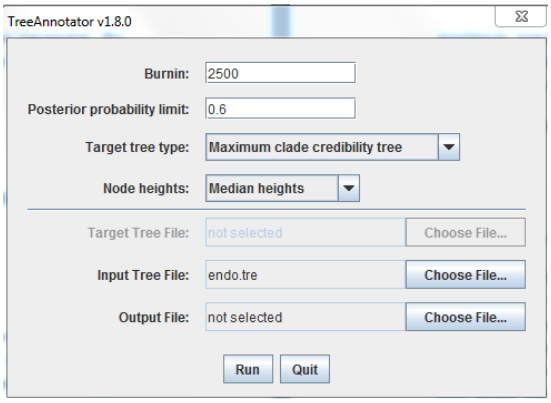


Figure 2.12. TreeAnotator graphical interface and its options. *This tool allows to select the most probable tree from the set of trees obtained by running the xml file in Beast.*

2.3.3.4. Tracer

Tracer is a graphical interface (**Fig. 2.13**) that makes possible the monitorization and analysis of the MCMC output carried out in BEAST. In order to perform the analysis, the log file obtained previously (POINT) that corresponds to the analysis of the phylogenetic computation was opened, many parameters related to MCMC analysis appeared on the left side of the interface, which were pondered by their Effective Sample Sizes (ESSs).

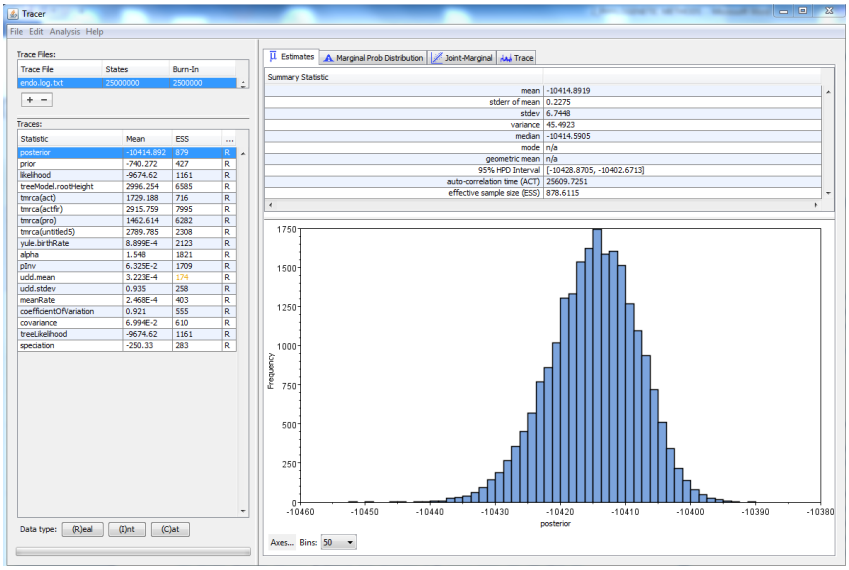


Figure 2.13. Tracer graphical interface. *Normal distribution of the probabilities is shown in this image.*

A low ESS means that the trace contained a lot of correlated samples and may not represent the posterior distribution well. It is advisable to run BEAST again until ESS reaches a value higher than 100.

2.3.3.5. Figtree

Finally, there are plenty of programs to draw the tree obtained by the previous steps. Figtree was used in this work, which is a program included in BEAST package.

This program is able to read tree files in both Nexus and Newick format. In the case of this format, Figtree has an extended version that includes parameters as fonts. It permits re-rooting, rotating

clades, showing or hiding labels etc. This is shown in **Figure 2.14**.

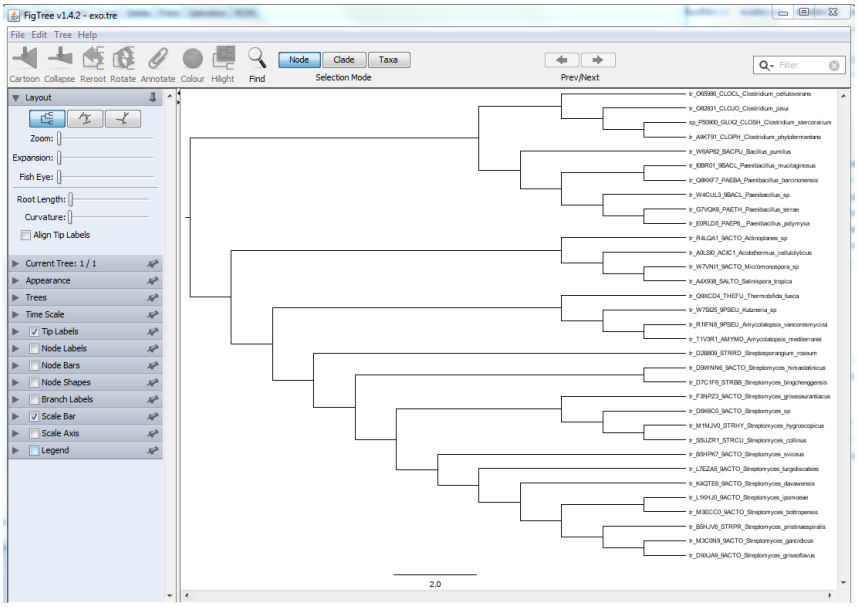


Figure 2.14. FigTree graphical interface and its options. *A consensus tree can be seen in the figure*

2.3.3.6. Treegraph

TreeGraph 2 is a graphical editor for phylogenetic trees which allows you to apply lots of graphical formats to the elements of your tree. Moreover, it supports several (visible or invisible) annotations (e.g. support values) for every branch or node. These annotations can be imported from Nexus tree files or text files containing data in a table (e.g. exported from a spreadsheet program).

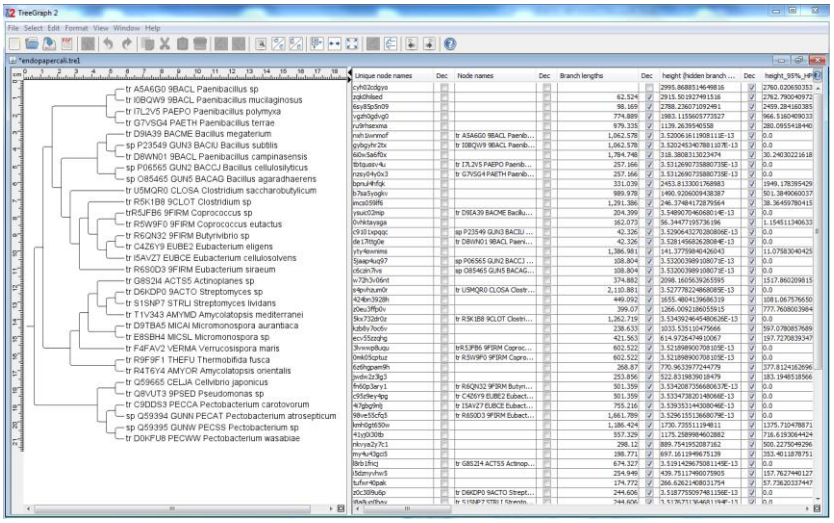


Figure 2.15. User interface of Treegraph. *In the image the tree and the length of the branches are shown.*

2.4. Reconstruction of ancestral sequences

Following the whole procedure described previously, a consensus tree was obtained and once being confident with the statistics, the ancestral sequences of interest were inferred. There are many algorithms and programs to obtain these sequences, but PAML (Phylogenetic Analysis Using Maximum Likelihood) [124, 125] was used to infer all the ancestral sequences in this work. PAML is based on the maximum likelihood algorithm mentioned in previous sections.

2.4.1. PAML

PAML is a package of programs for maximum likelihood analysis of protein and DNA sequences. In this thesis, Codeml was used in this thesis for the reconstruction of ancestral codons and proteins. In order to run this , it is needed to include in a new file a sequence data file, a tree file (in Newick format), a matrix file (Jones matrix in this case) and the control file (**Fig. 2.16**).

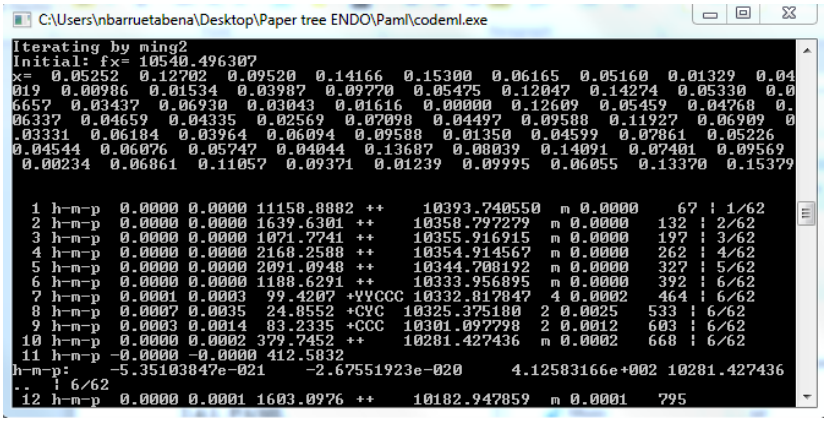


Figure 2.16. PAML program running the calculations for the ancestral reconstruction of proteins

Once everything was ready, the executable file was run and a rst file was created, containing all the information concerning the process the posterior probabilities and the joint and marginal protein reconstructions. **Figure 2.17** shows the information obtained in this type of files.

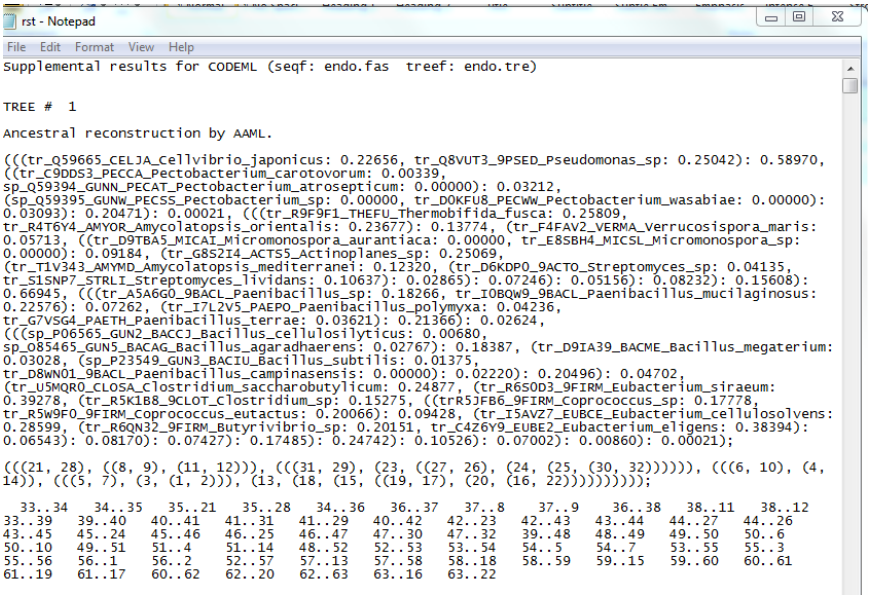


Figure 2.17. Rst file obtained in PAML. *All the information of the tree is contained in this file, including joint and marginal probabilities of each node.*

2.5. Applications

The applications for ancestral reconstruction have increased exponentially in the last two decades. In the field of molecular evolution the more outstanding advances have been in the optimization of the fluorescence performance of opsins [126] and GFP proteins [127], novel anticancer drug's mechanism and design [32], the uric acid and evolution in mammals [128], the amino acid persistence in proteins [57] or mammalian diving capacity evolution [129].

Regarding to other applications, the fields of usage have been such as, calculating spatial migration traits in order to infer the location of the ancestors [130], inferring ancestral ranges of species from phylogenetic trees in order to obtain historical biogeographic ranges [131] and genome rearrangements [132].

Chapter 3 : Experimental methods

3.1. Molecular biology techniques

The subsequent points explain the procedure of the molecular biology techniques used in this thesis in order to obtain both the ancestral and the extant cellulases in the laboratory. First, the DNA sequences that encode the protein of interest must be purchased. After that the genes need to be inserted into a bacterial expression vector, this is called ligation. Later, the plasmids are transformed into host bacteria in order to induce the protein expression. Once the protein of interest is expressed, the cells should be lysed to liberate the proteins. Finally, the resulting protein must be purified. This process is shown in **Figure 3.1**, step by step, since the cloning to the purify protein.

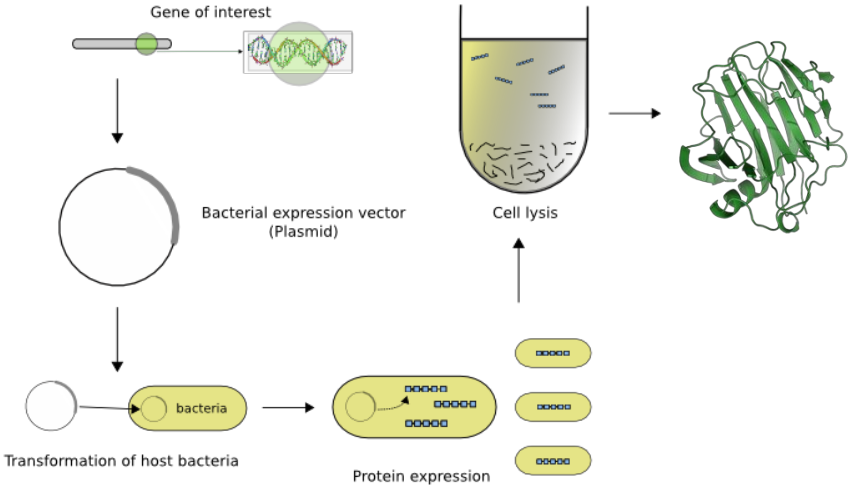


Figure 3.1. Schematic representation of the molecular biology techniques used to produce proteins. *First, the gene of interest is inserted in the expression plasmid and transformed in the bacteria. Then, the bacteria is grown and after the protein expression test a big culture is induced. Finally, the protein is obtained by cell lysis.*

3.1.1. Cloning of commercial plasmid

Cellulases encoding genes were codon optimized for E.coli and purchased in a commercial plasmid (Life Technologies). This plasmid contains an antibiotic resistance gene for the proper selection. This antibiotic was carbenicillin for all the genes but for the exoglucanase one that was kanamycin. The antibiotic ensures the proper selection of bacteria, being the only *E.Coli* colony grown in the plate. 1 µL of the commercial plasmid (50 ng/µL) was transformed into E.coli-XL1Blue competent cells (Agilent Technologies) following the manufacturer's protocol [133]. Once

transformation was performed, competent cells were grown in 400 µL of SOC medium (Invitrogen) for one hour and spread in LB-agar-antibiotic (the selected one in each case) plates and incubated overnight at 37 ºC.

Single colonies were isolated and grown in 10 mL of LB media + 1% 100 mg/mL kanamycin for 16 h at 37 ºC gently stirring. The harvesting of cells was made by centrifugation (14000 rpm, 10 min, 4 ºC, Eppendorf Centrifuge 5810R) and plasmids were extracted using a so called miniprep kit, DNA-plasmid extraction kit (Thermo Scientific) following the company's protocol [134]. Purified plasmids were eluted in 50 µl of nuclease-free water and their concentration was measured in the Nanodrop 2000L system.

3.1.2. Digestion of commercial plasmid

The enzymatic digestion of the commercial plasmid containing the cellulase gene was carried out after amplification. In order to perform the enzymatic digestion, a double digestion strategy with BamHI – KpnI cutting was used. BamHI and KpnI restriction sites are flanking the borders of the cellulase gene.

The enzymes used for the digestions were purchased from Thermo Scientific and the protocol used was the manufacturer´s Fast Digest protocol. The final digestion volume is adjusted to 50 µL and incubated at 37 ºC for one hour. The screening of the digestion products was made in a DNA-agarose gel (1%) in TAE buffer. The running of the DNA-agarose gel was carried out using the BioRad agarose electrophoresis equipment for approximately 90 min. After this time, the band corresponding to the cellulase was extracted from the gel and the gene was purified with a

DNA-extraction kit from Thermo Scientific following the usual protocol [135]. Concentrations were also measured using the Nanodrop 2000L.

The host used for the insert was pQE80 plasmid, it can be seen in **Figure 3.2**.

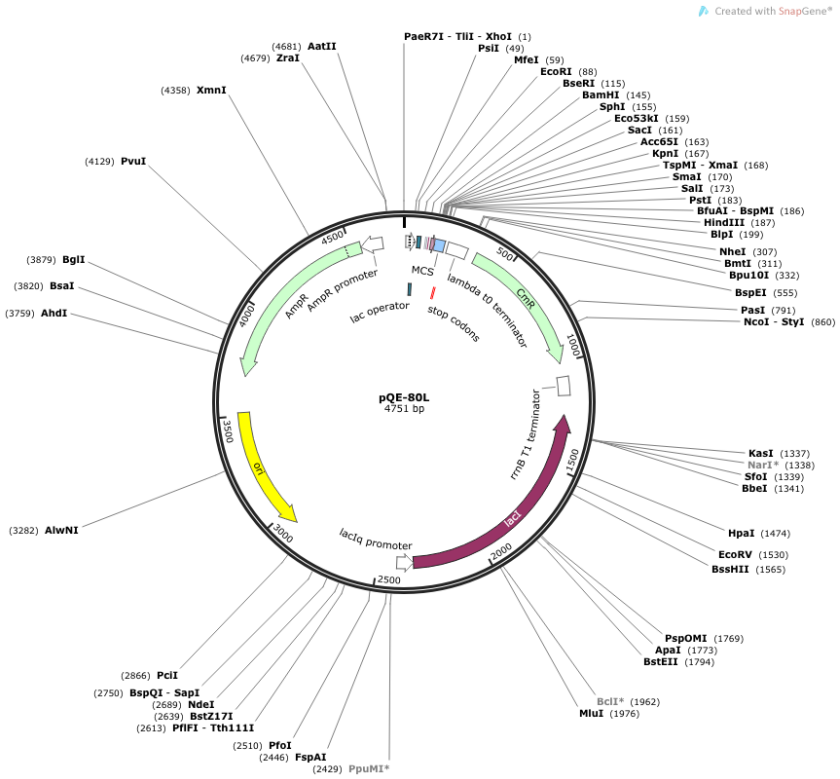


Figure 3.2. Scheme of the PQE80 plasmid use for hosting the cellulases.

In the section point (3.1.3) the protocol for the construction is explained.

3.1.3. pQE80-cellulase construct ligation

Once the digestion is made and the gene purified, the genes encoding the cellulase must be ligated onto a high-efficiency bacterial expression vector with compatible cohesive ends. The previously digested BamHI-pQE80-KpnI open plasmid was used (**Fig. 3.3**). This plasmid was a kind gift from Professor Julio Fernandez's lab at Columbia University. It also contains an ampicillin resistance gene. For the ligation of the gene encoding cellulase and the pQE80 plasmid Invitrogen's T4-DNA ligase protocol [136] was used. The mol ratio between the amount of plasmid vector and the cellulase gene insert is 3:1. With the following formula, the calculations for the needed amount of plasmid and DNA inserts were done. Ligations were incubated overnight at room temperature. Thereafter, to stop the process, ligations were diluted 5 times with deionized water.

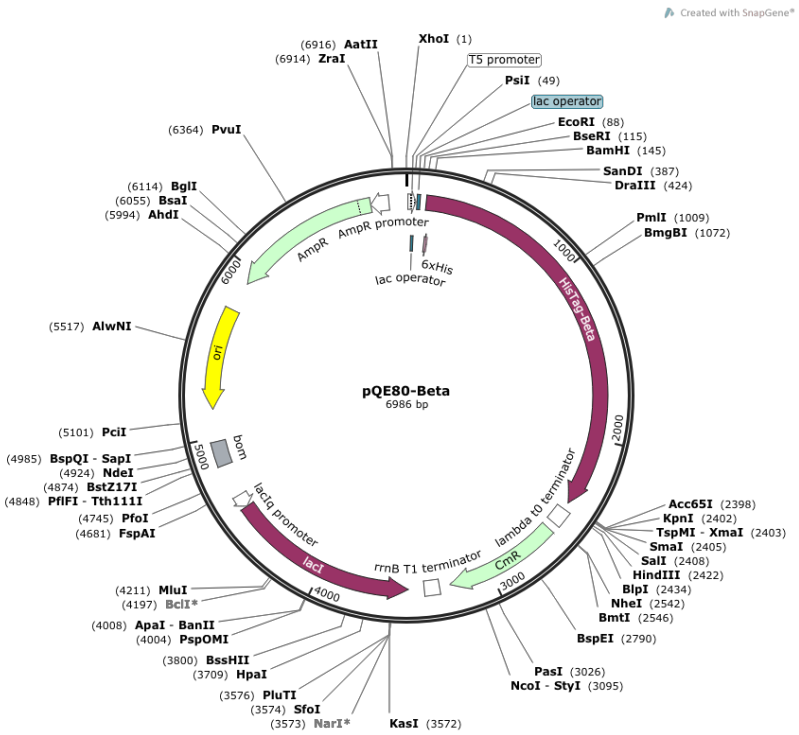


Figure 3.3. Schematic representation of the product of the ligation between the plasmid and the desired cellulase: *PQE80-beta-glucosidase ligation in this case.*

3.1.4. Cloning of pQE80-cellulase plasmid

5 µL of the recombinant plasmid (depending on the concentration) were transformed into E. coli-XL1Blue competent cells following the same protocol described in 3.1.1 section. Competent cells are later spread out onto LB-agar-ampicillin plates and incubated overnight at 37 ºC. In the same way previously described, single colonies were taken out and grown in 10 mL of LB media + 0.1% 100 mg/mL ampicillin for 16 h at 37 ºC. Finally, cells were harvested by centrifugation (14000 rpm, 10

min, 4 ºC) and plasmids were extracted using the same so called miniprep kit, DNA plasmid extraction kit. The purified plasmids were eluted in 50 µl nuclease-free water and their concentration was measured in the Nanodrop 2000L. The plasmids are screened and verified in a DNA-agarose (1%) gel and concentration is calculated using the Nanodrop 2000L system.

3.1.5. Screening

Once the pQE80-cellulase constructions were made and amplified, an amount between 1-10 µL of the plasmid was transformed onto E.coli-BL21 competent cells following the seller protocol [137]. After transformation, cells were grown as previously was made with EColi-Xl1blue in 400 µL of SOC medium for 1 hour at 37 ºC and spread out in LB-agar plates with the corresponding antibiotic. Plates were incubated overnight at 37 ºC to grow the colonies. Some single colonies were isolated and grown in 10 mL LB medium + antibiotic for 8 hours or until the optical density (OD) of the medium reached 0.6. ODs were measured with the Nanodrop 2000L.

In order to induce the overexpression of cellulases by T7 promoter activation, 5 µL of IPTG (isopropyl-β-D-thiogalactopyranosid, Sigma Aldrich) 100 mg/mL was added to the half of the volume of medium and the solution was incubated overnight at 37 ºC. 1 mL of each colony was taken then, to screen the overexpression. Bacteria were harvested by centrifugation (14000 rpm, 10 min, 20 ºC). Supernatant was discarded and bacteria were resuspended in 20 µL of extraction buffer. 20 µL of 2xSDS page Sample Buffer solution was added to each sample for the denaturation and charging of the protein in acrylamide electrophoresis gel separation. The samples were again centrifuged (14000 rpm, 30 min 20 ºC) and boiled at 95 ºC for 3 min.

The screening was carried out by running 20 µL of each of the solutions are run in an 8-12% acrylamide gel for approximately 1 hour in a BioRad acrylamide electrophoresis system. 12% gel has been used in the case of endoglucanase as it size is 33kDa. However in the case of exoglucanase 70kDa and beta-glucosidase 82kDa, 8% acrylamide gel has been used After the run, gels were cleaned in deionized water for 30 min. Proteins in the gel were stained with Bradford solution (Thermo Scientific) for 20 min and cleaned with deionized water again. Negative controls without IPTG are also added to the gel to visualize the overexpression better (**Fig. 3.4**).

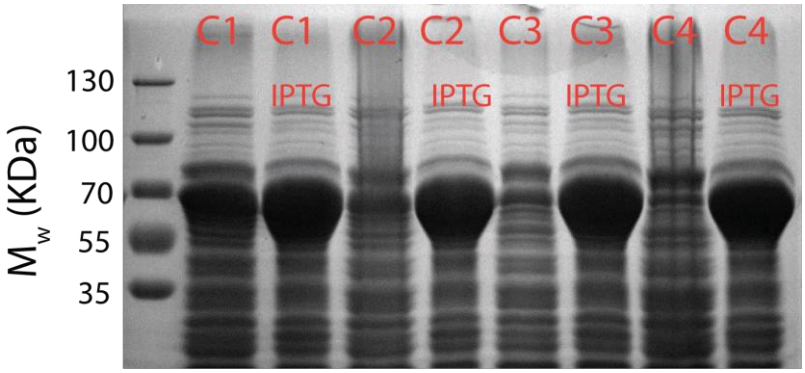


Figure 3.4. 8% Acrylamide electrophoresis gel for beta-glucosidase screening (~82000 KDa). Protein ladder (Thermofisher) can be visualized in the left side of the picture**.** C1-C4 nomenclature refers to the 4 colonies isolated. The ones in which iptg is written are the induce dones.

3.1.6. Protein production

The best overexpressed colony was selected and 1 mL of LB media with the desired bacteria was added to 1 L more LB media + 0.1% 100 mg/mL of the corresponding antibiotic + 0.1% 50

mg/mL chloramphenicol (it was added to maintain the ability of overexpression of the bacterial pLys system). The culture was incubated for about 8h until OD > 0.6 at 37 ºC shaking (250 rpm). Once the desired OD was reached, 0.1% 100 mg/mL IPTG was added to induce the overexpression of the protein. The culture was again incubated overnight (16 h more or less) at 37 ºC while shaking.

After doing this, bacteria were separated from the media by centrifugation (4000 rpm, 4 ºC, 20 min) and the supernatant discarded. The pellet was then resuspendend in 16 mL of extraction buffer and 160 µL of protease inhibitor (Merck Millipore) was added and incubated rocking (5 rpm) for 30 min at 4ºC with 160 µL of 100 mg/mL lysozyme (Thermo Scientific) solution for the enzymatic destabilization of the bacterial membrane. Once this is done, a series of reactives are added: 1.6 mL of 10% Triton X-100 (Sigma Aldrich) for the chemical destabilization of the bacterial membrane; 80 µL of 11 mg/mL DNAse I (Invitrogen) for the enzymatic degradation of DNA; 80 µL of 1 mg/mL RNAse A (Ambion) for the enzymatic degradation of RNA; 160 µL of 1M MgCl2 (Sigma Aldrich) as a catalyzer to increase the enzymatic activity of DNAse and RNAse. The suspension is incubated again for 10 min at 4 ºC with rocking prior to the cell lysis. Cell lysis was carried out by French press (G. Heinemann HTU DIGI-F Press). Cells were introduced in the press chamber and lysed at 18000 psi during 30 min. The lysis product obtained was then centrifuged in a high-speed centrifugation system (33000 rpm, 4 ºC, 90 min; Beckman Coulter Avanti J-26 XPI).

3.1.7. Protein purification

Regarding to the purification process, four different enzymes have been purified during this thesis and different purification processes have been carried out for them. The details have been described below.

3.1.7.1. Ancestral endoglucanase

The purification of ancestral endoglucanase was carried out first by temperature and then using a HisTrap column. After the centrifugation described in section 3.1.6 (in this case with 30min is enough) the supernatant was transferred to a 50 ml tube and it was incubated in a water bath at 50 ºC for 20 min. Then, the sample was cooled in ice for 5 min and centrifuged to eliminate debris at 4000xg for 10 min.

After the temperature step, the second step was carried out with the HisTrap cobalt affinity resin (Thermo Scientific). All the cellulase constructs contain a HisTag composed of 6 consecutive histidines in the N terminus of the construct which poses the ability to specifically bind to the cobalt affinity column. This binding was later eluted by adding imidazol in the buffer. A 150mM imidazole buffer was used for the elution.

3.1.7.2. Ancestral exoglucanase

For exoglucanase, the first purification process was carried out by means of a HisTrap nickel affinity resin (Thermo Scientific). In this case, niquel one was used, as the exoglucanase is harder to

purify. The niquel resin has a stronger affinity but it is not as specific as the cobalt one is. This binding can was later eluted by adding imidazol in the buffer. A 150mM imidazole buffer was used for the elution.

The second purificiation process used was by means of size exclusion and it was carried out with an ÄKTA pure fast protein liquid chromatography (FPLC) system (GE Healthcare) with a Superdex 200 column of 30 cm (GE Healthcare). Fractions of interest were collected from the chromatogram and stored in Acetate buffer 50mM (pH 5.5).

3.1.7.3. Ancestral beta-glucosidase

In the case of beta-glucosidase, the same process was used with some changes. In the first purification process instead of using niquel resin, cobalt resin was used.

Regarding to the second purification process, the buffer used for the elution in the size exclusion process was PBS (pH7).

3.1.7.4. Extant T.maritima

The extant *T.maritima* was purified in the same way of the ancestral endoglucanase, both for the first purification step and for the second purification step.

3.1.8. T.reesei cocktail protein determination

The determination of the protein content of the cocktail was first made by the dry weight method [138] for protein content determination. For that porpoise size exclusion chromatography was used, using a Superdex 200HR column, eluted in water. Then the sample was freeze dried and it was weighted. Second, absorbance at 280 was measured of a purified fraction and used densitometry and mass spectrometry for determining

concentration of endoglucanase. Moreover, the protein concentration was determined by the BCA assay (Pierce) [139] using a BSA standard supplied with the kit and a standard of our ancestral endoglucanase LFCA.

3.2. Biochemical assays

3.2.1 CMC

This assay is specific for measuring the endoglucanases activity. Endo-b-1,4-D-glucanase (EC 3.2.1.4) randomly cleaves accessible intermolecular b-1,4-glucosidic bonds on the surface of cellulose. Water-soluble derivatives of cellulose such as carboxymethylcellulose (CMC) and hydroxyethylcellulose (HEC) are commonly used for endoglucanase activity assays because insoluble cellulose has very low accessible fractionation of b-glucosidase bonds to cellulase [140-142]. The reaction of hydrolysis can be determined in different ways: by measuring the changes in reducing sugars, viscosity or color but the assay recommended for the endoglucanase (CMCase) assay is a fixed conversion method. This method, requires 0.5 mg of absolute glucose released under the reaction condition [143]. The reducing sugars concentration is finally measured by the DNS method [144].

Cellulolytic activity of ancestral endoglucanase (LFCA) was tested at 50 mM and pH 4.8 citrate buffer with 2 % CMC (Sigma), 30 min at various incubation temperatures. Cellulases from *T.maritima* and *T.reesei* (1,4-(1,3:1,4)-β-D-Glucan 4-glucano-hydrolase (EC 3.2.1.4), C2730 Sigma Aldrich) were used as controls. In addition two blanks were also prepared; the substrate blank (0.5 ml of CMC solution + 0.5 ml of citrate buffer) and the enzyme blank (0.5 ml of CMC solution + 0.5 ml

of dilute enzyme solution). Both the substrate and enzyme blanks were treated identically as the experimental tubes. Enzymatic reactions were terminated by placing the tubes into an ice-water bath. Enzymatic activity was determined quantitatively by measuring soluble reducing sugars released from the cellulosic substrate by the dinitrosalicylic acid (DNS) method. A volume of 3 ml of the DNS solution was added to each sample and the reaction mixtures were boiled for 5 min. After boiling, tubes were cooled and after adding 20ml of distilled water, absorbance was measured at 540 nm.

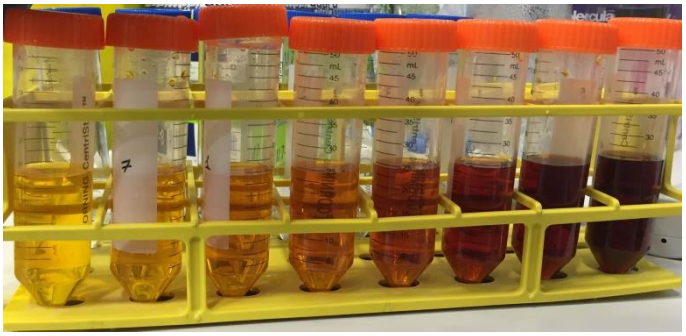


Figure 3.5. Color change between blanks and reactions with DNS method. *The yellow one is the blank, the darker the color is the bigger the reaction. It means that more sugar has been produced in the reaction.*

A glucose standard curve was used to determine the concentration of the released reducing sugars. For this porpoise, the following standards were prepared: GS1 – 0.125 ml of 2 mg/ml glucose + 0.875 ml of buffer. GS2 – 0.250 ml of 2 mg/ml glucose + 0.750 ml of buffer. GS3 – 0.330 ml of 2 mg/ml glucose + 0.670 ml of buffer. GS4 – 0.500 ml of 2 mg/ml glucose + 0.500 ml of buffer. The glucose released by the enzyme solutions was calculated with

deduction of the enzyme blank absorbance based on the glucose standard curve.

The determination of the pH dependence was done as following: purified enzymes were diluted in 50mM buffer at different pH values between 4 and 12. Activities were measured with 2% CMC at 70ºC for 30 min. All assays were performed in triplicate and the average value with standard deviation was determined.

3.2.1.1. Residual and long-term activity measurements

On the one hand the determination of the residual activity was carried out, to determine when the enzyme loses half of its activity. The enzymes diluted in citrate buffer 50 mM at their optimum pH, were incubated at different temperatures (60-90ºC). The residual activity was measured on 2% CMC for 30 min at 60ºC. The amount of reducing sugars was measured and quantified by the DNS method. The parameter T_{50} is defined as the temperature at which an enzyme loses 50% of its optimal activity after a 30 min heat treatment [145].

On the other hand, a study of the activity of the enzymes in different times was done, the long-term activity. In this case, all measurements were conducted in 50 mM citrate buffer, pH 4.8 on 2% CMC at 60°C for a period of 10 to 240 minutes. After hydrolysis, the reducing sugar concentration was measured by the DNS method.

3.2.1.2. Inactivation constant (Kin) determination

The objective of this assay was the determination of the inactivation constant, for this purpose, enzymes were incubated at

80ºC during different time intervals diluted in their optimum pH. The amount of reducing sugar was measured and quantified by the DNS method. The inactivation constant (K_{in}) was calculated using the equation *log* (% residual activity) = 2.303 x K_{in} x t, where t is time [11]. The half-lives of the enzymes were calculated from the plot.

3.2.2 CellG3

Endoglucanase activity was also measured using another different method, the CellG3 method of an endoglucanase assay kit (K-CellG3, Megazyme International, Ireland)[146]. As controls, cellulases from *T.maritima* and *T.reesei* (C2730, Sigma Aldrich) were used. Enzyme samples were diluted in acetate buffer (100 mM, pH 4.5) and after the addition of CellG3 substrate enzyme solutions were incubated at different pH´s and temperatures. The incubation was carried out for 10 min. Cellulase cleaved a bond within BClPNPβ-G3, the non-blocked reaction product containing the 2-chloro-4-nitrophenyl substituent was instantly cleaved to D-glucose and free 2-Cl-4-nitrophenol (ClPNP). Finally, the hydrolysis reaction was stopped by addition of Trizma base solution (pH 9) and the Cl-phenolate color was developed and measured at 400 nm (NanoDrop 2000C). CellG3 Unit was defined as the amount of enzyme required to release one micromole of 2-chloro-4-nitrophenol from CellG3 in one minute under the defined assay conditions, the enzyme activity was calculated multiplying the measured absorbance at 400 nm by 9.64 and by the dilution factor [146].

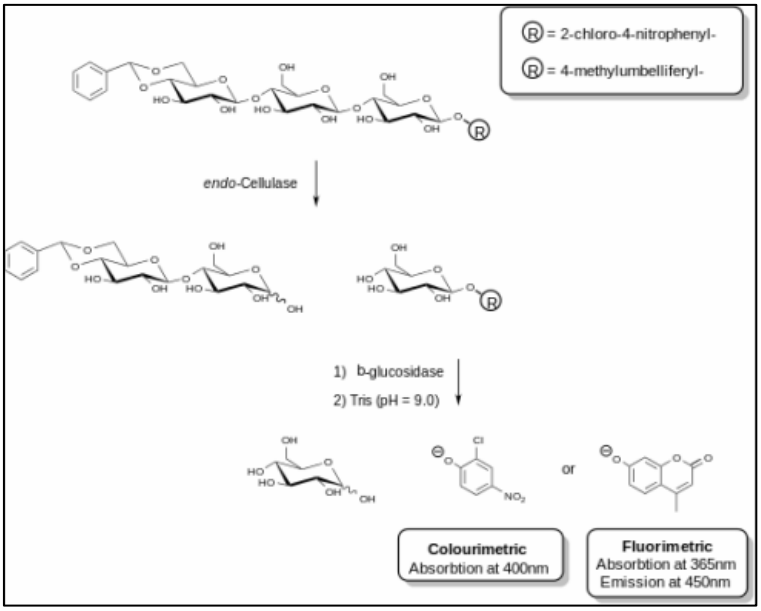


Figure 3.6. Colorometric change reaction of CellG3 kit [15]. *Cellulase cleaved a bond within BClPNPβ-G3, the non-blocked reaction product containing the 2-chloro-4-nitrophenyl substituent was instantly cleaved to D-glucose and free 2-Cl-4-nitrophenol (ClPNP).The color changed to yellow at 400nm absorbance in presence of beta-glucanase.*

3.2.3 Filter-paper

In this case, this assay was used for the determination of a total cellulase system made of three cellulases: endoglucanases, exoglucanases, and b-glucosidases. Total cellulase activities were measured using insoluble substrates, including pure cellulosic substrates such as Whatman No. 1 filter paper or any other lignocellulosic substrate [147]. Filter-paper assay FPA is the most common total cellulase activity assay recommended by IUPAC .

The assay based on a fixed conversion degree, measures the hydrolysis of both, crystalline and amorphous cellulose of the filter paper. In this case, the activity of the total cellulase is described in terms of filter-paper units (FPU).

The filter paper activity (FPA) of cellulase enzymes was carried out in a mixture containing 0.5 mL diluted enzyme by 50 mM citrate buffer (pH 4.8) and 50 mg of Whatman No. 1 filter paper and incubated at various temperatures for 1 h. CellicCTec2 (Novozymes) enzyme cocktail was used as a control. Apart from the reactions, three blanks were also prepared: Reagent blank (1.5 ml of 50 mM citrate buffer) enzyme blank (1.0 ml of 50 mM citrate buffer + 0.5 ml enzyme dilution) and substrate blank (1.5 ml of 50 mM citrate buffer + filter paper strip). All the blanks were treated identically as the experimental tubes. The reaction was finished placing the tubes on ice. The reducing sugars released were determined using the DNS method. 3 ml of DNS was added to all the tubes and after boiling for 5 min they were placed on ice again to stop the reaction. 0.5 ml of the colored solutions were withdrawn into 1.5-ml microcentrifuge tubes and centrifuged at 10000 g for 3 min. Finally, 2.5ml of distilled water was added to 0.2 ml of the supernatant and the absorbance was measured at 540 nm, where the absorbance of reagent blank was used as the blank.

In order to determine the released reducing sugars a standard curve was made by means of preparing the following standards: GS1: 1.0 ml of glucose standard + 4.0 ml buffer = 2 mg/ml (1.0 mg/0.5 ml). GS2: 1.0 ml of glucose standard + 2.0 ml buffer = 3.3 mg/ml (1.65 mg/0.5 ml). GS3: 1.0 ml of glucose standard + 1.0 ml buffer = 5 mg/ml (2.5 mg/0.5 ml). GS4: 1.0 ml of glucose standard + 0.5 ml buffer = 6.7 mg/ ml (3.35 mg/0.5 ml). Add 0.5

ml of GS1–4 solutions to 13 × 100 mm test tubes, and add 1.0 ml of 0.050 M citrate buffer.

Filter paper unit (FPU) is defined as 0.37 divided by the amount of enzyme that produces 2.0 mg glucose equivalents in 1 h from 50 mg of filter paper. All experiments were carried out in triplicates.

3.2.2.1. Lignocellulosic substrates hydrolysis

The protocol used for this assay was the same that the one for filter paper, the only difference is the substrate. But not only this, we have also added more cellulolytic enzymes such as laccase and xylanase. 50 mg of different lignocellulosic substrates in 50 mM citrate buffer at pH 4.8 were used. Enzyme hydrolysis was performed for 1 hour at 50°C. Endoglucanase alone or in combination with Laccase and Xylanase were used for hydrolysis of the lignocellulosic material. Three different enzyme combinations were used differing in the endoglucanase used: ancestral, *T. maritima* or *T. reesei*. Cellulose degradation was determined by determining percentage of hydrolysis as described elsewhere[148].

3.2.3 Avicel

In this case, a crystalline substrate was used for the cellulolytic activity with mixtures of the free enzymes (0.5 μM each) at 0.5 μM buffer acetate (50 mM final concentration) with 1 % Avicel (FMC, Delaware USA) at various temperatures and pH's for 24 hours. 0.4ml of the enzymes solutions was placed together with 1.6ml of Avicel solution. Also two blank were done: a substrate blank (1.6ml of Avicel solution + 0.4ml of acetate buffer) and an enzyme blank (1.6ml of acetate buffer + 0.4ml of

enzyme solution) [149]. Enzymatic reactions were stopped by placing the tubes into an ice-water bath, and the tubes were then be centrifuged for 2 min at 14,000 rpm at room temperature. Enzymatic activity was determined quantitatively by measuring soluble reducing sugars released from the cellulosic substrate by the dinitrosalicylic acid (DNS) method. A volume of 150 µL of the DNS solution was added to 100 µL of sample (supernatant fluids), and after boiling the reaction mixture for 10 min, absorbance at 540 nm was measured. Released sugar concentrations were determined using a glucose standard curve. Glucose concentration was determined using a glucose assay kit [150](GOD; Sigma-Aldrich) according to the manufacturer's instructions. All assays were performed at least twice in triplicate.

3.2.4. Thermal stability of the ancestral endoglucanase: Circular Dichroism

The thermal stability of the ancestral endoglucanase was determined by Circular dichroism (CD); measurements were made with a JASCO J-815 CD spectrophotometer. For each construct, spectra were generated by averaging five wavelength scans. Thermal unfolding transitions were monitored at 222 nm, with a 0.5°C step size, within the range of 55 to 110°C, in a thermal-resistant 10-mm quartz cuvette. Thermal denaturations at pH 4.8 were carried out in 50 mM citrate buffer both with 0.5M Glycerol and without glycerol [151].

3.2.5. Ancestral endoglucanases Kinetic parameters determination

In order to determine the kinetics parameters of the ancestral endoglucanase, Km and Vmax, ten different substrate concentrations were used in the range of 2 to 20 mg/ml CMC for endoglucanase. The Km and Vmax were determined

directly from the hyperbolic curve fitting of Michaelis-Menten equation generated using Phyton inhouse script. Kcat was determined by the formula Vmax/Et, where Et is the total enzyme concentration in μmol/ml [152].

3.3.Cellulosome

3.3.1. Minicellulosome

Two mini-scaffoldins were designed in this study consisting of components from *C. thermocellum* CipA scaffoldin.

3.3.1.1. Minicellulosome constructs

The X-module and type II dockerin dyad and the CBM were amplified from pET28-XDock and pET28-CBM, respectively (a kind gift by Prof. Ed Bayer, Weizmann Institute, Israel). Cohesin 7 was amplified from pAFM-c7A[153]. First, XDock was amplified with primers incorporating NdeI, NheI, KpnI and SpeI sites at the 5' end and 2 STOP codons and a XhoI site at the 3' end. The resulting fragment was cloned into pET28 vector using NdeI and XhoI sites. Then the CBM was amplified and cloned into the previous vector using NdeI and NheI sites. Next, cohesin 7 sequence was cloned using KpnI and SpeI sites to generate pET28-Scaf1. A second copy of cohesin 7 was then cloned into this vector in SpeI site to generate pETScaf2, containing 2 tandem cohesins. Both miniscaffoldins carried a hexa-histidine tag at the N-terminus.

Integration of the LFCA endoglucanase into the minicellulosome was accomplished by cloning the LFCA endoglucanase sequence into a pET28a vector between the NcoI and EcoRI sites. Then, the

sequence of *C. thermocellum* Cel8A dockerin (and N-terminal linker) was PCR amplified and cloned at the C-terminus of the LFCA sequence between EcoRI and XhoI sites thus generating pET28-LFCA_Dockerin that carries a C-terminal hexahistidine tag. LFCA_CBM was generated by replacing the Cel8A dockerin with a sequence containing the linker between Cel8A catalytic domain and dockerin, followed by the CipA CBM.Both mini-scaffoldins and LFCA endoglucanase fusion proteins were expressed in *E. coli* BL21 star. Expression of miniscaffoldins was carried out at 16ºC with 0.1 mM IPTG overnight, while LFCA fusions and Cel8A were expressed at 37ºC for 3 hours in 1 mM IPTG. Cultures were lysed by enzymatic means in 1 mg/ml lysozyme, 1% Triton X-100, 5 µg/ml DNAseI and 5 µg/ml RNAse A and centrifuged to remove cell debris. Clarified samples were incubated at 55ºC for 20 min, cooled in ice and centrifuged to eliminate aggregated proteins. Affinity purification was then carried out using HisTrap columns in an ÅKTA Purifier FPLC (GE healthcare). Sample purity was evaluated by SDS-PAGE and proteins were concentrated in Tris 50 mM, NaCl 300 mM, $CaCl_2$ 1 mM pH 7, quantified by absorbance at 280 nm with a NanoDrop (ThermoScientific) and stored in 50% glycerol. Mini-cellulosome assembly assays were performed by native-PAGE. Different relations of proteins were incubated in 50 mM Tris, 300 mM NaCl, 1mM $CaCl_2$ pH 7 at 37ºC for 1h before running the gel. SdbA cohesin was also added to block XDock in the scaffoldin. The true enzyme-scaffoldin ratio was determined from this analysis according to that ratio were no free protein was found in excess. This ratio was used in the following experiments.

Microcrystalline cellulose binding was assayed as described previously [21]. Briefly, 10 µg of protein was incubated with 10 mg of Avicel (SigmaAldrich) at 4ºC for 1h with gentle agitation. Samples were centrifuged and the supernatant was stored as the

unbound fraction. The pellet was washed three times and used as the bound fraction. Both samples were then analyzed by SDS-PAGE and BSA was used as a control.

3.3.1.2. Minicellulosome Activity assays

Proteins were incubated in Acetate buffer pH 5.5 containing 100 mM NaCl, 12 mM $CaCl_2$ and 2 mM EDTA for 1 h at 37ºC to allow complex formation. Enzymes were used at 0.5 μM (for Avicel and PASC analyses) and at 0.35 μM for CMC assays. Scaffoldins were added at equimolar concentration according to native-PAGE analysis. BSA was added in all samples to minimize unspecific enzyme-substrate interactions. Avicel assays were conducted for 24 h in an orbital shaker in 2-ml tubes containing a wing magnet to improve stirring so that this insoluble substrate did not precipitate. PASC was prepared as described elsewhere [154]. Assays in this substrate were conducted in similar tubes but in a heating block for 30 min. After incubation time, samples were centrifuged and the soluble sugars present in solution in the supernatant were determined by the DNS assay. Absorbance was measured in a 96-well plate using a FLUOstar fluorimeter (BMG Labtech, Germany) in the absorbance mode. CMC assays were conducted in a heating block using azo-CMC (Megazyme) as a substrate. The activity was determined according to the manufacturer's indications.

3.3.2. Cellulosome

3.3.2.1. Cellulosome design

Plasmid and Primers design for cloning the Ancestral cellulases in the cellulosome system.

3.3.2.2. Cellulosome construction

3.3.2.2.1. Cloning of the new designs in the expression plasmid by PCR

PCRs was performed with Phusion High Fidelity DNA polymerase F530-S (New England Biolabs, Inc), and PCR products and plasmids were restricted with Fastdigest enzymes (Thermo scientific, USA). T4 DNA ligase used for ligation (Fermentas UAB, Vilnius, Lithuania). PCR products were purified using a HiYield™ Gel/PCR Fragments Extraction Kit (Real Biotech Corporation, RBC, Taiwan), and plasmids were extracted using Qiagen miniprep kit (Valencia, CA) (**Fig 3.7**). Competent *Escherichia coli* XL1 cells were used for plasmid transformation.

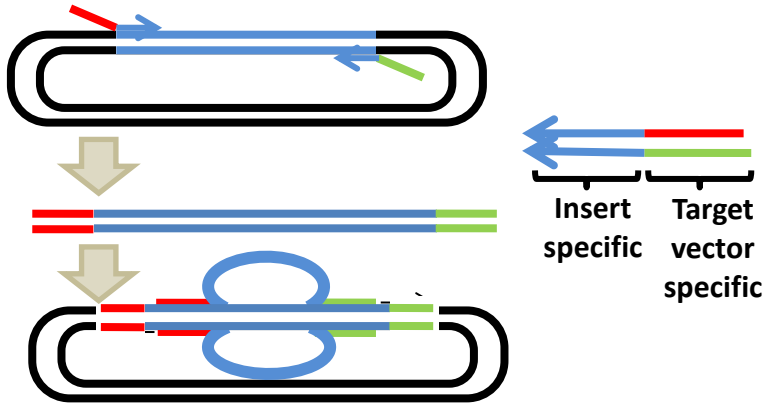


Figure 3.7. Phusion PCR. *Schematic representation of the procedure need for phusion PCR.*

3.3.2.2.2. Recombinant protein expression

We produced the recombinant proteins in *E. coli* BL21 (DE3) grown in 2 L LB (Luria Broth) and 2 mM $CaCl_2$ with the appropriate antibiotic at 37 °C until A_{600}≈0.8–1 and induced by adding 0.1 mM (final concentration) isopropyl-1-thio-β-D-galactoside (IPTG) (Fermentas UAB Vilnius, Lithuania). Cell growth were left at 16 °C overnight. Cells were harvested by centrifugation at 5000 rpm for 5 min. Pelleted cells were resuspended in 30 mL TBS containing 5 mM imidazole (Tris-buffered saline, 137 mM NaCl, 2.7 mM KCl, 25 mM Tris–HCl, pH 7.4). The His-tagged enzymes will be purified on a Ni–NTA column (Qiagen). Acrylamide gels SDS-PAGE (10 %) were used to assess the purity of the recombinant proteins and absorbance at 280 nm indicated their concentrations. We stored the proteins in 50 % (v/v) glycerol at −20 °C.

3.3.2.2.3. Gel electrophoresis

Each enzymatic component was first calibrated for its optimal ratio for full complex formation with the chimaeric scaffoldin. The three enzymes were then be mixed at their optimized ratio with the scaffoldin to ensure full complex formation. Protein mixtures supplemented with 12 mM $CaCl_2$ and 0.05 % Tween 20 and incubated for 2 h at 37 °C. The electrophoretic mobility of the proteins were then analyzed by PAGE under non-denaturing conditions with gels comprising a 4.3 % stacking gel and a 9 % separation gel. Migration were carried out at 100 V. The gels were stained using InstantBlue Coomassie-based staining (Expedeon, USA).

3.3.2.2.4. Affinity-based ELISA

The specificities of the cohesins for the chimeric dockerin-bearing enzymes were examined semiquantitatively by a sensitive enzyme-linked affinity assay in microtiter plates [155]. MaxiSorp ELISA plates (Nunc A/S, Roskilde,Denmark) were coated overnight at 4ºC with predetermined concentrations (designated below) of the desired CBM-Coh(100 ml/well) in 0.1M sodium carbonate (pH 9). The follow-ing steps were performed at room temperature with all reagents at a volume of 100 ml/well. The coating solution was discarded and blocking buffer (TBS, 10 mM CaCl2, 0.05% Tween 20, 2% BSA) was added (1 h incubation). The blocking buffer was discarded, and incremental concentrations of the desired EndoDockT or ExoDocG constructs, diluted in blocking buffer, were added. After a 1 h incubation period, the plates were washed three times with wash buffer (blocking buffer without BSA), and the primary antibody preparation was added. Following another 1 h incubation period, the plates were washed three times with wash buffer and the secondary antibody preparation was added. After another 1 h incubation, the plates were again washed (four times) with wash buffer and 100 ml/well TMB þ Substrate-Chromogen were added. Color formation was terminated upon addition of 1M H_2SO_4 (50 ml/well), and the absorbance was measured at 450 nm using a tunable microplate reader.

Chapter 4: Phylogenetic results

In this chapter I will describe the computational procedures utilized for the reconstruction of ancestral forms of cellulase enzymes. I have worked with three different cellulases enzymes, i.e., endoglucanase, exoglucanase and β-glucosidase. I will describe how the sequences for these enzymes are retrieved from internet databases and how they are handled in order to construct a phylogenetic chronogram. This chronogram is the base of the reconstruction process since it provides the overall framework of molecular relationships of all the sequence used in its construction. I will describe the different computational methodologies for tree building based on their statistical basis. These methods include parsimony, maximum likelihood and Bayesian inference. Although I have worked with all of them, I will mainly report the results on Bayesian inference [92-96]. In each phylogenetic tree we can find a series of internal nodes connecting different branches. Each one of these nodes represents the most probable sequence of the common ancestor of the connecting groups of species. I will select one node of each tree

based on different considerations such as age, position in the tree and importance, given the selected group of species. These genes encoding the sequences of the nodes will be synthesize, and reconstructed in the laboratory for testing.

4.1. Reconstruction of an ancestral bacterial endoglucanase

The first cellulase enzyme that we have reconstructed is a bacterial endoglucanase. This enzyme hydrolyzes amorphous and crystalline cellulose by randomly cutting the cellulose fibers giving rise to nanofibers that vary in length all the way from a single glucose molecule to a polymer composed by hundreds of glucose units. The first step in the reconstruction is collecting a number of sequences of endoglucanases from different species. This number varies depending on availability, but generally is recommended to be high enough as to provide a good representation of different phyla. In the case of our endoglucanase we searched in the Uniprot [110] database using BLAST [111] tool as described in the methods section (2.3.1). We searched the endoglucanases from family Cel5A with CAZy [156] identification code 3.2.1.4. This family is important from and industrial point of view, since there are examples of enzymes from this family with outstanding properties used in industrial applications.

I selected as query the Cel5A endoglucanase from *Bacillus Subtillis* using the default parameters in the Uniprot BLAST website. From this search I retrieved 32 homologous sequences that were available at the time of the search in august of 2014. All the sequences are available in Appendix I including also the Uniprot ID. Most sequences belonged to three different bacterial phyla, i.e., Firmicutes, Actinobacteria and Proteobacteria. The sequences were used to construct a multiple alignment using MUSCLE [115, 116] software. A fragment of this alignment can

be found in the material and methods section (2.3.2). Upon close inspection of the alignment, we can identify a well aligned block section that corresponds to the catalytic domain of the endoglucanases. This block shows no major gap or unstructured regions. This contrasts with the carbohydrate binding module (CBM) that the sequences show either in the C or N termini. The CBM is poorly aligned demonstrating a molecular diversity that might reflect different origins for this module. For this reason, I decided to focus the analysis on the catalytic domain of the homologous sequences.

Taking the aligned portion corresponding to the catalytic domain, the alignment was manually edited to remove long gaps or portions that are poorly represented in all the sequences. Once the alignment is edited, I used Bayesian inference, to infer the phylogenetic relation between the endoglucanase enzymes. I used BEAST [121, 157] software as described in the methods section. In the final tree, we can see the sequences belonging to the three phyla used in the construction of the chronogram (Actinobacteria, Firmicutes and Proteobacteria) correctly separated. We used fossil and genetic data from the Time Tree Of Life (TTOL) [31, 158] to calibrate the tree. This can be done either as a variable for several nodes in the estimation of the tree or by directly retrieving that age of nodes from the TTOL. In the case of the endoglucanase, I used age data for some nodes from the TTOL as calibration point and estimated the age for all other nodes (see methods section for details). As a result of a calibrated tree, we obtain a chronogram. In our case the mutation rate is not fixed and is uncorrelated for each brand. Thus, we obtain an uncorrelated relaxed clock chronogram that follows a lognormal distribution for node age. The estimated age for the oldest node is more than 3 By (~3000

million years). From the tree, we have identified the common ancestor corresponding to each phylum, i.e, LPCA (Last Proteobacteria Common Ancestor), LACA (Last Actinobacteria Common Ancestor) and LFCA (Last Firmicutes Common Ancestor) (**Fig 4.1**).

Finally, I used PAML [124, 125] software for the reconstruction of the most probable aminoacid sequence of each node. PAML assigns to each position of the inferred sequence the residue with the highest posterior probability. This sequence is not unique and depends on the sequence used for the tree. However, the phenotypes displayed by the protein or gene of this sequence must be robust and independent of the sequences used. In general, the true value of a reconstructed sequence lies on the phenotype rather than on the genotype. Finally, I selected the sequence of the node corresponding to the LFCA, for synthesis and laboratory resurrection. I chose this particular node because old enough and likely displays phenotypes that can provide information about life and environmental condition of our planet about 3 Bya, such as the high temperature of oceans.

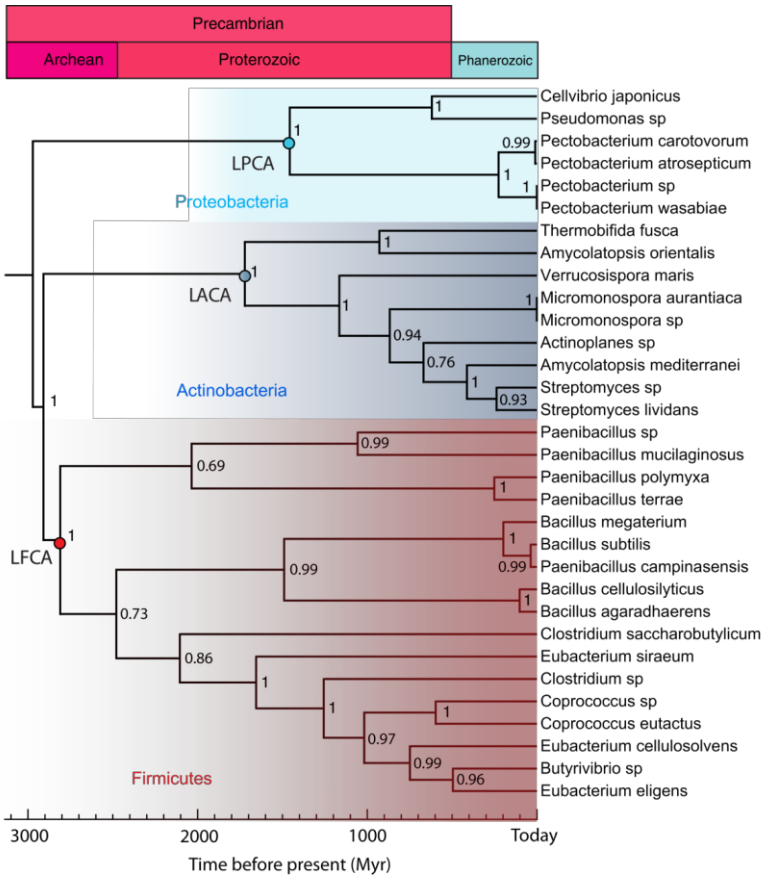


Figure 4.1. Uncorrelated relaxed clock chronogram for bacterial endoglucanases Cel5A*. A total of 32 sequences were used from three different phyla, Proteobacteria, Actinobacteria, and Firmicutes. The different species are indicated by UniProt identification codes. Divergence times were estimated using Bayesian inference and information from the TTOL. Geological scale and times are indicated. The internal node corresponding to the Last Firmicutes Common Ancestor (LFCA) was selected for reconstruction.*

4.1.1. Ancestral endoglucanase sequence analysis

From the tree in **figure 4.1** we selected the node that represents the last common ancestor of Firmicutes (LFCA) that lived ~2.8 Bya for the reconstruction. We speculate that this may have been one of the earliest cellulase enzymes. The ancestral reconstruction used a maximum likelihood [89-91, 159, 160] assignment at each site for the residue with the highest posterior probability. The posterior probabilities of all the aminoacids of the reconstructed sequence are shown in (**Fig 4.4**). The average posterior probability value is 0.95, which makes reliable the reconstruction.

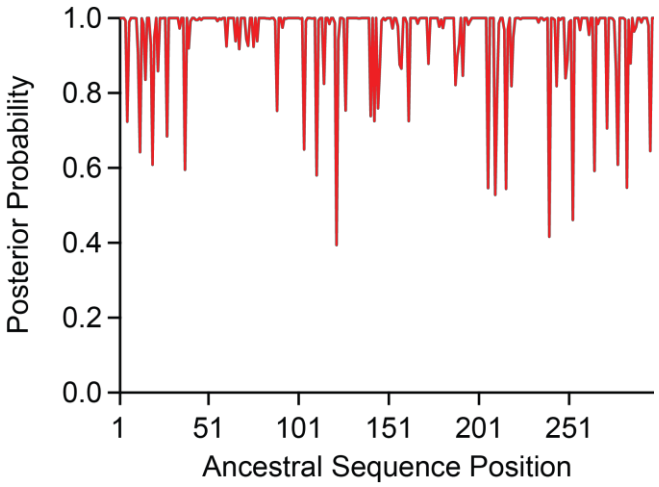


Figure 4.2. Posterior probability distribution for each inferred residue of the ancestral endoglucanase LFCA*. Each position corresponds to the residue with the highest posterior probability. The average posterior probability value is 0.95.*

Overall, the ancestral sequence from LFCA displays 71% identity with respect to their modern descendants. This implies that approximately 84 mutations occurred from the ancestral LFCA EG protein to the modern descendant that was used as a query.

4.2.1. Reconstruction of an ancestral bacterial exoglucanase

In the case of exoglucanase, I used the same procedure used in the case of the endoglucanase for the sequence selection; we took the sequences from the Uniprot database. The exoglucanase family I selected in this case was GH48 with CAZy identification code 3.2.1.176; I chose this family, because of its importance in industry. The selected query for the BLAST of the exoglucanase extant sequences was exoglucanase from *Thermobifida fusca.* Using this query, I selected 33 sequences for the alignment (See Appendix II). The sequences we chose, belonged to Actinobacteria and Firmicutes phyla. I generated a sequence alignment using the selected sequences and observed the same phenomena we previously saw for the endoglucanase; the catalytic domains of all of the sequences aligned correctly, forming a similar block of alignment than in the previous case. Nevertheless, it was not the case for the CBM, some sequences had the CBM at the C-termini and others at the N-termini, and there were numerous gaps. Thus, similarly to what we did for the endoglucanase, we made the alignment of the catalytic domain, without the CBM.

We used the sequences of the aligned catalytic domain to construct the phylogenetic chronogram of the exoglucanase CBHI. I inferred the tree using Bayesian inference (**Fig 4.2**). The root of the chronogram is dated 3 Bya and we could identify the common ancestors of the two phyla present in the tree, LACA (Last Actinobacteria Common Ancestor) and LFCA (Last Firmicutes Common Ancestor). I used PAML for the reconstruction of the most likely aminoacid sequence for each node and I selected the LACA node for the synthesis and laboratory experiments. I selected this exact node because of its

age and position in the tree. The age of the nodes was obtained using information from the TTOL.

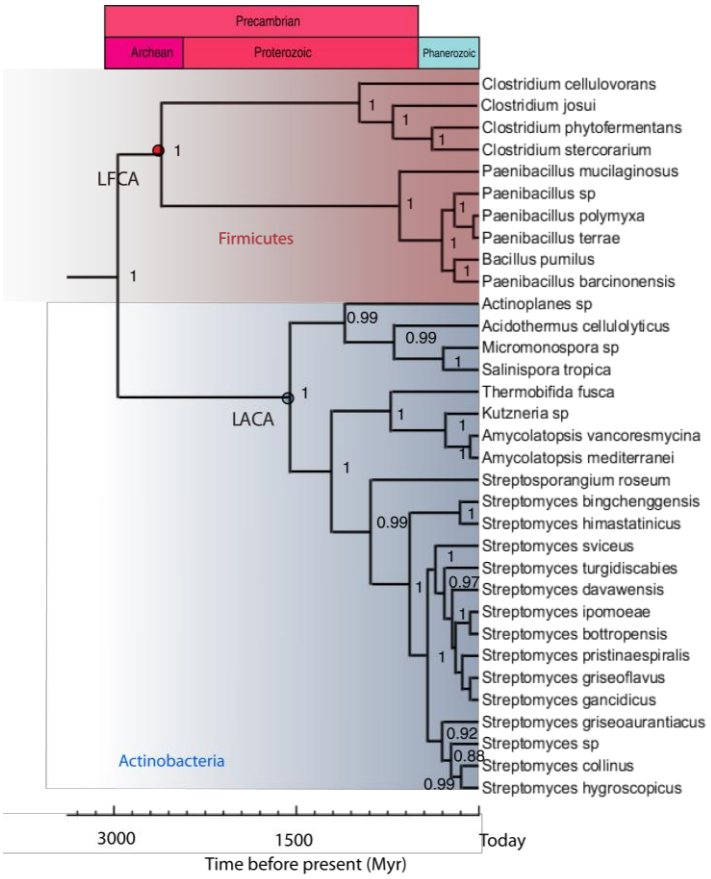


Figure 4.3. Uncorrelated relaxed clock chronogram for bacterial exoglucanase GH48*. A total of 33 sequences were used from Actinobacteria, and Firmicutes phyla. Divergence times were estimated using Bayesian inference and information from the TTOL[31]. Geological scale and times are indicated. The internal node corresponding to the Last Actinobacteria Common Ancestor (LACA) was selected for reconstruction.*

4.2.2. Ancestral exoglucanase sequence analysis

I made the reconstruction of the oldest exoglucanase ancestor that belonged to Actinobacteria phyla (LACA) of about 1300 Bya. We used maximum likelihood for the ancestral reconstruction, obtaining the residue with the highest posterior probability in each position. These posterior probabilities of all the aminoacids of the reconstructed sequence are shown in (**Fig 4.5**). The obtained average posterior probability value was 0.92, which makes reliable the reconstruction.

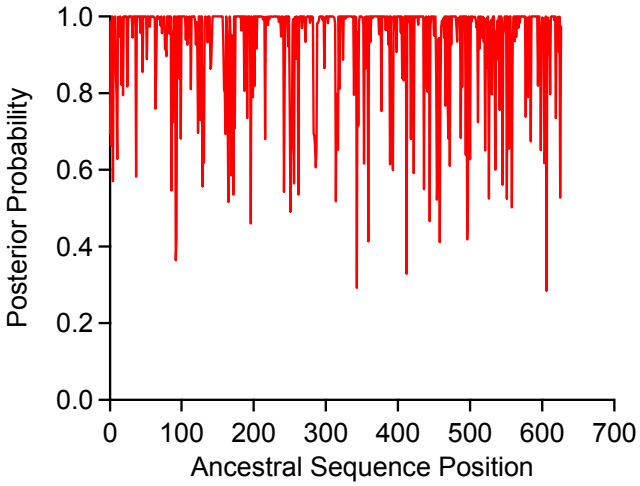


Figure 4.4. Posterior probability distribution for each inferred residue of the ancestral exoglucanase LACA*. Each position corresponds to the residue with the highest posterior probability. The average posterior probability value is 0.92.*

4.3.1. Reconstruction of an ancestral bacterial β-glucosidase

Regarding to the β-glucosidase chronogram, we used the same procedure described for the two previous enzymes. I retrieved the

sequences for the reconstruction from the Uniprot database. In this case, I selected bglII family of beta-glucosidases with CAZy identification code 3.2.1.21 as it is common to use enzymes from this family. I selected *Clostridium Thermocellum* as a query. I made the selection of the sequences in the same way of the previous cases, making a BLAST in Uniprot. 34 sequences of Actinobacteria and Firmicutes phyla were chosen for the reconstruction. As well as in the previous cases, we took the sequences for an alignment. The alignment was well-resolved without significant gaps or unstructured portions. The β-glucosidase cellulases do not have binding domains; they do not need this CBM as they are responsible of breaking the small cellobiose units and not crystalline cellulose regions. Due to the lack of this module, the alignment was easily resolved using the whole sequence of each specie for the alignment (All the sequences in appendix III).

I obtained a chronogram that diverged 3 Bya and we could identify the common ancestors of the two phyla present in the tree, LPCA (Last Proteobacteria Common Ancestor) and LFCA (Last Firmicutes Common Ancestor). I made the reconstruction of the most likely aminoacid sequence for each node using PAML and I selected the LACA node for the synthesis and laboratory experiments. The reason why I selected this node is its age and position in the tree. As well as in the other two cases, I determined the age of each nodes doing the datation with the TTOL.

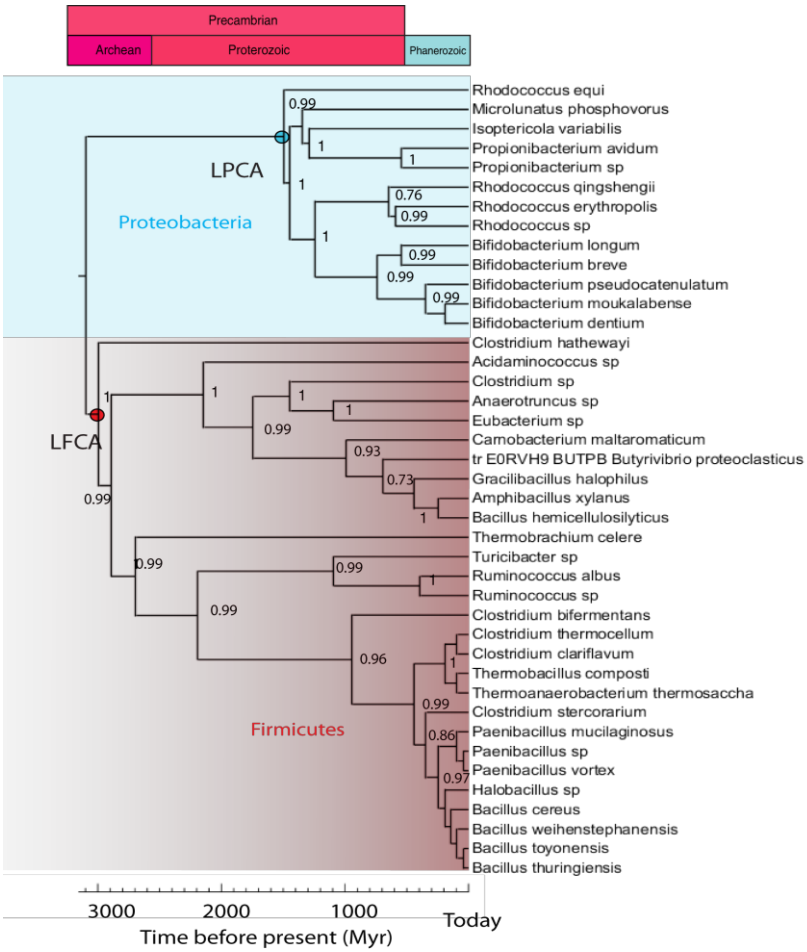


Figure 4.3. Uncorrelated relaxed clock chronogram for bacterial beta-glucosidase BglII*. A total of 34 sequences were used from Actinobacteria, and Firmicutes phyla. Divergence times were estimated using Bayesian inference and information from the TTOL. Geological scale and times are indicated. The internal node corresponding to the Last Firmicutes Common Ancestor (LFCA) was selected for reconstruction.*

4.3.2. Ancestral beta-glucosidase sequence analysis

Regarding to the beta-glucosidase, we selected the oldest ancestor of the Firmicutes phyla (LFCA) as well as in the endoglucanase case. We used maximum likelihood in this reconstruction too. The posterior probabilities for each aminoacid are represented in the (**Fig 4.6**). The value of the average of the posterior probabilities is 0.88.

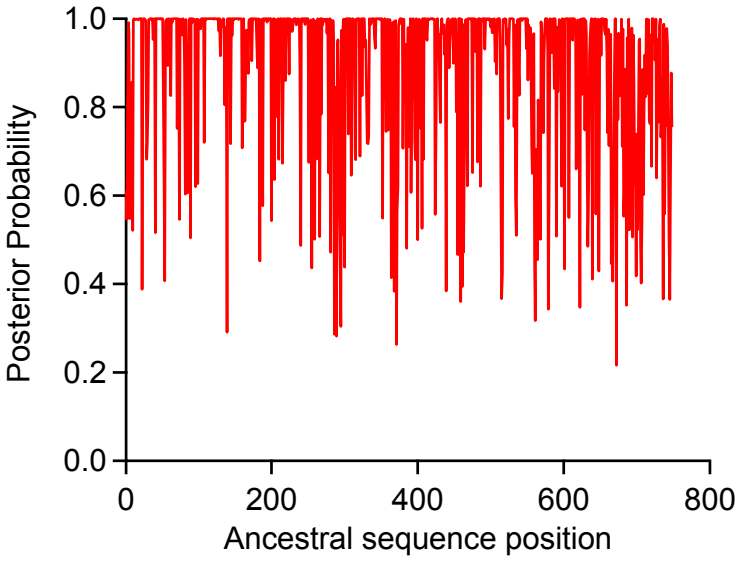


Figure 4.6. Posterior probability distribution for each inferred residue of the ancestral beta-glucosidase LFCA*. Each position corresponds to the residue with the highest posterior probability. The average posterior probability value is 0.88.*

Chapter 5: Experimental Results

In this chapter, I will describe the experimental results that show the performance and chemical properties of the reconstructed enzymes (endoglucanase, exoglucanase and β-glucosidase), obtained from the chronograms analyzed in Chapter 4. I will first describe the results of experimental synthesis and production of the ancestral enzymes using the standard molecular biology procedures. I will then report on the chemical performance of the resurrected enzymes under different conditions of temperature, pH and substrate. The ancestral enzymes will be compared individually and in a cocktail with extant endoglucanases used in biotechnological applications. Moreover, we tested the activity of the ancestral enzymes in a cellulosome, a bacterial molecular complex that incorporates several enzymes into scaffolding that enhances the performance of individual enzymes.

5.1 Ancestral cellulases production

In order to bring back to life the ancestral cellulases, we asked a company (Invitrogene) to synthesize the reconstructed sequences and we cloned them into an expression vector and expressed in the *E. coli* strain BL21. I carried out the procedure of cloning as I have explained in materials and methods section (3.1.6.). I show the high level of expression of the ancestral cellulases are in **Figure 5.1** SDS/PAGE acrylamide gel. When IPTG was added to induce the protein expression, an overexpressed protein band appeared at a determined molecular weight (endoglucanase ~33 kDa, exoglucanase ~69 kDa and β-glucosidase ~83 kDa). In all the cases this weight coincided with the molecular weight of the ancestral cellulase, which means that the transformation was correct and the bacteria was expressing the desired protein. Furthermore, it can be observed that in addition to the overexpressed band, there were other protein bands that were produced naturally by these bacteria.

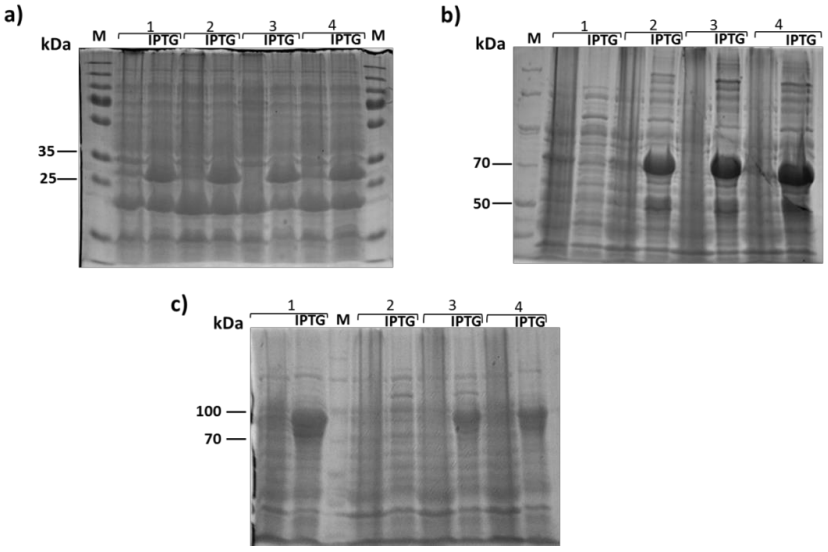


Figure 5.1. SDS acrylamide gel electrophoresis analysis of ancestral cellulases expression in E. coli bacteria. *(a) Endoglucanase expression analysis in 12% acrylamide gel. Lane 1, protein marker (M); lane 2 to 9, colonies 1 to 4 where the protein has been induced with IPTG in the second lane of each colony; lane 10, protein marker (M). (b) Exoglucanase expression analysis in 8% acrylamide gel. Lane 1, protein marker (M); lane 2 to 9, colonies 1 to 4 where the protein has been induced with IPTG in the second lane of each colony. (c) β-glucosidase expression analysis in 8% acrylamide gel. Lane 1 to 2, colony 1; lane 3, protein marker (M); lane 4 to 9, colony 2 to 4. The protein has been induced with IPTG in the second lane of each colony.*

The reason behind why the ancestral proteins expressed in such a big amount comparing with the extant ones is unknown, but it seems to be common in ancestral proteins [161]. In **Figure 5.2** we can see that the production of the ancestral cellulases is much

higher than that of the extant ones. In addition to running acrylamide gel of the reconstructed ancestral enzymes I run an acrylamide gel comparing the expression of the *T.maritima* endoglucanase and the ancestral endoglucanase LFCA (**Fig 5.2**). In this comparison, from a similar bacterial culture amount grown under the same conditions, the intensity of the band corresponding to LFCA endoglucanases is more than twice that of *T. maritima* endoglucanase.

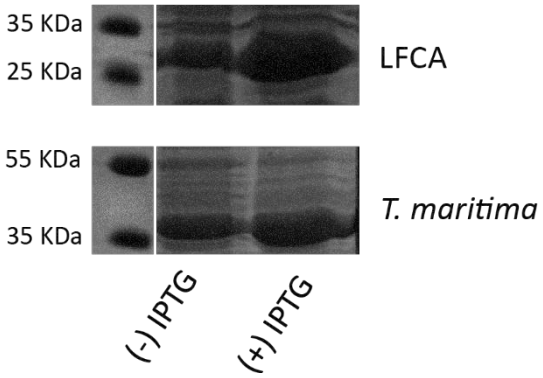


Figure 5.2. SDS-PAGE acrylamide 12% gel for the purified enzymes. *On the top panel, LFCA (33 kDa) and on the bottom panel, Endo-β-glucanase from T. maritima (37 kDa), with and without IPTG induction. After induction, the expressions level of LFCA EG is more than twice that of T. maritima, using the exact same protocol, amounts and expression system.*

Furthermore, I run an acrylamide gel of the commercial *T. reesei* mixtures. The results can be seen in **Fig 5.3a**. In this gel, there is no a single band, as expected from a commercial enzyme preparation, although a major band appears between 54 and 60 kDa [162, 163].

5.2. Mass spectroscopy and protein concentration determination

To further analyze the content of the enzyme preparation from *T. reesei*, we used mass spectrometry. Mass spectrometry (MS)is an analytical technique that ionizes molecules and sorts the ions based on their mass to charge ratio (m/z). For the application of the qualitative analysis of the enzymatic profiles, the mass analyzer that has the most impact is the time of flight (TOF). It is the most suitable analyzer in this type of studies due to its sensitivity, speed in full scan mode, high resolution and ability to identify unknown compounds. It is possible because TOF presents the possibility of measuring the exact mass of the detected ions using isotope distribution (True Isotopic Pattern, TIP). The time-of-flight analyzer discriminates according to the speed difference acquired by the ions inside a flight tube of known length as a function of its m/z ratio. TOF analyzer is based on the fact that all the ions generated in the ionization source have the same kinetic energy, their speed being inversely proportional to the square of their mass. A voltage (pulse) is applied to them to accelerate the ions, throwing them to a tube under high vacuum with a constant kinetic energy. Ions that have the same kinetic energy but different values of m/z will have different velocity. The higher m/z ions will travel at a slower speed than the lower m/z ions arriving later at the detector.

For the determination of the concentration of *T. reesei* enzyme, we have used two methodologies described in details in the material and methods section. The first method used was Pierce BCA Protein Assay Kit [139]using a BSA standard supplied with the kit and a standard of our ancestral endoglucanase and the second methods was the Difference Dry Weight method [138].These values have been also contrasted directly with other

researchers that use the same preparation [164]. We obtain a similar concentration that previously reported, around 125 mg/mL.

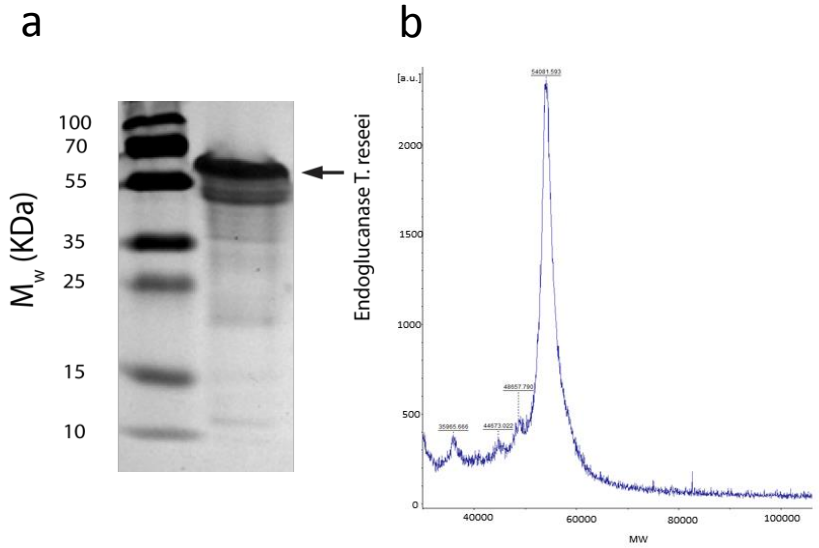


Figure 5.3. Endo-β-glucanase content determination for T.reesei preparation. *(**a**) present, the major component endo-β-glucanase represents about 70% of the total mix, as determined by gel densitometry. (**b**) Mass spectrum of T.reesei preparation in which endo-β-glucanase at 54 kDa represents also about 70 %, in close agreement with gel in (a).SDS-PAGE page acrylamide 8 % gel of T.reesei preparation. The main band represents the endoglucanase of Trichoderma reesei (~54 kDa). Although several enzymes are present, the major component endo-β-glucanase represents about 70% of the total mix, as determined by gel densitometry.*

5.3. Endoglucanase Assays

We carried out several assays in order to test the activity of the ancestral endoglucanase, first of all, a study of the specific activity was done. We measured the activity of the ancestral endoglucanase against two extant endoglucanases in a range of temperatures and pH values as it is shown in **Figure 5.4**.

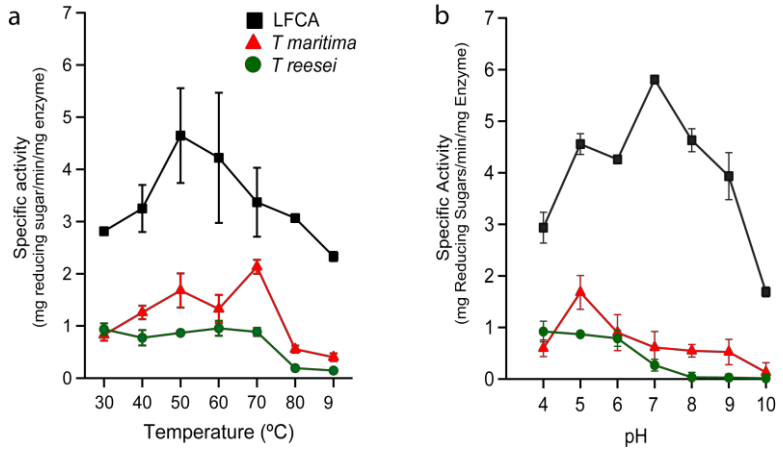


Figure 5.4. Activity assays for endoglucanase enzymes. (a) *Specific activity as a function of temperature for LFCA, T. maritima, and T. reesei cellulases at pH 4.8.* ***(b)*** *Specific activity as a function of pH(4-10) for LFCA, T. maritima, and T. reesei cellulases at 70°C.*

The first assay that we performed was the determination of the specific activity in a range of temperatures, from 30 to 90 ºC. This range is broader that the range generally used in industrial applications that does not go above 60 ºC. It is also broader than the typical range presented in the literature for improved endoglucanase testing, which is about 40-80 ºC [165, 166].

In the assay, I used a common soluble substrate such as carboxymethyl cellulose (CMC) [167]. As mentioned, I performed the assay at different temperatures and pH 4.8, incubating the substrate enzyme mix for 30 min and measured the reducing sugar concentration with the DNS method [168]. We determined the activity of the enzyme spectrophotometrically measuring the absorbance at 540 nm, as the amount of reducing sugar in mg released per min and per mg of enzyme used (see Materials and Methods). We show elevated activity at up to 90°C in the presence of 5% glycerol, which is generally used as stabilizer (**Fig 1b**). This working temperature is very high for endoglucanase, which generally operate in the range 40-60 °C.

The same measurements were performed in a broad pH range at 70ºC. **Figure 5.4** shows that the ancestral endoglucanase has higher specific activity with soluble CMC and 5% glycerol than bacterial and fungal cellulases at all temperatures tested. It also outperforms the assayed extant enzymes in all the range of pH. In **Figure 5.5** we show the same assays repeated at other temperatures and pH values.

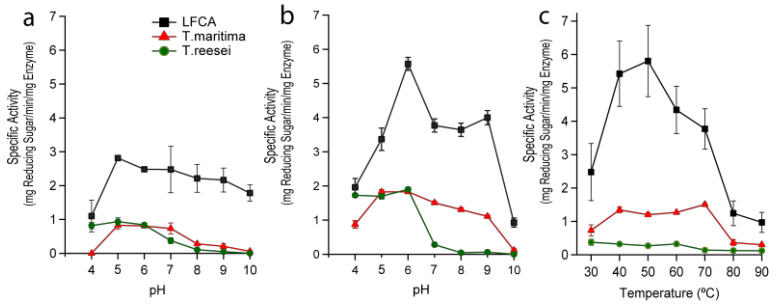


Figure 5.5 Endoglucanase activity measurements using CMC/DNS method. (a) *Specific activity assay at 30 ºC as a function of pH (4-12) for LFCA, T. maritima and T. reesei cellulases. We determined the reducing sugar mg equivalent released per minute and per mg of enzyme.* ***(b)*** *Specific activity assay at 50 ºC as a function of pH (4-12) for LFCA, T. maritima and T. reesei cellulases.* ***(c)*** *Specific activity assay in a range of temperatures (30-90 ºC) at pH 10 for LFCA, T. maritima and T. reesei cellulases. All assays were triplicated. Values are reported as average ±S.D.*

In **figure 5.5** three assays are shown, in the first one (**Fig 5.5a**) I show the activity assay I carried out at 30ºC; I run the same assay also at 50ºC (**Fig 5.5b**). In both cases, the ancestral endoglucanases outperforms the two commercial endoglucanases. The other assay that is shown is the one corresponding to pH 10 (**Fig 5.5c**) that I run in a range the temperatures 30-90ºC. As well as in the previous assays, at pH of 10 the endoglucanase shows a higher activity than the commercial endoglucanases from *T.maritima* and *T.reesei* in all the tested temperatures. The methodology used in those assays was the CMC method, the same described for **Figure 5.4**. In those cases, we see the exactly the same than in **Figure 5.4**, the ancestral endoglucanase shows a higher specific activity in all the tested conditions.

Another method available for the measurement of the specific activity of an endoglucanase is the use of a specific kit for endoglucanase activity. In this case (**Fig 5.6**) a CellG3 kit (Megazyme) [169] was used for the determination of the endoglucanase specific activity. I measured the specific activity of the endoglucanases at pH 5 and a range of temperatures (30-90ºC). In this case, I measured the activity of the ancestral endoglucanase against the endoglucanase of *Thermotoga Maritima*. The preparation *T.reesei* was not used in this case, as it is a mixture of enzymes and not a purified single enzyme. In all the temperatures I tested, the activity of the ancestral endoglucanase was much higher than the *T.maritima*, specially from 50ºC to 70ºC, where the difference is even higher.

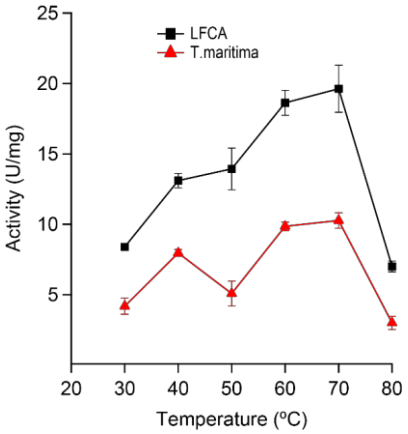


Figure 5.6. Endoglucanase activity measurements using CellG3 method. *Activity in CellG3 U/mg of two different endoglucanases: LFCA; Thermotoga maritima, measured in a range of temperatures (30-90ºC) at pH 5 with Megazyme endo-cellulase assay kit [4]. Both enzymes show similar profile but LFCA displays higher specific activity. All assays were triplicated. Values are reported as average ±S.D.*

The next property we evaluated for those endoglucanases was the long term activity. We define the long term activity as the reducing sugar production per minute. The hydrolysis rate was determined by measuring the activity at 60 °C and reaction times ranging from 10 to 250 min as shown in **Figure 5.7**, the ancestral enzyme reached over 90% reducing sugar production within 10 min.

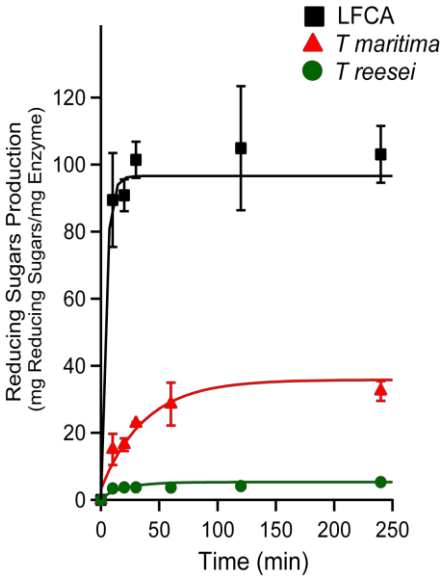


Figure 5.7. Long-time activity measurements*. Reducing sugars production was measured at different reaction times at 60 °C. Experimental details are provided in the Materials and Methods. In each case, three replicates were collected. The average ± S. D. values are shown for each measurement.*

We used exponential fits to determine a rate of 0.24 μg of reducing sugar/min for ancestral endoglucanase, 0.032 for *T. maritima* and 0.069 for *T. reesei.*

Another thing we determined when characterizing the enzyme was the stability of the ancestral endoglucanase to temperature

incubation compared to *T. maritima*, and *T. reesei* cellulases as **Figure 5.8** shows. By means of this graphic representation, we determined the temperature at which they lost half of their activity after 30 min of incubation and then ascertaining their residual activity at 60°C. We estimated a value of 85°C for *T. maritima*, 79°C for LFCA EG, and 72°C for *T. reesei*. In the case of the T.reesei preparation, it shows a biphasic-like decay, which we hypothesize that could be a consequence of the action of other enzymes in the preparation (**Fig 5.8**).

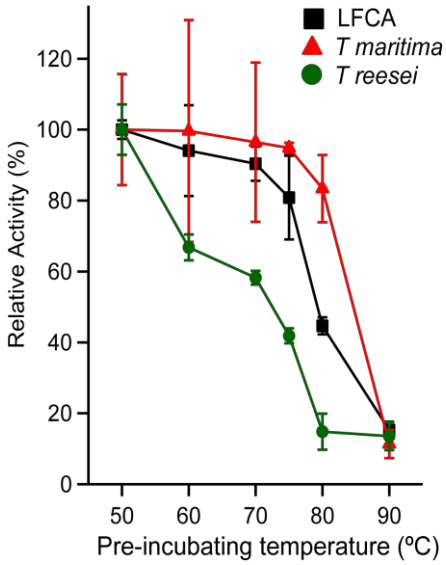


Figure 5.8. Pre-incubation experiments at different temperatures for the different endoglucanases conducted for 30 min. *Residual activity was determined on 2% CMC for 30 min at 60ºC using DNS. Relative activity is determined for each individual enzyme. Each enzyme was pre-incubated at its best performing pH value.*

Another relevant characteristic that we determined were the kinetics for the thermal inactivation of the enzymes at 80°C. In

order to study this, the residual activity (the remaining activity, after the loss of activity with the incubations) was plotted against the time, showing that it follows a clear first order kinetics for *T. maritima* and LFCA EG (**Fig 5.9**). In the case of the preparation of *T.reesei* we observe a biphasic behavior, the reason for this behavior was not clear, although we suspect that perhaps it could be an effect of the different enzymes present in the preparation.

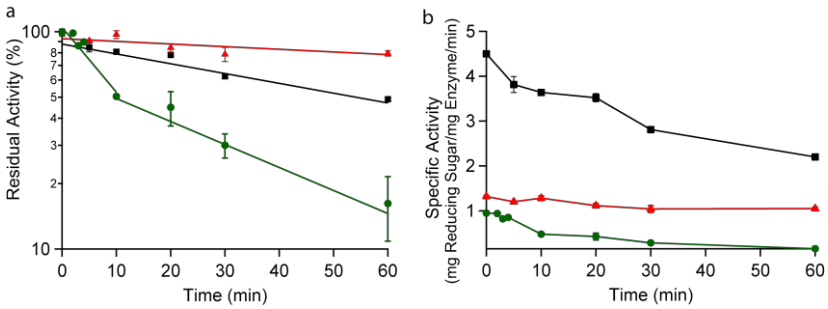


Figure 5.9. Endoglucanase inactivation at 80ºC. *(a) Residual activities were measure at different incubation times. (b) Specific activity was determined after incubation. The activity of non-incubated enzyme was used as a reference for 100% residual activity. Each assay was repeated five times. The values are presented as average ±S.D.*

From the plot (**Fig 5.9**) I determined, the inactivation constant (K_{in}) and half-life ($t_{1/2}$) [2], we used a linear fit for the determination. The values obtained in each case are shown in **Table 5.1**.

Enzyme	*Half-life (min)*	*Rate constant K_{in} (min^{-1})*
LFCA	56	0.29
T. marítima	178	0.10
T. reesei	9	2.26
		0.30

Table 5.1. Half-life and inactivation rate constant (K_{in}) of LFCA, T. maritima and T. reesei. *In the case of T.reesei the biphasic behavior observed could reflect the action of more than one enzyme in the preparation T.reesei.*

The values I obtained for the three enzymes are in consequence with what it was expected. The half-life for *T. maritima* and LFCA cellulases at 80°C was 178 and 56 min, respectively (**Table 5.1**). The $t_{1/2}$ value for *T. maritima* was high, as expected for an extreme thermophile, although it still displays lower specific activity than the ancestral LFCA endoglucanase. However, for *T.reesei* this value is smaller, 9 min. That means that it lost more than 50% of its activity within the first 10 min of incubation, with a slower decay after that, which again likely reflects the action of more than one enzyme.

Regading to the inactivation constant value (K_{in}) we calculated a K_{in} of 0.29 min^{-1} for LFCA EG, 0.10 min^{-1} for *T. maritima*, and 2.26 min^{-1} and 0.30 min^{-1} for the fast and slow phases of *T. reesei*, respectively.

The experiments described since now, have been performed using CMC, a soluble laboratory substrate useful for determining endoglucanase activity. However, cellulases must be able to

hydrolyze cellulose in lignocellulosic materials, such as agricultural, industrial, or city waste. To test this, we used cardboard as a source of cellulose. In cardboard, cellulose, lignin, and hemicellulose are present at approximately 60%, 15%, and 15%, respectively. We performed activity assays using isolated LFCA endoglucanase and in combination with laccase and xylanase, enzymes that can degrade lignin and hemicellulose, respectively. We determined the percentage of cellulose hydrolyzed in a 50 mg sample of cardboard within 1 h at 50°C and pH 4.8. As shown in **Figure 5.10**, the endoglucanase enzymes degraded very small amounts of cellulose on their own, no more than ~5%, with the commercial *T. reesei* being slightly more efficient, probably due to the action of other enzymes in the preparation.

5.3.1. Lignocellulosic substrates hydrolysis

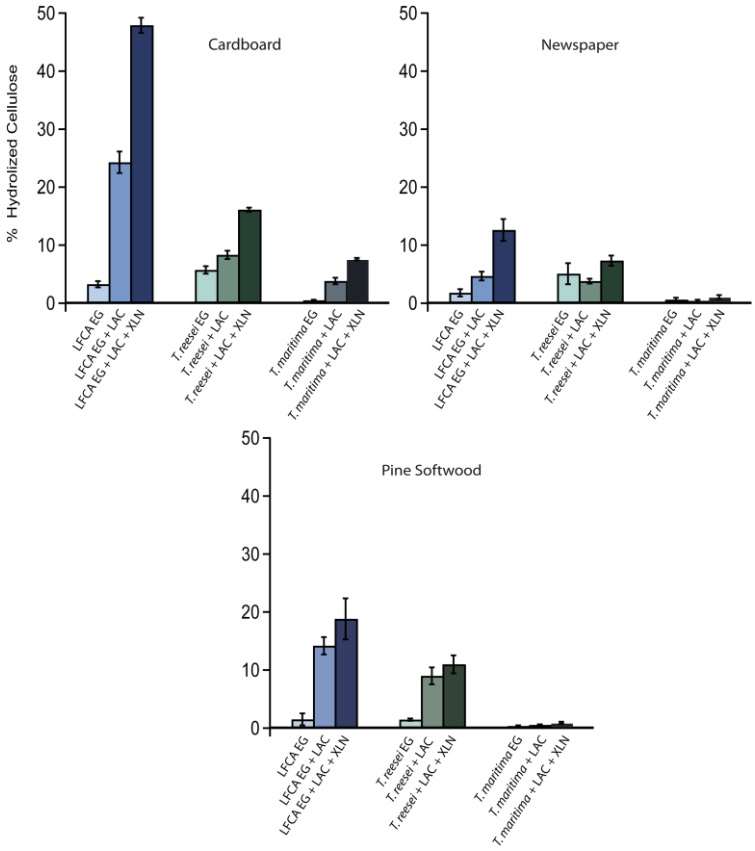


Figure 5.10. Hydrolysis of cardboard lignocellulosic material. *We used 50 mg of milled lignocellulosic material (cardboard, newspaper and pine softwood) in 50 mM citrate buffer at pH 4.8. Enzyme hydrolysis was performed for 1 hour at 50°C. Endoglucanase alone or in combination with laccase and xylanase were used for hydrolysis of the lignocellulosic material. Three different enzyme combinations were used differing in the endoglucanase used: ancestral, T. maritima or T. reesei. Released sugars are quantified with the DNS method. Cellulose hydrolysis yield was determined as described elsewhere [148, 170].*

The experiments described above were carried out using CMC, a soluble laboratory substrate useful for determining endoglucanase activity. However, for industrial applications, cellulases must be able to hydrolyze cellulose in lignocellulosic materials, such as agricultural, industrial, or city wastes in synergy with other enzymes such as lacasse and xylanase. This is important, for instance, for the pretreatment of lignocellusic biomass using enzymes. To test this aspect, we used cardboard, newspaper and softwood from pine tree as a source of cellulose. These three materials have different content of cellulose, kinging and hemicellulose. While cardboard contains around 60% cellulose and around 15% of lignin and hemicellulose, newspaper and pine softwood contain less cellulose, less than 50% [171-173] and more lignin, ~22 and ~30, respectively; and hemicellulose, ~18 and ~25, respectively . We performed activity assays using isolated LFCA EG and in combination with laccase from *Trametes pubescens* and xylanase from *Thricoderma viride*, enzymes that can break down lignin and hemicellulose, respectively. We determined the percentage of cellulose hydrolyzed in a 50 mg sample of lignocellulosic material [171-173], within 1 h at 50°C and pH 4.8. In the case of cardboard, the three EG enzymes degraded very small amounts of cellulose on their own, no more than ~5%, with the commercial *T. reesei* endoglucanase being slightly more efficient, probably due to the action of other enzymes in the preparation (**Fig 5.10a**). Conversely, LFCA EG worked best when used synergistically with laccase and xylanase hydrolyzing approximately half the cellulose present in the sample, as compared to *T. reesei* that degraded ~16% and *T. maritima* that degraded less than 10%. In the case of newspaper and softwood, the lower amount of cellulose and higher content of lignin is reflected in the lower efficiency of cellulose degradation, although still LFCA EG outperforms the other endoglucanases (**Fig 5.10a, and 5.10b**).

These results highlight not only the potential of LFCA EG to work with lignocellulosic substrates, but also the advantage of using multi-enzyme cocktails containing cellulases, laccases, xylanases and other enzymes for efficient pretreatment of raw materials and subsequent hydrolysis of cellulose.

As the graph in **Fig 5.10** shows, LFCA endoglucanase worked best used synergistically with laccase and xylanase and could hydrolyze approximately half the cellulose present in the sample, as compared to *T. reesei* that degraded ~16% and *T. maritima* that degraded less than 10%. These results show, the potential of the ancestral endoglucanase to work with lignocellulosic substrates and the advantage of using multi-enzyme cocktails containing cellulases, laccases, xylanases and other enzymes for efficient pretreatment of raw materials and hydrolysis of cellulose on the other. This synergy is really interesting under an industrial point of view, as using enzymes able to hydrolyze different substrates we can hydrolyze complex lignocellulosic materials.

5.3.2. Thermal stability of the ancestral endoglucanase: Circular Dichroism

In order to determine the thermal stability of the ancestral endoglucanase, we performed a circular dichroism experiment. (**Fig 5.11**). We examined the thermal denaturation of the ancestral endoglucanase versus the ancestral endoglucanase with 0.5% glycerol shifting the melting point. **Figure 5.11** shows elipticity indicating thermal denaturation transitions of endoglucanase (in blue) and the endoglucanase with 0.5% of glycerol (in green) at pH 4.8 from 55ºC to 110ºC. The circular dichroism at 222 nm should report transition midpoint temperatures (Tm) are reported

in **Table 5.2**. The signal was fitted to a two stated model using baselines for the unfolded and folded states and with the thermodynamics states adjusted by using the modified Gibbs-Helmholtz equation [174] from where we determined the thermodynamic values of enthalpy, entropy and the heat capacity.

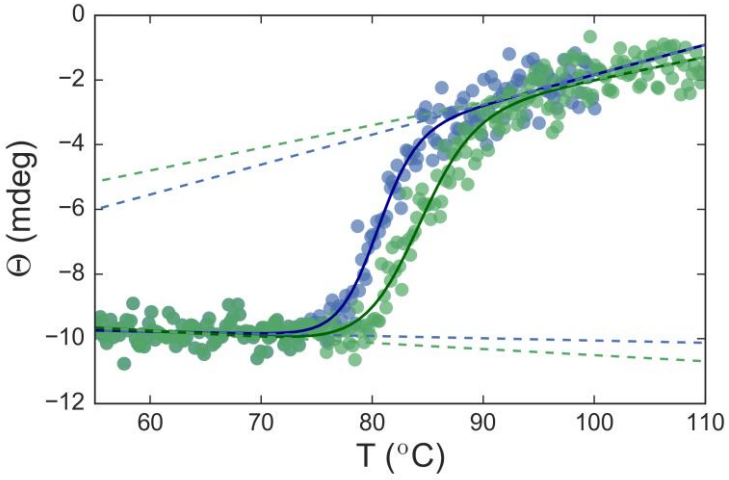


Figure 5.11. Thermal denaturation of ancestral reconstructed endoglucanase. *In blue the thermal denaturation of endoglucanase at pH 4.8 and 50mM citrate buffer from 55 to 110ºC. In green, the thermal denaturation of endoglucanase at pH 4.8 and 50mM citrate buffer from 55 to 110ºC in presence of 0.5% glycerol.*

In **Table 5.2** the values for the denaturing temperature and the thermodynamic constants are represented.

Enzyme	*Endoglucanase*	*Endoglucanase+ %0.5 Glycerol*
Tm (ºC)	**80.3**	**83.3**
ΔH (kJ/mol)	**592**	**428**
ΔS (kJ/mol/K)	**1.68**	**1.20**
Cp (kJ/mol/K)	**$9.8*10^{-6}$**	**$5.5*10^{-5}$**

Table 5.2. Thermodynamic constants of reconstructed ancestral endoglucanase

The Tm values (**Table 5.2**) show that the glycerol has a stabilizer effect as it was expected. By adding glycerol the Tm of the protein increased in 3ºC. Besides, it is a high value of Tm for this type of proteins that usually have values around 60-75ºC [175] obtaining similar enthalpy and entropy values [176]. We observe a marginal stabilization when using glycerol in the free energy and in the melting temperature with an increasing of 3ºC.

5.3.3. Ancestral endoglucanases Kinetic parameters determination

We further studied the reaction processes and catalytic events for the ancestral endoglucanase by determining K_M, a measure of substrate affinity, and Kcat/K_M, a measure of catalytic efficiency. In order to determine these kinetic parameters, Michaelis-Menten equation [177].

$$v = \frac{d[P]}{dT} = \frac{Vmax\ [S]}{K_M + [S]}$$

Where Vmax represents the maximum rate achieved at saturating substrate concentration and K_M (Michaelis constant) is the substrate concentration at which the reaction rate is half of the Vmax.

Michaelis–Menten saturation curve for an enzyme reaction shows as it is shown in (**Figure 5.12a**) the relation between the substrate concentration and reaction rate [178].

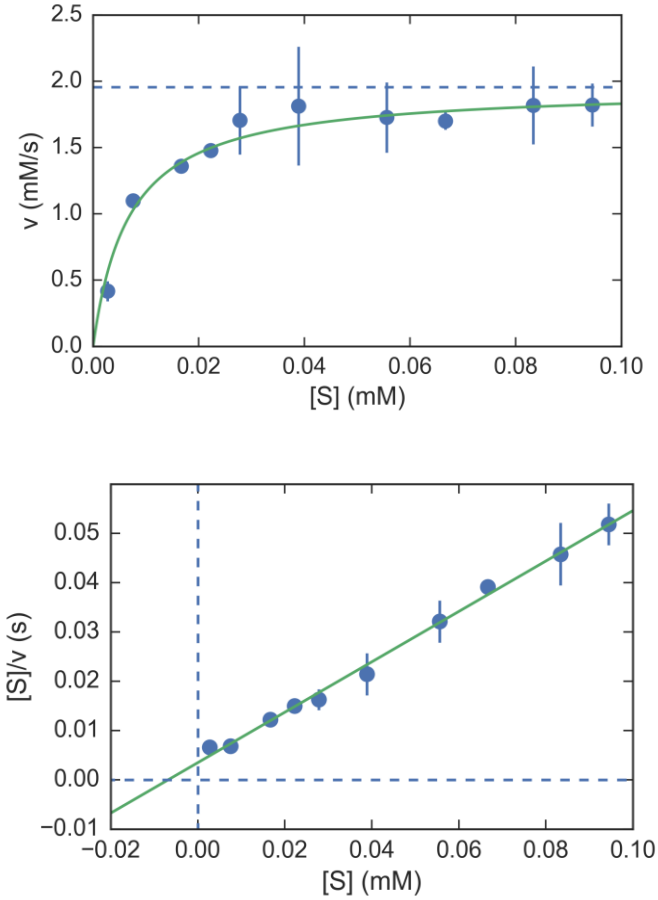


Figure 5.12. Hanes–Woolf plot and Lineweaver–Burk plot for reconstructed ancestral endoglucanase.

Results obtained for endoglucanase activity are shown in (**Table 5.3**).

Kinetic constants	*Endoglucanase*
K_M *(mM)*	**0.007**
V_{max} *(mM/s)*	**1.96**
K_{cat} *(s^{-1})*	**217**
K_{cat}/K_M*(mM/s)*	**$2.4*10^4$**

Table 5.3. Kinetic constants of ancestral endoglucanase

From the kinetic values determined from the plots, we observe that the enzyme has a high substrate affinity (K_M) and the measured catalytic efficiency is also high (K_{cat}/K_M) in comparisson with the constants measured in the literature for endoglucanases [179] [180, 181].

5.4. Enzyme Cocktail Assays

After studying the activity of the ancestral endoglucanase against some extant ones, I developed, a study of the ancestral cocktail against a commercial one, as it is explained in the next points of this work. The use of cellulase cocktails is really important in order to completely hydrolyze the cellulose into sugar monomers. Moreover, there is a huge industrial interest in the degradation of lignocellulosic materials that is why; we have included a laccase in our studies.

5.4.1. Ancestral Enzymes Cocktail

The first assays I carried out for the ancestral cocktail are shown in **Figure 5.13**. In this assays, I measured, the specific activity of the ancestral cocktail against a commercial one Ctec2 cocktail (**Fig 5.13**). We run the assay in three different temperatures 50ºC (**Fig 5.13a**), 60ºC (**Fig 5.13b**) and 70ºC (**Fig 5.13c**) and in three different pH values (5,7 and 11) in each case, As the figure

shows, the ancestral cocktail outperforms the commercial cocktail Ctec2 in all the cases (**Fig 5.13**). This significant difference in the activity is even bigger in the case of the neutral and the basic pH (**Fig 5.13b and 5.13c**), where the activity of the commercial cocktail is really small.

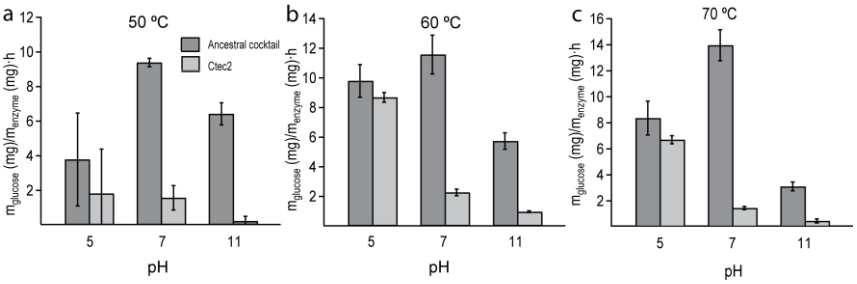


Figure 5.13. Specific activity as a function of pH for ancestral enzyme cocktail and commercial enzyme cocktail Ctec2. *a) 50ºC , b) 60ºc and c) 70ºC. Hydrolysis was carried out for 1 h using filter-paper as a substrate. All assays were triplicated. Values are reported as average ±S.D.*

Once I measured, the good performance of the ancestral cocktail in comparison with the commercial one Ctec2, I carried out a study of the stability. The study was developed in the same way we did in the case of the endoglucanases in section 5.3. For this purpose I performed several assays.

I determined the long term activity of both cocktails (ancestral cocktail and Ctec2 commercial cocktail) measuring the activity at 60 °C and reaction times ranging from 10 to 250 min as shown in **Figure 5.14**. In this figure, we see that the commercial cocktail Ctec2 reached almost the %100 of the reducing sugar production in a short time comparing with the ancestral cocktail. The plot shows (**Fig 5.14**) that the commercial cocktail reaches the %100

of its reducing sugar production in 50 minutes, in contrast, the ancestral one needs 250 minutes to reach it. However, the production of reducing sugars of the ancestral cocktail is higher than the commercial one from the very beginning.

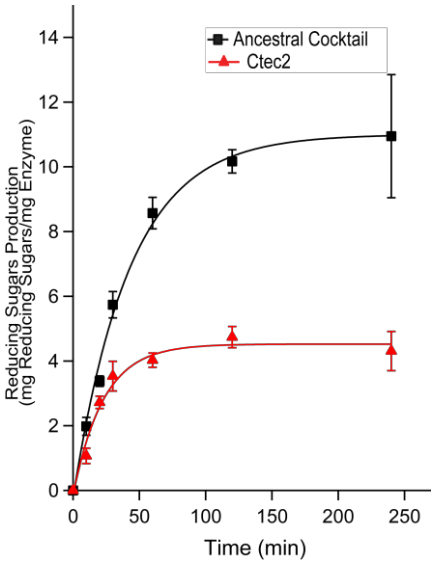


Figure 5.14. Long-time activity measurements for ancestral and commercial enzyme cocktail Ctec2*. Reducing sugars production was measured at different reaction times at 60 °C. Experimental details are provided in the Materials and Methods. In each case, three replicates were collected. The average ± S. D. values are shown for each measurement.*

From this plot (**Fig 5.14**) we calculated the hydrolysis rate of both cocktails. We obtained a value of 0.14µg of sugar per minute in the case of the ancestral cocktail and a rate of 0.067µg of sugar per minute for the commercial one Ctec2.

In addition, I also evaluated the stability for temperature incubation. This study is shown in **Figure 5.15** making the

comparison of the stability of the ancestral cocktail versus the commercial one Ctec2. By means of this graphic representation, we determined the temperature at which they lost half of their activity after 30 min of incubation, making after the measurement of the activity at 60ºC. The estimated values of temperature were the commercial Ctec2 and the ancestral cocktail lose half of their activity were 73ºC and 76ºC for each case (**Fig 5.15**).

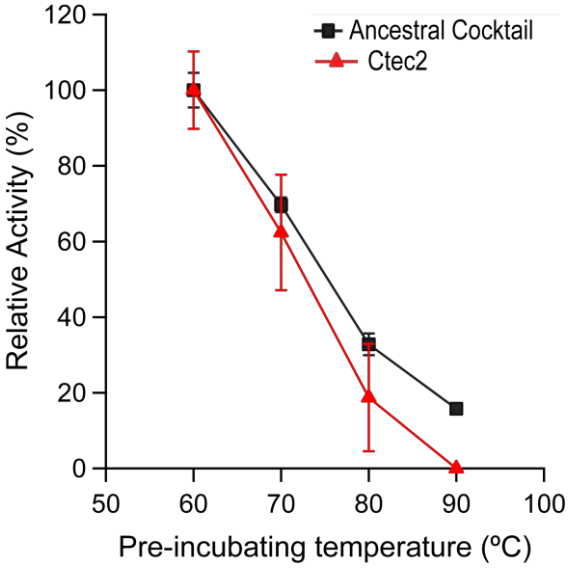


Figure 5.15. Pre-incubation experiments for ancestral and commercial enzyme cocktail Ctec2 at different temperatures conducted for 30 min. *Residual activity was determined on 2% CMC for 30 min at 60ºC using DNS. Relative activity is determined for each individual enzyme. Each enzyme was pre-incubated at its best performing pH value. All assays were triplicated. Values are reported as average ±S.D.*

Continuing with the thermal stability of the enzyme cocktails, I determined the kinetics for the thermal inactivation of the enzymes at 80°C. The residual activity plotted against the time

followed a clear first order kinetics for both ancestral and commercial cocktail Ctec2, it can be seen in **Figure 5.16**.

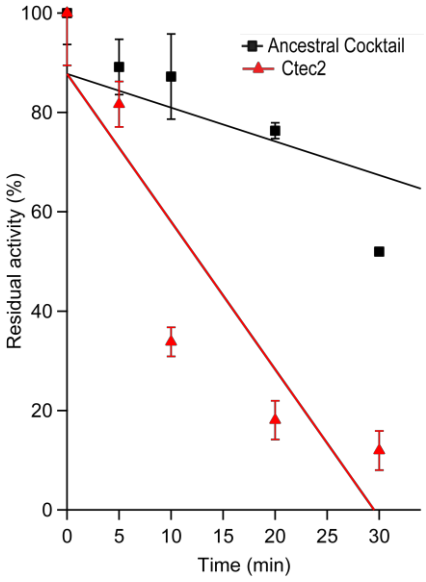


Figure 5.16. Ancestral and commercial enzyme cocktail Ctec2 inactivation at 80ºC. *(a) Residual activities were measured at different incubation times. (b) Specific activity was determined after incubation. The activity of non-incubated enzyme was used as a reference for 100% residual activity. Each assay was repeated five times. The values are presented as an average ±S.D. All assays were triplicated. Values are reported as average ±S.D.*

I calculated the inactivation constant (K_{in}) and half-life ($t_{1/2}$) from the plot, for what I used the same procedure used in point 5.2. The half-life for ancestral and commercial cocktails Ctec2 at 80°C was 55 and 13 min, respectively (**Table 5.2**).

Enzyme	*Half-life (min)*	*Rate constant K_{in} (min^{-1})*
Ancestral cocktail	55.6	0.39
Ctec2	12.7	1.72

Table 5.2. *Half-life and inactivation rate constant (K_{in}) of ancestral and commercial enzyme cocktails Ctec2.*

The values obtained in this case are similar to the previous ones, what makes sense. In the case of the commercial cocktail, we obtained a slightly higher value than for *T.reesei* but similar in magnitude. In the case of the ancestral cocktail is almost the same we measured for the ancestral endoglucanase. Regarding to K_{in}, the values also are similar that we obtained previously, 0.39 in the case of ancestral cocktail and 1.72 in the case of the commercial one.

We carried out all the experiments described until now in 5.3 point, using filter-paper, a common substrate very used for the determination of the activity of cellulase cocktails. Nevertheless, there is a huge interest in the hydrolyzation of crystalline cellulose in industry [182]. That is why, we decided to test the activity of the ancestral cocktail using a completely crystalline substrate as it is the case of Avicel. For this porpoise, we carried out the assay as shown in **Figure 5.17** at different temperatures (40-70ºC) in three different pH values (5,7 and 10). The incubation for this assay was performed for 24 hours in agitation. The figure clearly shows (**Fig 5.17**) that the specific activity of the ancestral cocktail is also higher in this case. This difference becomes even bigger when the temperature and the pH values are higher. So, this assay shows that the ancestral cocktail is not only

able to degrade amorphous cellulose in a better way, but also its activity is higher with crystalline cellulose. Although this are really interesting results, both filter-paper and avicel are %100 cellulose containing substrates and there is a need of hydrolyzing not only cellulosic substrates but also lignocellulosic materials as it was mentioned before. In this direction, there are several assays we performed in point 5.3.2 for those lignocellulosic materials used also before for the endoglucanase.

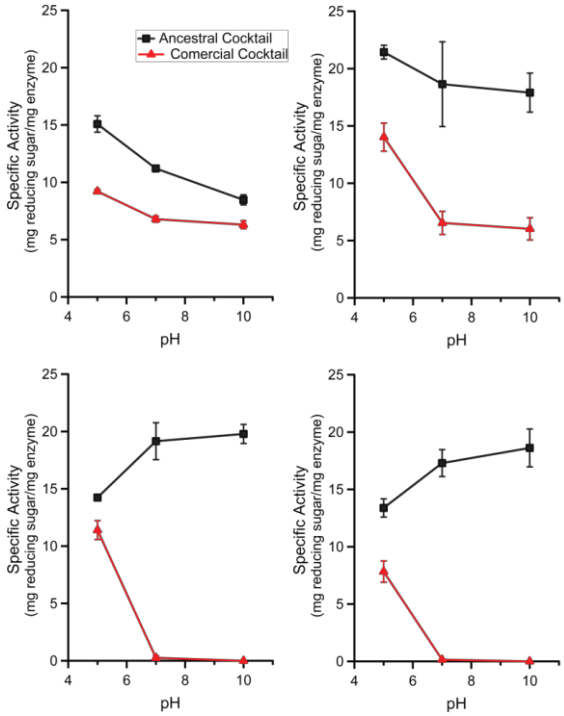


Figure 5.17. Ancestral and commercial enzyme cocktail Ctec2 activity measurements using avicel. *Specific activity assay at pH (5,7,10) for ancestral and commercial cocktail at different temperatures. a) 40ºC, b) 50ºC, c) 60ºC and d)70ºC We determined the reducing sugar mg equivalent released per minute and per mg of enzyme. All assays were triplicated. Values are reported as average ±S.D.*

5.4.2. Lignocellulosic substrates hydrolysis

We carried out activity assays with three different substrates (cardboard, newspaper and wrapping paper), values we obtained at 50 ºC and pH 4.8 can be seen in **Figure 5.18**. These values show that the ancestral cocktail has higher activity in all the substrates when we added laccase to the cocktail. As laccase degrades lignin, cellulose was more accessible for the cellulases present in the cocktail. In these conditions, where cellulose was more accessible, we measured the higher specific activity of the ancestral cocktail, almost doubling the activity of the commercial cocktail Ctec2. We expect that the commercial cocktail Ctec2 contains other enzymes such as xylanases and laccase in addition to the cellulases that favor the degradation. This can be the reason for the observed lower activities of the ancestral cocktail in absence of laccase.

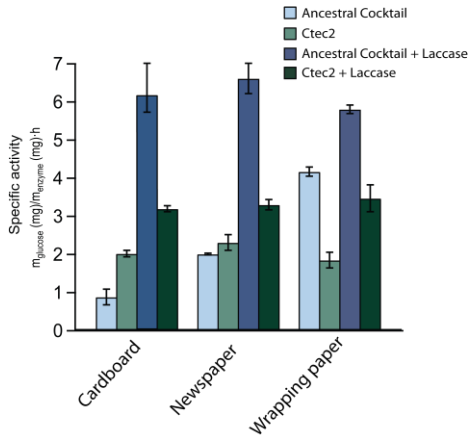


Figure 5.18. Activities of ancestral enzyme cocktail (CKA), commercial enzyme cocktail (CTec2), ancestral enzyme cocktail in presence of T. pubescens laccase (CKA + L) and commercial enzyme cocktail in presence of T. pubescens laccase (Ctec2 + L) at 50 ºC and pH 4.8. *Assays were carried out in three different substrates: cardboard, newspaper and wrapping paper.*

It can be also observed that the activity is substrate dependent. Substrates have different cellulose, hemicellulose and lignin content [183]. In addition, they went through different mechanical and chemical processes in their production. This may define the arrangement of the lignocellulosic fibers and therefore, as well as being different in composition they are different in structure. This diversity of substrate's characteristics may affect the ability of enzymes to reach their specific substrate and degrade it.

Comparing these results with the ones obtained in filter-paper ideal substrate (**Fig 5.13.**), lower values are obtained in lignocellulosic substrates. Filter-paper is pure cellulose that is synthetized in the laboratory. In contrast, lignocellulosic biomass is a complex substrate due to its structure and composition. Lignin and hemicellulose form a protective shell around cellulose, which obstructs enzymatic attack and thus, lower amount of glucose is released.

We repeated those assays in different temperatures; we plotted in (**Fig 5.19**) the activity values obtained at a temperature range of 50-70 ºC. In all the tested substrates, the highest activity was obtained at 50 ºC with the ancestral cellulases cocktail together with the laccase. As we saw before in **Figure 5.18**, the laccase influence was really positive and the activity of ancestral cellulases was higher than using the commercial ones Ctec2. Regarding temperature, its increment resulted in lower activities. At 70 ºC similar values were observed when the assay was carried out with or without laccase. This may happen due to the fact that laccase is not active at that temperature. If laccase losses its activity the accessibility of cellulose is reduced and thus, less cellulose is degraded to glucose.

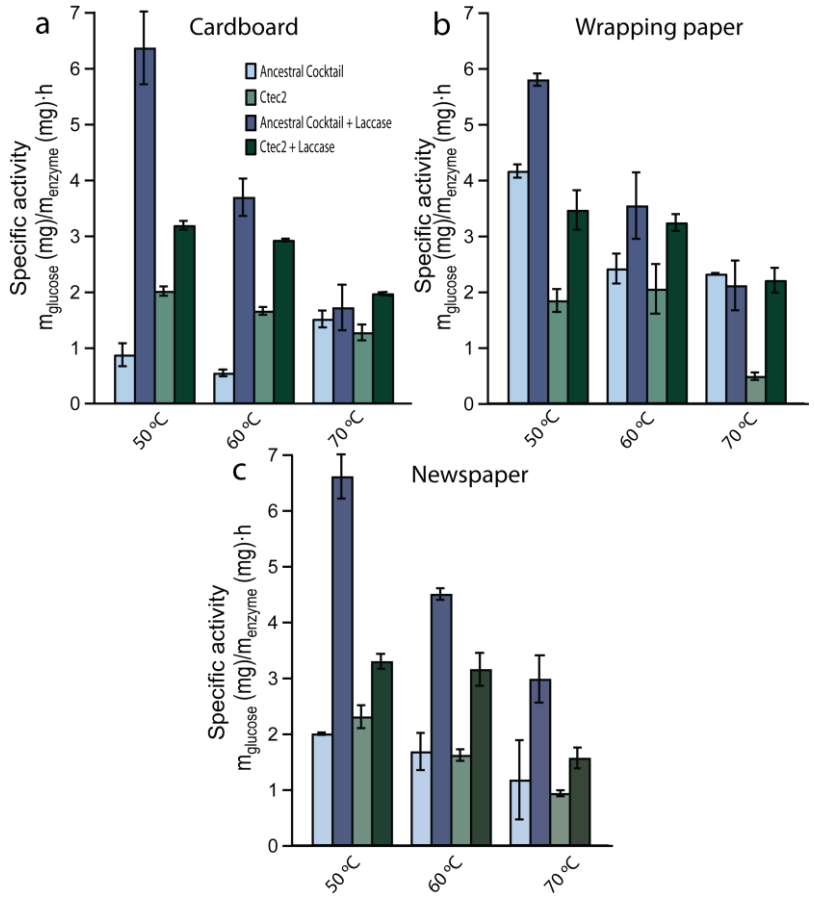


Figure 5.19. *Activities of ancestral enzyme cocktail (CKA), commercial enzyme cocktail (CTec2), ancestral cocktail in presence of T. pubescens laccase (CKA + L) and commercial enzyme cocktail in presence of T. pubescens laccase (CTec2 + L) at 50-70 ºC temperature range and pH 4.8. Assays were carried out in three different substrates: cardboard (a), newspaper (b) and wrapping paper (c).*

As mentioned before, the commercial cellulases cocktail Ctec2 contains other enzymes that favor the hydrolysis. Thereby, in some cases lower activities of the ancestral cocktail were observed when laccase was not added.

5.5. Minicellulosome

In this part of the thesis, we constructed different complexes with the ancestral endoglucanase. This work was carried out in collaboration with Mariano Carrión Vázquez group in the Cajal Institute (CNIC).

Apart from testing the activity of the ancestral endoglucanase as free enzyme, another interesting aspect was testing the performance of LFCA endoglucanase incorporated into a cellulosome, which is a macromolecular complex containing several lignocellulose-degrading enzymes attached to scaffoldin via dockerin protein domains. There are some organisms of cellulolytic bacteria such as *Clostridium thermocellum* that use the cellulosome to degrade cellulose in nature. The use of it has been suggested for industrial applications, due to the increased activity [184]. For this reason, we made different constructs fusing endoglucanase enzymes to domains present in the cellulosome. As it can be seen in **Figure 5.20** we bonded dockerin at the C-terminus of the ancestral endoglucanase (LFCA-Dock) to allow its incorporation into a mini-scaffoldin containing a single (Scaf1) or two tandem (Scaf2) cohesins. In this **Figure 5.20** there is a scheme of the different domains separately and also a schematic representation of the created complexes. Two different controls were used LFCA endoglucanase fused to a cellulose binding module (LFCA-CBM) and *C. thermocellum* Cel8A endoglucanase (CtCel8A), a major endoglucanase in its cellulosome [185].

5.5.1. Minicellulosome construction

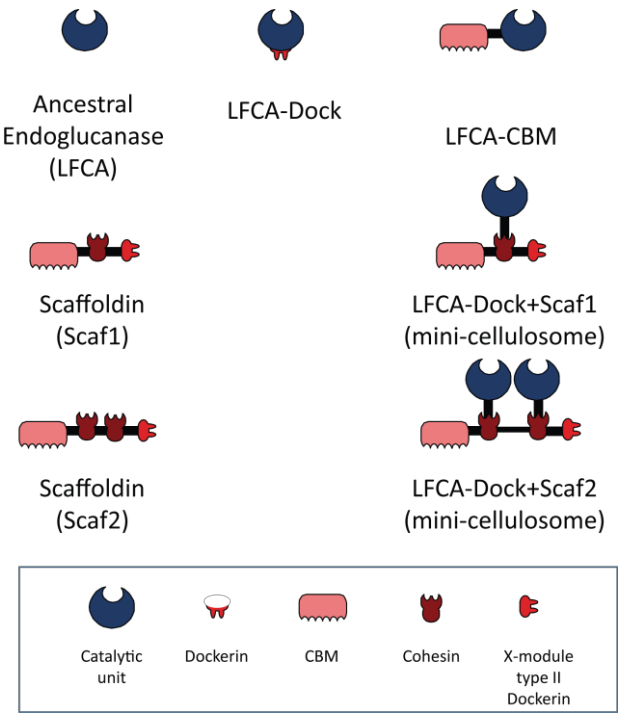


Figure 5.20. Cellulosome construction. *Schematic representation of the incorporation of ancestral endoglucanase (LFCA) into a mini-cellulosome. The different molecular elements assembled are represented.*

LFCA-Dock incorporation into two mini-scaffoldins occurred at molar ratios of 1.1:1 (LFCA-Dock:Scaf1) and 2:1 (LFCA-Dock:Scaf2), which was close to the expected ratio since cohesin-dockerin binding occurs in a 1:1 ratio[184], indicating precise complex formation (**Fig 5.22a**). Furthermore, LFCA-Dock incorporated into the cellulosome and LFCA-CBM was capable

of binding microcrystalline cellulose (**Fig 5.21**), while the other proteins were not. This indicates that, as expected, only when a CBM was present, specific microcrystalline cellulose binding could occur.

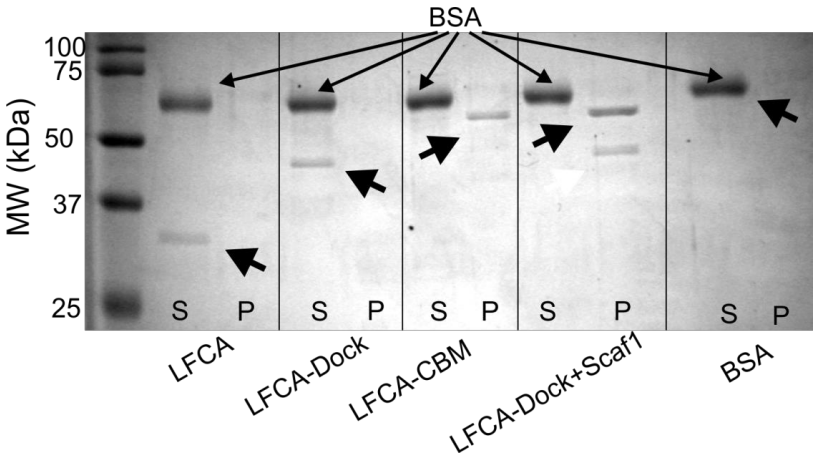


Figure 5.21. Controls of cellulose interaction of LFCA and variants. *Only LFCA-CBM and LFCA-Dock+Scaf1 bind microcrystalline cellulose (Avicel) and are found in the precipitated fraction (P) while other variants and the control protein BSA are found in the soluble (S) fraction. BSA was added to all samples to minimize nonspecific interactions.*

5.5.2. Minicellulosome activity assays

We studied the effect of the incorporation of the ancestral endoglucanase into the cellulosome carrying out activity assays with Avicel. Taking into account that this is a microcrystalline cellulose substrate this is targeted by the CBM used (**Fig 5.22b**). These assays were run at 70ºC. We chose this temperature taking into account that no major loss of activity was expected to happen during the long incubation time needed. Moreover, this was the temperature for which the highest activity was observed in the

CellG3 assay. Free LFCA endoglucanase showed a higher activity with this substrate than native CtCel8A but dockerin incorporation into LFCA resulted in a lower activity than that of the original LFCA endoglucanase, but it was still slightly higher than that of CtCel8A (**Fig 5.23c**). Importantly, when LFCA-Dock was incorporated into Scaf1, the resulting activity was extraordinarily improved.

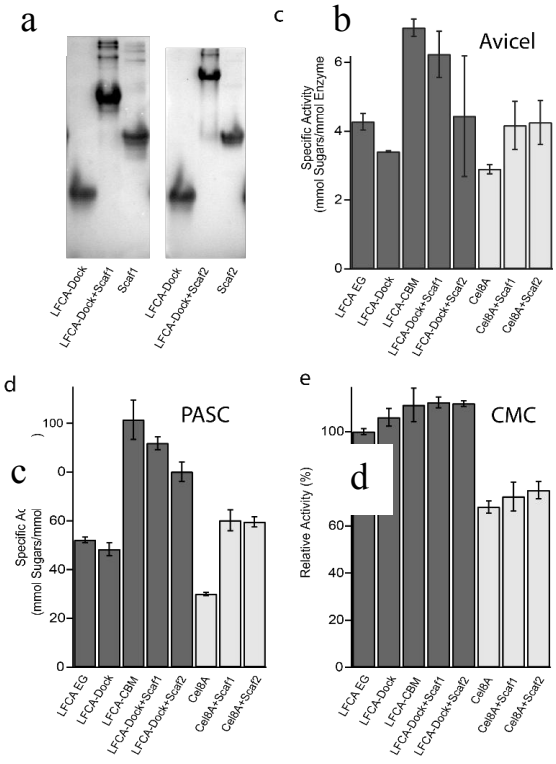


Figure 5.22. Cellulosome activity assays. *(a) Native-PAGE shows that a new band appears upon incubation of LFCA-Dockerin and a mini-scaffoldin, indicating complex formation. Activity of the free and mini-cellulosome bound LFCA on Avicel (b), PASC (d), and CMC (e). Each experiment was carried out in triplicate and the average ± S. D. values are shown.*

Concerning to the complex LFCA-CBM, we measured a similar activity than that of LFCA-Dock. This made us to think that the enhancement was due to a substrate targeting effect. Incorporation into Scaf2, whereby two tandem identical cohesins allow for the formation of a cellulosome with two enzymes, did not provide further activity improvement in either case, for LFCA endoglucanase and for CtCel8A. However, this result does not mean that further synergy could if we use different enzymes together with LFCA EG. Similar results were observed at all of the tested pH values (**Fig 5.23**) and at lower temperatures (**Fig 5.24**). At higher temperatures above 80 °C, though, the situation was reversed and CtCel8A showed higher activities (**Fig 5.24**), perhaps due to the long reaction times.

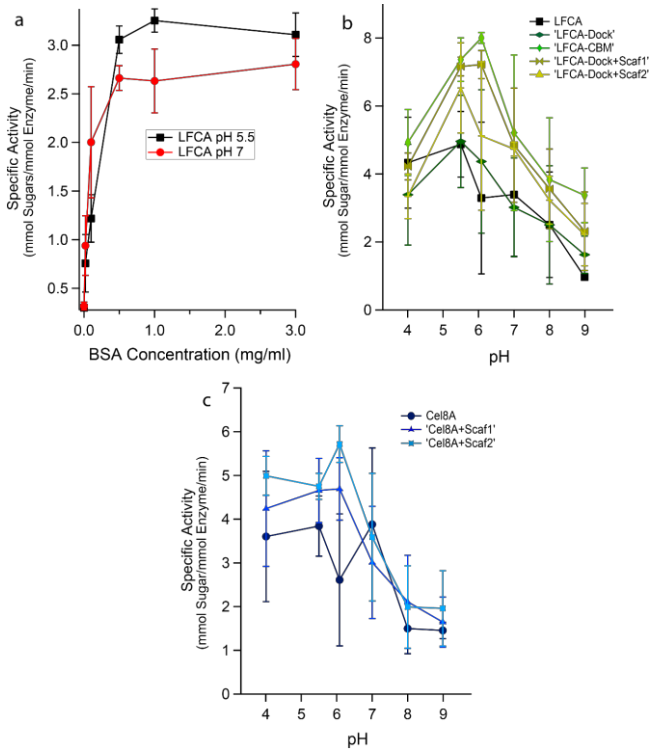


Figure 5.23. Activity of LFCA as a function of BSA concentration and pH in Avicel*. (a) Dependence of LFCA activity on the BSA concentration. (b) The activity of LFCA-variants and (c) CtCel8A at different pH values. Both assays were performed at 50ºC using Avicel as substrate in triplicate. Values show average ± SD.*

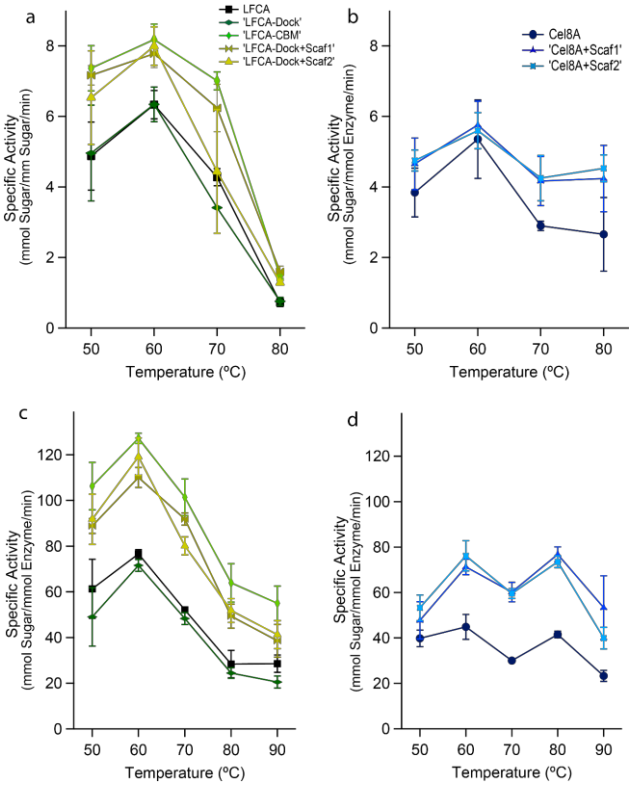


Figure 5.24. Temperature dependence of LFCA and CtCel8A enzymes free and integrated into a mini-scaffold in different substrates. *Avicel (a,b), PASC (c,d). Assays were done in triplicate. Values are shown as average ± SD.*

5.6.Cellulosome

I developed this work during a short stay in Pf Edward Bayer´s laboratory in the Weizmann Institute of Science as part of a collaboration with his group. The aim was to learn the designer cellulosome techniques and the implantation of our ancestral enzymes in their designer cellulosome. . The intention was to redesign and create a new cellulosome composed of ancestral

cellulases (endoglucanase, exoglucanase and beta-glucosidase) as it is shown in **Figure 5.25.**

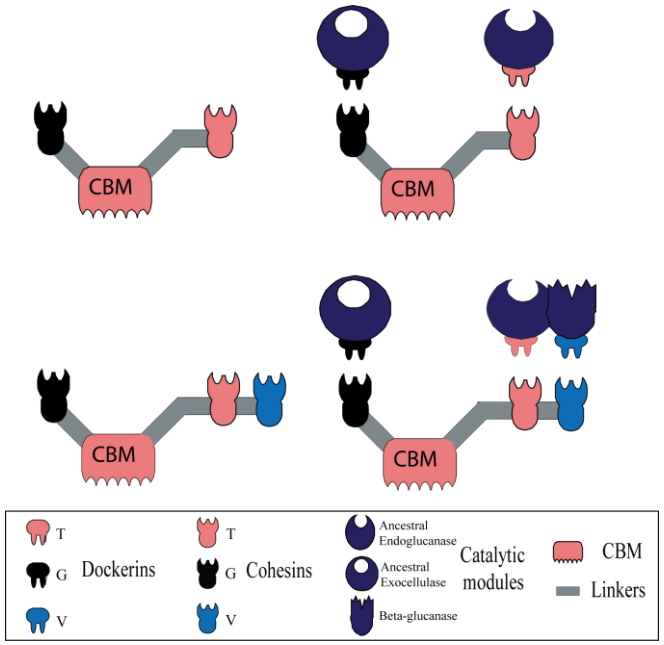


Figure 5.25. Schematic representation of the designed ancestral cellulosomes. *In the legend can be seen which was the meaning of each component. One of the designs was done to assemble the ancestral endoglucanase and the ancestral exoglucanase enzymes (the one on the top). The other one was designed in order to include the three ancestral enzymes (endoglucanase, exoglucanase and beta-glucosidase). The CBM chosen for those cellulosomes was the one from Clostridium Thermocellum.*

5.6.1. Recombinant protein expression

In order to produce designer cellulosomes of the ancestral enzymes, first of all, I carried out the production of both the scaffolds and the previously designed enzyme-dockerin complexes. The production was made as explained in material and methods. Once the production was done, I run acrylamide gels for each recombinant protein as shown in **Figure 5.26**.

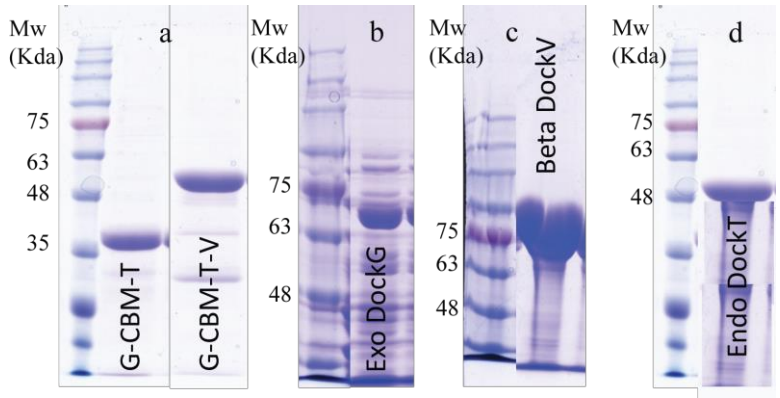


Figure 5.26. Screening of the recombinant proteins.

In those gels, the needed proteins and scaffolds can be seen in their correct sizes. After having all the proteins and scaffolds produced, I did the complexation for the designer cellulosome. For this porpoise, I used the previously described (material and methods) protocol.

5.6.2. Cellulosome construction

I tested the complexation running native gels of each enzyme with its corresponding dockerin and scaffolding. The one for beta-glucosidase was the first one and as **Figure 5.27** shows, I obtained a good complexation.

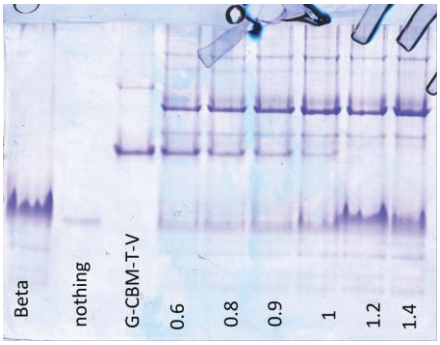


Figure 5.27. Native gel of the complex form between Beta-DockV and the cellulosome scafold. *The number below represents the scaffold/enzyme ratio used in each case.*

We need to test the enzyme/scaffold ratios used in **Figure 5.27**, in order to choose the most appropriate one, In this case, ratio 1 would be the best one as few enzyme and scaffold is left and a big quantity of the complex is formed, as it is shown in the gel (**Fig 5.27**).

In the case of the endoglucanase, **Figure 5.28** shows that we needed to try more ratios to see if the complexations took place or not. I made the same for both of the scaffolds.

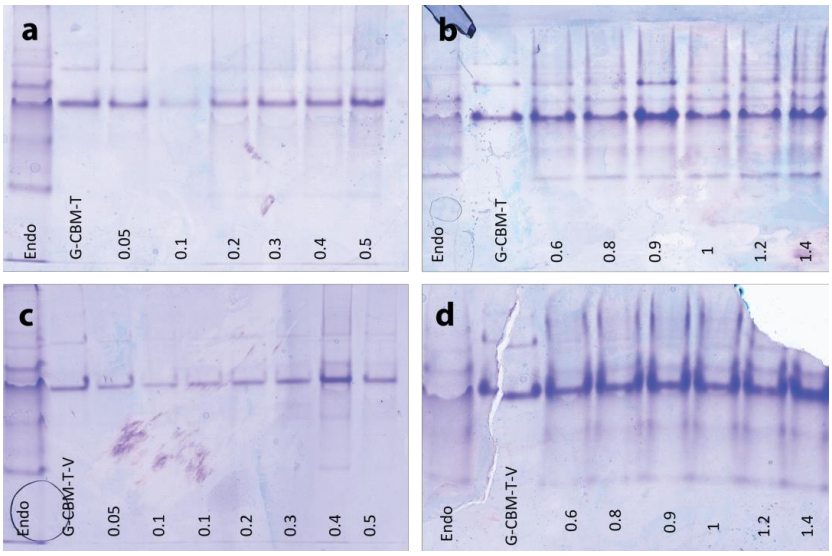


Figure 5.28. Native gel of the complex form between Endo-DockT and the cellulosome scaffold. *The number below represents the scaffold/enzyme ratio used in each case.*

In spite of the fact that, I tried more ratios of complexation, we did not manage to ensure that the complex was taking place. That is way, I carrie out an ELISA [155] affinity assay. All the cohesins in each scaffoldin bound their respective dockerin in a specific way and failed to bind (or bound very poorly) other nonmatching dockerin-bearing molecules. The scaffoldin-borne cohesins bound their matching dockerins as efficiently as the individual monovalent scaffoldins did, indicating that the binding capabilities of the scaffoldins were reliable and selective. All specific cohesin-dockerin interactions, for each scaffoldin, were of similar intensity as judged by the affinity enzyme-linked immunosorbent assay ELISA procedure, thus indicating that similar amounts of protein were bound in each well, suggesting a molar equivalent of the 1:1 scaffoldin (cohesin)-to-dockerin ratio.

By means of this assay (**Fig 5.30**) we conclude that we succeded with the complexation.

In the case of exoglucanase, the same native gels run for endoglucanase were done as **Figure 5.29** shows.

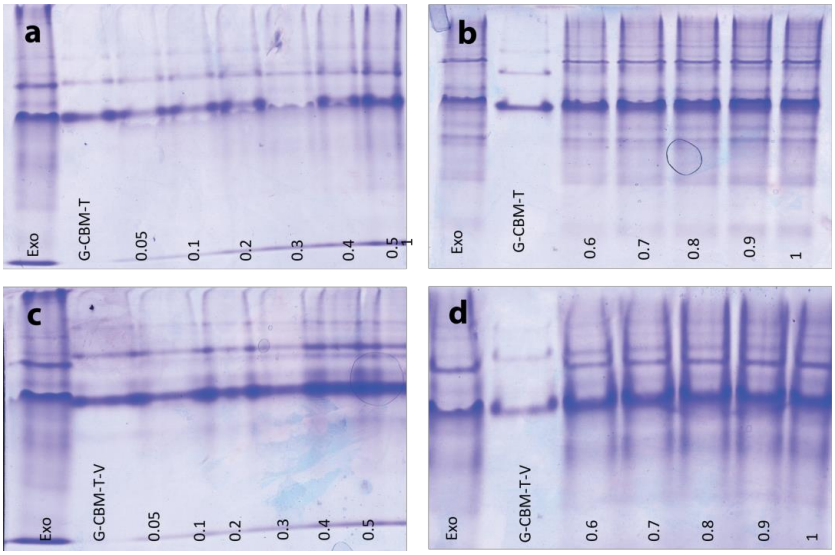


Figure 5.30. Native gel of the complex form between Exo-DockG and the cellulosome scaffold. *The number below represents the scaffold/enzyme ratio used in each case.*

As well as it happened for the endoglucanase, we were not able to see the complexation in the native gels. That is way; also in this case I performed an affinity ELISA. Nevertheless, in this case, the ELISA did not show positive results and that means that the complexation did not succeeded. We hypothesize that this could be because of steric effects and that there is a need on trying by the N-terminal of the ancestral exoglucanase. We designed new primers to a future approach.

In **Figure 5.30** the ELISA experiments results are shown, the endoglucanase-dockerinT complex (in red) was formed as the affinity is really similar to the control (in black). In contrast, the exoglucanase-dockerinG complex was not formed, as the line (in green) has nothing to do with the control.

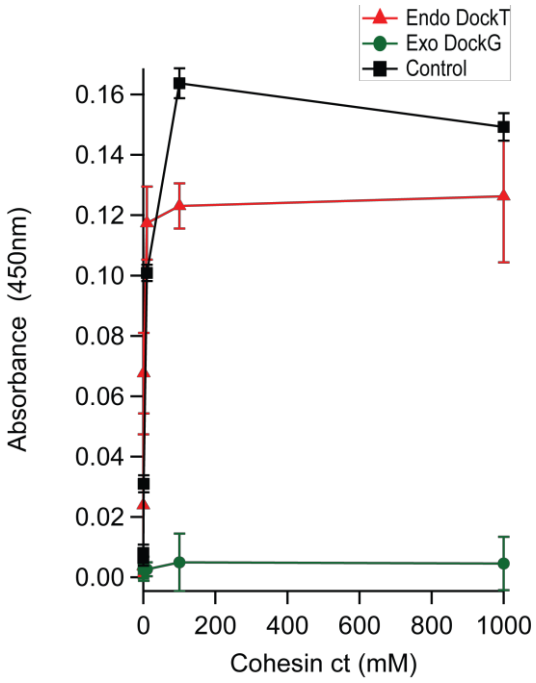


Figure 5.30.Affinity-based ELISA. *The absorbance is plot against the cohesion concentration. Black line represents the control, red one represents the Endo-dockT and green one Exo-DockG.*

Chapter 6: Discussion

In this thesis, I demonstrate that reconstructed ancestral enzymes have improved physicochemical properties that make them suitable for industrial applications. Overall, I demonstrate that the ancestral sequence reconstruction technique can be employed as a general technique for enzyme design I show the high performance over a broad range of temperatures and pH values, of three reconstructed ancestral cellulases (endoglucanase, exoglucanase and beta-glucosidase). The activity of the endoglucanase was notably improved, both as a free enzyme and as a part of a cellulosome complex. The activity of this endoglucanase was compared with two commercial endoglucanases, *T.maritima* and *T.reesei,* which are commonly used in industry. It is important to note that the comparison between ancestral endoglucanase and *T. maritima* endoglucanase is very significant taking into account that *T. maritima* is perhaps the most extremophile bacterium known. We also made a comparison between the ancestral enzymes cocktail and a commercial (Ctec2 cocktail) used in industry, in which our ancestral enzyme cocktail outperformed the commercial one. The specific activity of the ancestral cocktail was higher in all the tested conditions, not only using laboratory substrates as filter-paper but also using other lignocellulosic compounds such as cardboard.

The use of our technology, Ancestral Sequence Reconstruction, enables a bigger improvement of enzymes activity. Using this technique, phylogenetic relationship is studied by means of sequences of enzymes from modern organisms. This is the first time that such a work has been carried out explicitly focusing on industrial enzymes. Until now, ancestral proteins were reconstructed in order to study their evolution [21, 32]. We now extend the reach of the ancestral enzymes bringing real applications to industry such as the bioethanol production [44].

The ancestors of this modern proteins and enzymes often show exceptional properties related to its thermal stability, chemical or kinetic. High activities are expected because of the promiscuity, lower selectivity and therefore more effective with different types of substrates [2-8]. Ancestral enzymes are not necessarily more stable than modern extremophiles [22]. However, those enzymes living in the Hadean and Archean eons, with an estimated temperature of 60-70ºC in the oceans were thermophiles [20, 21, 186, 187]. . This thermophilic phenotype is captured by ancestral reconstruction and exhibited by our ancestral enzymes. Nevertheless, ancestral enzymes display other properties too, such as broad pH, higher expression yields or chemical promiscuity [20, 22, 24, 27, 161]. These properties make them more efficient that the contemporary enzymes, including extremophiles and make them a good alternative in industry.

Ancestral enzymes are known as generalists, which mean that they have a higher applicability comparing with the extant ones. Those extant enzymes are known as specialists, they have evolved to be more effective in a certain organisms and conditions [188]. For example, in the case of thermophile enzymes they are more effective than others under high temperatures, but they may show elevated substrate specificity. Therefore, ancestral reconstruction

is a good methodology for protein engineering having a high applicability in biotechnology [28, 32, 34].

The first enzyme we reconstructed was the endoglucanase, we achieved high specific activities under different conditions and substrates, only testing one enzyme, which makes ASR more efficient than any other protein engineering technique. Other currently available techniques like directed evolution need many variants through long cycles of evolution and testing. Moreover, improving the thermal stability of an enzyme maintaining its catalytic activity unchanged is a challenge in protein engineering.

In this thesis, I also reconstructed the catalytic domain of two other cellulases, i.e., exoglucanase and β-glucosidase. It was not possible to reconstruct the CBM of endoglucanase and exoglucanase, as the sequences of the selected species showed a large variability in these domains, including their position either in the C or N termini. The CBM is poorly aligned demonstrating a molecular diversity that might reflect different origins for this module. For this reason, I decided to focus the analysis on the catalytic domain of the homologous sequences. This procedure was used for both endoglucanase and exoglucanase. In the case of beta-glucosidase, we were able to align all the sequence together as they only had one domain. Taking into account that the CBM is the responsible of hydrolyzing crystalline cellulose, we hypothesize that a change in cellulose structure might be after the origin of the addition of this domain. The fact that beta-glucosidase does not have this domain can supports this idea, as the linkages that it breaks are not crystalline.

The initial results of this thesis were those for endoglucanase. In comparison with the enzymes commercially available and that are used in industry of *T. maritima* and fungal endoglucanase from *T. reesei*, our ancestral endoglucanase showed higher activity

especially at high temperatures and high pH values. Although it is true that the thermophilic endoglucanase from *T. maritima* is slightly more stable than the ancestral endoglucanase, the fact that the specific activity of the ancestral endoglucanase is higher in all conditions tested greatly compensates, and makes it suitable to be used even in the hardest conditions. Apart from testing the ancestral endoglucanase as a free enzyme, it was also tested as a part of a minicellulosome complex. In both cases, the ancestral endoglucanase outperformed the extant ones. It is remarkable, that in the case of the minicellulosome, not only amorphous cellulose was tested, but also crystalline one, which is of a big interest for industry [182].

Moreover, the ancestral endoglucanase also showed very good synergy with other lignocellulosic enzymes such as laccase and xylanase. This synergy was proved using cardboard as substrate.

We obtained similar results when using the ancestral cocktail of enzymes. Ancestral cellulases showed again, that they are much more active than commercial cellulases, exhibiting almost twice their specific activity. This result supports the theory that ancestral proteins can be adapted to harsh conditions obtaining better performances. These ancestral enzymes could obtain higher bioconversion yields and improved the efficiency of the process. Therefore, they represent a promising implementation for industrial usage to overcome the limitations of the current process.

Furthermore, it was observed that the addition of other lignocellulosic enzymes, such as laccase had a positive influence on the hydrolysis, leading to higher activity of the cellulases. This positive effect is caused because of the ability those lignocellulosic enzymes ability to break down the complex network of lignin and thereby, increase cellulose accessibility to

enzymatic attack. In the experiments presented here, it has been possible to observe the effect in different lignocellulosic materials, such as paper and cardboard. Even though different lignocellulosic substrates are composed of the same components, they differ in the percentage of the components and the structure. As a result, different performances are obtained in each substrate. Moreover, its recalcitrant structure and different components makes it difficult the hydrolysis, and lower activity values are obtained compared to the ones obtained in pure cellulose substrates such as filter paper. We expect that other lignocellulosic enzymes, such as laccases and xylanases, including fungal cellulases can benefit from ancestral reconstruction, which can help to generate highly efficient cocktails providing the improvement of the saccharification of cellulosic substrates for numerous industrial applications.

In the final part of my thesis, I show the addition of these improved enzymes to the designer cellulosome. Although it was not fully completed, we expect an optimized degradation of all types of lignocellulosic materials. The synergy of techniques, the ancestral sequence reconstruction and the designer cellulosome will suppose a biologic advance

Lots of efforts have been made in the last years in order to improve the performance and efficiency of enzymes in biotechnology. In fact, it has been one of the paradigms of modern molecular biology. There is still a need for further improvements, especially for industrial applications. Besides the improvement in the efficiency of enzymes, there is a need to upscale the production, in order to supply industrial demand. For that purpose, we can use different organisms that have been genetically modified to produce large amounts of enzymes e.g, *Trichoderma reesei* RUT C30 [189] or *Bacillus Subtilis* [190].

Furthermore, nowadays the possibility of designing an organism able to produce the desired improved enzymes is opened using CRISPR cas9 technique [191]. One can expect that in the future, the combination of existing techniques, such as directed evolution or rational design with ancestral sequence reconstruction could lead to novel enzymes with multiple improved properties and even new tailored functions.

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Appendix I

List of endoglucanase proteins from the species used in the construction of the phylogenetic tree

Q59665 *Cellvibrio japonicus*

ANGQPASFSGMSLFNTEWGGEYYNAQVVSWLKSDWNAKLVRAAM
GVEDGGYLTDPANKDRVTQVVDAAIANDMYVIIDWHSHNAHQYQS
QAIAFFQEMARKYGANNHVIYEIYNEPLQVSWSNTIKPYAQAVIAAIR
AIDPDNLIIVGTPTWSQDVDVAANDPITYQNIAYTLHFYAGTHGQYLR
DKAQTALNRGIALFVTEWGSVNANGDGAVANSETNAWVSFMKTNH
ISNANWALNDKVEGASALVPGASANGGWVNSQLTASGALAKSIIS

Q8VUT3 *Pseudomonas sp*

APISTNGNQLLFGGAVDSVAGPSLFNNGWGGEFYNAGAVASAQQD
WNAEI
RAAMGVDEGGYLEDASALNRVRAVVDAAIANDMYVIIDWHSHHAE
SYTQAAVSFFQQMASEYGQHDNVIYEIYNEPLSVSWSNTIKPYAEQVI
GAIRAVDPDNLIVVGTPTWSQDVDAAANDPITYNNIAYTLHFYAGTH
TQYLRDKAQYALDMGIPLFVTEWGTVNANGDGGVAYNETNTWMD
FLKANNISHANWALNDKAEGSSALVTGTNPSGNWADNQYTASGTFV
RDIVR

C9DDS3 *Pectobacterium carotovorum*

TPVETHGQLSIENGRLVDEQGKRVQLRGVSSHGLQWFGDYVNKDSM
KWLRDDWGINVFRVAMYTAADGYISNPSLANKVKEAVAAAQSLGV
YIIIDWHILSDNDPNIYKAQAKTFFAEMAGLYGSSPNVIYEIANEPNGG
VTWNGQIRPYALEVTDTIRSKDPDNLIIVGTGTWSQDIHDAADNQLP
DPNTMYALHFYAGTHGQFLRDRIDYAQSRGAAIFVSEWGTSDASGN
GGPFLPESQTWIDFLNNRGVSWVNWSLTDKSEASAALAPGASKSGG
WTEQNLSTSGKFVREQIR

Q59394 *Pectobacterium sp*

TPVETHGQLSIENGRLVDEQGKRVQLRGISSNGLQWVGDYVNKDSM
KWLRDDWGINVFRVAMYTAENGYIANPSLANKVKEAVAAAQGLGV
YIIIDWHTLSDNDPNTYKAQAKIFFAEMAGLYGNSPNVIYEIANEPNG
SVTWNGQIRPYALEVTDTIRSKDPDNLIIVGSGTWSQDIHDAADNQLP
DPNTLYALHFYAGTHGQFLRDRIDYAQSRGAAIFVSEWGTSDASGNG
GPFLPESQTWIDFLNNRGISWVNWSLSDKSETSAALVAGASKSGGWT
EQNLSTSGKFVREQIR

Q59395 *Pectobacterium atrosepticum*

TPVETHGQLSIENGRLVDEQGKRVQLRGVSSHGLQWFGDYVNKDSM
KWLRDDWGINVFRVAMYTAADGYISNPSLANKVKEAVAAAQSLGV
YIIIDWHILSDNDPNIYKAQAKTFFAEMAGLYGSSPNVIYEIANEPNGG
VTWNGQIRPYALEVTDTIRSKDPDNLIIVGTGTWSQDIHDAADNQLP
DPNTLYALHFYAGTHGQFLRDRIDYAQSRGAAIFVSEWGTSDASGNG
GPFLPESQTWIDFLNNRGVSWVNWSLTDKSEASAALAPGASKSGGW
TEQNLSTSGKFVREQIR

D0KFU8 *Pectobacterium wasabiae*

TPVETHGQLSIENGRLVDEQGKRVQLRGISSNGLQWVGDYVNKDSM
KWLRDDWGINVFRVAMYTAENGYIANPSLANKVKEAVAAAQGLGV
YIIIDWHTLSDNDPNTYKAQAKIFFAEMAGLYGNSPNVIYEIANEPNG
SVTWNGQIRPYALEVTDTIRSKDPDNLIIVGSGTWSQDIHDAADNQLP
DPNTLYALHFYAGTHGQFLRDRIDYAQSRGAAIFVSEWGTSDASGNG
GPFLPESQTWIDFLNNRGISWVNWSLSDKSETSAALVAGASKSGGWT
EQNLSTSGKFVREQIR

R9F9F1 *Thermobifida fusca*

TPVERYGKVQVCGTQLCDEHGNPVQLRGMSTHGIQWFDHCLTDSSL
DALAYDWKADIIRLSMYIQEDGYETNPRGTDRMHQLIDMATARGLY
VIVDWHILTPGDPHYNLDRAKTFFAEIAQRHASKTNVLYEIANEPNG
VSWASIKSYAEEVIPVIRQRDPDSVIIVGTRGWSSGPAEIAANPVNASN
IMYAFHFYAASHRDNYLNALREASELFPVFVTEFGTETYTGDGANDF
QMADRYIDLMAERKIGWTKWNYSDDFRSGAVFQPGTCASGGWSGS
SLKASGQWVRSKLQ

R4T6Y4 *Amycolatopsis orientalis*

TPVSINGKLHVCGVKLCNQYGKPIQLRGMSTHGIQWYSQCVKTASLD
ALANDWKADILRVAMYIQDDGYESNPRKTDMMHNYIEEATKRGMY

VLVDWHQLDPGDPNVNTDLAKTFFTEIAQRHKDKVNIIYDVANEPN
GVSWADVKRYAEEVIPVIRAQDPDSVVFLGTHGWSTDETDILNNPVN
ATNIMYTFHFYAASHQDEHYDALARTADKLPVFVTEFGTQTYTGDG
GNDFTYSQKYLDLLAAKKIGWTNWNFSDDFRSGAVFKTGTCAGNSF
TGTSLKPAGVWVRDRIR

F4FAV2 *Verrucosispora maris*

TPVQINGQLRVCGVNLCNQYGRPIQLRGMSTHGIQWFGNCYNNASL
DALATDWRADLFRIAMYVQEQGYETDPAGTNRVNNLVEEATRRGM
YAMIDFHLLTPGDPMFNLERAKTFFAAVSARHASKNNVIYEIANEPN
GVSWSTIKNYADQVIPVIRANDPDAVVIVGTRGWSSNHTEIVNNPVN
ASNVMYAFHFYAASHRDNYRAEVERAAARLPLFVTEFGTVDYTGDG
GVDLASSTQWLDLLDRLKIGYANWTFSDKAEGSAALRPGTCNGSNY
TGTSLTPSGVFMRERIR

D9TBA5 *Micromonospora aurantiaca*

TPVAINGQLQVCGVNLCNQYGRPIQLRGMSTHGLQWFANCYTDASL
DVLANEWRSDLLRISMYVQEQGYETNPAGTNQVNTLVDKAEARGM
YALIDFHTLTPGDPMYNLDRAKTFFANVSARNAAKKNVIYEITNEPN
GVSWSTIRNYAEQVIPVIRANDPDAVVIVGTRGWSSNSDEIVNNPVRA
QNIMYTFHFYAASHKDNYRNEVQRAASRLPLFVTEFGTVTYTGDGA
VDTASSNAWLDLLDRLKISYANWTLSDAPEGSAALRPGTCASGSFGG
TSLTESGAFMRERIR

E8SBH4 *Micromonospora sp*

TPVAINGQLQVCGVNLCNQYGRPIQLRGMSTHGLQWFANCYTDASL
DVLANEWRSDLLRISMYVQEQGYETNPAGTNQVNTLVDKAEARGM
YALIDFHTLTPGDPMYNLDRAKTFFANVSARNAAKKNVIYEITNEPN
GVSWSTIRNYAEQVIPVIRANDPDAVVIVGTRGWSSNSDEIVNNPVRA
QNIMYTFHFYAASHKDNYRNEVQRAASRLPLFVTEFGTVTYTGDGA
VDTASSNAWLDLLDRLKISYANWTLSDAPEGSAALRPGTCASGSFGG
TSLTESGAFMRERIR

G8S2I4 *Actinoplanes sp*

TPLAANGQLKVCGAGLCNQNGKKIQLRGVSSHGIHWFPGCYTGAAM
DALATDWNADLFRIAMYVQEGGYESDPTGTAKVNSLVDMAEAHGM
YALIDFHVLNPGDPNLNLARAKEFFAKVAARNAAKKNVIYEIANEPN
GVSWAGIKSYAEQVIPVIRANDPDGIVIIGTRGWSSSSAEIIDNPVNAT

NIMYAFHFYAASHKDDYRAEVQKAAASIPLFVTEFGTVSASGDGAV
DTAGTTAWLDLLDKLKISYANWNFGDKAEGSSILKPGSCNAGAFSGT
GLTPSGQLIRSRIR

T1V343 ***Amycolatopsis mediterranei***

TPLAANGQLHVCGVHLCNEANRAIQLRGMSTHGLQWFDSCYNDASL
DALANDWHADLLRIAMYVQEKGYETNPAWTDRVNSLVGEAEERGM
YAIVDFHTLTPGDPNYNLDRAKTFFAAVAARNAARKNVIYEIANEPN
GVSWGAIKSYAEQVIPVIRAADPDAVVIVGTRGWSSNETEIVNNPVN
AGNIMYTFHFYAASHKDNYRATVSRAATRLPLFVTEFGTVTATGGG
ALDQASTTAWLDLLDQLQISYANWTYSDADESSAALQPGTCAGGDY
GTGRLTASGALVRNRIN

D6KDP0 ***Streptomyces sp***

TPVGVNGQLHVCGVHLCNQYNHPIQLRGMSTHGIQWFSQCYNAASL
DALATDWKADLLRIAMYVQEDGYETDPAGTSRVNGLVDMAEARG
MYALIDFHTLTPGDPNYNLDRAKTFFASVAARNAAKKNVIYEIANEP
NGVSWAAIKNYAEQVIPVIRAADPDAVVIVGTRGWSSNESEIVNNPV
NAANIMYTFHFYAASHKDNYRSTVSRAASQLPLFVTEFGTVSATGGG
AVDQASSTAWLDLLDQLKISYANWTYSDAPEGSAALKPGTCGGSDY
GGSALTESGALVKSRIS

S1SNP7 ***Streptomyces lividans***

TPAAVNGQLHVCGVHLCNQYDRPIQLRGMSTHGIQWFGPCYGDASL
DALAQDWKSDLLRVAMYVQEDGYETDPAGTSRVNGLVDMAEDRG
MYAVIDFHTLTPGDPNYNLDRARTFFSSVAARNADKKNVIYEIANEP
NGVSWTAVKSYAEQVIPVIRAADPDAVVIVGTRGWSSNESEVVNNP
VNATNIMYAFHFYAASHKDDYRAAVSRAATRLPLFVSEFGTVSATG
GGAVDRSSSVAWLDLLDQLKISYANWTYSDADEGSAAFRPGTCEGT
DYSSSGLTESGALLKSRIS

A5A6G0 ***Paenibacillus sp***

GQLKVQGNQLVGQSGQAVQLVGMSSHGLQWYGNFVNKSSLQWMR
DNWGINVFRAAMYTAEDGYITDPSVKNKVKEAVQASIDLGLYVIID
WHILSDGNPNTYKAQSKAFFQEMATLYGNTPNVIYEIANEPNGNVSW
ADVKSYAEEVITAIRAIDPDGVVIVGSPTWSQDIHLAADNPVSHSNVM
YALHFYSGTHGQFLRDRITYAMNKGAAIFVTEWGTSDASGNGGPYL

PQSKEWIDFLNARKISWVNWSLADKVETSAALMPGASPTGGWTDAQ
LSESGKWVRDQIR

I0BQW9 ***Paenibacillus mucilaginosus***

AAAVPYGQLKVQGADLLGESGQRVQLRGMSSHGIHWYGDLVNPGS
LKWLKEDWNSNLFRVAMYTAEKGYITDPSVKEKVKEAVQAAIDLGL
YVIIDWHILTDGDPNTYKTQAKAFFQEMAALYGQYPNVIYELCNEPN
GNVTWAGQIKPYAQELTQAIRAIDPDNIIIVGTPNWSQDVNQAADSPL
PYGNIMYAAHFYAGTHGQWLRDKIDYARSKGAAVFVTEWGASDAS
GDGGPFLREAQEWIDFMNSRGISWANWSLADKEETSAALLPGANPS
GGWPASQLSASGQFVRSKLR

I7L2V5 ***Paenibacillus polymyxa***

TPVERYGQLSVKNGKLVDKNGQPVQLKGISSHGVQWFGDLVNQDT
MKWLRDDWGISVFRVALYTEENGYIANPSLKNKVKEAIEAAQKLGL
YVIIDWHILSDGDPNTHKNEAKAFFNEFSTKYGHLPNVIYELANEPNG
NVNWNNQIRPYASEVSQVIRAKDPDNIIIVGTGTWSQDVHDAADHPL
LDKNTMYTVHFYAGTHGQSLRDRIDYALNKGVGIFATEWGTSDASG
NGGPFLNESKVWTDFMASRKISWANWSLSDKNETSAALLPGADRKG
GWPDSQLTASGKFVKQAIL

G7VSG4 ***Paenibacillus terrae***

TPVERYGQLSVKNGKLVDKNGQPVQLKGISSHGVQWFGDLVNEDS
MKWLRDDWGISLFRVALYTEEDGYITNPSLKNKVKEAIEAAQKLGL
YVIIDWHILSDGDPNIHKNEAKAFFNEFATQYGNLPNVIYELANEPNG
NVNWNNQIRPYALEVSQVIRAKDPDNIIIAGTGMWSQDVHDAADNP
LPDKNTMYTVHFYAGTHGQYLRDRVDYALNKGVGIFATEWGTSDA
SGNGGPFLNESKVWTDFLASRGISWANWSLADKNETSAALLPGANR
KGGWPDSQLSSSGKFVKQAIL

D9IA39 ***Bacillus megaterium***

TPAAKNGQLSIKGTQLVNRDGKAVQLKGISSHGVRWYGDFVNKDSL
KWLRDDWGITVFRAAMYTADGGYIDNPSVKNKVKEAVEAAKELGI
YVIIDWHILNDGYPNQHKEKAKEFFKEMSSLCGNTPNVIYEIANEPNG
DVNWKRDIKPYAEEVISVIRKNDPDNIIIVGTGTWSQDVNDAADDQL
KDANVMYALHFYAGTHGQSLRDKANYALSKGAPIFVTEWGTSDAS
GNGGVFLDQSREWLNYLDSKNISWVNWNLSDKQETSSALKPGASKT
GGWPLTDLTASGTFVRENIL

P23549 ***Bacillus subtilis***

TPVAKNGQLSIKGTQLVNRDGKAVQLKGISSHGLQWYGEYVNKDSL
KWLRDDWGITVFRAAMYTADGGIIDNPSVKNKMKEAVEAAKELGIY
VIIDWHILNDGNPNQNKEKAKEFFKEMSSLYGNTPNVIYEIANEPNGD
VNWKRDIKPYAEEVISVIRKNDPDNIIIVGTGTWSQDVNDAADDQLK
DANVMDALHFYAGTHGQFLRDKANYALSKGAPIFVTEWGTSDASG
NGGVFLDQSREWLKYLDSKTISWVNWNLSDKQESSSALKPGASKTG
GWRLSDLSASGTFVRENIL

D8WN01 ***Paenibacillus campinasensis***

TPVAKNGQLSIKGTQLVNRDGKAVQLKGISSHGLQWYGEYVNKDSL
KWLRDDWGITVFRAAMYTADGGYIDNPSVKNKVKEAVEAAKELGI
YVIIDWHILNDGNPNQNKEKAKEFFKEMSSLYGNTPNVIYEIANEPNG
DVNWKRDIKPYAEEVISVIRKNDPDNIIIVGTGTWSQDVNDAADDQL
KDANVMYALHFYAGTHGQFLRDKANYALSKGAPIFVTEWGTSDAS
GNGGVFLDQSREWLKYLDSKTISWVNWNLSDKQESSSALKPGASKT
GGWQLSDLSASGTFVRENIL

P06565 ***Bacillus cellulosilyticus***

SVVEEHGQLSISNGELVNDRGEPVQLKGMSSHGLQWYGQFVNYESM
KWLRDDWGITVFRAAMYTSSGGYIEDPSVKEKVKEAVEAAIDLGIYV
IIDWHILSDNDPNIYKEEAKDFFDEMSELYGDYPNVIYEIANEPNGDV
TWDNQIKPYAEEVIPVIRNNDPNNIIIVGTGTWSQDVHHAADNQLTDP
NVMYAFHFYAGTHGQNLRDQVDYALDQGAAIFVSEWGTSEATGDG
GVFLDEAQVWIDFMDERNLSWANWSLTHKDESSAALMPGASPTGG
WTEAELSPSGTFVREKIR

O85465 ***Bacillus agaradhaerens***

SVVEEHGQLSISNGELVNERGEQVQLKGMSSHGLQWYGQFVNYESM
KWLRDDWGINVFRAAMYTSSGGYIDDPSVKEKVKEAVEAAIDLDIY
VIIDWHILSDNDPNIYKEEAKDFFDEMSELYGDYPNVIYEIANEPNGD
VTWGNQIKPYAEEVIPIIRNNDPNNIIIVGTGTWSQDVHHAADNQLAD
PNVMYAFHFYAGTHGQNLRDQVDYALDQGAAIFVSEWGTSAATGD
GGVFLDEAQVWIDFMDERNLSWANWSLTHKDESSAALMPGANPTG
GWTEAELSPSGTFVREKIR

U5MQR0 ***Clostridium saccharobutylicum***

ATTSFGGQLKVVGSQLCDSNGKPIQLKGMSSHGLQWYGQFVNYDSM
KFLRDKWGVNVIRAAMYTNEGGYISNPSSKEKIKKIVQDAIDLNMYV
IIDWHILSDNNPNTYKEQAKSFFQEMAEEYGKYSNVIYEICNEPNGGT
NWANDIKPYANYIIPAIRAIDPNNIIIVGTSTWSQDVDIAADNPLRYSNI
MYTCHFYAGTHTQSLRDKINYAMSKGIAIFVTEWGTSDASGNGGPYL
DESQKWVDFMASKNISWTNWALCDKSEASAALKSGSSTTGGWTDS
DLTTSGLFVKKSIG

R6S0D3 *Eubacterium siraeum*

TPVSQHGQLSVKNGQLVDKSGKGYQLRGMSTHGLTWFPEFVNESAF
KTLRDDWNTNVVRLAMYVDEGCYMGNKSGLELLEKGVDICIKLDM
YVIIDWHVLNPGDPSKYTNEAKSFFETVSKRYAKYPNVIYEICNEPNG
GASWSGNIKPYAEKIIPVIRKNAPNSVIIVGTPTWSQEIDKPLSDPLNY
KNVMYAFHFYAATHAGLRSNVENCVAQGLPVFVSEFGTCDASGGG
ANDFNETQKWLSYFDKQGISYCNWSICNKDETCSVLRPGTSANGNW
SESNLTENGKWMRNWFR

R5K1B8 *Clostridium sp*

TPLENHGALQVKGTQLVDKNGAPYQLKGVSTHGLAWFPEYVNKDA
FQTLRDDWGANVVRLAMYTDEGGYCNREQLKQLVSDGVEYATELG
MYVIIDWHILHDQDPTVYQGEAEDFFAEMSAKYAKYDNVIYEICNEP
NGGATWDGSVKPYAETIIPIIRKNDKDAIIIVGTPTWSQDVDAAADNPI
TQTNIMYAIHFYAATHTDGIRSKVSYALDKGLPVFVSEFSICDASGNG
SNDYDQAAKWFDLIDENQLSYCSWSLSNKAETSALISSGCSKTSGWS
EDDLSETGKWIRDQIL

R5JFB6 *Coprococcus sp*

TPVENHGKLSVKGTDLVDKNGDKYQLKGLSTHGMTWFPQYVSEETF
KTLRDDWGANLIRLAMYTDTGGYCDKAEIKKLLDDGVGYASDLGM
YVIIDWHILNDNNPNNHIEDAKDFFNTVSKEYAEYDNVLYEICNEPNG
GTTWTDVKSYAETIIPVIRANDKDAIIIVGTPTWSQDVDIASENPVTYD
NIMYAAHFYAATHKQELRDKISKAIDNGLPVFISEFSICDASGNGAID
YNEADAWFEFIDKYNLSYASWSLSNKAETASLFSSSSTTVSDFSESDIS
DTGKYIRDKIL

R5W9F0 *Coprococcus eutactus*

TPFDNHGQLSVKGTDIVDESGSKYQLKGVSTHGITWFPDYVNKDAFQ
SIRDDWDANLVRLAMYTDTGGYCDKDSIRGLVDAGVTAATELGMY

VIIDWHILNDNNPNSHIDDAKEFFDDVSAKYSSNHNVIYEICNEPNGG
TSWSDIKSYAEIIIPVIRKNDKNAIIIVGTPNWSQDVDIVSEDPITYDNI
MYAVHFYAATHKDDLRNKVKTAISNGLPVFVSEFSLCDASGNGGID
YDSSDVWFDLINDNNLSYASWSLCNKNETSALIKPDSTATSTITIDDLS
DTGKYVRDKIL

I5AVZ7 *Eubacterium cellulosolvens*

TPFENHGKLSVKGTDLVDESGKKYQLRGVSTHGLAWFPQYVNADAF
RTLRNDWGANLVRLAMYTDEGGYCNQGELKDLIDRGVNYASDLGM
YVIIDWHILYDNDPNQHKEEAKAFFDEMTKKYADRGNVLYEICNEPN
GATTWADVKKYAEEVIPVIRKNAPDAVIICGTPTWSQDVDQVAADPI
KGGNLMYTLHFYAATHKEDLRKKMQTAIASGTPVFISEFSICDASGN
GTLDYSSAEEWKNLIKEYNLSFAGWSLSNKDEASAIVRSGCDRLSDW
TEGELSDSGNWLKKLVS

R6QN32 *Butyrivibrio sp*

TPVGNHGQLSVKGVDLVDKNGSKYQLKGVSTHGLQWFPQYVNKDA
FKTLRDNWGANVVRLAMYTGENGYCSKADLEAKIDEGVKAASELG
MYVIIDWHILSDGNPNTYKDEAVKFFNKMSKKYSKNVNVIYEICNEP
NGGVDWNTIKTYADTIISTIRKNSPNAIILVGTPTWSQDVDAVAANPV
AKKNVMYTLHFYAGTHKDNIRNKLTAARNAGTPVFISEFSICDASGN
GGIDSTSANAWKKLINDNNVSYVGWSLCNKAETSALIVSSCSKLSGW
TDSELSETGKWLRNFIA

C4Z6Y9 *Eubacterium eligens*

VTTVPTGRLQVSGTKLTDESGNIIQLRGVSTHGISWFPDYVNYDAFAT
LRDDWGANVVRIAMYPEENGYLDKAALKQIIDNGVNYATELGMYVI
IDWHVLNYAPSRHTQEACDFFAEMASKYSGHDNVIYEICNEPVGAD
WNSDIKPYAETVIGTIRQFDDHALILVGTNTWSQDVDSVVGNTLDDG
NVMYVAHFYAGTHKENIRNKISTALNAGVPVFISECSICDASGNGGID
YASANEWLDFMNSNQLSFIAWSLSNKAETSALISSGCSAKSGWSDGD
LSETGRWFKSAIS

Resurrected endoglucanase sequence

**TPVETHGQLSVKGGQLVDENGKPVQLRGMSSHGLQWFGDFVNK
DSMKWLRDDWGINVFRVAMYTAEGGYITNPSVKNKVKEAVEAA
IDLGMYVIIDWHILSDNDPNTYKEQAKAFFQEMAAKYGNYPNVIY
EICNEPNGGVTWSNQIKPYAEEVIPAIRANDPDNIIIVGTPTWSQD**

VHDAADNPLPYSNIMYALHFYAGTHGQSLRDKIDYALSKGVAIFV
TEWGTSDASGNGGPFLNESQKWIDFMNSRNISWANWSLSDKSET
SAALMPGASPTGGWTDSNLSASGKFVREQIR

Appendix II

List of exoglucanase proteins from the species used in the construction of the phylogenetic tree

O65986 ***Clostridium cellulovorans***

VVPNNEYVQHFKDMYAKIHNANNGYFSDEGIPYHAVETLMVEAPDY
GHETTSEAFSYYMWLEAMNAKLTGDFSGFKKAWDVTEKYIIPGETD
QPSASMSNYDPNKPATYAAEHPDPSMYPSQLQFGAAVGKDPLYNEL
KSTYGTSQVYGMHWLLDVDNWYGFGGATSTSPVYINTFQRGVQESC
WETVPQPCKDEMKYGGRNGFLDLFTGDSQYATQFKYTNAPDADAR
AVQATYYAQLAAKEWGVDISSYVAKSTKMGDFLRYSFFDKYFRKV
GNSTQAGTGYDSAQYLLNWYYAWGGGISSNWSWRIGSSHNHFGYQ
NPMAAWILSNTSDFKPKSPNAATDWNNSLKRQIEFYQWLQSAEGGIA
GGASNSNGGSYQAWPAGTRTFYGMGYTPHPVYEDPGSNEWFGMQA
WSMQRVAEYYYSSKDPAAKSLLDKWAKWACANVQFDDAAKKFKIP
AKLVWTGQPDTWTGSYTGNSNLHVKVEAYGEDLGVAGSLSNALSY
YAKALESSTDAADKVAYNTAKETSRKILDYLWASYQDDKGIAVTET
RNDFKRFNQSVYIPSGWTGKMPNGDVIQSGATFLSIRSKYKQDPSWP
NVEAALA

O82831 ***Clostridium josui***

PVNKVYQERFESMYNKIKDPSNGYFSEEGIPYHSVETLMVEAPDYGH
VTTSEAMSYYMWLEAMYGRFTGDFSGFNKSWTTTEKYLIPTEKDQP
NSSMSRYDANKPATYAPEFQDPSKYPSPLDTSQPVGRDPINSQLTSAY
GTSMLYGMHWLLDVDNWYGFGVRADGTGKPSYINTFQRGEQESTW
ETIPQPCWDEHKFGGQYGFLDLFTKDTGTPAKQFKYTNAPDADARA
VQATYWANEWAKEQGKSVSTEVGKASKMGDYLRYSFFDKYFRKIG
QPTQAGTGYDSAHYLLSWYYAWGGGVDSTWSWIIGCSHNHFGYQN
PFAAWVLSTDSSFKPKSSNGATDWAKSLDRQLEFYQWLQSSEGAIAG
GATNSWNGRYESIPSGTSTFYGMGYVENPVYADPGSNTWFGMQVW
SMQRVAELYYKTGDTRAKNLLDKWAKWVNSEIKFNADGTFQIPGTL
DWEGQPDTWDPTQGYTGNPNLHVKVVNYNTDLGCASSLANTLTYY
AAKSGDTTSKENAKKLLDAMWNNYSDSKGISTIEQRGDYHRFLDQE

VYVPAGWTGKMPNGDVIKSGVKFIDIRSKYKQDPEWQTMVAALQA
GQVPTQRLHRFWAQSEFAVANGVYAILFP

P50900 ***Clostridium stercorarium***

SSDDPYKQRFLELWEELHDPSNGYFSSHGIPYHAVETLIVEAPDYGHL
TTSEAMSYYLWLEALYGKFTGDFSYFMKAWETIEKYMIPTEQDQPN
RSMAGYNPAKPATYAPEWEEPSMYPSQLDFSAPVGIDPIYNELVSTY
GTNTIYGMHWLLDVDNWYGFGRRADRISSPAYINTFQRGSQESVWE
TIPQPCWDDLTIGGRNGFLDLFVGDSQYSAQFKYTNAPDADARAIQA
TYWANQWAKEHGVNLSQYVKKASRMGDYLRYAMFDKYFRKIGDS
KQAGTGYDAAHYLLSWYYAWGGGITADWAWIIGCSHVHAGYQNP
MTAWILANDPEFKPESPNGANDWAKSLERQLEFYQWLQSAEGAIAG
GATNSYKGRYETLPAGISTFYGMAYEEHPVYLDPGSNTWFGFQAWT
MQRVAEYYYLTGDTRAEQLLDKWVDWIKSVVRLNSDGTFEIPGNLE
WSGQPDTWTGTYTGNPNLHVSVVSYGTDLGAAGSLANALLYYAKT
SGDDEARNLAKELLDRMWNLYRDDKGLSAPETREDYVRFFEQEVYV
PQGWSGTMPNGDRIEPGVTFLDIRSKYLNDPDYPKLQQAYNEGKAPV
FNYHRFWAQCDIAIANGLYSILFG

A9KT91 ***Clostridium phytofermentans***

TRGTYEQRFMDLWSDIKNPKNGYFSPQGIPYHSIETMIVEAPDYGHVT
TSEAMSYYMWLEAMYGKFTGDFSGYGTAWNVAEKYMIPTDADQPP
TSMSKYTPSKPATYAPEYQDPSQYPAKLDSSAPVGSDPIWSQLVAAY
NTIYGMHWLLDVDNWYGFGSRGDGTSKPSYINTFQRGEQESTWETIP
QPCWDTMKYGGTNGFLDLFTGDSSYAQQFKYTDAPDADARAIQAA
YWASEWAKDYGVNVDTYSSKATMMGDYLRYSMFDKYFRKIGNST
VAGTGYDASHYLLSWYYAWGGGITADWAWVIGSSHNHFGYQNPM
AAWVLSQNSKFKPKTTNGQADWATSLTRQLEFYQWLQSSEGGIAGG
ASNSKNGRYETWPAGTATFYGMGYEANPVYKDPGSNTWFGFQAWS
MQRVAYYYKTNDVKAKQILDKWVAWVKSVVVLKADGTFTIPSTLD
WSGQPDTWTGSYTGNSKLHVTVVDSGTDLGVTGSLANALLYYSKA
ANDVAAKNLAKELLDRVWKLYRDDKGVAAPEARADYKRFFEQTVY
VPSTFNGKMPNGDVIKSGIKFLDIRSKYLQDPSYPKLLAAYQSNKSPE
FIYHRFWAQCDVALANGVYALLYEN

W6AP62 ***Bacillus pumilus***

NKERFLTLYHQMKNDANGYFSKEGIPYHSVETLICEAPDYGHMTTSE
AYSYWLWLEVLYGHYTGDWSKLEAAWDNMEKFIIPVNEDGHDEQP
HMSSYNPSSPATYASEKPYPDQYPSQLTGARPAGQDPIDHELRSTYGT
NEYLMHWLLDVDNWYEYGNLLNPSHTAAYVNTFQRGPQESVWEAI
PHPSQDDKTAGKPNEGFMSLFTKENQAPAAQWRYTNATDADARAIQ
AMYWAKELGYNGSAYLDKAKKMGDFLRYGMYDKYFQTIGSGKQG
NPYPGNGKSACHHLMAWYTSWGGGLGEYANWSWRIGASHCHQGY
QNPVAAYALSSDKGGLKPSSPTGASDWEKTLKRQLEFYVWLQSKEG
AIAGGATNSWNGDYSAYPAGRSTFYDMAYEDAPVYHDPPSNNWFG
MQAWPIERVAELYYIFAKDGDKTSENFQMAKSVITKWVNYSLDYIFI
GTRPVSDQDGYFLDGQGQRILGGANVAVATTSGEFWIPGNIEWSGQP
DTWSGFQSATGNPNLTAVTKDPTQDTGVLGSLIKALTFFAAATQKET
GNYTALGIRAKETAAQLLEVAWNYNDGVGIVTEEDREDYHRFTGK
WENGAPSFTYHRFWSQVDMATAYAEYHRLIN

I0BR01 *Paenibacillus mucilaginosus*

DTTNKTRFLTLYNQIKDPANGYFSPEGIPYHAVETLLSEAPDHGHMTT
SEAYSYWLWLEALYGHHTGNWTRLEQAWDNMEQYIIPNASEQPTM
SGYNPASPATYAPEHRQPDQYPSQLGSSVTAGKDPLDAELKATYGSN
QTYLMHWLVDVDNWYGFGNSLNPSHTATYINTFQRGEQESVWEAIP
HPSQETFQFGKPGEGFATLFVKDSGAPAKQWRYTDATDADARVVQV
MYWAKSLGYSNPVYIEKAKKMGDYLRYGMYDKYFQQIGSAADGTP
SPGTGKDSSHYMAWYTAWGGGIGSNWAWRIGASHNHQAYQNPMA
AYALSEGGLAPKSPTAKQDWETSLKRQLEFYTWLQSSEGGIGGGATN
SKGGTYAPYPAGVSTFYGMAYEDAPVYHDPPSNTWFGFQAWPVERI
AEYYYAMAAKGDTASENFKMAKRVMDQWVKWALAYTTTPAPGQF
YILGGQEWTGQPDSWKGFSSFTGNPNYHVIAKGTSQDTGVLGSYIKL
LTFYAAGTQAENNGTLSAAGAQAKTTAEQLLNVAWNHNDGIGIAVP
EKRGDYSRYFTKEIYFPSGWSGTYGQGNALPGTGAVPSDPAKGGNG
VYLSYSELRPKIKQDPKWPYLENLYKSSYDPVTKKWTNGEPTFTYHR
FWAQVDMATAYA

Q8KKF7 *Paenibacillus barcinonensis*

DGINEARFLQLYAQLKDPANGYFSAEGIPYHSIETLLSEAPDYGHMST
SEAYSYWLWLETMYGHYTGDWSKLEAAWDNMEKYIIPVNEGDGNE
EQPTMSKYNPNSPATYAAEKPFPDQYPSNLNGQYAAGKDPLDAELK
ATYGNQTYLMHWLIDVDNWYGYGNLLNPSHTSTYVNTFQRGEQES
VWEAIPHPSQDDKSFGKAGEGFMSLFTKESQAPAQWRYTNATDADA
RAVQVLYWAKEMGYNNSEYLDKAKKMGDFLRYGMYDKYFQKVGS

AKNGTPTPGTGKDSNMYLMAWYTSWGGGLGQGGDWAWRIGASHT
HQAYQNPVAAYALSDPAGGLIPDSPTAKADWNATLKRQLEFYTWLQ
SHEGAVGGGATNSIGGSYAAYPAGVSTFYDMAYQEAPVYRDPDSNT
WFGFQAWPLERVAEMYYILAESGDLTSENFQMAKKVITKWVDWTK
DYVTTPAPGEFWIPGGQEWQGQPDKWNGFSTYTNNPNFHAITKDPV
QDTGVLGSYIKALTFFAAGTEAENGTLSAKGQEAKDLAQSLLDTAW
DYNDGVGIVTEEERKDYFRYFAKEIYLANWSGTFGQGNTIPGTAGVP
SDPAKGGNGVYIGYSDLRPAIKQDPAWAYLDNKYKTSYNPTTKQWE
NGAPTFTYHRFWSQVDMATAYG

W4CUL3 *Paenibacillus sp*

ASVEKTRFLQLYDQLKDPASGYFSAEGIPYHAVETLLSEAPNYGHMT
TSEAYSYWMWLEVLYGHNTGDWSKLESAWDNMEKYIIPINEGDGV
QEQPTMSSYNPNSPATYASELPQPDQYPSALNGKYTPGKDPLDAELK
STYGNNQTYLMHWLVDVDNWYGFGNLLNPTHTASYVNTFQRGVQE
SVWEAVGHPSQDNKTFGKSNEGFMSLFTKENSVPSAQWRYTNATDA
DARAVQAMYWAKDLGYTNTVYLNKAKKMGDFLRYGMYDKYFQKI
GSAANGSPQPGTGKDSSQYLLAWYTAWGGGLGTGGDWAWRIGASH
AHQGYQNVVAAYALSTAAGGLIPSSATAGTDWGKSLTRQLEFYNWL
QSAEGAIAGGATNSYGGSYSAYPAGKSTFYGMAYEEAPVYHDPPSN
NWFGMQSWSMERMAELYYILASSGDTSSDNFKMAKRVIENWINWS
KDYVFYILGGQEWTGQPDSWKGFSSFTGNPNYHVIAKGTSQDTGVL
GSYIKLLTFYAAGTQAENNGTLSAAGAQAKTTAEQLLNVAWNHND
GIGIAVPEKRGDYSRYFTKEIYFPSGWSGTYGQGNALPGTGAVPSDPA
KGGNGVYLSYSELRPKIKQDPKWPYLENLYKSSYDPVTKKWTNGEP
TFTYHRFWAQVDMATAYA

G7VQK6 *Paenibacillus terrae*

ATPESTRFLQLYKQLKDPASGYFSKEGIPYHSVETLLSEAPDYGHLTT
SEAYSYWMWLEVLYGNYTGDWGHLESAWDNMEKYIIPGKEEQPTM
SNYNPNSPATYAAEYSQPDLYPSRLSDQYSAGKDPLDSELKATYGNN
QTYMHWLLDVDNWYGFGNLLNPSHTATYVNTFQRGEQESVWEAIP
HPSQDNHKFGKSNEGFMSLFTKENNAPQQWRYTNATDADARAVQA
MYWAKELGYDNSVYLDKAKKMGDYLRYGMYDKYFQKAGSASKGS
PIAGTGKDASFYLMAWYTAWGGGLGQSGNWAWRIGASHAHQGYQ
NVVAAYALSDKDGGLIPNSPTAGQDWATSLKRQLEFYTWLQSDEGA
IAGGATNSWDGAYKAYPSGTSTFYGMAYTGAPVYQDPPSNNWFGM
QAWPVERVAELYYILAKKGDTSSEQFKMAKQVTENWIAWSKSYVK
GEFWLPSDLEWSGKPDTWSGFANHKGNTSLHVVTKKPVQDAGVLG

SYAKALTFFAAGTKAEKGDYSELGKEAKDLSKALLDAAWSYNDGIG
ITTKEAREEYYRYFSKEVYIPNGWSGKTGQGNTIPGKDATPSDPSKGG
NGTYSTYSDIRPNITKDPQWSYLKDKYTTSWNSQTQKWDKGAPQFT
YHRFWAQVDMATAYA

E0RLD5 *Paenibacillus polymyxa*

ATPESTRFLQLYKQLKDPASGYFSKEGIPYHSVETLMSEAPDYGHLTT
SEAYSYWMWLEVLYGHYTGDWGHLESAWDNMEKYIIPVNEGDGKE
EQPTMSNYNPNSPATYAAEYSQPDQYPSRLSGQYGAGKDPLDSELKA
TYGNNQTYLMHWLLDVDNWYGFGNLLNPSHTAAYVNTFQRGEQES
VWEAVPHPSQDNQKFGKLNEGFMSLFTKENNAPAQQWRYTNATDA
DARAVQAMYWAKELGYDNSVYLDKAKKMGDFLRYGMYDKYFQK
TGSASNGSPIAGTGKDASLYLMAWYTAWGGGLGQSGNWAWRIGAS
HAHQGYQNVVAAYALSNRDGGLIPNSPTAGQDWATSLKRQLEFYT
WLQSDEGAIAGGATNSWDGAYKAYPSGTSTFYGMAYTGAPVYNDP
PSNNWFGMQAWPVERVA
ELYYILAKKGDTSSEQFKMAKQVTENWIAWSKSYVTTAAKGEFWLP
SNLEWSGKPETWSGFANHKGNTNLHVVTKNPGQDAGVLGSYVKAL
TFFAAGTKAEKGDYSELGKEAKDLSKALLDAAWGYNDGIGITTKEA
REDYYRYFTKEVYIPNGWSGKTGQGNTIPGTDATPSDPSKGGNGTYS
SYSDIRPNITKDPQWSYLKDKYTTSWNKQTKKWDKGAPEFTYHRFW
SQVDMATAYA

R4LQA1 *Actinoplanes sp*

EGSADARFAQLYNDIKNPANGYFSPEGVPYHSVETLIDEAPDQGHET
TSEAFSYWLWLEAEHGRVAGDWTSFNAAWQTMEKYIIPSHADQPTN
DKYDPSKPATYAAEHPLPSQYPSQLDSSVSVGTDPLANELKSTYGTP
DIYGMWLLDVDNVYGFGHCGDKTSRVTYINTFQRGPQESTFETVPQP
SCDTFAAGGPNGYLDLFTKDTSYAKQWKYTDAPDADSRAVQAAYW
ALTWATEQGKASQVSASVANAAKMGDYLRYSFYDKYFKNPGCTST
GCAAGSGKSSSNNLMSWYYAWGGATDTSAGWAWRIGSSTSHFGYQ
NPMAAYVLSNNASMTPKSPTAKADWQASLNRQLEFYQWLQSSEGGI
AGGATNSWNGSYQAPPSGTPTFYGLSYVEAPVYEDPPSNRWFGMQT
WSLERLAEYYYLSGDTKAKAVLDKWVPWALDNSTIGATSFEIPSDLA
WSGKPATWNPSSPAANTGLHVTVSTKGQDLGVAGSFAKLLTYYAAK
SGNTRAKDAAKGLLDAIWAYKDSKGVSVTETRADYNRMDDVYNAS
TGQGIYIPPGWSGKMPNGDVIKPGVSFLDIRSWYKNDPDFAKVQSYL
DGGPAPTFNYHRFWAQVDVATGYA

A0LSI0 ***Acidothermus cellulolyticus***

NDPYIQRFLTMYNKIHDPANGYFSPQGIPYHSVETLIVEAPDYGHETT
SEAYSFWLWLEATYGAVTGNWTPFNNAWTTMETYMIPQHADQPNN
ASYNPNSPASYAPEEPLPSMYPVAIDSSVPVGHDPLAAELQSTYGTPD
IYGMHWLADVDNIYGYGDSPGGGCELGPSAKGVSYINTFQRGSQESV
WETVTQPTCDNGKYGGAHGYVDLFIQGSTPPQWKYTDAPDADARA
VQAAYWAYTWASAQGKASAIAPTIAKAAKLGDYLRYSLFDKYFKQ
VGNCYPASSCPGATGRQSETYLIGWYYAWGGSSQGWAWRIGDGAA
HFGYQNPLAAWAMSNVTPLIPLSPTAKSDWAASLQRQLEFYQWLQS
AEGAIAGGATNSWNGNYGTPPAGDSTFYGMAYDWEPVYHDPPSNN
WFGFQAWSMERVAEYYYVTGDPKAKALLDKWVAWVKPNVTTGAS
WSIPSNLSWSGQPDTWNPSNPGTNANLHVTITSSGQDVGVAAALAKT
LEYYAAKSGDTASRDLAKGLLDSIWNNDQDSLGVSTPETRTDYSRFT
QVYDPTTGDGLYIPSGWTGTMPNGDQIKPGATFLSIRSWYTKDPQWS
KVQAYLNGGPAPTFNYHRFWAESDFAMANA

W7VNI1 ***Micromonospora sp***

DNAYIKKFLDQYGKIKNSGYFSPEGVPYHSIETLIVEAPDHGHETTSE
AFSFWLWLEAQYGRVTQNWAPFNNAWTVMEKYIIPSHADQATAGSP
GTPQYAAEHNLPSQYPSTLDANVPVGQDPLRSELQSTYGTGDIYGMH
WLLDVDNTYGYGRCGDGTTRPAYINTFQRGTQESVWETVPQPSCDT
FKHGGPNGYLDLFVKESNAPAKQWK
YTNAPDADARAVQAAYWALTWAKAQGKAGDVAATVAKAAKMGD
YLRYALFDKYFKKIGNCVGASTCPAASGRDSAHYLLSWYYAWGGA
YDASQNWSWRIGSSHSHFGYQNPFAAWVMTNVAELKPKSPTAASD
WQKSLDRQLEFYTWLQSAEGGIAGGATNSWDGSYAQPPAGTATFYG
MFYDVDPVYNDPPSNQWFGMQAWSMQRIAELYLETGNAKAKALLD
KWVPWAIANTTLGTDWSIPSDMKWTGQPANWNPSSPQPNTNLHVEV
TVKGQDVGVAGAYARTLIAYAAKSGNTAAKDTAKGLLDALSAAAD
SKGVSTPEKRGDYKRFDDVYNAADGQGLYIPNGWTGKMPNGDVIAP
GKSFLDIRSFYKNDPDWPKVQAYLDGGPEPVFNYHRFWAQADIAMA
YA

A4X938 ***Salinispora tropica***

DNEYVARFLTQYGKIKNSGYFSSEGVPYHSIETLIVEAPDHGHETTSE
AFSFWLWLEAQYGRVTEDWAPFTNAWTVLENYIIPSSADQPTAGAS
GTAQYAAEYDLPSQYPAQLQPSVPVGQDPLRGELQSTYGTGDIYGM
HWLLDVDNTYGFGRCGDGTTRPAYINTFQRGQQESVWETVPQPSCE

TFTHGGQYGFLDISVQEQNAPAQQWKYT
NAPDADARAVQAAYWALTWAKQQGRAAEVAATVAKAAKLGDYL
RYAMFDKYFKQIGNCVGASTCPAGSGRESAHYLLSWYYAWGGAYE
SGQNWSWRIGSSHNHFGYQNPFAAWALTTVPELEPRSPSATTDWAR
SLERQLELYTWLQSAEGAIAGGATNSWGGRYAQPPAGTPTFYGMFY
DEKPVYHDPPSNQWFGMQVWSMHRIAELYLETGDARAEALLDRWV
PWAIANTRLGADWSIPAELTWTGQPNTWNPTNPEPNTDLHVEVTETG
QDVGAAAAYARTLIAYAARSGNVTAKTTAKGLLDALHAASDALGV
STVEKRGDYERFDDVYDASTGQGLYLPPGWTGTMPNGDVIEAGRSF
VEIRSFYLNDPDWPKVQAYLDGGAEPTFRYHRFWAQADVAMAYA

Q9XCD4 *Thermobifida fusca*

TSSYDQAFLEQYEKIKDPASGYFREFNGLLVPYHSVETMIVEAPDHG
HQTTSEAFSYYLWLEAYYGRVTGDWKPLHDAWESMETFIIPGTKDQ
PTNSAYNPNSPATYIPEQPNADGYPSPLMNNVPVGQDPLAQELSSTY
GTNEIYGMHWLLDVDNVYGFGFCGDGTDDAPAYINTYQRGARESV
WETIPHPSCDDFTHGGPNGYLDLFTDDQNYAKQWRYTNAPDADARA
VQVMFWAHEWAKEQGKENEIAGLMDKASKMGDYLRYAMFDKYFK
KIGNCVGATSCPGGQGKDSAHYLLSWYYSWGGSLDTSSAWAWRIGS
SSSHQGYQNVLAAYALSQVPELQPDSPTGVQDWATSFDRQLEFLQW
LQSAEGGIAGGATNSWKGSYDTPPTGLSQFYGMYYDWQPVWNDPPS
NNWFGFQVWNMERVAQLYYVTGDARAEAILDKWVPWAIQHTDVD
ADNGGQNFQVPSDLEWSGQPDTWTGTYTGNPNLHVQVVSYSQDVG
VTAALAKTLMYYAKRSGDTTALATAEGLLDALLAHRDSIGIATPEQP
SWDRLDDPWDGSEGLYVPPGWSGTMPNGDRIEPGATFLSIRSFYKND
PLWPQVEAHLNDPQNVPAPIVERHRFWAQVEIATAFA

W7SI25 *Kutzneria sp*

TSDYQVEFLKEYNKIKDPNSGYFRKFGNLLVPYHSVETLMVEAPDYG
HETTSEAFSYYLWLEASYGRITQDWAPFNAAWTSLETFAIPSDADQP
TNSGYNASKPATYAAEYPSPTKYPSQLQSGVAVGSDPIAGELKSTYG
TSSVYGMHWLFDVDNIYGFGHCEDGTNTTPAFINTFQRGSQESVWET
VTQPSCDMMKYGGKNGYLDLFTGDSSYAKQWKYTDAPDADARAV
QVAYQAEQWAKAQGKSSAVADVVKKASKMGDYLRYSLFDKYFKKI
GNCVGPSTCPAGSGKDSEHYLISWYYAWGGSADTSNAWAWRIGDG
AAHQGYQNPLAAYALSTDPGLKPLSATGSSDWATSLGRQLEFLQWL
QSSEGGIAGGATNSWDGQYGTPPSGDPTFYGMYYDQQPVWHDPPSN
QWFGFQTWGMERVAEYYQTTKDPRAKKVLDKWVPWAIANTTVGA
GGSFQIPSDLTWTGAPDTWNATSPGGNTGLHVAVKNYTQDVGVAGS

LAKTLMYYAAGSGDTKSRTVAEGLLTALTAHEDSLGIAVPETRTDYN
RFDDTYDAAADQGLYVPPGWTGTMPNGDPINSNSTFLSIRSFYKNDP
DWSKVQSYLNGGPAPTFTYHRFWAQSDIATAFA

R1IFN8 *Amycolatopsis vancoresmycina*

TSDYQLAFLTQYNKIKDPNNGYFRKFGNILVPYHSIETLIVEAPDHGH
ETTSEAFSYYLWLEAAYGRVTGDWSPFNQAWTSIETYAIPSAADQPG
NSGYNASKPATYAAEYPSPKQYPSQLQSGVSVGSDPIAAELKAAYGS
ADVYGMHWLLDVDNIYKFGHCEDGTNTAPAFINTFQRGSQESVWET
VTQPSCDLLKFGGKNGYLDLFTGDSSYAKQWKYTDAPDADARAVQ
VAFQAEKWAAAQGKSADVSAVVKKASKMGDYLRYSLFDKYFKKIG
NCVGASSCAAGTGKDSEHYLISWYYAWGGSMDSSSAWAWRIGDGA
AHQGYQNPLAAYALANDPGLKVTSATGAQDWATSLGRQLEFLQWL
QSSEGGLAGGATNSWDGQYGTPPSGTPTFYGMFYDYQPVWHDPPSN
QWFGFQTWGMERIAEYYQATNDARAKKILDKWVPWAIANTTVGAG
GSFQIPSDLTWSGAPDTWNATSPGANTGLHVTVKNFSQDVGVAASL
AKTLLYYASGSSNAQAKTVGEQLLTALTANADSKGIAVPETRTDYNR
FDDTYNATTDQGLYVPSGWSGTMPNGDPINANSTFLSIRSFYKSDPQ
WPKVQSYLDGGAAPTFTYHRFWAQSEIATAFA

T1V3R1 *Amycolatopsis mediterranei*

TSDYQLAFLTQYNKIKDPNNGYFRKFGNILVPYHSIETLIVEAPDYGH
ETTSEAFSYYLWLEASYGRVTGDWAPFNQAWTSIETYAIPSAADQPG
NSGYNASKPATYAAEYPSPKSYPSQLQSGVSVGSDPIAAELKAAYGS
PDVYGMHWLLDVDNIYKFGHCEDGTNTAPAFINTFQRGSQESVWET
VTQPSCDVLKFGGKNGYLDLFTGDSSYAKQWKYTDAPDADARVVQ
VAYQAEKWAQAQGKSADVAAVLKKASKMGDYLRYSLFDKYFKKIG
NCVGASSCPAGTGKDSEHYLISWYYAWGGSMDSSSAWAWRIGDGA
AHQGYQNPLAAYALSADPGLKVTSATGATDWATSLGRQMEFLQWL
QSSEGGLAGGATNSWDGQYGTPPAGTPTFYGMYYDWQPVWHDPPS
NQWFGFQTWGMERIAEYYQATGDARAKKILDKWVPWAIANTTVGA
GGSFQIPSDLTWSGAPDTWSATSPGSNTGLHVAVKNYSQDVGVAAS
LAKTLLYYASGSSNAQAKTVGEQLLTALSANADTKGIAVPETRSDYD
RFDDKYNATTDQGLYVPSGWTGTMPNGDPINANSTFLSIRSFYKSDP
QWPKVQSYLDGGAAPTFTYHRFWAQSEIATAFA

D2B809 *Streptosporangium roseum*

DNEYVKRFTTMYNKLKDPANGYFSPQGVPYHSVETFMVEAPDHGHE
TTSEAYSYYLWLEAAYGKVTGDWSRFNDAWASMEKYIIPATADQPT

NSFYNPSKPATYAGEWDDIKQYPSKLDGGVSVGSDPIANELKTAYGT
NDVYGMHWLLDVDNTYGFGRCGDGTTKPAYINTYQRGPEESVFETI
PQPSCDTFKHGGKNGYLDLFTGDSSYAKQWKYNAPDADARAVQVA
YWAHTWAKEQGKEAQVASSVTKAAKMGDYLRYAMYDKYFKKQG
CTSTTCPAGTGKDSSAYLLSWYYAWGGANDTSAGWAWRIGSSHNH
SGYQNPMAAWALSSVDALKPKGATAVQDWSTSLKRQLEFYRWLQS
SEGAIAGGATNSWQGHYAAPPSTLPTFYGMAYDWQPVYHDPPSNQ
WFGFQAWSMERVAELYYATGNADAKLVLDKWVKWATDNTTVNA
DGTFRIPSTLVWTGQPDTWNSGNPGPNAGLHVSIRDYTSDVGVAGSY
AKVLTYYAAKSGNATAKAVAKGLLDGLWKNNQDAKGVSVPETKA
DYNRLNDPVYVPPGWTGKMPNGDVIDSSSTFMSIRSFYKNDPDWPK
VDAYLKGTGPVPSFNYHRFWAQVDVAVALA

D9WNN6 *Streptomyces himastatinicus*

AKTYDARFLELYNKITAPSAGYFSPEGIPYHSVETLIVEAPDQGHETTS
EAYSYLIWLQAMYGKVTGDWTKFNSAWDIMEKYMIPTHADQPTNSF
YNASKPATYAPEWDQPSQYPSKLDGNVPVGQDPIAAELKSAYGTDDI
YGMHWIQDVDNAYGYGNSPGKCEAGPSDTGPSYVNTFQRGPQESV
WETVTQPTCDGFKYGGKNGYLDLFTGDASYSKQWKFTNAPDADAR
VVQAAYWASEWAKAQGKGSQISGNIAKAAKMGDYLRYAMYDKYF
KKVGNCAGETTCPAGSGKNSSSYLLSWYYAWGGATDTSAGWAWRI
GSSHAHGGYQNPMAAWALSAYADLKPKSATGASDWSTSLKRQLEF
YRWLQSSEGAIAGGATNSWQGRYATPPAGKSTFYGMYYDEKPVYH
DPPSNQWFGFQAWSMERVAEYYNRTGDASAKAVLDKWVTWALSK
TTINPDGTYQFPSNLQWSGQPDTWNASSPGANTGLHVTVTDYTNDV
GVAAAYAKTLSYYAAKSGHTAAKNTAKALLDGMWDHHQDALGIA
VPESRADYNRFDDPVYVPSGWTGTMPNGDKIDSGSTFLSIRSFYKND
PAWSKVEAYLKGGAVPSFTYHRFWAQADIALAMG

D7C1F6 *Streptomyces bingchenggensis*

SKAYDARFLDLYNKITAPGAGYFSPEGIPYHSVETLIVEAPDHGHETT
SEAYSYLIWLQAMYGKVTGDWSKFNSAWDTMEKYMIPTHADQPTN
SFYNASKPATYAPEWDLPSQYPAQLNGNVSVGNDPIAAELKSAYGTD
DIYGMHWIQDVDNVYGYGNSPGKCEAGPSDTGPSYVNTFQRGPQES
VWETVTQPTCDGFKYGGKNGYLDLFTGDASYAKQWKFTNAPDADA
RVVQAAYWAAEWAKAQGKGSQVSGNVAKAAKMGDYLRYAMYDK
YFKKVGNCVGETSCAAGSGKNSSHYLLSWYYAWGGATDTSAGWA
WRIGSSHAHGGYQNPMAAWALANYADLKPKSATGAADWSTSLKRQ
LEFYRWLQSSEGAIAGGATNSWQGRYATPPSGAATFYGMYYDEKPV

YHDPPSNQWFGFQAWSMERVAEYYNRTGDASAKTVLDKWVKWAL
SKTTINPDGTYRIPSTLQWSGQPDTWNASSPGANASLHVTVADYTND
VGVTAAYAKTLSYYAAKAGDTQARDTAKALLDGMWTNYQDSLGIA
VPETRADYNRFDDPVYVPSGWTGTMPNGDKVDSSSTFMSIRSFYKN
DPAWSKVEAYLKGGAVPSFTYHRFWAQADIALAMG

F3NPZ3 *Streptomyces griseoaurantiacus*

STAYDARFLDLYGKITDPANGYFSPDGVPYHSVETLIVEAPDHGHETT
SEAYSYLLWLQAMYGKVTGDWDKFNGAWDIMEKYMIPTHADQPTN
SFYDASKPATYAPEYDTPDEYPSALDTGASVGRDPIAAELKSAYGTS
DVYGMHWIQDVDNVYGYGNAPGKCEGGPTTSGPSYINTFQRGPQES
VWETVPQPTCDAFKYGGRNGYLDLFTGDSSYSKQWKYTDAPDADA
RAVQAAYWADVWAKEQGKGGEVSATVGKAAKMGDYLRYAMYDK
YFKKIGNCVGPSSCAAGSGKDASHYLLSWYYAWGGATDTSAGWAW
RIGSSHVHGGYQNPLAAYALSSYADLKPKSATGSADWATSLDRQLEF
YRWLQSDEGAIAGGATNSWQGRYATPPSGTPTFYGMYYDEAPVYH
DPPSNQWFGFQAWSMERVAEYYQQTGDADAKAVLDKWVDWALSE
TTINPDGTYRVPSTLQWSGKPDTWNASAPGDNSGLHVTVADYTDDV
GVAAAYAKTLTYYAAESGDTEAKSTAKALLDGMWDHYQDDLGIAV
PETRADYNRFEDSVYVPSGWTGTMPNGDTIDSSSTFASIRSFYKDDPA
WSKIESYLKGGAAPVFTYHRFWAQADIALAMG

D6K6C0 *Streptomyces sp*

SKAYDARFLDLYGKITNPANGYFSPEGIPYHSVETLIVEAPDYGHETT
SEAYSYLIWLQAMYGKVTGDWSKFNGAWDIMEKYMIPTHADQPTN
SFYNASKPATYAPEADTPNEYPAKLDTSVSVGSDPIAGELKSAYGTD
DVYGMHWIQDVDNVYGYGDEPGMCEAGPAATGPSYINTFQRGPQES
VWETVPQPTCDAFKYGGKNGYLDLFTGDSSYARQWKYTDAPDADA
RAVQAAYWADVWAKAQGKGGDVSATVGKAAKMGDYLRYAMYD
KYFKKIGNCTGPSTCAAGSGKDASHYLLSWYYAWGGATDTSAGWA
WRIGSSHVHGGYQNPLAAYALSSYADLKPKSATGASDWGTSLQRQL
EFYRWLQSSEGAIAGGATNSWQGRYATPPAGTPTFYGMYYDEAPVY
HDPPSNQWFGFQAWSMERVAEYYQQTGDAKAKTVLDKWVKWALA
NTTLNPDGSFLIPSTLKWSGKPDTWNAASPGANSSLHVTIADYTNDV
GVAAAYAKTLTYYAAKSGDAQAKSTAKALLDGMWANDQDALGVA
VPETRTDYSRFGDSVYVPSGWTGTMPNGDKIDSSATFSSIRSFYKNDP
AWVEDRGLPQGRGRPRLHVPPVLGPGGHSPRHG

M1MJV0 *Streptomyces hygroscopicus*

SKAYDARFLDLYGKITNPANGYFSPEGIPYHSVETLIVEAPDQGHETT
SEAYSYLLWLQAMYGKVTGDWSKFNGAWDIMEKYMIPTHADQPTN
SSYNASKPATYAPELDTPNEYPAKLDSSVSVGPDPIAGELKSAYGTDD
VYGMHWIQDVDNVYGYGNEPGKCEAGPTATGPSYINTFQRGSQESV
WETVPQPTCDAFKYGGRNGYLDLFTGDSSYAKQWKYTDAPDADSR
AVQAAYWADVWAKAQGKSADVSATVAKAAKMGDYLRYAMYDK
YFKKIGNCTSPSCPAGTGKDASHYLLSWYYAWGGATDTSAGWAWRI
GSSHVHGGYQNPLAAYALSSVADLKPKSATGATDWGKSLQRQLEFY
QWLQSSEGAIAGGATNSWLGRYAAPPAGASTFYGMYYDWQPVYHD
PPSNQWFGFQAWSMERVAEYYQQTGNASAKAILDKWVKWALSKTT
INPDGTYRIPSTLQWSGQPDTWNASSPGSNSGLHVTVADYTDDVGVA
AAYAKTLTYYAAKSGDSAAKSTAKALLDGMWNNYQDSLGIAVPET
RTDYSRFGDSVYVPSGWTGTMPNGDAINSSSTFASLRSFYKSDPNWS
KIEAYLKGGAAPVFTYHRFWAQADIALAMG

S5UZR1 *Streptomyces collinus*

SKAYDARFLDLYGKITNPANGYFSPEGIPYHSVETLIVEAPDHGHETT
SEAYSYLLWLQAMYGKVTGDWSKFNGAWDLMEKYMIPAHADQPT
SSFYNASKPATYAPELDTPNEYPAKLDTSVSVGPDPIAGELKTAYGTD
DVYGMHWIQDVDNVYGYGDEPGTCEAGPTATGPSYINTFQRGPQES
VWETVPQPTCDAFKYGGANGYLDLFTGDSSYARQWKYTDAPDADA
RAVQAAYWADVWAKAQGRSGEVSATVAKAAKMGDYLRYAMYDK
YFKKIGNCVGPSSCAAGTGKDASHYLLSWYYAWGGASDTSAGWAW
RIGSSHVHGGYQNPLAAYALSSNADLKPKSASGASDWGKSLQRQLEF
YQWLQSSEGAIAGGATNSWQGRYASPPAGTPTFYGMYYDWEPVYH
DPPSNQWFGFQAWSMERVAEYYQQTGSAAARTVLDKWVKWALSK
TTVNPDGTYRIPSTLQWSGRPDTWNAASPGGNSGLHVTVADYTDDV
GVAAAYAKTLTYYAARSGDAAAKSTAKALLDGMWGNYTDSLGIAV
PETRSDYGRFGDSVYVPSGWSGKMPNGDTAGASSTFSSIRSFYRNDP
AWSKIEAYLEGGAAPVFTYHRFWAEADIALAMG

B5HPK7 *Streptomyces sviceus*

TKAYDARFLDLYGKITNPANGYFSPEGIPYHSVETLIVEAPDYGHETT
SEAYSYLLWLQAMYGKVTGDWSKFNGAWEIMEKYMIPTHADQPTN
SFYNASKPATYAPELDTPNEYPAKLDSSVASGSDPLAGELKSAYGTD
DIYGMHWLQDVDNVYGFGNSPGKCEAGPTDTGPSYINTFQRGAQES
VWETVPQPTCDAFKYGGKNGYLDLFTGDSSYAKQWKFTDAPDADA
RAVQAAYWADIWAKQQGKGSDVSATVGKAAKMGDYLRYAMYDK
YFKKIGNCVGPSTCAAGTGKDASMYLLSWYYAWGGATDTSAGWA

WRIGSSHAHGGYQNPLAAYALSSYADLKPKSATGQSDWAKSLGRQL
EFYRWLQSDEGAIAGGATNSWAGRYAPPPAGKSTFYGMYYDEQPVY
HDPPSNQWFGFQAWSMERVAELYQQTGNAQAKAVLDKWVKWALS
KTTINPDGTYRIPATLQWSGQPDTWNASSPGANSGLHVTVADYTND
VGVAAAYAKTLSYYAAKSGDTAAKTTAKALLDGMWTNYQDSLGIA
VPEDRTDYNRFDDSVYVPSSFSGTMPNGDTINSSSTFASLRSFYKSDP
AWSKIEAYLKGGAVPSFTYHRFWAQADIALAMG

L7EZA5 *Streptomyces turgidiscabies*

SKVYDARFLDLYGKITNPASGYFSPEGIPYHSVETLIVEAPDQGHETTS
EAYSYLLWLQAMYGKVTGDWSKFNGAWSIMEQYMIPTHADQPTNS
FYNASKPATYAPEWDLPSQYPAKLDTGVSVGTDPIAAELKSAYGTDD
VYGMHWLQDVDNVYGYGNSPGKCEAGPTDTGPSYINTFQRGPQESV
WETVPQPTCDAFKYGGTNGYLDLFTGDASYAKQWKFTNAPDADAR
AVQAAYWADVWAKQQGKGADVSTTVGKAAKMGDYLRYSMYDKY
FKKIGNCVGPTACAAGTGKDASHYLMSWYYAWGGATDTSAGWAW
RIGSSHTHGGYQNPLAAYALSSSADLKPKSATGQADWSKSLGRQIEF
YRWLQSNEGAIAGGATNSWAGRYATPPAGTPTFYGMYYDEKPVYH
DPPSNQWFGFQAWSMERVAEYYQQTGNAAAKSVLDKWVDWALSK
TTINPNGTYQIPSTLQWSGAPDTWNATTPGANTGLHVTVADYTNDV
GVAAAYAKTLTYYADRSGDTEAATTAKALLDGMWSNYQDTLGIAV
PETRTDYNRFDDAVYVPSGWTGKMPNGDTVNSSSTFASIRSFYKNDP
NWSKIEAYLAGGAAPSFTYHRFWAQADIALAMG

K4QTE6 *Streptomyces davawensis*

SGEYDARFLELYGKITNPANGYFSPEGIPYHSVETLIVEAPDHGHETTS
EAYSYLLWLQAMYGKVTGDWTKFNGAWEIMEKYMIPTHADQPTNS
FYNASKPATYAPELDTPNEYPARLDTGVSVGPDPIAAELKSAYGTDD
VYGMHWLQDVDNVYGYGNSPGKCEAGPSDTGPSYINTFQRGAQES
VWETVPQPTCDAFKYGGTNGYLDLFTGDSSYAKQWKFTNAPDADA
RAVQAAYWADKWADAQGKGGDISATVAKAAKMGDYLRYAMYDK
YFKKVGNCVGPSACPAGTGKDSSFYLLSWYYAWGGATDTSAGWA
WRIGSSHAHGGYQNPMAAYALANYADLKPKSATGQADWAKSLERQ
IEFYRWLQSSEGAIAGGATNSWAGRYATPPAGKSTFYGMYYDEKPV
YHDPPSNQWFGFQAWSMERVAEYYQQTGNAAAKTVLDKWVDWAL
EHTTINPDGTYQIPSTLQWSGQPDTWNATSPGSNAGLHVTVADYTND
VGVAAAYAKTLTYYADRSGDTEAATTAKALLDGMWDNHQDALGIA
VPENRADYNRFDDSVYVPSGWTGTMPNGDAINASSTFESIRSFYEDD
PAWSKIESYLAGGAVPSFTYHRFWAQADIALAMG

L1KHJ0 ***Streptomyces ipomoeae***

SKTYDARFLELYGKITNPANGYFSPEGIPYHSVETLIVEAPDHGHETTS
EAYSYLLWLQAMYGKVTGDWSKFNNAWEIMEKYMIPTHADQPTNS
FYTANKPATYAPEHDTPGEYPAQLNTGVSVGSDPIAAELKSAYGTDD
IYGMHWLQDVDNVYGYGNSPGKCEAGPTDTGPSYINTFQRGPQESV
WETIPQPTCDQFKYGGKNGYLDLFTGDASYAKQWKFTNAPDADAR
AVQAAYWADIWAKQQGKGSDVSATIGKAAKMGDYLRYAMYDKYF
KRIGNCVGATSCPAGTGKDASHYLLSWYYAWGGATDTSAGWAWRI
GSSHTHGGYQNPLAAYALANYAPLKPKSTTGQADWAKSLDRQIEFY
RWLQSNEGGIAGGATNSWAGRYATPPAGTPTFYGMFYDEKPVYHDP
PSNQWFGFQAWSMERVAEYYQQTGNAAAKTVLDKWVDWALSKTT
INPDGTYRIPSTLQWSGAPDTWNASSPGANAGLHVTVADYTDDVGV
AAAYAKTLTYYADRSGDADAARVAKALLDGMWDHHQDGLGIAVP
ETRADYNRFDDRVYVPSGWTGTMPNGDAINSSSTFDSIRSFYEDDPA
WSKIEAYLAGGAAPSFTYHRFWAQADIALAMG

M3ECC0 ***Streptomyces bottropensis***

SSTYDERFLEMYGKITNPANGYFSPEGIPYHSVETLIVEAPDHGHETTS
EAYSYLLWLQAMYGKVTGDWSKFNGAWDIMEKFMIPTKADQPTTS
FYNASKPATYAPEHDTPNEYPAQLNTGVSVGPDPIAAELKTAYGTDD
VYGMHWLQDVDNVYGYGNSPGKCEAGPADTGPSYINTFQRGPQES
VWETVPQPTCDQFKYGGRNGYLDLFTGDASYAKQWKFTNAPDADA
RAVQAAYWADKWAKAQGKGGDVSATIGKAAKMGDYLRYAMYDK
YFKKIGNCVGATSCPAGTGKDSSHYLLSWYYAWGGATDTAAGWSW
RIGSSHTHGGYQNPLAAYALANYAPLKPKSATGQADWAKSLDRQIEF
YRWLQSDEGGIAGGATNSWAGRYATPPAGTPTFYGMFYDEKPVYH
DPPSNQWFGFQAWSMERVAEYYQQTGNAAAKTVLDKWVDWALSK
TTVNPDGTYRIPSTLQWSGAPDTWSASSPGANAGLHVTVADYTDDV
GVAAAYAKTLTYYADRSGDADAARVAKALLDGMWDHHQDGLGIA
VPETRADYNRFDDRVYVPSGWTGTMPNGDTINSSSTFESIRSFYEDDP
AWSKIEAYLAGGAAPSFTYHRFWAQADIALAMG

B5HJV6 ***Streptomyces pristinaespiralis***

SKEYDGRFLELYGKITDPANGYFSPEGIPYHSVETLIVEAPDHGHETTS
EAYSYLIWLQAMYGRITGDWTKFNGAWEIMEKYMIPTHADQATGSF
YDPNKPATYAPEHDQPSQYPAELQPSVTSGRDPIAAELKSAYGTDDV

YGMHWLQDVDNVYGYGNEPGKCEAGPSATGPSYINTFQRGPQESV
WETVPQPTCDRFAYGGTNGYLDLFTKDASYAKQWKYTNAPDADAR
AVQAAYWADLWAKEQGKGSQVSGTVAKAAKMGDYLRYAMFDKY
FKKVGNCVGPTTCPAGTGKDSSHYLLSWYYAWGGAADTSAGWAW
RIGSSHAHGGYQNPLAAYALSAYAPLKPKSATAQDDWAKSLDRQIEF
YRWLQADEGAIAGGATNSVGGRYEAPAAGTPTFYGMAYDEKPVYH
DPPSNQWFGFQAWSMERVAEYYQQTGDAQAKEVLDKWVDWALSE
TTVNPDGTFRIPSTLQWSGKPDTWNAANPGANAGLHVTVADYTQDV
GVAGAYAKVLTYYAARSGDTEAKSVAKALLDGMWDHHQDALGIA
VPETRTDYSRFDDPVYVPSGWTGTMPNGDTINSSSTFASLRSFYQDDP
AWSKIESYLAGGAAPEFTYHRFWAQADIALAMG

M3C0N9 *Streptomyces gancidicus*

AKTYESRFLELYDKITDPANGYFSPEGIPYHSVETLIVEAPDHGHETTS
EAYSYLLWLQAMYGRITGDWTRFNDAWATMERYAIPTHADQPTTSF
YDPSKPATYAPEHDTPDEYPSQLDSGVSVGRDPIAAELKSAYGTDDV
YGMHWIQDVDNVYGYGNSPGKCEAGPSDTGPSYINTFQRGPQESVW
ETVTQPTCDAFKYGGRNGYLDLFTKDASYARQWKFTNAPDADARA
VQAAYWADLWAKEQGKGGEVAGTVAKAAKMGDYLRYAMYDKYF
KKIGNCTSTSCPAGTGKDASHYLLSWYYAWGGATDTSAGWSWRIGS
SHAHGGYQNPLAAYALATYAPLKPKSATGAADWAKSYDRQLEFYR
WLQSDEGAIAGGATNSWAGRYTTPPSGTPTFYGMYYDEKPVYHDPP
SNQWFGFQAWSMERVAEVYQQTGNAQAKAVLDKWVDWALSKTTV
NPDGSFRIPSTLRWSGAPDTWNASSPGANRGLHVEVVDYTNDVGVA
GSYAKVLTYYAARSGDTEAADTAKALLDGMWANNQDDLGIAVPET
RTDYQRFDDPVHVPSGWTGTMPNGDRIDSSSTFLSIRSFYQDDPAWS
KIESYLEGGSAPVFTYHRFWAQADIATAMG

D9XJA9 *Streptomyces griseoflavus*

EKTYDARFLELYGKITNPANGYFSPEGIPYHSVETLIVEAPDHGHETTS
EAYSYLLWLQAMYGKVTGDWSKFNGAWDIMEKFMIPTHADQPTNS
FYNASKPATYAPEHDTPNEYPSQLDPGVSVGPDPIASELKSAYGTDD
VYGMHWIQDVDNVYGYGNSPGKCEAGPSDTGPSYINTFQRGPQESV
WETVPQPTCDAFKYGGTNGYLDLFTKDASYAKQWKFTNAPDADAR
AVQAAYWADLWAKDQGKGADVSATVAKAAKMGDYLRYAMYDK
YFKKIGNCTSPSCPAGTGKDASHYLLSWYYAWGGATDTSAGWAWRI
GSSHAHGGYQNPLAAYALSNYAPLKPKSATGADDWAKSMQRQLEF
YRWLQADEGGIAGGATNSWAGRYTTPPSGTPTFYGMHYDEKPVYH
DPPSNQWFGFQAWSMERVAELYQQTGNALAKSVLDKWVDWALSET

TVNPDGSFRIPSTLQWSGKPDTWNASSPGANSGLHVTVADYTNDVG
VAAAYAKTLTYYADRSGDTEAASTAKALLDGMWENNQDALGIAVP
ETRADYNRFDDPVHVPNGWSGTMPNGDAINSSSTFESIRSFYQDDPA
WSKIESYLAGGAAPTFTYHRFWAQADIALAMG

Resurrected exoglucanase sequence

**DNAYDQRFLTMYNKIKDPANGYFSPEGVPYHSVETLIVEAPDHGH
ETTSEAFSYYLWLEAMYGRVTGDWSPFNNAWDTMEKYIIPSHAD
QPTNSSYNPSKPATYAPEHPDPSQYPSQLDSSVPVGQDPIANELKS
TYGTNDIYGMHWLLDVDNVYGFGNSPGRCEDGDSTTRPAYINTF
QRGPQESVWETVPQPSCDTFKYGGPNGYLDLFTGDSSYPAKQWK
YTNAPDADARAVQAAYWAHEWAKEQGKASEVAATVAKAAKMG
DYLRYSMFDKYFKKIGNCVGASSCPAGTGKDSAHYLLSWYYAW
GGATDTSAGWAWRIGSSHSHFGYQNPMAAWALSNVAELKPKSP
TGASDWATSLKRQLEFYQWLQSSEGGIAGGATNSWNGSYATPPA
GTPTFYGMSYDEQPVYHDPPSNQWFGMQAWSMERVAEYYYAT
GDARAKAVLDKWVPWAIANTTINADGSFQIPSDLEWSGQPDTWN
ASSPGANTNLHVTVTNYGQDVGVAGSLAKTLTYYAAKSGNTTAK
DTAKGLLDALNNYQDSKGISVPETRADYNRFDDGVYVPPGWTGT
MPNGDVIKSGATFLDIRSFYKNDPDWPKVEAYLNGGPAPTFTYH
RFWAQVDIATAYARSCC**

Appendix III

List of beta-glucosidase proteins from the species used in the construction of the phylogenetic tree

U5DQJ4 *Rhodococcus equi*

MTMREQVSLTSGADFWHTHTPPVPGLPAIMLTDGPHGVRKQAATAA
GYSPESVPATCFPTASALAATWDVELLEEVGVALGTEARTEGVSVLL
GPGANIKRSALCGRNFEYFSEDPFLSSRMAAAWILGVQSTGVGASLK
HFAVNNQEFRRYSVDAVVDARALREIYLAGFEHAVVDARPATVMA
AYNRVGGTHCAENRWLLTDVLREQWGFDGLVVSDWGAVTRRSRCL
AAGLDLEMPGYGGLGDDDVLAAVGAGKLDPAAVARAAESVTRLIE
RTEAARTAAPGYDEAAHHALARRAAEAGTVLLRNDGVLPLAAAQV
AIVGEFAKQPRYQGAGSSGITPHRLDDAWTDLVEHLGAERLTYAPGY
RRASGRTDAALLDEARAVARDTDVTVVFAGLPDSYETEGVDRADLK
LPEGHDALIAAVAEVCPRVVVVLANGAPVTMPWHDDVAAIVECYLG
GQAAGSAIARILTGDAEPGGRLAETFPLHTSDNPVHVWPAGPSVVEY
RESIYVGYRYYDAAELDVRYPFGHGLSYTSFAWTELEVADVFDSTSE
DIEQRLDVSVRVTNTGDRPGSEVVQVYVRDVESTVFRPDQELAAFAK
VFLAPGESRRVTLHVDRRAFSFWDTTIDDWSIESGDFEIRVGASSRDIR
QSATVTLTSDRSGFDAGPLAYHGSPVFERAAFAELYGKPLPDNVVDA
PRHYSVDTPLADIRHPAAALLTRAMRRKVAATAPALDEDDPLSRLIE
RSLQELPPRMLPMLTQGGVTPAAAQAFVDICNGHTVRGGWALVAAL
RRK

F5XL24 *Microlunatus phosphovorus*

MAVGLDIPRLLGELTLAEKASLVSGSGFWFTQPVERLGIPAIMVSDGP
HGLRAQPPGGGDHVGLGSSLPATCFPTASAIASAWNPELLHRIGQAL
AQEARACNLSVILGSGINMKRSPLCGRNFEYFSEDPYLAGELAVGIVD
GIQSCGVGTSVKHWAANNQETDRLRCDSQVDERTLREIYFPAFARVV
EKSQPWTIMCSYNKVNGTSASENTWLLDTVLREEFGFEGLVVSDWG
AVYHPVPAVQAGCDLEMPPSKGRSEAAIVAAVESGELSVDVLDARV
RTVLELVAKGMHALELDESFDIDAHHALARQAAAESVVLLKNDGLL
PLTVEANIAVIGEFARTPRYQGAGSSQVVPTRLDTVLEELQTVYGELP
FAAAYGVGDTSNDAVLLVEAEQVAAAADIVVMLIGLPAAEESEGFD
RTHLNLPDNQLTALAAVAEANPNVVVVLANGSTVVLGDVLPRASAL
AEAWLGGQAAGGGIVDVLTGAVNPSGRLAETIPNRLEDNSSYLNFPG
EEQSVRYGEGIFIGYRGYDRQHLDVAFPFGFGLSYTSFELSDIKVRMR

GSVADETLGATVEVTVTNTGDVDGSEVVQVYVQDVVSTVARPVQEL
KGFAKVAVPAGGAVQVSIELDQRAFSYWSPRYRRWVVEAGDFEISV
GSSSRDLALSQSVTVEAATLLPPLTRDSTLQEWLADPAGRQLVEREV
AAGQPGAGMQEGLLEVIG
NFPMNALANLNLSLDHDSLDRVAAEWAGQQR

F6FVZ5 *Isoptericola variabilis*

MTTDTTPAPGTTPALTVEDVPRLVAELTLEEKASLCSGQDFWHTQAV
ERLGIPAVMVTDGPHGLRKQAGATDHVGLNESVPATCFPPAAGLGST
WDPELIWRVGEALGTETRANEVAVLLGPGVNMKRSPLCGRNFEYLA
EDPFLAGELAAPLVEGIQSKGVGTSLKHFAANNQETDRMRVDAQVD
ERTLREIYLPAFEKVVTRAQPWTVMCAYNKVNGTYASQHPWLLTEV
LRDEWGFEGLVVSDWGAVDDRVAGVKAGLDLEMPSSGGLNDARIV
EAVRSGHLSEADLDRAVTRVLTVVARSQAALAAPGEFDAEAHHALA
QEAATRAAVLLKNEGGVLPLSGDALGDVVVVGEMARTPRYQGAGS
SQVNPTRLVSALDALAERGLDVPFMPGYRLPEAEGKQGQDHPDDEL
RAEAVEAAAGKTAVVFLGLPAIDESEGYDREHMDLPASHTALLREVS
AAAERVIVVLSNGSAITVAGWQDQADAILETWLGGQAGGSATVALL
LGDAAPSGRLAESIPVRLEDVPAQLNFPGENGVVRYGEGIFIGYRGLD
ATRAEVSYPFGHGLTYTTFGFSDLAVDVAEVTEQTAADDVVVRVAL
TVTNTGEREGVAVPQLYVGRPASTVARAPRELRGFARVSLAPGASER
VELALTRRDLSHWDTLTHAWVVEPGALEVAVGASSRDLPLKATVEL
AAPALPRPLHRYSTIGEWRDQPEAWAALREKLGGFAELFEADSPDPA
MAAFLVEMPVIKVPMMGMASSLSIDDFETLLARFGRP

U1GC79 *Propionibacterium avidum*

MTPAEITDLISQLTLEEKASLCSGGDSWHLQTVERLGIPGPMVTDGPH
GLRKVADGSMAGIYDSVPATCFPTAAGLASTWDPELVHDIGVALGEE
TRAESVSVLLGPGINMKRSPLCGRNFEYFSEDPVLAGTLATELVRGIQ
SQGVGTSLKHFAANNQETDRLRVNAEIDERTLREIYLPAFEMVVKQA
DPWTVMCSYNAINGVYSSQNRWLLTELLHEEWGYKGLVISDWGAV
VNRVEGLRAGLDLEMPGDAPRNDARIVEAVRSGDLDEEILDTAVARI
LSLIARASAAMADPGSYDIEAHHQLARRAAAASVVLLKNDGDVLPLI
DPDDVVVIGEFARTPRYQGAGSSKVNPTRVDTALDSLRQTWDDVPF
APGFTLTDHPDPQLADEAVSLSRGKTAVLFLGLPAVAESEGYDRTNT
DLPTDQIDLLARVRKVASHTIVVLSNGSSVGTAEWDDQADAIVECWL
GGQASGSGVIDVLTGKVNPSGHLAETITRKLSDIPAQLNFPGEFQHVT
YGEGRYIGYRGLDATEREVAYPFGHGLSYTTFAYCDLDLLVRPVSDE
TAQDETVLTVSFTVSNTGDRAGATVPQVYLGFPDATVDRCVRELKA
FLRVELDPGESRSITIDLTRRDLSYWDILLHSWTVEPGTLRVEVGISSR

DLPLTGEVILDAPTVRHPLRRDSTVAEWMDADEEFAAKVRRATEQT
GIDLDSDPTTAAFILAMPAYKMLQMAPIMTPEELDKMLGD

S3WXI0 *Propionibacterium sp*

MTPAEITDLISQLTLEEKASLCSGGDSWHLQTVERLGIPGPMVTDGPH
GLRKVADGSMAGIYDSVPATCFPTAAGLASTWDPELVHDVGAALGE
ETRAEAVSVLLGPGINMKRSPLCGRNFEYFSEDPVLAGILATELVRGI
QSQGVGASLKHFAANNQETDRLRVSAGIDERTLREIYLSAFEMVVKH
AKPWTVMCSYNALNGVYCSQNRWLLTQVLRNEWGYDGLVISDWG
AVVDRVEGLRAGLNLEMPGDAPHNDARIVEAVRNGDLDEEVLDTA
VARILTLVAQASAAMADPGSYDIEAHHQLARRAAAASAVLLKNDGD
ALPLTGPDDVVVIGEFARTPRFQGAGSSKVNPTRVDTALDSLRHIWG
DVPFAPGFALTGESDPRLAEDAESLARGKTAVLFLGLPAVAESEGYD
RTNTDLPADQLDLLACVSKVASHTIVVLSNGSSVGMAEWDDQADAI
VECWLGGQASGSGVVDVLTGKVNPSGHLAETIAVTLSDIPAQLNFPG
EFQHVTYGEGRYIGYRGLDVIEREVAYPFGHGLSYTTFDYSDLDLAV
RPVTDETAQDESVLTVTFTISNTGDRTGSAVPQVYLGFPDATVNRCV
RELKAFRRVELAPGKSRSITIDLTRRDLSYWDILLQSWAVEPGTVRVE
VGRSSRDLPLTGVVILDAPTVHHPLRRDSTVAEWMAADEEFAAKVR
RATEQTGIDLDSDPTTAAFIL
AMPAYKMLQMAPIMTPEELDEMLGD

M2XAG3 *Rhodococcus qingshengii*

MTENYVDGLSLEEKASLTSGSDFWHSQSVPGIESILLTDGPHGVRKQP
EGGDALGLGHSIPATCFPPAVGLGSSWNLDLIRQVGEALGDEAKAEQ
VSVLLGPGINIKRSPLCGRNFEYVSEDPFLSGRVAAALITGIQSRGVGT
SLKHFAANNQEHDRMRVSADVDERTLREIYLAGFEYAVKTAAPTTV
MCSYNKINGVYSSQNHWLLTEVLREQWGFDGLVVSDWGAVNDRVA
ALAAGLDLEMPPTGTDTQIVDAVRGGDLDESVLTTAAERLATLVART
AAARTEGHTYDVERHHELARTAAAESAVLLANDGELLPLTPGGQTV
AVIGEFACSPRYQGAGSSQVVPTKLDNALDAILDREGADRVTFAPGF
TFDGTPDDDMVTEAVDAARRADVAVLFLGLPSATESEGFDRTDIELP
ADQIALLEAVHGANPNTVVVLANGGVVSIEPWKDHAAAILEGWLLG
QAGGSAIADLLFGITNPSGRLTETIPHRLQDNPSYLHFPGSQQHVRYG
EGLYVGYRYYDSALREVAYPFGFGLSYTTFDITDTSVEAGENSAEVT
VTVRNSGDRSGSTVVQVYVHDASASIDRPAQELKGFAKVHLDPDES
ATVTITLDTRAFAYWSVAAQDWAIEPGDYEIRVGFSSRDIATTDTITL
AGNVGVGTLDAMSTIGEWLAHPVGSAVLGAAMAAAAGDGAQAVSP
EMMALAGSMPLGKLATFGLGITEEQVEQLVAAAAQPAS

T1VRJ4 *Rhodococcus erythropolis*

MTENYVDGLSLEEKASLTSGSDFWHSQSVAGIESILLTDGPHGVRKQ
PEGGDALGLGHSIPATCFPPAVGLGSSWNLDLIRQVGEALGDEAKAE
QVSVLLGPGINIKRSPLCGRNFEYVSEDPFLSGRVAAALITGIQSRGVG
TSLKHFAANNQEHDRMRVSADVDERTLREIYLAGFEYAVKTAAPTT
VMCSYNKINGVYSSQNHWLLTEVLREQWGFDGLVVSDWGAVNDRV
AALAAGLDLEMPPTGTDTQIVDAVRGGDLDESVLTTAAERLATLVK
RTAAARTEGHTYDVERHHELARTAAAESAVLLANDGELLPLTPGGQ
TVAVIGEFACSPRYQGAGSSQVVPTKLDNALDAILDLEGADRVTFAP
GFTFDGTPDDHMVTEAVDAARRADVAVLFLGLPSATESEGFDRTDIE
LPADQIALLEAVHGANPNTVVVLANGGVVSIEPWKDHAAAILEGWL
LGQAGGSAIADLLFGITNPSGRLTETIPLRLQDNPSYLHFPGSQQHVRY
GEGLYVGYRYYDSALREVAYPFGFGLSYTTFDITDISVEAGENSAEVT
VTVRNSGDRSGSTVVQVYVHDASASIDRPAQELKGFAKVHLDPDES
ATVTITLDTRAFAYWSVAAQDWAIEPGDYEIRVGFSSRDIATTDTITL
AGNVGVGTLDAMSTIGEWLAHPVGSAVLGAAMAAAAGDGAQAVSP
EMMALAGSMPLGKLATFGLGITEEQVAQLVAAAAQPGS

U0EF81 *Rhodococcus sp*

MELTAATGRNDVTENYVDGLSLEEKASLTSGSDFWHSQSVAGIESIL
LTDGPHGVRKQPEGGDALGLGHSIPATCFPPAVGLGSSWNLDLIRQV
GEALGDEAKAEQVSVLLGPGINIKRSPLCGRNFEYVSEDPFLSGRVAA
ALITGIQSRGVGTSLKHFAANNQEHDRMRVSADVDERTLREIYLAGF
EYAVKTAAPTTVMCSYNKINGVYSSQNHWLLTEVLREQWGFDGLV
VSDWGAVNDRVAALAAGLDLEMPPTGTDTQIVDAVRGGDLDESVL
TTAAERLSTLVKRTAAARTEGHTYDVERHHELARTAAAESAVLLAN
DGELLPLTPGGQTVAVIGEFACSPRYQGAGSSQVVPTKLDNALDAIL
DLEGADRVTFAPGFTFDGTPNDDMVTEAVDAARRADVAVLFLGLPS
ATESEGFDRTDIELPADQIALLEAVHGANPNTVVVLANGGAVSTEPW
KDHAAAILEGWLLGQAGGSAIADLLFGITNPSGRLTETIPLRLQDNPS
YLHFPGSQQHVRYGEGLYVGYRYYDSALREVAYPFGFGLSYTTFDIT
DTSVEAGENSAEVTVTVRNSGDRSGSTVVQVYVHDASASIDRPAQEL
KGFAKVYLDPDESATVTITLDTRAFAYWSVAAQDWAIEPGDYEIRVG
FSSRDIATTDTITLAGNVGVGTLDAMSTIGEWLAHPVGSAVLGAAMA
AAAGDGAQAVSPEMMALAGSMPLEKLATFGLGITEEQVAQLVAAA
AQPGS

E8MQE8 *Bifidobacterium longum*

MEEPRTTARQSGRIGANAYRTTAQLKKLKERGIMSESTYPSVKDLTL
EEKASLTSGGDSWHLQGVESKGIPGYMITDGPHGLRKSLASSTGETD
LNNSVPATCFPPAAGLSSSWNPELIHKVGEAMAEECIQEKVAVILGPG
VNIKRNPLGGRCFEYWSEDPYLAGHEAVGIVAGVQSKGVGTSLKHF

AANNQETDRLRVDARISQRALREIYLPAFEHIVKTAQPWTIMCSYNRI
NGVHSAQNHWLLTDVLRDEWGFEGIVMSDWGADHDRVASLNAGL
NLEMPPSYTDDQIVYAARDGRIAPAQLDRMAQGMIDLINKACAAMSI
DGYRFDVDAHDEVAHQAAIESIVLLKNDDAILPLNADPAAARKIAVI
GEFARTPRYQGGGSSHITPTKMTSFLDTLAERGIKADFAPGFTLDLEP
ADPALESEAVETAKNADVVLMFLGLPEAAESEGFDRDTLDMPAKQI
ALLEQVAAANQNVVVVLSNGSVVSVAPWAKNAKGILESWLLGQSG
GPALADVIFGQVSPSGKLAQSIPLDINDDPSMLNWPGEEGHVDYGEG
VFVGYRYYDTYGKSVDYPFGYGLSYATFEIADVAAAKTGANTATVT
ATVTATVTNTSDVDAAETVQVYVAPGKADVARPKHELKGFTKVFLK
AGESKSVTIDL
DERAFAYWSEKYNDWHVESGEYAIEVGVSSRDIADTVVVTLEGDGK
SQPLTEWSTYGEWEADPFGAKIVAAVAAAGEAGELTKLPDNAMMRI
FLNSMPINSLSTLLGEGGKKIAKFMVDEYAKLAK

W6F3F9 *Bifidobacterium breve*

MSESTYPSVKDLTLEEKASLTSGGDAWHLQGVESKGIPGYMITDGPH
GLRKSLASSTGETDLDDSVPATCFPPAAGLSSSWNPELIHKVGEAMA
EECIQEKVAVILGPGVNIKRNPLGGRCFEYWSEDPYLAGHEAIGIVEG
VQSKGVGTSLKHFAANNQETDRLRVDARISPRALREIYFPAFEHIVKK
AQPWTIMCSYNRINGVHSAQNHWLLTDVLRDEWGFDGIVMSDWGA
DHDRGASLNAGLNLEMPPSYTDDQIVYAVRDGLITPAQLDRMAQGM
IDLVNKTRAAMSIDNYRFDVDAHDEVAHQAAIESIVMLKNDDAILPL
NAGPVANPSATPQKIAVIGEFARTPRYQGGGSSHITPTKMTSFLDTLA
ERGIKADFAPGFTLDLEPADPALESEAVETAKNADVVLMFLGLPEAA
ESEGFDRDTLDMPAKQITLLEQVAAANQNVVVVLSNGSVITVAPWA
KNAKGILESWLLGQSGGPALADVIFGQVSPSGKLAQSIPLDINDDPSM
LNWPGEEGHVDYGEGVFVGYRYYDTYGKAVDYPFGYGLSYATFEIT
GVAVAKTGANTATVNATVTNTSDVDAAETVQVYVVPGKADVARPK
HELKGFTKVFLKSGESKTVTIDLDERAFAYWSEKYNDWHVEAGEYA
IEVGVSSRD
IADTVAVALDGDGKTQPLTEWSTYGEWEADPFGAKIVAAVAAAGEA
GELPKLPDNAMMRMFLNSMPINSLPTLLGEGGKKIAQFMVDEYTKLS
K

J9XU17 *Bifidobacterium pseudocatenulatum*

MSEKTYPSINDLTLEEKASLTSGGDAWHLQGVEAKGIPGYMITDGPH
GLRKSNSATTGEVDLNNCVPATCFPPAAGLSSSWNPELIHQVGEAMA
EECIQEKVAVILGPGVNIKRNPLGGRCFEYWSEDPYLAGHEAIGIVAG
VQSKGVGTSLKHFAANNQETDRLRISANISQRALREIYFPAFEHIVKE
AQPWTIMCAYNCINGVHAAQDHWLLTDVLRDEWGFQGIVMSDWG

ADHDRVASLNAGLNLEMPPSYTDDQIVYAARDGRIQPAQLDRMAQG
MIDLVNKTRAAMSIENYRFDIEAHDEVAHQAAIESMVLLKNDDAILPI
AGDAKVTVIGEFARTPRYQGGGSSHITPTKMTSFLDTLTERGVDAKF
APGFTLDLEPADPALEAEAVDAAKGADVVLMFLGLPEAAESEGFDRE
TLDMPAKQIALLEAVAAENKNVVVVLSNGSVVTVAPWAKNAKGILE
SWLLGQSGGPALADVLFGKVSPSGKLAQTIPFDINDDPSTINWPGEEG
HVDYGEGVFVGYRYYDTYNKAVDYPFGFGLSYATFEVSDVKAVKT
GACTASVSAVVKNTSNVDAAETVQVYVAPGKADVVRPKHELKGFK
KVFLKAGESAEVSFELDDRAFAYWSERFNDWHVESGEYTIEVGTSSR
DIAGSAVVELDGDGKAQPLTEWSNFMEWRKDPLGSKVLEILRAEGE
AGRMPVVPDNDMTRLFLDSMPINSMSVLMGADGKQIFEYMLEKYAE
LTK

W4NB76 *Bifidobacterium moukalabense*

MSESTYPEVNDLTLEEKASLTSGGDAWHLQGVESKGIPGYMITDGPH
GLRKSLASSTGETDLNDSVPATCFPPAAGLSSSWNPELIHQVGEAMA
EECIQEKVAVILGPGVNIKRNPLGGRCFEYWSEDPYLAGHEAIGIVSG
VQSKGVGTSLKHFAANNQETDRLRVSANISQRALREIYFPAFEHIVKE
AQPWTIMCSYNRINGVHSAQNHWLLTDVLRDEWGFEGIVMSDWGA
DHDRVASLNAGLNLEMPPSYTDDQIVYAARDGRIQPEQLDRMAQGM
IDLVDKTRAAMSVEGYRFDVDAHDEVAHQAAVESMVLLKNDDAILP
VSSDAKVAVIGEFARTPRYQGGGSSHITPTRMTGFLDTLTARGVDVR
FAPGFTLDLEPADAAMAAEAVETAKGADVVLMFLGLPEAAESEGFD
RETLDIPAKQIELLEAVAAENRNIVVVLSNGSVVSVAPWAANAKGILE
SWLLGQAGGPALADVIFGHVSPSGKLAQTVPMDINDDPSMINWPGEE
GHVDYGEGVFVGYRYYDTYGKAVDYPFGYGLSYATFEVSDVKVAR
TGDNTASVSAVVKNVSDVDAAETVQVYVAPGKASVARPVHELKGF
RKVFLKAGESAEVSFDLDERAFAYWSEKFNGWHVESGAYTVEVGTS
SRDIAGTGV
VELDGDGKSEPLTEWSTFGEWSEDPTGSKIVASVYAEGEAGNLPKMP
DNDMMRMFLKSMPINSMPMLMSEGGKKITAFMLDEYAKVAK

D2Q5N4 *Bifidobacterium dentium*

MSESTYPEVNDLTLEEKASLTSGGDAWHLQGVEAKGIPGYMITDGPH
GLRKSLASSTGETDLNDSVPATCFPPAAGLSSSWNPELIHQVGEAMA
EECIQEKVAVILGPGVNIKRNPLGGRCFEYWSEDPYLAGHEAIGIVAG
VQSKGIGTSLKHFAANNQETDRLRVSANISQRALREIYFPAFEHIVKE
AQPWTIMCSYNRINGVHSAQNHWLLTDVLRDEWGFEGIVMSDWGA
DHDRVASLNAGLNLEMPPSYTDDQIVYAARDGRIQPEQLDRMAQGM
IDLVNKTRAAMSVEGYRFDVDAHDEVAHQAAVESMVLLKNDDAILP
VASDAKVAVIGEFARTPRYQGGGSSHITPTKMTSFLDTLTARGVDAK
FAPGFTLDLEPADAAMAADAVEVAKGADIVLMFLGLPEAAESEGFD

RETLDIPAKQVELLEAVAAENKNIVVVLSNGSVVSVAPWADNAKGIL
ESWLLGQAGGPALADVIFGNVSPSGKLAQTVPMDINDDPSMINWPGE
EGHVDYGEGVFVGYRYYDTYDKAVDYPFGYGLSYATFEVSDVKVA
KTGANTASVSAVVKNTSDVDAAETVQVYVAPGKSAVARPIHELKGF
RKVFLKAGESAEVSFDLDERAFAYWSEKFDDWHVESGEYAIEVGTSS
RDIAGTGM
VELDGDGKAEPLTEWSTFGEWSDDPVGSKIVASVYAEGEAGNLPKM
PDNDMMRMFLRSMPINSMPMLMSEGGKKITAFMLDEYAKVTE

R5T443 *Clostridium hathewayi*

MTDKIKDLVTAMTLEEKALLCSGKNFWQMEGIERLGIPSVMVTDGP
HGLRKQAGEADHLGLNQSVKATCFPPAVTSASSWDKAALYDMGQAI
GEECVQEEVAVVLGPGTNIKRSPLCGRNFEYFSEDPYLAGEMAAAWI
SGVQSKGIGTSLKHFAANNQEKARLVSNSVVDERALREIYLAPFEKA
VKQAQPWTVMCSYNRINGVYSCENEWLLTEVLRNEWGFQGLVMTD
WGAMNDRVKALKAGLELEMPGPDPYNDKKIVDAVRNGELDEAVLD
RAAERLLTVIMRAGEVHKKEYDAAAHHNLARRIAAESAVLLKNDG
MLPLKKENSYAVIGAFAETPRYQGAGSSRIHPHQIDCVLDSLRESGIA
FEYAPGYELEQDTINPVLLEEAAACAKGRDGVLVFAGLPDSYESEGF
DRTHLNLPKSHTALIEAVTAVNPHVTVVLLCGSAILMPWRDRVESILL
TYLGGEAAGSACADVLTGTVNPSGRLAETFPLSLEDTPCCENFAGEG
KDVEYRESILVGYRYYDWAEKPVAFPFGYGLSYTSFSYDSMEIVWDE
KEEKGEARITVTNTGETSGSEVVQLYIGKAQSGVMRAVRELKGFQKV
FLEPGELREVVIGLDRRSFSCYDANRSCWTVEAGTYQIYAASSSRDLK
LKKDLMLPGKELSHIPGYDAAETVKDGHFAADRKQFKRLFPDDLPLT
PDDGRITLNTTVKEIMASEKGKALLGGLVEGYSGRYSGDDDVSRMM
LAMLQDMPLRSLAMFGAVEMETLEEMVREIGRP

R7K9E6 *Acidaminococcus sp*

MDNKENSKQLTADNAVANVPDFDEILKQMTLEEKASLCSGKTFWLT
KEIKRLGVPSVLMTDGPNGLRKEKAGRGTNIMNESEPATCFPTAVTL
CSTWDPSLAEKAGKAIADEAKEQGQSTVLGPGVNIKRSPLCGRNFEY
FSEDPYLAGEIGKGWVHGVQSENIGVSLKHYCANNQEHIRMSVDTIA
DERALREIYLPAFENIVKDEQPTTVMASYNRLNGKYLCDNKRMLTEV
LRDEWGFKGIVVSDWGAVNDRVEGVKAGLDLEMPGNNGINDKLIVE
AVRNGTLDEADLDKVALRMIKFAFECKAKEVENHKADFEAHHTLAR
EIGAAGAVLLKNEENILPVKSGEKIAVIGQLAKVPRYQGAGSSNINPY
KKPVSFIEALSNANREYTYADGYTLKGNGYDKSLIKKAVKIAKDADK
VLLFIGLTDSYESEGFDRKHLNMPNGHEILFDAIQAVNSNVAVVFSGG
SPVDMREIAPAAKGLLCAYLGGQAVGEAVMDVIFGDVNPSGKLAET
WPLRLHDNIASKYFPMGPKTVEYRESVYVGYRFFDTAKRDVMFPFG
YGLSYTTFEYSDLKLSKEKFKDTDSVEISFKIKNTGSIDGAEVAQVYIS

DVESTIFRPEKELKRFSKVFLKAGESKEIKFTLDKRCFAYYNVDIKDW
HVESGDFKIMVGASSRDIRLEKTVSVESSAPSVKVPDYRESAPCYYTL
VDEKFDIPAEQFEVLYKAPLPDNSPYKKGEFNVNCTVGDVSISRWGK
FIQNLLHFGVKVVSRSSQNKDMLLASVDDMPIRSFYGFTGGMISAKS
VEGLIEMFNGKRGGFNKFIKGFKKPKEDKAKK

R6PJW2 *Clostridium sp*

MKNPKEILAQMTLEDKAALCDGADFWHLKGMEKYGIPSIMVCDGPH
GLRKKDYNKTGSSLSCSVPSICFPTAVTTACSWDPDLLHEMGVALGK
KCLKEEVGVLLGPGVNMKRSPLCGRNFEYFSEDPVLAGELAAGFIEG
VQSMGVGTSIKHFCANSQETRRMTCDSVVDERALREIYLTAFEIAVK
KAKPWTVMNSYNKINGAHGSENKHTQIEILRDEWGFDGVVVSDWG
AVNNRVLGLKNGNDLEMPSSAGSGAKKIVEAVKNGELDEAVVNER
ALNVLNLIKKAADGAKPGYEYNDADDQPLARKIAGQSMVLLKNDGI
LPLKKEGKIAVIGDFAKFPRHQGAGSSQINPTKMDNAYDELKELGYD
VEFVQGYERSAKKAAKNAAHIDAAAALAAKCDAAIVFVGLTDDYES
EGFDRTHMTLPEAHNKLVEEIVRVNKNVIVVLAGGSPVELPWNDSVR
AVLNSYLGGQAGAGAVADIISSKVNPSGKLAETYPVYYSDTPAVNNF
PGNPATVEYRESVYIGYRYYEKANKAVRYPFGFGLSYTTFGYSDIKL
DKSEMADTDTLKVSFKVKNTGSVAGAEIAQVYVSDKVSTIYRPVKEL
KGFKKVWLEPGEEKEVTVELCRRAFAFYNVKINDWCVESGEFDILVG
SSSADIRLS
ACVKVNAPEVEMPDYSKTAPDYYTGDVQNVPAAQFEAVLGRPLPPT
VKRKDIPIDVTDNFENAAHTKNGAKLYNTLKKLVPEGFAQAIALQTP
FRDFISMSGGVFSEDMAAGLLKILNGEKGGVRAILKCVPKAIKGIGPL
LKNI

R5XIE5 *Anaerotruncus sp*

MKHPEIVSKMSLEQKAKFVSGFDYWHLEEAPELGLPKIMITDGPHGL
RKQNTDKKASSGIGLGNSVPATCMPPAATSACSWDENLLREEGEALA
EECLQEKVSVILGPGTNIKRSPVCGRNFEYFSEDPLLAGKMSASLING
CQSKGVGNSLKHFACNSQEAFRMVLSEVIDERTMREIYFPAFEIAVKE
SQPWTVMNSYNRINGVYASQNEWLQEKVLRDEWGFKGLLVTDWG
ASVDRVPGLKYGTDLEMPSSGPLNTKRIIAAVNGGELDEAILDKRVD
NVVDLILKSKPALEQNNGYKFDVEAHHALSRKIAEGSMVLLKNDDKI
LPLKKGQKIAVIGEMAKSPRFQGAGSSVINPTKLDNAYDELVKLGAD
VTYAQGYYKSAPGKKDKDRKSDAQLVSEAVAAAKAADVAVVFVGL
TEEFEGEGYDRENINMPENHNKLVSEIAKANANTVVVLAGGSVVYIP
WLDEVKGLLNSGLGGQASGSAVANILTGAVNPSGKTAETYPVKYED
NPTFGNYPGGPVISEHKESVYIGYRYYDTAEKEVLFPFGYGLSYTTFE
YSDMKLSASDIKDTDTLKVSLKVKNTGDVDGAEIVQIYVADKESTIF

RPKKELRAFTKVFLKAGEEKEITLELGKRAFAYYNVKLGDWHVESG
EFEIIAAASSRDEKLKASVNVTSTVEAEVPDYRDIAPSYYTADIKDVD
DKQWGAVYGSELPARERDKNAKIDLYCCLNDARHTKWGGKLCRLIE
KIMSNMGSAENGDGKMLAAMATQIPIRNFVQMSMGVFSPKMAEGLL
KMLNDDESSFVGFNAIFWRLGGALTRLPSLLKSI

R5N0X1 *Eubacterium sp*

MKHKEIVEKMSLEQKAAFVSGYDYWHLEEAPELGLPKIMITDGPHGL
RKANPDKKSSTGGIGLGNSVPATCFPPAATSSCSWDPELLEQEGEAM
GEECLKEKVSTILGPGTNIKRAPVGGRNFEYFSEDPLLAGECAAAVIN
GVQSKGVGTSLKHFAANSQEAFRMVVNEVVDERTLRETYLTAFEIA
VKKAQPWTVMNSYNRINGVYASENEWLQQKVLRKEWGFEGLIVTD
WGASVDRIPGLKAGTDLEMPCSGDLNTNRIIAAVKDGTLDEKILDER
VDTVVDLIVKSKPALEKTHTYDVDAHHAIAQKIAEGSMQLLKNDDGI
LPLKDGQKVAVIGEMAKAPRFQGAGSSVINPTKLSNAFDELQKLGVD
ISYAQGYYKSAPSKKDKTPRKTSAELIAEAKEAASKADVAVVFVGLT
EEFEGEGYDREGIEIPAEHNELVAAVAEANPNTVVVIAGGSVILMPWL
KNVKGLLNSGLGGQAGGIAVANILTGKVNPSGKTSETYPRAFEDNPT
YGNFPGGPVTSEHRESVYIGYRYYDAADIDVEFPFGFGLSYTTFEYSD
IKLSKDKMKDTDTVTVSFKIKNTGSVDGAEIAEVYVADKESTIFRPKK
ELRGFKKVFLKAGEEKEVSVELSKRAFAFYNVELGDWMVETGEFDIL
VGSSSRDIKLTASMTVESTVTASIPDYRKTAPNYYNNVANITRDDFAA
VYGELPNPEIDKNKKIDLYCCLNDARHTKWGGKLCRLIEKIMSGMGS
DANGDGKMLAAMATQIPVRNFISMSMGAFSPKQAEGLLKMLNDDES
SFVGFNTIFWRLGGTLARLPKLLKSI

K8E314 *Carnobacterium maltaromaticum*

MKYQSLIEQMTLEEKASLMSGENFWNTKAIERLNIPSIMLTDGPHGLR
KQGGKADHLGLNASLPATCYPTAATLANSWDRELLNEIGQFLAAEC
VSENVSVILGPGLNLKRNPLCGRNFEYFSEDPYLTGELASQQVKGIQS
QGVAASPKHFAVNSQEHMRMTIDEVVDERSLRELYLEGFRRVVKQS
KPKTIMSSYNKINGIYANEHPHLMNDILYGEWQFDGVMVTDWGGNN
DRVAGLRAGNQLEMPSTNGITDQEIVVAIQTGELSEAILDQAVNQLL
KLVFETSSVTEKPVTIDYQKHHEKAVDAAKRSMVLLKNQKEILPLRA
SEKIALIGDFANKPRYQGAGSSLINPTQVPNFVEVLKESPLSIQGYAQG
YQRMGQRVKTKLVNEAIELAKKVDKVLLFVGLDESREAEGIDRHDL
KLPFNQLHLIEEITKVNPNVVIVLAGGGVLELPFEERVQGILHSYLAG
QGVAEALKEILLGTYNPSGKLSETYPLAYESVPSATYYPGKEATSEHR
EGLFVGYRYFDTANVPVKYSFGYGLSYTKFAYSDITREDLSVSFTVSN
SGKVAGEEVAQLYIEKQESKIIRAKRELKGFEKIYLEPGQSKVVTIQLT
EHDFSYYSLVNQDWAIEAGNYNIQIGSSIEDIRLTTMIEQVGKNESEEY

QLKKYPTYAQANVQRIPDAEFYQLLGYQPPALLWDPKKPLGMNDTI
AQARNKNWLGKSSYNIVIFVKNVLKIVKQPLWSNNVYFVLNMPFRQ
MERFTGGKISKKNVQRYLRWVNG
KN

E0RVH9 *Butyrivibrio proteoclasticus*

MKYQDIIEKMTIEEKAAFLSGKGEWQTRDFERLGIPSIFCSDGPHGIRK
QAGAGDHLGLNESVPATCFPTAATIANSWNEELGEELGKTLGEEAM
AEGVNVLLGPGLNIKRSPLCGRNFEYFSEDPYLAGKMAASYVKGIQS
QGVYACPKHFAVNSQELRRMAMNSVLDERTLREIYLTGFEIAVKEGK
AKTIMSAYNEVNGTYANENKHLLTDILRKDWGFDGIVITDWGASND
HALGVAAGSNLEMPNPGLDSARELIAAVESGKISIEDVDARVDELLD
AVMTLYVNSQNKANDFDKPAHHAVARKAATESTVLLKNEGSILPLK
PGAKVAVIGDFAFVPRYQGAGSSLVNPTKVETISEVIGSYDVQVIGSS
RGYSRTGEEDAATRKEALDIASRADIVLFFFGLNEDSESEGMDRTHM
RIPQNQINLLQELGQVNKNLVGIISAGSAIEMPWHHYFKAILHCYLNG
QAGAGAVMDILTGRVNPSGKLSETIPRRHEDTPAYRYYPSSQRTSEY
RESLYVGYRYYDTADIPVLYPFGFGLSYTKFEYDNLTVNEDGVSFDIK
NVGEVAGKEVAQLYVSLPGAKVFRPKKELKGFAKVSLEPGESKRVEI
AFDDKTFRYWNVKTDKWEVEGGEYQIRIGASSADIRLEGKILKSATT
DVLPYSEAELPSYYSGKIQTVEDAEFEKLLGRSIPDGRWSGQLTSNDA
ICQLYYAKSGLARFVYKRLTAMKKKADESGKPDLNILFIYNMPFRAM
AKMTGGAVSMDMVDGIVDLVNGHFF
GGLGKIISGYFRNSKLNKQYESRIKK

N4WNP3 *Gracilibacillus halophilus*

MKHKEIINKMTLEEKASLMSGKDFWQTQNIDRLGINSMFLADGPHGI
RKQAEAADHLGLNESIPATCFPTAATVANSWNPDLGEKIGDYLGREA
VAQNVNVLLGPGINMKRDPLCGRNFEYFSEDPYHAGKLAASYIRGIQ
SHGISACVKHFAANNQEARRMTIDTVVDERTLREIYLTAFEIAIQEGE
TKTVMSSYNKLNGEYTNENLHLMQRILRDEWGYNGVVVTDWGGSN
DRISGLIAGNELEMPTTAGETNKDIVQAIKNGTIKEEVLDVCVDRLLE
LMKSTEVSLQGNPQNNFDQDKHHQVAQQAAEESIVLLKNEDHILPLQ
KDVKVATIGDFAENPRYQGAGSSIVNPTKLDTTLEFMKDVNVQSIGY
AQGFERYGKRSKRKIKHACQLAKNADIVLLYMGLDEATEAEGLDRH
DMKIPENQIELLHALYEVNPNIVVVLSCGSAVEMPWIDKVKGLVHGY
LSGQAGAKAILRVLTGEVNPSGKLAETYPIRYQDTPAYNHFPGEEAS
VEYRESMYIGYRYYDTAHVDALFPFGYGLSYTTFEYSDLQVTQQGV
TFTITNNGEVAGSEVAQLYVSCSSGEIFRPSKELKGFSKVFLQPGETKK
VEIAFDDKTFRYFNVITNRWEVESAEYMIQIGASVEDIRLKNTIHIEGS
QAPQPYQKNQLPSYYSGEVNDVRQEEFEQLLGRRVPVSHWDRTRPL

GYNDTIAQCQYAKGAIARIAYHLIVFSHWFLRKIGKRSTANLIMMSV
YHMPFRGMARMTGGVINMSMLDGILQMVNGQFFKGLRHFLKERRK
MRQSQKQVPKSS

K0J833 *Amphibacillus xylanus*

MKYNTIINQMTLAEKVSLMSGKDFWQSENIDRLGIPSMFLADGPHGI
RKQAAAADHLGLNPSIPATCFPTAATVANSWDVNLINKMGQYLGIEA
ANQKVNVLLGPGINMKRNPLAGRNFEYFSEDPYLAGKLAASLIQGIQ
SKGIAASVKHFAVNNQEERRMAIDTIVDERTLREIYLTAFEIAIKEGKA
KTVMSAYNKLNGTYANENPHLLNTILRNEWDYDGVVVTDWGGNN
DRVAALKVGNELEMPTTNGETDQDIYQAIKSGELKEEVLDEAVDRLL
ELIMSTTSALKESNASVDEKKHHQLAQKIAEESIVLLKNEENILPLNN
DVNVAVIGDFAREPRYQGAGSSVVNPTILENTLDSLAQSGIKSIGFEPG
FNRYGKKSNRKIAKACALAEKAEVVLLYLGLDEVTEAEGLDRESIKIP
QNQIDLLNEIYQVNQNIVVILSSGAVVEMPWIDKVKGLLHGQLSGQA
GAKAILRILTGEVNPSGKLAESYPIRYEDTPSYHQFPGKEVSVEYREG
LFIGYRYYDTANVAVRFPFGYGLSYTTFEYSDLEVDRAGATFTITNTG
NLPGMEAAQLYVGCQSRAIFRPKKELKGFVKVSLQPGESKTVTIPFD
DKTFRYFNVKTNQWEIEEADYEIMIGSSSVDIQLSGVLFVEGTGAPLP
YDQTDIPSYYSGQVSNVGLEEFETLLGRKVPDAKWDRTKPLGYNDTI
AQCQYAKGLFARFAYQLIKFSHWFLRKIGKRSTANLIMMSVYHMPFR
GLARMTGGIMNMPMVDGVLMIVNGQFYKGLKHVLRERKQMIKAAK
VNN

W4QD03 *Bacillus hemicellulosilyticus*

MKYNDLIKKMTLEEKASLMSGKDFWQTESIERLGINSMFLADGPHGI
RKQAEAADHLGLNESIPATCFPTAATVANSWNEELVEKVGEYLGEEA
VSQKVNVLLGPGINMKRSPLAGRNFEYFSEDPYLAGKLAAGMIKGIQ
SHGISACVKHFAVNNQEERRMSIDTIVDERTLREIYLTAFEIAIKEGQS
KTVMSAYNMLNGAYTNENIHLMREILRDEWNYEGVVVTDWGGSND
RVAGLLAGNELEMPTTAGETDKEIIAAINKGHISEDILDECVDRLLDL
LFTTEKVFAKETKDFDIEEHHQMAQKVAEESIVLLKNEENILPLKQNE
KVAVIGDFAQEARYQGAGSSIVNPTILDNTLESLEESGLTYIGFEKGFD
RYGKKSKKQIDKACELAKEADVVLLYIGLDEVTEAEGLDRQSMSIPE
NQIELIHALHKVNVNIVAVLSCGAVVEMPWIGKVKGLLHSYLSGQAG
SKAILRAIVGEVNPSGKLAETYPIKHEDTPAYYHFPGKEVSVEYREGP
FIGYRYYDTANVNVLFPFGFGLSYTTFEYSEVQVDKSGVTFAITNTGD
YAGSEVAQLYVGCQSTNIFRPKKELKGFTKVFLNPGETKSVTIPFDDK
TFRYFNVKTNQWEIEEASYNIMIGASCSDIRLEETLVVEGTGAPLPYD
KDVLSSYYSGKANQVSIEEFEALLGYKVPVSTWDRTVPLGYNDTIAQ
CQYAKGLFARFAFRAITLSHSFLWKIGKRSTANLIMMSVYHMPFRGM

ARMTGGIMNMPMLDGVLMIVNGKFFKGLRHVLKERKKMRKAQKES
QQLINNRNKGEVL

R7RMQ6 ***Thermobrachium celere***

MNIDEIMNELTLEEKCSLLSGADFWNLKSIERLGIRKIMMTDGPHGLR
KQDMDASEIGLEKSVPATCFPSGAALASTWNKNLIKEVGKAIAKECL
DNDVDVLLGPAVNIKRSPLCGRNFEYYSEDPVLSSKIAKYFIKGVQSQ
GVSACIKHFAANNQEFRRLTTDVRVDERAFREIYLKSFEEAIKEAKPD
CVMCAYNKINGEYASDNKKLLNDILREEWGYEGVVISDWGAVNDR
VKSLEAGMDIEMPSCFGVNDRIVYEAVKSGKIKIEVLDRAVKRILKLI
LKHSNKSQTRCVDYEYNHRLASKVAEEAVILLKNDDDILPLNKDSRI
AIIGEFAKNPRFQGGGSSHVNPTKLECPIDEIKKYANSVVYARGFNSN
NDEIDEALIKEAVNLAKISDVVVLFLGLPERYESEGFDRADIKLPYNQ
NILVDEIYKVNKNIVVSLSVGSCVEMPWLDKVKAVLNGYLLGQAGG
SAIANILFGYSVPSGKLSETFIKRLEDNPSYINFPGSNDRVYYGESVFV
GYRYYDYKNIDVQFPFGHGLSYTRFEYSNLKIDKNDYFDDECIDISFK
LKNVGKYKAKEVVQVYISKPNSSIIRPIKELKEFEKIELDVGEEREVNL
KIKVDDLSYFDEQFNSFLVEEGEYKILIGSSSRDIRLEGSIKVKNRQKV
KRMYHINSTIMDVIKTEKGKELLKELVQMLDVDENNVQAKMMKEF
YLNMPLRGLIYYGNGKYDFEKLNEIIDLLNNEA

F0HG16 ***Turicibacter sp***

MRDIKQLINQMTLQEKAGMCSGLDFWRLKSVERLGIPKVMVSDGPH
GLRKQKDGASDVNDSIKAVCFPAGCAIACSFDRDLLYNLGILLGEEC
QAENVSILLGPAVNIKRSPLCGRNFEYFSEDPYLSSEMACNYIQGVQS
QGVGTSLKHFAANNQESRRMTASAQIDERTLREIYLASFENAVKVGK
PWAVMCSYNRINEVFASENKKLLTDILRHEWEFDGYVMSDWGAVN
NRVEGLKAGLDLEMPGSHGTNDKLIIEAINKGILDETTLDEAVERIVT
KIFEFVDNRQDAIFNRDVHHEAASKIATQSAVLLKNDGVLPLNKEAKI
AFIGAFAKSPRFQGGGSSHINTYKVNSALEAVSNVTSVSYAQGYELD
VDQINEELVQEAIQVAKSSEVAVIFAGLPDFFESEGYDRTHMQLPNCQ
NELIKEVVKVQPNTVVVLHNGSPVEMPWIHDVKGVLEMYLAGQAV
GLATIDLLFGRVNPSGKLAETFPLKLSDNPSYLNYQVVDDLIHYREGI
FVGYRYYDKKEMNVLFPFGYGLSYTTFEYHDLVLSRKEMLDTDELV
VSLKITNTGAVAGKEVVQLYVSDLTHLTIRPIKELKDFVKVELQAGET
KEVQMTLDKRAFAWYNETISDWYVGTGEYEILIGKSSREIVLKDVIK
VQSTVELPFKVTANTTFGDLMKHETLRPIIESLAKQIDISNVGQEIDFN
LELIQDTPLRELRTIQNIDNAMIEYLIQTFNHYLS

E6UA77 ***Ruminococcus albus***

MNIKKILKELTLEEKASLCSGADFWHTKAIERLAIPQIMVSDGPHGLR
KNAEDSSNPQEAIKAVCFPTASALACSFDRNLLTAMGKALGEECQAE
KVSVILGPGCNIKRSPLCGRNFEYFSEDPYLASEMAAAHIKGVQSKGV
GTSLKHFAANNQETRRLTINERIDERTLHEIYLAAFEGAVKQASPWTV
MCSYNRINGYHSAQNKWLLTDVLRDEWGYDGLVMSDWGAVDDRV
EGIKAGLDLEMPASFGKNDRLIVDAVNSGKLSMKALDKCVERVLKL
VDKAEESRTPTEWDMEAHHELAAKIAEQCAVLLKNDDAILPLSKED
KVCFIGEFAEKPRYQGGGSSHINSFKVTSALDAVKEFCKVEYAQGFIT
YEDRSDPKLLEQAVECAKYNDKVVIFAGLPDSFESEGFDRTHMRMPE
CQLELIREISKVNKNIVVVLHNGSPVELPFFDDIKGLLEVYLGGQAIGK
ATCDLLFGEAVPSGKLAESWCMKLEDNPSYLNFPGVVDELTYSEGIF
VGYRYYDKKKMAVRFPFGYGLSYTNFSYSDLVISASEINDDQTLTVS
CNITNTGNRTGMETVQLYVGDKESSVIRPVKELKGFEKVSLRPGETK
KVFFTLDKRAFAYYETTINDWFVEYGEFEIMIGASSRDIRLSGSVYVN
SKTKLPVQFTFNSTVGDVLSCSEGREVFGDFIEKFCRGMSDVSGDGFG
AVTMDMAMAMFKETPLRDIICYDERQEINRVWLGEMLDKLNSMLE
G

R6U2U6 *Ruminococcus sp*

MNIDKILKELTLEEKASLCSGSDFWHTEEIKRLDIPSIMVSDGPHGLRK
MRDDTDNPNEAIKAVCFPCACALACSFDRKLLTTLGKALGEECQAED
VSVILGPGCNIKRSPLCGRNFEYFSEDPYLASQLATAHIKGVQSKGVG
TSLKHFAMNNQETRRMSYSANVDERTFHEIYLSAFETPVKEAKPWTV
MCSYNRINGEYSSQNKLLLTDILRNEWGYEGLVVSDWGAVDDRPLG
VAAGLDLEMPTSNGKNDELIIEAVNNGSLSMKDLDKAVRNVLTLIQK
AEDGYAPATKWDKEKQHELAGKIESECAVLLKNDDKILPLDKSAKIA
FIGEFADKPRYQGGGSSHINSFKVTSALEAVKGMDNITYAQGFVTDR
DETVDELLDEAVELAKNSDVAVIFAGLPESFESEGFDRKHMRMPDCQ
LKLIDEVAKVNENVVIVLHNGSAVEMPFADKVKGILEMYLGGQNIGT
AEKALLFGEANPSGKLAETFPEKLSHNPSYLNFPGNMDDVDYTEGIF
VGYRYYDEKGIKPLFPFGHGLSYTTFEYSNLTVSENEIKDDKTLTVM
VDVTNTGDRDGMEIVQLYVSNKESSVRRPVRELKGFEKLFLKAGETK
KAVFHLDKRSFAYYEPDIHDWFVEYGEFIIEAGSSSRDIRLSTSVYVSS
DTKLPVHFTLNTTCGEINSIPEGRAMFEDILSKIDCGFGDTASDDLGAS
AKEMMEAMIRDMPLRTLVTFTNVPDITRAKMSEMVENLNEMLAEK

T4VHL0 *Clostridium bifermentans*

MNIKELIKQMSLEEKASLCSGLNFWNTKPIERLNIPSIMMTDGPHGLR
KQSEGADHLGINESVESTCFPTASALACSFDRDLVKELGIAIGEECQSE
NVSIVLGPGANIKRSPLCGRNFEYYSEDPYLSSEMAKNQIQGTQSQGI
GTSLKHFAANNQEHRRMTIDTIVDERTLREIYLASFETAVKEAQPWT

VMCAYNKLNGEYCSENYRLLTEILRNEWGFEGFVVSDWGAVNDRD
KGLYAGLELQMPADGGMGDALIVEAVKSNRLSEGVLDKAVERILNIT
FKAIENKRESVIYSKEKHHELARKIAGECMVLLKNEEKILPLKKEEKI
AVIGELATKVRYQGGGSSHINPTKLDNTYEEIVNFAGSENIRYARGYD
LSIDDTIYELTEEAKQLAIEADKVILFIGLPERYESEGFDRTHLNIPKNQ
YDLVKALKSVNENIVVILSNGSPIEMPFVSDVKAILEAYLTGQASGKA
ICDILYGEVNPSGKLAETFALKLSDNPSYLNFPGEVDKVEYKEGIFVG
YRYYDKKAMDVLFPFGYGLSYTNFEYSNLKISKNEIDDTEKVTVSVSI
KNIGDVFGKEIVQLYISDKESSVIRPEKELKGFEKIGLEPGEEKEVTFIL
NKRSFAYYNVDLGDWHVESGEFEILIGKSSREIVLKEVITVNTTSPIKT
IVTKNTALGDISHLPEVQQIMDAMIQSFGRDTSGLGEGNMFAEMMKF
MPLRALATFNPDGGQQLVDRIIESINS

U4MU82 *Clostridium thermocellum*

MAVDIKKIIKQMTLEEKAGLCSGLDFWHTKPVERLGIPSIMMTDGPH
GLRKQREDAEIADINNSVPATCFPSAAGLACSWDRELVERVGAALGE
ECQAENVSILLGPGANIKRSPLCGRNFEYFSEDPYLSSELAASHIKGVQ
SQGVGACLKHFAANNQEHRRMTVDTIVDERTLREIYFASFENAVKK
ARPWVVMCAYNKLNGEYCSENRYLLTEVLKNEWMHDGFVVSDWG
AVNDRVSGLDAGLDLEMPTSHGITDKKIVEAVKSGKLSENILNRAVE
RILKVIFMALENKKENAQYDKDAHHRLARQAAAESMVLLKNEDDVL
PLKKSGTIALIGAFVKKPRYQGSGSSHITPTRLDDIYEEIKKAGGDKVN
LVYSEGYRLENDGIDEELINEAKKAASSSDVAVVFAGLPDEYESEGFD
RTHMSIPENQNRLIEAVAEVQSNIVVVLLNGSPVEMPWIDKVKSVLE
AYLGGQALGGALADVLFGEVNPSGKLAETFPVKLSHNPSYLNFPGED
DRVEYKEGLFVGYRYYDTKGIEPLFPFGHGLSYTKFEYSDISVDKKD
VSDNSIINVSVKVKNVGKMAGKEIVQLYVKDVKSSVRRPEKELKGFE
KVFLNPGEEKTVTFTLDKRAFAYYNTQIKDWHVESGEFLILIGRSSRD
IVLKESVRVNSTVKIRKRFTVNSAVEDVMSDSSAAAVLGPVLKEITDA
LQIDMDNAHDMMA
ANIKNMPLRSLVGYSQGRLSEEMLEELVDKINNVE

G8LXR2 *Clostridium clariflavum*

MSRDIKKIIAEMTVEEKASLCSGFGNWHTKAVERLQIPPIMMVDGPH
GLRIQFKNADLSDTQNSLPATCFPTAVNMASTWDRNLVEEIGKAIGE
ECRAEEVSILLGPGANIKRSPLCGRNFEYFSEDPYLSSQMAASHIKGV
QSQGVGTSLKHFCANNQEHRRLTVDVKVDERTLREIYLASFEEAVKQ
AKPWTVMCSYNSVNGEFASENSYLLTHILRDEWGFEGFVVSDWGAV
NERVKGLKAGLDLEMPYSGGERDKQIADAVKNGELPEEVLDKAVER
LLKIIFKAIDNKKSGTTFDKKAHHELARRAARESMVLLKNEDGILPLK
KQGKIALIGAFAKNPRFQGGGSSHVNPTYLSNAYDAIVDLAGQKAEI

LYSPGYDLETDVVDEKLIDEAKEAAAKADVAVIFAGLTDSYESEGYD
RAHLRIPENHRLLIEAVAEVQGKTVVVLSNGSPIEMPWIDKVKAVLET
YLGGQAVGEAVADILFGEFSPCGKLAETFPEKLSHNPSYLNFPGEGDS
VEYREGLFVGYRYYDAKDIKPLFPFGFGLSYTDFEYSDIKLSKKEIND
NELLTVSVKIKNTGKMRGKEIVQLYVRDVEKSVIRPDKELKGFEKIEL
DAGEEKTVTFSLDKRAFAYYNTEIKDWHVESGEFEILIGKSSQDIVLK
EVVTVNSTIMIKKKFHMNTTLGDIMTYPGGLDKLNQYMHEYLKKHG
MDKGIKHMKQSMDTEMVKYTPLRCLPSFSGNEVSKETVIKLLEDLNS

L0EHB0 *Thermobacillus composti*

MPRDLKKLISEMTLEEKAGLCSGLNFWRTKPIERLGIPSIMMTDGPHG
LRKQQEDDGDHLVIGDSVPATCFPSGAGLAASWNRELVEKVGEALG
RECRAHGVSILLGPAMNIKRSPLCGRNFEYFSEDPYLTAKMAASHIRG
VQSQGVGTAAKHYAMNNQEHRRMNVDAIVDERTQREIYLAGFEGA
VRQAQPWAVMAAYNKVNGTYATENKTLLTDILREEWGFEGFVVSD
WGAVNDRVAGLAAGLDLEMPGNGGYRDKKIVEAVKSGLLPEELLD
RAVERILNIVFKAADAQQVNVSFDPDEHHRLARFVAAECMVLLKNE
DGILPLKKHGRIAVIGEMAKKPRYQGGGSSHVVPTKLDIPFDEMEAIV
QGGAELVYAQGYELDKDEPNEVMVEEAVRAVESADVAVIFAGLPDR
YESEGYDRKHMRLPDNHNRLIEAVAAVQPNVVVVLSNGSPVEMPWI
GQVKGVLEAYLGGQAAGGAIADLLFGESNPSGKLAETFPKSLRHNPS
YLNFPGESDRVEYREGLFVGYRHYEARGIEPLFAFGHGLSYTTFEYTE
ISLDKREMTDRETLRVCVKVKNTGNRAGKEVVQLYVRDTASSVTRP
EKELKGFQKVSLEPGEEKTVEFALDKRAFAYYNTDLRNWHVETGEF
EILAGGSSDRIALKAVVHVTSTEAVKKTFTPNSTLGDLLADPAGAQVI
QAMIQRMNSGWPVIDEETQMMMNAVMLDMPLRSLVAFSGGAFTEEI
MNGILRQLNGNVS

D9TTJ4 *Thermoanaerobacterium thermosaccha*

MKKDIKKLISEMTLEEKASLCSGLNLWQTKPIERLGIPSITMTDGPHG
LRKAKKSDNLGLDDSVPATCFPSGSALAASWDRDLIKKVGEAIGEECI
AEDVHILLGPAINIKRSPLCGRNFEYLSEDPYLISELAANYIKGVQSKG
VGTSIKHYAANNQEDYRMTVDVKVNERALREIYLTGFEGAIKQSQP
WTVMASYNKVNGVYATENEHLINEILRNEWKFDGIVISDWGAVNDR
VAALKAGLDIEMPGSGGEEDKKIVEAVKKGQISEEYLNSAVERILNIIF
KAYENKKKNQNYDAEKHHQLARQVASECMVLLKNEDEILPLKKQG
KIAIIGELAVKPRYQGGGSSHIVPTKLDIPYDKITKIAGNKAEIKYTPGY
ELEKDEVNNKLIEEAAYIAKNSDVAVIFAGLPDSYESEGYDREHMRIP
ESHNKLIQAIAEVQPNVVVVLCNGSPIEMPWVHQVKGILEAYLGGQA

SGGAIADILFGEKNPCGKLAETFPRELRNNPSYLNFPGEKNVVNYGEG
IFVGYRYYDTKGVEPLFPFGHGLSYTTFEYTDISTNKNKITEEETIEVR
VKVKNTGKRAGKEIIQLYVRDIQSSVTRPHKELKGFQKIYLEPGEEKT
VVFNLDKRSFAYYDVDSKDWYVETGDFEILVGSSSKNILLKTVIHITS
TTSVKKEYTRNSTINDVITNQYGRQIIYNIIKRIKSDTNDSQDMLNILK
KQLETNSQEISSSSQKDMMNAFLKNMPLRALTILSNGIFTEEMVNEVV
DGLNTISN

O08331 *Clostridium stercorarium*

MQRDIKKIISQMTLEEKASLCSALDAWTLKGVERLGIPSIMVSDGPHG
LRKRQRDPTDPGKKTTVPATCFPTAVGLASSWNRELVEKVGPALGEE
CQAEGIAVLLGPGTNIKLSPLSGRNFEYFSEDPYLSSEMARSHIKGVQS
RGVGTSLKHFAANNQEHRRMSVDAVIDERTLREIYLASFEGAVKKA
KPWTIMCSYNRVNGEYASENKFLLTDVLRNEWGFEGIVVSDWGAVN
ERVKGLEAGLDLEMPSSFGIGDQKIVEAVKKGELPEEVLDRTVERILN
LIFKAVDNRKENAGYDREAHHKLAREAARECMVLLKNEDKILPLRK
QGTIAVIGEFAKRPRYQGGGSSHVNPTIMDSPYEEIKKSAGNNADVIY
AQGYIIEKDEPDEKLLEEAKQTALKADVAVIFAGLPEHYECEGYDRT
HMRMPESHCTLIEEVAEVTTNVVVVLCNGSPVEMPWIDKVKGLLEA
YLGGQAMGGPLPIFCSETPIPGKLAETFPKQLSDNPSYLNFRERDRVE
YREGIFVGYRYYDKKNMEPLFPFGYGLSYTTFEYGDLKISRKEISDNE
TVTVSVKVKNTGDMAGKEIVQLYVRDIETSVIDRRRTEGFEKVELQP
GEEKTVVFELDKRAFAYTHRYKDWHVETGEFRDFIGRSSRDIVLKDK
IFVKSTVTIKKRWTVNTLVGDLLSDRVLEPVFREFIINEIKTWYLLDLL
DEDNHLLSVWMRYTPLRSLANSTGGELNEEKLNRLIDTLNANIK

F8FL27 *Paenibacillus mucilaginosus*

QRDIQELISQMTLEEKAGMCSGLDFWHLKGVERLGIPSVMVTDGPHG
LRKQKASADHLGLFDSVPATCFPSAAGLACSWDRELIRRVGVALGEE
CQAENVAVLLGPGANIKRSPLCGRNFEYFSEDPYLSSEMAASHIAGV
QSQGVGTSLKHFAVNNQEHRRMSVDAVVDERTLREIYLASFEGAVK
QSQPWTVMSSYNRVNGTYASENEFLLTDILKNEWGHEGFVVSDWGA
VDERADALAAGLELEMPASGGVGERKVVEAVQSGRLSMEALDRAV
ERLLTIIFRAVDHRKPGAAYDPAEHHRLAREVARESMVLLKNERSLL
PLSKGSHLAVIGAFADKPRYQGGGSSHIVPTQLDQPVEEIRKLADAVV
TYAQGYRLESDMADEALTEEAKRAASAADTAVIFAGLPDRYESEGY
DRTHLSLPANQIRLIEEVAAVQPKVVVVLLNGSPVEMPWIGSAQAVL
EGYLGGQAVGGAVADLLYGEANPCGKLAETFPEQLSDNPSYLFFPGE
GDRVEYREGIFVGYRYYDTKNVKPLFPFGYGLSYTTFEYTSLTLDKRI
QDTESVTVRVTVKNTGAAAGKEIVQLYVKDAESTVIRPAKELKGFAK

VFLQPGEERTVSFVLDKRAFAYYNVDLKDWHVETGEFHILAGSSSQH
IVLQDSVVVESTAALRKTYTRNTTLGDLLQDAAAREKAQGLLQAFQ
EASGFADDHADMMAAMMKYMPLRALVGFSQGALTEQTLEDLLKEL
NH

W4BGN2 *Paenibacillus sp*

MSEQRDIEKIINQMTLEEKAGMCSGLDFWNLKGVERLGIPSIMVTDG
PHGLRKQRQGADHLGIFDSVPSTCFPSAAGLASSWDRELIRQVGVAL
GEECQAEDVAVLLGPGANIKRSPVCGRNFEYFSEDPYLSSELAASHIQ
GVQSQGVGTSLKHFAANNQEHRRMTTDAVIDERTLREIYLASFEGAV
KKAKPWTVMCSYNQVNGTYASENPRLLTEILKDEWGHEGFVVSDW
GAVNQRDDALAAGMELEMPSSNGRGERKVIHAVQSGKLTEEALDRA
VARILRIIFMAVDHKKENAVYDQKEHHALARKVAGESMVLLQNEDQ
LLPLGQDSKIAVIGAFAKTPRFQGGGSSHINPTQLDTPYEEIVALSGHV
SNVTYAQGYQLENDAVDETLIHEALQAARQGQVAVVIAGLPDRYES
EGYDRTHLSLPANQTQLIDAIAEVQNNVVVVLLNGSPVEMPWLHRV
KAVIEGYLGGQAVGGAIADVLFGKKNPSGKLAETFPVKLSDNPSFLN
FPGEGDRVEYKEGIFVGYRYYDKKEMKTLFPFGHGLSYTTFEYSNLK
FSRTELADNETVEVSVTVTNTGHLAGKEIVQLYVRDVESLVIRPEKEL
KGFAKVDLEPGEHKTVSITLDKRSFAYYNVELKDWHVESGDFEVLIG
KSSQEIVLKDTLQVQSTVRLEQQYTLNSTIGELLSDPVTAETTGQLLK
KFQEASPMSGMAEDDS
HSELFAAMMKDMPLRNLLAFGGGAVKEETLLQLLDELNRR

E5YXP0 *Paenibacillus vortex*

MSEQRDIEKIINQMTLEEKAGMCSGLDFWNLKGVERLGIPSIMVTDG
PHGLRKQRQGADHLGIFDSVPSTCFPSAAGLASSWDRELIRQVGVAL
GEECQAEDVAVLLGPGANIKRSPVCGRNFEYFSEDPYLSSELAASHIQ
GVQSQGVGTSLKHFAANNQEHRRMTTDAVIDERTLREIYLASFEGAV
KKAKPWTVMCSYNQVNGTYASENPRLLTEILKDEWGHEGFVVSDW
GAVNQRDDALAAGMELEMPSSNGLGERKVIDAVQSGKLTEEALDRA
VARILRIIFMAIDHKKEKAVYDQKEHHALARKVAGESMVLLQNEDQ
LLPLGQDSKIAVIGAFAKTPRFQGGGSSHINPTQLDTPYEEIVALSGHV
SNVTYAQGYQLENDAVDETLIHEALQAARQGQVAVVIAGLPDRYES
EGYDRTHLSLPANQTQLIDAIAEVQNNVVVVLLNGSPVEMPWLHRV
KAVIEGYLGGQAVGGAIADVLFGKKNPSGKLAETFPVKLSDNPSFLN
FPGEGDRVEYKEGIFVGYRYYDKKEMKTLFPFGHGLSYTTFEYSNLK
FSRTELTDNETVEVSVTVTNTGHLAGKEIVQLYVRDVESLVIRPEKEL

KGFAKVDLEPGEHKTVSITLDKRSFAYYNVELKDWHVESGDFEVLIG
KSSQEIVLKDTLQVQSTVRLEQQYTLNSTIGELLSDPVTAETTGQLLK
KFQEASPMSGMAEDDSHSELFAAMMKDMPLRNLLAFGGGAVKEET
LLQLLDGLNRR

L5NBC9 *Halobacillus sp*

MDASIDHLIKKMTLEEKAGFLSGRDFWNLKGLERLDIPSVMVTDGPH
GLRKQAQGADHLGLNASVPATCFPSAAGLASTWDQDLICRVGEALG
EEAKTEEVAVLLGPGTNIKRSPLCGRNFEYFSEDPYLSSMMAASHIEG
VQSKGIGTSLKHFAANNQEHRRMSVDARVDERTLREIYLASFEHAVK
QAAPWTVMCSYNQVNGEYASESCRLLTEILRNEWGWDGVVVSDWG
AVNERVDGLNAGMDLEMPSTNGIHDREIVEAVKKGDLTEATIDTAV
GRVLGLIQKAVKNQQQTSYDKEAHHRLAREAAADGMVLLKNEGRIL
PLDKKASVALIGSFVKQPRIQGGGSSHINPTRVDDVVEEVKKTTGTDR
VTYAEGYPLENDAIDEAMIEEACTTAAAADVAVLFAGLPDRYESEGY
DRKHLELPENHRALIERVAAVQPNVIVVLSNGAPLEMPWLDEVPAVL
EGYLGGQAFGGAVADLLFGDKTPSGKLAETFPVRLVDNPSYLNFPGE
GDVVEYKEGLFVGYRHYDAKQIEPLFPFGHGLSYSTFEYSSLMIDSHE
IDDTEEVEVSVDVMNTGSVEAKETVQLYVRDTESSVIRPEKELKGFA
KVSLEPQEVKTISFTLDRRSFAYYNVELGDWQVETGDFDILIGKSSKD
IVLKDTIHVRSTTVISMPIHRNTLVGDLLKNPKTASVMKEFLAENNPF
GGMETEDGEMDDMMDAMMANLPLRALVNFSMGAFTEKHLQELIGS
LSRRI

C2Z9J7 *Bacillus cereus*

MKRDIKKIISQMTLEEKASLCSGLDFWNTKGIERLGIPSIMVTDGPHG
LRKQAEGADHLGIYNSIPSTCFPSAVGLASTWNKDLINQVGVALGEE
CQAENVGVLLGPGANIKRSPLCGRNFEYFSEDPYLSSQMAANHVKG
VQSQGIGTSLKHFAANNQEHRRMSVDAIVDERTLREIYLASFEDVIKE
AQPWTVMSAYNKINGEYASENNYLLNDILKDEWGFEGFVVSDWGA
VNERVASLANGLELEMPSSFGIGEKKIVDAVNGGELSVEKLDQSVER
LLNIIFKAVDNQLENAVYSKDAHHQLAREVASESMVMLKNEDSILPL
KKEGTVAIIGEFAKQPRYQGGGSSHINPTKLESILEEIEMVSGEKTNILF
EQGYNLASDDVDENMINEAKKIAESADTVVLFVGLPDRYESEGFDRK
HLQMPENHVQLIEAIAEVQSNIVVVLSNGAPIEMPWIGKVKGILEGYL
GGQALGGAIADLLFGDANPSGKLAETFPKVLSDNPSYLNFPGEGDKV
EYKEGVFVGYRYYDKKNVEPLFPFGFGLSYTNFEYSNLSVDKKEIKD
TETVSVTVNVKNIGSTVGKEIVQLYIKDVESTMIRPEKELKGFEKVEL
QPGEEKTVNFTLNKRSFAYYNVELKDWHVETGEFEILVGKSSREIVL

QDNMYVQSTTIIQKIVHRNTLLGDIFADPILAPIAKGLMEKALKDSPFG
SMAEGDSDVSEMMDAMLNYMPLRALVNFSAGAFTEEMLSKIIGTLN
DAQMN

W4R0J6 *Bacillus weihenstephanensis*

MKRDIKKIISQMTLEEKASLCSGLDFWNTKGIERLGIPSIMVTDGPHG
LRKQAEGADHLGIYNSIPSTCFPSAVGLASTWNKELIKQVGVALGEEC
QAENVGVLLGPGANIKRSPLCGRNFEYFSEDPYLSSQMAANHVKGV
QSQGVGTSLKHFAANNQEHRRMSVDAIVDERTLREIYLASFEYVIKE
AQPWTVMSAYNKVNGEYASENNYLLNDILKDEWGFEGFVVSDWGA
VNERVASLANGLELEMPSSFGIGEKKIVDAVNCGKLSVEKLDQAAER
LLYIIFKAYDNQLENAAYSKDAHHQLAREVASESMVMLQNEASILPL
KKEGTVAVIGGFAKQPRFQGGGSSHINPTNLESILEEIETVSGEKTNILF
AQGYDIASDDVNESMVNEAKKIAERADTAVLFVGLPDRYESEGFDR
KHLQMPENHVQLIEAIAEVQSNIVVVLSNGAPIEMPWIGKVKGILEGY
LGGQALGGAIADILFGDANPSGKLAETFPKVLSDNPSYLNFPGEGDK
VEYKEGVFVGYRYYDAKNIEPLFPFGFGLSYTNFEYSNLSINKKEITD
TETVSVSINVKNTGSRAGKEIVQLYIKDVESSMTRPEKELKGFEKVEL
QPGEEKTVSFTLNKRSFAYYNVELKDWHVETGEFEILVGKSSKEIVLH
DSMYVQSTTITQKPVHRNTLLGDIFTDPILAPIAKGLMEKALKDSPFG
SMTEGDSDASEMMDAMLNYMPLRALVNFSAGAFTEEMLSEIIGILND
AQMN

U5ZNE7 *Bacillus toyonensis*

MKRDIKKIISEMTLEEKASLCSGLDFWNTKGIERLGIPSIMVTDGPHGL
RKQAEGADHLGIYNSIPSTCFPSAVGLASTWNKDLIHEVGVALGEEC
QAEHVGVLLGPGANIKRSPLCGRNFEYFSEDPYLSSQMAINHVKGVQ
SQGIGTSLKHFAANNQEHRRMSVDAIVDERTLREIYLASFEDVIKEAQ
PWTVMSAYNKVNGEYASENNYLLHDILKDEWGFEGFVVSDWGAVN
ERVASLANGLELEMPSSFGIGEKKIIDAIHCGELSVEKLDQAVERLLYI
IFKAYDNQLENATYSKDMHHQLAREVASESMVMLQNEDSILPLKKE
GTVAVIGEFAKQPRYQGGGSSHINPTKLASIFEELEMVSGEKTNILFA
QGYDLASDDVDENLINEAKKIAESADTAVLFVGLPDRYESEGFDRKH
LQMPENHVQLIEAIAEVQSNIVVVLSNGAPIEMPWIGKVKGILEGYLG
GQALGGAIADLLFGDANPSGKLAETFPEVLSDNPSYLNFPGEGDKVE
YKEGVFVGYRYYDAKNIEPLFPFGFGLSYTNFEYSKLSIS
KNEIKDTDTVSVLVNVKNAGSIAGREIVQLYIKDVESSMIRPEKELKG
FEKIELQPGEEKTVSFTLNNRSFAYYNVEMKDWHVETGEFEILVGKSS
REIVLQDNIFVQSTTIIKKTVHRNTLLGDIFADRMLAPIAKELMEKALK
DSPFASMAEGDSDASEMMDAMLNYMPLRALVNFSAGAFTEEMLSEII
ELLKDAQMNQ

K0FNN0 ***Bacillus thuringiensis***

MKRDIKKIISEMTLEEKASLCSGLDFWNTKGIERLGIPSIMVTDGPHGL
RKQAEGADHLGIYNSISSTCFPSAVGLASTWNKDLIHEVGVALGEEC
QAEHVGVLLGPGANIKRSPLCGRNFEYFSEDPYLSSQMAINHVKGVQ
SQGVGTSLKHFAANNQEHRRMSVDAIVDERTLREIYLASFEDVIKEA
QPWTVMSAYNKVNGEYASENNYLLHDILKDEWGFEGFVVSDWGAV
NERVASLANGLELEMPSSFGIGEKKIIDAINCGELSVKKLNQAVERLL
YIIFKAYENQLENATYSKDTHHQLAREVASESMVMLQNEDSILPLKK
EGTVAVIGEIAKQPRYQGGGSSHINPTKLESIFEELEMVSGEKTNILFA
QGYDLASDDVDENLINEAKKIAESADTAVLFVGLPDRYESEGFDRKH
LQMPENHVQLIEAIAEVQSNIVVVLSNGAPIEMPWIGKVKGILEGYLG
GQALGGAIADLLFGDANPSGKLAETFPEVLSDNPSYLNFPGEGDKVE
YKEGVFVGYRYYDAKNIEPLFPFGFGLSYTNFEYSKLSISKNEIKDTD
TVSVLVNVKNAGSIAGKEIVQLYIKDVESSMIRPEKELKGFEKVELQP
GEEKTVSFTLNNRSFAYYNVELKDWHVETGEFEILVGKSSREIVLQD
NIFVQSTTIIKKTVHRNTLLGDIFADRMLAPIAKELMEKALKDSPFAS
MAEGGSDASEMMDAMLNYMPLRALVNFSAGAFTEEMLSEIIELLND
AQMNQ

Resurrected beta-glucosidase sequence

TDNIKELVNQMTLEEKASLCSGKDFWHTQSIERLGIPSIMVTDGP
HGLRKQAAEADHLGLNESVPATCFPTAAALASSWDPELLHEVGE
ALGEECRAENVSVLLGPGVNIKRSPLCGRNFEYFSEDPYLAGEMA
AAWISGVQSKGVGTSLKHFAANNQEHRRMTVDAVVDERTLREIY
LAAFENAVKQAQPWTVMCSYNRINGVYSSENKWLLTEVLRDEW
GFEGLVVSDWGAVNDRVKGLKAGLDLEMPSSGGLNDKQIVEAV
RNGELDEAVLDRAAERILTLIARAAAARKQNHTYDVEAHHALAR
RIAAESAVLLKNDDGILPLKKEAKIAVIGEFAKTPRYQGAGSSQIN
PTKLDNALDELRERGGADVTYAPGYELDGDRTDAALLEEAVEVA
KNADVVVVFAGLPDSYESEGFDRTHLNLPENHNALIEAVAEVNPN
VVVVLSNGSPVTMPWRDKVKAILESYLGGQAGGSAIADILTGEVN
PSGRLAETFPLRLEDNPSYLNFPGEPQHVEYRESIFVGYRYYDTAE
KDVAFPFGYGLSYTTFEYSDLKISKAADDNETVEVSVTVTNTGDR
AGSEVVQVYVGDAESTVFRPVQELKGFAKVFLEPGESREVTITLD
RRAFSYYNVKINDWTVESGDFEIRVGSSSRDIRLKATVTLNSTTPL
PATFTVNTTIGDIMASPAGKALLGALVQAVSAGSGAKDSVSRMM
MAMLQDMPLRSLAMFTGGAITPEMLEELVEMLNG

Appendix IV

List of sequences used in the cellulosome

Scaf1: Histag-CBM-COHESIN7-XDOCKERIN

MGSSHHHHHHSSGLVPRGSHMMANTPVSGNLKVEFYNSNPSD
TTNSINPQFKVTNTGSSAIDLSKLTLRYYYTVDGQKDQTFWCD
HAAIIGSNGSYNGITSNVKGTFVKMSSSTNNADTYLEISFTGGT
LEPGAHVQIQGRFAKNDWSNYTQSNDYSFKSASQFVEWDQVT
AYLNGVLVWGKEPGGSVVPASIGTAVRIKVDTVNAKPGDTVR
IPVRFSGIPSKGIANCDFVYSYDPNVLEIIEIEPGELIVDPNPTKSF
DTAVYPDRKMIVFLFAEDSGTGAYAITEDGVFATIVAKVKSGA
PNGLSVIKFVEVGGFANNDLVEQKTQFFDGGVNVGTSNKPVIE
GYKVSGYILPDFSFDATVAPLVKAGFKVEIVGTELYAVTDANG
YFEITGVPANASGYTLKISRATYLDRVIANVVVTGDTSVSTSQD
PIMMWVGDIVKDNSINLLDVAEVIRCFNATKGSANYVEELDIN
RNGAINMQDIMIVHKHFGATSSDYDAQ

Scaf2: Histag-CBM-COHESIN7-linker-COHESIN7-
XDOCKERIN

MGSSHHHHHHSSGLVPRGSHMMANTPVSGNLKVEFYNSNPSD
TTNSINPQFKVTNTGSSAIDLSKLTLRYYYTVDGQKDQTFWCD
HAAIIGSNGSYNGITSNVKGTFVKMSSSTNNADTYLEISFTGGT
LEPGAHVQIQGRFAKNDWSNYTQSNDYSFKSASQFVEWDQVT
AYLNGVLVWGKEPGGSVVPASAVRIKVDTVNAKPGDTVRIPV
RFSGIPSKGIANCDFVYSYDPNVLEIIEIEPGELIVDPNPTKSFDT
AVYPDRKMIVFLFAEDSGTGAYAITEDGVFATIVAKVKSGAPN
GLSVIKFVEVGGFANNDLVEQKTQFFDGGVNVGgssvpttqpnvpsd
gGTAVRIKVDTVNAKPGDTVRIPVRFSGIPSKGIANCDFVYSYD
PNVLEIIEIEPGELIVDPNPTKSFDTAVYPDRKMIVFLFAEDSGT
GAYAITEDGVFATIVAKVKSGAPNGLSVIKFVEVGGFANNDLV
EQKTQFFDGGVNVGTSNKPVIEGYKVSGYILPDFSFDATVAPL
VKAGFKVEIVGTELYAVTDANGYFEITGVPANASGYTLKISRA

TYLDRVIANVVVTGDTSVSTSQDPIMMWVGDIVKDNSINLLD
VAEVIRCFNATKGSANYVEELDINRNGAINMQDIMIVHKHFGA
TSSDYDAQ

LFCA-Dock: LFCA-linker-DOCKERIN-histag

MASMTGGQQMGRIRTPVETHGQLSVKGGQLVDENGKPVQLR
GMSSHGLQWFGDFVNKDSMKWLRDDWGINVFRVAMYTAEG
GYITNPSVKNKVKEAVEAAIDLGMYVIIDWHILSDNDPNTYKE
QAKAFFQEMAAKYGNYPNVIYEICNEPNGGVTWSNQIKPYAE
EVIPAIRANDPDNIIIVGTPTWSQDVHDAADNPLPYSNIMYALH
FYAGTHGQSLRDKIDYALSKGVAIFVTEWGTSDASGNGGPFLN
ESQKWIDFMNSRNISWANWSLSDKSETSAALMPGASPTGGWT
DSNLSASGKFVREQIREFpnplsdlsgqptppsnptpslppqvvyGDVNGDG
NVNSTDLTMLKRYLLKSVTNINREAADVNRDGAINSSDMTILK
RYLIKSIPHLPYLEHHHHHH

LFCA-CBM: LFCA-linker-CBM-histag

MASMTGGQQMGRIRTPVETHGQLSVKGGQLVDENGKPVQLR
GMSSHGLQWFGDFVNKDSMKWLRDDWGINVFRVAMYTAEG
GYITNPSVKNKVKEAVEAAIDLGMYVIIDWHILSDNDPNTYKE
QAKAFFQEMAAKYGNYPNVIYEICNEPNGGVTWSNQIKPYAE
EVIPAIRANDPDNIIIVGTPTWSQDVHDAADNPLPYSNIMYALH
FYAGTHGQSLRDKIDYALSKGVAIFVTEWGTSDASGNGGPFLN
ESQKWIDFMNSRNISWANWSLSDKSETSAALMPGASPTGGWT
DSNLSASGKFVREQIREFpnplsdlsgqptppsnptpslppqvvyTSMANTP
VSGNLKVEFYNSNPSDTTNSINPQFKVTNTGSSAIDLSKLTLRY
YYTVDGQKDQTFWCDHAAIIGSNGSYNGITSNVKGTFVKMSS
STNNADTYLEISFTGGTLEPGAHVQIQGRFAKNDWSNYTQSND
YSFKSASQFVEWDQVTAYLNGVLVWGKEPGGSVVPLEHHHH
HH

Cel8A: CEL8A-linker-DOCKERIN-histag

MGVPFNTKYPYGPTSIADNQSEVTAMLKAEWEDWKSKRITSN
GAGGYKRVQRDASTNYDTVSEGMGYGLLLAVCFNEQALFDD
LYRYVKSHFNGNGLMHWHIDANNNVTSHDGGDGAATDADE
DIALALIFADKLWGSSGAINYGQEARTLINNLYNHCVEHGSYV
LKPGDRWGGSSVTNPSYFAPAWYKVYAQYTGDTRWNQVAD
KCYQIVEEVKKYNNGTGLVPDWCTASGTPASGQSYDYKYDA
TRYGWRTAVDYSWFGDQRAKANCDMLTKFFARDGAKGIVD
GYTIQGSKISNNHNASFIGPVAAASMTGYDLNFAKELYRETVA
VKDSEYYGYYGNSLRLLTLLYITGNFpnplsdlsgqptppsnptpslppqv
vyGDVNGDGNVNSTDLTMLKRYLLKSVTNINREAADVNRDGA
INSSDMTILKRYLIKSIPHLPYLEHHHHHH

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Amaitzeko, gertukuez gogoratzia nahi dot, kuadrilakuez, beti hor egotiagaitxik, beti irrifarre bat ataratziagaitxik ta emondako animuengaitxik. Marta maitxia zelan ikasi dozun nere proiektua esplikatzen ta zelan lagundu dostazun deskonektatzen. Azpiri, beti hor egotiagaitxik ta babesa emotiagaitxik, beti zauzielako hor bixok bihar dan guztixandako. Eskerrik asko zuri be Amaia, beti hor egotiagaitxik ta zuri be Ale, entzutiagaitxik ta laguntziagaitxik, portadia primeran geratu da. Baita eskerrak emon nere taxista gogokuenei, astelehen goizak ez diralako berdinak zuek barik. Ta zelan ez etxekuei, beti zauzielako hor ta zuek barik hau ez zalako posible izango. Mila mila esker ama, laguntzagaitxik ta beti animuak emon ta entzutiagaitxik. Eskerrik asko aitxa ta Jon, bihar doten danandako hor zauzielako. Eskerrik asko bebai beste etxekuei, mila esker Itsaso zure laguntza ta animuengaitxik. Ta zelan ez zuri maitxia, eskerrik asko Gaizka, onduan euki leiken pertsona honena zaralako, zurekin hau ta nahi dotena eitxia posible dalako, ezebez esan barik be dana esanda dauelako…

Mila esker danori nerekin egotiagaitxik!