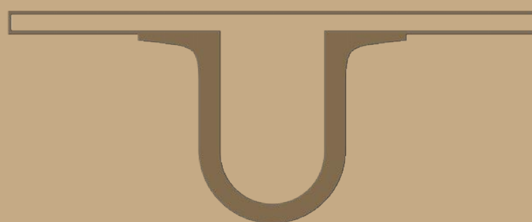

Inês Oliveira Costa

DEEP BIOSPHERE:
MICROBIAL POPULATIONS AS SOURCE OF BIOTECHNOLOGICAL SOLUTIONS

Dissertação no âmbito do Mestrado em Bioquímica orientada pelo Doutor Igor Tiago e apresentada ao Departamento de Ciência da Vida da Universidade de Coimbra.

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Abstract

Microorganisms constitute a large portion of the biosphere on Earth and part of them is present in the deep biosphere: a set of habitats physically located below the surface of continents and/or the bottom of the ocean. Microorganisms living in these environments can be a leverage to better understand strategies of survival/growth in extreme conditions; however, only a small percentage can be recovered and cultured on laboratory conditions. The set of microbial organisms that remains uncultured comprises the "microbial dark matter".

Alfaguara spring is a deep aquifer hyperalkaline spring, associated with serpentinization reactions, located at Peridotites of Ronda, South of Spain. To access the diversity of the aerobic heterotrophic bacteria present in this habitat, an extensive culture-dependent isolation process was conducted. Isolates were grouped by RAPD profiling, representative isolate(s) of each group were summarily characterized, and its phylogenetic placement was determined by 16S rRNA gene sequencing. The vast majority of the isolates were phylogenetically related to the phylum *Firmicutes*, namely with genus *Bacillus*. Others were related to phylum *Actinobacteria*, specifically with species belonging to genera *Microbacterium*, *Micrococcus* and *Kocuria*. The biotechnological potential of the isolates was accessed by screening their ability to degrade chitin, carboxymethyl cellulose, xylan and starch. All isolates were successful in the degradation of at least one of the substrates and some of them showed that ability at higher pH values. The major degradation activity (either by activity and number of isolates) was obtained for xylan. None of the isolates degraded chitin. The structural microbial diversity was also accessed by culture-independent methods, namely metagenomic 16S rDNA Illumina tags. *Hydrogenophaga/Serpentinomonas* was the most abundant genus found in the Alfaguara community, a common population found in this kind of environments. In addition, members of *Clostridia*, a class of strictly anaerobe microorganisms was also rather abundant. Moreover, *Candidatus nitrocosmicus* and *Candidatus nitrosoarchaeum* were the major archaeal populations detected. Despite their abundance, these two groups of aerobic archaea are not normally found at sites associated with serpentinization, making the Alfaguara deep aquifer community distinct from those already known.

Keywords: Archaea; Bacteria; Deep biosphere; Metagenomics; Biotechnological potential.

Resumo

Atualmente, acredita-se que os organismos que constituem maioritariamente a biosfera na Terra, os microrganismos, se encontram em elevada percentagem na biosfera de profundidade. Este termo é referente ao conjunto de habitats que se localizam abaixo da superfície dos continentes e do fundo dos oceanos e englobam microrganismos que podem ser utilizados para o estudo de estratégias de sobrevivência em condições extremas. No entanto, em escassas situações é possível simular todas as condições necessárias ao cultivo destes extremófilos em ambiente laboratorial e, por este motivo, a maioria destes microrganismos permanece por cultivar – designando-se por “microbial dark matter”.

A nascente de Alfaguara, localizada nos Peridotitos de Ronda, no sul de Espanha, está associada a um aquífero de profundidade gerado por reações de serpentinização. Com o objetivo de perceber qual a diversidade estrutural das populações bacterianas aeróbias heterotróficas presentes neste habitat, uma série de métodos dependentes de cultivo foi aplicada. Posteriormente, os isolados foram agrupados por perfil de RAPD, o(s) isolado(s) representativo(s) de cada grupo foram sumariamente caracterizados e sua filogenia foi determinada pela sequenciação do gene 16S rRNA. Com esta análise, determinou-se que a maioria dos isolados pertenciam ao filo *Firmicutes*, nomeadamente ao género *Bacillus*. Outros pertenciam ao filo *Actinobacteria*, sendo mais relacionados com espécies dos géneros *Microbacterium*, *Micrococcus* e *Kocuria*.

O potencial biotecnológico dos isolados também foi avaliado ao determinar a sua capacidade para degradar quitina, carboximetilcelulose, xilano e amido. Nenhum dos isolados testados degradou quitina, no entanto, todos os isolados apresentaram actividade de degradação positiva para pelo menos um dos outros três substratos testados. Alguns deles exibiram essa atividade a valores de pH mais elevados. Além disso, a principal atividade de degradação foi obtida para xilano.

Para determinar a diversidade estrutural o mais aprofundada possível, métodos independentes de cultivo, nomeadamente a plataforma de sequenciação massiva Illumina, foram aplicados. O género aeróbio facultativo *Hydrogenophaga/Serpentinomonas*, comumente identificado neste tipo de ambientes, foi identificado como sendo o mais

abundante na comunidade bacteriana, mas a classe *Clostridia*, associada a microrganismos estritamente anaeróbios também se mostrou bastante representada. Os géneros aeróbios *Candidatus nitrocosmicus* e *Candidatus nitrosoarchaeum*, foram o grupo de archaeas maioritariamente identificados. No entanto, apesar da sua abundância, estes grupos de archaeas não são normalmente encontrados em ecossistemas associados a serpentinização, tornando a comunidade de archaeas de Alfaguara distinta das já conhecidas e descritas na literatura.

Palavras-chave: Archaea; Bactéria; Biosfera profunda; Metagenómica; Potencial biotecnológico.

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Abbreviations

ABM2. Solid Alkaline Buffer Medium 2

BLAST. Basic Local Alignment Search Tool

BCYE. Buffered Charcoal Yeast Extract

bp. Base Pairs

C. Cytosine

ºC. Celsius Degrees

Ca^{2+}. Calcium

CD . Colony Diameter

CO_2 . Carbon Dioxide

CO. Carbon Monoxide

CRBG. Columbia River Basalt Group

DNA. Deoxyribonucleic Acid

dNTP. Deoxynucleotide

DNRA. Dissimilatory Nitrate Reduction to Ammonium

EC. Electric Conductivity

Fe^{2+}. Ferrous

g. Grams

HD. Degradation Halo Diameter

IC. Inorganic Carbon

kms. Kilometers

M. Molar

mM. Millimolar

mL. Milliliters

NGS. Next Generation Sequencing

O_2. Oxygen

OH^-. Hydroxide

OTUs. Operational Taxonomic Units

PCA. Principal Component Analysis

PCR. Polymerase Chain Reaction

PLFA. Polar Lipid Fatty Acid

RAPD. Random Amplified Polymorphic DNA

RNA. Ribonucleic Acid

rRNA. Ribosomal Ribonucleic Acid

R2A. Solid Reasoner's 2A Agar Medium

SLiMEs. Subsurface Lithoautotrophic Microbial Ecosystems

SRB2. Sulfate Reducing Bacteria

TAE. Tris-Acetate-EDTA

T. Temperature

TN. Total Nitrogen

TOC. Total Organic Carbon

UV. Ultraviolet

V. Volts

VAMPS. Visualization and Analysis of Microbial Population Structures

µL. Microliters

µm. Micrometer

µM. Micromolar

Chapter I - Introduction

1.1. Extreme environments and extremophiles

Extreme environments are characterized by having one or more extreme physical and/or geochemical conditions i.e. extremely high or low temperature, pressure, pH, salinity (Gomez, 2014). Thermal vents, hot springs and deep oceans are examples of extreme environments (Kushner, 1978) that have been increasingly studied for having so harsh conditions that are limiting for most living organisms(Kushner 1978). However, despite being considered unable of supporting life, some microorganisms can survive and grow in such environments and are, therefore, called extremophiles (Dhakar & Pandey, 2016).

The challenging lifestyle of these extremophiles has triggered a growing interest in both biotechnological and astrobiology fields of research. This particular group of microorganisms can thrive in conditions that restrict life for most living creatures since they possess enzymes that are able to function in those conditions (Charlesworth & P. Burns, 2016). This aspect is particularly important in the biotechnological area (González-Toril *et al.*, 2003) because some substrates can only be degraded or converted in such specific and extreme circumstances, namely high temperatures and/or very low or high pH values (Fujiwara, 2002).

Additionally, some studies focus on extremophiles microbial populations and to their energetic interaction with the environment, as models to understand early life in planet Earth and life elsewhere (Schulte *et al.*, 2006; Davies *et al.*, 2009).

1.2. Natural alkaline environments

Among natural alkaline environments, a rare type of extreme environments, soda lakes and deserts are the most commonly studied, representing high-pH environments on

Earth, with pH values comprised between 9.5-11, reaching values of 12 sporadically (Jones *et al.*, 1998; Sorokin *et al.*, 2014). Soda lakes are mainly found in depressions present in arid and semi-arid regions with high concentrations of sodium carbonates. These saline and alkaline lakes are formed when sodium-rich rocks are leached by groundwater with low concentrations of magnesium and calcium but abundant in carbon dioxide (Sorokin *et al.*, 2015; Sorokin *et al.*, 2014).

Other natural alkaline environments are the non-saline alkaline ecosystems. They are commonly associated to serpentinization reactions, a geochemical process that englobes the interaction between water and mafic/ultramafic rocks. This reaction leads to very specific chemical conditions (Tiago *et al.*, 2004) that were already found and well-studied in Lost City Hydrothermal Field-LCHF in the mid-Atlantic Ocean (Kelley *et al.*, 2005), the Coast Range Ophiolite (Twing *et al.*, 2017) and The Cedars in California (Suzuki *et al.*, 2013), Tablelands in Canada (Brazelton *et al.*, 2013), Voltri Massif in Italy (Brazelton *et al.*, 2017), the aquifer from Cabeço de Vide in Southeast of Portugal (Tiago *et al.*, 2004) and Oman springs (Chavagnac *et al.*, 2013).

1.1.1. Serpentinization reactions

The curiosity about environments allied to serpentinization has been increasing once it was discovered that molecular hydrogen and methane are formed during serpentinization reactions. These two gases have of particular interest since several microorganisms can use them as raw material to produce energy (Holm *et al.*, 2015).

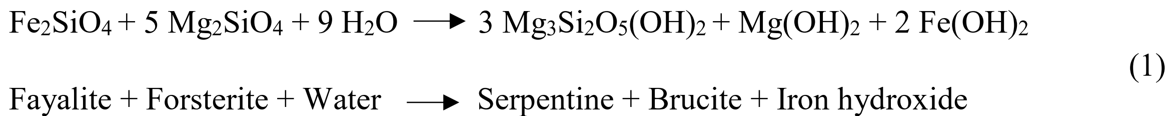
Fayalite (iron-rich olivine), forsterite (magnesium rich-olivine) and pyroxene are the minerals that constitute ultramafic rocks. Once exposed to surface or near- surface conditions, the components of these type of rocks become unstable and react to form new stable minerals while release energy (Cardace & Hoehler, 2009).

When meteoric waters or seawater interact with mafic/ultramafic rocks in conditions of pressure and temperature different from those of the upper mantle (where they are

characteristic) (Crespo-Medina *et al.*, 2014), serpentine minerals and hydroxides (Reaction 1) are formed and the alkalinity of the fluids increase (Cardace & Hoehler, 2009). This is possibly because these waters have large amounts of carbon dioxide which decomposes the silicate minerals and releases calcium (Ca^{2+}) and hydroxide (OH^{-}) (Tiago *et al.*, 2004).

Then, coupled to the oxidation of ferrous (Fe^{2+}) from iron hydroxide to magnetite, molecular hydrogen, is generated (Reaction 2) (Cardace & Hoehler, 2009).

These aqueous alterations are termed serpentinization since the serpentine minerals are often the dominant altering mineral formed.



$$Fe_2SiO_4 + 5\ Mg_2SiO_4 + 9\ H_2O \longrightarrow 3\ Mg_3Si_2O_5(OH)_2 + Mg(OH)_2 + 2\ Fe(OH)_2 \quad (1)$$

Fayalite + Forsterite + Water ⟶ Serpentine + Brucite + Iron hydroxide

(Cardace & Hoehler, 2009)

$$3\ Fe(OH)_2 \longrightarrow Fe_3O_4 + 2\ H_2O + H_2 \quad (2)$$

Iron hydroxide ⟶ Magnetite + Water + Hydrogen

(Cardace & Hoehler, 2009)

1.3. Deep biosphere

Nowadays, it is believed that microorganisms, including domains *Bacteria* and *Archaea*, constitute a major part of biosphere on Earth (Solden *et al.*, 2016) and that a considerable portion of them are present below the surface (Wu *et al.*, 2016), in the deep biosphere.

The term "deep biosphere" was proposed in 1992 by Gold (Gold, 1992) which suggested that life could extend for several kilometers in the subsurface while the temperature did not become too high for survival (Colman *et al.*, 2017). This deep, dark biosphere comprises the major set of habitats on Earth (Edwards *et al.*, 2012) however, one of the least studied too (Kadnikov *et al.*, 2017). Once located in the subsurface, light cannot reach these group of environments and, in most cases, photosynthesis cannot be the source of energy that support the community, unlike what happens on the surface. Thomas Gold believed that life in these conditions (light absence) was maintained by chemical sources of energy that come from the Earth's crust and from host rocks (Gold, 1992). Due to the physical, chemical and geological characteristics of these habitats, they are included in a specific set of extreme environments. As discussed above, some environments, and more specifically continental deep aquifers, are in permanent darkness, and water reaching them is depleted of any organic matter during the way down. For this reason, photosynthesis (or its sub-products) cannot be the subsistent mechanism to support the microbial community present in these deep habitats, and microorganisms must use the local geochemistry to survive (Shock & Holland, 2004).

Under reducing conditions, the hydrogen produced by serpentinization reactions combined with carbon monoxide or carbon dioxide liberated from the mantle, leads to the formation of methane and other hydrocarbons (Schrenk *et al.*, 2013). These reactions have the potential to provide the chemical energy that could sustain an ecosystem supported exclusively by chemolithoautotrophy.

Because continental deep-aquifers environments associated to serpentinization can persist without photosynthetic products, they are good candidates to be SLiMEs (subsurface lithoautotrophic microbial ecosystems) (Nealson *et al.*, 2005) . This is particularly fascinating since these environments can provide an idea of early Earth life where microorganisms depended solely on chemical energy sources (Russell *et al.*, 2010).

1.4. Structural microbial diversity characterization

Due to their interest, the study of microorganisms living in extreme environments, such as those with absence of light, has been increasing. Culture-dependent and/or -independent methods have been used, based on the analysis of the 16S rRNA gene sequence and/or shotgun-metagenomes, with the purpose of unveil the structural and/or functional diversity of these extremophiles.

1.4.1. 16S rRNA gene

16S ribosomal RNA (16S rRNA) gene is considered a good molecular marker in microbial ecology and it is the most commonly used (Case *et al.*, 2007). This gene is ubiquitous in prokaryotes, and due to specific characteristics, its sequence allows to identify prokaryotic species and perform taxonomic studies (Mizrahi-Man *et al.*, 2013). The prokaryotic 16S rRNA gene sequence (Figure1) is typically composed of nine hypervariable regions flanked with others highly conserved regions that allow primers binding for amplification (Claesson *et al.*, 2010). As the name implies, the conserved regions are very much identical in all prokaryotic organisms, however, hypervariable regions suffer mutations over time. Moreover, the gene is functionally conserved among all prokaryotic organisms. This suggest that alterations in hypervariable stretches are evolutionary since 16S rRNA gene sequences are more similar between organisms of the same genus and species but differ for organisms of different taxa. For these reasons, the comparison of these sequences makes possible to infer phylogenetic relations and characterize the structural diversity of the prokaryotic communities (Woo *et al.*, 2008).

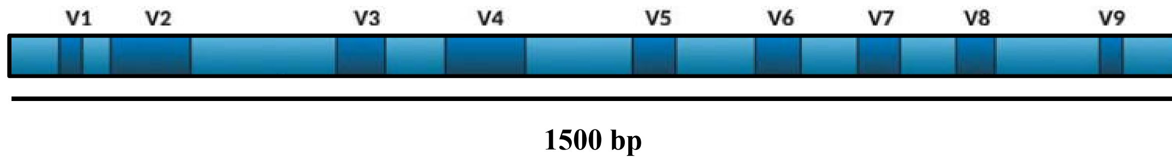


Figure 1. Representation of the 16S rRNA gene sequence. The dark blue sectors represent the nine hypervariable regions and conserved regions are represented by light blue sectors. Adapted from (Rapin *et al.*, 2017).

1.4.2. Culture-dependent methods

Formerly, the microbial diversity of a given environment would be solely determined by culture-dependent methods, which are based on the isolation of microorganisms in culture media. The aim of this approach is to recover as many isolates as possible in each different incubation conditions to get the closest possible idea of the microbial diversity. However, the more incubation conditions are used, greater the number of isolates to be obtained for screening and identification. In order to reduce the number of isolates to work with, molecular fingerprint techniques such as Random Amplification Polymerase Deoxynucleic acid (RAPD) are applied during the screening process. RAPD is a Polymerase Chain Reaction (PCR) based technique for the identification of genetic variations present in the DNA sequence (Kumari & Thakur, 2014) and allows the decrease of the number of isolates to be identified by grouping the isolates with the same RAPD profiles – RAPD typing (Power, 1996). Once the RAPD profiles are formed, PCR amplification of the 16S rRNA gene sequence of one or more representative isolates from each group is performed, and the PCR product is sequenced.

However, the development of molecular biology techniques applied to environmental studies such as massive parallel 16S rRNA gene tag sequencing, shown that only 1 to 4% of the existing microbial populations could be cultured – The Great Plate Count Anomaly (Harwani, 2012), revealing a newly unknown microbial diversity called "microbial dark

matter" (Hedlund & Dodsworth, 2014). This demonstrated that culture-dependent methods had several biases that prevented the correct determination of the structural diversity of a given environment (Carraro *et al.*, 2011). The fact that most of microorganisms cannot be grown under laboratory conditions can be explained with the difficulty in mimic the necessary grow conditions of each specific population thriving in each environment (Epstein, 2013).

Nowadays, it is possible (and desired) to use a combined approach of culture - dependent and -independent approaches for a correct characterization of the microbial diversity of an ecosystem. Although culture-dependent methods offer a low coverage assessment of the diversity (Orphan *et al.*, 2000), provides access to the actual living microorganism, allowing its study. In contrast, culture-independent methods provide high coverage results nevertheless, based only on genetic data (Riesenfeld *et al.*, 2004).

1.4.3. Biotechnological potential of the isolated populations

The conditions existing in serpentinization driven environments challenge the known limits of life therefore, determining the structural microbial diversity (by culture -dependent and -independent methods) is only the first step. Many of these extremophiles play fundamental rules in natural environmental processes and can be a source of new enzymes with potential for biotechnological applicability. These enzymes can be catalytically active under extreme conditions and may be useful in industrial procedures i.e. the production of biofuels or degradation of specific and degradation of recalcitrance carbon polymers, that depend on processes that occur in extreme conditions (Mirete *et al.*, 2016).

Plant biomass, the most abundant, accessible and economically advantageous biopolymer on Earth has high amounts of cellulose, xylan and starch that together account for 75% of terrestrial biomass (Thomas *et al.*, 2011; Rosado & Govind, 2003). These polysaccharides are part of plant cell wall (Kacuráková *et al.*, 2000) and not only are the main source of energy for several microorganisms but also are used as carbon source to produce

biofuel (Demura & Ye, 2010). Additionally, they are very recalcitrant for degradation and the use of new enzymes with special features can be a breakthrough for this process.

1.4.4. Culture-independent methods

Initially, the term “metagenomics” was used to describe random cloning of environmental DNA obtained from environmental samples (Handelsman *et al.*, 1998). On that period, metagenomics methodology relied on Sanger sequencing platform results for the sequencing of high numbers of cloned sequences, but the time/hands-on consuming workflow and limitations of this methodology lead to the necessity of creating new and improved technologies for sequencing large numbers of genomes. Nowadays, Sanger platform is classified as “first-generation” technology and subsequent new methods are designated as “next-generation sequencing” (NGS) technologies (Metzker, 2009). Currently, metagenomics comprises any molecular biology technique that involves the analyses of a total community genomics, from a given environment, without the need of previous cultivation (Thomas *et al.*, 2012). NGS technologies have the advantage to produce huge amount of data and are at the same time affordable and very fast to deliver results (Turumtay *et al.*, 2016). Illumina is one of the most commonly used NGS platform for ecological studies (Wen *et al.*, 2017).

1.5. Alfaguara deep aquifer

As discussed above, numerous studies are currently related to extreme environments associated with serpentinization. In some of these researches, access to the sample is possible through wells drilled into serpentines that provide direct access into the deep aquifers. However, in some cases, groundwater can be only be recovered from the subsurface through natural artesian springs, without anthropogenic action.

The Alfaguara spring (Figure 2), located at the Peridotites of Ronda in South of Spain, allows access to a deep aquifer since the groundwater circulates through the faults of the peridotites and is recovered at the surface.

Ronda Peridotites are a massif of continental lithospheric mantle emplaced into continental crust formed due to tectonic and igneous processes during the orogeny (Etiope *et al.*, 2016). This geologic structure is associated with mafic/ultramafic rocks which have low silica and high iron content (McCollom & Bach, 2009) that are called ophiolites - a section of the Earth's oceanic crust uplifted, exposed and emplaced onto continental crustal rocks (Ben-Avraham & Nur, 1982). These characteristics provide the conditions for serpentinization reactions to occur in depth when these specific rocks interacted with meteoric waters. This lead to the formation of a hyperalkaline deep groundwater, low saline, high pH, oligotrophic, CO_2 depleted groundwater with the presence of methane and hydrogen.

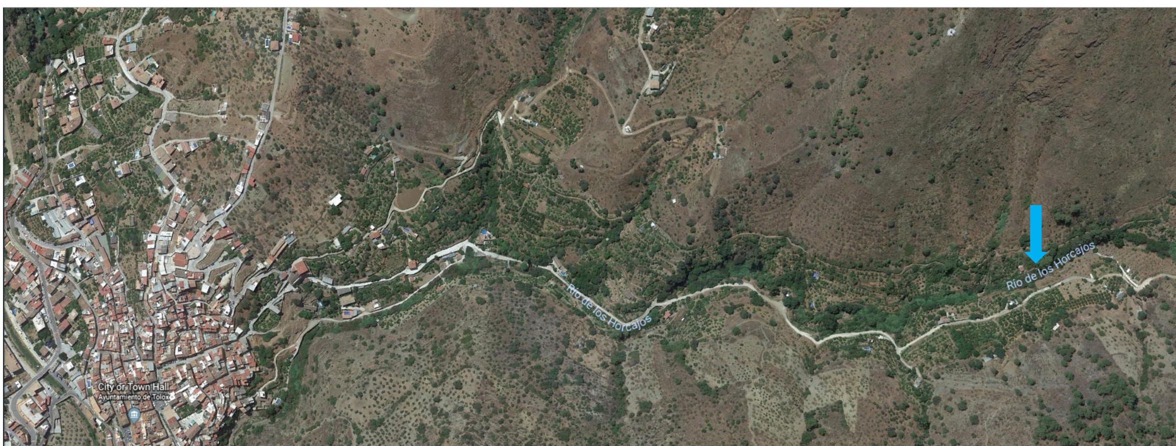


Figure 2. Alfaguara spring location in relation to the closest town Tolox. Tolox is located at approximately 60 kms West from Málaga, in the South of Spain.

1.6. Objectives

The Alfaguara deep aquifer is an extreme environment associated with serpentinization reactions. These reactions generate distinct chemical changes in the environment and culminate in the formation of deep-groundwater with high pH. Some microorganisms are capable of living and be active under these conditions and their presence in the subsurface at extreme temperatures and pH values raises the possibility that life, as we know, could have started differently. For this reason, the better the understanding of the organisms present in extreme environments, the better it will be possible to extrapolate about life on a primitive Earth and about life existence on other planets.

The main goal of this study was to determine the structural diversity of the Alfaguara deep aquifer ecosystem by culture dependent and independent methods.

Despite their importance in the astrobiology field, extremophiles have also become very useful in the biotechnology. Accordingly, the potential for biotechnological applicability of the microbial populations was also evaluated by testing the enzymatic activity of the isolates towards specific substrates.

Chapter II – Material and Methods

2.1. Samples and sampling site

The groundwater samples were recovered at the Ronda massif, South of Spain. This massif is composed of mafic/ultramafic rocks called peridotites and has several springs associated with deep aquifers, whose groundwater varies in their pH values (Etiope *et al.*, 2016). Of all the sampled springs, Alfaguara spring was the one that presented the most alkaline pH value (11.9) and, therefore, is the one that had the perspective of representing the most extreme environment. The groundwater was extracted with a peristaltic pump and filtered onsite.

Samples were collected for culture -dependent and -independent methodologies. Filters for culture independent methods were stored in sealed petri dishes and placed in "dry shipper" liquid nitrogen dewar, for further manipulation in Coimbra laboratory.

Filters used to collect samples for culture dependent methodologies were placed in a sterile plastic bag containing ~4 mL of spring water, vigorously shaken and rubbed to resuspend the biological material retained by filtration. Then, filters were removed and to the suspension it was added ~4 mL of a 30% glycerol solution. The final 15% glycerol suspension was aliquoted to several 1.5 mL cryotubes and stored in a "dry shipper" liquid nitrogen dewar for further manipulation in Coimbra laboratory.

The Alfaguara spring water chemical analysis was performed by Iñaki Vadillo and present here with permission.

2.2. Culture-dependent methods

2.2.1. Determination of the diversity of the aerobic heterotrophic bacterial populations

2.2.1.1. Isolation and cryopreservation

For isolation of cultivable aerobic heterotrophic bacterial populations, the cryopreserved samples from Alfaguara (section 2.1) were suspended in water prevenient from Cabeço de Vide, previously filtered through a 0.1 µm pore size filter and autoclaved at 121ºC for 30 minutes. This suspension was filtered with a filtration system through 47-mm-diameter filters (PALL Supor 200 sterile membrane; 0.2-µm pore size) attached to sterile shot bottle to collect the flow-through. The 0.2-µm pore size filtered suspension was filtered again through 0.1-µm pore size filters. All filters (0.2-µm and 0.1-µm pore size) were disposed on the surface of four different solid culture media in petri dishes (ABM2, BCYE, minimal media and R2A) (Tables I, II, III and IV, respectively) that were incubated at six diverse temperatures (15ºC, 22ºC, 30ºC, 37ºC, 45ºC and 50ºC) and five distinct pH (7, 8.5, 9, 10.5, 11.4). In total, 120 different conditions were used in the attempt to recover the most diverse bacterial populations. Cultures were observed every day and all colonies were isolated. Each single colony was subculture in the same medium, temperature and pH until a pure culture was obtained. After purification, each bacterial culture was cryopreserved at -80ºC in culture medium with 15% glycerol.

A medium for cryopreservation of the cultures was prepared with equal parts of the isolation medium twice concentrated adjusted to the corresponding pH and 30% glycerol solution autoclaved at 121ºC for 20 minutes for sterilization purposes.

2.2.1.2. DNA extraction of pure cultures

The DNA extraction of pure cultures was performed according to an adaptation to the (Wiedmann-Al-Ahmad et al., 1994) method. For each pure isolate, culture cell mass was suspended in 50 µL of lysis buffer (Table VIII) and subjected to temperatures of -80ºC and 70ºC alternately for fifteen minutes each. This step was performed twice to ensure the complete cell lysis. Then, 150 µL of sterile water and 300 µL of chloroform:isoamyl alcohol (24:1) were added and the suspension was centrifuged at 13 200 rpm for 15 minutes. The upper phase was collected and 500 µL of absolute ethanol was added. The suspension was centrifuged once more for 15 minutes, the supernatant was discarded, and the DNA was placed in an incubator at 50ºC until the ethanol evaporated. Once completely dry, it was re-hydrated with 30 µL sterile ultrapure water and store at -20ºC, until further use.

2.2.1.3. Random Amplified Polymorphic DNA (RAPD)

The genomic variety between the isolates was evaluated by a PCR based technique, Random Amplified Polymorphic DNA (RAPD). This method consists in the use of a single random short primer which anneals arbitrarily to several sites of the genomic DNA, amplifying it (Williams *et al.*, 1990). The main advantage is that it is possible to group isolates without previous knowledge of the DNA sequence – RAPD typing. The selected primer to these RAPD reactions was OPA3 (5’ - AGT CAG CCA C - 3’).

The amplification reactions were performed for a total volume of 30 µL and achieved on a thermal cycle with the conditions described below (Table XIII). The composition of the mixture was also described beneath (Table XII).

2.2.1.4. Visualization of the RAPD products by agarose gel electrophoresis

The visualization of the amplified products was achieved through an 2% agarose gel (subsection 2.2.4.11) electrophoresis. The agarose gel was stained with 17 μL of ethidium bromide and placed in Tris-acetate – EDTA (TAE) buffer. To the RAPD PCR products, 4 μL of loading buffer was added and the entire mixture was charged into a well of the gel. The electrophoresis occurs for 45 minutes at 50V.

2.2.1.5. Analyses of DNA profiles

Agarose gel was observed in a Gel Doc XR System (BioRad, Hercules, A, EUA), under UV light, and pictures were taken.

Clustering of the isolates was based on the similarities between the RAPD profiles observed in pictures taken, based on the number of bands, intensity and their migration when compared to the molecular weight marker (NZYDNA Ladder III, 200 to 10000 bp, NZYTech).

2.2.1.6. Amplification of the 16S rRNA gene by PCR

After the analyses of RAPD DNA profiles, one isolate of each RAPD group was selected and a PCR targeting the 16S rRNA gene was performed. The amplification was achieved using the specific universal bacterial primers 27F (5' – GAG TTT GAT CCT GGC TCA G – 3') and 1525R (5' – AGA AAG GAG GTG ATC CAG CC – 3').

Amplification reactions were performed for a total volume of 30 μL and achieved on a thermal cycle with the conditions described below (Table XV). The composition of the mixture was also described beneath (Table XIV).

2.2.1.7. Analysis of the amplification products

Analysis of the PCR products were performed by visualizing a 1% agarose gel (subsection 2.2.4.12). Molecular weight of the amplicons was determined by comparison with a molecular marker.

2.2.1.8. PCR products purification

For the purification of PCR products, the corresponding 1500 bp DNA bands were cut from the agarose gel. The following steps were performed according to the protocol of the NZYGelpure kit manufacturer.

2.2.1.9. 16S rRNA sequencing

For the sequencing of the PCR products, 5 µL of the purified DNA and 5 µL of the primer 519R (5' - CAG CMG CCG CGG TAA TWC – 3') (for a primer final concentration of 5 µM) were added to a multiwell plate. The mixture was sent to the commercial sequencing provider (GATC Biotech) and sequenced by the Sanger platform. The 16S rRNA sequences quality were evaluated with Sequence Scanner program and edited in BioEdit sequence alignment editor (Hall, 1999).

2.2.1.10. Phylogenetic analysis

To find the most closely related sequences, the 16S rRNA sequences from the isolates were submitted to a Blast-N in the GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) database.

All isolate sequences together with those of the closest relatives present in the ARB database, were aligned using SILVA (Web)Aligner (Pruesse *et al.*, 2007) and Clustal X2 program (Larkin *et al.*, 2007). The alignments were checked manually, and sequences were clustered into phylogenetic groups with a cutoff of 99.5%. Phylogenetic trees were constructed using Neighbor-Joining (Saitou & Nei, 1987) with Jukes–Cantor correction (Jukes & Cantor, 1969) and bootstrap analysis (Felsenstein, 1985) of 1000 resampling of the dataset.

2.2.2. Assessment of biotechnological potential of selected isolates

To determine the potential of the isolates to degrade cellulose, chitin, starch and/or xylan, one representative isolate of each phylogenetic group was selected. A bacterial suspension with optical density of 0.2 was prepared, and 5 µL of the suspension was disposed on the surface of petri dishes with different culture media. Minimal media and R2A were used (composition described in Tables III and IV), supplemented with each specific substrate (0.2% of carboxymethyl cellulose, 1.5% of colloidal chitin, 1% of starch or 0.5% of xylan). Minimal medium was also complemented with 0.005% of yeast extract. Four temperatures (20ºC, 26ºC, 37ºC and 45ºC) and four pH values (7, 8, 9 and 10) were tested, and cultures were incubated for 3 to 5 days for R2A or minimal media, respectively. After that, cell mass was extracted from the media surface, and for each substrate different staining solutions were used to ascertain the activity. Carboxymethyl cellulose cultures were stained with Congo Red 0.1% dye and discolored with NaCl solution. Cellulase expressing colonies will be surrounded by a yellow halo against a red background (Meddeb-Mouelhi *et al.*, 2014). Xylan (Meddeb-Mouelhi *et al.*, 2014) and starch (Mishra & Behera, 2008) cultures were stained with Lugol solution and amylolytic positive isolates and xylanase expressing colonies were identified by a colorless halo on a dark blue background . Chitin positive isolates were recognized by the presence of a clear halo around the colony (Suginta *et al.*, 2000)

To quantify the degradation activity of the isolates, both the colony and the degradation halo diameters were measured. Each condition was tested in duplicate, the mean activity was calculated, and the final values were normalized to compare the results. Normalization was

achieved by the formula 100-(CD*100/HD), where CD is the colony diameter and HD is the degradation halo diameter. Normalized values were obtained from the Table SI to SXIV of the supplementary data.

2.2.3. Culture media

2.2.3.1. Solid Alkaline Buffer Medium 2 (ABM2)

Table I. Solid Alkaline Buffer Medium 2 (ABM2) composition**.**

Composition	Quantity per L
Yeast extract	5 g
Tryptone	5 g
α – Ketoglutarate acid	1 g
Agar	15 g
Macronutrients solution 10x concentrated*	100 mL
Micronutrients solution 100x concentrated**	10 mL
Inorganic buffers (1M solution) ***	100 mL
Demineralized water	Up to 1000 mL

* Macronutrients solution composition is discriminated in the table V.

** Micronutrients solution composition is discriminated in the table VI.

*** According to the desirable pH value. Discriminated in the table VII.

Yeast extract, tryptone and α – ketoglutarate were weighted and dissolved in a solution with 100 mL of macronutrients, 10 mL of micronutrients and 100 mL of inorganic buffer for

pH values of 7 and 8.5. Demineralized water was added to a final volume of 1000 mL. The pH was adjusted to the desired values with hydrochloric acid solution or potassium hydroxide pellets. The solutions were transferred into shot-bottles with agar previously weighted and the medium was autoclaved at 121ºC for 20 minutes. For pH 9, 10.5 and 11.4 buffer solutions were autoclaved separately and only added to the medium after cooling to 50ºC. The medium was homogenized and distributed into petri dishes.

2.2.3.2. Buffered Charcoal Yeast Extract (BCYE) agar medium (Edelstein, 1981).

Table II. Buffered charcoal yeast extract agar medium (BCYE) composition.

Composition	Quantity per L
Yeast extract	10 g
Activated charcoal	2 g
α – Ketoglutarate	1 g
Agar	15 g
ACES	10 g
Ferric pyrophosphate*	10 mL
L-cysteine**	10 mL
Inorganic buffers	100 mL
Demineralized water	Up to 1000 mL

* Ferric pyrophosphate composition is discriminated in subsection 2.2.2.5.

** L-cysteine solution composition is discriminated in subsection 2.2.2.6.

Yeast extract, activated charcoal, α – ketoglutarate and ACES were weighted and dissolved in 100 mL of inorganic buffer for pH values of 7 and 8.5. Demineralized water was added to a final volume of 1000 mL. The pH's were adjusted to the desired values with hydrochloric acid solution or potassium hydroxide tablets. The solutions were transferred into shot-bottles with agar previously weighted and the medium was autoclaved at 121ºC for 20 minutes. For pH 9, 10.5 and 11.4 buffer solutions were autoclaved separately and only added to the medium after cooling to 50ºC. The medium was supplemented with 10 mL of ferric pyrophosphate, 10 mL of L-cysteine, homogenized and distributed into petri dishes.

2.2.3.3. Solid Minimal medium

Table III. Solid minimal medium composition.

Composition	Quantity per L
Macronutrients solution 10x concentrated*	100 mL
Micronutrients solution 100x concentrated**	10 mL
Agar	15 g
Inorganic buffers	100 mL
Demineralized water	Up to 1000 mL

* Macronutrients solution composition is discriminated in the table V.

** Micronutrients solution composition is discriminated in the table VI.

A solution with 100 mL of macronutrients, 10 mL of micronutrients and 100 mL of inorganic buffer for medium with pH values of 7 and 8.5 was prepared. Demineralized water was added to the previously mentioned solution plus 10% of R2A medium for a final volume of 1000 mL. The pH's were adjusted to the desired values with hydrochloric acid solution or potassium hydroxide tablets. The solutions were transferred into shot-bottles with agar

previously weighted and the medium was autoclaved at 121ºC for 20 minutes. For pH 9, 10.5 and 11.4 buffer solutions were autoclaved separately and only added to the medium after cooling to 50ºC. The medium was homogenized and distributed into petri dishes.

2.2.3.4. Solid Reasoner's 2A agar medium (R2A agar medium)

Table IV. Solid Reasoner's 2A agar medium (R2A) composition.

Composition	**Quantity per L**
Yeast extract	0.5 g
Proteose peptone No. 3	0.5 g
Casein hydrolysate	0.5 g
Glucose	0.5 g
Starch	0.5 g
Dipotassium phosphate	0.3 g
Magnesium sulfate	0.024 g
Sodium pyruvate	0.3 g
Agar	15 g
Inorganic buffers*	100 mL
Demineralized water	Up to 1000 mL

* According to the desirable pH value. Discriminated in the table VII.

Solid R2A medium was weighted into shot-bottles and hydrated with 100 mL of inorganic buffer for pH values of 7 and 8.5. Demineralized water was added to a final volume of 1000 mL. The pH was adjusted to the desired values with hydrochloric acid solution or potassium hydroxide pellets. The medium was autoclaved at 121ºC for 20 minutes. For pH 9, 10.5 and 11.4 buffer solutions were autoclaved separately and only added to the medium after cooling to 50ºC. The medium was homogenized and distributed into petri dishes.

2.2.4. Solutions and Reagents

2.2.4.1. Macronutrients solution

Table V. Macronutrients composition 10x concentrated.

Composition	Quantity per L
Nitriloacetic acid	1 g
Calcium sulfate	0.6 g
Magnesium sulfate heptahydrate	1 g
Sodium chloride	0.8 g
Potassium nitrate	1.03 g
N_2NO_3	6.89 g
N_2HPO	1.11 g
Demineralized water	Up to 1000 mL

All the previous compounds were dissolved in demineralized water for a final volume of 1000 mL.

2.2.4.2. Micronutrients solution

Table VI. Micronutrients solution composition 100x concentrated.

Composition	Quantity per L
Manganese (II) sulfate monohydrate	0.22 g
Zinc sulfate heptahydrate	0.05 g
Boric acid	0.05 g
Cobalt (II) chloride hexahydrate	0.0046 g
Copper (II) sulfate pentahydrate	0.0025 g
Sodium molybdate dihydrate	0.0025 g
Demineralized water	Up to 1000 mL

All the previous compounds were dissolved in demineralized water for a final volume of 1000 mL.

2.2.4.3. Inorganic buffers

Table VII. Inorganic buffers used on ABM2, R2A, BCYE and minimal media.

Composition	pH medium	Quantity per L
Tris-HCl	7 and 8.5	121.14 g
Carbonate-Bicarbonate	9.5 and 10.5	286.14 g - 84.01 g
Single carbonate KOH	11.4	286.14 g

All the previous compounds were dissolved in demineralized water for a final volume of 1000 mL.

2.2.4.4. Lysis buffer

Table VIII. Lysis buffer composition.

Composition	Quantity per L
Tween 20 2%*	500 mL
10x $NH4^{+}$ Buffer	100 mL
Demineralized water	Up to 1000 mL

* Previously sterilized by filtration (0.2 µm filter).

Lysis buffer solution was prepared with 500 mL of Tween 20 2%, 100 mL of 10x NH4+ buffer and ultrapure water, previously sterilized by filtration (0.2 µm filter) and autoclaving (121ºC, 20 minutes), to a final volume of 1000 mL. The final solution was sterilized by filtration (0.2 µm filter) and stored at 4ºC for further use.

2.2.4.5. Ferric pyrophosphate solution

A 2.5% solution of ferric pyrophosphate was prepared, by dissolving 25 g of ferric pyrophosphate in 1000 mL of demineralized water. The final solution was sterilized by filtration (0.2 µm filter) and stored at -20ºC.

2.2.4.6. L-cysteine solution

A 4% solution of L-cysteine solution was prepared, by dissolving 40 g of cysteine in 1000 mL of demineralized water. The final solution was sterilized by filtration (0.2 µm filter) and stored at -20ºC.

2.2.4.7. Tris-Acetate-EDTA (TAE) 50x solution

Table IX. Tris-Acetate-EDTA (TAE) 50x solution composition.

Composition	Quantity per L
Tris/base	242 g
Glacial acetic acid	57.1 mL
EDTA 0.5M	100 mL
Demineralized water	Up to 1000 mL

Tris was dissolved in 100 mL of EDTA and part of ultrapure water previously sterilized by autoclaving (121ºC, 20 minutes). After dilution, 57.1 mL of glacial acetic acid was added and ultrapure water was joined to a final volume of 1000 mL.

2.2.4.8. Ethidium bromide solution

A 0.5% aqueous solution of ethidium bromide was prepared, by dissolving 50 g of ethidium bromide powder in 1000 mL of distilled water. The solution was stored at 4ºC in a dark glass bottle covered with aluminum foil.

2.2.4.9. GES solution

Table X. GES solution composition.

Composition	Quantity per L
EDTA 0.5 M	200 mL
Guanidine thiocyanate	600 g
N-laurylsarcosine	10 g
Demineralized water	Up to 1000 mL

Guanidine thiocyanate was weighted and dissolved in 200 mL of EDTA and part of ultrapure water by heating to 65ºC. N-laurylsarcosine was added when the solution cooled. Ultrapure water was joined to a final volume of 1000 mL and the final solution was sterilized by filtration.

2.2.4.10. Loading buffer solution

Table XI. Loading buffer solution composition.

Composition	Quantity per L
Bromophenol blue	2.5 g
Sucrose	400 g
Ultrapure Milli-Q water	Up to 1000 mL

Bromophenol blue and sucrose were weighted and dissolved in ultrapure water to a final volume of 1000 mL. The final solution was sterilized by filtration (0.2 µm filter) and stored at 4ºC for further use.

2.2.4.11. 2% Agarose gel

For a final volume of 170 mL, 3.4 g of agarose was diluted in TAE 1x solution by heating. After cool, 17 µL of 0.5% solution of ethidium bromide was added. TAE 1x solution was prepared with 40 mL of TAE 50x solution (Table IX) diluted in 2000 mL of distilled water.

2.2.4.12. 1% Agarose gel

For a final volume of 150 mL, 1.5 g of agarose was diluted in TAE 1x solution by heating. After cool, 15 µL of 0.5% solution of ethidium bromide was added. TAE 1x solution

was prepared with 40 mL of TAE 50x solution (Table IX) diluted in 2000 mL of distilled water.

2.2.4.13. Colloidal chitin

Colloidal chitin was prepared with the slow addition of 5 g of chitin coming from crab shells to 60 mL of concentrated HCl. The mixture was kept at room temperature, stirred for 1 hour and then was filtered through glass wool. The filtrate was added to 200 mL of 50% ethanol and stirred again during the process. The precipitate was transferred to a glass funnel with a paper filter and washed with sterile distilled water (pH8) until the colloidal chitin became neutral (pH7). The colloidal chitin retained on the filter paper was removed with a spatula, weighted and stored in the dark at 4ºC.

2.2.4.14. Lugol solution

Lugol solution was prepared with 6.67 g of potassium iodide and 3.33 g of iodine dissolved in 1000 mL of distilled water. The solution was stored at room temperature in a dark bottle.

2.2.4.15. Congo red dye

Congo red dye was prepared with 1 g of congo red dissolved in 1000 mL of distilled water. The solution was prepared and stored at room temperature in a dark bottle.

2.2.5. PCR composition and conditions

Table XII. Random Amplified Polymorphic DNA (RAPD) composition.

Reagents	Volume (µL)	Final concentration
Ultrapure water	17.7	-
NH_4 reaction buffer (Bioline©)	70	10x
$MgCl_2$ solution (Bioline©)	30	50 mM
dNTPs (NZYTech®)*	6	10 mM
Primer OPA3 1:10 (NZYTech®)**	1	10 µM
Taq DNA polymerase (Bioline©)	0.3	5 $U.\mu L^{-1}$
DNA	2	-

* Deoxynucleotide (dNTP) work solution was prepared with 10 µL of each dNTP stock solution (100 mM) diluted in 90 µL of ultrapure water for a final concentration of 10 mM.

** OPA3 stock (100 µM) was hydrated in ultrapure water (sterilized by filtration and autoclaving at 121ºC for 20 minutes) to obtain the final concentration of 10 µM. Work solutions were reached with 10 µL of stock solution diluted in 90 µL of ultrapure water.

Table XIII. RAPD -PCR conditions.

Stage	Temperature (ºC)	Time (min)	Nº of cycles
Initial denaturation	94	5	1
Denaturation	94	1	
Annealing	40	1	35
Extension	72	2	
Final extension	72	10	1
Hold	4	∞	1

Table XIV. PCR composition.

Reagents	Volume (µL)	Final concentration
Ultrapure water	15.8	-
Buffer 10x (NZYTech®)	3	10x
$MgCl_2$ solution (NZYTech®)	2.4	50 mM
dNTPs (NZYTech®)*	6	10 mM
Primer 27F 1:1 (SIGMA-ALDRICH©)**	0.25	50 µL
Primer 1525R 1:1 (SIGMA-ALDRICH©)**	0.25	50 µL
Taq DNA polymerase (NZYTech®)	0.3	5 U.µL^{-1}
DNA	2	-

* Deoxynucleotide (dNTP) work solution was prepared with 10 µL of each dNTP stock solution (100 mM) diluted in 90 µL of ultrapure water for a final concentration of 10 mM.

** 27F and 1525R stock (100 µM) was hydrated in ultrapure water (sterilized by filtration and autoclaving at 121ºC for 20 minutes) to achieve the final concentration of 50 µM. Work solutions were reached with 10 µL of stock solution diluted in 10 µL of ultrapure water.

Table XV. PCR conditions.

Stage	Temperature (ºC)	Time (min)	Nº of cycles
Initial denaturation	94	4	1
Denaturation	94	1	
Annealing	55	1	30
Extension	72	1	
Final extension	72	10	1
Hold	4	∞	1

2.3. Culture-independent methods

2.3.1. DNA extraction

Total DNA was extracted from the filters with PowerSoil DNA isolation Kit (MoBio Laboratories) according to manufacturer protocol. DNA quantification and quality verification were determined by UV spectrophotometry at 260 nm (NanoDrop spectrophotometer). The resulting DNA was preserved at-20°C until further use.

2.3.2. Illumina sequencing

Genomic DNA was submitted to Josephine Bay Paul Center (Marine Biological Laboratory at Woods Hole) for Illumina sequencing. The structural diversity of bacterial and archaeal communities was assessed by sequencing the hypervariable regions V4-V5 of the

16S rRNA gene by Illumina MiSeq platform. Primers 518F (5' - CCAGCAGCYGCGGTAAN – 3') and 926R (5' - CCGTCAATTCNTTTRAGT – 3') (5' – CCGTCAATTTCTTTGAGT – 3') (5' - CCGTCTATTCCTTTGANT – 3') were used for the domain *Bacteria* and primers 517F (5' – GCCTAAAGCATCCGTAGC – 3') (5' - GCCTAAARCGTYCGTAGC – 3') (5' – GTCTAAAGGGTCYGTAGC – 3') (5' -GCTTAAAGNGTYCGTAGC – 3') (5' - GTCTAAARCGYYCGTAGC – 3') and 958R (5' – CCGGCGTTGANTCCAATT – 3') for the domain *Archaea*.

2.3.2.1. Analyses of Illumina 16S rRNA gene sequencing results

Raw data were analyzed with mothur v.1.40.0 software package (http://www.mothur.org/; Schloss *et al*., 2009). Initially, sequences reads were aligned and combined into contigs using the *make.contigs* command. All the sequences that did not assemble properly and have ambiguous bases were removed from the dataset using the *screen.seqs* command. Duplicates were merged through *unique.seqs* command and a table with the names of the sequences and groups was generated with *count.seqs* command. The resultant sequences were aligned by *align.seqs* command. Sequences that start and end in deviant mode positions were removed from the dataset using the *screen.seqs* command. Non-informative characters for the calculation of phylogenetic distances (".", "-") were also removed by *filter.seqs* command. Duplicates were merged once again with the *unique.seqs* command. Then, with *pre.cluster* command, the sequences were pre-clustered allowing for up to 2 differences per 100 b.p. between sequences. Chimaeras were also found and removed with *chimera.vsearch* and *remove.seqs* command, respectively. Sequences were classified with *classify.seqs* command, and sequences classified as representing mithocondrial and eukaryotic sequences were removed using the *remove.lineage* command. The resultant sequences were clustered in OUT's with a cutoff of 0.03 with *dist.seqs* and *cluster* commands. Singletons were removed by *remove.rare* command. Finally, sequences were phylogenetically classified using ARB-SILVA taxonomic database through the *classify.otu* command. Microbial diversity was also calculated with two different diversity indices (,

Chao, Shannon evenness) using the *summary.single* command. In addition, species richness was assessed by rarefaction analysis with *rarefaction.single* command.

2.3.2.2. Principal Component Analysis (PCA)

To understand which sites are most closely related to the diversity of the Alfaguara deep aquifer, a PCA was performed in CANOCO software package (Leps & Smilauer, 2003). For domain *Bacteria*, the treated results were compared with sequences related to serpentinization-associated sites and sequences belonging to sites where *Hydrogenophaga/Serpentinomonas* is the most abundant genus. In total, 41 samples were collected and serial PCA analyses were performed. Samples most dissimilar from Alfaguara site were removed sequentially. The last pruned version PCA was achieved with 15 samples, considered the most similar to the Alfaguara site.

For *Archaea* domain, the treated results were compared with serpentinization-associated sites and sequences belonging to the deep biosphere. In total, 13 samples were collected and multiple PCA analyses were also performed. Samples most dissimilar from Alfaguara site were removed sequentially. The last pruned version PCA was achieved with 7 samples, considered the most identical to the Alfaguara archaeal community.

All data was accessed and obtained from the Visualization and Analysis of Microbial Population Structures (VAMPS) website.

Chapter III: Results

3.1. Chemical characterization of the groundwater

Table XVI. Chemical analysis of six different sites associated with serpentinization.

Chemical parameters*	Cedars	AC3**	CROMO	TAB	VM	Alfaguara
EC (µS.cm^{-1})	444-3000	558	1560-5200	-	-	1153
IC (mg.L^{-1})	-	-	-	-	0.17-1.72	1.15
pH	8.36-11.9	11.4	7.9-12.2	7.82-12.63	8-12.3	11.9
T (ºC)	16-18	20.5	15.2-18.5	-	12.1-22.5	17.8
O_2 (mg.L^{-1})	<	<	0.03-1.05	-	-	3
TN (mg.L^{-1})	-	-	-	-	-	0.12
TOC (mg.L^{-1})	-	-	-	-	-	6.39

* EC, Electric Conductivity; IC, Inorganic Carbon; T, Temperature; TN, Total Nitrogen; TOC, Total Organic Carbon.

** All data was collected from (Tiago *et al.,* 2004) except the electric conductivity (Marques *et al.*, 2018).

The values reported for the Alfaguara spring are the average values over several sampling periods.

AC3, Cabeço de Vide; CROMO, The Coast Range Ophiolite; TAB, The Tablelands Ophiolite; VM, The Voltri Massif.

The "-" indicates data not found and "<" indicates that the parameter was below the detection limit.

The values variations were due to the different sampled spring (The Cedars, Tablelands and Voltri Massif) or different wells in (Cabeço de Vide and CROMO).

The chemical properties of the groundwater were analyzed and compared with other serpentinization-associated sites (Table XVI). The Alfaguara spring samples presented an EC, IC values and temperature consistent with groundwater allied with mafic/ultramafic rocks. Moreover, both the pH value obtained (11.9) and the presence of carbon are characteristic of this type of environments (Schrenk *et al.*, 2013). In addition, the oxygen levels were also measured and detected in values higher than those normally found in sites were serpentinization reactions occur. Data was collected from previous publications related to the Cedars (Suzuki *et al.*, 2013), Cabeço de Vide (Tiago *et al.*, 2004; Marques *et al.*, 2018), Coast Range Ophiolite (Twing *et al.*, 2017), Tableland (Brazelton *et al.*, 2013) and Voltri Massif (Brazelton *et al.*, 2017).

3.2. Culture-dependent methods

3.2.1. Bacterial recovery

The isolation procedure and the different culture conditions used allowed to obtain 995 isolates (Table XVII). As expected, most of the isolates were recovered from 0.2 µm pore size filter and in total 301, 294, 223 and 139 isolates were isolated from ABM2, R2A minimal and BCYE media, respectively. The ABM2 and R2A media showed the highest levels of recovery, and five of the six temperatures used favor the growth of the isolates at practically all pH values. However, the highest bacterial recovery, 39 isolates, was achieved in minimal medium at 37ºC and pH 9. Under the same conditions, by using R2A medium 19 isolates were covered, while in the ABM2 medium this only occur with 13 isolates. No isolate was recovered in any media at 50ºC.

Despite most of the isolates were recovered from 0.2 µm pore size filters, some were isolated by using 0.1 µm pore size filters. In this case, no substantial differences between results obtained were observed since all media, present a similar number of isolates. Moreover, although several temperatures and pH values were tested, only 18 conditions

allowed the isolation of the bacteria (Table XVIII). Minimal medium was the only to recover isolates at 15ºC and pH 11.4.

Table XVII. Total isolates recovered in the different culture conditions.

Filter pore size (µm)	Medium	Temperature (ºC)	pH	Total Isolates
0.2	ABM2	15	7	12
			8.5	7
			9	11
			10.5	11
			11.4	21
		22	7	24
			8.5	18
			9	13
			10.5	13
			11.4	3
		30	7	25
			8.5	31
			9	18
			10.5	11
			11.4	10
		37	7	18
			8.5	18
			9	13
			10.5	3
		45	7	7
			8.5	5
			9	8
			11.4	1

Table XVII (cont.). Total isolates recovered in the different culture conditions.

Filter pore size (µm)	Medium	Temperature (ºC)	pH	Total Isolates
0.2	BCYE	15	7	6
			8.5	7
			9	7
			10.5	10
			11.4	5
		22	7	4
			8.5	10
			9	4
			10.5	4
			11.4	8
		30	7	3
			8.5	24
			9	7
			10.5	14
			11.4	2
		37	7	4
			8.5	9
			9	5
			11.4	4
		45	7	1
			8.5	1

Table XVII (cont.). Total isolates recovered in the different culture conditions.

Filter pore size (µm)	Medium	Temperature (ºC)	pH	Total Isolates
0.2	Minimal	15	7	3
			8.5	6
			9	14
			10.5	4
			11.4	4
		22	7	24
			8.5	20
			9	15
			10.5	6
			11.4	2
		30	7	13
			8.5	27
			9	14
			10.5	3
			11.4	12
		37	7	5
			8.5	6
			9	39
			10.5	1
		45	8	5

Table XVII (cont.). Total isolates recovered in the different culture conditions.

Filter pore size (µm)	Medium	Temperature (ºC)	pH	Total Isolates
0.2	R2A	15	7	13
			8.5	16
			9	19
			10.5	15
			11.4	10
		22	7	12
			8.5	19
			9	3
			10.5	22
			11.4	8
		30	7	26
			8.5	30
			9	23
			10.5	7
			11.4	6
		37	7	1
			8.5	11
			9	19
			10.5	2
			11.4	2
		45	7	1
			8.5	7
			9	12
			10.5	9
			11.4	1

Table XVIII. Total isolates recovered in the different culture conditions.

Filter pore size (µm)	Medium	Temperature (ºC)	pH	Total Isolates
0.1	ABM2	15	8.5	2
		30	7	2
			9	1
			10.5	2
			11.4	1
		37	7	2
	BCYE	22	9	1
			10.5	2
		30	10.5	2
			11.4	1
		37	7	2
	Minimal	15	11.4	3
		22	9	1
	R2A	15	8.5	1
		30	7	1
		37	7	2
			8.5	1
		45	7	1

3.2.2. Grouping the isolates

To decrease the number of isolates to work with, they were first grouped by RAPD analysis (Tiago *et al.*, 2004). This molecular methodology allows to recognize the genetic variations present in the genomic DNA and, consequently, to find similar strains that may have been isolated numerous times. With this technique, 338 RAPD profiles where obtained and 188 different groups were identified (i.e. Figure 3). Furthermore, the representative

strains of each group were identified and classified phylogenetically by sequencing the 16S rRNA subunit encoding gene.

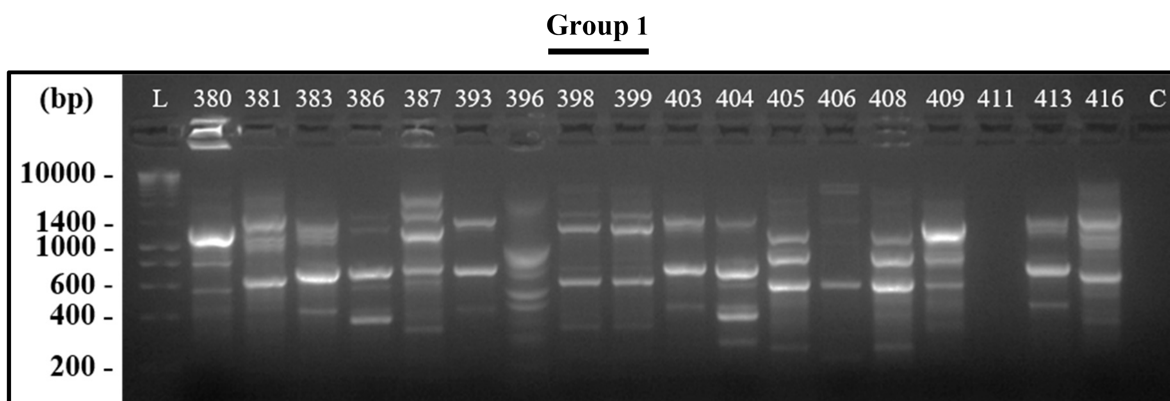


Figure 3. Example of RAPD profiles obtained for the isolates recovered from the Alfaguara deep aquifer. Isolates with the band patterns corresponding to the same base pairs were grouped (group 1). L represents the NZYDNA Ladder III; C represents the negative control and numbers match to isolates identification.

3.2.3. Phylogenetic analysis

To determine the structural diversity of the microbial community, the sequences of the 16S rRNA gene of each representative strain were compared with the closest relatives retrieved from SILVA database. This comparison allowed to obtain a phylogenetic tree to infer the isolates phylogenetic placement and affiliation. Based on this analysis 26 phylogenetic groups were formed, most of which belonging to the phylum *Firmicutes* (Figure 4). Of the 38 isolates represented in the phylogenetic tree, 34 are closely related to this phylum, mainly within genus *Bacillus* which represent 91.7% of the total of cultivable populations (Figure 4). Five other isolates, also belonging to this taxonomic division, are associated with *Jeotgalibacillus campisalis*, *Paenisporosarcina indica*, *Paenisporosarcina quisquiliarum, Staphylococcus epidermis* and *Staphylococcus aureus* species. These isolates represent approximately 3.85% of the cultivable populations (Figure 4).

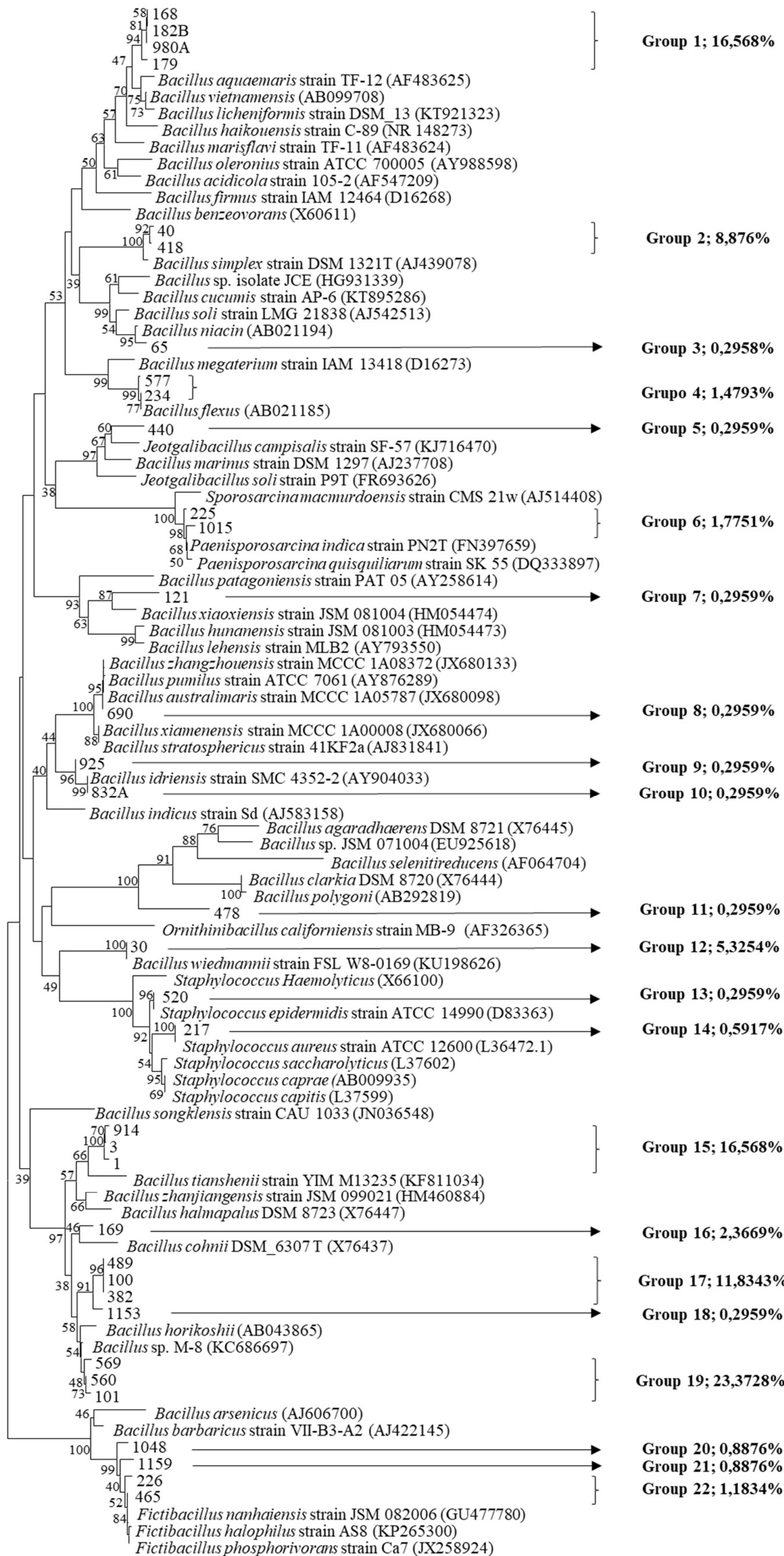


Figure 4. Phylogenetic tree based on the comparison of the 16S rRNA gene sequences of the isolates with the closest related organisms. The tree is based on the neighbor-joining algorithm and indicate the phylogenetic position of the isolates belonging to phylum Firmicutes. The scale bar represents 2% nucleotide sequence dissimilarity. The percentage represents the abundance of the cultivable populations. Table SXV in supplementary data show the correspondence between the isolates and their abundance.

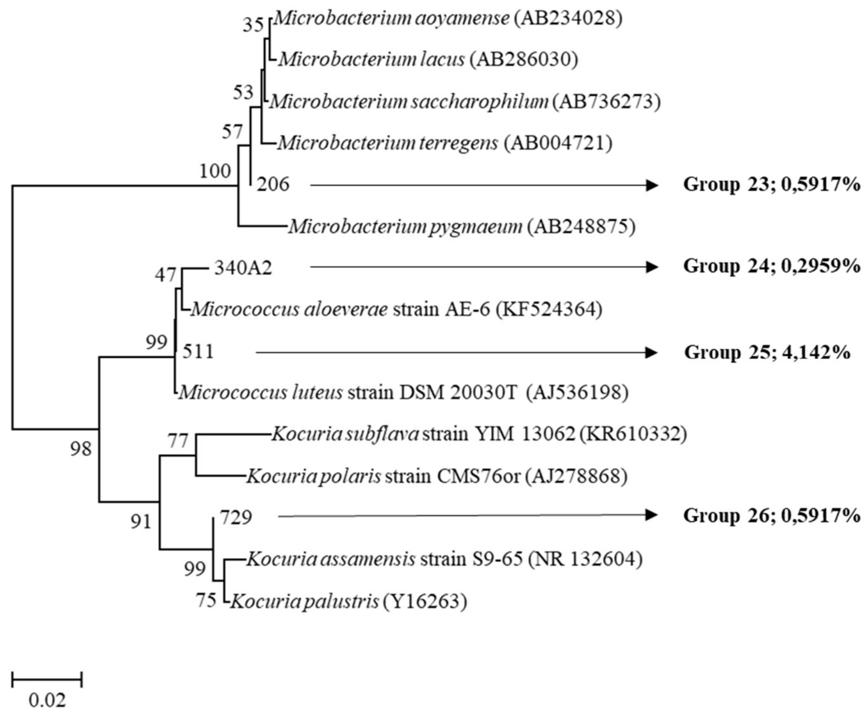


Figure 4 (cont.). Phylogenetic tree based on the comparison of the 16S rRNA gene sequences of the isolates with the related organisms. The tree is based on the neighbor-joining algorithm and indicate the phylogenetic position of the isolates belonging to phylum *Actinobacteria*. The isolates are represented numerically, and each phylogenetic group formed is represented with a different symbol. The scale bar represents 2% nucleotide sequence dissimilarity. The percentage represents the abundance of the cultivable populations. Table SXV in supplementary data show the correspondence between the isolates and their abundance.

Also present in the community are isolates belonging to phylum *Actinobacteria* (Figure 4 – Cont.). These are deeply related to different members of the genus *Microbacterium* and represent approximately 0.6% of the total cultivable bacteria. Isolates associated with *Micrococcus aloeverae*, *Micrococcus luteus* and *Kocuria assamensis* species are also present comprising almost 5% of the cultivable bacteria.

3.2.4. Assessment of biotechnological potential

To evaluate the biotechnological potential of the isolates obtained, one representative isolate of each phylogenetic group mentioned on 3.2.3. section was chosen. For each isolate, the capability for degradation of four different substrates was tested and results are presented below (Figures 1, 2 and 3).

The higher percentage of carboxymethyl cellulose degradation activity was obtained in minimal medium (Figure 5). All the tested conditions in this medium, except one (37ºC at pH 10), presented a greater number of isolates with activity than in R2A medium. Moreover, at 26ºC (pH 7 and 8) and 37ºC (pH 7, 8 and 9) all isolates showed the ability to degrade cellulose. Nevertheless, despite the obviously differences in the degradation activity, isolate 690 shown positive cellulolytic activity in both media under various conditions.

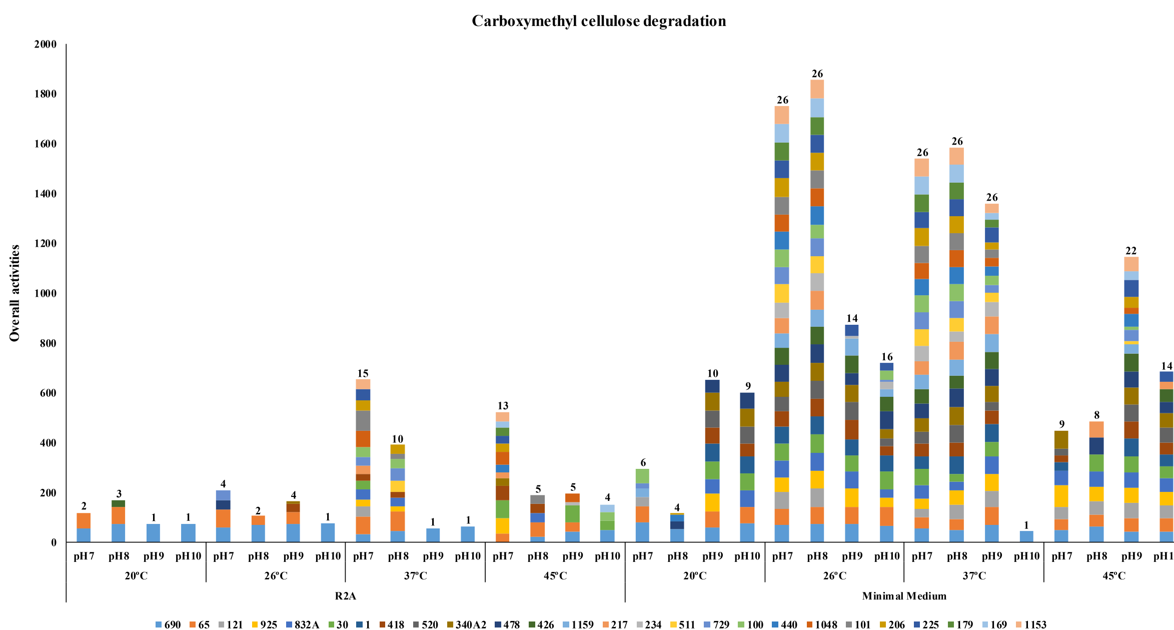


Figure 5. Comparison between the various activities of carboxymethyl cellulose degradation by the different isolates. The number at the top of the bars indicate the number of isolates with activity in the referent condition. Bars height is the sum of the activity value percentages determined for each of the isolates present in the figure.

Unlike cellulose, starch degradation activity was higher in R2A medium than in minimal medium. As shown, 11 out of the 16 conditions tested in R2A medium demonstrated a greater number of isolates capable of degrading starch when compared to minimal medium (Figure 6). Nevertheless, the most favorable conditions to the degradation of this substrate were attained in minimal medium at 26ºC in pH 9 and 10 (22 and 23 isolates, respectively) and in R2A medium at 37ºC in pH 9 (22 isolates). Due to constrains during the tests period, no results are shown for this substrate in minimal medium at 37ºC and pH 8.

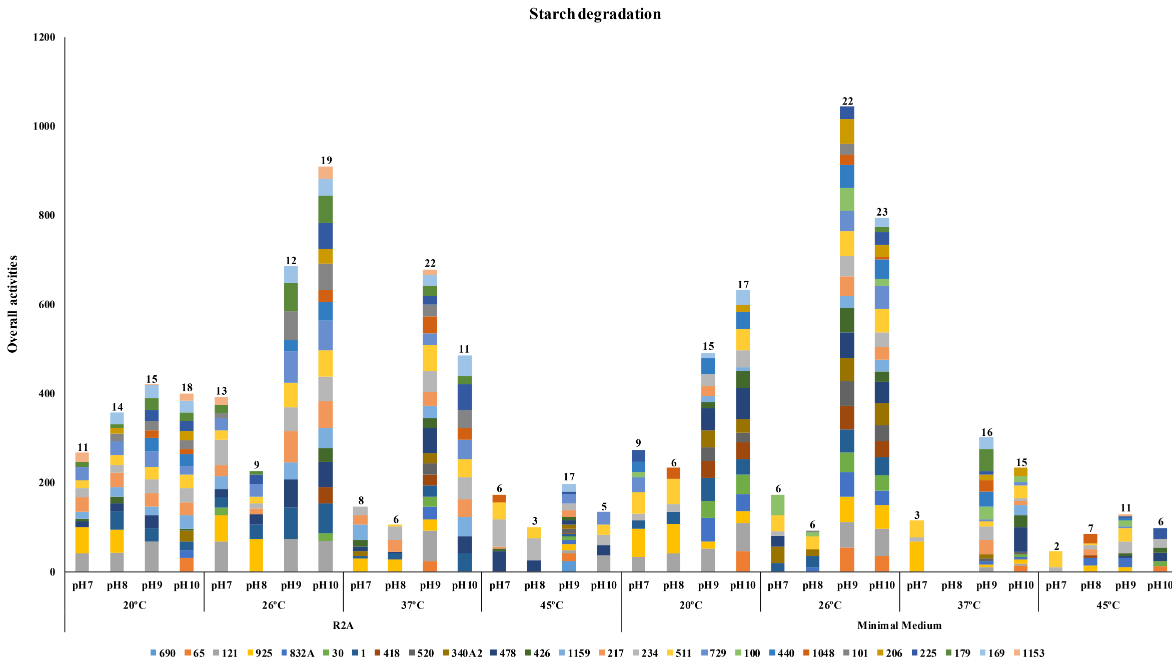


Figure 6. Comparison between the various activities of starch degradation by the different isolates. The number at the top of the bars indicate the number of isolates with activity in the referent condition. Bars height is the sum of the activity value percentages determined for each of the isolates present in the figure.

Of the three substrates referred, xylan is the one that the majority of the isolates shown the potential to degraded (Figure 7). The highest percentage of degradation activity was obtained in minimal medium, however, for both media a minimal number of 7 isolates with positive degradation capability was achieved for all tested conditions. Regardless, minimal medium shown the highest number of isolates with degradation activity at 20ºC and 26ºC at pH 10 (24 and 25 isolates, respectively) and at 37ºC at pH 9 (24 isolates).

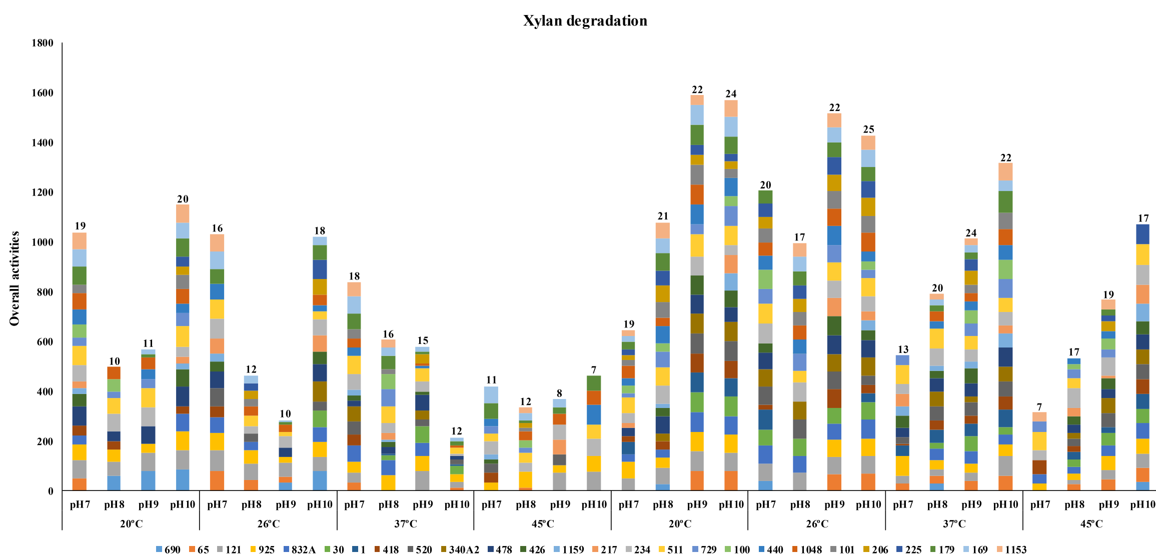


Figure 7. Comparison between the various activities of xylan degradation by the different isolates. The number at the top of the bars indicate the number of isolates with activity in the referent condition. Bars height is the sum of the activity value percentages determined for each of the isolates present in the figure.

Some isolates showed no degradation activity for one or more substrates. However, this lack of activity is only due to the inability to degrade the substrates since almost all isolates have grown in every condition (Tables SI to SVIII in the supplementary data).

The tests results related to the degradation of chitin did not present positive results and therefore were not presented here.

3.3. Culture-independent methods

3.3.1. Diversity indices

Table XIX. Diversity indices referring to the bacterial community present in Alfaguara deep aquifer.

Site	Coverage	Number of determined OTUs	Chao	Shannon Evenness
Alfaguara	0.99808	1890	1903,305882	0,619688

Bacterial diversity was evaluated with two different diversity indices and the resulting values are present in Table XIX. The coverage obtained indicates the percentage of total genera present in the sample, which is very close to 100%. The total number of determined operational taxonomic units (OTUs), richness, was identical to the value calculated by Chao index, which corroborates the coverage value. Moreover, Chao index indicates that Alfaguara community is composed by 1903 different OTUs classified at the genus level. Evenness in the sample represent the uniformity of distribution of OTUs abundance in a community. This parameter was calculated by Shannon evenness index and varies between 0, if the community is dominated by a single genus and 1, if all the genus were equally abundant (Faith & Du, 2017). Based on the obtained value, it was possible to determine that Alfaguara bacterial community has a small number of genera that are more abundant in the community. Moreover, the rarefaction curve was also constructed (Figures 8) and analyzed. The number of sequences found was related to the OTUs present in the sample. As demonstrated, the curve shows a high tendency for plateau, indicating that the richness detected is most likely representative of the bacterial diversity existent in the sample.

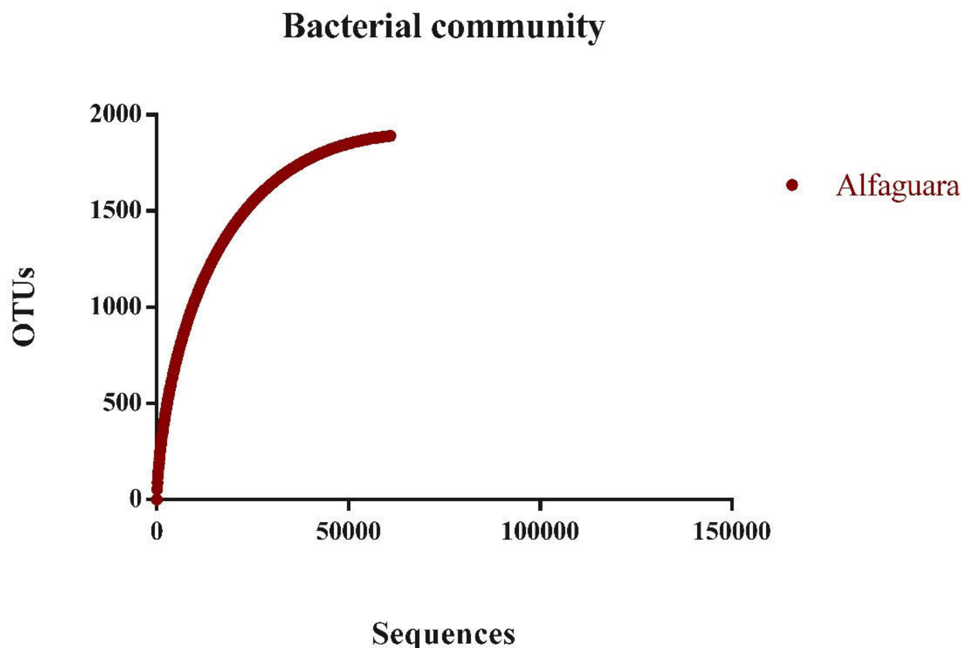


Figure 8. Rarefaction curve representative of Alfaguara bacterial richness.

Table XX. Diversity indices referring to the archaeal community present in Alfaguara deep aquifer.

Site	Coverage	Number of determined OTUs	Chao	Shannon Evenness
Alfaguara	0.999954	478	478,085714	0,156512

Archaeal diversity was also calculated through the same two diversity indices used for bacteria (Table XX). The coverage value obtained is very close to 100% as well. The total number of determined operational taxonomic units (OTUs), correspond to the value calculated by Chao index, which also corroborates the coverage. According to the Chao index, Alfaguara community presents a wide diversity of archaea, counting on 478 different genera approximately. Evenness was measured with Shannon evenness index, which demonstrate that the sample is dominated by a small number of genera that are more abundant

in the community. Additionally, the rarefaction curve was also constructed for archaeal communities (Figure 9). As shown, the richness detected is demonstrative of the archaeal diversity present in each sample since the saturation point was also reached for this group of microorganisms.

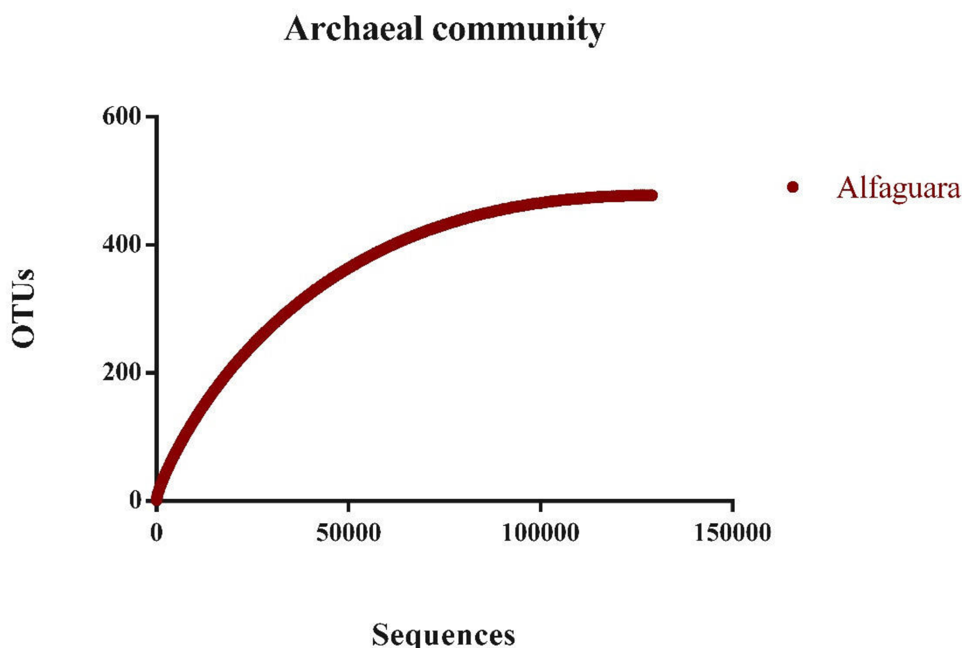


Figure 9. Rarefaction curve representative of Alfaguara archaeal richness.

3.3.2. Phylogenetic analyses of Illumina sequences

The microbial community present in the Alfaguara deep aquifer was also determined by total DNA sequencing and the predominant bacterial populations were characterized phylogenetically (Figure 10). The groundwater is mainly composed by members of the order *Betaproteobacteriales* (~53%), namely the genus *Hydrogenophaga/Serpentinomonas* (~47%) (family *Burkholderiaceae*). This group of hydrogen-oxidizing bacteria was isolated

for the first time in The Cedars, California (Suzuki *et al.*, 2013) and is found in several serpentinization systems. Further members of the family *Burkholderiaceae* also appear relatively abundant in the Alfaguara community however, they are not yet classified. *Clostridiales*, belonging to the class *Clostridia* (a strict anaerobic group), is the second most abundant order representing approximately 12% of the community. Members of this class were also detected in Cabeço de Vide, Portugal (Tiago *et al.*, 2004) in The Voltri Massif, Italy (Brazelton *et al.*, 2017)and in The Coast Range Ophiolite, California (Twing *et al.*, 2017) *Bacteroidales* (~10%), also present in Cabeço de Vide (Tiago & Veríssimo, 2013), is deeply related with the Alfaguara deep aquifer groundwater, being the third most abundant order in the community. Genus *Bacteroides* (family *Bacteroidaceae*) is dominant in this order however, other members of this group, belonging to genus *Parabacteroides* (family *Tannerellaceae*), are rather abundant. Order *Bifidobacteriales* (~5%), class *Actinobacteria*, is dominated by genus *Bifidobacterium* (family *Bifidobacteriaceae*) which also constitute an important part of the community. Equally significant are orders *Lactobacillales* (class *Bacilli*), *Sphingomonadales* (class *Alphaproteobacteria*) and *Enterobacteriales* (class *Gammaproteobacteria*), as well as families *SRB2* (a group of sulfur reducing bacteria) and *Xantomonadaceae*. Moreover, the presence of genera *Dethiobacter* and *Desulfonatronum*, associated to sulfur and sulfate reduction respectively, was detected.

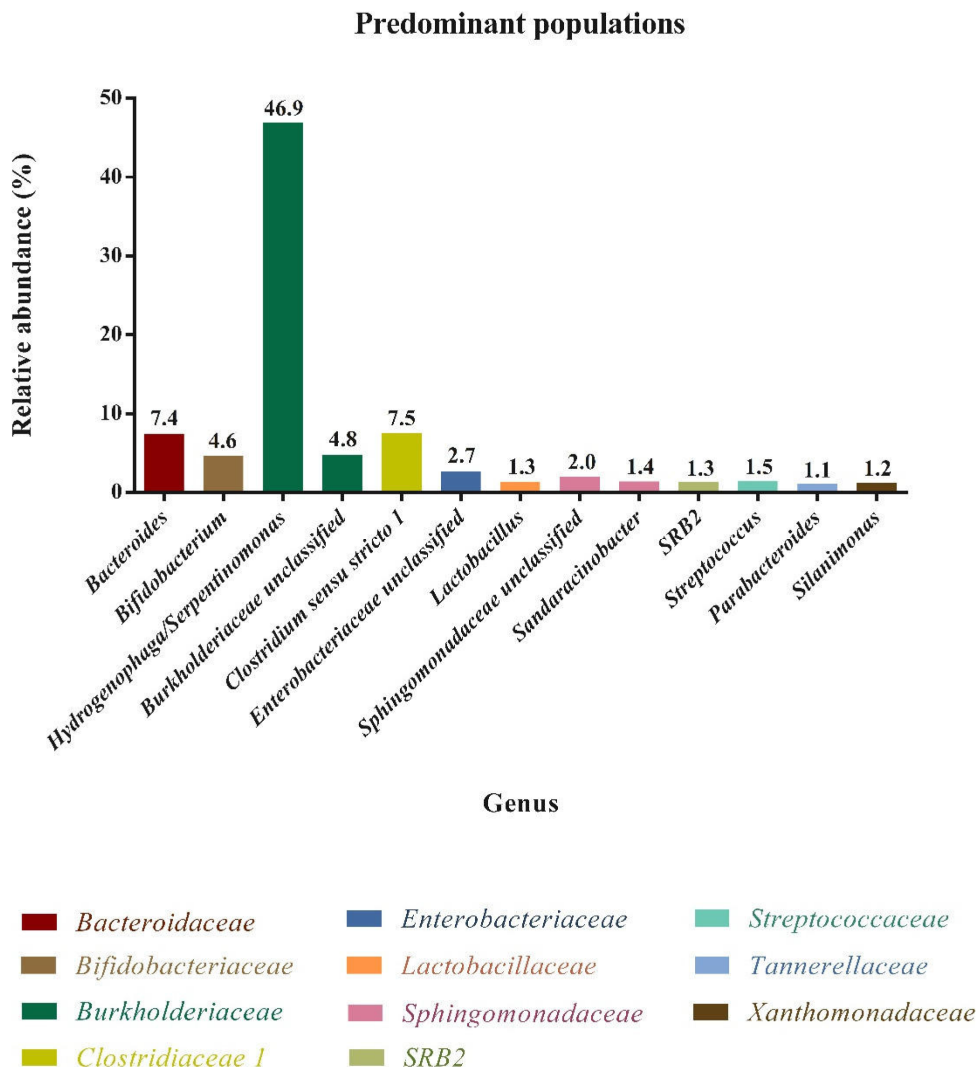


Figure 10. Phylogenetic characterization of the predominant bacterial populations assessed by culture-independent methods. The bacteria were classified at genus level and matched to the family taxonomic group through the color code represented in the graph bars. Phylogenetic description and abundance of bacterial predominant groups was discriminated in Table SXVI in supplementary data.

Besides *Bacteria*, members of domain *Archaea* also prevail in the groundwater sample. *Candidatus Nitrocosmicus* (order *Nitrososphaerales*, family *Nitrososphaeraceae*) is the most abundant genus with a representing approximately 61% of the archaeal community (Figure 11). *Candidatus Nitrosoarchaeum* (order *Nitrosopumilales*, family *Nitrosopumilaceae*) is

the second most represented group, accounting approximately 35% of the archaea populations (Figure 11). Both genera are associated to aerobic ammonia oxidation and, unlike other members of the community, are not normally detected in serpentine environments.

Genus *Hadesarchaeaeota*, is the third most represented genus in the Alfaguara community and is frequently associated with subsurface methane-rich environments (Jiang *et al*., 2007). Members of this group are involved in molecular hydrogen oxidation or production and may have the potential to coupling H_2 and the carbon monoxide (CO) oxidation to dissimilatory nitrate reduction to ammonium (DNRA). Also related with this serpentinization system is genus *ANME-1*, responsible for the anaerobic methane oxidation and genus *Methanobacter*, associated to methane reduction.

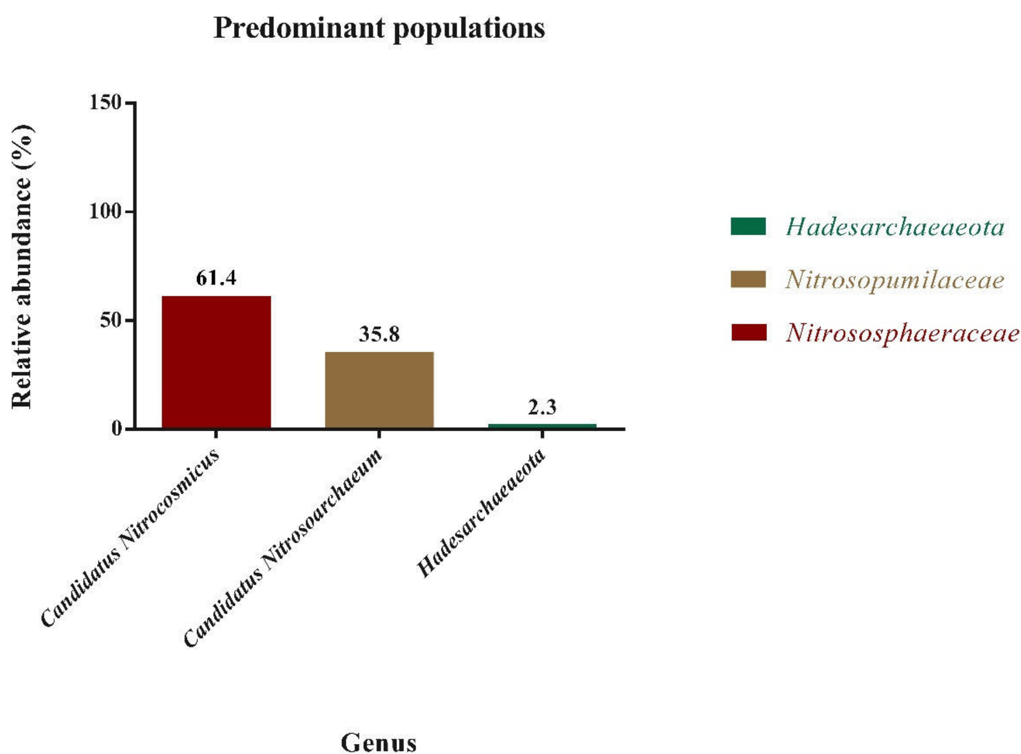


Figure 11. Phylogenetic characterization of the predominant archaeal populations assessed by culture-independent methods. The archaea were classified at genus level and matched to the family taxonomic group through the color code represented in the graph bars. Phylogenetic description and abundance of archaeal predominant groups was discriminated in Table SXVII in supplementary data.

3.3.3. Principal Component Analysis (PCA)

To determine the relation between Alfaguara bacterial diversity and fifteen other similar environments, a PCA was performed based on the results obtained from the culture independent methods (Figure 12). As shown, samples 1, 2, 3, 6, 8 and 9 are the most related with Alfaguara community. The first three samples are prevenient from the Coast Range Ophiolite in California. Samples 6 and 8 were collected from the Tablelands Ophiolite in Canada. Sample 9 was harvested in Wallula, Washington, from the Columbia River Basalt Group (CRBG).

Consequently, the samples 4, 5, 7, 10, 11, 12, 13, 14 and 15 are more distinct. The first two samples are prevenient from the Ligurian Ophiolite in Italy. Sample 7 was also collected in the Tablelands Ophiolite in Canada. Samples 10 and 11 are from the Onkalo tunnel in Finland. Samples 12 and 13 were prevenient from Olkiluoto groundwater also in Finland. Samples 14 and 15 were drilled in Cabeço de Vide, Portugal.

Samples from Olkiluoto groundwater, Cabeço de Vide aquifer and sample 11 from Onkalo tunnel showed the principal dissimilarity in relation to Alfaguara.

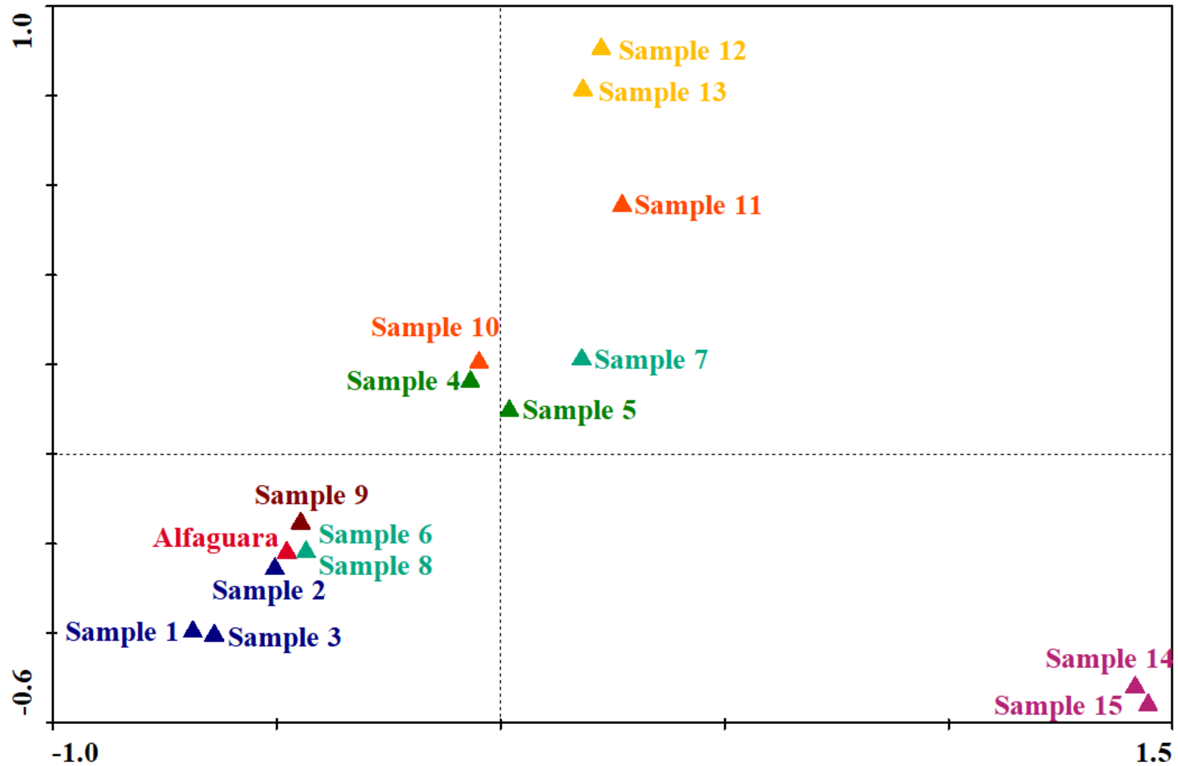


Figure 12. Principal component analysis based on the phylogenetic results of several samples from environments associated with serpentinization and from Hydrogenophaga/Serpentinomonas dominated habitats. Samples 1, 2 and 3 were collected from the Cost Range Ophiolite in California (Twing *et al*., 2017). Samples 4 and 5 were collected from Ligurian Ophiolite in Italy. Samples 7 and 8 were collected from the Tablelands Ophiolite in Canada (Brazelton *et al.*, 2013). Sample 9 was harvested in the CRBG in Wallula. Samples 10 and 11 were collected from Onkalo tunnel in Finland. Samples 12 and 13 were collected from Olkiluoto groundwater in Finland. Samples 14 and 15 were collected from Cabeço de Vide in Portugal (Tiago *et al*., 2004).

The relation between Alfaguara archaeal diversity and seven other similar environments, was also evaluated by PCA analyses (Figure 13). As shown, samples 1, 2, 3, 4, 5 and 6 are practically identical to each other. Samples 1 and 2 were prevenient from Haakon Mosby Mud Volcano in the Arctic Ocean. Samples 3 and 4 were collected from the

North American Basin and Range, Black Hills, and Canadian Shield. Samples 5 and 6 were harvested in the Eastern Mediterranean Sea and Black Sea.

Despite these similarities, the above-mentioned samples are very distinct from those of Alfaguara and Cabeço de Vide (sample 7). Nevertheless, the sample drilled in Cabeço de Vide is also dissimilar from the Alfaguara sample. This demonstrates that none of the sites has a diversity similar to that found in the Alfaguara groundwater.

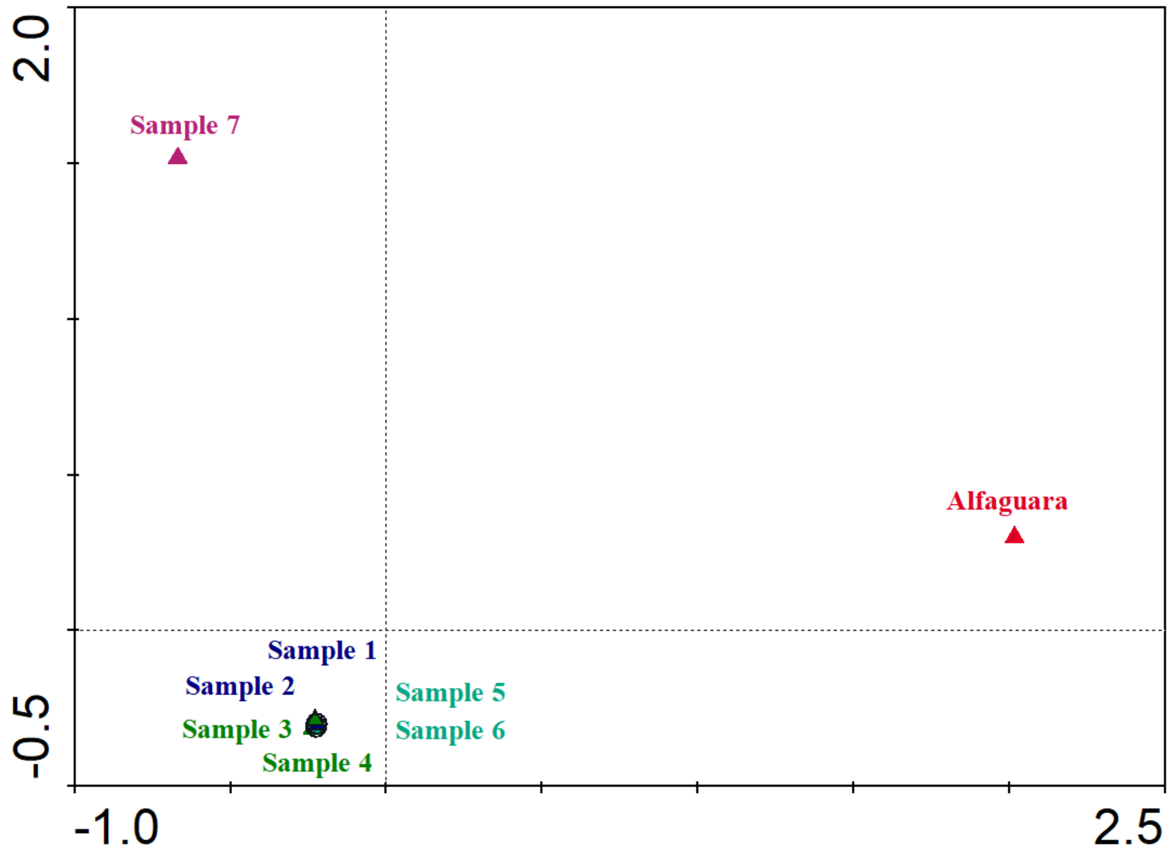


Figure 13. Principal component analysis based on the phylogenetic results of several samples from environments associated with serpentinization and deep biosphere. Samples 1 and 2 were collected in the Haakon Mosby Mud Volcano in the Arctic Ocean. Samples 3 and 4 were collected from the North American Basin and Range, Black Hills, and Canadian Shield. Samples 5 and 6 were collected at Eastern Mediterranean Sea and Black Sea. Sample 7 collected from Cabeço de Vide in Portugal (Tiago *et al.*, 2004).

Chapter IV – Discussion

Springs are natural systems that allow access to deep groundwater. Occasionally some sites can be related to deep aquatic environments were serpentinization reactions took place. These reactions, and the physical and chemical settings they generate, provide conditions for the existence of energy-generating reactions to occur, these being capable of producing compounds capable of supporting populations of microorganisms present in such habitats (Russell *et al.*, 2010). This aspect has aroused interest over time since extreme environments such as theses, so called SLIMEs, may be good analogues to understand early Earth life or live elsewhere (Nealson *et al.*, 2005).

The Alfaguara spring is associated to an artesian aquifer and, as referred, the groundwater flowing through it presents extreme parameters for life, namely, a high pH value (11.9). As shown, the EC, IC, pH and temperature present a range of similar values with other sites typically associated with peridotite environments. Nevertheless, the Alfaguara spring water distinguishes itself by the high levels of oxygen detected, being more than double, when compared with values determined for Cedars (Suzuki *et al.*, 2013), Cabeço de Vide (Tiago *et al*., 2004) and Cost Range Ophiolite waters (Twing *et al.*, 2017). This marked oxygenation can be explained by the fact that at some point in the rise for surface, the groundwater contacts with either oxygenated superficial water and/or atmospheric oxygen. Moreover, the high presence of nitrogen and TOC values also suggests that there may have been contact with water from the surface and/or soil. Most likely the Alfaguara groundwater interacts with both atmospheric oxygen and soil, rather than with superficial waters, since the pH value detected remains very high and stable over time, and waters such as those from Alfaguara springs have very low buffer capacity.

The main goal of this study was to determine the structural diversity of the Alfaguara deep aquifer by culture- dependent and -independent methods.

For this purpose, 120 different culture conditions were used to isolate 1206 bacteria, however, in successive attempts to reach pure cultures, 211 isolates were not able to be grown

again. The majority of the bacteria were isolated in ABM2 and R2A media, showing that these media were the best for the recovery of a larger number of isolates. Moreover, some isolates were recovered from 0.1-µm pore size filters (after the cell suspension was filtered through 0.2-µm pore size filters), which shows the presence of very small bacterial cells in the environment. In addition, this demonstrates that despite the believe that 0.2-µm pore size filters can produce sterile fluids, some bacteria can persist in the suspension. From the 995 remaining bacteria, despite several attempts and optimizations of the technique, RAPD profiles were obtained for only 388 strains. Nevertheless, this number of isolates seems to be significantly representative of the diversity existing for the aerobic cultivable populations of this environment (Figure SI in the supplementary data). The 338 isolates RAPD profiles were analyzed, and isolates were grouped according to the band pattern and their molecular weight. This methodology allowed the identification of 188 groups, whose representative(s) isolate(s) (depending on the number of bacteria per group) had the 16S rRNA gene sequenced. At the end, 26 phylogenetic groups were identified. With this analysis it was possible to verify that the greater diversity of species was recovered in the R2A medium. Of the phylogenetic groups attained, 22 were related to phylum *Firmicutes* and 4 to phylum *Actinobacteria*. These taxa were previously found in Cabeço de Vide (Tiago *et al.*, 2004*)*, although in this site most of the isolates belong to the phylum *Actinobacteria*. Furthermore, the presence of gram-negative species was observed in Cabeço de Vide but was not verified in Alfaguara. It is not clear if it was a bias of the sample collection and transport (that consisted in deepfreeze the suspension collected on spot) and/or the isolation conditions used, or if the unique conditions in Alfaguara springs favored the establishment of detected phylogenetic groups. Groups 16, 17, 18 and 19 (representing ~37% of the total bacterial populations) showed phylogenetic similarity with species extremely alkaliphilic or highly alkalitolerants, i. e. *Bacillus halmapalus*, *Bacillus cohnii* and *Bacillus horikoshii* (Nogi *et al.*, 2005). This along with results performed to access the biotechnological potential of the isolates (discussed below) we can conclude that most of the Alfaguara isolates are alkalitolerant, and that some can grow at high pH values and are well adapted to the extreme conditions existing in Alfaguara spring. Moreover, some isolates were associated with species commonly found in the soil, i.e. *Bacillus licheniformis* (Whitaker *et al.*, 2005),

Bacillus soli (Heyrman *et al.*, 2004). This aspect may be further evidence that the groundwater of Alfaguara interacts with soil at some point in its course towards the surface.

By culture independent methods, it was determined that genus *Hydrogenophaga/Serpentinomonas* is predominant in the Alfaguara deep aquifer. This genus encompasses extremely alkaliphilic and facultative aerobic bacteria, frequently identified in serpentinization-associated sites. In addition, some species belonging to this group, and found in the Cedars (Suzuki *et al.*, 2013), were able to use nitrate as electron accepter. These characteristics may be the reason for their abundance since Alfaguara water present high levels of nitrogen, O_2, and a high pH. Furthermore, it has been suggested that *Serpentinomonas* occupy transitional zones where deep-water mixes with superficial water and that these sites are distinct from those considered deep groundwater Cedars (Suzuki *et al.*, 2013). Nevertheless, as referred, possibly the oxygenation of this groundwater was prevenient from the atmospheric oxygen rather than superficial water, and the presence of both class *Clostridia* and genus *Bifidobacterium*, strictly anaerobic groups, also abundant in the community, corroborate that observation. In addition, *SRB2*, another anaerobic genus, related to sulfur reduction (Timmers *et al.*, 2015) and, therefore, suited to survive in anoxic alkaline environments. The presence of these strictly anaerobic populations indicates that the water collected came from deep, oxygen-depleted areas. Nevertheless, the association of these populations with aerobic bacteria raises the possibility that, not only deep-water bacteria were detected, but also those residing in sites along the rise of the water. The family *Enterobacteriaceae*, also detected in the bacterial community, is considered a marker usually associated to the presence of organic matter or soils. Thus, despite this not appear to be a very representative population, the presence of this group can be linked to the high nitrogen levels found in the groundwater, since it provides additional evidence that there was interaction between the Alfaguara water and superficial soil.

Members belonging to domain *Archaea* were also detected in the Alfaguara deep aquifer groundwater. *Candidatus Nitrocosmicus* and *Candidatus Nitrosoarchaeum* are the two most abundant genera, both commonly associated to aerobic ammonia oxidation events (Jung *et al.*, 2016; Hong *et al.*, 2014). Both archaeal genera are classified as aerobes, thus most probably are established at more oxygenated zones of the aquifer and, therefore, closer

to the surface. Nevertheless, they should be, most probably, adapted to live in high alkaline conditions, although the two groups are not normally associated with serpentine sites. In fact, the presence of these two genera of aerobic archaea in the Alfaguara groundwater differentiates Alfaguara environment from those found in the literature associated with serpentinization reactions. However, other similar environments (previously referred in section 3.1) related to mafic/ultramafic rocks normally do not have detectable concentrations of O_2 and the presence of this compound may be paramount to these archaeal populations to thrive. So, these results allow a better understanding of the community shifts (when comparing to the archaeal communities found in other serpentinization driven environments) when more adapt populations thrive taking advantage of the oxygen and nitrogen present in the groundwater and the H_2 provided by serpentinization reactions. Additionally, genus *Hadesarchaeota* was detected in Alfaguara spring. This specific group, commonly related with environments associated to mafic/ultramafic rocks, can produce H_2 and couple it with carbon monoxide to the reduction of nitrate to ammonium (Baker *et al.*, 2016). These compounds can be further used by other microorganisms present in the community. The presence of these populations provides strong evidence that the Alfaguara spring water came from deep, oxygen-depleted areas.

The biotechnological potential of the isolates was determined. The degradation tests were performed for a representative isolate of each phylogenetic group obtained by culture-dependent methods. For both carboxymethyl cellulose and xylan, the highest degradation activity occurred in minimal medium. This may be explained by the fact that when growing in R2A medium bacteria's use other carbon sources in medium constitution rather than the substrates in test. The higher activities in starch degradation were obtained at higher pH in R2A medium. In this case, as starch is a component of the medium, most probably we noticed a summed effect when calculating the activity values. Despite that, the overall activities increased from 20ºC to 26ºC but decreased with the increasing of incubation temperature. Nevertheless, despite this activity decrease, the degradation activity remained higher at alkaline pH values, namely pH 9 and 10. This may suggest an alkaline behavior of the amylases of these isolates. Of the 26 isolates tested, 17 or more presented degradation activities for xylan in minimal medium at all the conditions. The isolates showed higher activity at higher pH values in all the conditions and this activity also decreased with the

increasing of incubation temperature. However, the highest levels of degradation remained at pH 9 and 10 which, again, suggests an alkaline behavior for the enzymes of these isolates tested. Overall, it were identified several isolates that had degradative activities at interesting conditions, namely at high pH, reinforcing the knowledge that the isolates of this kind of environments can be a source of interesting enzymes, and that culture dependent methodologies are far from being obsolete. Additionally, future work will have to be done to confirm the activities and the novelty of the enzymes responsible for the activities tested.

Chapter V – Conclusion

Microorganisms that thrive in life-challenging conditions have become a major focus of study both in the astrobiology and biotechnological field. Most of these microorganisms, however, are not able to grow under artificial laboratory conditions, since it is practically impossible to simulate all the settings of their natural environments.

The culture-independent methods allow, in this sense, a greater perception of the microbial structural diversity of a given environment. However, it does not allow the direct contact with the microorganisms, which create great impediment in the study of several characteristics, i.e. the direct evaluation of the phenotypic characteristics of the populations detected and, more specifically their potential for degradation of specific substrates. For this reason, culture-dependent methods continue to be paramount in the study of microorganism and allied with culture-independent methods can provide the best possible view of the microbial diversity.

In the present study, many bacterial groups were isolated and their capacity to degrade four substrates was tested. Several sets of microorganisms showed degradation activity for the three substrates used and this activity was in the alkaline spectrum. This makes these strains interesting for further work in the area since some reactions only occur under extreme conditions.

Chapter VI – References

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Chapter VII - Supplementary data

Table SI. Diameter of the colonies of the isolates in the presence of chitin in minimal medium.

TºC	20				26				37				45			
pH	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**
690	0,6	0,7	0,7	0,6	0,8	0,9	0,7	0,7	0,9	1,1	0,8	0,6	0,9	1,6	0,8	0,9
65	0,8	0,7	0,7	0,7	0,8	1,0	0,8	0,8	1,0	1,0	0,8	0,9	0,9	1,4	0,8	0,8
121	0,7	0,8	0,7	0,7	0,9	1,1	0,7	0,8	1,2	1,0	0,9	0,9	1,0	1,6	0,7	0,8
925	0,7	0,9	0,7	0,7	0,9	1,1	0,7	0,7	1,1	1,0	1,0	0,7	1,0	1,6	0,8	0,8
832A	0,8	0,7	0,7	0,7	0,8	1,0	0,7	0,7	0,8	1,1	0,7	0,9	1,2	1,1	0,8	0,7
30	0,6	0,8	0,7	0,7	0,7	0,9	1,0	0,7	0,9	1,0	0,8	0,8	1,1	2,0	0,8	0,7
1	0,7	0,8	0,7	0,7	0,8	0,8	0,7	0,7	0,8	0,9	1,0	0,9	0,9	1,5	0,7	0,8
418	0,8	0,9	0,8	0,7	0,8	1,0	0,7	0,7	0,8	0,9	1,3	1,1	0,9	1,2	0,9	0,8
520	0,7	0,8	0,8	0,7	0,8	0,9	0,8	0,7	0,8	1,2	0,6	1,6	1,0	1,0	0,7	0,8
340A2	0,7	0,8	0,7	0,8	0,8	0,9	0,8	0,6	0,8	1,1	0,8	0,9	1,1	1,4	0,8	0,7
478	0,7	0,7	0,7	0,7	0,8	0,9	0,8	0,8	0,8	0,9	1,2	0,9	0,8	1,8	0,7	0,8
426	0,7	0,7	0,7	0,7	0,8	0,8	0,8	0,8	1,0	1,0	0,7	0,0	0,9	1,5	0,8	0,8
1159	0,7	0,7	0,5	0,7	0,7	0,8	0,7	0,6	0,9	1,0	1,0	1,0	0,4	1,2	0,6	0,0
217	0,6	0,6	0,6	0,3	0,6	0,7	0,7	0,6	0,6	0,7	0,6	1,1	0,6	1,0	0,8	0,4
234	0,9	0,9	0,7	0,7	1,2	1,2	0,8	0,7	1,3	0,9	1,1	1,4	0,6	1,0	1,2	0,9
511	0,8	0,7	0,6	0,6	0,7	0,8	0,6	0,6	0,8	0,9	0,8	1,3	0,0	0,3	1,0	0,4
729	0,6	0,6	0,6	0,6	0,7	0,7	0,9	0,7	1,4	0,6	0,7	1,4	0,6	0,0	1,2	0,3
100	0,0	0,3	0,3	0,3	0,7	0,7	0,7	0,7	0,6	0,7	1,1	1,1	0,8	0,0	1,1	0,3
440	0,6	0,7	0,7	0,7	0,7	0,7	0,8	0,7	1,3	0,8	1,1	1,1	0,5	1,4	1,1	0,0
1048	0,0	0,5	0,7	0,7	0,0	0,8	0,7	0,8	0,8	1,1	0,7	0,6	0,0	1,2	0,9	0,0
101	0,0	0,6	0,7	0,7	0,6	0,8	0,8	0,8	0,0	0,9	0,8	0,8	0,9	0,0	0,8	0,0
206	0,6	0,7	0,6	0,6	0,7	0,7	0,8	0,7	0,0	0,7	0,8	0,9	0,0	0,0	0,9	0,3
225	0,6	0,6	0,7	0,6	0,6	0,6	0,8	0,6	1,3	1,3	1,1	0,9	0,7	0,9	0,9	0,8
179	0,7	0,7	0,6	0,7	0,7	0,8	0,7	0,3	0,7	0,8	0,7	0,9	0,9	1,0	0,7	0,3
169	0,7	0,7	0,8	0,8	0,9	0,9	0,8	0,8	0,0	0,8	0,7	0,9	0,3	1,3	0,7	0,0
1153	0,6	0,7	0,6	0,6	0,8	0,7	0,7	0,8	0,3	0,8	0,6	0,9	0,3	0,6	1,0	0,0

Table SII. Diameter of the colonies of the isolates in the presence of chitin in R2A medium.

TºC	**20**				**26**				**37**				**45**			
pH	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**
690	1,1	1,1	0,8	0,8	1,5	1,2	0,8	0,8	1,0	1,5	0,0	1,3	1,6	0,6	1,0	0,0
65	0,9	0,8	0,7	0,8	1,4	1,2	0,7	0,7	0,8	1,3	0,3	1,3	1,1	1,5	1,2	0,6
121	0,9	0,8	0,9	1,0	0,8	0,8	0,9	0,9	0,5	1,1	0,3	0,6	0,6	0,0	0,3	0,0
925	0,9	1,0	1,0	0,7	0,9	0,9	0,8	0,8	0,5	1,3	0,0	1,4	1,1	1,1	1,0	1,4
832A	1,0	1,0	1,1	0,9	0,4	0,8	0,8	0,8	0,5	1,8	0,0	1,6	1,1	1,1	1,0	1,0
30	1,0	1,0	0,8	1,0	0,9	0,8	0,7	0,8	0,5	1,2	0,0	1,3	1,2	1,1	1,1	2,1
1	1,1	1,0	0,8	0,7	1,0	1,2	0,8	0,8	0,5	1,3	0,0	0,4	0,5	0,0	0,9	1,1
418	1,0	1,0	0,7	0,6	1,0	0,8	0,7	0,7	0,5	1,3	0,0	0,0	1,0	0,7	0,0	0,0
520	1,4	0,8	1,0	1,0	0,9	0,8	0,7	0,7	0,4	1,7	0,0	1,7	1,1	1,2	1,1	0,8
340A2	1,5	0,8	1,1	0,7	0,8	0,9	0,7	0,7	0,5	1,7	0,0	1,6	1,0	1,0	0,9	0,8
478	1,5	0,9	0,7	0,9	1,0	0,9	0,8	0,9	0,6	1,2	0,3	1,3	1,6	1,2	1,3	1,1
426	0,9	0,8	0,8	0,8	1,0	0,9	0,7	0,7	0,6	1,3	0,4	1,2	1,0	0,9	1,3	1,2
1159	1,1	0,8	1,0	0,8	0,8	0,8	1,1	0,9	0,5	1,4	0,4	1,5	1,0	1,0	1,1	0,6
217	0,9	1,0	0,8	0,8	1,0	1,0	0,8	0,8	0,5	1,3	0,3	1,5	1,0	0,9	1,1	0,9
234	1,0	1,1	0,9	0,7	1,1	1,4	0,9	0,8	0,6	1,6	0,3	1,1	1,6	1,2	1,0	0,9
511	1,2	0,9	0,8	0,8	1,0	1,2	0,8	0,8	0,6	1,1	0,3	1,1	1,3	1,3	0,9	1,3
729	1,1	1,0	0,8	0,9	1,0	0,8	0,7	0,7	0,5	1,1	0,4	1,1	1,0	0,6	0,7	0,8
100	1,7	0,8	0,7	0,9	0,9	0,8	0,7	0,7	0,5	1,3	0,5	1,3	1,0	1,1	0,8	0,9
440	1,7	0,8	1,2	0,7	1,0	0,7	0,7	0,7	0,5	1,4	0,0	0,0	0,0	0,0	0,9	0,0
1048	2,5	0,9	1,1	0,8	0,9	0,8	0,8	0,9	0,5	2,0	0,3	1,5	1,1	1,1	0,5	0,9
101	1,5	0,9	0,7	0,9	0,8	0,8	0,8	0,7	0,4	1,0	0,3	0,9	1,1	0,9	0,6	1,2
206	1,4	0,9	0,7	0,8	0,9	0,8	0,7	0,7	0,4	1,0	0,3	0,9	0,9	1,0	0,4	1,1
225	1,9	0,9	0,8	0,7	0,9	0,8	0,7	0,8	0,5	1,0	0,3	0,9	1,0	1,0	0,4	0,9
179	1,0	0,9	0,7	0,9	0,9	0,9	0,8	0,8	0,5	1,2	0,3	1,1	1,3	1,1	0,9	0,9
169	0,8	1,1	0,8	0,8	0,9	1,0	0,8	0,8	0,4	1,5	0,3	0,9	0,6	0,0	0,0	0,0
1153	1,3	1,0	0,8	0,9	1,0	0,9	0,8	0,7	0,5	1,4	0,3	1,6	1,1	1,1	0,9	0,8

Table SIII. Diameter of the colonies of the isolates in the presence of carboxymethyl cellulose in minimal medium.

TºC	**20**				**26**				**37**				**45**			
pH	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**
690	0,6	0,7	0,8	0,6	0,8	0,7	0,6	0,7	1,2	1,3	0,8	0,8	1,2	0,9	0,7	1,4
65	0,5	0,3	0,6	0,7	0,9	0,8	0,8	0,7	1,4	1,4	0,7	0,9	1,3	1,0	0,9	0,9
121	0,5	0,7	0,6	0,7	0,8	0,7	0,8	0,8	1,6	1,1	0,8	0,9	1,2	0,9	0,8	1,1
925	0,8	0,7	0,6	0,7	1,1	0,7	0,6	0,6	1,5	1,1	0,7	0,8	0,4	1,1	0,7	0,8
832A	0,7	0,7	0,7	0,7	0,8	0,7	0,7	0,6	1,1	0,4	0,8	0,7	0,9	1,0	0,8	0,9
30	0,0	0,0	0,6	0,7	0,8	0,7	0,8	0,7	0,9	0,5	1,1	0,7	0,0	0,9	0,8	1,2
1	0,0	0,0	0,6	0,7	0,9	0,7	0,8	0,7	1,2	0,7	0,7	1,3	0,5	0,0	0,7	1,1
418	0,8	0,8	0,6	0,7	1,0	0,7	0,6	0,7	1,3	1,2	0,8	1,5	0,8	0,5	0,7	1,1
520	0,0	0,0	0,7	0,6	1,1	0,7	0,7	0,7	1,3	0,8	1,0	0,9	0,6	0,0	0,7	0,7
340A2	0,3	0,0	0,6	0,6	0,9	0,7	0,7	0,7	1,2	0,7	0,7	0,7	0,7	0,0	0,8	0,9
478	0,3	0,6	0,8	0,6	0,8	0,7	0,8	0,6	1,1	0,6	0,8	1,0	0,0	0,9	0,7	1,3
426	0,5	0,0	0,7	0,6	0,9	0,7	0,7	0,7	1,1	1,3	0,8	1,3	0,0	0,7	0,7	1,1
1159	0,3	0,7	0,3	0,6	1,1	0,8	0,6	0,6	1,1	0,9	0,7	0,3	0,5	0,0	0,3	0,3
217	0,3	0,6	0,3	0,6	1,0	0,7	0,8	0,3	1,1	0,7	0,7	0,3	0,6	0,9	0,6	0,7
234	0,5	0,9	0,8	0,7	1,0	0,7	1,0	0,7	1,0	1,6	1,3	1,0	1,0	1,4	0,9	1,4
511	0,6	0,7	0,3	0,7	0,7	0,8	0,7	0,7	0,8	1,1	1,0	0,6	0,7	0,8	1,0	0,6
729	0,6	0,6	0,8	0,6	0,8	0,7	0,9	0,6	0,9	0,8	1,5	0,6	0,8	0,9	1,1	0,4
100	0,5	0,0	0,8	0,0	0,8	1,2	0,3	0,5	0,8	0,8	1,3	0,6	0,8	1,1	1,1	0,0
440	0,6	0,7	0,6	0,6	0,8	0,7	0,7	0,6	0,9	0,8	1,5	0,6	0,8	1,3	0,9	0,3
1048	0,5	0,4	0,6	0,7	0,8	0,7	0,8	0,8	0,9	0,8	1,1	0,7	0,8	1,3	0,7	0,5
101	0,6	0,7	0,6	0,7	0,7	0,7	0,9	0,7	0,8	0,8	0,9	0,8	0,8	0,8	0,9	0,8
206	0,6	0,7	0,7	0,7	0,7	0,7	0,7	0,3	0,8	0,8	1,0	0,6	0,8	0,8	0,9	0,4
225	0,6	0,7	0,3	0,6	0,7	0,7	0,8	0,3	0,9	0,8	1,2	0,4	0,8	0,9	0,8	1,2
179	0,6	0,7	0,6	0,7	0,7	0,7	0,8	0,3	0,8	0,8	1,2	0,3	0,8	0,8	1,1	0,6
169	0,7	0,7	0,7	0,8	0,7	0,7	0,8	0,8	0,7	0,7	1,1	0,8	0,7	0,9	0,6	0,5
1153	0,5	0,7	0,7	0,6	0,7	0,7	0,7	0,7	0,7	0,8	0,8	0,7	0,8	1,0	0,8	0,0

Table SVI. Diameter of the colonies of the isolates in the presence of carboxymethyl cellulose in R2A medium.

TºC	**20**				**26**				**37**				**45**			
pH	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**
690	0,9	0,8	0,7	0,6	1,0	1,1	0,8	0,8	2,1	1,7	1,1	1,2	2,1	2,3	1,0	1,0
65	0,7	0,6	0,0	0,0	0,7	0,7	0,0	0,0	1,0	0,9	0,0	0,0	0,8	1,3	0,3	0,0
121	0,7	0,6	0,8	0,9	0,8	0,7	1,0	1,0	0,6	0,0	0,7	0,7	0,3	0,6	0,6	0,7
925	0,8	0,8	0,7	0,6	0,9	1,0	0,8	0,8	1,0	1,2	0,9	0,9	1,1	0,7	0,6	0,7
832A	0,8	0,7	0,7	0,7	0,9	0,8	0,8	0,8	0,7	1,1	0,8	0,9	0,0	0,7	0,0	0,0
30	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,5	0,0	0,4	0,0	0,8	0,0	0,9	0,3
1	0,0	0,4	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,4	0,0	0,0	0,0	0,0	0,0	0,0
418	0,9	0,5	0,0	0,0	1,0	1,0	0,8	0,0	1,4	1,5	0,0	0,0	1,3	0,9	0,3	0,0
520	0,0	0,0	0,0	0,0	0,6	0,6	0,0	0,0	0,7	0,7	0,6	0,9	0,7	0,6	0,0	0,7
340A2	0,0	0,4	0,7	0,6	0,7	0,7	0,7	0,6	0,8	0,8	0,7	0,7	0,7	0,0	0,3	0,0
478	0,6	0,4	0,7	0,8	0,7	0,7	0,8	0,9	0,6	0,9	0,8	1,2	0,0	0,0	0,0	0,7
426	0,6	0,6	0,4	0,3	0,8	0,6	0,8	0,8	1,3	1,3	1,1	0,0	0,3	1,0	0,0	0,0
1159	0,6	0,7	0,0	0,0	0,7	0,7	0,6	0,0	1,0	1,1	0,8	0,5	0,3	0,6	0,6	0,5
217	0,6	0,7	0,6	0,6	0,7	0,7	0,6	0,7	0,8	0,8	0,7	0,6	0,4	0,7	0,6	0,3
234	0,8	0,9	0,7	0,7	1,1	1,2	0,8	0,7	1,8	1,7	1,7	1,5	0,4	1,9	1,7	1,2
511	0,8	0,7	0,7	0,6	0,9	1,0	0,8	0,7	1,0	1,1	1,1	0,8	0,0	0,8	0,7	1,0
729	0,7	0,7	0,6	0,6	0,7	0,7	0,7	0,6	0,8	0,8	1,3	0,7	0,0	0,0	0,0	0,0
100	0,6	0,7	0,7	0,7	0,8	0,7	0,8	0,8	0,3	0,4	1,0	1,0	0,5	0,6	0,4	0,8
440	0,7	0,7	0,7	0,7	0,8	0,8	0,9	0,8	0,0	0,5	0,8	0,6	0,5	0,0	0,0	0,0
1048	0,6	0,7	0,8	0,8	0,0	0,8	0,9	0,8	0,7	1,1	1,1	1,2	0,0	0,9	1,0	1,1
101	0,6	0,7	0,7	0,7	0,7	0,7	0,9	0,8	0,7	0,6	0,9	0,9	0,0	0,7	0,7	0,9
206	0,6	0,7	0,3	0,6	0,7	0,7	0,8	0,6	0,3	0,5	0,0	0,0	0,4	0,0	0,0	0,0
225	0,6	0,6	0,3	0,3	0,6	0,8	0,8	0,3	0,3	0,9	0,0	0,0	0,5	0,0	0,0	0,0
179	0,7	0,7	0,7	0,3	0,8	0,9	0,9	0,7	1,0	1,1	0,9	0,7	0,4	0,7	0,7	0,6
169	0,7	0,8	0,8	0,7	1,0	0,9	0,9	0,9	0,4	1,2	1,3	1,1	0,4	0,7	0,3	0,9
1153	0,6	0,9	0,8	0,8	0,7	0,8	0,8	0,7	0,7	1,0	1,1	1,0	0,3	0,7	0,7	0,9

Table SV. Diameter of the colonies of the isolates in the presence of starch in minimal medium.

T°C	20				26				37				45			
pH	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**
690	0,7	0,6	0,6	0,6	0,8	0,7	0,7	0,7	0,9	0,9	0,8	1,0	1,0	1,2	1,0	0,9
65	0,0	0,7	0,5	0,6	0,9	0,9	0,8	0,9	0,6	1,0	0,9	1,0	1,2	1,5	1,3	0,8
121	0,6	0,6	0,7	0,7	1,0	1,3	0,8	0,9	0,9	1,7	0,9	1,0	1,5	1,4	0,9	0,9
925	1,1	0,9	0,6	0,6	0,9	1,0	0,8	0,8	1,3	1,2	1,0	0,9	1,5	0,6	0,9	0,8
832A	0,7	0,8	0,6	0,7	0,7	0,8	0,8	1,0	1,0	1,2	1,2	0,9	1,0	0,6	0,8	0,8
30	0,0	0,3	0,6	0,7	0,8	0,8	0,8	0,9	0,3	1,2	1,1	1,0	0,0	1,1	1,0	0,8
1	0,3	0,3	0,5	0,8	0,8	0,8	0,8	0,8	0,5	1,4	1,0	0,9	0,0	1,3	0,8	0,8
418	0,4	0,9	0,5	0,6	0,8	1,0	0,7	0,8	1,0	0,8	1,0	0,9	1,5	1,5	1,2	0,8
520	0,0	0,0	0,6	0,8	0,8	0,9	0,8	0,8	0,5	0,7	0,9	0,9	0,0	1,1	1,5	0,9
340A2	0,6	0,3	0,6	0,6	0,7	0,8	0,8	0,8	0,8	1,2	1,0	0,8	0,6	0,9	0,8	0,4
478	0,5	0,5	0,6	0,7	0,8	0,9	0,8	0,9	0,4	1,2	1,1	1,1	0,4	0,6	1,1	1,1
426	0,6	0,3	0,6	0,6	0,7	0,7	0,8	0,8	1,5	1,3	0,9	0,9	0,6	1,1	1,0	0,8
1159	0,6	0,3	0,3	0,6	0,9	0,8	0,6	0,6	1,0	1,4	0,8	0,6	1,4	0,7	0,7	0,6
217	0,5	0,6	0,3	0,6	0,7	0,7	0,6	0,8	0,7	1,0	0,9	0,8	0,7	1,1	1,1	0,5
234	0,9	1,0	1,1	0,7	0,8	0,8	0,9	0,9	1,9	1,3	1,0	1,1	2,0	1,8	1,7	0,9
511	0,7	0,9	0,5	0,7	0,7	0,8	1,0	0,8	1,6	1,1	1,0	0,8	1,2	0,9	0,7	0,5
729	0,7	0,7	0,6	0,6	0,8	0,8	0,8	0,7	0,8	1,1	1,3	1,4	0,7	0,4	1,5	0,0
100	0,6	0,9	0,0	0,3	1,0	0,8	0,7	0,4	0,8	1,0	1,0	0,8	0,7	0,4	1,1	0,0
440	0,7	0,7	0,6	0,6	0,8	0,8	0,7	0,8	0,8	0,9	1,0	0,6	0,7	1,1	1,4	0,0
1048	0,7	0,7	0,7	0,7	0,8	0,8	0,8	0,8	0,8	0,9	1,0	0,9	0,7	1,1	0,9	0,6
101	0,7	0,7	0,6	0,7	0,7	0,7	0,8	0,9	0,8	0,8	0,9	0,9	0,6	0,4	1,2	0,0
206	0,7	0,7	0,3	0,7	0,8	0,8	0,7	0,6	0,8	0,9	1,2	0,8	0,6	0,4	0,8	0,4
225	0,6	0,6	0,5	0,6	0,8	0,8	0,7	0,5	0,8	0,8	1,0	1,1	0,6	1,5	1,4	0,3
179	0,7	0,7	1,0	0,6	0,7	0,8	0,8	0,7	0,8	0,8	0,8	0,9	0,6	0,5	0,4	0,0
169	0,7	0,7	0,7	0,7	0,7	0,8	1,3	0,8	0,8	0,9	0,9	0,8	0,6	0,4	0,6	0,0
1153	0,6	0,7	0,9	0,6	0,7	0,7	0,7	0,6	0,9	0,8	0,8	0,9	0,6	0,4	0,8	0,0

Table SVI. Diameter of the colonies of the isolates in the presence of starch in R2A medium.

T°C	**20**				**26**				**37**				**45**			
pH	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**
690	1,3	1,3	0,8	0,9	1,3	1,5	0,8	0,9	1,8	1,6	0,8	1,0	1,5	1,7	0,7	0,9
65	1,3	1,2	0,8	0,8	1,2	1,5	0,7	0,9	1,8	1,4	0,9	1,1	1,2	1,3	0,9	1,0
121	1,1	0,9	0,8	1,0	0,8	0,9	1,0	1,1	1,1	1,1	0,6	0,8	0,4	0,0	0,9	0,7
925	0,9	1,4	0,5	0,4	1,0	0,8	0,8	0,8	1,0	1,1	0,7	0,9	1,3	1,3	0,9	0,9
832A	1,0	1,1	0,5	1,0	1,0	0,9	0,7	0,8	1,1	1,2	0,7	0,9	1,1	1,1	0,9	0,9
30	0,9	1,0	1,0	0,8	1,0	0,9	0,8	0,9	1,0	1,0	0,7	0,8	1,1	1,1	1,0	0,9
1	1,0	1,2	0,8	1,2	1,2	1,2	0,8	0,9	1,0	1,3	0,3	0,9	0,4	0,4	1,0	0,3
418	0,9	1,1	0,7	0,9	1,0	0,9	0,7	0,7	1,0	1,1	0,3	0,8	0,8	1,1	1,0	0,0
520	0,8	0,9	0,7	0,7	0,9	0,9	0,7	0,7	1,0	1,1	0,7	0,8	1,1	1,2	0,9	0,9
340A2	0,8	1,1	4,9	0,9	0,9	1,1	0,8	0,7	1,1	1,6	0,7	0,9	1,0	1,1	0,9	0,9
478	1,1	1,1	0,9	0,5	1,1	1,1	0,9	1,0	1,0	1,1	0,8	1,1	1,5	1,5	1,1	1,2
426	0,8	0,8	0,7	0,9	0,8	0,8	0,8	0,8	1,0	1,3	0,7	0,7	1,2	1,1	0,9	0,7
1159	0,8	0,9	0,8	0.7	0,9	0,7	0,9	1,0	1,0	0,7	0,9	1,2	0,9	1,1	0,0	0,9
217	1,0	1,1	0,6	0,8	1,0	1,3	0,8	0,9	1,0	1,2	0,6	1,0	1,1	1,1	0,4	0,8
234	1,4	1,5	0,5	1,0	1,1	1,6	0,9	1,2	1,0	1,9	1,3	1,5	1,8	1,4	1,0	1,2
511	1,1	1,2	0,4	0,9	1,4	1,2	0,9	0,9	1,2	1,6	0,9	1,2	1,4	1,4	0,0	1,2
729	0,4	1,0	0,8	1,4	1,0	1,1	0,7	0,9	0,9	1,0	0,5	1,1	1,1	1,1	0,4	1,1
100	0,8	0,9	0,7	0,4	0,9	0,9	0,6	0,7	1,0	1,0	0,7	0,7	1,1	1,1	0,0	0,9
440	0,9	1,0	0,8	0,9	0,9	0.9	0,7	0,7	0,9	1,0	0,2	0,8	0,0	0,0	0,0	0,0
1048	0,9	1,0	1,0	0,8	0,8	0,9	0,7	0,7	1,0	1,1	0,9	0,9	1,1	1,1	0,9	0,9
101	0,8	1,0	0,9	0,9	1,0	0,9	0,8	0,9	1,0	1,1	0,8	1,0	1,2	1,1	0,7	1,1
206	0,8	0,9	0,8	0,9	0,8	0,8	0,7	0,8	0,9	1,0	0,4	0,7	1,0	1,0	0,0	0,8
225	0,8	0,9	0,9	0,9	0,9	0,8	0,7	0,7	0,9	1,0	0,8	0,8	1,1	1,1	0,5	0,9
179	0,8	1,0	0,7	1,0	1,0	1,2	0,8	0,8	1,0	1,1	0,7	1,1	1,0	1,1	0,0	0,8
169	0,3	0,9	0,4	0,8	0,8	1,0	0,9	0,8	0,9	1,2	1,0	1,1	0,0	0,0	0,6	0,7
1153	1,0	1,1	0,9	1,1	1,0	1,0	0,8	0,8	1,1	1,1	0,9	0,8	1,2	1,2	0,6	0,9

Table SVII. Diameter of the colonies of the isolates in the presence of xylan in minimal medium.

TºC	20				26				37				45			
pH	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**
690	0,65	0,65	0,525	0,65	0,7	0,75	0,35	0,7	1,1	1,15	1,15	0	1,1	1,4	1,1	1,6
65	0,0	0	0,525	0,6	0,65	0,75	0,725	0,75	1	0,9	1,2	0,9	1,3	1,25	1	1,1
121	0,55	0,5	0,525	0,675	0,75	0,775	0,75	0,8	0,95	0,75	1,65	0,5	0,5	1,5	0,95	1,1
925	0,6	0,6	0,525	0,6	0,8	0,85	0,7	0,7	0,95	1,2	1,35	1,2	0,4	0,5	0,85	1
832A	0,6	0,6	0,525	0,7	0,7	0,7	0,9	0,65	0,85	1	1,2	1,4	1,075	0,55	0,9	0,9
30	0,0	0	0,5	0,6	0,65	0,7	0,75	0,7	0,875	0,85	1	1,6	1,2	1,05	0,85	1,1
1	0,0	0	0,5	0,6	0,65	0,7	0,3	0,65	0,4	1,05	1,1	0,7	0,5	0,5	1,1	1
418	0,7	0,35	0,58	0,8	0,7	0,7	0,55	0,7	1,3	0,6	1,25	1,1	0,85	1,2	1,65	1
520	0,0	0	0,55	0,6	0,625	0,7	0,65	0,7	0,65	0,6	1,25	0,9	0,5	0,45	0,7	1
340A2	0,25	0,5	0,5	0,6	0,7	0,7	0,7	0,7	0,65	0,6	1,4	0,9	0,35	0,45	0,85	1
478	0,6	0,6	0,6	0,825	0,7	0,725	0,65	0,7	0,8	0,75	0,95	0,6	0	0,5	1	1
426	0,0	0,5	0,6	0,8	0,7	0,8	0,55	0,55	0,65	0,75	1,1	0	0	0,45	0,8	1,2
1159	0,3	0,5	0	0,55	0,8	0,85	0,35	0,6	0,75	0,6	0,8	1	0	0,4	1,15	0,7
217	0,55	0,5	0	0,55	0,65	0,7	0,65	0,65	0,3	0,75	0	1,5	0,3	1,05	0,7	0,6
234	0,95	0,95	0,55	0,7	0,7	0,725	0,8	1	1,15	0,85	1,25	1,1	1,65	1,05	0,7	0,5
511	0,7	0,7	0,25	0,65	0,725	0,75	0,6	0,7	0,9	1,3	0,9	1	1,7	0,8	0,35	0,6
729	0,6	0,8	0,5	0,6	0,75	0,75	0,7	0,8	0,875	0,9	0,95	0,5	0,7	0,8	1,35	1
100	0,7	0,6	0	0,3	0,75	3,05	0,4	0,8	0,8	0,8	1	0,6	0,7	1,15	1,05	0
440	0,6	0,6	0,5	0,6	0,725	0,75	0,625	0,65	0,85	0,85	1,15	0,9	0,75	1,1	1,4	0,8
1048	0,6	0,6	0,5	0	0,75	0,75	0,65	0,6	0,85	0,9	0,7	0,8	0,8	0,5	0,35	0
101	0,65	0,6	0,5	0,65	0,7	0,7	0,7	0,65	0,8	1	0,85	0,8	0,75	1,35	0,4	0
206	0,65	0,6	0,5	0,6	0,7	0,7	0,7	0,65	0,8	0,85	0,6	0,7	0,725	0,45	0,9	0
225	0,55	0,6	0,5	0,6	0,75	0,7	0,75	0,8	0,9	0,85	1,05	0,6	0,725	0,45	1,4	1,1
179	0,65	0,6	0,5	0,65	0,7	0,7	0,65	0,675	0,9	0,8	0,75	0,5	0,675	0,45	0,8	0
169	0,65	0,6	0,5	0,6	0,7	0,7	0,8	0,75	0,775	0,8	0,7	0,6	0,7	0,45	0,75	0
1153	0,6	0,6	0,6	0,65	0,7	0,75	0,65	0,7	0,8	0,85	0,7	0,7	0,7	0,45	0,55	0,7

Table SVIII. Diameter of the colonies of the isolates in the presence of xylan in R2A medium.

T°C	**20**				**26**				**37**				**45**			
pH	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**
690	1,0	0,9	0,7	0,6	1,7	1,3	0,8	0,9	1,7	1,7	1,5	1,1	1,3	1,0	1,2	1,2
65	0,6	0,7	0,0	0,0	0,7	0,7	0,7	0,0	1,1	1,6	0,5	1,0	1,0	1,2	0,9	0,0
121	0,6	0,6	0,7	0,7	0,7	0,8	0,9	1,0	0,3	0,4	0,7	0,7	0,0	0,8	0,7	0,6
925	0,8	0,8	0,7	0,6	0,9	1,2	0,8	0,8	1,3	1,2	1,0	1,1	0,6	1,0	0,8	1,5
832A	0,8	0,8	0,7	0,7	1,0	1,0	0,9	0,9	0,9	0,9	1,0	0,8	0,0	0,6	0,0	0,0
30	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,8	0,0	0,8	1,1	1,2	0,0	0,5	0,0	0,0
1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,9	0,0	0,5	1,0	0,6	0,0	0,0
418	0,9	0,9	0,6	0,3	1,0	1,1	0,7	0,3	1,7	0,7	0,6	0,3	1,1	1,3	0,7	0,0
520	0,0	0,0	0,0	0,0	0,6	0,7	0,3	0,4	0,9	0,7	1,3	0,9	0,6	1,2	1,2	0,0
340A2	0,6	0,0	0,6	0,6	0,7	1,0	0,8	0,7	0,9	0,7	0,7	0,7	0,0	0,0	0,7	0,0
478	0,6	0,7	0,8	0,7	0,7	0,7	1,1	1,0	1,0	0,8	1,2	1,1	0,4	0,5	0,9	0,8
426	0,6	0,6	0,6	0,6	0,8	0,7	1,0	0,8	1,7	1,4	1,0	0,8	0,7	1,3	0,7	0,0
1159	0,6	0,6	0,3	0,3	0,8	0,9	0,7	0,7	1,3	1,0	0,8	0,7	0,6	0,7	0,6	0,6
217	0,6	0,6	0,4	0,3	0,7	0,7	0,7	0,6	0,8	0,7	0,7	0,7	1,0	0,8	2,4	0,6
234	1,0	0,9	0,7	0,8	0,6	1,1	1,0	1,1	2,6	1,5	1,7	2,3	3,0	2,3	2,2	1,9
511	0,8	0,9	0,7	0,7	1,0	0,9	0,9	0,9	1,2	0,9	1,2	1,0	1,6	1,7	1,7	1,3
729	0,6	0,7	0,7	0,7	0,7	0,7	0,7	0,6	0,9	0,8	0,9	0,7	1,4	1,7	0,0	0,8
100	0,6	0,0	0,7	0,7	0,3	0,8	0,9	0,9	0,3	0,9	1,3	1,0	0,0	0,8	0,0	0,0
440	0,7	0,7	0,6	0,7	0,8	0,8	0,9	1,0	0,3	0,9	1,0	0,7	0,7	0,3	0,0	0,6
1048	0,6	0,8	0,8	0,9	0,3	1,2	1,1	1,1	0,3	1,0	1,4	1,4	0,3	1,4	1,4	1,3
101	0,6	0,7	0,8	0,7	0,6	0,9	1,0	1,0	0,8	0,6	1,1	1,0	0,5	1,2	1,0	1,0
206	0,6	0,7	0,6	0,6	0,7	0,9	0,8	0,7	0,5	0,9	1,1	0,0	0,0	0,4	0,0	0,0
225	0,6	0,6	0,6	0,6	0,8	0,8	0,7	0,7	0,4	0,4	0,8	0,3	0,0	0,5	0,0	0,0
179	0,8	0,8	0,7	0,6	0,9	0,9	1,0	0,8	1,2	0,9	1,1	0,9	1,0	0,8	0,9	0,8
169	0,7	0,7	0,8	0,8	0,9	0,7	1,1	1,2	0,8	0,9	1,4	1,3	0,7	1,3	0,7	0,6
1153	0,7	0,7	0,7	0,7	0,7	0,9	0,8	0,7	1,1	0,9	0,8	1,0	0,4	0,8	1,2	0,9

Table SIX. Diameter of the degradation halo of the isolates in the presence of carboxymethyl cellulose in minimal medium.

T°C	20				26				37				45			
pH	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**
690	77,7	51,5	57,1	76,0	68,0	72,0	71,4	64,6	54,0	48,0	68,8	44,4	50,1	60,9	41,3	42,5
65	67,0	0,0	66,7	65,8	66,0	70,0	68,2	74,3	44,0	44,0	70,1	0,0	42,5	50,0	56,0	55,1
121	36,1	0,0	0,0	0,0	68,0	72,0	0,0	0,0	36,0	58,0	66,4	0,0	49,0	55,0	60,8	50,0
925	0,0	0,0	70,6	0,0	58,0	72,0	74,0	38,0	40,0	58,0	69,0	0,0	86,0	56,0	59,8	54,1
832A	0,0	0,0	58,8	67,3	67,0	72,0	70,5	36,4	56,0	34,0	70,0	0,0	59,8	60,0	60,7	53,7
30	0,0	0,0	72,2	67,3	70,0	74,0	65,0	71,1	64,0	32,0	58,9	0,0	0,0	69,0	67,4	48,8
1	0,0	0,0	68,8	68,8	65,0	72,0	65,0	64,5	52,0	72,0	70,8	0,0	32,0	0,0	69,6	47,4
418	0,0	0,0	64,7	49,1	61,0	72,0	75,0	35,4	48,0	54,0	52,7	0,0	30,0	0,0	67,4	48,8
520	0,0	0,0	70,0	68,8	58,0	72,0	71,4	33,0	48,0	70,0	36,0	0,0	26,0	0,0	68,2	58,6
340A2	0,0	0,0	70,6	70,2	64,0	72,0	69,6	36,0	54,0	72,0	63,7	0,0	71,0	0,0	67,2	59,1
478	0,0	32,4	50,0	66,7	68,0	72,0	46,7	69,7	58,0	76,0	70,0	0,0	0,0	69,0	67,3	45,5
426	0,0	0,0	0,0	0,0	66,0	72,0	72,0	60,2	58,0	50,0	67,6	0,0	0,0	0,0	70,4	50,0
1159	33,3	0,0	0,0	0,0	58,0	70,0	68,4	30,0	58,0	64,0	69,8	0,0	0,0	0,0	37,5	0,0
217	0,0	0,0	0,0	0,0	62,0	72,0	0,0	0,0	56,0	73,0	70,1	0,0	0,0	64,0	0,0	30,0
234	0,0	0,0	0,0	0,0	61,0	72,0	9,1	31,0	62,0	38,0	58,0	0,0	0,0	0,0	0,0	0,0
511	0,0	0,0	0,0	0,0	74,0	70,0	0,0	0,0	67,0	56,0	38,0	0,0	0,0	0,0	14,7	0,0
729	22,7	0,0	0,0	0,0	70,0	72,0	0,0	7,1	66,0	68,0	32,5	0,0	0,0	0,0	44,4	0,0
100	57,1	0,0	0,0	0,0	70,0	54,0	0,0	36,1	68,0	68,0	36,8	0,0	0,0	0,0	13,6	0,0
440	0,0	26,7	0,0	0,0	70,0	72,0	0,0	0,0	66,0	68,0	37,4	0,0	0,0	0,0	50,3	0,0
1048	0,0	0,0	0,0	0,0	68,0	72,0	0,0	0,0	66,0	67,0	32,2	0,0	0,0	0,0	23,1	0,0
101	0,0	0,0	0,0	0,0	74,0	72,0	0,0	0,0	68,0	68,0	36,0	0,0	0,0	0,0	0,0	0,0
206	0,0	6,3	0,0	0,0	74,0	72,0	0,0	0,0	70,0	69,0	26,0	0,0	0,0	0,0	43,1	0,0
225	0,0	0,0	0,0	0,0	72,0	72,0	46,2	32,4	66,0	67,0	61,3	0,0	0,0	0,0	68,4	40,4
179	0,0	0,0	0,0	0,0	72,0	72,0	0,0	0,0	70,0	69,0	31,8	0,0	0,0	0,0	0,0	0,0
169	0,0	0,0	0,0	0,0	72,0	74,0	0,0	0,0	72,0	71,0	28,0	0,0	0,0	0,0	33,3	0,0
1153	0,0	0,0	0,0	0,0	73,0	74,0	0,0	0,0	72,0	69,0	36,0	0,0	0,0	0,0	60,4	0,0

Table SX. Diameter of the degradation halo of the isolates in the presence of carboxymethyl cellulose in R2A medium.

T°C	20				26				37				45			
pH	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**
690	57,0	73,1	72,0	73,3	58,3	68,1	71,3	74,5	32,9	45,0	55,8	63,6	0,0	20,9	42,8	50,0
65	61,0	67,5	0,0	0,0	73,0	38,5	50,0	0,0	71,4	77,0	0,0	0,0	35,0	59,4	38,0	0,0
121	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	39,4	0,0	0,0	0,0	0,0	0,0	0,0	0,0
925	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	28,6	21,4	0,0	0,0	62,6	0,0	0,0	0,0
832A	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	40,5	36,0	0,0	0,0	0,0	35,2	0,0	0,0
30	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	34,4	0,0	0,0	0,0	70,3	0,0	67,4	35,0
1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
418	0,0	0,0	0,0	0,0	0,0	0,0	32,6	0,0	25,0	21,4	0,0	0,0	58,5	37,5	0,0	0,0
520	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
340A2	0,0	0,0	0,0	0,0	0,0	0,0	11,1	0,0	0,0	0,0	0,0	0,0	30,9	0,0	0,0	0,0
478	0,0	0,0	0,0	0,0	37,9	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
426	0,0	26,9	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
1159	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
217	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	35,9	0,0	0,0	0,0	21,4	0,0	0,0	0,0
234	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	12,5	0,0
511	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	43,9	0,0	0,0	0,0	0,0	0,0	0,0
729	0,0	0,0	0,0	0,0	37,9	0,0	0,0	0,0	34,0	50,4	0,0	0,0	0,0	0,0	0,0	0,0
100	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	41,4	37,9	0,0	0,0	0,0	0,0	0,0	34,6
440	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	31,3	0,0	0,0	0,0
1048	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	63,5	0,0	0,0	0,0	50,0	0,0	34,1	0,0
101	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	81,1	21,1	0,0	0,0	0,0	35,4	0,0	0,0
206	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	41,7	38,2	0,0	0,0	37,1	0,0	0,0	0,0
225	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	41,9	0,0	0,0	0,0	30,4	0,0	0,0	0,0
179	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	32,5	0,0	0,0	0,0
169	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	23,3	0,0	0,0	31,5
1153	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	40,8	0,0	0,0	0,0	39,3	0,0	0,0	0,0

Table SXI. Diameter of the degradation halo of the isolates in the presence of starch in minimal medium.

TºC	20				26				37				45			
pH	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**
690	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,8	0,0	0,0	0,0	0,0	0,0	0,0
65	0,0	0,0	0,0	45,8	0,0	0,0	54,4	34,9	0,0	0,8	0,0	13,3	0,0	0,0	0,0	11,1
121	33,3	40,0	50,8	63,9	0,0	0,0	55,8	60,1	0,0	1,7	10,0	4,5	0,0	0,0	0,0	0,0
925	62,5	66,7	16,7	25,0	0,0	0,0	58,3	54,8	66,6	1,0	4,5	10,0	0,0	13,3	10,1	0,0
832A	0,0	0,0	52,3	39,7	0,0	10,0	54,4	32,1	0,0	1,0	9,1	5,6	0,0	16,7	20,0	0,0
30	0,0	0,0	39,2	43,2	0,0	0,0	44,2	33,9	0,0	1,1	0,0	5,0	0,0	0,0	0,0	11,1
1	18,8	27,3	50,2	34,2	18,3	24,1	51,1	39,7	0,0	1,1	0,0	0,0	0,0	0,0	0,0	0,0
418	0,0	0,0	38,2	37,5	0,0	0,0	54,6	36,7	0,0	0,1	0,0	0,0	0,0	5,6	0,0	0,0
520	0,0	0,0	31,9	22,5	0,0	0,0	55,0	35,9	0,0	0,9	5,6	5,0	0,0	0,0	0,0	0,0
340A2	0,0	0,0	36,7	30,6	36,4	16,3	50,8	49,5	0,0	1,1	8,3	0,0	0,0	0,0	0,0	0,0
478	0,0	0,0	51,3	70,5	25,0	0,0	57,8	48,7	0,0	0,9	0,0	56,8	0,0	0,0	3,8	20,0
426	0,0	0,0	12,5	36,7	0,0	0,0	54,6	22,2	0,0	1,1	0,0	26,5	0,0	0,0	5,6	11,1
1159	0,0	0,0	14,3	8,3	0,0	0,0	26,9	27,1	0,0	0,9	0,0	22,2	0,0	0,0	0,0	0,0
217	0,0	0,0	22,2	0,0	0,0	0,0	45,0	29,1	0,0	0,8	33,1	10,0	0,0	14,3	0,0	0,0
234	15,4	16,1	26,9	39,0	10,0	0,0	44,2	32,4	9,2	1,1	31,3	5,0	9,2	9,8	28,4	20,0
511	47,3	58,9	0,0	47,8	35,8	28,6	56,6	51,7	38,7	0,9	11,5	28,2	36,0	2,6	30,6	0,0
729	35,4	0,0	0,0	0,0	0,0	0,0	45,1	51,7	0,0	1,2	3,6	7,6	0,0	0,0	3,3	0,0
100	11,1	0,0	0,0	0,0	47,2	9,1	51,4	16,7	0,0	0,8	28,3	14,3	0,0	0,0	12,5	0,0
440	22,9	0,0	36,7	37,5	0,0	0,0	51,9	43,2	0,0	0,8	34,1	0,0	0,0	0,0	7,0	0,0
1048	0,0	23,1	0,0	0,0	0,0	0,0	23,1	5,6	0,0	0,8	25,0	0,0	0,0	21,7	0,0	0,0
101	0,0	0,0	0,0	0,0	0,0	3,1	25,0	0,0	0,0	0,8	0,0	0,0	0,0	0,0	0,0	0,0
206	0,0	0,0	0,0	15,0	0,0	0,0	54,6	26,9	0,0	0,9	12,5	19,2	0,0	0,0	0,0	0,0
225	26,9	0,0	0,0	0,0	0,0	0,0	28,1	29,2	0,0	0,8	9,1	0,0	0,0	0,0	2,6	25,0
179	0,0	0,0	0,0	0,0	0,0	0,0	0,0	10,0	0,0	0,8	49,8	0,0	0,0	0,0	0,0	0,0
169	0,0	0,0	11,1	35,0	0,0	0,0	0,0	21,1	0,0	0,9	25,0	0,0	0,0	0,0	0,0	0,0
1153	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,8	0,0	0,0	0,0	0,0	5,0	0,0

Table SXII. Diameter of the degradation halo of the isolates in the presence of starch in R2A medium.

TºC	**20**				**26**				**37**				**45**			
pH	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**
690	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	23,3	0,0
65	0,0	0,0	0,0	31,3	0,0	0,0	0,0	0,0	0,0	0,0	23,1	0,0	0,0	0,0	17,5	0,0
121	39,4	41,7	66,2	0,0	66,4	0,0	71,8	68,2	0,0	0,0	68,0	0,0	0,0	0,0	7,8	36,4
925	60,0	51,4	0,0	0,0	60,0	73,0	0,0	0,0	29,5	26,4	25,0	0,0	0,0	0,0	12,4	0,0
832A	0,0	0,0	0,0	16,7	0,0	0,0	0,0	0,0	0,0	0,0	28,6	0,0	0,0	0,0	10,6	0,0
30	0,0	0,0	0,0	0,0	16,7	0,0	0,0	18,8	0,0	0,0	22,7	0,0	0,0	0,0	6,7	0,0
1	0,0	42,2	31,0	18,2	22,5	32,5	70,8	66,4	4,5	13,6	25,0	39,5	0,0	0,0	5,0	0,0
418	0,0	0,0	0,0	0,0	0,0	0,0	0,0	35,0	0,0	0,0	25,0	0,0	0,0	0,0	2,5	0,0
520	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	25,0	0,0	0,0	0,0	9,6	0,0
340A2	0,0	0,0	0,0	25,0	0,0	0,0	0,0	0,0	11,5	0,0	22,7	0,0	0,0	0,0	9,6	0,0
478	14,3	17,2	28,9	0,0	20,6	22,1	62,8	58,7	10,4	4,2	57,9	38,0	46,0	24,7	10,4	22,7
426	5,0	15,0	0,0	4,5	0,0	0,0	0,0	29,2	14,2	0,0	20,0	0,0	6,7	0,0	7,5	0,0
1159	15,0	21,9	20,0	30,0	27,8	0,0	39,4	45,6	34,4	0,0	29,1	45,0	0,0	0,0	0,0	0,0
217	31,8	32,3	30,6	29,4	25,0	14,7	69,3	59,9	21,2	27,4	30,8	40,3	3,6	0,0	15,0	0,0
234	21,7	16,9	30,0	32,5	56,8	11,1	54,2	56,3	20,0	30,5	47,6	48,8	61,2	50,7	15,2	22,7
511	16,7	22,3	27,8	30,6	20,8	14,4	55,8	58,1	0,0	3,6	58,2	39,7	38,0	24,3	0,0	23,9
729	31,0	30,6	35,0	19,2	26,3	28,3	70,1	67,7	0,0	0,0	25,0	44,9	0,0	0,0	20,8	27,3
100	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
440	0,0	0,0	30,0	26,7	0,0	0,0	25,0	41,3	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
1048	0,0	0,0	17,2	10,0	0,0	0,0	0,0	26,7	0,0	0,0	38,4	26,5	16,7	0,0	0,0	0,0
101	0,0	18,3	21,9	19,2	12,5	0,0	65,2	58,3	0,0	0,0	27,8	39,3	0,0	0,0	0,0	0,0
206	0,0	13,6	0,0	21,9	0,0	0,0	0,0	33,3	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
225	0,0	0,0	25,0	23,3	0,0	21,4	0,0	59,2	0,0	0,0	17,9	57,9	0,0	0,0	5,0	0,0
179	11,4	7,7	26,5	18,8	17,9	7,1	62,5	61,3	0,0	0,0	23,1	18,8	0,0	0,0	0,0	0,0
169	0,0	25,0	27,8	26,7	0,0	0,0	38,6	38,5	0,0	0,0	25,1	45,6	0,0	0,0	18,8	0,0
1153	20,6	0,0	0,4	15,8	18,8	0,0	0,0	26,5	0,0	0,0	12,5	0,0	0,0	0,0	0,0	0,0

Table SXIII. Diameter of the degradation halo of the isolates in the presence of xylan in minimal medium.

TºC	20				26				37				45			
pH	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**
690	0,0	26,7	0,0	0,0	38,3	0,0	0,0	0,0	0,0	28,0	0,0	0,0	0,0	0,0	0,0	36,0
65	0,0	0,0	79,0	80,1	0,0	0,0	65,5	70,0	30,3	30,4	40,4	60,9	0,0	27,3	47,2	56,0
121	49,2	67,6	79,0	74,0	72,2	72,9	71,3	68,0	28,0	26,7	34,0	78,3	0,0	15,9	36,7	56,0
925	66,2	38,0	79,0	73,0	0,0	0,0	69,6	72,0	81,2	37,2	34,8	47,8	30,0	27,3	54,4	60,0
832A	29,2	33,3	79,0	70,8	71,9	66,6	62,6	74,0	0,0	46,7	50,1	39,1	37,0	25,0	45,8	64,0
30	0,0	0,0	80,0	79,7	62,5	68,5	63,2	72,0	0,0	25,0	59,2	30,4	0,0	32,0	47,2	56,0
1	50,0	0,0	80,0	76,0	79,4	0,0	0,0	36,0	42,3	50,5	50,0	69,6	0,0	28,3	23,5	60,0
418	25,0	32,5	76,8	69,2	20,8	0,0	75,1	34,0	7,7	38,6	34,1	52,2	57,3	23,8	0,0	60,0
520	0,0	0,0	78,0	78,9	74,8	78,3	72,3	36,0	25,0	56,4	52,5	60,9	0,0	29,5	57,2	60,0
340A2	0,0	30,8	80,0	75,0	68,5	73,0	70,1	72,0	0,0	58,3	23,6	60,9	0,0	25,0	58,5	60,0
478	33,3	69,3	76,0	61,8	67,4	0,0	76,7	72,0	37,2	55,7	54,8	73,9	0,0	30,0	36,7	60,0
426	0,0	33,3	76,0	65,3	35,7	0,0	76,1	40,0	50,0	29,4	59,3	0,0	0,0	33,3	45,7	52,0
1159	0,0	18,8	0,0	70,0	0,0	0,0	0,0	38,0	37,5	20,0	25,0	56,5	0,0	0,0	0,0	72,0
217	18,8	0,0	0,0	74,8	0,0	0,0	72,6	38,0	50,0	0,0	0,0	34,8	0,0	36,4	10,0	76,0
234	39,0	72,9	78,0	38,3	81,1	75,2	68,0	60,0	39,3	70,2	51,7	52,2	36,8	77,3	71,4	80,0
511	66,3	73,0	90,0	77,7	79,8	48,9	73,0	72,0	76,6	77,4	52,1	56,5	73,8	40,8	0,0	84,2
729	14,3	63,5	40,0	77,7	57,2	68,5	70,6	32,0	38,4	0,0	50,8	78,3	43,4	38,0	33,3	0,0
100	32,3	31,3	0,0	40,9	78,3	1,1	0,0	36,0	0,0	0,0	53,3	73,9	0,0	19,6	45,0	0,0
440	27,3	70,0	80,0	73,0	55,8	56,9	76,0	38,0	0,0	30,0	36,7	60,9	0,0	24,0	28,8	0,0
1048	50,3	35,0	80,0	0,0	55,3	56,3	71,0	76,0	0,0	41,5	32,4	65,2	0,0	0,0	0,0	0,0
101	22,7	63,3	80,0	36,0	53,3	53,3	69,0	68,6	0,0	0,0	32,5	65,2	0,0	0,0	0,0	0,0
206	20,0	65,0	40,0	30,0	49,7	53,3	68,2	72,8	0,0	0,0	56,4	0,0	0,0	0,0	40,4	0,0
225	25,0	60,0	40,0	30,0	50,0	53,3	68,1	65,0	0,0	0,0	48,4	0,0	0,0	0,0	23,7	78,8
179	30,0	70,0	80,0	72,4	56,1	56,1	62,5	58,9	0,0	23,3	26,5	84,8	0,0	0,0	23,3	0,0
169	22,7	61,3	80,0	76,9	0,0	59,2	57,4	70,0	0,0	23,3	27,8	45,5	0,0	0,0	0,0	0,0
1153	22,7	62,4	38,0	69,6	0,0	53,9	58,1	56,8	0,0	23,3	29,4	69,6	37,0	0,0	40,0	0,0

Table SXIV. Diameter of the degradation halo of the isolates in the presence of xylan in R2A medium.

TºC	**20**				**26**				**37**				**45**			
pH	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**	**7**	**8**	**9**	**10**
690	1,0	0,9	0,7	0,6	1,7	1,3	0,8	0,9	1,7	1,7	1,5	1,1	1,3	1,0	1,2	1,2
65	0,6	0,7	0,0	0,0	0,7	0,7	0,7	0,0	1,1	1,6	0,5	1,0	1,0	1,2	0,9	0,0
121	0,6	0,6	0,7	0,7	0,7	0,8	0,9	1,0	0,3	0,4	0,7	0,7	0,0	0,8	0,7	0,6
925	0,8	0,8	0,7	0,6	0,9	1,2	0,8	0,8	1,3	1,2	1,0	1,1	0,6	1,0	0,8	1,5
832A	0,8	0,8	0,7	0,7	1,0	1,0	0,9	0,9	0,9	0,9	1,0	0,8	0,0	0,6	0,0	0,0
30	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,8	0,0	0,8	1,1	1,2	0,0	0,5	0,0	0,0
1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,9	0,0	0,5	1,0	0,6	0,0	0,0
418	0,9	0,9	0,6	0,3	1,0	1,1	0,7	0,3	1,7	0,7	0,6	0,3	1,1	1,3	0,7	0,0
520	0,0	0,0	0,0	0,0	0,6	0,7	0,3	0,4	0,9	0,7	1,3	0,9	0,6	1,2	1,2	0,0
340A2	0,6	0,0	0,6	0,6	0,7	1,0	0,8	0,7	0,9	0,7	0,7	0,7	0,0	0,0	0,7	0,0
478	0,6	0,7	0,8	0,7	0,7	0,7	1,1	1,0	1,0	0,8	1,2	1,1	0,4	0,5	0,9	0,8
426	0,6	0,6	0,6	0,6	0,8	0,7	1,0	0,8	1,7	1,4	1,0	0,8	0,7	1,3	0,7	0,0
1159	0,6	0,6	0,3	0,3	0,8	0,9	0,7	0,7	1,3	1,0	0,8	0,7	0,6	0,7	0,6	0,6
217	0,6	0,6	0,4	0,3	0,7	0,7	0,7	0,6	0,8	0,7	0,7	0,7	1,0	0,8	2,4	0,6
234	1,0	0,9	0,7	0,8	0,6	1,1	1,0	1,1	2,6	1,5	1,7	2,3	3,0	2,3	2,2	1,9
511	0,8	0,9	0,7	0,7	1,0	0,9	0,9	0,9	1,2	0,9	1,2	1,0	1,6	1,7	1,7	1,3
729	0,6	0,7	0,7	0,7	0,7	0,7	0,7	0,6	0,9	0,8	0,9	0,7	1,4	1,7	0,0	0,8
100	0,6	0,0	0,7	0,7	0,3	0,8	0,9	0,9	0,3	0,9	1,3	1,0	0,0	0,8	0,0	0,0
440	0,7	0,7	0,6	0,7	0,8	0,8	0,9	1,0	0,3	0,9	1,0	0,7	0,7	0,3	0,0	0,6
1048	0,6	0,8	0,8	0,9	0,3	1,2	1,1	1,1	0,3	1,0	1,4	1,4	0,3	1,4	1,4	1,3
101	0,6	0,7	0,8	0,7	0,6	0,9	1,0	1,0	0,8	0,6	1,1	1,0	0,5	1,2	1,0	1,0
206	0,6	0,7	0,6	0,6	0,7	0,9	0,8	0,7	0,5	0,9	1,1	0,0	0,0	0,4	0,0	0,0
225	0,6	0,6	0,6	0,6	0,8	0,8	0,7	0,7	0,4	0,4	0,8	0,3	0,0	0,5	0,0	0,0
179	0,8	0,8	0,7	0,6	0,9	0,9	1,0	0,8	1,2	0,9	1,1	0,9	1,0	0,8	0,9	0,8
169	0,7	0,7	0,8	0,8	0,9	0,7	1,1	1,2	0,8	0,9	1,4	1,3	0,7	1,3	0,7	0,6
1153	0,7	0,7	0,7	0,7	0,7	0,9	0,8	0,7	1,1	0,9	0,8	1,0	0,4	0,8	1,2	0,9

Table SXV – Description and abundance of phylogenetic groups.

Groups	Number of Isolates	Abundance in the community (%)
1	56	16,56804734
2	30	8,875739645
3	1	0,295857988
4	5	1,479289941
5	1	0,295857988
6	6	1,775147929
7	1	0,295857988
8	1	0,295857988
9	1	0,295857988
10	1	0,295857988
11	1	0,295857988
12	18	5,325443787
13	1	0,295857988
14	2	0,591715976
15	56	16,56804734
16	8	2,366863905
17	40	11,83431953
18	1	0,295857988
19	79	23,37278107
20	3	0,887573964
21	3	0,887573964
22	4	1,183431953
23	2	0,591715976
24	1	0,295857988
25	14	4,142011834

26	2	0,591715976

Table SXVI. Phylogenetic description and abundance of bacterial groups detected in Alfaguara sample.

Domain	Phylum	Class	Order	Family	Genus	Abundance
Bacteria	Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium	7,468822945
	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	7,425233102
				Tannerellaceae	Parabacteroides	4,794624779
	Firmicutes	Bacilli	Clostridiales	Clostridiaceae 1	Clostridium sensu stricto 1	4,580802414
			Lactobacillales	Streptococcaceae	Streptococcus	2,652790137
		Clostridia		Lactobacillaceae	Lactobacillus	1,97830825
			Thermoanaerobacterales	SRB2	SRB2	1,538798829
	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonadaceae unclassified	1,278549413
					Sandaracinobacter	1,183373957
		Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	Hydrogenophaga/ Serpentinomonas	46,86037064
			Betaproteobacteriales	Burkholderiaceae	Burkholderiaceae unclassified	1,416025071
			Enterobacteriales	Enterobacteriaceae	Enterobacteriaceae unclassified	1,30021537
			Xanthomonadales	Xanthomonadaceae	Silanimonas	1,139526186

Table SXVII. Phylogenetic description and abundance of archaeal groups detected in Alfaguara sample.

Domain	Phylum	Class	Order	Family	Genus	Abundance
Archaea	*Hadesarchaeaeota*	*Hadesarchaeaeota*	*Hadesarchaeaeota*	*Hadesarchaeaeota*	*Hadesarchaeaeota*	2,312236287
	Thaumarchaeota	*Nitrososphaeria*	*Nitrososphaerales*	*Nitrososphaeraceae*	*Candidatus Nitrocosmicus*	61,36785577
					Candidatus Nitrosoarchaeum	35,76601458

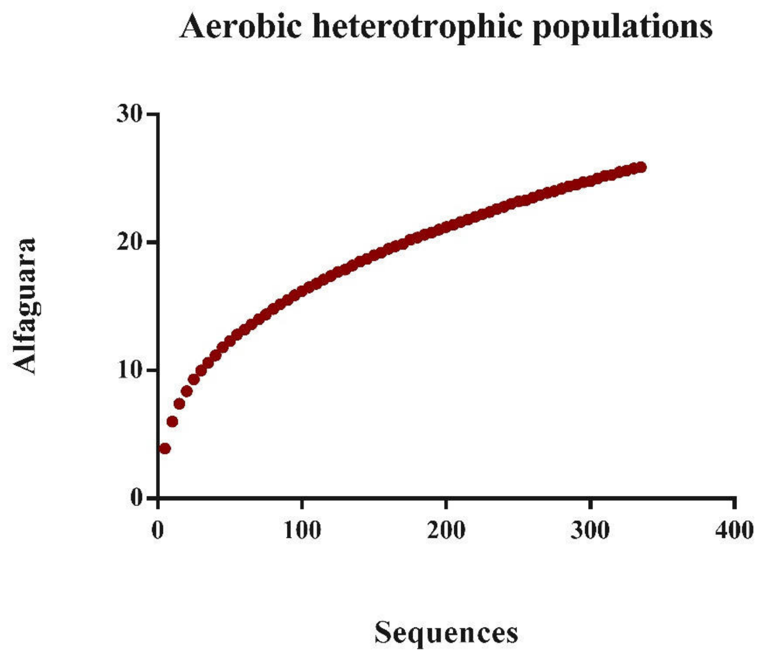

Figure SI. Rarefaction curve representative of Alfaguara archaeal richness.