PARTIAL PURIFICATION OF AGARASES FROM HALOPHILIC MICROORGANISMS ISOLATED FROM MARINE AND HYPERSALINE ENVIRONMENTS IN PUERTO RICO

By

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Resumen

Las agarasas son enzimas que son capaces de catalizar la hidrólisis de agar. Se clasifican en dos grupos, que son α-agarasa y β-agarasa, de acuerdo con el patrón de escisión. Varias agarasas se han aislado de diferentes géneros de bacterias que se encuentran en agua de mar y sedimentos marinos, así como microorganismos modificados genéticamente. El agar es un producto ficocoloide extraído de la pared celular de un grupo de algas rojas (Rhodophyceae) que incluye especies de *Gelidium* y *Gracilaria*. Estudios recientes han demostrado el gen de agarasa (AgaV) puede ser útil en dos aspectos: en primer lugar, como una enzima agarolítica, el AgaV recombinante purificada podría ser empleada en la recuperación de ADN a partir de geles de agarosa; en segundo lugar, como una proteína de secreción, AgaV se exploró en el nivel genético y se utilizó como un reportero en la construcción de una trampa de señal de secreción, que resultó ser una herramienta molecular simple y eficiente para la selección de genes que codifican proteínas de secreción de bacterias tanto en Gram-positivas y Gram-negativas. Según nuestro conocimiento, no se han publicado estudios sobre agarasas en Puerto Rico y debido a esto, el propósito de este estudio es examinar las muestras de agua que fueron tomadas de ambientes marinos (3.5% - 5.0% w / v de NaCl) y halófilicos (6% -30% w / v de NaCl) de Puerto Rico. Las muestras fueron recolectadas en bolsas estériles Whirl-pak® y fueron transportadas al laboratorio para ser procesadas. Las muestras de agua fueron concentradas por filtración con una membrana de 0.45 µm. Los aislados fueron crecidos en medio con extracto de levadura, medio sin extracto de levadura, medio con extracto de levadura y agarosa, y medio sin extracto de levadura con agarosa molecular, demostrando que pueden degradar el agar en medios con o sin nutrientes. El medio de crecimiento es una versión

modificada del medio “Synthetic Crenarcheota” (Könneke et al., 2005). Un total de 55 aislados fueron obtenidos del muestreo, 17 de éstos demostraron la capacidad de degradar agar. Los aislados fueron caracterizados utilizando propiedades físicas, moleculares y morfológicas. Se realizó un análisis del gen de ARN ribosomal 16S, luego se analizó el ADN metagenómico utilizando la reacción de polimerasa en cadena, lo cual demostró la presencia de bacterias y arqueas halofílicas. Los resultados preliminares indican que tenemos 17 aislados que pertenecen al dominio Bacteria y tienen actividad de agarasa. Este experimento puede contribuir al esclarecimiento sobre la diversidad y función de la enzima agarasa en ambientes halófilicos en Puerto Rico. El aislado MD25A produce una agarasa tipo beta, la cual exhibe una salinidad óptima de 1.5 M NaCl, una temperatura óptima de 37ºC, un pH de 8, es termoestable y tiene una vida media por 60 min a 50ºC. La producción inducida de la agarasa se hizo en el medio HA-I que contenía una solución al 0.2% de agarosa. La enzima purificada resultó ser homogénea y su peso molecular es de aproximadamente 80 kDa, como se observó en el “SDS-PAGE”. Los oligosacáridos producidos por la degradación del agar fueron analizados por cromatografía de capa fina. La agarasa generó los productos de degradación en el siguiente orden: neoagarohexaosa, neoagarotetraosa y una pequeña cantidad de neoagarobiosa. Estos resultados sugieren que nuestra enzima es una β-agarasa.

Abstract

Agarases are enzymes that can catalyze the hydrolysis of agar. They are classified in two groups, which are α-agarases and β-agarases, according to the cleavage pattern. Several agarases have been isolated from different bacterial genera found in seawater and marine sediments, as well as engineered microorganisms. Agar is a phycocolloid product extracted from the cell wall of a group of red algae (Rhodophyceae), including *Gelidium* and *Gracilaria.* Recent studies have demonstrated that the agarase gene *(Aga*V) is useful in two aspects: first, as an agarolytic enzyme, the purified recombinant AgaV could be employed in the recovery of DNA from agarose gels; second, as a secretion protein, AgaV was explored at the genetic level and used as a reporter in the construction of a secretion signal trap which proved to be a simple and efficient molecular tool for the selection of genes encoding secretion proteins from both Gram-positive and Gram-negative bacteria. To our knowledge, studies of agarases have not been conducted in Puerto Rico and because of that, the purpose of this study is to examine water samples that were taken from marine (3.5% - 5.0% w/v NaCl) and hypersaline (6.0% - 30.0% w/v NaCl) environments from Puerto Rico. The samples were collected in sterile bags Whirl-pak® and transported to the laboratory for processing. Additionally, water samples were concentrated by filtration using a 0.45 µm nitrocellulose membrane. The isolates were grown on media with yeast extract, media without yeast extract, media with yeast extract and molecular agarose, and media without yeast extract, but with molecular agarose to demonstrate agar hydrolisis even on media with or without nutrients. The culture medium is a modified version of Synthetic *Crenarcheota* (Könneke et al., 2005). A total of 55

isolates were obtained from these samplings, which 17 of these showed agarase activity. The isolates were characterized using physiological, molecular and morphological properties. PCR analysis of the 16S rRNA gene on the genomic DNA showed that isolates belong to the Bacteria domain. Preliminary results have shown that we have isolated 17 halophilic bacteria with agarase activity. This study can contribute in understanding the diversity and function of the enzyme agarase in saline to hypersaline environments from Puerto Rico. The isolate MD25A produces a β-type agarase that exhibits its best activity at 1.5 M NaCl, an optimal temperature of 37ºC, an optimal pH of 8, and has thermostability (half life for 60 min at 50ºC). The induced agarase production in the HA-I medium was made with a 0.2% agarose solution. The purified enzyme was homogeneous with a molecular weight of approximately 80 kDa, as presented by SDS-PAGE. The oligosaccharides produced by the degradation of agar were analyzed by Thin Layer Chromatography. The enzyme released its products in the following order: neoagarohexaose, neoagarotetraose and very small quantity of neoagarobiose. As a result, the data suggest that our enzyme is a β-type agarase.

I dedicate my thesis work to my family and many friends that were by my side during this chapter of my life. A special feeling of gratitude to my loving parents, Milagros Rivera Nieves and Miguel A. Oyola Velázquez, whose words of encouragement and trust helped me through this journey; without them I would have never reached this far. Thank you all for your support and for being there for me when things got rough. I also dedicate this thesis to my many friends from the Extremophile's Laboratory that have become like an extended family for me.

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1. Introduction

Agar is a polysaccharide that is obtained commercially from marine red algae (*Rhodophyceae*) specifically from the species in the genera *Gelidium* and *Gracilaria*, and it consists of two different components: agarose and agaropectin (Aoki et al., 1990; Rochas et al., 1994). Agarose (one of the main components of agar) consists of 3,6-anhydro-L-galactose and D-galactose alternately linked by α-1,3 and β-1,4 linkages (Kloareg and Quatrano, 1988). Agaropectin has the same basic disaccharide-repeating units as agarase with some hydroxyl groups of 3,6-anhydro-L-galactose residues replaced by sulfoxy or methoxy and pyruvate residues (Hamer et al., 1977). Agar is widely used as a gelling agent of microbiological culture media (Hehemann et al., 2012) as well as molecular sieving in DNA electrophoresis and gel-filtration chromatography (Kirimura et al., 1999). Although it has long been used as a gelling substance and a food or pharmaceutical ingredient stabilizer, its oligosaccharides derivatives have recently been found to possess various biological and therapeutic properties (Dong et al., 2006; Fernández et al.,1989).

Several studies have demonstrated the ability of microorganisms to degrade agar. Agar degrading bacteria were first described by Grant in 1902 (in Morrice et al., 1983). These organisms capable of agar degradation belong mainly to the Bacteria domain and have been isolated from marine environments. Examples of genera include *Acinetobacter, Agarivorans, Alteromonas, Bacillus, Cytophaga, Microbulbifer, Pseudoalteromonas, Pseudomonas, Salegentibacter, Thalassomonas, Vibrio,* and *Zobellia* (Hu et al., 2009; Fu and Kim, 2010). However, agar-degrading bacteria have also been isolated from other environments, such as soil (Suzuki *et al.*, 2002; Kirimura *et al.*, 1999; Voget *et al.*, 2003; Lakshmikanth *et al.*, 2006), sewage (Hofsten and Malmqvist, 1975), fresh water (Van der

Meulen *et al.,* 1976), the rhizosphere of spinach (Hosoda *et al.,* 2003), and plant roots (Hosoda and Sakai, 2006). Agar degrading bacteria are able to carry out this process through the use of an enzyme called agarase. Agarases are hydrolytic enzymes that degrade agar into oligosaccharides, which have various chemical properties and biological activities (Sugano et al, 1994). Agarases were characterized as either α- agarase (E.C. 3.2.1.158), that cleaves α-1, 3 linkages to produce a series of agarooligosaccharides related to agarobiose (Potin et al., 1993), or β-agarase (E.C. 3.2.1.81) that cleaves β-1, 4 linkages to produce neoagarooligosaccharides of series related to neoagarobiose (Kirimura et al., 1999). Both of these enzymes have been attracting keen interest in many fields of biochemistry and enzymology (Fu and Kim, 2010). The neoagarooligosaccharides have various special biological activities, such as inhibition of bacterial growth, slowing down starch degradation thereby reducing the calorific value of food, and providing anticancer, antivirus, and anti-oxidation activities (Giordano et al., 2006). The β-agarases are also used to recover DNA from agarose gel after electrophoresis (Finkelstein and Rownd, 1978; Burmeister and Lehrach, 1989).

Even though most agarases come from marine environments, there are no reports on this type of enzymes from halophilic bacteria that require high (more than 10%) NaCl concentrations for growth. The agar degrading process is exclusively performed by microorganisms and until recently it was believed to be exclusive of members of the Bacteria domain (Young et al., 1978). The Island of Puerto Rico, located in the Caribbean, has several hypersaline environments located in towns from the south of the island. There are no reports about agarases from hypersaline environments in Puerto Rico. Therefore, the main goal of this research project is to isolate and characterize halophilic

microorganisms containing agarases. The enzyme for an isolate was then partially purified and characterized biochemically. To achieve this, the study was focused in three hypersaline environments: The Cabo Rojo solar salterns, Guánica's solar salterns and the Peñuela's solar salterns. The geographical location, weather and water source of these sites are very different from equivalent ecosystems around the world (Litchfield and Gillevet, 2002; Zalar et al., 2005). This study constituted the first report of agar degrading populations in hypersaline environments from Puerto Rico.

2. Literature Review

2.1 Saline Environments

Saline environments are widely distributed on our planet and generally represented by the oceans, saline lakes and other water systems, as well as some saline soils. The most abundant water sources on our planet are saline environments. The oceans contain on average from 3-5% w/v of NaCl concentration. The saline environments are classified by their NaCl concentration into oligohaline (0.05% w/v NaCl), mesohaline (0.5% w/v NaCl), polyhaline (1.8% w/v NaCl), mixoeuhaline (3.0% w/v NaCl), metahaline (4.0% w/v NaCl), and hyperhaline (6.0% - 30.0% w/v NaCl) environments (Table 1) (Venice System, 1959). The oceans range between mixoeuhaline to metahaline environments, which means that the concentration of dissolved salt ranges from 3-4% w/v (Bromley et al., 1967). The lakes, ponds and salt works that have higher concentration of salt are classified as hypersaline. The Cabo Rojo Salt works (15-35% variable salinity), Dead Sea (34.2% salinity in 2010) and the Great Salt Lake (5–27% variable salinity) are examples of hypersaline environments found around the world.

Table 1. Classification of Thalassic Environments (Venice System, 1959).

Thalassic series	Parts Per Thousand	Salinity percent (% w/v NaCl)
Hyperhaline	60 o/oo - 300 o/oo	6% -30%
Metahaline	40 o/oo	4%
Mixoeuhaline	30 o/oo	3%
Polyhaline	18 o/oo	1.8%
Mesohaline	5 o/oo	0.5%
Oligohaline	0.5 o/oo	0.05%

2.2 Hypersaline Environments

The environments known as hypersaline are habitats that contain high salt concentration. They have been categorized in two types: thalassohaline and athalassohaline (Javor, 1989). Thalassohaline habitats are made from seawater and their solute composition is similar to it (Javor, 1989). The other type of habitat is the athalassohaline environment, which is rich in anions, other than chloride, and cations other than sodium such as calcium and magnesium (Demergasso et al., 2004). The solar salterns found around the world are an example of thalassohaline habitats. This extreme environment is created due to an extensive evaporation process. The high rate of evaporation stimulates the precipitation of gypsum, causing the decline of calcium and sulfate (Litchfield and Gillevet, 2002). Sodium chloride has been found to be the most abundant salt in these habitats (Oren, 2002). Additional vital ions present are: Mg^{+2}, SO_4^{2-}

, K^+, Ca2+, Br^-, HCO_3^- and F^- (Muhammmad Kaleem et al., 2017). These exact chemical and environmental restrictions are known to select for a limited but unique biodiversity in these environments in comparison to the sea ecosystem (Muhammmad Kaleem et al., 2017).

The microorganisms known as Halophiles have the ability of thriving at hypersaline environments. Several studies have confirmed that most of the dominant organisms present in hypersaline environments are the Archaea domain, although eukaryotic microorganism such as the algae Dunaliella salina and the fungus Wallemia have also been reported (Casamayor, 2002; Oren, 2002; Zalar et al., 2005). The microorganisms in these environments are in constant stress due to the low availability to nutrients restraining their diversity. There are two main mechanisms to manage the high salt concentrations in hypersaline environments: (1) the accumulation of inorganic solutes in the cytoplasm and (2) pumping ions out of the cell and storing organic compound such as sugars, amino acids and amino acid derivate (Oren, 1999, 2002). The last is the utmost effective mechanism. Bacteria can also be found in saline environments, but when the salinity goes up the diversity of this group is diminished mainly to the representatives of *Salinibacter ruber* (the only extreme halophilic bacteria) and members of the Archaea (Litchfield and Gillevet, 2002). The decrease in biodiversity is supporting evidence of the presence of microorganisms capable of performing most of the biochemical cycles (Oren, 2002; Litchfield and Gillevet, 2002).

2.2.1 The Island of Puerto Rico

The Commonwealth of Puerto Rico is composed of a number of islands in the northern Caribbean, including the island of Puerto Rico and offshore islands such as Culebra, Vieques, Mona, Monito, and Desecheo. Puerto Rico is a complex mosaic of interrelated habitats, including mangrove forests, seagrass beds, solar salterns, lagoons and coral reefs, as well as other coral communities (Ramos-Scharrón et al., 2015). The island has a tropical climate with a warm weather throughout the year. Due to the tropical climate and the variant landscape, the island contains dry, wet, marine, saline and hypersaline environments (Ramos-Scharrón et al., 2015). In the coasts of the island there is an abundant supply of red algae, which is suggestive of the presence of agar degrading microorganisms (Sakatoku et al., 2012). The marine and hypersaline environments are poorly understood.

The municipality of Guánica is located at the southern area of Puerto Rico, bordering the Caribbean Sea. Hills surround the town and harbor, including the 140 meters hill to the east of town, itself topped by the tiny Fort Caprón. East of the town there are some 200 acres (0.8 km^2) of land, including 5 km of beach line. It contains a tropical climate; the average annual temperature is 26.2°C. The average annual rainfall is 770 mm. The driest month is February, with 23 mm of rain. The Salt flats, belonging to the Montalva neighborhood, were famous since the time of Spanish domain, since they were the main supplier of salt to sailboats plying the Caribbean Sea (Guánica Municipality, 2015). The Montalva is a sandy land to the sea and full, deeper into barren rocks, covered with trees and populated by Caribbean mosquitos (Guánica Municipality, 2015).

Among the top biodiversity hotspots, we can find the Caribbean islands (Mittermeier et al., 2004) and their dry forests. The plants in the dry forests are considered

vastly more diverse at the family level than similar continental areas (Gentry, 1995). Studies about the biogeographic area have demonstrated that the Caribbean is one of the centers of high endemism and speciation in the world (Santiago-Valentín and Olmstead, 2004). In Guánica, we can find the Guánica State Forest which contains tropical dry forests as well as the abandoned Guánica Solar Salterns. However, there have been only few attempts to focus on the distinctive floristic composition of the state forest (Gentry, 1995). These salt flats have been abandoned for approximately a hundred years and there are no reports on halophilic microbial diversity.

The Cabo Rojo solar salterns are in the Cabo Rojo municipality, in the southwest area of Puerto Rico. The salterns are in the coastal zone and the specific coordinates are 17.9552° N, -67.2096° W. The Cabo Rojo Salt Flats are considered unique and irreplaceable. The coastline, mangroves, seagrass beds, and offshore coral reefs next to the area, are prime fish habitat, and are considered special aquatic sites (U.S. Fish and Wildlife Service, 2015). The salt flats are positioned in the Atlantic flyway and are a vital nesting ground and feeding area for the snowy plover, least tern, Wilson's plover, peregrine falcon, yellow-shouldered blackbird, brown pelican, and several species of sea turtles (U.S. Fish and Wildlife Service, 2015). Indeed, no fewer than 118 bird species have been recorded for the area. The climate is characterized by low precipitation, high temperatures and low humidity (Davis, 1978).

The Peñuelas municipality is located on the southern coastal plains, but the northern part of its territory is in the central mountain range. Elevations in this area exceed 800 meters above sea level (Colón et al., 2015). Two of the elevations are mounts Peñuelas and Garrote, two of the twenty highest elevations in Puerto Rico. Also, one of the seven sections

of the Guilarte State Forest is located in the northern part of this town (Barreal and Jaguas wards) (Colón et al., 2015). The Tallaboa Valley, which is comprised of the Tallaboa River and the surrounding alluvial plain, is also located in this municipality. The rest of the land is characterized by slight undulations and plains that reach into the lowlands in Tallaboa Poniente ward. This ward is located on the coast, were there are two promontories, Gotay and Guayanilla, as well as the Tallaboa Bay (Colón et al., 2015). Palomas, Parguera, Caribe, María Langa, and Rio (the largest) keys face the bay. There are also 30 hectares of red mangrove in this area (Colón et al., 2015). Nearby Ponce has winter highs averaging 30.55°C and summer highs of 33.89°C (The Weather Channel, 2015). The rainfall in Peñuelas varies both by season and locality. The municipality averages 978.41 mm of rain per year (The Weather Channel, 2015). The town contains an abandoned solar salterns located near “Encarnación” beach. In the Caribbean beaches, we find a high abundance of red algae which contain the polysaccharide known as agar.

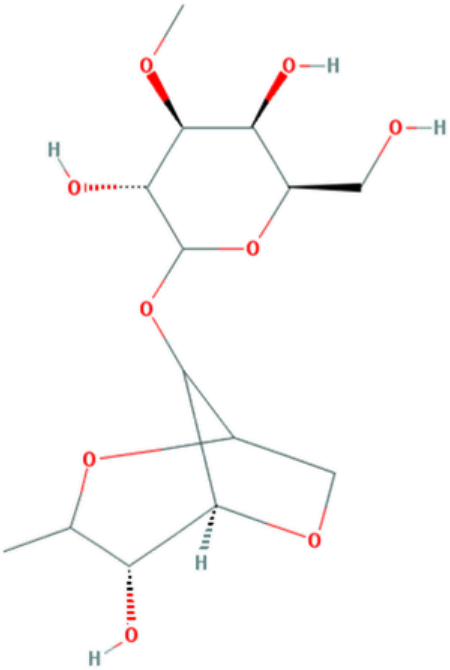

Figure 1. Original structure of agar repeat unit (Pubchem.ncbi.nlm.nih.gov, 2015).

The main components of the cell wall of red algae are agars and carrageenans (Craigie, 1990). This family of hydrocolloids consists of linear chains of galactose, with disaccharide repeating units of 3-linked β-D-galactose and 4-linked α-galactose units. This takes place as the L-isomer in agar and the D-isomer in carrageenan (Yaphe, 1957). In the carrageenans, the galactose units are substituted in a characteristic manner with sulphate groups. In contrast, the galactose units in agar are non-substituted or substituted with either O-methylether, pyruvic acid, ketal or sulphate groups (Roh et al., 2012). These substituent groups can alter the structural regularity of agar (Murano, 1995; Yaphe, 1957). The agar composition varies, depending not only on the particular strain or species, but also on the variations in nutrient levels, light levels and season (Cote and Hanisak, 1986). The quality of agar can be predicted from the type and content of sulphate esters, pyruvate ketals and methyl ethers (Murano, 1995).

2.2.2 Importance of *Gracilaria and Gelidium* as a Source of Agar

The top usage of red seaweeds is as food and as a source of two hydrocolloids, agar and carrageenan. Both hydrocolloids agar and carrageenan are water-soluble carbohydrates that are used to thicken aqueous solutions, to form gels of varying degrees of firmness, to form water-soluble films and to stabilize some food products (McHugh, 2003). Seaweeds as a source of these hydrocolloids, dates back to 1658 when the gelling properties of agar were discovered in Japan after Minoya Tarazaemon extracted agar for the first time from a red seaweed, using a freezethaw method (Armisen, 1995). Today agar is mainly extracted from two genera of red seaweed, *Gelidium* and *Gracilaria*. *Gelidium* gives the higher quality agar but unlike *Gracilaria*, all the *Gelidium* used for commercial agar extraction comes from natural resources. *Gelidium* is a small, slow growing plant and efforts to cultivate it have generally proved to be uneconomical (Figures 2 and 3) (Hatada et al., 2006). Before the 1950s, *Gracilaria* was considered inappropriate for agar production because the gel strength of the agar was too low. Nevertheless, in the 1950s it was discovered that pre-treatment of the seaweed with alkali before extraction lowered the yield but gave a good quality agar (McHugh, 2003). *Gracilaria* usually grows in the eulittoral zone, or just below it in the beginning of the sublittoral, on sandy or muddy sediments that are protected from waves. Sometimes it can be found free-floating in tidal lakes of salt or brackish water. The agar from *Gracilaria* has a high degree of sulphation and the gelling ability of *Gracilaria* agar is considerably improved by an alkali pretreatment with sodium hydroxide that converts β-L-galactose-6-sulphate into 3,6-anhydro-α-L-galactose (Murano, 1995; Armisen, 1995). Distinct to *Gracillaria*, *Gellidium* must however be processed within a few months and cannot be allowed to remain in storage for use during

years of lower availability (Armisen, 1995). The pre-treatment with alkali permitted the expansion of the agar industry and led to the harvesting of a variety of wild species of *Gracilaria*. Due to the latter, there was evidence of overharvesting of the wild crop and cultivation methods were then developed, both in ponds and in the open waters of protected bays. The supply of *Gracilaria* still comes primarily from natural populations, with the degree of cultivation depending on price fluctuations (McHugh, 2003).

Figure 2. *Gelidium*, rehydrated from dried material purchased by an agar producer. The coin diameter is 20mm (McHugh, 1984). © FAO 2010-2016. Fisheries and Aquaculture Department. Statutory bodies - Fisheries and Aquaculture Department. In: FAO Fisheries and Aquaculture Department [online]. Rome. Updated. [Cited 30 October 2016].

Figure 3. *Gracilaria*, rehydrated from dried material purchased by an agar producer. The coin diameter is 20 mm (McHugh, 1984)**.** © FAO 2010-2016. Fisheries and Aquaculture Department. Statutory bodies - Fisheries and Aquaculture Department. In: FAO Fisheries and Aquaculture Department [online]. Rome. Updated. [Cited 30 October 2016].

2.2.3 Porphyra spp.

Traditionally, in Eastern and Southeast Asia, the red algae *Porphyra* (Figure 4) has been cultivated and used as a supplement for its nutritional and medicinal purposes. It was commonly known as a Zicai or Ziyin. *Porphyra* is a red algae, belonging to the family *Bangiaceae*, order *Bangiales*, class *Protoflorideophyceae*, and phylum *Rhodophyta* (Cao et al., 2016). Currently, 279 species of *Porphyria* have been identified (www.algaebase.org/) worldwide, among which 15 species are found in China. When *Porphyra* is dried, it is known to contain nutritional and biofunctional components. It contains proteins, vitamins, minerals, dietary fiber, polyunsaturated fatty acids, carotenoids, saccharides and mycosporine-like amino acids (MacArtain et al., 2008; Smith et al., 2010). Recent studies have found a high-resolution structure of porphyran, which can be extracted from *Porphyra* sp. (Correc et al., 2011). The porphyran structure is

composed of 30% agarobiose and 70% porphyrobiose. The porphyran repetitions moieties are linked by α-1,3 linkage either to an agarose moiety or a porphyran moiety (Correc et al., 2011). Porphyran is a sulfated polysaccharide that demonstrates a wide variety of biological activities, including antitumor (Kwon and Nam, 2006), antioxidant (Zhang et al., 2003) and immunomodulating activities (Bhatia et al., 2013). Porphyran contains residues of D-galactose, L-galactose, 3,6-anhydro-l-galactose, 6-O-methyl-d-galactose, and ester sulfate. The hybrid structure is characteristic of agars, and even the agars from *Gelidium* and *Gracilaria spp*., which are considered "pure agarose" by commercial providers, contain a significant proportion of porphyrobiose moieties (Hehemann et al., 2010). The ratio of agarobiose to porphyrobiose increases in the following order: *Porphyran* spp., *Gracilaria* and *Gelidium* (Hehemann et al., 2010).

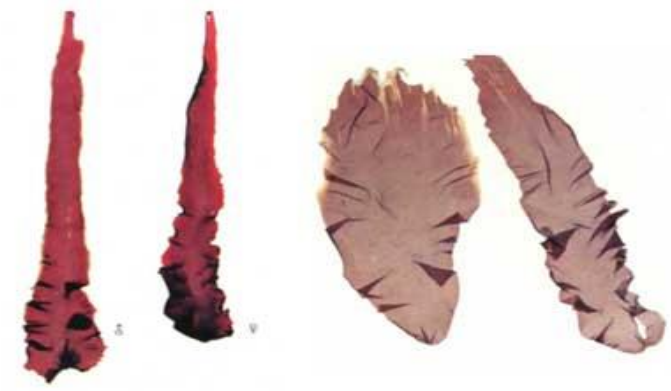

Figure 4. *Porphyra* sp. Cultured Aquatic Species Information Programme. *Porphyra* spp. Cultured Aquatic Species Information Programme. Text by Jiaxin Chen and Pu Xu. In: FAO Fisheries and Aquaculture Department [online]. Rome. Updated 18 February 2005. [Cited 30 October 2016].

2.2.4 Agarolytic Bacteria

The marine red algae (*Rhodophyta*) produce carrageenans and agars, which are the main constituents of their cell wall (Craigie, 1990). Bioconversion of carrageenans and agars is essentially performed by marine bacteria (Michel *et al.,* 2006). The first report of agar degrading bacteria was published in 1902 by Gran (Morrice *et al.,* 1983). Since then, a high quantity of bacteria has been reported with agar degradation capability. Strain isolation was performed mainly from marine environments, either in the water column, in coastal marine sediments or associated with marine red algae (Michel *et al.,* 2006). The agar degrading bacteria have also been identified from other sources, such as soil (Suzuki *et al.,* 2002; Kirimura *et al.,* 1999; Voget *et al.,* 2003; Lakshmikanth *et al.,* 2006), sewage (Hofsten and Malmqvist, 1975), fresh water (Van der Meulen *et al.,* 1974), the rhizosphere of spinach (Hosada *et al.,* 2003), and plant roots (Hosada and Sakai, 2006). Several halophilic and termophilic agar degrading bacteria have been isolated from hot springs (Shieh and Jean, 1998). Agarases are hydrolytic enzymes that degrade agar into oligosaccharides, which have various chemical properties and biological activities (Sugano et al., 1994). Several agarases have been identified in microorganism of various genera (Chi et al., 2012). They can be classified as α-agarase, β-agarase, and β-porphyranase, according to their cleavage pattern. The agarases that were characterized as α- agarase (E.C. 3.2.1.158) were those that cleave α-1,3 linkages to produce a series of agarooligosaccharides related to agarobiose (Potin et al., 1993), meanwhile the ones classified as β-agarase (E.C. 3.2.1.81) are able to cleave β-1,4 linkages to produce neoagarooligosaccharides of series related to neoagarobiose (Kirimura et al., 1999).

Glycoside hydrolases are defined as a widespread group of enzymes that hydrolyze the glycosidic bond between a carbohydrate and a non-carbohydrate moiety. Henrissat (1991) proposed a new classification system for glycosyl hydrolases, which was based on amino acid sequence similarities and not substrate specificity. An advantage of this system is that a protein or the translated sequence of even a domain can be classified before the enzyme activity is known (Henrissat *et al.,* 1993). By using this system agarases can be classified into three families of glycoside hydrolases, namely: GH-16, GH-50 and GH-86 (afmb.cnrsmrs.fr/CAZY) (Henrissat *et al.*, 1996).

2.2.5 Applications of Agarases

Previous studies have demonstrated that seaweeds contain useful substances such as vitamins, fatty acids, carotenoids and betaine (Fernández et al., 1989). The degradation of the polysaccharides in the cell wall of seaweeds is required during purification of these substances and degradation methods such as acid hydrolysis is so severe that the substances are not extracted intact. The use of enzymes such as agarases is a better alternative, because labile substances may be quite tolerant of the conditions produced during agarolytic degradation (Sugano *et al.*, 1993). Another use of agarases can be to degrade the cell walls of marine algae for production of protoplasts. Generation of large amounts of enzyme is needed for these purposes (Sugano *et al.*, 1994). In biotechnology, protoplasts can be used for biotechnology procedures such as agarophyte agar production from red algae (Araki and Arai, 1957). The agarase enzymes have potential applications in food, cosmetic and medical industries for production of oligosaccharides from agar (Lakshmikanth *et al.*, 2006). The oligosaccharides obtained by enzymatic hydrolysis of agarose have several properties, i.e. they are potent anti-oxidants and reactive free radical scavengers and can

inhibit lipid peroxidation (Wang *et al.*, 2004). These oligosaccharides can inhibit the growth of bacteria, slow the rate of degradation of starch, and are frequently used as low-calorie additives to improve food quality (Otha *et al.*, 2004). They have been shown to have antitumorigenic activity in mice (Fernández *et al.*, 1989). Studies have shown macrophage-stimulating activity and are suitable as a source of physiologically functional foods with protective and immunopotentiating activity (Yoshizawa *et al.*, 1995). One of the hydrolysis products of agarose following β-agarase degradation is neoagarobiose, which has both whitening and moisturizing effects on melanoma cells (Kobayashi *et al.*, 1997). Meanwhile, the other breakdown product of agar hydrolysis, neoagarohexaose, may probably be useful as a more effective skin moisturizer than smaller oligosaccharides, due to the viscosity of its hexamers is higher than that of smaller oligosaccharides (Otha *et al.*, 2004).

2.2.6 Characterized Agarases

The agarase activity has been reported in a wide range of microorganisms isolated from the previously mentioned environments. The characterized agarases are from the following microorganisms: *Pseudoalteromonas atlantica (*Yaphe, 1957*),* *Pseudoalteromonas antartica* (Vera et al., 1998), *Pseudomonas* sp. W7 (Kong et al., 1997), *Pseudomonas* sp. SK38 (Ryu et al., 2001), *Pseudomonas aeruginosa* AG LSL-11 (Lakshmikanth et al., 2006), a *Pseudomonas*-like bacterium (Hofsten and Malmqvist,, 1975), *Alteromonas agarlyticus* strain GJ1B (Young, et al.,1978), *Alteromonas* sp. strain C-1 (León *et al.*,1992), *Alteromonas* sp. E-1 (*Kirimura et al.*,1999), *Alteromonas sp.* SY37-12 (*Wang et al*., 2004), *Bacillus* sp. MK03 (Suzuki *et al.*, 2002), *Vibrio* sp. strain JT0107 (Sugano *et al.*,1993), *Vibrio* sp. AP-2 (Aoki *et al.*, 1990), *Vibrio sp. PO-303* (Araki *et al.*,

1998), *Cytophaga flevensis* (Van der Meulen *et al.,* 1976), *Streptomyces coelicolor* A3(2) (Kendall and Cullum, 1984), a *Microbulbifer* – like bacterium, strain JAMB-A94 (Ohta *et al.,* 2004), *Microbulbifer* strain JAMB-A7 (Otha *et al.,* 2004b), *Microscillla* sp. strain PRE1 (Zhong *et al.,* 2001), agarases from a mixed microbial population (Voget et al., 2003), *Thalassomonas* strain JAMB-A*33* (Otha *et al.*,2005), *Agarivorans* sp. JAMB-A11 (Otha *et al.,* 2005), *Acinetobacter* sp. AG LSL-1 (Lakshmikanth *et al.,* 2006), *Saccharophagus degradans 2-40* (Andrykovitch and Marx, 1988), *Zobellia galactanivorans* Dsij (Potin *et al.*,1991), *Halococcus* sp. 197A (Minegishi et al., 2013). Most of these microorganisms are gram-negative bacteria and most of the agarases are produced extracellularly, except a few agarases are produced intracellularly (Fu and Kim, 2010). There is only one report of an agar degrading archaeon from the genus *Halococcus* (Minegishi et al., 2013). Appendix 1 shows the classification of the sequenced and functionally active agarases from bacterial strains based on the glycosidase family. These are the characterized and sequenced strains from the Bacteria Domain and one from Archaea Domain, which is *Halococcus* sp.197.

2.2.7 Hydrolysis of Agar Pathway

To completely hydrolyze agar (which consists of complex polysaccharides) into the monomers β-D-galactopyranose, 3,6-anhydro-α-L-galactose and α-L-galactose-6-sulfate, we need several types of enzymes. The agarases are classified into three groups: α- agarase (EC 3.2.1.158), β-agarase (EC 3.2.1.81), and β- porphyranase (EC 3.2.1.) (Chi et al., 2012). The α- agarases can hydrolyze the α-1,3 glycosidic bonds of neoagarose and produce agaro-oligosaccharide. β -agarases can hydrolize the 1,4 glycosidic bonds. They recognize the

repetitions of agarobiose in agar. The degradation of these oligosaccharides produce neoagarooligosaccharides, with β-D-galactopyranose residues at their reducing ends (Araki, 1959). The third type of enzyme is the β-Porphyranases, which hydrolyze the β-(1,4) glycosidic bonds of the porphyrine. Recent studies suggest that there seem to exist three types of agar degrading pathways in nature (Minegishi et al., 2013; Chi et al., 2012).

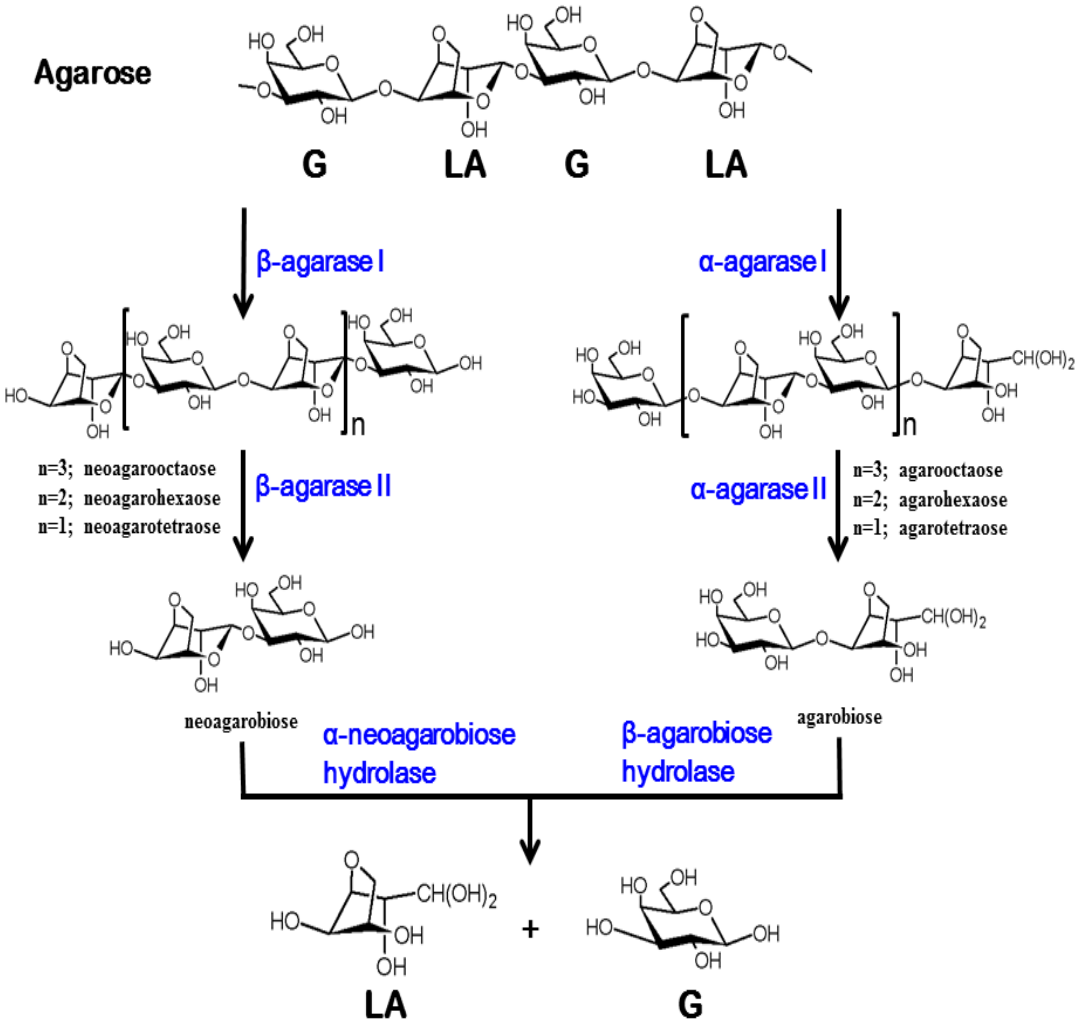

Figure 5. Schematic representation of α-agarose (right) and β-agarose (left) hydrolytic pathways. Oligomeric agarose is initially cleaved by the exported enzyme, α- or β-agarase (I). This enzyme cleaves agar at the α-(1,3) or β-(1,4) linkage, producing agarotetraose or neoagarotetraose, respectively. This tetrameric saccharide, like other oligomeric agarose, is then broken down to agarobiose or neoagarobiose through the action of α- or β-agarase (II), respectively. Finally, agarobiose or neoagarobiose is cleaved at the β-(1,4) or the α-(1,3) linkage by β-agarobiose hydrolase or by α-neoagarobiose hydrolase to produce G or LA, respectively. The classification of agarase (I), producing (neo) agarotetraose, and of agarase (II), producing (neo)agarobiose, follows the classification by Morrice et al. (1983). The LA at the reducing end of agaro-oligosaccharides forms an open, hydrated aldehydic structure (Rochas et al., 1994; Chi et al., 2012). Reprinted with permission Chi et al., 2012.

2.2.8 The α-agarolityc Pathway

The agarase enzyme has been found in different environments. However, the agarases seem to be produced by microorganism that use agar as their carbon and energy source. Many of the agar-degrading microorganisms have been found in marine environments but few have been isolated from terrestrial environments. All of the bacterial agarases that are functional and sequenced are listed in Appendix 1. α-agarases that are associated with the α-agar degrading pathway are the ones able to hydrolize α-(1,3) glycosidic linkages and have been found in marine environments. Agarases found in marine environments are produced by the microorganisms *Alteromonas agarlyticus* GJ1B (Potin et al., 1993) and *Thalassomonas* sp. JAM-A33 (Ohta et al., 2005). AgaA agarase was extracted and purified from *A. agarlyticus* and this enzyme can degrade agarose to agarotetraose and agarohexose (Potin et al., 1993). AgaA produces a native enzyme that is organized as a homodimer with an approximate molecular weight of 360kDa (Potin et al., 1993). AgaA33 was extracted from *Thalassomonas* sp. JAB-A33, which was renamed *Thalassomonas agarivorans*. The AgaA33 was isolated from deep-sea sediment at a depth of 230 m; further analysis determined that it has a molecular weight of 87kDa and a monomer structure. It is an endo α-type agarase that hydrolyses agarose to agarotetraose but can also hydrolyze agarohexaose, neoagarohexaose and porphyran (Ohta et al., 2005). Although the reducing sugar released from the degradation of porphyran with Aga33 α-agarase was not significant and even lower than that released upon treatment with AgaA β-agarase. The AgaA β-agarase belongs to the glycoside hydrolase (GH)-16 family. There are no other enzymes that have been functionally validated in the α-agarolytic pathway, therefore the direction of degradation pathway can be observed in above (figure 5). The

agarotetraose/agaroheaose must be further cleaved to agarobiose and afterward it is hydrolyzed to the monomers of β-D- galactopyranose and 3,6-anhydro-α-L-galactose in order to be used for metabolic processes (Chi et al., 2012). Hence, it is expected that enzymes such as agarotetraose/agarohexose hydrolases and β-agarobiose hydrolase should be identified in the nearby future (Chi et al, 2012).

2.2.9 The β-agarases in the β-agarolytic Pathway

The enzymes known as β-agarases can hydrolyze β-1,4 glycosidic linkages through several mechanisms. These enzymes have been found on many taxonomically diverse microbial genera. Among the genera we can find these enzymes are *Cytophaga* (van der Meulen and Harder 1976), *Pseudomonas* (Groleau and Yaphe 1977; Morrice et al., 1983), *Vibrio* (Araki et al., 1998; Dong et al., 2007; Fu et al., 2008; Liao et al., 2011; Sugano et al., 1993; Zhang and Sun 2007), *Alteromonas* (Kirimura et al., 1999; Wang et al., 2006), *Pseudoalteromonas* (Lu et al., 2009; Oh et al., 2010), *Flammeovirga* (Yang et al., 2011), and *Agarivorans* (Fu et al., 2010; Lee et al., 2006; Long et al., 2010; Ohta et al., 2004). Recent studies have found that the β-agarases can belong to four distinct GH families in the CAZy database: GH16, GH50, GH86 and GH118 (Michel et al., 2006). The biochemical characteristics of various β-agarases are listed and summarized well in the previous reviews (Fu and Kim 2010; Michel et al., 2006). The GH16 is the largest family and it consists of more than 2,800 members that are functionally heterogeneous such as β-agarases, β- porphyranases, endo-β-galactosidases, endo-β-1,3-glucanases (laminarinases), endo-β-1,3-1,4-glucanases (lichenases), κ-carrageenases, and xyloglucanases. The glycoside hydrolases of the family GH16 share the catalytic mechanism that leads to

overall retention of the anomeric configuration and transglycosylating activity (Henrissat and Davies 1997). The crystal structure of AgaD, that represents the third family of GH16 β-agarases. It contains three large insertions with respect to AgaA and AgaB (Hehemann et al., 2010). It was expected that this enzyme would have a different mode of action or a different specificity for natural agars because of divergence of amino acid sequence. The β-agarases belonging to GH16 appear to be diverse in structure and biochemical mode of action (Appendix 1). In the Appendix 1 we observe the validated agarases are similar in their properties but most of the GH16 B-agarases tend to degrade agar to longer oligosaccharides than neoagarobiose. The GH16, GH50 and GH86 families are comprised of only β-agarases and the GH16 includes 79 members; meanwhile, GH50 and GH86 contain 40 members among them. However, none of their 3D structures have been elucidated. So far, only nine agarases have been biochemically characterized (Appendix 1). The characterization shows if they have either exolytic or endo and exolytic activity and produce neoagarobiose as a major reaction product from neoagarohexaose or from agarose (Sugano et al., 1993; Ohta et al., 2005; Lee et al., 2008; Fu et al., 2008; Kim et al., 2010). In the GH86 family, we could find two agarases: AgaO from *Microbulbifer* sp. JAMB-A94 (renamed *Microbulbifer thermotolerans* JMBA- A94) and Aga86E from *S. degradans* 2-40; both have been functionally validated (Appendix 1). The β-agarase AgaO is an endo-β-agarase that can degrade agarose and agaro-oligosaccharides longer than hexamers, to neoagarohexaose as the main product (Ohta et al., 2004). On the other hand, Aga 86E has exo- β-agarase activity which produces neoagarobiose as the major reaction product form agarose (Ekborg et al., 2006). Futhermore, the GH118 family consists of only five members that include two putative and two functionally validated β-agarases.

Even though two of the β-agarases, AgaC from the marine *Vibrio* sp. PO-303 (Dong et al., 2006) and AgaB from the marine *Pseudoalteromonas* sp. CY24 (Ma et al., 2007), were classified within different genera, the amino acid sequenced deduced from their nucleotide sequences are identical, implying horizontal gene transfer between genera. Neither the AgaC or AgaB enzyme can hydrolyse neoagarooctaose or smaller neoagaro-oligosaccharides, resulting in the production of neoagarotetraose, neoagarohexaose and neoagarooctaose (major) as the end-product from agarose (Ma et al., 2007). In the β-agarolytic pathway, the agarose polymer is degraded by various β-agarases to oligosaccharides with various degrees of polymerization, such as neoagarotetraose, neoagaohexaose, and neoagarooctaose, but the overall reaction products converge to a disaccharide, α-neoagarobiose, through single or sequential reactions (Ekborg et al., 2006; Kim et al., 2010). The resulting α-neoagarobiose must be further degraded into β-D-galactopyranose and 3,6-anhydro-α-L-galactose in order to be metabolized (Figure 5, left). There have been many studies that tried to investigate the α-neoagarobiose hydrolase (NABH) responsible for the reaction, only a few biochemical experiments have demonstrated the activity of NABH in agarolytic microorganisms (van der Muelen and Harder, 1976; Suzuki et al., 2002; Day and Yaphe, 1957; Sugano et al., 1994). Even though the genetic information for NABHs is not available, they can be categorized into two biological classes depending on their cellular location: cytosolic NABH (Sugano et al., 1994; van der Muelen and Harder, 1976) and extracellular or periplasmic NABH (Suzuki et al., 2002; Dobs and Yaphe, 1975). Recently one extracellular NABH, designated 1,3-α-3,6- anhydro-L-galactosidase (AhgA), was reported from *Z. galactanivorans*- a marine flavobacterium isolated from red algae *Delesseria sanguinea*- with the capacity to

hydrolyse agars and carageenans (Barbeyron et al., 2001; Rebuffet et al., 2010). In addition, AhgA (Zq4663) is distantly related to the GH43 family but belongs to the new family GH117. In algal polysaccharides including agar and carrageenan the NABH enzyme was inactive. Furthermore, incubation of AhgA with neoagarotetraose, neoagarohexaose, and neoagarooctaose resulted in the release of a 3,6-anhydro-α-L-galactose unit, together with agarotriose, agaropentaose, and agaroheptaose, respectively. It was concluded that the enzyme AhgA catalyzes the last step in the β-agarolytic pathway, releasing 3,6-anhydro-α-L-galactose from the non-reducing end of neoagaro-oligosaccharides, including neoagarobiose. Three-dimensional structural analysis revealed that AhgA adopts the six bladed β-propeller fold, giving a putative active site with a funnel-like topology and supporting a strict exo-mode of action (Rebuffet et al., 2011). It was also assumed that AhgA formed a dimer by helix-turn-helix (HTH) domain swapping. Three acidic amino acids, Asp-97, Asp-252, and Glu-310, were thought to comprise the catalytic machinery of AhgA (Rebuffet et al., 2011). Consequently, a novel cytosolic NABH was reported from *S. degradans* 2-40 (Sde2657, EMBL ID- ABD81917). This enzyme was found to hydrolyze neoagarobiose, neoagarotetraose and neoagarohexaose and released a common product, 3,6-anhydro-α-L-galactose (Ha et al., 2011). The neoagarobiose was completely hydrolyzed into monosaccharides, indicating that Sde2657, like AhgA, is a glycosidase targeted to α-(1,3) linkages from the non-reducing end of neoagaro-oligosaccharides (Ha et al., 2011). The 3D structural analysis of Sde2657 revealed that the overall fold is homologous to that of AhgA. Particularly, Sde2657 is organized into an N-terminal helical extension and a C-terminal five-bladed β-propeller catalytic domain with three catalytic amino acids (Asp-52, Asp-207, and Glu-265), and it forms a dimer (Ha et

al., 2011). Not much data is available on NABH, but the basic biochemical properties of the two enzymes AhgA and Sde2657 seem to be almost identical. However, there are differences in the cellular location and the structural features of these two enzymes may reflect divergent evolution of NABH in the agarolytic pathway. Consequently, the fate of neoagarobiose seems to diverge along two paths, depending on the cellular locations of the NABH: (1) hydrolysis of neoagarobiose by AhgA in the extracellular space and subsequent transport of monosugar products into the cytosol (Figure 6b), or (2) transport of neoagarobiose into the cytosol before hydrolysis by Sde2657 (Figure 6a). According to the *in silico* analysis of the GH117 family proteins, 13 of the 24 members contain a signal peptide sequence for secretion, but the remaining 11 do not (Rebuffet et al., 2011). The former observation strongly supports the likelihood that two distinct paths for neoagarobiose metabolism may be widely distributed among microorganisms (Rebuffet et al., 2011).

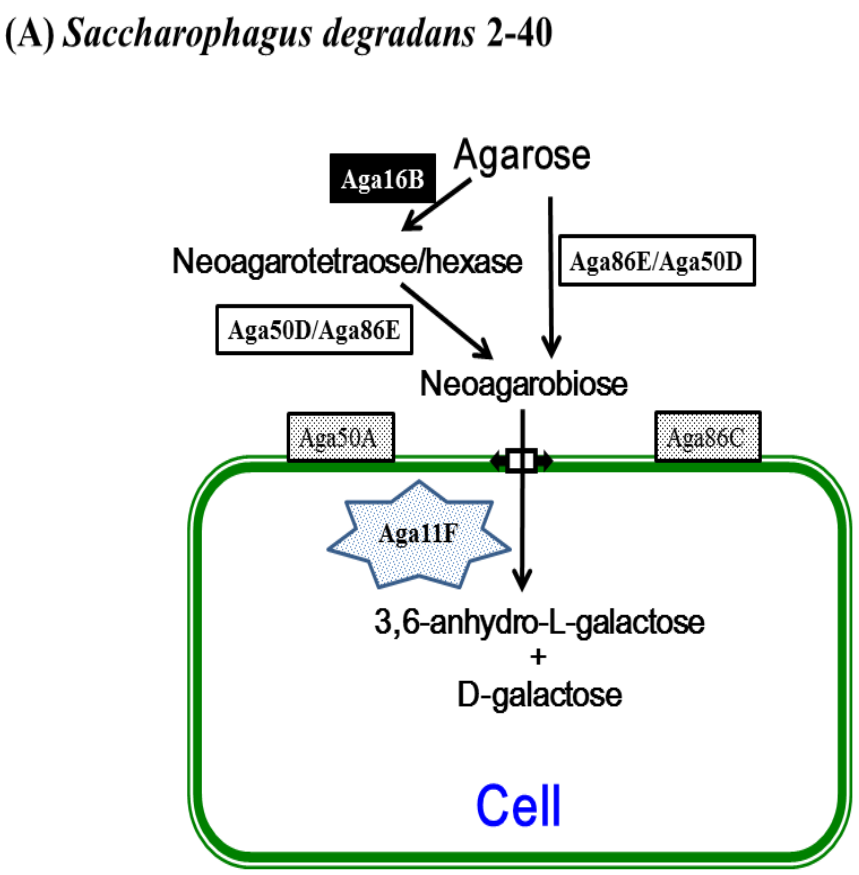

Figure 6a. Comparison of the agar hydrolytic pathway in two Gram-negative bacterial species, *Saccharophagus degradans* 2-40 (a) and *Zobellia galactanivorans* (b), and in one Gram-positive species, *Streptomyces coelicolor* A3(2) (c). This overall enzymology was constructed from the validated enzymatic properties and from the genetic information available for each strain, as detailed in the text. Black box: agarase (I), White box: agarase (II), dotted box: membrane-associated enzyme, and dotted heptagon: α-neoagarobiose hydrolase. The biochemical properties of the two putative membrane-associated proteins, Aga50A and Aga86C of *S. degradans* 2-40, have not been demonstrated. The two β-porphyranases, PorA and PorB, are expected to be periplasmic enzymes, and AgaD β-agarase is expected to be periplasmic or extracelular enzyme in *Z. galactanivorans*. The extracelular enzyme AgaD is shown for simplicity. Data extracted with permission from (Chi et al., 2012).

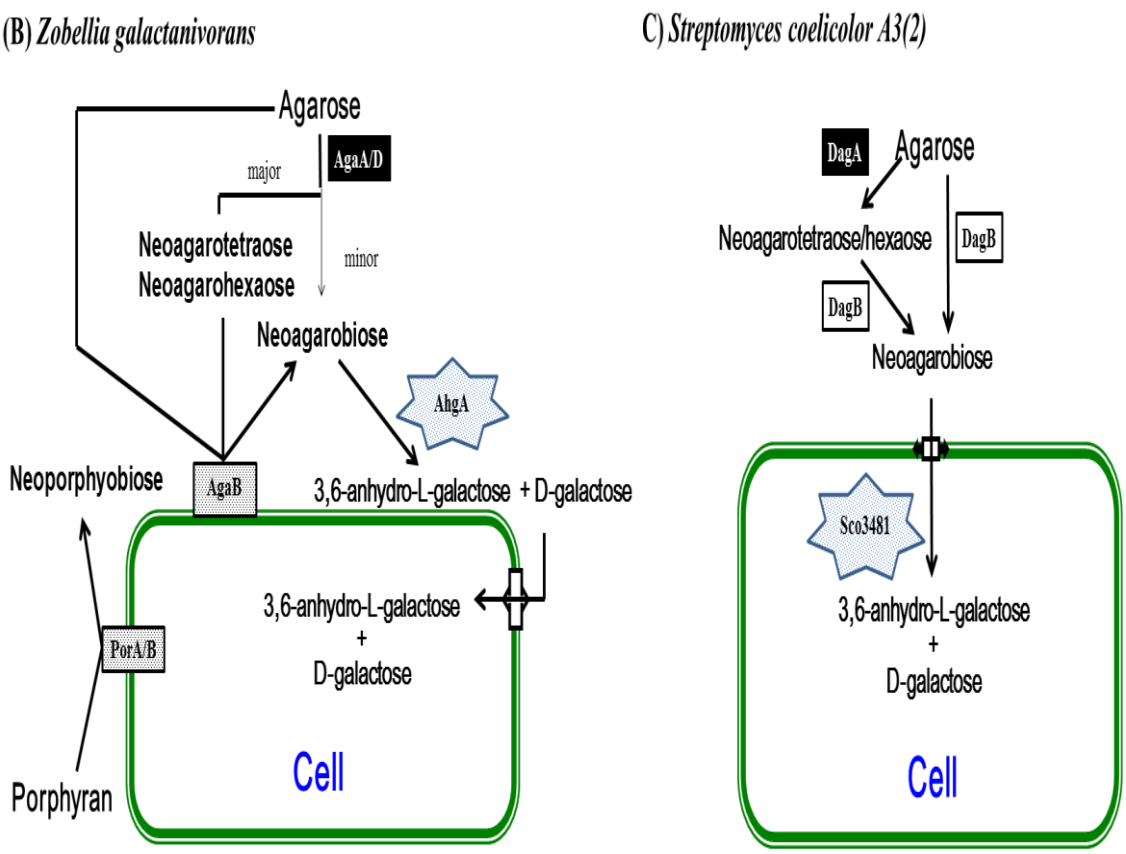

Figure 6b-c. Comparison of the agar hydrolytic pathway in two Gram-negative bacterial species, *Saccharophagus degradans* 2-40(a) and *Zobellia galactanivorans* (b), and in one Gram-positive species, *Streptomyces coelicolor* A3(*2)* (c). This overall enzymology was constructed from the validated enzymatic properties and from the genetic information available for each strain, as detailed in the text. Black box: agarose (I), White box: agarase (II), dotted box: membrane-associated enzyme, and dotted heptagon: α-neoagarobiose hydrolase. The biochemical properties of the two-putative membrane-associated proteins, Aga50A and Aga86C of *S. degradans* 2-40, have not been demonstrated. The two β-porphyranases, PorA and PorB, are expected to be peryplasmic enzymes, and AgaD β-agarase is expected to be periplasmic or extracelular enzyme in Z. *galactanivorans*. The extracelular enzyme AgaD is shown for simplicity. Data extracted with permission from (Chi et al., 2012).

2.3 The β-porphyranase Pathway

The β-porphyranases (EC 3.2.1.-) can hydrolyze the β-(1,4)-D-galactosidic linkages in porphyrans. In recent studies two β-porphyranases, PorA (Zg2600) and PorB (Zg1017), were identified and functionally validated in Z. *galactanivorans* (Hehemann et al., 2010). The two proteins that were identified belong to the GH16 family and signal peptide for localization to the periplasmic space (Appendix 1). Studies have shown that PorA and PorB lack activity toward commercial agarose or toward k-carrageenan. On the other hand, they show great activity toward purified porphyrans, with the major final product being the disaccharide porphyrobiose, indicating the cleavage of the β-(1,4)-glycosidic bond. The structural 3D analysis of PorA and PorB revealed that the structural differences in β-porphyranases that determine substrate recognition predominantly involve subsite -2, which binds an α-L-galactose-6-sulfate unit in PorA (β-porphyranase) and 3,6-anhydro-α-L-galactose unit in AgaA (GH16 β-agarase from the same bacterial species). In addition, the presence of a positively charged and hydrophobic pocket protruding into the substrate binding cleft is regarded as critical for porphyran recognition because it provides space for the bulky sulfate group substituted at carbon 6 of α-L-galactose-6-sulfate. Meanwhile, other glycoside hydrolases, including AgaA β-agarase, possess considerable steric constraints at this position that hinder productive binding and lead to an inability to hydrolyze pure porphyran. Recently, an *in-silico* analysis discovered that PorA and PorB constitute a monophyletic group distinct from the β-agarase and k-carrageenase subfamilies and forming a new subfamily within the GH16 with six other homologues (Hehemann et al., 2010). The β-porphyranase PorA almost completely degrades porphyran and predominantly produces products of the porphyrobiose series: the major product is

neoporphyrobiose and the minor product is neoporphyrotetraose. In addition, long hybrid neoporphyrobiose/neoagarobiose oligosaccharides, including the neoporphyrobiose-neoagarobiose-neoporphyrobiose hexasaccharides, are also produced by PorA. These hybrids oligosaccharides are systematically terminated at both ends, at least one neoporphyrobiose moiety, clearly demonstrating that PorA selectively cleaves the β-(1,4) glycosidic bond between the neoporphyrobiose moieties. The neoporphyrotetraose dimer can also be hydrolyzed into neoporphyrobiose, but the methylated tetramer neoporphyrobiose-C6 methylated-D-galactose-neoporphyrobiose is not futher degraded by PorA, which suggest that a demethylation step is necessary for metabolism of methylated oligosaccharides. In the hydrolysis of hybrid oligosaccharides, β-agarases are also needed to degrade the agarose repetition moieties of hybrid oligomers. The product of the degration, neoagarobiose, is likely further hydrolyzed to the monomers 3,6-anhydro-α-L-galactose and β-D-galactopyranose by an α-neoagarobiose hydrolase of the β-agarolytic pathway. There have been many reports of agar degrading microorganism as well as agarases and their genes. However, the results have been too fragmentary to interpret collectively and to overcome this limitation, researchers have adopted new techniques in proteomics, genomics and metabolomics.

3. Chapter I: Isolation, Characterization and Purification of an Agar-degrading Microorganism using Culture-dependent Techniques.

3.1 Introduction

Agar is a complex polysaccharide and the main cell wall component of red macroalgae, which is widely used as a gelling agent of microbiological culture media as well as molecular sieving in DNA electrophoresis and gel-filtration chromatography. Agar is produced comercially from the species of red algae genera *Gelidium* and *Gracilaria* and is composed of agarose and agaropectin (Aoki et al., 1990; Rochas et al., 1994). Agarases are the hydrolytic enzymes that degrade agar into oligosaccharides, which have various chemical properties and biological activities. Agarose consists of 3, 6-anhydro-L-galactose and D-galactose alternately linked by α-1, 3 and β-1, 4 linkages (Kloareg and Quatrano, 1988). Agaropectin has the same basic disaccharide-repeating units as agarase with some hydroxyl groups of 3,6-anhydro-L-galactose residues replaced by sulfoxy or methoxy and pyruvate residues (Hamer et al., 1977).

Agarases are hydrolytic enzymes that degrade agarose into oligosaccharides. They were characterized as either α-agarases (E.C. 3.2.1.158) that cleave α-1, 3 linkage to produce a series of agarooligosaccharides related to agarobiose (Potin et al., 1993), or β-agarases (E.C. 3.2.1.81) that cleave β-1,4 linkage to produce neoagarooligosaccharides of series related to neoagarobiose (Kirimura et al., 1999; Cui et al., 2014). Agarose-degrading enzymes have been attracting keen interest in many fields of biochemistry and enzymology (Fu and Kim, 2010). The neoagarooligosaccharides have various special biological activities, such as inhibition of bacterial growth, slowing down of starch degradation

thereby reducing the calorific value of food, and providing anticancer, antivirus, and anti-oxidation activities (Giordano et al., 2006). The β-agarases are also used to recover DNA from agarose gel after electrophoresis (Finkelstein and Rownd, 1978; Burmeister and Lehrach, 1989). There have been numerous reports on agarases from isolates belonging to genera of the domain Bacteria, including *Acinetobacter, Agarivorans*, *Alteromonas, Bacillus, Cytophaga, Microbulbifer, Pseudoalteromonas, Pseudomonas, Salegentibacter, Thalassomonas*, *Vibrio, Zobellia*, etc. (Hu et al., 2009; Fu and Kim, 2010), many of which are of marine origin. There have been no reports, however, on agarases from halophilic bacterial strains requiring high (more than 10 % w/v) NaCl concentrations for growth. On the other hand, only one report has been published on agarases from the domain Archaea. In this study, we isolated agarase-producing strains belonging to both domains (Bacteria and Archaea).

3.2 Materials and Methods

3.2.1 Sampling Sites

The Cabo Rojo solar salterns are in the island of Puerto Rico, which belong to the Greater Antilles located in the Caribbean. Cabo Rojo is a town in the southwest area of Puerto Rico. The salterns are in the coastal zone of Cabo Rojo and the specific coordinates are 17.9552° N, -67.2096° W. The climate is characterized by low precipitation, high temperatures, and low humidity (Davis, 1978). For samplings, the salterns were divided into three main areas: Bahía Sucia (beach), Fraternidad lagoon and crystallizers (ponds) (Figure 7).

The Guánica's abandoned salterns are in the coast to the southwest of the island (coordinates are described in Figure 3); this town also has a warm climate (Davis, 1978).

The final sampling site was the Peñuelas' abandoned salterns, which has the same climate as the other sampling sites (Figure 8). Although the sampling areas should be similar, two of the sampling sites have been abandoned for decades and; therefore, the geochemical cycles must be altered and the microbial communities should diverge.

Figure 7. Geographical location of the Cabo Rojo Solar Salterns. A. Crystallizers 17.951558N, -67.194683 W (20.0-32.0% w.v.) B. Fraternidad Lagoon 17.956523 N, -67.196832 W (8.0-15.0% w.v.) C. Bahía Sucia-Beach 17.951709 N, -67.192293 W (2.5-3.5% w.v.). Map data: Google, Google Maps ™

Figure 8. Geographical location of the Peñuelas Abandoned Solar Salterns. A. Beach 17.990332 N, -66.722512 W (3.0-3.5% w.v.) B. Crystallizer 1 17.991862 N, -66.721285 W (8.0-10.0% w.v.) C. Crystallizer 2 17.991455 N, -66.719602 W (9.5-10.5% w.v.) Map data: Google, Google Maps ™

Figure 9. Geographical location of the Guánica's Abandoned Solar Salterns. State Forest Shore sample (17.941770 N, -66.959294 W) (green arrow). Pink Pond, State Forest (17.941899 N, -66.959526 W) (red arrow), State Forrest Bay (17.942023 N, -66.959333 W)(blue arrow), Crystallizer B (17.941543 N,-66.954984 W), Crystallizer A (17.940913 N, -66.955034 W)(yellow arrow), Crystallizer C (17.940913 N, -66.955034 W)(orange arrow). Map data: Google, Google Maps ™

3.2.2 Sample Collection

Three independent samplings were performed at the extreme environments in Puerto Rico. Water samples were collected using sterile Whirl-pak®. The first sampling was carried out in the Cabo Rojo saltworks, where water samples from the "Bahia Sucia" beach, Fraternity Lagoon and the crystallizers were taken. The second sampling site was at the abandoned saltworks from Guánica, Puerto Rico in which samples were taken from the crystallizers, the hypersaline pond and the Guánica State Forest bay. The third set of samples came from the crystallizers and the "Encarnación" Beach from the Peñuela saltworks (Figure 9). All samples were transported to the laboratory for processing. The samples were filtered (volume) using 0.45µm nitrocellulose membranes and these were placed on growth media.

3.2.3 Isolation of Agar-degrading Microorganisms

The agar degrading microorganisms were screened on agar plates containing modified Synthectic *Crenarchaeota* medium (Könneke et al., 2005). The composition of the medium was as follows (in g per liter): 0.1 KBr, 1.5 $CaCl_2$, 5.0 $MgSO_4 \cdot 7H_2O$, 5.0 $MgCl_2 \cdot 6H_2O$, 1.0 yeast extract, 20.0 Bacto-agar (Difco), pH adjusted to 7.2 with 1 M NaOH and 25% HCl. Three different NaCl concentrations were used for isolation of halophilic/halotolerant microorganisms, which were (in g/L) 50.0 for 5%, 100 for 10% and 250 for 25% (w/v). A batch of the selective Synthetic *Crenarchaeota* medium was made with ampicilin to test for antibiotic resistance of the strains. Another batch of Synthetic *Crenarchaeota* medium was made without yeast extract to determine the main carbon source of the isolates.

3.2.4 DNA Extraction and Quantification

Genomic DNA extractions from pure cultures were performed using a specialized method developed in our laboratory for halophilic organisms. Briefly, cells were resuspended in 500µL of lysis buffer (0.75 M sucrose, 1% SDS, 40 mM Tris-acetate pH 7.8-8.0, 20 mM Sodium acetate pH 8.0, 1.0 mM EDTA pH 8.0), in 1.5mL microtubes with glass beads and placed in a water bath at 65°C for 10 min. Then, 20µL of lysozyme (10mg/mL Tris-Cl 10 mM) were added and incubated for 10 min at 37°C. Tubes were then placed in a horizontal platform adapter for vortex for 10 min at maximum speed. After this, 200µL of cold 5 M potassium acetate were added and the tubes incubated in ice bath for 5 min, centrifuged for 10 min at 13,000 rpm, and the supernatant transferred to a new micro centrifuge tube. Protein extractions were performed adding one volume of chloroform. Genomic DNA precipitation was achieved with 1 volume of cold 100% isopropyl alcohol and incubation at -80C for one h. DNA was finally resuspended in 50µL of TE 1X pH 8.0, added 5 µL of RNase (10mg/mL) and incubated at 37°C for 15 min. DNA quantifications were made using a NanoDrop spectrophotometer.

3.2.5 Archaeal and Bacterial 16S rDNA PCR reaction and electrophoresis

16S rDNA PCR Master Mix was made with 50µL of buffer, 25 µL of MgCl2, 5 µL of primer 7F, 5 µL of primer 927R, 10µL BSA, 10µL dNTP, 1.3µL of Taq polymerase and 177.3µL of distilled and deonized water. 1µL of DNA then was poured into each 0.5 microtubes and allowed one PCR reaction without DNA as negative control. The same 16S rDNA PCR procedure was made for bacteria, but in this case 5 µL of the primers 27F and

1492R were used. The conditions for the PCR were 95°C denature Bacteria 48°C / 52°C Archaea in annealing and 72°C extension for 35 cycles. Then conducted electrophoresis 0.8% agarose gel, where the first well contained 10 μL of genetic marker 1 kb, and the other was filled trenches were filled with 5 μL of DNA product and the chamber was filled with TAE 1X. Electrophoresis was run at 150V for 45 min.

Table 2. PCR primers for 16S phylogenetic analysis, colony PCR and the detection of genes encoding for the β-agarase

Enzyme	Gene	Group	Primer	Sequence (5'-3')	Reference	Amplicon Size	PCR Conditions
N/A	16S rRNA	Bacteria	27F	AGA GTT TGA TCC TGG CTC AG	Eden et al., (1991)	1465 bp	94°C-5 min, (94°C -1 min, 52°C -1 min, 72°C -3min) x 25 times, 72°C-10min
			1492R	GGT TAC CTT GTT ACG ACT			

Table 2. (Continued) PCR primers for 16S phylogenetic analysis, colony PCR and the detection of genes encoding for the β-agarase

Gene	Group	Primer	Sequence (5'-3')	Reference	Amplicon Size	PCR Conditions
	Archaea	7F	TTC CGG TTG ATC CTG CCG GA	Giovannoni et al., (1988)	920 bp	Same as above
		927R	CCC GCC AAT TCC TTT AAG TTT			
β-agarase	Bacteria	β-agaraseF	TAG GCT CTG CTC AGC CTT G T	Ma et al., (2006)	2411 bp	94°C-5 min, (94°C -1 min, 59°C -1 min, 72°C -3min) x 25 times, 72°C-10min
β-agarase		β-agaraseR	CGC GGT CGA GTG GTA GTT A			
	Bacteria	SP6	ATT TAG GTG ACA CTA TAG	pGEM® Vectors Promega (Promega Biotech USA)	1500 bp	95°C for 5 min, (95°C -1 min, 60°C -1 min, 72°C -3min) x 20 times, 72°C-10min
		T7	TAA TAC GAC TCA CTA TAG GG			

3.2.6 Cryopreservation of the Strains

Once the isolates were pure, we proceeded to freeze them to have them in stock for future studies. For this procedure, we began by filling 1.5 microtubes with 930 μL of the SC medium. Using a fully cultivated plate of each isolated, we scraped all the growth in the plate and placed it in the microtubes an incubated them for 2 h at 40°C. Finally, we added 70 μL DMSO and froze the strains at -80°C**.**

3.2.7 Partial Purification of the Agarases

The agarase producing isolate was cultured at 42°C with rotary shaking in 1 liter of a HA medium (in per liter): 150.0 g NaCl, 2.0 g KCl, 20.0 g $MgSO_4 \cdot 7H_2O$, 5.0 g yeast extract, 5.0 g casaminoacids, 3.0 g trisodium citrate, 1.0 g sodium glutamate, 36.0 mg $FeSO_4 \cdot 4H_2O$, 0.36 MG $MnCl_2 \cdot 4H_2O$, pH adjusted to 7.2 with 40% KOH. The cells at logarithmic phase were collected by centrifugation at 8000x g for 15 min, the pellet was seeded into 2 liters of an induction medium HA-I composed of salt and trace metal components of HA and 0.2% agarose as described by Minegishi et al., 2013. After incubation for 3 days with shaking at 42°C, the supernatant of the culture obtained by centrifugation was saturated with NaCl, and subjected to hydrophobic column chromatography using a column (2.5 X 15 cm) of TOYOPEARL Phenyl-650 M (TOSOH, Japan) equilibrated with 30% NaCl, 5 mM Tris–HCl, pH 7.0. After washing with the same buffer, elution was done with the Tris–HCl buffer of step-wise decreasing NaCl concentrations. Fractions with agarase activities were pooled, NaCl added to saturation, loaded to another column of TOYOPEARL Phenyl-650 M (1 X 10 cm), and eluted with a linear decreasing NaCl concentration, from 5.0 M to zero. Agarase-active fractions were

collected and dialyzed against 2.5 M NaCl, 5 mM Tris–HCl, pH 7.0, and concentrated with Amicon Ultra-15 Centrifugal Filter Units (Millipore). All procedures were done at 4°C and the relative protein content estimated by absorbance at 280 nm. Protein concentration of purified enzyme was determined by Lowry method, using bovine serum albumin as the standard.

3.2.8 Transformation of Competent Cells

Protocol extracted from Dagert and Ehrlich, (1979) and was used with minor modifications. The transformation was made using 50 ng of plasmid DNA containing 16S rDNA to 100 µL of competent cells (E. *coli* JM109) and left on ice for 10 min. The cells were then submitted to heatshock at 42ºC for 2 min or 37ºC for 5 min. Then, 0.9 mL of LB was added to allow expression at 37ºC for 30 to 60 min. 100 µL of cells were plated on LB containing antibiotic selection. The LB plates were incubated overnight at 37ºC.

3.2.9 Modified Ferricyanide Assay for Reducing Sugars (Park and Johnson, 1949)

The samples were made in duplicate; 100 µL of the culture supernatant was added to 100 µL of freshly-prepared 1% agarose substrate (20 mM PIPES solution 90 mL, agarose 1 g, water to 100 mL) and 200 µL 20 mM PIPES (piperazine-N-N'-bis (2-ethanesulfonic acid)) solution. Then the samples were incubated at 37 ˚C for 30 min. The reaction was terminated by adding 200 µL of Stop reagent ($Na_2HPO_4 \cdot 7H_2O$ (Saarchem) 1 g, NaOH (Saarchem) 1 g, water to 100 mL). This was followed by the addition of 300 µL Color reagent ($K_3(Fe(CN)_6)$ (Merck) 116 mg, NaOH 220 mg, water to 100 mL) and incubating the tubes in a boiling water bath for 2.5 min. The tubes were cooled at 22ºC and the

absorbance at 420 nm was determined. Agarolytic enzyme activity (agarolytic units: U) can be expressed as µg galactose produced per mL per h or per min. The concentration of agaro-oligosaccharides was determined from a standard curve of absorbance at 420 nm versus neo-oligosaccharides concentration (µg galactose/mL) to determine the type of agarase.

3.3 Polyacrylamide Gel Electrophoresis

To prepare a SDS-PAGE, the following reagents were combined: 12% separating gel mix in a glass beaker, 4 mL of 40% acrylogel, 2.5 mL of 4X separating gel buffer, sterile water to 9.95 mL, 50 µL of 10% ammonium persulfate, and 15 µL of TEMED. The separating gel mix was poured into the assembled gel plates, leaving sufficient space at the top for the stacking gel. The gel mix was gently overlaid with 0.1% SDS. After polymerization, the overlay was removed and the surface of the separating gel was rinsed to remove unpolymerized acrylamide. Then the 5% stacking gel was prepared as as follows: 625 µL of 40% acrylogel, 1.25 mL of 4X stacking gel buffer, 4.97 mL of sterile water, 25 µL of 10% ammonium persulfate and 15 µL of TEMED. The stacking gel mix was poured and the comb inserted immediately. After the stacking gel polymerized, the comb was removed and the wells were rinsed to remove any unpolymerized acrylamide. The assembled gel was placed into the electrophoresis apparatus and the tank filled with SDS-PAGE running buffer. The protein samples were prepared by adding 5 µL of SDS-PAGE sample buffer to 5-15 µL of protein sample. The protein samples were denatured by boiling for 3 min at 96ºC and loaded into the bottom of the wells and the gel ran at constant current of 15 mA in the stacking gel and 30 mA in the separating gel. After electrophoresis,

the visualization of the protein bands in the gel was achieved by staining with Coomassie blue dye for 15 min at 37ºC. The gel was placed in the destaining solution for 120 min.

3.3.1 Thin-Layer Chromatography Analysis (TLC)

The blot reaction mixes were added onto a Silica gel 60 (0.015-0.040 mm) covered in aluminum foil (20 cm x 20 cm) (Merck) in aliquots of 1 µL, until 3 µL were loaded per sample at the bottom of the gel. Samples were allowed to dry after each 1 µL blot. The Silica gel was placed in an upright position in a tank filled with 100 mL of the solvent n-butanol: acetic acid: water and the tank was sealed. The blots were allowed to develop until the solvent was 1-3 cm from the top of the gel. The visualization of the resulting saccharides was achieved by spraying the gel with the naphthoresorcinol reagent.

3.3.2 Agarase Enzyme Assay

Specific activity of purified agarase was determined according to a modified method of Ohta et al. (2004), using different substrates including 1% food-grade agar, 1% agarose and 1% carrageenan. Appropriately diluted enzyme solution was added to different substrates in phosphate buffer (pH 8.0) at 45°C, and was incubated at 45°C for 30 min. Activity was expressed as the initial rate of agar hydrolysis by measuring the release of reducing ends using the 3,5-dinitrosalicylic acid (DNS) procedure with D-galactose as the standard. One unit of the enzyme activity was defined as the amount of protein per min produced 1 µmol of reducing sugar as D-galactose under conditions of the assay.

4. Results

4.1 Isolation, purification and characterization of the microbial isolates based on their 16S rRNA gene.

The three beach samplings demonstrated an average NaCl concentration of 3.83% (w/v). The sampling was carried out at the Cabo Rojo, Guánica and Peñuelas Saltworks (Figures 7-9) and the salinities were measured in the laboratory using a hand refractometer (Table 3). From the samplings, we obtained 55 isolates from which initially 17 demonstrated agar degrading activity (Table 4). The isolates were grown on SC medium with molecular agarose, yeast extract and agar. They were also grown on the same medium without molecular agarose, yeast extract and agar. It was observed that the isolates contained a variety of pigmentation (Figures 10-12). A pattern of growth can be seen in Table 4, as salinity increased the archaea population increased, while the bacteria decreased.

The isolates were purified and selected by their agar degrading abilities. Then, a DNA extraction was performed from the purified isolates. Furthermore, the isolates were classified using the molecular markers for the 16S rRNA genes. The phylogenetic approach consisted of the use of the PCR technique with specific 16S rDNA primers (Table 2). This technique allows the differentiation between the archaea and bacteria. In Figure 15 the amplicon of the bacterial 16S rRNA gene with the expected molecular weight (1465 bp) can be observed. In addition, in Figure 16 the amplicon with the size of 920 bp can be seen. A 16S rRNA gene cloning was performed of the agar degrading isolates. An agarose electrophoresis was prepared to verify the inserts of the cloning (Figure 14). The clones were sent to the Macrogen® sequencing facility. Sequences were analyzed and compared in the Eztaxon database (Table 7).

Table 3. Sampling Sites Salinity Percentage in w/v at time of the sample collection.	
Gúanica Solar Saltern 1	12
Gúanica Solar Saltern 2	15
Gúanica Solar Saltern 3	14
Gúanica Solar Saltern 4	4
Lagoon Gúanica	14
Gúanica Pond	3
Candelaria	9
Crystallizer Private Salterns 1	32
Crystallizer Private Salterns 2	12
Crystallizer Private Salterns 3	35
Crystallizer Private Salterns 4	30
Bahía Sucia Beach	3.5
Candelaria Beach	4
Crystallizer Salterns 1	30
Crystallizer Salterns 2	30
Crystallizer Peñuela Saltern 1	7
Crystallizer Peñuela Saltern 2	8
Encarnación Beach	4

Table 4. Classification of the isolates from the three sampling sites organized by salinity.

Salinity Percentage (%NaCl w/v)	Total Isolates (per salinity)	Classification (16SrRNA)		Agar Degrading Isolates (per salinity)
		Bacteria	Archaea	
5	10	10	0	8
10	14	10	4	7
15	19	9	10	2
25	12	0	12	0
Total	**55**	**29**	**26**	**17**

Table 5. Characterization of the isolates based on their salinity, color, agar degradation and sampling site.

Isolates MD	Colony Color	Agar degradation	No YE Growth	0.1% YE Growth	Molecular Agarose	Sampling Site
23	Beige	+	+	+	+	Beach
24	Beige	+	+	+	+	Beach
25	White	-	+	+	+	Crystallizer
26	Beige	+	+	+	+	Beach
27	White	-	+	+	+	Fraternity
28	White	-	+	+	+	Fraternity
29	Beige	+	+	+	+	Fraternity
30	White	+	+	+	+	Fraternity
44	Beige	+	+	+	+	Beach
45	Beige	+	+	+	+	Beach
46	Beige	+	+	+	+	Beach
47	White	+	+	+	+	Beach
48	Beige	+	+	+	+	Beach
49	Beige	+	+	+	+	Fraternity
50	Beige	+	+	+	+	Beach
51	White	-	+	+	+	Fraternity

Table 6. Characterization of the Isolates from Guánica and Peñuelas Salterns

Isolates	Colony Color	Agar degradation	No YE Growth	0.1%YE Growth	Molecular Agarose	Gram Stain
MP2B	Beige	+	+	+	+	-
MD25A	Beige	+	+	+	+	-
MP4	White	-	+	+	-	+
MP2A	Beige	+	+	+	+	+
MP1B	Beige	+	+	+	+	+
PP1	Beige	+	+	+	+	+
PP2	Beige	+	+	+	+	-
GS3A	White	-	+	+	-	-
CSN4B	White	-	+	+	-	+

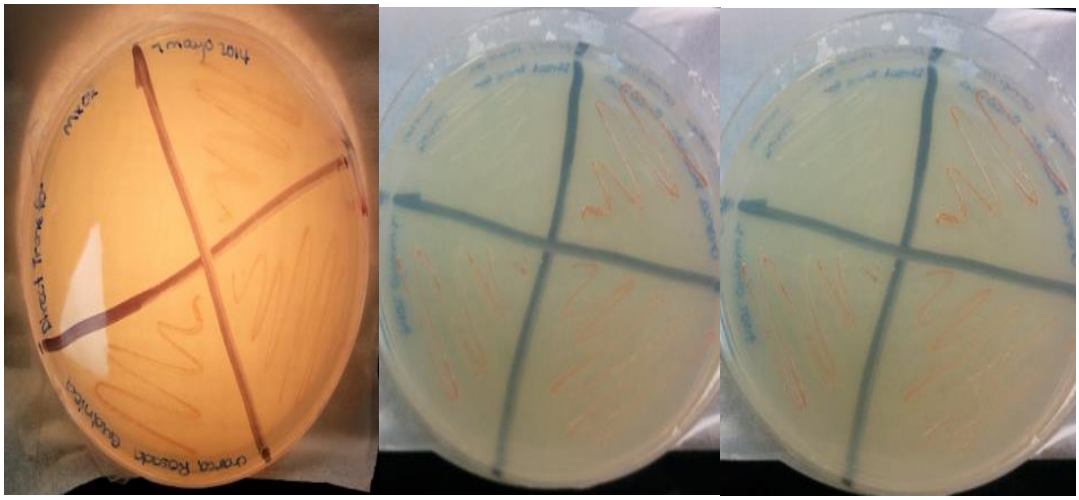

Figure 10. Morphology of the colonies in medium with 0.1% Yeast Extract at 5%, 10% and 25% NaCl (w/v). Isolated from the abandoned saltworks in Guánica, P.R.

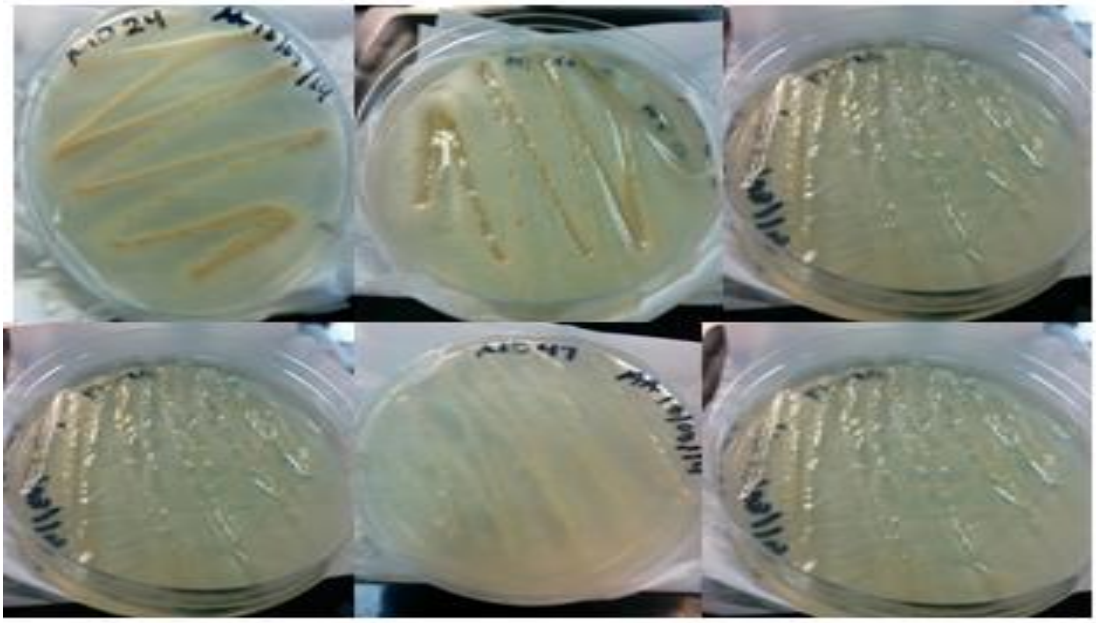

Figure 11. Morphology of the colonies in SC medium with 0.1% Yeast Extract at 5% and 10% NaCl (w/v). Isolated from the crystallizers, "Bahía Sucia" Beach and the Fraternity Lagoon from the Cabo Rojo saltworks.

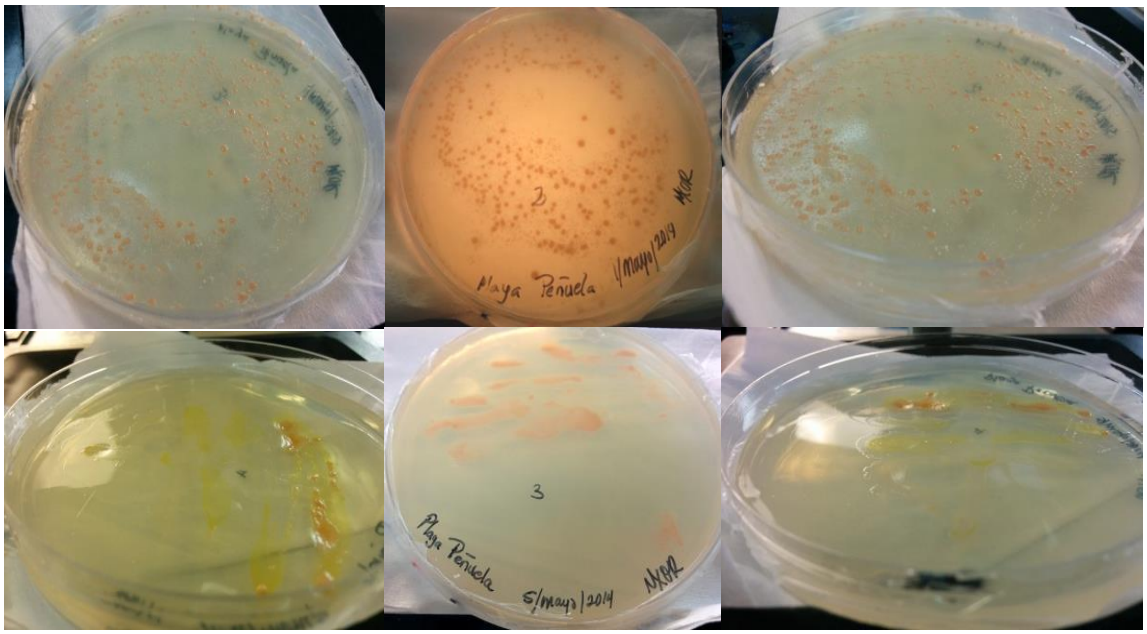

Figure 12. Morphology of the colonies in medium with 0.1% Yeast Extract at 5%and 10% NaCl (w/v). Isolated from the crystallizers and the “Encarnación” Beach from the Peñuelas saltworks.

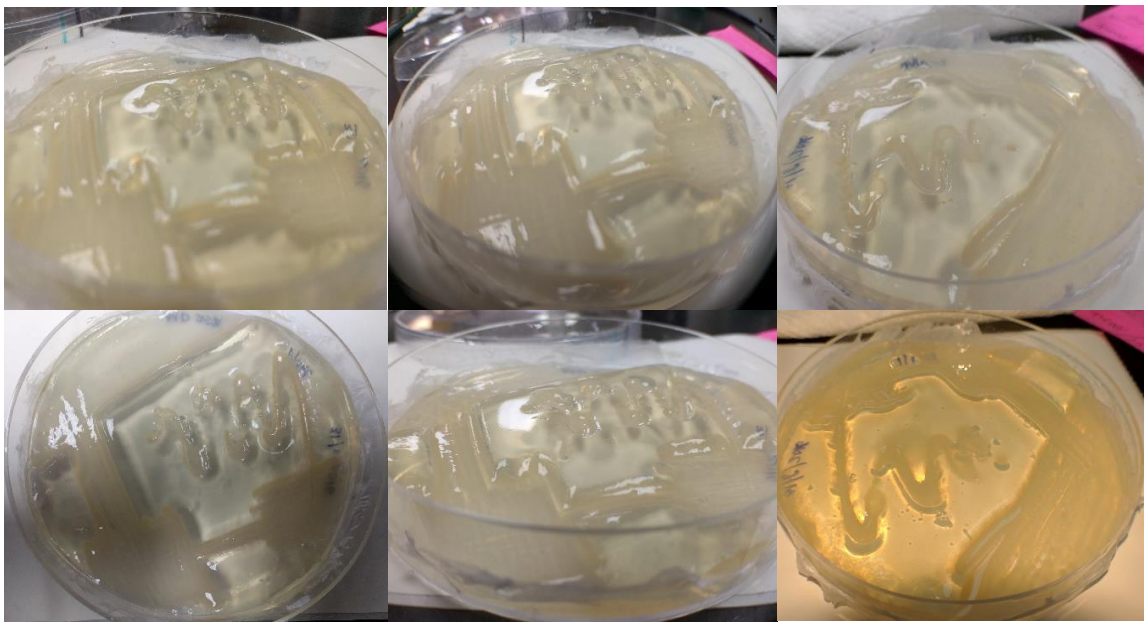

Figure 13. Agar degrading isolates from the Guánica and Peñuelas samplings. The images show the agarase enzyme degrading the agar in the medium.

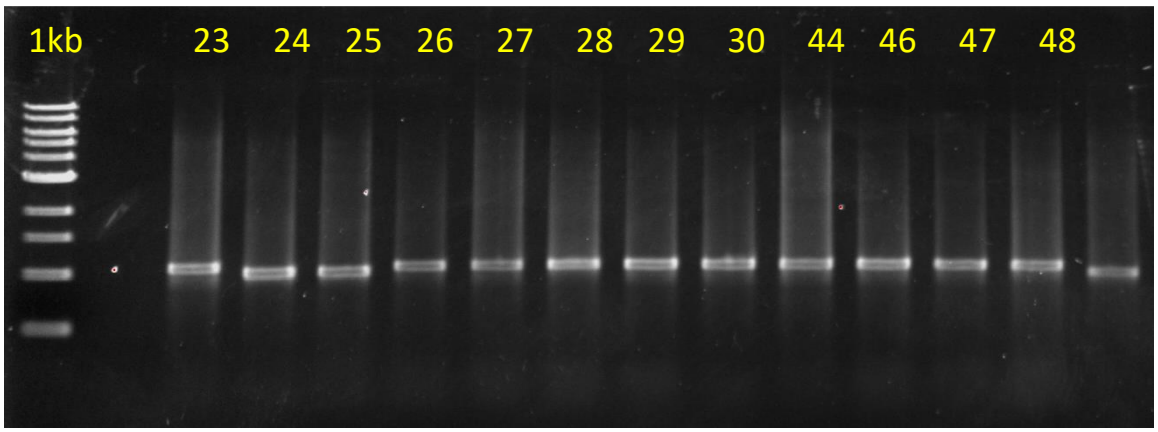

Figure 14. 16S rDNA Colony PCR agarose gel electrophoresis. The primer set used: Sp6 and T7. The expected amplicon for the primer set is 1200 bp; the agarose gel percent was 0.8%. MD23, MD27, MD28, MD29, MD44 and MD46 are isolates obtained from the "Bahía Sucia" beach (MD23, MD44-46), MD25 from the crystallizers, and the Fraternity Lagoon (MD27-29, 49-51). The DNA ladder used was 1kb.

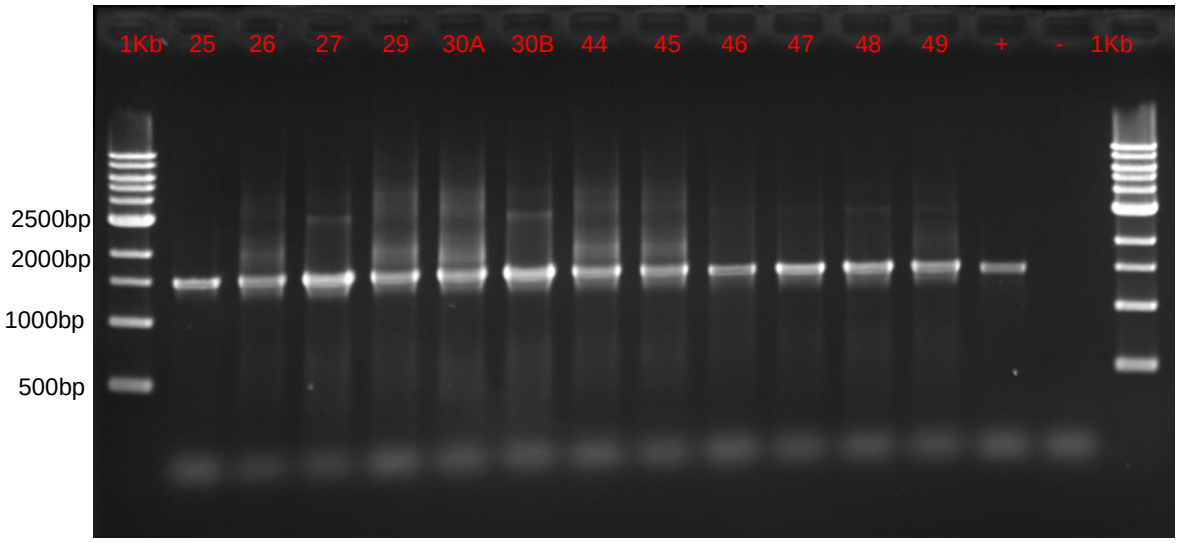

Figure 15. 16S rRNA PCR agarose gel electrophoresis. The primers set for bacteria were 27f and 1492r. The expected amplicon from bacteria primers is 1,465 bp for bacteria; the agarose gel percent was 0.8%. Wells contained the samples named MD 25-49 contained 5uL of genomic DNA of the isolates. A negative control without DNA was used. The DNA ladder used was 1kb.

Table 7. Phylogenetic identities of the isolates based on 16S sequences from the clones.

Isolate	Genebank no.	Sequence description	Sequence Size(bp)	Query Coverage (%)	E-value	Identity %
MD23	CP006884.1	*Haloarcula hispanica* N601 chromosome 1, complete sequence	1003	90	0.0	99
MD24	CP002921.1	*Haloarcula hispanica* ATCC 33960 chromosome I, complete sequence	960	90	0.0	96
MD25	EF153424.1	Uncultured *Haloarcula* sp. clone ntu13 16S ribosomal RNA gene, partial sequence	985	62	0.0	98
MD26	CP002921	*Haloarcula hispanica* ATCC 33960(T)	1023	73.73	0.0	89.41
MD29	AGIN01000009	Natrinema pellirubrum DSM 15624(T)	821	45	0.0	49.06
MD44	EF645681	*Haloarcula argentinensis* ICM9737(T)	956	65.54	0.0	96.66
MD47	AB010964	*Haloarcula quadrata* 801030/1(T)	1010	67.97	0.0	94.47
MD25A	AY526861	*Idiomarina fontislapidosi* F23(T)	1206	95	0.0	90.0
MP4	AJKS02000002	*Halomonas smyrnensis* AAD6(T)	1093	94	0.0	92.45

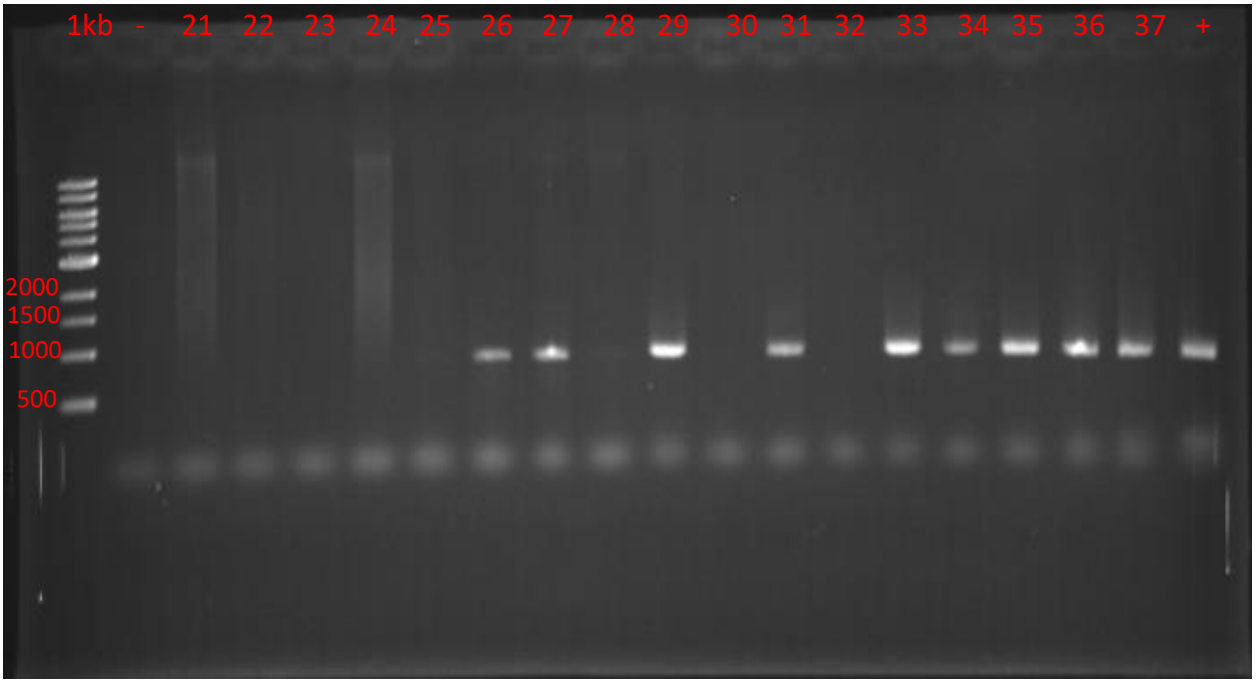

Figure 16. 16S rRNA PCR agarose gel electrophoresis. The primers set for Archaea were 7F and 927R. The expected amplicon from the Archaea primers is 920 bp; the agarose gel percent was 0.8%. A negative control without DNA (-) was used and the positive control was *H. borinquense* (+). The DNA ladder used was 1kb. Wells contained the samples named MD 21-37 contained 5uL of genomic DNA of the isolates from the three samplings.

4.2 Identification and Phylogenetic Analysis of the Isolates

We sequenced approximatedly 1200 bp of the 16S rRNA gene for each of the 8 bacterial isolates. The isolates were selected based on observed agar degrading activities. Three phylogenetic trees were constructed with the sequences obtained from our isolates and retrieved sequences from GeneBank database (Figures 17-19). Based on the similarity of the sequences, we used 4 of these sequences from our isolates (MP4, MD44, MD45, MD28C) to construct the Neighbor-joining phylogenetic trees (Figure 17). Meanwhile, the other 4 sequences from our isolates (MD25A, MD47, Playa Peñuelas 1, MP3B) that were related were used to construct the Neighbor-joining phylogenetic tree (Figure 18) and the Maximum Likelihood phylogenetic tree (Figure 19). It is shown that 4 of the isolates (MP4, MD44, MD45, MD28C) grouped with the genus *Halomonas*. The other 4 of the isolates (MD25A, MD47, Playa Peñuelas 1, MP3B) grouped with the genus *Idiomarina*. Strain MP4 was closely related to *Halomonas smyrnensis*. MD44, MD45 and MD28C were related to *Halomonas ventosae*. These isolates are related to known halophilic bacteria and they can grow in a highly selective medium that favors the growth of autotrophic microorganisms, as well as in nutrient-rich media. The MP4 and MD25A isolates were selected for further studies since results show the possibility of being new species.

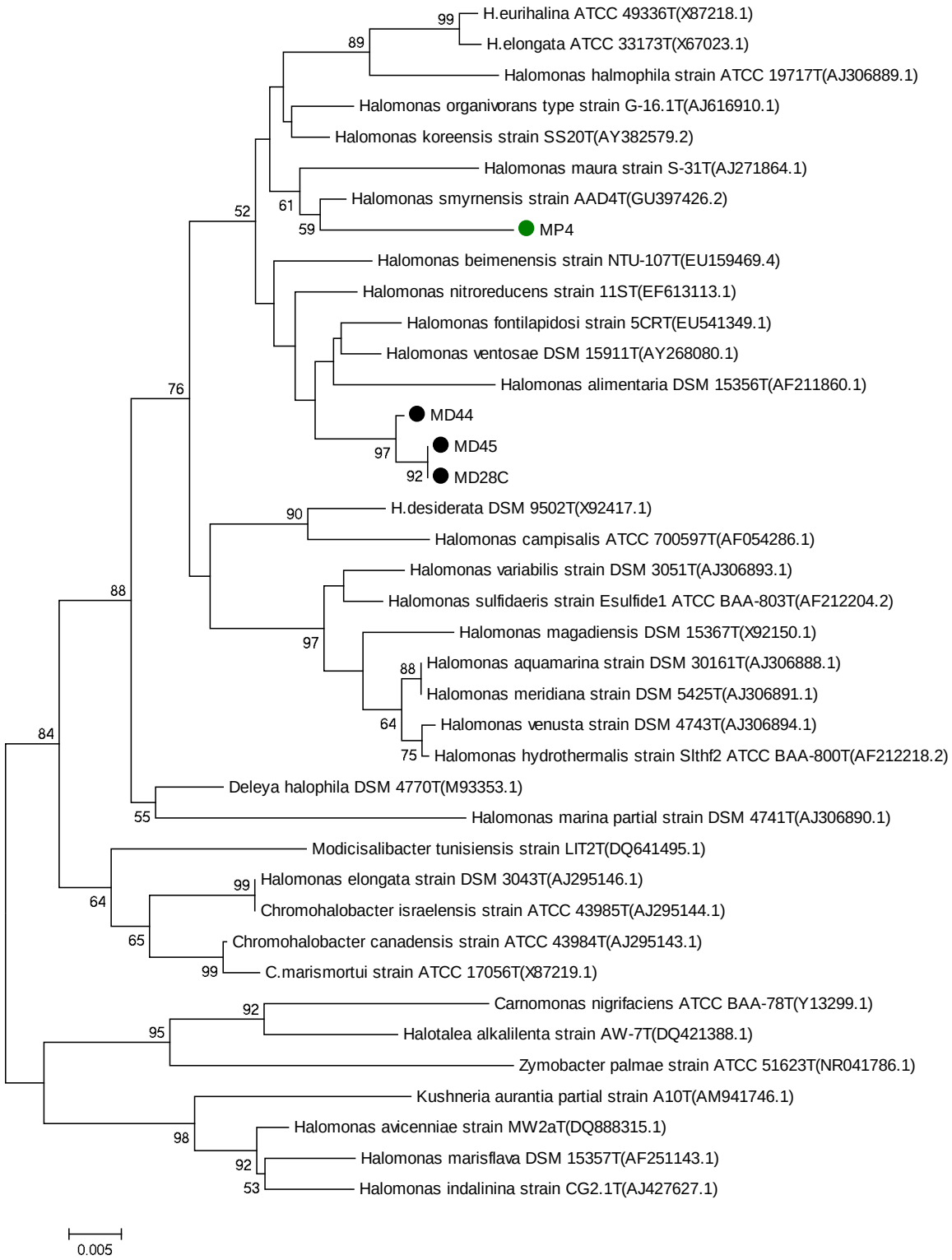

Figure 17. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of strains MP4, MD44, MD45, MD28C, and closely related species. Sequence accession numbers are shown in parentheses. Bootstrap values greater than 50% are indicated at branch points. *Zymobacter palmae* ATCC 51623T was used as an outgroup.

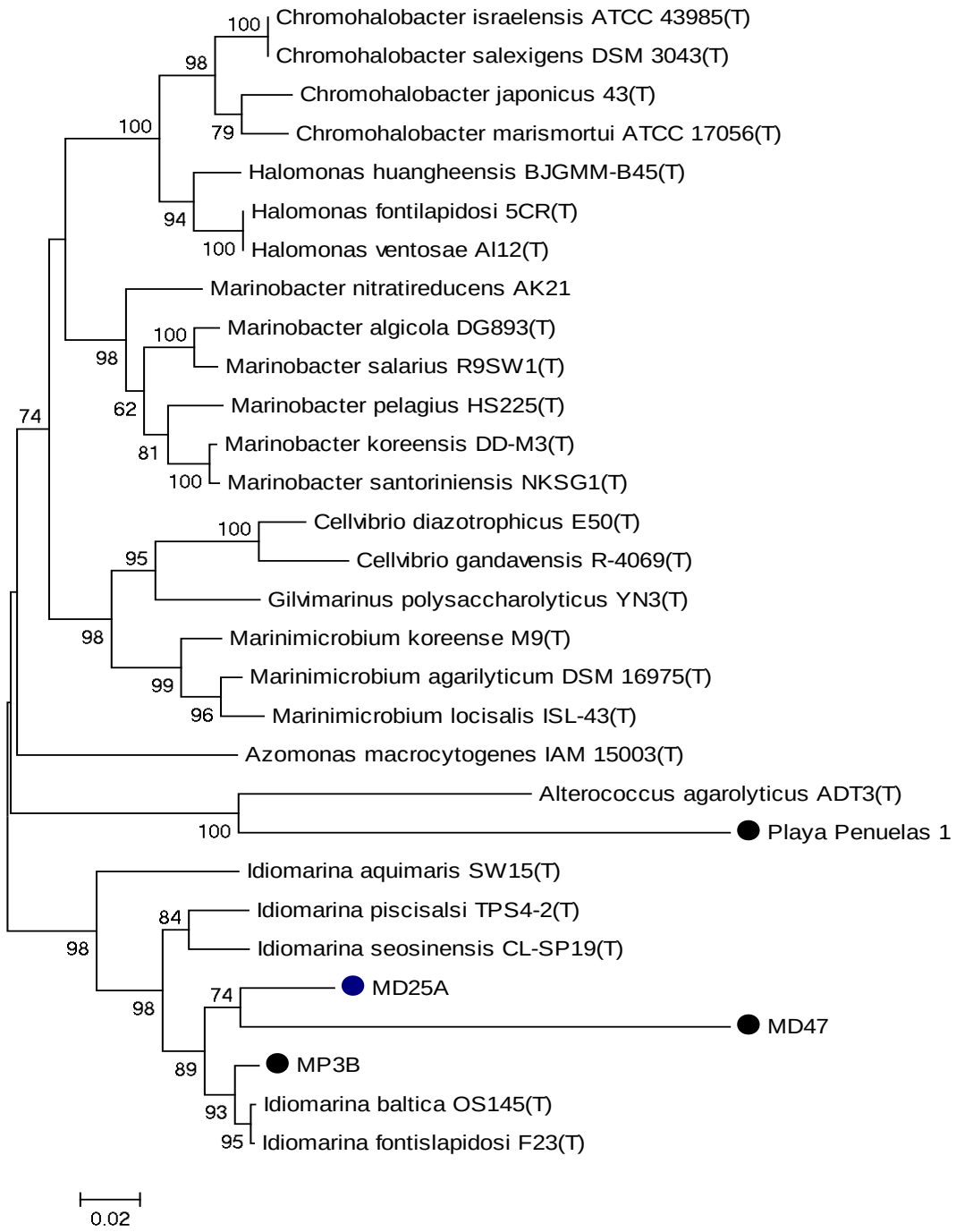

Figure 18. Neighbor joining phylogenetic tree based on 16S rRNA gene sequences of strain MD25A, MD47, Playa Peñuelas 1, MP3B which present agarase activity and closely related species. Sequence accession numbers are shown in parentheses. Bootstrap values greater than 50% are indicated at branch points.

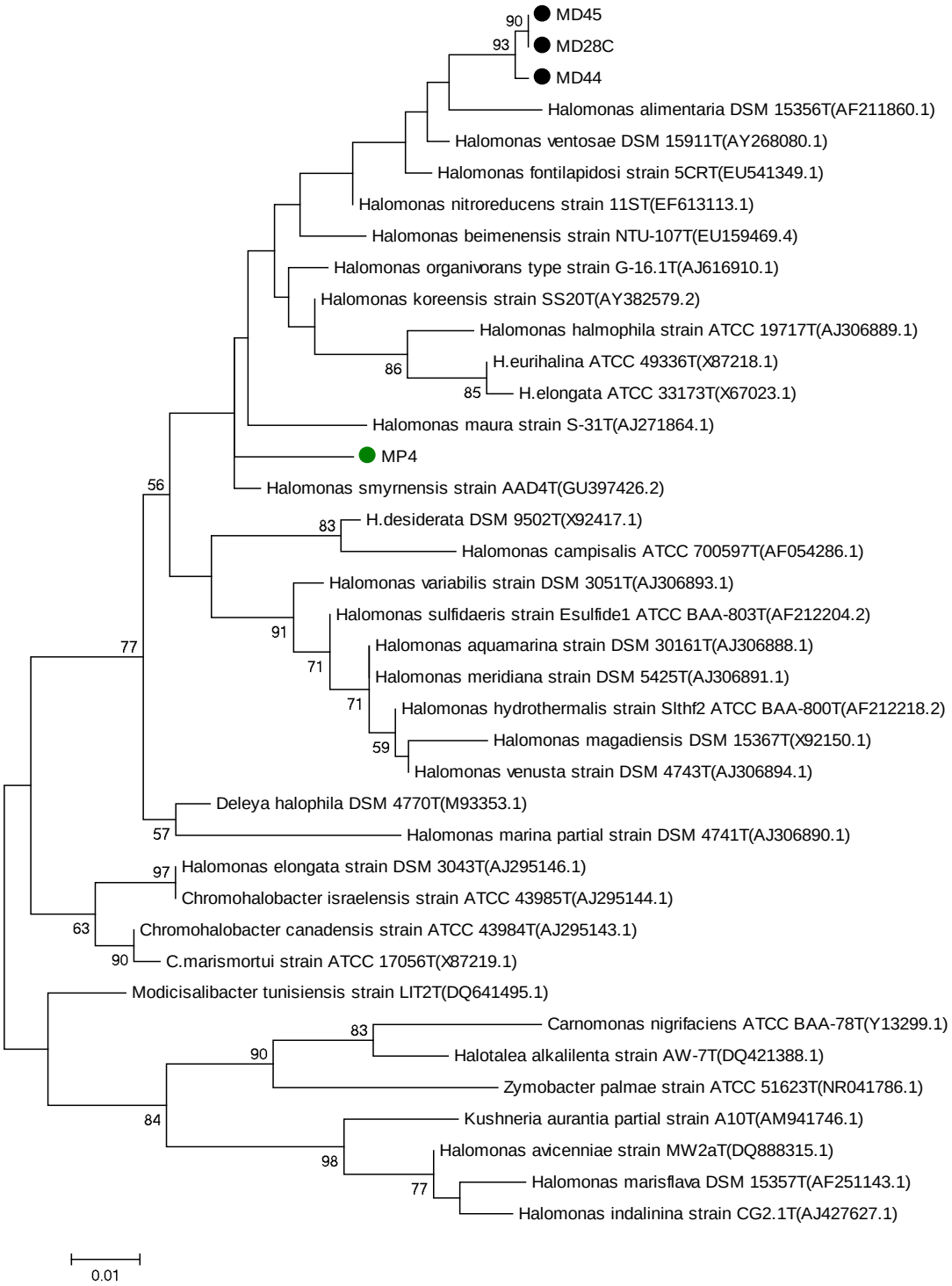

Figure 19. Maximum Likelihood phylogenetic tree based on 16S rRNA gene sequences of strains MP4, MD44, MD45, MD28C, and closely related species. Sequence accession numbers are shown in parentheses. Bootstrap values greater than 50% are indicated at branch points. *Zymobacter palmae* ATCC 51623T was used as an outgroup.

The previously selected isolates presented beige pigmentations and grew without yeast extract (Figures 10 and 13). To observe cellular morphology, a Gram staining of the isolates was performed. Strain MP4 was Gram-positive and MD25A was Gram-negative, although the cell morphology for both isolates was cocci. Tests such as optimum salinity, optimum temperature, growth inhibition by organic nutrients, hydrolysis of casein, catalase, and oxidase were also performed for strains MD25A and MP4. Results of these tests are presented in Table 6.

An antibiotic resistance profile was completed on isolates. As seen in Table 10, both isolates were susceptible to ciproflaxin (5 mg), nitrofuraton (300 mg) and ampicillin (50 mg). MD25 was resistant to gentamicin (50 mg), streptomycin (100 mg), rifampin (50 mg), sulfamethoxazole (25 mg), and erythromycin (25 mg). Meanwhile, MD25A was susceptible to tetracycline (50 mg), nalidixic acid (30 mg), chloramphenicol (25 mg), and vancomycin (25 mg). MP4 was resistant to nalidixic acid (30 mg), gentamicin (50 mg), streptomycin (100 mg), tetracycline (50 mg), vancomycin (25 mg), and chloramphenicol (25 mg). A carbohydrate hydrolysis test was also performed and the results are shown on Table 9. The carbohydrates used for these tests were sucrose, glucose, sorbitol, pyruvate, xylose, glycerol, lysine, mannitol, maltose, arabinose and fructose.

Table 8. Biochemical and molecular profiles for the isolates MD25A and MP4

Characteristics	MD25A	MP4
Colony Pigmentation	Beige	Beige
Gram Stain	-	+
Growth without yeast extract	+	+
Cell morphology	Cocci	Cocci
Optimal NaCl concentration	8	8
Optimal pH	8	8
Growth Temperature		
Range	20-60	20-60
Optimal	37	30
Casein hydrolysis	+	+
Catalase Test	+	+
Oxidase Test	+	+
β-agarase gene	+	-
Tween 80	-	-
Starch	-	+
Urea	-	-
Nitrate reduction	+	+
L – Arginine	+	+
Citrate	-	-

Table 9. Carbohydrate hydrolysis test of the isolates. (+) capable of degradation, (-) incapable of degradation

Sugar Hydrolysis	MD25A	MP4
Sucrose	+	-
Glucose	-	+
Sorbitol	+	+
Pyruvate	-	-
Xylose	-	-
Glycerol	+	+
Lysine	+	+
Mannitol	+	+
Maltose	+	-
Arabinose	+	+
Fructose	+	+

Table 10. Antibiotics resistance test (+ = resistant; - = susceptible) of the two possible new species of bacteria.

Antibiotic	MP4	MD25A
Ciproflaxin	-	-
Nalidixic Acid	+	-
Gentamacin	+	+
Streptomycin	+	+
Ampicillin	-	-
Tetracycline	+	-
Rifampin	+	+
Vancomycin	+	-
Chloramphenicol	+	-
Sulfamethoxazole	+	+
Nitrofuranton	-	-
Erythromycin	+	+

4.3 Biochemical Characterization of the Putative Agarase and Enzymatic Essays

Strain MD25A was selected for further analysis since it contained an enzyme capable of degrading agar. A PCR reaction was performed using β-agarase specific primers. The results of the PCR can be observed in Figure 20, where the amplification of the isolate's β-agarase gene (amplicon size 2,411 bp) can be noticed. Partial sequencing of the agarase gene was performed and the results of the analysis are summarized in Table 11. The sequence of this agarase enzyme was analyzed with BLAST database. Matching results based on sequence similarity are summarized in Table 11.

A growth curve analysis was performed to determine the physiological behavior of MD25A. The data of the growth kinetics are shown on Figure 21. The effects of salt concentration were measured using the following three salts: potassium chloride, sodium chloride and lithium chloride. The relative activity was measured and the highest activity was detected in potassium chloride (Figure 22). The optimal temperature of the agarase enzyme was determined in Figure 23. The residual activity and enzyme activity was also measured in five temperatures 35ºC, 40ºC, 50ºC, 60ºC and 70ºC (Figure 24). The optimal activity of the purified agarase was 37ºC and it was functional between the temperature range 35-60ºC (Figure 24).

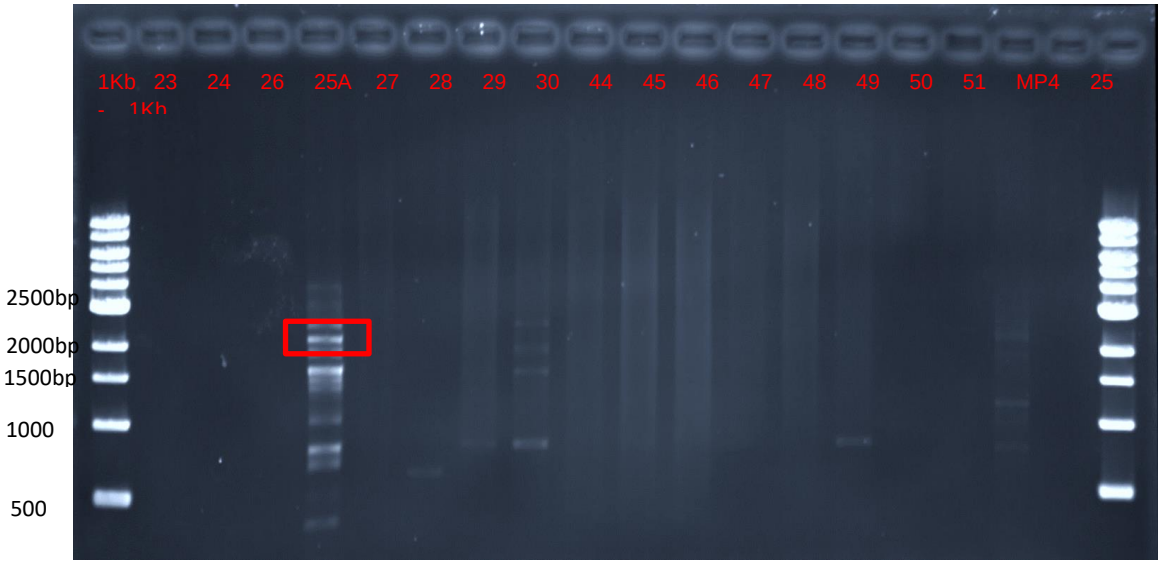

Figure 20. Preliminary identification of possible β-agarase gene. The primers set for bacteria were β-AgaF and β-AgaR. The expected amplicon from β-agarase primers is 2,411 bp; the agarose gel percent was 0.8%. 10µL of the genomic DNA of the isolates named MD23-51 and MP4 were added to the wells 23-51. These isolates were obtained from the Cabo Rojo and Guánica Saltworks. A negative control without DNA was used. The positive control was a mixture of DNA from several agarases. The DNA ladder used

Table 11. Similarity between the putative protein expressed by the agarase gene from the MD25A and agarases from other bacteria.

Accession number	Protein/Organism	Sequence identity (%)
WP_027330223.1	agarase [*Marinimicrobium agarilyticum*]	90
WP_066959277.1	agarase [*Microbulbifer* sp. Q7]	85
WP_020208742.1	hypothetical protein [*Gilvimarinus chinensis*]	83
WP_041522724.1	agarase [*Gilvimarinus agarilyticus*]	82
WP_049721026.1	agarase [*Gilvimarinus polysaccharolyticus*]	82
WP_055014349.1	agarase [*Pseudoalteromonas* sp. P1-13-1a]	63
WP_011575416.1	hypothetical protein [*Pseudoalteromonas atlantica*]	62
WP_039987978.1	agarase [*Paraglaciecola mesophila*]	61
GAC26688.1	beta-agarase B [*Paraglaciecola mesophila* KMM 241]	61
WP_008301658.1	beta-agarase B [*Paraglaciecola agarilytica*]	60

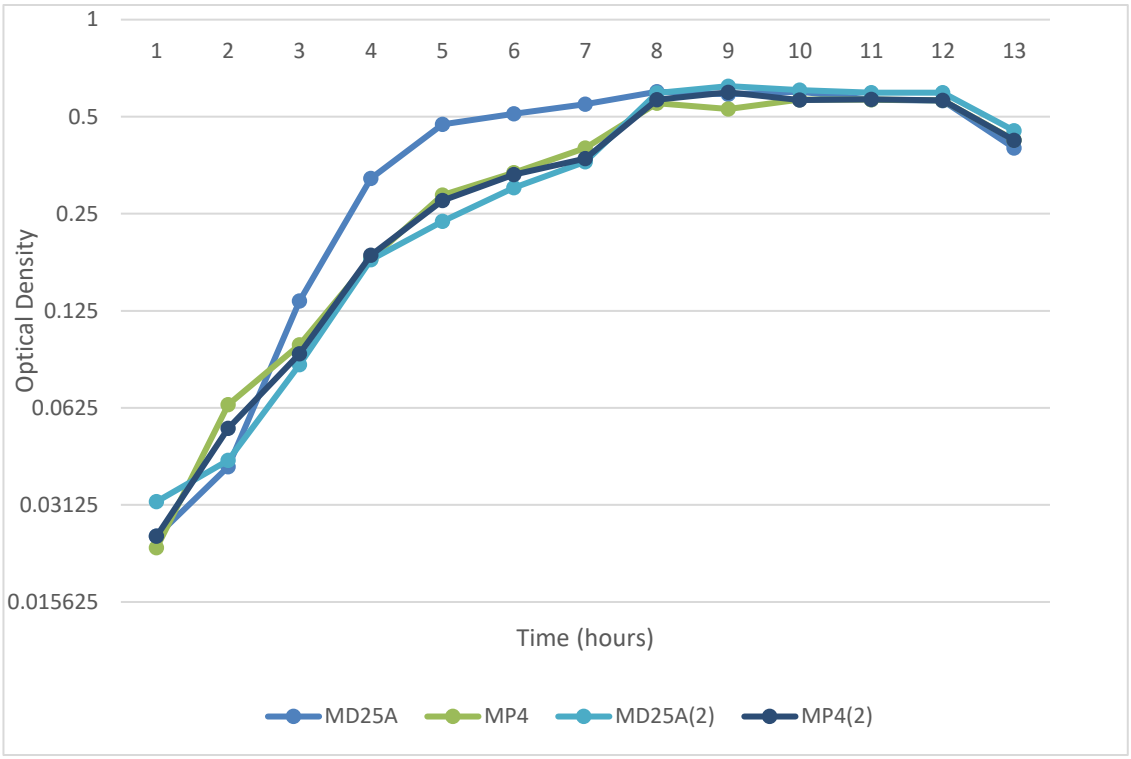

Figure 21. MD25A and MP4 strains growth curves. OD was measured at 600nm. Measurements were taken every four h. The measurements for the growth curves were made in triplicates.

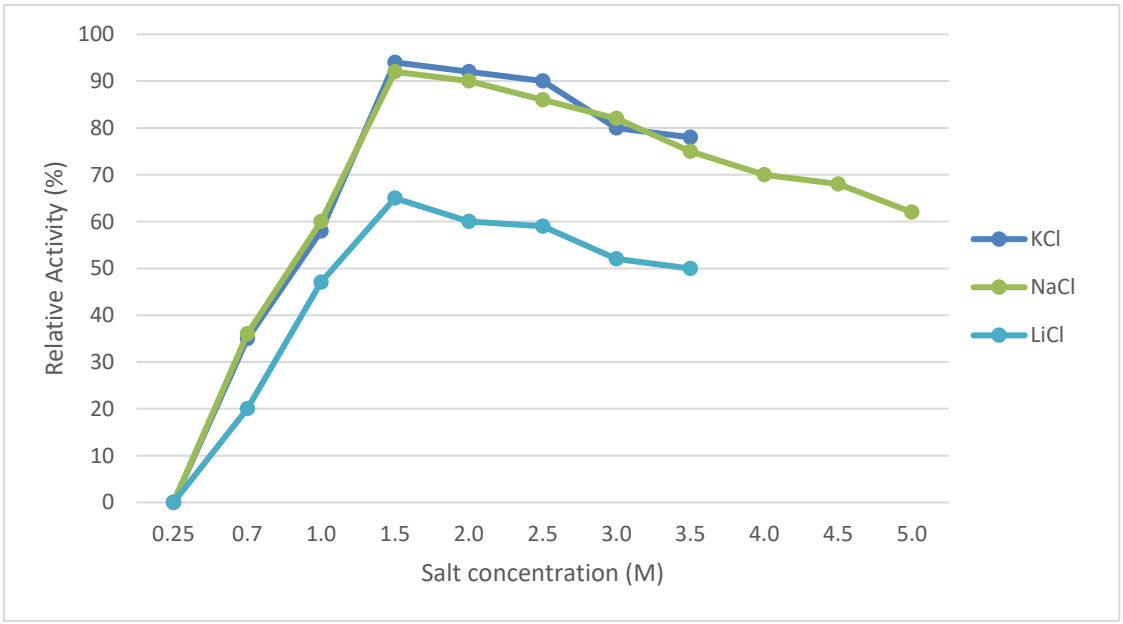

Figure 22. The effect of NaCl, KCl and LiCl concentrations on the activity of the agarase from MD25A determined at 37ºC using 0.3% agarose in the standard buffer (50 mM MES, pH 8.0) containing 0–5.0 M NaCl and 0–3.5 M of LiCl or KCl at intervals of 0.5 M. The measurements were made in triplicate.

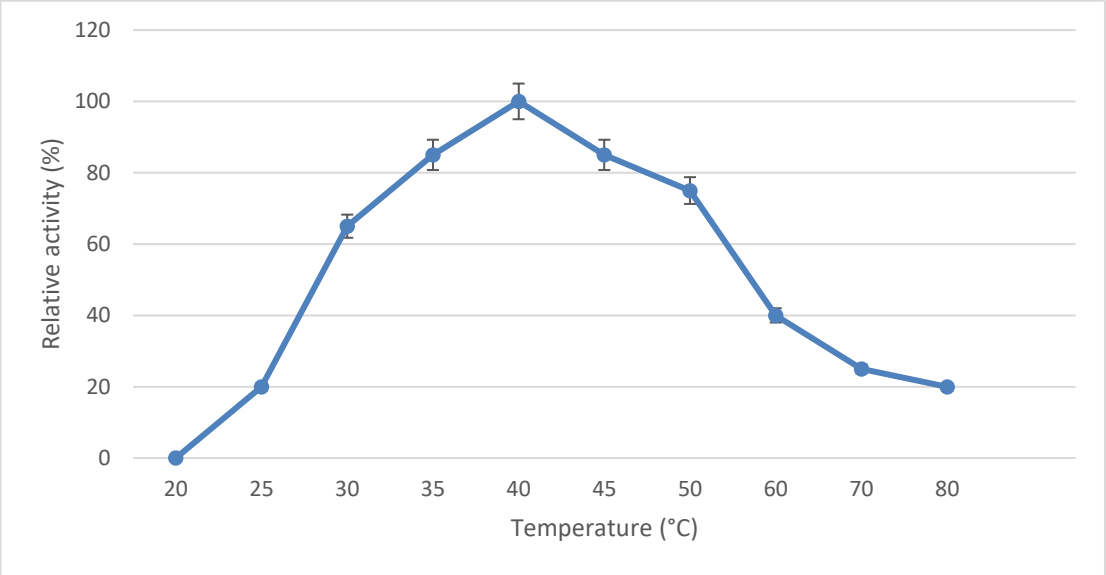

Figure 23. Effect of temperature on the enzyme activity and stability of purified agarase from MD25A. The enzyme activity was measured at temperatures ranging from 20 to 80°C using MES-NaOH buffer (pH 8.0) with DNS method at different temperatures (20–80°C). The maximum value (1.04 U ml^{-1}) observed at 37°C was considered 100%.

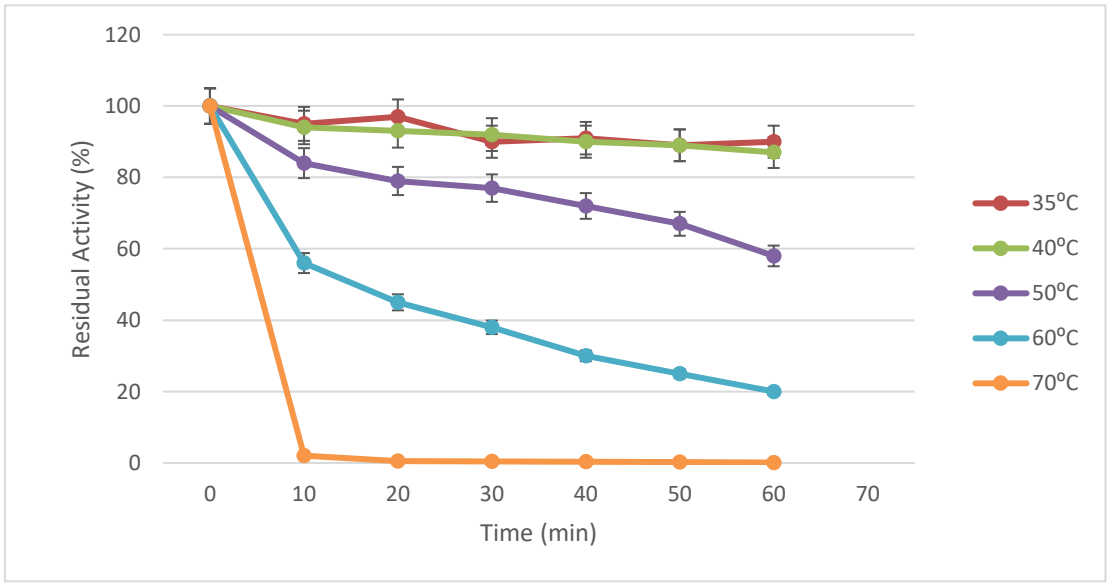

Figure 24. Effect of temperature on the enzyme activity and stability of purified agarase from MD25A. For the thermal stability of the agarase, the enzyme was incubated at indicated temperatures for 0–60 min. Thermostability of the enzyme was determined at 37°C after preincubation at different temperatures (35–70°C) for 1 h with a 1.5 M NaCl concentration.

4.4 Determination of Molecular Weight of the Purified Agarase Enzyme and DNS Hydrolysis Assay.

Figure 25 shows the result of the sodium dodecyl sulfate polyacrylamide gel electrophoresis in which the molecular weight of the agarase protein was determined. The weight of the purified protein was 97 kDa. The agarase activity assay was performed at 37°C at different pH conditions. The highest activity (0.96 U mL^{-1}) obtained at pH 8 was considered 100% (Figure 26). Thin-layer chromatogram of hydrolysis products by the agarase MD25A on food grade agar and neoagarooligosaccharides. TLC analysis results are shown in Figure 27. At the beginning of the reaction, the predominant product was the neoagarobiose (NA2). After 24 h, NA2 continued to be observed. After 24 h, two major spots (NA2 and NA4) were observed.

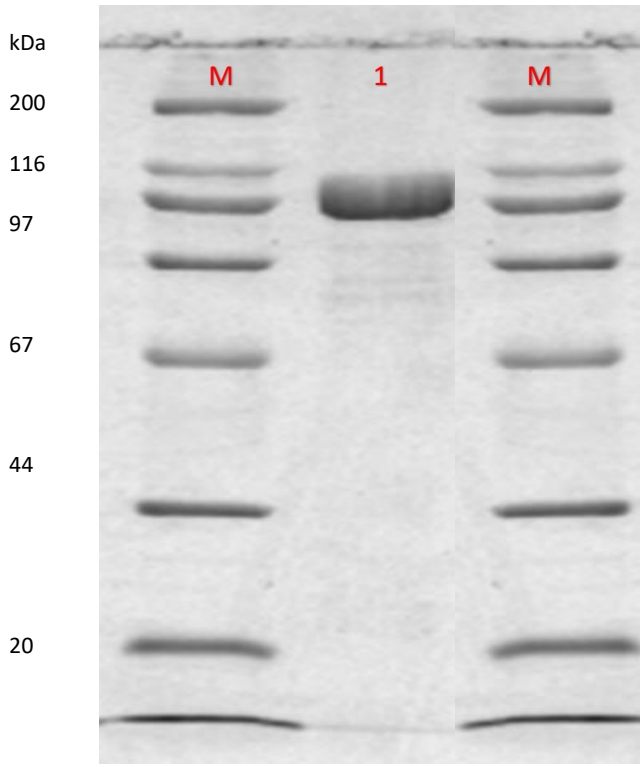

Figure 25. SDS-PAGE of the purified agarase from MD25A in 5–20% gradient polyacrylamide gel (lane P). Protein mass markers (in kDa) are indicated on the both side (lane 1). The protein was stained with Coomassie brilliant blue.

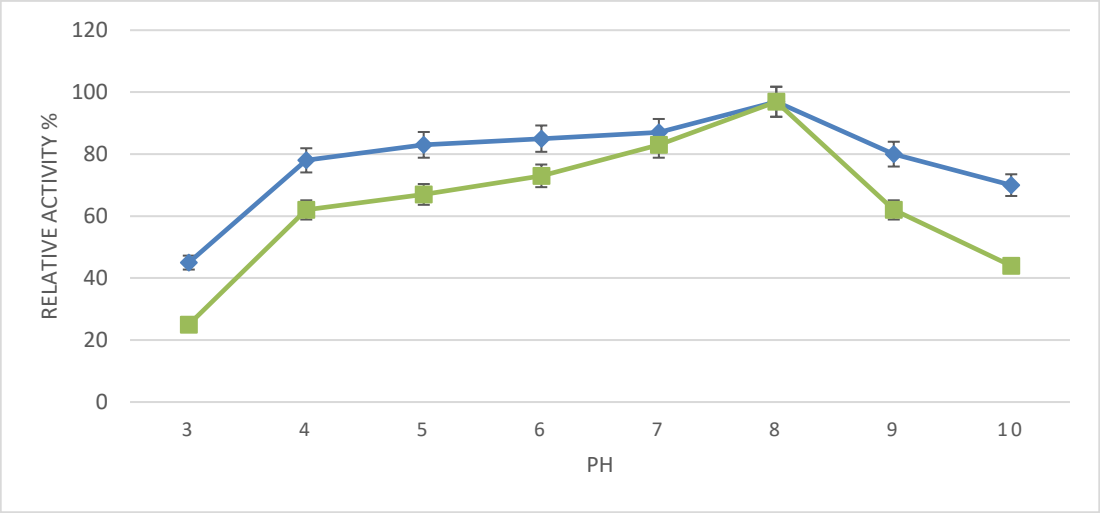

Figure 26. The effect of pH. The agarase activity assay was performed at 37°C at different pH conditions. The highest activity (0.96 U ml−1) obtained at pH 8 was considered 100%. 20 mM sodium acetate buffer (pH 3–6), 20 mM Tris/ HCl buffer (pH 7–9), and 20 mM glycine–NaOH buffer (pH 10). Filled squares, optimum pH; filled diamonds pH stability.

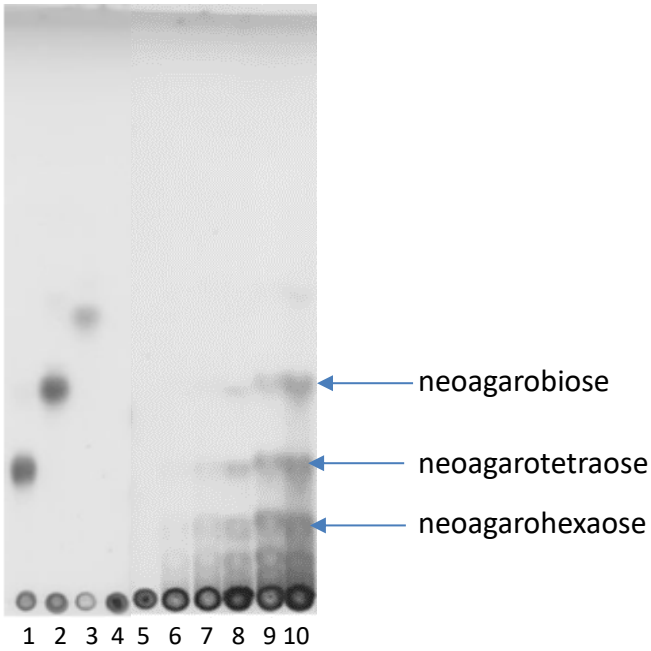

Figure 27. Thin-layer chromatogram of hydrolysis products by the agarase MD25A on food grade agar and neoagarooligosaccharides. Lanes 4–10, agarase MD25A (after incubation at 37ºC for 0h, 1h, 2h, 4h, 8h, 16 h, 24h), Markers were neoagarohexaose (lane 1), neoagarotetraose (lane 2), and galactose (lane 3). The developing solvent was chloroform: methanol: acetic acid (3:3:1, v/v), and products were visualized by spraying with 10% (v/v) H_2SO_4.

5. Discusion

5.1 Isolation, Purification and Characterization of the Microbial Isolates based on their 16SrRNA Gene

In this study the aim was to isolate agar-degrading microorganims from a saline environment in Puerto Rico. The decomposition of agar is of the upmost importance in the cycling of matter in the ocean, where agar and other polysaccharides are part of the carbohydrate constituents of many marine algae. In the summary performed by Fu and Kim (2010), many of the agar-degrading bacteria are of marine origin. Since solar salterns are filled with seawater, there is the possibility of finding this type of enzyme in these environments in Puerto Rico. Whereas numerous haloplilic Archaea and Bacteria have been isolated from solar salterns and lakes (Oren 2002; Enach et al. 2007; Fu and Kim, 2010; Sánchez-Porro et al., 2009; Soto-Ramírez et al., 2008), there have been no reports on agar-degrading microorganisms in Puerto Rico. The first sampling was at the Cabo Rojo Saltworks. From this sampling, we obtained 30 isolates from which initially 10 demonstrated agar-degrading activity. In the process of purification, some of the isolates lost their agar-degrading activity and we were left with 8 active strains. Figure 11 shows the colony color and morphology in SC medium with 0.1% Yeast Extract at 5% NaCl (w/v) of the isolates. These strains were isolated from the crystallizers, “Bahía Sucia” Beach and the Fraternity Lagoon located in the Cabo Rojo saltworks. The sampling in Peñuelas was also successful because we obtained 9 isolates from which 4 showed agar degrading activity.

The isolates were grown in media with yeast, without yeast, using agarose instead of agar and under these conditions, organisms grew and continued to degrade agar or agarose, respectively (Figure 11, Tables 5 and 6). These organisms could grow with or

without using yeast as their single-carbon source while keeping the agarase activity. The biomass generation time was reduced when using yeast extract in the media. Because the strains can grow in a culture medium that has bicarbonate and ammonium chloride as the only nitrogen and electron sources, their growth on the medium strongly suggests that these isolates are capable to degrade agar or agarose regardless of the nutrients available.

Tables 5 and 6 show the characterization of the isolates. The purified isolates were then analized by performing a 16S rRNA gene PCR. Hence, 29 of the isolates amplified the bacteria amplicon and 26 of the isolates amplified the Archaea amplicon (Figures 15-16). We also performed a β-agarase PCR and results indicate that we have two isolates with β-agarases (Figure 20). The PCR products were purified and sent to the Macrogen Corporation to be sequenced. The facility provided the sequencing data of the 16SrRNA, which we used for phylogenetic analysis. We generated several phylogenetic trees (Figures 17-19). The 16S rRNA sequences of isolates MD25A and MP4 were significantly different from those reported for the respective database matches.

The neighbor-joining tree topology suggests that the strain MP4 is related the *Halomonadaceae* and might be a new species of *Halomonas* (Figures 17, 19). Strain MP4 was closely related to *Halomonas smyrnensis*. MD44, MD45 and MD28C were related to *Halomonas ventosae*. Strain MP4 was isolated by culture on a modified Synthetic *Crenarchaeota* medium (pH 8.0) containing 8% NaCl from a water sample of the abandoned Guánica salt flats, which is situated in the south west in Guánica, Puerto Rico. This MP4 strain is moderately halophilic and can grow in SC medium containing NaCl concentrations ranging from 5% to 25% and it is consistent with the characteristics of the *Halomonas* spp. (Sánchez-Porro et al., 2009). The genus *Halomonas* is a diverse genus of

halophilic, alkalophilic gammaproteobacteria (Vreeland et al., 1980). The family *Halomonadaceae* includes four previously-known genera of halophilic bacteria: *Halomonas, Chromohalobacter, Alcanivorax, Kushneria*, and the recently described *Cobetia*, plus two genera of nonhalophilic bacteria, *Zymobacter* and *Carnimonas* (Arahal et al., 2002; Dobson and Franzmann, 1996; Garriga et al., 1998; Yakimov et al., 1998; Sánchez-Porro et al., 2009). The genus *Halomonas* was originally proposed to accommodate one species, *Halomonas elongata* (Vreeland et al., 1980). It now contains a large number of recently discovered species (Heyrman et al., 2002; Romanenko et al., 2002), as well as already known bacteria which were initially assigned to other genera, such as *Deleya, Volcaniella* and *Halovibrio* (Mellado et al., 1995; Quesada et al., 1984; Valderrama et al., 1998; Ventosa et al., 1998), but have since been included in *Halomonas*. The strain MP4 appears to be a new species of *Halomonas*, but further studies need to be performed to describe it.

Meanwhile, strain MD25A exhibited a similarity percent of 90.6 (EZtaxon database) using 1206 bp of its 16s rDNA gene, which together with their physiological profile may indicate that is a new species of *Idiomarina*. The organism demonstrated agar-degrading activity. It appears to be closely related to *Idiomarina fontislapidosi* F23 (Figure 18) and the phylogenetic tree was generated using four isolates and the sequences from the closest relatives. The tree topology suggests that the strain MD25A might be a new species related the *Idiomarina* and possibly with agarase activity which has not been described in the literature (Figure 18). The genus *Idiomarina* belongs to the halophilic bacteria and it currently comprises 26 species (Parte, 2013). All members of the genus *Idiomarina* possess characteristically high contents of iso-branched cellular fatty acids and have been isolated

from saline environments (Jean et al., 2009). These bacteria were isolated from saline habitats with a wide range of salinities, such as coastal and oceanic waters, coastal sediments, submarine hydrothermal fluids, solar salt-making works, and inland hypersaline wetlands (Gupta et al., 2011; Jean et al., 2009). Strain MD25A was isolated by culture on a modified Synthetic *Crenarchaeota* medium (pH 8.0) containing 8% NaCl from a water sample of the Cabo Rojo salt flats The MD25A strain is halophilic and can grow in SC medium containing NaCl concentrations ranging from 5% to 25%, and the charateristics are consistent with the *Idiomarina* spp. (Martínez-Canovas, 2004).

To further characterize both stains representing possible novel species, several tests were performed such as antibiotic resistant, carbohydrate dehydration, Gram-staining, and the traditional biochemical tests. The MP4 was Gram positive and MD25A was Gram negative, although they were both cocci. Gupta et al. (2011) compared several *Idiomarina* species based on the optimum salinity, optimum temperature, growth inhibition by organic nutrients, hydrolysis of casein, and catalase. The species treated in their study show similar characteristics to MD25A had (Gupta et al., 2011). Meanwhile, MP4 presented similarity in terms of phenotypic characteristics to the species of *Halomonas* (Martínez-Canovas, 2004).

MD25A was the isolate for which the β-agarase gene was amplified. A preliminary sequencing of the genome of MD25A was performed. The agarase gene from the sequencing was analized in the BLAST database. The respective matches obtained from the database demonstrated that our enzyme had a 90% similarity value when matched with the protein sequences available on the database. Based on the results, this suggest that the enzyme is a new agarase which had not been previously described.

5.2 Determination of Molecular Weight of the Purified Agarase Enzyme, TLC and DNS Hydrolysis Assay.

Then we proceeded to extract the agarase from the MD25A isolate using the protocol described by Minegishi et al. (2013). The purified agarase from our MD25A strain was halophilic and lost activity in absence of NaCl as do most enzymes from halophilic microorganisms. It is known that agarases from bacteria require low NaCl concentrations for their optimum activities when contrasted with other enzymes (Fu and Kim, 2010). For instance, optimum salinities are 0.15 M for β-agarase II from *Pseudomonas atlantica* (Morrice et al., 1983) and 0.9 M for β-agarase PjaA from *Pseudomonas* sp. strain W7 (Ha et al., 1997). The agarase from MD25A, besides of being from a bacterium, it had an optimal salinity of 1.5 M. Otherwise, the β-agarase from *Microbulbifer thermotolerans* (Thermostable β-agarase; Wako Pure Chemical; Industries, Ltd., Japan) showed activities up to 1.25 M NaCl, and decreasing activities were observed with increasing NaCl concentrations (Minegishi et al., 2013) (Figure 22). The agarases of bacterial strains usually work optimally below 40ºC (Fu and Kim, 2010). However, the agarase isolated from a marine environment from the microorganism *Microbulbifer thermotolerans* JAMB-A94 has an optimum temperature of 55ºC, which demonstrate that although the optimal temperature of most agarases is below 40ºC, there are agarases that their optimum temperature is above the average (Ohta et al., 2004). For instance, the moderately thermophilic bacteria, *Alterococcus agarolyticus*, growing at 38-58ºC, which was isolated by Shieh and Jean (1998), it was originally isolated from a hot spring. When *A. agarolyticus* was grown on agar medium it produced an extracellular agarase, but further studies about this agarase have not been published. The agarase produced by our organism

has an optimal temperature of 37ºC and it does not work below 35ºC because of the assay conditions that we used, since 0.3% agarose formed a soft gel below this temperature. The agarase from our isolate MD25A was quite stable at temperatures above 40ºC. For instance, in the presence of 1.5 M NaCl, pH 8, the enzyme retained approximately 60% of the initial activity after incubation for 1 h at 50ºC. Although most agarases of bacterial strains are stable below 50ºC, MD25A's agarase was stable at 50ºC for 1 h at half its relative activity (Fu and Kim, 2010) (Figure 24). Our enzyme did not work at 60ºC or 70ºC, which was expected because the isolate was not even moderately halophilic like the β-agarase-producing *Microbulbifer thermotolerans* JAMB-A94, whose agarase retained 10% of its activity at 70ºC for 15 min (Ohta et al., 2004). Similarly, the β-agarases from *Alteromonas* sp. SY37-12 (Wang et al., 2006), *Agarivorans albus* YKW-34 (Fu et al., 2008), *Acetinetobacter* sp. Ag LSL-1 (Lakshmikanth et al., 2006) and *Halococcus* sp. 197A retained activity after incubation as follows; 20% after 1 min at 70ºC, 10% after 60 min at 70ºC, and none after 60 min, respectively, with the exception of *Halococcus* sp. 197A which retained approximately 90% of its activity for 1 h at as high as 80ºC (Minegishi et al., 2013).

The isolate MD25A produces a β-type agarase which exhibits a halophilicity of 1.5 M NaCl, an optimal temperature of 37ºC, an optimal pH of 8 (Figure 26) and a thermostability (half life for 60 min at 50ºC) up to 50ºC. The oligosaccharides produced by the degradation of agar were analyzed by TLC. The degradation products were in the following order neoagarohexose, neoagarotetraose and a small quantity of neoagarobiose (lane 10) (Figure 27). The faint spot with slightly higher Rf value than galactose in lane 10 was neoagarobiose (Figure 27) (Otha et al., 2004). The data implies that our agarase is a β-

type agarase (Figure 27) based on the neoagarooligosaccharides produced. The induced agarase, produced in the HA-I medium with a 0.2% agarose, and later purified, was homogeneous with a molecular weight of approximately 80 kDa as presented in the SDS-PAGE (Figure 25). The aim of this study was to obtain a better understanding of the agarase enzyme in hypersaline environments in Puerto Rico. This was the first study about agarases performed in marine and hypersaline environments in Puerto Rico. Consequently, more extensive studies for agarase producing halophiles should be performed in Puerto Rico to determine if the decomposition of agar carries out the same steps as in marine environments.

6. Conclusion

The polysaccharide known as agar is an important source of biomass and it has been used in various fields throughout the years. The enzymatic degradation of agar is a key process for the practical use of marine biomass and for maintenance of the carbon cycle in the biosphere. There have been many reports of studies in saline environments related to the biodiversity and speciation, but there are not many studies of nutrient cycling and about the agar degradation pathway. After many efforts, remarkable progress has been made in the isolation of agarolityc microorganisms, the biochemical study of agarases and the degradation pathway (Chi et al., 2012). Although agarases have been used for many years in the recovery of DNA from agarose gels and instead of the degradation method, recently they have also been used for the preparation of agar-derived oligosaccharides. The previously mentioned agar-derivatives can be either neo-oligosaccharides or agaro-oligosaccharides. Usually the agaro-oligosaccharide were obtained by acid treatment, but recently they have been prepared using α-agarases. Nonetheless, the neoagaro-oligosaccharides can only be obtained by using a β-agarase (Araki, 1959). The neoagaro-oligosacchrides have been considered to be of high economic value, because of their physiological and biological activity, without toxicity, and they are classified under GRAS (Generally Recognized As Safe). These two oligosaccharides: neoagarotetraose and neoagarohexaose, exhibit antioxidative properties, scavenging the hydroxyl free radicals and superoxide anion radicals and inhibiting lipid peroxidation (Wang et al., 2004; Wu et al., 2005).

Furthermore, the neoagaro-oligosaccharides are excellent as a low-calorie additive to improve food quality, they can also inhibit the growth of bacteria, and slow down the degradation of starch; thus, they are vastly used in the food industry (Giordano et al., 2006). They are also used in the cosmetic and pharmaceutical company because of their moisturizing effect on skin and a whitening effect on melanoma cells (Kobayashi et al., 1997; Ohta et al., 2004). Similarly, the red algae can be used as a renewable and sustainable biomass for production of biofuels such as ethanol and butanol. To produce them, the agar must be hydrolysed completely into monomeric sugars, with the need to pass through a subsequent fermentation of 3-O-linked-β-D-galactopyranose (G) and 3,6-anhydro-α-L-galactose (LA) by microorganisms. Many ethanol-fermenting bacteria and yeasts can metabolize 3-O-linked-β-D-galactopyranose (G). Some of these include *E. coli*, *Zymomonas mobilis*, and *Saccharomyces cerevisiae* (Lindén et al., 1992; Wang et al., 2006; Yanase et al., 1991; Chi et al., 2012). However, there is no information available about the fermentability and metabolism of ,6-anhydro-α-L-galactose (LA) and L-galactose-6-sulfate. In addition, G and LA can also be converted into other chemicals. For example, G can be converted into D-tagatose, a low-calorie sweetener through the L-arabinose isomerase (Rhimi et al., 2011). The complete understanding of the agar-degrading system of microorganisms is utterly important for the use of red algal biomass. There are still many missing links in the agar-degradation pathway. Specifically, the agarotetraose hydrolase and β-agarobiose hydrolase in the α-agarolytic pathway have still not been reported. Moreover, studies should be performed on the sulfatase that hydrolyses the sulfate ester, to enable an efficient transformation from agaropectine to agarose. Although an arylsulfatase (arylsulfate hydrolase, EC 3.1.6.1), a class of glycosulfohydrolase has been

identified from a carrageenan-hydrolysis by *Alteromonas carrageenovora* (Barbeyron et al., 1995) and was used for the preparation of agarose from agaropectin (Cho et al., 2010).

The agarase pathway has not been studied in Puerto Rico and there are no reports about the abundaces of the types of agarases found on saline environments. Therefore, there are many challenges in completing the missing links of the agar-degrading pathway of the microorganisms as well as the elucidation of its regulatory network and the distribution of the enzymes in the diferent environments. If this knowledge could get completed, several industries would benefit from it. The aplications derived from the understanding of these pathways are essential for the health-food, pharmaceutical, cosmetic, and renewable energy industries. Consequently, it could also help reduce the effects of global warming by the development of red algae biofuels reducing the carbon footprint.

7. Recommendations

Since we have two isolates that strongly indicate that they might be new species, a genome sequencing should be performed to corfirm the new species. A comprehensive study should be performed to analize the types of agarases available throughout the island of Puerto Rico. A metagenomic analysis of the three different sampling areas (Cabo Rojo Salterns, Peñuelas Salterns, Guánica Salterns; Figures 7, 8 and 9) should be performed to look for the agarase genes. Preparing these metagenomes will give us access to the sequences of genes from the agarase pathway that can be used for phylogenetic analysis; while giving us an insight of the possible missing links in the agar-degrading pathway. Along with the latter, extracting metagenomic DNA and RNA to make studies about the relative abundance and expression of the agarase genes to confirm the presence of the different types of agar degrading enzymes present in these environments. Futhermore, a proteomics analysis could be performed to have a more comprehensive data to observe which enzymes are being produced. The data obtained could give us the missing links in the agar-degrading pathways.

8. References

1. Aoki, T., Araki, T., and Kitamikado, M. (1990). Purification And Characterization Of Beta-Agarases From Vibrio Sp. AP-2. *Nippon Suisan Gakkaishi* 56.5 (1990): 825-830. Web.

2. Andrykovitch, G. and Marx, I. (1988) Isolation of a new polysaccharide-digesting bacterium from a salt marsh. *Appl. Environ. Microbiol*. 54: 3-4

3. Arahal, D. R., Ludwig, W., Schleifer, K. H., and Ventosa, A. (2002). Phylogeny of the family Halomonadaceae based on 23S and 165 rDNA sequence analyses. *Int. J. Syst. Evol. Microbiol.*, *52*(1), 241-249.

4. Araki C (1959). Seaweed polysaccharides. In: Wolfrom ML (ed) Carbohydrate chemistry of substances of biological interest. Pergamon, London, pp 15–30

5. Araki, C. and Arai, K. (1957). Studies on the Chemical Constitution of Agar-agar. XX. Isolation of a Tetrasaccharide by Enzymatic Hydrolysis of Agar-agar. *Bull. Chem. Soc. Jpn*, *30*(3), 287-293.

6. Araki, T., Hayakawa, M., Lu, Z., Karita, S., Morishita, T. (1998) Purification and characterization of agarases from a marine bacterium, Vibrio sp. PO-303. *J. Mar. Biotechnol*. 6:260–265

7. Armisen, Rafael. (1995). World-Wide Use And Importance Of Gracilaria. *J. Appl. Phycol*. 7.3 (1995): 231-243. Web.

8. Barbeyron T, L'Haridon, S., Corre E, Kloareg B, Potin P. (2001). Zobellia Galactanovorans Gen. Nov., Sp. Nov., A Marine Species Of Flavobacteriaceae Isolated From A Red Alga, And Classification Of [Cytophaga] Uliginosa (Zobell And Upham 1944) Reichenbach 1989 As Zobellia Uliginosa Gen. Nov., Comb. Nov. *Int. J. Syst. Evol. Microbiol* 51.3 (2001): 985-997. Web.

9. Barbeyron T, Potin P, Richard C, Collin O, Kloareg B (1995). Arylsulphatase from Alteromonas carrageenovora. *Microbiology* 141:2897–2904

10. Bhatia, S., Rathee, P., Sharma, K., Chaugule, B., Kar, N., and Bera, T. (2013). Immuno-modulation effect of sulphated polysaccharide (porphyran) from Porphyra vietnamensis. *Int. J. Biol. Macromolec*, *57*, 50-56.

11. Belas R., Bartlett D., Silverman M. (1987). Cloning and gene replacement mutagenesis of a Pseudomonas atlantica agarase gene. *Appl. Environ. Microbiol*. 54: 30-37.

12. Bibb, M., Jones, G., Joseph, R., Buttner, M., and Ward, J. (1987). The Agarase Gene (dag A) of Streptomyces coelicolor A3(2): Affinity Purification and Characterization of the Cloned Gene Product. *Microbiology*, *133*(8), 2089-2096.

13. Bromley, L., De Saussure, V., Clipp, J., and Wright, J. (1967). Heat capacities of sea water solutions at salinities of 1 to 12% and temperatures of 2º degree to 80º degree. *J. Chem. Eng. Data*, *12*(2), 202-206.

14. Buttner, Mark J., Ian M. Fearnley, and Mervyn J. Bibb. (1987) The Agarase Gene (Daga) Of Streptomyces Coelicolor A3(2): Nucleotide Sequence And Transcriptional Analysis. *Mol. Gen. Genet.* 209.1 (1987): 101-109. Web.

15. Burmeister M and Lehrach H. (1989). Isolation of large DNA fragments from agarose gels using agarase. *Tren. Genet*. 5:41.

16. Cao, J., Wang, J., Wang, S., and Xu, X. (2016). Porphyra Species: A Mini-Review of Its Pharmacological and Nutritional Properties. *J. Med. Food. 19*(2), 111-119.

17. Casamayor, E.O., Massana, R., Benlloch, S., Ovreas, L., Diez, B., Goddard, V.J., Gasol, J.M., Joint, I., Rodriguez-Valera, F., and Pedros-Alio, C. (2002). Changes in archaeal, bacterial and eukaryal assemblages along a salinity gradient by comparison of genetic fingerprinting methods in a multipond solar saltern. *Environ. Microbiol.* 4: 338-348.

18. Chi, W., Chang, Y., and Hong, S. (2012). Agar degradation by microorganisms and agar-degrading enzymes. *Appl. Microbiol. Biotechnol.*, *94*(4), 917-930.

19. Chi, W., Park, J., Kang, D., and Hong, S. (2014). Production and Characterization of a Novel Thermostable Extracellular Agarase from Pseudoalteromonas hodoensis Newly Isolated from the West Sea of South Korea. *Appl. Biochem. Biotechnol.*, *173*(7), 1703-1716.

20. Cho, E., Kim, J., Kim Y., and Nam, S. (2010). Characterization of agarose produced by yeast cell surface displayed-arylsulfatase. *Kor. J. Microbiol. Biotechnol*. 38:428–433

21. Colón, A., Acevedo, M., and Acosta, I. (2015). *Municipalities: Peñuelas*. *Enciclopediapr.org*. Retrieved 22 February 2016, from http://www.enciclopediapr.org/ing/article.cfm?ref=08021701

22. Correc, G., Hehemann, J., Czjzek, M., and Helbert, W. (2011). Structural analysis of the degradation products of porphyran digested by Zobellia galactanivorans β-porphyranase A. *Carbohydr. Polym.*, *83*(1), 277-283.

23. Cote, G. and Hanisak, M. (1986). Production and properties of native agars from Gracilaria tikvahiae and other red algae. *Bot. Mar*. 29: 359-366.

24. Craigie, J.S. (1990) The cell wall. In KM Cole, RG Sheath, eds, Biology of the Red Algae. Cambridge University Press, Cambridge, UK, pp 221–257

25. Cui, F., Dong, S., Shi, X., Zhao, X., and Zhang, X. (2014). Overexpression and Characterization of a Novel Thermostable β-Agarase YM01-3, from Marine Bacterium Catenovulum agarivorans YM01T. *Mar. Drugs.*, *12*(5), 2731-2747.

26. Dagert, M. and Ehrlich, S.D. (1979). Prolonged incubation in calcium chloride improves the competence of Escherichia coli. *Gene*. 6: 23-28.

27. Davies, G., and Henrissat, B. (1995). Structures And Mechanisms Of Glycosyl Hydrolases. *Structure* 3.9 (1995): 853-859. Web.

28. Davis, J.S. (1978). Biological communities of nutrient enriched salina. *Aq. Bot*. 4: 23-42

29. Day, D. and Yaphe, W. (1975). Enzymatic hydrolysis of agar: purification and characterization of neoagarobiose hydrolase and p-nitrophenyl alpha-galactoside hydrolase. *Can. J. Microbiol.* 21:1512–1518

30. Demergasso, C., Escudero, L., Casamayor, E., Chong, G., Balagué, V., and Pedrós-Alió, C. (2008). Novelty and spatio–temporal heterogeneity in the bacterial diversity of hypersaline Lake Tebenquiche (Salar de Atacama). *Extremophiles*, *12*(4), 491-504.

31. Dobson, S. J. and Franzmann, P. D. (1996). Unification of the genera *Deleya* (Baumann et al. 1983), *Halomonas* (Vreeland et al. 1980), and *Halovibrio* (Fendrich 1988) and the species *Paracoccus halodenitrificans* (Robinson and Gibbons 1952) in to a single genus, *Halomonas*, and placement of the genus *Zymobacter* in the family *Halomonadaceae*. *Int. J. Syst. Bacteriol.* 46, 550–558.

32. Dong, J. Tamaru, Y., Araki, T. (2006). Cloning Of The Novel Gene Encoding -Agarase C From A Marine Bacterium, Vibrio Sp. Strain PO-303, And Characterization Of The Gene Product. *Appl. Environ. Microbiol.* 72.9 (2006): 6399-6401.

33. Dong J, Hashikawa S, Konishi T, Tamaru Y, Araki T (2006) Cloning of the novel gene encoding beta-agarase C from a marine bacterium, Vibrio sp. strain PO-303, and characterization of the gene product. *Appl. Environ. Microbiol.* 72:6399–6401

34. Eden P., Schmidt T., Blakemore R., Pace N. (1991). Phylogenetic Analysis of *Aquaspirillum Magnetotacticum* using polymerase chain reaction-amplified 16s rRNA-dpecific DNA. *Int. J. Syst. Evol. Microbiol.* 41: 324-325

35. Ekborg N, Taylor L, Weiner R, Hutcheson S (2006) Genomic and proteomic analysis of the agarolytic system of Saccharophagus degradans strain 2–40. *Appl. Environ. Microbiol.* 72:3396–3405

36. Enach M., Itoh T., Fukushima T., Usami R., Dumitru L. and Kamekura M. (2007). Phylogenetic relationships within the family Halobacteriaceae inferred from rpoB9 gene and protein sequences. *Int. J. Syst. Bacteriol.* 57, 2289–2295

37. Felsenstein, Joseph. (1985) Confidence Limits On Phylogenies: An Approach Using The Bootstrap'. *Evolution* 39.4: 783. Web.

38. Fernández, L., Valiente, O., Mainardi, V., Bello, J., Vélez, H., and Rosado, A. (1989). Isolation and characterization of an antitumor active agar-type polysaccharide of Gracilaria dominguensis. *Carbohydr. Res.*, *190*(1), 77-83.

39. Finkelstein, M., and Rownd, T. (1978). A rapid method for extracting DNA from agarose gels. *Plasmid*. ;1:557–562.

40. Flament, D., Barbeyron, T., Jam, M., Potin, P., Czjzek, M., Kloareg, B., and Michel, G. (2007). Alpha-Agarases Define a New Family of Glycoside Hydrolases, Distinct from Beta-Agarase Families. *Appl. Environ. Microbiol.*, *73*(14), 4691-4694.

41. Flament, D., Allouch, J., Potin, P., Thion, L., Kloareg, B., and Czjzek, M. (2005). The endo-β-agarases AgaA and AgaB from the marine bacterium Zobellia galactanivorans : two paralogue enzymes with different molecular organizations and catalytic behaviours. *Biochem. J.*, *385*(3), 703-713.

42. Fu, X.T., and Kim, S.M. (2010). Agarase: Review Of Major Sources, Categories, Purification Method, Enzyme Characteristics And Applications. *Mar. Drugs*. 8.1 (2010): 200-218. Web.

43. Fu X.T., Lin, H., and Kim. S.M. (2008). Purification and characterization of a novel beta-agarase, AgaA34, from Agarivorans albus YKW-34. *Appl. Microbiol. Biotechnol.* 78:265–273

44. Garriga, M., Ehrmann, M. A., Arnau, J., Hugas, M. and Vogel, R. F. (1998). *Carnimonas nigrificans* gen. nov., sp. nov., a bacterial causative agent for black spot formation on cured meat products. *Int. J. Syst. Bacteriol.* 48, 677–686

45. Giordano, A., Andreotti, G., Tramice, A., and Trincone, A. (2006). Marine glycosyl hydrolases in the hydrolysis and synthesis of oligosaccharides. *Biotechnol. J, 1*(5), 511-530.

46. Giovannoni, S.J., Delong, E.F., Olsen, G.J., and Pace, N.R. (1988) Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial-cells. *J Bacteriol* 170: 720–726

47. Gentry, A.H. (1995). Diversity and floristic composition of neotropical dry forests. Pp. 146–194. In: Bullock, S.H., Mooney, H.A. and Medina, E. (eds) Seasonally dry tropical forests. Cambridge University Press, Cambridge

48. Gonzalez, J. and Weiner, R. (2000). Phylogenetic characterization of marine bacterium strain 2-40, a degrader of complex polysaccharides. *Int. J. Syst. Evol. Microbiol*, *50*(2), 831-834.

49. Groleau, D., and W. Yaphe (1977). Enzymatic Hydrolysis Of Agar: Purification And Characterization Of B-Neoagarotetraose Hydrolase From Pseudomonas Atlantica. *Can. J. Microbiol.* 23.6 (1977): 672-679. Web.

50. Guánica Municipality. (2015). *Guánica Somos Todos - barrios*. *Guanicapr.net*. Retrieved 9 November 2015, from http://www.guanicapr.net/index.php/barrios

50. Gupta, H., Singh, A. and Sharma, R. (2011). Genome Sequence of Idiomarina sp. Strain A28L, Isolated from Pangong Lake, India. *J. Bacteriol., 193*(20), 5875-5876.

51. Ha, J., Kim G., Kim, S., Oh, T., Yu J., Kong, I. (1997) β-agarase from *Pseudomonas* sp. W7: purification of the recombinant enzyme from *Escherichia coli* and the effects of salt on its activity. *Biotechnol. Appl. Biochem*. 26:1–6

52. Ha, S., Lee, S., Lee, J., Kim, H., Ko, H., Kim, K. and Choi, I. (2011). Crystal structure of a key enzyme in the agarolytic pathway, α-neoagarobiose hydrolase from Saccharophagus degradans 2–40. *Biochem. Biophys. Res. Commun.* 412:238–244

53. Hatada, Y., Ohta, Y. and Horikoshi, K., (2006). Hyperproduction And Application Of A-Agarase To Enzymatic Enhancement Of Antioxidant Activity Of Porphyran. *J. Agric. Food Chem*. 54.26 (2006): 9895-9900. Web.

54. Hamer G., Bhattacharjee, S. and Yaphe, W. (1977) Analysis of the enzymic hydrolysis products of agarose by 13 C-n.m.r. spectroscopy. *Carbohydr. Res*. 54:7–l0

55. Hehemann, J., Correc, G., Thomas, F., Bernard, T., Barbeyron, T., Jam, M. and Czjzek, M. (2012). Biochemical And Structural Characterization Of The Complex Agarolytic Enzyme System From The Marine Bacterium Zobellia Galactanivorans. *J. Biol. Chem.* 287.36 (2012): 30571-30584. Web.

56. Hehemann, J., Michel, G., Barbeyron, T. and Czjzek, M. (2010). Expression, Purification And Preliminary X-Ray Diffraction Analysis Of The Catalytic Module Of A B-Agarase From The Flavobacterium Zobellia Galactanivorans. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* 66.4 (2010): 413-417. Web.

57. Hehemann, J., Correc, G., Barbeyron, T., Helbert, W., Czjzek, M., and Michel, G. (2010). Transfer of carbohydrate-active enzymes from marine bacteria to Japanese gut microbiota. *Nature*, *464*(7290), 908-912.

58. Henrissat, B. and Davies, G. (1997) Structural and sequence-based classification of glycoside hydrolases. *Curr. Opin. Struct. Biol.* 7:637–644

59. Henrissat, B. and Bairoch, A. (1993). New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J*, *293*(3), 781-788.

60. Henrissat, B. and Bairoch, A. (1996). Updating the sequence-based classification of glycosyl hydrolases. *Biochem. J*, *316*(2), 695-696.

61. Heyrman, J., Balcaen, A., De Vos, P. and Swings, J. (2002). Halomonas muralis sp. nov., isolated from microbial biofilms colonizing the walls and murals of the Saint-Catherine chapel (Castle Herberstein, Austria). *Int. J. Syst. Evol. Microbiol.* 52, 2049–2054

62. Hofsten, B. and Malmqvist, M. (1975). Degradation of Agar by a Gram-negative Bacterium. *J. Gen. Microbiol.*, *87*(1), 150-158.

63. Hosoda, A., Sakai, M. and Kanazawa, S. (2003) Isolation and characterization of agar-degrading Paenibacillus spp. associated with the rhizosphere of spinach. *Biosci. Biotechnol. Biochem.* 67, 1048–1055.

64. Hosoda, A. and Sakai, M. (2006). Isolation of Asticcacaulis sp. SA7, a novel agar-degrading alphaproteobacterium. *Biosci. Biotechnol. Biochem* 70, 722–725.

65. Hu, Z., Lin, B., Xu, Y., Zhong, M., and Liu, G. (2009). Production and purification of agarase from a marine agarolytic bacteriumAgarivoranssp. HZ105. *J. Appl. Microbiol.*, *106*(1), 181-190.

66. Javor B.J. (1989) Hypersaline Environments. *Microbiology and Biochemistry*. Berling: Springer-Verlag

67. Jean, W., Leu, T., Lee, C., Chu, T., Lin, S., and Shieh, W. (2009). Pseudidiomarina marina sp. nov. and Pseudidiomarina tainanensis sp. nov. and reclassification of Idiomarina homiensis and Idiomarina salinarum as Pseudidiomarina homiensis comb. nov. and Pseudidiomarina salinarum comb. nov., respectively. *Int. J. Syst. Evol. Microbiol.*, *59*(1), 53-59.

68. Kendall, K. and Cullum, J. (1984). Cloning and expression of an extracellular-agarase gene from Streptomyces coelicolor A3(2) in Streptomyces lividans 66. *Gene, 29*(3), 315-321.

69. Kim, H., Lee, S., Lee, D., Kim, H., Bang, W., Kim, K. and Choi, I. (2010). Overexpression and molecular characterization of Aga50D from Saccharophagus degradans 2–40: an exo-type β-agarase producing neoagarobiose. *Appl. Microbiol. Biotechnol*. 86:227–234

70. Kirimura K, Masuda N, Iwasaki Y, Nakagawa H, Kobayashi R, Usami S (1999) Purification and characterization of a novel beta-agarase from an alkalophilic bacterium, Alteromonas sp. E-1. J *Biosci. Bioeng*. 87:436–441

71. Kloareg, B, Quatrano, R. (1988). Structure of the cell walls of marine algae and ecophysiological functions of the matrix polysaccharides. *Oceanogr. Mar. Biol*. 26: 259–315.

72. Kobayashi, J., Mackinnon, S.E., Watanabe, O., Ball, D.J., Gu, X.M., Hunter, D.A. and Kuzon, W.M. (1997). The effect of duration of muscle denervation on functional recovery. *Muscle. Nerve. 20*, 858-866.

73. Kong J., Hwang S., Kim B., Bae S., Kim J. (1997) Cloning and expression of an agarase from a marine bacterium Pseudomonas sp. w7. *Biotechnol. Lett*. 19: 23-26.

74. Könneke, M., Bernhard, A., de la Torre, J., Walker, C., Waterbury, J. and Stahl, D. (2005). Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature, 437*(7058), 543-546.

75. Kwak, M., Song, J., Kim, B., Chi, W., Kwon, S., and Choi, S. et al. (2012). Genome Sequence of the Agar-Degrading Marine Bacterium Alteromonadaceae sp. Strain G7. *J. Bacteriol.*, *194*(24), 6961-6962.

76. Kwon M.J. and Nam T.J., (2006). Porphyran induces apoptosis related signal pathway in AGS gastric cancer cell lines. *Life. Sci.* 2006; 79:1956–1962.

77. Lakshmikanth, M., Manohar, S., Patnakar, J., Vaishampayan, P., Shouche, Y. and Lalitha, J. (2006). Optimization of culture conditions for the production of extracellular agarases from newly isolated Pseudomonas aeruginosa AG LSL-11. *World. J. Microbiol. Biotechnol*. 22, 531–537.

78. Leon, O., Quintana, L., Peruzzo, G. and Slebe, J. (1992) Purification and properties of an extracellular agarase from Alteromonas sp.strain C-1. *Appl. Environ. Microbiol*. 58:4060–406

79. Lee, D., Jang, M., Lee, O., Kim, N., Ju, S. and Lee, S. (2008) Overproduction of a glycoside hydrolase family 50 β-agarase from Agarivorans sp. JA-1 in Bacillus subtilis and the whitening effect of its product. *Biotechnol. Lett*. 30:911–918

80. Lee, D., Park, G., Kim, N., Lee, E., Jang, M., Shin, Y., Park, G., Kim, T., Lee, J., Lee, J., Kim, S. and Lee, S. (2006). Cloning, expression,and characterization of a glycoside hydrolase family 50 betaagarase from a marine Agarivorans isolate. *Biotechnol. Lett.* 28:1925–1932

81. Li, J., Sha, Y., Seswita-Zilda, D., Hu, Q., and He, P. (2014). Purification and Characterization of Thermostable Agarase from Bacillus sp. BI-3, a Thermophilic Bacterium Isolated from Hot Spring. *J. Microbiol. Biotechnol.*, *24*(1), 19-25.

82. Li, M., Li, G., Zhu, L., Yin, Y., Zhao, X., and Xiang, C. (2014). Isolation and Characterization of an Agaro-Oligosaccharide (AO)-Hydrolyzing Bacterium from the Gut Microflora of Chinese Individuals. *Plos ONE*, *9*(3), e91106.

83. Liao, L. Xu, X., Jiang, X., Cao, Y., Yi, N., Huo, Y., Wu, Y., Zhu, X., Zhang X., and Wu, M. (2011). Cloning, Expression, And Characterization Of A New -Agarase From Vibrio Sp. Strain CN41. *Appl. Environ. Microbiol*. 77.19: 7077-7079. Web.

84. Lin, B., Lu, G., Li, S., Hu, Z., and Chen, H. (2012). Draft Genome Sequence of the Novel Agarolytic Bacterium Aquimarina agarilytica ZC1. *J. Bacteriol.*, *194*(10), 2769-2769.

85. Lindén T, Peetre J., and Hahn-Hägerdal B. (1992). Isolation and characterization of acetic acid-tolerant galactose-fermenting strains of Saccharomyces cerevisiae from a spent sulfite liquor fermentation plant. *Appl. Environ. Microbiol*. 58:1661–1669

86. Litchfield, C. and Gillevet, P. (2002). Microbial diversity and complexity in hypersaline environments: A preliminary assessment. *J. Ind. Microbiol. Biotechnol.*, *28*(1), 48-55.

87. Long M, Yu Z, Xu X (2010). A novel β-agarase with high pH stability from marine Agarivorans sp. LQ48. *Mar. Biotechnol*. 12:62–69

88. Ma, C., Lu, X., Shi, C., Li, J., Gu, Y., and Ma, Y. (2007). Molecular Cloning and Characterization of a Novel beta-Agarase, AgaB, from Marine Pseudoalteromonas sp. CY24. *J. Biol. Chem.*, *282*(6), 3747-3754.

89. MacArtain, P., Gill, C., Brooks, M., Campbell, R., and Rowland, I. (2008). Nutritional Value of Edible Seaweeds. *Nutr. Rev.*, *65*(12), 535-543.

90. Martinez-Canovas, M. (2004). Halomonas ventosae sp. nov., a moderately halophilic, denitrifying, exopolysaccharide-producing bacterium. *Int. J. Syst. Evol. Microbiol.*, *54*(3), 733-737.

91. McHugh, D.J. (1984). Marine phycoculture and its impact on the seaweed colloid industry. *Hydrobiologia*, *116-117*(1), 351-354.

92. McHugh, D.J. (2003). A Guide to the Seaweed Industry. FAO Fisheries Technical Paper 441, Rome, 105 p.

93. Mellado, E., Moore, E. R. B., Nieto, J. and Ventosa, A. (1995). Phylo-genetic inferences and taxonomic consequences of 16S ribosomal DNAsequence comparison of Chromohalobacter marismortui, Volcaniellaeurihalina,andDeleya salina and reclassification of V. eurihalina asHalomonas eurihalina comb. nov. *Int. J. Syst. Bacteriol*. 45, 712–716.

94. Michel, W., Sandrine, D., Gilles, L., Alessia, M., Rodrigues, Y., and Philippe, D., (2006). Heavy Metals in the Sea Cucumber *Holothuriatubulosa* (Echinodermata) from the Mediterranean *Posidoniaoceanica* Ecosystem: Body Compartment, Seasonal, Geographical and Bathymetric Variations. *Environmental Bio-indicators*. 1: 268-285.

95. Minegishi, H., Shimane, Y., Echigo, A., Ohta, Y., Hatada, Y., and Kamekura, M. (2013). Thermophilic and halophilic β-agarase from a halophilic archaeon Halococcus sp. 197A. *Extremophiles*, *17*(6), 931-939.

96. Mittermeier R., Gil, P., Hoffmann, M., Pilgrim, J., Brooks, T., Mittermeier, C., Lamoreux, J., da Fonseca GAB. (2004). Hotspots Revisited: Earth's Biologically Richest and Most Endangered Ecoregions. Mexico City (Mexico): CEMEX

97. Morrice, L., McLean, M., Williamson, F., and Long, W. (1983). beta-Agarases I and II from Pseudomonas atlantica. Purifications and some properties. *Eur. J. Biochem.*, *135*(3), 553-558.

98. Muhammmad Kaleem, S., Iqra, A., and Tahir, I. (2017). Biology and Applications of Halophilic Bacteria and Archaea: A Review. *Electron. j. biol.*, *11*(3), 98-103.

99. Murano, E. (1995). Chemical structure and quality of agars from Gracilaria. *J. Appl. Phycol.* , *7*(3), 245-254.

100. Oh, C., Nikapitiya, C., Lee, Y., Whang I., Kim S., Kang D., Lee J (2010). Complete Genome Sequence Of The Agarase-Producing Marine Bacterium Strain S89, Representing A Novel Species Of The Genus *Alteromonas*. *J. Bacteriol*. 193.19 (2010): 5538-5538. Web.

101. Ohta, Y., Nogi, Y., Miyazaki, M., Li, Z., Hatada, Y., Ito, S., and Horikoshi, K. (2004). Enzymatic properties and nucleotide and amino acid sequences of a thermostable beta-agarase from the novel marine isolate, JAMBA94. *Biosci. Biotechnol. Biochem.* 68:1073–1081

102. Oren, A. (2002). Diversity of halophilic microorganisms: Environments, phylogeny, physiology, and applications. *J. Ind. Microbiol. Biotechnol. 28*(1), 56-63. doi:10.1038/sj.jim.7000176

103. Oren A., ed. (1999). Microbiology and biogeochemistry of hypersaline environments. Boca Raton: CRC Press.

104. Parte, A. C. (2013). LPSN—list of prokaryotic names with standing in nomenclature. *Nucleic Acids Res, 42*(D1), D613-D616.

105. Potin, P., Richard, C., Rochas, C., and Kloareg, B. (1993). Purification and characterization of the alpha-agarase from Alteromonas agarlyticus (Cataldi) comb. nov., strain GJ1B. *Eur. J. Biochem., 214*(2), 599-607.

106. Pubchem.ncbi.nlm.nih.gov,. (2015). *Agar (bacteriological)* | *C14H24O9* - *PubChem*. Retrieved 25 October 2015, from http://pubchem.ncbi.nlm.nih.gov/compound/71571511#section=Top

107. Quesada, E., Ventosa, A., Ruiz-Berraquero, F. and Ramos-Cormenzana, A. (1984). *Deleya halophila*, a new species of moderately halophilic bacteria. *Int. J. Syst. Bacteriol.* 34, 287–292

108. Ramos-Scharrón, C., Torres-Pulliza, D., and Hernández-Delgado, E. (2015). Watershed- and island wide-scale land cover changes in Puerto Rico (1930s–2004) and their potential effects on coral reef ecosystems. *Sci. Total. Environ., 506-507,* 241-251.

109. Rebuffet, E., Barbeyron, T., Jeudy, A., Jam, M., Czjzek, M., and Michel, G. (2010). Identification of catalytic residues and mechanistic analysis of family GH82 iota-carrageenases. *Biochemistry* 49:7590–7599

110. Rebuffet, E., Groisillier, A., Thompson, A., Jeudy, A., Barbeyron, T., Czjzek, M., and Michel, G. (2011) Discovery and structural characterization of a novel glycosidase family of marine origin. *Environ. Microbiol.* 13:1253–1270

111. Roh, H., Yun, E., Lee, S., Ko, H., Kim, S., and Kim, B.(2012). Genome Sequence of Vibrio sp. Strain EJY3, an Agarolytic Marine Bacterium Metabolizing 3,6-Anhydro-L-Galactose as a Sole Carbon Source. *J. Bacteriol.*, *194*(10), 2773-2774.

112. Rochas, C., Potin, P., and Kloareg, B.(1994) NMR spectroscopic investigation of agarose oligomers produced by an alpha-agarase. *Carbohydr. Res.* 253:69–77

113. Romanenko, L. A., Schumann, P., Rohde, M., Mikhailov, V. V. and Stackebrandt, E. (2002). Halomonas halocynthiae sp. nov., isolated from the marine ascidian Halocynthiae aurantium. *Int. J. Syst. Evol. Microbiol.* 52, 1773–1782.

114. Rhimi, M., Chouayekh, H., Gouillouard, I., Maguin, E., and Bejar, S. (2011). Production of D-tagatose, a low caloric sweetener during milk fermentation using L-arabinose isomerase. *Bioresour. Technol.* 102:3309–3315

115. Ryu, S., Cho, S., Park, S., Lim, W., Kim, M., and Hong, S. et al. (2001). Cloning of the cel 9A gene and characterization of its gene product from marine bacterium Pseudomonas sp. SK38. *Appl. Microbiol. Biotechnol.*, *57*(1-2), 138-145.

116. Saitou N. and Nei M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol*. 4:406-425.

117. Sakatoku, A., Wakabayashi, M., Tanaka, Y., Tanaka, D., and Nakamura, S. (2012). Isolation of a novel Saccharophagus species (Myt-1) capable of degrading a variety of seaweeds and polysaccharides. *Microbiologyopen*, *1*(1), 2-12.

118. Sanchez-Porro, C., de la Haba, R., Soto-Ramirez, N., Marquez, M., Montalvo-Rodriguez, R., and Ventosa, A. (2009). Description of Kushneria aurantia gen. nov., sp. nov., a novel member of the family Halomonadaceae, and a proposal for reclassification of Halomonas marisflavi as Kushneria marisflavi comb. nov., of Halomonas indalinina as Kushneria indalinina comb. nov. and of Halomonas avicenniae as Kushneria avicenniae comb. nov. *Int. J. Syst. Evol. Microbiol.*, *59*(2), 397-405.

119. Santiago-Valentin, E. and Olmstead, R. (2004). Historical Biogeography of Caribbean Plants: Introduction to Current Knowledge and Possibilities from a Phylogenetic Perspective. *Taxon*, *53*(2), 299.

120. Shi, X., Yu, M., Yan, S., Dong, S., and Zhang, X. (2012). Genome Sequence of the Thermostable-Agarase-Producing Marine Bacterium Catenovulum agarivorans YM01T, Which Reveals the Presence of a Series of Agarase-Encoding Genes. *J. Bacteriol.*, *194*(19), 5484-5484.

121. Shieh, W., and Jean, W. (1998). Alterococcus agarolyticus , gen.nov., sp.nov., a halophilic thermophilic bacterium capable of agar degradation. *Can. J. Microbiol.*, *44*(7), 637-645.

122. Smith, J.L., Summers, G. and Wong, R. (2010) Nutrient and Heavy Metal Content of Edible Seaweeds in New Zealand. New Zealand Journal of Crop and Horticultural Science, 38, 19-28.

123. Stanier, R. Y. (1941). Studies on Marine Agar-Digesting Bacteria . *J. Bacteriol*, *42*(4), 527–559.

124. Sugano, Y., Matsumoto, T., and Noma, M. (1994). Sequence analysis of the agaB gene encoding a new β-agarase from Vibrio sp. strain JT0107. *Biochimica Et Biophysica Acta (BBA) - Gene Structure And Expression*, *1218*(1), 105-108.

125. Sugano Y, Matsumoto T, Kodama H, and Noma M (1993). Cloning and sequencing of agaA, a unique agarose 0107 gene from a marine bacterium, Vibrio sp. strain JT0107. *Appl. Environ. Microbiol.* 59:3750–3756

126. Suzuki H, Sawai Y, Suzuki T, Kawai K (2002) Purification and characterization of an extracellular alpha-neoagarooligosaccharide hydrolase from Bacillus sp. MK03. *J. Biosci. Bioeng*. 93:456– 463

127. Tamura K., Stecher G., Peterson D., Filipski A., and Kumar S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution30: 2725-2729

128. Temuujin, U., Chi, W., Chang, Y., and Hong, S. (2011). Identification and Biochemical Characterization of Sco3487 from Streptomyces coelicolor A3(2), an Exo- and Endo-Type -Agarase-Producing Neoagarobiose. *J. Bacteriol*, *194*(1), 142-149.

129. U.S. Fish and Wildlife Service, 4. (2015). *Cabo Rojo (Salt Flats Unit) National Wildlife Refuge*. *Fws.gov*. Retrieved 9 November 2015, from http://www.fws.gov/caribbean/Refuges/Saltflat/default.htm

130. Valderrama, M. J., Monteoliva-Sanchez, M., Quesada, E. and Ramos-Cormenzana, A. (1998). Influence of salt concentration on the cellular fatty acid composition of the moderately halophilic bacterium Halomonas salina. *Res. Microbiol.* 149, 675–679

131. van der Meulen H.J., and Harder, W. (1976) Characterization of the neoagarotetra-ase and neoagarobi-ase of Cytophaga flevensis. Antonie van Leeuwenhoek 42:81–94

132. Venice system. (1959). Final resolution of the symposium on the classification of brackish waters. *Archo. Oceanogr. Limnol*., 11 (suppl): 243–248.

133. Vera, J., Alverez, R., Murano, E., Slebe, J.C. and Leon, O. (1998) Identification of a marine agarolytic *Pseudoalteromonas* isolate and characterization of its extracellular agarase. *Appl. Env. Microbiol.* **64:** 4378-4383.

134. Voget, S., Leggewie, C., Uesbeck, A., Raasch, C., Jaeger, K., and Streit, W. (2003). Prospecting for Novel Biocatalysts in a Soil Metagenome. *Applied And Environmental Microbiology*, *69*(10), 6235-6242.

135. Vreeland, R. H., Litchfield, C. D., Martin, E. L. and Elliot, E**. (**1980)**.** *Halomonas elongata*, a new genus and species of extremely salt-tolerant bacteria. *Int. J. Syst. Bacteriol.* 30, 485–495

136. Wang J, Jiang X, Mou H, Guan H (2004). Anti-oxidation of agaroligosaccharides produced by agarase from a marine bacterium. *J. Appl. Phycol*. 16:333–340

137. Wang J, Mou H, Jiang X, Guan H (2006) Characterization of a novel β-agarase from marine Alteromonas sp. SY37-12 and its degrading products. *Appl. Microbiol. Biotechnol*. 71:833–839

138. Wu, S.C., Wen, T.N., and Pan, C.L. (2005) Algal-oligosaccharide-lysates prepared by two bacterial agarases stepwise hydrolyzed and their anti-oxidative properties. *Fish. Sci.* 71:1149–1159

139. Yakimov, M. M., Golyshin, P. N., Lang, S., Moore, E. R. B., Abraham,W.-R., Lu¨ nsdorf, H. and Timmis, K. N. (1998).Alcanivorax borkumensisgen. nov., sp. nov., a new, hydrocarbon-degrading and surfactant-producing marine bacterium. *Int. J. Syst. Bacteriol*. 48, 339–348

140. Yanase, H., Fukushi, H., Ueda, N., Maeda, Y., Toyoda, A., and Tonomura, K. (1991). Cloning, sequencing, and characterization of the intracellular invertase gene from Zymomonas mobilis. *Agric. Biol. Chem.* 55:1383–1390

141. Yang JI, Chen LC, Shih YY, Hsieh C, Chen CY, Chen WM, Chen CC (2011) Cloning and characterization of β-agarase AgaYT from Flammeovirga yaeyamensis strain YT. *J. Biosci. Bioeng.* 112:225–232

142. Yaphe, W. (1957). The use of agarase from Pseudomonas atlantica in the identification of agar in marine algae (Rhodophyceae). *Can. J. Microbiol.*, *3*(7), 987-993.

143. Yasuike, M., Nakamura, Y., Kai, W., Fujiwara, A., Fukui, Y., Satomi, M., and Sano, M. (2013). Draft Genome Sequence of Agarivorans albus Strain MKT 106T, an Agarolytic Marine Bacterium. *Genome Announcements*, *1*(4), e00367-13-e00367-13.

144. Yoshizawa, F., Endo, M., Ide, H., Yagasaki, K., and Funabiki, R. (1995). Translational regulation of protein synthesis in the liver and skeletal muscle of mice in response to refeeding. *J. Nutr. Biochem.*, *6*(3), 130-136.

145. Young, K., Bhattacharjee, S., and Yaphe, W. (1978). Enzymic cleavage of the α-linkages in agarose, to yield agaro-oligosaccharides. *Carbohydrate Research*, *66*(1), 207-212.

146. Zalar, P., Sybren De Hoog, G., Schroers, H.J., Frank, J.M., and Gunde-Cimerman, N. (2005). Taxonomy and phylogeny of the xerophilic genus Wallemia (Wallemiomycetes and Wallemiales, cl. Et ord. Nov.). Antonie van Leeuwenhoek. 87 (4): 311–328.

147. Zhang, W., and L. Sun. (2007). Cloning, Characterization, And Molecular Application Of A Beta-Agarase Gene From Vibrio Sp. Strain V134. *Appl. Environ. Microbiol*. 73.9: 2825-2831. Web.

148. Zhang, Q., Li, N., Zhou, G., Lu, X., Xu, Z., and Li, Z. (2003). In vivo antioxidant activity of polysaccharide fraction from Porphyra haitanesis (Rhodephyta) in aging mice. *Pharmacol. Res*, *48*(2), 151-155.

149. Zhong, Z., Toukdarian, A., Helinski, D., Knauf, V., Sykes, S., Wilkinson, J.E., O'Bryne, C., Shea, T., Deloughery, C. and Caspi, R. (2001). Sequence analysis of 101-kilobase plasmid required for agar degradation by a Microcilla isolate. *Appl. Environ. Microbiol*. 67: 5771-5779.

9. Appendix

Appendix 1. Classification of the sequenced and functionally verified bacterial agarases based on the glycosidic hydrolase (GH) family.

Family	Bacterial Species	Protein	Accession no.	Localization	Product	Reference
GH16	*Streptomyces coelicolor* A3(2)	β-Agarase DagA	CAB61795	Extracellular	NA4, NA6	Temuujin et al.,2011
	Flammeovirga yaeyamensis strain YT	β-Agarase AgaYT	AEK80424	Extracellular	NA2, NA4	Yang et al.,2011
	*Pseudoalteromona*s sp.AG4	β-Agarse AgaP	ADD60418	Extracellular	NA4 ,NA6	Oh et al.,2010
	Agarivorans sp.LQ48	β-Agarase AgaA	ACM50513	Extracellular	NA4 ,NA6	Long et al.,2010
	Pseudoalteromonas sp.CY24	β-Agarase AgaA	AAN39119	Extracellular	NA4 ,NA6	Lu et al., 2009
	Agarivorans albus YKW-34	β-Agarase AgaB34	ABW77762	Extracellular	NA4	Fu et al., 2009
	Vibrio sp. strain PO-303	β-Agarase AgaA	BAF62129	Extracellular	NA2, NA4	Dong et al.,2007a
		β-Agarase AgaD	BAF34350	Extracellular	NA2, NA4	Dong et al.,2007b
	Vibrio sp. Strain V134	β-Agarase AgaV	ABL06969	Extracellular	NA4, NA6	Zhang and Sun 2007
	Saccharophagus degradans 2-40	β-Agarase Aga16B	AAT67062	Extracellular	NA4	Ekborg et al.,2006
	Zobellia galactanivorans	β-Agarase AgaA	AAF21820	Extracellular	NA4, NA6, NA2(minor)	JAM et al., 2005
		β-Agarase AgaB	AAF21821	Cytoplasmic membrane	NA2, NA4	JAM et al., 2005
		β-Agarase AgaD	CBM41186	Periplasmic space/ extracellular	NA4	Hehemann et al., 2010b
		β-Porphyranase PorA	CBM41182	Periplasmic space	NP2, NP4	Hehemann et al., 2010a
		β-Porphyranase PorB	CBM41181	Periplasmic space		Hehemann et al., 2010a
	Microbulbifer elongatus JAMB-A7	β-Agarase AgaA7	BAC99022	Extracellular	NA4	Ohta et al.,2004b
	Microbulbifer thermotolerans	β-Agarase AgaA	BAK08910	Extracellular	NA4(main),	Ohta et al.,2004c

	JAMB-A94				NA2, NA6	
GH50	*Streptomyces coelicolor* A3(2)	β-Agarase Sco3487	CAB61811	Extracellular	NA2	Temuujin et al.,2012
	Vibrio sp. CN41	β-Agarase AgaA41A	ADM25828	Intracellular	NA4	Liao et al.,2011
	Saccharophagus degradans 2-40	β-Agarase Aga50D	ABD81904	Extracellular	NA2	Kim et al., 2010
	Agarivorans albus YKW-34	β-Agarase AgaA34	P85974	Extracellular	NA2	Fu et al., 2008b
	Agarivorans sp.JA-1	β-Agarase	ABK97391	Extracellular	NA2	Lee et al.,2006
	Agarivorans sp. JAMB-AII	β-Agarase AgaA11	BAD99519	Extracellular	NA2	Ohta et al.,2005a
	Alteromonas sp. E-1	β-Agarase	BAE97587	Extracellular	NA2	Kirimura et al., 1999
	Vibrio sp. Strain PO-303	β-Agarase AgaB	BAG71427	Extracellular	NA2	Araki et al.,1998
	Vibrio sp. Strain JT0107	β-Agarase AgaA	BAA03541	Extracellular	NA2	Sugano et al., 1993
GH86	*Microbulbifer* sp. JAMB-A94	β-Agarase AgaO	BAK08903	Extracellular	NA6(main) NA4, NA2	Ohta et al.,2004a
	Saccharophagus degradans 2-40	β-Agarase Aga86E	ABD81915	Extracellular	NA2	Ekborg et al., 2006
GH96	*Thalassomonas* sp.JAMB-A33	α-Agarase AgaA33	BAF44076	Extracellular	A6, A4	Ohta et al.,2005b
	Alteromonas Agarilytica GJB	α-Agarase AgaA	AAF26838	Extracellular	A4	Potin et al., 1993
GH117	*Zobellia galactanivorans* DsiJT	α-NAH Zg4663	CBM41465	Outer membrane	G, LA	Rebuffet et al., 2011
	Saccharophagus degradans 2-40	α-NAH Aga117F	ABD81917	Intracellular	G, LA	Ha et al., 2011
GH118	*Pseudoalteromonas* sp. CY24	β-Agarase AgaB	AAQ56237	Extracellular	NA8,NA10	Ma et al.,2007
	Vibrio sp. PO-303	β-Agarase AgaC	BAF03590	Extracellular	NA4, NA6, NA8	Dong et al.,2006

*Data taken with authors permission from Chi et al. (2012).