

**CHARACTERIZATION OF MICROORGANISMS WITH CARBOHYDRASE
ACTIVITIES FROM TROPICAL ECOSYSTEMS**

By

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Abstract

Several microorganisms have been isolated from different tropical ecosystems (tropical rain forest, sea water, and marine sediments) from the island of Puerto Rico. These microorganisms were screened for carbohydrase activity using chromogenic substrates (amylose, pullulan, cellulose, dextran, arabinan, galactomannan, and xylan). The results indicate that 2 - 9 % of cultured organisms show carbohydrase activity. Bacterial isolates were identified by sequence analysis of the PCR amplified partial subunit rRNA sequence. NCBI^s database comparison of the most versatile strains gives a match with *Bacillus* sp., *Staphylococcus* sp., *Halomonas* sp., and a gamma proteobacterium, respectively. Growth curves indicate that all strains assayed can grow as well in at least one complex carbohydrate as in glucose. We have detected a high β -xylosidase activity from our *Bacillus* sp. strain crude enzyme preparation. Identification from isolated fungi indicates they belong to genus *Aspergillus* and *Microsporium* respectively. A yeast isolate was identified by D1/D2 domain of the large subunit (26S) ribosomal DNA sequence; it gives a match with *Glomus* sp. Our results show that tropical environments have potentially good sources of microorganisms with novel carbohydrases.

Resumen

Varios microorganismos fueron aislados en ecosistemas tropicales diferentes por ejemplo: (bosque tropical lluvioso, agua de mar, y sedimentos marinos) de la isla de Puerto Rico. Estos microorganismos fueron seleccionados por su actividad de carbohidrasas usando sustratos cromogénicos (amilosa, pululano, celulosa, dextrano, arabinano, galactomanano, y xilano). Los resultados indican que el 2 - 9 % de los organismos cultivados muestran actividad de carbohidrasas. Las bacterias aisladas fueron identificadas mediante el análisis de la secuenciación parcial de la subunidad pequeña de rRNA amplificado mediante RPC. La comparación de las cepas más versátiles con la base de datos de NCBI nos da un resultado de identificación con: *Bacillus* sp., *Staphylococcus* sp., *Halomonas* sp., y gamma proteobacteria, respectivamente. Las curvas de crecimiento indican que todas las cepas ensayadas pueden crecer tan bien en al menos un carbohidrato complejo como en glucosa. Pudimos detectar una actividad alta de β -xylosidasa en la preparación de la enzima cruda de la cepa identificada como *Bacillus* sp. La identificación de los hongos aislados indica que pertenecen al género *Aspergillus* y *Microsporium* respectivamente. Una levadura aislada fue identificada mediante secuenciación del dominio D1/D2 de la subunidad grande (26S) de DNA ribosomal; el resultado da una comparación con *Glomus* sp. Nuestros resultados muestran que los ambientes tropicales tienen el potencial para buenas fuentes de microorganismos con actividad de carbohidrasas poco comunes.

Dedication

To my family, the Tirado-Acevedo for teaching me the important things in life and for being all I want to be when I grow up.

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Table of contents

List of Tables.....	vii
List of Figures.....	viii
Fist of Appendixes.....	ix
Introduction.....	1
Literature Review.....	3
Cellulose degrading microorganisms.....	4
Xylan degrading microorganisms.....	5
Amylose degrading microorganisms.....	6
Pullulan degrading microorganisms.....	7
Galactomanan degrading microorganisms.....	8
Dextran degrading microorganisms.....	9
Arabinan degrading microorganisms.....	9
Materials and Methods.....	11
Sample collection and microorganism isolation.....	11
DNA Extraction.....	16
Polymerase chain reaction.....	16
Identification of isolates.....	17
Identification of fungal isolates.....	17
Growth curves.....	18
Enzyme assays.....	18
Results	21
Percentage of carbohydrate degraders in the total population.....	21
Sequencing and identification of bacterial isolates.....	25
Growth curves.....	25
Enzyme assays.....	29
Identification of fungal isolates.....	34
Discussion	36
Conclusions.....	41
References.....	42
Appendixes.....	51

List of Tables

Table 1: Carbohydrates used for essays as sole carbon source.....	14
Table 2: Carbohydrate degrading marine bacteria on AZCL insoluble chromogenic substrates at pH 4.7 and 7.0.....	22
Table 3: Carbohydrate degrading terrestrial bacteria on AZCL insoluble chromogenic substrates at pH 4.7 and 7.0.....	23
Table 4: Carbohydrate degrading yeast on AZCL insoluble chromogenic substrates at pH 4.7 and 7.0.....	24
Table 5: Carbohydrate degrading terrestrial fungi on AZCL insoluble chromogenic substrates on SD substrate at acidic pH.....	24
Table 6: 16S rRNA sequencing results for marine carbohydrate degrading bacteria.....	26
Table 7: 16S rRNA sequencing results for terrestrial carbohydrate degrading bacteria.....	27
Table 8: Generation time (hr) for selected strains on different substrates.....	28
Table 9: Final CFU/ml from growth curves for selected strains on different substrates.....	28
Table 10: α -Glucosidase assays from isolate Gamma proteobacterium Md 241.....	30
Table 11: Isolate <i>Bacillus</i> sp. Tf 1-40 α -glucosidase assays.....	31
Table 11: Isolate <i>Bacillus</i> sp. Tf 1-40 α -arabinosidase assays.....	32
Table 12: Isolate <i>Bacillus</i> sp. Tf 1-40 β -xylosidase assays.....	33

List of Figures

Figure 1: Methods flowchart.....12

Figure 2: Collection sites.....13

Figure 3: Colonies showing degradation of chromogenic carbon substrates15

Figure 4: Gycosidase detection flowchart.....20

List of Appendixes

Appendix 1: Culture Media.....51

Appendix 2: Partial 16S rRNA sequences.....54

Appendix 3: Isolated carbohydrate degrading fungi.....66

Introduction

In the searching of a cleaner and environmentally friendly fuel and concerned about the possibility of running out of fossil fuel some industries and laboratories have taken the task of looking for new alternatives. They have been working with the conversion of plant biomass to fuel ethanol. Plant biomass, the most abundant and renewable organic compound in nature can be converted to reducing sugars by means of hydrolysis by naturally produced enzymes. These sugars can eventually be used by yeast to produce ethanol. The aim of this work was to isolate and characterize microorganisms with novel carbohydrase activities from tropical ecosystems for their possible use in ethanol production and/or other industries.

Carbohydrates, sugar based compounds, are produced mostly by green plants and they serve as building blocks and energy source for plant growth and development. A large number of carbohydrates are present in plant cell walls as polysaccharides, which in turn are the most abundant organic compounds found in nature (de Vries and Visser, 2001). The two major components of plant cell walls are cellulose and hemicellulose, the latter are chemically complex. The hemicelluloses are numerous heteropolysaccharides such as arabinans, galactans, glucans, mannans, and xylans (Schäfer *et al.*, 1996). Since plant biomass is the major source of organic carbon for bacterial activity, it is not surprising that overwhelming quantities of microorganisms are capable of carbohydrase production.

Carbohydrases, enzymes that hydrolyze carbohydrates, represent one of the largest groups of industrial enzymes; second only to proteases, they account for about 28 % of the total worldwide sale of enzymes (Rao *et al.*, 1998.). These enzymes have

numerous applications in a number of industries. Some of these include baking, paper, detergent, textile, and the conversion of biomass to fuels and chemicals, thus reducing fossil energy dependency.

Screening of naturally occurring microorganisms may be the best way to obtain new strains and/or enzymes for commercial applications (Bothast & Saha, 1997). The tropics may be the ideal location for such screening since they have the most biologically diverse environments; Puerto Rico, a tropical island has an impressive level of biodiversity. There have been several studies on degradation of organic by microorganisms isolated in Puerto Rico. These organisms had the ability of degrading poly(β -hydroxybutyrate-co- β -hydroxyvalerate), poly(3-hydroxybutyrate-co-hydroxyvalerate) starch, toxic chemicals, and a variety of carbohydrates (Imam *et al.*, 1999; Leathers *et al.*, 2000; Franqui-Espiet, 2001; Zaidi *et al.*, 2003; Rosado & Govind, 2003). These results encourage the discovery of enzymes from tropical environments.

Literature Review

It has been demonstrated that microorganisms are efficient degraders of cellulose, starch and other carbohydrate components of plant hemicelluloses by production of carbohydrases. These enzymes can be used for the conversion of biomass to simple sugars and these sugars eventually to many chemicals. Over the past years there has been an intensive research movement to discover and isolate microorganisms with new and improved carbohydrase production because the enzymes known at the present have a low catalytic effect (Amano and Kanda, 2002) therefore do not make these processes cost effective. The enzymes of interest include cellulase, xylanase, and amylase among others. Microorganisms with such enzyme activities have been isolated in terrestrial as well as marine environments including bacteria, fungi and yeasts.

A number of studies on how cellulose hydrolysis by microorganisms especially fungi (*Trichoderma reesei*) have been done over the years. Cellulose consists of linear chains of β -(1-4)-linked D-glucose residues. Its complete hydrolysis requires the use of cellulase, a multi-enzyme system. This system consists of endo-(1-4)- β -D-glucanases, which act randomly along the cellulose chain and are more active on amorphous cellulose; exo-(1-4)- β -D-glucanases, which release cellobiose units mainly from the chain ends and degrade preferentially crystalline cellulose (Carrard *et al.*, 2000); and β glucosidase acting on cellobiose to yield two glucose molecules (Mansfield *et al.*, 1999).

Fungal cellulases are different from bacterial systems, while fungal enzymes can be produced as separate entities, each of them having a specific action. Cellulosomes, produced by some bacteria, consist of discrete multifunctional, multi enzyme complexes

(Boisset *et al.*, 1999). The most profound biochemical difference between fungal and bacterial systems is the end product produced during hydrolysis of cellulose. While fungal system produce solely glucose during cellulose degradation, bacterial system produce cellobiose, which is taken up to the cell and hydrolysed to glucose (Steenbakkers *et al.*, 2003).

The following is a brief description of some microorganisms that have been isolated and studied for their production of carbohydrases.

Cellulose degrading microorganisms

Cellulose is the most abundant polymer on earth (Murashima *et al.*, 2002) with an estimated synthesis rate of 4×10^9 tons/year (Parsiegla *et al.*, 1998). Based on this fact, one can suggest that cellulases are the most abundant carbohydrases in nature. Although cellulases are distributed throughout the biosphere, they are mostly manifested in microbial and fungal sources (Almin *et al.*, 1975). Recently, biotechnologists have shown great interest in cellulases for the production of a renewable source of fuels and chemicals (Béguin, 1990). *Clostridium thermocellum*, *Clostridium cellulovorans* (Murashima *et al.*, 2002), and *Clostridium celluloliticus* (Parsiegla *et al.*, 1998) are some of the documented bacteria having cellulase activities. Malekzadeh and colleagues (1993) isolated and identified three cellulolytic bacteria from forest humus soil. Two of those strains were characterized as new members of the genus *Cellulomonas*, *Cellulomonas persica* sp. nov. and *Cellulomonas iranensis* sp. nov., respectively (Elberson *et al.*, 2000). Cellulose degrading activity in the genus *Streptomyces* has also

been found. A new member of this genus called *Streptomyces cellulolyticus* sp. nov., was isolated from a soil sample (Li, 1997).

Two genera of fungi, *Trichoderma* and *Aspergillus* have been studied extensively for cellulase production (Bothast & Saha, 1997). *Trichoderma reesei* has become one of the most prolific sources of cellulase enzymes due to its secretion of a family of different cellulolytic enzymes (Fujita *et al.*, 2002). Several yeast genera have also been screened. These include *Candida*, *Debaromyces*, *Kluyveromyces*, and *Pichia*. *D. yamadae*, *K. marxianus*, and *C. chilensis* showed the presence of thermoactive enzymes, a desired characteristic for industrial application (Saha & Bothast, 1996).

Xylan degrading microorganisms

After cellulose, xylan is the most abundant renewable polysaccharide in nature (Bajpai, 1997). Xylan is a β -(1-4)-linked D-xylose polymer and the complete hydrolysis of its backbone involves β -(1-4)-xylanases and B-xylosidases. Agro-industries have been very interested in xylan degrading enzymes due to their applications in conversion of hemicellulosic biomass to fuels and chemicals, delignification of paper pulp, digestibility enhancement of animal feedstock, clarification of juices, and improvement in the consistency of beer (Saha & Bothast, 1999). Numerous genera of microorganisms have been documented to be capable of producing xylanases. Some of these are *Clostridium*, *Bacillus*, *Aeromonas*, *Ruminococcus*, and the thermophilics *Thermomonospora* and *Thermotoga* (Bothast & Saha, 1997; Saha & Bothast, 1999). *Trichoderma* and *Aspergillus* xylanases have also been reported (Bajpai, 1997). Some of these xylanases

are non-specific, attacking a number of compounds including cellulose, and cellobiose, arabinan, and xylan among others.

Amylose degrading microorganisms

Amylose is one of the two components of starch, the other being amylopectin. This carbohydrate is a polymer of glucose joined by linear α -(1-4)-glycosidic linkages. Amylases hydrolyze starch cleaving these linkages (Shaw *et al.*, 1995), generating linear maltodextrins (Rolfsmeier *et al.*, 1998). Amylases are among the most important commercial enzymes, having wide applications in starch processing, brewing, alcohol production, textile (Dong *et al.*, 1997) and detergent industries (Igarashi *et al.*, 1998). A vast number of these enzymes have been isolated from microorganisms. For example, *Streptococcus bovis*, an inhabitant of bovine rumen has been well studied for its amylase production. These enzymes have been purified, characterized (Freer, 1993), cloned in *E. coli* (Satoh *et al.*, 1993; Cotta and Whitehead, 1993), and sequenced (Satoh *et al.*, 1997). Several members of the *Bacillus* genus are well known for their amylolytic enzymes. Some of these include *B. amyloliquefaciens*, *B. stearothermophilus*, *B. subtilis* and *B. licheniformis*, widely used for technical application for their enzyme thermostability (Igarashi *et al.*, 1998; Dong *et al.*, 1997). Many hyperthermophiles have been screened for their variety of amylolytic enzymes (Adams, 1993). Several amylases from *Pyrococcus furiosus*, *Pyrococcus woesei* (Dong *et al.*, 1997), and *Thermotoga maritima* (Liebl *et al.*, 1997) have been described. Franqui-Espiet (2001), isolated and characterized a *Vibrio* species from a mangrove forest in Puerto Rico showing amylase

activity. Amylases from several *Aspergillus* species are well known, including *A. oryzae* (Nakajima *et al.*, 1986; Ohdan *et al.*, 1999) and *A. awamori* (Ohdan *et al.*, 1999).

Maltodextrins, short oligosaccharides with α -(1,4) and/or α -(1-6) glucosidic linkages produced by α -amylases are converted to glucose by the action of α -glucosidases (Kelly *et al.*, 1986). This enzyme is widely distributed among microorganisms. It has been characterized from a number of several bacteria such as *Lactobacillus brevis*, *Vibrio* sp. and especially *Bacillus* species including, *B. amylolyticus*, *B. brevis*, *B. cereus*, *B. megaterium*, and *B. subtilis* (Franqui-Espiet, 2001; Strzelecki *et al.*, 1993; Kelly *et al.*, 1986; Thirunavukkarasu and Priest, 1984). α -Glucosidases from archaeobacteria such as *Sulfolobus sulfataricus* and *P. furiosus* have been characterized (Rolfmeier *et al.*, 1998; Constantino *et al.*, 1990). An α -glucosidase has also been localized and characterized in *Brettanomyces lambicus*, a yeast used in the fermentation of a Belgian beer (Shantha Kumara *et al.*, 1993).

Pullulan degrading microorganisms

Pullulan is an α -glucan composed of α -(1-4) and α -(1-6) with maltotriose as its basic unit and it is produced by the yeast-like fungus *Aureobasidium pullulans*. Pullulanases are widely used in the starch processing industries along with amylases (Kim *et al.*, 2000). In the last decade new pullulanases have been found in a number of microorganisms, mainly thermophilic bacteria and archaea (Chen *et al.*, 2001). Highly thermostable pullulanases have been described in *P. woesei*, *P. furiosus*, *Thermococcus litoralis*, *Thermococcus celler*, *Thermococcus aggregans*, *Staphylothermus marinus*, *B. stearothermophilus* TS-23, *Thermus* sp. IM6501, and *Desulfurococcus mucosus* (Chen *et*

al., 2001; Duffner, *et al.*, 2000; Kim *et al.*, 2000; Niehaus *et al.*, 1999, and Canganella, *et al.*, 1994). Pullulanases have also been found in tropical environments. In 2001, Franqui-Espiet characterized a *Vibrio* species containing pullulanase activity (Franqui-Espiet, 2001).

Galactomanan degrading microorganisms

Galactomannans occur in the endosperms of a wide range of leguminous seed in amounts varying from 0.1 to 45 % (Kremnicky & Biely, 1997), and are the main softwood hemicelluloses (Brumer III *et al.*, 1999). The complete enzymatic degradation of galactomannan into monosaccharides involves endo-(1-4)- β -mannanase, β -mannosidase and α -galactosidase (Ademark *et al.*, 2001). The first two enzymes hydrolyze the β -D-mannoside linkages in β -(1-4)-D-mannan, producing small manno oligosaccharides and D-mannose, and the latter hydrolyses terminal α -(1-6)-linked galactose residues. These enzymes are widely used in the pulp bleaching and poultry industries, and have been reported from bacteria, fungi, and yeast. Wainø and Ingvorsen (1999) described a halostable mannanase from a novel halotolerant *Bacillus* sp. Thermostable manannases have been reported from *Bacillus stearothermophilus*, *Caldicellulosiruptor sacharolyticus*, and from a strain belonging to the genus *Thermotoga* (Wainø & Ingvorsen, 1999). *T. reesei* and *Phanerochaete chrysosporium* are some of the fungi reported to produce manannases (Brumer III *et al.*, 1999). In a study by Kremnicky and Biely (1997) the best producers of extracellular manannases among yeasts and yeast-like microorganisms, were found among the strains of

Aureobasidium pullulans. *Aspergillus niger* genes have been characterized for their production of α -galactosidase and β -mannosidase (Ademark *et al.*, 2001).

Dextran degrading microorganisms

Dextran is an exopolysaccharide consisting of α -1,6 linked glucose chains produced by numerous microorganisms, specially *Leuconostoc mesenteroides*. Dextranases have been shown to be useful in sugar industries eliminating difficult mucous residues and for dental industries in removing tartar on teeth (Khalikova *et al.*, 2003). Lately dextranases have been tested for its ability to enhance antibiotic activity (Mghir *et al.*, 1994). Various microorganisms are capable of producing dextran-degrading enzymes. For example, *Streptococcus downei*, *S. mutans*, and *S. salivarius*, *S. suis*, *Thermoanaerobacter* sp., and *Bacillus* sp. are among the bacteria producing dextranases (Khalikova *et al.*, 2003; Aoki & Sakano, 1997; Serhir *et al.*, 1997; Wynter *et al.*, 1996). Some dextranase producing fungi and yeasts include *Penicillium* sp., *Aspergillus* sp., *Thermomyces lanuginosus*, *Fusarium* sp., *Paecilomyces* sp., *Lipomyces* sp., and *Chaetomium gracille* (Clarke *et al.*, 1997; Jensen and Olsen, 1996).

Arabinan degrading microorganisms

Arabinan is a pectic polysaccharide found primarily in seeds, fruits, and vegetables (McKie *et al.*, 1997). L-arabinosyl residues constitute monomeric and/or oligomeric side chains on the β -(1-4)-linked xylose or galactose backbone in xylans, arabinoxylans, and arabinogalactans; they are the core in arabinan, forming α -(1-5)-linkages. α -L-Arabinofuranosidases and endo- α -(1-5)-arabinanases are the two major

enzymes that hydrolyze arabinan. The former are exo-type enzymes which hydrolyze terminal non-reducing α -L-arabinofuranosyl groups, while the latter is an endo-type enzyme specific for α -(1-5)-L-arabinofuranosidic linkages. These enzymes are investigated for their application in bio bleaching, and conversion of hemicellulose to fuels and chemicals. The arabinan degrading system of *Aspergillus niger* is one of the most extensively studied with one arabinanase and two arabinofuranosidases characterized (Skjøt *et al.*, 2001). Arabinofuranosidases have been characterized from *Bacillus stearothermophilus* T-6, *Clostridium acetobutylicum* ATCC824, *Streptomyces diasticus*, and *Aureobasidium pullulans* (Lee & Forsberg, 1987; Tjana *et al.*, 1992; Gilead & Shoham, 1995; Saha & Bothast, 1998). Arabinanases have been isolated from *Streptomyces chartreusis*, *S. diastatochromogenes*, *Aspergillus niger* and *A. awamory* among others (Matsuo *et al.*, 2000).

It has been documented in the literature that a whole number of novel enzymes can be isolated from natural isolates from different environments. The tropics, high carbon producing environments, are a promising source of enzymes with characteristics suitable for their use in different industries. Puerto Rico, is a Caribbean island surrounded by highly biodiverse waters and possesses El Yunque National Rain Forest. Tropical environments are considered the most productive and diverse (Star and Taggart, 1981). These characteristics make Puerto Rico ideal for the isolation of microorganisms with novel carbohydrase activities. The current research is an initial screen for potential carbohydrate degradation by microorganisms from tropical niches in the coastal and mountainous area of Puerto Rico.

Materials and Methods

Sample collection and microorganism isolation

For methods flow chart see figure 1. Seven seawater and sediments from southwest insular shelf edge and sixteen soil, plant litter, decaying wood, and water samples from El Yunque Tropical Rain Forest (Fig. 2), were collected in sterile 50 ml plastic tubes. These were immediately transported to the laboratory. Bacterial samples were plated out on solid rich nutrient medium Luria Agar or Bacto[®] Marine Agar at pH 4.7 or 7.0 at room temperature (23-25°C) as well as 45°C for three to seven days. Individual colonies were isolated and inoculated on seven different azurin insoluble crosslinked chromogenic carbon substrates, amylose, pullulan, cellulose, dextran, galactomannan, arabinan, and xylan (AZCL[®], Megazyme, Ireland limited) (1 % w/v) plates at 4.5 and 7.0 pH respectively (Table 1). Identification of carbohydrase activity was observed by substrate blue dye release (Fig 3).

Yeast and fungi were plated out on Yeast extract, Peptone, and Dextrose agar (pH 5.0) supplemented with Ampicillin (100 µg/ml) (YPD⁺). These isolates were tested on solid Synthetic Minimal Media (SD) containing 0.67 % yeast nitrogen base without aminoacids with AZCL[®] substrates (Appendix 1).

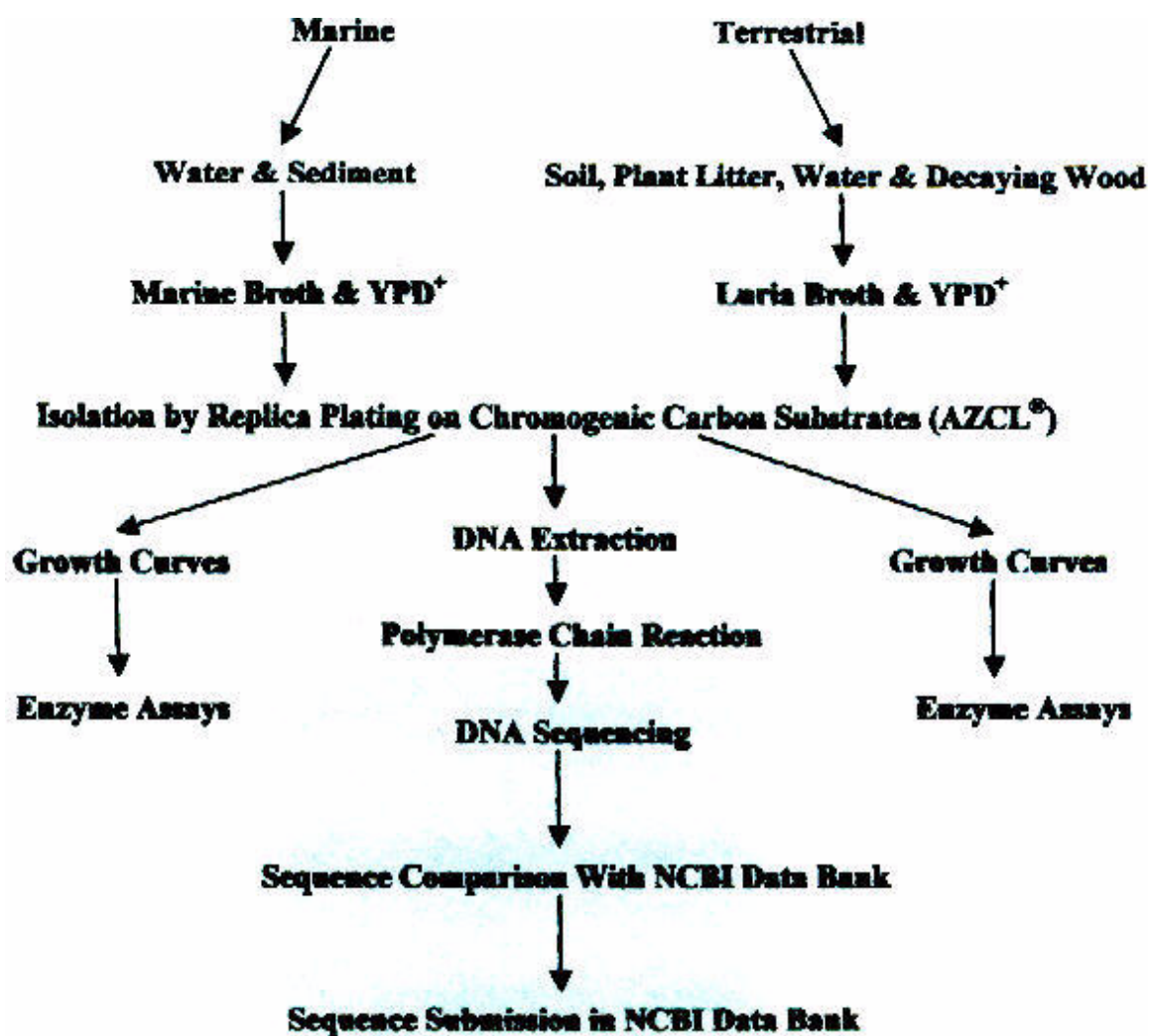


Figure 1. Methods flowchart.

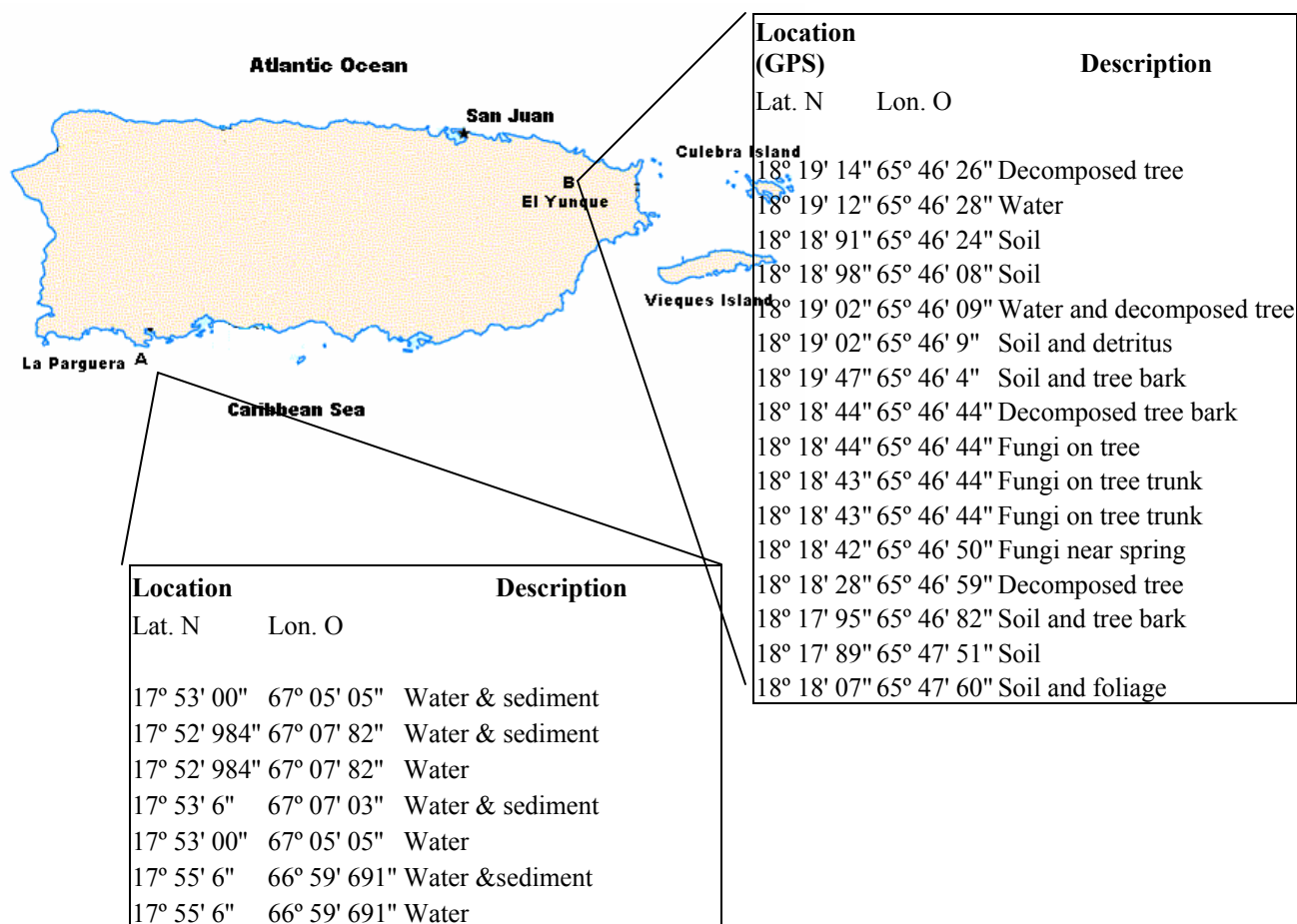


Figure 1. Collection sites. A) La Parguera insular shelf end, Lajas. B) El Yunque Tropical Rain Forest, Rio Grande.

Table 1. Carbohydrates used for essays as sole carbon source.

Carbohydrate	Linkage	Source
Amylose	α -(1-4)	Starch
Pullulan	α -(1-4), α -(1-6) branches	Aureobasidium pullulans
Cellulose	β -(1-4)	Plant biomass
Dextran	α -(1-6)	Soil bacteria
Galactomannan	β -(1-4), α -(1-6) branches	Wood and plant seeds
Arabinan	α -(1-5)	Plant cell walls
Xylan	β -(1-4)	Plant cell walls

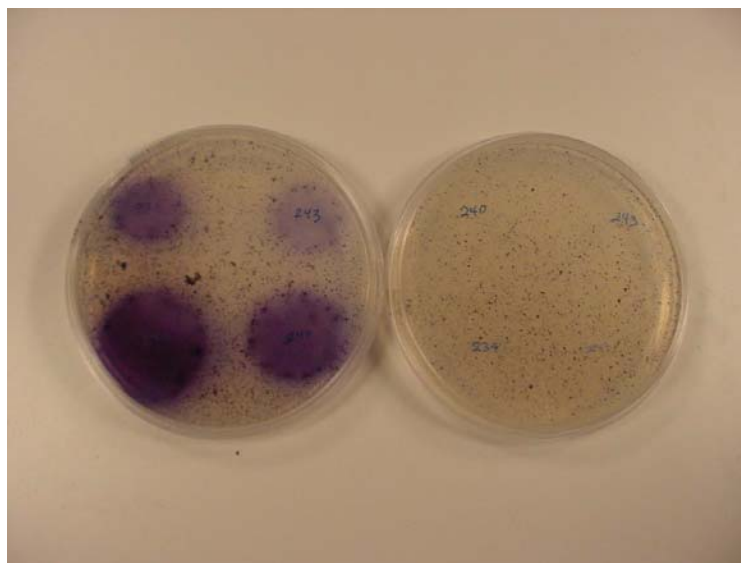


Figure 3. Colonies showing degradation of chromogenic carbon substrates. Blue dye release is indication of degradation.

DNA extraction

Bacterial genomic DNA was isolated using the technique described by Großkopf *et al.*, 1998. Briefly, the technique entails the following: 5 ml of mid to late log phase bacteria were collected by centrifugation and resuspended in 100 µl of deionized distilled water. Lysis of cells was achieved by boiling the samples for ten minutes. The solution was subsequently centrifuged and the supernatant collected. One microliter of supernatant was used directly for PCR amplification of the partial small subunit rRNA gene. Yeast DNA was isolated using the Y-DER[®] Yeast DNA Extraction Reagent Kit (Pierce, Illinois, U.S.A), according to the manufacturer's instruction. A concentration of 100 ng was used for PCR amplification of the divergent D1/D2 domains of the large subunit (LSU) rRNA gene (Kurtzman & Robnett, 1998).

Polymerase chain reaction

Genomic DNA of bacterial samples was used for PCR amplification. Approximately 526 base pairs (bp) of the 5' end of the 16S rRNA gene were amplified using highly conserved primers BSF8(s) 5'- AGAGTTTGATCCTGGCTCAG -3' (starting at nucleotide 8 on the *E. coli* numbering system) and BSR538(as) 5'- ATTACCGCGGCTGCTGGC - 3' (nucleotide 538 on the *E. coli* numbering system) synthesized by Genemed Synthesis, Inc. San Francisco, CA U.S.A. The amplification conditions were as follows: 30 amplifications cycles with denaturing at 95°C for one minute, annealing at 35°C for one minute, and extension at 72°C for one minute. The genomic DNA of yeast samples was used for PCR amplification as in Kurtzman &

Robnett, 1998. Approximately 579 bp of the 5' end of the large sub-unit (LSU) rRNA gene were amplified using primers NL-1 5'- GCATATCAATAAGCGGAGGAAAAG-3' (starting at nucleotide 63 for *Saccharomyces cerevisiae*) and NL-4 5'-GGTCCGTGTTTCAAGACGG – 3' (nucleotide 642 for *Saccharomyces cerevisiae*) (Kurtzman & Robnett, 1998) synthesized by Genemed Synthesis, Inc. San Francisco, CA U.S.A. The amplification conditions were as follows: 36 amplifications cycles with denaturing at 94°C for one minute, annealing at 52°C for two minutes, and extension at 72°C for two minutes (Kurtzman & Robnett, 1998).

Identification of isolates

Amplicons of isolates were purified using GeneClean[®] III DNA purification kit (BIO 101) according to manufacturer's specifications. Both strands of the amplified 16S rDNA templates were sequenced using ABI Prism[®] Big Dye[™] Terminator reaction and the ABI 310 genetic analyzer. Sequence electropherograms were edited using online tool Biology Workbench 3.2 (San Diego Supercomputer Center, <http://biowb.sdsc.edu>). The DNA sequence data was compared and analyzed using BLASTN (Altschul *et al.*, 1990).

Identification of fungal isolates

Fungal isolates were identified by morphological characteristics according to the taxonomic keys (Klich & Pitt, 1988; St-Germain & Summerbell, 1996).

Growth curves

Selected isolates were inoculated in 5 ml of liquid rich nutrient medium Luria Agar and Bacto[®] Marine Agar respectively (see Appendix 1) and left overnight. Two thousand cells of each isolate (1 O.D. 600nm = 2.5×10^8 cells/ml, Sambrook and Russell, 2001) inoculated in 50 ml standard medium (0.1 % Ammonium Chloride, 0.025 % HEPES, 0.01 % Yeast Extract as a vitamin source, and 1 % Glucose (control) or relevant carbohydrate substrates at both 4.7 as well as neutral pH. On a 24 hour basis, pH readings were taken and cells collected (100 μ l) and plated out on solid nutrient medium (see above). Plate counting was used for enumeration.

Enzyme assays

For an assay flow chart see figure 4. Selected isolates were inoculated in 5 ml of liquid rich nutrient medium Luria Agar or Bacto[®] Marine Agar respectively and left overnight. Four thousand cells of each isolate were inoculated in 100 ml standard medium (see above) at pH 7.0. Cells were harvested at logarithmic phase by centrifugation at 10,000 x g during 10 minutes using a Beckman J2-M6 centrifuge. The pellet was treated with Y-PER[®] Plus protein extraction reagent (Pierce, Illinois, USA) according to manufacturer's instructions. Cells were resuspended in an appropriate amount of Y-PER[®] Plus protein extraction reagent (2.5-5.0 ml for 1 gram of cell). The mixture was agitated gently at room temperature for 20 minutes. Cell debris was collected at 10,000 x g during 10 minutes. Culture supernatant, cytosolic fraction, and cell debris (resuspended in 0.05 M phosphate buffer) were used for glycosidase activities test with the *p*-nitrophenyl derivatives relevant monosaccharides (Sigma, St. Louis, M.O.,

U.S.A.): *p*-nitrophenyl- β -D-cellobioside, *p*-nitrophenyl- α -D-glucopyranoside, *p*-nitrophenyl- α -L-arabinofuranoside, *p*-nitrophenyl- β -D-xylopyranoside, *p*-nitrophenyl- β -D-mannopyranoside, and *p*-nitrophenyl- α -D-galactopyranoside according with the protocol by Ivanova *et al.* (2003) with some modifications. The reaction mixture consisted of 0.05 ml of culture supernatant, cytosol, or debris (diluted with 0.05 M phosphate buffer, pH 7.0, when needed) and 0.05 ml of corresponding *p*-nitrophenyl-glycoside (1 mg ml⁻¹ in 0.05 M phosphate buffer, pH 7.0), at 25°C. The reaction was stopped by addition of 1 M Na₂CO₃ (0.1 ml). One unit of glycosidase activity was defined as the amount of enzyme that liberated 1 μ mol of *p*-nitrophenol per minute at 25°C. Total protein concentration was measured using Sigma diagnostics[®] Protein assay kit (St. Louis, MO, USA) with bovine serum albumin as the standard.

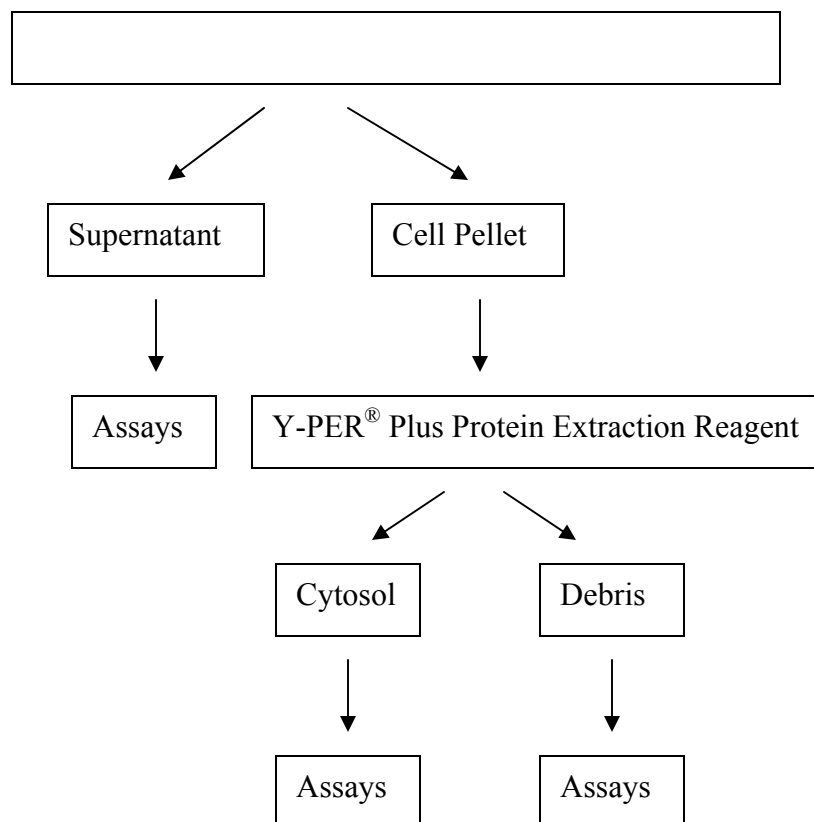


Figure 4. Glycosidase detection flowchart. After harvesting the cells, pellet was treated with Y-PER® Plus Protein Extraction Reagent. Supernatant, cytosol, and cell debris were used for enzyme assays.

Results

Percentage of Carbohydrate Degraders in the Total Population

Original bacteria on plates had an average population of 7.15×10^7 Colony Forming Units (CFU) in marine samples and 1.49×10^4 CFU in terrestrial samples. After replica plating from original population plates (MB, marine; LB, terrestrial) to AZCL[®] substrate plates, 2.12 % marine and 9.32 % terrestrial polysaccharides degraders were detected. Release of a soluble blue dye indicated the presence of carbohydrate degrading ability. A total of sixteen marine and nineteen terrestrial isolates were subsequently isolated and transferred to each AZCL[®] substrate (amylose, pullulan, cellulose, dextran, arabinan, galactomannan, and xylan), at both pH 4.7 and 7.0, and room temperature as well as 45°C. Most isolated marine bacteria degrade pullulan and arabinan at either pH, but not cellulose or galactomannan. Most isolated terrestrial bacteria degrade amylose and pullulan at both pH. Only one isolate showed cellulase activity. It degrades cellulose at room temperature as well as 45°C. No isolates were capable of dextran degradation (Table 2 - 3).

From YPD⁺ we were able to isolate eleven yeast and three fungal isolates. These isolates were transferred to each SD + AZCL[®] plates (Appendix 1), yeast at both pH 4.7 and 7.0, and fungal isolates at pH 4.7. Both, yeast and fungal isolates were tested at room temperature as well as 45°C. Only one yeast isolate showed carbohydrase activity degrading only amylose at acidic pH, and room temperature (Table 4). All fungal isolates were capable of degrading cellulose, galactomannan, and xylan. Two of these were capable of degrading the carbohydrates at room temperature as well as 45°C (Table 5). As with bacterial isolates no yeast or fungal isolate was capable of degrading dextran.

Table 2. Carbohydrate degrading marine bacteria on AZCL insoluble chromogenic substrates at pH 4.7 and 7.0.

Strain	Azurine-crosslinked Polysaccharide insoluble chromogenic substrates													
	Amylose		Pullulan		Cellulose		Dextran		Galactomannan		Arabinan		Xylan	
Isolate #	pH4.7	pH7.0	pH4.7	pH7.0	pH4.7	pH7.0	pH4.7	pH7.0	pH4.7	pH7.0	pH4.7	pH7.0	pH4.7	pH7.0
Md 208	-	+	+	+	-	-	-	-	-	-	-	-	-	-
Md 209	-	+	+	+	-	-	-	-	-	-	-	-	+	+
Md 210	-	-	+	+	-	-	-	-	-	-	-	-	-	-
Md 211	+	-	+	+	-	-	-	-	-	-	-	-	-	-
Md 213	-	-	+	+	-	-	-	-	-	-	+	+	-	-
Md 214	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Md 215	+	+	+	+	-	-	-	-	-	-	-	-	-	-
Md 216	+	+	+	+	-	-	-	-	-	-	-	-	-	-
Md 217	-	-	+	+	-	-	-	-	-	-	+	+	-	-
Md 218	+	+	+	+	-	-	-	-	-	-	-	-	-	-
Md 236	-	-	+	+	-	-	-	-	-	-	+	+	-	-
Md 237	+	+	+	+	-	-	-	-	-	-	+	+	-	-
Md 238	-	-	+	+	-	-	-	-	-	-	+	+	-	-
Md 239	-	-	+	+	-	-	-	-	-	-	+	+	-	-
Md 240	-	-	+	+	-	-	-	-	-	-	+	+	-	-
Md 241	+	+	+	+	-	-	-	-	-	-	-	+	-	-
Md 242	+	+	+	+	-	-	-	-	-	-	+	+	-	-
Md 243	-	-	+	+	-	-	-	-	-	-	+	+	-	-

Table 3. Carbohydrate degrading terrestrial bacteria on AZCL insoluble chromogenic substrates at pH 4.7 and 7.0.

Strain	Azurine-crosslinked Polysaccharide insoluble chromogenic substrates													
	Amylose		Pullulan		Cellulose		Dextran		Galactomannan		Arabinan		Xylan	
Isolate #	pH4.7	pH7.0	pH4.7	pH7.0	pH4.7	pH7.0	pH4.7	pH7.0	pH4.7	pH7.0	pH4.7	pH7.0	pH4.7	pH7.0
Tf 1-40	+	+	+	-	+	+	-	-	+	+	+	+	+	+
Tf 1-41	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Tf 1-42	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Tf 1-45	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Tf 228	+	+	+	+	-	-	-	-	-	-	-	-	-	-
Tf 229	+	+	+	+	-	-	-	-	-	-	-	-	-	-
Tf 230	-	+	-	+	-	-	-	-	-	-	-	-	-	-
Tf 231	+	+	+	+	-	-	-	-	-	-	-	-	-	-
Tf 232	+	+	+	+	-	-	-	-	-	-	-	-	-	-
Tf 233	-	-	+	+	-	-	-	-	-	-	-	-	-	-
Tf 234	+	+	+	+	-	-	-	-	-	-	-	-	-	-
Tf 246	-	-	-	-	-	-	-	-	-	-	+	+	-	-
Tf 247	+	+	+	+	-	-	-	-	+	+	-	-	-	-
Tf 248	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Tf 249	+	+	+	+	-	-	-	-	-	-	-	-	-	-
Tf 250	+	+	+	+	-	-	-	-	+	+	-	-	-	-
Tf 252	+	+	+	+	-	-	-	-	+	+	-	-	+	+
Tf 253	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Tf 254	+	+	+	+	-	-	-	-	+	+	-	-	+	+

Table 4. Carbohydrate degrading terrestrial yeast on AZCL insoluble chromogenic substrates at pH 4.7 and 7.0.

Isolate #	Azurine-crosslinked Polysaccharide insoluble chromogenic substrates													
	Amylose		Pullulan		Cellulose		Dextran		Galactomannan		Arabinan		Xylan	
	pH4.7	pH7.0	pH4.7	pH7.0	pH4.7	pH7.0	pH4.7	pH7.0	pH4.7	pH7.0	pH4.7	pH7.0	pH4.7	pH7.0
2-16	+	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 5. Carbohydrate degrading terrestrial fungi on AZCL insoluble chromogenic substrates on SD substrate at acidic pH.

Isolate	Azurine-crosslinked polysaccharide insoluble chromogenic substrates						
	Amylose	Pullulan	Cellulose	Dextran	Galactomannan	Arabinan	Xylan
Fdeg2	+	+	+	-	+	+	+
MD45	+	+	+	-	+	+	+
U45	-	-	+	-	+	-	+

Sequencing and Identification of Bacterial Isolates

Comparison of generated sequences with those in GenBank database indicates that the majority of the identified carbohydrate degrading marine isolates belong to the genus *Pseudoalteromonas* (Table 6). *Aeromonas* was the most predominant genus in terrestrial environment isolates with carbohydrase activities (Table 7); *Aeromonas* was also the only genus present in both marine and terrestrial environments. Complete sequences are presented in Appendix 1.

Growth curves

To better understand how well selected microorganisms degrade complex carbohydrates as compared to glucose we growth curves assays were done. Isolates Md 241, Md 242, Tf 1-40, and Tf 254 were selected for growth curves assays for their ability of degrading the most substrates. Generation time was calculated (Table 8). Isolate Tf 1-40 resulted to be the most versatile organism assayed. In addition to being the bacterial isolate degrading the most substrates, it was the only one capable of cellulose degradation. Isolate Tf 1-40 grew as well or better on amylose, galactomannan, and xylan as on glucose (Table 9). Isolates Md 241 and Tf 254 grew better on amylose than on glucose (Table 9). Isolate Md 242 grew as well on pullulan as on glucose (Table 9). None of the isolates showed growth at acidic pH in liquid media, possibly due to low aeration.

Table 6. 16S rRNA sequencing results for marine carbohydrate degrading bacteria.

Code no.	ID%	No. of base pairs sequenced	Best-matched organism	Class	GenBank Accession no.
Md 208	99	529	<i>Vibrio</i> sp.	γ-Proteobacteria	AY461666
Md 209	99	515	<i>Pseudoalteromonas</i> sp.	γ-Proteobacteria	AY461667
Md 210	99	537	Gamma proteobacterium	γ-Proteobacteria	AY461668
Md 211	99	529	Gamma proteobacterium	γ-Proteobacteria	AY461669
Md 213	99	528	<i>Pseudoalteromonas</i> sp.	γ-Proteobacteria	AY461670
Md 216	98	537	<i>Vibrio</i> sp.	γ-Proteobacteria	AY461671
Md 218	99	530	<i>Aeromonas</i> sp.	γ-Proteobacteria	AY461672
Md 236	99	520	<i>Pseudoalteromonas</i> sp.	γ-Proteobacteria	AY461673
Md 237	96	518	Gamma proteobacterium	γ-Proteobacteria	AY461674
Md 238	100	485	<i>Pseudoalteromonas</i> sp.	γ-Proteobacteria	AY461675
Md 239	98	526	<i>Pseudoalteromonas</i> sp.	γ-Proteobacteria	AY461676
Md 240	99	526	Gamma proteobacterium	γ-Proteobacteria	AY461677
Md 241	96	514	Gamma proteobacterium	γ-Proteobacteria	AY461678
Md 242	99	518	<i>Halomonas</i> sp.	γ-Proteobacteria	AY461679
Md 243	99	522	Gamma proteobacterium	γ-Proteobacteria	AY461680
Md 244	99	468	Alpha proteobacterium	α-Proteobacteria	AY461681

Table 7. 16S rRNA sequencing results for terrestrial carbohydrate degrading bacteria.

Code no.	ID%	No. of base pairs sequenced	Best-matched organism	Class	GenBank Accession No.
Tf 1 40	99	530	<i>Bacillus</i> sp.	Bacilli	AY461682
Tf 1 41	99	536	<i>Bacillus</i> sp.	Bacilli	AY461683
Tf 1 42	100	493	<i>Bacillus</i> sp.	Bacilli	AY461684
Tf 1 45	99	477	<i>Bacillus</i> sp.	Bacilli	AY461685
Tf 228	99	529	<i>Aeromonas</i> sp.	γ -Proteobacteria	AY461686
Tf 229	99	525	<i>Aeromonas</i> sp.	γ -Proteobacteria	AY461687
Tf 230	99	529	<i>Aeromonas</i> sp.	γ -Proteobacteria	AY461688
Tf 231	98	524	<i>Pseudomonas</i> sp.	γ -Proteobacteria	AY461689
Tf 232	99	529	<i>Aeromonas</i> sp.	γ -Proteobacteria	AY461690
Tf 233	99	529	<i>Aeromonas</i> sp.	γ -Proteobacteria	AY461691
Tf 234	99	529	<i>Aeromonas</i> sp.	γ -Proteobacteria	AY461692
Tf 235	96	515	Bacteroidetes bacterium	Unclassified	AY461693
Tf 246	99	520	Oxalobacteraceae bacterium	β -proteobacteria	AY461694
Tf 247	99	488	<i>Kluyvera</i> sp.	γ -Proteobacteria	AY461695
Tf 249	97	527	<i>Aeromonas</i> sp.	γ -Proteobacteria	AY461696
Tf 250	100	523	<i>Kluyvera</i> sp.	γ -Proteobacteria	AY461697
Tf 252	97	503	<i>Paenibacillus</i> sp.	Bacilli	AY461698
Tf 253	100	525	<i>Bacillus</i> sp.	Bacilli	AY461699
Tf 254	100	534	<i>Staphylococcus</i> sp.	Bacilli	AY461700

Table 8. Generation time (hr) for selected isolates on different substrates.

Isolate	Substrate						
	Glucose	Amylose	Pullulan	Cellulose	Arabinan	Galactomannan	Xylan
Md 241	1.74	1.60	3.44	n/d ^a	4.00	n/d ^a	n/d ^a
Md 242	1.00	10.5	1.65	n/d ^a	6.1	n/d ^a	n/d ^a
Tf 1-40	2.63	2.24	n/d ^a	5.87	10.80	2.22	2.03
Tf 254	3.44	2.18	4.40	n/d ^a	n/d ^a	4.20	5.98

(a) No degradation

Table 9. Final CFU/ml from growth curves for selected isolates on different substrates.

Isolate	Substrate						
	Glucose	Amylose	Pullulan	Cellulose	Arabinan	Galactomannan	Xylan
Md 241	5.56x10 ⁷	1.24x10⁸	5.00x10 ⁵	n/d ^a	5.76x10 ⁶	n/d ^a	n/d ^a
Md 242	6.2x10 ¹⁰	5.30x10 ⁷	9.30x10 ⁷	n/d ^a	1.42x10 ⁷	n/d ^a	n/d ^a
Tf 1-40	2.20x10 ⁶	6.70x10 ⁶	n/d ^a	1.15x10 ⁶	1.90x10 ⁶	7.08x10 ⁶	1.42x10⁷
Tf 254	5.12x10 ⁵	8.26x10⁶	7.57x10 ⁶	n/d ^a	n/d ^a	1.68x10 ¹⁰	1.10x10 ⁷

(a)No degradation

Enzyme assays

Isolates Md 241 and Tf 1-40 were selected for enzyme assays due to their versatile carbohydrate degrading capability. After the preparation of crude enzyme extracts, isolates Md 241 glycosidase production was tested with the *p*-nitrophenyl derivative monosaccharides of α -D-glucopyranoside and α -L-arabinofuranoside. Isolate Tf 1-40 glycosidase production was tested with the *p*-nitrophenyl derivatives monosaccharides of α -D-glucopyranoside, β -D-cellobioside, α -L-arabinofuranoside, β -D-mannopyranoside, α -D-galactopyranoside, and β -D-xylopyranoside. One unit (U) of glycosidase activity was defined as the amount of enzyme that liberated 1 μ mol of *p*-nitrophenol per minute at 25°C.

Isolate Md 241

α -Glucopyranosidase activity was found in the cytosol and the cell debris fraction (Table 10). The enzyme approached maximum activity (1.267 U/mg) after 48 hr of culture in the cell debris. No activity was detected on α -L-arabinofuranoside.

Isolate Tf 1-40

α -Glucopyranosidase activity was found exclusively in the cell debris. The enzyme approached maximum activity after 24 hr of culture; no activity was detected after 48 hr culture (Table 11). α -Arabinofuranosidase activity was found in the cytosol and cell debris fraction (Table 12).

Maximum enzyme activity was found in cell debris after 24 hr culture. β -Xylopyranosidase activity was found in all cell fractions, with most of the activity detected on the debris (Table 13), which approached maximum activity after 48 hr

Table 10. α -Glucosidase assays from isolate Gamma proteobacterium Md 241.

Culture time (hr)	Assayed fraction	Enzyme units (U) ^a	Total protein (mg/ul)	Specific activity (U/mg)
24	Supernatant	0.000	0.015	0.000
	Cytosol	0.003 \pm 0.000	0.038	0.079 \pm 0.000
	Debris	0.004 \pm 0.001	0.047	0.085 \pm 0.029
48	Supernatant	0.000	0.033	0.000
	Cytosol	0.079 \pm 0.000	0.189	.418 \pm 0.000
	Debris	0.136 \pm 0.018	0.107	1.267 \pm 0.165
72	Supernatant	0.000	0.051	0.000
	Cytosol	0.111 \pm 0.001	0.253	0.437 \pm 0.003
	Debris	0.157 \pm 0.000	0.183	0.858 \pm 0.000

(a) U: unit. One unit of glycosidase activity was defined as the amount of enzyme that liberated 1 μ mol of *p*-nitrophenol per minute at 25°C.

Table 11. Isolate *Bacillus* sp. Tf 1-40 α -Glucosidase assays.

Culture Time (hr)	Fraction Assayed	Enzyme Units (U)	Total Protein (mg/ul)	Specific Activity (U/mg)
24	Supernatant	0.000	0.068	0.000
	Cytosol	0.000	0.118	0.000
	Debris	0.769 \pm .001	0.236	3.259 \pm .012
48	Supernatant	0.000	0.056	0.000
	Cytosol	0.000	0.114	0.000
	Debris	0.000	0.267	0.000

Table 12. Isolate *Bacillus* sp. 1-40 α -Arabinosidase assays.

Culture Time (hr)	Fraction Assayed	Enzyme Units (U)	Total Protein (mg/ul)	Specific Activity (U/mg)
24	Supernatant	0.000	.115	0.000
	Cytosol	0.000	.045	0.000
	Debris	0.065 \pm 0.002	.011	5.909 \pm 0.257
48	Supernatant	0.000	.102	0.000
	Cytosol	0.109 \pm 0.001	.038	2.869 \pm 0.037
	Debris	0.0085 \pm 0.001	.075	0.114 \pm 0.009
72	Supernatant	0.000	.146	0.000
	Cytosol	0.025 \pm 0.000	.081	0.309 \pm 0.000
	Debris	0.013 \pm 0.006	.139	0.094 \pm 0.040

Table 13. Isolate *Bacillus sp.* Tf 1-40 α -xylosidase assays.

Culture Time (hr)	Fraction Assayed	Enzyme Units (U)	Total Protein (mg/ul)	Specific Activity (U/mg)
24	Supernatant	0.000	0.232	0.000
	Cytosol	0.094 ± .002	0.104	0.897 ± .021
	Debris	2.59 ± .014	0.096	27.121 ± .148
48	Supernatant	0.263 ± .012	0.249	1.053 ± .048
	Cytosol	0.209 ± .006	0.108	1.935 ± .052
	Debris	231.055 ± 2.864	0.323	715.031 ± 8.866
72	Supernatant	0.433 ± .002	0.276	1.570 ± .008
	Cytosol	0.131± .006	0.109	1.199 ± .052
	Debris	108.933 ± 3.582	0.303	359.515 ± 11.823

culture. No activity was detected on β -D-cellobioside, β -D-mannopyranoside, or α -D-galactopyranoside.

Identification of fungal isolates

The amylose degrading isolated yeast had a 94% similarity with *Glomus* sp. The fungal isolates were identified based on both macroscopic as well as microscopic characteristics.

Fdeg2 - *Microsporum* sp.

Macroscopic characteristics:

Colony diameter on Sabouraud dextrose agar (SDA), 70mm, powdery white-bone white on the surface; reverse: beige. Texture and colors on Mycological agar (MA) on the surface, as on SDA; reverse: brown-beige. Dermatophyte Test Medium positive (Appendix 3.1).

Microscopic characteristics:

Hyphae – hyaline and septate. Macroconidia – rough, thick walled, solitary, 2-4 celled.

MD45 – *Aspergillus* sp.

Macroscopic characteristics:

Colony diameter on Czapek yeast agar (CYA), 50mm (49.5 – 51mm). On MA, powdery gray green on the surface: reverse pale yellow. Texture and colors on SDA as on MA. Fast growth at 45°C (Appendix 3.2).

Microscopic Characteristics:

Stipes – hyaline and smooth walled. Aspergillia – uniseriate. Conidia – globose, smooth walled, and borne in columns. Foot cell – T-shaped.

U45 - *Aspergillus* sp.

Macroscopic characteristics:

Colony diameter on CYA, 51mm (48.5-52.5mm). On MA, powdery gray green on the surface: reverse pale yellow. Texture and colors on SDA as on MA. Fast growth at 45°C (Appendix 3.3).

Microscopic Characteristics:

Stipes – hyaline and smooth walled. Aspergillia – uniseriate. Conidia – globose, smooth walled, borne in columns. T-shaped foot cell.

Discussion

Microorganisms are widely known for their organic compounds degrading capabilities. Carbohydrate degrading microorganisms have been isolated in a broad number of environments, from Icelandic hot springs to ruminant guts (Perttula *et al.*, 1993; Freer, 1993). From our screening from insular shelf environments we only isolate a small number (2-9%) of bacteria able to produce carbohydrate-degrading enzymes. These numbers contrast with Franqui-Espiet (2001) findings, whose screenings of two mangrove swamp samples showed a high number of organisms with the ability of starch degradation compared with the total number of cultured cells. Our findings may indicate that in the environments tested, bacteria are not the major complex carbohydrate degraders. There are reports that show that activity of glycosidic enzymes decrease from eutrophic mangrove influenced samples as compared to oligotrophic samples (Rath *et al.*, 1993). Perhaps, since most microbes from nature (99%) are difficult to cultivate (Fuhrman & Campbell, 1998), our screening probably underestimates the diversity of carbohydrate degraders. The fact that 81% of isolated degrading bacteria were able of using pullulan as a sole carbon source may be indicative that pullulan is an abundant substrate in these environments. *Aureobasidium pullulans* is a ubiquitous species, and it has been isolated from Puerto Rican samples (Leathers, 1986).

The majority of marine isolates belong to the genus *Pseudoalteromonas*, this result is not surprising since bacteria from this genus are well distributed around marine environments (Ivanova *et al.*, 2002) and there are reports on a number of carbohydrases from this species (Holmström & Kjelleberg, 1999; Ivanova *et al.*, 2003). *Aeromonas*, the most abundant genus among our terrestrial isolates, is very common in a diverse number

of natural environments, including soil, fresh and brackish water, sewage, and waste water (Kühn *et al.*, 1997). Although most *Aeromonas* isolates are known for their virulence in animals and humans (Kaznowski, 1998), there are reports on members of this genus with capabilities of degrading organic compounds including hydrocarbons, chitin and xylan (Kubata *et al.*, 1995; Ramsay *et al.*, 2000; Wu *et al.*, 2001).

We found very interesting that some of the bacterial isolates grew better on complex carbohydrates than on glucose. For example, isolates Md 241 and *Bacillus* sp. Tf 1-40 grew much better on amylase and xylan respectively. These isolates had a final CFU/ml of an order of magnitude more on the mentioned substrates as compared to glucose. Although glucose is the preferred carbon source for most bacteria (Brückner and Titgermeyer, 2002), there are a vast number of reports on bacteria using other sugars and organic compounds over glucose. For example *Streptococcus thermophilus* and *Lactobacillus bulgaricus* are known for their preference of lactose over glucose (van den Vogaart *et al.*, 2000; Chervaux *et al.*, 2000; and Brückner and Titgermeyer, 2002). In an extreme case Dyksterhouse *et al.*, 1995 reported a new bacterium capable of growth on a number of organic substrates such aromatic hydrocarbons, fatty acids, and aminoacids but showed no growth on glucose. These cases indicate an adaptation to special ecological niches that may result in the choice of any carbohydrate as favored substrate (Brückner and Titgermeyer, 2002). So it may be concluded that isolates Md 241 and *Bacillus* sp. Tf 1-40 have adapted to specific tropical niches.

There is an assumption that an uptake of complex organic molecules by microorganisms is initiated by means of extracellular enzymes (Arnosti, 2000). Enzyme activities from both of our selected isolates, Md 241 and Tf 1-40, were detected mainly in

cytosol and cell debris fractions, possibly meaning that these enzymes are cell associated. Low or no production of extracellular enzymes of some marine organisms has also been reported in our laboratory (Rosado & Govind, 2003; Franqui-Espiet, 2001). This is probably a reflection of the nature of the environment in which secretion of large amounts of extracellular enzymes would be uneconomical for the organism (Perttula *et al.*, 1993), since marine currents and constant water runoff from the rain forest can wash away extracellularly produced enzymes impeding the substrate degradation by the organism. It is possible that these organisms employ a cellulosome-like system, where the cell has a non-enzymatic protein bound to its surface, and a number of enzymatic subunits attached to the other end. In addition to cellulose degradation, other cell wall material is known to be degraded by cellulosomes (Doi *et al.*, 2003). Although cellulosomes are known to be produced mainly by anaerobic bacteria such as *Clostridium*, there is evidence for cellulosomes in an aerobic bacterium (Shoham *et al.*, 1999). In the case of marine isolates, these cell associated enzymes may well serve as anchoring molecules.

Enzyme assays were done at neutral pH. Although there is the assumption that soil average pH is acidic, all the samples tested for pH resulted in neutral to basic readings. We were able to quantify only a low α -glucosidase activity from isolate Md 241. This may indicate high stability of the enzyme. Isolate Tf 1-40 showed a broader carbohydrate-degrading enzyme producing spectrum. We were able to detect α -glucosidase, α -arabinofuranosidase, and a very high β -xylopyranosidase activity. The low extracellular enzyme detection on this isolate is very interesting since this isolate's preliminary identification is *Bacillus subtilis* and among bacteria, *Bacillaceae* are the

most commonly used in industrial fermentations, since they secrete most of their enzymes (Morales *et al.*, 1993). Since these enzymatic assays were done with crude enzyme preparations, future isolation and characterization of these enzymes will be necessary to demonstrate if they can be used for industrial purposes.

Many studies on carbohydrase producing yeasts have been done with yeasts with winemaking importance and for ethanol production purposes. Manzanares and her collaborators (1999) detected β -D-xylosidase activity in eight strains belonging to the genera *Hanseniaspora* and *Pichia*. Saha & Bothast (1996) screened 48 yeast strains for production of β -glucosidase activities; all of them were able produce the enzyme. Our results of only one carbohydrate degrading yeast correlate with Buzzini & Martini's (2002) study where starch-degrading activity was rare among yeast isolated from tropical environments.

Fungi are some of the most thoroughly studied organisms that degrade cellulosic material (Chan & Au, 1987; MacKenzie *et al.*, 1987; Brumer III, *et al.*, 1999; Fujita *et al.*, 2002), because they appear to be the most efficient producers of extracellular enzymes (Jørgensen, *et al.*, 2003). Among fungi, *Trichoderma reesei* and *Aspergillus* spp. are some of the best studied. We have isolated three carbohydrate-degrading fungi, all of which are capable of cellulose degradation. Two of these isolates that were identified as *Aspergillus* spp., showed degradation on a wide variety of substrates at 45°C, including amylose, cellulose, and xylan among others. These properties make them interesting for industrial purposes. Dermatophytes, fungi belonging to the genera *Epidermophyton*, *Microsporum* and *Trichophyton*, are known for producing keratin degrading enzymes (Carlotti & Bensignor, 1999), infecting human, cats, and dogs among

other hosts. In our literature review we could not find any report on carbohydrate degrading capabilities of dermatophytes. Our third isolate, identified as *Microsporum* sp. could be the first report on a dermatophyte with carbohydrate degrading capabilities.

Conclusions

We have made an initial screening for potential carbohydrate degradation by microorganisms from tropical niches in the island of Puerto Rico. We have isolated a total of sixteen marine and nineteen terrestrial bacterial isolates, three fungi and a yeast capable of using complex carbohydrates as sole carbon source. Bacterial isolates Md 241 and *Bacillus* sp. Tf 1-40 showed better growth on some polysaccharides than on glucose indicating a possible adaptation to special ecological niches.

An α -glucopyranosidase was detected in the intracellular and cell surface fractions of isolate Md 241. Three carbohydrases were detected in isolate *Bacillus* sp. Tf 1-40. An α -glucopyranosidase was found only in the cell surface fraction, an α -arabinofuranosidase in the intracellular and cell surface fractions, and a highly active β -xylopyranosidase in all cell fractions tested.

All three isolated fungi showed degradation of most of the substrates tested including cellulose, supporting the idea that fungi are the principal cellulose degraders, since only one bacterial isolate was able of cellulose degradation. The isolation of only one carbohydrate-degrading yeast isolate correlate with other studies where carbohydrate degradation is rare among yeast from tropical environments.

This is the first attempt to characterized complex carbohydrate-degrading microorganisms from these environments in the island of Puerto Rico. Our results reveal that these tropical environments host a diversity of microorganisms capable of using these substrates as sole carbon source, some of them with unique characteristics. Further research must be done in pursuit of a more detailed characterization of these microorganisms, their enzymes, and possible use in the industry.

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Appendix 1: Culture Media

Czapek Yeast Extract (CYA)

	Amount
CYA Solution Agar, Difco (Detroit, MI, USA)	49g
Distilled Water	1L

Dermatophyte Test Medium (DTM)

DTM Agar, Difco (Detroit, MI, USA)	35g
Distilled Water	1L

Luria Broth (LB)

Yeast Extract	5 g
NaCl	10g
Tryptone	10g
Distilled Water	1L
Adjust pH to 7.0 or 4.5	
Agar (for solid media)	18g

Marine Broth (MB)

Bacto® Marine Broth 2216 (Difco Laboratories Detroit, MI, USA)	37.4 g
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Procedure:

Suspend the powder in 1L of distilled water and boil for 1-2 minutes. Autoclave at 121-124°C for 15 minutes.

Mycological Agar (MA)

Soy Peptone	10g
Dextrose	10g
Distilled Water	1L
Agar	16g

Saboraud Dextrose Agar (SDA)

Peptone	10g
Dextrose	40g
Distilled Water	1L
Agar	15g

Sole Carbon Source Media (AZCL[®])

For Bacteria:

Bottom layer:

NH ₄ Cl	1g
HEPES	.25g
Yeast Extract	.1g
.22um Filtered Sea Water or Distilled Water	1L
Adjust pH to 7.0 or 4.5	
Agar	18g

After autoclaving pour a layer of the medium in the Petri plate. Let it solidify.

Overlay:

AZCL [®] substrate (amylose, pullulan, cellulose, dextran, galactomannan, arabinan, or xylan)	.3g
NH ₄ Cl	1g
HEPES	.25g
Yeast Extract	.1g
.22um Filtered Sea Water or Distilled Water	1L
Adjust pH to 7.0 or 4.5	
Agar	18g

After autoclaving pour a layer of the medium over the bottom layer.

For Fungi and Yeasts:**Bottom Layer:**

Yeast Nitrogen Base Without Aminoacids	6.5g
Distilled Water	1L
Adjust pH to 5.0 or 7.0	
Agar (for solid media)	18g

After autoclaving pour a layer of the medium over the bottom layer.

Overlay:

AZCL [®] substrate (amylose, pullulan, cellulose, dextran, galactomannan, arabinan, or xylan)	.3g
Yeast Nitrogen Base Without Aminoacids	6.5g
Distilled Water	1L
Adjust pH to 5.0 or 7.0	
Agar (for solid media)	18g

After autoclaving pour a layer of the medium over the bottom layer.

Synthetic Minimal Media (SD)

Yeast Nitrogen Base Without Aminoacids	6.5g
Distilled Water	1L
Adjust pH to 5.0	
Agar (for solid media)	20g

Yeast, Peptone and Dextrose Media (YPD)

Yeast	10g
Peptone	20g
Dextrose	20g
Distilled Water	1L
Agar (For solid media)	18g

After autoclaving add ampicillin (100µg/ml).

Appendix 2: Partial 16S rRNA sequences from carbohydrate degrading bacterial isolates and partial 18S rRNA from yeast isolate.

Carbohydrate degrading marine bacteria.

Vibrio sp. Md 208.

AGAGTTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGC
 AAGTCGAGCGGAAACGAGTTATCTGAACCTTCGGGGAACGATAACGGCGTCCG
 AGCGGCGGACGGGTGAGTAATGCCTAGGAAATTGCCTTGATGTGGGGGATAA
 CCATTGGAAACGATGGCTAATACCGCATAATGCCTACGGGCCAAAGAGGGGG
 ACCTTCGGGCCTCTCGCGTCAAGATATGCCTAGGTGGGATTAGCTAGTTGGTG
 AGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCA
 GCCACACTGGAAGTGAACGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGG
 GGAATATTGCACAATGGGCGAAAGCCTGATGCAGCCATGCCGCGTGTATGAA
 GAAGGCCTTCGGGTTGTAAAGTACTTTCAGTTGTGAGGAAGGGGGTGTTCGTT
 AATAGCGGCATCTCTTGACGTTAGCAACAGAAGAAGCACCGGCTAACTCCGT
 GCCAGCAGCCGCGGGTAAT

Pseudoalteromonas sp. Md 209.

TAGAGTTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATG
 CAAGTCGAACGGTAACATTTCTAGCTTGCTAGAAGATGACGAGTGGCGGACG
 GGTGAGTAATGCTTGGGAACCTTGCCTTTGCGAGGGGGATAACAGTTGGAAAC
 GACTGCTAATACCGCATAATGTCTTCGGACCAAACGGGGCTTCGGCTCCGGC
 GCAAAGAGAGGCCCAAGTGAGATTAGCTAGTTGGTAAGGTAACGGCTTACCA
 AGGCGACGATCTCTAGCTGTTCTGAGAGGAAGATCAGCCCACTGGGACTGA
 GACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATG
 GGGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTG
 TAAAGCACTTTCAGTTGTGAGGAAAAGTTAGTAGTTAATACCTGCTAGCCGT
 GACGTTAACAACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTA
 AT

Gamma proteobacterium Md 210.

AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCA
 AGTCGAGCGGAAACGAGTTATCTGAACCTTCGGGGGACGATAACGGCGTCCGA
 GCGGCGGACGGGTGAGTAATGCCTAGGAAATTGCCCTGATGTGGGGGATAAC
 CATTGGAAACGATGGCTAATACCGCATAATGCCTACGGGCCAAAGAGGGGG
 ACCTTCGGGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAGTTGGTG
 AGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCA
 GCCACACTGGAAGTGAACGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGG
 GGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAA

GAAGGCCTTCGGGTTGTAAAGCACTTTCAGTCGTGAGGAAGGTaGGTGTAGTT
 AATAGCTGCATTATTTGACGTTAGCGACAGAAGAAGCACCCGGCTAACTCCGT
 GCCAGCAGCCGCGGTAAT

Gamma proteobacterium Md 211.

AGAGTTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGC
 AAGTCGAGCGGAAACGAGTTATCTGAACCTTCGGGGAACGATAACGGCGTCCG
 AGCGGCGGACGGGTGAGTAATGCCTAGGAAATTGCCCTGATGTGGGGGATAA
 CCATTGGAAACGATGGCTAATACCGCATAATGCCTACGGGCAAAGAGGGGG
 ACCTTCGGGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAGTTGGTG
 AGGTAATGGCTACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCA
 GCCACACTGGAAGTGAACGACGCTCCAGACTCCTACGGGAGGCAGCAGTGG
 GGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAA
 GAAGGCCTTCGGGTTGTAAAGCACTTTCAGTCGTGAGGAAGGTGGTGTAGTT
 AATAGCTGCATT

***Pseudoalteromonas* sp. Md 213.**

AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCA
 AGTCGAGCGGAAACGAAGAGGTGCTTGCACCTCTGGCGTCGAGCGGCGGAC
 GGGTGAGTAATGCTTGGGAATATGCCTTATGGTGGGGGACAACAGTTGGAAA
 CGACTGCTAATACCGCATGATGTCTACGGACCAAAGTGGGGGACCTTCGGGC
 CTCACGCCATAAGATTAGCCCAAGTGGGATTAGCTAGTTGGTGAGGTAATGG
 CTCACCAAGGCGACGATCCCTAGCTGGTTTGGAGAGGATGATCAGCCACACTG
 GGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTGGGGGAATATTG
 CACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTT
 CGGGTTGTAAAGCACTTTCAGTAAGGAGGAAAGGTTAAGTGTTAATAGCACT
 TAGCTGTGACGTTACTTACAGAAGAAGCACCCGGAGTAACTCCGGGCCAGCAG
 CCGCGGTAAT

***Vibrio* sp. Md 216.**

AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCA
 AGTCGAGCGGAAACGAGTTATCTGACCCTTCGGGTGACGATAACGGCGTCCGA
 GCGGCGGACGGGTGAGTAATGCCTGGGAAATTGCCCTGATGTGGGGGATAAC
 CATTGGAAACGATGGCTAATACCGCATAATAGCTTCGGCTCAAAGAGGGGGGA
 CCTTCGGGCCTCTCGCGTCAGGATATGCCAGGTGGGATTAGCTAGTTGGTGA
 GGTAAGGGCTACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAG
 CCACACTGGAAGTGAACGACGCTCCAGACTCCTACGGGAGGCAGCAGTGGG
 GAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAG
 AAGGCCTTCGGGTTGTAAAGTACTTTCAGCAGTGAGGAAGGTAGTAGTGTTA
 ATAGCACTATTTGACGTTAGCTGCAGAAGAAGCACCCGGCTAACTCCCGTG
 CCAGCAGCCGCGGTAAT

***Aeromonas* sp. Md 218.**

AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCA
 AGTCGAGCGGCAGCGGGAAAGTAGCTTGCTACTTTTGCCGGCGAGCGGCGGA
 CGGGTGAGTAATGCCTGGGAAATTGCCCAGTCGAGGGGGATAACAGTTGGAA
 ACGACTGCTAATACCGCATAACGCCCTACGGGGGAAAGCAGGGGACCTTCGGG
 CCTTGCGCGATTGGATATGCCCAGGTGGGATTAGCTAGTTGGTGAGGTAATG
 GCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACT
 GGAAGTCAAACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATT
 GCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCT
 TCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTTGATGCCTAATACGTA
 TCAACTGTGACGGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCA
 GCCGCGGTAAT

***Pseudoalteromonas* sp. Md 236.**

AGAGTTTGATCCTGGCTGAGATTGAACGCTGGCGGCAGGCCTAACACATGCA
 AGTCGAGCGGTAACAGAGAGTAGCTTGCTACTCTGGTGACGAGCGGCGGACG
 GGTGAGTAATGCTTGGGAATATGCCTTATGGTGGGGGACAACAGTTGGAAAC
 GACTGCTAATACCGCATGATGTCTACGGACCAAAGTGGGGGACCTTCGGGCC
 TCACGCCATAAGATTAGCCCAAGTGGGATTAGCTAGTTGGTGAGGTAATGGC
 TCACCAAGGCGACGATCCCTAGCTGGTTTGAGAGGATGATCAGCCACACTGG
 GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGC
 ACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTC
 GGGTTGTAAAGCACTTTCAGTAAGGAGGAAAGGTTAAGTGTTAATAGCACTT
 AGCTGTGACGTTACTTACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCC
 GCNGGTAAT

***Gamma proteobacterium* Md 237.**

AGAGTTTGATCCTGGCTGAGATTGAACGCTGGCGGCAGGCCTAACACATGCA
 AGTCGAACGGTAACAGGATGTGCTTGACATCGCTGACGAGTGGCGGACGGG
 TGAGTAATGCCTGGGAACTTGCCTTTGCGAGGGGGATAACAGTTGGAAACGA
 CTGCTAATACCGCATAATGTCTACGGACCAAACGGGGCTTAGGCTCCGGCGC
 AAAGAGAGGCCCAAGTGAGATTAGCTAGATGGTGAGGTAAAGGCTCACCAT
 GGCGACGATCTCTAGCTGTTCTGAGAGGAAGATCAGCCACACTGGGACTGAG
 ACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGG
 GGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGT
 AAAGCACTTTCAGTTGTGAGGAAAGGGTGTGGTTAATACCCAATATCTGTG
 ACGTTAACAAACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAA
 T

***Pseudoalteromonas* sp. Md 238.**

AGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGTAACAGA
 GAGTAGCTTGCTACTCTGCTGACGAGCGGCGGACGGGTGAGTAATGCTTGGG
 AATATGCCTTATGGTGGGGGACAACAGTTGGAAACGACTGCTAATACCGCAT
 GATGTCTACGGACCAAAGTGGGGGACCTTCGGGCCTCACGCCATAAGATTAG
 CCCAAGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATC
 CTAGCTGGTTTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCA
 GACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTG
 ATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTC
 AGTAAGGAGGAAAGGTTAAGTGTTAATAGCACTTAGCTGTGACGTTACTTAC
 AGAAGAAGCACCGGCTAACTCC

***Pseudoalteromonas* sp. Md 239.**

AGAGTTTGATCCTGGTTCACATTGAACGCTGGCGGCAGGCCTAACACATGCA
 AGTCGAGCGGTAACAAAGAGAAGCTTGCTNCTCTGCTGACGAGCGGCGGACG
 GGTGAGTAATGCTTGGGAATATGCCTTATGGTGGGGGACAACAGTTGGAAAC
 GACTGCTAATACCGCATGATGTCTACGGACCAAAGTGGGGGACCTTCGGGCC
 TCACGCCATAAGATTAGCCCAAGTGGGATTAGCTAGTTGGTGAGGTAATGGC
 TCACCAAGGCGACGATCCCTAGCTGGTTTGAGAGGATGATCAGCCACACTGG
 GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGC
 ACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTC
 GGGTTGTAAAGCACTTTCAGTAAGGAGGAAAGGTTAAGTGTTAATAGCACTT
 AGCTGTGACGTTACTTACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCC
 GCGGTAAT

***Gamma proteobacterium* Md 240.**

AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCA
 AGTCGAGCGGAAACGAAGAGGTGCTTGACCTCTGGCGTCGAGCGGCGGAC
 GGGTGAGTAATGCTTGGGAATATGCCTTATGGTGGGGGACAACAGTTGGAAA
 CGACTGCTAATACCGCATAATGTCTACGGACCAAAGTGGGGGACCTTCGGGC
 CTCACGCCATAAGATTAGCCCAAGTGGGATTAGCTAGTTGGTGAGGTAATGG
 CTCACCAAGGCGACGATCCCTAGCTGGTTTGAGAGGATGATCAGCCACACTG
 GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTG
 CACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTT
 CGGGTTGTAAAGCACTTTCAGTAAGGAGGAAAGGTTAAGTGTTAATAGCACT
 TAGCTGTGACGTTACTTACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGC
 CGCGGTAAT

Gamma proteobacterium Md 241.

AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCA
 AGTCGAACGGTAACAGGATGTGCTTGCACATCGNTGACGAGTGGCGGACGGG
 TGAGTAATGCTTGGGAACCTGCCTTTGCGAGGGGGATAACAGTTGGAAACGA
 CTGCTAATAACCGCATAATGTCTTTGGACCAAACGGGGCTTAGGCTCCGGCGC
 AAAGAGAGGCCCAAGTGAGATTAGCTAGATGGTGAGGTAAAGGCTCACCatG
 GCGACGATCTCTAGCTGTTCTGAGAGGAAGATCAGCCACACTGGGACTGAGA
 CACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGG
 GGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCCTTCGGGTTGTA
 AAGCACTTTCAGTTGTGAGGAAAGGGTGTGGTTAATACCCAATATCTGTGA
 CGTTAACAACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGGTAA
 T

***Halomonas* sp. Md 242.**

AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCA
 AGTCGAGCGGTAACAGATCCAGCTTGCTGGATGCTGACGAGCGGCGGACGGG
 TGAGTAATGCATAGGAATCTGCCCCGATAGTGGGGGATAACCTGGGGAAACCC
 AGGCTAATAACCGCATAACGTCCTACGGGAGAAAGGGGGCTCCGGCTCCCGCTA
 TNGGATGAGCCTATGTCCGATTAGCTAGTTGGTGAGGTAACGGCTCACCAAG
 GCCACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACATCGGGACTGAGA
 CACGGCCCGAACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGG
 GGAAACCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCCTTCGGGTTGTA
 AAGCACTTTCAGCGAGGAAGAACGCCTAGCGGTTAATACCCGCTAGGAAAGA
 CATCACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAAT

Gamma proteobacterium Md 243.

AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCA
 AGTCGAGCGGAAACATAGAGGTGCTTGCACCTCTGGCGTCGAGCGGCGGACG
 GGTGAGTAATGCTTGGGAATATGCCTTATGGTGGGGGACAACAGTTGGAAAC
 GACTGCTAATAACCGCATAATGTCTACGGACCAAAGTGGGGGACCTTCGGGCC
 TCACGCCATAAGATTAGCCCAAGTGGGATTAGCTAGTTGGTGAGGTAATGGC
 TCACCAAGGCGACGATCCCTAGCTGGTTTGGAGAGGATGATCAGCCACACTGG
 GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGC
 ACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTC
 GGGTTGTAAAGCACTTTCAGTAAGGAGGAAAGGTTAAGTGTTAATAGCACTT
 AGCTGTGACGTTACTTACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCC
 GCGGGTAAT

Alpha proteobacterium Md 244.

AGAGTTTGATCCTGGCTCAGAACGAACGCTGGCGGCAGGCCTAACACATGCA
 AGTCGAGCGAGACCTTCGGGTCTAGCGGCGGACGGGTAGTAACGCGTGGGA
 ACGTGCCCTTCTCTGCGGAATAGCCACTGGAAACGGTGAGTAATACCGCATA
 CGCCCTTCGGGGGAAAGATTTATCGGAGAAGGATCGGCCCGCGTTAGATTAG
 ATAGTTGGTGGGGTAACGGCCTACCAAGTCTACGATCTATAGCTGGTTTTAGA
 GGATGATCAGCAACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGG
 CAGCAGTGGGGAATCTTAGACAATGGGCGCAAGCCTGATCTAGCCATGCCGC
 GTGTGTGATGAAGGTCTTAGGATCGTAAAGCACTTTCGCCAGGGATGATAAT
 GACAGTACCTGGTAAAGAAACCCCGGCTAACTCCGTGCCAGCAGCCGCGGTA
 AT

Carbohydrate degrading terrestrial bacteria***Bacillus* sp. Tf 1-40.**

AGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCA
 AGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTG
 AGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGG
 GGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGC
 TTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGT
 AATGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCA
 CACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAA
 TCTTCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAG
 GTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAAT
 AGGGCGGTACCTTGACGGGACCTAACCAGAAAGCCACGGCTAACTACGTGCC
 AGCAGCCGCGGGTAAT

***Bacillus* sp. Tf 1-41.**

AGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCA
 AGTCGAGCGAATGGATTGAGAGCTTGCTCTCAAGAAGTTAGCGGCGGACGGG
 TGAGTAACACGTGGGTAACCTGCCATAAGACTGGGATAACTCCGGGAAACC
 GGGGCTAATACCGGATAACATTTTGAACCTGCATGGTTGCAAATTGAAAGGCG
 GCTTCGGCTGTCACTTATGGATGGACCCGCGTCCGATTAGCTAGTTGGTGAGG
 TAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCC
 AACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGA
 ATCTTCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAA
 GGCTTTCGGGTCGTAATAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAA
 TAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGC
 CAGCAGCCGCGGTAAT

***Bacillus* sp. Tf 1-42.**

AGAGTTTTGATCCTGGCTCAAGGAAGAACCTGCGGCGTCCTATACATGCAAG
 TCGAGCGAATGGATTGAGAGCTTGCTCTCAAGAAGTTAGCGGCGGACGGGTG
 AGTAACACGTGGGTAACCTGCCATAAGACTGGGATAACTCCGGGAAACCGG
 GGCTAATACCGGATAACATTTTGAAGTGCATGGTTCGAAATTGAAAGGCGGC
 TTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTA
 ACGGCTACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCAC
 ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAAT
 CTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGG
 CTTTCGGGTCGTAAAACCTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATA
 AGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCA
 GCAGCCGCGGTAAT

***Bacillus* sp. Tf 1-45.**

TAGAGTTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATG
 CAAGTCGAGCGAATGGATTGAGAGCTTGCTCTCAAGAAGTTAGCGGCGGACG
 GGTGAGTAACACGTGGGTAACCTGCCATAAGACTGGGATAACTCCGGGAAA
 CCGGGGCTAATACCGGATAACATTTTGAAGTGCATGGTTCGAAATTGAAAGG
 CGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGA
 GGTAACGGCTACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCG
 GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAG
 GGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGAT
 GAAGGCTTTCGGGTCGTAAAACCTCTGTTGTTAGGGAAGAACAAGTGCTAGTT
 AATAAGCTGGCACCTGACGGACCTACCAAAGCCCGGTATC

***Aeromonas* sp. Tf 228.**

AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCA
 AGTCGAGCGGCAGCGGAAAGTAGCTTGCTACTTTTGCCGGCGAGCGGCGGA
 CGGGTGAGTAATGCCTGGGGATCTGCCAGTCGAGGGGGATAACTACTGGAA
 ACGGTAGCTAATACCGCATAACGCCCTACGGGGGAAAGCAGGGGACCTTCGGG
 CCTTGCGCGATTGGATGAACCCAGGTGGGATTAGCTAGTTGGTGAGGTAACG
 GCTACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACT
 GGAAGTGCAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATT
 GCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCT
 TCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTCAGTAGCTAATATCTG
 CTGGCTGTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAG
 CCGCGGTAAT

***Aeromonas* sp. Tf 229.**

AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCA
 AGTCGAGCGGCAGCGGGAAAGTAGCTTGCTACTTTTGCCGGCGAGCGGCGGA
 CGGGTGAGTAATGCCTGGGAAATTGCCAGTCGAGGGGGATAACAGTTGGAA
 ACGACTGCTAATACCGCATAACGCCCTACGGGGGAAAGCAGGGGACCTTCGGG
 CCTTGCGCGATTGGATATGCCAGGTGGGATTAGCTAGTTGGTGAGGTAATG
 GCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACT
 GGAAGTGAAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATT
 GCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCT
 TCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTTGATGCCTAATACGTA
 TCAACTGTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAG
 CCGCGGGTAAT

***Aeromonas* sp. Tf 230.**

AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCA
 AGTCGAGCGGCAGCGGGAAAGTAGCTTGCTACTTTTGCCGGCGAGCGGCGGA
 CGGGTGAGTAATGCCTGGGAAATTGCCAGTCGAGGGGGATAACAGTTGGAA
 ACGACTGCTAATACCGCATAACGCCCTACGGGGGAAAGCAGGGGACCTTCGGG
 CCTTGCGCGATTGGATATGCCAGGTGGGATTAGCTAGTTGGTGAGGTAATG
 GCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACT
 GGAAGTGAAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATT
 GCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCT
 TCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTTGATGCCTAATACGTA
 TCAACTGTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAG
 CCGCGGTAAT

***Pseudomonas* sp. Tf 231.**

AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCA
 AGTCGAGCGGATGAAGGGAGCTTGCTCCTGAATTCAGCGGCGGACGGGTGAG
 TAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGC
 TAATACCGCATAACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGC
 TATCAGATGAGCCTAGGTTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCA
 AGGCGACGATCCGTAACCTGGTCTGAGAGGATGATCAGTCACACTGGAAGTGA
 GACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATG
 GGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTG
 TAAAGCACTTTAAGTTGGGAGGAAGGGTTGTAGATTAATACTCTGCAATTTTG
 ACCTTACCGACAGAATAAGCACCCGGCCTAACTCTGTGCCAGCAGCCGCGG
 TAAT

***Aeromonas* sp. Tf 232.**

TAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGC
 AAGTCGAGCGGCAGCGGGAAAGTAGCTTGCTACTTTTGCCGGCGAGCGGCGG
 ACGGGTGAGTAATGCCTGGGAAATTGCCAGTCGAGGGGGATAACAGTTGGA
 ACGACTGCTAATACCGCATAACGCCCTACGGGGGAAAGCAGGGGACCTTCGG
 GCCTTGCGCGATTGGATATGCCAGGTGGGATTAGCTAGTTGGTGAGGTAAT
 GGCTACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACAC
 TGGAAGTGAAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATT
 GCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCT
 TCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTTGATGCCTAATACGTA
 TCAACTGTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAG
 CCGCGGTAAT

***Aeromonas* sp. Tf 233.**

AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCA
 AGTCGAGCGGCAGCGGGAAAGTAGCTTGCTACTTTTGCCGGCGAGCGGCGGA
 CGGGTGAGTAATGCCTGGGAAATTGCCAGTCGAGGGGGATAACAGTTGGAA
 ACGACTGCTAATACCGCATAACGCCCTACGGGGGAAAGCAGGGGACCTTCGGG
 CCTTGCGCGATTGGATATGCCAGGTGGGATTAGCTAGTTGGTGAGGTAATG
 GCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACT
 GGAAGTGAAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATT
 GCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCT
 TCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTTGATGCCTAATACGTA
 TCAACTGTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAG
 CCGCGGTAAT

***Aeromonas* sp. Tf 234.**

AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCA
 AGTCGAGCGGCAGCGGGAAAGTAGCTTGCTACTTTTGCCGGCGAGCGGCGGA
 CGGGTGAGTAATGCCTGGGAAATTGCCAGTCGAGGGGGATAACAGTTGGAA
 ACGACTGCTAATACCGCATAACGCCCTACGGGGGAAAGCAGGGGACCTTCGGG
 CCTTGCGCGATTGGATATGCCAGGTGGGATTAGCTAGTTGGTGAGGTAATG
 GCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACT
 GGAAGTGAAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATT
 GCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCT
 TCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTTGATGCCTAATACGTA
 TCAACTGTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAG
 CCGTGGTAAT

Bacteroidetes bacterium Tf 235.

AGAGTTTGATCCTGGCTCAGGATGAACGCTAGCGGCAGGCTTAACACATGCA
 AGTCGAGGGGTATAGTTCTTCGGAATTAGAGACCGGCGCACGGGTGCGTAAC
 GCGTATGCAATCTACCTTTTACAGGGGGATAGCCCAGAGAAATTTGGATTAA
 TACCCCATAGTATACTGAGTTGGCATCAACATAGTATTAAGTCACAACGGT
 AAAAGATGAGCATGCGTCCATTAGCTAGTTGGTAAGGTAACGGCTTACCAA
 GGCTACGATGGGTAGGGGTCTGAGAGGGAGATCCCCACACTGGTACTGAG
 ACACGGACCAGACTCCTACGGGAGGCAGCAGTGAGGAATATTGGTCAATGG
 GCGCAAGCCTGAACCAGCCATGCCGCGTGACGGATGACGGTCTATGGATTG
 TAAACTGCTTTTGTACGAGAAGAAACTCCAACGTGTTGGAGCTTGACGGT
 ATCGTAAGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAAT

Oxalobacteraceae bacterium Tf 246.

AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCCTTACACATGCA
 AGTCGAACGGCAGCACGGAGCTTGCTCTGGTGGCGAGTGGCGAACGGGTGA
 GTAATATATCGGAACGTACCCTGGAGTGGGGGATAACGTAGCGAAAGTTACG
 CTAATACCGCATAACGATCTACGGATGAAAGTGGGGGATCGCAAGACCTCATG
 CTCGTGGAGCGGCCGATATCTGATTAGCTAGTTGGTAGGGTAAAAGCCTACC
 AAGGCATCGATCAGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGAAC TG
 AGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATG
 GGCGAAAGCCTGATCCAGCAATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTG
 TAAAGCTCTTTTGTACGGGAAGAAACGGTGAGGGCTAATATCTCTTGCTAAT
 GACGGTACCTGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTA
 AT

***Kluyvera* sp. Tf 247.**

AGATTGAACGGCTGGCGGCAGGCCTAACACATGCAAGTCGAACGGTAGCAC
 AGAGAGCTTGCTCTTGGGTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGA
 AACTGCCCGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATA
 ACGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCACACCATCGGATGTGC
 CCAGATGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCC
 CTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAAC TGAGACACGGTCCAG
 ACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGA
 TGCAGCCATGCCGCGTGATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCA
 GCGAGGAGGAAGGCATTAAGGTTAATAACCTTAGTGATTGACGTTACTCGCA
 GAAGAAGCACCGGCTAACTCC

***Aeromonas* sp. Tf 249.**

AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCA
 AGTCGAGCGGCAGCGGGAAAGTAGCTTGCTACTTTTGCCGGCGAGCGGCGGA
 CGGGTGAGTAATGCCTGGGAAATTGCCAGTCGAGGGGGATAGCAGTTGGAA

ACGACTGCTAATACCGCATAACGCCCTACGGGGGAAAGCAGGGGACCTTCGGG
 CCTTGCGCGATTGGATATGCCAGGTGGGATTAGCTAGTTGGTGGGGTCATG
 GCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACT
 GGAAGTGGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATT
 GCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCT
 TCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGGTAGCGGCTAATATCCG
 TTACCTGTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAG
 CCGCGGTAAT

***Kluyvera* sp. Tf 250.**

AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCA
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 TGAGTAATGTCTGGGAAACTGCCCCGATGGAGGGGGATAACTACTGGAAACGG
 TAGCTAATACCGCATAACGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTC
 ACACCATCGGATGTGCCAGATGGGATTAGCTAGTTGGTGAGGTAATGGCTC
 ACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAA
 CTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACA
 ATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGG
 TTGTAAAGTACTTTCAGCGAGGAGGAAGGCATTAAGGTTAATAACCTTAGTG
 ATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCG
 GTAAT

***Paenibacillus* sp. Tf 252.**

AGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGGCGTGCCTAATACATGCA
 AGTCGAGCGGATCTTGTCCTTCGGGACAAGGTTAGCGGCGGACGGGTGAGTA
 ACACGTAGGCAACCTGCCCTCAAGACTGGGATAACCTCCGGAAACGGATGCT
 AATACCAGATAAGCGATTTTCTCTCCTGGGAAGATCGAGAAAGACGGAGCAA
 TCTGTTACTTGAGGATGGGCCTGCGGCGCATTAGCTAGTTGGTGAGGTAACG
 GCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACT
 GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTC
 CGCAATGGACGCAAGTCTGACGGAGCAACGCCGCGTGAGTGAGGAAGGCCT
 TCGGGTCGTAAAGCTCTGTTGCCAGGGAAGAACGGGTGGAAGAGTAACTGCT
 TCCGCCATGACGGTACCTGAGAAGAAAACCCCGGCTAACTACGTGCCAGCAG
 CCGCGGTAAT

***Bacillus* sp. Tf 253.**

AGAGTTTNGATCCTGGCTCAGGATGAACGCTGGCGGGCGTGCCTAATACATGC
 AAGTCGAGCGAATGGATTGAGAGCTTGCTCTCAAGAAGTTAGCGGCGGACGG
 GTGAGTAACACGTGGGTAACCTGCCATAAGACTGGGATAACTCCGGGAAAC
 CGGGGCTAATACCGGATAACATTTTGAAGTGCATGGTTCGAAATTGAAAGGC
 GGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAG
 GTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGC

CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGG
 AATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGA
 AGGCTTTCGGGTCGTAAAACCTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGA
 ATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTG
 CCAGCAGCCGCGGGTAAT

***Staphylococcus* sp. Tf 254.**

AGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCA
 AGTCGAGCGAACAGATAAGGAGCTTGCTCCTTTGACGTTAGCGGCGGACGGG
 TGAGTAACACGTGGATAACCTACCTATAAGACTGGGATAACTTCGGGAAACC
 GGAGCTAATACCGGATAACATATTGAACCGCATGGTTCAATAGTGAAAGGCG
 GCTTTGCTGTCACTTATAGATGGATCCGCGCCGTATTAGCTAGTTGGTAAGGT
 AACGGCTTACCAAGGCAACGATACGTAGCCGACCTGAGAGGGTGATCGGCCA
 CACTGGAAGTGAAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAA
 TCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAG
 GTCTTCGGATCGTAAAACCTCTGTTATCAGGGAAGAACAATGTGTAAGTAAC
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 GCAGCCGCGGTAAT

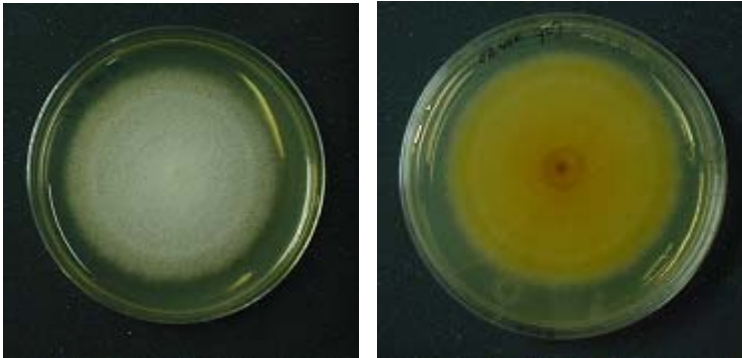
Carbohydrate degrading yeast

***Glomus* sp. Tf 2-16 (GenBank Accession No. AY522919)**

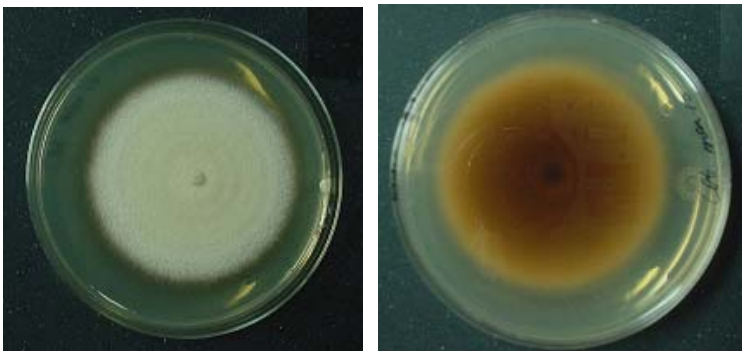
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 CACGGAGGGTGAGAGCCCCGTTTCGTACGAAGGCGGAAGCAGGATCCTTGTCT
 CGATGAGTCGAGTTGTTTGGGATTGCAGCTCTAAGTGGGTGGTAAATTCCATC
 TAAAGCTAAATATGGGTAGGAAACCGATAGCAAACAAGTACCGTGAGGGAA
 AGATGAAAAGAAGCTTTGAAAAGAGAGTGAAATAGTACCTGCAATTGCTGAA
 AAGGAACCGATGGGAAGCAGTGTATGGGGTCTGTCTGGTGTTACTGGTCGGA
 TCGCCGGTCTCGCTTCCTTGGCTGTGTGGGAAAAGGTTTCGTCCCTGTCACGCG
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 C

Appendix 3: Isolated carbohydrate degrading fungi.

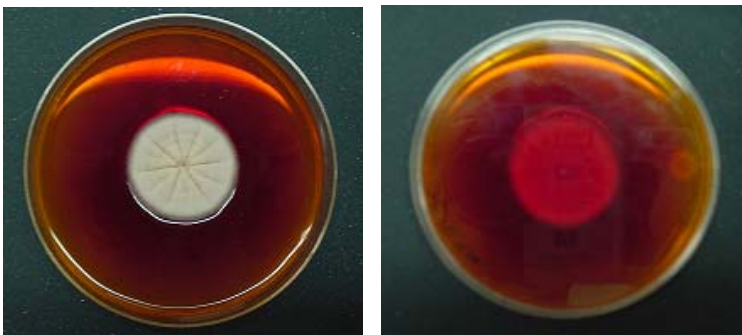
3.1 Fdeg2



Sabouraud dextrose agar at 25°C, 7 days (surface/reverse).

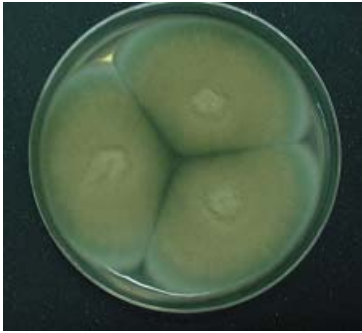


Mycological agar at 25°C, 7 days (surface/reverse).

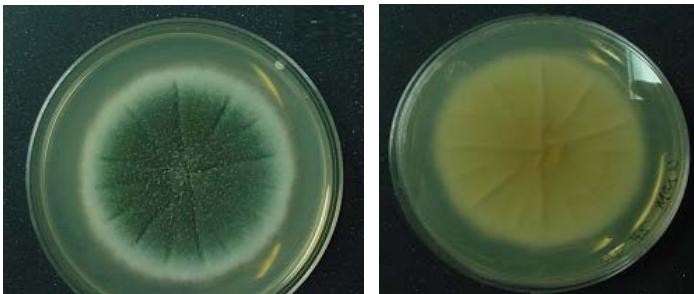


Dermatophyte test medium at 25°C, 7 days (surface/reverse).

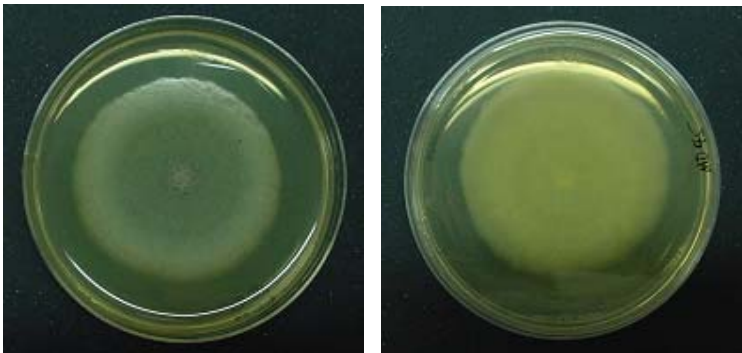
3.2 MD45



Czapek yeast extract agar, at 25°C, 7 days.

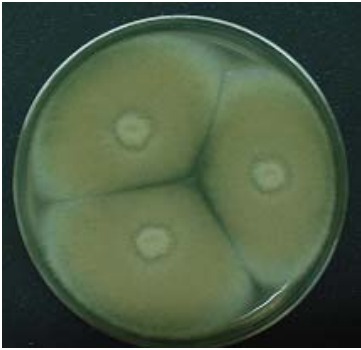


Mycological agar at 25°C, 7 days (surface/reverse).

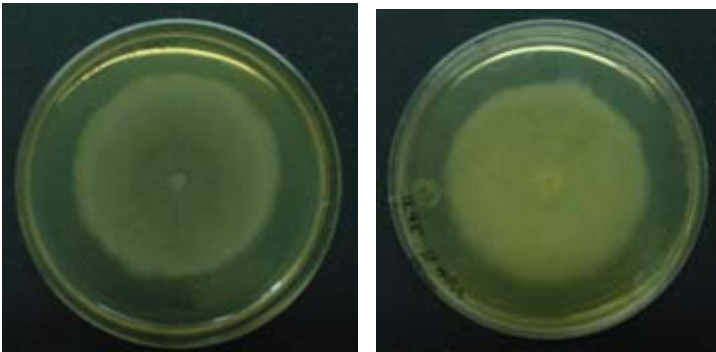


Saboraud dextrose agar at 25°C, 7 days (surface/reverse).

3.3 U45



Czapek yeast extract agar, at 25°C, 7 days.



Sabouraud dextrose agar at 25°C, 7 days (surface/reverse).