TITLE: Culturable heterotrophic bacteria from Potter Cove, Antarctica and their hydrolytic enzymes production

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Abstract

The affiliation of the dominant culturable bacteria isolated from Potter Cove, South Shetland Islands, Antarctica, was investigated together with their production of cold-active hydrolytic enzymes. A total of 189 aerobic heterotrophic bacterial isolates were obtained at 4°C and sorted into 63 phylotypes based on their Amplified Ribosomal DNA Restriction Analysis (ARDRA) profiles. The sequencing of the 16S rRNA genes of representatives from each phylotype showed that the isolates belong to the phyla *Proteobacteria* (classes *Alpha*- and *Gammaproteobacteria*), *Bacteroidetes* (class *Flavobacteria*), *Actinobacteria* (class *Actinobacteria*) and *Firmicutes* (class *Bacilli*). The predominant culturable group in the site studied belongs to the class *Gammaproteobacteria*, with 65 isolates affiliated to the genus *Pseudoalteromonas* and 58 to *Psychrobacter*. Among the 189 isolates screened, producers of amylases (9.5%), pectinases (22.8%), cellulases (14.8%), CM-cellulases (25.4%), xylanases (20.1%) and proteases (44.4%) were detected. More than 25% of the isolates produced at least one extracellular enzyme, with some of them producing up to six of the tested extracellular enzymatic activities. These results suggest that a high culturable bacterial diversity is present in Potter Cove and that this place represents a promising source of biomolecules.

Keywords: microbial enzymes, Antarctic bacteria, marine bacteria, cold enzymes, psychrophiles

Introduction

Potter Cove is a shallow Antarctic marine environment, located in King George Island (Isla 25 de Mayo), South Shetland Islands. The weather in Potter Cove is not as cold as in the continental Antarctica, with summer temperatures ranging from -3°C to 5°C and winter

temperatures not lower than -15°C to -20°C. In an open area on the southern margin of the cove, which stays free of ice during summer (facilitating research and diving activities), is located Carlini (Jubany) Argentinean Scientific Station (62° 14’ S 58° 40’ W). Because of its unique climate and its Antarctic location, the Potter Cove and Potter Peninsula have frequently been the focus of interest for scientist looking for a site for long-term ecological research and monitoring programs. Microorganisms were not the exception to these objectives. In recent years, several studies have been conducted to evaluate the effect of UV radiation on bacteria isolated from the water column of Potter Cove (Hernandez et al. 2007, 2009), to assess the biotechnological potential of autochthonous bacterial consortia for on-site bioremediation of hydrocarbon-contaminated soils (Ruberto et al. 2009; Vázquez et al. 2009; Ruberto et al. 2010), to detect the presence and diversity of naphthalene dioxygenase genes in soils (Flocco et al. 2009) and to screen for bacterial extracellular proteases for their use in laundry and food industry (Vázquez et al. 2004, 2005, 2008). Furthermore, a bacterial strain isolated from surface marine water of Potter Cove was described as a new species, *Bizionia argentinensis* (Bercovich et al. 2008) and its complete genome has been sequenced and is being thoroughly studied (Lanzarotti et al. 2011). Although this research have proven the ecological and biotechnological relevance of the culturable fraction of bacterial community of Potter Cove area, no study has been carried out to date involving a considerable amount of isolates, to gain insight into its taxonomic composition and their potential to be used in biotechnology. Extracellular hydrolytic enzymes have diverse potential applications in different industries, and its detection and characterization from extremophilic microorganisms are one of the most active fields of applied microbiology research (Kumar et al. 2011). In psychrophilic and psychrotolerant bacteria in particular, exoenzymes and other products are being studied for its use in a wide range of processes because they offer great advantages as the saving of energy expenses, the reduction in the loss of volatile compounds, the shortening

of time processes, the reduction of contamination risks or their high activity in processes requiring low temperatures, among others (Collins et al. 2007).

In this work we report the taxonomic affiliation of 189 bacterial isolates obtained from different samples taken from Potter Cove, with emphasis on providing an overview of the biodiversity of culturable bacteria and their capacity of production of hydrolytic enzyme activities with likely biotechnological application.

Materials and methods

Sampling area

Samples of seawater, marine sediment, algae and different marine animals were taken from Potter Cove and its shore, near Carlini (Jubany) scientific station (62° 14' S, 58° 40' W) in King George Island (Isla 25 de Mayo), South Shetland Islands, Antarctica; during Argentine summer Antarctic Research Expeditions (ARE) 1987, 2001, 2003 and 2005.

Isolation of bacteria

Small portions of the samples (except seawater, that was diluted directly) were placed in a screw-capped bottle containing 5 g of sterile sand and 15 ml of sterile diluent (1 g l^{-1} bacteriological peptone, Difco, diluted in 75 % v v^{-1} seawater) and vortexed for 5 min. After shaking, serial ten-fold dilutions were prepared in the same diluent and 0.1 ml of each dilution was spread onto the surface of half-strength Marine Agar 2216 (Difco) plates incubated for 96 h at 4°C. After incubation, the different morphotypes were picked out and isolates were obtained in pure culture by two successive transfers to the same media and finally preserved at -70°C in marine broth with 40 % v v^{-1} glycerol.

Identification of bacterial isolates

Isolates were identified on the basis of their 16S rRNA gene partial sequencing and phenotypic characteristics: colony and cell morphology, Gram stain affinity and metabolic profile (using standard tests and, in some cases, also the Analytical Profile Index API® 20 NE system, Biomerieux).

For the molecular identification, isolates were grouped in phylotypes according to their ARDRA profiles. Bacterial genomic DNA was extracted using the Illustra® Blood Genomic Prep Mini Spin Kit (GE Healthcare) according to the manufacturer's instructions. A fragment of the 16S rRNA gene of about 1500 bp was amplified using universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). PCR reaction mixture (50 µl) contained 1 µg of genomic DNA, 100 µM of each deoxynucleoside triphosphate, 0.4 µM of each primer, 1.5 mM of $MgCl_2$ and 1 U of Taq DNA polymerase (Invitrogen). Standard PCR consisted of an initial denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min, with a final extension at 72°C for 10 min. PCR products were examined by electrophoresis on 1% agarose gels stained with ethidium bromide and visualized under UV light. Amplified products were digested separately with the restriction enzymes *Alu*I and *Hpa*II and the fragments obtained were further separated by electrophoresis on a 10% polyacrylamide gel stained with ethidium bromide and visualized under UV light. ARDRA profiles in different gels were normalized through the use of the molecular weight marker 100 bp ladder (Invitrogen). Isolates were grouped in phylotypes by visual comparison of their ARDRA profiles, considering that two isolates belonged to the same phylotype if they shared both (*Alu*I and *Hpa*II) restriction profiles. Distinct cleavage patterns were considered as different phylotypes. According to the size of each ARDRA group, one, two or more isolates

from each unique phylotype were selected for sequencing. The selection was performed as to include at least one representative of each phenotypic pattern within each ARDRA group. Sequences from 16S rRNA genes were amplified from genomic DNA as described above and sent to Macrogen Inc. for further purification (Montage PCR Clean up kit, Millipore) and sequencing (Big Dye® terminator cycle sequencing kit, Applied BioSystems, USA). Products were resolved on an Applied Biosystems 3730XL automated sequencer.

Sequence analysis

Partial 16S rRNA gene sequences (ranging from 800 to 1400 nt) were edited using the BIOEDIT software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and compared online with homologous sequences deposited in databases, using the FASTA (http://www.ebi.ac.uk/fasta33/nucleotide.html) and BLAST (Megablast option) (http://www.ncbi.nlm.nih.gov/BLAST/) algorithms. Further analysis of the sequences was performed by comparison online against the 16S rRNA gene database (type strains) using the SeqMatch and Classifier tools from the Ribosomal Database Project Release 10 (http://rdp.cme.msu.edu/) and leBIBI web tool for bacteria identification (http://umr5558-sud-str1.univ-lyon1.fr/lebibi/lebibi.cgi). The integrative use of all the above mentioned tools made possible to arrive at a reliable identification of the isolates to the genus level.

Screening for extracellular hydrolytic enzymes production

A detection assay based on growth on solid media with single substrates as carbon source was conducted to evaluate the production of extracellular hydrolytic enzymes by the isolates. The strains were cultured by puncture in agar plates (75 % v v^{-1} sea water and 1.7 % w v^{-1} bacteriological agar) supplemented with 0.2 % w v^{-1} $(NH_4)_2SO_4$, 0.01 % w v^{-1} yeast

extract and the following substrates as the sole carbon sources (0.5 % w v^{-1}): crystalline cellulose and carboxymethyl-cellulose (CM-cellulose) (Baker) to detect cellulase production (Ulrich et al. 2007), xylan from birchwood (Sigma) to detect xylanase production (Li et al. 2008), citric pectin (Sigma) to detect pectinases (Sunnotel et al. 2002) and soluble starch (Baker) to detect amylases (Brizzio et al. 2007). In addition, extracellular protease detection was conducted by puncture the isolates in agar plates (50 % v v^{-1} sea water and 1.7 % w v^{-1} bacteriological agar) supplemented with skim milk (2.5 % w v^{-1}) (Dang et al. 2009). In all cases, pH was adjusted to 7.0-7.5 before sterilization and incubation was carried out at 16°C for 5-7 days, except for crystalline cellulose plates which were incubated for 3 weeks. For all the tested enzymes, positive reaction was considered when a clear halo around the colony was observed after incubation at 16°C for the indicated days. The clear zones of hydrolysis in media containing cellulose, CM-cellulose and xylan were developed by flooding the agar surface with an aqueous solution of Congo Red dye (1 mg ml^{-1}) for 15 min at room temperature. The stain solution was then poured off and plates were further treated by flooding with 1 M NaCl for 15 min. The developed zones of hydrolysis were stabilized for at least 2 weeks by further flooding the agar with 1 M HCl, which changes the dye colour from red to blue and inhibits enzyme activity. In the case of starch and pectin, the hydrolysis zones were developed by flooding the agar media with an iodine solution (Brizzio et al. 2007; Sunnotel et al. 2002).

Results

Diversity of marine isolates

Data about the 189 bacterial isolates obtained from the different biotopes explored are presented in Table 1. They were characterized on the basis of their ARDRA profiles obtained

separately with two restriction enzymes, generating 63 distinct cleavage patterns named phylotypes. Seventy-nine representative isolates of all phylotypes were selected for their 16S rRNA gene sequencing (Table 1). Most of the sequences obtained shared a quite high similarity (99-100%) with their nearest-neighbor sequences deposited in databases. Most of them were from isolates or clones from cold marine environments under environmental conditions comparable to those present in the studied area. Also the identity with homologous sequences from the closest type strains was generally greater than 97% (Table 2).

The taxonomic identification of the representative isolates from each of the 63 phylotypes indicated that 49 of them were represented by gram-negative bacteria and 14 by gram-positive bacteria. Out of the 14 gram-positive phylotypes, 12 belonged to the phylum *Actinobacteria*, represented by the genera *Arthrobacter, Salinibacterium, Kocuria* and *Williamsia*, and 2 belonged to the phylum *Firmicutes* (class *Bacilli*) represented by the genus *Planococcus.* On the other hand, out of the 49 gram-negative phylotypes, 45 belonged to the phylum *Proteobacteria* and 4 to the phylum *Bacteroidetes*. The phylum *Bacteroidetes* was represented for the class *Flavobacteria*, with isolates belonging to the genera *Polaribacter, Cellulophaga* and *Flavobacterium*. The phylum *Proteobacteria* was represented by 43 phylotypes belonging to the class *Gammaproteobacteria*, one to the class *Alphaproteobacteria* and one to the class *Betaproteobacteria.* Among the *Gammaproteobacteria*, a total of 12 different genera were identified (Table 2) being *Pseudoalteromonas* and *Psychrobacter* the most frequently recovered, representing the largest groups in terms of number of isolates (65 isolates belonged to the genus *Psychrobacter* and 58 to the genus *Pseudoalteromonas*). These two genera proved to be ubiquitous in Potter Cove, being recovered from almost all the samples screened: sediments, sea water, crustaceans, bivalves, ascidians, isopods, salps, amphipods, polychaetes, starfish, different kinds of fishes and algae.

Detection of hydrolytic enzyme activities

The production of extracellular hydrolytic enzymes by the 189 isolates is summarized in Table 2. In addition, the distribution of isolates producing the different extracellular hydrolytic enzymes screened among the recovered genera is shown in Fig. 1. Isolates that were able to hydrolyze starch, cellulose, CM-cellulose, pectin, xylan and casein represented, respectively, the 9.5%, 14.8%, 25.4%, 22.8%, 20.1% and 44.4 % of the total. Proteolysis was the most frequently detected activity, being expressed by 83 isolates belonging to the genera *Psychrobacter, Pseudoalteromonas*, *Pseudomonas, Colwellia, Salinibacterium, Shewanella, Cellulophaga* and *Hydrogenophaga* (Fig. 1). However, pectinase activity was detected in a greater number of genera than protease activity, with 43 isolates distributed in 11 different genera. The production of amylase was less frequently detected than the other hydrolytic activities. Nevertheless, the amylolytic group was quite diverse, being represented by 18 isolates belonging to the genera *Psychrobacter, Pseudoalteromonas, Arthrobacter, Salinibacterium, Shewanella* and *Planococcus*. The group able to hydrolyze crystalline cellulose was less abundant than the one with CM-cellulose hydrolytic activity, with 28 and 48 representative isolates, respectively. Moreover, while the cellulase-producing isolates were distributed between *Pseudoalteromonas, Psychromonas, Colwellia* and *Polaribacter* genera, the CM-cellulase producers belonged to these four genera plus to *Salinibacterium, Cellulophaga* and *Flavobacterium*. Although the detection of agarase production was not intended, the agarolytic activity of some isolates was evidenced as a softening of the agar around the colony observed after incubation in half-strength marine agar. This activity was found mainly in *Pseudoalteromonas* isolates from diverse biotopes.

It is important to remark that many isolates produced more than one extracellular enzyme; 2 were positive for the six enzymatic activities screened, 16 were positive for five of them, 12 produced three to four of the hydrolytic enzymes tested, 16 were positive for two of them and 51 produced only one enzyme activity. Taking this into account, we considered the

hypothesis of the success in the isolation of bacteria producing multiple hydrolytic enzymes through a strategy consisting in applying a selection pressure for a single activity in the initial isolation protocol. In order to analyse this possibility, the information obtained from the hydrolytic enzymes screening was used to calculate, for the total isolates producing each enzymatic activity tested, the percentage that also produced each one of the other enzymes screened (Fig. 2). In this sense, each group of isolates that were positive for a given enzyme represented the supposed outcome of an isolation procedure conducted by applying a selective strategy for that enzyme and therefore, they accounted for the 100% of the isolates belonging to the group. Then, the number of isolates in each group that also produced a second enzyme was recorded and the corresponding percentage related to the total number of isolates in the group was plotted. The analysis showed that protease activity was the most frequently found within the groups that had been obtained if initial isolation had been made on media selective for amylase, CM-cellulase and pectinase hydrolytic activities. In the groups simulating direct selection for cellulase and xylanase producers, CM-cellulase activity was the most abundant and protease activity was the second one. The uptake and hydrolysis of crystalline cellulose is more difficult for bacteria than that of its soluble derivative, the CM-cellulase (Ulrich et al. 2007) and then, it is not surprising that isolates producing cellulases were all able to hydrolyze CM-cellulase. Therefore, the production of proteases represented the main secondary activity produced by the isolates recovered from four out of the five pretended selective isolation conditions.

Discussion

The microorganisms used in this study were isolated over a period of ten years during different summer Antarctic Research Expeditions focused on different research objectives. Therefore, the samples from which the bacterial isolates were obtained were taken from

numerous and diverse marine sites and processed under different culture conditions, resulting in a different number of isolates from each studied biotope. Due to these reasons, in the present work, it is not possible to perform a quantitative description of diversity based on statistical analyses, like those reported in publications where microbial communities from unique biotopes were studied (Brinkmeyer et al. 2003; Dias et al. 2009; Schulze et al. 2006; Srinivas et al. 2009). On the contrary, our results allowed a qualitative description at the genus level of the diversity of culturable heterotrophic bacteria present in numerous marine biotopes, something that had been not assessed before in Potter Cove. Therefore, our approach was successful in disclosing the huge diversity of bacteria able to be cultured and producing bioactive molecules of industrial relevance. In this sense, there are some points that can be highlighted from the present study. One of them is the predominance of the class *Gammaproteobacteria* over the other classes recovered. This predominance, together with the presence of the classes *Flavobacteria* and *Bacilli*, agrees with that found in detailed studies of the bacterial Antarctic communities from sea water and marine ice (Acinas et al. 1999; Bowman et al. 1997; Brinkmeyer et al. 2003; Brown & Bowman 2001) and also from the Arctic coastal waters (Groudieva et al. 2004). In fact, *Gammaproteobacteria* is an important and widely distributed group in marine environments, frequently detected also in the analysis of the bacterial diversity from marine sediments (Bowman et al. 2003, Olivera et al. 2007; Srinivas et al. 2009; Yu et al. 2011; Zhou et al. 2009). In addition, the isolates belonging to the most recovered genera, *Pseudoalteromonas* and *Psychrobacter*, were retrieved from almost all the samples tested, showing their ubiquitous distribution in the coastal ecosystems of Potter Cove. These results agree with those reported by other authors who have isolated several species of these genera from many Antarctic environments (Bozal et al. 1997, 2003; Bowman et al. 1998; Vynne et al. 2011).

Among the isolates obtained in each Antarctic expedition, we found enzyme producers in all groups except NR, in which all isolates were obtained from the fish *Notothenia rossi*. A

high proportion of the enzyme-producers were affiliated with the more represented genera *Pseudoalteromonas*, *Pseudomonas* and *Psychromonas*, while only a few belonged to *Psychrobacter*. This last genus was almost the only one recovered from fishes, mainly *Notothenia*, explaining the absence of enzyme producers within the NR group as well as in almost all the *Notothenia coriceps* isolates obtained in 2005 expedition. Members of genus *Psychrobacter* were also reported as dominant among isolates from deep-sea sediments from the western Pacific (Dang et al. 2009), but those isolates were found to produce extracellular enzymes, mainly lipases and proteases, probably because the biotope represents an important site for particulate organic matter deposit that may stimulate the metabolic activity of sedimentary heterotrophic microorganisms.

In this work, we detected a high proportion of isolates that were positive for all the enzymatic activities screened. This provides only a small glimpse into the exceptional adaptation of the microbiota to the Antarctic environment. The *Pseudoalteromonas* isolates in particular showed a great potential for bioprospecting of all screened enzymatic activities, result that agrees with those from previous studies (Holmström et al. 1999; Hoyoux et al. 2001; Truong et al. 2001; Tutino et al. 2002; Zeng et al. 2006). Beyond the biotechnological potential, the high proportion of isolates belonging to the genus *Pseudoalteromonas* and the versatile hydrolytic activities detected in this group also suggest that these organisms may play an important role in polymer hydrolysis in cold environments. In these sense, and considering only the marine environment where these microorganisms thrive, it can be argued that those who express a great variety of bioactive molecules are those having the higher chances of adaptation and survival in a quite changing environment from the point of view of the availability of substrates for bacterial metabolism. These multiple-enzyme producers will be able to take advantage of a broader spectrum of substrates to incorporate as nutrients. Following this concept, providing that the purpose of a screening program is the screening for several enzymatic activities, the faster and less laborious strategy would be to perform the

initial isolation pushing for a single activity rather than performing the initial isolation in parallel using a different selective medium for each enzymatic activity. In the studied environment, the selective pressure for protease production can be a good choice to carry out the isolation of multiple producers, as it proved to be the most abundant extracellular enzyme detected in bacteria obtained from the explored biotopes. Although not related with their biotechnological potential, this fact was also observed by Groudieva et al. (2004) for bacteria from cold Arctic fjords, where protease was the most abundant hydrolytic enzyme produced by the bacteria isolated from sea ice.

Taking all the above mentioned into account, on the basis of the results presented here we proved the ecological relevance of the marine culturable heterotrophic bacterial community from Potter Cove, and representative isolates from several bacterial genera were recovered from it. Furthermore, the majority of the retrieved genera were ubiquitous, not displaying any evident distribution pattern among the explored biotopes. This brings to the light the presence of a remarkable bacterial diversity in Potter Cove, which undoubtedly contributes to varied processes of organic matter mineralization and nutrient recycling in the ecosystem, partly conditioning the biodiversity of phytoplanktonic, zooplanktonic, benthic and pelagic organisms that characterizes the cove and its shore (Schloss et al. 2002; Raes et al. 2009).

As a final remark, we consider that this work establishes a starting point for future programs on biomolecules discovery. These programs can be or not related to the search for those molecules detected in this study, as it was demonstrated that many isolates were producers of multiple bioactivities and therefore, the possibility that they may produce interesting molecules other than those detected in the present work is not rejected. We have also evidenced that the heterotrophic culturable microbiota of Potter Cove, an Antarctic location relatively easy to be accessed and studied since the Argentinean Scientific Station Carlini is settled on its shore, is a promissory source of biomolecules with potential industrial

interest. Further studies are being conducted to assess the potential of these marine isolates for their use in biotechnological applications.

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Table 1. Source, number of isolates obtained per sample in each summer Antarctic Research Expedition (ARE) using half-strength marine agar incubated at 4°C and their laboratory reference numbers.

ARE	Sample	Isolates	Laboratory reference number of isolates
1987	Fish (*Notothenia rossi*)		
	Stomach (NRE)	8	3NRE3, 5NRE1, 5NRE2, 5NRE6, 5NRE7, 8NRE2, 9NRE2, 9NRE8
	Intestine (NRI)	3	3NRI2, 3NRI4, 5NRI5
	Cloacae (NRC)	3	1NRC2, 2NRC2, 10NRC2
	Tegument (NRP)	6	2NRP2, 2NRP5, 3NRP10, 4NRP2, 4NRP4, 9NRP9
2001	Seaweed (*Adenocystis utricularis*)	3	S01-61, S01-62, S01-63
	Microalgae green mat	6	S01-64, S01-65, S01-66, S01-67, S01-68, S01-69
	Seawater	37	S01-70, S01-71, S01-72, S01-73, S01-74, S01-75, S01-76, S01-78, S01-99, S01-100, S01-101, S01-102, S01-103, S01-105, S01-106, S01-107, S01-108, S01-109, S01-110, S01-111, S01-112, S01-113, S01-114, S01-115, S01-116, S01-117, S01-118, S01-119, S01-120, S01-121, S01-122, S01-123, S01-124, S01-125, S01-126, S01-127, S01-128
	Surface marine sediment	8	S01-93, S01-94, S01-129, S01-130, S01-131, S01-132, S01-133, S01-134

	Bivalve (*Laternula elliptica)*	3	S01-96, S01-97, S01-98
	Fish (*Notothenia nudifrons)*		
	Stomach	1	S01-81
	Intestine	3	S01-82, S01-83, S01-84
	Fish (*Pagothenia bernacchii)*		
	Tegument	3	S01-85, S01-86, S01-87
	Stomach	1	S01-88
	Intestine	3	S01-89, S01-90, S01-91
	Limpet (*Nacella concinna*)	2	S01-79, S01-80
2003	Bivalve (*Laternula elliptica)*	9	S03-1, S03-2, S03-3, S03-4, S03-5, S03-6, S03-7, S03-8, S03-11
	Surface marine sediment	6	S03-9, S03-10, S03-12, S03-13, S03-14, S03-15
	Seawater	4	S03-21, S03-22, S03-23, S03-24
2005	Pelagic tunicate (*Salpa thompsoni*)	3	S05-48, S05-49, S05-53
	Surface marine sediment	9	S05-56, S05-60, S05-61, S05-62, S05-70, S05-72, S05-73, S05-74, S05-152
	Seawater	9	S05-88, S05-89, S05-90, S05-97, S05-99, S05-147, S05-148, S05-220, S05-221

Fish (*Notothenia coriceps)*		
Gills	4	S05-105, S05-106, S05-107, S05-109
Fins	6	S05-112, S05-113, S05-115, S05-116, S05-118, S05-119
Tegument	10	S05-120, S05-121, S05-122, S05-123, S05-124, S05-125, S05-126, S05-127,
Stomach	5	S05-128, S05-129
Intestine	2	S05-132, S05-133, S05-135, S05-136, S05-137
		S05-140, S05-145
Polichaete	4	S05-153, S05-154, S05-155, S05-156
Starfish (*Odontaster validus*)	4	S05-163, S05-164, S05-165, S05-166
Bivalve (*Laternula elliptica)*	5	S05-178, S05-179, S05-180, S05-185, S05-187
Microalgae red mat	4	S05-207, S05-208, S05-209, S05-210
Amphipod	2	S05-215, S05-216
Gastropod	3	S05-217, S05-218, S05-219
Isopod	7	S05-173, S05-174, S05-175, S05-222, S05-223, S05-224, S05-225
Giant isopod (*Glyptonotus antarcticus*)	2	S05-158, S05-159
Ascidian (*Molgula pedunculata*)	1	S05-94

Table 2. Distribution of the 189 proteolytic bacterial isolates into 63 ARDRA phylotypes, indicating their taxonomic affiliation and hydrolyzed substrates. The isolates selected for 16S rRNA gene sequencing are indicated in bold. [#]SM: skim milk; S: starch; X: xylan; P: pectin; C: cellulose; CMC: carboxymethyl-cellulose. ND: none enzymatic activity detected.

ARDRA phylotype	Number of isolates	Laboratory reference number of isolates	Hydrolysed substrates[#]	Taxonomic affiliation	Closest relative type strain 16S rRNA gene
F1	8	3NRE3, 5NRE1, **5NRE6**, 8NRE2, 5NRI5, 2NRP2, **9NRE8**, 9NRE2	ND	*Psychrobacter* sp.	*P. cibarius* (AY639871) (100%) *P. urativorans* (AJ609555) (99.9%)
F2	5	**3NRI2**, 3NRI4, 10NRC2, 2NRP5, **9NRP9**	ND	*Psychrobacter* sp.	*P. aquimaris* (AY722804) (99.1%)
F3	1	**1NRC2**	ND	*Psychrobacter* sp.	*P. fozii* (AJ430827) (99%)
F4	1	**4NRP2**	ND	*Psychrobacter* sp.	*P. luti* (AJ430828) (99.7%)
F5	7	**S01-115**, S01-120, S01-122, **S01-127**, S01-128, S01-134	S, SM	*Psychrobacter* sp.	*P. nivimaris* (AJ313425) (99.6-99.9%)
		S01-123	SM		
F6	34	**S05-48**, S05-105, S05-148, S05-121,	SM	*Psychrobacter* sp.	*P. fozii* (AJ430827) (99-99.2%)

		S01-124			
		S05-70, S05-94, S05-106, S05-107, S05-109, S05-113, S05-115, S05-116, S05-120, S05-122, S05-123, S05-125, S05-126, S05-127, S05-128, S05-129, S05-132, S05-133, S05-135, S05-136, **S05-137**, S05- 140, S05-145, **S05-152**, **S05-185**, **S05-187**, S05-147, S05-220, S05-221	ND		*P. cryohalolentis* (CP000323) (99.7-100%) *P. aquimaris* (AY722804) (99.7%)
F7	5	**S05-88** **S05-89** **S05-156**, S05-60, S05-61	ND	*Psychrobacter* sp.	*P. submarinus* (AJ309940) (99.7%) *P. faecalis* (AJ421528) (99.8-99.6%) *P. pulmonis* (AJ437696) (99.8%)
F8	3	**S05-56**, S05-119	ND	*Psychrobacter* sp.	*P. cryohalolentis* (CP000323) (99%)
		S05-118	SM		
F9	1	**S05-112**	ND	*Psychrobacter* sp.	*P. arcticus* (AY444822) (98.5%)

F10	11	**S01-65**	C, CMC, SM	*Pseudoalteromonas* sp.	*P. arctica* (DQ787199) (100%)
		S01-61, S01-125	S, C, CMC, P, X,		
		S01-64, S01-67, S01-68, S01-71,	SM		*P. paragorgicola* (AY040229) (99.8%)
		S01-99	C, CMC, P, X, SM		
		S01-70	S, C, CMC, P, SM		
		S01-113	CMC, P, X, SM		*P. elyakovii* (AF082562) (99.8%)
		S01-86	SM		
F11	7	S01-84, S01-110, **S01-117**	SM	*Pseudoalteromonas* sp.	*P. arctica* (DQ787199) (99.6%)
		S01-111	CMC, X, SM		
		S01-132	SM		*P. translucida* (AY040230) (99.2%)
		S01-129, S01-130	C, CMC, SM		
F12	4	**S01-121**	C, CMC, X, SM	*Pseudoalteromonas* sp.	*P. elyakovii* (AF082562) (99.7%)
		S01-66, S01-103	C, CMC, P, X, SM		
		S01-98	SM		
F13	9	**S01-81,** S01-82, S01-83, S01-88,	SM	*Pseudoalteromonas* sp.	*P. translucida* (AY040230) (99.4%)
		S01-89, S01-90, S01-91, S01-133			

		S01-114	CMC, SM	
F14	5	**S01-105**	CMC, P, SM	*Pseudoalteromonas* sp. *P. elyakovii* (AF082562) (99.9%)
		S01-108	CMC, SM	
		S01-109	C, CMC, SM	
		S01-118	C, CMC, X, SM	
		S01-119	C, CMC, P, X, SM	
F15	3	**S01-124**	S, P, SM	*Pseudoalteromonas* sp. *P. translucida* (AY040230) (99.1%)
		S01-100	S, C, CMC, P, SM	
		S01-126	P, SM	
F16	5	**S03-13**	CMC, P, X, SM	*Pseudoalteromonas* sp. *P. translucida* (AY040230) (99.1%)
		S03-1, S03-23	S, CMC, P, X, SM	
		S03-11	S, CMC	
		S03-22	S, CMC, P, SM	
F17	5	**S03-12**	SM	*Pseudoalteromonas* sp. *P. translucida* (AY040230) (99%)
		S03-2, S03-10	CMC, P, X, SM	
		S03-4	CMC, P, SM	

		S03-15	S, CMC, P, X, SM		
F18	1	**S03-24**	S, CMC, P, SM	*Pseudoalteromonas* sp.	*P. arctica* (AF529062) (99.7%)
F19	6	**S05-154**, S05-155, S05-173, S05-174	SM	*Pseudoalteromonas* sp.	*P. arctica* (AF529062) (99.7%)
		S05-153	P, SM		
		S05-180	S, P, SM		
F20	1	**S05-175**	SM	*Pseudoalteromonas* sp.	*P. arctica* (AF529062) (99.7%)
F21	1	**S05-223**	C, CMC, P, X, SM	*Pseudoalteromonas* sp.	*P. arctica* (DQ787199) (100%)
F22	9	**S01-63**, S01-79	ND	*Psychromonas* sp.	*P. arctica* (AF374385) (99.7%)
		S01-72	C, CMC, X		
		S01-116	P		
		S01-96, S01-106, S01-107, S01-112,	X		
		S01-131			
F23	2	**S01-62**	C, CMC	*Psychromonas* sp.	*P. arctica* (AF374385) (98.4-98.7%)
		S01-73	CMC		
F24	1	**S01-101**	C, CMC, P, X	*Psychromonas* sp.	*P. arctica* (AF374385) (99.2%)
F25	1	**S03-3**	ND	*Psychromonas* sp.	*P. arctica* (AF374385) (97.4%)

F26	2	**S05-166**	X	*Psychromonas* sp.	*P. arctica* (AF374385) (99.5%)
		S05-164	P, X		
F27	1	**S05-218**	ND	*Psychromonas* sp.	*P. arctica* (AF374385) (97.7%)
F28	1	**S05-224**	P, X	*Psychromonas* sp.	*P. arctica* (AF374385) (98.9%)
F29	1	**S01-85**	ND	*Pseudomonas* sp.	*P. migulae* (AF074383) (99.6%)
F30	4	**S05-49**	P	*Pseudomonas* sp.	*P. cedrella* (AF064461) (99%)
		S05-97, S05-99	SM		
		S05-178	ND		
F31	2	**S05-207**, S05-208	SM	*Pseudomonas* sp.	*P. brenneri* (AF268968) (99.6%)
F32	1	**5NRE2**	ND	*Arthrobacter* sp.	*A. gangotriensis* (AJ606061) (99.1%)
F33	1	**S01-102**	S, P	*Arthrobacter* sp.	*A. bergerei* (AJ609630) (98.6%)
F34	1	**S05-72**	ND	*Arthrobacter* sp.	*A. stackebrandtii* (AJ640198) (97.4%)
F35	1	**S05-210**	ND	*Arthrobacter* sp.	*A. gangotriensis* (AJ606061) (98.4%)
F36	1	**S05-215**	ND	*Arthrobacter* sp.	*A. gangotriensis* (AJ606061) (98.2%)
F37	1	**S05-163**	C, CMC, P, X, SM	*Colwellia* sp.	*C. aestuarii* (DQ055844) (97.7%)
F38	1	**S05-222**	ND	*Colwellia* sp.	*C. psychrerythraea* (AF001375) (99.4%)

F39	1	**S05-225**	C, CMC, P, X, SM	*Colwellia* sp.	*C. aestuarii* (DQ055844) (97.8%)
F40	2	**S01-93**	C, CMC, SM	*Colwellia* sp.	*C. aestuarii* (DQ055844) (97.6%)
		S01-94	C, CMC, X, SM		
F41	2	**S03-14**	S, CMC, X, SM	*Salinibacterium* sp.	*S. amurskyense* (AF539697) (99.9%)
		S03-9	CMC, P, X, SM		
F42	2	**3NRP10**, 5NRE7	ND	*Salinibacterium* sp.	*S. amurskyense* (AF539697) (99.8%)
F43	1	**S05-216**	ND	*Salinibacterium* sp.	*S. xinjiangense* (DQ515964) (98.2%)
F44	1	**S05-53**	P	*Salinibacterium* sp.	*S. amurskyense* (AF539697) (98.9%)
F45	1	**S05-73**	ND	*Salinibacterium* sp.	*S. amurskyense* (AF539697) (98.8%)
F46	4	**S05-158**, S05-209	SM	*Shewanella* sp.	*S. canadensis* (AY579749) (98.9%)
		S05-90	ND		
		S05-165	S, P, SM		
F47	2	**S01-69**, S01-87	C, CMC	*Polaribacter* sp.	*P. irgensii* (M61002) (97.3%)
F48	1	**S01-97**	ND	*Polaribacter* sp.	*P. irgensii* (M61002) (97.6%)
F49	2	**S05-62**	S, P	*Planococcus* sp.	*P. antarcticus* (AJ314745) (98.3- 99.4%)
		S05-74	S		

F50	1	**4NRP4**	ND	*Planococcus* sp.	*P. antarcticus* (AJ314745) (98.6%)
F51	1	**S05-159**	P	*Photobacterium* sp.	*P. indicum* (AB016982) (99.8%)
F52	1	**S05-179**	ND	*Photobacterium* sp.	*P. frigidiphilum* (AY538749) (99.1%)
F53	2	**S01-76**	P	*Kocuria* sp.	*K. palustris* (Y16263) (99.3%)
		S01-78	ND		
F54	2	**S03-6**	CMC	*Cellulophaga* sp.	*C. algicola* (AF001366) (98.5%)
		S03-8	P, SM		
F55	1	**2NRC2**	ND	*Enterobacter* sp.	*E. ludwigii* (AJ853891) (98.4%)
F56	1	**S01-74**	ND	*Acinetobacter* sp.	*A. lwoffii* (X81665) (99.6%)
F57	1	**S01-75**	ND	*Williamsia* sp.	*W. muralis* (Y17384) (99.9%)
F58	1	**S01-80**	ND	*Vibrio* sp.	*V. logei* (AJ437616) (99.7%)
F59	1	**S03-5**	CMC, X	*Flavobacterium* sp.	*F. algicola* (AB455265) (98.3%)
F60	1	**S03-7**	X, SM	*Hydrogenophaga* sp.	*H. taeniospiralis* (AF078768) (98.8%)
F61	1	**S03-21**	ND	*Cobetia* sp.	*C. marina* (AJ306890) (98.9%)
F62	1	**S05-217**	ND	*Sulfitobacter* sp.	*S. litoralis* (DQ097527) (99.8%)
F63	1	**S05-219**	ND	*Marinobacter* sp.	*M. maritimus* (AJ704395) (99.5%)

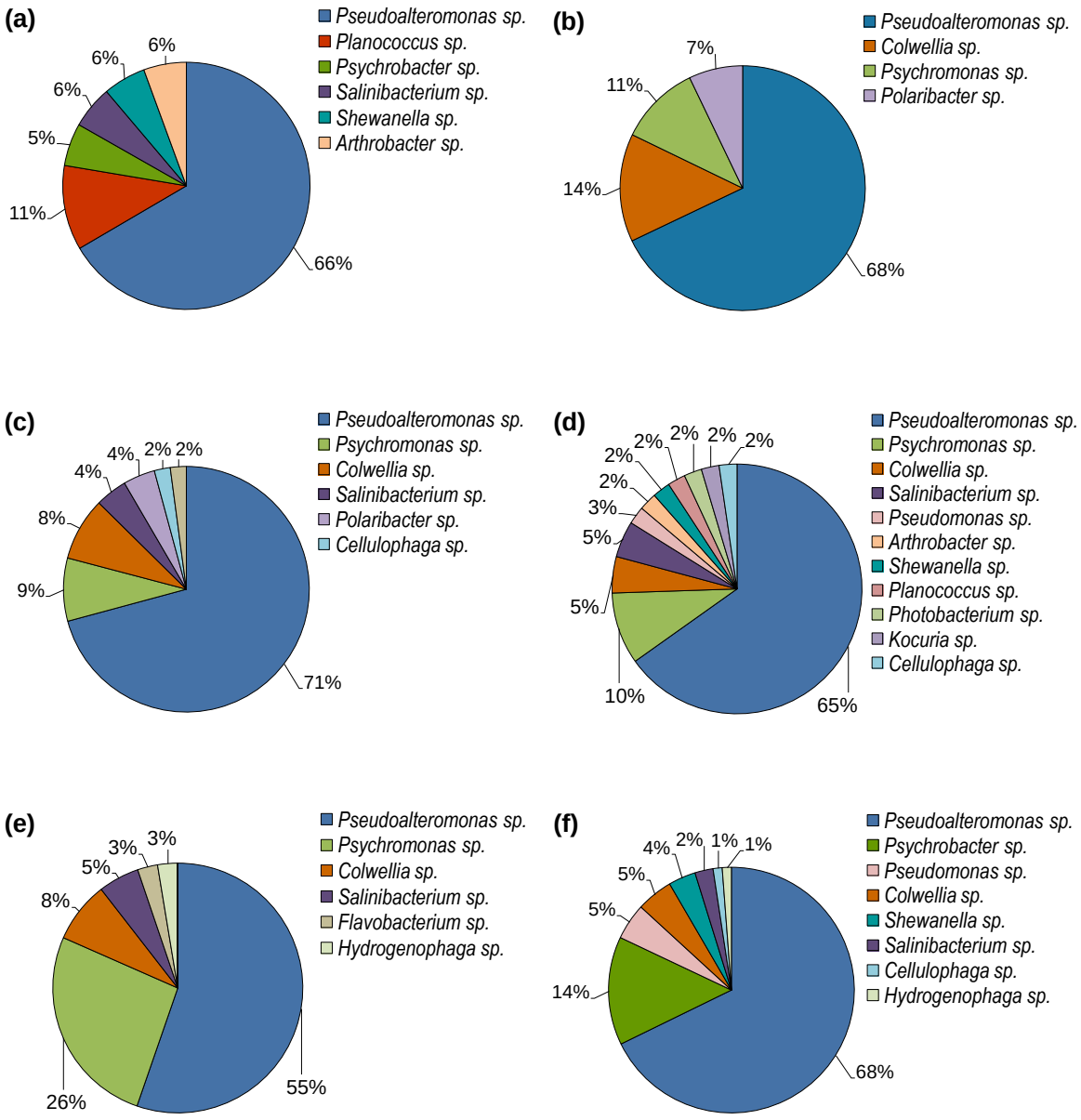

Figure 1. Abundance (%) of isolates affiliated with the different genera recovered for each detected enzymatic activity. **(**a) amylase, (b) cellulase, (c) carboxymethyl-cellulase, (d) pectinase, (e) xylanase, (f) protease.

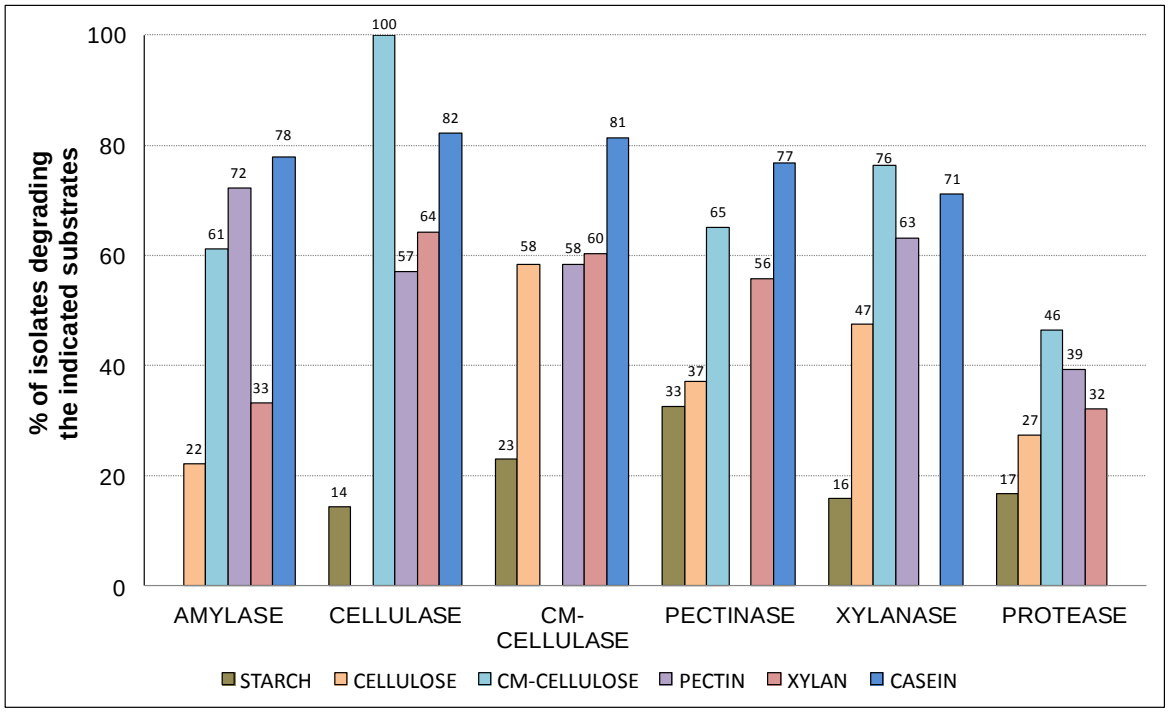

Figure 2. Percentage of isolates degrading additional substrates within the groups of producers of a particular enzymatic activity.