

Bacterial diversity and their adaptations in the shallow water hydrothermal vent at D. João de Castro Seamount (DJCS), Azores, Portugal

Chellandi MOHANDASS¹, Raju RAJASABAPATHY¹, Chinnarajan RAVINDRAN¹, Ana COLACO², Ricardo Serrao SANTOS², Ram Murthi MEENA¹

1) Microbial Technology Lab, Biological Oceanography Division, National Institute of Oceanography (CSIR), Dona Paula, Goa-403 004, India. Phone: 91(0)832 2450414, Fax: 91(0) 832-2450606. E-mail: cmohan@nio.org

2) IMAR - Department of Oceanography and Fisheries, University of Azores, Cais de Sta Cruz 9901-862, Horta, Portugal.

Abstract:

Bacterial diversity from the shallow vent of D. João de Castro Seamount (DJCS) Azores, Portugal and their adaptations to a nutrient rich environment was investigated from 2004 and 2005 cruise samples. Assessment of the qualitative composition of the heterotrophic bacterial diversity studied by various means like 16S rRNA, FAME and RFLP expressed the existence of consistent bacterial groups like *Bacillus*, *Staphylococcus*, *Micrococcus*, *Halomonas*, *Pseudoalteromonas*, *Alcaligenes* and *Brevibacterium*. *In situ* studies conducted in the laboratories with different metals, revealed that Fe, Mn and Pb reduces the bacterial growth period by 50% in some of the species like *Staphylococcus succinus*, *Bacillus subtilis* and *Bacillus* sp. About 55% of the organisms from the experimental site were able to hydrolyze carbohydrates, 27% of them were able to attack fatty acid and 45% were protein degraders. High temperatures (70 °C) and neutral pH (7) were opted by most of the vent bacteria to produce their enzymes. Adaptive mechanisms to detoxify the metals by producing H₂S gases, catalase and oxidase for decomposition of hydrogen peroxide and oxygen supply; extended survival in unfavourable conditions by sporulation were observed as a common characteristic features in most of the bacterial isolates of the shallow vent DJCS.

Key words: Diversity • Enzymes • FAME • Metals • RFLP • 16S rRNA

Introduction

Microbial community of hydrothermal vent systems is necessary to understand the functioning of the ecosystems and their impact to their surrounding environment (Sievert et al., 2000). Shallow water vents are different from deep ones because primary production is based on photosynthesis (Tarasov et al., 2005). Elements present in the shallow vents can be oxidized and used by many heterotrophic members of the Archaea and bacteria as electron acceptors for the anaerobic degradation of organic matter (Sievert et al., 2000). Vent associated microorganisms may be involved in microbially mediated transformation and precipitation of elements. Hydrothermal vents offer a variety of habitats to metabolically diverse microorganisms (Baross and Hoffman, 1985; Karl, 1995).

Cultivation based methods are not well suited for investigation of the general composition of microbial communities, since only a small percentage of the microorganisms are cultivable (Amann et al., 1995), however molecular tools such as 16S rRNA clone libraries made it possible to circumvent this limitation. Microbial investigation on the hydrothermal vents mostly deals with the selective isolation of chemosynthetic and thermophilic bacteria (Rusch et al., 2005). Although the trophic role of the heterotrophic bacteria in the vent ecosystem is not completely understood, roles in detoxification process by these microorganisms in association with polychaetes confirm the importance of these in the vent system (Jeanthon and Prieur, 1990b).

The D. João de Castro Seamount (DJCS) located in the North Atlantic, between the Azorean islands São Miguel and Terceira (38°13.3'N, 26°36.2'W), lies on the Terceira Rift (Vogt and Jung, 2004). The area of the DJCS shallower than 50 m is approximately 0.35 km², with 0.011 km² showing hydrothermal activity. Venting on the seamount can be divided in two main types; 'white' and 'yellow' vents, according to the bottom colour around the fluid exits. The venting fluids from the white zone (WZ) are significantly richer than those from the yellow zone (YZ) in dissolved sulphide [81.6 µM (WZ)/1.24 µM (YZ)]; H₂ [3183.5 µM (WZ)/84.4 µM (YZ)]; CH₄ [29.8 µM (WZ)/12.8 µM (YZ)] and the trace metal Pb [3.6 nM (WZ)/1.9 nM (YZ)], but poor in Fe [8.8 µM (WZ)/89.2 µM (YZ)] and Mn concentrations [0.5 µM (WZ)/ 6.8 µM (YZ)]. The fluids from the yellow zone are warmer (61.3 °C) than the ones from the white zone (35.8 °C) (Cardigos et al., 2005). These venting fluids have much higher trace metal and gas concentrations than the ambient Atlantic seawater (Colaco et al., 2006). The Atlantic seawater has 36.5 µM H₂ (Herr and Barger, 1978), 5.48 µM CH₄ (Conrad and Seiler, 1988) and 930 pmol of dissolved total sulphide (Andreae et al., 1991).

Hydrothermal vents are geological formations that release altered seawater which has been heated, often to temperatures exceeding 400 °C, by subterranean magma pockets as it circulates through the crust, mobilizing metals from basalt and acquiring elevated levels of sulphide from the reduction of sulphate by ferrous iron dissolved in the hot fluid (Vincent et al., 1994). When the superheated sea water mixes with cold oceanic sea water, the positively charged cations of metals, i.e. copper and zinc, leached out of the basalt precipitate as sulphides (Malahoff, 1985). Hydrothermal vents host unique communities of animals exposed to high concentrations of heavy metals and are covered with a community of metal-resistant bacteria (Jeanthon and Prieur, 1990a). Bacterial symbionts present with other animals in the vent utilize the metals like sulphide as an energy source to fix carbon (Vincent et al., 1994). Further, the basis of this association between bacteria and higher organisms in the hydrothermal vent is to detoxify invertebrate surfaces by symbiotic microorganisms (Alayse-Danet et al., 1987; Holmstrom and Kjelleberg, 1999; Jeanthon and Prieur, 1990b). Although the presence of large amounts of metals is a major feature of hydrothermal vent environments, relatively little is known about relationships between the metals and organisms living at these sites. Heterotrophic bacteria are generally ignored in the vent area and only the specialists like chemosynthetic and thermophilic bacteria are given more important.

Just a few studies on the diversity and adaptation of microorganisms have been carried out on the DJCS (filamentous bacteria from the white area at DJCS by Cardigos et al., 2005; assemblage of benthic diatoms and culturable heterotrophs by Raghukumar et al., 2008). The goal of the present study is to confirm the abundance of genetic diversity of the heterotrophic microbial population in metal rich environments and its adaptability for its better endurance.

Materials and Methods

Samples of water, sediment, bacterial and algal mats were collected in sterile containers from the DJCS (Fig. 1) during cruises on the research vessels R.V. Arquipelago (2004) and Águas Vivas (2005). Samples were collected by SCUBA diving. Pieces of algae, bacteria, and sediments were suspended in 50% sterile seawater, and then 100 µl was plated with solid nutrient agar media (HiMedia M001, Mumbai, India). Samples were stored in the dark at 4 °C on board and transported with ice packs to University of Azores, Horta, Portugal. Colonies appeared on the nutrient agar plates from various sampled sources like sediment, water, algal and bacterial mats were isolated and given numbers SV1 – SV23.

Isolation, purification and identification

Isolates obtained from the seawater nutrient agar plates were selected based on size, shape, and colour. Various morphological and biochemical tests like gram staining, spore staining, motility, H₂S production, catalase, oxidase, amylase, lipase and caseinase production were performed. All the tests have been carried out as per the standard microbiological protocol suggested by the supplier (HiMedia pvt ltd, Mumbai, India).

Bacterial identity based on Fatty acid Methyl Ester (FAME)

Approximately 30 – 40 mg of cells grown in Trypticase Soy Broth Agar (TSBA) media for one day was collected and the fatty acid preparation, extraction, purification, and methylation were accomplished using standard procedures (Sasser, 1990). The FAME profiles generated were compared against an inbuilt Sherlock TSBA Library version 4.0 B (MIDI Inc., DE, USA) and matched to a library of 700+ bacterial species representing over 180 genera. Cellular fatty acid composition analysis was done at Regional centre of Kochi, National Institute of Oceanography. Bacterial fatty acids were identified by comparison of the retention times of their methyl esters on the SE30 column.

Genomic DNA isolation

The genomic DNA was prepared from all the isolates by K- Cetyl Trimethyl Ammonium Bromide (CTAB) method (Sambrook and Russel, 2001) with some modifications. Sodium Dodecyl Sulphate (SDS) was replaced with CTAB in the extraction buffer; Ammonium acetate and proteinase K were eliminated from the original CTAB method since phenol was repeatedly used to remove proteins. Overnight grown bacterial cultures in LB broth (HiMedia M1245, Mumbai) were centrifuged at 8000 rpm for 10 min and the bacterial pellets were lyophilized. Approximately 500 mg of sample (dry weight) was re-suspended in 1.5 ml of extraction buffer (100 mM Tris HCl, 100 mM Na₂EDTA, 1.5 M NaCl and 1% CTAB). The extraction buffer was preheated at 60 °C for 60 min prior to treatment of the samples. After the addition of extraction buffer the samples were well mixed with sterile toothpicks and incubated at 65 °C for 60 min. The samples were then centrifuged at 12000 rpm for 10 min. The supernatant was collected and added with 500 µl of phenol: chloroform: iso-amyl alcohol mixture (25:24:1). The samples were then mixed for 30 min and centrifuged at 12000 rpm for 10 min. The same step was repeated once again and the supernatant was collected in a fresh tube. To the supernatant 500 µl of chloroform was added and centrifuged at 12000 rpm for 10 min. Nucleic acids in the supernatant fraction were

precipitated by adding 100% chilled ethyl alcohol. This was centrifuged and washed with 70% ethyl alcohol. The dried pellets were re-suspended in sterile double distilled water or TE buffer. The isolated DNA was run on 0.8% (w/v) Tris-Acetate-EDTA (TAE) agarose gel electrophoresis with ethidium bromide staining and visualized under UV.

Amplification of 16S rDNA

PCR amplification of 16S rRNA gene was done with the bacterial consensus primers 27F [5'-AGA GTT TGA TCC TGG CTC AG-3'] and 1492R [5'-GGT TAC CTT GTT ACG ACT T-3'] universal forward and reverse 16S rDNA primers (Lane, 1991). The PCR of the genomic DNA of 22 isolates were conducted in a final volume of 25 μ l. The reaction mixture contained 1x PCR buffer (Sigma, USA), 2.5 mM MgCl₂, 200 μ M DNTP's, 1 U of Taq DNA polymerase, 25 pmol of each forward and reverse oligonucleotide primers and approximately 20 ng of genomic DNA. The amplification profile consisted of an initial denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min. This was followed by a final extension step of 72 °C for 5 min. The samples were held at 4 °C until further analyse.

PCR-RFLP

RFLP marker was used to identify the variability among the bacterial groups from the shallow vent strains. The DNA obtained from all 22 bacteria were amplified using 16S primers and the PCR products were digested with Alu I with appropriate buffers (Fermentas) according to the manufacturer's protocol. The 20 μ l of above reaction mixture was incubated at 37 °C for 3 h. Restriction fragments were separated using 2.5% TAE-agarose gel at 80 V for 1 hour and visualized with UV light after staining with ethidium bromide (0.5 μ g ml⁻¹).

Sequencing

Sequencing of all the isolates was performed with an automatic sequencer Applied bio system. Foster City, USA. Dona-Paula, Goa. India.

Accession Numbers

The sequences were submitted to GenBank and accession numbers were assigned from GU143784 (SV1) to GU143805 (SV23).

BLAST Search and Phylogenetic Analysis

The PINTAIL program (Ashelford et al., 2005) was used to check chimera formations. No anomalies were detected. The partial 16S rRNA gene sequences of the isolated strains were compared with those available in the public databases. Identification to the species level was determined by a 16S rDNA sequence similarity of 99% with that of the prototype sequence in GenBank. Sequence alignment and comparison was performed using the multiple sequence alignment program Clustal X 1.81 (Thompson et al., 1997). Sequences were edited manually to remove the gaps. Neighbor-joining method was employed to construct the Phylogenetic tree using MEGA4 software (Tamura et al., 2007) and the maximum likelihood method was adopted for calculating the evolutionary distance (Tamura et al., 2004).

Metal studies

The growth preferences of bacterial cultures isolated from the DJCS vent site for Fe, Mn and Pb were tested. Zobell marine broth (M385 HiMedia, Mumbai) supplemented with one μM of filter sterilized FeCl_3 , MnCl_2 and one nM of $\text{Pb}(\text{CH}_3\text{COO})_2 \cdot \text{H}_2\text{O}$ were inoculated to the bacterial cultures having initial optical density (OD) of 0.01 – 0.02 at 600 nm. Medium without metal supplement was used as control. OD of the cultures was measured every 24 h till 8th day. From the growth curve of each isolate in each media, organisms and its preferences were recorded.

Combination of metals, pH and temperature on amylase production

Known amount of bacterial cells inoculated in to nutrient broth (HiMedia M088, Mumbai) with 1% starch, subsequently added with 1 μM Fe and Mn, and 1 nM of Pb. Inoculated flasks were incubated in a rotary shaker for 5 days. Media were centrifuged at 10,000 rpm for 10 minutes at 4 °C. The supernatants were assayed for amylase activity (Medda and Chandra, 1980). Nutrient broth with 1% starch without metal was used as blank. Effect of pH on the vent strains were studied at various pH from 3 – 9, by incubating 0.1 ml enzyme solution with 1 ml buffer and 1 ml of 1% starch. The various buffers used to obtain proper pH were citrate buffer for 3 – 6 pH; phosphate buffer for 7 & 8; carbonate - bicarbonate buffer for 9. Temperature effects on enzyme activity from 30 – 90 °C were determined by the standard procedure (Medda & Chandra, 1980).

Results

Bacterial diversity by 16S rRNA of the shallow vent

Shallow vent bacteria of the DJCS identified by 16S rRNA revealed seven major genera from this site. They belong to *Bacillus*, *Staphylococcus*, *Micrococcus*, *Halomonas*, *Pseudomonas*, *Alcaligenes* and *Brevibacterium*. Among the genera *Bacillus*, nine belongs to *B. subtilis*, one *B. licheniformis* and one *B. flexus*. *Staphylococcus arlettae* and *S. succinus*, *Micrococcus lutes*, *Alcaligenes faecalis* and *Brevibacterium casei* were some of the other bacteria retrieved from these vents (Fig. 2). Sequences obtained from the vent strains were shown 99 to 100% homology with the GenBank (Table 1).

Eighty six percent of the strains isolated from the shallow vent belonged to Gram-positive and spore producers. Most of the colonies were circular and creamy or dull white and only one is reported to be yellow in colour. Ninety percent of the strains were motile and the remaining was non motile (Table 2).

Bacterial strains isolated from the shallow vents of DJCS identified by FAME analysis resulted that, many of them belongs to *Bacillus* spp (15). *Brevibacterium casei*, *Staphylococcus cohinii*, *S. arlettae*, *Yersinia*, *Halomonas* and *Micrococcus* were some of the other type of bacteria identified by FAME (Table 3). The bacterial groups corresponds to the gas chromatographic profile of a bacterial species in the Sherlock TSBA Library version 4.0 B (Microbial ID, MIDI Inc.) with a similarity index of 0.3 – 0.8. High similarity index were noticed with *Yersinia*, 0.822; *Micrococcus*, 0.847 while the lowest index have been seen in SV17, *Bacillus* with 0.255 (Table 3).

High similarities of identity were noticed in 16S rRNA and FAME tests. Differences were observed in a few identities like *Alcaligenes* by 16S rRNA (SV1) while it was identified as being *Yersinia* in FAME (Table 3); this analysis did indicate *Alcaligenes* as a second option with the similarity index of 0.528 (data not shown).

Influence of metals on bacterial growth, diversity and its hydrolyses

Bacterial survival in the hydrothermal vents depends on the effective uptake and detoxification of the materials present over there. The shallow vent of Azores presents high content of H₂S, CH₄, H₂, Pb, Co, Fe, Ba and Mn (Cardigos et al., 2005). Systems for the detoxification of metals required enzymes for its reduction. Twenty two bacterial cultures isolated from the site were investigated adding Fe, Pb and Mn

in to their growth media. Around 90% of them enhanced their growth in the presence of these metals. Only 10% of them were able to grow in a better way without metals (Table 1). Different organisms preferred different metals for their growth. Seventy seven percent of the organisms grow well with Fe. Strains like SV14, SV19, SV3, SV11 and SV15 obtained their biomass earlier in the Fe enriched media when comparing to the media with no metals (Zobell broth). *Staphylococcus succinus* (accession no. GU143799) has reduced its growth time by 50% with Fe (Table 1). Eighteen percent of the vent organisms enhanced their growth in the presence of Mn. *Bacillus subtilis* (GU143798) reduced its growth time from 7 days to 3 days in presence of Mn to attain a close equivalent biomass (0.706 – 0.603). Overall growth studies with various metals indicated that most of the bacteria preferred Fe for their growth (Table 1).

The various shallow vent bacteria isolates were tested for enzyme production in their respective media. All the isolates produced catalase. Degradative enzymes like amylase, lipase and caseinase were produced by 55, 27 and 45% of the isolates respectively (Table 4). The high percentage (55%) of amylase producing strains (12 out of 22) conducted to further investigations on these enzymes under metal influence. Among the 12 strains exposed to the three different metals (Fe, Pb and Mn) in to their enzyme production medium (Medda & Chandra. 1980) only 4 strains had their activity augmented. *B. subtilis* (GU143788) yielded a high amylase activity with Fe (49.5 U/ml) (Fig. 3) subsequently the strains *Bacillus* sp (GU143797), *B. licheniformis* (GU143794) given around 12 U/ml and *B. subtilis* (GU143793) reported to produce only 2 U/ml more than the media without metal. Mn almost equalizes the enzyme production with no metals for *Bacillus* sp (GU143797) and Pb did not support the enzyme activity in a noticeable way (Fig. 3). Characterization studies showed that pH 7 and temperature 70 °C to be ideal for most of the strains. *B. subtilis* (GU143793) showed its maximum activity of 47.2 U/ml among the tested strains (Fig. 4 & 5).

Homogeneity of the bacteria by RFLP

Ten RFLP patterns were detected from the shallow vent strains using with Alu-I endonucleases (Fig. 6). Restriction fragments shorter than 100 bp were disregarded for better clarity. Nine cultures, of *B. subtilis* gave an identical RFLP pattern. Strains SV13 and 14 appear to be very close. SV6 and SV15 showed a strong similarity to *Bacillus fluxes*. SV1, SV2, SV9, SV17, SV20, SV21 and SV22 were not similar and appear to be seven different patterns of expressions (Fig. 6).

Discussion

Exploration of heterotrophic bacteria from DJCS vent region using 16S rRNA sequencing analysis expressed the dominance of *Bacillus* spp. More than 15 strains of 22 tested belong to the genus *Bacillus*. Seventy seven percent of the sequenced isolates were found to be *Firmicutes* phylum; 13.63% were *Proteobacteria* and the rest were *Actinobacteria*. Maximal occurrence of *Bacillus* sp, from the experimental site may be due to their ability to form spores, thus enabling them to withstand a wide range of environmental conditions like temperature 35.8 – 61.3 °C and the pH 5.01 – 5.09 prevailing over the DJCS site. The presence of the heterotrophic bacteria in low temperature diffuse fluids of the DJCS suggests that they were originated from the sub sea floor hydrothermal habitat where temperatures and the pH were within their growth range.

The heterotrophic bacterial strains identified (Gugliandolo and Maugeri, 1997) by classical taxonomy from the shallow water hydrothermal vent of Eolian Island Italy belong to the genera: *Flavobacterium*, *Acinetobacter*, *Vibrio*, *Pseudomonas*, *Alcaligenes*, *Micrococcus*, *Arthrobacter*, and Coryneforms. While comparing the heterotrophic diversity of the DJCS with other shallow vents like Eolian Island, these Azorean shallow vents were also showed similar groups of bacteria and did not varied much from others.

Bacteria which belongs to heterogeneous genera that often contains several FAME biotypes eg., *Bacillus*, *Micrococcus* and *Arthrobacter*, will not be identified with the existing program of TSBA 4.0 software, therefore additional technique will be needed to identify them at species level (Heyrman et al., 1999). This could be the reason that some of the bacteria identified by FAME did not match with 16S rRNA in DJCS samples.

RFLP patterns analysed from the DJCS samples did not expressed its similarity when relating species with enzyme production and metal preferences. The bacteria which were showing attraction towards a particular metal sometimes did not produce the same type of enzyme i.e., SV13 and SV14 preferred Fe mostly for its growth, but the bacterium SV13 produced only two enzymes (catalase and oxidase) and SV14 produced 4 enzymes (Table 2). Probably both organisms belong to same species but the strain may be different (Fig. 2). In some of the metal preference and enzyme synthesis investigations by different bacteria showed the same pattern like with SV6 and SV15. It can be said that, these organisms belong to the same strain (Fig. 2).

Enzymes play a vital role in breaking down the materials to simple and less toxic substance, helping the organisms on their system. Catalase is a common enzyme found nearly in all living organisms that are exposed to oxygen. Its function is to catalyze the decomposing of hydrogen peroxide to water and oxygen (Chelikani et al., 2004). Hydrogen peroxide is a harmful by product of many normal metabolic processes. This has to be quickly converted to other less dangerous substances. Our results can be interpreted in a way that all the 22 bacterial isolates from the DJCS tested for catalase production showed positive indication and these enzymes may help in the detoxification of the toxic materials produced from various metabolic processes and help them to better survive on the shallow vent region.

Neutral pH was found to be optimal for amylase production by *Bacillus thermooleovorans* and *B. coagulans* (Medda and Chandra, 1980), *B. licheniformis* (Krishnan and Chandra, 1983) and *B. brevis* (Tsvetkov and Emanuilova, 1989). Our results were also expressed in the same manner that minimum activity obtained by strain *B. subtilis* (GU143793) 3.4 U/ml at pH 3 raised up to 47.2 U/ml at pH 7. Most of the strains when increasing the pH presented the same behaviour of enhancement of the enzyme activities. This extends up to neutral pH. Afterwards the activities were not increasing. From the Shallow vent strains recorded high enzyme activities at 70 °C, which were higher or similar to what was described for other *Bacillus* alpha amylases (Bolton et al., 1997; Igarashi et al., 1998). Even though the sampling sites were showing pH of 5.01 to 5.09, neutral pH has been favoured for high enzyme synthesis. This differentiates the adaption and preference of pH over the bacteria on shallow vents.

Bacteria inhabiting the hydrothermal vents are well adapted to flourish in the diverse environment despite the presence of metals that would normally inhibit the growth of most bacteria. Only a small fraction (10%) of the bacteria *Staphylococcus arlettae* and *Halomonas* did not grow well in the presence of metals. The enriched growth of the bacterium *Staphylococcus succinus* in a shorter time with Fe expresses the significant adaptation of bacteria to the metal rich waters over DJCS. Microorganisms are known to employ a variety of mechanism to adapt to the presence of heavy metals. One of these is the production of hydrogen sulphide, which may act as a detoxification process through precipitation of metal sulphides (Priour and Jeanthon, 1987). Among the 22 retrieved organisms from the DJCS, 13 organisms were able to produce H₂S. This is a good indication and expresses elevated adaptation to the shallow vent condition. Probably this could be one of the essential mechanisms adapted by the shallow vent bacteria for its survival over DJCS. In addition, most of the heterotrophic bacteria

from the DJCS were able to produce no less than one of the physiological enzymes like protease, lipase and amylases. These bacterial enzymes may play a vital role in mineralization of organic material in this nutrient rich environment. As suggested by Maugeri (2009), microorganisms inhabiting shallow hydrothermal vents possess nutritional requirements and overall metabolic pathways ideally suited to such ecosystem that represents a clear example of the close connection between geosphere and biosphere. Cole (1977) stated that there was no significant inhibition of amylase activity by Pb but the quantity of amylase formed was reduced to 26% of control levels. Our results show the high inhibition of enzyme activity when we introduce Pb in to the enzyme production media. Enzyme productions were decreased by more than five times; even it shows ten times lower in *B. subtilis* GU143793 (Fig. 3).

In conclusion the DJCS vent is richly diversified by gram positive spore producing bacteria, specially dominated with *Bacillus* followed by *Staphylococcus*, *Pseudoalteromonas*, *Alcaligenes*, *Brevibacterium*, *Halomonas* and *Micrococcus*. Most of the bacterial communities which live on the shallow vent of DJCS were well adapted to Fe, Mn and Pb for their growth and metabolism. High indication of catalases and H₂S productions from the vent bacteria expresses its detoxification potentials over the toxic substances for their healthy survival. Producing more than two to three physiological enzymes by a single bacterium from the vents might have been the unique identity for their better survival and endurance. High temperature, neutrally active and metal induced amylases reported in the DJCS vent may be of better prospects in biotechnological applications.

Acknowledgements

This work was done under the Indo-Portuguese project in Science and Technology (GRICES/DST-India). We acknowledge GRICES, Portugal and Department of Science and Technology, India for funding the trips to the DJCS site for sampling. Thanks to the crew members of R.V. Arquipelago and Águas Vivas for their support. IMAR-DOP/UAç research activities are additionally supported through the pluri-annual and programmatic funding schemes of FCT (Portugal) and Azorean Regional Directorate for Science and Technology (DRCT, Azores, Portugal) as Research Unit #531 and Associate Laboratory #9 (ISR-Lisboa).

References

- Alayse-Danet A.M., Desbruyeres D. & Gaill F. 1987.** The possible nutritional or detoxification role of the epibiotic bacteria of *Alvinellid polychaetes*: review of current data. *Symbiosis* **4**: 51–62.
- Amann R.I., Ludwig W. & Schleifer K.H. 1995.** Phylogenetic identification and in-situ detection of individual microbial cells without cultivation. *Microbiological Reviews*, **59**: 143–169.
- Andreae T.W., Cutter G.A., Hussain N., Radford-knoery J. & Andreae M.O. 1991.** Hydrogen Sulfide and Radon in and over the Western North Atlantic Ocean. *Journal of Geophysical Research*, **96**(D10): 18753–18760.
- Ashelford K.E., Chuzhanova N.A., Fry J.C., Jones A.J., & Weightman A.J. 2005.** At least 1 in 20 16S rRNA sequence records currently held in public repositories is estimated to contain substantial anomalies. *Applied and Environmental Microbiology*, **71**, 7724–7736.
- Baross J.A. & Hoffman S.E. 1985.** Submarine hydrothermal vents and associated gradient environments as sites for the origin and evolution of life. *Origins of Life and Evolution of the Biosphere*, **15**: 327–345.
- Bolton D.J., Kelly C.T. & Fogarty W.M. 1997.** Purification and characterization of the α -amylase of *Bacillus flavothermus*. *Enzyme and Microbial Technology*, **20**: 340–343.
- Cardigos F., Colaco A., Dando P.R., Avila S.P., Sarradin P.M., Tempera F., Conceicao P., Pascoal A. & Santos R.S. 2005.** Shallow water hydrothermal vent field fluids and communities of the D. João de Castro Seamount (Azores). *Chemical Geology*, **224**: 153–168.
- Chelikani P., Fita I. & Loewen P.C. 2004.** Diversity of structures and properties among catalases. *Cellular and Molecular Life Sciences*, **61**(2): 192–208.
- Colaco A., Raghukumar C., Mohandass C., Cardigos F. & Santos R.S. 2006.** Effect of shallow-water venting in Azores on a few marine biota. *Cahiers de Biologie Marine*, **47**(4): 359–364.
- Cole M.A. 1977.** Lead inhibition of enzyme synthesis in soil. *Applied and Environmental Microbiology*, **33**(2): 262–268.
- Conrad R. & Seiler W. 1998.** Methane and hydrogen in seawater (Atlantic Ocean). *Deep-Sea Research*, **35**(12): 1903–1917.
- Felsenstein J. 1985.** Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, **39**: 783–791.
- Gugliandolo C. & Maugeri T.L. 1997.** Temporal variations in heterotrophic mesophilic bacteria from a marine shallow hydrothermal vent off the Island of Vulcano (Eolian Islands, Italy). *Microbial Ecology*, **36**: 13–22.
- Herr F. & Barger W. 1978.** Molecular Hydrogen in the near surface atmosphere and dissolved in waters of the Tropical North Atlantic. *Journal of Geophysical Research*, **83**(C12): 6199–6205.

- Heyrman J., Mergaert J., Denys R. & Swings J. 1999.** The use of fatty acid methyl ester analysis (FAME) for the identification of heterotrophic bacteria present on three mural paintings showing severe damage by microorganisms. *FEMS Microbiology Letters*, **181**(1): 55–62.
- Holmstrom C. & Kjelleberg S. 1999.** Marine *Pseudoalteromonas* species are associated with higher organisms and produce biologically active extracellular agents. *FEMS Microbiology Ecology*, **30**: 285–293.
- Igarashi K., Hatada Y., Hagihara H., Saeki K., Takaiwa M., Uemura T., Ara K., Ozaki K., Kawai S., Kobayashi T. & Ito S. 1998.** Enzymatic properties of a novel liquefying α -amylase from an alkaliphilic *Bacillus* isolate and entire nucleotide and amino acid sequences. *Applied and Environmental Microbiology*, **64**: 3282–3289.
- Jeanthon C. & Prieur D. 1990a.** Resistance to heavy metals of heterotrophic bacteria isolated from the deep sea hydrothermal vent polychaete, *Alvinella pompejana*. *Progress in Oceanography*, **24**: 81–88.
- Jeanthon C. & Prieur D. 1990b.** Susceptibility to heavy metals and characterization of heterotrophic bacteria isolated from two hydrothermal vent polychaete annelids, *Alvinella pompejana* and *Alvinella caudata*. *Applied and Environmental Microbiology*, **56**: 3308–3314.
- Karl D.M. 1995.** Ecology of free-living, hydrothermal vent microbial communities. In: *Microbiology of deep-sea hydrothermal vents* (Karl D.M. ed), pp. 35–124. CRC Press Inc: Boca Raton Florida.
- Krishnan T. & Chandra A.K. 1983.** Purification and characterization of α -amylase from *Bacillus licheniformis* CUMC305. *Applied and Environmental Microbiology*, **46**(2): 430–437.
- Lane D.J. 1991.** 16S/23S rRNA sequencing. In: *Nucleic acid techniques in bacterial systematics* (Stackebrandt E. and Goodfellow M. eds), pp. 115–175. John Wiley & Sons Inc: New York.
- Malahoff A. 1985.** Hydrothermal vents and polymetallic sulphides of the Galapagos and Gorda/Juan de Fuca ridge systems and of submarine volcanoes. In: *The hydrothermal vents of the Eastern Pacific: An overview* (Jones M.L. ed), pp. **6**: 19–41. Bulletin of the Biological Society of Washington.
- Maugeri T.L., Lentini V., Gugliandolo C., Italiano F., Cousin S. & Stackebrandt E. 2009.** Bacterial and archaeal populations at two shallow hydrothermal vents off Panarea Island (Eolian Islands, Italy). *Extremophiles*, **13**: 199–212.
- Medda S. & Chandra A.K. 1980.** New strains of *Bacillus licheniformis* and *Bacillus coagulans* producing thermo stable α -amylase active at alkaline pH. *Journal of Applied Bacteriology*, **48**: 47–48.
- Prieur, D. & Jeanthon C. 1987.** Preliminary study of heterotrophic bacteria isolated from two deep sea hydrothermal vent invertebrates; *Alvinella pompejana* (Polychaete) and *Bathymodiolus thermophilus* (Bivalve). *Symbiosis*, **4**: 87–98.

- Raghukumar C., Mohandass C., Cardicos F., Costa P.M.D., Santos R.S. & Colaco A. 2008.** Assemblage of benthic diatoms and culturable heterotrophs in shallow water hydrothermal vent of the D. João de Castro Seamount, Azores in the Atlantic Ocean. *Current Science*, **95**(12): 1715–1723.
- Rusch A., Walpersdorf E., DeBeer D., Gurrieri S. & Amend J.P. 2005.** Microbial communities near the oxic/anoxic interface in the hydrothermal system of Vulcano Island, Italy. *Chemical Geology*, **224**: 169–182.
- Sambrook J. & Russel D.W. 2001.** *Molecular cloning: A laboratory manual*. 3rd edn. CSH laboratory press, cold spring harbor, Newyork.
- Sasser M. 1990.** Identification of bacteria through fatty acid analysis In: *Methods in Phytobacteriology* (Klement Z., Rudolph K. and Sands D.C. eds), pp. 199–204. Akademiai Kiado: Budapest, Hungary.
- Sievert S.M., Ziebis W., Kuever J., & Sahn K. 2000.** Relative abundance of *Archaea* and *Bacteria* along a thermal gradient of a shallow water hydrothermal vent quantified by rRNA slot-blot hybridization. *Microbiology*, **146**: 1287–1293.
- Tamura K., Dudley J., Nei M. & Kumar S. 2007.** MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, **24**: 1596–1599.
- Tamura K., Nei M. & Kumar S. 2004.** Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences, USA*, **101**: 11030–11035.
- Tarasov V.G., Gebruk A.V., Mironov A.N. & Moskalev L.I. 2005.** Deep-sea and shallow-water hydrothermal vent communities: Two different phenomena? *Chemical Geology*, **224**: 5–39.
- Thompson J.D., Gibson T.J., Plewniak F., Jeanmougin F. & Higgins D.G. 1997.** The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, **24**: 4876–4882.
- Tsvetkov V.T. & Emanuilova E.I. 1989.** Purification properties of heat stable α -amylase from *Bacillus brevis*. *Applied Microbiology and Biotechnology*, **31**: 246–248.
- Vincent P., Pignet P., Talmont F., Bozzi L., Fournet B., Guezennec J., Jeanthon C. & Prieur D. 1994.** Production and characterization of an exopolysaccharide excreted by a deep-sea hydrothermal vent bacterium isolated from the polychaete annelid *Alvinella pompejana*. *Applied and Environmental Microbiology*, **60**: 4134–4141.
- Vogt P. & Jung W. 2004.** The Terceira Rift as hyperslow, hotspot dominated oblique spreading axis: a comparison with other slow spreading boundaries. *Earth and Planetary Science letters*, **218**: 77–90.

Table 1. 16S rRNA diversity and their growth with various metals in comparison with normal Zobell broth

Strain No.	16S rRNA Identity	Metals Preferred	Maximum biomass in the normal media in days (OD at 600nm)	Maximum biomass in days Enriched with metals (OD at 600nm)	Accession number	% Homology
SV1	<i>Alcaligenes faecalis</i>	Fe	6(0.689)	7(1.125)	GU143784	99
SV2	<i>Brevibacterium casei</i>		6(0.701)	5(0.984)	GU143785	99
SV3	<i>Bacillus subtilis</i>		7(0.521)	5(0.603)	GU143786	100
SV4	<i>Bacillus subtilis</i>		6(0.744)	5(1.042)	GU143787	100
SV6	<i>Bacillus flexus</i>		3(0.674)	5(1.188)	GU143789	100
SV8	<i>Bacillus subtilis</i>		7(0.551)	5(1.000)	GU143790	99
SV10	<i>Bacillus subtilis</i>		6 (0.666)	6(0.972)	GU143792	99
SV11	<i>Bacillus subtilis</i>		5(0.680)	4(0.645)	GU143793	100
SV13	<i>Bacillus</i> sp.		6(0.564)	5(1.094)	GU143795	99
SV14	<i>Bacillus</i> sp.		4(0.613)	3(1.033)	GU143796	100
SV15	<i>Bacillus</i> sp.		6 (0.674)	4(0.918)	GU143797	100
SV17	<i>Staphylococcus succinus</i>		6(0.721)	3(1.075)	GU143799	100
SV18	<i>Bacillus subtilis</i>		6(0.811)	6(1.625)	GU143800	99
SV19	<i>Bacillus subtilis</i>		6(0.741)	4(0.829)	GU143801	99
SV21	<i>Micrococcus luteus</i>		7(0.833)	6(1.137)	GU143803	99
SV22	<i>Pseudoalteromonas issachenkonii</i>		5(0.764)	6(1.067)	GU143804	99
SV23	<i>Bacillus subtilis</i>		6(0.596)	7(0.885)	GU143805	99
SV5	<i>Bacillus subtilis</i>	Mn	6 (0.698)	6(0.892)	GU143788	100
SV13	<i>Bacillus</i> sp.		6(0.564)	3(0.584)	GU143795	99
SV16	<i>Bacillus subtilis</i>		7(0.706)	3(0.603)	GU143798	100
SV22	<i>Pseudoalteromonas issachenkonii</i>		5(0.764)	6(0.841)	GU143804	99
SV12	<i>Bacillus licheniformis</i>	Pb	6 (0.841)	5(0.557)	GU143794	99
SV15	<i>Bacillus</i> sp.		6 (0.674)	5(0.808)	GU143797	100
SV9	<i>Staphylococcus arlettae</i>	Did not prepare any metals	6(0.629)		GU143791	99
SV20	<i>Halomonas</i> sp.		4(0.874)		GU143802	99

Table 2. Colony characteristics of various isolates obtained from the vent:

Culture	Spore	Gram ±	Colour	Size	Elevation	H ₂ S	Motility
SV1	+	-	Cream	5mm	Convex	-	Motile
SV2	+	+	Cream	4mm	Convex	+	Non-motile
SV3	+	+	Off white	Pinpoint	Raised	-	Motile
SV4	+	+	Cream	4mm	Raised	-	Motile
SV5	+	+	Cream	2mm	Raised	+	Motile
SV6	+	+	Cream	3mm	Raised	+	Motile
SV8	+	+	Cream	2mm	Raised	-	Motile
SV9	-	+	Off white	1mm	Raised	+	Motile
SV10	+	+	Off white	1mm	Flat	-	Motile
SV11	+	+	Cream	4mm	Raised	-	Motile
SV12	+	+	Cream	2mm	Flat	+	Motile
SV13	+	+	Cream	4mm	Convex	-	Motile
SV14	+	+	Cream	3mm	Raised	-	Motile
SV15	+	+	Cream	3mm	Raised	+	Motile
SV16	+	+	Off white	1mm	Flat	+	Motile
SV17	-	+	Off white	1mm	Flat	+	Motile
SV18	+	+	Off white	Pinpoint	Flat	+	Motile
SV19	+	+	Cream	1mm	Flat	+	Motile
SV20	-	-	Cream	Pinpoint	Flat	+	Motile
SV21	+	+	Yellow	1mm	Flat	+	Non-motile
SV22	+	-	Cream	5mm	Flat	+	Motile
SV23	+	+	Cream	2mm	Flat	-	Motile

Symbols: +, Positive; -, Negative.

Table 3. Bacterial identity by FAME

Strain number	Identity	Similarity Index
SV1	<i>Yersinia pseudotuberculosis</i>	0.822
SV2	<i>Brevibacterium casei</i>	0.605
SV3	<i>Bacillus subtilis</i>	0.746
SV4	<i>Bacillus sphaericus</i>	0.344
SV5	<i>Bacillus subtilis</i>	0.770
SV6	<i>Bacillus GC group 22</i>	0.552
SV8	<i>Bacillus subtilis</i>	0.303
SV9	<i>Staphylococcus arlettae</i>	0.661
SV10	<i>Yersinia pseudotuberculosis</i>	0.770
SV11	<i>Bacillus subtilis</i>	0.443
SV12	<i>Bacillus sphaericus</i>	0.403
SV13	<i>Bacillus sphaericus</i>	0.408
SV14	<i>Bacillus sphaericus</i>	0.517
SV15	<i>Bacillus flexus</i>	0.524
SV16	<i>Bacillus subtilis</i>	0.459
SV17	<i>Staphylococcus cohinii</i>	0.743
SV18	<i>Bacillus subtilis</i>	0.255
SV19	<i>Bacillus subtilis</i>	0.763
SV20	<i>Halomonas aquamarina</i>	0.767
SV21	<i>Micrococcus lysae</i>	0.847
SV22	<i>Bacillus lentimorbus</i>	0.353
SV23	<i>Bacillus subtilis</i>	0.413

Table 4. Various shallow vent bacteria tested for its enzyme production in their respective media.

Culture	Catalase	Oxidase	Amyalse	Lipase	Caseinase
SV1	+	+	+	+	-
SV2	+	+	+	-	+
SV3	+	-	-	-	-
SV4	+	+	+	-	+
SV5	+	+	+	+	+
SV6	+	+	-	-	-
SV8	+	+	-	+	-
SV9	+	-	-	-	-
SV10	+	+	+	-	+
SV11	+	+	+	-	-
SV12	+	+	+	-	-
SV13	+	+	+	-	-
SV14	+	+	-	-	+
SV15	+	+	+	-	-
SV16	+	+	-	+	+
SV17	+	-	-	-	-
SV18	+	+	+	-	-
SV19	+	-	+	+	+
SV20	+	+	-	-	-
SV21	+	+	-	-	+
SV22	+	+	-	-	+
SV23	+	+	+	+	+

Symbols: +, Production; -, No Production.

Fig.1.

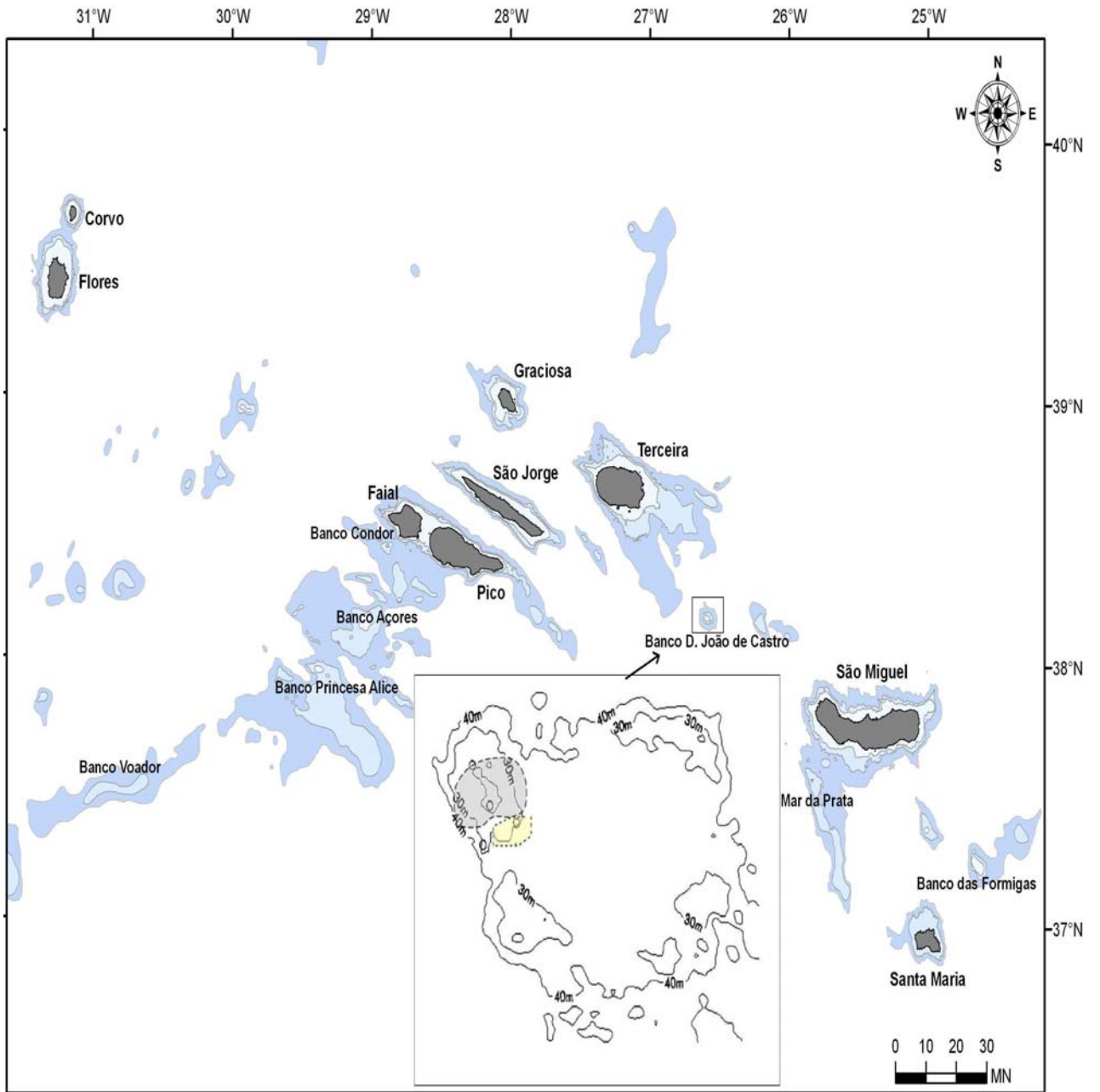


Fig. 2.

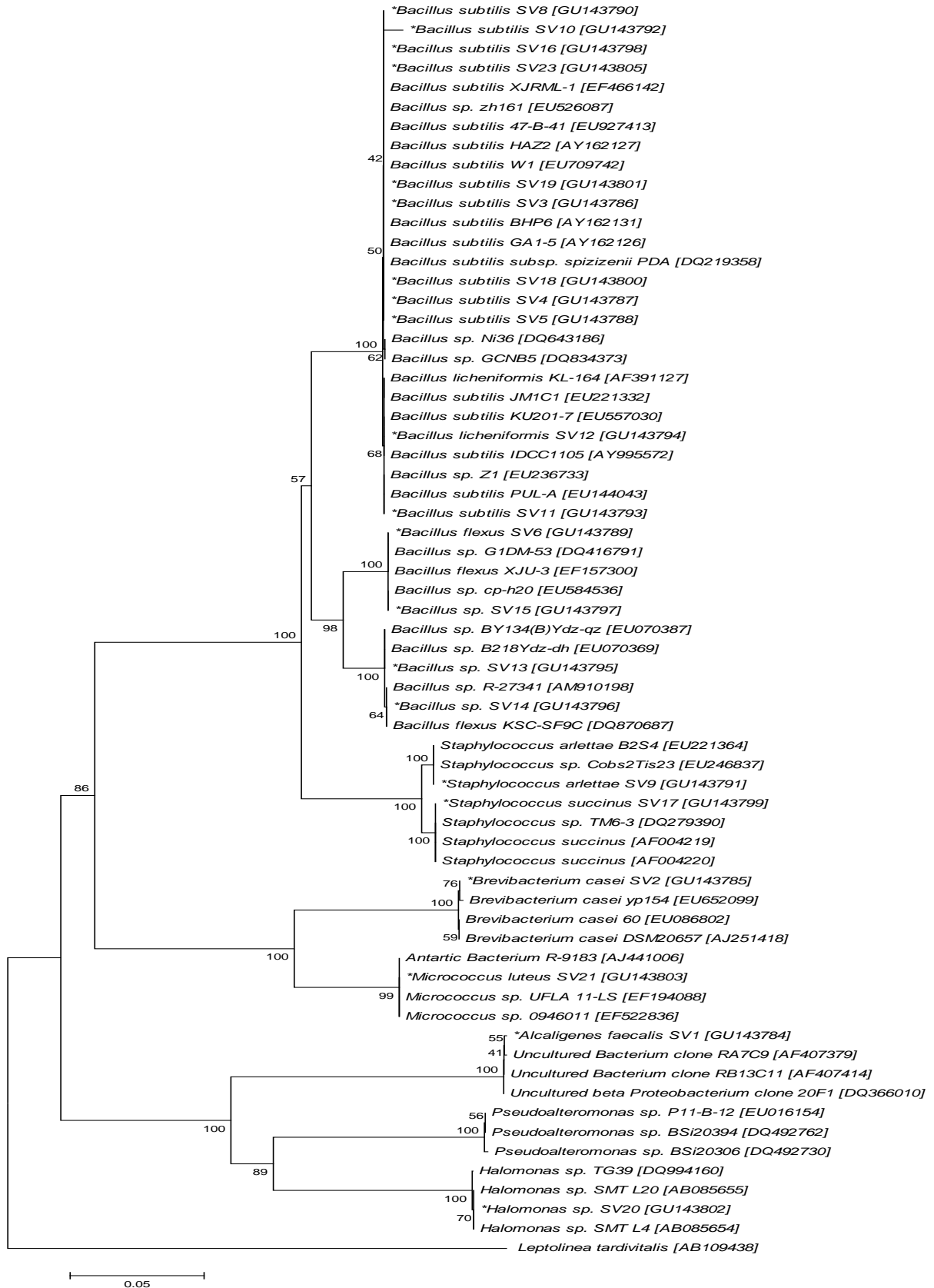


Fig. 3.

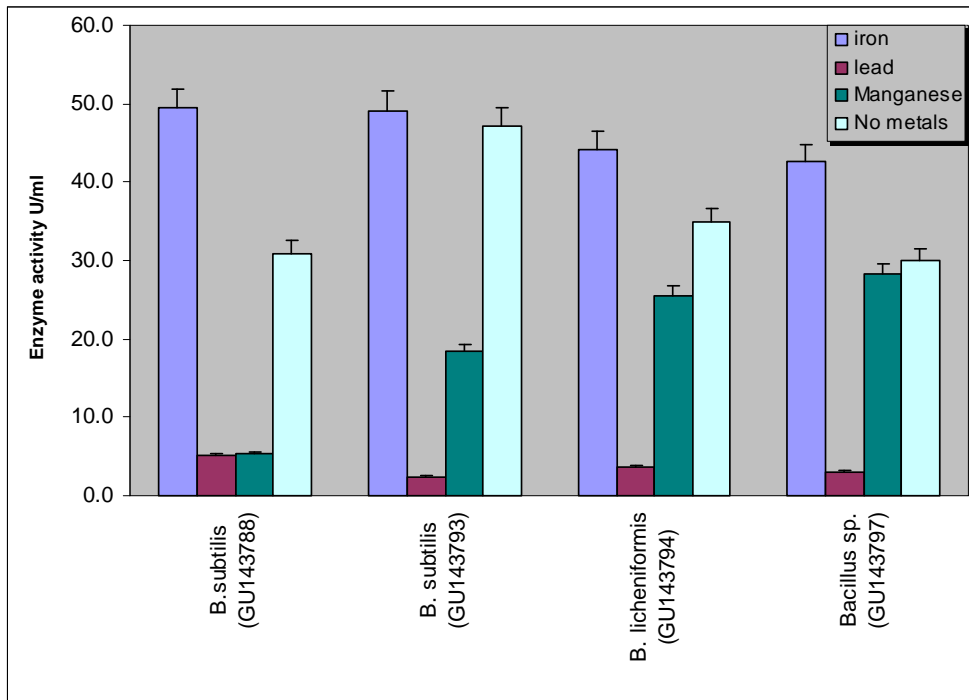


Fig. 4.

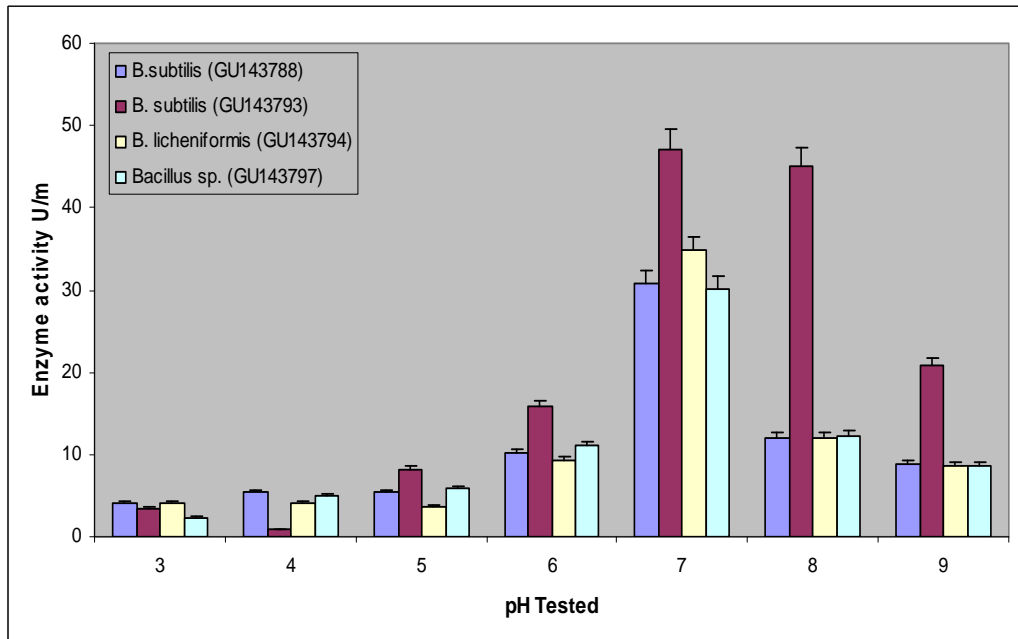


Fig. 5.

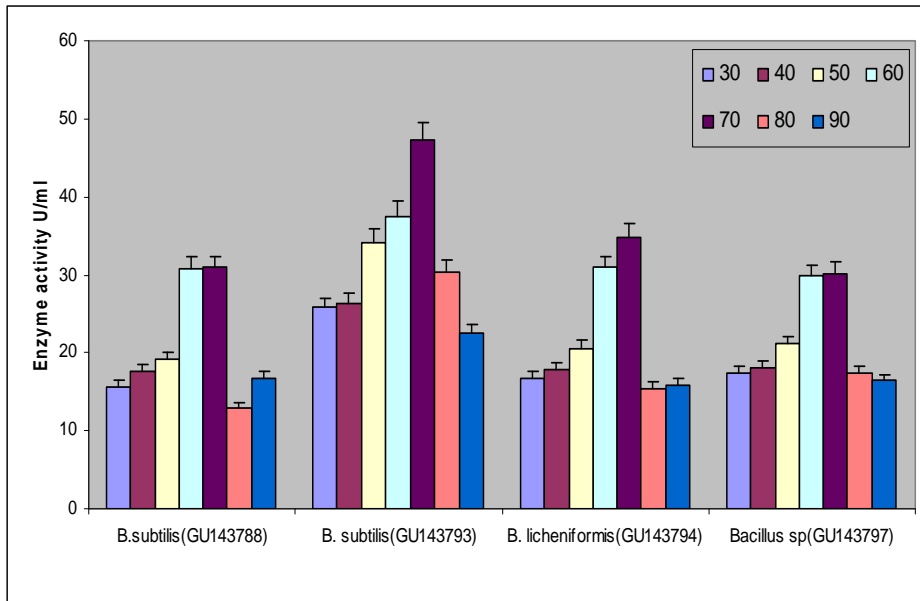
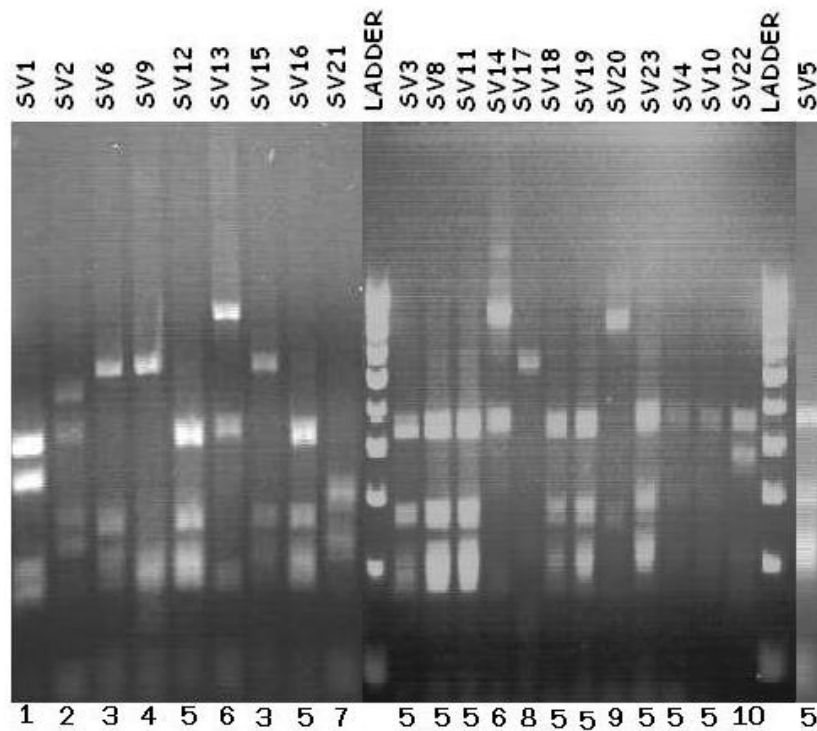


Fig. 6.



Legends to the Figures

Fig. 1. Sampling site of the shallow-water hydrothermal vent of the D. João de Castro Seamount (DJCS) in the Azores Islands, Portugal. The close up square in the picture is the enlargement of the DJCS vent with depth profile. The site lies between Terceira and São Miguel

Fig. 2. Neighbor-Joining tree based on evolutionary distances computed using Maximum Composite Likelihood method (Tamura et al., 2004) representing relationship between the 16S rDNA sequence of 21 bacterial isolates from DJCS. Bootstrap values generated from 500 replicates are shown at the nodes (Felsenstein, 1985); Bootstrap values above 40% are shown. *Leptollnea tartivitalls* AB109438 is used to assign an out group. Sequence <1000 bp is not included (SV22). Scale bar represents the 5% nucleotide substitution. Star indicates the bacteria isolated from DJCS

Fig. 3. Enzyme amylase activity on the selected vent strains while introducing the metals Fe, Pb and Mn in comparison with metal free enzyme production media

Fig. 4. pH effect on amylase activity on the selected vent strains

Fig. 5. Temperature effect on amylase activity on the selected vent strains

Fig. 6. Restriction patterns of PCR amplified 16S rRNA genes from the DJCS bacterial isolates digested with Alu I. Ladder used - 100 bp (Genei- Bangalore, India). Same number indicates similarity over the banding pattern.