Mytilus edulis associated bacteria Diversity and interactions based on bioactive molecules

Thesis

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Presented by

Asa Motiei

Kieler Wirkstoff-Zentrum (KiWiZ)

Helmholtz center for ocean research kiel (GEOMAR)

Christian-Albrechts-University of Kiel

Supervisor: Prof. Dr. Johannes F. Imhoff

Co-supervisor: Prof. Dr. Elena Gorokhova

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Summary

The marine environment is a habitat for many unique microorganisms, which produce biologically active compounds. This study was undertaken to survey culturable heterotrophic bacteria associated with *Mytilus edulis* as one of the most abundant cultured mussel in the Baltic Sea, Germany, with the purpose to find novel bioactive substances produced by these bacteria and also to test isolates for their antimicrobial activities. A total of 116 strains were selected to be investigated phylogenetically by 16S rRNA gene sequence analysis. Totally 14 genera belonging to 4 different classes including *Proteobacteria*, *Actinobacteria*, *Firmicutes* and *Bacteriodetes* were identified. The most bacteria were affiliated with *Gammaproteobacteria* and *Betaproteobacteria*, and a few also with *Actinobacteria*, *Firmcutes* and *Bacteriodetes*. 10 genera of Gram-negative bacteria including *Achromobacter*, *Pseudomonas*, *Shewanella*, *Enterobacter*, *Rahnella*, *Vibrio*, *Listonella*, *Aeromonas*, *Stenotrophomonas* and *Algoriphagus* and 4 genera of Gram-positive bacteria including, *Bacillus*, *Rhodococcus*, *Microbacterium* and *Agromyces* were detected. Bacterial strains affiliating to the genus *Pseudomonas, Bacillus and Microbacterium* were selected for further analysis in order to investigate their potential to produce bioactive substances. Three compounds assumed to be pyochelin, deoxyadenosin and 2, 4, 6-triacetylphloroglucinol produced by *Pseudomonas* sp. MB140 were dereplicated as well as heteroxanthin produced by *Bacillus* sp. MB028. The other substances produced by *Bacillus* sp. MB028 and *Microbacterium* sp. MB141, require further investigation and NMR analysis for identifying their chemical structure and properties. The most important antimicrobially active bacterium isolated from *Mytilus edulis*, was assigned to *Pseudomonas veronoii* (MB 140). The results of this study demonstrate that *Mytilus edulis* is a promising source for bacterial production of natural compounds.

INTRODUCTION

Introduction

Oceans cover more than 70% of earth‘s surface. They are complex ecosystems with an enormous diversity of life forms. Oceans bear almost unbelievably large diversity of microorganisms, with cell counts of 10^6- 10^9 cells per milliliter. Microorganisms inhabit all available niches from polar ice to hydrothermal vents, from the deep biosphere to mangrove forests and from oligotrophic open ocean waters to polluted coastal waters and sandy beaches (Imhoff et al., 2010). Microorganisms in marine ecosystem form a dominant biomass component and play an important and essential role in the marine food webs, biogeochemical cycles, metabolism, etc. The microbial diversity is presumed to be depicted in metabolic diversity, also resulting in a high potential for new bioactivities. Several studies show that bacterial communities associated with marine plants and organisms differ from the bacterial communities of surrounding seawater (Penesyan et al., 2009). Furthermore, there is evidence to support the involvement of host associated microorganisms in secondary metabolite production originally attributed to the host. Kobayashi and Ishibashi in their review (1993) provided several such examples which strongly suggest that microorganisms living in association with sponges are responsible for the production of many bioactive compounds. Unson and Faulkner (1994) isolated and separated a dominant prokaryotic endosymbiont the cyanobacterium *Oscillatoria spongeliae* in tropical Pacific Ocean from the sponge *Dysidea herbacea*. Using coupled gas chromatography—mass spectrometry and proton nuclear magnetic-resonance spectroscopy, they found that the major brominated compound isolated from the intact symbiotic association is found in the cyanobacterium and not in the sponge cells or heterotrophic bacteria. Also Schmidt et al., (2005) described patellamide A and C biosynthesis through a heterologous expression of the pathway, which was identified during a genome-sequencing project of the ascidian symbiont *Prochloron didemni*.

The exact relationship between microorganisms and their hosts are complex and usually poorly understood. Host organisms can provide a rich nutrient condition for their associated microbes, making them, attractive niches for several kinds of microbes and on the other hand leading a powerful competition between microbial communities. It has been hypothesized that the microbial partners construct chemical microenvironments with the eukaryotic host and live in syntrophy, participating in cycling of nutrients, as well

as preventing predation of the host via the production of bioactive molecules (Sharp et al. 2007). Thus, the production of bioactive molecules can be a result of interactions between hosts and their associated microorganisms as well as interactions among different microbial partners. Production of antibiotics by microbes can, for example, be a reaction to kill or slow down the growth of competitors and poisoning neighboring organisms (O'Brien & Wright 2011). In particular, the Gammaproteobacterium *Pseudoalteromonas tunicata*, known for the production of several bioactive compounds, is proposed to play a role in defending the host against surface colonisation by producing antimicrobial, antilarval and antiprotozoan compounds (Egans et al., 2001, 2002, 2006). Secondary metabolites, in contrast with primary metabolites, are produced by individual species and genera for specific physiological, social or predation reasons; therefore the production of bioactive compounds is strongly linked with the ecology of their producers (O'Brien & Wright 2011). The natural products isolated from marine organisms have been under investigation and focus in recent years. Discovered marine bioactive compounds are reviewed by Faulkner (1984-2002) and these studies are continued by Blunt et al. (2003-2013). Marine molluscs have become the focus of many chemical studies aimed at isolating and identifying novel natural compounds and the second largest molluscans class, the Bivalvia is relatively well presented in chemical studies (Benkendorff, K., 2009). In many cultures mussels feature in a range of tradition natural remedies; Prabhakar and Roy (2008) described the use of flesh of *Pila* and *Viviparous* sp. as medicine to cure asthma, swelling of joints, burns by aboriginal people of Kosi River Basin of North-Bihar in India. They are also used in Chinese traditional treatments (HU, 1980). Most of recent research in terms of natural compounds isolated from mussels is associated with sterols and bioaccumulated toxins responsible for paralytic shellfish poisoning (reviews by Bricelj & Shumway, 1998; Liewellyn, Negri &Robertsin, 2006; Cimminiello & Fattorusso, 2006). Also the involvement of antimicrobial peptids and proteins in humoral immunity of bivalves as chemical defense against pathogens was demonstrated recently (Mitta et al., 2000a, b; Cellura et al., 2007; Zhao et al., 2007; Li et al., 2009). Some interesting polyproniates and alkaloids (aromatic nitrogen compounds) have been also isolated from bivalves, although it is presently unclear if these play any defensive role. Despite the presence of shells, bivalves are soft bodied organisms which are sedentary filter feeders and often live in microbial rich habitats. Thus they are exposed to a large amount of microorganisms including pathogens and non-pathogens. Like all invertebrates, they don't have an acquired immunological memory (Sminia & Van Der Knaap, 1986. Hooper

et al, 2007) and also their innate immune system doesn't appear to have well-developed humoral component with biosynthesis of antimicrobial defense factors (Mitta et al., 2000a, b; Cellura et al., 2007; Zhao et al., 2007; Li et al., 2009). It can be assumed that they must have evolved alternative defense strategies to protect themselves against the microbial invasions. These protection mechanisms can involve production of bioactive substances as microbial secondary metabolites. Among microorganisms associated with marine organisms, bacteria and fungi associated with corals, sponges and algae are potent and well-studied producers of biologically active substances (Imhoff et al. 2011). However, not many studies have been undertaken on secondary metabolites and chemical structure of bacteria associated with bivalves. In a recent study, Romaneko et al. (2008), demonstrated antimicrobial activity among cultivated heterotrophic bacteria associated with the marine ark shell *Anadara broughtoni* in the Sea of Japan. In our study it has been focused on *Mytilus edulis*, a commercially important bivalve, which is grown in culture and thus is relatively abundant in supply for future investigations on chemical ecology and identification of natural compounds produced by associated microorganisms.

Aim of the thesis

The aim of this study is to characterize *Mytilus edulis* associated bacteria using a culture dependent method and 16S rRNA analysis. A further objective was to elucidate the potential of these bacteria to produce natural compounds by cultivation, extraction and analysis of the metabolite spectra. The effect of different cultivation conditions on the secondary metabolite production of the bacteria and the antimicrobial activity of selected strains against standard and environmental panels were also investigated.

MATERIAL AND METHODS

3.1. Sampling

All samples were collected from two different locations as described by Buer (2012): (1) a mussel and alga farm managed by, Coastal Research and Management Company (CRM) on the western shore of the Kiel Fjord and (2) a fish farm of Tassilo Jager-Klienicke on the eastern shore of the Kiel Fjord. (Figure 1)

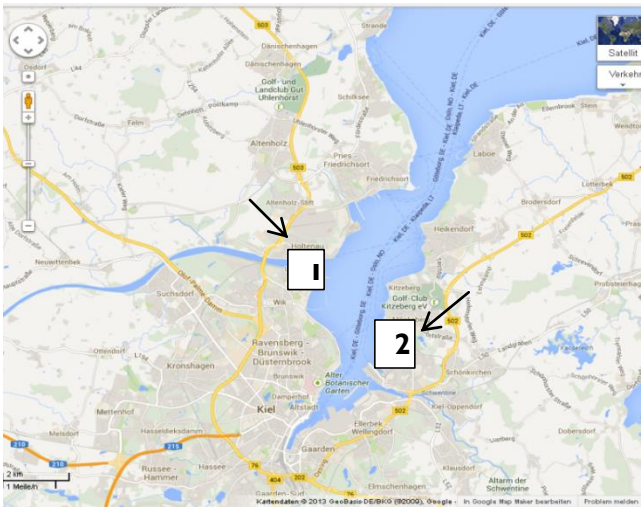


Figure 1: Sampling locations, 1- CRM and 2- Tassilo. (photo: Google Earth)

There were pilots installed to investigate the influence of coculture of the mussel (*Mytilus edulis*) and an alga (*Saccarina lattisma*) on microbial communities of these organisms (Buer, 2012). The experimental setup was consisting of four treatments, including only mussels, combination of mussel and alga at 2 different ratios and only the alga as control. Only the samples from the treatment of mussels were used in this study. All samples were taken under sterile conditions. All material and equipment for sampling was autoclaved for 20 min at 120°C. Sampling was carried out wearing disposal gloves and 70% ethanol was used for disinfection. To prepare mussel samples, the flesh was removed from the shells and transferred to a 50 ml falcon tube. An Ultra-Turrax was used to homogenize the samples. This suspension was used as stock solution of dilutions and 200 µl of different dilutions were pipetted to PCA plate (see Table 1) and spread. The plates were incubated at 28°C for 1-2 days and kept at 4°C in KiWiZ. Almost 9 month later on April 2013 we used these plates to obtain pure cultures by picking almost all colonies with different morphology and color and re-plating using the same medium as mentioned in Table 1.

3.2. Preparation of Medium

All ingredients mentioned in Table. 1 except agar were mixed using magnetic stirrer. Before adding agar, pH was measured by pH meter and adjusted to 7.0 with HCl or NaOH. The medium was autoclaved for 20 min at 120°C and transferred to different plates to be used for purification of the bacterial isolates. Each plate was labeled according to location and date of sampling (Figure 2).

Table 1: Non selective plate count agar (PCA) medium

Ingredients	g/L
Sodium Chloride	16
Peptone	5
Yeast (bacto)™	2.5
Glucose	1
Agar	15
Distilled Water	1 L

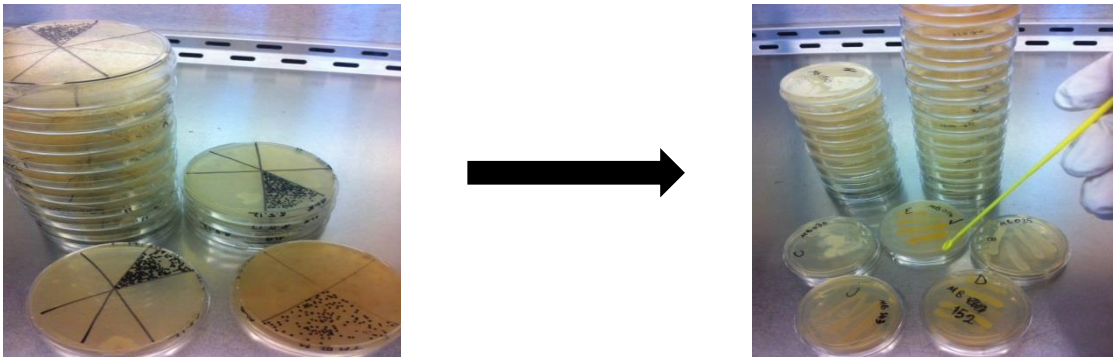


Figure 2: Making pure cultures from plates inoculated with environmental samples. Each plate was labeled according to date and location of sampling.

3.3. Storage and conservation

For storage, pure cultures were transferred to a mixture of 900 µl of liquid PCA medium (Table 1) and 100 µl DMSO, froze immediately at -180°C and also to the vials from the cryobank system according to the instructions of the manufacture (Mast Diagnostica GmbH, Reinfeld, Germany). After at least 1 hour, the liquid was removed from the cryobank vials and the cultures were frozen at -100°C.

3.4. DNA extraction and PCR

Each pure culture was inoculated in an Eppendorf vial containing100 µl of DNA-free water and froze for at least 1 hour at -20°C. A Biotherma Thermocycler was used to make a rapid

temperature changes from -20°C to 90°C for DNA extraction. Table 2 shows the temperature profile and condition to conduct polymerase chain reaction (PCR) using Biotherma Thermocycler. The samples were centrifuged afterward for 10 min, at 4°C, 8900 rpm. The supernatant was transferred in new Eppendorf vials. 1 µl of DNA extract was added to 24µl PCR master mix. The original concentration of primers was 100 pmol/µl and 1/10 fold dilution of original concentration was used for our experiment. The sequences for primers were 5'-GAG TTT GAT CCT GGC TCA-3' and 5'-GGT TAC CTT GTT ACG ACT T -3' for Eub27f and univ.1492r respectively (Table 3).

Table 2: Temperature profile of Biotherma thermocycler

	Temperature ° C	**Duration**
Denaturation	93	2 min
Amplification(30 cycles)	55 72 92	30 sec 30 sec 30sec
Last elongation	42 72	1 min 5 min
Cooling	15	

Table 3: PCR master mix

PCR master mix	**For each sample**	
DreamTaq™	12.5 µl	Fermentas, St Leon-Rot, Germany
Primer1 univ.1492r	1 µl	MWG- Biotech.AG, Germany
Primer2 Eub27f	1 µl	Metabion international AG, Germany
DNA free water	9,5 µl	Roth, Germany
Total volume	**24 µl**	

3.5. Electrophoresis

Electrophoresis was performed to verify the presence of PCR product. 1% agarose solution was made using 1x TBE buffer. Since agarose is normally not dissolved in TBE buffer, it needs to be heated frequently in microwave and shaken to dissolve and become a clear solution. Syber Safe DNA gel stain was added to agarose solution to intercalate the DNA and make the amplified fragments fluorescent under ultra violet light. This solution was placed in TBE buffer-filled box and used as electrophoresis gel when it was cool enough. A comb was used to make approximately 3 mm thick pockets in the gel and was

removed when the gel solidified. PCR products were centrifuged and 5µl of each sample was pipette to these pockets. Electrical field at 150V and 100 mA was applied for approximately 20 min in order to enable movement and separation of DNA fragments migrated toward the anode (Table 4). Also, 5 µl of DNA marker (Peglab, 1kb DNA-feiter, 0.5 µg/lane) was used in one of the pockets to provide a scale for estimating the size of amplified DNA fragments.

Table 4: Electrophoresis Gel

Substance	Amount	Ingredients
Agarose powder	0,4 g	
1x TBE Buffer	40 ml	189 mM TRIS Base 89 mM Boric acid 2 mM EDTA-Na_2
SYBR® Safe DNA gel stain	4 µl	

Ultra violet light was used to visualize the DNA fragments.

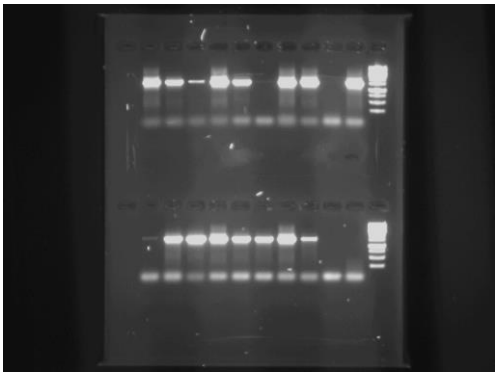


Figure 3: The efficiency of PCR was assessed by agarose gel electrophoresis

3.6. Sequencing

The samples which showed good fragments of DNA in electrophoresis were prepared for sequencing by dilution of PCR products. The amplified 16S rDNA- fragment has a length of approximately 1500 bp. The PCR products were diluted using 15, 20, 25 and 30 µl of DNA-free water, depending on the amount of product estimated as density of fragments and were pipetted to 96 well plates, centrifuged and frozen at -18°C until they were sent to Institute for clinic molecular biology (ikmb) at Christian Albrechts University of Kiel for sequencing. Each plate was named and an excel sheet was prepared for each plate to label

the place of each sample. The primers used for sequencing were 342f, 534r and 790f. Sequences were edited by ChromasPro v.1.33 (Technelysium Pty Ltd, Helensvale, Australia) and were compared to the NCBI database using BLAST (see http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al., 1990) and Ribosomal Database Project (RDP) sequence match (See http://rdp.cme.msu.edu) was used to determine the similarity level of species.

3.7. Cultivation of liquid cultures

The isolates with a good quality of sequences and also with more capability to produce natural compounds according to the previous studies, were selected for analysis of secondary metabolites profile. Strains were cultured on agar plate with the same recipes as mentioned in Table 1. They were incubated for 3- 4 days at 28°C in the dark. 1-cm^2 of each preculture was used for the cultivation of different liquid media as it is described in Table 5. All media were autoclaved before cultivation for 20 min at 120°C and inoculation was performed using clean bench. Three approaches using different scales were applied in this experiment, 100 ml, 1 L and 10 L. For each, 300 ml, 2 L and 10*2 L Erlenmeyer flasks were used, respectively, to make different liquid media. Selected pure cultures were inoculated in specific media (Table 5). The liquid cultures were incubated in the dark on shakers with 120 rpm at 28° C for 2 days.

The strains used for cultivation in 100 ml approach and the media in which they are cultivated are summarized in Table 5.

Table 5. Selected strains cultivated in different media

Strains	Medium
Bacillus sp.	GYM4, MB
-MB011	
-MB028	
-MB079	
Pseudomonas sp.	GYM4, GP
-MB034	
-MB140	
Microbacterium sp. MB141	GYM4, Oat Medium(OM)
Rhodococcus sp. MB056	GYM4, MB, M410
Rahnella sp. MB100	GYM4, MB
Shewanella sp. MB045	GYM4, MB

3.8. Extraction of liquid cultures (100 ml approach)

For HPLC analysis of the metabolite profiles and for antimicrobial assays, culture extracts of all selected strains were prepared. The entire culture broth (100 ml) was mixed with 100 ml of ethyl acetate and homogenized using Ultra-Turrax T25 basic disperser (IKA-Werke GmbH and Co., Staufen, Germany) at 13,000 rpm for 30 sec. The mixtures were transferred to a 500 ml separation funnel and remained until 2 separation phases were appeared. More ethyl acetate was added if phase separation didn't occur. The lower phase was discharged and afterward 50 ml distilled water was added to separation funnel and after shaking, the lower phase was discharged again. The upper phase was transferred to a rotary evaporator at 40°C and 150 rpm to get drained. The dried extract was resolved in methanol and transferred to HPLC-vial screw cap tubes using syringe filter with 0.2 µm PTFE membrane. The tubes were labeled and weighed when they were empty. After adding extract solution, they were dried under nitrogen again to get the weight of the dried extract. 1 mg/ml and 10 mg/ml solution in methanol were used for HPLC analysis and antimicrobial assays, respectively.

3.9. Chemical analysis of culture extracts

For analyzing the metabolite profile of the strains, the culture extracts were resolved in methanol to make 1 mg/ml solution and dereplication of substances was performed by comparison of mass spectra (MS) and UV data obtained by high-performance liquid chromatography (HPLC)-UV/Mass analysis. Reversed-phase HPLC experiments were performed using a Phenomenex Onyx Monolithic C_{18} column (100 by 3 .00 mm) and applying an H_2O (Solvent A)-acetonitrile (MeCN; Solvent B) gradient with 0.1% HCOOH added to both solvents (gradient program: 0 min, 5% solvent B; 4 min, 60% solvent B; 6 min, 100% solvent B; flow, 2 ml/min) on a VWR Hitachi Elite LaChrom system coupled to an electrospray ionization (ES1) ion trap detector (Esquire 4000; Brukar Daltonics). The results were compared with data from the Antibase (Laatsch, H. 2007) and the Chapman & Hall/CRC Chemical Database Dictionary of Natural Products (Backingham, J. 2009).

3.10. 1 liter approach

In this approach, the strains were selected from 100 ml approach according to their metabolite profile in HPLC chromatograms and also considering their bioactivity in antimicrobial test. For instance, *Microbacterium* sp. MB141 showed obviously better mass and UV spectra in HPLC analysis after growth in oat medium. There was no significant difference between the productions of natural compounds for *Bacillus* sp. MB028 in both media. There was no significant metabolite activity detected in HPLC chromatograms of *Bacillus* sp. MB011 and *Pseudomonas* sp. MB034. But they showed slight inhibition activity against some test strains in bioassays and therefore were selected for larger scale culture and extraction. Even if almost nothing was detected in *Rhodococcus* sp. MB056 HPLC chromatograms, it was selected for 1 liter approach since it is widely distributed in marine ecosystems and as far as known there is no bioactive metabolite described for it in literature. Therefore it was selected to have chance to detect metabolites by a 10 fold concentration. *Pseudomonas* sp. MB140 selected for next culture scale, due to its metabolite profile in HPLC analysis and also high inhibition activity against both environmental and standard panel in bioassays.

The strains and media in which they were grown are listed in Table 6.

Table 6. Strains and media for 1 liter approach

Strains	**Medium**
Bacillus sp.	
-MB011	GYM4
-MB028	MB
Pseudomonas sp.	
-MB034	GYM4
-MB140	GP
Microbacterium sp. MB141	Oat Medium(OM)
Rhodococcus sp. MB056	GYM4

In 1L approach, 2 L Erlenmeyer flasks were used for the bacterial culture and the same processes of extraction of liquid culture and chemical analysis of culture extracts as mentioned in 100 ml approach was performed. Just the extraction and chemical analysis were done for 3 days to detect the optimal time scale for optimal compound production. Every day 20 ml cultures were mixed and homogenized with 20 ml ethyl acetate using Ultra-Turrax T25 and when the separation occurred the upper phase was directly transferred to a 100 ml round flasks. Rotary evaporator was used to drain extract. The dried extract was resolved in 300 ml methanol and HPLC analysis was done. For final extraction, 500 ml ethyl acetate was used and homogenized mixture was transferred into 2 L separation funnel. The upper phase was discharged to 500 ml round flasks and drained using rotary evaporator.

3.11. 10 Liter approach

10 of 2 L Erlenmeyer flasks were used for bacterial culture which contain 1 L bacterial culture each and the rest of processes were the same as mentioned in100ml and 1 liter approach for extraction of liquid culture and chemical analysis of culture extract. Two strains were selected for 10 L approach including *Pseudomonas* sp. MB140 and *Bacillus* sp. MB028 in GP and MB medium, respectively. 2 cm^2 of preculture of each strain on agar plates used for inoculation of every 1 L medium.

3.12. Antimicrobial activities of culture extracts

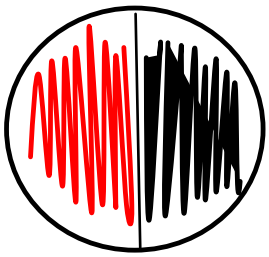
Antimicrobial activities were evaluated against the following standard panel including: *Bacillus subtilis* (DSM 347), *Escherchia coli* (DSM 498) and *Candida albicans* (DSM1386). All strains were obtained from the German Collection of Microorganisms and Cell Cultures, DSMZ, Braunschweig, Germany. Also, 3 different environmental bacteria from our samples were used: *Bacillus* sp. MB011, *Pseudomonas* sp. MB140 and *Shewanella* sp. MB045. The bioactivity tests were modified according to Schneemann et al. (2010). Briefly, 100 µl methanolic solutions of the extracts with concentration of 10 mg/ml were added into a 96-well microtiter plate and dried in a vacuum centrifuge. An overnight cultures of test organisms were prepared in different medium and diluted to OD_{600} of 0.01 to 0.03 as following: *Bacillus subtilis* and *Escherchia coli* in TSB medium with OD_{600} of 0.01, *Candida albicans* in M186/3 medium with OD_{630} of 0.03 and the environmental isolates in PCA liquid medium with OD_{600} 0f 0.02. These overnight cultures were added to the wells. After incubation time of 5 hours at 37°C, 200rpm, 10 µl of resazurin solution (0.2 mg/ml phosphate-buffered saline) was added to each well and plates were incubated at 28°C until the color of negative control changed from blue to pink. The chloramphenicol solution was used as positive control and bacterial cells without extracts as negative controls. For evaluation of cell viability, transformation of resazurin to resorufin was assayed by measuring the fluorescence at 560 nm after excitation at 590 nm. The data were compared to optical density measurements before incubation with bacterial cells and also with negative and positive controls on the same plate. The natural absorbance of the extract fractions was corrected by subtracting initial extract-only blank values from values obtained for treatments according to Lane et al. (2009). The tests were performed in 3 replicates.

3.13. Co-cultivation

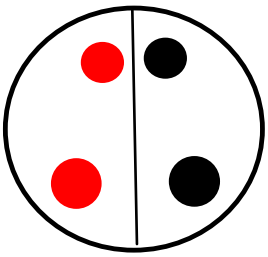
9 strains were selected for co-cultivation: *Pseudomonas* sp. MB034 and MB140, *Bacillus* sp. MB011, MB028 and MB079, *Microbacterium* sp. MB141, *Rahnella* sp. MB100, *Rhodococcus* sp. MB056 and *Shewanella* sp. MB045. The strains' stocks on PCA plates (Table 1) were used to make co-cultivation on new PCA plates. Co-cultivation was performed using *Pseudomonas* sp. MB140, *Bacillus* sp. MB011 and *Shewanella* sp. MB045

against all the other strains mentioned above. Two methods were used for co-cultivation. In the first one, each strain was cultured and full streaked on half of the each plate (Figure 4). Another method was point (dotted) culture, in which two colonies of each strain were inoculated on agar plates in close and far distances (Figure 5). The bacterial inoculation was performed using sterilized microbial loop and clean bench. After incubation for 2-3 days at 28 °C in the dark, the cultures were checked for inhibition activity zone with observation of agar plates to see if growth of bacteria is inhibited by another strain or not and also for morphological changes including changes in form, mobility and size of cells, using microscope Axioscop 40 (Zeiss, Germany). To prepare the samples, one drop of distilled water was put on a slide and inoculated with a small amount of co-cultivated bacterial sample. The samples were taken from both close proximity and distally from another strain. The samples were covered by cover slip and the bubbles under cover slip were removed. Three magnifications were used to adjust the observation of samples under microscope, 10x, 40x and 100x. A drop of oil on cover slip was used for magnification of 100x to prevent refraction.

Figure 4 and 5: Different methods of bacterial culture for co-cultivation



(4): Full streaked Culture



(5): Dotted form culture

3.14. Extraction of co-cultured bacteria:

The plates with full streaked cultures were used for extraction to analyze their HPLC chromatograms in order to investigate the secondary metabolites of co-cultivated bacteria. The middle part of plates to a width of 4 cm, were cut and transferred to 250 ml bottles. 100 ml ethyl acetate were added to the bottles and after that Ultra Turrax was done for 1-2 min. Then the liquid part was discharged directly to rotary evaporator at 40° C and 150 rpm to get drained. The dried extract was resolved in methanol and transferred to HPLC-vial screw cap tubes using syringe filter with 0.2 µm PTFE membrane. The tubes

were labeled and weighed when they were empty. The solution in these tubes got dried under nitrogen again to get the weight of dried extract to make 1 mg/ml solution in methanol for HPLC analysis.

RESULTS

4.1. Isolation and characterization of bacteria associated with *Mytilus edulis*

16S rDNA sequences of 116 bacterial strains were classified to species level using NCBI Blast and RDP sequence match. The majority of strains were affiliated to the class *Proteobacteria* (75%) (87 of 116) which also showed the highest diversity (9 genera). The next most abundant were isolates belonging to *Actinobacteria* (15% of all strains), while *Firmicutes* and *Bacteroidetes* were noticeably less than the other two groups (9% and 1% of all isolates). *Bacillus* sp. and *Algoriphagus* sp. are representatives of *Firmicutes* and *Bacteriodetes*, respectively. *Achromobacter* was the most dominant group in *Proteobacteria* (32% of *Proteobacteria*) and the only genus belonging to *Betaproteobacteria.* *Shewanella*, *Pseudomonas* and *Stenotrophomonas* were predominant in *Gammaproteobacteria* (15%, 13% and 13% respectively). Members of *Aeromonas*, *Rahnella* and *Enterobacter* represented less than 13% of all isolates. *Microbacterium* was predominant among *Actinobacteria* (82% of *Actinobacteria*) while *Rhodococcus* and *Agromyces* represented 12% and 6%, respectively. Figure 6- 9 show the classification of strains derived from *Mytilus edulis.* Strains with close relationship to known species are summarized in Table 7.

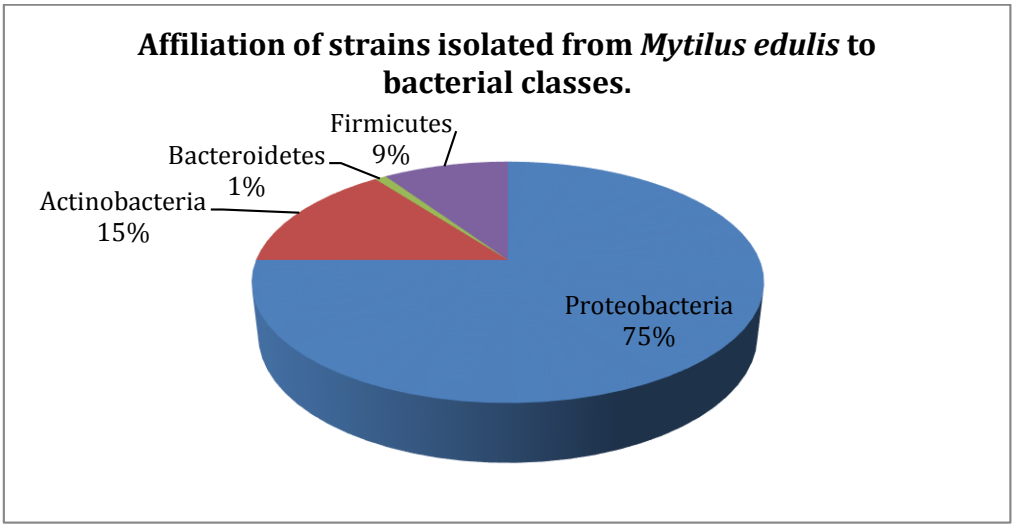


Figure 6: Diversity of bacteria isolated from *Mytilus edulis*

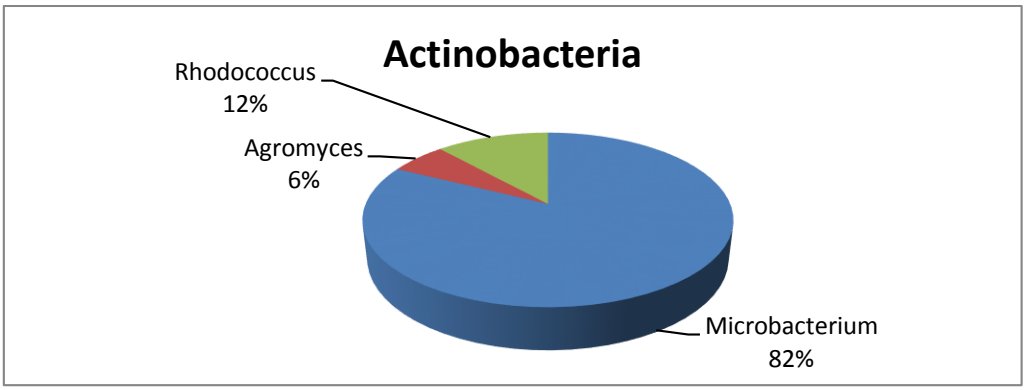


Figure 7: Affiliation of strains to genera of the class *Actinobacteria*

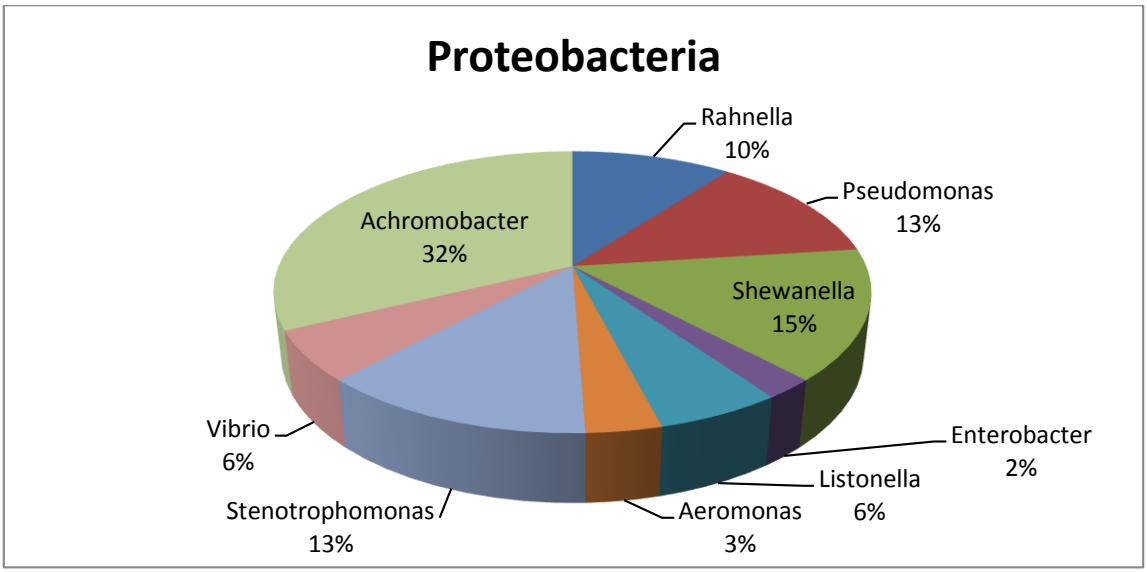


Figure 8: Affiliation of strains to genera of the class *Proteobacteria* (percentage)

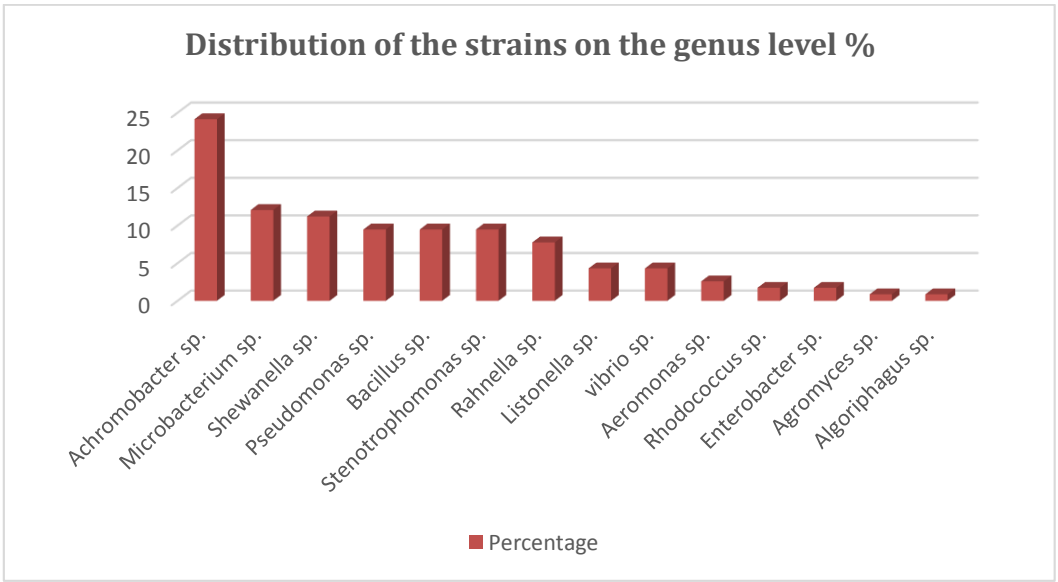


Figure 9: Distribution of the strains on genus level associated with *Mytilus edulis*.

Table 7: Next related validly described type strains to the isolates

Isolates	Length (bp)	Next related type strain	Strain number	Accession number	Similarity (%)	Location and date
Betaproteobacterium						
MB095	798	*Achromobacter denitrificans*	DSM 30026^T	AJ278451	91,0	CRM,11/06/2012
MB102	911	*Achromobacter denitrificans*	DSM 30026^T	AJ278451	95,2	CRM,11/06/2012
MB092	1443	*Achromobacter denitrificans*	DSM 30026^T	AJ278451	95,2	CRM,11/06/2012
MB105	798	*Achromobacter denitrificans*	DSM 30026^T	AJ278451	91,0	CRM,11/06/2012
MB131	1405	*Achromobacter denitrificans*	DSM 30026^T	AJ278451	94,9	CRM,11/06/2012
MB154	920	*Achromobacter denitrificans*	DSM 30026^T	AJ278451	86,8	Tassilo,11/06/2012
MB155	1296	*Achromobacter denitrificans*	DSM 30026^T	AJ278451	82,9	Tassilo,11/06/2012
MB001	1395	*Achromobacter piechaudii*	EY3860 = ATCC43552^T	AB010841	98,6	CRM, 07/05/2012
MB044	1429	*Achromobacter piechaudii*	EY3860 = ATCC43552^T	AB010841	98,6	Tassilo,07/05/2012
MB122	1450	*Achromobacter piechaudii*	EY3860 = ATCC43552^T	AB010841	98,8	CRM,11/06/2012
MB031	1399	*Achromobacter piechaudii*	EY3860 = ATCC43552^T	AB010841	98,7	CRM, 07/05/2012
MB008	1432	*Achromobacter spanius*	LMG 5911^T	AY170848	99,6	CRM, 07/05/2012
MB054	1483	*Achromobacter spanius*	LMG 5911^T	AY170848	98,6	Tassilo,07/05/2012
MB057	1429	*Achromobacter spanius*	LMG 5911^T	AY170848	99,2	Tassilo,07/05/2012
MB061	1492	*Achromobacter spanius*	LMG 5911^T	AY170848	98,8	Tassilo,07/05/2012
MB085	903	*Achromobacter spanius*	LMG 5911^T	AY170848	88,8	CRM,11/06/2012
MB003	1414	*Achromobacter spanius*	EY3860 = ATCC43552^T	AY170848	99,5	CRM, 07/05/2012
MB005	1413	*Achromobacter spanius*	LMG 5911^T	AY170848	99,5	CRM, 07/05/2012
MB032	1492	*Achromobacter spanius*	LMG 5911^T	AY170848	98,6	CRM, 07/05/2012
MB033	1412	*Achromobacter spanius*	LMG 5911^T	AY170848	99,1	CRM, 07/05/2012
MB046	1433	*Achromobacter spanius*	LMG 5911^T	AY170848	99,6	Tassilo,07/05/2012
MB064	1431	*Achromobacter spanius*	LMG 5911^T	AY170848	99,0	Tassilo,07/05/2012
MB088	1382	*Achromobacter spanius*	LMG 5911^T	AY170848	99,0	CRM,11/06/2012
MB152	1494	*Achromobacter spanius*	LMG 5911^T	AY170848	99,6	Tassilo,07/05/2012
MB159	1441	*Achromobacter spanius*	LMG 5911^T	AY170848	94,6	Tassilo,11/06/2012
MB020	1413	*Achromobacter spanius*	LMG 5911^T	AY170848	99,6	CRM, 07/05/2012
MB023	1422	*Achromobacter spanius*	LMG 5911^T	AY170848	98,8	CRM, 07/05/2012
MB144	1409	*Achromobacter spanius*	LMG 5911^T	AY170848	96,6	Tassilo,11/06/2012
Gammaproteobacterium						
MB118	1374	*Shewanella algidipiscicola*	S13 ^{T}	AB205570	99,0	CRM,11/06/2012
MB013	1391	*Shewanella baltica*	NCTC 10735^T	AJ000214	98,4	CRM, 07/05/2012
MB043	1299	*Shewanella baltica*	NCTC 10735^T	AJ000214	97,5	Tassilo,07/05/2012
MB083	1233	*Shewanella baltica*	NCTC 10735^T	AJ000214	91,6	CRM,11/06/2012
MB096	811	*Shewanella baltica*	NCTC 10735^T	AJ000214	98,1	CRM,11/06/2012
MB010	1492	*Shewanella baltica*	NCTC 10735^T	AJ000214	98,5	CRM, 07/05/2012
MB086	1510	*Shewanella baltica*	NCTC 10735^T	AJ000214	98,5	CRM,11/06/2012
MB106	1511	*Shewanella baltica*	NCTC 10735^T	AJ000214	98,1	CRM,11/06/2012
MB045	1420	*Shewanella baltica*	NCTC 10735^T	AJ000214	98,2	Tassilo,07/05/2012
MB153	1516	*Shewanella baltica*	NCTC 10735^T	AJ000214	97,9	CRM,07/05/2012
MB017	1499	*Shewanella baltica*	NCTC 10735^T	AJ000214	98,7	CRM, 07/05/2012
MB166	1505	*Shewanella colwelliana*	ATCC 39565^T	AY653177	99,8	Tassilo,11/06/2012
MB026	1504	*Shewanella vesiculosa*	M7^T	AM980877	97,1	CRM, 07/05/2012

Table 7: Continued

Isolates	Length (bp)	Next related type strain	Strain number	Accession number	Similarity (%)	Location and date
Gammaproteobacterium						
MB075	946	*Stenotrophomonas maltophilia*	IAM 12423^T	AB294553	96,8	CRM,11/06/2012
MB090	1261	*Stenotrophomonas maltophilia*	IAM 12423^T	AB294553	93,1	CRM,11/06/2012
MB098	1474	*Stenotrophomonas maltophilia*	IAM 12423^T	AB294553	99,1	CRM,11/06/2012
MB113	1339	*Stenotrophomonas maltophilia*	IAM 12423^T	AB294553	93,8	CRM,11/06/2012
MB119	1425	*Stenotrophomonas maltophilia*	IAM 12423^T	AB294553	99,3	CRM,11/06/2012
MB120	1325	*Stenotrophomonas maltophilia*	IAM 12423^T	AB294553	98,3	CRM,11/06/2012
MB133	892	*Stenotrophomonas maltophilia*	IAM 12423^T	AB294553	93,7	CRM,11/06/2012
MB021	1357	*Stenotrophomonas maltophilia*	IAM 12423^T	AB294553	99,0	CRM, 07/05/2012
MB072	1413	*Stenotrophomonas maltophilia*	IAM 12423^T	AB294553	98,9	Tassilo,07/05/2012
MB074	1391	*Stenotrophomonas maltophilia*	IAM 12423^T	AB294553	96,2	CRM,11/06/2012
MB077	1392	*Stenotrophomonas maltophilia*	IAM 12423^T	AB294553	99,1	CRM,11/06/2012
MB132	802	*Stenotrophomonas maltophilia*	IAM 12423^T	AB294553	91,5	CRM,11/06/2012
MB040	1325	*Pseudomonas cuatrocienegasensis*	1N^T	EU791281	97,6	Tassilo,07/05/2012
MB006	1371	*Pseudomonas guineae*	LMG 24016^T = M8	AM491810	98,6	CRM, 07/05/2012
MB009	1424	*Pseudomonas guineae*	LMG 24016^T = M8	AM491810	99,3	CRM, 07/05/2012
MB047	1013	*Pseudomonas guineae*	LMG 24016^T = M8	AM491810	91,3	CRM,07/05/2012
MB012	1445	*Pseudomonas jessenii*	CIP 105274^T	AF068259	92,9	CRM, 07/05/2012
MB070	1094	*Pseudomonas rhodesiae*	CIP 104664^T	AF064459	97,6	Tassilo,11/06/2012
MB128	1499	*Pseudomonas veronii*	CIP 104663^T	AF064460	99,9	CRM,11/06/2012
MB140	1507	*Pseudomonas veronii*	CIP 104663^T	AF064460	99,1	Tassilo,11/06/2012
MB007	1485	*Pseudomonas veronii*	CIP 104663^T	AF064460	99,7	CRM, 07/05/2012
MB027	1358	*Pseudomonas xanthomarina*	KMM 1447^T	AB176954	97,8	CRM, 07/05/2012
MB034	1384	*Pseudomonas xanthomarina*	KMM 1447^T	AB176954	97,3	CRM, 07/05/2012
MB065	1441	*Rahnella aquatilis*	DSM 4594^T	AJ233426	97,8	Tassilo,07/05/2012
MB100	1506	*Rahnella aquatilis*	DSM 4594^T	AJ233426	98,5	CRM,11/06/2012
MB093	1506	*Rahnella aquatilis*	DSM 4594^T	AJ233426	99,3	CRM,11/06/2012
MB103	1506	*Rahnella aquatilis*	DSM 4594^T	AJ233426	99,3	CRM,11/06/2012
MB129	1492	*Rahnella aquatilis*	DSM 4594^T	AJ233426	98,6	CRM,11/06/2012
MB138	1476	*Rahnella aquatilis*	DSM 4594^T	AJ233426	99,1	Tassilo,11/06/2012
MB142	1506	*Rahnella aquatilis*	DSM 4594^T	AJ233426	99,0	Tassilo,11/06/2012
MB160	1478	*Rahnella aquatilis*	DSM 4594^T	AJ233426	98,9	Tassilo,11/06/2012
MB163	1310	*Rahnella aquatilis*	DSM 4594^T	AJ233426	92,9	Tassilo,11/06/2012
MB068	1471	*Aeromonas piscicola*	S1.2^T	FM999971	99,3	Tassilo,07/05/2012
MB018	1379	*Aeromonas salmonicida*	JCM7873^T	AB027542	99,7	CRM, 07/05/2012
MB165	1509	*Aeromonas salmonicida*	JCM7873^T	AB027542	99,8	Tassilo,07/05/2012
MB117	1406	*Listonella anguillarum*	NCIMB 6^T	AM235737	98,5	CRM,11/06/2012
MB127	1503	*Listonella anguillarum*	NCIMB 6^T	AM235737	98,7	CRM,11/06/2012
MB123	1393	*Listonella anguillarum*	NCIMB 6^T	AM235737	98,9	CRM,11/06/2012
MB124	1507	*Listonella anguillarum*	NCIMB 6^T	AM235737	99,3	CRM,11/06/2012
MB091	884	*Listonella anguillarum*	NCIMB 6^T	AM235737	88,0	CRM,11/06/2012
MB067	1157	*Vibrio cyclitrophicus*	LMG 21359^T	AM162656	95,9	Tassilo,07/05/2012
MB109	876	*Vibrio diazotrophicus*	ATCC 33466^T	X74701	96,4	CRM,11/06/2012
MB110	908	*Vibrio ordalii*	ATCC 33509^T	X74718	99,1	CRM,11/06/2012
MB114	827	*Vibrio ordalii*	ATCC 33509^T	X74718	90,1	CRM,11/06/2012
MB115	889	*Vibrio ordalii*	ATCC 33509^T	X74718	82,5	CRM,11/06/2012

Table 7: Continued

Isolates	Length (bp)	Next related type strain	Strain number	Accession number	Similarity (%)	Location and date
Gammaproteobacterium						
MB015	1484	*Enterobacter amnigenus*	JCM1237^T	AB004749	99,5	CRM,07/05/2012
MB082	1356	*Enterobacter ludwigii*	EN-119^T = DSMZ 16688^T= CIP 108491^T;			CRM,11/06/2012
Actinobacteria						
MB002	1248	*Microbacterium maritypicum*	DSM 12512^T	AJ853910	99,8	CRM,07/05/2012
MB076	1403	*Microbacterium maritypicum*	DSM 12512^T	AJ853910	99,1	CRM,11/06/2012
MB104	1434	*Microbacterium maritypicum*	DSM 12512^T	AJ853910	99,9	CRM,11/06/2012
MB146	1448	*Microbacterium maritypicum*	DSM 12512^T	AJ853910	98,5	Tassilo,11/06/2012
MB019	1166	*Microbacterium maritypicum*	DSM 12512^T	AJ853910	99,7	CRM, 07/05/2012
MB038	1399	*Microbacterium maritypicum*	DSM 12512^T	AJ853910	99,4	Tassilo,07/05/2012
MB094	780	*Microbacterium maritypicum*	DSM 12512^T	AJ853910	99,9	CRM,11/06/2012
MB101	1488	*Microbacterium maritypicum*	DSM 12512^T	AJ853910	99,4	CRM,07/05/2012
MB141	1479	*Microbacterium maritypicum*	DSM 12512^T	AJ853910	99,5	Tassilo,11/06/2012
MB161	1473	*Microbacterium maritypicum*	DSM 12512^T	AJ853910	98,7	Tassilo,11/06/2012
MB022	1490	*Microbacterium maritypicum*	DSM 12512^T	AJ853910	99,2	CRM, 07/05/2012
MB089	1493	*Microbacterium oxydans*	DSM 20578^T	Y17227	99,1	Tassilo,07/05/2012
MB004	1463	*Microbacterium paraoxydans*	CF36^T	AJ491806	99,7	CRM, 07/05/2012
MB136	1320	*Microbacterium paraoxydans*	CF36^T	AJ491806	97,4	Tassilo,11/06/2012
MB041	1389	*Rhodococcus qingshengii*	djl-6^T	DQ090961	88,4	Tassilo,07/05/2012
MB056	1351	*Rhodococcus qingshengii*	djl-6^T	DQ090961	99,9	Tassilo,07/05/2012
MB024	1468	*Agromyces mediolanus*	DSM 20152^T	X77449	99,3	CRM, 07/05/2012
Firmicutes						
MB037	1503	*Bacillus anthracis*	ATCC 14578^T	AB190217	99,4	Tassilo,07/05/2012
MB028	1425	*Bacillus cereus*	ATCC 14579^T	AE016877	99,8	CRM,07/05/2012
MB053	1438	*Bacillus cereus*	ATCC 14579^T	AE016877	99,2	Tassilo,07/05/2012
MB170	1483	*Bacillus cereus*	ATCC 14579^T	AE016877	99,8	Tassilo,11/06/2012
MB030	1489	*Bacillus cereus*	ATCC 14579^T	AE016877	99,6	CRM, 07/05/2012
MB051	1522	*Bacillus cereus*	ATCC 14579^T	AE016877	98,0	Tassilo,07/05/2012
MB011	1481	*Bacillus pumilus*	ATCC 7061^T	AY876289	99,7	CRM, 07/05/2012
MB079	1298	*Bacillus weihenstephanensis*	DSM11821^T	AB021199	98,6	CRM,07/05/2012
MB039	1357	*Bacillus weihenstephanensis*	DSM11821^T	AB021199	98,3	Tassilo,07/05/2012
MB048	1514	*Bacillus weihenstephanensis*	DSM11821^T	AB021199	99,9	Tassilo,07/05/2012
MB139	1506	*Bacillus weihenstephanensis*	DSM11821^T	AB021199	99,7	Tassilo,11/06/2012
Bacteroidetes						
MB134	1485	*Algoriphagus winogradskyi*	LMG 21969 ^{T}	AJ575263	99,5	Tassilo,07/05/2012

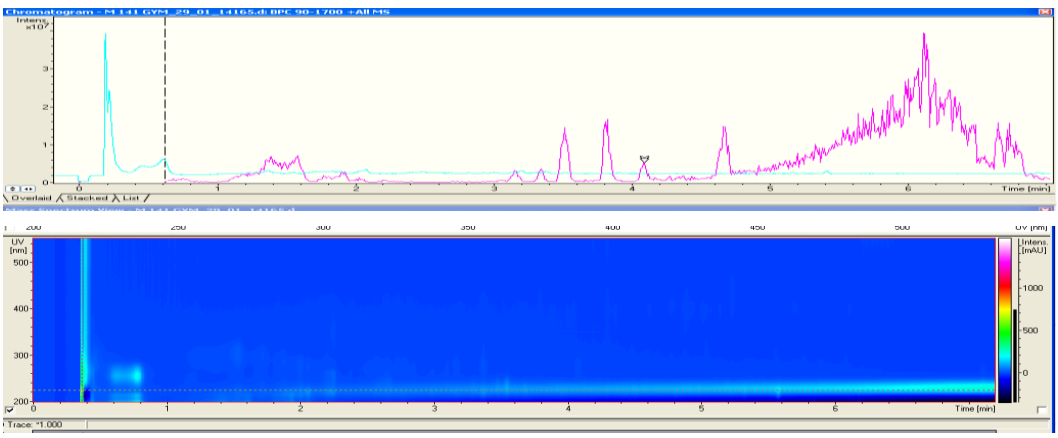
4.2. Comparison of the production of compounds in different media

In the 100 ml approach, the first screening of compound production in several bacterial strains isolated from *Mytilus edulis* was performed. Also, the production of compounds in different media was compared. To compare the production status of each strain in different media, HPLC chromatograms were analyzed and mass peaks in a time range between 2 and around 6 min and also with intensity of more than $0.2*10^7$ were counted (Table 8).

By counting the number of mass peaks produced by each strain and more importantly considering their UV signals, a suggestion could be made about the efficiency of different media for production of natural compounds. Some specific media were considerably better for culture of some strains in order to achieve bioactive metabolites; such as Gym4 for *Bacillus* sp. MB011 and MB079 and also for *Pseudomonas* sp. MB034 than MB medium. Interestingly, *Bacillus* sp. MB028 had almost the same profile for mass chromatogram and UV spectra in both media. *Pseudomonas* sp. MB140 showed better natural compound production in GP medium while the culture of *Microbacterium* sp. MB141 was more effective in OM medium (Figure 10.2). No UV signals could be seen in secondary metabolite profiles of *Microbacterium* sp. MB141 in GYM4 (Figure 10.1). No metabolite was detected in extracts of *Shewanella* sp. MB010 and MB045, *Rahnella* sp. MB100 and *Rhodococcus* sp. MB056.

Figure 10: Comparison of secondary metabolite profiles in GYM4 and OM medium for *Microbacterium* sp. MB141. (1) Mass and UV chromatogram in GYM4; (2) Mass and UV chromatogram in OM

(1) Mass and UV spectra of *Microbacterium* sp. MB141 extract in GYM4 medium



(2) Mass and UV spectra of *Microbacterium* sp. MB141 extract in oat (OM) medium

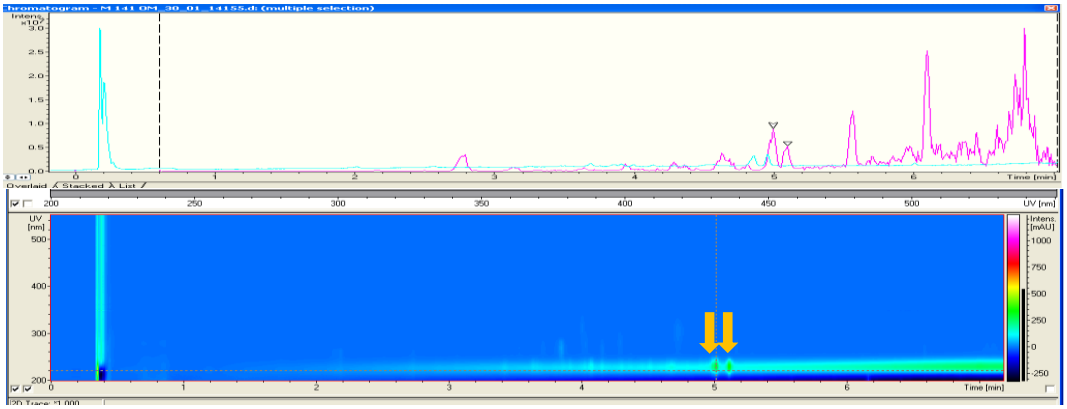


Table 8: Mass peaks and UV spectra of culture extracts

Strain	Medium	No. of mass peaks	Mass	Intensity	UV spectra
Bacillus sp. MB079	GYM4	1 1	197.2 211.2	~0.85*10^7 ~0.35*10^7	- -
Bacillus sp. MB079	MB	-		-	-
Bacillus sp. MB028	GYM4	1 1 1 1 1 1	600.4 713.5 681.4 699.4 695.4 713.5	~1.8*10^8 ~0.5*10^8 ~0.5*10^8 ~1.9*10^8 ~1.9*10^8 ~3.6*10^8	225-280-290-311 225-280-290-311 225-280-290-308 225-284-320 225-280-290-308 225-280-290-311
Bacillus sp. MB028	MB	1 1 1 1 1 1 1	681.5 727.5 281.2 713.5 695.5 600.4 713.5	~0.25*10^8 ~0.25*10^8 ~0.25*10^8 ~0.6*10^8 ~1*10^8 ~1.8*10^8 ~3.6*10^8	280-290 - - 225-280-290-311 225-280-290-308 225-280-290-308 225-280-290-311
Bacillus sp. MB011	GYM4	1 1 1	423.2 438.2 444.3	~2.8*10^7 ~2.8*10^7 ~0.5*10^7	215-315-350 215-315-350 -
Bacillus sp. MB011	MB	1 1 1	406.1 423.2 438.2	~1*10^7 ~1.5*10^7 ~2*10^7	225-314-370 225-314-370 225-314-370
Pseudomonas sp. MB034	GYM4	1 1 2 1 1 1 1 1	269.2 318.2 278.2 298.2 453.3 315.2 291.2 479.3	~0.6*107 ~0.6*107 ~0.8*107 ~1*10^7 ~1.4*10^7 ~1.6*10^7 ~4.6*10^7 ~5*10^7	- - - - 225-400-432 - - -
Pseudomonas sp. MB034	GP	1 1 1	253.2 279.2 281.3	~1*10^7 ~1.2*10^7 ~6*10^7	- - -
Pseudomonas sp. MB140	GYM4	2 1 1 1 1	234.2 253.2 255.2 207.2 241.2	~1*10^7 ~1*10^7 ~1.8*10^7 ~2*10^7 ~3*10^7	- - - - 225-275-330

Table 8: continued

Strain	Medium	No. of mass peaks	Mass	Intensity	UV spectra
Pseudomonas sp. MB140		1 1 1	213.2 281.2 281.2	~5*10^7 ~6*10^7 ~6.8*10^7	- - -
Pseudomonas sp. MB140	GP	1 1 1 1 1 1 1	417.3 419.3 225.2 324.1 227.2 252.1 251.2	~1.5*10^7 ~2*10^7 ~2*10^7 ~4.5*10^7 ~5.8*10^7 ~7*10^7 ~8.8*10^7	- - - 220-254-316-360 230-312-350 228-335-360-397 228-335-360-397
Shewanella sp. MB010	GYM4	-			-
Shewanella sp. MB010	MB	-			-
Shewanella sp. MB045	GYM4	1 1 1 1	453.3 479.3 451.2 281.3	~0.5*10^7 ~0.5*10^7 ~1.5*10^7 ~2.8*10^7	- - 227, 280 -
Shewanella sp. MB045	MB	-			-
Microbacterium sp. MB141	GYM4	1 1 1 1	556.2 439.2 634.3 728.3	~1.6*10^7 ~1.6*10^7 ~1.8*10^7 ~0.5*10^7	225-290 - - -
Microbacterium sp. MB141	OM	2	297.2	~1*10^7	230-263
Rahnella sp. MB100	GYM4	-	-	-	-
Rahnella sp. MB100	MB	-	-	-	-
Rhodococcus sp. MB056	GYM4	-	-	-	-
Rhodococcus sp. MB056	MB	-	-	-	-
Rhodococcus sp. MB056	M410	-	-	-	-

4.3. Chemical analyses of culture extracts

The chemical analyses of secondary metabolites of strains revealed different metabolic profiles even for closely related strains. Three isolates showed exceptionally similar and biotechnologically attractive metabolite profile within the screening in both 100ml and 1 L approach and were selected for detailed investigation, including *Bacillus* sp. MB028, *Pseudomonas* sp. MB140 and *Microbacterium s*p. MB141. Dereplication of the produced compounds was made. *Bacillus* sp. MB028 produced specific substances in both media of GYM4 and MB in 100 ml approach as well as 1 L (Figure 10, Table 9). The substance A with molecular weight of around 600.4 and UV peaks of 225, 280 and 311nm is assumed to be heteroxantin which was isolated from *Mytilus edulis* before (Buckingham, 2009), but it was not clear whether the substance originated from a symbiotic microorganism. For

the other substance with molecular weight of 713.5 and UV peaks at 225, 280 and 311 nm, there was no match found in either Natural Compound Dictionary or in Antibase. The same was true for *Microbacterium* which produced substance C representative in two mass peaks with molecular weight of 297.2 and UV peaks at 231, 265 nm with no match found from the same origin(Figure 11, Table 10). Although it is assumed to be derived from a marine *Micromonospora* sp. (*Actinobacteria*), previously, as 2-Ethyl-1-hydroxy-8-methoxy-3-methylanthraquinone (Buckingham, 2009).

Figure 10. Mass spectrometry and UV data obtained by HPLC analysis for substances, produced by *Bacillus* sp. MB028

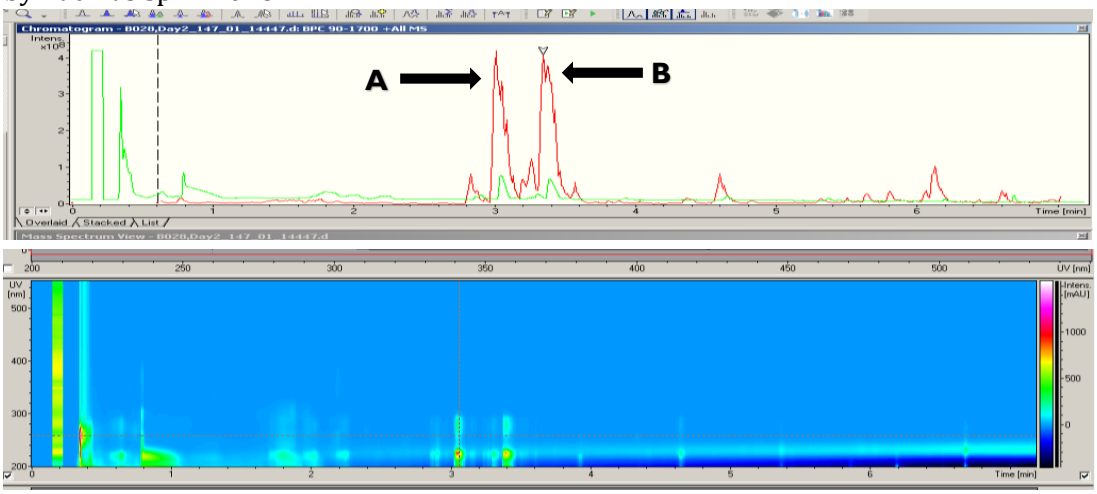


Table 9: Information about substances produced by *Bacillus* sp. MB028

Peak	Mass(m/z)	UV Peaks(nm)	Substances	Origin of substance
A	600.4	225, 280, 311	Heteroxanthin	*Mytilus edulis*
B	713.5	225,280,311	No match found	-

Figure 11: Mass spectrometry and UV data obtained by HPLC experiments for substances, produced by *Microbacterium* sp. MB141

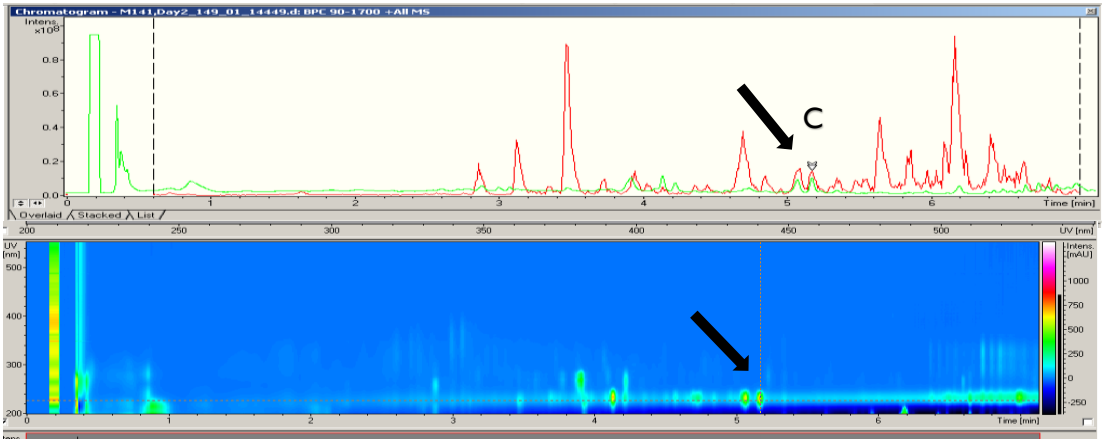
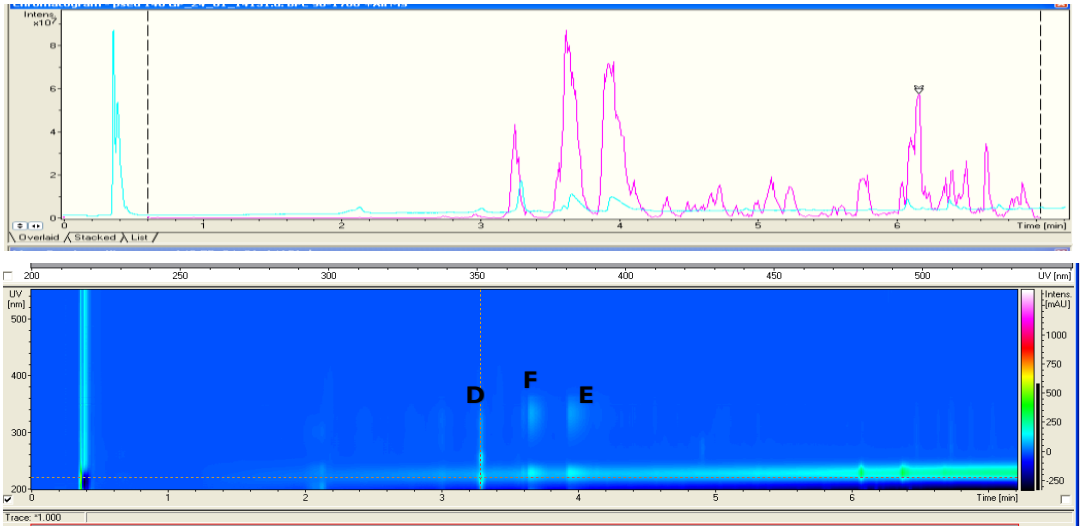


Table 10: Information about substances produced by *Microbacterium* sp. MB141

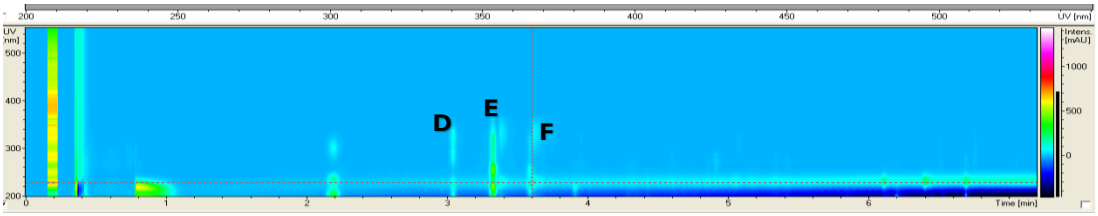
Peak	Mass (m/z)	UV Peaks (nm)	Substances	Origin of substance
C	297.2	231,265	No match found	-

Also *Pseudomonas* sp. MB140 produced the same substances in both approaches with molecular weight of 324.1, 252.1 and 251.1. The substance D is recognized to be pyochelin ($C_{14}H_{16}N_2O_3S_2$) with molecular weight of 324.418 and UV peaks at 220, 255, 316 and 360 nm. The original source of this substance was *Pseudomonas aeruginosa*, *Pseuodomonas cepacia* (Ankenbauer, R.G. et al. 1988). The second one, substance E is assumed to be 2, 4, 6-triacetylphloroglucinol ($C_{12}H_{12}O_6$) which was derived before from *Pseudomonas.fluorescens* with mass of 252.2 (De Bernardi, M et al. 1976). The third one was deoxyadenosin with mass of 251.1 and UV peaks at 225, 336, 360 and 390 nm which is isolated for the first time from *Pseudomonas amygdali* (Evidente et al., 1989). Figure 12 and Table 11 show the information about bioactive metabolites produced by *Pseudomonas* sp. MB140.

Figure 12. Mass spectrometry and UV data obtained by HPLC experiments for substances, Produced by *Pseudomonas* sp. MB140



1 liter



10 Liter

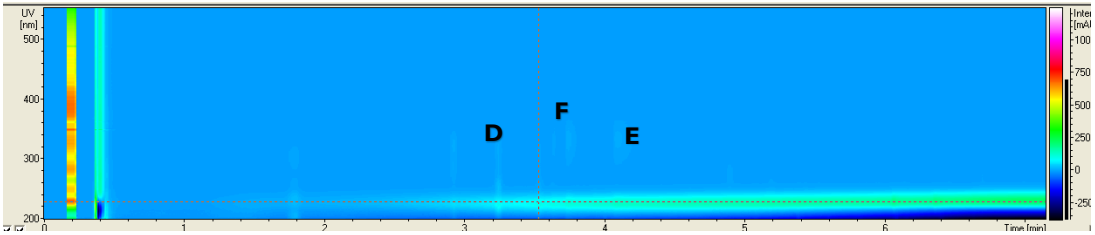


Table 11: Information about substances produced by *Pseudomonas* sp. MB140

	Mass(m/z)	**UV Peaks(nm)**	**Substances**	**Origin of substance**
D	324.1	220, 255,316,360	Pyochelin	*Pseudomonas aeruginosa*, *Pseudomonas cepacia*
E	252.1	220,255,316,360	2,4,6-Triacetylphloroglucinol	*Pseudomonas fluorescens*
F	251.2	225, 336,360,390	Deoxyadenosin	*Pseudomonas amygdali*

Figure 13: Chemical structure of pyochelin

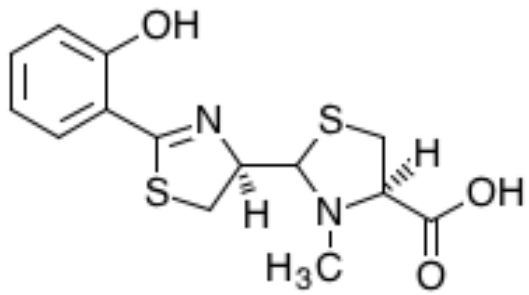


Figure 14: Chemical structure of 2, 4, 6-triacetylphloroglucinol

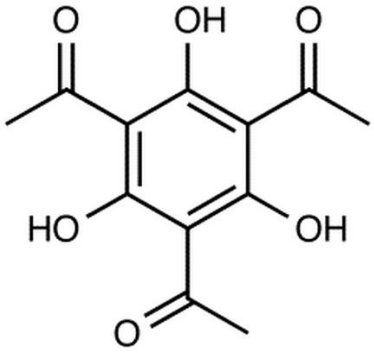
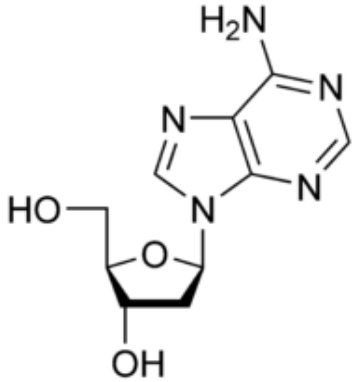


Figure 15: Chemical structure of deoxyadenosin



4.4. 10 L approach

In the 10 L approach we focused on *Bacillus* sp. MB028 and *Pseudomonas* sp. MB140. The substances mentioned in Table 9 and 11 were produced by *Bacillus* sp. MB028 and *Pseudomonas* sp. MB140, respectively, in all 3 approaches described in this study. *Bacillus* sp. MB028 had almost the same secondary metabolite profile in different approaches. The retention time for substances E and F produced by *Pseudomonas* sp. MB140, didn't follow the same pattern in different approaches and also the strength and intensity of UV spectra for these substances was not the same at similar conditions (Figure 12).

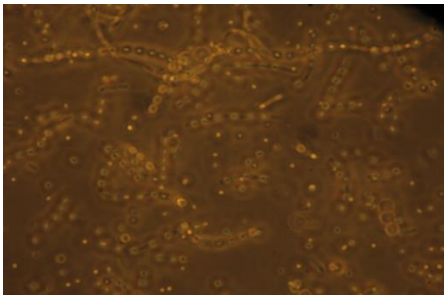
4.5. Antimicrobial activities of the culture extracts

The extracts of 10 strains isolated from *Mytilus edulis* were tested for their antimicrobial activity against environmental and standard panels of microorganisms after cultivation in different media. The antimicrobial assay showed, that the extract of *Pseudomonas* sp. MB140 in GP medium strongly inhibited the environmental test organisms including *Bacillus* sp. MB011 and *Shewanella* sp. MB045 (growth inhibition: ~100% and ~98%, respectively) and also members of standard panel including *Bacillus subtilis* and *Candida albicans* (growth inhibition: ~107% and ~80.4%, respectively). The extract of *Pseudomonas* sp. MB140 grown in GYM4 medium presented only slight inhibition against the test organisms. Some strains showed slight inhibition against some environmental samples and not against the standard panel. For example, *Bacillus* sp. MB011, in both media (GYM4 and MB), and *Rhodococcus* sp. MB056 in GYM4 medium, inhibited *Pseudomonas* sp. MB140. *Microbacterium* sp. MB140 grown in oat medium inhibited *Shewanella* sp. MB 045 and also *Pseudomonas* sp. MB140. The only strain which could well inhibit the metabolic activity of *E. coli* was *Microbacterium* sp. MB140 cultured in oat medium (metabolism inhibition: ~32.7%). Information about inhibition activity of extracts is summarized in Figure II.9, II.10 and appendices 2 and 3.

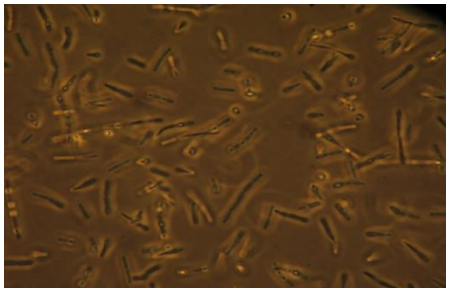
4.6. Co-cultivation

No inhibition zones in growth of bacterial strains were detected after co-cultivation on agar plates and also no significant morphological changes e.g., shape, size and mobility of cells were detected by microscopic observation, except some changes in the cell form and shape in *Bacillus* sp. MB079 co-cultivated with *Shewanella* sp. MB045 (Figure 16). No clear and significant mass peaks and UV spectra could be detected in HPLC chromatograms and secondary metabolite profiles of co-cultivated strains.

Figure 16: Morphological changes in cells of *Bacillus* sp. MB079 from close (1) and far (2) distance, respectively, to *Shewanella* sp. MB045



(1)



(2)

Figure 17: Inhibition activity of culture extracts against three environmental isolates. : Growth inhibition, : Metabolism inhibition

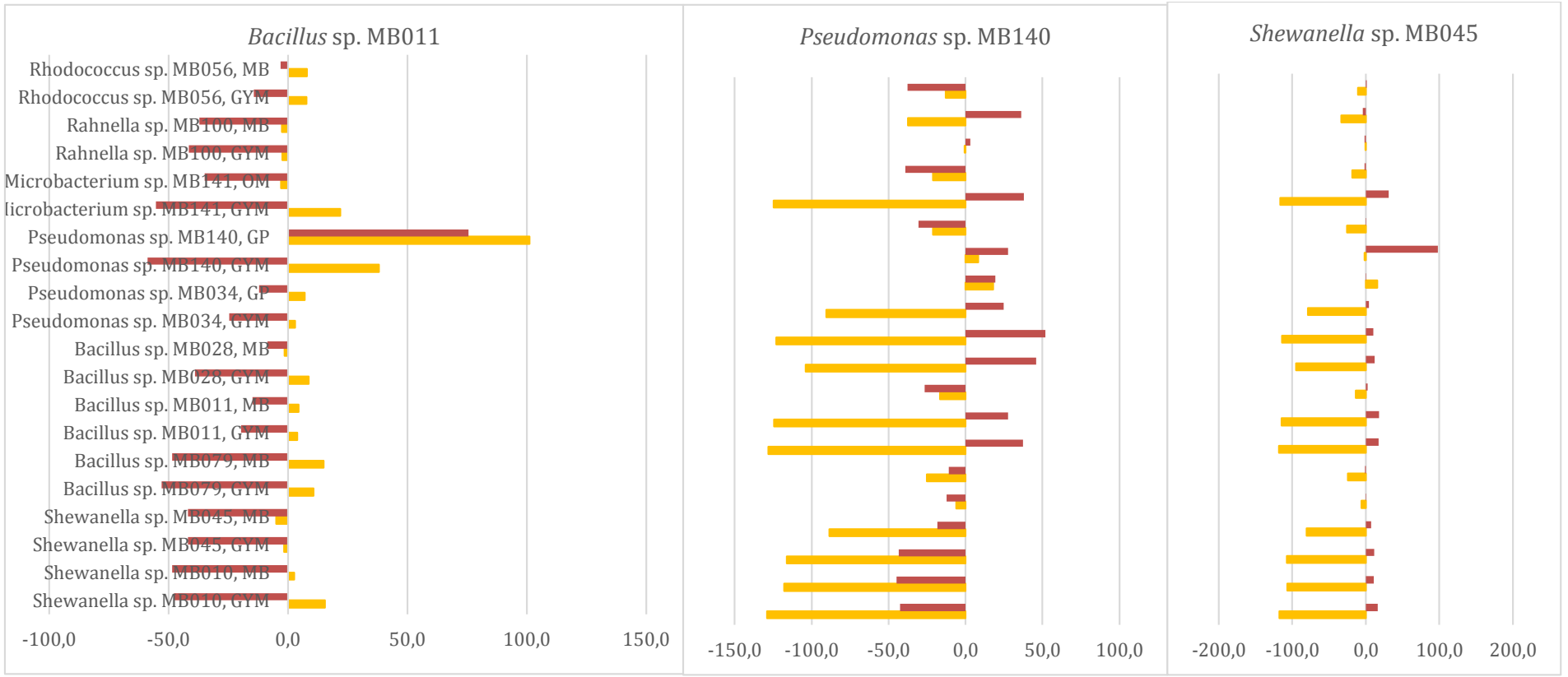
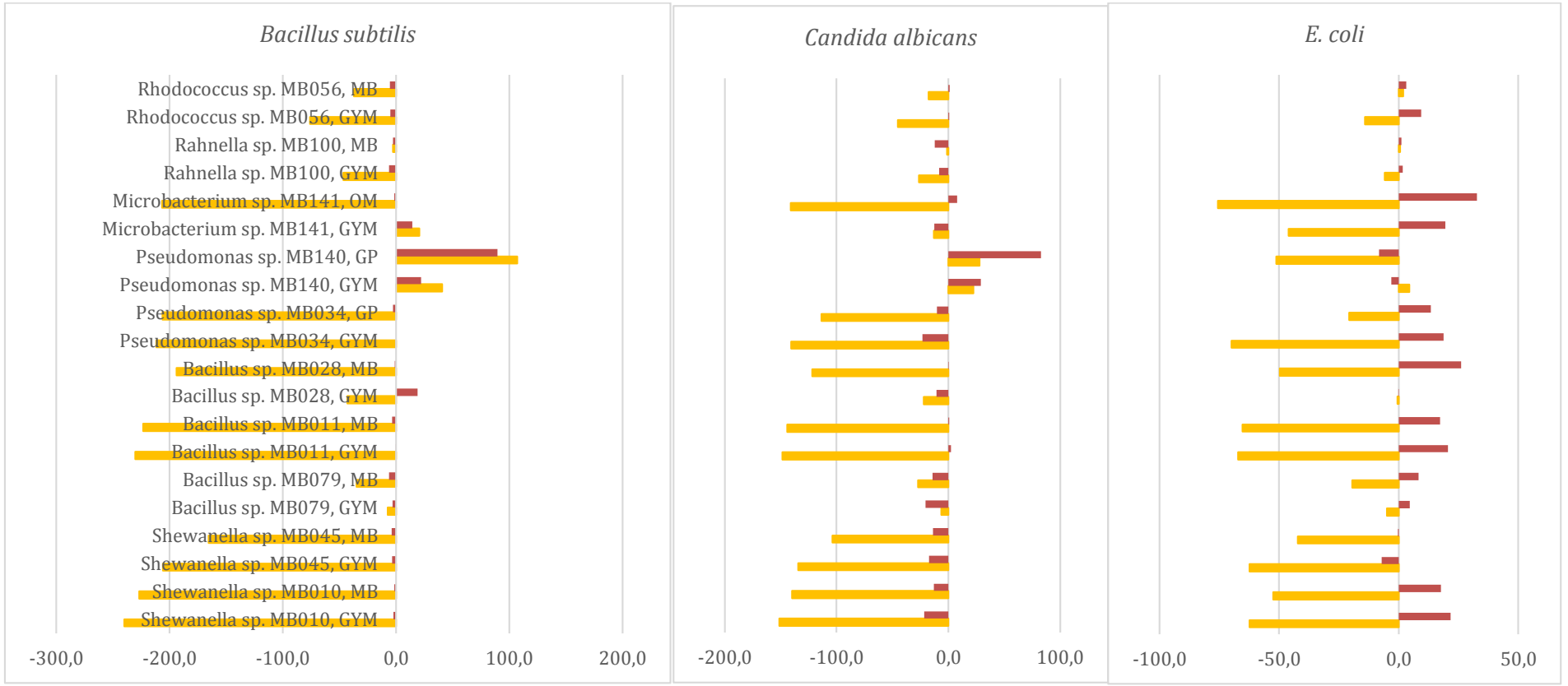


Figure 18: Inhibition activity of culture extracts against standard strains. : Growth inhibition : Metabolism inhibition



DISCUSSION

5.1. Isolation and characterization of *Mytilus edulis* associated bacteria

Taking into consideration the 16S rRNA gene sequence similarity value of 97% as an accepted criterion for differentiation of bacterial species (Stackebrandt and Goebel, 1994) most of the isolates obtained in this study could be assigned to previously described bacteria species. In total 14 genera belonging to 4 classes (*Proteobacteria*, *Actinobacteria*, *Firmicutes* and *Bacteriodetes*) were identified. Among *Gammaproteobacteria* representatives of *Shewanella*, *Stenotrophomonas*, *Pseudomonas*, *Vibrio*, *Listonella*, *Rahnella* and *Aeromonas* species were detected, while *Achromobacter* was the only representative of *Betaproteobacterium.* Members of *Actinobacteria* affiliated to the genera *Microbacterium*, *Rhodococcus* and *Agromyces*. *Firmcutes* and *Bacteriodetes* representatives are only *Bacillus* and *Algoriphagus*, respectively. This bacterial diversity was higher than that reported by Beleneva et al. (2003), who isolated 526 strains of heterotrophic bacteria from a natural and cultured population of the mussel *Mytilus trossulus* in Peter the Great Bay in Russia and detected 6 genera of Gram-negative bacteria including *Pseudoalteromonas*, *Pseudomonas*, *Vibrio*, *Photobacterium*, *Cytophaga*/*Flavobacterium*/*Bacteroides* and *Moraxella*, as well as *Streptomyces* representative of Gram-positive bacteria. Moreover, in their study, the taxonomic composition of natural and cultured bacteria from mussels were similar. Cavallo et al. (2008), also reported different genera of *Aeromonas*, *Moraxella*, *Pseudomonas*, *Alcaligenes*, *Acinetobacter*, *Flavobacterium*, *Chromobacterium*, *Photobacterium* and *Flexibacter*, *Lucibacterium* and *Vibrio* among Gram-negative bacteria and *Bacilli* among Gram-positive bacteria in their samples isolated from *Mytilus galloprovincialis* in Northern Ionian Sea.

Gammaproteobacteria were clearly dominant among the strains isolated from the *Mytilus edulis* in this study. This is in accordance with other studies on microbial diversity in different marine ecosystems such as water samples of Bermuda Atlantic (Cho and Giovanni, 2004) and North Sea (Eilers, 2000) or intestine of marine creatures (Kurahashi and Yokota, 2002) regardless whether cultivation-dependent or cultivation-independent approaches were applied. Romaneko et al. (2008) in their study of cultured heterotrophic bacteria associated with the marine ark shell *Anadara broughtoni* in the Sea of Japan, isolated and identified 149 phenotypically and 27 strains among were identified phylogenetically by 16S rRNA gene sequence analysis. In their study, most bacteria were affiliated with *Gammaproteobacteria* and *Alphaproteobacteria*, and less with *Firmicutes*, *Actinobacteria*, and *Cytophaga- Flavobacterium- Bacteroides* (CFB) group. Also Kurahashi

and Yokota (2002), have reported that most of their 116 isolates from intestine of some marine organisms including molluscs, pisces and protochordata collected at the coasts of the Kanto area in Japan belonged to the *Gammaproteobacteria* (*Proteobacteria*: 74 strains) and CFB group including *Cytophaga- Flavobacterium- Bacteroides* (12 strains) with the rest belonging to the B*acillus/Clostridium* (2 strains), and *Actinobacteria* (2 strains). The remaining strain of their study was found to belong to yeast *Ascomycota*, based on 18S rDNA. Of 74 of *Proteobacteria* strains, 42 were identified as *Vibrio.* The most commonly reported genera of gut bacteria in invertebrates are *Vibrio, Pseudomonas*, *Flavobacterium*, *Micrococcus* and *Aeoromonas* (Harris, 1993). Lhafi, S.K., Kühne, M. (2007) also indicated that blue mussels from shellfish growing area in German Wadden Sea contain pathogens relevant to public health and *Vibrio sp*. was detected in 74.4% of the samples analyzed in their study. Moreover, a correlation between water temperature and occurrence of *Vibrio* sp. has been reported with the highest bacteria occurrence at 13° C to 22° C, while temperature below 8.5° C considerably weaken the pathogens (McLaughlin et al., 2005; HØi et al., 1998; Kasper et al., 1993). The occurrence of *Vibrio* sp. in the range of temperature between 10 and 16 (Buer. 2012) in our study is in accordance with previous studies, particulary when taken into account a cold adaptation for *Vibrio* sp. (Wright et al. 1996).

Bacteria of *Aeromonas* species are widely distributed in marine environments, especially in Mollusca, but can also be isolated from clinical samples. The number of *Aeromonas* strains found among isolates was relatively low (2.6 %). Since the composition of the microbial communities of hydrobionts is largely determined by condition of the surrounding water, which is due to their suspension filtering activity (Ugolev, 1985., Beleneva et al., 2003), the low content of bacteria from the family *Enterobacteriaceae* (1.7%) and *Aeromonas* (2.6%) in microbial communities isolated from the mussels is a reflection of almost ecological condition of surrounding water, although it cannot be accepted for edible mussels in aquaculture ecosystems. According to Cavallo et al. (2009), *Aeromonas* prevailed both in their water (18%) and mussel (*Mytilus galloprovincialis*) samples (40%) and the other genera such as *Moraxella, Pseudomonas, Alcaligenes, Acinetobacter, Flavobacterium, Chromobacterium, Photobacterium* and *Flexibacter* were present with different percentage of isolation.

By application of culture-based methods we could survey a part of the bacterial groups, not microbial community taken as a whole and to get a wide range and variety of bacteria,

several kinds of media should be used. However, despite using just one medium and in comparison with previous studies, a great variety of bacterial strains were obtained.

5.2. *Mytilus edulis* associated bacteria as producer of natural product

Among the 116 strains that were isolated and phylogenetically classified, 5 strains, including members of the genera *Pseudomonas, Bacillus*, *Rhodococcus, Shewanella* and *Microbacterium*, each cultured in two different media were selected for further analysis for their potential to produce natural compounds. Known substances were identified, but likely also new substances were detected. Three compounds including pyochelin, deoxyadenosin and 2, 4, 6-triacetylphloroglucinol produced by *Pseudomonas* sp. MB140 were dereplicated as well as heteroxanthin produced by *Bacillus* sp. MB028. Pyochelin is a structurally unique phenolate siderophore (Cox and Graham, 1979) which is also produced by *Pseudomonas aeruginosa* (Ankenbauer et al., 1998), *Pseudomonas cepacia* (Sokol, 1984, 1986) and *Pseudomonas fluorescens* (Sokol, 1984). Pyochelin has been assigned the chemical structure 2-[2-(o-hydroxyphenyl)-2-thiazolin-4-ylI-3-methyl-4-thiazolidinecarboxylic acid (Cox et al., 1981). It plays an important role in the interaction of *P. aeruginosa* with the mammalian nutritional immunity system (Cox, 1985). Pyochelin has been shown to facilitate the removal of iron from the critical component of the nutritional immunity system, transferrin (Sriyosachati and Cox, 1986), and is also capable of stimulating bacterial growth during infections in mice (Cox, 1982). Derivation of deoxyadenosin for the first time was reported by Evidente et al. (1989) from *Pseudomonas amygdali* as a new cytokinin and production of 2, 4, 6-triacetylphloroglucinol by *Pseudomonas fluorescens* was reported previously by (Buckingham, 2009). The production of the substance which is assumed to be heteroxanthin by *Bacillus* sp. MB028 (assigned to *Bacillus cereus*) was previously reported in *Mytilus edulis* (Buckingham, 2009). The other substances produced by *Bacillus* sp. MB028 and *Microbacterium* sp. MB141 are needed to be further investigated using NMR analysis for identifying their chemical structure and properties.

In terms of antimicrobial activity, *Mytilus edulis* associated bacteria showed a high potential of growth and metabolic inhibition activity, which *Mytilus edulis* may use for its own benefit. Particularly strong antimicrobial activity against standard and environmental strains was detected in *Pseudomonas* sp. MB140 grown in GP medium.

This was not surprising since *Pseudomonas* is a very potent genus of marine bacteria known to produce a variety of bioactive compounds (Bhatnagar and Kim, 2010). It has been reported that *Pseudomonas* produces 795 bioactive substances covering 610 antibiotic compounds and 185 compounds encompassing bioactivities apart from antibiotic property (Berdy, 2005). From a marine *Pseudomonas* sp. isolated from California tide pool, Wratten et al. (1977), isolated three antibacterial substances, 4-hydroxy-benzaldehyde, 2-*n*-heptyl-4-quinolinol, and 2-*n*-pentyl-4-quinolinol which inhibited the growth of *Vibrio anquillarum*, *Vibrio harveyi* and *Staphylococcus aureus*. It is still not clear which specific compound (or a combination) is responsible for such an inhibitory effect in *Pseudomonas* sp. MB140 assigned to *Pseudomonas veronoii*. Moreover, since the extract of this strain in GYM4 medium showed slighter inhibition activity compared to GP medium, it can be assumed that the same substances are produced in both media but in lower amount in GYM4. Romaneko et al.; (2008) studied bacteria associated with the marine ark shell *Anadara broughtoni* in the Sea of Japan for their antimicrobial, hemolytic and surface activity. A high level of antimicrobial activity was detected among Gram positive bacteria assigned to *Bacillus pumilus*, *Bacillus licheni* and *Paenibacillus polymixa* isolated from intestine tissue of mussels. These strains had also been demonstrated to be efficient producers of many biologically active compounds (Von Do¨hren, 1995). They also reported the antimicrobial activity of strain related to *Pseudomonas fulva* (Romaneko et al., 2008). MB011 assigned to *Bacillus pumilus*, just showed a slight metabolic inhibition against *Pseudomonas* sp. MB140. The similar inhibition was detected for *Bacillus* sp. MB028 in our study. *Microbacterium* sp. MB140 was the only strain which showed metabolic inhibition (~33%) against *E. coli*. Blunt et al. (2013) in their review of marine natural product reported two epimeric quinazolinone alkaloids isolated from *Bacillus cerus* (sea mud, Baimajin, Hainan, China) which showed moderate activity against *Candida albicans*, but in our study no activity was detected by *Bacillus* sp. MB028 assigned to *Bacillus cerus*. Although different antimicrobial activities of culture extracts were detected in the bioassays, almost no morphological changes or inhibition zone could be seen in observation of co-cultivations on agar plates or under a light microscope. The conclusion can be made that growth inhibition is a more sensitive parameter than changes in morphologies of cells and colonies.

In general, bivalves as sedentary suspension feeders which are exposed to a large amount of microorganism through their gills, have developed efficient chemical defense against pathogens. This has been demonstrated in recent studies on the involvement of

antimicrobial peptides and proteins in humoral immunity of bivalves (Mitta et al., 2000a, b; Cellura et al., 2007; Zhao et al., 2007; Li et al., 2009). Some interesting polyproniates and alkaloids (aromatic nitrogen compounds) have been also isolated from bivalves, although it is presently unclear if these play any defensive role. For example Mytilins A and B, cationic cysteine-rich AMPs, were isolated and characterized from *Mytilus edulis* (Mitta et al., 2000). In addition to the characterized defensins and mytilins, an antifungal peptide named mytimycin, also isolated from *M.edulis*, was shown to delay the growth of *Neurospora crassa* and *Fusarium culmorum* (Charlet, et al., 1996). Among bacteria associated with marine organisms, members of *Actinobacteria* such as *Streptomyces* and *Actinomycete* species and also *Bacillus* sp. are the most capable and well-studied bacteria in the production of natural compounds and secondary metabolites (Imhoff et al. 2011). But as far as known, not so many secondary metabolites produced by bacteria associated to mussels especially *Mytilus edulis*, have been described in the literature.

It is supposed that associated microorganisms protect their host from colonization and against pathogenic microorganisms by bioactive metabolites, thus contributing to vital organismal functions and chemical protection. These functions and natural products mediating them can have large effects on ecological interactions at microscopic to ecosystem scales, affecting multiple trophic levels and biogeochemical processes. This area of research is a rich source of ecological insights, new chemistry and biotechnological applications.

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APPENDICES

APPENDIX 1: Recipe of all media used during the experiments.

OM (oat medium)

20g oat bran

15g tropic marine salt

1000ml Deion water

pH: 7. 0

MB (Marine Broth)

37.4g marine broth (Difco)

1000ml Deion water

GYM4

4g glucose

4g yeast extract

4g malt extract

15g tropic marine salt

1000ml Deion water

pH: 7.2

GP

10g L-glutamic acid sodium salt monohydrate

2g KH_2PO_4

0.5g $MgSO_4$

1000ml Deion water

pH: 7.2

APPENDIX 2: Inhibition activity of all extracts in different media against environmental isolates.

Yellow cells show the inhibition activity more than 30%, which is considered as inhibited criteria.

Extract of strains	**Medium**	***Bacillus* sp. MB011**				***Pseudomonas* sp. MB140**				***Shewanella* sp. MB045**			
		Growth (%)		Resazurin (%)		Growth (%)		Resazurin(%)		Growth (%)		Resazurin (%)	
***Shewanella* sp. MB010**	GYM	14,5	16,6	-46,4	-49,2	-63,8	-68,6	-42,5	-42,6	-169,7	-173,3	18,5	14,3
***Shewanella* sp. MB010**	MB	3,3	2,1	-48,7	-48,3	-101,6	-102,3	-44,7	-44,8	-171,3	-182,5	9,1	12,3
***Shewanella* sp. MB045**	GYM	4,2	-7,3	-41,5	-42,3	-102,8	-101,3	-42,9	-43,6	-184,3	-187,9	10,9	11,9
***Shewanella* sp. MB045**	MB	-5,2	-4,9	-41,6	-42,3	-96,4	0,7	-43,8	7,0	-152,2	-180,2	1,7	13,2
***Bacillus* sp. MB079**	GYM	5,9	15,7	-54,9	-50,9	-2,2	-43,8	-2,5	-21,8	-55,7	-29,8	1,6	-1,3
***Bacillus* sp. MB079**	MB	16,6	13,1	-48,1	-49,0	-33,5	-24,7	-9,1	-12,7	-43,8	-39,9	-1,1	-0,8
***Bacillus* sp. MB011**	GYM	5,6	2,1	-11,2	-28,2	-65,9	-65,1	40,0	34,9	-173,5	-175,9	17,1	17,8
***Bacillus* sp. MB011**	MB	4,9	3,9	14,4	-44,3	-71,7	-73,8	28,9	26,6	-169,1	-169,0	18,2	18,0
***Bacillus* sp. MB028**	GYM	7,2	10,3	-38,1	-39,8	-81,3	-87,3	-20,5	-32,5	-71,5	-53,5	3,0	2,4
***Bacillus* sp. MB028**	MB	-1,9	-0,9	-6,0	-11,3	-102,2	-101,3	46,8	44,9	-164,0	-171,9	11,5	12,3
***Pseudomonas* sp. MB034**	GYM	2,9	3,1	-26,6	-22,5	-99,9	-97,3	54,7	49,3	-172,8	-175,9	3,2	17,7
***Pseudomonas* sp. MB034**	GP	5,4	8,7	-8,1	-16,0	-97,9	4,1	46,0	3,7	-60,6	-2,3	9,1	-0,9
***Pseudomonas* sp. MB140**	GYM	38,3	38,1	-60,3	-57,3	-89,4	0,7	37,5	1,2	-28,7	-1,8	0,3	0,5
***Pseudomonas* sp. MB140**	GP	100,8	101,6	77,5	73,6	13,8	23,0	21,3	33,9	97,3	100,2	95,7	100,2
***Microbacterium* sp. MB141**	GYM	23,3	20,6	-57,0	-53,8	-45,5	-32,5	-36,8	-24,1	-12,6	-45,5	0,3	0,6
***Microbacterium* sp. MB141**	OM	-0,1	-5,8	-32,1	-37,7	-99,6	-98,9	37,6	38,2	-171,6	-172,1	33,4	29,0
***Rahnella* sp. MB100**	GYM	-3,0	-1,9	-41,7	-41,4	-45,2	-47,5	-40,9	-37,4	-50,7	-25,9	-2,6	-0,9
***Rahnella* sp. MB100**	MB	-2,2	-3,0	-36,2	-37,9	4,6	3,8	8,7	-2,5	2,8	1,2	-1,8	-1,6
***Rhodococcus* sp. MB056**	GYM	7,3	8,3	-27,7	-0,8	-125,9	-120,7	31,5	40,6	-117,3	-127,8	-4,0	-3,7
***Rhodococcus* sp. MB056**	MB	9,9	5,9	-4,2	-1,8	-98,8	-95,5	-32,4	-42,8	-64,8	-87,3	-3,0	5,4

APPENDIX 3: Inhibition activity of all extracts in different media against standard set of organisms.

Extract of strains	**Medium**	***Bacillus subtilis***				***Escherchia coli***				***Candida albicans***			
		Growth (%)		Resazurin (%)		Growth (%)		Resazurin (%)		Growth (%)		Resazurin (%)	
***Shewanella* sp. MB010**	GYM	-235,1	-244,8	-2,2	-2,7	-62,3	-62,3	21,8	21,7	-52,6	-115,3	-20,1	-23,1
***Shewanella* sp. MB010**	MB	-215,8	-238,0	-1,6	-1,8	-50,4	-54,6	16,8	18,5	-73,9	-95,3	-13,9	-12,3
***Shewanella* sp. MB045**	GYM	-192,6	-219,5	-4,2	-3,3	-66,0	-58,9	-6,1	-8,0	-114,5	-126,6	-15,5	-19,1
***Shewanella* sp. MB045**	MB	-168,7	-162,4	-3,3	-4,4	-41,1	-43,4	5,3	-6,1	61,5	-20,7	-14,2	-13,5
***Bacillus* sp. MB079**	GYM	11,6	-26,8	-1,5	-4,9	-12,2	2,5	18,2	-9,0	11,4	-15,2	-30,6	-10,4
***Bacillus* sp. MB079**	MB	-38,5	-31,2	-6,0	-6,6	-27,4	-11,5	9,3	7,2	-34,5	-65,3	-18,6	-9,3
***Bacillus* sp. MB011**	GYM	-228,2	-232,3	-0,6	0,4	-66,7	-67,6	23,5	17,7	-157,1	-179,7	1,0	3,8
***Bacillus* sp. MB011**	MB	-218,3	-228,1	5,3	-12,2	-66,3	-64,5	21,4	13,2	-54,8	-40,5	-0,6	2,0
***Bacillus* sp. MB028**	GYM	-40,8	-45,7	12,9	24,6	-1,2	0,3	-0,5	0,4	-45,8	-33,5	-9,6	-11,2
***Bacillus* sp. MB028**	MB	-187,3	-200,7	0,4	-3,2	-45,7	-53,5	18,5	33,5	-43,2	-43,1	1,6	-1,1
***Pseudomonas* sp. MB034**	GYM	-207,7	-215,6	-1,0	1,3	-69,0	-70,9	21,3	16,3	-53,4	-10,2	-29,2	-17,1
***Pseudomonas* sp. MB034**	GP	-204,7	-208,2	-2,7	-3,1	-1,6	-39,9	3,0	24,0	-15,3	-11,5	-11,5	-9,0
***Pseudomonas* sp. MB140**	GYM	36,2	44,8	24,9	19,0	5,4	3,6	-0,9	-5,3	24,5	22,6	21,5	36,7
***Pseudomonas* sp. MB140**	GP	106,3	107,9	87,4	91,3	-51,8	-50,5	-3,0	-13,5	61,9	98,9	79,6	86,0
***Microbacterium* sp. MB141**	GYM	25,6	15,2	13,2	15,0	-40,8	-51,4	21,3	17,7	9,3	24,0	-10,6	-14,2
***Microbacterium* sp. MB141**	OM	-202,5	-210,9	-2,7	-0,8	-75,9	-75,3	31,2	34,3	15,2	7,6	7,8	8,2
***Rahnella* sp. MB100**	GYM	-44,0	-49,2	-6,6	-5,9	-6,1	-5,6	2,2	1,2	-12,2	-13,5	-8,1	-8,8
***Rahnella* sp. MB100**	MB	4,0	-9,2	-5,2	-0,6	0,2	1,1	4,0	-1,9	-22,8	-38,2	-8,6	-16,1
***Rhodococcus* sp. MB056**	GYM	-108,2	-44,0	-5,3	-5,2	-17,0	-11,2	9,0	9,6	-4,3	36,5	-2,9	4,2
***Rhodococcus* sp. MB056**	MB	-28,3	-46,2	-5,4	-5,9	4,1	-0,4	2,3	4,2	-26,5	18,6	-0,9	3,1

9- Declaration

Hereby, I declare that I myself have prepared and performed the master thesis by title of „*Mytilus edulis* associated bacteria: Diversity and interaction based on bioactive compounds“, presented here without the use of other resources than the stated ones. All positions where I have used existing publications, literally or analogously, are marked as citations.

This thesis has not been handed in at any other authority neither in the same form nor similar or in extracts.

The printed version and the electronic version handed in are identical.

10/01/2014
Asa Motiei