UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS

DEPARTAMENTO DE BIOLOGIA VEGETAL

Novel Screening Platforms Construction for Detection of New Marine Microbial Bioactive Compounds

Gabriela Alexandra Rodrigues Simões

Mestrado em Biologia Molecular e Genética

Dissertação orientada por:

Professora Doutora Helena Vieira

2018

Agradecimentos

Este foi um ano cheio de experiências, aprendizagem e momentos stressantes. E como diz o provérbio "A união faz a força". Assim, nesta secção, quero deixar o meu enorme agradecimento a todas as pessoas que contribuíram, direta e indiretamente, para a concretização e conclusão de mais uma etapa da minha vida.

Em primeiro lugar, quero deixar um enorme agradecimento à minha orientadora Doutora Helena Vieira cuja orientação, assistência e incentivo foram cruciais para a materialização desta dissertação de mestrado.

Um agradecimento mais do que especial aos meus pais, Tina e Gabriel. Não há palavras para vos agradecer tudo o que fizeram por mim. Um obrigada cheio de carinho e amor!

Um reconhecimento muito especial ao meu irmão, Joel, que passou os últimos meses da tese a perguntar-me "Então, Gabi quando é que entregas essa tese?". "Já está, Joel!".

Aos meus amigos de sempre, Andreia Guilherme, Andreia Carvalho, Cristiana Vieira e Miguel Oliveira, os meus mais sinceros agradecimentos. Vocês foram, sem dúvida, imprescindíveis para a conclusão e realização desta etapa. Obrigada pela paciência e pela amizade.

À Filipa Antunes, chefe do laboratório, um especial agradecimento por me ter recebido bem e pela ajuda prestada.

Aos meus colegas do mestrado e do laboratório obrigada pela companhia.

A todos, os meus mais sinceros agradecimentos.

Abstract

Most life on Earth exists within the oceans that hold a great deal of biodiversity. Marine organisms show a wide bioactive diversity, with more than 1000 new compounds being discovered each year. Microorganisms, despite being deeply exploited in terrestrial environments, are still a relatively minor source of marine-based commercial compounds compared to other marine organisms. One way to maximize the chemical diversity available from marine sources, and their commercial potential, is to focus on microorganisms, as they are easier to collect and reproduce. Marine microorganisms can sense, adapt and respond to their environment quickly and can compete for defence and survival in extreme habitats, like deep sea hydrothermal vents, by producing exclusive secondary metabolites. The major goal of this thesis work was to develop assay platforms to screen bioactive compounds with antimicrobial and antioxidant potential from marine sources. As screening material a 227 marine microbial extracts library was used. Five screening assays were developed and validated for anti-microbial bioactivity screening against different strains, namely *Enterococcus* sp. (VanA+), *Klebsiella oxytoca*, *Salmonella enteritidis, Salmonella typhimurium* and *Shigella* sp. Out of these, two failed to be fully validated, the *Enterococcus faecalis* and *Staphylococcus aureus*. From the initial 1135 marine extracts tested, 69 hits were found to inhibit the growth of specific pathogenic bacteria. In parallel, two antioxidant assays were set-up, validated and implemented in the lab to evaluate antioxidant properties of the marine compounds. The DPPH assay was optimized and validated through DPPH scavenger activity measurements and hit LSWA081 was found to be over 100% better than the current gold standard. To increase the biological significance, a SOD1 screening platform was also implemented from a pre-existing SOD1 mutant yeast strain. A primary SOD1 screening assay was performed and 8 new target specific hits identified, that still remain to be validated in a secondary dose-response secondary screening. The majority of the identified hits, both antimicrobial and antioxidant are derived from Menez Gwen hydrothermal vent, a very harsh acidic and very high temperature deep sea habitat. The work performed in this thesis can be the basis of novel marine natural products and bioactives development programmes with relevance to a variety of industries.

Keywords: marine microbial; bioactivity; antimicrobial screening; antioxidant screening; commercial value

Resumo

Uma parte considerável da biodiversidade da Terra está presente em ecossistemas marinhos. Esta elevada e única biodiversidade marinha tem sido reconhecida como uma importante fonte de variabilidade química, estimando-se que anualmente são descobertos cerca de 1000 compostos de origem marinha. No entanto, o seu conhecimento científico e exploração comercial é ainda reduzido. Uma acumulação de dados científicos que comprovam os benefícios destes compostos marinhos nos mais variados sectores, desde os biocombustíveis, biomateriais, cosmética, alimentação funcional, têxtil, aplicações industriais para além da farmacêutica, tem conduzido a um aumento significativo dos esforços para investigar e isolar mais bioativos presentes nesta biodiversidade marinha. Dentro desta, os microrganismos marinhos têm sido alvo recente de uma maior atenção. Os microrganismos são já amplamente explorados em ambientes terrestres, mas ainda representam uma pequena parte dos compostos de origem marinha em comparação com outros organismos marinhos, como é o caso das macroalgas ou esponjas. A sua fácil manipulação e baixo impacto que a sua produção poderá ter no ecossistema são vistos como vantagens na exploração destes microrganismos. Uma outra forma de maximizar a diversidade química disponível de origem marinha, e seu potencial comercial, passa por estudar os microrganismos que habitam ambientes com condições de sobrevivência extremas, como fontes hidrotermais e oceano profundo. Nestes habitats, os microrganismos marinhos têm de possuir habilidades únicas, que lhes permitem adaptarem-se e responder ao seu ambiente de uma forma rápida, produzindo metabolitos secundários exclusivos que permitem a sua sobrevivência, defesa e competição. Estes metabolitos secundários são definidos como "armas bioquímicas" necessárias para competir, sobreviver e defenderem-se contra predadores e presas, sendo aceite pela comunidade científica que organismos que habitam as fontes hidrotermais deverão apresentar metabolitos secundários exclusivos. O principal objetivo desta tese foi desenvolver e implementar várias plataformas de rastreio rápido para identificar compostos bioativos com potencial antimicrobiano e antioxidante. A avaliação da qualidade global dos ensaios antimicrobianos e do ensaio da SOD1 para antioxidantes foi realizada utilizando o fator *Z-prime*, onde um ensaio excelente apresenta valores superiores a 0,5. Como fonte de potenciais bioativos foi utilizada uma biblioteca única de 227 extratos de microrganismos marinhos. Estes microrganismos foram isolados de 4 fontes hidrotermais: Menez Gwen, Rainbow, Monte Saldanha e Lucky Strike.

A atividade antimicrobiana de compostos marinhos pode ser detetada através da observação da inibição de crescimento de vários microrganismos-teste na presença destes extratos naturais. Atualmente, existem diversos métodos usados para detetar bioatividade antimicrobiana, sendo que o ensaio de rastreio rápido antimicrobiano selecionado foi o método de diluição, onde o crescimento microbiano é observado através de sucessivas medições de densidade ótica. Sete estipes bacterianas foram selecionadas, tendo em conta uma listagem de bactérias cuja a descoberta de antibióticos é assumida como crucial, publicada pela *World Health Organization*, e a listagem de bactérias existentes no laboratório. Ao todo das 7 bactérias selecionadas, cinco ensaios de rastreio rápido foram primariamente desenvolvidos e validados para identificar bioatividade antimicrobiana contra diferentes estirpes, nomeadamente: *Enterococcus* sp. (VanA +), *Klebsiella oxytoca*, *Salmonella enteritidis*, *Salmonella typhimurium* e *Shigella* sp. Estas sete bactérias foram submetidas a um rastreio primário, de onde resultaram 155 bioativos positivos. Estes primeiros bioativos positivos foram sujeitos a um rastreio secundário, onde se testou várias concentrações para um efeito dose-resposta, tendo sido identificados um total de 61 extratos bioativos positivos capazes de inibir 5 das 7 espécies de bactéria selecionadas: *Enterococcus* sp. (VanA+), *Klebsiella oxytoca*, *Salmonella typhimurium* e *Salmonella enteritidis*. Estes bioativos positivos reconfirmados serão no futuro submetidos a mais estudos, nomeadamente para elucidação da sua estrutura, e a técnicas de dereplicação para despistar compostos já conhecidos. No

entanto, duas plataformas para rastreio antimicrobiano não foram totalmente validadas para *HTS*, a plataforma para *Enterococcus faecalis* e para *Staphylococcus aureus*, uma vez que os valores de Z' foram inferiores ou iguais a zero durante o decorrer da experiência.

Paralelamente, dois ensaios antioxidantes foram preparados, validados e implementados em laboratório para avaliar as propriedades antioxidantes dos compostos marinhos em estudo. As reações oxidativas podem envolver a produção de radicais livres, que podem desenvolver reações em cadeia perigosas e como tal há necessidade permanente de novos, e mais sustentáveis compostos bioativos antioxidantes.

O teste do DPPH (1,1-difenil-2-picrilhidrazil) foi otimizado e validado para a atividade antioxidante, através de medidas de atividade do DPPH *scavenger*. Este teste simples e rápido é baseado na inibição da acumulação de produtos oxidados, uma vez que a geração de radicais livres é inibida pela adição de antioxidantes e captura de radicais livres. O DPPH é um radical livre estável devido à deslocalização do eletrão sobresselente sobre a molécula como um todo e não pode dimerizar. Assim, evitando a sua agregação, é possível usá-lo para medir a atividade de *scavenger* de radicais livres de um composto, sendo que este radical na presença de um antioxidante captura o electrão livre. Foram identificados 30 extratos bioativos positivos que apresentam uma atividade scavenger superior a 80 %, sendo que o extrato LSWA081 se destacou por ter uma taxa de DPPH *scavenger* acima de 100%, superando inclusive um dos melhores antioxidantes conhecidos, o ácido ascórbico. Este composto foi coletado da fonte hidrotermal *Lucky Strike*. Cerca de 63 % destes poderosos DPPH *scavengers* foram extraídos, no entanto, de microrganismos isolados de Menez Gwen.

Para aumentar o significado biológico do rastreio de antioxidantes, foi também implementada uma plataforma para identificar extratos que consigam substituir a função da enzima SOD1, usando uma estirpe de levedura mutante de SOD1 pré-existente. Este gene codifica uma enzima que converte aniões superóxido em H_2O_2 e oxigénio, protegendo assim as células contra danos oxidativos. A levedura é um organismo modelo, adequado para programas de descoberta de bioatividade para aplicações antioxidantes, devido ao seu alto grau de conservação de processos biológicos e sua facilidade para manipulação genética, tempos de geração curtos, adaptabilidade genética e escalabilidade. Inicialmente, testes de validação foram realizados para avaliar a viabilidade do uso da *Sacharomyces cerevisae* sod1Δ:: URA3 como uma plataforma de rastreio rápido. Assim, foi realizada uma avaliação do crescimento em meio líquido suplementado com H_2O_2 e ácido ascórbico para caracterizar a estirpe de *Saccharomyces cerevisae* mutante. Foi observado que a levedura mutante na presença de H_2O_2, um indutor de stress, apresenta um retardamento no crescimento em comparação com a estirpe selvagem. Também no âmbito da caracterização das estirpes, procedeu-se à amplificação do gene SOD1 por PCR que confirmou que o gene SOD1 está presente na linhagem selvagem, correspondente a 609 pb, e não na estirpe sod1Δ :: URA3, como esperado. O fator Z 'foi calculado em cada ponto do tempo para a curva de crescimento da levedura inoculada, sendo que o fator Z considerado para a seleção do momento do ensaio no qual os resultados podem ser confiáveis e analisados corresponde a 24 horas desde o início do ensaio, onde o fator Z' é de 0,60. Depois da validação do ensaio, foi realizado um primeiro ensaio de rastreio rápido, onde 8 bioativos positivos foram identificados. Estes terão de ser submetidos a um rastreio secundário posterior para serem revalidados em estudos dose-resposta, evitando, assim, possíveis falsos positivos. A maioria dos extratos identificados como bioativos positivos, tanto antimicrobianos como antioxidantes, tiveram origem na fonte hidrotermal Menez Gwen, um habitat marinho profundo muito ácido e de temperatura muito alta. É importante mencionar que alguns bioativos positivos apresentam tanto bioatividade antimicrobiana, como antioxidante e, portanto, são extratos microbianos marinhas com potencial elevado valor comercial que poderá valer a pena explorar no futuro. Os compostos MGBA001 e MGMS166O2 destacam-se por serem bioativos positivos para as

três plataformas de rastreio rápido desenvolvidas. O MGBA001, apesar de ter uma baixa atividade antioxidante no ensaio SOD1, possui uma excelente atividade do DPPH (> 80%) e possui propriedades antimicrobianas contra as bactérias gram-positivas, *Enterococcus* sp. (VanA +) e *Staphylococcus aureus*. O trabalho realizado nesta tese pode ser a base de novos produtos naturais marinhos e programas de desenvolvimento de bioativos com relevância para uma variedade de indústrias, que incluem medicina e cosmética.

Palavras-Chave: microrganismos marinhos; bioatividade; rastreio de antimicrobianos; rastreio de antioxidantes; valor comercial

Table of contents

List of Figures

List of Tables

List of Equations

List of Abbreviations and Acronyms

ALS	Amyotrophic lateral sclerosis
BHI	Brain-heart infusion
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
dNTP	Deoxyribonucleotide triphosphate
DPPH	2,2-diphenyl-1-picrylhydrazyl
EDTA	Ethylenediamine tetraacetic acid
FDA	Food and Drug Administration
FRAP	Ferric Reducing Ability of Plasma
HTS	High Throughput Screening
LB	Luria-Miller
LDL	Low-density lipoprotein
Leu	Leucine
LEU2	Leucine locus
LiAc	Lithium acetate
LPO	Lipid peroxidation
MAR	Mid-Atlantic Ridge
Min	Minute
NDA	New Drug Application
OD600	Optical density at 600 nm
ON	Overnight
PCR	Polymerase chain reaction
ROS	Reactive oxygen species
ROS	Reactive oxygen species
Rpm	Rotations per minute
SC	Synthetic Complete
SOD1	superoxide dismutase 1 yeast gene
sod1Δ	Yeast strain carrying a deletion of SOD1 ORF
TBE	Tris/Borate/EDTA
TLC	Thin-layer-chromatography
Ura	Uracil
URA3	Uracil locus
UV	Ultraviolet light
WHO	World Health Organization
Z’ factor	Z prime factor

Chapter 1. Introduction

1.1.The Marine hidden biodiversity as a hot spot for novel compounds

An enormous proportion of all life on Earth exists within the oceans[1]. Currently described marine species represent only 15% of all presently known species[2] and it is predicted that around 32% still remains to be discovered just in Europe' seas[3]. Even though **biodiversity within marine ecosystems** is only partly explored, it has been clearly identified to be an enormous source of innovation. The study of marine organisms increased significantly the amount of known natural products, with around 25 000 compounds of marine origin known[4] to date and, since 2008, more than 1 000 newly compounds are discovered each year[5], These data makes it clear that the marine environment is becoming a top spot of bioactive compounds. Searching in oceans for new sources of such innovative compounds may therefore open fresh perspectives in bioactive discovery[6] and subsequently an urge to find new and better ways to explore those compounds is constantly growing.

Marine bioactives have been known to be safer, cheaper, and less toxic[7]. In addition, Kong *et al*[8] showed that marine natural products are superior to the terrestrial in terms of chemical novelty as they are exclusively used by marine organism, rendering them unique survival skills needed in those extreme environments. Others have pointed an higher incidence of significant bioactivity in marine sources when compared with terrestrial organisms[9]. A great number of current drugs and other substances have their grounds in marine environment. Cytarabine, an anticancer agent, and vidarabine, an antiviral agent, for example, are one of the first synthetic marine derived drugs developed from extracts isolated from a Caribbean sponge *Cryptotethya crypta*, approved by the FDA in the 1970's[10]. One of the latest addition to the pharmaceutical market from the marine environment is Eribulin mesylate, which gained FDA approval in November 2010 for metastatic breast cancer[11]. Many other marine or marine-derived drugs are currently in clinical trials[12].

It is noteworthy that marine sources have demonstrated tremendous abilities as producers of pharmacological compounds, but other valuable bioactivities with other useful applications can also be found in marine environment. For example, in the food field, marine substances are being added to nutritional enrichment of regular food products[13]. Proteins from marine sources are being used in food products due to their film foaming capacity and gel forming ability[14]. In the cosmetic field, anti-aging, photo protective and anti-wrinkle marine microbial derived molecules were also discovered and developed[15]. Many more application examples exist, ranging from household products (like enzymes as laundry detergent additives[16]), textile applications (with alternatives to synthetic dyes[17]), agriculture applications (as antimicrobial for silkworm disease[18]) or even in civil construction (marine shells as a replacer of cement[19]) or in biomaterials (dental implants from chitosan[20] or bioplastics). Another great application of marine bioactivity is biofuels since algae biomass is already used to generate biodiesel, bioethanol, biogas, biohydrogen, and other biofuels[21].

Marine-based compounds currently known were isolated from a great variety of organisms that range from microorganisms, such as bacteria, to macroorganisms, such as fish. Microorganisms, despite being deeply exploited in terrestrial environments[22], are still a relatively minor source of marine-based compounds compared to other marine organisms[23].One way to maximize the chemical diversity available from marine sources, and their commercial potential, is to target microorganisms (as they are easier to collect and more suitable as to ecosystem impact) in new extreme habitats. Deep-sea hydrothermal vents are an example of extreme marine environment which are known to be an oasis of life and of unique biodiversity. These microorganisms live in a biologically competitive environment, characterized by physical extremes of temperatures (from 3ºC up to 400 ºC) and pressure, a complete absence of light and chemical variety, pH and temperature gradients. Marine microorganisms have

single abilities, which allow them to sense, adapt and respond to their environment quickly and can compete for defence and survival by producing exclusive secondary metabolites[24]. By definition, secondary metabolites are "compounds that are not involved in primary metabolism, but have a secondary role in the life of the producing organism", such as defensive chemicals[6]. Studies defined these metabolites as "chemical weapons" used to inhibit physical processes in preys, predators or other competitors, to survive in the marine habitat. Hence, it's only intuitive to argument that vent microorganisms must produce even more exclusive secondary metabolites to survive in these extreme habitats. So, in 2002, during the Portuguese research mission SEAHMA-I, five MAR (Mid Atlantic Ridge) sites along the Azores archipelago (Menez Gwen, Lucky Strike, Mount Saldanha, Rainbow and Menez Hom) were sourced and samples were collected. A series of microorganisms were isolated and propagated in the lab and extracts produced using different growth conditions. This library is a potential source of novel chemical bioactives and is currently screened for several market applications. This thesis work focused on a subset of a collection of extracts from these samples and developing assays to determine their bioactivity potential in anti-microbial and anti-oxidant applications. Previous work[25] by the group leader team has already confirmed bioactivity within this collection but much more possibilities exist yet.

1.2.Searching for innovative bioactive compounds

Searching for bioactives is a routine work for many labs and companies. According to Hu *et al.* (2015)[5], the most common **bioactivity** searched for is anti-cancer with more than 50 % of compounds found, followed by antibacterial compounds. Others like antioxidants, anti-inflammatory or unique and single targeted bioactivities are also on the radar. These type of bioactives have several commercial applications and their development path is different. Still, the initial phases of bioactive discovery are quite common across different types of applications.

The **bioactive discovery process** starts with a clinical and/ or commercial need that leads to the identification of a target[26]. Hughes et al. (2011)[26] defined target as a "range of biological entities such as proteins, genes and RNA with a specific role of interest". Once identified, the target is submitted to several validation techniques, and its role in the desired end need is established. After this process, the target can be used as a model against which to find new bioactives. At this stage a screening of a library of compounds with **biochemical or cell-based assays** is performed. The purpose of this stage is to find a **hit**, "a compound which has the desired activity in a compound screen"[26]. These hit compounds are then submitted to an optimization stage, to be evaluated in terms of potency, cytotoxicity and selectivity until a "lead compound" is selected for the development phase[26]. This stage is vital to anticipate possible side effects and, subsequently, to avoid costly downstream failures. The current drug discovery and development process is essentially divided into two phases: drug discovery and clinical drug development[27] (Figure 1.1). The initial drug discovery stage follows the same steps as described above and it is common to most market applications. The clinical stages include animal and human testing and these latter stages are very different in relation to other non-pharmaceutical applications.

Once a candidate compound meets all the desired criteria, it can proceed to the next development phases and from this phase onwards, specific methodologies are needed depending on to the market application. In the pharmaceutical and nutraceutical pipelines, the clinical phases are sequentially ordered to test safety in humans, first in a small non-patient and healthy group (Phase I), then in larger groups of patients (Phase II and III). When clinical phase trials are completed, a large set of data relating to safety, toxicity windows, efficacy levels and dosages, among others, are compiled in a New Drug Application (NDA) report, which is submitted to regulatory agencies[23].

On the other hand, the cosmeceutical development phase is shorter, with less costly and lower time-consuming too. Efficacy and safety trials are performed using cell-based and 3D printed skin models, since animal testing is abolished in most countries. Once a cosmeceutical is considered safe follows testing in a small group of healthy humans for a short period of time to evaluate the performance and efficacy of the product under study. Finally, the product formulation is registered by the owner and introduced in the market[23].

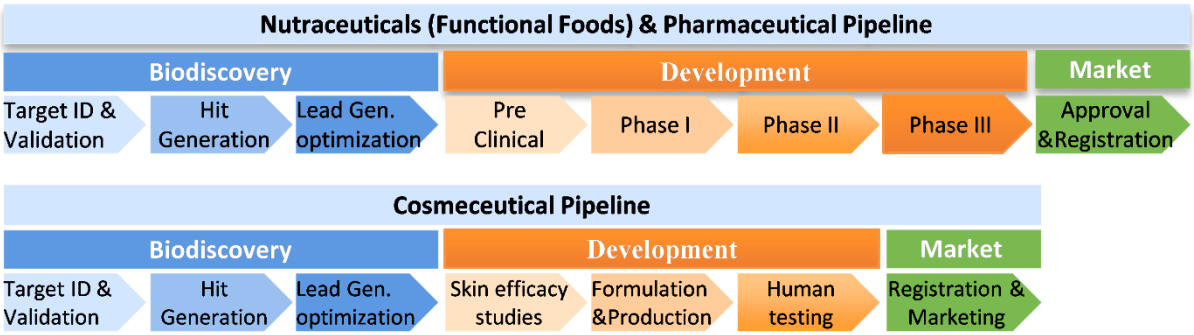

Figure 1.1 - Nutraceutical, pharmaceutical and cosmeceutical discovery and development process phases. Source: Grand Challenges in Marine Biotechnology, 2018

The search for bioactivity can be highly costing and time consuming[28], thus screening methods have evolved towards **High Throughput Screening (HTS)**. High throughput screening (HTS) is a new "faster" bioactive-discovery approach, that can help the industries reduce the final costs in the bioactive development phase[29]. The main goal of the HTS technique is to accelerate bioactive discovery by **screening large compound libraries in a shorter period** with the highest possible content of information. It involves several steps such as target identification, reagent preparation, compound management, assay development and high-throughput library screening as well as data analysis.

The pathway for bioactive discovery from natural sources faces several **challenges**, starting with the access to the marine environment followed by efficient screenings of the promising natural products, which are often isolated in very small amounts[6]. Sampling in difficult accessible spots can be a hardback, but the development of new sophisticated equipment, as remotely operated vehicles (ROVs) has been a great help[30]. However, this new technology is very expensive and only a small number of laboratories have access to it[31]. Another avenue researchers are tackling to increase success rates is to increasingly focus on microorganisms, since only a small sample is needed initially, and propagation is possible and more sustainable. Regarding supply there is also the challenge of manufacturing enough quantities to ensure a sustainable supply[32]. Total chemical synthesis[633] and microbial fermentation[34], as well molecular biology tools[35] are potential solutions being deployed and developed to solve the supply problem. Still a major challenge persists as most of the marine microbes are still hard to cultivate in the laboratory due to the difficulty in reproducing deep ocean characteristics.

Finally, one additional limitation of bioactivity discovery is the repeated discovery of known compounds due to the existing assay methods, leading to a need to increase the availability of novel techniques to maximize the discovery of new compounds[36].

1.3.Route to an HTS assay design

Compound screening can be laborious and time consuming, therefore in the recent years many developments have been made in order to foster, facilitate and obtain more information with less cost. High throughput screening is defined by the number of compounds tested to be in the range of 10 000 to 100 000 per day[37]. Several **criteria** must be considered to take a simple screening assay to the next level as a High Throughput Screening (HTS) assay, independently of the assay format. The factors to be considered are the following[26,38]:

i. Pharmacological relevance: prior to screening campaigns, the assay should be validated. When available, known ligands with activity at the target under study can be tested, in order to demonstrate if the assay is capable to identify compounds with the desired bioactivity;
ii. Reproducibility of the assay: the assay must be reproducible across assay plates, screening days and the entire drug discovery program;
iii. Assay costs: screening format (96, 384 or 1536-well microplates), reagents and assay volumes should be optimized in order to minimize the costs of the assay, maintaining the good quality of the assay;
iv. Assay quality: assay robustness can be determined by Z prime (Z') factor, which is a dimensionless parameter that considers the signal window and the variance that exists around such window;
v. Effect of compounds in the assay: chemical libraries are usually stored in dimethyl sulphoxide (DMSO), which is known to be toxic to cells at a certain concentration, therefore, the assay validation must be performed taking into account the solvent of the compounds as well the final concentration of the solvent in the assay. This factor can aid to identify false negative and positive compounds, and, therefore, to reduce further costs; if, for instance, the aim is to find an inhibitory action, without this solvent test, it's not possible to access if it's the solvent that is responsible by the inhibition or if it's the test compound.

Although an HTS assay comprises the screening of a large number of compounds per day [37], a simpler assay with less compounds tested should be performed in order to advent a transformation to a high-screening assay and check the criteria mentioned above. Hence, a validation of a non-HTS assay should be also performed based on its Z prime factor[39].

A screening assay should be divided into two stages: **the primary and secondary screening**. In the primary screening, a rapid screening is performed to narrow a compounds library to a number of compounds where it is most possible to get a hit. At this stage, the screenings are often performed using a single replica to diminish the costs. In the secondary screening, a dose-response assay and a triplicate replica are performed. To the hits identified in the primary screening, a confirmatory dose-response secondary screening is performed aiming to eliminate false positives and to define which hits were the best candidates for further studies and/or move on to a pre-clinical/development stage.

In this study we have applied the concept of HTS to all our screens and, whenever possible, performed primary and secondary screens.

1.4.Antimicrobial Screenings

Antibiotics or antimicrobial agents are "substances of biological, semi-synthetic or synthetic origin that inhibit the growth or kill bacteria" (ISO 20776-1:2006.).

Since Alexander Fleming and the discover of penicillin[40], the search for other similar antimicrobial compounds in nature proceeded, both in terrestrial[41] and marine[42] environments. However, the development of antibiotics has been followed by the emergence of bacterial strains resistant to such antibiotics[43]. Antibiotic resistance has an impact not only on healthcare, but it also has an important economic impact[36], making the development of new antibiotics a top priority. In February 2017, the World Health Organization (WHO) published a list of bacteria for which new antibiotics are urgently needed, to help in prioritizing the research and development of new and effective antibiotics. The list underline Enterobacteriaceae family pathogens (as *Klebsiella*, *Enterococcus* and *Shigella* genus) and *Staphylococcus aureus* as critical pathogens[44]. This list helps guiding research related to new antibiotic by allowing research to target just some specific bacterial strains with medical needs.

Antimicrobial activity of natural compounds can be detected by observing the growth response of various microorganisms to natural extracts samples that are placed in contact with them. Currently, there are several methods for antimicrobial discovery being performed and it is a continuously evolving field[23]. For a long time, the platform that delivered the majority of antibiotic to medicine was the Waksman platform. This consists on the screening of microorganisms from soil by growth in different culture media followed by bioactivity assays to identify the compounds that inhibit the growth of specific bacteria[45]. However, after sometime this platform started to return old known compounds, which reduced the efficiency and adaptations of this screening platform arised[42,46]. Maes *et. al* (2006)[47] classified the antimicrobial test methods into three main groups: diffusion, dilution and bio-autographic methods. In the diffusion method, filter paper discs containing the test compound at a known concentration are brought in contact with an inoculated medium and the diameter of the inhibition growth zone around the compound is used as measure of antibacterial activity. In the dilution methods, test compounds are mixed with a medium that has previously been inoculated with the test organism, and microbial growth can be measured by turbidity (optical density) or redox-indicators. At last, bio-autography methods measure antimicrobial activity on a chromatogram[47], where the most con is the limitation to microorganisms that easily grow on thin-layer-chromatographic (TLC) plate. Today, target specific screens are more widely used.

In this thesis work, the chosen anti-microbial screening assay was the dilution method with microbial growth followed by the optical density measurements. Primary and secondary screenings were performed.

1.5.Antioxidant Screenings

Oxidation is a chemical reaction that transfers hydrogen or an electron from a substance to an oxidising agent, and this type of reaction can generate free radicals (also called reactive oxygen species or ROS). ROS include superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\cdot}$), hypochlorite (OCl^-) and peroxynitrite ($ONOO^-$) [48]. Imbalance of free radicals can cause structural and functional changes in biomolecules (DNA, lipids, proteins). In humans, it can lead to a great number of pathologies, chronic diseases such as cardiovascular diseases, cancer, diabetics and aging, and some degenerative diseases as Parkinson's and Alzheimer's disease[49] or Amyotrophic Lateral Sclerosis (ALS)[50]. Antioxidants are compounds capable of helping the organism own defences against free radicals damage. The organism itself has its own mechanisms to fight ROS, by producing own endogenous antioxidants. Enzymes, such as catalases, glutathione peroxidases, superoxide dismutases (SOD), or nonenzymic compounds, such as uric acid or metallothionein are known examples. Insufficient levels of endogenous antioxidants can cause oxidative stress, an imbalance between oxidants and antioxidants resulting in cellular damage. Moreover, when these endogenous mechanisms fail to ensure the protection of the organism against ROS, a need for exogenous antioxidants arises. Exogenous antioxidants[51] can

be synthetic compounds, as butylhydroxyanisole but can also be found in natural sources, as vitamin C found in citrus fruits.

Besides the medical application, **antioxidants** have many other applications such as cosmetics or food related applications. ROS molecules can damage the human skin, leading to skin disorders[52] and premature aging of the skin[53] and antioxidants can help protect the skin against these effects[54]. There are some synthetic commercial antioxidants in the market being used to help protect the skin against oxidation, but there are some health hazards associated to them[55], hence the search for natural antioxidants can be a safer alternative in the cosmetic field. Oxidation can also arise in foods during harvesting, processing and storage, giving rise to the development of unpleasant flavours[56], loss of essential fatty acids, fat-soluble vitamins and other bioactives, and formation of potentially toxic compounds, consequently making the lipid or lipid-containing foods inappropriate for consumption[57]. As in cosmetic, the use of some synthetic antioxidants in conservation of food are considered harmful and have been limited. Thus, nowadays there are substantial need for natural antioxidants applied in this industry[58–61].

There are several methods used to find antioxidant property in unknown bioactive samples. The most popular strategy to determine the antioxidant activity of a certain compound *in vitro* is to directly measure the ability to scavenge specific free radicals. The **DPPH test** is a rapid, simple (not involving many steps and reagents) and less expensive method in comparison to other methods[62], which makes this one of the most widely chosen methods to study the antioxidant activity of natural compounds and therefore one of the selected assays to be used under this study.

However, the immediate translation of the antioxidant results from these type of *in vitro* biochemical assays towards applications in humans can be dubious and unpredictable, Thus, cell- or organisms-based models for screening antioxidants have been more recently considered a better choice over *in vitro* testing. In these, biological models, such as yeast, can succeed to the preliminary, but not necessarily dispensable due to cost constraints, *in vitro* chemical tests in the assessment of natural antioxidants compounds[63]. In *in vivo* methods, the samples that are to be tested are usually administrated to either living cell culture or testing animals (as mice, rats) with a specific dosage. These can include methods as lipid peroxidation (LPO) assay[64], the most used *in vivo* assay, and LDL assay[65]. Other assays, with specific unique targets can also be devised.

SOD1 is an antioxidant gene and its absence can be used to test antioxidant activity, as described in literature[66–68]. The superoxide dismutases (SODs) are the first and most important line of antioxidant enzyme defence system against ROS in a cell. This enzymatic complex is responsible for metabolizing superoxide radicals into oxygen and H_2O_2, which is further converted to water by catalase, peroxiredoxins and glutathione peroxidases[69], as shown in Figure 1.2. There are three mainly distinct isoforms of SOD identified in mammals: SOD1, SOD2 and SOD3. SOD1 is the major intracellular SOD, mainly localized in cytosol[70] with a smaller fraction in the intermembrane space of mitochondria. This enzyme binds to molecules of copper and zinc to break down toxic and charged oxygen molecules called superoxide radicals[71]. The most commonly studied connections between SOD1 and human diseases proposes a relation between SOD1 mutations and a neurogenerative disease called amyotrophic lateral sclerosis (ALS) [72].

SOD1 is highly conserved across all eukaryotic phyla and is present in all cells and tissues, where it is believed to act as a first line of defence against toxicity of superoxide anion radicals[73]. *Saccharomyces cerevisiae* is a unicellular eukaryotic organism widely used as a model for cellular and molecular biology research, since it's easily cultured and divide quickly in a short generation time of a matter of hours[63]. *Saccharomyces cerevisiae* is, therefore, considered to be an optimal model to study stress responses

because of the following factors: i) budding yeast genome presents a high degree of homology with the human genome; ii) there are many proteins that show an elevated functional homology with specific human proteins; iii) it is a system whose genetic manipulation is reasonably easy and cheaper than other higher eukaryotic models; iv) having an haploid state facilitates the study of multiple processes, especially to study gene function –a single copy of the genome, being impossible to mask the effect of mutations; v) is one of the few eukaryotic organisms with a most complete database[74].

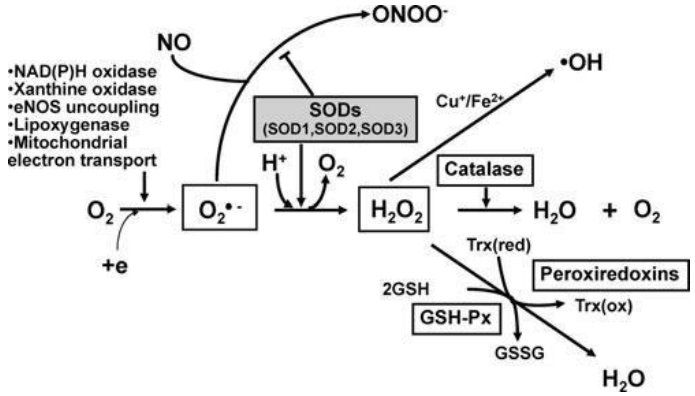

Figure 1.2 - Generation and metabolism of reactive oxygen species. Source: Fukai and Ushio-Fukai, 201163

1.6.Major applications and development paths

There is a continual need for new **bioactives** agents, with **application** in many different **market** sectors, from material sciences to industrial applications, ranging from cosmetics, nutraceutical and especially pharmaceutical applications, namely to treat a large variety of diseases for which there are no effective therapies. The answer to these needs can be in unique marine environments, and most likely in microorganisms, as they can survive in such severe conditions and can more easily guarantee a sustainable collection and manufacturing of such bioactives.

Marine microorganisms constitute a new promising source of a unique and diverse bioactivity. Some of the currently known applications of marine microorganisms derived bioactives are shown in Table 1.1.

Table 1.1 – Some examples of applications of marine microbial bioactives

Name of bioactive	Isolated from	Mechanism of action	Reference
3,4-dihydroxyphenylalanine (DOPA)-melanin	*Aeromonas media*	Photoprotective agent for bioinsecticides	[75]
Abyssine® (EPS HYD657)	*Alteromonas* spp. extract	Used in cosmetic for its anti-inflammatory and anti-UVB properties	[76]
Actiporine 8G®	Microalgae *Jania rubens* active	Anti-ageing, antipollution, detox and slimming activity	[76]
Aquastatin A	*Cosmospora* sp. SF-5060	Antidiabetic activity, by inhibition of protein tyrosine phosphatase 1B (PTP1B)	[77]
Aspergiolide A	*Aspergillus glaucus*	Anti-tumour activity	[78]
Cellynkage®	*Halomonas eurihalina* EPS	Menopausal rejuvenator, collagen inducer	[76]
Macrolactin S	*Bacillus* sp.	Antibacterial activity against *E. coli*, *S. aureus* and *B. subtilis*	[79]
Mycosporine-like amino acids (MAAs)	Actinomycetales Microorganisms	Antioxidant activity, scavenging activity of superoxide anions and inhibition of lipid peroxidation	[80]
Prodigiosin-like pigment	*Serratia* sp. BTWJ8	Used to dye textile materials	[17]
SILIDINE®	Microalgae *Porphyridium cruentum* extract	Shooting, anti-inflammatory, heavy legs (endothelin -I stimulator) anti-Rosacea, (endothelin-I stimulator)	[76]

1.7.Main goal and specific objectives of this thesis

The major goal of this thesis work was to develop assay platforms to screen bioactive compounds with antimicrobial and antioxidant potential from marine sources.
Specifically, this work aims to:

i. Develop assay platforms to screen antimicrobial and antioxidant bioactivity, exploring microbial, biochemistry and molecular biology tools;
ii. Validate the newly implemented platforms and protocols of each screening assay;
iii. Screen a large collection of marine natural extracts from the Portuguese ocean and hydrothermal vents;
iv. For positive hits extracts, further development was designed targeting dose-response studies with various bioactive concentrations to study their efficacy and toxicity;
v. Statistical analysis and hit prioritization for subsequently commercial prioritization development.

Chapter 2. Material and Methods

The following sections list the items necessary for performing all experiments in the scope of this work.

2.1. Material

2.1.1. Reagents

Concerning molecular biology procedures, the reagents used were acquired to several suppliers. The Taq DNA Polymerase, dNTPs, PCR buffer, O´Gene Ruler 1 Kb DNA ladder,1 kb plus DNA ladder, Ultrapure agarose and 5x TBE (Tris/Borate/EDTA) were obtained from Invitrogen Life Technologies, ThermoFisher Scientific, Waltham, MA, USA. The loading buffer was purchased from Takara, Kusatsu, Japan. For the SOD1 assay, the ascorbic acid was acquired from Merck Millipore, Billerica, MA, USA. Restriction enzyme Hind III and the respective buffer were purchased from New England Biolabs (NEB), Ipswich, MA, USA.

For microbiology procedures (bacterial and yeast cultures), brain-heart infusion (BHI) medium, yeast extract, bacteriological agar type E were purchased from Biokar Diagnostics, France. Bacto yeast nitrogen base without amino acids and Luria-Miller (LB) medium were purchased from Sigma-Aldrich, St. Louis, MO, USA. D-Glucose anhydrous was acquired from Merck Millipore, Billerica, MA, USA.

For biochemical procedures, the compound 2,2-diphenyl-1-picrylhydrazyl (DPPH) was acquired from Sigma-Aldrich (St, Louis, MO, USA) and the methanol was acquired from Carlo Erba, Chaussée du Vexin, France.

2.1.2. Cells

2.1.2.1. Bacterial Strains

Microorganisms were obtained from the culture collections of the Microbiology & Biotechnology group of Biosystems and Integrative Sciences Institute (M&B-BioISI). The microorganisms used are: *Escherichia coli* ATCC 25923, *Salmonella typhimurium* ATCC 14028, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* DSMZ 20376T, *Enterococcus* sp. (*vanA*+), *Klebsiella oxytoca*, *Shigella* sp., and *Salmonella enteritidis*.

2.1.2.2. Yeast Strains

The yeast strains used are described in Table 2.1 and were generously provided by Dr. Lisete Fernandes (Faculty of Sciences, University of Lisbon). The Δsod1 single null mutant[81] was made by the one-step gene disruption procedure described previously[82].

Table 2.1 - Yeast strains used in this study

Yeast Strain	Genotype	Reference
EG103	(DBY746) MATα leu2-3, 112 his3Δ1 trp1-289 ura3-52 GAL+	81
EG118	EG103 with sod1Δ:: URA3	81

2.1.3. Plasmid

The yeast high-copy bi-directional expression episomal plasmid pESC-LEU (Stratagene, La Jolla, CA, USA). This plasmid contains two promoters, GAL1 and GAL10, in opposing directions, and the

auxotrophic selection market LEU2. Culture media for yeast strains transformed with this plasmid lacks, therefore, the amino acid leucine.

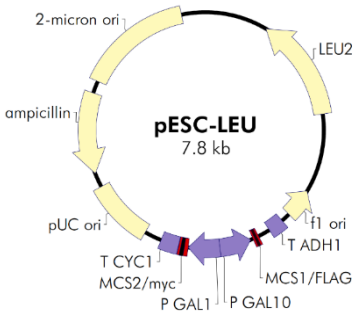

Figure 2.1 - Map of the pESC-LEU vector. Source: https://www.agilent.com/cs/library/usermanuals/public/217451.pdf

2.2.Methods

2.2.1. Cells media, growth and storage

2.2.1.1.Bacterial Strains and growth

Bacterial cells were grown in BHI. Bacterial cells were routinely cultivated at the optimal growth temperature of 37ºC for 16-18h (overnight, ON). For liquid cultures, agitation at 160 rpm was used. For growth in solid media, agar was added to the media (15 g/l). For long-term storage, bacterial strains were cryopreserved with glycerol (15% final concentration) and kept at -20 ºC and -80ºC.

2.2.1.2.Yeast media and growth

Yeast strains were cultured in Synthetic Complete (SC) media (6,7 g/l Bacto-yeast nitrogen base without amino acids; 20 g/l glucose; 2 g/l drop out mix). *S. cerevisae* ΔSOD1 colonies were isolated on SC media minus uracil. Yeast cells were routinely cultivated at the optimal growth temperature of 28ºC for 2-3 days. For liquid cultures, agitation at 220 rpm was used. For growth in solid media, agar was added to the media (20 g/l). For long term storage, yeast strains were cryopreserved with glycerol (15% final concentration) and kept at -20 ºC and -80 ºC.

2.2.2. Molecular biology methods

The molecular cloning of SOD1 gene of *S. cerevisae* was initiated to revert sod1Δ:: URA3 yeast and some steps are described below.

2.2.2.1.DNA extraction

2.2.2.1.1. Genomic DNA extraction

Yeast DNA was isolated by GES Method adapted from Pitcher et al. 1989[83]. Several colonies of yeast cells were resuspended in lysis buffer (50 mM Tris; 250 mM NaCl; 50 mM EDTA; 0,3 % SDS; pH8,0). A volume correspondent to 100 µl of microspheres was added and then vortex with maxim velocity for 2 minutes. The suspension was incubated at 65 ºC for 30 minutes, and then agitated by vortexing with maximal velocity for 2 minutes. After that, 250 µl of GES reagent (5 mM Guanidium Thiocyanate; 100 mM EDTA pH 8,0; 0,5% v/v Sarcosil) were added to the mixture, then shake by inversion and cooled on ice for 10 minutes. Subsequently, an aliquot of 250 µl of cold 10 mM NH_4Ac was added and cooled on ice for another 10 minutes. The mixture was added with 1 ml chloroform/ isoamyl alcohol (24:1), mixed by inversion and then centrifuged (maximal velocity, 10 min). The supernatant (the upper layer of fluid) was decanted into a new tube, added and mixed with one half amount of isopropanol and DNA. The DNA pellet was washed with 70% ethanol and centrifuged. The supernatant was discarded and, after air drying it was dissolved in 100 µL of Tris-EDTA (TE) buffer.

2.2.2.2.Plasmid DNA extraction

The plasmid DNA extraction was performed using JetStar 2.0 Plasmid Purification MiniPrep Kit (Genomed, USA), following recommended guidelines. This kit allows the DNA extraction by gravity flow. The DNA was dissolved in a suitable TE buffer (10 mM Tris; 1mM EDTA; pH 8).

2.2.2.3.Quantification of DNA concentration

Quantification of DNA was performed by fluorometry using the Nanodrop Spectrophotometer ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA), using1 µl of the DNA sample, following manufacturer's instructions. Quantification of DNA thought analysis of agarose gel lanes with adequate agar concentration loads was performed using also ImageJ software.

2.2.2.4.Agarose gel electrophoresis and DNA gel extraction and purification

Routine analysis of DNA was performed using agarose gels (0.7% - 1.5% w/v, depending on application) cast with 1x TBE (Tris/Borate/EDTA) buffer. Electrophoresis was performed using mini or medium EasyCast horizontal apparatus (OWL Separation system, Thermo Fisher Scientific, Waltham, MA, USA) at a constant voltage of 70 V-90 V (depending on the gel concentration and size of the gel) until the desired separation was achieved. Agarose gel was stained in an 0,5 µg/ml ethidium bromide solution. DNA fragments size was estimated by including in each DNA electrophoresis 5 µl of O´Gene Ruler 1 Kb DNA ladder and/or Invitrogen 1 Kb plus DNA ladder. DNA was visualised using the Uvitec image acquisition system (UVItec Cambridge, Cambridge, UK). Excision of DNA fragments of the desired size from the agarose gels was routinely performed on an UVIvue transilluminator (UVItec Cambridge, Cambridge, UK) with the help of a blade. DNA extraction and purification from low-melt agarose gels in 1x TBE was performed with Freeze 'N Squeeze DNA gel extraction spin columns (Bio-Rad Laboratories, Berkeley, California, USA), following manufacturer's instructions.

2.2.2.5.Polymerase Chain Reaction (PCR)

PCR was used for amplification of DNA from a variety of sources.

For routine use, Taq DNA polymerase was used. Synthetic oligonucleotides (primers) were resuspended in sterile MilliQ H2O at the final concentration of 50 µM. Typical PCR reactions contained 50-100 ng of template DNA, 0.2 mM of each dNTP, 0.5 µM of each primer, 1x PCR reaction buffer (as supplied by the manufacturer) and 0.5 units of Taq DNA polymerase per 50 µl reaction. Since the reaction buffer did not contain magnesium chloride, it was added usually to a final concentration of 0.2 mM. A negative control (PCR reaction without DNA) was routinely performed for each PCR mix.

Reactions were performed using a T Gradient (Biometra, Germany) and UNO II (Biometra, Germany) thermocycler. Typical cycling conditions consisted of 94ºC denaturation step for 10 min, followed by 35 cycles of denaturation at 94ºC for 30 secs, annealing for 40 sec, with temperatures depending on the pair of primers melting temperature, and extension at 68ºC for high fidelity polymerases or 72ºC, for routine use polymerase, for 60-90 sec (depending on the size of the amplicon). A final extension step of 72ºC for 10 min was included. Successful amplification was confirmed via agarose DNA electrophoresis.

2.2.2.6.Restriction digestion

Single restriction digestion reactions were performed using HindIII according with the manufacturer's instructions. Successful digestion of DNA was confirmed via agarose DNA electrophoresis relative to undigested DNA.

2.2.2.7.Competent DH5-α *E. coli* cells

Chemically competent *E. coli* were generated by treatment with calcium chloride, using standard protocol in Sambrook and Russel [84]. A 100 ml starter culture of cells DH5-α was grown in LB media at 37 ºC, 180 rpm agitation for 3 hours, until it reached an optical density at 600 nm (OD_{600}) inferior to 0.4. The starter culture was divided in two ice-cold 50 ml Falcon tubes. Cells were cooled on ice for 10 min and pelleted at 2700 g for 10 min at 4 ºC. The supernatant was removed, and the pellet gently resuspended in 30 ml of ice-cold $MgCl_2$-$CaCl_2$ solution (80 mM MgCl2; 20 mM $CaCl_2$). Cells were pelleted by centrifugation at 2700 g for 10 min at 4 ºC and gently resuspended in 2 ml of ice-cold 0.1M $CaCl_2$ for each 50 ml of the starter culture. To storage the cells, 140 µl of DMSO were first added to each 4 ml of resuspended cells and the suspension were cooled on ice for 15 min. More 140 µl of DMSO were added to the suspension and aliquots of 50 µl, 100 µl and 200 µl were dispensed into pre-chilled and sterile microcentrifuge tubes and snap-frozen in dry-ice prior to storage at -80 ºC. When needed the frozen tubes should first be thawed by holding the tubes with the palm of the hand and when the cells are unfrozen, they should be storage in ice for 10 min before using it.

2.2.2.8.Transformation of *E. coli*

For transformation of *E. coli*, frozen 100 µl of competent *E. coli* were thawed with the hand and then keep it on ice. 5 µl of DNA solution (pESC-leu) was added to 100 µl of competent *E. coli* cells in ice-cold microcentrifuge tubes, and cells were incubated on ice for 15 min. Cells were heat shocked for 45 sec in a 42 ºC water bath and then cooled on ice for 2 min. Cells were recovered by incubation in 900 µl of Super Optimal broth with Catabolite repression (SOC) medium, at 37 ºC, 225 rpm agitation, for 1 h. Cells were plated onto selective LB media plates with 100 µg/ml ampicillin. Positive colonies were observed after overnight incubation at 37 ºC. To determine the transformation efficiency, pUC19 control DNA was used. Protocol described at MCLAB – Molecular Cloning Laboratories (www.mclab.com).

2.2.3. Characterization of yeast strains

To confirm the genotype of the sod1 yeast strains, microbiology and molecular biology techniques were performed.

2.2.3.1.Yeast growth analysis

Prior to the screening, a liquid growth evaluation assay was performed to confirm the phenotype of the yeast strains and to choose the best starting OD_{600} to perform future bioactive discovery screenings. The growth of yeast strain sod1Δ::URA3 versus the WT strain (DBY746), was evaluated in culture media containing or not uracil and supplemented, or not, with peroxide hydrogen, a stress inducer, (H_2O_2 1mM), and ascorbic acid (AA; 5 mM).

Yeast strains were pre-inoculated on liquid SC media, lacking the uracil amino acid, depending on the strain. Cultures were incubated at 28ºC with agitation (220 rpm). Yeast OD_{600} was monitored using an WPA UV 1101 Spectrophotometer (OD measure single cuvette). After ON growth, yeasts were inoculated at a starting OD_{600} 0.1 in SC-URA media, and SC+URA, respectively, and incubated at 30 ºC or 37ºC in 96-well plates (200 µl final volume). Yeast cells were grown in a LiCONiC STX40 Automated Incubator (Perkin Elmer, Waltham, MA, USA) and growth was manually monitorized by measuring OD_{595} using a Victor 3V microplate reader (Perkin Elmer, Waltham, MA, USA).

2.2.3.2.Confirmation of SOD1 deletion

For genetic confirmation of yeast strains, standard PCR reactions were used to amplify the coding sequences of interest, SOD1 gene, with appropriate primers (Table 2.3). To evaluate the quality of the extracted genomic DNA, a PCR amplification of the internal control gene, Small Subunit of Ribosomal RNA (SSU), was performed, using specific primers. For each pair of primers, a PCR negative control without gDNA was performed.

Table 2.2 - Primer and PCR protocols of SOD1 gene and small subunit of rRNA

Locus	Primer	Primer Sequence 5' – 3'	Direction	PCR protocol	Reference
SOD1 gene (~600 bp)	SOD1F	CAGGCAAGAAAGCAATCGCG	Forward	1. 94 ºC – 5 min 2. 95 ºC – 1 min 3. 57 ºC – 1 min 4. 72 ºC – 1 min 5. Repeat 2-4 for 30 cycles 6. 72 ºC – 1 min 7. 4 ºC on hold	Design primers
	SOD1R	GGACATAAATCTAAGCGAGGG	Reverse		
Small Subunit (SSU, 18S) of the rRNA (~1200 bp)	NS1	GTA GTC ATA TGC TTG TCT C	Forward	1. 95˚C – 5 min 2. 94˚C – 30 s 3. 52˚C – 30 s 4. 72˚C – 1 min 5. Repeat 2–4 for 35 cycles 6. 72˚C – 8 min 7. 4˚C on hold	[85]
	NS4	CTT CCG TCA ATT CCT TTA AG	Reverse		

2.2.4. Bioactivity Screenings

2.2.4.1.PharmaBug marine microbial collection

The extracts used in this study are part of a marine bacteria collection, named PharmaBUG. The samples of this collection were isolated from four hydrothermal fields in the Mid-Atlantic Ridge (MAR) north of the Azores archipelago, within the Portuguese Continental Shelf and under Portuguese Jurisdiction. The five hydrothermal fields are called (1) Menez Gwen, that is characterized by small chimneys that result from calcium sulphate precipitation, with temperatures that can reach 265-281 ºC and with pH values between 4.2 and 4.8; (2) Rainbow, has the higher hydrothermal activity, localizes 2300 meters deep and also has black smoker type chimneys, expelling fluids at 360 ºC that are rich in H_2, CH_4 and metals such as copper and zinc; (3) Lucky Strike, one of the biggest hydrothermal fields known, localizing 1550 to 3000 meters deep and can reach 333 ºC; and (4) Monte Saldanha, is a 2200 meters deep vent, characterized by low temperature and high levels of methane, where the fluids expelled are rich in metal oxides and sulphates. After isolation, bacterial isolates were grown in a commercial culturing media (0.5% peptone (w/v), 0.3% meat extract (w/v)) supplemented with 3% sea salts) and their biomass production optimized for potential scale up.

The selected isolates were adapted to controlled laboratory growth conditions and both aqueous and organic extracts present a 25 mg/ml final concentration.

Table 2.3 – Characteristics of the hydrothermal vents. Mbsl – meters below sea level. ND – not defined

Hydrothermal vent	Depth (mbsl)	Maximum temperature (º C)	pH	Constituents	Type of samples collected
Menez Gwen	840 - 865	281	4,2 – 4,8	Small chimneys that result from calcium sulphate Precipitation	Water, sediments, small animals, rocks and chimney samples
Rainbow	2270 - 2320	362	2,8	Enriched concentrations of H2, CH4, Fe, and chloride	Water, small animals and chimney samples
Lucky Strike	1600 - 1740	333	3,8 – 4,5	Fluids depleted in sulfides but enriched in methane	Sediments, Small animals and chimney samples
Monte Saldanha	2300	7 - 9	ND	Deposits on serpentinized peridotite and gabbro	Water and sediments

2.2.4.2.Antimicrobial Screening

2.2.4.2.1. Selection of bacteria

Starting from the WHO list of critical pathogens cited previously[44], of multidrug resistant bacteria, including carbapenems and 3rd generation cephalosporines, and the ones available in our laboratory, seven bacterial strains were selected. The WHO list is organized in a priority scale: priority 1 (critical), priority 2 (high) and priority 3 (medium), based on how deadly the infections they cause are, the treatment duration, the frequency of the resistance in the community, the tenderness to spread between humans and animals, and other criteria, but special urgency of the need for new antibiotics. The *Enterococcus faecalis*, *Enterococcus* sp. vancomycin resistant (VanA+) and *Klebsiella oxytoca* were chosen since they belong to the Enterobacteriaceae family, included in the priority 1 list. The *Staphylococcus aureus*, *Salmonella typhimurium* and *Salmonella enteritidis* are included in priority 2 group. And finally, *Shigella* sp. was chosen in representation of priority 3 group.

2.2.4.2.2. Primary screening

Marine microbial extracts bioactivity against known bacterial pathogens was tested by using broth method as described below. The chosen microorganisms to screen were: *Salmonella typhimurium* ATCC 14028, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* DSMZ 20376T, *Enterococcus* sp. (*vanA*+), *Klebsiella oxytoca*, *Shigella* sp., and *Salmonella enteritidis*.

An overnight (ON) 30 ml culture from each bacterial strain was grown until it reached an optical density at 600 nm (OD_{600}) of ≥ 1. The organic and aqueous extracts of the marine derived library were then screened in 96-well assays, using 20 µl of bacterial culture and the appropriate dosage of marine extract in a final volume of 200 µl. Organic extracts (OE) and aqueous extracts (AE) were tested at a concentration of 0,5 mg/ml and 0,25 mg/ml, respectively for the primary screening. Negative control corresponds to ON culture plus media, without extract. The antibacterial activity was determined by measuring the Optical Density (OD) at 595nm of the assay plates at 0h, 2h, 4h, 6h, 8h and 24 hours after the beginning of the experiment. Positive control corresponds to a concentration of antibiotic capable of inhibiting the bacterial growth plus ON culture plus media. All the assays were made with a proportion of ON culture/media of 1:10. Positive hits were selected using the criteria shown in Table 2.5.

Table 2.4 - Hit classification of primary antimicrobial screening

	Ranking
Extract OD_{595} ≤ positive control OD_{595} at 8 h and/or at 24 h	Excellent
Extract OD_{595} ≤ positive control OD_{595} + 0.2 at 8 h and/or at 24 h	Good
Extract OD_{595} ≤ positive control OD_{595} + 0.3 at 8 h and/or at 24 h	Fair

2.2.4.2.3. Secondary Screening

The hits identified in the primary screening were confirmed in a dose-response assay, where marine extracts were tested in three to five concentrations, depending on extract availability: 0.1, 0.25, 0.5, 0.75 and 1 mg/ml.

To be considered a hit at the secondary screening, an extract had to inhibit the growth of bacteria above a certain threshold OD_{595}, represented by Equation 2.1.

$$\boldsymbol{Threshold = (M + SD)NC}$$

Equation 2.1 - Equation used to calculate the minimal threshold (OD_{595}) for determination of marine microbial hits in the secondary antimicrobial assay. M stands for minimum, SD for standard deviation and NC for negative control

Inhibition rates (%) were calculated in the secondary screening at different periods of time (0h, 2h, 4h, 6h, 8h and 24h), using the following equation:

$$\boldsymbol{Inhibition\ rate}\ (\%) = \mathbf{100} - \frac{(\boldsymbol{OD_{595}(extract)} - \boldsymbol{OD_{595}(positive\ control)})}{\boldsymbol{OD_{595}(negative\ control)} - \ (\boldsymbol{OD_{595}(positive\ control)}} \times \mathbf{100}$$

Equation 2.2 - Formula to calculate the inhibition rate (%) of the microbial growth

2.2.4.3.Antioxidants Screenings

2.2.4.3.1. DPPH assay

The DPPH assay was done according to the method of Brand-Williams *et al.* (1995)[86] with some modifications. Optimization of the test was made using ascorbic acid with several concentrations (0; 10; 20; 40; 60; 80 and 100 µg/ml) and DPPH 0,5 mM. The extracts were screened in 96-well plates at final concentration of 1mg/ml with a final assay volume of 50 µl. In summary, the extracts were incubated with DPPH for 10 min in the dark at room temperature and the resulting reaction measured spectrophotometrically at 520nm, using a Microplate Reader Zenyth 3100 (Salzburg, Austria).

DPPH reduction rate were calculated using the following formula:

$$\boldsymbol{DPPH\ reduction}\ (\%) = \frac{\boldsymbol{Abs\ control} - (\boldsymbol{Abs\ sample} - \boldsymbol{Abs\ blank})}{\boldsymbol{Abs\ control}} \times \mathbf{100}$$

Equation 2.3 - Formula to calculate the DPPH reduction rate, Where Abs control - DPPH without sample and with 20µl H2O; Abs sample - Test sample plus DPPH; Abs blank - Test sample plus methanol

2.2.4.3.2. SOD1 assay

Saccharomyces cerevisae model system[66–68] was adapted to the yeast strains existing in the laboratory, shown in Table 2.1, in order to study the antioxidant potential of the compound library in study. The yeast strain sod1Δ::URA3 was used as a screening system for the identification of molecules with a bioactivity capable of, fully or partially, replace the SOD1 antioxidant function. The screening was designed to search for natural products that restored the growth of sod1Δ::URA3 yeast strain closer to the levels of the control strain DBY746, by calculating the growth recovery percentage. To be considered a hit, an extract had to rescue the growth of sod1Δ::URA3 above a certain threshold OD_{595}.

The yeast strain sod1Δ::URA3 was pre-inoculated on liquid SC media lacking the uracil amino acid at 28ºC with agitation (220 rpm) on an overnight period. Thereafter, yeast cells were reinoculated in fresh media with a ratio of pre-inoculum/ media of 1:10 in 96-well plates. The extracts were screened at a concentration of 0,5 mg/ml with one replica. The positive control corresponds to the *S. cerevisae* growth in H_2O_2 supplemented with acid ascorbic 50 mM and the negative control corresponds to the same conditions without ascorbic acid. All reagents were added to the plates using multichannel electronic pipettes. Inoculated 96-well plates were incubated in a LiCONiC STX40 Automated Incubator (Perkin Elmer, Waltham, MA, USA), with continuous agitation, and growth was manually monitored by measuring OD_{595} using a Victor 3V microplate reader (Perkin Elmer, Waltham, MA, USA) for 54 hours.

To be considered a hit, an extract had to rescue the growth of sod1Δ yeast above a certain threshold OD_{595}, considering values higher than (M+SD) sod1Δ yeast + H_2O_2 (negative control) plus 25% of the signal dynamic range (M+SD) sod1Δ yeast + AA (positive control) - (M+SD) sod1Δ yeast + H_2O_2 (negative control).

$$\boldsymbol{Threshold} = \boldsymbol{(M+SD)NC} + \frac{[(M+SD)PC - (M+SD)NC]}{4}$$

Equation 2.4 - Equation used to calculate the minimal threshold (OD_{595}) for determination of marine microbial hits in the antioxidant discovery assay using sod1Δ yeast strain. M stands for maximum, SD for standard deviation, NC for negative control and PC for positive control

The recovery rate (RR (%)) was calculated according to the following equation:

$$\boldsymbol{RR}\ (\%) = \frac{\boldsymbol{OD_{595}(E, t = x) - OD_{595}(E, t = 0h)}}{\boldsymbol{OD_{595}(NC, t = x) - OD_{595}(NC, t = 0h)}} \times \mathbf{100} - \mathbf{100}$$

Equation 2.5- Formula to calculate the recovery rate (RR) of the yeast, Where E corresponds to sample extract and NC to negative control

2.2.4.4.Data Analysis

The evaluation of the overall quality of the antimicrobial and SOD1 assay was performed using the Z-prime factor (also known as Z'-factor)[39]. The Z'-factor is a dimensionless parameter that considers the assay signal dynamic range and the data variation associated with samples, without intervention of test compounds[38], as shown in the equation below.

$$\mathbf{Z'\text{- factor}} = \mathbf{1} - \frac{\mathbf{3}\left(\boldsymbol{SD_p} + \boldsymbol{SD_n}\right)}{\left|\boldsymbol{A_p} - \boldsymbol{A_n}\right|}$$

Equation 2.6 - Z prime factor (Z') equation applied in the antimicrobial and SOD1 assay, Where "SD" stands for standard deviation, "A" for average, "p" for positive control and "n" for negative control. Positive control defines the set of individual assays from control wells that gives the maximum signal and negative control refers to the set of individual assays from control wells that gives the minimum signal

According to Zhang *et al.*[38], a higher Z' factor corresponds to an higher confidence on the data obtained in the assay performed as HTS. Z'- factor values equal to 1 correspond to the ideal assays, with high signal dynamic range and low variation of references measurements. Z' values below 1 and superior or equal to 0.5 are considered excellent assays. Z' values bellow of 0.5 are considered marginal assays, but some assays can still be used with care; and negative values classify assays as unsuitable for HTS, since there is an overlapping of positive and negative control values[38].

Chapter 3. Results and Discussion

3.1.Study collection analysis

A total of 227 marine microbial extracts were submitted to bioactivity screening tests in this work. Figure 3.1 represents the distribution of the analysed extracts by hydrothermal vent source.

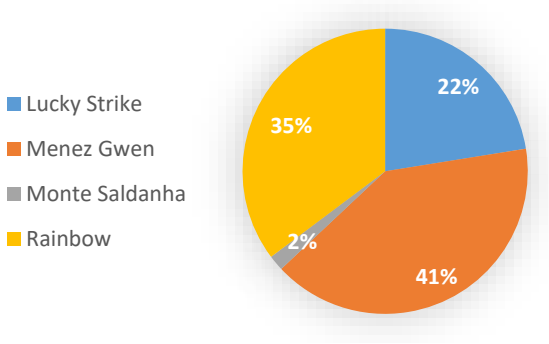

Figure 3.1 – Distribution of the extracts by hydrothermal vent

In Figure 3.2 is represented the total types of sample collected in the four hydrothermal vents in the Mid-Atlantic Ridge as mentioned in Section 2.2.4.1. From this analysis, it can be concluded that the majority of the extracts were collected from a mollusc *Bathymodiolus azoricus*, a typical bivalve from the Azores deep-sea, followed by water filtered extracts. All the other extracts were distributed in similar fractions around 2 to 11 %. The minor fractions were obtained from Chimney A, Sediment A and Gastropods.

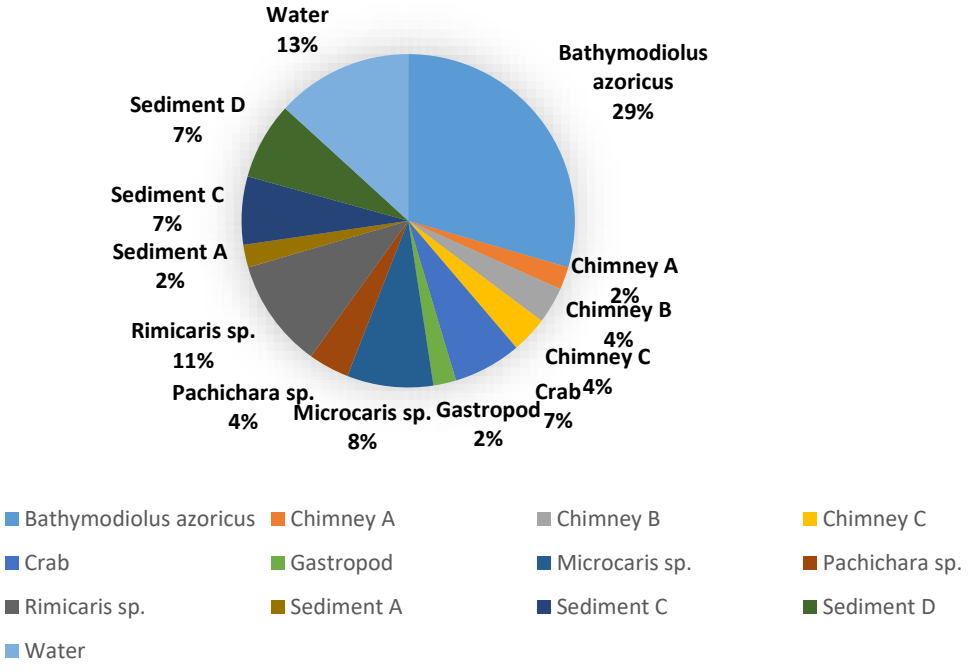

Figure 3.2 - Total types of samples collected distribution

3.2.Antimicrobial assay

The search for new antibiotics is an important element in the fight against the increasing number of infections caused by antibiotic-resistant pathogens. Additionally, anti-microbial bioactives have many other applications like detergents, functional textiles or cosmeceuticals just to name a few. In this work the antimicrobial assay described earlier was used to test the potential bioactivity of a natural compound library of 227 extracts of marine origin. Their capacity to reduce or inhibit the bacterial growth of preselected species in liquid media was evaluated in primary and secondary assays, after the assay platforms were set up and validated.

3.2.1. Set up and quality assessment of the assay

After selection of the strains to be tested, a growth curve assessment of each bacterial strain was performed (see Section 2.2.1.1). For each strain a total of 4 assays were executed, initially the two-fold-dilutions were tested in Erlenmeyer flasks followed by 2 miniaturization assays in 96-well plates. Two-fold-dilutions were experimented, 1:5 and 1:10, and as shown in Figure 3.3 there was no significant different between both growth curves, which guided us to choose the higher dilution, 1:10, allowing the use of a lower quantity of pre-inoculum.

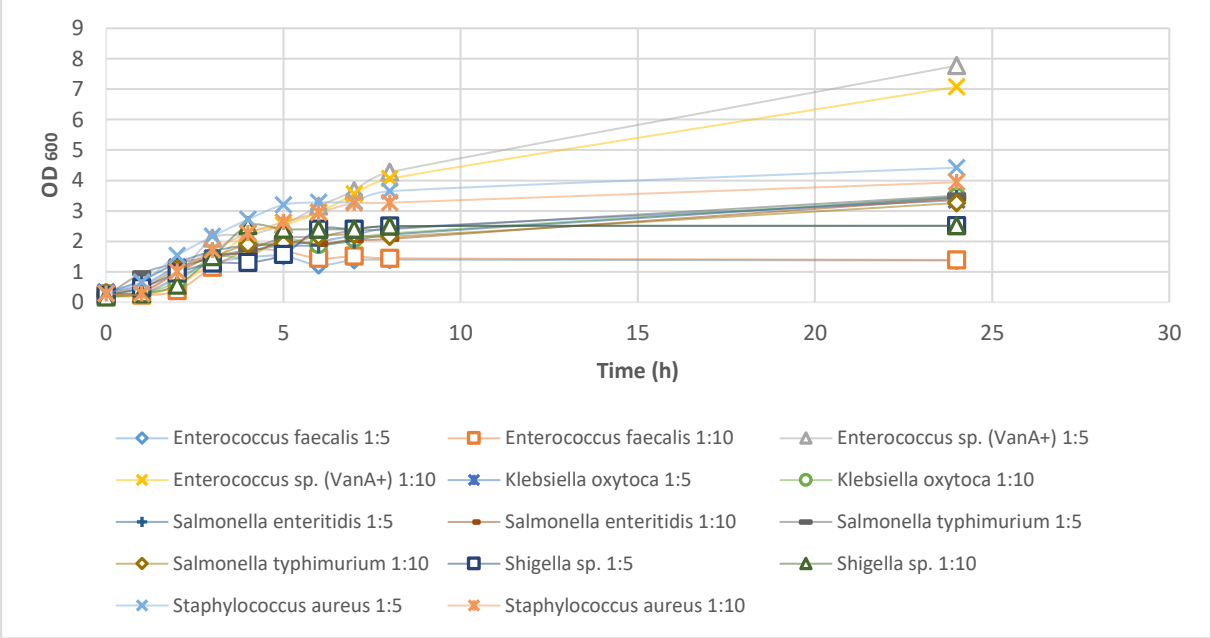

Figure 3.3 – Monitorization of the growth of the 7 selected bacteria strains for 24 hours, using two different dilutions 1:5 and 1:10

The minimal inhibitory concentration of their known antibiotics was found in order to establish a positive control, as Table 3.1 show.

Table 3.1 - Growth inhibitory concentrations of the bacterial strains used in the antimicrobial assay

Strains	Antibiotic	Inhibition Concentration (µg/ml)
S. typhimurium	Gentamycin	10
S. enteritidis	Gentamycin	10
Staphylococcus aureus	Gentamycin	5
Enterococcus faecalis	Gentamycin	100
Enterococcus sp. (VanA+)	Rifampicin	40
Klebsiella oxytoca	Ciprofloxacin	4
Shigella sp.	Ciprofloxacin	15

Considering the Z prime factor as a measure of the assay quality, a calculation of Z' for all strains and for all hours of the assay (Table 3.2) was performed. This data suggested that not all assays/strains are suitable for HTS screening assays, namely *Enterococcus faecalis*, since it has Z' values below zero for all times tested. For screening purposes, only the Z' values between 0 and 1 were considered, where the Z'-factor considered for selection of the assay time-point at which the results can be trusted and analysed corresponds to the highest. The *Enterococcus* sp. assay validation has a Z' = 0,61, at 4 hours from the starting point. For the *Klebsiella oxytoca* and *Salmonella enteritidis* a valid Z' factor was obtained starting at 2 hours, reaching the maximum points at 24h for the first strain and 6 hours for the last one. These were both considered excellent screening assays (Z' values > 0,5). For the *Salmonella typhimurium* and *Shigella* sp., the time point chosen were 24 h and 8 h, with Z' values of 0,77 and 0,64, respectively. For the *Staphyloccoccus aureus*, despite it being considered a marginal assay for HTS, since all the Z' values were below 0,5, it was decided to be used for low to medium level screenings, and we selected the 4h spot, since it corresponds to its best Z' value (0,13).

Table 3.2 - Z'-factor values for all antimicrobial assays

		Hour from the beginning of the assay					
		0	**2**	**4**	**6**	**8**	**24**
Z'-factor value	*E.faecalis*	-18,42	-2,84	-1,88	-3,46	-5,42	-1,03
	Enterococcus sp. (VanA+)	-16,45	0,04	**0,61**	0,45	0,26	0,01
	Klebsiella oxytoca	-2,55	0,73	0,80	0,82	0,86	**0,92**
	Salmonella enteritidis	-0,83	0,81	0,95	**0,95**	0,93	0,90
	Salmonella typhymurium	-15,54	-0,54	-0,21	-0,16	0,26	**0,77**
	Shigella sp.	-2,55	0,01	0,63	0,61	**0,64**	0,60
	Staphylococcus aureus	-12,96	-0,89	**0,13**	0,04	0,04	-0,41

3.2.2. Screening for antimicrobial potency

To search for the antimicrobial bioactivity amongst the marine collection of extracts PharmaBUG, a total of 3 560 screening tests were performed, using 227 marine microbial extracts screened against 7 bacterial strains, as mentioned in section 2.2.4.2.1. At the primary screening stage, and as described previously, just one replicate of each sample was adopted. From these 227, 155 natural extracts were classified as good candidates in this primary screen. This corresponds to a hit ratio of 68%, which may seem too high. However, having in consideration the ranking classification mentioned in section 2.2.4.2.1, which is not too stringent, it is acceptable. The hits identified in the primary screening were subjected to a confirmatory dose-response secondary screening aiming to eliminate false positives and to define which hits were the best candidates for development of potential bioactives for inhibiting antimicrobial growth, since it allowed to classify hits according to their potency.

The parameters used to determine the adequate threshold OD_{595} of the secondary antimicrobial screening for hit selection are shown in Table 3.3. Only the bacteria strain's whose final result became hit, have their hit selection parameters presented above.

Table 3.3 - Parameters used for hit determination in the secondary antimicrobial screening

	Strains					
Parameters	*Enterococcus* sp. (VanA+)	*Klebsiella oxytoca*		*Salmonella enteritidis*	*Salmonella typhimurium*	*Staphylococcus aureus*
Time (h)	4	2	8	2	24	4
(M) Minimum OD_{595}	0,220	0,13	0,118	0,111	0,383	0,192
(SD) Standard Deviation OD_{595}	0,017	0,011	0,012	0,004	0,011	0,020
Threshold	0,49	0,35	0,66	0,37	0,820	0,361
Z' factor	0,61	0,73	0,86	0,81	0,77	0,13

In the secondary screening a threshold value was calculated in order to evaluate the best hits to move on to a next phase and, for these, the inhibition growth rate was subsequently calculated (see section 2.2.4.2.2). Out of 155 hits taken to the dose response confirmatory secondary assay, we have confirmed 69 potential hit candidates. This corresponds to a final hit ratio of 30,4%. These results are summarized in the follow chart flow and further detail in the Annex of this thesis (Chapter 6).

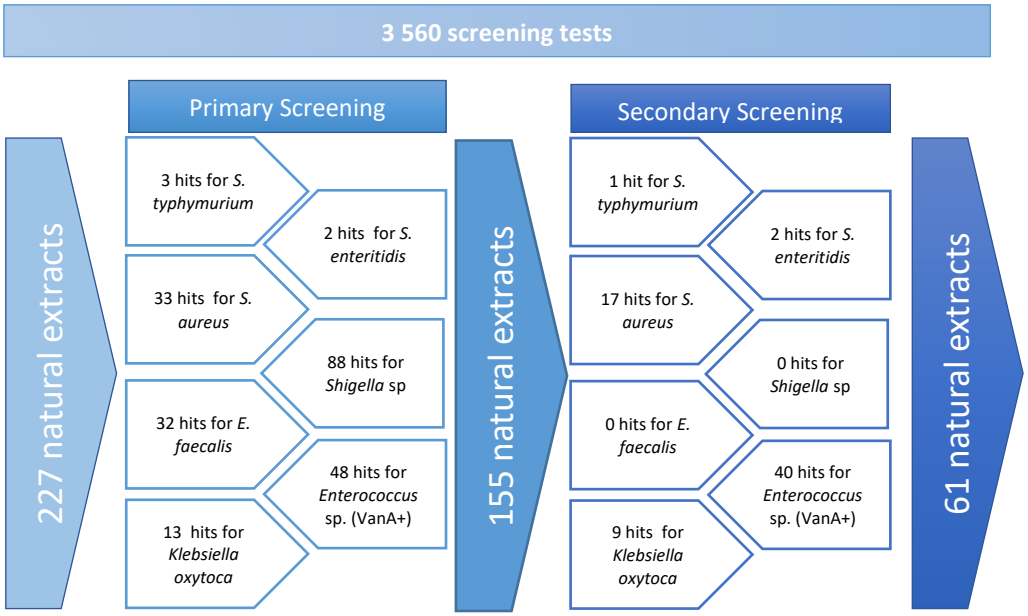

Figure 3.4 – Anti-microbial marine natural extracts screening work flow chart and results.

As said before, of the 155 hit extracts from the primary screening, 69 hits were confirmed as good candidates to follow to the next stage of the bioactivity discovery routine. In the case of the bacterial strain *Shigella* sp., it appears that the 88 hit extracts in the primary screening were all false positives, but for *Salmonella enteritidis* it was verified the opposite, where the 2 primary hits are also hits in the secondary screening. These "false positives" hits in the first run may have failed to validate on the second run merely because of random measurement error[87]. Therefore, a way to reduce this hit variation between the two screening campaigns can go through by increasing the number of replicas in the first screen.

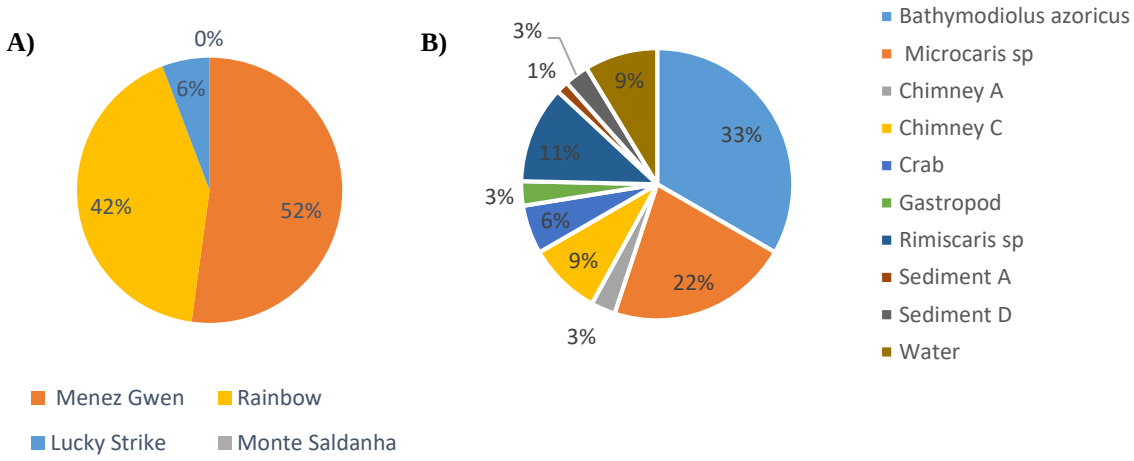

Figure 3.5 – a) Distribution of the antimicrobial hits by hydrothermal vent; b) Distribution of the antimicrobial hits by type of sample

Comparing the distribution of the total analysed extracts (Figure 3.5A) with the distribution of the antimicrobial hits (Figure 3.5B), it can be observed that the majority of the antimicrobial bioactives are mostly represented in samples collected from *Bathymodiolus azoricus* and *Microcaris* sp, with 33 % and 22 % of representability, respectably. A transcriptome sequencing and analysis of gill tissues from the bivalve *Bathymodiolus azoricus* revealed the existence of enzymes involved in sulfur and methane oxidation[88], which does not represent a novelty since this organism lives in symbiosis with two types of chemosynthetic Gammaproteobacteria (a sulfur oxidizer and a methane oxidizer). This fact reinforces the idea that the organisms living in deep-sea environments have unique mechanisms to help them survive in these harsh conditions. Another same-based analysis identified a great number of putative genes involved in animal physiological responses, particularly immune and stress-related responses, as a gene that codes for antibacterial protein defensin, providing evidence of *B. azoricus* functional immune system[89].

The 61 final natural extracts selected at the end of the secondary screening were distributed as 62,8 % of organic extracts and 37,2 % of aqueous extracts. Gram-negative bacteria, as is the case of *Salmonella* and *Klebsiella* genus, have only organic extracts, also hydrophobic, as positive hits. These bacteria are known to be very efficient at keeping out drugs, since their outer membrane is a barrier for amphipathic compounds[45]. Since uptake of nutrients is essential for bacterial survival, a bacteria cannot be entirely impermeable, thus Wiener and Horanyi, 2011[90] addressed some possible explanations to justify hydrophobic transit. Therefore, being able to pass through the outer membrane, the inner membrane despite being an efficient barrier to hydrophilic substances, it isn't necessary to hydrophobic molecules[45]. Noteworthy to mention that all bacterial strains tested are facultative anaerobic and 64,3% of the "secondary" extracts were collected from strains classified as facultative anaerobic as well.

Drug discovery programs focused on antimicrobial activity are gaining momentum and will open new possibilities for therapeutic developments in the fight against antibiotic-resistant bacterial strains. There are several screening assays to seek for drug discovery for antimicrobials. In this work thesis, a screening assay was adapted and coupled with a unique library of marine bacteria extracts, allowing the identification of 69 natural extracts capable of inhibit the growth of diverse bacteria, such as *Staphylococcus aureus*, *Enterococcus* sp. (VanA+), *Salmonella enteritidis*, *Salmonella typhimurium* and *Klebsiella oxytoca*. These extracts can be the basis of new bioactive development programs for medical and pharmaceutical applications but also for disinfectant/cleaning product applications.

3.3.DPPH test

Oxidative reactions can involve the production of free radicals, which can be followed by dangerous chain reactions. The selected way to evaluate antioxidant capacity of the marine microbial collection in this study was by a rapid DPPH staining method as primary screening, in order to reduce the potential number of samples to be tested in more complex, and biologically relevant, assays. This method is typically based on an inhibition of the accumulation of oxidized products, since the generation of free radicals is inhibited by the addition of antioxidants and scavenging of the free radicals. DPPH (1,1-diphenyl-2-picrylhydrazyl) is a stable free radical due to the delocalisation of the spare electron over the molecule as a whole and it cannot dimerise (Figure 3.6), avoiding its aggregation which allowed it to be used as scavenger. If aggregation occurred it would lead to radical neutralization. This specific stable radical in the presence of an antioxidant captures the free electron, being possible to measure radical scavenging activity.

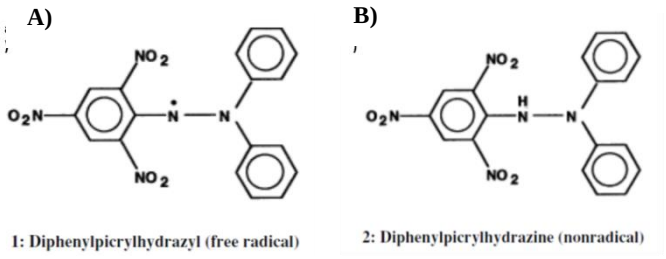

Figure 3.6 - DPPH molecule A) free radical; B) nonradical – reduced form

3.3.1. Set up and optimization of the DPPH assay

A calibration curve with ascorbic acid (AA), one of the most potent antioxidants, was performed using DPPH as the test compound (Figure 3.7). The DPPH reduction percentage was calculated considering the OD_{520} values after incubation of AA with DPPH after a specific time in a dark environment. OD measurements were performed in different times to establish the best incubation time: 10 min, 20 min, 30 min and 1h. Data shows that there is no significant difference between the 10 min and 20 min experience times, which led us to choose the smallest incubation time. After 30 min and 1h the DPPH reduction has a value very different from zero at concentration equal to 0µg/ml, which led us to conclude that after these period, DPPH assay has reached a plateau and no longer is accurate.

Figure 3.7 - DPPH percentage versus acid ascorbic concentration during different incubation times

3.3.2. Screening for antioxidant potency

A total of 227 marine microbial extracts were screened for antioxidant activity by a rapid DPPH test, as described in section 2.1.1.1.2. Figure 3.8 represents the distribution of these extracts by hydrothermal vent source (A) and by DPPH reduction percentage (A). From these results, one can conclude that the major hydrothermal vent source of antioxidant bioactives is the Menez Gwen, followed by Rainbow. This is in accordance with data from deletion SOD1 strain antioxidative potential determination too (see section 3.4.3).

From the DPPH reduction percentage, it's possible to conclude that 13% of the extracts exhibit a reduction potential of over 80%. Additionally, 44 % of the analysed extracts exhibit a DPPH reduction between 20 - 50 %.

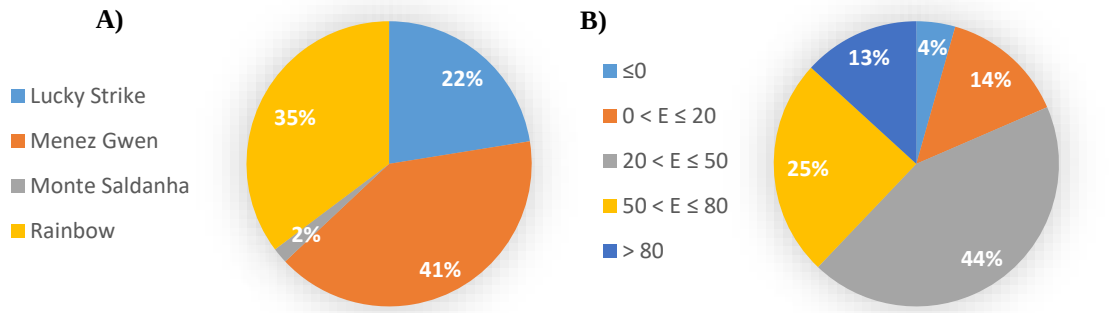

Figure 3.8 – A) Distribution of hydrothermal vents sources; B) Distribution of DPPH reduction percentages, where "E" represents extract

A more detailed analysis of the best antioxidants' extracts, namely the ones between 50 and 80 % showed that again, the two main hydrothermal sources contributing to these bioactives, are Rainbow and Menez Gwen, as shown in Figure 3.9.a. For the top antioxidant activity (above 80 %), the analysed extracts were mainly (63%) isolated from the Menez Gwen hydrothermal vent.

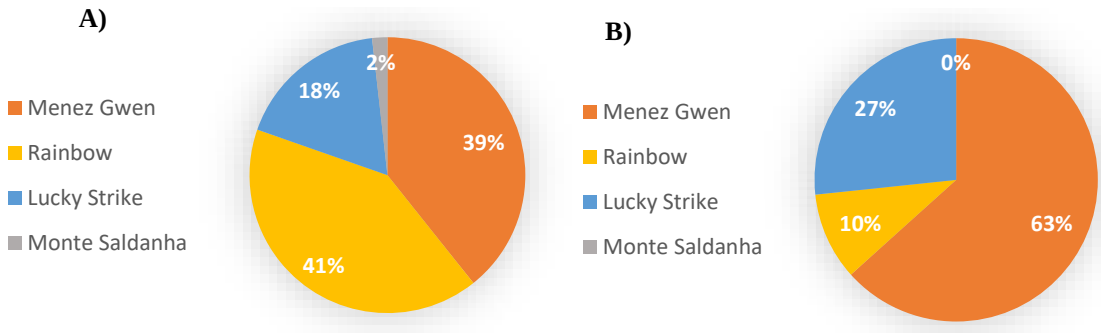

Figure 3.9 – a) Distribution of extracts with a DPPH reduction between 50 – 80 % by hydrothermal vents source; b) Distribution of extracts with a DPPH reduction above 80 % by hydrothermal vents source

Noteworthy mention that the extract AEWC081 stands out as the most potent DPPH reductor with a reduction rate of 103%. However, and strikingly, this extract was isolated from the Lucky Strike hydrothermal vent, from marine strain LSWA081. Therefore, this may be a unique compound not present in most other extracts. The Lucky Strike vent is characterized by a mild acidic medium (pH 3,8 to 4,5) and an environment depleted in sulphides, but rich in methane, which can be considered a hostile oxidant environment. As explained previously, these harsh conditions may conduct to the development of a particular antioxidant activity.

A correlation evaluation between sources and antioxidative potential was performed (comparison of the ones with best performance (> 80%) in terms of DPPH reduction) (Figure 3.10). From this distribution it can be observed that the majority of the antioxidant bioactives are also derived from the most abundant source: the *Bathymodiolus azoricus* and water filtered extracts are ahead in terms of activity, with the last having an increase of 54 % in terms of representability. It can be highlighted in terms of representability the *Microcaris* sp., with 112 %, and gastropod, with 400 % of increase, comparing to their percentage representative in the overall distribution (see section 3.1).

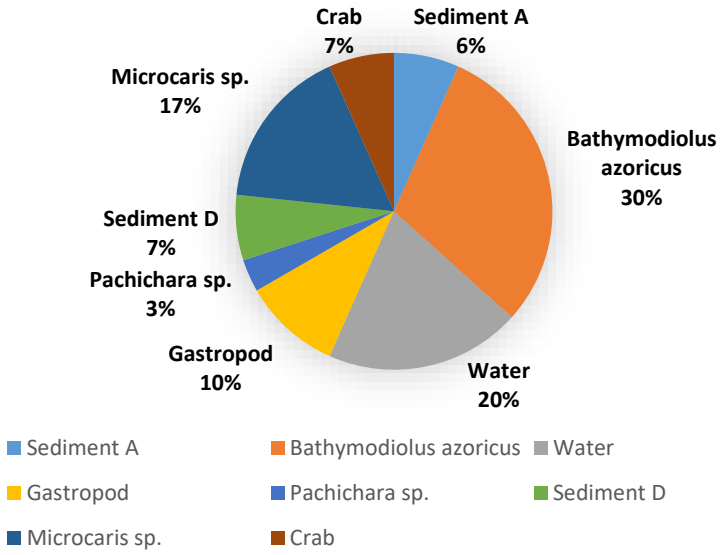

Figure 3.10 – Types of sample collected with DPPH reduction above 80 % distribution

These results demonstrate that the DPPH test is a simple and easy test to determine the antioxidant potency of a compound. For this study DPPH assay was optimized for a smaller amount of final volume, having in mind potential HTS scalability, on the contrary to most performed studies published where a larger sample extract and reagents were needed. Additionally, this test has the intent to complement the yeast-based screening platform mentioned. Nevertheless, there was no expectation that both antioxidant assays have the same results, as it was indeed observed, since the two assays are associated to different anti-oxidant action mechanisms.

3.4. SOD1 assay

It is widely known that yeast is a suitable organism model for HTS bioactivity discovery programs for antioxidant applications, due to its high degree of conservation of biological processes and its acquiescence for genetic manipulation, short generation times, genetic tractability and scalability[74]. Besides, yeast-based screening systems are very informative and are cost-competitive, allowing short time frames for hit identification. These platform types further maximize yeast usefulness by refining the screening criteria to develop stringent screening tools that potentially reduce attrition rates in subsequent phases of bioactivity discovery.

Bioactivity discovery programmes focused on antioxidant activity are very common, however, they are mostly performed by rapid screening, as DPPH or FRAP (Ferric Reducing Ability of Plasma) test, and there is a significant amount of studies using, for example plant samples, but very few using marine microbial samples. One of the specific goals of this thesis work was to develop a robust yeast-based platform for the identification of new bioactives of marine origin as potentials antioxidants. The yeast strain sod1Δ has a compromised stress response function, due to the deletion of the gene SOD1. This gene codes for an enzyme that converts superoxide anions into H_2O_2 and oxygen, thus protecting cells against oxidative damage. Its absence in the cell can be a way of testing antioxidant activity of certain

compounds as the cell is impaired to deal with that kind of stress. In this *in vivo* assay, the antioxidant activity was detected by monitorization of the yeast growth via measuring optical density.

3.4.1. Characterization of yeast strains

In present work, validation tests were performed to evaluate the viability of using sod1Δ::URA3 as a screening system. Firstly, a liquid growth evaluation in SC media supplemented with H_2O_2 (1 mM) and ascorbic acid was performed to characterize the deleted *Saccharomyces cerevisae* strains. Cells were treated with hydrogen peroxide 1mM, an oxidative stress inducer (negative control), that caused a growth delay in ΔSOD1 yeast strain compared with DBY746 wild type strain. However, upon the addition of ascorbic acid, which is a well-known antioxidant compound (positive control), all yeast strains recovered, in presence of H_2O_2, as shown in Figure 3.10. This validated the assay functionality and allowed us to perform the screening campaigns.

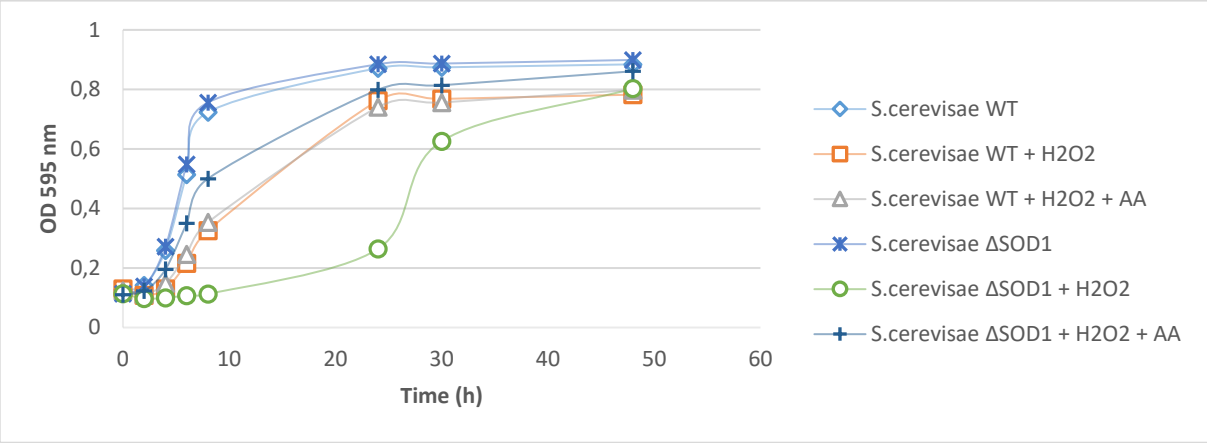

Figure 3.11 - Yeast strains were tested for peroxide hydrogen, a stress inducer, (H2O2 1mM) and ascorbic acid (AA; 5 mM) sensitivity

When working with yeast, the growth of the yeast strain must be characterized. For this growth curves are built, by taking OD measurements at different time intervals. This curve could be divided into three major phases: i) lag phase, ii) logarithmic phase, and iii) stationary phase. During the first phase, yeast cells are acclimating to the environment and are growing but are not replicating. In the second phase, cells are actively dividing thus leading to cell doubling. Finally, when available nutrients are exhausted, yeast cells enter stationary phase, the third phase, where cell division slows down, and the cell population remains constant. Using the growth curves analysis, it's possible to calculate the doubling time of a strain, by the following equation: $Doubling\ time = \frac{Ln\,(2)}{r}$, where the $r = \frac{Ln\left(\frac{OD1}{OD2}\right)}{t1-t2}$. The doubling time is defined as the time required for a cell population to double in cell number. The doubling time calculated for the growth curves of the Figure 3.11 are represented in Table 3.3 and shows a substantial growth delay difference between WT and ΔSOD1 strain under oxidative stress.

Table 3.4 - Doubling time (h) of yeast strains

	Generation time (h)	
	WT	ΔSOD1
Normal conditions	2,15	1,98
Plus H_2O_2	3,00	15,24
Plus H_2O_2 and AA	3,18	2,65

In parallel, amplification of the SOD1 gene by PCR confirmed that the gene SOD1 is present in the WT strain, correspondent to 609 bp, and not in the sod1Δ::URA3 strain, as expected. At the same time, amplification of an internal gene, the small subunit of ribosomal RNA, was performed to guarantee the quality of the extracted DNA. Amplification of the internal gene corresponded to a lane with 1200 base pairs, and is present in both strains as expected, as seen in Figure 3.12A.

After PCR confirmation, a restriction digestion with *Hind* III was executed. The SOD1 amplicon (marked with a "red rectangle") was cut and purified from the gel, followed by restriction enzyme digestion. From this single restriction digestion, two sequences with, respectively, 245 and 363 pair bases were expected. As it shows in Figure 3.11B, 3 lanes appeared, product of the Hind III digestion: the first lane is the DNA not digested, the second and third lane, although very vanished (marketed by asterisks) can be positively identified has the two sequences resulted from the digestion.

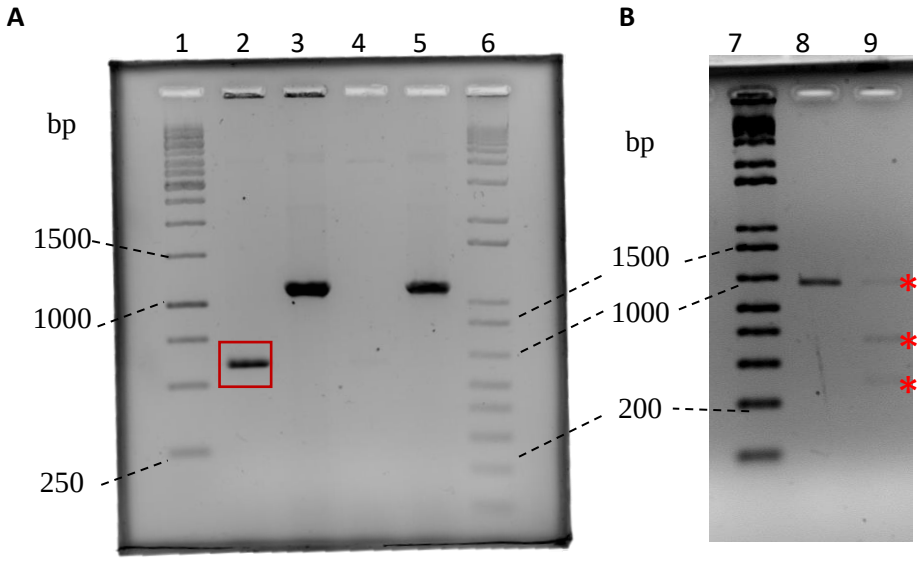

Figure 3.12: A: Agarose gel electrophoresis of PCR results with SOD1 primers (lanes 2 and 4) and NS1/NS4 primers (lanes 3 and 5); Lane 2 and 3 corresponds to *S. cerevisae* WT genomic DNA and lanes 4 and 5 correspond to *S. cerevisae* ΔSOD1 genomic DNA; B: "red rectangle" indicates the band that was cut and purified to further Hind III digest to confirm the presence of the gene, where the lane 8 corresponds to SOD1 amplicon and lane 9 to SOD1 amplicon digested

As seen in previous works[66–68], yeast lacking the SOD1 gene present a phenotype that could be used as a read-out in a screening system, once the lack of expression of this particular gene doesn't compromise the antioxidant function of the cells, but only slows its recovery rate in oxidative stress environment (see Figure 3.11) but without cell's death. Besides, the sod1Δ yeast has the same growth behaviour as the WT strain in normal conditions. To identify antioxidant bioactivity, an *in vivo* screening system using yeast as a model was adapted and optimized with an unique library of marine microorganisms' extracts.

At this stage, DMSO tolerance was assessed at 0,5 mg/ml concentration, the same used for the extracts to be tested in this assay, and no significant effects on the yeast's growth were observed (data not shown).

3.4.2. The yeast strain sod1Δ::URA3 was suitable for drug discovery screenings

The Z' factor was calculated at each time point for the growth curve of yeast inoculated. Negative values of Z'-factor were obtained during the lag growth phase, since there was no difference between the

growth of yeast in stress oxidative with (positive control) and without ascorbic acid (negative control), indicating that data obtained at these time-points cannot be considered for screening assay points. However, after 6h incubation, the control strain entered in the exponential growth phase.

Table 3.5 - Z' factor values at all time-points of the yeast sod1Δ::URA3

	Time (h)							
	0	**2**	**4**	**6**	**8**	**24**	**30**	**48**
Z' factor value	-11,5474	-0,9071	0,2264	0,5323	0,6396	**0,6401**	-0,8365	-1,1902

The overall quality of the screening system was assessed using a variation of screening window coefficient, denoted Z prime factor (Z'- factor), that takes into account the assay signal dynamic range and the data variation of the controls without need of a positive control compound[38]. In this assay, the Z'-factor that should be considered for selection of the assay time-point at which the results can be trusted and analysed corresponds to 24 hours from the beginning of the assay. The Z' factor obtained was 0,6401 which means that it was an excellent assay, and 24 hours is the assay time-point that should be used to draw further conclusions from the antioxidant activity of the compounds tested. The Z'- factor enable the classification of this platform as excellent screening assay, being liable to be adapted for HTS assays.

3.4.3. Screening for antioxidant potency of various bioactive compounds

Once confirmed that this screening system was robust to perform reliable bioactivity assays, a proof-of-concept screen was performed using a unique collection of 227 marine microbial extracts from hydrothermal vents. The screening platform was designed to select compounds capable of rescuing the growth of sod1Δ yeast in an oxidative environment to the levels of the same strain in the same conditions but in presence of a very strong antioxidant, the ascorbic acid, above a strictly defined threshold.

A total of 227 extracts were screened in the same initial conditions (see section 2.2.4.3.2) and 8 hits were selected to be validate in future secondary screening.

The parameters used to determine the adequate threshold OD_{595} for hit selection and the robustness of the assay at time-point 24h are shown in Table 3.6.

Table 3.6 - Parameters used for hit determination in the primary screening with sod1Δ drug discovery platform

	Controls	
Parameters (t = 24h)	sod1Δ::URA3 + H_2O_2	sod1Δ::URA3 + H_2O_2 +AA
(A) Average OD_{595}	0,109	0,718
(M) Maximum OD_{595}	0,121	0,846
(SD) Standard Deviation OD_{595}	0,010	0,072
Threshold	0,131/ 0,327	
Z' factor	0,60	

The Z' factor of the primary screening assay calculated was 0.60 at 24h incubation, classifying the screening assay as excellent[38] and indicating that the results obtained can be trusted. The natural extracts able to rescue the growth of sod1Δ yeast strain in presence of stress oxidative to OD_{595} values equal or superior to the threshold of 0,131 were classified as hits, following the reasoning described in section 2.1.1.1.1 of Chapter 2. To further increase stringency, another threshold (2) was calculated by adding to the first threshold 25% of the signal dynamic between the positive and negative controls. The threshold

2 was 0,327 and identified the hits with a greater antioxidant activity. In this case, just one extract seems to have an exceptional antioxidant potency.

A total of 8 hits out of 227 natural extracts tested were selected as the most potent hits, representing the primary hit rate of 3,5 %, which is in line with current hit rates for cellular based HTS.

As an example, the growth of sod1Δ yeast strain with the best hit, the aqueous extract AEWC184, in liquid selective media relative to that of the positive control (sod1Δ + H_2O_2 +AA) and of the negative control (sod1Δ + H_2O_2) is presented in Figure 3.12. As it is possible to see, the addition of the extract AEWC184 was able to rescue the growth of sod1Δ yeast in stress oxidative to near normal levels, at the time-point of analysis (t=24 h).

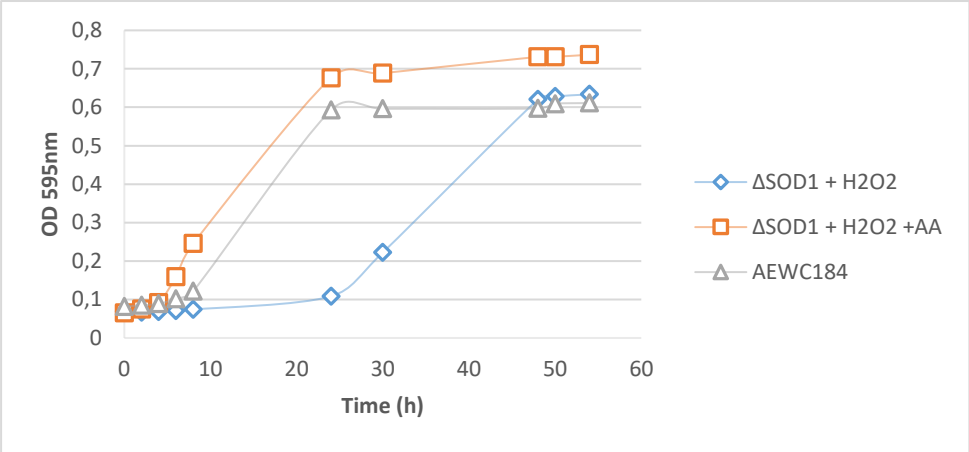

Figure 3.13: Hit of the primary screening with sod1Δ::URA3 strain

The primary screening resulted in the identification of 8 hits, where one (AEWC184) seems to have the best antioxidant capacity tested since is the only one that surpasses above the more restricted selected threshold and restores normal function to the deleted strain. This may be a potential candidate for a bioactive commercial development programme.

The hits were ranked according to their antioxidant capacity, depending of the ratio between the OD_{595} and the threshold less restrictive (0,131) obtained at the time-point 24h for each natural extract. In Table 3.6, some parameters about each hit, such as the specific hydrothermal vent where it was extracted from, are presented.

Table 3.7 - Marine bacterial strain information about the hit (* facultative anaerobic). MG – Menez Gwen; RB – Rainbow; MS - *Microcaris* sp.; O2 - Facultative anaerobic; BA - *Bathymordiolus azoricus*; SD – Sediment D; SC – Sediment C; SA - Sediment A; CR -Crab

Ranking	Hit ID	Marine Strain	Hydrothermal vent	Temperature of collection point (º C)	OD_{595} (T=24h)	Recovery (%)
#1	AEWC184	MGCR184O2*	Menez Gwen	8,2	0,593	65,1
#2	AEWC082	MGSD082	Menez Gwen	9,1	0,299	24,6
#3	AEWC166	MGMS166O2*	Menez Gwen	8,2	0,252	18,1
#4	AEWEC98	MGMS098O2*	Menez Gwen	8,2	0,2	10,9
#5	AEWC248	RBRS248O2*	Rainbow	3,7	0,181	8,3
#6	AEWC083	MGSA083	Menez Gwen	8,7	0,138	2,3
#7	AEWC168	MGMS168O2*	Menez Gwen	8,2	0,132	1,5
#8	AEWC001	MGBA001	Menez Gwen	8,4	0,132	1,5

From the above observations it can be seen that in these 8 primary hits, the majority were collected from the same hydrothermal vent (Menez Gwen), which is characterized by an acidic environment in calcium sulphate deposits. It can also be observed that the majority of hits were extracted from marine facultative anaerobic bacteria from the same hydrothermal vent. One hit is from the Rainbow vent, which like the previous is characterized for expelling chimneys from which water and other compounds are present at very high temperatures (low oxygen content). All these conditions are aggressive conducting to survival resistant and may explain the powerful antioxidant capacity of the extracts obtained from these organisms. Another observation is the fact that most hits correspond to aqueous extracts, where more hydrophilic molecules are present, making possible the assumption that the antioxidant hit compounds are more water-soluble and this may help their isolation procedures afterwards. Noteworthy to mention that some hit extracts have double bioactivity and therefore are potentials marine microbial extracts worth to commercially explore in the future.

Table 3.8 - Hit marine microbial extracts activity summary (*previously identified bioactivity - not published work from the lead-team group)

Marine Strain	Antioxidant activity		Antimicrobial activity	Other activity*
	DPPH	SOD1		
MGBA001	✓	✓	✓	Anti-UVC
MGBA003	✓		✓	Anti-UVC
MGBA005	✓		✓	
MGCR019	✓		✓	
MGMS008	✓		✓	
MGMS011	✓		✓	
MGMS098O2		✓	✓	
MGMS166O2	✓	✓	✓	Anti-UVC
MGMS168O2		✓	✓	Anti-UVC, Cosmetic
RBBA051	✓		✓	
RBRS248O2	✓	✓	✓	Anti-UVA
RBWC199O2	✓		✓	Anti-UVC

From all the screening extracts, MGBA001 and MGMS166O2 stand out for being positive hits for 3 screening platforms developed. MGBA001 despite having a low antioxidant activity in SOD1 assay, has an outstanding DPPH activity (> 80 %) and has antimicrobial properties against the gram-positive bacteria, *Enterococcus* sp. (VanA+) and *Staphylococcus aureus*. The extract MGMS166O2 has a good antioxidant activity for both antioxidant platforms and can inhibit the growth of *S. aureus*. These hits are very promising in terms of broad bioactive potential development programmes.

Chapter 4. Conclusion and future work

The ocean plays a critical role in supporting human well-being, from providing food and nutrients to being a source of pleasure for tourism while acting as a carbon storage[91], and an interest in the study and exploration of marine microorganisms as potential producers of new compounds for applications in diverse areas, where extreme marine environments, as deep-sea vents, represents a promising source of novel bioactive compounds.

During this thesis work, 5 screening assays were efficiently developed and validated for anti-microbial bioactivity screening against *Enterococcus* sp. (VanA+), *Klebsiella oxytoca*, *Salmonella enteritidis*, *Salmonella typhimurium* and *Shigella* sp. Out of these, 2 failed to be fully validated, the *Enterococcus faecalis* and *Staphylococcus aureus*. This is due to their Z' values being close or inferior to 0. These screening assays should be subject to more optimization work, as described in Nalo *et al*., 2006[87].From

the initial 227 marine extracts tested in each platform, 69 hits were found that can inhibit the growth of specific pathogenic bacteria: 40 hits were found for *Enterococcus* sp. (VanA+), 9 hits for *Klebsiella oxytoca*, 2 hits for *Salmonella enteritidis*, 1 hit for *Salmonella typhimurium*.

The 61 extracts founded to have antimicrobial activity should be subjected to further studies, that should include bioguided fractionation, positive fractions identification and final bioactive compound chemical structure elucidation. Repeated discovery of known compounds can be a possibility and in order to minimize time, effort and cost, it is usually advised to confirm if the bioactive discovered has or not been already identified. Data bases matching and biochemical techniques like High Performance Liquid Chromatography-Mass Spectrometry, HPLC-Solid Phase Extraction, and Ultra High Performance Liquid Chromatography, or bioactivity fingerprints, such as cytological profiling or BioMAP[36] dereplication tools may aid this process. Other dereplication techniques can include molecular methods such as 16S-Internal Transcribed Spacer RFLP, partial 16S rDNA sequencing, and repetitive extragenic palindromic-PCR of the BOX DNA element [36].

In parallel, two antioxidant assays were set-up, validated and implemented in the lab to evaluate antioxidant properties of the marine compounds. The DPPH test was optimized and validated for antioxidant activity, through DPPH scavenger activity measurements. From the initial 227 tested marine extracts, 30 extracts were identified with great antioxidant potential, having a percentage of DPPH scavenger activity above 80 %. Hit LSWA081 was actually above 100% being better than the current gold standard of ascorbic acid. Although DPPH test is simple and rapid, just one replica was used in this preliminary work, and a confirmation assay with at least triplicates should be considered.

To increase the biological significance of antioxidant screening, a SOD1 screening platform was also implemented from a pre-existing SOD1 mutant yeast strain. This platform was characterized, and its high throughput usage optimized and validated. A primary SOD1 screening assay with 227 extracts was performed and 8 new target specific hits identified. These 8 extracts remain to be further re-validated through a secondary dose response screening aiming to eliminate false positives and to define if these hits can be good candidates for development of potential drugs for replacing SOD1 antioxidant function. This work should be, also, addressed with the identification of the bioactive and its structure elucidation.

The majority of the identified hits, both antimicrobial and antioxidant, derived from mostly of Menez Gwen hydrothermal vent, a very harsh acidic and very high temperature deep sea habitat.

The work performed in this thesis can be the basis of novel marine natural products and bioactives development programmes with relevance to a variety of industries, starting at the most needed pharmaceutical application with new antibiotics development, but also relevant for cosmetics (anti-oxidants and anti-ageing) or household cleaning (anti-microbial/detergent products) applications.

Chapter 5. Bibliography

1. Mora C, Tittensor DP, Adl S, Simpson AGB, Worm B. How many species are there on earth and in the ocean? *PLoS Biol*. 2011;9(8). doi:10.1371/journal.pbio.1001127.

2. Costello MJ, Wilson S, Houlding B. Predicting total global species richness using rates of species description and estimates of taxonomic effort. *Syst Biol*. 2012;61(5):871-883. doi:10.1093/sysbio/syr080.

3. Costello MJ, Wilson SP. Predicting the number of known and unknown species in European seas using rates of description. *Glob Ecol Biogeogr*. 2011;20(2):319-330. doi:10.1111/j.1466-8238.2010.00603.x.

4. Blunt JW, Copp BR, Keyzers RA, Munro MHG, Prinsep MR. Marine natural products. *Nat Prod Rep*. 2016;33:382-431. doi:10.1039/C5NP00156K.

5. Hu Y, Chen J, Hu G, et al. Statistical research on the bioactivity of new marine natural products discovered during the 28 years from 1985 to 2012. *Mar Drugs*. 2015;13(1):202-221. doi:10.3390/md13010202.

6. Montaser R, Luesch H. Marine natural products : a new wave of drugs ? *Futur Med Chem*. 2011;3(12):1475-1489. doi:10.4155/fmc.11.118.Marine.

7. Adnan M, Alshammari E, Patel M, Ashraf SA, Khan S, Hadi S. Significance and potential of marine microbial natural bioactive compounds against biofilms/biofouling: necessity for green chemistry. *PeerJ*. 2018;6. doi:10.7717/peerj.5049.

8. Kong DX, Jiang Y-Y, Zhang H-Y. Marine natural products as sources of novel scaffolds: Achievement and concern. *Drug Discov Today*. 2010;15(21-22):884-886. doi:10.1016/j.drudis.2010.09.002.

9. Munro MHG, Blunt JW, Dumdei EJ, et al. The discovery and development of marine compounds with pharmaceutical potential. *J Biotechnol*. 1999;70:15-25.

10. Bergmann W, Feeney RJ. Contributions to the study of marine products. XXXII. The nucleosides of sponges. I. *J Org Chem*. 1951;16(6):981-987. doi:10.1021/jo01146a023.

11. Huyck TK, Gradishar W, Manuguid F, Kirkpatrick P. Eribulin mesylate. *Nat Rev Drug Discov*. 2011;10:173-174. doi:10.1038/nrd3389.

12. Mayer AMS, Glaser KB, Cuevas C, et al. The odyssey of marine pharmaceuticals: a current pipeline perspective. *Trends Pharmacol Sci*. 2010;31:255-265. doi:10.1016/j.tips.2010.02.005.

13. Ngo D-H, Wijesekara I, Vo T-S, Ta Q, Kim S-K. Marine food-derived functional ingredients as potential antioxidants in the food industry: An overview. *Food Res Int*. 2011;44:523-529. doi:10.1016/j.foodres.2010.12.030.

14. Rasmussen RS, Morrissey MT. Marine Biotechnology for Production of Food Ingredients. *Adv Food Nutr Res*. 2007;52:237-292. doi:10.1016/S1043-4526(06)52005-4.

15. Corinaldesi C, Barone G, Marcellini F, Dell'Anno A, Danovaro R. Marine microbial-derived molecules and their potential use in cosmeceutical and cosmetic products. *Mar Drugs*. 2017;15(118). doi:10.3390/md15040118.

16. Ali NE-H, Hmidet N, Ghorbel-Bellaaj O, Fakhfakh-Zouari N, Bougatef A, Nasri M. Solvent-stable digestive alkaline proteinases from Striped Seabream (Lithognathus mormyrus) viscera: Characteristics, Application in the Deproteinization of shrimp waste, and evaluation in laundry commercial detergents. *Appl Biochem Biotechnol*. 2011;164(7):1096-1110. doi:10.1007/s12010-011-9197-z.

17. Krishna JG, Basheer SM, Beena PS, Chandrasekaran M. Marine Bacteria As Source of Pigment for Application As Dye in Textile Industry. *Proc Int Conf Biodivers Conserv Manag*. 2008:743-750.

18. Kumar SR, Ramanathan G, Subhakaran M, Inbaneson SJ. Antimicrobial compounds from marine halophytes for silkworm disease treatment. *Int J Med Med Sci*. 2009;1(5):184-191.

19. Adarsh S, Kumar S. Role of marine shell (Meretrix casta: Bivalve) in cement mortar preparation: An experimental study. *Int J Res Eng Appl Manag*. 2018;4(1):7-11. doi:10.18231/2454-9150.2018.0077.

20. Husain S, Al-Samadani KH, Najeeb S, et al. Chitosan biomaterials for current and potential dental applications. *Materials (Basel)*. 2017;10(6):1-20. doi:10.3390/ma10060602.

21. Demirbas MF. Biofuels from algae for sustainable development. *Appl Energy*. 2011;88(10):3473-3480. doi:10.1016/j.apenergy.2011.01.059.

22. Kasanah N, Hamann MT. Development of antibiotics and the future of marine microorganisms to stem the tide of antibiotic resistance. *Curr Opin Investig drugs*. 2004;5(8):827-837.

23. Rampelotto PH, Trincone DA, eds. *Grand Challenges in Marine Biotechnology*. Naples, Italy: Springer International Publishing; 2018. doi:https://doi.org/10.1007/978-3-319-69075-9.

24. Zhang L, An R, Wang J, et al. Exploring novel bioactive compounds from marine microbes. *Curr Opin Microbiol*. 2005;8(3):276-281. doi:10.1016/j.mib.2005.04.008.

25. Martins A, Tenreiro T, Andrade G, et al. Photoprotective bioactivity present in a unique marine bacteria collection from Portuguese deep sea hydrothermal vents. *Mar Drugs*. 2013;11:1506-1523. doi:10.3390/md11051506.

26. Hughes JP, Rees S, Kalindjian SB, Philpott KL. Principles of early drug discovery. *Br J Pharmacol*. 2011;162:1239-1249.

27. Mohs RC, Greig NH. Drug discovery and development: Role of basic biological research. *Alzheimer's Dement Transl Res Clin Interv*. 2017;3(4):651-657. doi:10.1016/j.trci.2017.10.005.

28. Marilynn Larkin. Breaking bottlenecks in drug discovery and development.

29. Martis EA, Radhakrishnan R, Badve RR. High-Throughput Screening: The Hits and Leads of Drug Discovery- An Overview. *J Appl Pharm Sci*. 2011;1(11):2-10. http://www.academia.edu/871020/High-Throughput_Screening_The_Hits_and_Leads_of_Drug_Discovery-_An_Overview.

30. Dias DA, Urban S, Roessner U. A Historical overview of natural products in drug discovery. *Metabolites*. 2012;2(2):303-336. doi:10.3390/metabo2020303.

31. Martins A, Vieira H, Gaspar H, Santos S. Marketed marine natural products in the pharmaceutical and cosmeceutical industries: Tips for success. *Mar Drugs*. 2014;12(2):1066-1101. doi:10.3390/md12021066.

32. Molinski TF, Dalisay DS, Lievens SL, Saludes JP. Drug development from marine natural products. *Nat Rev Drug Discov*. 2009;8(1):69-85. doi:10.1038/nrd2487.

33. Grondal C, Jeanty M, Enders D. Organocatalytic cascade reactions as a new tool in total synthesis. *Nat Chem*. 2010;2:167-178. doi:10.1038/nchem.539.

34. Pettit RK. Mixed fermentation for natural product drug discovery. *Appl Microbiol Biotechnol*. 2009;83:19-25. doi:10.1007/s00253-009-1916-9.

35. Lane AL, Moore BS. A sea of bisynthesis: marine natural products meet the molecular age. *Nat Prod Rep*. 2011;28(2):411-428. doi:10.1039/c0np90032j.A.

36. Rocha-Martin J, Harrington C, Dobson ADW, O'Gara F. Emerging strategies and integrated systems microbiology technologies for biodiscovery of marine bioactive compounds. *Mar Drugs*. 2014;12(6):3516-3559. doi:10.3390/md12063516.

37. Mayr LM, Bojanic D. Novel trends in high-throughput screening. *Curr Opin Pharmacol*. 2009;9(5):580-588. doi:10.1016/j.coph.2009.08.004.

38. Zhang J-H, Chung TDY, Oldenburg KR. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J Biomol Screen*. 1999;4(2):67-73.

39. Iversen PW, Eastwood BJ, Sittampalam GS, Cox KL. A Comparison of Assay Performance Measures in Screening Assays: Signal Window, Z' Factor, and Assay Variability Ratio. *J Biomol Screen*. 2006;11(3):247-252. doi:10.1177/1087057105285610.

40. Tan SY, Tatsumura Y. Alexander Fleming (1881–1955): Discoverer of penicillin. 2015;56(7):366-367. doi:10.11622/smedj.2015105.

41. Giudice A Lo, Bruni V, Michaud L. Characterization of Antarctic psychrotrophic bacteria with antibacterial activities against terrestrial microorganisms. *J Basic Microbiol*. 2007;47:496-505. doi:10.1002/jobm.200700227.

42. Hughes CC, Fenical W. Antibacterials from the Sea. *Chemestry Eur J*. 2010;16:12512-12525. doi:10.1002/chem.201001279.

43. Saga T, Yamaguchi K. History of Antimicrobial Agents and Resistant Bacteria. *JMAJ*. 2009;52(2):103-108.

44. WHO publishes list of bacteria for which new antibiotics are urgently needed. https://www.who.int/news-room/detail/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed. Accessed October 10, 2017.

45. Lewis K. Platforms for antibiotic discovery. *Nat Rev Drug Discov*. 2013;12(5):371-387. doi:10.1038/nrd3975.

46. Wang G, Hosaka T, Ochi K. Dramatic activation of antibiotic production in Streptomyces coelicolor by cumulative drug resistance mutations. *Appl Environ Microbiol*. 2008;74(9):2834-2840. doi:10.1128/AEM.02800-07.

47. Cos P, Vlietinck AJ, Berghe D Vanden, Maes L. Anti-infective potential of natural products: How to develop a stronger in vitro "proof-of-concept." *J Ethnopharmacol*. 2006;106(3):290-302. doi:10.1016/j.jep.2006.04.003.

48. Aruoma OI. Nutrition and health aspects of free radicals and antioxidants. *Food Chem Toxicol*. 1994;32(7):671-683. doi:10.1016/0278-6915(94)90011-6.

49. Uttara B, Singh A V, Zamboni P, Mahajan RT. Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. *Curr Neuropharmacol*. 2009;7(1):65-74. doi:10.2174/157015909787602823.

50. Bozzo F, Mirra A, Carrì MT. Oxidative stress and mitochondrial damage in the pathogenesis of ALS: New perspectives. *Neurosci Lett*. 2017;636:3-8. doi:10.1016/j.neulet.2016.04.065.

51. Lü J-M, Lin PH, Yao Q, Chen C. Chemical and molecular mechanisms of antioxidants: experimental approaches and model systems. *J Cell Mol Med*. 2010;14(4):840-860. doi:10.1111/j.1582-4934.2009.00897.x.

52. Bickers DR, Athar M. Oxidative stress in the pathogenesis of skin disease. *J Invest Dermatol*. 2006;126:2565-2575. doi:10.1038/sj.jid.5700340.

53. Rinnerthaler M, Bischof J, Streubel MK, Trost A, Richter K. Oxidative stress in aging human skin. *Biomolecules*. 2015;5:545-589. doi:10.3390/biom5020545.

54. Pouillot A, Polla LL, Tacchini P, Neequaye A, Polla A, Polla B. Natural Antioxidants and their Effects on the Skin. In: *Formulating, Packaging, and Marketing of Natural Cosmetic Products*. ; 2011:239-257. doi:10.1002/9781118056806.ch13.

55. Services USD of H and H. Fourteenth Report on Carcinogens. 14th Report on Carcinogens. http://ntp.niehs.nih.gov/go/roc14. Published 2016.

56. Hans Lingnert. Influence of Food Processing on Lipid Oxidation and Flavor Stability. In: *Lipid Oxidation in Food*. Vol 500. ; 1992:292-301. doi:10.1021/bk-1992-0500.ch016.

57. Shahidi F, Zhong Y. Lipid oxidation and improving the oxidative stability. *Chem Soc Rev*. 2010;39(11):4067-4079. doi:10.1039/b922183m.

58. Karaosmanoglu H, Kilmartin PA. *Tea Extracts as Antioxidants for Food Preservation*. Elsevier Ltd; 2015. doi:10.1016/B978-1-78242-089-7.00009-9.

59. Berdahl DR, McKeague J. *Rosemary and Sage Extracts as Antioxidants for Food Preservation*. Elsevier Ltd; 2015. doi:10.1016/B978-1-78242-089-7.00008-7.

60. Weng CJ, Yen GC. *Natural Plant Extracts as Antioxidants for Food Preservation*. Elsevier Ltd; 2015. doi:10.1016/B978-1-78242-089-7.00010-5.

61. Shahidi F, Chandrasekara A. *The Use of Antioxidants in the Preservation of Cereals and Low-Moisture Foods*. Elsevier Ltd; 2015. doi:10.1016/B978-1-78242-089-7.00017-8.

62. Alam MN, Bristi NJ, Rafiquzzaman M. Review on in vivo and in vitro methods evaluation of antioxidant activity. *Saudi Pharm J*. 2013;21(2):143-152. doi:10.1016/j.jsps.2012.05.002.

63. Meng D, Zhang P, Li S, Ho C, Zhao H. Antioxidant activity evaluation of dietary phytochemicals using Saccharomyces cerevisiae as a model. *J Funct Foods*. 2017;38:36-44. doi:10.1016/j.jff.2017.08.041.

64. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem*. 1979;95(2):351-358. doi:10.1016/0003-2697(79)90738-3.

65. El-Saadani M, Esterbauer H, El.Sayed M, et al. A spectrophotometric assay for lipid peroxides in serum

lipoproteins using a commercially available reagent. *J Lipid Res*. 1989;30:627-630.

66. Subhaswaraj P, Sowmya M, Bhavana V, Dyavaiah M, Siddhardha B. Determination of antioxidant activity of Hibiscus sabdariffa and Croton caudatus in Saccharomyces cerevisiae model system. *J Food Sci Technol*. 2017;54(9):2728-2736. doi:10.1007/s13197-017-2709-2.

67. Silva CG, Herdeiro RS, Mathias CJ, et al. Evaluation of antioxidant activity of Brazilian plants. *Pharmacol Res*. 2005;52(3):229-233. doi:10.1016/j.phrs.2005.03.008.

68. Silva CG, Raulino RJ, Cerqueira DM, et al. In vitro and in vivo determination of antioxidant activity and mode of action of isoquercitrin and Hyptis fasciculata. *Phytomedicine*. 2009;16(8):761-767. doi:10.1016/j.phymed.2008.12.019.

69. Fridovich I. Superoxide Radical and Superoxide Dismutases. *Annu Rev Biochem*. 1995;64:97-112.

70. Crapo JD, Oury T, Rabouille C, Slot JW, Chang LY. Copper, zinc superoxide dismutase is primarily a cytosolic protein in human cells. *Proc Natl Acad Sci U S A*. 1992;89(21):10405-10409. doi:10.1073/pnas.89.21.10405.

71. SOD1 gene.

72. Bunton-Stasyshyn RKA, Saccon RA, Fratta P, Fisher EMC. SOD1 Function and Its Implications for Amyotrophic Lateral Sclerosis Pathology: New and Renascent Themes. *Neuroscientist*. 2015;21(5):519-529. doi:10.1177/1073858414561795.

73. Leitch JM, Yick PJ, Culotta VC. The right to choose: Multiple pathways for activating copper, zinc superoxide dismutase. *J Biol Chem*. 2009;284(37):24679-24683. doi:10.1074/jbc.R109.040410.

74. Torre-Ruiz MA de la, Pujol N, Sundaran V. Coping With Oxidative Stress. The Yeast Model. In: *Current Drug Targets*. Vol 16. ; 2015:2-12. doi:10.2174/1389450115666141020160105.

75. Wan X, Liu HM, Liao Y, et al. Isolation of a novel strain of Aeromonas media producing high levels of DOPA-melanin and assessment of the photoprotective role of the melanin in bioinsecticide applications. *J Appl Microbiol*. 2007;103(6):2533-2541. doi:10.1111/j.1365-2672.2007.03502.x.

76. Calado R, Leal MC, Gaspar H, et al. Grand Challenges in Marine Biotechnology. In: Rampelotto PH, Trincone A, eds. *Grand Challenges in Marine Biotechnology*. 1st ed. Cham: Springer International Publishing AG; 2018:317-403.

77. Seo C, Sohn JH, Oh H, Kim BY, Ahn JS. Isolation of the protein tyrosine phosphatase 1B inhibitory metabolite from the marine-derived fungus Cosmospora sp. SF-5060. *Bioorganic Med Chem Lett*. 2009;19(21):6095-6097. doi:10.1016/j.bmcl.2009.09.025.

78. Sun X, Zhou X, Cai M, Tao K, Zhang Y. Identified biosynthetic pathway of aspergiolide A and a novel strategy to increase its production in a marine-derived fungus Aspergillus glaucus by feeding of biosynthetic precursors and inhibitors simultaneously. *Bioresour Technol*. 2009;100(18):4244-4251. doi:10.1016/j.biortech.2009.03.061.

79. Lu XL, Xu QZ, Shen YH, et al. Macrolactin S, a novel macrolactin antibiotic from marine Bacillus sp. *Nat Prod Res Formely Nat Prod Lett*. 2008;22(4):342-347. doi:10.1080/14786410701768162.

80. Miyamoto KT, Komatsu M, Ikeda H. Discovery of gene cluster for mycosporine-like amino acid biosynthesis from Actinomycetales microorganisms and production of a novel mycosporine-like amino acid by heterologous expression. *Appl Environ Microbiol*. 2014;80(16):5028-5036. doi:10.1128/AEM.00727-14.

81. Gralla EB, Valentine JS. Null Mutants of Saccharomyces cerevisiae Cu , Zn Superoxide Dismutase : Characterization and Spontaneous Mutation Rates. *J Bacteriol*. 1991;173(18):5918-5920.

82. Rothstein RJ. One-Step Gene Disruption in Yeast. *Methods Enzymol*. 1983;101(C):202-211. doi:10.1016/0076-6879(83)01015-0.

83. Pitcher; DG, Saunders NA, Owen RJ. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. 1989:151-156.

84. Sambrook J, Russel RW. *Molecular Cloning: A Laboratory Manual*. 3rd ed. New York: Cold Spring

Harbor Laboratory Press; 2001.

85. Raja HA, Miller AN, Pearce CJ, Oberlies NH. Fungal Identification Using Molecular Tools: A Primer for the Natural Products Research Community. *J Nat Prod*. 2017;80(3):756-770.

86. Brand-Williams W, Cuvelier ME, Berset C. Use of a Free Radical Method to Evaluate Antioxidant Activity. *LWT - Food Sci Technol*. 1995;28(1):25-30. doi:https://doi.org/10.1016/S0023-6438(95)80008-5.

87. Malo N, Hanley JA, Cerquozzi S, Pelletier J, Nadon R. Statistical practice in high-throughput screening data analysis. *Nat Biotechnol*. 2006;24(2):167-175. doi:10.1038/nbt1186.

88. Egas C, Pinheiro M, Gomes P, Barroso C, Bettencourt R. The Transcriptome of Bathymodiolus azoricus Gill Reveals Expression of Genes from Endosymbionts and Free-Living Deep-Sea Bacteria. *Mar Drugs*. 2012;10(8):1765-1783. doi:10.3390/md10081765.

89. Bettencourt R, Pinheiro M, Egas C, et al. High-throughput sequencing and analysis of the gill tissue transcriptome from the deep-sea hydrothermal vent mussel Bathymodiolus azoricus High-throughput sequencing and analysis of the gill tissue transcriptome from the deep-sea hydrothermal vent mussel. *BMC Genomics*. 2010;11(1):1-17.

90. Wiener MC, Horanyi PS. How hydrophobic molecules traverse the outer membranes of Gram-negative bacteria. *PNAS*. 2011;108(25):10929-10930. doi:10.1073/pnas.1106927108.

91. Halpern BS, Longo C, Hardy D, et al. An index to assess the health and benefits of the global ocean. *Nature*. 2012;488(7413):615-620. doi:10.1038/nature11397.

Chapter 6. Annexes

Table 6.1 - Ranking of hits obtained after the secondary antimicrobial screening against *Enterococcus* sp. (VanA+) anaerobic; BA - Bathymordiolus azoricus; RS - Rimicaris sp; WC- water ; PS - Pachichara sp.; SD – Sediment D; SC – Sedicment C; SB – Sediment B; SA - Sediment A ; CR -Crab; CA -Chimney A; CB -Chimney B; CC – Chimney C; GA -Gastropode

			Ihnibition growth (%)		
	Hit ID	**Marine Strain**	0,1 mg/ml	0,25 mg/ml	0,5 mg/ml
Enterococcus sp. (VanA+) (T = 4h)	OE001	MGBA001	23,6		77,1
	OE003	MGBA003	22,7		40,8
	OE005	MGBA005	23,5		38,3
	OE007	MGMS007	33,9	41,5	73,6
	OE011	MGMS011	25,8	21,2	34,8
	OE015	MGGA015	25,2	21,9	22,6
	OE017	MGSA017	29,9	22,9	
	OE019	MGCR019	24,8	25,0	
	OE0051	RBBA051	27,9		
	OE055	RBBA055	24,7		20,9
	OE216	RBBA216O2	26,4		
	OE220	RBWC220O2	31,1		
	OE227	LSCA227O2	30,6		
	OE228	LSCA228O2	26,0		
	OE252	RBRS252O2	25,4	29,2	
	AEWC001	MGBA001	31,5		
	AEWC002	MGBA002	21,0		
	AEWC005	MGBA005	30,0		
	AEWC006	MGBA006	28,6		
	AEWC007	MGMS007	23,9		
	AEWC008	MGMS008	26,5		
	AEWC009	MGMS009	27,0		21,9
	AEWC011	MGMS011	31,6	21,1	24,6
	AEWC012	MGMS012	32,4		
	AEWC014	MGGA014	28,7	21,5	
	AEWC124	RBBA124O2	23,5		
	AEWC125	RBBA125O2			21,5
	AEWC194	MGCC194O2	24,5		
	AEWC199	RBWC199O2	30,4		
	AEWC200	RBWC200O2	23,2		20,5
	AEWC201	RBWC201O2	25,9		21,5
	AEWC202	RBWC202O2	34,9		
	AEWC213	MGCC213O2	27,4		
	AEWC216	RBBA216O2	23,3		
	AEWC219	RBBA219O2	29,4		20,6
	AEWC232	LSSD232O2	25,7		
	AEWC235	MGCC235O2	31,4		26,1
	AEWC240	MGMS240O2	28,3		

	AEWC247	RBRS247O2	27,8		
	AEWC248	RBRS248O2	95,0		

Table 6.2 - Ranking of hits obtained after the secondary antimicrobial screening against Salmonella enteritidis. MG – Menez Gwen; RB – Rainbow; LS – Lucky Strike; MS -Monte Saldanha; MS - Microcaris sp.; O2 - Facultative anaerobic; BA - Bathymordiolus azoricus; RS - Rimicaris sp; WC- water ; PS - Pachichara sp.; SD – Sediment D; SC – Sedicment C; SB – Sediment B; SA - Sediment A ; CR -Crab; CA -Chimney A; CB -Chimney B; CC – Chimney C; GA -Gastropode

			Ihnibition growth (%)				
	Hit ID	**Marine Strain**	0,1mg/ml	0,25 mg/ml	0,5 mg/ml	0,75 mg/ml	1 mg/ml
Salmonella enteritidis	OE232	LSSD232O2		50,4 (T=2h)	51,5(T=2h)	48,5 (t=2h)	46,9 (T=2h)
	OE235	MGCC235O2		52,8 (T=2h)	57,1 (T=2h)	53,1 (T=2h)	54,3 (T=2h)

Table 6.3 - Ranking of hits obtained after the secondary antimicrobial screening against Salmonella typhimurium. MG – Menez Gwen; RB – Rainbow; LS – Lucky Strike; MS -Monte Saldanha; MS - Microcaris sp.; O2 - Facultative anaerobic; BA - Bathymordiolus azoricus; RS - Rimicaris sp; WC- water ; PS - Pachichara sp.; SD – Sediment D; SC – Sedicment C; SB – Sediment B; SA - Sediment A ; CR -Crab; CA -Chimney A; CB -Chimney B; CC – Chimney C; GA -Gastropode

			Inhibition growth (%)		
	Hit ID	**Marine Strain**	0,1mg/ml	0,25 mg/ml	0,5 mg/ml
Salmonella typhimurium	OE007	MGMS007	22,8 (T= 24h)		

Table 6.4 - - Ranking of hits obtained after the secondary antimicrobial screening against Klebsiella oxytoca. MG – Menez Gwen; RB – Rainbow; LS – Lucky Strike; MS -Monte Saldanha; MS - Microcaris sp.; O2 - Facultative anaerobic; BA - Bathymordiolus azoricus; RS - Rimicaris sp; WC- water ; PS - Pachichara sp.; SD – Sediment D; SC – Sedicment C; SB – Sediment B; SA - Sediment A ; CR -Crab; CA -Chimney A; CB -Chimney B; CC – Chimney C; GA -Gastropode

		Inhibition growth (%)			
Strain	**Hit ID**	**Marine Strain**	0,1mg/ml	0,25 mg/ml	0,5 mg/ml
klebsiella oxytoca	OE128	RBBA128O2	23,1 (T=2h)		
	OE131	RBBA131O2	21,3 (T =2h)		
	OE168	MGMS168O2	20,4 (T =2h)		
	OE194	MGCC194O2	19,8 (T =2h)		
	OE203	MGCR203O2	25,9 (T =2h)		
	OE209	MGCR209O2	24,6 (T =2h)		
	OE210	MGCB210O2	31,4 (T =8h)		9,8 (T = 2h)
	OE218	RBBA218O2	25,5 (T =8h)		
	OE220	RBWC220O2	27,6 (T =8h)		

Table 6.5 - Ranking of hits obtained after the secondary antimicrobial screening against Staphylococcus aureus. MG – Menez Gwen; RB – Rainbow; LS – Lucky Strike; MS -Monte Saldanha; MS - Microcaris sp.; O2 - Facultative anaerobic; BA - Bathymordiolus azoricus; RS - Rimicaris sp; WC- water ; PS - Pachichara sp.; SD – Sediment D; SC – Sedicment C; SB – Sediment B; SA - Sediment A ; CR -Crab; CA -Chimney A; CB -Chimney B; CC – Chimney C; GA -Gastropode

			Inhibition growth (%)		
Strain	**Hit ID**	**Marine Strain**	0,1mg/ml	0,25 mg/ml	0,5 mg/ml
Staphylococcus aureus (T=4h)	OE001	MGBA001			88,9
	OE003	MGBA003			87,8
	OE007	MGMS007	77,1		89,1
	OE098	MGMS098O2	63,6	65,3	93,2
	OE125	RBBA125O2			87,4
	OE127	RBBA127O2	78,3		96,3
	OE128	RBBA128O2	80,3	68,9	101,4
	OE166	MGMS166O2			84,7
	OE168	MGMS168O2	55,0		93,9
	OE217	RBBA217O2			96,9
	OE241	MGMS241O2			80,8
	OE247	RBRS247O2			75,7
	OE248	RBRS248O2			74,8
	OE249	RBRS249O2			78,9
	OE250	RBRS250O2			68,1
	OE253	RBRS253O2	99,2		86,6
	AEWC250	RBRS250O2		76,7	