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Physiological assessment of non-pigmented extremophilic yeasts from the Atacama Desert as models for space exploration

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"The roots of education are bitter, but the fruit is sweet."

(ARISTÓTELES)

RESUMO EXPANDIDO

Introdução

Ainda que a vasta maioria de microrganismos extremófilos pertence aos Domínios Bacteria e Archaea, microrganismos eucariotos também podem apresentar mecanismos de resistência a uma variedade de condições extremas. Mais especificamente, leveduras são constantemente ressaltadas devido a sua flexibilidade fisiológica e alta sobrevivência sob uma variedade de parâmetros extremos. Compostos fotoprotetores, dentre eles melaninas e carotenoides, são comumente associados a uma elevada resistência a parâmetros ambientais extremos, devido aos seus efeitos diretos e indiretos associados à tolerância. De forma contrária, compostos fotoprotetores não-pigmentados costumam ser menos estudados, ainda que compreendendo importantes mecanismos de resistência frente à radiação ultravioleta e demais condições extremas, dentre elas os estresses osmótico e oxidativo, bem como estresse termal, salino e nutricional.

Objetivos

Esta tese teve como objetivo investigar leveduras não-pigmentadas, isoladas do Deserto do Atacama, de acordo com sua resistência a fatores ambientais extremos para avaliar seu potencial para estudos em astrobiologia e exploração espacial. Dentre os objetivos específicos, incluem-se a identificação de novas cepas de leveduras não pigmentadas, isoladas de amostras de solo do Deserto do Atacama; avaliar sua resistência a radiação ultravioleta e dessecação, bem como variações de temperatura e salinidade; e analisar a potencial aplicação destas leveduras em futuros estudos de astrobiologia e exploração espacial.

Metodologia

O primeiro capítulo desta tese de doutorado tem como objetivo revisar a função dos principais compostos fotoprotetores já estudados -- melaninas, carotenoides e micosporinas -- e comparar as diferenças de resistência entre leveduras pigmentadas e não pigmentadas, bem como discutir o potencial biotecnológico de compostos fotoprotetores não pigmentados, justificando maiores estudos na área. O segundo capítulo desta tese compreende a avaliação da resistência de seis leveduras não-pigmentadas isoladas de amostras de solo do Deserto do Atacama -- um ambiente extremo com severas condições ambientais. As leveduras foram identificadas através do sequenciamento da região intergênica ITS, e uma árvore filogenética foi reconstruída a partir do método de máxima verossimilhança. Além disso, os isolados foram estudados de acordo com sua resistência à radiação ultravioleta (UVC), ciclos de dessecação e reidratação, temperatura e diferentes condições salinas, reforçando a importância do Deserto do Atacama como nicho de microrganismos eucarióticos extremófilos e a necessidade de estudos aprofundados para um maior entendimento dos aspectos moleculares, ecológicos e fisiológicos destes organismos. Por fim, o terceiro e último capítulo dessa tese associa a possibilidade de utilização de leveduras extremófilas não-pigmentadas em estudos no campo da Astrobiologia, com especial atenção ao recente desenvolvimento de técnicas em biologia sintética como potencial mecanismo para a otimização de espécies extremófilas como organismos modelo para a utilização de recursos in situ durante futuras operações de exploração espacial frente ao objetivo de exploração do planeta Marte.

Resultados e Discussão

Os resultados obtidos através desta tese de doutorado possibilitaram a identificação de novas leveduras extremófilas não pigmentadas isoladas do Deserto do Atacama. Dentre elas, *Naganishia onofrii* (ATA13A) e *Papiliotrema laurentii* (ATA13B), apresentaram sobrevivência frente aos parâmetros ambientais extremos analisados, radiação UVC e dessecação, e salinidade, e crescimento máximo entre 20-30 °C. Microorganismos extremófilos não-pigmentados vem sendo estudados apenas nas últimas décadas, e estudos recentes indicam uma ausência de correlação entre pigmentação e resistência a fatores ambientais extremos. Compostos fotoprotetores não-pigmetados, dentre eles, as micosporinas, poderiam explicar a resistência destes microorganismos frente a exposição à radiação UV, dessecação, e demais fatores ambientais. Como extremófilos eucariontes, as espécies aqui identificadas reforçam a dominância de leveduras basidiomicetas em ambientes de alta elevação. Já seu fenótipo não-pigmentado reforça a necessidade de maiores estudos visando a compreensão de mecanismos alternativos de defesa a fatores ambientais extremos. Por fim, este estudo possui importância também para o campo da astrobiologia, uma vez que amplia nossa compreensão acerca dos aspectos moleculares, ecológicos, e fisiológicos por trás da resistência de microorganismos à fatores ambientais extremos, bem como das biomoléculas com potencial extremofílico para futura exploração espacial.

Considerações Finais

De acordo com os resultados obtidos nesta tese, os fatores combinatórios específicos que resultam em uma maior resistência e sobrevivência ambiental – dentre eles os fatores analisados em *N. friedmannii*, *Holtermaniella wattica,* e *P. laurentii* – devem ser melhor estudados para avaliar a implementação prática de leveduras extremófilas não-pigmentadas visando a implementação de parte da estrutura necessária para uma exploração espacial de longo-termo.

Palavras-chave: extremófilos, leveduras não-pigmentadas, radiação ultravioleta, astrobiologia.

ABSTRACT

Although the vast majority of extremophilic microorganisms belong to the Bacteria and Archaea Domains, eukaryotic microorganisms may also present resistant mechanisms to a variety of extreme conditions. In particular, yeasts are highlighted for their physiological flexibility and survivability under a wide range of extreme parameters. Photoprotective compounds, such as melanins and carotenoids, are usually associated with increased resistance to extreme parameters due to their direct and indirect tolerance-associated effects. In contrast, non-pigmented photoprotective compounds are less studied albeit also comprising important mechanisms of resistance against ultraviolet radiation and other extreme conditions including osmotic and oxidative stress as well as salinity, temperature, and nutritional stress. The first chapter of this thesis aimed to understand the role of the main photoprotective compounds -- melanin, carotenoids, and mycosporines -- and compare the differences found in resistance between pigmented and non-pigmented yeasts, as well as discuss the biotechnological potential of non-pigmented photoprotective compounds, justifying further studies in this topic. The second chapter of the thesis aimed to evaluate six non-pigmented yeasts isolated from soil samples from the Atacama Desert -- a harsh environment with extreme environmental conditions. Yeasts were identified by ITS sequencing and a phylogenetic tree was reconstructed based on maximum likelihood. In addition, the yeast isolates were assessed in terms of their resistance to ultraviolet radiation (UVC), desiccation-rehydration cycles, temperature, and different salinity concentrations, reinforcing the importance of the Atacama Desert as a niche of eukaryotic extremophilic microorganisms and the need for further studies aimed at clarifying their molecular, ecological and physiological aspects. Finally, the third and final chapter of this thesis associates the potential implementation of non-pigmented extremophilic yeasts in Astrobiology studies, in particular with the recent advances in synthetic biology as a potential methodology for the optimization of extremophilic species aimed to be used for *in situ* resource utilization during future missions toward space exploration aimed to explore the planet Mars.

Keywords: Extremophiles, non-pigmented yeasts, ultraviolet radiation, astrobiology.

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LISTA DE ABREVIATURAS E SIGLAS

6-4PPs	(6-4) - Pyrimidone photoproducts
ANOVA	Analysis of variance
ARC	Astrobiology Research Center
ASTROLAB	Laboratory of Astrobiology
BLSS	Biological Life Support Systems
CFU	Colony-Forming Units
CPD	Cyclobutane Pyrimidine Dimers
DHN	1,8-dihydroxynaphthalene
HSD	Honestly Significant Difference
ISRU	*In Situ* Resource Utilization
ISS	International Space Station
L-DOPA	L-3,4-dihydroxyphenyl-alanine
LEMEx	Laboratory of Molecular Ecology and Extremophiles
MGG	Mycosporine-Glutaminol-Glucoside
NaCl	Sodium Chloride
NASA	National Aeronautics and Space Administration
NCBI	National Center for Biotechnology Information
OD	Optical Density
PFA	Planetary Field Analogue
PPCs	Photoprotective Compounds
ROS	Reactive Oxygen Species
UVA	Ultraviolet Radiation A
UVB	Ultraviolet Radiation B
UVC	Ultraviolet Radiation C
UVR	Ultraviolet Radiation
YM	Yeast

SUMÁRIO

1 INTRODUCTION

Microorganisms can be found in virtually all habitats throughout our planet, leveraging critical ecological functions such as primary colonization, energy supply and nutrients recycling. In addition, microorganisms participate in symbiotic and pathogenic interactions and offer a wide biotechnological applicability (DUARTE, 2010). More recently, the development of metagenomic techniques provided the research of microorganisms with the ability to survive under atypical conditions in environments previously considered unsuitable for life (CARDOSO et al., 2011). Later studies revealed a whole class of microorganisms now called extremophiles, being adaptable to at least one extreme physical or chemical condition. Extremophiles can be found from glaciers and polar environments (psychrophilic) to volcanos (thermophiles), hypersaline lagoons (halophiles), and high-radiation environments (radioresistant microorganisms), among others (DUARTE et al., 2012; LÓPEZ-GARCÍA, 2001).

The vast majority of extremophilic microorganisms currently known belong to the Bacteria and Archaea Domains. However, the Domain Eukarya also comprises microorganisms resistant to a variety of extreme conditions (ROTHSCHILD; MANCINELLI, 2001), among which yeasts are highlighted for their high number of extremophilic species. Although vastly distributed within Earth's habitats, yeasts have not been extensively studied until the last few decades, with a recent shift towards the publication of scientific studies highlighting their physiological characteristics and their survivability under a wide range of parameters (BUZZINI et al., 2018; VISHNIAC, 2006; YURKOV, 2018).

The resistance to extreme environmental features involves an intricate web of evasion, protection and repair mechanisms with different species evolving different defense mechanisms to their own survival. As non-motile microorganisms, yeasts cannot leverage evasion mechanisms to survive extreme conditions. In contrast, a recent number of studies indicate the synthesis and accumulation of primary and secondary compounds among extremophilic yeasts' key strategies of defense. More specifically, radioresistant yeasts can produce photoprotective compounds (LIBKIND et al., 2004; MOLINÉ et al., 2014; SINGH; GABANI, 2011) that indirectly reflect into different pigmentation levels to these organisms. Examples include the melanins (CORDERO; CASADEVALL, 2017; EISENMAN; CASADEVALL, 2012) and carotenoids (BHOSALE; BERNSTEIN, 2005; BRITTON, 2008; MOLINÉ et al., 2014),

extra- and intracellular compounds, respectively, that function primarily as ultraviolet radiation (UVR) protection factors, as well as in response to temperature and other stress factors.

On the other hand, a specific type of photoprotective compounds includes those that do not reflect into pigmentation to their producers, including carotenoid precursors phytoene and phytofluene (BUZZINI et al., 2018; MELÉNDEZ-MARTÍNEZ et al., 2015), and mycosporines (BUZZINI et al., 2018; GABANI; SINGH, 2013; OREN; GUNDE-CIMERMAN, 2007). Besides protecting against UVR, there is evidence supporting the effects of such compounds against osmotic and oxidative stress, leading to hypothesis in terms of their function as biocompatible solutes, as well as against water, salinity, temperature, and nutritional stress (BUZZINI et al., 2018; OREN; GUNDE-CIMERMAN, 2007).

Practical examples highlighting the function of non-pigmented photoprotective compounds include the observation of a higher resistance to UVB in non-pigmented *Cryptococcus neoformans* and *Cryptococcus laurentii* compared to their melanized counterparts (SCHIAVE et al., 2009). In addition, Villarreal et al. (2016) did not observe a clear correlation between pigment production and UVC resistance in several yeast species. The latter research group also demonstrated an increased amount of mycosporines in species with the highest resistance levels. More recently, Pulschen et al. (2015) isolated two pigmented and two non-pigmented yeast species (*Naganishia friedmannii* e *Holtermaniella wattica*) from the Atacama Desert (Chile). Given the high resistance of both pigmented and non-pigmented species to UVB, UVC and solar radiation (UVA+UVB), the authors suggest the involvement of complementary mechanisms (GAO; GARCIA-PICHEL, 2011; PULSCHEN et al., 2015; 2018).

Researching extreme environments and their microorganisms opens new opportunities for the discovery of novel compounds and protection mechanisms, which can be leveraged to biotechnological processes favoring the development of biomedicine and pharmacology applications. In addition, the study of extremophilic microorganisms can give us hints to survival abilities that could be useful for the exploration of extraterrestrial planets, an emerging area of study within astrobiology research groups. After the isolation and identification of non-pigmented yeasts *N. friedmannii* and *H. wattica* by Pulschen et al. (2015), continued research resulted in the isolation of new non-pigmented yeast strains from the same soil samples. Therefore, this doctoral thesis presents a work with the goal of further understanding

the physiology and extremophilic capabilities of non-pigmented yeasts isolated from the Atacama Desert in order to provide insights into their applicability within biotechnology and space exploration studies.

2 GOALS

2.1 GENERAL GOALS:

- To investigate the potential of non-pigmented yeasts isolated from the Atacama Desert for astrobiology and space exploration studies according to their resistance to extreme environmental factors.

2.2 SPECIFIC GOALS:

- To identify new strains of non-pigmented yeasts isolated from soil samples isolated from the Atacama Desert.
- To evaluate the resistance of isolated strains to ultraviolet radiation (UVC) and desiccation.
- To evaluate the resistance of isolated strains to temperature and salinity extremes.
- To assess the potential application of the non-pigmented yeast strains for studies of astrobiology and space exploration.

3 CHAPTER I: REVIEW ON PHOTOPROTECTIVE COMPOUNDS IN PIGMENTED AND NON-PIGMENTED YEASTS

The first chapter of this thesis refers to a published review paper about photoprotective compounds in pigmented and non-pigmented yeasts. This chapter serves as the state of the art of the thesis, describing the different pigmented and non-pigmented photoprotective compounds and their direct and indirect protective effects against radiation in radioresistant yeasts. The review was published in the "*Applied Microbiology and Biotechnology*" journal (ISSN:0175-7598).

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MINI-REVIEW

Photoprotective compounds and radioresistance in pigmented and non-pigmented yeasts

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ABSTRACT

Ultraviolet radiation, continuously reaching our planet's surface, is a type of electromagnetic energy within the wavelength range of 10 to 400 nm. Despite essential for all life on Earth, ultraviolet radiation may have severe adverse cellular effects, including DNA dimerization and production of reactive oxygen species. Radioresistant microorganisms can survive under high doses of ultraviolet radiation, enduring the direct and indirect effects on nucleic acids and other biomolecules. The synthesis and accumulation of photoprotective compounds are among the main strategies employed by radioresistant yeast species to bear the harmful effects of ultraviolet radiation. A correlation between pigments and resistance to ultraviolet radiation has been widely recognized in these microorganisms; however, there is still some debate on this topic, with non-pigmented strains sometimes being more resistant than their pigmented counterparts. In this review, we explore the role of photoprotective compounds - specifically, melanin, carotenoids, and mycosporines - and compare the differences found in resistance between pigmented and non-pigmented yeasts. We also discuss the biotechnological potential of these photoprotective compounds, with special emphasis on those produced by non-pigmented yeast strains, such as phytoene and phytofluene. The use of "-omics" approaches should further unveil the radioresistance mechanisms of non-pigmented yeasts, opening new opportunities for both research and commercial applications.

Key Points:

a) Updated knowledge on photoprotective compounds from radioresistant yeasts;
b) Differences on radioresistance between pigmented and non-pigmented yeasts;
c) Future prospects over the study of non-pigmented photoprotective compounds.

Keywords: Carotenoids; Extremophiles; Melanin; Mycosporines; Photoprotective compounds; Ultraviolet radiation.

3.1 INTRODUCTION

Radiation refers to the energy in transit throughout space in the form of particles (particulate radiation) or waves (electromagnetic radiation). Ultraviolet radiation (UVR), continuously reaching our planet's surface, is a type of electromagnetic energy within the wavelength of 10 to 400 nm (GABANI; SINGH, 2013; ROTHSCHILD; MANCINELLI, 2001). The Sun is the main source of incoming UVR, which can be divided into three bands according to their wavelength spectrum, namely UVA (315–400 nm), UVB (280–315 nm), and UVC (100–280 nm), the latter being efficiently absorbed by ozone and oxygen molecules in the stratosphere layer and, therefore, not entering Earth (MOLINÉ et al., 2014).

Even though UVR represents only a small part of the solar radiation spectrum, its effects on Earth's life are still of high importance. UVA represents a large proportion of UVR wavelengths -- around 95% of total ultraviolet radiation that reaches Earth's surface. Nonetheless, UVB is the most biologically active and responsible for the main biological effects observed (BRAGA et al., 2015). Despite essential for photosynthetic organisms and consequently all life on Earth, UVR may have severe adverse effects. UVR-induced damage can directly reach nucleic acids and other biomolecules or indirectly through the promotion of reactive oxygen species (ROS), both leading to loss of biological function (BUZZINI et al., 2018; DIFFEY, 1991; MOLINÉ et al., 2014; ROTHSCHILD; MANCINELLI, 2001).

Direct DNA damage is the most common deleterious effect attributed to UVR. Changes on DNA molecular structure, by dimerization between adjacent pyrimidine bases, will result in impaired DNA transcription, translation, and replication (GABANI; SINGH, 2013; GAO; GARCIA-PICHEL, 2011; ROTHSCHILD, 1999). The two major types of dimerizations are cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4)-pyrimidone photoproducts (6-4PPs). Despite less recurrent, 6-4PPs are much more mutagenic than CPDs (MOLINÉ et al., 2014). UVA-induced dimerization occurs much less efficiently than that induced by UVB.

On the other hand, UVA-induced DNA damage occurs primarily through the promotion of ROS, a group of chemically reactive molecules such as hydrogen peroxide and superoxides. While ROS are naturally generated with metabolism and almost instantaneously converted to more benign compounds (*e.g.*, water and oxygen), their increase and accumulation could result in significant cell damages. ROS

reacts with nucleic acids and macromolecules such as lipids and proteins; therefore, higher stimulation of ROS production will inevitably result in cellular impairment (CADET et al., 2012). Nonetheless, UVR may also cause direct damage to proteins, being absorbed by chromophores and consequently causing fissures within the amino acid cross-links (DIFFEY, 1991; MOLINÉ et al., 2014).

Eventually, all direct and indirect effects caused by UVR may lead to cell damage and even death. Therefore, exposure to UVR imposes a detrimental component to all organisms' ecology (GAO; GARCIA-PICHEL, 2011). This is especially true for microorganisms living on surfaces open to sunlight. Beyond being exposed to higher UVR intensities, microorganisms are also more vulnerable due to their smaller size and, therefore, the higher ratio between genetic material and cellular volume (AGOGUÉ et al., 2005).

UVR's incidence greatly varies around different environments on Earth, intensifying at lower latitudes, like Arctic and Antarctic regions, and higher elevations, such as the mountainous environments of the Atacama Desert and the Alps (BRAGA et al., 2015; BUZZINI et al., 2018; CABROL et al., 2014; MOLINÉ et al., 2014; PULSCHEN et al., 2015). The irradiation doses received by these habitats are enough to classify them as extreme environments; thus, radiation becomes a restrictive parameter for most living beings, promoting the selection of extremophilic microorganisms (LYNCH et al., 2012).

3.1.1 Extremophiles

UVR is only one of many challenging physicochemical factors that represent a barrier to life, and environments with high UVR irradiance will most probably present other extreme conditions to the microorganisms living there, such as temperatures, salt, pH, water content, or nutrient availability (BUZZINI et al., 2018; PÉTER et al., 2017). Organisms coping with such extreme aspects of life are classified as extremophiles. Some extremophiles tolerate certain conditions and are subclassified as extremotolerants, while others not only survive but also require those conditions to live, being subclassified as true extremophiles (ROTHSCHILD; MANCINELLI, 2001).

When surviving under high doses of UVR, extremophiles are classified as radioresistants (GABANI; SINGH, 2013). Radioresistant microorganisms have been reported from various extreme environments, particularly above-cited Antarctica and

the Atacama Desert (ÓRDENES-AENISHANSLINS et al., 2016; REIS-MANSUR et al., 2019; PAULINO-LIMA et al., 2016; PULSCHEN et al., 2015). To survive the direct and indirect deleterious effects imposed by UVR, radioresistant extremophiles developed different protective mechanisms, most of them related to the production and accumulation of primary and/or secondary metabolic compounds, called extremolytes (GABANI; SINGH, 2013).

One of the main functions of extremolytes is the absorption of radiation, conserving the extremophiles' genetic material. Awareness of such metabolites is recent, and an increasing number of papers have been revealing not only their cellular, molecular, physiological, and ecological effects but also their potential for biotechnology. For instance, some extremolytes have been explored as new sources of antioxidants, cell cycle-blocking agents, and sunscreens (RADDADI et al., 2015; RASUK et al., 2016; WILSON; BRIMBLE, 2009). Furthermore, recent knowledge on cellular and molecular characteristics of extremolytes has contributed to new insights on the evolution of extremophiles, consequently expanding our understanding of the real limits for life.

3.1.2 Radioresistant Yeasts

Most species of extremophiles currently being studied belong to the prokaryotes; however, extremophilic microorganisms can be found amid all three domains of life (ROTHSCHILD; MANCINELLI, 2001). Yeasts are no exception, as this versatile group of eukaryotes presents a growing number of extremophilic - and, most specifically, radioresistant - species living under heavily stressed habitats. This polyphyletic group comprises basidiomycetous and ascomycetous fungi with a main saprophyte role in the environment (BUZZINI et al., 2018; KUTTY; PHILIP, 2008). Although universally distributed among Earth's extreme environments, extremophilic yeasts were not noticed until the last decades, with recent papers demonstrating community differences correlating with physicochemical parameters (BUZZINI et al., 2018; VISHNIAC, 2006; YURKOV, 2018).

For Buzzini et al. (2018), a yeast species can be precisely recognized as extremotolerant if, besides being isolated from an extreme environment and growing under extreme conditions (or growing optimally, in the case of true extremophiles), it demonstrates a physiological capacity to withstand those conditions. In extreme

habitats, selective pressure under high UVR has culminated in the emergence of yeast species with defense mechanisms to cope with the harmful effects of radiation (BRAGA et al., 2015). Such mechanisms may involve molecular repair or antioxidative systems, such as enzymes for DNA repair or ROS scavenge, respectively (GABANI; SINGH, 2013; MOLINÉ et al., 2014; SCHIAVE et al., 2009). However, as the saying goes, prevention is better; therefore, pigment production is a typical prevention mechanism found in radioresistant yeasts.

3.2 PIGMENTS AS PHOTOPROTECTIVE COMPOUNDS

Resistance to UVR involves a multifactorial network of avoidance, screening, and repairing mechanisms, with different species bearing distinct defensive systems to evade the harmful effects of UVR. Being non-motile, yeasts cannot indulge in the benefits of some avoidance methods, such as moving from a place with high UVR doses to a more shaded position or averting surfaces during the day (ROY, 2000; SCHIAVE et al., 2009). However, growing evidence indicates the synthesis and accumulation of photoprotective compounds (PPCs), widespread among microorganisms living in high-UVR environments, as one of the main strategies employed by radioresistant microorganisms (LIBKIND et al., 2004; MOLINÉ et al., 2014; SINGH; GABANI, 2011).

PPCs can act on protecting microorganisms by direct or indirect mechanisms. Indirect mechanisms include the abovementioned antioxidative systems, while the optical screening of ultraviolet wavelengths, that is, the efficient block of incoming solar rays, comprises the direct mechanisms of PPCs (LIBKIND et al., 2004). If a given metabolic compound acts directly as a PPC, absorbing a range of light spectrum and being sufficiently accumulated to reduce the harmful UVR effects, it can be considered as an ultraviolet sunscreen (or UV-screening compound). Due to this need to be sufficiently accumulated to function properly, the benefits of sunscreen production are typically observed in large-celled microorganisms, as a considerable amount of energy and space may need to be invested for their biosynthesis and storage (GAO; GARCIA-PICHEL, 2011).

Other important characteristics of sunscreens are the dissipation of absorbed energy in the absence of any disturbance to the cell, and the so-called loss of protection after molecular or physical removal, resulting in metabolic consequences,

such as growth impairment, under UVR (BRAGA et al., 2015; GAO; GARCIA-PICHEL, 2011). The consistent exposure of microorganisms to a wide spectrum of wavelengths has been shown to stimulate the synthesis and accumulation of PPCs, in special of sunscreens. This pattern has also been reported for some fungal species (AVALOS; ESTRADA, 2010; FULLER et al., 2013; LIBKIND et al., 2004; 2005), therefore regarded as a typical response of radioresistant microorganisms.

The amount of UVR penetrating the cells can be reduced by the accumulation of PPCs intracellularly or extracellularly, and to date, a considerable amount of secondary metabolites with photoprotective functions has been extracted from radioresistant microorganisms. Extracellular compounds are located within the cell wall and serve as a first physical barrier to incoming sun rays, while intracellular compounds are located in the cytoplasm, absorbing varying spectrums in the ultraviolet range (Roy 2000). Melanin is the most known extracellularly accumulated PPCs, while carotenoids and mycosporines are examples of intracellularly accumulated metabolites functioning as sunscreens (GAO; GARCIA-PICHEL, 2011; PÉREZ et al., 2006). Different PPCs will present different wavelength ranges (Fig. 1), and despite generally regarded as a passive defense mechanism (BRAGA et al., 2015; GAO; GARCIA-PICHEL, 2011), some PPCs may act more than shielding cells from incoming radiation, possessing also anti- oxidative abilities (LIBKIND et al., 2009; MOLINÉ et al., 2009; TSIMAKO et al., 2002).

Microbial PPCs have been gaining attention in the last few years as natural alternatives to synthetic sunscreens, with considerable applications in the cosmetics, pharmaceutical, textile, and food industries (AKILANDESWARI; PRADEEP, 2016). Recently published new information about the physiology and molecular biology of sunscreens has increased our knowledge on their biotechnological applications and may facilitate settlement of sustainable production of these compounds. As single-celled organisms with high production of PPCs, microalgae, and cyanobacteria are candidates for in vitro supply of sunscreens, however, cultivation of such microorganisms may not always be economically profitable due to their complex circadian rhythms. Yeasts, on the other hand, are easily cultivated under laboratory conditions and have a short and simple cell cycle, therefore appropriate for in vitro cultivation, and biotechnological production of a reasonable amount of sunscreens via recombinant biosynthesis may be a reality in the next few years (DZEHA et al., 2019; GAO; GARCIA-PICHEL, 2011).

3.2.1 Melanins

Melanins are a group of extracellular heterogeneous UV-absorbing compounds conferring brown to black pigmentation in a high number of species (Figure 2). Melanins are produced through the polymerization of phenolic and indolic monomers, complexing into a highly insoluble assemble, and usually aggregated with proteins and/or carbohydrates (BUTLER; DAY, 1998). The sunscreen capacity of melanins is observed from far UV to the infrared, with a maximum absorbance in the UV region between 200 and 300 nm (BRAGA et al., 2015; GAO; GARCIA-PICHEL, 2011; LIBKIND et al., 2009; SUWANNARACH et al., 2019). The first report of melanin in a yeast species was given by Roy et al. (1989) in the budding yeast *Exophiala jeanselmei*, with numerous papers for the characterization of new mesophilic and radioresistant yeast and fungal melanins being published every year (EISENMAN; CASADEVALL, 2012; GESSLER et al., 2014).

Two distinct pathways lead to the production of melanin in microorganisms: the L-DOPA (L-3,4-dihydroxyphenyl-alanine) and the DHN (1,8-dihydroxynaphthalene) pathways (EISENMAN; CASADEVALL, 2012). The L-DOPA pathway may generate eumelanins -- by which tyrosine is oxidized into DOPA - or pheomelanin - through which DOPA suffers cysteinization before being polymerized. In fungal species, however, mela- nins are mostly produced through the DHN pathways, leading to polymerization of allomelanins. Although highly heterogeneous, allomelanins are the least investigated group of melanins. Besides biosynthesis pathways, these subclassifications of melanin also differ in composition, with eumelanin and pheomelanin being mostly composed of nitrogen and sulfur, respectively, and allomelanins containing only a trace of nitrogen (BUTLER; DAY, 1998; GAO; GARCIA-PICHEL, 2011; EISENMAN; CASADEVALL, 2012; PLONKA; GRABACKA, 2006).

In addition to absorbing UVR and, therefore, functioning as UV-screening compounds, fungal melanins may also be involved in the protection against other environmental parameters, among them are elevated temperatures and different oxidizing parameters (CORDERO; CASADEVALL, 2017; EISENMAN; CASADEVALL, 2012). Melanins may also be a factor of virulence for some black yeast species, such as the case of the pathogens *Sporothrix schenckii* and *Cryptococcus neoformans*,

protecting the yeast against common medical treatments (MORRIS-JONES et al., 2003; NOSANCHUK et al., 2000). Nevertheless, their immuno-pharmacological, anti-inflammatory, and antioxidative abilities have enabled innumer- able applications in medical, agriculture, and cosmetic industries (NOSANCHUK et al., 1998).

3.2.2 Carotenoids

Carotenoids are intracellular liposoluble aliphatic polyenes composed of 40 conjugated atoms of carbon, therefore classified as tetraterpenoid pigments. These secondary compounds are associated with lipids in the cellular membrane (Figure 3) and efficiently absorb light in the high-energy spectrum of light, between 400 and 500 nm, harmlessly dissipating the energy as heat. Currently, there are more than 750 reported carotenoids, which can be grouped either as carotenes, containing only carbon and hydrogen atoms, or xanthophylls, consisting of carbon, hydrogen, and also oxygen atoms. β-Carotene and torulene are classified as carotenes, while astaxanthin and canthaxanthin are examples of xanthophyll carotenoids (AVALOS; LIMÓN, 2015; BHOSALE; BERNSTEIN, 2005; BRITTON, 1995; 2008). Their numerous conjugated double bonds act as chromophores and are responsible for the impressive natural red to yellow coloration of plants, fungi, and microorganisms, which are the exclusive producers of such sunscreens (MOLINÉ et al., 2014; NELIS; DE LEENHEER, 1991).

Most carotenoids will be indirectly responsible for the coloration of microorganisms. However, the precursors of all other carotenoids, phytoene, and phytofluene are carotenoids with specific chemical structures. It is known that a minimum of seven conjugated double bonds is needed for a carotenoid to display its characteristic colors. Phytoene and phytofluene, however, possess only three and five conjugated double bonds, respectively, and are therefore classified as colorless carotenes (BUZZINI et al., 2018; MELÉNDEZ-MARTÍNEZ et al., 2015). Nonetheless, these distinct carotenes still exhibit the ability of light absorption, with spectra ranging from 276 to 297 nm for phytoene and from 331 to 367 nm for phytofluene (BRITTON, 1995).

Coloration is only a secondary role derived from carotenoids, which can present several alternative functions besides radiation absorption. These PPCs have long been known for their versatility, presenting important functions in different cellular and membrane compounds. However, their role as antioxidative compounds is the

main studied function besides protecting the cells against incoming UVR. This secondary protection of carotenoids prevents oxidative damage, thus protecting cells against the indirect effects of radiation. Among microorganisms, yeasts are major carotenoid producers, with high quantities of β-carotene, γ-carotene, torulene, and canthaxanthin being produced by some species, i.e., *Rhodotorula* sp. and *Sporobolomyces* sp. Species of the genera *Phaffia* and *Xanthophyllomyces*, on the other hand, are known producers of astaxanthin (BHOSALE; GADRE, 2001; MATA-GÓMEZ et al., 2014; MOLINÉ et al., 2014). Previous experiments have shown their antioxidative role through quenching and scavenging ROS; however, the number of reports on the antioxidative function of carotenoids in yeasts is still scarce (BRITTON, 2008; MATA-GÓMEZ et al., 2014; MOLINÉ et al., 2009), and different carotenoid molecules may have evolved, contrasting protective roles (KHANEJA et al., 2010).

Due to their association with improved immune systems and the prevention of health disorders - such as cancer and heart diseases - much focused have been given to carotenoid research and production for pharmaceutical, nutrition, food, and cosmetic industries (BRITTON, 2008; MELÉNDEZ-MARTÍNEZ et al., 2015; PRAKASH; GUPTA, 2014). Growing commercialization, however, has increased costs for extraction from plants, traditionally yielded as dried powder extracts. Natural extraction also suffers from unstable supplies due to climatic and geographic conditions. Chemical synthesis, on the other hand, presents hazardous wastes that also increase costs for an environmentally safe industry (MATA-GÓMEZ et al., 2014). Due to their safety and easy management, yeasts have been presented as an option of reduced cost for carotenoid biosynthesis. In these microorganisms, carotenoids can be accumulated under UVR or other sets of stresses or highly induced via genetic manipulation, therefore presenting a viable option for culture production of these secondary compounds (BRITTON, 2008; GAO; GARCIA-PICHEL, 2011; MALDONADE et al., 2008; ROY, 2000). In particular, recent attention has been given to extremophilic yeasts from unexplored extreme habitats, in search of hyper-producing strains that could further reduce production costs.

3.2.3 Mycosporines

Mycosporines are intracellular PPCs containing small, low-molecular-weight, and water-soluble molecules with a diverse chemical structure (Figure 4).

Mycosporines are composed of an aminoxyclohexenone or an aminocycloheximine central ring, with substitutions on C1 and/or C3 reflecting on the high diversity of this class of UV-absorbing compounds. An amino compound will always be the substitute for C3, while C1 may be substituted with either an oxo or an imino moiety. Two to four double bonds are also present in the chemical composi- tion of mycosporines. Contrary to melanin and most caroten- oids, mycosporines are colorless compounds (BUZZINI et al., 2018; DZEHA et al., 2019; GAO; GARCIA-PICHEL, 2011; OREN; GUNDE-CIMERMAN, 2007; SINGH; GABANI, 2011). Still, they present a UV absorption spectrum between 296 and 360 nm and release the absorbed energy as heat without further cellular harm, therefore being also classified as sunscreen compounds. The absorption spectra of mycosporines are close banded with its chemical structures, and different substitutions on C1 and C3 may alter the wavelength range of absorbed energy, with minimum absorptions at 210 nm and maximum at 400 nm (BUZZINI et al., 2018; GABANI; SINGH, 2013; GAO; GARCIA-PICHEL, 2011; LIBKIND et al., 2006).

The first of these sunscreens was described by Leach (1965) in the mycelia of sporulating basidiomycetous fungi, hence the name mycosporine, but the first report for a yeast species was published only in the mid-2000s (SOMMARUGA et al., 2004). Widespread within microorganisms, mycosporines may be found in photosynthetic species, as in the case of cyanobacteria, dinoflagellates, microalgae, and macroalgae, as well as in non-photosynthetic species, such as fungi. In yeasts, in particular, mycosporines are thought to be even more common than carotenoids for both pigmented and non-pigmented species (BUZZINI et al., 2018; OREN; GUNDE-CIMERMAN, 2007). Mycosporine production seems to be restricted to species with the shikimic acid pathway, which is analogous and possibly evolutionarily connected to the biosynthesis of flavonoids - a class of UV-screening compounds mainly found in plants (MOLINÉ et al., 2014; ROY, 2000; ROZEMA et al., 1997).

It is worth mentioning that there are still some nomenclature complications with this class of PPCs, diverging between different authors. Some may name mycosporines with amino acid residues as mycosporine-like amino acids (GAO; GARCIA-PICHEL, 2011; OREN; GUNDE-CIMERMAN, 2007). Others refer to mycosporine-like amino acids for those sunscreens produced by aquatic organisms, whereas mycosporines would be exclusively produced by fungal species (BANDARANAYAKE, 1998; LIBKIND et al., 2006). Other authors, still, utilize both

mycosporines and mycosporine-like amino acids as synonyms, with no clear distinction between any substitution in the chemical structure nor from who produced it (GAO; GARCIA-PICHEL, 2011).

Research on the health effects of mycosporines is recent, and papers on their therapeutic and pharmaceutical advantages are still scarce (GABANI; SINGH, 2013). Just as melanin and carotenoids, biosynthesis and accumulation of mycosporines may be stimulated with UVR in yeasts (LIBKIND et al., 2004), so some species have been studied for future production for biotechnological applications as a replacement for UV-absorbing petrochemical- or synthetic-based compounds (DZEHA et al., 2019; MÉNDEZ-ÁLVAREZ et al., 2000). However, the sole presence of mycosporines may not be enough to prevent the negative effects of incoming UVR into the cells. Some authors suggest that cell size is determinant for the efficiency of mycosporine accumulation, as cells would need to be at least 10 to 100 μm in size for mycosporines to provide an effective shielding response (GARCIA-PICHEL, 1994; ROY, 2000; OREN; GUNDE-CIMERMAN, 2007). On the other hand, community accumulation of mycosporines has shown an effective blockage of UVR, compared to individual accumulation of such compounds, at least for aquatic microorganisms (SINHA et al., 1998). Nevertheless, mycosporines have also shown to be indirectly related to survival under high UVR or other environmental stresses, providing at least some degree of protection for those who synthesize and accumulate it.

Evidence for the antioxidative effects of these compounds is increasing, such as mycosporine-Gly extracted from marine species (BUZZINI et al., 2018; GUNDE-CIMERMAN, 2007; DUNLAP; YAMAMOTO, 1995; OREN; GABANI; SINGH, 2013). Another secondary function provided by mycosporines would be their role in the osmotic balance. When faced with salt stress, most resistant microorganisms synthesize and ac- cumulate uncharged organic molecules, the so-called compatible solutes, to reduce osmotic pressure within the cells. Some halophilic yeast species from the genera Hortaea and Phaeotheca have been shown to accumulate mycosporines under high-salt conditions, raising the question of whether these would also function as compatible solutes. Although their benefits do not compare with genuine compatible solutes -- such as trehalose, glycine, and betaine -- they may act as supplementary compounds and thus still present benefits to those species (GAO; GARCIA-PICHEL, 2011; OREN; GUNDE-CIMERMAN, 2007). To a lesser extent, mycosporines are also studied for their valuable effects under drought and thermal

stress, as intracellular nitrogen reserves, as well as their function in fungal reproduction (BUZZINI et al., 2018; OREN; GUNDE-CIMERMAN, 2007).

3.3 PHOTOPROTECTIVE COMPOUNDS AND RADIORESISTANCE IN PIGMENTED AND NON-PIGMENTED YEASTS

Resistance to UVR is species dependent, and although experiments often attempt to test radiation as an independent environmental parameter, in nature, it will never be truly found separately from other physicochemical elements, such as temperature, moisture, or availability of nutrients. Comparing results from UVR experiments is very challenging, as no standard is established and different laboratories will rely on distinct exposure times, radiation wavelengths, doses, or irradiances, reflecting on totally contrasting final methodologies (SCHIAVE et al., 2009; WONG et al., 2019). Nonetheless, our bulk knowledge was built onto these papers, and to date, there are no other reliable approaches for comparing the responses of microorganisms to UVR. In this matter, yeasts have been shown as great model organisms, with rapid growth and easily measurable responses to stress conditions, therefore simplifying the comparison of results.

As mentioned above, the rule of thumb is that more pigments will reflect in higher UVR resistance, not only due to direct “barrier” effects, blocking the entrance of incoming radiation, but also as a result of indirect, experimentally observed oxidative effects. In the 1960s, experiments with the basidiomycete Rhodotorula glutinis irradiated with gas lamps revealed a higher photosensitivity of carotenoidless mutants compared to the wild strain (MAXWELL et al., 1966). However, as older cultures of the non-pigmented strains revealed higher resistance than younger ones, the authors suggested that other physical and/or physiological features should also account for the resistance in this species. Although classified as carotenoidless, mutant strains still presented some concentration of the colorless carotenoid phytoene, which could at least partly explain the higher resistance observed in older cultures.

Albeit irradiated with a different wavelength (UVC), melanized *C. neoformans* (ATCC 24067) cells also presented higher resistance than their non-melanized counterparts (WANG; CASADEVALL, 1994). These authors also observed a higher tolerance in older, heavily melanized cultures, with higher concentrations of melanin possibly resulting in a more effective shielding effect than that observed in young, less

melanized cultures. More recently, Moliné et al. (2009) demonstrated the resistance to radiation in the basidiomycetous *Sporobolomyces ruberrimus* and *Cystofilobasidium capitatum*, with albino strains (CBS 7501 and 7420, respectively) more susceptible to UVB than carotenoid-pigmented wild strains (CRUB 1040 and 1047, respectively). Resistance was higher for cells exposed during the stationary phase of growth, compared to the exponential phase, reflecting once again the increment in protection with higher carotenoid accumulation.

Antarctic yeast species were also shown to be highly resistant to UVB and UVA, with resistance associated with the concentration of carotenoids (TSIMAKO et al., 2002) or melanin (ONOFRI et al., 2007). Production of mycosporines was detected for yeast isolates from Antarctica (VAZ et al., 2011) as well as from the Arctic (KOGEJ et al., 2006). In Antarctica, mycosporine production was shown to be species specific, identified in both pigmented and non-pigmented isolates. In freshwater yeasts from high-elevation Patagonian lakes (Argentina), the production of pigment compounds was found to be positively correlated with water transparency (LIBKIND et al., 2004, 2006; 2009). Authors suggested that highly transparent lakes present an environment with higher incoming UVR, selecting for yeasts with a higher constitutive concentration of carotenoids, which are, therefore, more resistant than non-pigmented yeasts. Similarly, Brandão et al. (2011) observed that yeasts from oligotrophic Patagonian lakes also produce more PPCs than yeasts collected from the littoral sites of the same lake, probably due to the increased UVR exposure caused by low turbidity of these sites. However, carotenoid content in these Patagonian yeasts was highly variable, and mycosporine production was higher for yeasts with lower carotenoid content. Libkind et al. (2006) found out that only 8% of pigmented yeasts were also mycosporine producers, against 70% of the non-pigmented yeast species. Still, mycosporine production was also correlated with a higher UVB resistance.

Melanin has been shown to protect yeast and filamentous fungal cells from gamma radiation, and increasing numbers of melanized species have been found in environments contaminated with radionuclides, such as the Chernobyl Nuclear Power Plant (Ukraine), and the Nevada Test Site (USA), suggesting melanin production as an evolutive advantage over non-pigmented species (DIGHTON et al. 2008). Some studies suggest that melanin not only protects yeast and filamentous fungi from the harmful effects of gamma radiation, but also that these microorganisms may benefit from it. Exposure of melanized *C. neoformans* (Cap 67) to visible light led to an ATP

reduction, while its non-pigmented counterpart presented elevated levels of ATP under the same conditions (BRYAN et al., 2011). As growth increase and radiotropism were already observed for melanized fungi exposed to gamma radiation (DADACHOVA et al., 2007; DIGHTON et al., 2008), the authors suggested the reduction in ATP levels could be a reflection of the capability of melanized *C. neoformans* in utilizing radiation energy as an energy transporter for metabolic processes. However, the molecular mechanisms behind such propositions have not yet been shown.

Pigmentation may even protect from the harsh conditions of space. The cryptoendolithic Antarctic black yeast-like fungus *Cryomyces antarcticus* (CCFEE 534 and CCFEE 515) was extensively studied in relation to its strongly melanized cells and radioresistance, surviving high doses of ultraviolet rays, gamma rays, deuteron rays, and X-rays, besides a combination of space and extraterrestrial conditions (ONOFRI et al., 2012; 2019; PACELLI et al., 2017a, b, c; SELBMANN et al., 2011). Unsurprisingly, *C. antarcticus* has become one of the best eukaryotic models for astrobiological studies, with remarkable growth, DNA, and ultrastructural preservation mainly associated with its thick, melanized cell wall. *Friedmanniomyces endolithicus* (CCFEE 5208) is another Antarctic melanized fungi for which melanin is associated with resistance to gamma radiation (PACELLI et al., 2018). Despite the huge load of results that correlates cellular pigment production and higher resistance to UVR, there are still some examples contrary to this. Schiave et al. (2009) found no significant difference in resistance of melanized and non- melanized cells of different strains of *C. neoformans* (strains 968, CRN 20, ATCC 28957, ATCC 90112) and *Cryptococcus laurentii* (strains CRN6, CRN9, CRN10, CRN21) after UVB irradiation. Contrary to what was observed until then, strains with slow melanization presented the higher resistance, compared to strains with a faster and more intense melanization. Resistance was also reduced in older cells, as opposed to what was previously observed by Maxwell et al. (1966) and Wang and Casadevall (1994). In this sense, Schultzhaus et al. (2019) observed similar survival rates between melanized and non-melanized *C. neoformans* (H99) cultures exposed to gamma radiation, arguing that melanization may only contribute with minor protection in radioresistance, as melanized cells present a slower growth and, therefore, are favored with more time for recovery mechanisms such as DNA repair enzymes. A slower growth also favored recovery from double-strand DNA breaks in C. antarcticus (CCFEE 515) cultures exposed to deuteron rays (PACELLI et al., 2017b).

Villarreal et al. (2016) did not observe a clear correlation between production of PPCs and UVC resistance in several yeast species. A strain of the ascomycetous *Leuconeurospora* sp. (T27Cd2), for example, presented high UVC tolerance; however, its carotenoid content was the lowest between carotenogenic yeasts. Its mycosporine content, on the other hand, was the highest among mycosporine-producing species, thus probably supporting such high resistance.

Although UVB irradiation of hyper-pigmented mutant strains of *Rhodotorula mucilaginosa* (CRUB 138) revealed a higher survival, carotenoidless mutant strains presented survival rates comparable to those presented by the wild type (MOLINÉ et al., 2010). Authors suggested this may have derived from the mutagenesis process, in which UVB exposure may have selected for carotenoidless strains with higher resistance. Nonetheless, it still indicates that other molecular processes may be behind radiation resistance. Dadachova et al. (2007) observed that non-pigmented *C. neoformans* (ATCC 24067) cells grew faster than melanized cells. Colorless cells also incorporated more 14C-acetate, indicating that melanization may reduce the cell wall porosity and, therefore, limit the incorporation of nutrients. Moreover, the process of producing and accumulating melanin contains oxidation reactions, consequently demanding higher metabolic costs that will reflect on a slower growth for melanized cells.

The theory of thermal melanism was postulated long ago but only recently experimentally confirmed for yeast species: organisms with darker pigmentation will absorb a higher amount of radiation - therefore, heating at a faster pace -- compared to light-pigmented or non-pigmented organisms. For *C. neoformans* (ATCC 208821) and *Candida* sp., an association between melanin content and reduced or no growth at higher temperatures was found. The carotenogenic *R. mucilaginosa* also presented similar heating rates as the aforementioned melanogenic species (CORDERO et al., 2018). Therefore, a pattern for pigmented species being found in higher latitudes and for non-pigmented species being found in the tropics is expected. Some exceptions to this pattern will still happen, as pigmentation exerts functions other than thermoregulation, such is the case for blocking incoming radiation. Thus, melanization - and, more broadly, pigmentation - is a species-specific microbial property resulting from endemic selective pressures that vary from each geographical location.

Pulschen et al. (2015) isolated pigmented and non- pigmented yeast strains from the Atacama Desert (Chile), all of which presented high resistance to UVB, UVC,

as well as solar radiation (UVA + UVB). Later experiments revealed *Naganishia friedmannii* (KM243310, previously classified as *Cryptococcus friedmannii*), one of the non-pigmented isolated strains, also presenting resistance to high-altitude atmospheric environment, combining other environmental factors, such as desiccation, to the harmful effects of UVR (PULSCHEN et al., 2018). Although no pigment could be identified with the Raman spectroscopy, authors suggested that some pigment may still be present in minor concentrations, below the technique detection limits. Still, as the direct shielding effects of melanin and carotenoids present some controversial results, complementary mechanisms are probably involved in radiation resistance for non-pigmented species. Besides the possession of colorless antioxidative compounds, like mycosporines, photorepair and other molecular mechanisms should be involved (GAO; GARCIA-PICHEL, 2011; PULSCHEN et al., 2015; 2018).

3.4 BIOTECHNOLOGY OF NON-PIGMENTED PHOTOPROTECTIVE COMPOUNDS

Pigmented photoprotective compounds have a high value for human health and are therefore highly appreciated in a wide range of industrial areas, *e.g.*, chemical, pharmaceutical, and cosmetics. *In vitro* cultivation of pigmented microorganisms is a feasible solution for alternatively increasing the production of such important compounds, and yeasts have been proved as low-cost models with excellent capacity for biosynthesis of carotenoids (MATA-GÓMEZ et al., 2014). Nevertheless, although the biotechnological aspects of pigmented PPCs are well known, the same does not hold true for non-pigmented PPCs.

The direct and indirect effects of non-pigmented PPCs have been proved useful for skin protection and their potential as sunscreen formulations started to be acknowledged with applications for patents two decades ago (BANDARANAYAKE, 1998; OREN; GUNDE-CIMERMAN, 2007). A study with mycosporines isolated from scallops (indirectly derived from microalgae) demonstrated the protective effects of three mycosporines in human skin cells, not only with UV shielding but also stimulating cell proliferation (OYAMADA et al., 2008). Studies on yeast-derived mycosporines, on the other hand, are still scarce. The basidiomycetous *Xanthophyllomyces dendrorhous* is a rare example of yeast applied in the production of non-pigmented PPCs. This species is known for the accumulation of mycosporine-glutaminol-glucoside (MGG), uniquely found in yeasts (JOHNSON, 2013).

X. dendrorhous is also studied for the production of pigmented carotenoids (LIBKIND et al., 2011), as well as the non-pigmented carotenoid phytoene (POLLMANN et al., 2017). This species was the first target-mutagenized yeast for production of phytoene (NIKLITSCHEK et al., 2008) and is currently being studied for increased production through knockout mutants for the phytoene desaturase gene (crtI) (POLLMANN et al., 2017). Besides its potential application as natural sunscreens, phytoene, as well as the other non-pigmented carotenoid phytofluene, offers potential for cosmetic and overall health applications (MELÉNDEZ-MARTÍNEZ et al., 2015; MIRAS-MORENO et al., 2019). Noteworthy, these compounds have also presented positive results over different types of cancer (*e.g.*, skin, prostate, and breast cancers). Altogether, the biological effects of phytoene and phytofluene have increased their added value, and higher efforts are necessary for meeting the current market demands. Therefore, the expanding field of in vitro production of phytoene and phytofluene, as well as mycosporines, presents a great perspective over the biotechnological future of non-pigmented yeast-derived PPCs (MELÉNDEZ-MARTÍNEZ et al., 2018; MIRAS-MORENO et al., 2019).

3.5 CONCLUSIONS AND FUTURE PROSPECTS

The selection of colorless strains via mutagenic agents is a laborious work and may not reflect the true nature of radioresistant microorganisms. The exploration of extreme environments should help unravel new natural colorless lineages, such as those isolated by Pulschen et al. (2015), opening new opportunities for the discovery of novel metabolic pathways that may be at least partly responsible for the higher radiation resistance of such microorganisms. Moreover, the protective and antioxidative capabilities of colorless carotenoids, such as phytoene and phytofluene, have been widely neglected, and these may hold important biological effects over a wide range of health conditions. This review presented the current state-of-the-art over-pigmented and non-pigmented photoprotective compounds and their importance for yeasts' radioresistance, as well as the current biotechnological aspects of mycosporines and the non-pigmented carotenoids phytoene and phytofluene. Future genomic, transcriptomic, and proteomic studies should reveal novel cellular and molecular mechanisms, as well as physiological systems, further elucidating UVR resistance in pigmented and non-pigmented yeast species.

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4 CHAPTER II: IDENTIFICATION AND PHYSIOLOGICAL ASSESSMENT OF NON-PIGMENTED EXTREMOPHILIC YEASTS ISOLATED FROM THE ATACAMA DESERT (CHILE)

The second chapter of this thesis refers to the study of six non-pigmented yeasts isolated from soil samples from the Atacama Desert. Yeasts were studied in terms of their resistance to UVR, desiccation, temperature, and different salinity concentrations. Part of the results from this chapter (Section B) were included in a manuscript recently submitted to the journal "Extremophiles" (ISSN:1431-0651).

SECTION A: Identification and physiological evaluation of non-pigmented extremophilic yeasts isolated from the Atacama Desert (Chile)

SECTION B: Resistance Profile of Two Non-Pigmented Extremophilic Yeasts Isolated from the Atacama Desert (Chile)

4.1 SECTION A: IDENTIFICATION AND PHYSIOLOGICAL EVALUATION OF NON-PIGMENTED EXTREMOPHILIC YEASTS ISOLATED FROM THE ATACAMA DESERT (CHILE)

4.1.1 Introduction

A wide range of biotic and abiotic factors reflects in the current yeast biodiversity found in extreme environments (FOTEDAR et al., 2018; GUNDE-CIMERMAN et al., 2000). Radiation, salinity and desiccation are among the main factors that interact to select poly-extremophilic organisms in the Atacama Desert. The results presented in this section correspond to the identification and characterization of yeast strains isolated from the Atacama Desert, indicating their potential for resistance to ultraviolet radiation, desiccation, salinity and temperature, as well as their potential application for further studies on biotechnology and astrobiology.

4.1.2 Methodology

4.1.2.1 Collection, Isolation, and Culturing Conditions

All yeast isolates included in this study were collected from the Atacama Desert (PULSCHEN et al., 2015). The microorganisms were isolated from the superior layer of soil samples at three distinct collection sites close to the Sairecabur Volcano (Table 1), in January 2012, and provided by Dr. Fábio Rodrigues and Dr. Ana Carolina de Carvalho, from the Laboratory of Astrobiology (ASTROLAB, Institute of Chemistry, University of São Paulo, Brazil). Yeast strains were transported to the Laboratory of Molecular Ecology and Extremophiles (LEMEx, UFSC) and stored with YM broth (peptone 5 $g.L^{-1}$, yeast extract 3 $g.L^{-1}$, malt extract 3 $g.L^{-1}$, glucose 10 $g.L^{-1}$) with glycerol 20% at -20 °C). Routine counting was maintained in YM broth at 25 °C with constant agitation (150 rpm).

Table 1 – Collection date, site, and altitude of each yeast isolate.

Yeast	Collection Date	Latitude	Longitude	Altitude (m)
Naganishia friedmannii Strain 16Lv2	08/01/2012	-22.706917	67.996050	3981
Holtermaniella wattica Strain 16Lv1	08/01/2012	-22.706917	67.996050	3981
Strain ATA13A	08/01/2012	-22.716812	-67.923690	5047
Strain ATA13B	08/01/2012	-22.716812	-67.923690	5047
Strain ATA16A	08/01/2012	-22.706917	67.996050	3981
Strain ATA16BAB	08/01/2012	-22.706917	67.996050	3981
Strain ATA16BC	08/01/2012	-22.706917	67.996050	3981
Strain ATA37BB	13/01/2012	-26.295352	-70.659348	83

4.1.2.2 Molecular Identification

The genetic material utilized for the molecular identification of the yeast isolates was extracted according to the rapid yeast DNA extraction protocol developed by Green and Sambrook (2018), with modifications. Briefly, yeast isolates were cultivated in YM solid plates until late exponential phase (approximately 72h), transferred to microtubes containing 1000 µL of CTAB buffer and maintained under 37°C for 1 hour to loosen the cell wall. Then, 60 µL of phenol: chloroform: isoamyl ethanol solution (25:24:1) were added to the tubes with sterile 2 mm glass beads. After vortexing each sample for 60 seconds, each microtube was centrifuged (5 minutes, 15.000 rpm, room temperature) and the superior aqueous layer was transferred to new clean microtubes. A chloroform: isoamyl ethanol solution (24:1) was added to the sample to further separate proteins from DNA, followed by a new centrifugation step using the same conditions above. Total genomic DNA was precipitated by ethanol

100% with sodium acetate (3M) for 15 minutes on ice, followed by removal of the supernatant aqueous phase and new centrifugation (10 minutes, 15.000 rpm, 4 °C). DNA pellets were then washed with 100 µL of ethanol 70%, centrifuged, and dried under room temperature. The genetic materials were finally ressuspended by 50 µL of MilliQ water (pH 8,0). Samples were quantified by NanoDrop (ThermoFisher, US) and stored under refrigeration (-20 °C) until subsequent analysis.

Yeast isolates were identified by sequencing the internal transcribed spacer (ITS) of rRNA genes amplified by primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (WHITE et al., 1990). PCRs reactions consisted of 2.5 µL of 10x buffer, 1 µL of 50 mM $MgCl_2$, 0.2 µL of 100 mM dNTPs (25 mM each), 0.5 µL of each primer (20 µM each), 0.2 µL of 5 U/µL Taq polymerase (Invitrogen), 1 µL of diluted genomic DNA (~10 ng/µL), and sterile ultrapure water to a final volume of 25 µL. PCR started with an initial 94°C for 3 min, followed by 35 cycles of denaturation (94°C for 1 min), annealing (55°C for 1 min), and extension (72°C for 2 min), and then a final extension of 72°C for 10 minutes. PCR amplicons were purified using the ethanol precipitation method described by Green and Sambrook (2016). The purified amplicons were sequenced at Macrogen, Inc (South Korea). The obtained sequences were analyzed by BLAST with references from the GenBank database.

4.1.2.3 Phylogenetic Analysis

For the phylogenetic analysis, ITS sequences from all yeast isolates were identified altogether with the reference sequences obtained from the GenBank database. Reference sequences were selected from type strains with high similarity against the tested yeast isolates, excluding environmental non-cultivated organisms. A merged FASTA file containing the yeast isolates and reference sequences was aligned on MUSCLE using the default parameters. A phylogenetic tree was constructed on software MEGA 11 (STECHER et al., 2020; TAMURA et al., 2021) based on Maximum Likelihood method, using Kimura 2-parameters as substitution model and bootstrap test with 1000 replications.

4.1.2.4 Growth Curves

Yeasts' growth curves were estimated from the assessment of strains cultivated for 80 hours in YM broth (25°C, constant agitation of 150 rpm). Strains were selected from a single colony cultivated on agar plates, cultured for 24 hours. Then, 30 µL were transferred to 3 mL of YM broth as the initial culture of the experience. After every 4 hours, 200 µL aliquots were quantified with OD_{595}, in triplicate.

4.1.2.5 UVC Experiments

Radiation experiments were conducted according to the protocol developed by Pulschen et al. (2015), with modifications. Yeast strains were cultivated from a single colony, as previously described, until the late exponential phase (OD_{595} = 1.0-1.1, approximately 48 hours of culture). Then, 1000 µL were centrifuged (3000 rpm, 5 minutes) and washed with sterile saline solution. Pellets were dispersed in sterile saline solution to a volume of 1 mL of cell suspension and transferred to a 10 cm diameter sterile Petri dish and irradiated with a Philips (Philips, Eindhoven, The Netherlands) TUV-20W low-pressure Hg lamp (253.7 nm), with samples placed at 22 cm from the lamp. Cells were irradiated for 30 and 60 seconds, in triplicate. Serial dilutions were performed (10^{-1} to 10^{-6}) and 10 µL aliquots were transferred to solid YM plates, in triplicate, to viability assessments through colony-forming units (CFU) account after incubation for 48 h at 25°C. Control samples were not exposed to UVC.

4.1.2.6 Salinity Experiments

Yeast strains were cultivated from a single colony, as previously described, until the late exponential phase (OD_{595} = 1.0-1.1, approximately 48 hours of culture). Then, 30 µL were transferred to 3 mL of liquid YM broth with NaCl solution under increasing concentrations (1, 2, 3, 4, and 5%) for 48 h. After the experiment, samples were quantified, in triplicate, by OD_{595} measurement.

4.1.2.7 Desiccation Experiments

Yeast strains were cultivated from a single colony, as previously described, until the late exponential phase (OD_{595} = 1.0-1.1, approximately 48 hours of culture). Then, 50 µL aliquots of washed early stationary phase cells were transferred to 96-well plates, in triplicate, and dried under oxygenation for 24 h periods. After each period, cells were rehydrated with PBS solution and diluted in serial dilutions (10^{-1} to 10^{-6}) for CFU count. In total, five desiccation-rehydration cycles were completed.

4.1.2.8 Statistical Analysis

Data obtained by the previously described experiments was analyzed through ANOVA (analysis of variance) with post-hoc Tukey HSD (Honestly Significant Difference) to verify the statistical significance of results obtained from UVC, salinity, and desiccation treatments with their respective controls. All analyses were conducted by JMP version 16 (SAS Institute Inc., Cary, NC, US).

4.1.3 Results and Discussion

4.1.3.1 Molecular Identification

The sequencing of isolated yeasts provided the identification of three different species from the Basidiomycota Phylum (Table 2). As expected, most of the isolated yeasts were identified as *Naganishia friedmannii* (strains ATA13A, ATA16B, ATA16BAB, and ATA37BB), while a fifth yeast strain (ATA16BC) was identified as *Holtermaniella wattica*. Both *N. friedmannii* and *H. wattica* were initially identified from antarctic environments (VISHNIAC, 1985; GUFFOGG et al., 2004) and are commonly isolated from extreme temperature environments. In addition, yeasts from the *Naganishia* genus (previously classified as *Cryptococcus*) were identified from different cold environments, including Iceland and Russia (VISHNIAC, 2006), as well as from deserts, as initially described by Vishniac (1998). Besides temperature extremes, extremophilic *Naganishia* species are also usually associated with high desiccation and low nutritional environments (VISHNIAC, 2006), being considered among the microorganisms with highest UVR resistance to date (SCHMIDT et al., 2017).

Lastly, our molecular analysis identified yeast strain ATA13B as *Papiliotrema laurentii*, a species commonly isolated from environmental samples and previously classified as *Cryptococcus laurentii*. *P. laurentii* strains were identified from soil and moss samples from Livingston Island in the Antarctic (PAVLOVA et al., 2001). However, to our knowledge, this is the first identification of the species from a soil sample from a desert environment. Even though melanized species were previously identified, *P. laurentii* does not usually present a pigmented phenotype (ASADZADEH et al., 2020). The same holds true to the specimens identified in this study. Previous studies demonstrate the acidophilic, alkalophilic, halophilic and psychrophilic capacity of *P. laurentii* strains (BUZZINI et al., 2018). The yeast was also demonstrated to grow under low nitrogen concentrations (SARKAR et al., 2017). Finally, recent studies indicate the biotechnological potential of *P. laurentii* with the production of lipids with high industrial applicability (VIEIRA et al., 2020; WANG et al., 2018).

Table 2 – Molecular identification of yeast strains isolated from the Atacama Desert.

Yeast	BLAST	Strain	identity	Number of Base Pairs	Access
ATA13A	*Naganishia friedmannii*	XZY80-3	99.66%	587	MW710873.1
ATA13B	*Papiliotrema laurentii*	FC7-7A	100%	487	MW894949.1
ATA16B	*Naganishia friedmannii*	XZY80-3	100%	587	MW710873.1
ATA16BAB	*Naganishia friedmannii*	XSR18-1	100%	576	MW710076.1
ATA16BC	*Holtermaniella wattica*	XZY548-1	100%	521	MW710762.1
ATA37BB	*Naganishia friedmannii*	XZY80-3	100%	587	MW710873.1

4.1.3.2 Phylogenetic Analysis

From the identified yeast species, a selection of 26 high similarity reference species were utilized for the reconstruction of a phylogenetic tree. Selected species included: four *Cryptococcus*, four *Holtermaniella*, two *Holtermannia,* five *Kwoniella,* six *Naganishia* and five *Papiliotrema* species (Table 3). *Kwoniella* and *Cryptococcus* species were included as outgroup species to the tree, with the exception of *Cryptococcus antarticus*. The Maximum Likelihood analysis of the intergenic sequence combined into a reasonably parsimonious phylogenetic tree, with bootstrap values between 98 and 100% to the genus level (Figure 1).

All species analyzed refer to Basidiomycota Division and Tremellomycetes Class. *Cryptococcus* (VUILLEMIN, 1901), *Papiliotrema* (SAMP; WEISS; BAUER, 2002), and *Kwoniella* (STATZELL; FELL, 2008), rely among the Tremellales Order, with both *Cryptococcus* and *Kwoniella* under the Cryptococcaceae Familty. It is thus observed that species from the three genera were placed next to the root of the tree. In contrast, *Holtermaniella* and *Holtermannia* species are identified among the Holtermanniales Order (BOEKHOUT, 2011; LIBKIND; WUCZKOWSKI; TURCHETTI; 2011), and were placed to the center of the tree, with clear partition between both genera. Finally, *Naganishia* species (Filobasidiales Order) are localized to the apical region of the phylogenetic tree, together with *Cryptococcus antarticus.*

Similarly, to the molecular identification, isolates were clearly classified among the three genera *Naganishia*, *Holtermaniella*, and *Papilitorema*. On the other hand, all species were classified with a low bootstrap value within the species level, indicating the need for further studies to group or differentiate these strains at the species level. Yeast isolates from the *Naganishia* genus (ATA13A, ATA16B, ATA16BAB, and ATA37BB) form with an 88% bootstrap, a large group together with *N. friedmannii* and *N. onofrii*; however, their identification to the species level remains unclosed based on the evidence provided by this study.

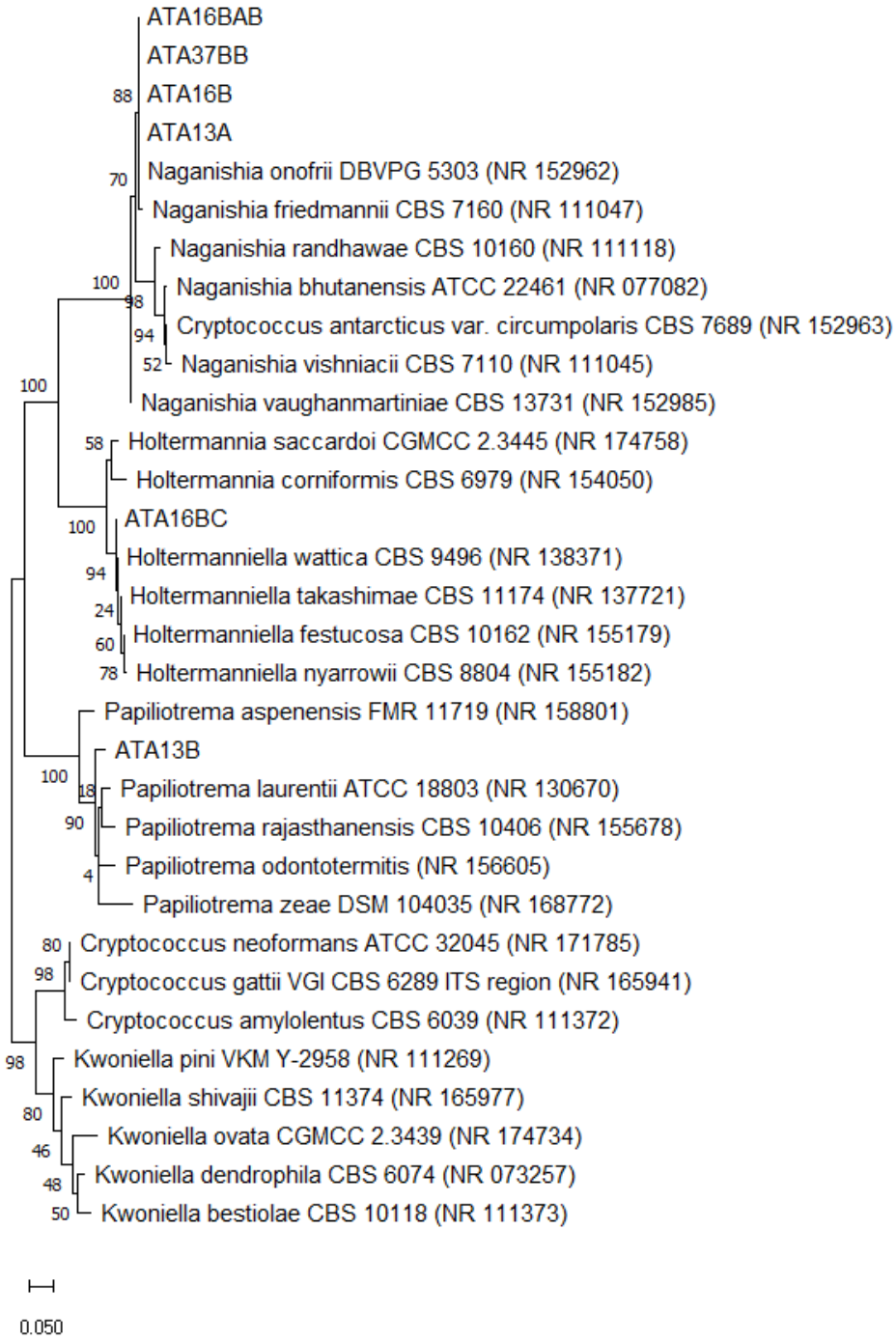
Previous studies indicate a clear dominance of psichrophylic *Naganishia* species in high altitude soils at the Atacama Desert (COSTELLO et al., 2009; LYNCH et al., 2012). These species are highly adapted to the low temperature extremes of the desert, as well as to other stress factors identified in such environments. These harsh environments might select for a high genic mutation rate (TURCHETTI et al., 2015), consequently reflecting high intra-genus variability. Such variability increases the

challenge on how to differentiate species, as cryptic mutations cannot be detected by traditional gene markers (SCHLICHTING, 2008). The precise determination of phylogenetic relations among *Naganishia* species are crucial for the understanding of its ecological dispersion throughout extreme environments. Thus, besides the physiological, metabolic, and cellular characteristics, different gene markers (***e.g.,*** RPB1, RPB2, TEF1, CYTB, 18S, LSU and 3 rDNA) should be implemented for the reliable identification of these species (SCHMIDT et al., 2017; TURCHETTI et al., 2015).

Table 3 – Reference species selected for the reconstruction of a phylogenetic tree.

Reference Species	Strain	Access
Cryptococcus amylolentus	*CBS 6039*	*NR111372*
Cryptococcus antarcticus	CBS 7689	NR152963
Cryptococcus gattii	CBS 6289	NR165941
Cryptococcus neoformans	ATCC 32045	NR171785
Holtermannia corniformis	CBS 6979	NR154050
Holtermannia saccardoi	CGMCC 2.3445	NR174758
Holtermanniella festucosa	CBS 10162	NR155179
Holtermanniella nyarrowii	CBS 8804	NR155182
Holtermanniella takashimae	CBS 11174	NR137721
Holtermanniella wattica	CBS 9496	NR138371
Kwoniella bestiolae	CBS 10118	NR111373
Kwoniella dendrophila	CBS 6074	NR073257
Kwoniella ovata	CGMCC 2.3439	NR174734
Kwoniella pini	VKM Y-2958	NR111269
Kwoniella shivajii	CBS 11374	NR165977
Naganishia bhutanensis	ATCC 22461	NR077082
Naganishia friedmannii	CBS 7160	NR111047
Naganishia onofrii	DBVPG 5303	NR152962
Naganishia randhawae	CBS 10160	NR111118
Naganishia vaughanmartiniae	CBS 13731	NR152985
Naganishia vishniacii	CBS 7110	NR111045
Papiliotrema aspenensis	FMR 11719	NR158801
Papiliotrema laurentii	ATCC 18803	NR130670
Papiliotrema odontotermitis	N/A	NR156605
Papiliotrema rajasthanensis	CBS 10406	NR155678
Papiliotrema zeae	DSM 104035	NR168772

Figure 1 – Phylogenetic tree reconstructed from the ITS sequence of six yeast isolates and 26 reference species. The scale refers to the number of substitutions per site.

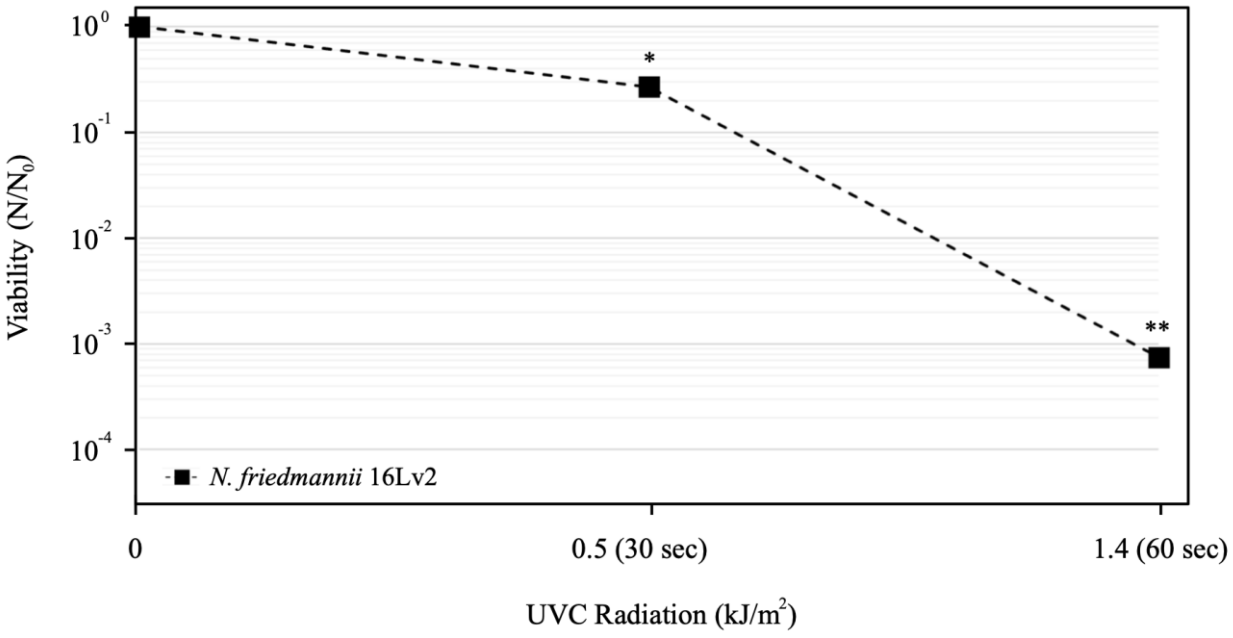


Maximum likelihood phylogenetic tree constructed with the ITS sequence of the six yeast isolates from Atacama Desert and 26 type strain sequences. Bootstrap values calculated from 1000 replications are shown next to the branches. Scale bar represents the number of substitutions per site.

4.1.3.3 UVC Resistance

Ideal conditions for radiation assays require a radiometer equipment to conduct the quantification of exact dosages to which microorganisms are exposed to. Because our laboratory does not provide radiometer equipment, cultures of *N. friedmannii* 16Lv2 (PULSCHEN et al., 2015) were implemented as an alternative normalization method of the radiation dosage to our experiments (Figure 2). Results indicated similar viabilities between the yeast cultures utilized in this work and the yeast cultures exposed by Pulschen et al. (2015), with a significant reduction in viability after 30 and 60 seconds of exposure ($p < 0,0001$, F ratio = 94,9464) referring to an approximate dosage of 0.5-0.6 kJ/m^2 and 1.2-1.4 kJ/m^2, respectively.

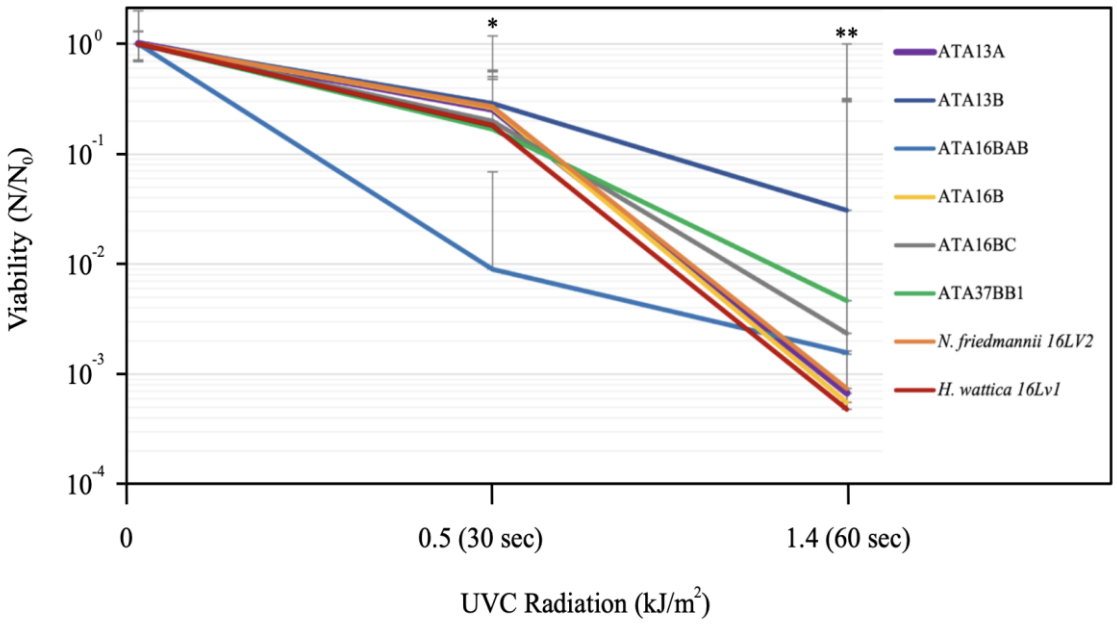
Figure 2 – Viability of *N. friedmannii* 16Lv2 after UVC exposure.



After normalizing the exposure time, we conducted the further assessment of all isolated yeasts (Figure 3), with results indicating a significant reduction in viability as compared to each of the yeasts' respective control samples ($p < 0,0001$, F ratio = 653,8679). Yet, all samples survived to both radiation exposure times, without significant differences being observed between each yeast strain ($p = 0.8991$, F ratio = 0.3995). Similarly, to Pulschen et al. (2015) results, *H. wattica* (average viability of 18% after 30 seconds of UVC exposure) presented a reduced viability compared to *N. friedmannii* (average viability of 27%). It is worth mentioning that although presenting a similar viability after 30 seconds of UVC exposure, the yeast strain ATA13B (*P.*

laurentii) presented the highest resistance among all tested yeasts after 60 seconds of UVC exposure.

Figure 3 – Viability of yeast strains after 30 and 60 seconds of UVC exposure. Symbols indicate significant differences according to the ANOVA and Tukey tests ($p \leq 0.05$).



As reinforced by Villarreal et al. (2016), the scientific literature about yeasts' resistance to UVR is still scarce. Even less studies can be found about the resistance of non-pigmented yeasts compared to those providing for at least some degree of pigmentation. As the selective mechanisms are imposed to yeasts and other microorganisms in a similar manner, it becomes evident the potential for selection of radioresistant yeast species in environments where high altitudes reflect into a higher incidence of radiation -- among which the Atacama Desert (CABROL et al., 2014). Combined with other environmental factors observed in such extreme environments, the impact of UVR is modulated such that the selection of resistant organisms reflects the exposure time, wavelengths, and the incidence of cell-reaching photons (WONG et al., 2018). Therefore, the results observed in this study indicate the presence of new non-pigmented yeasts providing mechanisms of resistance to UVR, with a particular highlight to *P. laurentii* ATA13B.

DNA represents a key target for the mutagenic effects of high UVR exposure, particularly UVC (BEBLO et al., 2011; SELBMANN et al., 2011). A critically deleterious effects from which microorganisms have to protect after UVR exposure is the direct

damage to their genetic material, including structural changes in the conformation of DNA molecules (*e.g.,* pyrimidine dimerization), thus altering transcription, translation and replication mechanisms that are essential for cellular organization (GABANI; SINGH, 2013; GAO; GARCIA-PICHEL; 2011; ROTHSCHILD, 1999). In this context, molecular repair mechanisms and antioxidative systems are crucial for the survival of radioresistant species, as commonly observed among pigmented and non-pigmented yeasts studies thus far.

On the other hand, differently from pigmented microorganisms, in which the production of photoprotective pigments constitutes a key barrier to UVR, non-pigmented yeasts are primarily dependents of alternative, non-pigmented compounds to reduce UVR-induced damage. Although different UV-absorbing compounds were already identified from yeasts, the physiological mechanisms behind these compounds are not completely understood yet (WONG et al., 2018), as well as their potential applicability throughout biotechnological applications. Over the last few years, these precursor compounds have been strongly neglected by studies evaluating UVR resistance among pigmented microorganisms. With the discovery of new non-pigmented species, their ecological relevance and protective capacity becomes more and more evident.

Similarly, to carotenoid precursors, mycosporines and mycosporine-like amino acids also have low molecular weight and do not reflect into visible pigmentation. In addition, mycosporines absorb wavelengths similar to carotenoid precursors, between 250 and 360 nm (BUZZINI et al., 2018; WONG et al., 2018). In contrast, mycosporines have been more well studied over the last years, with further recognition of their functions other than energy absorption, including their antioxidative properties (MOLINÉ et al., 2010). Villarreal et al. (2016) describes a practical example of mycosporines' protective effects, providing for high UVC resistance in *Leuconeurospora* yeasts with low pigmented carotenoid content and high mycosporine concentration. In addition, these authors discussed the inverse correlation between the content of carotenoids and mycosporines as indication of the accumulation of the latter as alternative protective compounds. Even though not quantified by this study, it is worth noting the strong potential production of such compounds in the non-pigmented radioresistant yeasts isolated from the Atacama Desert, thus opening up new possibilities for studies using these yeasts.

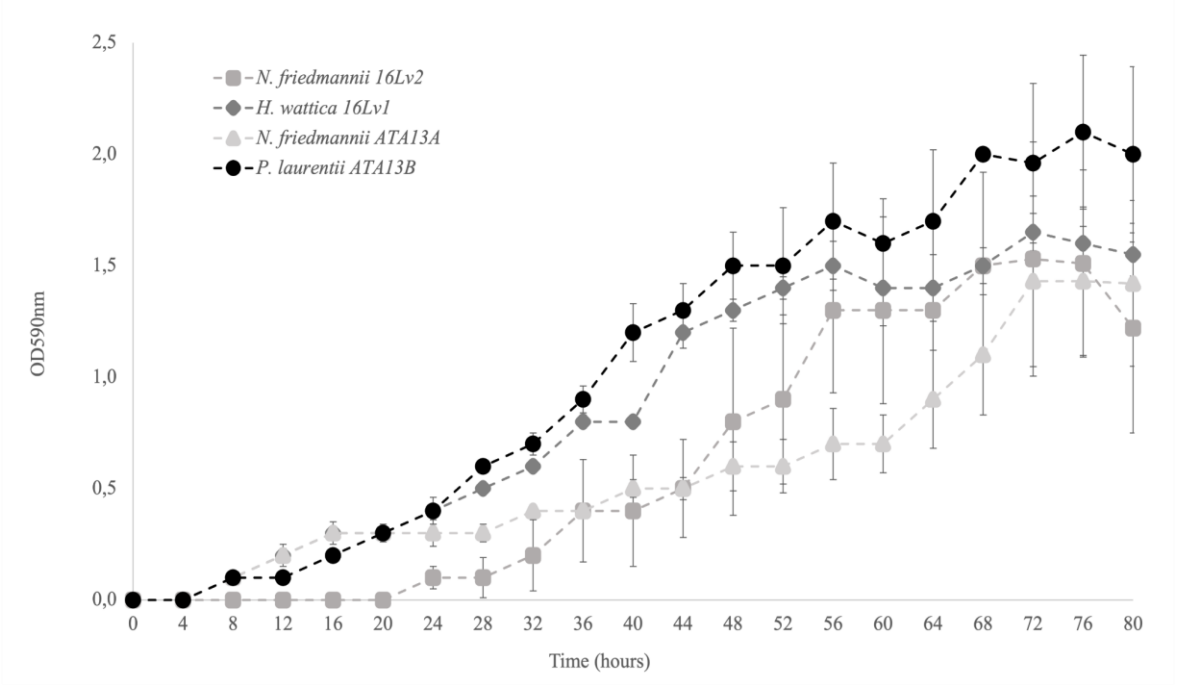
4.1.3.4 Selection of Yeasts for Further Studies

Based on the previous experiments, we selected two yeast strains to be further studied in conjunction with *N. friedmannii* 16Lv2 and *H. wattica* 16Lv1 (PULSCHEN et al., 2015): ATA13A (*N. friedmannii*), as a *Naganishia* species isolated from a collection site from a superior altitude (5047 m) compared to the collection site of *N. friedmannii* 16Lv2 (3981 m); and ATA13B (*P. laurentii*) for representing a new species not previously identified in soil samples from the Atacama Desert.

4.1.3.5 Growth Curves

Growth curves were established for each of the four yeast strains studied to establish the cultivation times necessary for obtaining the different growth stages (Figure 4). *P. laurentii* ATA13B presented the fastest growth compared to the other strains, reaching the late exponential phase (OD= 1.0) after approximately 38 hours of cultivation. *P. laurentii* ATA13B was followed by *H. wattica* 16Lv1 (approximately 42 hours), *N. friedmannii* 16Lv2 (approximately 52 hours) and *N. friedmannii* ATA13A (approximately 64 hours).

Figure 4 – Growth curves of *N. friedmannii* 16Lv2, H. wattica 16Lv1, *N. friedmannii* ATA13A, and *P. laurentii* ATA13B after 80 hours of culture in YM broth (25 ˚C, 150 rpm).

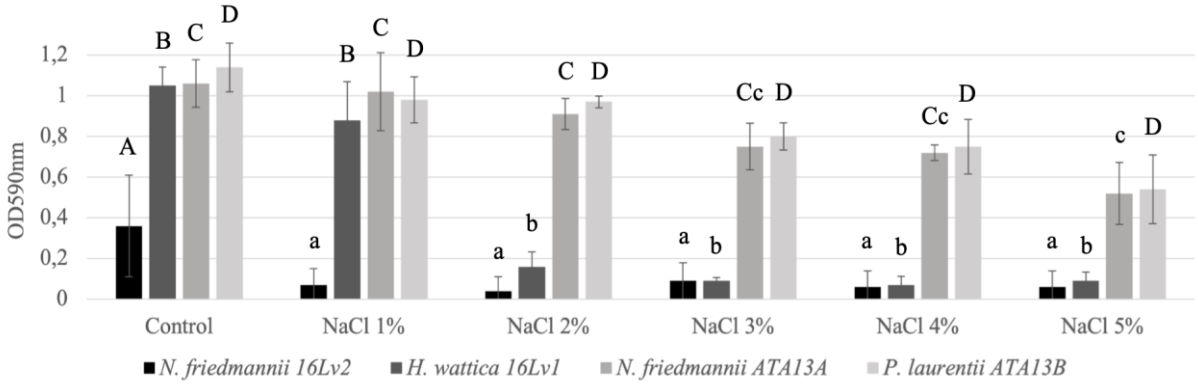


4.1.3.6 Salinity Resistance

Contrary to our observations with the UVC resistance experiments, the four yeast strains presented different tolerance to increasing concentrations of NaCl (Figure 5). In this experiment, *N. friedmannii* 16Lv2 presented slower growth, reaching a maximum OD of 0.36 after 48 hours of culture without any additional NaCl. This yeast strain presented practically no growth (OD<0.1) in all salinity concentrations (1%, 2%, 3%, 4%, and 5%). Results indicate a significantly lower growth compared to the control ($p < 0,0001$).

All other yeast strains presented similar growth at the control conditions (OD between 1.0-1.1 after 48 hours), as well as after cultivation under 1% of salinity (OD between 0.8 and 1.0 after 48 hours). However, *H. wattica* 16Lv1 did not grow in cultures with additional NaCl concentrations (2%, 3%, 4%, and 5%), showing a significantly reduced growth ($p < 0,0001$) compared to control and 1% cultures. In contrast, both *N. friedmannii* ATA13A and *P. laurentii* ATA13B presented growth under all salinity concentrations tested. Specifically, *N. friedmannii* ATA13A presented average reduction of 3.5%, 14.2%, 29.5%, 32.5%, and 50.7% after cultivation under 1%, 2%, 3%, 4%, and 5% of salinity, respectively, with significant reduction observed only after cultivation at 5% NaCl ($p = 0,0022$). Finally, *P. laurentii* ATA13B also survived all NaCl concentrations tested, without significant reduction in growth. Growth reductions for this yeast strain were of 14.1%, 14.7%, 29.4%, 34.5%, and 52.8%, after culture under 1%, 2%, 3%, 4%, and 5% of salinity, respectively.

Figure 5 – Survival of *N. friedmannii* 16Lv2, *H. wattica* 16Lv1, *N. friedmannii* ATA13A, and *P. laurentii* ATA13B after cultivation in YM broth with 1%, 2%, 3%, 4%, and 5% of NaCl addition. Different letters represent statistical differences according to ANOVA and Tukey tests ($p \leq 0.05$).



Similar to all other extreme conditions, a reduced microbial growth is usually observed in environments with increased salinity concentrations (FOTEDAR et al., 2018). Halotolerant microorganisms are classified as those that survive and grow under salinity concentrations higher than 1%, whereas halophiles thrive under extreme concentrations around 15% (BUZZINI et al., 2018) up to 20-25% (NAZARETH et al., 2012). Previous studies have shown the presence of basidiomycetous yeasts in hypersaline environments. The so-called black yeast (*Hortea weneckii*) is highlighted among the most emblematic halophilic yeasts (GOSTINCAR et al., 2021; GUNDE-CIMERMAN et al., 2009). However, as indicated by its name, a strong melanization is primarily responsible for its high salinity tolerance, whereas non-pigmented halotolerant or halophilic yeasts are more scarcely studied.

According to Silva-Graça and Lucas (2003), halophilic microorganisms present not only survivability and growth under elevated salinity conditions, but also a preference for such extreme conditions compared to non-saline growth sites. Since both strains presented growth inversely correlated with salinity additions in this study and were studied only up to 5% salinity concentrations, *N. friedmannii* ATA13A and *P. laurentii* ATA13B can be classified as halotolerant species. New experiments are suggested to demonstrate their potential under higher salinity concentrations - *e.g.,* under extreme concentrations of up to 15% NaCl - potentially evidencing these strains as halophilic microorganisms. Yet, their survival under the experimental conditions tested in this study strongly suggest the selection of physiological mechanisms in response to salinity stress for *N. friedmannii* ATA13A and *P. laurentii* ATA13B compared to *H. wattica* 16Lv1 and, in particular, to *N. friedmannii* 16Lv2. Key microbial resistance mechanisms are historically classified as an increased production of osmolytes (BLOMBERG; ADLER, 1992), and the presence of pump channels for passive and active transport of ions and osmolytes, as well as a specific membrane lipid composition with high capacity of retention of osmolytes and other antioxidative compounds (YOSHIKAWA et al., 1995).

More specifically, osmotic pressure maintenance occurs through two main strategies: the "salt-in" strategy refers to the accumulation of inorganic salts -- *e.g.,* potassium chloride -- as a way to manipulate the osmotic balance inside the cells. On the other hand, the compatible solutes strategy refers to the accumulation of organic compounds with low molecular weight as a means to maintain a more steady cellular and enzymatic functioning (BROWN; SIMPSON 1972; GUNDE-CIMERMAN et al.,

2018). Currently, a wide range of organic compounds have been observed in association with the compatible solutes strategy by halotolerant and halophilic microorganisms, including sugars and amino acids, and, more specifically, glutamate, mannitol, and trehalose (ZEIDLER et al., 2017). Glycerol, arabitol, sorbitol, and trehalose are the most commonly observed among salt resistant yeasts (WELSH, 2000).

Although a more energetically costly strategy compared to the salt-in strategy (OREN, 2011), compatible solutes represent a good cost benefit due to their low molecular weight with ubiquitous protective effects. Through the stabilization of membranes and proteins, compatible solutes offer a protective effect to other environmental extremes, including temperature and desiccation (WELSH, 2000; ZEIDLER; MULLER, 2019). For this reason, it is worth highlighting again the fact that *H. wattica* 16Lv1, and, more specifically, *N. friedmannii* 16Lv2 presented a low salt resistance according to the results of this study. *Naganishia* yeasts are commonly isolated from hot and cold extreme environments, usually presenting some degree of salinity resistance -- although lower than the resistance presented by halotolerant and halophilic species (SCHMIDT et al., 2017). In fact, yeasts from the genus *Naganishia* and *Holtermaniella* have been isolated from hypersaline seas (FOTEDAR et al., 2018) and soils (MOKHTARNEJAD et al., 2016), among which the psychrotolerant species *N. friedmannii*, *Naganishia albidosimilis*, *Naganishia albida*, *Naganishia qatarensis*, *H. wattica* and *Holtermanniella takashimae*. In addition to these studies, Pulschen et al. (2015) described the observational growth of *N. friedmannii* 16Lv2 and *H. wattica* 16Lv1 under maximum salinity concentration of 1.75 and 2.25 mol/M, corresponding to approximately 10 and 12% of NaCl.

However, the methodology utilized in the present study differed from that of Pulschen et al. (2015), which can partly explain the varied results observed. While Pulschen et al. (2015) evaluated growth by means of solid agar plates through CFU counts of up to 20 days of culture, our study assessed yeasts' growth through OD measurements of liquid cultures after 48 hours under constant agitation. According to Gunde-Cimerman and Plemenitas (2005), yeast species adapted to extreme environmental conditions usually present a reduction in metabolism and overall growth, a fact that commonly hinders their identification compared to bacteria and other microorganisms. Since CFU observation represents a qualitative analysis of tolerance to a given environmental factor, it becomes evident the key difference between the

mere survival and actual physiological growth of these yeasts under increased salinity concentrations. Therefore, although the experiments from Pulschen et al. (2015) demonstrate the survivability of *N. friedmannii* 16Lv2 and *H. wattica* 16Lv1 under NaCl concentrations of up to 10% (1.75 mol. L^{-1}) and 12% (2.25 mol. L^{-1}), our experiments demonstrate that both yeast strains present a reduced growth capacity under concentration 10-15-fold lower (1% and 2% NaCl, respectively).

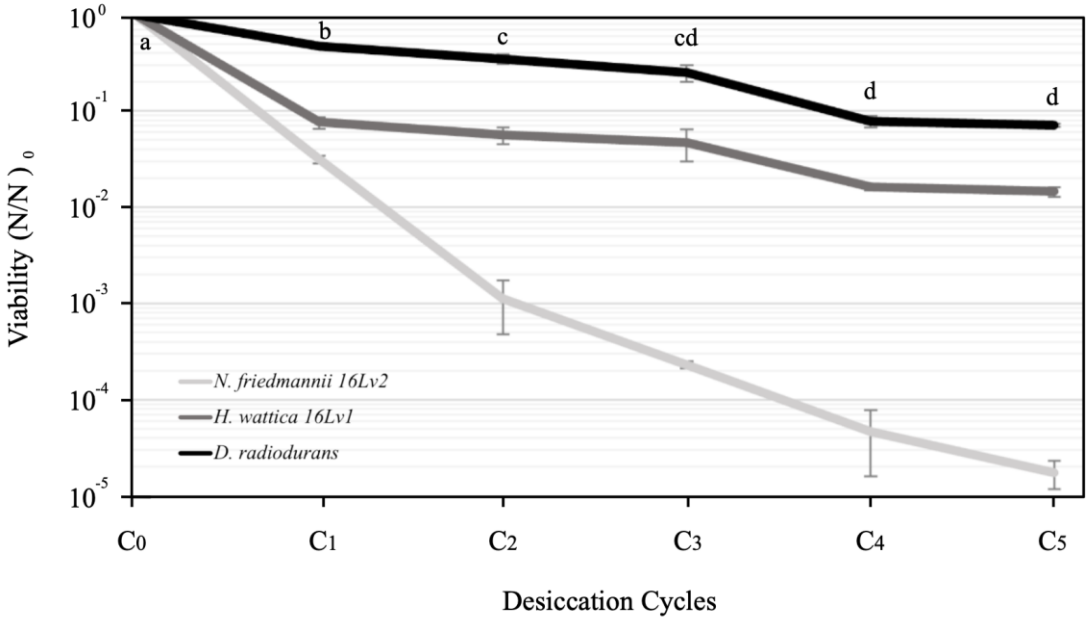
4.1.3.7 Desiccation Tolerance

Yeasts from extreme environments are constantly submitted to desiccation-dehydration cycles according to the sazonal cycles of the habitat. Resistance to the desiccation process requires a reduction in water accumulation and cellular metabolism via the so-called anhydrobiosis process (REN et al., 2020; TAPIA et al., 2015). More important than the overall time to which microorganisms are exposed to the anhydrobiosis process, however, is the number of desiccation-rehydration cycles to which these organisms have to withstand, since those are the critical moments were key ultrastructural alterations occur, leading to significant cellular and physiological effects. For example, Ren et al. (2020) revealed significant changes during the desiccation process in *Saccharomyces cerevisiae*, including a reduced size of vacuoles and lipid droplets, as well as alterations in the mitochondrial cristae and endoplasmic reticulum membrane. Therefore, our experiment tested the yeasts' resistance through five desiccation-dehydration cycles. For each cycle, three yeast aliquots were resuspended and plated for CFU count, while the other samples were rehydrated and desiccation again.

Due to its high radiation and desiccation resistance, the extremophilic bacteria *Deinococcus radiodurans* (MATTIMORE; BATTISTA, 1996) was used as a positive control organism to the desiccation experiments of this study. Our results indeed reveal *D. radiodurans* as the highest resistance among all tested organisms, with 46.9%, 34.8%, 25.0%, 7.8%, and 7.1% viability after all subsequent desiccation cycles, respectively (Figure 6). This is in high accordance with previous studies that demonstrate the outstanding ability of *D. radiodurans* in terms of desiccation resistance. Although our statistical analysis reveals no significant difference between the two tested yeasts, these microorganisms presented varied results in terms of their desiccation resistance. Whereas *H. wattica* 16Lv1 presented viabilities of 7.6%, 5.6%,

4.7%, 1.6% and 1.4%, after all five desiccation cycles, respectively, *N. friedmannii* 16Lv2 presented a reduced resistance, with 3.1% viability after the first cycle, and less than 1% after all four subsequent desiccation cycles. Altogether, our results indicate a significant resistance of *D. radiodurans* compared to the two tested yeasts ($p < 0,0001$, F ratio = 11,0756), as well as between the different desiccation cycles - particularly cycle 1, 2 and 4-5 ($p < 0,0001$, F ratio = 179,3663).

Figure 6 – Viability of *D. radiodurans* versus yeasts *N. friedmannii* 16Lv2 and *H. wattica* 16Lv1 after five desiccation-rehydration cycles. Different letters represent statistical differences according to ANOVA and Tukey tests ($p \leq 0.05$).

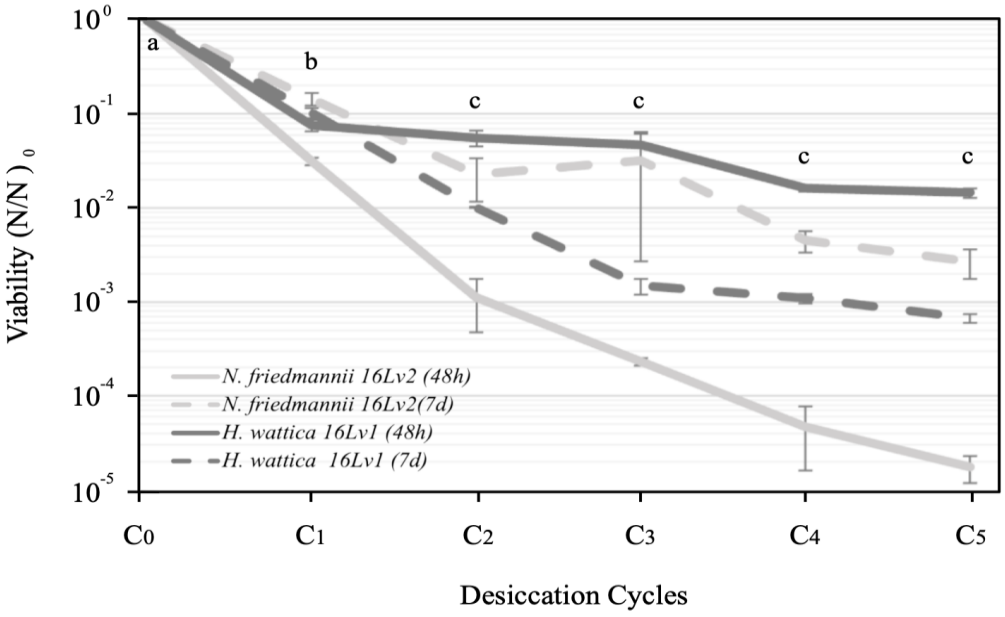


Both desiccation and radiation stresses can lead up to oxidative damage through an increased amount of reactive oxygen species (ROS) among their main deleterious mechanisms (FARRUGIA; BALZAN, 2012; FRANÇA et al., 2007). In addition, both stresses cause direct molecular damage through the promotion of double strand breaks (DSBs) and denatured proteins (CALAHAN et al., 2011). Previous studies have shown a correlation between the cellular and molecular mechanisms of resistance to radiation and desiccation stress, particularly for the extremophilic bacteria *D. radiodurans* (MATTIMORE; BATTISTA, 1996; UJAONEY et al., 2017). However, the resistance to one or both factors varies and it's not always correlated within different microorganisms. For example, Beblo-Vranesevic et al. (2018) demonstrated an independence between resistance mechanisms to desiccation and ionizing radiation in anaerobic bacteria isolated from different extreme

environments. As previously described, the number of studies that tested the potential correlation between radiation and desiccation in extremophilic yeasts is scarce, particularly within non-pigmented yeasts.

The results from this study indicate that *H. wattica* 16Lv1 presents a more robust desiccation resistance compared to *N. friedmannii* 16Lv2. In contrast, *N. friedmannii* 16Lv2 presents overall higher resistance to UVC, as well as to UVB and solar radiation, as observed by Pulschen et al. (2015), whereas *H. wattica* 16Lv1 can survive and grow under higher salinity concentrations. Aiming to further understand the different mechanisms of resistance between these two species, we further investigated their resistance to desiccation-rehydration cycles, now comparing the viability of cultures under the exponential growth phase (48h of culture) and long-term culturing (7 days of culture). Interestingly, the desiccation of long-term cultures resulted in an increased resistance for *N. friedmannii* 16Lv2, whereas the desiccation of long-term culture cells resulted in an increased mortality for *H. wattica* 16Lv1 (Figure 7). Similar to the previous experiment, our statistical analysis revealed a significant difference between the desiccation treatments, particularly between the first and second cycles ($p < 0{,}0001$, F ratio = 3497,753) compared to the control.

Figure 7 – Viability of *N. friedmannii* 16Lv2 and *H. wattica* 16Lv1, both young (exponential growth phase, 48h) and old cultures (long-term culture, 7 days), after five desiccation-rehydration cycles. Different letters represent statistical differences according to ANOVA and Tukey tests ($p \leq 0.05$).



An increased stress resistance capacity is usually observed in cultures reaching the stationary growth phase due to the positive regulation of genes responsible for the production and accumulation of proteins and other resistance compounds (JACOBS et al., 2012; SOARES et al., 2010; ZEIDLER; MULLER, 2019). Even though we didn't observe significant differences between the two yeasts ($p = 0,9944$, F ratio = 0,0256), our results reinforce the hypothesis that *N. friedmannii* and *H. wattica* present different resistance mechanisms to radiation and desiccation stress. The results from this study highlights the necessity of further research to elucidate the specific resistance mechanisms selected between these two yeast species. For example, the resistance of *N. friedmannii* 16Lv1 may be correlated with a higher production of antioxidative compounds at the stationary growth phase - similar to what the previously mentioned authors describe in their studies - whereas *H. wattica* 16Lv2 presents a more acute resistance mechanism, with higher resistance over exponential growth phases.

Another hypothesis can be evaluated in terms of the types of compounds being produced by each yeast. Since neither of these species produce pigmented compounds and both present some degree of resistance to UVR, the production of secondary - non-pigmented - resistance compounds it is highly probable (PULSCHEN et al., 2015), including those with photoprotective and/or antioxidative effects, such as mycosporines (MOLINÉ et al., 2014; OREN; GUNDE-CIMERMAN, 2007), particularly in the case o *N. friedmannii* 16Lv2, which presented higher resistance to UVR. In contrast, previous studies have demonstrated the protective effects of compatible solutes, including threalose, in *Acinetobacter baumannii* strains exposed to high salt concentrations (ZEIDLER et al., 2017) as well as desiccation stress (ZEIDLER; MULLER, 2019). Such compounds have also been observed in the bacteria species *Bradyrhizobium japonicum, Rhodococcus jostii* and *Escherichia coli* with high desiccation resistance (CYTRYN et al., 2007; LEBLANC et al., 2008; ZHANG; YAN, 2012). Thus, the increased resistance to salt stress presented by *H. wattica* 16Lv1 can be an indication of a higher production of compatible solutes.

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4.2 SECTION B: RESISTANCE PROFILE OF TWO NON-PIGMENTED EXTREMOPHILIC YEASTS ISOLATED FROM THE ATACAMA DESERT (CHILE)

Submission Confirmation

Thank you for your submission

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ABSTRACT

The Atacama Desert (Chile) is a hyper-arid desert combining extreme ultraviolet radiation, high daily temperature variations, and a low humidity state that conditions microbial life to long desiccation periods. Although an increasing number of studies have clarified the resistance mechanisms of prokaryote species living in such extreme environments, only a few have focused on the study of extremophilic eukaryotic microorganisms such as yeasts. In this study, we report the characterization of two non-pigmented, extremophilic yeast isolates isolated from the topsoil layer of a volcano slope in the Atacama Desert. Yeast isolates were identified by ITS sequencing and a phylogenetic tree was reconstructed. Although the identification of yeast isolate ATA13A could not be confirmed between *Naganishia friedmannii* or *Naganishia onofrii*, its resistant properties were further reinforced by a closeby allocation with other cold and hot desert environments, whereas isolate ATA13B was identified as Papiliotrema laurentii species with 100% identity with isolate FC7-7A. Both isolates demonstrated resistance to UVC, desiccation, and salinity stresses, with an overall optimal growth between 20-30 °C, reinforcing the importance of the Atacama Desert as a niche of eukaryotic extremophilic yeasts and the need for further studies aimed at clarifying their molecular, ecological and physiological aspects.

4.2.1 Introduction

The selection of microorganisms in environments with high solar exposure culminated in extremophiles with the ability to survive the direct and indirect effects imposed by ultraviolet radiation (UVR). The so-called radioresistant microorganisms developed a wide range of protective mechanisms, usually relying on the production and accumulation of photoprotective compounds (GABANI; SINGH, 2013). Pigmentation comprises the main mechanism underlying photoprotection, and microbial pigments such as melanin and carotenoids have been gaining attention over the last few years as natural alternatives to synthetic sunscreens (AKILANDESWARI; PRADEEP, 2016).

Among radioresistant yeasts, although most authors correlate microbial pigmentation with increased UVR resistance (*e.g.*, DIGHTON et al., 2008; GESSLER et al., 2014; ONOFRI et al., 2019; PACELLI et al., 2018), a few studies recognize the existence of non-pigmented specimens with similar or even higher resistance than their pigmented counterparts. Examples include similar resistance to UVB (SCHIAVE et al., 2009) and gamma radiation (SCHULTZHAUS et al., 2019) between melanized and non-melanized cells of *Cryptococcus neoformans*, high UVC tolerance in low-carotenoid content *Leuconeurospora* sp. (VILLARREAL et al., 2016), and high survival rates in carotenoidless *Rhodotorula mucilaginosa* exposed to UVB radiation (MOLINÉ et al., 2010).

Exploring environments with extreme UVR exposure has opened opportunities to further understand the mechanisms and pathways involved in non-pigmented radioresistance. The Atacama Desert (Chile) is a highlighted example of extreme environments on Earth. Multiple harsh characteristics in this hyper-arid desert combine into a challenging location for life to flourish, leaving space for a few extremophilic microorganisms (NAVARRO-GONZALEZ et al., 2003). In particular, the Sairecabur volcano (San Pedro de Atacama) offers extreme UVR exposure (LYNCH et al., 2012; CABROL et al., 2014) and high daily temperature variations, as well as a low humidity state that conditions microbial life to long periods of desiccation stress (COSTELLO et al., 2009; LYNCH et al., 2012).

Although most ecology studies have determined the diversity of prokaryotic communities along the Atacama Desert (AZUA-BUSTOS et al., 2012; NEILSON et al., 2012; OKORO et al., 2009; WIERZCHOS et al., 2006), demonstrating the

extraordinary resistance capacity of different species to a variety of extreme conditions (COCKELL et al., 2008; PAULINO-LIMA et al., 2016; RIVADENEYRA et al., 1999), a few recent research groups have focused on the investigation of extremophilic eukaryotic species and their resistance mechanisms (CONLEY et al., 2006; PULSCHEN et al., 2015). In particular, Pulschen et al. (2015) demonstrated the UVR resistance of two non-pigmented yeast species (*Naganishia friedmannii* 16Lv2 and *Holtermaniella wattica* 16Lv1) alongside physiological characteristics of both yeasts compared to pigmented species (*Exophialla sp* 15Lv1 and *Rhodosporidium toruloides* 16Lv3), indicating similar resistance to UVC and even higher resistance to solar radiation (UVA+UVB) compared to the carotenogenic species *R. toruloides*.

After Pulschen et al. (2015) publication, two non-pigmented yeast strains, ATA13A and ATA13B, were further isolated from closeby soil sites at 5047 meters of altitude at the Sairecabur volcano, Atacama Desert. In this study, we report the characterization of non-pigmented, extremophilic isolates ATA13A and ATA13B in terms of their resistance to UVR, desiccation, salt and temperature stresses.

4.2.2 Material and Methods

4.2.2.1 Collection Site, Isolation and Culture Conditions

Samples were collected from the top layer of soil from volcano slope (latitude 22.716945°S, longitude 67.923690°W, altitude 5047 m) at the Sairecabur volcano, Atacama Desert, in January 2012, using sterile tools, and kept refrigerated until the analysis. Measurements of UVR fluxes at the volcano, as well as pH, salinity, and the elemental composition of soil samples are described in Pulschen et al. (2015). Yeast samples were isolated through the dilution of 1 g of soil samples in 10 mL of sterile saline solution (0.9%) and further cultivation of 100 µL in Rose bengal agar plates (48h at 30 °C). Then, selected yeast colonies were stored in glycerol 20% at -20 °C, and transferred to YM broth (peptone 5 g.L-1, yeast extract 3 g.L-1, malt extract 3 g.L-1, glucose 10 g.L-1) for routine cultivation (25 °C with constant agitation of 150 rpm).

4.2.2.2 Molecular Identification and Phylogenetic Analysis

Total genomic DNA of the yeast strains was extracted according to the rapid isolation of yeast DNA protocol established by Green and Sambrook (2018). Yeast identification was carried out by sequencing the internal transcribed spacer (ITS) of rRNA genes amplified by primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990). PCRs reactions consisted of 2.5 µL of 10x buffer, 1 µL of 50 mM MgCl2, 0.2 µL of 100 mM dNTPs (25 mM each), 0.5 µL of each primer (20 µM each), 0.2 µL of 5 U/µL Taq polymerase, 1 µL of diluted genomic DNA (~10 ng/µL), and sterile ultrapure water to a final volume of 25 µL. PCR started with an initial step of 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min, and then a final extension of 72 °C for 10 minutes. PCR amplicons were purified using the ethanol precipitation method described by Green & Sambrook (2016). The PCR fragments were sequenced at Macrogen, Inc. (South Korea). The obtained sequences were analyzed using BLAST against the GenBank database. The most similar sequences from BLAST analysis were selected for the phylogenetic analysis, including type strains and environmental sequences. Multiple alignments were performed using MUSCLE (Edgar, 2004). A phylogenetic tree was constructed on software MEGA 11 (Tamura et al. 2021; Stecher et al. 2020) based on Maximum Likelihood method, using Kimura 2-parameters as substitution model and bootstrap test with 1000 replications.

4.2.2.3 UVC Resistance Experiment

UVC resistance of the yeasts was evaluated in comparison to *Naganishia friedmannii*, strain 16Lv2 (PULSCHEN et al., 2015), used as a high-radiation resistant yeast model. Yeasts were grown in liquid YM broth to early stationary phase (OD_{595} of 1.0-1.1), centrifuged (5 minutes, 3000 rpm), and washed with 0.9% w/v NaCl. The pellet was dispersed in sterile saline solution to a volume of 1 mL of cell suspension and transferred to a standard 09 cm diameter sterile Petri dish and irradiated with a Philips (Philips, Eindhoven, The Netherlands) TUV-20W low-pressure Hg lamp (253.7 nm), with samples placed at 22 cm from the lamp. Cells were irradiated for 30 and 60 seconds, in triplicate. Preliminary protocols demonstrated the correlation of irradiance times to a final UVC dose of 0.5 kJ/m^2 (30 seconds) and 1.4 kJ/m^2 (60 seconds). After

the different fluences, the colony-forming units (CFU) were evaluated by incubation on YM agar plates at 25 °C for 48 h.

4.2.2.4 Desiccation Resistance Experiment

Desiccation resistance was evaluated in comparison to *Deinococcus radiodurans*, used as a high-desiccation resistant model. Yeasts were grown under similar conditions as previously described. Then, 50 µL aliquots of washed early stationary phase cells were transferred to 96-well plates, in triplicate, and dried under oxygenation for 24 h periods. After each period, cells were rehydrated with PBS solution for CFU count. In total, four desiccation-rehydration cycles were completed.

4.2.2.5 Temperature and Salt Resistance Experiments

Temperature experiments were performed with cultures on YM agar plates incubated at 10, 15, 20, 25, 30, 35, and 40 °C for 48 h. Growth was compared qualitatively based on CFUs. For halotolerance experiments, 30 µL of early stationary cells were cultivated in YM broth with different NaCl concentrations (1%, 2%, 3%, 4%, and 5%), in triplicate. After 48 h, 200 µL aliquots were quantified by OD measurement. Standard YM broth cultures were considered as control samples.

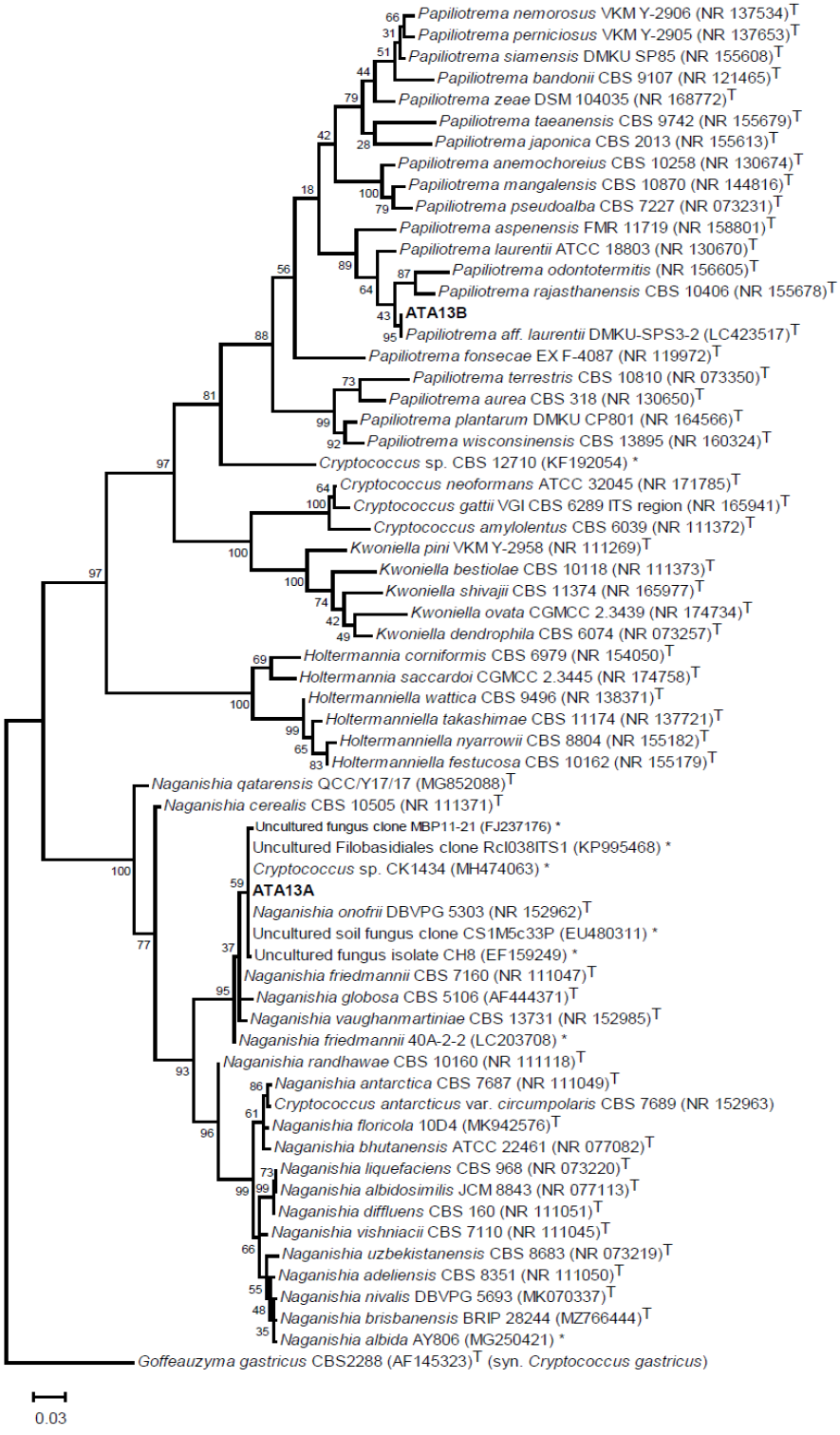
4.2.3 Results

4.2.3.1 Molecular Identification and Phylogenetic Tree

Analysis of the ITS sequence from ATA13A and ATA13B isolates revealed high sequence similarity to *Naganishia onofrii* DBVPG 5303 (99.65% identity) and *Papiliotrema laurentii* FC7-7A (100% identity) species, respectively. The resulting phylogenetic tree (Figure 8) agrees with the classification of ATA13B isolate within the *Papiliotrema* cluster, positioning together with *P. laurentii* DMKU-SPS3-2 supported by 95% bootstrap value. Isolate ATA13A, however, is positioned in the *Naganishia* cluster, being similar to *Naganishia* isolates from desert or other extreme arid environments. Examples include *Naganishia* CH8 (99.65% identity) from a semiarid park in New Mexico, *Naganishia* CK1434 (99.64%) from arid grassland in Utah, and *Naganishia* MBP11-21 (99.29%) from an alpine glacier forefield in Austria. In addition to the mentioned high similarity shared by ATA13A and *N. onofrii*, the Atacama isolate

also showed a 98.76% identity with type strain *N. friedmannii* CBS 7160. The tree branch lengths among the *Naganishia* was relatively short (< 0.03 substitutions per site, in average), which suggests the ITS sequence has no phylogenetic resolution to precisely classify species in this group. However, our results indicate the potential allocation of isolate ATA13A as either *N. friedmannii* or *N. onofrii* species, as well as suggesting the possibility of its identification as a new *Naganishia* species.

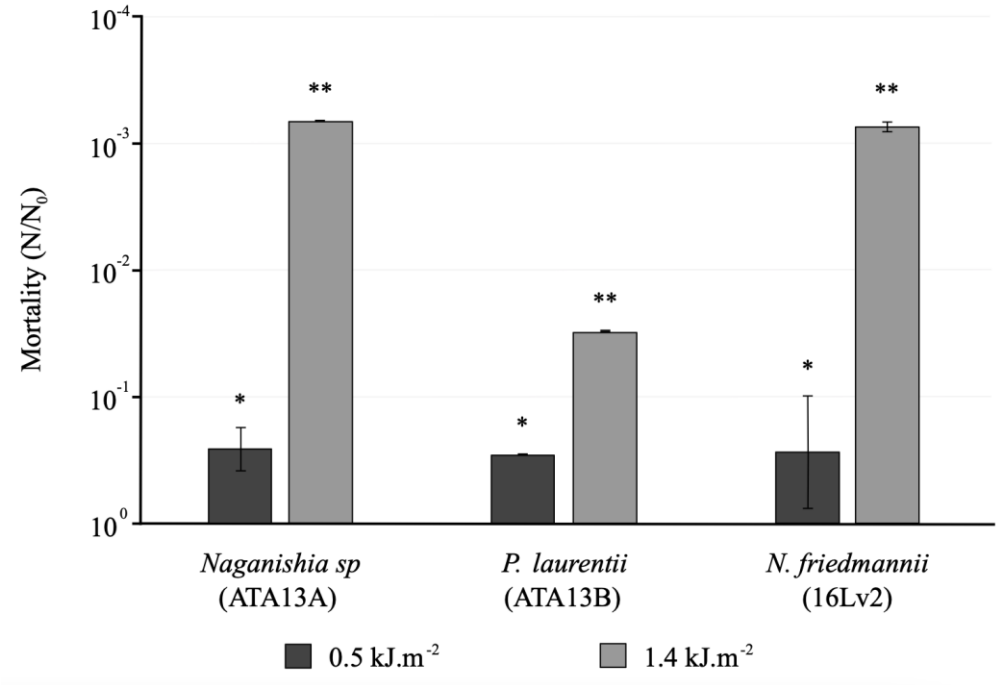
Figure 8 – Maximum likelihood phylogenetic tree constructed with the ITS sequence of yeast isolates ATA13A and ATA13B (in bold) and other similar sequences according to BLAST results. *Goffeauzyma gastricus* (synonym *of Cryptococcus gastricus*) was chosen as root taxa. Bootstrap values calculated from 1000 replications are shown next to the branches. An asterisk (*) indicates environmental sequences or isolates recovered from deserts, glacier fields, or other extreme arid environments. Letter T represents sequences from type strains. Scale bar represents the number of substitutions per site.



4.2.3.2 UVR and Desiccation Resistance

To determine the ability of the two yeast isolates to tolerate different extreme environmental conditions, we conducted physiological assays against UVC radiation, desiccation-rehydration cycles, extreme salt concentrations, and different temperature conditions. Results from the UVC experiments (Figure 9) demonstrate a similar resistance ability between the two isolates from our study compared to *N. friedmannii* 16Lv2 isolated and identified by Pulschen et al. (2015), with overall reductions between 71.1-74.4% after exposure to 0.5 kJ/m^2. However, increased exposure time indicates an optimized resistance of *P. laurentii*, although not statistically significant, with a reduction of 96.9% (about 1.5 log of viability) of culture viability compared to 99.9% (about 3 log of viability) of both *Naganishia* strains.

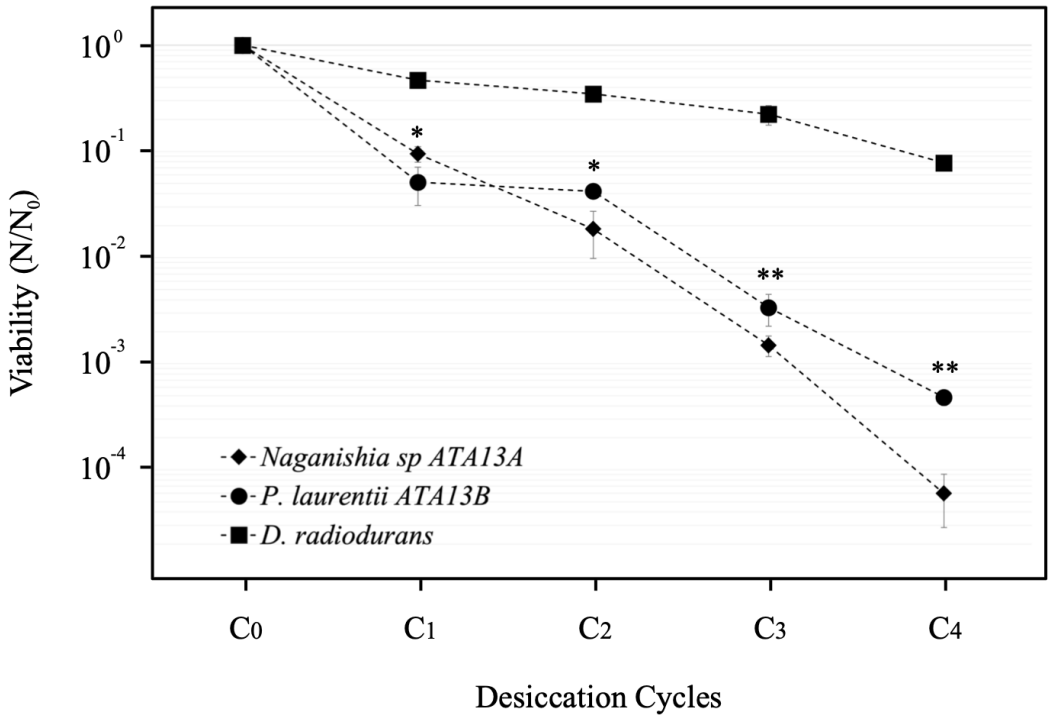
Figure 9 – Mortality of *Naganishia* sp ATA13A, *P. laurentii* ATA13B, and *N. friedmannii* 16Lv2 after exposure to UVC radiation. Symbols represent statistical differences according to ANOVA and Tukey tests ($p \leq 0.01$).



Since the desiccation tolerance of *N. friedmannii* 16Lv2 is not yet established, we decided to use the extremophilic bacteria *D. radiodurans* as a high-desiccation high-desiccation resistant model due to its well-established ability to survive prolonged desiccation (MATTIMORE; BATTISTA, 1996). As expected, *D. radiodurans* demonstrated strong desiccation resistance, reaching about one log of viability

reduction (92.1%) only after the fourth desiccation cycle (Figure 10). In contrast, the exposure of *N. friedmannii* ATA13A and *P. laurentii* ATA13B to desiccation-rehydration cycles reduced their viability under a higher progression level, reaching about one log of viability reduction after the first desiccation cycle. More specifically, *N. friedmannii* ATA13A and *P. laurentii* ATA13B lost 90.5% and 94.8% viability, respectively, with viability losses higher than 95% for all other desiccation cycles tested.

Figure 10 – Viability of *Naganishia* sp ATA13A, *P. laurentii* ATA13B, and *D. radiodurans* after exposure to four desiccation-dehydration cycles. Symbols represent statistical differences according to ANOVA and Tukey tests ($p \leq 0.01$).



4.2.3.3 Temperature Growth Range and Halotolerance

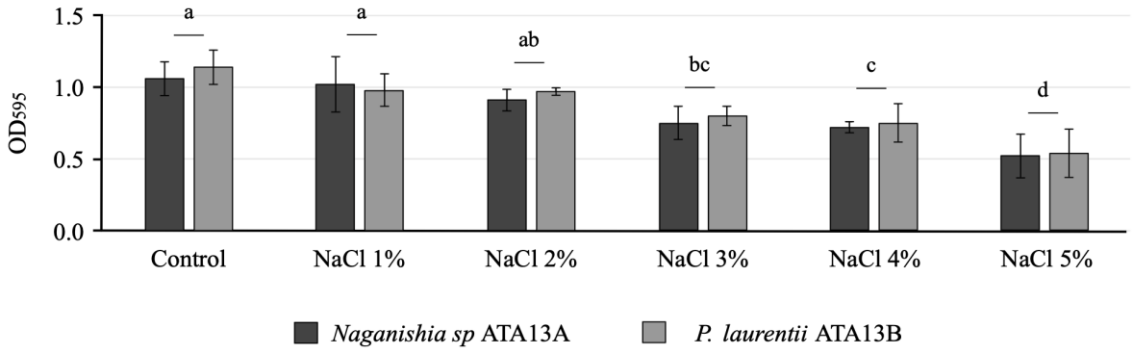
Temperature experiments demonstrate further shared similarities between the two non-pigmented yeast species, with a similarly higher growth at temperatures between 20-30°C (Table 4). However, *P. laurentii* ATA13B demonstrated further ability to grow at the lower and higher extremes, with higher colony growth at 10 and 15°C, and a dense colony growth after cultivation at 35°C compared to *Naganishia sp.* ATA13A. None of the yeast strains could grow under a temperature of 40°C.

Table 4 – Temperature profile of *Naganishia* sp ATA13A and *P. laurentii* ATA13B after 48h of cultivation in agar YM plates at different temperatures.

Species	Strain	Culture Temperature						
		10 °C	15 °C	20 °C	25 °C	30 °C	35 °C	40 °C
Naganishia sp	ATA13A	+	++	+++	+++	+++	++	-
Papiliotrema laurentii	ATA13B	++	++	+++	+++	+++	+++	-

In regards to their salinity tolerance, both yeast strains tolerated up to 5% NaCl (w/v), thus providing a resistance profile within the classification as halotolerant microorganisms (Figure 4). More specifically, *N. friedmannii* ATA13A and *P. laurentii* ATA13B demonstrated a significant growth reduction (29.2% and 29.8%, respectively, compared to control samples) after cultivation under 3% NaCl (w/v), with further reductions of 33-35% and 51-53% after cultivation under 4% and 5% of NaCl.

Figure 11 – Optical density of *Naganishia* sp ATA13A and *P. laurentii* ATA13B after culture in YM broth under increasing salinity concentrations. Different letters represent statistical differences according to ANOVA and Tukey tests ($p \leq 0.01$).



4.2.4 Discussion

Understanding the tolerance limits of extremophilic microorganisms provides a reference to a wide range of research topics. Firstly, biomolecules produced by extremophiles are widely implemented within biotechnology and industrial processes (RASUK et al., 2016). Secondly, such incredible organisms may also accumulate compounds with pharmaceutical and biomedical applications (KOUR et al., 2019). Finally, extremophiles are the best model organisms to understand the emergence and evolution of life on our planet, as well as the possibility of finding indigenous life outside Earth (*i.e.*, astrobiology) or transporting Earth's life into extraterrestrial habitats for further space exploration (ROTHSCHILD; MANCINELLI, 2001).

This study describes the identification of two yeast strains with extremophilic abilities from soil samples collected from the Atacama Desert. Analysis of the ITS sequence exhibited the highest similarity to *N. onofrii* (ATA13A, 99.65% identity) and *P. laurentii* (ATA13B, 100% identity). Although the phylogenetic tree agrees with ATA13B isolate as a *P. laurentii* species, it was allocated close to *P. laurentii* DMKU-SPS3-2 isolated from an acidic peatland area in tropical Thailand, characterized by high rainfall and organic matter concentration (SATIANPAKIRANAKORN et al., 2020) in contrast to Atacama's low water availability and scarce sources of organic carbon (NAVARRO-GONZALEZ et al., 2003). On the other hand, although without a consensus regarding its classification within *N. friedmannii* or *N. onofrii* species, yeast isolate ATA13A was closely identified among *Cryptococcus/Naganishia* species isolated from extreme arid environments including glacier forefield at the Austrian Central Alps (MBP11-21, KUHNERT et al., 2012), semiarid soil from the Chilean matorral (KP995468, unpublished), and a semiarid grassland in central New Mexico, USA (EU480311, PORRAS-ALFARO et al., 2011), reinforcing its adaptation to extreme environmental conditions.

Both *Naganishia* and *Papiliotrema* genera were previously allocated within the conventional classification of *Cryptococcus* species based on morphological, reproductive, and physiological features (MORALES-LÓPEZ et al., 2021). More recently, two key turning points derived from further taxonomic studies of the group. The first provided reclassification of *Cryptococcus* species complex based on their huge genetic and phenotypic heterogeneity, resulting in the proposition of new species and variations (HAGEN et al., 2015). Then, the taxonomic revision of *Cryptococcus*

was performed towards the phylogenetic integration of all five major Tremellomycetes lineages, optimizing the previously artificial inclusion criteria taken into account for the classification of the genus (LIU et al., 2015). Currently, *Papiliotrema* species were reclassified based on their closed phylogenetic relationship with the type species of this genus, *Papiliotrema bandonii* (CBS 9107), whereas *Naganishia* species are referenced on the unofficial type species *Naganishia friedmannii* isolated from Antarctic cryptoendolithic samples (SCHMIDT et al., 2017).

As such, both *Naganishia* and *Papiliotrema* species are expected to provide some degree of taxonomic, morphology or physiological similarity. Indeed, *N. friedmanii* and *P. laurentii* have been highlighted among key polyextremotolerant yeast species with the ability to withstand salt and cold temperature extremes (BUZZINI et al., 2018). Our study indicates similar macroscopic features after cultivation in YM liquid broth under 25°C, with both yeasts presenting pinpoint, white-to-creamy cultures, and no diffusible or apparent pigmentation. Moreover, both species demonstrated similar abilities in terms of their tolerance to UVR and desiccation stresses, as well as similar tolerance to increasing salt concentrations and temperature ranges.

Although not statistically significant, our study still indicates a trend towards higher UVR and desiccation resistance for *P. laurentii* ATA13B compared to *Naganishia sp* ATA13A, particularly at higher stress levels (*i.e.,* after 1.4 kJ/m^2 of UVC radiation exposure and after the fourth desiccation-rehydration cycle). Both desiccation and radiation stresses can lead up to cellular damage through direct molecular damage (CALAHAN et al., 2011) as well as indirectly increasing the amount of reactive oxygen species (ROS) (FRANÇA et al., 2007; FARRUGIA; BALZAN, 2012). Furthermore, yeasts can overcome mortality under low water activity by means of a temporary and reversible reduction of their metabolism (RAPOPORT et al., 2016), as well as diversified secondary abilities. For example, *Naganishia albida* (previously *Cryptococcus albida*) demonstrated tolerance to different drying levels by increasing the production of a polysaccharide capsule (AKSENOV et al., 1973).

However, although previous studies have shown a correlation between the cellular and molecular mechanisms of resistance to radiation and desiccation stress, particularly for the extremophilic bacteria *D. radiodurans* (MATTIMORE; BATTISTA, 1996; UJAONEY et al., 2017), studies that tested the potential correlation between radiation and desiccation in extremophilic yeasts are currently scarce. One lesson learned from bacterial studies is that resistance to one or both factors is species-

specific and does not always correlate among different microorganisms. For example, Beblo-Vranesevic et al. (2018) demonstrated independence between resistance mechanisms to desiccation and ionizing radiation in anaerobic bacteria isolated from different extreme environments.

Moreover, our results reinforce the presence of resistance mechanisms to UVR and desiccation within eukaryotic species, particularly within non-pigmented yeasts that do not make use of pigmented compounds among their primary resistance strategies. The crucial importance of pigmentation for yeast survival under harsh UVR conditions is only recently being investigated, and studies have demonstrated similar viability levels between melanized and non-melanized strains of *P. laurentii* after UVB exposure (SCHIAVE et al., 2009). Similar results have been observed among psychrotolerant yeasts species with reduced production of carotenoids that have been exposed to UVC radiation (VILLARREAL et al., 2016), with increased production of non-pigmented protective compounds -- such as mycosporines -- highlighted as key mechanisms potentially corresponding to non-pigmented yeasts' increased survivability (LIBKIND et al., 2004).

In terms of their temperature profile, our results suggest higher growth between 25-30 °C for *Naganishia sp* ATA13A, and between 20-35 °C for *P. laurentii* ATA13B. These results are surprising given the apparently well-established maximum temperature values between 25 °C (PULSCHEN et al., 2015) and 27 °C (VIMERCATI et al., 2016) for high-elevation *Naganishia* isolates, and 28 °C for *P. laurentii* strains from the McMurdo Dry Valleys, Antarctica (BRUNATI et al., 2009), in addition to an overall optimal growth temperature between 20-25 °C for yeast species (KURTZMAN et al., 2011). In fact, the absence of growth above 25-28 °C has been used as evidence of *Naganishia* and *Papiliotrema* psychrophilic abilities to function within colder temperature ranges that predominate at higher elevations. Our results suggest a higher temperature adaptation in the strains isolated at >5000 meters of altitude at the Atacama Desert. Yet, their ability to grow below 10 °C reinforces their psychrotolerant nature with adaptations to survive within the colder temperatures observed at high-elevation volcano soils, particularly for *P. laurentii* ATA13B.

Finally, cultivation under increasing NaCl concentrations indicates the halotolerance of both studied species. *Naganishia* species, including *N. friedmannii*, *N. antarticus,* and *N. vishniacii* have been previously shown as halotolerant microorganisms (SCHMIDT et al., 2017). The study of secondary molecules produced

by a variety of halotolerant fungi species has shown great potential in terms of their applicability to pharmaceutical and biotechnology industries. However, melanized species, such as *Hortaea werneckii*, have been more commonly studied for their withstanding ability to produce bioactive compounds and enzymes with activity under hypersaline conditions (ANWAR et al., 2020). In contrast, more recent studies have demonstrated the potential application of *P. laurentii* strains for the production of eco-friendly biosurfactants as promising substitutes for synthetic surfactants (CHAVES et al., 2021) and lipids with potential application for the biodiesel industry (VIEIRA et al., 2020; WANG et al., 2018), as well as the production of enzymes that promote the remediation of synthetic plastic wastes through the biodegradation of polyester and polyurethane (HUNG et al., 2019).

To the best of our knowledge, this is the first time that a strain of *P. laurentii* (ATA13B) was found from sample soils collected from the Atacama Desert. In addition, our research group isolated and identified a different strain of *Naganishia* species (ATA13A) with halotolerant abilities. As eukaryotic extremophiles, both species further demonstrate the dominance of basidiomycetous yeasts -- particularly from the *Cryptococcus albidus* clade -- in some of our planet's highest elevations soil environments (SCHMIDT et al., 2017). As non-pigmented strains, both reinforce the hypothesis that further resistance mechanisms may be responsible for extremophiles' great tolerance to various environmental factors besides the commonly studied pigmented compounds (PULSCHEN et al., 2015). As such, our study reinforces the importance of the Atacama Desert as a niche of extremophilic microorganisms and the need for further studies aimed at clarifying the molecular, ecological and physiological aspects of extreme environments, as well as treasured biomolecules with a potential application within the biotechnological, pharmaceutical, and astrobiological research spaces.

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5 CHAPTER III: NON-PIGMENTED EXTREMOPHILIC YEASTS AS MODEL ORGANISMS FOR ASTROBIOLOGY AND SPACE EXPLORATION

The final chapter of this thesis refers to concluding remarks in terms of the potential application of non-pigmented extremophilic yeasts to studies on astrobiology and space exploration. The chapter covers the development of Astrobiology as an effective research area, the constraints involved in space exploration, with particular focus on Mars, and the development of synthetic biology techniques for the optimization of non-pigmented extremophilic yeasts as model organisms for *in situ* resource utilization.

5.1 ASTROBIOLOGY

Whilst Biology concerns the study of Earth's living organisms, Astrobiology studies life as a planetary phenomenon, with the overarching goal of understanding the possibility of finding living organisms beyond our planet's biosphere. Although human curiosity has always led to questions representing the core of Astrobiology research, only recently has humanity gained enough technological and scientific advancements that unlocked the first tangible steps towards understanding life elsewhere in our universe (BLUMBERG, 2003).

The first exact mention of the term Astrobiology is not known. Among the registered pioneers are Janet Morrison's paper "Astrobiology" in the Astronomical Society of the Pacific (LAFLEUR, 1941), Gabriel Tikhov's mention of Astrobotany in a Moscow publication (TIKHOV, 1953), and Flávio Pereira's book "Introduction to Astrobiology" published in Brazil (PEREIRA, 1956). As a scientific endeavor, however, Astrobiology was first described under the term "Exobiology", as an active effort from the National Aeronautics and Space Administration (NASA) aimed to study the origins of life on Earth and elsewhere (BLUMBERG, 2003). The first Exobiology studies were deemed inconclusive, particularly after NASA's missions Viking 1 and Viking 2 conducted on Mars surface, waning off any collective efforts for the next two decades.

Astrobiology got a second shot at demonstrating its value as a scientific query in the mid-1990s, after further sophistication of laboratory infrastructure and improvements in space-borne equipment, as well as with the recent shift towards the discovery of more and more planets orbiting stars other than our own Sun. Ultimately, the first official Astrobiology conference was conducted in 1996 at the Astrobiology Research Center -- ARC (BLUMBERG, 2003). In spite of the former introduction of Astrobiology by the above-mentioned book by Flávio Pereira, only in the middle 2000s did significant interest in Astrobiology arise among the Brazilian scientific community (DUARTE et al., 2012).

Astrobiology's foundational questions revolve around the possibility of life existing on planets other than our own (ABBOTT; PEARCE, 2020). Given that Earth is the only place where there's scientific evidence for life, understanding the origin, evolution and distribution of life in the "pale blue dot" is the most effective methodology towards scientific advancement and thus a required step to understanding whether we are alone in the universe or not (BILLI, 2019). In addition to these questions,

Astrobiology also studies the potential for cross-contamination as we directly reach out and investigate extraterrestrial environments. As such, planetary protection explores the possibility of forward contamination – that is, the transfer of terrestrial microorganisms to other planetary bodies – as well as the possibility of Earth's contamination with materials returned from extraterrestrial missions – backward contamination (ABBOTT; PEARCE, 2020).

To test whether a planet can be proved inhabited or not, we must first define what makes a planet habitable (BILLI, 2019). According to Cockell et al. (2016), an environment can be considered habitable if it supports the activity of at least one live organism. Despite extreme physicochemical characteristics pointing out the minute potential of life on extraterrestrial planets, terrestrial life has been proven a stubborn entity that flourishes even on habitats previously thought hostile (Harrison et al. 2013). In line with this finding, Tom Brock's pioneer discovery of life in Yellowstone's hot springs (BROOK; FREEZE, 1969) uncovered the study of microorganisms living under extreme environmental conditions – the so-called extremophiles.

The discovery of microorganisms surviving and flourishing under extreme environmental conditions expanded the limits of habitability over a broader range of physicochemical parameters, opening space for further recognition of extraterrestrial life as a plausible hypothesis on our universe (CAVICCHIOLI, 2002; ROTHSCHILD; MANCINELLI, 2001). Laboratory simulations exposing extremophilic microorganisms to a wide range of conditions are now used as a well-established methodology to understand the biological arsenal behind some microorganisms' resistance (ABBOTT; PEARCE, 2020; BILLI, 2019).

The combination of various extremes creates a distinct mixture of factors that intensify the selection of polyextremophiles armed with specific survival toolkits for their environments. By studying extreme habitats on Earth and their polyextremophilic microorganisms, one can understand the required mechanisms, strategies and adaptations within specific niches of habitability, thus expanding our knowledge base about where and how life could be most probably found (CASSARO et al., 2021).

In particular, the Atacama Desert is a key example of an extreme environment providing similar conditions to extraterrestrial habitats (CASSARO et al., 2021). The Atacama's extreme UVR exposure, significant daily variations in temperature, high saline concentration, low water activity and low nutrient availability (NAVARRO-GONZÁLEZ et al., 2003) combine into a similar mix of conditions to what can be found

in some specific habitats of Mars – thus being called a Planetary Field Analogue – PFA (CASSARO et al., 2021). Moreover, the Atacama Desert represents a rich source of extremophilic microorganisms able to simultaneously survive several of the aforementioned extreme parameters.

5.2 MARS

Mars is the closest planetary body to Earth, and it's been long studied for its potential suitability to harbor other forms of life. Astronomy and Astrobiology research, alongside non-tripulated planetary missions, have determined the environmental conditions of various habitats within the red planet (ABBOTT; PEARCE, 2020). Mars contains surface and underground water sources (DUNDAS et al., 2018; NANGLE et al., 2020; OROSEI et al., 2018), as well as essential elements for life dispersed throughout its atmosphere and regolith (MESLIN et al., 2013). Moreover, studies have shown a substantial possibility of hydrological cycles and even water bodies in past Mars (CHANGELA et al., 2021; REDD, 2020; SCHELLER et al., 2021; WORDSWORTH, 2016).

In contrast, Mars offers an average equatorial temperature of –14°C (NANGLE et al., 2020), a reduced atmospheric pressure between 0.6 and 0.8 kPA, a dry and salt-rich surface, as well as high UVR exposure. Although Mars receives 57% less radiation than Earth, its CO_2-rich, thin atmosphere – about 100-fold thinner than Earth's – results in extreme daily fluxes of radiation, particularly UVC wavelengths (ROTHSCHILD; MANCINELLI, 2001). Therefore, microorganisms potentially surviving life under the extremes of a Martian habitat would be better represented by psychrophiles (able to maintain their metabolites and overall cell structure under extremely low temperatures), anhydrobiosis (able to survive desiccation-rehydration cycles or extended periods under dried forms without significant ultrastructural damage), halophiles (able to control osmotic pressure maintenance under high salt concentration), and radioresistant microorganisms (able to shield solar wavelengths or diminish & regenerate the direct and indirect damage caused by UVR).

To date, a wide variety of microorganisms have been shown to possess the molecular and physiological mechanisms necessary to survive conditions similar to those observed on the surface or subsurface of Mars (CHANGELA et al., 2021). Examples predominantly include prokaryotes such as bacteria and archaea

(ABREVAYA et al., 2011; GÓMEZ et al., 2010; PAULINO-LIMA et al., 2013), or cyanobacteria and microalgae (GAO et al., 2013; de VERA et al., 2014), but also eukaryotic microorganisms primarily represented by yeasts and overall fungi (BLACHOWICZ et al., 2019; PULSCHEN et al., 2018).

In accordance, the recent development of high-throughput sequencing and metagenome-assembly techniques has revealed an even more extensive diversity of prokaryotes and eukaryotes in Earth's most extreme environments, expanding our ability to isolate and characterize a larger amount of polyextremophilic microorganisms that could reinforce the potential for life to surge and evolve within the harsh conditions of extraterrestrial planets.

Yet, although most studies focus on the potential current or past habitability of Mars, facing the likely possibility of never finding extant life on the red planet can raise even more crucial questions in Astrobiology (CHANGELA et al., 2021). Apart from philosophical interrogations regarding terrestrial life as a single, unique event in an ever-expanding universe, a habitable, but inhabited Mars could represent a major experimental site for the development of terraforming and space exploration research. Although a seemingly impossible quest, the urge to explore new territories has inspired public and, more recently, private funding to excel the infrastructure necessary for space exploration. As Earth's neighbor planetary body, Mars is "the next most important frontier" of humanity's exploration efforts (NANGLE et al., 2020).

5.3 SPACE EXPLORATION

The "study of the living universe" (CHYBA; HAND, 2005) as a prime target by Astrobiologists is not limited by the potential distribution of life throughout natural events (*e.g.,* comets, bolide impacts, solar winds, etc.). Space exploration is an exceptional achievement in human history and could be responsible, in the near future, for the distribution of human and microbial life through extraterrestrial planets (NANGLE et al., 2020). After a few waxing and waning financial periods, technological advancements towards the exploration of the universe – or, at least, of the closest planetary bodies to our home planet – it's getting close to their prime time: the exploration of a neighbor planet by means of a manned mission (LOPEZ et al., 2019; VERSEUX et al., 2016a). Aside from its proximity to Earth (NANGLE et al., 2020),

studies have recently identified habitable environments with amenable conditions for humans and microorganisms on Mars (COCKELL, 2021; MORGAN et al., 2021;).

As a consequence, while none of the exploration efforts conducted so far have resulted in the discovery of significant hints for the existence of life within Martian habitats, the planet's habitable conditions still bear potential for scientific and technological efforts toward creating a second home for humanity (CHANGELA et al., 2021; NANGLE et al., 2020). With that in mind, NASA has established the land of humans on Mars as a medium-term target (*i.e.*, mid-2030s), and several projects are currently being developed with the goal of designing and manufacturing the infrastructure necessary for the successful settlement of humanity on Martian territory (CHANGELA et al., 2021; VERSEUX et al., 2016b).

Several countries have sent their own orbiters and landers to the red planet (SMITH et al., 2020). Recent examples include NASA's fifth Martian rover, Perseverance (POO, 2020), the Chinese orbiter Tianwen-1 (WAN et al., 2020), United Arab Emirates' Mission Hope (SHARAF et al., 2020), and the European Space Agency's rover ExoMars Rosalind Franklin (CHANGELA et al., 2021), all designed to characterize current geology and climate conditions at the surface and near-surface of the planet. In addition to public funding for Mars exploration, private enterprises are also growing in this space. SpaceX, Blue Origin, and Virgin Galactic are representative examples of aerospace and commercial spaceline companies working on the design, manufacture, engine, and launch of civil spaceflights (LOPEZ et al., 2019), as well as human settlements on Mars (CHANGELA et al., 2021).

Although undoubtedly noteworthy, the technological advancements in space engineering (NANGLE et al., 2020) that support human missions to Mars are only a small step toward a much bigger endeavor: once launched, manned missions must be able to surpass payload challenges for dozens of humans throughout an eight-month flight, as well as address the even more challenging demands of assisting all basic human needs to be produced *in situ* after humanity arrives on the red planet (CHANGELA et al., 2021). Aside from courage, humans designated to complete the paramount mission of Mars exploration must carry out the most superior innovations in infrastructure, automation, and resource production (SZOCIK et al., 2020; TIEZE et al., 2020).

5.4 IN SITU RESOURCE UTILIZATION

Long before the idea of mass colonization was effectively pursued, the International Space Station (ISS) conducted experiments that indicated the unrealistic value of a "carry it with you" plan to support life outside Earth. Current space missions are already too expensive: around $150 million to support a crew of six ISS astronauts per year, according to Revellame et al. (2021). Mars missions must maintain their crews with minimal Earth supply requirements and the most basic human needs met on location, particularly for long-term missions aiming to establish permanent Martian colonies (BILLI, 2019, CHANGELA et al., 2021; VERSEUX et al., 2016a).

Thus, Earth-independent human settlement will require the maximization of technological advancements toward Mars *in situ* resource utilization – ISRU – for food, water, medicines, clothing, and habitat, as well as waste recycling and removal (ROTHSCHILD, 2016; VERSEUX et al., 2016a). In fact, Martian rocks provide most elements required for life, from the most crucial elements – the so-called CHONPS – to trace elements necessary for the fine-tuning of a healthy, sustainable life (*e.g.,* Mg, Fe, Ca, Na, K, Mn, Cr, Ni, Mo, Cu, Zn), as well as additional gaseous carbon (*e.g.,* CO2, CH4) and even water sources (COCKELL, 2014; VERSEUX et al., 2016a). ISRU is, therefore, a feasible future approach for the sustainable establishment of human settlements on Mars (CHANGELA et al., 2021).

However, most current ISRU methodologies still rely on physicochemical processing units that would not be able to sustain long-term missions without constant resupply from Earth (BILLI, 2019; VERSEUX et al., 2016b). A more innovative approach that has been extensively explored in recent years is the combination of self-sustaining biological life support systems (BLSS) for Mars ISRU (REVELLAME et al., 2021). These are focused on an extensive reduction of mass (thus, reducing resupply needs) and optimization of autonomous systems able to operate under extreme environmental conditions (ROTHSCHILD, 2016) that highlight the valuable implementation of microorganisms – in particular, extremophilic microorganisms – for bioproduction, conversion, and recycling based on Martian resources (NANGLE et al., 2020; ROTHSCHILD, 2016; VERSEUX et al., 2016b).

On Earth, bioproduction still represents an expensive and fancy approach that cannot financially compete with less intricate industries such as forests- or petrochemical-based manufacturing. On the other hand, the manufacturing of

biological materials through the capabilities of extremophilic microorganisms may be the only viable option for the sustainable settlement of the red planet (ROTHSCHILD, 2016). From food and pharmaceuticals to bioplastics and construction materials, microorganisms will play a pivotal role in the success of early-stage ISRU-based Martian missions, eventually enabling the adaptation of complex efforts toward complete Earth independence. Closing the loop of Martian life-supporting systems, microorganisms will also be implemented in liquid and solid waste recycling (CHANGELA et al., 2021; NANGLE et al., 2020). Eventually, ISRU BLSS is envisioned through a custom and on-demand system with multiple bioreactor modules combining specific microbial consortia for specific production and recycling applications (NANGLE et al., 2020).

Identifying extremophiles able to adapt and live within Mars's extreme conditions is essential, and multiple experiments have been conducted to that end. Examples include experiments aboard satellites (*e.g.,* EXPOSE and BIOPAN) to test the survivability of microbial species under space conditions (ABBOTT; PEARCE 2020; LOPEZ et al., 2019). Although such experiments successfully demonstrate the potential of prokaryotic and eukaryotic species to survive under specific conditions found outside our planet, the ideal solution of a polyextremophilic microorganism with outstanding fitness capabilities able to perform toward specific bioproduction applications may still rely on the hands of bioengineering magnifications that have been recently achieved with the establishment of synthetic biology (NANGLE et al., 2020; VERSEUX et al., 2016a).

5.5 SYNTHETIC BIOLOGY

The genetic material of Earth’s microorganisms has been long manipulated, aiming the fine-tuning of a variety of bioprocesses towards increasing or optimizing the manufacture of food, drinks, drugs and overall chemicals, as well as biomaterials and biofuels (PATRA et al., 2021; VERSEUX et al., 2016b). More recently, advancements in multi-omics and bioinformatics methodologies enabled the advent of a new research area called synthetic biology. Together with the holistic view of a biological system – systems biology – synthetic biology provides the ability to predict and calibrate cellular functions, as well as to refine genetic inefficiencies and even construct new molecular pathways from scratch (NANGLE et al., 2020; PATRA et al., 2021). Even more recent

developments allowing for efficient and versatile genome-editing tools, such as the CRISPR/Cas9 system, have made genetic modifications relatively easy and straightforward, elevating the achievements of systems and synthetic biology to a whole new level (HANCZYC, 2020; PATRA et al., 2021).

Based on genetic engineering, synthetic biology will enable Mars' ISRU through the development of microorganisms optimized for two key, convoluted areas: the fine-tuning of extremophilic characteristics (*e.g.,* optimizing extremophiles for the specific extreme temperature, salt, and radiation conditions of Mars), and the optimization of metabolites manufacturing (*e.g.,* magnifying food or plastics bioproduction), thus overcoming the mass and volume challenges of a manned mission (ROTHSCHILD, 2016) while enabling the achievement of a simpler BLSS possessing the essential characteristics according to a targeted product (NANGLE et al., 2020; PATRA et al., 2021; VERSEUX et al., 2016a).

Given the potential of synthetic biology for enabling human missions on Mars, NASA has extensively invested in this research approach. Strategies include the transfer of genes from one organism into another to increase the latter's fitness under extreme conditions through an improved stress response system (HANCZYC, 2020; PATRA et al., 2021; VERSEUX et al., 2016b). However, given the multifactorial nature of some specific resistance mechanisms, the overexpression of genes from an already resistant microorganism may provide better phenotypic results. One example is the complex and intricate combination of DNA repair mechanisms, antioxidative defenses, and morpho-physiological characteristics responsible for the resistance to extreme radiation and desiccation exposure. To that end, specific genetic engineering approaches may be required, such as modifying gene expression activity through promoters, repressors, and reporters (HANCZYC, 2020) or subjecting microbial populations to multiple rounds of mutagenesis and artificial selection toward the optimization of complex features (*i.e.,* directed evolution) (VERSEUX et al., 2016b).

In short, synthetic biology represents a key strategy allowing the feasible implementation of on-demand bioproduction and bioreclamation systems towards ISRU on Mars (CHANGELA et al., 2021; NANGLE et al., 2020; ROTHSCHILD, 2016). However, scientific advancements still lack in terms of which specific microorganisms we should focus on for each case application (CHANGELA et al., 2021). The implementation of what Billi (2019) named "a robust chassis for synthetic biology under extreme conditions" is crucial for engineering-driven methodologies optimizing

microbial organisms for bioproduction and bioreclamation. Although prokaryote organisms have been established among the main microbes studied for optimization of bioprocesses through genetic manipulation, we envision the manipulation of the molecular features of extremophilic yeasts to render space synthetic biology aiming for even more functional BLSS in the extreme environmental conditions of Mars.

5.6 YEAST OPTIMIZATION FOR BLSS AND ISRU

Prokaryotic microorganisms have long been used as cell factories for the bioproduction of metabolites on Earth. Examples include the well-studied bacteria *Escherichia coli* and other bacterial genera *Clostridium* spp. and *Corynebacterium* spp. (PATRA et al., 2021), as well as the more recently studied cyanobacterial genus *Chroococcidiopsis* (WITTHOHN et al., 2021). However, prokaryotic species present disadvantages in terms of their sub-optimal tolerance to high metabolite concentration or pH variations. In addition, the inability to adhere to compartment-specific reaction and increased phage infection susceptibility also reflect drawbacks of prokaryotes compared to eukaryotes for specific bioprocessing systems (BLOUNT et al., 2012; PATRA et al., 2021; RUNGUPHAN; KEASLING, 2014).

In contrast, yeasts present more robust morpho-physiological characteristics, such as easily trackable genomes, inherent fermentation features, optimized tolerance to harsh environmental conditions, as well as the ability to perform post-translational modifications that allow producing human proteins not achievable with bacterial cultures (CHEN; NIELSEN, 2013; PATRA et al., 2021; VOGL et al., 2013; WALSH, 2010), whilst performing significantly faster growth culture and requiring significantly less space than other robust bioproduction systems such as higher fungi and plants (REVELLAME et al., 2021). Consequently, yeasts are among the most well-studied eukaryotic organisms for the bioproduction of food, pharmaceuticals, and overall biochemicals (PATRA et al., 2021). Moreover, the ability of yeasts to survive prolonged periods of desiccation also promotes these organisms among the most well-suited for space flights and the long-term goals of Mars exploration through BLSS (BILLI, 2019; TIEZE et al., 2020).

Although *Saccharomyces cerevisiae* has been widely applied as a conventional model yeast for bioproduction aiming at human healthcare and industrial studies (PATRA et al., 2021) and even for space research seeking to understand its

responses after exposure to radiation and other extreme conditions (TIEZE et al., 2020), recent studies have defended a more practical implementation of non-conventional yeast extremophilic species due to their unique inherent phenotypical properties (GALANIE et al., 2015; QIAO et al., 2017). Although offering less traceable genetic footprints, non-conventional yeasts' utilization of unusual energy sources and increased endurance to specific processing conditions are preferred as these usually combine into a generally higher titer of desired metabolites, particularly non-ethanol products. Examples of non-conventional yeasts include oleaginous *Y. lipolytica* and *R. toruloides* cultures storing cellular lipids as cheap carbon sources and thermotolerant *K. marxianus* used for saccharification and fermentation under high temperatures (PATRA et al., 2021).

Recent advancements in multi-omics techniques enable the study of a greater diversity of non-conventional yeast species from extreme environments. Examples include psychrophiles, thermophiles, halotolerant, and radioresistant yeasts from hot and cold deserts such as the Atacama Desert and Antarctica (CONNELL et al., 2014; PULSCHEN et al., 2015) and highlight the ever-increasing opportunity to learn new metabolic capabilities aimed to a plethora of bioprocesses (PATRA et al., 2021; WENDLAND, 2020). Based on the combinatorial properties of extreme conditions found at these and other PFAs, researchers can study and suggest ideal extremophilic yeast candidates for genetic optimization toward biotechnological processes useful for developing BLSS on Mars.

5.7 PIGMENTED AND NON-PIGMENTED YEASTS AS MODEL ORGANISMS FOR SPACE EXPLORATION

Pigmentation has always been stated as a helpful characteristic increasing the survivability of microorganisms under extreme environmental conditions. Extensive scientific literature has demonstrated the beneficial effects of pigments such as melanin and carotenoids for extremophilic species living not only under extreme radiation exposure, primarily due to the shielding effects concerning the absorption and dissipation of electromagnetic radiation waves, but also under salinity, water, temperature, and nutritional stress – more correlated explicitly with the indirect effects of scavenging of reactive species, thus reducing overall cellular damage (CHANGELA et al., 2021).

A key example of the protective effects of pigmentation of eukaryotic species is the Antarctic rock-inhabiting yeast *Cryptococcus antarticus*. Commonly known as black rock fungi, this species has been mapped and characterized after the discovery of the first cryptoendolithic communities in Northern Victoria Land (ONOFRI et al., 2021) and is commonly well-accepted as a eukaryotic model for investigations concerning Martian missions (ONOFRI et al., 2012; SCALZI et al., 2012; SELBMANN et al., 2015; de VERA et al., 2014). *C. antarticus*'s thick melanized cell walls are described among the crucial characteristics that enable the yeast's strong resistance to UV radiation, salinity and temperature extremes, desiccation, and freeze/thawing cycles (ONOFRI et al., 1999, 2021; SELBMANN et al., 2015), and even extraterrestrial conditions such as simulated Martian habitats (CHANGELA et al., 2021; SELBMANN et al., 2015; ONOFRI et al., 2008).

As stated by Cordero et al. (2020), "melanization also comes with a cost." One of the key properties of pigmentation – particularly melanized cells – towards effective survival in extreme conditions may be the very responsible for melanin's unfavorable characteristics under Martian environmental conditions. Although the thermal melanism hypothesis predicts melanin's adaptive ability to convert solar radiation into heat in favor of better growth conditions under cold climates with restricted solar radiation (CORDERO et al., 2018), it may also lead to darkly pigmented microorganisms heating up faster than their non-pigmented, radioresistant counterparts. In addition, free radicals and other by-products created during melanin's biosynthesis may increase the vulnerability of darkly pigmented species (CORDERO et al., 2020), besides the reduced cell wall porosity that may limit the incorporation of nutrients (DADACHOVA et al., 2007; KREUSCH; DUARTE, 2021).

Altogether, these characteristics lead to darkly melanized yeasts at a disadvantage compared to light-pigmented or non-pigmented extremophilic species with better nutrient incorporation and faster growth under the extreme conditions of Mars (KREUSCH; DUARTE, 2021). Therefore, better studies supporting the idea of growing non-pigmented, extremophilic yeast species are required to demonstrate these yeasts' ability to grow and participate in bioproduction systems under Martian conditions.

5.8 CONCLUDING REMARKS

Although accurate access to galactic cosmic rays and specific Martian characteristics is only possible through direct, *in situ* exposure, the study of model organisms through laboratory analogues of planetary environments provides an irreplaceable methodology for current investigations concerning astrobiology and space exploration (BILLI, 2019). In line with the results found in this thesis, the specific combinatorial effects that account for higher resistance and survivability of non-pigmented, extremophilic yeast species – such as *N. friedmannii*, *H. wattica*, and *P. laurentii* – should be pursued to evaluate further the practical implementation of the aforementioned species towards the BLSS and ISRU infrastructure necessary for the long-term goal of effective Martian exploration.

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6 FINAL CONSIDERATIONS

Exploring extreme environments and their microorganisms opens new opportunities for discovering novel compounds and protection mechanisms, as biomolecules produced by extremophiles are widely implemented in biotechnology and industrial processes, as well as pharmaceutical and biomedical applications. Moreover, extremophiles are the best model organisms to study the possibility of finding indigenous life outside Earth (*i.e.,* astrobiology) or transporting Earth's life into extraterrestrial habitats (*i.e.,* space exploration).

Most extremophilic microorganisms currently known belong to the Bacteria and Archaea Domains. In addition, the protective and antioxidative capabilities of colorless microorganisms have been widely neglected. However, eukaryotes also comprise microorganisms resistant to a variety of extreme conditions, with yeasts, in particular, being constantly highlighted for their physiological flexibility and survivability under a wide range of extreme parameters. Therefore, better studies supporting the idea of growing non-pigmented, extremophilic yeast species are required to demonstrate these yeasts' ability to thrive in harsh environments on Earth, as well as extraterrestrial planetary bodies, as means to facilitate space exploration.

To assess the potential of non-pigmented yeasts for biotechnology, astrobiology, and space exploration studies according to their resistance to extreme environmental factors, this study investigated new strains of non-pigmented yeasts isolated from the Atacama Desert to their resistance to UVC, desiccation, temperature and salinity extremes.

Our results reinforce the resistance to UVR and desiccation within eukaryotic species, particularly within non-pigmented yeasts that do not use pigmented compounds among their primary resistance strategies. In particular, our results further demonstrate the dominance of basidiomycetous yeasts - particularly from the *Cryptococcus albidus* clade - in extreme environments and reinforce the hypothesis that different resistance mechanisms may be responsible for extremophiles' tolerance to environmental factors besides pigments.

Finally, this thesis opens space for the investigation of the specific combinatorial effects that account for higher resistance and survivability of non-pigmented, extremophilic yeast species, with the ultimate goal of their practical implementation towards BLSS and ISRU infrastructure necessary for long-term space exploration.