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Release of cell-free enzymes by marine pelagic fungal strains

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Fungi are ubiquitous organisms that secrete different enzymes to cleave large molecules into smaller ones so that can then be assimilated. Recent studies suggest that fungi are also present in the oceanic water column harboring the enzymatic repertoire necessary to cleave carbohydrates and proteins. In marine prokaryotes, the cell-free fraction is an important contributor to the oceanic extracellular enzymatic activities (EEAs), but the release of cell-free enzymes by marine fungi remains unknown. Here, to study the cell-free enzymatic activities of marine fungi and the potential influence of salinity on them, five strains of marine fungi that belong to the most abundant pelagic phyla (Ascomycota and Basidiomycota), were grown under non-saline and saline conditions (0 g/L and 35 g/L, respectively). The biomass was separated from the medium by filtration (0.2 μm), and the filtrate was used to perform fluorogenic enzymatic assays with substrate analogues of carbohydrates, lipids, organic phosphorus, sulfur moieties, and proteins. Kinetic parameters such as maximum velocity (V_{max}) and half-saturation constant (K_m) were obtained. The species studied were able to release cell-free enzymes, and this represented up to 85.1% of the respective total EEA. However, this differed between species and enzymes, with some of the highest contributions being found in those with low total EEA, with some exceptions. This suggests that some of these contributions to the enzymatic pool might be minimal compared to those with higher total EEA. Generally, in the saline medium, the release of cell-free enzymes degrading carbohydrates was reduced compared to the non-saline medium, but those degrading lipids and sulfur moieties were increased. For the remaining substrates, there was not a clear influence of the salinity. Taken together, our results suggest that marine fungi are potential contributors to the oceanic dissolved (i.e., cell-free) enzymatic pool. Our results also suggest that, under salinity changes, a potential effect of global warming, the hydrolysis of organic matter by marine fungal cell-free enzymes might be affected and hence, their potential contribution to the oceanic biogeochemical cycles.

KEYWORDS

marine fungi, cell-free enzymatic activity, kinetics, maximum velocity, half-saturation constant

1 Introduction

Low-molecular-weight (LMW) molecules are operationally defined as molecules with a molecular weight below 1000 Da, whereas high-molecular-weight (HMW) molecules comprise those larger than 1000 Da (Benner et al., 1992; Amon and Benner, 1996; Benner, 2002). In marine ecosystems, the majority of dissolved organic matter (DOM) is composed of LMW molecules, but the bioavailable ones are mainly HMW molecules (Wheeler, 1976; Amon and Benner, 1994; Amon and Benner, 1996; Benner, 2002). In order to take them up, microorganisms need to hydrolyze them into smaller molecules (<600 Da) (Weiss et al., 1991).

Osmotrophy is a feeding strategy that involves the secretion of different enzymes to transform large molecules into smaller ones which can then be absorbed by osmosis (Richards et al., 2012; Muszewska et al., 2017). For the fungal kingdom, osmotrophy is a distinctive feature that has allowed them to use largely inaccessible nutrients and conquer diverse environments (Dix and Webster, 1995; Webster and Weber, 2007; Richards and Talbot, 2013), including marine ones. Here, the fungal species can be obligate or facultative (Jennings, 1983). Obligate marine fungi are only capable of living in marine environments, while facultative species have a terrestrial origin, but are capable of also living in them (Raghukumar, 2017).

Total extracellular enzymatic activities (EEAs) are a combination of cell-attached and cell-free enzymatic activities (Wetzel, 1991; Baltar, 2018). Operationally defined, cell-attached enzymes are those retained on a 0.2 μm filter, whereas cell-free enzymes pass this filter (Baltar, 2018). In marine environments, the first ones are linked to the cell wall or periplasmic space, whereas cell-free enzymes are dissolved in the immediate waters (Hoppe et al., 2002). As cell-attached enzymes are tightly linked to the cell, and respond to substrates outside the cell, they represent a chemical communication with the surrounding environment (Chróst, 1990). In contrast, cell-free enzymes are released by the cells into the surrounding environment (Priest, 1977; Chróst, 1990; Wetzel, 1991). As these are not linked anymore to the cell, they are not metabolically controlled by the cell (Kamer and Rassoulzadegan, 1995). However, in the case of substrate limitation, cell-free enzymes can be used as a strategy “to find food fast” (Chandrasekaran and Kumar, 1997), and also to utilize other polymeric compounds which are otherwise non-usable (Chróst, 1990; Chróst, 1992). The occurrence of cell-free enzymes in marine environments might be crucial as these can access distant substrates, and influence the kinetics of organic matter (Kamer and Rassoulzadegan, 1995). This mobilization might also result in an improvement in substrate availability (Wetzel, 1991). As coined by Baltar (2018), cell-free enzymes are a kind of “living dead”, as they are not attached anymore to the cell, but can still perform their respective function. Moreover, as suggested by Arnosti (2011), cell-free enzymes can influence the carbon cycle at different times and spaces where they were originally produced. Therefore, a dissociation between marine microorganisms and enzymatic activities might exist (Arnosti, 2011; D’ambrosio et al., 2014; Baltar et al., 2016; Muszewska et al., 2017; Thomson et al., 2019).

Marine microbial EEAs contribute significantly to the breakdown of organic substrates (Hoppe et al., 2002). The

majority of them were originally believed to be cell-attached rather than cell-free (Hoppe, 1983; Rego et al., 1985; Chróst, 1990; Chróst and Rai, 1993; Hoppe et al., 2002). Contrary, other studies suggested that the cell-free EEAs can be similar to or even higher than the cell-attached enzymatic pool (Kamer and Rassoulzadegan, 1995; Keith and Arnosti, 2001; Baltar et al., 2010; Duhamel et al., 2010; Allison et al., 2012; Baltar et al., 2013; Baltar et al., 2016; Baltar, 2018). Additionally, some studies pointed out that the sources of marine cell-free enzymes can be numerous (Kamer and Rassoulzadegan, 1995; Allison et al., 2012; Baltar et al., 2013), but believed to be mostly of bacterial origin (Hollibaugh and Azam, 1983; Chróst, 1990; Hoppe and Ullrich, 1999; Obayashi and Suzuki, 2008b; Baltar et al., 2010; Bong et al., 2013; D’ambrosio et al., 2014; Li et al., 2019). However, in a study on the upwelling ecosystem of Chile, Gutiérrez et al. (2011) found potential evidence of an active contribution of marine fungi to the total enzymatic pool, suggesting that they could also be involved in the breakdown of organic matter in the ocean.

As shown by Vetter and Deming (1999), cell-free enzymes act at a distance from the microorganism that originally released them, and in the absence of dissolved organic matter, these enzymes used particulate organic matter which provided enough hydrolysate to support microbial growth. Therefore, cell-free enzymes might be secreted to increase the chance of survival of the cell that originally produced them (Chróst, 1986; Chróst and Overbeck, 1987). But compared to the cell-attached enzymes, cell-free enzymes might also benefit other cells of the surrounding environment (Luo et al., 2009).

Due to anthropogenic and natural causes, salinity fluctuations have been reported in different oceanic regions (Skiris et al., 2014). These changes can lead microorganisms to experience osmotic and ionic stress (Gladfelter et al., 2019), and influence their extracellular enzymatic activities (Chróst, 1990; Caruso and Zaccone, 2000; Salazar-Alekseyeva et al., 2022)[in revision]. Marine fungi seem to tolerate a wide range of salinities (Jennings, 1983), but they are probably not halophilic (Gladfelter et al., 2019). Hence, it is currently unknown how changes in oceanic salinities might affect fungi and their EEAs, especially, the cell-free fraction.

Compared to bacteria, marine fungi are less studied, so here we investigated their secretion of cell-free enzymes using five species as representatives of the most dominant marine pelagic fungal phyla: Ascomycota, and Basidiomycota (Taylor and Cunliffe, 2016; Amend et al., 2019; Morales et al., 2019). These species were grown in non-saline and saline media to resemble conditions of freshwater and marine environments, respectively. The cell-free fraction EEA was determined and the potential effect of salinity on the kinetic parameters such as maximum velocity (V_{max}) and half-saturation constant (K_m) was analyzed.

2 Methods

The marine fungal species *Blastobotrys parvus* (HA 1620), *Metschnikowia australis* (HA 635), *Rhodotorula sphaerocarpa* (HB 738), and *Sakaguchia dacryoidea* (HB 877) were obtained from the Austrian Center of Biological Resources (ACBR), but were

originally isolated from the Antarctic Ocean (Fell and Hunter, 1968; Newell and Fell, 1970; Fell and Statzell, 1971; Fell et al., 1973). In the case of *Rhodotorula mucilaginosa*, this was isolated from the Atlantic Ocean during the Poseidon Cruise in March 2019. All these pure isolates were maintained on yeast malt extract agar (Wickerham, 1939; Wickerham, 1951) at room temperature, and were renewed monthly.

To culture these species, the protocols of Salazar Alekseyeva et al. (2021) and Salazar-Alekseyeva et al. (2023) [in revision] were followed. One medium containing 2 g/L of glucose, malt extract, peptone, yeast extract, and 0.5 g/L of chloramphenicol was prepared and further divided into two media. The first one contained 35 g/L of artificial sea salts (S9883 Sigma-Aldrich), and the second one did not contain salts (0 g/L). In a vertical laminar airflow cabinet (Steril Bio Ban 72), with a sterile loop, an arbitrary amount of one-week-old pure isolates was transferred into autoclaved artificial seawater (35 g/L sea salts S9883 Sigma-Aldrich) until an optical density of ≈ 1 at 660 nm wavelength (OD_{660}) was reached. This was measured with a UV-1800 Shimadzu spectrophotometer and represented a correlation with the cell count. Per every 1 L of the autoclaved medium, 0.01 L of this fungal dilution was added. Afterwards, 150 mL of this mixture (medium and fungal dilution) were put in Schott bottles and incubated at 5°C on a rotary shaker (Jeio Tech ISS-7100 Incubated Shaker) until the exponential phase was reached. Three bottles with similar OD_{660} values (biological triplicates) were chosen for further analyses (EEAs and biomass).

To estimate the fungal biomass that was releasing cell-free enzymes, 40 mL of the sample was vacuum filtered onto a pre-weighed and combusted (450°C for 6 h) Whatman GF/F filter (WHA1825047 Sigma-Aldrich, 47 mm diameter), and for 3 days, this filter was dried at 80°C. Finally, the filter was weighed again, and the fungal biomass was estimated from the difference between the pre-weighed filter and the dried one.

To estimate the fungal abundance that was releasing cell-free enzymes, 1.5 mL of the liquid media containing fungi was used. For a single-cell suspension, this volume was filtered onto a pluriStrainer Mini (43-10040-50 pluriSelect, 40 μ m mesh size), fixed with a final concentration of 0.5% glutaraldehyde for 10 minutes, and lastly frozen at -80°C until further processing. As *B. parvus* has a filamentous structure, its abundance was not possible to estimate with this method. For the other species, depending on the OD_{660} value, 10 to 40 μ L of the thawed sample and 5 μ L of SYBR[®] Green 100x (S9430, Sigma-Aldrich) were added, and completed with Tris EDTA buffer (TE) to obtain a final volume of 500 μ L. Finally, the sample was measured with a BD Accuri[™] C6 Plus Flow Cytometry set at 'Run with limits' of 10,000 events and 'Medium', and the cell abundance was estimated with the BD Accuri C6 Software.

To determine the cell-specific biomass, 1.5 mL of sample were filtered with a pluriStrainer Mini (43-10040-50 pluriSelect, 40 μ m mesh size) to obtain a single-cell suspension. The sample was fixed in the dark with 0.5% (final conc.) glutaraldehyde for 10 minutes, and subsequently frozen at -80°C until further processing. Due to the multicellular structures of the filamentous species *B. parvus*, its

cell abundance could not be determined. For the other species, depending on the optical density, 10 to 40 μ L of the sample was diluted with TE to obtain a final volume of 500 μ L which was later stained with 5 μ L SYBR[®] Green 100x (S9430, Sigma-Aldrich). The cell abundance was determined using a BD Accuri[™] C6 Plus Flow Cytometry set at 'Run with limits' of 10,000 events and 'Medium' and the cell abundance was obtained with the BD Accuri C6 Software.

To obtain the cell-free fraction, the protocols of Hollibaugh and Azam (1983) and Kim et al. (2007) together with the suggestion of Obayashi and Suzuki (2008a) were followed. This was obtained by separating the biomass through vacuum filtration on Whatman Track-Etched Membranes with a pore size of 0.22 μ m (WHA10417012 Sigma-Aldrich, 47 mm diameter). To maintain the integrity of the cells, the pressure did not exceed 100 mbar (Karner and Herndl, 1992).

To estimate the enzymatic activity, the protocols of Hoppe (1983); Salazar Alekseyeva et al. (2022), and Salazar-Alekseyeva et al. (2023) [in revision] were followed. The fluorogenic substrate analogues 4-methylumbelliferyl β -D-glucopyranoside (M3633 Sigma-Aldrich), 4-methylumbelliferyl β -D-xylopyranoside (M7008 Sigma-Aldrich), 4-methylumbelliferyl *N*-acetyl- β -D-glucosaminide (M2133 Sigma-Aldrich), 4-methylumbelliferyl-oleate (75164 Sigma-Aldrich), 4-methylumbelliferyl phosphate (M8883 Sigma-Aldrich), 4-methylumbelliferyl sulfate potassium salt (M7133 Sigma-Aldrich), *N*-succinyl-Ala-Ala-Pro-Phe-7-amido-4-methylcoumarin (L2145 Sigma-Aldrich), and *t*-butyloxycarbonyl-L-phenylalanyl-L-seryl-L-arginine-7-amido-4-methylcoumarin (3107-v PeptaNova) were used to determine the potential activity of the enzymes β -glucosidase (BGL), β -xylosidase (BXY), *N*-acetyl- β -D-glucosaminidase (NAG), lipase (OLE), alkaline phosphatase (APA), sulfatase (SUL), leucine aminopeptidase (LAP), and trypsin (TRY), respectively (Table 1). According to the targeted substrate, the enzymes were classified as cleaving carbohydrates (BGL, BXY, and NAG); lipids, phosphorus and sulfur moieties (OLE, APA, and SUL, respectively), and proteins (LAP and TRY). Consistently, methylcoumaryl amide (MCA) (A9891 Sig-ma-Aldrich) and methylumbelliferone (MUF) (M1381 Sigma-Aldrich) were used to normalize the emitted fluorescence by the potential activities mentioned. Both, substrates analogues and standards were dissolved in 2-methoxyethanol. Briefly, in sterile 96 well microplates with F bottom and low protein binding (XT64.1, Carl Roth), 300 μ L of only liquid culture was added as blank. Moreover, 15 μ L of the respective standard was added to 285 μ L of liquid culture. For MCA, the final concentrations were 100 μ M, 50 μ M, 10 μ M, and 1 μ M, and for MUF, 2000 μ M, 1000 μ M, 100 μ M, and 50 μ M. Finally, 30 μ L of the respective fluorogenic substrate was added to 270 μ L of liquid culture and serially diluted to obtain 12 final concentrations from 1000 to 0.5 μ M, except trypsin, from 500 to 0.2 μ M. The volume was completed with an additional 150 μ L of liquid culture. All these enzymatic assays were done with biological triplicates, The emitted fluorescence was measured hourly with FluoroLog[®] Horiba at an excitation wavelength of 365 nm and an emission wavelength

TABLE 1 Enzymes targeted with fluorogenic substrate analogues of three categories, and their respective fluorogenic standards (MUF methylumbelliferyl, and MCA methylcoumarylamide).

| Category | ID | Enzyme | Standard |
|--|-----|--------------------------------------|----------|
| Carbohydrates | BGL | β -glucosidase | MUF |
| | BXY | β -xylosidase | MUF |
| | NAG | N-acetyl- β -D-glucosaminidase | MUF |
| Lipids, phosphorus and sulfur moieties | OLE | Lipase | MUF |
| | APA | Alkaline phosphatase | MUF |
| | SUL | Sulfatase | MUF |
| Proteins | LAP | Leucine aminopeptidase | MCA |
| | TRY | Trypsin | MCA |

The abbreviations of the respective enzyme used throughout the text are given in the column ID.

of 445 nm for 3 hours (T_0 , T_1 , T_2 , and T_3). Between measurements, the microplates were incubated in the dark at 5°C.

To calculate the kinetic parameters such as maximum velocity (V_{max}) and half-saturation constant (K_m), the hydrolysis rates obtained from the change of fluorescence over time in the samples with the fluorogenic substrate, were fitted directly to the Michaelis-Menten equation using nonlinear least-squares regression analysis in RStudio (Huitema and Horsman, 2018). For the biomass-specific activity, the V_{max} was normalized by the dry weight [$\mu\text{mol/g biomass}\cdot\text{h}$], and for the cell-specific activity, it was normalized by the cell abundance [$\text{amol/cell}\cdot\text{h}$]. The V_{max} and K_m values provided in this study refer only to the cell-free fraction, as the total EEA values are from Salazar-Alekseyeva et al. (2023) [in revision]. The percentage of the cell-free fraction to the total EEA (cell-attached plus cell-free) was calculated by comparing the cell-free rates from this study to the total rates from the mentioned study of Salazar-Alekseyeva et al. (2023) [in revision], all of them normalized by the biomass. To evaluate the distribution of the obtained data, Shapiro-Wilk test was used. Additionally, the significance between fungal species of these kinetic parameters and percentages was analyzed with one-way Analysis of Variance (ANOVA). Finally, Tukey's Honestly Significant Difference (Tukey's HSD) and Student-T were performed to identify significance at species level. All statistical analysis were ran in RStudio.

3 Results

Remarkably, all the studied fungal species were capable to release cell-free enzymes to enzymatically hydrolyze carbohydrates (Figure 1), lipids, phosphorus and sulfur moieties (Figure 2), and proteins (Figure 3). Generally, the contribution of cell-free enzymes to the total EEA (V_{max}) varied among the fungal species, as well as between the non-saline and saline conditions (Figure 4 and Table 2),

similar to the K_m values (Table 3). The percentage of cell-free enzyme secretion represented up to 85.1% of the total EEA (Figure 5).

3.1 Cleavage of carbohydrates

3.1.1 β -glucosidase

In all the fungal species studied, the V_{max} of cell-free BGL was significantly higher in the non-saline than in the saline medium (t -test; $p < 0.001$; Figure 1.1), but there was no significant difference in the K_m (t -test; $p = 0.3$; Table 3). The highest V_{max} value was detected in *S. dacryoidea* under non-saline conditions (t -test; $p < 0.001$; $1.7 \pm 0.6 \mu\text{mol/g biomass}\cdot\text{h}$ and $87.9 \pm 29.0 \text{amol/cell}\cdot\text{h}$). The percentage of cell-free relative to the total BGL by *S. dacryoidea* only represented $1.0 \pm 0.4\%$ and $2.6 \pm 1.5\%$ in the non-saline and saline medium, respectively (Figure 5A). In contrast, in the two species of the genus *Rhodotorula*, *R. mucilaginosa* under non-saline conditions and *R. sphaerocarpa* under saline conditions, the BGL rates were low, but the proportion of cell-free fraction represented $78.3 \pm 14.3\%$, and $73.6 \pm 13.7\%$, respectively, of the total BGL. For the remaining species, the contribution of cell-free to the total BGL activities ranged between 16.0% and 52.1%.

3.1.2 β -xylosidase

Similar to BGL, in all the fungal strains, the V_{max} of cell-free BXY was significantly higher under non-saline than under saline conditions (t -test; $p = 0.005$; Figure 1.2), but no significant difference was detected in the K_m (t -test; $p = 0.5$; Table 3). In the non-saline medium, the highest V_{max} values were detected for *R. sphaerocarpa* and *S. dacryoidea* (t -test; $p = 0.001$), with values of $0.3 \pm 0.2 \mu\text{mol/g biomass}\cdot\text{h}$ and $12.4 \pm 6.7 \text{amol/cell}\cdot\text{h}$, and $0.2 \pm 0.2 \mu\text{mol/g biomass}\cdot\text{h}$ and $11.7 \pm 8.5 \text{amol/cell}\cdot\text{h}$, respectively. Consistent with what was observed for BGL, even though *S. dacryoidea* exhibited one of the highest V_{max} , the contribution of the cell-free fraction to the total EEA was low with percentages of $2.4 \pm 1.9\%$ and $2.2 \pm 0.8\%$ under non-saline and saline conditions, respectively (Figure 5B). For the remaining fungal strains, the contribution of cell-free to the total BXY varied between 10.4 and 51.8%.

3.1.3 N-acetyl- β -D-glucosaminidase

Similar to BGL and BXY, the V_{max} of cell-free NAG was significantly higher in the non-saline than in the saline medium for all the species studied (t -test; $p < 0.001$; Figure 1.3). However, as shown in Table 3, there was no significant difference in the K_m for all the species (t -test; $p = 0.3$), except for *B. parvus*, where it was significantly higher under saline conditions (t -test; $p = 0.02$; $631.5 \pm 141.5 \mu\text{M}$). When the V_{max} was normalized by the biomass, *B. parvus* had the highest V_{max} under non-saline conditions (t -test; $p < 0.001$; $0.6 \pm 0.1 \mu\text{mol/g biomass}\cdot\text{h}$). But when the V_{max} was normalized by the cell abundance, *R. sphaerocarpa* exhibited the highest V_{max} also in this medium (t -test; $p = 0.003$; $12.7 \pm 3.8 \text{amol/cell}\cdot\text{h}$). The contribution of the cell-free fraction to the total NAG varied from 12.5 to 68.8% (Figure 5C).

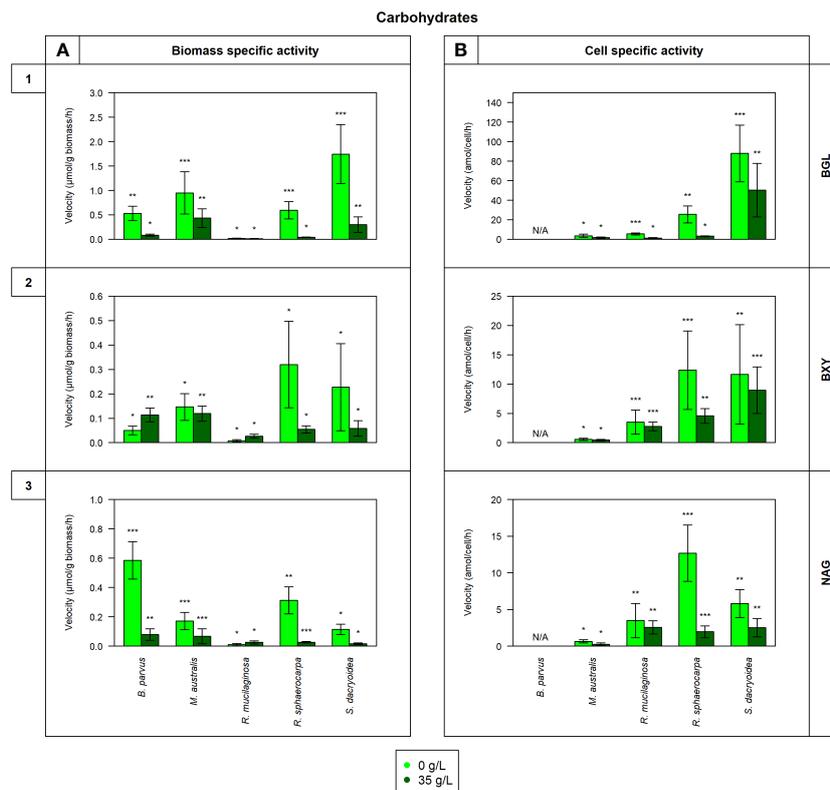


FIGURE 1

Maximum velocity (V_{max}) of the cell-free enzymatic activity obtained from the filtrate of biological triplicates of the fungal strains *B. parvus*, *M. australis*, *R. mucilaginosa*, *R. sphaerocarpa*, and *S. dacryoidea* grown under non-saline (light green) and saline (dark green) conditions, and normalized by the (A) dry weight in $\mu\text{mol/g biomass}\cdot\text{h}$ and by the (B) cell abundance in $\text{amol/cell}\cdot\text{h}$. For *B. parvus*, B was not possible to calculate, so it is represented by "N/A". The substrates hydrolyzed denoted the use of carbohydrates by (1) β -glucosidase (BGL), (2) β -xylosidase (BXY), and (3) *N*-acetyl- β -D-glucosaminidase (NAG). Moreover, Tukey's HSD was calculated by salinity where * represents $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$.

3.2 Cleavage of lipids, phosphorus and sulfur moieties

3.2.1 Lipase

The V_{max} values were significantly higher in the saline than in the non-saline medium for all the fungal strains (*t*-test; $p = 0.05$; Figure 2.1). The K_m , however, was not significantly different between both media (*t*-test; $p = 0.4$; Table 3). *S. dacryoidea* exhibited significantly higher V_{max} than the other species (*t*-test; $p < 0.001$), with values of $32.1 \pm 6.5 \mu\text{mol/g biomass}\cdot\text{h}$ and $1676.5 \pm 333.4 \text{ amol/cell}\cdot\text{h}$ under non-saline conditions, and $54.2 \pm 30.3 \mu\text{mol/g biomass}\cdot\text{h}$ and $8319.8 \pm 3229.3 \text{ amol/cell}\cdot\text{h}$ under saline conditions. The contribution of cell-free to the total OLE oscillated from 8.6 to 85.1% where the highest percentages corresponded to species with generally low OLE activity, such as *M. australis*, *R. mucilaginosa*, and *R. sphaerocarpa* (Figure 5D).

3.2.2 Alkaline phosphatase

The V_{max} of cell-free APA was significantly higher under non-saline than under saline conditions for all the fungal strains (*t*-test; $p < 0.001$; Figure 2.2), but no significant difference was detected in the K_m (*t*-test; $p = 0.6$; Table 3). Normalizing APA activity to the biomass revealed a significantly higher V_{max} in *M. australis* under both, non-saline and saline conditions (*t*-test; $p < 0.001$; 10.1 ± 5.5

$\mu\text{mol/g biomass}\cdot\text{h}$ and $5.9 \pm 1.1 \mu\text{mol/g biomass}\cdot\text{h}$, respectively). Normalizing the V_{max} to the cell abundance exposed *S. dacryoidea* in the saline medium as the species with the highest V_{max} (*t*-test; $p < 0.001$; $132.5 \pm 21.1 \text{ amol/cell}\cdot\text{h}$). The contribution of cell-free to the total APA varied between 16.9 and 41.7% (Figure 5E).

3.2.3 Sulfatase

All the marine fungal strains expressed cell-free SUL activity with no significant difference between the V_{max} in the non-saline and saline medium (*t*-test; $p = 0.5$; Figure 2.3). The highest SUL activity detected among all the fungal strains was in *S. dacryoidea* under saline conditions (*t*-test; $p = 0.04$; $0.4 \pm 0.2 \mu\text{mol/g biomass}\cdot\text{h}$ and $64.6 \pm 28.6 \text{ amol/cell}\cdot\text{h}$). Contrary, in the case of the K_m , these values were significantly higher under saline than under non-saline conditions for all the species (*t*-test; $p < 0.001$; Table 3). The proportion of the cell-free fraction of SUL to the total SUL activity varied between 18.5 and 82.3% (Figure 5F).

3.3 Cleavage of proteins and peptides

3.3.1 Leucine aminopeptidase

There was no significant difference in the V_{max} nor the K_m of cell-free LAP between the non-saline and saline medium for none of the

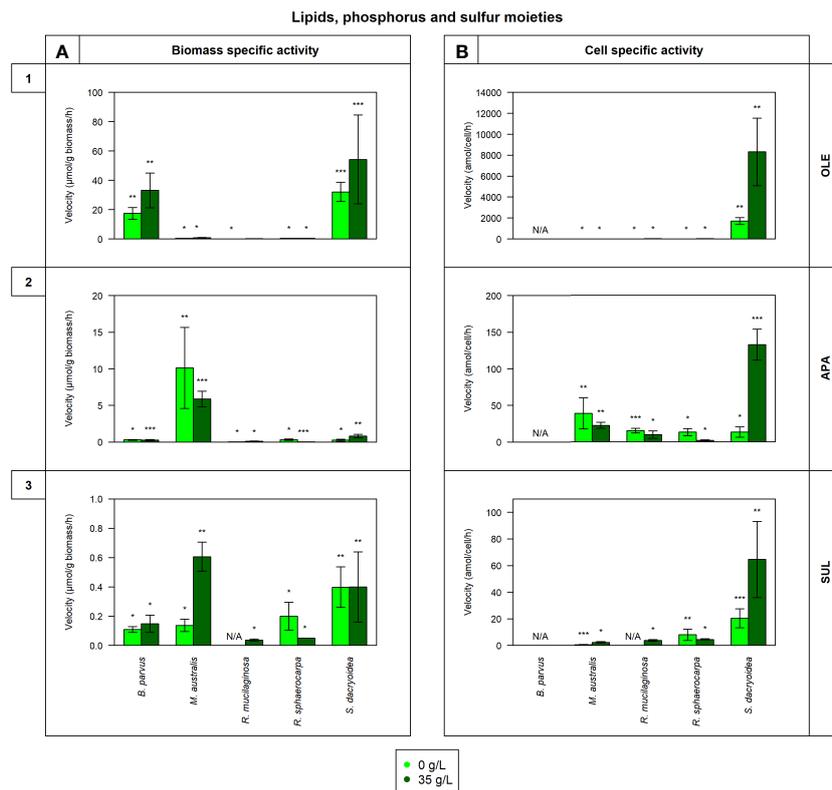


FIGURE 2 Maximum velocity (V_{max}) of the cell-free enzymatic activity obtained from the filtrate of biological triplicates of the fungal strains *B. parvus*, *M. australis*, *R. mucilaginosa*, *R. sphaerocarpa*, and *S. dactyloidea* grown under non-saline (light green) and saline (dark green) conditions, and normalized by the (A) dry weight in $\mu\text{mol/g biomass}\cdot\text{h}$ and by the (B) cell abundance in $\text{amol/cell}\cdot\text{h}$. For *B. parvus*, B was not possible to calculate, so it is represented by "N/A". The substrates hydrolyzed denoted the use of lipids, phosphorus and sulfur moieties by (1) lipase (OLE), (2) alkaline phosphatase (APA), and (3) sulfatase (SUL), respectively. *R. mucilaginosa* did not exhibit any SUL activity under non-saline conditions, so it is also represented by "N/A". Moreover, Tukey's HSD was calculated by salinity where * represents $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$.

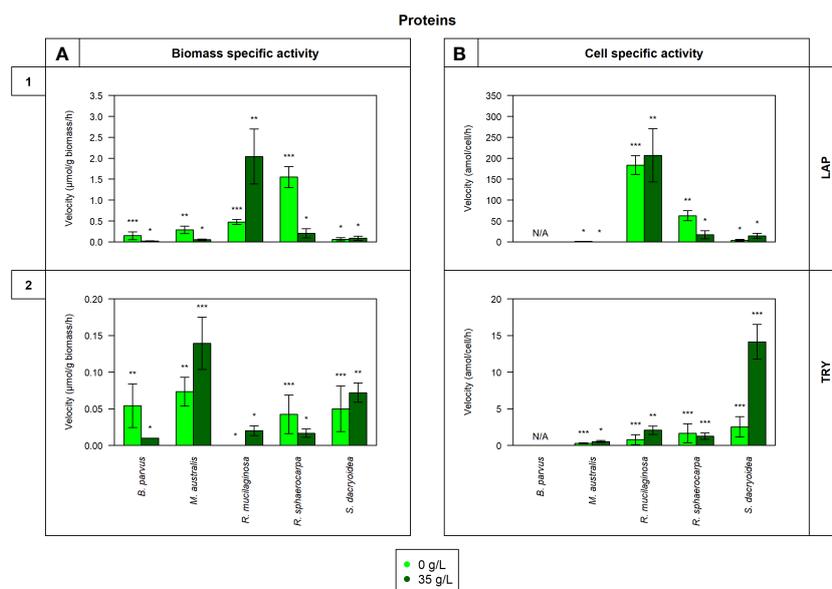


FIGURE 3 Maximum velocity (V_{max}) of the cell-free enzymatic activity obtained from the filtrate of biological triplicates of the fungal strains *B. parvus*, *M. australis*, *R. mucilaginosa*, *R. sphaerocarpa*, and *S. dactyloidea* grown under non-saline (light green) and saline (dark green) conditions and normalized by the (A) dry weight in $\mu\text{mol/g biomass}\cdot\text{h}$ and by the (B) cell abundance in $\text{amol/cell}\cdot\text{h}$. For *B. parvus*, B was not possible to calculate, so it is represented by "N/A". The substrates hydrolyzed denoted the use of proteins by (1) leucine aminopeptidase (LAP), and (2) trypsin (TRY). Moreover, Tukey's HSD was calculated by salinity where * represents $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$.

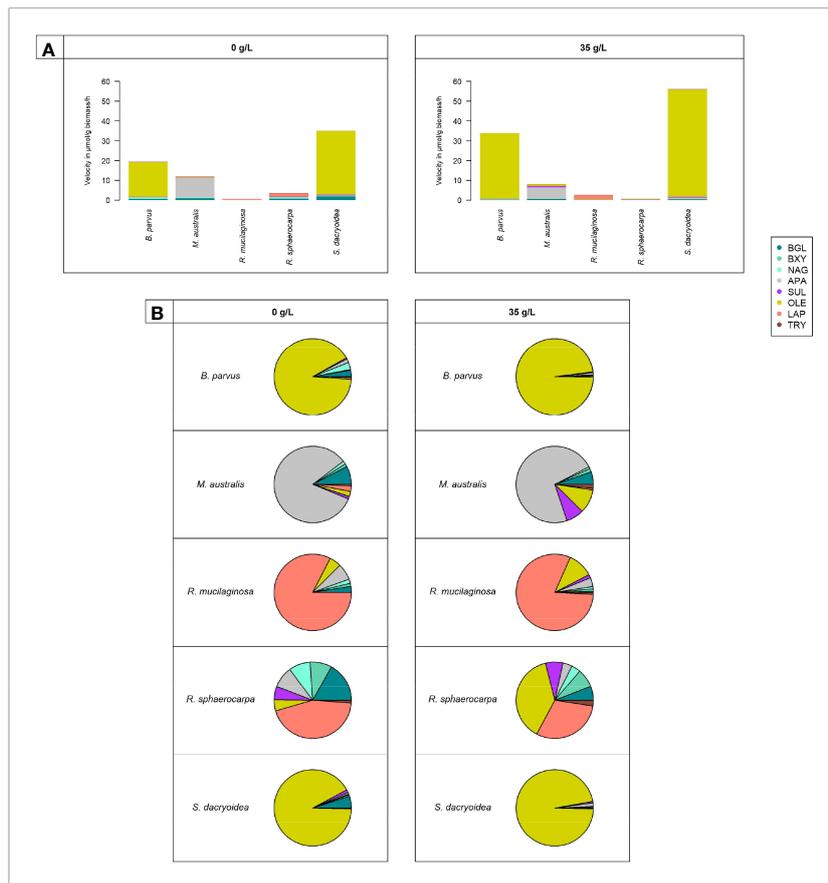


FIGURE 4 Cell-free enzymatic activities by fungal species: *B. parvus*, *M. australis*, *R. mucilaginosa*, *R. sphaerocarpa*, and *S. dacryoides* for β -glucosidase (BGL), β -xylosidase (BXY), *N*-acetyl- β -D-glucosaminidase (NAG), lipase (OLE), alkaline phosphatase (APA), sulfatase (SUL), leucine aminopeptidase (LAP), and trypsin (TRY), under non-saline (0 g/L), and saline (35 g/L) conditions. **(A)** Sum of all cell-free V_{max} in $\mu\text{mol/g biomass}\cdot\text{h}$ and **(B)** Percentage of all cell-free V_{max} in %.

fungal species (*t*-test; $p=0.4$; Figure 3.1 and *t*-test; $p=0.9$; Table 3, respectively). The highest LAP activity detected among all the fungal strains was in *R. mucilaginosa* under saline conditions (*t*-test; $p < 0.001$; $2.0 \pm 0.7 \mu\text{mol/g biomass}\cdot\text{h}$ and $206.8 \pm 63.6 \text{ amol/cell}\cdot\text{h}$). The

highest contribution of cell-free LAP to the total LAP activity was also detected in *R. mucilaginosa* ($20.6 \pm 6.3\%$). The other fungal species exhibited generally a low contribution of cell-free LAP activity to the total LAP activity ranging from 0.1 to 10.9% (Figure 5G).

TABLE 2 Main location ($\geq 50\%$), cell-attached (CA) or cell-free (CF), of the enzymes degrading carbohydrates by β -glucosidase (BGL), β -xylosidase (BXY), and *N*-acetyl- β -D-glucosaminidase (NAG); lipids, phosphorus and sulfur moieties by lipase (OLE), alkaline phosphatase (APA), and sulfatase (SUL), respectively; and proteins by leucine aminopeptidase (LAP) and trypsin (TRY).

| Enzyme | Carbohydrates | | | | | | Lipids, phosphorus and sulfur moieties | | | | | | Proteins | | | | |
|------------------------|---------------|----|-----|----|-----|----|--|----|-----|----|-----|----|----------|----|-----|----|----|
| | BGL | | BXY | | NAG | | APA | | SUL | | OLE | | LAP | | TRY | | |
| Condition | N | S | N | S | N | S | N | S | N | S | N | S | N | S | N | S | |
| <i>B. parvus</i> | CA | CA | CA | CA | CA | CA | CA | CA | CA | CA | CA | CA | CA | CA | CA | CA | CF |
| <i>M. australis</i> | CA | CA | CA | CA | CF | CA | CA | CA | CA | CF | CF | CF | CA | CA | CA | CA | CA |
| <i>R. mucilaginosa</i> | CF | CA | CF | CA | CA | CA | CA | CA | N/A | CF | CF | CF | CA | CA | CA | CA | CA |
| <i>R. sphaerocarpa</i> | CF | CF | CA | CF | CA | CF | CA | CA | CA | CF | CA | CF | CA | CA | CA | CA | CA |
| <i>S. dacryoides</i> | CA | CA | CA | CA | CF | CA | CA | CA | CA | CA | CA | CF | CA | CA | CA | CA | CA |

These were investigated in five marine fungal isolates *B. parvus*, *M. australis*, *R. mucilaginosa*, *R. sphaerocarpa*, and *S. dacryoides* grown under non-saline (N) and saline (S) conditions. *R. mucilaginosa* did not exhibit any SUL activity under non-saline conditions, so it is indicated below by "N/A".

TABLE 3 Average and standard deviation of K_m in μM calculated from the cell-free enzymatic activity fraction of biological triplicates of five marine fungal isolates *B. parvus*, *M. australis*, *R. mucilaginosa*, *R. sphaerocarpa*, and *S. dacryoidea* grown under non-saline (N) and saline (S) conditions.

| Species | | <i>B. parvus</i> | | <i>M. australis</i> | | <i>R. mucilaginosa</i> | | <i>R. sphaerocarpa</i> | | <i>S. dacryoidea</i> | |
|--|-----|------------------|---------------|---------------------|---------------|------------------------|---------------|------------------------|--------------|----------------------|---------------|
| Condition | | N | S | N | S | N | S | N | S | N | S |
| Carbohydrates | BGL | 288.7 ± 111.1 | 472.6 ± 171.1 | 466.8 ± 59.3 | 366.7 ± 98.7 | 363.3 ± 215.8 | 8.3 ± 4.1 | 26.6 ± 4.8 | 12.1 ± 5.3 | 635.9 ± 81.3 | 398.1 ± 81.5 |
| | BXY | 40.2 ± 24.0 | 13.7 ± 8.7 | 10.5 ± 3.3 | 6.7 ± 2.6 | 11.4 ± 2.9 | 16.7 ± 11.2 | 6.7 ± 1.6 | 8.7 ± 3.7 | 16.2 ± 6.4 | 25.0 ± 14.8 |
| | NAG | 100.9 ± 27.1 | 648.6 ± 120.5 | 39.4 ± 7.8 | 27.4 ± 7.4 | 214.0 ± 57.9 | 294.0 ± 127.9 | 0.8 ± 0.2 | 6.6 ± 1.8 | 39.8 ± 18.9 | 13.8 ± 4.4 |
| Lipids, phosphorus and sulfur moieties | APA | 24.4 ± 8.6 | 300.9 ± 161.4 | 117.8 ± 37.8 | 47.5 ± 7.8 | 379.3 ± 173.9 | 115.9 ± 64.0 | 27.7 ± 13.3 | 1.3 ± 0.4 | 4.3 ± 2.0 | 9.2 ± 3.7 |
| | SUL | 32.4 ± 15.2 | 384.7 ± 146.8 | 121.5 ± 65.1 | 413.7 ± 200.8 | N/A | 566.0 ± 189.3 | 1.1 ± 0.3 | 1.5 ± 0.6 | 345.4 ± 135.5 | 572.9 ± 171.8 |
| | OLE | 60.9 ± 16.1 | 59.8 ± 21.6 | 404.1 ± 203.3 | 161.7 ± 63.1 | 430.4 ± 165.6 | 602.2 ± 222.8 | 3.3 ± 1.4 | 146.1 ± 32.7 | 15.8 ± 4.2 | 61.2 ± 18.9 |
| Proteins | LAP | 316.1 ± 154.8 | 66.2 ± 24.8 | 48.0 ± 14.4 | 109.4 ± 3.3 | 74.1 ± 12.6 | 108.0 ± 44.2 | 133.2 ± 19.0 | 109.8 ± 26.2 | 347.5 ± 146.3 | 462.7 ± 140.1 |
| | TRY | 136.1 ± 79.8 | 3.1 ± 1.9 | 10.7 ± 5.0 | 58.3 ± 6.4 | 12.2 ± 2.9 | 13.2 ± 7.3 | 15.6 ± 1.9 | 3.2 ± 0.4 | 333.6 ± 119.7 | 83.1 ± 43.9 |

The substrates used were indicative of cleavage of carbohydrates by β -glucosidase (BGL), β -xylosidase (BXY), and *N*-acetyl- β -D-glucosaminidase (NAG); lipids, phosphorus and sulfur moieties by lipase (OLE), alkaline phosphatase (APA), and sulfatase (SUL), respectively; and proteins by leucine aminopeptidase (LAP), and trypsin (TRY). *R. mucilaginosa* did not exhibit any SUL activity under non-saline conditions, so it is indicated below by "N/A".

3.3.2 Trypsin

There was no significant difference between the V_{\max} obtained in the non-saline and the saline medium (*t*-test; $p = 0.08$; Figure 3.2). However, as shown in Table 3, *M. australis*, *R. mucilaginosa*, and *R. sphaerocarpa* exhibited a significantly higher K_m in the saline than in the non-saline medium (*t*-test; $p = 0.04$). The contrary occurred for *B. parvus* and *S. dacryoidea* (*t*-test; $p = 0.003$). Similar to APA, TRY activity normalized by the biomass resulted in a significantly higher V_{\max} for *M. australis* in both media than for the remaining species (*t*-test; $p < 0.001$). However, normalizing the V_{\max} to cell abundance revealed that *S. dacryoidea* in the saline medium exhibited a higher V_{\max} than the other fungal species (*t*-test; $p < 0.001$; 14.1 ± 2.4 amol/cell*h). Interestingly, TRY was the only enzyme studied where a significant difference in the contribution of cell-free TRY activity to the total TRY activity was detectable between the non-saline and saline medium (Figure 5H). The contribution of the cell-free fraction to the total TRY activity was significantly higher under saline with 16.5 to 84.9% than under non-saline conditions with 2.5 to 10.4% (*t*-test; $p < 0.001$).

3.4 Cleavage of proteins and peptides

Compared to the other EEAs tested, the contribution of cell-free OLE to the cell-free enzymatic pool was significantly higher (*t*-test; $p < 0.001$; Figure 4). Cell-free OLE exhibited the highest V_{\max} values, and these were mainly found in *S. dacryoidea* and *B. parvus*. Contrarily, the contribution of cell-free TRY to the cell-free

enzymatic pool was significantly lower than the other EEAs (*t*-test; $p < 0.001$; Figure 4).

4 Discussion

4.1 Release of cell-free enzymes by pelagic fungal strains

It is remarkable that all the species studied of marine pelagic fungi, *B. parvus*, *M. australis*, *R. mucilaginosa*, *R. sphaerocarpa*, and *S. dacryoidea*, released cell-free enzymes. These species seem to be versatile as they exhibited a spectrum of cell-free enzymes capable of hydrolytically cleaving carbohydrates, proteins, lipids, and moieties of phosphorus and sulfur at a distance from the cell that originally produced them. Nonetheless, species and enzymes were affected differently by salinity changes.

4.1.1 Release of cell-free enzymes cleaving carbohydrates

Cellulose is a polymeric substrate that cannot be transported across the fungal cell wall, requiring at least partial extracellular hydrolysis (MacDonald and Speedie, 1982). The release of cell-free β -glucosidase by fungi has been reported in terrestrial species like *Sporotrichum thermophile* (Bhat et al., 1993; Gaikwad and Maheshwari, 1994) and marine species associated with decaying estuarine and marine plants (MacDonald and Speedie, 1982), driftwood (MacDonald and Speedie, 1982), mangroves (Pointing

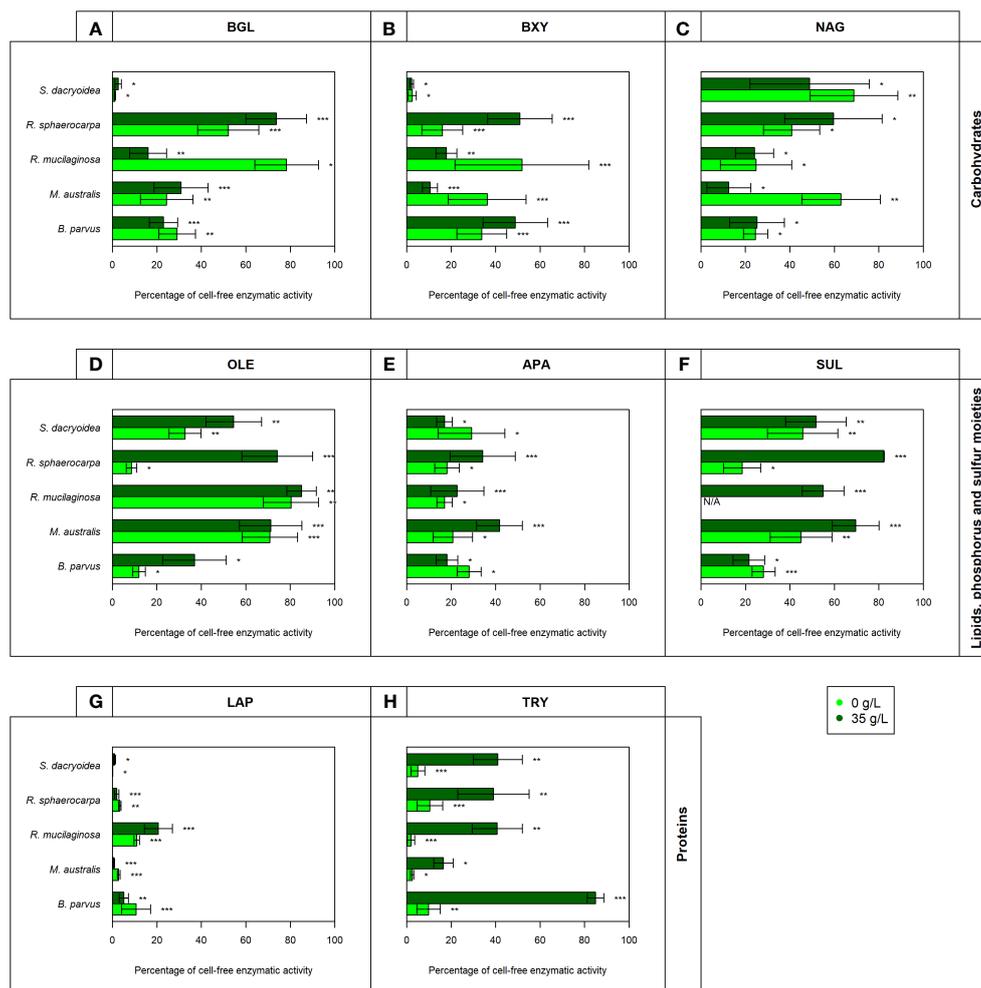


FIGURE 5

Contribution of cell-free enzymatic activity as a percentage to the total extracellular enzymatic activity normalized by the biomass for the enzymes (A) β -glucosidase (BGL), (B) β -xylosidase (BXY), (C) *N*-acetyl- β -D-glucosaminidase (NAG), (D) lipase (OLE), (E) alkaline phosphatase (APA), (F) sulfatase (SUL), (G) leucine aminopeptidase (LAP), and (H) trypsin (TRY) of biological triplicates of the fungal strains *B. parvus*, *M. australis*, *R. mucilaginosa*, *R. sphaerocarpa*, and *S. dacryoidea*. The species *R. mucilaginosa* did not exhibit any SUL activity under non-saline conditions, so it is represented by "N/A". According to the salinity, non-saline (light green) and saline (dark green), Tukey's HSD was performed and * represents $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$.

et al., 1999), sediments (Elyas et al., 2010), and macroalgae (Lee et al., 2019). In the present study, the level of secretion of cell-free β -glucosidase differed among the species studied (Table 2). Compared with the rest of the fungal species, *S. dacryoidea* was the species that exhibited the highest V_{max} of cell-free (Figure 1.1), but the lowest cell-free contribution to the total β -glucosidase activity has also been reported for species like *Trichocladium achrasporum* (MacDonald et al., 1985). For the other fungal species tested except the ones of the genus *Rhodotorula*, *R. mucilaginosa* and *R. sphaerocarpa*, the cell-free fraction represented up to 30.8% of the total EEA suggesting that the β -glucosidase activity is mainly cell-associated (Table 2 and Figure 5A). This is consistent with previous reports on other marine fungi species (MacDonald and Speedie, 1982; MacDonald et al., 1985; Pointing et al., 1999).

Like cellulose, xylan also cannot penetrate the cell due to its polymeric structure (Biely, 1985; Lenartovicz et al., 2003; Collins

et al., 2005), so β -xylosidases can be cell-attached or cell-free (Lenartovicz et al., 2003). Reese et al. (1973) reported that in the early growth stages of fungal cultures, xylosidases were cell-attached, but later on, these enzymes were released into the medium either by true secretion or cell lysis. Interestingly, similar to BGL, although *S. dacryoidea* displayed one of the highest V_{max} values (Figure 1.2), cell-free β -xylosidase was low compared to the total EEA (Figure 5B). Hence, we conclude that β -xylosidases released by the studied marine fungi species were also mostly cell-attached (Table 2). This was also found for some widespread fungi species like *Cryptococcus albidus* (Defaye et al., 1992), *Aspergillus fumigatus* (Lenartovicz et al., 2003), *Thermomyces lanuginosus* (Singh et al., 2003), *Aureobasidium pullulans* (Ohta et al., 2010), and some other species (Reese et al., 1973).

In the study of Matsumoto et al. (2004), *Verticillium lecanii*, originally isolated from *Lecanium corni*, produced extracellular *N*-acetyl- β -D-glucosaminidase from shrimp waste. Fungi can degrade

chitin and use it as a carbon and nitrogen source (Gaderer et al., 2017), but in contrast to bacteria, fungi can also use it as a building block for the synthesis of new chitin (Edson and Brody, 1976). Thus, the number of chitinases produced by fungi has been related to their chitin content and growth mode (Hartl et al., 2012). For instance, as the cell wall of filamentous fungi consists of 10 to 20% of chitin (Ruiz-Herrera, 1991), the number of chitinases is normally 10 to 30 (Seidl, 2008; Kubicek et al., 2011). The cell wall of yeast fungi consists of only 0.5 to 5% of chitin (Garcia-Rubio et al., 2020), so the number of chitinases might be lower in yeast than in filamentous fungi. Even though the overall *N*-acetyl- β -D-glucosaminidase activity was low, compared with other species, *B. parvus*, a filamentous species, exhibited the highest V_{\max} of cell-free (Figure 1.3) similar to what was reported by Salazar-Alekseyeva et al. (2022) and Salazar-Alekseyeva et al. (2023) [in revision]. As shown in Table 2, the main location of *N*-acetyl- β -D-glucosaminidase might be cell-attached.

Marine microorganisms require diverse substrates which are normally polymeric (Wang et al., 2016). In these environments, carbohydrates are the largest macromolecular compound class of DOC (Benner et al., 1992). According to Biely (1985), microorganisms that compete for carbon sources, secrete enzymes that are mainly cell-attached. As mentioned above, cellulose, chitin, and xylan are polymeric structures, so for these carbohydrates, fungal cell-attached enzymes might dominate the hydrolytic cleavage of this abundant macromolecular compound class.

4.1.2 Release of cell-free enzymes cleaving lipids, phosphorus and sulfur moieties

Lipids are high energy sources (Parrish, 2013), and also building blocks for organisms (Bergé and Barnathan, 2005), so the degradation of lipids like phospholipids might be a means to obtain both, carbon and phosphorus (Celussi and Del Negro, 2012). According to Singh and Mukhopadhyay (2012) and Duarte et al. (2021), the majority of lipases in fungi were released into the extracellular medium. In the studies of Papanikolaou et al. (2007) and Louhasakul et al. (2016), the marine yeast *Yarrowia lipolytica*, produced only cell-attached lipase, but in the study of Scioli and Vollaro (1997) this same species produced both, cell-attached and cell-free OLE. In another study, other 9 marine yeast strains, including one used in our study, *R. mucilaginosa*, produced cell-attached lipase, and only *Aureobasidium pullulans* produced cell-free lipase (Wang et al., 2007). Table 2 and Figure 5D indicate that the lipase activity of *M. australis* and *R. mucilaginosa* was mainly cell-free, whereas for the species *R. sphaerocarpa*, and *S. dacryoidea* the cell-free fraction depended mainly on the salinity, where higher percentages of cell-free enzymatic activity were found under saline conditions. *B. parvus* was the only species where the majority of lipase activity was cell-attached. Hence, we suggest that the release of cell-free lipase might be species-specific and dependent on the salinity.

Phosphorus is an essential element required for many biological processes (Colman et al., 2005; Paytan and McLaughlin, 2007;

Brembu et al., 2017; Lockwood et al., 2022). In aquatic environments, low phosphate concentrations have been related to the expression of alkaline phosphatase (Hassan and Pratt, 1977; Colman et al., 2005; Srivastava et al., 2021). However, in carbon-limited environments, microorganisms might use APA not only to obtain phosphate, but also to access the carbon moieties from organic matter (Hoppe and Ullrich, 1999; Colman et al., 2005). In bacteria, APA is generally located in the periplasmic space, whereas in fungi, it is generally attached to the cell surface (Chrost et al., 1984). In soil studies, six fungal species, one of them a widespread fungus as it is *Aspergillus niger*, released approximately 22% of extracellular alkaline phosphatase (Tarafdar et al., 2002). These authors suggested that this low release was related to the fungal structure, and a low membrane permeability for this enzyme (Tarafdar et al., 2002). Our study on marine fungi also suggests that APA activity is mostly cell-attached (Table 2), as the activities of the cell-free APA fraction amounted to at most 41.7% of the total APA activity (Figure 5E).

Sulfur is also an essential element required for numerous biological molecules (Klotz et al., 2011; Helbert, 2017). In marine environments, polysaccharides can be highly sulfated (Kloareg and Quatrano, 1988; Wang et al., 2016; Helbert, 2017), probably as a physiological adaptation to the high environmental ionic strength (Kloareg and Quatrano, 1988; Aquino et al., 2005; Aquino et al., 2011; Ciancia et al., 2020). In these environments, the cleavage of sulfate groups might be necessary not only to obtain sulfur, but also to access the carbohydrates (Schultz-Johansen et al., 2018; Hettler et al., 2022). For the widespread fungi species *Neurospora crassa*, cell-attached, and cell-free sulfatase were reported by Scott and Metzner (1970). We also found these two types of extracellular enzymes in our studied marine fungi species. Interestingly, all the species, except *B. parvus*, released a higher percentage of cell-free enzymes in the saline than in the non-saline medium (Table 2 and Figure 5F). Therefore, fungi might be capable to secrete cell-free sulfatase as a strategy to remove sulfates and gain access to other carbohydrates (Salazar-Alekseyeva et al. 2023) [in revision].

4.1.3 Release of cell-free enzymes cleaving proteins and peptides

Grazing and viral lysis are the main sources of proteins and peptides released into the seawater (Repeta, 2015). Proteases hydrolytically cleave them to obtain both, carbon and nitrogen (Li et al., 2019), and this can take place inside or outside the cell (Pantoja et al., 1997; Gupta et al., 2002). Nitrogen is an essential element, especially for the growth and function of enzyme-dependent microorganisms (Allison, 2005). Hydrolysis rates are mainly influenced by the size and chemical structure of the substrate, where peptides are hydrolyzed much faster than proteins (Pantoja and Lee, 1999). Bacterial leucine aminopeptidase has been widely reported to be mainly cell-attached in seawater (Pantoja et al., 1997; Obayashi and Suzuki, 2008b; Bong et al., 2013), as well as in freshwater (Chróst and Rai, 1993; Millar et al., 2015). Remarkably, in all the marine fungal strains studied, this was the only enzyme where the majority of enzymatic activity was cell-attached (up to 79.4%) in both non-saline and saline medium (Table 2 and

Figure 5G). *S. dacroioidea*, however, exhibited the lowest contribution of cell-free to the total LAP activity (Figure 5G). Trypsin also exhibited a high cell-attached enzymatic activity (up to 89.6%), but only under non-saline conditions (Figure 5H).

4.1.4 Influence of salinity on cell-free enzymes in marine fungi

Diverse environmental factors can influence microbial cells, and also the subsequent secretion of enzymes (Chróst, 1990). In the case of cell-free enzymes, as these are released from the cell into the ambient water, their fate will depend on the conditions of the water (Hoppe and Ullrich, 1999; Baltar, 2018). Therefore, cell-free enzymes might be susceptible to degradation and chemical changes (MacDonald and Speedie, 1982; Wetzel, 1991).

Enzymes are strongly associated with water as it tends to bind to the hydrophobic groups located on the enzyme surface (Saenger, 1987; Kornblatt and Kornblatt, 2002; Zaccai, 2004; Rezaei et al., 2007). This allows the enzyme to maintain its native structure, and hence, it can function properly (Kuntz Jr, 1971; Saenger, 1987; Karan et al., 2012). As salts promote ionic and hydrophobic effects, salinity is considered an important environmental factor that can influence the solubility and stability of enzymes (Baxter, 1959; Lanyi, 1974; Karan et al., 2012) and thus, their functions (King, 1986; Caruso and Zaccone, 2000). Though, the magnitude of these effects will depend on the salt concentration as well as the chemical composition of the enzyme (Sinha and Khare, 2014). Some marine studies have reported salinity as an important environmental factor influencing microbial enzymatic activity (Caruso and Zaccone, 2000; Salazar-Alekseyeva et al. 2023) [in revision]. However, the influence of salinity on fungal cell-free enzymatic activities has not been reported yet. Based on our results, we suggest that the salinity effect on the kinetic parameters such as maximum velocity (V_{max}) depends on the enzyme as well as on the species (Figures 1–5). Under saline conditions simulating marine environments, we found that the V_{max} of the enzymes BGL, BXY, NAG, and APA was reduced, with only one exception, the APA of *S. dacroioidea*. This reduction was different for each species, similar to what was reported for the total EEA by Salazar-Alekseyeva et al. (2023) [in revision].

Certain enzymes can be salt-tolerant or halophilic (Larsen, 1967). Here, amino acid residues located on the enzyme surface tend to bind to hydrated cations (Lanyi, 1974; Jin et al., 2019). This creates a large multilayered shell that keeps the enzyme hydrated (Karan et al., 2012), and also allows it to adopt a flexible conformational structure (Baxter, 1959; Hutcheon et al., 2005; Sinha and Khare, 2014; Karan et al., 2020). In the present study, under saline conditions, we detected potentially halophilic cell-free enzymes which were APA, SUL, and TRY for *S. dacroioidea*, SUL for *R. mucilaginoso*, and OLE for all the species (Figures 2, 3). *S. dacroioidea* is a widespread species (Fell et al., 1973; Gadanho et al., 2003; Allen et al., 2004; Francis et al., 2016), that has been identified as a potentially facultative marine species adapted to varying salinities Salazar-Alekseyeva et al. (2023) [in revision]. As the available nutritional sources are found in high concentrations of

osmolytes, fungi probably evolved enzymes to successfully compete for their uptake with other microorganisms (Gladfelder et al., 2019; Gonçalves et al., 2021). Curiously, cell-free lipase was the only enzyme where the V_{max} of all the studied fungal species was enhanced under saline conditions (Figure 2.1). Comparable results were supported by Kiran et al. (2009) showing that the production of lipase and other biosurfactants by the marine fungi *Aspergillus ustus* isolated from a symbiosis with a marine sponge, was higher under saline conditions. In the lipases of a pathogenic species, *Candida rugosa*, structural differences in the flap, substrate-binding pocket, and mouth of the hydrophobic tunnel, were responsible for three isoenzymes with different substrate specificity and catalytic properties (Mancheño et al., 2003). Moreover, the species *Yarrowia lipolytica* was reported to encode lipase isoenzymes with different substrate specificities (Fickers et al., 2005). On the other hand, interestingly, trypsin was the only enzyme that exhibited a higher percentage of cell-free activity under saline than under non-saline conditions (Figure 5H). In coastal seawater, cell-free trypsin has been reported to be between 40 to 80% of the total trypsin activity (Obayashi and Suzuki, 2008b). As a result, the release of cell-free enzymes into the surrounding water might be an advantage in marine environments allowing fungi to access substrates away from them.

In the open ocean, microbial cell-free enzymatic activities have been reported to be lower than in nearshore waters (Li et al., 2019). The reason for this might be the input of terrestrial substrates (Allison et al., 2012; Millar et al., 2015) and other organisms that stimulate the enzymatic activities in coastal waters (Li et al., 2019). For marine aggregates, Ziervogel and Arnosti (2008) stated that salinity or other chemical and physical factors can affect the lifetime of cell-free enzymes, as longer active lifetimes were found in nearshore waters than in offshore waters of the Gulf of Mexico. Our results indicate that salinity can influence the kinetics of cell-free enzymes, but marine fungi might be capable to produce different enzymes adapted to different salinities. For instance, in a study of fungi inhabiting mangrove forests, one fungal species was able to secrete two enzymes under saline conditions, and a different one in the absence of salt (Li et al., 2002). As proposed by Zaccai (2004), each enzyme might evolve a stable form specific to an environment, so isoenzyme expression might be a strategy to adapt to different salinities (Arfi et al., 2013; Salazar-Alekseyeva et al. 2023) [in revision]. From our study, it appears that the marine fungi species are adapted to a wide range of salinities, probably with different enzymes, also known as isoenzymes, capable to perform the same reaction under different salinities.

4.2 Location of marine fungi enzymes

The location of extracellular enzymes, either cell-attached or cell-free, influences the degradation and subsequent utilization of the substrates (Parawira et al., 2005), and this might depend on the lifestyle (Traving et al., 2015). For particle-associated microorganisms, cell-free enzymes could be favourable due to

their proximity to the substrates (Vetter et al., 1998). Contrarily, for free-living microorganisms, cell-attached enzymes could be beneficial (Chróst, 1990; Traving et al., 2015) as the substrates in marine environments are generally highly diluted (McCarthy et al., 1996; Amon and Benner, 2003). For fungi, the location might be specially important (Dubovenko et al., 2010). In the case of marine fungi, a high secretion of CAZymes was associated with a preferential particle-associated lifestyle (Baltar et al., 2021). In the present study, we found that the majority of EEAs were cell-attached (Table 2), which suggests a free-living lifestyle. However, salinity might have influenced the switch to cell-free. For example, under non-saline conditions, the release of SUL and OLE was mainly cell-attached, but under saline conditions, the main location of these enzymes changed to cell-free. As mentioned before, highly sulfated polysaccharides are present in marine environments (Kloareg and Quatrano, 1988; Wang et al., 2016; Helbert, 2017), so a high salinity might induce a higher expression of cell-free enzymes like sulfatase. This also suggests that the fungal lifestyle might depend on the available substrates.

Unlike bacteria, fungi are capable to penetrate solid substrates (Raghukumar, 2017), specially the filamentous ones (Souza et al., 2015). In the present study, *B. parvus* was the only species that had this structure, and the majority of its EEAs were cell-attached (Table 2). These enzymes are capable of degrading substrates until they can be assimilated by the cells that originally synthesized them (MacDonald and Speedie, 1982; Biely, 1985; Grant and Rhodes, 1992; Confer and Logan, 1998; Lenartovicz et al., 2003; Collins et al., 2005). As the hyphal growth has been highlighted as an important characteristic to colonize substrates (Zalar et al., 2005), *B. parvus* might be using cell-attached enzymes to hydrolytic cleave them.

In marine environments, as bacteria and fungi might have similar functions like decomposers and their close spatial proximity, might lead to antagonistic or synergistic interactions (Velicer, 2003; Romani et al., 2006). In marine bacteria, the direct release of cell-free enzymes has been reported as a response to the presence of substrate (Alderkamp et al., 2007), starvation (Albertson et al., 1990; Alderkamp et al., 2007; Bong et al., 2013), changes in the cell permeability (Chróst, 1990), or due to cell decay (Baltar et al., 2019). However, when the substrate becomes limited, but the secretion of cell-free enzymes by marine fungi remains unknown. In freshwater studies, Millar et al. (2015) reported contributions of microbial cell-free enzymatic activities of 15.5, 32.6, 32.9, 82.5, and 24.4% for BGL, NAG, APA, SUL, and LAP, respectively, of the total EEA. In contrast, Kamer and Rassoulzadegan (1995) and Baltar (2018) suggested that the marine microbial cell-free fraction can represent up to 100% of the total EEA. These authors reported microbial cell-free enzymatic activities ranging from 0, 37, and 34% for BGL, APA, and LAP, respectively, and up to 100% of the total EEA (Baltar et al., 2010; Baltar et al., 2013; Baltar et al., 2016). In the present study, we report that depending on the species and also on the salinity, the fungal cell-free fraction can vary from 0.1 to 85.1% of the total EEA with some enzymes more likely to produce cell-free enzymes like OLE and SUL.

4.3 Potential environmental implications

Climate change is influencing the freshwater input from ice, riverine, and precipitation into oceans, hence, also its salinity (Myers et al., 1990; Munk, 2003; Reid et al., 2009; Hutchins and Fu, 2017; Kohler et al., 2020). The desalination of the oceans, also known as ocean freshening, can intensify the stratification which decreases the vertical mixing of the water column and affects the transport of nutrients (Reid et al., 2009; Balaguru et al., 2016; Hutchins and Fu, 2017). As suggested by Arnosti et al. (2014), nutrient availability can influence the magnitude and distribution of extracellular enzymatic activities. Accordingly, a different nutrient availability might lead to expression of other enzymes, which might also influence the potential role of marine fungi in the oceanic biogeochemical cycles. Moreover, most marine fungi are adapted to tolerate high salinity (Jennings, 1983), so salinity can influence the fungal community composition (Rojas-Jimenez et al., 2019). Our results indicate that the studied marine fungal species might be adapted to different salinities, but their extracellular enzymatic activities, both, cell-attached and cell-free, might be affected by salinity.

5 Conclusions

Based on our results, the marine fungi species studied are capable to secrete an array of cell-free enzymes, and this can represent up to 85.1% of the respective total EEA. Though, it is important to consider that some of these high values were found in fungal strains and enzymes with low total EEA, so their contribution to the cell-free enzymatic pool might be minimal compared to others. Additionally, the release of these extracellular enzymes can be influenced by environmental parameters such as salinity, despite being species-specific. As fungi are undeniably widespread in marine environments, their cell-free enzymes might also be an important part of the oceanic enzymatic pool. Nonetheless, as oceans are complex and diverse ecosystems, other abiotic and biotic variables should also be included in future studies.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

Conceptualization, KS-A, and FB; data curation, KS-A; formal analysis, KS-A; funding acquisition, FB; investigation, KS-A; methodology, KS-A, and FB; project administration, FB; resources, GH, and FB; software, KS-A; supervision, FB; validation, KS-A; visualization, KS-A; writing original draft, KS-A; writing, review, and editing, KS-A, GH, and FB. All authors have read and agreed to the published version of the manuscript.

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