



Formation and characterization of biofilms formed by salt-tolerant yeast strains in seawater-based growth medium

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Received: 14 October 2020 / Revised: 30 December 2020 / Accepted: 20 January 2021 / Published online: 25 February 2021
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Abstract

Yeast whole cells have been widely used in modern biotechnology as biocatalysts to generate numerous compounds of industrial, chemical, and pharmaceutical importance. Since many of the biocatalysis-utilizing manufactures have become more concerned about environmental issues, seawater is now considered a sustainable alternative to freshwater for biocatalytic processes. This approach plausibly commenced new research initiatives into exploration of salt-tolerant yeast strains. Recently, there has also been a growing interest in possible applications of microbial biofilms in the field of biocatalysis. In these complex communities, cells demonstrate higher resistance to adverse environmental conditions due to their embedment in an extracellular matrix, in which physical, chemical, and physiological gradients exist. Considering these two topics, seawater and biofilms, in this work, we characterized biofilm formation in seawater-based growth media by several salt-tolerant yeast strains with previously demonstrated biocatalytic capacities. The tested strains formed both air-liquid-like biofilms and biofilms on silicone surfaces, with *Debaryomyces fabryi*, *Schwanniomyces etchellsii*, *Schwanniomyces polymorphus*, and *Kluyveromyces marxianus* showing the highest biofilm formation. The extracted biofilm extracellular matrices mostly consisted of carbohydrates and proteins. The latter group was primarily represented by enzymes involved in metabolic processes, particularly the biosynthetic ones, and in the response to stimuli. Specific features were also found in the carbohydrate composition of the extracellular matrix, which were dependent both on the yeast isolate and the nature of formed biofilms. Overall, our findings presented herein provide a unique data resource for further development and optimization of biocatalytic processes and applications employing seawater and halotolerant yeast biofilms.

Key points

- Ability for biofilm formation of some yeast-halotolerant strains in seawater medium
- ECM composition dependent on strain and biofilm-forming surface
- Metabolic enzymes in the ECM with potential applications for biocatalysis

Keywords Biofilms · Extracellular matrix composition · Seawater · *Debaryomyces* · *Schwanniomyces* · *Kluyveromyces*

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Introduction

Virtually, all living microorganisms have the capacity to form biofilms that represent communities, in which living cells embedded in an extracellular matrix (ECM) are attached to various biotic and abiotic surfaces (Flemming and Wingender 2010; Strieth et al. 2018). The ECM of biofilms consists of self-produced secreted polymeric substances. Within the biofilm ECM, chemical gradients are generated. These gradients determine the existence of heterogeneous populations with distinct phenotypes and result in manifestation of diverse metabolic pathways, stress responses, and other biological activities (Stewart and Franklin 2008). Biofilm formation is complex and is influenced by multiple biological, chemical, and

physical factors. In fact, numerous physiological states can be distinguished during biofilm formation (Halan et al. 2012). Initial interactions of planktonic cells with a surface depend on the availability of nutrients. During proliferation and maturation phases, cells then adhere to it and begin to reproduce and to make the ECM that embeds the cells attached to the surface and to each other. Such a convoluted development leads to the formation of a complex three-dimensional structure with water channels and pores through which nutrients and waste can diffuse. Finally, a pseudo-steady state is established, in which dispersion or release of biofilm biomass particles or individual cells allows for the re-initiation of the cycle.

Cellular organization in biofilms provides enormous advantages, such as colonization of host tissues; expression of virulence characteristics; metabolic cooperation; efficient nutrient capture; cellular communication; and increased tolerance to chemical, physical, and biological stress conditions (Castrillón et al. 2013). These properties make biofilms potentially harmful to human health; in fact, multiple biofilm-associated bacterial and fungal organisms have been shown to be responsible for the majority of microbial infections in humans (Inigo and Del Pozo 2018). While prokaryotic biofilms seem to be more extensively studied (Bales et al. 2013; Cheng et al. 2019; Colvin et al. 2011, 2012), a substantial amount of scientific data on fungal biofilms exists. Research studies have been carried out in the eukaryotic biology model organism *Saccharomyces cerevisiae* (Andersen et al. 2014), as well as in a few human pathogenic organisms, such as *Aspergillus fumigatus*, *Cryptococcus neoformans*, and various *Candida* species (Dominguez et al. 2018, 2019; Le Mauff et al. 2019; Martinez and Casadevall 2015; Zarnowski et al. 2014). With bacterial biofilms, the secreted high molecular weight exopolysaccharides constitute the scaffold for the formation of this structure, to which other carbohydrates adhere, as well as proteins, lipids, and nucleic acids present in smaller quantities (Hall-Stoodley et al. 2004). The data available for fungal biofilms, obtained in a great extent from *Candida* spp., revealed the presence of a high content of proteins and polysaccharides with the ECM composition similar to that of the cell wall of the microorganism that forms it, indicating that broad diversity may exist (Mitchell et al. 2016).

The abovementioned features of biofilms can hinder some industrial processes, while also be beneficial and valuable for others, like those related to the biotechnological production of organic compounds and the modification of several foods (Berlanga and Guerrero 2016; Muffler et al. 2014; Strieth et al. 2018). For decades, biofilms have been applied to bioremediation, particularly in the treatment of wastewater and off-gas (Gross et al. 2007).

Biofilms have also been applied to biocatalytic processes, in which natural enzymes or whole cells are used to perform chemical transformations of organic compounds. They have

been particularly used for fermentative production of bulk chemicals, such as acetic acid, ethanol, and butanol (Demirci et al. 1997; Ho et al. 1997; Qureshi et al. 2004), as well as in the production of organic acids (citric, fumaric, and succinic acids), enzymes (lignin peroxidase, manganese peroxidase, cellulase), bioactive compounds (bacteriocins such as pediocin or nisin and antibiotics such as cephalosporin C), and fine chemicals (Gross et al. 2007, 2013; Halan et al. 2012, 2014; Muffler et al. 2014; Romero et al. 2018; Willrodt et al. 2017).

Over the last several years, we have demonstrated the usefulness of some *Saccharomyces* and non-*Saccharomyces* yeast strains for the stereoselective production of several compounds of chemical and pharmaceutical interest (Andreu and del Olmo 2014, 2018, 2019, 2020; Andreu et al. 2016). A considerable problem faced when whole cells are used as biocatalysts in industrial processes is related to a massive consumption of water due to the low solubility of the hydrophobic substrates. This concern can be addressed by the utilization of seawater as a sustainable alternative to freshwater in chemical reactors (Andreu and del Olmo 2018, 2019, 2020). Finding halotolerant microbial species that could carry out biocatalytic processes in this medium is another logical step in the implementation of this strategy at an industrial scale. In this work, we analyzed the capability of several salt-tolerant yeast strains useful as biocatalysts for biofilm production in seawater medium. The strains considered have been *Torulaspora delbrueckii*, *Kluyveromyces marxianus*, *Debaryomyces hansenii*, *Debaryomyces fabryi*, *Schwanniomyces etchellsii*, *Schwanniomyces polymorphus*, *Meyerozyma guilliermondii*, *Pichia glucozyma*, *Pichia fermentans*, *Pichia jadinii*, and *Saccharomyces cerevisiae* Ethanol Red. We also characterized in-depth the composition of biofilm ECMs in those biofilm-forming halotolerant yeast species.

Materials and methods

Yeast strains and growth conditions for biofilm production

The yeast strains used in this work are described in Table 1. To determine the halotolerance of yeast strains, growth experiments were carried out in culture plates of YPD-based medium that contained 2% (w/v) agar supplemented with NaCl to a final concentration between 3.5 and 7.5% (w/v).

The seawater used in the experiments came from the El Perelló beach (Mediterranean Sea), València (Spain). It was sterilized by autoclaving under standard conditions. The water salinity for this area was around 4% (w/v), according to the determination made by weighing the solid residue after lyophilization. The sodium content, analyzed by flame emission spectroscopy (589.0 nm, Thermo Fisher Scientific

Table 1 Strains used in this work

Strain	Origin
<i>Debaryomyces hansenii</i>	CBS767[a]
<i>Debaryomyces fabryi</i>	CECT[b] 10099
<i>Schwanniomyces etchellsii</i>	CECT[b] 11406
<i>Schwanniomyces polymorphus</i>	CECT[b] 10060
<i>Pichia jadinii</i>	CECT[b] 1060
<i>Kluyveromyces marxianus</i>	CECT[b] 1018
<i>Torulaspota delbrueckii</i>	CECT[b] 1015
<i>Meyerozyma guilliermondii</i>	CECT[b] 11379
<i>S. cerevisiae</i> Ethanol Red®	Leaf[c]
<i>Pichia glucozyma</i>	CECT[b] 11449T
<i>Pichia fermentans</i>	CECT[b] 1455

[a] Provided by Dr. José Ramos (Universidad de Córdoba, Spain)

[b] CECT: Colección Española de Cultivos Tipo, Servei Central de Suport a la Investigació, Universitat de València, Paterna, Spain

[c] Lesaffre Advanced fermentations, Marcq-en-Barœul, France

(Waltham, USA) model iCE 3000 Series), was 12.46 ± 0.17 g/L, and pH ranged from 7.5 to 8.5, depending on the batch.

For biofilm production, cells inoculated in YPD medium prepared in seawater (SW-YPD) (1% (w/v) yeast extract, 2% (w/v) bacto peptone, 2% (w/v) glucose) were incubated overnight at 30 °C in an orbital shaker (200 rpm). Then, the cultures were diluted in the same medium without any carbon source (SW-YP) to a final OD₆₀₀ of 0.3. Fifty milliliters of these suspensions was placed in 250-mL glass beakers and incubated at 30 °C for 14 days without shaking. Alternatively, silicone tubes (length of 25 cm; external diameter of 12 mm; internal diameter of 8 mm) were filled with 10 mL of the same solution, closing both ends by joining them with a polypropylene pipette tip, and kept under the same conditions.

Microscopic visualization of biofilms

The coverslip assay was used for in vitro biofilm imaging (Mitchell et al. 2015). Briefly, in vitro biofilms were grown on sterile coverslips (Thermanox, Thermo Fisher Scientific, Waltham, USA) in 12-well polystyrene plates that were each pre-coated with 10 µL of human NaEDTA plasma and allowed to dry at 30 °C. Fungal cell inocula (10^6 cells/mL) were prepared out of overnight yeast cultures in artificial SW-YPD (1% (w/v) yeast extract, 2% (w/v) bacto peptone, 2% (w/v) glucose, 2% (w/v) NaCl) at 30 °C followed by cell counting with an automated Countess™ II cell counter (Invitrogen, Carlsbad, USA). Yeast inocula were applied to each coverslip at 30 °C for 60 min. The initial inoculum was then removed, and 1 mL of fresh artificial SW-YPD was added to each well. Lastly, the plates were incubated at 30

°C for 48 h. Samples were imaged on a Leo 1530-1 FESEM/EDS/EBSB scanning electron microscope system (Carl Zeiss Microscopy, White Plains, USA).

Determination of the biofilm formation capacity

For each one of the tested strains growing in glass beakers or silicone tubes under the conditions described above, biofilm and planktonic cells were separated. In the case of experiments carried out with glass beakers, the suspension was passed through a sterile stainless steel colander in which the biofilm was trapped, allowing the separation of the two pools. For silicone tube experiments, planktonic cell solutions were initially obtained by emptying the tubes and, after several washes, the biofilm biomass attached on the silicone was scraped off and collected by adding water. In both cases, the material of each fraction was centrifuged at $2500 \times g$ for 3 min, washed with water, and lyophilized. Then, the weights of biofilm and planktonic fractions were determined, and the ratio between them was considered the biofilm formation capacity.

Isolation of the biofilm ECM

Biofilm ECM was obtained as described previously (Zarnowski et al. 2014) for *Candida* species with minor modifications. Briefly, biofilms were separated from planktonic cells as described above, collected by centrifugation, and washed. Then, the biomass was resuspended in 30 mL of water, and the solution was sonicated for 5 min at 20 kHz in a Vibra Cell VCX-500 sonicator (Sonics & Materials Inc., Newtown, USA). Cells were collected by centrifugation, and the matrix (supernatant) was filtered through 0.22-µm PES filtration units (VWR, Radnor, USA). Sodium azide was added to a final concentration of 0.02% (w/v), and the solution was frozen and lyophilized. After resuspension in water and exhaustive dialysis in 50 mM ammonium bicarbonate buffer, the solution was used for further chemical analyses.

Analyses of the composition in carbohydrates of the biofilm ECM

Carbohydrates in biofilm ECMs were analyzed based on the modified procedures reported elsewhere (Zarnowski et al. 2014). Monosugars were converted to alditol acetate derivatives (Blakeney et al. 1982) and then identified and quantified by gas chromatography on a GC-2010 system (Shimadzu, Kyoto, Japan). A Crossbond® 50% cyanopropylmethyl/50% phenylmethyl polysiloxane column was used (15 m × 0.25 mm with 0.25-µm film thickness, RTX-225, Restek, Bellefonte, USA). The GLC (gas liquid chromatography) conditions were as follows: injector at 220 °C, FID detector at 240 °C, and a temperature program of 215 °C for 2 min, then 4 °C

min^{-1} up to 230 °C before holding for 11.25 min, run at constant linear velocity of 33.4 cm/s and split ratio of 50:1.

Analyses of the ECM proteomes

Enzymatic “in liquid” digestion and mass spectrometric analysis were done at the Mass Spectrometry Facility, Biotechnology Center, University of Wisconsin—Madison. Two hundred micrograms of proteins was extracted by precipitation with 15% TCA (trichloroacetic acid)/60% acetone and then incubated at -20 °C for 30 min. The ECM preparation was centrifuged at $16,000\times g$ for 10 min, and the resulting pellets were washed twice with ice-cold acetone, followed by an ice-cold MeOH wash. Pelleted proteins were resolubilized and denatured in 10 μL of 8 M urea in 100 mM NH_4HCO_3 for 10 min, then diluted to 60 μL for tryptic digestion with the following reagents: 3 μL of 25 mM DTT, 4.5 μL of acetonitrile, 36.2 μL of 25 mM NH_4HCO_3 , 0.3 μL of 1M Tris-HCl, and 6 μL of 100 ng/ μL Trypsin Gold solution in 25 mM NH_4HCO_3 (Promega, Madison, USA). Digestion was conducted in two stages. First, the digestion was incubated overnight at 37 °C. Then, an additional 4 μL of trypsin solution was added, and the mixture was incubated at 42 °C for an additional 2 h. The reaction was terminated by acidification with 2.5% TFA (trifluoroacetic acid) to a final concentration of 0.3% and then centrifuged at $16,000\times g$ for 10 min. Trypsin-generated peptides were analyzed by nanoLC-MS/MS using the Agilent 1100 nanoflow system (Agilent, Santa Clara, USA) connected to a hybrid linear ion trap-orbitrap mass spectrometer (LTQ-Orbitrap, Thermo Fisher Scientific, Waltham, USA) equipped with a nanoelectrospray ion source. Capillary HPLC was performed using an in-house fabricated column with an integrated electrospray emitter, as described elsewhere (Martin et al. 2000). Sample loading and desalting were achieved using a trapping column in line with the autosampler (Zorbax 300SB-C18, 5 μm , 5×0.3 mm, Agilent, Santa Clara, USA). The LTQ-Orbitrap was set to acquire MS/MS spectra in a data-dependent mode as follows: MS survey scans from 300 to 2000 m/z were collected in profile mode with a resolving power of 100,000. MS/MS spectra were collected on the five most abundant signals in each survey scan. Dynamic exclusion was employed to increase the dynamic range and maximize peptide identifications. Raw MS/MS data were searched against a species-select concatenated amino acid sequence database using an in-house MASCOT search engine (Perkins et al. 1999). The data were searched against concatenated *D. fabryi* and *K. marxianus* amino acid sequence databases, whereas the proteomes of *S. etchellsii* and *S. polymorphus* were searched against the proteome of *D. hansenii*. These two species were initially classified within the *Debaryomyces* genus, but recently reassigned to *Schwanniomyces* (Kurtzman and Suzuki 2010). Identified proteins were further annotated and filtered

to 1.5% peptide and 0.1% protein false-discovery-rate with Scaffold Q+ version 4.10.0 (Proteome Software Inc., Portland, USA) using the protein prophet algorithm (Keller et al. 2002). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al. 2019) partner repository with the dataset identifier PXD022284 and 10.6019/PXD022284.

The ECM proteomes of the tested halotolerant yeast species were analyzed functionally using the UniProt Knowledgebase data, which contains reviewed UniProtKB/Swiss-Prot entries (Uniprot Consortium 2019). Functional Gene Ontology (GO) terms were assigned for each protein identified based on the respective halotolerant yeast genome data. Two sets of hierarchies (“biological process” and “molecular function”) were used to prepare the visualizations of relative quantities of biofilm proteins. On the basis of this hierarchical classification scheme, Voronoi treemaps were constructed (Bernhardt et al. 2009). This approach divides screen space according to hierarchy levels in which the main functional categories determine screen sections on the first level, subsidiary categories on the second level, and so forth. Voronoi treemaps were prepared with Paver version 2.1.9 (DECODON Software UG, Greifswald, Germany).

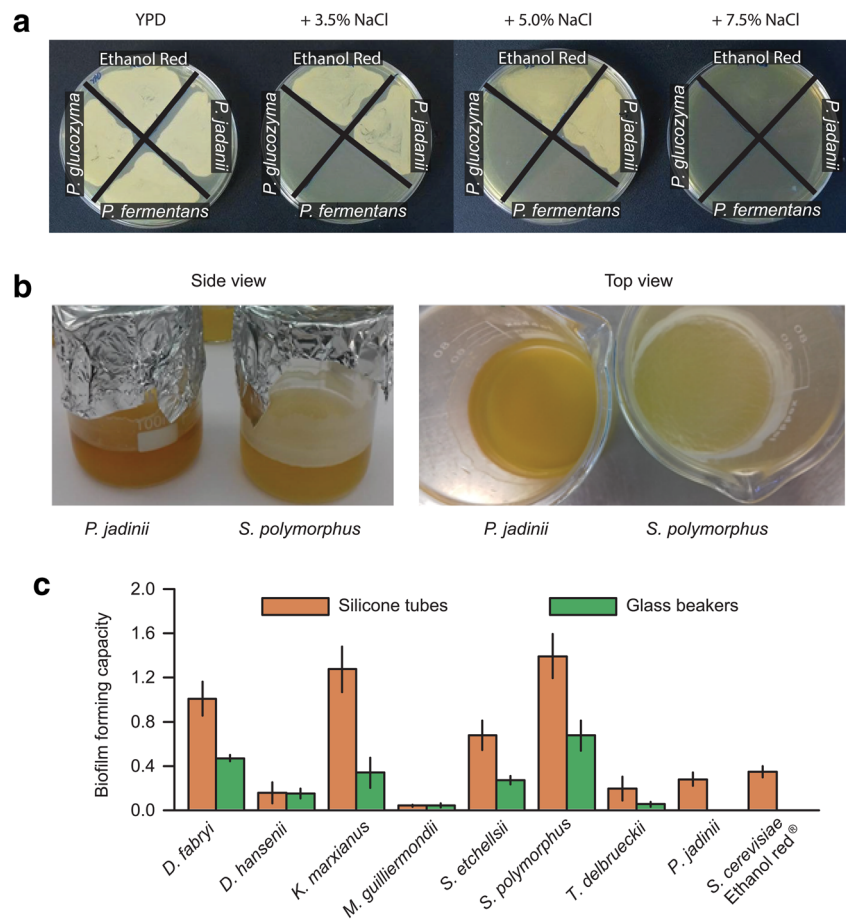
Results

The ability of halotolerant yeast strains for biofilm and ECM production in seawater

The requirement for large quantities of water at the industrial scale makes the use of seawater as a sustainable, freely available solvent, more conveniently. Recently, we have reported the versatility of several halotolerant strains for biocatalytic processes in the presence of seawater (Andreu and del Olmo 2018, 2019, 2020). Some of the tested yeasts such as *T. delbrueckii* and *K. marxianus* were capable of growing in YPD supplemented with NaCl up to 5% (w/v), while *D. hansenii*, *D. fabryi*, *S. etchellsii*, *S. polymorphus*, and *M. guilliermondii* tolerated even 7.5% NaCl (Andreu and del Olmo 2018, 2020). In this work, we have expanded this growth characteristic analysis to four additional strains of biotechnological interest: *P. glucozyma*, *P. fermentans*, *P. jadinii*, and *S. cerevisiae* (Ethanol Red) (Table 1). Only the latter two tolerated salt concentrations up to 5% (w/v) and were thus also considered for additional analyses in this work (Fig. 1a).

We further characterized the ability of the tested halotolerant strains to form biofilms in seawater-growth media on two types of solid surfaces: in glass beakers and in silicone tubes. These complex microbial community structures appeared mainly as a white veil of cell aggregates forming air-liquid biofilms on the liquid surface as well as to some extent on the glass walls (Fig. 1b). In silicone tubes, the biofilms

Fig. 1 Salt resistance and biofilm-forming capacity in seawater-based medium for the strains considered in this work. **Panel a:** Growth of several yeast strains on the YPD-derived plates supplemented with NaCl concentrations of 3.5, 5, or 7.5% (w/v), or without salt (YPD). 10^6 cells from the overnight cultures in YPD were spread on plates, which were then incubated for 48 h at 28 °C. This figure shows the result of a representative experiment. **Panel b:** Comparison of the 14-day growth appearance of one strain capable of biofilm formation in glass beakers (*Schwanniomyces polymorphus*) and another in which only planktonic cells are observed (*Pichia jadinii*). **Panel c:** Biofilm formation capacity was determined as the ratio between the weight of the dry biomass corresponding to biofilm respect to the weight of the dry planktonic cells after 14-day incubation. The values shown correspond to the average of three experiments



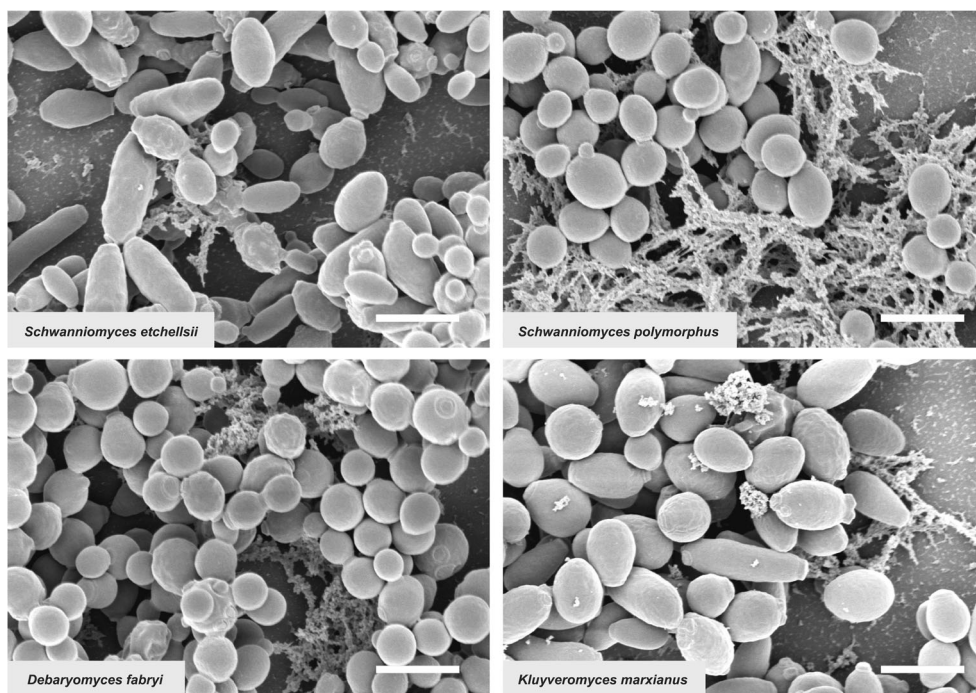
were observed attached to the silicone surface. Except for *P. jadinii* and *S. cerevisiae* Ethanol Red, all the tested yeast strains formed visible biofilms both on silicone and glass, but their ability to grow as biofilms varied (Fig. 1c). The highest biofilm-forming capacities were determined for both *Schwanniomyces* species, *D. fabryi*, and for *K. marxianus*. Microscopic studies of biofilm architecture revealed the presence of visible ECM deposits (Fig. 2). These four strong biofilm producers were subjected to biochemical analyses of the ECM composition. It is noteworthy that no biofilm-like structures were observed in yeast cultures grown in freshwater-based media.

Glycosyl composition of the biofilm ECM of halotolerant yeast strains

Fungal biofilm functional biology has been linked to the ECM quantity and its composition, in which carbohydrates constitute a considerable fraction (Reichhardt et al. 2015; Zarnowski et al. 2014). The ECM carbohydrate profiles of the tested halotolerant yeast strains consisted of three pentoses (ribose, arabinose, and xylose) and two hexoses (mannose, glucose), but their ratios varied between species and, even to a higher degree, growth

conditions (Fig. 3). Mannose represented the most abundant sugar in the biofilm ECMs formed in silicone tubes for *K. marxianus* and *S. polymorphus*, with percentages reaching 50%, whereas comparable amounts of mannose and arabinose were found in *D. fabryi* and *S. etchellsii*. In the case of biofilms formed in glass beakers, mannose was present at lower concentrations, while arabinose was usually the most abundant carbohydrate found. In fact, this monosaccharide was especially predominant in air-medium biofilms formed by *D. fabryi*, *S. polymorphus*, and *K. marxianus*, where its content reached up to 80%. The amount of arabinose in the *S. etchellsii* ECMs was low, whereas higher ribose concentration was detected in comparison with the other strains. Interestingly, significant variability in the content of this monosaccharide was observed, with percentages usually higher in the exopolysaccharides of the biofilms formed on silicone. The highest glucose content was found in the *S. etchellsii* ECMs (around 25%), while in the other cases, percentages between 2 and 15 were detected. Two additional monosaccharides, xylose and rhamnose, were identified in the tested ECMs, but in low or even trace amounts with percentages up to 11 in silicone biofilms and 4 in glass beaker biofilms, respectively.

Fig. 2 Scanning electron microscopy of extracellular matrix deposits present in the halotolerant yeast biofilms of *Schwanniomyces etchellsii*, *Schwanniomyces polymorphus*, *Debaryomyces fabryi*, and *Kluyveromyces marxianus*. Forty-eight-hour-old biofilms were grown in seawater-YPD medium (SW-YPD). Scale bars represent 10 μm



Proteomic analysis of the biofilm ECM of halotolerant yeast strains

Proteins are the major component of the ECM in yeast biofilms (Mitchell et al. 2016). In this work, proteins that represented $> 0.05\%$ of the respective proteome were considered (Table 2). Bearing in mind this cutoff, the analyzed

proteomes of biofilm ECMs of the halotolerant yeasts contained between 220 and 390 proteins depending on the strain and growth condition. Unlike the observed differences in the ECM carbohydrate profiles, the analysis of the ECM proteomes revealed striking similarities with reference to the most abundant functional categories and subcategories. Gene Ontology-based functional predictions grouped most of these

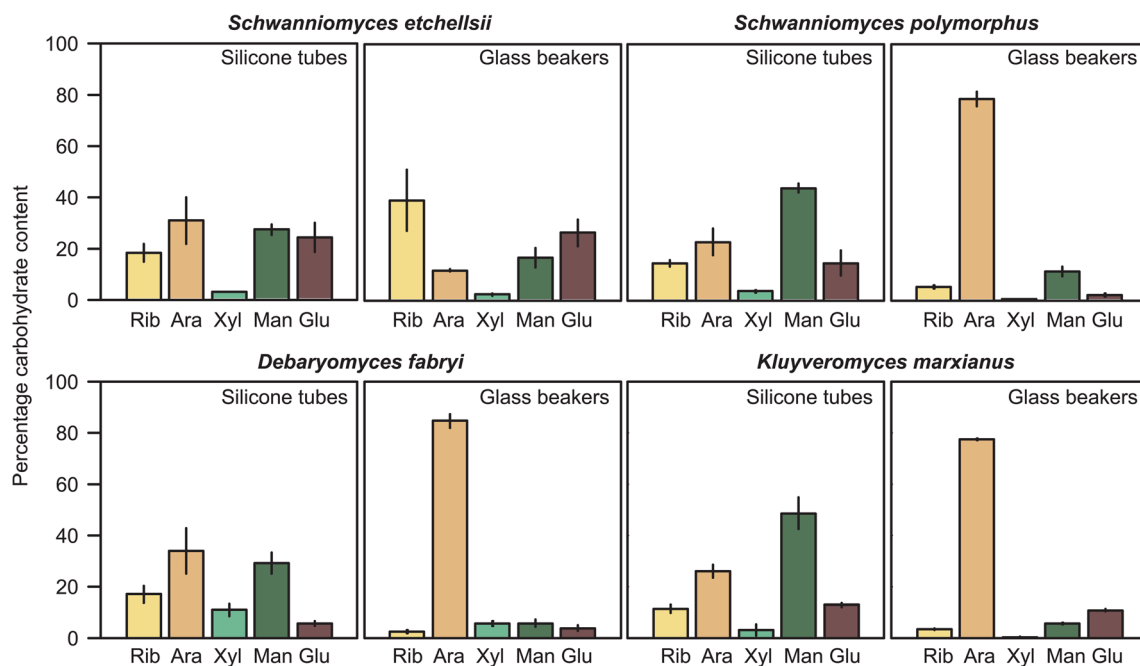


Fig. 3 Monocarbohydrate profiles of the extracellular matrices in biofilms formed by the halotolerant yeasts *Schwanniomyces etchellsii*, *Schwanniomyces polymorphus*, *Debaryomyces fabryi*, and

Kluyveromyces marxianus. Data correspond to the average and standard deviation from 5 independent experiments

Table 2 Number of proteins identified for each strain and growth condition and in the Gene Ontology category of metabolic process

	<i>D. fabryi</i>		<i>S. etchellsii</i>		<i>S. polymorphus</i>		<i>K. marxianus</i>	
	st ¹	gb ²	st ¹	gb ²	st ¹	gb ²	st ¹	gb ²
> 0.05%	293	390	279	281	229	282	220	349
Metabolic process	169	229	191	189	161	195	158	233
Metabolic process (percentage, %)	58	59	68	67	70	69	72	67

¹ st and ² gb refers to silicone tubes and glass beakers, respectively

proteins into the “metabolic process” category with a larger number classified as “biosynthetic process” when compared to “catabolic process,” which reflects a relevant molecular “building” activity in these structures (Table 2 and Fig. 4). Three other major functional protein groups, including “establishment of localization,” “response to stress,” and “cellular component organization,” were also abundant (Figs. 4, 5, 6, 7, and 8 and Fig. S1 in the Supplementary Material). Tables S1 to S8 in the Supplementary Material compile the information about the proteins found in diverse categories and subcategories ordered by their weight percentage.

Our detailed analysis revealed the presence of multiple enzymes participating in glycolysis/gluconeogenesis and in other processes strictly related to carbohydrate metabolism. For example, about 9% and 12% of the ECM proteins of *D. fabryi* grown on silicone and glass, respectively, were functionally classified as such (Tables S1 and S2 in the Supplementary Material), while these numbers were higher for *K. marxianus* and amounted 23% and 18%, respectively (Tables S3 and S4 in the Supplementary Material). Proteins involved in the biosynthesis of nitrogen compounds such as

amino acids, proteins, and nucleotides were also found in abundance. Among the proteins involved in the response to stress, heat shock proteins and enzymes participating in the decomposition of reactive oxygen species were prevalent. In the functional categories of establishment of localization (localization) and cellular component organization (and biogenesis) proteins involved in transport processes, folding and ribosomal structure are mainly found (Figs. 4, 5, 6, 7, and 8 and Fig. S1 and Tables S1 to S8 in the Supplementary Material).

A compelling correlation between the ECM of the biofilms formed on silicone and glass beakers was observed with reference to the most abundant proteins with percentages higher than 0.7–1% (proteins in bold in Figs. 4, 5, 6, 7, and 8). The vast majority of those proteins were represented by heat shock proteins (such as SSA1, SSA2, and sphingolipid long chain base-responsive protein PIL1) or enzymes involved in glycolysis/gluconeogenesis (glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, and enolase), pentose phosphate pathway (6-phosphogluconate dehydrogenase), Krebs cycle (malate dehydrogenase), and ATP synthesis (several

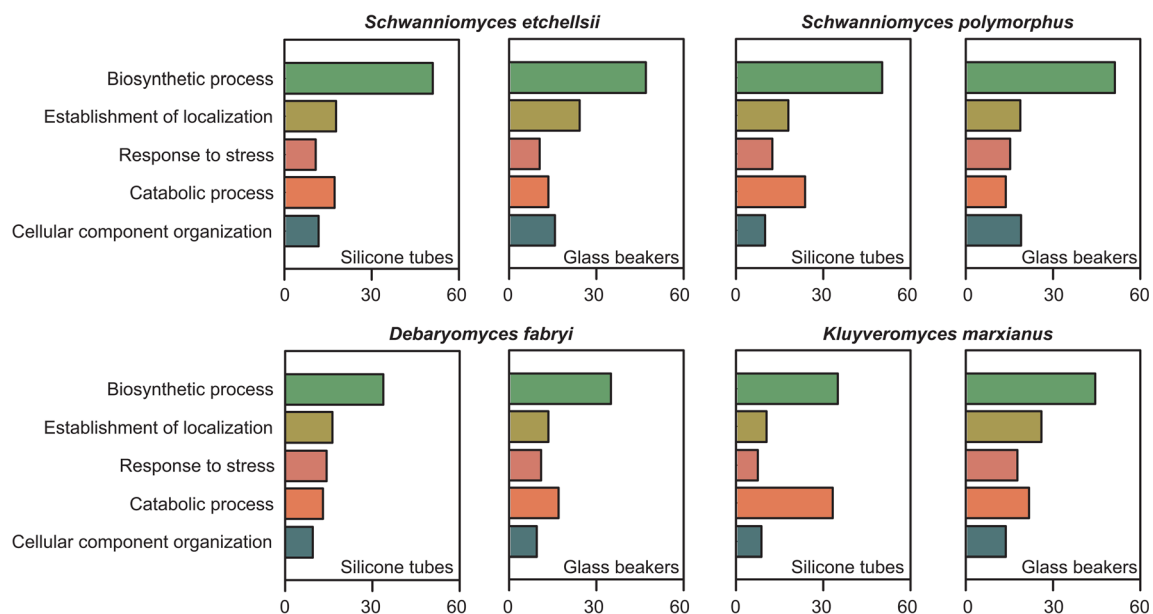
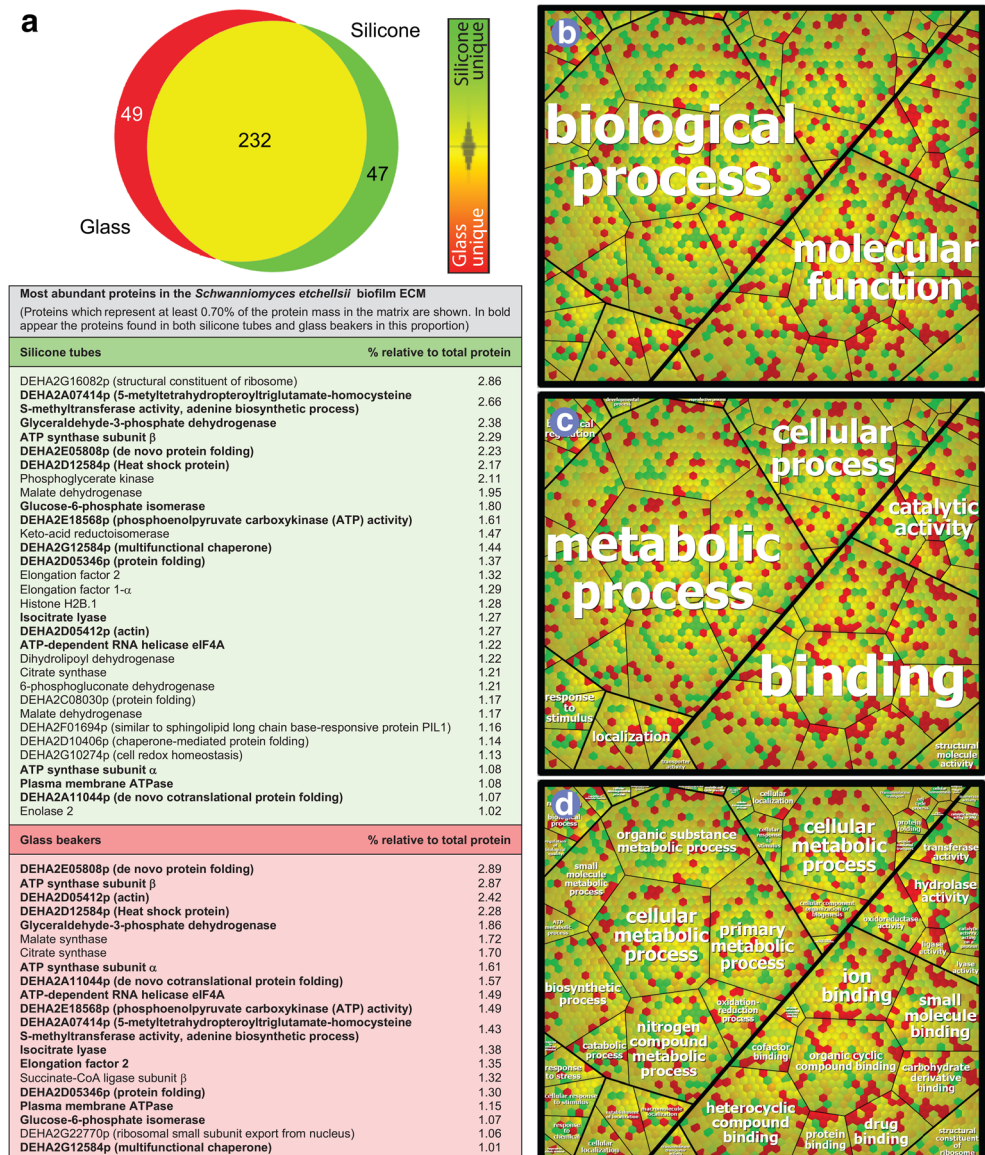


Fig. 4 Distribution of main functional categories of proteins identified in the biofilm ECMs of the halotolerant yeasts, *Schwanniomyces etchellsii*, *Schwanniomyces polymorphus*, *Debaryomyces fabryi*, and

Kluyveromyces marxianus. Functional protein assignments were done based on the Gene Ontology data in the UniProt Knowledgebase

Fig. 6 Comparative ECM proteomics of *Schwanniomyces etchellsii* biofilms grown in glass beakers and silicone tubes. **a** A Venn diagram illustrating the number of exclusive (green and red) and common (yellow) biofilm ECM proteins. **b, c, d** Smallest clusters represent individual identified proteins and are arranged inside higher level regions according to their GO biological process and molecular function assignments. Z-scores were calculated based on log₂ silicone tube/glass beaker ratios of biofilm proteins and mapped to a color ramp starting with red (more protein in glass beaker biofilms), passing yellow (similar protein proportions under both growth conditions), and reaching green (more protein in silicone tube biofilms)

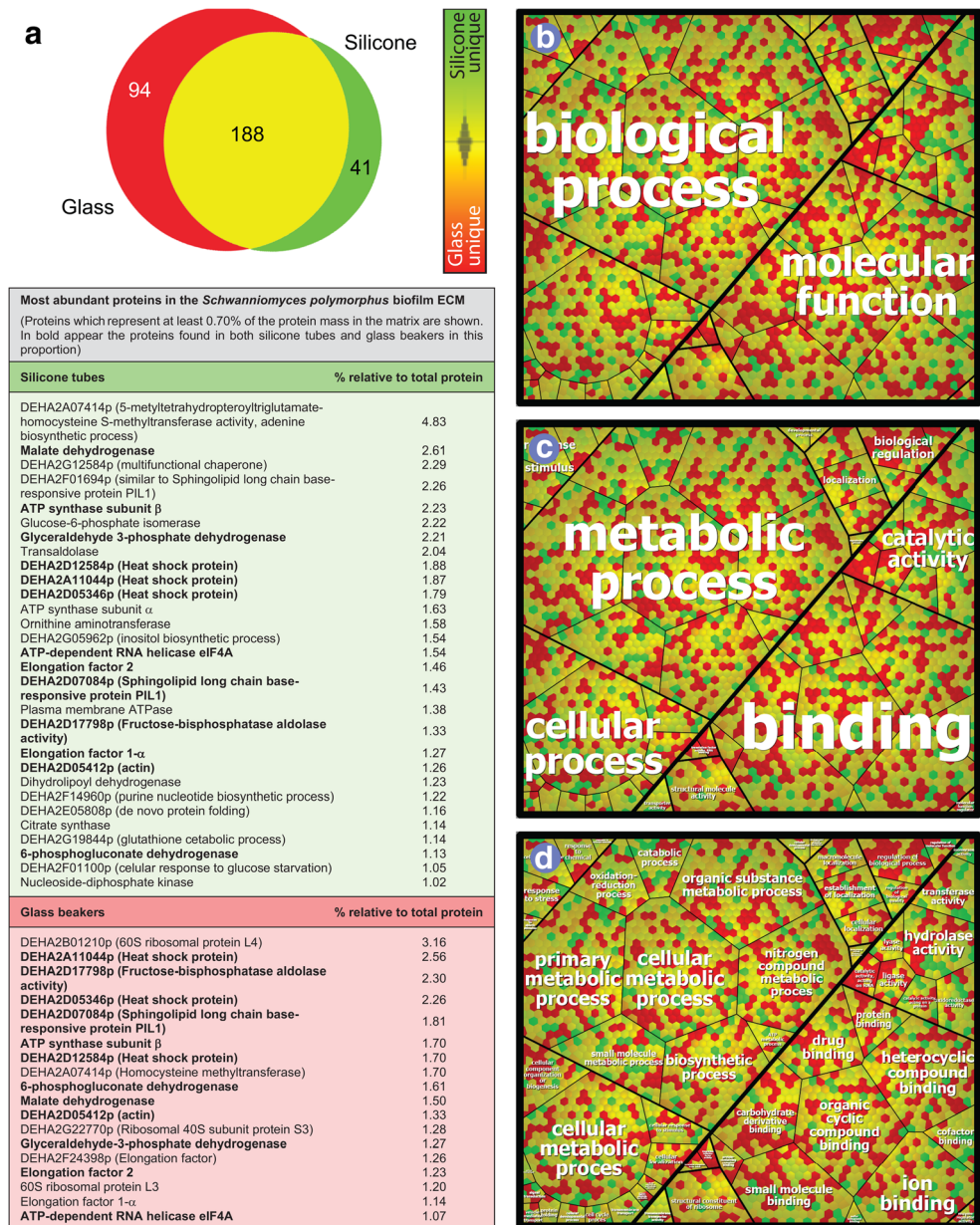


components of the proteasome endopeptidase complex appeared uniquely in those formed by *S. etchellsii* and *S. polymorphus*.

Our in-depth analysis of the ECM proteomes laid a solid base for potential protein-based discriminatory studies among the halotolerant yeast species considered herein. For instance, *D. fabryi* had the lowest percentage of proteins involved in metabolism (Table 2). This value was 58–59%, whereas this number was around 70% in other species. In addition, proteins involved in lipid metabolism were found in higher amounts in the *K. marxianus* biofilms grown on silicone and glass (see Supplemental Tables S3 and S4 in comparison with S1, S2, and S5 to S8). Enzymes involved in amino acid catabolism were also more abundant in the ECM of these biofilms. As mentioned above, some proteins were abundantly formed under both biofilm growth conditions (Figs. 5, 6, 7, and 8 and

Table S9 in the Supplementary Material). For instance, β-glucosidases, previously reported in the *C. albicans* biofilm ECM (Taff et al. 2012; Zarnowski et al. 2014), were detected in the biofilms formed only in silicone tubes by the tested *Schwanniomyces* species (Supplemental Tables S1–S9). Regulatory subunits of 26S protease as well as some ribosomal proteins, D-3-phosphoglycerate dehydrogenase, GTP-binding nuclear protein, and homocitrate synthase were found only in these *K. marxianus* biofilm ECMs. In the case of the other strains, catalase (*D. fabryi*), cystathionine beta-synthase and glutamate-5-semialdehyde dehydrogenase (*S. etchellsii*), and 3-isopropylmalate dehydrogenase and calmodulin (*S. polymorphus*) were unique in the biofilm ECMs formed in this material. Likewise, several proteins were only identified in the biofilms formed by individual strains in glass beakers. Catalase, some components of the cytochrome b-c 1, and

Fig. 7 Comparative ECM proteomics of *Schwanniomyces polymorphus* biofilms grown in glass beakers and silicone tubes. **a** A Venn diagram illustrating the number of exclusive (green and red) and common (yellow) biofilm ECM proteins. **b, c, d** Smallest clusters represent individual identified proteins and are arranged inside higher level regions according to their GO biological process and molecular function assignments. Z-scores were calculated based on log₂ silicone tube/glass beaker ratios of biofilm proteins and mapped to a color ramp starting with red (more protein in glass beaker biofilms), passing yellow (similar protein proportions under both growth conditions), and reaching green (more protein in silicone tube biofilms)



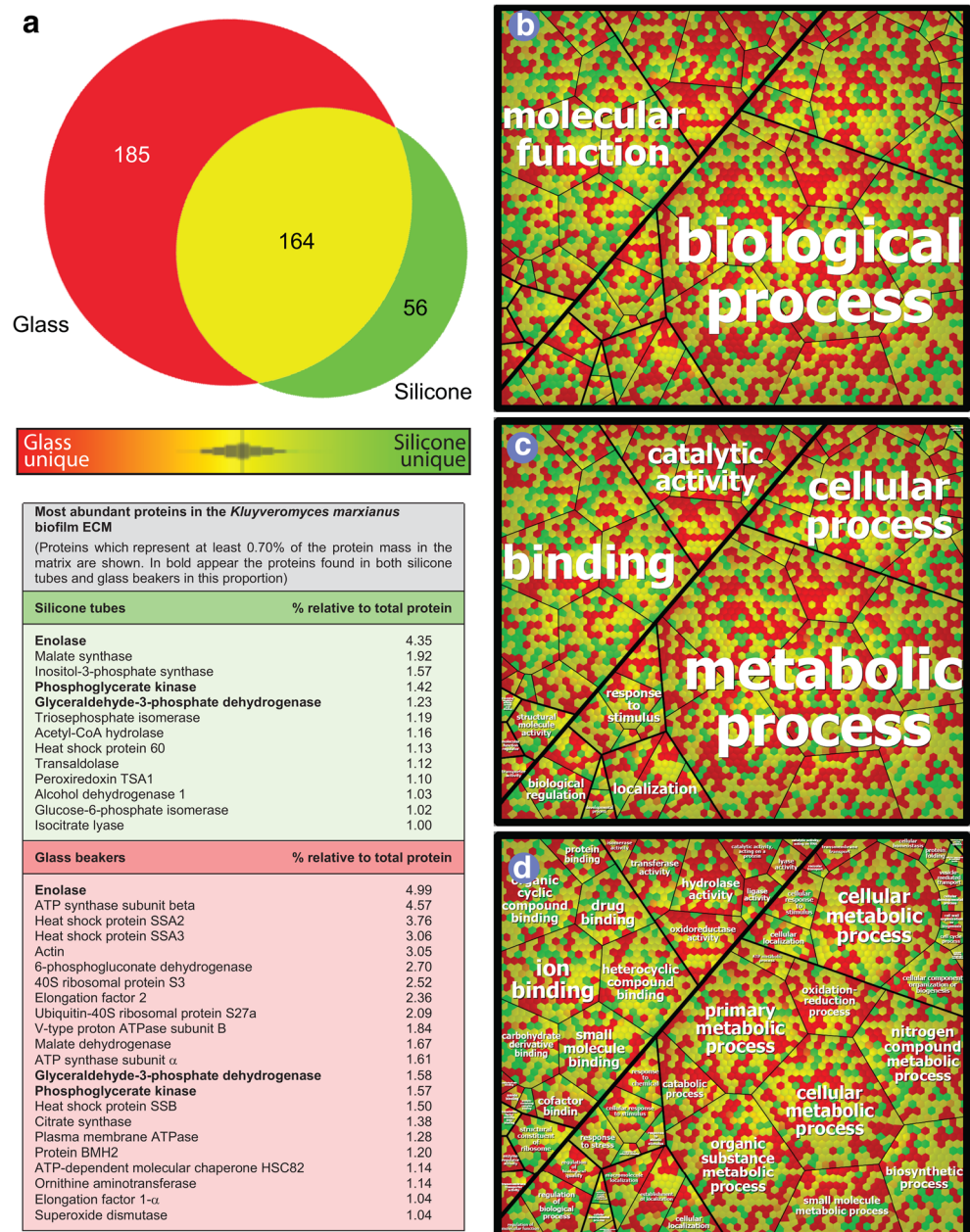
the proteasome endopeptidase complexes were formed in *K. marxianus*, whereas several nucleolar proteins, the ubiquitin-activating enzyme E1 and the pyruvate dehydrogenase E1 component subunit beta were also unique of these structures in *D. fabryi*.

Discussion

Biofilm formation is an elegant, powerful, and inexpensive solution for cell immobilization. One of the preferred strategies involves physical entrapment of the cells in a polymeric matrix (Halan et al. 2012; Muffler et al. 2014; Rosche et al. 2009). In the biocatalysis field, cell immobilization by means

of biofilms provides some benefits, such as the ease of their formation; the low cost of the process compared to other methods of immobilizing enzymes or whole cells, especially for large-scale use; and the increased stability and robustness of these structures to high reactants/product concentrations (Winn et al. 2012). In addition, this approach offers use of high cell densities, in which enzymes can remain active at higher levels over long periods of operation and formed products can be easily recovered from the cells (Muffler et al. 2014). Unfortunately, certain disadvantages can also be experienced in these communities when used for biocatalysis, including undesired side reactions, potential contamination due to cell leakage, or limited mass transfer of substrates and products through the cell membrane and immobilizing matrix

Fig. 8 Comparative ECM proteomics of *Kluyveromyces marxianus* biofilms grown in glass beakers and silicone tubes. **a** A Venn diagram illustrating the number of exclusive (green and red) and common (yellow) biofilm ECM proteins. **b, c, d** Smallest clusters represent individual identified proteins and are arranged inside higher level regions according to their GO biological process and molecular function assignments. *Z*-scores were calculated based on log₂ silicone tube/glass beaker ratios of biofilm proteins and mapped to a color ramp starting with red (more protein in glass beaker biofilms), passing yellow (similar protein proportions under both growth conditions), and reaching green (more protein in silicone tube biofilms)



(Muffler et al. 2014). Although the use of biofilm communities for the production of fine chemicals has not yet been widely implemented at an industrial scale, several research studies have been reported within the last few years, indicating its great future potential. For example, native and recombinant strains of *Pseudomonas* sp. have been used to catalyze the styrene asymmetric epoxidation, the selective octane hydroxylation to octanol, and the production of (*R*)-(+)-perillic acid from (*R*)-(+)-limonene (Gross et al. 2007, 2013; Halan et al. 2014; Willrodt et al. 2017). Besides, *Bacillus* sp. Mcn4 biofilms have been demonstrated as an excellent support for enzymes of its extracellular matrix (Romero et al. 2018). Based on these studies, it is imperative to gain more detailed

information on the ability of biocatalysts to form biofilms under potentially advantageous growth conditions leading to production of desired fine chemicals.

Both formation and composition of fungal biofilms have been extensively studied for more than three decades, with special emphasis on *C. albicans* biofilms due to their role in colonization and infections in humans. Thus, over the last several years, many research groups have provided important information about the *C. albicans* biofilm life cycle and its clinical consequences (Wall et al. 2019). Furthermore, one of the first studies on *S. cerevisiae* biofilms revealed its capability to adhere to polystyrene and polypropylene surfaces at low-glucose concentrations and demonstrated the requirement

of Flo11 in this attachment process (Reynolds and Fink 2001). The flor or velum formation by the *S. cerevisiae* flor strains is also a form of air-liquid interfacial biofilm that is essential for aerobic growth during the production of sherry or sherry-like wines (Zara et al. 2011). The abovementioned protein Flo11 was also found to be essential in this biological system (Zara et al. 2005). The ability of some commercial wine yeasts to form biofilms has been described with the presence of cells with different lifestyles and growth modes, such as invasive growth, bud elongation, and sporulation (Tek et al. 2018). These authors have proposed that such cellular organization allows for yeasts to colonize the winery environment. In this work, we report on the biofilm formation and composition of the halotolerant yeast strains, *K. marxianus*, *D. fabryi*, *S. etchellsii*, and *S. polymorphus*, that are of great importance in several essential industrial and biotechnological processes. We demonstrate that they can form two kinds of such communities in the presence of seawater: air-liquid biofilms on the liquid surface in glass beakers and biofilms attached to silicone tubes.

The biofilm ECM is not a passive structural material in biofilms; rather, it is dynamic and involved in nutrient sequestration, water adsorption, protection of free-living cells from environmental stress, signaling, migration, and genetic exchange (Dragos and Kovacs 2017). Both its structure and composition depend on the organism; the type of media; if any, supporting biofilm growth; and environmental or process characteristics (Edel et al. 2019). Overall, fungal biofilm ECMs appear to be complex blends of all types of biologics: proteins, carbohydrates, lipids, and nucleic acids (Mitchell et al. 2016). In all the cases described up to now, both protein and carbohydrates were the main components (40% and 43% in *A. fumigatus*, and 55% and 25% in *C. albicans*, respectively) (Reichhardt et al. 2015; Zarnowski et al. 2014).

Polysaccharides represent a key component of ECMs in all the biofilms described so far. In the yeast strains considered herein, mannose represented a very abundant monosugar in the biofilms formed in silicone tubes, and its weight percentages were around 50% for *K. marxianus* and *S. polymorphus* (Fig. 3). Arabinose was usually the most abundant carbohydrate in biofilms formed on glass beakers, whereas xylose and rhamnose were present in all tested biofilm ECMs, but at very low concentrations. The content of two other identified carbohydrates, ribose and glucose, varied significantly in the analyzed ECMs. The monosaccharide profiles of the biofilm ECMs formed in silicone tubes were similar for the four analyzed strains and completely different to those observed in glass beakers. In this sense, the main distinctive feature among the tested yeast strains was found for *S. etchellsii* biofilms grown in glass beakers, which had noticeably lower levels of arabinose. In addition, higher ribose and lower arabinose concentrations were determined in these biofilms when compared to their silicone counterparts. Overall, we concluded that the

measured monosaccharide profiles were relatively simple yet offering some potential for discriminatory studies.

Proteins represent a large portion of the biomass in most of the reported ECM analyses (Branda et al. 2005; Branda et al. 2006; Flemming and Wingender 2010). In all of the proteomes analyzed in this work (Figs. 5, 6, 7, and 8, Tables 2 and Fig. S1 and Tables S1–S9 in the Supplementary Material), the overrepresented functional protein categories corresponded to metabolism, particularly carbohydrate and amino acid biosynthesis, and response to stimuli. Glyceraldehyde-3-phosphate dehydrogenases (GAPDH), along with other glycolytic and gluconeogenic enzymes, several heat shock proteins, and antioxidant enzymes such as superoxide dismutase and catalase, were particularly abundant. These results are in agreement with previously published data on protein composition in yeast biofilm ECMs. For instance, Lattif et al. (2008) showed GAPDH to be the only upregulated enzyme of the glycolysis/gluconeogenesis pathways during the early phase of biofilm formation by *C. albicans*, and its expression level was 9.1 fold higher when compared to cell walls. Thus, GAPDH was identified as a key player during biofilm formation. Two other enzymes belonging to these pathways, phosphoglycerate kinase and fructose biphosphate aldolase, were also upregulated in maturing biofilms (Lattif et al. 2008). In addition, glutamate and nitrogen metabolism pathways were represented only in early phase biofilms, while proteins involved in purine metabolism, glycine/serine/threonine metabolism, carbon fixation, and inositol metabolism were primarily upregulated in mature phase biofilms. In the ECM of *S. cerevisiae* biofilm-like mats, GAPDH was also found (Faria-Oliveira et al. 2015) along with other proteins that were grouped into distinct functional classes, mostly including metabolism, protein fate/remodeling, and cell rescue and defense mechanisms. Interestingly, heat shock chaperones, metalloproteinases, superoxide dismutase, broad signaling cross-talkers, and other putative signaling proteins were present in abundance. In the case of *A. fumigatus* biofilm-like structures, catalase B was detected as one of the major proteins in the ECM; the presence of this enzyme was associated with its protective role against reactive oxygen species, which microbes encounter in excessive amounts while infecting a host (Reichhardt et al. 2015). In fact, catalases have been reported to play a critical role in other microbial biofilms, such as those formed by *Pseudomonas aeruginosa* (Elkins et al. 1999). It is worth mentioning that elevated expression levels of heat shock proteins have been reported during *C. albicans* biofilm formation (Becherelli et al. 2013). The similarities found between the protein composition of the biofilm ECMs of non-pathogenic yeasts tested in this work and those causing infection suggest that the presence of antioxidant enzymes and other stress response proteins is not only relevant to yeast protection during infection but also from the environmental conditions in which biofilms exist.

Many of the proteins identified herein did not contain secretion signals, and their extracellular location could not be easily explained. This striking observation is in agreement with previously published studies characterizing biofilm ECM composition. The presence of these proteins in the biofilm extracellular milieu could be due to either the use of a non-secretory pathway, which does not require leading secretion sequences, or leakage of proteins from cells undergoing cellular death (Nickel and Rabouille 2009; Nombela et al. 2006; Nosanchuk et al. 2008; Zarnowski et al. 2014). However, the existence of several hundred moonlighting proteins, which in many cases perform multiple Gene Ontology-based unpredictable functions, has been documented recently (Jeffery 2018). Often, these classically intracellular proteins are secreted extracellularly by unknown mechanisms and become involved in completely different, functionally distinct processes. For example, cellular ribosomal proteins have been identified in the *Staphylococcus aureus* biofilm ECM, where they are responsible for physical stabilization of the microbial community (Graf et al. 2019). According to these authors, the oxygen limitation in biofilms determined the release of fermentation products like formate, lactate, and acetate, which resulted in an acidic environment, while the strong positive charge located on those specific ECM proteins probably mediated electrostatic interactions with anionic cell surface components and anionic metabolites leading to strong aggregation and biofilm stabilization. The glycolytic enzyme GAPDH, overexpressed in many yeast biofilms as commented above, has also been shown to play an important role in the organization of these communities by *C. albicans*; the ability of this microorganism to form early and mature phase biofilms was significantly reduced in the presence of sodium iodoacetate, which specifically and irreversibly inhibits this enzyme (Lattif et al. 2008). It is hence difficult to assign a proper function that an individual protein could have once located in the biofilm ECM; actually, it could not be related to its cellular role.

The results described in this work reveal important differences in the content of the biofilm ECM considered both in monosugars and proteins. It is worth mentioning that the atomic composition in both materials is the same, silicon and oxygen, but their synthesis, structure, and other features are very different. In glass, atoms are linked in a crisscross network; in silicone, long lineal chains are formed and joined by cross links. Physical properties (hardness, elasticity, porosity, rugosity ...) are also very different. In fact, in glass beakers, biofilms are not attached to the glass surface (they are mainly formed in the interface between the liquid medium and air), while in silicone tubes, biofilms are made on the solid material.

Many of the enzymes found in the biofilm ECM function as an external digestive system that breaks down extracellular biopolymers later used as energy sources (Zarnowski et al. 2014). In fact, other studies of bacterial biofilms have

demonstrated their ECMs to be enzymatically active (Absalon et al. 2012; Chen et al. 2013; Jiao et al. 2011; Romani et al. 2008; Sutherland 2001). Relatively high percentages of metabolic enzymes in the biofilm ECMs of *K. marxianus* and both *Schwanniomyces* strains used in this work suggest that these yeast biofilms are more suitable as potential biocatalysts, although more thorough experimental verification would be required. In addition, differences were found with reference to the enzymes that are unique or present in higher percentages in the ECM of biofilms formed in silicone tubes and in glass beakers (see Figs. 5, 6, 7, and 8, and Table S9 and Fig. S1 in the Supplementary Material) for each one of the tested strains. This information could be helpful for the selection of the most appropriate surface for biofilm formation depending on the biocatalytic purposes.

The halotolerant yeast strains tested in this work have been extensively used in our laboratory for the stereoselective synthesis of compounds of interest for the pharmaceutical and chemical industries, using seawater in some cases as a sustainable solvent (Andreu and del Olmo 2018, 2019, 2020). Their ability to form biofilms in seawater-based growth medium as well as both the abundance and the complexity of enzymes located in these extracellular structures offer unlimited possibilities for their use as a convenient strategy for cell immobilization for biocatalytic purposes. Compared to planktonic cells, the ability of microorganisms to live in complex biofilms results in higher stability, physical robustness, and increased resistance to toxic compounds (Halan et al. 2012). All these factors strongly support the idea of using immobilized biofilms in biocatalytic applications. Although this strategy has not been widely implemented at an industrial scale, these biofilm systems have already been used for the production of valuable chemicals or manufacturing of value-added products (Edel et al. 2019). With reference to novel industrial applications, it is still necessary to gain more in-depth knowledge about and understanding of long-term operability and techniques to steer the desired processes and large-scale reactor systems. In fact, existing membrane biofilm reactors and bioelectrochemical systems appear to be promising tools for highly productive biofilms (Edel et al. 2019; Ontiveros-Valencia et al. 2018). Future studies will determine factors related to the applicability of individual biofilm structure types in existing and developing biocatalytic processes and provide better understanding of some aspects related to biotechnological capabilities of these halotolerant yeast-based biofilms.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00253-021-11132-1>.

Acknowledgements We gratefully acknowledge Dr. J. Ramos for providing us with the *Debaryomyces hansenii* strain CBS767 and the SCSIE (Universitat de València) for access to its instrumental facilities. We thank Ms. Marienela Y. Heredia for comments on this manuscript.

Author contribution Conceptualization and supervision by C.A. and M.O. R.Z.; C.A. and M.O. conceived, designed, performed research and analysis of data and wrote the paper. H.S. contributed with some experiments. D.A. was responsible for the discussion of results and supervision of the paper.

Funding This work was supported by grants from the Universitat de València (UV-INV-AE15-323062) and the National Institutes of Health (R01 AI073289).

Data availability Exhaustive information about the proteins identified in the extracellular matrix of the biofilms formed by the yeast strains considered in this work is included in the Supplementary Material.

Declarations

Ethics approval The article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare no conflict of interest.

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