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Review

The Extracellular Matrix of Yeasts: A Key Player in the Microbial Biology Change of Paradigm

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Abstract

Microbes are traditionally regarded as planktonic organisms, individual cells that live independently from each other. Although this is true, microbes in nature mostly live within large multi-species communities forming complex ecosystems. In these communities, microbial cells are held together and organised spatially by an extracellular matrix (ECM). Unlike the ECM from the tissues of higher eukaryotes, microbial ECM, mostly that of yeasts, is still poorly studied. However, microbial biofilms are a serious cause for concern, for being responsible for the development of nosocomial infections by pharmacological drugs-resistant strains of pathogens, or for critically threatening plant health and food security under climate change. Understanding the organization and behaviour of cells in biofilms or other communities is therefore of extreme importance. Within colonies or biofilms, extremely large numbers of individual microbial cells adhere to inert surfaces or living tissues, differentiate, die or multiply and invade adjacent space, often following a 3D architectural programme genetically determined. For all this, cells depend on the production and secretion of ECM, which might, as in higher eukaryotes, actively participate in the regulation of the group behaviour. This work presents an overview of the state-of-the-art on the composition and structure of the ECM produced by yeasts, and the inherent physicochemical properties so often undermined, as well as the available information on its production and delivery pathways.

Keywords: extracellular matrix; yeasts; multicellular aggregates; polysaccharides; structure; physicochemical properties

1. Introduction

Microbial cell communities occurring in biofilms, mats, flocs or colonies, are possibly the oldest, most successful and widespread form of life on Earth [1]. Nevertheless, it is a fairly new notion that, although microorganisms are able to live as individual cells, in nature they rather live in large, organized communities, often containing multiple species from very diverse levels of organization, which help them cope with constantly changing environments, and biotic and abiotic stressors. This notion raises major questions, like how these communities are structured, how do microorganisms organize collectively, providing access to food and water to the whole community, and how they deal with competitors or predators. Ultimately, how do microorganisms communicate intra- and inter-species? Answers to these questions may provide some insight on the very origin of multicellularity, while simultaneously it requires that we look at microbial communities from an ecological point of view, as being part of a greater ecosystem, be it located in a pond or a river, in a pipe of any industrial infrastructure, or co-habiting higher order organisms as part of a plant or animal microbiota.

Biofilms in particular have driven considerable attention from researchers, mostly because of the undesirable effects associated to their development. Most often they are formed by a variety of species from one or more distinct group or kingdom, including algae and microscopic animals [2]. In specific niches, they can also be formed by a single type of microorganism, like bacteria [3], filamentous fungi [4] or yeasts [5]. Importantly, biofilms are found attached to biotic or abiotic surfaces in almost all humid or aqueous environments which include urban, agricultural or industrial facilities [6–10] as well as naval and sea or river-associated infrastructures [11]. Their presence causes process inefficiency, energy waste and economic losses and contributes to the spread of invasive species in many river and estuarine ecosystems with disastrous consequences for the environment [12]. Nevertheless, biofilms are most studied in regard to human health, because of their insidious development on medical devices and hospital equipment and facilities [13,14]. In these locations, biofilms are vehicles for the development and spread of opportunistic microorganisms, which can originally be commensal, but shift into living tissue-invading virulent forms [15]. Biofilms are thus frequently the cause of nosocomial infections [16,17]. The colonization of hospital-related surfaces also favours the contact of infectious microorganisms with low and erratic doses of pharmaceuticals, contributing to the development of multiple resistances. The most well-studied of these biofilm-forming microorganisms are the bacteria *Staphy-*

lococcus aureus [3] and *Pseudomonas aeruginosa* [18], as well as the yeasts from *Candida* genus [19]. In addition, biofilms are also associated with pathologies in non-human animals [20] and plants [21], but in spite of the increased frequency and devastating consequences of these diseases [22–26], the associated knowledge is very disproportionate when compared to what is known about human diseases.

Biofilms provide cells with communal benefits, such as increased resistance to antibiotics, antifungals or other drugs, as well as to host defences [27–30]. Also, external *stimuli* derived from climate or other origins, such as temperature, osmotic or oxidative stress, or nutrient deficiency, cause a much lesser impact on the survival of individual cells within biofilms when compared to planktonic cells [31–33]. Cells within a biofilm display altered plasma membrane sterol composition [34], and an increased expression of multidrug-resistance pumps [35,36], both contributing to the higher resistance to drugs and pharmaceuticals. In these environments, cells also tend to differentiate into more invasion-prone forms, like fungal penetrating hyphae or haustoria [37,38]. Most importantly, they secrete abundant extracellular matrix (ECM) [5]. This provides physical and life support while promoting cell-cell communication and the control of differentiation, community expansion/cell division, *quorum* sensing (QS) and invasion of adjacent or farther locations, all of which are former prerogatives of the ECM from animal tissues. Microbial ECM is often designated as extracellular polymeric substance (EPS), although this designation is also very often used to refer to the actual polymers that compose the ECM.

Globally taken, the knowledge on microbial ECM composition, architecture and biological roles is rather scattered and incomplete, especially in what concerns yeasts [39,40]. Moreover, discrepancies are also found in the chemical and molecular characterization of the components of a same microorganism ECM. This has primarily to do with the different conditions in which the cells are cultivated, but it also derives from the different methodologies applied to extract and fractionate the ECM for chemical characterization [41]. For example, harvesting fungal ECM by concentrating it with ethanol, as used by Beauvais *et al*. [42], is good for precipitating polysaccharides, and has also been used to analyse the polysaccharide fraction from the biofilm ECM of other mucous-producing fungal species [43]. Nevertheless, this method is not appropriate to allow the detection/quantification of the glycoproteins in the same samples, which amount can thus end up very underestimated as discussed by Singh *et al*. [43]. On the other hand, the solubilization of the ECM deproteinized fraction in trichloroacetic acid (TCA) or other organic solvents [1,41–44] can yield different compounds. N-acetylglucosamine (GlcNAc) is virtually insoluble in water [45], while glucose and other common sugars or melanin cannot be quantified in TCA-solubilized samples. Moreover, some authors use very diverse methods for identification and quantification of the compounds in each fraction which differ substantially in accuracy and detection levels. These vary from classical chemical methods, and enzymatic or immunoenzymatic assays, to spectrophotometry [43] or complex chromatography (reviewed by [41]). Chemical fractionation is unavoidable, but the procedure may condition the analytical results, namely by jeopardizing the detection of all the compounds, especially those in smaller amounts, or by biasing their relative concentrations.

Yeasts' ECM is basically not yet biochemically and molecularly understood. What molecules and what compounds form the ECM? Which could be their specific roles in the several stages of the establishment of a biofilm or a colony? What would be their contribution to the physical properties of the biofilm and in commanding the fate and the behaviour of the cells within? What pathways and molecular mechanisms are involved in their production and secretion? These questions are mostly unanswered. Yet, the knowledge from their answers is indispensable to meet the demands of the increasingly more spread and more virulent fungal diseases in plants and animals, including humans [46–48]. This work gathers the information available regarding the ECM from yeasts, from the most well-known human pathogens, like *Candida albicans* and *Cryptococcus neoformans*, to the industrially important exopolysaccharide producer *Aureobasidium pullulans*, and the unavoidable model yeast *Saccharomyces cerevisiae*.

2. Yeasts' ECM Exopolysaccharides

The exopolysaccharides produced and secreted by yeasts, either as part of biofilms or colonies ECM, or simply accumulating in the growth media of planktonic cultures, differ in composition, structure and molecular weight (MW). Information on their chemical structure can be found in the literature for 12 species of Ascomycetes and 27 of Basidiomycetes (Table 1, Ref. [17,42,43,49–121]). In total, 21 different exopolysaccharide core structures were established for Ascomycetes, and 34 for Basidiomycetes. From these last, 95% are branched, from which, 15% are highly branched. The available information on the structure of these branches does not always match the information available on the core structure (Table 1). The (1,3)/(1,6) and α/β types of linkages, shape the 3D structure of the polymers, their branched or linear structure, and this ultimately has consequences at the level of the physicochemical properties of the ECM. Core structures from Basidiomycetes displayed 59% α- and 41% β-glycosidic bonds, while the correspondent branches displayed 78% β-bonds. These percentages were calculated using the information in Table 1. Otherwise, for Ascomycetes, the number of α- and β-bonds in the core structures was more similar, respectively 43% and 33%, but in opposition to Basidiomycetes, their branches displayed 75% α-bonds. Moreover, the core structures of the exopolysaccharides produced by both groups of yeasts contain mostly 1,3 and 1,6 glycosidic link-

ages (Table 1), which confer more flexibility to the polymer [122], while the correspondent branches contain, respectively, very variable, or mostly 1,2 linkages which confer more stiffness [122].

Glucose is the predominant sugar residue present in Basidiomycetes and Ascomycetes exopolysaccharides, seconded by mannose (Fig. 1A), *i.e.*, it exists in most of the exopolysaccharides that were characterised. Otherwise, each one of these sugars is present in different amounts in the different polymers. Mannose is the sugar residue present in higher amounts in Basidiomycetes' exopolysaccharides, while in Ascomycetes, which present a larger variety of composing sugars, including N-acetylglucosamine (GlcNac) and unspecified hexosamines not found in Basidiomycetes (Fig. 1A), glucose is the dominant monomer (Table 1). Moreover, uronic acids, which are key components of mammalian ECM polysaccharides, have also been reported to occur in some yeasts' exopolysaccharides. The basidiomycetous *Cryptococcus* and *Tremella* spp., *Rhodotorula minuta* and *Trichosporum asahii* (Table 1), all produce glucuronic acid (GlcA), and the ascomycetous *C. tropicalis* produces uronic acids which were not yet identified (Table 2, Ref. [88,90,113,123,124]). Additionally, the exopolysaccharides from both groups of yeasts also largely differ in MW (Table 1), with values globally ranging from $\leq$8 kDa to $>$7 MDa (Fig. 1B).

Many of the compounds that were listed in Tables 1 and 2 are exopolysaccharides produced by cells from planktonic culture conditions, in which cells are multiplying while submersed. Exceptions are *C. albicans* [107–109, 111,113], *C. tropicalis* [111], *C. neoformans* [17], *Knufia petricola* [116], and *S. cerevisiae* [120] whose polysaccharides were extracted from the biofilm-like cultures ECM. One single work, that of Beauvais *et al*. [42], characterized the exopolysaccharides from flocs produced by a *S. cerevisiae* naturally flocculating wild-type strain. Additionally, other yeasts unmentioned in Tables 1 and 2 were reported to produce exopolysaccharides in very low amounts ($\leq$0.2 g/L) when cultivated in wine fermentation conditions Comitini *et al*. [125]. Those are 34 yeasts strains from 11 species of *Candida*, plus *Torulaspora delbrueckii*, *Lachancea thermotolerans*, *Metchnikowia pulcherrima* and *S. cerevisiae*. Another case also not included in Tables 1 and 2 is that of *Rhodotorula acheniorum* [126], which when co-cultivated in whey ultrafiltrate with *Lactobacillus casei* or *Kluyveromyces marxianus*, was reported to produce up to 9 g/L exopolysaccharides. These examples stress how much more common than usually thought the production and secretion of exopolysaccharides by yeasts may be, even if not in the frame of multicellular aggregates.

The emerging picture from all these works suggests that Basidiomycetes tend to produce high MW highly mannosylated and highly branched polysaccharides, while Ascomycetes tend to produce smaller and less branched or linear highly glycosylated polysaccharides. The branches of Basidiomycetes polysaccharides are mostly β-(1,2) linked and those of Ascomycetes are mostly α-linked. These differences inevitably correspond to significant differences in physicochemical properties, to which add the different amounts that accumulate in the extracellular environment. Together they condition the biology of the cellular community.

3. Physicochemical Properties of Yeasts' ECM

Two important roles of microbial ECM are those of providing a support scaffold, adherent to a living tissue or an inert surface, and contributing for efficient water/nutrients supply to all the cells in the community. The nature, concentration and solubility of the components within the ECM are under the influence of environmental factors like temperature, pH and humidity and accordingly influence these multicellular aggregates physicochemical properties. These properties include viscosity and gelation that impact directly in cells access to nutrients, and exposure to aggressors, including pharmacological drugs. Most importantly, they also impact in the ability of films and colonies to retain water and allow proper hydration to the inner cell layers. In this context, the balance between several physical features of polysaccharides commands their ability to establish intermolecular associations, which strongly translate into more or less solubility [122] and into water mobilization, and consequently to more or less stiffness of the ECM.

The solubility of exopolysaccharides is mostly commanded by their MW, as well as their components and their molecular structure. Basic glycoside chemistry [122,127, 128] dictate that (i) low MW polysaccharides are more soluble than high MW ones (a polysaccharide with more than 20 oside units is supposedly insoluble in water due to a high exclusion volume [129]); (ii) for a same MW, the more soluble molecules are those bearing branched and irregular structure, while more linearity or group repetitivity leads to more insolubility, (iii) the chemical nature of monomers influences solubility. Polysaccharides containing uronic acids are more prone to interact with water. Moreover, the existence of charged residues, both positively or negatively, or chemical substitution, consisting of the addition of carboxyl, sulfuric ester or other chemical groups to the sugar monomers [122], tend to increase the interaction with water and therefore solubility when compared to neutral molecules. Finally, (iv) the type of glycosidic bond influences the behaviour of the polymer in solution. The most common linkages found in ECM polysaccharides introduce diverse degrees of flexibility to the molecules by commanding their degree of order/disorder and their state of lower energy. Solubility is higher for (1,6), followed by (1,3) and (1,4) glycosidic bonds. All these features are synergistically intercorrelated, influencing each other and the ability of a certain ECM to retain water through the solubilization

Table 1. Chemical composition and structure of the polysaccharides from yeasts' extracellular matrix.

Yeast species	Designation	Core structure	Branch structure	Size	Compostion	Ref.
Basidiomycetes						
Bullera alba	GXM	α-(1→3)-Man*p*	• β-(1→2)-Glc*p*A • β-(1→4)-Xyl*p*	-	D-Man*p*, D-Glc*p*A, D-Xyl*p*	[49]
	GalXM	-	-	-	-	
Bullera tsugae	Mannan	• β-(1→3)-D-Man • β-(1→4)-D-Man	-	20–40 kDa	D-Man*p*	[50]
Cryptococcus (Papiliotrema) flavescens	GXM	α-(1→3)-D-Man*p*	• β-(1→2)-[Xyl*p*]$_n$-α-(1→2)-Man*p* • β-(1→2)-Xyl*p*-α-(1→2)-Man*p* • β-(1→2)-[Xyl*p*]$_n$ • β-(1→2)-GlcA*p*	-	Man*p*=Xyl*p* (GlcA, Glc?)	[51]
Cryptococcus flavus	-	-	-	1 MDa	Man>Glc>>Xyl>Gal	[52]
Cryptococcus laurentii	-	-	-	4.2 MDa	Ara>>Man>Glc>Gal Rha	[53]
	Heteropolysacch.	-	-	-	Man>Xyl>Gal>Glc>GlcA	[54]
	Heteropolysacch.	-	-	8 kDa	Xyl>Man>Glc>other	[55]
	GalGXM	α-(1→6)-D-Man	Highly branched • [α-(1→2 →3 →4)-D-Man]$_{2,3>>4,5}$ with sub-branches of β-D-Xyl, β-D-Glc, β-D-Gal • α-(1→6)-D-Gal	ca 19 kDa	D-Man>>D-Gal>D-Glc=D-Xyl	[56]
	GXM	α-(1→3)-Man	• D-Xyl • D-GlcA	-	D-Man, D-Xyl, D-GlcA	[57]
	GXM	Xyl-[Man]-GlcA	-	-	D-Man>D-Xyl, D-GlcA	[58]
	Glucan	(1→2 →3 →4 →6)-Glc	-	-	D-Glc	
	GXM	α-(1→2)-α-(1→3)-Man	-	-	D-Man, D-Xyl, D-GlcA	[59]
	GM	α-(1→6)-D-Man	α-(1→3)-D-Man-[α-(1→2)-Man]$_{2,3,4}$ with sub-branches ofα-(1→2)-D-Glc	-	D-Man>>>D-Glc	[60]
	Amylose	-	-	-	Glc	[61]
	GXM	α-(1→2 →3)-D-Man	α-(1→2)-D-Man	• 1 MDa	D-Man>D-Xyl>D-Gal	[62]
	GalGXM	α-(1→6)-D-Man		• 60–400 kDa	GlcA	
	GM	α-(2→6) (3→6)-D-Man		• 25–60 kDa	β-Xyl, β-GlcA	
	Amylose	α-(1→6)-D-Gal				

Table 1. Continued.

Yeast species	Designation	Core structure	Branch structure	Size	Compostion	Ref.
Crytococcus neoformans	GXM GalXM	α- and β x *p* and *f*	-	-	Man>>Xyl>GlcA>Gal	[63]
	GXM and others	-	-	-	Man≈Glc≈Xyl>>Rha> Fuc>Gal>GlcA>Rib	[17]
	GMX	[α-(1→3)-D-Man*p*]$_n$	• [β-(1→2)-D-Xyl*p*]$_{0-2}$ • [β-(1→4)-D-Xyl*p*]$_{0-2}$ • β-(1→2)-D-Glc*p*A	-	Man>>Xyl>GlcA	[64–67][4]
Kazachstania unispora	-	-	-	-	Gal≥Glc>Man	[68]
Kuraishia capsulata	Mannan Phosphomannan	[→6)-α-Man*p*-(1→2)-α-Man*p*-(1→2)-α-Man*p*-(1→] β-Man*p*-(1→2)-[α-Man*p*-(1→2)-(1,6-*P*)→2Man*p*]	-	360 kDa 635 kDa	Man	[69]
Moniliella pollinis	-	α and β linked		-	Glc>>Man>Gal	[70]
*Pseudozima sp.*NII 08165	-	-	-	1.7 MDa	Man>Glc=Gal	[71]
Rhodosporidium babjevae	-	β-linked	-	• 1 MDa • 500 kDa • 200 kDa	Man, Glc	[72]
Rhodosporidium paludigenum	Pullulan	[→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→]n [3]	no	-	Glc	[73]
Rhodotorula acheniorum	Mannan I	-	-	• 310 kDa	• Man>>Glc	[74]
	Mannan II	-	-	• 249 kDa	Man>>Glc>Gal>Fuc>Ara	[75]
	-	-	-	-		
Rhodotorula bacarum	Pullulan	-	-	-	Glc	[76]
Rhodotorula glutinis	-	-	-	-	Man>Glc>Ara	[77]
	-	-	-	-	Man>Glc>Gal>Xyl	[78]
	-	-	-	-	Man>Gal>Glc>Xyl	
	-	alternate β-(1→3) and β-(1→4)-D-Man	-	-	Man*p*	[79]
	-	-	-	100–380 kDa	Man>>Fuc>Glc=Gal	[80]
Rhodotorula minuta	-	β-linked	-	-	Man, Glc	[81]
	-	-	-	-	Glc>Man>Rha	[82]
Rhodotorula mucilaginosa	-	β-D-Glc*p*	(1→2 →3 →6)-D-Glc*p* and D-Man*p*	84 kDa	Glc>>Man	[83]
	RESP2-A	β-(1→3)-Gal	Highly branched • β-(1→2 →3)-Glc • β-(1→4 →4,6)-Man • β-(1→2,3,4)-Ara Highly branched	7.1 MDa	Gal>>Glc=Man>>Ara	[84]
Rhodotorula rubra	Rhodexman	• β-(1→3)-D-Man • β-(1→4)-D-Man	-	300–500 kDa	D-Man*p*	[50] [85]
	-	-	-	-	Man>>GlcA>Glc>Gal>Xyl>Ara	[86]

Table 1. Continued.

Yeast species	Designation	Core structure	Branch structure	Size	Compostion	Ref.
Sporidiobolus pararoseus	-	-	Highly branched	7.4 MDa	Gal>Glc>Man	[87,88]
Sporobolomyces salmonicolor	Mannan	β-Man	-	>1 MDa	Man*p*>>Glc>Gal	[89]
	Mannan	-	-	-	Man≈Glc	[90]
Sporobolomyces singularis[1]	Trisacch. Tetrasacch.	[β-(1→4)-D-Gal*p*]$_{2-3}$-β-(1→4)-D-Glc*p*	-		Gal*p*, Glc*p*	[91]
Tremella sp[2]	-	α-(1→3)-Man	β-D-Xyl (inc)	162 kDa (av)	Man, Xyl, GlcA	[92]
Tremella aurantialba	TAPS	-	-	2.9 MDa	Man>> GlcA>Man-N>Glc>Xyl	[93]
Tremella fuciformis	-	α-(1→3)-Man – highly branched at C2	• β-(1→2)-D-Xyl • D-GlcA	-	Man*p*> Xyl, GlcA	[92,94]
	Tremellan	-	-	-	• Man*p*> Xyl, GlcA • GlcA>Man, Xyl	[95]
Tremella mesenterica	Tremellan	α-(1→3)-Man*p* – 80% branched at C2	• β-(1→2)-D-GlcA*p* • β-(1→2, 4)- D-Xyl*p*	-	Man, Xyl, GlcA	[92,96]
	Pullulan	[→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→]$_n$ [3]	no	ca 36 kDa	Glc	[97,98]
Trichosporon asahii	GXM	[α-(1→3)-D-Man]$_6$	• β-(1→2, →6)-D-Glc*p* • β-(1→2, →6)-D-Xyl*p* • β-(1→2)-D-Xylp-β-(1→4)-D-Xyl*p* • β-(1→2)-D-Xyl*p*	-	Man>Xyl>Glc≈GlcA	[99,100]
	GXM	-	-	-	-	[101]
	Glucan	β-(1→3)-D-Glc	-	-	-	
Ustilago maydis	Glucan	β-(1→4)- β-(1→3)-D-Glc	Interlinked arrangements	• 27–65 kDa • 44 kDa	Glc	[102]
Ascomycetes						
Aureobasisium pullulans	Pullulan	[→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D--Glc*p*-(1→]$_n$ [3]	no	260 kDa	Glc	[43,98,103,104]
	Aubasidian	β-(1→3)-D-Glc*p*	α-(1→6)-D-Glc-[α-(1→4)-D-Glc]	-	Glc	Elinov *et al.,* 1975 *in* [43,105,106]
Candida albicans	GMCx	β-(1→6)-Glc-[α-(1→6)-Man]$_9$	• [α-(1→2)-D-Man]$_{2-3}$ • [α-(1→2)-D-Man]$_{3-4}$	• High MW • Low MW	Ara>Man>Glc=Xyl	[107]
		β-(1→3)-Glc	-	-	-	
	MGCx	α-(1→2)-Man α-(1→6)-Man 6-α-(1→6)-Man (2→6)-α-(1→6)-Man	α-(1→2)-Man-α-(1→2) α-(1→2)-Man-α-(1→3) 3-Man-α-(1→2) β-(1→2)-Man-α-(1→2) Man-α-(1→2) Man-β-(1→2)	• High MW • Low MW	-	[108]

Table 1. Continued.

Yeast species	Designation	Core structure	Branch structure	Size	Compostion	Ref.
	Glucan	β-(1→3)-Glc	-	-	-	[109]
	-	β-(1→6)-α-D-Glc β-(1→3)-β-D-Glc, -α-D-Rha, β-D-GlcNAc	-	300 kDa	Glc >> Man, Rha, GlcNac	[110]
	Glucan	β-(1→3)-Glc	-	-	Glc>>Hexosamine>UAs	[111]
	-	-	-	-	Glc; Gal; Hexosamine	[112]
	Monosacch.	-	-	-	Glc	
Candida famata	Glucan	α-(1→4)-Glc	-	-	Hexosamine>>UAs	[113]
Candida glabrata	MGCx	α-(1→2)-Man α-(1→6)-Man 6-α-(1→6)-Man (2→6)-α-(1→6)-Man	α-(1→2)-Man-α-(1→2) β-(1→2)-Man-α-(1→2) Man-α-(1→2)	• High MW • Low MW		[108]
Candida parapsilosis	MGCx	α-(1→2)-Man α-(1→6)-Man 6-α-(1→6)-Man (2→6)-α-(1→6)-Man	α-(1→2)-Man-α-(1→2) α-(1→2)-Man-α-(1→3) 3-Man-α-(1→2) β-(1→2)-Man-α-(1→2) Man-α-(1→2) Man-β-(1→2)	• High MW • Low MW		[108]
Candida tropcalis	GM	α-(1→2)-D-Man and/or α-(1→3)-D-Man	• α-D-Man*p*-(1→3)-D-Glc • α-D-Man*p*-(1→4)-D-Glc • α-D-Man*p*-(1→3)-[D-Man*p*-(1→4)]-D-Glc • α-D-Man*p*-(1→6)-D-Man*p*-(1→3 or 4)-D-Man*p*-(1→4 or 3)-D-Glc • α-D-Man*p*-(1→3)-[D-Man*p*-(1→4 or 6)]-D-Man*p*-(1→6 or 4)-D-Man*p*	74 kDa	Glc>Man*p*	[111]
	MGCx	α-(1→2)-Man α-(1→6)-Man 6-α-(1→6)-Man (2→6)-α-(1→6)-Man	α-(1→2)-Man-α-(1→2) β-(1→2)-Man-α-(1→2) Man-α-(1→2) Man-β-(1→2)	• High MW • Low MW		[108]
Candida utilis	Pullulan Gal*f*M	α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4) α-(1→2)-Man*p*	- β-(1,6)-Gal*f*	- -	D-Glc Man, Gal	[114,115]
Knufia petricola	Mannan PM	[→6)-α-Man*p*-(1→2)-α-Man*p*-(1 →2)-α-Man*p*-(1→]$_n$[3] [β-Man*p*-(1→2)-(6P)-Man*p*-(1]	-	-	-	[116]
Kuraishia capsulata	Glucan Mannan Rhamnan	α- and β-(1→6)-D-Glc α-(1→6)-D-Man α-(1→3)-(1→2)-Rha*p*	-	-	Glc, Man, Rha, Rib, Fuc, Xyl, D-Gal	[69]

Table 1. Continued.

Yeast species	Designation	Core structure	Branch structure	Size	Compostion	Ref.
Lipomyces starkeyi	Heteropolysacch.	α-(1→3)-(1→2)-Rha*p* α-(1→6)-Man α and β (1→3)-Glc	-	-	α-, β-D-Glc, α-D-Man, α-L-Rha	[117]
Nakazawaea (Hansenula, Pichia) holstii	PM	α-(1→6)-D-Man	• [-6-*O*-PO_3H_2-[α-D-Man*p*(1→3)]$_3$-α-D-Man*p*-(1→2)-α-D-Man*p*(1→] • [-2-Man*p*(1→]	1 MDa	Man*p*	[118,119]
Saccahromyces cerevisiae	- Monosacch. Mannan Monosacch.	- - (1→6)-Man -	- - • β-(1→3)-D-Man • β-(1→2)-D-Man -	35–40 kDa <8 kDa - -	Glc>Man>>Gal Glc • Glc>>Man • Man>>Glc Glc	[120] and our group unpublished results [42]
Schizosaccharomyces pombe	GalM	$Hex_9[GlcNac]_2$ $Hex_8[GlcNac]_2$	-	-	Man>Gal>>GlcNac	[121]
Schizosaccharomyces japonicus	GalM	• α-(1→2)-D-$Man_9[GlcNac]_2$ • $Gal4Man_{10}[GlcNac]_2$ • $Hex_8[GlcNac]_2$	-	-	Man≠Gal>>GlcNac	[121]

All the results from *Basidiomycetes* were obtained in planktonic cultures, in batch or fermentors. Exceptions are (i) *Cryptococcus neoformans* [17] that was cultivated to form a biofilm, and (ii) *Tremella fuciformis* which consisted of a commercial sample of this fungus fruiting body [94]. Otherwise, the results presented for *Ascomycetes* vary. Some were obtained using liquid cultures and others biofilms, and yet the results from *Saccharomyces cerevisiae* from Beauvais *et al*. [42] were obtained from flocs under fermentative conditions. Some abbreviations: GM, glucomannan; Glc*p*, glucopyranosyl; GlcA, glucuronic acid; GalM, galactomannan; Gal*f*M, Galactofuromannan; PM, phosphomannan; Man-N, mannosamine; GXM, glucuronoxylomannan; GalXM, galactoxylomannan; GalGXM, galactoglucoxylomannan; GMCx, glucan-mannan complex; GlcNac, N-acetylglucosamine; UAs, Uronic acids; (inc), incomplete information; - unknown; no – non-existing.

[1] The yeast was cultivated in yeast extract with lactose instead of glucose as sole carbon source.

[2] *Tremella aurantia, T. globospora, T. foliacea. T. indecorata, T. encephala, T. cinnabarina, T. brasiliensis* [92].

[3] Linear maltotriose homopolymer linked 1-6, with a variable number and position of maltotetrose units identically linked.

[4] Authors describe GXM as a heteropolysacchride from the capsule of *C. neoformans.*

Table 2. Yeasts that were reported to produce large amounts of exopolysaccharides, which were not chemically and/or structurally characterized.

Yeast species	Carbon sources yielding exopolysaccharides maximum production	Optimal Production (g/L)	Ref.
Candida famata	Maltose	≈2.0	[113]
Candida guillermondii		≈3.0	
Cryptococcus flavus (3 strains)	Sucrose	2.7–3.9	[90]
Debaryomyces hansenii		≈4.0	
Pseudozyma antartica		3.9	
Rhodotorula glutinis		3.8	
Rhodotorula minuta (15 strains)		1.9–2.6	
Rhodotorula mucilaginosa		134.8	[123]
Rhodotorula sp CAH2		7.5	[124]
Sporobolomyces roseus (2 strains)		≈5.0	[90]
Sporodiobolus pararoseus	Glucose	13.1	[88]

of its components, in particular at a microscale. An example of a readily water-soluble polysaccharide is that of pullulan. In spite that it usually consists of a very large number of maltotriose repeating units, and therefore has a large MW and repetitive structure, it is a polydisperse molecule in which the α-(1,4) links within maltotriose units alternate with α-(1,6) links connecting them, which confers more flexibility to the polymer, even at high polymer concentrations [130]. All considering, the highly branched structure of Basidiomycetes polysaccharides should correspond to a more disordered conformation, due to the absence of repetitive periodic molecular features, promoting the interaction with water molecules. This is though counteracted by the tendentially high MW, since the heavier the polysaccharide, the less soluble it is. The opposite should be true for Ascomycetous linear or low branched but lower MW polysaccharides.

The retention of water at a microscale within biofilms and colonies is allowed by the ECM, therefore maintaining the hydration of the cells [131]. Together, all the physicochemical features above-mentioned concur to affect solubility, but may also contribute to the formation of hydrophobic pockets, caging water molecules, which play an important role in the ECM gelling process [132], providing lowest entropy stabilization to the ECM. Pore-scale water content is acknowledged to control the hydraulic connectivity of bacterial communities, and the retention of water in the soil at a microscale is promoted by bacterial EPS [122,131]. Accordingly, soil bacteria contribute positively to the maintenance of moisture by producing ECM/EPS, although the actual physical influence of those polysaccharides in soil aggregation and structure is not yet fully understood [133]. Hydrophobic pockets can also be generated by the presence of rhamnose residues [127,128], which were so far only found in a few yeasts (Table 1). Noticeably, *Lipomyces starkeyi* produces rhamnan [117], which is an exception in all the yeasts' ECM so far characterized. This yeast is a probiotic, which ECM/EPS production was enhanced with the goal of prolonging effective adhesion to the gastrointestinal tract [117]. This work was based on the knowledge that the ECM/EPS from lactic acid bacteria probiotic strains, namely *Lactobacillus lactis*, has the physiological function of improving bacteria colonization of the mucosae, besides other important roles like those of antitumor and immunomodulating substances [134].

According to Sutherland [127,128] the polysaccharides from microbial ECM may adopt more or less disordered forms according to the external physical conditions, including temperature and pH, but they tend to stabilize. The water retention by ECM depends, namely, on the ability of the sugar molecules to establish several types of chemical bonds with the water molecules, like London forces established with water dipoles, or H bonds established with the more hydrophilic sites of each osidic unit of both neutral or ionic polysaccharides [135]. Ionic polysaccharides establish more H bonds, building hydration spheres around each ionic group, which in turn contribute to the formation of double H bonds with the remaining hydrophilic groups of the glucopyranosyl unit. This is further influenced by the presence of ions in solution, like Ca^{2+} [122,131], as well as pH. Generally, higher concentrations of monovalent ions are needed to yield gels equivalent in strength to those induced by divalent counterions. Therefore, these ions tend to promote cross-linking between the polymers. Most of exopolysaccharides in microbial ECM adopt a helical configuration, often double or triple, which interaction with each other is facilitated by these types of weak chemical bonds, which are more easily formed when the polysaccharides contain anionic groups derived from uronic acids, phosphate groups or their amphipathic nature like that of L-rhamnose (Table 1) or L-fucose [127,128]. Microbial ECM, can thus basically be considered as a hydrogel of entangled polymers maintained by weak non-covalent interactions, which should attain for poor tensile strength and low resistance to shear mechanical forces. Intermolecular bonding associated with gel formation is also highly governed by the presence of auxiliary (guest) molecules, like for example pyruvate, acetate

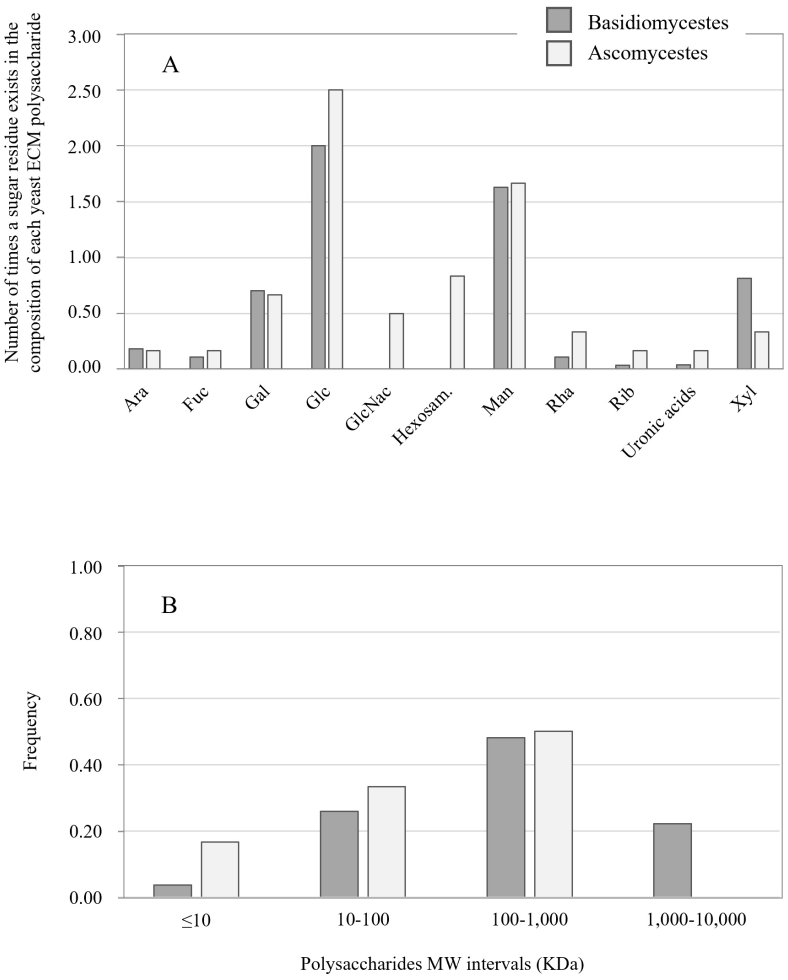

Fig. 1. Analysis of the data in Table 1 on the exopolysaccharides secreted by Basidiomycetous and Ascomycetous yeast species. (A) Number of times a given sugar is present in the polysaccharide molecule composition. (B) Distribution of the exopolysaccharides molecular mass.

or other acids, which may also cross-link the ECM polymers, potentially increasing its stiffness. That occurs in bones' mineral platelets [136]. Yeasts secrete considerable amounts of citrate [137] or acetate [138] during fermentation. These acids influence the pH and may constitute a resource carbon source. Importantly, in view of all the above, they may also actively participate in the chemical structuring of microdomains/niches with specific properties within the biofilm or colony. Intermolecular bonding is therefore ultimately potentiated by the abundance or scarcity of water, ions or guest molecules in the close vicinity of the ECM. In extensively branched polymers, when interacting polymers are different, the stability of the interactions is higher, generating a stiffer gel.

Exopolysaccharides produced by yeasts may in addition suffer a yet unknown degree of chemical substitution, e.g., sulfation. This is a common feature of the ECM exopolysaccharides from other species prompting their commercial exploitation for pharmacological applications. An example is that of ascidians which ECM provides heparan sulfate with anticoagulant and anti-cancer properties [139]. Importantly, the presence of chemical groups can easily be reverted, which transforms the ECM into a chemically and physically very dynamic environment, able to rapidly adjust to shearing forces, as well as demanding pressure, temperature or pH conditions. In biofilms formed by the filamentous fungus *Aspergillus fumigatus*, polysaccharides GAGs are deacetylated in order to turn them cationic, a property that was found to be required for adherence and biofilm onset [140]. Accordingly, a deacetylase complex from *C. albicans* that operates regularly on histones was found to mediate this yeast biofilm dispersal and drug resistance [141]. Notably, in biofilm-like mats of *S. cerevisiae*, the purified ECM polysaccharides induced metachromatic shift, which is suggestive of chemical substitution, corroborated by their separation by diaminopropane agarose gel electrophoresis [120]. Although the authors clearly associated these properties with the deletion of the *O*-acyltransferase-encoding *GUP1*, the subject was not further explored.

The ECM though contains large amounts of other molecules [127,128], notably proteins that will certainly not only functionally contribute to a high microenvironmental

diversity, but also contribute to shape the physicochemical properties in every niche. Different polysaccharides interacting with several amounts of different proteins, as well as lipids [107] and e-DNA [142,143] will undoubtedly generate a dynamic environment with space-specific properties, including rigidity/strength and sheer stress resistance. The ECM mechanical properties must therefore be envisaged as multifactorial and sensibly a synergistic interaction of complex microsystems of matter and energy transfer.

Sheer stress is caused by fluid flow, which is a common feature to microbial biofilms adhering to ship's hulls, pipes, catheters or blood vessels, where it translates into localized dynamic combinations of tensile and compression forces to the microbial cells. In animal cells the transduction of mechanical stress into chemical signals (mechanotransduction) is specifically conducted through protein connections between the cell and the ECM (mechanotransduction foci) [144]. Although the correspondent signalling pathways are still mostly unknown, mechanotransduction does cause changes in gene expression [145]. A number of ion channels have been shown to be gated by mechanical stimuli and playing putative roles in mechanotransduction, like the degenerins or the Na^{2+} channels (ENaCs) from *Caenorhabditis elegans* [146,147] or the ENaCs from the human epithelium Na^{2+} channels [148,149]. In yeasts there are multiple mechanic force-dependent channels and sensors with mechanosensitive extracellular domains like the Wsc1-4, Mtl1-2, Mid2 and Dfi1 from *S. cerevisiae*, *C. albicans* and *Schizosaccharomyces pombe* [144]. All of these sensors activate conserved signalling pathways/MAPK cascades regulating the expression of sets of genes that are mostly involved in the cell wall integrity (CWI) and maintenance. Additionally, yeasts also contain Mid1 stretch-activated Ca^{2+} channel sensing multiple mechanical signals. Ca^{2+} influx regulates calcineurin pathway and cell cycle through the transcription factor Crz1 which has a very important role in cell polarization processes mandatory for survival in biofilms [144].

Another way that yeasts display to sense mechanical stress occurs through osmosensors, the cell surface mucins Hkr1 and Msb2 and the transmembrane Sln1/Sho1. These primordially sense a_w/osmotic stress/shock and activate HOG pathway and other cross-talking signalling pathways to control filamentation and the production and retention of the osmolyte glycerol [144,150,151]. HOG thus contributes to regulate internal yeast cell hydrostatic pressure/turgor (also designated as osmotically active cell volume or intracellular osmolarity). Specific kinds of mechanical stress, like stretching, will also be sensed at the level of the membrane eisosomes. When these are flattened, the associated TORC effectors Slm1 and Slm2 are released and TORC2 is activated, which operates as a master regulator of membrane and wall-related processes [152]. TORC2 controls membrane and protein homeostasis [144,153], including the synthesis of sphingolipids and cytoskeleton reorganization which is also indispensable for polarization and invasion, as well as differentiation into pseudo or true hyphae. But maybe more important is that TORC2 and the ESCRT complex [154] are functionally related in the dependence of calcineurin [155]. Ultimately, any form of mechanical stress [144] may trigger an ensemble of pathways, the CWI, Calcineurin, HOG and TORC2 and Ras/cAMP/PKA, all converging to obtain a concerted and coherent cellular response, which eventually re-establishes a liveable homeostasis that includes adaptations in cell volume and morphology/differentiation demanding plasma membrane and wall remodelling.

One single protein has so far been clearly associated with yeast's ECM physicochemical properties, the *S. cerevisiae O*-acyltransferase Gup1 [156]. Biofilm-like mats of Δ*gup1* null mutant, in opposition to the wild type strain, produce a sludge-like ECM which lacks one polysaccharide compared to wild type [120], and harbours approximately 15% less proteins, 26% of which are not found in the *wt* ECM [157]. The Gup1 protein is a putative regulator of multiple cellular interwined functions (reviewed by [156]). Accordingly, it is found in multiple subcellular localizations, mostly at the plasma membrane, where it associates with the Pil1 eisosomes structural protein [158]. Eisosomes have been suggested to serve as membrane reservoirs for turgor-derived stretching, which would be consistent with the exacerbated membrane proliferation induced by Gup1 over-expression [159]. Anyway, the tripartite connection between *GUP1*, eisosomes and the ECM could rather relate with these structures mediating the exit of proteins important for the ECM generation/remodelling. Accordingly, the proteome from the ECM generated by the mutant Δ*gup1* was much smaller than the one from the correspondent *wt* strain [157].

Other proteins that could be related to yeast ECM construction and remodelling are the yeast counterparts of the human ECM metalloproteases. These enzymes have a prominent role in human tissues ECM remodelling, which is associated with normal physiology and pathology, as well as embryonic development, wound healing and metastasis [160,161]. They regulate tissue density and stiffness, by modulating the size, structure and amounts of ECM components under the control of major regulatory signalling pathways (namely TGFβ, insulin-like growth factor (IGF) and PI3K/Akt), or sensors which transduce mechanic signals into molecular responses [162]. The yeast *S. cerevisiae* genome encodes 47 metalloproteases. From these, Prd1 (a metallo-endopeptidase) and Ape2 (an aminopeptidase) belong to the mammalian ECM Zn-containing metalloproteinases superfamily 55486, grouping enzymes involved in collagen processing and degradation [163]. Others belong to the Zn-Dependent Exopeptidases Superfamily 53187, Lap4 (a Zn-metalloproteinase), Dug1 (a metallo-dipeptidase) and Ecm14 (a Zn-carboxipeptidase). All these proteins are found in the ECM from *S. cerevisiae* biofilm-

like mats, some only in the ECM and not in liquid media planktonic cultures (Pdr1, Ape2, Dug1) and others (Lap4 and Ecm14) in putatively higher amounts than in those cultures [157]. Whether any of these enzymes does participate in the ECM remodelling, remains to be seen. Moreover, several α-saccharide remodelling enzymes were present, like the Glc3 (glycogen branching enzyme), the Mnn2 (α-(1,2)-mannosyltransferase) and the Sga1 (glucoamylase involved in glycogen degradation). This is consistent with the idea that the synthesis of the ECM polysaccharides occurs extracellularly suggested by several authors (e.g., [154]).

4. Conditions for Exopolysaccharides Production by Yeasts

The structure and composition of microbial exopolysaccharides as well as their production yield depend on features of the culture cultivation conditions and the medium composition. Some of the environmental requirements mentioned in the literature for the production of exopolysaccharides by yeasts regard general cultural specificities of yeasts. These include cultivation temperature, aeration and initial pH. Many yeasts species grow optimally at temperatures ranging 25–30 °C. Fewer species are known prefer lower or higher temperatures, like the human pathogens which optimal temperature for growth is 37 °C. Although many of the production conditions reported fall within that temperature range, the optimal temperature for yeast growth does not necessarily match the optimal temperature for the production of exopolysaccharides by the same yeast. For example, *R. acheniorum* optimal growth temperature is rather exceptionally at 20 °C but production is maximum at 26 °C [74].

Another relevant factor is that of O_2 availability during culture growth. Literature often refers the requirement of abundant aeration for yeasts to produce exopolysaccharides [164,165]. The best producers, *Aureobasidium*, *Candida*, *Cryptococus*, *Pichia* and *Rhodotorula* spp, are respiratory yeasts [166], which batch cultures naturally require a high air/liquid ratio (1:5–1:10) often obtained by high orbital shaking speed (150–200 rpm). Still, the highest exopolysaccharide production by *R. acheniorum* [167] or *Sporidiobolus salmonicolor* [168] were obtained at higher aeration, achieved by up to 500 rpm orbital shaking [165]. Vlaev *et al.* [168] showed that increasing orbital shaking even more (up to 800 rpm) was counterproductive.

The initial pH of a culture, for most of yeast common media naturally lie around 5–6. During yeast population increase it will rapidly achieve values as low as pH 1–2. Several works mention that an initial pH of 4–5 is mandatory for exopolysaccharide production [169–171]. Nevertheless, this is probably not a generalizable requisite [41,165,170]. For example, *Rhodotorula mucilaginosa* was reported to produce most at initial pH 6.0 [83]. Although different optimal pH values have been reported [170], the production of exopolysaccharides could be related with the very low pH achieved at the end of culture growth. Cho *et al.* [80] showed that *Rhodotorula glutinis* only produced exopolysaccharides if the pH of the culture was allowed to lower beyond pH 2.0. Identical claim was made by Pavlova and Grigorova [167] for the production of exopolysaccharides by *R. acheniorum*. This is concomitant with the realization that exopolysaccharide production only occurs during pre-stationary/stationary growth phase, *i.e.,* when pH is lowest. In that phase, according to several authors [75,80,165,167], the nitrogen source should be exhausted or near exhaustion, but the carbon source should still exist, a condition that is only achieved if the C/N ratio of the culture media is very high. This agrees with so many other authors claiming the need for a very disproportionate C/N molar ratio like the $\pm$ 15:1 suggested by Cho *et al.* [80] for *R. glutinis*, the 20:1 suggested by Gientka *et al.* [165] for *R. acheniorum*, or the 10:1 suggested by Seo *et al.* [170] for *A. pullulans*. Otherwise, a higher proportion of N source would lead to considerably higher amounts of biomass being produced. Nevertheless, this requirement cannot be generalized. *R. minuta* and *R. mucilaginosa* were best at producing exopolysaccharides at C/N ratios of 0.2–0.3 [81,83,123]. Moreover, the pH and the type of N source are linked. Mineral sources like ammonium salts, in opposition to organic nitrogen sources like yeast extract, promote the achievement of a lower pH and concomitantly a higher production of exopolysaccharides [75,80].

The possible reasons for the pH-N link are well documented in *S. cerevisiae*. Although one should be cautious to generalize the information available for *Saccharomyces* spp to less-conventional yeasts, specifically because their metabolism is often respiratory and not respiro-fermentative, the proteins involved in ammonium transport are extremely conserved throughout plants, animals and microbes of diverse types [172,173]. This way, in *S. cerevisiae*, at ammonium concentrations $\leq$5 mM, the uptake of this ion, and consequent depletion from the medium, is performed by the Mep1 and Mep2 proteins from the AMT-Mep-Rh superfamily of ammonium transporters. The transport mechanism involves the high affinity binding of NH_4^+ by the hydrophilic periplasmic domain of the transporter, followed by its deprotonation into NH_3 and H^+ inside the protein which is compatible with the medium pH not influencing the NH_4^+ deprotonation and transport [173,174]. The transmembrane channel of these permeases is rather narrow [175,176], allowing only the entry into the cell of either NH_3 alone or NH_3 together with H^+ [174,177]. The hypothesis of a H^+ symport has not been ascertained yet, but is favoured by evidence from plant and bacteria AMTs [172], and because all H^+ symporters work at their best at medium pH $\leq$5.0, favoured by a high proton gradient. Additionally, voltage-clump experiments using oocytes-reconstituted plant Amts revealed currents concomitant with ammonium transport, and a rise of internal pH was observed in proeolipossomes-reconstituted

AmtB from *Escherichia coli* consistent with the inwards co-transport of H^+ [173]. Ammonium-derived ammonia has been described as mediating inter-colony communication [178]. Ammonia is produced in pulses by one colony, diffuses and reaches the neighbouring colonies. Amazingly, the first pulse is non-directed, but the second targets the neighbour colony, and causes growth inhibition of the facing parts of both colonies. Although the experimental framework is absent, these observations are consistent with the production of ECM being inhibited by the presence of ammonium, therefore impairing colony enlargement and ensuring directionality. Since Mep1 and Mep2 ammonium transporters are also ammonium sensors, it is possible that they ensure a back-and-forth *trade* of ammonium between the two colonies influencing both at the same time into not producing ECM. In opposition, the microbial ECM supposedly dictates the amounts and diffusion extent of QS molecules [179], whether these are restricted to ammonia or not.

In yeasts, the performance of H^+ symporters is generally coupled with that of Pma1, the yeast plasma membrane H^+-ATPase pump, which exports H^+ to maintain the proton gradient functional, causing medium acidification [180–182]. This concurs with the observation that growing the yeasts in inorganic ammonium salts increases medium acidification as they are exhausted, their transport inwards being favoured by the negative in-out pH gradient, followed by H^+ extrusion by H^+-ATPase. The high oxygenation requirement for the exopolysaccharide production cultures might contribute to increase mitochondrial respiration and ATP supply in those circumstances, although it does not explain why the amount of available O_2 must be so high. In the end, the medium acidifies as N is exhausted, which might be needed for gene expression purposes, since the N-depletion responding signalling pathways command filamentation and cell wall integrity. They might concomitantly command ECM formation.

In line with the above, the chemical composition of the culture media is probably the most demanding condition for optimal exopolysaccharide production. The yield is influenced not only by mineral *versus* organic nitrogen sources but also by carbon source. Yeasts produce exopolysaccharides while growing on many sugars, hexoses, pentoses and disaccharides, which could mean that yeasts produce exopolysaccharides in basically every sugar they can metabolize. Yet, most studies consider sucrose as the ideal carbon source promoting the best yields [113,165,168]. Importantly, the carbon and nitrogen sources can affect not only the yield but also the composition of the exopolysaccharide [113,165,183]. Grigorova *et al*. [75] showed that *R. acheniorum* MC cultivated in media with different carbon sources presented changes in yield, which was highest when cells were cultivated on sucrose, as well as in the chemical composition of the exopolysaccharides. The relative percentages of the composing sugar monomers varied, particularly those of fucose (35% variation between results obtained using glucose, galactose, sucrose or dextrose syrup, Sirodex®), and glucose (31% variation in the same media), although mannose and glucose kept being the most represented monomers. The possibility should therefore be considered that ammonium salts might also interfere with the composition of exopolysaccharides, and/or their structure, according to the fact that in yeasts, several major cross-talking signalling pathways respond to ammonium exhaustion in concert with carbon source availability.

5. Biosynthesis of Exopolysaccharides by Yeasts

Most of the yeast species which ECM components have been identified (Table 1) have no information available on what enzymes/genes are involved in their production and how they are regulated. An exception is that of pullulan production by *A. pullulans*, although pullulan is mostly secreted during planktonic growth. Pullulan is a linear α-(1,6)-glucan, made of repeating maltotriose (α-(1,4)-glucose) units. The pathway displayed in Fig. 2A was proposed for *A. pullulans* [164,184]. It still contains many unanswered questions, namely, (i) which lipid is targeted by the enzyme responsible for Step 1, (ii) how the addition of the 3rd glucose monomer after Step 3 is achieved, and (iii) how elongation as a whole is achieved and controlled. Additionally, evidence concerning which enzymes would be responsible for all those reactions is also missing. On the other hand, α-glucans like pullulan are part of some yeasts' cell wall, namely those of *S. pombe* [185], and several dimorphic human pathogens, *Histoplasma capsulatum, Paracoccidioides brasiliensis* and *Blastomyces dermatitidis* [186]. In *S. pombe,* α-glucans are synthesized via the integral membrane multidomain α-glucan synthetase *AGS* [184,185]. This protein has three distinct domains, (i) the intracellular domain with α-amylase activity (Amy-D), which is responsible for the synthesis of long chain α-glucans, (ii) the extracellular domain, a glycogen synthetase (Gys-D) that acts as an α-glucan remodelase, and (iii) the transmembrane domain (EPST-D), which permeates outwards long chain α-glucans [187]. Based on the evidence from *S. pombe*, Wei *et al*. [188] proposed a new pathway for the production of pullulan by *A. melanogenum* (Fig. 2B), different from that proposed for *A. pullulans*, based on the multifunctional AmAgs2. The synthesis of a long α-(1,4)-glucan precursor chain from smaller primers occurs intracellularly and precedes the formation of the maltotriose units. This long chain is then transported into the periplasm where it is subsequently fractioned and combined to form pullulan. The pullulan primers are short chain α-glucans which are synthesized by enzymes that usually perform the synthesis of ceramides and phospholipids intermediates, namely Gcs1 and Sgt1, respectively ceramide and sterol β-glycosyltransferases, as well as the glyconenins Glg1 and Glg2 [188]. In *S. pombe*, although the three domain Ags

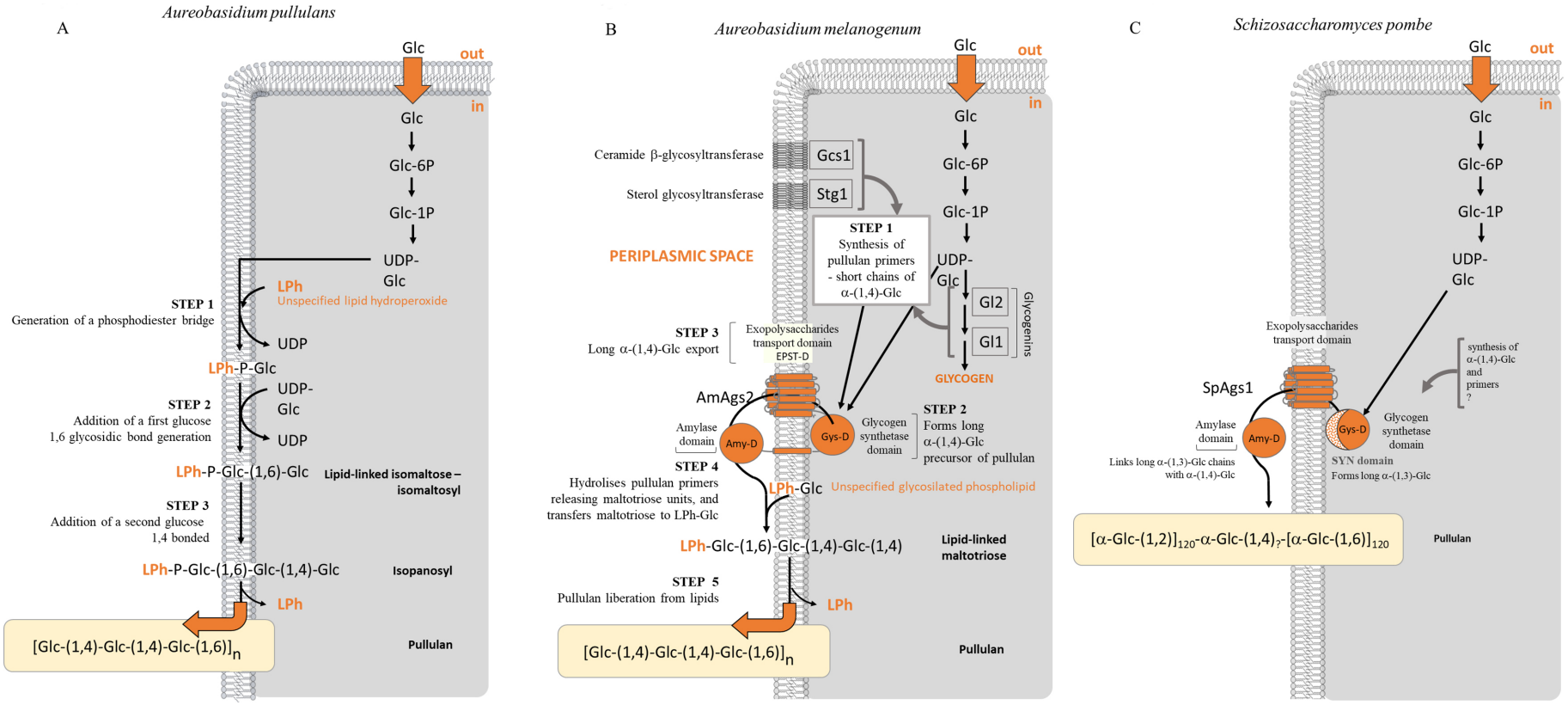

Fig. 2. Metabolic pathways proposed for the production of pullulan. (A) *Aureobasidium pullulans*, (B) *Aureobasidium melanogenum* and (C) *Schizosaccharomyces pombe*.

enzyme has been identified, the information on its structure and function and exopolysaccharides synthesis pathway is scarcer (Fig. 2C) [185], and even more so in the case of the dimorphic human pathogens above mentioned, which α-glucan synthesis pathway is suggested to differ considerably [186]. Importantly, the existence of α-glucans in the cell wall of a particular yeast is not necessarily directly associated with their presence in the ECM, whose composition and synthesis and secretion pathways are largely unknown.

Unlike the cases above, the ECM from *S. cerevisiae* was described to contain high amounts of glucose monosaccharides [42,120] which origin or biological purpose is still unknown. Moreover, Beauvais *et al.* [42] also reported the presence of β-linked branched mannosides. Our group's research on the ECM from biofilm-like mats (unpublished results) otherwise showed the presence of α-linked branched mannosides and small size α-(1,4)-glucan (amylose). The same genetic background was used, the haploid S288C-derived BY4741, nevertheless Beauvais *et al.* [42] extracted the ECM from flocs produced by two galactose/raffinose-grown strains, respectively deleted and overexpressing the lectin-encoding ORF *FLO1*. Regardless of this unique strategy, these results suggest that *S. cerevisiae* can secrete into its ECM either α- or β-glucans. The cell wall of this yeast contains β-(1,3) and β-(1,6)-glucans [189], identically to *C. albicans* and plenty other yeast species [190,191]. Therefore, the presence of β-glucans in *C. albicans'* ECM could be associated with the enzymatic machinery involved in cell wall maintenance and integrity. It is interesting to note that the Fks1 and Gsc2/Fks2 membrane multispanning proteins that are found in the β-(1,3)-glucan synthetases complexes were suggested to form pores that may guide the newly formed or in-formation glucan chain through the plasma membrane to the outside [191]. Synthesis and secretion are thus intimately orchestrated. Otherwise, the presence of α-glucans would require the chemical remodelling of the β-forms or *de novo* synthesis. The enzymatic interconversion $\alpha \leftrightarrow \beta$ of D-glucose anomers is performed by aldose 1-epimerases which function as mutarotases. *S. cerevisiae* has four ORFs encoding aldose 1-epimerases. The *GAL10*/YBR019C catalyses the interconversion of UDP-galactose and UDP-D-glucose at the level of galactose metabolism, but has a second catalytic function as UDP-glucose 4-epimerase [192]. The other three ORFs putatively encode a Glc-6P 1-epimerase (YMR099C), and two aldose 1-epimerases (YHR210C and YNR071C). The true relation of these ORFs and proteins with the synthesis and secretion of the yeast ECM remains unknown.

It is possible that some interchange between α- and β-forms occurs during the process of ECM formation, and that some of the glucans actually derive directly from the outer cell wall. Fungal cell walls in general are composed of basal layers of chitin (N-acetylglucosamine polymer), and β-(1,3) and β-(1,6)-glucans, these last organised as parallel β-(1,3) strands connected by bridging covalently attached short β-(1,6)-glucan polymers. Outer layers of the fungal cell wall are formed by mannoproteins abundantly *O*- and *N*-mannosylated, most of which are GPI-anchored proteins covalently linked to β-(1,6)-glucan, and to a lesser extent, Pir proteins attached to β-(1,3)-glucan via alkali-sensitive bonds. Many of these proteins are cell wall-remodelling enzymes [190]. It is therefore important to bear in mind that the interconversion between α- and β-D-glucose anomers does not necessarily require enzymes. It can also occur spontaneously in aqueous solution at a rate that may prove significant [193]. These authors report that at pH 6.0 and 20 °C the spontaneous anomerization of α-glucose can occur at the rate of 0.4×10^{-3} μmol β-Glc/sec/μmol α-Glc, and chemical equilibrium between the two anomers is reached at approximately $1\alpha/3\beta$ [193]. Mutarotation α-/β- equilibrium is dependent on a number of factors affecting the individual behaviour of the electrons in the glucan molecules like pH, but not on temperatures ranging 0–37 °C [194]. At human plasma physiological pH ($\pm$7.4) this α-/β- equilibrium somewhat favours the β-form [195]. At yeasts' physiological pH (2.0–5.0), the β-form-favouring equilibrium should be even more accentuated [196]. But *in vivo*, the ECM from yeasts' biofilms and colonies is a very complex and dynamic environment which most probably contains niches with specific environmental characteristics that would allow a constant and dynamic back and forth interchange between the two forms. The importance of this discussion has to do with the fact that α- and β-anomers display distinct physicochemical properties. In particular, they differ considerably in solubility in water, which at 20 °C is 82.5 (α-) and 178 g/100 mL (β-) (e.g., [197]). The presence of both forms and their constant interchange, regardless of the enzymes available for cell wall remodelling, would allow to meet cells' requirements while changing water chemical availability/retention and adjusting viscosity.

The ECM from biofilms formed by the human pathogen *C. albicans* also displays β-glucans (Table 1) identically to this species' cell wall. From a large number of proteins involved in this yeast cell wall integrity/maintenance and remodelling [189], very few were established a possible specific role in the synthesis of matrix glucans. Taff *et al.* [109] suggested the involvement of three *C. albicans* proteins in the modification and delivery of β-glucans to the ECM, the CaPhr1 cell surface glycosidase, the CaBgl2 β-(1,3) and β-(1,6)-glucosyltransferase, and the CaXog1 exo-(1,3)-β-glucanase. These enzymes apparently act independently from the transcription regulator Zap1, a Zn-finger protein that regulates negatively the synthesis and accumulation of β-glucans in the ECM [198,199] and also controls the equilibrium between *C. albicans* yeast and hyphae forms [108,109]. They are instead regulated by the alkaline pH response transcription factor Rim101 [200]. Mitchel *et al.* [201] showed that in the

CaPHR1 null mutant the synthesis of β-(1,6)-glucan was reduced to $\pm$10%, and to even less when *CaBGL2* was further deleted. The CaPhr1/2 proteins belong to a family of carbohydrate activated enzymes encompassing glycoside hydrolases (GH72) which includes enzymes from yeasts and fungi that localize at the cell periphery which actual biochemical role is unknown. Therefore, their role as regulators cannot be excluded. Accordingly, the expression of *CaPHR1* was shown to condition the amounts of ECM β-glucan produced in a biofilm, and the overexpression of *CaPHR2* was implicated in the enhancement of biofilm occupancy as a whole, but not that of biofilm biomass, which suggests that the amounts of ECM produced are not affected [200]. This means that the biofilm ECM-mediated biological processes governing occupancy, namely filamentation invasiveness and mating, must be indirectly influenced by the Phr proteins [200]. Importantly, these proteins further influence the cell wall integrity (CWI) and stress responses.

On the other hand, previous work had attempted the identification of the genetic determinants from *C. albicans* genome that were associated with the positive and negative regulation of ECM deposition in biofilms [198,202,203]. A few genes, classically ascertained to the cell wall mannan and glucan pathways, were identified in this yeast which disruption impairs both the deposition of mannans and glucans in the ECM and appear to be active and critical for the assembly of the ECM [204]. Some of these genes are under the negative control of the Zap1 transcription factor. From these, only two of them, encoding the glucoamylases Gca1 and Gca2, have actually been implicated in the formation of the ECM [202,203]. Although the exact mechanism through which these enzymes might operate is unknown, they have been suggested to promote the release of soluble β-(1,3)-glucan fragments from longer glucan chains.

Two other proteins were suggested to govern the levels of β-(1,6)-glucans from *C. albicans* biofilm ECM, Big1 and Kre5 [201]. Kre5, for being a UDP-glucose:glycoprotein glucosyltransferase, could be directly involved in the exopolysaccharide synthesis. Concurrently, the *kre5* null mutant displays less than 50% β-(1,6)-glucans compared to the *wt* [201]. Nevertheless, it also displays less 30-40% mannans and β-(1,3)-glucans. Moreover, the ECM from biofilms made from mixed cultures of this mutant together with the null mutants defective on the Golgi mannosyltransferase complex subunit *MNN9,* or the β-(1,3)-glucan synthase expression regulator *TET-FKS1*, presented overwhelming amounts of each exopolysaccharide compared to *wt* and to each mutant cultured alone. This behaviour is more compatible Kre5 being a regulator than a synthesis enzyme, sharing partially overlapping regulatory functions of ECM production with Mnn9 and Tet-Fks1. Importantly, the assembly of *C. albicans* ECM is claimed to occur extracellularly [201,204], which is consistent with other lines of evidence.

Pullulan production by *A. pullulans* has been reported as being best on sucrose, not glucose. The underlying reason comes from that pullulan production enzymes are repressed by glucose at high concentration [188]. It is though not certain how this occurs. Several authors suggest that the global glucose repressor Mig1 is involved, based on that the deletion of this gene homologue in *A. melanogenum* increased pullulan production. This may be less straightforward than it seems, since some strains of *A. melanogenum* were shown to produce pullulan in media with high amounts of fructose and glucose as it occurs in honey [205]. Authors associated this apparent constitutive derepression with the high osmotic tolerance of the strains and their concurring ability to produce and accumulate large amounts of the osmolyte glycerol, although no more data or detailed explanation are available.

Moreover, the requirement for ammonium exhaustion for highest production of exopolysaccharides strongly suggests the involvement of major N-dependent signalling pathways, which control bulk physiological responses including yeast cell wall remodelling and filamentation [206, 207]. Being exopolysaccharides a pre-requisite for the establishment of a biofilm, in which cells often differentiate into hyphae or pseudo-hyphae, it is plausible that those pathways are somehow involved.

6. Delivery of Exopolysaccharides to Yeasts' ECM

Cells from all kingdoms of life produce and secrete extracellular vesicles as a means to excrete proteins and other macromolecules. Additionally, all cells that are walled require a process that allows the passage of macromolecules through the cell wall barrier. That is the case of yeasts. Extracellular vesicles (EVs) were found to be released by yeast human pathogens, including *C. albicans* [154,208,209], as well as the model yeast *S. cerevisiae* [210,211]. Their role has been firmly associated with the biogenesis of ECM in *C. albicans* biofilms, in particular with the deposition of matrix exopolisaccharides [154]. Mutants defective in proteins involved in late endosome sorting complexes required for transport (ESCRT proteins) displayed reduced matrix exopolysaccharides and biofilm-forming ability, and consequently increased sensibility to fungicides. These defects were all fully complemented by externally adding EVs isolated from the *wt* strain biofilms [154]. These EVs are specific of biofilms, differing from others that are released by planktonic cell cultures. In such cultures, both *S. cerevisiae* [210] or *C. albicans* [154] secrete two distinct sized populations of vesicles, one clearly smaller than the other, although the sizes were different from one species to the other and from one strain to the other within the same species. *C. albicans* biofilms only present the smaller EVs. The time course of its formation and release followed biofilm-development, but their composition is slightly different from the biofilm ECM. Mannan/glucan proportion in EVs is approximately 5 times higher than in the ECM [154], es-

sentially due to a much higher presence of α-(1,2)-mannan. Associated with the extremely large proteome carried by the EVs [154], authors reasoned that the vesicles might carry the enzymes needed for macromolecular synthesis, namely those responsible for ECM building through glucan and mannan remodelling. Accordingly, in *C. neoformans*, a trans-cell wall vesicular transport mediates the deposition of the glucuronoxylomannan (GXM) at this yeast capsule [212].

The β-(1,3)-glucanosyltransferase Gas1 from *S. cerevisiae* required for cell wall assembly is heavily represented in the proteome of the ECM from this yeast biofilm-like mats [157]. This protein has been traditionally described as located in the cell surface *via* a GPI anchor. Nevertheless, Gas1 was recently found in a group of proteins from the cell wall that lack the lipid part of their anchor [213]. Proteins that suffered the delipidation of their anchors stay in the wall covalently or non-covalently attached to the β-glucans through their mannans. Rekstina *et al*. [213] speculated that these delipidated proteins relate to lipid vesicles that are found inside the cell wall, which location could precede their excretion to the extracellular space [213]. The yeast cell wall architecture is not well understood, in spite that its molecular composition has been often determined. It has been largely considered like a *fence* with large holes, allowing the indiscriminate passage of large molecules regardless of their shape or charge. The suggestion that it allows the permeation of molecules up to 600 Da in a specific manner made by de Nobel and Barnett [214], could correspond to the formation of pores achieving de diameter of 400 nm reported by de Sousa-Pereira and Geibel [215], which size depend on oxidative stress, Ca^{2+} and other factors. The emerging model is that the EVs formed within the cell permeate the cell wall and are eventually freed, carrying enzymes that can perform the delivery of the ECM polysaccharides in their final form.

7. Yeasts' ECM Proteome

The chromatographic analysis of the proteome of yeasts ECM revealed this is an extremely proteinaceous environment [107,157,216]. *S. cerevisiae* ECM contained 614 well-identified proteins that were not found in the control supernatant of the same strain grown in liquid medium [157], which have well-characterized roles allowing their distribution into several functional groups. The largest group aggregated enzymes from several central metabolic paths, including the full sets from glycolysis, fermentation and gluconeogenesis [157]. The presence of these two metabolically antagonistic sets of enzymes suggests that biofilms harbour two metabolically different populations of cells [217]. The second largest group includes numerous enzymes from the HSP70 family involved in the synthesis, folding and degradation of other proteins [157].

A crucial similarity is found between the proteomic analyses, that which studied the ECM extracted from *S. cerevisiae* biofilm-like mats [157], and which that studied this same yeast planktonic cultures EVs [210]. The most abundant functional class of EVs' proteome contained many enzymes from metabolic processes including carbohydrate metabolism, followed by proteins involved in other proteins biosynthesis, degradation and transport [210]. This remarkable resemblance suggests that the proteome within the yeast ECM indeed might be, at least in part, delivered by EVs. Accordingly, these vesicles also contained glucanases and glucanosyl transferases which are probably involved in cell wall remodelling [210,218]. These might also be involved in the synthesis of the ECM polysaccharides. Moreover, *S. cerevisiae* secretes two sizes of EVs [210], the smaller of which specifically carries enzymes from gluconeogenesis: Fbp1 (fructose 1,6-biphosphatase), Mdh1 (malate dehydrogenase), Icl1 (isocitrate lyase), Pck1 (phosphoenolpyruvate carboxykinase) as well as Tdh1/2 (glyceraldehyde 3-P dehydrogenase) [209,211]. This specificity agrees with the existence of two populations of cells, each secreting one type of EVs.

Otherwise, the filtered supernatant of *Saccharomyces* planktonic cultures presented a very reduced proteome (app. 87% less proteins) [157], suggesting that in planktonic cultures most proteins are secreted to the extracellular medium *via* EVs and not individually, which is consistent with the fact that many of the proteins identified in association with EVs are predicted to harbour an ER and secretory pathway signal peptide instead of a secretion signal [210]. The same was observed with the proteins from EVs from *C. albicans* [208]. These authors showed that in this yeasts' planktonic cultures, the proteome from EVs harboured 40% proteins that are predicted to not have a signal peptide, while in the same cultures' vesicles-free supernatant, the number of proteins actually possessing a secretion signal was 90%. The hypothesis that the yeast ECM proteome is secreted via EVs is consistent with Thomas *et al*. [219] describing that most of the proteins they identified in *C. albicans* biofilm ECM had no secretion signal. For this reason, Gil-Bona *et al*. [208] suggested that *C. albicans* has two different secretory pathways, a classical one requiring an export signal peptide, which secretes mostly cell wall remodelling and maintenance enzymes (49%), followed by hydrolases and adhesins (21%), metabolism enzymes and virulence factors, and a second non-classical protein export system, not requiring an export signal peptide that occurs through the secretion of EVs [208]. The proteome in the biofilms ECM must derive from either or both secretion systems. Actually, the possibility that EVs are part of the yeast ECM derives from the fact that a small fraction of lipids is found in *C. albicans* biofilms ECM (15% according to Zarnowski *et al*. [107], which composition (89.2% of neutral lipids and 85.6% of the polar lipid fraction consisting of phosphatidylethanolamine (PE) [107]) is identical to that of this yeast plasma membrane (90% neutral lipids and 70.0% of the polar lipid fraction consisting of PE [220]).

Although membranes' lipid composition may vary according to the strain, yeast-hyphae differentiation and environmental cues [209,221], this remains a prominent similarity, consistent with the EVs deriving from plasma membrane as previously suggested by Vargas *et al*. [209].

As in *Saccharomyces cerevisiae*, also the proteome from *C. albicans* biofilms ECM was characterized [107, 109,219]. They identically included proteins that could be ascertained to 485 different proteins [107], whose most abundant group (351 proteins) corresponded to enzymes from carbohydrate, amino acids and energy metabolism pathways. The biggest of these groups was that from carbohydrate metabolism, with 177 enzymes from TCA cycle, pyruvate metabolism and, importantly, glycolysis/gluconeogenesis. Moreover, the ECM from *C. albicans*, identically to that of *S. cerevisiae*, also presented a set of HP70 family, as well as the enzymes above mentioned (Fks1, Bgl2, Xog1 and Phr1), involved in polysaccharides synthesis and remodelling [109,219].

The presence of so many proteins with well-established intracellular functions, in particular the entire sets of enzymes from main metabolic pathways raises important questions. They could ensure metabolism extracellularly, which could be supported by the presence in the ECM from both *S. cerevisiae* and *C. albicans* of large amounts of monosaccharides (Table 1). Accordingly, the bacterial biofilm matrix was suggested to correspond to an external digestion system promoted by the immobilization of enzymes which may participate in general metabolic reactions as well as in the very remodelling of the ECM structural components [40]. Alternatively, those enzymes could provide other presently unknown functions as moonlighters. This terminology was applied to several yeast enzymes that perform utterly distant tasks than the one they were primarily described for. As an example, the glycolytic and/or gluconeogenic enzymes fructose 1,6-bisphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase and alcohol dehydrogenase, were found to bind fibronectin, laminin, plasminogen or to complement regulators with high specificity, and were identified as important virulence factors in *C. albicans* [222–225]. Although *S. cerevisiae* does not behave identically to *C. albicans*, all those proteins were equally identified in that yeast biofilms ECM [157]. No study was done so far characterizing the metabolome of yeasts' ECM. Otherwise, the biofilms of *C. albicans* were shown to be characterized by yeast cells displaying low rates of TCA cycle and mitochondrial activity as well as high levels of several amino acids and glycerol [226], suggesting a tendency for conservation of energy together with the accumulation of protective metabolites [227]. Identically, the cells of *C. neoformans* within biofilms were reported to shift from TCA cycle towards pyruvate utilization in particular through fermentation [216]. Whether the massive secretion of metabolism enzymes relates with these changes in metabolism will have to be explored in the future.

8. Specificities of Biofilm-Forming Model Yeasts

8.1 Candida albicans

Human pathogenic biofilm-forming yeasts include many *Candida* species. These include *C. glabrata*, *C. parapsilosis*, *C. dublinensis*, *C. tropicalis* or *C. auris*, and are often referred as NCAC species (non-*Candida albicans Candida* species) [228]. In most individuals with a healthy immune system, these yeasts are harmless commensal populations existing in equilibrium with other members of the microbiota and colonizing many areas of the human body, especially mucosae of the gastrointestinal and genitourinary tracts. However, disturbances caused by shifts in, namely, pH, O_2 or nutrition, as well as a depression of the immune system can enable *Candida* yeasts to rapidly proliferate [229,230].

C. albicans infections are associated with the proliferative colonization through the formation of highly structured biofilms [231]. Within these, yeast cells can differentiate into pseudo-hyphae, but mostly they differentiate into penetrative invasive true hyphae [232]. This yeast-to-hyphae transition is required for infection to develop and commands virulence [233–236]. Importantly, it is also required for the formation of biofilms [5,203]. It derives from extensive changes in gene expression [237–241] accompanied by high levels of horizontal gene transfer [242–244]. The genes that govern *C. albicans* biofilm formation are not limited by the above-mentioned *PHR1, XOG1, BGL2*, as well as *ZAP1* and *RIM101* transcription factors [109]. They cover a broad diversity of functional categories, including, namely, the yeast-to-hyphae morphological shift and filamentation growth [198,202,203].

Importantly, some of those genes encode drug efflux pumps like *MDR1* [203], contributing to the biofilm increased resistance to antifungal drugs. Concomitantly, *C. albicans* biofilms are mostly resistant to the currently available antifungal drugs. Several mechanisms underlie this resistance. A first consists in the increased expression of drug efflux pumps, including *CDR1*, *CDR2* and *MDR1* [239,245,246]. These genes become upregulated within the first few hours of yeast/surface contact and remain that way throughout biofilm development, whether or not an antifungal drug is present [239,247–251] A second mechanism corresponds to the existence of persistent cells in the biofilm. These are metabolically quiescent cells that resist to a range of drug concentrations otherwise lethal to planktonic cells [252,253]. They can revert back to metabolically active cells and contribute to the reformation of the biofilm once the antifungal drug has been removed [254]. A third mechanism is the physical barrier to drug penetration provided by the ECM itself [111,112,255–257]. The β-1,3-glucan polysaccharides present in the matrix have been

found to contribute to antifungal resistance, since treatment of biofilms with β-1,3-glucanase or deletion of genes that encode enzymes involved in the synthesis of β-1,3-glucan increases the sensitivity to antifungal drugs [255]. Finally, the fourth mechanism is provided by the presence of eDNA. This has been observed to mediate, at least partially and through an unknown mechanism, the resistance to some antifungal drugs [258]. Accordingly, DNAse treatment was shown to enhance drug susceptibility. eDNA, being an acid, interferes with ECM physicochemical properties, promoting considerable changes in the ECM structure and properties. It may even provide the cells with nutrients as suggested by Karygianni *et al*. [40].

8.2 Cryptococcus neoformans

Another human pathogenic biofilm-forming species is *C. neoformans*. This is an opportunistic yeast-like fungus that causes several pathologies (Cryptococcosis) in mammalian hosts, mainly if they are immunocompromised, from which the most severe is meningoencephalitis [259, 260]. *C. neoformans* distinguishes from *Candida* species namely because it is not able to form pseudo-hyphae. This species has a very complex life cycle [261]. According to several host and environmental cues, it may encompass yeasts or hyphae, haploid, diploid or polyploid cells, as well as chlamydospores, basidium and basidiospores. It may multiply endlessly as a heterothallic budding yeast, which may belong to either of two mating types a and α, identically to *S. cerevisiae*. In response to nutrient limitation, yeast cells may fuse and form regular diploids, or large polyploids known as Titan cells. But it may also happen that nuclear fusion is delayed, and the resulting dikaryon initiates filamentous growth, identically to filamentous fungi. Hyphae can be true diploids or dikarions and ultimately produce haploid spores bearing the a or the α genomes [261].

C. neoformans unique morphological feature is a polysaccharide capsule involving the yeast cells [262], which is the most important virulence factor during infection [263], since non-encapsulated mutants are avirulent [264]. It is also important in the dispersal of this pathogen in the environment [265]. The capsule is composed primarily of two polysaccharides, glucuronoxylomannan (GXM) and glucuronoxylomannogalactan (GXMGal; previously called galactoxylomannan) [263,266,267]. The capsule confers protection to the yeast during infection as it has been shown to inhibit phagocytosis [268,269], but also has a role in virulence, since both GXM and GXMGal are potent inducers of apoptosis [270–272]. Besides, these polysaccharides also induce an immunological unresponsiveness by, among others, inhibiting the production of antibodies [273,274]. In addition, the capsule presents a small proportion of mannoproteins [275,276], as well as, importantly, hyaluronic acid (HA) [277,278]. The presence of HA apparently facilitates the adhesion of the fungal cells to the tissues [277] and its interaction with the human cells HA receptor CD44 initiates the events of invasion process in the human brain microvascular endothelial cells [278,279]. The HA receptors CD44 and RHAMM to a lesser extent, are present in human cells plasma membrane lipid rafts, which absence, inactivation or displacement cause a very significant decrease in *Cryptococcus* infection ability [278]. Although there is no direct implication of HA, or any other capsule component, in the development of biofilms by *C. neoformans* yeasts, it appears that the capsule is also important for that purpose, since acapsular strains do not develop biofilm [280]. Accordingly, it cannot be ignored that CD44 in response to HA has been associated with uncontrolled cell proliferation as well as invasiveness and migration in cancer [281–283].

Similar to *Candida* yeasts, also *C. neoformans* cells within biofilms are less susceptible to antifungal agents as well as to the host immune system [284,285]. A cryptococcal biofilm consists of a complex network of only yeast cells, enmeshed in a substantial amount of ECM [284,285]. Its adhesion process was shown to be mediated by the release of one of the capsule polysaccharide, initiating the creation of the biofilm ECM [280,286]. Whether this is GXM, the same that *C. neoformans* releases into the colonised tissues, is not known, although both capsule and biofilm have GXM [17]. Accordingly, specific antibodies against GXM inhibited biofilm formation [286]. The capsule could thus act as a facilitator for the biofilm initiation.

The secreted GXM, also designated exo-GXM, has important roles in the development of Cryptococcosis, which include the formation of biofilm and concomitantly protection of the fungal cells against anti-fungal drugs [286,287], given that exo-GXM-deficient mutant is inefficient in biofilm formation and is avirulent [288]. Exo-GXM accumulates in cerebrospinal fluid, serum and tissue of infected patients [289–291] and presents several immunomodulatory properties (reviewed in [292]), including that of inhibiting phagocytosis. This occurs probably because GXM is polyanionic. This feature has been associated with the possibility of GXM causing an electrostatic repulsion, preventing host immune cells from interacting with and eliminating fungal cells [293].

8.3 Saccharomyces cerevisiae

S. cerevisiae is the best-studied yeast ever. It is considered a model for human biology due to a high degree of evolutionary conservation of many proteins and molecular processes between humans and yeast. Besides aggregating onto colonies [294] or biofilms/mats [295–297], *S. cerevisiae* can also aggregate onto flocs [298]. All provide survival advantages due to the cooperative behaviour of cells [299,300], and the establishment of common protective mechanisms [301], namely the production and accumulation of the ECM [301,302]. *S. cerevisiae* multicellular aggregates of all types produce ECM [42,303–305]. This, as for other yeast species, is crucial to prevent desiccation [306], while providing protection against xenobiotics

[304], and ultimately a nutrient reservoir [217], as discussed above.

Yeasts essentially develop two major types of colonies. Smooth colonies are formed by most laboratory or domesticated strains [303]. They are composed of tightly attached cells unable to invade semisolid supports, such as agar, and do not harbour any ECM [307,308]. Alternatively, cells on the surface of the colony pack together forming a protective thin layer of tightly connected cells [303,307]. Structured colonies formed by wild strains are also designated in the literature as *colony biofilms* [303]. These are composed by an aerial part, formed mostly of oval shaped cells above the agar, and the subsurface "roots", formed by pseudohyphae invading the agar [304,306]. Cells forming *colony biofilms* are connected by extracellular fibres [303,304,309] and are embedded in an abundant ECM which functions as a nutrient reservoir and as a low-permeability barrier, blocking the contact of compounds diffusing through the agar with the cells [304].

S. cerevisiae colonies also differ in size. In fact, they can grow disproportionally on low-density (0.3%) YPD agar, becoming giant colonies that can achieve almost the full size of a Petri dish which are also called mats [295,310]. These resemble biofilms in terms of spatial organization and properties. They contain distinct cell populations: a central hub composed of adherent cells, from which emanates a network of "cables" that culminates in the formation of spokes bordered by a rim of non-adherent cells [295]. Analogously, Varon and Choder [311], reported that starving cells in the centre of colonies were covered with ECM and united by continuous extensions of the mucous substance forming fibrils, in increasingly higher amounts when cells were purposedly starved. The nature of these fibrils is still unknown, as it is whether they allow actual connection /communication between the cells. Besides forming concentric gradients of nutrients (glucose) and pH [296], which can influence cell properties within the hub and rim of a colony, these also form gradients of ammonia. These signal and confine the apoptotic death of the cells to the centre of the colony [312,313], thus contributing to the enlargement of the cell population with healthy, reproducing cells at the periphery.

The *S. cerevisiae* ECM regulates the transition from a relatively slow-growing colony to a rapidly expanding mat [313,314], as well as cell-cell communication, cell-cell and cell-substrate adhesion, and spatial heterogeneity-mediated cell morphology, growth and multiplication [304,315,316]. These processes are accompanied by considerable changes in the expression of many genes, the most studied of all being the adhesin/flocculin (Flo) proteins [297,303,317–321]. These are glycoproteins, similar to the adhesion proteins from other yeasts, including human pathogens such as *C. albicans* and *C. glabrata* [316,319,320,322,323]. Flocculins share a common structure [323–325] but different functions [318,319], and contribute to the development of different types of flocs [324,326]. The most well-known gene associated with cell adhesion to agar and plastic surfaces is *FLO11* [318]. This gene encodes a GPI-anchored cell surface flocculin that contains a specific domain responsible for cell-surface adhesion [316,317] and required for the yeast differentiation into pseudohyphae [327,328]. It is a key protein, involved in the formation of structured *colony biofilms* [329], *flor* [330], biofilms [331], and mats [295,332]. Concurrently, any *flo11*Δ mutant is unable to form a biofilm [295] resulting in the formation of smooth colonies in various non-isogenic wild strains isolated from different habitats [306]. Furthermore, the expression of Flo11 and the consequent biofilm formation, are influenced by gradients of glucose and pH which are established by the growing cell population [296]. High pH in the biofilm decreases the adherence capacity of Flo11 and allows the detachment of the cells [296]. Furthermore, mutants defective on the *SNF1*, *YAK1* and *RAS2* genes from SNF1/AMPK, Yak1/PKA and Ras/PKA pathways, that respond to changes in glucose levels, showed blocked biofilm formation and decreased expression of *FLO11,* [296,333–336] namely through the intervention of the Tup1 and Cyc8 transcription regulators. While Cyc8 prevents the formation of structured *colony biofilms* by repressing *FLO11* expression, Tup1 antagonizes Cyc8p-mediated *FLO11* repression and stabilizes the Flo11 protein preventing its degradation [333]. In addition to Flo11, other features that are important for *colony biofilm* formation, such as cell invasiveness, adhesion to solid surfaces and the presence of fibres connecting the cells, are also antagonistically regulated by Cyc8 and Tup1 [333,334]. Conversely, features that are related to other types of multicellularity, such as cell flocculation, are co-repressed by both regulators [333]. The expression of *FLO11* is also controlled by epigenetic mechanisms, including histone deacetylation, chromatin remodelling, non-coding RNAs and prion formation [316,319,337–340].

Several other genes besides *FLO* genes participate in the establishment of *S. cerevisiae* multicellular forms. The gene *BTN2* expresses a v-snare interacting protein involved in intracellular protein trafficking [341]. When this gene was deleted in a *Sacharomyces cerevisiae flor* strain, the adhesion to plastic was impaired, but the yeast formed a consistently larger biofilm with concomitant increased transcription of *FLO11* [342]. Besides, *HSP12*, which encodes a heat-shock protein, was also reported to have a role in cell adhesion, since the mutation or deletion of this gene resulted in the failure to form a biofilm [343]. Furthermore, Hsp70 molecular chaperones play key roles in mat formation with the assistance of the nucleotide exchange factors Fes1 and Sse1 and the Hsp40 family member Ydj1 since the disruption of these cofactors completely abolished mat formation [344]. Glycosylated cell surface proteins that contribute to the cell surface hydrophobicity, like Ccw14, also contribute to *S. cerevisiae* biofilms. Deletion of *CCW14* resulted in the decrease of the weight of biofilm formed, as

well as of the cell adherence to polystyrene [345].

9. Final Considerations

The study of yeast ECM components from planktonic cultures or from biofilms have very distinct purposes. One is identifying economically viable biotechnological processes for pharma or food industries, and another is identifying the molecular players governing the establishment and the biology of yeast biofilms, mainly those that are formed by pathogens. The exopolysaccharides that are produced in planktonic cultures meet the first goal, but cannot be considered to have the same physiological roles as the ones produced in the context of biofilms or other large multicellular aggregates. Therefore, the actual knowledge on the yeast's ECM composition, supporting multicellular communities and promoting cell differentiation, communication, spatial organization and controlling replication and death, is much scarcer. To this situation also contribute the technical difficulties associated with obtaining substantial ECM amounts, compatible with common analytical procedures, and the use of very different ways to culture the cells, which do not replicate one another. In this regard some efforts are noticeable [309,310,345,346], but much remains to be done.

Each yeast species' ECM has a characteristic glycoside composition. Polysaccharides differ in size, structure and composing monomers, and in the majority of the studied cases they are chemically and structurally different from those found in the cell wall. Additionally, in some cases, the ECM has been reported to contain large amounts of monosaccharides, which origin remains obscure in view of the often-abiotic biofilm-supporting surroundings. Another important feature of yeasts' ECM consists in the existence of a large proteome, that was only fully characterized in *S. cerevisiae* and in *C. albicans*. The two proteomes are very similar. Noticeably both yeasts share the presence of full metabolic sets from main metabolic pathways like glycolysis, gluconeogenesis, TCA and respiration. The results from both studies also concur in pointing to the putative involvement of acetic and citric acid in the ECM formation, but critical information is absent. For the time being none of these components has been associated to specific functions or physicochemical properties of the ECM, but rather to pharmacological bioactive properties sustaining their possible commercialization [41]. The matrixome of yeast communities needs to be extensively addressed if answers to fundamental questions are to be answered.

The most fundamental of all questions relates with the acquisition of obligatory multicellularity. Yeasts display facultative multicellularity, in the sense that they may form multicellular aggregates either by clonal or aggregation strategies [347]. They may not split after mitosis, remaining ligated [348], or they modulate their adhesive properties by changing the expression of the *FLO* genes (reviewed by [347]) and stick together. Experimentally, it was possible to turn this facultative into an obligatory multicellular aggregate (called *snowflake* yeast), which grows spatially in a geometric coherent way, where cells diversify roles and interact socially, and replicates through the formation of multicellular propagules [348,349]. Does ECM play any role in this behaviour, mirroring the higher eukaryotes cell-ECM mutual regulation? How does a population of millions of yeast cells which are not mobile achieves the orchestrated collective behaviour, spatially oriented? Is ammonia the single communication path between yeast cells or is the ECM a central hub for more complex molecular signals identical to higher eukaryotic tissues? These questions sum to many others underlying all that was summed up in this review. A deeper understanding of the ECM produced by yeasts and its components, as well as its biology, genetics and molecular effectors, and how they change according and react to external stimuli, will be instrumental to a deeper understanding of yeast biology, supporting their many new utilizations in perspective, and the continuous utilization of yeasts as a scientific fundamental model for Biology.

Author Contributions

CL—conceptualization, bibliography review and manuscript writing and editing; CS—bibliography review and manuscript writing and editing. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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