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Proteomics characterization of biofilm formation by salt-tolerant *Schwannyomyces etchellsii* in seawater-based growth medium

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ABSTRACT

Microbial biofilms are complex communities with cells embedded in an extracellular matrix. We previously discovered that salt-tolerant yeasts *Debaryomyces fabryi*, *Schwanniomyces etchellsii*, *Schwanniomyces polymorphus* and *Kluyveromyces marxianus* were able to form biofilms when grown in seawater- but not in freshwater-based media. The extracellular matrices of these biofilms were composed mainly of carbohydrates and proteins involved in metabolic processes and the response to stimuli. We herein focus on one of these yeasts, *S. etchellsii*, to explore its molecular determinants for biofilm formation in depth. We describe new analyses in which the proteome of cells in the biofilm network formed in seawater-based media is compared to that of the planktonic cells co-existing with them and with cells suspended in freshwater-based growth media. According to our data, in both cases biofilms cells contain overexpressed proteins involved in protein biosynthesis, in membrane structures and in transport mediated by vesicles. The great number of proteins with higher expression in these cells participating in translation and located in ribosomes indicate that they are more engaged in protein biosynthesis than their counterparts. Analyses carried out with the STRING database reinforced these results. Cell viability was also wider in biofilm cells. Our analyses have also allowed us to detect in *S. etchellsii* a homolog of the *Candida albicans* Spf1p. This protein is an ion transporter P-type ATPase in this microorganism, which participates in several processes, including cellular adhesion and cell wall organization and biogenesis. Our work provides a dataset with a large number of unknown proteins of *S. etchellsii* that show sequence similarity to proteins from other yeasts; this knowledge will help to better understand the proteome of this yeast and to look for future biotechnological applications.

1. Introduction

Biofilms are communities of living organisms in which cells embedded in an extracellular matrix (ECM) are attached to certain surfaces (Flemming and Wingender, 2010; Strieth et al., 2018). The formation of such structures provides microorganisms with advantages such as good ability to colonize host tissues, the expression of virulence characteristics, and increased tolerance to chemical, physical and biological stress conditions (Castrillón et al., 2013). All these properties increase the ability of these microorganisms to cause human infections. However, such communities are useful in some industrial and biotechnological processes related to the production of foods, bulk and fine chemicals, enzymes, and so on (Berlanga and Guerrero, 2016; Demirci et al., 1997; Gross et al., 2007, 2013; Halan et al., 2012, 2014; Ho et al., 1997; Muffler et al., 2014; Qureshi et al., 2004; Romero et al., 2018; Strieth et al., 2018; Willrodt et al., 2017).

The composition of the ECM in prokaryotic biofilms has been widely characterized (Bales et al., 2013; Cheng et al., 2019; Colvin et al., 2011, 2012), and reveals a scaffold formed by exopolysaccharides to which other carbohydrates bind, as do proteins, lipids and nucleic acids albeit to a lesser extent. The result is a complex 3D structure with channels and pores that allow the diffusion of water, nutrients and waste substances. Biofilm thickness is constantly maintained by a dynamic process between detachment of cells and re-growth (Halan et al., 2012).

In fungal biofilms, most available information has been obtained from human pathogenic organisms, including *Aspergillus fumigatus*,

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Cryptococcus neoformans, and various *Candida* species (Dominguez et al., 2018, 2019; Le Mauff et al., 2019; Malinovská et al., 2023; Martinez and Casadevall, 2015; Zarnowski et al., 2014; Zore et al., 2023). For these biofilms, diversity in the particular composition has been described, but with high common content in proteins and polysaccharides. It is worth mentioning that some moonlighting proteins have also been identified in these structures (Zarnowski et al., 2014; Zarnowski et al., 2021a). Extracellular vesicles are an important component involved in biofilm formation, dynamics and resistance to drugs (Abels and Breakefield, 2016; Brown et al., 2015; Brown Harding et al., 2024; Zaborowski et al., 2015; Zarnowski et al., 2021b, 2022). They mediate the unconventional transport of proteins, nucleic acids, carbohydrates, and lipids to facilitate cell-to-cell communication through their delivery in the ECM, in which chemical gradients are generated (Stewart and Franklin, 2008). Data about the biofilms formed by halotolerant yeasts and their biotechnological applications have been recently compiled by Andreu and del Olmo (2023).

Biofilm formation is a complex process that can be influenced by multiple biological, chemical, physical and environmental factors (Halan et al., 2012). Biofilms-forming cells can be differentiated for their suspended analogs or planktonic cells, and not only for their ability to generate the ECM, but also for their growth rate and the up- and down-regulation of specific genes (Donlan, 2002). Several studies have compared the proteomes between both kinds of cells to better understand these differences. In eukaryotic microorganisms, most of these studies have focused on flor yeasts, and on pathogenic yeasts (especially, on *Candida* species), because of their respective industrial or pathogenic relevance. Regarding flor yeasts, the most abundant proteins detected in biofilm cells are involved in respiration, translation, stress damage prevention and repair, amino acid metabolism, glycolysis/gluconeogenesis and biosynthesis of vitamin B9 (Moreno-García et al., 2015, 2017). It has also been shown that proteins Ccw14 and Ygp1 influence the weight of the biofilm and cell adherence (Moreno-García et al., 2018).

One of the first comparative studies between planktonic and biofilm cells in *C. albicans* revealed that the former differ mostly in cytoplasmic profiling while the latter diverge mainly in surface profiling (Martínez-Gomariz et al., 2009). These authors observed differential expression in enzymes of respiratory and fermentative metabolism of pentoses and glucose, and in folate interconversions, and proteins associated with counteracting stress conditions, host response and multi-organism interaction. Later studies about the same organism have shown that the biofilm proteome contains a bigger proportion of proteins associated with the endomembrane system, mitochondrion and cytoplasm. These proteins are involved in the regulation of cellular macromolecule biosynthesis and metabolism, transcription and gene expression (Munusamy et al., 2021). It is worth mentioning the increased incorporation into the biofilm cells of stress response proteins, including alkyl hydroperoxide reductase, thioredoxin peroxidase and thioredoxin, involved in oxidative stress defenses (Seneviratne et al., 2008, 2010), and specific proteases Sap5 and Sap 6 (Winter et al., 2016). In *Candida orthopsilosis*, Pires et al. (2016) describe glycolytic flux changes to the synthesis of glycogen and trehalose. Recently, studies with *Cryptococcus gattii*, a fungus that causes pneumonia, indicate that biofilm cells are maintained by the induction of the electron transport chain for re-oxidation, and by alternative energy metabolites like succinate and acetate (Santi et al., 2024). Finally, but not less importantly, several studies point out the involvement of flocculins, adhesins and other related proteins in biofilm development in pathogenic yeasts, such as *C. auris* (Santana et al., 2023), *C. glabrata* (Fernández-Pereira et al., 2021; Wang et al., 2024) and *Pichia kudriavzevii* (Alvarado et al., 2023). Overall, data about this topic are very limited, particularly in non pathogenic yeasts, and more information is needed to understand the molecular mechanisms underlying biofilm formation and stability.

Recently, we described how salt-tolerant yeast strains *Kluyveromyces marxianus*, *Debaryomyces fabryi*, *Schwanniomyces etchellsii* and *Schwanniomyces polymorphus*, with potential interest in the stereoselective synthesis of chemical and pharmaceutical compounds (Andreu and del Olmo, 2014, 2018, 2019, 2020; Andreu et al., 2016), have the ability to form biofilms at air–liquid interfaces and on silicone surfaces when culture media based in seawater are employed (Zarnowski et al., 2021a). Besides, in this study the ECM composition of these communities was thoroughly characterized. In the present manuscript, we made proteomic comparisons between biofilm and planktonic cells and between cells in biofilm and cells grown under non-biofilm forming conditions. Their purpose is to examine in more depth the molecular mechanisms that lie behind the ability of these yeasts to form biofilms under these conditions. As a model for the study, we took yeast *Schwannyomyces etchellsii*, which has been widely used in our laboratory with very good results in different biocatalytic processes such as the production of (*R*)-(−)-phenylacetylcarbonil, a chiral intermediate precursor of ephedrine, or the enantioselective reduction of several prochiral ketones, both in freshwater and seawater (Andreu and del Olmo, 2014, 2018, 2019). This strain was isolated from fermenting cucumbers and it was previously known as *Debaryomyces etchellsii* until its inclusion in the genus *Schwannyomyces*. We showed that is is be able to grow in the presence of NaCl concentrations up to at least 7.5 % (w/v) (Andreu and del Olmo, 2018), and it is more resistant to organic solvents/compunds and has greater metabolic activity in seawater than in freshwater (Andreu and del Olmo, 2019).

2. Materials and methods

2.1. Yeast strain and growth conditions

Sachwannyomyces etchellsii (CECT 11406) was obtained from the "Colección Española de Cultivos Tipo, Servei Central de Suport a la Investigació", Universitat de València, Paterna (Spain).

The seawater used in experiments came from the El Perelló beach (Mediterranean Sea), València (Spain), whose treatment and composition have been explained elsewhere (Zarnowski et al., 2021a).

The following experiments were carried out in triplicate. The cells inoculated in YPD medium prepared in seawater (SW-YPD, 1 % (w/v) yeast extract, 2 % (w/v) bactopeptone, 2 % (w/v) glucose) were incubated overnight at 30 °C in an orbital shaker (200 rpm). Cultures were diluted in the same medium without any carbon source (SW-YP) to a final OD_{600} of 0.3. Then 50 mL of these suspensions were placed inside 250 mL glass beakers and incubated at 30 °C for 14 days without shaking. At the end of this time period, a biofilm at the air–liquid interface and a free-cell (planktonic) population at the bottom of the glass were present. Hereinafter, we refer to these conditions as biofilm formation conditions (BFCs). In parallel, the same procedure, but using YP prepared in freshwater in all cases, was followed. In this case, no biofilm formation was observed, and only free cells were present in the medium. We refer to these conditions as no biofilm formation conditions (NBFCs).

2.2. Cells preparation

The cells from the samples under NBFCs (hereafter NBF-cells) were recovered by centrifugation (3 min × 3000 g). The biofilm and planktonic cells (from now BF-cells and PT-cells respectively) coming from the samples under BFCs were separated. For this purpose, the suspension was passed through a sterile stainless-steel colander in which the biofilm was trapped. Then PT-cells were recovered by centrifuging from the filtered medium (3 min × 3000 g). The biomass retained in the colander was resuspended in 30 mL of water and sonicated for 5 min at 20 kHz in a Vibra Cell VCX-500 sonicator (Sonics & Materials Inc., Newtown, USA). Under these conditions, the material that formed the ECM remained in solution, while BF-cells were sedimented and collected by centrifugation.

2.3. Viability determination

PT- and BF-cells, collected as described in the previous paragraph, were resuspended in water, and the OD_{600} was measured for both populations. Appropriate dilutions according to it were carried out for plating an equivalent amount of both in YPD solid medium (containing 2 % (w/v) glucose and 2 % (w/v) agar). After two incubation days at 30 °C, the number of colony forming units (cfu) was counted. The data was finally expressed as cfu/OD_{600}. These experiments were carried out at least in triplicate.

2.4. Proteome analyses

NBF-, BF- and PT-cells were used to prepare the protein extracts as follows. After three washes with cold water, the cells corresponding to 150–200 OD_{600} units were resuspended in 500 μL of lysis buffer (140 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris HCl pH 8, 0.5 % (v/v) NP-40 and EDTA-free protease inhibidor cocktail *cOmplete* (Roche). The same volume of glass beads was added, and the mix was vortexed 10 times for 15 s (with another 15-second period on ice in between). Samples were centrifuged at 4 °C for 15 min at 15,000 g, and supernatants were collected and quantified by using bicinchoninic acid (Pierce BCA Protein Assay Kit, Thermo Scientific, Rockford, USA). The integrity of the protein extracts was assessed by SDS-PAGE.

Proteome analyses were carried out in the Proteomics facility of the SCSIE (Universitat de València) following the SWATH-MS (Sequential Window Acquisition of all THeoretical Mass Spectra, Ludwig et al., 2018, and references therein) methodology. The samples from each individual condition and a sample of a spectral library (60 μg), built by mixing aliquots of an equivalent amount of the samples from each condition, were analyzed by 1D_SDS_PAGE, and each one was digested with trypsin (Promega) following the protocol described elsewhere (Shevchenko et al., 1996; Pinheiro et al., 2020).

For spectral library building, 5 μL of each digested sample were desalted using a trap column (3μ C18-CL, 350 μm × 0.5 mm; Eksigent) with 0.1 % TFA (5 μL/min for 5 min), and were then loaded onto an analytical column (3μ C18-CL 120 Ă, 0.075 × 150 mm; Eksigent). Peptide elution was carried out with a linear gradient from 7 % to 40 % B in A for 60 min (A: water, 0.1 % formic acid; B: acetonitrile, 0.1 % formic acid) at a flow rate of 300 nL/min. Peptides were analyzed in a mass spectrometer nanoESI qQTOF (6600plus TripleTOF, ABSCIEX). Ionization was carried out in a Source Type Optiflow < 1 μL Nano applying 3.0 kV to the spray emitter at 200 °C. Analysis was carried out in a data-dependent mode. Survey MS1 scans were acquired from 350–1400 *m/z* for 250 ms. The quadrupole resolution was set to 'LOW' for MS2 experiments, which were acquired from 100–1500 *m/z* for 25 ms in 'high sensitivity' mode. Following switch criteria were used: charge: 2+ to 4+; minimum intensity; 250 counts per second (cps). Up to 100 ions were selected for fragmentation after each survey scan. Dynamic exclusion was set to 15 s.

ProteinPilot default parameters were used to generate the peak list directly from 6600 TripleTof wiff files. The Paragon algorithm (Shilov et al., 2007) of ProteinPilot v 5.0 was used to search the Uniprot-Yeast database (parameters: Trypsin specificity, IAM cys-alkylation, taxonomy restricted to Yeast, and the search effort set to through and FDR correction). The Pro group algorithm was employed for protein grouping.

For SWATH analysis 5 μL of each digested peptide from individual samples bands were chromatographically analyzed as spectral library but the tripleTOF was operated in swath mode, in which a 0.050-s TOF MS scan from 350–1250 *m/z* was performed, followed by 0.080-s product ion scans from 350–1250 *m/z*. 100 variable windows from 400 to 1250 *m/z* were acquired throughout the experiment. The total cycle time was 2.79 secs.

The wiff files obtained from the Swath experiment were analyzed by Peak View 2.1. The area data obtained with Peak View were analyzed with Marker View (Sciex). First, the calculated protein areas were normalized by the total sum of the areas of all the quantified proteins. Next the statistical tests of reduction of dimensionality, PCA and DA (both with Pareto scaling) were carried out. The proteins showing unused score > 1.3 were identified with ≥ 95 % confidence.

The mass spectrometry proteomics data are deposited in the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD050945.

Proteomes were analyzed functionally using the UniProt Knowledgebase data, which contains reviewed UniProtKB/Swiss-Prot entries (Uniprot Consortium, 2019). Functional Gene Ontology (GO) terms were assigned for each identified protein based on the yeast genome data. The hierarchy "Biological process" was used to prepare the visualizations of the relative quantities of biofilm proteins.

Protein sequence alignments were carried out using the CLUSTAL O (1.2.4) multiple sequence alignment from EMBL's European Bioinformatics Institute (Sievers et al., 2011). The EMBOSS Matcher algorithm from the same institution was used for the pairwise comparisons (Waterman and Eggert, 1987). To analyze the funcional categories involved in the expression changes and the network of interactions between proteins identified in this study by their similarity to their *Debaryomyces hansenii* counterparts, the STRING (Search Tool for the Retrieval of Interacting Genes) was used (Szklarczyk et al., 2023).

3. Results

3.1. Overview of the experimental conditions followed to understand the proteome response to biofilm formation

In our experiments, we compared the proteome of cells in three different metabolic states. In all cases, they were inoculated in a medium without any carbon source, but there were differences in the origin of the water employed for preparing media. When fresh water was used (NBFCs), a single cell population was observed (NBF-cells). Conversely in the medium based in seawater (BFCs), cells built a complex lattice with a matrix produced by themselves (BF-cells), which lived together with free (planktonic) cells (PT-cells).

To simplify the experiment, we first compared the proteome of NBF- and BF-cells, which were separated from the ECM following the procedure described in the Materials and Methods section. In a second analysis the protein extracts obtained from BF- and PT-cells, both from the same medium based in SW-YP, were compared. All the experiments were performed in triplicate.

3.2. Comparison between NBF- and BF-cells

In order to systematize the results, we considered differences in the expression levels between both populations to be equal to or higher than 1.3, and a p-value equal to or lower than 0.1 for the analyses of the overrepresented categories. With these criteria, 96 proteins were more expressed under the NBFCs (NBF-cells). Four of them could not be mapped by UniProt. Of those remaining, assignations could be made for the molecular process in 84, for the biological process in 63 and for the cellular component in 30. The distribution of these proteins in the Biological Process Gene Ontology categories is shown in Fig. 1 and Table S2 in the Supplementary Material. The proteins with differential expression levels above 2 (67) are described in Table S1 in the Supplementary Material.

Under BFCs (BF-cells), 296 proteins were found to be more expressed. For 292 of them, information was obtained by using UniProt tools. For 270, data about molecular function were found. The number was smaller for the biological process and the cellular location (229 and 200, respectively). Fig. 2 and in more detail Table S4 of the Supplementary Material contains information about the distribution of these proteins in the Biological Process Gene Ontology categories. Table S3 in the Supplementary Material collect only those proteins with fold change

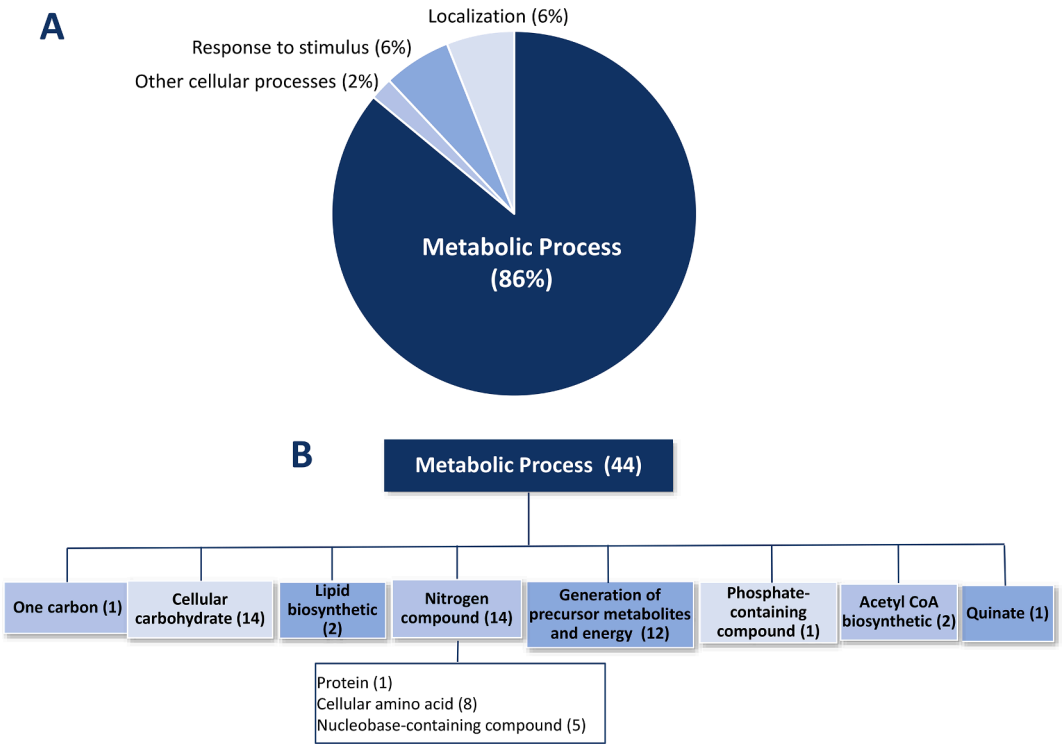


Fig. 1. Distribution of the proteins with higher expression levels in NBF- than in BF-cells according to their biological process categories. Graphic A indicates the percentage of each process. Graphic B shows the number of identified proteins corresponding to metabolic process. The numbers shown are approximate because some proteins appear in several subcategories and others belong to categories less represented that have not been included in this table. Proteins considered show fold change equal or higher than 1.3 and p-value lower than 0.1.

values above 2 (235). For the analyses herein described, we focused on the information about biological processes and the subcellular localization in which the overexpressed proteins participate.

With the NBF-cells (Fig. 1 and Tables S1 and S2 in the Supplementary Material), the observed greater metabolic activity corresponded to the metabolism of carbohydrates and of nitrogen compounds (14 proteins in both cases) and, to a lesser extent, to the generation of precursor metabolites and energy (with 12 entries related mainly to TCA cycle enzymes isocitrate dehydrogenase and malate dehydrogenase). Inside the category of metabolism of nitrogen compounds the main group corresponds to amino acid metabolism with 8 proteins involved in the biosynthesis of cysteine, glycine, methionine, threonine, isoleucine and proline identified. According to the obtained results, some glycogen mobilization could probably occur with further glycolysis and Krebs cycle. In line with this, it is important to point out the rising levels of some of the proteins involved in the oxidative stress response (peroxiredoxin and superoxide dismutase). Some overexpressed proteins were also related to acetyl-CoA and inositol biosynthesis. It is noteworthy the detection of catabolic 3-dehydroquinase of *Candida albicans*, an enzyme of the 3,4-dihydroxybenzoate biosynthesis pathway; this molecule is produced from yeasts to humans, results from the degradation of phenolic compounds, and has pharmacological potential for its antioxidant properties (Kakkar and Bais, 2014).

In the BF-cells, when a comparison was made to the NBF-cells, the overrepresented categories corresponded once again to metabolic process (Fig. 2 and Tables S3 and S4 in the Supplementary Material). In this case, however, the number was much bigger than in the NBF- vs BF-comparison (140 and 44 respectively). The most represented subcategory related to the nitrogen compound metabolic process (with more than 110 entries), particularly translation (66 proteins). Proteins participating in metabolism of amino acids such as cysteine, glutamine, proline, lysine, glycine, histidine, threonine, isoleucine, valine and leucine were also found. It is important to point out the identification of the proteins involved in lipid biosynthesis (fatty acids, isoprenoid and steroid), including the subunits of the fatty acid synthase. Besides, the category of the generation of precursor metabolites and energy was represented by 10 entries related to the tricarboxylic acid and/or glyoxylate cycles (i.e. malate synthase and succinate dehydrogenase) and the electron transport chain. Some of the proteins participating in the response to abiotic stimuli (oxidative stress, heat and glucose starvation) were also more expressed under this adverse growth condition. Localization/transport seems to be more active in these cells according to the higher levels of proteins involved in mitochondrial and vesicle-mediated transport.

Regarding the subcellular location of the overexpressed proteins (Fig. 3) and, according to the biological processes in which they participated, for the NBF-cells most proteins were cytosolic and mitochondrial, with only a few located in ribosomes, the nucleus, the vacuole or the plasma membrane. For the BF-cells, the majority of proteins that were more expressed than in the NBF-cells were located in organelles, mainly ribosomes, and also in the cytosol. Approximately 19 % of them were associated with the cell surface or membrane structures.

3.3. Comparison between BF- and PT-cells

Following the previously defined criteria (differences in the expression levels between both populations equal to or higher than 1.3, and a p-value equal to or lower than 0.1), 227 proteins were more expressed in the biofilm cells than in the planktonic ones. To them, a molecular function category could be assigned to 207, a biological process was found for 163, and information about cellular component was obtained in 101 cases. The distribution of these proteins in the Biological Process Gene Ontology categories is shown in Fig. 4 and Table S6 in the Supplementary Material. The proteins with differential expression levels above 2 (97) are described in Table S5 in the Supplementary Material.

The planktonic cells overexpressed 165 proteins compared to the

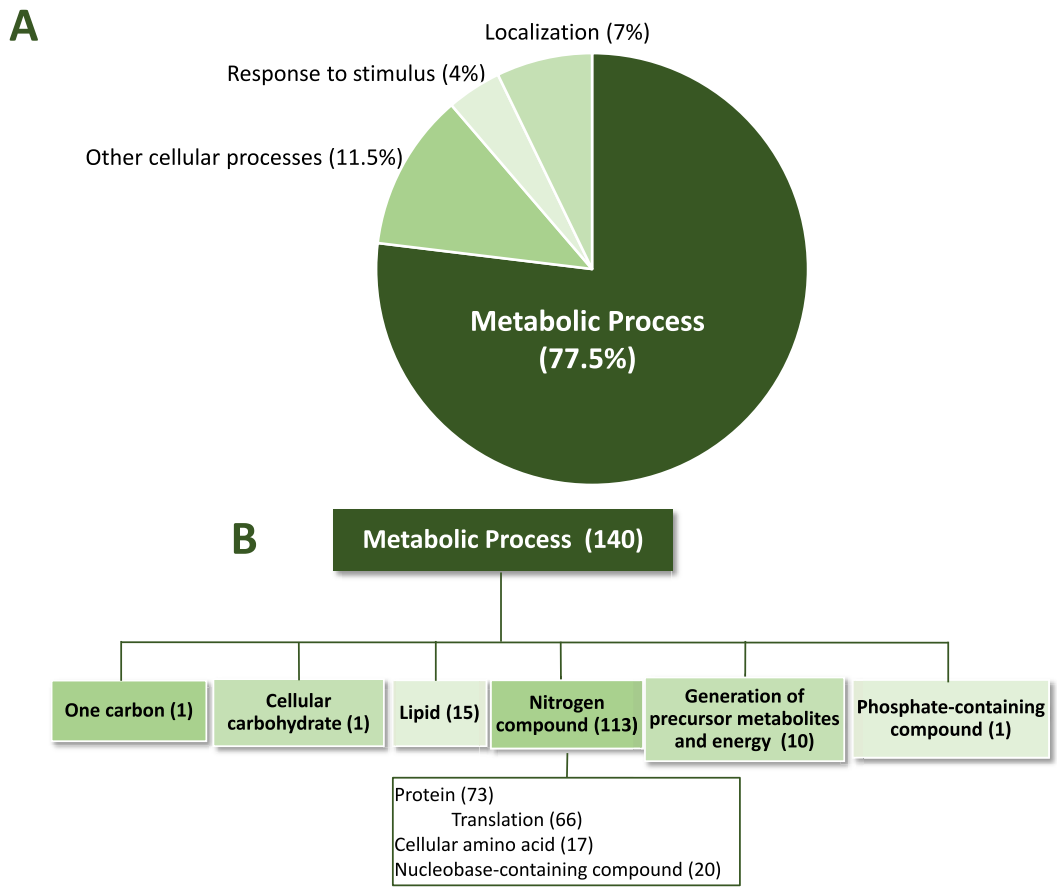


Fig. 2. Distribution of the proteins with higher expression levels in BF- than in NBF-cells according to their biological process category. Graphic A indicates the percentage of each process. Graphic B shows the number of identified proteins corresponding to metabolic process. The numbers shown are approximate because some proteins appear in several subcategories and others belong to categories less represented that have not been included in this table. Proteins considered show fold change equal or higher than 1.3 and p-value lower than 0.1.

biofilm cells. Of them, 149 were related to a particular category of molecular function, and 137 to a biological process. For 75 proteins, information about an association with some cellular component is available. Fig. 5 and Table S8 in the Supplementary Material contains information about the distribution of these proteins in Biological Process Gene Ontology categories. Those proteins showing differential expression values above 2 (81) are included in Supplementary Table S7.

As shown in Tables S6 and S8, regarding carbohydrate metabolism, a higher expression of the enzymes involved in the pentose-phosphate shunt for NADPH regeneration was found in the biofilm cells, while the enzymes of glycolysis were comparatively overexpressed in the case of planktonic cells. However in both cases, the enzymes involved in this pathway were detected. A bigger number of lipid metabolic proteins was observed in the biofilm cells than in the planktonic cells. They appeared to be essentially biosynthetic (of inositol, propionyl-CoA and sterol in the biofilm cells, and phosphatidylcholine for the planktonic cells).

In both comparisons, the most represented metabolic category was that related to nitrogen compounds (Figs. 4 and 5), but several interesting differences are worth mentioning (see also Tables S6 and S8). The most significant was to find 51 translation-associated proteins and simply one proteolytic overrepresented in BF cells, whereas 9 proteins related to essentially proteolytic metabolism and only 1 involved in translation were found in PT cells. This fact allows us to deduce an essentially biosynthetic protein metabolism for the former and a catabolic protein metabolism for the planktonic ones. Nevertheless, some aminoacyl-tRNA synthetases were found to be overexpressed in both cell types (9 in PT- cells and 5 in BF cells), and the level of synthesis of certain amino acids (arginine, proline, asparagine, lysine, cysteine, methionine, threonine and glycine) appeared to be higher in planktonic cells, in which enzymes involved in their biosynthesis were found to be overexpressed. As for nucleobase-containing compounds, more highly expressed proteins were also detected in both cell types (18 in the biofilm versus 21 in the planktonic cells, Figs. 4 and 5).

Another interesting category that was overrepresented in both comparisons referred to the generation of precursor metabolites and energy, with 21 entries in the BF-cells and 30 in the PT ones (Figs. 4 and 5). They corresponded mainly to TCA and/or glyoxylate cycles and ATP synthesis coupled electron transport (Tables S6 and S8 in the Supplementary Material). Malate dehydrogenase and malate synthase showed a higher expression in the planktonic cells, while the opposite occurred for isocitrate lyase, succinate-CoA ligase, oxoglutarate dehydrogenase and subunits alpha and gamma of ATP synthase (Tables S5 and S7).

The detection of the overexpressed proteins that participate in protein folding in the PT-cells suggested that the control of this process could be more relevant than in their biofilm counterparts (Tables S6 and S8). More proteins were seen to be involved in protein transport with differential expression in the BF-cells. In both cases, one protein related to the oxidative stress response was identified.

It is also worth mentioning that the same above-described enzyme (catabolic 3-hydroquinase) which is involved in 3,4-dihydroxybenzoate biosynthesis was overexpressed in the PT-cells.

All these data indicate marked metabolic activity in both cell kinds, as well as some variations between them regarding the balance of biosynthesis, particularly of proteins, toward the biofilm cells.

For the subcellular localization of the overexpressed proteins (Fig. 3), the major group for the BF-cells was represented by those found

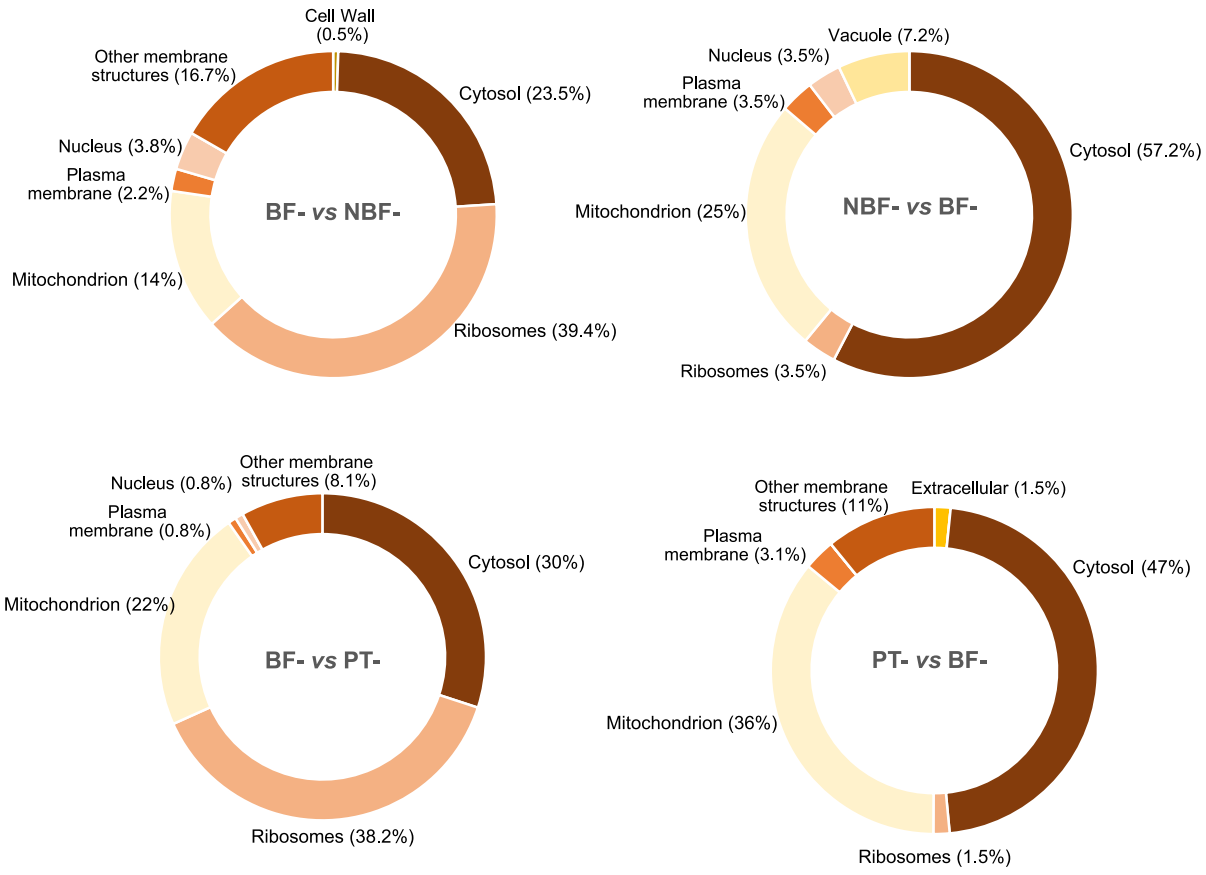


Fig. 3. Distribution of the upregulated proteins according to their subcellular location.

in organelles, mainly ribosome (38 %); 9 % of proteins were associated with the surface or membrane structures. For the PT-cells, cytosolic proteins represented 47 % of the total, and only one was found in the ribosome. In both cases, and according to the data obtained about biological processes, many mitochondrial proteins were detected (22 % and 36 % respectively). One extracellular protein was more expressed in the planktonic cells than in the biofilm ones.

3.4. Common proteins overexpressed in BF-cells vs both NBF- and PT-cells, and in NBF- and PT-cells vs BF-cells

Although the metabolic situation of the cells under non biofilm forming conditions and of the planktonic cells is not probably the same, we considered interesting to identify which common proteins were overexpressed in biofilm cells when compared to them. These analyses allowed the detection of 28 proteins, that were mainly components of the ribosome engaged in translation, but other proteins involved in metabolism, transport and organelle organization were also found (Table 1). On the other hand, 9 common proteins were found to be overexpressed in NBF- and PT-cells when both were compared to BF-cells. These proteins corresponded to enzymes participating in different metabolic processes (Table 2).

3.5. Functional analysis of the D. hansenii proteins with similarity of sequence with those corresponding to the S. etchellsii identified in the proteomic analyses

Based on studies by Kurtzman and Suzuki (2010) on D1/D2 LSU (D1/D2 domain of the large subunit of ribosomal DNA) and SSU rDNA (small subunit of ribosomal DNA) by neighbour-joining and maximum parsimony analysis, the species known as *Debaryomyces etchellsii* was tentatively introduced into the genus *Schwannyomyces*. However, *S. etchellsii* and *D. hansenii* are considered members of the family Debaryomycetaceae and a close phylogenetic relationship exists between the two species, as evidenced by the studies carried out by Thanh et al. (2002). The results shown in Tables S1, S3, S5 and S7 show that more than 10 % of the *S. etchellsii* proteins found in this study were identified by their sequence similarity with *D. hansenii* proteins: 40 of 296 in the BF vs NBF-cells comparison, 34 of 227 in the BF vs PT, 11 of 96 in the case of the NBF vs BF and 20 of 165 in PT vs BF. As shown in Table 1, seven of these proteins were found to be commonly overexpressed in BF- cells vs NBF- and PT- cells. Of these, two had enzymatic activity related to the metabolism of nitrogen compounds, and five were ribosomal proteins.

In view of the relationship between the two species, and since little is known about *S. etchelsii*, additional analyses were carried out about these proteins and their role in *D. hansenii*. The Search Tool for the Retrieval of Interacting Genes (STRING) was used to obtain further information on the relationship between the *D. hansenii* proteins identified in each of the comparisons. This is a database of known or predicted direct (physical) and indirect (functional) protein–protein interactions obtained from various types of quantitatively integrated sources (Szklarczyk et al., 2023). Analyses performed on comparisons between NBF- or PT-cells vs BF-cells did not provide relevant information. In contrast, an important network of interactions was found considering the proteins identified in the comparison between BF-cells and NBF- or PT-cells (Fig. 6, panel A and panel B respectively).

In the first case (BF- vs NBF-cells), and, according to the information provided by STRING, more interactions than expected were found (87 vs. 64, see legend of Fig. 6). Such enrichment is considered as an indication that the proteins are at least partially biologically connected as a group. Among all these interactions, those that gave rise to three networks formed by 11, 7 and 3 proteins stood out. Their components (Table S9) were mainly related to translation, vesicle-mediated transport and protein folding in response to stress, respectively. These three

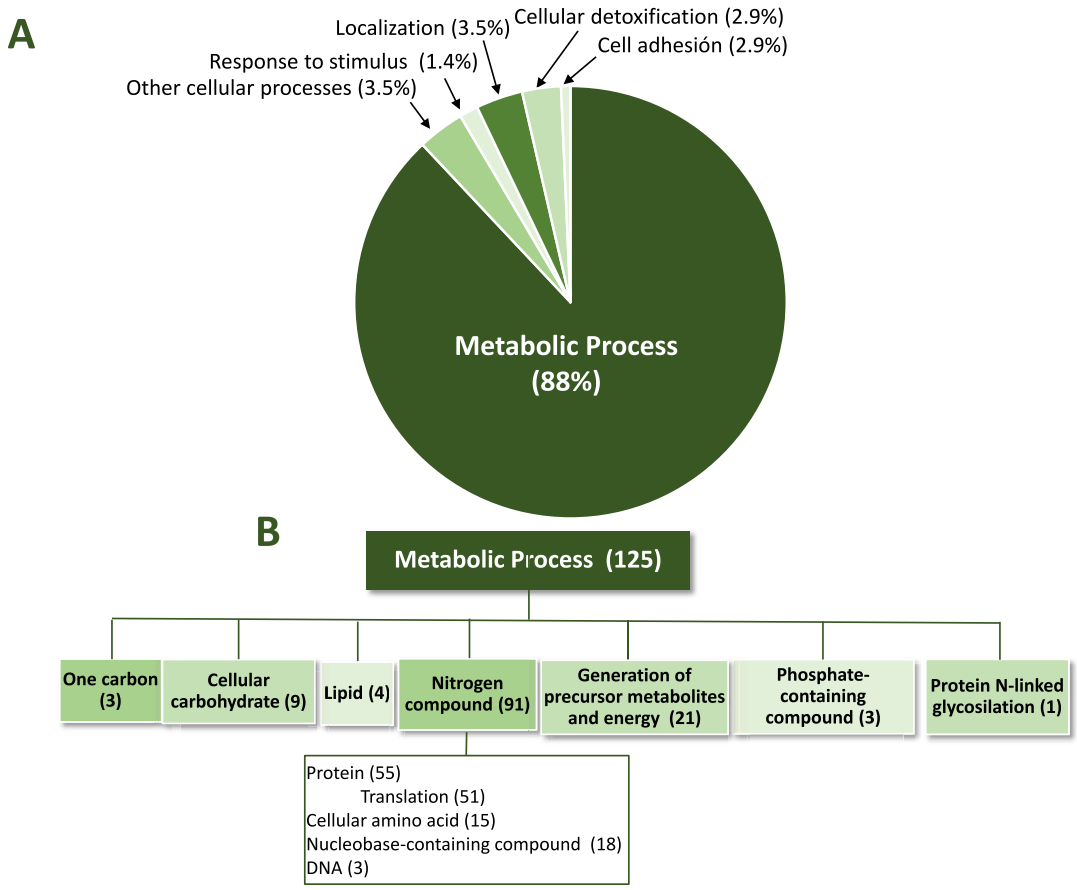


Fig. 4. Distribution of the proteins with higher expression in BF- than in PT-cells in biological process categories. Graphic A indicates the percentage of each process. Graphic B shows the number of identified proteins corresponding to metabolic process. The numbers shown are approximate because some proteins appear in several subcategories and others belong to categories less represented that have not been included in this table. Proteins considered show fold change equal or higher than 1.3 and p-value lower than 0.1.

categories were enriched in previous studies performed by Uniprot with yeast proteins as described above. Analysis of the functional enrichments by other STRING tools (Gene Ontology, local network clustering, KEGG pathways, reactome pathways and subcellular localization) again revealed the role of overexpressed proteins in ribosomal function. Information on the reactome pathways (Fabregat et al., 2017) is included in Table S10.

Regarding the BF- vs PT-cells comparison, more interactions than expected (112 vs 70) were also found, highlighting a very strong interaction between 14 proteins, whose components are related to the ribosome and translation (see Table S11). Analysis of the functional enrichments revealed as overrepresented other interesting categories such as: rRNA export from the nucleus, cellular amino acid biosynthetic (Gene Ontology, biological function), sorbitol dehydrogenase-like and D-xylose NADP:reductase activity, glycolysis (cluster of local networks), pentose and glucuronate interconversions (KEGG pathways) and signalling and carbohydrate metabolism (reactome pathways, Table S10).

3.6. Biofilm cells show higher viability than planktonic cells

Experiments were carried out to determine the number of colonies that the samples from the biofilms and the planktonic cells corresponding to the same optical density value, formed on rich medium plates. The results of these studies revealed that viability in the biofilm cells was 2-fold higher than in the planktonic cells. In the former, $1.56 \times 10^7 \pm 1.31 \times 10^6$ cfu were measured per OD_{600} unit. In the latter, $6.89 \times 10^6 \pm 1.08 \times 10^5$ cfu/OD_{600} unit were counted. It is important to point out that differences were statistically significant according to the *t*-test (p-value of 0.088).

These comparative experiments were not carried between the NBF- and BF-cells because the growth in the former was very low.

3.7. A homolog of the yeast Spf1 protein is more expressed in the biofilm cells than in the planktonic cells in S. etchellsii

The proteomic analyses carried out in this work allowed as to identify the protein Q59Q34_CANAL among those overexpressed in the biofilm cells, with a fold-change of 1.43. This protein is included in the following GO categories: single-species biofilm formation and flocculation on inanimate substrate, cell adhesion involved in single-species biofilm formation, flocculation, fungal-type cell wall organization, protein N-linked glycosylation, transmembrane transport, calcium ion transport from the cytosol to the endoplasmic reticulum and cellular cation homeostasis.

According to our studies, hence, there is a homolog of *Candida albicans* Spf1p in *S. etchellsii*. This protein has been characterized as an ion transporter P-type ATPase in this microorganism, involved in protein glycosylation, cellular adhesion, transmembrane transport, and cell wall organization and biogenesis (Gaudet et al., 2011; Yu et al., 2012, 2013), and is located in the endoplasmic reticulum and plasma membranes (Cabezón et al., 2009; Gaudet et al., 2011). It contains 1223 amino acids with 10 putative transmembrane domains.

Table 3 shows the results of the pairwise alingments between the Spf1 proteins found in yeasts capable of biofilm formation (*C. albicans* and *K. marxianus*) and *S. cerevisiae* strain S288c. Fig. S1 in the Supplementary Material includes the multiple alignment between all these

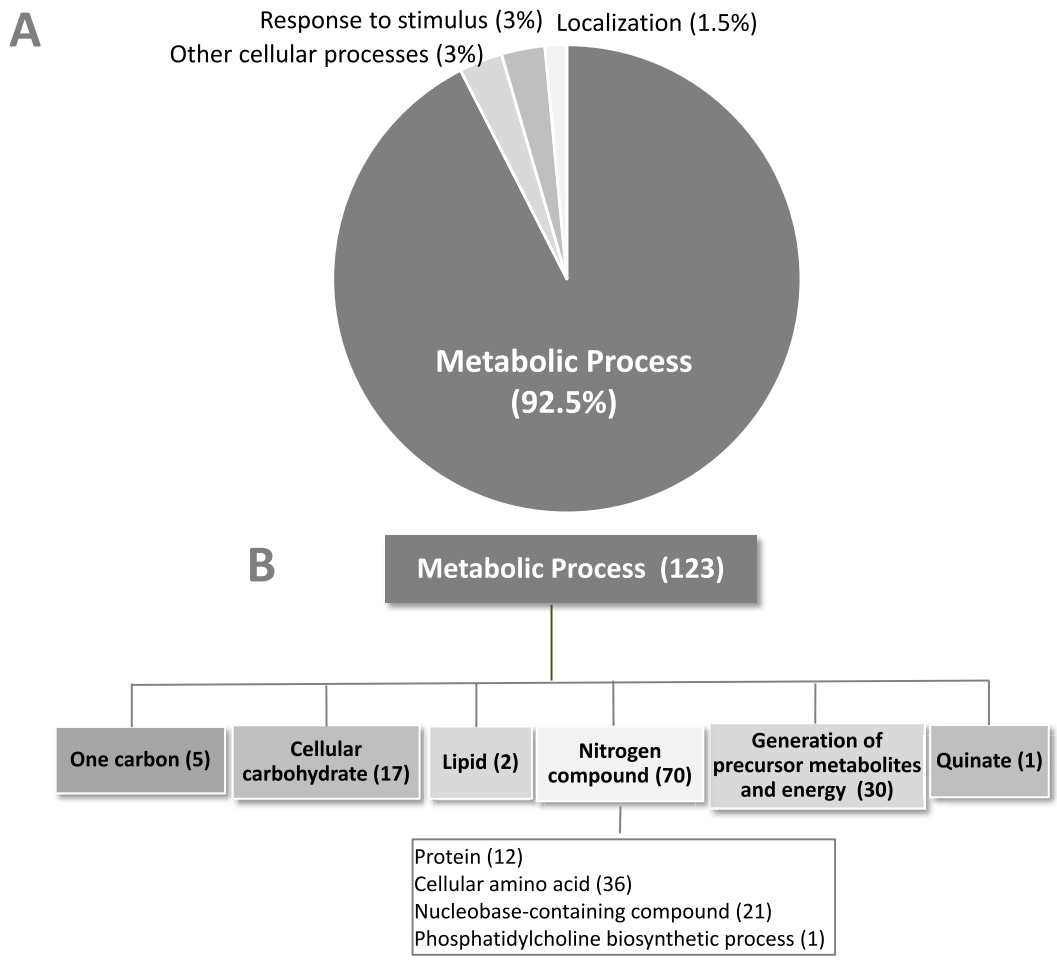


Fig. 5. Distribution of the proteins with higher expression in PT- than in BF-cells in biological process categories. Graphic A indicates the percentage of each process. Graphic B shows the number of identified proteins corresponding to metabolic process. The numbers shown are approximate because some proteins appear in several subcategories and others belong to categories less represented that have not been included in this table. Proteins considered show fold change equal or higher than 1.3 and p-value lower than 0.1.

sequences and that of another *S. cerevisiae* strain, Lalvin EC1118, as well as the location of the peptides encoded by the *S. etchellsii* genome identified in our analyses whose sequences match that of the *C. albicans* Spf1p protein. The *C. albicans* Spf1p protein shows between 90 % and 100 % of sequence identity with the protein of other *Candida* species (data not shown). Besides, it has an identity of around 64 % with the corresponding *S. cerevisiae* protein. The considered strains of the *Saccharomyces* genus contain almost identical sequences between them (99.84 % of identical amino acids, and only 2 different amino acids). The identity between the *K. marxianus* and *S. cerevisiae* Spf1 proteins is around 75 %. All these data indicate important sequence conservation of this protein in yeasts.

4. Discussion

Biofilms constitute a very interesting natural cell immobilization mode to be used in the reactions catalyzed by microorganisms (Halan et al., 2012; Strieth et al., 2018; Todhanakasem, 2017). For this reason, and to fully exploit all the possibilities they can offer, it is important to understand not only the physiological characteristics of cells under these conditions, but also the molecular mechanisms involved in the formation of such communities. This work presents the results of a proteomic study of the biotechnologically relevant *S. etchellsii* yeast. We analyzed and compared for the first time the protein expression in this microorganism among the BF- NBF- and PT-cells. Overall, some common proteins were overexpressed in the biofilm cells regardless of whether the comparison was made to the cells under the non biofilm formation conditions or to the planktonic cells. This was particularly the case of those involved in translation and in accordance with their location in ribosomes. It is also important to highlight that more proteins related to lipid metabolism, membrane structures and transport mediated by vesicles were detected overexpressed. Under all the analyzed conditions, indications of oxidative metabolism were found mainly due to the identification of the proteins belonging to the next three groups: 1) TCA cycle, electron transport chain, and mitochondrial ADP/ATP transmembrane transport; 2) response to oxidative stress and removal of superoxide radicals; 3) the proteins located in mitochondria.

Our results are consistent with those described in proteomic studies carried out by other research groups using yeasts capable of biofilm formation. In analyses with flor yeasts, Moreno-García et al. (2015, 2017) observed that the most abundant proteins under biofilm forming conditions were involved in respiration, translation, stress damage prevention and amino acid metabolism, among other categories. In the studies carried out with *C. albicans* to compare planktonic cell and biofilm proteomes, Seneviratne et al. (2010) described that the biofilm proteome showed a down-regulation of glycolytic enzymes, a trait which has also been observed in our studies. More recently, Munusamy et al. (2021) described in this yeast that cellular macromolecule biosynthesis regulation is a major biological process associated with biofilm cells. Besides, as we observed, the biosynthesis of particular amino acids is related to one or other cells. In accordance with the data presented by these authors and Martínez-Gomariz et al. (2009), our

Table 1
Common proteins overexpressed in *S. etchellsii* BF-cells vs NBF- and PT-cells.

Protein	Description according to Uniprot
tr|A0A167DRI2|A0A167DRI2_9ASCO	Ribosomal 60S subunit protein L32
tr|A0A1E3P112|A0A1E3P112_WICAA	Small ribosomal subunit protein uS17 N-terminal domain-containing protein
tr|A0A1E4S0U0|A0A1E4S0U0_CYBJN	40S ribosomal protein S4
tr|A3GHI2|A3GHI2_PICST	Peroxisomal membrane protein PMP47
tr|A3GHY7|A3GHY7_PICST	60S ribosomal protein L27
tr|A3LUS1|A3LUS1_PICST	Protein involved in nonallelic heterokaryon incompatibility
tr|A5DVG0|A5DVG0_LODEL	60S ribosomal protein L6
tr|B9WIE6|B9WIE6_CANDC	Ribosomal protein, small subunit, putative
tr|G3AWI7|G3AWI7_CANTC	Ribosomal protein, small subunit, putative
tr|G3AWR0|G3AWR0_CANTC	Ribosomal protein L31e
tr|G3B4L9|G3B4L9_CANTC	Ribosomal protein L2
tr|G8XZ95|G8XZ95_PICSO	GTPase activity. Organelle organization
tr|G8Y284|G8Y284_PICSO	Mitochondrial ADP/ATP translocase
tr|G8Y804|G8Y804_PICSO	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial
tr|G8YL02|G8YL02_PICSO	60S ribosomal protein L27
tr|G8YN47|G8YN47_PICSO	Structural constituent of the ribosome
tr|H8WXD6|H8WXD6_CANO9	Rpl21a ribosomal protein
tr|K0KNJ6|K0KNJ6_WICCF	60S ribosomal protein L17
tr|M7WNA8|M7WNA8_RHOT1	40S ribosomal protein s13
tr|Q5AJ93|Q5AJ93_CANAL	Small ribosomal subunit protein eS7
tr|Q6BGY4|Q6BGY4_DEBHA	40S ribosomal subunit S3
tr|Q6BH88|Q6BH88_DEBHA	Delta-1-pyrroline-5-carboxylate dehydrogenase (proline catabolic process to glutamate
tr|Q6BHV6|Q6BHV6_DEBHA	Structural constituent of ribosome
tr|Q6BI01|Q6BI01_DEBHA	Ribosome component
tr|Q6BI46|Q6BI46_DEBHA	Component of the ribosomal small subunit
tr|Q6BMX8|Q6BMX8_DEBHA	Catalyzes the conversion of inosine 5′-phosphate (IMP) to xanthosine 5′-phosphate (XMP), the first committed and rate-limiting step in the de novo synthesis of guanine nucleotides
tr|Q6BYA0|Q6BYA0_DEBHA	Component of the ribosomal small subunit
tr|Q6CJL7|Q6CJL7_KLULA	Ribosomal protein L15

Table 2
Common proteins overexpressed in *S. etchellsii* NBF-and PT-cells vs BF-cells.

Protein	Description according to Uniprot
tr|A0A0H5C453|A0A0H5C453_CYBJN	Malate dehydrogenase
tr|A3GEU3|A3GEU3_PICST	Fructose-bisphosphatase
tr|A3LNE3|A3LNE3_PICST	Aldehyde dehydrogenase
tr|A5DD25|A5DD25_PICGU	Dihydrolipoyl dehydrogenase
tr|A5DGY9|A5DGY9_PICGU	Malate dehydrogenase
tr|A5DPU3|A5DPU3_PICGU	Aminotransferase class I/classII domain-containing protein
tr|C5M2D7|C5M2D7_CANT	Malate dehydrogenase
tr|C5MIT2|C5MIT2_CANTT	Nucleoside diphosphate kinase
tr|W3VUP7|W3VUP7_PSEA5	UTP–glucose-1-phosphate uridylyltransferase

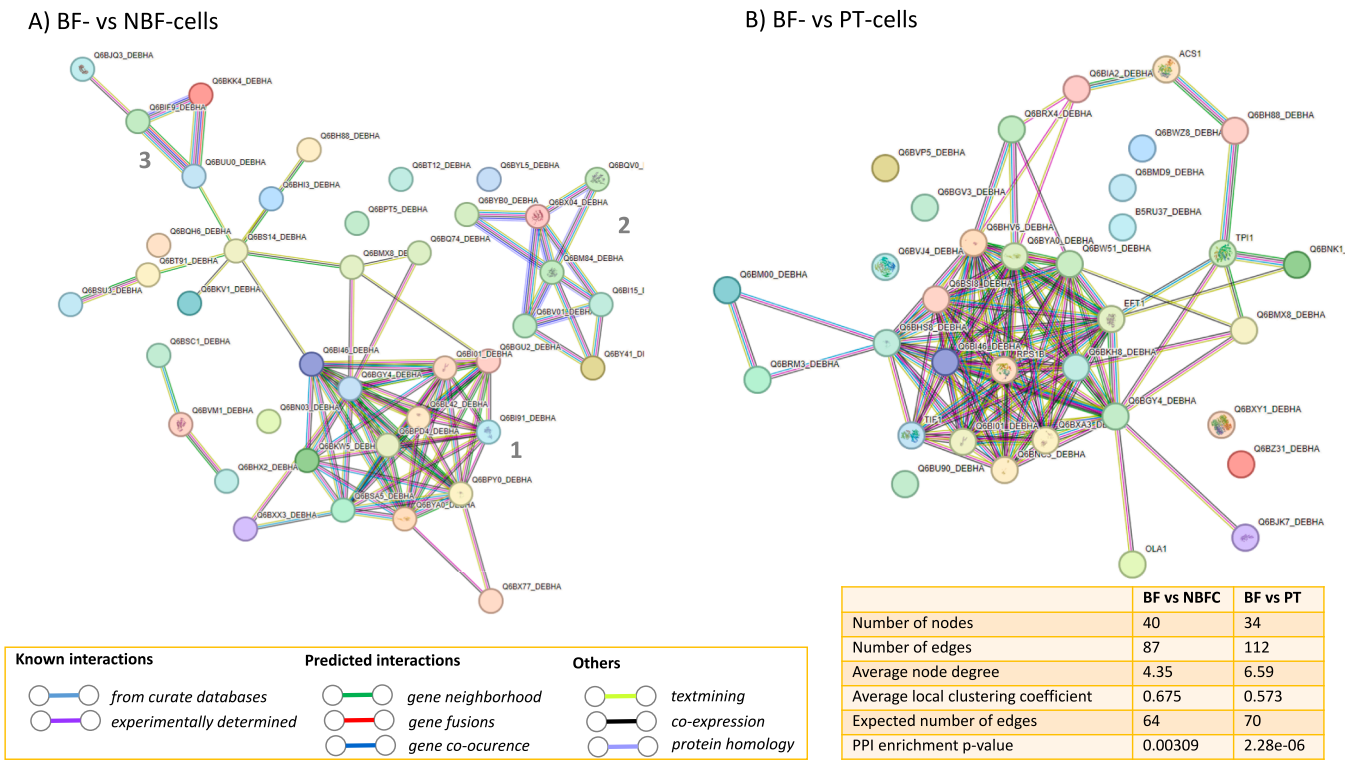
results also indicate enrichment in the cytoplasmic proteins in the planktonic cells and in the endomembrane system in the biofilm cells. The results of these authors and ours allow a conclusion to be reached: there is a common pattern of protein expression in biofilm cells compared to their counterparts, which is probably not limited to pathogenic yeasts.

The *S. etchellsii* genome has not yet been sequenced and very little information is available about its genes and proteins. This work allowed us to identify in this microorganism a protein that is involved in cell adhesion, biofilm formation and intracellular transport according to its homology with *S. cerevisiae* and *C. albicans* Spf1p. This protein is of interest because of its multiple functions in yeast. The *S. cerevisiae SPF1* gene was cloned by complementation of the phenotype of mutants resistant to SMKT, a killer toxin produced by the halotolerant yeast *Pichia farinosa* KK1 (Suzuki and Shimma, 1999). These researchers found that this gene was not essential for viability, but its disruption mutant exhibited phenotypes characteristic of glycosylation-defective mutants. Subsequent studies by Cronin et al. (2002) and Sørensen et al. (2019) showed that this protein has a Mg^{2+} binding site and is a P-type ATPase localized to the endoplasmic reticulum (ER) membrane whose activity is stimulated by phosphatidylinositol 4-phosphate. Other mutant phenotypes of this gene have been identified: impaired calcium homeostasis (with increased transcription of calcium-regulated genes, defective ER function (constitutive activation of the unfolded protein response, hypersensitivity to tunicamycin (glycosylation inhibitor)), and changes in sterol homeostasis (increased sensitivity to sterol production inhibitors, change in ergosterol/lanosterol ratio, accumulation of sterols in the plasma membrane) (Cronin et al., 2002; Sørensen et al., 2019). Some of these phenotypes are similar to those of mutants that do not express the Golgi apparatus pump Pmr1, leading to the suggestion that both cooperate in maintaining the homeostasis of the organelles in which they reside (Cronin et al., 2002; Vashist et al., 2002). Interestingly, the human ATPase protein ATP13A1, implicated in neurological disorders such as Parkinson's disease, complements the *S. cerevisiae SPF1* gene deletion, is located in the ER and has a function in this structure similar to that of Spf1 (Sørensen et al., 2018). Analyses of the related protein in *C. albicans* revealed its P-type ATPase activity and localization in the ER (Yu et al., 2012, 2013). The disruption mutant studies carried out by these authors not only revealed defects in glycosylation and calcium homeostasis, but also others related to cell wall integrity and morphogenesis: increased sensitivity to cell wall stress conditions, abnormal cell wall composition, delayed cell wall reconstruction, defects in hyphal development, reduced flocculation, adhesion and biofilm formation, as well as attenuation of virulence.

Previous studies by other authors have demonstrated the relevance of adhesins, flocculins and other related proteins in biofilm formation. It is worth mentioning the increased incorporation into the biofilm cells of *C. glabrata* of putative adhesins, such as Epa22, Awp14 and Awp2e, and the role in biofilm development of at least one of them, Awp14 (Fernández-Pereira et al., 2021). Greater abundance of flocculins, mainly Flo110, has also been found in the floating biofilm compared to the exponential cells of pathogenic yeast *Pichia kudriavzevii* (Alvarado et al., 2023). Analyses of the adhesins in *C. auris* have demonstrated that it contains an apparently specific protein, surface colonization factor (Scf1), which is required for biofilm formation, colonization of skin and inserted medical devices, virulence and systemic infection (Santana et al., 2023). Recently, Mss11, a transcription factor already known for its role in filamentous growth in other yeasts, has been found to play a crucial role in adhesion and biofilm formation, and virulence in *C. glabrata* (Wang et al., 2024). Further studies will be required to understand the particular role of *S. etchellsii* Spf1, and to identify other proteins involved in biofilm formation and other cellular processes in this yeast.

The information provided in this manuscript contains many unknown proteins of *S. etchellsii* that have been identified by means of their similarity of sequence with other yeast proteins. Particularly interesting are those related to proteins from the yeast *D. hansenii*, on which more information is available and which we have focused on using the STRING analyses described in the results section.

Among the proteins identified thanks to this study in *S. etchellsii*, we consider it interesting to highlight those involved in the generation of energy and metabolic precursors or in survival under conditions of oxidative stress generated by these processes. By means of sequence similarity analysis with proteins from *Candida*, *Pichia*, *Phaffia*, *Lachancea*, *Cyberlindnera*, *Meyerozyma*, *Scheffersomyces* or *Metschnikowia* species, we have found in this work most of the proteins of the tricarboxylic acid cycle (citrate synthase, aconitase, isocitrate dehydrogenase, a-ketoglutarate dehydrogenase, succinyl-CoA synthetase, succinate dehydrogenase and malate dehydrogenase), as well as the beta subunit of



	BF vs NBFC	BF vs PT
Number of nodes	40	34
Number of edges	87	112
Average node degree	4.35	6.59
Average local clustering coefficient	0.675	0.573
Expected number of edges	64	70
PPI enrichment p-value	0.00309	2.28e-06

Fig. 6. STRING protein–protein interaction network of *D. hansenii* proteins showing sequence similarity with those overexpressing in BF- vs NBF-cells (panel A) or in BF- vs PT-cells (panel B). The nodes indicate proteins, and edges indicate the number of interactions. The number of average interactions per node is indicated by the node degree. The clustering coefficient specifies the average node density of the map and is a measure of how connected the nodes in the network are. The expected number of edges gives how many edges are expected for a random set of proteins of the same size and grade distribution extracted from the genome. A small PPI enrichment p-value indicate that the nodes are not random and that the observed number of edges is significant. Numbers 1, 2 and 3 in grey in panel 1 indicate the 3 main networks found.

the E1 component of pyruvate dehydrogenase, an enzyme necessary for the entry of pyruvate from glycolysis into the cycle. It is important to note that some of these proteins are also part of the glyoxylate cycle, along with others also identified in this study (malate synthase and isocitrate lyase). The functioning of this cycle is fundamental in the conditions faced by the yeast cells in the experiments described in this paper, since it allows the link between anabolic and catabolic metabolism under conditions of glucose deprivation and the assimilation of alternative carbon sources. The two key enzymes involved in this cycle are highly conserved in plants, bacteria and fungi (Dunn et al., 2009), which was a key factor in their identification.

S. etchellsii proteins have also been found that are part of the electron transport chain (succinate dehydrogenase, cytochrome *b* and cytochrome oxidase subunit 2), as well as components of ATP synthase responsible for coupling the proton flow generated in the respiratory chain to ATP synthesis (alpha and gamma subunits). These proteins are also highly conserved between yeast and mammals (Pagadala et al., 2011). Respiratory metabolism leads to the generation of reactive oxygen species and it is essential for yeast to have mechanisms to respond to this stress condition (reviewed in Yaakoub et al., 2022). Four proteins identified in *S. etchellsii* on the basis of similarity to their counterparts in *Pichia sorbitophila* are essential for this purpose: peroxidase (G8XYZ0_PICSO), catalase (G8Y7H6_PICSO), peroxiredoxin (G8YBX3_PICSO) and superoxide dismutase (G8YQ50_PICSO). Peroxidase and catalase are responsible for the breakdown of hydrogen peroxide, peroxiredoxin is a thiol-specific peroxidase involved in the reduction of organic hydroperoxides and hydrogen peroxide, and superoxide dismutase accelerates the reaction of the superoxide anion with itself to produce hydrogen peroxide and oxygen. These *P. sorbophila* proteins share at least 50 % sequence identity with the corresponding proteins of yeast genera such as *Meyerozyma*, *Debaryomyces*, *Candida*, *Scheffersomyces* or *Metschnikowia* according to Uniprot (data not shown).

According to this, more information about the proteome of *S. etchellsii* is provided and all this data could be useful for other research groups/projects in the future. Besides, the analysis carried out with all these proteins to determine its molecular function reveals that more than 150 of them have enzymatic activity, which opens the possibility to identify proteins with particular properties that could be useful for biotransformations. As explained in the Introduction section this strain has interesting catalytic properties that should lie in its enzymes and a

Table 3
Data from the pairwise alignments of yeast Spf1 proteins according to the EMBOSS Matcher facility.

A) Identity (%)

Strain	*C. albicans*	*K. marxianus*	*S. cerevisiae* S288c
C. albicans	100	64.5	63.7
K. marxianus	64.5	100	75.2
S cerevisiae S288c	63.7	75.2	100

B) Similarity (%)

Strain	*C. albicans*	*K. marxianus*	*S. cerevisiae* S288c
C. albicans	100	77.3	78.2
K. marxianus	77.3	100	85.7
S cerevisiae S288c	78.2	85.7	100

C) Gaps (%)

Strain	*C. albicans*	*K. marxianus*	.*S cerevisiae* S288c
C. albicans	0	2.1	2.0
K. marxianus	2.1	0	0.2
S cerevisiae S288c	2.0	0.2	0

better knowledge of them is needed.

5. Conclusions

In a previous work (Zarnowski et al., 2021a) we described the ability of *S. etchellsii* to form biofilms in seawater medium and carried out an analysis of the composition of their extracellular matrix in terms of monosaccharides and proteins. Herein, we have carried out new analyses in order to characterize the proteome of the biofilm cells embedded in this extracellular matrix and to determine molecular mechanisms that could explain the ability of this yeast to form such structures. The present work reports differences between the proteome of the biofilm cells and the planktonic or non-biofilm forming cells of yeast *S. etchellsii*. Besides dissimilarities in many other metabolic (carbohydrate, lipid and amino acid biosynthesis or catabolism) and non-metabolic (response to stress, transport) processes, these cells display increased activity in protein biosynthesis, which should lie behind their higher viability. This is in accordance with the location of a large number of overexpressed proteins in ribosomes, and the results obtained with the use of the STRING database in analyses carried out with yeast *D. hansenii* proteins with which sequence similarity was found. Our studies demonstrate the upper expression in the biofilm cells of a homolog of *S. cerevisiae* and *C. albicans* Spf1 protein, which has been implicated in cell adhesion, biofilm formation and intracellular transport in these yeasts. It is worth mentioning that in this work a list of *S. etchellsii* proteins with counterparts in other yeasts is provided; considering the low information available about the proteome of this yeast it could be helpful for the future characterization of other proteins, particularly those with catalytic activity.

CRediT authorship contribution statement

Both authors have contributed in all the roles required for the herein presented work: conceptualization, data analysis, funding acquisition, investigation, and writing and editing the original draft.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crbiot.2024.100242.

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