Applied Microbiology and Biotechnology
https://doi.org/10.1007/s00253-021-11090-8

APPLIED GENETICS AND MOLECULAR BIOTECHNOLOGY

Surface display of HFBI and DewA hydrophobins on *Saccharomyces cerevisiae* modifies tolerance to several adverse conditions and biocatalytic performance

Cecilia Andreu[1] · Javier Gómez-Peinado[2] · Lex Winandy[3] · Reinhard Fischer[3] · Marcel li del Olmo[2]

Received: 29 October 2020 / Revised: 14 December 2020 / Accepted: 3 January 2021

Abstract

Hydrophobins are relatively small proteins produced naturally by filamentous fungi with interesting biotechnological and biomedical applications given their self-assembly capacity, efficient adherence to natural and artificial surfaces, and to introduce modifications on the hydrophobicity/hydrophilicity of surfaces. In this work we demonstrate the efficient expression on the S. cerevisiae cell surface of class II HFBI of *Trichoderma reesei* and class I DewA of *Aspergillus nidulans*, a hydrophobin not previously exposed, using the Yeast Surface Display a-agglutinin (Aga1-Aga2) system. We show that the resulting modifications affect surface properties, and also yeast cells' resistance to several adverse conditions. The fact that viability of the engineered strains increases under heat and osmotic stress is particularly interesting. Besides, improved biocatalytic activity toward the reduction of ketone 1-phenoxypropan-2-one takes place in the reactions carried out at both 30 °C and 40 °C, within a concentration range between 0.65 and 2.5 mg/mL. These results suggest interesting potential applications for hydrophobin-exposing yeasts.

Key points

- *Class I hydrophobin DewA can be efficiently exposed on S. cerevisiae cell surfaces.*
- *Yeast exposure of HFBI and DewA increases osmotic and heat resistance.*
- *Engineered strains show modified* biocatalytic behavior

Keywords Hydrophobin · HFBI · DewA · Biocatalysis · Stress resistance

Introduction

Biocatalysis is considered a powerful tool to perform chemical transformations of organic compounds under very mild conditions and with high chemo-, regio-, and stereoselectivity. Its specificity results in the production of a few side products, and water is employed as an environmentally friendly solvent (Ni et al. 2014), with minimum waste pollutant production (Crabtree 2009; Heus et al. 2015; Tao and Kazlauskas 2011). For all these reasons, biocatalysis has been broadly used as a green alternative to classic chemical methods in pharmaceutical, agrochemical, and food industries (Andreu and Del Olmo 2014; Andreu et al. 2016; Andreu and del Olmo 2018a, 2019, 2020).

✉ Marcel li del Olmo
m.del.olmo@uv.es

[1] Departament de Química Orgànica, Facultat de Farmàcia, Universitat de València, Burjassot, València, Spain

[2] Departament de Bioquímica i Biologia Molecular, Facultat de Ciències Biològiques, Universitat de València, Burjassot, València, Spain

[3] Department of Microbiology, Karlsruhe Institute of Technology (KIT)-South Campus, Fritz-Haber-Weg 4, D-76131 Karlsruhe, Germany

Published online: 23 January 2021

Whole cells of *Saccharomyces cerevisiae* have been widely employed in asymmetric transformations, especially in the synthesis of chiral alcohols by bioreduction reactions (Bariotaki et al. 2012; Moore et al. 2007; Pscheidt and Glieder 2008). Employing whole cells as biocatalysts offers several advantages over the use of isolated enzymes: the natural environment in cells increases enzyme stability; no additional costs are needed for enzyme isolation and purification; finally, and most importantly, the use of expensive external cofactors is unnecessary because cells have systems for their own efficient in situ recycling (Kisukuri and Andrade 2015; Robertson and Steer 2004).

Yeast surface display (YSD) technology allows the expression of peptides and proteins of interest in the yeast cell wall (for recent reviews see Andreu and del Olmo 2018b; Linciano et al. 2019; Urbar-Ulloa et al. 2019). It is based on a type of protein characteristic of the most superficial cell wall layer, mannoproteins such as Sag1 (AGα1), a-agglutinin (Aga1-Aga2), Fig2, Flo1, Sed1, Spi1, Cwp1/2, Tip1, Tir1, and Pir1/2/3/4. The protein to be exposed on the cell surface is associated with one of them, it comes in the form of a fusion protein, and its expression can be regulated by different systems. The choice of one or another depends on the yeast species, the required expression level, and the size of the protein to be placed on the cell surface (Andreu and del Olmo 2018b). The widely used a-agglutinin system consists of two subunits linked through two disulfide bridges: Aga1 is anchored via a GPI to the cell surface, while Aga2 is used to immobilize the protein of interest on the cell surface (Cappellaro et al. 1994). pYD1 and pYD5 are vectors used for YSD with this system (Kieke et al. 1997; Wang et al. 2005); in both, gene fusions are expressed under the control of the *GAL1* gene promoter and are, hence, induced by galactose. The YSD methodology has allowed different biomedical and biotechnological applications to be developed, including biocatalysis (Benjaphokee et al. 2012; Boder et al. 2012; Kumar and Kumar 2019; Park 2020; Perpiñá et al. 2015; Tabañag et al. 2018; Takayama et al. 2006; Traxlmayr and Shusta 2017; Wasilenko et al. 2010; Wu et al. 2015).

Hydrophobins are relatively small proteins produced naturally by filamentous fungi, whose properties allow these organisms to survive and adapt to the environment (Sammer et al. 2016; Wessels 1997; Wösten 2001). They show interesting physicochemical properties, such as surface activity and self-assembly, and can adhere efficiently and stably to natural and artificial surfaces. With these capacities, they are able to convert hydrophobic surfaces into hydrophilic or hydrophilic into hydrophobic (Linder et al. 2005; Wessels 1997). Their homology is not great, except they contain many hydrophobic amino acids and eight cysteine residues with conserved spacing, which form a total of four disulfide bridges. Two classes are differentiated: type I hydrophobins, which contain between 100 and 125 amino acids, can be glycosylated and can only be dissociated from membranes by agents such as formic acid (FA) or trifluoroacetic acid (TFA); type II hydrophobins are formed by 50-100 residues and are soluble in ethanol or SDS (Wessels 1997; Wösten 2001). These molecules have attracted much interest in biotechnology for their potential applications. In fact, it has been described that type I hydrophobins in the soluble form are able to stabilize some compounds of medical interest (Zhao et al. 2016) and can increase the solubility of organic compounds (Valo et al. 2010), properties that have been applied to obtain pharmaceutical preparations.

Among the hydrophobins described to date, we find the hydrophobin of class II HFBI of *Trichoderma reesei* and that of class I DewA of *Aspergillus nidulans*. The former is expressed at very high levels when that fungus is grown in glucose-containing media (Nakari et al. 1993) and has been genetically and biochemically characterized (Nakari-Setälä et al. 1996). HFBI has been previously exposed on the surface of yeast cells by using the YSD methodology to obtain slightly less negatively charged cells with a more apolar cover (Nakari-Setälä et al. 2002). There are reports showing that the co-display of this hydrophobin with the lipase B of *Candida antarctica* in *Pichia pastoris* allows this lipase greater activity due to the changes introduced in the structure and the hydrophobic properties of the cell surface (Wang et al. 2016; Zhang et al. 2017).

Hydrophobin DewA was identified by Stringer and Timberlake (1995). It has been described to be actively expressed in late conidiation stages in *A. nidulans* (Grünbacher et al. 2014; Stringer and Timberlake 1995), downregulated in early vegetative growth, and differentially expressed during the isotropic-to-polar growth switch, which suggests that it may function in isotropic expansion during both vegetative growth and asexual reproduction (Breakspear and Momany 2007). It has been subsequently expressed in *T. reesei* (Schmoll et al. 2010). Some more recent studies demonstrate its utility for the functionalization of surfaces to which it is capable of adhering. In this sense, variants of DewA that contain binding sites for integrin receptors, such as the Arg-Gly-Asp sequence (RGD) or the globular domain of laminin LG3, and can present greater adhesion capacity of mesenchymal stem cells, osteoblasts, fibroblasts, and chondrocytes at no higher risk of bacterial infections may be useful for developing medical implants (Boeuf et al. 2012). Fokina et al. (2016) have described how biotechnologically relevant enzymes, such as laccases, can be immobilized on particular surfaces by their expression as fusion proteins with DewA. These authors thus obtained interesting results for the LccC protein of *A. nidulans* in modified hydrophilic polystyrene.

Winandy et al. (2018) expressed and purified these two, and other soluble class I Dew hydrophobins of *A. nidulans* from *Escherichia coli* to compare their surface binding

properties. These authors demonstrated that all these proteins are efficient in forming glass surface coatings to thereby increase the hydrophobicity of glass. Besides, they organized a uniform layer, except for DewE, which formed protein aggregates. The results reported agree with previous studies demonstrating that hydrophobins from a single organism belonging to the same class can exhibit various surface binding characteristics (van Wetter et al. 2000). Indeed, only DewA protein layers showed typical class I high resistance to water, ethanol, detergent, and temperature treatments, while the HFBI class II hydrophobin formed less stable layers on glass surfaces. More recently, this research group also revealed that DewA and HFBI are able to coat and reduce the water absorption of different lithotypes, independently of their chemical nature and structure. DewA especially generated strong water repellency on all three lithotypes without decreasing the vapor permeability of stone samples (Winandy et al. 2019).

In this work, the cloning of the genes encoding both HFBI and DewA hydrophobins in plasmid pYD5 was carried out to achieve efficient exposure on the cell surface of the yeast *S. cerevisiae.* The properties of the resulting strains were characterized, and their potential application as biocatalysts was also evaluated and compared to the unmodified one.

Materials and methods

Cloning of hydrophobin genes in the pYD5 vector

Genes *hfbI* and *dewA* were cloned in pYD5 (Wang et al. 2005). Previously, and in order to increase the versatility of this vector for genetic engineering purposes, unique restriction sites for *Sma*I and *Nco*I were added close to the recognition site for *Eco*RI by site-directed mutagenesis, using the "QuikChange Lightning Site-Directed Mutagenesis Kit" (Stratagene, La Jolla, USA) following a previously described procedure (Andreu and del Olmo 2013). The employed oligonucleotides are included in Table S1 in the Supplementary Material. The resulting plasmid was designed by pYD5M (Fig. 1a).

For *hfbI* cloning, the coding region for the mature protein was amplified by PCR from plasmid pTNS23 (Nakari-Setälä et al. 2002) using oligonucleotides HFBSMA and HFBECO (described in Table S1 in the Supplementary Material) and introduced between the *Sma*I and *Eco*RI sites of pYD5M (Fig. 1b). pTNS23 was previously isolated from yeast strain VTT-C-99315 provided by the VTT Technical Research Centre (Finland) and propagated through *E. coli* strain DH5α (New England Biolabs, Ipswich, Massachusetts, USA). The resulting plasmid was named pYD5M-HFBI.

For *dewA* cloning, the coding region was amplified from plasmid pLW58 (Winandy et al. 2018) using oligonucleotides DEWAECORVF and DEWAECORVR (Table S1 in the Supplementary Material), digested with *Eco*RV, and introduced into the *Sma*I site of pYD5M (Fig. 1b). The resulting plasmid was named pYD5M-DewA.

The Gibson Assembly methodology was used to introduce a second copy of the hydrophobin-coding genes into pYD5M-HFBI and pYD5M-DewA (Gibson et al. 2009). To this end, these plasmids were linearized with *Sma*I and *Eco*RI, respectively. PCR products *hfbI* and *dewA* were generated using oligonucleotides HfbIGibA/B and DewAGibA/B (Table S1 in the Supplementary Material), and the procedure described in the *Gibson Assembly® Cloning Kit* (New England Biolabs, Ipswich, Massachusetts, USA) was followed with the reagents supplied by the manufacturer. The resulting plasmids were called pYD5M-HFBI-2c and pYD5M-DewA-2c.

The introduction of the desired sequences and their location in the frame with the Aga2 coding sequence in the resulting plasmids were confirmed in all cases by sequencing with oligonucleotides AGA2SEQ and SEQPYD5DIR (also described in Table S1 in the Supplementary Material).

Yeast strains and growth conditions

All the experiments were carried out with the *S. cerevisiae* EBY100 strain (*MATa trp1 leu2Δ1 his3Δ200 pepA:HIS3 prb1D1.6R can1 PGAL-AGA1*, Wang et al. 2005) transformed with the above-described pYD5-derived plasmids. Strains were grown in SC-trp minimal medium (0.17% (*w*/*v*) nitrogen base without amino acids and ammonium sulfate, 0.5% (*w*/*v*) $(NH_4)_2SO_4$, 0.2 % (*w*/*v*) drop out mix without Trp), containing 2% (*w*/*v*) glucose (Glu) or galactose (Gal) as a carbon source. The solid medium also contained 2% (*w*/*v*) agar and 2% glucose as a carbon source. Liquid cultures were incubated at 30 °C in an orbital shaker (180 rpm). For most experiments, an overnight liquid culture was prepared on SC-Trp Glu medium up to an OD_{600} between 2 and 5; then, cells were collected, washed, and transferred to fresh SC-Trp Gal medium, and incubation continued for 24 h.

To carry out the growth kinetic experiments, the cells from the overnight cultures in the SC-Trp containing glucose were transferred to a fresh medium with glucose or galactose at an

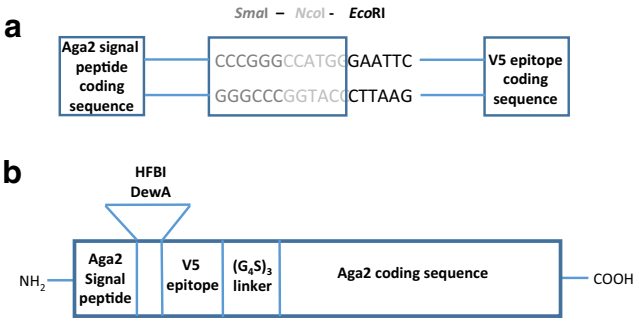


Fig. 1 Scheme of the sequence modifications introduced into the pYD5 vector (on the gray scale) to generate pYD5M (a) and the protein fusions expressed under the control of the *GAL1* promoter in pYD5M-HFBI and pYD5M-DewA (b)

OD_{600} of 0.2, and incubation continued for 2 days at 30 °C. In some experiments, growth was also followed under several stress conditions (37 °C or 0.5 M NaCl). In these cases, the initial OD_{600} after cell transfer from glucose to galactose medium was approximately 0.3, and the stress condition was applied 6 to 8 h later to allow the expression of the fusion proteins. Experiments were carried out in triplicate.

Protein analysis

Samples were taken from the cultures of the strains considered grown in glucose or galactose. Protein extracts were prepared and analyzed by western blotting as described in Perpiñá et al. (2015). Prior to immunodetection, the transfer of proteins to the nitrocellulose filters was confirmed by staining with 0.1% (*w*/*v*) Ponceau S (Sigma, St. Louis, USA) in 5% acetic acid. Anti-V5 (Invitrogen, San Diego, USA) was employed to detect the proteins expressed from plasmid pYD5 using a dilution of 1 : 5000 in PBS, 0.05% (*v*/*v*) Tween 20, 5% (*w*/*v*) nonfat dry milk.

Flow cytometry

The flow cytometry experiments were carried out as described in Perpiñá et al. (2015) with the samples obtained from the cultures of strains after growth under the previously described conditions.

Fluorescence microscopy

To detect Aga2 on the cell surface by fluorescence microscopy, cells were prepared in a similar fashion to that used for the FACS (fluorescence-activated cell sorting) analyses and they were finally observed under a fluorescence microscopy Axioskop 2 (Zeiss Inc., Jena, Germany) using the GFP filter. Pictures were taken with a SPOT camera (Diagnostic Instruments Inc., Sterling Heights, USA).

Assay of cell surface hydrophobicity

The cell surface hydrophobicity analysis done with the strains herein used was carried out according to the procedures described by Wang et al. (2016) and Zhang et al. (2017), in which this parameter is determined from the proportion of the yeast cells passed to an organic phase (constituted by butanol in our case). For these experiments, cells were grown in SC-Trp medium with galactose as the only carbon source for 24 h after previous growth in Sc-Trp Glu. Experiments were carried out 5 times.

Contact angle determination

The yeast cells from 24-h cultures in SC-Trp Gal medium were freeze-dried, resuspended in 100% EtOH (*w*/*w* ratio 1 : 10 yeast/EtOH), and applied on both "normal" hydrophilic glass and silanized hydrophobic glass. After complete solvent evaporation, WCA were measured with an OCA20 and v3.12.11 of the SCA 202 software (both DataPhysics Instruments GmbH, Filderstadt, Germany), as described in Winandy et al. (2018).

Flocculation assay

The flocculation ability of the strains considered in this work was determined by the method described by Bony et al. (1997) and Nayyar et al. (2014). The yeast cells grown in SC-Trp Gal for 24 h were harvested by centrifugation, washed in de-flocculation buffer (50 mM sodium acetate pH 4.5, 5 mM EDTA), incubated in this medium for 30 min, and washed again (twice in this buffer and twice in double-distilled water). Cells were resuspended at OD_{600} of 2 in 5 mL of flocculation buffer (50 mM sodium acetate, 5 mM $CaCl_2$) and placed inside test tubes (15 mm diameter, 50 mm height). They were sealed and left in a shaking incubator at room temperature and 140 rpm for 30 min. Then, the cell suspension was left undisturbed for 6 min in a vertical position and OD_{600} was determined by taking the samples just below the meniscus. The percentage of flocculated cells was calculated by subtracting the fraction of cells that remained in suspension from the total cell count. Experiments were carried out 5 times.

Viability determination under several stress conditions

The percentage of viable cells was determined from the cultures grown for 24 h in SC-Trp medium containing galactose as the only carbon source. Five OD_{600} units were collected by centrifugation, washed with distilled water, and resuspended in 1 mL of this solution containing the considered concentration of the reagent to be tested. The analyzed conditions were temperature (37, 40, 42, and 44 °C), presence of salt (NaCl concentrations between 0.7 and 1.5 M), ethanol (6 to 10%, *v*/*v*), acetonitrile (3 to 9%, *v*/*v*), dimethylformamide (DMF, 6 to 25%, *v*/*v*), and dimethylsulfoxide (DMSO, 12–30%, *v*/*v*). Viability was determined by using a trypan blue reagent as previously described (Andreu and del Olmo 2019). Experiments were carried out in triplicate.

The viability for organic compound phenoxyacetone was also determined. It was also used to analyze the ability of these strains as biocatalysts. In this case, ten OD_{600} units were employed instead of five, to work under the same conditions in both experiments. The concentration range was between 0.65 and 5.3 mg/mL.

General procedure for biocatalytic processes using cell-exposing hydrophobins HFBI and DewA

The cells from the overnight cultures in SC-Trp Glu were transferred to a fresh medium containing galactose as the only carbon source and incubation at 30 °C continued for another 24-h period. The volume that corresponded to 100 OD_{600} units from these cultures was centrifuged. Cells were washed and suspended in 2% galactose prepared in distilled freshwater (9 mL) and transferred to a 50-mL Erlenmeyer flask. The mixture was incubated at 30 °C or 40 °C for 30 min with orbital shaking (180 rpm). Then, 1 mL of a freshly prepared suspension of phenoxyacetone (1-phenoxypropan-2-one, 1) in water at a 10-fold higher concentration than the desired final one was added. The mixture was maintained with orbital shaking for 24 h at the same temperature. Afterward, it was centrifuged (3 min at 3000 rpm) and the aqueous supernatant was extracted with methylene chloride (2 × 8 mL). The organic phases were combined and dried over sodium sulfate. After solvent evaporation, the crude material was analyzed by ^{1}HNMR (in a Bruker DRX 300 spectrometer; Bruker, Billerica, MA) and integrated to quantify the percentage of all the compounds. Chiral High-Performance Liquid Chromatography (HPLC, Merck Hitachi Lachrom system, Darmstadt, Germany) using a Chiralcel ODH column (Hex/*i*prOH 95:5, flow = 1 mL min^{-1}, wavelength = 214 nm) was performed to determine enantiomeric excess. In all cases, and according to the retention times (Andreu and del Olmo 2019), the enantiomer that was mainly obtained was that with *S* configuration (ee 85%). The NMR data corresponding to the purified compound by flash chromatography (Merck silica gel 60, particle size: 0.040-0.063 mm) were fully consistent with those described in the literature (Andreu and del Olmo 2019).

Results

Hydrophobins HFBI from *T. reesei* and DewA from *A. nidulans* can be efficiently expressed on the cell surface of *S. cerevisiae* strain EBY100

In order to understand how the exposure of hydrophobins of classes I and II could affect the features of the *S. cerevisiae* cell surface, the coding sequence of two well-known representatives of these groups (class I DewA from *A. nidulans* and class II HFBI from *T. reesei*) were cloned into the pYD5 yeast display vector, as described in "Materials and Methods." In this way, after the transformation of strain EBY100 with the resulting plasmids (pYD5M-HFBI and pYD5M-DewA), the expression of the gene fusions should occur in the presence of galactose as the only carbon source, and fusion proteins Aga2-HFB1 and Aga2-DewA would remain attached to the cell surface through the disulfide bridges established between agglutinins Aga1 and Aga2.

The western blot shown in Fig. S1 in the Supplementary Material demonstrates that both proteins were expressed in the transformed yeast cells as fusions with V5 epitope-Aga2. Migration of proteins was affected by their glycosylation, which resulted in several bands that appeared rather fuzzy. Flow cytometry analyses were carried out to determine the level of expression of these fusion proteins on the cell surface. The presence of the V5 epitope in the fusion proteins provides an accurate determination of the percentage of cells displaying HFBI and DewA in populations after 24 h of growth in a minimal medium containing galactose as a carbon source. The obtained results (Fig. 2a) indicated that efficient exposure had been achieved. According to the integration of the peaks corresponding to the fluorescent (the right one) and nonfluorescent (the left one) cells, around 71% of them exposed Aga2 in the strain transformed with pYD5M and a slightly lower percentage (about 67–69% approximately) was detected for Aga2-HFBI and Aga2-DewA. The analysis of the mean fluorescent intensity, shown as M in this figure, indicated certain displacement to lower values in DewA, and in HfbI to a greater extent. According to this, the number of molecules exposed per cell lowered to some extent when hydrophobins were introduced into protein V5-Aga2, but the values were indicative of a marked expression on the cell surface.

Exposure of fusion proteins with hydrophobins was also confirmed by fluorescence microscopy observation (Fig. 2b).

Exposure of hydrophobins HFBI from *T. reesei* and DewA from *A. nidulans* affects cell surface properties

Hydrophobins have been described to be able to convert hydrophobic surfaces into hydrophilic or hydrophilic into hydrophobic (Wessels 1997; Linder et al. 2005).

Figure 3 shows the hydrophobicity profile provided by the *Protscale* software (Gasteiger et al. 2005) for the fusion proteins of Aga2 with the two hydrophobins considered in this work compared to that corresponding to Aga2 when they are expressed from the pYD5-derived vectors herein used.

According to these profiles, both proteins should increase the hydrophobicity of the cell surface. This parameter was also determined experimentally following the procedure of Wang et al. (2016) and Zhang et al. (2017), as described in "Materials and Methods." In this methodology, the hydrophobicity index (HI) is determined through the cell population being distributed between butanol (organic phase) and water. The obtained results were 0.022 ± 0.012 for the EBY100/pYD5M strain, 0.109 ± 0.027 for EBY100/pYD5M-HFBI, and 0.072 ± 0.012 for EBY100/pYD5M-DewA. The

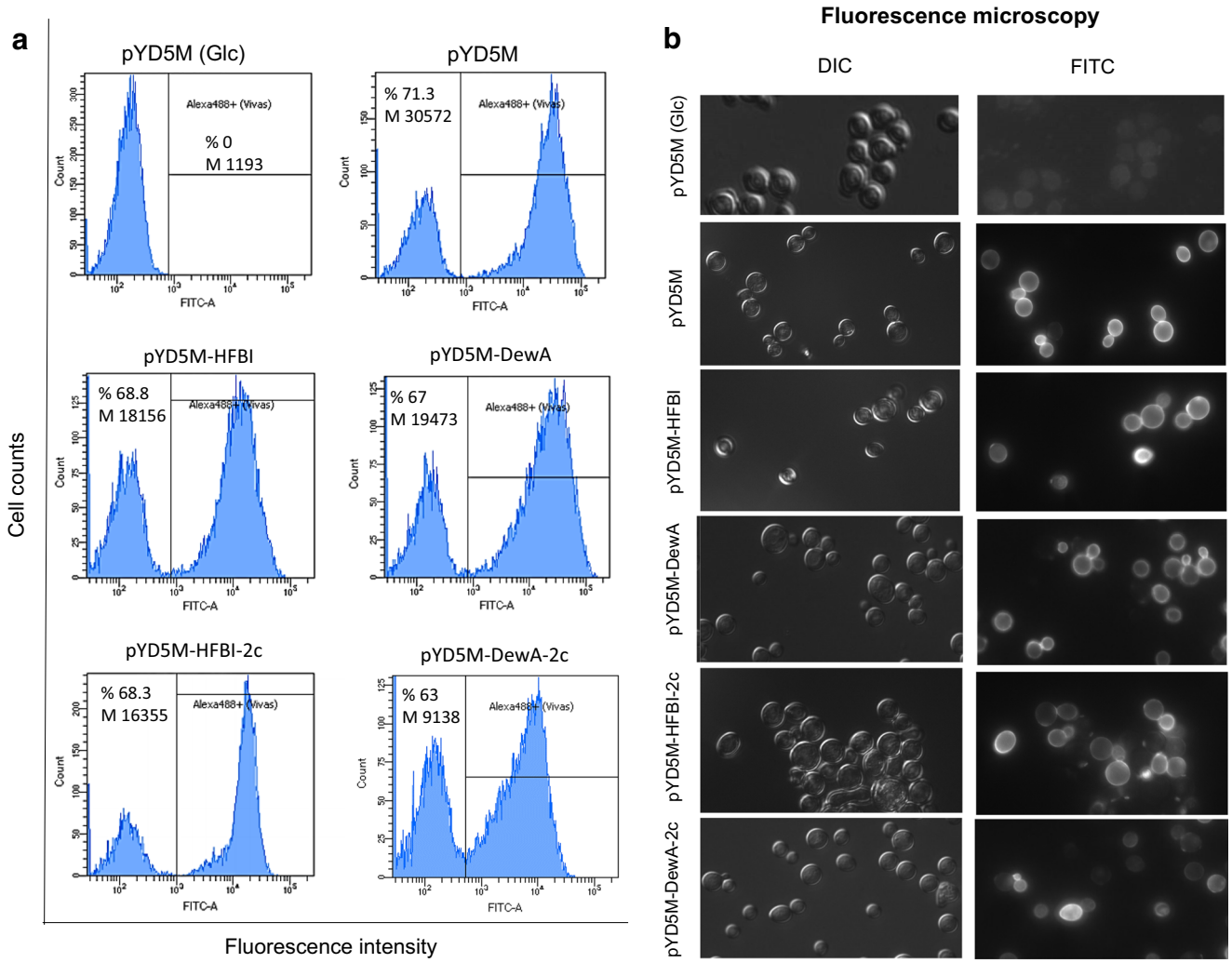
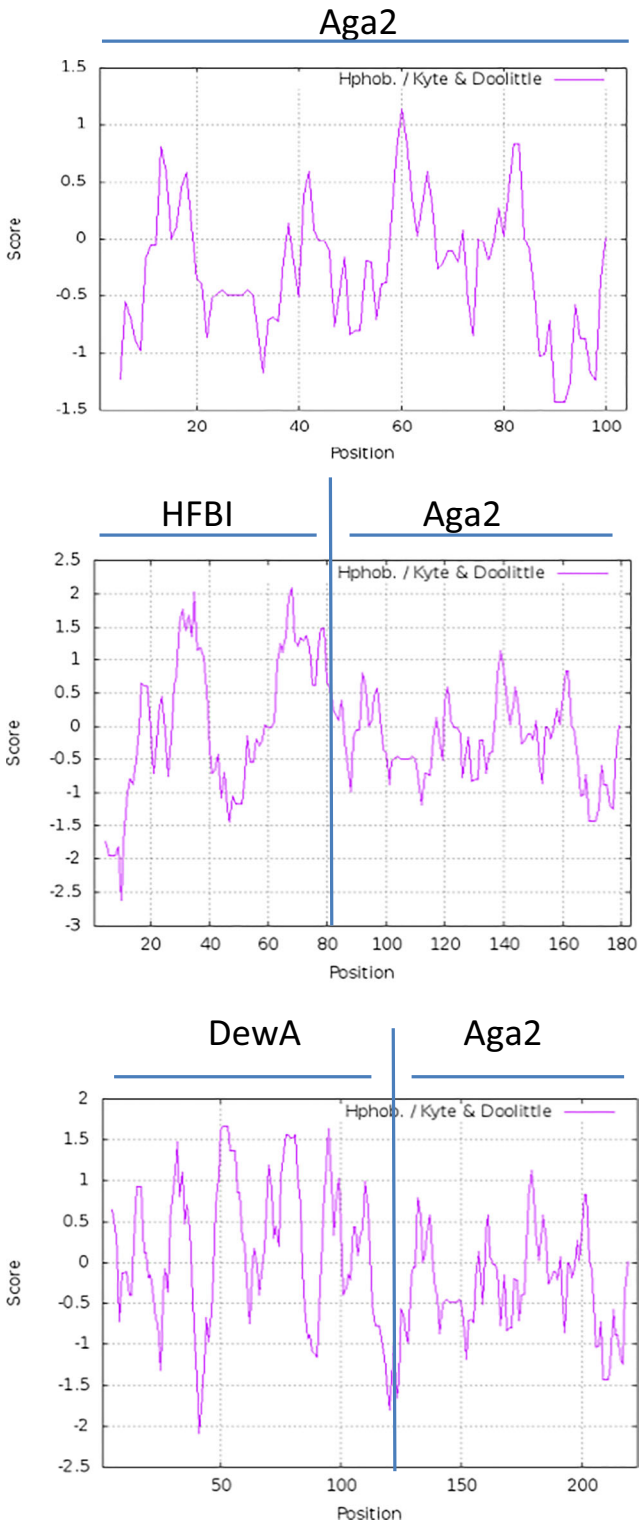


Fig. 2 Flow cytometry (a) and fluorescence and DIC microscopy (b) analyses of the cultures corresponding to the EBY100-derived strains expressing the Aga2 (pYD5M) or Aga2 fusion proteins with HFBI (pYD5M-HFBI, pYD5M-HFBI-2c) or DewA (pYD5M-DewA, pYD5M-DewA-2c). Strains were grown O/N in SC-Trp Glu at 30 °C. Then, they were transferred to the same medium, but it contained galactose as a carbon source, and were incubated for another 24-h period. An accessible display of the Aga2 fusion proteins was evaluated by immunofluorescence labeling with an anti-V5 antibody. Experiments were repeated 3 times with similar results. The figure shows the result of a representative experiment of each strain. The first panel in each figure is a control that corresponds to a sample of the EBY100/pYD5 cells grown in glucose; in b, the FITC image was overexposed

differences between the modified and the control strain were statistically significant considering the p values found (7.287×10^{-5} and 8.019×10^{-5}, respectively). According to this, the exposure of the hydrophobins herein considered increased the hydrophobicity of the cell surface, almost 5-fold in the case of HFBI and 3.27-fold for DewA.

The contact angles with hydrophilic and hydrophobic glass were determined for the cells grown for 24 h in a minimal medium with galactose. According to the results shown in Fig. 4, no significant differences were found between the considered strains. This suggests that the addition of hydrophobins to the yeast surface had no effect on surface hydrophobicity, unlike evidences for the opposite situation provided by the above-described analyses.

Another feature of yeast cells that can provide information about the changes on the surface associated with hydrophobin exposure is their flocculation ability. This property was measured by determining the percentage of cells which did not remain on the top of the solution after flocculation induction in the 24-h cultures in a galactose-containing medium. The results were 7.40 ± 1.63% for the EBY100/pYD5M strain, 27.39 ± 3.42% for EBY100/pYD5M-HFBI, and 17.07 ± 2.70% for EBY100/pYD5M-DewA. The differences between the modified and the control strain were statistically significant considering the p values found (8.30×10^{-4} and 2.22×10^{-3}, respectively). These data indicate that the modified strains displayed greater flocculation capacity, which resulted in bigger differences when class II hydrophobin was considered. Indeed, the



◀ **Fig. 3** Protein profile of the fusion proteins expressed from plasmids pYD5M, pYD5M-HFBI, and pYD5M-DewA. The amino acid scale used to determine profiles was defined by the numeric value assigned to each one. The *Protein Translate* facility was employed for the translation of the nucleotide sequence and the *Protscale* to generate the protein profile (*Swiss Institute of Bioinformatics ExPASy*). Vertical lines separate the part corresponding to the introduced sequences and the Aga2 protein. According to the scale used (Hphob. / Kyte and Doolittle 1982), the individual value for each amino acid is as follows: Ala: 1.800; Arg: −4.500; Asn: −3.500; Asp: −3.500; Cys: 2.500; Gln: −3.500; Glu: −3.500; Gly: −0.400; His: −3.200; Ile: 4.500; Leu: 3.800; Lys: −3.900; Met: 1.900; Phe: 2.800; Pro: −1.600; Ser: −0.800; Thr: −0.700; Trp: −0.900; Tyr: −1.300; Val: 4.200

percentage of flocculated cells in the strains exposing DewA and HFBI was increased 2.31- and 3.70-fold, respectively, compared to that of the control. This result is consistent with the greater hydrophobicity of the cells of these two strains obtained from the butanol extraction experiments.

HFBI and DewA exposure on *S. cerevisiae* influences resistance under several adverse conditions for yeast growth

The modification of the yeast surface associated with the exposure of hydrophobins of classes I and II could result in changes in the resistance to stress conditions produced by physicochemical agents, including the substrates and solvents used for biocatalysis with yeast whole cells. In this section, viability in the presence of adverse conditions was analyzed

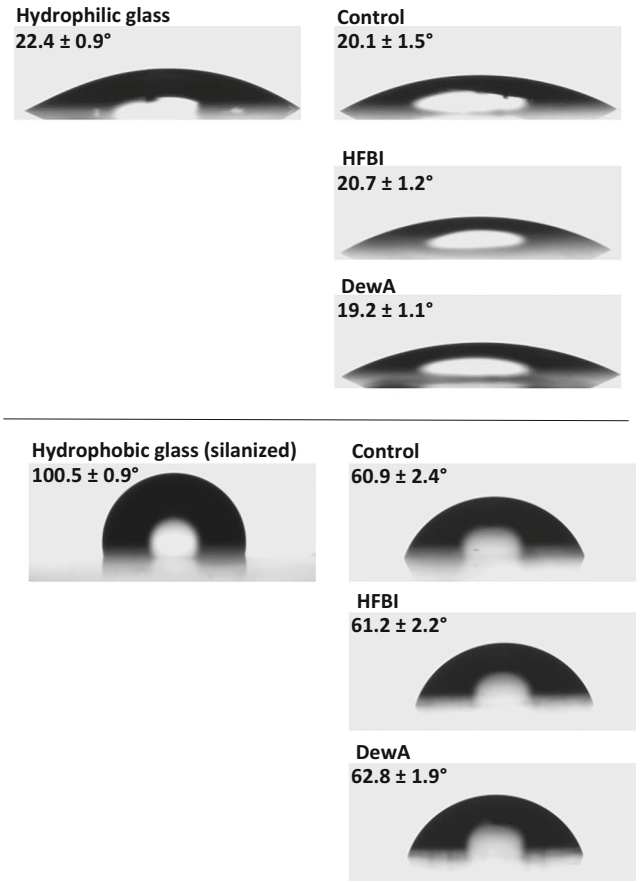


Fig. 4 Water contact angle measurements of the yeast-coated glass slides. The images on the left are the WCA of the glass with no yeast on it. The shown values correspond to mean and standard deviation, with $n = 10$

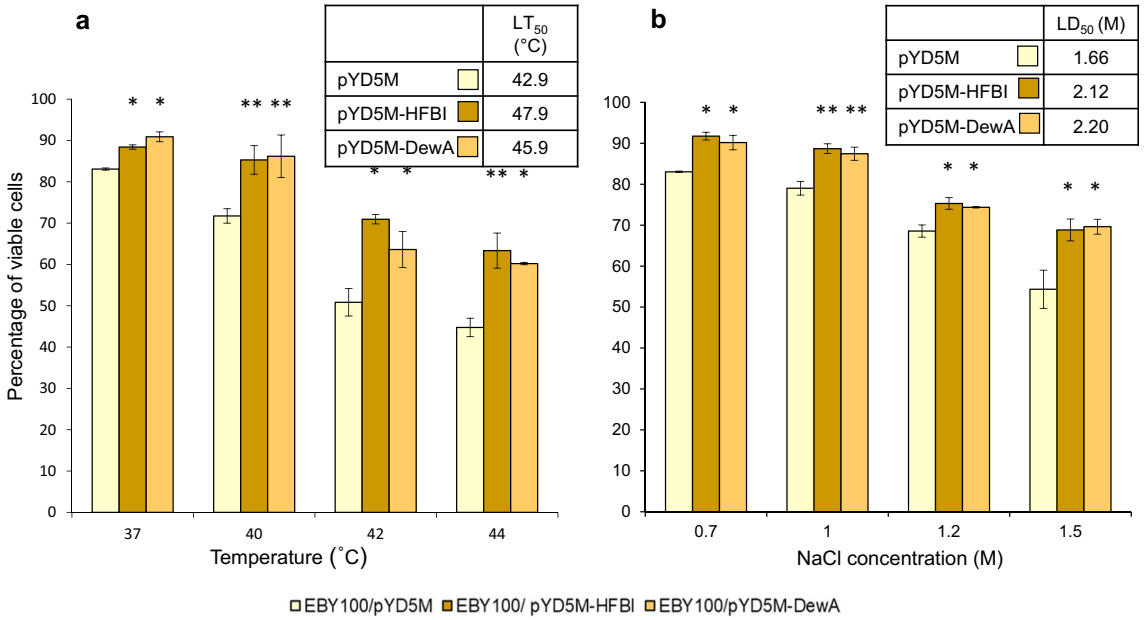


Fig. 5 Resistance of the yeast cells exposing Aga2 (vector pYD5M, left bar), Aga2-HFBI (middle bar), and Aga2-DewA (right bar) to high temperatures and salt concentrations. Five OD_{600} units of yeast cells from the 24-h cultures in galactose were incubated under the conditions described in "Materials and Methods." The percentage of viable cells was determined in each case by the trypan blue exclusion assay. Experiments were carried out 5 times, and the figure shows the mean value and the standard deviation. * indicates those cases in which the differences compared to the strain transformed with vector pYD5M were statistically significant with a p value lower than 0.05; ** is included in those cases in which the p value was lower than 0.01. The LT_{50} and NaCl LD_{50} values are also shown; standard deviations were always lower than 5% of the mean value

by the trypan blue exclusion assay following the procedure described in "Materials and Methods." The obtained results are shown in Figs. 5 and 6 and S2 and S3 in the Supplementary Material.

First, the viability of all the strains was determined at different temperatures (37, 40, 42, and 44 °C). As seen in Fig. 5a, the modified ones always showed more thermoresistance than the control (EBY100/pYD5M), with statistically significant

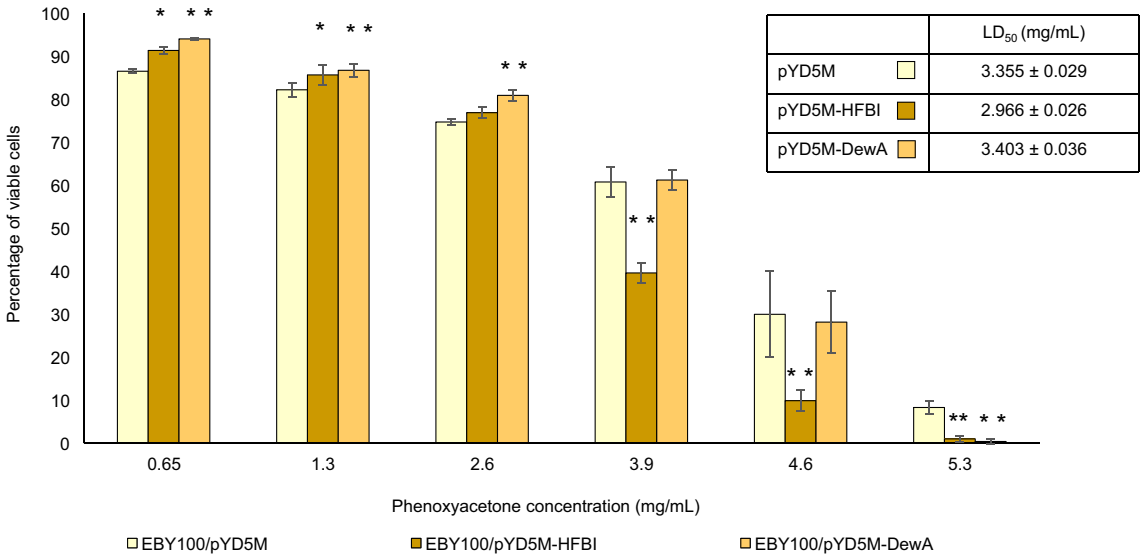


Fig. 6 Resistance of the yeast cells exposing Aga2 (vector pYD5M), Aga2-HFBI, and Aga2-DewA to phenoxyacetone. Ten OD_{600} units of yeast cells from the 24-h cultures in galactose were incubated under the conditions described in "Materials and Methods" with the concentrations of the compound indicated in the figure. The percentage of viable cells was determined in each case by the trypan blue exclusion assay. Experiments were carried out 5 times, and the figure shows the average value and the standard deviation. * indicates those cases in which the differences in relation to the strain transformed with the vector pYD5M were statistically significant with a p value lower than 0.05; ** is included in those cases in which the p value was lower than 0.01. The LD_{50} values for this compound are also shown

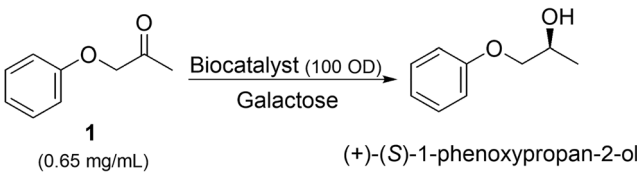
differences. Overexposure of DewA, and particularly of HFBI, resulted in greater resistance and mainly at the highest temperatures. Accordingly, the LT_{50} value was determined for each one (temperature at which 50% viable cells were found).

Next, salt resistance was also checked and, once again, the two strains displaying hydrophobins were more viable, with statistically significant differences (Fig. 5b). Under this stress condition, both showed a similar pattern with quite close LD_{50} values.

Growth experiments were also carried out with strains EBY100/pYD5M, pYD5M-HFBI, and pYD5M-DewA under the two conditions explained above. The growth in the SC-trp medium containing galactose as the only carbon source at 30 °C was followed as the control. As shown in Fig. S2 in the Supplementary Material, the HFBI-expressing cells grew slightly more slowly compared to the other strains, which showed quite similar behavior to one another. However, when growth was followed at 37 °C, the strains exposing hydrophobins displayed more growth, particularly that overexpressing DewA on the cell surface. In the experiments carried out in the presence of 0.5 M NaCl, both strains exposing hydrophobins also showed improved growth compared to the unmodified one, in which case the better results were found by HFBI exposure.

Resistance to several organic solvents was also analyzed. As shown in Fig. S3 in the Supplementary Material, and after considering all the data, no consistent and significant differences were found among the strains for ethanol, acetonitrile, dimethylformamide, and dimethylsulfoxide under the concentrations tested in these experiments (6–10%, 3–9% *v*/*v*, 6–25% *v*/*v*, and 12–30% *v*/*v*, respectively). However, it is worth mentioning that in some cases, exposure of hydrophobins (particularly HFBI) resulted in lower resistance to these agents (e.g., see the data for 6% (*v*/*v*) ethanol, 7–9% acetonitrile, and 30% (*v*/*v*) DMSO).

The organic substrates that can be used in biocatalytic processes carried out by yeast whole cells could be toxic for them, which limits the charge that can be applied in the corresponding reactions. Figure 6 shows the results of the viability of the three considered strains in the presence of different amounts of one of these substrates: phenoxyacetone 1. We can see that the effect of the exposure of hydrophobins on the cell surface depends on the considered substrate concentration. Increased resistance of yeast cells to this compound was found when present from 0.65 to 2.6 mg/mL of the final concentration. However, viability of the HFB1 expressing strain was more negatively affected from around 3.9 mg/mL compared to the other two. From these data, LD_{50} was calculated and included in the same figure.



Scheme 1 Reduction of phenoxyacetone 1 to (+)-(*S*)-1-phenoxy-2-propanol biocatalyzed by strains EBY100/pYD5M/pYD5M-HFBI and pYD5M/DewA in the presence of galactose. OD refers to OD_{600} units

Introduction of a second copy of the HFBI and DewA hydrophobins does not affect the properties of the yeast cell surface and viability under adverse conditions

Improvement in the cell surface properties and stress resistance associated with the exposure of one copy of the hydrophobins herein considered prompted us to analyze if the introduction of a second copy fused to the first one could provide additional positive effects. The Gibson Assembly strategy was used to construct the corresponding strains as explained in "Materials and Methods." The efficient expression of the two copies of HFBI or DewA on the cell surface was confirmed by western blot, flow cytometry, and microscopy (Fig. S1 in the Supplementary Material, and Figs. 2a and 2b, respectively). The data obtained in the last analyses indicated that the percentage of cells exposing hydrophobins was not affected by the addition of the second copy. However, the mean fluorescence intensity decreased, which suggests that the number of proteins per cell lowered. The experiments carried out with these strains revealed no significant differences in terms of the above-described properties, such as the HI, flocculation ability, and heat resistance (data not shown). Accordingly, further analyses were restricted to the strains exposing one copy of the herein analyzed hydrophobins.

Behavior of hydrophobin-exposing strains EBY100/pYD5M-HFBI and EBY100/pYD5M-DewA in biocatalysis

The effect of the hydrophobin expression on cells' surface during the biocatalytic processes carried out by yeast whole cells in an aqueous medium was determined. For this purpose, the reaction reduction of the ketone 1-phenoxypropan-2-one 1 (Scheme 1) was taken as a benchmark.

When the reaction was carried out at the optimal growth temperature for *S. cerevisiae* (around 30 °C), the transformation was more marked with the modified strains than in the control one, particularly with EBY100/pYD5M-HFBI, with a statistically significant difference in this case (Fig. 7a). Given the greater viability of the YSD modified strains at 40 °C, the ability of whole cells to reduce this substrate was also tested at this temperature. Under this condition, transformation was lower in all cases, although these two strains showed enhanced reactivity compared to the control one, with statistically significant differences once again for the HFBI-exposing strain (Fig. 7b).

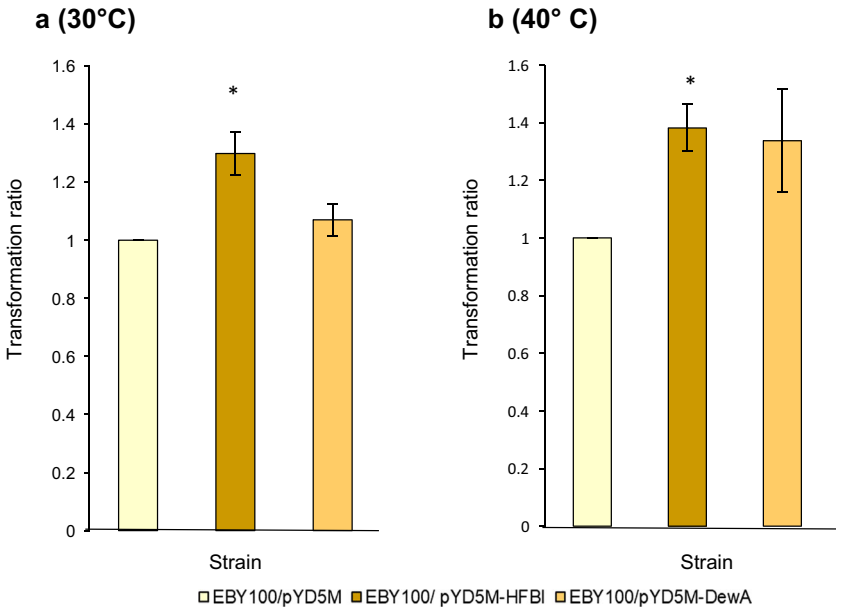


Fig. 7 Comparative results among the three strains studied for the reduction reaction of ketone 1. Experiments were carried at 30 °C or 40 °C for 24 h using 100 OD_{600} units of whole cells in 10 mL of 2% galactose and 0.65 mg/mL of a substrate. The mean value and standard deviation for the transformation ratio of five independent experiments are shown in this figure. The transformation ratio was determined as the relation between the conversion (expressed as μmol of product/mg cells dry weight) obtained with the hydrophobin-modified strains vs. the control one, for which a value of 1 was assigned. Conversion was calculated based on the integration of ^{1}HNMR spectra. Cell dry weight was determined after the lyophilization of the aliquots containing 100 OD_{600} units of whole cells

The effect of the substrate charge on the development of the reaction was also analyzed. As shown in Fig. 8, biocatalytic activity was greater at 30 °C in both hydrophobin-exposing strains than in the control one by at least up to 2.5 mg/mL of the substrate final concentration, with statistically significant differences in all cases. Higher substrate concentrations were not considered because of the sharp drop in cell viability described in a previous section (Fig. 6).

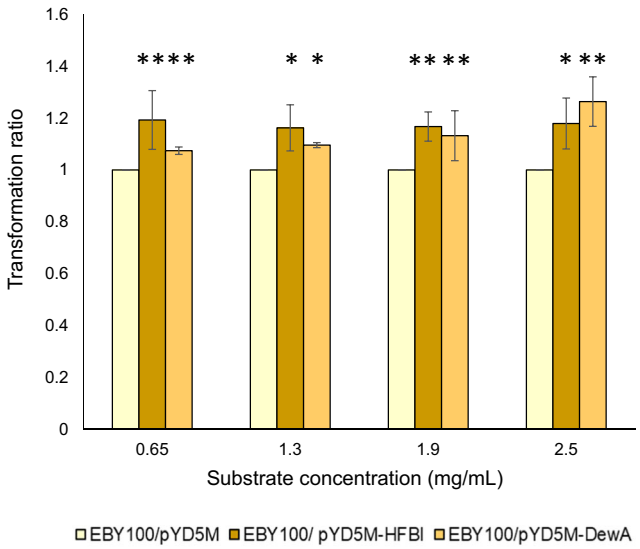


Fig. 8 Effect of the substrate concentration on the biocatalytic transformation by the yeast whole cells of the strains herein considered. Reactions were carried out for 24 h using 100 OD_{600} units of whole cells in 10 mL of 2% galactose. Substrate 1 was used at the final concentration indicated in each case. The mean value and standard deviation of five independent experiments are shown in the figure. The transformation ratio was determined as in Fig. 7

Discussion

Hydrophobins are proteins with interesting biotechnological and biomedical applications, and research about their properties and utilities has increased in the last few years (Bayry et al. 2012; Berger and Sallada 2019; Khalesi et al. 2015a, 2015b; Maiolo et al. 2017; Niu et al. 2012; Valo et al. 2010; Wösten and Scholtmeijer 2015). Several reports have analyzed the effects of displaying hydrophobins of class I (SC3 of *Schizophyllum commune*) and class II (HFBI de *Trichoderma reesei*) on the surface of yeast cells belonging to the *Saccharomyces cerevisiae* and *Pichia pastoris* species (Nakari-Setälä et al. 2002; Wang et al. 2016; Zhang et al. 2017).

The results described in this work indicate that the two considered hydrophobins, HFBI and DewA (a hydrophobin of class I of *A. nidulans* not previously exposed), were accurately displayed on the yeast surface of *S. cerevisiae* (Fig. 2).

In previous studies, higher hydrophobicity was observed in EBY100 cells overexpressing Aga2 in the cell surface (Andreu and del Olmo 2013). Herein, we found that the exposure of both hydrophobins, especially HFBI, resulted in an additional increase of the hydrophobicity of the cell surface

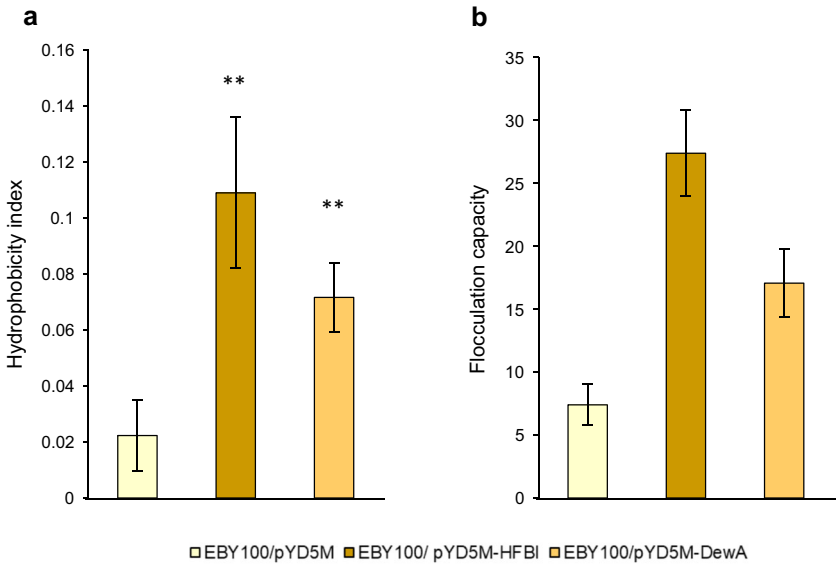


Fig. 9 Comparison between the data obtained for the hydrophobicity index and flocculation capacity for the strains herein considered. * indicates those cases in which the differences compared to the strain transformed with vector pYD5M were statistically significant with a p value lower than 0.05

(Fig. 9). Nakari-Setälä et al. (2002) described the display of HFBI on *S. cerevisiae* and also found that hydrophobin-expressing yeast was much less hydrophilic than the control ones. Unfortunately, it is not possible to compare our results to those found by these authors for different reasons; e.g., they were unable to determine the level of HFBI exposure by FACS and used a different yeast display system based on Flo1 anchoring and the constitutive *ADH1* promoter to regulate expression; besides, the methodology followed to determine this property was different. Wang et al. (2016) also exposed HFBI in *Pichia pastoris* and observed a higher HI value (1.68-fold change). We also found a greater tendency of flocculation for the strains exposing hydrophobins, mainly HFBI, which is consistent with hydrophobicity differences (Fig. 9). Jin and Speers (1998) reported that a high cell surface hydrophobicity (CSH) level facilitates better cell-cell contact in an aqueous medium, which results in a more specific lectin-carbohydrate interaction. Nayyar et al. (2014) also described a correlation between increased CSH and the initiation of flocculence during fermentation for four industrial *S. cerevisiae* strains used to produce beer, champagne, wine, and fuel alcohol.

Despite these results, all the strains considered herein obtained quite close water contact angle values (WCA, around 19–20) on hydrophilic glass, which were similar to those found for other unmodified yeast strains reported in the literature (20.7 for H2155, Nakari-Setälä et al. 2002; 18.4 for NCYC 1681, White and Walker 2011). It is difficult to explain the reason for the discrepancies that appear among the hydrophobicity data obtained from solvent extraction analyses and WCA determinations, but they have also been reported by other authors (White and Walker 2011). Nakari-Setälä et al. (2002) only detected quite a modest increase, which was probably not statistically significant, in the contact angle in water when expressing protein HFBI on the surface by the Flo1 anchoring system (32.8 ± 2.1 compared to 30.0 ± 1.9). We cannot rule out that the treatment with ethanol used to prepare samples could bring about changes in the association or conformation of the hydrophobins that could affect the WCA determinations. Besides, there is the possibility of a capillary effect due to spaces between cells or channel-like structures that form between multiple yeast layers depending on coating density.

We also analyzed the effect of displaying these proteins on cell surfaces on their resistance to stress conditions, like high temperatures and presence of salt or organic compounds. As expected, the increased stressing agent diminished cell viability, but this effect was less marked on the strains exposing hydrophobins than on the control one in all cases. By means of transmission electron microscopy, Wang et al. (2016) observed that exposure of HFBI and type I hydrophobin SC3 resulted in cell wall structure differences. These authors found that the cell wall inner layers of the recombinant strains displaying these hydrophobins were thicker than those of the reference strain. There were also slightly longer mannan fibrils on the cell wall outer layer of the strain exposing SC3 and shorter ones in that displaying HFBI. Perhaps, these differences lie behind the increased resistance to high temperatures and salt solutions of the EBY100 cells transformed with pYD5M-HFBI and pYD5M-DewA reported in this work (Fig. 5, S2 and S3 in the Supplementary Material).

Exposure of a second copy of HFBI or DewA hydrophobins fused to the first one did not provide any additional improvement to surface properties and resistance to high temperatures. Although this second copy introduced more hydrophobic residues, the structure adopted when the whole protein was associated extracellularly to the cell wall by the linking Aga1-Aga2-hydrophobin is not known. Besides, the flow cytometry analyses revealed fewer copies

per cell when the second copy was introduced (see the M value in Fig. 2a).

Improved biocatalytic activity in lipases co-expressed with hydrophobins SC3 or HFBI on the cell surface has been demonstrated (Wang et al. 2016; Zhang et al. 2017). These authors considered that this can be explained by the change in the surface structure and hydrophobic characteristics, which resulted in an effect on the catalytic features of the lipase displayed. In this work, we analyzed how exposing proteins HFBI and DewA on the yeast cell surface influenced the biocatalytic reduction reaction of ketone 1 carried out by whole cells. Thus, we demonstrated that the exposure of both proteins resulted in enhanced activity when phenoxyacetone concentrations went up to 2.5 mg/mL, possibly because greater surface lipophilicity makes its entry in the cell easier. No reactions were carried out at higher substrate charges given the toxic effect on cells (Fig. 6).

Relevant results were found when reactions were carried out at 40 °C (Fig. 7). At this temperature, reactions were slower than at 30 °C, but stereoselectivity remained (ee 85%). Once again, greater thermotolerance was observed for the hydrophobin-exposing strains as in the viability experiments. This result is important for the biocatalytic processes that employ whole cells because it offers the possibility of using high lipophilic substrates in an aqueous medium to increase their solubility by raising the reaction temperature. Further analysis should be performed to understand the applications of the new strains described herein and the possibility of introducing these modifications into other more convenient yeast species for biotechnological processes.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00253-021-11090-8.

Acknowledgments We are indebted to Dr. Markku Saloheimo for providing us with the *S. cerevisiae* strain VTT-C-99315 carrying the pTNS23 plasmid. We gratefully acknowledge SCSIE (Universitat de València) for providing access to its instrumental facilities for DNA sequencing and flow cytometry.

Author contribution C.A and M.O conceived, designed, and performed research and data analyses, performed conceptualization and supervision, and wrote the paper. L.W and J.G contributed with some experiments. R.F was involved in the discussion of results and the supervision of the paper. C.A and M.O have contributed equally to this work.

Funding This work was supported by grants from the Universitat de València: UV-INV-AE15-323062 and UV-19-INV-AE19.

Data availability All the data generated from this study are included in this article or in its supplementary information files.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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

References

Andreu C, del Olmo M (2014) Potential of some yeast strains in the stereoselective synthesis of (*R*)-(-)-phenylacetylcarbinol and (*S*)-(+)-phenylacetylcarbinol and their reduced 1,2-dialcohol derivatives. Appl Microbiol Biotechnol 98:5901–5913. https://doi.org/10.1007/s00253-014-5635-5

Andreu C, Peña M, del Olmo M (2016) Biocatalytic reduction of racemic 2-arenoxycycloalkanones by yeasts *P. glucozyma* and *C. glabrata*: one way of achieving chiral 2-arenoxycycloalcohols. Appl Microbiol Biotechnol 100:4865–4873. https://doi.org/10.1007/s00253-015-7261-2

Andreu C, del Olmo M (2018a) Biotransformation using halotolerant yeast in seawater: a sustainable strategy to produce *R*-(-)-phenylacetylcarbinol. Appl Microbiol Biotechnol 102:4717–4727. https://doi.org/10.1007/s00253-018-8945-1

Andreu C, del Olmo M (2019) Improved biocatalytic activity of the *Debaryomyces* species in seawater. Chemcatchem 11:3085–3092. https://doi.org/10.1002/cctc.201900558

Andreu C, del Olmo M (2013) Yeast arming by the Aga2p system: effect of growth conditions in galactose on the efficiency of the display and influence of expressing leucine-containing peptides. Appl Microbiol Biotechnol 97:9055–9069. https://doi.org/10.1007/s00253-013-5086-4

Andreu C, del Olmo M (2018b) Yeast arming systems: pros and cons of different protein anchors and other elements required for display. Appl Microbiol Biotechnol 102:2543–2561. https://doi.org/10.1007/s00253-018-8827-6

Andreu C, del Olmo M (2020) Whole-cell biocatalysis in seawater: new halotolerant yeast strains for the regio- and stereoselectivity reduction of 1-phenylpropane-1,2-dione in saline-rich media. Chembiochem 21:1621–1628. https://doi.org/10.1002/cbic.202000023

Bariotaki A, Kalaitzakis D, Smonou I (2012) Enzymatic reductions for the regio- and stereoselective synthesis of hydroxy-keto esters and dihydroxy esters. Org Lett 14:1792–1795. https://doi.org/10.1021/ol3003833

Bayry J, Aimanianda V, Guijarro J, Sunde M, Latgé J (2012) Hydrophobins—unique fungal proteins. PLoS Pathog 8:e1002700. https://doi.org/10.1371/journal.ppat.1002700

Benjaphokee S, Hasegawa D, Yokota D, Asvarak T, Auesukaree C, Sugiyama M, Kaneko Y, Boonchird C, Harashima S (2012) Highly efficient bioethanol production by a *Saccharomyces cerevisiae* strain with multiple stress tolerance to high temperature, acid and ethanol. New Biotechnol 29:379–386. https://doi.org/10.1016/j.nbt.2011.07.002

Berger B, Sallada N (2019) Hydrophobins: multifunctional biosurfactants for interface engineering. J Biol Eng 13:10. https://doi.org/10.1186/s13036-018-0136-1

Boder ET, Raeeszadeh-Sarmazdeh M, Price JV (2012) Engineering antibodies by yeast display. Arch Biochem Biophys 526:00–106. https://doi.org/10.1016/j.abb.2012.03.009

Boeuf S, Throm T, Gutt B, Strunk T, Hoffmann M, Seebach E, Mühlberg L, Brocher J, Gotterbarm T, Wenzel W, Fischer R, Richter W (2012) Engineering hydrophobin DewA to generate surfaces that enhance adhesion of human but not bacterial cells. Acta Biomater 8:1037–1047. https://doi.org/10.1016/j.actbio.2011.11.022

Bony M, Thines-Sempoux D, Barre P, Blondin B (1997) Localization and cell surface anchoring of the *Saccharomyces cerevisiae* flocculation protein Flo1p. J Bacteriol 179:4929–4936. https://doi.org/10.1128/jb.179.15.4929-4936.1997

Breakspear A, Momany M (2007) *Aspergillus nidulans* conidiation genes *dewA*, *fluG*, and *stuA* are differentially regulated in early vegetative growth. Eukaryot Cell 6:1697–1700. https://doi.org/10.1128/EC.00189-07

Cappellaro C, Baldermann C, Rachel R, Tanner W (1994) Mating type-specific cell-cell recognition of *Saccharomyces cerevisiae*: cell wall attachment and active sites of a- and alpha-agglutinin. EMBO J 13:4737–4744. https://doi.org/10.1002/j.1460-2075.1994.tb06799.x

Crabtree RH (2009) Handbook of green chemistry Volume 3. Biocatalysis. Wiley-VCH, Weinheim

Fokina O, Fenchel A, Winandy L, Fischer R (2016) Immobilization of LccC laccase from *Aspergillus nidulans* on hard surfaces via fungal hydrophobins. Appl Environ Microbiol 82:6395–6402. https://doi.org/10.1128/AEM.01413-16

Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, Bairoch A (2005) Protein identification and analysis tools on the ExPASy server. In: Walker JM (ed) The proteomics protocols handbook. Humana Press, Totowa, pp 571–607

Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA, Smith HO (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods 6:343–345. https://doi.org/10.1038/nmeth.1318

Grünbacher A, Throm T, Seidel C, Gutt B, Röhrig J, Strunk T, Vincze P, Walheim S, Schimmel T, Wenzel W, Fischer R (2014) Six hydrophobins are involved in hydrophobin rodlet formation in *Aspergillus nidulans* and contribute to hydrophobicity of the spore surface. PLoS One 9(4):e94546. https://doi.org/10.1371/journal.pone.0094546

Heus S, Meynial-Salles I, O'Donohue MJ, Dumon C (2015) White biotechnology: state of the art strategies for the development of biocatalysts for biorefining. Biotechnol Adv 33:1653–1670. https://doi.org/10.1016/j.biotechadv.2015.08.004

Jin Y-L, Speers RA (1998) Flocculation of *Saccharomyces cerevisiae*. Food Res Int 31:421–440. https://doi.org/10.1016/S0963-9969(99)00021-6

Khalesi M, Gebruers K, Derdelinckx G (2015a) Recent advances in fungal hydrophobin towards using in industry. Protein J 34:243–255. https://doi.org/10.1007/s10930-015-9621-2

Khalesi M, Mandelings N, Herrera-Malaver B, Riveros-Galan D, Gebruers K, Derdelinckx G (2015b) Improvement of the retention of ocimene in water phase using class II hydrophobin HFBII. Flavour Fragr J 30:451–458. https://doi.org/10.1002/ffj.3260

Kieke MC, Cho BK, Boder ET, Kranz DM, Wittrup KD (1997) Isolation of anti-T cell receptor scFv mutants by yeast surface display. Protein Eng 10:1303–1310. https://doi.org/10.1093/protein/10.11.1303

Kisukuri C, Andrade L (2015) Production of chiral compounds using immobilized cells as a source of biocatalysts. Org Biomol Chem 13:10086–10117. https://doi.org/10.1039/c5ob01677k

Kumar R, Kumar P (2019) Yeast-based vaccines: new perspective in vaccine development and application. FEMS Yeast Res 19:foz007. https://doi.org/10.1093/femsyr/foz007

Kyte J, Doolittle RF (1982) A simple method for displaying the hydropathic character of a protein. J Mol Biol 157:105–132. https://doi.org/10.1016/0022-2836(82)90515-0

Linciano S, Pluda S, Bacchin A, Angelini A (2019) Molecular evolution of peptides by yeast surface display technology. MedChemComm 10:1569–1580. https://doi.org/10.1039/c9md00252a

Linder MB, Szilvay GR, Nakari-Setälä T, Penttilä ME (2005) Hydrophobins: the protein-amphiphiles of filamentous fungi. FEMS Microbiol Rev 29:877–896. https://doi.org/10.1016/j.femsre.2005.01.004

Maiolo D, Pigliacelli C, Sánchez Moreno P, Violatto MB, Talamini L, Tirotta I, Piccirillo R, Zucchetti M, Morosi L, Frapolli R, Candiani G, Bigini P, Metrangolo P, Baldelli BF (2017) Bioreducible hydrophobin-stabilized supraparticles for selective intracellular release. ACS Nano 11:9413–9423. https://doi.org/10.1021/acsnano.7b04979

Moore JC, Pollard DJ, Kosjek B, Devine PN (2007) Advances in the enzymatic reduction of ketones. Acc Chem Res 40:1412–1419. https://doi.org/10.1021/ar700167a

Nakari T, Alatalo E, Penttilä ME (1993) Isolation of *Trichoderma reesei* genes highly expressed on glucose-containing media: characterization of the *tef1* gene encoding translation elongation factor 1 alpha. Gene 136:313–318. https://doi.org/10.1016/0378-1119(93)90486-m

Nakari-Setälä T, Aro N, Kalkkinen N, Alatalo E, Penttilä M (1996) Genetic and biochemical characterization of the *Trichoderma reesei* hydrophobin HFBI. Eur J Biochem 235:248–255. https://doi.org/10.1111/j.1432-1033.1996.00248.x

Nakari-Setälä T, Azeredo J, Henriques M, Oliveira R, Teixeira J, Linder M, Penttilä M (2002) Expression of a fungal hydrophobin in the *Saccharomyces cerevisiae* cell wall: effect on cell surface properties and immobilization. Appl Environ Microbiol 68:3385–3391. https://doi.org/10.1128/AEM.68.7.3385-3391.2002

Nayyar A, Walker G, Canetta E, Wardrop F, Adya AK (2014) Cell surface properties and flocculation behavior for industrial strains of *Saccharomyces cerevisiae*. Int J Appl Microbiol Biotechnol Res 2:64–72. https://doi.org/10.33500/ijambr.2014.02.007

Ni Y, Holtmann D, Hollmann F (2014) How green is biocatalysis? To calculate is to know. ChemCatChem 6:930–943. https://doi.org/10.1002/cctc.201300976

Niu B, Wang D, Yang Y, Xu H, Qiao M (2012) Heterologous expression and characterization of the hydrophobin HFBI in *Pichia pastoris* and evaluation of its contribution to the food industry. Amino Acids 43:763–771. https://doi.org/10.1007/s00726-011-1126-5

Park M (2020) Surface display technology for biosensor applications: a review. Sensors 20:2775. https://doi.org/10.3390/s20102775

Perpiñá C, Vinaixa J, Andreu C, del Olmo M (2015) Development of new tolerant strains to hydrophilic and hydrophobic organic solvents by the yeast surface display methodology. Appl Microbiol Biotechnol 99:775–789. https://doi.org/10.1007/s00253-014-6048-1

Pscheidt B, Glieder A (2008) Yeast cell factories for fine chemical and API production. Microb Cell Factories 7:25–61. https://doi.org/10.1186/1475-2859-7-25

Robertson DE, Steer BA (2004) Recent progress in biocatalyst discovery and optimization. Curr Opin Chem Biol 8:141–149. https://doi.org/10.1016/j.cbpa.2004.02.010

Sammer D, Krause K, Gube M, Wagner K, Kothe E (2016) Hydrophobins in the life cycle of the ectomycorrhizal basidiomycete *Tricholoma vaccinum*. PLoS One 11:e0167773. https://doi.org/10.1371/journal.pone.0167773

Schmoll M, Seibel C, Kotlowski C, Wöllert Genannt Vendt F, Liebmann B, Kubicek CP (2010) Recombinant production of an *Aspergillus nidulans* class I hydrophobin (DewA) in *Hypocrea jecorina* (*Trichoderma reesei*) is promoter-dependent. Appl Microbiol Biotechnol 88:95–103. https://doi.org/10.1007/s00253-010-2710-4

Stringer MA, Timberlake WE (1995) *dewA* encodes a fungal hyddrophobin component of the *Aspergillus* spore wall. Mol Microbiol 16:33–44. https://doi.org/10.1111/j.1365-2958.1995.tb02389.x

Tabañag IDF, Chu IM, Wei YH, Tsai SL (2018) The role of yeast-surface-display techniques in creating biocatalysts for consolidated bioprocessing. Catalysts 8:94. https://doi.org/10.3390/catal8030094

Takayama K, Suye SI, Kuroda K, Ueda M, Kitaguchi T, Tsuchiyama K, Fukuda T, Chen W, Mulchandani A (2006) Surface display of organophosphorus hydrolase on *Saccharomyces cerevisiae*. Biotechnol Prog 22:939–943. https://doi.org/10.1021/bp060107b

Tao J, Kazlauskas RJ (2011) Biocatalysis for green chemistry and chemical process development. John Wiley & Sons, Hoboken

Traxlmayr MW, Shusta EV (2017) Directed evolution of protein thermal stability using yeast surface display. Methods Mol Biol 1575:45–65. https://doi.org/10.1007/978-1-4939-6857-2_4

Urbar-Ulloa J, Montaño-Silva P, Ramírez-Pelayo AS, Fernández-Castillo E, Amaya-Delgado L, Rodríguez-Garay B, Verdín J (2019) Cell surface display of proteins on filamentous fungi. Appl Microbiol Biotechnol 103:6949–6972. https://doi.org/10.1007/s00253-019-10026-7

Valo H, Laaksonen P, Peltonen L, Linder M, Hirvonen J, Laaksonen T (2010) Multifunctional hydrophobin: toward functional coatings for drug nanoparticles. ACS Nano 4:1750–1758. https://doi.org/10.1021/nn9017558

van Wetter MA, Wösten HA, Wessels JG (2000) SC3 and SC4 hydrophobins have distinct roles in formation of aerial structures in dikaryons of *Schizophyllum commune*. Mol Microbiol 36:201–210. https://doi.org/10.1046/j.1365-2958.2000.01848.x

Wang P, He J, Sun Y, Reynolds M, Zhang L, Han S, Liang S, Sui H, Lin Y (2016) Display of fungal hydrophobin on the *Pichia pastoris* cell surface and its influence on *Candida antárctica* lipase B. Appl Microbiol Biotechnol 100:5883–5895. https://doi.org/10.1007/s00253-016-7431-x

Wang Z, Mathias A, Stavrou S, Neville DM (2005) A new yeast display vector permitting free scFv amino termini can augment ligand binding affinities. Protein Eng Des Sel 18:337–343. https://doi.org/10.1093/protein/gzi036

Wasilenko JL, Sarmento L, Spatz S, Pantin-Jackwood M (2010) Cell surface display of highly pathogenic avian influenza virus hemagglutinin on the surface of *Pichia pastoris* cells using α-agglutinin for production of oral vaccines. Biotechnol Prog 26:542–547. https://doi.org/10.1002/btpr.343

Wessels JG (1997) Hydrophobins: proteins that change the nature of the fungal surface. Adv Microb Physiol 38:1–45. https://doi.org/10.1016/s0065-2911(08)60154-x

White JS, Walker GM (2011) Influence of cell surface characteristics on adhesion of *Saccharomyces cerevisiae* to the biomaterial hydroxylapatite. Antonie Van Leeuwenhoek 99:201–209. https://doi.org/10.1007/s10482-010-9477-6

Winandy L, Hilpert F, Schlebusch O, Fischer R (2018) Comparative analysis of surface coating properties of five hydrophobins from *Aspergillus nidulans* and *Trichoderma reseei*. Sci Rep 8:12033. https://doi.org/10.1038/s41598-018-29749-0

Winandy L, Schlebusch O, Fischer R (2019) Fungal hydrophobins render stones impermeable for water but keep them permeable for vapor. Sci Rep 9:6264. https://doi.org/10.1038/s41598-019-42705-w

Wösten HA (2001) Hydrophobins: multipurpose proteins. Annu Rev Microbiol 55:625–646. https://doi.org/10.1146/annurev.micro.55.1.625

Wösten H, Scholtmeijer K (2015) Applications of hydrophobins: current state and perspectives. Appl Microbiol Biotechnol 99:1587–1597. https://doi.org/10.1007/s00253-014-6319-x

Wu C, Sun H, Li Y, Liu X, Du X, Wang X, Xu P (2015) Biosensor based on glucose oxidase-nanoporous gold co-catalysis for glucose detection. Biosens Bioelectron 66:350–355. https://doi.org/10.1016/j.bios.2014.11.037

Zhang K, Jin Z, Wang P, Zheng S-P, Han S-Y, Lin Y (2017) Improving the catalytic characteristics of lipase-displaying yeast cells by hydrophobic modification. Bioprocess Biosyst Eng 40:1689–1699. https://doi.org/10.1007/s00449-017-1824-9

Zhao L, Xu H, Li Y, Song D, Wang X, Qiao M, Gong M (2016) Novel application of hydrophobin in medical science: a drug carrier for improving serum stability. Sci Rep 6:26461. https://doi.org/10.1038/srep26461