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Cell wall alterations occurring in an evolved multi-stress tolerant strain of the oleaginous yeast *Rhodotorula toruloides*

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The oleaginous yeast species *Rhodotorula toruloides* is a promising candidate for applications in circular bioeconomy due to its ability to efficiently utilize diverse carbon sources being tolerant to cellular stress in bioprocessing. Previous studies including genome-wide analyses of the multi-stress tolerant strain IST536 MM15, derived through adaptive laboratory evolution from a promising IST536 strain for lipid production from sugar beet hydrolysates, suggested the occurrence of significant modifications in the cell wall. In this study, the cell wall integrity and carbohydrate composition of those strains was characterized to gain insights into the physicochemical changes associated to the remarkable multi-stress tolerance phenotype of the evolved strain. Compared to the original strain, the evolved strain exhibited a higher proportion of glucomannans, fucogalactomannans, and chitin relative to (1→4)-linked glucans, and an increased presence of glycoproteins with short glucosamine derived oligosaccharides, which have been found to be associated to ethanol stress tolerance and physical strength of the cell wall. Furthermore, the evolved strain cells were found to be significantly smaller than the original strain and more resistant to thermal and mechanical disruption, consistent with higher proportion of beta-linked polymers instead of glycogen, conferring a more rigid and robust cell wall. These findings provide further insights into the cell wall composition of this basidiomycetous red yeast species and into the alterations occurring in a multi-stress tolerant evolved strain. This new information can guide yeast genome engineering towards more robust strains of biotechnological relevance.

The oleaginous yeast species *Rhodotorula toruloides* has attracted significant interest as a cell factory for biotechnological applications due to its exceptional ability to accumulate lipids (up to 70% of its dry weight)[1,2]. Moreover, this yeast species is known for its efficient growth on a wide variety of carbon sources (C-sources), tolerance to inhibitory compounds and other stresses associated to industrial bioprocesses, and ability to produce triacylglycerols, like vegetable oils that can be used in biodiesel production[1–3]. This red yeast also produces other value-added bioproducts, such as carotenoids, valuable as natural colorants and antioxidants in the food and pharmaceutical industries[3,4].

The promising *R. toruloides* strain IST536 was previously selected in our lab to produce lipids from sugar beet hydrolysates through the complete consumption of all the major C-sources present, including D-galacturonic acid (GalA)[5]. To improve this strain's performance for bioprocesses based on lignocellulosic biomass hydrolysates (LCH) an adaptive laboratory evolution (ALE) approach was employed[6]. The evolved strain, IST536 MM15, was developed by imposing selective pressure through increasing concentrations of methanol in a medium with a high concentration of glycerol (5% v/v) as the sole C-source, additionally leading to osmotic stress[6]. This strain demonstrated a multi-stress tolerance phenotype, exhibiting resilience to a wide range of stress factors. These included the four major inhibitory compounds present in LCH (acetic acid, formic acid, hydroxymethylfurfural,

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and furfural), as well as methanol-, osmotic-, salt-, oxidative-, genotoxic-, ethanol-, and medium-chain fatty acid-induced stresses[6,7]. Moreover, the evolved multi-stress tolerant strain is a better lipid producer in terms of lipid productivity and total lipid content (24.9% and 36.2% of dry cell biomass for the original strain compared with the evolved strain, respectively, in synthetic lignocellulosic hydrolysate supplemented with the typical inhibitory compounds). Regarding lipid profiling, the main difference observed was the higher percentage of C18:1 in the evolved strain (56% compared to 41% in the original strain)[6], pointing out this yeast strain as a highly promising lignocellulosic-biomass-based oil producer. Considering the higher tolerance to methanol and osmotic stress, among other stresses, of this evolved strain capable of using glycerol as the sole carbon source, this strain is also promising for the valorization of crude glycerol, a methanol contaminated byproduct of the biodiesel industry[2,8]. In summary, and although *R. toruloides* is not able to catabolize methanol, this evolved strain is a robust and versatile promising alternative for microbial oil production using a wide range of low-cost, renewable feedstocks.

Genome-wide analyses of the evolved strain, compared to the original strain, enlighten the molecular mechanisms underlying its robust multi-stress tolerance phenotype[7]. Results revealed the convergence from a triploid in the original strain to a diploid in the evolved strain and uncovered several genomic variants and alterations in transcript levels of genes primarily linked to cell wall biogenesis, integrity, and remodeling and involved in stress-responsive pathways[7]. The global transcriptional response suggested that coordinated changes occurred in the cell wall of the evolved strain[7]. A previous characterization of the evolved multi-tolerant strain indicated a cell wall less susceptible to zymolyase and a decreased cell permeability in the absence or presence of inhibitors to which the strain is tolerant[6]. However, its cell wall composition structure is still uncharacterized.

The yeast cell wall is essential to maintain cell shape and integrity and is the first line of defense against diverse environmental stress factors[9]. Most fungal cell walls are bi-layered structures composed of an inner and an outer layer[10]. The inner layer of most fungal species primarily consists of glucans composed of →3)-D-Glc*p*-(*β*1→ chains, which may be connected to the →2)-D-Man*p*-(*α*1→ and →6)-D-Man*p*-(*α*1→ units of mannoproteins and to the →4)-D-Glc*p*NAc-(*β*1→ units of chitin through a glycosylphosphatidylinositol (GPI) anchor, providing mechanical strength to the cell wall[10,11]. Under stress conditions, this mechanical strength is increased by promoting interchain glycosidic linkages between glycogen and *β*-glucans through (*β*1→4)-glucose linkages[12]. The outer layer composition is more variable among fungal species; for instance, in *Saccharomyces* and *Candida* species it is composed of mannoproteins covalently linked to (1→3)-linked glucans through →6)-D-Glc*p*-(*β*1→ chains, conferring a protective role and being essential for cell-to-cell interactions[10,11]. In response to environmental stress conditions and osmotic challenges, the cell wall and cell volume can be rapidly altered[13]. The major role of the cell wall as the first line of yeast defense against a wide range of environmental stresses is reflected by the intricacy of the underlying regulatory networks under stress[9,14]. Understanding the composition of the cell wall and the alterations occurring at this level under stress can provide mechanistic insights to improve tolerance to industrially relevant stress conditions aiming for high productivity and product yields[9,14]. A substantial amount of information is available regarding the structure, biosynthesis, regulation, and composition of the cell wall in the model yeast *Saccharomyces cerevisiae*[9,11,15]. However, this information is still limited for non-conventional yeasts, particularly *R. toruloides*[15].

To date, only a few studies have focused on investigating the cell wall composition of *R. toruloides*[16,17]. The yeast *R. toruloides* belongs to the order Microbotryales in the subphylum Pucciniomycotina. Members of this order are known for their distinctive cell wall compositions, characterized by mannose as the predominant sugar, the common presence of fucose (Fuc), occasional presence of rhamnose (Rha), and absence of xylose (Xyl)[16,18]. Based on the results from previously conducted genome-wide analyses[7], it is hypothesized that *R. toruloides* cell wall polysaccharides may be modified as a response to the stress conditions imposed during ALE. Accordingly, this study aimed to gain insights into the physicochemical properties of the cell wall of the multi-stress tolerant evolved *R. toruloides* strain compared with the original strain to uncover mechanistic insights underlying its remarkable robust phenotype.

Results

The evolved multi-stress tolerant strain has a smaller cell size than the parental strain

The cell size of *R. toruloides* IST536 and the derived evolved strain, IST536 MM15, were assessed using flow cytometry and electron microscopy. In flow cytometry, forward scatter (FSC) values provide relative measurements of cell size or granularity. FSC measures light scattered in the forward direction as cells pass through a laser beam, correlating with their size. Larger cells scatter more light forward than smaller ones. The distribution and mean FSC values from 50,000 cells of IST536 or IST536 MM15 sampled during exponential growth are shown in Fig. 1. For the timepoints 0 h and 2 h, while the OD_{600} increased, the FSC was found to decrease given that budding yeast divides asymmetrically which results in mother and daughter cells of different sizes, with daughter cells typically being smaller at birth. To ensure that these smaller daughter cells growth is sufficient before progressing through the cell cycle, size control mechanisms such as an extended G1 phase are adopted[19]. This extension of the G1 phase is reflected in FSC measurements for cell size assessment. The increase of culture OD_{600} is essentially due to the accumulation of cells from ongoing division, rather than to changes in the size of individual cells. Notably, cells of the original strain appeared consistently larger than cells of the evolved strain during exponential growth (from 2 h to 6 h of cultivation in YNB medium at 30 °C). It should be noted that FSC readings may be influenced by other factors like cell shape and optical properties and thus require cautious interpretation.

For this reason, a microscopy approach was also used. Both strains were cultivated in YNB medium at 30 °C and harvested during the exponential phase. Direct microscopic examination was performed using scanning electron microscope (SEM). Cell sizes were determined by measuring individual cell diameters using ImageJ (Fig. 2). Results highlight a statistically significant difference in cell size between IST536 and IST536 MM15, with

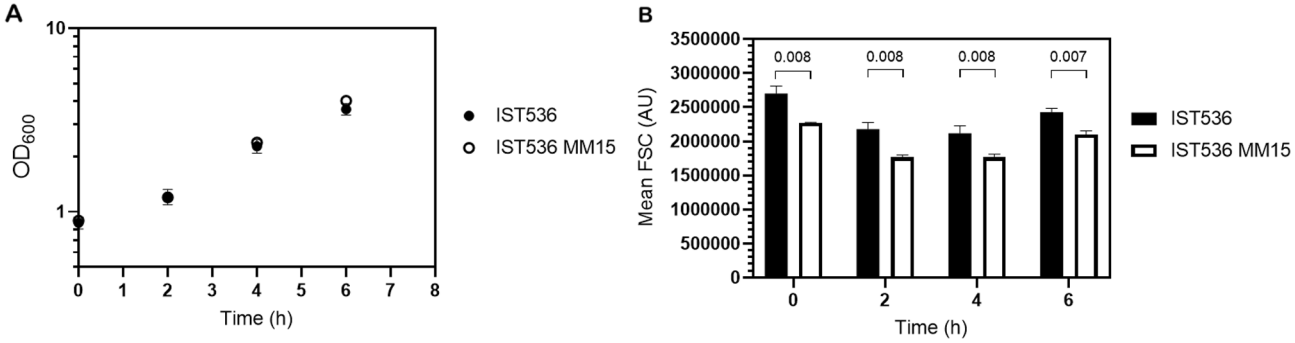

Fig. 1. Cell size estimations from flow cytometry were based on forward scatter (FSC) values. A fixed total of 50,000 events per sample were acquired using a slow flow rate (14 μL/min). Optical density (OD) values from *R. toruloides* IST536 (closed circles) and IST536 MM15 (open circles) (**A**) correspondent to the sampling time points for cell size estimations; Barplots displaying mean FSC values for different culturing time points during the exponential growth phase of *R. toruloides* IST536 cells (black bars) and IST536 MM15 (white bars) (**B**). Adjusted p-values are indicated above the bars.

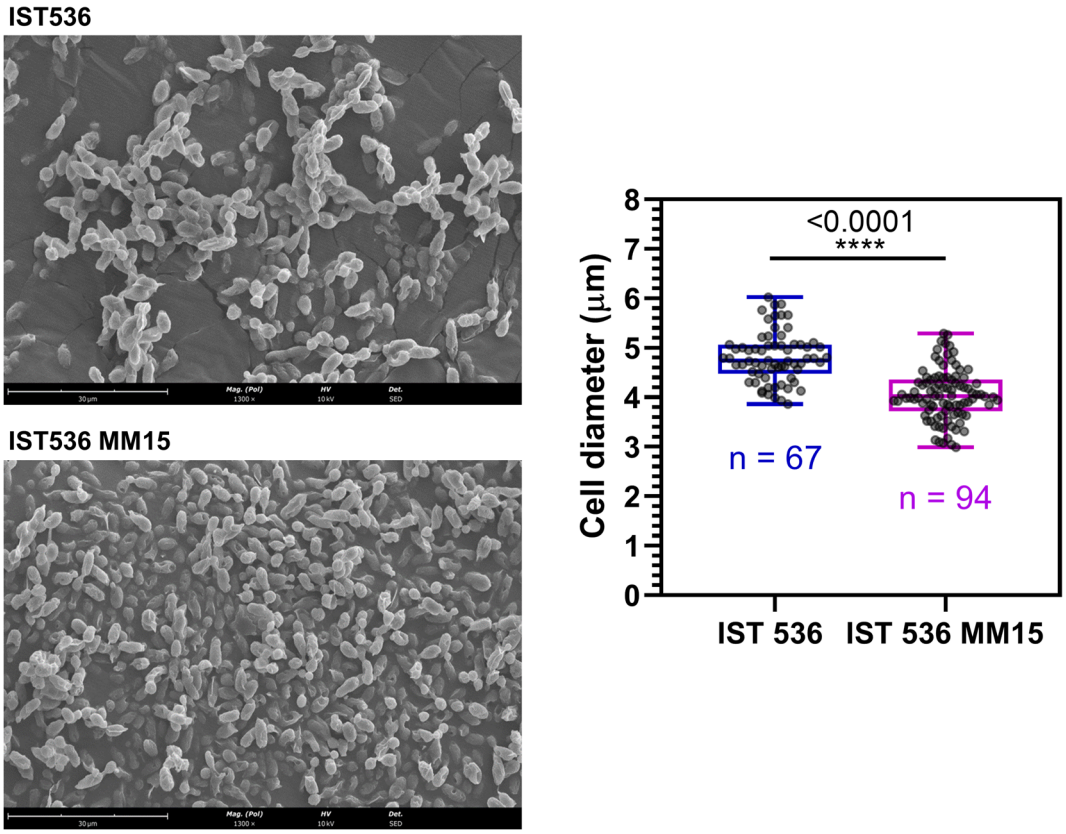

Fig. 2. Scanning electron microscopy (SEM) images depicting IST536 and IST536 MM15 cells with a magnification of 1300× (left panel). Barplots on the right panel show the cell size estimations based on measurements of the SEM images using ImageJ software, where the longest diameter of individual cells was manually measured. Statistical significance was assessed through an unpaired t-test. n denotes the number of individual cells measured.

the former exhibiting larger cells. These findings are consistent with those observed by flow cytometry analysis, reinforcing the idea that the evolved multi-stress tolerant strain has a smaller cell size than the parental strain.

Cell wall chemical composition and integrity of the evolved strain versus the parental strain

The analysis of the sugar composition from the cell walls of *R. toruloides* IST536 revealed that they are composed of 23.9 (w/w) polysaccharides (Table 1), with glucose (Glc; 44 mol%), mannose (Man; 27 mol%), and galactose

Strain	mol % (mean ± SD)							Total (mg/g)
	Fuc	Xyl	Man	Gal	Glc	GlcN	UA	
IST 536	4 ± 0	1 ± 0	27 ± 1	12 ± 1	44 ± 3	7 ± 0	5 ± 1	239 ± 5
IST 536 MM15	9 ± 1	1 ± 0	31 ± 1	16 ± 1	32 ± 3	9 ± 1	7 ± 2	223 ± 11

Table 1. Cell wall sugar composition in sugars (mol %) and total polysaccharide (mg/g) of *R. toruloides* IST536 and IST536 MM15. Results are expressed as mean ± standard deviation values. Fuc – fucose, Xyl – xylose, Man – mannose, Gal – galactose, Glc – glucose, GlcN – glucosamine, UA – uronic acids.

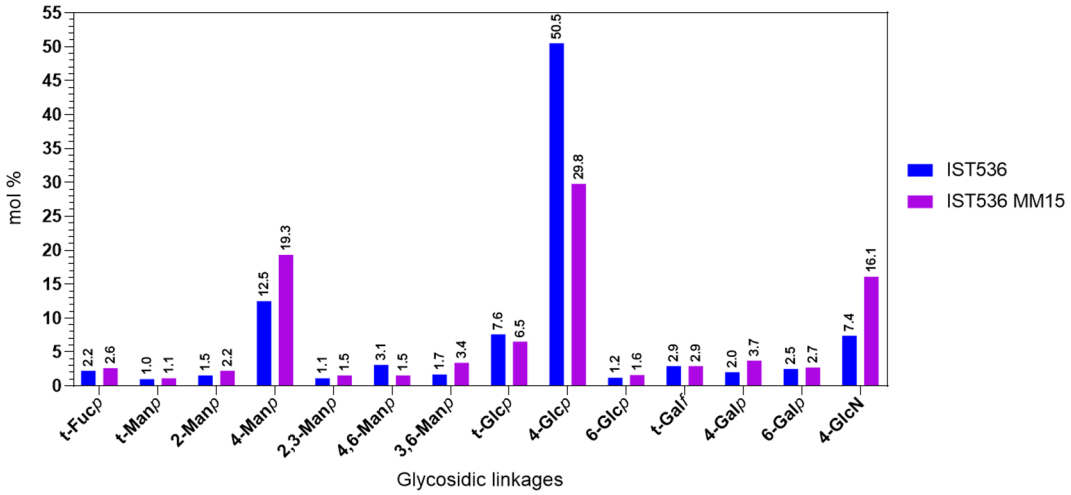

Fig. 3. Glycosidic linkages from the cell walls of *R. toruloides* IST536 and IST536 MM15. Only linkages with mol% > 1 were included.

(Gal; 12 mol%) as the predominant components. Glucosamine (GlcN), derived from chitin, accounted for 7 mol%. Fucose (Fuc) was present in minor proportions (4 mol%), whereas xylose (Xyl) accounted for a minor sugar constituent (1 mol%). Moreover, uronic acids (UA, 5 mol%) were also found present as constituents of *R. toruloides* IST536 cell wall.

Comparing the parental and evolved strains, the proportion of Glc was notably lower in the evolved strain (32 mol% versus 44 mol%), indicating a lower content in glucans (Table 1). Based on the analysis of glycosidic linkages, these consist of (1→4)-linked glucans, comprising 51 mol% of the glycosidic linkages found in the parental strain and 30 mol% in the evolved strain (Fig. 3). Glycosidic linkages of (1→6)-Glc*p* were also found in minor amounts in both strains. Both strains exhibited a similar amount of Man (27–31 mol%) mostly found as (1→4)-linked mannans: 13 mol% in the parental strain and 19 mol% in the evolved strain. These mannans are composed of mixed linkages (1→4)-Man*p*-(1→3)-Man*p* at a 1:9 proportion in the parental strain and 1:6 in the evolved strain. The (1→4)-Man*p* and (1→3)-Man*p* residues are substituted at *O*-6 positions by t-Glc*p* or (1→6)-Glc*p*. These linkages are characteristic of a glucomannan, described to occur in *R. toruloides*[20]. However, these glucomannans are reported to display a high proportion of 3-Man*p*:4-Man*p* residues (1:1). The proportion of Gal was notably higher in the evolved strain (16 mol% versus 12 mol%), indicating increased galactosylation compared to cell wall of the parental strain (Table 1). The Gal residues are found as t-Gal*f*, (1→4)-Gal*p*, and (1→6)-Gal*p* (Fig. 3). The occurrence of t-Fuc, t-Gal*f*, and (1→3)-Man*p* residues substituted at *O*-2 suggests the presence of fucogalactomannans, as described for *Rhodotorula glutinis* K-24 [21]. Regarding chitin, both strains exhibited a similar amount: 21 and 23 mg/g in the parental and evolved strain, respectively. The proportion of (1→4)-Glc*p*N linkages was considerably higher in the evolved strain (16 mol%) compared to the parental strain (7 mol%). Besides chitin, the (1→4)-Glc*p*N linkages could arise from glycoproteins, as described for *Rhodotorula rubra*[21]. The (1→4)-Glc*p*N, together with the higher proportion of (1→4)-Man*p* and (1→3,6)-Man*p* residues, is characteristic of the glycosidic moiety of these glycoproteins[21]. The *N*-acetylglucosamine (Glc*p*NAc) residues, associated to these glycoproteins as short chain oligosaccharides, are degraded by the harsh acid hydrolysis conditions used for chitin determination, explaining its assessment by methylation analysis.

Cell wall integrity was compared by subjecting IST536 and IST536 MM15 cells, previously cultured in minimal medium, to thermal and mechanical disruption followed by their visual inspection through scanning electron microscopy. Results indicate a heightened mechanical resistance of the evolved strain compared to the parental strain (Fig. 4).

Discussion

The size of the evolved strain cells compared to the parental strain was found to be substantially smaller. Yeast cell size control is a critical factor for cellular adaptation to fluctuating environmental conditions[22]. It impacts

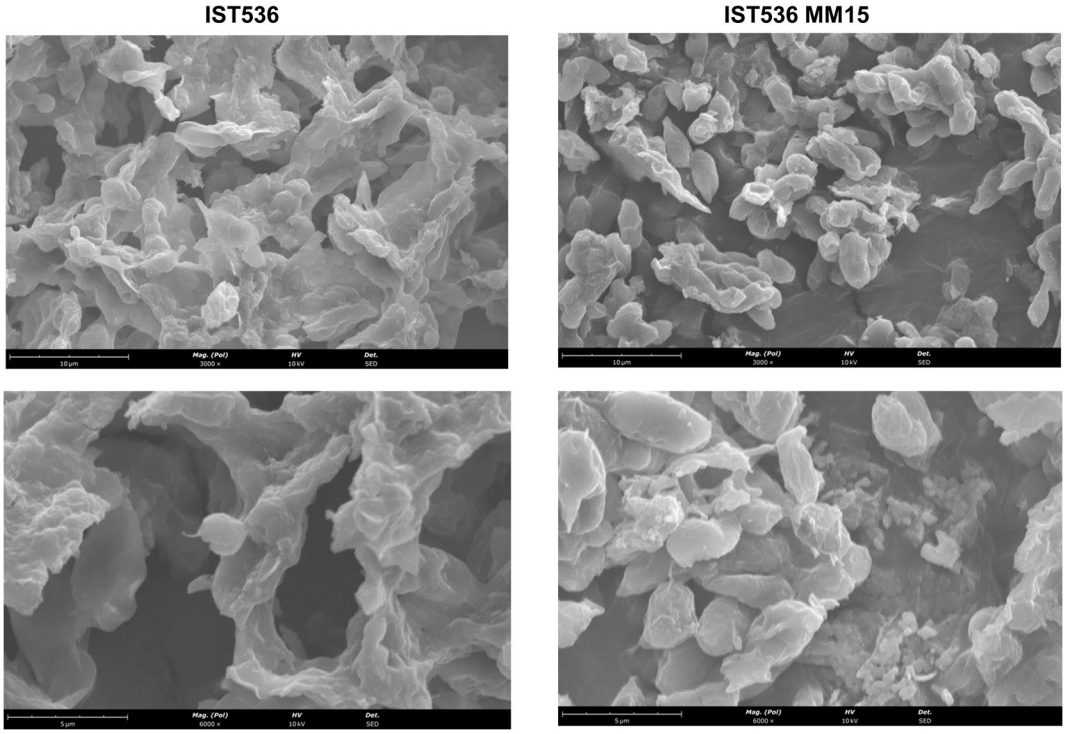

Fig. 4. Scanning electron microscopy (SEM) images depicting the cell walls of IST536 (left panel) and IST536 MM15 (right panel) strains post an identical mechanical disruption procedure. The top images showcase a magnification of 3000×, while bottom images provide a closer view at 6000× magnification.

physiological and molecular processes such as nutrient uptake, cell cycle progression, and gene expression[23,24]. The differences identified may be at least in part attributed to variations in ploidy between the two strains[7], that is, the convergence from a triploid parental strain to a diploid evolved strain. In fact, cell size was shown to be linearly proportional to DNA content in yeast cells, with diploid cells being about twice as large as the haploid counterparts[22].

The knowledge of cell wall composition of oleaginous yeast species, such as the basidiomycetous yeast *R. toruloides*, is substantially limited when compared to other yeast species such as *S. cerevisiae*[9,15]. The carbohydrate proportion in the cell walls of IST536 and IST536 MM15 cells were found to be lower than those reported for other *R. toruloides* strains (580 mg/g)[16–18]. Furthermore, the most representative sugar in both the parental and evolved strains was glucose, which contrasts with mannose, commonly the most abundant sugar in the cell wall of *Rhodotorula* species. Compared to the original strain, the evolved multi-stress tolerant strain exhibited a higher proportion of glucomannans, fucogalactomannans, and chitin relative to (1→4)-linked glucans. The evolved strain also appears to display a high proportion of glycoproteins substituted with short chain oligosaccharides composed of Glc*p*N. The presence of glucomannans and fucogalactomannans, as a highly branched polysaccharide, have been linked to play a role in conferring tolerance to ethanol stress[20,25].

The observed increase in the proportion of glucomannans and fucogalactomannans in combination with carbohydrate structures composed of (1→4)-Glc*p*N linkages, may potentially explain the evolved strain's multi-stress tolerance phenotype across various environmental conditions and stress factors[6,7]. In fact, chitin contributes to the rigidity and physical strength of the cell wall through covalent cross-linking to $\beta-glucans$. In response to stress, chitin levels are increased via cell wall integrity (CWI) pathways[9,26]. Glycosyl phosphatidylinositol (GPI)-linked cell wall proteins (CWPs) play essential roles in the cell wall by modulating its structure and acting as active catalysts in cell wall biogenesis[27]. These proteins determine cell wall permeability and surface charge and can function as extracellular mechanosensors that activate CWI pathways in response to stress conditions[9,26,27].

The observed increase in (1→4)-Glc*p*N linkages, associated to the presence of higher amounts of chitin and/or glycoproteins in the cell wall of the evolved strain, correlates well with the genome-wide alterations described for this strain[7]. Notably, genes associated with chitin metabolism, including three chitin synthases, a chitin synthase activator, and a putative chitinase, exhibited either increased transcriptional levels or missense mutations, while a gene encoding a chitin deacetylase displayed significantly reduced transcript levels[7]. Additionally, genes associated with protein glycosylation and glycosylphosphatidylinositol (GPI) anchors showed several missense mutations and transcriptional alterations in the evolved strain. These genes include a probable GPI lipid remodelase and a subunit of the GPI transaminase complex, which exhibited increased transcription levels, possibly due to amplified copy numbers. Furthermore, several genes encoding proteins involved in the synthesis of GPI anchors and their attachment to proteins displayed numerous missense mutations in their nucleotide sequences. Moreover, a substantial number of genes (14) with missense variations were associated with protein glycosylation, including a GDP-mannose pyrophosphorylase involved in cell wall biosynthesis, and

mannosyltransferases[7]. These modifications and remodeling of the evolved strain are consistent with the shift observed in the cell wall polysaccharides to increment the production of polymers with higher resistance and the decrease of (1→4)-linked glucans. These results allow to infer that the increment of beta-linked polymers such as mannans (glucomannans and fucogalactomannans), and chitin, instead of glycogen (1α→4-linked glucose), promotes a more rigid and robust cell wall in the evolved strain cells. The evolved strain has previously been shown to display a cell wall that is less susceptible to lysis by zymolyase, whose major activities are β-1,3-glucanase and β-1,3-glucan laminaripentaohydrolase[5]. This agrees with the observed absence of (1→3)-linked glucose[5].

The prolonged exposure to methanol of the evolved strain might have induced cell wall remodeling processes that resulted in a more robust cell wall with increased resistance to zymolyase treatment[6]. In agreement with a more rigid and robust cell wall in the evolved strain cells, a higher resistance to non-selective mechanical disruption of the cell wall compared to the original strain was observed in this study. Zymolyase resistance has been reported for cells previously exposed to ethanol stress[9,28,29], with zymolyase resistance showing a positive correlation to ethanol concentration and exposure time[29,30]. Moreover, zymolyase resistance, observed in cell wall polysaccharides of *S. pastorianus* of brewer's spent yeast submitted to ethanol stress conditions, is due to a decrease in (1→3)-linked glucose, as well as the establishment of novel linkages of (β1→3)-glucans with glycogen[12]. Mannans and phosphomannans, in the form of capsular exopolysaccharides, may also offer further protection from environmental stress conditions[15,31].The response of *R. toruloides* cell wall polysaccharides to stress conditions does not result in an increase in storage carbohydrate content, as observed in *S. pastorianus* and *S. cerevisiae* under ethanol stress[12,32]. This is possibly because, as an oleaginous yeast, *R. toruloides* accumulates other sources of storage compounds, likely in the form of triacylglycerols.

Results from this study are consistent with recent publications reporting improved cell wall integrity in more tolerant strains of the model yeast *S. cerevisiae*, developed through evolutionary engineering approaches, to a broad range of stresses, including caffeine, silver, 2-phenylethanol, iron, and oxidative stress[33–37]. In those evolved strains, improved cell wall integrity was shown based on lyticase/zymolyase sensitivity assays and mutations and/or upregulation of genes related to cell wall integrity were identified. Our study, dedicated to a nonconventional yeast species provides a deeper elucidation of cell wall integrity and carbohydrate composition to gain insights into the physicochemical changes associated to the remarkable multi-stress tolerance phenotype occurring in the evolved strain. These results also constitute the starting point for further characterization of cell wall composition regulation and remodeling of *R. toruloides* based on varying medium compositions and exposure to industrially relevant environmental stress conditions, providing a deeper elucidation of these processes, which can be leveraged to engineer more biotechnologically robust strains.

Methods

Yeast strains and growth conditions

The yeast strain *Rhodotorula toruloides* IST536 (PYCC 5615) and the derived mutant *R. toruloides* IST536 MM15, obtained through Adaptive Laboratory Evolution (ALE)[6], were used in this study. Strain IST536 is a conjugated strain derived from IFO 0559 × IFO 0880 [38]. IFO 0559 was originally isolated in Japan from wood-pulp, whereas IFO 0880 was originally isolated in Japan from the air[38]. The strains IST536 and IST536 MM15 were maintained at 4 °C in YPD agar plates (2% glucose (NZYtech), 1% yeast extract (Difco), 2% peptone (Difco), 2% agar). For long-term storage, the strains are preserved at -80 °C in YPD medium supplemented with 15% glycerol (v/v).

All pre-cultures were started from yeast isolated colonies grown on YPD agar plates and cultivated in 100 mL shake flasks containing 50 mL of YPD medium, incubated at 30° C, for 24 h with orbital shaking (250 rpm). For main yeast cultivation, pre-cultured cells were harvested by centrifugation at 4600 × *g* for 5 min at 4 °C, washed twice in sterile water and then inoculated in 20 mL of minimal medium (0.67% Yeast Nitrogen Base (YNB) (Difco), 2% glucose (NZYtech)) used in 100 mL shake flasks, at an initial OD_{600} of 1.0 (approximately 7×10^6 and 8×10^6 cells/mL for IST 536 and IST 536 MM15, respectively). Yeast growth was performed at 30 °C with orbital agitation (250 rpm) and monitored by measuring the optical density at $\lambda = 600$ nm (OD_{600}) using a U-2000 spectrophotometer - HITACHI.

Yeast cell size estimation

Flow cytometry

Flow cytometry was used for the estimation of the relative cell sizes of *R. toruloides* IST536 and the evolved strain IST536 MM15. Yeast cells were inoculated in 20 mL of minimal medium at an initial OD_{600} of 1.0 (approximately 7×10^6 and 8×10^6 cells/mL for IST 536 and IST 536 MM15, respectively). Cells were harvested from 0 to 6 hours (2 h intervals) of cultivation. Flow cytometric analyses were performed using a BD Accuri C6 Plus (BD Biosciences). Relative cell sizes were obtained based on the mean values of the forward scatter (FSC) signal. All experiments were repeated using three biological replicates. A fixed total of 50,000 events per sample were acquired using a slow flow rate (14 μL.min^{-1}). Statistical significance was assessed using multiple t-tests and p-values were adjusted using the Holm-Sidak method, with alpha = 0.05.

Scanning electron microscopy (SEM)

To determine alterations in cell size, *R. toruloides* IST 536 and IST 536 MM15 strains were cultivated with an initial $OD_{600} = 1.0$ (approximately 7×10^6 and 8×10^6 cells/mL for IST 536 and IST 536 MM15, respectively) in YNB medium at 30 °C with orbital shaking (250 rpm) and were harvested by centrifugation (4600 × g for 5 min, at 4 °C) after reaching an OD_{600} of 5.0. Cells were washed with water, resuspended in YPD medium and frozen at -80 °C in 15% (v/v) glycerol. Direct microscopic examination was performed using scanning electron microscope (SEM). Cell sizes were determined by manually measuring the longest diameter of individual cells using ImageJ software.

Cell wall integrity assessment through SEM

The cell wall integrity from IST536 and the evolved strain IST536 MM15 was assessed through scanning electron microscopy (SEM). Cells were cultivated in YNB medium with an initial $OD_{600} = 1.0$ (approximately 7×10^6 and 8×10^6 cells/mL for IST 536 and IST 536 MM15, respectively) at 30 ºC and were harvested by centrifugation (4600 × *g* for 5 min, at 4 ºC) after reaching an OD_{600} of 5.0 (corresponding to 4.7×10^7 and 4.8×10^7 cells/mL for IST 536 and IST 536 MM15, respectively). To perform the autolysis, each cell suspension was placed in a 60 ºC bath and stirred for 2 h. After that, the temperature was increased to 80 °C for 10 min to inactivate hydrolytic enzymes. The samples were then centrifuged (24,700 × *g*, 4 °C, 15 min), the precipitate with cell walls was washed three times with distilled water (resuspended and centrifuged at 24,700 × *g*, 4 °C, 15 min). The autolysis precipitate, in a ratio of 1:16 (mg dry basis/mL H_2O), was stirred in a hotplate (15 min, 100 °C), cooled, centrifuged (24,652 × *g*, 4 °C, 20 min) and the residue were collected. The residue was additionally subjected to a step of mechanical lysis using glass beads (10 cycles, of two minutes each, of vortex and immediate incubation in ice). Cells residues were lyophilized for 24 h, and this biological material was examined microscopically by SEM. Samples were placed on an Al stub using double-sided carbon tape and sputter coated with a thin Au/Pd film in a Quorum Technologies coater, model Q150T ES. They were then analysed in a ThermoScientific desktop SEM, model Phenom ProX G6, with a CsB6 filament and equipped with a light elements energy dispersive spectroscopy (EDS) detector.

Preparation of cell wall material

Cell wall material was isolated following an autolysis procedure. To achieve this, an *R. toruloides* cells suspension was heated up to 60 °C and left to stand, with stirring, for 2 h, as described by Reis et al.[32]. The temperature of the cell suspension was increased to 80 °C for 10 min to achieve enzyme inactivation. The sample was then centrifuged (24,700 × *g*, 4 °C, 15 min). The isolated precipitate, composed of yeast cell walls, was washed three times and centrifuged under the same conditions. The obtained cell wall material was frozen, freeze dried and stored at room temperature in a desiccator until further analysis.

Cell wall carbohydrate composition

Cell wall carbohydrate composition was determined following methodologies for neutral sugar, chitin, and uronic acids quantification. Neutral sugars were analyzed by gas chromatography with a flame ionization detector (GC-FID) following a previous pre-hydrolysis with 72% sulfuric acid, Saemen acid hydrolysis, and derivatization to alditol acetates step[39]. Chitin was quantified considering the amount of GlcN determined by gas chromatography–mass spectrometry (GC–MS, GCMS-QP2010 Ultra, Shimadzu) of the alditol acetates obtained after an acid hydrolysis step with 6 M HCl for 6 h. The phenylphenol colorimetric method, using galacturonic acid (GalA) as standard, was used to estimate the uronic acids[40]. Glycosidic-linkage analysis was performed by gas chromatography–mass spectrometry (GC–MS, GCMS-QP2010 Ultra, Shimadzu) of partially methylated alditol acetates (PMAA) obtained after a methylation, acid hydrolysis, reduction and acetylation steps[41].

Data availability

All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

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Author contributions

M.A. and M.M. performed the experimental work related to yeast cells under the supervision of I.S-C., and P.F. the chemical analysis of the provided cell walls under the supervision of E.C. and M.A.C. I.S-C. designed and coordinated the research work and with M.A. wrote the manuscript with contributions from all the authors in their areas of expertise. All authors revised and agreed on the final manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

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