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Reliable Approach for Pure Yeast Cell Wall Protein Isolation from *Saccharomyces cerevisiae* Yeast Cells

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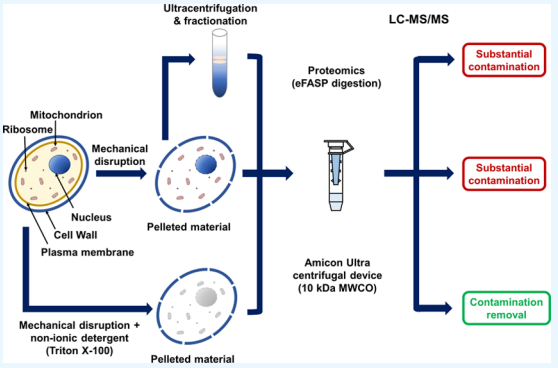
ABSTRACT: *Saccharomyces cerevisiae* yeast is a fungus presenting a peripheral organelle called the cell wall. The cell wall protects the yeast cell from stress and provides means for communication with the surrounding environment. It has a complex molecular structure, composed of an internal part of cross-linked polysaccharides and an external part of mannoproteins. These latter are very interesting owing to their functional properties, dependent on their molecular features with massive mannosylations. Therefore, the molecular characterization of mannoproteins is a must relying on the optimal isolation and preparation of the cell wall fraction. Multiple methods are well reported for yeast cell wall isolation. The most applied one consists of yeast cell lysis by mechanical disruption. However, applying this classical approach to S288C yeast cells showed considerable contamination with noncell wall proteins, mainly comprising mitochondrial proteins. Herein, we tried to further purify the yeast cell wall preparation by two means: ultracentrifugation and Triton X-100 addition. While the first strategy showed limited outcomes in mitochondrial protein removal, the second strategy showed optimal results when Triton X-100 was added at 5%, allowing the identification of more mannoproteins and significantly enriching their amounts. This promising method could be reliably implemented on the lab scale for identification of mannoproteins and molecular characterization and industrial processes for "pure" cell wall isolation.



INTRODUCTION

Saccharomyces cerevisiae, also known as baker's or brewer's yeast, is the most common budding yeast species of the genus *Saccharomyces*. Although largely exploited since the Neolithic age by humans in diverse applications, mainly fermented food and beverage production, *S. cerevisiae* remained an unknown basic ingredient for a long time.[1] Belonging to the Fungi kingdom, *S. cerevisiae* is a unicellular eukaryotic microorganism, comprising multiple cellular compartments or organelles, each exerting particular functions to maintain cellular homeostasis.[2] The outermost of these latter is the yeast cell wall (YCW), representing 15−30% of the yeast dry weight and regulated by approximately 1200 genes (20% of the total number of *S. cerevisiae* genes).[3,4] The YCW is a rigid protecting shell with a thickness ranging from 100 to 200 nm.[5] Besides having a major physicochemical barrier role aiming to maintain morphological, osmotic integrity, and molecular accessibility control, YCW constitutes an important center of cell−cell and cell-environment interactions.[6,7] All these vital roles can be attributed to its complex macromolecular composition, dynamically changing depending on multiple factors, including environmental and stress conditions such as nutrient availability, temperature, and pH.[8] Generally, YCW comprises an internal cross-linked polysaccharide lattice, containing mainly β-glucans (up to 60% of YCW dry weight) and a minor amount of chitin (Figure 1). To this inner layer are bound mannoproteins (MNPs), considered to be the second most abundant YCW component (up to 40% of YCW dry weight), forming the YCW fibrillary external layer (Figure 1).[4,5,7,9]

YCW MNPs are increasingly gaining attention owing to their biotechnological importance in a wide range of industrial applications. Due to their molecular structure being highly mannosylated, YCW MNPs are integrated as additives in the food industry. Besides their use as bioemulsifiers[10,11] and food stabilizers,[12] they are mainly known for their enological application,[13] thanks to their complexation with phenolic compounds,[14,15] the inhibition of tartrate salt crystallization, and the influence of wine aspect both by prevention of haze formation and promotion of yeast flocculation.[16] Moreover, YCW MNPs have shown interesting health-promoting features

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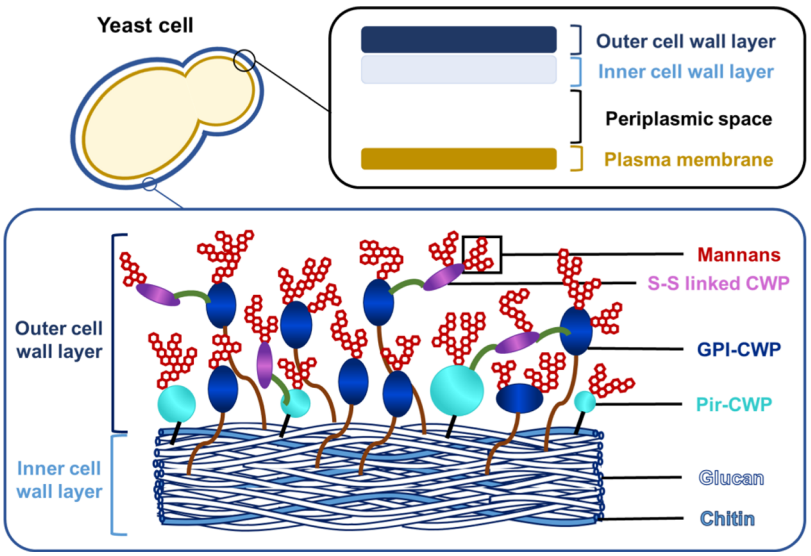

Figure 1. *S. cerevisiae* YCW molecular structure. The YCW is separated from the lipid bilayer of the plasma membrane by a periplasmic space. It is composed of two layers: the inner polysaccharide layer to which are bound mannoproteins forming the outer layer.

by various mechanisms, such as their antioxidant[17] and immunomodulatory roles through interactions with the host immune system,[18] as well as a reported antitumor action.[19,20] Their regulatory activity concerning microbial growth and equilibrium in the gastrointestinal tract stimulates lactic acid bacteria proliferation and inhibits that of pathogenic bacteria.[21,22] This justifies their use as adjuncts for animal feed[23,24] and supplementation for humans.[25] These and other roles of YCW MNPs resulting from their molecular structure remain far from being fully understood, strengthening the need to enrich them through appropriate extraction procedures, in addition to their structural characterization through well-established workflows.

The mannoproteins can be covalently or noncovalently bound to the YCW polysaccharide layer. The covalently bound ones, also referred to as cell wall proteins (CWPs) can be classified into three main groups according to their molecular linkage type: GPI-CWP group including the majority of CWPs linked through a glycosylphosphatidylinositol (GPI) remnant, PIR-CWP group comprising CWPs linked through an alkali-sensitive bond, and the group of proteins linked by disulfide bridges to other CWPs (Figure 1).[26] For the proper extraction of each of the aforementioned mannoprotein groups, various dedicated methods are adapted, whether based on physical, chemical, or enzymatic treatments.[26,27] These procedures can be applied either directly on whole yeast cells or on isolated YCWs. The characterization of YCW MNPs on the proteomic level was accomplished using a myriad of biochemical, biophysical, bioinformatic, and molecular biology tools.[7] Among others, mass spectrometry-based proteomic approaches have widely allowed YCW MNPs' identification and quantitation.[28] The impressive evolvement of the proteomics field over the past 2 decades was also reflected by the extent of gathered information concerning YCW MNPs. While the first reports—limited in their identifications—relied on gel-based separation of extracted MNPs followed by peptide mass fingerprinting and sequencing analysis with MALDI-TOF/TOF,[29] the commonly used approach representing the golden standard method is based on the direct in-solution tryptic digestion of YCW isolated by mechanical disruption of yeast cells.[30] This latter allowed the identification of additional CWPs.[30] Another study led by the same research group permitted the estimation of surface densities by absolute quantitation of individual CWPs and monitoring the dynamics of CWP population by their relative quantitation using isobaric tagging (ITRAQ).[31]

Nevertheless, all of these previous studies mentioned the presence of contamination by proteins originating from other organelles. Indeed, our proteomic analysis showed that subsequent to mechanical disruption, YCW proteins are minor; the majority of the detected proteins are cytosolic and mitochondrial proteins. The presence of cytosolic proteins in the wall was explained by the possibility that they reach the wall via a nonconventional export pathway, acting as "moonlighting" proteins having a specific function on the cell surface,[32] or regarded simply as contamination due to permeation of the biological bilipid membranes as the plasma membrane and mitochondrial membranes during the preparation procedure.[33] Some suggested that subcellular fractionation by ultracentrifugation might be an option to minimize the contamination during YCW isolation following disruption of whole cells.[2,34] Despite this suggestion, proteomics of ultracentrifugation resulting in YCW has never been applied for YCW proteome analysis.

In this current study, we describe a reliable strategy to obtain nearly pure YCW isolates following mechanical disruption. The proteomics characterization showed that the purification method based on ultracentrifugation had limited outcomes. On the other hand, adding Triton X-100 (a nonionic detergent) at a concentration of 5% demonstrated a significant reduction in both the number and the relative abundance of contaminants, mainly including mitochondrial proteins. This was concomitantly accompanied by an enhancement in the number and the abundance of identified CWPs that became the most abundant proteins.

EXPERIMENTAL SECTION

S288C Strain Fed-Batch Culture. For the seed development stage, the *S. cerevisiae* S288C strain (ATCC: 204 508, MATα SUC2 gal2 mal2 mel flo1 flo8-1 hap1 ho bio1 bio6)

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was inoculated initially in a 250 mL Erlenmeyer flask containing 150 mL of sterilized standard YPD medium composed of 1% yeast extract (212 750, Gibco Bacto Yeast Extract, Life Technologies Miami, FL), 2% bactopeptone (Gibco Bacto Peptone Life Technologies, Detroit, MI), and 2% glucose (Carlo Erba reagents, Var de Reuil, France) and grown for 24 h at 30 °C with shaking at 120 rpm. After centrifugation and pellet washing, the concentrated seed at 100 g/L was injected into a 7 L bioreactor (ez-Control autoclavable Bioreactor 7 L, Applikon Biotechnology, Delft, The Netherlands) containing 1.5 L of sterilized YPD medium without glucose (1% yeast extract, 2% bactopeptone). The fed-batch bioreaction was carried out in three independent experiments for 48 h at 30 °C with an airflow of 1 VVM, and the medium pH was controlled at 5.0 by the automatic addition of 10% H_2SO_4 and 10% NaOH. Sterile solutions of the yeast extract (80 g/L), bactopeptone (160 g/L), and glucose (2 solutions at 110 and 710 g/L) were continuously added to the bioreactors to finally simulate a 4-fold concentrated YPD with oxidative respiration as the main metabolic pathway. During the bioreaction, samples were taken to realize a dry matter determination following desiccation at 105 °C, and the spent medium containing the cells was filtered and served to determine the concentrations of glucose and ethanol by HPLC ion-exchange chromatography using a Prominence HPLC system (Shimadzu) equipped with an Aminex HPX-87H (1 250 140, Pkg of 1, 300 mm × 7.8 mm, Biorad, CA). The monitoring of these cultures showed high reproducibility in terms of growth, glucose consumption (residual glucose < 0.2 g/L) as well as ethanol profile (<1 g/L). In general, the glucose converted to ethanol represents less than 3% of the fed glucose, indicating that the growth is mainly oxidative (SI, Figure S1A–D).

YCW Isolation by Mechanical Disruption. This method is based on the frequently cited protocol for YCW isolation by mechanical disruption by de Groot et al.[35] The yeast cell suspension (100 mg/mL) was transferred to 2 mL of ice-cold lysis buffer containing 10 mM Tris-HCl of pH 7.5 supplemented with a 1× complete protease inhibitor cocktail (#11697498001, Roche Diagnostics, Basel, Switzerland), in BeadBug triple-pure prefilled tubes with 1 g of 0.5 mm glass beads (Benchmark Scientific, Sayreville, NJ). Mechanical disruption was realized 15 times through a cycle-based method, knowing that a cycle includes a homogenization step of 1 min using BeadBug (Benchmark Scientific) followed by a resting step on ice for 5 min. Then, the cell lysate was filtered from beads that were washed three times with 1 mL of NaCl 1 M solution. The cell lysate and the bead washings were pooled and centrifuged at 4 °C for 10 min at 4000*g* (Centrifuge 5430 R, Eppendorf, Hamburg, Germany). The resulting pellet was further washed three times with 1 mL of NaCl 1 M solution to reduce positively charged intracellular proteins interacting by electrostatic adsorption to the negatively charged YCW, and recuperate them in the supernatant that was discarded. Afterward, the pellet was extracted with 1 mL of SDS extraction buffer (50 mM Tris-HCl pH 7.5, 100 mM EDTA, 150 mM NaCl, 100 mM β-mercaptoethanol, 2% SDS) and heated for 10 min at 100 °C. This step was repeated once again, before centrifuging for 5 min at 20 000*g* (Allegra 64R Centrifuge, Beckman Coulter, Brea, CA). The resulting pellet was washed several times with 1.5 mL of ultrapure water and it consists of YCW isolates, while the supernatant contains indirectly covalently linked YCW proteins (by a disulfide bond). The YCW-washed pellet was dried in a Concentrator plus (Concentrator Savant ISS110, Eppendorf), weighed, and stored at −20 °C.

Purification of YCW Isolates by Ultracentrifugation. The applied protocol is adapted from Kurita et al.[34] Briefly, 100 mg of yeast cells was suspended in 2 mL of ice-cold lysis TNE buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM EDTA, supplemented with a 1× complete protease inhibitor cocktail). The cells were mechanically disrupted with a BeadBug homogenizer in the presence of 1 g of 0.5 mm glass beads, as previously described. Subsequently, the cell lysate was filtered from beads that were washed three times with 1 mL of NaCl 1 M solution. The cell lysate and the bead washings were pooled and centrifuged at 4 °C for 10 min at 4000*g* (Centrifuge 5430 R, Eppendorf): the resulting pellet was further washed three times with 1 mL of NaCl 1 M solution. The washed pellet of nuclei and YCW was suspended in 1 mL of the suspension buffer (10 mM Tris-HCl pH 7.4, 15.25% sorbitol, 10 mM EDTA, supplemented with a 1× complete protease inhibitor cocktail), to be transferred to a 12 mL continuous density gradient of Optiprep (D1556; Sigma-Aldrich, Saint-Louis, MI) (18−48%). Ultracentrifugation was performed at 4 °C for 19 h at 155 000*g* using an SW41Ti swinging bucket rotor (Beckman Coulter, Brea, CA); 24 fractions of 0.5 mL each were sequentially collected from the gradient top.

Purification of YCW Isolates with Triton X-100. The mitochondrial lysis and YCW isolates' purification was realized during the mechanical disruption for YCW isolation, by adding Triton X-100 (9036-19-5; Sigma-Aldrich Saint-Louis, MI) at concentrations ranging from 0 to 20% to the lysis buffer. The usual disruption with glass beads is performed but followed by a 30 min resting step on ice allowing the solubilization of mitochondria. Thereafter, the upcoming steps of washing and delipidation proceeded as for the previously described mechanical disruption for YCW isolation.

Proteomics Experiments. The protein concentration of samples was determined using a Pierce BCA protein assay kit (Thermo Scientific). A classical bottom-up proteomics workflow was performed according to the eFASP method.[36] Briefly, tryptic digestion was carried out overnight at 37 °C in an ammonium bicarbonate buffer of pH 8.8 inside Amicon 10 kDa MWCO filtration devices (Millipore), through the addition of 1 μg of sequencing grade trypsin (V5111, Promega, Madison, WI) to 50 μg of proteins from YCW isolates or ultracentrifugation fractions. For complete peptide recovery, filtration units were subsequently washed twice with 50 μL of 50 mM ammonium bicarbonate and extracted with ethyl acetate (270 989; Sigma-Aldrich). Peptides were dried in a Concentrator plus (Concentrator Savant ISS110, Eppendorf). The peptide concentration was determined after adding 10 μL of 0.1% formic acid using the absorbance measurement at 214 nm with a spectrophotometer (Denovix DS-11 + spectrophotometer; Denovix Inc., Wilmington, NC). A nanoflow HPLC instrument (U3000 RSLC ThermoFisher Scientific, Waltham, MA) was used, coupled on-line to a Q Exactive Plus mass spectrometer (ThermoFisher Scientific) with a nanoelectrospray ion source. Then, 1 μg of peptides was loaded onto the preconcentration trap (ThermoFisher Scientific, Acclaim PepMap100 C18, 5 μm, 300 μm i.d × 5 mm) using partial loop injection, for 5 min at a flow rate of 10 μL/min with buffer A (5% acetonitrile and 0.1% formic acid) and separated on a reversed-phase column (Acclaim PepMap100 C18, 3 μm,

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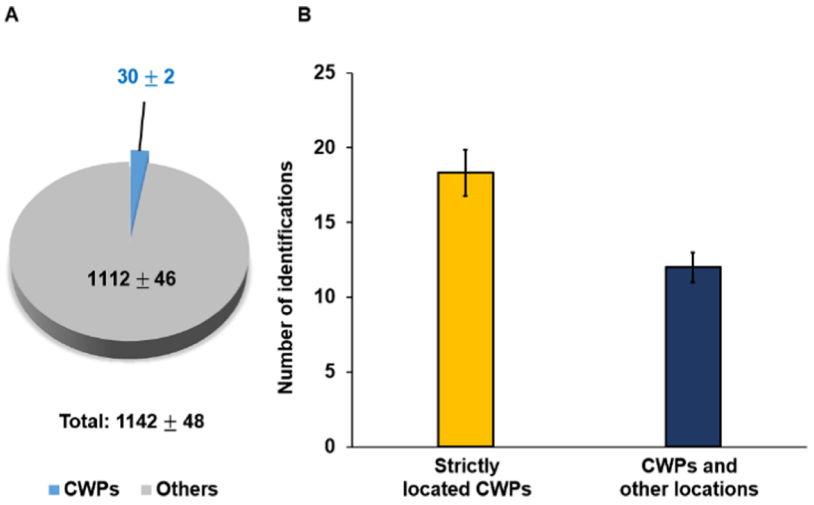

Figure 2. Protein identification by bottom-up proteomics applied to YCW isolated by mechanical disruption from S288C yeast cells cultured in fed-batch mode. (A) Pie chart showing the total number of identified proteins. (B) Histogram indicating the number of identified CWPs according to the gene ontology annotation. The data is represented as the average ± standard deviation for three independent experiments.

75 mm i.d. × 500 mm) with a linear gradient of 5−50% buffer B (75% acetonitrile and 0.1% formic acid) at a flow rate of 250 nL/min and at 45 °C. The gradient length was 160 min. The column was washed with 99% of buffer B for 10 min and reconditioned with buffer A. The total time for an LC-MS/MS run was about 180 min long.

Bioinformatic and Statistical Analysis. The acquired raw files were analyzed with Proteome Discoverer 2.2 software (ThermoFisher Scientific) with a Sequest search engine against the *S. cerevisiae* S288C strain dataset (orf_trans_all) from the Saccharomyces Genome Database (SGD) (last modified in January 2015, verified with the last released database in April 2021). The mass tolerance for peptides was specified at 10 ppm and 0.01 Da for mass spectroscopy (MS)/MS. Variable modifications included were as follows: the search included variable modifications of methionine oxidation and asparagine deamidation. Proteins were identified with two unique peptides. A label-free quantification method using the Minora algorithm was implemented in data processing. Gene Ontology (GO) analysis using the Uniprot Knowledgebase (UniprotKB) and SGD GO slim mapper tool was performed, specifically in what related to the cellular component category permitting the study of the subcellular location of proteins. Statistical analysis was performed by a one-way ANOVA test using XLSTAT software, with a significance threshold of 0.05. The experiments were performed in three independent biological replicates.

RESULTS AND DISCUSSION

Direct Proteomics of Mechanical Disruption YCW Isolates. Over the past 2 decades, various studies were carried out for the proteomics characterization of *S. cerevisiae* YCW. Among multiple strategies of cell lysis and YCW isolation,[37,38] the most commonly used approach for lab-scale YCW preparation relies on the mechanical disruption using glass beads.[35] In our work, we first isolated YCW from fed-batch cultured S288C yeast cells by mechanical disruption using glass beads according to the protocol described by de Groot et al.[35] The proteomics of the isolated YCW was performed following an eFASP method. We saw a high total number of identified proteins reaching 1142 on average (Figure 2), among which an average of 30 CWPs was observed (Figure 2).

These results show that mechanical disruption enables the isolation of YCW proteins but is heavily contaminated by proteins from other organelles. From these identified proteins, an average of 18 strictly located CWPs were detected (Figure 2B). The rest consisting of 12 CWPs can be found in other subcellular locations (Figure 2B). The outcomes in terms of strictly located CWPs are quite similar to what was reported in the study of Yin et al.,[30] carried out on YCW isolated through the same mechanical disruption from the FY833 yeast strain (*MATa his*3Δ300 *ura*3−52 *leu*2Δ1 *lys*2Δ202 *trp*1Δ63) in YPD medium in batch culture. The aim of this study was to directly identify CWPs without any prior release step from the YCW. It proved to be efficiently capable to identify, while they are still linked to YCW, 19 CWPs, with 12 GPI-modified proteins, 4 proteins of the PIR family, and 3 proteins alkali-sensitive linked to the YCW (Scw4, Scw10, and Tos1). One member of the PIR family proteins, Pir3, was only identified in stationary-phase cells while other identified proteins are the same as log-phase cells. This is in agreement with our results except for five proteins which are Tip1, Tir1 (required for anaerobic growth), Plb2, Pry3 (daughter cell-specific cell wall protein required for efficient export of lipids), and Pir3 proteins. In this work, the proteins from other subcellular locations were not listed. At this stage, it is difficult to say if the discrepancy in both study results is linked to cell culture mode or sample preparation; as in the study of Yin et al.,[30] the tryptic digestion was performed in solution without any detergent, in contrast to our digestion led according to the eFASP method that employs deoxycholate and other detergents to enhance protein solubility.

As the low number of identified CWPs does not reflect their real abundance in the YCW preparation—compared to the total number of identified proteins—we adopted the label-free quantification approach that considers the abundance of identified proteins based on their peptide intensities and sequences. The label-free quantification indicated that the most abundant 100 proteins represent an average of 85% of the protein content at the end of the fed-batch culture. Most importantly, 8 CWPs representing 34.1% of the protein content in terms of abundance are identified among the most

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100 abundant proteins (Figure 3). These proteins are mainly highly abundant glycolytic enzymes (Tdh3, Tdh2, Tdh1, and

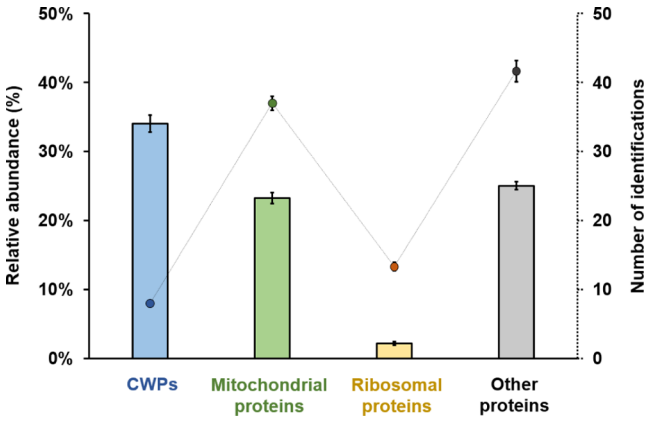

Figure 3. Label-free quantitative proteomics of YCW isolated by mechanical disruption from S288C yeast cells cultured in fed-batch mode, showing the most 100 abundant proteins. Graph showing the number (filled dots) and the relative abundance (histogram) of the identified CWPs, mitochondrial proteins as well as ribosomal proteins classified according to the GO annotation. The data is represented as the average ± standard deviation for three independent experiments.

Fba1) and heat shock proteins (Hsc82, Hsp82) that can also be located in other organelles. Despite this, these proteins are widely described as CWPs, and this classification is yet not certain. These CWPs that can be located elsewhere in the yeast cell constitute the major amount of the CWPs, with a relative abundance reaching an average of 29.2% among the relative abundance of 34.1%. The remaining 4.9% stand for the two heat shock proteins Ssa1 and Ssa2 considered being mainly located in the CWPs (SI, Figure S2). Accordingly, we examined the number and relative abundance of the mitochondrial proteins among these 100 most abundant proteins. As displayed in Figure 3, a substantial number (37 ± 1) and abundance (23.3 ± 0.8%) of mitochondrial proteins were found. However, the ribosomal proteins were the minor group, accounting for an average of 13 proteins representing 2.2% of the protein content in terms of abundance.

Summed up, these quantitative results point out two important considerations. The first is related to YCW isolation preparation, where it clearly shows that the mechanical disruption yields in addition to the YCW organelle other subcellular compartments, mainly mitochondria. The second consideration is related to the cell programming in fed-batch culture depending on the nutrient availability and deprivation. At the end of the fed-batch culture, where a low growth rate occurs, the ribosomal proteins are consequently decreased. Moreover, the metabolism is purely oxidative, characterized by an important mitochondrial presence and activity, being the organelle responsible for respiration. These findings quite resemble the end of a batch culture, where the cells enter the quiescence or the stationary phase, generally marked as the most stress and starvation tolerant state. The proteome is significantly remodeled, generally characterized by stress-response proteins' (as chaperones and heat shock proteins) upregulation[39,40] and growth-related proteins' (as ribosomal proteins) downregulation,[41] along with an important glycolytic activity and storage carbohydrate synthesis.[42] Apart from the low growth rate and the limited supply of nutrients during the fed-batch culture, the high cell population density is another causal factor of stress, making the continuous nutrient supplementation insufficient. These results highlight the significance of the culture mode and conditions, proving that the proteome is highly dependent on the growth rate regulated by nutrient supply rates along the fed-batch culture course.

In Table 1, the identified CWPs and their relative abundance are listed. The list demonstrates the high abundance of CWPs that are also located in other cellular compartments, especially glycolytic enzymes due to their involvement in neogluco-genesis, the reverse pathway of glycolysis. This pathway synthesizes glucose to be incorporated in storage carbohydrates as glycogen and trehalose in the fed-batch culture conditions, and in addition, a high expression of starvation-induced expression of heat shock proteins, Ssa1 and Ssa2. Some CWPs are only found in starvation phases, like among others Suc2, Pho5, and Exg1. However, other known stationary-phase and starvation-induced proteins such as Ygp1 and Pir3 were not observed.

The undesirable enrichment in mitochondrial and ribosomal proteins in our YCW preparation proves that the mechanical disruption method not only allows us to isolate YCW but also other organelles that are left and highly interfere with the study of mannoproteins. Strictly located CWPs of interest were quantitatively scarcely represented in these experiments and so were suppressed by the most abundant proteins from other organelles. Thus, we decided to address this issue by minimizing the contamination by other organelles.

Proteomics of Mechanical Disruption YCW Isolates Purified by Ultracentrifugation. Although previously proposed,[2] ultracentrifugation has rarely been applied for YCW study purposes and usually is combined to YCW prior degradation with glucanases for subsequent intracellular organelle segregation. Hence, we chose to use a continuous density gradient of iodixanol for this ultracentrifugation according to an adapted method of Kurita et al.[34] Following 19 h of ultracentrifugation, we saw an only band in the middle of the density gradient (Figure 4A). Then, 26 fractions of 500 μL each from the top to the bottom of the density gradient were carefully recuperated and analyzed by the bottom-up proteomics approach using the eFASP method. We will only showcase the data of the most enriched fraction (fraction 12) in CWPs, according to their maximum number and relative abundance. The remaining data of the other fractions can be found in the supporting information (SI, Figures S3 and S4). For the total number of identified proteins (Figure 4B), the results showed a slightly reduced overall number of identified proteins with an average of 1097 compared to 1142 in the case where ultracentrifugation was not applied (Figure 2A). This reflects the fact that the application of ultracentrifugation has not allowed a substantial reduction, in terms of the number of identifications, YCW preparation contaminants. Nonetheless, the ultracentrifugation step showed its interest in augmenting the number of identified strictly located CWPs, attaining an average of 22 proteins, in addition to an average of 11 CWPs that can be located in other organelles (Figure 4C).

Consequently, when ultracentrifugation was implemented, the identification of additional CWPs was allowed compared to the direct proteomics of YCW isolates following mechanical disruption (Table 1). These proteins are involved in cell wall remodeling such as Scw11, a probable glucanase allowing cell separation;[43] Gas3, a probable glycosyltransferase elongating YCW β-1,3-glucan chains;[44] and Ecm33 protein important for proper YCW biogenesis and integrity.[45] Other identified

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Table 1. List of Identified and Quantified CWPs Following Bottom-Up Label-Free Proteomics Approach Application to Different YCW Isolates[a]

Accession number SGD	Accession number Uniprot	Gene name	Direct Proteomics (Yin *et al*, 2005)	Relative abundance (This study)		
				Direct Proteomics	Ultracentrifugation	Mitochondrial lysis (5% Triton X-100)
YGR192C	P00359	*TDH3*	-	1.07E-01 ± 4.95E-03	1.03E-01 ± 3.09E-03	2.17E-02 ± 2.36E-03
YJR009C	P00358	*TDH2*	-	9.25E-02 ± 4.44E-03	8.98E-02 ± 3.51E-03	1.81E-02 ± 1.45E-03
YJL052W	P00360	*TDH1*	-	7.43E-02 ± 3.68E-03	6.98E-02 ± 9.99E-04	2.08E-02 ± 1.29E-03
YKL060C	P14540	*FBA1*	-	1.10E-02 ± 2.03E-03	8.98E-03 ± 1.19E-03	1.66E-03 ± 5.34E-04
YMR186W	P15108	*HSC82*	-	3.65E-03 ± 2.63E-04	4.31E-03 ± 1.22E-04	5.17E-03 ± 5.96E-04
YPL240C	P02829	*HSP82*	-	3.59E-03 ± 2.16E-04	4.15E-03 ± 2.54E-04	4.93E-03 ± 5.00E-04
YKL157W	P32454	*APE2*	-	2.86E-04 ± 5.15E-05	2.10E-04 ± 2.19E-05	2.20E-05 ± 1.87E-05
YHR107C	P32468	*CDC12*	-	9.09E-05 ± 2.37E-05	8.97E-05 ± 1.15E-05	1.33E-04 ± 2.99E-05
YKR042W	P36135	*UTH1*	-	8.73E-05 ± 1.73E-05	6.48E-05 ± 6.12E-06	6.99E-04 ± 1.25E-04
YBR162C	P38288	*TOS1*	+	3.21E-05 ± 1.02E-05	**4.16E-05**	6.57E-04 ± 1.38E-04
YLR314C	P32457	*CDC3*	-	2.78E-05 ± 7.47E-06	3.62E-05 ± 4.05E-06	**1.81E-05**
YDR321W	P38986	*ASP1*	-	**1.39E-05**	3.01E-05 ± 1.54E-05	-
YJR076C	P32458	*CDC11*	-	**1.01E-05**	-	-
YLR377C	P09201	*FBP1*	-	**5.52E-06**	-	-
YMR008C	P39105	*PLB1*	-	-	-	4.11E-05± 3.97E-05
YAL005C	P10591	*SSA1*	-	2.82E-02 ± 5.37E-04	2.49E-02 ± 5.48E-04	5.02E-03 ± 9.45E-04
YLL024C	P10592	*SSA2*	-	2.04E-02 ± 7.02E-04	1.95E-02 ± 8.75E-04	4.52E-03 ± 7.70E-04
YGR282C	P15703	*BGL2*	-	8.86E-04 ± 4.04E-05	5.39E-03 ± 5.11E-04	3.73E-04 ± 3.93E-05
YDR032C	Q12335	*PST2*	-	5.98E-04 ± 6.62E-05	1.13E-03 ± 1.74E-04	**5.48E-06**
YGR279C	P53334	*SCW4*	+	1.01E-03 ± 1.39E-04	7.76E-04 ± 1.20E-04	1.12E-02 ± 7.24E-04
YLR390W-A	O13547	*CCW14*	+	9.15E-04 ± 9.69E-05	4.49E-04 ± 3.02E-04	2.48E-03 ± 2.19E-03
YBR093C	P00635	*PHO5*	-	5.36E-05 ± 3.09E-05	7.79E-04 ± 8.57E-05	**4.06E-05**
YOL030W	Q08193	*GAS5*	+	9.82E-05 ± 7.42E-06	4.37E-04 ± 1.95E-05	2.36E-03 ± 1.77E-04
YMR307W	P22146	*GAS1*	+	1.17E-04 ± 2.20E-05	4.09E-04 ± 8.28E-05	4.84E-03 ± 7.03E-04
YJL159W	P32478	*HSP150*	+	7.49E-05 ± 9.32E-05	3.02E-04 ± 1.53E-04	1.24E-01 ± 2.08E-02
YJR004C	P20840	*SAG1*	-	3.54E-04 ± 9.29E-05	3.47E-04 ± 3.13E-05	1.08E-02 ± 2.97E-03
YAR071W	P35842	*PHO11*	-	-	3.67E-04 ± 7.90E-05	-
YIL162W	P00724	*SUC2*	-	2.05E-04 ± 1.64E-05	2.09E-04 ± 1.37E-05	2.99E-04 ± 3.16E-05
YBR078W	P38248	*ECM33*	+	**7.80E-05**	2.30E-04 ± 2.17E-05	1.34E-03 ± 2.64E-04
YNL160W	P38616	*YGP1*	-	-	8.93E-05 ± 6.14E-05	2.04E-04 ± 1.22E-04
YMR215W	Q03655	*GAS3*	+	**1.73E-05**	8.49E-05 ± 2.27E-05	1.75E-03 ± 3.43E-04
YGR189C	P53301	*CRH1*	+	3.49E-05 ± 8.37E-06	6.74E-05 ± 2.91E-05	3.84E-03 ± 6.72E-04
YKL096W	P28319	*CWP1*	+	7.60E-05 ± 2.22E-05	4.01E-05 ± 2.56E-05	3.65E-03 ± 1.49E-04
YJL171C	P46992	*YJR1*	-	-	6.44E-05 ± 1.41E-05	1.48E-04 ± 8.00E-05
YJL158C	P47001	*CIS3*	+	-	3.19E-05 ± 8.26E-06	9.07E-02 ± 1.35E-02
YLR300W	P23776	*EXG1*	-	1.43E-05 ± 1.57E-06	**2.32E-05**	-
YMR305C	Q04951	*SCW10*	+	2.24E-05 ± 5.77E-06	1.90E-05 ± 5.53E-06	4.84E-04 ± 7.61E-05
YIL123W	P40472	*SIM1*	-	1.84E-05 ± 8.83E-06	**1.26E-05**	1.50E-04 ± 4.21E-05
YGL028C	P53189	*SCW11*	-	-	6.79E-06 ± 2.25E-07	4.37E-05 ± 1.29E-05
YHR215W	P38693	*PHO12*	-	-	**2.31E-04**	-
YEL040W	P32623	*UTR2*	+	1.79E-05 ± 1.13E-05	**2.44E-05**	5.50E-04 ± 1.30E-04
YNR067C	Q04438	*DSE4*	-	1.02E-06 ± 2.93E-08	-	-
YKL164C	Q03178	*PIR1*	+	-	-	1.09E-01 ± 1.86E-02
YKL163W	Q03180	*PIR3*	+	-	-	8.53E-02 ± 1.36E-02
YMR006C	Q03674	*PLB2*	+	-	-	8.62E-05 ± 7.91E-06
YDR055W	Q12355	*PST1*	-	-	-	1.99E-05 ± 3.95E-06
YBR067C	P27654	*TIP1*	+	-	-	-
YER011W	P10863	*TIR1*	+	-	-	-
YJL078C	P47033	*PRY3*	+	-	-	-

[a]The yellow-highlighted rows correspond to strictly located CWPs, whereas blue-highlighted ones refer to CWPs that can be located in other subcellular organelles. (+) Identified protein and (−) unidentified protein. Relative abundance is calculated as the ratio of the protein abundance (emPAI) to the sum of all identified protein abundances and is represented in the table as the mean ± standard deviation of three independent experiments. The bold relative abundances indicate the proteins identified in one of the three replicates only.

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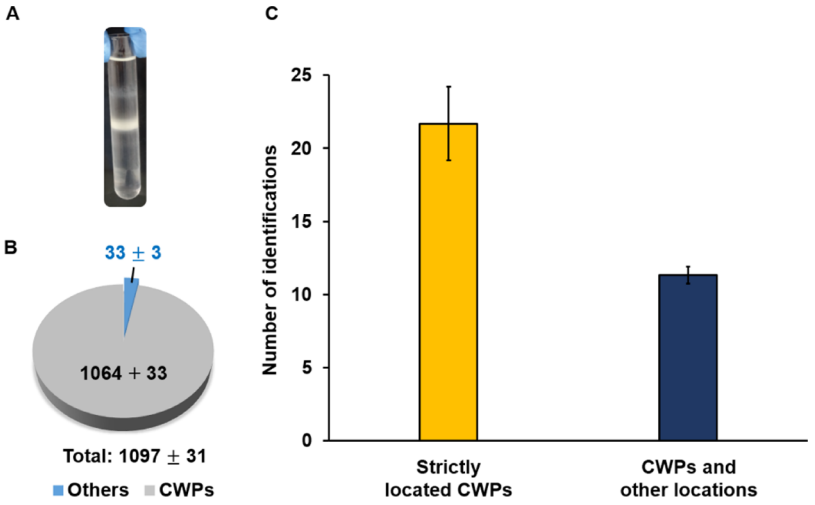

Figure 4. Protein identification by bottom-up proteomics applied to YCW isolated by mechanical disruption from S288C yeast cells cultured in fed-batch and followed by ultracentrifugation for purification. (A) Macroscopic view of the ultracentrifugation tube showing one central thick band. (B) Pie chart showing the total number of identified proteins in the YCW-enriched fraction (fraction 12). (C) Histogram indicating the number of identified CWPs in the YCW-enriched fraction (fraction 12) classified according to the Gene Ontology annotation. The data is represented as the average ± standard deviation for three independent experiments.

proteins required for the adaptation to cell wall stress and stationary phase were detected through this method like Yjr1 induced upon cell wall damage and DNA replication stress[46] and Ygp1 induced in response to nutrient limitations and involved in adaptations to the stationary phase.[47] Cis3 was also identified following ultracentrifugation. This structural mannoprotein is important for YCW stability and optimal growth.[48] The repressible acid phosphatase Pho11 is another newly identified mannoprotein induced upon phosphate deprivation.[49]

However, other CWPs identified in the absence of ultracentrifugation were not detected when ultracentrifugation was applied (Table 1). This group includes three cell wall remodeling enzymes: the cross-linking enzyme chitin transglycosylase encoded by the gene *UTR2*,[50] the probable secreted β-glucosidase Sim1 from the SUN protein family,[51] and the endo-1,3-β-glucanase Dse4 localized at the side of the daughter cell and involved in the degradation of the cell septum separating the daughter cell from the mother cell during septation.[52]

From a quantitative point of view, the most 100 abundant proteins in the YCW-enriched fraction when ultracentrifugation was applied constitute an average of 83% of the protein content. Concerning CWPs, the results were similar to when ultracentrifugation was not applied, where 33% of the protein content in terms of abundance was represented by 9 CWPs (Figure 5).

The majority are CWPs that can be located in other locations, with six proteins representing 28.0% of the protein content (SI, Figure S5). In addition to stress-response glycolytic enzymes and heat shock proteins, one additional strictly located CWP, the Bgl2 protein, was identified compared to the direct proteomics case. The Bgl2 protein is known to be a major protein of the YCW with an endo-β-1,3-glucanase activity. This remodeling enzyme is involved in both cell wall maintenance and mannoprotein incorporation into the YCW.

The ultracentrifugation did not allow for reducing the mitochondrial protein content. Conversely, we can see in Figure 5, an increase in their number and relative abundance

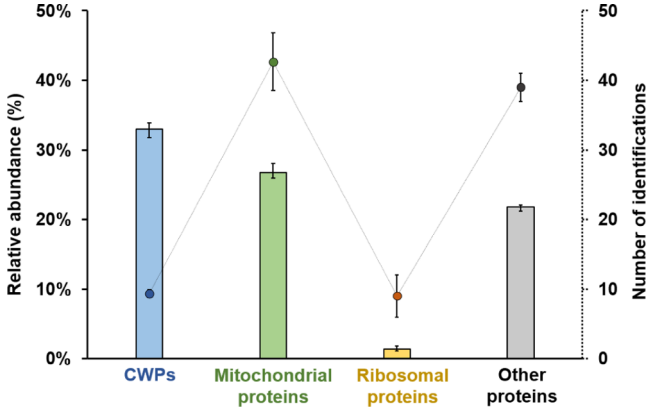

Figure 5. Label-free quantitative proteomics of YCW isolated by mechanical disruption from S288C yeast cells cultured in fed-batch mode and followed by ultracentrifugation for purification. A graph showing the number (filled dots) and the relative abundance (histogram) of the identified CWPs, mitochondrial proteins as well as ribosomal proteins classified according to the GO annotation among the most 100 abundant proteins. The data is represented as the average ± standard deviation for three independent experiments.

attaining an average of 43 and 26.8%, respectively. For ribosomal proteins, an important reduction in both the number (9 ± 3) and the relative abundance (1.4 ± 0.4%) levels was observed (Figure 5). Ultracentrifugation helped to decrease the ribosomal protein contamination, whereas it had a limited effect on the mitochondrial proteins.

Ultracentrifugation permitted the identification of additional CWPs that direct proteomics could not. Nonetheless, it was not efficient in the clearance of YCW preparations obtained by mechanical disruption from other organelles' contaminants.

Proteomics of Mechanical Disruption YCW Isolates Purified with Triton X-100. Facing the substantial presence of mitochondrial proteins in YCW isolates obtained by mechanical disruption, even after ultracentrifugation application, we were concerned about finding a strategy that enables us to remove this contamination. This aims to obtain a "pure" YCW preparation, which can be further employed as a YCW

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model rich in mannoproteins and glucans and suitable for molecular characterization studies. The bibliography showed us that mitochondrial lysis can be achieved using deoxycholate or a nonionic detergent, such as Triton X-100, octylglucoside, digitonin, urea, and thiourea.[53−56] In a classical proteomic workflow, deoxycholate is one important constituent of the lysis, exchange, and digestion buffers. This fact explains our results enriched in mitochondrial proteins where the application of proteomics protocols engenders mitochondrial solubilization (due to deoxycholate) following YCW isolation by mechanical disruption. Thus, we decided to perform mitochondrial lysis during the mechanical disruption course before applying a proteomic workflow. In this regard, we chose to add Triton X-100 to the mechanical disruption lysis buffer in different concentrations ranging from 0 to 20%. This choice was guided by what was shown previously as being the most efficient, the less denaturing at low concentrations,[57,58] and the most widely used detergent for mitochondrial lysis compared to other detergents.[53]

In the absence of Triton X-100, the total number of identified proteins was an average of 940 proteins (Figure 6A),

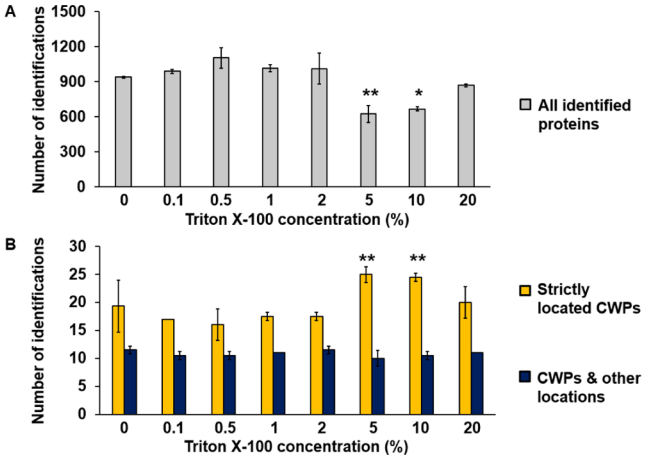

Figure 6. Protein identification by bottom-up proteomics applied to YCW isolated by mechanical disruption in the presence of variable added amounts of Triton X-100 from S288C yeast cells cultured in fed-batch. (A) Histogram showing the total number of identifications depending on the Triton X-100 concentration. (B) Histogram indicating the number of identified CWPs is classified according to the gene ontology annotation depending on the Triton X-100 concentration. The data is represented as the average ± standard deviation for two independent experiments. Statistical significance was determined by the one-way ANOVA test (*p-value < 0.05 and ** p-value < 0.01).

among which an average of 18 were strictly located CWPs and an average of 12 CWPs that can be located in other subcellular compartments (Figure 6B). This total number of identification increases with the addition of up to 2% Triton X-100 (Figure 6A). This can be related to incomplete lysis of the membrane, due to the presence of the YCW organelle. A significant increase was shown at 0.5% Triton X-100 attaining an average of 1105 proteins (Figure 6A) accompanied by a slight decrease in the number of identified CWPs to an average of 27 proteins (Figure 6B).

When the concentration of Triton X-100 was further augmented to 5 and 10%, we observed a significant decrease in the total number of identified proteins to reach on average 626 and 669 proteins, respectively (Figure 6A). This reduction was concomitant to a significant increase in the number of strictly located CWPs to an average of 25 in both cases (Figure 6B). The addition of 20% Triton X-100 did not yield either an additional reduction in the total number of proteins or an improvement in the CWP identifications. The high viscosity of the corresponding buffer can be the cause of this result. These outcomes suggest that the addition of 5 or 10% of Triton X-100 to the lysis buffer during the mechanical disruption of yeast cells for YCW isolation is advantageous. This optimization allows the significant reduction of identified non-YCW proteins and increase of identified CWPs.

The label-free quantification results suggest that the most abundant 100 proteins represent on average almost 80% of the protein content for all of the used concentrations of Triton X-100, except for the preparation with 5 and 10% of the detergent, showing a significant increase to more than 90% of the protein content in terms of abundance compared to other preparations (Figure 7).

Most importantly, the number and the relative abundance of CWPs identified among the most 100 abundant proteins were significantly increased in YCW isolated upon adding 5% of Triton X-100 during the mechanical disruption, which attain an average of 20 proteins corresponding to 53% of the protein content in terms of abundance (Figures 7 and S6A). An increase in the number of the relative amount of CWPs was also shown for the concentration of 10% Triton X-100; however, it was not statistically significant (Figures 7 and S6A). Unlike the case of proteomics applied to YCW isolated without performing mitochondrial lysis—in the absence or presence of an ultracentrifugation step—we found that the majority of these CWPs are strictly located in the YCW when 5% Triton X-100 was added, with an average of 14 mannoproteins representing an average of 45.9% of the protein content in terms of abundance (SI, Figure S7). The relative abundance of the six identified CWPs that can be located in other subcellular organelles among the most 100 abundant proteins significantly decreased when 5% Triton X-100 was added to reach an average of 7.2% in terms of abundance (SI, Figure S7).

This result shows that the CWPs that can be located in other subcellular organelles are mainly removed by this mitochondrial lysis step. The remaining identified proteins in this category are truly present on the YCW and are mainly well covered (8 proteins identified with a sequence coverage exceeding 30%). In contrast, the number and the relative abundance of mitochondrial proteins were concomitantly decreased in YCW preparation obtained with 5 and 10% of Triton X-100. In the case of the addition of 5% Triton X-100, their values were significantly diminished reaching an average of 17 mitochondrial proteins representing 8.4% of the protein content (Figures 7 and S6B).

When 10% of Triton X-100 was added, the relative abundances of mitochondrial proteins were more dispersed among the triplicates leading to a p-value greater than 0.05. For the 42 ribosomal proteins detected among the most 100 abundant proteins, their relative abundance did not exceed 15.2% of the protein content (Figures 7 and S6C).

Hence, these results further support the advantages of Triton X-100 addition at a concentration of 5% for mitochondrial lysis, making it the optimal concentration for the higher qualitative and quantitative enrichment in YCW during the mechanical disruption of S288C yeast cells.

The list of identified CWPs and relative abundance following the addition of 5% Triton X-100 during YCW

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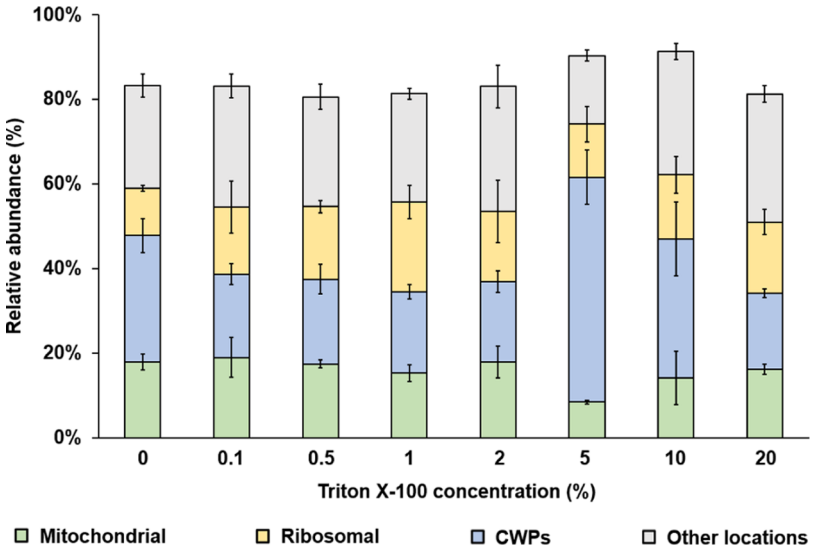

Figure 7. Label-free quantitative proteomics of YCW isolated by the mechanical disruption of S288C yeast cells cultured in fed-batch in the presence of variable added amounts of Triton X-100. Histogram showing the relative abundance of the identified CWPs, mitochondrial proteins as well as ribosomal proteins classified according to the GO annotation among the most 100 abundant proteins. The data is represented as the average ± standard deviation for three independent experiments.

preparations is shown in Table 1. In the case of strictly located CWPs, the most important feature was the identification of mannoproteins Hsp150, Cis3, as well as the newly identified Pir1 and Pir3, which are members of the PIR family of CWPs. These mannoproteins are the most abundant ones (Table 1). This was totally opposite to the case of proteomics of YCW in the absence of Triton X-100, with or without ultracentrifugation, where heat shock proteins were predominant. This amelioration was also reflected by the increased sequence coverage of the identified strictly CWPs where 12 of these proteins showed a sequence coverage exceeding 20% (data not shown). These outcomes prove that the preparation of YCW isolates is crucial and has important effects on the identification and quantification of CWPs. These PIR family proteins are highly homologous structural proteins and are extensively *O*-glycosylated. They are required for cell wall stability, optimal growth, and tolerance to heat shock.[59] A recent study of the *Candida albicans* cell wall architecture by transmission electron microscopy and tomography showed their entrapment in the internal layer of the YCW.[60] Knowing that the *C. albicans* cell wall architecture is a similar structure to that of *S. cerevisiae*,[61] this finding might explain the improvement in the identification of these YCW intrinsic proteins upon Triton X-100 addition: the removal and reduction of signal suppressing contamination from abundant proteins, which are not located in YCW, encountered in YCW standard isolation. Besides, three additional CWPs were exclusively identified following 5% Triton X-100 addition. This group consists of two phospholipases Plb1 and Plb2 involved in fatty acid metabolism,[62] in addition to the Pst1 protein that functionally is redundant to the Ecm33 protein in damaged cell wall repair and integrity maintenance.[45]

Nonetheless, some identified CWPs in standard proteomics applied to YCW isolated by mechanical disruption without the addition of Triton X-100 were lost upon the addition of 5% Triton X-100, such as the Dse4 protein, Pst2 protein, the exo-1,3-β-glucanase Exg1, and the three repressible acid phosphatases (Pho5, Pho11, and Pho12) (Table 1). This loss of identification might be due to the fact that these proteins are secreted into the extracellular medium, thus their binding to the cell wall is weak and removable by the addition of a nonionic detergent.

All in all, the presented results provide evidence about the advantages of adding a detergent for solubilization of mitochondria as other membranous organelles, aiming to reduce undesirable protein contaminants from other cell compartments and yield a better enrichment in YCW for subsequent fractionation and extraction of mannoproteins, destined as a model for further structural studies.

CONCLUSIONS

In summary, this work presents the first detailed investigation of nearly pure YCW preparation from S288C whole yeast cells cultured in YPD medium through oxidative growth in a fed-batch regimen. By applying bottom-up proteomics to YCW isolated by mechanical disruption, we identified a considerable amount of non-YCW proteins, which were not previously reported by the application of the same method.[30] Although demonstrating the metabolic and replicative regimen of the cells during the fed-batch culture, their presence influences the characterization of CWPs. The high abundance of contaminants suppresses the detection of CWP peptides. Thus, this contamination of YCW preparation by mechanical disruption should be removed to improve CWP mapping. Purification steps seem to be required to yield a pure YCW preparation.

As a first means to further purify the standard YCW isolates, ultracentrifugation using a continuous density gradient of iodixanol was carried out. Despite the insignificant effect of ultracentrifugation in non-YCW protein contamination reduction, ultracentrifugation combined with proteomics allowed the identification of more CWPs, which were not detected in its absence.

Another strategy to "clean" YCW isolates from mitochondrial and other organelle proteins was the adaptation of the YCW mechanical disruption method, through the addition of the Triton X-100 detergent reported to be efficient in mitochondrial lysis. The choice of Triton X-100 among a myriad of detergents was supported by a previous work

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claiming its superior efficiency in mitochondrial lysis. Different concentrations of Triton X-100 were assayed. An optimal efficiency in the reduction of non-CWPs' number and abundance, especially mitochondrial proteins, accompanied by the significant qualitative and quantitative enrichment in CWPs, was obtained with 5% Triton X-100 addition. This concentration is a bit higher than that usually used to solubilize native proteins.[57,58] The optimal concentration of added Triton X-100 to fully preserve the activity of the YCW proteins deserves to be thoroughly assessed. This simple adaptation can be so useful for the production of the prototype analytical preparation of YCW. This kind of preparation can serve as an enriched raw material for further fractionation and extraction yielding mannoproteins. The latter can be used as a control or model for structural studies. An upscale of this preparation procedure for industrial production could also be envisaged, taking into account the use of another biocompatible, bioderived, and green detergent. In this regard, different classes of microbial biosurfactants can be tested. These are generally secondary metabolites, such as lipopeptides, glycopeptides, glycolipids, and glycolipopeptides, produced by a wide variety of microorganisms including bacteria, yeast, and fungi.[63]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c02176.

Proteomics data comprising raw and result files for all of the YCW isolates have been deposited to the ProteomeXchange consortium via the PRIDE[64] partner repository with the dataset identifier PXD032386 (PDF)

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Author Contributions

The manuscript was written through contributions of all authors and all authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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