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Identification of Proteins Involved in pH Adaptation in Extremophile Yeast *Yarrowia lipolytica*

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1. Introduction

Extremophile yeast *Yarrowia* is now commonly acknowledged as a prospective industrial microorganism and a highly promising cell model. This is due to several unique properties of this organism. First, it is able to grow rapidly on a broad range of organic substrates including waste water, oil paraffin and non-natural substances. This property, due to the presence of peroxisomes, allows the application of *Y. lipolytica* for waste management, water and soil bioremediation and for conversion of fossil organic compounds and pollutants to feed ingredients. Second, due to a high metabolic activity and resistance to chemical stresses, *Y. lipolytica* is able to produce aggressive organic compounds (e.g. succinate) at high yields. Third, *Y. lipolytica* provides a well-established model of a dimorphic transition between a yeast-like state and a mycelium forming fungi. This property enables the application of *Y. lipolytica* as a model for drug discovery for therapeutic control of *Candida albicans* (the most common fungal pathogen in humans) and other pathogenic fungi. In contrast to *C.albicans*, *Y.lipolytica* is easily cultivated and has a complete sexual cycle. Thus, genetic mating analyses are readily applicable. Dimorphic transition in *Y. lipolytica* is also considered as the simplest model of cell differentiation in eukaryotes. Taken together, these factors provided the incentive for complete sequencing of the *Y. lipolytica* genome. This has been carried out by GenoLevures Consortium in France (Dujon et al, 2004). Availability of the complete genomic sequence opened access to proteomic assays to be combined with functional studies of *Y. lipolytica*. The proteomic approach has been successfully used by Morin et al (2007) for the identification of major proteins involved in the dimorphic transition of *Y. lipolytica*.

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Beyond the abovementioned properties, *Y. lipolytica* remains the only known ascomycetes yeast readily growing on alkaline media and in the presence of salts at near-saturating concentrations. These phenomena have been studied by both genetic and biophysical methods. Genetic and molecular biology data implicated the involvement of Rim101- and calcineurine-dependent signal pathways in the high pH adaptation (Lambert et al, 1997). Biophysical data by Zvyagilskaya et al (2000) demonstrated the exchange of proton-dependent machineries involved in metabolite symport (e.g. phosphate ion) through the plasma membrane as a mechanism for Na^+-dependent adaptability. Rim101- and calcineurine-dependent regulatory pathways as well as the proton/Na^+ symport switch are ubiquitous in all studied yeast including *Saccharomyces cerevisiae*. Under normal conditions, these mechanisms usually provide a launch of emergency responses to stress, allowing only a short-term survival of the cells under the alkaline / high salt conditions. In contrast, *Y. lipolytica* permanently grows on media with a pH up to 10. On the other hand, similar to other ascomycetes, *Y. lipolytica* is considered to prefer an acidic pH media. Many strains of this species demonstrate an exclusive resistance to low pH (Yuzbashev et al, 2010). Taken together, these data show that the ambivalent pH adaptation molecular mechanisms in *Y. lipolytica* coupled to an extreme halotolerance, remains obscure. Their discovery may significantly contribute to practical applicability of *Y. lipolytica.*

2. Research objectives

Taking into account the availability of a complete genomic sequence, we aimed to apply proteomics technique for the identification of *Y. lipolytica* proteins whose occurrence depends on pH medium and apparently contributes to global mechanisms of pH adaptation.

3. Methods

3.1 Yeast strain and culture conditions

Y. lipolytica strain PO1f (MatA, leu2-270, ura3-302, xpr2-322, axp-2) was purchased from CIRM-Levures collection (France) where it was deposited under accession number CLIB-724. The strain differs from the wild type *Y. lipolytica* by auxotrophy towards Leu and Ura and by an ability to grow on sucrose. Y. lipolytica basic strain was maintained on solid media of the following composition (g/l): yeast extract – 2.5; bactopeptone – 5.0; glycerol – 15.0; malt-extract– 3.0; agar – 20.0; pH 4.0-4.2 or 8.9-9.0. Liquid nutrient broths were prepared as follows (g/l): - $MgSO_4 \times 7H_2O$ - 0.5; NaCl - 0.1; $CaCl_2$ - 0.05; KH_2PO_4 - 2; $K_2HPO_4 \times 3H_2O$ – 0,5; $(NH_4)_2SO_4$ - 0.3; Ca pantotenate - 0.4; inositol - 2.0; nicotinic acid - 0.4; n-amino benzoic - 0.2; pyridoxine -0.4; riboflavin - 0.2; thiamine - 0.1; biotin - 0.002; folic acid - 0.002; H_3BO_4 -0.5; $CuSO_4 \times 5H_2O$ -0.04; KI - 0.1; $FeCl_3 \times 6H_2O$ - 0.2; $MnSO_4 \times H_2O$ - 0.4; $NaMoO_4 \times 2H_2O$ - 0.2; $ZnSO_4$ - 0.4; pH – 4.0-4.2 or 8.9-9.0, yeast extract "Difco" - 2.0. 1% glycerol was used as a principal carbon and energy supply. pH was controlled permanently during cultivation.

3.2 Cell extract preparation

Cell cultures (24 h) were used for proteomic studies (average A_{590} =7.5-8.0). The biomass was harvested by centrifugation at 4000g for 10 min. The cells were washed twice with ice-cold deionized water and eventually pelleted.

To prepare protein extracts, 100 mg of the cell pellet was transferred to a vial containing 2ml lysis buffer (9M urea, 5% β-mercaptoethanol, 2% Triton X-100, and 2% ampholytes, pH 3.5-10 (Sigma, USA)) and thoroughly suspended. The sample was either immediately heated in a boiling bath for 3-5 min or placed on ice and sonicated in an ultrasonic desintegrator (MSE-Pharmacia) for 2 min (4 cycles 30 sec each). In both cases the homogenate was clarified by centrifugation in a microfuge for 20 min at maximum speed. The pellet was discarded and 100 µl of the clear supernatant was used for isoelectrofocusing (IEF).

3.3 Two-dimensional gel electrophoresis (2DE)

The first dimension separation employed IEF in glass tubes (2.4 × 180mm) filled with 4% polyacrylamide gel prepared with 9M urea, 2% Triton X-100 and 2% ampholyte mixture. Ampholytes of 5-7 and 3.5-10 pH ranges mixed at 4:1 ratio were used in all experiments. The protein extracts (100µl) were applied at the acidic end of the gel, and IEF was carried out using a Model 175 electrophoretic cell (Bio-Rad, USA) until 2400 V/h was achieved. The polyacrylamide gel columns with protein samples separated by IEF were applied as a starting point for separation in the second dimension, for which slab electrophoresis in polyacrylamide gel (200 × 200 × 1 mm) was used with a linear 7.5-20% gradient of acrylamide in the presence of 0.1% SDS using a vertical electrophoretic cell (Helicon Company, Russia). A well was created for protein marker application at the edge of each gel slab. Further details of the modified 2DE approach are described earlier (Kovalyova et al, 1994; Laptev et al, 1995; Kovalyov et al, 1995).

For protein visualization, the polyacrylamide gel slabs were stained with Coomassie Blue R-250 and then with silver nitrate according to the well-described methods (Blum et al, 1987) and modified by the addition of 0.8% acetic acid to sodium thiosulfate. The stained gels were documented by scanning on an Epson Expression 1680 scanner, and densitometry was carried out using the Melanie software (GeneBio, Switzerland) according to the manufacturer's protocol.

Molecular masses (*M*) of the fractionated proteins were determined by their electrophoretic mobility in the second dimension as compared to protein markers from standard heart muscle lysates (Kovalyova et al, 1994). The results of the mass determinations were verified by a calibration curve plotted using a marker kit (MBI Fermentas, Lithuania) with *M* ranging 10-200kDa. Isoelectric points (p*I*) of fractionated proteins were determined from their electrophoretic location in the first dimension, as described earlier (Kovalyova et al, 1994; Laptev et al, 1995), taking into account the known localization of identified reference proteins. Theoretical values of *M* were also taken from the Swiss-Prot database taking into account evidence for posttranslational processing of signal sequences (when available).

3.4 Protein identification by mass spectrometry

Isolation of protein fractions from polyacrylamide gel slabs, hydrolysis with trypsin, and peptide extraction for protein identification by matrix assisted laser desorption/ionization time of flight mass-spectrometry (MALDI-TOF MS) were carried out according to published protocols (Shevchenko et al, 1996) with some modifications (Govorun et al, 2003). A sample (0.5 µl) was mixed on the target with equal volume of 20% acetonitrile containing 0.1% trifluoroacetic acid and 20 mg/ml of 2,5-dihydroxybenzoic acid (Sigma-Aldrich, USA) and air dried. Mass spectra were recorded on a Reflex III MALDI-TOF mass spectrometer (Bruker Daltonics, USA) equipped with a UV-laser (336nm) in the positive mode with masses ranging

from 500-8000Da. The mass spectra were internally calibrated using trypsin autolysis products. The proteins were identified with Mascot software (Matrix Science, USA) using databases of the US National Center of Biotechnological Information (ncbi.nhm.nih.gov).
The NCBI database was searched within a mass tolerance of ±70 ppm for the appropriate species proteins; with one missed cleavage allowed. Protein score > 84 are significant ($p<0.05$). Carbamidomethylation ion of a cysteine residue and the oxidation of methionine are considered modification. Proteins were evaluated by considering the number of matched tryptic peptides, the percentage coverage of the entire protein sequence, the apparent MW, and the pI of the protein.

4. Results

4.1 Equalizing culture growth conditions

Previously we reported data about pH adaptation of *Y. lipolytica* carried out in minimal synthetic medium with succinate as the single source of carbon and energy (Guseva et al, 2010). However, elucidation of principles enabling *Y. lipolytica* to survive under strong alkaline conditions requires discrimination of partial physiological reactions of certain media components. This is possible only if several media pairs (each with acidic and alkaline pH) are compared. Therefore, we aimed to reproduce the experiments in a complete liquid medium containing 2% yeast extract and 1% glycerol. It was prepared in three versions with pH 4.0, 5.5 and 9.0. Growth curves were plotted using A_{600} as a criterion (Fig. 1). The inoculums for each culture were produced on a solid medium using the same pH as the main experiment. Inoculation dosage was $\approx 10^4$ cells per ml.
Surprisingly, retardation of *Y. lipolytica* growth at pH 4.0 and 5.5 versus pH 9.0 was found during periods of 1-20 h after inoculation. During periods of 20-24 h A_{600} as well as cfu contents, determined by microbiological method, were the same in all three cases. Consequently, only 24 h old cultures were subjected to further proteomic studies.

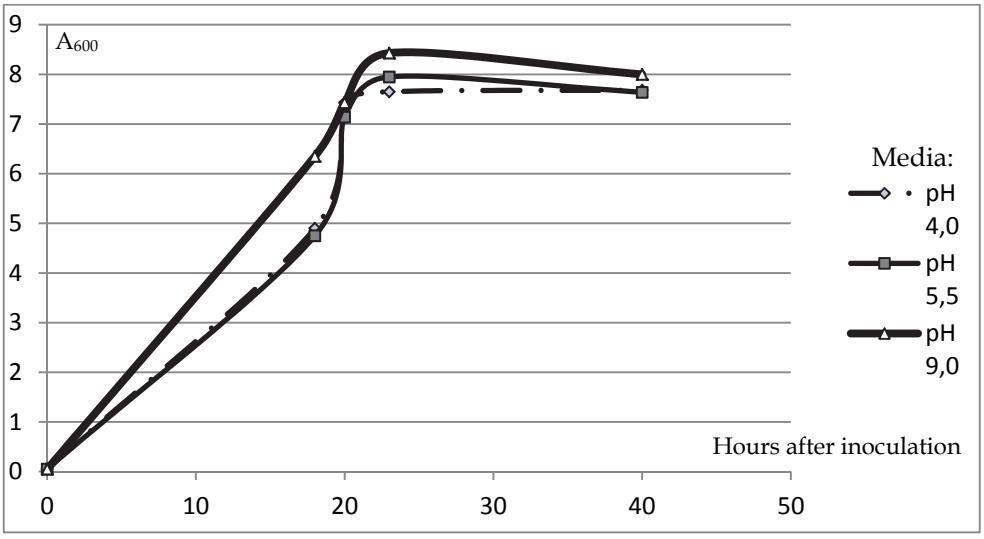

Fig. 1. Growth curves of *Y. lipolytica* at rich media with different pH's.

4.2 Analysing morphological differences of *Y. lipolytica* culture by microscopy

Measuring A_{600} of the culture is a precise and simple qualitative technique. However, it does not allow the visualization of putative morphological cell changes under different pH conditions. These changes may compromise the accuracy of A_{600} data conversion to cell number.

In order to track morphological changes in *Y. lipolytica* cells in liquid media at pH 4.0 and 9.0 cultures were subjected to visual phase-contrast microscopy (100^{x} magnification) with no fixation. The data (Fig. 2) demonstrate that average cell volume was 2-4 times larger in the culture at pH 4.0 when compared to pH 9.0. The cells grown in alkaline media contained massive vacuoles occupying most cell volume.

Taken together, these observations lead to conclusion that the volume of the cytoplasm relative to the total volume of the cells is much reduced when growing under alkaline conditions. One could also presume that the ratio between proteins in the cytoplasm and intracellular membrane compartments (vacuoles, mitochondria, Golgi apparatus) may also be altered (Brett & Merz, 2008).

4.3 Preparing *Y. lipolytica* protein extracts

Accurate pair-wise comparison of proteomes requires thorough equalizing and normalizing of source biological material. Massive and tightly cross-linked polysaccharide cell walls are a specific attribute of all yeast species including *Y. lipolytica*. It protects the cells from rapid changes in environmental conditions but also substantially hinders experimental processing of yeast samples (Dagley et al, 2011). This problem is commonly addressed in transcriptomic studies, but proteomic research also requires optimal extraction procedures. Fortunately, even mechanically durable cell walls are susceptible to mechanical crushing (ultrasonic treatment, French-press, glass beads) but such procedures take time. In the course of mechanical homogenization, intracellular lysosomes are broken, and thus incapsulated cathepsins come in contact with cytoplasmic proteins. Taken together these issues may result in the degradation of proteins that intend to be subjected to further analysis. On the other hand, many membrane and cell-wall associated proteins are poorly extracted by water or buffers. Moreover, detergent treatment does not always provide an exhaustive extraction technique. Heavily glycosylated proteins located in ER, Golgi apparatus and in the cell wall are often excluded by such processes (Morelle et al, 2009; Pascal et al, 2006).

These two problems substantially preclude complete characterization of the yeast proteome and may compromise validity of the obtained data. Thus far, only a single report has undertaken a proteomic study of *Y. lipolytica* (Morin et al, 2007). These authors analyzed proteins from water soluble cell fractions produced by mechanical disintegration and the subsequent removal of the insoluble fraction by centrifugation. Hence, the membrane, cell-wall and cytoskeleton associated proteins were excluded from consideration. Taking into account presumed contribution of membrane transport machinery to pH adaptation in *Y. lipolytica* (Zvyagilskaya et al, 2000) a complete proteome assay seemed to be more relevant to our research objectives.

To address this problem, we proposed two modifications of a chemical lysis method adapted from the preparation of human muscle tissue (Kovalyova et al, 2009). The first modification (Fig. 3, 4 and 5) included the instant resuspension of the yeast cells in a hot lysis buffer containing urea, reducing agent, Triton X-100 and ampholytes. The second included a preliminary ultrasonic treatment of the cells suspended in the same buffer on ice

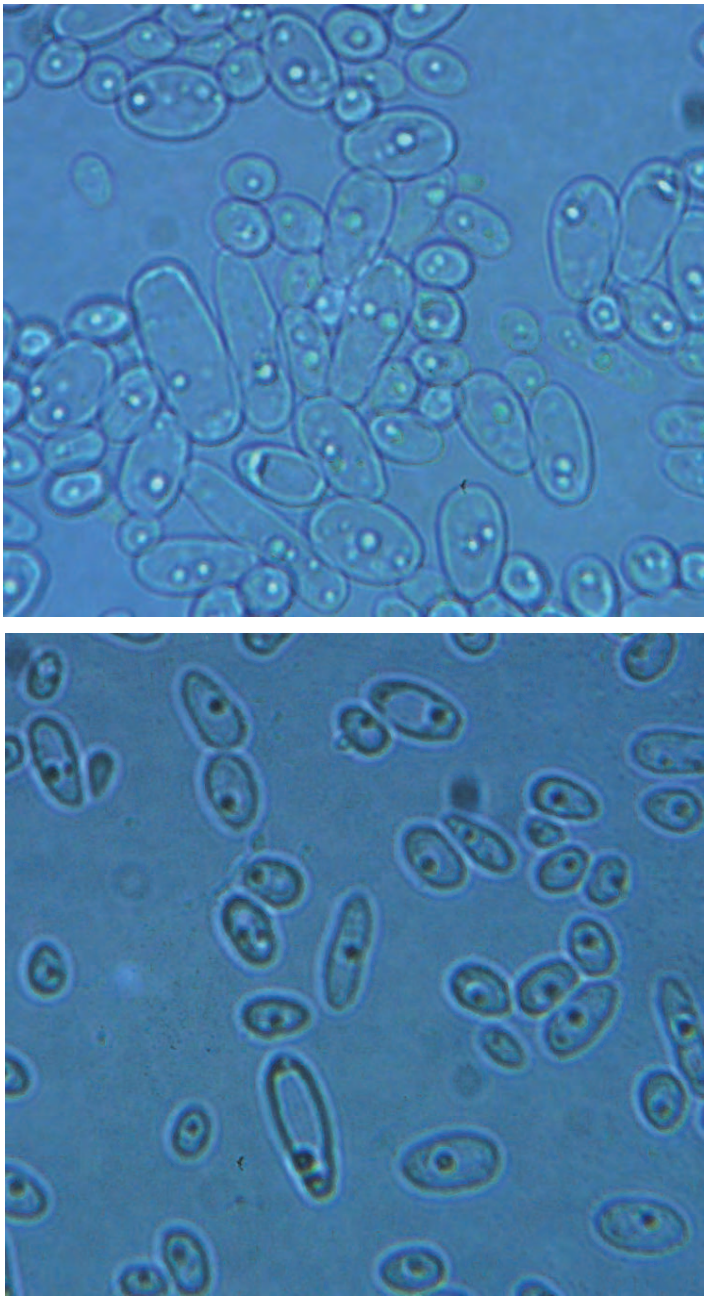

Fig. 2. Y. lipolytica cells cultured in growth media under acidic (A; pH 4.0) and alkaline (B; pH 9.0) conditions (growth time 24 h). Images from an optical microscope with 100x magnification.

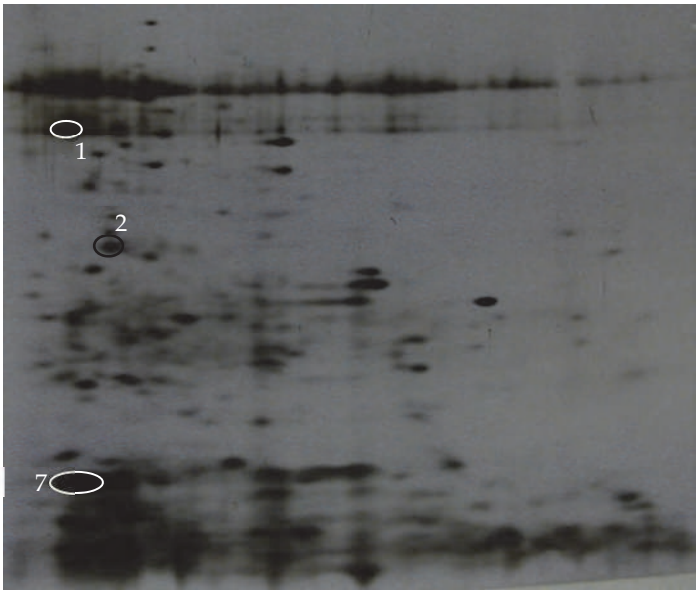

Fig. 3. 2D electophoregarm of *Y. lipolytica* proteome cultured on pH 4.0 medium (double silver/Coomassie R-250 staining). The cells were lysed in the denaturing buffer without mechanical disintegration. MALDI-TOF MS analysis of the spots specific for this specimen (not found in Fig. 4 or 5).

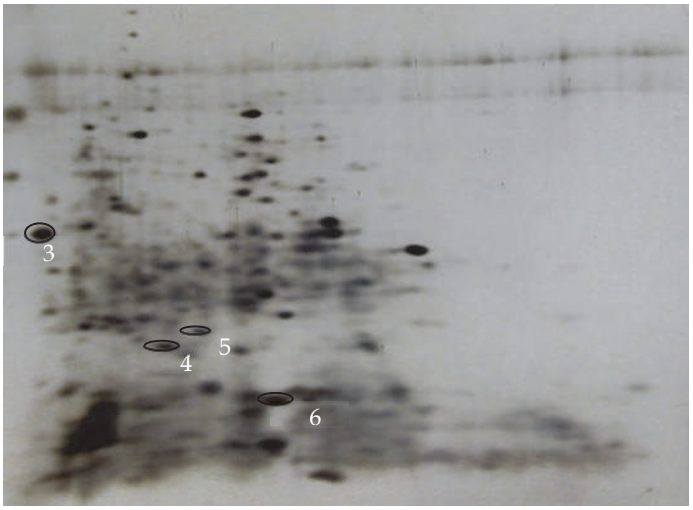

Fig. 4. 2D electophoregarm of *Y. lipolytica* proteome cultured on pH 5.5 medium (double silver/Coomassie R-250 staining). The cells were lysed in the denaturing buffer without mechanical disintegration. MALDI-TOF MS analysis of the spots specific for this specimen (not found on Fig. 3 and 5).

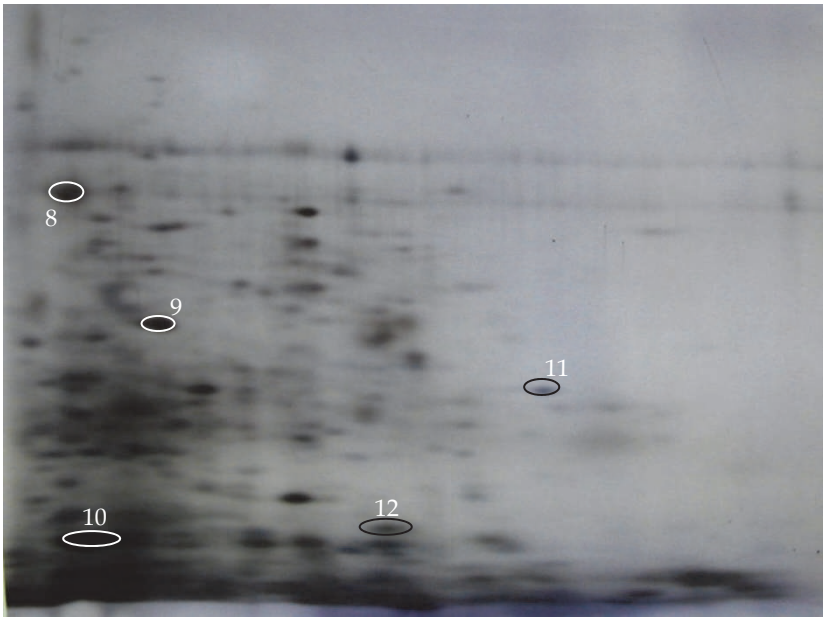

Fig. 5. 2D electophoregarm of *Y. lipolytica* proteome cultured on pH 9.0 medium (double silver/Coomassie R-250 staining). The cells were lysed in the denaturing buffer without mechanical disintegration. MALDI-TOF MS analysis of the spots specific for this specimen (not found on Fig. 3 and 4).

(Fig. 6 and 7). The volume ratio between cell pellet and the lysis buffer must be about 1:20. The cells must be placed into a vial containing the buffer to provide instant resuspension of the sample. After homogenization, the non-soluble pellet containing polysaccharides must be discarded by an intensive centrifugation step to avoid clogging of IEF tubes.
Both methods resulted in gels that produced ≈1000 individual spots, compared to other tested methods which rendered <100 spots (data not shown). However, the overall spot pattern obtained by two methods from the same biological material was significantly different (compare Fig. 3 to Fig. 6 and Fig. 5 to Fig. 7). Moreover, the quality of the protein extract produced under alkaline conditions was always less than in samples produced under acidic conditions. However, the results were highly reproducible for the same method even when applied to independently cultured material.

4.4 Studies of *Y. lipolytica* protein extracts by 2DE and MALDI-TOF MS

A total of 5 types of extracts were analyzed. Three samples were produced using hot buffer extraction from whole cells (the cultures were produced in media at pH 4.0, 5.5 and 9.0). Two samples were obtained from the cells subjected to ultrasonic disintegration directly in the ice-cold lysis buffer (the cultures were produced in media at pH 4.0 and 9.0). The unique spots specific for each sample were identified by comparison with the samples obtained by the same technique. Only intense spots corresponding to abundant cell proteins were analyzed by MALDI-TOF MS. Although cultures produced at pH 4.0 and 5.5 were analyzed separately, we suggest that differences between them must be considered as the "base-line

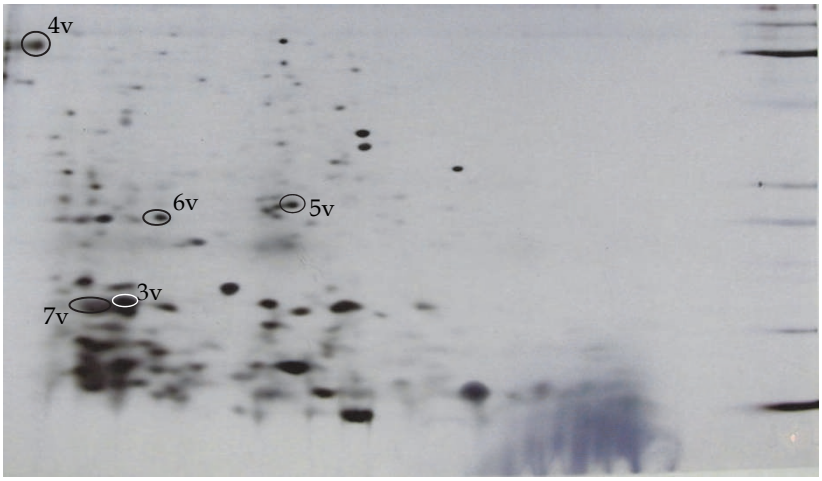

Fig. 6. 2D electophoregarm of *Y. lipolytica* proteome cultured on pH 4.0 medium (double silver/Coomassie R-250 staining). The cells were homogenized by ultrasonic treatment with subsequent denaturing buffer without mechanical disintegration. MALDI-TOF MS analysis of the spots specific for this specimen (not found on Fig. 7).

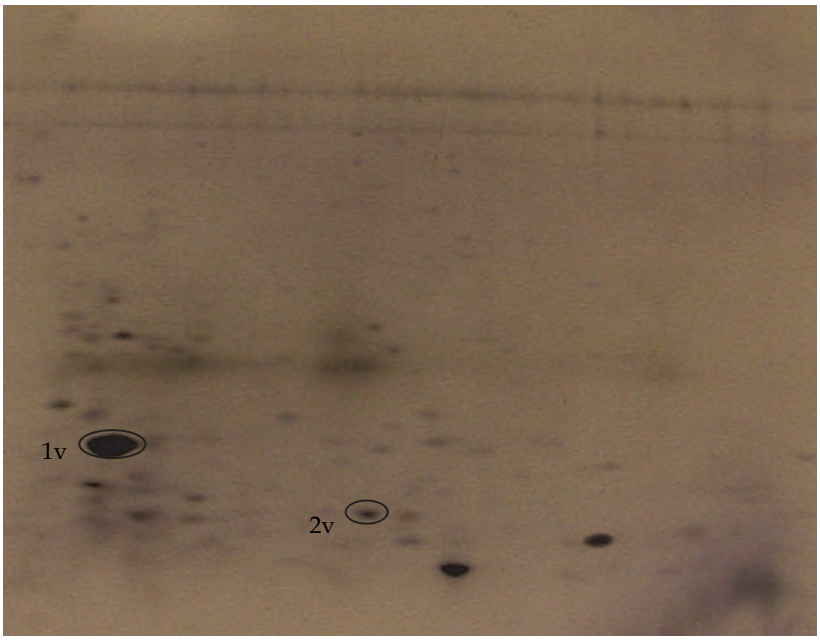

Fig. 7. 2D electophoregarm of *Y. lipolytica* proteome cultured on pH 9.0 medium (double silver/Coomassie R-250 staining). The cells were homogenized by ultrasonic treatment with subsequent denaturing buffer without mechanical disintegration. MALDI-TOF MS analysis of the spots specific for this specimen (not found on Fig. 6).

fluctuation" since both pH ranges are considerably below the pH of the cytoplasm. Comparison within this pair may allow an estimation of the reproducibility of the employed techniques e.g. as described by (Huang et al, 2011).

The data shown demonstrates that many selected spots from the 2D electophoregrams were not able to be identified by MALDI-TOF MS analysis (Table 1). Consequently, only two

Code	Exp. Mr kDa	YL protein identified by Mascot	Mascot Score	Calc. Mr Da	Homologue with known function
1	72	Invalid data			
2	48	Invalid data			
7	12	Invalid data			
3	39	YALI0B03564p	106	34031	P43070 C. albicans Glucan 1,3- □-glucosidase precursor (EC 3.2.1.58) (Exo-1-3-β- glucanase)
4	23	YALI0B15125p	247	21311	P34760 S. cerevisiae YML028w TSA1 thiol-specific antioxidant
5	25	Invalid data			
6	13	YALI0F09229p	99	17031	P36010 S. cerevisiae YKL067w YNK1 nucleoside diphosphate kinase
8	75	Invalid data			
9	38	Invalid data			
10	11	Invalid data			
11	29	YALI0F17314p	163	29514	P04840 S. cerevisiae YNL055c POR1 mitochondrial outer membrane porin
3v	13	YALI0E19723p	95	17290	P04037 S. cerevisiae YGL187c COX4 cytochrome-c oxidase chain IV
5v	24	YALI0F05214p	151	26679	P00942 S. cerevisiae YDR050c TPI1 triose-phosphate isomerase singleton
6v	21	YALI0B03366p	97	20957	P14306 S. cerevisiae YLR178C carboxypeptidase Y inhibitor (CPY inhibitor) (Ic)(DKA1/NSP1/TFS1)
7v	12	Invalid data			
1v	72	Invalid data			
2v	12	YALI0D20526p	106	13681	P22943 S. cerevisiae YFL014W 12 kDa heat shock protein (Glucose and lipid-regulated protein)

Table 1. 2DE protein spots subjected to identification by MALDI-TOF MS

clearly alkaline-inducible proteins were identified. The most prominent candidate proteins exhibiting great pH-inducibility and high overall expression levels (e.g. 1v, 8, 9 and 10) could not be identified. A higher proportion of spots were successfully identified from the samples originating from pH 5.5 medium compared to the samples from pH 4.0 medium. Furthermore, gel resolution and total number of resolved spots also increased under pH 5.5 conditions. This could be explained by the observation that the share of cytoplasm proteins in the total cell volume is proportionally higher under optimal conditions (pH 5.5) and decreases under acidic or alkaline stress in favor of the membrane compartments (vacuoles, mitochondria, ER, Golgi apparatus) (see Fig. 2). This idea is supported by observation that 6 out of 8 proteins represented in Table 1 are "pH-reactive" and are allocated to non-cytoplasm compartments. It is also in a good agreement with numerous communications about involvement of ER and mitochondria to anti-stress adaptation of organisms from all kingdoms (Hoepflinger et al 2011; Rodriguez-Colman et al, 2010). Reactive oxygen species (ROS) formation accompanies all responses to stresses and cross-talk between ER and mitochondria contributes to abatement of damage caused by uncontrolled oxidation (Bravo et al, 2011; Tikunov et al, 2010).

4.5 Functions and genomic organisation of the genes encoding potential "pH-reactive proteins" in *Y. lipolytica*

In order to systematically assess properties of the up-and down-regulated alkaline-sensitive proteins, we arranged the available functional data from Swiss-Prot records for each identified protein (Table 2).

Genomic localization of the pH-regulated proteins is not uniform. However, one can make an observation that no pH-reactive genes were found on chromosomes A or C.

The data demonstrate an important role of non-cytoplasmic cell compartments in the pH adaptation of *Y. lipolytica*. Only two proteins (4 and 5v) from the eight identified have annotated subcellular locations corresponding to the cytoplasm. While it is possible that adaptation to the acidic and alkaline pH depends on these polypeptide structures, one must take into account that many potentially important pH-reactive proteins failed to be identified. Therefore, we cannot conclude that all major pH-reactive proteins were found. It is worth noting that this and other studies (Guseva et al, 2010) have failed to identify plasma membrane components (ATPase subunits and pumps) responsible for direct ion exchange between the cytoplasm and the environment.

A comparison of this study with pH-reactive proteins identified previously (Guseva et al, 2010) in *Y. lipolytica* cultivated on a minimal medium with succinate was undertaken. Two proteins YALI0F17314p and YALI0B03366p were found in both cases. YALI0F17314p (outer membrane mitochondrial porin, VDAC) was the only alkaline-inducible protein found in both cases. In contrast, YALI0B03366p (carboxypeptidase Y inhibitor, a lysosomal component) was found to be an alkaline-inducible on minimal medium with succinate and alkaline-repressible in complete medium with glycerol (present study). This comparison leads to the conclusion that the outer membrane mitochondrial porin is possibly an essential part of *Y. lipolytica* pH-adaptation machinery, independent of the utilized nutrient source.

Another identified alkaline-inducible component of *Y. lipolytica*, Hsp12 is an intrinsically unstructured stress protein that folds upon membrane association and modulates membrane function (Welker et al, 2010). Hsp12 of *S. cerevisiae* is upregulated several 100-fold in response to stress. Our phenotypic analysis showed that this protein is important for survival under a variety of stress conditions, including high temperature. In the absence of

a. Alkaline-inducible proteins

Code	YL Swiss-Prot acc. #	Function	Protein cell localization	Gene (Gene Bank acc. Number)	Chromosomal localization
11	YALI0F17314p	POR1 mitochondrial outer membrane porin	Outer mebrane of mitochondria	gi|50556244	F (2311796-2313207)
2v	YALI0D20526p	12 kDa heat shock protein	Cytoplasm/internal membrans	gi|50551205	D (2604298-2604907)

b. Alkaline-repressible proteins

Code	YL Swiss-Prot acc. #	Function	Protein cell localization	Gene (Gene Bank acc. Number)	Chromosomal localization
3	YALI0B03564p	Glucan 1,3- beta - glucosidase precursor	Golgi appartus	gi|50545854	B (498811-499752)
4	YALI0B15125p	Peroxiredoxin (PRX) family, Typical 2-Cys PRX subfamily	Cytoplasm	gi|50546891	B (2015063-2015653)
6	YALI0F09229p	nucleoside diphosphate kinase	Mitochondria matrix	gi|50555578	F (1287080-1287730)
3v	YALI0E19723p	COX4 cytochrome-c oxidase chain IV	Inner membrane of mitochondria	gi|50553496	E (2354436-2354924)
5v	YALI0F05214p	TPI1 triose-phosphate isomerase singleton (glycolysis)	Cytoplasm	gi|50555229	F (783915-784734)
6v	YALI0B03366p	carboxypeptidase Y inhibitor	Vacuole	gi|50545840	B (481138-482256)

Table 2. Functions and genomic organisation of the genes encoding potential "pH-reactive proteins" in *Y. lipolytica*

Hsp12, we observed changes in cell morphology under stress conditions. Surprisingly, in the cell, Hsp12 exists both as a soluble cytosolic protein and associated with the plasma membrane. The *in vitro* analysis revealed that Hsp12, unlike all other Hsps studied so far, is completely unfolded; however, in the presence of certain lipids, it adopts a helical structure.

The presence of Hsp12 does not alter the overall lipid composition of the plasma membrane but increases membrane stability (Welker et al, 2010). This information allows us to hypothesize that the biological function of Hsp12 is in rearranging and repairing membrane compartments under the stress conditions. This point of view is in perfect agreement with observations about the key role of the inner membrane compartments in the alkaline adaptation in *Y. lipolytica*. Unfortunately the involvement of this protein in many types of stress responses may result in data concerning its expression pattern poorly reproducible.

5. Conclusion

A new yeast cell extraction procedure enabled the resolution of more than 1000 individual protein spots of *Y. lipolytica* samples for each gel. This is ~2-fold more than in outlined by previous studies (Morin et al, 2007) where water soluble cell fractions were analyzed. In total, two proteins were up-regulated at pH 9.0, the mitochondrial outer membrane porin (VDAC) and 12 kDa heat shock protein.

These data complement the conclusions by Morin et al (2007) who emphasized the occurrence of energy metabolism proteins within the proteome portion as up-regulated in *Y. lipolytica* hyphae during the dimorphic transition. Similar conclusions were reported for stress adaptation in *S. cerevisiae* (Martínez-Pastor et al, 2010; Rodriguez-Colman et al, 2010) and *Candida albicans* (Dagley et al, 2011). VDAC is not only responsible for protein import into mitochondria but essentially contributes to antioxidant resistance of mitochondria (Tikunov et al, 2010).

To the best of our knowledge, we provide the first report about the application of proteomic techniques to address the problem of *Y. lipolytica* adaptation to growth in alkaline conditions. In contrast to the previously hypothesized involvement of plasma membrane transporters and global transcription regulators (e.g. Rim101) in high pH adaptation, our study elucidated a key role for mitochondrial proteins and represents a new result for *Y. lipolytica*. On the other hand, this observation is in a good agreement with reports concerning the pivotal role of non-cytoplasmic compartments in stress adaptation in other biological systems e.g. yeast and plants (Bravo et al, 2011; Hoepflinger et al 2011; Rodriguez-Colman et al, 2010).

In our opinion, this work exemplifies a prompt and inexpensive study which could be easily undertaken for any physiological experiment with the organisms whose genome has been recently sequenced. Finally, it should be noted that a total cell proteome assay is strongly recommended for this kind of study, although some effort to determine an appropriate lysis buffer for protein extraction must be undertaken.

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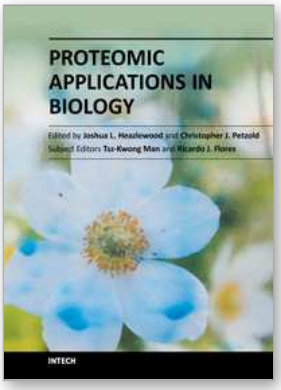

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The past decade has seen the field of proteomics expand from a highly technical endeavor to a widely utilized technique. The objective of this book is to highlight the ways in which proteomics is currently being employed to address issues in the biological sciences. Although there have been significant advances in techniques involving the utilization of proteomics in biology, fundamental approaches involving basic sample visualization and protein identification still represent the principle techniques used by the vast majority of researchers to solve problems in biology. The work presented in this book extends from overviews of proteomics in specific biological subject areas to novel studies that have employed a proteomics-based approach. Collectively they demonstrate the power of established and developing proteomic techniques to characterize complex biological systems.

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