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Effect of different glucose concentrations on proteome of *Saccharomyces cerevisiae*

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ABSTRACT

We performed a proteomic study to understand how *Saccharomyces cerevisiae* adapts its metabolism during the exponential growth on three different concentrations of glucose; this information will be necessary to understand yeast carbon metabolism in different environments. We induced a natural diauxic shift by growing yeast cells in glucose restriction thus having a fast and complete glucose exhaustion. We noticed differential expressions of groups of proteins. Cells in high glucose have a decreased growth rate during the initial phase of fermentation; in glucose restriction and in high glucose we found an over-expression of a protein (Peroxiredoxin) involved in protection against oxidative stress insult. The information obtained in our study validates the application of a proteomic approach for the identification of the molecular bases of environmental variations such as fermentation in high glucose and during a naturally induced diauxic shift.

1. Introduction

Saccharomyces cerevisiae is a facultative anaerobe able to live on various fermentable and non-fermentable carbon sources. When yeast is grown on fermentable substrates such as glucose, the metabolic energy essentially originates from glycolysis. The Pasteur effect relates oxygen with sugar catabolism, hence glycolysis in resting cells proceeds more rapidly under anaerobic conditions than under aerobic conditions [1]. Fermentation is the predominant route of sugar metabolism in growing cultures. If the glucose concentration is high, the Pasteur effect is no longer operable and is replaced by the Crabtree effect, by which cells continue to ferment [2]. The Crabtree effect can be either a short-term or a long-term effect [3]. The short-term effect is characterized by its capability of triggering alcoholic fermentation upon a sudden glucose excess condition whereas the long-term effect is characterized by the respiratory fermentative metabolism observed in batch cultivation or in continuous culturing [4]. In *S. cerevisiae*, growth in rich media with low glucose concentration increases both replicative and chronological longevity [5,6]. The beneficial effects of glucose restriction in yeast are related to the increase in respiratory rates occurring when glucose levels in the media are low [7,8]. These enhanced respiratory rates increase intracellular NAD^+ levels which may be involved in the regulation of replicative lifespan. This modulates the activity of sir2 family proteins [9] and reduces the releasing of mitochondrial reactive oxygen species (ROS) [10]. ROS are normal by-products of cell metabolism; however, an increase in their production can cause oxidative modifications in cell macromolecules such as proteins, DNA and lipids. A great number of recent reports seem to confirm the Harman "free radical theory of ageing" [11] proposed some 50 years ago notwithstanding the many still remaining unknown details. In our previous study, a proteomic approach was used to evaluate the redox state of yeast protein cysteines during chronological ageing [12]. We demonstrated that glucose restriction and growth on glycerol supplemented media extend *S. cerevisiae* lifespan and that oxidative damage in cells grown on high glucose mostly affects glycolytic enzymes. Studying how yeast adapts to environmental changes is important, for the optimization of its industrial applications and also because knowledge gained during such experiments could help understand some important eukaryotic cells biological processes. Comprehensive transcriptome analyses have been performed to study the effects of different nutrient conditions on yeast [13–16]. These genome-wide expression profiles show how they respond to different nutritional environments at transcriptional level. However, increasing evidence shows that mRNA abundance is not always correlated with protein expression levels [17–21]. Therefore, it is essential to study yeast adaptation and other biological processes with proteome studies, that allow the analysis of all proteins present in a certain condition. Two-dimensional reference maps have been constructed for important industrial yeast strains [22–25]. Many quantitative 2D-gel-based studies analyzed yeast growth under different environmental conditions [26–28]. We performed a proteomic study to understand how *S. cerevisiae* adapts its metabolism during the exponential growth in medium supplemented with three different concentrations of glucose: 2%, 0.5% and 20%. A medium with 20% glucose concentration is very similar to natural must.

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Throughout alcoholic fermentation *S. cerevisiae* cells must cope with several stress conditions that could affect their growth and viability. Glucose *per se* is a powerful signaling molecule in yeast. When glucose concentration falls below 0.2%, cells stop dividing for a few hours. After this lag phase, cells start to consume ethanol (obtained from the former glucose catabolism) by respiration (natural diauxic shift) [29]. A yeast model of glucose restriction (0.5% glucose) was chosen in order to study the effect of glucose limitation on its proteome. The aim of this study was to analyze the proteome changes during growth under both high glucose concentration and glucose restriction in yeast. As a result, we noticed differential expressions of groups of proteins. Yeast grown in these conditions shows different growth rates and fermentative behaviors. The different expression patterns could be related to the stress caused by high glucose concentration and to the previously demonstrated beneficial effect of glucose restriction.

2. Materials and methods

2.1. Strains

The *S. cerevisiae* strain used in this study is wild-type strain W303-1A, MATa, ura3-52, trp1Δ2, leu2-3_112, his3-11, ade2-1, can1-100 (accession no. 20000A; EUROSCARF, Frankfurt a.M., Germany).

2.2. Growth conditions

Yeast cells were grown at 30 °C in synthetic complete (SC) medium containing 0.67% w/v of Yeast Nitrogen Base without Amino Acids (USbiological) supplemented with complete Amino Acid dropout solution (USbiological). 0.5% glucose, 2% glucose and 20% glucose were used as carbon sources. Cells were picked from fresh colonies and grown overnight in SC medium with 0.5%, 2% and 20% glucose. Cells were then diluted in their respective fresh media to an optical density at 600 nm of 0.2 O.D./ml using flasks with volume/medium ratio of 3:1. Growth was monitored by measuring the turbidity of the culture at 600 nm (OD600) on a spectrophotometer until they reached the cellular density of 0.8 O.D./ml.

2.3. Ethanol and glucose measurement

For ethanol and glucose determination, cells were grown in SC medium containing a fermentable carbon source (0.5% glucose, 2% glucose and 20% glucose) to an optical density (600 nm) of 0.8 O.D./ml. One milliliter of the growth was harvested at exponential phase and then centrifuged. The cleared supernatant was collected to estimate the ethanol production and the residual glucose. Ethanol was determined using the alcohol-dehydrogenase/aldehyde-dehydrogenase method (the assay was performed according to the "K-etoh" kit from Megazymes (Ireland)). Glucose was determined according to the Accu-Chek® Active Glucose (Roche Diagnostics) protocol.

2.4. Intracellular ROS evaluation

To evaluate the ROS production, cells were grown in SC medium supplemented with 0.5%, 2% and 20% glucose and were harvested when they reached the cellular density of 0.8 O.D./ml. One O.D. of cells was washed twice in 10 mM HEPES buffer, then resuspended in the same buffer and incubated at 30 °C in the dark for 2 h with dihydrorhodamine 123 (Molecular Probes) in order to highlight ROS production on a Leica TCS SP5 confocal microscope.

2.5. Sample preparation and 2D-GE

For two-dimensional experiments, cells were harvested during the exponential phase at a cellular density of 0.8 O.D./ml. Cells were lysed in RIPA buffer (50 mM Tris–HCl pH 7, 1% NP-40, 150 mM NaCl, 2 mM EGTA, 100 mM NaF) plus a cocktail of yeast protease inhibitors (Sigma) with glass beads in a Fastprep instrument (Savant). Protein extracts were clarified by centrifugation at 8000×*g* for 10 min. Proteins were precipitated following the chloroform/methanol protocol [30] and the pellet was resuspended in 8 M urea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1- propanesulfonate (CHAPS) and 20 mM dithiothreitol (DTT). For each experimental condition at least three samples were run in order to assess biological and analytical variation. Isoelectrofocusing (IEF) was carried out on nonlinear wide-range immobilized pH gradients (pH 3–10; 18 cm long IPG strips; GE Healthcare, Uppsala, Sweden) and achieved using the EttanTM IPGphorTM system (GE Healthcare, Uppsala, Sweden). MS-Preparative-run IPG-strips were rehydrated at 16 °C with 350 μg of proteins in 350 μl of lysis buffer and 0.2% carrier ampholyte for 1 h at 0 V and for 8 h at 30 V. The strips were then focused at 16 °C according to the following electrical conditions: 200 V for 1 h, from

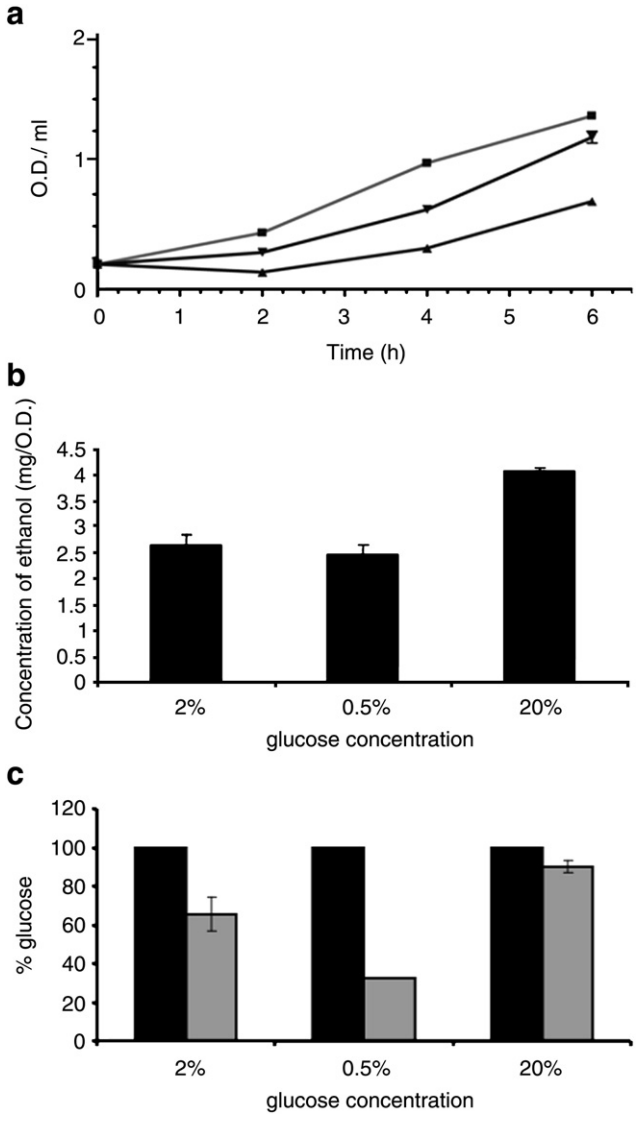

Fig. 1. (a) Aerobic exponential growth of W303 1A in SC medium supplemented with: ■, 2% glucose; ▲, 20% glucose; ▼, 0.5% glucose. Growth rate of cells was determined by O.D. measurements at 600 nm for the indicated times. (b) Ethanol concentration (mg/O.D.) was evaluated at the cellular density of 0.8 O.D./ml on SC plus 2%, 0.5% and 20% glucose. (c) Percentage of residual glucose evaluated at 0.8 O.D./ml on SC medium containing an initial glucose concentration of: 2% glucose; 0.5% glucose and 20% glucose. Black bars indicate the amount of initial glucose in each medium which is to be considered 100%. Gray bars represent the % of residual glucose in each medium. Data represent the mean and standard deviation obtained from three independent experiments.

Table 1

A. Total, residual and used glucose in SC medium supplemented with 2%, 0.5% and 20% glucose measured at the cellular density of 0.8 O.D./ml. Glucose is expressed in mg/O.D. and in percentage (relatives and absolute (in brackets)).

Glucose	2%		0.5%		20%	
	mg/O.D.	%	mg/O.D.	%	mg/O.D.	%
Total	22.7	100 (2.0)	5.3	100 (0.5)	217	100 (20.0)
Residual	14	65.4 (1.3)	1.8	32 (0.17)	195	90.1 (18.0)
Consumption	8	34.6 (0.7)	3.6	68 (0.33)	21.8	9.9 (2.0)

B. Correlation between ethanol production, expressed in mg/O.D. and in mol/O.D., and glucose consumption. The percentage represents the rate of glucose used for fermentation in comparison to total glucose consumption.

	Ethanol		Glucose	
	mg/O.D.	mol/O.D.	Consumption (mol/O.D.)	% used for ethanol production
2%	2.6	5.6×10^{-5}	4.4×10^{-5}	63.0
0.5%	2.4	5.2×10^{-5}	2.0×10^{-5}	100
20%	4.2	9.1×10^{-5}	12.1×10^{-5}	37.6

300 to 3500 V in 30 min, 3500 V for 3 h, from 3500 to 8000 V in 30 min, 8000 V until a total of 80,000 Vh was reached. After focusing MS-preparative IPG strips were equilibrated for 12 min in 6 M urea, 30% glycerol, 2% sodium dodecylsulfate (SDS), 0.05 M Tris–HCl, pH 6.8, 2% DTT, and subsequently for 5 min in the same urea/SDS/Tris buffer solution but substituting the 2% DTT with 2.5% iodoacetamide. The second dimension was carried out on 9–16% polyacrylamide linear gradient gels (18 cm × 20 cm × 1.5 mm) at 40 mA/gel constant current and 10 °C until the dye front reached the bottom of the gel. The MS-preparative gels were stained with colloidal Coomassie [31].

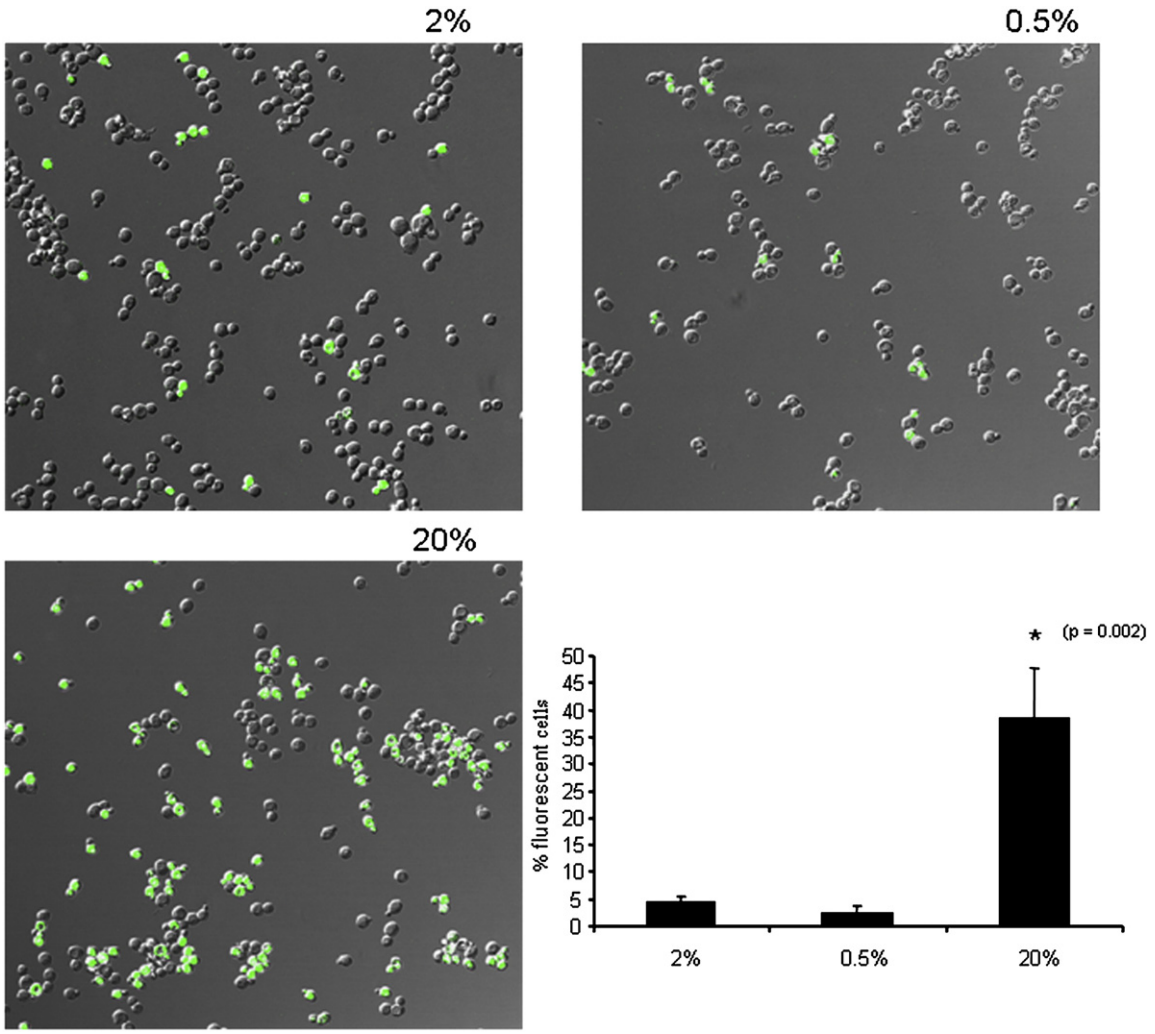

Fig. 2. ROS production, evaluated using the DHR123 fluorescent probe in cells grown exponentially (0.8 O.D./ml) on different glucose concentrations: figures represent a merge between DIC and fluorescent images obtained with a 40× objective. In the histogram, the percentage of fluorescent cells stained with DHR123 at the cellular density of 0.8 O.D./ml is shown. A two-tailed non-paired Student's *t*-test was performed using ORIGIN 6.0 (Microcal Software, Inc.) to determine if the relative change in the conditions of 0.5% and 20% glucose was statistically significant in comparison to the control cells. Differences were considered statistically significant when $p<0.01$ (*).

2.6. Image analysis and statistics

Gels were acquired with an Epson expression 1680 PRO scanner. For each strain condition, 2D gels were run in triplicate and only spots present in all the replicates were taken into consideration for subsequent analysis. Computer-aided 2D image analysis was carried out using ImageMaster 2-D Platinum software version 6.0 (GE Healthcare). Relative spot volume ($\%V = 100 \times V$ single spot$/V$ all spots, where V = integrated OD over the spot area) was used for quantitative analysis in order to reduce experimental errors. The normalized intensity of spots on three replicates of 2-D gels was averaged and standard deviation was calculated for each condition. A two-tailed non-paired Student's *t*-test was performed using ORIGIN 7.5 (Microcal Software, Inc.) to determine if the variations in relative spot volume were statistically significant.

2.7. In-gel trypsin digestion and MALDI-TOF Mass spectrometry

Protein spots were manually excised from the gel, washed with high-purity water and with 50% acetonitrile/water and dehydrated with 100% acetonitrile. The gel slices were swollen at room temperature in 20 μl of 40 mM NH_4HCO_3/10% acetonitrile containing 25 ng/μl trypsin (Trypsin Gold, mass spectrometry grade, Promega). After 1 h, 50 μl of 40 mM NH_4HCO_3/10% acetonitrile was added and digestion proceeded overnight at 37 °C. The generated peptides were than extracted with 50% acetonitrile/5% trifluoroacetic acid (TFA, 2 steps, 20 min each at room temperature), dried by vacuum centrifugation, suspended in 0.1% TFA, passed through micro ZipTip C18 pipette tips (Milllipore, Bedford, MA, U.S.A.) and directly eluted with the MS matrix solution (10 mg/ml α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/1% TFA). Mass spectra of the tryptic peptides were obtained using a Voyager-DE MALDI-ToF mass spectrometer (Applied Biosystems). Peptide mass fingerprinting database searching was performed using the MASCOT search engine (http://www.matrixscience.com) in NCBInr/Swiss-Prot databases. Parameters were set to allow one missed cleavage per peptide, a mass tolerance of 0.5 Da and considering carbamidomethylation of cysteines as a fixed modification and oxidation of methionines as a variable modification.

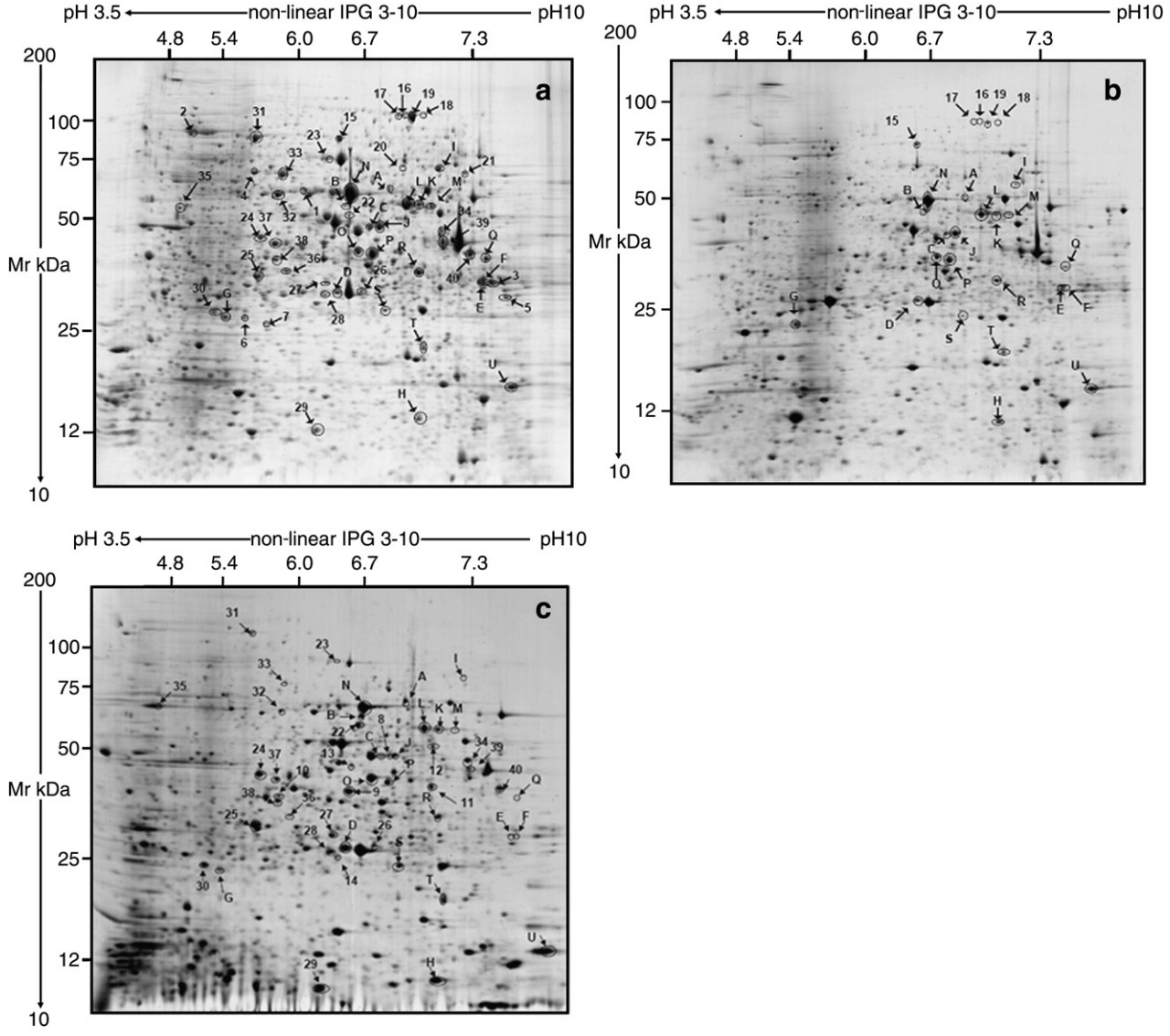

Fig. 3. Representative Blue Coomassie 2D gels of total protein extract from cells grown exponentially in 2% glucose (a), 0.5% glucose (b) and 20% glucose (c). Quantitative and qualitative variations are displayed with circles. Quantitative variations between cells grown in 0.5% glucose (panel b) and 20% glucose (panel c) vs. 2% glucose (panel a) are indicated by capital letters. They are listed in Table 2. Qualitative variations identified are displayed with numbers and represent conditions described in Sections 3.5. They are listed in Table 3.

3. Results and discussion

3.1. Growth and fermentation profile during aerobic cultivation

The W303-1A yeast strain was aerobically cultivated in SC medium supplemented with 2%, 0.5% and 20% glucose. The strain is Crabtree positive and to analyze its behavior, we first evaluated cell growth rate. The three different growth behaviors are shown in Fig. 1a. In 2% and 0.5% glucose the yeast enters the exponential phase more rapidly than in 20% glucose. We choose the standard condition of 2% glucose concentration as control. We found that cells grown in glucose restriction show a higher growth rate compared to cells grown in high glucose. After cellular acclimation the exponential growth begins. When the cultures reach a cellular density of 0.8 O.D./ml (i.e. 8×106 cells/ml) we evaluate cellular ethanol production and glucose consumption. We choose this particular cellular density because in yeast grown under glucose restriction, this point corresponds to the onset of the natural diauxic shift [32] (glucose concentration in the medium reaches 0.2%, see Table 1A). In this particular moment cells undergo a metabolic adaptation accompanied by a global reprogramming of gene expression (including mitochondrial biosynthesis) which precedes complete glucose exhaustion. The ethanol production in the three different amounts of glucose is shown in Fig. 1b. The ethanol concentration at 0.8 O.D./ml is: 2.6 mg/O.D. for 2% glucose; 2.4 mg/O.D for 0.5% glucose and 4.2 mg/O.D. for 20% glucose. Under high glucose concentration (20%) ethanol production is not as high as expected. Pham T. K. et al. [33,34] had previously reported that to minimize the osmotic stress caused by a high glucose concentration, *S. cerevisiae* accelerates its production of acidic compounds and glycerol rather than synthesize ethanol. The differences related to sugar consumption among the cultures are made evident in Fig. 1c: at 0.8 O.D./ml a low percentage of glucose consumption (9.9%) is observed in yeast grown in 20% glucose; at that cellular density these cells leave approximately 90% residual glucose. On the contrary, glucose consumption percentually increases in cells grown in glucose restriction: cells leave about 32% residual glucose in the medium. In Table 1 the differences in fermentative abilities are shown using two parameters: glucose consumption (A) and ethanol production (B). As is evident in Table 1A and Fig. 1c, the cells in glucose restriction are the ones to use the most glucose. In high glucose concentration ethanol is the major product but as shown in Table 1B and Fig. 1c, the percentage of glucose used for fermentation, in comparison to the total glucose consumption (9.9%), is only 37.6%.

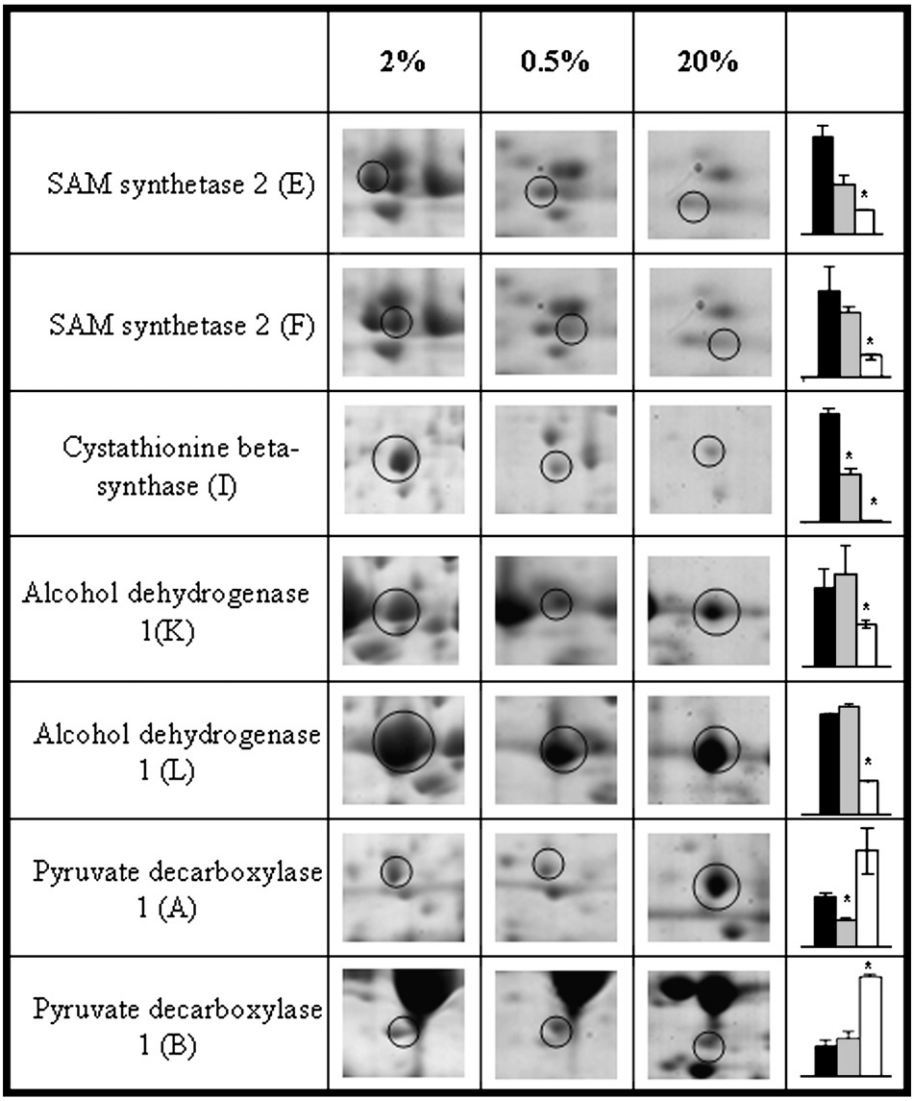

Fig. 4. Magnified regions of 2D gel images of spots corresponding to some identified proteins. Spots of interest are indicated with circles, with the corresponding protein names given on the left. In the column on the right the relative histograms are reported. Black bars, gray bars and white bars represent 2%, 0.5% and 20% glucose, respectively.

3.2. Evaluation of intracellular generation of free radicals

ROS production in the three different glucose concentrations (2%, 0.5% and 20%) was measured with dihydrorhodamine (DHR123) when the cells reach a cellular density of 0.8 O.D./ml [35,36]. Yeast cells grown in 0.5% and 2% glucose concentration show a relatively low and comparable ROS production. Oxidation of DHR123 results considerably higher in yeast grown in 20% glucose. Fig. 2 shows that approximately 50% of cells grown in 20% glucose are stained with fluorescent Rodamine at 0.8 O.D./ml. thus indicating a high level of ROS production. On the contrary, the percentage of fluorescent cells at the same growth point is less than 10% in 0.5% and 2% glucose. Considering that intracellular levels of ROS depend on the balance between their production and scavenging, the high level of ROS production in cells grown in 20% glucose indicates an alteration of this equilibrium.

3.3. Proteomic analysis

To investigate *S. cerevisiae* protein expression during exponential growth under different glucose concentrations we compared the proteomes of yeast grown in 0.5% and 20% to that grown in 2% glucose (as control). Yeast cells were harvested during exponential phase (0.8 O.D./ml) and proteins were extracted and resolved by two dimensional SDS-PAGE (2D-GE). Fig. 3 shows typical 2D-GE images of soluble proteins from yeast cells grown in 2% (control cells, panel a), 0.5% (panel b), and 20 % (panel c) glucose concentration. The pattern of protein distribution and relative abundance displays corresponds well with yeast 2D-GE protein profiles produced previously by our laboratory [37] and to a 2D-GE *S. cerevisiae* reference gel available on the internet (www.expasy.org). An average of about 1000 spots is detected in each 2D-GE gel stained with Coomassie. The computer analysis points out a total of 156 protein spots changing significantly. Of these protein spots, 82 are differentially expressed (quantitative difference) whereas 74 are not detected in all the analyzed strains (qualitative difference). The general pattern of migration is maintained between replicate gels, thus indicating the high degree of reproducibility of sample preparation and of 2D-GE procedures. MALDI-TOF mass spectrometry was used to identify proteins of interest. It is important to remember that proteins are affected by posttranslational regulation and that, although we characterized only the spots with different abundance levels, we found several isoforms of proteins. The results reported in this study indicate that, during exponential phase, there are significant alterations in protein expression profile of cells grown on different glucose concentrations.

3.3.1. Identification of differentially expressed proteins (quantitative difference)

A total of 21 differentially expressed proteins were successfully identified by MS analysis. The locations of these spots are marked with circles and capital letters in the representative gels in Fig. 3 (panels a, b and c). In Fig. 4 an enlargement of spots corresponding to some identified differentially expressed proteins is shown with the corresponding changes in expression rate. Fourteen spots in cells grown in 0.5% glucose (spots A, C, E, H, I, J, M, O, P, Q, R, S, T and U) and 12 in cells grown in 20% glucose (spots C, E, F, I, J, K, L, M, N, P, Q, and R) are down-regulated in comparison to cells grown in 2% glucose. Of these: 8 spots (C, E, I, J, M, P, Q and R) show a down-regulation in both 0.5% and 20% glucose *versus* control cells. Nine spots are up-regulated (spots A, B, D, O, G, H, S, T and U) in 20% glucose grown cells in comparison to control cells. Spot G, corresponding to peroxiredoxin TSA1, is upregulated in both 0.5% and 20% glucose in comparison to the control. This protein is involved in protection against oxidative stress and its expression depends on nutrient availability or on stress conditions. Peroxiredoxin belongs to a set of stress defense proteins known as "environmental stress response," thus suggesting the importance a redox control complex has in stress conditions [38]. In 0.5% glucose grown cells 6 spots (spots B, D, F, K, N and L) show no

Table 2
Relative protein expression changes of 2% glucose vs. 0.5% glucose and 20% glucose.

Spot no.	Protein name	AC[a]	Score[b]	No. of matching peptide[c]	Sequence coverage[d] (%)	%V (x10^{-4}) mean (±SD)[e]			Fold change[f]	
						Glucose 2%	Glucose 0.5%	Glucose 20%	2%/0.5%	2%/20%
A	Pyruvate decarboxylase isozyme 1	P06169	102	8	22	998 ± 74	521 ± 37[h]	1891 ± 463	1.9	0.52
B	Pyruvate decarboxylase isozyme 1	P06169	100	6	25	554 ± 141	733 ± 96	1916 ± 6[g]	0.75	0.29
C	Pyruvate decarboxylase isozyme 1	P06169	105	8	23	2845 ± 90	1037 ± 142	1527 ± 170[g]	2.7	1.9
D	Adenylate kinase cytosolic	P07170	73	6	31	3681 ± 1870	3560 ± 70[g]	7763 ± 472	1	0.47
E	S-adenosylmethionine synthetase 2	P19358	86	11	33	2493 ± 280	1270 ± 220	331 ± 20[g]	1.9	7.5
F	S-adenosylmethionine synthetase 2	P19358	89	12	35	1373 ± 408	1027 ± 113	603 ± 50[g]	1.3	2.2
G	Peroxiredoxin TSA1	P34760	104	8	51	436 ± 244	1820 ± 80[h]	1063 ± 85	0.23	0.41
H	Peptidyl-prolyl cis-trans isomerase	P14832	83	9	43	2042 ± 274	920 ± 32[g]	9392 ± 1100	2.2	0.21
I	Cystathionine beta-synthase	P32582	93	15	34	2908 ± 107	1280 ± 120[h]	25 ± 12[g]	2.3	11.8
J	Phosphoglycerate kinase	P00560	111	13	42	9666 ± 3460	3515 ± 25	2074 ± 43	2.7	4.7
K	Alcohol dehydrogenase 1	P00330	104	12	47	3058 ± 682	3605 ± 1025	1629 ± 169	0.84	1.9
L	Alcohol dehydrogenase 1	P00330	111	13	50	16,641 ± 117	17,850 ± 350	5548 ± 348[g]	0.93	2.9
M	Heat shock protein homolog SSE1	P32589	66	10	19	1940 ± 1	788 ± 98	331 ± 15[g]	2.4	6
N	Enolase 2	P00925	150	20	58	46,594 ± 8776	52,400 ± 10,400	24,660 ± 5927	0.88	1.9
O	Enolase 2	P00925	148	17	51	4884 ± 401	1375 ± 115	8955 ± 379[g]	3.5	0.54
P	Glyceraldehyde-3-phosphate dehydrogenase 3	P00359	69	9	39	11,174 ± 813	3125 ± 315	2601 ± 145[g]	3.5	4.3
Q	Glyceraldehyde-3-phosphate dehydrogenase 3	P00359	68	9	40	2268 ± 588	540 ± 15	102 ± 82[h]	4.2	22.2
R	Glyceraldehyde-3-phosphate dehydrogenase 3	P00359	70	10	43	5383 ± 598	1690 ± 80[h]	1576 ± 79	3.2	3.4
S	Glyceraldehyde-3-phosphate dehydrogenase 3	P00359	65	7	42	1331 ± 40	341 ± 97	2504 ± 134	3.9	0.53
T	Glyceraldehyde-3-phosphate dehydrogenase 3	P00359	68	10	42	2195 ± 193	840 ± 115	3638 ± 268[h]	2.6	0.6
U	Glyceraldehyde-3-phosphate dehydrogenase 3	P00359	70	11	37	6042 ± 1453	1725 ± 125[g]	16,573 ± 1	3.5	0.36

[a] Swiss-Prot/TrEMBL accession number.
[b] MASCOTscore (Matrix Science, London, UK; http://www.matrixscience.com).
[c] Number of peptide masses matching the top hit from Ms-Fit PMF.
[d] Percentage of amino acid sequence coverage of matched peptides in the identified proteins.
[e] Each value represents the mean 6 SD of individually computed %*V* (*V* = integration of OD over the spot area; %*V* = *V* single spot/*V* total spots) in three different gels of control, 0.5% and 20%.
[f] Fold change (control vs. 0.5% and control vs. 20% glucose) was calculated dividing %*V* from control by the %*V* from 0.5% glucose and from 20% glucose.
[g] *p*-value 6<0.01.
[h] *p*-value 6<0.05.

variation in comparison to the control cells. This suggests that an increase in the expression level of a number of specific proteins is a dominant phenomenon in cells grown in high glucose concentration. The identities of the found proteins are summarized in Table 2. We identified 3 spots (spots A, B and C) corresponding to pyruvate decarboxylase 1 (pdc-1p); 2 spots corresponding to enolase 2 (spots O and N); 6 spots (S, T, U and P, Q, R) corresponding to glyceraldehyde-3-phosphate dehydrogenase 3; 2 spots that correspond to alcohol dehydrogenase 1 (adh-1p) (spots K and L) and finally 2 spots (E and F) that correspond to S-adenosylmethionine synthetase 2. Indeed, according to Petrak et al. [39], quantitative alterations of glycolytic enzymes are frequently observed following a generic stress event.

3.3.2. Identification of qualitative differences

A computer analysis pointed out a total of 40 qualitative spot variations between control cells and cells grown in 0.5% and 20% glucose. These spots were all identified by MS analysis. The data are summarized in Table 3. The location of the spots is marked with circles and numbers in the representative gels shown in Fig. 3 (panels a, b, and c). Seven spots (spots 1 to 7; Fig. 3a) are detected exclusively in the control cells and 7 spots (spots 8 to 14; Fig. 3a and b) are detected exclusively in the 20% glucose yeast cells. Seven spots (spots 15 to 21; Fig. 3c) are detected both in the control cells and in the 0.5% glucose yeast cells while 19 spots (spots 22 to 40; Fig. 3a and c) are detected both in the control cells and in the 20% glucose yeast cells.

3.3.2.1. Proteins detected exclusively in 2% glucose. Seven proteins are detected exclusively in cells grown in 2% glucose. Of these, a majority is involved in cell's glucose metabolism and protein synthesis (Fig. 3a and Table 3). Among these we identified the enolase 2 enzyme (spot 1). This enzyme catalyses the first common step of glycolysis and gluconeogenesis; its expression is glucose induced. We also identify pyruvate kinase1 (pyk-1, spot 3), catalyst in the final step of glycolysis. At this glucose concentration we identified 2 more spots (spots 6 and 7) as bmh1 and bmh2 which are the yeast members of

Table 3
MS identification of qualitative differences.

Spot no.	Protein name	AC[a]	Score[b]	No. of matching peptide[c]	Sequence coverage[d]
Proteins detected exclusively in glucose 2%					
1	Enolase 2	P00925	148	17	51
2[e]	**HSP82**	P10591	70	7	14
3	**Pyruvate kinase 1**	P00549	67	6	19
4	**Eukaryotic translation initiation factor 4B**	P34167	98	10	21
5	**Ketol-acid reductoisomerase, mitochondrial precursor**	P06168	63	8	31
6	**Protein BMH1**	P29311	63	6	31
7	**Protein BMH2**	P34730	72	5	27
Proteins detected exclusively in glucose 20%					
8	Phosphoglycerate kinase	P00560	148	17	51
9	Glyceraldehyde-3-phosphate dehydrogenase 3	P00359	68	9	40
10	Pyruvate decarboxylase isozyme 1	P06169	102	8	22
11					
12	**YNN4**	P53912	56	6	25
13	**Elongation factor 2**	P32324	62	10	12
14	**ATP-dependent RNA helicase MSS116, mitochondrial precursor**	P15424	75	5	10
Proteins detected exclusively in glucose 2% and glucose 0.5%					
15	**Polyadenylate-binding protein, cytoplasmic and nuclear (Poly(A) binding protein) (PABP)**	P04147	64	7	18
16	**5methyltetrahydropteroyltriglutamate-homocysteine methyltransferase**	P05694	70	9	14
17					
18					
19					
20	**GMP synthase [glutamine-hydrolyzing]**	P38625	55	7	14
21	**ATP synthase subunit alpha, mitochondrial precursor**	P07251	91	14	26
Proteins detected exclusively in glucose 2% and glucose 20%					
22	Pyruvate decarboxylase isozyme 1	P06169	102	8	22
23	Pyruvate decarboxylase isozyme 1	P06169	100	6	20
24	**Fructose-bisphosphate aldolase**	P14540	94	8	50
25	**Fructose-bisphosphate aldolase**	P14540	56	5	23
26	Adenylate kinase cytosolic	P07170	59	6	31
27	Enolase 2	P00925	150	20	58
28	Enolase 2	P00925	150	20	58
29	Enolase 2 fragment				
30	**Heat shock protein SSA2**	P10592	74	12	23
31	**Heat shock protein SSB1**	P11484	93	17	35
32	**Heat shock protein SSB1**	P11484	80	15	30
33	**NADP-specific glutamate dehydrogenase 2**	P39708	68	9	27
34	Phosphoglycerate kinase	P00560	117	13	42
35	**Small glutamine-rich tetratricopeptide repeat-containing protein 2**	Q12118	65	7	29
36	**(dl)-glycerol-3-phosphatase 1**	P41277	78	7	35
37	**Inorganic pyrophosphatase**	P00817	94	14	57
38	**NADPH dehydrogenase 2**	Q03558	88	10	34
39	Glyceraldehyde-3-phosphate dehydrogenase 3	P00359	117	12	53
40	Glyceraldehyde-3-phosphate dehydrogenase 3	P00359	68	9	40

[a] Swiss-Prot/TrEMBL accession number.
[b] MASCOTscore (Matrix Science, London, UK; http://www.matrixscience.com).
[c] Number of peptide masses matching the top hit from Ms-Fit PMF.
[d] Percentage of amino acid sequence coverage of matched peptides in the identified proteins.
[e] "Bold" indicates proteins with spots found exclusively as qualitative difference.

the highly conserved 14-3-3 protein family present in all eukaryotic organisms. These two proteins are involved in cells growth control, in post-transcriptional regulation and in the regulation of many processes [40].

3.3.2.2. Proteins detected exclusively in 20% glucose. The 7 spots detected exclusively in yeast cells during exponential growth in 20% glucose are listed in Table 3. The locations of the identified proteins are marked with circles and numbers in the representative gel shown in Fig. 3c. Some of these proteins are present in multiple forms. We found the enzyme phosphoglycerate kinase (spot 8) expressed in 20% glucose; this is a glycolitic/gluconeogenic enzyme. When cells produce a high amount of ethanol, they use this metabolite as a carbon source and they convert it into glucose through gluconeogenic pathway. We also identified 2 spots of the pyruvate decarboxylase 1 enzyme (spots 10 and 11). Pyruvate decarboxylase 1 is the first enzyme of the fermentation pathway and its expression in cells grown in 20% glucose agrees with the high rate fermentation that we evaluated in these cells. Furthermore we saw that the translation protein elongation factor 2 (spot 13) and the ATP-dependent RNA helicase (spot 14) which is involved in mitochondrial splicing and also required for efficient mitochondrial translation, are expressed exclusively in 20% glucose.

3.3.2.3. Proteins exclusively detected both in 2% glucose and 0.5% glucose. Among the 7 protein spots, whose expression during the exponential growth resulted detectable exclusively both in 2% and in 0.5% glucose (Fig. 3a and b), is the 5-methyltetrahydropteroyl-triglutamate-homocysteine-methyltransferase which is present in multiple forms. We identified, in fact, 4 spots (spots 16, 17, 18 and 19) that correspond to this enzyme which is involved in amino acids metabolism. We also identified the poly-(A)-binding protein (spot 15), which is part of the 3′-end RNA processing complex and interacts with translation factor eIF-4G, and the alpha subunit of mitochondrial F1F0 ATP synthase (spot 21), which is a large, evolutionarily conserved enzyme complex required for ATP synthesis.

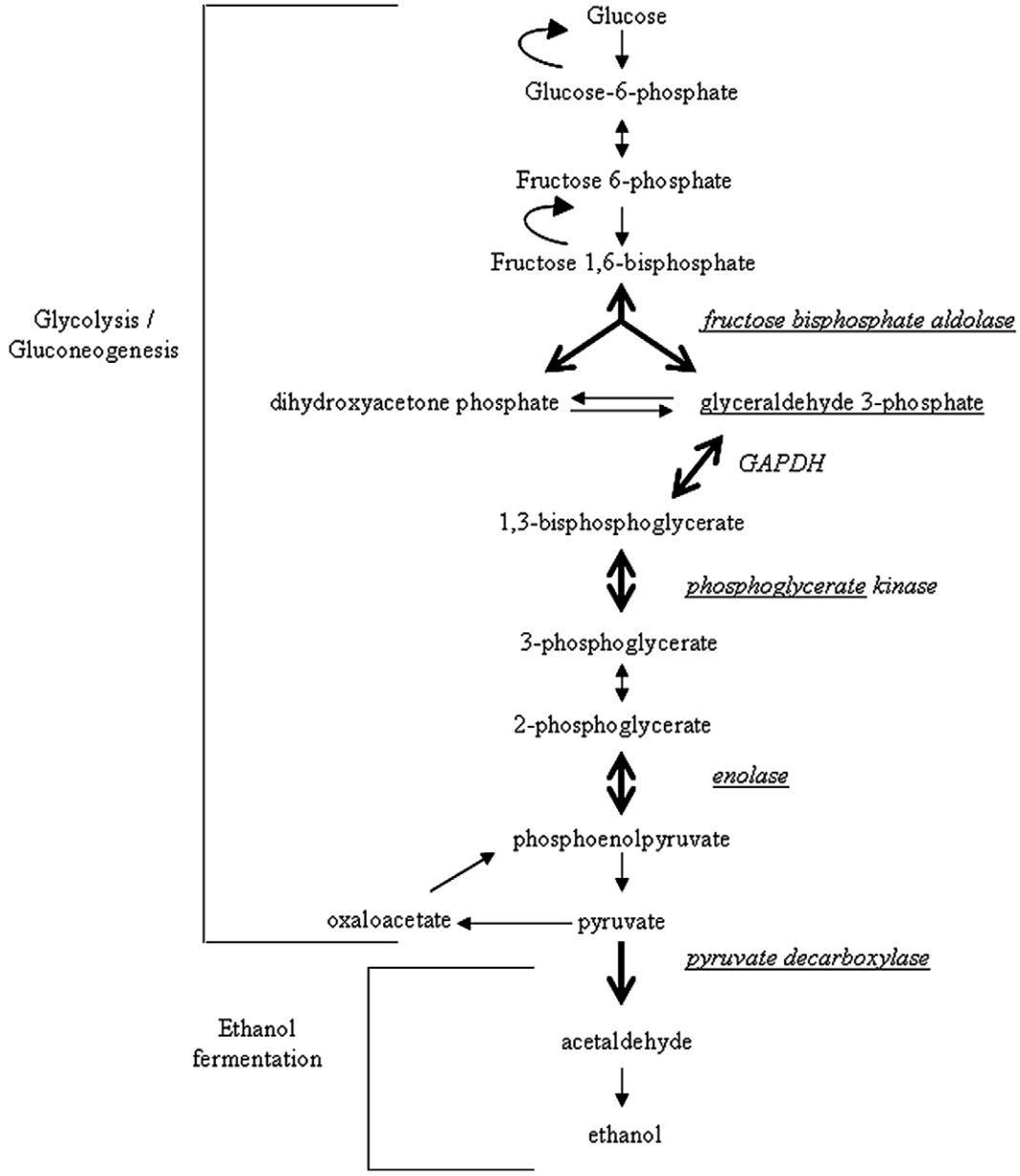

Fig. 5. Scheme of glycolysis, gluconeogenesis and ethanol fermentation. Enzymes written in bold correspond to spots exclusively present in 2% and 20% glucose.

3.3.2.4. Proteins exclusively detected both in 2% glucose and 20% glucose. Of the 18 proteins (Fig. 3a and c) whose expression results inhibited in 0.5% glucose we identified several proteins related to glycolysis and alcoholic fermentation. Two spots (spots 22 and 23) correspond to the enzyme pyruvate decarboxylase 1. One spot corresponds to phosphoglycerate kinase (spot 34), a key enzyme in glycolysis. Two spots (spots 24 and 25) correspond to fructose 1,6-bisphosphate aldolase, which is required for glycolysis and 2 spots correspond to enolase 2 (spots 27 and 28) whose expression is glucose-induced. We also identified 2 spots corresponding to glyceraldehyde-3-phosphate dehydrogenase 3 (spots 39 and 40). Fig. 5 highlights the major metabolic pathways affected and the protein spots exclusively detected in 2% and 20% glucose concentrations.

Among the proteins detected in 0.5% glucose we identified a protein involved in the synthesis of amino acids: 5-methyltetrahydropteroyltriglutamate-homocysteinemethyltransferase. Moreover, we found the cytoplasmatic and nuclear poly (A)-binding protein involved in the regulation of peptide formation on ribosomes. In our study the naturally induced diauxic shift resulted in an induction of the global protein synthesis. This, in turn, allows the *de novo* biosynthesis of functional mitochondria. Glucose restriction also induces the synthesis of a few proteins necessary for respiratory growth such as the mitochondrial ATP synthase subunit alpha that produces mitochondrial ATP. This confirmed the induction of respiration during the diauxic shift.

In the current study we detected that, during the first hours of fermentation, the expression of proteins involved in response to oxidative stress is increased in yeast cells. In particular, we found two members of the heat shock protein 70 family (hsp70) which are stress-induced and not detected in 0.5% glucose: ssa2p and ssb1p. These stress response proteins can indicate a better adaptation during fermentation and be important for maintaining the viability of cells during fermentative conditions. Changes in proteins involved in energetic metabolism and oxidative stress control may reflect a proteome adaptation as a response to perturbation of protein levels caused by stimuli of different origin [41].

In our study we see that, in high glucose, the percentage of glucose used for fermentation is low compared to total glucose consumption and that the ethanol production is inhibited. We deduced that glucose was also used to produce other molecules, such as glycerol. Further evidence was observed, since adh1p, an enzyme related to ethanol production, was down-regulated. Moreover, we found an increase in the expression of many proteins involved in glycerol biosynthesis. We determined that DL-glycerol-3-phosphatase1 (involved in glycerol biosynthesis) was expressed exclusively in this condition. This is induced in response to osmotic stress. These results confirmed the theory according to whom *S. cerevisiae* accelerates its production of glycerol and acidic compounds rather than synthesizing ethanol to minimize the effects of the high osmotic conditions generated by a high glucose concentration and ethanol production [33]. We can conclude that yeast cells in high glucose concentration have a decreased growth rate during the initial phase of fermentation because part of their metabolism is occupied in the synthesis of compounds to resist osmotic stress.

4. Conclusions

The information obtained in our study validates the application of a proteomic approach for the identification of the molecular bases of environmental variations such as fermentation in high glucose and during a naturally induced diauxic shift. Identifying the functionally modulated proteins involved in glucose induced yeast response, will lead to a better comprehension of the mechanisms underlying the effects of different glucose concentrations and will contribute to the complete understanding of yeast fermentation and respiration metabolism.

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