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Research article

Comparative proteome analysis of *Brettanomyces bruxellensis* under hydroxycinnamic acid growth

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ABSTRACT

Background: *Brettanomyces bruxellensis* is an important spoilage yeast in the winemaking process. The capacity of this yeast to generate an undesired off-flavor constitutes a significant loss in the Chilean wine industry.
Results: The proteomic profile of *B. bruxellensis* in the presence of *p*-coumaric acid was determined by 2D gel electrophoresis, gel image analysis and differential spot selection. A set of 41 proteins showed a differential accumulation of ± 2 and a *p*-value ≤ 0.0001. The homology sequence analysis was performed using the databases available. Differential proteins belonged to the categories of 'energy production and conversion' and 'amino acid transport and metabolism'.
Conclusions: The proteomic profile of *B. bruxellensis* cultivated in the presence of *p*-coumaric acid in synthetic wine, agrees with the hypothesis of metabolic flux regulation, allowing a better conditioning to an adverse environment. This study involved the translational level of *B. bruxellensis* in the production of ethylphenols and corroborated that this yeast presented an advantage in these stress conditions. Thus, this work will allow an understanding of the regulation and processes involved in the production of ethyl-derivate compounds by *B. bruxellensis*. Furthermore, it allows the development of newer and better techniques for spoilage yeast control.

1. Introduction

Wine alterations by yeast still constitute a potential threat, particularly for products aged in wood barrels, where there is an increased interest in spoilage by yeasts belonging to the genus *Brettanomyces*. Among the species of this genus, *Brettanomyces bruxellensis* is considered to be the worst contaminant not only in the wine industry, but also in other processes such as bioethanol production [1,2,3]. The presence of this yeast in wine has been associated with the appearance of phenolic aromas described as 'medical', 'horsy' or 'smoky', which are considered strongly detractive for the sensorial characteristics of the product. These off-odors result from the metabolization of hydroxycinnamic acids naturally present in grape must by *B. bruxellensis*. These hydroxycinnamic acids present antimicrobial effects since microorganisms metabolize them to much less toxic compounds such as vinyl or ethyl derivatives [4] The latter are phenolic compounds such as 4-vinylphenol and 4-ethylphenol from *p*-coumaric acid, or 4-vinylguaiacol and 4-ethylguaiacol from ferulic acid [3]. The mechanism of action of hydroxycinnamic acids is similar to that of weak acids, which can disturb intracellular pH and hence cell metabolism [5]. In order to counter the pH effects, the cells reduce the concentration of protons by using a proton pump (Pma1p) in the presence of different weak acids such as cinnamic, ascorbic, octanoic, succinic or acetic acids [6,7]. An increase of H^+-ATPase pump activity has been described during the *lag* growth phase of *B. bruxellensis* in the presence of *p*-coumaric acid, suggesting an early adaptation mechanism against this hydroxycinnamic acid [8].

Different studies revealed that *B. bruxellensis* strains varied in their production of phenolic substances in wine [9,10]. This variability in sensory descriptions of *B. bruxellensis* has been related to genetic strain variation. In fact, it is known that this yeast is a highly diverse microorganism both genetically [11,12] and physiologically [9,13]. Genetic studies in this yeast have shown an unusual variability in chromosome number and genome rearrangements which confer the ability to survive and proliferate after alcoholic fermentation [11,14, 15]. Studies of different *B. bruxellensis* strains (CBS) belonging to the same group display a very similar off-flavor production [9]. However, the isolates from Tuscan wines showed a wide biodiversity within the species, despite the limited geographic area [16]. This shows that the relationship between the genomic diversity of *B. bruxellensis* and its ability to produce phenols is still unclear.

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The *B. bruxellensis* strain LAMAP2480, an isolate from Chilean wines, displays a high production of phenolic compounds, representing a potential risk as wine-spoilage yeast. This isolate shows a very efficient adaptation mechanism against the action of *p*-coumaric acid during growth in synthetic wine [17].

Recently, genomic and transcriptomic approaches provided a set of genes in *B. bruxellensis* LAMAP2480 which is highly represented when the strain is grown in the presence of *p*-coumaric acid [18]. Thus, in order to elucidate the mechanism used by *B. bruxellensis* LAMAP2480 in 4-ethylphenol production, the objective of this work was to evaluate the proteomic response during yeast growth in the presence or absence of *p*-coumaric acid.

2. Materials and methods

2.1. Microorganism

B. bruxellensis LAMAP2480 was obtained from the strain collection of the Applied Microbiology and Biotechnology Laboratory (LAMAP) of the Universidad de Santiago de Chile (USACH). The strain was maintained on YPD medium (0.5% peptone, 0.5% yeast extract, 4% glucose, 4% agar, pH 6.0) until use.

2.2. Pre-culture and culture conditions

2.2.1. Pre-culture conditions

Before performing synthetic wine assays, an adaptation step was carried out as described by Sturm et al. [19]. *B. bruxellensis* LAMAP2480 was grown in YPD medium for 10 d at 28°C. Colonies from YPD agar were inoculated into 10 mL of YPD1 (0.5% peptone, 0.5% yeast extract, 8% glucose, pH 6) supplemented with 6% (*v*/v) of ethanol and grown at 28°C for 48 h. A total volume of the inoculum was added to fresh medium prepared in a 1:1 proportion of SW (0.2% yeast extract, 0.12% glucose, 0.24% fructose, 0.06% trehalose, 0.1% $(NH_4)_2SO_4$, 0.8% $MgSO_4$, 0.2% KH_2PO_3, 0.25 mg/L of biotin, 0.0045 mg/l of thiamin,10% ethanol (*v*/v), pH 3.8 adjusted with HCl), YPD2 (0.5% peptone, 0.5% yeast extract, 4% glucose, pH 4). The culture was incubated for 3 d at 28°C. Finally, 9 vol of synthetic wine was added to 1 vol of culture and yeasts were grown until a concentration of 10^8 cells/mL.

2.2.2. Culture conditions

A volume from the adapted culture was used to inoculate synthetic wine to a final concentration of 10^6 cells/mL, containing 100 mg/L of *p*-coumaric acid (Sigma-Aldrich, USA). A control without acid was also used.

2.3. Growth kinetic

Growth of *B. bruxellensis* LAMAP2480 in synthetic wine in the presence or absence of *p*-coumaric acid was performed at 28°C for 8 d with shaking at 150 rpm. Growth was determined by measuring absorbance at 640 nm. A total of 6 biological replicates of each treatment were collected by centrifugation at exponential phase. The cell pellets were washed with ice-cool Milli-Q water, and stored at −-80°C until protein extraction.

2.4. Protein extraction and quantification

Each sample of *B. bruxellensis* was submitted to protein extraction by a modified protocol [20]. Cultures of 100 mL were collected at exponential phase, six samples for each treatment: in the presence or absence of 100 mg/L of *p*-coumaric acid. Samples were thawed on ice and collected by centrifugation at 800 × g for 5 min at 0°C. The sediment was washed with 30 mL phosphate buffer saline (PBS: 140 mM NaCl, 2 mM KCl, 10 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 4.0) and incubated on ice for 10 min. Cells were then recovered by centrifugation at 800 × *g* for 5 min at 0°C. These wash and centrifugation steps were performed twice. Subsequently, cells were resuspended in 20 mL stabilization buffer A (1 M sorbitol, 10 mM MgCl2, 25 mM potassium phosphate buffer solution, pH 7.8; 10.2 g/mL phenyl-methyl-sulfonyl fluoride (PMSF), 2 mM dithiothreitol) and incubated at 30°C for 10 min. Following centrifugation at 800 × g for 5 min, the pellet was resuspended in 20 mL stabilization buffer B (1 M sorbitol, 10 mM $MgCl_2$, 25 mM potassium phosphate buffer solution, Ph 7.8; 25 mM sodium succinate buffer solution, pH 5.5; 10.2 mg/mL PMFS, 2 mM dithiothreitol) and incubated for 10 min at 30°C. Then, 20 μg/μL zymolyase (Seikagaku Corporation, Tokyo, Japan) was added and incubated at 37°C for 3 h. Once the spheroplasts were obtained, they were collected by centrifugation at 200 × g for 10 min at 4°C. The pellet was resuspended in 3 mL of lysis buffer (50 mM HEPES buffer solution, 17 mg/mL PMFS, 20 μg/mL aprotinin, 30 μL NP-40) and incubated on ice for 1 h. Finally, it was centrifuged at 6200 × g for 10 min at 4°C and the supernatant collected. The total protein concentration was determined using the method described by Bradford [21], using BSA as standard. To remove salts and other substances which could interfere with the labeling, electrophoresis and improve spot resolution, we used the Ettan 2-D Clean-up kit (GE Healthcare, 80-6484-51) to precipitate the proteins. Therefore, proteins were suspended in the DIGE labeling buffer (7 M urea, 2 M thiourea, 4% CHAPS and 20 mM Tris). Finally, the protein concentration was determined with the RC-DC (BioRad Protein Assay) method using BSA as standard. Integrity of proteins was checked in 12% SDS-PAGE gels.

2.5. Labeling protein and 2D gel electrophoresis

In order to prevent protein binding affinity to fluorochromes, the samples collected were labeled with fluorescent dyes using the minimal labeling technique. Half of the biological control samples and half of the treated samples were labeled with Cy3 and the other half were labeled with Cy5, by incubating 50 μg of proteins with 400 pmol of Cy3 or Cy5, respectively. The labeling reaction was conducted at 4°C in the dark for 60 min. An internal standard (IS), composed of equal amounts of all the samples, was labeled with Cy2.

Isoelectric focusing (IEF) was performed in 24 cm Immobiline Dry-Strips (BioRad), immobilized in a pH gradient 3–10 and hydrated in a rehydration solution (8 M urea, 4% CHAPS, DeStreak (12 μg/L), 65 mM DTT and 1% ampholytes (pH 3–11). The IEF was performed at 20°C in the strips: i) 300 V for 4 h, ii) 1000 V for 6 h, iii) 8000 V for 3 h and iv) 8000 V–32,000 V/h. Strips were equilibrated through a process of reduction in 50 mM Tris buffer, 6 M urea, 30% glycerol, 2% SDS, followed by alkylation in buffer containing 2.5% of iodoacetamide (IAA), before the second dimension. Electrophoresis was performed in 25 cm × 21 cm × 1 mm homogeneous polyacrylamide 12.5% gels in two steps: i) 2 W/gel for 1 h and ii) 15 W/gel for 6 h.

2.6. Gel image analysis and differential spot selection

Gel scanning was carried out on a Typhoon™ 9400 Variable Mode scanner (Amersham Biosciences, Uppsala, Sweden). An image file analysis was performed with the DeCyderTM 6.0 Differential Analysis software (Amersham Biosciences, Uppsala, Sweden), which obtains data on protein levels with statistically significant differences. Interferences were manually minimized and the differentially accumulated spots were selected using a Student t-Test (p-value ≤0.05) and False Discovery Rate (FDR), in order to prevent false positives.

2.7. Differential spot identification and annotation

Selected spots were manually excised from gels and digested with trypsin. LC MS/MS spectra and MS/MS were obtained in an Autoflex III

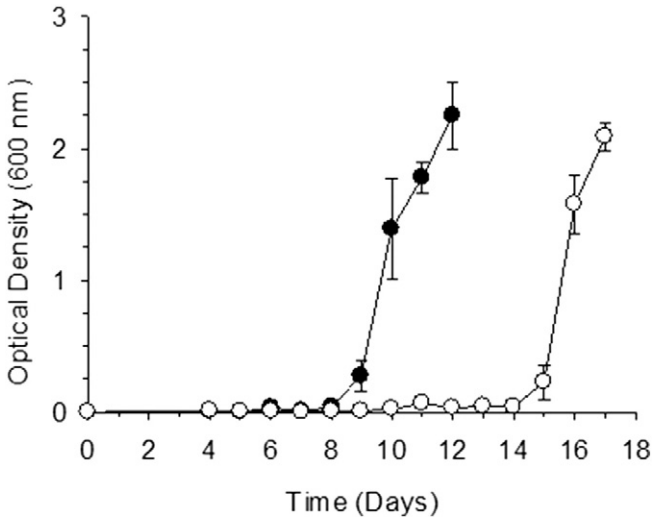

Fig. 1. Growth curves of *B. bruxellensis* cells in synthetic wine with 100 mg/L of *p*-coumaric acid.

MALDI-Tof/Tof spectrometer. Spectra were putatively identified by using local ascot software methods of peptide mass fingerprinting for MS data and ion search for MS/MS data, against *B. bruxellensis* AWRI_1499 protein database obtained from NCBI and UniProt. Putative annotated protein sequences were retrieved in FASTA format and grouped by molecular function and biological process Clusters of Orthologous Groups (COGs).

3. Results

3.1. Profile yeast growth in the presence of p-coumaric acid

As expected, the growth assays showed that the wine strain *B. bruxellensis* LAMAP2480 grew with a significant delay (over 7 d) in the presence of *p*-coumaric acid. This was despite yeast growth in the presence of acid reaching similar values to the control treatment (Fig. 1). A normal consumption of glucose was observed in both conditions (data not shown). Samples were taken when the cultures reached a similar exponential growth phase, to avoid any variability in the comparative proteomic analysis.

3.2. Proteomic analysis

To identify proteomic changes caused by the presence of *p*-coumaric acid, protein extracts were separated by 2D electrophoresis. A total of six protein samples were analyzed for each treatment: with or without *p*-coumaric acid. On average, the comparative analysis of 2D electrophoresis gel images provided the detection of 925 differentially expressed proteins (Fig. 2). During the analysis with DeCyder™ Differential Analysis software, spots with quantitative changes (p-value ≤0.001) were considered. Based on this analysis, a total of 179 proteins showed significant changes in abundance under the different treatments. Finally, in order to narrow the data, a new selection was performed by applying a 99.99% confidence level (p-value ≤0.0001) and an abundance of ≥2. Under these conditions, a total of 41 proteins were detected and selected to be identified (Table 1). The analysis of proteins from the total proteins accumulated differentially in the presence of *p*-coumaric acid revealed that the majority showed an upregulated accumulation with a fold change variation between 2.02 and 9.79 (Table 1). Interestingly, only one protein identified as a phosphoglycerate mutase (spot 2206), involved in the glycolysis pathway displayed a downregulation in the presence of *p*-coumaric acid; all other proteins upregulated (Table 1).

3.3. Analysis of differentially accumulated proteins

The sequence homology analysis was performed using the *B. bruxellensis* AWRI1499 (Australian strain) databases available, which were then compared with genomic data of the Chilean strain LAMAP2480. The 41 differentially accumulated and identified proteins were divided into different categories according to biological processes and molecular function by COGs. It can be observed that the proteins are clustered in three biological processes: (A) metabolism, (B) cellular processes and signaling and, (C) information storage and processing (Fig. 3). Moreover, the functional category distribution for molecular processes showed that the addition of *p*-coumaric acid led to the accumulation of proteins belonging to the category of 'translational, ribosomal structure and biogenesis' (26.7%) and 'post-translational modification, protein turnover and chaperones' (20.6%) (Fig. 4). Interestingly, the third and fourth main group of this distribution was 'energy production and conversion' and 'amino acid transport and metabolism', which include 14.7% and 8.8% of the total

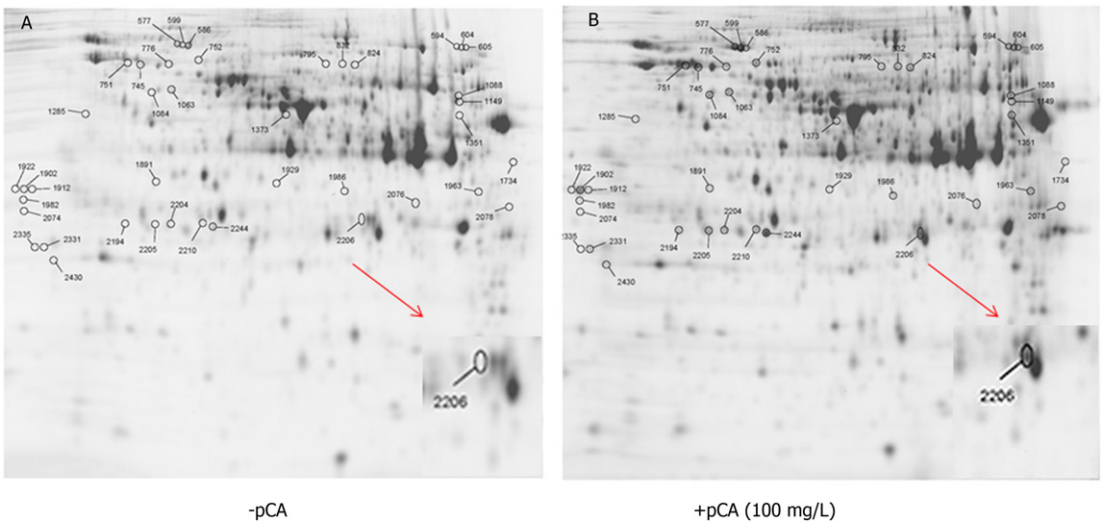

Fig. 2. Differentially accumulated proteins detected in 2D-PAGE. 2D-gel showing proteome profile of *B. bruxellensis* cells and numbered spots corresponding to the proteins identified during growth in synthetic wine without (A) and with (B) 100 mg/L of *p*-coumaric acid.

Table 1
Proteins of *Brettanomyces bruxellensis* differentially accumulated during growth in synthetic wine with 100 mg/L of *p*-coumaric acid. Numbered spots correspond to proteins observed in Fig. 2.

Ot	NCBI Acc. no. *D. bruxellensis* AWRI1499	% seq. Cov.	Acc. UniProt	Protein description blast *D. bruxellensis* AWRI1499	Fold change	Acc. no. *D. bruxellensis* LAMAP2480	Score bits	E value	Identity (%)	Protein descption blast *D. bruxellensis* LAMAP2480
577	gi|385,303,999	25	I2JYI5	Heat shock protein Hsp88	3,67	DEB_0656	1097	0	98	Heat shock protein homolog Sse1
586	gi|385,303,999	26	I2JYI5	Heat shock protein Hsp88	3,16	DEB_0656	1097	0	98	Heat shock protein homolog Sse1
594	gi|385,304,066	17	I2JYP9	Translation initiation factor eif3 subunit	2,46	DEB_5225	1393	0	95	eIF3b subunit of the core complex of translation eIF3
599	gi|385,303,999	24	I2JYI5	Heat shock protein Hsp88	3,01	DEB_0656	1097	0	98	Heat shock protein homolog Sse1
604	gi|385,304,182	15	I2JZ09	Glutamyl-tRNA synthetase	2,29	DEB_3410	1329	0	94	Glutamyl-tRNA synthetase (GluRS)
605	gi|385,303,590	9	I2JXF2	Aconitase mitochondrial precursor	2,46	DEB_2803	1533	0	97	Aconitase
745	gi|385,303,397	15	I2JWX0	Heat shock protein Ssb1	3,35	DEB_0154	1167	0	94	Cytoplasmic ATPase Ribosome-associated molecular chaperone
751	gi|385,303,397	38	I2JWX0	Heat shock protein Ssb1	3,42	DEB_0154	1167	0	94	Cytoplasmic ATPase Ribosome-associated molecular chaperone
752	gi|385,304,308	13	I2JZC9	Mitochondrial matrix ATPase	2,02	DEB_3227	1192	0	93	Hsp70 family ATPase
776	gi|385,303,397	16	I2JWX0	Heat shock protein Ssb1	2,17	DEB_0154	1167	0	94	Cytoplasmic ATPase: Ribosome-associated molecular chaperone
795	gi|385,305,672	11	I2K328	Polyadenylate-binding protein	2,24	DEB_6112	1226	0	90	Poly(A) binding protein
824	gi|385,305,672	23	I2K328	Polyadenylate-binding protein	2,36	DEB_6112	1226	0	90	Poly(A) binding protein
832	gi|385,305,672	11	I2K328	Polyadenylate-binding protein	2,52	DEB_6112	1226	0	90	Poly(A) binding protein
1063	gi|385,302,952	28	I2JVQ3	Vacuolar ATP synthase subunit b	2,04	DEB_8230	903	0	100	Subunit B of the vacuolar H^+-ATPase (V-ATPase)
1084	gi|385,302,952	25	I2JVQ3	Vacuolar ATP synthase subunit b	3,85	DEB_8230	903	0	100	Subunit B of the vacuolar H^+-ATPase (V-ATPase)
1088	gi|385,303,472	19	I2JX43	Delta-1-pyrroline-5-carboxylate dehydrogenase	2,52	DEB_1480	1069	0	99	Delta-1-pyrroline-5-carboxylate dehydrogenase
1149	gi|385,305,298	15	I2K234	Serine mitochondrial precursor	2,02	DEB_0153	841	0	86	Mitochondrial serine hydroxymethyltransferase
1285	gi|385,303,397	22	I2JWX0	Heat shock protein Ssb1	2,05	DEB_0154	1167	0	94	Cytoplasmic ATPase. Ribosome-associated molecular chaperone
1351	gi|385,301,161	49	I2JQX1	Isocitrate mitochondrial precursor	3,18	DEB_9391	914	0	97	Mitochondrial $NADP^+$-specific isocitrate dehydrogenase
1373	gi|385,301,947	10	I2JT02	Translation elongation factor 2	2,06	DEB_5012	1523	0	96	Elongation factor 2 (EF-2)
1734	gi|385,304,846	6	I2K0U7	Malate NAD^+-dependent	2,05	DEB_6563	659	0	99	Mitochondrial malate dehydrogenase
1891	gi|385,303,892	22	I2JY86	Sphingolipid long chain base-responsive protein Pil1	2,33	DEB_9433	466	e-132	83	Primary component of eisosomes
1902	gi|385,304,034	13	I2JYL8	Elongation factor 1-β	9,79	DEB_5213	235	6,00E-63	64	Elongation factor 1-β
1912	gi|385,304,034	3	I2JYL8	Elongation factor 1-β	8,12	DEB_5213	235	6E-63	64	Elongation factor 1-β
1922	gi|385,306,057	6	I2K441	60s ribosomal protein l8-b	6,89	DEB_7740	472	e-134	90	Ribosomal protein L4 of the large (60S) ribosomal subunit
1929	gi|385,305,338	2	I2K272	40s ribosomal protein s5	2,41	DEB_3786	423	e-129	100	Protein component of the small (40S) ribosomal subunit
1973	gi|385,301,952		I2JT04	YMR226c-like protein	2,25	DEB_3523	534	e-153	98	$NADP^+$-dependent dehydrogenase
1982	gi|385,306,057	9	I2K441	60s ribosomal protein l8-b	5,71	DEB_7740	472	e-134	90	Ribosomal protein L4 of the large (60S) ribosomal subunit
1986	gi|385,303,283	28	I2JWL7	Sphingolipid long chain base-responsive protein Pil1	2,36	DEB_9433	395	e-111	74	Primary component of eisosomes
2074	gi|385,306,053	4	I2K437	60s ribosomal protein l2	4,43	DEB_7737	484	e-137	94	Protein component of the large (60S) ribosomal subunit
2076	gi|385,301,733	14	I2JSF0	Alcohol dehydrogenase	2,15	DEB_7598	453	e-128	64	Mitochondrial alcohol dehydrogenase
2078	gi|385,303,430	2	I2JX01	Glyceraldehyde-3-phosphate dehydrogenase	5,38	DEB_4294	674	0	98	Glyceraldehyde-3-phosphate dehydrogenase
2194	gi|385,305,335	3	I2K269	Heat shock protein Hsp20	3,74	DEB_3791	364	e-101	89	Small heat shock protein C4
2204	gi|385,304,076	17	I2JYQ7	Mitochondrial peroxiredoxin Prx1	2,99	DEB_0579	471	e-134	99	Mitochondrial peroxiredoxin
2205	gi|385,305,335	5	I2K269	Heat shock protein Hsp20	3,47	DEB_3791	364	e-101	89	Small heat shock protein C4
2206	gi|385,300,983	13	I2JQG8	Phosphoglycerate mutase	-2,11	DEB_6902	483	e-137	95	Tetrameric phosphoglycerate mutase
2210	gi|385,305,359	27	I2K291	Vacuolar ATP synthase subunit E	5,95	DEB_11809	350	2,00E-97	93	Subunit E of the vacuolar H^+-ATPase (V-ATPase)
2244	gi|385,304,076	11	I2JYQ7	Mitochondrial peroxiredoxin Prx1	3,82	DEB_0579	471	e-134	99	Mitochondrial peroxiredoxin
2331	gi|385,303,768	3	I2JXW9	Translationally controlled tumor protein	2,44	DEB_9730	320	1,00E-88	93	Protein that associates with ribosomes
2335	gi|385,305,439	4	I2K2G2	Eukaryotic translation initiation factor 5a	2,46	DEB_1606	313	1,00E-86	99	Translation elongation factor eIF-5 A
2430	gi|385,303,843	6	I2JY42	Peroxiredoxin Tsa1	4,02	DEB_7532	404	e-113	100	Thioredoxin peroxidase

proteins, respectively. These latter groups can be related to energetic imbalance that supposes the maintenance of intracellular pH.

4. Discussion

In this study, we carried out an analysis of the proteome changes occurring in yeast during the exponential growth phase, in the presence of *p*-coumaric acid. Within the cells exposed to *p*-coumaric acid, we detected an increased accumulation of proteins related to stress proteins and chaperones such as Hsp20, 88 and several Hsp70 isoforms. The small heat shock proteins (sHSP) have been described in different tissues and organisms only in stress conditions [22]. They are induced by exposure to a range of stress factors, such as Hsp26, which is induced by osmostress [23], heat shock [24], H_2O_2 [25] or exposure to sorbic acid [26]. Hsp20 (spots 2194 and 2205), is a well characterized protein in plants and is induced by heat-stress [22]. In

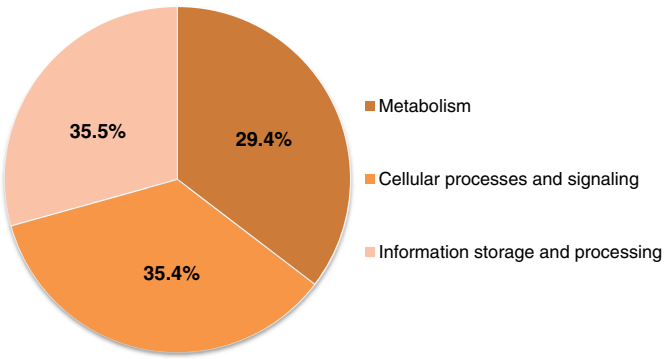

Fig. 3. Functional category distribution of identified differential proteins associated by Clusters of Orthologous Groups for biological processes.

stress conditions, Hsp20 aggregate to form complexes such as intracellular matrices to prevent protein denaturation [27]. However, the precise role and function of Hsp20 under weak acid stress in yeast remains unknown. In *Saccharomyces cerevisiae*, it has been proposed that Hsp26 could be used to prepare denatured proteins for refolding or degradation under sorbic acid stress [26]. In our case, Hsp20 could exert a similar role in the resistance to *p*-coumaric acid. In this regard, the data suggests that an inhibitory action of *p*-coumaric acid could be intracellular protein denaturation, as has been described for sorbic acid [26]. In this context, other HSP were also induced under *p*-coumaric acid stress, such as Hsp70 isoforms (spots 745, 751, 776 and 1285). Hsp70 are specialized proteins involved in translation and early polypeptide folding processes [28]. It is known that Ssb1 (Hsp70) is induced by H_2O_2 [25] in response to sorbic acid [26] and its deletion produces mutants which are more sensitive to low temperatures and salt [29]. Furthermore, we observed the accumulation of Hsp88 isoforms (spots 577, 586 and 599) or Sse1p in synthetic wine in the presence of ethanol. Previous works have described Sse1p as proteins involved in environmental stress responses, which are accumulated in the presence of ethanol, and prevent protein unfolding [30]. The increase of these proteins in the presence of *p*-coumaric acid could be indicative of an increase in damaged intracellular proteins or a major protein expression and/or turnover in *p*-coumaric acid stress.

Several ribosomal proteins (spots 1922, 1924, 2074) and translation elongation factors (spots 1373, 1912), along with different translation initiation factors (spots 594, 2335) (Table 1), show a greater accumulation under *p*-coumaric acid conditions, suggesting that the synthesis of most general proteins is activated under these conditions. Translation elongation factors such as EF-3A, similar to the ones detected in this work, have been described in *S. cerevisiae* under sorbic acid stress conditions [26]. Studies on the adaptive response to acetic acid in the highly resistant yeast *Zygosaccharomyces bailii,* have shown an increased accumulation of the translation initiation factor eIF-5A and protein component of the small (40S) ribosomal subunit [31], similar to the proteins detected in *B. bruxellensis* in the presence of the weak *p*-coumaric acid [31]. The accumulation of these proteins, in association with the overproduction of chaperones, may stimulate protein synthesis rate for the cells to grow even under unfavorable conditions of *p*-coumaric acid. In fact, the data suggests the synthesis of most proteins activated by *p*-coumaric acid is to restructure the mechanism for more energy production (see below).

Metabolism was the principal biological process determined in this study (Fig. 3), energy production and conversions were included as the principal molecular functions (Table 1). This category can be related to an energetic imbalance that supposes the maintenance of intracellular pH under weak acid conditions. The dissociation of weak acids in the cell leads to the accumulation of protons and of the corresponding anions resulting in internal acidification [32]. Hydroxycinnamic acids diffuse into the cell affecting cellular pH, blocking transport and inhibiting growth [5]. To counteract the weak acid induced intracellular acidification, an increase in the activity of the plasma membrane H^+-ATPase and of the vacuolar ATP synthase (V-ATPase) is observed under weak acid stress [32]. An H^+-ATPase pump which supports a mechanism to counteract the decrease in internal pH caused by the presence of this acid, has been described in *B. bruxellensis* in response to *p*-coumaric acid growth [8]. Two different V-ATPase subunits were spotted in our study (spots 1063, 1084 and 2210), both included in the molecular category energy production and conversion (Fig. 4). V-ATPases are large, complex enzymes responsible for the translocation of protons into the lumen from the cytoplasm. This action is very important for many cellular processes, such as endocytosis, cytoplasmic pH homeostasis, protein processing, and the coupled transport of small molecules. Moreover, it plays an important role in stress tolerance [33,34]. These data suggest a role for V-ATPases in tolerance to *p*-coumaric acid maintaining cytoplasmic pH homeostasis. Additionally, the treatments with sorbic acid in *S. cerevisiae* promoted the accumulation of Atp2, a mitochondrial ATPase β-subunit [26]. The apparent increase of energy generation during adaptation to *p*-coumaric acid growth is supported by previous investigations, which show the excess consumption of intracellular ATP pools as an attempt to restore the homeostasis under weak acid conditions [35,36]. Interestingly, different works have shown the upregulation of Atp2 by ethanol [37]. In our study *B. bruxellensis* was grown in SW containing 10% ethanol, which could be contributing to the accumulation of V-ATPases observed.

In addition to the accumulation of mitochondrial proteins involved in energy generation, we also detected three proteins that belong to

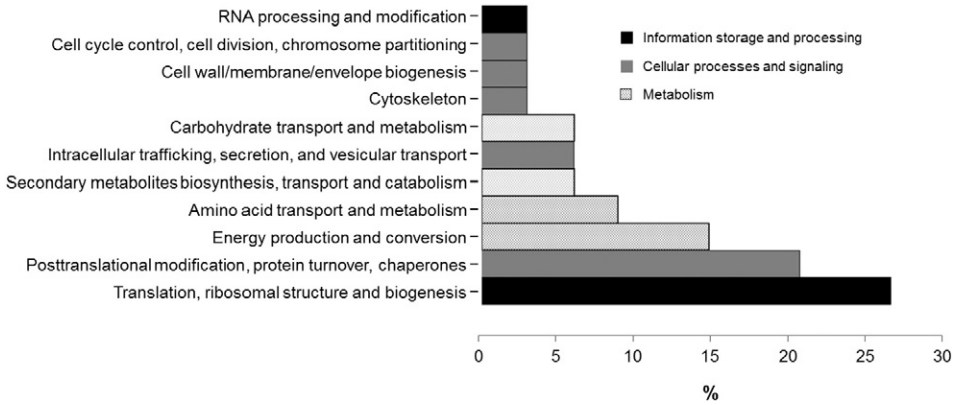

Fig. 4. Functional category distribution of identified differential proteins associated by Clusters of Orthologous Groups for molecular function.

the tricarboxylic acid cycle (TCA): aconitase (spot 605), malate dehydrogenase (spot 1734) and $NADP^+$-specific dehydrogenase (spot 1351). The stimulation of TCA flux in *p*-coumaric acid stressed *B. bruxellensis* cells allows the enhancement of the ATP pool. Previously, malate dehydrogenase has been found to be required for the first step of weak acid metabolism in the resistant yeast *Z. bailii* [31,38]. This yeast is resistant to acetate and has the ability to metabolize the acid in the presence of glucose, while in similar conditions malate dehydrogenase was depressed in *S. cerevisiae*. Considering this data, the increase of this enzyme in *B. bruxellensis* is consistent with the high accumulation of V-ATPases, due to an increase of ATP synthesis required in the presence of weak acids. This supports the energy-consuming mechanism necessary to counteract its deleterious action, as has been reported in *S. cerevisiae* [31,32,39,40].

In relation to energy generation, other enzymes belonging to the dehydrogenase family have been detected during growth of *B. bruxellensis* under *p*-coumaric acid stress (spots 1973, 2076 and 2078). In *S. cerevisiae* these enzymes form complexes in the mitochondria. One of these complexes has three NADH-dehydrogenases, a glycerol-3-phosphate dehydrogenase, an acetaldehyde dehydrogenase and TCA enzymes (malate dehydrogenase, citrate synthase, succinate dehydrogenase and fumarate hydratase) [41] In our work, a variety of these enzymes have been detected, suggesting an increase of NADH and ATP pools, which corroborate the resulting high energy demand for growth under *p*-coumaric acid conditions. These assumptions are in agreement with the increased accumulation of glyceraldehyde-3-phosphate dehydrogenase (spot 2078) (Table 1), a glycolytic enzyme responsible for formation of 1,3-bisphosphoglycerate and NADH. Furthermore, an alcohol dehydrogenase (spot 2076) (Table 1) was present in our study under *p*-coumaric acid conditions. This fact may be related to an increase of glycolytic flux through glucose to compensate for the energetic imbalance caused by the weak acid. Other works described evidence of an enhancement of the key glycolytic enzymes [26], which support previous observations stressing the importance of an energy-generating metabolism to weak acid adaptation [35,36]. Moreover, in previous studies with nitrate, *B. bruxellensis* presented a metabolic flux regulation, with an increase of ATP synthesis, TCA and glycolytic enzymes and an overproduction of alcohol dehydrogenase in order to compensate for the energetic imbalance caused by nitrate assimilation [42]. These data suggest that *B. bruxellensis* could present similar behavior in the presence of *p*-coumaric acid, which induces the same metabolic responses in order to obtain the necessary energy to cover the energetic demand caused by growing under these stress conditions. On the other hand, the only enzyme not accumulated was a phosphoglycerate mutase (spot 2206) (Table 1) responsible for the conversion of 3-phosphoglycerate to 2-phosphoglycerate.

In this work we detected an increased accumulation of the Δ^1-pyrroline-5-carboxylate dehydrogenase (spot 1088) in *B. bruxellensis* under *p*-coumaric acid stress. Structurally, this enzyme has been well characterized in *S. cerevisiae* [41,43] and is involved in the conversion of the excess proline to glutamate in the mitochondria [44]. Proline catabolism intermediate Δ^1-pyrroline-5-carboxylate is toxic to yeast cells because of the formation of ROS [45] and directly inhibits mitochondrial respiration [46]. This compound is metabolized by Δ^1-pyrroline-5-carboxylate dehydrogenase into a non-toxic intermediate, the amino acid glutamate [43,45,46]. Moreover, the enzyme is required in different species to regulate redox state, homeostasis and virulence [47,48]. Even in plants, it has been described as playing an important role in protection from proline metabolism toxicity [49]. On the other hand, it has been described that proline-accumulating strains displayed tolerance to acetic acid, whereas strains with compromised proline metabolism displayed sensitivity [50]. Furthermore, sensitivity to weak acids appears to be reduced with the addition of proline. The data suggests that the increased accumulation of Δ^1-pyrroline-5-carboxylate dehydrogenase for glutamate formation could be a strategy of *B. bruxellensis* to obtain precursors for TCA and an increase of ATP, in order to equilibrate the homeostasis in *p*-coumaric acid conditions. In our study, a mitochondrial peroxiredoxin Prd1 (spots 2204 and 2244) and a peroxiredoxin Tsa1 (spots 2430) were accumulated during stress conditions (Table 1). The first presented thioredoxin peroxidase activity and is induced during respiratory growth and oxidative stress [51]. The second, considered as the major yeast peroxiredoxin, acts as both a ribosome-associate and a free cytoplasmic antioxidant. It functions as a specific antioxidant in the cytoplasm to protect the cell against the oxidative stress caused by nascent-protein misfolding and aggregation [52]. Transcript analysis carried out in *S. cerevisiae* grown in sorbic acid displayed many enzymes involved in reducing oxidative stress, which were not detected in the proteomic study. However, our work revealed different proteins related with these processes and apparently induced by *p*-coumaric acid stress.

Within the differentially accumulated proteins, it was not possible to find Pad1p, enzyme responsible for decarboxylating *p*-coumaric acid and converting it into 4-vinylphenol [20]. It has been reported that 4-ethylphenol formation is growth associated, and occurs roughly between mid-exponential growth phase and the beginning of the stationary phase [53]. This could explain the absence of the Pad1 protein within the differentially accumulated proteins, therefore, this enzyme decarboxylated the *p*-coumaric acid in the early stages of growth. Our results show proteins accumulated during the exponential phase, wherein the second reaction step occurs, corresponding to the reduction of 4-vinylphenol to 4-ethylphenol.

In this work we have explored 2DE-based expression proteomics focusing on the growth of *B. bruxellensis* LAMAP2480 in the presence of hydroxycinnamic acids namely *p*-coumaric acid, to extend the knowledge on the mechanisms underlying its response to weak acid. The results indicate that the response to *p*-coumaric acid involves an increased activity of different metabolic processes to improve the requirements of ATP and NADH production, in order to assure cell detoxification. Results also suggest that there is an induction of protective mechanisms in response to the acid, in particular, through the induction of RNA machinery, oxidative stress response and an increase of TCA flux. Taken together, data obtained from this study provides insights into *p*-coumaric acid adaptation in the wine spoilage yeast *B. bruxellensis*.

Conflict of interests

The authors declare that they have no competing interests.

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