



# Proteome and fermentative parameters of *Saccharomyces cerevisiae* CAT-1 under Very High Gravity Fermentation (VHGF) using sugarcane juice

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## ABSTRACT

Alcoholic fermentation is an important process in the modern world, allowing the production of ethanol for several applications. Different *Saccharomyces cerevisiae* strains have been used for this purpose, such as CAT-1, a strain resistant to different stress factors. Hence, our aim was to analyze some fermentative parameters and the proteome of *S. cerevisiae* CAT-1 under Very High Gravity Fermentation (VHGF) using sugarcane juice as fermentative medium. The yeast was cultured in the must with the sucrose concentration adjusted to 2%, 14%, 21% and 30%, for 10 h at 30 °C. The cell viability was 96-100% for all sucrose concentrations analyzed and the biomass increased for each condition as time function. The highest ethanol recovery was obtained under 30% sucrose. Considering the *S. cerevisiae* CAT-1 proteome under 14% and 30% sucrose, qualitative and quantitative differences were found in the protein expression. Important enzymes for fermentation, such as enolase and one alcohol dehydrogenase isoform were more expressed at 30% sucrose than with 14% sucrose. The yeast *S. cerevisiae* CAT-1 is an interesting strain to be used for fermentation under VHGF technology using sugarcane juice, allowing high ethanol recovery with increased expression of proteins related to alcoholic fermentation and viability as well.

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## 1. INTRODUCTION

Alcoholic fermentation is an important process nowadays, allowing the production of ethanol for several applications, especially as fuel. This process has been conducted using different strains of the yeast *Saccharomyces cerevisiae*. When a microorganism is submitted to the anaerobic condition or to the medium containing high sugar concentration, the fermentation can be conducted effectively [1]. The fermentative process used in the Brazilian industries, which uses sugar-cane juice and/or molasses as fermentative medium, is characterized by high cellular density, short period of fermentation (6-11 h) at 32-35°C and recycling of yeast cells, with recovery of 8-11% (v/v) of

ethanol concentration [1]. However, an important challenge in this scenario is to increase the ethanol production in the fermentative process. Focused on this aim, different wild *S. cerevisiae* strains, such as CAT-1 and PE-2, isolated from Brazilian distilleries as indigenous, have been used by different industries from the alcoholic sector [2]. The choice of a yeast strain for a fermentative process depends on different microbial characteristics as the velocity of transformation of the sugars into ethanol, resistance to high ethanol concentration, resistance to modifications of pH and temperature, genetic stability and insensitivity to antibiotics [3]. The yeast *S. cerevisiae* CAT-1, isolated from a Brazilian distillery in 1998, is an important strain that can be used for alcoholic fermentation because of its resistance to different stress factors such as increased temperature and high concentration of sugars in the medium [4, 5]. In addition, it is a flocculent microorganism with reduced production of spume during the industrial process [4].

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The genome (~ 12-Mb) analysis of the CAT-1 strain indicated that this yeast is a heterozygous diploid with low occurrence of transposable elements, gene duplication and deletions as well. Some genes are involved with important ways for ethanol production [6]. Recently, a quantitative proteomic analysis for this strain was performed, showing high fermentative performance and robustness when compared to PE-2 strain. Additionally, proteins associated to trehalose synthesis and to oxidative stress were abundant under batch fermentation [2]. Considering the robustness of the CAT-1 strain, engineered *S. cerevisiae* strains able to ferment D-xylose were reported, as well as their potential for ethanol production using corn cob hydrolysate [7].

According to Carvalho and Sato (2011) [8], the microbial metabolism can be dislocated to a specific metabolic way by controlling the volume of the substrate in the reactor, allowing the accumulation of the product (in this case, ethanol) and reducing the production of vinasse. However, this process uses around of 21% of dissolved solids (21° BRIX) in the medium, what produces a wine with low ethanol concentration and high volumes of vinasse that can cause serious environmental problems [9, 10]. This technology is known as High Gravity Fermentation (HGF), recovering 10-12% ethanol at the end of the process. An interesting alternative to HGF is the Very High Gravity Fermentation (VHGF) technology, which uses a concentration of dissolved solids  $\geq 27\%$  (v/v), allowing the reduction of bacterial contamination, ethanol recovery of 15-18% at the end of the process, reduction in the volume of vinasse produced and water and energy economy [12]. Under this scenario, the analysis of the metabolic behavior of the fermentative microorganisms under VHGF is a key step to improve the alcoholic fermentation process. With this aim, the identification and the importance of different *S. cerevisiae* proteins during fermentation such as heat shock proteins and enzymes from the glycolytic pathway, differently expressed according to the fermentation conditions, have been reported [13-15]. Despite the description of the *S. cerevisiae* KAY446 proteome under VHGF [16], this manuscript describes, for the first time, the proteome analysis of the robust yeast *S. cerevisiae* CAT-1 under VHGF aiming to understand the metabolic differences compared to the fermentation at low sugar concentration (14%). Some fermentative parameters such as cell growth and viability, consumption of sugars and ethanol production were evaluated during the fermentation using sugar-cane juice.

## 2. MATERIAL AND METHODS

### 2.1 Microorganism and culture conditions

The yeast *Saccharomyces cerevisiae* strain CAT-1 was maintained in slants of YPD-agar medium (yeast extract 10g/L, peptone 20g/L, glucose 20 g/L and agar 20 g/L) previously autoclaved at 120°C, 1.5 atm for 20 min. After growth at 37°C for 48 h, the cultures were maintained at 4°C for up to 15 days, a period that was considered appropriate for the maintenance of the cell viability. The pre-inoculum was prepared according to the

process described by Moreira *et al.* (2015) [17] with some modification. Yeast cells from the slants were added to a tube containing 4 mL of distilled water sterilized; after homogenization of the suspension, 1 mL was added to Erlenmeyer flasks containing YPSac 10% medium (yeast extract 10 g/L, peptone 10 g/L and sucrose 100 g/L), with pH adjusted to 5.0. The medium was previously autoclaved in the same conditions described above. The medium containing the yeast cells was maintained at 30°C at 250 rpm for 20 h to obtain the cell concentration to conduct the fermentation with high cell density. After this period, the medium containing the cells was centrifuged at 3,100xg for 10 min at 4°C. The cells obtained were washed with a cold solution of NaCl (0.85% m/V), submitted to the vortex and centrifuged in the same conditions described previously. Thereafter, the cells were inoculated in 125 mL Erlenmeyer flasks containing 25 mL of sugarcane juice with the content of sucrose adjusted to 21% and 30% or without adjustment (14%), or to the medium containing 2% sucrose. The cultures were maintained at 37.5°C for 10 h. Samples (300  $\mu$ L) were withdrawn at 2.5 h intervals and used for the analysis of the fermentative parameters.

### 2.2 Obtainment of cells, lysis and protein extract

After cultivation, the culture medium containing the yeast cells was centrifuged at 3,100xg for 15 min at 4°C. The cells obtained were separated from the liquid, washed with a solution of NaCl (0.85%, m/V) and centrifuged in the same condition described above. This procedure was performed three times. After the last centrifugation step, the cells were submitted to maceration using liquid nitrogen in a porcelain mortar. The proteins extracted were solubilized using an extraction buffer composed of 100 mM Tris-HCl pH 8.0, 1 mM PMSF, 1 mM EDTA, 2 mM DTT and 2 mM benzimidazole (modified from Moazed and Johnson) [18]. After solubilization, the content was centrifuged at 3,100xg for 15 min at 4°C and the supernatant was used as protein crude extract.

The protein extract was precipitated with 40% TCA for 24 h at 4°C. Thereafter, the material was centrifuged at 9,300xg for 20 min at 4°C. The protein pellets obtained were washed two times with cold acetone and maintained at room temperature for 5h to enable the total evaporation of the acetone. Two  $\mu$ g of protein was used for 1 mL of re-hydration buffer (urea 8M, CHAPS 2%, 50 mM DTT, Bio-Lyte 0.2% and bromophenol blue 0.001%). After solubilization, the extract was aliquoted and stored at -20°C for use in the 2D electrophoresis.

### 2.3 Protein quantification

The quantification of total proteins present in the crude extract used for proteomic analysis was carried out according to the methodology described by Bradford [19] using bovine serum albumin as standard. The protein concentration was expressed as mg of protein per mL of sample.

### 2.4 Electrophoresis

The protein extracts yielded after the lysis of cells, obtained for each cultivation condition, were used for SDS-PAGE

electrophoresis according to Laemmli (1970) [20] using gradient gels (4-15%) (Mini-PROTEAN TGX Gel, BioRad). The power source was adjusted to 120V, 8 W and 20 mA/gel and electrophoretic run was conducted using a Mini-PROTEAN Tetra Cell (Bio-Rad) apparatus. After running, the gels were removed and stained with Coomassie Brilliant Blue R-250. The gels were decolorized using a solution of methanol (45%, v/v) and acetic acid (10%, v/v) until the visualization of the protein bands.

## 2.4 2D-Electrophoresis

The isoelectric focusing was conducted using 7cm IPG strips at pH range 3.0 to 10.0 (BioRad). 125  $\mu$ L of protein extract (250  $\mu$ g of protein) was used to re-hydrate the strips passively for 18 h. After this step, the isoelectric focusing was conducted in the Protean IEF Cell (BioRad) adjusted at 50  $\mu$ A and with 4 steps, as follows: 250 V for 15 min; 4,000 V for 1 h; 4,000V to 15,000 for 1h and 500 V as the rold condition. The focusing was conducted for 6 h. After this step, the IPG strips were removed and immersed for 15 min in the equilibrium buffer I (BioRad) (375 mM Tris-HCl pH 8.8, 6 M urea, 2% DTT, 2% SDS and 0.001% bromophenol blue and 30% glycerol). Thereafter, the strips were immersed for 15 min in the equilibrium buffer II (BioRad) (375 mM Tris-HCl pH 8.8, 6 M urea, 2% SDS, 30% glycerol and 25 mg/mL iodecetamide). After the re-hydration, the strips were immersed in the running buffer composed of 25 mM Tris-HCl, 1.92 mM glycine and 0.1% SDS for 5 min under agitation and used in the second dimension step in a 12% SDS-PAGE. The run was conducted for 2 h under 8W, 120 V and 20 mA using a PowerPac Universal Power Supply (BioRad). After the run, the gel was washed three times with milli-Q water (5 min each) and fixed using a solution of 2% phosphoric acid and 2% methanol for 1 h, washed with milli-Q water and stained with Blue Silver [21] for 12 h. The gels were decolorized with milli-Q water until the visualization of the protein spots. The gel images were captured using a densitometer GS-800 (BioRad) and the Quantity One software (BioRad). The analysis of the images was made using the PDQuest software (BioRad). Four biological replicates were considered for each condition analyzed. Statistical analyses were performed using ANOVA with 95% confidence level. Differences among protein spots with *p* value less than 0.05 were considered significant (*p* < 0.05).

## 2.5 Trypsin digestion and Mass Spectrometry

After the analysis of the images, the protein spots differently expressed for each condition (14% and 30% sucrose) were excised, inserted in a 1.5-mL micro tube and added with 250  $\mu$ L of a solution composed of (v/v) 50% ammonium bicarbonate (0.1 M, pH 8.0) and 50% acetonitrile P.A. for 3 h. Thereafter, the solution was removed and 700  $\mu$ L of this same solution was added, maintained for 12 h and then removed. For full dehydration, the tubes were added with 250  $\mu$ L of acetonitrile P.A. and maintained for 2 h without agitation. After this period, the solution was removed and the tube was maintained at room temperature for 24 h. The dehydrated gels were transferred to tubes of 0.5 mL and

added with 2.5  $\mu$ L Trypsin (Promega) and 17  $\mu$ L ammonium bicarbonate buffer (1 M, pH 8.0), and maintained at 37°C for 2 h. After this period, the tubes were added with 150  $\mu$ L of ammonium bicarbonate and maintained at room temperature for 22 h. The tryptic digestion was stopped by the addition of 4  $\mu$ L formic acid P.A. for 2 h. Previously to the analysis, each material digested was loaded in a Poros 50 R2 reverse phase column (PerSeptive Biosystems). The purified peptides were hydrated with 6  $\mu$ L of matrix solution composed of 5 mg/mL  $\alpha$ -cyano-4-hydroxycinnamic acid in 50% acetonitrile (v/v) and 0.1% trifluoroacetic acid (v/v). Thereafter, 2  $\mu$ L of each protein sample was applied in the MALDITOF/TOF plate (Axima performance, Kratos - Shimadzu, Manchester, UK.) and the MS/MS profile for each protein digested was analyzed using the MASCOT software (Matrix Science, London, UK) and NCBIInr database.

## 2.6 Analysis of the Fermentative Parameters

### 2.6.1 Cell growth and viability

The cell growth was determined through the biomass determination for each fermentative condition (2% sucrose as control; 14% and 21% sucrose as HGF; 30% sucrose as VHGF) at 540 nm according to the equation:

Cell concentration = Abs 540 nm x dilution x f (0.67  $\pm$  0.05 for *S. cerevisiae*)

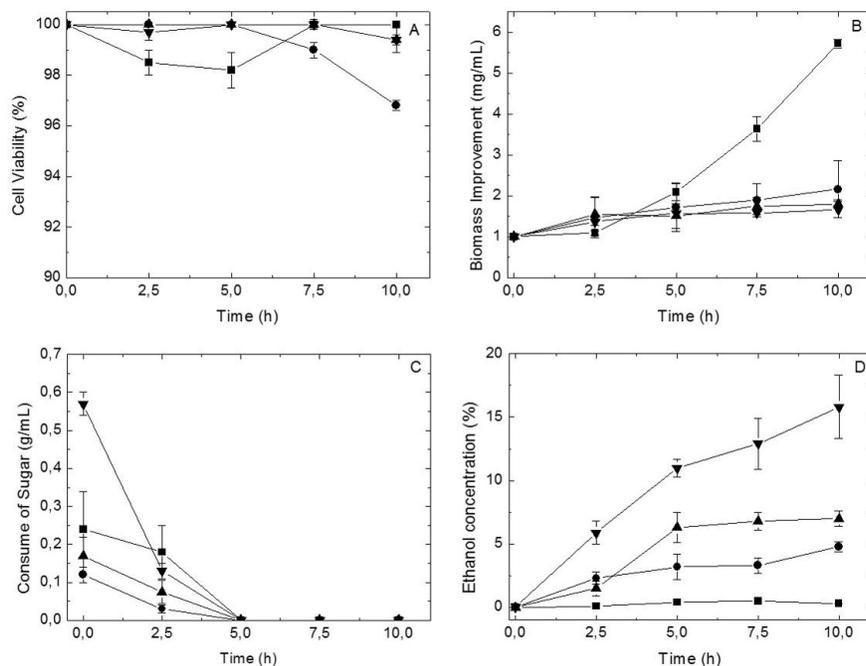
The cell viability was determined using methylene blue according to Shen *et al.* (2014) [22]. The analysis was performed on a microscope using an objective lens of 40X.

### 2.6.2 Determination of the consumption of carbon source and ethanol production

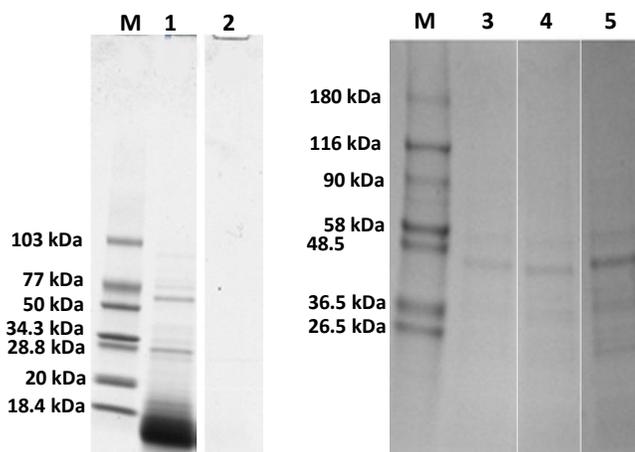
The evaluation of the consumption of the carbon source for each fermentative condition, as described above, was performed using DNS (3, 5-Dinitrosalicylic acid) according to Miller (1959) [23], at 546 nm. For ethanol quantification, the ethanol determination kit (Sigma-Aldrich) was used, according to the methodology described by Zanon *et al.* (2006) [24], at 340 nm.

## 3. RESULTS AND DISCUSSION

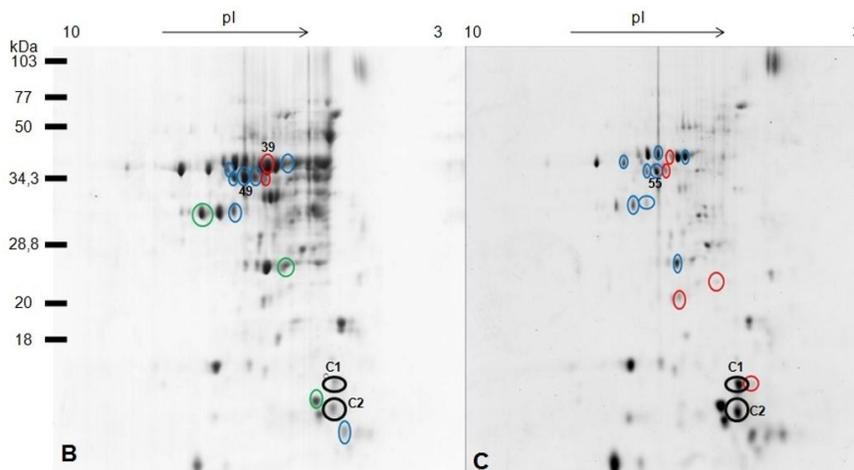
The yeast *S. cerevisiae* CAT-1 was able to grow under all sucrose concentrations (2%, 14%, 21% and 30%) used for the fermentative process. As it can be observed in the figure 1A, the cell viability was not drastically modified (96-100%) considering the different sucrose concentration adjusted in the fermentative medium for all periods analyzed. However, it is evident that the viability found at 30% sucrose after 10 h of fermentation was higher than that observed for the conditions containing 14% and 21% sucrose. The cell viability is an important factor for the alcoholic fermentation with direct relation with the product obtained. Drastic cell death can compromise ethanol production. According to the figure 1B, it is possible to observe an increase in the dried biomass for each fermentative condition (Figure 1B) considering that in the periods of fermentation, a 5.4-fold biomass increase is compared to the initial period.



**Fig. 1:** Cell viability (A), biomass increasing (B), consume of sugar (C) and ethanol production (D) for *S. cerevisiae* CAT-1 cultured in sugar cane juice containing 2% (square), 14% (circle), 21% (up triangle) and 30% (down triangle) sucrose for different periods.



**Fig. 2:** Electrophoretic profile at 4-15% SDS-PAGE for intracellular proteins obtained through culturing *S. cerevisiae* CAT-1 in the sugar cane juice containing 2% (lane 1), 14% (lane 3), 21% (lane 4) and 30% (lane 5) sucrose. M = molecular mass markers; Lane 2 = control (most without sucrose).



**Fig. 3:** Two-dimensional electrophoresis profile for proteins obtained from the *S. cerevisiae* CAT-1 cultured in sugar cane juice containing 30% sucrose (A) and 14% sucrose (B). The red and blue circles indicate the protein spots quantitatively different and the green circles indicate the protein spots qualitatively different.

These data indicate that the metabolic way was directed to cell growth and development. At 21% and 30% sucrose, there is no significant difference in the dried biomass. The figure 1C presents the use of sucrose during the fermentative process. It can be noted that, for 5-hour fermentation, the sucrose was fully consumed regardless of its initial concentration. The figure 1D shows the ethanol production under each fermentative condition used. The highest ethanol recovery was obtained under 30% sucrose in the fermentative medium. Under this condition, the ethanol recovery was 2.5-, 3.5- and 53-fold higher than that found for 21%, 14% and 2% sucrose in the fermentative medium. The recovery obtained at 30% sucrose was  $15.99 \pm 1.38\%$ , near the theoretic value (Table 1). When the sucrose was fully consumed (5 h), the ethanol production continued, but its slope was reduced. The same phenomenon was observed for *S. cerevisiae* and *Saccharomyces ludwigii* in the presence of additional glucose in the fermentative medium [25]. Mash with high sugar concentration containing glucose as carbon source added with maize syrup, urea and metallic ions promoted ethanol recovery of 19% [26].

Supplementation of the medium with the nitrogen source available and amino acids has also been applied to improve ethanol recovery [27]. Alternatively, the use of inoculum with high cell density has been mentioned as a good option for the supplementation of the fermentative medium [28]. According to Betite *et al.* (2012) [29], supplementation of VHGF with peptone and ammonium sulphate as nitrogen sources promoted an increase in the biomass and improved the fermentation. In the present work, 16% of ethanol recovery was obtained using *S. cerevisiae* CAT-1 under VHGF without the addition of nitrogen sources, which is a very interesting value considering the theoretic value and reduction of the fermentative process cost. It is important to remember that the ethanol concentration during the process can be inhibitory to the microorganism, but as presented in the viability data, the yeast *S. cerevisiae* CAT-1 has good tolerance to ethanol.

**Table 1:** Alcohol recovery for fermentation using 14%, 21% and 30% of dissolved solids in the sugar cane juice.

Dissolved solids (%)	Ethanol Recovery (%)	
	Real	Theoretical
14	$4.80 \pm 0.22$	7
21	$7.06 \pm 0.06$	7-11
30	$15.99 \pm 1.38$	17-19

After studying some fermentative parameters, the aim was focused on the analysis of proteome of *S. cerevisiae* CAT-1 cultured in both 14% and 30% sucrose in the medium to observe the yeast responses when submitted to VHGF. Most protein spots were obtained at a pI range 5.0-8.0 for both conditions. Considering the replicates for each culture condition, at 14% of sucrose, 107 protein spots were obtained, while at 30% of sucrose, 137 protein spots were obtained. Some of these protein spots are present in both conditions, but others are specific for 14% of sucrose and for 30% of sucrose.

The quantitative analysis, *i.e.*, the evaluation of the expression level (increase in the expression of a specific protein

when both conditions are compared), showed 15 different protein spots while the qualitative analysis indicated the presence of 3 different spots. In total, 27 protein spots with significant differences (statistical, quantitative and qualitative) were submitted to the identification and only 17 protein spots were effectively identified according to their tryptic peptides using the Mascot software (Table S1). Properties for each protein spot identified, such as pI and molecular mass, as well as similarity and function, are reported in the Table 2.

Most proteins identified are involved in glycolysis and fermentation pathways, showing differences in their expression levels under VHGF and HGF, as also pointed out by Pham *et al.* (2006) [16] for the proteomic analysis of *S. cerevisiae* KAY446. The protein identified as phosphopiruvate hydratase (enolase) ENO2p is an important enzyme in the glycolytic pathway responsible to convert the 2-phosphoglycerate into phosphoenolpyruvate. According to this, the ENO2p presents a direct impact on the ethanol production during the fermentative process [13, 30], what can explain its increased expression in the condition of 30% of sucrose, 2-fold higher than that observed in the condition with 14% of sucrose. Another important protein for the ethanol production, the alcohol dehydrogenase (ADH1p), is responsible for the reduction of the acetaldehyde to ethanol [31], what justifies the presence of multiple protein spots for this enzyme.

In general, the protein spots identified as ADH1p were more expressed under 30% of sucrose than 14% of sucrose. The presence of multiple ADH1p can be explained by the post-translational processing as acetylation and phosphorylation [13]. In addition, it is possible to indicate that under high sucrose concentration, the glucose concentration obtained from sucrose hydrolysis will increase and, consequently, the production of ethanol will as well, highlighting the importance of the increased levels of ADH1p in the presence of 30% of sucrose in the culture medium. Other proteins were also identified in spite of the inexistence of quantitative or qualitative differences when compared to the protein spots obtained for both 14% and 30% of sucrose as, for example, the dithiol glutaredoxine (GRX 1) and the restriction of telomere-capping protein 3 (Rtc3p), among others. The former is an important glutathione-dependent protein from the oxidoreductase family, responsible for the regulation of the reduced state in yeast cells, protecting these cells from the oxidative stress in the culture medium [32]. The presence of proteins involved in the responses to oxidative stress is important to understand the stress tolerance observed for CAT-1 strain, as also reported by Santos *et al.* (2016) [2]. The protein Rtc3p is related to the RNA metabolism [33]. However, Zhao *et al.* (2014) [34] demonstrated that this protein, together with other proteins, has a key function in the nitrogen metabolism in the yeast cells. Triose phosphate isomerase (TPI) is another enzyme identified with great importance in the glycolysis pathway. This enzyme is responsible for the 1,2-hydrogen shift at dihydroxyacetone phosphate (DHAP) to produce glyceraldehyde 3-phosphate (GAP) [35].

**Table S1:** Identified peptides for each spot with protein match by MS/MS.

Spot	Identified peptides	Protein (similarity)
1	LVSWYDNEYGYSTR	Unnamed protein product
2	TFFVGGNFK	Chain A – triosephosphate isomerase
40	SISIVGSYVGNR VLGIDGGEGKEELFR	Alcohol dehydrogenase – ADH1
43	DGKYDLDFK IGSEVYHNLK NVNDVIAPAFVK VNQIGTLESSEIK LGNAILGVSLAASR AAQDSFAAGWGMVSHR SIVPSGASTGVHEALEMR	Enolase
45	YVLEHHPR ELPGVAFLESEK ASAPGSVILLENLR	Phosphoglycerate kinase
49	SISIVGSYVGNR VLGIDGGEGKEELFR	Alcohol dehydrogenase – ADH1
50	MVSMILSIIVFGKSVR	Hut1p
52	TFFVGGNFK SYFHEDDEFIADK	Chain A Triose phosphate isomerase Tpi1p
53	YHIEEGSR ELPGVAFLESEK ASAPGSVILLENLR	Phosphoglycerate kinase
54	GVIFYESHGK SISIVGSYVGNR VLGIDGGEGKEELFR	Alcohol dehydrogenase – ADH1
55	GVIFYESHGK SISIVGSYVGNR VLGIDGGEGKEELFR	Alcohol dehydrogenase – ADH1
56	SISIVGSYVGNR VLGIDGGEGKEELFR	Alcohol dehydrogenase – ADH1
58	LVSWYDDEYGYSTR	Glyceraldehyde phosphate dehydrogenase - GAPDH
59	FAEQYSDAAFYK AEVSSMPTLIFYK	Chain A of Mxr1-Trx2
A	GFGYAGSPFHR	Peptidylprolyl isomerase CPR1
C1	HIGGNDLQELR	Dithiol glutaredoxin GRX1
C2	AQVENEFVK IEEVIDLILR	Rtc3p

Other peptide matches were found but they were not assigned to protein hits using the Mascot software.

**Table 2:** Identification of the protein spots from *S. cerevisiae* CAT-1 cultured under 14% and 30% sucrose.

	Spot	Hits	Score	Mr	pI	Protein (similarity)	Function	Organism
VHGF (30% sucrose)	1	gi 3720	41	35.823	6.46	Unnamed protein product	glycolysis	<i>S. cerevisiae</i>
	2	gi 230405	78	26.762	5.75	Chain A – triosephosphate isomerase	glycolysis	<i>S. cerevisiae</i>
	40	gi 3339	51	37.256	6.26	Alcohol dehydrogenase – ADH1	fermentation	<i>S. cerevisiae</i>
	43	gi 171455	171	46.830	10.16	Enolase	glycolysis	<i>S. cerevisiae</i>
	45	gi 10383781	85	44.768	7.11	Phosphoglycerate kinase	glycolysis	<i>S. cerevisiae</i>
	49	gi 3339	73	37.256	6.26	Alcohol dehydrogenase – ADH1	fermentation	<i>S. cerevisiae</i>
HGF (14% sucrose)	50	gi 6325012	46	38.335	9.76	Hut1p	Protein folding	<i>S. cerevisiae</i>
	52	gi 230405	103	26.762	5.75	Chain A Triose phosphate isomerase	glycolysis	<i>S. cerevisiae</i>
		gi 323309847	156	26.873	5.53	Tpi1p		
	53	gi 10383781	79	44.768	7.11	Phosphoglycerate kinase	glycolysis	<i>S. cerevisiae</i>
	54	gi 3339	231	37.256	6.26	Alcohol dehydrogenase – ADH1	fermentation	<i>S. cerevisiae</i>
	55	gi 3339	241	37.256	6.26	Alcohol dehydrogenase – ADH1	fermentation	<i>S. cerevisiae</i>
	56	gi 3339	114	37.256	6.26	Alcohol dehydrogenase – ADH1	fermentation	<i>S. cerevisiae</i>
Both	58	gi 229428	94	35.638	9.17	Glyceraldehydophosphate dehydrogenase - GAPDH	glycolysis	Saccharomycetales
	59	gi 122920148	126	12.264	6.02	Chain A of Mxr1-Trx2	Cell protection	<i>S. cerevisiae</i>
	A	gi 6320359	72	17.494	6.9	Peptidylprolyl isomerase CPR1	Protein folding and sporulation	<i>S. cerevisiae</i>
	C1	gi 6319814	41	12.486	4.98	Dithiol glutaredoxin GRX1	Cell protection	<i>S. cerevisiae</i>
	C2	gi 6321879	68	12.002	5.05	Rtc3p	Nitrogen metabolism	<i>S. cerevisiae</i>

Only 27 protein spots with significant differences (statistical, quantitative and qualitative) were submitted to the identification and 17 protein spots were effectively identified. Identification of protein spots with score value lower than 20 were not considered.

The phosphoglycerate kinase (PGK) is another important enzyme in the glycolysis pathway responsible to catalyze the transfer of a phosphate group from 1,2-bisphosphoglycerate to ADP producing ATP and 3-phosphoglycerate. In the gluconeogenesis, this enzyme catalyzes the reverse reaction producing ADP and 1,2-bisphosphoglycerate. It has been demonstrated that the PGK over expression could be related to the higher production of intracellular ATP in pre-apoptotic yeast cells, restoring the normal aging of the *Saccharomyces* cells [36]. Triose phosphate isomerase is a key enzyme in both glycolysis and gluconeogenesis. This enzyme catalyzes the reversible reaction of glyceraldehyde 3-phosphate to dihydroxyacetone phosphate. The protein identified as Hut1p, an UDP-galactose transporter was shown to be involved in the maintenance of the optimal environment for protein folding in the endoplasmic reticulum in *S. cerevisiae* cells and in *Schizosaccharomyces pombe* cells [37]. Peptidyl prolyl isomerase CPR1 is a conserved cyclophilin from yeast to human, but it is not essential. This enzyme is related to both the peptidyl-prolyl isomerization during protein folding or conformational changes and the meiosis to promote an efficient sporulation when associated with other two proteins from the histone deacetylase complexes and in the glucose-stimulated transport of fructose-1,6-bisphosphatase into Vid vesicles [38, 39]. The protein Mxr1-Trx2 is a methionine S-sulfoxide reductase that uses thioredoxin as electron donor [40, 41]. Both thioredoxin-1 (Trx1) and thioredoxin-2 (Trx2) were previously identified in the quantitative proteome analysis of *S. cerevisiae* CAT-1, constituting important elements in the stress tolerance observed for this yeast [2]. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), an important enzyme in the carbohydrate metabolism, is responsible for the oxidative phosphorylation of glyceraldehyde-3-phosphate into 1-3-diphosphoglycerate in the presence of nicotinamide adenine dinucleotide (NAD<sup>+</sup>). Despite its importance for glycolysis and gluconeogenesis, studies have presented other roles of GAPDH such as DNA replication and repair, membrane fusion and transport and tRNA export, [42].

Different authors have reported the analysis of the *S. cerevisiae* proteome [15, 34, 43], but not specifically for the CAT-1 strain. Recently, Santos et al. (2016) [2] analyzed the proteome of *S. cerevisiae* CAT-1 and they found 16 proteins up regulated when the yeast was maintained under batch fermentation using a synthetic medium. Proteins, heat shock, cell membrane and cell wall proteins, among others, were identified. Differently, we described the proteome analysis under VHGF using sugarcane juice as the fermentative medium, finding especially proteins involved in the fermentation.

#### 4. CONCLUSION

The proteome analysis of the yeast *S. cerevisiae* CAT-1 submitted to VHGF using high cellular density revealed qualitative and quantitative differences in the protein expression when compared to HGF condition. Most of the proteins identified have an important role in the glycolysis pathway. The increased

expression of some proteins under VHGF, such as ENO2p, can explain the improvement in the ethanol production. In spite of the high ethanol concentration under VHGF condition, the cell viability was also improved, what is important to the fermentative process. The robustness of the CAT-1 strain was confirmed, reinforcing its potential to be used for high ethanol recovery, using VHGF technology with high cell density, from sugar cane juice, what can positively impact on the ethanol market.

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