Brazilian Journal of Microbiology 45, 2, 475-483 (2014)
ISSN 1678-4405

Research Paper

Biomarkers to evaluate the effects of temperature and methanol on recombinant *Pichia pastoris*

Andrea B. Zepeda[1,2,*], Carolina A. Figueroa[1,2,*], Dulcineia S.P. Abdalla[3], Andrea Q. Maranhão[4], Patricio H. Ulloa[1], Adalberto Pessoa Jr[2], Jorge G. Farías[1]

[1]Departamento de Ingeniería Química, Facultad de Ingeniería, Ciencias y Administración, Universidad de La Frontera, Temuco, Chile.
[2]Departamento de Tecnologia Bioquímico-Farmacêutica, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, SP, Brazil.
[3]Departamento de Análises Clínicas e Toxicológicas, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, SP, Brazil.
[4]Departamento de Biología Celular, Instituto de Ciências Biológicas, Universidade de Brasilia, Brasília, DF, Brazil.

Submitted: May 27, 2013; Approved: September 9, 2013.

Abstract

Pichia pastoris is methylotrophic yeast used as an efficient expression system for heterologous protein production. In order to evaluate the effects of temperature (10 and 30 °C) and methanol (1 and 3% (v/v)) on genetically-modified *Pichia pastoris*, different biomarkers were evaluated: Heat stress (HSF-1 and Hsp70), oxidative stress (OGG1 and TBARS) and antioxidant (GLR). Three yeast cultures were performed: 3X = 3% methanol-10 °C, 4X = 3% methanol-30 °C, and 5X = 1% methanol-10°C. The expression level of HIF-1α, HSF-1, HSP-70 and HSP-90 biomarkers were measured by Western blot and *in situ* detection was performed by immunocytochemistry. Ours results show that at 3% methanol -30 °C there is an increase of mitochondrial OGG1 (mtOGG1), Glutathione Reductase (GLR) and TBARS. In addition, there was a cytosolic expression of HSF-1 and HSP-70, which indicates a deprotection against nucleolar fragmentation (apoptosis). On the other hand, at 3% methanol -10 °C and 1% and at methanol -10 °C conditions there was nuclear expression of OGG1, lower levels of TBARS and lower expression of GLR, cytosolic expression of HSF-1 and nuclear expression HSP-70. In conclusion, our results suggest that 3% methanol-30 °C is a condition that induces a strong oxidative stress and risk factors of apoptosis in modified-genetically *P. pastoris*.

Key words: oxidative stress, recombinant yeast, heat stress protein, DNA damage.

Introduction

The pharmaceutical industry uses different types of cell cultures to produce biological drugs in high quantities (Panagiotou *et al.*, 2011), such as monoclonal antibodies which appear as a prominent therapeutic intervention but requires appropriate post-translational modifications to be effective and safe for the human (Sohn *et al.*, 2010).

Currently, there are various expression systems for recombinant antibodies such as *Escherichia coli*, insect cells, yeast and mammalian cells (Hayden *et al.*, 1997). Within the broad strain of yeasts used as expression systems, the methylotrophic yeast *Pichia pastoris* has emerged as an important host for heterologous protein expression in both biomedical research and industrial biotechnology (Solà *et al.*, 2004; Ahn *et al.*, 2009) and recently in metabolic engineering applications (Sohn *et al.*, 2010), because it is considered as a economic system of well-defined fermentation process, growth to high cell density has been shown to substantially improve the production of heterologous proteins (Whyteside *et al.*, 2011) and ease the genetic manipulation (van der Klei *et al.*, 2006). This system

Send correspondence to J.G. Farías. Departamento de Ingeniería Química, Facultad de Ingeniería, Ciencias y Administración. Universidad de La Frontera, Casilla 54-D, Temuco, Chile. E-mail: jorge.farias@ufrontera.cl.
* These authors contributed equally to this work.

also has a process of post-translational modifications similar to humans which allows a simple purification system of heterologous proteins due to secrete low levels of endogenous proteins (Macauley-Patrick *et al.*, 2005; Damasceno *et al.*, 2012), beside the production of heterologous proteins is associated with strong inducible promoter by methanol, alcohol oxidase gene 1 (*AOX1*) (Arakawa *et al.*, 2006; Xuan *et al.*, 2009; Ahn *et al.*, 2009).

The expression level of proteins in *P. pastoris* depends critically on growth conditions (Solà *et al.*, 2007), such as temperature, carbon source (Ni *et al.*, 2008) and oxygen (Verbelen *et al.*, 2009), which have been proven to be cell type-specific and varied depending on the product that is generated, so it has not yet been achieved a specific method, like biomarkers, to determine the effects of these conditions to find the optimal parameters for each model. Among the adverse consequences caused by disregard the effects of these parameters on the cultivation of yeast, temperature conditions would affect the expression of the transcription factor known as factor of response to heat stress (HSF-1) (Gasser *et al.*, 2007), so it would regulate HSP-70 expression. Heat shock proteins (HSPs) are a family of molecular chaperones, which are indispensable in physiological states and exhibit a protective role in pathological processes; there are conditions of environmental stress such as heat shock and some pathological states that induce the expression of HSPs (Khalil *et al.*, 2011).

Another effect of environmental conditions is the generation of reactive oxygen species (ROS), which can be generated endogenously in the presence of aerobic metabolism from mitochondria or generated during the metabolism of methanol when this is oxidized to formaldehyde (HCHO), producing high levels of hydrogen peroxide (H_2O_2) which has the ability to easily become a more damaging species (Bener *et al.*, 2008), resulting in membrane lipid peroxidation (Priault *et al.*, 2002), carbonylation and oxidation of residues of proteins and causing oxidation of nitrogen bases and strand breaks in DNA (Cash *et al.*, 2007). One of the most common oxidative damage that affects the nitrogen bases is known as 7,8-dihydro-8-oxoguanine (8-oxoguanine) and it is the result of guanine oxidation (Sandigursky *et al.*, 1997). However, there is an enzyme responsible for 8-oxoguanine repair known as OGG1 (8-oxoguanine DNA glycosylase) and this has OG-glycosylase/AP ligase activity (Leipold *et al.*, 2003; Solà *et al.*, 2004). There is an antioxidant enzyme that protects the cells from oxidative stress, Glutathione Reductase (GLR) converts the oxidized form (GSSG) to reduced form of Glutathione (GSH) (Yano *et al.*, 2009b). GSH scavenges cytotoxic H_2O_2 and maintains a redox balance in the cellular compartments (Blokhina *et al.*, 2003). Despite this information, temperature and methanol effects on the heat and oxidative stress biomarkers in *P. pastoris* cultures during monoclonal antibodies production has not yet been determined. For this reason, the aim of this study is to determine the optimal conditions using biomarkers related with heat stress and oxidative stress on genetically-modified *P. pastoris*.

Materials and Methods

Reagents

The solvents used were of analytical grade. The culture medium and glycerol were autoclaved at 121 °C for 20 min at 1 atm and glucose at 0.5 atm. Solutions and buffers were prepared with deionized water, besides methanol, biotin, ampicillin, casamino acids, YNB medium ("Yeast Nitrogen Base") and other reagents were sterilized by filtration on membrane of 0.22 microns.

Strain

We used lineage SMD1168 (Invitrogen ®): Δ*pep*4::*URA*3 Δ*kex*1::*SUC*2*his*4*ura3* with His^-Mut^+ phenotype which was used for the expression of scFv. The genetically modified strain was provided by the research group of Professor Dr. Dulcineia Saes Parra Abdalla of the Department of Clinical and Toxicological Analysis of the Ciências Farmacêuticas-USP and was built by the group of Prof. Dr. Andrea Maranhão of the Department of Molecular Biology of Universidade de Brasília.

Maintenance and reactivation of *P. pastoris*

For the preservation of cells of *P. pastoris*, the colonies were replicated every three months on YPD solid medium (Yeast Extract Peptone Dextrose) (yeast extract 1% (w/v), casein peptone 2% (w/v), glucose 2% (w/v), bacteriological agar 1.2% (w/v) and incubated at 30 °C for 24 h. After that period, the colonies were removed from plates and inoculated into Erlenmeyer flasks of 500 mL capacity, containing 100 mL of YPD liquid medium at 30 °C and 250 rpm for 24 h. Then, the colonies were stored at 4 °C and -70 °C in YPD medium containing 20% sterile glycerol. For the reactivation step, 1 mL of frozen material was inoculated in Erlenmeyer flasks of 500 mL capacity containing 100 mL growth medium BMGY (Buffered Glycerol Complex Medium) (YNB medium 0,34% (w/v) + ammonium sulfate 1% (w/v), yeast extract 1% (w/v), casein peptone 2% (w/v), buffer potassium phosphate (100 mM) pH 6.0, biotin $4x10^{-5}$% (w/v), glycerol 1% (v/v), casamino acids 2% (w/v) and incubated at 30 °C and 250 rpm for 16 h.

Preparation of *P. pastoris* inoculum in shaker (growth phase)

For inoculum stage in a stirrer, it was prepared BMGY medium through five solutions (1: 2 g Yeast Extract, 4 g peptone, dilute to 50 mL with deionized water, 2: 20 mL buffer phosphate, 2 g glycerol, 3: 2.68 g Yeast Nitrogen Base and dilute to 50 mL with deionized water, 2 g of ammonium sulfate and dilute to 40 mL with deionized water, 4: 4 g casamino acids and dilute to 40 mL with

deionized water, 5: 400 mL of biotin) to 200 mL in a 500 mL Erlenmeyer flask and withdrew 10% of the initial volume (20 mL) which was used to cultivate 200 mL of *P. pastoris* strain genetically modified and incubated at 30 °C at 250 rpm for 16 h. Subsequently, the inoculum is transferred to 180 mL of BMGY medium and incubated at 30 °C at 250 rpm for 24 h.

Induction phase in a shaker

After the growth phase (40 h) it was added 1% and 3% methanol. To inhibit the production of protease was also added 1 mM PMSF (phenylmethanesulfonylfluoride). Before the addition of methanol, the temperature was adjusted to 10 °C and 30 °C. This induction phase was carried out after 24, 48 h and 72 h. The total culture time was 96 h.

Experimental Design After 96 h each culture was centrifuged at 1957 x g for 30 min where aliquots of 2 mL were obtained for its use in subsequent trials. The samples for analysis corresponded to: 3X: 3% methanol -10 °C; 4X: 3% methanol -30 °C and 5X: 1% methanol -10 °C.

Quantification of proteins

The cell lysate was performed by ultrasonication for 30 min in ultrasonicator bath Elmasonic E 60 H (Elma, Singen, Germany). Quantification of proteins was performed through the Coomassie blue method (Bradford, 1976). The calibration curve was performed with BSA (stock 2 mg/mL) to a standard concentration of 100 µg/mL and the dilution was made with distilled water, the absorbance measurement at 595 nm was performed in Spectrophotometer Optizen 3220 UV (Mecasys Co., Daejeon, Rep. of Korea) and its concentration was calculated according to the initial ratio volume and initial concentration *vs.* the volume and final concentration. According to the method of cell lysate, it was used 5 to 20 µL of these cells within UV Macro 3.5 mL (Arquimed) then it was added distilled water to 100 µL plus 1 mL of 1X Bradford, mixed and allowed to incubate for 5 min at room temperature and then was measured. The results were expressed as mg protein / mL of cells.

Determination of lipid peroxidation

Lipid peroxidation was quantified by measuring thiobarbituric acid reactive substances (TBARS) produced from the reaction of TBA with malondialdehyde (MDA) (Farías *et al.*, 2012). Cell disruption was performed by using lysis buffer. Subsequently the samples were washed twice with distilled water and suspended at a concentration of 5 x10^8 cells/mL. To each sample it was added cold trichloroacetic acid (TCA) 10% (v/v), then incubated for 15 min on ice and centrifuged for 15 min at 2367 x g at 4 °C (Eppendorf 5804R, Germany). Subsequently the supernatant was incubated with twice the volume of TBA 0.67% (v/v) for 20 min at 95 °C in thermostated bath (YCW-0125, Gemmy Industrial Corporation, Taiwan), then the mixture was cooled on ice, centrifuged 4 s to 1380 x g and the absorbance was measured at 532 nm in the Spectrophotometer 3220-UV Optizen (Mecasys Co., Daejeon, Republic of Korea). We performed a calibration curve using 1,1,3,3-tetramethoxypropane 500 µM as standard. The results were expressed as micromoles of TBARS by 5x 10^8 cells (Kwolek-Mirek *et al.*, 2009). The concentrations were determined in triplicate for each sample.

SDS / PAGE and Western blot analysis

To evaluate and correlate the expression levels of 8-oxoguanine DNA glycosylase (OGG1), Glutathione Reductase (GLR), factor of response to heat stress (HSF-1) and heat shock protein 70 (HSP-70), the levels of all proteins were measured to different growing conditions in a shaker. The protein samples were separated by 10% SDS/PAGE (HSF-1), 12% SDS-PAGE (HSP-70 and OGG1) and 5% SDS-PAGE (GLR), then these were transferred to membrane Hybond-C (Amersham Pharmacia, Piscataway, NJ, USA) using a transfer cell Transfer-blot SD Semi-dry (Bio-Rad, Tokyo, Japan). The membranes were then blocked by incubation with skim milk 5% (w/v) in PBS, pH 7.2 for 1 h at room temperature (RT) under mild agitation. Subsequently, the membranes were incubated with rabbit anti-(rat-OGG1) IgG (1:50 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-(-rat-GLR) IgG (1:50 dilution), rabbit anti-(rat-HSF-1) IgG (1:50 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti-(rat-HSP-70) IgG (1:50 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 16 h at 4 °C. After the membranes were washed 5 times for 10 min with PBS, pH 7.2, containing Tween-20 0.1% (v/v), a fourth wash was carried out only with PBS pH 7.2 to remove all traces of detergent, then were incubated with peroxidase-conjugated goat anti-(rabbit IgG) IgG (dilution 1:500) (Jackson Laboratories ImmuneResearch, PA, USA) and peroxidase-conjugated goat anti-(mouse IgG) Ig (dilution 1:500) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h. After being washed 5 times for 10 min with PBS, pH 7.2, containing Tween-20 0.1% (v/v) and once for 5 min with PBS pH 7.2 only, the peroxidase activity was detected by a chemiluminescent method using an ECL Plus kit (Amersham Pharmacia, Buckinghamshire, UK). The β-actin antibody (Sigma) was used as loading control in Western blot in dilution 1:50.

Immunocytochemistry

Cells were fixed in formalin 3.7% (v/v) and permeabilized with cold methanol (-20 °C). Endogenous peroxidase was blocked with H_2O_2 0.3% (v/v) in dark for 30 min at RT. The cells were blocked with bovine serum albumin (BSA, Sigma-Aldrich) 5% at RT for 15 min and washed for 5 min with PBS. Then they were incubated with rabbit anti-(rat-HSF-1) IgG (1:100 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti-(rat-

HSP-70) IgG (1:100 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at 37 °C in a humid chamber. Later were incubated with peroxidase-conjugated goat anti-(rabbit IgG) IgG (dilution 1:1000) (Jackson Laboratories ImmuneResearch, PA, USA) and peroxidase-conjugated goat anti-(mouse IgG) IgG (dilution 1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min at RT. The peroxidase activity was visualized using 1,3-diaminobenzidine (DAB) and were counterstained with hematoxylin. The slides were immersed in a series of alcohols, in increasing order (70°, 95° and 100°) for 5 min each one, then allowed to soak for 10 min in xylol and finally made the final assemble end where the slides were observed under the BX43 Optical microscope (Olympus, Tokyo, Japan).

Data analysis

The results show that normal distribution was analyzed using One Way test - ANOVA followed by Tukey analysis. ANOVA analysis was performed to determine significant interaction between the expression of proteins-OGG-1, GLR, HSF-1 and HSP-70 - and different culture conditions, because this could indicate a difference in the expression of these molecules. We also analyzed using ANOVA, the level of lipid peroxidation in different cultures. The statistical significance level used was $p < 0.05$ for all tests. Results are presented as mean ± standard deviation.

Results

Oxidative stress

In order to evaluate the effects of temperature and methanol, TBARS was analyzed. For the recombinant yeast, in the three conditions, TBARS shows in the 4X (3% (v/v) Methanol -30 °C) condition an increase of oxidative stress in comparison to 3X (3% (v/v) Methanol -10 °C) and 5X (1% (v/v) Methanol -10 °C) conditions ($p < 0.05$; Figure 1).

Expression level of nuclear repair proteins, antioxidant and heat stress protein

To determine oxidative DNA damage, OGG1-1a and OGG1-2a were evaluated. OGG1 is an enzyme responsible for repairing the DNA damaged by oxidative stress. Our results showed that in 3X and 5X cultures OGG1-1a was expressed (nuclear); while in 4X culture both subunits OGG1-1a (nuclear) and OGG1-2a (mitochondrial) (Figure 2) were expressed.

To determine antioxidant defense, GLR was evaluated. GLR is an enzyme that maintains the reduced levels of Glutathione, acting as antioxidant protection. Our results showed that in 4X culture was observed higher expression of GLR in comparison with 3X and 5X cultures ($p < 0.05$; Figure 3).

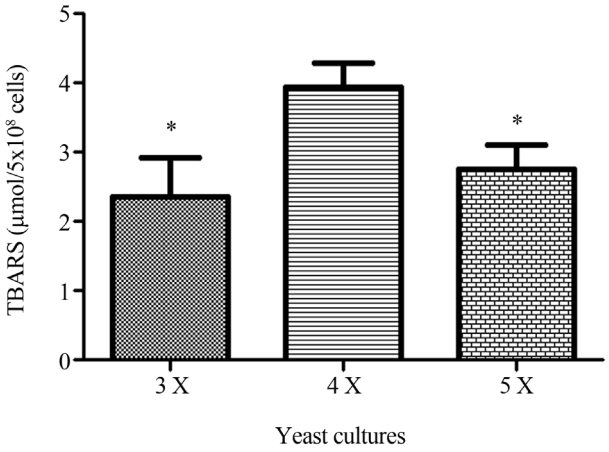

Figure 1 - Protein expression level of OGG1. Western blotting and band intensity obtained from samples with different conditions of temperature and concentration of methanol were analyzed. The bars indicate mean ± standard deviation of n = 3. Statistical analysis: ANOVA one-way followed by Tukey analysis. $p < 0.05$, * indicates there was significant difference in respect to OGG1-1a subunit of 4X, ** indicates there was significant difference in respect to OGG1-1a subunit of 5X. 3X = 3% (v/v) Methanol -10 °C, 4X = 3% (v/v) Methanol -30 °C, 5X = 1% (v/v) Methanol -10 °C.

The expression of HSF-1 did not shown significantly difference between all culture conditions ($p > 0.05$; Figure 4). HSF-1 protein expression was mainly found in the cytoplasm of yeast under all three culture condition. Also, in the 5X culture HSF-1 was found in the nucleus, indicating that it might be acting in the transcription of various proteins in response to heat stress. These proteins would act as chaperones in the regulation of protein stability to achieve the conformation necessary to fulfill their role (Figure 5). On the other hand, HSP-70 was mainly expressed in the nucleus in 3X and 5X culture conditions; while that in 4X culture was found in the cytoplasm (Figure 5).

Discussion

Methylotrophic yeast *P. pastoris* has several characteristics that allow it to be a more optimal host for heterologous protein production and/or monoclonal antibodies. Perhaps the most important for the industry is that allows a faster and more efficient purification process because of the low concentration of endogenous proteins that it normally produces. In the search for biomarkers, this property is of great concern because these characteristics are objectively measured and evaluated as an indicator of normal biological processes or pharmacologic responses to therapeutic intervention (van Lammeren *et al.*, 2011). Nowadays, production of high amounts of proteins of therapeutic interest has generated the need to scale *P. pastoris* cultures to increase the cell density and the product of interest (Arakawa *et al.*, 2006). However, *P. pastoris* has presented problems when scaled, in terms of cell instability in the density of the culture. This has directly affected the production of

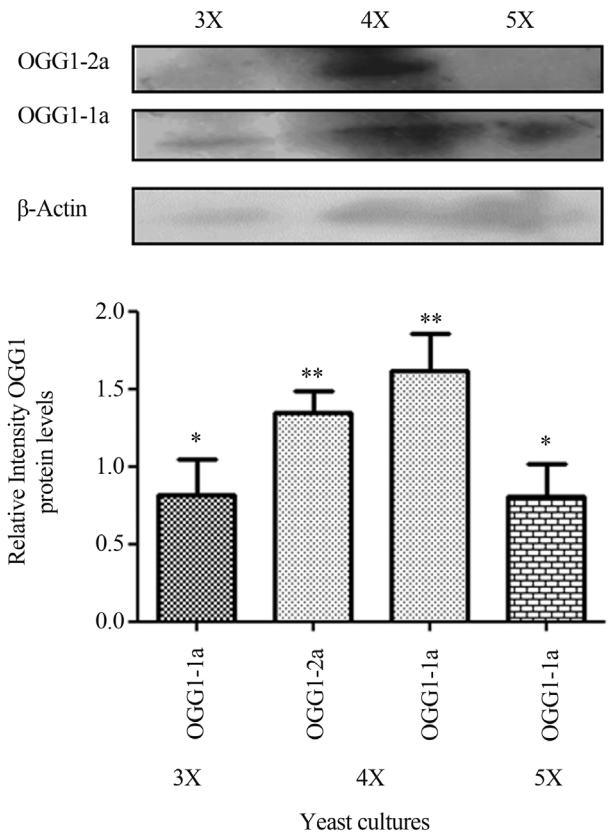

Figure 2 - Protein expression level of OGG1. Western blotting and band intensity obtained from samples with different conditions of temperature and concentration of methanol were analyzed. The bars indicate mean ± standard deviation of n = 3. Statistical analysis: ANOVA one-way followed by Tukey analysis. $p < 0.05$, * indicates there was significant difference in respect to OGG1-1a subunit of 4X, ** indicates there was significant difference in respect to OGG1-1a subunit of 5X. 3X = 3% (v/v) Methanol -10 °C, 4X = 3% (v/v) Methanol -30 °C, 5X = 1% (v/v) Methanol -10 °C.

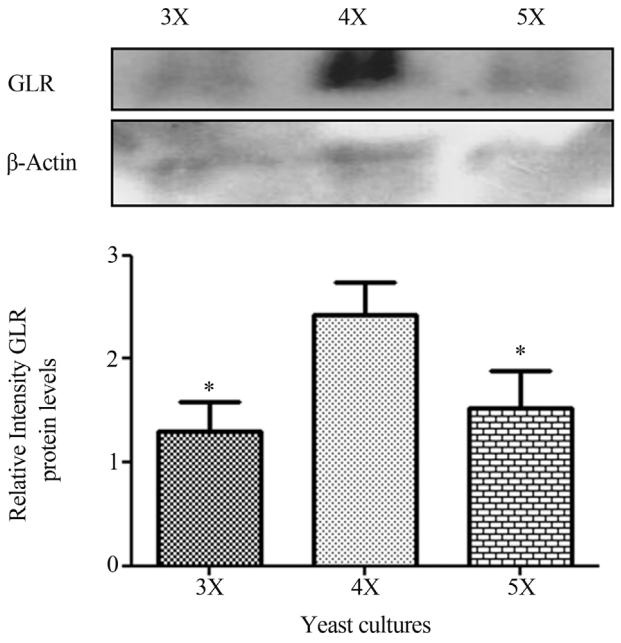

Figure 3 - Protein expression level of GLR. Western blotting and band intensity obtained from samples with different conditions of temperature and concentration of methanol were analyzed. The bars indicate mean ± standard deviation of n = 3. Statistical analysis: ANOVA one-way followed by Tukey analysis. * $p < 0.05$, indicates there was significant difference in respect to 4X. 3X = 3% (v/v) Methanol -10 °C, 4X = 3% (v/v) Methanol -30 °C, 5X = 1% (v/v) Methanol -10 °C.

heterologous proteins that requires maintaining a constant specific growth rate. Our results shown a higher concentration when the yeast was grown at 10 °C, differing from those observed at 15 °C in such yeasts as *P. pastoris* and *Saccharomyces cerevisiae*, where the biomass was reduced (Steels *et al.*, 1994; Pizarro *et al.*, 2008) and where methanol could have negative effects on cell growth compared to other inductors such as glycerol and glucose (Dragosits *et al.*, 2011).

Regarding to the extent of lipid peroxidation in recombinant yeasts, our results showed higher levels of TBARS under conditions of methanol 3% (v/v) and at 30 °C. These could be related to those reported by several authors, where *P. pastoris* cultures at 20 °C induce the transcription of genes that encode enzymes which regulates oxide/reduction homeostasis, such as glutathione reductase (GLR1) and thioredoxin reductase (TRR1) related to the antioxidant protection (Dragosits *et al.*, 2009). However, the exposure of the yeast to methanol also generates meta-

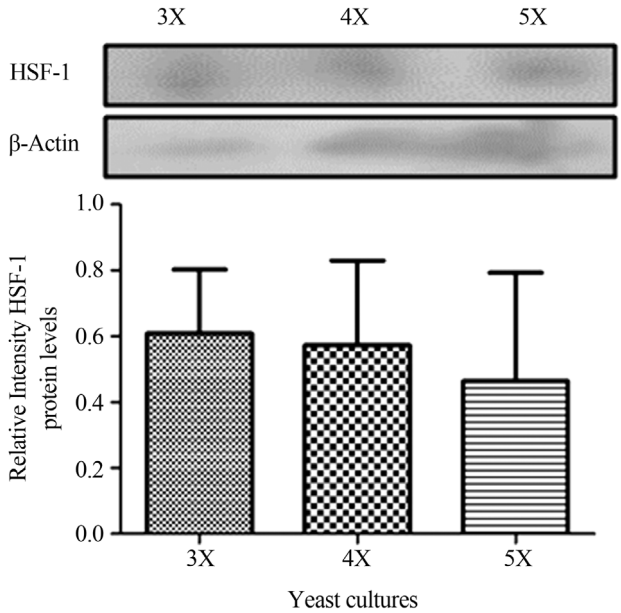

Figure 4 - Protein expression level of heat stress factor. Western blotting and band intensity of HSF-1 obtained from samples with different conditions of temperature and concentration of methanol were analyzed. The bars indicate mean ± standard deviation of n = 3. Statistical analysis: ANOVA one-way followed by Tukey analysis. There was no significant difference between either group ($p > 0.05$). 3X = 3% (v/v) Methanol -10 °C, 4X = 3% (v/v) Methanol -30 °C, 5X = 1% (v/v) Methanol -10 °C.

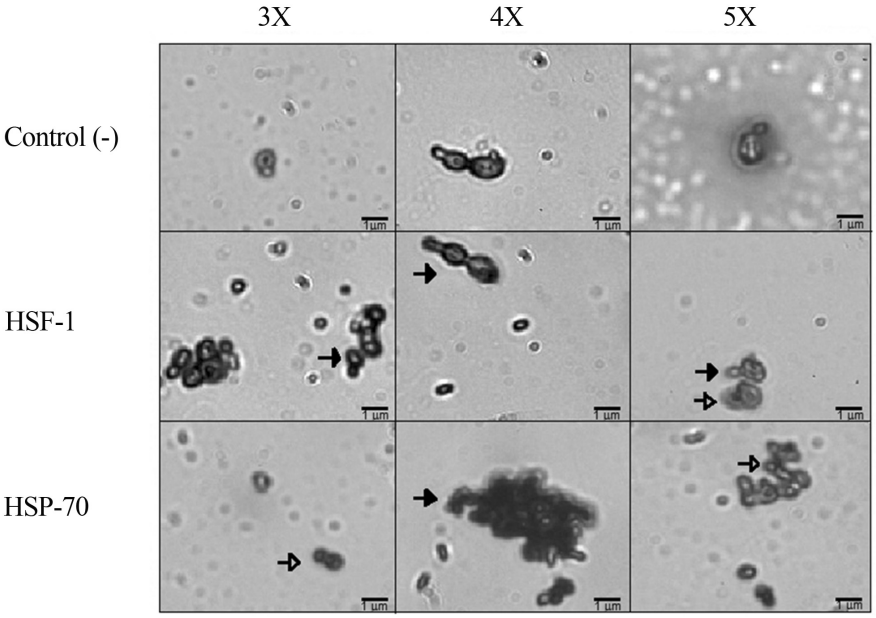

Figure 5 - Presence *in situ* of heat shock proteins. Immunolocalization of HSF-1 and HSP-70 in *P. pastoris* yeast cells under different conditions of temperature and concentrations of methanol. 3X = 3% (v/v) Methanol -10 °C, 4X = 3% (v/v) Methanol -30 °C, 5X = 1% (v/v) Methanol -10 °C. Closed arrows indicate cytoplasm and open arrows indicate nucleus.

bolic products which act as reactive oxygen species, such as hydrogen peroxide. It can become a harmful molecule capable for generating membrane lipid peroxidation (Jin *et al.*, 2011).

Among the considerations to be taken into account when analyzing both the effects of the inductor and temperature, is the composition of lipid membranes that are essential for the cell stability and are seriously affected by oxidative damage. In accordance to that, the culture at 30 °C and 3% (v/v) of methanol presented the higher increase of lipid peroxidation (Figure 1) produced by a high production of ROS derived from the metabolism of methanol and high temperature. Besides it must be considered that aerobic metabolism generates increased production of ROS in the mitochondria. This would happen because the electron transport in mitochondria consumes most cellular oxygen and generates superoxide anion, which is converted to H_2O_2 from mitochondria by superoxide dismutase (SOD2) (Davidson and Schiestl, 2001). However, ROS production can act as an intracellular signal that promotes the nuclear translocation of transcription factor PpYap1, which induces the expression of genes involved in antioxidant defense (Yano *et al.*, 2009). The culture of *P. pastoris* in methanol medium induces nuclear localization of Yap1 and activates the expression of GLR (Yano *et al.*, 2009a). It would indicate that the culture at 3% (v/v) of methanol would not efficiently induces cell antioxidant system, probably in this condition with higher level of methanol could induce an increased production of ROS in comparison to 1% (v/v) methanol medium. Nevertheless, in the culture exposed to 30 °C and 3% (v/v) methanol was observed higher level of GLR expression, probably it could not protect completely to the cells when they were exposed to high levels of methanol and temperature. Furthermore, it has been reported that products of lipid peroxidation induce cytotoxicity, in contrast to that; sublethal concentrations of methanol induce cellular responses related in enhancing the adaptation and tolerance against oxidative stress by up-regulation of antioxidant enzymes (Niki, 2009).

It has been observed in *S. cerevisiae* that the oxidative stress tolerance and the stress induced by temperature depend on the lipid composition of the membrane, there being a positive correlation between the cellular damage and increased unsaturation of fatty acids (Leipold *et al.*, 2003). In fact, *P. pastoris* is characterized by high levels of Poly Unsaturated Fatty Acids (PUFA), therefore in a stress condition (30 °C and 3% (v/v) methanol) it would be more susceptible to oxidative stress, raising the levels of lipid peroxidation and DNA damage that can be observed indirectly in the detection of relative expression of enzymes that are part of this repair machinery, as OGG1. The results determine that in the recombinant yeast, in all growth conditions, actives the nuclear DNA repair machinery (OGG1-1a), which may be indicative of a normal situation that occurs in the DNA of all organisms or that none of the conditions studied is suitable for yeast, so this will be constantly generating oxidative damage on the genetic material and the repair machinery is protecting the DNA from oxidative damage. However, the condition of 30 °C and 3% (v/v) methanol concentration, produce fairly high levels of oxidative stress that affects both the nuclear and mitochondrial DNA, because in these conditions is when we ob-

served the presence of OGG1-2a (Figure 2A). This occurs probably because there is an increased oxidative damage caused by temperature and higher level of methanol, as demonstrated indirectly by TBARS (Figure 1). This situation could also be explained by a mechanism related to the control of the location of OGG1, in which the oxidative damage in the nuclear DNA indicates the recruitment of OGG1 in the nucleus; while oxidative damage in mitochondrial DNA induces the recruitment of OGG1 in the mitochondria (Alseth *et al.*, 1999). It is also important to mention that the culture that produced the highest concentration of heterologous proteins showed a higher amount of oxidative damage, which may be affecting the genomic and mitochondrial DNA of recombinant *P. pastoris.* On the other hand, the oxidative damage could affect the expression of endogenous proteins involved in the antioxidant protection systems of this yeast, showing an inefficient protection against oxidative stress induced in these conditions. Consequently it would negatively affect the count of the yeast and may affect the production of recombinant proteins.

In almost all living systems, upshifts in temperature cause a heat stress response that leads to a strong induction of a conserved group of proteins called heat shock proteins (HSPs) (Guerra *et al.*, 2005). These have shown a decrease in protein folding at low temperatures and thus, a possible correlation with the beneficial effect on protein secretion (Dragosits *et al.*, 2010). In accordance to that, the culture at 30 °C - 3% (v/v) of methanol, in comparison with the cultures at 10 °C - 3% (v/v) of methanol and 10 °C - 1% (v/v) of methanol, is the one with a lower concentration of total protein. This would indicate that the heat stress generated by 30 °C would act beneficially on the production of proteins to activate the chaperone, which may be protecting peptides to acquire the proper conformation of a mature protein. In relation to heat stress, HSF-1 does not show to have high transcriptional activity in the cultures because that should be found into the nucleus; only the culture at 10 °C - 1% (v/v) of methanol would be slightly presenting this activity because their presence was found near the nucleus. This localization could be related to the decrease in the concentration of methanol or interaction with other protein that would act mainly on the protection of endogenous protein production over the heterogeneous proteins, or just these are more sensitive to changes in the medium. The culture at 30 °C - 3% (v/v) of methanol should have displayed a greater presence and nuclear localization considering that it was the highest temperature, and its presence or more specifically its role as a transcription factor is related to this type of stress. Despite this, it didn't showed significant difference in comparison at 10 °C, which could indicate that at 30 °C the yeast *P. pastoris* isn't found in a stress condition but within the range of normal growth of it. Regarding to proteins response to heat stress, HSF-1 in all the cultures would induce the transcription of HSP-70 but the difference is due to the functionality of the last one. Wang *et al.* (2012) founded that cells can increase their apoptosis when the nucleolar fragmentation increases in presence of high oxidative stress and HSP-70 will prevent nucleolar fragmentation induced for this stress. In accordance to that, our results showed HSP-70 in the nucleus of the cultures at 10 °C. This means that these cells would activate their anti-apoptotic system due to the protection of the nucleolus and avoiding its fragmentation and subsequent apoptosis. At 30 °C, HSP-70 was founded in the cytoplasm, indicating that the anti-apoptotic system was not efficiently activated since this culture showed high levels of oxidative stress and more oxidative damage in comparison at 10 °C. In this manner, HSP-70 and maybe others HSPs could be acting only to low oxidative stress induced by 10 °C and favored by 1% (v/v) of methanol but in presence to high oxidative stress these proteins would not be functional or would be used in other pathways, like to control the respiratory metabolism.

Conclusions

The measure of OGG-1, GLR, HSF-1 and HSP-70 can be useful to identify the optimal conditions for a culture of genetically -modified *P. pastoris*, where we determined that at 30 °C - 3% (v/v) of methanol it would increase the apoptosis induced for a strong oxidative stress. Because there is little information about the effect of the recombination on *P. pastoris,* more research is needed to evaluate the influence of this process in front of different culture conditions on the physiology, metabolism and cell communication mechanisms of this expression system, which would give us knowledge about the functioning of the yeast and then design and devise strategies to improve the production of heterologous proteins from *P. pastoris* recombinant.

Acknowledgments

The authors are sincerely thankful for support provided by CONICYT doctoral fellowship to A.Z. and C.F., FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo, Brasil, Processo Número: 2012/50210-9), International Cooperation Program of Universidad de La Frontera DI12-4001 and by DIUFRO grant DI12-2007

Competing Financial Interests

The authors declare no competing financial interests.

References

Ahn J, Hong J, Park M, Lee H, Lee E, Kim C, Lee J, Choi ES, Jung JK, Lee H (2009) Phosphate-responsive promoter of a *Pichia pastoris* sodium phosphate symporter. Appl Environ Microbiol 75:3528-3534.

Alseth I, Eide L, Pirovano M, Rognes T, Seeberg E, Bjørås M (1999) The Saccharomyces cerevisiae homologues of endonuclease III from *Escherichia coli*, Ntg1 and Ntg2, are

both required for efficient repair of spontaneous and induced oxidative DNA damage in yeast. Mol Cell Biol 19:3779-3787.

Arakawa K, Abe M, Noda Y, Adachi H, Yoda K (2006) Molecular cloning and characterization of a *Pichia pastoris* ortholog of the yeast Golgi GDP-mannose transporter gene. J Gen Appl Microbiol 52:137-145.

Bener Aksam E, Jungwirth H, Kohlwein SD, Ring J, Madeo F, Veenhuis M, van der Klei IJ (2008) Absence of the peroxiredoxin Pmp20 causes peroxisomal protein leakage and necrotic cell death. Free Radic Biol Med 45:1115-1124.

Blokhina O, Virolainen E, Fagerstedt KV (2003) Antioxidants, oxidative damage and oxygen deprivation stress: a review. Ann Bot 91 Spec No:179-194.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254.

Brauchi S, Cea C, Farias JG, Bacigalupo J, Reyes JG (2006) Apoptosis induced by prolonged exposure to odorants in cultured cells from rat olfactory epithelium. Brain Res 1103:114-122.

Cash TP, Pan Y, Simon MC (2007) Reactive oxygen species and cellular oxygen sensing. Free Radic Biol Med 43:1219-1225.

Damasceno LM, Huang CJ, Batt CA (2012) Protein secretion in *Pichia pastoris* and advances in protein production. Appl Microbiol Biotechnol 93:31-39.

Davidson JF, Schiestl RH (2001) Mitochondrial respiratory electron carriers are involved in oxidative stress during heat stress in *Saccharomyces cerevisiae*. Mol Cell Biol 21:8483-8489.

Dragosits M, Frascotti G, Bernard-Granger L, Vázquez F, Giuliani M, Baumann K, Rodríguez-Carmona E, Tokkanen J, Parrilli E, Wiebe MG, Kunert R, Maurer M, Gasser B, Sauer M, Branduardi P, Pakula T, Saloheimo M, Penttilä M, Ferrer P, Luisa Tutino M, Villaverde A, Porro D, Mattanovich D (2011) Influence of growth temperature on the production of antibody Fab fragments in different microbes: a host comparative analysis. Biotechnol Prog 27:38-46.

Dragosits M, Stadlmann J, Albiol J, Baumann K, Maurer M, Gasser B, Sauer M, Altmann F, Ferrer P, Mattanovich D (2009) The effect of temperature on the proteome of recombinant *Pichia pastoris*. J Proteome Res 8:1380-1392.

Dragosits M, Stadlmann J, Graf A, Gasser B, Maurer M, Sauer M, Kreil DP, Altmann F, Mattanovich D (2010) The response to unfolded protein is involved in osmotolerance of *Pichia pastoris*. BMC Genomics 11:207.

Farías JG, Zepeda AB, Calaf GM (2012) Melatonin protects the heart, lungs and kidneys from oxidative stress under intermittent hypobaric hypoxia in rats. Biol Res 45:81-85.

Gasser B, Maurer M, Rautio J, Sauer M, Bhattacharyya A, Saloheimo M, Penttilä M, Mattanovich D (2007) Monitoring of transcriptional regulation in *Pichia pastoris* under protein production conditions. BMC Genomics 19:179.

Guerra E, Chye PP, Berardi E, Piper PW (2005) Hypoxia abolishes transience of the heat-shock response in the methylotrophic yeast *Hansenula polymorpha*. Microbiology 151:805-811.

Hayden MS, Gilliland LK, Ledbetter JA (1997) Antibody engineering. Curr Opin Immunol 9:201-212.

Jin H, Liu G, Dai K, Wang H, Li Z, Shi Z (2011) Improvement of porcine interferon-α production by recombinant *Pichia pastoris* via induction at low methanol concentration and low temperature. Appl Biochem Biotechnol 165:559-571.

Khalil AA, Kabapy NF, Deraz SF, Smith C (2011) Heat shock proteins in oncology: Diagnostic biomarkers or therapeutic targets? Biochim Biophys Acta 1816:89-104.

Kwolek-Mirek M, Bednarska S, Bartosz G, Bilinski T (2009) Acrolein toxicity involves oxidative stress caused by glutathione depletion in the yeast *Saccharomyces cerevisiae*. Cell Biol Toxicol 25:363-378.

Leipold MD, Workman H, Muller JG, Burrows CJ, David SS (2003) Recognition and removal of oxidized guanines in duplex DNA by the base excision repair enzymes hOGG1, yOGG1, and yOGG2. Biochemistry 42:11373-11381.

Macauley-Patrick S, Fazenda ML, McNeil B, Harvey LM (2005) Heterologous protein production using the *Pichia pastoris* expression system. Yeast 22:249-270.

Niki E (2009) Lipid peroxidation: physiological levels and dual biological effects. Free Radic Biol Med 47:469-484.

Ni Z, Zhou X, Sun X, Wang Y, Zhang Y (2008) Decrease of hirudin degradation by deleting the KEX1 gene in recombinant *Pichia pastoris*. Yeast 25:1-8.

Panagiotou V, Love KR, Jiang B, Nett J, Stadheim T, Love JC (2011) Generation and screening of *Pichia pastoris* strains with enhanced protein production by use of microengraving. Appl Environ Microbiol 77:3154-3156.

Pizarro FJ, Jewett MC, Nielsen J, Agosin E (2008) Growth temperature exerts differential physiological and transcriptional responses in laboratory and wine strains of *Saccharomyces cerevisiae*. Appl Environ Microbiol 74:6358-6368.

Priault M, Bessoule JJ, Grelaud-Coq A, Camougrand N, Manon S (2002) Bax-induced cell death in yeast depends on mitochondrial lipid oxidation. Eur J Biochem 269:5440-5450.

Sandigursky M, Yacoub A, Kelley MR, Xu Y, Franklin WA, Deutsch WA (1997) The yeast 8-oxoguanine DNA glycosylase (Ogg1) contains a DNA deoxyribophosphodiesterase (dRpase) activity. Nucleic Acids Res 25:4557-4561.

Sohn SB, Graf AB, Kim TY, Gasser B, Maurer M, Ferrer P, Mattanovich D, Lee SY (2010) Genome-scale metabolic model of methylotrophic yeast *Pichia pastoris* and its use for in silico analysis of heterologous protein production. Biotechnol J 5:705-715.

Solà A, Jouhten P, Maaheimo H, Sánchez-Ferrando F, Szyperski T, Ferrer P (2007) Metabolic flux profiling of *Pichia pastoris* grown on glycerol/methanol mixtures in chemostat cultures at low and high dilution rates. Microbiology 153:281-290.

Solà A, Maaheimo H, Ylönen K, Ferrer P, Szyperski T (2004) Amino acid biosynthesis and metabolic flux profiling of *Pichia pastoris*. Eur J Biochem 271:2462-2470.

Steels EL, Learmonth RP, Watson K (1994) Stress tolerance and membrane lipid unsaturation in Saccharomyces cerevisiae grown aerobically or anaerobically. Microbiology 140:569-576.

van der Kemp PA, Thomas D, Barbey R, de Oliveira R, Boiteux S (1996) Cloning and expression in Escherichia coli of the OGG1 gene of *Saccharomyces cerevisiae*, which codes for a DNA glycosylase that excises 7,8-dihydro-8-oxoguanine and 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine. Proc Natl Acad Sci USA 93:5197-5202.

van der Klei IJ, Yurimoto H, Sakai Y, Veenhuis M (2006) The significance of peroxisomes in methanol metabolism in methylotrophic yeast. Biochim Biophys Acta 1763:1453-1462.

van Lammeren G, L Moll F, Borst GJ, de Kleijn DP, P M de Vries JP, Pasterkamp G (2011) Atherosclerotic plaque biomarkers: beyond the horizon of the vulnerable plaque. Curr Cardiol Rev 7:22-27.

Verbelen PJ, Depraetere SA, Winderickx J, Delvaux FR, Delvaux F (2009) The influence of yeast oxygenation prior to brewery fermentation on yeast metabolism and the oxidative stress response. FEMS Yeast Res 9:226-239.

Wang K, Deng G, Chen G, Liu M, Yi Y, Yang T, McMillan DR, Xiao X (2012) Heat shock protein 70 inhibits hydrogen peroxide-induced nucleolar fragmentation via suppressing cleavage and down-regulation of nucleolin. Cell Stress Chaperones 17:121-130.

Whyteside G, Alcocer MJ, Kumita JR, Dobson CM, Lazarou M, Pleass RJ, Archer DB (2011) Native-state stability determines the extent of degradation relative to secretion of protein variants from *Pichia pastoris*. PLoS One 6:e22692.

Xuan Y, Zhou X, Zhang W, Zhang X, Song Z, Zhang Y (2009) An upstream activation sequence controls the expression of AOX1 gene in *Pichia pastoris*. FEMS Yeast Res 9:1271-1282.

Yano T, Takigami E, Yurimoto H, Sakai Y (2009a) Yap1-regulated glutathione redox system curtails accumulation of formaldehyde and reactive oxygen species in methanol metabolism of *Pichia pastoris*. Eukaryot Cell 8:540-549.

Yano T, Yurimoto H, Sakai Y (2009b) Activation of the oxidative stress regulator PpYap1 through conserved cysteine residues during methanol metabolism in the yeast *Pichia pastoris*. Biosci Biotechnol Biochem 73:1404-1411.