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Free fatty acids reduce metabolic stress and favor a stable production of heterologous proteins in *Pichia pastoris*

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ABSTRACT

The growth of yeasts in culture media can be affected by many factors. For example, methanol can be metabolized by other pathways to produce ethanol, which acts as an inhibitor of the heterologous protein production pathway; oxygen concentration can generate aerobic or anaerobic environments and affects the fermentation rate; and temperature affects the central carbon metabolism and stress response protein folding. The main goal of this study was determine the implication of free fatty acids on the production of heterologous proteins in different culture conditions in cultures of *Pichia pastoris*. We evaluated cell viability using propidium iodide by flow cytometry and thiobarbituric acid reactive substances to measure cell membrane damage. The results indicate that the use of low temperatures and low methanol concentrations favors the decrease in lipid peroxidation in the transition phase from glycerol to methanol. In addition, a temperature of 14 °C + 1%M provided the most stable viability. By contrast, the temperature of 18 °C + 1.5%M favored the production of a higher antibody fragment concentration. In summary, these results demonstrate that the decrease in lipid peroxidation is related to an increased production of free fatty acids.

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Introduction

Pichia pastoris is a methylotrophic yeast used as a heterologous host for the production of recombinant proteins.[1] An attractive feature of *P. pastoris* is that, as a eukaryote microorganism, heterologous proteins are more likely to be soluble, correctly folded and to have undergone the post-translational modifications required for activity.[2] Because of these characteristics, many studies have been performed to enhance the production of recombinant proteins. However, the effect of changes in the culture media, temperature, pH, type of inductor, among others, has not been widely tested on the yeast, and has not taken into account that a negative effect on the yeast metabolism may have a direct impact on the quality of heterologous proteins.

Methylotrophic yeasts catalyze the oxidation of short aliphatic alcohols, such as methanol, ethanol and 1-propanol due to the enzyme alcohol oxidase (AOX). This enzyme is found mainly in the peroxisome matrix, and it is key in the oxidation of methanol to formaldehyde resulting in the production of H_2O_2.[3] It is important to consider that glycerol, ethanol and methanol can be used as a sole carbon source for *P. pastoris* growth. Arias et al.[37] reported that glycerol is a better carbon source than glucose to preculture these cells, allowing for high biomass productivity, lower duplication time and greater final concentration and productivity of the antibody fragment. Diauxic growth is also possible, especially when the substrate is composed of a mixture of ethanol-glycerol or ethanol–methanol.[4] When the expression of heterologous proteins is associated with cell growth under aerobic conditions, then it is important to prevent the production of ethanol, which inhibits cell growth.[5] Additionally, glucose and ethanol act as negative effectors, reducing AOX expression by catabolite repression and end product inhibition.[4,5]

Proteolytic degradation of recombinant proteins expressed in *P. pastoris* has been attributed to the metabolism of methanol to ethanol, which induces cell lysis particularly late in the fermentation. It has also been reported that proteolytic degradation can be minimized through the manipulation of pH and temperature,[6] with cell viability enhanced for over 24 h in batch fermentations at very low (10 °C) and very high temperatures (37 °C).[7] Conversely, growth using high concentrations of methanol as the sole carbon source at high temperatures induces strong oxidative stress[8] due to autoxidation of polyunsaturated fatty acids (PUFAs) or lipid peroxidation that reduces biomass production.[9] PUFA residues of membrane phospholipids are very sensitive to oxidation and the action of ROS,[34] wherein PUFAs easily undergo non-enzymatic oxidation and fragmentation during lipid peroxidation and form numerous toxic products. For example, hydroxyl radical (•OH) can easily "steal" an electron from such unsaturated fatty acids to give rise to a carbon-centered lipid radical. Lipid radical can further interact with molecular oxygen (O_2) to produce lipid peroxyl radical (fatty acid peroxyl radical). Being an unstable species, lipid peroxyl radical interacts with another free fatty acid to produce a different fatty acid radical and lipid peroxide (LOOH). The formed fatty acid radical reacts again with molecular oxygen to produce fatty acid peroxyl radical, etc., creating the cycle which continues, as the new fatty acid radical reacts in the same way.[35] However, it is unknown what effects induce the oxidative stress following the growth of *P. pastoris* at high temperatures using methanol as the sole carbon source. For this reason, in this study we investigated methanol catabolism through the pyruvate pathway in *P. pastoris*, and monitored the effects of this metabolism on the heterologous expression of recombinant antibody fragment protein.

Materials and methods

Strain

P. pastoris strain SMD1168 (Invitrogen®): Δ*pep4*::*URA3*Δ*kex1*::*SUC2his4ura3* with His^-Mut^+ phenotype was used for the expression of antibody fragment scFv. The genetically modified version of this strain was provided by the research group of Prof. Dr. Dulcineia S. P. Abdalla of the Departamento de Análises Clínicas e Toxicológicas, Faculdade de Ciências Farmacêuticas at the Universidade de São Paulo, and was constructed by the group of Prof. Dr. Andrea Q. Maranhão of the Department of Molecular Biology at the Universidade de Brasília, Brazil.

Reagents

The solvents were of analytical grade. The components of culture medium were autoclaved at 121 °C for 20 min, except for yeast nitrogen base, ammonium sulfate and casamino acids, which were autoclaved at 110 °C for 5 min. Biotin was sterilized by filtration through a 0.22 μm pore diameter membrane. Solutions and buffers were prepared by using deionized water. Propidium iodide (PI), 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimi-dazolylcarbocyanine iodide (JC-1) and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich (St Louis, MO, USA). The Mitochondrial Membrane Potential Detection Kit was purchased from Biotium (Hayward, CA, USA).

Maintenance and reactivation of P. pastoris

For the preservation of *P. pastoris*, the colonies were replicated every three months on YPD solid medium (1% (w/v) yeast extract, 2% (w/v) casein peptone, 2% (w/v) glucose, 2% (w/v) bacteriological agar) and incubated at 30 °C for 24 h. After that period, the colonies were removed from plates and inoculated in 500 mL Erlenmeyer flasks 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 500 mL Erlenmeyer flasks containing 100 mL of the growth medium BMGY (buffered glycerol complex medium) (1% (w/v) yeast extract, 2% (w/v) casein peptone, 0.34% (w/v) yeast nitrogen base, 1% (w/v) ammonium sulfate, 100 mM buffer potassium phosphate pH 6.0, 4×10^{-5}% (w/v) biotin, 1% (v/v) glycerol, 2% (w/v) casamino acids with deionized water) and incubated at 30 °C and 250 rpm for 19 h in a shaking incubator (Model SI-300, Jeio Tech, Seoul, Korea).[8] The reactivation step should be realized once because

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