Experimental design for heat-shock proteins from *Candida albicans*: An application for vaccines

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ABSTRACT Immunotherapy in the fight against *Candida albicans* infections is a powerful tool to modulate the immune system permanently and definitively against sensitizing antigens in immunosuppressed individuals. This study proposes the investigation of the cellular behavior of the fungus *Candida albicans* (ATCC® 10231) when submitted to thermal stress, to evaluate the synthesis of HSPs, for application in vaccines. Method: The fungus strain was cultivated, and factorial experimental design was delineated, having as factors temperature (38, 46, and 55 °C), time (15, 52.5, and 90 min), and agitation. Cell mass (mg/mL) and glucose intake (mg/mL) were considered response variables. The obtained data show that there was no synthesis of HSPs below 46.5 °C, however, this was considered an optimal temperature for the expression of HSPs of 33, 40, and 44 kDa. In temperatures above 47 °C, glucose consumption was reduced to 15.5 %, until it was no longer metabolized by the fungus at 55 °C, suggesting a condition of defense against thermal stress and subsequent cell death. In the contour region of the bands applied to the study, it can be considered that the Heat-shock proteins process was optimized, however, genetic factors are determinants for specific expressions of HSPs, being intrinsic characteristics of the strains under study.

KEYWORDS *Candida albicans*; Heat Shock Protein; Experimental design; Process optimization; Application in vaccines

1. Introduction

The fungus Candida albicans can trigger superficial infections in the mucosa, and skin and in more serious situations, infections in internal organs in immunosuppressed and vulnerable (Swidergall and LeibundGut-Landmann, 2022). In these cases, the fungus does not find resistance from the immune system (IS), triggering chronic sensitivity, a fact that can be circumvented through immunomodulatory vaccines, making the SI tolerant to this pathogen (Vonk et al., 2006).

The significant relevance of thermal shock proteins (HSPs) (Puustinen and Sistonen, 2020) has applications in the immunotherapy area and signals ways to understand many pathologies (Matthews and Burnie, 1989; Magliani et al., 2002). Several scientific studies report that HSPs proteins of the fungus *C. albicans* have virulence potential and the development of antifungal therapies (Burnie et al., 2006; Gong et al., 2017). HSPs can be expressed under thermal shock, osmotic, oxidative, and heavy metal stress (Fu et al., 2012). In aggressive temperature conditions, *C. albicans* releases proteins that perform powerful interest functions in the medical, clinical, and therapeutic fields (Singh, 2020). Knowledge of the conditions in the thermal shock process such as induction time, temperature, and agitation, for example, provides information that can infer in obtaining these proteins more efficiently. However, the scientific literature does not establish criteria that outline the favorable conditions of the thermal shock process and the factors that influence it. Thus, the present study aims to investigate the variables of the "Heat shock" process for the fungus *C. albicans*.

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2. Methods

2.1 Cultivation

For the study, the lineage of fungus *C. albicans* ATCC® 10231, the first generation was used. The vial with lyophilized material was initially hydrated with 400 µL of solution and sterile sodium chloride of 0.9 % (w/v). An aliquot of 50 µL of (from hydrated fungal suspension) hydrated was inoculated in 5 mL of BHI broth (Brain Heart Infusion) for the formation of a pre-inoculum. The vials were incubated for 24 h at 35 °C and 110 rpm. After the vial was poured in 100 mL with Smith broth and incubated for another 24 h until the exponential growth phase, with immediate application of the thermal shock process. However, initially, a wild strain was isolated and thermal shock tests were performed, and later the experiment was standardized with the strain ATCC® 10231.

2.2 HSP factorial design for *C. albicans*

For factorial design (2^3) low, central, and high levels respectively, temperature (38 °C; 46.5 °C and 55 °C); incubation time (15 min; 52.5 min and 90 min) and agitation of the system (Universal 320R, Benchtop centrifuge). The presence and absence of agitation (middle aeration) were considered independent qualitative variable, assigned the values of -1 (without agitation) and +1 (with agitation).

The rotation (rpm) was based on a rotor com diameter of 6 cm, but rotation values can be converted considering: Relative Centrifugal Force (RCF) or G-force using the equation $RCF = 1.12 \times Radius \times (rpm/1000)^2$. The parameters evaluated as response variables were glucose content (mg/mL) consumed and the mass cellular (mg/mL) obtained over the thermal shock. The results were analyzed by analysis of variance (ANOVA) using *Statistics 8* software. The determinations were performed with 3 repetitions, totaling 30 trials.

2.3 Determination of glucose content

Glucose content (mg/mL) was determined by the reaction between reducing sugar and 3,5-dinitrosalic acid, from a calibration curve with standard glucose (α-D-Glucosand anhydrous, 96 % - Sigma-Aldrich) as absorbance function at 540 nm, according to the procedure described in the literature (Miller, 1959).

2.4 Obtaining the cell content

Cell mass was obtained after centrifugation of 50 mL of the cell medium after the process of thermal shock and subsequent filtration on Whatman filter paper®. The mass was kiln-dried at 40 °C for 24 h, after sterilization with a diluted solution of formaldehyde at 10 % (v/v) (da Silva et al., 2014).

2.5 Characterization by polyacrylamide gel electrophoresis in denaturalizing conditions (SDS-PAGE)

Aliquots of the supernatants for each set of experiments were characterized by electrophoresis under denaturing conditions, using polyacrylamide gel 12.5 % (v/v). The samples were prepared by 1:1 dilution in a sample buffer containing 2-mercaptoethanol and Bromophenol and submitted to 90 °C for 5 min. 20 µL of the sample were applied to each gel well and the electrophoretic run was initially performed at 70 V for 1 h and after 200 V for 2 h. Samples were revealed by impregnation with silver.

2.6 Feasibility test

The aliquots removed after thermal shock were evaluated for viability using Trypan blure exclusion test considering the integrity of the cell membrane. The contingent of dead cells of *C. albicans* was determined by the Neubauer Assembly count, where the dead cells, in view of the loss of selectivity, absorb the trypan blue dye into their cytoplasm, contrary to live cells, according to the literature (Naves et al., 2013).

3. Results

Before he application of the factorial experimental design (PEF), a preliminary test was performed with a strain of wild *C. albicans*, in a transient sequence of temperature shock, as shown in Fig. 1, where it was observed that the efficiency for expression of HSPs, it results from the second thermal shock (ray 1.2(+)) in addition to the direct influence of agitation.

Using the standard strain ATCC® 10231 of *C. albicans* the effects of the combinations of each controlled variable, T (°C), t (min) and agitation (rpm) in the PEF obtained by analysis of variance (ANOVA), where the

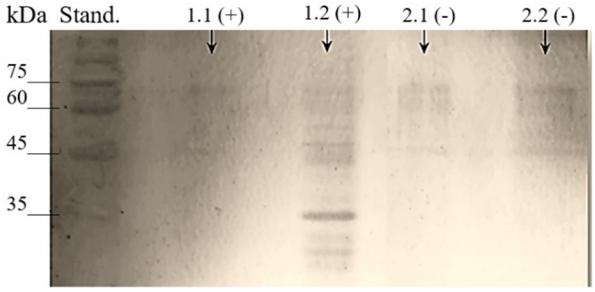

Figure 1: Thermal shock for wild candida (37 °C, 15 min) SDS-PAGE 12 % acrylamide/bis-acrylamide for *C. albicans*. In gel: A) Page Ruler protein pattern™; 1.1(+) first thermal shock and 1.2(+) second thermal shock with agitation; 2.1(-) first thermal shock and 2.2(-) second thermal shock without agitation. (+) medium agitated and (-) without agitation.

response variable, cell mass (mg/mL), presented curvature with significant statistics ($p - level \geq 0.05$), which indicates a close approximation between the factors that optimize the thermal shock process, according to Equation 1, generated by *Statistics 8* software.

$$Cellular\ content\ (\text{mg/mL}) = 1.97 + 10.59 \cdot T + 9.84 \cdot t + 193 \cdot A + 0.01 \cdot Tt + 16.78 \cdot TA + 28.19 \cdot tA \quad (1)$$

with a coefficient of determination, R^2 = 0.9801 and R^2 adj = 0.9832.

For glucose consumption, the equation obtained according to Eq. 2:

$$Glucose\ concentration\ (\text{mg/mL}) = 3.99 \cdot t - 1.56 \cdot A + 3.73 \cdot Tt - 2.16 \cdot TA + 0.31 \cdot tA - 0.61 \cdot TtA \quad (2)$$

with a coefficient of determination, R^2 = 0.9969 and R^2 adj = 0.957. Where: T: temperature (°C); t: time and A: agitation.

Pareto charts (Fig. 2A and 2B) show the contribution of the variable to interaction (interaction 3), has greater significance for the synthesis of new cells of *C. albicans*, considering the m/v ratio, obtained after thermal shock (Fig. 2B). The contribution of the variable time indicates that the permanence of *C. albicans* cells at a given shock temperature, print a more pronounced effect on glucose consumption in the non-lethal temperature range (Fig. 2A). The system with agitation of the oxygen uptake is favored contributing to cellular development. Thus, in agitated medium, the average consumption of glucose, used as a carbon source, was 52.8 % (± 0.529) at 38 °C (15 min), showing 2.75 x 107 cells after 90 minutes of cultivation. In conditions more aggressive temperatures at 55 °C for 90 min, the number of cells was 1.00 x 107 and glucose consumption was reduced to 15.5 % (±0.301) (Fig. 3.), which characterizes cell lethality under these imposed conditions.

The marginal means (Fig. 4) present the cellular behavior of *C. albicans* regarding the biomass obtained from

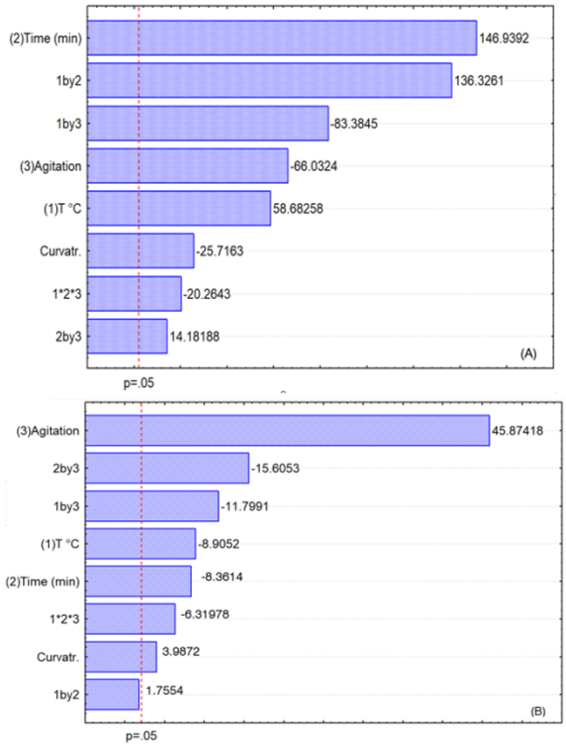

Figure 2: Pareto chart for magnitude and importance of thermal shock process variables. A) Glucose consumption (mg/mL) and B) Cell mass production (mg/mL).

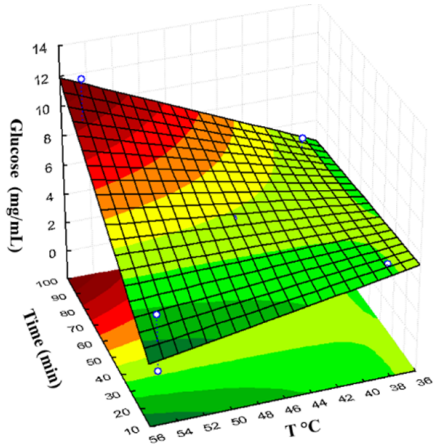

Figure 3: Fitted surface for glucose consumption considering the temperature of thermal shock (38 °C, 46.5 °C and 55 °C), time of permanence in thermal shock (15, 52.5 and 90 min) in agitated medium.

the second thermal shock. The experiments were carried out in the exponential phase mean growth, thus justifying the variation of the cell content (mg/mL), observed at 38 °C, and can be visualized under optical microscope germination of blastoconidia (Fig. 4.1A). However, this was not considered a critical temperature for protein synthesis, even though it was above the cultivation temperature of 35 °C. This was also observed by Naves et al. (2013), which reports yeast growth can run between 20 °C to 38 °C.

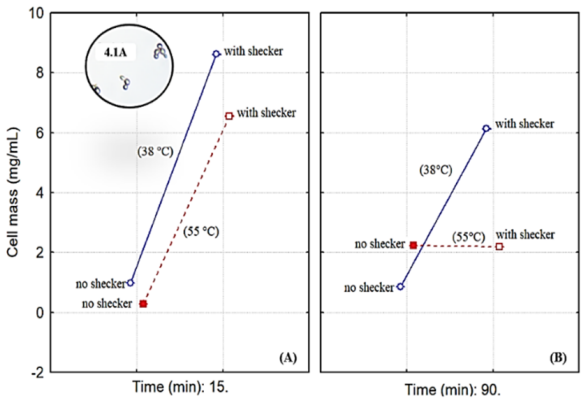

Figure 4: Plots of Marginal Means of 95 % confidence limit, showing cell trends (mg/mL) in relation to temperature, time and agitation in the thermal shock process.

As shown by the electrophoresis gel (Fig. 5) the HSPs are expressed at 46.5 °C (52.5 min, with agitation) in defense action against the dangers of the cellular to thermal shock, identified in approximately 44 kDa, 40 kDa, and 33 kDa. The biosynthesis of these proteins is regulated by genetic factors of specific transactions, therefore it shows the peculiarity of the ATCC®10231 strain, in view of the activation of thermal shock genes (Prohászka, 2003). The expressed proteins are multifunctional and may be related to the potential for virulence and drug resistance against *C. albicans* (Chun et al., 2022). They are classified as chaperones Hsp12 and Hsp21 of low molecule mass *r* between 12 to 42 kDa. According to Fu et al. (2012), these proteins are induced during the growth of the stationary phase. According to Gong et al. (2017), they can also be produced by oxidative stress, and participate as regulators of glycerol homeostasis at high temperatures.

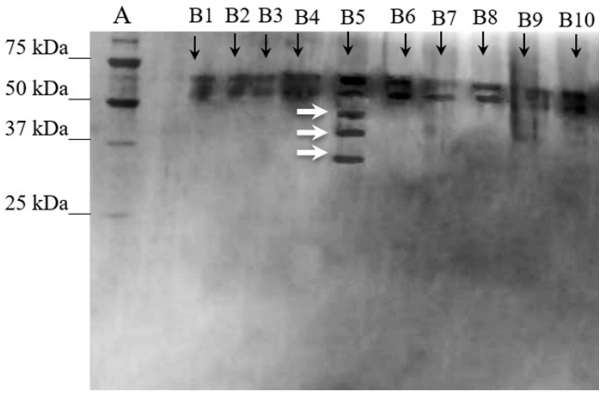

Figure 5: Electrophoresis SDS-PAGE for thermal shock process. (A) Stan: Precision Plus Protein™ Dual Color Standards (Bio-Rad). Response to experiment design B1: 38 °C, 15 min, -1; B2: 38 °C, 15 min, +1; B3: 38 °C, 90 min, -1; B4: 38 °C, 90 min, +1; B5: 46.5 °C, 52.5 min, +1; B6: 46.5 °C, 52.5 min, -1; B7: 55 °C, 15 min, -1; B8: 55 °C, 15 min, +1; B9: 55 °C, 90 min, -1; B10: 55 °C, 90 min, +1. Protein profile: (a): 70 kDa; (b): 60 kDa; (c): 40 kDa; (d): 38 kDa (e): 30 kDa.

The thermal shock proteins of *C. albicans* are inserted in increasing dilution in the treatment of cases of recurrent candidiasis, through a deep subcutaneous approach, making the immune system tolerant to the presence of these proteins, thus preventing the inflammatory process from being initiated (Oliveira et al., 2021; Magliani et al., 2002). Still for the PEF ranges under study, protein synthesis is not observed at temperatures below 46.5 °C, however, to survive lethal exposure (55 °C for 15 min), the fungus *C. albicans* synthesized proteins in low concentrations, being visualized in weak bands to the left of an electrophoresis gel. Low concentrations, being visualized in weak bands to the left of electrophoresis gel. This fact can be suggested as a study target for better understanding, using intermediate incubation temperatures between 46 °C and 55 °C. However, in a time longer than this, cell death occurred, a phenomenon confirmed in feasibility tests, which justifies the dashed line in the graph of marginal means in Fig. 4B. Through the results obtained, the idea is reinforced that not only the lysates of *C. albicans* cells are interesting for the composition of fungal suspensions in the preparation of vaccines, but also the application of proteins obtained in the heat shock method proposed here, enhancing the immunomodulatory power, since antibodies (IgE) are trained for the recognition of HSPs.

However, in a time longer than this, cell death occurred, a phenomenon confirmed in feasibility tests, which justifies the dashed line in the graph of marginal means in Fig. 4B.

4. Conclusions

The optimization of the Heat-shock process to facilitate the obtaining of specific proteins, with virulence potential, can be obtained from an experimental contour of the process variables, thus promoting data scarce in the literature. Incubation at a high temperature, however, induces synthesis and minimizes lethality problems. A factor, important for future studies and the kinetic behavior for the maximum synthesis of these proteins, to determine the conditions of processes that govern the synthesis, accumulation, and stabilization of HSPs, which would reduce time and energy expenditure. However, although this study provides information that can be applied in the optimization of Heat-shock proteins, it is important to highlight the intrinsic factor genetic and phenotype of the species of *C. albicans*, a fact that has been considered and investigated by ongoing research, between the different strains.

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Conflicts of interest

The authors declare that there are no conflicts of interest. Authors are solely responsible for the content and writing of the article.

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