EXPRESSION OF A 100kDa RECOMBINANT PROTEIN FROM *Histoplasma capsulatum* IN THE METHYLOTROPHIC YEAST *Pichia pastoris*

Purpose

Histoplasmosis is a systemic and endemic mycosis widely distributed in the Americas caused by the dimorphic fungus *Histoplasma capsulatum*. The infection is usually asymptomatic in immunocompetent individuals. However, immunocompromised patients may contract the disseminated form of the disease, which has a bad prognosis and requires rapid diagnosis and treatment. The definitive diagnosis involves the isolation of *H. capsulatum* by culture from clinical specimens, which may take up to 4 weeks. In addition, molecular methods are expensive and have low sensitivity and immunoassays present many false-positive results.

Thus, the aim of this work is to express Hc100, a specific protein of 100kDa of *H. capsulatum*, to develop a novel direct immunoassay and to perform characterization studies of this protein as a first approach for the potential development of novel therapeutic strategies.

Materials and Methods

Transformation of Pichia pastoris: The gene that encodes for Hc100 was optimized for expression in *P. pastoris* and constructed with a secretory signal (α-MF) and a polyhistidine-tag. This construction was under the control of the methanol inducible promoter *aox* in the plasmid pPICZα. *Pichia pastoris* X-33 strain was then transformed by electroporation and genotype of five clones was analyzed by PCR. Phenotype was analyzed by growing the clones and the X-33 strain in Minimal Dextrose Medium (MMD) and Minimal Methanol Medium (MMM) agar plates for 48h.

Expression of Hc100: *P. pastoris* X-33 strain and Hc100 clones were grown in BMMY,BMM, MM and MBS media with and without protease inhibitors for 72h with pulses of 0.5% methanol every 24h to maintain induction. Growth rate and pH of cultures were measured at 16, 24, 48 and 72h. Cell culture supernatants collected at different time points of induction were analyzed by 8% SDS-PAGE followed by Western bloting. The identity of the Hc100 was confirmed by Western blot and mass spectrometry.

Purification of Hc100: Purification of Hc100 from a 72h cell culture supernatant was carried out using a Ni-NTA affinity chromatography column. The column was washed 2 times and the Hc100 was eluated first with a buffer containing 100mM Imidazol and second with a buffer containing 250mM Imidazol. Flow-thorws and eluates were analyzed by 8% SDS-PAGE followed by Coomasie-blue R250 staining and Western bloting.

Results

Transformation of Pichia pastoris: Several colonies were obtained from *Pichia pastoris* X-33 transformation and the presence of the Hc100 gene was confirmed by colony-PCR. All clones grew as the same rate as the X-33 strain, suggesting that all the clones had phenotype Mut+ (Methanol utilization Plus) (Figure 1).

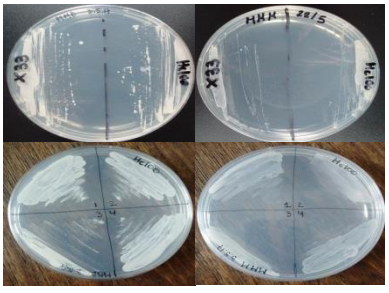

Figure 1. Phenotype of *P. pastoris* Hc100 transformants

Expression of Hc100: Different growth rates and pH were observed between the grow media (Figures 2 and 3). A band of the expected size was observed in the supernatants of BMMY and MBS media at 16, 24, 48 and 72h of methanol induction with the highest expression and lowest degradation levels at 72h of induction in MBS media with protease inhibitors (Figures 4). The identity of the Hc100 was confirmed by Western blot and mass spectrometry. Also, a lower molecular weight band was observed at 48 and 72h of induction, probably due to degradation processes. No Hc100 expression was observed when growing in BMM and MM media.

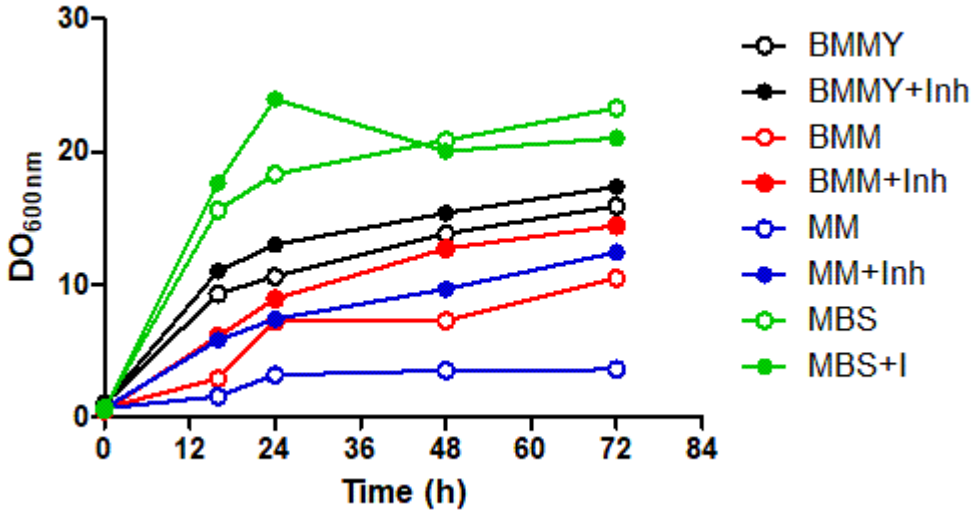

Figura 2. Expression of Hc100. Growth rate was estimated by measuring the OD_{600nm}. The highest biomass was obtained by growing in MBS medium.

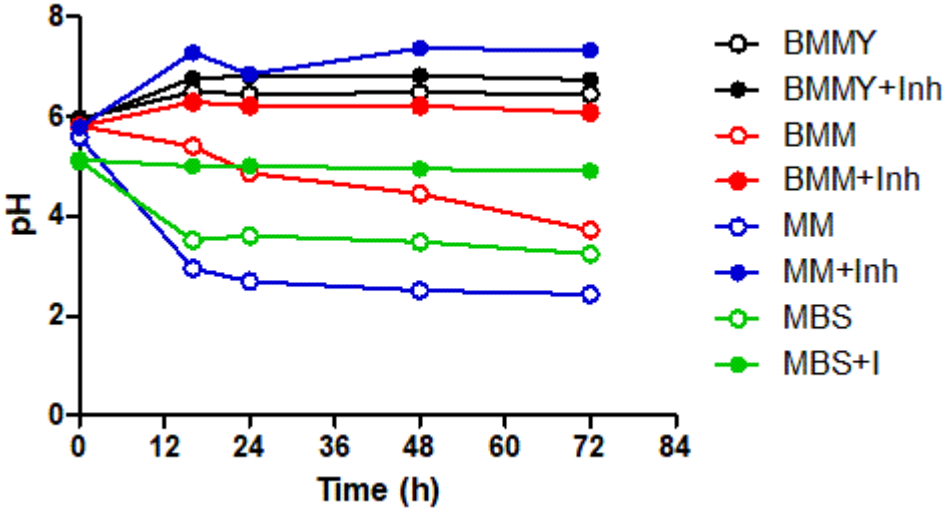

Figura 3. Expression of Hc100. Culture pH was measured at different time points.

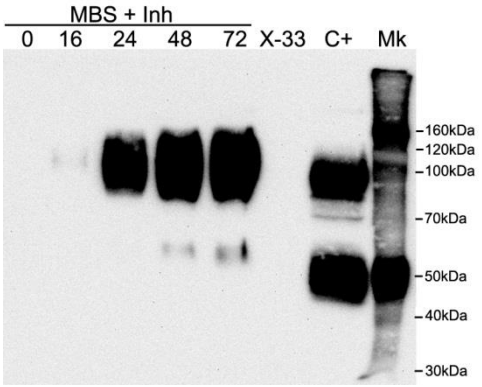

Figure 4. Expression of Hc100 in MBS media with protease inhibitors. Culture supernatant obtained at different time points were analyzed by Western bloting with anti-polyhistidine-tag antibodies.

Purification of Hc100: A concentration of 0.8mg/L of culture supernatant of Hc100 was obtained with a purity of ~90% (Figures 5 and 6).

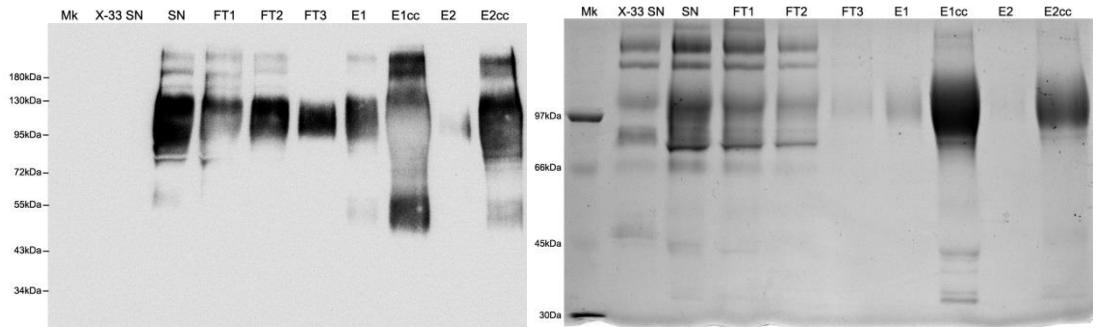

Figure 5. Purification of Hc100. *P. pastoris* X-33(X-33 SN) and Hc100-transformed (SN) culture supernatants, flow-throws from washes (FT1, FT2, FT3) and eluates (E1: 100mM Imidazole; E2: 250mM Imidazole) were analyzed by 8% SDS-PAGE followed by Coomasie-blue R250 staining.
Figure 6. Purification of Hc100. *P. pastoris* X-33(X-33 SN) and Hc100-transformed (SN) culture supernatants, flow-throws from washes (FT1, FT2, FT3) and eluates (E1: 100mM Imidazole; E2: 250mM Imidazole) were analyzed by Western bloting.

Conclusions

The Hc100 from *H. capsulatum* was successfully expressed in the cell culture supernatant of the methyilotrophic yeast *P. pastoris*.
The identity of the Hc100 was confirmed by Western blot and mass spectrometry.
The purification process of Hc100 based on a Ni-NTA affinity chromatography reached a purity of ~90%.
P. *pastoris* proved to be a valid biotechnological tool for the expression of the Hc100, thus encouraging the national production of novel fungal antigens for the potential development of new rapid diagnostic tests for this clinical relevant form of the histoplasmosis disease.