Full Length Research Paper

Utilization of experimental design and surface response methodology to study the influence of glucose and ammonium sulphate in the chlamydosporulation of *Candida albicans* FMT123-05

João Vicente Braga de Souza[1]*, Carolina Talhari[2], Dieter Reinel[3] and Sinésio Talhari[2]

[1]Department of Mycology, Institute of Tropical Medicine of Amazonas, Brazil.
[2]Department of Dermatology, Institute of Tropical Medicine of Amazonas, Brazil.
[3]Bernhard-Nocht-Institute for Tropical Medicine, Hamburg, Germany.

The aim of this work was to evaluate the influence of glucose and ammonium sulphate in the chlamydosporulation of *Candida albicans* FMT123-05. Different media were produced and inoculated with *C. albicans* FMT123- 05. The glucose and ammonium sulphate content were defined using an experimental design (2^2 + star). Glucose content inhibits *C. albicans* chlamydosporulation two times more than ammonium sulphate. The mathematical model and the surface response demonstrated that the total absence of carbon and nitrogen sources was the best condition for chlamydosporulation of *C. albicans* 123-05 in the experimental conditions.

Key words: Chlamydosporulation, glucose, ammonium sulphate, experimental design.

INTRODUCTION

The incidence of cutaneous, subcutaneous and invasive fungal infections has increased in recent years. Invasive fungal infections are usually correlated with morbidity and mortality especially in immunocompromised patients such as recipients of bone marrow transplants, patients with hematological malignancies with or without chemotherapy and AIDS patients (Jarvis, 1995; Jitsurong, 1993; Weig et al., 1998; Toscano and Jarvis, 1999; Trost et al., 2004). *Candida* species are one of most commonly opportunistic fungi (Trost et al., 2004; Farina et al., 1995). In particular, *Candida glabrata, Candida krusei, Candida lusitaniae* and *Candida guilliermondii* are of special concern because of their intrinsic resistance to Fluconazole (Pfaller et al., 1999).

Fast and specific methods are required to identify *Candida* species in order to guide the antifungal therapy.

Carbohydrates assimilation tests (API20C/32C), macroscopic morphology in CHROMagar and micromorphology in special culture media have routinely been used (Bale et al., 1997; Jarvis et al., 1995; Sidrin and Rocha. 2004)

C. albicans is the most frequently isolated species being found in 60 - 75% of the sites of infection. This specie is pleiomorphic and has the ability to switch its mode of growth from budding yeast-cells (blastospores) to a filamentous form in response to a wide variety of environmental signals (Odds, 1985; Gow 1997; Kurtzman and Fell, 1998; Berman and Sudbery, 2002). The filamentous form presenting pseudohyphae hyphae with terminal chlamydospores is conventionally used for *C. albicans* identification.

Several studies were carried out in order to obtain an adequate culture media for micromorphologic identifycation of *C. albicans.* Most of them evaluated complex culture media derived from natural sources (Beheshti, 1975; Casal and Linares, 1981; Hayes, 1966; Hunppert, 1975; Jansons and Nickerson, 1970; Jitsurong et al., 1993; Odds et al., 1997; Kumar et al., 2006). The culture

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*Corresponding author. E-mail: joaovicentebragasouza@yahoo.com.br. Tel: +55 92 21273555.

Table 1. Level and factors used in the experimental design.

Level	-1,41	-1	0	+1	+1,41
Glucose (g/L)	0	3.5	12	20.5	24
Ammonium sulphate (g/L)	0	0.9	3	5	5.9

media that have been conventionally used for the last three decades for *Candida* species identification are corn meal and rice agar, however more studies evaluating the culture media composition for faster micromorphological identification are still required (Irobi et al., 1999).

Factorial experimental design and surface response methodology are statistical tools that have been used in multivariated studies allowing the determination of the influence of the variables, their interaction and their statistical significance (Barros Neto, 1995). No study was carried out using these statistical tools to evaluate the influence of nutrients in the micromorphology of pathogenic *Candida* species.

The aim of this work was to evaluate the influence of glucose and ammonium sulphate in the chlamydo-sporulation of *C. albicans* 123-05.

MATERIALS AND METHODS

Candida albicans strain

This experiment was carried out employing *C. albicans* strain FMT123-05. This strain was isolated from a patient with fungemi and it was identified by its macroscopic, microscopic, reproductive and physiological characteristics according to the methods established by Fell Kurtzman and Fell (1998) . This strain was obtained from the mycological culture collection of the Foundation of Tropical Medicine of Amazon.

Culture medium, inoculum and growth conditions

The solid basal medium was composed by glucose, $(NH_4)_2 SO_4$, $MgSO_4.7H_2O$ (0, 5 g/L), KH_2PO_4 (1 g/L) and agar (15 g/L). The concentration of glucose and $(NH_4)_2SO_4$ varied according to the experimental design.

The cell inoculum was obtained according to Sidrin and Rocha (2004). 25 L of a cell suspension (1×10^4), originated from a 48 h culture, was dropped onto the culture medium surface and covered with a cover slip. Direct microscopy, through the cover slip, was carried out in order to quantify the chlamydospores.

Experimental design and surface response methodology

In order to verify the influence of glucose (g/L) and ammonium sulphate (g/L) content in the chlamydospores production (Y), a 2^2 + star experimental design with three repetitions in the central point was employed (Barros Neto, 1995). Table 1 shows the level and factors used in the design.

All eight experiments were performed as well as three assays representing the central point (coded value 0). A statistical model to

determine chlamydospores number was determined by the response regression procedure.

The statistical analysis was performed using STATGRAPHICS statistical software version 6.0 and STATISTICA program version 5.0.

Chlamydospores quantification

The chlamydospores number was determined by microscopy with magnification of 400x. Ten microscopy fields were used to calculate the mean value of chlamydospores per microscopy field.

RESULTS

In order to determine the influence of glucose and ammonium sulphate in the chlamydospores production, a 2^2 + star experimental design was used. Table 2 shows the results of the experimental design. The main effects and their respective interactions calculated from the data of Table 2 are shown in Table 3.The standard errors for the effects are shown in Table 3. Barros Neto (1995) only considers significant (95% confidence) the effects with values higher than $t_v \, x$. The t_v value is t test for v freedom degree. In this study the t test, for 2 freedom degree (95% confidence), was 4.3025.

The linear effects of glucose (A), ammonium sulphate (B) and the quadratic interaction of glucose (A*A) were significant and a linear model was adjusted (Equation 1).

Y= 8.57387 – 0.713854.[Glucose] – 0.527857.[Ammonium sulphate] + 0.0185223.[Glucose]. [glucose]
$$(1)$$

Table 4 shows the variance analysis, ANOVA, for the linear effects shown in Equation 1.

The F test (Fisher) was used to evaluate the regression and the lack-of-fit of the model (Barros Neto, 1995). The P- value for all the considered factors was near or inferior to 0.05 showing that these effects have significant regression. The P-value of lack -of-fit was 0.2355 which shows that the lack-of-fit was not significant. The absence of lack-of-fit, the significant regression and the high variance percentage explained, showed that the model, presented in Equation 1, could be used to explain the studied region. The surface response created using the model (Equation 1) is shown in the Figure 1. Figure 2 shows the micromophology of *C. albicans* 123-05 in two different mediums studied in this work.

Table 2. Results of the 2^2 + star experimental design with three repetitions in the central point.

Test	Glucose (g/L)	Ammonium sulphate (g/l)	Chlamydospores number per microscopy field
1	20.5	5.0	0
2	12	3.0	1
3	12	5.9	0
4	12	0.0	4
5	12	3.0	1
6	20.5	0.9	1
7	3.5	0.9	6
8	0.0	3.0	8
9	3.5	5.0	3
10	24.02	3.0	0
11	12	3.0	0

The chlamydospores number ranged from 0 to 8 per microscopy field.

Table 3. Variables affecting the chlamydospores production by *Candida albicans* 123-05 as revealed by the 2^2 + star experimental design.

Variables	Estimated effects ± standard error
Average	0.66 ± 0.33*
X1:	-4.57 ± 0.40*
X2:	-2.16 ± 0.40*
X1*X1	2.95 ± 0.48*
X1*X2	0.5 ± 0.57
X2*X2	0.95 ± 0.48

Standard error estimated from pure error with 2d.f. *Significative effects at the 5% level (t = 4.3025). [A] = dextrose content; [B] = sulfate ammonium content.

Table 4. Analysis of variance for evaluation of the model (Equation 2).

Source of variation	Sum of squares	df	Mean square (MQ)	F-ratio	P-value
A: Glucose	41.9239	1	41.9239	125.77	0.0079
B: Ammonium sulphate	9.36764	1	9.36764	28.10	0.0338
AA	11.0708	1	11.0708	33.21	0.0288
Lack-of-fit	5.87994	5	1.17599	3. 53	0.2355
Pure error	0.666667	2	0.333333		
Total	68.9091	10			

R^2 = 90.4997%; R^2 (adjusted for d.f).

DISCUSSION

It is effortless to find in the literature, the affirmation that chlamydospores are refractile, thick-walled cells that are produced under nutrient poor; oxygen limited conditions at low temperatures (Kumar et al., 2006). However other conditions as nutrients sources and content have decisive influence in the chlamydosporulation. (Jansons and Nickerson, 1970).

Our experiment allows us to quantify the influence, effect of the nutrients, and their interaction. Even in low concentration, glucose and ammonium sulphate
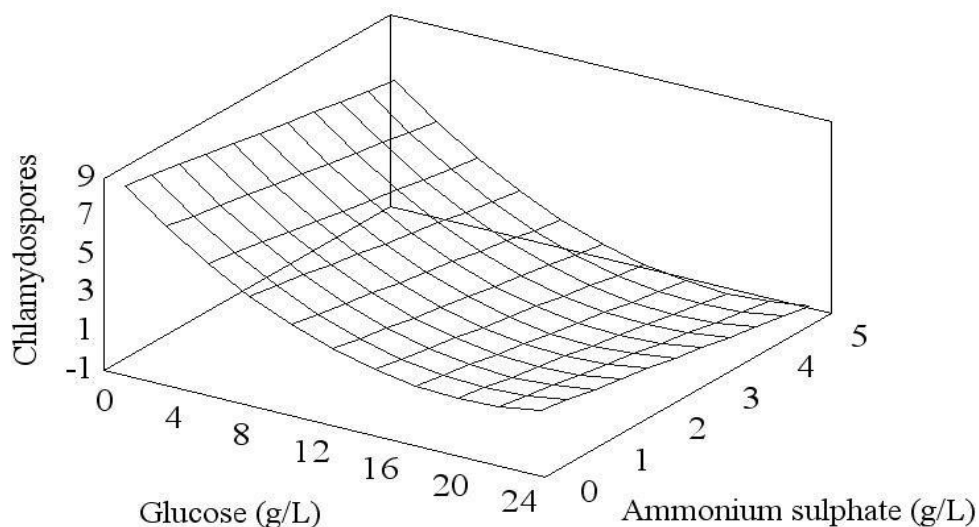
Figure 1. Influence of the contents of dextrose and ammonium sulphate in the chlamydospores production by *C. albicans* 123-05.
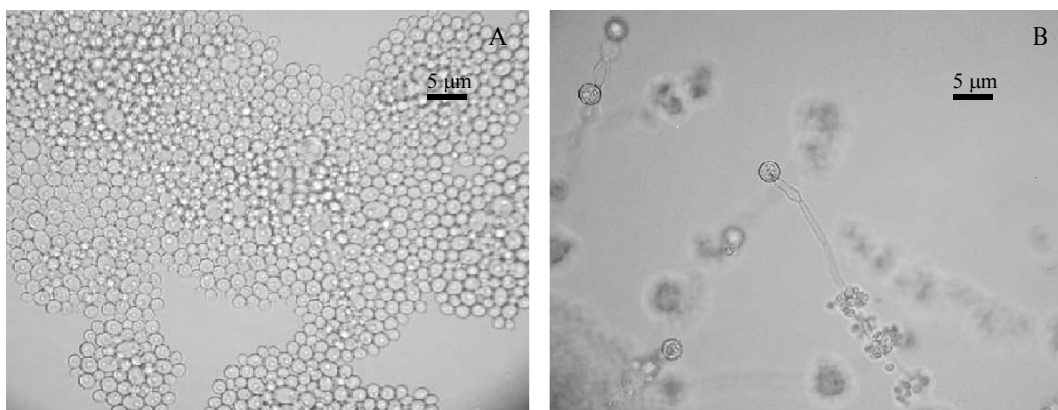

Figure 2. Micromorphology of *C. albicans* 123-05 (400×). A) 12 and 3 g/L of dextrose and ammonium sulphate, respectively (Medium 2); B) 0 and 3 g/L of dextrose and ammonium sulphate (Medium 8).

decreased the formation of chlamydospores. Glucose content inhibited *C. albicans* chlamydosporulation two times more than ammonium sulphate. The negative influences of glucose in chlamydospore production have been described since 1970 (Jansons and Nickerson, 1970) . However, this was the first study that quantified it using an experimental design and surface response methodology.

These powerful statistical tools were especially useful for this culture medium optimization (Barros, 1995). The maximum chlamydospores production predicted by the model was 10 cell per microscopic field corresponding to the point defined by the contents of glucose (A = -1.41) and ammonium sulphate (B = -1.41) of 0 g/l and 0 g/l (v/v), respectively (Figure 1).

Culture medium with low or none nutrient content have

been used for microorganism identification. An example of that is the water agar, a culture medium without carbon and nitrogen sources that have been used to induce spores production of filamentous fungi and actinomycetes (Sidrin and Rocha, 2004).

This preliminary finding shows that it is possible to use a simple culture media without any carbon and nitrogen source for the identification of the *C. albicans* strain used in this work. However, the main contribution of this work is the utilization of the statistical tools in order to improve the knowledge about a culture medium used for diagnosis.

ACKNOWLEDGEMENT

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