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OPTIMAL CONDITIONS FOR INDUCTION OF MYCELIAL FORMATION BY *CANDIDA ALBICANS* (ATCC 10231)

SALEH A. KABLI

Department of Biological Sciences, Faculty of Science, King Abdulaziz University, P.O. Box: 80203, Jeddah 21589, Saudi Arabia.

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Abstract. Four liquid media were tested for their ability to promote filamentation in ***Candida albicans*** (ATCC 10231). The proportion of mycelium produced was highest in modified Sabouraud's broth medium (MSB), comprising 1% mycological peptone (Oxoid) and 0.2% glucose, pH 7.4, and to lesser extent horse serum medium, pH 6.8. The peak of mycelium production appeared between 1.5 and 7.5h after inoculation of MSB medium and between 1.5 and 6.0h for the second medium. The shift of pH of MSB from acidity to neutrality favored more yeast growth than the shift from neutrality to alkalinity. The pH range of 5.4 to 8.4 favored filamentation in *C. albicans* while 7.4 proved to be the most inductive for filamentation percentage (100%). Incubation temperature of 37°C was optimal for maximum mycelial formation and yeast growth. Lower temperature (34° C) had suppressive effect on filamentation than the higher temperatures (40° and 43°C). The phenotypic switching of *C. albicans* on six solid media under aerobic and anaerobic (7% CO_2) conditions indicated that the yeast emerged feet appendages at the colony edges on horse serum medium, in absence or presence of 7% CO_2, and on Lee's, blood agar and chocolate agar media under 7% CO_2 only. However, it failed to form feet appendages on both MSB and Winge media either in presence or absence of 7% CO_2.

Key Words: *Candida albicans* ✱ Phenotype ✱ Mycelial formation ✱ Yeast phase ✱ Filamentation

INTRODUCTION

Candida albicans is an opportunistic fungal pathogen in humans and can cause either systemic or mucosal infection. In immunocompromised patients, this organism can progress to severe systemic invasion, leading to life- threatening circumstances (Odds, 1988, 1994). *C. albicans* is a polymorphic fungus capable of converting its cell shape from budding yeast to filamentous form, including pseudohyphae and true hyphae. This morphological transition has been strongly associated with pathogenicity (Calderone, 2002). *C. albicans* exhibits the ability to grow in either a yeast- like or germ tube and mycelial (pseudomycelial) form in response to different environmental factors. In vivo, the yeast of mycelium transition appears to play a role in pathogenesis and is considered as an important virulence factor (Cutler, 1991; Yang, 2003). In vitro, the morphological transition can be easily induced by environmental and / or nutritional conditions (i.e., fermentation medium, temperature, and pH), (Casanova *et al*, 1997; Johnson *et al*, 2005).

This paper describes the growth and induction of mycelial development in *Candida albicans* as influenced by different fermentation media, growth temperature and pH value, as well as, the yeast phenotypic switching on solid media, under aerobic and anaerobic (7% CO_2) conditions.

MATERIALS AND METHODS

Organism

Candida albicans (ATCC 10231) used in this study was obtained from the hospital of King Abdulaziz University, Jeddah, Saudi Arabia. It was kept on Sabouraud's glucose agar slopes at 4°C. Inocula were prepared from cultures on Sabouraud's agar slopes incubated at 37°C for 16-18h. The yeast cells were washed in water, centrifuged, and resuspended in water (under aseptic conditions). The number of blastospores / ml of suspension was determined by haemocytometer counting, and a suitable volume of suspension was added to 250 ml Erlenmeyer flasks containing 100 ml of broth to yield an initial concentration of 10^6 blastospores / ml (Evans *et al*, 1974).

Growth media and culture conditions

The four media tested for their ability to stimulate mycelium production in *Candida albicans* (ATCC 10231) were; modified Sabouraud's glucose broth (MSB), comprising 1% mycological peptone (Oxoid) and 0.2% glucose, adjusted to pH 7.4 (Evans *et al*, 1975), Lee's medium (amino acid/ synthetic medium) , which consisted of (g/l): $(NH_4)_2\ SO_4$, 5; K_2HPO_4, 2.5; NaCl, 5; $MgSO_4.7H_2O$, 0.2; glucose, 12.5; biotin, 0.001; and L-form of alanine, 0.5; leucine, 1.3; lysine, 1.0, methionine, 0.1; ornithine, 0.071; phenyl alanine, 0.5; thereonine, 0.5., pH 6.8. To the autoclaved ingredients, biotin (sterilized by Seitz filter) was added, (Lee *et al*, 1975), and Winge medium that contained (g/l): glucose, 20; yeast extract (Oxoid), 3.0, pH 5.6 (Mattia and Cassone, 1979), as well as, horse serum medium (Oxoid, SROO 35° C), pH 6.8. Media were warmed to their incubation temperature before inoculation. The flasks were then agitated on a rotary shaker (150 rpm) for 12 h. The obtained growth and the ability to form germ tubes and pseudomycelium (mycelium or filamentation) were recorded at 1.5 h intervals.

Growth and mycelial formation

The cell concentration was determined as single cells (blastospores) by direct count in a Burke haemocytometer. Germ tube and pseudomycelium (mycelium or filamentation) formation were measured by counting the number of individual cells showing a definite outgrowing tube and expressing this as percentage of the total cell population.

Total cell population = Number of blastospores + Germ tube forming cells.

Effect of pH value

The influence of different pH values (3.4 – 9.4) of the selected modified Sabouraud's glucose broth (MSB) was tested by the initial adjustment of aliquots of the medium to pH values range (3.4 – 9.4) by either 1 N HCl or NaOH.

Effect of incubation temperature

Candida albicans (ATCC 10231) was allowed to grow under the best pH value (pH 7.4) that induces mycelial formation and at incubation temperatures of 34°, 37°, 40°, and 43° C.

Phenotypic switching of Candida albicans:

Candida albicans (ATCC 10231) was allowed to grow on solid media of MSB, Lee's, Winge, blood agar base (HIMEDIA, Mo 89), chocolate agar (HIMEDIA), and horse serum, aerobically and anaerobically (in presence of 7% CO_2) for 48h at 37°C. Thereafter, the colony growth was described, either grows/h as normal yeast growth or forming feet appendages at the colony edges (Soll, 2002).

RESULTS AND DISCUSSION

Effect of growth media

The growth and filamentation pattern of *Candida albicans* (ATCC 10231) in the tested media (Table 1) indicates that ingredients of Winge medium favored yeast growth and not yeast filamentation, while the formatted of the horse serum medium promoted properly mycelial development rather than the yeast growth. However, ingredients of modified Sabouraud's broth (MSB) medium stimulated relative high growth values and high yeast filamentation. Lee's medium formulation favored moderate yeast growth and low mycelial formation. The pattern of filamentation in the 4 media was recorded in the early stages of growth. After a period, differed according to the type of the medium, the new blastospores were however continued to reproduce as budding yeasts but not give rise to further filaments developing on the type of the medium.

Table (1). Effect of different growth media on mycelial production in *Candida albicans* (ATCC 10231) incubated at 37°C for 12h

Incubation period (h)	Horse serum		MSB		Lee's		Winge	
	Total yeast count/ml x10^4	%of cells forming germ tubes	Total yeast count /ml x10^4	% of cells forming germ tubes	Total yeast count /ml × 10^4	% of cells forming germ tubes	Total yeast count / ml x 10^4	% of cells forming germ tubes
1.5	118	100.0	152	100.0	104	24.90	230	12.42
3.0	154	100.0	244	98.39	232	22.23	442	8.67
4.5	207	96.43	364	95.24	282	18.34	667	5.95
6.0	282	84.62	460	92.31	360	14.54	955	4.17
7.5	340	77.78	680	82.35	450	12.24	1200	3.34
9.0	396	76.92	770	72.74	680	8.60	1350	2.98
10.5	489	73.68	990	56.82	898	6.54	1495	2.71
12.0	571	66.67	1215	48.56	1186	4.97	1580	2.58

Media were inoculated with 10^6 blastospores/ ml.

On horse serum medium the proportion of inoculum's cells which initially gave rise to filaments was maximum, and the peak of mycelium production appeared between 1.5 and 4.5h after inoculation. Similar profile appeared with MSB medium, though the filamentation peak appeared between 1.5 and 7.5h. Instead of low mycelium formation by the yeast on both Lee's and Winge media still the filamentation phase was achieved between 1.5 to 4.5h after inoculation.

Horse serum medium proved to be the most inductive tested medium for filamentation. This may be attributed to its contents of hemin, hormones, and other natural ingredients of blood serum that are necessary to germ tube formation and hence increased yeast pathogenicity to their host. The superiority of horse serum medium and hemin, as well as, natural hormones and other ingredients found in the blood serum was reported by many workers (Casanova *et al,* 1997; Brown, 2002; Gow, 2002; Clemons *et al,* 2004; Johnson *et al,* 2005). Our results revealed also that the tested yeast failed to form germ tubes when grow on Sabouraud's medium. However, modified Sabouraud's broth (MSB) medium, which contained only 0.2% glucose noticeably stimulated mycelial formation, this finding may be related to glucose starvation. The induction of mycelial formation due to starvation and lower glucose concentration was previously indicated by some workers (Sabie and Gadd, 1988; Paranijape and Datta, 1991; Johnson *et al*, 2005)

Lowered mycelial formation in the tested *Candida albicans,* when grown in Lee's and Winge media may due to the synthetic nature of Lee's medium and its content of amino acids, biotin, high phosphate (0.25%) and relatively high glucose content (1.25%), while Winge medium contained high glucose level (2%) . In accordance with these findings it was reported that *Candida albicans* responds to amino acids starvation by activating hyphal development (Tournu *et al,* 2005). Furthermore, glucose and phosphate are known to regulate dimorphism in *Candida albicans.* The high concentration of phosphate in Lee's medium may be the reason for the reduced ability of starved cells to form germ tubes (Paranijape and Datta, 1991). These results assessed the importance of nutritional factors in induction of germ tube formation by *C. albicans* (Mattia and Cassone, 1979; Paranijiape and Datta, 1991; Casanova *et al,* 1997; Westwater *et al,* 2005).

Effect of pH value

Impressed by the fact that *Candida albicans* is an opportunistic fungal pathogen that invades any organ with pH ranges from acidity to alkalinity (Cornet *et al,* 2005), the effect of the starting pH value of MSB medium on yeast mycelial formation was tested. The results (Table 2) indicate that the yeast growth and germ tube formation responded differently to the pH value. The highest yeast growth was attained with pH range from 4.4 to 7.4. While, alkaline pHs (8.4 and 9.4) were responsible for lower growth values. This indicates that the shift of pHs from acidity to neutrality favored more yeast growth than the shift from neutrality to alkalinity. The pattern of filamentation percentage was observed at the early stages of growth, where the peak of germ- tube formation appeared between 1.5 to 3h, for the acidic pHs (3.4 to 6.4, respectively). Where as at the neutral and alkaline pHs (7.4 to 9.4) the peak was

Table 2. Effect of pH value on mycelial production in *Candida albicans* (ATCC 10231)cultivated in modified Sabouraud's broth (MSB) medium at 37°C for 12 h

Incubation period (h)	pH value													
	3.4		4.4		5.4		6.4		7.4		8.4		9.4	
	TYC/ ml × 10^4	% of CFGT	TYC/ ml × 10^4	% of CFGT	TYC/ ml × 10^4	% of CFGT	TYC/ ml × 10^4	% of CFGT	TYC/ ml × 10^4	% of CFGT	TYC/ ml × 10^4	% of CFGT	TYC/ ml × 10^4	% of CFGT
1.5	112	12.05	132	27.96	146	77.78	168	86.84	152	100	108	70.11	102	30.0
3.0	168	80.4	172	41.84	186	77.14	230	90.00	224	98.39	205	81.08	164	74.36
4.5	202	6.07	216	38.36	278	64.28	380	75.58	364	95.00	346	95.24	224	91.30
6.0	380	3.57	500	33.34	550	52.24	650	57.50	460	92.31	445	73.89	360	85.71
7.5	532	2.55	700	23.85	830	34.69	900	48.77	680	82.35	598	55.03	420	75.00
9.0	840	1.63	1002	16.68	1020	28.23	1160	37.84	770	72.74	720	45.74	610	51.80
10.5	1100	1..25	1200	13.95	1280	22.5	1320	33.25	990	56.82	800	41.18	725	43.72
12.0	1230	1.12	1380	12.16	1473	19.6	1592	27.58	1215	48.56	960	34.35	900	35.33

TYC = Total yeast count
CFGT = Cells forming germ tube.

recorded within incubation period of 1.5 to7.5h. The pH value of 7.4 of MSB medium was the most inductive for filamentation (100%) percentages. It was reported that the extracellular pH value is one of the environmental factors that modifies the physiology and morphology of the cell (Cornet *et al*, 2005). It was also indicated that the gene of cell wall protein is expressed at a pH ≥ 5.5 and is required for systemic candidiasis (blood pH is near neutrality), whereas its paralogue gene is expressed only at acidic pH (pH ≤ 5.5) and is required for vaginal candidiasis (vaginal pH is around 4.5) (Saporito–Irwin *et al*, 1995; De Bernardis *et al*, 1998). The role of pH regulation in morphogenesis and germ tube formation was reported by many workers (Pollack and Hashimoto, 1987, Sabie and Gadd, 1988; Casanova *et al,* 1997; Tournu *et al*, 2005; Johnson *et al*, 2005; Westwater *et al*, 2005).

Effect of incubation temperature

The influence of temperature range (34° – 43°C) on the formation of the germ tube revealed that 37°C was optimal for maximum filamentation percentages and yeast growth as compared to the other incubation temperatures. However, higher temperatures (40°– 43°C) were concomitant with lower yeast growth, and high mycelial formation (Table 3). The pattern of filamentation percentage was in the early stages of growth and the peak of mycelium production was after 1.5h of incubation at temperature of 34° C and appeared to be between 1.5 to 4.5h at 37° and 40°C. These findings assessed that the human body temperature (37°C) favors filamentation and hence pathogenicity of *Candida albicans*. In accordance with these findings, it was reported that 37°C was optimum for germ tube formation in *Candida albicans* and its adherence to the host cells (Mattia and Cassone, 1979; Hrmova and Drobnica, 1981; Casanova *et al*, 1997; Samaranayake and Samaranayake, 2001; Westwater *et al*, 2005).

Phenotypic switching of C. albicans (ATCC 10231) in solid media

The phenotypic of *Candida albicans* (ATCC 10231) grown on solid media of MSB, Lee's, Winge, chocolate, blood agar, and horse serum agar incubated in presence of 7% CO_2 incubator (anaerobic treatment) and in usual incubator (aerated treatment) for 48h at 37°C, recorded normal yeast growth without feet appendages at the colony edges when yeast was grown on the first five tested media in absence of 7% CO_2 (anaerobic treatment). However, on horse serum medium feet appendages emerged in both conditions (absence and presence of 7% CO_2). While on MSB and Winge medium, the yeast failed to form feet appendages. The phenotypic switching in colony form of *C. albicans* in aerobic and anaerobic conditions is used as indication for its virulence and pathogenicity, as well as, resistance to fungal antibiotics. *C. albicans* strains isolated from candidiasis ills have the ability for phenotypic switching, while those inhabiting healthy persons lack this phenomenon (Soll, 2002; Cetinkaya and Kiraz, 2005) Phenotypic switching of *C. albicans* is one of the major virulence factors and has been shown to be effective in defense of the immune system, increase in adherence, increase in enzyme secretion, and decrease in susceptibility to antifungal (Slutsky *et al*, 1985; Soll, 2002; Yang, 2003).

Table (3). Effect of incubation temperature in mycelial production in *Candida albicans* (ATCC 10231) cultivated in modified Sabouraud's broth (MSB) medium at pH 7.4 for 12h.

Incubation period (h)	Incubation temperature (°C)							
	34		37 (basal)		40		43	
	TYC/ ml $\times 10^4$	% of CFGT	TYC/ ml $\times 10^4$	% of CFGT	TYC/ ml $\times 10^4$	% of CFGT	TYC/ ml $\times 10^4$	% of CFGT
1.5	164	27.45	152	100	113	72.19	103	61.43
3.0	313	21.74	224	98.39	192	79.31	173	66.26
4.5	408	19.67	364	95.24	280	85.50	243	54.75
6.0	500	16.20	460	92.31	430	64.10	396	33.61
7.5	730	11.10	680	82.35	560	56.25	445	29.93
9.0	848	9.61	770	72.74	706	48.72	680	19.70
10.5	937	8.65	990	56.82	816	42.15	740	18.12
12.0	1132	7.17	1215	42.56	915	37.60	826	16.25

TYC = Total yeast count
CFGT = Cells forming germ tube.

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