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Effect of Incubation Temperature and Human Serum on Yeast to Hyphal Morphogenesis in Vaginal *Candida albicans* and its Correlation to Virulence Markers

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Authors' contributions

This work was carried out in collaboration between all authors. Author TAE designed the study, and wrote the first draft of the manuscript. Author RAA participated in planning of the study and managed the manuscript analyses. Author AHA participated in its final designing, provided reagents for assessment of exoenzymes activity and manage statistical analysis. Author ZZA, performed the practical laboratory activities and participated in the interpretation of the results. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: To investigate the effect of human serum, starvation and /or variation in incubation temperature on yeast and pseudo-hyphae and/ or hyphal cell differentiation in vaginal *Candida albicans* strains and, its correlation to exoenzymes productivity.
Study Design: A total of 31 *C. albicans* strains previously isolated from high vaginal swab specimens of pregnant Saudi women, as well as the *C. albicans* QC strain ATCC 10231 were recruited from Brain Heart Infusion-glycerol stock cultures(-80ºC) & included in the study.
Place and Duration of Study: Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, King Saud University, Riyadh, Saudi Arabia, between September 2013 and December 2013.
Methodology: Each of thirty one vaginal *C. albicans* strains and the QC strain (ATCC

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10231) was grown in Modified Sabouraud Broth (MSB) at 25ºC and at 37ºC with or without addition of 20 % human serum; and morphological growth was observed at 2 hours intervals by phase contrast microscopy. Selected *C. albicans* strains that showed ability and/or weak-ability of yeast-hyphal transition were also tested for their exo-hydrolytic enzymes of phospholipase, and proteinase as caseinase, & gelatinase, and coagulase, virulence markers.

Results: Showed that at 25ºC 28/31(90.3%) strains were non filamentous, 3/31(9.6%) strains were moderately filamentous, and 0.0% strong filamentous, in comparison, at 37ºC those numbers were 19/31(61.3%), 10/31(32.3%), and 2/31 (6.4%) respectively, suggesting that mere increase in temperature from 25ºC to 37ºC remarkably increases yeast morphogenesis to filamentous forms. Such increase was significantly ($P<0.001$) more pronounced upon the addition of 20% serum at either incubation temperature of 25ºC or 37ºC as expected. Generally the presence of serum and/or incubation at high temperature (37ºC), speeds off hyphal growth formation. Additionally, results also showed that 8/31 (25.8%) strains exhibited transition to hyphal forms only in presence of serum, whereas 7/31 (22.6%), apparently lost their capacity to switch to hyphal forms even in presence of serum and/or at temperature of 37ºC incubation. In contrast three strains 3/31, (9.7%) expressed such ability of filamentous growth in presence or absence of serum at 37ºC as well as 25ºC. These strains also showed enhanced secretion of exoenzymes. Therefore, these strains would be the most virulent ones. Whereas those strains (7/31, 22.6%) that did not show filamentous growth at any of the examined growth conditions would be considered as less virulent strains. However, considering the limited number of strains tested in this study, these findings require further substantiation by large sample size and *in vivo* animal studies.

Conclusion: Results obtained suggest that vaginal *C. albicans* strains are heterogenous in their potency to switch from yeast to hyphae. Strains which show morphogenesis in absence of serum and/or at low temperature (25ºC) exhibit higher exoenzymes activity suggesting that these strains are more pathogenic.

Keywords: Candida albicans; vaginal strains; yeast-hyphal forms transition; exoenzymes activity; virulence factors.

1. INTRODUCTION

Candida albicans is usually encountered among the majority of the healthy individuals as a harmless commensal fungus. However, in less immune-competent persons, it became an opportunistic invasive pathogen, to the extent that it has been described as the fourth leading cause of nosocomial blood stream infections [1], where mortality might reach up to 37-44% [2,3].

C. albicans is a polymorphic fungus that has the ability to rapidly switch between yeast and filamentous forms [4]. The organism is able to grow in different morphological forms such as oval budding yeast cells (blastoconidia),and a range of filamentous forms that include true hyphae (without constrictions) as well as pseudohyphae, (with constrictions) [5]. The yeast form is apparently important for dissemination through the bloodstream [6,7] and adherence to endothelial surfaces [8]. The filamentous forms, on the other hand, are more adapted for invasion through the host epithelial tissue [9], and filamentous forms have a higher resistance to neutrophil killing [10]. Indeed, an engulfed *C. albicans* yeast cell can destroy macrophage if filamentous growth is triggered after phagocytosis [11,12].

A variety of environmental factors such as serum, ambient pH above 6.5, temperature of 37°C and above, and low dissolved oxygen can trigger transitions from the yeast form of growth to a pseudohyphae and hyphal growth forms *In vitro* [13]. These morphological differentiations promote colonization and invasion at different anatomical sites. *C. albicans* strains seem to adhere equally well to both exfoliated vaginal and epithelial cells [14,15]. Its virulence is enhanced by toxins, proteolytic and phospholipase elaborated by yeast [16].

The secreted aspartyl-proteinases elaborated by pathogenic *Candida* species have been identified in vaginal secretions in women with symptomatic vaginitis but not in those with asymptomatic colonization [17]. Several genes that govern proteinase production (SAP1, SAP2, and SAP3) have been cloned, and a strong correlation exists both *In vitro* and in experimental vaginitis between gene expression, aspartyl-proteinase secretion, and the ability to cause disease [18,19]. Accordingly, the present study was undertaken to investigate the effect of human serum, starvation and /or variation in incubation temperature on yeast and pseudo-hyphae and/ or hyphal cell differentiation in vaginal *C. albicans* strains and, its correlation to exo-hydrolytic enzymes of phospholipase and proteinase as caseinase, & gelatinase and coagulase, virulence markers.

2. MATERIALS AND METHODS

2.1 Isolation and Identification

All of the thirty one vaginal *C. albicans* strains used in this study, were recruited from Brain Heart Infusion stock cultures containing 20% glycerol and maintained at (-80ºC) which were previously isolated from high vaginal swab specimens of Saudi pregnant women as reported previously [20,21]. Presumptive and laboratory identification of these *C. albicans* strains was previously carried out by colony morphology on Sabouraud dextrose agar (Fig.1A) and CHROMagar Candida (Fig. 1B) medium (CHROMagar, Paris, France), germ tube test (Fig.1C) and chlamydospore formation on corn meal agar (Fig.1D), as well as API 20C (bioMerieux) identity confirmation. Briefly, as reported previously [20], the API 20C uses disposable plastic strips containing 20 cupules. The first cupule is a negative control and second cupule contain glucose positive control. The remaining 18 cupules each contain a specific substrate that may be assimilated by the test organisms. The strips were incubated at 29-30ºC and read at 48-72 hours. Sugars assimilations reactions were read by comparing the strip to growth controls and identification was obtained by referring to the analytical profile index. A profile number was generated for each strip depending upon the reactions it produced.

In parallel to the 31 randomly selected vaginal *C. albicans* strains, the *C. albicans* ATCC 10231 was also included as (QC) reference strain.

2.2 Inoculum Preparation and Testing Efficiency of Filamentous Growth Forms

The modified Sabouraud glucose broth (MSB) medium, 1% mycological peptone (Oxoid, UK), and 0.2% glucose [21,22] was used to test the ability of filamentous formation in 31 randomly selected *C. albicans* strains. Each of the selected 31 *C. albicans* isolates or QC strain was grown in MSB (5ml) under static condition at 30ºC for 18 hours (Fig. 2), then cultures were diluted to 1:50 in fresh MSB and incubated with shaking at 37ºC for 4-6

hours. After incubation cells were recovered by centrifugation at 4000 rpm for 5 min and washed with sterile water to remove the entire medium. The pellets of cells were then re-suspended in sterile water and adjusted to O.D at 620 nm, 0.3 to 0.5 and incubated for 2 hours at 30ºC in order to exhaust and stop the cell cycle of all the yeast-cells. After these steps, the cells were kept in the refrigerator at 4ºC for 72 hours in order to induce a metabolic starvation. Thereafter cells were harvested by centrifugation for 5 minute at 4000 rpm and washed twice with sterile saline. The pellets were then re-suspended in sterile saline, and turbidity adjusted to O.D 620 nm=0.5 [21,23,24]. To each well of the microtiter plates (flat bottom) 180µl of MSB and inoculated with 20µl of the above yeast cell suspension. Yeast-free medium controls were also included. Each plates-set was performed in triplicate in relation to 4 experimental conditions by adding to each well: i-saline (50µl) at 25ºC and 37ºC; and ii-human serum (50µl) at 25ºC and 37ºC. These experimental steps are illustrated in Fig. 2.

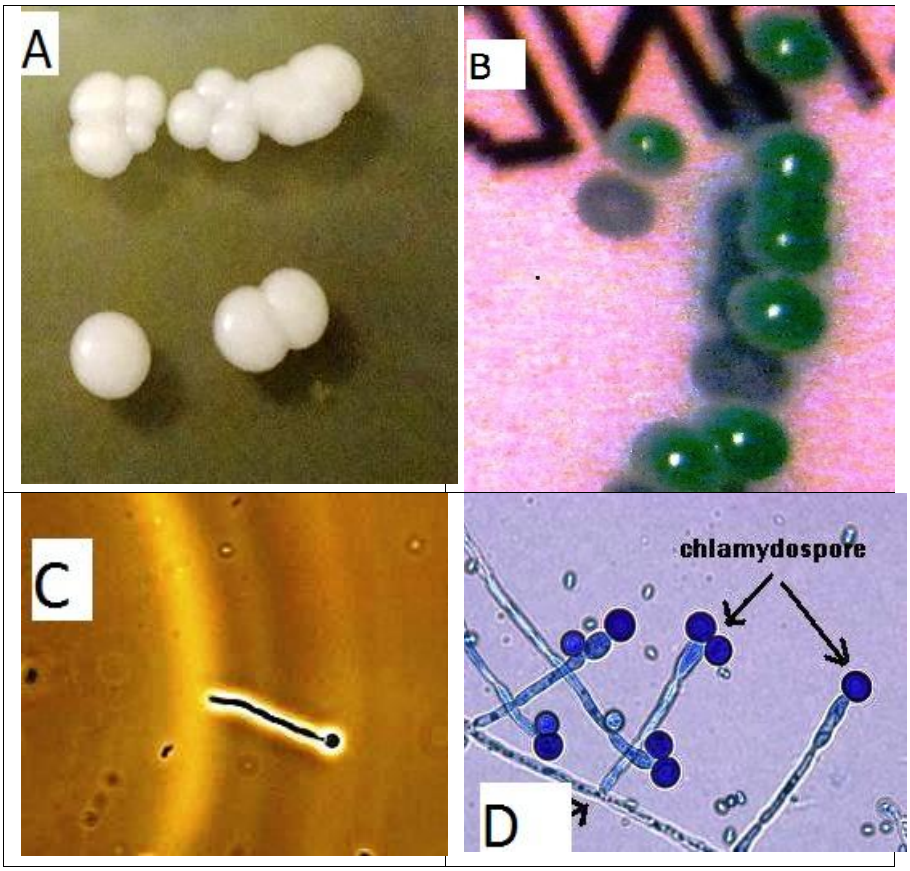

Fig. 1. Colonies of *C. albicans* appear with smooth surfaces on Sabouraude dextrose agar, A; Dark green colonies of *C. albicans* as appear on CHRO Magar Candida medium, B; photographs of *C. albicans* positive germ tube test (C), and its chlamydospores (D) on corn meal agar medium

2.3 Detection of Exoenzymes Activity

The inoculum preparation of selected represented *C. albicans* strains as well as QC strain and media used for the assessment of exoenzymes productivity of phospholipase, proteinase (as caseinase, and gelatinase) and coagulase were carried out essentially as previously described by several investigators [16,25,26,27]. Results interpretation of precipitation and/or clearance zone around the colony (triplicate plates) was measured according to the method described by Price et al. [26], where It was define as the ratio of the diameter of the colony to the total diameter of the colony plus the precipitation and /or clearance zone. A Pz value of 1 indicated no enzyme activity, Pz<1 denoted enzyme activity. The lower the Pz value, the higher the enzyme activity [16].

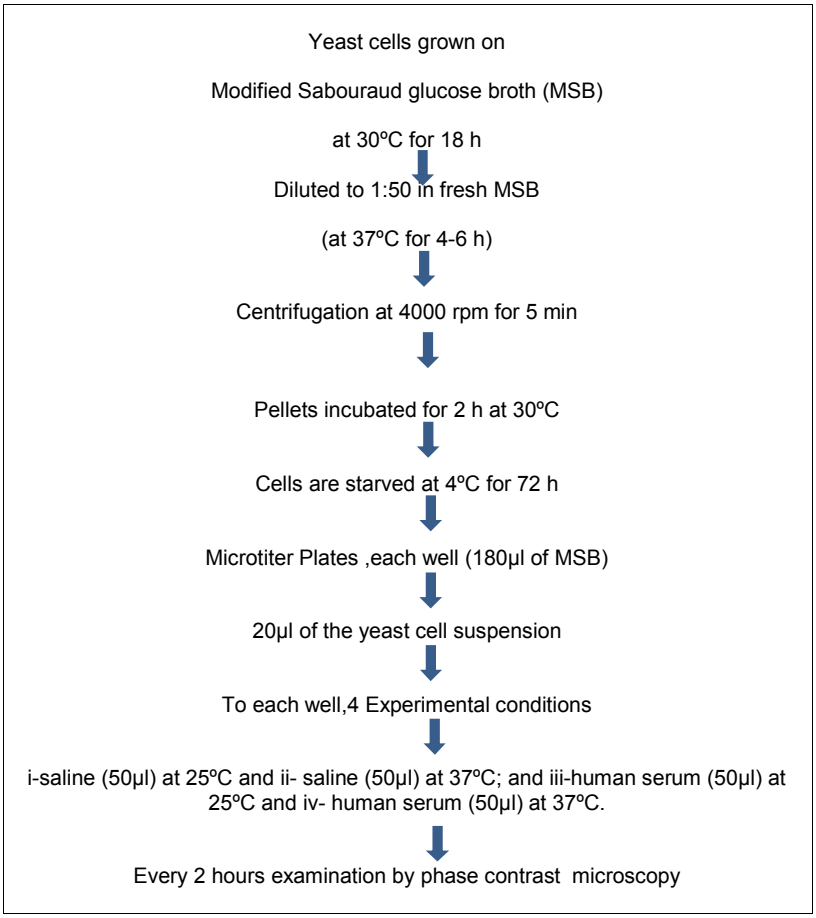

Fig. 2. A flow chart illustrating the experimental steps of the study

2.4 Statistical Analysis

The results were analyzed using SPSS 19 (Statistical Package for Social Science; release 19.0). Chi-square test and Fishers exact test were used to compare association between proportions and a P-value < 0.05 was considered as significant.

3. RESULTS

As presented in Table (1), each of the examined 31 *C. albicans* strains as well as the QC strain ATCC 10231, was grown in MSB with and without serum at 25ºC and at 37ºC and examined by phase contrast microscopy at 2 hours intervals as illustrated in (Fig.2) flow chart. The summarized results in Table (2) showed that at 25ºC 28/31(90.3%) strains were non filamentous, 3/31(9.6%) strains were moderately filamentous, and 0.0% strong filamentous, in comparison, at 37 ºC those numbers were 19/31(61.3%), 10/31(32.3%), and 2/31 (6.4%) respectively, suggesting that mere increase in temperature from 25ºC to 37ºC remarkably increases yeast morphogenesis to filamentous forms. Such increase was significantly ($P<0.001$) more pronounced upon the addition of 20% serum at either incubation temperature of 25ºC or 37ºC as expected. This difference in this enhancement proved (Table 2) significantly ($P<0.001$) as compared to same respective results without serum.

Table 1. The tested *C. albicans* strains (n=31) and QC ATCC 10231 strain arbitrary categorized on bases of their filamentous capacity on modified Sabouraud broth medium with or without human serum at 25ºC and 37ºC temperatures of incubation

C. albicans strains	Filamentous potency			
	25ºC	25ºC+Serum	37ºC	37ºC+ Serum
ATCC 10231	-	+	+	++
5	-	+	-	+
27	-	+	+	++
89*	-	+	+	++
116	-	+	+	++
118	-	-	-	-
120	-	-	-	-
126	-	+	-	+
128	-	-	-	+
172	-	+	-	++
178	-	+	-	+
192	-	+	-	+
226	-	-	-	+
238	-	+	+	+
241	-	+	-	++
260	-	++	+	++
261	-	++	+	+
294	-	-	-	-
304	-	+	+	++
307	-	+	+	++
327	+	++	+	++
354	-	+	+	++
373	-	+	-	+
403	-	+	-	+
530*	-	-	-	-
532	-	-	-	-
593	-	-	-	-
594	-	-	-	+
623*	-	-	-	-
640	-	+	-	+
646	+	++	++	++
695*	+	++	++	++

-: non filamentous strain; +: moderately filamentous strain; ++: strongly filamentous strain;
** represented strains*

Generally, as expected, the presence of serum and/or incubation at high temperature (37ºC) speeds off mycelia formation as long as it is expressed. Additionally, Table (1) also showed that strains {126, 172, 178, 192, 241, 373, 403, and 640}, 8/31 (25.8%) exhibited transition to hyphal forms only in presence of serum, whereas strains {118, 120, 294, 530, 532, 593, and 623}, 7/31 (22.6%), apparently lost their capacity to switch to filamentous forms even in presence of serum and/or at temperature of 37ºC incubation. Meanwhile strains {128, 226, and 594}, 3/31 (9.6%) displayed transition to hyphal forms only in presence of serum and incubation at temperature of 37ºC. In contrast strains {327, 646, and 695}, 3/31 (9.6%) expressed such ability of filamentous growth in presence or absence of serum at 37ºC as well as 25ºC (Table 1). Thus as illustrated in Figs. 3,4,& 5, the *C. albicans* strain 304, strain 126,& strain 623 would be considered as strong, moderate, and poor for their respective filamentous potency.

Table 2. Overall assessment of Influence of incubation temperature and 20% serum on filamentous growth in tested *C. albicans* strains (n=31)

Conditions	Filamentous potency of *C. albicans* (n=31)			
	Strongly filamentous strains	**Moderately filamentous strains**	**Non-filamentous strains**	**P- value**
25ºC	0	3	28	<0.0001
25ºC+20% serum	5	16	10	0.012
37ºC	2	10	19	<0.0001
37ºC+20%serum	12	12	7	0.298

The peak of filamentous formation by *C. albicans* was observed between 2-4 hours of incubation in MSB medium with 20% serum, whereas it was between 2 and 6 hours in MSB medium without serum. Nevertheless, reversion to secondary blastoconidia began at a mean of 2.5 hours after inoculation in both conditions.

In the present study, an attempt was also made to test the correlation of potency of morphological differentiation from yeast to hyphal forms in randomly represented *C. albicans* strains and their exoenzymes activity in parallel with the QC ATCC10231 strain; as it has been reported [28,29] that genes leading hyphal phase differentiation are co-regulated with those genes governing virulence factors such as cell attachment-adhesins and exo-hydrolytic enzymes of phospholipase, and proteinase as caseinase, & gelatinase, and coagulase. The obtained results showed remarkable difference in various hydrolytic enzymes productivity among tested strains as presented in Table (3) and Fig. (6 & 7).The discriminative enzymes that correlate the potency of yeast-hyphal transition were in order phospholipase, gelatinase, coagulase, and to a lesser extent caseinase. Considering these exoenzymes activity of tested represented strains, results seem to suggest that the strains {327, 646, and 695}, 3/31 (9.6%) which expressed ability of filamentous growth in presence or absence of serum at 37ºC as well as 25ºC, also showed enhanced secretion of exoenzymes. Hence, these strains would be the most virulent ones. Whereas those strains {118, 120, 294, 530, 532, 593 and 623}, 7/31 (22.6%) that did not show filamentous growth at any of the examined growth conditions would be considered as less virulent strains. The QC reference strain and strain 89 lies in between those two extremes, again for both characters i.e. morphogenesis efficacy and hydrolytic enzymes activity (Tables 1 and 2 and Figs. 6 and 7).

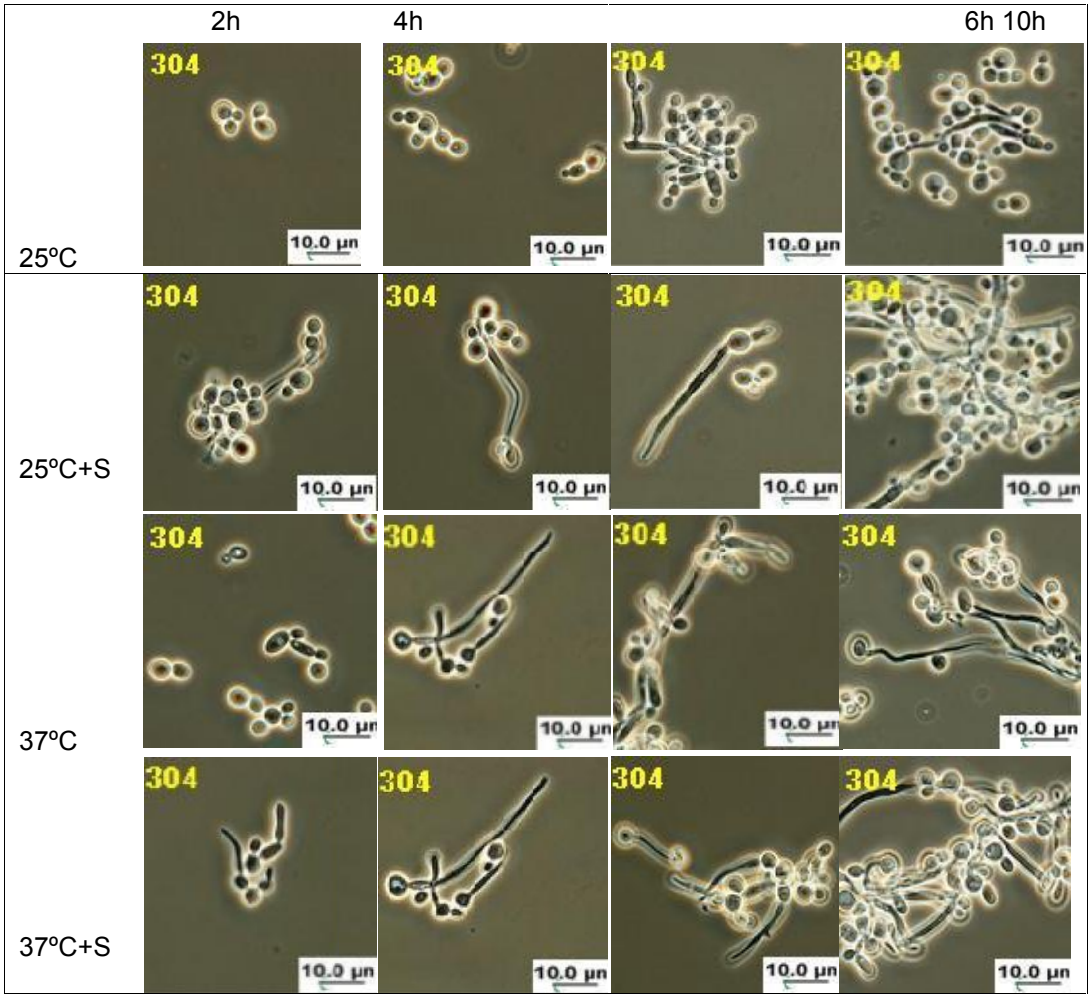

Fig. 3. Phase contrast microscopic observation (×100) at 2 hours intervals, of filamentous range of *C. albicans* (strain, 304), strong filaments producer with or without (S) serum at 25ºC and 37ºC

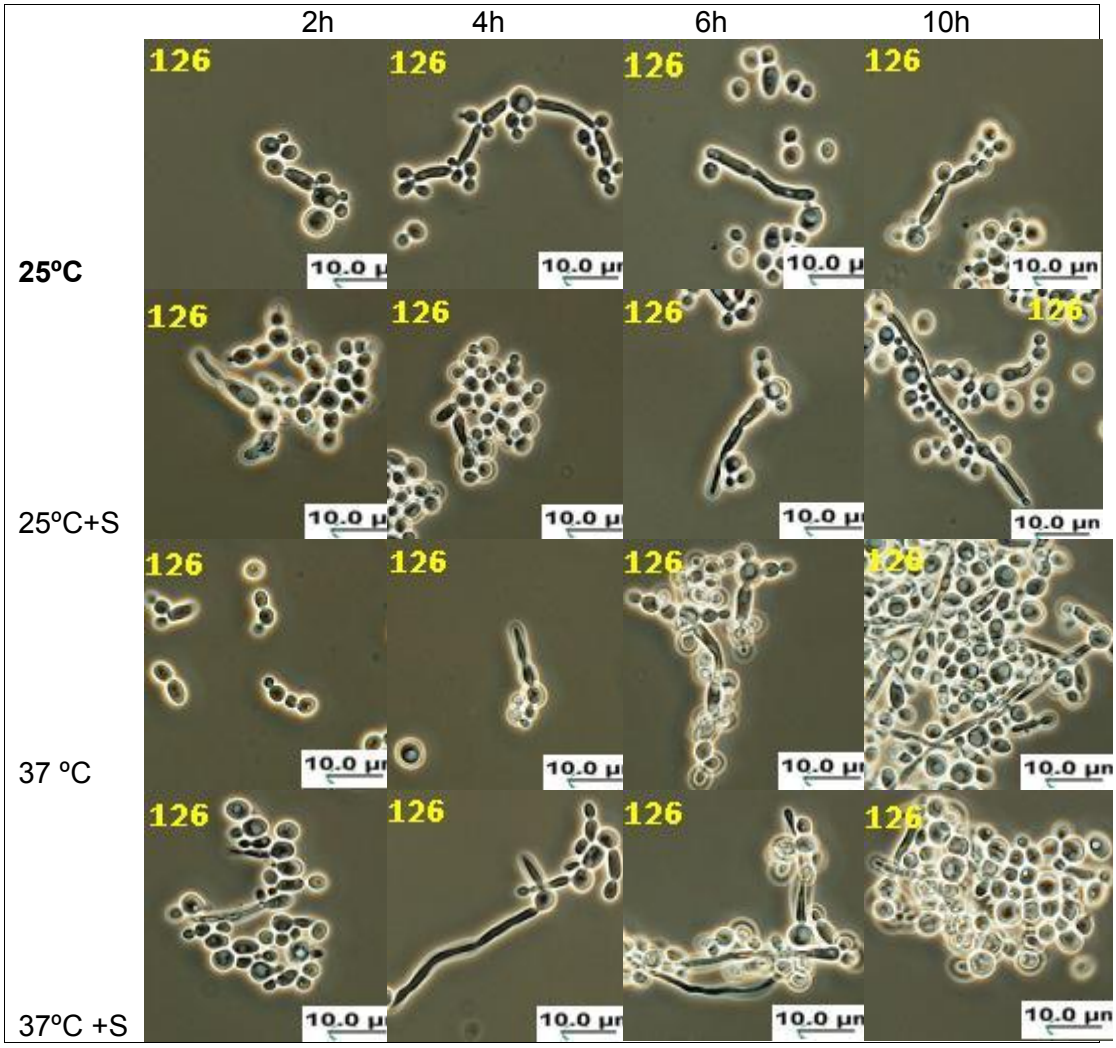

Fig. 4. Phase contrast microscopic (×100) observation of filamentous range of *C. albicans* (strain, 126). Moderate filamentous potency with or without (S) serum at 25ºC and 37ºC

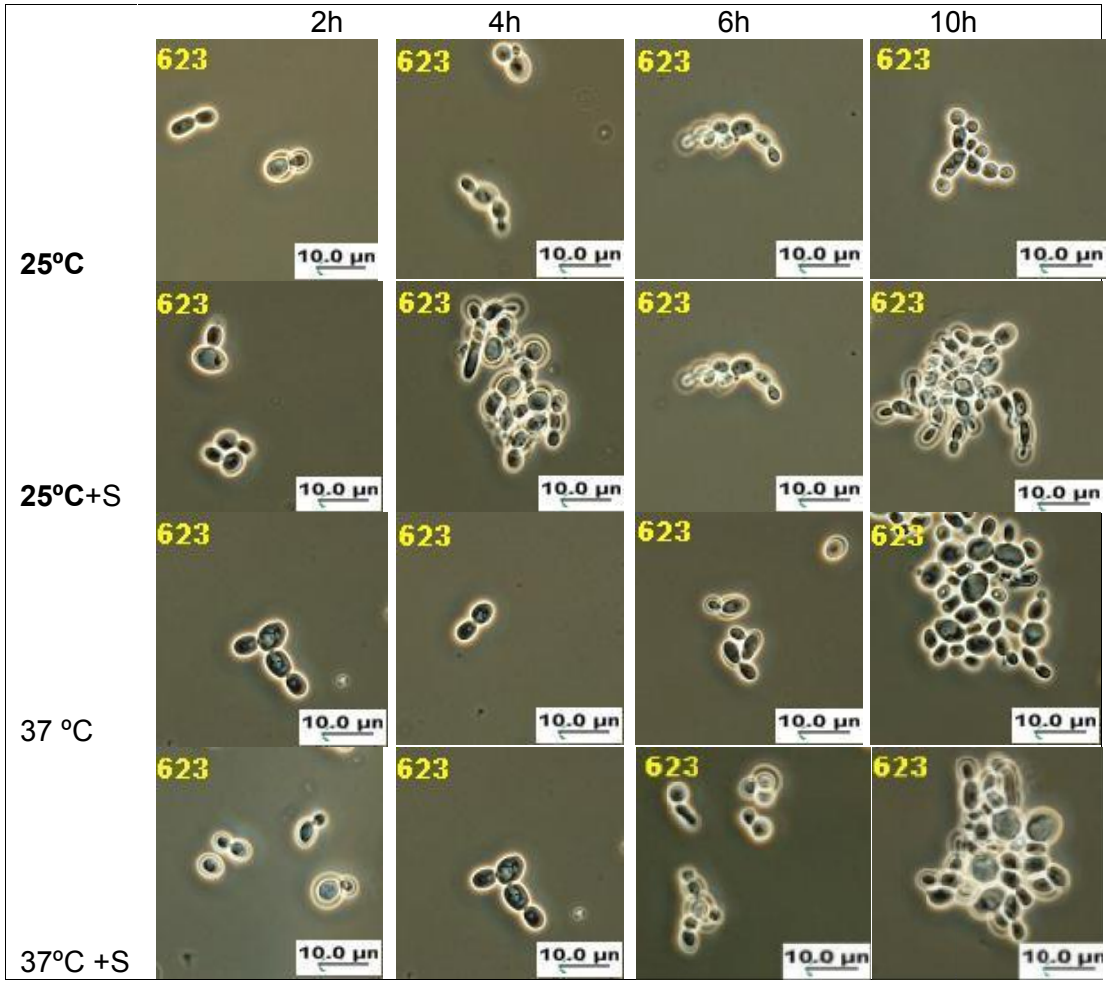

Fig. 5. Phase contrast microscopic (×100) observation of filamentous range of *C. albicans* (strain, 623) with almost no filamentous potency with or without (S) serum at 25ºC and 37ºC)

Table 3. The exoenzymes activity of randomly represented* *C. albicans* strains of varied filamentous capacity

Strains*	Pz** value on medium of		Gelatinase	Coagulase
	Egg yolk	Casein		
ATCC10231	0.4	0.46	±	-
530	0.34	0.44	±	-
695	0.24	0.32	++	++
89	0.47	0.46	-	-
623	1.0	0.5	±	±

** C. albicans control strain and strain, 89(moderate filamentous transition); strain 695(strong); and strains 530 &623 (relatively poor)-, No activity; ±, weak activity; ++, strong activity;, all results are mean of three experiment's ;**,A Pz value of 1 indicated no activity, and less than one (Pz< 1) specified the phospholipase and / or caseinase*

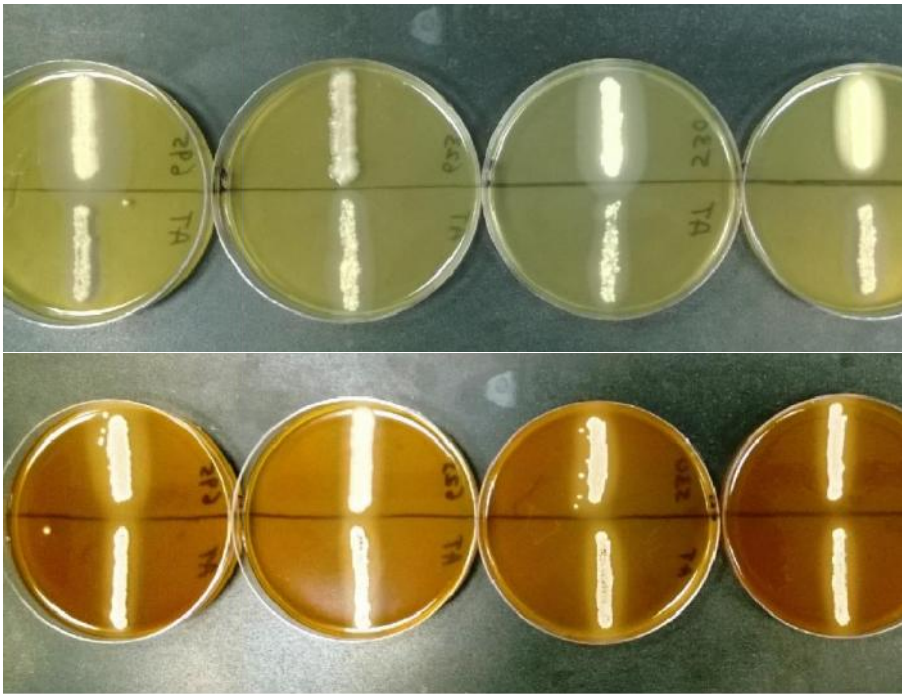

Fig. 6. Top row clockwise the phospholipase activity of *C. albicans* strains: 695, 623, 530, and 89 as compared to QC ATCC10231 and bottom row showed respective protease activity of the same strains; **C. albicans* control strain and strain, 89 (moderate filamentous transition); strain 695 (strong); and strains 530 & 623 (relatively poor)

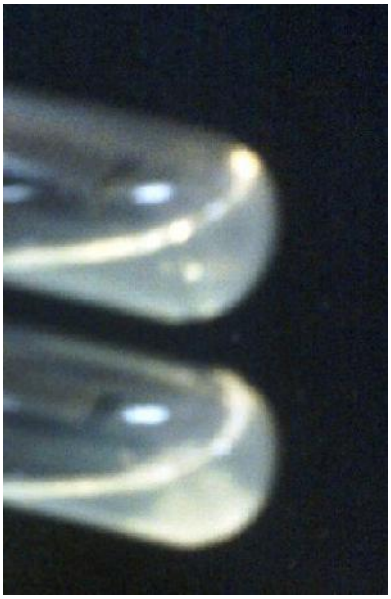

Fig. 7. Photograph of top tube *C. albicans* ATCC 10231(negative coagulase) and bottom one of *C. albicans* strain 695(positive coagulase)

4. DISCUSSION

The interactions between host and pathogen are very important in determining whether an infection ensues or the host retains a healthy constitution. The balance is truly fine especially in the case of opportunistic pathogens as *C. albicans*, where slightest reduction in the host defenses can allow initiation of infection while the healthy individual can harbor the pathogen keeping it at bay. Hence the present study was initiated to investigate the effect of human serum, starvation and /or variation in incubation temperature on yeast and pseudo-hyphae and/ or hyphal cell differentiation in vaginal *C. albicans* strains and, its correlation to exo-hydrolytic enzymes of phospholipase, and proteinase as caseinase, gelatinase and coagulase, virulence markers.

The peak of filamentous formation by *C. albicans* was noticed between 2-4 hours of incubation in MSB medium with 20% serum and between 2 and 6 hours in mere MSB medium for the examined strains. In a similar study, Nadeem et al.,[24) reported that the peak of germ tube production by *C. albicans* appeared between 1.5 to 6.0 hours observed in different growth media and at different temperature and pH values and it gradually decreases after 6.0 hours. The MSB was used with several researchers to investigate the morphogenesis of *C. albicans* strains [22,24]. Nevertheless, Nadeem et al. [24] reported that this medium showed 40% filamentation after 4.5 hours of inoculation of culture and it gave comparable low filamentation than RPMI-1640 medium. In agreement with these authors, hyphae are readily induced from un- budded yeast cells by a growth temperature of 37°C [30,31] and according to the previous paper [22] 37°C was optimal for filamentation, while Nadeem et al. [24] reported that higher temperature (40°C) resulted in gradual decrease in fungal growth. Hence the role of incubatory temperature in polymorphism of *C. albicans* cannot over-emphasize as it has been reported by many workers [30,31,]. The importance nutritional factors that induces germ-tube formation by *C. albicans* strains was reported by Casanova et al. [32] and Westwater et al. [33]. While Villar et al. [34] recognized that the filamentous form is responsible for the pathogenesis of the fungal infection. Phenotypic plasticity is the major virulence factors, hyphal form of *C. albicans* could penetrate to epithelial layer to cause infection. While the yeast form remained on the surface, defense the immune system, increase the enzyme secretion and decrease in susceptibility to antifungal agents [22].

On the other hand as demonstrated in this study human serum not only promoted *C. albicans* morphogenesis but also it has been shown to promote *C. albicans* biofilm growth and virulence gene expression on silicone biomaterial [28]. The current study explored time course intervals with phase microscopy to fellow the efficacy of the examined starved (to by pass glucose repression of germ tube formation) vaginal *C. albicans* strains to switch from yeast form to hyphal forms in presence and absence of human serum. In agreement with several researchers [22,28.], generally serum speeds off hyphal transition in a much more pronounced fashion as compared to those strains incubated without its inclusion and this difference was significant (p=0.001). Additionally, the present study also showed that 8/31 (25.8%) strains displayed transition to hyphal forms only in presence of serum, whereas 7/31 (22.6%), did not express such switch at all, regardless of promoted conditions of serum and optimum incubatory temperature of 37ºC. In contrast three strains (3/31, 9.6%) expressed such ability of filamentous growth in presence or absence of serum at 37ºC as well as 25ºC, and also displayed higher exoenzyme activities as compared to represented strains of the former group. Therefore, these strains (3/31, 9.6%) would be the most virulent ones, and thereby eligible for invasive infections rather than the usual commensal nature. Whereas those strains (7/31, 22.6%) that did not show filamentous growth (less virulent) at

any of the examined growth conditions, and with low and/ or no hydrolytic enzyme activity , would be considered as less virulent strains. Several investigators [28,29] have documented that genes coding hyphal phase differentiation are co-regulated with those genes governing virulence factors such as cell attachment -adhesins and different hydrolytic enzymes(virulence factors), the present findings seem to strengthen such phenotypic link [35]. Hence, one might postulate that it is possible by simple laboratory testing to highlight the virulence status of clinically recovered *C. albicans* strains, and thereby prophylactic treatment and /or decolonization intervention can be instructed as preventive measures for possible eventual invasive infections; especially in pregnant women and /or immune compromised hosts. In this context, García-Ruiz et al. [36] and Pazos et al. [37] have demonstrated the applicability of detection of antibodies to *C. albicans* germ tubes for diagnosis and therapeutic monitoring of invasive candidiasis in patients with hematologic malignancies and neutropenia in adult patients. However, considering the limited number of strains tested in this study, these findings require further substantiation by large sample size and *in vivo* animal studies.

5. CONCLUSION

Results obtained suggest that vaginal *C. albicans* strains are heterogenous in their potency to switch from yeast to hyphae. Strains which show morphogenesis in absence of serum and/or at low temperature (25ºC) exhibit higher exoenzymes activity suggesting that these strains are more pathogenic.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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