Extracellular phospholipase activity in the environmental strains of *Cryptococcus neoformans* and *Cryptococcus gattii* isolated from Betul city of Madhya Pradesh

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Abstract

Cryptococcosis accounts for significantly life-threatening diseases in healthy and immunocompromised individuals by the production of extracellular enzymes in host cell. In the present study we focused on the extracellular phospholipase (PLP) activity which contributes to the most widely concerned issue of these enzymes as prominent virulence factors. For the screening of phospholipase producing strains, 45 environmental isolates of both *Cryptococcus neoformans* and *C. gattii* strains were point inoculated on egg yolk agar. In reference to *C. neoformans* and *C. gattii* isolated from tree samples, 17 (62.9%) strains showed high phospholipase production on 5^{th} day and 18 (66.66 %) on 8^{th} day of incubation with low Pz value ($Pz \leq 0.6$). However, in case of yeast strains obtained from pigeon samples showed high phospholipase production that is 10 (55.55 %) and 11 (61.11 %) on 5^{th} and 8^{th} day of incubation. As per the statistical analysis using Independent "t" test, no significant difference was observed between phospholipase production by *C. neoformans and C. gattii* strains.

Keywords: *Cryptococcus neoformans*, *C. gattii*, phospholipase activity, virulence factors

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Introduction

The potential *Cryptococcus* virulence determinants play crucial roles in the fungal pathogenesis, these include extracellular enzymes production, the release of polyol metabolites, interaction with hormones, adherence, and production of mannoproteins (Kronstad *et al.*, 2011).

Cryptococcus neoformans species complex produces phospholipase enzyme which is pathogenic and the mechanism to decline its activity *in-vivo* is a subject less explored.

Phospholipases are the enzymes which are found to hydrolyze ester-linkages in glycerophospholipids and disruption of host cell-membranes (Nawange *et al,* 2020).

A phospholipase is an enzyme that hydrolyzes phospholipids into fatty acids and other lipophilic substances (Dennis,1994). There are four major classes, termed PL A, B, C and d distinguished by what type of reaction they catalyze. Sharon *et al.*, (2000), illustrated multimeric structure (comprising two (or three) subunits) of cell-associated PLB purified from *S. pombe* and *T delbrueckii.*

Price *et al.,* (1982) has given a reliable semi-quantitative egg yolk plate method for the rapid screening of *Cryptococcus neoformans* for extracellular phospholipids production. however, phospholipase production was also examined on agar containing Tween and olive oil (*Werner et al.,* 2011).

Vidotto *et al.*, (1996), was the first who reported the secretion of phospholipase by 22 strains of *C. neoformans*. Furthermore, Chen and co-workers (1997) reported phospholipase, lysophospholipase and lysophospho lipase-transacylase activity on 50 strains of *C. neoformans* grown on egg yolk agar and later four strains were used to study virulence in BALB/c mice with varying levels of phospholipase activity. Their investigation suggests that extracellular phospholipase activity may disrupt mammalian cell membranes and allow the yeast cells to penetrate into host tissues.

Later the role of phospholipase as a virulence factor in *C. neoformans* has been confirmed by analyzing the phospholipase B gene, *plb1* in *C. neoformans. C. neoformans var. neoformans* and *C. gattii* shows a significant difference in their phospholipase activity (Ruma-Haynes, 2000).

According to the Casadevall and Pirofski, (2006), Virulence factors are the determinants of pathogenicity including genes or gene products such as enzyme molecules involved in this relationship, producing superficial to invasive infections in humans. Both the species of *Cryptococcus neoformans* species complex produces panoply of virulence factors.

Material and methods

In the present study we studied phospholipase production in the total of 45 environmental isolates of *C. neoformans* and *C. gattii* strains isolated from both the tree and pigeon excreta samples. Phospholipase activity was analysed essentially according to egg-yolk plate method given by Price *et al.,* (1982) with slight modifications.

The media contained 1M sodium chloride, 0.005 M calcium chloride, 8% sterile egg yolk emulsion, supplemented with 50 mg chloramphenicol (to inhibit bacterial growth). To avoid diffusion of the precipitation zone modifications were done in which the basal SDA medium was increased to 1.75% by adding Bacto agar (HiMedia Mumbai).

This molten SDA medium was poured in petri plates and allowed to solidify. The fresh cultures of the *Cryptococcus* sp. strains were spot inoculated and the plates were incubated at 37°C for 5 days. The entire experiment was performed in triplicates. Pz (precipitation zone) value was measured which is the ratio of colony diameter to total diameter of colony and precipitation zone, and the scoring was done according to method described previously (Price *et al.*, 1982).

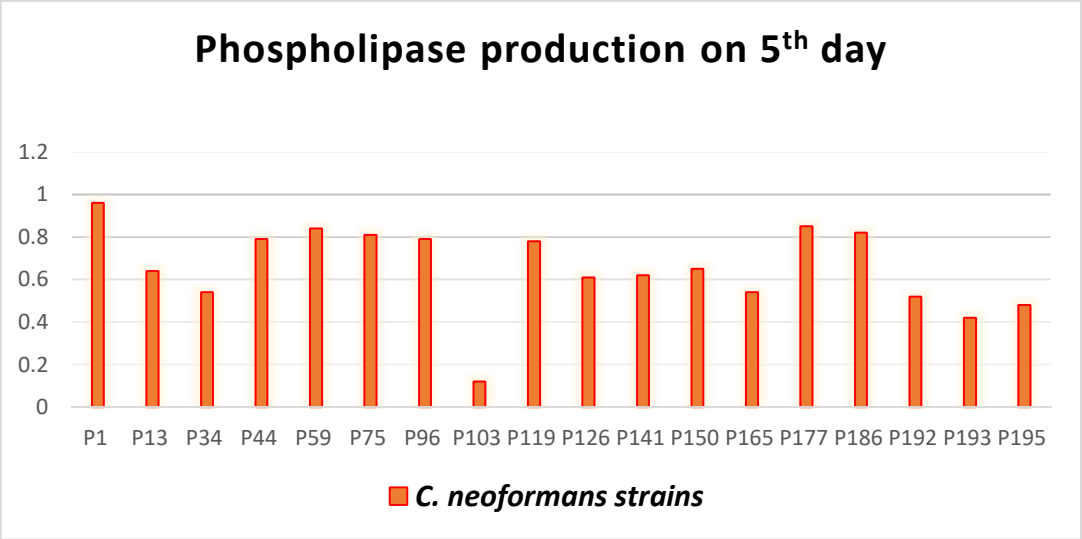
On the basis of enzymatic activity and their Pz values strains has been divided into five ranges belonging to respective classes, Pz group and enzyme production i.e, Pz=1 (class, very high Pz group, no enzyme production), Pz= 0.9. – 0.99 (+/-, high, very low), Pz= 0.8. – 0.89 (++, Intermediate, Low), Pz= 0.70 – 0.79 (+++, low, high) and Pz= ≤0.69 (++++, very low, very high). The reference strain, *C. albicans* MTCC/ATCC 10231, served as the positive control while the *C. glabrata* IHEM 22129 strain from our laboratory stock collection served as the negative control.

Result and discussion

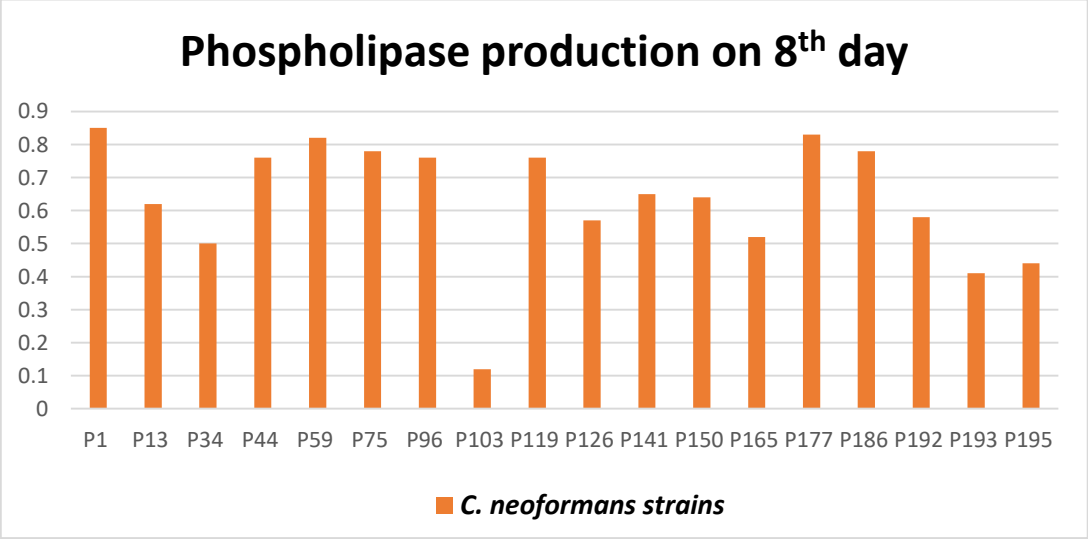
As the result suggests out of total 45 environmental samples, 30 *C. neoformans* and 15 *C. gattii* samples belongs to 27 tree species and 18 pigeon habitats. In reference to *C. neoformans* and *C. gattii* isolated from tree samples showed high phospholipase production was observed by 17 (62.9%) strains on 5^{th} day and 18 (66.66 %) on 8^{th} day of incubation with low Pz value (Pz ≤ 0.6). Whereas very low enzyme production was recorded for 1 (3.7 %) environmental strain with high Pz value (Pz=1). However, in case of yeast strains obtained from pigeon samples showed high phospholipase production that is 10 (55.55 %) and 11 (61.11 %) on 5^{th} and 8^{th} day of incubation.

It was very interesting to find that Pz was found more on the 8^{th} day of incubation as compared to the 5^{th} day of incubation. As per the PL activity, some *C. neoformans* strains isolated form pigeon samples including P13, P103, P126, P141, P150, P165, P192, P193, P195 showed low Pz values on 5^{th} day of incubation and high phospholipase production indicates the virulent nature of these strains.

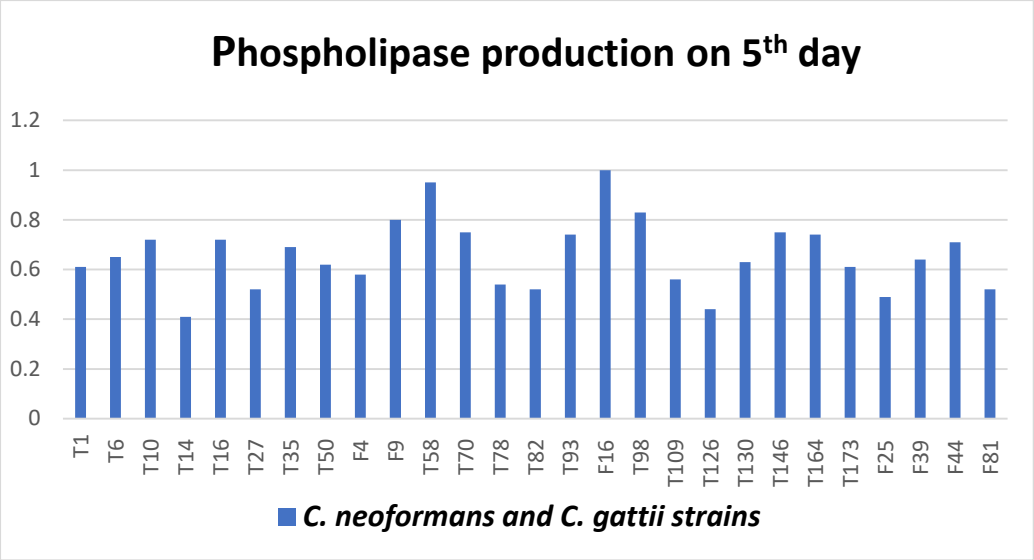
Furthermore, very high phospholipase production was obtained by *C. neoformans and C. gattii* strains (T1, T6, T14, T27, T35, T50, F4, T78, T82, T109, T126, T130, T173, F25, F39, F81) isolated from tree samples on 5th day of incubation. Independent "t" test was used and revealed no significant difference between phospholipase production by *C. neoformans and C. gattii* strains on 5th and 8th day of incubation.



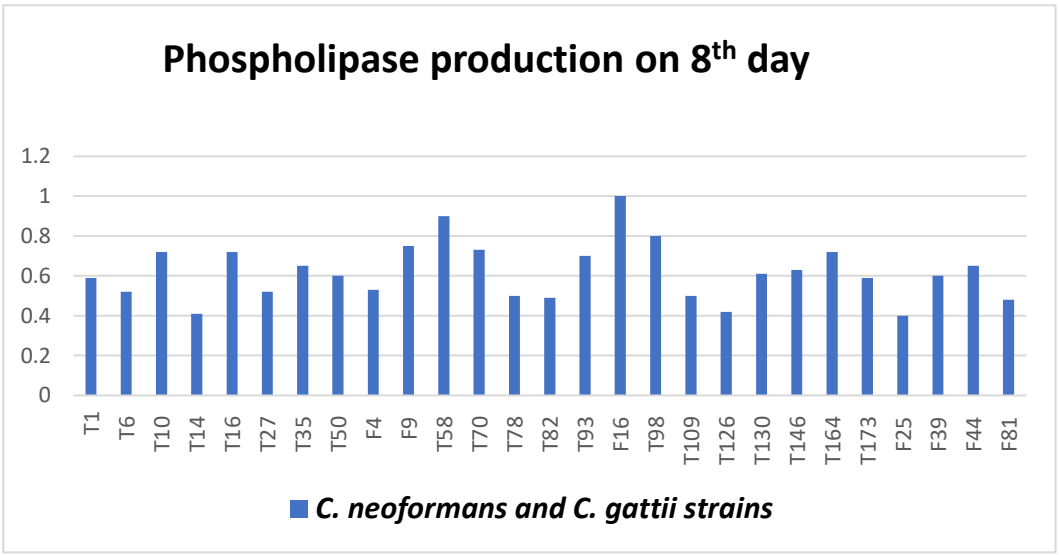
Graph 1: Showing Pz values of *C. neoformans* isolated from pigeon samples on 5th day of incubation



Graph 2: Showing Pz values of *C. neoformans* strains isolated from pigeon samples on 8th day of incubation



Graph 3: Showing Pz values of *C. neoformans and C. gattii* strains isolated from tree samples on 5th day of incubation



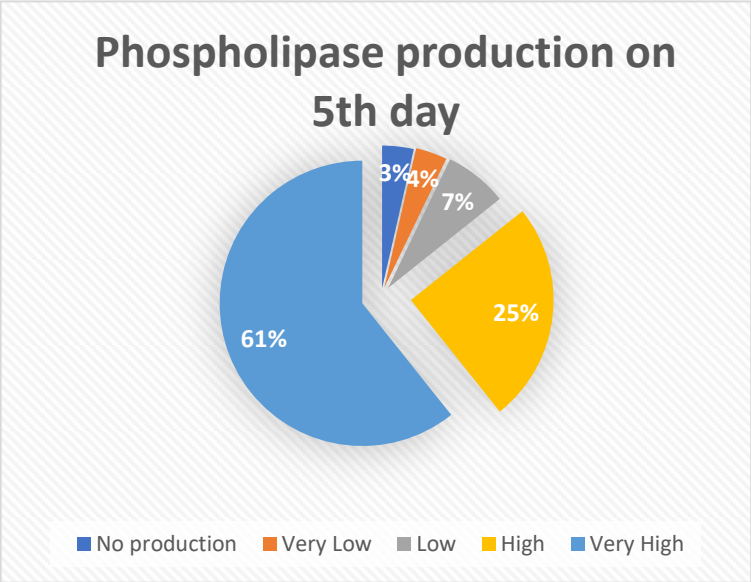
Graph 4: Showing Pz values of *C. neoformans and C. gattii* strains isolated from tree samples on 8th day of incubation

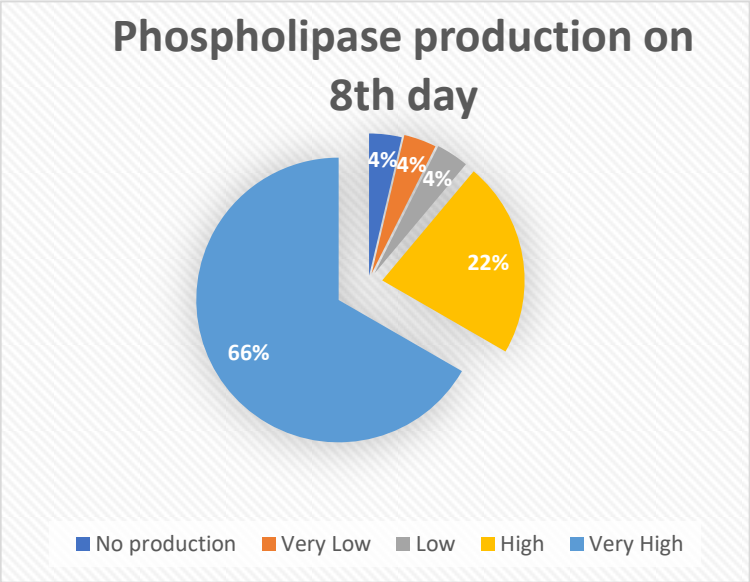
Table 1: categorization of all the tree isolates based on their Pz values calculated on 5th and 8th day

Range	Class	Pz Group	Enzyme Production	Frequency of tree isolates	
				5th day	**8th day**
Pz=1	-	Very High	No production	1 (3.7 %)	1 (3.7 %)
Pz= 0.9. – 0.99	+/-	High	Very Low	1 (3.7 %)	1 (3.7 %)
Pz= 0.8. – 0.89	++	Intermediate	Low	2 (7.40 %)	1 (3.7 %)
Pz= 0.70 – 0.79	+++	Low	High	7 (25.9%)	6 (22.22 %)
Pz= ≤0.69	++++	Very Low	Very High	17 (62.9%)	18 (66.66 %)

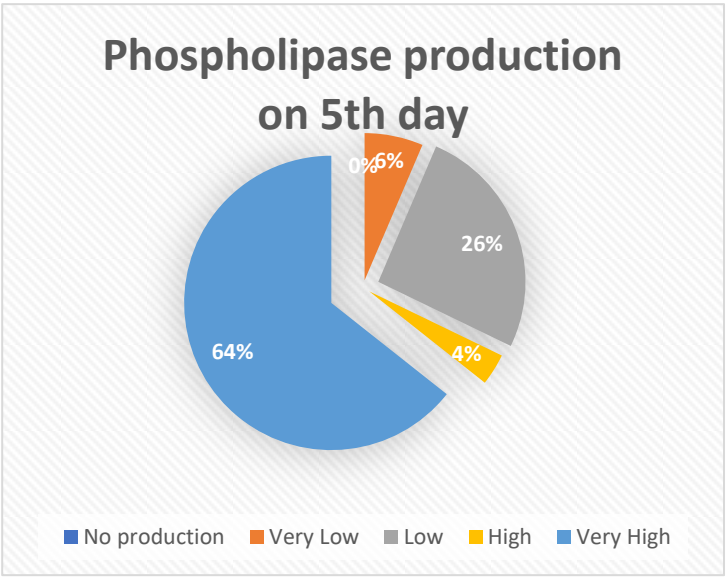
Table 2: categorization of all the pigeon isolates based on their Pz values calculated on 5th and 8th day

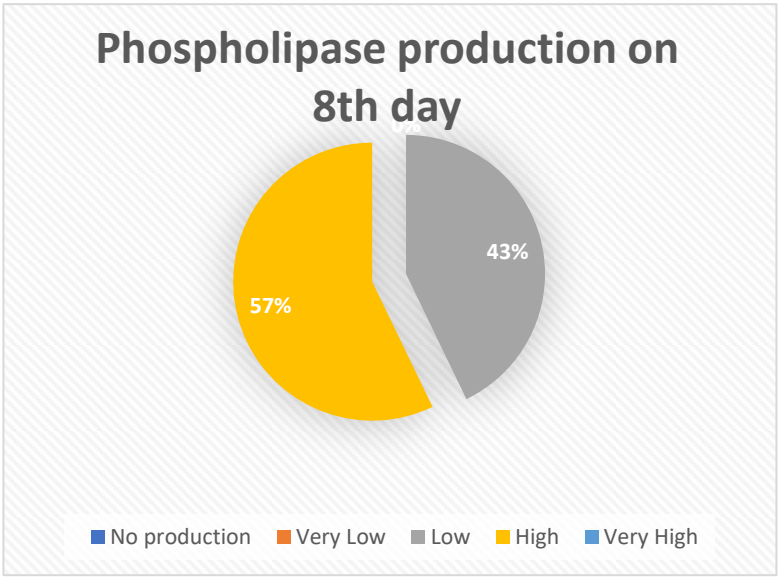
Range	Class	Pz Group	Enzyme Production	Frequency of tree isolates	
				5th day	**8th day**
Pz=1	-	Very High	No production	0 (0 %)	0 (0 %)
Pz= 0.9. – 0.99	+/-	High	Very Low	1 (5.55 %)	0 (0 %)
Pz= 0.8. – 0.89	++	Intermediate	Low	4 (22.22 %)	3 (16.66 %)
Pz= 0.70 – 0.79	+++	Low	High	3 (%)	4 (22.22 %)
Pz= ≤0.69	++++	Very Low	Very High	10 (55.55 %)	11 (61.11 %)





Graph 4.2e: Showing different range of phospholipase producing *C. neoformans and C. gattii* strains isolated from tree samples on 5th and 8th day of incubation





Graph 4.2f: Showing different range of phospholipase producing *C. neoformans* strains isolated from pigeon excreta samples on 5th and 8th day of incubation

Figure 4.2a: Sabouraud's egg yolk agar plates showing phospholipase precipitation zone around the colony of environmental strains of *C. neoformans and C. gattii* isolated from tree samples.

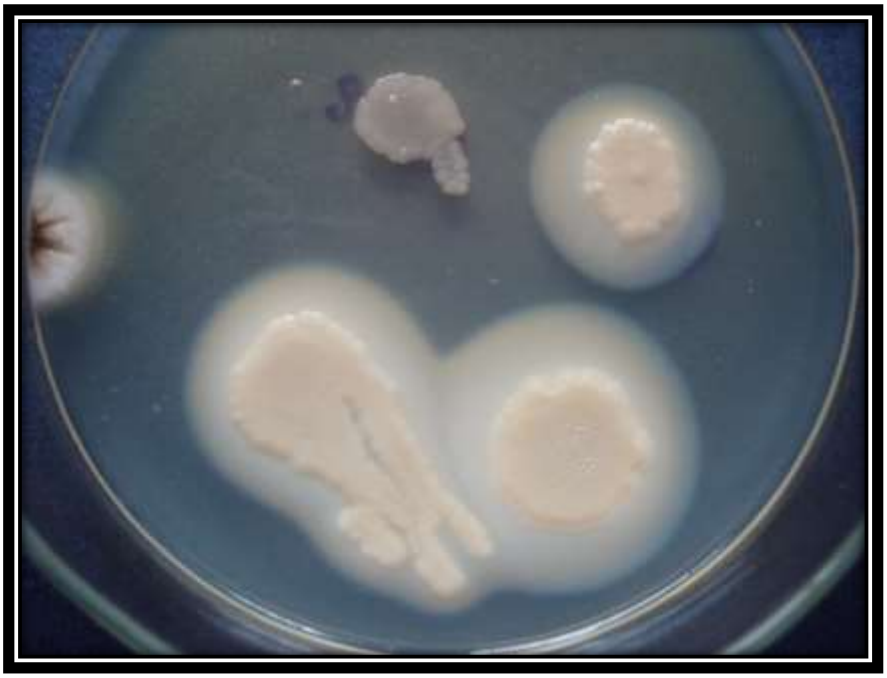


Figure 4.2b: Showing phospholipase precipitation zone around the colony of environmental strains of *C. neoformans* isolated from pigeon excreta samples.

Previous investigations were using the same *in-vitro* method given by Price *et al.*, (1982), for the screening of phospholipase enzyme by opportunistic pathogens. Vidotto *et al.* (1998), screened 46 bird dropping strains by using the egg-yolk plate method to measure phospholipase production, in accordance with this we studied phospholipase activity in 18 *C. neoformans* isolated from pigeon excreta samples**.**

Furthermore, opportunistic pathogens other than *Cryptococcus neoformans species complex were also investigated for* phospholipase production. Nawange *et al.,* (2020), reported a total of 141 (74.2%) clinical samples were positive for the production of phospholipase (Pz ≤ 0.6) including 86.92% belongs to *C.albicans* and 46.6% of non-*albicans Candida* species.

A significant high phospholipase production was observed in both the environmental isolates of *Cryptococcus neoformans* and *C. gattii* strains which may represent the high pathogenicity and virulent phenomenon of these opportunistic yeast (Djordjevic, 2010).

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