

***In vitro* characteristics of fungal biofilm formation and the influence of physiological stress on biofilm growth**

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Abstract: Fungal biofilms are a growing clinical concern associated with high mortality rates. This study included three fungal groups, dimorphic fungi (*Candida albicans*), Dermatophytes (*Trichophyton mentagrophytes*) and non-dermatophytes (*Acremonium sclerotigenum*, *Aspergillus niger*). This research describes the *in vitro* characteristics of biofilm formation in three fungal groups. The influence of osmotic, oxidative and pH stress environment on biofilm growth was also focused. Biofilm characteristics in *A. sclerotigenum* and *A. niger* were studied for the first time. *In vitro* qualitative and quantitative approaches were used to evaluate biofilm development including the test tube method, tissue culture plate method in addition to staining with crystal violet and safranin. All the isolates were able to form biofilm. Biofilm development under different pH range showed maximum growth at neutral pH. At a concentration of 5mM hydrogen peroxide and 2M NaCl biofilm formation was maximum for all three fungal groups under an oxidative and osmotic stress respectively. Study revealed that biofilm production was increased under osmotic and oxidative stress. All isolates respond to oxidative and osmotic stress by changing the cell wall composition with a rich exopolymeric matrix in order to survive in stress environment.

Keywords: Biofilm, *candida albicans*, dermatophytes, non-dermatophytes, oxidative stress, osmotic stress.

INTRODUCTION

Biofilms are three dimensional structure of microorganisms either clustered together or adhered to a surface (biotic or abiotic) composed of polymeric mass enclosed in extracellular matrix providing shield to themselves (Ramage *et al.*, 2009). Biofilm structure is often made up of active and dead cells, components containing water channel, proteins, carbohydrate and lipids, extracellular matrix ECM secreting nucleic acid and extracellular DNA (Cavalheiro and Teixeira, 2018, Wilson *et al.*, 2017). As a result of genetic modifications, the cells in a biofilm show changes in growth rate and gene transcription. It is involved in increased tolerance to antifungal agents, chemical, biological or physiological stress as compared to their individual planktonic cells. Some other benefits may include surface attachment, enhanced intercellular communication, dispersal and colonization with host tissue which lead them capture nutrients more efficiently (Harding *et al.*, 2009). Biofilms impact the health of a number of people, particularly in HIV, cancer, transplant recipients and new born babies.

Biofilm detection and analysis can be performed using a number of methodologies, each based on a distinct cell or extracellular matrix response. This includes tissue culture plate method, tube method, Congo red agar method, bioluminescent assay and piezoelectric sensors (Hassan *et al.*, 2011). The culture medium RPMI 1640 (Roswell Park Memorial Institute Medium) is demonstrated as optimum medium for biofilm growth. This medium is extensively employed in the *in vitro* biofilm production of numerous

fungal species (Costa-Orlandi *et al.*, 2014). Crystal violet (CV) is the most prominent quantification assay for staining polysaccharide matrix in biofilm formation (Silva *et al.*, 2009, Goodman, 1957).

Biofilms are produced by many medically significant fungus, including *Candida* (Finkel and Mitchell, 2011), *Aspergillus* (Beauvais *et al.*, 2008), *Cryptococcus* (Martinez *et al.*, 2007), *Trichosporum* (Di Bonaventura *et al.*, 2006) and *Pneumocystis* (Davis *et al.*, 2002). It is reported that dermatophytes belonging to trichophyton species (*T. rubrum* and *T. mentagrophytes*) can form a mature biofilm within 72 hours (Costa-Orlandi *et al.*, 2014). But rare biofilm studies were performed on non-dermatophyte species. Both osmotic and oxidative stress attributes are important in fungal pathogenicity and antifungal susceptibility. Osmotic stress results in reduced membrane permeability, a drop in turgor pressure and reduced cell size. While, oxidative stress confronts reactive oxygen species (ROS). According to a recent biofilm literature, eDNA creation occurs as a result of cell lysis through H₂O₂ production. (Pemmaraju *et al.*, 2016). The relationship between stress adaptation and biofilm production in eubacteria has been explained, specifically in *Staphylococcus aureus* and *Streptococcus* species, in which osmotic and oxidative stress stimulate biofilm production (Wen *et al.*, 2005). The major constituents of biofilm are EPS, that works as a gel-like matrix which links cells with each other to create aggregates and protects microbes from excessive salt stress (You *et al.*, 2015). Biofilms enable microorganisms to resist the effects of extreme acidic and basic pH. It has been discovered that under excessively acidic pH, heavy metals' solubility increases that boost the toxicity index in cells. The

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combination of highly acidic pH and heavy metals was discovered to cause a considerable shift in the EPS constituents of the biofilm that plays a significant role in microbe adaption to harsh conditions. The research reveals that biofilms provide protection in extremely acidic settings, which is facilitated by specific polysaccharide (Yin *et al.*, 2019).

This Study focused on three fungal groups, dimorphic fungi (*Ca20, Ca23*), dermatophyte (*Tm25, Tm26*) and non dermatophytes (*A.niger, A.sclerotignum*) using different qualitative and quantitative methods to evaluate biofilm formation capability in all fungal isolates. In addition, the influence of varying stress environment on biofilm growth were also investigated.

MATERIALS AND METHODS

Isolates and growth conditions

The study was conducted in three fungal groups obtained from Civil hospital Bahawalpur, identified as dimorphic fungi (*Candida albicans*), Dermatophytes (*Trichophyton mentagrophytes*) and Non-dermatophytes (*Accremonium sclerotigenum, Aspergillus. niger*). All isolates were inoculated on Sabouraud dextrose agar (SDA) media containing 0.5g/liter chloramphenicol and 0.40g/liter cyclohexamide to inhibit the unwanted microbial contamination. The incubation time was 24-48 hours for *Candida albicans*, 7-10 days for *T. mentagrophytes*, *Accremonium. sclerotigenum* and *Aspergillus. niger* at 25-30°C.

Qualitative tube assay

To assess biofilm formation, a qualitative assay was performed as reported by (Christensen *et al.*, 1982). Freshly grown culture isolates were inoculated in sterile test tubes containing 10ml of tryptic soya broth and incubated overnight for dimorphic fungi and 7 days for dermatophytes and non-dermatophytes at 37°C. After complete incubation, media was poured out of the tubes and washed with Phosphorous buffered saline (PBS) and dried at room temperature. Then, 0.1% Crystal violet was used to stain the dried test tubes. The excess stain was removed with distilled water and tubes were dried again. Experiments were repeated three times.

Biofilm formation assay

To assess early biofilm formation, a 24 well plates method was adopted as reported by (Mowat *et al.*, 2007) with some alterations. The isolates were inoculated on potato dextrose agar PDA g/L for 5 days or until sporulation at 28°C. After incubation, the spores were shredded and washed with 5ml of PBS and diluting the spore's suspension to endmost concentration of 1×10^6 CFU per ml by using hemocytometer. Subsequently, biofilms were allowed to form by pouring 100µl of spores' suspension into wells of micro-titer plates and incubated at 37°C for 3 hours for biofilm pre adhesion. After, supernatant was aspirated

delicately from the wells and washed three times with PBS to remove planktonic cells. Then, 200µl of RPMI 1640 medium with glutamine was poured into micro-titer wells. The incubation time was followed as 24 hours for dimorphic fungi and 72 hours for dermatophytes and non-dermatophyte species at 37°C.

Quantification of biofilm by crystal violet staining

A quantification method for biofilm biomass formed was performed as described by (Mowat *et al.*, 2007). Culture medium was removed from the pre-formed mature biofilms in micro-titer plates and adhered cells were washed thrice with PBS and wells were air dried for 20 minutes. Then, 0.5% crystal violet was poured in each well in a volume containing 100µl of solution and left for 15 minutes for staining. The wells were de-stained with distilled water followed by washing with 100µl of pure ethanol solution to thoroughly diffuse crystal violet. Then, 200µl of solution was transferred to a new micro titer plate and plate was read on ELISA reader at wavelength of 592nm for *Candida albicans* and 570nm for all other fungal isolates (Seidler *et al.*, 2008). Experiment was repeated in triplicates along with one positive and one negative control. *Candida albicans* was used as a positive control and RPMI medium without spores as a negative control. Biofilm growth in micro-titer plate was evaluated by optical density (OD) of fungal biofilm isolates and compared with OD control. The biofilm formation was calculated as specified by given formula (Tulasidas *et al.*, 2018).

$OD \leq ODC = \text{non-adherent}$

$ODC < OD \leq 2 ODC = \text{weakly adherent}$

$2ODC < OD \leq 4 ODC = \text{moderately adherent}$

$4ODC < OD = \text{strongly adherent}$

Quantification of the extracellular matrix by safranin staining

Biofilm were formed in micro- titer plates as previously described, then culture media was removed from each well and 50µl (safranin 0.5%) was added in each well to stain ECM for 5 minutes. Supernatant was gently discarded and wells were washed three times with PBS. Finally, readings were taken at 492nm wavelength on ELISA plate reader (Seidler *et al.*, 2008).

Oxidative stress effect on biofilm growth

An oxidative stress susceptibility assay was performed on pre-formed biofilms with some modifications as reported by (Costa-Orlandi, 2020 #1). Biofilms were formed in micro- titer plates as described earlier. Then, 200µl of RPMI 1640 growth medium containing 1-9mM H_2O_2 concentration was transferred to pre-formed biofilms in separate wells of micro-titer plates. A negative control was also included containing only the RPMI 1640 with no fungal cells. The incubation time was followed as 24 hours for dimorphic fungi and 72 hours for dermatophytes and non-dermatophytespecies at 37°C. After incubation, all the samples were stained with safranin and plates were read using ELISA plate reader at 492nm (Mowat *et al.*, 2007).

Osmotic stress effect on biofilm growth

An osmotic stress susceptibility assay was performed on pre-formed biofilms with some modifications as reported by (Pierce, 2008 #2, Zmantar, 2010 #3). Then, 200µl of RPMI media containing 1-3 M NaCl concentration was transferred to pre-formed biofilms in separate wells of micro-titer plates. A negative control was also included containing only the RPMI 1640 with no fungal cells. The incubation time was followed as 24 hours for dimorphic fungi and 72 hours for dermatophytes and non-dermatophytes species at 37°C. After incubation, all the samples were stained with safranin and plates were read using ELISA plate reader at 492nm (Mowat *et al.*, 2007).

pH effect on biofilm growth

The effect of pH on biofilm growth was assessed as reported by (Zmantar *et al.*, 2010). To examine biofilm growth pattern, pre-formed biofilms were treated with RPMI media at different pH levels from acidic to alkaline (1, 3, 5, 7, 9 and 12). Biofilms grown at RPMI media's pH i.e. 6.3 were considered as control. The incubation time was followed as 24 hours for dimorphic fungi and 72 hours for dermatophytes and non-dermatophytes species at 37°C. After incubation, all the samples were stained with safranin and plates were read using ELISA plate reader at 492nm (Mowat *et al.*, 2007).

STATISTICAL ANALYSIS

All data analysis and statistical procedures were performed using Origin software version 2018. The results were presented as mean $\pm$ standard deviation of mean values.

RESULTS

Isolates and growth conditions

All strains were grown on Sabouraud dextrose agar medium at 27°C. After 24-48 hours *Candida albicans* showed optimum growth with creamy white appearance. Dermatophytes and non-dermatophytes were grown after 7 days of incubation at 27°C. *T. mentagrophytes* were characterized as white or beige in colour, cottony appearance with distinctive odour. *Accremonium sclerotigenum* was characterized as white sporulated growth and *Aspergillus niger* was characterized with a cottony look, white to yellow at first, later it turns black as shown in fig. 1.

Qualitative tube assay

All fungal isolates showed biofilm formed in a ring form around walls and bottom surface of test tube. A distinct line surrounding the bottom and walls of test tubes represent clear biofilm formation. Among all isolates, *Candida albicans* formed strongest biofilm as shown in fig. 2. The assay was performed in triplicates.

Quantification assay

The absorbance values of biofilm biomass stained with crystal violet and extracellular matrix stained with safranin

revealed thick and strongest biofilm production in *Candida albicans* among all groups. While, *Accremonium sclerotigenum* gave strong positive biofilm mass. *Trichophyton mentagrophyte* gave moderate positive biofilm formation and *Aspergillus niger* moderate biofilm production as represented in table no.2. The average absorption values obtained by staining with 0.5% crystal violet and 1% safranin has been represented in fig. 3 and 4.

pH stress effect

The effect of varying pH applied on biofilm showed that all fungal isolates produce maximum biofilm at a neutral pH 5-7. *Candida albicans* at PH 6.3 and 7, *Trichophyton mentagrophytes* 25at skin PH 5 to 7, *Accremonium sclerotigenum* and *Aspergillus niger* at PH 6.3 showed maximum biofilm formation. At acidic value PH (3) and alkaline PH value (12) all the strains showed restricted biofilm growth as shown in fig. 5.

Oxidative and osmotic stress

The quantitative safranin staining and absorbance results showed that biofilm production was maximum at a concentration of 5mM H₂O₂ and 2M NaCl as compared to control, where no stress effect was induced. *Candida albicans* produced thick biofilm under osmotic and oxidative stress as compared to other fungal isolates as represented in fig. 6 and fig. 7.

DISCUSSION

The association and adherence of pathogenic fungus to host tissues is directly related to the development of mycosis (Vermout *et al.*, 2008). The factors that cause dermatophyte adherence to diverse surfaces are poorly known. Biofilms are three dimensional structured group of microorganisms either clustered together or adhered to surface (biotic or abiotic) composed of polymeric mass enclosed in extracellular matrix and providing shield to themselves. Biofilm production in dermatophytes has been offered as a possible explanation for dermatophytomas, in which confined dense white fungal masses reside within and beneath the nail plate, however this has yet to be proven with fungal groups (Burkharta *et al.*, 2002).

In dermatophytes, polysaccharides contents are responsible for cell or surface adhesion (Sauer *et al.*, 2002). Hydrophobicity of candida cell wall is the most distinct reason leading to surface adhesion and biofilm formation due to specific cell wall proteins.

Previous research on the cell wall of *Candida albicans* has revealed a link between cell surface hydrophobicity (CSH) and adhesion capacity (Henriques *et al.*, 2002). The mechanism behind biofilm adhesion in non-dermatophytes has not been reported. Biofilm regulation mechanism and treatment sources remains incredibly challenging due to the high resistance under extreme environmental stresses to the available antifungals.

Table 1: Fungal isolates used in this study.

Isolates	Identified Species	Accession number	Class group	Clinical types
<i>Trichophyton mentagrophytes</i> 25 (Tm 25)	<i>Trichophyton mentagrophytes</i>	MN661259.1	Dermatophytes	Tinea Capitis
<i>Trichophyton mentagrophytes</i> 26 (Tm 26)	<i>Trichophyton mentagrophytes</i>	MN661259. 1	Dermatophytes	Tinea Capitis
<i>Aspergillus niger</i> (A.Niger)	<i>Aspergillus Niger</i>	MG654699.1	Non-dermatophyte	Tinea Capitis
<i>Accremonium Sclerotignum</i> (A.Scl)	<i>Accremonium Sclerotignum</i>	MK732096.1	Non-dermatophyte	Tinea Pedis
<i>Candida Albicans</i> 20(Ca 20)	<i>Candida Albicans</i>	Not identified	Dimorphic Fungi	Tinea Ungium
<i>Candida Albicans</i> 23(Ca 23)	<i>Candida Albicans</i>	Not identified	Dimorphic Fungi	Tinea Ungium

Table 2: Biofilm production in fungal isolates.

Fungal Isolates	Biofilm Producer
<i>Candida albicans</i> (Ca20)	strong biofilm producer
<i>Candida Albicans</i> (Ca23)	strong biofilm producer
<i>Trichophyton Mentagrophyte</i> (Tm 25)	moderate biofilm producer
<i>Trichophyton Mentagrophyte</i> (Tm 26)	moderate biofilm producer
<i>Accremonium Sclerotignum</i> (A.scl)	strong biofilm producer
<i>Aspergillus niger</i> (A.niger)	Moderate biofilm producer

Fungal Isolates	Front View	Reverse View	Microscopic View	Accession number
<i>Trichophyton mentagrophytes</i> 25 (Tm 25)				MT371234.1
<i>Trichophyton mentagrophytes</i> 26 (Tm 26)				MT371233. 1
<i>Aspergillus Niger</i>				MG654699.1
<i>Accremonium sclerotignum</i>				MK732096.1
<i>Candida Albicans</i> 20 (Ca 20)				Not identified
<i>Candida Albicans</i> 23 (Ca 23)				Not identified

Fig. 1: Isolates growth using SDA media incubated at 27°C after 24 hours (*Candida Albicans*) and 7 days (*Trichophyton. Mentagrophytes*, *Aspergillus. Niger*, *Accremonium. sclerotignum*). Colonies are creamy white (*Candida albicans*), white cottony with smooth surface (*Trichophyton Mentagrophytes*) white sporulated growth (*Accremonium sclerotignum*) and dark green to black cottony appearance (*Aspergillus niger*).

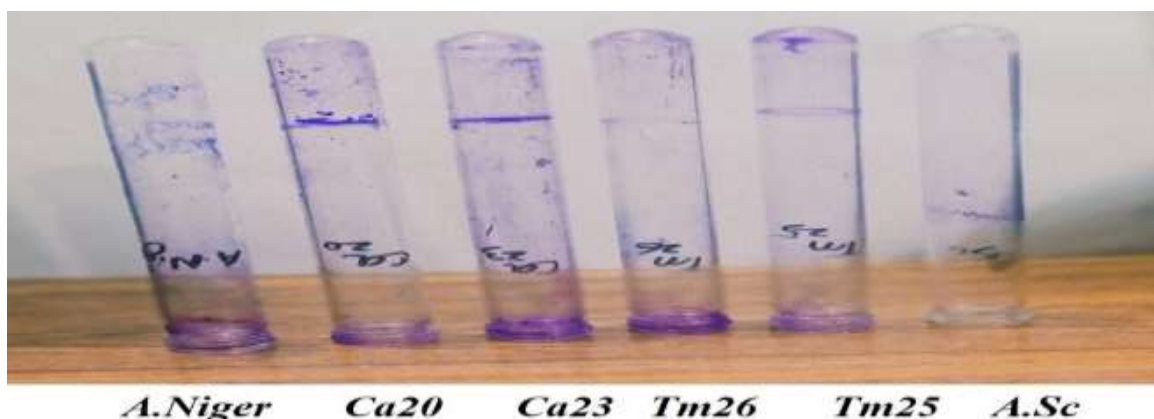


Fig. 2: Biofilm ring formation in test tubes around walls and bottom surface of tubes. Ca 20: strong biofilm producer, Ca 23: strong biofilm producer, Tm 26: Weak biofilm producer, Tm 25: moderate biofilm producer, A. *Sclerotignum*: moderate biofilm producer and A. *niger*: moderate biofilm producer.

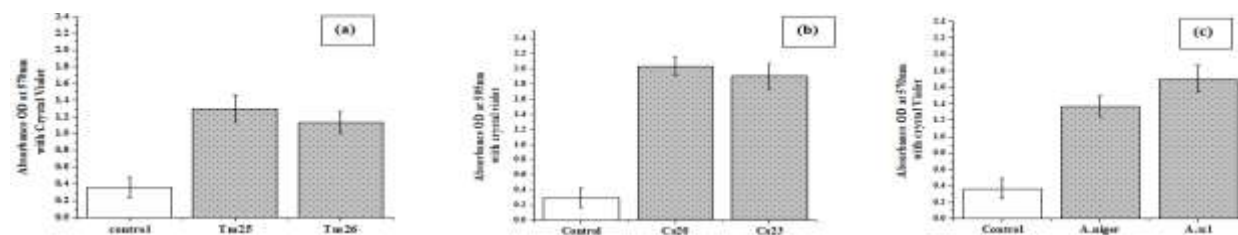


Fig. 3: Biofilm biomass quantification with crystal violet. (a) Dermatophyte isolates (b) Dimorphic fungal isolates (c) Non-dermatophyte isolates stained with crystal violet. Biomass production is in an order. *Ca20>Ca23>A.scl>Tm25>A.niger>Tm26*. Statistical Analysis: Data represent mean $\pm$ standard deviation values using Origin 2018 software.

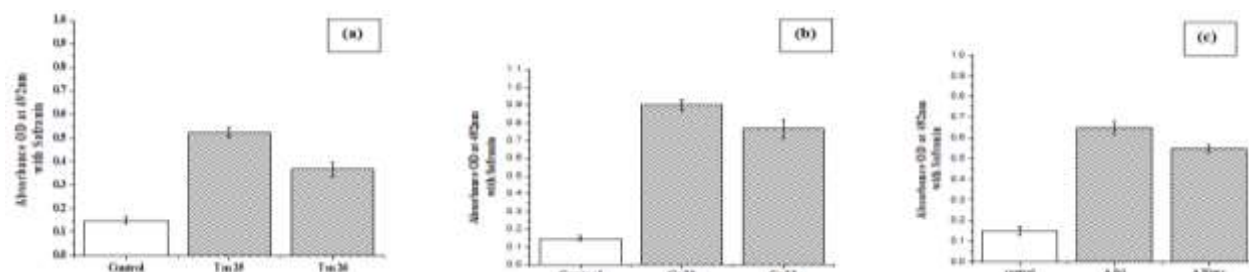


Fig. 4: Extracellular matrix quantification with safranin staining. (a) Dermatophyte isolates (b) Dimorphic fungi isolates (c) Non-dermatophyte isolates stained with safranin. Biofilm ECM production is in order *Ca20>Ca23>A.scl>Tm25>A. niger >Tm26*. Statistical Analysis: Data represent mean $\pm$ standard deviation values using origin 2018 Software.

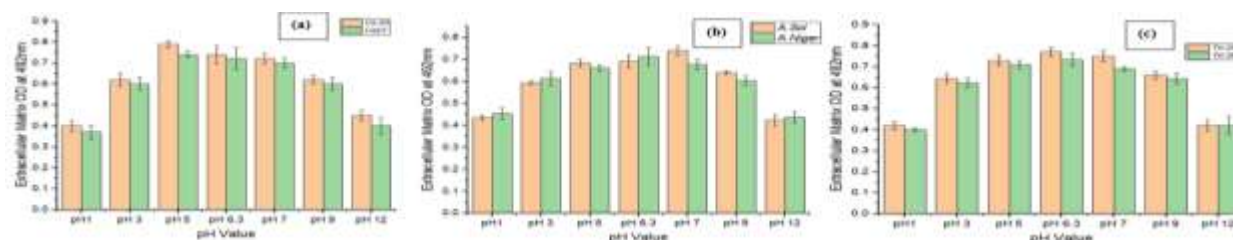


Fig. 5: Extracellular matrix quantification with safranin at varying pH values. Maximum biofilm formation is as (a) *Candida Albicans* at pH 5, (b) *Accremonium Sclerotignum* at pH 7, *Aspergillus Niger* at pH 6.3 (c) *Trichophyton mentagrophyte* at PH6.3. Statistical Analysis: Data represent mean $\pm$ standard deviation values using origin 2018 Software.

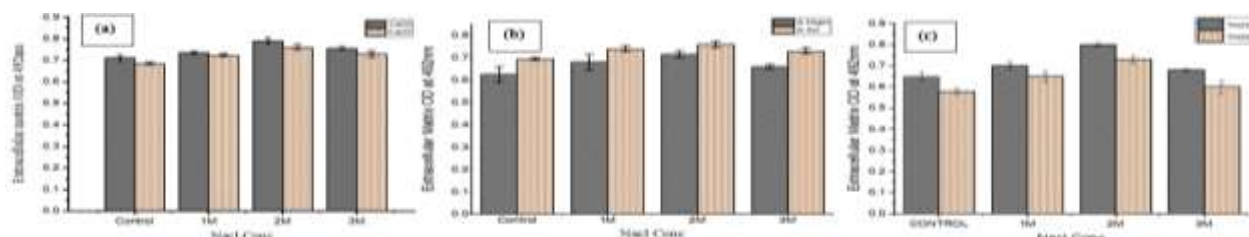


Fig. 6: Effect of osmotic stress applied on pre-formed biofilms measured using safranin staining. (a) Dimorphic fungi (b) Non-dermatophytes (c) Dermatophyte isolates. Maximum biofilm growth shown at 2M NaCl concentration. Statistical Analysis: Data represent mean $\pm$ standard deviation values using Origin 2018 Software.

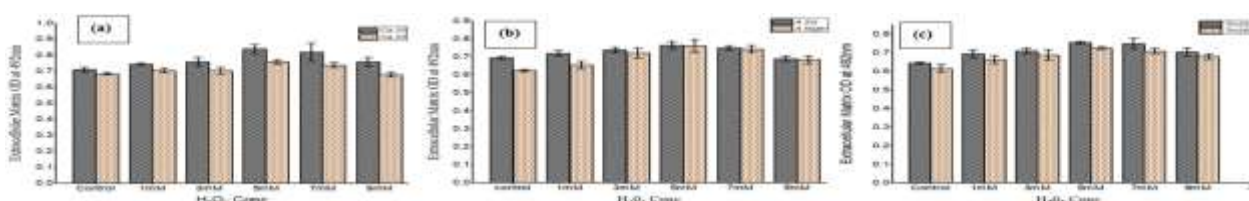


Fig. 7: Effect of oxidative stress applied on pre-formed biofilms measured using safranin staining. (a) Dimorphic fungi (b) Non-dermatophytes (c) Dermatophytes isolates. Maximum biofilm growth shown at 5mM H₂O₂ concentration. Statistical Analysis: Data represent mean $\pm$ standard deviation values using Origin 2018 Software.

Previous research on the cell wall of *Candida albicans* has revealed a link between cell surface hydrophobicity (CSH) and adhesion capacity (Henriques *et al.*, 2002). The mechanism behind biofilm adhesion in non-dermatophytes has not been reported. Biofilm regulation mechanism and treatment sources remains incredibly challenging due to the high resistance under extreme environmental stresses to the available antifungals.

Study put forward *in vitro* qualitative as well as quantitative assays providing favorable environment for biofilm development and quantification. Six isolates belonging to three fungal groups, dimorphic fungi (*Candida albicans*), dermatophytes (*Trichophyton mentagrophyte*) and non-dermatophyte (*Aspergillus niger*, *Accremonium sclerotignum*) were studied. In this study, we selected three characterization methodologies, test tube method was used for phenotypic biofilm formation. Crystal Violet and Safranin staining were utilized for *in vitro* quantification of biofilm produced. Study represented that *Candida albicans* produced more polysaccharide structures and extracellular matrix as compared to other isolates.

Oxidative and osmotic stress is a malicious adaptation with extracellular matrix formation. It promotes fungal pathogen survival and biofilm formation. Oxidative stress produces reactive oxygen species (ROS) and promote eDNA secretion. This stress adaptation of pathogens sustain biofilm integrity and enhance biofilm virulence activity (Pemmaraju *et al.*, 2016). The results showed that dimorphic fungi had the strongest biofilm adherence capability. Dermatophytes and non-dermatophytes had moderate to strong biofilm adherence capability respectively.

Study indicated that increased pH is associated with an increase in biofilm development in all fungal groups studied. However, oxidative and osmotic stress resulted in significant biofilm production by adapting stress environment. Thus, Study suggested that alkaline pH and physiological stresses positively contribute towards increased biofilm production in fungi.

CONCLUSION

The production of biofilms by pathogenic fungi is a global health problem. Fungal infections caused by biofilms are connected to serious clinical consequences. The present study demonstrated that all fungal groups have ability to form biofilm. In qualitative and quantitative biofilm assays performed, dimorphic fungi produced thick biofilm biomass as compared to other groups. Biofilms formation was maximum at neutral PH. Biofilm production was increased under osmotic and oxidative stress as compared to control. Results revealed that dimorphic fungi have more tendency to produce biofilm formation under the

physiological stress applied. Thus, this study suggest that dimorphic fungi is more vulnerable to produce biofilm production under physiological stresses in immuno-compromised individuals. Study suggest that structural analysis of fungal biofilms under the oxidative and osmotic stress profile may contribute to a better understanding of the mechanisms that may assist in the development of new techniques to treat biofilm infections.

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