

Full Length Research Paper

Indole, a bacterial signaling molecule, exhibits inhibitory activity against growth, dimorphism and biofilm formation in *Candida albicans*

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A wide variety of signaling molecules produced by bacteria may influence physiology of neighboring microorganisms. Indole, a small molecule secreted by bacteria is known to control growth, physiology as well as biofilm formation in various bacteria. Effects of indole on eukaryotic microorganisms are not known. The objective of this study was to analyze response of *Candida albicans*, commensal yeast of the humans, to indole. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of indole for the growth of *C. albicans* was studied as per CLSI guidelines. Effect of indole on morphogenesis and adhesion was analyzed by using microplate based standard methodologies. Activity of indole on biofilm development and mature biofilms was studied in an *in vitro* biofilm model using XTT-metabolic assay and microscopic observations. Indole exhibited fungistatic as well as fungicidal properties to inhibit planktonic and biofilm growth in two strains of *C. albicans*. Interestingly, the inhibitory effects were obtained at concentrations which may exist *in vivo*. Indole prevented serum induced filamentous growth at concentrations lower than the growth inhibitory concentration. Inhibition of growth, morphogenesis, biofilm development and mature biofilms, for the first time suggests anti- *C. albicans* potential of indole, which may have significant consequences in bacterial-fungal interactions *in vivo*.

Key words: Antifungal, biofilms, *Candida*; drug resistance, indole, microbial interactions, morphogenesis, small molecules.

INTRODUCTION

Candida albicans, the most common fungal pathogen of humans, is associated with high morbidity and mortality in immunocompromised patients (Kim and Sudbery, 2011; Pfaller and Diekema, 2007). *C. albicans* biofilms on implanted medical devices and host epithelial cell surfaces favor establishment of infection (Andes et al., 2007; Iliknur et al., 2012). Biofilm related infections show different properties from those of planktonic cells, such as, increased resistance to antimicrobial agents, multiple drug resistance and tolerance to the host immune defenses. Biofilms formed by *C. albicans* may be up to 2000 fold more resistant to antifungal drugs than the

planktonic cells (Chandra et al., 2001; Shinde et al., 2012). Options of the antifungal drugs available for the treatment of systemic and invasive candidiasis are restricted to polyenes, allylamines, azoles and echinocandin class of molecules (Cannon et al., 2009; Mishra et al., 2007). High toxicity, narrow spectrum and emergence of drug resistance put limitations on the effective use of these drugs (Lattif et al., 2004; Mishra et al., 2007). As such, search for novel molecules to combat biofilm associated *C. albicans* infections need to be continued (Odds, 2003).

A wide variety of extracellular signaling molecules (including acyl homoserine lactones, butyrolactones, furanosyl borate diester, oligopeptides, hydroxyketones, derivatives of fatty acids, cyclic nucleotides and indole) produced by bacteria, are known to influence physiology and life style of the producer organism as well as that of

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others (Mueller et al., 2009). Indole is produced as a byproduct of the breakdown of tryptophan through tryptophanase activity (Martino et al., 2010). At least eighty five species of bacteria, including Gram positive and negative, are known to produce indole (Hu et al., 2010). Work done on *Escherichia coli* have established indole as a signaling as well as a quorum sensing molecule. Indole was shown to control expression of genes involved in amino acid metabolism, motility, chemotaxis, adhesion, biofilm formation, drug efflux and drug resistance in bacteria (Lee et al., 2011; Martino et al., 2010; Mueller et al., 2009). Reports have suggested that indole plays an important role in inhibition of bacterial biofilms (Bansal et al., 2010), while few cases of enhancement of biofilms are also observed (Martino et al., 2010). Recently, indole has been considered as an inter-species biofilm signal for interactions not only among bacteria but also between bacteria and human epithelial cells in the gastrointestinal tract (Lee et al., 2007a, b; Lee et al., 2011). Its role in inter-kingdom interactions between eukaryotic and prokaryotic microorganisms is not investigated. Indole produced by bacterial residents of the human body (for example those in oropharyngeal cavity, genitourinary tract, gastrointestinal tract) may exert various effects on eukaryotic commensal like *C. albicans*. Only one report is available, which described that indole produced by plants exert fungistatic effect on planktonic growth of *C. albicans* (Himejima and Kubo, 1993). In this study, for the first time, we report a potential role for indole in regulating the growth, morphogenesis and biofilm formation by *C. albicans*.

MATERIALS AND METHODS

Cultures

C. albicans, ATCC 90028 and 10231 strains were obtained from the Institute of Microbial Technology, Chandigarh, India. The strains were maintained on Yeast–Peptone–Dextrose (YPD) agar slants at 4°C.

Media, chemicals and culture conditions

Yeast–Peptone–Dextrose (YPD) medium was prepared by dissolving individual components (Yeast extract 1%, Peptone 2% and Dextrose 2%) in distilled water, and pH was adjusted to 6.5. Solid medium was prepared by adding 2.5% agar powder to YPD broth. A single colony from the YPD plates was inoculated in 50 ml of YPD broth in a 250 ml Erlenmeyer flask and incubated at 30°C on an orbital shaker at 120 rpm for 24 h. Cells from the activated culture were harvested followed by centrifugation for 5 min at 2000 g speed, washed three times with PBS (10 mM phosphate buffer, 2.7 mM potassium chloride and 137 mM sodium chloride, pH 7.4) and resuspended in PBS. Twenty percent solution of horse serum was prepared in sterile deionized distilled water. RPMI-1640 medium with L-glutamine and without sodium bicarbonate and buffered with 165 mM MOPS (3-[N-morpholine] propane sulphonic acid) pH 7, was filter-sterilized. Various concentrations of indole from 0.062 to 2 mg/ml were prepared in RPMI-1640 medium, using

DMSO as a solvent. Highest concentration of DMSO in the assay system was 1% v/v. All the media components and chemicals were from Hi-Media Laboratories Ltd., Mumbai, India. Indole (AR) was purchased from sd fine-Chem Ltd., Mumbai. XTT [that is, 2, 3-bis (2-methoxy-4-nitro-sulphophenyl)-2H-tetrazolium-5-carboxanilide] and menadione were purchased from Sigma-Aldrich Chem. Ltd. Mumbai, India.

Growth assay

Effect of indole on the growth of planktonic cells of *C. albicans* was studied by using the standard broth micro dilution methodology, as per CLSI guidelines (Routh et al., 2011). Briefly, various concentrations of indole ranging from 0.062 to 2 mg/ml were prepared in RPMI-1640 medium, in 96 well plates. Wells without indole served as a control. Inoculum of 1×10^3 cells/ml was added to each well and the plates were incubated at 37°C for 48 h. To analyze the growth, absorbance was read spectrophotometrically at 620 nm, using a microplate reader (Multiskan EX, Thermo Electron Corp., USA). The lowest concentration of indole which caused fifty percentage reduction in the absorbance compared to that of control, was considered as minimum inhibitory concentration (MIC) for growth of *C. albicans*.

Minimum fungicidal concentrations (MFC)

To determine the minimum *Candida*-cidal concentrations of indole, cells from the wells, where MIC was detected and the wells above that were selected. An aliquot of 10 µl cell suspension from each well was spread on YPD agar plates. The plates were incubated for 48 h at 30°C temperature and observed for the presence of colonies. Absence of visual growth (no appearance of colonies) was considered as fungicidal concentration (Routh et al., 2011).

Germ tube formation assay

Germ tube formation was studied using micro plate assay in 96-well plates (Chauhan et al., 2011). Cell density of the stock suspension was determined by hemocytometer count. Cells were inoculated in 20% serum prepared in deionized distilled water to get 1×10^6 cells/ml and various concentrations of indole were added. Wells without indole were kept as a control. Final volume of assay system in each well was kept at 200 µl. The plates were incubated at 37°C at 200 rpm on an orbital shaker for 2 h and cells were observed microscopically. Every time 100 cells were counted and the numbers of yeast and germ tube forms were noted. Percentage of germ tube formation in each well compared with that of control was calculated.

Adhesion assay

Effect of indole on adherence of *C. albicans* to a solid surface that is (polystyrene) was studied by using micro plate based assay (Camacho et al., 2007). Various concentrations of indole were prepared in the wells of microplate by double dilution. Wells without indole were kept as a control. Fifty microlitre of cell suspension was added to each well so that the final cell number was 1×10^7 cells/ml. Final volume of assay system in each well was kept at 100 µl. The plates were incubated at 37°C for 90 min at 100 rpm in an orbital shaking incubator to allow attachment of cells on the surface. After the incubation, wells were washed with PBS to remove nonattached cells. Density of the adherence in each well was analyzed by using XTT- metabolic assay and percentage of adhered cells was calculated compared to that of control.

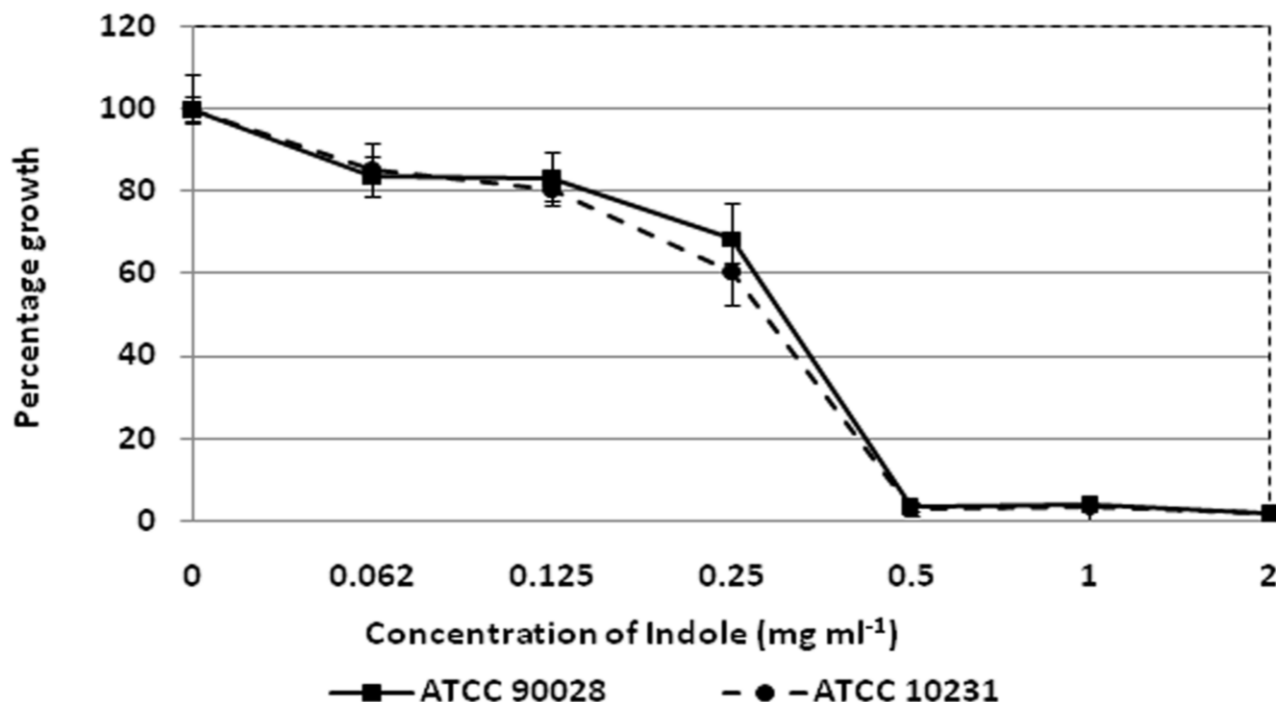


Figure 1. Growth of *C. albicans* (ATCC 90028 and ATCC 10231), in presence of indole. Growth, after 48 h, was analyzed in terms of absorbance at 620 nm and percentage of growth compared to that of control was calculated.

Biofilm formation

C. albicans biofilms were developed on polystyrene surface of 96-well plates as per standard methodologies (Bachmann et al., 2003; Shinde et al., 2012). A cell suspension of 1×10^7 cells/ml was prepared in PBS and 100 μ l was inoculated in each well. In the adhesion phase, plates were incubated at 37°C for 90 min to allow attachment of cells on the surface. Non-adhered cells were removed by washing the wells with sterile PBS, two to three times. Two hundred microlitre of the RPMI-1640 medium was added to each well and the plates were incubated at 37°C for 48 h to allow biofilm formation. To observe its effect on development of biofilms, RPMI-1640 medium along with various concentrations of indole (from 0.062 to 2 mg/ml), was added to each well immediately after adhesion phase and incubated for 48 h at 37°C. To analyze activity against mature *C. albicans* biofilms, indole was added to 24 h mature biofilms and incubated further for 48 h. After incubation, wells were washed to remove any planktonic cells, and biofilms were observed using an inverted light microscope. Biofilm growth was analyzed with XTT metabolic assay.

Biofilm quantitation by XTT assay

Biofilm growth was quantitated using XTT that is [2, 3-bis (2-methoxy-4-nitro-sulphophenyl)-2H-tetrazolium-5-carboxanilide] metabolic assay (Shinde et al., 2012). XTT solution was prepared by mixing 1 mg/ml XTT salt in PBS and stored at -20°C. Prior to use, menadione solution prepared in acetone was added to XTT to a final concentration of 4 μ M. The wells containing biofilms were washed with PBS to remove non adhered cells and incubated for 5 h in 100 μ l of XTT-menadione solution in dark, at 37°C at 100 rpm. The color formation by water soluble formazan product was measured at 450 nm using a microplate reader (Multiskan EX,

Thermo Electron Corp., USA). Wells without indole were considered as control, while those without biofilms were the blank.

Statistical analysis

All the experiments were done with two strains and values mentioned are the mean of triplicate observations with standard deviation.

RESULTS

Growth of *C. albicans* was inhibited in presence of indole

Indole inhibited *C. albicans* growth in a concentration-dependent manner. Fungistatic effect of indole on the growth of ATCC 90028 and 10231 was observed at 0.5 mg/ml concentration, after 24 and 48 h of incubation. Exposure to 1 mg/ml of indole was fungicidal and killed the planktonic cells and no viable cells were detected (Figure 1).

Indole is an inhibitor of morphogenesis

Indole inhibited yeast to hyphae dimorphism induced by 20% serum, in a concentration-dependent manner. At 0.125 mg/ml of indole, around 25% inhibition of germ tube forms was seen, while 0.25 mg/ml of it caused 77

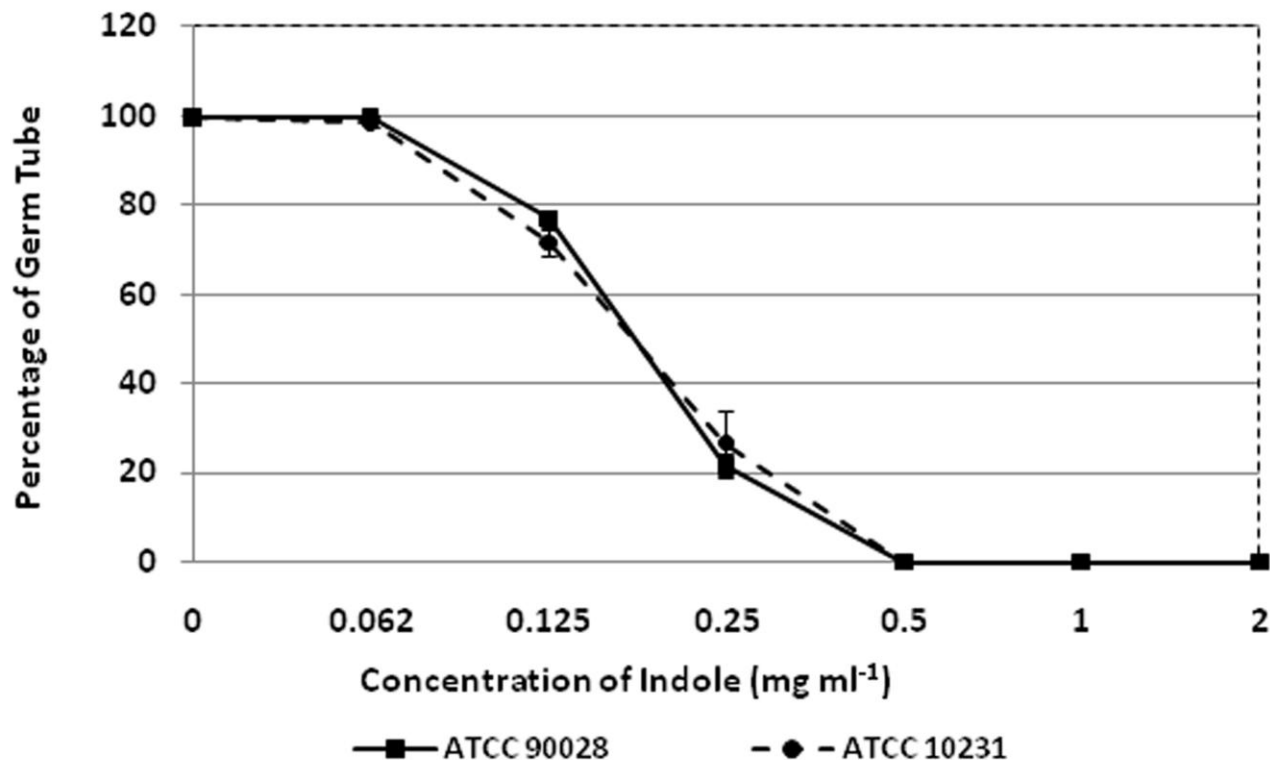


Figure 2. Effect of Indole on serum induced yeast to hyphae dimorphism in *C. albicans* (ATCC 90028 and ATCC 10231). Germ tube formation decreases in presence of increasing concentration of indole.

and 72% inhibition in ATCC 90028 and ATCC 10231, respectively. Response of both strains was more or less similar. Complete inhibition of germ tubes occurred at 0.5 mg/ml in both the strains (Figures 2 and 5A).

Adhesion to solid surface was less sensitive

C. albicans adhesion to polystyrene was unaffected by addition of indole up to 1 mg/ml concentration. Adherence of the cells was not influenced by indole concentrations of 0.062 to 1 mg/ml and showed XTT metabolic activity similar to that of control (data not shown). Analyzing the adhered cells with XTT-reduction assay showed that 2 mg/ml indole inhibited adhesion to the solid surface.

Biofilm development was inhibited by indole

Addition of indole at 0 time point of biofilm that is (immediately after adhesion phase of 90 min) prevented normal biofilm formation. 0.25 mg/ml of indole did not affect development of biofilms, while 0.5 mg/ml showed a significant (70 to 80%) reduction in biofilm growth compared to that of control biofilms. At 1 mg/ml of indole, only adhered yeast cells were observed indicating that

biofilm development was completely inhibited (Figures 3 and 5B).

Indole eradicated mature biofilms

Mature biofilms of *C. albicans* were sensitive to indole's inhibitory activity, at relative high concentrations. At 0.5 mg/ml concentration, indole showed significant (47 and 59%) inhibitions of *C. albicans* ATCC 90028 and 10231, respectively. One mg/ml of indole caused complete eradication of mature biofilms and only adhered yeast cells remained on the polystyrene surface (Figures 4 and 5C).

DISCUSSION

Microorganisms residing as commensal in the human body may interact with other organisms and modulate their community behavior (Morales and Hogan, 2010; Shirtliff et al., 2009). A diversity of small molecules produced by bacteria play important roles in cooperative or competitive microbial interactions. Also, it was suggested that host bacterial community may control fungal proliferation and infection through the molecules secreted by them (Wargo and Hogan, 2006). Indole

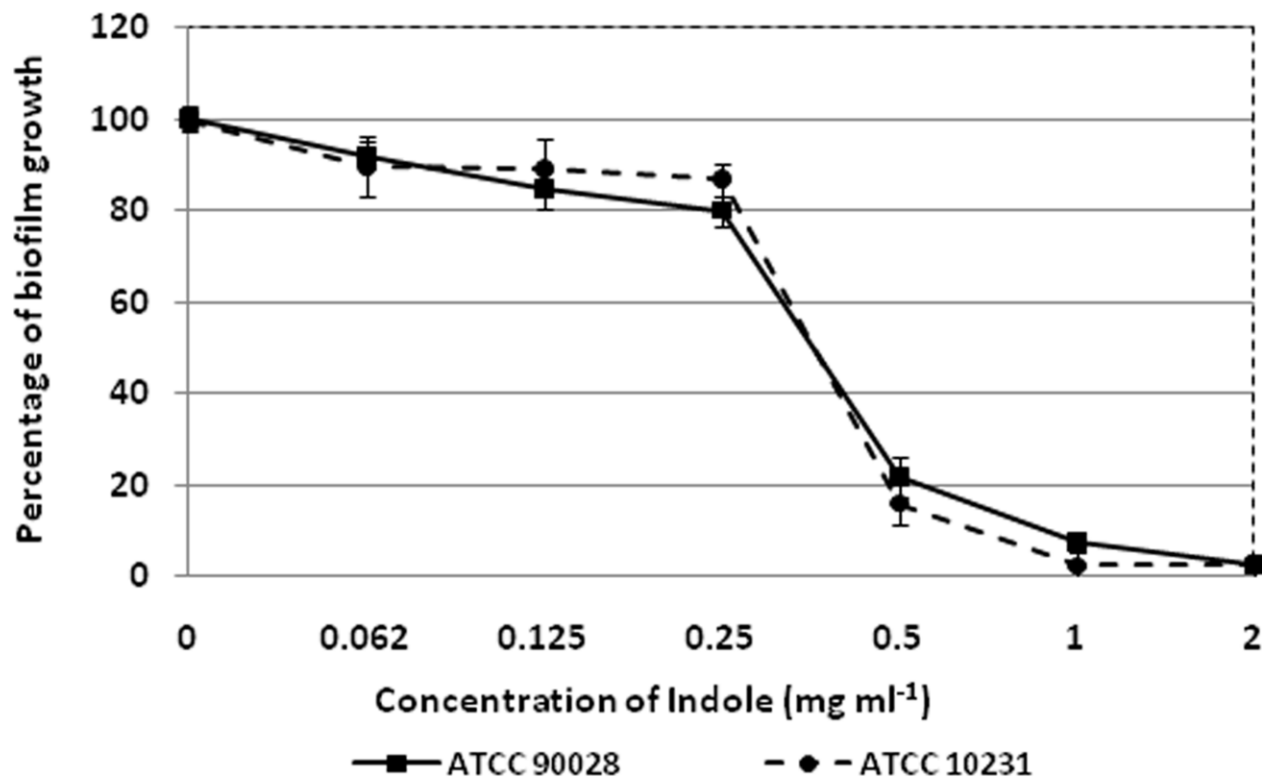


Figure 3. Biofilm formation of *C. albicans* (ATCC 90028 and ATCC 10231) in presence of indole. Effect on biofilm development was analyzed in terms of percentage of relative metabolic activity (RMA) by XTT-assay.

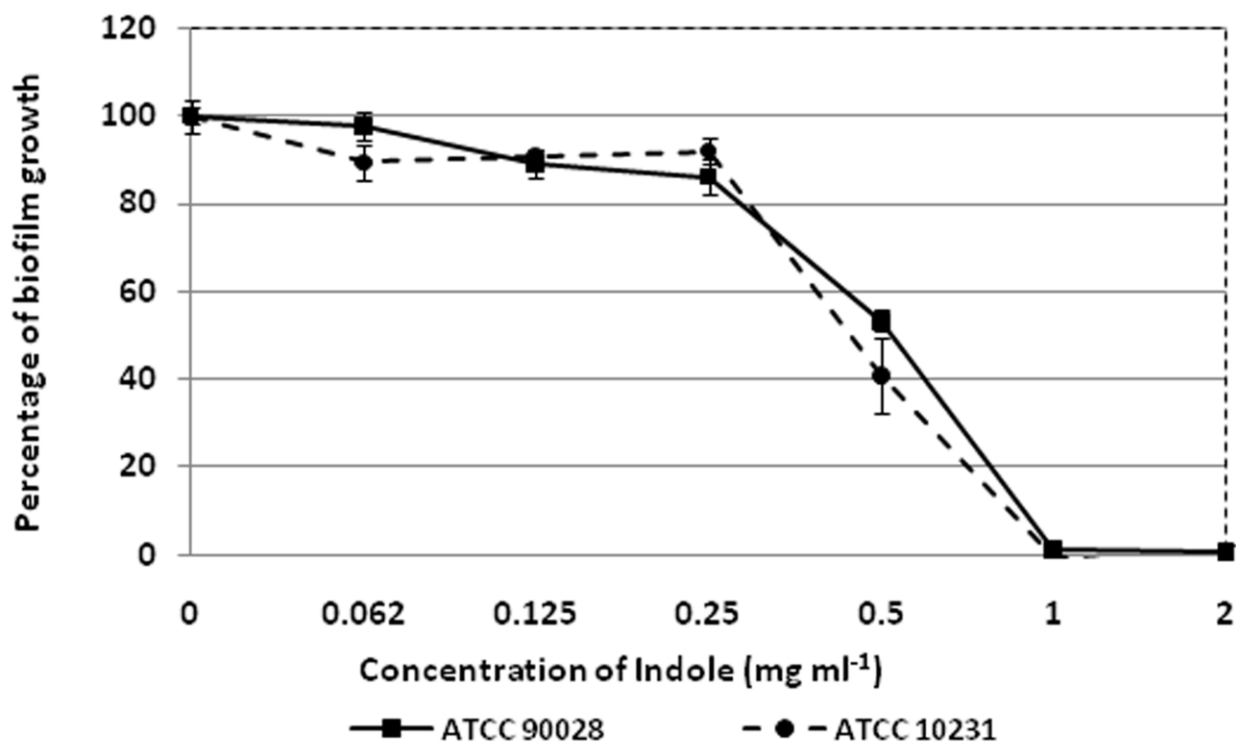


Figure 4. Effect of indole on the mature biofilms of *C. albicans* (ATCC 90028 and ATCC 10231). Reduction in biofilm growth was analyzed in terms of percentage of relative metabolic activity (RMA) by XTT-assay.

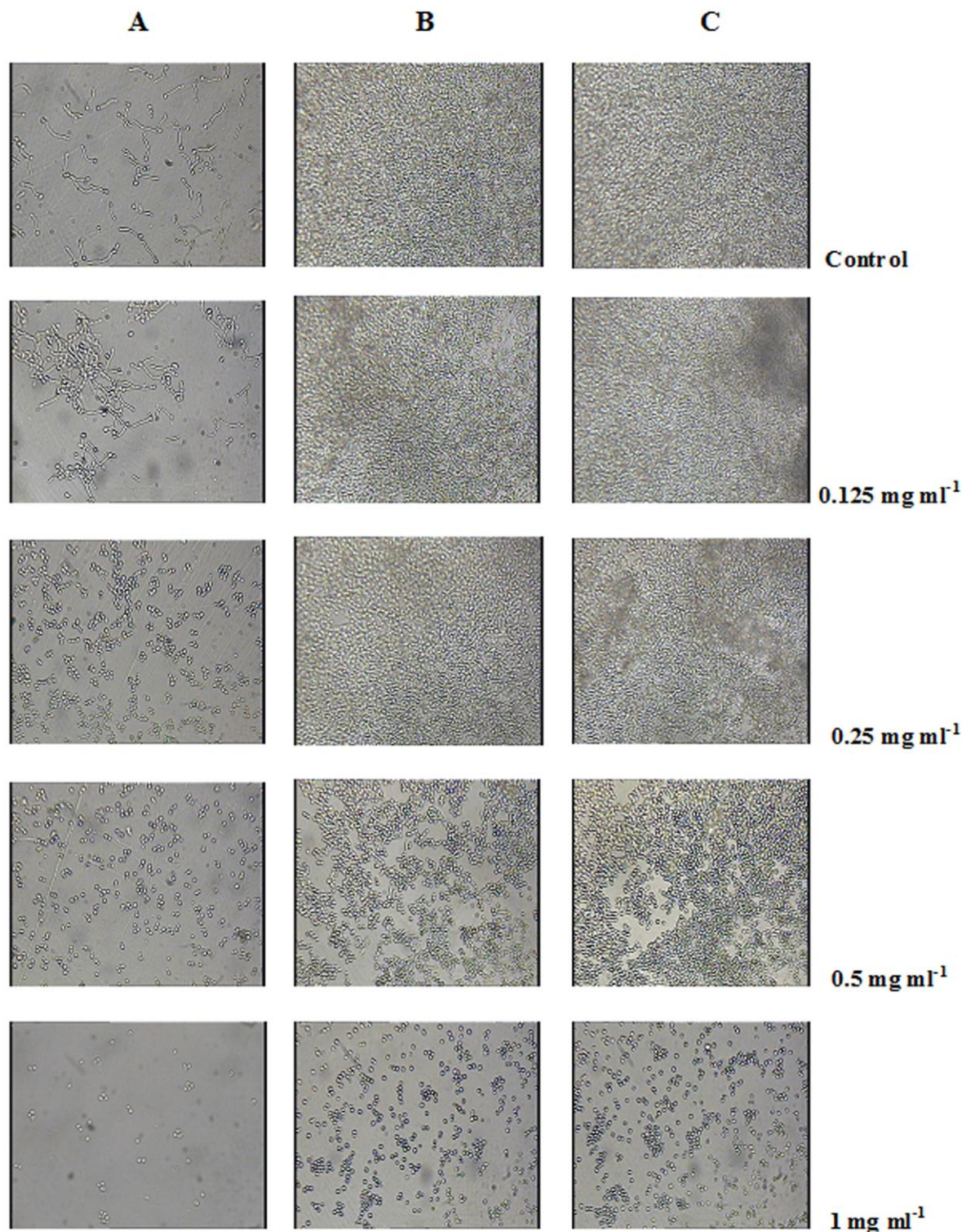


Figure 5. Photomicrographs in panels A, B, C describe morphogenesis and biofilm growth of *C. albicans* ATCC 90028, in presence of various concentrations of indole ranging from 0.125 to 1 mg/ml (top to bottom). (A) Indole prevents serum induced yeast to hyphae morphogenesis at 0.25 mg/ml concentration. (B) No dense biofilm matrix was observed at 0.5 mg/ml concentration indicating prevention of biofilm development which was confirmed by XTT assay. At 1 mg/ml of indole, only adhered cells were observed showing complete inhibition of biofilms. (C) Growth of mature biofilms was inhibited at 0.5 mg/ml of indole, while 1 mg/ml of it eradicated biofilm matrix and only adhered cells remained on the polystyrene surface. These microscopic observations were confirmed and supported by reduction or no activity in XTT metabolic assay. First photograph in each panel shows control without indole. (Magnification $\times 200$).

produced by bacteria residing in the gastrointestinal (GI) tract is known to act as a signal and control community growth of competing bacteria (Martino et al., 2010). However, activity of indole on eukaryotic microorganisms like *C. albicans*, commensal yeast of the humans, is unknown. The aim of this study was to investigate the effects of indole on *C. albicans* growth, morphogenesis and biofilm formation. Early report described that plant molecules like polygodial, in combination with 400 to 800 µg/ml indole, have fungistatic activity against *C. albicans* (Himejima and Kubo, 1993). However, effects of indole on important attributes of growth that is, yeast to hyphae morphogenesis and adhesion to solid surfaces have not been studied. Effects of indole on the community growth that is, (biofilms) of *C. albicans* were unexplored. Results of our study on two standard strains of *C. albicans*, established the MIC of indole at 500 µg/ml, while high concentration that is, 1 mg/ml exerted fungicidal effect. The sub MIC concentration of 250 µg/ml exerted a significant (> 70%) inhibition of serum induced yeast to hyphae conversion. Specific activity against morphogenesis is interesting because it is an important virulence attribute of *C. albicans*. Around 1.1 mM concentration of indole (which is equivalent to 130 µg/ml) was reported to be present in human feces (Lee et al., 2007a), most of which may be of bacterial origin. *Escherichia coli* is known to produce 500 to 700 µM of indole in cultures supernatant (Lee et al., 2007b). Actual concentration of indole accumulated in GI tract and in microenvironments like biofilms may be several folds more than this. It was reported that 1 mM indole is nontoxic to human epithelial cells (Lee et al., 2007a). Its derivative found in plants that is, 3-indolyl acetonitrile (IAN) was not teratogenic to rat fetus up to 200 mg/kg dose, when administered subcutaneously (Lee et al., 2011).

Small molecules that play signaling roles at low concentrations may have antibiotic potential at high concentrations (Dietrich et al., 2008; Mlot, 2009). We propose that *in vivo*, gastrointestinal indole may control *C. albicans* proliferation and filamentation, thereby avoiding invasion of epithelial tissues. Extending our work to adhesion and biofilm formation showed that adhesion to a solid substrate was not sensitive to inhibitory activity of indole. Although, very high concentration (2 mg/ml) prevented adhesion of *C. albicans*, it remained unaffected up to 1 mg/ml of indole. Interestingly, development of biofilms after adhesion of cells to the surface was readily inhibited by indole, at 500 µg/ml concentration. Similarly, addition of 500 µg/ml of indole resulted in significant (50 to 60%) removal of mature biofilms. Hence, indole may be playing a role to prevent *C. albicans* biofilms on host tissues and associated infections.

Exact mechanism of action behind indole mediated inhibition of *C. albicans* growth and biofilms is still unclear. Various genes in *C. albicans* are differentially

regulated during filamentation and biofilm formation. There is possibility that indole secreted by bacteria interfere in signaling events like Ras- cAMP pathway or modulate expression of important genes like *EFG1*, *ALS1*, *ALS3*, *HWP1* and *BCR1*, in *C. albicans* (Nobile and Mitchell, 2006; Peleg et al., 2010). In *E. coli*, it acts as a quorum sensing molecule (QSM) to inhibit motility, chemotaxis and biofilm formation as well as cause delay in cell division. High concentration of indole that is, 4 mM (which is equivalent to 500 µg/ml), was reported to block *E. coli* cell division (Lee et al., 2007a). Similar mechanisms need to be investigated in *C. albicans*. To summarize, our work showed that indole inhibits planktonic as well as biofilm growth of *C. albicans* and also acts as an inhibitor of yeast to hyphae morphogenesis. Work in this direction may provide novel approaches against *C. albicans*, especially biofilm associated infections.

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