Effect of alcohols on filamentation, growth, viability and biofilm development in *Candida albicans*

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Abstract

In this study we report the potential of alcohols as morphogenetic regulators in *Candida albicans*. All the alcohols tested influenced various modes of growth like planktonic as well as biofilm forms. Viability was affected at high concentrations. Among the alcohols, the response of *C. albicans* to amyl alcohol (pentanol) was noteworthy. Amyl alcohol at a concentration 0.5% which was not inhibitory to growth and viability specifically inhibited morphogenetic switching from yeast to hyphal forms. It also inhibited normal biofilm development favoring yeast dominated biofilms. Based on this study we hypothesize that alcohols produced under anaerobic conditions may not favor biofilm development and support dissemination of yeast cells. Since anaerobic conditions are not found to favor production of quorum sensing molecules like farnesol, the alcohols may play a role in morphogenetic regulation.

Key words: alcohols, signalling, morphogenesis, biofilms, yeast metabolism, *Candida albicans*.

Introduction

*Candida albicans* is considered as a serious pathogen in immunocompromised patients causing high mortality rate (Fridkin and Irwin, 1983; Jean-Marcel, 1998; Virudes et al., 2003; Parahym et al., 2009). Several studies have shown that in *C. albicans*, filamentation is a virulence factor (Lo et al., 1997; Saville et al., 2003; Yang, 2003). It also plays a critical role in biofilm formation (Bailie and Douglas, 1999). Thus, inhibition of filamentation may reduce pathogenesis and biofilm formation in *C. albicans* (Lo et al., 1997; Bailie and Douglas, 1999; Lopez-Ribot, 2005).

Metabolites produced under different environmental conditions are reported as virulence molecules in microbes (Dufour and Rao, 2011). Different alcohols are produced by the yeast *Saccharomyces cerevisiae* and *C. albicans in vitro* (Ghosh et al., 2008; Hazelwood et al., 2008). For example in presence of leucine, valine, phenylalanine, tyrosine, and tryptophan as nitrogen sources, *Saccharomyces cerevisiae* produces isomethyl alcohol, isobutanol, tyrosol, phenylethanol, and tryptophol respectively (Dickinson, 2008; Hazelwood et al., 2008). Alcohols such as ethanol, propanol, isopropanol, 1-butanol, 2-butanol, isomethyl alcohol, and tert-amyl alcohol are reported to induce filamentation in *S. cerevisiae* (Lorenz et al., 2000). Attachment to tissue culture dishes and polystyrene dishes in *Pseudomonas* species is affected by methanol, ethanol, propanol, and butanol (Fletcher, 1983). *C. albicans* in presence ethyl alcohol, dodecanol, isoamyl alcohol, and nerolidol failed to switch from yeast to hyphae under standard induction conditions (Martins et al., 2007; Davis-Hanna et al., 2008; Chauhan et al., 2010). Short chain n-alkanols are reported to affect the morphogenesis in the dimorphic fungus, *Aureobasidium pullulans* (Moragues et al., 1998). Studies are lacking on the effect of propanol, butanol, isopropanol, pentanol and isobutanol in *C. albicans*. In this paper we report for the first time the effect of short chain alcohols on induced morphogenesis, growth, and biofilm formation in *C. albicans*.

Materials and Methods

Organism, media and culture conditions

*Candida albicans* ATCC 90028 (MTCC 3017) was used throughout the study. The culture was maintained on Yeast-Peptone-Dextrose (YPD) agar slants at 4 °C. Yeast-Peptone-Dextrose medium (YPD) was prepared by dissolving individual components (Yeast extract 1%, Peptone 2%...
and Dextrose 2%) in distilled water. pH was adjusted to 6.5. Solid medium was prepared by adding 2.5% agar powder to YPD broth. Two inducers i.e. 10% horse serum was prepared in deionized distilled water while, RPMI -1640 medium with L-glutamine w/o sodium bicarbonate buffered with 165 mM MOPS (3-[N-morpholine] propane sulphonic acid) was added in sterilized distilled water and pH was adjusted to 7. All the media components and chemicals were from Hi-Media Laboratories Ltd. Mumbai, India. For culture activation, a single colony from the YPD plates was inoculated in 50 mL of YPD broth, in a 250 mL conical flask and incubated at 30 °C on an orbital shaker at 120 rpm for 24 h. Cells from the activated culture were harvested by centrifugation for 5 min at 2000 g speed and washed three times with PBS (10 mM Phosphate buffer, 2.7 mM Potassium chloride and 137 mM Sodium chloride pH 7.4) and re-suspended in PBS.

Filamentation assay

Filament formation was studied using microtiter plate assay in 96 -well microtiter plates as described previously (Chauhan et al., 2010). Briefly, cells from stock were inoculated in various inducer media to get 1 x 10^6 cells ml^-1. Various concentrations of butanol, isobutanol, tertiary-butanol, propanol, isopropanol and pentanol to give final concentrations of 0.06, 0.12, 0.25, 0.5, 1, 2, and 4% (v/v) were added. Wells without alcohol were kept as control. Final volume of assay system in each well was kept 200 μL. The plates were incubated at 37 °C at 200 rpm on an orbital shaker for 4 to 8 h. After incubation, 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 was calculated compared to that of control with the following formula:

\[
\% \text{ formation} = \frac{\text{no. treatment}}{\text{no. control}} \times 100
\]

where % formation = percentage of germ tube formation, no. treatment = no. of germ tubes in treatment and no. control = no. of germ tubes in control.

Growth and viability assay

Effect of alcohols on growth of C. albicans yeast phase cells was determined by standard CLSI M27-A microbroth dilution method (NCCLS, 1997). Various concentrations of butanol, isobutanol, tertiary-butanol, propanol, isopropanol, and pentanol (amyl alcohol) ranging from 0.025 to 4% v/v were added to RPMI-1640 medium along with various concentrations of butanol, isobutanol, tertiary-butanol, propanol, isopropanol, and pentanol ranging from 0.025 to 4% v/v and one control without alcohols was added to each well. The plates were incubated at 37 °C for 24 h to allow biofilm formation. After incubation, wells were washed to remove any planktonic cells and biofilms were observed under an inverted light microscope (Metzer, India). Measurement of metabolic activity of biofilm was done by MTT assay with little modification (Hawser and Douglas, 1997). The tetrazolium salt 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT, Hi-media, Mumbai, India) was used in an assay. 50 μL of MTT solution (stock solution containing 5 mg MTT mL^-1 PBS, diluted 1:5 in prewarmed 0.15 M PBS prior to addition) was added in each well. The plate was incubated for 5 h at 37 °C. Dimethyl sulfoxide (200 μL) was added to each well to solubilise MTT formazan product and optical density was measured at 450 nm using a 96-well microplate reader (Multiscan Ex-Thermo Electron Corp. USA). Percentage of metabolic activity in biofilm formation was calculated compared to that of control (without alcohol).

Statistical analysis

All the experiments were done in triplicates and standard deviation from the mean was calculated. Effect of alcohols on biofilm formation was analyzed using Two-way Anova (Bonferroni posttests) by GraphPad Prism 5 software. P < 0.05 was considered statistically significant.

Results

Effect of various alcohols on induced morphogenesis

All the alcohols inhibited filamentation in a concentration dependent manner induced by two standard inducers (Figure 1). Responses of C. albicans to alcohols were found to be similar for both the inducers at all time points studied. Pentanol at a concentration of 0.5% showed com-
plete inhibition of filamentation, while 1% of butanol and propanol completely blocked hyphae. Isobutanol, isopropanol and tertiary-butanol at 2% completely halted yeast to hyphal form transitions (Figure 3). The efficacy of alcohols towards induced morphogenesis is as follows:

Pentanol < Butanol, Propanol < Isobutanol, Isopropanol, Tertiary-Butanol

Growth and viability of *C. albicans* cells in presence of alcohols

Alcohols up to 0.5% of concentration did not cause any effect on viability. Addition of 4% of various alcohols inhibited growth by 60-80%. 2% of butanol, isobutanol, tertiary-butanol, and pentanol reduced growth by 15-20%, whereas propanol and isopropanol at the same concentration inhibited growth by 20-25%. 4% of butanol, isobutanol, and propanol were found to be toxic to *Candida* cells, while isopropanol, tertiary-butanol, and pentanol caused 35-50% reduction in viability at 4% (Table 1). The sensitivity of *Candida* cells for alcohols is as follows:

Butanol < Propanol < Isobutanol < Isopropanol < Pentanol < Tertiary-butanol

*Candida* biofilms in presence of various alcohols

All the alcohols studied significantly (*p = 0.0005*) inhibited biofilm formation in a concentration dependent manner. Treatment with 4% of alcohols caused 50-60% reduction in metabolic activity in *Candida* biofilm (Figure 2). Addition of 2% of butanol, isobutanol, tertiary-butanol, propanol, and isopropanol after adhesion phase inhibited biofilm development and patches of few adhered yeast cells were observed, whereas similar effect was seen at 1% of pentanol (Figure 4). 1% of butanol, isobutanol, tertiary-butanol, propanol, and isopropanol inhibited filamentation in biofilms and favored ‘yeast only’ biofilm. Pentanol caused considerable inhibition of biofilm at 0.5% concentration leading to yeast only biofilm formation.

Discussion

The behavior and production of metabolites by *Candida albicans in vivo* may greatly vary depending on the niches it colonizes in the human body like gastrointestinal tract, vaginal, or oral cavities (Ghosh *et al.*, 2008). In deep seated mycosis or in biofilms on prosthetic devices it may live in a polymicrobial environment and may be subject to anaerobic conditions or exposed to the metabolites of its own or that of other organisms (Dumitru *et al.*, 2004; Dufour and Rao, 2011). In gastrointestinal tract, deep

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<th>Concentrations of alcohols (% v/v)</th>
<th>% Viability</th>
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<tr>
<td></td>
<td>Propanol</td>
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<td>0</td>
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<td>0.062</td>
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<td>1</td>
<td>80 (± 6)</td>
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<tr>
<td>2</td>
<td>70 (± 6)</td>
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<td>4</td>
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*Values are the mean of triplicate viable counts. Cells were incubated at 35 °C at various time intervals on an orbital shaking incubator in RPMI-1640 medium containing various concentrations of alcohols. Viability count was done after 48 h. Values in parenthesis indicate standard deviation.*
seated mycosis, and biofilms it may produce considerable amount of alcohols, because of the anaerobic conditions. The quorum sensing molecule, farnesol is undetectable under anaerobic conditions of growth, suggesting signalling roles for other metabolites (Hornby et al., 2011). Alcohols are very common metabolites produced by yeast under anaerobic conditions. Interestingly, the concentrations of al-

Figure 2 - Effect of various alcohols on biofilm formation by Candida albicans. In vitro biofilm development in the presence of alcohols was quantified by using MTT assay. RPMI-1640 medium containing various concentrations of respective alcohols and one control without alcohol was incubated at 37 °C for 24 h.

Figure 3 - Morphology of Candida albicans cells in presence of pentanol. Briefly cells were incubated in serum at 37 °C for 4, 6, and 8 h respectively and morphology was assessed after incubation and photographs were taken by Labomed imaging device. (Magnification x100).

Figure 4 - Candida albicans biofilm development in presence of various alcohols. In vitro biofilms were developed on 96-well polystyrene surfaces in the presence of alcohols and one control without alcohol was incubated at 37 °C for 24 h. After incubation biofilms were observe microscopically and photographs were taken by Labomed imaging device. (Magnification x200).
Roles of alcohols in C. albicans

cohols produced under anaerobic conditions are more in comparison to yeast cells produced under aerobic growth (Ghosh et al., 2008). Most of the alcohols tested by us inhibited yeast to hyphal form switching.

We have earlier reported the morphogenetic regulatory properties of ethyl alcohol (Chauhan et al., 2010) and its oxidative product, acetaldehyde in C. albicans (Chauhan et al., 2011). In this paper we report for the first time the effect of pentanol, butanol, propanol, isobutanol, isopropanol, and tertiary-butanol on morphogenetic switching, biofilm formation, growth, and viability in C. albicans. Among the various alcohols studied, pentanol (amyl alcohol) was found to be very effective. 0.5% of pentanol completely blocked induced morphogenesis without affecting viability of C. albicans. The hyphal inhibitory concentration of pentanol did not alter growth and viability of Candida cells. The same concentration of pentanol showed around 50% reduction in metabolic activity in biofilm development (Figure 2). While for other alcohols the hyphal inhibitory concentration showed 10-30% reduction in viability and 35-45% decreased in metabolic activity was observed in Candida biofilms compared to that of control (Table 2). Based on our results, we have proposed a model for the role of alcohols in C. albicans biofilms (Figure 5).

Deeper regions of mature biofilms may produce anaerobic conditions (Dumitru et al., 2004). Anaerobic conditions also persist in deep seated mycosis. The type of alcohol produced may vary depending on the amino acid availability. For example, availability of leucine, isoleucine, valine, and threonine may favor the production of amy alcohol, isoamyl alcohol, butanol, and propanol respectively (Dickinson et al., 1998; Lorenz et al., 2000; Hazelwood et al., 2008). The alcohols may exert effects depending on its concentrations. At lower concentrations like 0.5% in case of amyl alcohol it may inhibit morphogenetic switching and support yeast phase growth. While at high concentrations it may slow down the growth rate. Most of the alcohols may exert similar effect like ethyl alcohol, amyl alcohol, isoamyl alcohol, etc. Favoring of yeast phase growth may support disseminative mode of growth and also aid to escape from the alcohol poisoned environment. It may also offer ecological advantages for C. albicans over other competing organisms which are more susceptible to alcohols than Candida under mixed species of populations.

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References


![Figure 5](image_url) - A model for the role of alcohols favoring disseminative mode of growth in Candida albicans. Planktonic cells get attached to surfaces of polystyrene, catheter, silicone, etc. The adhered cells start to develop biofilms. Biofilm development leads to matured biofilm which include the mixture of yeast cells (green colored), hyphae (red and blue colored) and extracellular polymatrix (yellow colored). Deeper region of matured biofilm favors anaerobic conditions which gives rise to the production of alcohols. These alcohols inhibit filamentation and favors disseminative growth which allows Candida to escape from the alcohol poisoned environment.
century of research on *Saccharomyces cerevisiae*. Appl Environ Microbiol 74:2259-2266.


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