Microbiological Research 207 (2018) 19–32

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

5-hydroxymethyl-2-furaldehyde from marine bacterium *Bacillus subtilis* inhibits biofilm and virulence of *Candida albicans*

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ARTICLE INFO

Keywords:
5-hydroxymethyl-2-furaldehyde
Antibiofilm activity
Antivirulence activity
Bacillus subtilis
Candida albicans
Yeast to hyphae

ABSTRACT

Candida albicans is considered as the primary etiologic agent of candidiasis, a very common fungal infection in human. The yeast to hyphal transition and ability to form hypoxic biofilm on medical devices is well allied with virulence and antifungal resistance of *C. albicans*. Antagonistic agents that inhibit biofilm formation and alter susceptibility of *C. albicans* to conventional antifungals is of profound need. The present study explores the antibiofilm efficacy of *Bacillus subtilis*, a marine bacterial isolate from Palk Bay against *C. albicans*. Mass spectrometric analysis of ethyl acetate extract of *B. subtilis* unveiled 5-hydroxymethyl-2-furaldehyde (5HM2F) as one of its major components. 5HM2F demonstrated concentration dependent biofilm inhibition, which was also corroborated through microscopic analysis. Furthermore, 5HM2F was effective in inhibiting other virulence factors of *C. albicans* such as morphological transition and secreted hydrolases production. Fourier transform infrared spectroscopic analysis showed alteration in amide bond region. The reduction in ergosterol content and increased antifungal susceptibility was well allied with real time PCR result, which showed down regulation of genes involved in drug resistance mechanisms. *In vivo* study using *Caenorhabditis elegans* also substantiated the antivirulence efficacy of 5HM2F at *in vivo* condition. Thus, the present study reports the therapeutic potential of 5HM2F against *C. albicans* infections.

1. Introduction

The commensal fungus, *Candida albicans* is attained in earlier neonatal life through childbirth and remains as a common inhabitant, colonizing the mucosal lining of human oral, gastrointestinal and genitourinary tracts (Pierce et al., 2015). Lately, it has been deliberated to be an opportunistic pathogen wherein, its innocuous nature is disgruntled by multiple reasons such as impaired immune system, nutritional deprivation, use of corticosteroids and or inappropriate intake of antibiotics (Dutton et al., 2016). *C. albicans* has been reported to be the third leading pathogen of blood stream infections, which begets a variety of human ailments ranging from minor superficial skin infections to fatal oropharyngeal, vulvovagainal and invasive candidiasis (Rajendran et al., 2016; Kong et al., 2016). Furthermore, the most endemic candidiasis is estimated to be the fourth major instigator of hospital acquired infections that account for nearly fifty percent of mortality rate in United States and besides, it has the predilection for patients with weakened immune system such as AIDS victims, cancer patients undertaking chemotherapy, underweight neonates and transplant recipients (Morrell et al., 2005; Kabir et al., 2012).

Emerging infectious diseases remain to be a prime threat to mankind, which is liable for 15 million deaths worldwide every year (Fauci et al., 2005). However, the infective potential of an opportunistic pathogen involved in infectious diseases is mainly attributed to its virulence traits, which decide the type and severity of host infection (Jabra-Rizk et al., 2016). The putative virulent traits of *C. albicans* involved in host infections include morphological transition from yeast to hyphae (morphogenesis), metabolic adaption, phenotype swapping from white to opaque cells, production of degradative enzymes such as proteases, lipases and phospholipases, expression of adhesins and invasins on its cell surface and biofilm formation (Calderone and Fonzi, 2001; Muthamil and Pandian, 2016).

C. albicans biofilms are established by the initial sessile aggregation of yeast cells on host surface, which is subsequently trailed by hyphal

Abbreviations: CFCS, cell free culture supernatant; EA, ethyl acetate; MBIC, minimum biofilm inhibitory concentration; 5HM2F, 5-hydroxymethyl-2-furaldehyde; EPS, extracellular polymeric substances

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https://doi.org/10.1016/j.micres.2017.11.002
Received 24 May 2017; Received in revised form 20 October 2017; Accepted 4 November 2017
Available online 08 November 2017
0944-5013/

formation (Kong et al., 2016). The ability of *C. albicans* to form biofilm on biotic and abiotic surfaces together with morphogenesis remains to be the foremost cause of pathogenesis that facilitates resistance to host barricades and antifungal treatment (Yano et al., 2016). In addition, the surface associated virulence factors such as adhesins and invasins are supportive for the attachment and invasion into the host cells, which momentously aids *C. albicans* in evolving sophisticated mechanisms to escape host immune responses (Brunke et al., 2016). Additionally, phospholipases and proteases, the most prominent degradative enzymes of *C. albicans* are mainly implicated in degrading lipids of cell membrane and vital proteins of skin where, lipids and proteins are entailed in sustentation of body's physiology (de Paula Menezes et al., 2016).

The treatment of *C. albicans* infections includes several antifungal classes such as polyenes, triazoles, alkyl amines and echinocandin wherein, each class accomplishes its requisite action through specific modes (Sanglard and Odds, 2002). Numerous reports suggest that inappropriate and overuse of antibiotics creates a selective pressure on microbes, augmenting the evolution of microbes to sustain in the adverse environment (Penesyan et al., 2015). In the recent past, the development of resistance by *C. albicans* against conventional antifungal therapies have been reported, which incapacitate the management of infections and also originate an exceptional menace to mankind (Ellepola et al., 2016).

Recently, antivirulence therapies have gained more attention in clinical settings, in which, the virulence of pathogen is concentrated instead of its growth for hindering microbial infections. Consecutively, the less virulent pathogen has little chance to develop resistance as well as becomes more susceptible to antibiotics and host immune system (Silva et al., 2016). In this midst, the less explored marine microbial consortia have been reported to possess many quintessential therapeutic assets such as antimicrobial, antibiofilm and antivirulence properties (Thenmozhi et al., 2009). Thus, the focus of the present study is to delineate the antibiofilm and antivirulence potential of marine bacterium, *Bacillus subtilis* (identified by 16S rRNA sequencing; Genbank Accession No. KC433737) against the infective potential of *C. albicans*.

2. Materials and methods

2.1. Microbial strains and culture conditions

In the current study, fungal strain *C. albicans* ATCC 90028 was maintained in yeast extract peptone dextrose (YEPD) (1% yeast extract, 2% peptone and 2% dextrose) agar plates (1.8% agar) and cultured routinely in YEPD broth. The broth cultures were incubated in shaker incubator (160 rpm) at 37 °C. One percent of overnight culture with optical density (OD_{600nm}) of 1.5 was used for performing all *in vitro* assays. Spider broth comprising 1% of mannitol, 0.2% of dipotassium hydrogen phosphate and 1% of nutrient broth (Muthamil and Pandian, 2016) was used in biofilm assay for augmenting hyphal formation.

The marine bacterial isolate from Gulf of Mannar, *B. subtilis* (Genbank Accession No. KC433737) was maintained in Zobell Marine Agar (ZMA, Hi-Media, India) and cultured in Zobell Marine Broth (ZMB, Hi-Media, India). The culture was incubated in shaker incubator (160 rpm) with optimal temperature of 28 °C.

2.2. Bacterial extract preparation

Bacterial extract preparation and purification was carried out as suggested by Thenmozhi et al. (2009) with minor modifications. The cell free culture supernatant (CFCS) of *B. subtilis* grown in ZMB at 28 °C for 48 h was collected by centrifugation (15,294 × *g* for 20 min at 4 °C). Membrane filter (0.2 µm pore size) was used to filter the CFCS. The filtered CFCS was extracted twice using equal volume of ethyl acetate and subsequently dried at room temperature under reduced pressure in rotary vacuum drier (Christ RVC 2-18, Germany) to obtain crude extract. The crude ethyl acetate (EA) extract was weighed and re-dissolved in methanol for carrying out bioassays.

2.3. Evaluation of antibiofilm activity of EA extract

The antibiofilm activity of crude extract was assessed against *C. albicans* in 24-well microtitre plate (MTP) (Subramenium et al., 2015a). Briefly, increasing concentrations of crude extract (50–200 µg/ml) was added individually to wells containing 1 ml of spider broth. One percent inoculum of overnight *C. albicans* culture was added to the wells and incubated at 37 °C for 24 h in static condition. Wells containing spider broth alone and wells containing methanol (vehicle) were considered as blank and control respectively. After incubation, the planktonic cells were discarded and the wells were washed twice with sterile distilled water to remove loosely bound cells and allowed to air dry. Dried wells were then stained with 0.4% crystal violet for 10 min to quantify biofilm formation. The unbound excess stain was removed by washing the wells twice with sterile distilled water and allowed to dry. The cell bound stain was extracted using 1 ml of 10% glacial acetic acid and its absorbance was measured at $OD_{570\ nm}$. The amount of cell bound crystal violet in a well is directly proportional to the biofilm formation. The least concentration of extract which exhibited more than 80% of biofilm inhibition was considered as minimum biofilm inhibitory concentration (MBIC) (Kwasny and Opperman, 2010; Haque et al., 2016; Muthamil and Pandian, 2016). Percentage of biofilm inhibition was calculated using the following formula:

$$\% \text{ of biofilm inhibition} = [(\text{Control } OD_{570\ nm} - \text{Treated } OD_{570\ nm})/\text{Control } OD_{570\ nm}] \times 100$$

2.4. Effect of EA extract on growth of C. albicans

The antifungal effect of crude extract against *C. albicans* was evaluated using microbroth dilution method in 24-well MTP as per the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2006). Wells containing 1 ml of YEPD broth was supplemented with 1% of overnight *C. albicans* culture and varying concentrations of crude extract (50–200 µg/ml). Wells holding 1 ml of plain YEPD broth served as blank. The plate was incubated at 37 °C for 24 h. After incubation, absorbance of the plate was measured at $OD_{600\ nm}$.

2.5. Partial purification and GC–MS analysis of active fraction

For partial purification and GC–MS analysis of crude EA extract, a protocol described by Viszwapriya et al. (2016) was followed with slight modifications. The partial purification of crude EA extract was aided by pre-coated thin layer chromatography (TLC) plate using chloroform: ethyl acetate: methanol (1:1:0.1) solvent system. Distinct bands were scraped and extracted with methanol concisely after derivatization of TLC plate with iodine vapors. The extracted bands were evaluated for antibiofilm activity. Further, GC–MS analysis of active fraction was accomplished using AccuTOF Gcv equipment. The compound separation was performed in hp1 capillary column of 30 m length and 0.25 µm diameter. Temperature range was set between 100–280 °C with 5 °C gradient per min. Helium was employed as carrier gas, which was passed at the rate of 1 ml per min through the column. The run was performed in EI + ionization mode. The obtained chromatogram was compared with NIST library to identify the compounds on the basis of spectral match.

2.6. Effect of 5HM2F on biofilm and growth of C. albicans

5-(Hydroxymethyl)-2-Furancarboxaldehyde (5HM2F; Catalogue no. H40807-1G, Sigma Aldrich, Switzerland) was prepared as 20 mg/ml