Yeast Biofilms in the Context of Human Health and Disease

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Abstract Microbial biofilms play important roles in ecology, industry and most importantly in the human health. Extensive research is being done to study their involvement in chronic infectious diseases. Yeasts, members of the Kingdom fungi, are no exception and flourish as biofilms in their natural habitats. Yeasts either exist as a part of human microbiota or reside in close proximity environment and may turn pathogenic to cause superficial or systemic infections. The majority of these infections involve growth in biofilm form. Particularly, a large population of immunocompromised individuals and patients using prosthetic devices are susceptible to biofilm related infections. *Candida*, *Cryptococcus* and *Histoplasma* are the major yeast species responsible for high morbidity and mortality associated with mycoses. Interestingly, these yeasts colonize host tissues or medical devices to form biofilms which are highly resistant to antifungal drugs. Also, biofilms may act as a reservoir for recurrent infections and consequently complicate the antifungal therapy. Efforts are being done to characterize biofilms as an important virulence factor in fungi. This review, with a special emphasis on *Candida albicans*, discusses biofilm formation and associated drug resistance. Also, the involvement of yeast biofilms in human diseases and the therapeutic strategies are briefly reviewed.

Keywords Antifungal · Biofilm · *Candida* · Drug resistance · Infection · Prostheses · Virulence · Yeast

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1 Introduction

Approximately 1.2 billion people are estimated to suffer from fungal diseases worldwide (Denning and Bromley 2015). Most of these (around 1 billion) are cutaneous/superficial infections involving the skin, nail, hair and oral or urinogenital mucosa. Although cured by antifungal therapy, superficial infections have a substantial effect on the quality of life (FIT 2012). Remaining are the systemic/invasive infections, which are hard to diagnose, difficult to treat, and hence a threat to the patients. Up to two million people die of severe fungal infections every year. Various species of yeast and filamentous fungi are known to be human pathogens, and more are being reported to be associated with mycoses. However, species belonging to the genera *Aspergillus*, *Candida*, *Cryptococcus, Pneumocystis*, and *Histoplasma* are the major fungal killers (Brown et al. 2012a). It would be interesting to note that three of the major culprits are yeasts. Few of them are dimorphic in nature and can switch between yeast and hyphal morphologies.

Over the last 30 years, incidences of fungal infections have greatly increased parallel with advances in medical technology and an increase in the population of immunocompromised patients. Prolonged lives of old age people, increase in the number of ICU patients, prolonged stay in ICU, HIV/AIDS-infected, organ transplant, cancer, and neonatal patients, people undergoing surgeries; all this coincided with a rise in the use of broad-spectrum antibiotics, prolonged chemotherapy, immunosuppression therapy, indwelling catheters and medical devices, culminating into rising of opportunistic fungal pathogens (Diekema et al. 2012; Pfaller et al. 2012). More importantly, there has been an increase in the fungal infections related to the biofilm growth form (Mathe and Van Dijck 2013).

Biofilm is a community of microorganisms characterized by cells which are irreversibly attached to a surface and embedded in a matrix of extracellular polymeric substances. Cells in a biofilm (sessile cells) exhibit altered phenotype compared to the free-living (planktonic) cells, due to surface induced gene expression (Donlan and Costerton 2002). Living as a community is a survival strategy which provides several ecological benefits to microorganisms; like, escape from host immune defense, protection from environmental stress, better acquisition of nutrients, metabolic cooperation, and persistence in unfavorable niches (West et al. 2007). Hence, the majority of the microorganisms, in their natural habitats, live as aggregated communities attached to a surface (Donlan and Costerton 2002). Unfortunately, human pathogens adapt this strategy to colonize host tissues or indwelling prostheses, survive the attack of the defensive immune system, resist the antimicrobial agents/antibiotics and flourish to cause infections (Costerton et al. 1999). It is believed that in humans, 80% of all microbial infections are biofilm-related. Efforts are being done to study the involvement of biofilms in chronic infectious diseases, medical device associated infections and the drug resistance associated with them (Harriott et al. 2010; Fox and Nobile 2012).

This is also true for yeasts, which can colonize human host leading to recalcitrant infections. Yeast infections on moist surfaces, mucosal tissues and prosthetic

devices inside the body involve biofilm formation (Cuellar-Cruz et al. 2012). For example, oropharyngeal candidiasis, vaginitis, and native valve endocarditis infections are associated with *Candida* biofilms. Similarly, *Candida* biofilms are the third leading cause of catheter-related fungemia (Ganguly and Mitchell 2011; Desai et al. 2014). Overall, biofilm formation is a crucial step in yeast infections (d'Enfert 2009). Most of the knowledge on the fungal biofilms has been obtained from studies on yeasts. *Candida albicans* is capable of formation of highly structured biofilms and has emerged as a model system to study pathogenic biofilms. Various in vitro and in vivo studies on *C. albicans* have contributed significant information on biofilm formation and associated characteristics (Shinde et al. 2012b; Nett and Andes 2015). In this chapter, we discuss biofilm formation in yeasts, particularly pathogenic yeasts and its consequences on the human health. Biofilm-related drug resistance, underlying mechanisms and various strategies to overcome biofilm associated infections are also discussed, with a special emphasis on *C. albicans* biofilm.

2 Yeasts as Human Pathogens

Unicellular fungi, yeasts as they are commonly known, belong to the kingdom fungi which occupy a diverse range of environments with an estimated 1.5 million species (Hawksworth 2001). Only a fraction of these are yeasts, but they exhibit recognizable effects on the human life. Selective yeasts are applicable in human welfare. A well-known example is commercial use of different strains of budding yeast, *Saccharomyces cerevisiae*. It is used to ferment sugars in the production of alcoholic beverages and also routinely applied in bakery industry and food industry. *S. cerevisiae* is often taken as a vitamin supplement because it is a rich source of B vitamins, niacin, and folic acid. It is also popular as a model organism in cell and molecular biology and hence indirectly contributes to advances in human medicine (d'Enfert 2009; Alexandre 2013). However, few of the yeasts are human pathogens and exert substantial effects on human health.

Recent reports on host-fungal interactions have revealed that fungi are an integral part of the human microbiome, and must be playing an important role in defining commensal microbial communities (Huffnagle and Noverr 2013). The interactions among commensal yeasts or bacteria and yeast and their consequences to the host are being explored actively (Klotz et al. 2007; Underhill and Iliev 2014). For example, the interplay between *C. albicans* and bacterial pathogens such as *Pseudomonas aeruginosa*, *Acinetobacter baumanii*, and *Salmonella enteric* in the gastrointestinal tract has been demonstrated either in vitro or in invertebrate models of infection (Davis-Hanna et al. 2008). Few bacterial pathogens may limit the infectivity of *C. albicans* through the secretion of small molecules such as homoserine lactones; while, secretion of quorum sensing molecules like farnesol by *Candida* may influence bacterial colonization (Hogan 2006).

Table 1 Most significant fungal infections and their estimated worldwide burden

Infection	Causative fungal pathogen	Predominant morphology (yeast/filamentous)	Estimated infections worldwide/year	Associated mortality (%)
Cryptococcosis	*Cryptococcus neoformans*; *C. gattii*	Yeast	>10,00,000	20–70
Candidiasis	*Candida albicans* and non-albicans *Candida* species (NACS)	Yeast	>4,00,000	46–75
Pneumocystis	*Pneumocystis jirovecii*	–	>4,00,000	20–80
Aspergillosis	*Aspergillus fumigatus*	Filamentous	>2,00,000	30–95
Histoplasmosis	*Histoplasma capsulatum*	Yeast	25,000	28–50

Even though very few of the fungi are pathogenic to insects, amphibians, plants, animals and humans; they have a pronounced effect on the global biota (Fisher et al. 2012; Gundacker and Baddley 2015). Fungi are supposed to cause billions of infections every year and estimated to kill around two million people Worldwide (Table 1) (Brown et al. 2012a). Unfortunately, their influence on the human health is still under-recognized. For example, the fungal infections have not been mentioned by World Health Organization in their program. Almost everybody experience superficial fungal infection at least once in a lifetime, the majority of which are cured easily in healthy individuals. However, millions of immunocompromised individuals contract life-threatening invasive infections which are much harder to be cured. In many cases, the rate of mortality often exceeds as high as 50%, with total deaths exceeding that of associated with TB and malaria (Brown et al. 2012a).

About 300 fungal species are well recognized to be associated with human diseases and infections, only a few of these are yeasts and only a minor of the latter are human pathogens; but, exhibit the substantial effect on the human health and disease. Approximately 90% of the deaths related to fungal infections involve species belonging to five genera i.e. *Aspergillus*, *Candida*, *Cryptococcus, Histoplasma* and *Pneumocystis* (Table 1) (Brown et al. 2012b). Three of this i.e. *Candida* spp., *Cryptococcus* spp., and *Histoplasma* spp. are yeasts or dimorphic fungi predominantly existing in yeast morphological form.

Yeasts can be found in different natural habitats such as plants, soil, water, animals and importantly humans. Two important yeasts, *Cryptococcus neoformans* and *Histoplasma caspulatum*, are naturally found in the soil and other environmental niches. They frequently get access to the human host and may reside inside the body for a long time without causing any harm. However, being opportunistic, they take advantage of a weak immune system and proliferate in the human host to cause infections (d'Enfert 2009). Few members such as *C. albicans* and non-albicans *Candida* species (NACS) are commensal and grow on the skin surfaces, mucous membranes, oropharyngeal, urinogenital and gastrointestinal tracts as normal microbiota; but, may turn pathogenic to invade tissues and proliferate to cause disease in immunocompromised patients.

The opportunistic behavior of pathogenic yeasts like *Candida* and *Cryptococcus* is responsible for a sharp rise in the infections to a population of immunocompromised individuals such as HIV-infected people, cancer patients, diabetics, patients under long term antibiotic treatment or chemotherapy, people undergoing organ transplantation or heart surgeries, hospitalized patients using catheters, bone implants and other prostheses (Raut and Karuppayil 2016; Polvi et al. 2015). It has been estimated that in the United States the cost burden of fungal infections, the majority of which are due to yeasts, may be as high as $2.6 billion per year (Wilson et al. 2002).

3 Biofilm Formation in Yeasts

Biofilm formation represents an important intrinsic property of most of the microorganisms. Fungi are no exception to this and are capable of biofilm formation in vitro and in vivo. Fungal species, particularly those which are involved in human disease are being studied in detail for their biofilm forming abilities (Table 2) (Desai et al. 2014). Most common fungal pathogens belong to phyla Ascomycota or Basidiomycota. Major pathogenic yeasts belong to the phylum Ascomycota (including species from the genera *Candida* and *Histoplasma*) and the Basidiomycota (include the genera *Cryptococcus* and *Trichosporon*). Biofilm formation and biofilm mediated pathogenesis have been studied in only a few of these pathogens (Desai et al. 2014; Fox et al. 2015).

3.1 Candida

Candida species are the most common fungal pathogens responsible for the superficial and life-threatening systemic infections. Candidiasis is prevalent in immunocompromised patients, people undergoing chemotherapy, invasive clinical procedures, major trauma and prolonged stay in intensive care units. Advanced medical procedures such as the use of catheters, neonatal intensive care, gut surgeries, or organ transplantation are predisposing factors to disseminated *Candida* infections (Calderone and Clancy 2012). *Candida* species are the fourth most common cause of nosocomial (hospital-acquired) bloodstream infections and the third major reason for catheter-related infections (Pfaller and Diekema 2007). The estimated annual global incidence of *Candida* bloodstream infections is approximately 400,000 cases, per year, with very high mortality rates of 30–40% (Brown et al. 2012a). Mucosal *Candida* infections of the oral and genital tracts are very common. For example, 50–75% of women suffer from at least one episode of vulvovaginal candidiasis and 5–8% (75 million) experience at least four episodes annually (Sobel 2007). Also, there are at least 10 million cases of oral thrush in HIV/AIDS patients, cancer patients and other immunocompromised patients (Pfaller and Diekema 2007). More than 20 species of *Candida* have been found to be involved in human disease. However, *C. albicans* predominates and is the

Table 2 Biofilm formation by important yeast pathogens and involvement in human infections

Pathogenic yeast	Biofilm formation		Biofilm associated infections	Drug resistance	References
	In vitro	In vivo			
Candida albicans; non-albicans *Candida* species (NACS) like, *glabrata; parapsilosis; dubliniensis; krusei; tropicalis*	Yes	Yes	Superficial and systemic candidiasis; mucosal infections; invasive tissue infections; candidemia; colonization of catheters, endotracheal tubes, cardiac devices, implants, voice prostheses, joint prostheses and bone implants	Yes	Chandra et al. (2001a), Donlan and Costerton (2002), Kojic and Darouiche (2004), Kaur et al. (2005), Kumar and Menon (2006), Ramage et al. (2006), Al-Fattani and Douglas (2006), Shinde et al. (2012b) and Raut et al. (2013b)
Cryptococcus neoformans; C. gattii	Yes	Yes	Meningoencephalitis and pulmonary infections; device related infections such as cardiac valves, peritoneal dialysis equipments, ventriculoatrial shunt	Yes	Braun et al. (1994), Banerjee et al. (1997), Martinez and Casadevall (2007) and Robertson et al. (2012)
Histoplasma capsulatum	Yes	Yes	Histoplasmosis; human respiratory system (lung) infections; device associated infections	Yes	Pitangui et al. (2012), Pierce et al. (2013) and Brilhante et al. (2015)
Trichosporon asahii	Yes	Yes	Mainly device associated infections of dialysis grafts, breast implants	Yes	Reddy et al. (2002), Krzossok et al. (2004) and Bonaventura et al. (2006)

most common species associated with human infections. Medically important species other than C. albicaus i.e. NACS include *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, *Candida dubliniensis*, *Candida krusei*, *Candida rugosa* and *Candida lusitaniae* (Calderone and Clancy 2012).

Candida can cause a wide spectrum of clinical manifestations and attack nearly every organ in the human body. It is the most common yeast species isolated from blood and mucosal surfaces. How it remains at mucosal surfaces in the presence of adaptive immunity is still not known. It has been speculated that ability to produce

immunomodulatory compounds (such as oxylipins) and to adhere various surfaces leading to biofilm formation helps *Candida* to persist various niches for prolonged periods (Huffnagle and Noverr 2013). In general, *Candida* species can colonize various mucosal surfaces at oral and nasal cavities, gastrointestinal and urogenital tract and develop a community structure (Samaranayake et al. 2009; Sardi et al. 2013). *Candida* readily adheres to prosthetic devices implanted in a patient and form biofilms leading to a device associated infections (Kojic and Darouiche 2004). Formation of biofilm is being thoroughly investigated in *C. albicans* and various in vitro and in vivo studies have contributed to our understanding of biofilm mode of growth (Nett and Andes 2015). Obviously, it has been considered as a model to study the formation of fungal biofilms and various manifestations associated with it.

Candida albicans biofilm is not just an aggregation of cells; but, is a heterogeneous structure developed by cells interacting as a community (Nickerson et al. 2006). The behavior of individual cells in a biofilm is regulated by diffusible molecules. A signal transduction process which involves the production, release and response to signalling molecules secreted by the microbial cells themselves, is called quorum sensing. The diffusible molecules involved in quorum sensing are called autoinducers or quorum sensing molecules (QSMs) (Hornby et al. 2001). QSMs accumulate in the medium as a microbial population grows and may convey the physiological changes to individual cells in response to the density of population. *C. albicans* biofilm development is also regulated through quorum sensing; however, the molecular mechanisms behind it are not fully understood.

Farnesol, a QSM in *C. albicans*, is a sesquiterpene continuously produced during growth of *C. albicans* cells. Extracellular farnesol accumulates in the culture to exert various physiological effects on individual cells as well as community growth. For example, at a cell density of $>10^6$ cells/ml, it reaches to a threshold concentration and prevents yeast to hyphal morphogenesis. Exogenously added farnesol (2–250 μM) prevents morphogenesis induced by various inducers (Mosel et al. 2005; Rathod et al. 2013). Tyrosol is another QSM which acts opposite to farnesol and is known to enhance germ tube formation during growth. At low cell densities, it reduces lag phase in the growth and promotes the formation of hyphae (Chen et al. 2004). In addition, few more molecules like nerolidol, isoamyl alcohol, dodecanol, ethanol and acetaldehyde were detected in *Candida* cultures and known to act as morphogenetic signalling molecules to inhibit filamentation (Chauhan et al. 2011a, b).

Formation of biofilm by *C. albicans* takes place through three distinct stages such as early, intermediate and maturation phases (Chandra et al. 2001a). Early phase extends over 0–6 h approximately. It consists of adhesion of blastospores/yeast form cells to a surface (0–2 h), the formation of micro-colonies and dimorphic transition to give rise hyphal forms (3–6 h). Intermediate phase (6–18 h) is characterized by cellular growth, an increase in cell density, the formation of multiple layers of cells, and elongation of filaments to form a mesh-like network of yeast, hyphae and pseudohyphae. In the last maturation phase (18–48 h), multiple layers of cells start depositing extracellular polymeric matrix (EPM). A dense network of filamentous and yeast cells embedded in EPM gives a

three-dimensional, heterogeneous community structure. At the end of the maturation phase controlled dispersion of planktonic cells from community takes place (Raut 2014).

Adhesion of blastospores (yeast cells) to a solid surface is of prime importance in biofilm formation. During initial attachment which is reversible, nonspecific interactions like *van der waals* forces and electrostatic forces between cells and abiotic surface are involved (Klotz 1990). Cell surface hydrophobicity and hydrophobic interactions play important role in this stage (Panagoda et al. 2001; Raut et al. 2010). In later stages, anchoring of the cells takes place by means of specific cell surface molecules called adhesins (Verstrepen and Klis 2006). Binding through adhesins is irreversible i.e. if there are no strong physical/chemical forces acting, the cells cannot be removed easily. Proteins and mannoproteins present in *Candida* cell wall are involved in binding to host tissue surface as well as abiotic surfaces. Ability to bind to abiotic surfaces is important in device-related infections. The peptide portion of cell surface mannoproteins, particularly the exposed hydrophobic domains may be involved in binding to plastic materials (Chaffin et al. 1998). The Agglutinin like sequence genes (*ALS*) are known to code for adhesins in *C. albicans*. Out of the eight different proteins encoded by *ALS* gene family, Als3p protein shows stronger adhesive properties and is involved in adhesion to plastic. *BCR1* gene is found to act as a transcription factor and regulate expression of Als3p surface protein (Nobile and Mitchell 2006). Increased expression of drug efflux pumps in response to contact and adhesion of *C. albicans* to the surface plays an important role in antifungal resistance in biofilms (Kumamoto and Vinces 2005).

Formation of hyphae is another important event in *C. albicans* biofilm development and maturation. The presence of multiple layers of filamentous growth is important in typical *Candida* biofilm structure. Mutants of *C. albicans* that are unable to form hyphae were observed to form only a basal layer of biofilm (Nickerson et al. 2006). Biofilms formed in vitro and in vivo were detected to secrete farnesol and tyrosol, which suggests that *C. albicans* biofilms are regulated by various QSMs. Tyrosol is shown to enhance filamentation in early and intermediate biofilms, while farnesol is found dominant in maturation phases to overcome tyrosol activity and inhibit mycelial growth (Chen et al. 2004).

Specific genes are expressed during biofilm development and maturation (Garcia-Sanchez et al. 2004). Northern blot analysis of sessile and planktonic cells of *C. albicans* showed differential gene expression. Particularly, *ALS* gene family and genes belonging to drug efflux pump proteins, *CDR1 and CDR2* are found to be over-expressed (Chandra et al. 2001b). Analysis of 1850 different genes showed that 325 genes are differentially expressed in biofilm phenotype compared to that of planktonic. Two hundred fourteen of 325 genes were shown to be over expressed (Garcia-Sanchez et al. 2004). These genes were from various functional categories of metabolism, cell cycle, DNA processing, protein synthesis, cell signalling and transport. Among all, 34 genes involved in protein synthesis were up-regulated significantly. Genes for synthesis of aromatic amino acid and sulfur amino acids were overexpressed indicating their importance in the biofilm mode of growth. Expression of a set of genes for lipid synthesis like ergosterol, sphingolipids and

phospholipids was increased. Genes that control cell wall synthesis and organization and genes involved in adhesion were up-regulated significantly. Also, the hyphal regulatory genes were found to be differentially regulated indicating the importance of filamentous forms in normal biofilm development (Garcia-Sanchez et al. 2004).

Biofilm-associated infections of *C. albicans* range from superficial oral thrush, denture stomatitis, ophthalmic infections, and wound/burn infections, to severe candidaemia and colonization of internal tissues and organs (Ramage et al. 2006). Many non-albicans *Candida* species are also involved in clinical biofilm-related infections. For example, *C. glabrata*, *C. dubliniensis*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei*, have been observed to cause biofilm-associated infections (Ramage et al. 2006; Silva et al. 2011). Interestingly, few studies have reported that *C. albicans* biofilm production is significantly less frequent than non-albicans *Candida* spp. (Ramage et al. 2014). *C. dubiliensis* is capable of formation of a complex biofilm structure, consisting of blastospores, pseudohyphae, and hyphae, as seen in typical *C. albicans* biofilm (Ramage et al. 2001). Same is the case with *C. tropicalis*, which is able to form heterogeneous biofilms consisting of hyphae and filamentous forms, enclosed in a matrix layer (Bizerra et al. 2008). However, the EPM content shows the presence of hexosamine, small amounts of protein, phosphorous, and more uronic acid than that in *C. albicans* biofilms (Al-Fattani and Douglas 2006). *C. glabrata* readily form biofilms on biotic as well as abiotic surfaces in the host body, although in vitro biofilms show reduced thickness. Another characteristic feature of *C. glabrata* biofilm is that there is no morphogenetic switching of cells to give rise filamentous growth. Instead, cells adhered to a surface form layered clusters of blastospores. This community is covered by the EPM which exhibit comparatively higher concentration of carbohydrate and protein than biofilm formed by other NACS (Silva et al. 2009; Kucharíkova et al. 2011).

Similarly, *C. parapsilosis* also develop into a biofilm devoid of hyphae formation, hence the three-dimensional community possesses only layers of clustered yeast form cells. EPM of these biofilms is prominently rich in carbohydrates, while protein content is comparatively less (Silva et al. 2009). Strain-dependent variation in biofilm formation has been observed in *C. parapsilosis* isolates (Lattif et al. 2010; Silva et al. 2011). Although these two NACS do not form true hyphae, their biofilms may show the presence of elongated yeast cells resembling pseudohyphae (Ramage et al. 2014). *C. parapsilosis* and *C. glabrata* are responsible for 13 and 24% of *Candida* bloodstream infections, respectively. *C. parapsilosis* is most commonly infects neonates, transplant patients, and patients receiving parenteral nutrition. *C. glabrata* is known to form biofilms on voice prostheses. Such infections are clinically important as they hamper normal work of the device, restrict airflow and impede normal activities like speech, swallowing and respiration (Fanning and Mitchell 2012).

The gastrointestinal tract of 30–80% of healthy individuals is colonized by *Candida* and at many instances may enhance the inflammation (Kumamoto 2011). Colonization of percutaneous endoscopy gastronomy tubes by *C. albicans* and *C. tropicalis* may contribute to the degradation of the polyurethane to cause

diarrhoea and sepsis (Trevisani et al. 2005). Colonization of mucosal layers of urinary/vaginal tract leading to vulvovaginal candidiasis is common. *Candida* biofilms on urethral stents are associated with pyelonephritis, cystitis and prostatitis (Sobel 2011). It has been reported that pathogenic fungal species play a role in wound infections and in combat trauma cases. Moreover, molecular analysis of chronic wound infections, including ulcers, non-healing surgical wounds and venous leg ulcers, showed that *Candida* spp. were the most abundant fungal pathogens (Branski et al. 2009; Paolino et al. 2012; Ramage et al. 2014).

3.2 *Saccharomyces*

Baker's yeast, *S. cerevisiae*, is known to form biofilms on solid surfaces and is being developed as an in vitro model system for biofilms. These biofilms are characterized by a thin matrix of budding yeast cells and elongated pseudohyphal cells (Reynolds and Fink 2001). *S. cerevisiae* can undergo a transition from budding yeast form to a filamentous multicellular community (Bastidas and Heitman 2009). Haploid cells show invasive growth to form biofilms on semisolid agar medium upon carbon starvation (Reynolds and Fink 2001); while diploid cells form pseudohyphae in response to nitrogen starvation (Gimeno et al. 1992). The filamentous transition is regulated by cyclic adenosine monophosphate (cAMP)—protein kinase A (PKA) pathway and a mitogen-activated protein kinase (MAPK) pathway (Ryan et al. 2012). Downstream of these pathways is *FLO11* gene which encodes a cell-surface protein involved in haploid invasive growth, biofilm formation, and diploid pseudohyphal growth (Guo et al. 2000). The expression of *FLO11* is controlled by numerous transcriptional regulators which are being investigated.

Biofilm formation in *S. cerevisiae* takes place through specific steps in which cell-to-cell interactions and cell-to-surface interaction occur simultaneously to result in adhesion and colonization of cells (Bojsen et al. 2012). It has the ability to adhere biotic and abiotic surfaces such as polystyrene, silicone, polypropylene, and polyvinylchloride. Besides its role in colonization of semisolid and solid agar, Flo11p directly plays a significant role in the adhesion to solid surfaces and is responsible for hydrophobic properties of the cell wall (Reynolds and Fink 2001). The same protein (Flo11p) also contributes to the yeast biofilm formation at the air-liquid interphase. For example, 'flor' observed in some alcoholic beverages is nothing but *S. cerevisiae* biofilm on air-liquid interface. It is useful in the aerobic growth of yeast and synthesis of specific metabolites in the production of sherry wines (Vallejo et al. 2013). Generally, *S. cerevisiae* is not observed to be involved in human infections; however, on rare occasions, it is reported as a member in mixed-species biofilm infections on catheters in ICU patients. Hence, it is speculated that *S. cerevisiae* is capable of biofilm formation in vivo and may be associated with infections in severely immunocompromised patients (Fox et al. 2015).

3.3 *Cryptococcus*

Cryptococcus species rank high among the prominent fungal pathogens of the humans. Cryptococcosis is mainly caused by *C. neoformans* and a closely related species *C. gattii*. These species which mainly resides in soil and avian habitats has a worldwide distribution and are frequently involved in meningoencephalitis and severe pulmonary infections (Gullo et al. 2013). Ability to form biofilms may play an important role in the survival of *C. neoformans*, in its environmental niche (Pierce et al. 2013). *Cryptococcus* biofilms have a well organized structure consisting of yeast cells. Layers of yeast cells are surrounded by the matrix material which mainly contains glucuronoxylomannan and galactoxylomannan and various sugars such as xylose, mannose, and glucose (Martinez and Casadevall 2007). Exposure to *Cryptococcus* mainly occurs by inhalation of airborne organisms into the lungs. It can cause local as well as systemic infections and mainly invades the central nervous system to cause meningoencephalitis. Both the species can form biofilms which is a threat not only to immunocompromised but also immunocompetent individuals (Alvarez et al. 2008; Fox et al. 2015).

Quantitative or qualitative defects in cellular immune functions, particularly in CD4+ lymphocytes due to AIDS, immunosuppressive medications, and solid organ transplantation are the major risk factors for cryptococcal infection; while biofilm formation on host tissues further complicates it (Park et al. 2009). Similarly, *C. neoformans* is reported to form biofilms on prostheses such as ventricular shunts, cardiac valves and peritoneal dialysis equipments to cause device related infections (Ramage et al. 2009a). The biofilm growth of *Cryptococcus* is very well tolerant to the attack of immune cells and various antifungal drugs. The estimated yearly global burden of Cryptococcal meningitis is around 1 million cases, with more than 620,000 deaths in sub-Saharan Africa. Mortality rates associated with *Cryptococcus* infections in AIDS patients are estimated to be 15–20% in the United States and 55–70% in Latin America and sub-Saharan Africa, despite the availability of the treatment (Brown et al. 2012a).

3.4 *Histoplasma*

Histoplasma capsulatum, the causative agent of histoplasmosis is an opportunist which infects the human respiratory system, primarily in immunocompromised patients. *H. capsulatum* var. capsulatum is a dimorphic fungus and exists as a filamentous form in the environment and predominates as a yeast-form in vivo (McKinsey and McKinsey 2011). Pitangui et al. (2012) have reported a dense community of yeast-form cells in vitro and suggested that same architecture may be prevalent in vivo, depicting biofilm formation abilities of *Histoplasma* (Pitangui et al. 2012). Such a biofilm growth may be responsible for clinical infections of

Histoplasma and exhibit resistance to antifungal drugs (Pierce et al. 2013; Brilhante et al. 2015).

Conidia (spores) of *Histoplasma* when inhaled, germinate in the lungs to give budding yeast form which has infective abilities. Cells ingested by pulmonary macrophages can survive and multiply within phagolysosomes to turn pathogenic under favorable conditions (Nucci and Marr 2005). Histoplasmosis may range from localized tissue infection to a lethal disseminated infection. Dissemination of the cells to various tissues results in damage to multiple organs and proves fatal to severely immunocompromised individuals (Kauffman 2007; McKinsey and McKinsey 2011).

3.5 Trichosporon

Infections caused by other yeasts, such as *Trichosporon* species, are also on the rise (Kontoyiannis et al. 2004). *Trichosporon asahii* is an emerging fungal pathogen and majorly infects patients with suppressed immune status. For example, disseminated *Trichosporon* infections are mainly observed in organ transplant patients (Ramage et al. 2009a). Trichosporonosis have been observed associated with implanted medical devices and is supposed to colonize there as biofilm growth forms. Biofilms formed are typical complex structures consisting of yeast and hyphal cells. This network is embedded in protective EPM (Bonaventura et al. 2006). *Trichosporon* biofilms are mainly found associated with dialysis graft and breast implants (Reddy et al. 2002; Krzossok et al. 2004).

4 Biofilm as a Virulence Factor in Pathogenic Yeasts

Cellular aggregation and surface colonization by fungi, particularly yeasts, was reported as early as in 1938 (Vallejo et al. 2013). The intrinsic ability of microorganisms to group and form communities is widely distributed in nature. It is supposed to play crucial roles in reproduction, colonization, pathogenesis, and survival under environmental stress (Costerton et al. 1999). Primary colonization of yeasts in the human host is through the acquisition of maternal flora in the perinatal period and later human contact, like in the case of *C. albicans*; or it is through interaction/exposure with surrounding environment; for example, *Cryptococcus* infection (Alvarez et al. 2008). Once a fungal cell reaches the mucosal surface or blood stream, it colonizes a tissue to survive there either as a commensal or a pathogen. The commensal association doesn't cause any damage to the host unless the immune status or the microbiota of the host is disturbed (Casadevall and Pirofski 2007).

Relatively little is known about the molecular requirements for commensalism of yeasts, as no reliable animal models are available which mimics in vivo conditions of the human host (Miceli et al. 2011). Similarly, details about mutualistic/beneficial fungal colonization and its relationship with the human host are not known. Only one

example is of *Saccharomyces cerevisiae var. boulardii*, which is considered a beneficial fungus. It is well-described as a probiotic for the relief of gastroenteritis (Dinleyici et al. 2012). However, few reports have described that it can grow on indwelling catheters and form biofilms to cause fungemia. This may happen when the catheters are contaminated through accidental aerosolization of probiotic preparation intended to be given to the patients (Cassone et al. 2003).

Usually, yeasts follow strategies like, persistence in macrophages, commensalism with other microorganisms or colonization and formation of the community known as 'biofilms'; which allow them to survive and flourish in the host (d'Enfert 2009). Also, yeasts can readily adhere to abiotic surfaces of indwelling medical devices. Colonization of mucosal layers, tissues and prostheses, may result in subsequent biofilm formation; which leads to either asymptomatic persistence of the pathogen or extensive association and overgrowth culminating in an infection (Casadevall and Pirofski 2007). Yeast infections associated with biofilm growth have been observed in oral soft tissues, teeth, skin, wounds, the middle ear, the gastrointestinal tract, the urogenital tract, airway/lung tissue, heart valves, the eyes, dental implants, urinary tract prostheses, the peritoneal membrane and peritoneal dialysis catheters, indwelling catheters for hemodialysis and for chronic administration of chemotherapeutic agents, cardiac implants such as pacemakers, prosthetic heart valves, ventricular assist devices, and synthetic vascular grafts and stents, internal fixation devices, and percutaneous sutures, and tracheal and ventilator tubing, penile implants, hip and joint prostheses (Kojic and Darouiche 2004; Desai et al. 2014).

Yeast biofilms on human skin have been linked to the development of many dermatologic conditions or diseases (Kong and Segre 2012; Nusbaum et al. 2012). For example, *Candida* is reported to be involved in the development of atopic dermatitis (AD) (Zhang et al. 2011). Particularly, *C. albicans*, *Cryptococcus diffluans*, and *Cryptococcus liquifaciens* are the yeast species which have been found to colonize skin of AD patients (Sonesson et al. 2013). Quantification of microbial flora has revealed that fungi contribute to >50% of the microbial burden at the majority of wounds. *Candida* biofilms have been associated with the delayed healing of chronic wounds (Leake et al. 2009). Although microbial communities in the oral cavity are dominated by bacteria, considerable fungal organisms are also detected which may have significant effects on oral microbiota and overall health. *Candida* and *Cryptococcus* are the yeasts most frequently colonizing the oral cavity; and the species majoraly present are *C. albicans*, *C. parapsilosis*, *C. tropicalis*, and *C. neoformans* (Ghannoum et al. 2010).

The lungs harbor a low level of microflora and little is known about the fungal burden of the lungs. However, the presence of yeast like fungi, *Pneumocystis* spp. is most frequently observed, which proliferates to cause pneumonia in immunocompromised patients (Huffnagle and Noverr 2013). Similarly, limited information is available on the fungal communities of the gastrointestinal tract. It harbors low pH tolerant yeasts such as *Candida* species. *C. albicans* have been isolated from the sites of gastric ulceration in addition to *Helicobacter* and *Lactobacillus* bacteria. It is now being realized that the fungal/yeast biofilms may

play a decisive role in overall health, especially in patients where normal bacterial biota is disturbed. Various prostheses, notably different type of catheters, are readily colonized by yeasts leading to biofilm formation (Kojic and Darouiche 2004). Strikingly, yeasts (mainly *C. albicans*) are the third leading cause of catheter-related infections (Crump and Collignon 2000). *C. neoformans* frequently form biofilms on ventricular shunts, cardiac valves and peritoneal dialysis equipment (Ramage et al. 2009b). The presence of indwelling prostheses is considered as a risk factor for the development of *C. glabrata* infections. It readily forms biofilm on venous catheters, prosthetic joints and peritoneal dialysis systems. Also, *C. parapsilosis* is found to colonize indwelling catheters in neonates, prosthetic knees in old age people, hip joint and breast implants (Ramage et al. 2014). Other biofilm forming yeasts involved in device-related clinical infections include species of *Histoplasma, Cryptococcus and Trichosporon* (Table 2) (Fanning and Mitchell 2012; Pierce et al. 2013).

Biofilm-related infections are difficult to treat and hard to eradicate, hence considered as a clinical threat. Besides the reasons like poor diagnosis, lack of effective therapy, and the emergence of resistant strains, biofilm formation is also the main reason behind high mortality and morbidity related to fungal infections. Biofilm formed by pathogenic yeasts display elevated resistance to most of the antifungal drugs available for the treatment and hampers the normal treatment procedures. It has been reported that biofilms formed by members of genus *Candida*, *Cryptococcus*, *Histoplasma,* and *Trichosporon* show reduced susceptibility to various antifungal agents compared to their planktonic growth (Pettit et al. 2010; Ramage et al. 2012; Zhang et al. 2012; Brilhante et al. 2015). Moreover, this community structure can very well withstand host immune defense (Fanning and Mitchell 2012). Further, severity increases as biofilms may act as reservoirs to keep releasing the cells which cause repeated infections when antibiotic therapy is discontinued or immune system is compromised.

Colonization of prostheses like catheters, heart valves, pacemakers and another bio-medical-assist devices in patients can compromise the normal function of the device or even may lead to its failure (Srinivasan et al. 2014). It was found that involvement of biofilm forming strain in nosocomial infections increased the risk of death as compared to non-biofilm forming isolates. It has been revealed that *Candida* clinical isolates which are able to form biofilms, have significantly more contribution to hospital mortality, costs of antifungal therapy, and increased the length of hospital stay of the patients (Ramage et al. 2014). Overall, biofilm formation is an important virulence factor in yeasts; and hence need to be studied thoroughly for its role in human health and disease.

5 Drug Resistance and the Mechanisms Involved

5.1 Drug Resistance

Antifungal drugs available for the treatment of candidiasis are mainly confined to four classes of molecules i.e. polyenes, 5-fluoro-cytosine (5-FC), azoles and echinocandins (Nosanchuk 2006). Nucleoside analogue, 5-flucytosine (5-FC) is converted to

5-fluorouracil (5-FU) when enters a fungal cell. 5-FU mimics a pyrimidine analogue to interfere with nucleic acid synthesis and inhibit cell cycle. Although 5-FC was found promising against *Candida* in the initial period, its use was limited by the high prevalence of resistance in *C. albicans* isolates (White et al. 1998). Polyenes represented by two heterocyclic molecules, amphotericin B and nystatin, are amphipathic in nature. Polyenes get intercalated into lipid bilayers, bind to membrane sterols and aggregate, which ultimately causes the formation of pores and leakage of cellular ions resulting in cell death. Polyenes also cause oxidative damage to the *Candida* cells. Although resistance to polyenes is not very common, it may be evident in few mutant populations (*ERG3* mutants) or growth forms like late biofilms where cells have decreased ergosterol content. The main limiting factor for polyenes is the severe toxicity associated with them (Xie et al. 2014).

The azole antifungals include some of the most widely prescribed drugs like fluconazole, active against *Candida* and other yeasts. Initially derived imidazoles, for example, miconazole and ketoconazole, have been replaced with less toxic and more efficient triazoles like fluconazole, itraconazole, and voriconazole. Azole drugs mainly interfere in ergosterol biosynthetic pathway. They inhibit the cytochrome P_{450} enzyme, 14α-lanosterol demethylase encoded by the *ERG11* gene. Depletion of membrane ergosterol affects membrane fluidity and integrity to cause loss of membrane function. Intervention in sterol synthesis may result in the synthesis of alternate toxic sterols resulting in inhibition of *C. albicans* growth (Cannon et al. 2009). Yeasts like *C. krusei* and *Cryptococcus* are resistant to azoles, while the emergence of drug resistance in susceptible yeasts (e.g. *C. albicans*) has been reported from all over the world (Rathod et al. 2012; Ghannoum and Rice 1999; Mishra et al. 2007). Echinocandins such as caspofungin, micafungin and anidulafungin, inhibit the enzyme 1,3-β-glucan synthetase resulting in a reduction of 1,3-β-glucan in the cell wall. Although recent, clinical resistance to echinocandin has been reported; a point mutation in 1,3-β-glucan synthase subunit was found responsible for echinocandin resistance in *C. albicans* (Xie et al. 2014).

A characteristic feature of yeast biofilms is its resistance to most of the available antifungal drugs including the widely prescribed azoles. It has been reported that biofilms formed by members of genus *Candida*, *Cryptococcus*, *Histoplasma* and *Trichosporon* show reduced susceptibility to various antifungal agents compared to their planktonic growth (Bonaventura et al. 2006; Pettit et al. 2010; Pitangui et al. 2012; Ramage et al. 2012). Susceptibility studies have revealed that biofilms formed by *C. albicans* may be up to 2,000 times more resistant to antifungal drugs than that of planktonic cells (Baillie and Douglas 2000; Shinde et al. 2012b). Also, biofilms of NACS show enhanced drug resistance to antifungal (Ramage et al. 2012; Desai et al. 2014; Fox et al. 2015). Resistance to antifungal drugs increases with the development of biofilm structure making mature biofilms totally non-responsive to the drug therapy (Shinde et al. 2012b), thus administration of very high doses of antifungals for a prolonged time is usually required to treat such infections. However, side effects due to toxicity put limitations on the effective use of antifungal drugs against biofilms.

5.2 Mechanisms of Drug Resistance

Based on *C. albicans* biofilm studies, various reasons have been proposed to be responsible for drug resistance associated with yeast biofilms. No single reason could fully explain the antifungal resistance exhibited by biofilms, hence considered as a multifactorial phenomenon. General mechanisms supposed to be responsible include, sequestration of drugs by extracellular polymeric matrix (EPM), enhanced drug efflux, high cell density, changes in metabolic state, the presence of persister cells and activation of the stress-responsive pathway (Mathe and Van Dijck 2013; Taff et al. 2013).

Formation of EPM is an important characteristic of biofilm formation. Individual cells remain embedded in this matrix which is composed of carbohydrates, proteins, and nucleic acids, often secreted by the biofilm cells (Al-Fattani and Douglas 2006; Martins et al. 2012). It was found that reduced drug diffusion may not be a problem in *Candida* biofilms and drugs like fluconazole could diffuse through normally. Instead, specific components of the matrix must be contributing to the resistance (Al-Fattani and Douglas 2004). Interestingly, treatment of biofilms with DNAse was found to enhance the sensitivity of *C. albicans* biofilm to the activity of caspofungin and amphotericin B. Hence, extracellular DNA in association with other components must be providing structural integrity and strength to EPM and contributing to drug resistance (Martins et al. 2012; Rajendran et al. 2013). Similarly, both, biofilm cells and biofilm matrix contain higher levels of β-1,3-glucans in their cell wall, compared to planktonic cells. The glucan was observed to bind four- to five-fold more drug than that of planktonic and contribute sequestering of antifungal azoles and polyenes (Nett et al. 2007; Mitchell et al. 2013). Disruption of β-1,3-glucans by glucanase treatment resulted in increased drug susceptibility of biofilms. Further evidence comes from an observation where low expression of glucan synthase gene was found to enhance the antibiofilm efficacy of amphotericin B, anidulafungin, and flucytosine (Nett et al. 2010). Overall, glucan-mediated binding/sequestering of drugs is an important resistance mechanism in biofilm growth form.

Up-regulation of drug efflux protein after exposure to antifungal drugs is a well-known mechanism of resistance in planktonic cells (Xie et al. 2014). ATP-binding cassette (ABC) transporter superfamily (e.g. *CDR1* and *CDR2*) and the major facilitator (MF) class (e.g. *MDR1*) are two main types of efflux pump proteins in *C. albicans* (Akins 2005; Cowen et al. 2014). Overexpression of these transporter proteins was observed in both, in vitro and in vivo biofilms, even in the absence of drug. Hence, upregulation of efflux pumps seems to be a normal mechanism associated with biofilm development (Ramage et al. 2002). Adhesion of *C. albicans* to a solid surface is sufficient to activate expression of the genes encoding the efflux pumps (Mateus et al. 2004). The efflux of drugs entering the cells remains active in mature biofilms too and continue to be a cause of biofilm-related drug resistance (Nobile et al. 2012).

Biofilm is an aggregated community of the cells attached to a surface, and cell density in that microenvironment is high. In the microplate based in vitro biofilm model for susceptibility testing, cell density ranges between 10^6 and 10^8 cells/ml. If the biofilm community is dispersed the cells with lower density exhibit increased sensitivity. Even, in the planktonic cell assays, it has been observed that increasing cell concentration results in reduced susceptibility to the drugs, fluconazole, ketoconazole, caspofungin and amphotericin B (Perumal et al. 2007; Mathe and Van Dijck 2013). Moreover, there is density-dependent secretion of quorum sensing molecules in biofilms; for example farnesol in *C. albicans* (Hornby et al. 2001). The presence of molecules like farnesol influences the overall gene expression of individual cells and may contribute to lower drug susceptibility (Cao et al. 2005; Garcia-Sanchez et al. 2004).

Reduced rate of metabolic activity of bacterial cells could contribute to the low drug sensitivity. In the bacterial biofilms, there is a limitation of nutrients so the cells may exhibit lower growth rates resulting in resistance to drugs which are effective against actively growing (like planktonic) cells (Martinez and Rojo 2011). But this may not be true for fungi; for example, biofilms were found equally resistant to amphotericin B, over a range of growth rates. Similarly, limitation to important nutrients like glucose or elements like iron did not cause changes in *Candida* biofilm susceptibility to amphotericin B (Baillie and Douglas 1998a, b). However, the role of altered metabolism in fungal resistance is not well investigated. Persister cells are a subset of cells which are phenotypically dormant and highly tolerant to the antimicrobial drugs. Bacterial biofilms harbor a notable (1%) percentage of persister cells which contribute to overall antibiotic resistance (Lewis 2010).

Persister cells have been observed in *C. albicans* biofilms too, and are highly resistant to antifungal agents (Khot et al. 2006). These are supposed to be phenotypic variants of the wild type exclusively present in biofilms and which gives rise to subpopulations of cells to form a new biofilm. Persisters act as a reservoir to initiate a new biofilm cycle and their drug tolerant nature is an important reason for the failure of antifungal treatment in clinical settings (LaFleur et al. 2006). Furthermore, *C. albicans* persister cells are exclusively recovered from biofilms and not from planktonic populations, regardless of their growth phase, and require attachment to a substrate to initiate the dormant phenotype. Biofilms of *C. krusei* and *C. parapsilosis* have been observed to harbor persisters and may be contributing to tolerance to drugs like amphotericin B (Al-Dhaheri and Douglas 2008). The molecular mechanisms underlying the drug refractory characteristic of fungal persisters is not investigated in detail.

Adhesion to a surface after initial contact is first important step in biofilm formation. The reversible attachment to a substrate results in activation of various signalling pathways. For example, the protein kinase C (PKC) pathway is an important pathway activated in response to cell wall stress (Kumamoto and Vinces 2005). Activation of such a stress-responsive pathway in fungal cells turns them drug tolerant. Mkc1 is the terminal mitogen-activated protein (MAP) kinase in PKC cascade. Deletion of *MKC1* gene has been shown to form abnormal *C. albicans* biofilms (Kumamoto and Vinces 2005). Interestingly, such a biofilm was found several times more sensitive to the antifungal activity of azoles.

Activation of a heat shock protein Hsp90 also contributes to azole and echinocandin resistance. This is through calcineurin pathway for stress responses (Cowen 2009; Singh et al. 2009). Inhibition of the protein phosphatase i.e. calcineurin or intervention of Hsp90 results in sensitization of *C. albicans* biofilm to various antifungal drugs (Uppuluri et al. 2008; Shinde et al. 2012a). Overall, drug resistance exhibited by yeast biofilms is governed by a complex network of multiple factors.

6 Therapeutic Strategies

6.1 Therapeutics

Current therapeutics against yeast biofilm includes the use of antifungal drugs to achieve inhibition of sessile cells and eradicate biofilm mass from the surface of biomaterials (Ramage et al. 2013). However, prevention of biofilm appears to be the best strategy because the drug resistance comes into picture once biofilms are developed and complicate the treatment. In fact, once biofilm is formed, removal of a colonized device (mainly catheters) is a strategy applied whenever suitable and helps to reduce mortality in device-associated infections (Andes et al. 2012; Cornely et al. 2012). Removal of the medical device may not be always possible, as the surgical procedures involve risk and increased costs. In such scenario, use of antifungal agents is necessary. Antifungal lock therapy (ALT) is one of the initial options for the treatment of catheter-related infections (Walraven and Lee 2013). Usually, polyenes and echinocandins are applied; for example, amphotericin B and its liposomal form are two agents commonly used for ALT purpose (Cornely et al. 2012). Similarly, caspofungin has been used to deal with catheter-related *Candida* biofilms (Ozdemir et al. 2011).

Various in vitro studies have indicated the efficacy of polyenes and echinocandins against *C. albicans* biofilm; hence, it would be useful to treat the biofilm infections in vivo (Kuhn et al. 2002). Although ALT using caspofungin and micafungin have shown high efficacy, it fails to completely eradicate the biofilm growth (Cateau et al. 2011). Liposomal AMB exhibited better antibiofilm activity than echinocandins (Ramage et al. 2013). Animal catheter models studies suggested that azoles are ineffective against biofilm growth, while liposomal AMB significantly reduced *C. albicans* biofilm infection. Similarly, AMB deoxycholate and caspofungin have been observed to achieve 80–100% removal of *C. albicans* colonization from catheters in rabbit models (Shuford et al. 2006). Infections associated with medical devices other than catheters, for example, prosthetic heart valves, knee implants or pacemakers, are hard to deal with; because removal of such a device is not easy and it involves a risk. For example, *Candida* related infective endocarditis is difficult to treat and involves mortality rates around 50%. Such infections can be treated with liposomal amphotericin B or caspofungin (Ellis et al. 2001; Falcone et al. 2009).

The combination of polyenes and azoles has been found useful in the inhibition of wound-related biofilms. *Candida* biofilm infections at wounds and joints can be efficiently treated with a combination of liposomal AMB and voriconazole or posaconazole. Combinatorial therapy is also applied to treat oral fungal biofilms like denture-related stomatitis and oral candidiasis (Rautemaa and Ramage 2011). Despite the available options of biofilm therapy, treatment of biofilm infections remains a challenge. In the majority of cases, complete removal of colonized growth is not achieved and may result in recurrent infections (Ramage et al. 2014; Fox et al. 2015). Hence, there is need to find alternative therapeutic options for the treatment of yeast biofilms.

6.2 *Future Strategies*

Development of an antifungal agent is difficult as fungi are eukaryotic organisms and share many similarities with the human host (Routh et al. 2011). Hence, to find a cellular mechanism that can be specifically targeted in the fungal cells and use it from the drug discovery point of view is relatively complicated. This becomes a more difficult task when the infections are biofilm-associated and exhibit increased resistance to antifungal agents. Various approaches are being followed to increase the antifungal arsenal.

Rational drug designing is one of the approaches which target a specific protein or biochemical pathway (Srinivasan et al. 2014). For example, identification of the mechanisms behind biofilm dispersal may help for developing a compound that dismantles biofilm community. Similarly, a better understanding of the proteins involved in the transformation of a sessile cell into a persister during biofilm formation would allow devising strategies to reverse their physiology. The combination of such a strategy with available antifungals would successfully remove biofilms, kill the released planktonic cells and prevent recurrence of infections (Fox et al. 2015). A diverse range of genes involved in adherence, morphogenesis, quorum sensing, matrix production, cell wall biosynthesis, and metabolism have been found to play important roles in biofilm formation and regulation (Garcia-Sanchez et al. 2004; Nobile et al. 2012; Desai et al. 2014). Various proteins are differentially expressed between biofilms and planktonic cells. Many of these proteins may be enzymes resulting in a different metabolic state of biofilms. This may be used to target a metabolic pathway important for biofilm growth and can be used as drug targets (Fox et al. 2015).

A systems biology study to target important protein involved in biofilm formation is another approach. For example, Nobile et al. (2012) have identified transcription factors regulating biofilm growth of *C. albicans* (Nobile et al. 2012). The study identified six main regulators of transcription, Bcr1, Tec1, Efg1, Ndt80, Rob1 and Brg1. They are involved in controlling the expression of at least 1000 target genes. Deletion of *ALS1*, *HWP1*, and *CAN2* genes has been found to result in the defective biofilm. It has been proposed that Als1 and Hwp1 which are cell

surface proteins involved in adhesion, Can2 and Tpo4 (probably play a role in transport), and Eht1 protein involved in fatty acid synthesis and morphogenesis may be explored as antibiofilm targets (Fox and Nobile 2012; Nobile et al. 2012). A study has identified transcription regulators Bcr1, Ace2, Snf5, and Arg81 important for adhesion to silicone and subsequent biofilm formation (Finkel et al. 2012). Zap1 is another important regulator of extracellular matrix production and also govern the synthesis of β-1,3-glucan and other matrix constituents (Nobile et al. 2009).

However, rational drug designing is time-consuming and involves a lot of money. Many researchers are following an empirical approach for antifungal discovery through screening of synthetic/semi-synthetic chemicals (Srinivasan et al. 2014). Plant extracts, essential oils and their constituent molecules exhibit novel antimicrobial and antifungal properties (Raut et al. 2013a; Raut 2014; Raut and Karuppayil 2014a). Most importantly, phytochemicals have been found to possess inhibitory potential against drug-resistant biofilms of bacterial and fungal pathogens (Raut and Karuppayil 2014b). Efforts are being done to identify molecules with antibiofilm potential through random screening of small molecules of natural origin including phytochemicals. It includes a search for plant actives which can prevent biofilm development as well as those which disrupt mature biofilms (Raut et al. 2012, 2013b, 2014; Raut and Karuppayil 2016).

Phytochemicals or other synthetic molecules can be used in combination with existing drugs so that to potentiate the activity of available antifungal agents. The combinatorial approach may be useful to mitigate the drug-resistance associated with biofilm communities. Drug efflux inhibitors or cell sensitizer molecules may be used to overcome the problem of biofilm mediated resistance (Shinde et al. 2013; Doke et al. 2014). Other miscellaneous approaches include combination of biofilm disruptive agents with drugs so that EPM surrounding the biofilm is disturbed. For example, combinatorial therapy of AMB and CSP with DNase significantly disrupted EPM and sensitized *C. albicans* biofilm to antifungal drugs (Martins et al. 2012).

Use of broad-spectrum antimicrobial metal ions like silver or nanoparticles of silver is another interesting way. It can be used for coating a catheter surface or medical device to prevent adhesion and biofilm formation by yeasts. Silver interferes with DNA replication, denatures proteins, and inhibit oxidative enzymes (Rai et al. 2009). Its combination with antifungal drugs can be very effective. Silver nanoparticles have been shown to inhibit *C. albicans* and *C. glabrata* biofilms at various stages of development. These have been utilised in hydrogels used to treat chronic wounds and also in denture prostheses (Monteiro et al. 2011, 2012). Molecules that interfere with the quorum sensing involved in biofilm formation and regulation are also an attractive alternative for biofilm mitigation (Nickerson et al. 2006; Kalia 2013). Screening of clinical and preclinical non-antifungal drugs, drug compound libraries, and repurposing of them against fungal biofilms is a recent approach being investigated (Routh et al. 2011; Shinde et al. 2013; Pierce and Lopez-Ribot 2013).

7 Conclusions

Biofilms of pathogenic yeasts are increasingly being recognized for their involvement in the human health and disease. Yeast biofilms on tissue surfaces and/or indwelling prostheses and drug-resistant infections associated with these have emerged as a serious threat to a large population of immunocompromised individuals. The available arsenal of antifungal agents is not sufficient to successfully mitigate biofilms; hence, there is an urgent need to search for novel therapeutic agents. Further understanding of the mechanisms involved in biofilm formation and regulation may provide clues to the development of antibiofilm strategies for the prevention and treatment of yeast infections.

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