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REVIEW

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Unlocking the potential of fungal extracts as inhibitors of biofilm formation and improving human health

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The emerging threat of antibiotic resistance and the formation of resilient biofilms pose a challenge to contemporary healthcare systems. This review dives into the interplay between antibiotic resistance mechanisms and biofilm production. Many pathogenic bacteria have an inherent ability of adhering tightly to a surface forming a complex matrix of extracellular polymeric substances (EPS) surrounding their cells. This is called a biofilm which allows pathogenic bacteria to survive in unsuitable environment. The adaptive nature of biofilms provides a protective shield against conventional antimicrobial agents, promoting chronic infections and complicating medical interventions. This phenomenon further adds to the ever-increasing problem of antibiotic resistance. Thus, there is an immediate necessity in developing novel strategies to deal with bacterial biofilms. In terms of human health, biofilms can be formed on mucosal surfaces and on surfaces of medical equipment. They are also a major reason for causing 'biofouling'. Different approaches have been undertaken to counteract the menace of biofilms encompassing physical, chemical as well as biological methods. However, recent studies have shown that natural bioactive compounds found in fungal extracts, which has already been gaining attention due to their various properties like immunomodulatory activity, anti-tumor activity, antimicrobial activity etc., have the ability to prevent the formation as well as viability of biofilms through numerous mechanisms. This article thus explores the nuances of biofilm formation and its effects, and further delves deep into the convincing potential of the different components in fungal extracts against bacterial biofilms.

Key words: Bacterial biofilms, Antibiotic resistance, Fungal extracts, Fungal metabolites, Antibacterial activity, Quorum sensing, Metabolic inhibition, Enzymatic degradation, Eco-friendly

Antibiotic resistance poses a significant challenge to global public health, rendering the once-effective antibiotics ineffective and compromising our ability to treat bacterial infections. This phenomenon has escalated into a critical worldwide threat and has been characterised by the World Health Organisation (WHO) as one of the most pressing issues in modern medicine, emphasizing the urgent need for concerted international action (World Health Organization: WHO, 2023).

Antibiotic resistance is a serious problem as a result of the inappropriate and excessive use of antibiotics in both human and animal healthcare. The inherent ability of bacteria to adapt and evolve makes this problem worse (Spellberg *et al.*, 2008; Ventola, 2015). When the first antibiotics, such as penicillin, were discovered in the early 20th century, antibiotic resistance essentially started (Fleming, 1929). Antibiotics were formerly believed to be amazing drugs that could revolutionize healthcare and save many lives. However, overuse and abuse of antibiotics swiftly led to the emergence of resistant strains, which ushered in a new era of medicine (Davies and Davies, 2010). The severity of the antibiotic resistance crisis is highlighted statistically. The World Health Organisation (WHO) estimates that antibiotic resistance causes 700,000 deaths globally each year, and if the current trend continues, this number is expected to increase to 10 million deaths annually, by the year 2050 (World Health Organization: WHO, 2023). Furthermore, compared to susceptible strains, antibiotic-resistant infections are linked to greater fatality rates, lengthier hospital stays, and higher healthcare expenses. Moreover, the problem of antibiotic resistance is further complicated by the formation of bacterial biofilms which is an alternate survival strategy. Within biofilms, bacteria exhibit increased tolerance to antibiotics, often needing concentration that are multiple orders of magnitude higher than those effective against the free-floating bacteria (Langsrud, Sidhu, Heir, & Holck, 2003; Simões & Vieira, 2009; Simões, Simões, Machado, Pereira, & Vieira, 2006).

Biofilm-forming bacteria are a major problem in healthcare settings because they can colonise medical devices and tissues and cause chronic infections that are resistant to host immune responses and medications (Muhammad *et al.*, 2020). Famous instances include osteomyelitis, otitis media, periodontitis, infective endocarditis, infections linked to cystic fibrosis, and chronic wounds (Muhammad *et al.*, 2020). Bacteria with a reputation for producing biofilms, such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Pseudomonas aeruginosa*, are the main culprits behind such infections (Muhammad *et al.*, 2020). Alternative antibacterial agents, such as natural products and synthetic compounds, are being investigated in light of the increasing rate of antibiotic resistance and the sluggish pace of antibiotic development (Mishra *et al.*, 2020). With a variety of modes of action, including enhanced membrane permeabilization, rupture of extracellular matrix, and suppression of quorum sensing, fungal extracts have shown great promise as anti-biofilm agents (Mishra *et al.*, 2020). These extracts have the potential to prevent implant-associated bacterial infections and fight biofilm-associated infections in conjunction with synthetic compounds (Roy *et al.*, 2018; Mishra *et al.*, 2020). Combination treatments that incorporate several anti-biofilm substances from several suppliers may increase effectiveness and lower the possibility of resistance (Chung &Toh, 2014; Jiang *et al.*, 2020). Fungal extracts, including nisin, endolysins and polysaccharides, have been shown in recent research to be beneficial in reducing the formation of biofilm on biomedical surfaces (Mishra *et al.*, 2020; Choi *et al.*, 2022). All aspects considered, fungal extracts offer new therapeutic approaches to treat illnesses linked to bacterial biofilms, which presents a promising way to address the problems caused by these biofilms in medical settings.

BACTERIAL BIOFILMS

Single-cell organisms can undergo transition to a transient multicellular lifestyle by forming biofilms, where the expression of “group behaviour” helps their survival in harsh environment. Biofilm formation is a diverse and dynamic developmental procedure. Multiple regulatory networks mediate the shift from planktonic growth to biofilm in response to environmental changes. These

networks translate signals into co-ordinated changes in gene expression, which in turn mediate the temporal and spatial reorganization of a bacterial cell (Pratt and Kolter 1998; O'Toole *et al.* 2000; Prigent-Combaret *et al.* 2001; Parsek and Singh 2003; Lenz *et al.* 2008; Monds and O'Toole 2009). By changing the expression of surface molecules, virulence factors and nutrient utilization, this cellular reprogramming gives bacteria an arsenal of traits that allow them to survive under adverse conditions (Lenz *et al.* 2008;Zhang and Mah 2008). Bacteria are cocoons in a self-produced extracellular matrix made of extracellular polymeric substances (EPS) that serve as a stabilising scaffold for the three-dimensional biofilm structure along with components like pili, flagella and other adhering fibres. While hydrophilic polysaccharides help retain water through hydrogen bond interactions, the matrix of biofilms effectively retains nutrients for metabolic utilisation by resident bacteria. In response to nutrition availability, bacteria release enzymes that change the composition of EPS, which shapes the architecture of biofilms (Sauer *et al.*, 2004; Ma *et al.*, 2009). This leads to a well-hydrated, strong and highly elastic framework that promotes close communication between cells, DNA exchange and defence against external stimuli. The "division of labour" within communities is fueled by environmental gradients inside biofilms, which cause variable gene expression in response to local nutrition and oxygen availability.

Though they share genetic similarities with the rest of the bacterial population, recent investigations have shown the presence of nondividing, metabolically inactive persister cells inside biofilms that display tolerance to a variety of antibiotics (Lewis 2005, 2008). In clinical settings, these persister cells are thought to be essential for the reseeding of biofilms after antibiotic therapy is stopped (Lewis 2005, 2008). The biofilm matrix protects biofilm bacteria from innate immune defence like opsonization and phagocytosis as well as antibiotic treatments within the host environment. Furthermore, interactions between bacteria inside biofilms might promote the spread of additional virulence factors and drug-resistance indicators (Vuong *et al.* 2004). As a result, pathogens that form biofilms remain in the host, resulting in the development of persistent and resistant infections. Examples of these infections include urinary tract infections (UTIs) caused by uropathogenic *Escherichia coli* (UPEC) and *Klebsiella pneumoniae* (Foxman 2010), periodontitis linked to mixed biofilms of *Streptococcus mutans* and other bacteria, infections brought about by catheters and other devices that are linked to pathogens like *E. coli* and *Enterococcus faecalis*. Particularly in immunocompromised patients, infections caused by opportunistic biofilm-forming pathogens can have devastating consequences, often resulting in severe symptoms and, in many cases, fatal outcomes.

BACTERIAL BIOFILM FORMATION

On any surface, biofilm growth primarily occurs in three steps. Cells attach to a surface in the first stage, assemble to create microcolonies, and then differentiate into a mature structure called a biofilm. Following full development, biofilms disintegrate via active and mechanical ways (Boles & Horswill, 2008). In particular, sedimentation, Brownian motion and hydrodynamic forces govern the deposition of bacteria, while Lifshitz-Van der Waals, hydrophobic, acid-base, and electrostatic interaction forces govern the adhesion to the substratum (Van Oss *et al.*, 1986). The formation of biofilm is aided by a number of surface-associated proteins, including OmpA, fibronectin-binding proteins, protein A, SasG, biofilm-associated protein (BAP) and numerous other elements, especially during the early attachment stages (Fuqua *et al.*, 1994). Certain species have the ability to adhere to surfaces, but they can also attach themselves directly to the earlier colonies or to the matrix. This colonisation is mediated by small signalling molecules working with cell-cell communication mechanisms. Most people refer to this phenomenon as "quorum sensing" (Fuqua *et al.*, 1994). One important quorum-sensing-controlled trait is biofilm development. The extracellular matrix, a complex and highly polar collection of biomolecules comprising proteins, polysaccharides, nucleic acids, and lipids, surrounds the bacterial cells in biofilms (Overhage *et al.*, 2008). The matrix offers defence against a range of stressors, including exposure to antibiotics and immune cell attacks. Nevertheless, the matrix of the biofilm does not serve as the mechanical barrier against antimicrobial

agents. This was corroborated by a study that demonstrated that ampicillin could penetrate biofilm formed by a *Klebsiella pneumoniae* strain deficient in β-lactamase, but ampicillin was unable to do so in biofilm formed by a wild type strain of the bacteria that possessed β-lactamase. This suggests that in the latter scenario, ampicillin was quickly broken down by β-lactamase prior to infiltrating the wild type biofilm. The second stage of biofilm growth begins when the bacteria begin to secrete EPS. This is an irreversible process. Up to the third stage of development, EPS is continuously secreted, guaranteeing that bacteria can safely adhere to the surface inside of a densely packed biomolecular layer. The fully developed biofilm now has a three-dimensional, tower-like structure. These towers are made up of tiny channels that move waste, water and nutrients. The planktonic bacteria are housed in the tiny cavities of towers. Research findings indicate that there are significant variations in the organisation and architecture of biofilms among various bacteria. The precise cause of this mutation is still unknown. However, biofilm development in *P. putida* is controlled by the adhesive protein LapA (Gjermansen *et al.*, 2010), whereas *P. aeruginosa* and other pseudomonads are governed by the exopolysaccharides Pel and Psl in terms of biofilm formation. Therefore, differences in the extracellular matrix (ECM) component may be the cause of the variations in biofilm structure. Finally, the cavities holding bacteria that are not attached to the surface are emptied when these towers either erode (small sections) or are sloughed off (big parts) and become detached. The discharge of new germs into the environment comes next (Costerton *et al.*, 1987; Purevdorj-Gage *et al.*, 2005).

Recent research on a variety of bacterial species, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Pseudomonas fluorescens*, *Yersinia pestis*, *Escherichia coli*, *Vibrio cholerae*, *Burkholderia cenocepacia*, *Salmonella enterica*, *Clostridium difficile*, *Klebsiella pneumoniae*, *Vibrio cholerae* and *Bacillus subtilis* have shown that an increase in the intracellular secondary messenger c-di-GMP level signals the start of biofilm development and pathogenicity (Gjermansen *et al.*, 2010); c-di-GMP was initially identified as a unique secondary messenger in allosteric stimulation of cellulose synthase in *Gluconacetobacter xylinus*. 55 different c-di-GMP circuits use different forms of phosphodiesterases and diguanylate cyclases that are produced by bacteria (Massie *et al.*, 2012). In order to carry out its biological activity, c-di-GMP binds to a variety of receptors, such as transcription factors, enzymes, adaptor proteins and switches. Additionally, it has been documented that a number of environmental factors and transducer processes raise the quantity of c-di-GMP within the cell. This promotes the secretion of ECM and results in the formation of adhesins. *P. aeruginosa* produces ECM components like CdrA adhesin, alginate exo-polysaccharide, Pel, and Psl in response to positive regulation of their levels of c-di-GMP. Small regulatory RNAs (sRNA) also control the production of biofilm in a number of bacterial species in addition to c-di-GMP (Chambers & Sauer, 2013).

Planktonic aggregates are formed by some bacterial strains, although they rely on specific growth conditions. Previous research indicates that some *S. aureus* strains produce huge aggregates, with the formation process beginning early in the exponential development phase. When cell density is low, a cluster of roughly 20 cells forms an organised population. These larger structures, however, produce aggregates up to 1000 mm in diameter at higher densities. The mass aggregation is said to be caused by extracellular polysaccharide intracellular adhesin called Polymers of β 1-6 N-acetylglucosamine (PNAG) when the chemical structure was determined (Antonelli *et al.*, 2012), and spa encoding Protein A.

ANTIBIOFILM STRATEGIES

Antibiotics have long been the first choice for treating bacterial illnesses; however, due to their widespread usage, the host microbiota is negatively impacted, which increases the growth of opportunistic pathogens and antibiotic resistance (Dethlefsen *et al.*, 2008; Ubeda *et al.*, 2010). Even though they work well against general infections, they do not provide much protection against biofilm-related instances, particularly when implants or prosthetics are involved (Secinti *et al.*, 2011). Because of persister cells, matrix transport constraints, and

resistance transfer, biofilm bacteria present a special difficulty (Lewis, 2005; Costerton *et al.*, 2007). New therapeutic strategies are required because this resistance can be up to 1000 times greater than that of planktonic cells (Hoiby *et al.*, 2010).

FREQUENTLY ENCOUNTERED ANTIBIOFILM STRATEGIES

- Phage Therapy: Because phages are self-replicating and selective, they present a promising alternative to traditional antibiotics (Donlan, 2009). Because they can encode enzymes that break down EPS and specifically target stationary-phase bacteria within the biofilm, lytic phages are an excellent means of targeting biofilm bacteria (Burrowes *et al.*, 2011).
- Silver Nanoparticles: Using silver nanoparticles to impregnate medical equipment has become popular as a means of preventing biofilm formation (Fey, 2010). Silver ions have a strong antibacterial effect by rupturing bacterial membranes and blocking essential biological functions (Kim *et al.*, 2007). The use of nanotechnology has brought silver back into vogue because of its great biocidal efficacy against biofilms in nanoparticle form (Morones *et al.*, 2005).
- Antimicrobial Peptides: Peptides with antibiofilm potential include cathelicidins and lytic peptides. According to Pompilio *et al.* (2011), cathelicidins show quick bactericidal action against strains that are resistant to multiple drugs, indicating possible therapeutic use. Lytic peptides damage bacterial membranes, inhibiting the production of biofilms and efficiently penetrating already-formed ones.
- Antiadhesion agents: Mannosides, pilicides, and curlicides are antiadhesion agents. Mannosides, which impede type 1 pili assembly and UPEC adherence, are a result of efforts to stop bacterial adhesion. They have shown promise in stopping the formation of biofilms and upsetting preexisting ones (Cusumano *et al.*, 2011). Targeting type 1 pili and curli biogenesis, pilicides and curlicides efficiently prevent the formation of biofilms and improve bacterial clearance.
- Polysaccharides: According to Qin *et al.* (2009), some bacterial exopolysaccharides, such as Pel and Psl in *P. aeruginosa*, interfere with the ability of other species to form biofilms, indicating that they may have antibiofilm properties. These polysaccharides provide broad-spectrum biofilm suppression by changing the properties of bacterial cells, functioning as signalling molecules, or competitively inhibiting carbohydrate-protein interactions.
- Interference with signal transduction: It appears possible to interrupt the beginning of biofilms without causing bacterial resistance by focusing on bacterial signaling cascades, namely the QseBC two-component system (Clarke *et al.*, 2006). Inhibiting QseC phosphatase activity successfully decouples gene expression in pathogens carrying QseC, thereby decreasing pathogenicity.
- Enzymes and chelating agents as "Antimatrix" agents: Biofilm components are broken down by enzymes such as DNase I and dispersin B, which efficiently disperse the biofilms produced (Kostakioti *et al.*, 2013). Biofilm disruption is improved by including dispersin B into phages (Kostakioti *et al.*, 2013). Chelating drugs have been shown to be effective in treating and preventing diseases connected to biofilms, as they disrupt the structure of biofilms by interfering with metal cations.

These new tactics address the shortcomings of traditional antibiotic treatments and provide novel ways to fight biofilm-associated infections while causing the least amount of harm to the host microbiota. It is highly promising that more study and advancement in these fields may lead to better clinical outcomes for illnesses linked to biofilms.

INTRODUCTION TO FUNGAL EXTRACTS

Out of all the strategies available for dealing with biofilms, great potential lies in the exploitation of fungal extract in terms of both their efficacy as well as their relatively simpler accessibility. Before diving deeper into the topic, it is relevant to discuss more on the nuances of fungal extracts.

Encompassing about 144 thousand species, the kingdom of fungi consists of multicellular eukaryotes with their distinct feature of cell walls being made of chitin, one of the most resistant organic materials in the world. The kingdom fungi includes various organisms like molds, yeasts, rusts, smuts, mildews and mushrooms, and are very widely distributed on Earth (Ahmadjian *et al.*, 2023). The term 'fungus' is often associated by the common masses as something negative, be it molds spoiling the bread or the dermatophytes, resulting in dermatological problems. Fungi have always been of great environmental, industrial, scientific and medicinal importance. Humans have been able to harness its power from the age-old making of bread and wine to full-on fungal-derived pharmaceuticals and crop enhancement practices. The fungus can be a great source of nutrients in the form of mushrooms or single cell proteins (SCPs) like yeasts. They have also been used for their medicinal properties like antibiotics (penicillin being the most notable), enzyme production to be used in detergents, textiles and laboratories, as biopesticides as well as biofertilizers and also as very efficient eukaryotic hosts for expression of proteins. It can be said without any doubt that fungi have been always more of a boon than a curse to humans as well as the environment.

Now, for most of these uses, we have been able to utilise intact fungal cells. However, if one can identify the bioactive compounds and find a way to apply them either in the form of crude extracts or as purified compounds, it is possible to completely omit the caveats that usage of a living cell brings into the equation. There are concrete reasons for using fungus as our main source of bioactive compounds, those being:

- Profuseness – Fungi belong to an extremely biodiverse category of organisms which are further capable of producing tons of secondary metabolites, providing nearly endless resources to contribute to a huge library of bioactive compounds when compared to their prokaryotic counterparts.

- Ease of access – Fungal extracts make it possible to bypass the complex downstream processes required to isolate and purify specific products. This greatly reduces the cost as well as the time required to obtain the final product starting from the point of synthesis.

- Sustainability - Fungal extracts are also renewable (large-scale cultures possible) and eco-friendly, being biological in origin, thereby reducing the negative impact which residual chemicals might have on the environment.

PREPARATION OF FUNGAL EXTRACTS

First in the line of making a fungal extract is the identification of the desired bioactive compound. These bioactive compounds are most notably secondary metabolites in nature which function in times of stress and are in general compounds which are not directly associated with growth (Calvo *et al.*, 2002). However, we are not only interested in metabolites for the fulfilment of our purpose. The plethora of primary metabolites and enzymes produced by fungi are also of scientific importance (Orgaz *et al.*, 2006; Kües, 2015, Daley *et al.*, 2017). Secondary metabolites can be predominantly classified into four classes viz., polyketides, non-ribosomal peptides, terpenes and indole alkaloids (Keller *et al.*, 2005). The study of fungal metabolites has been in full force since the early 21st century and different approaches have been used for this purpose. The transition from genomics to more modern approaches like metabolomics has vastly improved the efficacy of the identification of novel compounds. The latter studies involve sample collection from desired places, most often from soil or other ecological niches but can include biological samples from blood or tissue as well. However, keeping our objective in mind, the fungus should ideally be isolated from areas with a higher tendency to be occupied by biofilm-forming pathogens. So, under those unfavorable conditions, fungal colonies that have been able to grow without the interference of the biofilms would have a high-likelihood of containing anti-biofilm compounds. The focus should thus ideally be to isolate samples from wastewater, contaminated pipelines, hospital effluents (highly enriched in antibiotic-resistant pathogenic bacteria) (Nath *et al.*, 2020) or from macro-fungi fruiting bodies, exposed to wet climate (Estrela and Abraham,

2016). A good source of these bioactive compounds is from endophytic fungi, which are a group of symbiotic fungi residing within the different tissues of plants. These fungi form intimate associations with their host plant and contribute to plant development as well as defence against pathogens and other environmental stresses.

This is followed by various extraction methods using appropriate hydrolytic enzymes like chitinase to digest the cell wall and further chemical or physical means of shearing cells. Sonication is one such strategy, very popular in recent times that does not seem to chemically alter or physically destroy our desired compounds which was tested for fungus in the late 90s (Smedsgaard, 1997). The extracts are prepared through extraction of the bioactive compounds via means of a suitable solvent like ethanol, methanol or water where they are dissolved, based on their polarity. Supercritical fluid extraction using carbon dioxide is a more recent method that allows for the extraction of heat-sensitive compounds (Mazzutti *et al.*, 2012). These series of steps however are not necessary for extracellular/secretory pigments or metabolites. Removal of biomass by simple biological filters will leave the bioactive compound in the spent broth which can be concentrated by evaporating the solvent or purified via centrifugation or chromatographic methods, as required. After extraction, we are left with the analysis of our sample. The most commonly used technique to separate the components of the sample is via gas chromatography (to separate volatile molecules) (Stoppacher *et al.*, 2010; Kadhim *et al.*, 2016) or liquid chromatography (LC) / high-performance liquid chromatography (HPLC) (Feussner and Feussner, 2019), followed by identification/screening via a mass spectrophotometer (referred to as GC-MS or LC-MS analysis). The data is analysed to gain insight into the potential target molecules through similarity search protocols for pre-existing molecules in the database or through novel findings of potential compounds with active pharmacophores. The latter can be done directly through different assays. For example, a DNA protection assay can be used to screen for components of the extract having antioxidative properties (Poorniammal *et al.*, 2018). This forms the basis for further research to test and validate the efficacy of the compounds, followed by further optimization to get our desired product with significant biological activity.

DOCUMENTED PROPERTIES OF FUNGAL EXTRACTS

As the research on fungal extracts and their application is a dynamic field, numerous instances have been found of its potential uses. A few noteworthy ones include:

MEDICINAL APPLICATIONS

i. Antioxidant Property – A very relevant role of fungal extracts comes in the form of their antioxidant properties. The metabolites work by scavenging for reactive oxygen and nitrogen species and donating hydrogen atoms or electrons to neutralize the free radicals, thereby suppressing their oxidative properties. Reactive oxygen species (ROS) are produced as a byproduct of metabolism and can cause severe damage to DNA if left unchecked including mutations and even DNA strand breaks. The main source of these metabolites mainly comes from macro-fungi belonging to basidiomycetes. Radical scavenging assays and DNA nicking assays have proved efficiency of fungal extracts from different species like *Cryptoporus volvatus, Gloeophyllum abietinum, Stereum hirsutum* etc. as viable sources of antioxidants (Lee *et al.*, 2013). DPPH is another such free radical which was subjected to different concentrations of fungal pigments extracted in methanol. This experiment was done with extracts from microfungi, and the pigments isolated from *Thermomyces* sp. and *Chaetomium* sp. showed sufficient DPPH scavenging activity. The results were further complimented by performing a series of other assays like the ferric reducing antioxidant power (FRAP) assay and DNA protection assays from chemical modifiers like $FeCl_2$ and H_2O_2 (Poorniammal *et al.*, 2018). This property can be utilized in cosmetic products to create antioxidant creams and moisturizers safe to apply on skin.

ii. Immunomodulatory Effects – Components of

fungal extracts like beta-glucans (components of the cell wall), triterpenoids (secondary metabolites), etc. have immunomodulation effects and after extensive research, it is now clear that they can have prominent effects on both innate and adaptive branches of immunity including both humoral and cell-mediated branches. The effects include induction of hematopoiesis for the 'production' of immune cells, 'maturation and differentiation' of T-cells into T¬H and T¬C cells as well as the 'activation' of lymphocytes, macrophages, natural killer cells NKCs, complement system and secretion of certain cytokines, all induced by fungal metabolites such as beta-glucans through an array of different mechanisms (Lull *et al.*, 2005; Meng *et al.*, 2013; Lu *et al.*, 2016; Cai *et al.*, 2019; Llauradó *et al.*, 2021). Fungal extracts have also been seen to exhibit immunosuppressive activity (Ujam *et al.*, 2021).

Secretions of pro-inflammatory molecules like interleukins, tumor necrosis factor, prostaglandins, etc. if uncontrolled, can cause severe cell damage and initiate acute and chronic inflammation. Fungal metabolites have been seen to downregulate certain genes in the human body responsible for inflammatory reactions. These genes involve *iNOS, COX-2, IL-1β, and TNF-α* which are downregulated following the suppression of the transcription factor NF-κB (Lull *et al.*, 2005). Methanol extract obtained from an edible mushroom *Pleurotus florida* was able to combat acute inflammation induced by carrageenan as well as chronic inflammation induced by formalin. The result obtained was also seen to be comparable to the marketed drug diclofenac. The same extract was also seen to inhibit aggregation in human platelets induced by adenosine diphosphate by as much as 88% to 95% at standardized concentrations and time periods (Jose *et al.*, 2004). Furthermore, some fungal extracts like that of *Fomitopsis pinicola* have also been shown to exhibit anti-inflammatory responses (Cheng *et al.*, 2008; Taofiq *et al.*, 2016). These observations can thereby shed light on the treatment of auto-immune disorders and inflammatory bowel diseases.

A chloroform extract of an endophytic fungus *Entrophospora infrequens* obtained from the plant *Nothapodytes foetida* contained a compound called captothecin (CPT) that was able to show a dose-related decrease in the synthesis of primary antibody while the methanolic extract at lower doses showed a high degree of antibody stimulatory activity (Pur *et al.*, 2007), proving that the nature of extract can also dictate the different bioactive components present in it and ultimately affect the physiological implications.

iii. Anticancer Property – Fungal metabolites have also been investigated for anti-tumour/anticancer activity in animal-tissue culture and mice models. One such observation has been the water and ethanol extracts of *Formitopsis pinicola* showing notable anti-cancer activity (growth inhibitory ratio 82.8%, $p < 0.001$), which was seen to decrease the tumor size and increase the life span of test mice with sarcoma tumors by inducing cell apoptosis (Wu *et al.*, 2014). Studies are being conducted in-vitro and in-vivo at a rapid pace to finally be able to progress on to human clinical trials.

NON-MEDICINAL APPLICATIONS

i. Industrial Use – Fungal extracts have been used for their plethora of pigments like melanin, carotenoids, flavins, indigo, etc. on an industrial scale in the manufacturing of different textiles, food preservatives and cosmetics (Sajid and Akbar, 2018). Secondary metabolites are used generally as preservatives for their anti-microbial nature and pigments for dyeing, while fungal enzymes like lipase, laccase, protease, chitinase and xylanase among others are commercially produced for juice production, stain removal, bio-bleaching, industrial tanning, biosensor manufacturing and majorly in paper and textile industries (Dhevagi *et al.*, 2021).

ii. Agricultural Use – Endophytic fungi living in close association with plants, produce certain secondary metabolites which benefit the plant in certain ways. It can stimulate the growth of the plant or might contain specific components to stimulate the plant defence or even work directly as anti-microbial agents. While the utilization of intact cells till now has been the primary choice to be used as plant growth modulators, extracts of fungi have shown promise as biocontrol agents. A case can be seen in the hydroalcoholic extract of a mushroom *Lactarius deliciosus* (L.) S.F. Gray, which is

able to function as a fungicide against the phytopathogen *Monilinia fructicola* at concentrations of 1.25 mg/mL.

iii. Bioremediation – Fungal enzymes like laccases present abundantly in white-rot fungi can function as oxidizing agents by abstracting electrons from a variety of substrates, a lot of which are well-known environmental pollutants. They can act on phenolic as well as non-phenolic recalcitrant industrial wastes and oxidize them to non-harmful products (Viswanath *et al.*, 2014). This again, is not an explored field as most of the researches have been done using intact cells or through synthetic membranes and nanoparticles coated with the bioactive components. The results however are remarkable as practically a lot of xenobiotic components like heavy metals, pesticides, aromatic hydrocarbons, residual drugs and plastics have been remedied from the environment by different metabolites and enzymes produced in fungi (Viswanath *et al.*, 2014; Joshi *et al.*, 2011; Vaksmaa *et al.*, 2023; Karas *et al.*, 2011; Salvadori *et al.*, 2014).

FUNGAL EXTRACTS AGAINST BIOFILM

Fungal extracts are preferred when dealing with non-living targets like prevention or elimination of biofilms from medical devices. However, extrapolating this idea for use in clinical scenarios would involve a thorough assessment of extracts to identify the relevant bioactive molecules which can subsequently be purified and evaluated on living targets. Extracts themselves might be used owing to their low cost and simplicity but it might lead to certain cytotoxicity and selectivity issues, discussed later.

We want to address the problem of dealing with bacterial biofilms in three stages, dealing with the bacteria before the biofilm is formed involving bactericidal approaches and immunomodulatory functions, mechanisms to prevent the formation of biofilms, and, dealing with the bacteria after the biofilm matrix has been secreted which involves approaches like the enzymatic degradation of the matrix components.

APPROACH 1: ANTIMICROBIAL ACTIVITY

While we discuss the potency of fungal extracts in the prevention of biofilm formation by several mechanisms, it is relevant enough to discuss their anti-bacterial activity. It has been noted how the immunomodulatory properties of fungal extracts can stimulate/enhance the immune system. These observations showcase a potent method of getting rid of biofilm-forming bacteria in the human body which is to target and eliminate them by our immune system before they can form biofilms. This, however, is very much hypothetical and a better and simpler mechanism would be to exploit the antibacterial nature of fungal extracts both in clinical scenarios and also on biofilms formed on artificial surfaces. Biofilm is a resistant complex that most antibiotics cannot penetrate; therefore, the most effective approach would be to eliminate the bacteria before it can begin quorum sensing and subsequently biofilm formation. Fungi are prevalent in a variety of ecological niches and thus have to compete with a wide range of bacteria and other organisms for the same resources. This has led them to develop various antimicrobial compounds which can exert both bacteriostatic as well as bactericidal effects commonly referred to as antibiotics. They operate in several ways, the most common of which are inhibiting bacterial cell-wall synthesis, interfering with protein production, altering membrane permeability and directly affecting the bacterial nucleic acids. The recent generation of antibiotics is mostly semi-synthetic and synthetic analogues, owing to the rapid rise of resistance against their natural precursors due to their widespread and indiscriminatory usage (Hutchings et al, 2019).

Penicillin is the most widely known bioactive antimicrobial compound (antibiotic) isolated from *Penicillium sp*. which inhibits the growth of bacteria through the prevention of new cell wall formation (Kardos and Demain, 2011). This penicillin belongs to the category of a non-ribosomal peptide which is one of the many different types of metabolites produced in the fungi like β-glucans (Chamidah *et al.*, 2017), terpenoids (Abdel-Rahman *et al.*, 2019), polyketides (Shah *et al.*, 2020), chitosans (Chang *et al.*, 2019) and alkaloids (Pinheiro *et al.*, 2013). Some of these are documented to be produced by fungi to exclusively kill microbes while

the potency of others has been identified through several in-vitro experiments. These compounds often act on a broad range of microorganisms including both bacteria and fungi. However, our interest lies purely in their ability to deal with biofilm-forming bacteria. Certain species of *Trichoderma* and *Aspergillus* are very common biocontrol agents and can produce a variety of mycotoxins like aflatoxins which also inhibit the growth of microbes. Fungal mycelial extracts have been tested against Gram-positive and Gram-negative bacteria and have been shown to inhibit their growth. This has been tested in wet-lab experiments using modified paper disc assays. The activity varies with the nature of solvents, producing strains of the fungus and the concerned microbes (Synytsya *et al.*, 2017). Chitosan is a sugar found in the cell walls of fungi like *Rhizopus* that can inhibit the growth of Gram-positive bacteria (Jeihanipour *et al.*, 2007).

Extract from an endophytic fungus of the ethnomedicinally important plant *Cordyline fruitcosa* was seen to have strong antibacterial activity similar to that of the leaf extracts from the host plant. The compounds responsible for this were identified as 4-hydroxy-5-phenyl penta-1,3-dien-1-yl acetate and ergosterol (Elfita *et al.*, 2019). In another study, the antibacterial properties of extracts from five endophytic fungi were determined by an agar well diffusion technique where it was seen that *Cochliobolus sp.* APS1 was able to show broad-spectrum antibacterial ability against Gram-positive MRSA (Methycillin-resistant *Staphylococcus aureus*), VRSA (Vancomycin-resistant *Staphylococcus aureus*), *Bacillus subtilis* as well as Gram-negative *Pseudomonas aeruginosa* and *E. coli*, all of which are known to be biofilm-forming bacteria (Santra *et al.*, 2022). Additionally, a phenyl derivative from the endophytic fungus *Aspergillus flavipes* called flavipesin A identified from ethyl acetate extracts of the fungus was also seen to penetrate the mature biofilm matrix of *S. aureus* and show significant bactericidal activity (Bai *et al.*, 2014).

It is thus observed that the metabolites in fungal extracts can carry anti-bacterial properties which can eliminate the bacteria before it has begun forming biofilms. Instances have also been seen of fungal metabolites being able to penetrate preformed mature biofilms. However, this anti-bacterial property can be utilized better in combination with techniques involving the degradation of biofilm-matrix through methods such as enzymatic degradation of EPS (discussed later). These synergistic effects can massively improve the overall efficacy of the anti-biofilm activity of the relevant fungal extracts.

APPROACH 2: PREVENTION OF BIOFILM FORMATION

Against quorum sensing: Fungal extracts, especially terpenes have shown anti-biofilm properties by preventing bacterial biofilm formation of human pathogens through various mechanisms. One notable compound is farnesol, which is sesquiterpene derived from fungi such as *Candida* species. Research indicates that farnesol inhibits biofilm formation by pathogenic fungi and bacteria, including *Candida albicans* and *Staphylococcus aureus*, by disrupting quorum sensing and virulence factor production (Estrela & Abraham, 2016). Additionally, farnesol has been shown to reverse antibiotic resistance in bacterial biofilms, making them more susceptible to antibiotic treatment. Other fungal terpenes, such as mevalonolactone and ophiobolins, have also exhibited biofilm-modulating activities against various bacterial pathogens, including *Staphylococcus epidermidis*, *Mycobacterium smegmatis* and *Bacillus subtilis*, by preventing biofilm formation at minimal inhibitory concentrations (Estrela & Abraham, 2016).

These fungal extracts can be incorporated into different methods to prevent bacterial biofilm formation:

1. Direct Application: Fungal terpenes, such as farnesol, can be directly applied to cultures of pathogenic bacteria at early stages of biofilm formation to inhibit quorum sensing and virulence factor production, thus preventing biofilm formation (Estrela & Abraham, 2016).

2. Combination Therapy: Fungal terpenes, when combined with conventional antibiotics like fluconazole, can synergistically inhibit biofilm formation and kill drug-resistant pathogens. This approach enhances the efficacy of antibiotic treatment against biofilm-associated infections (Li *et al.*, 2015).

3. Surface Coating: Fungal terpenes can be incorporated into coatings or surface treatments for medical devices, implants or hospital surfaces to prevent bacterial colonization and biofilm formation, thereby reducing the risk of healthcare-associated infections.

4. Antimicrobial Formulations: Fungal terpenes can be formulated into antimicrobial agents, such as disinfectants, creams or ointments, for topical application to prevent bacterial biofilm formation on skin wounds, catheters or other medical devices.

5. Nanoencapsulation: Fungal terpenes can be encapsulated into nanoparticles for controlled release and targeted delivery to sites of bacterial infection or biofilm formation, enhancing their bioavailability and therapeutic efficacy.

QUORUM QUENCHING

In healthcare settings, bacterial biofilms are a serious concern because they can result in antibiotic resistance and persistent infections, especially on medical devices. Through a mechanism known as quorum sensing, these biofilms offer bacteria a protective habitat while enabling them to coordinate their behavior and communicate, thus promoting the production of biofilms and pathogenicity. Nevertheless, by focusing on quorum sensing pathways, fungal extracts, which are abundant in bioactive substances like terpenes and diketopiperazines, present a viable way to reduce the production of bacterial biofilms.

A prominent metabolite of fungi is farnesol, a sesquiterpene produced from the genus *Candida*. Studies have demonstrated its ability to interfere with quorum sensing and prevent the formation of biofilms in bacteria and fungi, including *Staphylococcus aureus* and *Candida albicans* (Estrela & Abraham, 2016). Farnesol lowers the risk of device-related infections by disrupting bacterial communication, which stops the coordinated actions needed for biofilm growth. Furthermore, it has been shown that farnesol can increase the susceptibility of bacterial biofilms to antimicrobial drugs by reversing antibiotic resistance (Jabra-Rizk *et al.*, 2006).

Additionally, by inhibiting the formation of biofilms at low inhibitory concentrations, other fungal terpenes like mevalonolactone and ophiobolins have demonstrated biofilm-modulating activities against a variety of bacterial pathogens, such as *Staphylococcus epidermidis*, *Mycobacterium smegmatis* and *Bacillus subtilis* (Estrela & Abraham, 2016). These substances work by interfering with quorum sensing mechanisms and inhibiting virulence factors important for biofilm formation. By interfering with quorum sensing systems, these substances prevent the production of virulence components that are essential for the formation of biofilms.

In addition, fungi that produce metabolites of polyketides like cytosporone E, patulin and penicillic acid, have demonstrated encouraging biofilm-inhibitory effects by disrupting quorum sensing pathways and lowering the production of virulence factors in bacteria like *Burkholderia cenocepacia* and *P. aeruginosa*. For instance, penicillic acid and patulin increase the susceptibility of *P. aeruginosa* biofilms to antibiotics and stimulate the human immune system, which helps eradicate biofilms (Estrela & Abraham, 2016). Furthermore, artificial diketopiperazines sourced from fungus have exhibited strong biofilm-inhibitory characteristics through disruption of bacterial quorum sensing systems. These substances, which include cyclo (L-Tyr-L-Leu) and cyclo (L-Leu-L-Pro) prevent *Staphylococcus aureus* and *Staphylococcus epidermidis* from forming biofilms, which lowers the risk of infections linked to devices.

Biofilm formation on medical devices can be efficiently controlled by adding fungal extracts into a variety of applications, including direct application, combination therapy with antibiotics, medical device surface coating, antimicrobial formulations, and nanoencapsulation (Estrela & Abraham, 2016). For example, bacterial adhesion and biofilm formation can be inhibited by directly applying fungal terpenes such as farnesol to medical device surfaces, and the effectiveness of antimicrobial treatment against device-related infections can be increased through combination therapy with conventional antibiotics.

Fungal terpenes can be used to coat the surface of medical devices or added to antimicrobial formulations to prevent bacterial colonisation and biofilm development, hence lowering the risk of infections linked

to the use of medical devices. Furthermore, fungal extracts can be delivered to infection locations with targeted and regulated release due to nanoencapsulation, which maximises their therapeutic efficiency and reduces any possible negative effects.

Thus, by focusing on quorum sensing processes, fungal extracts present a viable method of avoiding the formation of bacterial biofilms on medical devices. Fungal metabolites can reduce the incidence of device-related infections and enhance patient outcomes in healthcare settings by interfering with bacterial communication and suppressing the expression of virulence factors essential for biofilm formation. To fully realise the potential of fungal extract formulations in fighting against biofilm-associated infections, more investigation into their clinical uses and formulation optimisation is needed.

Two marine fungal extracts have effectively prevented quorum sensing and in turn bacterial biofilm formation.

It was discovered that marine fungal extract of *Pestalotiopsis sydowiana* PPR had potent antipathogenic properties. The minimum inhibitory concentration (MIC) of the fungal extract against the test pathogen *Pseudomonas aeruginosa* strain PAO1 (*Pseudomonas aeruginosa* original 1) was measured at 1,000 µg/ml. Sub-MIC concentrations of fungal extract (250 and 500 µg/ml) decreased Quorum sensing (QS)-regulated virulence phenotypes by 84.15%, 73.15%, 67.37%, 62.37%, and 33.65% in *P. aeruginosa* PAO1, including pyocyanin, chitinase, protease, elastase and staphylolytic activity (Parasuraman *et al.*, 2020). Additionally, it lowered the bacterial synthesis of exopolysaccharides, rhamnolipids and alginate by 74.99%, 68.01%, and 54.98%, respectively and prevented the bacteria from forming biofilms by 90.54%. The metabolite of *Pestalotiopsis sydowiana* PPR interacts to the bacterial QS receptor proteins (LasR and RhlR) in a manner akin to those of their corresponding natural signalling molecules, according to an in-silico analysis. Among the metabolites of *Pestalotiopsis sydowiana* PPR, cyclo (-Leu-Pro) (CLP) and 4-hydroxyphenylacetamide (4-HPA) were shown to be strong bioactive chemicals (Parasuraman *et al.*, 2020). The findings suggest that the metabolites of *Pestalotiopsis sydowiana* PPR can be employed as promising QS inhibitors that target pathogenic bacteria.

Pseudomonas aeruginosa is an opportunistic human pathogen that can lead to a number of clinical issues, such as a persistent lung infection in people with cystic fibrosis. A number of virulence traits, including elastase, lipopolysaccharide, rhamnolipids, pyocyanin, cyanide, and exotoxin, as well as flagellar motility, biofilm maturation, antimicrobial resistance and alginates, which promote biofilm formation, are expressed via QS in *P. aeruginosa* infections. Rhamnolipids are essential for the bacteria to successfully establish the infection and to elude the host immune response. Acyl-homoserine lactone (AHL) genes for the two QS systems, LasIR and RhlIR, coordinate the infection process of *P. aeruginosa*. Their respective particular signaling molecules, 3-oxo-C12-HSL and C4-HSL, respectively, activate both of these systems in a cascade fashion. Drugs are being developed to effectively target the QS mechanism in order to control the *P. aeruginosa* infection.

The objective of the study was to separate the fungi from the dry wood samples that were taken from the coast, close to the Tamil Nadu town of Muthupet, India, and to test the capacity of the fungi to prevent *P. aeruginosa* PAO1 from forming biofilms by inhibiting quorum sensing (QS).

- Fungi isolation and screening: Using the single spore isolation technique on malt extract agar, fungi were isolated from samples of dry wood. Isolated, morphologically unique colonies were kept in -80°C environment with 25% glycerol.
- Making crude extract: After culturing a few chosen fungal isolates in broth made of malt extract, crude metabolites were extracted. To create crude extracts, the cell-free supernatant of the culture broth was gathered, combined with ethyl acetate, and dried.
- Screening for anti-QS activity: The violacein inhibition assay was used to evaluate the anti-QS potential of fungal crude extracts. A biosensor strain called *Chromobacterium violaceum* was employed to assess the violacein production inhibition. Using the double-layer agar diffusion method, *C.*

violaceum was cultured for 24 hours at 37°C in wells containing varying quantities of fungal crude extract. By assessing the zone of inhibition, violacein production was found to be inhibited.

Ability of fungal extract to stop biofilm formation and quorum sensing

- Reduction of violacein synthesis: Crude fungal extracts have been shown to be able to stop *C. violaceum* from producing violacein. The anti-QS action of fungal extract was demonstrated by the zone of inhibition surrounding the wells that contained them.
- Determining the minimum inhibitory concentration (MIC): The microdilution method was employed to ascertain the MIC of fungal crude extracts in order to examine their impact on QS-regulated virulence components of P. *aeruginosa* PAO1.
- Growth curve analysis: For duration of 24 hours, the impact of sub-MIC concentrations of fungal crude extracts on *P. aeruginosa* PAO1 growth was assessed.
- Evaluation of QS-regulated virulence factors: Pyocyanin synthesis, protease synthesis, elastase activity, chitinase activity, and LasA protease behavior were among the QS-regulated virulence factors of *P. aeruginosa* PAO1 that were found to be affected by fungal extract.
- Biofilm inhibition: Using crystal violet staining and microscopic examination, the impact of fungal extracts on *P. aeruginosa* PAO1 biofilm growth was assessed.
- Gene expression studies: Reverse Transcription Polymerase Chain Reaction (RT-PCR) was used to examine how fungal bioactive substances affected virulence genes of *P. aeruginosa* PAO1.

Overall, the findings showed that fungal crude extracts had strong anti-QS activity, which prevented *P. aeruginosa* PAO1 biofilm formation and QS-regulated virulence factors. These results demonstrate the possibility of fungal metabolites as viable options for the creation of anti-QS drugs that could prevent biofilm-related problems and bacterial infections. Another suitable example of fungal extracts effectively preventing quorum sensing was performed by Martín-Rodríguez *et al.* in 2014. 75 fungal isolates were recovered from reef organisms (endophytes), saline lakes and mangrove rhizosphere (Martín-Rodríguez *et al.*, 2014). Their QS inhibitory activity was evaluated in *Chromobacterium violaceum* CVO26. Four strains of endophytic fungi stood out for their potent activity at concentrations from 500 to 50 μg mL^{-1}. The molecular characterization, based on the internal transcribed spacer (ITS) region sequences (ITS1, 5.8S and ITS2) between the rRNA of 18S and 28S, identified these strains as belonging to four genera, *Sarocladium* (LAEE06), *Fusarium* (LAEE13), *Epicoccum* (LAEE14) and *Khuskia* (LAEE21) (Martín-Rodríguez *et al.*, 2014).

Using conventional isolation methods, marine fungal endophytes were extracted from a variety of aquatic settings. Fungal colonies with unique morphologies were chosen and cultivated for additional examination. After culturing fungal isolates in the proper conditions, solvent extraction techniques were used to derive crude extracts. For additional analysis, the organic phase containing bioactive chemicals was gathered and dried. To evaluate quorum sensing inhibition, bacterial biosensor strains such as *P. aeruginosa* and *Chromobacterium violaceum* were used (Martín-Rodríguez *et al.*, 2014). Conventional tests were employed to ascertain the capacity of fungal extracts to suppress the synthesis of violacein in *C. violaceum* or quorum sensing-regulated virulence factors in *P. aeruginosa*. The diameter of the inhibition zone around fungal extract wells indicated the extent of quorum sensing inhibition.

Biofilm inhibition evaluation

- Microtiter plate assay: Using microtiter plate tests, the impact of fungal extracts on biofilm formation was assessed. Fungal extracts were applied to bacterial cells and biofilm development was measured by crystal violet staining.
- Confocal Laser Scanning Microscopy (CLSM): To determine the degree of biofilm inhibition, the structural integrity of biofilms treated with fungal extracts was observed using CLSM.

Determination of the mechanism of action:

- Gene expression studies: Using molecular biology methods including RT-PCR and RNA sequencing, the effect of fungal extracts on the expression of quorum sensing-regulated genes in bacteria was examined.
- Protein analysis: Western blotting or proteomics methods were used to examine alterations in the expression of proteins linked to quorum sensing.

The synthesis of violacein and other quorum sensing-regulated virulence factors was reduced, indicating that fungal extracts significantly inhibited quorum sensing in bacterial biosensor strains. Fungal extract treatment led to a dose-dependent inhibition of biofilm development in bacterial culture, as evidenced by decreased biomass in the biofilm and structural changes that could be seen with CLSM. The study showed how fungal extracts from marine endophytes could stop the production of biofilms and disrupt bacterial quorum sensing. These results emphasize the value of looking for novel antimicrobial agents in natural sources and show that marine fungus has therapeutic promise in the fight against infections linked to biofilms. To clarify the precise bioactive substances in charge of the effects seen and to maximize their effectiveness for therapeutic uses, more investigation is necessary.

PREVENTION OF ADHESION OF BIOFILMS

Using KB22 gingival epithelial cells as a model system, a series of experiments were carried out to evaluate the potential of fungal extracts in preventing bacterial biofilm formation by reducing adhesion and invasion, which is the first stage of biofilm formation. Initially, trypan blue exclusion assay was used to assess the toxicity of the fungal extract at different doses towards KB cells. Only the mushroom extract of the studied extracts demonstrated toxicity, and it was ruled out for more testing. Following that, various bacterial strains cultivated under suitable circumstances were used for bacterial growth and labelling. The radiolabeled bacteria were removed and resuspended in growth medium or phosphate buffer for the next tests (Glasenapp *et al.*, 2019). Researchers looked into the effectiveness of fungal extracts, particularly those derived from Shiitake mushrooms (*Lentinula edodes*). The purpose of the experiment was to determine whether these extracts could prevent bacteria from adhering to and internalising into the monolayers of KB22, a type of gingival fibroblast cell line (Glasenapp *et al.*, 2019). *Actinomyces naeslundii* and other labeled bacterial suspensions were made in phosphate-buffered saline (PBS) with varying doses of the Shiitake mushroom extract in order to assess the inhibitory effects on bacterial adhesion. Following the addition of aliquots of the bacterial suspensions, KB monolayers were incubated for one hour at 37°C in an environment containing 5% CO_2. Following the incubation period, the number of bacteria per monolayer was determined by measuring the radioactivity after the cells were broken up. The comparison between treated and untreated controls allowed for the determination of the inhibitory activity of the Shiitake mushroom extract. In a similar manner, the inhibition of bacterial internalisation was evaluated. *Prevotella intermedia* and *A. naeslundii* bacterial suspensions were made in KB cell growth medium with different amounts of Shiitake mushroom extract. Following KB monolayer incubation, non adherent bacteria were eliminated, and the total amount of cultivable bacteria in each monolayer was measured. Antibiotics with bactericidal concentrations were used to eliminate external bacteria, and the number of internal cultivable bacteria per monolayer was then used to assess the internalised bacteria. The inhibitory action of the extract was evaluated by treated and untreated samples. The outcomes showed that, in a dose-dependent way, the shiitake mushroom extract efficiently suppressed bacterial adhesion and internalisation into KB22 monolayers. This implied that the extract has anti-adhesive qualities that inhibit the attachment of bacteria such as *A. naeslundii* to gingival epithelial cells. Additionally, the extract did not exhibit any toxicity towards KB cells, suggesting that it may be safe to use orally. All things considered, our results demonstrate the medicinal potential of shiitake mushroom extracts in avoiding the production of bacterial biofilms and the related oral illnesses.

Therefore, it appears that fungal extracts have the

potential to act as preventive agents against the formation of bacterial biofilms by demonstrating encouraging inhibitory effects on bacterial adhesion and invasion into gingival epithelial cells. The comprehensive experimental methodology and outcomes showcased here offer significant perspectives on the advancement of fungal-derived medicines to tackle illnesses linked to biofilms.

APPROACH 3: DEGRADATION OF BIOFILM MATRIX AND DISRUPTION OF EPS COMPONENTS:

Fungi produce a plethora of different hydrolytic enzymes like proteases, lipases, nucleases and glycosidases which target different components of a bacterial biofilm matrix such as proteins, lipids, nucleic acids and polysaccharides, respectively. An added benefit of this strategy lies in the fact that a lot of these hydrolytic enzymes are secreted extracellularly and can be simply isolated from pure cultures of the fungi without further steps involving downstream processing. The enzymatic treatment cannot only prevent the formation of biofilms by getting rid of the secreted EPS before the bacteria have multiplied to their maximum potential, but they can also eliminate already established biofilms.

These enzymes are often not produced by the fungi for the primary purpose of degradation of biofilms. They are utilized in its metabolism like the degradation of complex plant-cell wall materials to be resourced as food. These compounds are also often present in biofilms and so repurposing the enzymes for the role of biofilm degradation from the extracts of the fungus could prove to be an effective strategy. The complexity of bacterial biofilms means that different fungal enzymes can be used alone or in combination, to effectively target the different components of the biofilm, leading to its ultimate degradation (Kaur *et al.*, 2020). Breakdown of the polysaccharide component of the fungi would get rid of the main glue holding the biofilm together and can lead to the dispersion of biofilms from abiotic surfaces.

Biofilms are very notorious for causing biofouling, a complex interaction seen between microbial biofilms, dissolved substances, and the material on which the biofilm is formed (Flemming, 2002). This deposits layers of slime-like gunk on the inner surface of different components like drainage pipes, where both intra and inter-species interaction take place forming heterogeneous biofilms. Surface fermentation under laboratory conditions of a certain strain of *Aspergillus niger* grown in a mixture of substrates produced a cocktail of enzymes at different standardized conditions. The enzymes were subsequently tested on both Gram-positive and Gram-negative biofilm-forming bacteria and both showed high efficacy in biofilm degradation. The Gram-negative bacteria however was seen to be affected to a higher degree possibly due to capsular polysaccharides being hydrolysed which are produced by the tested organisms of *E. coli* and *Salmonella enterica* (Kaur *et al.*, 2020). The results of biofilm degradation can be visualized in the form of morphological anomalies using different microscopic techniques. In this particular case, Field Emission Scanning Electron Microscopy (FESEM) was used to confirm the loss of biofilms after the treatment. Another fungal strain, *Aspergillus clavatus* MTCC1323 growing under solid substrate fermentation conditions produced ample amounts of protease, amylase and pectinase which were able to degrade biofilms of *P. aeruginosa* and *B. subtilis* at an efficiency of 82% and 75%, respectively, as analysed through Fourier transform infrared (FTIR) spectroscopy technique (Singh *et al.*, 2015).

A study was done on the fungal extracts used as cleaning agents on glass containing *Pseudomonas fluorescens* biofilms on its surface. The fungi were initially allowed to grow on various media to facilitate the induction of different enzymes which were then tested separately to measure their effectiveness in the removal of biofilms. The extract from *A. niger* grown on gum arabic showed a high degree of protease and cellulase activity and had a high biofilm removal efficiency of 65 ± 5%. Significant level of glucuronic acid in the gum arabic is also found in the biofilm EPS of *Pseudomonas.* Thus, certain enzymes produced by the fungus growing on gum arabic can attack the glucuronic acid components of the bacterial biofilm matrix. *Trichoderma viride* growing in pectin showed undetectable levels of protease; however, the cellulase and pectin esterase

activity was seen to be high. Here the biofilm removal efficiency was also seen to rise to 84 ± 2% and it could be theorized that pectin esterase was able to deacetylate alginate-like polysaccharides in the biofilm matrix, causing it to become softer or more porous, which allowed the other enzymes to penetrate the framework of the gel (Orgaz *et al.*, 2006). However, a direct correlation of anti-biofilm activity cannot be established purely based on interactions of enzymes on purified components of biofilms (Ellis *et al.*, 2023). All extracts/supernatants of fungi need to be assayed on naturally formed complex biofilms, to assert whether they can truly be used for the fulfilment of our purpose. The enzyme α-xylosidase from *Aspergillus thermomutatus* showed biofilm-degrading activity against *Staphylococcus* biofilms and yet two other β-xylosidases (similar family of enzymes) failed to show any significant effect on the same (Ellis *et al.*, 2023).

The efficacy of this approach can be further enhanced using combination techniques, involving enzymes as well as antibiotics. The enzymes serve to physically remove the EPS components, while the antibiotics target the bacterial pathogens directly. Furthermore, the approach can be utilized in clinical scenarios using enzymes purified from the extracts. A case was seen in the use of a cellulase enzyme combined with the antibiotic ceftazidime, which significantly reduced the biofilm mass of *P. aeruginosa* in a dose-dependent manner. Concentrations of 2.5, 5 and 10 U/mL of the cellulase enzymes effectively reduced the minimum biofilm eradication concentration (MBEC) value of the antibiotic from 32 to 128 folds (Kamali *et al.*, 2021).

CONCLUSION AND FUTURE PROSPECTS

In the realm of different possibilities for combating bacterial biofilms, fungal extracts stand out as potentially key players, offering a wide range of mechanisms against both biofilm formation and biofilm degradation. The advantage of fungal extracts is clear in terms of broad-spectrum specificity, compatibility with combinatorial methods as well as highly reduced likelihood of resistance against them. The three approaches discussed in this chapter open up a lot of avenues to optimize our outlook and methodically plan a line of attack against these biofilm-forming bacteria, depending on the predicament that lies before. A collaboration of two or more outlooks can potentially result in a more fruitful outcome. Furthermore, a cocktail of extracts or extracts used in synergy with already available natural or synthetic antibiofilm agents can prove to be more efficient as well. Prospects of using recombinant DNA technology to create super strains producing multiple fungal metabolites in high concentrations can improve the potency of extracts obtained from them.

However, while fungal extracts definitely have their own advantages in terms of simplicity and cost-effectiveness both with regard to their preparation and application, it still seems advantageous to purify our compound of interest to mitigate unnecessary outcomes and opt for more advanced methods. The utility of nanoparticles (NPs) has gained some spotlight over the years due to the enhanced penetration of preformed biofilm matrix that they offer as well as more directed and localized delivery and far fewer side-effects/toxicity-related issues which becomes imperative in clinical settings. Zinc oxide nanoparticles (ZnO NPs) have been produced using a green synthesis protocol from a rhizosphere fungus *Trichoderma asperellum* growing on a substrate of zinc nitrate hexahydrate. The NPs were seen to be efficient as antibacterial agents and could also effectively prevent *S. auereus* formation and adherence (Shobha *et al.*, 2023). Nanoparticles of gold and silver among others have also been seen to have similar effects (Soliman *et al.*, 2023). This can be levelled up further by coating the NPs using fungal components like chitosan. The results, as expected showed boosted qualities in terms of antibacterial and antibiofilm activity against certain biofilm-forming Gram-positive and Gram-negative bacteria. The same product could even target fungal biofilms of *Candida albicans* and eliminate them by as much as 92% (Thaya *et al.*, 2016). Oral biofilms have also been addressed using novel techniques like pH-activated NPs, containing fungal metabolite farnesol which showed 4-fold more effectiveness than free farnesol against *Streptococcus*

mutans biofilms (Horev *et al.*, 2015). Additionally, purified components of the fungal extract can be coated on to the surfaces of medical devices and other types of equipment to prevent the growth of bacteria and the formation of biofilms. Evidently, it has been seen that chitosan-coated surfaces have been able to reduce viable cell numbers of different bacteria like *S. auereus, Klebsiella pneumoniae, P. aeruginosa and C. albicans* for viable biofilm formation by as much as 95 to 99.99% (Carlson *et al.*, 2008). Water extracts obtained from *Trichoderma terreum* containing high phenolic content were added to the aforementioned chitosan films and were seen to add vastly to the elasticity, antioxidative and antimicrobial properties of the films along with improved anti-quorum sensing activity (Koc *et al.*, 2020).

Overall, in this review, we discussed some of the advances that have been made in the usage of fungal extracts as well as the advanced approaches employing specific fungal components. This area of research is still mostly unexplored considering the diversity of fungi and fungal metabolites, and the possibilities are endless for advances both in medical as well as non-medical fields.

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