Anti-microbial and anti-biofilm compounds from Indonesian medicinal plants

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Chapter 1

General introduction

In recent times there has been an increased urgency to find new anti-microbial compounds as a growing number of microorganisms develop resistance to many commonly used anti-microbial agents upon exposure to anti-microbials. Infections caused by antibiotic-resistant microorganisms complicate conventional treatment, resulting in prolonged illness and greater risk of death (Barza and Travers, 2002).

The problem of microbial resistance to antibiotics appeared as soon as the mass-production of penicillin was started in 1945 (Shaughnessy, 2007), and has now become a major concern in medicine throughout the world. Bacterial resistance not only can occur to a single class of antibiotic, but also to multiple classes of antibiotics. The susceptibility of microorganisms to antibiotics can be categorized in two types:

- innate resistance, which means that some microorganisms are inherently resistant to many antibiotics;
- Acquired resistance, which can arise by a number of diverse mechanisms such as mutation and horizontal gene transfer.

Horizontal gene transfer can occur by several different processes such as bacterial conjugation, transfer of genetic material between bacterial cells by direct cell-to-cell contact. Also DNA can be transferred from one bacterium to another via a viral vector, or directly taken up from its surrounding through the cell membrane (Tenover, 2006).

One of the most serious cases of multidrug resistance is the resurgence of tuberculosis caused by the bacterium *Mycobacterium tuberculosis*, which results in around 2 million deaths per year. Multi-drug resistant *Mycobacterium* strains and the spread of the HIV/AIDS pandemic have contributed to this mortality (Amin, 2006). Tuberculosis, combined with other mycobacterium-based infections, has killed over 30 million people in the last decade, highlighting the need for new anti-bacterial compounds.

According to an annual World Health Organization (WHO) report (WHO, 2014), half of all *Staphylococcus aureus* infections in the United States (US) are resistant to penicillin, methicillin,

tetracycline and erithromycine. This left vancomycin as the only effective agent available at the time. However, glycopeptide intermediate-resistant *S. aureus* (GISA) or vancomycin intermediate-resistant *S. aureus* (VISA) strains began to appear in the late 1990s. The first identified case was in Japan in 1996, and thereafter strains have been found in hospitals in England, France and the US. The first documented strain with complete resistance to vancomycin, termed vancomycin-resistant *S. aureus* (VRSA) appeared in the US in 2002.

Resistance to antibiotics of pathogenic fungi is even a more serious problem, due to the limited number of available anti-fungal drugs. Fungi can counteract the fungicidal or fungistatic effects of all anti-fungals classes by three major mechanisms, namely: (i) reducing the accumulation of the drug within the fungal cell, (ii) decreasing the affinity of the drug for its target, and (iii) modifications of metabolism to counterbalance the drug effect (Vandeputte *et al*., 2012).

Taken together the increase in (multi) drug resistance of pathogenic microbes and the limited amount of antibiotics available for certain classes of microorganisms requires the discovery of new methods and drugs to defend humans and animals against infection by the pathogenic organisms. Another important reason to discover new classes of antibiotics is the recent recognition that a "biofilm" is the most common living environment of microorganisms (including those causing diseases) and that in this environment microorganisms are much more resistant against antibiotics. Biofilms were actually already discovered in 1650s by Anthonie van Leeuwenhoek when he examined the "animalcules" in the dental plaque on his own teeth, however the theory describing the biofilm process was not developed until Costerton and colleagues published their study in 1978. Costerton's theory states that the majority of bacteria grow in matrix-enclosed biofilms that adhere to biological or non biological surfaces in aqueous environments, and that these sessile bacterial cells differ greatly from their planktonic counterparts. Biofilms have been intensively studied in recent years due to increased awareness of the impact of biofilms on human health and industrial productivity (Costerton *et al.,* 1978; Donlan and Costerton 2002; Hall-Stoodley *et al.,* 2004).

Biofilms are defined as a community of cells that are attached to a surface, grow on this surface and can form structured layers. Within these layers cells develop properties as free living (planktonic) cells. One of these traits is the development of an increased antibiotic resistance and the expression of pathogenicity properties. Bacterial biofilm formation is likely to involve a cascade of gene expression events associating with a crossover of many sensing systems directed against environmental changes (Yamanaka *et al*., 2009). *Streptococcus mutans* biofilm proved to exhibit a different expression of some proteins involved in cell signalling, peptidoglycan biosynthesis, motility, and adhesion in comparison to its planktonic cultures (Svensäter *et al*., 2001). In the study by

Kreikemeyer *et al*. (2011) concerning biofilm structure, the determinants of *Streptococcus* pathogenicity are related to the discovery of long filamentous structures similar to the pilus observed on bacteria surfaces. These structures exhibit adhesive properties and may play a key role in adhering to host cells and tissues, as well as in biofilm formation by pathogens of the *Streptococcus* species. Exopolysaccharide (EPS) is one of the main constituents of the biofilm extracellular matrix (Flemming et al., 2007), and recent investigations have revealed that each biofilm-forming bacterium produces distinctive EPS components *e.g*. alginate and/or Psl found in *Pseudomonas aeruginosa* (Ryder *et al*., 2007), and polysaccharide intercellular adhesin (PIA) of *Staphylococcus* (Gotz, 2002). This EPS provides a protection for the biofilm microorganisms from a variety of environmental threats, such as UV radiation, pH shifts, osmotic shock, desiccation, and toxic compounds, and creates a micro niche favorable for long term survival and functioning of the microbial communities (Baumgartner *et al*., 2008). Therefore, when microorganisms grow as a biofilm they are less susceptible against antibiotics, biocides and other chemical and physical challenges (Lewis, 2001).

It is estimated that at least 65% of all hospital infections involve biofilms, since biofilms are not only efficiently formed on human tissues, but also on medical equipment such as catheters and biomaterials such as implants (Donlan, 2002). Furthermore, it is shown that biofilms are involved in developing resistance of bacterial infections (Furukawa *et al*., 2006) as biofilms form a very suitable environment for the selection and emergence of multi-drug resistant strains.

Previously, antibiotic discovery and characterization has been studied mostly with free living bacteria. Since bacterial cells in a biofilm are physiologically so different, it is clear that specific compounds that are able to degrade biofilms still need to be discovered. The anti-biofilm compounds are important tools in the successful treatment of infections and to prevent the development of resistance.

Quorum sensing (QS)

Microorganisms can communicate with one another. One of the communication mechanisms, called quorum sensing, has been named so after discovery of the mechanism underlying cell-density-dependent behavior (Nobile and Mitchell, 2007; Atkinson and Williams, 2009). It has been shown that bacteria assess their population density by producing and detecting the concentration of small, diffusible cell-to-cell signal molecules (auto-inducer), such as an *N*-acylhomoserine lactone (AHL), that they export into their environment. At high population density, a surplus of signaling molecules accumulates in the environment causing diffusing back of these molecules into the cell (Bassler and

Losick, 2006; Atkinson and Williams, 2009). When the signal molecules reaches a threshold level inside the cell, they activate (or repress) a response regulator. This regulator controls expression of quorum-sensing dependent genes such as genes encoding virulence factors or genes encoding proteins involved in biofilm formation (Shih and Huang, 2002; Hentzer *et al*., 2003; Atkinson and Williams, 2009), antibiotic production (Liu *et al*., 2007; Duerkop *et al*., 2009), pigment production, bioluminescence (Fuqua *et al*., 2001), and surface motility (Rasmussen *et al*., 2000) as well as gene transfer systems (Rodelas *et al*., 1999; Schaefer *et al*., 2002).

In Gram-negative bacteria, one of the best studied quorum-sensing systems is the LuxR/I system which consists of a four-component circuit: an AHL (*N*-Acyl homoserine lactones)-signal molecule, a LuxI-type signal synthase, a LuxR-type signal receptor, and the target gene(s) The AHL-signal is synthesized at a low basal level by the AHL-synthase, the product of the *luxI* gene. The AHL-signaling molecules diffuses out of the cell, and when the cell density is high, AHL diffuses back into the cell where it binds to and activates the transcriptional activator LuxR, which then stimulates transcription of *luxI* (Hentzer and Givskov, 2003).

In Gram-positive bacteria, the most common type of signal molecules is oligopeptides that are being exported from the bacterium by transporters (Miller and Bassler, 2001). These peptides will interact with two-component systems ultimately regulating gene transcription. Interaction with the peptide ligand initiates a series of phosphorylation events that culminate in the phosphorylation of a cognate response regulator protein. This phosphorylation allows the response regulator to bind to the promoter region of the quorum sensing–controlled target gene(s) and to alter in this way their transcription (Reading and Sperandio, 2005).

The existence of fungal QS systems was revealed after the discovery that farnesol inhibit biofilm formation in the pathogenic polymorphic fungus *Candida albicans*. In addition to farnesol, the aromatic alcohol tyrosol was also found to be a *C. albicans* QS-molecule (QSM) controlling growth, morphogenesis and biofilm formation. In *Saccharomyces cerevisiae*, two other aromatic alcohols, phenylethanol and tryptophol, were found to be QSMs regulating morphogenesis during nitrogen starvation conditions. Additionally, population density-dependent behaviors that resemble QS have been described in several other fungal species (Albuquerque and Casadevall, 2012).

There is evidence that interspecies communication via QS can occur. This is known as QS cross talk. Cross talk plays a significant role in microbiology as in nature micrrorganisms almost always exist in

mixed species communities. Among the many virulence factors and phenotypes that are controlled by quorum-sensing, there is a clear focus on biofilm formation (Kjelleberg and Molin, 2002).

Biofilm: a novel target

For decades, microbes are mostly considered as free-floating single-celled (planktonic) organisms living an independent lifestyle, rapidly multiplying and described based on their growth characteristic in nutritionally rich culture media (Donlan, 2002; Schachter, 2003). But in fact in nature, most of microbes live together in large aggregates, are attached to a surface, are growing on this surface and often form structured layers. This way of occurance of microbial populations is known as a biofilm. A biofilm community can comprises a single kind of microorganism, but in nature biofilms almost always consist of mixtures of many species of bacteria, as well as fungi, algae, yeasts, protozoa, and other microorganisms, along with non-living debris and corrosion products (Liu *et al*., 2000; Sutherland 2001). Biofilms are found in almost every habitat where the combinations of moisture, nutrient, and surface are available, such as in pipe-lines of industrial or drinkable water, on rocks, at the grime in the toilet bowls and showers or in moss or lichen on tree trunk. Growth as biofilms is likely to provide a better opportunity to adapt to changing conditions collectively than as single cells. Advantages of microbial life in a biofilm comprise protection from host defense and antibiotics, increased availability of nutrients by sequestration to nutrient-rich areas, and cooperative benefits of life in a community in general (Leid, 2009).

Biofilms also have an important impact on human health. The National Institutes of Health (NIH) estimated that biofilms are involved in at least more than 65% of nosocomial infections and up to 75% of microbial infections that occur in the human body (Richards *et al*., 2009). In human tissue for instance, biofilms are produced on the surface of teeth as dental plaque, lungs in cystic fibrosis patients, and as fungal infection on the woman reproductive system. Biofilms formed on medical instruments such as catheters, artificial heart valves, contact lenses, and artificial joints, putting patients at risk for local and systemic infection complications, including local-site infections, catheter-related bloodstream infections, and endocarditis (Donlan, 2002; O'grady *et al*., 2002). The establishment of microbial biofilms is often considered a pathogenicity trait during chronic infections, and biofilm formation has been recognized as an important virulence mechanism of many bacterial pathogens (Donlan and Costerton, 2002).

Biofilm communities can develop within hours. There are five stages of biofilm development, as depicted schematically in figure 1. The first step of biofilm formation is attachment of free-floating

cells, referred as ‘pioneer cells’ to a surface associated with proteins, glycoproteins, humic-like substances (HULIS), and other dissolved or colloidal organic matters (the so called "conditioning layer"). This conditioning layer is consider to play a vital role in initial microbial attachment as they neutralizes excessive surface charge and surface free energy which may prevent a microbial cell from approaching near enough to initiate attachment (Schneider and Leis, 2002; Palmer *et al.,* 2007; Renner and Weibel, 2011).

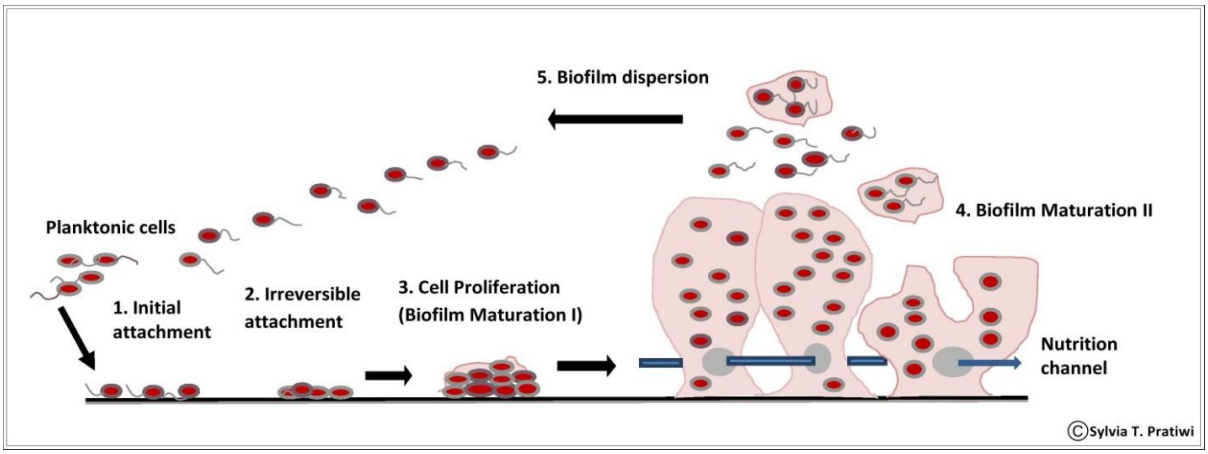

Fig. 1: Five stages of biofilm development: 1. Initial attachment of planktonic cells to the surface, 2. Irreversible (permanent) attachment, 3. Cells proliferation and formation of EPS (maturation I), 4. Formation of biofilm structure (maturation II), 5. Biofilm spreading (dispersion).

During initial attachment, the ‘pioneer cells’ adhere to the surface initially through weak, reversible Van der Waals forces. The bacteria may then form a transient association with the surface, and facilitate the arrival of other cells by providing more diverse adhesion sites to yield a basal layer and forms a stable association as a member of a microcolony (Watnick and Kolter, 1999). Afterwards, the attachment of the cells becomes irreversible as the microcolonies start joining together above the substratum to form a continuous layer of extracellular polymeric substances (EPS) that run into a complex, three-dimensional mushroom-like structure stick out from the surface, separated by “voids” through which the medium (e.g., water with nutrients) may flow relatively uninhibited. This EPS holds the biofilm together and serves as a barrier to defend the microbial colonies from anti-microbials. The last stage in the development of biofilms is the detachment of small or large clumps of cells, or by a type of "seeding dispersal" from the biofilm that releases individual cells**.** These cells move on to further colonize sites, and repeat the development cycle. Dispersal of cells from the biofilm colony is an essential stage of the biofilm lifecycle because it enables biofilms to spread and colonize new surfaces (Hall-Stoodley *et al.,* 2004; Richards *et al.*, 2009).

The EPS consists of polysaccharides, proteins, glycoproteins, glycolipids and contribute to biofilm integrity. Extracellular DNA (eDNA) has also been found in the EPS and is considered as a remnant of lysed cells (Cheng *et al*., 2011) All EPS biopolymers are highly hydrated. About 97% of the biofilm consists of water and forms a matrix that keeps the biofilm cells together and prevents desiccation (Zhang *et al*, 1998; Shuterland, 2001). It is clear from a number of studies that mutants unable to synthesize the EPS are unable to form biofilms although they may still attach to surfaces and form micro-colonies to a limited extent (Flemming and Wingender, 2010; Xiao *et al*., 2012).

Biofilm communities of bacteria are different from planktonic cells, most importantly being their increased resistance to anti-microbial agents. The dense EPS matrix serves as an initial barrier to prevent penetration of anti-microbial agents at the full depth of biofilm. As a consequence, cells inside the biofilm are about 10 to 1.000 times more resistant to anti-microbial agents than planktonic cells. Cells in biofilms are also extraordinarily resistant to phagocytosis, making biofilms extremely difficult to eradicate from living host (Stewart *et al.,* 2001). Hentzer, *et al.* in their experiments (2003), showed that classical antibiotics used to treat *P. aeruginosa* infections, e.g., tobramycin and piperacillin are required at concentrations 100-to-1000-fold higher in order to kill biofilm bacteria than in order to kill their planktonic counterparts.

Generally, the most rapid cell growth in a biofilm occurs at the biofilm surface where the oxygen and nutrients are plentiful. Cells that are embedded deep inside the biofilm might grow in a slow or starved state due to nutrients and oxygen limitations and accumulation of toxic metabolic product (Wimpenny and Kinniment, 1995). Slower growing or non-growing cells are not very susceptible to many anti-microbial agents. Penicillin which target cell wall synthesis kills only growing bacteria (Tuomanen *et al*., 1986; Stewart and Costerton, 2001). Also, anti-microbials hardly affect all bacteria within the biofilm due to heterogeneity within the biofilm, because unlike the growth of bacteria in liquid cultures which produce homogeneous populations of genetically identical cells, growth in biofilms generates a large amount of genetic diversity (Stewart, 1998; Costerton *et al*., 1999). Heterogeneity within the biofilm caused by cellular diversity formed by gradients of nutrients, waste products and signaling factors which can develop microniches within the biofilm (Mehrotra, 2007).

Biofilms also accommodate heterogeneous populations coming from both epigenetic and genetic phenomena. Epigenetic heterogeneity is the production of persister cells as well as in stationary-phase planktonic culture. Persister cells are cells that neither grow nor die in the presence of bactericidal agents (dormant), and tolerant to a lethal stress such as anti-microbial treatment, heat shock, cold shock, changes in pH and some chemicals that are lethal to growing cells (Hengge-Aronis,

1996; Lewis, 2007). The presence of persister cells explains the resistance of biofilms-based infection to anti-microbial therapy (Nobile and Mitchell, 2007).

The role of QS in biofilm

The precise role for QS in biofilm formation and maturataion is not always clear. Multiple reports have discussed the involvement of QS in biofilm formation, and conflicting conclusions have been drawn regarding the importance of QS in microbial biofilm formation. QS regulatory networks are very intricate and may include several genes whose products affect biofilm development at different stages (Solano *et al.*, 2014). The effect of AHL QS on *P. aeruginosa* biofilms appears to depend on different growth conditions, such as static or under flow, different growth phases observed, different supply of nutrients, or strain differences (Heilmann and Götz, 2010). It was shown that QS affected *P. aeruginosa* biofilms only in the presence of glucose and not citrate (De Kievit *et al*., 2001). Schaber *et al*., (2007) found that biofilms formed by *P. aeruginosa* in acute burn wound infections in mice to be unaffected by mutation of the *lasI* and *rhlI* QS genes, despite the fact that the mutant strains were significantly less virulent than their wild-type counterpart.

In *Listeria monocytogenes*, the effect of the agr (accessory gene regulation) system is to induce the cells to bind to a surface (Rieu *et al.*, 2008). A mutation in the *agrD* gene reduced its ability to attach to a PVC surface, thus makes it difficult to form biofilm. Similarly in *Enterococcus faecalis*, the *fsr* peptide base QS system has also been shown to positively influence biofilm formation (Hancock and Perego, 2004; Nakayama *et al*., 2006).In *Aeromonas hydrophila*, the deletion of the AHL synthase gene *ahyI* responsible for the synthesis of both C4 and C6 HSL results in the reduction of biofilm differentiation and the failure of the bacteria to form microcolonies (Lynch *et* al., 2002).

In several cases, such as in *P. aeruginosa* and a common co-habitat of Cystic Fibrosis (CF) patient lung infections, *Burkholderia cenocepacia*, QS does not appear to affect the initial attachment of cells to a surface, but rather the maturation of biofilm structure. Mutations in the QS systems of these bacteria result in the formation of flat, undifferentiated biofilms (Huber *et al.,* 2001). In *Serratia liquefaciens* and *S. marcescens*, QS system regulates the maturation stages of biofilm formation. A mutation in the AHL synthase gene *swrI* results in biofilm development which is apparently halted after 24 hours, forming a flat and thinner biofilm, with more homogenous cells which are poorly attached and less cellular aggregation. This is in contrast to the wild type, which forms aggregate cluster biofilms (Labbate *et al*., 2004; Rice *et al*., 2005).

Staphylococcus uses the *agr* system to regulate biofilm formation. The agr system downregulates the expression of genes encoding colonization factors, such as the microbial surface components recognizing adhesive matrix molecules (MCSRAMMS) and other adhesin that promote attachment and biofilm accumulation, or to induce the dispersal cells from a biofilm by initiate secretion of a variery of toxins (α-toxins, δ-toxins, and toxic shock syndrome toxins-1), and ezymes (such as proteases, lipases, hyaluronidase, and nuclease) that are involved in tissue destruction and/or biofilm detachment probably requires for dissemination of the infection and colonization of new sites (Heilmann and Götz, 2010).

However, not all microorganisms positively regulate biofilm formation using QS. High cells densities of *Vibrio cholerae* are known to inhibit biofilm formation (Karatan and Watnick, 2009), whilst studies have shown that *V. cholera* "locked" in a low-cell density mode, produce more pronounced biofilms (Teschler *et al*., 2015).

Model organism: *P. aeruginosa*

P. aeruginosa (figure 2), is a Gram-negative bacteria rod-shaped, about 1-5 µm in length and about 0.5-1.0 µm in breadth (Prescott *et al*., 2009). This bacteria is an obligate aerobe, however *P. aeruginosa* is considered by many as a facultative anaerobe, as it is well adapted to proliferate in conditions of partial or total oxygen depletion (Kelli *et al*., 2007; Prescott, *et al*., 2005).

P. aeruginosa produces two types of water-soluble pigments: pyocyanin, a blue-green, water- and chloroform-soluble, non-fluorescent pigment, and pyoverdine, a yellow-green, water-soluble, fluorescent pigment which are released in the medium resulting yellow-green iridescent colonies on plate agar. Pyocyanin (from "pyocyaneus") refers to "blue pus", which is a characteristic of suppurative infections caused by *P. aeruginosa*. Pyoverdin is most often produced by *P. aeruginosa* and other Pseudomonads commonly isolated from humans. In contrast, *P. aeruginosa* is the only *Pseudomonas* spp. known to produce pyocyanin, a pigment that diffuses into the surrounding medium. Definitive clinical identification of *P. aeruginosa* often includes identifying the production of pyocyanin and pyoverdine, as well as its ability to grow at 42°C (Forbes *et al*., 2007; Todar, 2011; Tamang, 2014).

P. aeruginosa has an outer membrane which contains Protein F (OprF) OprF functions as a porin that enable certain molecules and ions to come into the cells. OprF also act as a structural protein to maintain the bacterial cell shape (Nestorovich *et al*., 2006; Fito-Boncompte, 2011). *P. aeruginosa* is

motile by single polar flagellum. The flagellum is very important during the early stages of infection, as it is required for the attachment and invasion of the tissues of the hosts. This bacterium also has *N*-methyl-phenyl-alanine (NMePhe) or type IV pili which contribute greatly to its ability to adhere to mucosal surfaces and epithelial cells (Mattick, 2002; Klaussen *et al*., 2003).

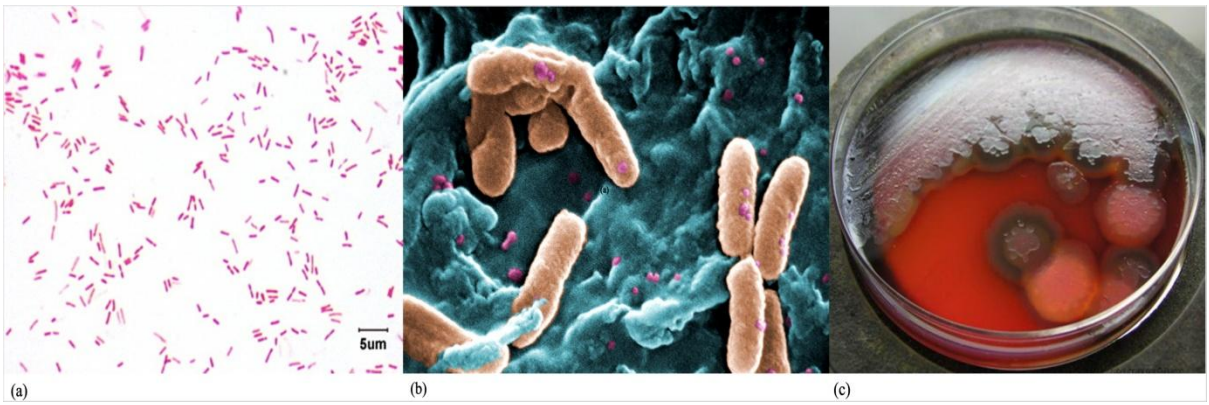

Fig. 2: Multiple views of *Pseudomonas aeruginosa*. (a) Gram stain of *P. aeruginosa*, (b) scanning electron micrograph (SEM) of *P. aeruginosa,* (c), colonies of *S. aureus* on blood agar plates showing hemolysis (Kaiser, 2009; Carr, 2006).

Under oxidative-stress conditions, *P. aeruginosa* synthesizes Fe- or Mn-containing superoxide dismutase (SOD) enzymes, which catalyze the very reactive O- to H_2O_2 and O_2. *P. aeruginosa also* produces several catalases that detoxify H_2O_2, leaving oxygen and water (Hassett *et al.,* 1993; Elkins *et al.,* 1999).

***P. aeruginosa* virulence factors and disease association**

P. aeruginosa is an opportunistic human pathogen that is commonly found in the environment e.g. soil, water and other moist locations. It is the most common Gram-negative bacterium found in nosocomial infections. For most healthy people, this bacterium seldom poses a problem. *P. aeruginosa* causes urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteremia, bone and joint infections, gastrointestinal infections, and a variety of systemic infections, particularly in patients with severe burns and in cancer or AIDS patients who are immunosuppressed. In the expanding AIDS population, *P. aeruginosa* bacteremia is associated with 50% of deaths. CF patients are characteristically susceptible to chronic infection by *P. aeruginosa*, which is responsible for high rates of illness and death in this population (Todar, 2011).

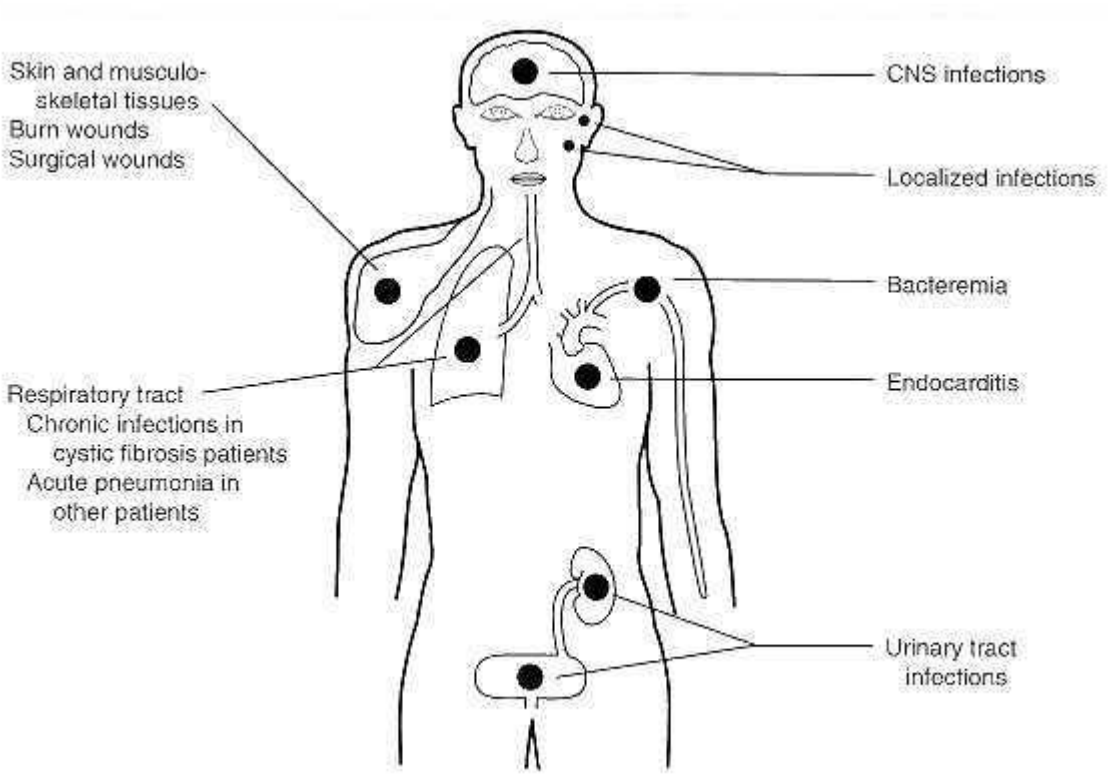

Fig. 3: Various sites of *P. aeruginosa* infection (Iglewski, 1996).

The capability of *P. aeruginosa* to produce infections is due to several extracellular and cell-associated virulence factors, (e.g, adhesins, invasins, toxins, antiphagocitic surface properties, defense against serum bactericidal reactions, and defense against immune responses) which play major roles in the toxicity and aggressivity of the bacteria. The bacterial capsule or alginate slime layer that forms matrix of the *Pseudomonas* biofilm effectively protects the bacteria from the opsonization by antibodies, complement deposition, and phagocyte engulfment (Todar, 2011).

Elastase and alkaline protease, are two extracellular proteases which have been associated with virulence that during the invasive stage. Elastase cleaves collagen, IgGs, IgAs, elements of the complement system. It also lyses fibronectin to expose receptors for bacterial attachment on the mucosa of the lung. Elastase activity also disrupts the respiratory epithelium and interferes with ciliary function. Alkaline protease interferes with fibrin formation and will lyse fibrin. It destroys the ground substance of the cornea and other supporting structures composed of fibrin (Todar, 2011).

P. aeruginosa secretes a number of toxins into the extracellular environment, but one set of toxins is injected directly into host cells. This occurs through a macromolecular syringe consisting of a type III secretion system (TTSS) that inject at least four toxins into host cells. Injection of these toxins leads

to disruption of Ras- and Rho-mediated signal transduction pathways, collapse of the actin cytoskeleton, and cell death (Frank, 1997; Engel and Balachandran, 2009).

Exotoxin A is the most potent toxic factor which is responsible for local tissue damage, bacterial invasion and immunosuppression. Exotoxin A catalyzes ADP-ribosylation and inactivation of elongation factor 2, an essential component of the protein synthesis machinery which leads to inhibition of protein biosynthesis and cell death (Todar, 2011).
About 60% of people with cystic fibrosis have a chronic respiratory infection caused by *P. aeruginosa* biofilm-growing mucoid (alginate-producing) strains that settle into the thick mucus trapped in the respiratory passages. *P. aeruginosa* mucoid biofilms allow bacterial cells to escape from the innate and adaptive defense mechanisms of the lungs, and intense antibiotic therapy (Høiby *et al*., 2010).

***P. aeruginosa* QS and biofilm formation**

There are two QS systems in *P. aeruginosa*, which have been extensively studied. The *las* system consists of the LasR transcriptional regulator and the LasI synthase protein. LasI is essential for the production of the AHL signal molecule *N*-(3-oxododecanoyl)-L-homoserine lactone (3O-C_{12}-HSL). LasR requires 3O-C_{12}-HSL in order to become an active transcription factor. A second QS system in *P. aeruginosa* consists of the RhlI and RhlR proteins. The RhlI synthase produces the AHL *N*-butyryl-L-homoserine lactone (C_4-HSL), and RhlR is the transcriptional regulator. Only when RhlR is complexed with C_4-HSL it is activated. Both 3O-C_{12}-HSL and C_4-HSL have been shown to freely diffuse out of bacterial cells; however, 3O-C_{12}-HSL diffusion is significantly slower than that of C_4-HSL. Recently, a third LuxR homologue termed QscR was identified, which has been shown to regulate the transcription of both *lasI* and *rhlI*. Although QscR exhibits significant homology to LasR and RhlR, it is currently unknown whether an AHL or similar molecule is needed to stimulate QscR function. Data indicate that *qscR* is important in regulating the production of several virulence factors but whether this regulation occurs through controlled expression of both the *las* and the *rhl* systems is currently unknown (Smith and Iglewski, 2003; Bjarnsholt, 2010).

At least three exo-polysaccharides, Psl, Pel, and alginate contribute to biofilm formation in *P. aeruginosa* that responsible to protect bacteria from environmental stressed and is required for bacterial cells to adhere to a substratum and maintaining biofilm structure (Matsukawa and Greenberg, 2004; Ryder *et al.,* 2007). The *Psl* (*p*olysaccharide *s*ynthesis *l*ocus) containing the *pslA*-*O* genes, is responsible for mannose and galactose rich EPS, whereas *pel* (referring to *pel*licle, a biofilm formed at the air-medium interface), containing the genes *pelA*-*G*, is responsible for synthesis of a glucose-rich component of the matrix (Khan *et al*., 2010).

The dispersal mechanism for bacterial cells to escape the biofim is induced by unfavorable conditions and important to spawn new communities of bacterial cells at new locations. It involves a reduction mechanism of bacterial adhesiveness, and a breakdown of the biofilm matrix (Harmsen *et al.,* 2010; Gjermansen *et al.,* 2005). Davies and Marques (2009) found that the compound *cis*-2-decenoic acid (CDA), an unsaturated fatty acid produced by *P. aeruginosa* is capable of inducing the dispersal of established biofilms and of inhibiting biofilm development. The authors suggested that this molecule is produced continuously by *P. aeruginosa* during growth in biofilms and that small microcolonies do not disperse because CAD is removed through diffusive and advective transport; However dispersal from larger microcolonies occurs because the rate of production of this molecule exceeds the rate of diffusion.

Model organism: *S. aureus*

S. aureus, means the "golden cluster seed" is a Gram-positive coccus, non-motile, non-spore forming bacteria, facultative anaerobic, with the diameter of 0.5-0.15 µm, that usually occur single, in pairs, tetrads, short chains (three or four cells) or irregular grape-like clusters. Typical colonies are yellow to golden-yellow in color, smooth, entire, slightly raised, often with hemolysis when grown on blood agar plates (figure 4). *S. aureus* is able to produce enzyme catalase (catalase positive) and able to convert hydrogen peroxidase (H_2O_2) to water and oxygen. The catalase test is useful to distinguish Staphylococci from Enterococci and Streptococci. The ability of *S. aureus* to clot blood plasma by the presence of enzyme coagulase is the main criteria to identify this bacterial, separating the virulent pathogen of *S. aureus* from the less virulent coagulase-negative Staphylococci species (Prescott *et al.,* 2005).

Resistance of *Staphylococcus* to many commonly used antibiotics started as soon as the first antibiotic, penicillin, was introduced in 1943. By 1950, 40% of hospital *S. aureus* isolates were penicillin-resistant (Chambers, 2001). The resistance is mediated by penicillinase, an enzyme that breaks-down the β-lactam ring of the penicillin molecule. β-Lactamase-resistant penicillins such as methicillin, oxacillin, cloxacillin, and fluconacillin that are able to resist degradation by staphylococcal penicillinase were developed to treat penicillin-resistant *S. aureus*. Methicillin was the first antibiotic in this class to be used in 1959, but only two years later, the first case of methicillin-resistant *S. aureus* (MRSA) was found and became endemic in 1990 (Givney *et al*., 1997).

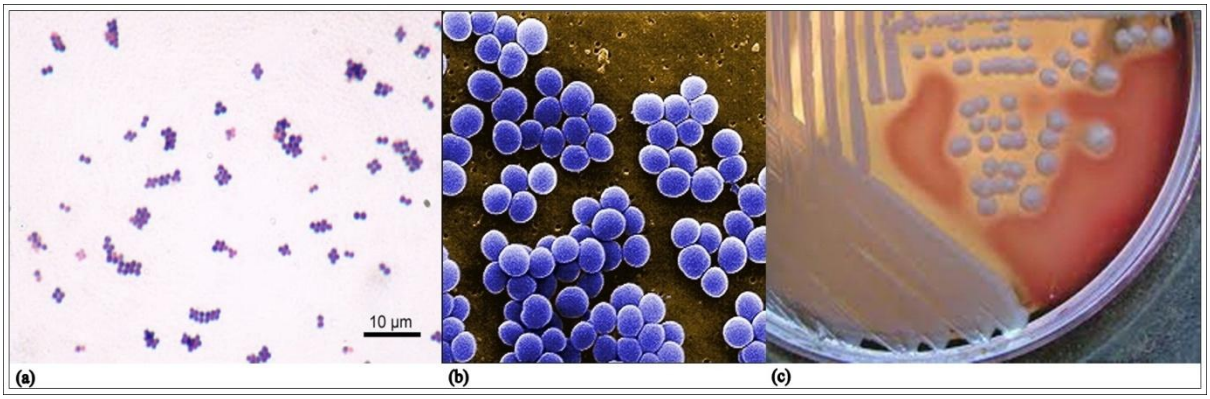

Fig. 4: Multiple views of *Staphylococcus aureus*. (a) Gram stain of *S. aureus*, (b). scanning electron micrograph (SEM) of *S. aureus*, (c) colonies of *S. aureus* on blood agar plates showing hemolysis (Tambe, 2005[a]; Carr, 2007; Buxton, 2013).

First line treatments for infections caused by MRSA are currently glycopeptide antibiotics (vancomycin and teicoplanin). Most of *S. aureus* isolates are susceptible to vancomycin. *Staphylococcus* bacteria are classified as vancomycin intermediate resistant *S. aureus* (VISA) if the MIC for vancomycin is between 4-8 µg/mL, and classified as vancomycin resistant *S. aureus* (VRSA) if the vancomycin MIC is ≥16µg/ml (Walters *et al*., 2015). The first case of vancomycin-intermediate *S. aureus* (VISA) was reported in Japan in 1996 (Hiramatsu *et al*., 1997), and the first case of *S. aureus* resistant to glycopeptide antibiotics was only reported in 2002 (Chang *et al.,* 2003). Surveillance data shows VISA and VRSA infections are uncommon, and only seen in people with significant underlying health conditions. However, the prevalence has been gradually rising and reports of cases have spread throughout the United States. Due to the high rates of vancomycin clinical treatment failure and associated morbidity and mortality rates of 60%–70%, these VISA and VRSA strains deserve serious attention (Walters *et al.,* 2015).

Virulence factors and disease association

S. aureus is one of the most common causes of nosocomial infection. *S. aureus* infections are often acute and pyogenic and, if untretated, may spread to surroundings tissue or, via bacteremia, to metastasic sites (involving organs) such as skin which is the cause Staphylococcal scalded skin syndrome (SSSS) in infants. Infection from Staphylococci can also cause life-threatening diseases such as pneumonia, meningitis, endocarditis, bacteremia, septicemia, cervicitis, and toxic shock syndrome (TSS) (Prescott *et al*., 2005).

The diverse range of *S. aureus* diseases has been attributed to its ability to produce an array of virulence factors. *S. aureus* expresses many potential virulence factors, such as surface proteins that promote colonization of host tissues; invasins that promote bacterial spread in tissues (i.e.

hyaluronidase); surface factors that inhibit phagocytic engulfment (capsule, Protein A); biochemical properties that enhance their survival in phagocytes (i.e. catalase production); immunological disguises (Protein A, coagulase); membrane-damaging toxins that lyse eucaryotic cell membranes (hemolysins, leukotoxin, leukocidin); exotoxins that damage host tissues or otherwise provoke symptoms of disease: Staphylococcus enterotoxins (SEs), TSST, exfoliative toxins (ET); and inherent and acquired resistance to anti-microbial agents (Todar, 2011; Cheung *et al.*, 2004).

Bacterial surface protein adhesins, known as MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) mediates *S. aureus* adherence to extracellular matrix (ECM) components to initiate colonization to the host colonize and resist physical removal (Foster and Höök, 1998). Certain MSCRAMMs acting as invasins, an adhesin (a surface protein in the cell wall of bacteria) molecule, can facilitate bacterial entry into the cell by phagocytosis, and place the bacteria in a vacuole. By entering the cytoplasm of the host cell, it has a ready supply of nutrients and is protected from complement, antibodies, and certain other body defenses. Once the bacterium escapes from the vacuole and is able to replicate in the cytoplasm of the host cell (Prescott *et al.,* 2005). This interaction is critical for infection and virulence and also permits internalization of pathogens into non-phagocytic host cells (Cambronne *et al.,* 2005; Niemann *et al*., 2004).

S. aureus is a clinically important capsule-forming bacterium, i.e., they produce uronic acid-containing, extracellular polysaccharides, which enhance microbial virulence by rendering the bacterium resistant to phagocytosis. The capsular polysaccharide (CPs) production appears to be an important virulence determinant, and occurs as different chemical structures depending on the serotype of the organism (Cook, *et al.,* 2009). This CPs are found in 90% of the clinical strains. Eleven serotypes have been described, type 5 and 8 are the most common among human isolates (80%) (Poutrel *et al.,* 1988).

Some pathogens can hide their unique antigenic surface components from the immunological system. *S. aureus* produces cell-bound coagulase and clumping factor that cause fibrin to clot and to deposit on the cell surface. It is possible that this disguises the bacteria immunologically so that they are not readily identified as antigens and targets for an immunological response (Todar, 2011).

According to the presence of enzyme coagulase, the virulence-pathogen *S. aureus* can be separated from the less virulent coagulase-negative Staphylococci species. There are three coagulase-positive Staphylococci species: *S. aureus* in humans and mammals, and *S. intermedius* and *S. hyicus* in vertebrates (Murray *et al.*, 1995). Coagulase negative staphylococci (CoNS) such as *S. epidermidis* do

not produce coagulase, are nonpigmented, and are generally less invasive but have increasingly been associated as opportunistic pathogens with serious nosocomial infections (Prescott *et al.,* 2005).

Two different forms of coagulase can be produced, free and bound. While free coagulase is an extracellular enzyme that is secreted extracellularly, bound coagulase is part of the larger family of MSCRAMMs. Bound coagulase, also known as clumping factor, reacts with fibrinogen to cause organisms to aggregate. Another factor, extracellular staphylocoagulase, reacts with prothrombin to form staphylothrombin, which can convert fibrinogen to fibrin. This results in clotting of the blood. Approximately 97% of human *S. aureus* isolates possess both of free and bound forms of coagulase. There are two different tests that can be performed to detect the presence of coagulase: a tube test to detect the presence of free coagulase, and a slide test to detect bound coagulase. Isolates that do not produce clumping factor must be tested for the ability to produce extracellular coagulase (free coagulase). Another way of *S. aureus* to hide its unique antigens from opsonizing antibodies or complement is by producing Protein A which is located on cell wall. Protein A is an IgG-binding protein which binds to the Fc end of an antibody, thus coating the bacteria with antibodies that helps inhibit phagocytic engulfment and acts as an immunological disguise (Todar, 2011).

Depending on the strain, *S. aureus* is capable of secreting several toxins that can be categorized into three groups: Pyrogenic toxin superantigens (PTSAgs), Exfoliative toxins (ET), and other toxins. Toxic shock syndrome (TSS) is associated with strains that produce and secrete exotoxin toxic shock syndrome toxin 1 (TSST-1), a member of PTSAgs family that has the ability to stimulate T-cells. Although first described in children, it was most frequently associated with women using tampons during menstruation. The staphylococcal enterotoxins which cause food poisoning also belong to PTSAgs family. These enterotoxins are divided into 6 types of heat-stable toxins, i.e. A, B, C1, C2, D, E, responsible for the gastrointestinal upset typical of food poisoning (Prescott *et al.,* 2005). Exfoliative toxins are implicated in the disease staphylococcal scalded-skin syndrome (SSSS), also known as Ritter disease, which occurs most commonly in infants and young children. The protease activity of exfoliative toxins causes peeling of the skin observed with SSSS (Bukowski *et al*., 2010).

Other Staphylococcal toxins that act on cell membranes include alpha hemolysin-toxin, beta hemolysin-toxin, delta hemolysin-toxin, and gamma hemolysin-toxin which are destroy erythrocytes, and several bicomponent toxins such as toxin Panton-Valentine Leukocidin (PVL), a pore forming toxin in the membranes of the infected cells, causing the cell's contents to leak out and cause cell death.

S. aureus is normal inhabitants of the upper respiratory tract, skin, intestine, and vagina, and known as the most important human staphylococcal pathogen. This bacterium, altogether with pneumococci and streptococci, is member of a group of invasive Gram-positive bacteria known as the pyogenic (or pus-producing) cocci, causing various supurative, or pus-forming diseases (e.g., boils, carbuncles, folliculitis, impetigo contagiosa, scalded-skin syndrome) in humans. *S. aureus* also cause medical device-related infections such as intravascular line sepsis and prosthetic joint infections (Prescott *et al.,* 2005).

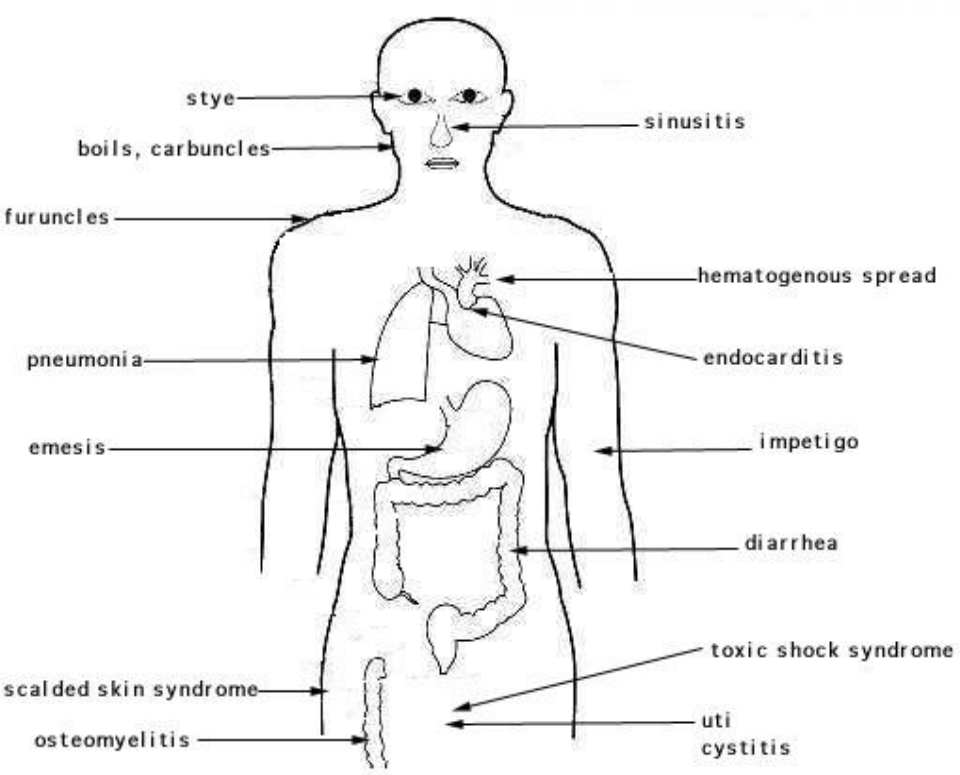

Fig. 5: Various sites of *S. aureus* infection (Todar, 2011).

***S. aureus* QS and biofilm**

The *S. aureus* quorum-sensing system is encoded by the accessory gene regulator (*agr*) locus, which consists of two divergent transcripts, RNAII and RNAIII, driven by the P2 and P3 promoters (Yarwood *et al*., 2004). RNAII contains four open reading frames designated *agrA* to *agrD,* that encode factors required to synthesize **a**uto-**i**nducing **p**eptide (AIP). The current working model suggests that the autoinducer peptide is generated from *agrD* and then processed, post-translationally modified, and secreted by a membrane-bound protein bearing an ABC-type transporter system known as AgrB. The sensing apparatus consists of the transmembrane receptor-histidine kinase AgrC. If sufficient

quantities of the appropriate AIP ligand have accumulated, AgrC phosphorylates AgrA. which then binds to the RNAIII transcript that encodes a small RNA that functions to modulate gene expression of *S. aureus* gene (Figure 6) (Chegelski *et al*., 2008).

A second quorum-sensing cascade, **R**NAIII **a**ctivating **p**rotein (RAP) and the **t**arget of **R**NAIII **a**ctivating **p**rotein (TRAP), is thought to serve as a precursor to the Agr system (Balaban and Novick, 1995). The RAP is secreted and accumulates outside the cell, and at a threshold concentration, RAP triggers the phosphorylation of the cytoplasmic protein TRAP, which induces expression of the RNAII transcript (Gov *et al*., 2004). Interestingly, a linear heptapeptide called RIP is known to block the activity of RAP, enabling small-molecule control over *S. aureus* pathogenicity (Balaban *et al.,* 1998).

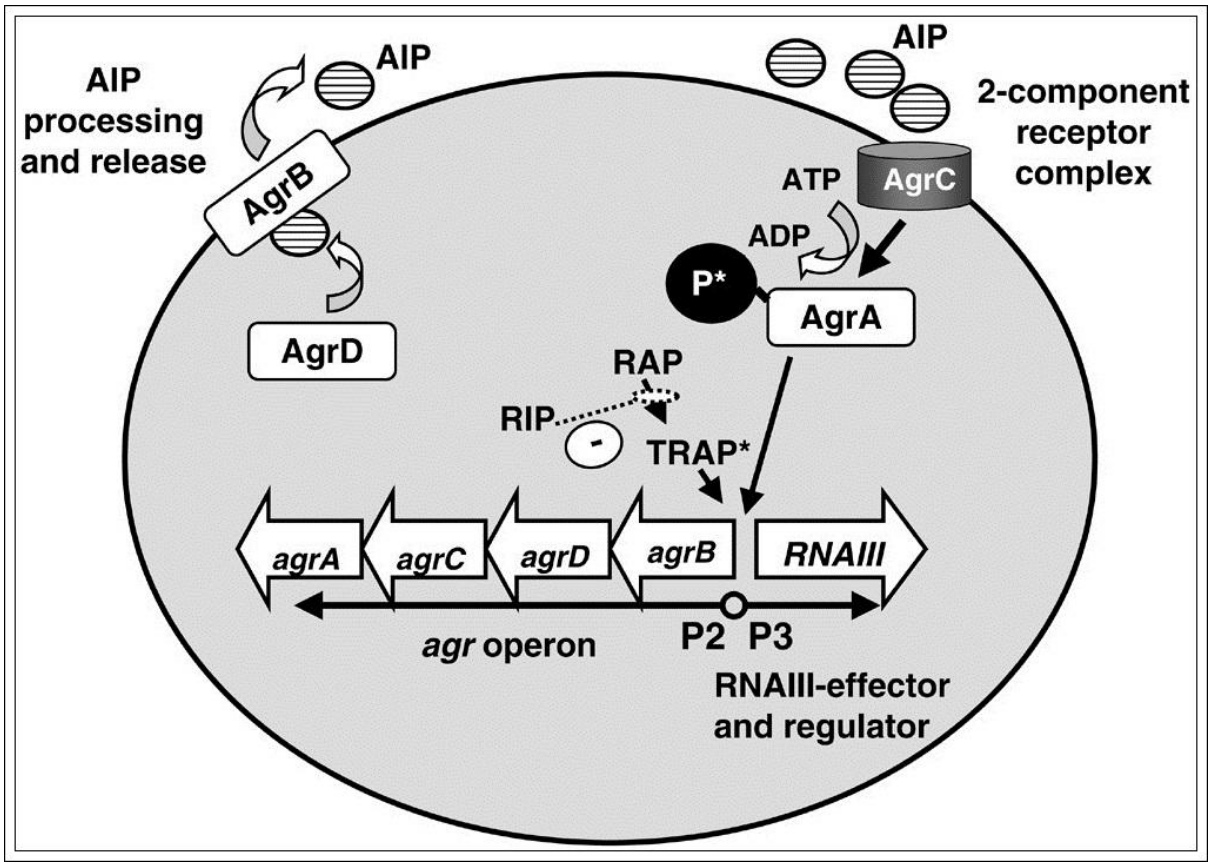

Fig. 6. Schematic diagram of quorum-sensing systems of *S. aureus*. The gene locus for the *agr* system contains two divergent transcripts, RNAII and RNAIII, driven by the P2 and P3 promoters. The RNAII transcript encodes the *agrBDCA* operon, which encodes the signal, processing and detection components for quorum-sensing in *S. aureus*. The RNAIII transcript is a regulatory RNA that up-regulates and down-regulates all genes related to quorum-sensing. Agr, accessory gene regulator; AIP, autoinducer peptide; P, promoter site; P*, phosphorylated protein; RAP, RNAIII-activating protein; RIP, RNAIII-inhibiting peptide; TRAP, target of RNAIII-activating protein (Asad and Opal, 2008).

The capability of *S. aureus* to form biofilms is one of the defense mechanisms that makes this bacterium difficult to eradicate with standard antibiotic treatment and makes it inherently resistant to host immune responses. The biofilm formation then consequently impedes the treatment of many chronic *S. aureus* biofilm related infections, including endocarditis, osteomyelitis and indwelling medical device infections (Costerton *et al*., 1999).

As well as *P. aeruginosa* biofilm, the development of a biofilm in *S. aureus* involves an initial attachment, maturation and detachment phase. Initial attachment can occur on tissues or after covering of an abiotic surface by host matrix proteins in the human body (specific, protein-protein interaction) or directly to an abiotic surface (nonspecific interaction) by surface hydrophobicity. MSCRAMMMs expressed by *S. aureus* have the capacity to bind to human matrix proteins such as fibrinogen or fibronectin (Patti *et al.,* 1994; Otto, 2008).

The major functional component responsible for intercellular adhesion essential for formation of multilayered *S. aureus* biofilm is the **p**olysaccharide **i**ntercellular **a**dhesin (PIA) which is also called **p**oly-***N***-**a**cetyl**g**lucosamine (PNAG). Together with other polymers such as **t**eichoic **a**cids (TA) and proteins, PIA forms the major part of slime, the extracellular matrix of biofilm-forming staphylococci (Otto, 2008). In *S. aureus* isolates from animals suffering from mastitis, a cell wall-bound surface protein named biofilm-associated protein, Bap, is involved in adherence to a polystyrene surface, intercellular adhesion, and biofilm formation (Cucarella *et al.,* 2001).

Biofilm detachment which may occur by the detachment of single cells or larger cell clusters is crucial for the dissemination of bacteria to other colonization sites. Some factors such as mechanical forces (e.g. blood vessel flow), cessation of the production biofilm building material such as exo-polysaccharide, and detachment factors such as enzymes that destroys the matrix, or surfactants-like molecules (e.g. δ-toxin or quorum-sensing-controlled surfactant peptides known as **P**henol-**s**oluble-**m**odulins (PSMs)) contributing to the detachment of biofilm (Boles and Horswill, 2008; Otto, 2008).

PSMs is a novel class of small peptides with an amphipathic α-helical structure and strong surfactant-like properties, are produced by most staphylococci, especially pathogenic *S. aureus* and *S. epidermidis*. PSMs can induce the production of proinflammatory cytokines; recruit, activate and lyse neutrophils to help staphylococci evade immune damage; lyse erythrocytes, and are associated with the hemolysis of staphylococcal disease; facilitate the structuring and detachment of

staphylococcal biofilms and disseminate biofilm-associated infection; and kill competing microbes and act as weapons in interbacterial warfare. Therefore, PSMs are considered to be critical virulence-associated factors and to play important roles in the pathogenesis of staphylococci. PSMs can be subdivided in two classes: an alpha-type that is 20 amino acids in length and a beta-type that is approximately 40-45 amino acids in length. Under biofilm conditions, PSM expression is shifted to the beta-type of PSM peptides, which are encoded in an operon (Yao *et al.,* 2005). After attachment to a surface and actively expressing PSM beta-type peptides, some cell clusters discontinue expressing PSM beta-type for yet unknown reasons, possibly due to limited oxygen concentration. Cell clusters with active PSM beta expression detach, leaving gaps in the biofilm, which ultimately leads to the typical structure of a biofilm with mushroom-likes structure and fluid-filled channels that are believed to have a vital function in delivering nutrients to cells in deeper biofilm layers (Costerton *et al.,* 1995; Otto, 2008).

An anti-biofilm drug in staphylococci that would inactive a factor required for biofilm formation that appears to be involved in biofilm-associated infection such as polysaccharide intercellular adhesin (PIA), is worth considering. *Actinobacillus actinomycetemcommitans* produces PIAse, named dispersin B which is a potential anti-biofilm drug (Kaplan *et al*., 2003). Lysostaphin, a peptidoglycan-degrading enzyme, although not biofilm-specific, is being evaluated for therapeutic use against staphylococci biofilm (Wu *et al.,* 2003; Otto, 2008).

Model organism: *C. albicans*

C. albicans belongs to the Ascomycota class of fungi and the family *Saccharomycetaceae*. *C. albicans* is a diploid fungus that can exist in two morphological states (dimorphism): as unicellular yeast (blastopores and chlamydospores), as well as in filamentous forms: hyphae, or pseudohyphae, a chain of distinct cells formed by the failure of mother and daughter to separate after each cell division. This dimorphism is depending upon environmental factors such as nutrients, CO_2, neutral pH, high temperature (37^{o}C), and cell population density (Kohler and Fink, 1996; Brown and Gow, 1999; Romano, 2008). *C. albicans* switched to the chlamydospore morphology when cultured under certain nutrient-poor media (Staib and Morschhäuser, 2007). Among *Candida* species, *C. albicans* has the strongest tendency to form hyphae, which parallels its role as the most virulent *Candida* species. *C. albicans*mutants defective in hyphal formation have almost completely lost their virulence (Sonneborn *et al*., 1999) Strains of *C. albicans* which are 'locked' into either yeast or hyphal morphology are dramatically attenuated in their virulence (Lo *et al.,* 1997).

C. albicans is also well known for its capability to undergo a process called phenotypic switching, which is superficially resembles dimorphism, and is thought to be important for *Candida*'s virulence (Thompson *et al*., 2011). Switching usually is generated spontaneously and discriminated by colony morphology and in some cases by the phenotype of cells in the budding phase. For example *C. albicans* strains WO-1 produces two different colonies: one that grows as round cells in smooth white colonies and one that is rod-like and grows as flat gray colonies. The switching is reversible, and colony type can be inherited from one generation to another (Tao *et al*., 2014).

The capability of *C. albicans* to change its morphological pattern from the yeast (Y) form to the mold or mycelial (M) form in the external environment (YM shift) is important in pathogenesis of candidiasis. In the yeast state, *Candida* is a non-invasive, sugar-fermenting organism, while in mycelia state it is invasive and can produce rhizoids, a filament used to anchor the fungus down. Rhizoids can penetrate mucosa or intestinal walls, leaving microscopic holes and allowing toxins, undigested food particles, bacteria, and yeast to enter the bloodstream (Ryan and Ray, 2004).

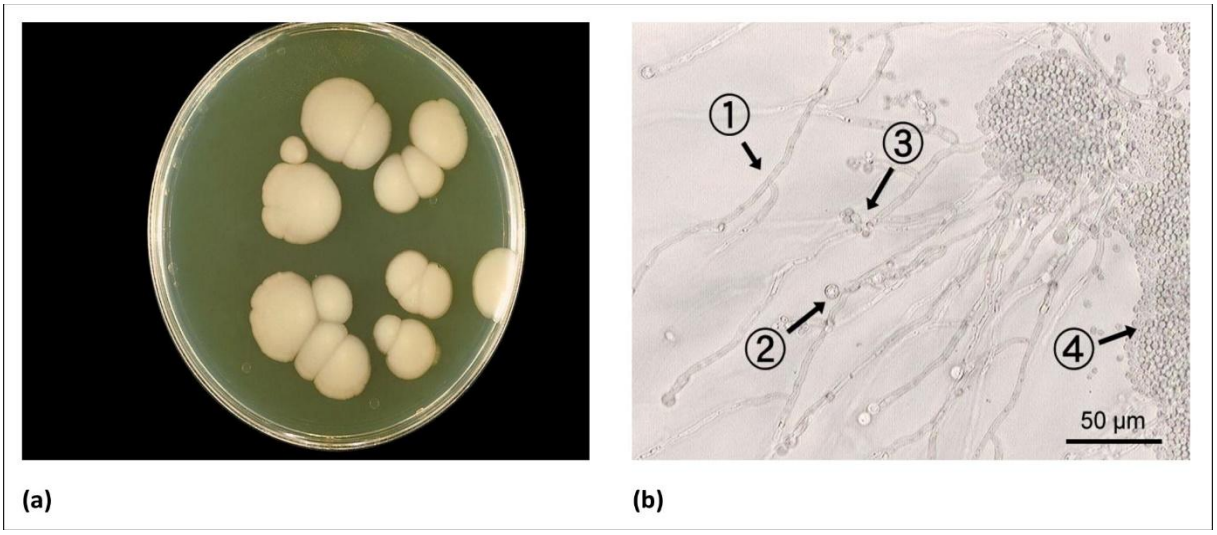

Fig. 7. Multiple views of *C. albicans*. (a) SABHI agar plate culture of the fungus *C. albicans* grown at 20°C, (b) Microscopic image (200-fold magnification) of *C. albicans* ATCC 10231, grown on cornmeal agar medium with 1% Tween80 : 1. Pseudohypha, 2. Chlamydospore, 3. Blastospore, 4. Yeast-like cells (Tambe, 2005[b]; Kaplan, 2009).

C. albicans virulence factors and disease association

Candida is the most common cause of fungal disease of mammals. Mycoses range from the common mild cutaneous or subcutaneous skin infections, to the potentially lethal acute or chronic infection of deep tissue. Of the *Candida* species afflicting humans, *C. albicans* is the most abundant and significant species, and the most commonly studied because it causes a variety of mycotic infections

in humans (Siqueira and Sen, 2004). This fungus has been claimed to be the fourth most common cause of hospital-acquired infectious disease in the United States (Miller *et al*., 2001).

One of the major reasons for *C. albicans* to be such an effective opportunist is that the organism has at its disposal an arsenal of virulence traits to cause a persistent infection in susceptible individuals. These traits include production of secreted aspartyl proteases and phospholipases, host recognition biomolecules (adhesins), phenotypic switching and the ability to undergo morphogenic shift from yeast-to-hyphae (Navarro-Garcia *et al.,* 2001; Si *et al*., 2013). The transition from unicellular yeast form to filamentous form (pseudohyphae or hyphae) is strongly required for *C. albicans* pathogenesis. Yeast forms are more suited for dissemination in tissues and to other hosts, whereas hyphal forms are required for tissue damage and invasion. Yeast cells, when phagocytosed by macrophages, produce hyphae and secrete hyphae-associated proteinases that kill macrophages. These factors prevent hyphal cells from being killed by neutrophils. In addition, hyphal cells have been shown to induce epithelial cell endocytosis. *C. albicans* cells induce epithelial cells to produce pseudopods that surround them, and pull them into the cell, helping *Candid*a cells to invade epithelial cells (Gow *et al*., 2012). The ability of *Candida* to form biofilms on catheters, endotracheal tubes, pace-makers, and other prosthetic devices has contributed to its predominant prevalence in nosocomial infections. Dental plaque is a well-known example of biofilm formation from *Candida* cells, and is responsible for oral candidiasis. Biofilm formation on such tissues is favored by a high concentration of glucose, serum, and other proteins (Calderone and Fonzi, 2001).

Normal amounts of *Candida* live in the mouth, stomach, and vagina, and do not cause infections. Candidiasis (*Candida* infections) can occur in the mouth, vagina, skin, stomach, and urinary tract when there is an overgrowth of *Candida*. Causes may include taking certain drugs (especially antibiotics, corticosteroids, and some birth control pills), pregnancy, being overweight, having a bacterial infection, or several different health conditions (for example, a weakened immune system, diabetes, and psoriasis). About 75% of women will get a vaginal yeast infection during their lifetime, and 90% of all people with HIV/AIDS develop *Candida* infections. Oral infections, called oral thrush, are most common in infants, elderly people, and those with a weakened immune system (Calderone and Fonzi, 2001).

***Candida* QS and biofilm**

QS has a contribution to morphogenic control in *C. albicans*. The yeast-to-hyphal shift *in vitro* is dependence on cell density, a phenomenon called the inoculum size effect. The inoculum effect is seen when yeast cell are diluted to concentrations less than 10^6 CFU in culture medium, under

conditions which affect the cells to germinate into the hyphal form (pH 7.5, 37^{o}C). Inoculation of the cells at higher concentration will maintain the cells in yeast morphology (Kruppa, 2008).

The basis for this cell-density-dependent control of morphogenesis is similar to that which is seen with bacterial cells regulating their activities via QS. The QS molecule in *C. albicans* is farnesol [(2E,6E)-3,7,11-trimathyldodeca-2,6,10-trien-1-ol], farnesoic acid [(2E,6E)-3,7,11-trimethyldodeca-2,6,10-trienoic acid], and tyrosol [4-(2-hydroxyethyl)phenol]. Farnesol was shown to prevent the germination of yeast cells into mycelia, a phenomenon that may be pertinent to *C. albicans* biofilm formation. Farnesoic acid, like farnesol, acts to block the yeast to filament transition, but it is far less active than farnesol and produced by only one known strain of *C. albicans* 10231, while tyrosol is a promoter of hyphal development, shortens the lag-time for cells to begin germinating when present under hyphal-inducing conditions (Oh *et al.,* 2001; Hornby *et al.,* 2001; Alem *et al.,* 2006).

The capacity of *C. albicans* to cause disease is closely associated with their ability to grown as biofilm communities, in contrast to planktonic growth. The biofilm of *C. albicans* formed in almost any medical devices including catheters, joint prostheses, cardiac valves, artificial bypass devices, pacemakers, contact lens, dentures, and intrauterine devices (IUDs). Candidiasis associated with indwelling devices can result in serious medical complications, expensive care and limiting the prolonged use of the devices, since removal of the device is nearly always necessary to cure the infections (Nett and Andes, 2006; Lal *et al.,* 2008).

Candida biofilm formation occurs in three developmental phases over a period of 24-72 hours. The initial phase (0-11 h) begins with the adherence of yeast cells to the substrate. The intermediate phase (12-30 h) involves the attached cells proliferations to form microcolonies and producing carbohydrate-rich extracellular matrix. The maturation phase (31-72 h) occurs when adherent cells are achieved with a biofilm consisting of interspersed yeast/hyphae cells encased in the thick extracellular matrix. Finally, newly formed yeasts on the surface of biofilm are released from the biofilm which allows further dissemination of the organism (Blankenship and Mitchell, 2006).

Biofilm-associated fungal cells are much more resistant to the traditional anti-fungal agents. Resistance of biofilm cells can be up to 1000-fold greater than that of planktonic cells and can also resist host immune factors (Hawser and Douglas, 1995). Several factors have been proposed for the increased anti-fungal resistance of *C. albicans* biofilms. These include altered growth／metabolic rate of cells within biofilm, presence of extracellular matrix with delayed penetration of the anti-

microbial agent, expression of resistance genes and presence of persister (drug tolerant) cells (Tobudic *et al.,* 2011). A biofilm consists of a heterogeneous population of cells with different growth rates; therefore, a subpopulation of cells could also confer anti-fungal resistance because of their slower growth rate (Kumamoto, 2002). Extracellular matrix can act as a physical barrier that prevents the access of anti-microbials to cells embedded in the biofilm community. This barrier is depends on both, amount and nature of the extracellular matrix, as well as the physicochemical properties of the drug (Tobudic *et al.,* 2011).

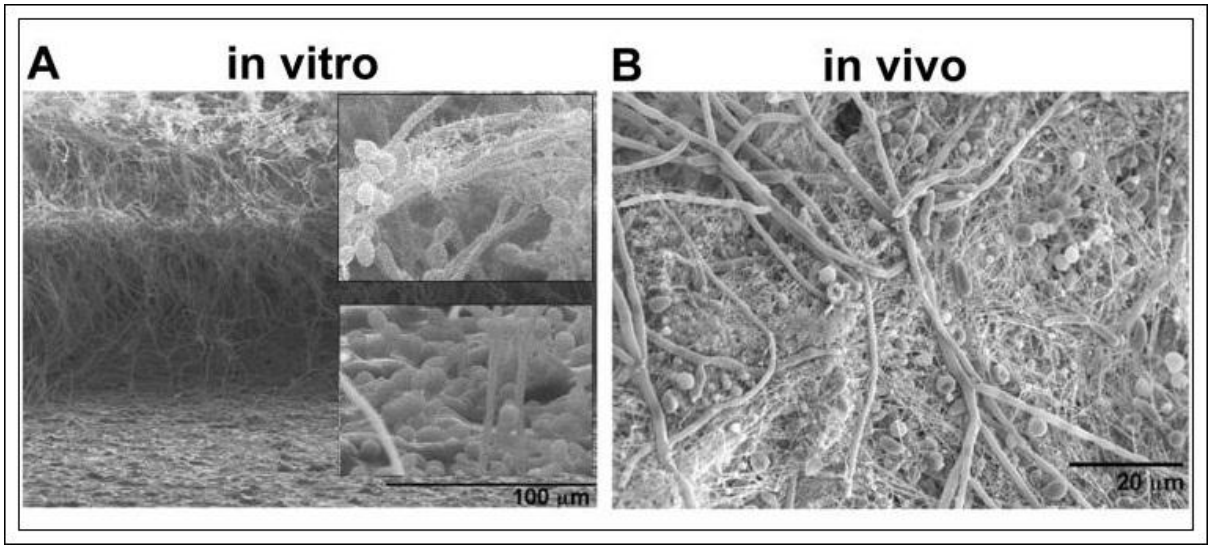

Fig. 8: *C. albicans* biofilm structure *in vitro* and *in vivo.* A) Scanning electron micrograph (SEM) of an *in vitro* biofilm. The biofilm sample was sliced to reveal three layers in a cross-sectional view. The basal layer includes primarily yeast cells, as evident in the lower enlarged inset. The central layer is mainly hyphae. The upper layer has yeast cells budding from the hyphae. The upper enlarged inset shows extracellular matrix material, which appears fibrous in this preparation. B) SEM of an *in vivo* biofilm from the rat catheter model. Yeast cells, hyphae, and some pseudohyphal cells are evident, along with extracellular matrix material (Finkel and Mitchell, 2011).

The involvement of two different types of membrane-localised efflux pumps, adenosine triphosphate-binding cassette transporters encoded by the ***C****andida* **d**rug **r**esistance (CDR) genes and major facilitators encoded by the **m**ulti-**d**rug **r**esistance (MDR) genes increased azole anti-fungal resistance in planktonically grown of *C. albicans*. The expression of MDR and CDR genes in biofilms is phase-specific, contributing to azole resistance only during the early phases of biofilm growth. Moreover, changes in the membrane sterol composition also contribute to azole resistance in the intermediate and mature phases of C*. albicans* biofilm (Mukherjee *et al*., 2003).

Drug discovery from natural sources

Natural products are products from natural origin, including an entire organisms (plants, animals, or microbes) that have not been subjected to any kind of processing or treatment other than a simple process of preservation (e.g., drying); part of an organism (e.g., leaves or flowers of a plant, an isolated animal organ); an extract of an organism or part of an organism, exudates, and pure compounds (e.g., alkaloids, coumarins, flavonoids, glycosides, lignans, steroids, sugars, terpenoids, etc.) isolated from plants, animals, or microorganisms (Sarker *et al*., 2006).

Traditional medicinal use of natural products plays a major role since in ancient civilizations and in many parts of the world. The importance of natural products as a source of medicines is acknowledged by the World Health Organization (WHO). There are many example of drugs derived from natural product, such as compounds from plants: artemisinin from *Artemisia annua* (Weathers *et al.,* 2011), Taxol from *Taxus baccata* (Malik *et al*., 2011); compounds from microbes: menisporopsin B from *Menisporopsis theobromae* (Chinworrungsee *et al.,* 2006), hirsutellide A from *Hirsutella kobayasii* (Vongvanich *et al*., 2002); and compounds from animals (Trabectedin from *Ecteinascidia turbinata* (Monk *et al*., 2012), and Ziconotide from *Conus magus* (McGivern, 2007).

Natural products remain to play an important role in the discovery of new drugs in the coming years as a rich source of novel chemotypes. It is because of plants and other organisms produce many biologically active compounds for defense and other purposes that are often very complex and offer incomparable structural diversity. Synthetically prepared drugs can never compete with the natural variation therefore the screening and isolation from natural sources is the only feasible way to access them. Natural products have also been an invaluable source to synthesize novel drug candidates. There are three roles of natural products to the development of new drugs; by acting as new drugs to be used directly for the treatment of disease; by indicating new modes of pharmacological action which allow complete synthesis of novel analogs (e.g. synthesis analogs of penicillin from *Penicillium notatum*); and by providing natural blueprint used for the synthesized of more complex molecules (e.g. diosgenin from *Dioscorea floribunda* for the commercial synthesis of cortisone, pregnenolone, progesterone, and other steroid products) (Sarker *et al.,* 1999; Li and Vederas, 2009). The structural analysis of natural compounds and the ability to synthesize them allowed chemist to modify them in order to suppress or enhance certain characteristics such as solubility, efficiency or stability in the human body (Shelley, 2009; Ji *et al.,* 2009).

Plants as anti-microbial therapies

The search for pharmaceutically active compounds to combat new and existing diseases is ongoing, and higher plants have often been targeted as part of this search because they contain an abundance of potentially active secondary metabolites. It is estimated that there are 250.000 to 500.000 species of plants on Earth (Boris, 1996). About 155.000 seed plants occur in the tropics, with about 120.000 in the tropical moist forest alone (only 7-8% of the land surface on earth). This is due to the high humidity, elevated temperature, species density and a continuous growing season. The result is an enormous diversity of chemical structures, including secondary metabolites involved in many biological processes and interactions, e.g., as attractants of pollinators, signal products, defensive substances against predators and parasites, or in resistance against pests and diseases. Since only 5-15% of the higher plants have been systemically investigated for the presence of the bioactive compounds, nature's biodiversity remains largely unexplored (Cragg *et al*., 1997; McGee, 2006).

Plants have always played a central role in the prevention and treatment of disease since the ancient time. Many modern medicines are derived from traditional medicinal plants. The illnesses treated by medicinal plants cover an incredibly broad range, from minor ailments such as cuts and skin infections, to more severe diseases including typhoid fever, diabetes, and cancer-like conditions (Clardy and Walsh, 2004). The goals of using plants as sources of therapeutic agents are: to isolate bioactive compounds for direct use as drug, e.g., atropine, scopolamine, digoxin, digitoxin, morphine, reserpine, taxol, vinblastine, vincristine; to produce bioactive compounds from novel or known structures, using them as lead compounds for (semi)synthesis of novel patentable entities with better activity and/or lower toxicity, e.g., metformin, nabilone, oxycodone, taxotere, taniposide, terapamil, podophyllotoxin; to use natural product as pharmacological tools, e.g., lysergic acid diethylamide, mescaline, strychnine, yohimbine; and to use the whole plant or part of it as a herbal remedy, e.g., cranberry (*Vaccinium macrocarpon*), *Echinacea*, feverfew (*Tanacetum parthenium*), garlic (*Allium sativum*), Ginkgo (*Ginkgo biloba*), St. John's wort (*Hypericum perforatum*), and saw palmetto (*Serenoa repens*). There are four standard approaches available for selecting plants for medicinal purposes: random selection followed by chemical screening, random selection followed by anti-microbial assay, follow-up of anti-microbial assay reports, and follow-up of ethnomedical or traditional uses of plants against infectious diseases (Fabricant and Farnsworth, 2001).

Following the Amazon rain forests, Indonesia has the second biggest biodiversity in the world

expressed by a high number of indigenous medicinal plants. As an archipelagic state with thousands of islands, Indonesia is endowed with a rich and unique biodiversity. The area of Indonesian tropical forests covers about 143 million hectares and is inhabited by about 80% of the world's medicinal plants. It is estimated that the Indonesian tropical forests inhabit 28,000 plant species. There are various reports concerning the inventory of higher plant in Indonesia. The Indonesian Country Study on Biodiversity (ICSBD 1993) puts the number of flowering plants species in Indonesia between 25,000 and 30,000. Some 40 million Indonesians depend directly on the country's biodiversity, and the Indonesian community makes use of around 6,000 plant species. Data of the number of medicinal plants also vary. Zuhud *et al.* (2001) identified 1,845 species with medicinal potential in the forests of Indonesia. These numbers are potentially to be updated due to the continuing inventory and investigation of yet unidentified species (Damayanti *et al.,* 2011).

A large number of anti-microbial agents derived from traditional medicinal plants are available for treating various diseases caused by microorganisms. There are vast amount of published scientific information from around the globe describing the anti-microbial activities of plant extracts against a range of bacteria, or fungi. The anti-bacterial activity also could be due to various chemical components. The useful major groups of anti-microbial phytochemicals can be divided into several categories that include alkaloids, flavones (flavonoids, flavonols, quinones), essential oils, lectins, polypeptides, phenolics, polyphenols, tannins and terpenoids (Samy and Gopalakrishnakone, 2008).

The use of medicinal plants in Indonesia has always been a part of culture that has been passed down from generation to generation. Throughout the centuries, Indonesia's indigenous people developed traditional medicines from plants identified by their forefathers for curing illness and keeping their health (Bermawie, 2004). By trial-and-error the country's early inhabitants learns how to distinguish useful plants with beneficial effects from those that were either toxic or non-active. They picked, kept and used medicinal plants to satisfy their basic needs and even experimented on combinations of plants or processing methods to gain optimal results. The abundantly available medicinal plants in Indonesia might be the great potential source for the isolation of novel, naturally-occuring anti-microbial active compounds with high activity against microbial biofilms.

Main hypotheses and aims of this thesis

Hypothesis: Some traditionally used Indonesian medicinal plants have anti-infective properties which may affect biofilm formation of human pathogenic microorganisms such as *P. aeruginosa*, *S. aureus* and *C. albicans*.

Specifically this research will investigate of the presence of anti-microbial and anti-biofilm activity of Indonesian medicinal plants against *P. aeruginosa*, *S. aureus* and *C. albicans* planktonic growth and biofilm. The construction of extract libraries of plant material will provide the source for screening and elucidation of anti-microbial (anti-biofilm) compounds. The capability of these plants in inhibiting QS systems also will be determined. Purification and characterization together with determination of the mode of action of the active compound(s) from these plants species is important in order to obtain compound(s) that may be used for drug candidate against biofilm infection and will be powerful tools in the successful treatment of microbial infections.

Aim and Thesis Outline

Microbial biofilms are responsible for several chronic diseases that are difficult to treat. Biofilm protects the microbial from killing by antibiotics. Even sensitive microbials that do not have a known genetic basis for resistance can have profoundly reduced susceptibility when they form a biofilm. In biofilms, poor antibiotic penetration, nutrient limitation and slow growth, adaptive stress responses, and formation of persister cells are hypothesized to constitute a multi-layered defense. Disabling biofilm resistance may enhance the ability of existing antibiotics to clear infections involving biofilms (Stewart and Costerton, 2001; Stewart, 2002). This thesis aims to discover new anti-microbials from medicinal plants with a high activity against microbial biofilms and to analyze if such compounds will exhibit a synergistic effect with other known antibiotics.

Chapter 1 reviews the discovery of drugs from natural sources, the use of plants as anti-microbial therapies, the quorum sensing mechanism in microbial related to biofilm formation, biofilm involved infections, antibiotic resistance of biofilm cells and emergence of multi-resistant microbial pathogens and strategies to discover anti-biofilm therapeutics.

Chapter 2 describes the screening for anti-microbial compounds on microbial biofilms. Compounds are isolated from Indonesian medicinal plants by ethanol extraction to discove new candidate antibiotics to be used against microbial biofilms.

Chapter 3 and 4 are focused on the discovery of novel essential oils as anti-microbials from Indonesian medicinal plants. The effect of the plant essential oils on bacterial cells and microbial biofilms is studies using viability staining, fluorescence microscopy analysis and colony forming unit (CFU) determination along with toxicity assay of the isolated compound(s).

Chapter 5 focuses on the screening of the Indonesian medicinal plants ethanol extract and essential oils for their capability to inhibit the quorum sensing mechanisms.

The purification and structural identification of the most potential compounds to obtain a novel anti-biofilm compound along with the analysis of a synergistic effect of novel anti-biofilm activities isolated from medicinal plants in combination with known antibiotic used to treat biofilms is described in **Chapter 6**.

In **Chapter 7** a general discussion is given about the results of the research described in this thesis.

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