MOLECULAR CHARACTERIZATION OF RHAMNOLIPID AND ITS EFFECT ON CANDIDA BIOFILM

A THESIS

Submitted in partial fulfilment of the requirements for the award of the degree

of

DOCTOR OF PHILOSOPHY

in

CHEMISTRY

by

NIVEDITA

DEPARTMENT OF CHEMISTRY
INDIAN INSTITUTE OF TECHNOLOGY ROORKEE
ROORKEE - 247 667 (INDIA)
DECEMBER, 2011

INDIAN INSTITUTE OF TECHNOLOGY ROORKEE
ROORKEE

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled, **"Molecular characterization of rhamnolipid and its effect on Candida biofilm"** in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy and submitted in the Department of Chemistry of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during the period from January 2007 to December 2011 under the supervision of Dr. G. Bhattacharjee, Professor, Dr. A. K. Singh, Professor, Department of Chemistry and Dr. Vikas Pruthi, Associate Professor, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee, India.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other institute.

Nivedita

(NIVEDITA)

This is to certify that the above statement made by the candidate is correct to the best of our knowledge.

(A. K. Singh)	(Vikas Pruthi)	(G. Bhattacharjee)
Supervisor	Supervisor	Supervisor

Date: 23/12/11

The PhD Viva-Voce Examination of **Ms. Nivedita,** Research Scholar has been held on...4th..April..,.2012

Supervisors

Chairman SRC

External Examiner

Head of the Department & Chairman ODC

ABSTRACT

Biosurfactants are amphipathic compounds possessing both hydrophilic and hydrophobic moieties that partition preferentially at the interface between two different phases in a heterogeneous system. In past two decades, these surface active compounds have gained significant commercial importance as compared to their synthetic chemical counterpart due to their tailor-made multifaceted diversity, eco-friendly nature, higher biodegradable ability, low toxicity, effectiveness at extreme pH and temperature suitability for large scale production and selectivity. Among various types of biosurfactant available rhamnolipid, glycolipid-type biosurfactant, specifically produced by *Pseudomonas aeruginosa* are most effective surfactants which have great potential for biomedical and industrial applications. Rhamnolipid have also been found to have antagonistic effects on economically important zoosporic plant pathogens, thus opening their avenues as biocontrol agents. Recently, rhamnolipid has emerged as a promising multipurpose ingredient, which exhibit emulsifying, anti-adhesive and antimicrobial activities simultaneously and are consequently suitable for many food applications. However, despite their potential and biological origin, their role in commercialized usage as an effective therapeutic agent to combat infectious diseases is still to be established. This lacuna of their usage in the biomedical field prompted us to undertake the present investigation on *in vitro* studies on rhamnolipid medicated biofilm inhibition using *Candida albicans* as a model system. *C. albicans* is an opportunistic dimorphic pathogen which commonly affect immuno-compromised individual and is capable of causing life threatening infection. In fact, *C. albicans* is ranked fourth in causing nosocomial infections, third in catheter-related infections, second highest in colonization to infection rate and highest overall in crude mortality. It is believed that *C. albicans* in its biofilm mode consists of a structured communities of cells embedded in biomatrix. The process of biofilm

formation has been divided into different stages involving initial attachment to surface, formation of microcolonies on the surfaces and finally differentiation of microcolonies into exopolysaccharide-encased mature biofilm. Such biofilm can cause potentially enormous damage specially when formed on medical devices which can result in the failure of these devices and serve as a reservoir or source for infections. Thus, it becomes important to control primary colonization of fungal growth as an effective therapeutic solution not only to removal such fungal biofilm but also to eradicate them from surfaces. The objective of present work was thus outlaid to isolate, screen, purify and assess the ability of rhamnolipid as pointed below, as potential alternative to the available conventional therapies for the inhibition of *C. albicans* biofilm. From the petroleum oil contaminated sites, soil samples were taken and screened for native bacterial population for biosurfactant production. Through, screening on minimal media supplemented with different hydrocarbons as sole organic source, 25 isolates (NSVP1-NSVP10, DSVP11-DSVP20 and SSVP21-SSVP25) were selected. These 25 bacterial isolates were then tested for haemolytic ability, drop collapse assay, emulsification assay and surface tension reduction as an index for surface active agent production. Of these four isolates namely NSVP2, DSVP11, DSVP17 and DSVP20 were found to pose biosurfactant producing ability and among them DSVP20 was found to be potential producer of biosurfactant. Through 16S rRNA sequencing and taxonomic characterization, it was found out that DSVP20 exhibited 97-99% similarity with *Pseudomonas sp.*. Further, biochemical tests and cultural characteristics established DSVP20 as *Pseudomonas aeruginosa* (GenBank accession no. GQ865644). Extraction of biosurfactant was done from the cell free broth through acidification followed by organic extraction with ethyl acetate. The extracted biosurfactant was further purified through absorption chromatography. The FTIR spectrum of bisourfactant was recorded in the spectral region

of 4000–500 cm^{-1} which exhibited specific absorption bands at 3381 cm^{-1}, 2927 cm^{-1}, 2859 cm^{-1}, 1735cm^{-1}, 1635 cm^{-1}, 1300–1100 cm^{-1}, 1013 cm^{-1}, 906 cm^{-1} and 840 cm^{-1} indicating the chemical structure to be di-rhamnolipid (RL2). The weak broad band located at 3364 cm^{-1} can be attributed to the O–H stretching vibrations of hydroxyl groups, while the strong absorption peaks at 2927 cm^{-1} and 2859 cm^{-1} are assigned to the C–H stretching vibrations of the hydrocarbon chain positions. Stretching band of C=O at 1730 cm^{-1} is characteristic of ester bonds and carboxylic acid groups. The carbonyl stretching peak was observed at 1640 cm^{-1}, which is characteristic of ester compounds. The C–O stretching bands in the range of 1457–1100 cm^{-1} related to the bonds formed between carbon atoms and hydroxyl groups in the chemical structures of the rhamnose rings and the ester carbonyl group was also confirmed from the peak at 1057 cm^{-1}, which corresponds to C-O stretching vibration. Relatively strong sorption bands of pyranyl at 906 cm^{-1} and 840 cm^{-1} suggest it to be RL2 in nature. MALDI-TOF mass spectrum analysis of the sample gave intense signals at *m/z* 672.81 corresponding to the sodium adduct $[M + Na]^+$ of the RL2 which is reported to be m/z 649. The characteristic chemical shifts observed from ^1H NMR analysis were 0.895 ppm (for $–CH_3$), 1.262 ppm (for $–(CH_2)_6$), 2.552 ppm (for $–CH_2–$ COO–), 4.878 ppm (for –O–CH–), and 5.243 ppm (for –COO–CH–). The ^{13}C NMR also displayed chemical shifts of 102.317 and 94.450 ppm which is a characteristic peak of RL2. The RL2 present in the cell free broth of *Pseudomonas sp.* showed thermal, pH and salt stability when tested at different time intervals. The surface tension and critical micelle dilution (CMD) values remained stable after exposure to high temperatures (100°C) even after 4 h. The surface activities were retained over a pH range of 4–12 with minimal deviation in surface tension and CMD^{-1} values, whereas CMD^{-2} showed a slight and gradual increase in surface tension with increasing pH values. No appreciable changes were observed on surface tension and

CMD values with the addition of NaCl up to 10% in cell free broth. Scanning electron microscopy, confocal laser scanning microscopy and atomic force microscopy were used in the study to explore the structural integrity and developmental characteristics of *C. albicans* biofilm. SEM observations provided useful information on the different cellular morphologies present in the biofilm structure. The mature biofilm consist of a mixture of yeast and filamentous forms embedded within exopolymeric material. Confocal laser scanning microscopy of *C. albicans* biofilm combined with fluorescent stains, FITC-ConA and PI showed presence of EPS in cell bound as well as secreted form. It demonstrated that *C. albicans* possess structural heterogeneity and displays architectural similarity to that of bacterial biofilm. Atomic force microscopy provided a surface view of the *C. albicans* biofilm providing resolution at the atomic level. AFM was shown to be sensitive enough to dissect the effect of subtle changes in overall *C. albicans* cell surface composition on the initial interaction with biomaterials. XTT (2, 3-bis [2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) reduction assay data showed maximum adherence with 48 h grown culture. Data also suggested that biofilm were highly metabolically active in its first 24 h. However, as the *Candida* biofilm matures and become more complex (48 to 72 h), the metabolic activity reached its plateau, reflecting high number of cells that constitute the mature biofilm. The minimum inhibitory concentrations (MICs) of RL2 were evaluated against *C. albicans* by standard broth dilution assay on different stages of *C. albicans* biofilm formation formed on 96 well microtitre plates. *In vitro* studies showed that anti-adhesive activity of RL2 to inhibit the *C. albicans* adhesion to be concentration dependent. Our observation showed about 50% of candidal cells attached to 96-well plate after 2 h of treatment with 0.16 mg mL^{-1} RL2. RL2 (5.0 mg mL^{-1}) isolated from *P. aeruginosa* DSVP20 was able to reduce 96% of pre-biofilm formed on 96 well microtitre plate. Cell surface hydrophobicity data recorded

also indicated that with increase in RL2 concentration, there is a marked reduction in cell surface hydrophobicity of *C. albicans* biofilm. *C. albicans* is the most common human fungal pathogen associated with device related infections mainly in its biofilm mode of growth. As biofilm residing *Candida* cells achieved much more resistance against antimicrobials than their planktonic counterparts, the fact that the RL2 was able to inhibit *C. albicans* biofilm adhesion as well as formation asserts on the possible use of RL2 as an alternative antifungal agent.

ACKNOWLEDGEMENT

First and foremost I would like to thank Prof G. Bhattacharjee Professor, Department of Chemistry, IIT Roorkee, Roorkee, for his ever enthusiastic spirit and constant support, which has contributed to the successful completion of this thesis.

My sincere thanks to Dr. A. K. Singh, Professor, Department of Chemistry, IIT Roorkee, Roorkee, for his valuable comments and suggestions towards the improvement of my Ph.D. work right from the beginning. He has been outstandingly supportive throughout.

Words are insufficient to convey gratitude to my IIT supervisor, Dr. Vikas Pruthi, Associate Professor, Department of Biotechnology, IIT Roorkee, Roorkee. It is my proud privilege to express my deep sense of gratitude to him for his keen interest, valuable suggestions, critical comments from time to time and constant encouragement during the entire course of my Ph.D. His guidance helped throughout my research and writing of this thesis.

I am thankful to my SRC (Student Research Committee) members: Prof. S. M. Sodhi, Dr. R.K. Peddinti, Associate Professor (Department of Chemistry) and Prof. I. D. Mall, (Department of Chemical Engineering), IIT Roorkee, Roorkee, for their input, valuable discussions and accessibility. Their critical comments have always been for the betterment of my own work. They have shared valuable insights relevant to the study in basic education and not just in the technology sector.

I acknowledge MHRD (Ministry of Human Resources and Development), Government of India, New Delhi, for providing financial assistance during my research period.

I would like to express my earnest gratitude and thanks to Dr. Naveen Kumar Navani and Dr Ranjana Pathania, Assistant Professors, Department of Biotechnology, IIT, Roorkee. I am thankful that they gave me a chance to work in their laboratory for a period of six months. I owe to them and their team an important component in my research. I would especially like to thank students of the CBDD group without whose intellectual and scientific contribution I would have never been able to complete my degree.

It was a pleasure to share doctoral studies and life with wonderful people like Dr. Sameena Ma'am, Dr. Jitendra Sir, Dr. Prerna, Koteshwar, Anjali and Manoj, my first lab mates.

I am grateful to Suma, Sonal, Priya and Richa who provided for some much needed humour and entertainment in what could have otherwise been a somewhat stressful laboratory environment and also for their friendship and for sharing the glory and sadness of conference deadlines and day-to-day research.

I take this privilege to thank all the Chemistry and Biotechnology office staff for their academic support and for tirelessly doing the much needed paper work.

My heartfelt thanks to all my friends and colleagues: Dr. Nidhi, Dr. Manali, Preety, Santosh, Rajni, Tapas, Dr. Rashmi Lorish and Dr. Jyoti Agrawal, who have helped by sparing their valuable time by sharing their thoughts with me and also providing some light moments amidst the stress of the project.

Also, the warmth and care extended by my Mama Dr. Dharmendra Singh, Associate Professor, Department of Electronics and Computer Engineering, IIT Roorkee, Roorkee, his loving wife Mrs. Preeti Singh who always supported me emotionally, and his adorable children Vatsala and Varu is worth mentioning and is thanked for. I am indebted to them for taking care of me and for giving me constant

encouragement and support without which I would not have been able to come this far, it was like a home away from home.

I am indebted to many of my colleagues for their support, a few who deserve special mention are Satya, Iyer, Abhishek, Parmesh and Manasi for the stimulating discussions, for the sleepless nights we were working together before deadlines, and for all the fun we have had in the last years for creating a pleasant working atmosphere. They were always by my side whenever I needed them. I will forever fondly remember them, their smiles and their silly jokes.

I owe sincere thanks to my sweet roommates (friends) Dr. Rashmi and Dr. Prachi who made my stay here at Roorkee the most memorable one. They always stood by me, understood me, faced my anger with a smile and helped me believe in myself whenever I failed to do so. Their love and care will always be cherished.

To my best friend Jyoti, who will always be my no. one critic and silent supporter. Thank you for being with me in happiness and pain. I wish we will be friends and live together, forever.

There were few people outside the IIT campus who kept my connection with the rest of the world alive. I owe thanks to the love, care and support extended by my Bua Mrs. Shanti Singh and Manasi's parents Professor P.K. Gupta and Mrs. Mamta Gupta.

My Chacha Dhirendra Singh and Mikki mausi, deserve a special mention for always having taken care of family problems which left me free to focus on my research.

Last but not the least, where would I be without my family? My parents deserve special mention for their inseparable support and prayers for giving birth to me at the first place and supporting me spiritually throughout my life. My Father, Dr. S. B. Singh, who is also my role model, is the person who taught me to lead life amongst

difficulties and obstacles with a smile. He showed me the joys of intellectual pursuit ever since I was a child and always supported me in all my decisions throughout my life. My Mother, Sheela Singh, is the one who sincerely raised me with her caring and gentle love. Rahul, my brother for his support and care, behind his tough exterior.

Words fail me to express my appreciation for my husband Avijit whose dedication, love and persistent confidence in me, have taken the load off my shoulder. I owe him for selflessly letting his intelligence, passions, and ambitions collide with mine I would also thank my father-in-law Dr. Ashok Shahi, my mother-in-law Mrs. Vibha Shahi and my brother-in-law Ashutosh, for supporting me selflessly throughout my research.

Finally, my greatest regards to the Almighty for bestowing upon me the courage to face the complexities of life and completing this project successfully. Thank you so much God for giving me this skill in writing. I hope I didn't fail you, I am freer now and have become a fearless and stronger woman.

LIST OF PUBLICATIONS/PAPERS PRESENTED IN CONFERENCES/SYMPOSIA

- **Nivedita Singh,** Vishnu Agarwal, Suma C. Pemmraju, Richa Panwar and Vikas Pruthi (2010). Impact of infectious *Candida albicans* biofilm on biomaterials. ***Indian Journal of Biotechnology*** 10:417-422.

- **Nivedita Singh,** Swaranjit S Cameotra, Gurudas Bhattacharjee, Ashok k Singh, Vikas Pruthi. Influence of di-rhamnolipid from *Pseudomonas aeruginosa* on *Candida* Biofilm. (Manuscript submitted)

- **Nivedita Singh,** Akhansha Nayyar, G. Bhattacharjee, A. K. Singh, Vikas Pruthi Assessment of herbal dentifrices against *Candida* biofilm. (Manuscript submitted)

- **Nivedita Singh,** G. Bhattacharjee, A. K. Singh, Vikas Pruthi Eugenol mediated eradication of *Candida albicans* biofilm formed on surgical sutures. (Manuscript submitted)

- **Nivedita Singh,** Vishnu Agarwal, Vikas Pruthi (2010) Impact of infectious *Candida albicans* biofilm on biomaterials. International Conference on Genomic Sciences, Tamilnadu.

- **Nivedita Singh,** Suma C Pemmaraju, A K Singh, G Bhatacharjee, Vikas Pruthi (2011) Prevention of *Candida albicans* biofilm on contact lenses using Eugenol. 52nd Annual

AMI conference International Conference on Microbial Biotechnology for sustainable development, Chandigarh.

- **Nivedita Singh**, Akhansha Nayyar, G. Bhattacharjee, A. K. Singh, Vikas Pruthi (2011) Assessment of herbal dentifrices against *Candida* biofilm. NHBT, Trivandrum.

- Suma C Pemmaraju, Deepak Sharma, **Nivedita Singh**, Richa, Vikas Pruthi (2011) Production of microbial surfactants from oily sludge contaminated soil by *Bacillus subtilis* DSVP23. NHBT, Trivandrum.

- **Nivedita Singh,** Vishnu Agrawal, Suma C Pemmaraju, S. Jain, G. Bhattacharjee, A. K. Singh, V. Pruthi (2011) Inhibition of *Candida albicans* biofilm formed on biopolymeric surfaces by plant oils. International Conference on Yeast Biology, Mumbai.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

%	:	Percentage
µl	:	Microlitre
µm	:	Micrometer
°C	:	Degree centigrade
AFM	:	Atomic force microscopy
BATH	:	Bacterial adherence to hydrocarbons
BLAST	:	Basic local alignment search tool
cfu	:	Colony forming unit
CLSM	:	Confocal laser scanning microscopy
CMC	:	Critical micelle concentration
CMD	:	Critical micelle dilution
Con A	:	Concavalin A
conc.	:	Concentration
Da	:	Dalton
E_{24} (%)	:	Emulsification index
EPS	:	Extracellular polymeric substances
FITC	:	Fluorescein isothiocynate
FTIR	:	Fourier transform infrared spectroscopy
Fig	:	Figure
g	:	Gram
h	:	Hour
HIC	:	Hydrophobic interaction chromatography
i.e.	:	That is
IIT	:	Indian Institute of Technology
IMTECH	:	Institute of Microbial Technology
IUD	:	Intra uterine device
kHz	:	Kilohertz
kb	:	Kilobasepair
L	:	Litre
M	:	Molar
mg	:	Milligram
MIC	:	Minimum inhibitory concentration
MALDI-TOF-MS	:	Matrix assisted laser desorption ionization time-of-flight mass spectrometry
min	:	Minute
mL	:	Millilitre
mM	:	Millimolar
MM	:	Molecular weight marker
MTCC	:	Microbial type culture collection
MTP	:	Microtitre plate
MOPS	:	3-(*N*-morpholino) propanesulfonic acid

N	:	Normal
mg/mL		Microgram/milliletre
mN/m	:	Millinewton/meter
NCCLS	:	National Committee for Clinical Laboratory Standards
nm	:	Nanometer
NMR	:	Nuclear magnetic resonance
OD	:	Optical density
PAC	:	Pruthi and Cameotra media
PBS	:	Phosphate buffer saline
PI	:	Propidium iodide
ppm	:	Parts per million
PS	:	Polystyrene
rRNA	:	Ribosomal ribose nucleic acid
RPMI	:	Roswell park memorial institute
RP	:	Replica plate test
R_f	:	Retention factor
rpm	:	Rotations per minute
RT	:	Room temperature
RL2	:	di-rhamnolipid
SEM	:	Scanning electron microscopy
SAT	:	Salt aggregation test
sp.	:	Species
Spp.	:	Species
TES	:	Trace element substance
TLC	:	Thin layer chromatography
viz	:	That is to say
v/v	:	Volume/volume
vol	:	Volume
vvm	:	Volume/volume/minute
w.r.t.	:	With respect to
w/v	:	Weight/volume
XTT	:	2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2H- tetrazolium-5-carboxanilide
YEP	:	Yeast extract phosphate
YPD	:	Yeast extract peptone dextrose
ZOI	:	Zone of inhibition

CHAPTER 1

INTRODUCTION

Biosurfactants are amphipathic compounds possessing both hydrophilic and hydrophobic moieties that partition preferentially at the interface between two different phases in a heterogeneous system (Li *et al.*, 2011; Makkar *et al.*, 2011; Mnif *et al.*, 2011; Batista *et al.*, 2006). These amphiphilic compounds accommodate a wide structural diversity exhibited by biomolecules such as glycolipids, lipopeptides, biopolymers, acylglycerols. In past two decades, these surface active compounds have gained significant commercial importance as compared to their synthetic chemical counterpart due to their tailor-made multifaceted diversity, eco-friendly nature, higher biodegradable ability, low toxicity, effectiveness at extreme pH and temperature suitability for large scale production and selectivity (Sánchez *et al.*, 2009; Rodrigues *et al.*, 2006b; Singh and Cameotra, 2004; Cameotra and Makkar, 1998; Desai and Banat, 1997). Among various types of biosurfactants, rhamnolipid is a glycolipid based biosurfactant specifically produced by *Pseudomonas aeruginosa*, with huge potential as surfactants in biomedical and industrial applications (Dusane *et al.*, 2010; Sotirova *et al.*, 2010; Muthusamy *et al.*, 2008; Whang *et al.*, 2008; Nitschke and Costa, 2007; Rodrigues *et al.*, 2006a,b; Irie *et al.*, 2005; Urum *et al.*, 2005; Abalos *et al.*, 2004). Rhamnolipids belongs to a group of glycolipid based biosurfactants and are amphipathic in nature. It comprises of a hydrophilic head, which is formed by one or two rhamnose moieties (respectively called monorhamnolipid and dirhamnolipid) and a hydrophobic tail containing one or two fatty acid chains. Rhamnolipid plays an important application in petroleum-related industries which is used in enhanced oil

recovery, cleaning oil spills, oil-contaminated tanker cleanup, viscosity control, oil emulsification and removal of crude oil from sludges (Whang *et al.,* 2008; Bertrand *et al.,* 1993). Rhamnolipid also imparts antagonistic effect on harmful algal blooms (HABs), which in turn promotes their usage as biocontrol agents (Wang *et al.*, 2005; Stanghellini and Miller, 1997). The emulsifying, anti-adhesive and antimicrobial activity of rhamnolipid makes them suitable for many food applications (de Araujo *et al.*, 2011; Dusane *et al.*, 2010; Nitschke and Costa, 2007; Irie *et al.*, 2005). However, despite their potential and biological origin, their role in commercialized usage as an effective therapeutic agent to combat infectious diseases is still to be established. The antimicrobial basis for rhamnolipid could be accounted by the physiological role it plays i.e. maintaining biofilm porosity through localized abrogation of biofilm. This feature rationalizes rhamnolipid usage as potential antibiofilm agent when used at concentrations much higher than the physiological ones. Their antibiofilm activity had been reported against biofilms of bacterial origins (de Araujo *et al.*, 2011; Dusane *et al.*, 2010; Irie *et al.*, 2005) but against the biofilm of fungal origins, reports are sparse. Surfactin produced from *Bacillus subtilis* and surlactin from *Lactobacillus sp.* are other known examples of biosurfactants acting heterogeneously among various species. This prompted us to undertake the present investigation through *in vitro* studies on rhamnolipid medicated biofilm inhibition using *Candida albicans* as a model system. Although the majority of implant related infections are caused by bacteria like *Staphylococcus epidermidis, Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli*, fungal infections are becoming increasingly important. They are most often caused by pathogenic *Candida* genus, particularly *Candida albicans. C. albicans* is an opportunistic dimorphic pathogen which commonly affect immuno-compromised individual and is capable of causing life threatening

infection (Finkel and Mitchell, 2011; Nett *et al.*, 2008; Ruhnke, 2006; Douglas, 2003). In fact, *C. albicans* is ranked fourth in the causing nosocomial infections, third in catheter-related infections, second highest in colonization to infection rate and highest overall in crude mortality (Subha and Gnanamani, 2008). The increase in *Candida* infections in last decades has almost paralleled the increased usage in broad range of medical implant devices in patients with impaired host defenses. Even with current antifungal therapy, mortality of patients with invasive candidiasis can be as high as 40% (Dominic *et al.*, 2007). It is believed that *C. albicans* in its biofilm mode, consists of a structured communities of cells embedded in biomatrix (Lal *et al.*, 2010; Donlan, 2001). The process of biofilm formation has been classified into different stages involving initial attachment to surface, formation of microcolonies on the surfaces and finally differentiation of microcolonies into exopolysaccharide-encased mature biofilm (Agarwal *et al.*, 2008; Chandra *et al.*, 2001). Biofilm formation is a clinically significant process in view of the fact that (1) they are resistant to antimicrobial agents; (2) they may be a persistent source of infection; (3) they may harbor pathogenic organisms, and (4) they may allow exchange of resistance plasmids (Donlan, 2001). Biomaterials such as stents, shunts, prostheses (voice, heart valve and knee prostheses), implants (lens and breast implants and dentures), endotracheal tubes, pacemakers and various types of catheters have all been shown to facilitate *C. albicans* colonization and biofilm formation (Cao *et al.*, 2008). Such biofilm can cause potentially enormous damage specially when formed on medical devices which can result in the failure of these devices and serve as a reservoir or source for persistent and systemic infections (Cameotra *et al.*, 2009; Uppuluri *et al.*, 2009; Agarwal *et al.*, 2008; Al-Fattani and Douglas, 2006; Mukherjee *et al.*, 2003; Baillie *et al.*, 2000). As factors responsible for *C. albicans* pathogenesis is still unknown, it

becomes extremely vital to control primary colonization of fungal growth. The prophylaxis of inhibiting fungal colonization is an only therapeutic solution for removal as well as abrogation of fungal biofilm from biological surfaces. Since years, azole drugs and derivatives continue to dominate as antifungal agents of choice against *Candida* related infections, as topical applications or as oral drugs. Even though very widely acclaimed for their efficacy, these drugs are known to have side effects (Bruzual *et al.*, 2007; Jain *et al.*, 2007; Sheehan *et al.*, 1999). Besides this, the action of antifungal may be limited by their penetration and chemical reaction into biofilm matrix (Jain *et al.*, 2007).

The objective of the investigation as listed below was to do molecular characterization of surface active agent and to assess its ability as potential alternative to the available conventional therapies for the inhibition of *C. albicans* biofilm.

I. Screening and isolation of surface active agent producing microorganisms
II. Extraction, purification and molecular characterization of di-rhamnolipid from culture broth of *P. aeruginosa* DSVP20
III. Physico-chemical analysis and optimization of growth conditions for di-rhamnolipid production from *P. aeruginosa* DSVP20
IV. To study the effect of di-rhamnolipid on the *C. albicans* biofilm

CHAPTER 2

LITERATURE REVIEW

2.1 Biosurfactant and its nature

Biosurfactant produced by microbes are structurally diverse and heterogeneous groups of surface-active amphipathic molecules which partition preferentially at the interface between fluid phases such as oil/water or air/water interfaces (Sen *et al.*, 2010; Luna *et al.*, 2009; Wang *et al.*, 2007; Rodrigues *et al.*, 2006a; Desai and Banat, 1997; Neu, 1996). The polar, water soluble part of a biosurfactant may consists of simple groups such as carboxylate or hydroxyl or a complex mixture of phosphate, amino acids or peptides, anions or cations, or mono-, di- or polysaccharides. The lipophilic portions comprise hydrocarbon tail that usually made of long chain, saturated or unsaturated, hydroxyl or α-alkyl-β-hydroxy fatty acids and may contain cyclic structures (Banat, 1995). The fatty acid of lipophilic moiety is linked to the hydrophilic group by a glycosidic, ester or amide bond (Rosenberg and Ron, 1999). Most biosurfactants are either neutral or negatively charged. The negatively charged biosurfactants are anionic in nature due to presence of carboxylate, phosphate or sulphate group. Least number of cationic biosurfactants contains amine group (Cooper, 1986). A special property associated with biosurfactant is their ability to lower the surface tension of water from 72 mN m^{-1} to below 35 mN m^{-1} (Desai and Banat, 1997; Cooper, 1986).

Effective physicochemical properties, such as low interfacial tensions, critical micelle concentration (CMC) and also temperature stability are characteristics of these compounds. The heterogeneous groups of these surface-active molecules also reduce the CMC and interfacial tension in both aqueous solutions and in hydrocarbon mixtures. These properties create micro emulsions which lead to micelle formation where

hydrocarbons can solubilize in water or water-in-hydrocarbons (Banat, 1995). As compared to chemical surfactants, these compounds have several advantages such as lower toxicity, higher biodegradability and effectiveness at extreme temperatures or pH values. Furthermore, these molecules can be tailor-made to suit different applications by changing the growth substrate or growth conditions. Besides this, some biosurfactants have proven to be suitable alternatives to synthetic medicines and antimicrobial agents and may therefore be used as safe and effective therapeutic agents.

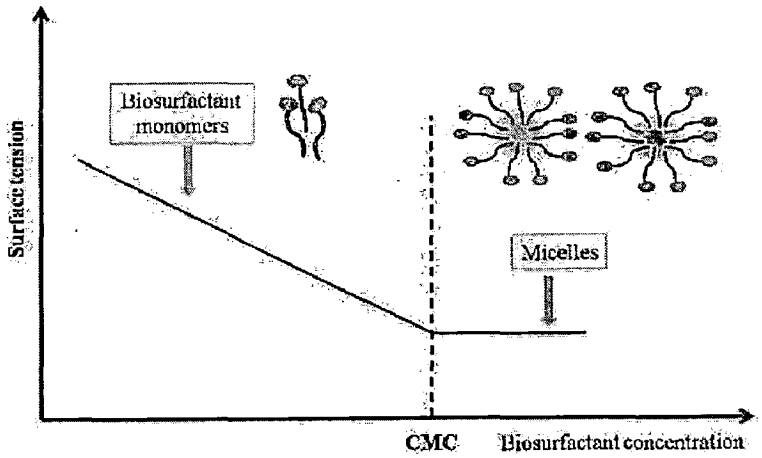


Figure 1: The relationship between biosurfactant concentration, surface tension and formation of micelles

Most biosurfactants are considered secondary metabolites, though some may play essential roles for the survival of the producing-microorganisms either through facilitating nutrient transport, microbe-host interactions or as biocide agents (Sen *et al.*, 2010; Toribio *et al.*, 2010; Rodrigues *et al.*, 2006b). The role of biosurfactant also include increasing the surface area and bioavailability of hydrophobic water-insoluble substrates, heavy metal binding, bacterial pathogenesis, quorum sensing and biofilm formation (Rodrigues and Teixeira, 2010).

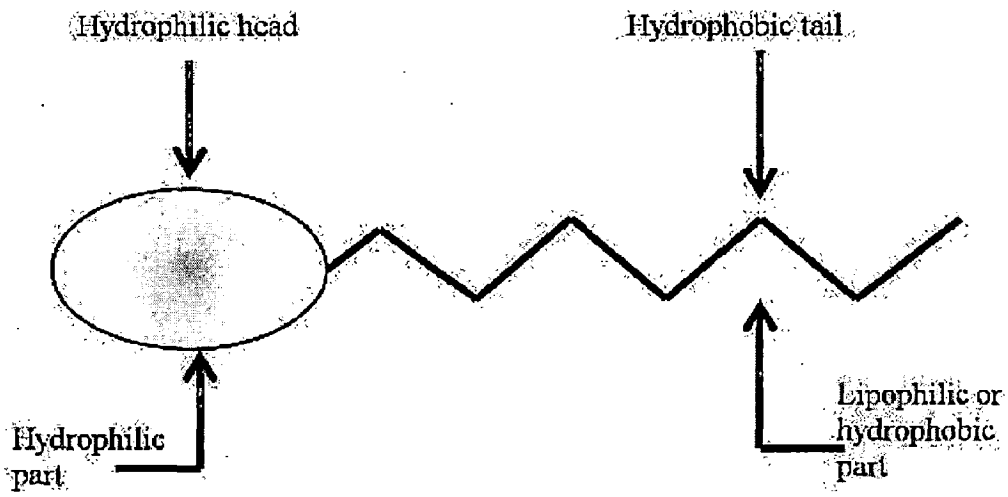


Figure 2: Structure of surfactant molecule

2.2 Classification of biosurfactants

Biosurfactants are classified according to the nature of their polar grouping and categorized mainly by their chemical composition and microbial origin. The major classes of biosurfactant include glycolipids, lipopeptides and lipoproteins, phospholipids, hydrosylated and crosslinked fatty acids, polymeric surfactants and particulate surfactants and it is presumed that diverse properties and physiological functions have been exhibited by different groups of biosurfactants (Sen *et al.*, 2010; Rodrigues and Teixeira, 2010; Rodrigues *et al.*, 2006a; Cameotra and Makkar, 2004; Ron and Rosenberg, 2001).

2.2.1 Glycolipids

Glycolipids are the most known class of biosurfactants. Being a structural element of cell membranes, they are widely distributed across all life forms. They are glycosyl derivatives of various classes of lipids such such as an acylglycerol, a sphingoid, a ceramide (*N*-acylsphingoid) or a prenyl phosphate. Based on the hydrophobic moiety, glycolipids are further classified majority into glycoglycerolipids, glycophosphoinositols and glycosphingolipids. sterolipids, prenolipids, saccharolipids and ketolipids. In saccharolipids, mono, di or oligosaccharides conjugates with beta hydroxy fatty acids

through glycosidic linkage. Based on the sugars involved, saccharolipids are named, for instance; Rhamnolipids derivative of rhamnose, Sophorolipids derivative of sophorose, Trehalolipids derivative of trehalose and Fructose-lipids derivative of fructose (Mulligan, 2005; Kitamoto *et al*, 2002; Rosenberg and Ron, 1999; Desai and Banat, 1997, Lang and Wagner, 1987).

Saccharolipids are widely distributed among prokaryotic genera. In Gram negative bacteria, lipid A which is the part of lipopolysaccharide layer of outer membrane comprises of *N*-Acetylated glucosamines based saccharolipids.

Trehalose lipids (mainly diacyl trehaloses) are found in various Gram positive eubacteria belonging to the mycolates group, the order Actinomycetales (*Mycobacterium, Rhodococcus, Nocardia and Gordonia)*, in fungi, algae and in insects where they may have a role as an energetic reserve compound.

Sophorolipids (SLs) are a group of microbial glycolipids produced by yeasts, such as *Candida bombicola*, *Yarrowia lipolytica*, *Candida apicola*, and *Candida bogoriensis*

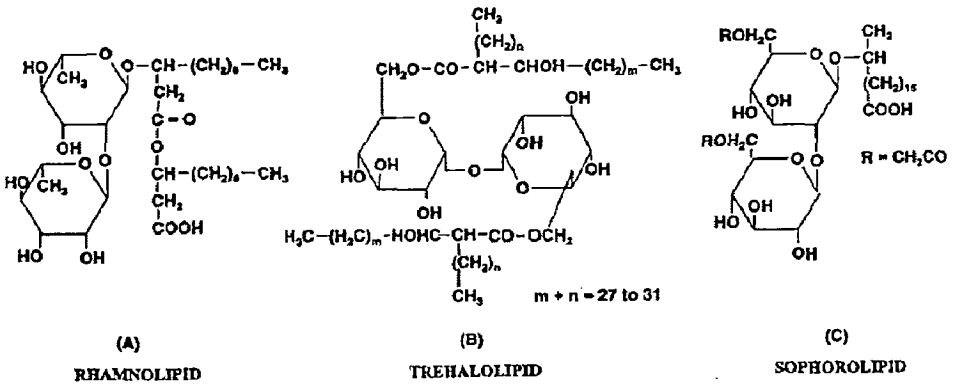


Figure 3: Structure of some common glycolipids

Table 1. Major types of biosurfactant produced by microorganisms

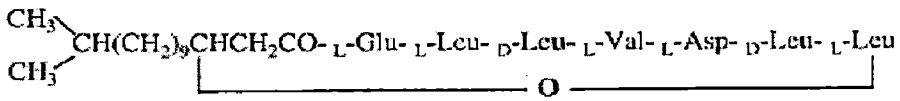
Biosurfactant types	Microorganisms	References
Glycolipids		
Rhamnolipids	*P. aeruginosa* *Pseudomonas* sp.	Robert *et al.*, 1989 Lang *et al.*, 1989
Trehalolipids	*R. erythropolis* *N. erythropolis* *Mycobacterium* sp.	Rapp *et al.*, 1979 Margaritis *et al.*, 1980 Cooper *et al.*, 1989
Sophorolipids	*T. bombicola* *T. apicola* *T. petrophilum*	Cooper *et al.*, 1989 Gobbert *et al.*, 1984 Hommel *et al.*, 1987
Cellobiolipids	*U. zeae*, *U. maydis*	Boothroyd *et al.*, 1956 Syldatk *et al.*, 1985
Lipopeptides and lipoproteins		
Peptide-lipid	*B. licheniformis*	Yakimov *et al.*, 1995
Serrawettin	*S. marcescens*	Matsuyama *et al.*, 1991
Viscosin	*P. fluorescens*	Neu and Poralla 1990
Surfactin	*B. subtilis*	Bernheimer and Avigad, 1970
Gramicidins	*B. brevis*	Marahiel *et al.*,1977
Polymyxins	*B. polymyxa*	Suzuki *et al.*, 1965
Subtilisin	*B. subtilis*	Bernheimer and Avigad, 1970
Fatty acids, neutral lipids, and phospholipids		
Fatty acids	*C. lepus*	Cooper and Paddock, 1983
Neutral lipids	*N. erythropolis*	Lin *et al.*, 1994
Phospholipids	*T. thiooxidans*	Koch *et al.*, 1988
Polymeric surfactants		
Emulsan	*A. calcoaceticus*	Zosim *et al.*, 1982
Biodispersan	*A. calcoaceticus*	Rosenberg *et al.*, 1988
Mannan-lipid-protein	*C. tropicalis*	Kappeli *et al.*, 1984
Liposan	*C. lipolytica*	Cirigliano and Carman, 1985
Carbohydrate-protein-lipid	*P. fluorescens* *D. polymorphis*	Persson *et al.*, 1988 Singh and Desai, 1989
Protein PA	*P. aeruginosa*	Hisatsuka *et al.*,1977
Particulate biosurfactants		
Vesicles and fimbriae	*A. calcoaceticus*	Gutnick and Shabtai, 1987

2.2.2 Lipopeptides

Lipopeptides usually appear as mixtures of closely related compounds which show slight variations in their amino acid composition and/or lipid portion which is mostly a hydroxy fatty acid. A family of cyclic lipopeptides consists of 8 to 17 amino acids and a

lipid portion which is composed of 8 to 9 methylene groups and a mixture of linear and branched tails (Desai and Banat, 1997). Major research has been focused on class of lipopeptides formed by certain strain of *B. subtilis* and *B. pumilis* (Mulligan, 2005; Cameotra and Makkar, 2004).

Different strains of *B. licheniformis* produce lipopeptide biosurfactants e.g. lichenysin A (Yahkimov *et al.*, 1995), lichenysin B (Lin *et al.*, 1994), lichenysin C (Jenny *et al.*, 1991), biosurfactant BL-86 (Horowitz *et al.*, 1990).



Surfactin (SF)

Figure 4: Structure of cyclic lipopeptide surfactin produced by *B. subtilis*

A large number of cyclic lipopetides including decapeptide antibiotics (gramicidins) and lipopeptide antibiotics (polymyxins) produced by *B. brevis* (Marahiel *et al.*, 1977) and *B. polymyxa* (Suzuki *et al.*, 1965) respectively, possess remarkable surface active properties. Other types of lipopeptides include ornithine containing lipids from *P. rubescens* (Yamane, 1987) and Cerilipin an ornithine and taurine containing lipid from *Gluconobacter cerinus IFO 3267* (Tahara *et al.*, 1976). Other lipopeptides include viscosin produced by *P. fluorescens* (Laycock *et al.*, 1991) and arthrofactin, produced by *Arthrobacter* spp. Strain MIS38 (Morikawa *et al.*, 1993).

2.2.3 Fatty acids, phospholipids and neutral lipids

Several bacteria and yeast that produce fatty acids and phospholipids while growing on n-alkanes, have received considerable attention as surfactants (Rosenberg and Ron, 1999). These biosurfactants are able to produce optically clear microemulsions of alkanes

in water (Desai and Desai, 1993). The hydrophilic lipophilic balance (HLB) of fatty acids is related to the length of the hydrocarbon chain. Examples of microorganisms that produce these types of biosurfactants include sulphur-reducing bacteria, *Thiobacillus thiooxidans* (Desai and Banat, 1997) and *Corynebacterium lepus* (Rosenberg and Ron, 1999; Cooper and Paddock, 1983).

2.2.4 Polymeric biosurfactants

Many bacterial species from different genera produce exocellular polymeric surfactants composed of proteins, polysaccharides, lipopolysaccharides or complex mixture of these biopolymers (Rosenberg and Ron, 1999). Fatty acids are covalently linked to the polysaccharides through *o*-ester linkages (Desai and Banat, 1997; Zukerberg *et al.*, 1979). The best-studied polymeric biosurfactants are emulsan, liposan and mannoprotein (Rosenberg and Ron, 1999; Desai and Banat, 1997).

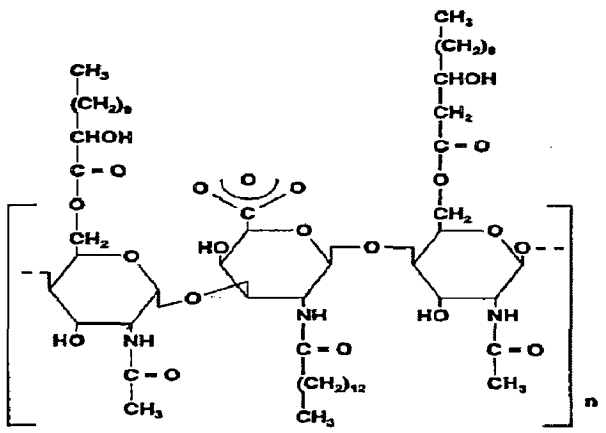


Figure 5: Structure of emulsan produced by *Acinetobacter calcoaceticus*

2.2.5 Particulate biosurfactants

Particulate biosurfactant includes vesicles and fimbriae produced by *Acinetobacter* sp. (Kappeli and Finnerty, 1980). The purified vesicles are composed of proteins, phospholipids and lipopolysaccharides. This extracellular membrane vesicles partition

hydrocarbons, and form a microemulsion, which plays an important role in alkane uptake by microbial cells (Desai and Banat, 1997).

2.3 Rhamnolipid: evaluation and production

Rhamnolipid is a glycolipid that contains one or two molecules of rhamnose that are linked to one or two molecules of β-hydroxydecanoic acid. Up to seven homologues have been identified (Abalos *et al.*, 2001). L-Rhamnosyl-L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate and L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate, referred to as rhamnolipid 1 and 2, respectively, are the principal glycolipids produced by *P. aeruginosa* (Desai and Banat, 1997). Rhamnolipid from *P. aeruginosa* have shown to lower the surface tension from 72 to 26 mN m^{-1} and the interfacial tension against *n*-hexadecane to 1 mN m^{-1} (Mulligan, 2005; Lang and Wagner, 1987).

2.3.1 Physico-chemical characterization of rhamnolipid

Crude rhamnolipid extract from microbial sources appeared as viscous sticky oily residue with amber to yellowish brown in color and fruity pineapple in odor (Abdel-Mawgoud *et al.*, 2009). Rhamnolipid is soluble in aqueous solution at pH>4 with optimum solubility at pH 7–7.5. It is soluble in organic solvents like methanol, chloroform, ethylether, and ethylacetate. It was observed that rhamnolipid have their optimum aqueous solubility at neutral to alkaline pH (Silva *et al.*, 2010). This phenomenon is attributed to their acidic nature. Rhamnolipid contain a single free carboxylic acid group corresponding to the β- hydroxy fatty acid moiety, and this confers the anionic nature of rhamnolipid. It was reported that as the pH increases from 5 to 8, the negative charge of the polar head increases (Abdel-Mawgoud *et al.*, 2009), and this is reflected by increased aqueous solubility. Rhamnolipid is usually produced as a mixture of homologues, whose similarity and abundance hinder their separation. On the other

hand, the use of the whole mixture as a sole component enhances the surface active properties of rhamnolipid (Guo *et al.*, 2009). Rhamnolipid's hydrophilicity is due to rhamnosyl groups while the long fatty acid chains bestow it with hydrophobicity. The carboxylic moieties confer the rhamnolipid with negative charge depending on the subphase conditions such as pH and electrolytic strength.

2.3.2 Surface activities

2.3.2.1 Surface tension

Surface tension is a phenomenon involving the cohesive force between liquid molecules. Surface tension can be defined in terms of work, W as follows (Garret, 1972):

Surface tension, $\gamma = W/\Delta A$, where ΔA is the change in surface area.

Plots of the surface tension (γ) at 25 °C versus logarithmic concentration in mg L^{-1} for the different biosurfactants revealed surface tension values are decreased gradually with increasing concentrations until a constant value is obtained. When a rhamnolipid is added to air /water or oil/water system at increasing concentration, reduction of surface tension is observed only up to a critical level beyond which micelle formation takes place (Makkar and Cameotra, 1997). This break point, after which surface tension becomes constant, was taken as the critical micelle concentration (CMC) and is determined from the break point of the surface tension versus concentration curve. This had been used previously to quantify surface activities of biosurfactants such as surfactin (Moran *et al.*, 2002) and rhamnolipid (Johnson and Boese-Marrazzo, 1980). For practical purposes, it is important to distinguish between an effective biosurfactant and an efficient biosurfactant. Effectiveness is measured by the minimum value to which the surface tension can be reduced, whereas efficiency is measured by the biosurfactant concentration required to produce a significant reduction in the surface tension of water. The latter can be known

from the CMC of the biosurfactant (Abdel-Mawgoud *et al.*, 2009; Haba *et al.*, 2003; Parkinson, 1985). In general, biosurfactants are more effective and efficient and their CMC is about 10–40 times lower than that of chemical surfactants, i.e. less surfactant is necessary to get a maximum decrease in surface tension (Muthusamy *et al.*, 2008).

2.3.2.2 Interfacial tension

The direct measurement of the interfacial activity of the culture supernatant is also the most straightforward screening method and very appropriate for a preliminary screening of biosurfactant producing microbes (Lin, 1996). This gives a strong indication on biosurfactant production. The interfacial of a liquid can be measured by a variety of methods. However, there is a restriction in the range of measurement (Morikawa *et al.*, 2000; Makkar and Cameotra, 1997). A good surfactant can lower the interfacial tension of water/hexadecane from 40 to 1 mN m^{-1} (Pacwa-Płociniczak *et al.*, 2011). Surfactin from *B. subtilis* can reduce the interfacial tension of water/hexadecane to <1 mN m^{-1} (Desai and Banat, 1997). Rhamnolipid from *P. aeruginosa* decrease the interfacial tension of water/hexadecane to <1 mN m^{-1} (Desai and Banat, 1997). The sophorolipids from *T. bombicola* have been reported to reduce the interfacial tension to 5 mN m^{-1} (Desai and Banat, 1997).

2.3.2.3 Critical micelle dilution (CMD)

The critical micelle dilution (CMD) method, which was determined by measuring the surface tension of serial dilutions of supernatant from fermentation broth, was used as a criterion for selecting high production strain. The two cultures with very different concentrations of biosurfactant may display the same surface tension. This problem can be solved by serial diluting until a sharp increase in surface tension is observed (Batista *et*

al., 2006; Makkar and Cameotra, 1997). The corresponding dilution of the supernatant is called critical micelle dilution (CMD) and correlates to the concentration of biosurfactant.

2.3.3 Rhamnolipid production

Rhamnolipid is an effective biosurfactant and is well suited for applications in bioremediation of oil pollutants (Mulligan, 2005), the major hurdle for commercial application of the rhamnolipid has been its low yield and high production cost (Wei *et al.*, 2005). Although the potential for rhamnolipid production is determined by the genetics of the microorganisms, other factors such as environmental conditions and the nature of the substrate also influence the yield of rhamnolipid. Hence, optimization of these conditions may lead to high and safe rhamnolipid production. Most of the studies to date describe rhamnolipid production by bacteria grown on hydrocarbons, but a few have reported rhamnolipid produced by bacteria growing on carbohydrates (Haferburg *et al.*, 1986). Most of the world's total oil and fat production is derived from plants. Hence, these hydrophobic substrates may be used for bulk production of rhamnolipid. There are reports regarding the production of rhamnolipid from different substrates such as glycerol (Arino *et al.*, 1996), soybean oil (Lang and Wullbtandt, 1999), olive oil (Mercade´ *et al.*, 1993), corn oil (Linhardt *et al.*,1989), canola oil (Sim *et al.*, 1997), ethanol (Matsufuji *et al.*, 1997), sucrose, and whey (Babu *et al.*, 1996) by different strains of *P. aeruginosa*. Use of water miscible wastes such as molasses and whey and distillery wastes, wastewater from olive oil processing, has also been reported (Daniel *et al.*, 1998; Patel and Desai, 1997; Babu *et al.*, 1996; Mercade *et al.*, 1993;). In earlier studies, *P. aeruginosa* GS9-119 and *P. aeruginosa* DS10-129 produced 4.3 and 7.5 g L^{-1} of crude biosurfactant, respectively, when grown on a glucose plus glycerol medium (Rahman *et al.*, 2002a). The use of renewable low-cost substrates, such as plant oil and grain starch,

and even lignocellulosic biomass, could dramatically increase the economics of rhamnolipid production (Mukherjee *et al.*, 2006; Nitschke *et al.*, 2005). Therefore, there is an urgent demand to develop an efficient biosurfactant producer and a cost-effective bioprocess for the production of rhamnolipid. The types of the rhamnolipid produced depend on several factors: the bacterial strain, the carbon source used, as well as the culture conditions (Soberón-Chávez *et al.*, 2005). Concerning its production, high yields are obtained as compared to other biosurfactants; furthermore, the carbon source can be supplied by several raw materials like, for instance, used oils or wastes from the food industry (Soberón-Chávez *et al.*, 2005; Banat *et al.*, 2000; Lang and Wullbrandt, 1999), and therefore the overall procedure for the bacterial production of rhamnolipid can be classified as a green process (Sánchez *et al.*, 2009). Soybean oil waste is found as the best substrate for rhamnolipid production by *P. aeruginosa* LBI, exhibiting therefore the greatest potential as an alternative economically viable carbon source (Nitschke *et al.*, 2005). Attempts have also been made to engineer microbes for production of rhamnolipid as an agent for enhanced oil recovery (Wang *et al.*, 2007). Indigenous *P. aeruginosa* EM1 isolated from oil-contaminated site has also been used for production of rhamnolipid (Wu *et al.*, 2008).

2.3.4 Biosynthetic routes of Rhamnolipid

The rhamnolipid were first described in 1949 (Jarvis and Johnson, 1949), and their biosynthesis was studied *in vivo* by using radioactive precursors (Hauser and Karnovsky, 1957). Rhamnolipid, being glycosyl derivatives of β-hydroxyl acids, are synthesized through sequential glycosyl transfer reactions, each catalyzed by a specific rhamnosyltransferase with TDP-rhamnose acting as a rhamnosyl donor and 3-hydroxydecanoyl-3-hydroxydecanoate acting as the acceptor (Wins *et al.*, 2009; Soberón-

Chávez *et al.*, 2005; Rahim *et al.*, 2001; Kanehisa and Goto, 2000; Ochsner and Reiser, 1995; Burger *et al.*, 1963). *P. aeruginosa* produces two major types of rhamnolipid in liquid cultures namely, monorhamnolipid, rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (Rha-C10-C10) and the dirhamnolipid, rhamnosyl-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (Rha-Rha-C10-C10) (Deziel *et al.*, 2000). However, 25 rhamnolipid congeners have been described in *P. aeruginosa*, varying in chain length and/or extent of saturation, showing that the addition of a hydrocarbon chain to dTDP-L-rhamnose is not specific to the carbon chains (Deziel *et al.*, 1999). The initial precursor for rhamnolipid biosynthesis, a β-hydroxyl acid, is synthesized through condensation of malonyl Co-A and acetyl Co-A to form acetoacetyl-CoA through FabH (Campos-garci'a *et al.*, 1998). This ketoester is further reduced by FabG, a NADPH dependant enzyme, to produce a β-hydroxy acid. The β-hydroxy acid is then dehydrogenated to produce a trans-enoyl-ACP complex by Fab A and Fab Z. This trans-enoyl acid is then reduced by FabI, with NADH as cofactor, to produce acyl-ACP complex. Through repetition of this four chemical reaction long cycle elongation occurs *i.e.* longer aliphatic chains of β-hydroxy acids are generated. These high molecular weight β-hydroxy acids act as precursors for the synthesis of polyhydroxy alkonates (PHA) which acts as bacterial storage compounds. This polymerization is brought about by PHA synthases (PhaC) using coenzyme A linked monomeric β-hydroxyl fatty acids as substrates. The dimeric β-hydroxyl fatty acids (For example β-hydroxydecanoyl-β-hydroxydecanoate) act as substrate for rhamnose transferase (Rt) which in turn catalyses the transfer of rhamnose units to its substrate to synthesize rhamnolipid (Miller *et al.*, 2006). There are two kinds of rhamnotransferase known, one is Rt1 which catalyses the transfer of single rhamnose unit to dimeric β-hydroxyl fatty acids to create monorhamnolipid while the other, Rt2 catalyses the transfer of the rhamnose unit to

mono-rhamnolipid for the synthesis of di-rhamnolipid. For both enzymes, TDP-L rhamnose acts as rhamnose donor and rhamnose subunits are bonded to β-hydroxyl acids through glycosidic linkages (Deziel *et al.*, 2003; Rahim *et al.*, 2001). The biosynthetic pathways responsible for generating PHA are well studied in *E. coli* and homologs of enzymes involved in the biosynthetic pathway are found in other bacteria (Park and Lee, 2003). Such as *rhlG*, a homologue of *fabG*, found in *Pseudomonas* spp. is dedicated for the synthesis of β-hydroxy moiety of rhamnolipid. Genes coding for biosynthesis, regulation, and induction of Rtl enzyme are organized in tandem in the *rhlABRI* gene cluster around min 38 of the *P. aeruginosa* chromosome (Campos-garci'a *et al.*, 1998; Ochsner and Reiser, 1995). The genes encoding Rt2 have yet to be described.

2.4 Potential applications of rhamnolipid

Rhamnolipid are naturally produced as a mixture of mono- and di-rhamnolipid. Rhamnolipid are being used in a wide range of applications (Deziel *et al.*, 2000). As a surfactant or emulsifier, rhamnolipid can be used as natural "green" ingredient in cosmetics, detergents, shampoos and soaps as well as for environmental cleanup (Bioremediation), enhanced oil recovery (EOR), sludge removal, pesticide dispersal and wetting agent. When used as a surfactant, only low concentrations are necessary. As a fungicide and antibiotic, rhamnolipid can be used as a pesticide, food preservative, and medical device and implant coating. As a pharmacological agent, rhamnolipid can be used for age marks, bedsores, burn repair, wound healing, psoriasis, sun damage, and wrinkle reduction. As an anionic complexation agent, rhamnolipid foaming can be used to remove heavy metal contaminants from soils, wastewater, and other liquids.

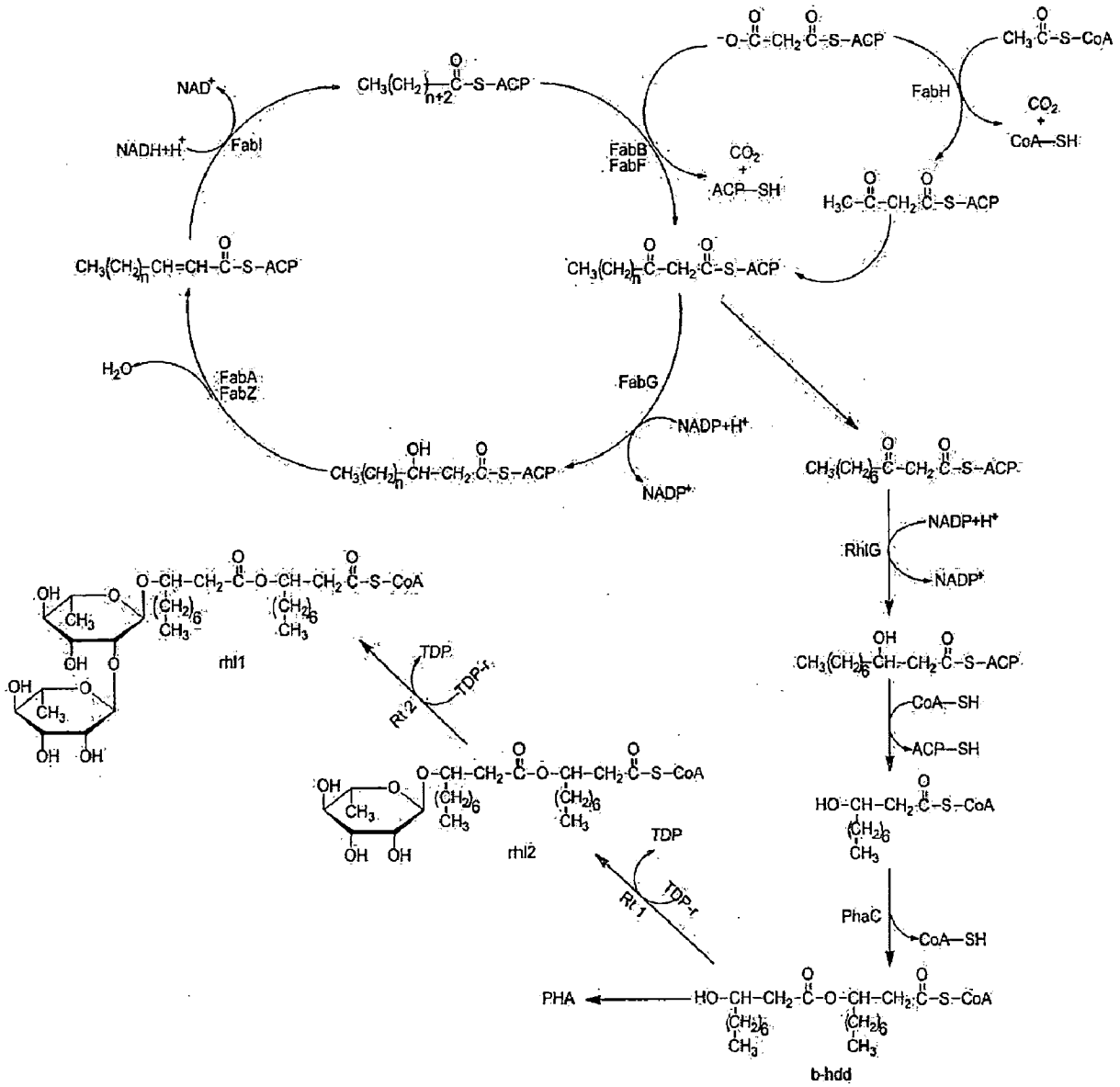


Figure 6: Schematic representation of the fatty acid biosynthetic pathway showing the deduced role of the RhlG protein in the production of rhamnolipid and PHAs (Campos-Garcia *et al.*, 1998)

2.4.1 Environmental applications

With the use of rhamnolipid, the biodegradation of the pollutants can be significantly enhanced. It has been found that the biodegradation of Casablanca crude oil was accelerated in the presence of rhamnolipid produced by *P. aeruginosa* AT10 (Abalos *et al.*, 2004). Zhang *et al.*, 1997 reported that rhamnolipid increased the solubility of phenanthrene (polycyclic aromatic hydrocarbons) in a test solution, resulting in the enhancement of the phenanthrene biodegradation rate. In addition, rhamnolipid produced

by *P. aeruginosa* UG2 was found to increase the solubilization of pesticides, resulting in the stimulation of biodegradation rate and extent (Mata-Sandoval *et al.*, 2001; 2000). The enhancement of hexadecane biodegradation by rhamnolipid has also been reported (Noordman *et al.*, 2002). Besides their use as a pure culture, rhamnolipid can stimulate the biodegradation of contaminated soil and water. Rahman *et al.*, 2002b showed that rhamnolipid-containing additives had positive effects on the bioremediation of gasoline-contaminated soil. The potential use of rhamnolipid produced by *P. aeruginosa* J4 for the biodegradation of diesel-contaminated water and soil has also been reported (Wang *et al.*, 2008). Clifford *et al.*, 2007 found that rhamnolipid produced by *P. aeruginosa* ATCC 9027 significantly improved the solubilization of tetrachloroethylene (PCE), a common ground water pollutant, indicated the potential use of the tested biosurfactant in surfactant-enhanced aquifer remediation (SEAR) applications. Cassidy *et al.*, 2002 also suggested that rhamnolipid might be applied in intrinsic bioremediation using in situ rhamnolipid production at an abandoned petroleum refinery. In some cases, biodegradation processes are too slow or infeasible, so it is necessary to remove the contaminants from the environment (Maier and Soberon-Chavez, 2000). Urum *et al.*, 2005 investigated the removal of crude oil from soil in air sparging assisted stirred tank reactors using two surfactants, sodium dodecyl sulfate (SDS) and rhamnolipid.

Table 2. Applications of rhamnolipid

Industry	Applications	Role of rhamnolipid
Petroleum industry	Enhanced oil recovery	Improving oil drainage into well bore, stimulating release of oil entrapped by capillaries, wetting of solid surfaces, Reduction of oil viscosity and oil pour point, lowering of interfacial tension, dissolving of oil
	De-emulsification	De-emulsification of oil emulsions, oil solubilization, viscosity reduction, wetting agent
Environmental industry	Bioremediation	Emulsification of hydrocarbons, lowering of interfacial tension, metal sequestration
	Soil remediation and flushing	Emulsification through adherence to hydrocarbons, dispersion, foaming agent, detergent, soil flushing
Food industry	Emulsification and de-emulsification	Emulsifier, solubilizer, demulsifier, suspension, wetting, foaming, defoaming, thickener, lubricating agent
	Functional ingredient	Interaction with lipids, proteins and carbohydrates, protecting agent
Biological industry	Microbiological	Physiological behaviour such as cell mobility, cell communication, nutrient accession, cell–cell competition, plant and animal pathogenesis
	Pharmaceuticals and therapeutics	Antibacterial, antifungal, antiviral agents, adhesive agents, immunomodulatory molecules, vaccines, gene therapy
Agricultural industry	Biocontrol	Facilitation of biocontrol mechanisms of microbes such as parasitism, antibiosis, competition, induced systemic resistance and hypovirulence
Bioprocessing industry	Downstream processing	Biocatalysis in aqueous two-phase systems and microemulsions, biotransformations, recovery of intracellular products, enhanced production of extracellular enzymes and fermentation products
Cosmetic industry	Health and beauty products	Emulsifiers, foaming agents, solubilizers, wetting agents, cleansers, antimicrobial agents, mediators of enzyme action

The results indicated that rhamnolipid removed oil from the contaminated soil sample comparable to the tested synthetic surfactant (Urum *et al.*, 2005). Bai *et al.*, 1997 reported that mono-rhamnolipid produced by *P. aeruginosa* ATCC 9027 displayed efficiency in the removal of residual hexadecane from soil higher than three synthetic surfactants: SDS, polyoxyethylene and sorbitan monooleate. Noordman *et al.*, 1998 showed that rhamnolipid produced by *P. aeruginosa* UG2 effectively removed phenanthrene from soil. Mulligan, 2005 found that rhamnolipid foam effectively removed inorganic heavy metal, including cadmium and nickel, from a contaminated soil sample. The removal of copper, (Mulligan *et al.*, 2001) zinc and lead (Herman *et al.*, 1995) by rhamnolipid has also been reported. In soil remediation applications, one of the important considerations is the size of the surfactant microstructures. Because contaminants are often found in very small soil pores, the movement of surfactant molecules through the soil can be easily limited by the pore size. Therefore, the size of the rhamnolipid microstructures should be studied closely for their effective use. It was previously reported that rhamnolipid could form various types of microstructures in an aqueous media (including lamellar sheets, vesicles and micelles), depending on concentration and pH 1-6, (Champion *et al.*, 1995). The size of these rhamnolipid microstructures ranged from less than 50 nm to larger than 1 μm, while the smaller-sized soil pores was in the range of 2 μm-0.2 mm. Thus, the appropriate size of rhamnolipid microstructure could be achieved by controlling the concentration and pH.

2.4.2 Food applications

Biosurfactants can be explored for several food-processing applications. In this section we emphasize their potential as food-formulation ingredients. Apart from their obvious role as agents that decrease surface and interfacial tension, thus promoting the

formation and stabilization of emulsions, surfactants can have several other functions in food. For example, to control the agglomeration of fat globules, stabilize aerated systems, improve texture and shelf-life of starch-containing products, modify rheological properties of wheat dough and improve consistency and texture of fat-based products (Nitschke and Costa, 2007). In bakery and icecream formulations biosurfactants act by controlling consistency, retarding staling and solubilizing flavour oils; they are also utilized as fat stabilizers and antispattering agents during cooking of oil and fats. Improvement in dough stability, texture, volume and conservation of bakery products is obtained by the addition of rhamnolipid surfactants (Van Haesendonck and Vanzeveren, 2004). The study also suggested the use of rhamnolipids to improve the properties of butter cream, croissants and frozen confectionery products. L-Rhamnose has considerable potential as a precursor for flavouring. It is already used industrially as a precursor of high-quality flavour components like furaneol (Linhardt *et al.*, 1989; Lang and Wullbrandt, 1999).

2.4.3 Therapeutic and biomedical applications

Rhamnolipid obtained from *P. aeruginosa* AT10 showed excellent antibacterial activity against *Escherichia coli*, *Micrococcus luteus*, *Alcaligenes faecalis*, *Serratia arcescens*, *Mycobacterium phlei*, *Staphylococcus epidermidis* and antifungal activity against *Aspergillus niger*, *Chaetonium globosum*, *Enicillium crysogenum*, *Aureobasidium pullulans*. Besides this, it also showed phytopathogenic activity against *Botrytis cinerea* and *Rhizoctonia solani* (Abalos *et al.*, 2001). During *P. aeruginosa* biofilm development, due to continuous accumulation of the exopolymeric substances the porosity and dynamism of biofilm is completely blocked. At this stage, *P. aeruginosa*, rhamnolipid acts as surface active molecule which modulates biofilm architecture through its

membrane destabilizing activity, thereby maintaining the biofilm porosity and enabling the water channels not to be blocked (Davey *et al.*, 2003). Previously, researchers have also reported the antagonistic effects of rhamnolipid on the economically important zoosporic plant pathogens (Stanghellini and Miller, 1997). Wang *et al.*, 2005 during their studies on algicidal activity of rhamnolipid biosurfactants produced by *Pseudomonas aeruginosa* have shown that it had potential algicidal effects on the harmful algal bloom (HAB) species, *Heterosigma akashiwo* and *Protocentrum dentatum*. Earlier researchers have also shown the inhibitory potential of the rhamnolipid on *P. aeruginosa* biofilm development when added during the initial stages of biofilm formation (de Araujo *et al.*, 2011; Dusane *et al.*, 2010). Davey *et al.*, 2003 observed that rhamnolipid interferes with the microbial adherence to substratum and it also retards biofilm growth by its membrane destabilizing activity. However, at later stages when maturation of biofilm had taken place, rhamnolipid exhibits no potential antibiofim activity (Rodrigues *et al.*, 2006a; b). Rhamnolipid affects both planktonic as well as sessile cells through altering the lipopolysaccharide content of cell wall and outer membrane protein composition (Dusane *et al.*, 2010; Sotirova *et al.*, 2010; Irie *et al.*, 2005). Additionally, rhamnolipid also exhibit antibiofilm activity on other microorganisms (Dusane *et al.*, 2010). *Bordetella bronchiseptica*, a human pathogen, infects the respiratory extract of wide range of mammals owing to its biofilm forming tendency and recently rhamnolipid had been shown to inhibit its biofilm which further validates its potency as antimicrobial agent (Irie *et al.*, 2005). As *C. albicans*, mediates many of its infection through biofilm formation which is well resistant to conventional antifungal agents, we investigated the antibiofilm activity of rhamnolipid on *Candida* biofilm. Moreover, being therapeutically effective rhamnolipid biosurfactant is a suitable alternative to synthetic medicines and

antimicrobial agents, and may be used as safe and effective therapeutic agents (Benincasa *et al.*, 2004; Singh and Cameotra, 2004; Makkar and Cameotra, 2002; Flasz *et al.*, 1998).

2.5 Biofilm formation by *Candida albicans*

2.5.1 Biofilm

Regulated life cycle switching of microorganism from nomadic unicellular state to a sedentary multicellular state where subsequent growth result in structured communities and cellular differentiation had shown the scientific world that biofilm formation once considered being the domain of a few species is now really a universal attribute of microorganisms (Yang *et al.*, 2011; Yergeau *et al.*, 2010; Read *et al.*, 2010; Flemming *et al.*, 2007). In fact, biofilm formation is a complex dynamic process which consists of interdependent communities, microbial species embedded in a biopolymer matrix (containing polysaccharides, protein and DNA) on either biotic or abiotic substrata (Sauer, 2003; Hall-Stoodley and Stoodley, 2002) and able to respond and adapt to changes in environment or perform highly specialised tasks similar to multi-cellular organisms (Hall-Stoodley and Stoodley, 2002; O'Toole *et al.*, 2000; Costerton *et al.*, 1999). Microorganisms embedded in the biofilm have characteristic architecture, phenotypic properties and slow growth than planktonic cells. This matrix encapsulated biofilm provide slow access to nutrients and oxygen supply leading a decrease in the rate of macromolecular synthesis and as a consequence, to get it protected from the onslaught of both host immune response and antimicrobial agents (Nava-ortiz *et al.*, 2010; Smith, 2005; Donlon and Costerton, 2002).

2.5.2 *C. albicans* biofilm on medical implant

Approximately 65% of hospital-acquired infections are related to surface-attached microbes (Resch *et al.*, 2005). Cauda, 2009 pointed out that *Candida* spp. are responsible for a great number of infections related to implanted devices, especially intravascular and urinary catheters. It is estimated that infection is associated to biofilm formation in case of urinary catheters, bone fixation materials, central venous catheters, pace-makers, vascular grafts, joint prosthesis and mechanical cardiac valves. Furthermore, biofilm formation is very frequent in dental and laryngeal prostheses and in other biomedical devices that are used in contact with skin or mucosal surfaces (Kojic and Darouiche, 2004). Biofilm forming strains of *C. albicans*, *C. parappsilosis*, *C. tropicals* and *C. glabrata* have been associated with considerably high mortality rates (Hasan *et al.*, 2009; Trofa *et al.*, 2008; Tumbarello *et al.*, 2008). The ARTEMIS global antifungal surveillance program showed that *C. albicans* was the most common (63-70%) cause of invasive fungal infections, followed by *C. glabrata* (44%), *C. tropicalis* (6%) and *C. parapsilosis* (5%) (Pfaller and Diekema, 2007). *C. albicans* is an opportunistic dimorphic pathogen which commonly affects immune-compromised individual and is capable of causing life threatening infections (Rosenbach *et al.*, 2010; Wang *et al.*, 2010; Imamura *et al.*, 2008). Importantly, *C. albicans* is ranked fourth in causing nosocomial infections, third in catheter-related infections, second highest in colonization to infection rate and highest overall in crude mortality is of immense concern (Subha and Gnanamani, 2008). It is believed that *C. albicans* biofilm, a structured communities of cells embedded in biomatrix, formed on medical devices can result in the failure of the device and serve as a reservoir or source for infections (Lal *et al.*, 2010; Martins *et al.*, 2010a).

2.5.3 Architecture and development of *C. albicans* biofilm

Although research on bacterial biofilm has been fairly advanced (Donlan and Costerton, 2002; Costerton *et al.*, 1999; 1994; 1987) studies on fungal biofilm have only recently been initiated (Chandra *et al.*, 2001; Martinez and Casadevall, 2005). Similar to bacterial biofilm, fungal biofilm poses heterogeneous architecture with increased stress tolerance and properties quite distinct as compared to their planktonic counterparts. Analogous to quorum sensing signals (homoserine lactones) observed in case of bacteria biofilm, fungal biofilm are also regulated through signalling molecules such as farnesol and tyrosol (Ramage *et al.*, 2002a). Being a part of human normal microbe flora, *C. albicans* are exposed to a variety of implants utilized as medical devices and eventually these biomaterials used as substrates for biofilm formation which in turns leads to infection and implant discard (Ramage *et al.*, 2005; 2002b; Alem and Douglas, 2005). To understand the architecture of *C. albicans* biofilm, both *in vivo* and *in vitro* models have been studied (Seneviratne *et al.*, 2008; Thein *et al.*, 2007; Kuhn *et al.*, 2002; Chandra *et al.*, 2001). Comprehensive studies utilizing these models revealed that *Candida* biofilm is an organized three dimensional structure comprising of yeast cells, hyphae as well as pseudohyphae cells embedded in a fungal derived extracellular matrix consisting of polysaccharides and proteins. This heterogenous architecture of candidal biofilm well accords with the biofilm of bacterial origin with the exception of hyphae which are predominantly observed in fungal biofilm (Ramage *et al.*, 2005). Conventional assays for assaying biofilm include calorimetric tests based on dyes such as crystal violet, MTT, XTT, fluorescein diacetate (FDA), resazurin, 1,9-dimethyl methylene blue (DMMB) etc (Al-Dhaheri and Douglas, 2010; Agarwal *et al.*, 2008; Chandra *et al.*, 2008; Peeters *et al.*, 2008; Jin *et al.*, 2004; Nikawa *et al.*, 1996). Although aforementioned calorimetric assays and techniques have been extensively used to quantify growth kinetics of *Candida*

biofilm, they don't reveal any information regarding biofilm architecture. With different forms of vegetative and germ cells scattered in the biofilm, the CFU counts lacks reproducibility and thus one has to rely on imaging technologies to understand biofilm morphology. Various microscopic techniques such as scanning electron microscopy (SEM), fluorescence microscopy, and confocal scanning laser microcopy (CLSM) have been employed for direct observation of developing *Candida* biofilm (Thein *et al.*, 2007; Chandra *et al.*, 2001) SEM has been extensively used by previous workers to obtain high-resolution images of the surface topography of *Candida* biofilm but involves degradation of the native hydrated structural features (particularly of the matrix) due to the fixation and dehydration steps performed during sample preparation (Thein *et al.*, 2007; Samaranayake *et al.*, 2005; Chandra *et al.*, 2001). Moreover, efficient software programmes that couples SEM imaging and quantify architectural parameters of candidal biofilm have not been developed. Among the other techniques, fluorescence microscopy reveals only surface morphology of biofilm whereas CLSM provides useful information about 3D architectural properties of bio-volume, average thickness, biofilm roughness, surface area/volume ratio and the proportion of live/dead cells in the different biofilm development stages. In contrast, recently developed mathematical modelling software COMSTAT coupled with CLSM has been successfully utilised to quantify architectural parameters of bacterial biofilm (Hansen *et al.*, 2007; Heydorn *et al.*, 2000). Seneviratne *et al.*, 2009 revealed that CLSM coupled with COMSTAT software analyses yields reliable information regarding growth kinetics; architecture and viability of biofilm. CLSM examination of *C. albicans* biofilm in combination of the fluorescent dyes FUN-1 and ConA (both from Molecular Probes, Inc., Eugene, Oreg.) have shown the importance of these dyes in casting the position and proportion of living and dead cells found in biofilm (Lal *et al.*, 2010; Chandra *et al.*, 2001; Haugland, 1996). *In vitro* experiments had shown

that *C. albicans* biofilm formation proceeds in three distinct developmental phases namely early, intermediate and mature (Mukherjee *et al.*, 2003; Chandra *et al.*, 2001).

Early phases (0-11 h) which involves adsorption of *C. albicans* cells to a surface, initial adhesion to the surface, formation of basal layers of *C. albicans* microcolonies and an initial switch to a hyphal mode of growth (Samaranayake *et al.*, 1995; Samaranayake and MacFarlane, 1981).

Intermediate phases (12-30 h) at which microcolonies inhabits basal layers, hyphal layers are formed above the basal layers, extracellular matrix material is secreted which surrounds *C. albicans*, host cells and hyphae (Douglas, 2003; Chandra *et al.*, 2001).

Mature phases (38-72 h) mature biofilm contain numerous microcolonies with extensive hyphae and pseudohyphae surrounded by exopolysaccharide layer and interspersed with water channels to allow circulation of nutrients, and dispersal of cells from the mature biofilm (Wilcox *et al.*, 2004; Douglas, 2003; Stoodley *et al.*, 2002; Chandra *et al.*, 2001). In a typical biofilm formed on a hydrophobic surface such as silicon elastomer, a basal layer of blastospores covered by hyphal layer is embedded in exopolysaccharide layer. Adhesion is initially through non-specific interactions, such as hydrophobic and electrostatic forces, between the cells and the substratum (Nett and Andes, 2006; Donlan and Costerton, 2002; Samaranayake, 1990) but eventually specific adhesion molecules are expressed to facilitate stronger adhesion. Amongst these specific adhesions molecules, one well known group belongs to cell-surface glycoproteins that are encoded by the agglutinin-like sequence (ALS) gene family (Zhao *et al.*, 2005). Once the blastospores adhere, distinct boundaries are established which manifests to biofilm architecture. Morphogenesis of these cells in the nascent community depends on various factors such as carbon source, substratum, and species. Water channels between hyphal

cells facilitate the diffusion of nutrients from the environment through the biomass to the bottom layers and also permits waste disposal. Considerable similarities are observed in biofilm formed on both *in vitro* and *in vivo* models but maturation is faster and thickness is greater in *in vivo* biofilm as compared to those grown on *in vitro* systems. The thickness of a biofilm grown *in vitro* can range from 25 μm to 450 μm (Kuhn *et al.*, 2002; Chandra *et al.*, 2001; Ramage *et al.*, 2001a); whereas it usually exceeds 100 μm in *in vivo* models (Andes *et al.*, 2004).

2.5.4 Factors favouring *C. albicans* biofilm formation

Candida biofilm formation may be affected by many physical, biological, chemical and environmental factors like hydrodynamic shear, mass transfer, detachment, substratum texture, physiology of cells, microbial population, EPS, physico-chemical environment, type of substrate and nutrients affect the biofilm structure to various extents (Rosenberg and Kjelleberg, 1986). Conceptual and mathematical modelling was described by Picioreanu *et al.*, 2000c and Taylor *et al.*, 1990 to explain the effect of different factors on biofilm structure.

2.5.4.1 Substratum effect

Virtually any type of surface on Earth can be colonised by bacteria, yeasts, viruses or fungi (de Carvalho, 2007) but the factors influencing microbial adherence to a surface include chemical composition of the material, surface charge, hydrophobicity, surface roughness, the wettability, the preconditioning of the surface and contact angle measurements (Mauclaire and Egli, 2010). Rough surfaces have higher surface to volume ratio hence provide a larger surface for microbial adhesion (Teughels *et al.*, 2006; Scheuerman *et al.*, 1998). Microbes prefer to attach to low-energy, hydrophobic surfaces

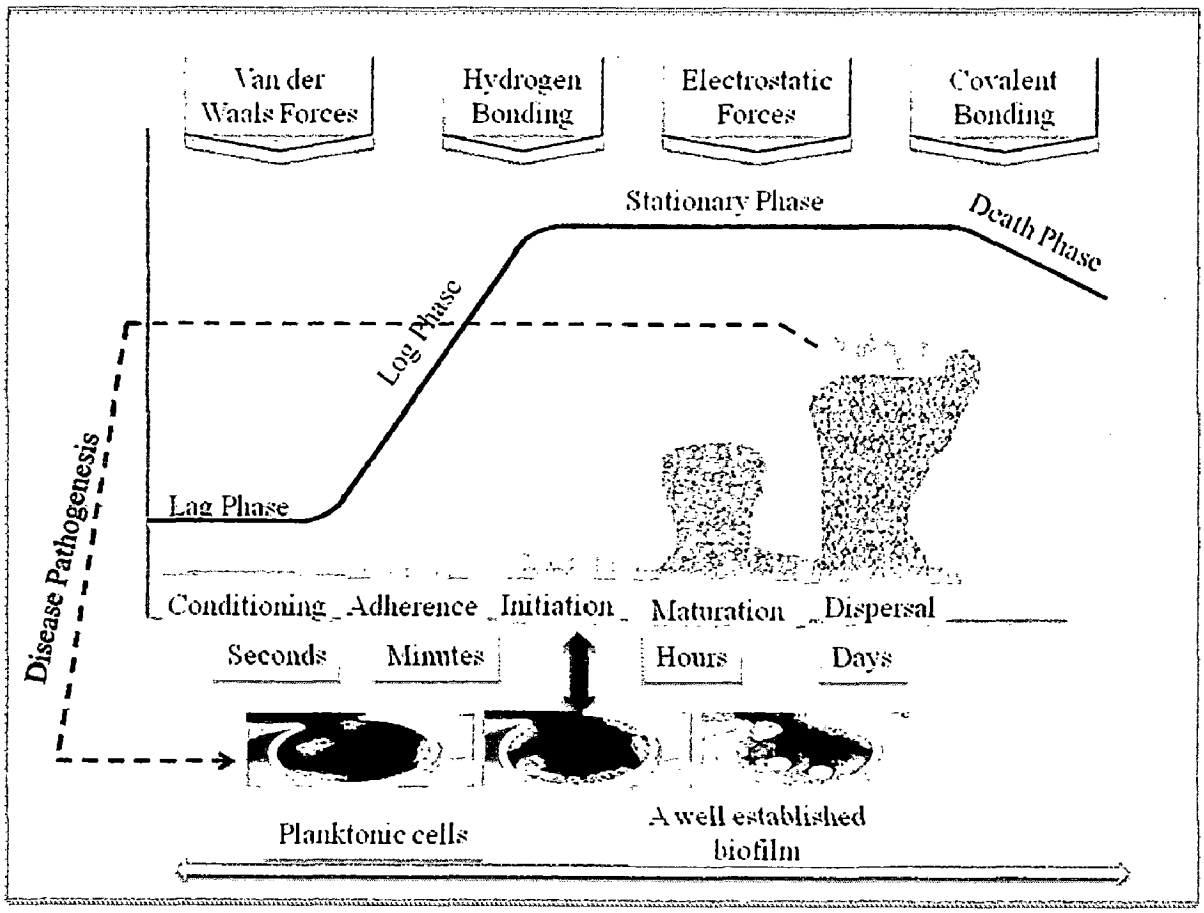


Figure 7: Different stages of biofilm development

such as polystyrene, Teflon rather than to high-energy, hydrophilic surfaces such as stainless steel, glass (Hong-Geller *et al.*, 2010; Pagedar *et al.*, 2010; Chandra *et al.*, 2005; Gottenbos *et al.*, 2000). This observation accords with the fact that biofilm formation by each *Candida* species varies depending on the materials (Estivill *et al.*, 2011; Tumbarello et al., 2008). Different researchers earlier reported that *C. albicans* adherence to biomaterials such as denture (Frade and Arthington-Skaggse, 2011; Singh *et al.*, 2011; Chandra *et al.*, 2005). The implant site infection rates are different both in case of porous and dense materials and increase with porosity. Microbes prefer grooved and porous materials to adhere due to increased surface area (Kinnari *et al.*, 2009; Medilanski *et al.*, 2002). Researchers also noticed that modification of surface properties of biomaterials prevent or reduce biofilm formation on biomaterials (Frade and Arthington-Skaggs, 2011; Chandra *et al.*, 2005). Furthermore, biofilm formed on rougher surfaces tends to have higher resistance towards antibiofilm agents as compared to biofilm formed on less rougher or polished surfaces (Singh *et al.*, 2011; Nevzatoğlu *et al.*, 2007). The chances of *Candida* spp. mediated stomatities increases with the usage of liners, especially in the case of dentures, with extremely porous surfaces i.e, increased surface energy (Pereira-Cenci *et al.*, 2008; Nevzatoğlu *et al.*, 2007). Enviornmental factors such as moisture, solar radiation, and corrosive nature of various metals and alloy surfaces also affect microbial adhesion (de Carvalho, 2007).

2.5.4.2 Acquired pellicle

Acquired pellicle refers to a layer of biological material conditioned on the surface of implants through body fluids such as urine, blood, saliva and synovial fluid (Honraet *et al.*, 2005; Xiong *et al.*, 2005; Byers and Ratner, 2004; Jin *et al.*, 2004; Kojic and Darouiche, 2004; Nemet and Zagar, 2000; Nikawa *et al.*, 2000, 1997). The composition

of the pellicle is related to the nature of the substratum (Edgerton *et al.*, 1996) and varies with the biological fluid to which it is exposed (Edgerton *et al.*, 1996). The chemical modification of implants surfaces alters the property of microbial adherence. Random binding of host proteins on to the implants surfaces is responsible for acquired pellicle formation which further accelerates biofilm formation by assisting in initial adhesion (Kojic and Darouiche, 2004). The effect of acquired pellicles on candidal biofilm formation has not been widely studied but sporadic studies indicate that coating substrata with human whole saliva quantitatively promotes *Candida* biofilm formation (Ramage *et al.*, 2005; 2001; Nikawa *et al.*, 1997). Acquired pellicle enhances receptor mediated adhesion and various host proteins coated on implants acts as docking agents for initial adherence. Glycoprotein on the yeast surface may be involved in this event, since protease and glycosidase treatment of yeast cells can significantly reduce adhesion to fresh human submandibular-sublingual saliva (HSMSL)-coated polymethylmethacrylate (Edgerton *et al.*, 1993). On the other hand, mucins in HSMSL and four proteins of 17, 20, 24 and 27 kDa in human parotid saliva (HPS) have been identified as receptors for *C. albicans* adhesion (O'Sullivan *et al.*, 1997). Even though acquired pellicle increase the frequency of adherence, a rate limiting step in biofilm formation, it showed little effect on overall biofilm physiology (Ramage *et al.*, 2005). In addition, the effect of acquired pellicles on candidal biofilm formation seems to vary from species to species (Baboni *et al.*, 2010). Nikawa *et al.*, 1997 during *in vitro* biofilm formation studies on soft lining materials observed that coating acrylic sheets with serum or saliva did not enhance biofilm formation of most tested isolates of *C. glabrata* or *C. tropicalis*, but showed promoting effect when noted with *C. albicans*. Interestingly, in contrast to saliva's innate immune function, coating of implants with salivary fluids facilitates adherence of *C. albicans* (Moura *et al.*, 2006; Elguezabal *et al.*, 2004; Nikawa *et al.*, 2000; Millsap *et al.*,

1999). Recently studies have indicated that aromatic hydrocarbons released through cigarette smoke can interfere with biofilm formation on various orthodontic materials (Baboni *et al.*, 2010).

2.5.4.3 Physicochemical factors

Biofilm formation by microbes is affected by range of different physiochemical factors including temperature, time of exposure, microbial concentration, intermicrobial co-adhesion, pH, oxygen, nutrients and presence of toxin (Bandara *et al.*, 2010; Filoche *et al.*, 2010; *ten* Cate *et al.*, 2009; Bruzual *et al.*, 2007; Sissons *et al.*, 2007; Sampermans *et al.*, 2005; Jin *et al.*, 2004; Verstrepen *et al.*, 2003; McWhirter *et al.*, 2002; Nikawa *et al.*, 1997; 1996). Studies on the effect of carbon source on *C. albicans* biofilm have yielded conflicting results (Jin *et al.*, 2004). Nutritional stress is one of the prominent factors regulating biofilm formation as it regulates the synthesis of autoinducers which signals for biofilm formation through specific and diverse signal transduction pathways (Jin *et al.*, 2004). Environmental factors operating within the oral milieu such as dietary sugars and saliva could modify candidal adhesion and biofilm formation to varying extents (Jin *et al.*, 2004). Concentrations of nutrients, electrolytes and pH value in the culture environment also induce adhesion by activating adhesion gene (Kucharikova *et al.*, 2011; Sampermans *et al.*, 2005; Verstrepen *et al.*, 2003). Chemical nature of the carbon source also affects biofilm synthesis as indicated by increase in biofilm formation under the presence of glucose (Al-Fattani and Douglas, 2006). Beside this, numerous environmental factors such as pH, temperature and inter species competition also influence adhesion and biofilm formation (Bandara *et al.*, 2010; Pan *et al.*, 2010; Bunt *et al.*, 1993). Presence of functional groups on cell surface enhances biofilm adhesion through increased non-bonding interactions. Uncharged bacterial cell surface associated at pH ranging from 2.2

to 4.0 and 1M ionic strength exhibits enhanced adhesion to hydrophobic surfaces (Katsikogianni and Missirils, 2004). *C. albicans* is able to grow either aerobically or anaerobically, and only a few studies have so far specifically focused on *C. albicans* biofilm formation in anaerobic conditions (Thein *et al.*, 2007). Contrastingly, studies done by of Biswas and Chaffin, 2005 suggested the inability of *C. albicans* to form biofilm under strict anaerobic conditions.

2.5.4.4 Exopolymeric substances

Candidal exopolymeric substances (EPS) are an inherent part of a biofilm and bestow biofilm with structural stability as well as acts as a structural barrier between sessile cells & environment (Lal *et al.*, 2010; Knoshaug *et al.*, 2000). EPS form a three-dimensional, gel-like, highly hydrated structure having dynamic environment in which microbial cells are immobilised. These substances have electric charge (due to functional groups) and are of hydrophobic nature which enables them to interact with variety of substrates which in turn facilitates adhesion, an initial and crucial step in biofilm formation (Saravanan and Jayachandran, 2008). Moreover, these substances by acting as a barrier between sessile microbial cells and environment (Allison *et al.*, 1999), restricts the diffusion of various antibiotics and protects the cells from host immune response (Hall-Stoodley and Stoodley, 2009). The EPS are mainly constituted by simple sugars, polysaccharides, proteins, eDNA and trace elements like phosphorus, sulphur etc. Al-Fattani and Douglas, 2006 during study on *Candida sp.* biofilm showed glucose (32.2% *w.r.t.* entire biofilm content) and hexosamine (27.4% *w.r.t.* entire biofilm content) as a major carbhohydrate content. Apart from this they also reported small amounts of phosphorus, hexosamine, and uronic acid in EPS content of *Candida sp.* biofilm (Al-Fattani and Douglas, 2006). Although polysaccharides and proteins are the more extensively studied substances of

Candida biofilm EPS, other molecules such as lipids and nucleic acids play a crucial role on EPS functions (Nobile and Mitchell, 2007). Earlier, different research groups have identified eDNA in the surrounding milieu as part of the EPS of biofilm formed by bacteria (Allesen-Holm *et al.*, 2006; Vilain *et al.*, 2009). Allesen-Holm *et al.*, 2006 have proposed the mechanisms implicated in eDNA release which include cell lysis, quorum sensing, and excretion from DNA containing vesicles. Besides the role of eDNA as a structural component of bacterial biofilm EPS, its role at the initial stage of biofilm formation and antibiotic resistance is still in its infancy (Tetz *et al.*, 2009; Izano *et al.*, 2008 ; Mulcahy *et al.*, 2008; Whitchurch *et al.*, 2002). Recently, Martins *et al.*, 2010b has been studied the role of eDNA in *C. albicans* biofilm structure and formation, with eDNA being a key element of the EPS in mature *C. albicans* biofilm and playing a predominant role in biofilm structural integrity and maintenance. Some of the components found in biofilm are also secreted by the planktonic cells into their medium (Thomas *et al.*, 2006) which indicates that these are the metabolic by-products. Previous studies had shown that EPS production is also determined by environmental conditions such as carbon source, stress and flow rate of nutrients (Al-Fattani and Douglas, 2006; Hawser *et al.*, 1998). Generally, EPS contain a wide variety of macromolecules and ions (Sutherland, 2001), which have multiple forms of interactions, like hydrogen bonding between electronegative atoms and polymer bridging between divalent cations. The combination of these interactions contributes to the biofilm's overall cohesiveness (Ahimou *et al.*, 2007; Chaw *et al.*, 2005).

2.6 Genetic regulation of *C. albicans* biofilm

Inspite of being the most common opportunistic human pathogen, information with respect to genetic architecture and complexity of *C. albicans* biofilm is limiting. Genome

sequence of *C. albicans* strain SC5314 (Jones *et al.*, 2004) have shown that around two-thirds of its open reading frames bears orthology with open reading frames of *Saccharomyces cerevisiae* genome (d'Enfert *et al.*, 2005; Ihmels *et al.*, 2005; Goffeau *et al.*, 1996). Since, *S. cerevisiae*, is a widely studied model organism, extensive homology of *C. albicans* genome besides taxonomic level enables easy adaptation of the genetic tools and information for better understanding of *C. albicans* biology and pathogenesis (Wang *et al.*, 2009). Though molecular basis of *C. albicans* biofilm formation and development is less understood it is believed that the interaction through substratum causes differential gene expression for biofilm genesis (Nailis *et al.*, 2006).

A list of transcription factors (Table 3), either repressors or activators, regulates biofilm development (Finkel and Mitchell, 2011; Wächtler *et al.*, 2011; Nobile *et al.*, 2006). Zap1 is a Zinc finger DNA binding domain containing transcription factor which negatively regulate the biofilm formation probably in according to the ambient zinc levels (Heller *et al.*, 2009). The azole efflux genes *CDR1, CDR2* and *MDR1* are induced early in biofilm formation and might contribute to overall azole resistance (Mansfield *et al.*, 2010). Transcription factors such as Efg1, Cph 1and Tec1 positively regulates candidal morphogenesis and biofilm development and null mutants of Efg1 and Cph1 were not able to mediate hyphal growth (Biswas *et al.*, 2007). Transcription factors modulating surface properties of hyphae (Bcr1) and controlling their morphogenesis (*CHK1*) are also critical for biofilm formation (Biswas *et al.*, 2007). *BCR1* proved to be a novel regulator of hyphal cell surface properties, thus suggesting that hyphal adherence specifically is critical for biofilm formation. Deletion of the histidine kinase gene *CHK1* renders both hyphal morphogenesis and biofilm formation refractory to farnesol inhibition. Alcohol dehyrogenases like Csh1 yields a matrix-inhibitory signal and retards biofilm development whereas Adh5 might function preferentially to yield a matrix-stimulatory

signal and enhance biofilm development (Finkel and Mitchell, 2011; Ramage *et al.* 2005). A cell wall protein which anchors the biofilm to substratum also regulates biofilm development. Ywp1/Pga24 negatively regulates adherence of yeast cells and deletion of *YWP1* improves adherence; over-expression of *YWP1* inhibits adherence (Granger *et al.*, 2005). Ywp1 is covalently linked to the cell wall through a glycophosphotidylinositol (GPI) anchor-derived linkage and has some structural similarity to the *S. cerevisiae* adhesin Flo1 (*de* Groot *et al.*, 2004; 2003). The largest family of known adhesins in *C. albicans* is the *ALS* (agglutinin-like sequence) gene family, and at least one family member, *ALS3*, is upregulated during hyphal morphogenesis (Hoyer *et al.*, 1998). Als family members are known to interact with several substrates, including host cells and proteins (Kumamoto and Vinces, 2005; Sheppard *et al.*, 2004; Sundstrom, 2002; Hoyer, 2001). The *ALS* family has been implicated specifically in biofilm formation through the finding that expression of *ALS1* and other family members increases during biofilm development *in vitro*.

Table 3. Genes involved in biofilm and associated drug resistant functions in *Candida*[#]

Gene	Description	Mutant phenotype for hyphal growth, biofilm formation and virulence	References
ACE2	Transcription factor	severe biofilm defect, aberrant hyphae formation	Kelly *et al.*, 2004
ADH1	Alcohol dehydrogenase	increased biofilm biomass	Mukherjee *et al.*, 2006
ADH5	Alcohol dehydrogenase	increased biofilm matrix	Nobile *et al.*, 2009
ALS3	Adhesin, invasin, ferritin receptor	abnormal biofilm formation	Almeida *et al.*, 2008
BCR1	C2H2 zinc finger transcription factor, biofilm and cell wall regulator	abnormal biofilm formation	Nobile *et al.*, 2006
BUD2	GTPase activating protein for Rsr1, involved in polar bud site selection and hyphal growth guidance, thigmotropism	abnormal filamentous growth	Hausauer *et al.*, 2005
CAT2	Carnitine acetyltransferase	reduced biofilm formation and biofilm thickness	Strijbis *et al.*, 2008
CBK1	Protein kinase	severe biofilm defect and defective hyphae formation	Blankenship *et al.*, 2010
CDR1,CDR2	Drug efflux pump	inhibited biofilm growth in the presence of azoles	Viudes *et al.*, 2002
CHK1	Histidine kinase	forms farnesol-resistant biofilm	Kruppa *et al.*, 2004
CKA2	Catalytic subunit (alpha-subunit) of protein kinase CK2, central role in governing	normal filamentous growth, decreased virulence	Bruno and Mitchell, 2005

	calcium homeostasis		
CPH1	Transcription factor required for mating and hyphal growth on solid media	abnormal biofilm formation	Alem *et al.*, 2006
CPH2	Myc family bHLH transcriptional activator of hyphal growth	normal filamentous growth, normal virulence	Chamilos *et al.*, 2009
CSA1	Cell wall protein	defect in biofilm formation, additive with *pga10-* and *rbt5-*	Perez *et al.*, 2006
CSH1	Aryl-alcohol dehydrogenase	decreased biofilm matrix in *zap1-*	Nobile *et al.*, 2009
CZF1	Zink finger protein, regulator of hyphal growth	abnormal filamentous growth	Chamilos *et al.*, 2009
EAP1	Cell wall protein	severe defect in biofilm formation	Li *et al.*, 2003
ECE1	Putative transmembrane protein	restores defect in *bcr1-* biofilm	Nobile *et al.*, 2006
ECM33	GPI-anchored cell wall protein, involved in cell wall integrity	abnormal filamentous growth, decreased virulence	Martinez-Lopez *et al.*, 2004
EFG1	Transcription factor with bHLH required for hyphal growth and metabolism; cell-wall gene regulator	abnormal filamentous growth, abnormal biofilm formation, decreased virulence	Alem *et al.*, 2006
FKS1	Beta-1,3 glucan synthase	reduced matrix production, reduce biofilm resistance to fluconazole	Nett *et al.*, 2011

FLO8	Transcription factor	defect in biofilm formation, defective hyphae formation	Cao *et al.*, 2005
GCA1,GCA2	Gluco-amylase	increased biofilm matrix	Nobile *et al.*, 2009
GCN4	Transcription factor	decreased biofilm biomass	Garcia-Sanchez *et al.*, 2004
GIN4	Protein kinase	severe biofilm defect, defective hyphae	Blankenship *et al.*, 2010
GPD2	Similar to glycerol 3-P dehydrogenases	normal filamentous growth	Fan *et al.*, 2005
GPP1	Putative glycerol 3-phosphatase	normal filamentous growth	Fan *et al.*, 2005
HGC1	Hypha-specific G1 cyclin-related protein, regulation of hyphal morphogenesis	abnormal filamentous growth, decreased virulence	Wang *et al.*, 2009
HWP1	Hyphal cell wall protein, adhesin	decreased biofilm formation, decreased virulence	Nobile *et al.*, 2006
HWP2	Cell wall protein	decreased biomass	Ene and Bennett, 2009
HYR1	GPI-anchored cell wall protein, hyphal-induced, macrophage-induced	normal filamentous growth	Bailey *et al.*, 1996
ICL1	Isocitrate lyase of the glyoxylate cycle, involved in utilization of alternative carbon metabolism	decreased virulence	Lorenz and Fink, 2001
IFD6	Aryl-alcohol dehydrogenase	decreased biofilm matrix in *zap1-*	Nobile *et al.*, 2009
IPF946	Epithelial escape and dissemination	abnormal filamentous growth	Zakikhany *et al.*, 2007

IRE1	Protein kinase	severe biofilm defect and hyphae formation	Blankenship *et al.*, 2010
KEM1	Exo-RNase	severe medium-dependent defect in biofilm formation and hyphal formation	Richard *et al.*, 2005
MDR1	Drug efflux pump	inhibited biofilm growth in the presence of azoles	Mukherjee *et al.*, 2003
MDS3	Unknown	severe medium-dependent defect in biofilm formation and hyphal formation	Richard *et al.*, 2005
MKC1	MAP kinase of the cell wall integrity pathway, contact induced filamentation	abnormal biofilm formation, decreased virulence	Kumamoto, 2005
NDH51	Subunit of nicotinamide adenine dinucleotide dehydrogenase complex I	decreased biofilm mass	Liu *et al.*, 2009
NRG1	Transcription factor	decreased release of dispersal cells	Uppuluri *et al.*, 2010
NUP85	Nuclear pore protein	severe medium-dependent defect in biofilm formation and hyphal formation	Richard *et al.*, 2005
OCH1	Alpha-1,6-mannosyltransferase	defect in biofilm formation, cellular aggregation, cell wall defects	Stichternoth and Ernst, 2009
PBR1	Unknown	forms a thin biofilm with reduced matrix production	Sahni *et al.*, 2009
PDX1	Pyruvate dehydrogenase	biofilm with reduced density	Liu *et al.*, 2009

PES1	Pescadillo homologue	reduced cell dispersion	Uppuluri *et al.*, 2010
PGA1	Cell wall protein	reduced metabolic activity	Hashash *et al.*, 2010
PGA10	Cell wall protein	defect in biofilm formation, additive with *rbt5-* and *csa1-*	Perez *et al.*, 2006
PLB1	Phospholipase B	decreased virulence	Leidich *et al.*, 1998
PMT1	Mannosyltransferase	biofilm with reduced biomass, defective hyphae	Peltroche-Llacsahuanga *et al.*, 2006
PMT2	Mannosyltransferase, involved in cell wall regeneration (β-1,6-glucan and mannoprotein levels)	homozygous mutant not viable, heterozygote abnormal filamentous growth, decreased biofilm formation	Prill *et al.*, 2005
PMT4,PMT6	Mannosyltransferase	moderate defect in biofilm formation	Peltroche-Llacsahuanga *et al.*, 2006
RAS1	RAS signal transduction GTPase; regulates cAMP and MAP kinase pathways	abnormal filamentous growth, decreased virulence	Feng *et al.*, 1999
RBT1	Cell wall protein	reduced biomass	Ene and Bennett, 2009
RBT5	Cell wall protein	defect in biofilm formation, additive with *pga10-* and *csa1- RBT5*-oe, restores biofilm in *bcr1-*	Nobile *et al.*, 2006
RIM101	Transcription factor involved in alkaline pH response	abnormal filamentous growth, decreased invasive growth	Davis *et al.*, 2000

RIX7	AAA ATPase	defect in biofilm formation	Melo *et al.*, 2006
RSR1	GTP/GDP cycling, involved in polar bud site selection and hyphal growth guidance, thigmotropism	abnormal polar bud site	Hausauer *et al.*, 2005
SOD5	Copper- and zinc-containing superoxide dismutase, required for oxidative stress tolerance	decreased virulence	Martchenko *et al.*, 2004
SUN41	Cell wall protein	severe biofilm defect, aberrant hyphae	Norice *et al.*, 2007
SUV3	Mitochondrial RNA helicase	severe medium-dependent defect in biofilm formation and hyphae formation	Richard *et al.*, 2005
TEC1	TEA/ATTS transcription factor involved in regulation of hypha-specific genes, regulates Bcr1	abnormal filamentous growth, decreased biofilm formation, decreased virulence	Nobile *et al.*, 2006
TOR1	Phosphatidylinositol kinases	rapamycin treatment inhibits *TOR1* resulting in decreased adhesion of cells and loss of filamentation	Bastidas *et al.*, 2009
TPK1	Catalytic subunit of cAMP-dependent protein kinase (PKA), isoform of Tpk2p	abnormal filamentous growth	Park *et al.*, 2005
TPK2	Catalytic subunit of the cAMP-dependent	abnormal filamentous growth, reduced virulence	Park *et al.*,2005

	protein kinase A (PKA)		
TUP1	Repressor of filamtous growth	hyperfilamentous growth	Braun *et al.*, 2000
UME6	Transcription factor	reduced biomass biofilm and defective hyphae formation, increased cell release from biofilm	Uppuluri *et al.*, 2010
VAM3	Vacuolar trafficking	biofilm defect, fragile biofilm, reduced biomass and abnormal hyphae	Palanisamy *et al.*, 2009
VPS1	Dynamin-family GTPase-related protein	rudimentary biofilm composed primarily of yeast and pseudohyphae	Bernardo *et al.*, 2008
VPS11	Involved in protein trafficking; putative role in vesicle-target membrane fusion, required for vacuole formation	abnormal filamentous growth	Palmer *et al.*, 2005;2003
YAK1	Protein kinase	severe biofilm defect and hyphal formation	Goyard *et al.*, 2008
YHB1	Nitric oxide dioxygenase, required in nitric oxide scavenging/detoxification	abnormal filamentous growth	Hromatka *et al.*, 2005
YWP1	Cell wall protein	increased adhesion, biofilm formation by only yeast-form cells	Granger *et al.*, 2005
ZAP1	Transcription factor	increased production of biofilm matrix	Heller *et al.*, 2009
orf19.851	Protein of unknown function; transcription is negatively regulated by Rim101p	/	

	(Eukaryot Cell 2(4):718-28)		
orf19.2833	PGA34, putative GPI-anchored protein of unknown function; transcription is repressed in response to alpha pheromone in SpiderM medium	/	
orf19.3459	Not characterized	/	
orf19.3600	Not characterized	/	

[#] (Finkel and Mitchell, 2011; Wächtler *et al.*, 2011; Nobile and Mitchell, 2006)

2.7 *C. albicans* biofilm resistance

The mechanisms that contribute to drug resistant phenotype in eukaryotic cells include reduction in the import of the drug into the cell; modification or degradation of the drug once it is inside the cell; changes in the interaction of the drug with the target enzyme (binding, activity); changes in other enzymes of the same enzymatic pathway and an increased efflux of the drug from the cell (White *et al.*, 1998). Studies done by Anderson, 2005 too have shown that growth of *C. albicans* in biofilm mode had increased antifungal resistance, in the expanding population of immunocompromised persons. Presence of such 'antifungal persister' cells and inhibition of diffusion of antifungals by the extracellular biofilm matrix may also offer resistance (Al-Dhaheri and Douglas, 2010; Al-Fattani and Douglas, 2006; LaFleur *et al.*, 2006), despite the fact that the molecular mechanisms underlying the antifungal resistance had not been fully understood (d'Enfert, 2006; Mukherjee *et al.*, 2005; Ramage *et al.* 2005).

2.7.1 Factors attribute to antifungal resistance

2.7.1.1 Biofilm growth

Progression of drug resistance of the *C. albicans* biofilm is associated with parallel increase in metabolic activity of the developing biofilm (Nett *et al.*, 2011; Bruzual *et al.*, 2007). Increased drug resistance is not due to lower metabolic activity of cells in maturing biofilm, but is more related to the maturation process (Watamoto *et al.*, 2011; Ferreira *et al.* 2010). As biofilm consists of a heterogeneous population of cells with different growth rates; therefore, a subpopulation of cells could also confer antifungal resistance because of their slower growth rate (Kumamoto, 2002).

2.7.1.2 Genetic basis of antifungal resistance

Molecular mechanisms conferring antifungal resistance in *C. albicans* biofilm have not been fully elucidated. Majority of the drug resistance genes are those coding for multidrug efflux pumps, the upregulation can result in cell to become a multidrug-resistant phenotype (Douglas, 2002; Ramage *et al.*, 2002b; Chandra *et al.*, 2001). *C. albicans* possess two different types of efflux pumps: adenosine triphos-phate–binding cassette (ABC) transporters encoded by the *CDR* genes (*CDR1* and *CDR2*) and major facilitators encoded by the *MDR* genes (Harry *et al.*, 2002; Ramage *et al.*, 2002b). The overexpression of the *MDR1* gene leads to exclusively fluconazole resistance (Chandra *et al.*, 2001; Moran *et al.*, 1998). Studies have shown that ATP-binding cassette (ABC) and major facilitator superfamily (MFS) drug efflux pumps are involved in increased azole antifungal resistance in only early phase. Microarray analysis of *C. albicans* biofilm further confirmed expression of *MDR* and *CDR* genes in biofilm is phase-specific, contributing to azole resistance only during the early phase, whereas changes in sterol composition are involved in the resistance in the mature phase (Mukherjee *et al.*, 2003). A study using an *in vivo* biofilm model has yielded different results, the expression of *CDR1* and *CDR2* is up-regulated in biofilm compared with planktonic cells, but that *EFG11* and *MDR1* expression is similar in both biofilm and planktonic cells (Andes *et al.*, 2004). Recently, the role of β-1,3-glucan in antifungal resistance was postulated as it has shown that cell walls from biofilm cells could bind (and hence neutralize) antifungals better than planktonic variants, and that exogenous β-1,3-glucan reduced the activity of fluconazole against planktonic *C. albicans* cells (Nett *et al.*, 2008). Another contributing factor might be the role of cross-resistance. It has been frequently observed that mild forms of stress may prepare cells for subsequent (increased) stress conditions of a different nature (ten Cate *et al.*, 2009; Kara *et al.*, 2006). Biofilm cells live in a nutrient-

poor, hypoxic environment, and it is conceivable that the suboptimal growth conditions in biofilm may result in increased tolerance to various forms of stress, including antimycotics-related cellular stress. More sophisticated *in vitro* and *in vivo* genome wide expression analyses are needed to elucidate the role of genes that confer higher antifungal resistance in Candida biofilm. Phenotypic switching of *C. albicans* (Balan *et al.*, 1997) and *C. glabrata* (Lachke *et al.*, 2002) also regulates the expression of some genes such as *CDR* gene, which is closely associated with azole resistance of *C. albicans*.

2.8 Antifungal susceptibility of *C. albicans* biofilm

Since decades many drugs and derivatives continue to dominate as an antifungal arsenal to combat *C. albicans* infections (Pusateri *et al.*, 2009; Agarwal *et al.*, 2008; Seyfarth *et al.*, 2008). Even though they are very widely acclaimed for their efficacy, these drugs are known to have side effects and become ineffective when the therapies are delayed or when the organism switched to its biofilm mode (Bruzual *et al.*, 2007; Jain *et al.*, 2007). Besides this, the action of these drugs may be limited by their penetration and chemical reaction into biofilm matrix, the extracellular polymeric material (Lal *et al.*, 2010; Nett *et al.*, 2010; Perlin *et al.*, 2009; Jain *et al.*, 2007).

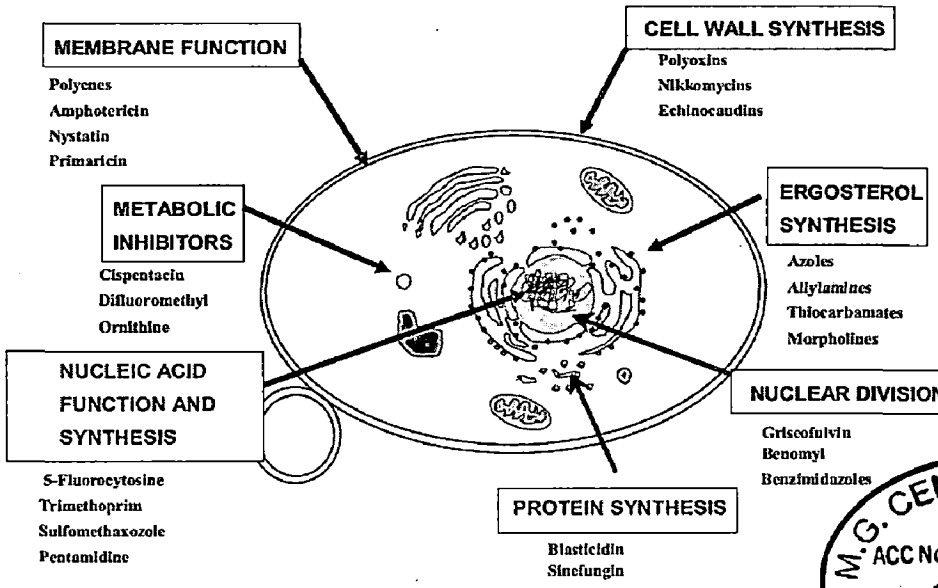


Figure 8: Different sites of action of antifungal agents (Mishra *et al.*, 2007)

The most prominent drug which is used widely for combating *C. albicans* caused infections is azole based. Ergosterol is a structural component of fungal cell membranes as cholesterol is in animal cell membranes. Azoles inhibits the cytochrome P450 dependant lanosterol demethylase which eventually leads to the accumulation of methylated 14α sterols which are toxic to the cells (Clark *et al.*, 1996). The other class of drugs inhibiting the ergosterol pathway is morpholines, allylamines and thiocarbamates. The other important classes of anticandidal drugs include polyenes, amphotericin B, nystatin and primaricin. These drugs destabilize the cell membrane of fungi by intercalating into the ergosterol containing phospholipid bilayer and creating channels which consequently abolishes the proton gradient required for oxidative phosphorylation (Mishra *et al.*, 2007). Even though, several classes of drugs as depicted in Fig. 8 could be potentially useful in prophylaxis. They are completely ineffective once candidal infection is established as they are often associated with biofilm formation. *Candida* biofilm are resistant to plethora of

antifungal agents and drug concentrations required for 50% reduction in metabolism were five to eight times higher for biofilm that for planktonic cells, and 30-2000 times higher than the corresponding minimum inhibitory concentration (Ramage *et al.*, 2002a; 2002b; Baillie and Douglas, 2000). There are various factors responsible for antifungal resistance in biofilm. The major factor is the up regulation of many drug efflux pumps such as ABC transporters, MFS pumps in the cells anchored in biofilm. These efflux pumps reduce the bioavailability of the drugs and effectively increase the drug concentrations by many folds (Ramage *et al.*, 2002a). The other factor alters the pharmacokinetics of drug by reduced diffusion or increased expulsion of drugs through biofilm. Moreover, the slow growth rate of sessile cells in biofilm brings about changes in their cell membrane which in turn reduce their susceptibility towards drugs acting at cell membrane such as amphotericin B (Nett *et al.*, 2007). The selective evolution and proliferation of population with mutation in the target genes or enzymes in biofilm also concurs with its resistance towards antifungal agents acting on metabolic pathways; for instance: point mutations occurring in lanosterol methylase gene *ERG11* brings about resistance towards azoles (Lamb *et al.*, 1997). Biofilm of non *C. albicans* species, such as *C. tropicalis* and *C. parapsilosis*, were also drugs resistant (Kuhn *et al*, 2002). Many studies have demonstrated drug resistance for *Candida* biofilm grown on cellulose (Baillie and Douglas, 1998), polysytrene (Ramage *et al.*, 2001a; 2001b), silicone elastomer (Chandra *et al.*, 2001) and denture acrylic (Chandra *et al.*, 2001). Recently, however, it has been reported that some of the newer antifungal agents are active against *Candida* biofilm (Douglas, 2003; 2002). Although biofilm of *C. albicans* and *C. parapsilosis* were clearly resistant to two new triazoles (voriconazole and revuconazole), there appeared to be some antibiofilm activity with lipid formulations of amphotericin B and two echinocandins, caspofungin and micafungin (Kuhn *et al.*, 2002). The

efficacy of caspofungin against *C. albicans* biofilm *in vitro* has been confirmed by other workers (Ramage *et al.*, 2002b). Caspofungin, is an antifungal agent that inhibits the synthesis of β-1,3-glucan, the major structural component of *Candida* cell wall and its biofilm matrix and has proved to be an important agent for biofilm removal (Baillie and Douglas, 2000). Recent preventive trials against *C. albicans* biofilm demonstrated that peppermint, eucalyptus, ginger grass and clove oils act as potent antifungal agents against *C. albicans* biofilm and eucalyptus oil was found to be a potentially superior antifungal agent in compared to fluconazole (Agarwal *et al.*, 2008). It is believed that plant derived substance owing to their versatile application could justify the search for noval antifungal agent. Eugenol [2-methoxy-4-(2-propenyl) phenol], the major phenolic component of clove (*Eugenia caryophillis*) oil belongs to a new class of microbicidal phenylpropanoids which possesses potent fungicidal, bactericidal, anesthetic, anti-oxidant and anti-inflammatory properties (Guenette *et al.*, 2006; Nangle *et al.*, 2006). The increasing resistance of biofilm residing microbes towards these compounds and the reduced number of available drugs asserts on the need for the search and development of novel therapeutic alternatives which is indeed the aim and goal of the present study.

CHAPTER 3

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Microbial strains

a) Bacterial strains: *Pseudomonas aeruginosa* (DSVP20) and besides this, 309 bacterial strains were collected from contaminated oil sludge samples (Ankleswar, Gujarat) and from local fuel filling stations.

b) Fungal strains: *Candida albicans* (MTCC 227) was procured from Institute of Microbial Technology (IMTECH), Chandigarh, India.

3.1.2 Chemical reagents and diagnostic kits

Some of the fine chemicals and kits used in the study have been listed below:

- 2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT); Menadione; Ethidium Bromide (EtBr) from Sigma chemicals, USA.
- Silica gel 60-200 from Merck, Germany.
- Fluoranthene, Pristine, Hexadecane from Acros organics, Belgium.
- Propidium Iodide (PI); Fluorescein Isothiocynate (FITC) and Concanavalin A (ConA) from Himedia chemicals, India.
- All culture media and solvents were obtained from Himedia chemicals and Ranbaxy, India.

3.1.3 Culture media

Yeast extract peptone dextrose (YPD), Yeast extract phosphate (YEP) and Roswell Park Memorial Institute (RPMI) 1640 medium were purchased from

Himedia Chemicals, India.

3.1.4 Indwelling medical devices (IMDs)

- Surgical sutures: Nonabsorbable sutures Prolene* NW843 (Ethicon; Johnson & Johnson), Sutupak* SW216 (Ethicon; Johnson & Johnson) and absorbable sutures Chromic NW4241 (Mersutures; Johnson & Johnson), Plain W2004 (Ethicon; Johnson & Johnson).
- Soft contact lenses: Etafilcon A (Johnson & Johnson), Galyfilcon A (Johnson & Johnson), and Hilafilcon A (Bosch & Lomb).
- Commercially available biopolymer, polystyrene (PS) was cut into 1 cm^2 and sterilized before use.

3.2 METHODS

3.2.1 Isolation and screening of rhamnolipid producing microorganism

Oil sludge samples were collected from contaminated sites viz. Ankleshwar, Gujarat. Besides this, samples contaminated with diesel oil and motor oil from local fuel filling stations were also used in this study. The samples were stocked in a sterile container and stored at 4°C until analysed.

3.2.2 Media preparation

"Pruthi and Cameotra" (PAC) salt medium used in this study contained the following nutrients (mg L^{-1}), which were found optimal in biosurfactant synthesis: KH_2PO_4 2000; $Na_2HPO_4 \cdot 2H_2O$ 2000; K_2SO_4 350; $NaNO_3$ 2000; $MgSO_4 \cdot 7H_2O$ 150; NaCl 100; $FeSO_4 \cdot 7H_2O$ 50; $CaCl_2$ 50; 1 mL of the trace elements (TES) per liter and 2% glycerol as sole carbon source. TES contained (mg L^{-1}): $ZnSO_4 \cdot 7H_2O$ 525; $MnSO_4 \cdot 4H_2O$ 200;

$CuSO_4 \cdot 5H_2O$ 705; $Na_2MoO_4 \cdot 2H_2O$ 15; $CoCl_2 \cdot 6H_2O$ 200; H_3BO_3 15; $NiSO_4 \cdot 6H_2O$ 27 (Pruthi and Cameotra, 2003) .

3.2.3 Bacterial enumeration

Soil samples (10 g) were shaken with 200 mL distilled water for 30 min at 200 rpm and allowed to settle. The supernatant was collected and centrifuged at 3000 rpm for 5 min before plating on PAC salt medium supplemented with 2% crude oil for detection of bacterial isolates as colony forming unit (cfu) / g of soil (Kiyohara *et al.*, 1982). The purified bacterial colonies obtained were tested for their ability to grow on 2% hydrocarbon substrates like dodecane, hexadecane, pristine, toluene and fluoranthene (Himedia, Acros).

3.2.4 Screening of selected isolates for biosurfactant production

3.2.4.1 Surface tension measurement

Surface tension of *P. aeruginosa* cell-free broth was measured using Wilhelmy plate measurement technique as described earlier by Pallas and Pethica, 1983 at various time intervals. The surface tension of PAC salt medium at 25°C was used as control. Cell free broth was taken in a 50 mL glass beaker and placed onto the tensiometer platform. A platinum plate was touched the liquid-air interface, to measure the surface tension (mN m^{-1}) by a Du-Nouy tensiometer (CSC No 70535, USA). Between each measurement, the platinum plate was rinsed three times with water, three times with acetone, and was then allowed to dry. The instrument was calibrated using water to a reading of 72 mN m^{-1} and all the measurements were taken in triplicate (Cameotra and Singh, 2009). For determining the CMD, the cell-free broth was diluted 10-fold (CMD^{-1}) and 100-fold (CMD^{-2}) with distilled water (Rivardo *et al.*, 2009; Rahman *et al.*, 2002a).

3.2.4.2 Haemolytic activity

Screening of isolated biosurfactant producing strain was determined by haemolytic activity. The fresh single colony of cultures was streaked on blood agar plates containing 5% (v/v) blood (Himedia, India), respectively and incubated at 37°C for 48-72 h. Haemolytic activity was detected by presence of a clear zone around the bacterial colony after incubation for 48 h at 37°C which is an indicative of biosurfactant production (Plaza *et al.*, 2006; Mulligan *et al.*, 1984).

3.2.4.3 CTAB agar plate assay

The CTAB agar plate method is a semi-quantitative assay for the detection of extracellular glycolipid biosurfactants of various isolates developed by Seigmund and Wagner, 1991. Each pre-grown culture (24 h) from MSM medium was spot inoculated onto CTAB agar plates and incubated at 37°C for 48 h to detect dark blue halos around the culture spot.

3.2.4.4 Drop collapse assay

The drop collapse assay was performed in the polystyrene 96-well microtitre plate as reported previously (Bodour and Miller, 1998). Bacteria were subcultured from overnight cultures into PAC salt medium and grown for 16 h at 30°C. Cells were removed by centrifugation, and the supernatant was filtered through a 0.22-µm membrane and serially diluted in dH_2O. Aliquots of 5µL were spotted into the centre of the well using a 25 µL glass syringe (Hamilton, USA) by holding the syringe at an angle of 45°C and assayed for bead formation. Samples that did not form a bead were defined as having drop collapse activity (Caiazza *et al.*, 2005; Deziel *et al.*, 2003).

3.2.4.5 Emulsification test

A qualitative biosurfactant activity assay was performed using a modified method emulsification test (Cameron *et al.*, 1998).The bacterial broth was centrifuged and 2 mL cell free broth with 3 mL of n-hexadecane in the screw cap test tube was then added. The mixture was vortexed at high speed for 2 min and left at room temperature. The result was observed after 24 h for the stability of emulsion. The total volume of the mixture, volume of emulsified and volume of non-emulsified phase was observed (Chen *et al.*, 2007). The emulsification index E_{24} (%) was calculated dividing the height of the emulsion layer by the mixture's total height and multiplying by 100.

E_{24} (%) = (The height of emulsion layer / The height of total solution) × 100

3.2.5 Cell surface hydrophobicity technique

Bacterial strains selected on basis of above screening methods (3.2.4), were subjected to cell surface hydrophobicity technique for further assessment of biosurfactant production. In this technique, an array of tests namely Hydrophobic Interaction Chromatography (HIC): Bacterial Adherence to Hydrocarbons (BATH): Adherence is Polystyrene; Replica Plate Test (RP) was performed.

3.2.5.1 Hydrophobic interaction chromatography (HIC)

In this method, phenyl Sepharose CL-4B, bed volume approximately 0.6 mL was used as the column packing matrix. The column was equilibrated with a solution of 4.0 M NaCI in 0.5 M citrate buffer. Cell suspension was prepared in the same solution which served for equilibrating the gel and 0.1 mL was introduced on the gel followed by 3 mL of the equilibrating solution. Elute (non retained bacteria) was compared with the original

bacterial suspension by measuring the absorbance at 540 nm and the results recorded as a percentage of retained bacteria (Hydrophobic Index, HI) (Pruthi and Cameotra, 1997).

3.2.5.2 Bacterial adherence to hydrocarbons (BATH)

BATH assays were performed to determine changes in cell surface hydrophobicity during growth on PAC salt medium as previously described (Zhang 1992; Rosenberg *et al.*, 1980). Briefly, cells were grown for 24 h at 30°C in PAC salt medium, washed twice in phosphate buffer and resuspended in the same buffer to reach OD_{600nm} of about 0.6. The cell suspension (1.2 mL) was then mixed with 0.2 mL of hydrocarbon (dodecane, hexadecane, pristane) and vortexed for 2 min. It was then allowed to stand at room temperature for 15 min. The aqueous phase was recovered, and its optical density was measured at 600 nm. The difference between the OD of the aqueous phase before and after mixing time was calculated: $100\times[1-(OD_{600}$ after mixing/OD_{600} before mixing)]. The results were expressed as the percentage of cells bound to hydrocarbon (Caiazza *et al.*, 2005; Christova *et al.*, 2004).

3.2.5.3 Adherence to polystyrene: replica plate (RP) test

In this technique, 25 mm diameter coupons were cut from sterile disposable polystyrene petri dishes. They were pressed firmly on to confluent agar surface growth and the replica colonies obtained on the polystyrene surface were washed in running water for 2 min. To facilitate visualization and comparison with the original colonies the replica was fixed by dipping in methanol and staining with crystal violet. Greater than 50% coverage of the disc by adherence cells was scored as positive. This procedure was repeated at different time intervals so as to check the age dependent hydrophobicity of the bacterial colonies (Pruthi and Cameotra, 1997).

3.2.6 Extraction and purification of biosurfactant from culture broth

To extract biosurfactant, the *P. aeruginosa* DSVP20 was cultivated at 30°C for 96 h at rotary shaking (150 rpm) in a 250 mL Erlenmeyer flask containing 100 mL of the PAC salt medium. Cell free broth of the *P. aeruginosa* so obtained was then centrifuged (9000×g for 20 min, 4-8°C). The broth was then acidified to pH 2 with 6M H_2SO_4 and allowed to stand overnight at 4°C. The precipitate was harvested by centrifugation (5000×g, 20 min) and extracted twice with ethyl acetate. The organic phase was collected and the solvent was removed in a rotary evaporator (Yamto, Japan) to obtain crude biosurfactant which was again precipitated with acid, re-dissolved in deionized water followed by lyophilization. The surface active properties of above extracted biosurfactant were then measured as described in section 3.2.4. For further purification, Sánchez *et al.*, 2007 method was used. Briefly, about 6 g of crude extracted mixture was re-dissolved in 10 ml of chloroform ($CHCl_3$) and subjected to chromatography on 85 g ultra pure flash silica-gel 60, which has a particle size of 40–63 μm, 230–400 mesh, and a surface area of 500 $m^2\ g^{-1}$. The loaded column (2.8 cm× 35 cm) was rinsed with $CHCl_3$ at a flow rate of approximately 1 mL min^{-1} until neutral lipids were totally eluted. $CHCl_3$–MeOH (5:1 to 1:5, v/v) were then applied as mobile phase in sequence and 15 mL fractions were collected by an auto fraction collector, followed by TLC detection.

3.2.7 Structural analysis of biosurfactant

3.2.7.1 Thin layer chromatography (TLC)

The initial characteristic of biosurfactant was done using thin layer chromatography (TLC) on silica gel 60 plates (5553, Merck, Darmstadt, Germany) with solvent system comprising of chloroform/methanol/acetic acid (15:5:1) and visualized by orcinol-sulfuric

acid staining (Christova *et al.*, 2004). Spots so obtained were then scraped and extracted with 3 mL of chloroform/menthol (2:1, v/v) in an elution column.

3.2.7.2 Fourier transforms infrared spectroscopy (FTIR)

For FTIR analysis, dried biosurfactant was ground with KBr powder and was dispersed uniformly in a matrix of dry nujol (paraffin) mar, compressed to form an almost transparent disc for FTIR spectra measurement in the frequency range of 4000-500 cm^{-1}. The biosurfactant sample was characterized using FTIR spectrophotometer (Thermo-Nicolet, USA) equipped with OMNIC software for data analysis (Worakitsiri *et al.*, 2011).

3.2.7.3 Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF-MS)

For mass spectrometric analysis of isolated biosurfactant was dissolved in anhydrous methanolic 5% HCl, heated at 100°C for 2 h, then neutralized with 0.5 M $NaHCO_3$ and extracted with $CHCl_3$. The $CHCl_3$ extract containing fatty acid methyl esters was dissolved in CH_3OH then 0.5 µL of purified biosurfactant were spotted onto an anchor chip positions on a MALDI plate. 0.5 µL of matrix was added to the sample spot which consist of saturated solution of 2,5-dihydroxybenzoic acid (DHB) in water and 0.3 mg mL^{-1} α-cyano-4-hydroxycinnamic acid in acetone: ethanol (2:1, v/v). A peptide standard was also spotted for external calibration. The spots were left at room temperature to dry and analyzed on Applied Biosystems Voyager System 4402 mass spectrometer in delayed mode, with an acceleration voltage 20 kv (Wang *et al.*, 2005).

3.2.7.4 Nuclear magnetic resonance spectroscopy (NMR)

Spectra of the purified biosurfactant was recorded with ^{1}H and ^{13}C NMR using 500

MHz Bruker AV500 OFT-NMR spectrometer (Thanomsub *et al.*, 2006; Wie *et al.*, 2008). The samples were prepared as solutions in 100% $CDCl_3$ using approximately 1-3 mg of biosurfactant for ^1H NMR analysis.

3.2.8 Physico-chemical properties of di-rhamnolipid (RL2)

3.2.8.1 Surface active properties of RL2

The surface active properties (Surface tension, CMD^{-1}, CMD^{-2} and emulsification index) of above extracted purified RL2 were measured as per the protocols described in section 3.2.4.

3.2.8.2 Determination of sugar concentration

Substrate concentration (total sugars) was estimated by Anthrone reaction (Roe *et al.*, 1955). In brief, 4 mL of Anthrone Reagent (2.0 g of Anthrone dissolved in 1L of conc. sulfuric acid) was added to 1 mL of sample or standard solution and rapidly mixed in an ice bath. Tubes were incubated in a boiling water bath for 10 min., cooled and read at 620 nm using a reagent blank. Sugar in the sample was determined from calibration curve plotted using rhamnose as the standard (Dubois *et al.*, 1956).

3.2.8.3 Determination of lipid content of the isolated RL2

To determine the lipid content, the biosurfactant was extracted by gently refluxing with diethyl ether for 1 h. This step was repeated thrice. The ether extract was dried with anhydrous Na_2SO_4 and clarified, followed by gravimetric estimation of the crude lipid.

3.2.8.4 Stability studies of purified RL2

Aqueous solution of 0.1% purified RL2 was used to study its stability effect at different temperature, pH and salinity.

3.2.8.4.1 Stability at different temperature

To check the stability of RL2 at different temperature, RL2 solutions (0.1%) was exposed to temperature (4-121°C) for 10 min and the surface activities (surface tension, CMD^{-1} and CMD^{-2}) and emulsification activity of the resultant solutions were then measured (Abdel-Mawgoud *et al.*, 2009).

3.2.8.4.2 Stability at different pH

To monitor the stability of RL2 at different pH, a set of RL2 solutions (0.1%) was adjusted to pH values of 4, 6, 8, 10 and 12. The surface activities (surface tension, CMD^{-1} and CMD^{-2}) and emulsification activity of the resultant solutions were then measured (Zhang and Miller, 1992; Cooper and Goldenberg, 1987).

3.2.8.4.3 Stability at different salinities

To test the stability of RL2 at different salinity, RL2 solutions (0.1%) were prepared containing different NaCl concentrations (1, 2, 4, 6, 8, 10%, w/v). The surface activities (surface tension, CMD^{-1} and CMD^{-2}) and emulsification activity of the resultant solutions were then measured (Helvaci *et al.*, 2004).

3.2.9 Optimization of physico-chemical parameters for RL2 production

Optimization of physico-chemical parameters for maximum production of RL2 was done using different parameters such as incubation period, aeration and agitation rate, temperature, type of substrates and substrate concentration.

3.2.9.1 Effect of incubation period on RL2 production

To optimize *P. aeruginosa* DSVP20 growth at 37°C for RL2 production at different intervals time (24-144 h) changes in surface tension values, RL2 production and dry biomass were recorded as has been described by Praveesh *et al.*, 2010.

3.2.9.2 Effect of aeration and agitation rate on RL2 production

To study the effect of aeration and agitation on optimum RL2 production, different aeration (0.25-1.5 vvm) and agitation rates (50-200 rpm) were used. Changes in surface tension values, RL2 production and dry biomass were measured after 96 h of growth at 37°C (Wei *et al.*, 2005).

3.2.9.3 Effect of pH and temperature on RL2 production

To study the effect of pH and temperature on optimum RL2 production, wide range of pH (4-12) and temperatures (20-50°C) were used. Changes in surface tension values, RL2 production and dry biomass were measured after 96 h of growth (Wei *et al.*, 2005).

3.2.9.4 Effect of carbon source on RL2 production

To study the effect of carbon sources on optimum RL2 production, PAC salt medium supplemented with 2% (w/v) glycerol, glucose, mannitol, starch, sucrose, maltose, oleic acid, olive oil and soyabean oil was used. Changes in surface tension values, dry biomass and RL2 production were measured after 96 h of growth at 30°C.

3.2.9.5 Effect of glycerol concentration on RL2 production

To investigate the effect of glycerol concentration on RL2 production, glycerol was added at different conc. 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 % (w/v) into PAC salt medium. Changes in surface tension values, dry biomass and RL2 production were measured after 96 h of growth at 30°C.

3.2.9.6 Effect of inorganic and organic nitrogen sources on RL2 production

0.5% (w/v) of inorganic (ammonium sulphate, ammonium nitrate, ammonium chloride, sodium nitrate, sodium nitrite and potassium nitrate) and organic nitrogen (urea and yeast extract) sources, were added separately in PAC salt medium having 2% (w/v)

glycerol. Changes in surface tension, dry biomass and RL2 production were then measured as described previously (Silva *et al.*, 2010).

3.2.10 *C. albicans* biofilm formation and quantification

3.2.10.1 *C. albicans* biofilm formation

The *C. albicans* culture broth was centrifuged (5000×g for 15 min at 4°C) and pellet was washed twice with sterile PBS buffer (phosphate buffered saline, pH 7.2). The cell pellet was then diluted to obtain an inoculum of 1×10^7 blastospores mL^{-1} in RPMI 1640-MOPS medium for experimental work. *C. albicans* biofilm formation and quantification was done on commercially available, presterilised, polystyrene, flat-bottomed, 96-well microtitre plates (MTP), as described earlier (Bruzual *et al.*, 2007). Standard cell suspensions (200 µL containing 1×10^7 blastospores mL^{-1} in RPMI 1640-MOPS medium) were seeded in MTP wells at 37°C for 90 min (adhesion phase), the wells were then washed with sterilized PBS to remove loosely adhered cells, followed by addition of sterilized RPMI 1640-MOPS medium (200 µL) for 72 h at 37°C incubation without agitation to form the biofilm. Quantification of biofilm was done using XTT reduction assay and measuring the OD_{492nm} using MTP reader (Oasys UVM 340). All assays were carried out in triplicate (Bruzual *et al.*, 2007; Silva *et al.*, 2008).

3.2.10.2 XTT reduction assay

The metabolic activity of candidal cells were measured using the tetrazolium (XTT) assay based upon the reduction of XTT tetrazolium to tetrazolium formazan by mitochondrially active cells in the presence of menadione, an electron-coupling agent. Briefly, from each well RPMI-1640 medium was aspirated and rinsed twice with PBS. The plates were then incubated for 1.5 h at 37°C in the dark with 40 µL of XTT (1 mg mL^{-1} in PBS) and 2 µL of menadione (0.4 mM in acetone) in 158 µL of PBS. The

absorbance of XTT formazan was then measured at 492 nm (microplate reader, Spectra Max M2) and correlated with the metabolic activity of Candidal cell within the biofilm (Silva *et al.*, 2008; Bruzual *et al.*, 2007).

3.2.10.3 Scanning electron microscopy (SEM)

SEM studies on C. albicans biofilm were performed using PS coupons as described by Singh *et al.*, 2011. Briefly, PS discs (0.5 cm^2), were fixed with 2.5% (v/v) glutaraldehyde in PBS for 2 h at room temperature. They were then treated with 1% (w/v) uranyl acetate for 1 h, and washed with distilled water. The samples were dehydrated with ethanol series (30%, 50%, 70%, 90% and 100%). All samples were dried to critical point by Polaron critical point drier, coated with gold and viewed under SEM (Leo 435, England) for analysis.

3.2.10.4 Confocal laser scanning microscopy (CLSM)

CLSM was performed to visualize *C. albicans* biofilm on PS coupons by fixing it with 2.5% glutaraldehyde in 0.1 M phosphate buffered saline (PBS; pH 7.2) for 1.5 h. After washing with PBS, coupons were transferred to 12-well plates and incubated for 45 min at 37°C in 4 mL of PBS containing the fluorescent stains Propidium iodide (PI; 8 μM) and (FITC-ConA; 50 μg mL^{-1}) in 10 mM hydroxyethylpiperazine ethanesulfonic acid (HEPES)/ 0.1 M NaCl, containing 0.1 mM Ca^{2+} and 0.01 mM Mn^{2+}. PI (long-pass filter; excitation wavelength, 543 nm; emission, 617 nm) intercalates into double-stranded nucleic acids to metabolically inactive cells and emit red florescence, while FITC-Con A (long-passfilter; excitation wavelength, 490 nm; emission, 525 nm) binds to glucose and mannose residues of fungal cell wall polysaccharides and emits green fluorescence. After incubation with the dyes, the PS coupons were flipped and placed on a 35-mm-diameter glass-bottom petri dish. Stained biofilm were observed by using an Olympus FluoView™

FV1000 confocal laser scanning microscope (objective Plan Apo 60X1.4 oil, Japan) (Lal *et al.*, 2010).

3.2.11 Effect of RL2 on *C. albicans* biofilm

3.2.11.1 Minimum inhibitory concentration (MIC) determination of RL2

The MIC of RL2 against the *C. albicans* was determined by using the standard broth microdilution method (NCCLS, 2002). Different RL2 concentrations (0.78–400 μg mL^{-1}) were prepared in RPMI 1640-MOPS medium and added to each MTP wells. An overnight grown culture of *C. albicans* was diluted to final concentration of 0.5×10^3 to 2.5×10^3 blastospores mL^{-1} in RPMI 1640-MOPS medium and then inoculated up to 200 μL into above MTP wells as has been done by Park *et al.*, 2006; Bruzual *et al.*, 2007. The plates were incubated at 37°C for 24 h without agitation. The MIC was defined as the lowest drug concentration at which there was complete inhibition of *C. albicans* growth when estimated using MTP reader at OD_{530nm}. Wells without RL2 and those lacking the cells were used as controls. All assays were carried out in triplicate.

3.2.11.2 Anti-adhesive action of RL2 on *C. albicans*

The anti-adhesive activity of RL2 against *C. albicans* was estimated by adding 200 μL of Candidal cells (1×10^7 blastospores mL^{-1} in RPMI 1640-MOPS medium) in 96-well plate. Cells were allowed to adhere for 4 h at 37°C, then medium was aspirated and wells were washed with sterilized PBS to remove loosely adhered cells. Different concentrations of RL2 (0.04-5.0 mg mL^{-1}) were then added and the plates were further incubated at 37°C for 2 h. The plates were read at OD_{492nm} by performing XTT reduction assay. The results were expressed in term of percent cell adhesion compared to rhamnolipid-untreated wells, which were used as control. All assays were carried out in triplicate.

3.2.11.3 RL2 treatment of pre-formed biofilm

C. albicans biofilm was allowed to form in 96-well polystyrene MTP as described earlier (Agarwal *et al.*, 2008). After 48 h incubation period, planktonic cells were removed and the medium was replaced with RPMI 1640-MOPS medium containing different concentrations of RL2 (0.04–5.0 mg mL^{-1}). These plates were further incubated at 37°C for 12 h. The biofilm formed on MTP wells were quantified by using the XTT reduction assay (Silva *et al.*, 2008; Bruzual *et al.*, 2007). The values are expressed in terms of percent biofilm formed in comparison to untreated biofilm as control (Dusane *et al.*, 2010; Bruzual *et al.*, 2007).

3.2.11.4 Cell surface hydrophobicity

Cell surface hydrophobicity of *Candida* biofilm was assessed by the biphasic separation method in RPMI 1640-MOPS medium at 37°C as described previously (Klotz *et al.*, 1985). Briefly, *C. albicans* exposed to different concentrations (0.04-5.0 mg mL^{-1}) of RL2 were incubated for 48 h to form biofilm. Biofilm formed was then removed from the MTP well surfaces with a sterile scraper to prepare a cell suspension (OD_{600nm} of 1.0 in RPMI 1640-MOPS medium). A total of 1.2 mL of the suspension from each group was drawn into a clean glass tube and overlaid with 0.3 mL of octane. The mixtures were vortexed for 3 min and then the phases were allowed to separate. The OD_{600nm} was determined for the aqueous phase soon after the two phases had separated. OD values of strains in RPMI 1640-MOPS medium without octane overlay were used as a negative control. Three repeats were performed for each group. The relative hydrophobicity was recorded as [(OD_{600} of the control–OD_{600} after octane overlay)/OD_{600} of the control]×100.

3.2.11.5 Scanning electron microscopy (SEM)

Effect of RL2 (5.0 mg mL^{-1}) on *C. albicans* adhesion and pre-formed *C. albicans* biofilm in comparison to control formed on PS coupons were visualized by SEM as described above in section 3.2.10.3.

3.2.11.6 Atomic force microscopy (AFM)

Micrographs of untreated and 12 h RL2 (5.0 mg mL^{-1}) treated preformed *C. albicans* biofilm on PS coupons were obtained with AFM (NTEGRA NT-MDT, Russia). All micrographs were collected in semi-contact mode using sharpened silicon nitride cantilevers NSG10S with spring constant about 10 N m^{-1}. The cantilevers had an amplitude range 5–15 nm, tip radius 10 nm and cone angle of 22 degree. Height and deflection images were simultaneously acquired at a scan rate of 250 kHz. Data analysis was done using NOVA software (Lal *et al.*, 2010).

3.2.11.7 Confocal laser scanning microscopy (CLSM)

CLSM was performed to visualize the impact of RL2 (5.0 mg mL^{-1}) on *C. albicans* adhesion and pre-formed *C. albicans* biofilm formed on PS coupons as described above in section 3.2.10.4.

CHAPTER 4

RESULTS

4.1 Isolation and screening of bacterial isolates for biosurfactant production

Total bacterial population obtained from oil sludge samples and fuel filling stations 1.8×10^{7} cfu/g and 2.4×10^{10} cfu/g respectively after 48 h of incubation at 30°C (Table 4). The bacterial strains so obtained were then purified and checked for their ability to grow on different hydrocarbons *viz.* dodecane, n-hexadecane, pristane, eicosane, toluene and fluoranthene as sole carbon source in "Pruthi and Cameotra" salt medium. Among, 310 purified bacterial isolates obtained, 25 isolates (NSVP1-NSVP10, DSVP11-DSVP20, SSVP21-SSVP25) showed the ability to grow on these tested hydrocarbons (Table 5). These 25 bacterial isolates were then tested for hemolytic ability, CTAB agar plate assay, drop collapse assay, emulsification assay, and surface tension reduction as an index for biosurfactant production (Fig. 9). Of these four isolates namely NSVP2, DSVP11, DSVP17 and DSVP20 were found to be potential biosurfactant producer (Table 6). *P. aeruginosa* DSVP20 grown on "Pruthi and Cameotra" salt medium with 2% glycerol showed maximum biosurfactant production (5.8 g L^{-1}) after 96 h of growth (Fig. 10). The surface activities measured in terms of surface tension revealed maximum reduction in its value from 72.0 mN m^{-1} to 28.5 mN m^{-1} in cell free broth of *P. aeruginosa* while CMD^{-1} and CMD^{-2} values recorded were 39.50 mN m^{-1} and 42.50 mN m^{-1} respectively after 96h.

Table 4: Bacterial population in the original natural habitats and after isolation and selection on 2% hydrocarbon as substrate

Isolation source	**Type of contamination**	**Total bacterial population (cfu/g)**	**Number of purified strains isolated**	**Hydrocarbon utilizing isolates**
Oily sludge, Ankleswar, Gujarat	Hydrocarbon contaminated	1.8×10^{7}	101	16
Fuel filling stations Roorkee, India	Gasoline, Diesel fuel	2.4×10^{10}	209	9
Total			310	25

Table 5: Growth of bacterial isolates on different hydrocarbons

Isolation Source	**Isolates**	**Dodecane**	**Hexadecane**	**Pristane**	**Toluene**	**Fluoranthene**
Oily sludge	NSVP1	++	++	-	-	-
	NSVP2	+++	++	++	+++	++
	NSVP3	++	++	++	-	-
	NSVP4	++	++	-	-	-
	NSVP5	++	++	++	+	-
	NSVP6	++	++	++	-	-
	NSVP7	++	++	++	+	+
	NSVP8	++	++	-	-	-
	NSVP9	++	++	++	+	-
	NSVP10	++	++	++	-	-
	DSVP11	++	+++	++	++	+++
	DSVP12	++	++	+	+	-
Fuel filling stations	DSVP13	+	+	-	-	-
	DSVP14	++	++	+	+	+
	DSVP15	++	++	++	++	-
	DSVP16	++	++	+	+	-
	DSVP17	+++	+++	++	++	++
	DSVP18	++	++	++	++	++
	DSVP19	++	++	++	++	-
	DSVP20	+++	+++	+++	+++	+++
	SSVP21	++	++	++	++	++
	SSVP22	++	++	++	+	-
	SSVP23	++	++	-	-	-
	SSVP24	++	++	+	-	-
	SSVP25	++	++	++	+	-

(+) indicates growth, (++) good growth, (+++) excellent growth and (–) no growth

Table 6: Screening of isolates for biosurfactant production

Isolates	Drop Collapse	Blood Haemolysis	Emulsification Index, E_{24} (%)	Surface Tension (mN m^{-1})	CTAB
NSVP1	—	Nil	8	65	Nil
NSVP2	**+**	**8 mm**	**65**	**34.6**	**8.9 mm**
NSVP3	+	Nil	20	44	Nil
NSVP4	—	2.6 mm	60	39.6	Nil
NSVP5	—	Nil	14	61.2	Nil
NSVP6	+	Nil	10	60.3	Nil
NSVP7	+	2.45 mm	9.3	61.6	Nil
NSVP8	+	Nil	21.5	44	Nil
NSVP9	+	Nil	8	65	Nil
NSVP10	+	Nil	12	59	Nil
DSVP11	**+**	**10 mm**	**72**	**32.4**	**10.3 mm**
DSVP12	+	Nil	9.1	35	Nil
DSVP13	+	Nil	7.9	66	Nil
DSVP14	+	Nil	10.1	63	Nil
DSVP15	—	Nil	11	64	Nil
DSVP16	—	Nil	9	61	Nil
DSVP17	**+**	**12 mm**	**83**	**27.6**	**11.4 mm**
DSVP18	+	Nil	22	56	Nil
DSVP19	+	Nil	29	55	Nil
DSVP20	**+**	**16 mm**	**94**	**26.8**	**17.2 mm**
SSVP21	+	Nil	25	51	Nil
SSVP22	—	Nil	16	38	Nil
SSVP23	—	3.6 mm	39	65	Nil
SSVP24	+	Nil	9	35	Nil
SSVP25	—	Nil	41	61	Nil

(+) indicates growth and (—) no growth

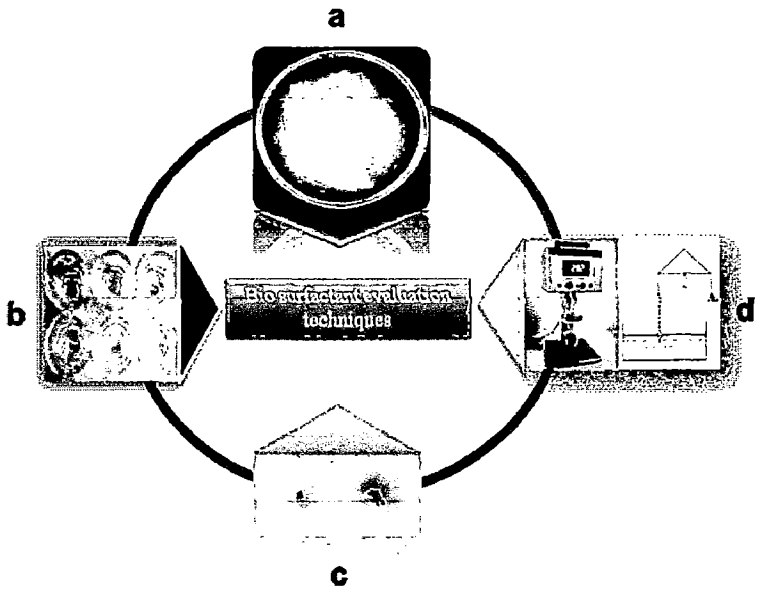


Figure 9: Screening methods used for evaluating biosurfactant production: a) Haemolysis on Blood agar plate b) Drop collapse assay c) CTAB agar plate assay d) Surface tension reduction using Du-Nouy-ring-type tensiometer

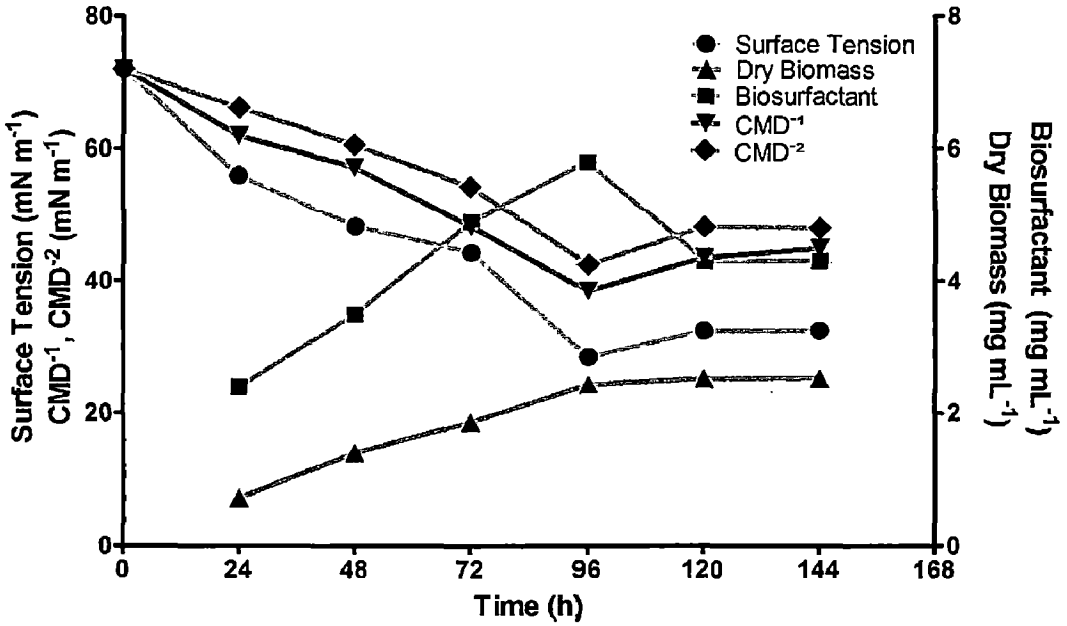


Figure 10: Surface tension, CMD^{-1}, CMD^{-2}, dry biomass and biosurfactant production by *P. aeruginosa* DSVP20 at different time intervals (0-144 h) at 37°C

4.1.1 Cell surface hydrophobicity studies

A convenient way for confirmation of biosurfactant production by above bacterial isolates was performed using cell surface hydrophobicity studies. This technique includes various assays like hydrophobic interaction chromatography (HIC), salt aggregation test (SAT), bacterial adherence to hydrocarbon (BATH), and adhesion to polystyrene by replica plate test (RP). Results of these tests as shown in Table 7 confirmed the biosurfactant producing ability of bacterial isolates: NSVP2, DSVP11, DSVP17 and DSVP20. Data showed among these isolates, DSVP11 and DSVP20 were scored positive for the adherence to polystyrene coupons when tested using replica plate method. The result of HIC for these isolates showed that the bacterial strain DSVP20 had maximum HI (98%) while BATH assay based on decrease in absorbance of lower aqueous phase of cell suspension depicted that DSVP20 had 14, 12, 14% absorbance for dodecane, hexadecane, pristine respectively.

Table 7: Cell surface hydrophobicity studies of isolates

ASSAY						
STRAINS	**HIC**		**RP**	**BATH**		
				DODECANE	**HEXADECANE**	**PRISTANE**
NSVP2	91		—	14	18	19
DSVP11	95		+	80	76	79
DSVP17	28		—	16	15	18
DSVP20	98		+	14	12	14

4.2 Characterization and identification of strain DSVP20

The selected bacterial strain DSVP20 was characterized using microscopic studies, various biochemical tests and molecular analysis using 16S rRNA amplification. On

microscopic examination, DSVP20 appeared as Gram-negative, aerobic, motile, rod shaped bacteria (Fig. 11). On PAC salt medium, DSVP20 showed large creamish colonies having undulate margin with circular form and flat elevation (Fig. 11). Biochemical analysis was performed using Bergey's manual of systematic bacteriology, the strain was tentatively identified as *Pseudomonas aeruginosa* (Table 8). To confirm the identity of the isolate, PCR amplification and sequencing of 16S rRNA gene was done. The results revealed that the PCR amplified product yielded an amplicon of 1.5 kb (Fig. 12) which corresponds to 16S rRNA gene. Alignment of the 16S rRNA gene sequences of bacterial isolate DSVP20 with sequences obtained by doing a BLAST searching revealed 100% similarity to *P. aeruginosa*. The tree was constructed using the neighbour-joining method (Felsenstein, 1993). Dendrogram showing phylogenetic relationships derived from 16S rRNA gene sequence analysis of strain DSVP20 (GeneBank accession no. GQ865644) with respect to other *Pseudomonas* species is shown in Fig. 13.

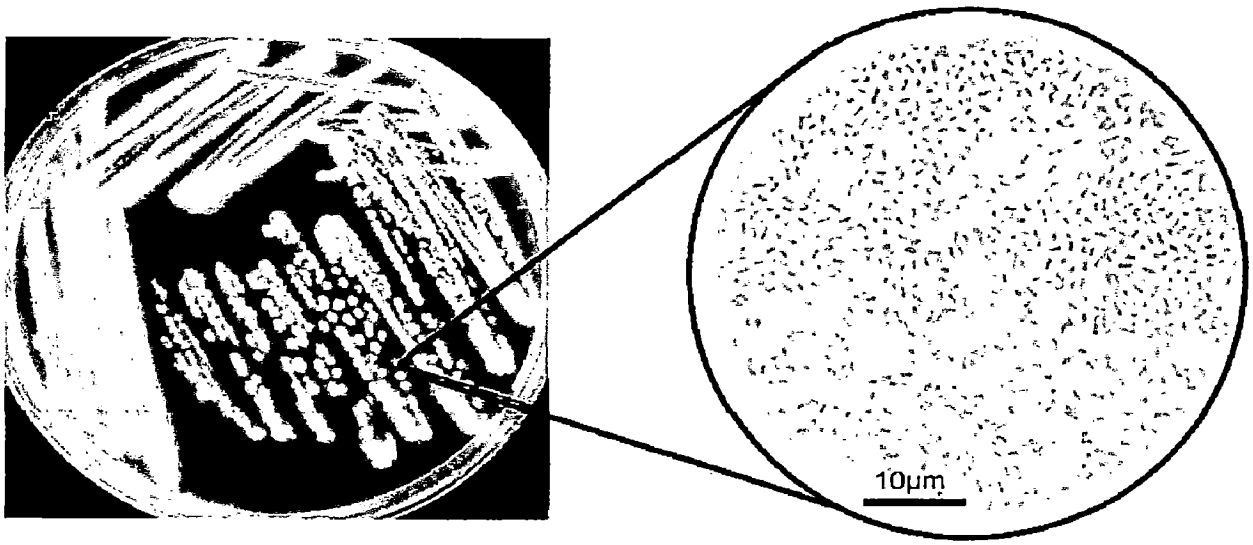


Figure 11: (a) Gram staining and (b) Plate showing colony morphology of strain DSVP20

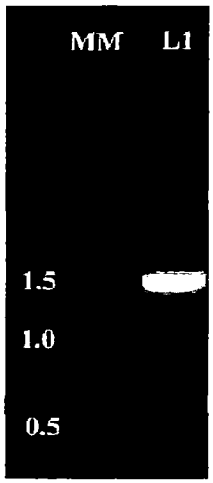


Figure 12: PCR amplified product (1.5 kb) of *P. aeruginosa* DSVP20, where MM represents molecular weight marker

Table 8: Biochemical analysis of strain DSVP20

Biochemical characterization of strain DSVP20	
Gram stain	Gram -ve
Shape	Rod
Motility	+
Spore formation	-
VP test	-
Indole production	-
Reduction of nitrate	+
Citrate	+
Catalase test	+
Oxidase test	+
Utilization of glucose	-
Utilization of glactose	-

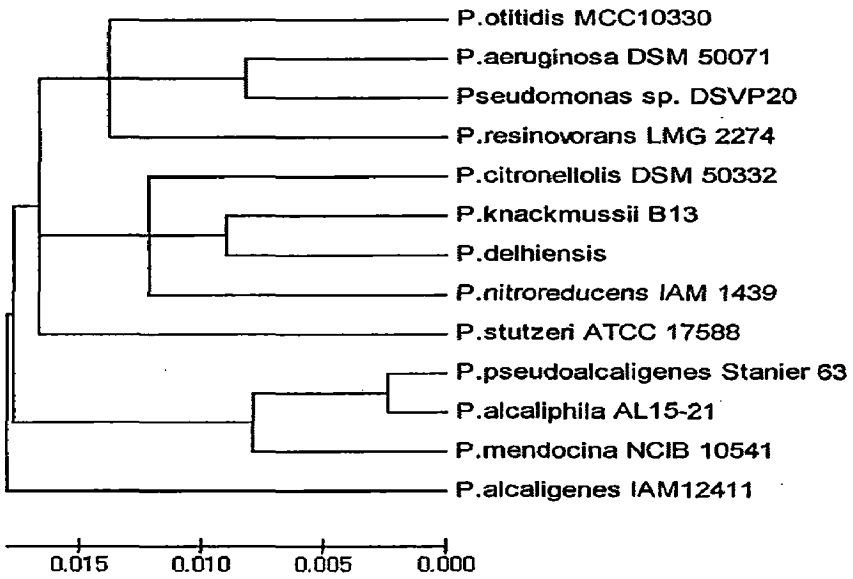


Figure 13: Dendrogram showing phylogenetic relationships derived from 16S rRNA gene sequence analysis of strain DSVP20 with respect to *Pseudomonas* species

4.3 Extraction and purification of biosurfactant from culture broth

Maximum surface tension reduction, dry biomass and rhamnolipid values obtained from cell free broth of *P. aeruginosa* DSVP20 were found to be 28.5 mN m^{-1}, 2.43 mg

mL^{-1} and 5.8 mg mL^{-1} respectively after 96 h of growth (Fig. 10). After preliminary isolation in the silica-gel column to remove neutral lipids, the partially purified biosurfactant was then separated by TLC (Fig. 14) which suggested that the purified biosurfactant obtained from cell free broth *P. aeruginosa* to be glycolipid in nature having R_f valve of 0.415 in the solvent system chloroform/methanol/acetic acid (15:5:1) when sprayed with orcinol-sulfuric acid stain.

4.3.1 Fourier transform infrared spectroscopy (FTIR)

The FTIR spectrum of RL2 were recorded in the spectral region of 4000–500 cm^{-1} (Fig. 16) which exhibited specific absorption bands at 3381 cm^{-1}, 2927 cm^{-1}, 2859 cm^{-1}, 1735cm^{-1}, 1635 cm^{-1}, 1300–1100 cm^{-1}, 1013 cm^{-1}, 906 cm^{-1} and 840 cm^{-1} indicating the chemical structure to be di- rhamnolipid (RL2). The weak broad band located at 3364 cm^{-1} can be attributed to the O–H stretching vibrations of hydroxyl groups, while the strong absorption peaks at 2927 cm^{-1} and 2859 cm^{-1} are assigned to the C–H stretching vibrations of the hydrocarbon chain positions. Stretching band of C=O at 1730 cm^{-1} is characteristic of ester bonds and carboxylic acid groups. The carbonyl stretching peak was observed at 1640 cm^{-1}, which is characteristic of ester compounds. The C–O stretching bands in the range of 1457–1100 cm^{-1} related to the bonds formed between carbon atoms and hydroxyl groups in the chemical structures of the rhamnose rings and the ester carbonyl group was also confirmed from the peak at 1057 cm^{-1}, which corresponds to C-O stretching vibration. Relatively strong sorption bands of pyranyl at 906 cm^{-1} and 840 cm^{-1} suggest it to be RL2 in nature.

4.3.2 Matrix assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS)

Data obtained from MALDI-TOF mass spectrum analysis of the purified bio-

-surfactant showed well-resolved groups of peaks at *m/z* values between 600-2500. It gave intense signals at *m/z* 672.81 corresponding to the sodium adduct $[M + Na]^+$ of the RL2 which had m/z 649 (Fig. 15).

Figure 14: TLC profile of eluted biosurfactant from *P. aeruginosa* DSVP20

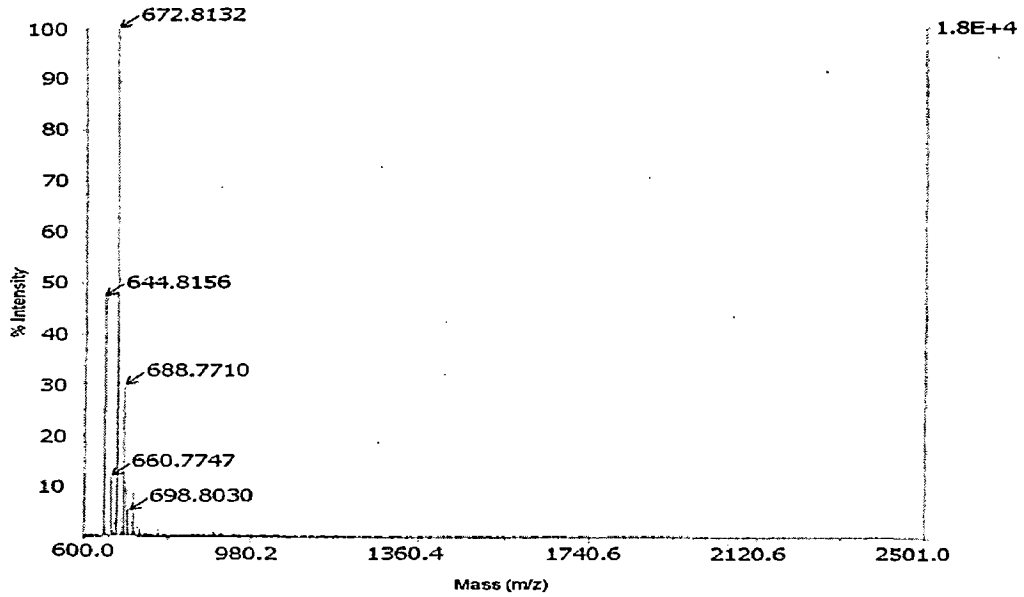


Figure 15: MALDI-TOF-MS profile of biosurfactant from *P. aeruginosa* DSVP20

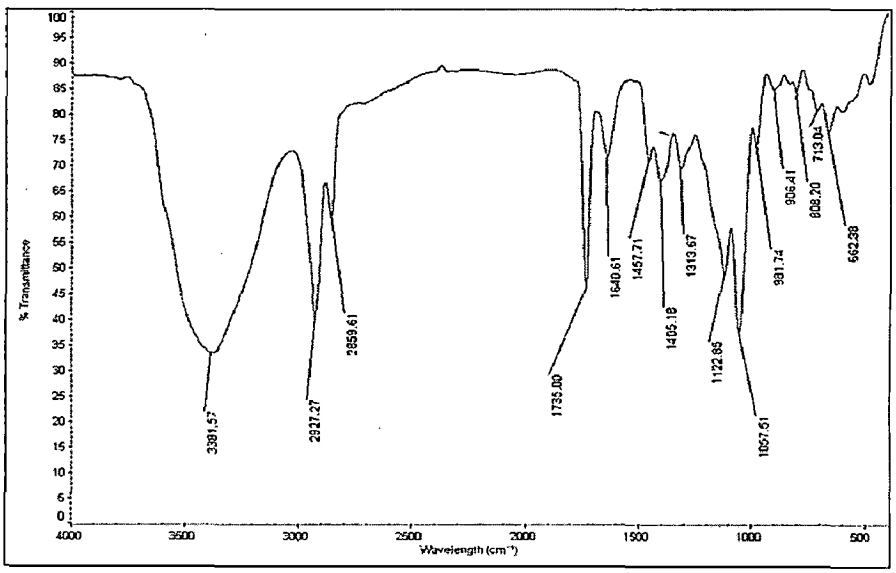


Figure 16: FTIR spectrum of biosurfactant from *P. aeruginosa* DSVP20

4.3.3 Nuclear magnetic resonance (NMR)

The characteristic chemical shifts observed from ^{1}H NMR analysis were 0.895 ppm (for $-CH_3$), 1.262 ppm (for $-(CH_2)_6$), 2.552 ppm (for $-CH_2-$ COO–), 4.878 ppm (for –O–CH–), and 5.243 ppm (for –COO–CH–) (Fig. 17). The ^{13}C NMR also displayed chemical shifts of 102.317 and 94.450 ppm which is a characteristic peak of RL2 (Fig. 18).

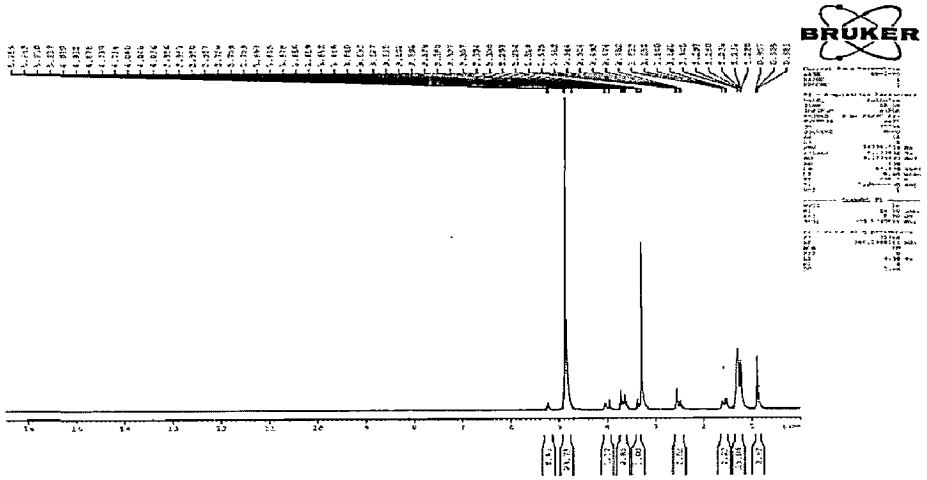


Figure 17: ^{1}H NMR spectrum of biosurfactant from *P. aeruginosa* DSVP20

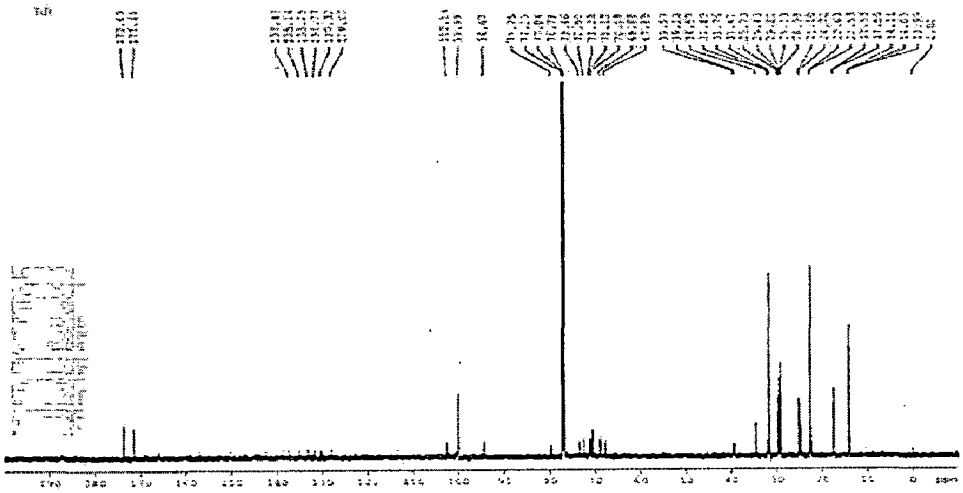


Figure 18: ^{13}C NMR spectrum of biosurfactant from *P. aeruginosa* DSVP20

4.4 Surface active properties of RL2

The surface active properties such as surface tension, CMD^{-1}, CMD^{-2} and emulsification index of purified RL2 (0.1 mg mL^{-1}) obtained from *P. aeruginosa*

DSVP20 were found to be 26.9 mN m^{-1}, 30.0 mg mL^{-1}, 36.0 mg mL^{-1} and 98% respectively.

4.5 Determination of lipid and sugar content of RL2

Isolated rhamnolipid (RL2) was mainly composed of carbohydrates and lipids 14.7% carbohydrate, while lipid content was 29.3% respectively. The remaining constituents 54.2% were dialyzable (inorganic salts) which had precipitated along with the surface active compound. The proportion of the surfactant ratio of carbohydrate and lipid remain constant in the acid precipitate at different periods of harvesting.

4.6 Stability studies of purified RL2

The aqueous solution of the RL2 (0.1%) from *P. aeruginosa* DSVP20 when incubated for 10 min at different temperatures (4-100°C) showed little variation in surface properties and emulsification values. Similarly, a wide range of pH values (4-12) had minimal effect on the surface properties and emulsification values of the RL2. Data showed no appreciable changes in reduction of the surface properties and emulsification value of RL2 were recorded when the NaCl concentration were varied from 2-10% (Table 9). The surface active properties such as surface tension, CMD^{-1}, CMD^{-2} and emulsification index of purified RL2 (0.1 mg mL^{-1}) obtained from *P. aeruginosa* DSVP20 were found to be 26.8 mN m^{-1}, 30 mg mL^{-1}, 36 mg mL^{-1} and 98% respectively.

Table 9. Stability studies of RL2

Effect of pH on RL2				
pH	Surface tension ($mN\ m^{-1}$)	CMD^{-1} ($mN\ m^{-1}$)	CMD^{-2} ($mN\ m^{-1}$)	E_{24} (%)
4	32.7	36.9	40.6	69
6	26.8	30.4	33.6	94
8	27.1	32.8	34.9	86
10	32.2	36.8	40.4	68
12	32.5	36.8	40.4	68.5
Effect of temperature on RL2				
Temperature (°C)	Surface tension ($mN\ m^{-1}$)	CMD^{-1} ($mN\ m^{-1}$)	CMD^{-2} ($mN\ m^{-1}$)	E_{24} (%)
4	30.6	34.2	39.0	70
20	27.2	32.8	34.9	86
40	27.4	32.8	34.9	86
60	28.2	33.2	35.4	78
80	30.2	34.0	39.0	69
100	30.3	34.5	39.0	70
121	30.3	34.6	39	71
Effect of saline concentration on RL2				
NaCl (%)	Surface tension ($mN\ m^{-1}$)	CMD^{-1} ($mN\ m^{-1}$)	CMD^{-2} ($mN\ m^{-1}$)	E_{24} (%)
1	27.2	32.8	34.9	86
2	28.4	33.2	35.4	78
4	29.5	34.3	38.3	74
6	29.8	34.5	39.0	70
8	32.1	36.8	40.4	68
10	35.7	38.2	42.5	65

4.7 Optimization of RL2 production

4.7.1 Effect of incubation period on RL2 production

The effect of incubation period on RL2 production was determined by incubating the culture medium at different time intervals (24-198 h). Maximum RL2 production (6.4 mg mL^{-1}), dry biomass (2.71 mg mL^{-1}) and surface tension reduction value (27.6 mN m^{-1}) were recorded after 96 h of growth as shown in Fig. 19.

4.7.2 Effect of aeration and agitation rate on RL2 production

Results obtained using different aeration (0.25-1.5 vvm) and agitation rates (50–250 rpm) on RL2 production showed that as the agitation rate increased from 50 to 150 rpm, an increase in RL2 production (7.50 mg mL^{-1}) also was observed (Fig. 20 & Fig. 21). However, the RL2 productions start diminishing thereafter with increase in agitation rate. Effect of aeration (0.5 vvm) showed maximum RL2 production (7.55 mg mL^{-1}) when the culture was grown for 96 h at 30°C.

4.7.3 Effect of pH and temperature on RL2 production

The results presented in (Fig. 22), showed that higher and lower pH values (12.0 and 4.0) resulted in major reduction in RL2 production while maximum RL2 (6.83 mg mL^{-1}) was obtained at pH 6. Similarly, it was observed that temperature values above 35°C resulted in lower RL2 production (Fig. 23). Increase in incubation temperature to 45°C also decreases RL2 production to 2.35 mg mL^{-1} with negligible dry biomass (0.72 mg mL^{-1}).

4.7.4 Effect of carbon source on RL2 production

Optimization of RL2 production using different type of carbon sources such as carbohydrates (i.e., glucose, glycerol, mannitol, sucrose, maltose, glycerol and starch), vegetable oils (i.e., olive oil and soybean oil), and fatty acid (i.e., oleic acid) as a substrate in PAC salt medium was carried out in 500 ml flask. Results revealed an increase in RL2 production (2.78 mg mL^{-1}) and biomass (7.31 mg mL^{-1}) with decrease in surface tension value (26.9 mN m^{-1}) using glycerol as a carbon source in comparison to control (Fig. 24). Optimization of substrate concentration for RL2 production was carried out in 500 mL flask containing different conc. (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 %) of glycerol as a carbon source in PAC salt medium.

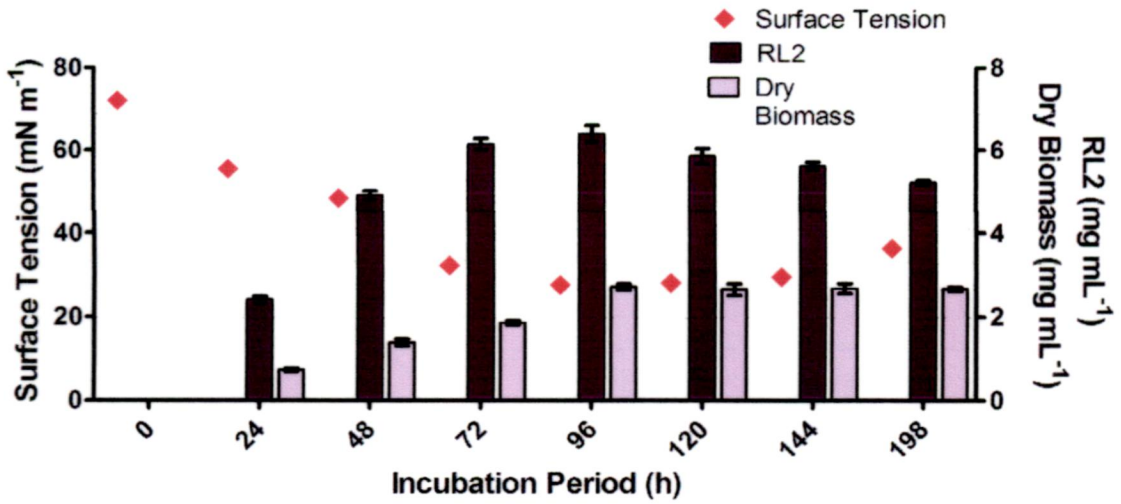


Figure 19: Effect of incubation period on surface tension, dry biomass and RL2 production

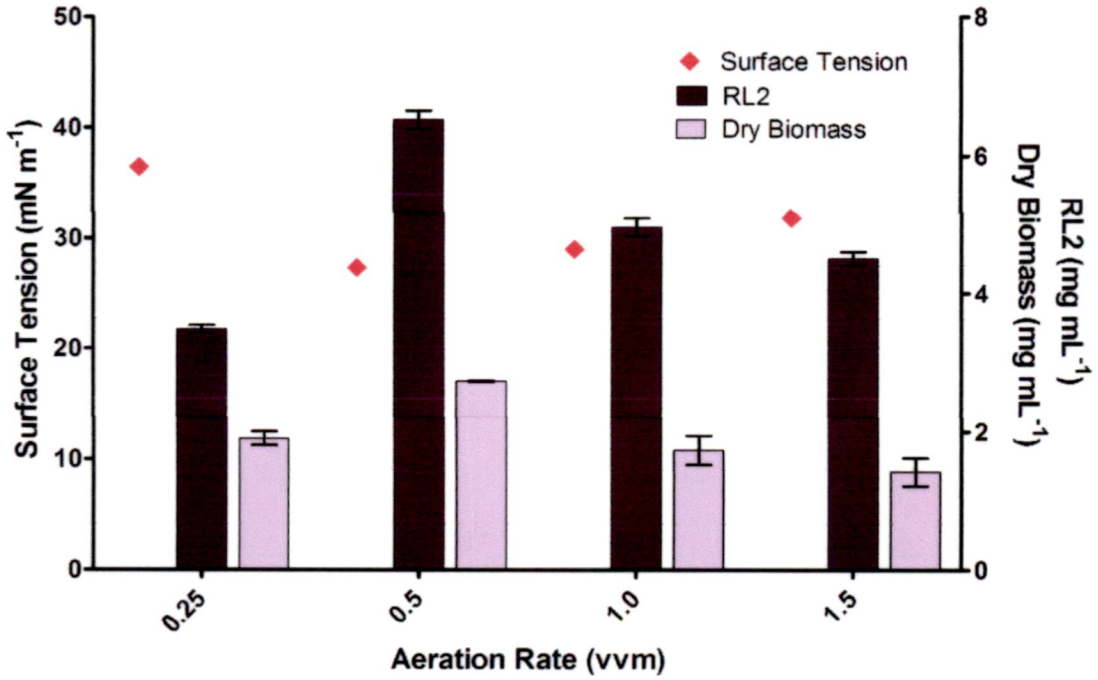


Figure 20: Effect of aeration on surface tension, dry biomass and RL2 production

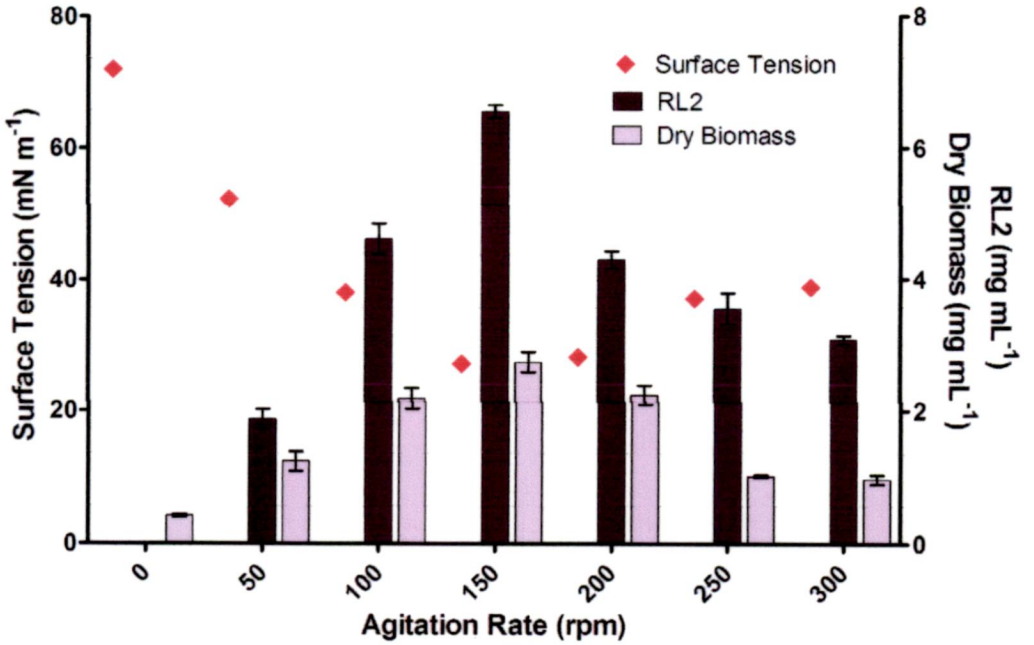


Figure 21: Effect of agitation on surface tension, dry biomass and RL2 production

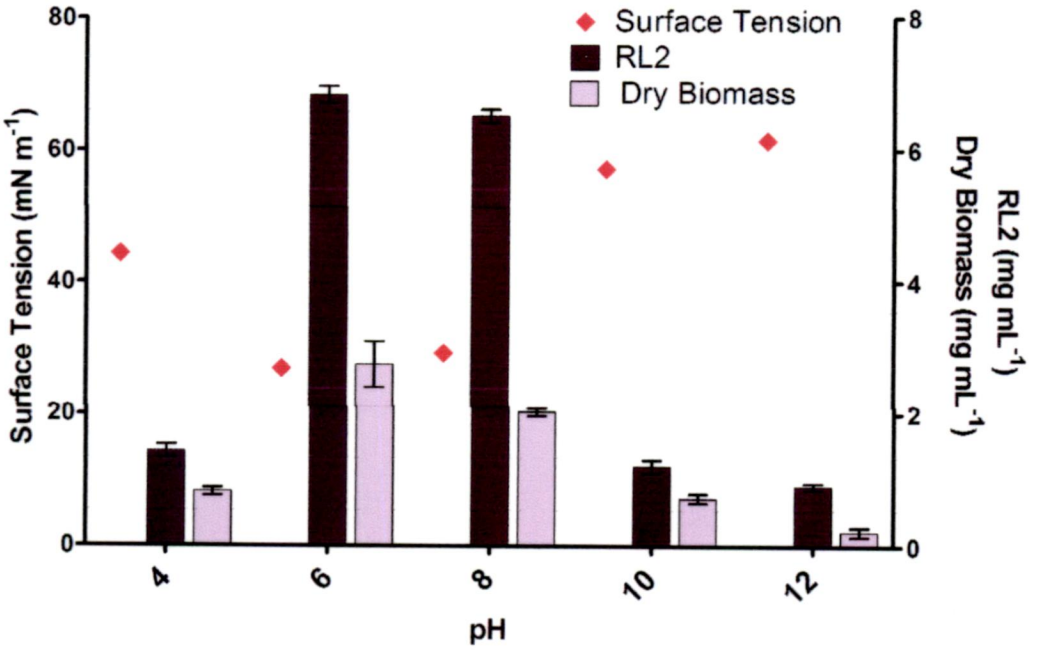


Figure 22: Effect of pH on surface tension, dry biomass and RL2 production

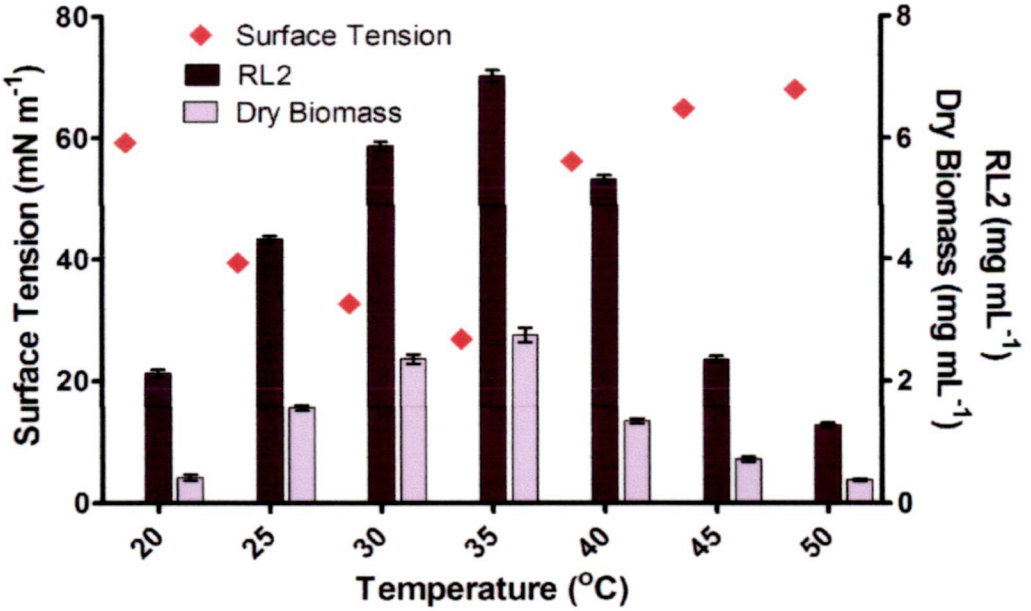


Figure 23: Effect of temperature on surface tension, dry biomass and RL2 production

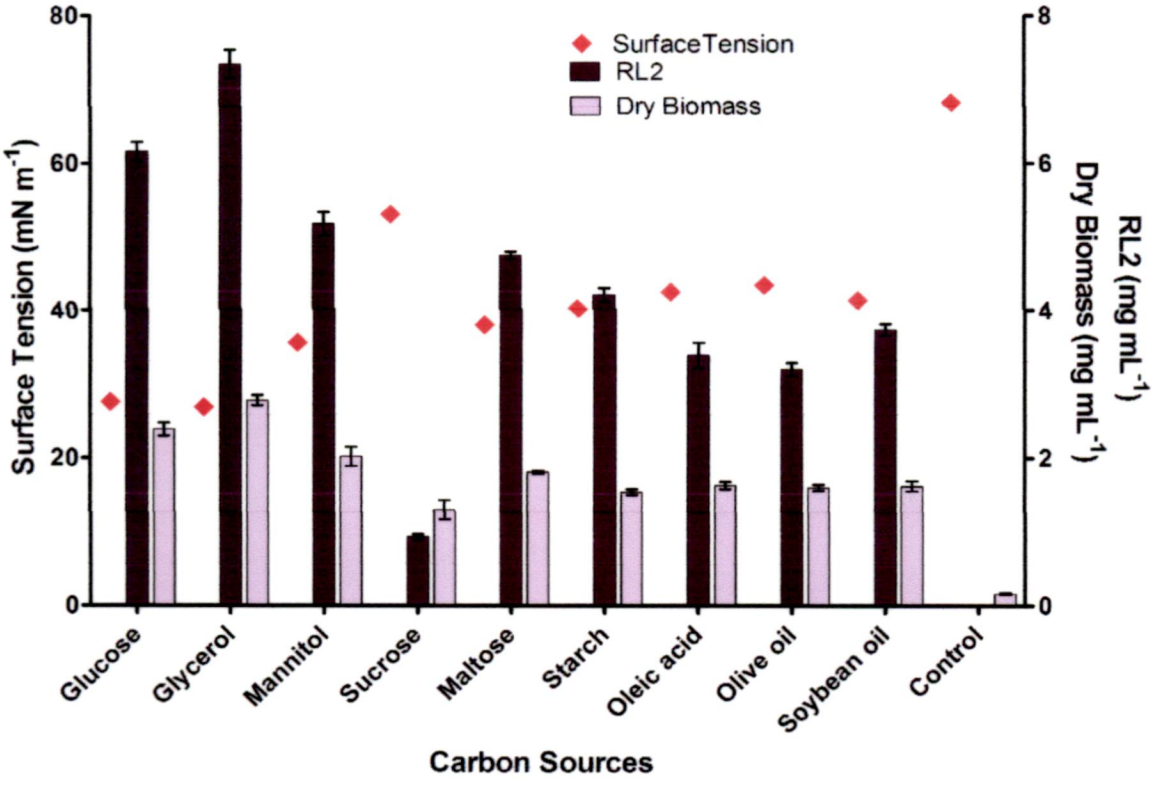


Figure 24: Effect of additional carbon source on surface tension, dry biomass and RL2 production

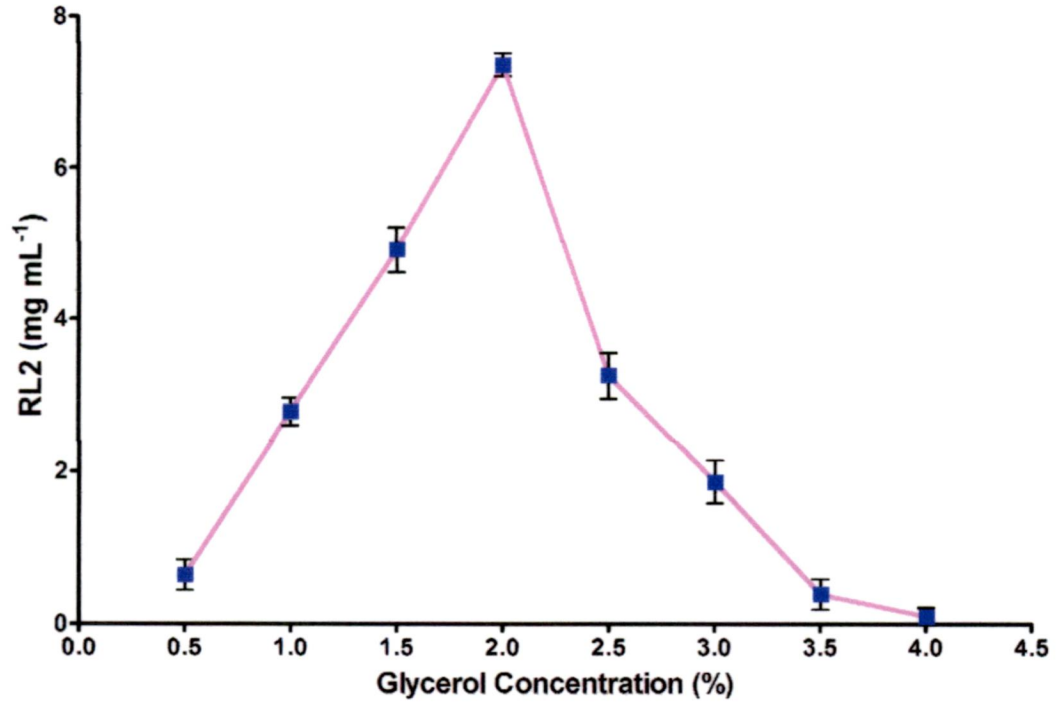


Figure 25: Effect of glycerol concentration on RL2 production.

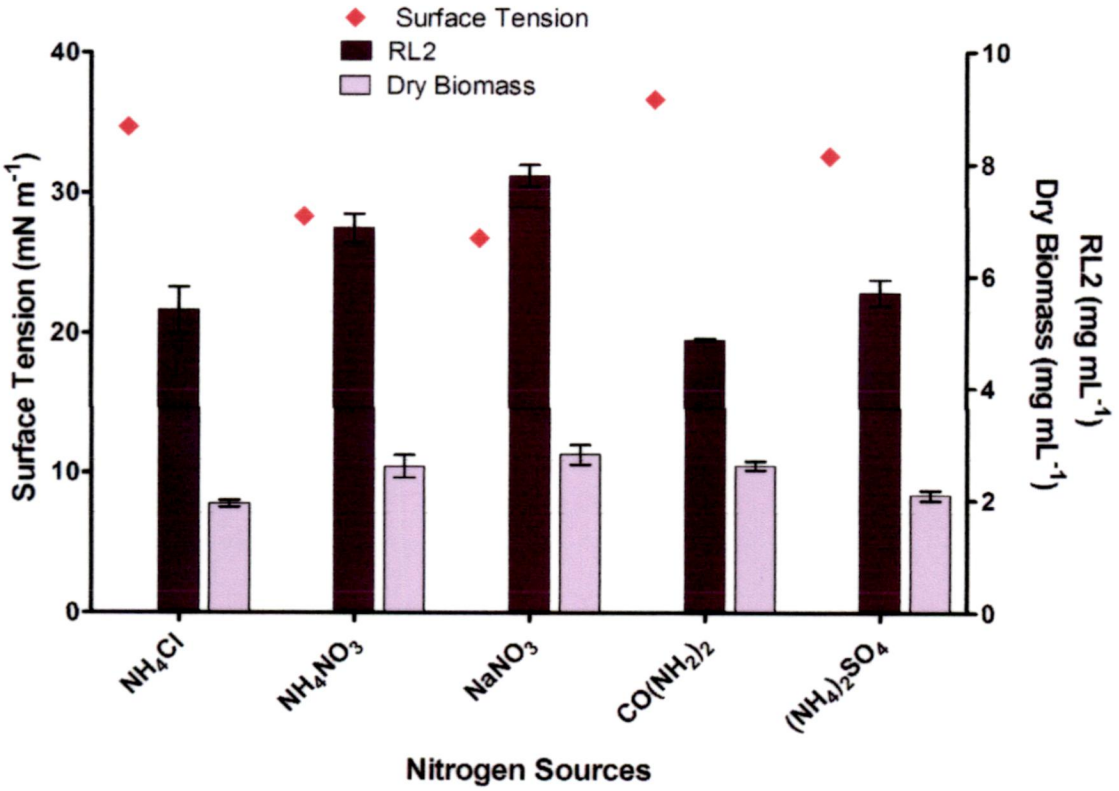


Figure 26: Effect of organic and inorganic nitrogen sources on surface tension, dry biomass and RL2 production

4.7.5 Effect of substrate concentration on RL2 production

Maximum biosurfactant production (7.35 mg mL^{-1}) by *P. aeruginosa* DSVP20 took place after 96 h of growth using 2% glycerol (Fig. 25). Inhibitory effect of glycerol on RL2 production was observed when the glycerol concentration exceeded ≥4%.

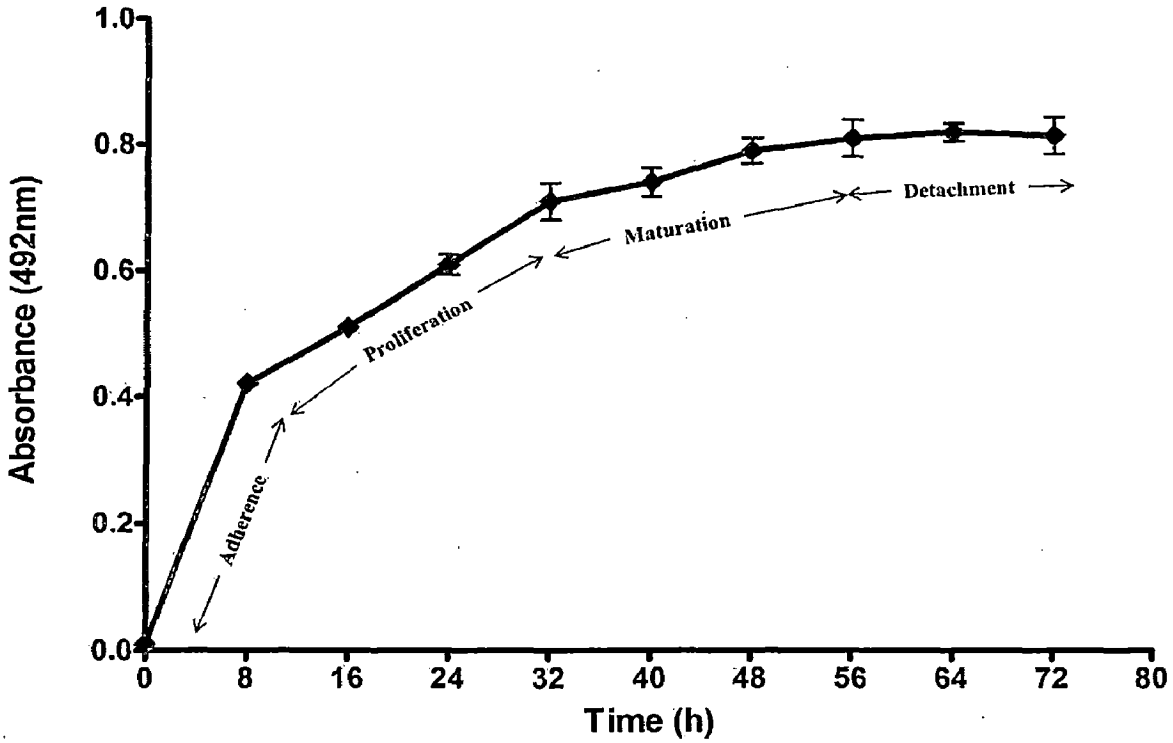
4.7.6 Effect of carbon and nitrogen sources on RL2 production

Among nitrogen sources used, $NaNO_3$ gave maximum RL2 yield (7.8 mg mL^{-1}) followed by $(NH_4)_2SO_4$ (5.71 mg mL^{-1}) and NH_4Cl (5.41 mg mL^{-1}). Minimum surface tension values 26.78 mN m^{-1} and 28.2 mN m^{-1} were achieved using sodium nitrate and ammonium sulphate respectively (Fig. 26). Nitrogen sources like urea, sodium nitrate and ammonium chloride depicted least reduction in surface tension value compared with nitrogen free medium therefore, were not suitable for RL2 production. Fig. 26, represents the surface tension value and RL2 production by *P. aeruginosa* DSVP20 using different of nitrogen sources at 48 h of growth at 35°C.

4.8 *C. albicans* biofilm formation and quantification

4.8.1 *C. albicans* biofilm formation

The adherence and subsequent biofilm formation by *C. albicans* on the surface of MTP wells over 72 h were determined by XTT reduction assay (Fig. 27). In the initial phase (8 h), *C. albicans* biofilm were highly metabolically active but the complexity increases as the biofilm matures (24 to 48 h) and the metabolic activity reached a plateau, reflecting the increased number of cells. These mature biofilm as demonstrated by SEM were characterized by a mixture of yeasts, germ tubes and young hyphae, and an intracellular matrix of EPS (Fig. 8C). Biofilm with less cell density were observed after 56 h depicting biofilm detachment and reduction due to accumulation of metabolites and waste products.



(A) XTT reduction assay

Figure 27: *C. albicans* biofilm formation at different time intervals and its biofilm quantification by XTT reduction assay

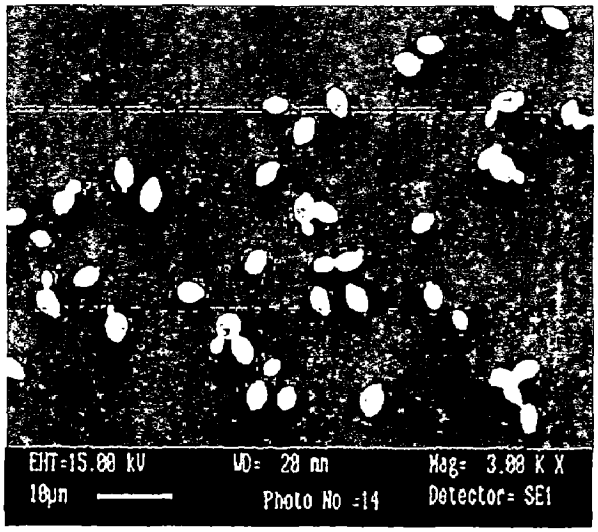
4.8.2 SEM and CLSM visualization of *C. albicans* biofilm

Biofilm formation by *C. albicans* on PS discs was monitored by SEM (Fig. 28). Despite its destructive nature, SEM observations provided useful information on the different cellular morphologies present in the biofilm structure. Initial adherence of yeast cells was followed by germ tube formation and subsequent development of hyphae (12 h and 24 h). Mature biofilm consisted of a dense network of cells of all morphologies, deeply embedded in matrix consisting of expolymeric material (24 h and 48 h). Biofilm with less cells density was observed after 56 h depicting biofilm detachment and reduction due to accumulation of metabolites and waste products.

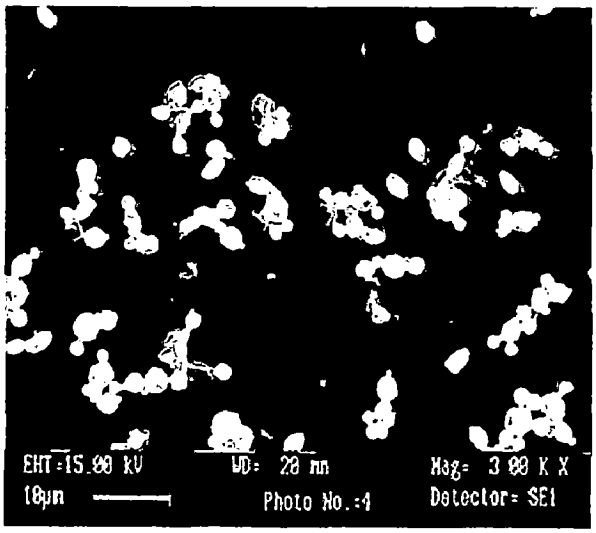
The non-invasive CLSM technique enabled imaging of intact biofilm and visualization of the three dimensional distribution of labeled *C. albicans* cells in the

context of the complex biofilm community. Significant channeling and porosity were observed. Overall, results indicated that mature *C. albicans* biofilm displayed typical microcolony/water channel architecture with extensive spatial heterogeneity. A three dimensional reconstruction of a 48 h old, 30.6 μm thick *C. albicans* biofilm after staining showing FITC-ConA (green) and PI (red) resulting from the compilation of a series of individual *xy* sections taken across the *z* axis (Fig. 29).

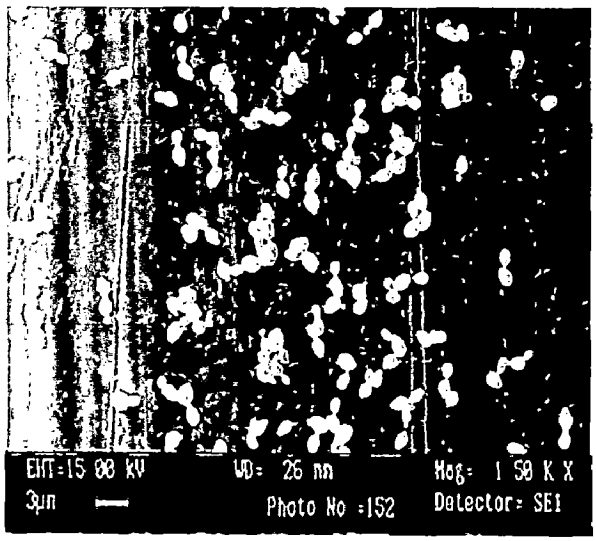
Further analysis of typical micro-colony inside the 48 h grown biofilm showed that the structures are covered with dense EPS matrix (FITC-ConA, green color) produced by cells inside them (Fig. 29). Staining of the micro-colony with PI showed the cells as red color and EPS containing spaces or cavities in black color spot (Fig. 29) suggesting secretion of EPS as cell over-layers.



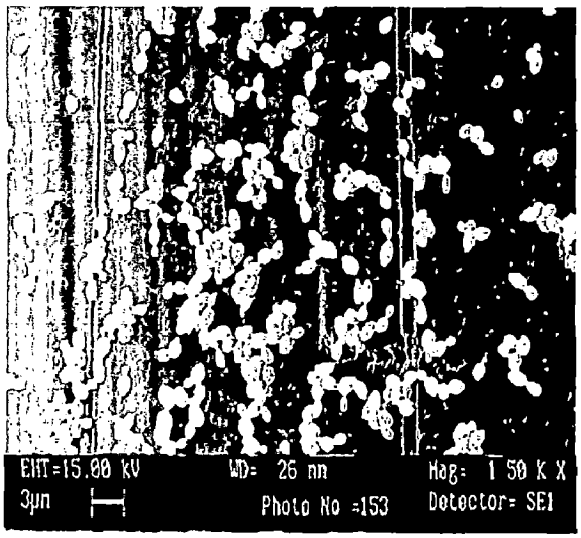
2h



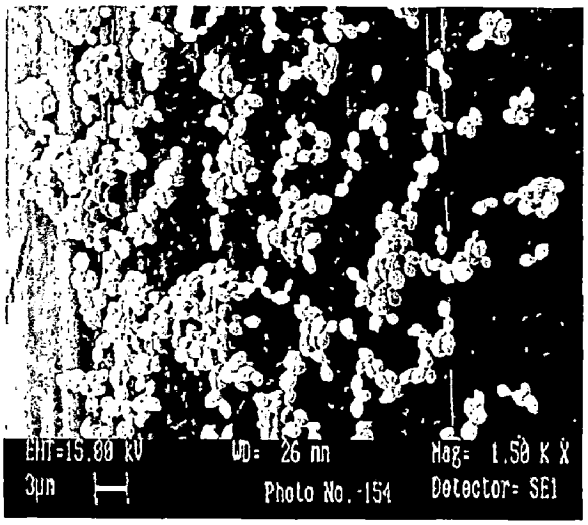
4h



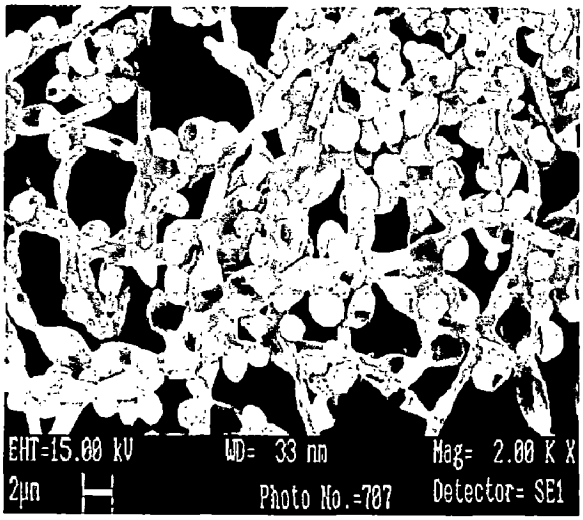
6h



8h



12h



24h

Contd...

Contd...

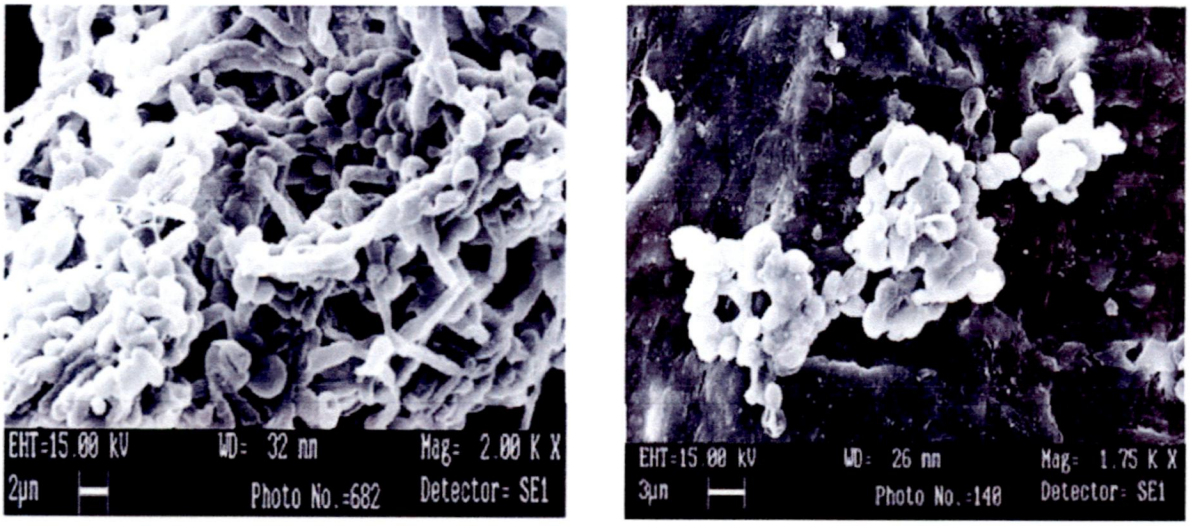


Figure 28: Scanning electron micrographs of *C. albicans* biofilm at different time intervals

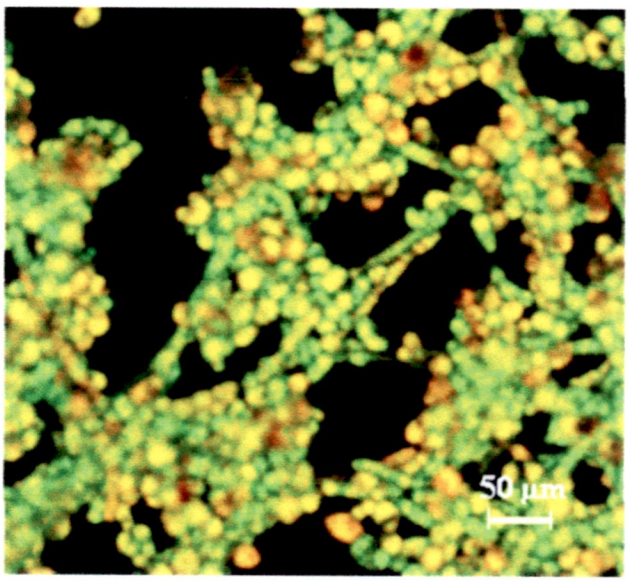


Figure 29: CLSM micrograph of 48 h old *C. albicans* biofilm after staining with FITC-ConA (green) and PI (red)

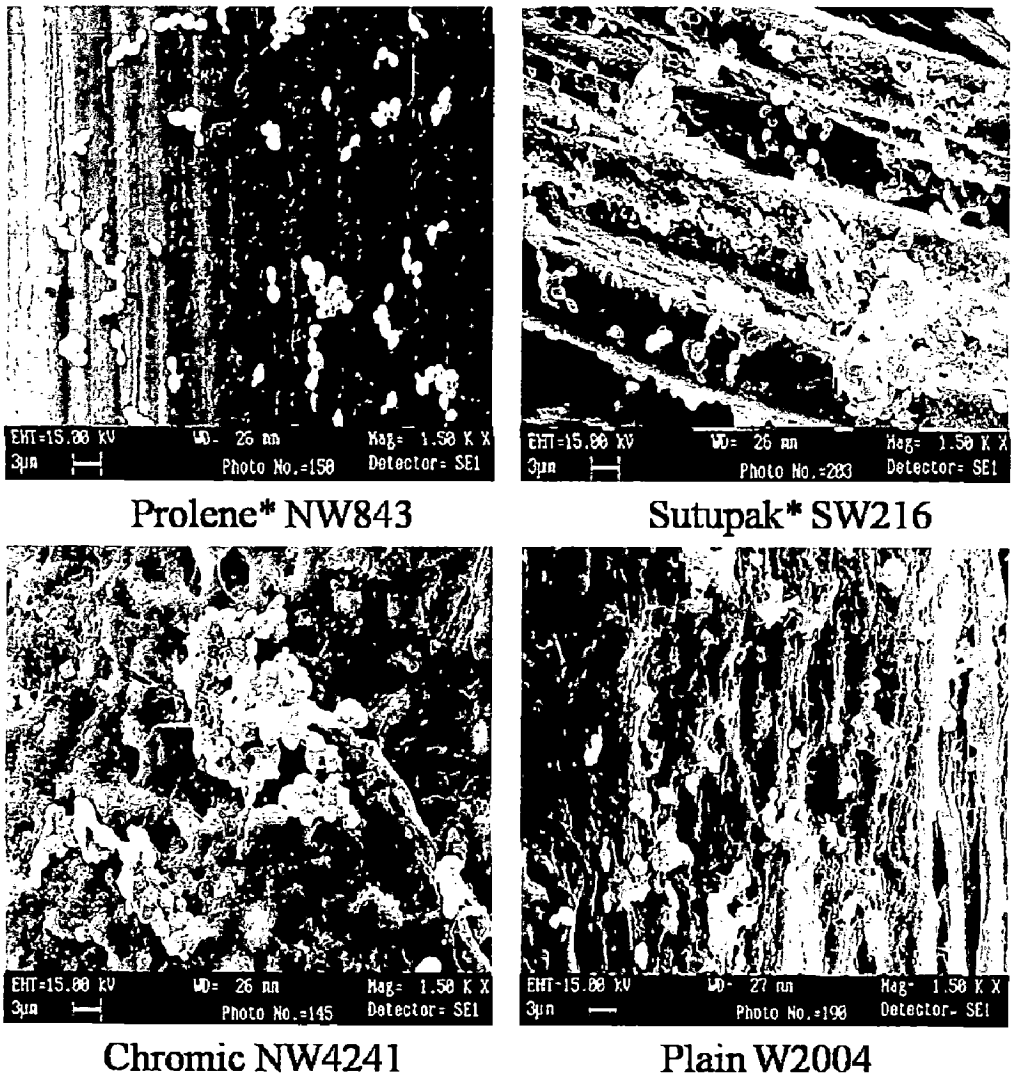
4.8.3 *C. albicans* biofilm on different indwelling medical devices

Data obtained from *C. albicans* biofilm formed on different surgical sutures (Fig. 30A) revealed that maximum biofilm formation was observed by nonabsorbable sutures Sutupak* SW216) and Prolene* NW843, followed by absorbable sutures Plain W2004 and Chromic NW4241 respectively. Similar studies were done on contact lenses showed that maximum *C. albicans* biofilm formation was on Etafilcon followed by Galycofilcon, Hilafilcon respectively after 48h biofilm growth, when quantified using XTT reduction assay. The topology of *C. albicans* biofilm on different absorbable and nonabsorbable suture materials as well as on contact lenses was also observed by SEM (Fig. 30B). Results showed that maximum biofilm formation was observed on Sutupak* SW216, containing dense hyphae and pseudohyphe network of *Candida* cells encased in extracellular matrix as compared to biofilm formed on absorbable sutures, Chromic NW4241 and Plain W2004 respectively (Fig. 30A). Interestingly least biofilm formation was observed on sutures Prolene* NW843. Visualization of 48 h grown SEM micrograph of *C. albicans* biofilm on contact lenses showed maximum biofilm formation on Etafilcon followed by Galycofilcon and Hilafilcon (Fig. 30B).

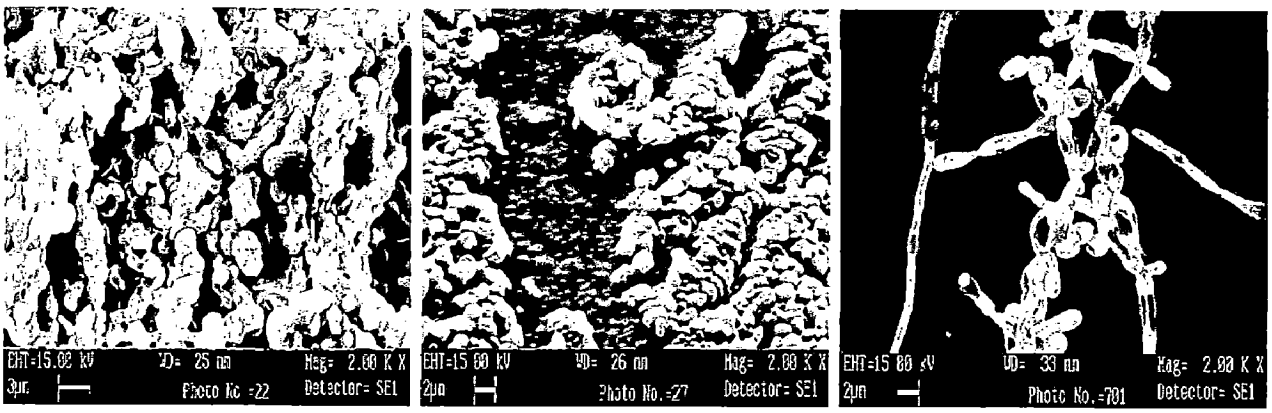
4.9 Effect of RL2 on *C. albicans* biofilm

4.9.1 Minimum inhibitory concentration (MIC) of RL2

The antifungal activity of purified RL2 from *P. aeruginosa* when checked using the standard broth microdilution method showed inhibition of *C. albicans* growth to be dose dependent. Data recorded showed that the MIC of RL2 to inhibit growth of *C. albicans* to be > 100 μg mL^{-1} (Fig. 31). The growth of *C. albicans* was poorly inhibited upto 0.78 μg mL^{-1} and 50% reduction was observed when > 3.12 μg mL^{-1} of RL2 was used.



(A)



(B)

Figure 30: *C. albicans* biofilm on different indwelling medical devices, (A) Surgical sutures and, (B) Contact lenses

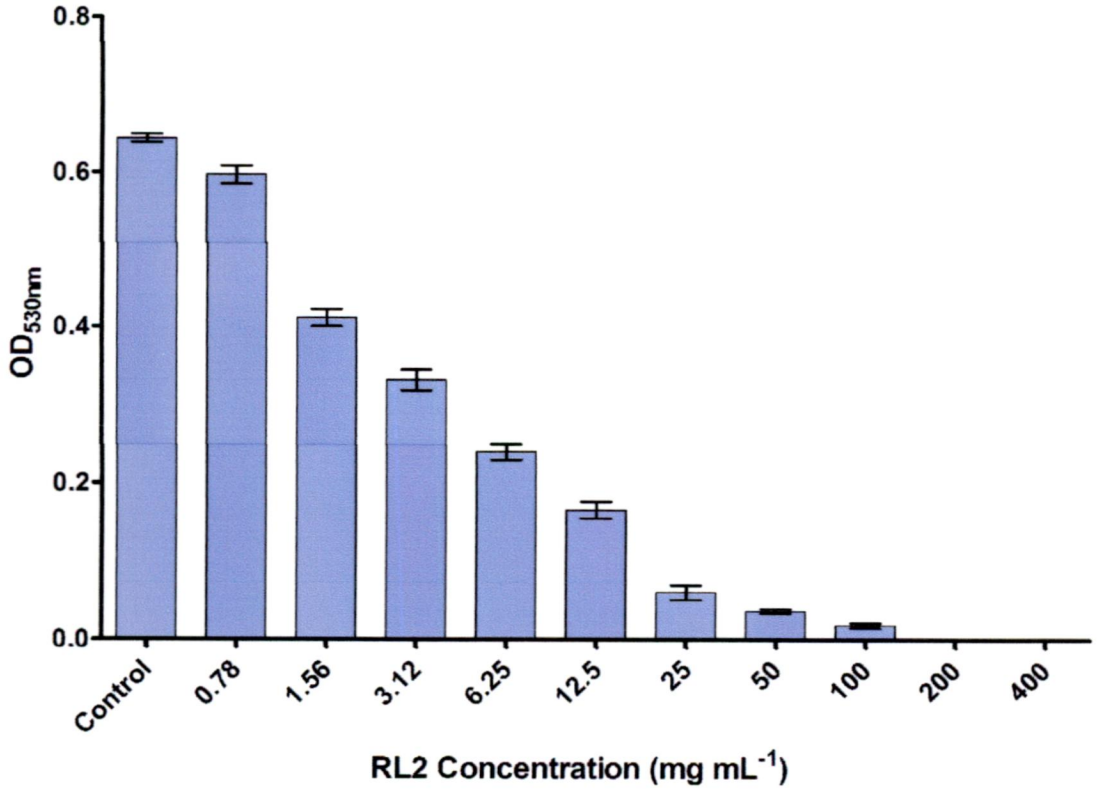


Figure 31: Minimum inhibitory concentration of RL2 on *C. albicans* after 48 h at 37 °C

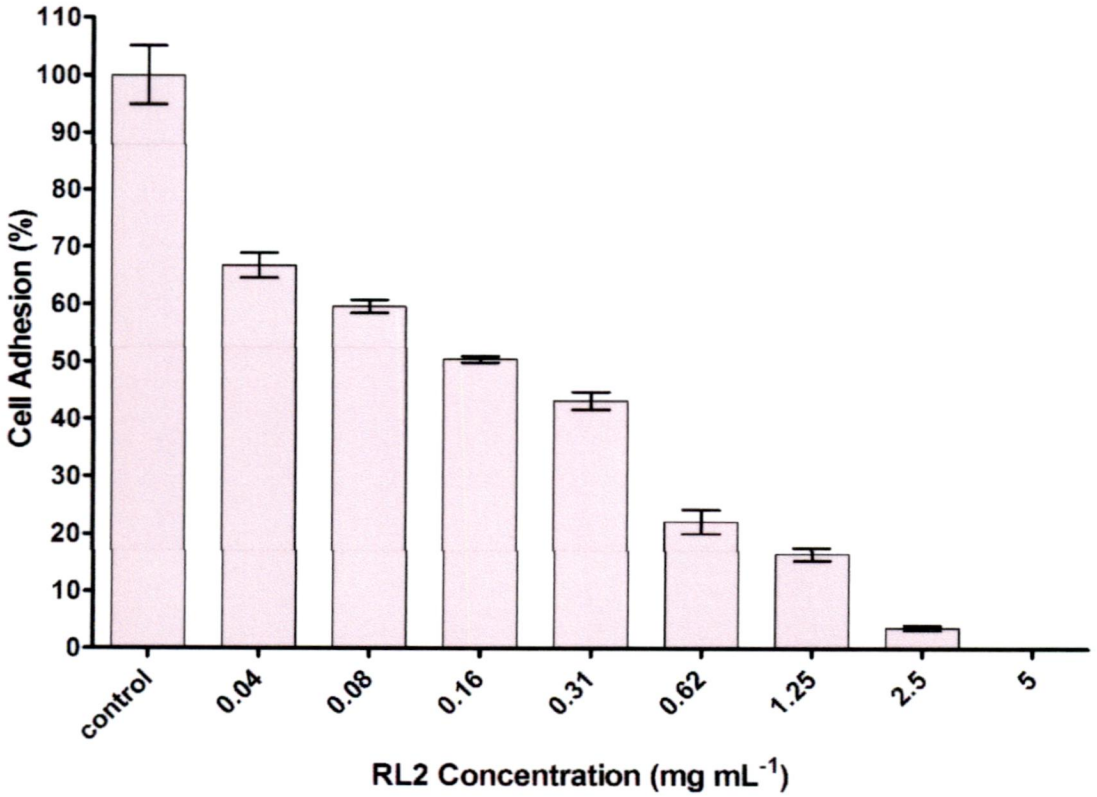


Figure 32: Effect of different RL2 concentrations on candidal cells adhesion after 2 h

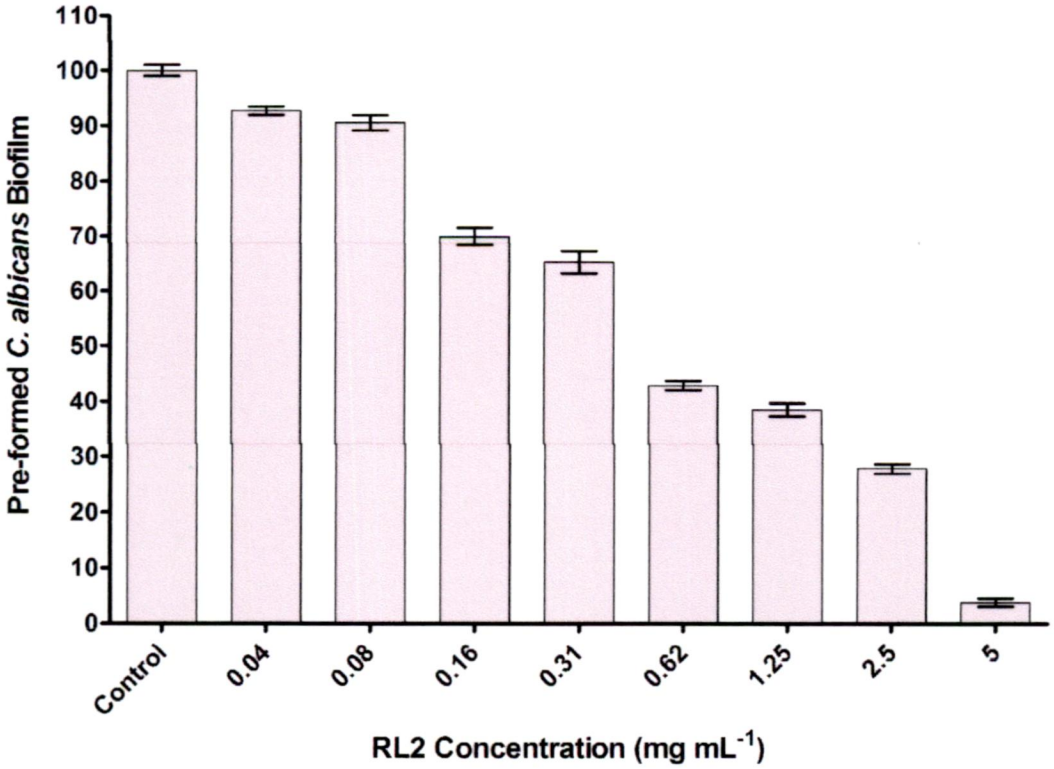


Figure 33: Effect of different concentrations of RL2 on percentage reduction of pre-formed *C. albicans* biofilm after 12 h at 37 °C

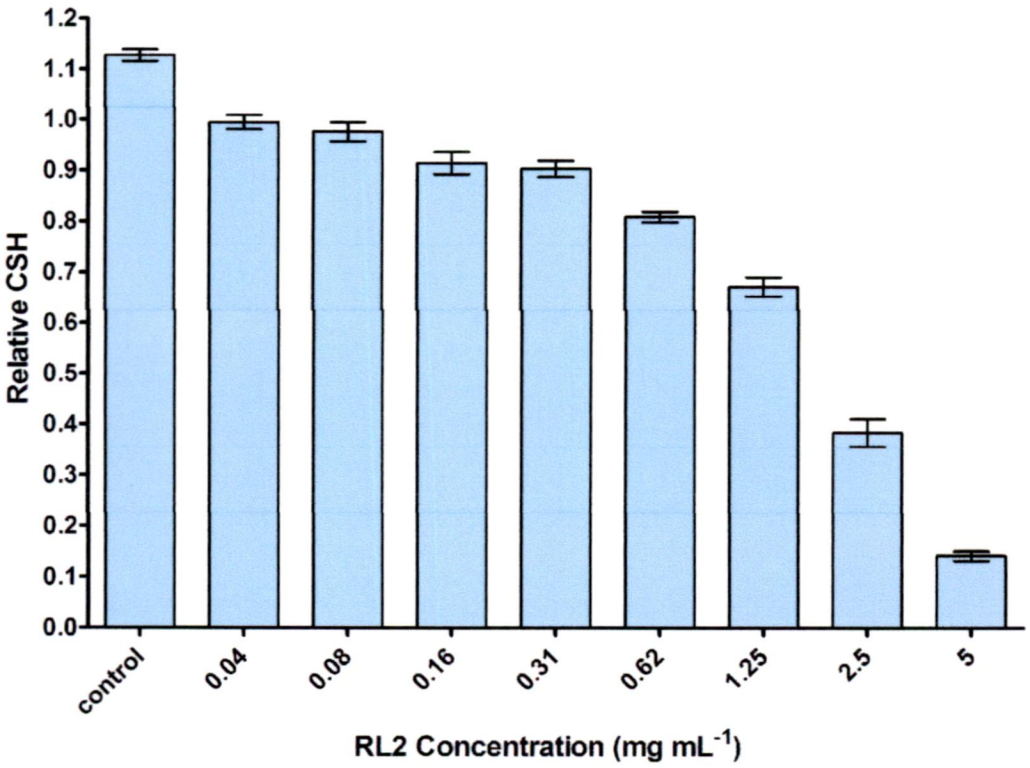


Figure 34: Effect of different concentrations of RL2 on cell surface hydrophobicity of *C. albicans* biofilm

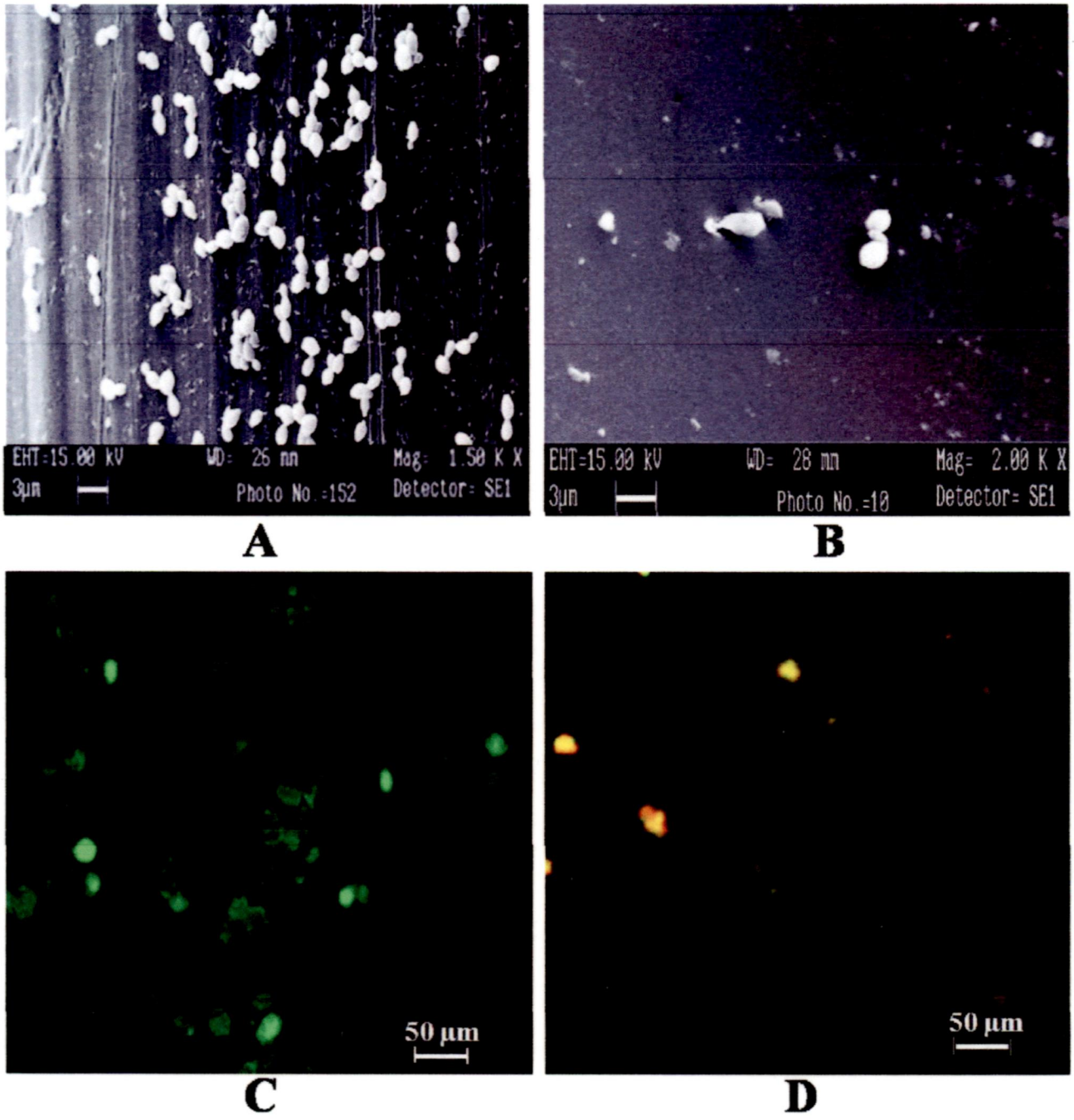


Figure 35: SEM micrographs (A-control and B- RL2 treated) and CLSM micrographs (C-control and D- RL2 treated) of *C. albicans* cells

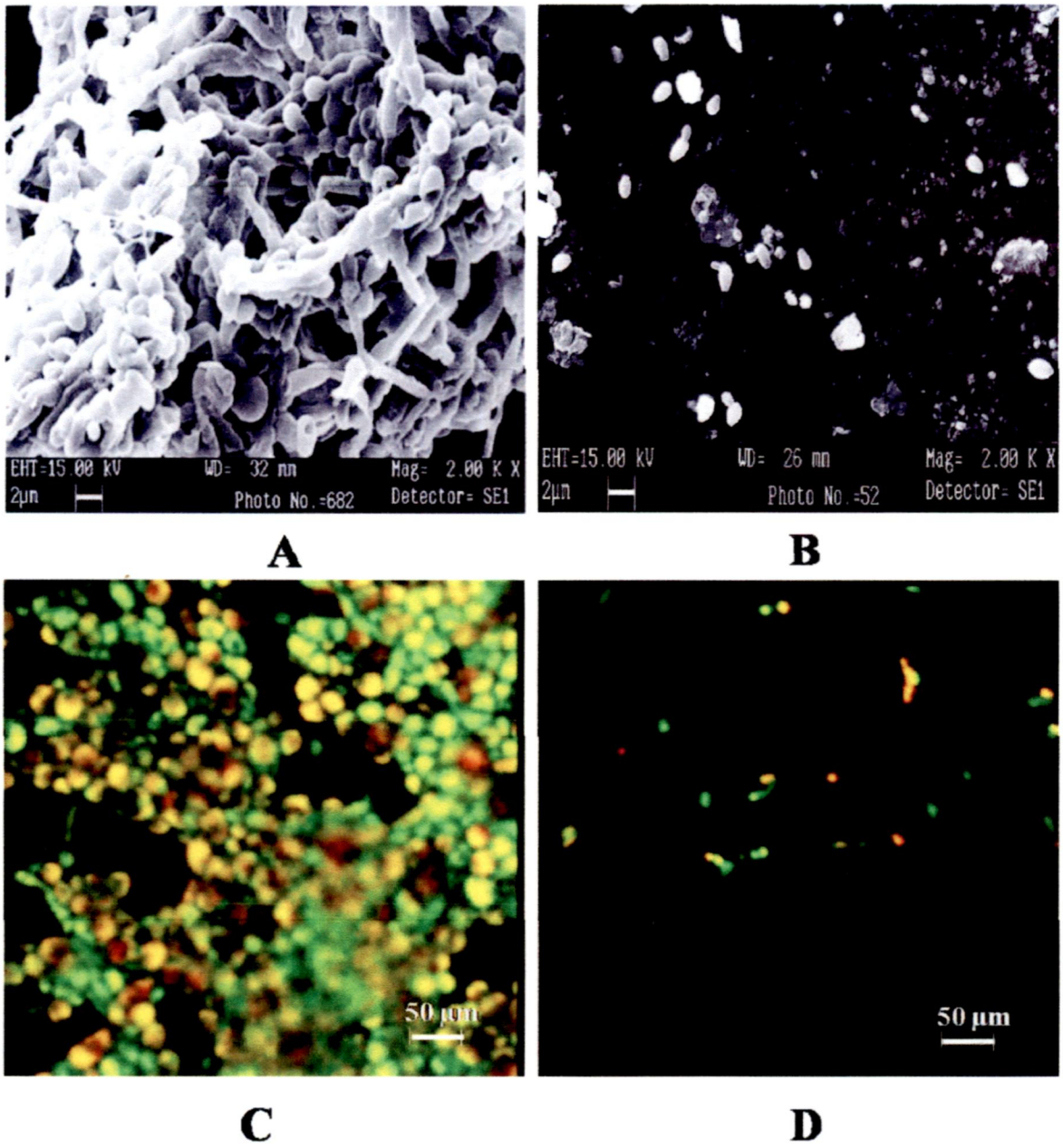


Figure 36: SEM micrographs (A-control and B- RL2 treated) and CLSM micrographs (C-control and D- RL2 treated) of pre-formed *C. albicans* biofilm

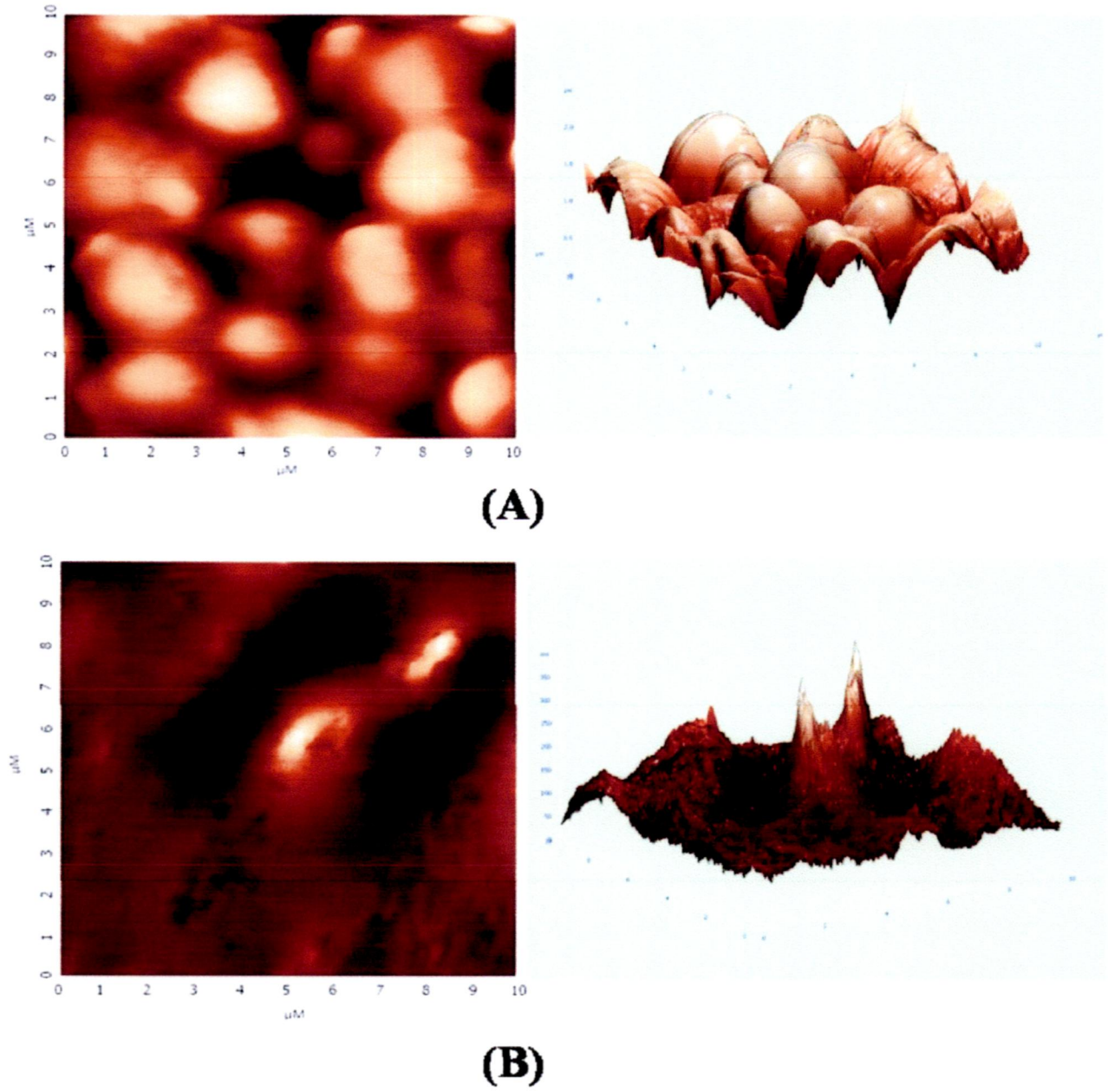


Figure 37: AFM micrographs showing roughness of (A) pre-formed *C. albicans* biofilm (222 nm) and (B) after treatment for 12 h with 5.0 mg mL^{-1} concentration of RL2 preformed *C. albicans* biofilm (31 nm). X–Y scales 10 µm

4.9.2. Anti-adhesive activity of RL2

In vitro studies on effect of different concentration (0.04-5.0 mg mL^{-1}) of RL2 on candidal cell adhesion after 2 h showed it to be concentration dependent (Fig. 32). Data showed about 50% of *Candida* cells remain adhered to 96-well plate after 2 h of treatment with 0.16 mg mL^{-1} RL2.

4.9.3. RL2 treatment of pre-formed biofilm

Pre-formed *C. albicans* biofilm on PS surface were reduced upto 70% and 90% with RL2 treatment at a concentration of 2.5 mg mL^{-1} and 5.0 mg mL^{-1} respectively. Data revealed that at lower concentrations $\leq$ 0.312 mg mL^{-1}, the biofilm reduction by the RL2 was not quite evident. However, at higher concentrations there was a dose-dependent increase in biofilm reduction caused by the RL2 (Fig. 33). Statistical analysis result obtained using student's t-test showed significant difference ($p < 0.05$) at higher concentrations of RL2 (>1.25 mg mL^{-1}) when compared with untreated biofilm.

4.9.4. The cell surface hydrophobicity of *C. albicans* biofilm

Cell surface hydrophobicity results showed reduction in *C. albicans* biofilm formed on MTP wells when exposed to different concentration of RL2 (Fig 34). The relative cell surface hydrophobicity was 0.4 and 0.14 at RL2 concentration of 2.5 and 5.0 mg mL^{-1}, respectively as depicted in Fig 34. Data recorded also indicated that with increase in RL2 concentration, there is a marked decline in cell surface hydrophobicity of *C. albicans* biofilm.

4.9.5. Scanning electron microscopy (SEM)

SEM micrograph results showed a noticeable reduction in the dense network of candidal cells, germ tube, hyphae and pseudohyphae enclosed in exopolysaccharides

matrix in preformed *C. albicans* biofilm treated with RL2 (5.0 mg mL^{-1}) when compared with its control. Effect of RL2 on *C. albicans* adhesion visualized by SEM micrographs also showed similar reduction in number of candidal cells and alteration in structural design of *C. albicans* when compared with its control (Fig. 35A, 35B & Fig. 36A and 36B).

4.9.6. Atomic force microscopy (AFM)

AFM data obtained on the roughness of pre-formed *C. albicans* biofilm and RL2 (5.0 mg mL^{-1}) treated *C. albicans* biofilm on PS surface were 222 nm and 31 nm respectively as shown in Fig. 37.

4.9.7. Confocal laser scanning microscopy (CLSM)

Through CLSM micrographs it was established that *C. albicans* biofilm architecture was disturbed on RL2 treatment and fewer numbers of candidal cells were seen adhering to the PS surface ((Fig. 35C, 35D & Fig. 36C, 36D). The green fluorescence from FITC-ConA binding indicates dense network of polysaccharides and areas of red fluorescence was due to PI binding which represent dead cells. The yellow color formed as a result of overlapping of red and green images depicts production of exopolysaccharides as capsular components.

CHAPTER 5

DISCUSSION

Interest in biosurfactants, the amphiphatic molecules, had steadily increased due to their diversity, environmentally friendly nature, potential in microbial enhanced oil recovery, biomedical applications, and food processing industries (Makkar *et al.*, 2011; Mnif *et al.*, 2011; Sánchez *et al.*, 2009; Wang *et al.*, 2008; Batista *et al.*, 2006; Rodrigues *et al.*, 2006a; Singh and Cameotra, 2004). Biosurfactants, have distinct advantages over their chemical counterparts in biodegradability, effectiveness at extreme temperature, pH and in having lower toxicity (Sánchez *et al.*, 2009; Rodrigues *et al.*, 2006a; Singh and Cameotra, 2004; Banat *et al.*, 2000; Cameotra and Makkar, 1998).

In present investigation, intent was to isolate a novel biosurfactant producing microorganism from oil sludge samples from contaminated sites *viz.* Ankleshwar, Gujarat. Besides this, samples contaminated with diesel oil and motor oil from local fuel filling stations were also used in this study. The samples from these sites were gathered because the probability of finding biosurfactant producing native bacterial population mineralizing crude oil hydrocarbons in petroleum oil contaminated site is higher (Parreira *et al.*, 2011; Arutchelvi and Doble, 2010; Rajan, 2010; Zhang *et al.*, 2010; Ganesh and Lin, 2009). A total of 25 morphologically distinct bacterial isolates were selected on their basis of ability to grow on different hydrocarbons using enrichment techniques. The indigenous population of hydrocarbon degrading bacteria was found to be 2×10^4 cfu/g of soil. Similar results, confirming low indigenous population of hydrocarbon degrading bacteria in soil (10^3 to10^4 cfu/g of soil) have been obtained during evaluation studies of inoculum addition in oily sludge contaminated soil by Mishra *et al.*, 2001. Such

enrichment methods are highly selective, resulting in the isolation of new microbial species from various natural habitats as has been reported by Jacques *et al.*, 2009.

Based on various physical and chemical properties employed to measure the efficiency for biosurfactant production such as haemolytic activity, CTAB assay, drop collapse assay, surface tension reduction, and emulsification activity, a total of four strains namely, NSVP2, DSVP11, DSVP17 and DSVP20 were selected. Similar studies, to screen and evaluate potential biosurfactant producing microorganisms have been done by Hazra *et al.*, 2011, Techaoei *et al.*, 2011, Anandaraj and Thivakaran, 2010. Studies have documented these methods to be rapid, simple and reliable for screening and selection of microbes producing biosurfactants (Hazra *et al.*, 2011; Liu *et al.*, 2011; Techaoei *et al.*, 2011; Anandaraj and Thivakaran, 2010; Nie *et al.*, 2010; Balogun and Fagade, 2008; Bodour *et al.*, 2004; Banat *et al.*, 2000). Besides this, cell surface hydrophobicity studies (HIC, BATH, RP assay) were further exploited for confirmation of biosurfactant production by these isolates (Pruthi and Cameotra, 1997; Neu and Poralla, 1990). These assay procedures suggest that hydrophobic nature of cell surface is a prerequisite for the identification of biosurfactant producing strain.

On comparative analysis of physiochemical parameters especially hydrophobic properties, bacterial isolate DSVP20 was selected as an active biosurfactant producer. Earlier, Rosenberg and Kjelleberg, 1986, too had recommended the use of cell surface hydrophobicity tests as a characteristic feature of biosurfactant producing microbes. The selected bacterial strain DSVP20 when visualized microscopically appeared as Gram negative, motile rod shaped with circular undulated margins.

The phylogenetic tree was prepared based on neighbor joining analysis of the 16S rRNA gene nucleotide sequences to understand the taxonomic position of DSVP20. On

pair wise alignment of the 16S rRNA gene nucleotide sequence, maximum similarity was observed with the organisms of class γ – Proteobacteria and family Pseudomonadaceae. Among the species of genus *Pseudomonas,* a detailed phylogenetic study was performed. The sequence analysis revealed that DSVP20 16S rRNA gene nucleotide sequence showed 97% to 99% identity with *P. aeruginosa*. Phylogram (Fig. 13) constructed on the basis of multiple sequence alignment from selected BLAST hits, revealed the evolutionary distance between DSVP20 and other members of *Pseudomonas* species. Thus, based on its morphological, physiological and biochemical features, as well as its 16S rRNA sequence analysis, strain DSVP20 was classified as *P. aeruginosa* (GenBank accession no. GQ865644).

The reduction of the surface tension values and increase in the yield of biosurfactant during late log phase of *P. aeruginosa* growth indicates the isolated biosurfactant to be a secondary microbial metabolite as reported for *P. aeruginosa* by different research groups (Cameotra and Singh, 2009; Wei *et al.*, 2008). Our data revealed similar trends in surface tension reduction, dry biomass and rhamnolipid values obtained from cell free broth of *P. aeruginosa* DSVP20 which were 28.5 mN m^{-1}, 2.43 mg mL^{-1} and 5.8 mg mL^{-1} respectively during the late log growth phase (96 h) of *P. aeruginosa* DSVP20. Acid precipitation and ethyl acetate extraction yielded an extract which was subjected to absorption chromatography for the purification of rhamnolipid. Preliminary chemical characterization of the isolated biosurfactant suggested it to be a glycolipid in nature. Thin-layer chromatography revealed R_f values 0.415 when sprayed with orcinol reagent. It is in agreement with the literature studies where the standard glycolipid with a retention index of 0.42 was found out to be a rhamnolipid (Abdel-Mawgoud *et al.*, 2009). FT-IR spectrum of isolated rhamnolipid when compared with previously reported biosurfactants from *Pseudomonas* species, showed band characteristic of –OH bond, aliphatic chain,

C=O stretching for ester bond and –COO stretching for carbonyl bond this further confirmed that isolated biosurfactant to be a glycolipid in nature (Bharali and Konwar, 2011; Pornsunthorntawee *et al.*, 2009; Tuleva *et al.*, 2002). Analysis of spectrum showed typical broad absorption band at 3364 cm^{-1} for O-H stretching vibrations confirms the presence of hydroxyl group in the rhamnolipid, whereas the bond formation between the carbon and hydroxyl group in the ring of rhamnolipid was confirmed by the presence of C-O stretching vibrations. These findings are in accordance with previously reported literature (Worakitsiri *et al.*, 2011). Relatively strong absorption bands of pyranyl at lower energy 906 cm^{-1} and 840 cm^{-1} in the IR spectrum of the biosurfactant revealed it to be a RL2 in nature which is in accordance with previous reports of Hazra *et al.*, 2011; Worakitsiri *et al.*, 2011. MALDI-TOF mass spectrometry is an emerging technique offering promise for the fast and accurate determination of molecular mass of biomolecules. The mass spectrum of biosurfactant showed an intense peak at *m/z* 672.81, this can be assigned to sodiated adduct of RL2 (Wang *et al.*, 2005; Deziel *et al.* 2000). Nuclear Magnetic Resonance (NMR) spectroscopy is the most powerful tool available for determining the structure of organic compounds. This technique relies on the ability of atomic nuclei to behave like a small magnet and align them with an external magnetic field. When irradiated with a radio frequency signal the nuclei in a molecule can change from being aligned with the magnetic field to being opposed to it. The most common nuclei observed using this technique are ^{1}H and ^{13}C. Analysis of proton NMR spectra suggests the two α-rhamnose and two β-hydroxy fatty acid moieties and provided the information about the length of the fatty acid chains. ^{13}C NMR signals for CH_3 and CH_2 are similar to lipid structure and the signals at 171.4 and 173.6 ppm, corresponds to ester and carboxylate moieties. The ^{13}C NMR also displayed chemical shifts at 102.5, 94.4 ppm, this indicate that the molecular structure is identical to RL2. Similar NMR

spectroscopy analysis of di-rhamnolipid production has been reported by Wei *et al.*, 2008 and Ganesh *et al.*, 2010 using indigenous isolates, *P. aeruginosa* J16 and *P. aeruginosa* BS-161R respectively. Earlier, literature findings showed that *P. aeruginosa* could produce different rhamnolipid homologues, which may differ in the chain length of fatty acids or in the number of rhamnose units (Deziel *et al.*, 1999). Interestingly, amphipathic nature of rhamnolipid was observed to be proportional to fatty acid chain length and number of rhamnose units respectively (Wei *et al.*, 2008; Nitschke *et al.*, 2005). The two common structures in rhamnolipid family are mono-rhamnolipid and di-rhamnolipid, composed of monomer or dimer of L-rhamnose molecules connected with β-hydroxydecanoyl-β-hydroxydecanoate respectively (Soberon-chavez *et al.*, 2005).

The surface active properties of purified RL2 (0.1 mg mL^{-1}) obtained from *P. aeruginosa* DSVP20 showed maximum surface tension reduction to be 26.9 mN m^{-1} while critical micelle dilution (CMD^{-1} and CMD^{-2}) values of purified RL2 obtained from DSVP20 were 30 mN m^{-1} and 36 mN m^{-1} respectively suggesting it to be an effective surfactant. In comparison to this, CMD values of rhamnolipid were reported to be 13.4 mN m^{-1} and 50 mN m^{-1} by Abdel-Mawgoud *et al.*, 2009 and Wei *et al.*, 2005 respectively while working on *P. aeruginosa*. Purified RL2 (0.1 mg mL^{-1}) obtained from DSVP20 also exhibited excellent emulsification activity as the E_{24} (%) value was found out to be 98%. Similar bio-emulsification properties of rhamnolipid have been reported by earlier by Abdel-Mawgoud *et al.*, 2009.

Interestingly, the surface tension, critical micelle dilution (CMD) and emulsification index values of the purified RL2 from *P. aeruginosa* DSVP20 were retained over a wide pH range (4-12), with minimal deviation in surface active properties. However, CMD^{-2} showed a slight and gradual increase on surface tension with increasing pH values.

Besides this, purified RL2 at different thermal conditions (4-121°C) was also found stable and no appreciable changes were observed on surface tension and CMD values with the addition of NaCl up to 10% in aqueous solution of RL2 thereby demonstrating the potential commercial applications of RL2. Results obtained above were in agreement with previous findings by Abdel-Mawgoud *et al.*, 2009. They too in their study have shown that rhamnolipid produced by *P. aeruginosa* isolate Bs20 exhibited excellent stabilities at high temperatures (heating at 100°C for 1 h and autoclaving at 121°C for 10 min), salinities (up to 6% NaCl), and pH values (up to pH 13).

One of the important strategies to improve RL2 production is to optimize the constituents of growth media. Several studies have demonstrated that the medium composition and growth conditions can influence the type and yield of biosurfactant (Makkar *et al.*, 2011; Lotfabad *et al.*, 2010; Prieto *et al.*, 2008; Rashedi *et al.*, 2005). With a view to develop an economically feasible technology to improve RL2 production, a number of factors which includes optimization of environmental and fermentation parameters such as aeration, agitation, pH, temperature, incubation period, effect of carbon and nitrogen sources were under taken. Results obtained using different aeration (0.25-1.5 vvm) and agitation (50–250 rpm) on RL2 production showed that these two parameters are highly correlated with the oxygen transfer efficiency as has been observed by other researchers (Yeh *et al.*, 2006). Results obtained during studies on the effect of pH and temperature on RL2 production clearly indicates that the pH of the medium and incubation temperature did play a major role in RL2 production by *P. aeruginosa* DSVP20. These results are in good agreement with the results obtained earlier for biosurfactant from *P. aeruginosa* which suggested that it would be more economical to use 30°C in practical applications for biosurfactant from *P. aeruginosa* (Praveesh *et al.*, 2010).

Literature revealed that the type of carbon substrates markedly affected the production yield of rhamnolipid (Wu *et al.*, 2008; Maier and Chavez, 2000; Mata-Sandoval *et al.*, 2000, Lang and Wullbrandt, 1999). In light of this, we investigated the role of different carbon sources on rhamnolipid production. The carbon sources tested included carbohydrates (i.e., glucose and sucrose), glycerol, vegetable oils (i.e., olive oil and soybean oil) and fatty acid (i.e., oleic acid). Data indicated that glucose and glycerol were effective carbon substrates for RL2 production (Fig. 24). After cultivation for 4 days, the culture with glucose and glycerol produced 6.16 and 7.31 mg mL^{-1} of RL2, respectively. Surprisingly, *P. aeruginosa* DSVP20 was unable to utilize sucrose efficiently, resulting in a poor RL2 production (0.93 mg mL^{-1}) as shown in Fig. 24. It is likely that the DSVP20 strain may lack the enzyme (i.e., invertase) for sucrose hydrolysis. Although vegetable oils have been frequently used as the carbon substrates for rhamnolipid production with *P. aeruginosa* strains (Maier and Chavez, 2000; Rahman *et al.*, 2002b; Sim *et al.*, 1997), the DSVP20 strain attained a lower RL2 production from olive oil and soybean oil than that from glucose and glycerol (Fig. 24). Data showed that RL2 production was 3.2 and 3.74 mg mL^{-1} for olive oil and soybean oil, respectively (Table 3). It was observed that the direct use of fatty acid (i.e., oleic acid) as the carbon source did not improve RL2 production (Fig. 24), suggesting that hydrolysis of the oils was not the bottle- neck step. Some reports show that vegetable oils were more efficient substrates in rhamnolipid production from *P. aeruginosa* strains when compared with glucose, glycerol, and hydrocarbons (Maier and Chavez, 2000; Mata-Sandoval *et al.*, 2001). However, from data obtained from our studies on *P. aeruginosa* DSVP20 revealed that it preferred glycerol when tested for RL2 production (Fig. 24) suggesting thereby that the carbon source preference for rhamnolipid production seems to be strain dependent. Since, lipids are acylglycerols, usage of glycerol as carbon source may increase lipid

production which in turn could accelerate rhamnolipid production. It is interesting to note that as the RL2 production decreased sharply as glycerol concentration went over 2% (Fig. 25). Silva *et al.*, 2010 during their studies on *P. aeruginosa* UCP0992 too have reported glycerol (3%) to be as a suitable substrate for production of biosurfactant. Though, the significant inhibitory effect of glycerol has not yet been revealed in the literature. In fact, there is little information regarding rhamnolipid production from a high level (4% or above) of glycerol, but Hauser and Karnovsky, 1957 did report a 2000 mg L^{-1} yield of rhamnolipid from a *P. aeruginosa* strain grown at 3% glycerol.

With respect to nitrogen sources, rhamnolipid production by *P. aeruginosa* varied with both the quality and quantity of nitrogen source assimilated (Fig. 26). Nitrogen starvation plays an important role in the production of surface-active compounds by microorganisms (Wu *et al.*, 2008). Keeping the optimum glycerol concentration, the effect of the nitrogen source on RL2 production was examined for most commonly used organic and inorganic nitrogen sources reported in the literature. Among the five nitrogen sources utilized *i.e.* ammonium chloride, ammonium nitrate, ammonium sulphate, sodium nitrate and urea, it was observed that sodium nitrate ($NaNO_3$) was the most efficient nitrogen source (Silva *et al.*, 2010). Thus, sodium nitrate was selected for our experimental work, considering its availability and low cost as has been ably supported by the other research groups (Sim *et al.*, 1997; Wei *et al.*, 2005). Urea when used as the organic nitrogen sources led to reduce RL2 production (4.87 mg mL^{-1}) despite good cell growth (2.62 mg mL^{-1}) when compared with $NaNO_3$. In fact, Kim *et al.*, 2006, too had reported that the organic nitrogen source could help cell growth but was unfavorable to production of glycolipid biosurfactant.

According to Mulligan and Gibbs, 1989, *P. aeruginosa* uses nitrates, ammonia and amino acids as nitrogen sources. Irrespective of nitrogen sources, ammonia acts as a common principle precursor molecule for initiating the nitrogen assimilation pathway. Ammonia could be assimilated either by glutamate dehydrogenase (EC 1.4.1.4) to form glutamate or by glutamine synthetase (EC 6.3.1.2) to form glutamine. Glutamate and glutamine then underwent trans-amino reaction to generate other sets of amino acids. As nitrates had to be reduced to nitrite and then to ammonia, their usage as nitrogen source creates a nitrogen limiting condition, which could be favorable for RL2 biosynthesis as confirmed by the results. Under nitrogen limiting condition, enhanced lipid synthesis could occur which in turn would potentially accelerate rhamnolipid biosynthesis. Therefore, nitrogen-limiting condition created by using nitrate as nitrogen source is favorable for rhamnolipid production (Silva *et al.*, 2010).

Quantification of *C. albicans* biofilm formation assessed over a range of time intervals by an XTT metabolic reduction assay was done using 96-well microtiter plate in this study. This allowed us to more easily observe metabolic activity of biofilm growth and perform multiple experimental parameters in a reproducible manner as has been reported earlier by Agarwal *et al.*, 2008. Data obtained from our results (Fig. 27) clearly showed that the formation of brown colored water-soluble tetrazolium formazan produced was due to the reduction of XTT, by mitochondrial dehydrogenase of *C. albicans*. This depicts a linear relationship with the metabolic activity of *C. albicans* biofilm as has been reported by other researchers (Lal *et al.*, 2010; Nett *et al.*, 2008; Ramage *et al.*, 2005). Studies have shown that initially, there was a period of adherence (0-2 h) and subsequent microcolony formation (2-4 h). Dimorphic switching occurred thereafter with a transition from budding-yeast forms to filamentous pseudo- and true-hyphal forms (4-6 h). Micro-colonies then became interlinked by the hyphal extensions, forming a confluent

monolayer (6-8 h). The complexity of the biofilm increased with time, taking on a three-dimensional architecture with spatial heterogeneity as it matured (8-48 h). The biofilm after 24 h and 48 h consisted of a mixture of yeast cells, pseudohypahe and true hyphae. Production of EPS and microcolony formation were the most important factor in the three-dimensional architecture, with yeast cells located in the basal layer as also reported by Baille and Douglas, 1999. Earlier, researcher while working on different substrates such as intravascular catheters, dentures, heart valves, implanted devices and contact lenses too have reported that architecture of *C. albicans* biofilm was influenced by surface properties of materials (Nikawa *et al.*, 1996; Chandra *et al.*, 2005; 2008; Imamura *et al.*, 2008; Estivill *et al.*, 2011; Frade *et al.*, 2011) which supports our investigation results that suture material of different surface properties have marked influence on the buildup of *C. albicans* biofilm formed on them.

The barrier posed by the basic functional properties of surgical suture such as biological inertness, sufficient surgical knot strength and reliability, thromboresistance, atraumaticity, absence of wicking has now been dethroned by microbial adherence in the form of biofilm on them (Zhukovskii, 2005). As the sutures tested in this study differ in their surface properties, we hypothesized that the ability of *C. albicans* to form biofilm would also vary with different sutures. To test this hypothesis, we quantified biofilm formed on IMDs such as sutures and contact lenses using the XTT-based reduction method. Data revealed that of the four commonly used surgical sutures maximum biofilm formation was observed by nonabsorbable sutures Sutupak* SW216 and Prolene* NW843, followed by absorbable sutures Plain W2004 and Chromic NW4241 respectively (Fig. 30A). As the contact lenses used in this study differ in their surface properties, *C. albicans* biofilm formed on all three type of lenses exhibited structural and topological variations. Similar results were noticed by Imamura et al., 2008 during their studies on

Fusarium and *Candida albicans* biofilm formed on soft contact lenses. Recently, it had been shown that biofilm formation by *Candida* spp., varies depending on substratum physical and chemical properties (Estivill *et al.*, 2011). Earlier, researchers have shown that modification in surface properties of biomaterials influences the ability of *C. albicans* to form biofilm (Chandra *et al.*, 2005). Besides this, it has also been observed that hydrophilic coated device surfaces are key players in reduction of bacterial colonization (Okajima *et al.*, 2006; Cagavi *et al.*, 2004).

RL2 isolated from *P. aeruginosa* DSVP20 exhibits a dose dependent antimicrobial action with increasing RL2 concentrations. MIC_{90} of RL2 obtained in this study against planktonic (free floating) *C. albicans* was found to be at 100 $\mu g\ mL^{-1}$ which was much elevated as compared to MIC_{90} values observed against bacteria (Dusane *et al.*, 2010; Lang *et al.*, 1989).

Different group of biosurfactants exhibit diverse antimicrobial properties as reported in case of biosurfactant 'Lunasan' produced by *C. sphaeria* (Luna *et al.*, 2011), 'Rufisan' from *C. lipolytica* (Rufino *et al.*, 2011) and 'Mannosylerythritol lipids' from *C. antarctica* (Arutchelvi *et al.*, 2008). Significant antimicrobial properties were also recorded by lipopeptide surfactants depending on the way which interact with the cell membrane of the target cells (Monteiro *et al.*, 2011; Sriram *et al.*, 2011). It is hypothesized previously that antimicrobial property of rhamnolipid is due to the modification of cell permeability which may be release of lipopolysaccharides from outer membrane of Gram-negative bacteria by solubilization or due to formation of transmembrane pores as channels to the periplasm in the surface of Gram-positive bacteria (Sotirova *et al.*, 2010). Similarly, we too believe that *C. albicans* RL2 obtained being amphipathic could destabilize the cytoplasmic membrane by inserting themselves in membrane phospholipid bilayer which

in turn could bring about the anticandidal action. Earlier, researchers have shown that adhesion is a prerequisite parameter for colonization of surface–attached microbes leading to the development of mature biofilm. Besides this, they have shown that correlation exists between factors such as surface hydrophobicity, biofilm development, and the anti-adhesive activity of RL2 to control infection (de Araujo *et al.*, 2011; Dusane *et al.*, 2010; Rodrigues *et al.*, 2006a). In earlier reports, the preconditioning of 96-well plate with a rhamnolipid, produced by *P. aeruginosa* was recorded to inhibit the adhesion of *Listeria monocytogenes* by about 82% (de Araujo *et al.*, 2011). Similarly, 46-99% inhibition of *B. pumilus* cells attachment to 96-well plate was observed when a low concentration (0.05-100 mM) of commercially available rhamnolipid was used by Dusane *et al.*, 2010. Rhamnolipid was also reported to interfere in adhesion of bacteria and yeasts isolated from explanted voice prostheses to silicon rubber (Rodrigues *et al.*, 2006a). The disruption studies on *C. albicans* biofilm which was recorded to be uniformly resistant to a wide spectrum of conventional antifungal agents by purified RL2 were experimentally validated using XTT based colorimetric assay which is based on formation of tetrazolium formazan product (Kuhn *et al.*, 2002; Chandra *et al.*, 2001; Hawser *et al.*, 1998). Our microscopic analysis studies (SEM, AFM and CLSM) and tetrazolium salt based XTT assay also confirms that RL2 obtained from *P. aeruginosa* can lead to a significant disruption of the *C. albicans* biofilm architecture and design *in vitro*. Recently, Fracchia *et al.*, 2010, while working on *Lactobacillus*-derived biosurfactant too have reported the inhibition of human pathogenic *C. albicans* biofilm formation by it. Apart from external addition of biosurfactant for removal of pre-formed biofilm, surface conditioning or coating of biosurfactant could prove effective in preventing bacterial adhesion (Rodrigues *et al.*, 2006a; Mireles *et al.*, 2001) Although, biofilm inhibition mechanism of RL2 is still to be decoded but it is hypothesized that the mechanism is most likely related to the

inhibition of the cell to cell interactions as well as cell to substrate interaction (Davey *et al.*, 2003) or it may be attributed to the destabilizing action on cell membranes caused by interactions between phospholipids and biosurfactants (Carrillo *et al.*, 2003). Biofilm inhibition activity of rhamnolipid is believed to be based on altering the physical properties, the chances of resistance development against it through spontaneous mutations are remote (Carrillo *et al.*, 2003; Davey *et al.*, 2003). The hydrophobic nature of biosurfactants enables them to maintain porosity in developing biofilm through open channel formation. Such activities further confer biosurfactants with antimicrobial activity against fungi and bacteria, when they are used at concentration far above than physiological concentrations. Previous reports have shown that rhamnolipid is effective in preventing or slowing the formation of, or accelerating the dispersion phase of biofilm development of bacterial species (Dusane *et al.*, 2010; Stanghellini *et al.*, 1997). Similarly, cell surface hydrophobicity, which contributes to the interaction between the cells and the surface, is an important and vital factor in the adhesion of *C. albicans* biofilm, as has also been shown by other research groups (Borecka-Melkusova and Bujadakova, 2008; Li *et al.*, 2003; el-Azizi *et al.*, 1999; Silva *et al.*, 1995).

From our results on cell surface hydrophobicity also it could be interpolated that RL2 treated *C. albicans* biofilm inferred a dose dependent correlation. In fact, these results are in agreement with other research groups who have shown that biosurfactant had less supportive to play for adhesion and further biofilm formation (Cao *et al.*, 2009; Klotz *et al.*, 1985). Data obtained by visualization of SEM micrographs studies showed marked reduction in intense arrangement of *C. albicans* cell adhesion and biofilm formation depicting alteration in structural design of *C. albicans* when compared with its control. Similar results were recorded by de Araujo *et al.*, 2011 while working on *Listeria monocytogenes* whose adhesion was inhibited by RL2 and surfactin. RL2 have also

shown to disrupt preformed marine biofilm in case of *Bacillus pumilus* (Dusane *et al.*, 2010). Thus, confirming the excellent potential of RL2 as biofilm reducing agent (Fig. 35A & 35C). CLSM micrograph data obtained revealed marked difference between RL2 untreated and treated *C. albicans* biofilm (Fig. 35B & 35D) as the former biofilm was less structured and contained fewer cells. The green fluorescence from FITC-ConA binding indicates live cells and dense network of polysaccharides whereas red fluorescence due to PI binding represents dead cells. The yellow color formed as a result of overlapping of red and green images depicts production of exopolysaccharides as capsular components (Fig. 35B & 35D) (Lal *et al.*, 2010). Through CLSM micrographs it was established that *C. albicans* biofilm architecture was disturbed on RL2 treatment and fewer number of candidal cells were seen adhering to the surface. Similar studies on rhamnolipid ability to disperse *Bordetella bronchiseptica* biofilm, which in turn causes a significant reduction in viable cell count, has been recorded earlier (Irie *et al.*, 2005) RL2 seems to show better potential as an anti-adhesion and anti-biofilm formation agent, especially for food applications, when it can be utilized as a multipurpose formulation ingredient (Dusane *et al.*, 2010; de Araujo *et al.*, 2011). Our results on AFM micrograph of pre-formed *C. albicans* biofilm depicted high number of grooves and crusts in comparison to RL2 treated *C. albicans* micrograph (Fig. 36). Similarly, reduction in roughness during AFM studies on a *C. albicans* was recorded by the researcher while working on lemon grass oil as an antimicrobial agent (Tyagi *et al.* 2010).

Overall, the results prove the biomedical potential of rhamnolipid against *C. albicans*. Growth inhibition studies prove that the RL2 could act against both planktonic as well as sessile cells of *C. albicans*. The potential antibiofilm activity of RL2 which

makes it far superior than other antifungal agents was uniquely proved through differential as well as advanced microscopic studies.

CHAPTER 6

CONCLUSION

The foremost task undertaken in this investigation dealt with the screening of bacterial strains capable of producing maximum biosurfactant. Among 310 isolates from different samples, 25 bacterial isolates showed the ability to grow on different hydrocarbons *viz.* dodecane, n-hexadecane, pristane, eicosane, toluene and fluoranthene as sole carbon source in PAC salt medium. These 25 bacterial isolates were then tested using simple, rapid and efficient techniques such as haemolytic ability, CTAB agar plate assay, drop collapse assay, emulsification assay and surface tension reduction as an index for biosurfactant production. From them four strains namely NSVP2, DSVP11, DSVP17 and DSVP20 were selected and screened for potential biosurfactant production. Biosurfactant producing ability of these isolates was further confirmed by assaying cell surface hydrophobicity which exhibited direct correlation between biosurfactant production by the isolates and their cell surface hydrophobic properties. Among these four bacterial isolates, DSVP20 was found to be a potential biosurfactant which when grown on PAC salt medium with 2% glycerol showed maximum biosurfactant production (5.8 g L^{-1}) after 96 h of growth. The surface activities measured in terms of surface tension revealed maximum reduction in its value from 72.0 mN m^{-1} to 28.5 mN m^{-1} in cell free broth of *P. aeruginosa* while CMD^{-1} and CMD^{-2} values recorded were 39.50 mN m^{-1} and 42.50 mN m^{-1} respectively after 96 h. Morphological, biochemical and molecular (16S rRNA) characterization of above bacterial strain DSVP20 revealed that the strain resembles *Pseudomonas aeruginosa* (GenBank accession no. GQ865644). Biochemical characterization using TLC and FTIR of biosurfactant from *P.*

aeruginosa DSVP20 indicated it to be glycolipid in nature with 14.7% carbohydrate content and lipid content was 29.3% respectively. Analysis by MALDI-TOF-MS and NMR further revealed that the purified biosurfactant to be di-rhamnolipid (RL2) in nature with m/z range of 649 Da. Nutritional requirements for optimum production of RL2 by *P. aeruginosa* DSVP20 showed 2% glycerol as a suitable carbon source for maximum growth and RL2 production. The yield of RL2 (7.8 mg mL^{-1}) was recorded maximum at optimum conditions of 96 h, 150 rpm, 35° C and pH of 6.0. The surface activities of the purified RL2 remained unaltered within a wide pH (4-12), temperature (4-121°C) ranges and under NaCl concentrations up to 10%. The anti-adhesion effect of the purified RL2 suggested its potential as an alternative antifungal agent. The *C. albicans* biofilm disrupting ability of RL2 was qualitatively observed microscopically using SEM, AFM and CLSM. Microscopic studies sought out the low cell density, structural deformation and increased proportion of dead cells over live cells in RL2 treated *C. albicans* biofilm. Quantitative estimation of the biofilm reduction action was assessed over a range of time intervals by XTT metabolic reduction assay revealed that RL2 acted in a dose dependant manner. Moreover, it was found out that 90% biofilm mitigation could be achieved by 5.0 mg mL^{-1} of purified RL2. The results obtained suggest the possible use of RL2 as an alternative antifungal agent in the medical field for application against *C. albicans* biofilm, responsible for medical implant related infection, through coating or imprinting the implant surfaces with RL2. It was possible to conclude that RL2, an antibiofilm antifungal agent, could be a suitable alternative to conventional therapies for inhibiting *C. albicans* biofilm based infections.

References

1. Abalos A, Pinazo A, Infante M R, Casals M, Garcýa F, Manresa A. 2001. Physicochemical and antimicrobial properties of new rhamnolipids produced by *Pseudomonas aeruginosa* AT10 from soybean oil refinery wastes. *Langmuir* 17:1367-1371.
2. Abalos A, Viñas M, Sabaté J, Manresa M A, Solanas A M. 2004. Enhanced biodegradation of casablanca crude oil by a microbial consortium in presence of a rhamnolipid produced by *Pseudomonas aeruginosa* AT10. *Biodegradation* 15:249-260.
3. Abdel-Mawgoud A M, Aboulwafa M, Hassouna N. 2009. Characterization of rhamnolipid produced by *Pseudomonas aeruginosa* isolate Bs20. *Appl Biochem Biotechnol* 157:329-345.
4. Agarwal V, Lal P, Pruthi V. 2008. Prevention of *Candida albicans* biofilm by plant oils. *Mycopathologia* 165:13-19.
5. Ahimou F, Semmens M J, Novak P J, Haugstad G. 2007. Biofilm cohesiveness measurement using a novel atomic force microscopy methodology. *Appl Environ Microbiol* 73:2897-2904.
6. Al-Dhaheri R S, Douglas L J. 2010. Apoptosis in *Candida* biofilms exposed to amphotericin B. *J Med Microbiol* 2:149-157.
7. Alem M A, Douglas L J. 2005. Prostaglandin production during growth of *Candida albicans* biofilms. *J Med Microbiol* 54:1001-1005.
8. Alem M A, Oteef M D, Flowers T H, Douglas L J. 2006. Production of tyrosol by *Candida albicans* biofilms and its role in quorum sensing and biofilm development.

Eukaryot Cell 5:1770-1779.

9. Al-Fattani M A, Douglas L J. 2006. Biofilm matrix of *Candida albicans* and *Candida tropicalis*: chemical composition and role in drug resistance. *J Med Microbiol* 55:999-1008.

10. Allesen-Holm M, Barken K B, Yang L, Klausen M, Webb J S, Kjelleberg S, Molin S, Givskov M, Tolker-Nielsen T. 2006. Characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. *Mol Microbiol* 59:1114-1128.

11. Allison D G, Heys S J D, Willcock L, Holah J, Gilbert P. 1999. Cellular detachment and dispersal from bacterial biofilms: role for quorum sensing? In: Biofilms: The Good, the Bad and the Ugly, Wimpenny J W T, Gilbert P, Walker J T, Brading M E, Bayston R (eds), Bioline, Cardiff, p 279–293.

12. Almeida R S, Brunke S, Albrecht A, Thewes S, Laue M, Edwards J E, Filler S G, Bernhard Hube B. 2008. The hyphal-associated adhesin and invasin Als3 of *Candida albicans* mediates iron acquisition from host ferritin. *PLoS Pathog* 4: e1000217.

13. Anandaraj B, Thivakaran P. 2010. Isolation And Production Of Biosurfactant Producing Organism From Oil Spilled Soil. *J Biosci Tech* 1:120-126.

14. Andes D, Nett J, Oschel P, Albrecht R, Marchillo K, Pitula A. 2004. Development and characterization of an *in vivo* central venous catheter *Candida albicans* biofilm model. *Infect Immun* 72:6023-6031.

15. Arino S, Marchal R, Vandecasteele J P. 1996. Identification and production of rhamnolipidic biosurfactants by a *Pseudomonas* species. *Appl Microbiol Biotechnol* 45:162-168.

16. Arutchelvi J I, Bhaduri S, Uppara P V, Doble M. 2008. Mannosylerythritol lipids: A review. *J Ind Microbiol Biotechnol* 35:1559-1570.

17. Arutchelvi J, Doble M. 2010. Characterization of glycolipid biosurfactant from *Pseudomonas aeruginosa* CPCL isolated from petroleum-contaminated soil. *Lett Appl Microbiol* 51:75-82.

18. Baboni F B, Filho O G, Moreno A N, Antonio E, Rosa R. 2010. Influence of cigarette smoke condensate on cariogenic and *Candida* 1 biofilm formation on orthodontic materials. *Am J Orthod Dentofacial Orthop* 138:427-434.

19. Babu P S, Vaidya A N, Bal A S, Kapur R, Juwarkar A, Khanna P. 1996. Kinetics of biosurfactant production by *Pseudomonas aeruginosa* strain BS2 from industrial wastes. *Biotech Lett* 18:263-268.

20. Bai G, Brusseau M L, Miller R M. 1997. Biosurfactant-enhanced removal of residual hydrocarbon from soil. *J Contain Hydrol* 25:157-170.

21. Bailey D A, Feldmann P J, Bovey M, Gow N A, Brown A J. 1996. The *Candida albicans* HYR1 gene, which is activated in response to hyphal development, belongs to a gene family encoding yeast cell wall proteins. *J Bacteriol* 178:5353-5360.

22. Baillie G S, Douglas L J. 1998. Effect of growth rate on resistance of *Candida albicans* biofilms to antifungal agents. *Antimicrob Agents Chemother* 42:1900-1905.

23. Baillie G S, Douglas L J. 1999. *Candida* biofilms and their susceptibility to antifungal agents. *Meth Enzymol* 310:644-656.

24. Baillie G S, Douglas L J. 2000. Matrix polymers of *Candida biofilms* and their possible role in biofilm resistance to antifungal agents. *J Antimicrob Chemother* 46:397-403.

25. Balan I, Alareo A, Raymond M. 1997. The *Candida albicans* CDR3 gene codes for an opaque-phase ABC transporter. *J Bacteriol* 179:7210-7218.

26. Balogun S A, Fagade O E. 2008. Screening for surface-active agent producing bacteria from diesel oil polluted tropical soil. *World Appl Sci J* 3:930-933.

27. Banat I M, Makkar R S, Cameotra S S. 2000. Potential commercial application of microbial surfactants. *Appl Microbiol Biotechnol* 53:495-508.

28. Banat I M. 1995. Biosurfactants production and possible uses in microbial enhanced oil recovery and oil pollution remediation: a review. *Biores Technol* 51:1-12.

29. Bandara H M H N, Yau J Y Y, Watt R M, Jin L J, Samaranayake L P. 2010. *Pseudomonas aeruginosa* inhibits *in vitro Candida* biofilm development. *BMC Microbiol* 10:125-133.

30. Bastidas R J, Heitman J, Cardenas M E. 2009. The protein kinase Tor1 regulates adhesin gene expression in *Candida albicans*. *PLoS Pathog* 5:e1000294.

31. Batista S B, Mounteer A H, Amorim F R, Totola M R. 2006. Isolation and characterization of biosurfactant/bioemulsifier-producing bacteria from petroleum contaminated sites. *Bioresour Technol* 97:868-875.

32. Benincasa M, Abalos A, Oliveira I, Manresa A. 2004. Chemical structure surface properties and biological activities of the biosurfactant produced by *Pseudomonas aeruginosa* LBI from soapstock. *Antonie van Leeuwenhoek* 85:1-8.

33. Bernardo S M, Khalique Z, Kot J, Jones J K, Lee S A. 2008. *Candida albicans* VPS1 contributes to protease secretion, filamentation, and biofilm formation. *Fungal Genet Biol* 45:861-877.

34. Bernheimer A W, Avigad L S. 1970. Nature and properties of a cytolytic agent produced by *Bacillus subtilis*. *J Gen Microbiol* 61:361-369.

35. Bertrand J C, Bonin P, Goutx M, Mille C. 1993. Biosurfactant production by marine microorganisms: potential application to fighting hydrocarbons marine pollution. *J Mar Biochtechnol* 1:126-129.

36. Bharali P, Konwar B K. 2011. Production and physico-chemical characterization of a biosurfactant produced by *Pseudomonas aeruginosa* OBP1 isolated from petroleum sludge. *Appl Biochem Biotechnol* 164:1444-1460.

37. Biswas S K, Chaffin W L. 2005. Anaerobic growth of *Candida albicans* does not support biofilm formation under similar conditions used for aerobic biofilm. *Curr Microbiol* 51:100-104.

38. Biswas S, Van Dijck P, Datta A. 2007. Environmental sensing and signal transduction pathways regulating morphopathogenic determinants of *Candida albicans*. *Microbiol Mol Biol* 71:348-376.

39. Blankenship J R, Fanning S, Hamaker J J, Mitchell A P. 2010. An extensive circuitry for cell wall regulation in *Candida albicans*. *PLoS Pathog* 6:e1000752.

40. Bodour A A, Maier R M. 1998. Application of a modified drop-collapse technique for surfactant quantification and screening of biosurfactant-producing microorganisms. *J Microbiol Methods* 32:273-280.

41. Bodour A A, Barajas C G, Jiorle B V, Malcomson M E, Paull A K, Somogyi A,Trinh L N, Bates R B, Maier R M. 2004. Structure and characterization of flavolipids, a novel class of biosurfactants produced by *Flavobacterium* sp. strain MTN11. *Appl Environ Microbiol* 70:114-120.

42. Boothroyd B, Thorn J A, Haskins R H. 1956. Biochemistry of the ustilaginales. XII. Characterization of extracellular glycolipids produced by *Ustilago sp. Can J Biochem Physiol* 34:10-14.

43. Borecka-Melkusova S, Bujadakova H. 2008. Variation of cell surface hydrophobicity biofilm among genotypes of *Candida albicans* and *Candida duliniensis* under antifungal treatment. *Can J Microbiol* 54:718-724.

44. Braun B R, Head W S, Wang M X, Johnson A D. 2000. Identification and characterization of TUP1-regulated genes in *Candida albicans*. *Genetics* 156:31-44.

45. Bruno V M, Mitchell A P. 2005. Regulation of azole drug susceptibility by *Candida albicans* protein kinase CK2. *Mol Microbiol* 56:559-573.

46. Bruzual I, Riggle P, Hadley S, Kumamoto C A. 2007. Biofilm formation by fluconazole-resistant *Candida albicans* strains is inhibited by fluconazole. *J Antimicrob Chemother* 59:441-450.

47. Bunt C R, Jones D S, Tucker I G. 1993. The effects of pH, ionic strength and organic phase on the bacterial adhesion to hydrocarbons (BATH) test. *Intern J Pharm* 99:93-98.

48. Burger M M, Glaser L, Burton R M. 1963. The enzymatic synthesis of rhamnose-containing glycolipids by extracts of *Pseudomonas aeruginosa*. *J Biol Chem* 238:2595-2602.

49. Byers J D, Ratner B D. 2004. Bioinspired implant materials befuddle bacteria. *ASM News* 70:232-237.

50. Cagavi F, Akalan N, Celik H. 2004. Effect of hydrophilic coating on microorganism colonization in silicone tubing. *Acta Neurochir* 146:603– 610.

51. Caiazza N C, Shanks R M Q, O'Toole G A. 2005. Rhamnolipids modulate swarming motility patterns of *Pseudomonas aeruginosa*. *J Bacteriol* 187:7351-7361.

52. Cameotra S S, Makkar R S. 1998. Synthesis of biosurfactants in extreme conditions. *Appl Microbiol Biotechnol* 50:520-529.

53. Cameotra S S, Makkar R S. 2004. Recent applications of biosurfactants as biological and immunological molecules. *Curr Opin Microbiol* 7:262-266.

54. Cameotra S S, Singh P. 2009. Synthesis of rhamnolipid biosurfactant and mode of hexadecane uptake by *Pseudomonas* species. *Microbial Cell Factories* 8:16.

55. Cameron D R, Cooper D G, Neufeld R J. 1998. The manoprotein of *Saccharomyces cerevisae* is an effective bioemulsifier. *Appl Environ Microbiol* 54:1420-1425.

56. Campos-Garciá J, Caro A D, Na'jera R, Miller-Maier R M, Al-Tahhan R A, Sobero'n-Cha'vez G. 1998. The *Pseudomonas aeruginosa rhlG* gene encodes an NADPH dependent b-ketoacyl reductase which is specifically involved in rhamnolipid synthesis. *J Bacteriol* 180:4442-4451.

57. Cao X H, Liao Z Y, Wang C L, Yang W Y, Lu M F. 2009. Evaluation of a lipopeptide biosurfactant from *Bacillus natto* TK-1 as a potential source of anti-adhesive, antimicrobial and antitumor activities. *Braz J Microbiol* 40:373-379.

58. Cao Y Y, Dai B D, Wang Y, Huang S, Xu Y G, Cao Y B, Gao P H, Zhu Z Y, Jiang Y Y. 2008. *In vitro* activity of baicalein against *Candida albicans* biofilms. *Int J Antimicrob Agents* 32:73-77.

59. Cao Y, Cao Y B, Zheng X, Kang Y, Yao L, Xie Y, Zhu Y, ChenW S, Jiang Y. 2005. cDNA microarray analysis of differential gene expression in *Candida albicans* biofilm exposed to farnesol. *Antimicrob Agents Chemother* 49:584-589.

60. Carrillo C, Teruel J, Aranda F, Ortiz A. 2003. Molecular mechanism of membrane permeabilization by the peptide antibiotic surfactin. *Biochim Biophys Acta* 1611:91-97.

61. Cassidy D P, Hudak A J, Werkema D D, Atekwana E A. 2002. In situ rhamnolipid production at an abandoned petroleum refinery. *Soil Sediment Contam* 11:769-787.

62. Cauda R. 2009. Candidaemia in patients with an inserted medical device. *Drugs* 69:33-38.

63. Chamilos G, Nobile C J, Bruno V M, Lewis R E, Mitchell A P, Kontoyiannis D P. 2009. *Candida albicans* Cas5, a regulator of cell wall integrity, is required for virulence in murine and toll mutant fly models. *J Infect Dis* 200:152-157.

64. Champion J T, Gilkey J C, Lamparski H, Retterer J, Miller R M. 1995. Electron microscopy of rhamnolipid (biosurfactant) morphology: effects of pH, cadmium and octadecane. *J Colloid Interface Sci* 170:569-574.

65. Chandra J, Kuhn D M, Mukhrjee P K, Hoyer L L, McCormick T, Ghannoum M A. 2001. Biofilm formation by the fungal pathogen *Candida albicans:* Development, architecture, and drug resistance. *J Bacteriol* 183:5385-5394.

66. Chandra J, Mukherjee P K, Ghannoum M A. 2008. *In vitro* growth and analysis of *Candida* biofilms. *Nature Protocols* 3:1909-1924.

67. Chandra J, Patel J D, Li J, Zhou G, Mukherjee P K, McCormick T S, Anderson J M, Mahmoud A, Ghannoum A M. 2005. Modification of surface properties of biomaterials influences the ability of *Candida albicans* to form biofilms. *Appl Environ Microbiol* 71:8795-8801.

68. Chaw K C, Manimaran M, Tay F E H. 2005. Role of silver ions in destabilization of intermolecular adhesion forces measured by atomic force microscopy in *Staphylococcus epidermidis* biofilms. *Antimicrob Agents Chemother* 49:4853-4859.

69. Chen S Y, Wei Y H, Chang J S. 2007. Repeated pH-stat fed-batch fermentation for rhamnolipid production with indigenous *Pseudomonas aeruginosa* S2. *Appl Microbiol Biotechnol* 76:67-74.

70. Cheng S, Clancy C J, Checkley M A, Handfield M, Hillman J D, Proqulske-Fox A, Lewin A S, Fidel P L, Nquyen M H. 2003. Identification of *Candida albicans* genes induced during thrush offers insight into pathogenesis. *Mol Microbiol* 48:1275-1288.

71. Christova N, Tuleva B, Nikolova-Damyanova B. 2004. Enhanced hydrocarbon biodegradation by a newly isolated *Bacillus subtilis* strain. *Z Naturforsch* 59c:205-208.

72. Cirigliano M C, Carman G M. 1985. Purification and characterization of liposan, a bioemulsifier from *Candida lipolytica. Appl Environ Microbiol* 50:846-850.

73. Clark F S, Parkinson T, Hitchcock C A, Gow N A R. 1996. Correlation between rhodamine 123 accumulation and azole sensitivity in *Candida* species – possible role for drug efflux in drug resistance. *Antimicrob Agents Chemother* 40:419-425.

74. Clifford J S, Ioannidis M A, Legge R L. 2007. Enhanced aqueous solubilization of tetrachloroethylene by a rhamnolipid biosurfactant. *J Colloid Interface Sci* 305:361-365.

75. Cooper D G and Goldenberg B G. 1987. Surface-Active Agents from Two *Bacillus* Species. *Appl Environ Microbiol* 53:224-229.

76. Cooper D G, Liss S N, Longay R, Zajic J E. 1989. Surface activities of *Mycobacterium* and *Pseudomonas. J Ferment Technol* 59:97-101.

77. Cooper D G, Paddock D A. 1983. *Torulopsis petrophilum* and surface activity. *Appl Environ Microbiol* 46:1426-1429.

78. Cooper D G. 1986. Biosurfactants. *Microbiol Sci* 3:145-149.

79. Costerton J W, Cheng K J, Geesey G, Ladd T L, Nickel J C, Dasgupta M, Marrie T J. 1987. Bacterial biofilms in nature and disease. *Ann Rev Microbiol* 41:435-464.

80. Costerton J W, Lewadowski Z, DeBeer D, Caldwell D, Korver D, James G. 1994. Biofilms, the customized microniche. *J Bacteriol* 176:2137-2142.

81. Costerton J W, Stewart P S, Greenberg E P. 1999. Bacterial biofilms: a common cause of persistent infections. *Science* 284:1318-1322.

82. Costerton J W. 1999. Introduction to biofilm. *Int J Antimicrob Agents* 11:217-221.

83. Davis D, Wilson R B, Mitchell A P. 2000. RIM101-dependent and-independent pathways govern pH responses in *Candida albicans*. *Mol Cell Biol.* 20:971-978.

84. Daniel H J, Otto R T, Reuss M, Syldatk C. 1998. Sophorolipid production with high yields on whey concentrate and rapeseed oil without consumption lactose. *Biotechnol Lett* 20:805-807.

85. Davey M E, Caiazza N C, OToole G A. 2003. Rhamnolipid surfactant production affects biofilm architecture in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* 185:1027-1036.

86. de Araujo L V, Abreu F, Lins U, de Melo Santa Anna L M, Nitschke M, Freire D M G. 2011. Rhamnolipid and surfactin inhibit *Listeria monocytogenes* adhesion. *Food Res Int* 44:481-488.

87. de Carvalho C C C R. 2007. Biofilms: Recent developments on an old battle. *Recent Patents on Biotechnology* 1:49-57.

88. de Groot P W J, de Boer A D, Cunningham J, Dekker H L, de Jong L, Hellingwerf K J, de Koster C, Klis F M. 2004. Proteomic analysis of *Candida albicans* cell walls reveals covalently bound carbohydrate-active enzymes and adhesins. *Eukaryot Cell* 3:955-965.

89. de Groot P W, Hellingwerf K J, and Klis F M. 2003. Genome-wide identification of fungal GPI proteins. *Yeast* 20:781-796.

90. d'Enfert C. 2006. Biofilms and their role in the resistance of pathogenic *Candida* to antifungal agents. *Curr drug targets* 7:465-470.

91. d'Enfert C, Goyard S, Rodriguez-Arnaveilhe S, Frangeul L, Jones L, Tekaia F, Bader O, Albrecht A, Castillo L, Dominguez A, Ernst J F, Fradin C, Gaillardin C, Garcia-Sanchez S, de Groot P, Hube B, Klis F M, Krishnamurthy S, Kunze D, Lopez M C, Mavor A, Martin N, Moszer I, Onésime D, Perez Martin J, Sentandreu R, Valentin E, Brown A J P. 2005. *Candida* DB: a genome database for *Candida albicans* pathogenomics. *Nucleic Acids Res* 33:353-357.

92. Desai J D, Banat I M. 1997. Microbial production of surfactant and their commercial potential. *Microbiol Mol Bio Rev* 61:47-64.

93. Desai J D, Desai A J. 1993. Production of biosurfactants. In: Biosurfactants: production, properties, applications, Kosaric N (eds), Marcel Dekker, New York, p 65-97.

94. Déziel E, Lépine F, Dennie D, Boismenu D, Mamer O A, Villemur R. 1999. Liquid chromatography/mass spectrometry analysis of mixtures of rhamnolipids produced by *Pseudomonas aeruginosa* strain 57RP grown on mannitol or naphthalene. *Biochim Biophys Acta* 1440:244-252.

95. Déziel E, Lépine F, Milot S, Villemur R. 2000. Mass spectrometry monitoring of rhamnolipids from a growing culture of *Pseudomonas aeruginosa* strain 57RP. *Biochim Biophys Acta* 1485:145-152.

96. Deziel E, Lepine F, Milot S, Villemur R. 2003. rhlA is required for the production of a novel biosurfactant promoting swarming motility in *Pseudomonas aeruginosa*:

3-(3-hydroxyalkanoyloxy) alkanoic acids (HAAs), the precursors of rhamnolipids. *Microbiology* 149:2005-2013.

97. Donlan R M. 2001. Biofilms and device-associated infections. *Emerg Infect Dis* 7:277-281.

98. Dominic R M, Shenoy S, Baliga S. 2007. *Candida* biofilms in medical devices: evolving trends. *Kathmandu Univ Med J* 5:431-436.

99. Donlan R M, Costerton J W. 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* 15:167-193.

100. Douglas L J. 2002. Medical importance of biofilms in *Candida* infections. *Rev Iberoam Micol* 19:139-143.

101. Douglas L J. 2003. *Candida* biofilms and their role in infection. *Trend Microbiol* 11:30-36.

102. Dubois M, Gilles K A, Hamilton J K, Rebers P A, Smith F. 1956. Colorimetric method for determination of sugars and related substances. *Anal Chem* 28:350-356.

103. Dusane D H, Nancharaiah Y V, Zinjarde S S,Venugopalan V P. 2010. Rhamnolipid mediated disruption of marine *Bacillus pumilus* biofilms. *Colloids Surf B Biointerfaces* 81:242-248.

104. Edgerton M, Lo S E, Scannapieco F A. 1996. Experimental salivary pellicles formed on titanium surfaces mediate adhesion of streptococci. *Int J Oral Maxillofac Implants* 11:443-449.

105. Edgerton M, Scannapieco F A, Reddy M S, Levine M J. 1993. Human submandibular-sublingual saliva promotes adhesion of *Candida albicans* to polymethylmethacrylate. *Infect Immun* 61:2644-2652.

106. el-Azizi M, Khardori N. 1999. Factors influencing adherence of *Candida* spp. to host tissues and plastic surfaces. *Indian J Exp Biol* 37:941-951.

107. Elguezabal N, Maza J L, Ponton J. 2004. Inhibition of adherence of *Candida albicans* and *Candida dubliniensis* to a resin composite restorative dental material by salivary secretory IgA and monoclonal antibodies. *Oral Dis* 10:81-86.

108. Ene I V, Bennett R J. 2009. Hwp1 and related adhesins contribute to both mating and biofilm formation in *Candida albicans*. *Eukaryot Cell* 8:1909-1913.

109. Estivill D, Arias A, Torres-Lana A, Carrillo-Muñoz A J, Arévalo M P. 2011. Biofilm formation by five species of *Candida* on three clinical materials. *J Microbiol Methods* 86:238-242.

110. Fan J, Whiteway M, Shen S H. 2005. Disruption of a gene encoding glycerol 3-phosphatase from *Candida albicans* impairs intracellular glycerol accumulation-mediated salt-tolerance. *FEMS Microbiol Lett* 245:107-116.

111. Feng Q, Summers E, Guo B, Fink G. 1999. Ras signaling is required for serum-induced hyphal differentiation in *Candida albicans*. *J Bacteriol* 181:6339–6346.

112. Ferreira C, Silva S, Faria-Oliveira F, Pinho E, Henriques M. 2010. *Candida albicans* virulence and drug-resistance requires the O-acyltransferase Gup1p. *BMC Microbiology* 10:238-251.

113. Filoche S, Wong L, Sissons C H. 2010. Oral biofilms: emerging concepts in microbial ecology. *J Dent Res* 89:8-18.

114. Finkel J S, Mitchell A P. 2011. Genetic control of *Candida albicans* biofilm development. *Nature Rev Microbiol* 9:109-118.

115. Flasz A, Rocha C A, Mosquera B, Sajo C A. 1998. Comparative study of the toxicity of a synthetic surfactant and one produced by *Pseudomonas aeruginosa* ATCC 55925. *Med Sci Res* 26:81-185.

116. Flemming H C, Neu T R, Wozniak D J. 2007. The EPS matrix: the "house of biofilm cells". *J Bacteriol* 189:7945-7947.

117. Fracchia L, Cavallo M, Allegrone G, Martinotti M G. 2010. A Lactobacillus-derived biosurfactant inhibits biofilm formation of human pathogenic *Candida albicans* biofilm producers. *Current Research Technology and Education Topics in Applied Microbiology and Microbial Technology.* A. Mendez-Vilas(Ed.). p 827-837.

118. Frade J P, Arthington-Skaggse B A. 2011. Effect of serum and surface characteristics on *Candida albicans* biofilm formation. *Mycoses* 54:154-162.

119. Ganesh A, Lin J. 2009. Diesel degradation and biosurfactant production by Gram-positive isolates. *Afr J Biotechnol* 8:5847-5854.

120. Ganesh K C, Suman K M, Biswanath D, Sridhar B, Sarala D G, Mallampalli S K. 2010. Synthesis of biosurfactant-based silver nanoparticles with purified rhamnolipids isolated from *Pseudomonas aeruginosa* BS-161R. *J Microbiol Biotechnol* 20:1061-1068.

121. García-Sánchez S, Sylvie A, Ismaïl I, Guilhem J, Jean-Marc Ghigo C. 2004. *Candida albicans* biofilms: a developmental state associated with specific and stable gene expression patterns. *Eukaryot Cell* 3:536-545.

122. Garret H E. 1972. Surface active chemicals. Oxford: Pergamon Press Ltd, p 1-60.

123. Gobbert U, Lang S, Wagner F. 1984. Sophorose lipid formation by resting cells of *Torulopsis bombicola. Biotechnol Lett* 6:225-230.

124. Goffeau A, Barrell B G, Bussey H, Davis R W, Dujon B, Feldmann H, Galibert F, Hoheisel J D, Jacq C, Johnston M, Louis E J, Mewes H W, Murakami Y, Philippen P, Tettelin H, Oliver S G. 1996. Life with 6000 genes. *Science* 274:546, 563-567.

125. Gottenbos B, van der Mei H C, Busscher H J. 2000. Initial adhesion and surface growth of *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* on biomedical polymers. *J Biomed Mater Res* 50:208-214.

126. Goyard S, Knechtle P, Chauvel M, Mallet A, Prévost C, Proux C, Coppée J, Schwartz P, Dromer F, Park H, Filler G, Janbon G, d'Enfert C. 2008. The Yak1 kinase is involved in the initiation and maintenance of hyphal growth in *Candida albicans*. *Mol Biol Cell* 19:2251-2266.

127. Granger B L, Flenniken M L, Davis D A, Mitchell A P, Cutler J E. 2005. Yeast wall protein 1 of *Candida* albicans. *Microbiology* 151:1631-1644.

128. Guenette S A, Beaudry F, Marier J F, Vachon P. 2006. Pharmacokinetics and anesthetic activity of eugenol in male Sprague–Dawley rats. *J Vet Pharmacol Ther* 29:265-270.

129. Guo Y P, Hu Y Y, Gu R R, Lin H. 2009. Characterization and micellization of rhamnolipidic fractions and crude extracts produced by *Pseudomonas aeruginosa* mutant MIG-N146. *J Colloid Interface Sci* 331:356-363.

130. Gutnick D L, Shabtai Y. 1987. Exopolysaccharide bioemulsifiers. In Biosurfactants and biotechnology, Kosaric N, Cairns W L, Gray N C C (eds), Marcel Dekker, New York, p 211-246.

131. Haba E, Pinazo A, Jauregui O, Espuny M J, Infante M R, Manresa A. 2003. Physicochemical characterization and antimicrobial properties of rhamnolipids produced by *Pseudomonas aeruginosa* 47T2 NCBIM 40044. *Biotechnol Bioeng* 81:316-322.

132. Haferburg D, Hommel R, Claus R, Kleberh P. 1986. Extracellular microbial lipids as biosurfactants. *Adv Biochem Eng/Biotechnol* 33:53-93.

133. Hall-Stoodley L, Stoodley P. 2002. Developmental regulation of microbial biofilms. *Curr Opin Biotechnol* 13:228-233.

134. Hall-Stoodley L, Stoodley P. 2009. Evolving concepts in biofilm infections. *Cellular Microbiology* 11:1034-1043.

135. Harry J B, Song J L, Lyons C N, White T C. 2002. Transcription initiation of genes associated with azole resistance in *Candida albicans*. *Med Mycol* 40:73-81.

136. Hansen S K, Rainey P B, Haagensen J A, Molin S. 2007. Evolution of species interactions in a biofilm community. *Nature* 445:533-536.

137. Hasan F, Xess I, Wang X, Jain N, Fries B C. 2009. Biofilm formation in clinical *Candida* isolates and its association with virulence. *Microbes Infect* 11:753-761.

138. Hashash R, Younes S, Bahnan W, El Koussa J, Maalouf K, Dimassi HI, Khalaf RA. 2010. Characterisation of Pga1, a putative *Candida albicans* cell wall protein necessary for proper adhesion and biofilm formation. *Mycoses* 54:491-500.

139. Haugland R P. 1996. Handbook of fluorescent probes and research chemicals. *Sixth Edition*, M.T.Z. Spence, Ed., Molecular Probes, Inc.

140. Hausauer D L, Gerami-Nejad M, Kistler-Anderson C, Gale C A. 2005. Hyphal guidance and invasive growth in *Candida albicans* require the Ras-like GTPase Rsr1p and its GTPase-activating protein Bud2p. *Eukaryot Cell* 4:1273-1286.

141. Hauser G, Karnovsky M L. 1957. Rhamnose and rhamnolipid biosynthesis by *Pseudomonas aeruginosa*. *J Biol Chem* 224:91-105.

142. Hawser S P, Baillie G S, Douglas L J. 1998. Production of extracellular matrix by *Candida albicans* biofilms. *J Med Microbiol* 47:253-256.

143. Hazra C, Kundu D, Ghosh P, Joshi S, Dandia N, Chaudharia A. 2011. Screening and identification of *Pseudomonas aeruginosa* AB4 for improved production, characterization and application of a glycolipid biosurfactant using low-cost agro-based raw materials. *J Chem Technol Biotechnol* 86:185-198.

144. Heller K. 2009. Zap1 sticks it to *Candida* biofilms. *PLoS Biol* 7(6): e1000117.

145. Helvaci S S, Peker S, Ozdemir G. 2004. Effect of electrolytes on the surface behavior of rhamnolipids R1 and R2. *Colloids Surf B Biointerfaces* 35:225-233.

146. Herman D C, Artiola J F, Miller R M. 1995. Removal of cadmium, lead and zinc from soil by a rhamnolipid biosurfactant. *Environ Sci Technol* 29:2280-2285.

147. Heydorn A, Nielsen A T, Hentzer M, Sternberg C, Givskov M, Ersboll B K, Molin S. 2000. Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology* 146:2395-2407.

148. Hisatsuka K, Nakahara T, Minoda Y, Yamada K. 1977. Formation of protein-like activator for *n*-alkane oxidation and its properties. *Agric Biol Chem* 41:445-450.

149. Hommel R K, Stuwer O, Stuber W, Haferburg D, Kleber H P. 1987. Production of water-soluble surface-active exolipids by *Torulopsis apicola*. *Appl Microbiol Biotechnol* 26:199-205.

150. Hong-Geller E, Valdez Y E, Shou Y, Yoshida T M, Marrone, B L, Dunbar J M. 2010. Evaluation of *Bacillus anthracis* and *Yersinia pestis* sample collection from nonporous surfaces by quantitative real-time PCR. *Lett Appl Microbiol* 50:431-437.

151. Honraet K, Goetghebeur E, Nelis H J. 2005. Comparison of three assays for the quantification of *Candida* biomass in suspension and CDC reactor grown biofilms. *J Microbiol Methods* 63:287-295.

152. Horowitz S, Gilbert J N, Griffin W M. 1990. Isolation and characterization of a surfactant produced by *Bacillus licheniformis* 86. *J Ind Microbiol* 6:243-248.

153. Hoyer L L, Payne T L, Bell M, Myers A M, Scherer S.1998. *Candida albicans* ALS3 and insights into the nature of the ALS gene family. *Curr Genet* 33:451-459.

154. Hoyer L L. 2001. The *ALS* gene family of *Candida albicans*. *Trends Microbiol* 9:176-180.

155. Hromatka B S, Noble S M, Johnson A D. 2005. Transcriptional response of *Candida albicans* to nitric oxide and the role of the YHB1 gene in nitrosative stress and virulence. *Mol Biol Cell* 16:4814-4826.

156. Ihmels J, Bergmann S, Berman J, Barkai N. 2005. Comparative gene expression analysis by differential clustering approach: application to the *Candida albicans* transcription program. *Plos Genet* 1:e39.

157. Imamura Y, Chandra J, Mukherjee P K, Lattif A A, Szczotka-Flynn B L, Pearlman E, Lass J H, O'Donnell K, Ghannoum M A. 2008. *Fusarium* and *Candida albicans* biofilms on soft contact lenses: model development, influence of lens type, and susceptibility to lens care solutions. *Antimicrob Agents Chemother* 52:171-182.

158. Irie Y, O'Toole G A, Yuk M H. 2005. *Pseudomonas aeruginosa* rhamnolipids disperse *Bordetella bronchiseptica* biofilms. *FEMS Microbiol Lett* 250:237-243.

159. Izano E A, Amarante M A, Kher W B, Kaplan J B. 2008. Differential roles of poly-N acetylglucosamine surface polysaccharide and extracellular DNA in *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. *Appl environ microbiol* 74:470-476.

160. Jarvis, F G, Johnson M J. 1949. A glyco-lipid produced by*Pseudomonas aeruginosa*. *J. Am. Chem. Soc* 71:4124-4126.

161. Jacques R J S, Okeke B C, Bento F M, Peralba M C R. 2009. Improved enrichment and isolation of polycyclic aromatic hydrocarbons (PAH)-degrading microorganisms in soil using anthracene as a model PAH. *Curr Microbiol* 58:628-634.

162. Jain N, Kohli R, Cook E, Gialanella P, Chang T, Fries B C. 2007. Biofilm formation by and antifungal susceptibility of *Candida* isolates from urine. *Appl Environ Microbiol* 73:1697-1703.

163. Javaheri M, Jenneman G E, McInnerney M J, Knapp R M. 1985. Anaerobic production of a biosurfactant by *Bacillus licheniformis* JF-2. *Appl Environ Microbiol* 50:698-700.

164. Jenny K, Kappeli O, Fiechter A. 1991. Biosurfactants from *Bacillus licheniformis*: structural analysis and characterization. *Appl Microbiol Biotechnol* 36:5-13.

165. Jin Y, Samaranayake L P, Samaranayake Y, Yip H K. 2004. Biofilm formation of *Candida albicans* is variably affected by saliva and dietary sugars. *Arch Oral Biol* 49:789-798.

166. Johnson M K, Boese-Marrazzo D. 1980. Production and properties of heat stable extracellular hemolysis from *Pseudomonas aeruginosa. Infect Immun* 29:1028-1033.

167. Jones T, Federspiel N A, Chibana H, Dungan J, Kalman S, Magee B B, Newport G, Thorstenson Y R, Agabian N, Magee P T, Davis R W, Scherer S. 2004. The diploid genome sequence of *Candida albicans. PNAS* 19:7329-7334.

168. Kanehisa M, Goto S. 2000. KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Research* 28: 27-30.

169. Kappeli O, Finnerty W R. 1980. Characteristics of hexadecane partition by the growth medium of Acinetobacter sp. *Biotechnol Bioeng* 22:495-503.

170. Kappeli O, Walther P, Mueller M, Fiechter A. 1984. Structure of cell surface of the yeast *Candida tropicalis* and its relation to hydrocarbon tranport. *Arch Microbiol* 138:279-282.

171. Kara D, Luppens S B, Ten Cate J M. 2006. Differences between single- and dual-species biofilms of *Streptococcus mutans* and *Veillonella parvula* in growth, acidogenicity and susceptibility to chlorhexidine. *Eur J Oral Sci* 114:58-63.

172. Katsikogianni M, Missirlis Y F. 2004. Concise review of mechanisms of bacterial adhesion to biomaterials and of techniques used in estimating bacteria-material interactions. *Eur Cell Mat* 8:37-57.

173. Kelly M T, MacCallum D M, Clancy S D, Odds F C, Brown A J, Butler G. 2004. The *Candida albicans CaACE2* gene affects morphogenesis, adherence and virulence. *Mol Microbiol* 53: 969-983.

174. Kim H S, Jeon J W, Kim B H, Ahn C Y, Oh H M, Yoon B D. 2006. Extracellular production of a glycolipid biosurfactant, mannosylerythritol lipid, by *Candida* sp. SY16 using fed-batch fermentation. *Appl Microbiol Biotechnol* 70:391-6

175. Kinnari T J, Esteban J, Gomez-Barrena E, Zamora N, Fernandez-Roblas R, Nieto A, Doadrio J C, Lopez-Noriega A, Ruiz-Hernandez E, Arcos D, Vallet-Regi M. 2009. Bacterial adherence to SiO2-based multifunctional bioceramics. *J Biomed Mater Res A* 89:215-223.

176. Kitamoto D, Isoka H, Nakahara T. 2002. Functions and potential applications of glycolipid biosurfactants-from energy-saving materials to gene delivery carriers. *J Biosci Bioeng* 94:187-201.

177. Kiyohara H, Nagao K, Yana K. 1982. Rapid screen for bacteria degrading water-insoluble, solid hydrocarbons on agar plates. *Appl Environ Microbiol* 43:454-457.

178. Klotz S A, Drutz D J, Zajic J E. 1985. Factors governing adherence of *Candida* species to plastic surfaces. *Infect Immun* 50:97-101.

179. Knoshaug E P, Ahlgren J A, Trempy J E. 2000. Growth-associated exopolysaccharide expression in *Lactococcus lactis* subspecies *cremoris* Ropy 352. *J Dairy Sci* 83:633-640.

180. Koch A K, Reiser J, Kappeli O, Fiechter A. 1988. Genetic construction of lactose-utilizing strains of *P. aeruginosa* and their application in biosurfactant production. *Nat Biotechnol* 6:1335-1339.

181. Kojic E M, Darouiche R O. 2004. *Candida* infections on medical devices. *Clin Microbiol Rev* 17:255-267.

182. Kruppa M, Bastiaan P, Neeraj C, Adrienne V, Ronald L, Richard A. 2004. The two-component signal transduction protein Chk1p regulates quorum sensing in *Candida albicans*. *Eukaryot Cell* 3:1062-1065.

183. Kucharikova S, Tournu H, Lagrou K, Dijck P V, Bujdakova H. 2011. Detailed comparison of *Candida albicans* and *Candida glabrata* biofilms under different conditions and their susceptibility to caspofungin and anidulafungin. *J Med Microbiol* 60:1261-1269.

184. Kuhn D M, George T, Chandra J, Mukherjee P K, Ghannoum M A. 2002. Antifungal susceptibility of *Candida biofilms*: unique efficacy of amphotericin B lipid formulations and echinocandins. *Antimicrob Agents Chemother* 46:1773-1780.

185. Kumamoto C A, Vinces M D. 2005. Contributions of hyphae and hypha-co-regulated genes to *Candida albicans* virulence. *Cell Microbiol* 7:1546-1554.

186. Kumamoto C A. 2002. *Candida* biofilms. *Curr Opin Microbiol* 5:608-611.

187. Kuyukina M S, Ivshina I B, Makarov S O, Litvinenko L V, Cunningham C J, Philp J C. 2005. Effect of biosurfactants on crude oil desorption and mobilization in a soil system. *Environ Int* 31:155-161.

188. Lachke S A, Joly S, Daniels K, Soll D R. 2002. Phenotypic switching and filamentation in *Candida glabrata*. *Microbiology* 148:2661-2674.

189. LaFleur M D, Kumamoto C A, Lewis K. 2006. *Candida albicans* biofilms produce antifungal-tolerant persister cells. *Antimicrob Agents Chemother* 50:3839-3846.

190. Lal P, Sharma D, Pruthi P, Pruthi V. 2010. Exopolysaccharide analysis of biofilm-forming *Candida albicans*. *J Appl Microbiol* 109:128-136.

191. Lamb D C, Kelly D E, Baldwin B C, Gozzo F, Boscott P, Richards W G, Kelly S. 1997. Differential inhibition of *Candida albicans* CYP51 with azole antifungal stereoisomers. *FEMS Microbiol Lett* 149:25-30.

192. Lang S, Katsiwela E, Wagner F. 1989. Antimicrobial effects of biosurfactants. *Fat Sci Technol* 91:363-366.

193. Lang S, Wagner F. 1987. Structures and properties of biosurfactants. In: Biosurfactants and Biotechnology, Kosaric N, Cairns W L, Gray N C C (eds), Surfactant Science Series, Marcel Dekker, New York,p 21-47.

194. Lang S, Wullbrandt D. 1999. Rhamnose lipids biosynthesis, microbial production and application potential. *Appl Microbiol Biotechnol* 51:22-32.

195. Laycock M V, Hildebrand P D, Thibault P, Walter J A, Wright J L C. 1991. Viscosin, a potent peptidolipid biosurfactant and phytopathogenic mediator produced by a pectolytic strain of *Pseudomonas fluorescens*. *J Agric Food Chem* 39:483-489.

196. Leidich S D, Ibrahim A S, Fu Y, Koul A, Jessup C, Vitullo J, Fonzi W, Mirbod F, Nakashima S, Nozawa, Ghannoum M A. 1998. Cloning and disruption of caPLB1, a phospholipase B gene involved in the pathogenicity of *Candida albicans*. *J Biol Chem* 273:26078-26086.

197. Li H, Xu M, Sun W, Sun G. 2011. Rhamnolipid production by *Pseudomonas aeruginosa* GIM 32 using different substrates including molasses distillery wastewater. *Appl Biochem Biotechnol* 163:600-611.

198. Li X, Yan Y, Xu J. 2003. Quantitative variation of biofilms among strains in natural populations of *Candida albicans*. *Microbiology* 149:353-362.

199. Lin S C, Minto M A, Sharma M M, Georgiou G. 1994. Structural and immunological characterization of a biosurfactant produced by *Bacillus licheniformis* JF-2. *Appl Environ Microbiol* 60:31-38.

200. Lin S. 1996. Biosurfactants: Recent advances. *J Chem Tech Biotech* 66:109-120.

201. Linhardt R J, Bakhit R, Daniels L, Mayerl F, Pickenhagen W. 1989. Microbially produced rhamnolipid as a source of rhamnose. *Biotechnol Bioeng* 33:365-368.

202. Liu G, Vellucci V F, Kyc S, Hostetter M K. 2009. Simvastatin inhibits *Candida albicans* biofilm *in vitro*. *Pediatr Res* 66:600-604

203. Liu T, Hou J, Zuo Y, Bi S, Jing J. 2011. Isolation and characterization of a biosurfactant producing bacterium from Daqing oil-contaminated sites. *Afr J Microbiol Res* 5:3509-3514.

204. Lorenz M C, Fink G R. 2001. The glyoxylate cycle is required for fungal virulence. *Nature* 412:83-86.

205. Lotfabad T B, Abassi H, Ahmadkhaniha R, Roostaazad R, Masoomi F, Zahiri H S, Ahmadian G, Vali H, Noghabi K A. 2010. Structural characterization of a rhamnolipid-type biosurfactant produced by *Pseudomonas aeruginosa* MR01: Enhancement of di-rhamnolipid proportion using gamma irradiation. *Colloids Surf B Biointerfaces* 81:397-405.

206. Luna J M, Rufino R D, Sarubbo L A, Rodrigues L R, Teixeira J A, de Campos-Takaki G M. 2011. Evaluation antimicrobial and anti-adhesive properties of the

biosurfactant Lunasan produced by *Candida sphaerica* UCP 0995. *Curr Microbiol* 62:1527-1534.

207. Luna J M, Sarubbo L, de Campos-Takaki G M. 2009. A new biosurfactant produced by *Candida glabrata* UCP 1002: Characteristics of stability and application in oil recovery. *Braz Arch Biol Techn* 52:785-793.

208. Maier R M, Soberón-Chavéz G. 2000. *Pseudomonas aeruginosa* rhamnolipids: biosynthesis and potential applications. *Appl Microbiol Biotechnol* 54:625-633.

209. Makkar R S, Cameotra S S, Banat I M. 2011. Advances in utilization of renewable substrates for biosurfactant production. *AMB Express* 1:5-24.

210. Makkar R S, Cameotra S S. 2002. An update on the use of unconventional substrates for biosurfactants production and their new applications. *Appl Microbiol Biotechnol* 58:428-434.

211. Makkar R, Cameotra S. 1997. Biosurfactant production by a thermophilic *Bacillus subtilis* strain. *J Ind Microbiol Biotechnol* 18:37-42.

212. Mansfield B E, Oltean H N, Oliver B G, Hoot S J, Leyde S E, Hedstrom L, Theodore C. 2010. Azole drugs are imported by facilitated diffusion in *Candida albicans* and other pathogenic fungi. *PLoS Pathogens* 6:e1001126.

213. Marahiel M, Denders W, Krause M, Kleinkauf H. 1977. Biological role of gramicidin S in spore functions. Studies on gramicidin-S negative mutants of *Bacillus brevis* 9999. *Eur J Biochem* 99:49-52.

214. Margaritis A, Kennedy K, Zajic J E. 1980. Applications of an air lift fermenter in the production of biosurfactants. *Dev Ind Microbiol* 21:285-294.

215. Martchenko M, Alarco A M, Harcus D, Whiteway M. 2004. Superoxide dismutases in *Candida albicans*: transcriptional regulation and functional characterization of the hyphal-induced SOD5 gene. *Mol Biol Cell* 15:456-467.

216. Martinez L R, Casadevall A. 2005. Specific antibody can prevent fungal biofilm formation and this effect correlates with protective efficacy. *Infect Immun* 10:6350-6362.

217. Martinez-Lopez R, Monteoliva L, Diez-Orejas R, Nombela C, Gil C. 2004. The GPI-anchored protein CaEcm33p is required for cell wall integrity, morphogenesis and virulence in *Candida albicans*. *Microbiology* 150:3341-3354.

218. Martins M, Henriques M, Azeredo J, Rocha S M, Coimbra M A, Oliveira R. 2010a. *Candida* species extracellular alcohols: production and effect in sessile cells. *J Basic Microbiol* 50:89-97.

219. Martins M, Uppuluri P, Thomas D P, Cleary I A, Henriques M, Lopez-Ribot J L, Oliveira R. 2010b. Presence of extracellular DNA in the *Candida albicans* biofilm matrix and its contribution to biofilms. *Mycopathologia* 169:323-331.

220. Mata-Sandoval J C, Karns J, Torrents A. 2000. Effect of rhamnolipids produced by *Pseudomonas aeruginosa* UG2 on the solubilization of pesticides. *Environ Sci Technol* 34:4923-4930.

221. Mata-Sandoval J C, Karns J, Torrents A. 2001. Influence of rhamnolipids and triton X-100 on the biodegradation of three pesticides in aqueous phase and soil slurries. *J Agric Food Chem* 49:3296-3303.

222. Matsufuji M, Nakata K, Yoshimoto A. 1997. High production of rhamnolipids by *Pseudomonas aeruginosa* growing on ethanol. *Biotechnol Lett* 19:1213-1215.

223. Matsuyama T, Sogawa M, Yano I. 1991. Direct colony thin-layer chromatography and rapid characterization of *Serratia marcescens* mutants defective in production of wetting agents. *Appl Environ Microbiol* 53:1186-1188.

224. Mauclaire L, Egli M. 2010. Effect of simulated microgravity on growth and production of exopolymeric substances of *Micrococcus luteus* space and earth isolates. *FEMS Immun Med Microbiol* 59:350-356.

225. McInerney M J, Javaheri M, Nagle D P. 1990. Properties of the biosurfactant produced by *Bacillus licheniformis* strain JF-2. *J Ind Microbiol* 5:95-102.

226. McWhirter M J, McQuillan A J, Bremer P J. 2002. Influence of ionic strength and pH on the first 60 min of *Pseudomonas aeruginosa* attachment to ZeSe and to TiO2 monitored by ATR-IR spectroscopy. *Colloids Surf B Biointerfaces* 84:17-25.

227. Medilanski E, Kaufmann K, Wick L, Wanner O, Harms H. 2002. Influence of surface topography of stainless steel on bacterial adhesion. *Biofouling* 18:193-203.

228. Melo A S A, Padovan A C B, Serafim R C, Puzer L , Carmona A K, Juliano Neto L, Brunstein A, Briones M R S. 2006. The *Candida albicans* AAA ATPase homologue of *Saccharomyces cerevisiae* Rix7p (YLL034c) is essential for proper morphology, biofilm formation and activity of secreted aspartyl proteinases. *Genet Mol Res* 5:664-687.

229. Mercadé M E, Manresa M A, Robert M, Espuny M J, Andrés C, Guinea J. 1993. Olive oil mill effluent (OOME): new substrate for biosurfactant production. *Biores Technol* 43:1-6.

230. Miller D J, Zhang Y, Rock C O, Stephen W. 2006. Structure of RhlG, an essential-ketoacyl reductase in the rhamnolipid biosynthetic pathway of *Pseudomonas aeruginosa. J Biol Chem* 281:18025-18032.

231. Millsap K W, Bos R, van der Mei H C, Busscher H J. 1999. Adhesion and surface-aggregation of *Candida albicans* from saliva on acrylic surfaces with adhering bacteria as studied in a parallel plate flow chamber. *Antonie Van Leeuwenhoek* 75:351-359.

232. Mireles J R, Toguchi A, Harshey R M. 2001. *Salmonella enterica* serovar typhimurium swarming mutants with altered biofilm-forming abilities: surfactin inhibits biofilm formation. *J Bacteriol* 183:5848-5854.

233. Mishra N N, Prasad T, Sharma N, Payasi A, Prasad R, Gupta D K, Singh R. 2007. Pathogenicity and drug resistance in *Candida albicans* and other yeast species. *Acta Microbiol Immunol Hung* 54:201-235.

234. Mishra S, Jyot J, Kuhad R C, and Lal B. 2001. Evaluation of inoculum addition to stimulate in situ bioremediation of oily-sludge-contaminated soil. *Appl Environ Microbiol* 67:1675-1681.

235. Mnif S, Chamkha M, Labat M, Sayadi S. 2011. Simultaneous hydrocarbon biodegradation and biosurfactant production by oilfield-selected bacteria. *J Appl Microbiol* 111:525-536.

236. Monteiro A S, Miranda T T, Lula I, Denadai A M L, Sinisterra R D, Santoro M M, SantosV L. 2011. Inhibition of *Candida albicans* CC biofilms formation in polystyrene plate surfaces by biosurfactant produced *by Trichosporon montevideense* CLOA72. *Colloids Surf B Biointerfaces* 84:467-476.

237. Moran A C, Martinez M A, Sineriz F. 2002. Quantification of surfactin in culture supernatant by hemolytic activity. *Biotechnol Lett* 24:177-180.

238. Moran G P, Sanglard D, Donnelly S M, Shanley D, Sullivan D J, Coleman D C. 1998. Identification and expression of multidrug transporters responsible for fluconazole resistance in *Candida dubliniensis*. *Antimicrobial Agents Chemother* 42:1819-1830.

239. Morikawa M, Daido H, Takao T, Murata S, Shimonishi Y, Imanaka T. 1993. A new lipopeptide biosurfactant produced by *Arthrobacter* sp. strain MIS38. *J Bacteriol* 175:6459-6466.

240. Morikawa M, Hirata Y, Imanaka T. 2000. A study on the structure-function relationship of lipopeptide biosurfactants. *Biochim Biophys Acta* 1488:211-218.

241. Moura J S, Silva W J, Pereira T, Del Bel Cury A A, Rodrigues Garcia R C. 2006. Influence of acrylic resin polymerization methods and saliva on the adherence of four *Candida* species. *J Prosthet Dent* 96:205-211.

242. Mukherjee P K, Chandra J, Kuhn D M, Ghannoum M A. 2003. Mechanism of fluconazole resistance in *Candida albicans* biofilms: Phase-specific role of efflux pumps and membrane sterols. *Infect Immun* 71:4333-4340.

243. Mukherjee P K, Zhou G, Munyon R, Ghannoum M A. 2005. *Candida* biofilm: a well-designed protected environment. *Med Mycol* 43:191-208.

244. Mukherjee S, Das P, Sen R. 2006. Towards commercial production of microbial surfactants. *Trends Biotechnol* 24:509-515.

245. Mulcahy H, Charron-Mazenod L, Lewenza S. 2008. Extracellular DNA chelates cations and induces antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *PLoS Pathog* 4:e1000213.

246. Mulligan C N, Gibbs B F. 1989. Correlation of nitrogen metabolism with biosurfactan production by *Pseudomonas aeruginosa. Environ Microbiol* 55:3016-3019.

247. Mulligan C N, Yong R N, Gibbs B F. 2001. Heavy metal removal from sediments by biosurfactants. *J Hazard Mater* 85:111-125.

248. Mulligan C N. 2005. Environmental applications for biosurfactants. *Environ Pollut* 133:183-198.

249. Mulligan C, Cooper D, Neufeld R. 1984. Selection of microbes producing biosurfactants in media without hydrocarbons. *J Fermentation Technol* 62:311-314.

250. Muthusamy K, Gopalakrishnan S, Ravi T K, Sivachidambaram P. 2008. Biosurfactants: Properties, commercial production and application. *Curr Sci* 94:736-747.

251. Nangle M R, Gibson T M, Cotter M A, Cameron N E. 2006. Effects of eugenol on nerve and vascular dysfunction inmstreptozotocin-diabetic rats. *Planta Med* 72:494-500.

252. Nailis H, Coenye T, Van Nieuwerburgh F, Deforce D, Nelis H J. 2006. Development and evaluation of different normalization strategies for gene expression studies in *Candida albicans* biofilms by real-time PCR. *BMC Mol Biol* 7:25-33.

253. Nava-Ortiz C A, Burillo G, Concheiro A, Bucio E, Matthijs N, Nelis H, Coenye T, Alvarez-Lorenzo C. 2010. Cyclodextrin-functionalized biomaterials loaded with miconazole prevent *Candida albicans* biofilm formation *in vitro*. *Acta Biomater* 6:1398-1404.

254. Nemet B, Zagar Z. 2000. Fibronectin concentrations in catheter sepsis. *Clin Microbiol Infect* 6:121-124.

255. Nett J E, Guite K M, Ringeisen A, Holoyda K A, Andes D R. 2008. Reduced biocide susceptibility in *Candida albicans* biofilms. *Antimicrob Agents Chemother* 52:3411-3413.

256. Nett J, Lincoln L, Marchillo K, Massey R, Holoyda K, Hoff B, Van Handel M, Andes D. 2007. Putative role of β-1, 3 glucans in *Candida albicans* biofilm resistance. *Antimicrob Agents Chemother* 51:510-520

257. Nett J E, Sanchez H, Cain M T, Andes D R. 2010. Genetic basis of *Candida biofilm* resistance due to drug-sequestering matrix glucan. *J Infect Dis* 202:171-175.

258. Nett J E, Sanchez H, Cain M T, Ross K M, David R, Andes D R. 2011. Interface of *Candida albicans* biofilm matrix-associated drug resistance and cell wall integrity regulation. *Eukaryot Cell* 10:1660-1669.

259. Nett J, Andes D. 2006. *Candida albicans* biofilm development, modeling a host–pathogen interaction. *Curr Opin Microbiol* 9:340-345.

260. Neu T R, Poralla K. 1990. Emulsifying agent from bacteria isolated during screening for cells with hydrophobic surfaces. *Appl Microbiol Biotechnol* 32:521-525.

261. Neu T R. 1996. Significance of bacterial surface-active compounds in interaction of bacteria with interfaces. *Microbiol Rev* 60:151-166.

262. Nevzatoğlu E U, Ozcan M, Kulak-Ozkan Y, Kadir T. 2007. Adherence of *Candida albicans* to denture base acrylics and silicone-based resilient liner materials with different surface finishes. *Clin Oral Investig* 11:231-236.

263. Nie M, Yin X, Ren C, Wang Y, Xu F, Shen Q. 2010. Novel rhamnolipid biosurfactants produced by a polycyclic aromatic 2 hydrocarbon-degrading bacterium *Pseudomonas aeruginosa* strain NY3. *Biotechnol Adv* 28:635-643.

264. Nikawa H, Nishimura H, Yamamoto T, Hamada T, Samaranayake L P. 1996. The role of saliva and serum in *Candida albicans* biofilm formation on denture acrylic surfaces. *Microbial Ecol Heath Dis* 9:35-48.

265. Nikawa H, Hamada T, Yamamoto T, Kumagai H. 1997. Effects of salivary or serum pellicles on the *Candida albicans* growth and biofilm formation on soft lining materials *in vitro*. *J Oral Rehabil* 24:594-604.

266. Nikawa H, Jin C, Hamada T, Makihira S, Kumagai H, Murata H. 2000. Interactions between thermal cycled resilient denture lining materials, salivary and

serum pellicles and *Candida albicans in vitro*. Part II. Effects on fungal colonization. *J Oral Rehabil* 27:124-130.

267. Nitschke M, Costa S G, Haddad R, Goncalves L A, Eberlin M N, Contiero J. 2005. Oil wastes as unconventional substrates for rhamnolipid biosurfactant production by *Pseudomonas aeruginosa* LBI. *Biotechnol Prog* 21:1562-1566.

268. Nitschke M, Costa S. 2007. Biosurfactants in food industry. *Trends Food Sci Technol* 18:252-259.

269. Nobile C J, Andes D R, Nett J E, Smith F J, Yue F, Phan Q T, Edwards J E, Filler S G, Mitchell A P. 2006. Critical role of Bcr1-dependent adhesins in *C. albicans* biofilm formation *in vitro* and *in vivo*. *PLoS Pathog* 2:e63.

270. Nobile C J, Mitchell A P. 2007. Microbial biofilms: e pluribus unum. *Curr Biol* 17:349-353.

271. Nobile C J, Nett J E, Herndday A D, Homann O R, Deneault J, Nantel A, Andes D R, Johnson A D, Mitchell A P. 2009. Biofilm matrix regulation by *Candida albicans* Zap1. *PLoS Biol* 7:e1000133.

272. Noordman W H, Ji W, Mark L, Brusseau M L, Janssen D B. 1998. Effects of rhamnolipid biosurfactants on removal of phenanthrene from soil. *Environ Sci Technol* 32:1806-1812.

273. Noordman W H, Wachter J H J, de Boer G J, Janssen D B. 2002. The enhancement by surfactants of hexadecane by *Pseudomonas aeruginosa* varies with substrate availability. *J Biotechnol* 94:195-212.

274. Norice C T, Smith F J, Solis N, Filler S G, Mitchell A P. 2007. Requirement for *Candida albicans* Sun41 in biofilm formation and virulence. *Eukaryot Cell* 6:2046-2055.

275. Ochsner U A, Reiser J. 1995. Autoinducer-mediated regulation of rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 92: 6424-6428.

276. Okajima Y, Kobayakawa S, Tsujin A, Tochikubo T. 2006. Biofilm formation by *Staphylococcus epidermidis* on intraocular lens material. *Invest Ophthalmol Vis Sci* 7:2970-75.

277. O'Sullivan J M, Cannon R D, Sullivan P A, Jenkinson H F. 1997. Identification of salivary basic proline-rich proteins as receptors for *Candida albicans* adhesion. *Microbiology* 143:341-348.

278. O'Toole G, Kaplan H B, Kolter R. 2000. Biofilm formation as microbial development. *Annu Rev Microbiol* 54:49-79.

279. Pacwa-Płociniczak M, Płaza G A, Piotrowska-Seget Z, Cameotra S S. 2011. Environmental applications of biosurfactants: recent advances. *Int J Mol Sci* 12:633-654.

280. Pagedar A, Singh J, Batish V K. 2010. Surface hydrophobicity, nutritional contents affect *Staphylococcus aureus* biofilms and temperature influences its survival in preformed biofilms. *J Basic Microbiol* 50:98-106.

281. Palanisamy S K, Ramirez M A, Lorenz M, Lee S A. 2009. *Candida albicans* PEP12 is required for biofilm integrity and *in vivo* virulence. *Eukaryot Cell* 9:266-277.

282. Pallas N R, Pethica B A. 1983. The surface tension of water. *Colloids and Surfaces* 6:221-227.

283. Palmer G E, Cashmore A, Sturtevant J. 2003. *Candida albicans* VPS11 is required for vacuole biogenesis and germ tube formation. *Eukaryot Cell* 2:411-421.

284. Palmer G E, Kelly M N, Sturtevant J E. 2005. The *Candida albicans* vacuole is required for differentiation and efficient macrophage killing. *Eukaryot Cell* 4:1677-1686.

285. Pan Y, Breidt F, Gorski L. 2010. Synergistic effect of sodium chloride, glucose, and temperature on biofilm formation by *Listeria monocytogenes* serotype 1/2a and 4b strains. *Appl Environ Microbiol* 76:1433-1441.

286. Park B J, Arthington-Skaggs B A, Hajjeh R A, Iqbal N, Ciblak M A, Lee-Yang W, Hairston M D, Phelan M, Plikaytis B D, Sofair A N, Harrison L H, Fridkin S K, Warnock D W. 2006. Evaluation of amphotericin b interpretive breakpoints for *Candida bloodstream* isolates by correlation with therapeutic outcome. *Antimicrob Agent Chemother* 50:1287-1292.

287. Park H, Myers C L, Sheppard D C, Phan Q T, Sanchez A A, Edwards J E, Filler S G. 2005. Role of the fungal Ras-protein kinase A pathway in governing epithelial cell interactions during oropharyngeal candidiasis. *Cell Microbiol* 7:499-510.

288. Park S J, Lee S Y. 2003. Identification and characterization of a new enoyl coenzyme a hydratase involved in biosynthesis of medium-chain-length polyhydroxyalkanoates in recombinant *Escherichia coli*. *J Bacteriol* 185:5391-5397.

289. Parkinson M. 1985. Biosurfactants. *Adv Biotechnol* 3:65-83.

290. Parreira A G, Tótola M R, Jham G N, Da Silva S L, Borges A C. 2011. Microbial biodegradation of aromatic compounds in a soil contaminated with gasohol. *Br J Biotechnol* 1:18-28.

291. Patel R M, Desai A J. 1997. Biosurfactant production by *Pseudomonas aeruginosa* GS3 from molasses. *Lett Appl Microbiol* 25:91-94.

292. Peeters E, Nelis H J, Coenye T. 2008. Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. *J Microbiol Meth* 72:157-165.

293. Peltroche-Llacsahuanga H, Goyard S, d'Enfert C, Prill S K, Ernst J F. 2006. Protein O-mannosyltransferase isoforms regulate biofilm formation in *Candida albicans*. *Antimicrob Agents Chemother* 50:3488-3491.

294. Pereira-Cenci T, Deng D M, Kraneveld E A, Manders E M, Del Bel Cury A A, ten Cate J M. 2008. The effect of *Streptococcus mutans* and *Candida glabrata* on *Candida albicans* biofilms formed on different surfaces. *Arch Oral Biol* 53:755-764.

295. Pérez A, Ramage G, Blanes R, Murgui A, Casanova M, Martínez J P. 2006. Biofilm formation by *Candida albicans* mutants for genes coding fungal proteins exhibiting the eight-cysteine-containing CFEM domain. *FEMS Yeast Res* 6:1074-1084.

296. Perlin D S. 2009. Antifungal drug resistance: do molecular methods provide a way forward? *Curr Opin Infect Dis* 22:568-573.

297. Persson A, Oesterberg E, Dostalek M. 1988. Biosurfactant production by *Pseudomonas fluorescens* 378: Growth and product characteristics. *Appl Microbiol Biotechnol* 29:1-4.

298. Pfaller M A, Diekema D J. 2007. Epidemiology of invasive candidiasis: a persistent public health problem. *Clin Microbiol Rev* 20:133-163.

299. Picioreanu C, van Loosdrecht M C M, Heijnen J J. 2000c. Modelling and predicting biofilm structure. In: Community Structure and Co-operation in Biofilms, Allison D G, Gilbert P, Lappin-Scott H M, Wilson M (eds), Cambridge University Press, p 129-166.

300. Plaza G, Zjawiony I, Banat I. 2006. Use of different methods for detection of thermophilic biosurfactant-producing bacteria from hydrocarbon-contaminated bioremediated soils. *J Petro Science Eng* 50:71-77.

301. Pornsunthorntawee O, Maksung S, Huayyai O, Rujiravanit R, Chavadej S. 2009. Biosurfactant production by *Pseudomonas aeruginosa* SP4 using sequencing batch reactors: Effects of oil loading rate and cycle time. *Biores Technol* 100:812-818.

302. Praveesh B V, Soniyamby A R, Mariappan C, Kavithakumari P, Palaniswamy M. 2010. Production of biosurfactant using cashew nut shell liquid as the carbon source from *Pseudomonas aeruginosa* MTCC 424. *Global J Biotechnol Biochem* 5:216-219.

303. Prieto L M, Michelon M, Burkert J F M, Kalil S J, Burkert C A V. 2008. The production of rhamnolipid by a *Pseudomonas aeruginosa* strain isolated from a southern coastal zone in Brazil. *Chemosphere* 71:1781-1785.

304. Prill S K, Klinkert B, Timpel C, Gale C A, Schroppel K, Ernst J F. 2005. PMT family of *Candida albicans*: five protein mannosyltransferase isoforms affect growth, morphogenesis and antifungal resistance. *Mol Microbiol* 55:546-560.

305. Pruthi V, Cameotra S S. 1997. Rapid identification of biosurfactant-producing bacterial strains using a cell surface hydrophobicity techniques. *Biotechnology techniques* 11:671-674.

306. Pruthi V, Cameotra S S. 2003. Effect of nutrients on optimal production of biosurfactants by *Pseudomonas putida*- A Gujarat oil field isolate. *J Surfact Detergents* 6:65-68.

307. Pusateri C R, Monaco E A, Edgerton M. 2009. Sensitivity of *Candida albicans* biofilm cells grown on denture acrylic to antifungal proteins and chlorhexidine. *Arch Oral Biol* 54:588-594.

308. Rahim R, Ochsner U A, Olvera C, Graninger M, Messner P, Lam J S, Soberon-Chavez G. 2001. Cloning and functional characterization of the *Pseudomonas aeruginosa* rhlC gene that encodes rhamnosyltransferase 2, an enzyme responsible for di-rhamnolipid biosynthesis. *Mol Microbiol* 40:708-718.

309. Rahman K S, Banat I M, Thahira J, Thayumanavan T, Lakshmanaperumalsamy P. 2002a. Bioremediation of gasoline contaminated soil by a bacterial consortium amended with poultry litter, coir pith and rhamnolipid biosurfactant. *Biores Technol* 81:25-32.

310. Rahman K S, Rahman T J, McClean S, Marchant R, Banat I M. 2002b. Rhamnolipid biosurfactant production by strains of *Pseudomonas aeruginosa* using low cost raw materials. *Biotechnol Prog* 18:1277-1281.

311. Rajan A P. 2010. Isolation and characterization of oil degrading bacteria from oil contaminated soils of Vellore district, Tamil Nadu, India. *J Environ Eng Sci* 52:113-116.

312. Ramage G, Saville S P, Thomas D P, Lo´pez-Ribot J L. 2005. *Candida* biofilms: an update. *Eukaryot cell* 4:633-638.

313. Ramage G, Saville S P, Wickes B L, Lopez-Ribot J L. 2002a. Inhibition of *Candida albicans* biofilm formation by farnesol, a quorum-sensing molecule. *Appl Environ Microbiol* 68:5459-5463.

314. Ramage G, Vande Walle K, Wickes B L, Lopez-Ribot J L. 2001a. Standardized method for *in vitro* antifungal susceptibility testing of *Candida albicans* biofilms. *Antimicrob Agents Chemother* 45:2475-2479.

315. Ramage G, Vande Walle K, Wickes B L, Lopez-Ribot J L. 2001b. Biofilm formation by *Candida dubliniensis*. *J Clin Microbiol* 39:3234-3240.

316. Ramage G, VandeWalle K, Lopez-Ribot J L, Wickes B L. 2002b. The filamentation pathway controlled by the Efg1 regulator protein is required for normal biofilm formation and development in *Candida albicans*. *FEMS Microbiol Lett* 214:95-100.

317. Rapp P, Bock H, Wray V, Wagner F. 1979. Formation, isolation and characterization of trehalose dimycolates from *Rhodococcus erythropolis* grown on n-alkanes. *J Gen Microbiol* 115:491-503.

318. Rashedi H, Assadi M, Bonakdarpour B, Jamshidi E. 2005. Environmental importance of rhamnolipid production from molasses as a carbon source. *Int J Environ Sci Tech* 2:59-62.

319. Read S T, Dutta P, Bond P L, Keller J, Rabaey K. 2010. Initial development and structure of biofilms on microbial fuel cell anodes. *BMC Microbiol* 10:98-107.

320. Resch A, Rosenstein R, Nerz C, Götz F. 2005. Differential gene expression profiling of *Staphylococcus aureus* cultivated under biofilm and planktonic conditions. *Appl Environ Microbiol* 71:2663-2676.

321. Richard M L, Nobile C J, Bruno V M, Mitchell P. 2005. *Candida albicans* biofilm-defective mutants. *Eukaryot Cell* 4:1493-1502.

322. Rivardo F, Turner R J, Allegrone G, Ceri H, Martinotti M G. 2009. Anti-adhesion activity of two biosurfactants produced by *Bacillus* spp. prevents biofilm formation of human bacterial pathogens. *Applied Microbiol Biotechnol* 86:541-553.

323. Robert M, Mercade M E, Bosch M P, Parra J L, Espuny M J, Manresa M A, Guinea J. 1989. Effect of the carbon source on biosurfactant production by *Pseudomonas aeruginosa* 44T. *Biotechnol Lett* 11:871-874.

324. Rodrigues L R, Banat I M, van der Mei H C, Teixeira J A, Oliveira R. 2006a. Interference in adhesion of bacteria and yeasts isolated from explanted voice

prostheses to silicone rubber by rhamnolipid biosurfactants. *J Appl Microbiol* 100:470-480.

325. Rodrigues L R, Teixeira J A. 2010. Biomedical and therapeutic applications of biosurfactants. *Adv Exp Med Biol* 672:75-87.

326. Rodrigues L, van der Mei H, Banat I M, Teixeira J, Oliveira R. 2006b. Inhibition of microbial adhesion to silicone rubber treated with biosurfactant from *Streptococcus thermophilus* A. *FEMS Immunol Med Microbiol* 46:107-112.

327. Roe J H. I955. Analysis of total hexoses found in glycoprotein. *J Biol Chem* 212:335-338.

328. Ron E Z, Rosenberg E. 2001. Natural roles of biosurfactants. *Environ Microbiol* 3:229-236.

329. Rosenbach A, Dignard D, Pierce J V, Whiteway M, Kumamoto A C. 2010. Adaptations of *Candida albicans* for growth in the mammalian intestinal tract. *Eukaryot Cell* 9:1075-1086.

330. Rosenberg E, Ron E Z. 1999. High and low-molecular-mass microbial surfactants. *Appl Microbiol Biotechnol* 52:154-162.

331. Rosenberg E, Rubinovitz A, Gottlieb A, Rosenhak S, Ron E Z. 1988. Production of biodispersan by *Acientobacter calcoaceticus* A2. *Appl Environ Microbiol* 54:317-322.

332. Rosenberg M, Gutnick D, Rosenberg E. 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surfacehydrophobicity. *FEMS Microbiol Lett* 9:29-33.

333. Rosenberg M, Kjelleberg S. 1986. Hydrophobic interactions in bacterial adhesion. *Adv Microbial Eco* 9:353-93.

334. Rufino R D, Luna J M, Sarubbo L A, Rodrigues L R, Teixeira J A, Campos-Takaki GM. 2011. Antimicrobial and anti-adhesive potential of a biosurfactant Rufisan produced by *Candida lipolytica* UCP 0988. *Colloids Surf B Biointerfaces* 84:1-5.

335. Ruhnke M. 2006. Epidemiology of *Candida albicans* infections and role of non-*Candida albicans* yeasts. *Curr Drug Targets* 74:495-504.

336. Sahni N, Yi S, Daniels K J, Srikantha T, Pujol C, Soll D R. 2009. Genes selectively up-regulated by pheromone in white cells are involved in biofilm formation in *Candida albicans*. *PLoS Pathog* 5:e1000601.

337. Samaranayake L P, MacFarlane T W. 1981. The adhesion of the yeast *Candida albicans* to epithelial-cells of human-origin *in vitro*. *Arch Oral Biol* 26:815-820.

338. Samaranayake Y H, Wu P C, Samaranayake L P, So M. 1995. Relationship between the cell surface hydrophobicity and adherence of *Candida krusei* and *Candida albicans* to epithelial and denture acrylic surfaces. *APMIS* 103:707-713.

339. Samaranayake Y H, Ye J, Yau J Y Y, Cheung B P K, Samaranayake L P. 2005. *In vitro* method to study antifungal perfusion in *Candida* biofilms. *J Clin Microbiol* 43:818-825.

340. Samaranayake, L P. 1990. Introduction and Historical Aspects. In: Oral Candidosis, Samaranayake L P, MacFarlane T W (eds). London, Wright-Butterworth, p 1-9.

341. Sampermans S, Mortier J, Soares E V. 2005. Flocculation onset in *Saccharomyces cerevisiae*: the role of nutrients. *J Appl Microbiol* 98:525-531.

342. Sánchez M, Aranda F J, Espuny M J, Marqués A, Teruel J A, Manresa A, Ortiz A. 2007. Aggregation behaviour of a dirhamnolipid biosurfactant secreted by *Pseudomonas aeruginosa* in aqueous media. *J Colloid Interface Sci* 307:246-253.

343. Sánchez M, Aranda F J, Teruel J A, Ortiz A. 2009. Interaction of a bacterial dirhamnolipid with phosphatidylcholine membranes: abiophysical study. *Chem Phys Lipids* 161:51-55.

344. Saravanan P, Jayachandran S. 2008. Preliminary characterization of exopolysaccharides produced by a marine biofilm-forming bacterium *Pseudoalteromonas ruthenica* (SBT 033). *Lett Appl Microbiol* 46:1-6.

345. Sauer, K. 2003. The genomics and proteomics of biofilm formation. *Genome Biol* 4:219.

346. Scheuerman T R, Camper A K, Hamilton M A. 1998. Effect of substratum topography on bacterial adhesion. *J Colloid Interface Sci* 208:23-33.

347. Sen R, Sivapathasekaran C, Mukherjee S. 2010. Biosurfactant production and growth kinetics of bacteria in a designer marine medium: Improved physiochemical properties. *Biotechnol J* 5:1060-1068.

348. Seneviratne C J, Jin L, Samaranayake L P. 2008. Biofilm lifestyle of *Candida:* a mini review. *Oral Dis* 14:582-590.

349. Seneviratne C J, Silva W J, Jin L J, Samaranayake Y H, Samaranayake L P. 2009. Architectural analysis, viability assessment and growth kinetics of *Candida albicans* and *Candida glabrata* biofilms. *Arch Oral Biol* 54:1052-1060.

350. Seyfarth F, Schliemann S, Elsner P, Hipler U C. 2008. Antifungal effect of high- and low-molecular-weight chitosan hydrochloride, carboxymethyl chitosan, chitosan oligosaccharide and *N*-acetyl-d-glucosamine against *Candida albicans*, *Candida krusei* and *Candida glabrata. Int J Pharma* 353:139-148.

351. Sheehan D J, Hitchcock C A, Sibley C M. 1999. Current and emerging azole antifungal agents. *Clin Microbiol Rev* 12:40-79.

352. Sheppard D C, Yeaman M R, Welch W H, Phan Q T, Fu Y, Ibrahim A S, Filler S G, Zhang M, Waring A J, Edwards J E. 2004. Functional and structural diversity in the Als protein family of *Candida albicans*. *J Biol Chem* 279:30480-30489.

353. Silva S N R L, Farias C B B, Rufino R D, Luna J M, Sarubbo L A. 2010. Glycerol as substrate for the production of biosurfactant by *Pseudomonas aeruginosa* UCP0992 S.N.R.L. *Colloids Surface B Biointerfaces* 79:174-183.

354. Silva T M, Glee P M, Hazen K C. 1995. Influence of cell surface hydrophobicity on attachment of *Candida albicans* to extracellular matrix proteins. *J Med Vet Mycol* 33:117-122.

355. Silva W J, Seneviratne J, Parahitiyawa N, Rosa E A, Samaranayake L P, Cury A A. 2008. Improvement of XTT assays performance for studies involving *Candida albicans* biofilms. *Braz Dent J* 19:364-369.

356. Sim L, Ward O P, Li Z Y. 1997. Production and characterization of a biosurfactant isolated from *Pseudomonas aeruginosa* UW-1. *J Indust Microbiol Biotechnol* 19:232-238.

357. Singh M, Desai J D. 1989. Hydrocarbon emulsification by *Candida tropicalis* and *Debaryomyces polymorphus*. *Indian J Exp Biol* 27:224-226.

358. Singh N, Agarwal V, Chaitanya S, Panwar R and Pruthi V. 2011. Impact of infectious *Candida albicans* biofilm on biomaterials. *Ind J Biotechnol* 10:417-422.

359. Singh P, Cameotra S S. 2004. Potential applications of microbial surfactants in biomedical sciences. *Trends Biotechnol* 22:142-146.

360. Sissons C H, Anderson S A, Wong L, Coleman M J, White D C. 2007. Microbiota of plaque microcosm biofilms: effect of three time's daily sucrose pulses in different simulated oral environments. *Caries Res* 41:413-422.

361. Smith A W. 2005. Biofilms and antibiotic therapy: Is there a role for combating bacterial resistance by the use of novel drug delivery systems? *Adv Drug Deliv Rev* 57:1539-1550.

362. Soberon-Chavez G, Lepine F, Deziel E. 2005. Production of rhamnolipids by *Pseudomonas aeruginosa*. *Appl Microbiol Biotechnol* 68:718-725.

363. Sotirova A V, Spasova D I, Galabova D N, Karpenko E, Shulga A. 2010. Rhamnolipid–biosurfactant permeabilizing effects on gram-positive and gram-negative bacterial strains. *Curr Microbiol* 56:639-644.

364. Sriram M I, Kalishwaralal K, Deepak V, Gracerosepat R, Srisakthi K, Gurunathan S. 2011. Novel lipopeptide biosurfactant produced by hydrocarbon degrading and heavy metal tolerant bacterium *Escherichia fergusonii* KLU01 as a potential tool for Bioremediation. *Colloids Surf B Biointerfaces* 85:174-181.

365. Stanghellini M E, Miller R M. 1997. Biosurfactants: their identity and potential efficiency in the biological control of zoosporic plant pathogens. *Plant Dis* 81:4-12.

366. Stichternoth C, Ernst J F. 2009. Hypoxic adaptation by Efg1 regulates biofilm formation by *Candida albicans*. *Appl Environ Microbiol* 75:3663-3672.

367. Stoodley P, Sauer K, Davies D G, Costerton J W. 2002. Biofilms as complex differentiated communities. *Annu Rev Microbiol* 56:187-209.

368. Strijbis K, Carlo W T , Wouter F, Els C, Janny V, Donna M, Frank C, Ekaterina P, Bastiaan P, Ben D. 2008. Carnitine-dependent transport of acetyl coenzyme A in *Candida albicans* is essential for growth on nonfermentable carbon sources and contributes to biofilm formation. *Eukaryot Cell* 7:610-618.

369. Subha T S, Gnanamani A. 2008. Perfusion of antifungal agents through biofilms of *Candida* sp. *Curr Sci* 94:74-779.

370. Sundstrom P. 2002. Adhesion in *Candida* spp. *Cell Microbiol* 4:461-469.

371. Sutherland I W. 2001. Biofilm exopolysaccharides: a strong and sticky framework. *Microbiology* 147:3-9.

372. Suzuki T, Hayashi K, Fujikawa K, Tsukamoto K. 1965. The chemical structure of polymyxin E. The identies of polymyxin E1 with colistin A and polymyxin E2 with colistin B. *J Biol Chem* 57:226-227.

373. Syldatk C, Lang S, Wagner F. 1985. Chemical and physical characterization of four interfacial-active rhamnolipids from *Pseudomonas* sp. DSM 2874 grown on *n*-alkanes. *Z Naturforsch* 40:51-60.

374. Tahara Y, Yamada Y, Kondo K. 1976. A new lipid; the ornithine and taurine-containingerilipin. *Agric Biol Chem* 40:243-244.

375. Taylor S W, Milly P D, Jaffe P R. 1990. Biofilm growth and the related changes in the physical properties of a porous medium permeability. *Wat Resour Res* 26:2161-2169.

376. Techaoei S, Lumyong S, Prathumpai W, Santiarwarn D, Leelapornpisid P. 2011. Screening characterization and stability of biosurfactant produced by *Pseudomonas aeruginosa* SCMU106 isolated from soil in Northern Thailand. *Asian J Biolol Sci* 4:340-351.

377. *ten* Cate J M , Klis F M, Pereira-Cenci T, Crielaard W, Groot P W J. 2009. Molecular and cellular mechanisms that lead to *Candida* biofilm formation. *J Dent Res* 88:105-115.

378. Tetz G V, Artemenko N K, Tetz V V. 2009. Effect of DNase and antibiotics on biofilm characteristics. *Antimicrob Agents Chemother* 53:1204-1209.

379. Teughels W, Assche N V, Sliepen I, Quirynen M. 2006. Effect of material characteristics and/or surface topography on biofilm development. *Clin Oral Implants Res* 17:68-81.

380. Thanomsub B, Pumeechockchai W, Limtrakul A, Arunrattiyakorn P, Petchleelaha W, Nitoda T. 2006. Chemical structures and biological activities of rhamnolipids produced by *Pseudomonas aeruginosa* B189 isolated from milk factory waste. *Biores Technol* 97:2457-2461.

381. Thein Z M, Samaranayake Y H, Samaranayake L P. 2007. *In vitro* biofilm formation of *Candida albicans* and non-albicans *Candida* species under dynamic and anaerobic conditions. *Arch Oral Biol* 52:761-767.

382. Thomas D P, Bachmann S P, Lopez-Ribot J L. 2006. Proteomics for the analysis of the *Candida albicans* biofilm lifestyle. *Proteomics* 6:5795-5804.

383. Toribio J, Escalante A E, Soberón-Chávez G. 2010. Rhamnolipids: Production in bacteria other than *Pseudomonas aeruginosa. Eur J Lipid Sci Tech* 112:1082-1087.

384. Trofa D, Gacser A, Nosanchuk J D. 2008. *Candida parapsilosis*, an emerging fungal pathogen. *Clin Microbiol Rev* 21:606-625.

385. Tuleva B K, Ivanov G R, Christova N E. 2002. Biosurfactant production by a new *Pseudomonas putida* strain. *Z Naturforsch C* 57:356-360.

386. Tumbarello M, Sanguinetti M, Trecarichi E M, La Sorda M, Rossi M, de Carolis E, de Gaetano Donati K, Fadda G, Cauda R, Posteraro B. 2008. Fungaemia caused by *Candida glabrata* with reduced susceptibility to fluconazole due to altered gene expression: risk factors, antifungal treatment and outcome. *J Antimicrob Chemother* 62:1379-1385.

387. Tyagi A K, Malik A. 2010. Liquid and vapour-phase antifungal activities of selected essential oils against *Candida albicans*: Microscopic observations and

chemical characterization of *Cymbopogon citratus*. *BMC Complementary and Alternative Medicine* 10:65.

388. Uppuluri P, Chaturvedi A K, Srinivasan A, Banerjee M, Ramasubramaniam A K, Köhler J R, Kadosh D, Lopez-Ribot J L. 2010. Dispersion as an important step in the *Candida albicans* biofilm developmental cycle. *PLoS Pathog.* 6:e1000828.

389. Uppuluri P, Pierce C G, Lopez-Ribo J L. 2009. *Candida albicans* biofilm formation and its clinical consequences. *Future Microbiol* 4:1235-1237.

390. Urum K, Pekdemir T, Ross D, Grigson S. 2005. Crude oil contaminated soil washing in air sparging assisted stirred tank reactor using biosurfactants. *Chemosphere* 60:334-343.

391. Van Haesendonck I. P. H., Vanzeveren E. C. A. 2004. Rhamnolipids in bakery products. W.O. 2004/040984, International application patent (PCT).

392. Verstrepen K J, Derdelinckx G, Verachtert H, Delvaux F R. 2003. Yeast flocculation: what brewers should know? *Appl Microbiol Biotechnol* 61:197-205.

393. Vilain S, Pretorius J M, Theron J, Brozel V S. 2009. DNA as an adhesin: *Bacillus cereus* requires extracellular DNA to form biofilms. *Appl Environ Microbiol* 75:2861-2868.

394. Viudes A, Pemán J, Cantón E, Úbeda P, López-Ribot J, Gobernado M. 2002. Candidemia at a tertiary-care hospital: epidemiology, treatment, clinical outcome and risk factors for death. *Eur J Clin Microbiol Infect Dis* 21:767-774.

395. Wächtler B, Wilson D, Haedicke K, Dalle F, Hube B. 2011. From attachment to damage: defined genes of *Candida albicans* mediate adhesion, invasion and damage during interaction with oral epithelial cells. *PLoS One* 6:e17046.

396. Wang A, Raniga P P, Lane S, Lu Y, Liu H. 2009. Hyphal chain formation in *Candida albicans*: Cdc28-Hgc1 phosphorylation of Efg1 represses cell separation genes. *Mol Cell Biol* 29:4406-4416.

397. Wang D, Liu Y, Lin Z, Yang Z, C H. 2008. Isolation and identification of surfactin producing *Bacillus subtilis* strain and its effect of surfactin on crude oil. *Wei Sheng Wu Xue Bao* 48:304-311.

398. Wang Q, Fang X, Bai B, Liang X, Shuler P J, Goddard III W A, Tang Y. 2007. Engineering bacteria for production of rhamnolipid as an agent for enhanced oil recovery. *Biotechnol Bioeng* 98:842-853.

399. Wang X, Gong L, Liang S, Han X, Zhu C, Li Y. 2005. Algicidal activity of rhamnolipid biosurfactants produced by *Pseudomonas aeruginosa*. *Harmful Algae* 4:433-443.

400. Wang Y C, Lan C Y, Hsieh W P, Murillo L A, Agabian N, Chen B S. 2010. Global screening of potential *Candida albicans* biofilm-related transcription factors via network comparison. *BMC Bioinformatics* 11:53.

401. Watamoto T, Samaranayake L P, Egusa H, Yatani H, Seneviratne C J. 2011.Transcriptional regulation of drug-resistance genes in *Candida albicans* biofilms in response to antifungals. *J Med Microbiol* 60:1241-1247.

402. Wei Y H, Cheng C L, Chien C C, Wan H M. 2008. Enhanced di-rhamnolipid production with an indigenous isolate *Pseudomonas aeruginosa* J16. *Process Biochemistry* 43:769-774.

403. Wei Y H, Chou C L, Chang J S. 2005. Rhamnolipid production by indigenous *Pseudomonas aeruginosa* J4 originating from petrochemical wastewater. *Biochem Eng J* 27:146-154.

404. Whang L M, Liu P W, Ma C C, Cheng S S. 2008. Application of biosurfactants, rhamnolipid, and surfactin, for enhanced biodegradation of diesel-contaminated water and soil. *J Hazard Mater* 151:155-163.

405. Whitchurch C B, Tolker-Neilson T, Ragas P C, Mattick J S. 2002. Extracellular DNA required for bacterial biofilm formation. *Science* 295:1487.

406. White T C, Marr K A, Bowden R A. 1998. Clinical, cellular and molecular factors that contribute to antifungal drug resistance. *Clin Microbiol* 2:382-402.

407. Wilcox C M, Rodgers W, Lazenby A. 2004. Prospective comparison of brush cytology, viral culture, and histology for the diagnosis of ulcerative esophagitis in AIDS. *Clin Gastroenterol Hepatol* 2:564-567.

408. Wins, Van Rossum T, Lo R, Khaira B, Whiteside M D, Hancock R E W, Brinkman F S L. 2009. *Pseudomonas* genome database: facilitating user-friendly, comprehensive comparisons of microbial genomes. *Nucleic Acids Res* 37:483-488.

409. Worakitsiri P, Pornsunthorntaweea O, Thanpitcha T, Chavadej S, Weder C, Rujiravanit R. 2011. Synthesis of polyaniline nanofibers and nanotubes via rhamnolipid biosurfactant templating. *Synthetic Met* 161:298-306.

410. Wu J Y, Yeh K L, Lu W B, Lin C L, Chang J S. 2008. Rhamnolipid production with indigenous *Pseudomonas aeruginosa* EM1 isolated from oil-contaminated site. *Biores Technol* 99:1157-1164.

411. Xiong Y Q, Willard J, Kadurugamuwa J L, Yu J, Francis K P, Bayer A S. 2005. Real-time *in vivo* bioluminescent imaging for evaluating the efficacy of antibiotics in a rat *Staphylococcus aureus* endocarditis model. *Antimicrob Agents Chemother* 49:380-387.

412. Yakimov M M, Timmis K N, Wray V, Fredrickson H L. 1995. Characterization of a new lipopeptide surfactant produced by thermotolerant and halotolerant subsurface *Bacillus licheniformis* BAS50. *Appl Environ Microbiol* 61:1706-1713.

413. Yamane T. 1987. Enzyme technology for the lipid industry: an engineering overview. *J Am Oil Chem Soc* 64:1657-1662.

414. Yang L, Liu Y, Wu H, Hoiby N, Molin S, Song Z J. 2011. Current understanding of multi-species biofilms. *Int J Oral Sci* 3:74-81.

415. Yeh M S, Wei Y H, Chang J S. 2006. Bioreactor design for enhanced carrier-assisted surfactin production with *Bacillus subtilis*. *Process Biochem* 41:1799-1805.

416. Yergeau E, Lawrence J R, Waiser M J, Korber D R, Greer C W. 2010. Metatranscriptomic analysis of the response of river biofilms to pharmaceutical products, using anonymous DNA micro arrays. *Appl Environ Microbiol 76:5432-*5439.

417. Zakikhany K, Naglik J R, Schmidt-Westhausen A, Holland G, Schaller M, Hube B. 2007. *In vivo* transcript profiling of *Candida albicans* identifies a gene essential for interepithelial dissemination. *Cell Microbiol* 9:2938-2954.

418. Zhang G , Wu Y, Qian X, Meng Q. 2005. Biodegradation of crude oil by *Pseudomonas aeruginosa* in the presence of rhamnolipids. *J Zhejiang Univ SCIB* 6:725-730.

419. Zhang Y, Maier W J, Miller R M. 1997. Effect of rhamnolipids on the dissolution, bioavailability and biodegradation of phenanthrene. *Environ Sci Technol* 31:2211-2217.

420. Zhang Y, Miller R M. 1992. Enhanced octadecane dispersion and biodegradation by a Pseudomonas rhamnolipid surfactant (biosurfactant). *Appl Environ Microbiol* 58:3276-3282.

421. Zhang Z, Gai L, Hou Z, Yang C, Ma C, Wang Z, Sun B, He X, Tang H, Xu P. 2010. Characterization and biotechnological potential of petroleum-degrading bacteria isolated from oil-contaminated soils. *Biores Technol* 10:8452-8456.

422. Zhao X, Oh S H, Yeater K M, Hoyer L L. 2005. Analysis of the *Candida albicans* Als2p and Als4p adhesins suggests the potential for compensatory function within the Als family. *Microbiology* 151:1619-1630.

423. Zheng X, Wang Y, Wang Y. 2004. Hgc1, a novel hypha-specific G1 cyclin-related protein regulates *Candida albicans* hyphal morphogenesis. *Embo J* 23:1845-1856.

424. Zhukovskii V A. 2005. Current status and prospects for development and production of biologically active fibre materials for medical applications. *Fibre Chem* 37:352-354.

425. Zosim Z, Gutnick D L, Rosenberg E. 1982. Properties of hydrocarbon-in-water emulsions stabilized by *Acinetobacter* RAG-1 emulsan. *Biotechnol Bioeng* 24:281-292.

426. Zukerberg A, Diver A, Peeri Z, Gutnick D L, Rosenberg E. 1979. Emulsifier of *Arthrobacter* RAG-1: chemical and physical properties. *Appl Environ Microbiol* 37:414-420.