

<b>Contents</b>	<b>Page no.</b>
Title Page	i
Dedication	ii
Approval of the viva-voce board	
Certificate by the Supervisor	iii
Acknowledgements	iv
Declaration	v
List of Symbols and Abbreviations	vi-viii

## **Chapter 1: Introduction and literature survey**

Introduction	
1.1. Introduction to infertility	3
1.2. Literature survey	4
1.3. Marine biosurfactants	6
1.3.1.Glycolipids	7
1.3.2.Exopolysaccharides	7
1.3.3. Glycolipoproteptides	7
1.3.4.Lipopeptides	8
1.4. Physiochemical properties	9
1.5. Biological activities	9
1.6. Production of biosurfactants	10
1.6.1. Growth associated production	11
1.6.2. Mixed growth associated production	11
1.6.3. Non-growth associated production	12
1.6.4. Production under growth-limiting conditions	12
1.6.4.1. Carbon source	12
1.6.4.2 Nitrogen source	13
1.6.4.3. Role of precursor in biosurfactant production	13
1.7. Fermentation processes	14
1.8. Bioprocess optimization	15
1.8.1. Plackett-Burman design	16
1.8.2. Taguchi experimental design	16
1.8.3. Fractional factorial design	16
1.8.4. Single variable at a time experiments	17
1.8.5. Response surface methodology	17
1.8.6. Artificial Neural Network modeling (ANN) and Genetic algorithm (GA) optimization	19
1.9. Batch and Fed batch/semi-continuous operations	20
1.10. Downstream processing and characterization	21
1.11. Purity determination	23
1.12. Lacuna and Challenges	23

<b>Objectives</b>	25
<b>Chapter 2: Analytical method development for the assay of biosurfactants in high performance thin layer chromatography (HPTLC) and high performance liquid chromatography (HPLC)</b>	
2.1. Background	29
2.2. Materials and Methods	30
2.2.1. Microorganism	30
2.2.2. Inoculum preparation and medium	30
2.2.3. Growth conditions, biomass and surface tension measurement	30
2.2.3.1. Biomass and biosurfactant quantification	31
2.2.3.2. Glucose estimation	31
2.2.4. Isolation and extraction of biosurfactant	31
2.2.5. Analytical method	32
2.2.5.1.1. Chemical nature of the biosurfactant	32
2.2.5.1.2. Quantification of biosurfactant	32
2.2.6. High Performance Liquid Chromatography (HPLC)	32
2.2.6.1. Method development in HPLC	32
2.2.7. Method validation	34
2.2.7.1. HPTLC	34
2.2.7.2. HPLC	34
2.2.8. Surface tension measurement of HPLC purified surface-active compounds	34
2.3. Results and discussion	35
2.3.1. Growth pattern and biosurfactant production	35
2.3.2. Chemical nature of the biosurfactant and method development for analysis, purification and quantification	36
2.3.3. Quantification of biosurfactant	38
2.3.4. Method development for analysis and purification of isoforms by HPLC	40
2.3.4.1. Initial method	40
2.3.4.2. Stages I-III methods	41
2.3.4.3. Final optimized method	42
2.3.4.4. Validity of the optimized method	42
Conclusion	44
<b>Chapter 3: Formulation of production medium and its optimization</b>	
3.1. Background	47
3.2. Materials and Methods	48
3.2.1. Medium development	48

3.2.2. Effects of carbon, nitrogen sources and various marine-derived salts on biosurfactant production	48
3.2.3. Modified Marine Medium composition	49
3.2.4. Biosurfactant quantification	49
3.2.5. Kinetics study	49
3.2.6. RP-HPLC analysis	49
3.2.7. Medium optimization	50
3.2.7.1. Single-factor-at-a-time experiment	50
3.2.7.2. Experimental design	50
3.2.7.3. Response surface methodology	51
3.2.7.4. Artificial Neural Network linked with Genetic Algorithm (ANN-GA) as a modeling and optimization tool	52
3.2.7.4.1. Feed forward back propagation network	53
3.3. Results and discussion	56
3.3.1. Effect of carbon source	56
3.3.2. Effect of nitrogen source	56
3.3.3. Effect of various marine-derived salts on biosurfactant production	57
3.3.4. Biosurfactant production in MMM	59
3.3.5. RP-HPLC	61
3.3.6. Optimization of the critical medium components	61
3.3.6.1. Single-factor-at-a-time optimization strategy	61
3.3.7. Response surface methodology	63
3.3.7.1. Validation studies	67
3.3.8. Optimization of critical medium components using ANN-GA	67
3.3.8.1. Model Validation	70
3.3.8.2. Validation studies	70
3.3.9. Comparison between RSM and ANN-GA optimization	72
Conclusion	72

#### **Chapter 4: Development of a bioprocess in bench top fermenter using batch and semi-continues operation**

4.1. Background	75
4.2. Materials and Methods	76
4.2.1. Medium and inoculum preparation	76
4.2.2. Biosurfactant production in bench-top fermenter	76
4.2.3. Intermittent feeding for unsteady state fed-batch (Semi-continuous) operation	77
4.2.4. Determination of kinetics parameters	79

4.2.5. Mixing efficiency of the bioreactor: Theory	79
4.2.5.1. Experimental procedure	80
4.2.6 Determination of oxygen transfer rate	82
4.2.6.1. Experimental procedure	83
4.2.7. Biomass, biosurfactant and glucose estimation	83
4.3. Results and discussions	83
4.3.1. Batch fermentation	83
4.3.1.1. Validation of growth associated production by Luedeking- Piret (L-P) model	84
4.3.1.2. Mixing efficiency of the fermenter	85
4.3.1.3. Determination of KLa	86
4.3.2. Unsteady state fed-batch (USFB) operation	87
4.3.2.1. Unsteady state fed batch (USFB)-I	88
4.3.2.2. Unsteady state fed batch (USFB) – II	89
Conclusion	92

**Chapter 5: Experimental process optimization in batch fermenter for the enhanced production of the biosurfactants**

5.1. Background	95
5.2. Materials and Methods	95
5.2.1. Biosurfactant production and quantification	95
5.2.2. Process Optimization	96
5.2.2.1 Single-factor-at-a-time experiments and experimental design	96
5.2.3. Artificial Neural Network linked with Genetic Algorithm (ANN-GA) as a modeling and optimization tool	98
5.2.3.1. Feed forward back propagation network	98
5.3. Results and discussion	100
5.3.1. Optimization of process parameters using ANN-GA	100
5.3.1.1. ANN model –GA optimization validation and confirmative studies	103
5.3.2. Percentage enhancement in biosurfactant production	104
Conclusion	105

**Chapter 6: Purification of biosurfactant products by chromatographic and ultrafiltration methods and their characterization**

6.1. Background	109
6.2. Materials and methods	110
6.2.1. Thin layer chromatography (TLC) purification	110
6.2.2. High Performance Liquid Chromatography (HPLC) purification	110

6.2.3. Ultrafiltration	111
6.2.4. Purity determination	112
6.2.4.1. Critical micelle concentration (CMC)	112
6.2.5. Characterization of biosurfactants	112
6.2.5.1. Fourier Transform-Infrared Spectrophotometry (FTIR)	112
6.2.5.2. MALDI-ToF mass spectral analysis	113
6.3. Results and discussion	113
6.3.1. TLC purification	113
6.3.2. HPLC purification	114
6.3.3. Recovery of biosurfactant using ultrafiltration	115
6.3.3.1. Effect of permeate flux	115
6.3.3.2. Recovery of biosurfactant	117
6.3.4. Characterization of the lipopeptides	119
6.3.4.1. FTIR analysis	119
6.3.4.1.1. TLC purified product	119
6.3.4.1.2. HPLC purified products (A–D)	120
6.3.4.1.3. HPLC purified products (E–F)	121
6.3.4.1.4. Ultrafiltered products	122
6.3.4.2. MALDI-ToF analysis	124
6.3.4.2.1. TLC purified product	124
6.3.4.2.2. HPLC purified isoforms (A–D)	126
6.3.4.2.2.1. HPLC purified isoforms (E–F)	128
6.3.4.2.3. Ultrafiltered products	131
Conclusion	134

## **Chapter 7: Physiochemical properties and biological activities of the lipopeptide biosurfactant**

7.1. Background	137
7.2. Material and Methods	138
7.2.1. Physiochemical properties	138
7.2.1.1. Biosurfactant stability	138
7.2.1.2. Emulsification study	138
7.2.2. Biological activities	138
7.2.2.1. Antimicrobial activities	138
7.2.2.2. Antiproliferative activity	139
7.3. Results and Discussion	140
7.3.1. Stability analysis	140
7.3.1.1. Effects of NaCl	140
7.3.1.2. Effects of pH	141
7.3.1.3. Emulsification activity	141

7.3.2. Antimicrobial activity of the HPLC purified isoforms	143
7.3.2.1. Fengycin lipopeptides	143
7.3.2.2. Surfactin lipopeptides	145
7.3.3. Antiproliferative activity of TLC purified lipopeptide	147
Conclusion	148
<b>Summary and Conclusion</b>	149
<b>References</b>	155
<b>Appendix</b>	171
<b>Publications/contributions</b>	
<b>Curriculum vitae</b>	