



Ain Shams University  
Faculty of Pharmacy  
Microbiology & Immunology dept.

# **Improvement and Scaling Up of Rhamnolipid Production by a *Pseudomonas aeruginosa* Isolate**

**A Thesis**

Submitted in Partial Fulfillment of the Requirements for the

**PhD degree**

In

Pharmaceutical Sciences

**(Microbiology and Immunology)**

By

**Ghadir Saeed Mohammed EL-Housseiny**

Master of Pharmaceutical Sciences, Microbiology &

Immunology,

Faculty of Pharmacy, Ain Shams University, 2009

**2016**



Ain Shams University  
Faculty of Pharmacy  
Microbiology & Immunology dept.

**Improvement and Scaling Up of Rhamnolipid Production by a  
*Pseudomonas aeruginosa* Isolate**

**A Thesis**

Submitted in Partial Fulfillment of the Requirements for the

**PhD degree**

In

Pharmaceutical Sciences

**(Microbiology and Immunology)**

By

**Ghadir Saeed Mohammed EL-Housseiny**

Master of Pharmaceutical Sciences, Microbiology & Immunology,

Faculty of Pharmacy, Ain Shams University, 2009

Under Supervision of

**Prof. Dr. Nadia A. El-Haleem Hassouna**

Professor of Microbiology and Immunology,

Faculty of Pharmacy, Ain Shams University

**Prof. Dr. Mohammad Mabrouk Aboulwafa**

Professor and Head of Microbiology and Immunology Department,

Faculty of Pharmacy, Ain Shams University

**Ass. Prof. Khaled Mohamed Anwar Aboshanab**

Assistant Professor of Microbiology and Immunology,

Faculty of Pharmacy, Ain Shams University

**2016**

# Acknowledgements

*First, I would like to thank **Prof. Dr. Nadia Hassouna**, Professor of Microbiology and Immunology, Faculty of Pharmacy, Ain Shams University, for her sincere support, valuable advice and continuous guidance throughout the work.*

*I am deeply grateful to **Prof. Dr. Mohammed Mabrook Aboulwafa**, Professor and Head of Microbiology and Immunology Department, Faculty of Pharmacy, Ain Shams University for choosing this topic, scientific supervision and thorough revision of this thesis.*

*I am greatly indebted to **Assis. Prof. Khaled Anwar Aboshanab**, assistant Professor of Microbiology & Immunology, Faculty of Pharmacy, Ain Shams University for his constant effort, encouragement and follow up throughout this work.*

*A special thanks goes to **Assis. Prof. Rania Hathoot**, assistant Professor of Pharmaceutics, Faculty of Pharmacy, Ain Shams University for her help in the Design Expert and Graph Pad Prism softwares.*

*I would also like to thank all my **colleagues** and all **workers** in the Microbiology and Immunology Department, Faculty of Pharmacy, Ain Shams University for their help and support.*

*I cannot end without thanking my **family** for their endless love and encouragement which helped me to concentrate on my study.*

والحمد لله رب العالمين.....

**Ghadir Saeed El-Housseiny**

# Table of Contents

<b>TABLE OF CONTENTS</b>	<b>i</b>
<b>LIST OF ABBREVIATIONS</b>	<b>xi</b>
<b>LIST OF FIGURES</b>	<b>xiii</b>
<b>LIST OF TABLES</b>	<b>xx</b>
<b>ABSTRACT</b>	<b>1</b>
<b>INTRODUCTION</b>	<b>3</b>
<b>LITERATURE REVIEW</b>	<b>5</b>
<b>1. Biosurfactants</b>	<b>5</b>
<b>2. Chemical structure of RLs</b>	<b>6</b>
<b>3. Properties of RLs</b>	<b>8</b>
<b>4. Physiological functions of RLs for the producing organisms</b>	<b>9</b>
4.1 Uptake of hydrophobic substrates	9
4.2 Antimicrobial activity	10
4.3 Immune modulation and virulence	10
4.4 Swarming	11
4.5 Biofilm development	11
<b>5. Biosynthesis and regulation of RL production</b>	<b>12</b>
5.1 Biosynthesis of RLs	12
5.1.1 Biosynthesis of the Sugar Moiety of RLs	12
5.1.2 Biosynthesis of the Lipid Moiety of RLs	13
5.1.3 Last Enzymatic Reactions in RLs Biosynthesis	14
5.2 Regulation of RL Biosynthesis	15
5.2.1 Regulation of RL Production by Genetic Factors	15

# Table of Contents

---

5.2.2	Regulation of RL Production by Environmental Factors	18
<b>6.</b>	<b>Detection and quantification of RLs</b>	<b>18</b>
6.1	Qualitative Methods	19
6.2	Quantitative Methods	20
6.2.1	Spectrophotometric Methods	20
6.2.2	Chromatographic Methods	20
<b>7.</b>	<b>Potential Applications of RLs</b>	<b>22</b>
7.1	Cosmetic industry	22
7.2	Bioremediation	23
7.3	Food Process Engineering	24
7.4	Household and Cleaning Applications	25
7.5	Pharmaceuticals	26
7.6	Agriculture	26
7.7	Microbial enhanced oil recovery (MEOR)	27
<b>8.</b>	<b>Factors affecting RL production</b>	<b>27</b>
8.1	Nutritional factors	27
8.1.1	Carbon Source	28
8.1.2	Nitrogen, Minerals, and Iron Sources	28
8.2	Environmental factors	29
<b>9.</b>	<b>RL production by Fermentation</b>	<b>30</b>
9.1	Fermentation strategies	30
9.1.1	Batch and fed batch fermentation	31
9.1.2	Resting Cells fermentation	32
9.1.3	Semicontinuous fermentation	32
9.1.4	Continuous fermentation	33

# Table of Contents

---

9.1.5	Solid State fermentation	33
9.2	Kinetics of the RL production process	34
<b>10.</b>	<b>Recovery and purification of RLs</b>	<b>36</b>
10.1	Batch-wise separation of RLs from culture broth	37
10.1.1	Precipitation	37
10.1.2	Solvent extraction	37
10.1.3	Selective crystallization	37
10.2	Continuous separation of RLs from culture broth	38
10.2.1	Adsorption	38
10.2.2	Ion exchange	38
10.2.3	Membrane filtration	39
10.2.4	Foam fractionation	39
10.2.5	Chromatographic separation of RL mixtures	39
10.2.5.1	Preparative thin-layer chromatography	40
10.2.5.2	Normal phase column chromatography	40
10.2.5.3	Reversed phase column chromatography	40
<b>11.</b>	<b>Strategies towards commercial production of RLs</b>	<b>41</b>
11.1	Optimizing the producer microorganism	42
11.1.1	Alternative strains for RL production	42
11.1.2	Engineering next-generation RL producing strains	42
11.2	Optimizing the fermentation medium	44
11.3	Optimizing the fermentation process	45
11.3.1	Process optimization: the best combination of essential factors	45
11.3.2	Downstream processing: fast, efficient and cheap product recovery	46

# Table of Contents

---

<b>MATERIALS AND METHODS</b>	<b>47</b>
<b>1. Bacterial isolates</b>	<b>47</b>
<b>2. Chemicals</b>	<b>47</b>
<b>3. Media</b>	<b>48</b>
3.1 Readymade media	48
3.2 Mineral Salts Medium (MSM)	48
3.3 Soybean oil Mineral Salts Medium (SMSM)	49
3.4 Siegmund Wagner (SW) Agar	50
3.5 Glycerol stock medium	50
3.6 Media for SSF	50
<b>4. Reagents, buffers and solutions</b>	<b>51</b>
4.1 Orcinol reagent	51
4.2 HCl solution	51
4.3 NaHCO <sub>3</sub> solution	51
4.4 NaOH solution	51
<b>5. Collection of soil samples</b>	<b>51</b>
<b>6. Recovery, preliminary identification and maintenance of <i>Pseudomonas</i> isolates</b>	<b>52</b>
<b>7. Screening of the recovered <i>Pseudomonas</i> isolates for RL production</b>	<b>53</b>
7.1 Primary screening using SW agar	53
7.2 Secondary screening using liquid media	53
<b>8. Identification of the selected <i>Pseudomonas</i> isolate P6</b>	<b>54</b>
8.1 DNA sequencing of 16S rRNA	54
8.2 Sequence analysis	54
<b>9. Bacterial culture for RL production in shake flasks</b>	<b>54</b>

## Table of Contents

---

9.1	Determination of bacterial count	54
9.2	Seed culture preparation	55
9.3	Production conditions	55
<b>10. Studying the factors affecting RL production by <i>Pseudomonas</i> isolate P6 using SLF in shake flasks</b>		<b>56</b>
10.1	Batch culture	56
10.1.1	Time course of growth and RL production in MSM	56
10.1.2	Effect of different media components	56
10.1.2.1	Effect of replacement of glucose in MSM with other carbon sources and testing the production in MSM and SMSM at different concentrations of two selected carbon sources.	56
10.1.2.2	RL production in MSM and SMSM with dual carbon sources	56
10.1.2.3	Effect of different concentrations of NaNO <sub>3</sub> and different C/N ratios	57
10.1.3	Effect of different environmental fermentation conditions	58
10.1.3.1	Response surface methodology (RSM) for the optimization of RL production	58
10.1.3.2	Experimental verification test for RSM results	58
10.2	Fed batch culture	60
<b>11. Improvement of RL production of <i>Pseudomonas</i> isolate P6 by mutation</b>		<b>61</b>
11.1	Treatment with UV	61
11.2	Treatment with gamma radiation	61
11.3	Screening of the selected colonies for their RL production	62
<b>12. Studying the RL production using SLF in a laboratory fermentor</b>		<b>62</b>
12.1	Production by the parent <i>Pseudomonas</i> isolate P6	63



## Table of Contents

---

12.1.1	Preparation of seed culture	63
12.1.2	Fermentation processes	63
12.1.2.1	Batch fermentation	63
12.1.2.1.1	Studying the time course of RL production	64
12.1.2.1.2	Effect of inoculum size	64
12.1.2.1.3	Effect of aeration rate	64
12.1.2.1.4	Effect of pH	64
12.1.2.1.5	Effect of agitation rate	64
12.1.2.2	Fed batch fermentation	65
12.1.2.3	Semicontinuous fermentation	65
12.2	Production by the <i>Pseudomonas</i> mutant 15GR	65
12.2.1	Preparation of seed culture	65
12.2.2	Fermentation processes	65
12.2.2.1	Batch fermentation	65
12.2.2.2	Fed batch fermentation	66
12.2.2.3	Semicontinuous fermentation	66
12.3	Kinetics of the fermentation process	66
12.3.1	Determination of fermentation parameters	66
12.3.2	Fermentation kinetics models	67
<b>13.</b>	<b>Studying the RL production by <i>Pseudomonas</i> mutant 15GR using solid state fermentation (SSF)</b>	<b>69</b>
13.1	Production of RL by SSF using different solid substrates	69
13.2	Extraction and quantification of RLs	70
13.3	Studying the factors affecting RL production by <i>Pseudomonas</i> mutant 15GR using SSF	71

## Table of Contents

---

13.3.1	Studying the time course of RL production in SSF using the selected substrate and comparing it to the production in SLF	71
13.3.2	Effect of using variable concentrations of glycerol in impregnating solution	71
13.3.3	Effect of some fermentation conditions on RL production	72
13.3.3.1	Response surface methodology (RSM) for the optimization of RL production in SSF	72
13.3.3.2	Experimental verification test for RSM results	73
13.4	Studying the time course of RL production by <i>Pseudomonas</i> mutant 15GR using optimized SSF conditions	73
<b>14. Analytical methods</b>		<b>73</b>
14.1	Determination of biomass	74
14.2	Colorimetric determination of RL concentration	75
14.2.1	Sample preparation	75
14.2.2	Orcinol assay	75
14.3	Determination of residual glycerol concentration	76
14.4	Determination of residual nitrate concentration	77
<b>15. Statistical and graphical analysis</b>		<b>78</b>
<b>RESULTS</b>		<b>79</b>
<b>1. Recovery and preliminary identification of <i>Pseudomonas</i> isolates from soil samples</b>		<b>79</b>
<b>2. Screening of the recovered <i>Pseudomonas</i> isolates for RL production</b>		<b>79</b>
2.1	Primary screening using SW agar	79
2.2	Secondary screening using liquid media	81
<b>3. Identification of the selected <i>Pseudomonas</i> isolate P6</b>		<b>81</b>
<b>4. Factors affecting RL production by <i>P. aeruginosa</i> isolate P6 using SLF in shake flasks</b>		<b>81</b>

# Table of Contents

---

4.1	Batch culture	81
4.1.1	Time course of growth and RL production in MSM	81
4.1.2	Effect of different media components	82
4.1.2.1	RL production in MSM with different carbon sources and in SMSM	82
4.1.2.2	Effect of variable concentrations of the selected carbon sources	84
4.1.2.3	RL production in optimized GSM and SMSM with dual carbon sources	85
4.1.2.4	Effect of different concentrations of NaNO <sub>3</sub> and different C/N ratios	85
4.1.3	Effect of different environmental fermentation conditions	87
4.1.3.1	RSM for the optimization of RL production	87
4.1.3.2	Model diagnostics	97
4.1.3.3	Experimental verification test for RSM results	104
4.2	Fed batch culture	104
4.2.1	Feeding with C source (glycerol)	105
4.2.2	Feeding with N source (sodium nitrate)	107
4.2.3	Feeding with dual substrate (glycerol and sodium nitrate)	109
<b>5.</b>	<b>Improvement of RL production of <i>P. aeruginosa</i> isolate P6 by mutation</b>	<b>112</b>
<b>6.</b>	<b>RL production using SLF in a laboratory fermentor</b>	<b>116</b>
6.1	Production by the parent <i>P. aeruginosa</i> isolate P6	116
6.1.1	Batch fermentation	116
6.1.1.1	Time course of RL production	116
6.1.1.2	RL production using different inoculum sizes	118
6.1.1.3	RL production using different aeration rates	121

## Table of Contents

---

6.1.1.4	RL production using different pH	123
6.1.1.5	RL production using different agitation rates	126
6.1.2	Fed batch fermentation	129
6.1.3	Semicontinuous fermentation	132
6.2	Production by <i>P. aeruginosa</i> mutant 15GR	135
6.2.1	Batch fermentation	135
6.2.2	Fed batch fermentation	137
6.2.3	Semicontinuous fermentation	138
6.3	Kinetics of the fermentation process	140
6.3.1	Determination of fermentation parameters	140
6.3.2	Fermentation kinetics models	142
<b>7.</b>	<b>RL production by <i>P. aeruginosa</i> mutant 15GR using SSF</b>	<b>147</b>
7.1	Production of RL by SSF using different solid substrates	147
7.2	Studied factors affecting RL production by mutant 15GR using SSF	148
7.2.1	Time course of RL production in SSF using the selected solid substrate mixture and in SLF	148
7.2.2	Effect of variable concentrations of glycerol in impregnating solution	149
7.2.3	Effect of some fermentation conditions on RL production	150
7.2.3.1	Response surface methodology (RSM) for the optimization of RL production in SSF	150
7.2.3.2	Model diagnostics	154
7.2.3.3	Experimental verification test for RSM results	156
7.3	Time course of RL production by <i>P. aeruginosa</i> isolate 15GR using optimized SSF conditions	156

## Table of Contents

---

<b>DISCUSSION</b>	<b>157</b>
<b>SUMMARY</b>	<b>190</b>
<b>REFERENCES</b>	<b>195</b>
<b>APPENDIX</b>	<b>233</b>

## List of Abbreviations

<b>Adeq precision</b>	Adequate precision
<b>Adj R<sup>2</sup></b>	Adjusted R <sup>2</sup>
<b>ANOVA</b>	Analysis of variance
<b>BBD</b>	Box behnken design
<b>BLAST</b>	Basic Local Alignment Search Tool
<b>cfs</b>	Cell free supernatant
<b>cfu</b>	Colony forming units
<b>CMC</b>	Critical micellar concentration
<b>C/N ratio</b>	Carbon/ nitrogen ratio
<b>CTAB</b>	Cetyltrimethylammonium bromide
<b>C- source</b>	Carbon source
<b>CV</b>	Coefficient of variation
<b>3D</b>	Three dimensional
<b>DO%</b>	Dissolved oxygen %
<b>ECH/I</b>	Enoyl-CoA hydratases/isomerases
<b>EDTA</b>	Ethylene diamine tetra acetic acid
<b>FAS</b>	Fatty acid synthesis
<b>GC</b>	Gas chromatography
<b>GMSM</b>	Glycerol-mineral salts medium
<b>Gy</b>	Gray
<b>HAQ</b>	4-hydroxy-2-alkylquinolones
<b>HLB</b>	Hydrophilic lipophilic balance
<b>HPLC</b>	High performance liquid chromatography
<b>HSL</b>	Homoserine lactone
<b>IDS</b>	Initial dry solids
<b>IS</b>	Impregnating solution
<b>ISPR</b>	In situ product removal
<b>LB</b>	Luria Bertani
<b>LC</b>	Liquid chromatography

## List of Abbreviations

---

<b>MEOR</b>	Microbial enhanced oil recovery
<b>MS</b>	Mass spectrometry
<b>MSM</b>	Mineral salts medium
<b>N- source</b>	Nitrogen source
<b>OD</b>	Optical density
<b>PQS</b>	<i>Pseudomonas</i> quinolone signal
<b>Pred R<sup>2</sup></b>	Predicted R <sup>2</sup>
<b>QS</b>	Quorum sensing
<b>RI</b>	Refractive index
<b>RL</b>	Rhamnolipid
<b>rpm</b>	Revolutions per minute
<b>rRNA</b>	Ribosomal ribonucleic acid
<b>RSM</b>	Response surface methodology
<b>RTD</b>	Resistance temperature detector
<b>SMSM</b>	Soybean oil-mineral salts medium
<b>SLF</b>	Submerged liquid fermentation
<b>SSF</b>	Solid state fermentation
<b>SW agar</b>	Siegmund Wagner agar
<b>TLC</b>	Thin layer chromatography
<b>TSB</b>	Trypticase soy broth
<b>UV</b>	Ultraviolet
<b>v/v</b>	Volume per volume
<b>WAC</b>	Wood activated charcoal