



# **Production of Cholinesterase Inhibitors from Irradiated Endophytic Microbes**

Ph.D. Thesis Submitted by

**Amira Gamal Zaki Mohamed**

Ass. Lecturer, Plant Research Department,  
Nuclear Research Center, Egyptian Atomic Energy Authority  
(B.Sc. Microbiology, 2010; M.Sc. Microbiology, 2015)

**Supervised by**

**Dr. Einas Hamed El-Shatoury**

Ass. Professor of Microbiology, Department of Microbiology,  
Faculty of Science, Ain Shams University

**Dr. Ashraf Sabry Ahmed**

Ass. Professor of Microbiology, Plant Research Department,  
Nuclear Research Center, Egyptian Atomic Energy Authority

**2019**

Ain-Shams University  
Faculty of Science  
Microbiology Department



## Approval Sheet

# **Production of Cholinesterase Inhibitors from Irradiated Endophytic Microbes**

**Ph.D. Thesis**

**Submitted by / Amira Gamal Zaki Mohamed**

(B.Sc. Microbiology, 2010)

(M.Sc. Microbiology, 2015)

### Thesis Supervisors

**Dr. Einas Hamed El-Shatoury** .....

Assistant Professor of Microbiology, Faculty of Science,  
Ain-Shams University

**Dr. Ashraf Sabry Ahmed** .....

Assistant Professor of Microbiology, Nuclear Research Center,  
Atomic Energy Authority

### Examination Committee

**Prof. Dr. Ayman Farrag Ahmed** .....

Professor of Microbiology, Faculty of Science, Al-Azhar University.

**Prof. Dr. Soad Ahmed Abdallah** .....

Professor of Microbiology, Faculty of Women for Arts, Science and  
Education, Ain Shams University

**Dr. Einas Hamed El-Shatoury** .....

Assistant Professor of Microbiology, Faculty of Science,  
Ain-Shams University.

**Dr. Ashraf Sabry Ahmed** .....

Assistant Professor of Microbiology, Nuclear Research Center,  
Atomic Energy Authority.

**Examination Date: 7 / 8 / 2019**

## **ACKNOWLEDGMENT**

*All the Praise and thanks to God for guiding and assisting me to finish this work and without whose mercy and guidance this work would neither has been started nor completed.*

*I would like to express my thanks and sincere gratitude to my supervisor **Dr. Einas Hamed El-Shatoury**, Assistant Professor of Microbiology, Microbiology Department, Faculty of Science, Ain Shams University for helping during the experimental work, valuable advice, constructive criticism, patience, kind care during the progress of this work and continuous encouragement. Her effort in revising the manuscript of this thesis is greatly appreciated.*

*Special thanks to **Dr. Ashraf Sabry Ahmed**, Assistant Professor of Microbiology, Plant Research Department, Nuclear Research Center, Atomic Energy Authority for his help, offering the facilities, interest, continuous encouragement, valuable suggestions and helpful advice from the beginning to the end of this work, I am deeply indebted for his supervision.*

*I wish to express my thanks to **Dr. Ola E.A Al-Hagar** lecturer of Microbiology, Plant Research Department, Nuclear Research Center, Atomic Energy Authority for her help, interest, and support during the experimental work, I am thankful for her supervision.*

*All Thanks to **Dr. El-Sayed R, El-Sayed**, lecturer of Microbiology, Plant Research Department, Nuclear Research Center, Atomic Energy Authority of Egypt for suggesting the point of research, continuous support, encouragement, and help during the experimental work specially in the extraction and TLC analyses.*

*All thanks to **Prof. Dr. Mohamed Abd El-Montaser Abouzeid** Head of Microbiology Department, Faculty of Science, Ain Shams University for his help, support, and encouragement.*

*Special thanks to Dr. Mohamed Ayaad, lecturer in Nuclear Research Center, Atomic Energy Authority for providing the plant samples.*

*Deep thanks to Dr. Hany Aglan, lecturer in Nuclear Research Center, Atomic Energy Authority for his assistance in performing the HPLC analysis.*

*I extend my appreciation to Al-Azhar Center for fermentation Biotechnology and Applied Microbiology, Prof. Dr. Ayman A. Farrag the manager, and Dr. Mahmoud Esmail for allowing me into the bioreactor facility.*

*To the staff at Microbiology Department, Faculty of Science, Ain Shams University, Thank you—especially to Dr. Ali M. Saeed, Lecturer of Microbiology for his help in the molecular identification part.*

*Deep thanks to my colleague Dr. Gharib El-Sayyad, lecturer in the National Center for Radiation and Nuclear research for his help during the publication steps, valuable advice, and encouragement.*

*Special thanks to Dr. Ehab El-Belefy, lecturer of applied Phycology at Botany and Microbiology Department, Faculty of Science, Al-Azhar University and Dr. Baraa Elsaied, lecturer at Botany and Microbiology Department, Faculty of Science, Al-Azhar University for their effort in explaining the statistical factorial designs.*

*All thanks to my close friends and colleagues, Samah Yousef, Dr. Yasmine Hassanin, Engy Yousery, Amr Khaled, Mahmoud Omar, Mahmoud mosleh, Mohamed E. Elmehy, and Hamed Ali, for their continuous help, advice, support and encouragement.*

*Finally, it is always hard to look back and remember every one who helped me. I wish to thank everybody who participated in completion of this work*

*Amira Gamal Zaki*



*Dedication*

*I dedicate this work to,  
My beloved parents,  
My brother and my sister,  
A special dedication to,  
My kind grand mother  
Finally, to my family.*

# **Statement of Originality**

All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references. None of the work has been submitted for another degree in this or any other University.

*Amira Gamal Zaki*

# Contents

Page

<b>Introduction</b>	<b>1</b>
<b>Aim of study</b>	<b>4</b>
<b>Review of literature</b>	<b>5</b>
1. Alzheimer's Disease (AD)	5
1.1. Common symptoms of AD	5
1.2. Pathogenesis of AD	6
1.3. Epidemiology of AD	6
1.4. Current therapeutic strategies for AD	7
2. Acetylcholine and acetylCholinesterase	8
3. Cholinesterase inhibitors	9
4. Alkaloids as acetylcholinesterase inhibitors	11
5. The approved medications for AD	11
5.1. Huperzine A as useful and prospective anti-AD drug	14
5.2. Clinical trials of HupA	17
5.3. Sources of HupA	18
6. Endophytic microbes	19
6.1. Endophytic fungi with anti-AchE activity	22
6.2. Endophytic actinobacteria	23

7. Optimization strategies	24
7.1. Conventional optimization method	24
7.2. Factorial design or Design of Experiment (DoE)	25
7.3. Types of designs in DoE	24
8. Non ionizing and Ionizing radiation	28
8.1. Microbial susceptibility to radiation	28
8.2. Use of radiation in microbial strains improvement for production enhancement of industrial products	29
9. Fermentation processes and fermenters	31
9.1. Batch culture technique	31
<b>Material and Method</b>	<b>34</b>
<b>I- Materials</b>	<b>34</b>
1. Plant samples for endophytes isolation	34
2. Solutions for surface sterilization of the plant samples	34
3. Media composition used in the present investigation	35
3.1. Potato Dextrose Agar (PDA) medium	35
3.2. Potato dextrose broth	35
3.3. Modified potato dextrose broth	35
3.4. Starch Nitrate Casein agar media	36
3.5. Starch Nitrate Casein broth media	36
3.6. Malt extract (ME) agar	36
3.7. Czapek's Yeast Dox agar medium	37

3.8. Medium used during cytotoxicity evaluation	37
4. Used Buffers	39
5. Experimental chemicals and solvents	39
<b>II-METHODS</b>	<b>40</b>
1. Isolation and preserving of endophytic fungi from plant samples	40
2. Isolation and preserving of endophyte actinomycetes from plant samples	41
3. Alkaloids analysis in metabolites of isolated endophytes (fungi and actinbacteria)	41
3.1. Extraction method	41
3.2. Preparation of TLC plates	43
3.3. Development of TLC plates for qualitative alkaloids analysis	43
4. Acetylcholinesterase inhibitory assay	44
5. Screening the production of HupA between the obtained cholinesterase inhibitors	45
6. High Performance Liquid Chromatography (HPLC) analysis of HupA	46
7. Morphological identification of endophyte isolates	46
8. Molecular identification of the HupA producing fungal isolates	47
9. Statistical designs for optimization of culture conditions and media components for maximum HupA production by <i>A. brassicae</i>	49
9.1. Plackett–Burman (PB) design	50
9.2. Two level full factorial experiments	55

9.3. Response Surface Methodology (RSM)	56
10. Strain improvement through irradiation mutagenesis	58
10.1. Spore suspension preparation	58
10.2. UV irradiation	58
10.3. <sup>60</sup> Co gamma irradiation	59
10.4. Estimation of survival levels resulted from irradiation	59
10.5. Effect of radiation on HupA production using OFAT method	60
10.6. Factorial experimental designs for studying the interaction effect between irradiation factors	60
10.6.1. Two level full factorial model	60
10.6.2. Response Surface Methodology (RSM)	62
11. Production of HupA by endophytic <i>A. brassicae</i> in Fermenter (Batch fermentation processes) under optimum conditions	63
12. Cytotoxicity assay of the extracted HupA	65
12.1. Preparation of HupA working solution and propagation of the human cell lines	65
12.2. Cytotoxicity evaluation by viability assay	66
<b>EXPERIMENTAL RESULTS</b>	<b>69</b>
<b>Part 1: Screening the production of acetylcholinesterase inhibitors by endophytic microbial isolates.</b>	<b>69</b>
1. Isolation of endophytic microbes (actinobacteria and fungi)	69

2. Screening the endophytic microbial isolates (fungi and actinobacteria) for AchE inhibitory activity	<b>76</b>
3. Screening of the production of HupA by the endophytic isolate AGF041	<b>78</b>
3.1. TLC analysis	<b>78</b>
3.2. UV spectroscopic analysis of hupA	<b>81</b>
3.3. HPLC analysis of hupA	<b>81</b>
4. Identification of the HupA-producing fungal isolate	
4.1. Morphological identification of the HupA-producing isolate	<b>84</b>
4.2. Molecular identification of HupA-producing isolates	<b>84</b>
<b>part 2: Statistical optimization for maximum HupA Production under liquid fermentation conditions using design of experiment (DoE) method</b>	<b>89</b>
1. Plackett-Burman (PB) experimental design	<b>89</b>
2. Full factorial design	<b>99</b>
3. Central Composite design	<b>105</b>
<b>Part 3: Irradiation mutagenesis for enhancing HupA production and a bioreactor application</b>	<b>112</b>
1. Effect of UV light irradiation on growth and HupA production by <i>A. brassicae</i> AGF041	<b>112</b>
2. Effect of <sup>60</sup> Co gamma irradiation on growth and HupA production by <i>A. brassicae</i> AGF041	<b>113</b>
3. Studying the interacted effect of both UV and gamma irradiation on HupA production by <i>A. brassicae</i> using factorial design methodology	<b>110</b>

3.1. Full factorial design	116
3.2. Central composite design	123
<b>Part 4: large scale production of HupA using a batch culture-type fermenter</b>	<b>129</b>
<b>Part 5: Cytotoxicity evaluation of the extracted HupA</b>	<b>132</b>
<b>Discussion</b>	<b>134</b>
<b>Summary and conclusion</b>	<b>149</b>
<b>References</b>	<b>152</b>
<b>Arabic abstract</b>	

# List of Tables

No.	Title	Page
<b>Table 1</b>	The investigated variables with their codes and levels for the PB design	<b>54</b>
<b>Table 2</b>	The investigated irradiation variables with their codes and levels for the factorial design	<b>61</b>
<b>Table 3</b>	Endophytic microbes isolated from different plant species and their anticholinesterase activity.	<b>73</b>
<b>Table 4</b>	Relative frequency of each identified endophytic fungal isolate showing the dominance of genera, <i>Aspergilli</i> and <i>Alternaria</i> from all selected plant species.	<b>74</b>
<b>Table 5</b>	PB design matrix showing the observed dry weight values of <i>A. brassicae</i> ATCC 204363 exerted by the trails.	<b>93</b>
<b>Table 6</b>	PB design matrix showing the observed HupA yield. from <i>A. brassicae</i> ATCC 204363 exerted by the trails.	<b>94</b>

<b>Table 7</b>	Statistical analysis of the main effect of each tested factor on the dry weight of <i>A. brassicae</i> using PB design.	<b>89</b>
<b>Table 8</b>	Regression Statistics and model <i>P</i> value for dry weight of <i>A. brassicae</i> by PB-design.	<b>89</b>
<b>Table 9</b>	Statistical analysis of the main effect of each tested factor on HupA production by <i>A. brassicae</i> using PB design.	<b>97</b>
<b>Table 10</b>	Regression Statistics and model <i>P</i> value for HupA production by PB-design.	<b>97</b>
<b>Table 11</b>	The tested factors with their low, high level and center point for $2^3$ full factorial design.	<b>99</b>
<b>Table 12</b>	The full factorial design matrix with three replications of center point showing the dry weight values and the observed HupA concentrations by <i>A. brassicae</i> comparing with the expected data.	<b>102</b>
<b>Table 13</b>	Statistical analysis of the main effect and the interacted effect between the three selected factors on HupA production by <i>A. brassicae</i> using a $2^3$ full factorial model.	<b>103</b>

<b>Table 14</b>	Regression Statistics and model <i>P</i> -value for HupA production by the full factorial design.	<b>103</b>
<b>Table 15</b>	CCD design matrix showing the observed and predicted HupA production exerted by the model trails	<b>107</b>
<b>Table 16</b>	Statistical analysis of the main, squared and interacted effects of the three selected factors on HupA production by <i>A. brassicae</i> using the CCD.	<b>108</b>
<b>Table 17</b>	Regression Statistics and model <i>P</i> value for HupA production by the CCD.	<b>108</b>
<b>Table 18</b>	Effect of UV irradiation on dry weight and HupA production by <i>A. brassicae</i> .	<b>115</b>
<b>Table 19</b>	Effect of gamma irradiation on dry weight and HupA production by <i>A. brassicae</i> .	<b>115</b>
<b>Table 20</b>	Full factorial design matrix of irradiation factors on HupA production by <i>A. brassicae</i>	<b>120</b>
<b>Table 21</b>	Analysis of Variance for HupA yield by <i>A. brassicae</i> affected by irradiation factors using full factorial design.	<b>121</b>