



Treatment of different types of cancer cells using bacterial L-asparaginase

Thesis Submitted for
The award of the degree of doctor philosophy in microbiology

By

Noha Emad El Din Abdel Razek

(M.Sc. Microbiology, Faculty of Science, Ain Shams University, 2011)

Supervisors

Dr.Khaled Zakaria El-Baghdady

Associate Professor of Microbiology Microbiology Department, Faculty of Science, Ain Shams University

Dr. Einas Hamed El-Shatoury

Associate Professor of Microbiology Microbiology Department, Faculty of Science, Ain Shams University

Dr. Nahla Gamal El Din Mohamed

G.M of Rabies research Lab The Holding Company for Biological Products and Vaccines (VACSERA)

> Microbiology Department Faculty of Science Ain Shams University 2019

Acknowledgments

Praise to Allah the lord of the universe and creation, the merciful and kindness, and blessing upon Mohammed prophet of Allah and upon his family and companions.

First I would like to owe our profound gratitude to **Dr.Khaled**Zakaria El-Baghdady, Associate Prof of Microbiology, Microbiology

Department, Faculty of Science, Ain Shams University, who has been a source of inspiration and guiding light of me throughout my dissertation. His careful corrections during regular meetings and discussions always ensured a more thoughtful approach towards problem solving.

Deep thanks conveyed to **Dr. Einas Hamed El-Shatoury,**Associate Prof of Microbiology, Microbiology Department, Faculty of
Science, Ain Shams University, for her useful advises, for her great
help to finish this study.

My Grateful thanks to **Dr. Nahla Gamal El Din Mohamed** G.M of Applied research Lab (VACSERA) for all kinds of help and facilities she offered to accomplish this work and supports all the time.

Special thanks are also to my family, and every one gave me a hand to complete this work.

Last but not least, I would like to pay thanks to all staff members of Microbiology Department, Faculty of Science, Ain Shams University for their sincere help and support.

Thank, you

Declaration

I declare that this thesis has been composed solely by myself and it has not been submitted, in whole or in part, in any previous application for a degree. The work presented is entirely my own.

CONTENTS

Title	Page
	No.
List of Tables	I
List of Figures	IV
Abbreviations	VII
Abstract	1
Introduction	3
Aim of the work	6
Review of Literature	7
1.Bacteria in cancer therapy: a novel experimental strategy	7
1.1. Background	8
1.2. Bacterial therapy	9
2. Microbial L-Asparaginase: A potent anti-tumor enzyme	12
3. Role of L-asparagine in normal and tumor cells	15
4. Mechanism of action of L-asparaginase	17
5. Biochemical and enzyme kinetic properties	19
6. Microbial sources of L-asparaginase	20
6.1. L-asparaginase by bacteria	21
6.2. L-asparaginase from fungi	21
6.3. L-asparaginase from yeast	22
6.4. L-asparaginase from actinomycetes	22
6.5. L-asparaginase from algae	22
7. L-asparaginase types and structures	23
8. Intracellular and extracellular L-asparaginase	24
9. Comparative evaluation of <i>E. coli</i> and <i>Erwinia</i> L-asparaginase	25
10. Properties of L-asparaginase	26
11. Industrial production and Optimization process of L-asparaginase.	27

12. Recombinant L-asparaginase	30
13. Toxicity of L-asparaginase to normal cells	31
14. Application of L-asparaginase	34
14.1. Application of L-asparaginase in food industry	34
14.2. In biosensors	36
14.3. L-asparaginase as antioxidant	36
15. Introduction to apoptosis	36
15.1. Definition	36
15.2. Morphological features of apoptosis	37
Materials and Methods	41
2.1 Materials	41
2.1.1 Soil samples	41
2.1.2 Microorganisms	41
2.1.3 Bacteriological media	41
2.1.4 Preparation of reagents, buffers and solutions	46
2.1.5 Chemicals	52
2.1.6 Cell lines	53
2.2 Methods	53
2.2.1 Collection of soil samples	53
2.2.2 Isolation of L-asparaginase producing bacteria	53
2.2.3 Screening of L-asparaginase producing bacteria	54
2.2.4 Preliminary identification of L-asparaginase producing	54
bacterial isolates	
2.2.4.1 Gram staining	55
2.2.4.2 Biochemical test	55
2.2.5 Semi-quantitative determination of L-asparaginase	58
production using well diffusion method	56
2.2.6 Identification of the most potent isolates using Biolog	59
system	<i>(</i> 2
2.2.7 Quantitative assay of L-asparaginase production	62
2.2.7.1 Enzyme production	62

2.2.7.2 Determination of L-asparaginase activity	62
2.2.7.3 Standard curve of ammonium sulfate	63
2.2.8 Estimation of protein Concentration	65
2.2.8.1 Standard curve of bovine serum albumin (BSA)	65
2.2.8.2 Total protein estimation method	67
2.2.9 Determination of enzyme activity	68
2.2.10 Optimization of Enzyme production	69
2.2.10.1 Effect of temperature	69
2.2.10.2 Effect of pH	69
2.2.10.3 Effect of Incubation period	70
2.2.10.4 Effect of carbon source	70
2.2.10.5 Effect of nitrogen source	71
2.2.11 Production of L-asparaginase at optimum conditions	71
2.2.12 Partial purification of L-asparaginase enzyme	71
2.2.13Sodium dodecyl sulfate-polyacrylamide gel	73
electrophoresis (SDS PAGE)	
2.2.14 Cell culture of MCF-7, HepGII (cancerous cell lines)	73
and WISH (non cancerous cell line) cells	
2.2.15 MTT Cytotoxicity assay for L- asparaginase enzyme	74
2.2.15.1 Principle	74
2.2.15.2 Procedure	75
2.2.16 Treatment of MCF-7, HepGII (cancerous cell lines)	77
and WISH (non cancerous cell line) with purified L -	
asparaginase enzyme	
2.2.17 Gene expression using RT- PCR technique	77
2.2.18 RNA extraction	77
2.2.19 cDNA Preparation	79
2.2.20 RT-PCR assay	80
Results	82
3.1. Isolation of L-asparaginase producing bacterial isolates	82
3.2. Preliminary identification of the isolates	83

3.3 Semi-quantitative determination of L-asparaginase	85
production by plate method assay	83
3.4. Identification of most potent isolates	87
3.5. Quantitative production of L-asparaginase by	90
Pectobacterium carotovorum and Serratia marcescens	90
3.6. Optimization for L-asparaginase production by	
Pectobacterium carotovorum and Serratia marcescens	91
isolates	
3.6.1 Effect of different incubation temperatures	91
3.6.2 Effect of initial pH on L-asparaginase production	93
3.6.3 Effect of incubation periods on L-asparaginase	94
production	74
3.6.4 Effect of carbon sources on L-asparaginase production	95
3.6.5 Effect of nitrogen sources on L-asparaginase production	97
3.7 Production of L-asparaginase by <i>Pectobacterium</i>	
carotovorum and Serratia marcescens isolates under	98
optimum conditions	
3.8 Partial purification of L-asparaginase enzyme	101
3.9 <i>In vitro</i> cytotoxicity assay of extracted L-asparaginase on	104
MCF-7 (human breast cancer) cell line	
3.9.1 Cell viability determined by MTT assay	104
3.9.2 Morphological changes of <i>MCF-7</i> induced by L-	111
asparaginase.	
3.10 RT-PCR for P53, BCL2 and Bax genes for treated cancer	113
cell lines (MCF-7 and HepGII) with purified L-asparaginase.	_
3.11 RT-PCR for P53, BCL2 and Bax genes for treated	
normal human epithelial cells (WISH cell line) with purified	115
L-asparaginase.	
Discussion	116
Conclusion	128
Recommendations	129

Summary	130
References	132
ملخص عربي	

LIST OF TABLES

Table No.	Title	Page No.
(1)	Sampling sites.	41
(2)	Layout of assays in the MicroPlate.	61
(3)	Construction of standard graph for Ammonium Sulfate.	64
(4)	Construction of standard curve for protein estimation.	66
(5)	Components volume of SDS – polyacrylamide gel solutions.	73
(6)	The thermal cycler reaction conditions.	79
(7)	Volumes recommended for PCR reaction.	80
(8)	Primer sequences of human apoptosis related genes and reference endogenous control gene.	81
(9)	L-asparaginase producing bacteria from different soils.	82
(10)	Morphological characteristics of L-asparaginase producing bacterial isolates.	83
(11)	Biochemical analysis and motility test of the selected isolates.	84
(12)	Screening of L-asparaginase activity on modified M9 medium	86
(13)	Results illustration for Biolog System Identification Plate for isolate FS-4.	88
(14)	Results illustration for Biolog System Identification Plate for isolate GL-7.	89

		1
(15)	Activity and specific activity (U/ml) of L-asparaginase produced by <i>Pectobacterium carotovorum</i> and <i>Serratia marcescens</i> isolates.	90
(16)	Activity (U/ml) of L-asparaginase produced by <i>P. carotovorum</i> and <i>S. marcescens</i> using different incubation temperatures.	92
(17)	Activity (U/ml) of L-asparaginase produced by <i>P. carotovorum</i> and <i>S. marcescens</i> using different pH values.	93
(18)	Activity (U/ml) of L-asparaginase produced by <i>P. carotovorum</i> and <i>S. marcescens</i> using different incubation periods.	95
(19)	Activity (U/ml) of L-asparaginase produced by <i>P. carotovorum</i> and <i>S. marcescens</i> using different C-sources.	96
(20)	Activity (U/ml) of L-asparaginase produced by <i>P. carotovorum</i> and <i>S. marcescens</i> using different N-sources.	97
(21)	The optimum conditions for L-asparaginase production.	99
(22)	Activity and specific activity (U/ml) of L-asparaginase produced by <i>Pectobacterium carotovorum</i> and <i>Serratia marcescens</i> isolates.	99
(23)	L-asparaginase activity produced by <i>Pectobacterium</i> carotovorum and <i>Serratia marcescens</i> isolates before and after optimization.	100
(24)	Evaluation of different concentrations of L-asparaginase extracted from <i>S. marcescens</i> and <i>P. carotovorum</i> on <i>MCF-7</i> . Cell viability determined by MTT assay.	105

(25)	Evaluation of different concentrations of L-asparaginase extracted from <i>S. marcescens</i> and <i>P. carotovorum</i> on <i>HepGII</i> Cell viability determined by MTT assay.	107
(26)	Evaluation of different concentrations of L-asparaginase extracted from <i>S. marcescens</i> and <i>P. carotovorum</i> on <i>WISH</i> Cell viability determined by MTT assay.	109

LIST OF FIGURES

Figure No.	Title	Page No.
(1)	Schematic overview of role of bacteria in cancer therapy.	11
(2)	Showing biosynthetic pathway of L-asparagine	16
(3)	Antineoplastic action of l-asparaginase	18
(4)	Schematic illustration of the reaction mechanism of L-asparaginase	19
(5)	Illustration of the formation of acrylamide.	35
(6)	Hallmarks of the apoptotic and necrotic cell death process	39
(7)	MicroPlate of GEN III identification System.	59
(8)	Standard curve of ammonium sulfate measured at wavelength 450 nm.	65
(9)	Standard curve of bovine serum albumin.	67
(10)	Morphological characteristics of isolates FS-4 (A) and GL-7 (B).	85
(11)	L-asparaginase production by bacterial isolates.	87
(12)	Biolog System Identification Plate for isolate FS-4.	88
(13)	Biolog System Identification Plate for isolate GS-7.	89
(14)	L-asparaginase activity produced by <i>P. carotovorum</i> and <i>S. marcescens</i> isolates.	90

(15)	L-asparaginase production by <i>P. carotovorum</i> and <i>S. marcescens</i> isolates under different incubation temperatures.	92
(16)	L-asparaginase production by <i>P. carotovorum</i> and <i>S. marcescens</i> isolates under different pH values.	94
(17)	L-asparaginase production by <i>P. carotovorum</i> and <i>S. marcescens</i> isolates under different incubation periods.	95
(18)	L-asparaginase production by <i>P. carotovorum</i> and <i>S. marcescens</i> isolates under different C- sources.	96
(19)	L-asparaginase production by <i>P. carotovorum</i> and <i>S. marcescens</i> isolates under different N-sources.	98
(20)	L-asparaginase activity produced by <i>P. carotovorum</i> and <i>S. marcescens</i> isolates under optimum conditions.	100
(21)	L-asparaginase activity produced by <i>P. carotovorum</i> and <i>S. marcescens</i> isolates before and after optimization.	101
(22)	Sodium dodecyl sulfate polyacrylamide gel electrophoreses (SDS-PAGE) of purified L-asparaginase enzyme from <i>Pectobacterium carotovorum</i> isolate.	102
(23)	Sodium dodecyl sulfate polyacrylamide gel electrophoreses (SDS-PAGE) of purified L-asparaginase enzyme from <i>Serratia marcescens</i> isolate.	103
(24)	Cytotoxicity effects of different concentrations of L-asparaginase extracted from <i>S. marcescens</i> and <i>P. carotovorum</i> on <i>MCF-7</i> Cell lines and their IC ₅₀ values.	106

(25)	Cytotoxicity effects of different concentrations of L-asparaginase extracted from <i>S. marcescens</i> and <i>P. carotovorum</i> on <i>HepGII</i> Cell lines and their IC ₅₀ values.	108
(26)	Cytotoxicity effects of different concentrations of L-asparaginase extracted from <i>S. marcescens</i> and <i>P. carotovorum</i> on <i>WISH</i> Cell lines and their IC ₅₀ values.	110
(27)	Photomicrographs of morphological changes of <i>MCF</i> -7, <i>HepGII</i> and <i>WISH</i> cells in response to L-asparaginase extracted from <i>S. marcescens</i> and <i>P. carotovorum</i> at 40X 10 magnification microscopy.	112
(28)	Agarose gel (2%) separating RT-PCR of pro-apoptotic and anti-apoptotic genes in <i>MCF-7</i> and <i>HepGII</i> cells for 24 h (A), 48 h (B) and 72 h (C) respectively.	114
(29)	Agarose gel (2%) separating RT-PCR of pro-apoptotic and anti-apoptotic genes in <i>WISH</i> cell for 48 h.	115

LIST OF ABBREVIATIONS

Abbreviate Meaning

°C Degree Celsius µl Micro liter

ALL Acute lymphoblastic leukemia

APS Ammonium per sulfate
ATP Adenosine triphosphate
BSA Bovine serum albumin

BUG agar Biolog universal growth agar

cDNA Complementary DNA CFU Colony forming unit

cm Centimeter

DCA Dichloroacetate

DEAE cellulose Diethylaminoethyl cellulose column

DMSO dimethyl sulfoxide

DNA Deoxyribonucleic acid

EDTA Ethylene diamine tetra acetic acid

FBS Fetal bovine serum

g Gram h Hour

HAMLET Human alpha-lactalbumin made lethal

to tumor cells

HepGII human hepatocellular carcinoma

IC₅₀ Half maximal inhibitory concentration

IU/ml International unit per milliliter

KDa Kilodalton

MCF-7 Human breast adenocarcinoma cell line

mm Millimeter mM Millimolar