## Microbiological Studies on the Production of Fungal Proteases

Thesis
Submitted for the Partial Fulfillment
of The Degree of Master of Science
In Microbiology

By

Sanaa Khamis Gomaa Said

(B.Sc.Microbiology-2003)

## Supervised by

Prof. Dr.

Fawkia Mohamed EL-Beih Prof. of Microbiology Microbiology Department Faculty of Science Ain Shams University Prof. Dr.

Nadia Naim Ahmed
Prof. of Chemistry of
Natural and Microbial
Products
National Research Center

Dr.Adel Ahmed EL-Mahalawy
Assistant Prof. of Microbiology
Faculty of Science
Ain Shams University

Microbiology Department Faculty of Science Ain Shams University

2010

بسم الله الرحمن الرحيم (هالم سبحانك لا علم لنا إلا ما علمتنا انك أنت العليم الحكيم)

حدق الله العظيم

(سورة البقرة أية ٣٢)

This thesis has not been previously submitted for a degree at this or any other university and it is original work of the writer

## Acknowledgment

I wish to express my sincere gratitude to **Prof. Dr. Fawkia Mohamed EL-Beih** Professor of

Microbiology, Faculty of Science, Ain Shams University

For her kind supervision, support and careful revision of the manuscript.

I am also indebted to **Prof. Dr. Nadia Naim**Ahmed Professor of Chemistry of Natural and Microbial

Products in National Research Center for suggesting the topic of this work, continuous encouragement.

I wish to express my sincere gratitude to **Dr.Adel**A. EL-Mahalawy Assistant Prof. of Microbiology,

Faculty of Science, Ain Shams University for his kind supervision in this work.

My deepest thanks are also extended to Dr. Mona S. Shafei Assistant Prof. of Chemistry of Natural and Microbial Products, National Research Center, for her personal supervision during the work.

Gratefulness and deepest thanks are extended to **Prof. Dr. Abdel – Mohsen Saber** Professor of Chemistry of Natural and Microbial Products in National Research Center for his skillful assistance during the application experiment.

Thanks are also extended to the colleagues of the department of Nature and Microbial Products in National Research Center for kind help and facilities provided.

Last but not least, I would like to thank my mother and my father for their dedication and patience all over the time needed to prepare & present this thesis.

Gratefulness and deepest thanks are extended to my husband for his support and patience all over the time needed to prepare & present this thesis.

Gratefulness and deepest thanks are extended to my brother for his continuous encouragement.

|          | CONTENTS   | page |
|----------|--|------|
|          | List of Tables   |      |
|          | List of Figures  |      |
|          | List of abbreviations                                    |      |
|          | Abstract   |      |
| 1. INTRO | DUCTION  | 1    |
| 2. REVIE | W OF LITERATURES   |      |
| 2.1.     | History of proteases                                     | 3    |
| 2.2.     | Classification of proteases                              | 6    |
| 2.3.     | Sources of proteases                                     | 7    |
| 2.4.     | Applications of proteases                                | 10   |
| 2.5.     | Factors affecting the proteases production               | 14   |
| 2.6.     | Properties of proteases                                  | 20   |
| 2.7.     | Partial purification of proteases                        | 24   |
| 2.8.     | Immobilization of proteases                              | 25   |
|          | 3.MATERIALS AND METHODS                                  |      |
| 3.1.     | Materials  | 29   |
| 3.1.1.   | Fungi  | 29   |
| 3.1.2.   | Chemicals  | 29   |
| 3.2.     | Methods  | 30   |
| 3.2.1.   | Maintenance of fungi                                     | 30   |
| 3.2.2.   | Preparation of inoculum.                                 | 30   |
| 3.2.3.   | Detection of zeralenon toxin, aflatoxins and ochratoxins | 30   |
| 3.2.4.   | Proteases production media                               | 33   |
| 3.2.5.   | Cultivation methods                                      | 33   |
| 3.2.6.   | Quantitative estimation of the biomass                   | 33   |
| 3.2.7    | Estimation of proteases activity                         | 34   |
| 3.2.7.1. | Enzyme source  | 34   |
| 3.2.7.2. | Determination of the proteolytic activity                | 34   |
| 3.2.7.3  | Determination of extracellular protein                   | 35   |

|            | CONTENTS  | page |
|------------|---|------|
| 3.2.8.     | Partial purification of Aspergillus flavus proteases      | 35   |
| 3.2.8.1.   | Fractional precipitation by ethanol                       | 36   |
| 3.2.8.2.   | Fractional precipitation by acetone                       | 36   |
| 3.2.8.3.   | Fractional precipitation by ammonium sulphate             | 37   |
| 3.2.9.     | Immobilization of partially purified Aspergillus flavus   |      |
|            | proteases   | 38   |
| 3.2.9.1.   | Physical adsorption                                       | 38   |
| 3.2.9.2.   | Ionic binding   | 38   |
| 3.2.9.3.   | Entrapment  | 38   |
| 3.2.9.3.1. | On agar and agarose                                       | 38   |
| 3.2.10.    | Properties of the free and immobilized extracellular      |      |
|            | proteases   | 39   |
| 3.2.10.1.  | Effect of pH on proteases activity                        | 39   |
| 3.2.10.2   | Effect of pH stability on proteases activity              | 39   |
| 3.2.10.3.  | Effect of assayed temperature on proteases activity       | 40   |
| 3.2.10.4.  | Effect of thermal stability                               | 40   |
| 3.2.10.5.  | Effect of incubation period on proteases activity         | 40   |
| 3.2.10.6.  | Effect of different metal chlorides on proteases activity | 41   |
| 3.2.10.7.  | Effect of CaCl <sub>2</sub> concentration                 | 41   |
| 3.2.10.8.  | Effect of enzyme concentration                            | 41   |
| 3.2.10.9   | Effect of different substrates on proteases activity      |      |
|            | (substrate- specificity)                                  | 42   |
| 3.2.10.10. | Effect of substrate concentration                         | 42   |
| 3.2.10.11. | Operational stability of the immobilized 20 - 40% acetone |      |
|            | proteases fraction  | 42   |
| 3.2.11.    | Application of Aspergillus flavus proteases               | 42   |
| 4.EXPERI   | MENTAL RESULTS  |      |
| 4.1.       | Screening the collected fungi for proteases production    | 44   |
| 4.2        | Detection of toxins.                                      | 46   |
|            |   |      |

|           | CONTENTS   | page |
|-----------|--|------|
|           |  |      |
| 4.3.      | Physiological and biochemical factors affecting proteases                          |      |
|           | production   | 46   |
| 4.3.1.    | Effect of different media on proteases production                                  | 46   |
| 4.3.2.    | Effect of inoculums size on proteases production                                   | 49   |
| 4.3.3.    | Effect of incubation period on proteases production                                | 52   |
| 4.3.4.    | Effect of incubation temperature on proteases production                           | 52   |
| 4.3.5.    | Effect of different carbon sources on proteases production                         | 57   |
| 4.3.6.    | Effect of different concentrations of glucose on proteases                         |      |
|           | production   | 60   |
| 4.3.7.    | Effect of different nitrogen sources on proteases production                       | 60   |
| 4.3.8.    | Effect of soybean concentration on proteases production                            | 61   |
| 4.3.9.    | Effect of initial pH on proteases production                                       | 68   |
| 4.3.10.   | Effect of different buffering pH on proteases production                           | 71   |
| 4.3.11.   | Effect of different concentrations of KCl on proteases                             |      |
|           | production   | 74   |
| 4.3.12.   | Effect of different concentrations of KH <sub>2</sub> PO <sub>4</sub> on proteases |      |
|           | production   | 74   |
| 4.3.13.   | Effect of different concentrations of MgSO <sub>4</sub> .7H <sub>2</sub> O on      |      |
|           | proteases production   | 79   |
| 4.3.14.   | Effect of some additives on proteases production                                   | 82   |
| 4.3.14.1. | Effect of different metal chlorides on proteases production                        |      |
|           | by Aspergillus flavus  | 82   |
| 4.3.14.2  | Effect of different concentrations of Tweens                                       | 85   |
| 4.4.      | Partial purifiction of Aspergillus flavus proteases                                | 85   |
| 4.4.1.    | Fractional precipitation by acetone  | 85   |
| 4.4.2.    | Fractional precipitation by ethanol  | 90   |
| 4.4.3.    | Fractional precipitation by ammonium sulphate                                      | 92   |
| 4.5.      | Immobilization of partially purified Aspergillus flavus                            |      |
|           | proteases  | 95   |

|         | CONTENTS   | page |
|---------|--|------|
| 4.5.1.  | By ionic binding   | 95   |
| 4.5.2.  | By physical adsorption                                     | 95   |
| 4.5.3.  | By entrapment  | 100  |
| 4.6.    | Properties of the free and immobilized Aspergillus flavus  |      |
|         | proteases  | 100  |
| 4.6.1.  | Effect of different pHs on proteases activity              | 103  |
| 4.6.2.  | Effect of pH stability                                     | 103  |
| 4.6.3.  | Effect of assayed temperatures on proteases activity       | 108  |
| 4.6.4.  | Thermal stability  | 112  |
| 4.6.5.  | Effect of reaction time on proteases activity.             | 113  |
| 4.6.6.  | Effect of different metal chlorides on proteases activity  | 120  |
| 4.6.7   | Effect of different CaCl <sub>2</sub> concentrations       | 120  |
| 4.6.8.  | Effect of enzyme concentrations on proteases activity      | 125  |
| 4.6.9   | Effect of substrate specificity                            | 125  |
| 4.6.10. | Effect of different substrate (casein) concentrations      | 130  |
| 4.6.11. | Operational stability of the immobilized Apergillus flavus |      |
|         | proteases  | 130  |
| 4.7.    | Application of Aspergillus flavus proteases in batting     |      |
|         | process  | 139  |
| 5.      | DISCUSSION   | 143  |
| 6.      | SUMMARY  | 166  |
| 7.      | REFERENCES   | 171  |
|         | ARABIC SUMMARY   |      |

|           | List of Tables  | Page |
|-----------|---|------|
| Table (1) | Screening the collected fungi for proteases production  | 45   |
| Table (2) | Production of proteases by <i>Aspergillus flavus</i> using different media  | 47   |
| Table (3) | Effect of inoculum size on proteases activity and S.E.A by  Aspergillus flavus  | 50   |
| Table (4) | Effect of incubation period on biomass, proteases activity and S.E.A <i>by Aspergillus flavus</i>                                       | 53   |
| Table (5) | Effect of incubation temperature on proteases activity and S.E.A <i>by Aspergillus flavus</i>   | 55   |
| Table (6) | Effect of different carbon sources on proteases activity and S.E.A by <i>Aspergillus flavus</i>   | 58   |
| Table (7) | Effect of different glucose concentrations on proteases production  | 62   |
| Table (8) | Effect of different nitrogen sources on proteases activity and S.E.A by <i>Aspergillus flavus</i>                                       | 64   |
| Table (9) | Effect of different concentrations of soybean on proteases activity and S.E.A by <i>Aspergillus flavus</i>                              | 66   |
| Table(10) | Effect of different initial pH values on proteases activity and S.E.A by <i>Aspergillus flavus</i>                                      | 69   |
| Table(11) | Effect of different buffering pH medium on proteases activity and S.E.A by <i>Aspergillus flavus</i>                                    | 72   |
| Table(12) | Effect of different concentrations of KCl on proteases activity and S.E.A by <i>Aspergillus flavus</i>                                  | 75   |
| Table(13) | Effect of different concentrations of KH <sub>2</sub> PO <sub>4</sub> on proteases activity and S.E.A by <i>Aspergillus flavus</i>      | 77   |
| Table(14) | Effect of different concentrations of MgSO <sub>4</sub> .7H <sub>2</sub> O on proteases activity and S.E.A by <i>Aspergillus flavus</i> | 80   |
| Table(15) | Effect of different metal chloride on proteases activity and S.E.A by <i>Aspergillus flavus</i>   | 83   |
| Table(16) | Effect of different concentrations of Tweens on proteases activity and S.E.A by <i>Aspergillus flavus</i>                               | 86   |

| Table(17)        | Fractional precipitation of Aspergillus flavus culture filtrate                                 |     |
|------------------|---|-----|
|                  | by acetone  | 88  |
| <b>Table(18)</b> | Fractional precipitation of Aspergillus flavus culture filtrate                                 |     |
|                  | by ethanol  | 91  |
| <b>Table(19)</b> | Fractional precipitation of Aspergillus flavus culture filtrate                                 |     |
|                  | by ammonium sulphate  | 93  |
| <b>Table(20)</b> | Immobilization of Aspergillus flavus proteases by ionic   |     |
|                  | binding   | 96  |
| <b>Table(21)</b> | Immobilization of Aspergillus flavus proteases by physical                                      |     |
|                  | adsorption  | 98  |
| Table(22)        | Immobilization of Aspergillus flavus proteases by   |     |
|                  | entrapment  | 101 |
| Table(23)        | Effect of different pH values of the reaction mixture on  |     |
|                  | the free and immobilized <i>Aspergillus flavus</i> proteases activity                           | 104 |
| Table(24)        | pH stability of the free and immobilized <i>Aspergillus</i>                                     | 104 |
| 1 abic(24)       | flavus proteases  | 106 |
| <b>Table(25)</b> | Effect of the assayed temperatures on the free and  |     |
|                  | immobilized Aspergillus flavus proteases activity   | 109 |
| <b>Table(26)</b> | Thermal stability of the free and immobilized Aspergillus                                       |     |
|                  | flavus proteases  | 114 |
| <b>Table(27)</b> | Activation energy, half-life and deactivation rate constant                                     |     |
|                  | of the free partially purified and immobilized of   |     |
|                  | Aspergillus flavus proteases  | 117 |
| <b>Table(28)</b> | Effect of reaction time on the free and immobilized   |     |
|                  | Aspergillus flavus proteases activity   | 118 |
| <b>Table(29)</b> | Effect of different metal chlorides on the free and   |     |
|                  | immobilized Aspergillus flavus proteases activity   | 121 |
| Table(30)        | Effect of different CaCl <sub>2</sub> concentration on the free and                             |     |
| ,                | immobilized Aspergillus flavus proteases activity   | 123 |
| Table(31)        |   | 123 |
| Table(31)        | Effect of free and immobilized enzyme concentration on<br>Aspergillus flavus proteases activity | 126 |
|                  | risperguius jiuvus proteases activity   | 120 |

| Table(32) | Effect of substrate specificity  | 128 |
|-----------|--|-----|
| Table(33) | Effect of different substrate concentration on the free and immobilized <i>Aspergillus flavus</i> proteases activity | 132 |
| Table(34) | Determination of kinetic constants (K <sub>m</sub> and V <sub>max</sub> )  |     |
|           | using Linweaver-Burk plot  | 134 |
| Table(35) | Operational stability of immobilized Aspergillus flavus  |     |
|           | proteases  | 137 |

|           | List of Figures   | page |
|-----------|---|------|
| Fig. (1)  | Production of <i>Aspergillus flavus</i> proteases by usingdifferent media   | 48   |
| Fig. (2)  | Effect of inoculum size on proteases production by Aspergillus flavus   | 51   |
| Fig. (3)  | Effect of incubation period on proteases production by Aspergillus flavus   | 54   |
| Fig. (4)  | Effect of incubation temperature on proteases production by <i>Aspergillus flavus</i>   | 56   |
| Fig. (5)  | Effect of different carbon sources on proteases production by <i>Aspergillus flavus</i>   | 59   |
| Fig. (6)  | Effect of different glucose concentrations on proteases production by <i>Aspergillus flavus</i>                                 | 63   |
| Fig. (7)  | Effect of different nitrogen sources on proteases production by <i>Aspergillus flavus</i>                                       | 65   |
| Fig. (8)  | Effect of different concentrations of soybean on proteases activity and S.E.A by <i>Aspergillus flavus</i>                      | 67   |
| Fig. (9)  | Effect of different initial pH values on proteases production by <i>Aspergillus flavus</i>                                      | 70   |
| Fig. (10) | Effect of different buffering pH on proteases production by Aspergillus flavus  | 73   |
| Fig. (11) | Effect of different concentrations of KCl on proteases production by <i>Aspergillus flavus</i>                                  | 76   |
| Fig. (12) | Effect of different concentrations of KH <sub>2</sub> PO <sub>4</sub> on proteases production by <i>Aspergillus flavus</i>      | 78   |
| Fig. (13) | Effect of different concentrations of MgSO <sub>4.7</sub> H <sub>2</sub> O on proteases production by <i>Aspergillus flavus</i> | 81   |
| Fig. (14) | Effect of different metal chlorides on proteases activity and S.E.A by Aspergillus <i>flavus</i>                                | 84   |
| Fig. (15) | Effect of different concentrations of tweens on proteases production by <i>Aspergillus flavus</i>                               | 87   |
| Fig. (16) | Fractional precipitation of <i>Aspergillus flavus</i> culture filtrate by acetone   | 89   |
| Fig. (17) | Fractional precipitation of <i>Aspergillus flavus</i> culture filtrate by ethanol   | 92   |
| Fig. (18) | Fractional precipitation of <i>Aspergillus flavus</i> culture filtrate by ammonium sulphate                                     | 94   |
| Fig. (19) | Immobilization of <i>Aspergillus flavus</i> proteases by ionic binding  | 97   |

|           | List of Figures  | page |
|-----------|--|------|
| Fig. (20) | Immobilization of <i>Aspergillus flavus</i> proteases by physica adsorption  | 99   |
| Fig. (21) | Immobilization of <i>Aspergillus flavus</i> proteases by entrapment  | 102  |
| Fig. (22) | Effect of different pH values on the free and immobilized<br>Aspergillus flavus proteases activity                           | 105  |
| Fig. (23) | pH stability of the free and immobilized <i>Aspergillus flavus</i> proteases   | 107  |
| Fig. (24) | Effect of assayed temperature on the free and immobilized proteases activity of <i>Aspergillus flavus</i>                    | 110  |
| Fig. (25) | Log of relative activity as a function of temperature for the free and immobilized <i>Aspergillus flavus</i> proteases       | 111  |
| Fig. (26) | Thermal stability of the free and immobilize of <i>Aspergillus flavus</i> proteases activity                                 | 115  |
| Fig. (27) | Log of activity retained as a function of time for the free and immobilized <i>Aspergillus flavus</i> proteases              | 116  |
| Fig. (28) | Effect of reaction time on the free and immobilized<br>Aspergillus flavus proteases activity                                 | 119  |
| Fig. (29) | Effect of different metal chlorides on the free and immobilized <i>Aspergillus flavus proteases</i> activity                 | 122  |
| Fig. (30) | Effect of different CaCl <sub>2</sub> concentration on the free and immobilized <i>Aspergillus flavus</i> proteases activity | 124  |
| Fig. (31) | Effect of free and immobilized enzyme concentration on <i>Aspergillus flavus</i> proteases activity                          | 127  |
| Fig. (32) | Effect of substrate specificity  | 129  |
| Fig. (33) | Effect of different substrate concentrations on the free and immobilized <i>Aspergillus flavus</i> proteases activity        | 133  |
| Fig.(34a) | Lineweaver-Burk plot for the free A. flavus proteases  | 135  |
| Fig.(34b) | Lineweaver-Burk plot for the immobilized A. flavus proteases   | 136  |
| Fig. (35) | Operational stability of immobilized <i>Aspergillus flavus</i> proteases   | 138  |
| Fig. (36) | SEM for leather samples treated with local unhairing agent with "Na <sub>2</sub> S Local 3 %" and bated with lab enzymes     | 140  |
| Fig. (37) | SEM for leather samples treated with local unhairing agent with "Na <sub>2</sub> S Local 4 %" and bated with lab enzymes     | 141  |

| List of Figures  |         |  |  |  | page |  |  |  |     |
|--|---------|--|--|--|------|--|--|--|-----|
| Fig. (38) SEM for leather samples treated with local unhearing agent with "Na <sub>2</sub> S Local 5 %" and bated with lab |         |  |  |  |      |  |  |  |     |
|  | enzymes |  |  |  |      |  |  |  | 142 |