STUDIES ON PRODUCTION, ANALYSIS AND CYTOTOXICITY OF INDOLIZIDINE ALKALOID, SWAINSONINE, FROM AN INSECT PATHOGENIC FUNGUS *METARHIZIUM ANISOPLIAE*

A THESIS

Submitted for the partial fulfillment of requirements for the award of

DOCTOR OF PHILOSOPHY

Under the supervision of

Dr. Gurvinder Kaur Saini

by

Digar Singh

Roll No: 09610606

December 2013

Department of Biotechnology

Indian Institute of Technology Guwahati

Guwahati – 781039

Assam, India

Dedicated To

My Parents, Family, Loved Ones

And The Almighty

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

Department of Biotechnology

STATEMENT

I do hereby declare that the content embodied in this thesis is the result of investigations carried out by me in the Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati, India, under the supervision of Dr. Gurvinder Kaur Saini.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work of other investigators are referred.

Digar Singh
December, 2013

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

Department of Biotechnology

CERTIFICATE

It is certified that the work described in this thesis entitled **"Studies on production, analysis and cytotoxicity of indolizidine alkaloid, swainsonine, from an insect pathogenic fungus *Metarhizium anisopliae*"** by Mr. Digar Singh for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under my supervision at the Department of Biotechnology, Indian Institute of Technology Guwahati, India and this work has not been submitted elsewhere for a degree.

Dr. Gurvinder Kaur Saini
Associate Professor
(Supervisor)
December, 2013

ACKNOWLEDGEMENTS

The results embodied in this thesis are the outcome of cooperation of many helping hands and it could not have seen the light of the day without their voluntary involvement. Here I take the pleasant opportunity to express my gratitude to all of them.

First and foremost, with a deepest sense of respect, I would like to express my sincere gratitude to my supervisor, Dr. Gurvinder Kaur Saini, for her valuable suggestions, encouragement and inspiration. I am indebted to her for introducing me into the realm of this interesting work, training me with the techniques, giving me the freedom in designing and conducting the experiments. I earnestly thank her for imbibing scientific temperament and appreciable work ethics in me which enhanced my grasp of knowledge and made my learning pursuit exciting. I acknowledge her crucial role in sprucing up of my overall personality. I would also like to thank wholeheartedly my administrative supervisor, Prof. Vikash Kumar Dubey, for his kind support in conducting my Ph.D. viva-voce examination successfully.

I would like to acknowledge my sincere gratitude to all my doctoral committee members, Dr. K. Pakshirajan, Dr. V.V. Dasu and Dr. C.V. Sastri for their valuable suggestions and precious time.

My sincere thanks go to faculty members of the department, Professor Arun Goyal and Dr. Nitin Chaudhary, for their great help in materialization of some of my personal and professional endeavors. I thank Dr. Dubey, Dr. Goswami, Dr. Trivedi and Dr. Swaminathan for their instructions during course work.

I remain thankful to my seniors Dr. Uzma Mustafa and Dr. Priyanka Dhar for their guidance and training. I am immensely thankful to my research group members Manoj, Surjya, Balwant, Abhishek, Pradeep, Puneet and Dhana. Heartly thanks to Himangshu, Mohan and Brijesh Kumabhani (ECE) for their adept hands in fruition of some of my crucial experiments.

A special thanks goes to my dearest friend, Ravi Manu at IIT Guwahati, I would like to thank him for his kind support and encouragement during my entire journey of PhD life. I like to thank my batch mates Himangshu, Adi Moolam and Ayan for their academic emotional support.

I am thankful to Mr. Nurul, Mrs. Prarthana, Mrs. Anita, Mrs. Rashmi, Mr. Yadav, Mr. Dipankar, Mr. Bidyut, Mr. Pankaj, Mrs. Parnita, Mr. Chandan and Mr. Niranjan for their technical help and assistance. I sincerely thank security and cleaning staff of the Department of Biotechnology.

I am thankful to the Indian Institute of Technology Guwahati (IITG) for providing me with the state of the art infrastructure for advance level of research. I also thank Central Instruments Facility (CIF) at IITG for providing the instrumental facilities and National Centre for Cell Sciences (NCCS), Pune, for providing the cell lines for my research work. I wholeheartedly thank Dr. Richard Humber, United States Department of Agriculture–Agriculture Research facility (USDA-ARS) for gifting the Metarhizium cultures. The financial support for scholarship from the Council of Scientific and Industrial Research (CSIR), New Delhi, India is duly acknowledged.

Finally, my PhD. endeavor could not have been successful without the unfathomable love, trust, support and blessings of My Parents. I owe my achievements to my parents and elder Brothers. I convey my special thank to my dearest friend Damu, without whom, I would have not have been able to cope up the stress of my Ph.D. life.

I would also like to pay my gratitude to the omnipotent and omniscient GOD, whose wish governs the ultimate entropy of all the matter and energy of the known and unknown universe.

Digar Singh
December, 2013

CONTENTS

Synopsis v

Chapter 1. Introduction, Review of Literature and Objectives 1-39
1.1. Introduction 2
1.2. Entomopathogenic fungi 3
1.3. Bioactive secondary metabolites from *Metarhizium* 4
1.3.1. Viridoxin 7
1.3.2. Ovalicin 7
1.3.3. Destruxin 7
1.3.4. Swainsonine 8
1.4. Fermentative production of swainsonine from *Metarhizium anisopliae* 9
1.4.1. Parameters and optimization models for fermentative production 10
1.4.1.1. One factor at a time and Plackett-Burman screening and optimization 10
1.4.1.2. Statistical optimization models: Response surface methodology 13
1.4.1.3. Artificial intelligence based optimization model 14
1.4.1.4. Hybrid genetic algorithms: Artificial neural network-genetic algorithm 17
1.5. Downstream recovery of swainsonine 21
1.5.1. Partial purification and solvent extraction 21
1.5.2. Chromatographic purification 22
1.5.2.1. Ion Exchange chromatography 22
1.5.2.2. Thin layer chromatography 23
1.6. Analysis and detection of swainsonine 24
1.6.1. Enzymatic Assay 24
1.6.3. Thin layer chromatography 24
1.6.3. Gas chromatography and gas chromatography-mass spectrometry 25
1.6.4. Liquid chromatography 26
1.6.5. Liquid chromatography-mass spectrometry 27
1.6.6. Flow injection mass spectrometry 27
1.7. Therapeutic applications of swainsonine 29
1.7.1. Anticancer activities and cancer chemotherapy 30
1.8. Lacunae 33
1.9. Objectives of the study 34
Figures 35

Chapter 2. Screening of *Metarhizium* strains, media components and physiological culture conditions for fermentative swainsonine production 40-65
2.1. Introduction 41
2.2. Materials and Methods 44
2.2.1. Fungal strains, culture and storage conditions 44
2.2.2. Chemicals and Reagents 44
2.2.3. Sterilization and aseptic techniques 46
2.2.4. Dry cell weight method for growth profile studies 46
2.2.5. Mannosidase inhibition assay for the quantification of swainsonine 47
2.2.6. Screening of *Metarhizium* strains and growth profile studies 48
2.2.7. One factor at a time approach screening 48
2.2.8. Plackett Burman screening 49
2.3.Results and Discussion 50
2.3.1. Screening of *Metarhizium* strains 50
2.3.2. One factor at a time screening 50
2.3.2.1. Screening of the basal fermentative media 50
2.3.2.2. Screening of the best carbon sources 51
2.3.2.3. Screening of the best nitrogen sources 52
2.3.2.4. Screening of the best pH range 53
2.3.3. Plackett-Burman screening 53
2.4. Conclusions 55
Tables 56
Figures 59

Chapter 3. Optimization of media components, operation conditions and scale up parameters from shake flask to bench-top bioreactor levels 67-104
3.1. Introduction 67
3.2. Materials and Methods 70
3.2.1. Fungal strain and culture conditions 70
3.2.2. Chemicals and Reagents 70
3.2.3. Instrumentation for stirred tank fermentation 71
3.2.4. Parameters selected for optimization 71
3.2.5. Mannosidase inhibition assay 71
3.2.6. Total saccharide estimation 71
3.2.7. Growth rate analysis 72
3.2.8. Analysis of medium viscosity 72
3.2.9. Gassed power per unit volume 72
3.2.10. Determination of volumetric oxygen transfer coefficient 72

3.2.11. Morphological analysis 74
3.2.12. Optimization methodologies 74
3.2.12.1. Response surface methodology 74
3.2.12.2. Sequential screening cum optimization of agitation and aeration 75
3.2.12.3. The hybrid artificial neural network and genetic/evolutionary algorithm 75
3.2.13. Experimental validation of the optimized model 77
3.3. Results and Discussion 78
3.3.1. Response surface methodology based statistical optimization of the fermentative medium components 78
3.3.2. Shake flask evaluation of swainsonine production as a function of DCW, saccharide concentration and pH 80
3.3.3. The artificial neural network-real encoded genetic algorithm based optimization of medium components 81
3.3.4. Optimization of agitation and aeration rates at the bench top bioreactor level 82
3.3.5. Sequential OFAT screening cum optimization of agitation-aeration rates, analysis of scale up parameter and pellet morphologies 82
3.3.6. Optimization of agitation-aeration rates using artificial neural network-evolutionary algorithm 84
3.3.7. Validation and analysis of ANN-EA optimized model 84
3.3.8. Analysis of scale up parameters (Pg/V_L and K_La) for ANN-EA optimized fermentative model 85
3.4. Conclusions 87
Tables 88
Figures 94

Chapter 4. Purification, characterization and quantitative analysis of swainsonine 105-127
4.1. Introduction 106
4.2. Materials and Methods 109
4.2.1. Fungal strain and culture conditions 109
4.2.2. Chemicals and Reagents 109
4.2.3. Swainsonine production 109
4.2.4. Swainsonine extraction and partial purification 109
4.2.5. Qualitative mass spectrometry instrumentation and analysis of partially purified samples 110
4.2.6. Experimental and blank samples for high performance liquid chromatography 110
4.2.7. Analytical HPLC Instrumentation and operation conditions 111

4.2.8. Mass spectrometry analysis of HPLC fractions 111
4.2.9. Preparative cum quantitative liquid chromatography-mass derived instrumentation and operation conditions 112
4.2.10. Analytical LC-MS validation of MSD purified broth fractions 112
4.3.Results and Discussion 113
4.3.1. Solvent extraction, enzymatic quantification and MS analysis 113
4.3.2. Quantitative HPLC analysis and method optimization 113
4.3.3. Liquid chromatography-mass derived purification 115
4.3.4. LC-MSD ESI-MS+ quantification 116
4.3.5. Comparative evaluation of conventional HPLC and mass derived quantitative methods 116
4.3.5.1. Limit of detection 116
4.3.5.2. Linearity 117
4.3.5.3. Selectivity and precision 117
4.3.5.4. Inter-method accuracy 117
4.3.5.5. Ruggedness 117
4.4. Conclusions 118
Tables 119
Figures 120

Chapter 5. Analysis of *in situ* entomotoxic and anticancer activities of swainsonine 128-154
5.1. Introduction 129
5.2. Materials and Methods 132
5.2.1. Fungal strain and culture conditions 132
5.2.2. Chemicals and Reagents 132
5.2.3. Cell culture and treatments 132
5.2.4. MTT cell viability assay of *Sf*-21 and HL-60 cells 133
5.2.5. Tryphan blue cell death assay of *Sf*-21 and HL-60 cells 133
5.2.5. Statistical analysis 134
5.2.6. Inverted phase contrast microscopy of *Sf*-21 and HL-60 cells 134
5.2.7. Fluorescence microscopy and differential staining using Annexin V-FITC/ PI staining of *Sf*-21 and HL-60 cells 134
5.2.8. Fluorescence activated cells sorting analysis 135
5.2.8.1. Apoptosis analysis of *Sf*-21 cells 135
5.2.8.2. DNA cell cycle analysis of HL-60 cells 135
5.2.9. Ultramorphodimensional analysis of *Sf*-21 cells 136
5.2.9.1. Field emission-scanning electron microscopy 136
5.2.9.2. Atomic force microscopy 137
5.3. Results and Discussion 138
5.3.1. Cytotoxicity analysis of swainsonine for *Sf*-21 cells 138
5.3.2. Cytotoxicity analysis of swainsonine for HL-60 cells 138

5.3.3. Inverted phase contrast microscopy 139
5.3.3.1. Microscopic examination of *Sf*-21 cells 139
5.3.3.2. Microscopic examination of HL-60 cells 139
5.3.4. Fluorescence microscopy and differential staining 140
5.3.4.1. Fluorescence microscopy of *Sf*-21 cells 140
5.3.4.2. Fluorescence microscopy of HL-60 cells 140
5.3.5. Fluorescence activated cell sorting analysis 141
5.3.5.1. Analysis of apoptosis in *Sf*-21 cells 141
5.3.5.2. Analysis of DNA cell cycle in HL-60 cells 141
5.3.6. Ultramorphodimensional analysis of swainsonine induced apoptosis in *Sf*-21 cells 142
5.4. Conclusions 144
Figures 145
References 155-182

Appendix I - List of Abbreviations vi
Appendix II - Mathematical Symbols and Greek Letters vii
List of Publications viii
List of Conferences ix
Awards and Fellowships x
Vitae xi

SYNOPSIS

Introduction

Agriculture is the backbone of Indian economy which witnessed multi-fold increase in the last four decades, leading to an era of food security to the nation. This remarkable growth was achieved through the uptake of newer technologies in the form of high yielding crop varieties, chemical fertilizers and pesticides, as well as from the expansion of cultivation land for agriculture. During the Green Revolution, achieving high crop yields at any cost was the ultimate goal. The emphasis now is on sustainable agriculture-increasing yields without harming the environment. Biopesticides are one of the vital components of sustainable agriculture and it aims to avoid or reduce the use of harmful chemical pesticides toxic to humans or livestock in environment. The specificity of biopesticides towards a specific insect host is attributed to the specific metabolites or products which selectively enhance its entomotoxic behaviour and virulence.

The entomopathogenic fungus *Metarhizium anisopliae* is a well studied and field applied species for microbial control of insect pests (Schrank and Vainstein, 2010). *Metarhizium* spp. produce a variety of secondary metabolites in several chemical classes, including cytochalasins C and D, myroridins, destruxins A, B and E, viridoxin, helvonic acid, 12-hydroxyovalicin, hydroxyfungerin, 7-desmethyl analogues of fusarin C and (8Z)-fusarin C, serinocyclins A and B and aurovertins. These metabolites are toxic to a broad range of animals and microbes, including insects, fungi, bacteria and viruses (Carlos *et al.*, 2010). One of those is swainsonine, a trihydroxy indolizidine alkaloid with selective inhibition property against Golgi α-mannosidase II that blocks the abnormal formation of complex β-1, 6 branched N-linked glycans, leading to reduced metastasis and tumour growth (Thompson *et al.*,

2012). Swainsonine is described for its apoptotic anticancer properties against cancerous A549 cells, antivirus and antitumor drug application (Li *et al.*, 2012). Swainsonine has also been reported to reduce the growth rate of human melanoma, colon and gastric carcinoma cells and C6 glioma cells *in situ* and *ex situ* (Sun *et al.*, 2009). However, swainsonine production still remains a challenge from both biological as well as chemical synthetic routes, due to its high processing cost.

Microbial biosynthesis of this alkaloid through fermentative means is being investigated as an alternative to the chemical process. The RSM (response surface methodology) is the most preferred and worldwide practiced optimization method which is a collection of statistical techniques for designing experiments, building models, evaluating the effect of factors and searching for the optimum conditions (Tabandeh *et al.*, 2008). It also includes studying the effects of several factors by varying them simultaneously under limited number of experiments. However, in the last two decades artificial intelligence based computational programmes such as ANN (artificial neural networks) coupled genetic or evolutionary programme based optimization methods has evolved more efficiently for empirical modelling, especially in case of nonlinear biological systems. ANNs can learn from examples and are fault tolerant, in the sense that they are able to handle noisy, incomplete and nonlinear data, and can perform prediction and generalization at high speed (Haykin, 2008). On the other hand, GA is inspired by the theories of natural selection and evolution. Recently many studies have been performed on GA with real encoding, which outperforms conventional binary coded representation (Yoon and Kim, 2012). Optimization of swainsonine production is being studied in the context of varying physico-chemical properties of the production media (C-N source, pH, inoculum size and trace elements e.g., $CaCl_2$ conc.), operation parameters (agitation and aeration), vessel types (airlift,

batch and fed-batch etc), volumes (2-20 L), geometries (impeller types) and physico-chemical properties of the production media (Patrick *et al.*, 1993, 1995, 1996; Tamerler *et al.*, 1997; 1998). Among these, agitation and aeration are the most basic parameters in the fungal aerobic fermentation process. The efficiency of aeration is directly influenced by agitation, resulting in disintegration of air bubbles into smaller subunits and thus increased interfacial area between the gas and liquid phases (Jafari *et al.*, 2007). Hence the optimization of bioreactor operations can favour the economic scale and production of swainsonine through fermentative means.

The extraction and purification of swainsonine has been reported from the plant sources, *Astragalus* (Zhao *et al.*, 2009) and *Oxytropis* (Gardner *et al.*, 2003; Wang *et al.*, 2011). Recently, swainsonine was extracted and purified with various polar solvent systems, physiological conditions and ion exchange resins (Gardener and Cook, 2010). However, the use of polar solvents like ethanol or water also imposes another problem of co-extraction of broth components like glucose, amino acids and other such hydrophilic metabolites. Hence, this necessitates the development of significant extraction and purification methods to remove the low molecular weight extraneous compounds. Several methods have been reported for the analysis and quantification of swainsonine including thin-layer chromatography (TLC) (Molyneux *et al.*, 1991), capillary gas chromatography (Molyneux *et al.*, 1989), LC-MS/MS (Gardner *et al.*, 2001) and HPLC with evaporative light-scattering detector (Yang *et al.*, 2012). Liquid chromatography will undoubtedly become the greatest area of growth in this respect because of its ability to analyze swainsonine without derivatization, but conventional HPLC analysis has rarely been applied to swainsonine. There are two reasons for this: (i) the lack of suitable chromophore and (ii) the extreme hydrophilicity and polarity of swainsonine (Stockigt *et al.*, 2002).

Preparative LC-MS methods are advantageous over destructive low pH conditions in ion-exchange chromatography (Donaldson *et al.*, 1990; Molyneux *et al.*, 1995). Mass spectrometry is considered to be a specific and universal detection method (Molyneux *et al.*, 2002). However, mass derived quantification for swainsonine was limited to the knowledge. Liquid chromatography-mass derived (LC-MSD) purification is a combination of liquid chromatography and mass spectrometry and provides a high throughput rapid purification and quantification of diverse library compounds in nature and its chemical characterization in a non-destructive manner (Hendricks *et al.*, 2011) contrary to the earlier reported methods (Donaldson *et al.*, 1990; Gardener *et al.*, 2001; Molyneux *et al.*, 1991).

Swainsonine's capacity in control practices is a less studied matter. Increasing sensitivity towards secondary metabolites from fungal biological control agents (BCAs) has prompted the toxicological risk assessment of metabolites produced by *M. anisopliae* (Skrobek and Butt, 2005). *In situ* assays are important and useful tools in toxicity assessment of various classes of environmental contaminants including fungal metabolites because they significantly reduce evaluation time and also provide information about the mode of action of the toxicant (Fornelli *et al.*, 2004). The interest in developing *in situ* cytotoxicity tests has increased in recent years as they are usually less expensive, more quantitative, more reproducible and more rapid than *ex situ* studies.

OBJECTIVES

The present study describes a complete process developed for the production, purification and *in situ* entomotoxic and therapeutic potentials of the purified swainsonine. The overall work is divided into the following six objectives;

1. Screening of *Metarhizium* strains, media components and physiological culture conditions for fermentative swainsonine production.
2. Shake flask optimization of production media components.
3. Optimization of operational conditions and scale up parameters at bench-top bioreactor level.
4. Purification, characterization and quantitative analysis of swainsonine.
5. Analysis of *in situ* entomotoxic effects of swainsonine in *Spodoptera frugiperda* cells, *Sf*-21.
6. Analysis of *in situ* anticancer activities of swainsonine in human promyelocytic leukemia cells, HL-60.

PROPOSED SCHEME OF CHAPTER DIVISIONS FOR PH.D THESIS

Chapter 1: Introduction, Review of Literature and Objectives.

Chapter 2: Screening of *Metarhizium* strains, media components and physiological culture conditions for fermentative swainsonine production.

2.1. Introduction
2.2. Materials and methods
2.3. Results and Discussion
2.4. Conclusions
Tables and figures

Chapter 3: Optimization of media components, operation conditions and scale up parameters from shake flask to bench-top bioreactor levels

3.1. Introduction
3.2. Materials and methods
3.3. Results and Discussion
3.4. Conclusions
Tables and figures

Chapter 4: Purification, characterization and quantitative analysis of swainsonine

4.1. Introduction
4.2. Materials and methods
4.3. Results and discussion
4.4. Conclusions
Tables and figures

Chapter 5: Analysis of *in situ* entomotoxic and anticancer activities of swainsonine

5.1. Introduction
5.2. Materials and methods
5.3. Results and discussion
5.4. Conclusions
Tables and figures

REFERENCES
APPENDIX I: List of abbreviations.
APPENDIX II: List of mathematical symbols and Greek letters
RESEARCH OUTPUT

BRIEF DESCRIPTION OF THE RESEARCH WORK

Chapter 1: Introduction, Review of Literature and Objectives.

The chapter describes the role of fungal biocontrol agents and their efficacy in modern agriculture. The application mechanisms and formulation studies reported worldwide for *Metarhizium anisopliae* are discussed in detail. Here, the role of various physiological, mechanical and especially biochemical (enzymes, toxins and metabolites) aspects in the virulence of *M. anisopliae* is emphasized based on the available literature. The chapter also discusses the importance of the novel secondary metabolites from *Metarhizium*, like swainsonine in the diverse areas of therapeutic, glycobiology and the potential entomotoxic applications relevant to the framed objectives of the proposed research work.

Chapter 2: Screening of *Metarhizium* strains, media components and physiological culture conditions for fermentative swainsonine production

Overall ten *Metarhizium anisopliae* strains - ARSEF-2735 (UM2), 2135 (UM3), 2424 (UM4), 3210 (UM5), 2596 (UM6), 1724 (UM8), 549 (UM15), 1744 (UM 16), 23 (UM17) and 2575 (UM 18) were screened and enzyme assayed for the production of swainsonine in different media, Complex oatmeal, Czapekdox media with and without lysine (0.08 % w/v) and SDB - sabouraud dextrose broth (Sim and Perry, 1995). The composition of fermentative media including macronutrients (C-N sources), micronutrients ($CaCl_2$, $MgSO_4$) and pH values were screened using OFAT (one factor at a time) and PB (Plackett-Burman) screenings.

OFAT-screening (Production media, C-N sources and pH)

The present study shows that the nutrient requirement is specific for each strain of *Metarhizium*. Here, the effect of only one of the variable (component) was

measured with respect to the response (swainsonine production), while keeping the rest of the variables fixed. Although the OFAT screening seems to be tedious but it still has some advantages over rest of the statistical methods of screening, these are fewer level changes and protection against the premature termination of experiments in a sequential way.

PB Screening (oatmeal, glucose, inoculum size, L-lysine, $CaCl_2$, $MgSO_4.7H_2O$, pH)

The factors were selected based upon the OFAT-screening results and literature available for fermentative production of swainsonine using *M. anisopliae* in a very limited number of experimental sets. The OFAT method can screen the simultaneous screening of factors and their individual effects on swainsonine production in a very limited number of experimental sets. The levels of significance for each variable were determined using the lack of fit test (F-test) at $p < 0.05$.

Results:

a. *M. anisopliae* ARSEF-1724 (UM8) was screened as the best strain with an average swainsonine production of 1.34 µg/ml after 72 h of incubation at 180 rpm and 28 ºC (**Fig. 1a**).

b. The growth rate kinetics study for *M. anisopliae* ARSEF-1724 (UM8) showed that the strain has doubling time (T_d) of 27 h with stationary phase ranging from 72 h-96 h of incubation (**Fig. 1b**).

c. Oatmeal (6 %) and glucose (2 %) were screened as the best production medium composition with an overall swainsonine production of 1.60±0.32 µg/ml.

d. Addition of inorganic nitrogen sources to the production media had no significant effect on the rate of swainsonine production.

e. The pH range of 5-5.5 was found as optimal for the swainsonine production in the finally screened oatmeal based production medium by *Metarhizium* strain (ARSEF 1724). The overall optimization and screening steps are shown in **Fig. 2**

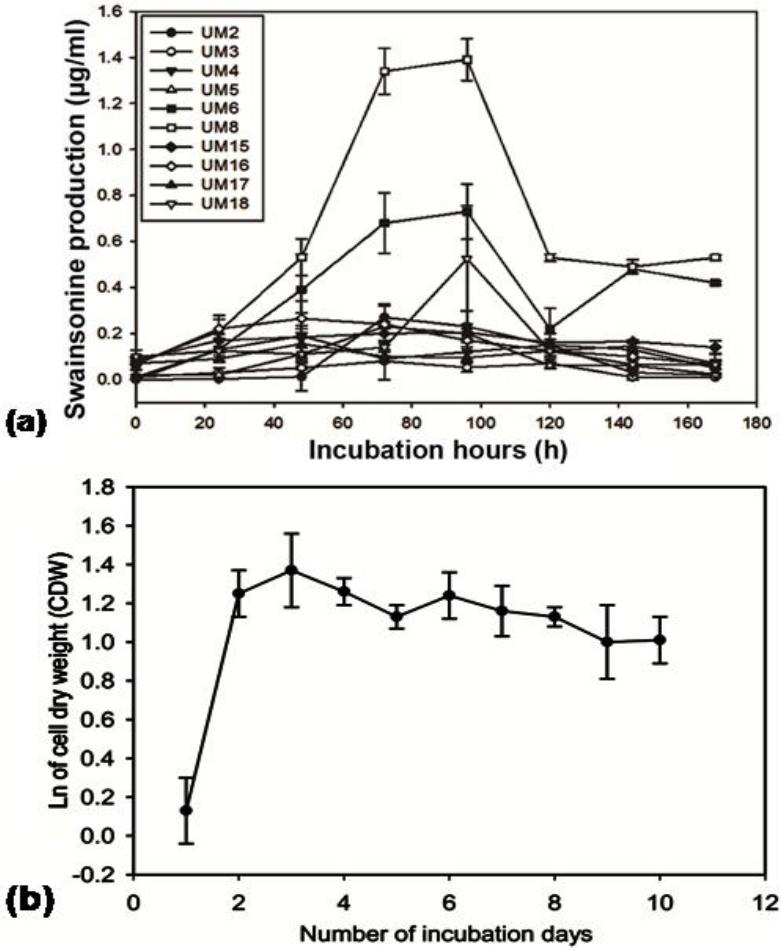


Fig. 1. (a) Screening of *M. anisopliae* strains for swainsonine production in 2 % (w/v) oatmeal fermentation medium. Here, UM2: ARSEF-2735, UM3: ARSEF-2135, UM4: ARSEF-2424, UM5: ARSEF-3210, UM6: ARSEF-2596, UM8: ARSEF-1724, UM15: ARSEF-549, UM16: ARSEF-1744, UM17: ARSEF-23 and UM18: ARSEF-2575. **(b)** Growth curve of *M. anisopliae* ARSEF-1724 (UM8).

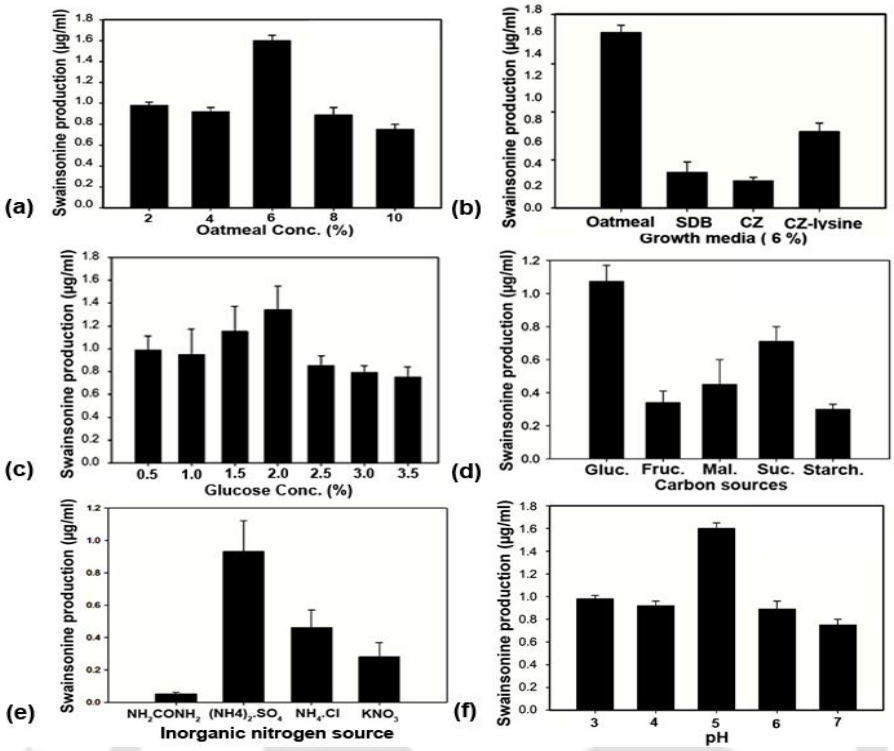


Fig. 2. Screening of for the fermentative production of swainsonine from *M. anisopliae* strain ARSEF-1724 (UM8). at optimal 72 h of incubation **(a)** 2-10 % oatmeal concentrations **(b)** Different growth medium at 6 % (w/v) concentration **(c)** 0.5-3.5 % (w/v) glucose concentrations in 6 % oatmeal **(d)** 2 % (w/v) different carbon sources in 6 % oatmeal **(e)** 0.5 % (w/v) of different inorganic nitrogen sources and **(f)** different pH conditions

f. The PB screening resulted in the four most significant factors for fermentative production of swainsonine; glucose, $CaCl_2$, oatmeal and inoculum size (**Fig. 3**).

g. The production range was varied from 0.55 to 1.55 µg/ml, this further confirmed the need for optimization.

h. ANOVA provided the regression equation for the model with significant variables at (P<0.05):

$$Y = 0.885 - 0.235A + 0.081C + 0.191E - 0.088G$$

Here A = glucose, C = oatmeal, G = inoculum size and E = $CaCl_2$.

Chapter 3: Optimization of media components, operation conditions and scale up parameters from shake flask to bench-top bioreactor levels.

Shake flask optimization of production media components

Response surface methodology (RSM) and artificial neural network-real encoded genetic algorithm (ANN-REGA) were employed to develop a process for fermentative swainsonine production from *M. anisopliae* ARSEF 1724 (UM8) under shake flask conditions. The effect of finally screened process variables viz. inoculum size, oatmeal extract, glucose, and $CaCl_2$ were investigated through central composite design-CCD (**Fig. 3**).

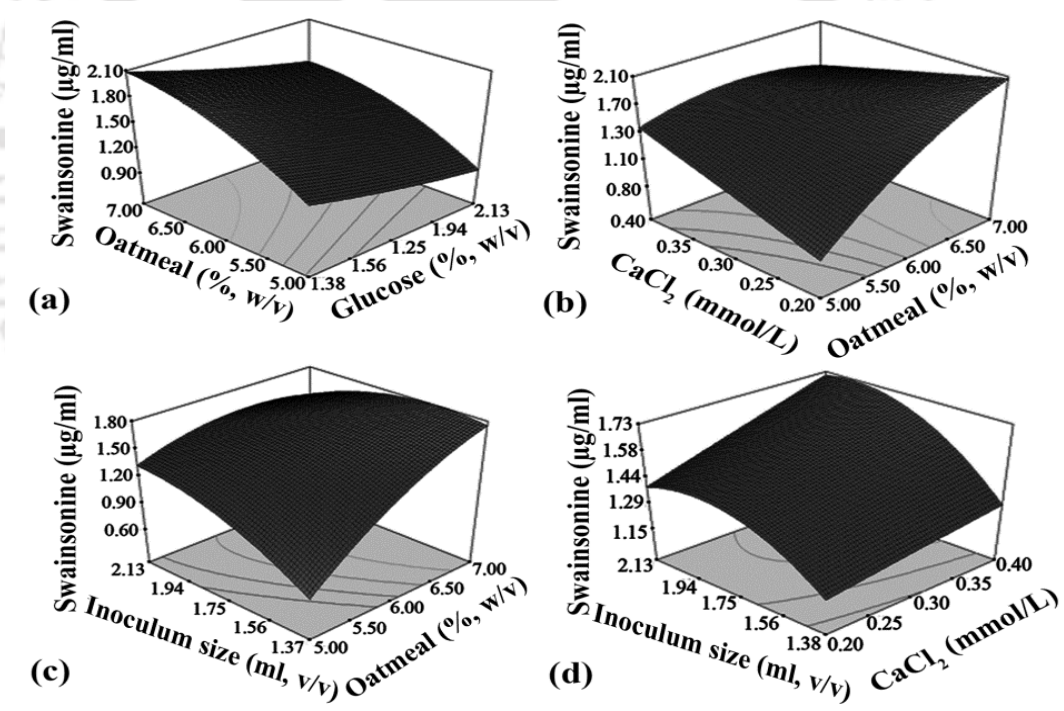


Fig. 3. Three dimensional response surface plots for swainsonine production **(a)** glucose and oatmeal, (**b)** oatmeal and $CaCl_2$, **(c)** oatmeal and inoculum size and **(d)** $CaCl_2$ and inoculum size.

The CCD results were further utilized for training sets in ANN with training and test R values of 0.99 and 0.94, respectively. ANN-REGA was finally employed to simulate the predictive swainsonine production with best evolved media composition. ANN-REGA predicted a more precise fermentation model with 103 % (shake flask) increase in alkaloid production compared to 75.62 % (shake flask) obtained with RSM model upon validation (**Fig. 4**).

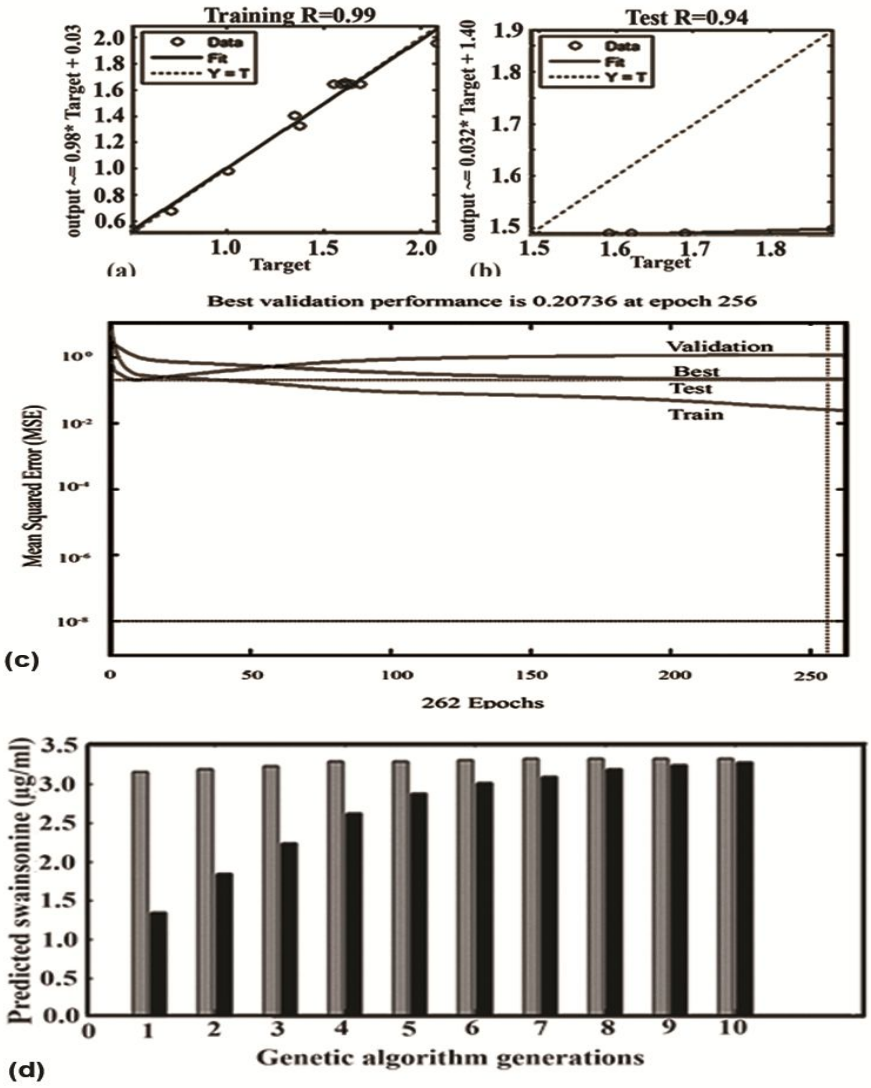


Fig. 4. The Training **(a)** test **(b)** and performance **(c)** plots for ANN-model. The GA evolution plot **(d)** for best (▌) and average fitness (▌) values of swainsonine production (µg/ml) over ten generations in ANN-GA optimization model.

Results:

The optimal media composition for fermentative swainsonine production using *M. anisopliae* (ARSEF-1724) under shake flask conditions was optimized as;

a. RSM based - 1.38 % (w/v) glucose, 6.96 % (w/v) oatmeal, 0.2 mM of $CaCl_2$ and 1.38 % (v/v) of inoculum size with predicted and experimental swainsonine production of 2.77 µg/ml and 2.81±0.17 µg/ml respectively.

b. ANN-REGA based - 2.90 % (w/v) glucose, 7.89 % (w/v) oatmeal, 0.21 mM $CaCl_2$ and 1.22 % (v/v) of inoculum size with predicted and experimental swainsonine production of 3.32 µg/ml and 3.25±0.39 µg/ml respectively.

Hence, further scale up studies for swainsonine production could provide the more appreciable results at bioreactor levels with controlled agitation and aeration conditions using advanced algorithm based optimization models.

Digar Singh and Gurvinder Kaur **(2013)** ***Folia Microbiologica,*** 58; 393-401.

Optimization of operational conditions and scale up parameters at bench-top bioreactor level

The optimal control of operation conditions for fermentative production of swainsonine was developed using evolutionary algorithm (EA) and compared with conventional one-factor-at-a-time (OFAT) method. The effects of varying agitation (300-500 rpm) and aeration (0.5-2.0 vvm) rates for different incubation hours (72-108 h) were evaluated in 3 L bench top bioreactor with glucose-oatmeal based production media. Prominent scale up parameters, gassed power per unit volume (Pg/V_L, W/m^3) and volumetric oxygen mass transfer coefficients (K_La, s^{-1}) were evaluated at optimized conditions (**Table 1**). Hence, the study aims the comparative optimization

cum scale up analysis for swainsonine production with advanced computational EA and conventional OFAT- optimization methods. The proposed model worked efficiently with real value inputs and drastically reduced computational load.

Results:

A maximum of 6.59±0.10 µg/ml of swainsonine production was achieved at 400 rpm and 1.5 vvm after 84 h of incubation with OFAT optimization model.

a. The corresponding Pg/V_L and K_La values were 91.66 W/m^3 and 341.48×10^{-4} s^{-1} respectively.

b. The OFAT experimental sets were further utilised in EP simulations for optimal solutions. A maximum of 7.93±0.52 µg/ml of swainsonine production was experimentally observed at EP-simulated bioreactor conditions of 325 rpm and 2 vvm after 80 h of incubation with nearly constant K_La (349.25×10^{-4} s^{-1}) and significantly reduced Pg/V_L (33.33 W/m^3) drawn by the impellers.

c. The proposed model worked efficiently with real value inputs and drastically reduced computational load.

Table 1. Evolutionary program (EP) based simulations (1-10), experimental validation of EP-optimized (11) and OFAT-optimized (12) models at varying agitation (N) and aeration (Q) rates for different incubation hours (h) with respect to swainsonine production. The production was correlated with corresponding power consumed per unit volume (Pg/V) under aeration and volumetric oxygen mass transfer coefficient (K_La).

EP simulations	Agitation N (rpm)	Aeration Q (vvm)	Incubation hours (h)	Swainsonine production (µg/ml)
1	425.71	1.98	74.81	6.56
2	300.58	1.37	85.15	7.72
3	302.61	1.51	77.95	7.22
4	441.11	1.98	107.91	4.28
5	303.71	1.78	97.46	6.31
6	392.10	1.95	107.96	7.55
7	411.47	1.63	100.57	6.67
8	459.28	1.68	107.85	8.21
9	300.10	1.98	103.33	8.43
10*	325.47	1.99	80.75	10.08
EA	**325**	**2**	**80**	**7.93±0.52**
			At K_La = 349.25×10^{-4} s^{-1} and Pg/V = 33.33 W/m^3	
OFAT	**400**	**1.5**	**84**	**6.59±0.10**
			At K_La = 341.48×10^{-4} s^{-1} and Pg/V = 91.66 W/m^3	

***Best evolved run after EP- simulations.**

Digar Singh and Gurvinder Kaur **(2013) Bioprocess and Biosystems Engineering** (communicated)

Chapter 4: Purification, characterization and quantitative analysis of swainsonine.

Metarhizium fermentation broth was subjected to basic downstream steps- filtration, centrifugation and chemical precipitation methods etc., before applying the conventional partial purification strategies as shown in the **Fig. 5**.

Partial purification and solvent extraction

Results:

a. A prior subtractive purification strategy was applied to precipitate endogenous proteins, sugars and nucleotides etc. in fermentation broth using organic solvents under chilled conditions.

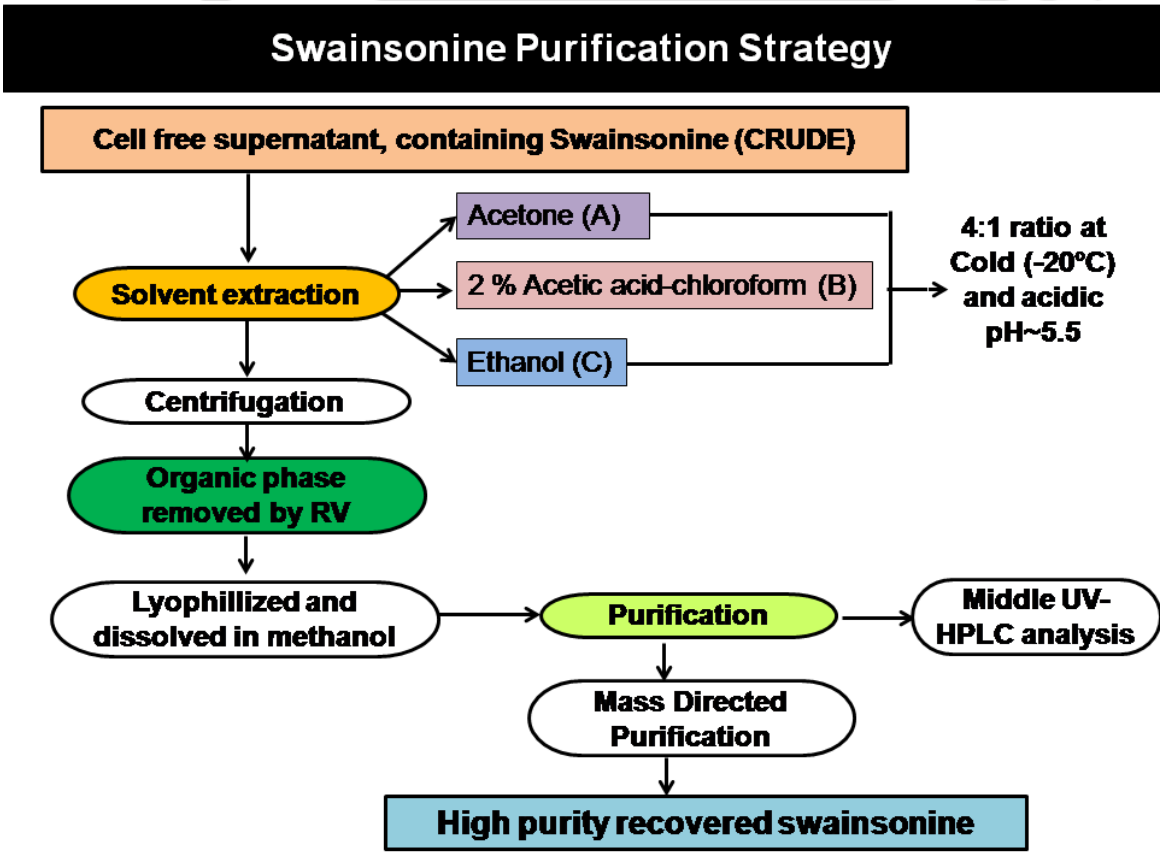


Fig. 5. Analytical and preparatory methods for swainsonine.

Table 2. Extraction of swainsonine from *M. anisopliae* ARSEF-1724 with three different organic solvents.

Sample	Broth volume (ml)	Yield (µg/ml)	Total (µg)	Yield (%)	Fold purification
A. Acetone extraction					
Crude	85	2.86	243.10	-	-
Extract supernatant	6	7.16	42.96	17.67	2.5
Extract pellet	1	0.69	0.69	0.28	0.24
B. 2 % acetic acid in chloroform extraction					
Crude	90	2.75	247.50	-	-
Extract supernatant	6	5.19	31.14	12.58	1.88
Extract pellet	1	0.57	0.57	0.23	0.20
C. Ethanol extraction					
Crude	90	2.88	259.20	-	-
Extract supernatant	6	3.35	20.10	7.75	1.16
Extract pellet	1	0.04	0.04	0.01	0.01

b. An average of 2.5-folds purification was achieved using solvent A (acetone), with 7.16 µg/ml of swainsonine in extract supernatant. However, only 1.88 and 1.16 fold purification of swainsonine was observed with solvents B (5.19 µg/ml) and C (3.35 µg/ml), respectively (**Table 2**).

Liquid chromatographic operations for swainsonine

Analytical middle UV-HPLC

A simple, rapid and fairly reliable UV-HPLC analytical method was developed for swainsonine in *Metarhizium* fermentation broth. The partially purified broth samples were analysed using reverse phase HPLC method at middle UV range (205 nm) with three different mobile phases and optimal gradient programme.

Results:

a. The best chromatographic resolution for swainsonine was observed between elution time t_e = 6.0-6.9 min (**Fig. 6**).

b. The corresponding HPLC-peak fraction was further characterized using mass spectrometry to observe significant abundance of MS1 abundance of $[M+H]^+$ 174.30, specific to swainsonine (inset of **Fig. 6**).

c. The swainsonine concentration in the *Metarhizium* fermentation broth was determined as 4.04±0.52 µg/ml, which is very close to that determined by mannosidase inhibition assay (3.87±0.45 µg/ml).

d. The optimal mobile phase for gradient HPLC elution of swainsonine was 0.1 % TFA in water and acetonitrile.

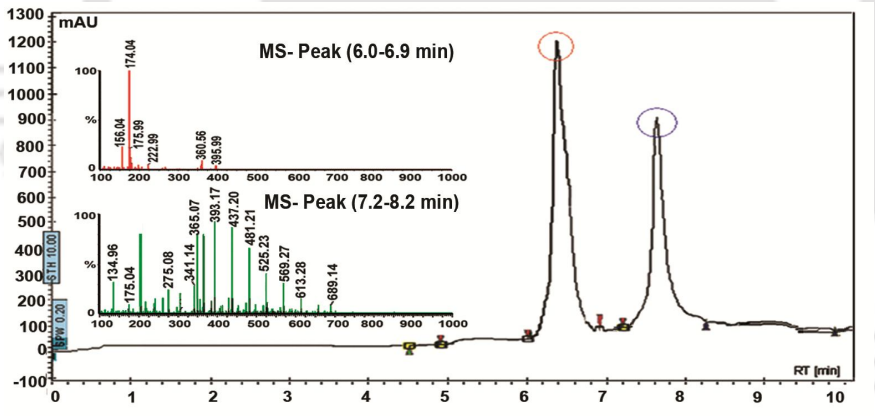


Fig. 6. HPLC chromatograms and corresponding mass spectrum (inset) for the major peaks collected partially purified broth fraction at flow rates Fr = 1 ml/min from 0-5 min and 0.25 ml/min from 5-10 min, all at mUV range of 205 nm.

Digar Singh and Gurvinder Kaur **(2013)** ***Journal of Biosciences*** (Submitted)

Preparative cum quantitative mass derived liquid chromatography

The partially purified broth was further subjected to mass-derived preparative-cum-quantitative analysis. Mass spectrometry is considered to be a specific and

universal detection method. Liquid chromatography-mass derived (LC-MSD) purification provides a high throughput rapid purification cum quantification of diverse library compounds under selected ion monitoring (SIM) mode. Moreover, the procedure was sensitive, selective and non-destructive method of analysis for swainsonine.

Results:

a. The mass derived (m/z)-based rapid preparatory analytical method was developed for swainsonine in broth fractions, where the alkaloid was eluted as MS1 fraction $[M+H]^+$ 174.36±0.21 at 4.91±0.04 min.

b. The purified fractions were further analyzed and validated for swainsonine purification with analytical LC-MS system. Swainsonine was eluted as a single peak at t_e = 6.38±0.04 min (**Fig. 7a**)

c. The mass (m/z) based quantification method was developed based on the abundance of ESI+ MS1 ion corresponding to the concentration. The average swainsonine concentration in the purified fractions was determined as 7.85±1.59 µg/ml corresponding to 3.74×10^5 MS1 counts (**Fig. 7b**).

d. The method was found to be quite reliable in the linear range (1-8 µg/ml), precise (relative SD values of 0.79 % for swainsonine MS1 count intensities) and accurate.

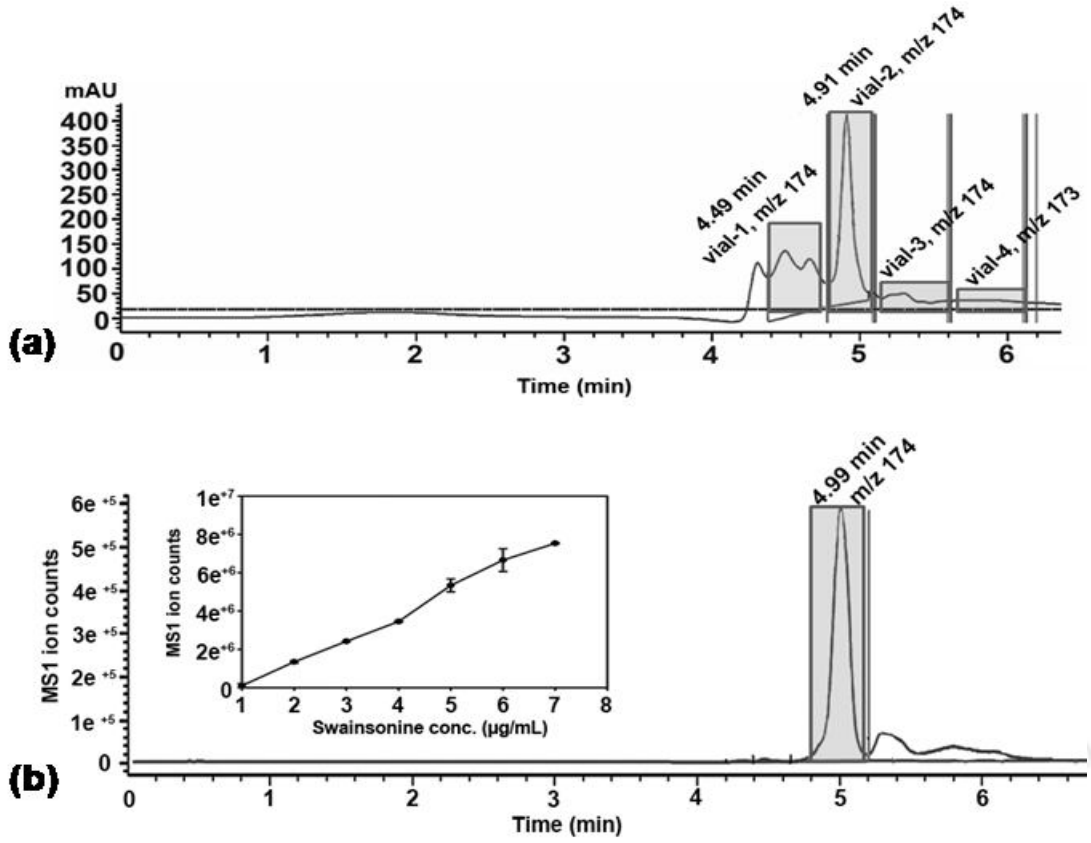


Fig. 7.(a) DAD chromatogram of acetone extracted swainsonine fractions in LC-MSD purification, **(b)** Intensity ion counts vs. retention time (min) curve for mass derived quantification in SIM-mode. Calibration curve of swainsonine for precursor ion $[M+H]^+$ 174.36±0.21 as an inset figure.

Digar Singh and Gurvinder Kaur **(2013)** ***FEMS Microbiology Letters*** 347, 7-13.

Chapter 5: Analysis of *in situ* entomotoxic and anticancer activities of swainsonine.

The bioactivities of swainsonine (standard and purified) were examined through *in situ* assays to establish its potential applications in the diverse areas of agriculture and therapeutics. This chapter describes the two different aspects regarding the *in situ* toxicological assessment of swainsonine towards lepidopteran cell line *Sf*-21 and cancerous human promyelocytic leukemia cells, HL-60. The swainsonine induced cytotoxic effects were assessed using the MTT cell viability and Tryphan blue cell

death assays. The FE-SEM and AFM based ultra-morphological examination of the swainsonine treated cells showed characteristic apoptotic features. The quantitative analysis of apoptotic/cell cycle regulatory effects of swainsonine in the *Sf*-21 and HL-60 cells were evaluated with FACS (fluorescence activated cell sorting) based methods.

Results:

The entomotoxic potentials of swainsonine

a. The alkaloid showed very low IC_{50} values based on the MTT cell proliferation and TB-tryphan blue dye exclusion cell death assay towards *Sf*-21 cells (**Table 3**).

Table 3. Toxicological assays for preparative LC-MSD purified swainsonine fractions in two independent sets of experiments.

Toxicological assay	Mass derived fractions	Standard swainsonine
MTT- cell proliferation assay	5.27 µM	3.90 µM
Tryphan blue cell death assay	8.67 µM	6.91 µM

b. The ultra-morphological examination of the swainsonine treated (standard and MSD purified) *Sf*-21 cells showed the characteristic apoptosis markers like membrane blebbings, ruptures and cell shrinkage (**Fig. 8**).

c. The fluorescence activated cell sorting (FACS) analysis concluded a significantly high percentage of swainsonine treated cells in the early and late apoptotic (~25 % for standard and 20 % for purified, swainsonine respectively) phases after 36 h of post-treatment incubation.

d. The study thus confirmed the potential entomotoxic effects of swainsonine and its role in entomopathogenic virulence of *M. anisopliae* ARSEF-1724 (UM8).

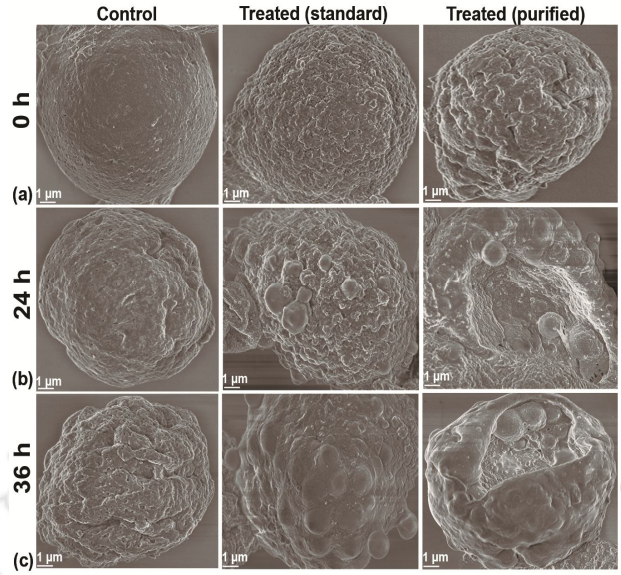


Fig. 8. Ultramorphological FE-SEM analysis (15KX) of the control and swainsonine treated (standard and purified), *Sf*-21 cells at 0 h, 24 h and 36 h post-treatment incubations.

Digar Singh and Gurvinder Kaur **(2013)** ***Biotechnology Progress*** (communicated)

Results:

The therapeutic potentials of swainsonine

a. Swainsonine did not show any significant anti-proliferative activity towards HL-60 cells after 36-48 h of incubation (doubling time). The MTT based assay showed the some activity only at 48^{th} h ($IC_{50\ standard}$ = 6.96 µM).

b. However, swainsonine ($IC_{50\ standard}$ = 6.96 µM and $IC_{50\ purified}$ = 9.50 µM) exhibited considerable cell cycle regulatory activities in HL-60 cells at around 36-48 h of post- treatment incubation.

c. The Mod-fit analysis of swainsonine treated HL-60 cell cycle showed a significant S-phase arrest at 36 h (48 % cells) and 48 h (60 % cells) of post treatment incubations respectively for purified swainsonine samples (**Fig. 9**).

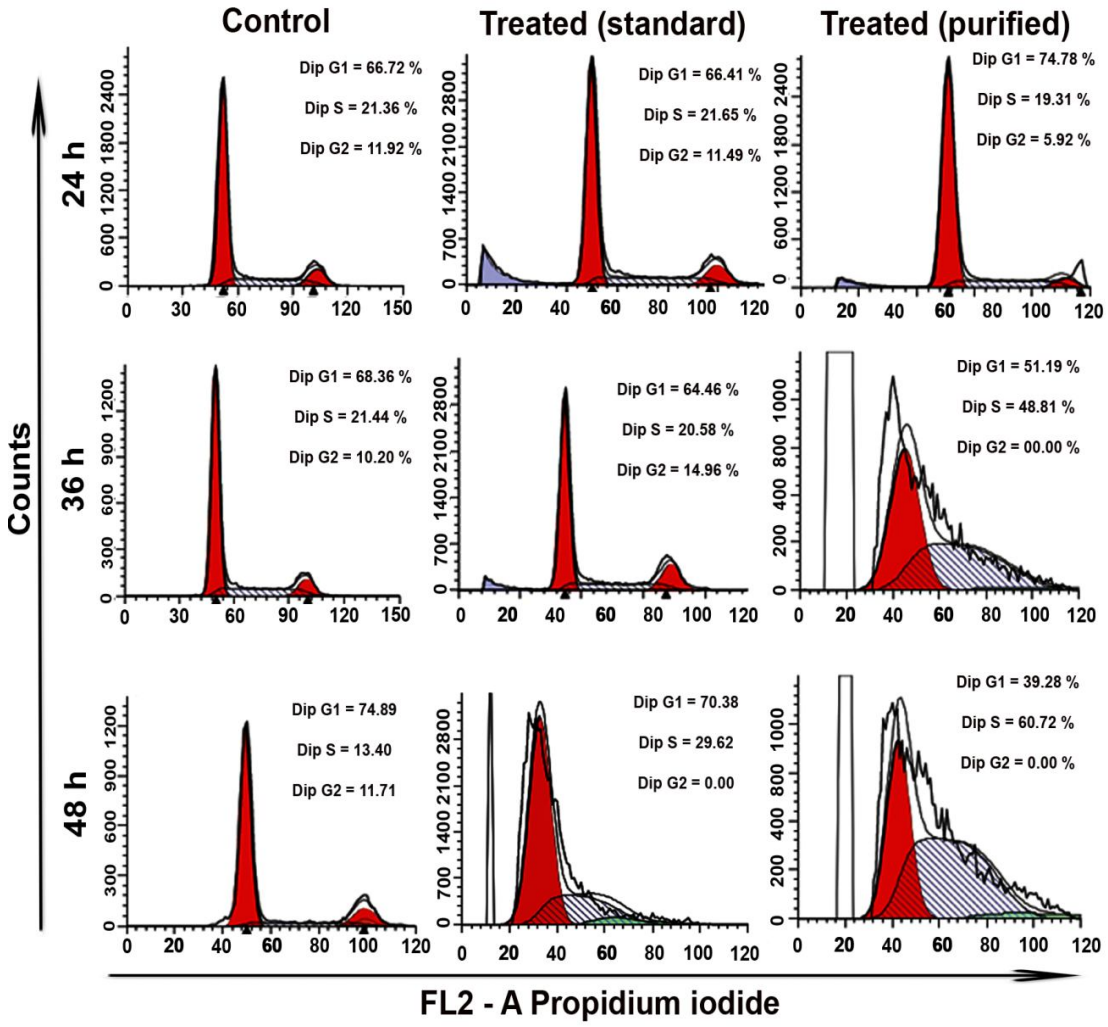


Fig. 9. Analysis of cells cycle progressions in control and swainsonine treated (standard and purified) HL-60 cells.

SIGNIFICANT FINDINGS AND FUTURE PROSPECTS

Significant findings

1. *Metarhizium anisopliae* ARSEF-1724 (UM8) was screened as the highest swainsonine (1.6 µg/ml) producing strain.
2. Hybrid artificial neural network (ANN) coupled with genetic or evolutionary algorithms (GA/EA) were superior compared to convention RSM (response surface methodology) and OFAT (one factor at a time) statistical methods for enhanced swainsonine production through optimal fermentation models.
3. A prior subtractive purification strategy was applied to precipitate higher MW endogenous proteins, sugars and nucleotides etc. in *Metarhizium* fermentation broth for swainsonine purification using acetone-methanol solvent systems.
4. Swainsonine was fairly detected and analysed at middle UV range (205 nm) in reverse phase HPLC analysis.
5. Partially purified (solvent extracted) swainsonine was purified by mass (m/z) derived preparative cum analytical high throughput LC method.
6. The Mass based quantification method was first applied for swainsonine. Swainsonine concentrations were calculated based on the abundance of MS1 counts $[M+H]^+$ in MS-ESI+ under SIM (selected ion monitoring) corresponding to the swainsonine concentrations injected, under a given sets of MS-conditions.
7. Swainsonine showed significant *in situ* entomotoxic activities against lepidopteran, *Sf*-21 cell line through apoptotic mode of cell death. The study further signifies the role of secondary metabolites in the virulence of entomopathogenic fungi.
8. Swainsonine showed the considerable *in situ* cell cycle regulatory activities in leukemic, HL-60 cell line which further suggests its therapeutics potentials.

Future prospects

1. Identification and analysis of specific mechanism of swainsonine triggered apoptosis in *Sf*-21 cells at proteomic and genomic levels.
2. Molecular characterization of the swainsonine induced cell cycle arrest at proteomic and genomic levels in leukemic HL-60 cells.

CHAPTER 1

Introduction, Review of Literature and Objectives

1.1. Introduction

Agriculture is the backbone of Indian economy which witnessed multi-fold increase in the last four decades, leading to an era of food security to the nation. This remarkable growth was achieved through the uptake of newer technologies in the form of high yielding crop varieties, chemical fertilizers and pesticides, as well as from the expansion of cultivation land for agriculture. During the Green Revolution, achieving high crop yields at any cost was the ultimate goal. The emphasis now is on sustainable agriculture-increasing yields without harming the environment. Biopesticides are one of the vital components of sustainable agriculture, which avoid and reduce the use of harmful chemical pesticides toxic to humans and livestocks in environment. Amongst the virus, bacteria and fungi applicable in pest control the latest are well studied and widely used. The reasons are their efficiency in killing the hosts, the great biodiversity of the fungi that represent the many possibilities of finding the most appropriated isolates to develop competitive biological control agents and their relative environmental safety (Thomas and Read, 2007). Mycobiocontrol is the use of fungi in biological processes to lower the insect density with the aim of reducing disease-producing activity and consequently crop damage. All groups of insects may be affected and over 700 species of fungi have been recorded as pathogens (Chet, 1987; Chet *et al.*, 1993). Mycobiocontrol is an environmentally sound and effective means of reducing or mitigating insect-pests and its effects through the use of natural enemies. Pest-related damages result in a heavy loss, approximately estimated to be US $10,000 million annually in agricultural production in the field and storage in India.

1.2. Entomopathogenic fungi

The potential of entomopathogenic fungi as pest control agent has been realised since the advent of the 19th century when Metchnikoff (1879) and Krassilstschik (1888) independently discovered and formulated the use of *Metarhizium anisopliae* in the control of pest like *Anisoplia austriaca*, *Asproparthenis punctiventris* and *Cleonus punctiventris*. Earlier in 20th century, Berger (1909) worked extensively on *Aschersonia* in the United States, and this fungus was used until 1920's when the effective chemical pesticide agents became available to the modern agriculture. However, the adoption of biocontrol agents was still tedious and laborious task for actual field formulations. The extensive research on the variety of fungal biocontrol agents like *Beauveria*, *Trichoderma* and *Metarhizium* finally paved the way for their efficient field applications. Fungi in the genus *Metarhizium* causes fatal mycoses in a wide variety of important insect pest species (Lepidoptera, Helicoverpa, Coleoptera, *etc.*) and, consequently, occupy the forefront of efforts to develop entomopathogenic fungi as biological control agents.

The genus *Metarhizium* can be taxonomically classified as:

Kingdom	Fungi
Phylum	Basidiomycota
Class	Agaricomycetes
Order	Cantharellales
Family	Ceratobasidiaceae

Metarhizium spp. belongs to a group of entomopathogenic fungi used worldwide as alternative or additional to chemical insecticides in agricultural pest and disease vector control programs (Roberts and St. Leger, 2004). In addition to being an

entomopathogen, *M. anisopliae* also can colonize plant roots and is a common component of the rhizosphere (root-soil interface).The mechanisms involved in the rhizosphere competence of *M. anisopliae* or in its fungal-plant interactions are only partially understood (Hu and St. Leger, 2002; Wang and St. Leger, 2007). The specificity of entomopathogens towards a specific insect host is attributed to the specific metabolites or products which selectively enhance its entomotoxic behaviour and virulence. The wide utilization of *M. anisopliae* as bioinsecticide has increased interest in its basic biology, including its fungal secondary metabolites. The secondary metabolites are natural products which are not essential for the normal growth, development or reproducibility of an organism. However, these bioactive secondary metabolites play a vital role in fungal survival, interspecies interactions, signalling as well as defense against predation and pathogens in their environment.

1.3. Bioactive secondary metabolites from *Metarhizium*

The fungal entomopathogens including *Metarhizium* produce an array of secondary metabolites in trace quantities whose production varies between species, strains and cultural or environmental conditions (Kershaw *et al.*, 1999; Amiri-Besheli *et al.*, 2000; Quesada-Moraga and Vey, 2003; Strasser *et al.*, 2000; Vey *et al.*, 2001; Wang *et al.*, 2004). Fungal secondary metabolites are potentially toxic to antagonistic arthropods, the massive biosynthetic machinery driving the formation of mycotoxins, which provides the direct chemical shield against invading pests. Hence, these bioactive molecules with selective biological activities may provide a novel source of natural insecticidal or other toxicological agents (Mollier *et al.*, 1994). Recently, *Metarhizium* metabolites have received increasing attention from the researchers of the diverse fields of studies. The biosynthesis of a still underexplored diversity of

secondary metabolites is an outstanding hallmark of this fungus. There are number of secondary metabolites and bioactive products reported from *Metarhizium* spp. having a broad range of toxic and therapeutic applications (Fig. 1.1).

The secondary metabolites from fungal entomopathogens including *Metarhizium* spp. play an important role in their entomotoxic virulence and hence, are important agents for pest biocontrol studies in the integrated pest management (IPM) programmes. There are various metabolites from entomopathogenic fungi, which exhibit the significant insecticidal properties. The biosynthesis of the secondary metabolites is very strictly regulated by the molecular genetics mechanisms enabling the fungal survival under challenged environmental conditions. The few of these metabolites from entomopathogenic fungi exhibits immunosuppressive effects (Pedras *et al.*, 2002; Vey *et al.*, 2002; Fiolka, 2008; Pal *et al.*, 2007; Vilcinskas and Gotz, 1999). Hence, interactions with the host immune system likely constitute an important interface, where secondary metabolites may serve as a means to resist or suppress animal defences (Gillespie *et al.*, 2000). Therefore, measurements of their invasive effects on arthropods should be evaluated in greater detail at the ‘‘fungal secondary metabolite-host immune system” interface. The apparent induction of apoptosis in insect host hemocytes by the destruxin metabolites of *M. anisopliae* (Vilcinskas *et al.*, 1997; Gillespie *et al.*, 2000) and the cyclosporin A mediated arrest of host detoxification mechanisms by *Beauveria bassiana* (Podsiadlowski *et al.*, 1998) are two examples of how fungal secondary metabolites may act to incapacitate the immune systems of arthropod hosts (Gillespie *et al.*, 2000). In some other studies, aflatoxins produced by *Aspergillus flavus* (Diener *et al.*, 1987), ochratoxins produced by *A. ochraceus* (Myokey *et al.*, 1969), destruxins from *Metarhizium* (Jegorov *et al.*, 1995) and beauvericin from *Beauveria* (Kucera and Sansinakowa, 1968) *etc.* have

shown to play an important role in fungal infection and can generate a toxic response in different pest species (Turner *et al.*, 2009). Fornelli *et al.* (2004) reported *in vitro* entomotoxic effects of various *Metarhizium* metabolites against the lepidopteran cell lines. However, the actual mechanisms of induced cytotoxicity against lepidopterans were not studied in detail. The fungal secondary metabolites although have no major role in growth, reproduction and development of an organism but critically influence the survival, inter- and intra-species interactions and defence against predation or pathogens in the environment by changing host development, behavior, reproduction, and the onset of host morbidity (Andersen *et al.*, 2009; Roy *et al.*, 2006; Lefevre *et al.*, 2009; Krasnoff *et al.*, 2007; Pal *et al.*, 2007; Xu *et al.*, 2008, 2009), so understanding their effects on host regulatory networks and signal transduction pathways are of paramount importance. However, there are also certain consumer concerns regarding the various secondary metabolites produced by *Metarhizium* in the environments, prompting their detailed scrutiny. The secondary metabolites from *Metarhizium* spp. are usually low molecular weight molecules (< 2 kDa), derived initially from the primary metabolites and manifested from the complex multienzyme biosynthetic pathways. The role of secondary metabolites from *Metarhizium* is not completely understood, however, the extracellular metabolites *viz.* destruxins, acts as antimicrobial or anti-immune agents eliminating the pathogens or potential competitors of nutrients was well studied (Roberts *et al.*, 1992).

A number of studies have shown that some of the fungal metabolites are also toxic to human cell lines *in vitro* (Dumas *et al.*, 1996; Fornelli *et al.*, 2004), whereas others shows antibiotic, fungicidal, antiviral or entomotoxic activities (Terry *et al.*, 1992; Amiri *et al.*, 1992; Bandani *et al.*, 2000; Vey *et al.*, 2001). These studies further emphasize the need of biological risk assessment associated with the use of biological

control agents (BCA's) towards their safe applications in the environment. A few the well studied secondary metabolites produced by *Metarhizium* spp. were discussed below.

1.3.1. Viridoxin

The two of the prominent viridoxins (viridoxin A and B) were first reported from *Metarhizium flavoviridiae* isolated from Czechoslovakia. These compounds are closely related to plant toxins "colletotrichins" with insect toxic activities in the range of 40-50 ppm (Gupta *et al*., 1993). Although, the viridoxins have some structural similarity with colletotrichins except esterified alcoholic groups, contrarily the former shows selective toxicity to insect and not to the plants.

1.3.2. Ovalicin

Metarhizium isolate from Japan was reported to produce a novel bioactive compound ovalicin (12-hydroxy ovalicin) (M.Wt. = 311 Da) with potential immunosuppressive activities (Kuboki *et al.*, 1999). The compound also showed selective inhibitory activities against mouse leukemia cells (L-1210) without affecting the growth of normal cells. The strain was also reported to produce a functionally similar compound, metacytofilin (M.Wt. = 305 Da) with immunosuppressive and delayed-type hypersensitivity against mixed lymphocytes and no anticancer effects (Iijima *et al.*, 1992).

1.3.3. Destruxin

Destruxins (Dxs) produced by *M. anisopliae* in fermentation and, by far, the most exhaustively researched toxins of the entomopathogenic fungi characterized as important virulence factors accelerating the deaths of infected insects. Dxs are

composed of an α-hydroxy acid and five amino acid residues joined by amide and ester linkages to form cyclic structures. There are 38 Destruxins or Dx analogs (Pedras *et al.*, 2002), which is double the earlier reported, 19 types (Gupta *et al.*, 1989). They are divided chemically into five basic groups labelled from A to E, plus several subgroups of each. Dxs A and B were initially isolated in Japan during 1960's from *M. anisopliae* strains which they synonymously called 'Oospora destructor' and hence the compound was first named destruxin. These cyclic depsipeptides have been extensively researched by plant pathologists, microbiologists and natural products chemists for their toxic biochemical activities. The biosynthetic pathway is assumed to be through a non-ribosomal multifunctional enzyme system (Kleinkauf and von Dohren, 1987, 1996; Peeters *et al.*, 1990; Turner, 2000). The toxicity to insects of Dxs A, B, and E is well documented. The toxin causes acute muscular paralysis in arthropods attributed to the muscle cells depolarization due to reversible opening of Ca^{+2} channels in the membrane (Dumas *et al.*, 1996; Samuels *et al.*, 1988). The ionophoric properties of Dx A allow calcium ion mobilization across liposomal membrane barriers (Hinaje *et al.*, 2002). The half life of Dxs in certain lepidopteran species, *Galleria mellonella* is very low (< 1 h) due to degradation of its unstable cyclic structure. Dx B is toxic to plants, and its detoxification in the plant has been followed in a series of experiments also summarized by Pedras *et al.* 2002.

1.3.4. Swainsonine

Swainsonine, a sugar analogue, is an indolizidine alkaloid molecule with a fused piperidine and pyrrolidine ring system. It was first isolated from the fungal plant pathogen *Rhizoctonia leguminicola*, but its structure was not correctly assigned until it had been rediscovered in an Australian plant, *Swainsona canescens* (Colegate *et al.*,

1979). It is found in a number of different plants that cause a lysosomal storage disease (Dorling *et al.*, 1980) which is known as locoism in the western USA (Molyneux and James, 1982). Locoism is a chronic disease and develops in live-stock after grazing of *Astragalus* or *Oxytropis* plants that contain swainsonine (locoweeds) for an extended period of several weeks. This chronic disease characterized by weight loss, depression, altered behavior, decreased libido, infertility, and death, resulting in significant economic losses on an annual basis. Swainsonine was first reported from *M. anisopliae* isolate F-3622 by Hino *et al.* (1985). This compound is also an effective inhibitor of both lysosomal α-mannosidase and mannosidase II. The first of these is involved in cellular degradation of polysaccharides and the latter is a key enzyme in the processing of asparagine-linked glycoproteins (Elbein *et al.*, 1981). Swainsonine is the first glycoprotein processing enzyme inhibitor to be used for clinical trials for its potential anticancer, antimetastatic, antiproliferative and immulomodulatory applications with over 24 other mammalian oriented studies on the biological effects of swainsonine (Nemr, 2000). There are large numbers of bioactive metabolites reported from various *Metarhizium* spp., which are being studied for their therapeutic, industrial and toxicological applications. However, not much of the scientific studies have been carried out towards the microbial production, purification and application on swainsonine.

1.4. Fermentative production of swainsonine from *Metarhizium anisopliae*

In despite of extraction from plants and chemical modes of synthesis, the fermentative production of swainsonine from fungal culture *i.e.*, *Metarhizium* or *Rhizoctonia* has received a very less attention. The fermentative production of swainsonine is advantageous over the rest of the methods on account of two major

reasons: (i) there are four chiral centres in the molecular structure of swainsonine, hence the synthesis of isomeric forms is disproportionate with respect to the required swainsonine. (ii) the process is multistep, hence uneconomic at pilot scales. Hence, fermentative biosynthesis of swainsonine is being investigated as an alternative to the chemical synthesis process. The submerged fermentative production of swainsonine using *M. anisopliae* strains is being reported at shake flask, bench top and pilot scale bioreactor levels (Patrick *et al.*, 1993; 1995). Optimization of swainsonine production is being studied in the context of varying physico-chemical properties of the production media (C-N source, pH, inoculum size and trace elements *viz.*, $CaCl_2$ conc.), operation parameters (agitation and aeration), vessel types (airlift, batch and fed-batch etc), volumes (2-20 L), geometries (impeller types), physico-chemical properties and *Metarhizium* strain selections (Patrick *et al.*, 1993, 1995, 1996; Tamerler., 1997, 1998; Justo *et al.*, 2007; Singh and Kaur, 2012). The development of proper screening and optimizations methods is the important step in the efficient utilization of fermentation technology for the swainsonine production.

1.4.1. Parameters and optimization models for fermentative production

1.4.1.1. One factor at a time and Plackett-Burman screening and optimization

The screening of process parameters (factors) is primarily carried out using one factor at a time (OFAT) and Plackett-Burman (PB) screening methods. The parameter designing is the essential step to determine the nominal values for the process parameters. The OFAT method involves the experiments, where only one of the process variables is evaluated under a given range with rest of the parameters kept constant. This approach is simplest to implement, and primarily helps in selection of significant parameters affecting the fermentative production. The OFAT approach is

specifically applied when the optimum values for process parameters are evaluated under an experiment with interactions among the parameters (Friedman and Savage, 1947). The OFAT approach concentrates on the observations in the region which contains optimum values that may be preferred when an experimenter wishes to react more quickly to a data. It can safely be used in those cases in which factor effects are three or four times' standard deviation due to experimental error (Daniel, 1973). However, this method is not only time restrictive, but also ignores the combined interaction(s) among various physical and nutritional parameters (Vishwanatha *et al.*, 2010). Hence, the more advanced screening and optimization procedures based on various statistical models are being used.

Design of experiments (DOE) provides a powerful means to predict the improvements in product quality and process efficiency in a process that involves multiple effective parameters. The Plackett-Burman (PB) design matrix is the statistical DOE that is widely used to study the individual impact of most significant process parameters towards fermentation process. PB is a set of small and efficient experimental designs, which is very much reliable, widely applicable and especially well suited for bioresearch and development (Plackett and Burman, 1946; Haaland, 1989). In recent years, the optimization research has encouraged the use of statistical methods like regression or factorial designs and evolutionary operations (EVOP). However, these models also involve large number of experiments when dealing with higher number of parameters with undefined range. Hence, if the less significant parameters happen to be chosen for optimization studies, the study will be more tedious and less statistically significant. The PB design enables the screening of most significant process variables out of the large number of probable parameters in a very limited number of experiments. The PB design of optimum multifactor screening

experiment based on balanced incomplete blocks with each design of *N* experiments is useful for studying *N-1* variables.

The fermentative production of swainsonine using *M. anisopliae* depends upon the number of process parameters which significantly affects the overall production rate. The process may involve the screening of most efficient *Metarhizium* strains, production media components, physiological and culture conditions (Singh and Kaur, 2012). Tamerler *et al.* (1998) have suggested the correlation between the *Metarhizium* pellet morphologies and the swainsonine production as a function of pH changes. The authors observed a steep decline in the pH profile of the fermentation media from initial 6.5 to the lowest of 3.8 units, and reported to have a steep decline in the swainsonine production rates when the pH was externally maintained (43.30 mg/ml when the pH was not maintained and 8.4 mg/ml when the pH was externally maintained). The finding also suggested a late pH control strategy (preferably after the late log phase, 72 h) for the optimal production of swainsonine from *Metarhizium* fermentations. Optimal pH requirements can be different for each stage of microbial growth and sudden pH changes can cause temporary or permanent perturbations in metabolism of the products of interest. The swainsonine production in stirred tank and airlift of 2-20 L was also assessed under varying agitation rates with respect to the corresponding fungal morphologies (Tamerler and Keshavarz, 1999). The study evaluates the role of shear stress induced by the higher agitation rates on the culture physiology of filamentous fungi and its role on product yield. The lower agitation rates results in the inadequate supply of metabolic oxygen, lower growth rates and entangled hyphae, whereas the higher agitation rates results in the fragmented hyphal bodies and the overall reduction in the yield. However, the effect of operational parameters (agitation rate) depends upon several factors including the fungal strain,

the seed culture (spore, mycelia or pellets), the nature of the growth media and the hydrodynamic condition in a bioreactor (Metz and Kossen, 1977; Metz *et al.*, 1979). The swainsonine production was also studied in context of the various vessel geometries (airlift, batch, fed-batch etc.) and impeller types as a function of broth rheologies, culture physiology and aeration conditions (Patrick *et al.*, 1993; 1995; 1996).

1.4.1.2. *Statistical optimization models: Response surface methodology*

The optimal production of swainsonine from the submerged *Metarhizium* fermentation needs the systematic screening and optimization of critical parameters *viz.*, type of strain, culture conditions, bioreactor operation conditions (agitation, aeration, vessel and impeller types), medium rheology and culture physiology *etc.* The response surface methodology (RSM) is the commonly used statistical optimization method which provides a polynomial model with universal approximation properties for any number of input and output variables in a very limited number of experiments (Prakasham *et al.*, 2007). Hence, RSM is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors, and analyzing optimum conditions of factors for desirable responses. The RSM based methods has been successfully applied for the medium optimization towards the fermentative production of swainsonine from *M. anisopliae* at the shake flask (Singh and Kaur, 2013). The primary step in any optimization procedure involves the identification of suitable approximations for a relationship *i.e.* independent variable inputs and response. The most common approximations in RSM are low order polynomials (first and second order) which encourage the suitable design of experiment or DOE (Box and Draper, 1987). The fermentative production of

swainsonine has been evaluated in different production media *viz.* complex oatmeal extract (Patrick *et al.*, 1996; Justo *et al.*, 2007; Singh and Kaur, 2012; 2013), modified starch-casein supplemented with L-lysine (Tamerler and Keshavarz, 1999) and Czapekdox medium with and without lysine (Singh and Kaur, 2012). The statistical optimization of fermentative media components and inoculum size has significantly increased the swainsonine production both at the shake flask and bioreactor levels. However, the systematic optimization and scale up studies regarding the *Metarhizium* fermentation models are rarely been studied.

1.4.1.3. *Artificial intelligence based optimization models*

The development of an efficient fermentation model for the lab and industrial scale production of swainsonine is of critical importance because the media components, physiological conditions, bioreactor operational parameters (agitation-aeration) and scale up parameters eventually affects the overall economy of a bioprocess. The last two decades have witnessed the development of the more efficient optimization methods such as artificial intelligence based artificial neural networks (ANNs), Darwinian evolution based genetic algorithms and Fuzzy logics, based on logical reasoning and perceptions.

ANN has emerged as an attractive tool for non-linear multivariate optimizations, having a black-box concept with no prior knowledge of the process. It allowed learning a process model based on input-output response or behaviour. The power of ANN is that it is generic in structure and exhibits the ability to learn from data gathered by users, thus widely known as a reliable modelling tool for process that is experienced lack of information (Ahmad *et al.*, 2010). Furthermore, ANN can also work well even with relatively less data in which experimental data of RSM should be

sufficient to build an effective ANN model (Desai *et al.*, 2008; Singh and Kaur, 2013). Hence, ANN is a learning system based on a computational technique that can simulate the neurological processing ability of the human brain and can be applied to quantify a non-linear relationship between input variables (process parameters) and swainsonine production through *Metarhizium* fermentation modelling by means of iterative training of data obtained from a designed experiment. A typical ANN is made of the following basic components:

(i) Processing element

The unit analogous to the biological neuron is a processing element (PE). Each PE has many inputs and outputs. The network consists of many units or neurons, each possibly having a small amount of local memory. The units are connected by unidirectional communication channels "connections" which carry numeric data. The units operate only on their local data and on the inputs they receive from connection.

(ii) Connection weight

The output path of a processing element is connected to input paths of other PEs through connection weights, analogous to the synaptic strength of neural connections.

(iii) Input, output and hidden layers

A network consists of a sequence of layers with connections between successive layers. Data to the network is presented at input layer and the response of the network to the given data is produced in the output layer. There may be several layers between these two principal layers, which are called hidden layers.

(iv) Training

Most neural networks have some sort of "training" rule whereby the weights of connection are adjusted on the basis of presented patterns. In other words, neural network patterns "learn from example".

(v) Error

It is defined as the total sum of the difference between desired output and output produced by the network for the set of inputs.

(vi) Learning rate

A learning rate regulates the connection weights within the network in response to the example inputs and desired outputs. The training of neural network model is similar to the way humans or animals are trained by reinforcement technique, where certain synapses that connect the neurons selectively get strengthened leading to increase in the gain.

(vii) Recall

Recall refers to how the network processes a data set presented at its input layer and produces a response at the output layer. The weights are not changed during the recall process.

Derived from their biological counterparts, ANNs are based on the concept that a highly inter-connected system of simple processing elements can learn complex inter relationships between independent and dependent variables. ANNs offer an attractive approach to the black-box modelling of highly complex, nonlinear systems having a large number of inputs and out puts in the form of massively connected parallel structures. It has three-layered system as input layer, intermediate hidden

layer and an output layer (Fig. 1.2). Each layer contains a number of neurons. The number of neurons in the input layer equals the number on inputs to the neural network while the number of neurons in the output layer equals the number outputs in the system. Although numerous guidelines have been proposed for selecting the number of units in the hidden layer, they do not work in all situations, and the number is often determined heuristically. Each neuron is connected to all the neurons in the next layer by means of a "connection weight". The output from neurons can be calculated by suitable "transform equations" provided the inputs and the connection weights are known. The sequence of neural network modelling is to assume a set of weights initially, compute the outputs and the predict error, and then adjust the weights according to an error minimization technique until the prediction error falls to an acceptable level. This activity of finding optimal weight is called network training. Once the network is so trained, the black-box model is ready, and may be used to predict outputs for a set of new inputs, not originally part of those used in training.

1.4.1.4. *Hybrid genetic algorithms: Artificial neural network- genetic algorithm*

In recent years, genetic algorithm (GA) based on ANN model as an objective or fitness function has been applied successfully in optimizing the input space of various bioprocess studies (Singh and Kaur, 2013; Zafar *et al.*, 2012). GA is an artificial intelligence based stochastic non-linear optimization technique which solves optimization problems based on natural selection or Darwin's theory of evolution (Desai *et al.*, 2008). In hybrid ANN-GA optimization model, The ANN derived fuzzy population (collection of individual sets of experiment) is set to undergo the various genetic operations *i.e.* selection, crossover, mutation etc. to finally produce a most evolved or optimal sets of solution over successive generations. Hence, in a particular

environment, the individuals (solutions) who better fit the environment will be able to survive and hand down their chromosomes (optimal inputs variables) to their descendants, while the less evolved individuals will become extinct. In the original genetic algorithm an individual chromosome is represented by a binary string. The bits of each string are called genes and their varying values as alleles. A group of individual chromosomes are called a population. Basic genetic operators include reproduction, crossover and mutation. Genetic algorithms are especially capable of handling problems in which the objective function is discontinuous or non differentiable, non-convex, multimodal or noisy. Since the algorithms operate on a population instead of a single point in the search space, they climb many peaks in parallel and therefore reduce the probability of finding local minima (Whitley, 1988). Nagata and Chu (2003) analysed RSM data (presented by Achary *et al.*, 1997) using neural network and GA to predict optimum conditions and reported that feed-forward neural network and GA (FFNN-GA) is a more efficient optimization model. A more advanced form of Real Coded GA is also practiced instead of conventional Binary Coded GA. To compensate for the excessive computation time required by the BGA, the real encoded genetic algorithm (REGA) emphasizing on the coding of the chromosomes with floating point representation was introduced and proven to have significant improvements on the computation speed and precision (Gen and Cheng 2000; Goldberg 1991). The appropriate implementation of GA includes the definition of the objective function, definition and implementation of the genetic representation, and definition and implementation of the genetic operators. The basic operation scheme for a typical GA is shown in Fig 1.3. At the same time, much effort was imposed to improve computation performance of GAs and avoid premature convergence of solutions. GA is capable of exploring large input variables' space

through the search operators *viz.*, selection, crossing over, mutation and elitism (Weuster-Botz, 2000). These GA operators are as discussed below:

(i) Selection (of the mating pool)

The ANN generated population/chromosomes (experimental sets) undergoes the selection procedure to seek out the best fitted individuals for mating to generate the consecutive populations.

(ii) Crossover

The algorithm exchanges the parent chromosomes (if binary coded) between pairs of solutions in mating pool with some probability (p_{cross}) to produce the candidates for the next generation population. For the real encoded population individuals, the random combination of two best candidates from the mating pool is set to undergo random cross over with some defined probability for the consecutive iterations

(iii) Mutation

The binary coded population individuals are mutated by flipping bit values by some defined or random probability value (p_{mut}). The real encoded population individuals are mutated with some random integers replacing the integer values in another individual, preferably in a very low frequency. The final result of the mutation is only selected if it is a feasible candidate solution.

(iv) Elitism

This step of the algorithm is only employed if the population individuals generated by crossover and mutation steps are worse or less evolved than the parent

individuals to prevent the less evolved candidates to be passed to the next generation. In this situation, the whole steps are repeated to seek the better evolved candidates.

The biochemical engineering applications of artificial intelligence based ANN-GA (real or binary coded) optimization models would present an economic incentive towards the fermentative production of swainsonine. Singh and Kaur (2013) have described an ANN combined with real encoded genetic algorithm (ANN-REGA) to optimize the production media components for swainsonine production at shake flask levels, and compared the optimization efficiency of the model with that of statistical RSM- optimization model.

The optimization of bioreactor operation conditions *i.e.*, agitation, aeration and incubation time is also studied using the real value encoded evolutionary programming (EP) of the similar nature and correlated successfully with the scale up parameters (oxygen mass transfer coefficient K_La s^{-1} and gassed power per unit of volume Pg/V_L W/m^3) for swainsonine production. The nonlinear behaviour and time varying properties make bioreactors difficult to control with traditional techniques. In this context, there is a need to consider quantitative mathematical models, capable of describing the process dynamics and the interrelation among relevant variables. Additionally, robust optimization techniques must deal with the complexity of models, the environment constraints and the inherent noise of the experimental process. GA has proven to be extremely suitable for the optimization of highly non-linear problems with many variables associated to a variety of microbial fermentation models. There are numerous reports regarding the successful applications of these heuristic optimization models for the optimization of various fermentation models, such as the three folds increase in mevinolin biosynthesis by *Aspergillus terreusi* (Zuzek *et al.*, 1996), nearly two fold increase in the scleroglucan production (Desai *et*

al., 2008), 6 % increase in glucansucrase activities (Singh *et al.*, 2008) and about 70 % enhancement in marine biosurfactant production (Sivapathasekaran *et al.*, 2010) etc. Process modelling is a combination of both science and art, because a good dose of creativity is required to make assumptions for a computationally simple yet predictive model. Modelling inherently involves a compromise between accuracy (complexity), cost and effort involved in developing a model. There are many optimum design methods which combine the optimization algorithms with the computer simulations that have been reported for fermentation modelling (Abakarov *et al.*, 2009; Guo *et al.*, 2010).

1.5. Downstream recovery of swainsonine

1.5.1. Partial purification and solvent extraction

The extraction and purification of swainsonine have always posed a challenge, principally because of its chemical structure and properties. The three hydroxyl groups in swainsonine make the compound extremely soluble in polar solvents with little or very low solubility in organic solvents. The significant degree of hydrophilicity and lack of differential solubility between the typical chloroform and water solvent systems make the extraction of such compounds very difficult using the basified aqueous solutions, commonly employed for the extraction of alkaloids. The polyhydroxy alkaloids of similar class have traditionally been extracted (from plant or fungal sources) using 25-50 % methanol or ethanol in water or the water alone under low pH conditions (Fellows *et al.*, 1979). Swainsonine was initially extracted with ethyl acetate (Colegate *et al.*, 1979), acetone and alcohol-water mixtures (Molyneux and James, 1982), but in general the use of alcohol-water solvent mixtures is most suitable because of its high solubility in polar sovents. Recently, swainsonine was

extracted with four different polar solvent systems (a. Chloroform and 2% acetic acid, b. 2% acetic acid and chloroform, c. 2% acetic acid and d. methanol) under different physiological conditions (Gardener and Cook, 2011). Although the use of aqueous or alcoholic solvents results in the co-extraction of many other polar constituents *viz*. sugars or low molecular weight amino acids etc., together with the desired alkaloid. Wang *et al.* (2010) has proposed an ultrasound assisted mechanical extraction method for swainsonine extraction from *Oxytropis ochrocephala* Bunge followed by the thin layer chromatography (TLC) and gas chromatography (GC) characterization of the extracts. Gardener and Cook (2011) reported the Strata-X-C solid-phase extraction of swainsonine.

1.5.2. Chromatographic purification

The use of various solvent systems resulted in the co-extraction of impurities along with the desired alkaloid, so the next step will be the use of various chromatographic techniques to achieve maximum purification of swainsonine.

1.5.2.1. *Ion Exchange chromatography*

Ion exchange chromatography was reported for the removal of extraneous compounds optimally. A variety of ion exchange resins including both cationic and anionic have been used. Dowex 50 and Amberlite CG 120 with NH^{+4} or H^{+1} ion forms were commonly employed for swainsonine purification (Hohenschutz *et al.*, 1981). Weak cation exchange resin CM Sepharose CL-6B by a linear gradient of sodium chloride (0–1 M) in sodium acetate (10 mM, pH 5) was also used for swainsonine (Colegate *et al.*, 1979) The polar packing materials are usually avoided for column purification of swainsonine because the highly basic and hydrophilic nature of these compounds resulting in their strong adsorption on the polar matrices like silica gel,

making the elution process extremely difficult (Saul *et al.*, 1983). However, Hino *et al.* (1985) have reported the column purification of swainsonine from *Metarhizium* spp. using silica gel matrix and elution using n-butanol-ethanol-chloroform-28 % ammonium hydroxide (4:4:4:1), but purification efficiency is still need to be analysed. The simultaneous extraction-purification was also reported for swainsonine from *Oxytropis*, involving liquid-liquid extraction with chloroform and 2 % acetic acid, followed by rapid concentration of acidic layer with Dowex 50W-X8 column and elution with 1M NH_4OH (Gardner *et al.*, 2001).

1.5.2.2. *Thin layer chromatography*

The preparative thin layer chromatography (TLC) is also applicable for swainsonine purification in a very efficient way, provided the concentration of the compound is exceptionally high in the extracts. The TLC method is more rapid, economic and convenient to perform under laboratory conditions. Although the preparative TLC method is more convenient, the major problem arises in developing the alkaloid spots with very similar R_F values and its separation from the stationary phase. However, centrifugal radial TLC combines high separation speed with flexibility in fraction sizes collected, and alkaloids with very similar R_F values may be resolved (Stahl and Muller, 1982). Swainsonine was purified by TLC from the extracts of *Rhizoctonia leguminicola* on silica gel-G plates using acetone-chloroform-50 % aqueous diethylamine at 60:20:20 ratios of mobile phases (Guengerich *et al.*, 1973). The preparative TLC is advantageous to purify larger (grams) quantities of the crude alkaloid extracts once a suitable procedure is developed, while the primary disadvantage is the primary optimization of the method and requirement of large amount of solvents as mobile phase. Another major hurdle encountered is the

development and resolution of the alkaloids on the preparative TLC plates. The acetate derivatives of swainsonine have been reported as less polar and relatively more mobile on the non-polar mobile phase used in TLC separation. However, the acetate derivatives need to be again deacetylated for obtaining the biochemically active swainsonine again after elution (Harris *et al.*, 1988).

1.6. Analysis and detection of swainsonine

There has been a continued interest in the study of swainsonine because of its wide therapeutic applications. The lack of suitable chromophore in swainsonine renders the use of conventional methods of chromatography with ultraviolet (UV) detector difficult (Pastuszak *et al.*, 1990). For this researchers have always been in the search of sensitive, undemanding and high throughput analytical methods for swainsonine analysis. There are several methods developed and also novel techniques will increasingly be applied for swainsonine analysis.

1.6.1. Enzymatic Assay

Enzymatic assay is the most rapid, simplest procedure for swainsonine analysis. Sim and Perry (1995) described a novel and simple enzymatic assay method for swainsonine analysis, based upon its specific inhibitory properties towards α-mannosidase. The assay developed was applied to culture supernatants to investigate the production of swainsonine by *M. anisopliae*.

1.6.3. Thin layer chromatography

Analytical thin layer chromatography (TLC) has proved to be applicable to the polyhydroxy alkaloids for purity determination and detection. Chromatographic conditions were similar as for preparative TLC (Section 1.5.2.2). However,

swainsonine is quite insensitive in low concentration to commonly used reagents for alkaloid visualization such as Dragendorff's or iodoplatinate. A highly sensitive dual reagent spray system consisting of 10% acetic anhydride in benzene, followed by Ehrlich's reagent (p-dimethylaminobenzaldehyde in ethanol containing boron trifluoride etherate was developed for swainsonine analysis with a minimum detection level of about 0.5 mg (Saul *et al.*, 1983; Molyneux *et al.*, 2007).

1.6.3. Gas chromatography and gas chromatography-mass spectrometry

The structural relationship of polyhydroxy alkaloid, swainsonine to sugars has pointed the way to gas chromatography (GC) as a technique for analysis. GC analysis of swainsonine was initially performed in the serum of cancer patients under clinical trials (Bapista *et al.*, 1994). As with the sugars, the underivatized alkaloids are too polar for GC in their native form and hence require derivatization for GC analysis. The polyhydroxy alkaloids including swainsonine are first needed to be derivatized using trimethylsilyl (TMS) or N-methyl-N-trimethylsilyltrifluoracetamide (MSTFA) in pyridine to provide the necessary volatility and stability (Molyneux *et al.*, 2002). Trimethylsilylation of polyhydroxy alkaloids belonging to several different structural classes was first achieved with trimethylchlorosilane-hexa methyldisilazane-pyridine (1:3:9) at 50 °C (Nash *et al.*, 1986). In combination with mass spectrometry, gas chromatography-mass spectrometry (GC-MS), the GC technique shows greater potential for analysis of polyhydroxy alkaloids in the plant, microbial and animal tissue samples. A considerable degree of structural information may be deciphered from the GS-MS analysis of the derivatized alkaloids. The fragmentation pattern of the TMS derivative of swainsonine and castanospermine is analogous to that of the underivatized alkaloids showing cleavage of the pyrolidine ring at the β-bonds to the

nitrogen atom. Thus, the method is also helpful in the identification, structural elucidation and quantitative analysis of the polyhydroxy alkaloids. However, GC-MS method seems to be having certain drawbacks regarding the complexities of pre column derivatization is the major and the tedious methodology involved (Gardner *et al.*, 2001).

1.6.4. Liquid chromatography

Liquid chromatography (LC) will be undeniably the most suitable technique for swainsonine analysis because it requires no derivatization. However, it is very difficult to detect swainsonine by conventional UV detector due to lack of chromophore group. A more serious problem is its extreme hydrophilicity and generally low solubility in non-polar mobile phases impeding the development of suitable solvent system and choice of column matrices. Donaldson *et al.* (1990) have described the pulsed amperometric LC analysis for swainsonine in high performance ion exchange chromatography (HPIC) under isocratic elution using Dionex CS3 cation exchange column. The method involved the post column elution with mild basic sodium hydroxide, with swainsonine elution in the linear range of 10 ng/ml-20 µg/ml. However, the lower detection limit is substantially high (10–50 ng/ml) and hence, the sensitivity of method is comparatively low.

Evaporative light scattering detector (ELSD) is considered as a universal detection mode suitable for non-chromophoric compounds (Wang *et al.*, 2006). Since the response of ELSD depends on the size, shape and number of eluate particles rather than the structure and chromophore of analytes, any compound less volatile than the mobile phase can be detected by ELSD (Li *et al.*, 2001). The term hydrophilic interaction chromatography was first coined by Alpert (1990) to distinguish this

technique from normal-phase chromatography. It is a useful technique for the increased retention of polar analytes that offers a difference in selectivity compared to traditional reversed-phase chromatography (Grumbach *et al.*, 2004). Recently Yang *et al.* (2012) have described an isocratic high performance liquid chromatography-evaporative light scattering detector (HPLC-ELSD) method for swainsonine detection and quantification from fungal endophytes, *Undifilum oxytropis*.

1.6.5. Liquid chromatography-mass spectrometry

Mass spectrometry (MS) provides a next-generation platform that overcomes many of the limitations of enzymatic, TLC, HPLC and GC techniques, and provides new capabilities for swainsonine analysis. The liquid chromatography-mass spectrometry (LC-MS) analysis with electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) proved to be useful for the analysis and identification of polyhydroxy alkaloids of plants and microbial origins. A practical application of LC-MS and LC-MS/MS methods for the analysis of swainsonine has been first demonstrated in the plant extracts from Locoweed genus O*xytropis* (Gardner *et al.*, 2001). The analysis was performed on the reverse phase C_{18} (100×32 mm) column using the isocratic mobile phase (5 % CH_3OH in 20 mM aqueous $CH_3COO.NH_4$) with APCI in positive ion mode with sequential tandem mass spectrometry (MS/MS). The first order mass spectrum of swainsonine showed highest counts for protonated MS $[M+H]^+$, m/z = 174 and considerable counts for the dehydroxylated MS/MS ionic species $[M-OH]^+$, m/z 156 in positive ion mode.

1.6.6. Flow injection mass spectrometry (FIMS)

A direct flow injection mass spectrometry (FIMS) can be a method of choice for the automation analysis of swainsonine. FIMS method is the simplest form of

rapid sample introduction into the mass spectrometer, contrary to tedious and time-consuming sample preparation and a long laborious LC-MS runs (Zhang *et al.*, 2004). FIMS is a selected ion monitoring (SIM) mode depending on the principle that the MS-counts (MS or MS/MS) for a particular species are selectively extracted from the total composite ion peaks in the MS spectra, which is specifically correlated with the corresponding concentration of a particular ion using a regression curve under a given set of conditions (Fattorusso and Taglialatela-Scafati, 2007). The feasibility of the method was demonstrated using both atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) (Zhang *et al.*, 2004). The characteristic MS protonated ion counts for swainsonine, $[M+H]^+$, m/z = 174, will be considered for qualitative as well as quantitative analysis. The FIMS quantification method is recently been applied for the quantification of polyethylene glycol (PEG-300) in drug formulations (Zhang *et al.*, 2004), sulfonyl urea and carbamate class of agrochemicals (Nanita *et al.*, 2009), organophosphorus pesticides dimethoate and omethoate in porcine plasma and urine (John *et al.*, 2010), polyethylene glycol (PEG-400) excreted in human urine after oral administration (Ashirua *et al.*, 2011; Bhaskara *et al.*, 2013), multiple chain length ceramides in biological samples (Chen *et al.*, 2012) and compared well with the conventional LC-MS/DAD methods. There is a growing interest in the research related to the analysis of swainsonine in plants, microbes and animal tissues. The development of simple, rapid and high throughput methods of purification analysis would facilitate to catalogue the swainsonine content and fulfil its necessary supply for biological evaluations.

1.7. Therapeutic applications of swainsonine

Swainsonine is a well known potent and specific inhibitor of lysosomal, cytosolic α-mannosidases, as well as Golgi α-mannosidase II (Gerber-Lemaire and Juillerat-Jeanneret, 2010). The mannosidase inhibition of swainsonine is extremely specific for acidic α-mannosidases and not even towards other acid glycosidases including β-mannosidase (Fig. 1.4). The three hydroxyl groups of swainsonine are oriented in the same spatial configuration as those of the corresponding substrate, α-D-mannopyranoside in such a way that inhibitor is quite specific for this enzyme. The Lineweaver-Burk plots of substrate concentration curves in the presence of various concentrations of swainsonine (inhibitor) showed considerable curvature at the high substrate concentrations and suggesting its competitive inhibition mechanism of action towards α-mannosidases (Kang and Elbeing, 1983).

The therapeutic application of swainsonine has been mainly linked to the inhibition of Golgi α-mannosidase II by blocking the production of the complex β-1,6-branched N-linked glycans (Wrodnigg *et al.*, 2008). Swainsonine acts as an inhibitor of Golgi α-mannosidase II, an enzyme involved in the glycoprotein processing of mannose residues during N-linked oligosaccharide maturation on the surface of tumor cells. This leads to an increase in high mannose oligosaccharides and decrease in the normally formed complex type oligosaccharides at the cell surface (Elbein *et al*, 1981). Cells that grow with these hybrid surface oligosaccharides are reported to show the altered functional properties depending upon the interactions of surface receptors with ligand molecules (Andrzej *et al.*, 1989). Since these alterations in the normal glycosylation patterns are associated with tumorous cells undergoing metastatic dissemination and similar disease progressions. The malignant transformations are often accompanied by the altered expression levels of complex glycans which might

be regulatory biomolecules for cell to cell adhesion, recognition, migration and apoptosis. Hence, the controlled the modulation of glycan expressions with certain interfering compounds has promising potential in cancer chemotherapy (de-Freitas-Junior *et al.*, 2012). The authors have found a significant cytotoxic activity of N-glycan biosynthesis inhibitors, swainsonine and tunicamycin against HT-116 colon cancer cell line. These inhibitors drastically influenced cell to cell adhesions and migrations in cancerous cells *in vitro*. Therefore, inhibition of Golgi α-mannosidase II offers a potentially effective target for cancer chemotherapy. In this context, swainsonine is notable as being the first glycoprotein processing inhibitor selected for clinical evaluation having promising future. There are number of studies which also suggested that swainsonine inhibits the normal α-mannosidase II activity, involved in the asparagine linked glycoprotein processing. When canine kidney cells were incubated in swainsonine and labeled with ^{3}H mannose, there was a considerable decrease in the amount of radioactivity incorporated into the complex types of glycoproteins, whereas the radioactivity incorporated into the high-mannose glycoproteins was either unchanged or somewhat elevated (Elbein *et al.*, 1981).

1.7.1. Anticancer activities and cancer chemotherapy

Oligosaccharides on glycoproteins mediate a dynamic protein state, involving folding, quality control, secretion and catabolism (Varki, 1993; Dwek, 1996). Glycans are also related to tumor progression and metastasis as well as to immune system activity (Hakomori, 1996). Recently, the potential relationship of glycans with chemoresistance has been evaluated, suggesting the use of swainsonine as adjuvant in chemotherapy for cancer treatment (Hamaguchi *et al.*, 2007; Kudo, 2007). Swainsonine has also been shown to promote anti-tumor immunomodulatory activities

of immune system and protect both murine and human bone marrow against the toxicity of chemotherapeutic drugs (Klien *et al.*, 1999; Oredipe *et al.*, 2003). Swainsonine also enhances the effectiveness of anti-tumor agent, 5-fluorouracil (5-FU) by indirectly reducing the drug resistance in the target malignant cells (Hamaguchi *et al.*, 2007). The effective inhibition of both lysosomal α-mannosidase and Golgi α-mannosidase II by swainsonine is of paramount importance in cancer therapies. Swainsonine has been shown to reduce tumor cell invasion in Phase I human cancer trials and to enhance the natural antitumor defenses of the body, such as lymphokine activated killer cell (LAK) and natural killer cell (NK) cytotoxicity (Galustian *et al.*, 1994; Goss *et al.*, 1994). However, in the Phase II trials, no antitumor activity was observed in patients with locally advanced or metastatic renal cell carcinomas, and the treatment was discontinued due to disease progression and the toxicity of the compound (Shaheen *et al.*, 2005). Analogues of swainsonine have been shown to have a reduced toxicity, but they also have a diminished antitumor effect (Gerber-Lemaire and Juillerat-Jeanneret, 2010). However, swainsonine has been shown to increase tumor cell sensitivity to cytotoxic drugs and to reduce resistance to anticancer drugs in multistage malignancies.

Swainsonine was also verified to decrease expression of various apoptosis inhibiting genes in different tumor cells, which culminated with the induction of apoptosis *in vitro* and *in vivo* (Sun *et al.*, 2007, 2009). When swainsonine was administered as an adjuvant with chemotherapeutic drug cisplatin, antitumor effects like DNA damage, apoptosis and cell cycle arrest were observed to be enhanced *in vitro* (Santos *et al.*, 2011). In another study of swainsonine induced apoptosis in human gastric carcinoma cells SGC-7901, the intracellular overload of Ca^{2+} was observed. Ca^{2+} ion act as a second intra-cellular messenger of signal transduction and

participates in many kinds of physiological activity such as muscle contraction, nerve conduction, cell proliferation and differentiation. High level Ca^{2+} ions interferes severely with the mitochondrial oxidative phosphorylation, down-regulates production of adenosine triphosphate, disorganizes mitochondrium, activates phospholipases and even promotes membrane phospholipid hydrolization. This in turn, causes cellular membrane and organ damage, activating proteinase free radical multiplication, or expression of gene to apoptotic mode of cell death (Sun *et al.*, 2007). Similar studies have shown that anticancer and tumor suppressive affects of swainsonine both *in vitro* and *in vivo* against human melanoma, colon, gastric, lung cancer, C6 glioma and A549 cancer cells through a variety of altered gene expression mechanisms (Dennis *et al.*, 1989a, b, 1990; Li *et al.*, 2012).

The detailed analysis of apoptotic cell death induction in human oesophageal squamous carcinoma (Eca-109) cells by *in vitro* treatment of swainsonine revealed a complex cascade of genes triggered mechanism (Fig. 1.5). The study demonstrated a mitochondria mediated-caspase dependent pathway, where swainsonine treatment up-regulated Bax, down-regulated Bcl-2 expression, triggered Bax translocation to mitochondria, destructed mitochondria integrity and activated mitochondria-mediated apoptotic pathway, followed by the release of cytochrome C. These biochemical signals activated caspase-9 and caspase-3 genes, resulting in the cleavage of poly (ADP-ribose) polymerases-PARP, essential for various functions related to cell physiology and maintenance. This chain of events finally results in a state of apoptotic cell death induction in Eca-109 cells (Li *et al.*, 2012). The several lines of evidence suggesting the toxicological application of swainsonine ranging from the diverse fields of agriculture to the medicine prompted us to explore its dynamic application possibilities.

1.8. Lacunae

As is evident from the literature, the work on swainsonine production, quantification and cytotoxic application is very scarce, which has motivated us to work in this area. The earlier studies are mostly concentrated to the swainsonine extraction from the plants and very few reports describe its fermentative mode of production through systematic and advanced optimization models. The swainsonine production still remains a challenge from both biological as well as chemical synthetic routes, due to its high downstream processing cost. Hence, efficient extraction, purification and analytical methodology are needed to develop for the economic recovery of swainsonine from the *Metarhizium* fermentation broth. However, the recent reports also suggested its various therapeutic applications which are still to be elucidated in detail. Moreover, the role of swainsonine in the chemical ecology of *Metarhizium*-arthropod interaction and its entomotoxicity are never been addressed, and hence needs a greater attention.

1.9. Objectives of the study

The present study describes a complete process developed for the production, purification of swainsonine from *Metarhizium anisopliae*. *In situ* entomotoxic as well as therapeutic potentials of the purified swainsonine were also studied. The overall work is divided into the following six specific objectives to address the earlier proposed lacunae (Secion 1.8).

1. Screening of *Metarhizium* strains, media components and physiological culture conditions for fermentative swainsonine production.
2. Shake flask optimization of production media components.
3. Optimization of operational conditions and scale up parameters at bench-top bioreactor level.
4. Purification, characterization and quantitative analysis of swainsonine.
5. Analysis of *in situ* entomotoxic effects of swainsonine in *Spodoptera frugiperda* cells, *Sf*-21.
6. Analysis of *in situ* anticancer activities of swainsonine in human promyelocytic leukemia cells, HL-60.

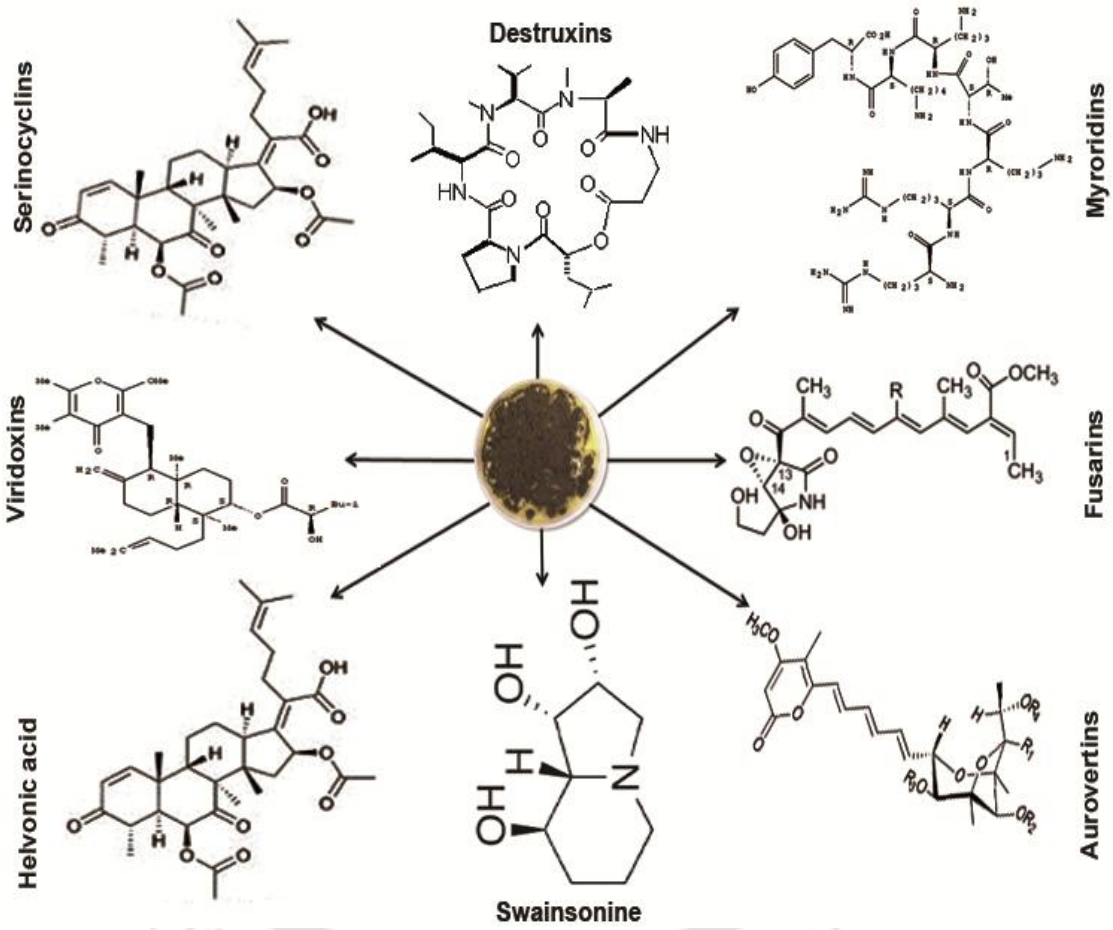
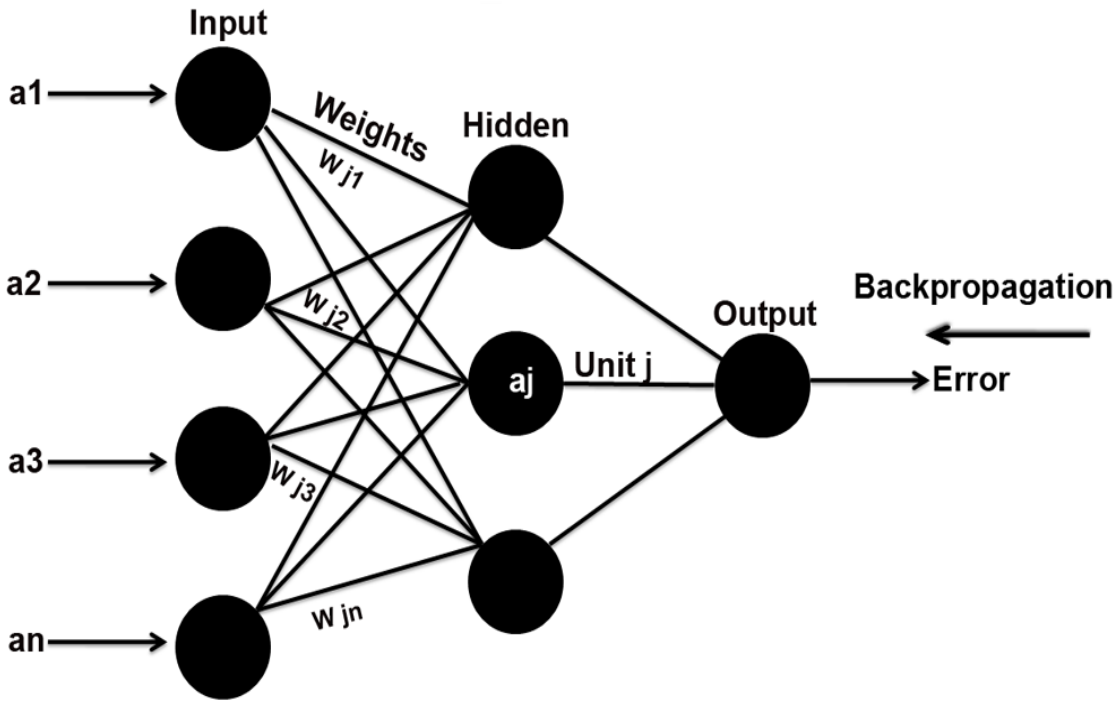


Fig. 1.1. Bioactive secondary metabolites reported from *Metarhizium* spp.



$$S_j = \sum_{i=0}^{n} W_j \; a_j$$

$$a_j = \frac{1}{1+e^{-s_j}}$$

Fig. 1.2. A typical feedforward neural network (FFNN) where process parameters are described as input variables (a1, a2, a3...an) with their corresponding weights (Wj1, Wj2...Wjn) (Adopted from Ahmed, 2005). Here the corresponding equations are representing the sum of the input function **(S_j)** with the corresponding input's weight (**W_j**), with **(a_j)** is the activation value for the unit **(*i*)** and **(*n*)** stands for number of inputs that send connections to unit **(*j*)**.

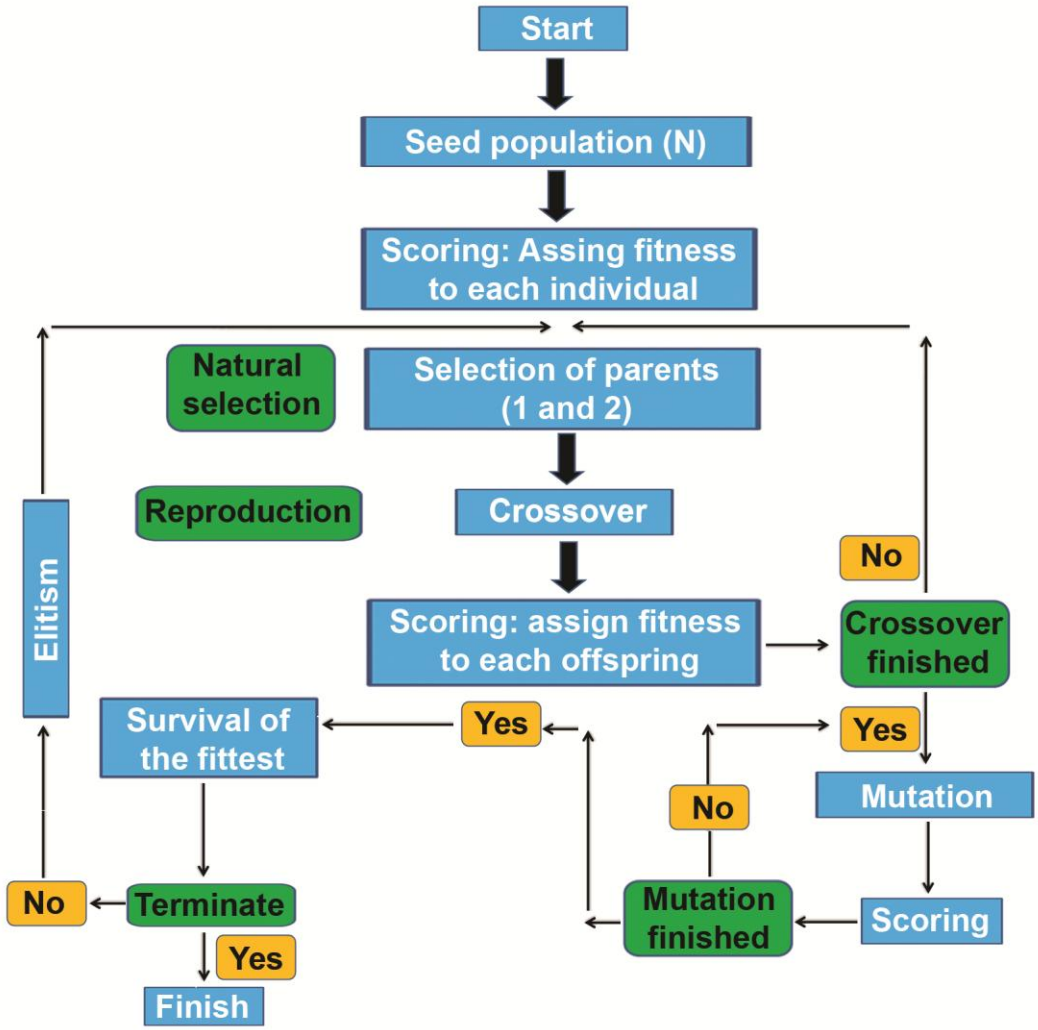


Fig. 1.3. Schematic diagram for a typical genetic algorithm (GA) operation.

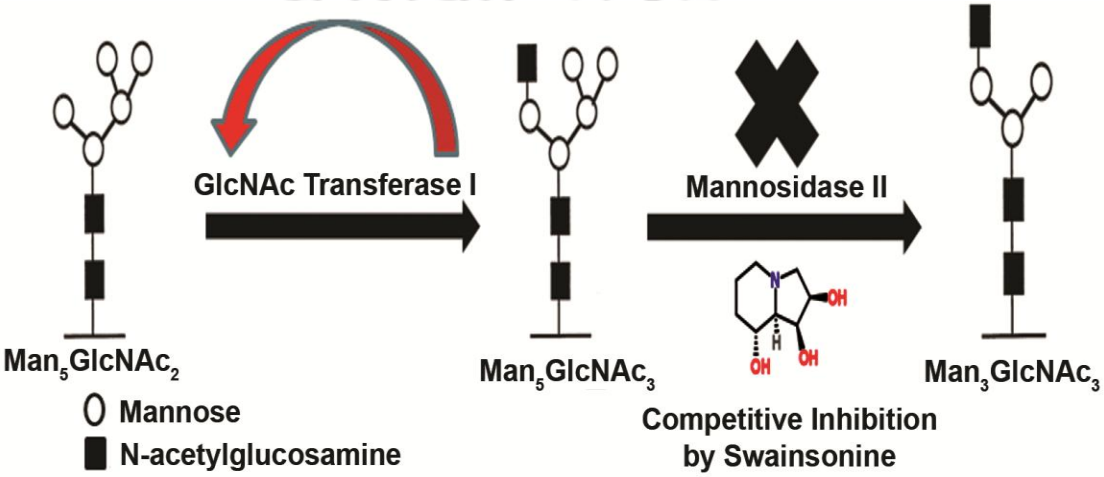


Fig. 1.4. Inhibition of Golgi oligosaccharide processing by swainsonine (adopted from Houston *et al.* (1997).

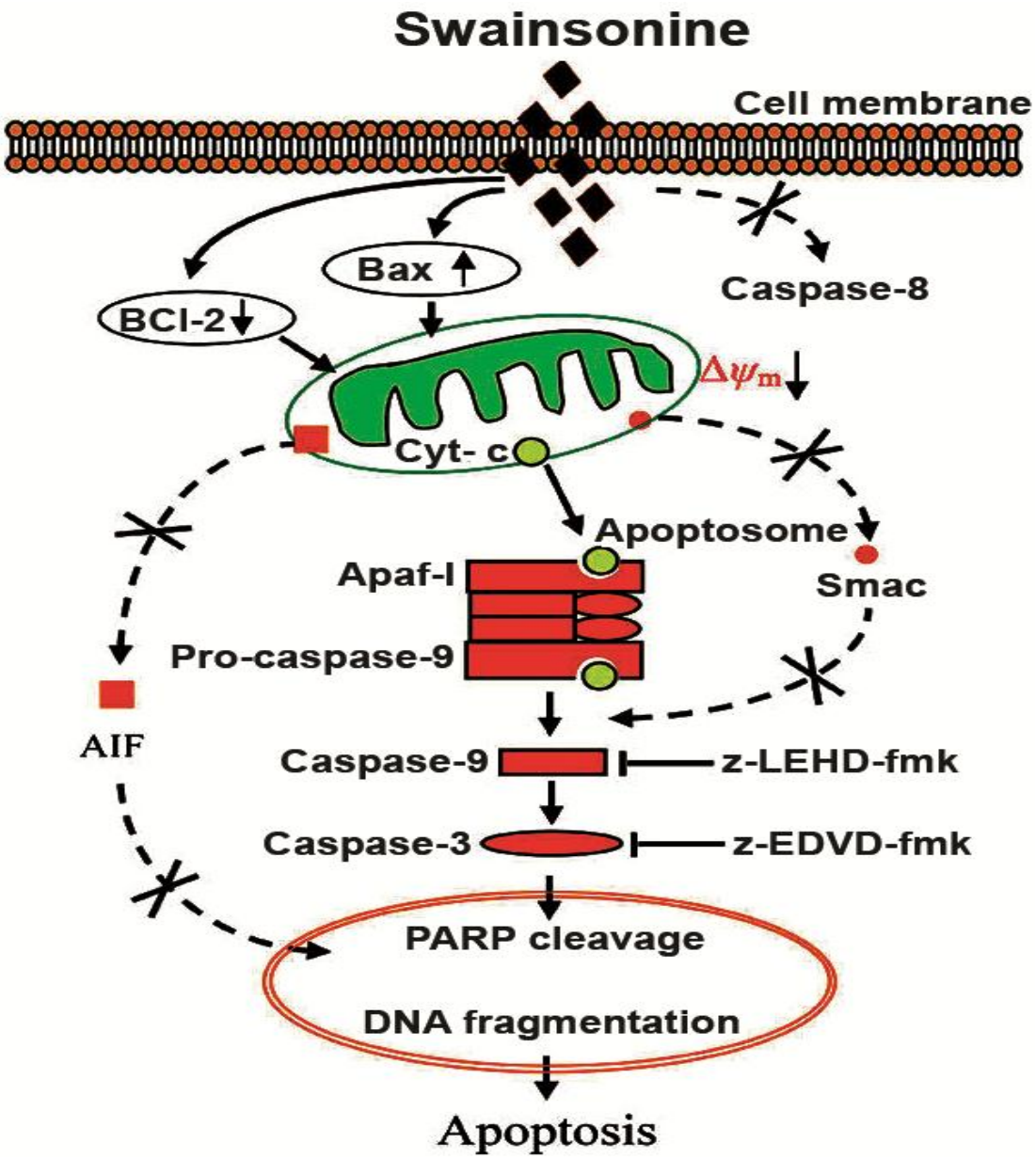


Fig. 1.5. Proposed model of molecular mechanism of swainsonine-induced apoptosis in Eca-109 cells (Adopted from Li *et al.*, 2012).

CHAPTER 2

Screening of *Metarhizium* strains, media components and physiological culture conditions for fermentative swainsonine production

2.1. Introduction

Swainsonine has attracted significant attention in recent years because of its antimetastatic, antiproliferative and immunomodulatory properties finding its potential applications in the malignant chemotherapies (Hino *et al.*, 1985). Swainsonine is mainly extracted from locoweed species, but extraction from plants is uneconomic on account of the necessity of large cultivable lands as well as tedious extraction procedures (Tamerler *et al.*, 1997). Chemical synthesis of swainsonine has also been developed, but it is very expensive due to the difficulties created by their four chiral centers resulting in more than twenty reaction steps and in racemic mixtures. Moreover, chemical synthesis is low yielding in efficiency (Nemr, 2000). Microbial production of swainsonine is being investigated as an alternative route to its chemical synthesis and extraction from plants, which has been reported from *Rhizoctonia leguminocola* (Schneider *et al.*, 1983), *Metarhizium anisopliae* (Hino *et al.*, 1985; Patrick *et al.*, 1995, 1996;) and by the fungal endophytes of locoweed (Braun *et al.*, 2003). The shake flask production of swainsonine was first scaled up to different bioreactor levels by Patrick *et al.* (1993, 1996; Patrick *et al.*, 1996). The fermentative production of swainsonine has been reported to be influenced by different strains, production media components, inducers, metal ions, pH and physiological conditions (Patrick *et al.*, 1995; Justo *et al.*, 2007; Tamerler *et al.*, 1998). However, the detailed analyses of these significant parameters were not evaluated individually and/or cumulatively with respect to the fermentative production of this alkaloid. The *Metarhizium* fermentation process was primarily carried out using complex oatmeal medium at 28±2 °C and 180-200 rpm. The culture growth and metabolite production rates were also evaluated in glucose and inducer (L-lysine) supplemented media for different incubation periods with respect to different

Metarhizium strains (Justo *et al.*, 2007). A linear correlation has been established between the culture physiology in fermentation process and the metabolite production rates, signifying the impact of operational conditions on the yield. A regulatory influence of pH shift on the production of both primary and secondary metabolites has been demonstrated. The pH of the culture broth is one of the several parameters, which affect morphology and productivity. Dramatic changes in cell size or shape are the responses of microbes to changes in pH (James *et al.*, 1991; Tseng and Montville, 1992; Tamerler *et al.*, 1998).

The past two decades have witnessed numerous efforts regarding the fermentative production of swainsonine, but none of the reports have ever described the systematic optimization procedures and models in this direction. The optimization of production media and fermentation conditions plays an important role in developing bioprocesses and improving their performance which can be manipulated by the conventional or statistical methods. Therefore, optimization of nutritional and physical parameters for maximum swainsonine production is of utmost importance for making an economic and cost-effective production at industrial level. It is therefore essential to identify, screen and optimize the primary process parameters, which can significantly manoeuvre the efficient as well as economic production of swainsonine through fermentation means. In order to perform the identification and screening of most effective parameters in fermentative production of swainsonine, the major nutritional factors (C-N sources, growth factors, inducers, metal ions, *etc.*) and physiological culture conditions (pH and temperature) towards *Metarhizium* cultivations first need to be addressed. Besides these parameters, the operational parameters (agitation, aeration, mass transfer) and incubation hours significantly affects the fermentation process. In conventional ‘one factor at a time’ (OFAT)

approach, the nutritional/cultural factors are optimized by changing one factor at a time, and keeping other variables constant. Optimization of a bioprocess using one OFAT method is well studied but has certain disadvantages such as time consumption, more number of experimental runs and lack of knowledge about the interaction between the variables involved in the process (Abdel-Fattah and Olama, 2002). This demerit can be overcome by the use of the Plackett-Burman (PB) screening of process parameters, which involves the evaluation of individual process parameters and the effects of their interaction towards a process (fermentative production of swainsonine). The '*N*' number of process parameters can easily be evaluated in '*N+1*' experimental runs using PB design of experiment (DOE) (Naveena *et al.*, 2005). The design matrix involves both the screening of parameters and optimization of their effective value range. The statistical DOE can overcome the drawbacks encountered in convention methods with a detailed analysis of statistical significance values assigned to each of the parameter. An optimally balanced culture medium is a mandatory prerequisite for the maximal production of the secondary metabolites through fungal fermentations.

Although there are number of reports available regarding the chemical synthesis of swainsonine apart from its extraction from plants, only few are applicable for its scale up because they are either highly expensive or less efficient. Hence, there is a need for improvement in its production of swainsonine through fermentation. The present study aimed at the identification, screening and analysis of individuals as well as interactive effects on the fermentative production of swainsonine from *Metarhizium anisopliae* strains.

2.2. Materials and Methods

2.2.1. Fungal strains, culture and storage conditions

Nineteen different *Metarhizium* strains were procured from The Plant Protection Unit, United States Department of Agriculture (USDA), New York, Ithaca. The individual strains were named with prefix ARSEF - 'Agriculture research service for entomopathogenic fungi' followed by the isolate number. The cultures were again named in the lab with the particular prefix UM- '*Metarhizium* from USA' followed by a unique numeral. All the cultures with their specific codes, geographical origin and host insects are described in the Table 2.1. The cultures were grown on sabouraud dextrose agar (SDA) slants at 28 °C and maintained at 4 °C. The cultures were propagated by sub-culturing every 4 weeks. Ten day old SDA slants were used for the preparation of conidial suspension at 4×10^8 spores/ml for inoculation.

2.2.2. Chemicals and Reagents

Swainsonine standard compound (from *M. anisopliae*), α-D-Mannosidase (from Jack beans), p-nitrophenyl-α-D-mannopyranoside, α-L-fucosidase (from bovine kidney), p-nitrophenyl-α-D-fucopyranoside, L-glutathione reduced were all obtained from Sigma Aldrich, USA. All the growth media (oatmeal, sabouraud dextrose yeast extract agar and potato dextrose agar) and their components were purchased from Himedia, India. All the chemicals used were of analytical grade.

Citrate Buffer (0.5 M, pH 4.5)

Solution I: 0.5 M citric acid (M.W. 192.12); 9.6 g of citric acid was dissolved in 100 ml of double distilled water (ddH_2O).

Solution II: 0.5 M sodium citrate dihydrate (M.W. 258.06); 25.80 g of sodium citrate dehydrate was dissolved in 100 ml of ddH_2O.

Procedure: The pH of solution II was adjusted to 4.5 by gradually adding the solution I and the volume of the mixture were finally adjusted to 100 ml by adding ddH_2O.

Borate Buffer (0.2 M, pH 9.8):

Solution I: 0.2 M Boric acid (M.W. 61.83); 1.36 g of boric acid was added to 100 ml of ddH_2O.

Solution II: 0.2 M sodium tetraborate (M.W. 381.37); 7.62 g of sodium tetraborate (Borax) was added to 100 ml of ddH_2O.

Procedure: The pH of the solution II was adjusted to 9.8 by gradually adding the solution I and the final volume was adjusted to 100 ml by adding ddH_2O.

Sabouraud dextrose yeast extract (SDY) broth, pH 5.5:

Sabouraud dextrose yeast extract (SDY) broth contained (%, w/v): dextrose, 4 %; peptone, 1 and yeast extract, 1. The SDY agar at 6.5 % (w/v) was prepared in ddH_2O.

Czapek dox (CZ) broth, pH 5.5

Medium M_1: Medium M_1 contained (%, w/v): sucrose, 3; $NaNO_3$, 0.3 %; K_2HPO_4, 0.1; $MgSO_4$, 0.05 %; KCl, 0.05 and $FeSO_4$, 0.001 %

Medium M_2: The composition was same as M_1, but was supplemented with L-lysine (0.01 to 0.10 %, w/v). Lysine was filter sterilized (0.2 µm) and added to the medium after sterilization.

Potato dextrose broth (PDB)/agar, pH 5.5

Potato dextrose broth (PDB) contained (%, w/v): potato infusion, 0.4 and dextrose, 2. Potato dextrose agar (PDA) at 2.4 % (w/v) was prepared in ddH_2O.

Oatmeal media

Oatmeal powder was used in various concentrations (2-10 %, w/v) for screening and optimization experiments.

2.2.3. Sterilization and aseptic techniques

All the culture media were sterilized by autoclaving at a steam pressure of 10.3 kPa (15 lb/in^2), and a temperature of 121°C, for 20 min. All inoculum preparations and culture transfers were carried out under aseptic conditions using laminar air flow chambers.

2.2.4. Dry cell weight method for growth profile studies

Biomass was estimated by quantifying dry cell weight (DCW) from the duplicate 250 ml Erlenmeyer flasks containing 6 % (w/v) complex oatmeal at every 24 h of incubation at 28 °C and 180 rpm. The biomass was filtered through pre-weighted Whatman No. 1 filter paper and washed with distilled water. The filtered biomass was dried overnight at 100 °C and finally weighed to estimate the growth pattern. The DCW of the biomass was measured against every 24 h of post inoculation incubation. The slope of the exponential phase was measured which represents the specific growth rate (μ) of the culture ant the doubling time (T_d) for the culture was calculated using the following formula.

$$T_d = \frac{(log)_e}{\mu} \qquad (Eq.\ 2.1)$$

2.2.5. Mannosidase inhibition assay for the quantification of swainsonine

The swainsonine concentration in the culture supernatant was determined using α-mannosidase inhibition assay (Sim and Perry, 1995). The assay relies on the potent and specific inhibition of jack bean α-mannosidase by the low concentrations of swainsonine. The broth samples (1ml) were withdrawn at indicated time intervals and centrifuged at 16,000*g* for 20 min at 4 °C and then the supernatants were filtered using Whatman No. 1 filter paper to separate the cells. The reaction mixture was miniaturized to 50 µl volume to carry out the experiment in 96-well microtitre plates. The reaction mixture was constituted with 19.71µl citrate buffer (0.05 M, pH 4.5), 5µl of reduced glutathione (0.1mM), 5 µl $ZnCl_2$ (0.1mM), 5.29 µl of α-mannosidase enzyme (0.03 U/ml) and 10 µl of culture supernatants. All the components were added sequentially and incubated for 10 min at room temperature. Then 5 µl of substrate, p-nitrophenyl-α-D-mannopyranoside (3mM) was added and the reaction was further incubated for 4 min. The enzymatic reaction was finally stopped by adding 100 µl Borate buffer (0.2M, pH 9.8). The release of p-nitrophenol from the substrate by mannosidase activity was colorimetrically measured at absorption wavelength of 405 nm in ELISA plate reader (Tecan, Infinite 200, USA). A calibration curve was obtained by plotting the ratio of enzyme activity in the absence to the presence of swainsonine (V_C/V_I) against the inhibitor concentrations [I], ranging from 0.1 to 0.5 µg/ml (Fig. 2.1). Swainsonine concentration was measured by the correlation of absorbance with the corresponding calibration curve equation. The inhibitor concentrations lying outside the sensitivity range of the assay were determined after appropriate dilutions.

To ensure that the α-mannosidase inhibition by a sample was caused due to specific inhibition by swainsonine, rather than by non-specific effect on enzyme

activity, a control enzyme, α-fucosidase was incorporated into the procedure. If a reaction mixture is found to be inhibit both the classes of glycosidases (mannosidases and fucosidases), then the process would be inferred as a non-specific kind of inhibition.

2.2.6. Screening of *Metarhizium* strains and growth profile studies

Strains of *M. anisopliae* were initially screened for their growth and sporulation characteristics on oatmeal broth media and the ten selected strains were finally evaluated for swainsonine production in 2 % (w/v) oatmeal broth (Justo *et al.*, 2007). The culture inoculum of 4×10^8 spores/ml was inoculated in 250 ml production media in duplicate Erlenmeyer flasks. The cultures were incubated at 180 rpm and 28 °C for 168 h. Samples were removed after every 24 h and assayed for swainsonine production as described in the Section 2.2.5. The growth profile of finally screened *Metarhizium* strain was evaluated in optimal oatmeal concentration using DCW method as described in Section 2.2.4.

2.2.7. One factor at a time approach screening

One factor at a time (OFAT) approach was used for optimizing the swainsonine production from *M. anisopliae* ARSEF-1724 (UM8). Apart from the earlier known physical parameters for growth conditions, all other chemical parameters were optimized individually at a time keeping the other parameters constant. The effects of different oatmeal concentrations (2-10 %, w/v), growth media (SDB, CZ M_1 and M_2), carbon sources (glucose, fructose, maltose, sucrose and starch), glucose concentrations (0.5-3.5 %, w/v), 6 % (w/v) complex nitrogen sources (oatmeal, peptone, beef extract and yeast extract), 0.5 % (w/v) of simple nitrogen sources (urea,

ammonium sulphate, ammonium chloride and potassium nitrate) and pH (3-7) were screened for optimal swainsonine production from *M. anisopliae* fermentation.

2.2.8. Plackett-Burman screening

Overall seven parameters were screened using Plackett Burman-design of experiment (PB-DOE) (Plackett and Burman 1946) in the "Design Expert software DX7" for their cumulative effects towards fermentative production of swainsonine from *M. anisopliae*. Based on PB factorial design, each factor was examined in two levels: -1 for low level and +1 for high level (Plackett and Burman, 1946). Table 2.2 shows levels of each factor used in the experimental design and the design matrix. These factors used in PB with their high and low range values were selected based upon the OFAT screening experiments (described in Section 2.2.7) and reports. The parameters for PB screening were (A) glucose, (B) lysine, (C) oatmeal extract, (D) $MgSO_4.7H_2O$, (E) $CaCl_2$, (F) pH and (G) inoculum size. The design of experiment (DOE) study ranges and significant values for each parameter are shown in Table 2.2. The level of significance for each variable was determined using the lack of fit test (F-test) with at $p < 0.05$.

2.3. Results and Discussion

2.3.1. Screening of *Metarhizium* strains

Out of the nineteen strains, only ten *Metarhizium* strains ARSEF-2735, 2135, 2424, 3210, 2596, 1724, 549, 1744, 23 and 2575 were finally selected based upon their growth characteristics in swainsonine producing media. In the screening experiment, a non linear relationship was unexpectedly observed between the growth rates and metabolite production. The fungal strains with superior growth and sporulation characteristics *viz.*, ARSEF-2735, 549, 1744, 23 and 2575 were not producing the proportional quantities of swainsonine. However, the strains with moderate growth characteristics (ARSEF-1724 and 2596) were found to produce considerably higher titres of the alkaloid of interest (Fig. 2.2). Hence, it can be inferred that the conditions favouring only the culture growth are not the sole determinants for the fermentative swainsonine production from *M. anisopliae*, as also reported by Tamerler *et al.* (1997). *M. anisopliae* ARSEF-1724 (UM8) was finally screened to produce the highest titres of the swainsonine 1.34 µg/ml, between 72-96 h of incubation.

2.3.2. One factor at a time screening

2.3.2.1. *Screening of the basal fermentative media*

The fermentative production of swainsonine was evaluated using the best screened *M. anisopliae* ARSEF-1724 (UM8). The optimal swainsonine production of 1.60±0.32 µg/ml was achieved using 6 % (w/v) oatmeal as the sole production medium (Fig. 2.3.a). The alkaloid production was significantly lesser in 6 % (w/v) CZ M_2 media (containing 0.08 %, w/v lysine) with an average yield of 0.69±0.09 µg/ml. This ruled out the possibilities of L-lysine as a key inducer in the biosynthetic

pathway for swainsonine production in *M. anisopliae*, as reported in case of *Rhizoctonia* (Guengerich *et al.*, 1973; Harris *et al.*, 1988). The production was further decreased in other fermentative media such as SDB and CZ M_1 at the similar ratios (Fig. 2.3.b).

The growth profile analysis *M. anisopliae* ARSEF-1724 (UM8) was carried out at 6 % (w/v) of oatmeal fermentative medium. The culture showed the specific growth rate (μ) of 0.61 per day with the doubling time (T_d) of nearly 27 h (1.12 d). The growth curve analysis showed that the culture attained the stationary phase at 72 h and extends upto 96 h of post inoculation incubation (Fig. 2.4). Many commercially important secondary metabolites from microbial sources are known to produce during stationary phase (Shaikh *et al.*, 2010). The growth curve analysis has further showed the optimal incubation period for the production and recovery of swainsonine from the *Metarhizium*. Hence, the further screening experiments for the swainsonine production were carried out at 72 h from *Metarhizium* fermentation broth.

2.3.2.2. *Screening of the best carbon sources*

The screening of different carbon sources in oatmeal medium showed 2 % (w/v) of glucose as the best medium supplement with closely similar but slightly lesser swainsonine production of 1.34±0.21 µg/ml, probably as a consequence of repeated subcultures to obtain the inoculum of similar spore age. The production was significantly reduced at glucose concentration above and below this optimum level. The lowest swainsonine production of 0.75±0.09 µg/ml was obtained at 3.5 % (w/v) glucose concentration with a sharp 44% reduction (Fig. 2.5a). This further signifies the inhibitory effects of glucose at higher concentrations. The substitution of glucose at the equivalent concentrations (2 %, w/v) by other carbon sources *viz.*, sucrose,

maltose, fructose and starch, resulted in the reduction of swainsonine production considerably (Fig. 2.5b).

2.3.2.3. *Screening of the best nitrogen sources*

Complex nitrogen sources

Certain complex nitrogen sources were also systematically evaluated to augment the alkaloid yield, since these nutrients play a key role in growth and sporulation of entomopathogenic fungi (Tseng *et al.*, 1992). Although complex oat meal can be considered as the basal media rich in nitrogen content, but other complex nitrogen sources at 6 % (w/v) concentration such as peptone, beef extract and yeast extract were also examined for swainsonine production as the potential basal media in combination with 2 % (w/v) of glucose. The maximum swainsonine production of 1.48±0.14 µg/ml from *M. anisopliae* ARSEF-1724 (UM8) was again observed with oatmeal (6 %, w/v) medium supplemented with glucose (2 %, w/v). However, the combination of beef extract and glucose also resulted in the comparatively similar swainsonine production of 1.39±0.12 µg/ml, but the combination was not further considered on account of its three-fold higher expenditure. The rest of the combinations, peptone and yeast-extract each with glucose reduced swainsonine production by 31.75 % and 45.94 %, when compared with oatmeal (Fig. 2.5c).

Simple nitrogen sources

The effects of 0.5 % (w/v) simple nitrogen sources such as NH_2CONH_2, $(NH_4)_2SO_4$, NH_4Cl and KNO_3 as the potential process parameters were examined for fermentative swainsonine production from *M. anisopliae* ARSEF-1724 (UM8). Only $(NH_4)_2SO_4$ showed some considerable results with 0.93±0.19 µg/ml of alkaloid production (Fig. 2.5d). However, the production rate was considerably lesser than that

of the oatmeal (6 %, w/v) medium supplemented with glucose (2 %, w/v). Moreover, the swainsonine production was even lesser with other simple nitrogen sources *viz.*, NH_2CONH_2, NH_4Cl and KNO_3. Hence, the addition of simple nitrogen sources was not considered for swainsonine production from *M. anisopliae* ARSEF-1724 (UM8).

2.3.2.4. *Screening of the best pH range*

Many biological systems involve acid-base equilibrium and therefore, depend critically on the pH of environment. The pH drastically affects the permeability of cell membrane and solubility of the components in growth medium. Thus, a modification in the pH might also cause some macro or micronutrients to precipitate and become impossible to be assimilated (Watson *et al.*, 2001). A mild acidic pH value of 5 was observed to be optimum for swainsonine production of 1.18±0.15 µg/ml in oatmeal medium (6 %, w/v) supplemented with glucose (2%, w/v) from *M. anisopliae* ARSEF-1724 (UM8) (Fig. 2.6).

2.3.3. Plackett-Burman screening

There was a wide variation of swainonine production from 0.40±0.11 µg/ml to 1.55±0.16 µg/ml was observed in the 12 trials (Table 2.2). This variation reflected the importance of medium optimization to attain optimal yields of swainsonine. Using PB design out of seven, four components *viz.*, glucose, oatmeal, $CaCl_2$ and inoculum size were actually found to be the best screened process parameters towards the fermentative production of swainsonine (Table 2.3). The ANOVA provided the regression equation for the model with significant variables at ($p < 0.05$):

$$Y = 0.885 - 0.235\,A + 0.081\,C + 0.191\,E - 0.088\,G \qquad (Eq.\,2.2)$$

Here, Y = swainsonine production (µg/ml), A = glucose (%, w/v), C = oatmeal (%, w/v), E = $CaCl_2$ (mM) and G = inoculum's size (%, v/v).

The fit of the model was expressed by its high coefficient of determination R^2 (0.99), indicating that 99% of the variability in the response could be explained by the model. High model F-value (112.67) implies that model is significant. F-value is calculated as ratio of mean square regresion and mean square residual. Model *p*-value ($p > F$) is very low (0.0002) that again signifies the model (Table 2.3). The *p*-values were used as a tool to check the significance of each of the coefficients, which are necessary to understand the pattern of the mutual interactions between the test variables. The smaller the magnitude of the *p*, the more significant is the corresponding coefficient. Values of $p < 0.05$ indicate model terms as significant. On this basis $CaCl_2$ and glucose were the most significant parameters, followed by oatmeal and inoculum size at their high confidence level of ≥ 95 %. However, lysine, pH and $MgSO_4$ $7H_2O$ were not found significant for swainsonine production (Table 2.3). The Pareto chart showed the ranking of these variables in accordance to the absolute values of standarized effects (Fig. 2.7). Calcium is structural component of the cell wall, cofactor of many enzymes and has prominent role in cell growth, cell division and hyphal growth of many mycorrhiza (Jackson and Heath 1993), which might be one of the reason of enhancement in swainsonine production.

2.4. Conclusions

The use of entomopathogenic fungus, *M. anisopliae* in the fermentative biosynthesis of swainsonine involves a number of decisive variables such as the appropriate growth media for a specific fungal strain, medium ingredients (C and N sources), culture conditions and physiology. The absolute understanding of all these parameters serves as the building blocks for an efficient fermentation model. Using one factor at a time (OFAT) approach, the range of effective variables including the best medium composition, pH and incubation time were determined for the best screened *Metarhizium* strain. Among nineteen strains, *Metarhizium anisopliae* ARSEF-1724 (UM8) was found to produce highest swainsonine concentration of 1.34 µg/ml. Oatmeal medium was found to be the best for swainsonine production, when compared with SDB and CZ M_1 and CZ M_2 media. The optimum conditions for swainsonine production were oatmeal (6 %, w/v) medium supplemented with glucose (2 %, w/v) at pH ~ 5, 180 rpm and 28 °C after 72 h. The OFAT approach as a whole proved to be useful for the stepwise and systematic optimization of effective variables for enhancing the swainsonine, resulting in 20 % increase in the swainsonine yield. Furthermore, the statistical PB screening revealed glucose, $CaCl_2$, oatmeal and inoculum size as the most effective process parameters in the statistical hierarchy for swainsonine production. In PB-DOE, the experimental swainsonine production varied widely from 0.55 to 1.55 µg/ml, further necessitating the need for development of efficient fermentation modelling. The present study aimed to lay the foundation for stepwise optimization of factors involved in swainsonine production from *M. anisopliae* ARSEF-1724 (UM8).

Table 2.1. Different strains of *Metarhizium anisopliae* used in the present study, their host insects and geographical locations.

Strains (Lab codes)	Accession no. (ARSEF codes)	Host insect	Geographical location
UM1	ARSEF 1745	*Nilaparvata lugens*	India
UM2	ARSEF 2735	*Spodoptera* sp.	Philippines
UM3	ARSEF 2153	*Nephotettix virescens*	Indonesia
UM4	ARSEF 2424	Lepidoptera larva	Indonesia
UM5	ARSEF 3210	Coleoptera	India
UM6	ARSEF 2596	*Pyrausta machaeralis*	India
UM7	ARSEF 1080	*Helicoverpa zea*	USA
UM8	ARSEF 1724	*Nilaparvata lugens*	India
UM9	ARSEF 1727	*Nilaparvata lugens*	India
UM10	ARSEF 3295	*Anticarsia gemmatalis*	Mexico
UM11	ARSEF 1729	*Nilaparvata lugens*	India
UM12	ARSEF 1744	*Nilaparvata lugens*	India
UM13	ARSEF 1823	*Nilaparvata lugens*	India
AR1	Local	Unknown	India
UM 14	ARSEF 2575	Curculionidiae	USA
UM15	ARSEF 549	Galacticidae	Brazil
UM 16	AESEF 1744	*Nilaparvata lugens*	India
UM 17	ARSEF 23	*Conoderus* sp.	USA
UM 18	ARSEF 2575	*Curculio caryae*	USA

Table 2.2. Plackett-Burman (PB) design matrix in real and coded (in brackets) values along with the predicted and experimental swainsonine production from *Metarhizium anisopliae* ARSEF 1724 (UM8).

Run order	Variables with their real values											Swainsonine (µg/ml)	
	Glucose (%, w/v)	Lysine (%, w/v)	Oatmeal (%, w/v)	$MgSo_4$ (mM)	$CaCl_2$ (mM)	pH	Inoculum size (%, v/v)	D1	D2	D3	D4		
												Exp*	Prd**
1	2.5 (+1)	0.1 (-1)	10 (+1)	0.5 (+1)	0.1 (-1)	6.5 (+1)	0.5 (-1)	+1	+1	-1	+1	0.55±0.11	0.58
2	2.5 (+1)	1 (+1)	10 (+1)	0.1 (-1)	0.5 (+1)	6.5 (+1)	0.5 (-1)	+1	+1	+1	-1	1.06±0.11	1.08
3	0.5 (-1)	1 (+1)	2 (-1)	0.1 (-1)	0.1 (-1)	6.5 (+1)	2.5 (+1)	+1	+1	-1	-1	0.79±0.21	0.82
4	0.5 (-1)	1 (+1)	10 (+1)	0.5 (+1)	0.1 (-1)	6.5 (+1)	2.5 (+1)	+1	-1	-1	-1	0.89±0.08	0.86
5	2.5 (+1)	1 (+1)	2 (-1)	0.5 (+1)	0.1 (-1)	4.5 (-1)	0.5 (-1)	+1	-1	+1	+1	0.40±0.16	0.39
6	0.5 (-1)	0.1 (-1)	2 (-1)	0.1 (-1)	0.1 (-1)	4.5 (-1)	0.5 (-1)	+1	-1	+1	+1	0.99±0.19	1.00
7	2.5 (+1)	0.1 (-1)	10 (+1)	0.1 (-1)	0.1 (-1)	4.5 (-1)	2.5 (+1)	-1	-1	-1	-1	0.54±0.19	0.52
8	2.5 (+1)	0.1 (-1)	2 (-1)	0.1 (-1)	0.5 (+1)	6.5 (+1)	2.5 (+1)	-1	+1	+1	+1	0.77±0.16	0.75
9	0.5 (-1)	0.1 (-1)	10 (+1)	0.5 (+1)	0.5 (+1)	4.5 (-1)	2.5 (+1)	-1	+1	+1	-1	1.21±0.14	1.24
10	2.5 (+1)	1 (+1)	2 (-1)	0.5 (+1)	0.5 (+1)	4.5 (-1)	2.5 (+1)	-1	-1	+1	-1	0.58±0.18	0.59
11	0.5 (-1)	0.1 (-1)	2 (-1)	0.5 (+1)	0.5 (+1)	6.5 (+1)	0.5 (-1)	-1	-1	-1	+1	1.29±0.24	1.27
12	0.5 (-1)	1 (+1)	10 (+1)	0.1 (-1)	0.5 (+1)	4.5 (-1)	0.5 (-1)	-1	+1	-1	+1	1.55±0.16	1.53

* Experimental swainsonine production; ** Model predicted swainsonine production; D1, D2, D3 and D4 are the dummy variables; {+} represents the high and {–} represents the low values for each variable in the coded form.

Table 2.3. Statistical analysis of variance (ANOVA) for Plackett Burman design matrix towards fermentative swainsonine production from *Metarhizium anisopliae* ARSEF 1724 (UM8).

Source	SS*	DF**	MS***	F value	*p*-value *p* > F
Model	**1.32**	**7**	**0.18**	**119.90**	**0.0002 (Significant)**
Glucose	0.66	1	0.66	418.54	<0.01e-^{2} [a]
Lysine	5.33e^{-004}	1	5.33e^{-004}	0.33	0.59 [c]
Oatmeal	0.08	1	0.08	50.54	0.21e-^{2} [a]
$MgSO_4.7H_2O$	0.05	1	0.05	32.01	0.48e^{-2} [b]
$CaCl_2$	0.44	1	0.44	278.42	< 0.01e^{-2} [a]
pH	5.33e^{-004}	1	5.33e^{-004}	0.33	0.59 [c]
Inoculum size	0.09	1	0.09	59.13	0.15e^{-2} [a]
Residual	6.33e^{-003}	4	1.58e^{-003}	-	-
Core Total	1.33	11	-	-	-

* sum of squares; ** degrees of freedom; *** Mean of squares

[a] most significant at '*P*>F<0.05; [b] least significant at '*p* > F >0.05'; [c] Non significant at '*p* < F > 0.05' with model regression values $R^2 = 0.99$ and $R^2_{adj.} = 0.98$.

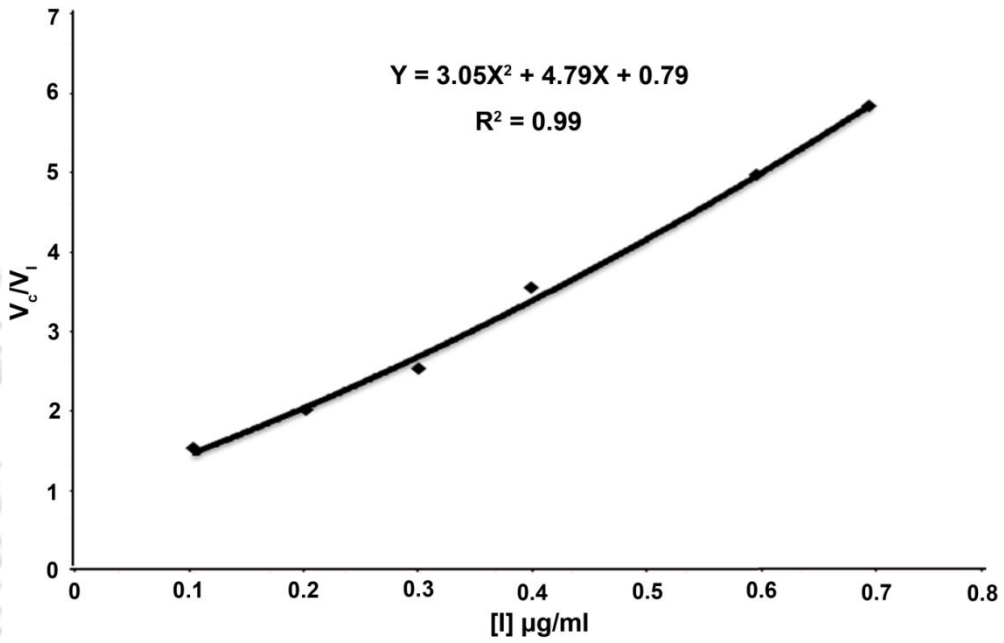


Fig.2.1. Calibration curve of the mannosidase inhibition assay for the quantification of swainsonine. Here, 'Y' represents V_C/V_I, the ratio of α-mannosidase activities in the absence (V_C) to the presence of swainsonine (V_I) and 'X' represents the swainsonine concentration (I).

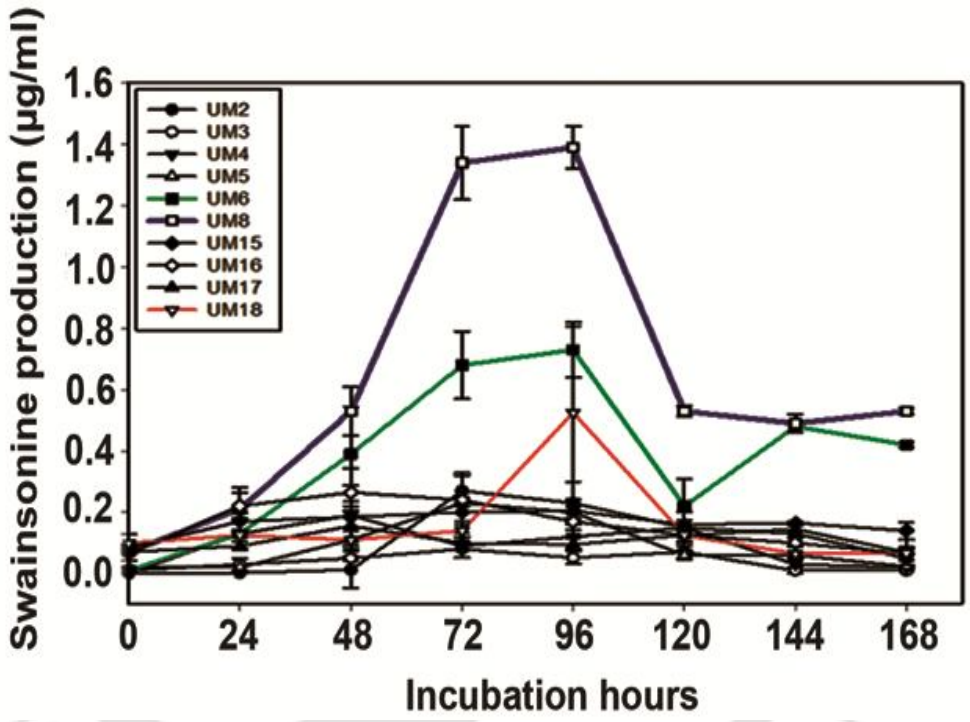


Fig. 2.2. Screening of *Metarhizium anisopliae* strains for swainsonine production in 2 % (w/v) oatmeal fermentation medium. Here, UM2: ARSEF-2735, UM3: ARSEF-2135, UM4: ARSEF-2424, UM5: ARSEF-3210, UM6: ARSEF-2596, UM8: ARSEF-1724, UM15: ARSEF-549, UM16: ARSEF-1744, UM17: ARSEF-23 and UM18: ARSEF-2575.

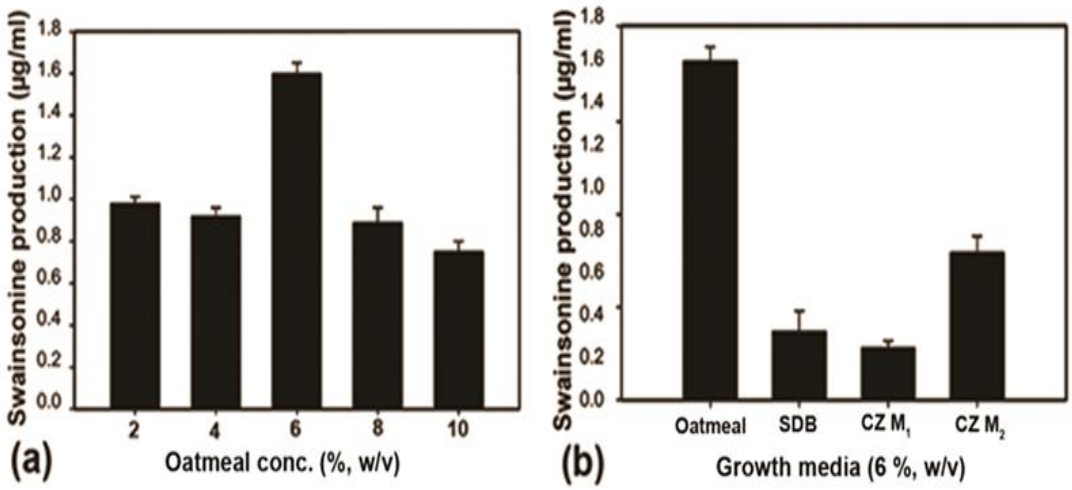


Fig. 2.3. Screening of basal media **(a)** oatmeal concentration, and **(b)** other growth media including oatmeal, SDB (sabouraud dextrose broth), CZM_1 and CZM_2 each at 6 % w/v concentrations, towards the fermentative production of swainsonine from the *Metarhizium anisopliae* ARSEF-1724 (UM8).

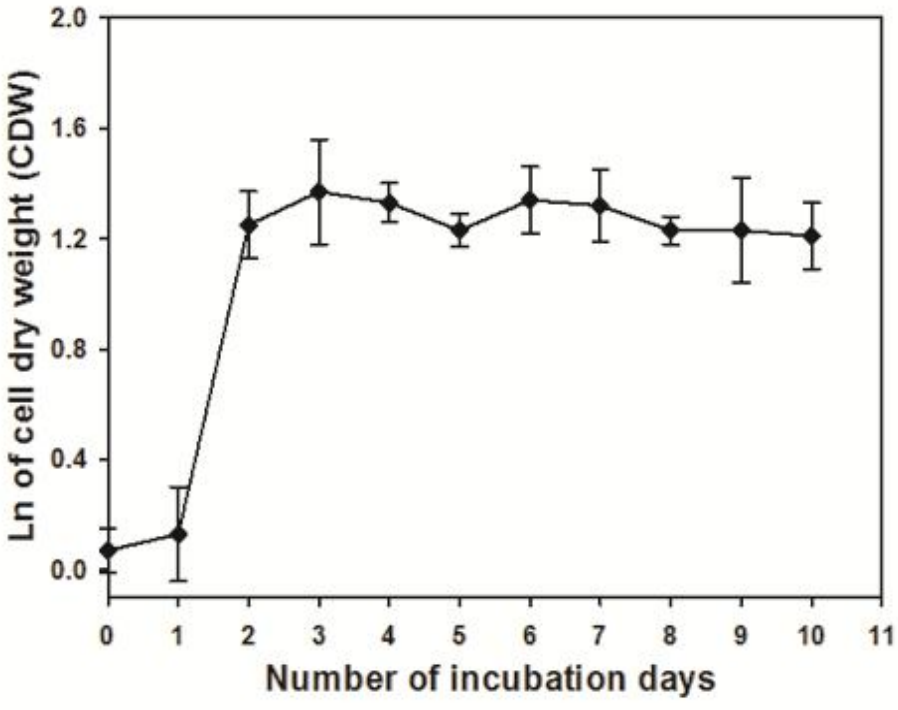


Fig. 2.4. Growth profile of *Metarhizium anisopliae* ARSEF-1724 (UM 8).

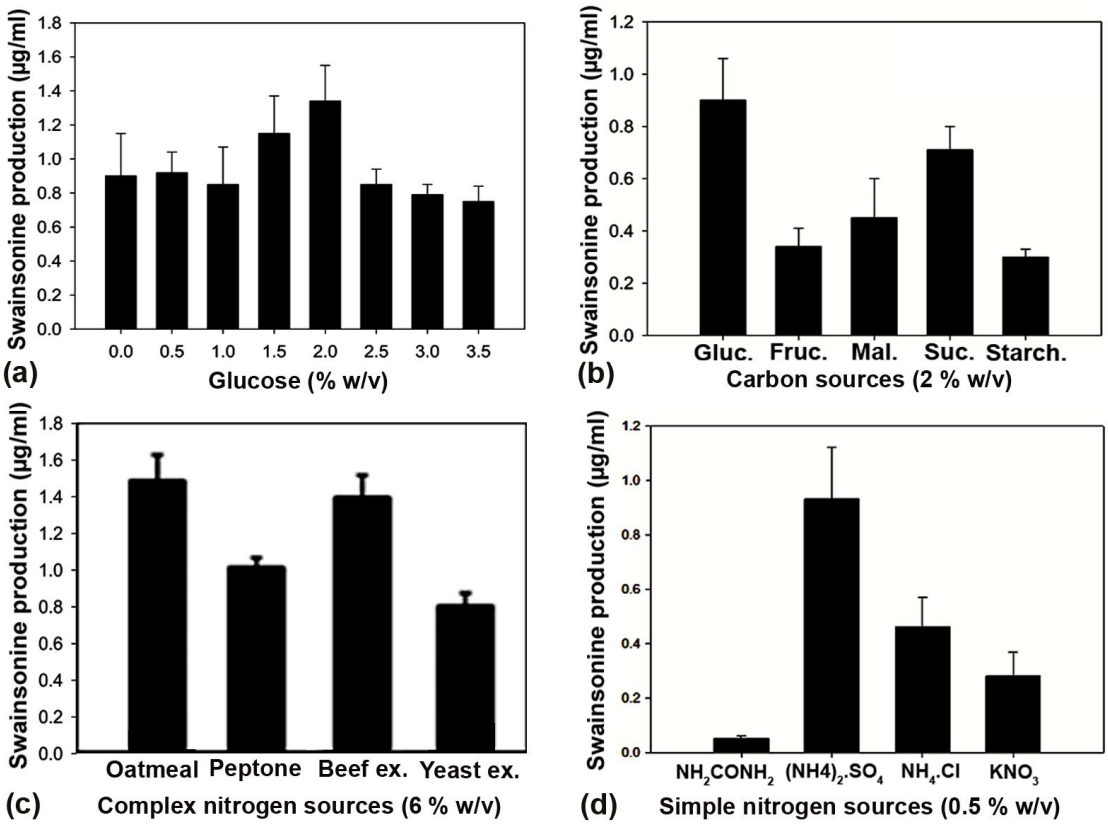


Fig. 2.5. Screening of **(a)** glucose concentrations, **(b)** different carbon sources, **(c)** complex nitrogen sources, and **(d)** simple nitrogen sources, for the fermentative production of swainsonine from *Metarhizium anisopliae* ARSEF-1724 (UM8).

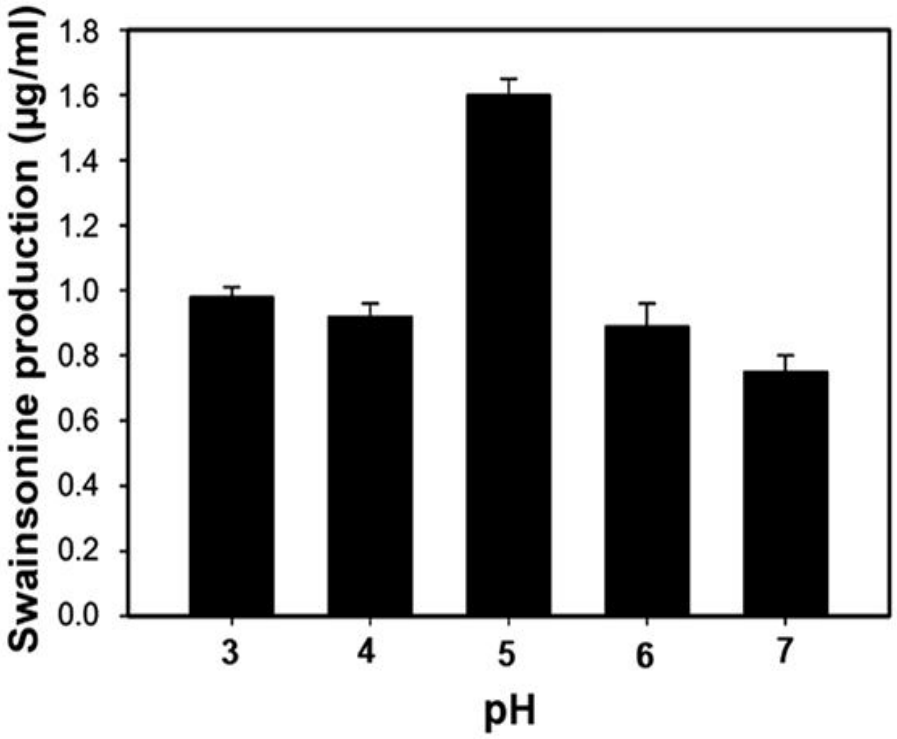


Fig. 2.6. Screening of optimal pH conditions in the oatmeal (6 %, w/v) and glucose (2 %, w/v) medium for the fermentative production of swainsonine from *Metarhizium anisopliae* ARSEF-1724 (UM8).

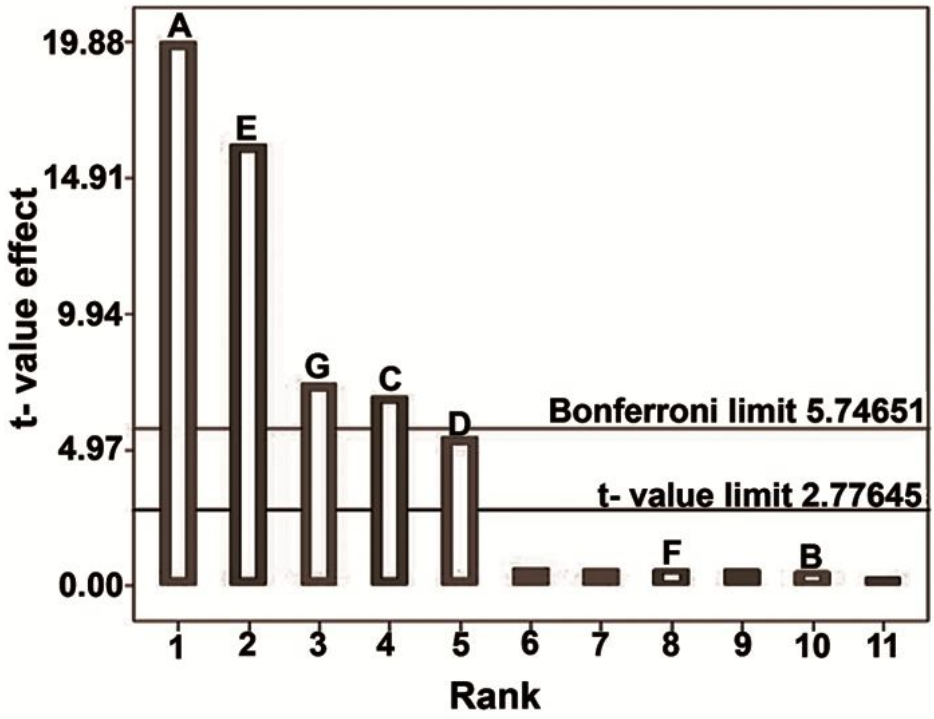


Fig. 2.7. Pareto chart of the standardized effects of the factors in Plackett Burman design of experiment (PB-DOE) with (A) glucose, (B) lysine (C) oatmeal (D) $MgSO_4.7H_2O$ (E) $CaCl_2$ (F) pH and (G) inoculum size, for the fermentative production of swainsonine from *Metarhizium anisopliae* ARSEF-1724 (UM8).

CHAPTER 3

Optimization of media components, operation conditions and scale up parameters from shake flask to bench-top bioreactor levels

1. Introduction

At present, the swainsonine production is mainly derived from chemical synthesis or plant sources. However, the submerged fermentation process using *Metarhizium anisopliae* offers an efficient method, which requires the less technical skills, lower energy, easy maintenance and controlled production. The submerged fermentation process for *M. anisopliae* towards swainsonine production is deeply influenced by various medium components, culture conditions and operation parameters. A fermentation system is a complicated multi-phase, multi-component system. Growth and production are affected by a wide range of parameters including cultivation medium, inoculum, pH, temperature, aeration, agitation and shear stress, *etc.* In many of the fungal fermentation, a high agitation rate is necessary to provide an adequate mixing and mass transfer, especially when the fungal cells grow in a freely dispersed form which results in a non-Newtonian broth and a high apparent viscosity. However, the resulting mechanical forces can cause mycelia damage and thus, the agitation rate is limited to a range that avoids exerting high shear stresses on fungal mycelia. Oxygen is often a limiting component in fermentations because of its low solubility and the low volumetric mass transfer in bioreactors. In the culture of aerobic microorganisms, oxygen serves as a substrate for energy generation and especially in a non Newtonian broth, oxygen is an important nutrient that is used by microorganisms for growth, maintenance and metabolite production, and hence the scarcity of oxygen affects the process performance (Garcia-Ochoa *et al.*, 2000). Consequently, the incubation time for maximum recovery of the compound varies in accordance to the number of other parameters *viz.*, internal composition of cells and their morphological states. These variable parameters turn into an extremely dynamic

environment within an operational bioreactor, which can only be simulated with the efficient optimization and quantitative model.

The classical optimization procedures involving the sequential manipulation of individual parameters do not take into account the influence of intra-parameter interaction on the product yield. The statistical optimization procedure response surface methodology (RSM) has been proved as a valuable optimization tool to evaluate the relative significance of several parameters in a bioprocess by performing very limited sets of experiments (Elibol, 2004; Piyushkumar *et al.*, 2007; Gao *et al.*, 2009).

In the last two decades, artificial neural network-genetic algorithm (ANN-GA) has evolved as a more efficient method for empirical modelling, especially in case of nonlinear biological systems. ANNs can learn from examples and are fault tolerant, in the sense that they are able to handle noisy, incomplete and nonlinear data, and can perform prediction and generalization at high speed (Haykin, 2008). Genetic algorithm (GA) is inspired by natural selection and genetic programming. In the recent years, hybrid approaches namely ANN-GA have been successfully used for the optimization of various bioprocess systems (Desai *et al.*, 2008; Zhang *et al.*, 2010; Caldeira *et al.*, 2011). Recently, many studies have been performed on GA with real encoding, which outperforms conventional binary coded representation (Yoon and Kim, 2012). Real encoded genetic algorithm (REGA) emphasizing on the coding of the chromosomes with floating point representations is proven to have significant improvements on the computation speed and precision. At the same time, much effort was imposed to improve computation performance of GA and to avoid premature convergence of solutions. REGA is capable of exploring large input variables space

through the search operators such as selection, crossing over, and mutation (Gen and Cheng, 2000; Goldberg, 1991).

A more sophisticated version of GA with the data structures and algorithm operators are developed for real time specific optimization problem such as shake flask or bioreactor fermentations, are often termed as evolutionary program or algorithms (EP or EA). Modern EP with diminished computational load, flexible constraint handling, low model complexities and higher limits of control variables can provide the optimal solutions to the complex fermentation models (Mandli and Modak, 2012). The EP parameters are defined in real form (numerals) rather than binary codes. The binary representation used in classical GAs has some drawbacks when applied to multidimensional, high precision numerical problems. The bit-strings become very long and the search-space is blown up. Further, much computation time is lost in conversion of the bit-strings to real values and vice-versa. Hence, EPs are proved to be more appropriate for parameter optimization problems with variables over continuous domains (Abdul-Rahman *et al.*, 2013).

The present chapter describes the comparative optimization and modelling of *Metarhizium* fermentation systems for the enhanced production of swainsonine, and the development of a scale up fermentative bioprocess from the shake flask to the bench-top bioreactor levels.

3.2. Materials and methods

3.2.1. Fungal strain and culture conditions

Metarhizium anisopliae (ARSEF 1724) was procured from "The Plant Protection Unit, United States Department of Agriculture (USDA), New York, Ithaca. The culture was maintained in sabauraud dextrose agar (SDA) slants at 4 °C. A ten day old SDA slant was used for the preparation of conidial suspension of 4×10^8 spores/ml for inoculation (Section 2.2.1, Chapter 2).

3.2.2. Chemicals and Reagents

All the chemicals and reagents used were same as described in Section 2.2.2 of Chapter 2. The PB-screened oatmeal production medium was used for the *Metarhizium* cultivation (Section 2.3.3, Chapter 2). The optimal media components were further proportionally varied at shake flask and bench bioreactor levels.

3.2.3. Instrumentation for stirred tank fermentation

A 3 L dished bottom, stirred tank Bioreactor (ADI 1010, Applikon Biotechnology, Holland) containing 1.2 L of production medium was inoculated with 72 h pre-culture inoculum. The proper mixing was achieved with two Rushton disc turbine impellers (D = 0.045 m), impeller diameter/tank diameter ratio (D_i/D_t) of 0.34 and impeller blade width/impeller diameter ratio (W_i/D_i) of 0.26. In all fermentations, the cultures were maintained at 28±2 °C. Dissolved oxygen tension (% DOT air saturation), pH, temperature and air flow-rate were continuously monitored for 168 h fermentations. *In situ* sterilization of bioreactor with fermentation media was achieved at 121 °C and 15 lb/inch^2 for 20 min. Initially, the pH was not maintained but monitored throughout the course of fermentation as reported by Tamerler *et al.*,

(1998). Foaming was not observed and thus no antifoam was used. Samples were removed aseptically after every 12 h intervals for analyses.

3.2.4. Parameters selected for optimization

The OFAT and PB screened process parameters *viz.*, oatmeal, glucose, $CaCl_2$ and inoculum size were evaluated at their optimum levels for swainsonine production. The fermentation scale up at bench-top bioreactor levels involved the further optimization of agitation (N) and aeration rates (Q) with their influence on the swainsonine production rate as a function of incubation hours (h).

3.2.5. Mannosidase inhibition assay

Swainsonine production in culture supernatants was determined using the α-mannosidase inhibition assay (Sim and Perry, 1995) as described in Section 2.2.5 of Chapter 2.

3.2.6. Total saccharide estimation

The total saccharide (glucose) estimation was performed using the phenol-sulphuric acid method (Dubois *et al.*, 1956) in a micro-titre plate (Fox and Robyt, 1991). To 25 µl of broth sample in a microtitre plate, 25 µl of 5 % (v/v) phenol (Merck chemicals) was added and mixed by shaking the plate on a vortex mixer for 30 s. Then the plate was placed on an ice bath and 125 µl of concentrated sulphuric acid was added to each well containing the mixture. The plate was again shaken for 30 s to ensure proper mixing of the contents of the wells. Then the plate was wrapped in cling film and incubated in water bath at 80 °C for 30 min. After cooling to room temperature, the absorbance was determined at 490 nm on an ELISA plate reader (Tecan, Infinite 200, USA). A standard graph was plotted using glucose in the concentration range 0.25-200 µg/ml (Fig. 3.1).

3.2.7. Growth rate analysis

Growth rate was estimated as a function of dry cell weigh (DCW) for the fungal mycelium (Section 2.2.4).

3.2.8. Analysis of medium viscosity

The shear viscosity was measured *ex-situ* from the calibration curve between average shear stress (τ_{av}) around the impeller and average shear rate (γ_{av}) for the broth samples removed after every 12 h at 25 ºC using rheometer (Thermo Electron, model Haake rheostress RSI) interfaced with a HAAKE RheoWin 323 software (Fig. 3.2). The applied shear rate was in the range of 0.1–100 s^{-1}.

3.2.9. Gassed power per unit volume

Gassed (Pg) to un-gassed (P) power per unit volume (Pg/V_L, W/m^3) drawn by impellers was estimated using Hughmark correlation (1980). Although the relation is more appropriate for single impeller system but is less specific for a wide range of vessel geometries (Eq. 3.1). For Pg (gassed) to P (un-gassed) power consumptions ratio, the relation is:

$$\frac{P_g}{P} = 0.10 \left(\frac{F_g}{N_i V}\right)^{-0.25} \left(\frac{N_i^2 D_i^4}{g W_i V^{2/3}}\right)^{-0.20} \qquad \text{(Eq. 3.1)}$$

Here, F_g = gas flow rate (m^3/s), W_i = impeller blade width (m), V = medium volume (m^3); N_i = rotations/second (rps), g = acceleration due to gravity (m/s^2) and D_i = impeller's diameter (m).

3.2.10. Determination of volumetric oxygen transfer coefficient

Volumetric mass transfer coefficient (K_La, s^{-1}) was experimentally determined for actively growing culture in bioreactor from 78 h to 85 h at EP-optimized agitation and aeration conditions using dynamic method (Taguchi and Humphrey, 1966). The

dynamic method involves the measurement of dissolved oxygen concentration in the medium by absorption or desorption of oxygen. The oxygen uptake rate (OUR or $Q_{O2}.X$) and K_La are determined after a step change in the concentration/nature of the inlet gas followed by analysis of the dynamic change in the dissolved oxygen concentration. The aeration supply and agitation are stopped momentarily to the actively growing culture, so that the rate of decrease of dissolved oxygen concentration is caused entirely by the culture respiration (index of OUR). The decrease in DOT is usually linear and the slope of the C_L (dissolved oxygen concentration, mol/m^3) as the function of time provides a direct estimate of OUR. The fate of the DOT in a submerged fermentation bioreactor system is mathematically defined as:

$$d\frac{C_L}{d_t} = K_L a\,(C_L^* - C_L) - Q_{O_2}.X \qquad (Eq.3.2)$$

Here, C_L = dissolved oxygen concentration (mol/m^3), C_L^* = dissolved oxygen concentration with mean gaseous oxygen concentration (mol/m^3).

Before the dissolved oxygen concentration reaches its critical lower level, the aeration and agitation are again resumed so that the DOT level again reaches the normal level. Now, the K_La can be derived using the Eq. 3.2, when substituted in terms of DOT as follows:

$$C_L = C_L^* - \frac{1}{K_L a}\left(\frac{dC_L}{d_t} + Q_{O_2}.X\right) \qquad (Eq.3.3)$$

The slope of the linear fit for the Eq. 3.3 represents the K_La (Fig. 3.3a). The estimation of $Q_{O2}.X$ and K_La depends on the section of the curve (time points) used for the

evaluation (Fig. 3.3b). The theoretical K_La value for all other experimental sets was derived using Ogut and Hatch equation (1988):

$$K_La = 23.1\left(\frac{P_g}{V_L}\right)^{0.016}(V_g)^{0.85}(\mu_a)^{-0.60} \qquad (Eq.3.4)$$

Here, Pg/V_L = power per unit of liquid volume under aeration condition (W/m^3), V_g = gas superficial velocity for aeration rates (m/s) and μ_a = apparent viscosity for the fermentation medium under aeration condition (Pa.s).

3.2.11. Morphological analysis

The culture morphology (pellet) was investigated under a light microscopy after staining the broth samples with lactophenol cotton blue (LCB). The image was captured with a digital camera mounted on an inverted phase contrast microscope (Nikon Eclipse, E 200) at 40X magnification.

3.2.12. Optimization methodologies

3.2.12.1. *Response surface methodology*

The levels of four independent variables, *viz.*, (A) glucose, (B) oatmeal, (C) $CaCl_2$ and (D) inoculum size were finally considered. The coded and real values for each of the variables are shown in Table 3.1. Each factor was studied at five different levels (-2, -1, 0, +1, +2), with all the factors with their central coded values as zero. A 2^4 full factorial central composite design (CCD) was used to determine the optimum concentrations of these four variables in 30 run orders ($2^k + 2k + 6$), in duplicates at the optimum vicinity. The statistical software package 'DESIGN-EXPERT®7.0' (StatEase, Inc., Minneapolis, USA) was used to analyse the experimental design and

statistical analysis of the model. The relationships among the variables were determined by fitting the second-order polynomial equation to the experimental data:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i} \sum_{j} \beta_{ij} X_i X_j \qquad \text{(Eq. 3.5)}$$

Here, Y = predicted response (swainsonine production), k = number of factor variables, β_o = model constant, β_i = linear coefficient, β_{ii} = quadratic coefficient, β_{ij} = interaction coefficient and X_i = variable in its coded form.

3.2.12.2. *Sequential screening cum optimization of agitation and aeration*

Three different agitation rates 300, 400 and 500 rpm at the varying aeration rates of 0.5, 1, 1.5 and 2 vvm, were evaluated sequentially for swainsonine production with only one factor varying at a time (OFAT) optimization. Swainsonine production was monitored for 156 h at every 12 h interval in bench top bioreactor level by using α-mannosidase assay as described in the Section 2.2.5 of Chapter 2.

3.2.12.3. *The hybrid artificial neural network and genetic/evolutionary algorithm*

The ANN is a predictive model loosely based on the working of biological neurons. The two different ANN constructs were created depending upon the experimental requirements. First a feed foreword neural network (FFNN) with four neurons (glucose, oatmeal, $CaCl_2$ and inoculum size) in the input, six neurons in the hidden and one in the output layer (swainsonine production) was created for the approximation of medium components. The training was carried out by 'Levenberg-Marquardt' algorithm with 300 epochs and learning rate (lr) of 0.07 using the training experimental sets from CCD experiment (Section 3.2.12.1). In an another attempt, a FFNN was constructed with three input neurons, *viz.*, agitation (N), aeration (Q) and

incubation hours (a) as input, six hidden neurons (one hidden layer) with one output for swainsonine production using 'gradient decent back propagation with adaptive learning' algorithm. The 200 epochs were tuned for the lr of 0.12. For training and validation, the ANN was subjected to input test sets experimental data (Section 3.2.12.2).

Initially the weights and bias values were selected randomly but adjusted later on in accordance with the convergence in target and test values for swainsonine production. Weights were corrected using the equation:

$$\Delta W = (J^T + \lambda I)^{-1} J^T \nabla E \qquad \text{(Eq. 3.6)}$$

Here, J = Jacobian matrix of errors for each one of the weights, ∇E = gradient of error function, λ = positive scalar, I = identity matrix and E = errors vector. This E (error vector) is defined by another mathematical relation:

$$E = \sum_{k=1}^{m} (yk - y\kappa)^2 \qquad \text{(Eq. 3.7)}$$

Here, yk = real or target (swainsonine production, µg/ml) value, yκ = value predicted by ANN and m = number of samples used to train the neural network (12 experimental sets).

"Goodness of fit" was determined using mean squared error (MSE) and correlation coefficient (R) values for the ANN-model, describing the variance in the input and output variables.

The GA/EA is the optimization procedure that search for an optimal value of a complex function by adopting the process of natural evolution (Goldberg, 1989).

Based upon the specific optimization experiments, the two different GA models were constructed with following operators:

Generation of population: A population size of 1000 chromosomes, each representing an individual set of experiment was created and reproduced up to ten generations to provide a best evolved optimal solution. Here, each chromosome is represented by the real values of indicating the individual variables in specific optimization experiment, *viz.*, the optimal values for medium components and bioreactor operation parameters as described in Section 3.2.12.1 and 3.2.12.2.

Selection of best fitted individuals: Best fitted individuals were selected from the current population using the selection operator.

Genetic crossover: Simple heuristic crossover at the rate of less than 0.07 was applied to build the new chromosomes.

Mutation: Randomly selected uniform genes were mutated from the domain of 1000 chromosomes (genes = number of variables×1000) in the population. The mutation rate was set low at ≤ 0.1 %.

Elitism: The best evolved individuals (optimal solutions) were passed on to the next generation without being affected by mutation, so that the evolution achieved in a generation shouldn't fall below a minimum level. The hybrid ANN-GA/EA model was created in MATLAB version 7.6.0 (Mathworks Inc 2008, Natick, USA).

3.2.13. Experimental validation of the optimized model

All the validation experiments at the shake flask and bioreactor levels were carried out in triplicate and the average of the solutions are expressed with the standard deviation (±SD).

3.3. Results and discussion

3.3.1. Response surface methodology based statistical optimization of the fermentative medium components

The 3D response surface plots and ANOVA of CCD further established oatmeal and glucose at their optimum levels as sole production media components, in agreement with earlier results (Section 2.3.3, Chapter 2). Based on the full factorial 2^4 CCD matrices (Table 3.1), a quadratic polynomial relation was established between the swainsonine production and the medium components. The resulting RSM model equation was as follows:

$$Y = 1.62 - 0.18\,A + 0.26\,B + 0.03\,C + 0.08\,D + 0.03\,A^2 - 0.14\,B^2 - 0.003\,C^2 - 0.14\,D^2 - 0.01\,AB + 0.08\,AC + 0.24\,AD - 0.27\,BC - 0.23\,BD + 0.04\,CD \quad (Eq.\ 3.8)$$

Here, A = glucose (%, w/v), B = oatmeal (%, w/v), C = $CaCl_2$ (mM) and D = inoculum size (%, v/v).

The results were analyzed using the ANOVA to the experimental design used (Table 3.2). A model is considered to be significant if its *p*-value is lower than 0.05. The 'adequate precision' of the statistical data was indexed by 'signal to noise' ratio, which should be greater than 4. Here, this ratio was 63.49 which confirmed the adequacy of signal. Based on the quadratic relation (Eq. 3.8), the 3D response surface curves (Fig. 3.4) were plotted between the swainsonine production (Z-axis) and any two independent variables, maintaining rest of the variables at their optimum levels. The effects of glucose and oatmeal on swainsonine production at a fixed concentration of $CaCl_2$ (0.3 mM) and inoculum size (1.75 % v/v) are shown in Fig. 3.4a. It was observed that the swainsonine production increased significantly between oatmeal

concentration ranges of 5.5-7 % (w/v). However, with increasing glucose concentration, the production attained saturation. This result inferred the lesser degree of interaction between glucose and oatmeal (p = 0.19). The effect of oatmeal and $CaCl_2$ together had prominent interaction level (p = 0.0001). Swainsonine production enhanced continuously with elevation in oatmeal and $CaCl_2$ concentrations and achieved a maximum level at 6 % (w/v) and 0.4 mM, respectively (Fig. 3.4b). The alkaloid production as a function of oatmeal and inoculum size showed a very prominent interaction (p = 0.0001) with a steep rise in its concentration. Highest production predicted at nearly 6 to 6.5 (%, w/v) of oatmeal concentration and inoculum size of 2 % (v/v) as shown in Fig. 3.4c. Product concentration was influenced by $CaCl_2$ and inoculum size (Fig. 3.4d). It was also observed that inoculum size has a significant effect with prominent interaction (p = 0.005) whereas, $CaCl_2$ had a lesser effect. However, $CaCl_2$ was also significantly affecting the production, as was evident from the PB screening and its higher degree of interaction with rest of the variables in CCD experiment (p = 0.0001). The small concentrations of $CaCl_2$, might be providing calcium (Ca^{+2}) as trace element and two chloride (Cl^-), which play an important role in the nutrient uptake by influencing the transport channels across the fungal cell membrane. Apart from this, calcium is the structural component of cell wall, cofactor of many enzymes, has prominent role in cell growth, cell division and hyphal growth of many mycorrhiza (Jackson and Heath, 1993).

The maximization of the regression equation (Eq. 3.5) was carried out using iterative methods to obtain the optimum levels of variables (medium components). The maximum predictable response (swainsonine production) was estimated by substituting these optimum levels of variables into the regression equation (Eq. 3.8). Hence, the optimum levels of each variable were determined as: 1.38 % (w/v) glucose,

6.96 % (w/v) oatmeal, 0.2 mM $CaCl_2$ and 1.38 % (v/v) inoculum size. With optimized levels, the average swainsonine production was found to be 2.81±0.17 µg/ml, which was in accordance to the model predicted value 2.77 µg/ml. An overall 75.62 % increase in the swainsonine production from *M. anisopliae* ARSEF-1724 was achieved after statistical medium optimization.

3.3.2. Shake flask evaluation of swainsonine production as a function of DCW, saccharide concentration and pH

Swainsonine production in the optimized medium was finally evaluated as the function of parameters pH, total saccharides (glucose) and DCW at shake flask level (Fig.3.5). Production reached a maximum level with 2.67±0.41 µg/ml after 72 h of incubation and thereafter decreased. Depletion of saccharide content in the media was followed by reduction in swainsonine production sharply after 96 h. Initial medium pH 5.8 was slightly lowered and then maintained at 5.3 up to 72 h and reduced to pH 4.8 after 144 h followed by rapid decrease in the alkaloid production. Under shake flask evaluation of RSM-optimized results, alkaloid production fell rapidly after 72-96 h, followed by decrease in glucose and pH of the fermentation media. This indicated some 'diauxic' like behaviour of the fungal strain (ARSEF 1724), which can be hypothesized to consume swainsonine (including other metabolites) with the decrease in saccharide concentration below a critical level in the media. This is simultaneously accompanied by the reduction in pH of media from 5.5 to 4.5. This reduction in pH can be attributed to the production of some organic acids (Tamerler and Keshavarz, 1999). Since, indolizidine and pyrollidine class of alkaloids are more stable at low pH (Fellows and Fleet, 1989), hence the probability of low pH related degradation of the alkaloid is not considerable.

3.3.3. The artificial neural network-real encoded genetic algorithm based optimization of medium components

The non-linear relationship between the medium components (oatmeal, glucose, $CaCl_2$ and inoculum size) with swainsonine production was further modelled by ANN-REGA. The ANN model was trained and validated based upon the obtained experimental results of CCD. Twenty CCD experimental data sets were used to adjust the weights of the model for training and the remaining ten for testing the network's performance after each iteration. The model was found to be highly significant with the R^2-value of 0.99 for the training data and 0.94 for the test data (Fig. 3.6a and 3.6b). The results showed that ANN prediction was closer to the experimentally validated values for the CCD experiments as well as the regression prediction (Fig. 3.6c). Using the trained ANN as a fitness function, the medium optimization was carried out with REGA. The algorithm was run five times and the results at the tenth generation for each run were reported along with the experimentally validated value for swainsonine production (Table 3.3). The average of the best runs of the algorithm was considered for experimental validation at shake flask level. The evolution of healthy individuals achieved stagnation by the end of tenth generation and remained constant thereafter (Fig. 3.7a). The ANN-REGA predicted an average 3.32 µg/ml of swainsonine production with medium compositions: 2.90 % (w/v) glucose, 7.89 % (w/v) oatmeal, 0.21 mM $CaCl_2$ and 1.22 % (v/v) inoculum size. The shake flask validation of ANN-REGA model resulted in an average swainsonine yield of 3.25 µg/ml, in close agreement with the predicted yield (Fig. 3.7b). The REGA optimized solutions showed an overall increase of 103 % in alkaloid production compared to the unoptimized model previously reported (Section 2.3.1, Chapter 2). Although, the interaction between the medium components cannot be exactly

described by computational algorithms, but it can be assumed that there is a lack of interaction between these components, if they converge to an optimum value (Haider *et al.*, 2008). Hence, further scale up studies for swainsonine production could provide the more appreciable results at bioreactor levels with controlled agitation, aeration and pH conditions.

3.3.4. Optimization of agitation and aeration rates at the bench top bioreactor level

Swainsonine production through submerged aerobic fermentation of filamentous fungi, *M. anisopliae* is affected significantly by agitation and aeration rates (Patrick *et al.*, 1995; Tamerler and Keshavarz, 1999). Hence, a series of batch fermentations were carried out for determining the optimal range of the process parameters.

3.3.5. Sequential OFAT screening cum optimization of agitation-aeration rates, analysis of scale up parameter and pellet morphologies

An agitation-aeration rate of 400 rpm-1.5 vvm was found optimal with maximum swainsonine production of 6.39±0.19 µg/ml at 84 h (Fig. 3.8a and 3.8b). The optimal production was achieved at the expense of 91.66 W/m^3 of power drawn by the impellers which increased linearly to maintain the constant stirrer speeds at altered culture conditions (Table 3.4). The relationship between the gassed power consumption per unit volume (Pg/V_L) is directly related to volumetric oxygen transfer coefficient (K_La), hence, it is an important scale up parameter (Vilaca *et al.*, 2000). In case of non-Newtonian fermentation media, the broth apparent viscosity (μ_a) negatively and gas superficial velocity (V_g) positively affects K_La as is shown by correlation proposed by many researchers as described in Table 3.5. Here, the optimal swainsonine production was followed by the K_La value of 341.48×10^{-4} s^{-1} which

decreased thereafter due to increase in broth apparent viscosity. However, a sharp increase in mass transfer values was again observed probably due to sporulation at around 108 h resulting in slight decrease in μ_a values. In contrast, Pg/V_L values were increased linearly with due course of fermentation and was not reduced significantly even after the culture sporulation (at 108 h) and drop in broth apparent viscosity (μ_a).

The higher aeration and agitation rates have two main effects on the cultivation of filamentous fungi in a stirred tank reactor on the one hand, enhanced oxygen supply and better mixing as well as mass and heat transfer. On the other hand, higher aeration and agitation intensities lead to higher energy input connected with higher mechanical stress on the fungal pellets, causing morphological changes, variations in biomass growth and product formation (Amanullah *et al.*, 2000; Rosa *et al.*, 2005; El-Enshasy *et al.*, 2006; Zmak *et al.*, 2006). The optimal production of swainsonine at different agitation and aeration rates was also correlated with the *Metarhizium* pellet morphology after 72 h of incubation (Fig. 3.9). The too much loosened, fluffy and exposed hyphae can easily be fragmented from the pellet surface due to mechanical stress encountered at increasing agitation rates (Fig. 3.9a-c), resulting in highly concentrated and mechanically injured pellets (Nielsen *et al.*, 1995; Cui *et al.*, 1997). However, at optimal combination of agitation and aeration rates *viz.*, 400 rpm-1.5 vvm, the pellet micro-morphology showed a more even, circular, relatively smaller and healthy pellets (Fig 3.9d-g). The ideal pellet morphologies for maximum fermentative yield are relatively smaller pellets, loosened enough for proper nutrient and oxygen transport within the core of the culture (Pazouki and Panda, 2000).

3.3.6. Optimization of agitation-aeration rates using artificial neural network - evolutionary algorithm

Neural network training and validation was performed with a total of 12 sets of training and test data each. Network's performance was assessed using MSE (mean squared error) and Regression analysis. The ANN training was performed for 200 epochs after which the MSE value of 0.0091 was observed. The training (0.96) and test regression (0.93) values further confirmed the networks efficacy (Fig. 3.10). Once trained, the neural network served as fitness function for the creation of a fuzzy set of large population (1000 experimental sets) in evolutionary programming (EP). Optimization of the agitation (N) and aeration (Q) rates was repeatedly performed 10 times and 10 generations in each case, unless a fermentation model was achieved to emulate the optimum production of swainsonine (Fig. 3.11a). EA optimized fermentation model predicted the swainsonine production to be 10.08 µg/ml at 325.47 rpm and 1.99 vvm after 80.75 h of incubation (Table 3.6).

3.3.7. Validation and analysis of ANN-EA optimized model

EP simulated fermentation model was lab validated at bioreactor operational conditions of 325 rpm and 2 vvm (Table 3.6). The swainsonine production was monitored for 156 h at every 12 h interval (including the optimal 80 h) and correlated with pH profile, saccharide consumption rate and growth rate kinetics (Fig. 3.11b). The swainsonine production was significantly less (7.93±0.52 µg/ml) compared to the EP simulated optimized production of 10.08 µg/ml. The swainsonine production was optimally achieved in the early stationary phase between 72 to 96 h and maintained thereafter up to 120 h with an average production of 7.53±0.52 µg/ml of alkaloid. However, the production fell steeply thereafter with depletion of saccharides (< 4

mg/ml) and decrease in pH values (< 4), below an assumed critical limit as reported earlier at shake flask level (Section 3.3.2). These conditions might have altered the functioning of trans-membrane sensor/transport proteins in fungi towards nutrients and metabolites in broth. In the absence of any other assimilable carbon source, these regulatory mechanisms together may resort diauxic growth of *Metarhizium* on secondary metabolites including swainsonine to obtain metabolic carbon (Young *et al.*, 2010). Hence, optimization of fed batch operation with altered carbon sources and pH conditions can be another area of research for enhanced production of swainsonine.

3.3.8. Analysis of scale up parameters (Pg/V_L and K_La) for ANN-EA optimized fermentative model

The EP simulated bioreactor conditions were also correlated with scale transformation dimensions. The theoretical K_La value at 80 h of 349.25×10^{-4} s^{-1} was in close agreement with corresponding experimental value of 352.16×10^{-4} s^{-1} (Table 3.6). Although, a marginal 20.33 % increase in swainsonine production was achieved at a nearly constant volumetric mass transfer rate (K_La) but at the expense of only one-third of the power (Pg/V_L) drawn by the impellers compared to that of OFAT optimization model. In case of non-Newtonian fermentation broth comprising filamentous fungi, there are higher shear rates (γ_{av}) and hence low apparent viscosities (μ_a) near the impeller (Rushton turbines, with low Di/Dt = 0.34) resulting in homogenous mixing and higher K_La (Olmos *et al.*, 2013). In contrast, there is a sharp decrease in turbulence, shear rate and rise in apparent viscosities beyond the limits of impeller radius which negatively affects K_La at the varying hydrodynamic conditions, broth rheologies, vessel geometries and culture conditions (Garcia-Ochoa *et al.*, 2000;

Shin *et al.*, 1996; Wang *et al.*, 1979; Gavrilescu *et al.*, 1993; Vilaca *et al.*, 2000; Linek *et al.*, 2004; Moo-Young and Blanch, 1981; van't Riet, 1979). This causes the channeling of sparged air towards the impeller and hence its poor distribution throughout the reactor. The condition can be overcome at higher agitation rates, which in turn increases significant power consumption and its loss as dissipated heat. The average power consumption increased logarithmically at the rate of 37.49±10.75 W/m^3 for 300 rpm, 91.66±28.05 W/m^3 for 400 rpm, 222.91±51.08 W/m^3 for 500 rpm with constant aeration rates (0.5 vvm) and with due course of incubation time (72-108 h) in bioreactor (Table 3.4). Here, an increase in gassed power consumption relative to agitation rates was not accompanied with any significant rise in production rate on account of the probable hydrodynamic stress to the fungal pellets (Benchapattarapong *et al.*, 2005). Hence, the proper elucidation and analysis of the scale up parameters (Pg/V_L and K_La) at optimal levels for swainsonine production was performed using evolutionary program based optimization model.

3.4. Conclusions

The two different optimization models for the optimal medium composition (at shake flask and bioreactor levels) and bioreactor operational conditions have been developed and comparatively analysed in the present chapter. The statistical RSM and artificial intelligence based ANN-REGA/EA were employed to develop a production process for swainsonine using *Metarhizium anisopliae* ARSEF 1724 (UM8). The validation experiments for optimal medium components (glucose, oatmeal, $CaCl_2$ and inoculum size) inferred that the ANN-REGA predicted a more efficient model with an average 103 % increase in swainsonine production compared with 75.62 % using statistical RSM model. The scale up production of swainsonine from the shake flask to bench top bioreactor levels involved the further optimization of agitation and aeration at different incubation hours. The initial OFAT screening of effective variable range (agitation and aeration) followed by their ANN-EA optimization resulted in an average 144 % increase in the alkaloid production with healthy culture physiologies (pellet morphology). Moreover, the ANN-EA optimization also inferred a 20.33 % increase in swainsonine production at almost same volumetric oxygen mass transfer coefficient (K_La) and one third of power consumed (Pg/V_L) by the impellers, as compared to the initial OFAT-screening of aeration and agitation rates. As the proposed optimization models works efficiently with real value inputs at low computational load, ANN-EA can potentially be used to simulate other scale transformation studies involving aerobic fermentation.

Table 3.1. Full factorial 2^4 CCD matrix of the four variables in real and coded values along with experimentally observed (mean±SD), regression predicted and ANN predicted response values for swainsonine production from *Metarhizium anisopliae* ARSEF-1724 (UM8).

Glucose (%, w/v)	Oatmeal (%, w/v)	$CaCl_2$ (mM)	Inoculum size (%, v/v)	Swainsonine production (µg/ml)		
Data sets used for ANN model training				**Experimental**	**Regression**	**ANN**
1.37(-1 α)	5(-1 α)	0.20(-1 α)	1.37 (-1 α)	1.04±0.07	1.00	1.11
2.12(+1 α)	5(-1 α)	0.20(-1 α)	1.37 (-1 α)	0.02±0.01	0.02	0.01
1.37(-1 α)	7(+1 α)	0.20(-1 α)	1.37(-1 α)	2.59±0.45	2.59	2.51
2.12(+1 α)	7(+1 α)	0.20(-1 α)	1.37(-1 α)	1.61±0.19	1.53	1.65
1.37(-1 α)	5(-1 α)	0.40(+1 α)	1.37(-1 α)	1.35±0.39	1.36	1.40
2.12(+1 α)	5(-1 α)	0.40(+1 α)	1.37(-1 α)	0.72±0.16	0.73	0.68
1.37(-1 α)	7(+1 α)	0.40(+1 α)	1.37(-1 α)	1.88±0.28	1.84	1.72
2.12(+1 α)	7(+1 α)	0.40(+1 α)	1.37(-1 α)	1.08±0.32	1.14	1.04
1.37(-1 α)	5(-1 α)	0.20(-1 α)	2.12(+1 α)	1.12±0.43	1.05	1.16
2.12(+1 α)	5(-1 α)	0.20(-1 α)	2.12(+1 α)	1.01±0.25	1.04	0.98
2.12(+1 α)	7(+1 α)	0.40(+1 α)	2.12(+1 α)	1.38±0.01	1.41	1.32
1.00(-2α)	6(0)	0.30(0)	1.75(0)	2.08±0.43	2.11	1.98
2.50(+2α)	6(0)	0.30(0)	1.75(0)	1.41±0.42	1.39	1.25
1.75(0)	4(-2α)	0.30(0)	1.75(0)	0.52±0.34	0.50	0.53
1.75(0)	6(0)	0.30(0)	1.75(0)	1.62±0.36	1.62	1.64
1.75(0)	6(0)	0.30(0)	1.75(0)	1.55±0.06	1.62	1.64
1.75(0)	6(0)	0.30(0)	1.75(0)	1.59±0.33	1.62	1.64
1.75(0)	6(0)	0.30(0)	1.75(0)	1.68±0.29	1.62	1.64
1.75(0)	6(0)	0.30(0)	1.75(0)	1.64±0.28	1.62	1.64
1.75(0)	6(0)	0.30(0)	1.75(0)	1.69±0.15	1.62	1.64
Data sets used for ANN model testing						
1.37(-1 α)	7(+1 α)	0.20(-1 α)	2.12(+1 α)	1.73±0.15	1.71	1.84
2.12(+1 α)	7(+1 α)	0.20(-1 α)	2.12(+1 α)	1.65±0.45	1.67	1.71
1.37(-1 α)	5(-1 α)	0.40(+1 α)	2.12(+1 α)	1.53±0.22	1.60	1.47
2.12(+1 α)	5(-1 α)	0.40(+1 α)	2.12(+1 α)	1.95±0.04	1.93	1.85
1.37(-1 α)	7(+1 α)	0.40(+1 α)	2.12(+1 α)	1.18±0.19	1.15	1.21
1.75 (0)	6(0)	0.10(-2α)	1.75(0)	1.47±0.28	1.54	1.52
1.75(0)	6(0)	0.50(+2α)	1.75(0)	1.75±0.29	1.68	1.71
1.75(0)	6(0)	0.30(0)	1.00(-2α)	0.86±0.40	0.87	0.92
1.75(0)	6(0)	0.30(0)	2.50(+2α)	1.21±0.05	1.20	1.34
1.75 (0)	8(+2α)	0.30(0)	1.75(0)	1.55±0.26	1.57	1.68

Table 3.2. ANOVA for quadratic model and its coefficients estimated by linear multiple linear regression.

Source	SS*	DF**	MS***	F-value	Pro. $p > F$	Significance
Model	**7.04**	**14**	**0.50**	**153.88**	**<0.0001**	**Significant**
A	0.78	1	0.78	239.11	$< 0.01e^{-2}$	
B	1.72	1	1.72	526.89	$< 0.01\ e^{-2}$	
C	0.03	1	0.03	9.62	$0.73\ e^{-2}$	
D	0.15	1	0.15	48.52	$< 0.01\ e^{-2}$	
A^2	0.02	1	0.02	7.91	$1.31\ e^{-2}$	
B^2	0.59	1	0.59	180.79	$< 0.01\ e^{-2}$	
C^2	$0.02e^{-2}$	1	$0.02e^{-2}$	0.07	$7.85\ e^{-1}$	
D^2	0.59	1	0.59	180.79	$< 0.01\ e^{-2}$	
AB	$0.06e^{-1}$	1	$0.06e^{-1}$	1.84	$19.39\ e^{-2}$	
AC	0.12	1	0.12	36.89	$< 0.01\ e^{-2}$	
AD	0.92	1	0.92	283.57	$< 0.01\ e^{-2}$	
BC	1.21	1	1.21	372.05	$< 0.01\ e^{-2}$	
BD	0.85	1	0.85	263.05	$< 0.01\ e^{-2}$	
CD	0.03	1	0.03	10.78	$0.05\ e^{-1}$	
Residual error	0.04	15	0.003			
Lack-of-fit	0.03	10	0.003	1.21	$43.89\ e^{-2}$	Non-significant
Pure error	0.01	5	0.002			
Total	7.09	29				

* sum of squares; ** degrees of freedom; *** Mean of squares

A: Glucose, B: Oatmeal, C: $CaCl_2$ and D: Inoculum size

With model regression values $R^2 = 0.99$ and $R^2 = 0.98$

Table 3.3. ANN-REGA optimized concentration of medium components along with their predicted and experimental swainsonine production from *Metarhizium anisopliae* ARSEF-1724 (UM8).

ANN-REGA run	Glucose (%, w/v)	Oatmeal (%, w/v)	$CaCl_2$ (mM)	Inoculum size (%, v/v)	Swainsonine production (µg/ml)
1	2.92	7.91	0.20	1.11	3.06
2	2.82	7.97	0.23	1.31	3.20
3	2.96	7.91	0.20	1.15	3.06
4	2.92	7.95	0.21	1.16	3.15
5	2.89	7.73	0.25	1.40	3.14
Average model predicted	2.90	7.89	0.21	1.22	**3.12**
Experimental	2.90	7.89	0.21	1.22	**3.25±0.39**

Table 3.4. Effects of varying agitation (N) and aeration (Q) rates at varying incubation hours (72-96), with respect to the power consumed per unit volume (Pg/V_L) under aeration and volumetric oxygen mass transfer coefficient (K_La) for the swainsonine production from *Metarhizium anisopliae* ARSEF-1724 (UM8).

S. No.	N (rpm)*	Q (vvm)**	Incubation time (h)	Pg (W)	Pg/V_L (W/m^3)	K_La (s^{-1})	Swainsonine Production (µg/ml)	
							Y_{EX} [a]	Y_{EP} [b]
1	300	0.5	72	0.03	25.00	116.43×10^{-4}	3.74±0.02	2.89
2	300	0.5	84	0.04	33.33	123.12×10^{-4}	3.76±0.07	2.95
3	300	0.5	96	0.05	41.66	101.02×10^{-4}	3.33±0.03	3.12
4	300	0.5	108	0.06	50.00	119.07×10^{-4}	3.56±0.03	3.60
5	400	0.5	72	0.06	50.00	142.18×10^{-4}	2.99±0.09	2.97
6	400	0.5	84	0.13	108.33	136.21×10^{-4}	3.99±0.02	3.92
7	400	0.5	96	0.12	100.00	144.25×10^{-4}	4.29 ±0.11	5.24
8	400	0.5	108	0.13	108.33	154.71×10^{-4}	4.86±0.003	6.31
9	500	0.5	72	0.18	150.00	173.94×10^{-4}	3.81±0.06	4.33
10	500	0.5	84	0.27	225.00	168.74×10^{-4}	4.65±0.10	5.01
11	500	0.5	96	0.31	258.33	155.25×10^{-4}	5.05±0.19	5.28
12	500	0.5	108	0.31	258.33	166.85×10^{-4}	5.08±0.12	5.37
13	400	1	72	0.06	50.00	242.64×10^{-4}	3.33±0.31	4.16
14	400	1	84	0.12	100.00	257.43×10^{-4}	3.18±0.21	5.52
15	400	1	96	0.11	91.66	235.14×10^{-4}	4.59±0.39	6.34
16	400	1	108	0.12	100.00	245.20×10^{-4}	4.57±0.33	5.76
17	400	1.5	72	0.09	75.00	350.72×10^{-4}	5.95±0.06	5.29
18	400	1.5	84	0.11	91.66	341.48×10^{-4}	6.59±0.10	6.74
19	400	1.5	96	0.12	100.00	336.19×10^{-4}	6.39±0.19	6.17
20	400	1.5	108	0.13	108.33	365.76×10^{-4}	6.36±0.12	6.35
21	400	2	72	0.06	50.00	479.06×10^{-4}	5.55±0.07	5.44
22	400	2	84	0.08	66.66	462.24×10^{-4}	5.83±0.02	5.55
23	400	2	96	0.11	91.66	469.90×10^{-4}	5.49±0.007	5.66
24	400	2	108	0.12	100.00	521.03×10^{-4}	5.88±0.03	5.82

*, ** 'rpm and vvm' were changed to 'rps and vvs' for calculation purpose.

[a] Experimental swainsonine production observed for OFAT screening and optimization.

[b] Predicted swainsonine production for optimized EP simulations.

Table 3.5. Reported correlations for volumetric oxygen mass transfer coefficient (K_La, s^{-1}) observed with varying fermentation media and vessel dimensions.

References	Vessel diameter (m)	Impeller characteristics		Proposed K_La (s^{-1}) correlations and type of fluids
		Type	Di/Dt	
Present work	0.13	Rushton	0.34	$K_La = 23.1\,(\frac{P_g}{V_L})^{0.016}\,(V_g)^{0.85}\,(\mu_a)^{-0.60}$
*Ogut and Hatch, 1988	0.14	Rushton	Not available	Non Newtonian and high broth density ~193.97±16.24 kg m^{-3} between 72h to 108h.
Garcia-Ochoa *et al.*, 2000	0.13	Rushton	0.34	$K_La = 6.14 \times 10^{-3}\,(\frac{P_g}{V_L})^{0.60}\,(V_g)^{0.50}\,\eta_{eff}{}^{-0.50}$ Non Newtonian, non coalescent fermentation broth
Shin *et al.*, 1996	0.13	Rushton	0.34	$K_La = 0.019\,(\frac{P_g}{V_L})^{0.55}\,(V_g)^{0.64} \times (1 + 2.12x + 0.20x^2)^{-0.25}$ *E. coli* fermentation broth, high density and non Newtonian
Wang *et al.*, 1979	Not available	Rushton	Not available	$K_La = 8.42\,(\frac{P_g}{V_L})^{0.33}\,(V_g)^{0.56}$ Non Newtonian fermentation broth (20-30 m^3)
Gavrilescu *et al.*, 1993	Not available	Rushton	Not available	$K_La = 0.025\,(\frac{P_g}{V_L})^{0.40}\,(V_g)^{0.50}(N)^{0.50}(\mu_a/\mu_g)^{0.65}$ Non Newtonian *Actinomycetes* fermentation broth (20-100 m^3)
Vilaca *et al.*, 2000	0.21	Rushton	0.40	$K_La = 6.76 \times 10^{-3}\,(\frac{P_g}{V_L})^{0.94}\,(V_g)^{0.65}$ Air-water-sulfite solution
Linek *et al.*, 2004	0.29	Rushton	0.33	$K_La = 0.01\,(\frac{P_g}{V_L})^{0.69}\,(V_g)^{0.58}$ Air-water (Newtonian)
Moo-Young and Blanch, 1981	Not available	Rushton	Not available	$K_La = 1.80 \times 10^{-3}\,(\frac{P_g}{V_L})^{0.70}\,(V_g)^{0.30}$
van't Riet, 1979	Various	Various	various	$K_La = 2 \times 10^{-3}\,(\frac{P_g}{V_L})^{0.70}\,(V_g)^{0.20}$ Air-water with ions

Table 3.6. Evolutionary program (EP) based simulations (1-10), experimental validation of the EP and OFAT - optimized models at varying agitation (N) and aeration (Q) rates for different incubation time (h) with respect to swainsonine production from *Metarhizium anisopliae* ARSEF-1724 (UM8). The production was correlated with corresponding power consumed per unit volume (Pg/V_L) under aeration and volumetric oxygen mass transfer coefficient (K_La).

EP simulations	Agitation N (rpm)	Aeration Q (vvm)	Incubation time (h)	Swainsonine production (µg/ml)
1	425.71	1.98	74.81	6.56
2	300.58	1.37	85.15	7.72
3	302.61	1.51	77.95	7.22
4	441.11	1.98	107.91	4.28
5	303.71	1.78	97.46	6.31
6	392.10	1.95	107.96	7.55
7	411.47	1.63	100.57	6.67
8	459.28	1.68	107.85	8.21
9	300.10	1.98	103.33	8.43
10*	325.47	1.99	80.75	10.08
Experimental validation of EP-optimized model	325	2	80	7.93±0.52
			At K_La = 349.25×10^{-4} s^{-1} and Pg/V_L = 33.33 W/m^3	
OFAT-optimized model	400	1.5	84	6.59±0.10
			At K_La = 341.48×10^{-4} s^{-1} and Pg/V_L = 91.66 W/m^3	

* Finally evolved EP simulation chosen for experimental validation.

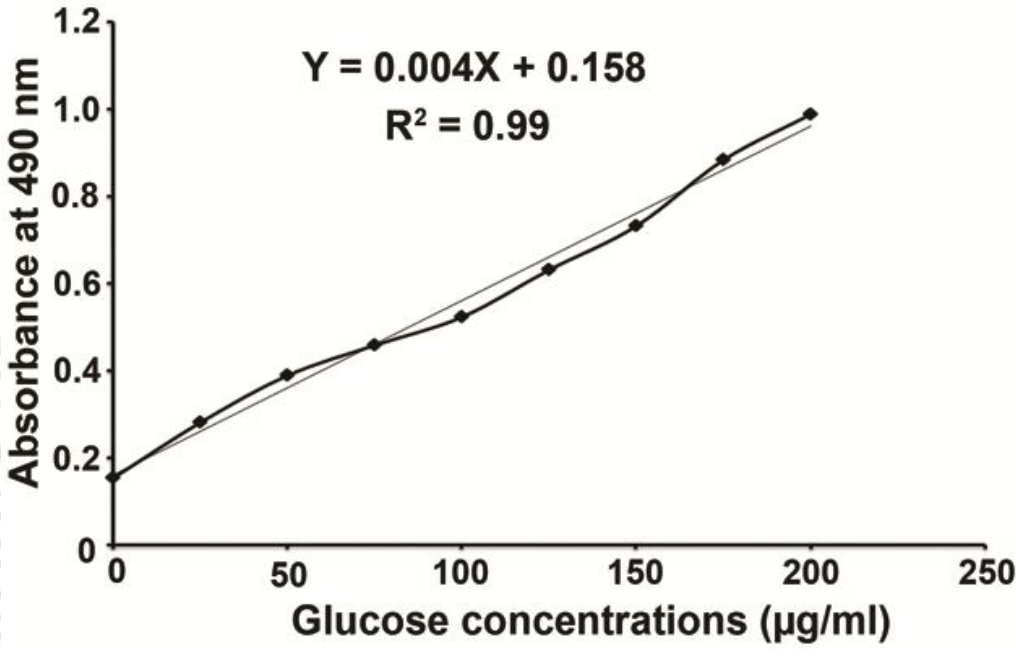


Fig. 3.1. Calibration curve for the total glucose estimation using phenol-sulphuric acid method. Here, 'Y' represents the absorbance values for the reaction at 490 nm and 'X' represents the glucose concentration in the fermentation broth.

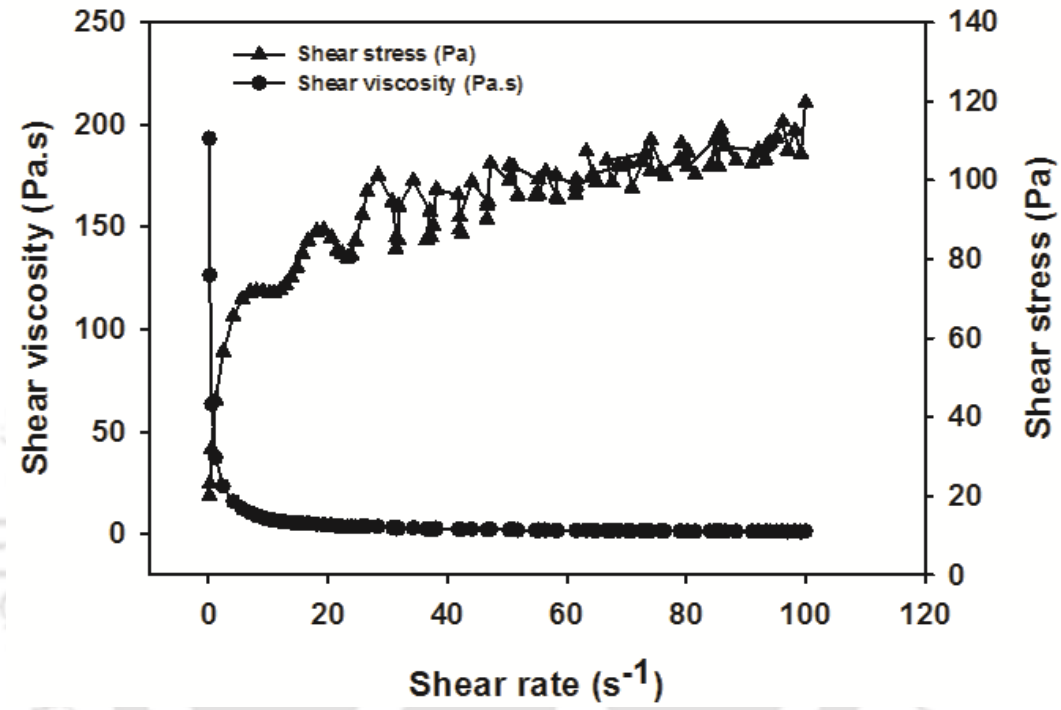


Fig. 3.2. Correlation of shear viscosity and shear stress as a function of shear rate in the optimized fermentation medium for swainsonine production from *Metarhizium anisopliae* ARSEF-1724 (UM8) at bench top bioreactor level.

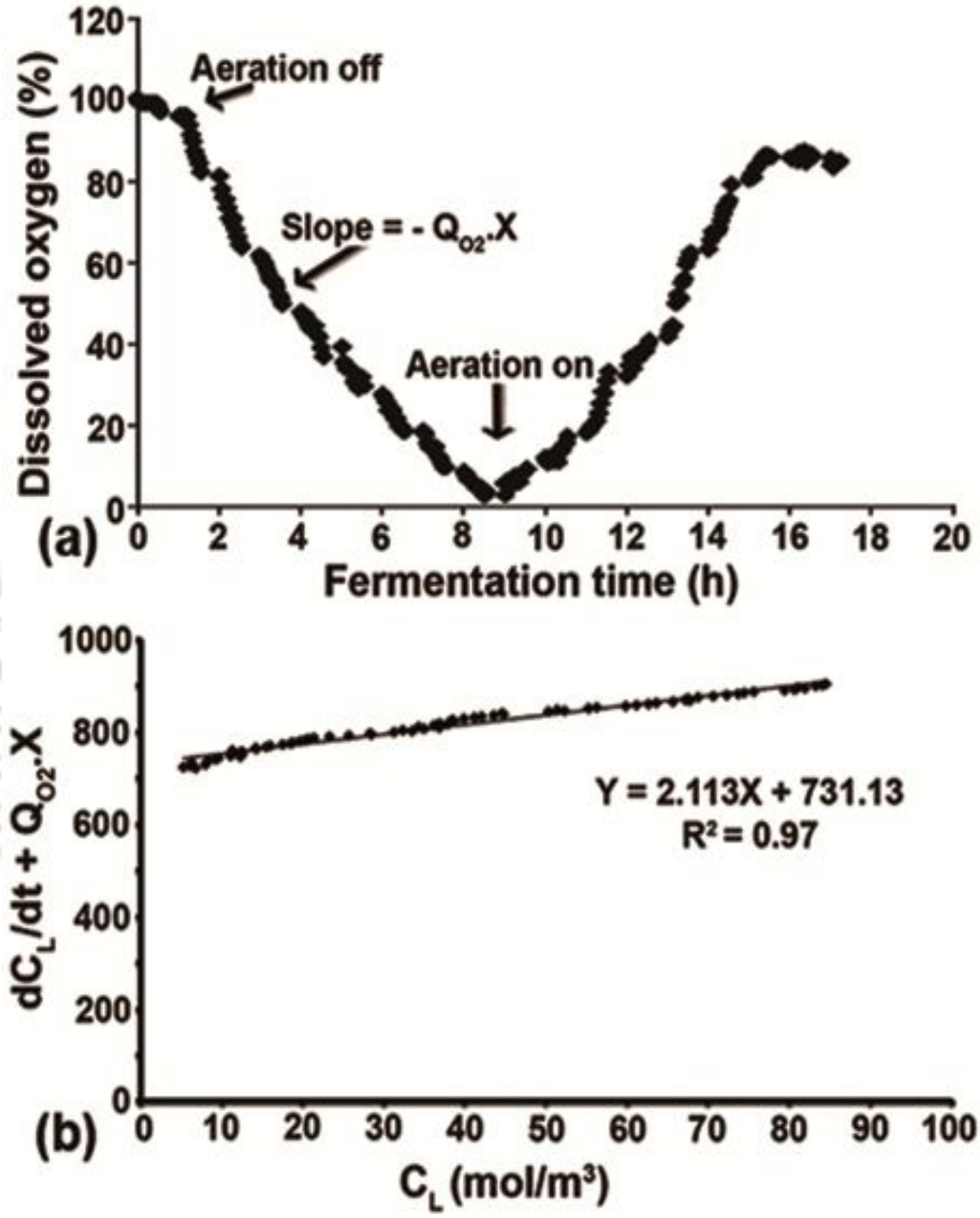


Fig. 3.3. (a) The evaluation of total oxygen consumption rate, and **(b)** the determination of volumetric oxygen mass transfer (K_La, s^{-1}) coefficient as the slope of equation 3.3 for the incubation period 72-90 h during *Metarhizium anisopliae* ARSEF-1724 (UM8) fermentation in dynamic method.

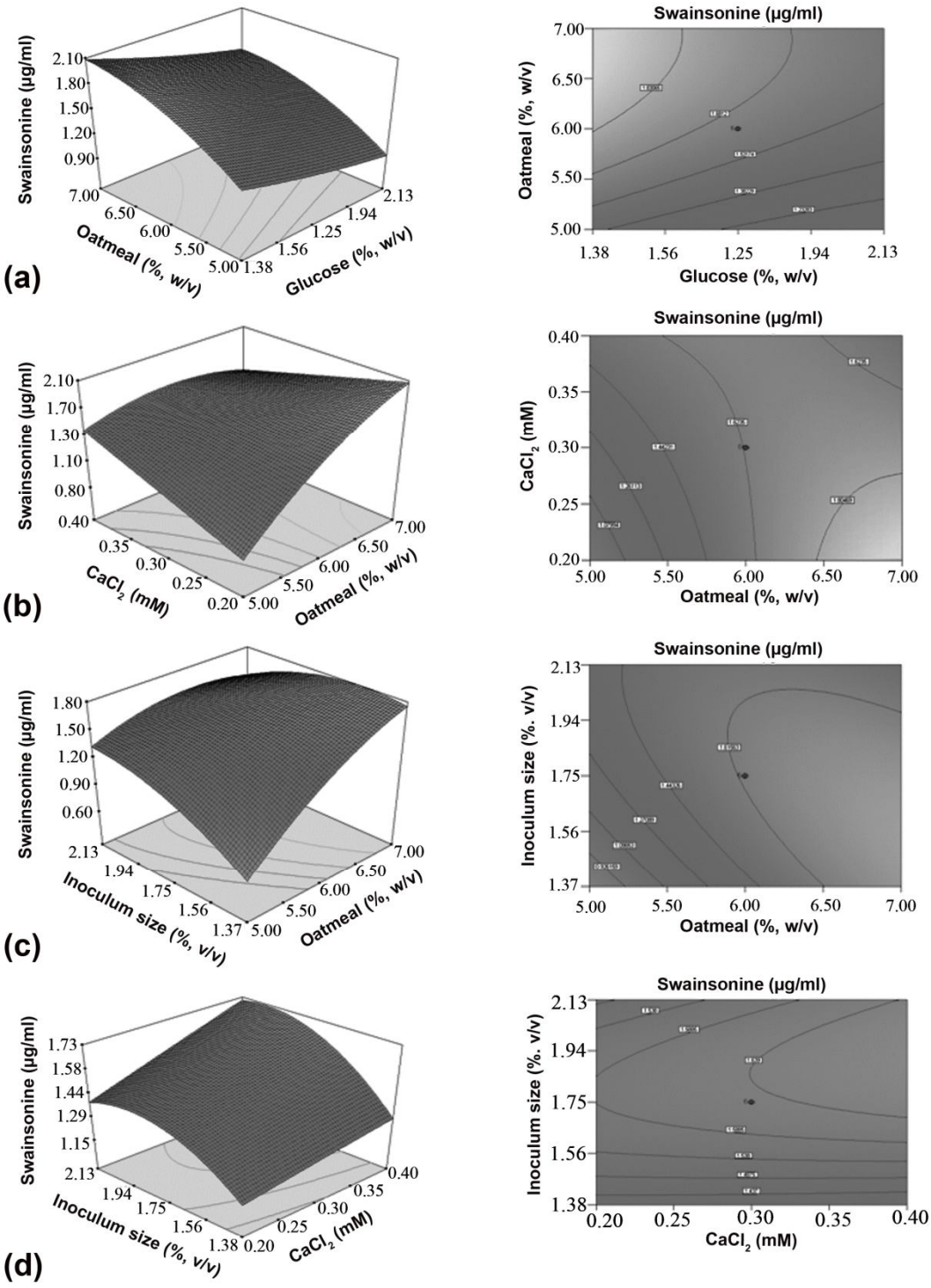


Fig. 3.4. 3D response surface and contour plots for swainsonine production from *Metarhizium anisopliae* ARSEF 1724 (UM8) **(a)** glucose and oatmeal, (**b)** oatmeal and $CaCl_2$, **(c)** oatmeal and inoculum size and **(d)** $CaCl_2$ and inoculum size.

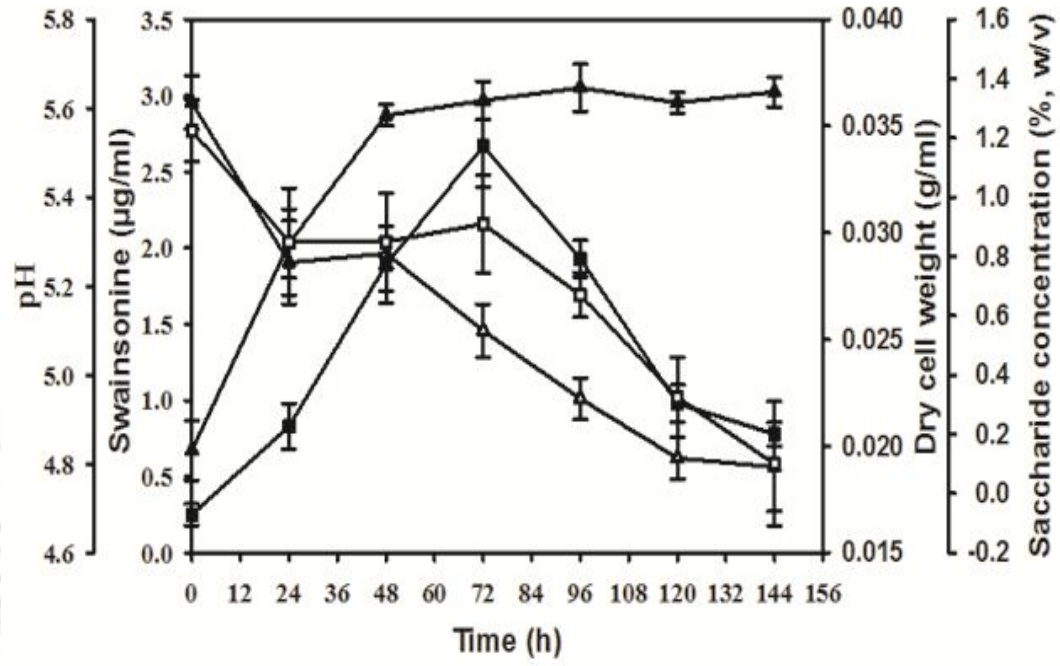


Fig. 3.5. Shake flask evaluation of swainsonine production from *Metarhizium anisopliae* ARSEF-1724 (UM8) (─■─) as a function of CDW (─▲─), saccharide concentration (─Δ─) and pH (─□─) on RSM optimized medium.

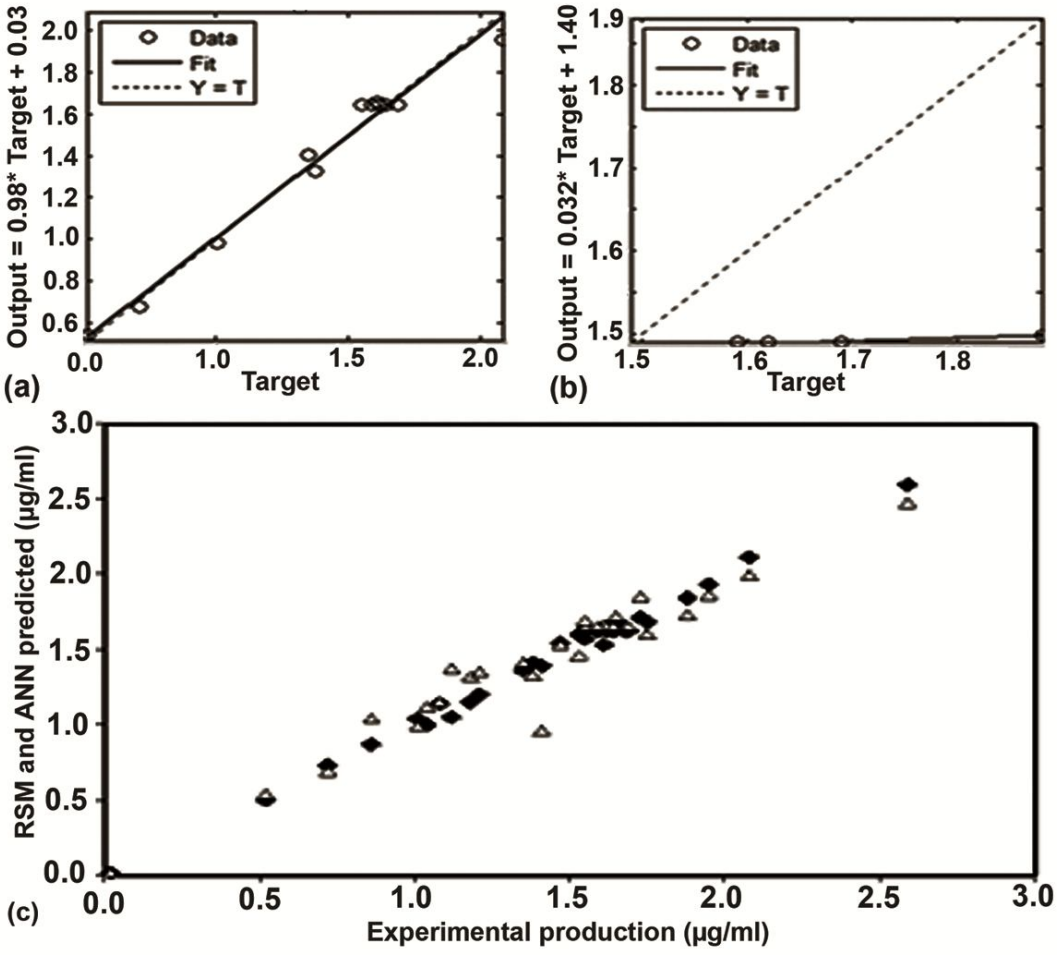


Fig. 3.6. Regression curves for the ANN-generated sets of experiments (population individuals in genetic algorithm) and their solutions **(a)** training, R^2 = 0.99 **(b)** test, R^2 = 0.94 and **(c)** model predicted (ANN ─Δ─, RSM─♦ ─) versus experimental swainsonine production from *Metarhizium anisopliae* ARSEF-1724 (UM8).

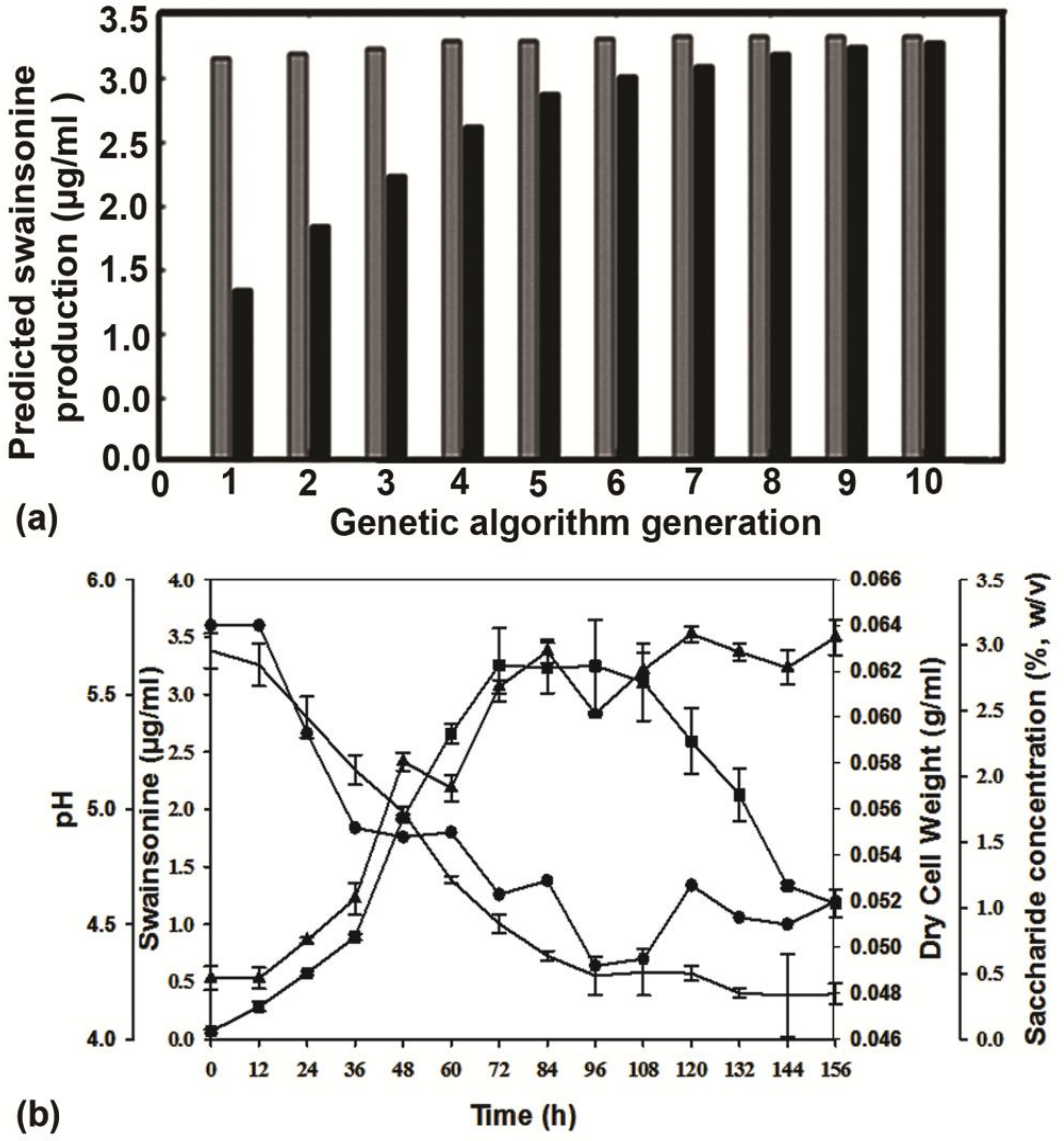


Fig. 3.7. (a) Evolution of best (▌) and average (▌) predicted values for swainsonine production (µg/ml) from *Metarhizium anisopliae* ARSEF 1724 (UM8) over ten generations in genetic algorithm and **(b)** shake flask evaluation of swainsonine production (—■—) as a function of CDW (—▲—), saccharide concentration (——) and pH (—●—) in ANN-REGA optimized medium.

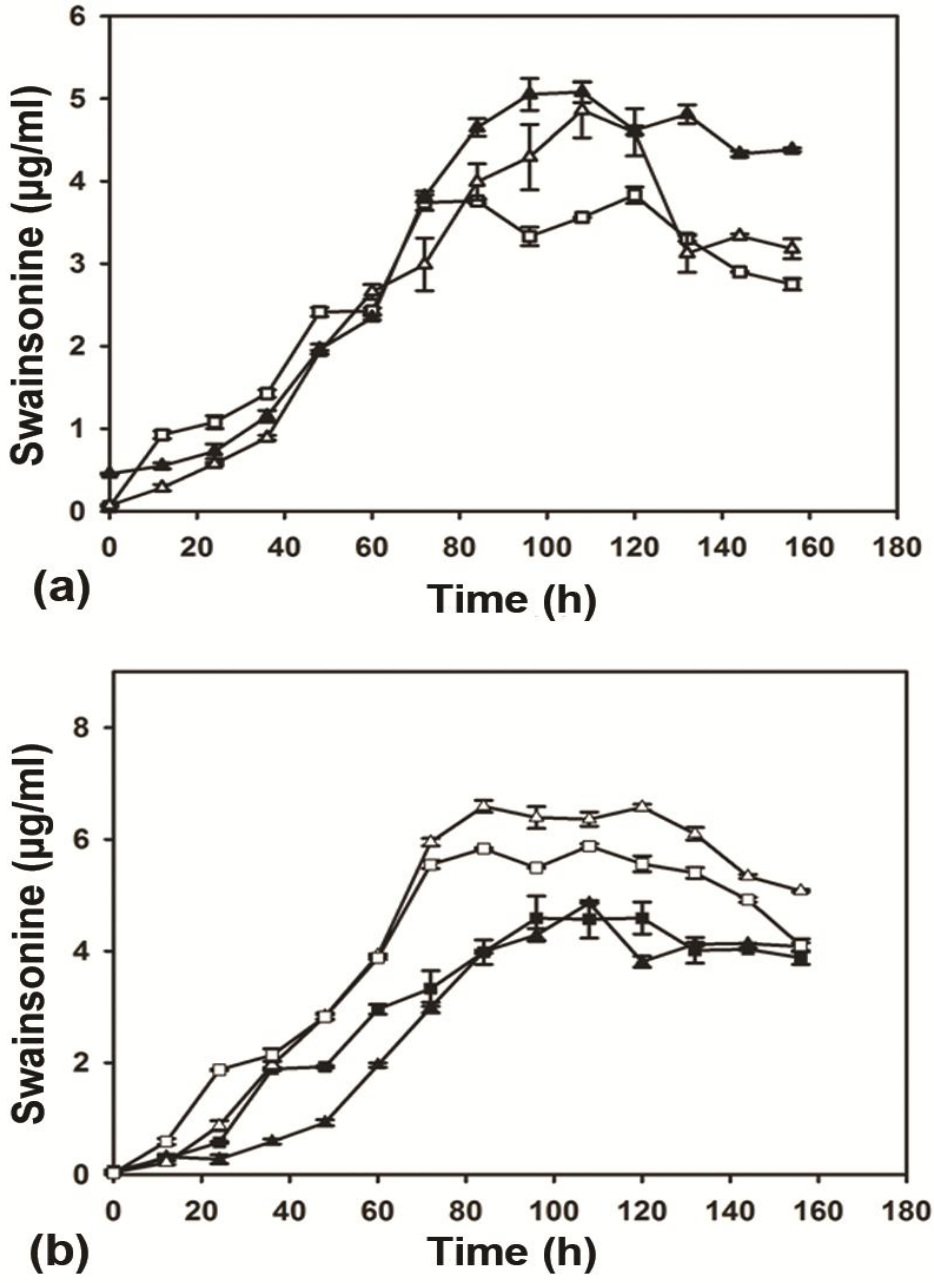


Fig. 3.8. One factor at a time (OFAT) screening cum optimization of swainsonine production from *Metarhizium anisopliae* ARSEF-1724 (UM8) in a bench top bioreactor in due course of fermentation (156 h) at **(a)** varying agitation rates 300 rpm (—□—), 400 rpm (—Δ—) and 500 rpm (—▲—) with a constant aeration of 0.5 vvm, and **(b)** varying aeration rates 0.5 vvm (—▲—), 1 vvm (—■—), 1.5 vvm (—Δ—) and 2 vvm (—□—) with a constant agitation of 400 rpm.

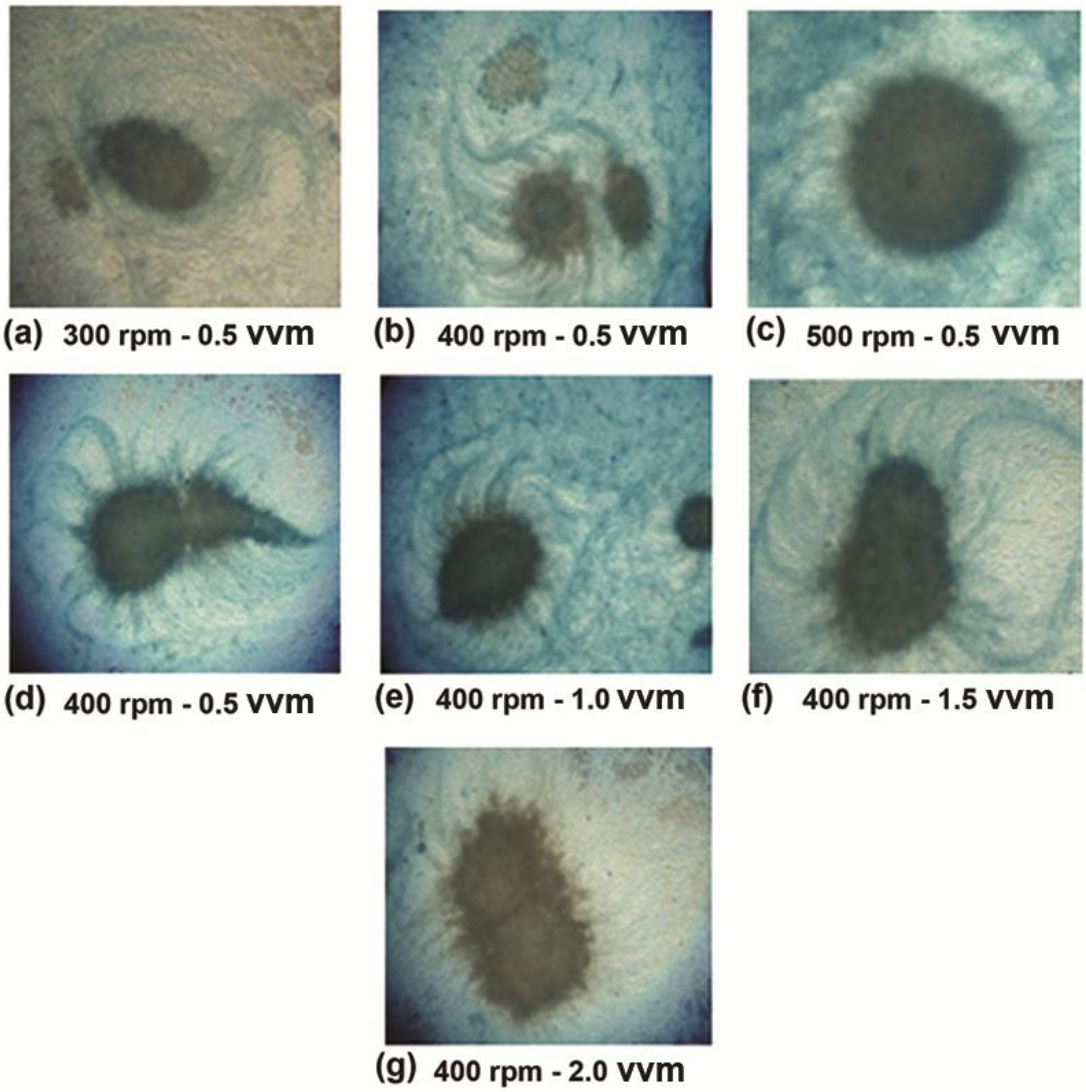


Fig. 3.9. The 40X microscopic images of *Metarhizium anisopliae* ARSEF 1724 (UM8) pellet morphologies at different combinations of agitation and aeration rates in bench top bioreactor.

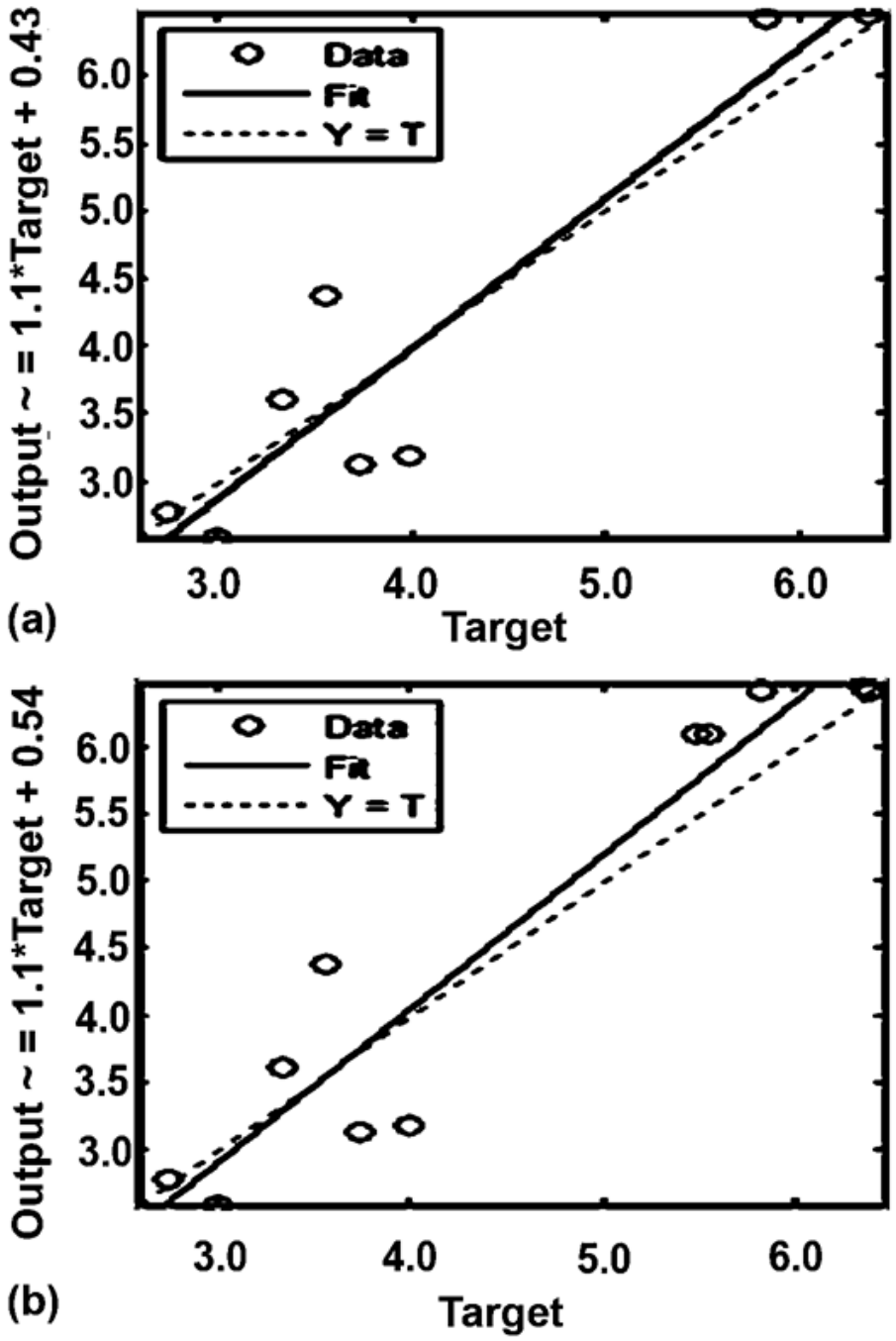


Fig. 3.10. Regression curves of the ANN-generated sets of experiments (population individuals in evolutionary algorithm) and their solutions (Target) **(a)** training, $R^2 = 0.96$, and **(b)** test, $R^2 = 0.93$.

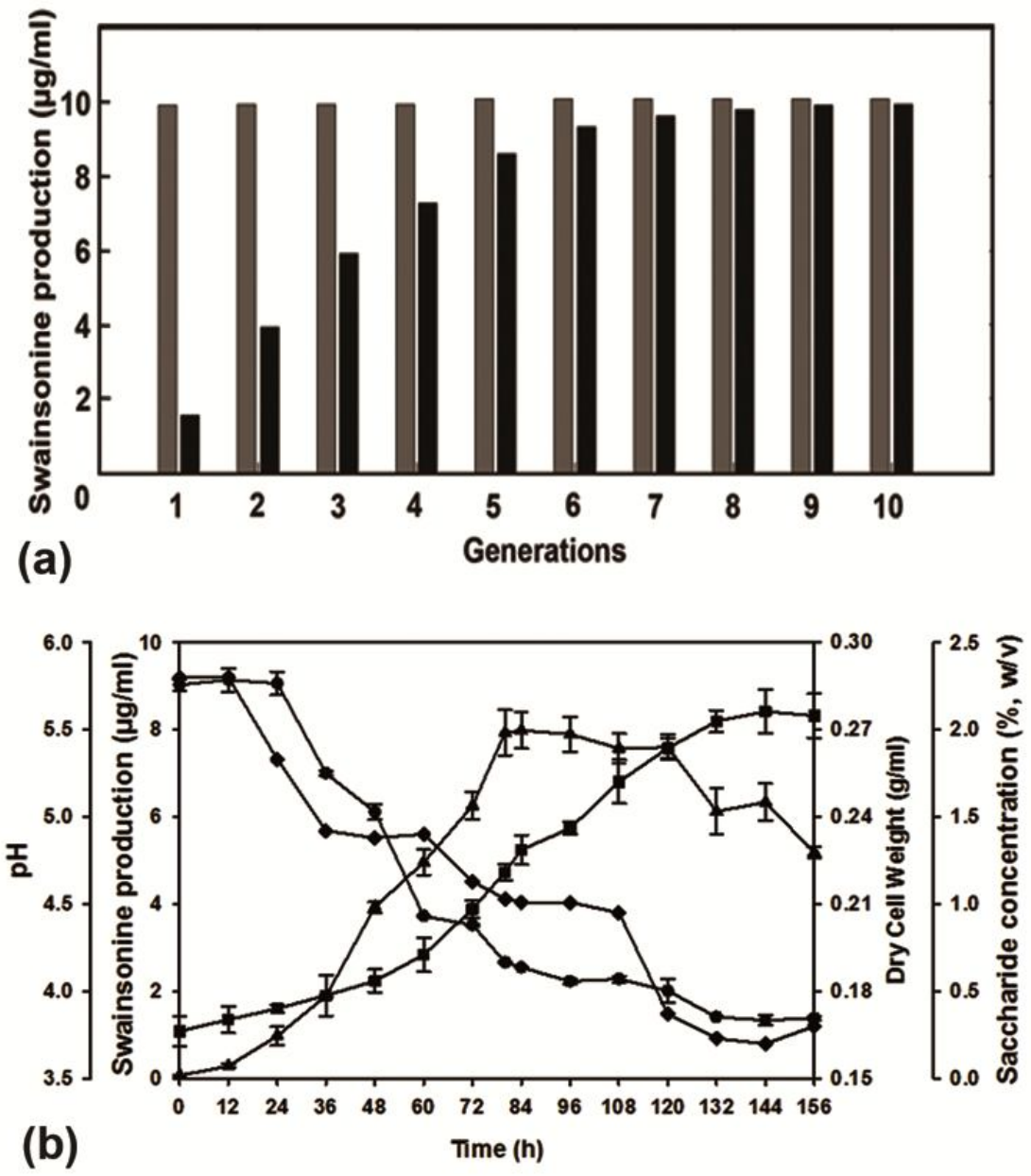


Fig. 3.11. (a) Evolution of best (▌) and average (▌) predicted values for swainsonine production (µg/ml) from *Metarhizium anisopliae* ARSEF-1724 (UM8) over ten generations and **(b)** bench top bioreactor evaluation for swainsonine production (—▲—) with respect to cell dry weight (—■—), medium pH (—●—) and saccharide consumption (—♦—) with due course of fermentation (156 h) at ANN-EA optimized conditions of agitation and aeration rates.

CHAPTER 4

Purification, characterization and quantitative analysis of swainsonine

4.1. Introduction

The primary goal of the natural products' research is to extract, isolate and characterize specific analytes from the complex mixture of microbial or plant matrices. The purification of swainsonine still remains a challenge from both biological as well as chemical synthetic routes, due to its high processing cost making the alkaloid highly expensive (50-200 USD/mg from Sigma-Aldrich, USA). The extraction and purification of swainsonine has been reported from the plant sources *Astragalus* (Zhao *et al.*, 2009) and *Oxytropis* (Gardner *et al.*, 2003; Wang *et al.*, 2011). Recently, swainsonine was extracted and purified with various polar solvent systems, physiological conditions and ion exchange resins (Gardener and Cook, 2010). However, the use of polar solvents like ethanol or water also imposes another problem of co-extraction of broth components like glucose, amino acids and other such hydrophilic metabolites. Moreover, such purification strategies are tedious and lack sufficient literature from microbial sources such as *M. anisopliae*. Hence, this necessitates the development of significant extraction and purification methods to remove the low molecular weight extraneous compounds.

Several methods have been reported for the analysis and quantification of swainsonine including enzymatic assay (Sim and Perry, 1995), thin-layer chromatography (TLC) (Molyneux *et al.*, 1991), capillary gas chromatography (Molyneux *et al.*, 1989), liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Gardner *et al.*, 2001) and high performance liquid chromatography (HPLC) with evaporative light-scattering detector (Yang *et al.*, 2012). Liquid chromatography will undoubtedly become the greatest area of growth in this respect because of its ability to analyze swainsonine without derivatization, but conventional HPLC analysis has rarely been applied to swainsonine. There are two reasons for this: (i) the lack of

suitable chromophore and (ii) the extreme hydrophilicity and polarity of swainsonine (Stockigt *et al.*, 2002). However, despite of certain limitations the readily available UV-HPLC method has the best combination of sensitivity, linearity and reliability. The alkaloids with weak or no chromophore groups can also be analysed at middle-UV (mUV) range (~200 nm) but this spectral range is also sensitive for few mobile phase impurities, which might interfere with the detection signals, which needs to be properly corrected (Gao *et al.*, 2008). Liquid chromatography-mass derived (LC-MSD) purification or preparative LC-MS, a combination of liquid chromatography and mass spectrometry adds a new dimension to traditional purification strategies. It provides a high throughput rapid purification and quantification of diverse library compounds in nature and its chemical characterization in a non-destructive manner allowing selective mass-derived fractionation (Hendricks *et al.*, 2011) contrary to the earlier reported methods (Donaldson *et al.*, 1990; Gardener *et al.*, 2001; Molyneux *et al.*, 1991).

Mass spectrometry (MS) is considered to be a specific and universal detection method (Molyneux *et al.*, 2002). MS will provide a next generation stage for swainsonine analysis with higher precision, sensitivity, and throughput. However, mass derived quantification for swainsonine was limited to the knowledge. The application of newer-generation MS with electrospray ionization will allow the development of targeted alkaloid (swainsonine) quantification. The underlying concept is that swainsonine may be quantified by using the selected ion monitoring (SIM) mode. The rapid evolution of analysis methods and software for targeted quantification, together with the widespread accessibility of applicable MS instruments, offers biochemists a transformative platform for systematic, reliable, and essentially universal quantification method.

There is increased international interest in swainsonine producing plants and microorganisms. North America has about 350 different species of *Astragalus* and *Oxytropis* while China has about 300 different reported species (Cao *et al.*, 1992; Ping *et al.*, 2009). A collaborative program between the USDA Poisonous Plant Research Laboratory and Chinese researchers is underway to catalogue the swainsonine content of many of these plants. Microbial production of swainsonine is also being investigated from *Rhizoctonia leguminocola* (Schneider *et al.*, 1983), *Metarhizium anisopliae* (Hino *et al.*, 1985; Patrick *et al.*, 1995, 1996) and by the fungal endophytes of locoweed (Braun *et al.*, 2003). Therefore, development of a simple and rapid method for the extraction and analysis of swainsonine would facilitate processing the large number of samples in this endeavour. The purpose of the present study was to develop an efficient purification and quantification method for swainsonine from *Metarhizium anisopliae* ARSEF-1724 using different solvent extraction procedures followed by comparative LC based analysis and purification methods.

4.2. Materials and Methods

4.2.1. Fungal strain and culture conditions

Metarhizium anisopliae ARSEF-1724 (UM8) was procured from "The Plant Protection Unit, United States Department of Agriculture (USDA), New York, Ithaca. The culture was maintained in SDA slants at 4 °C. A ten day old SDA slant was used for the preparation of conidial suspension of 4×10^8 spores/ml for inoculation as described in Section 2.2.1 of Chapter 2.

4.2.2. Chemicals and Reagents

1 mg of standard swainsonine crystals (Sigma-Aldrich) were dissolved in 1 ml of HPLC grade water to prepare stock (5.78 mM) and working (10-100 µM) standard solutions. All standards were then stored at 2-8 ºC for 3-6 months. All HPLC grade solvents such as acetonitrile, methanol and triflouroacetic acid (TFA) were obtained from Merck, Germany. All other chemicals used were of analytical grade as described in Section 2.2.2 (Chapter 2).

4.2.3. Swainsonine production

Swainsonine production was carried out in a 3 L bench top bioreactor in replicates using complex oatmeal based-genetic algorithm optimized medium (Section 3.3.3, Chapter 3).

4.2.4. Swainsonine extraction and partial purification

The 72 h culture broth was centrifuged at 16,000*g* for 10 min at 4 °C to separate the cells. The supernatant was mixed with three different solvent systems: (A) acetone, (B) 2 % acetic acid in chloroform and (C) ethanol, at the fixed proportion of 1:4. Cold (-20 ºC) and acidic (pH~5.5) conditions were employed upto 8 h for

efficient extraction (Fellows and Fleet, 1989; Wessel and Flugge, 1984). The mixture was immediately centrifuged to remove the high molecular weight components. The extract was then concentrated in vacuum rotary evaporator (Ika HB 10) at 45 °C and freeze-dried. Finally, the dry extract was reconstituted with 1 ml of methanol for further MS and LC-MSD analyses. The swainsonine concentration was determined in the culture supernatant and the extracted samples using α-mannosidase inhibition assay as described in Section 2.2.1 of Chapter 2.

4.2.5. Qualitative mass spectrometry instrumentation and analysis of partially purified samples

Qualitative swainsonine detection was carried out by electrospray ionization-time of flight mass spectrometry (ESI-TOF MS) (Waters, model Q-TOF micromass HAB273, USA) in positive ion mode. The MS-ESI+ ion source parameters were adjusted for source and desolvation gas temperatures at 100 ºC and 250 ºC, respectively. The sampling, capillary and extraction cone voltages were set at 35 V, 3 KV and 3 V respectively, with ion guide at 1 V. The flow injection rate was fixed at 20 µl/min. The MS data were obtained in full scan mode (mass range 100-1,000 Da). Instrument control and data acquisition was performed with MassLynx analytical software. The solvent extracted broth samples were further subjected to comparative LC based methods of analysis and purification.

4.2.6. Experimental and blank samples for high performance liquid chromatography

Experimental samples, different solvent extracted swainsonine as described in Section 4.2.4 were purified using high performance liquid chromatography (HPLC). The HPLC grade water was maintained as blank for chromatographic runs. The low

range (2-10 µg/ml) HPLC standard curve was prepared for swainsonine standard solutions in triplicates.

4.2.7. Analytical HPLC Instrumentation and operation conditions

The partially purified solvent extracted broth fractions were analysed qualitatively and quantitatively for swainsonine titres in the Varian Prostar HPLC system. The system was equipped with a binary pump, UV-visible detector, and a 20 µl injection loop. Hypersil BDS RP-C18 column (250×4.6 mm) of particle size 5 Aº (Thermo, Waltham, USA) was used for chromatographic separation. The eluted samples were detected at middle-UV (mUV) wavelength of 205 nm and confirmed with the retention time of standard swainsonine in triplicate runs (Kim and Nho, 2004; Dennis *et al.*, 2002). Samples (crude and standard) were prepared by filtration through 0.22 µm cellulose nitrate membrane filters prior to their injection into the analytical column. The regression curve was obtained by plotting log of concentration versus log of peak area. Three different mobile phases (pH~5.5), (i) 5 % methanol in ammonium acetate buffer (Solvent-A) and water (Solvent-B): Method A, (ii) acetonitrile (Solvent-A) and water (Solvent-B): Method B, and (iii) actetonitrile (Solvent-A) and 0.1 % TFA in water (Solvent-B): Method C, were evaluated for chromatographic separation of swainsonine. The gradient programme employed for HPLC operations involved: 100 % of Solvent-B from 0-3 min (Fr = 1ml/min) and 100 % of solvent-A from 3-10 min (Fr = 0.5-0.25 ml/min).

4.2.8. Mass spectrometry analysis of HPLC fractions

The HPLC fractions at stipulated elution time were collected and analysed using ESI-TOF MS. The MS instrumentation and operation conditions were same as described in Section 4.2.5.

4.2.9. Preparative cum quantitative liquid chromatography-mass derived instrumentation and operation conditions

The preparatory LC-MSD system (Agilent technologies Inc., 1260 Infinite series, USA) consists of binary solvent pump and autosampler (ALS) with thermostatted oven, reverse phase C18 column (21.2×250 mm) of particle size 7 A°, and diode array (DA) detector. Swainsonine was eluted using linear gradient programme (100 % water from 0-5 min and 100 % acetonitrile from 5-20 min) at a flow rate of 10 ml/min. The optimized capillary voltage 3 KV, fragmentor voltage 70 KV and collision energy 25 KV values were applied. The mass spectrometer was run in selected ion monitoring (SIM) mode for the mass range 173 and 174.36±0.21. Instrument control and data acquisition was performed with Chemstation analytical software. LC-chromatogram of total ion count (TIC) versus elution time (t_e) was analyzed in commercial MassHunter software (Agilent Technologies Inc. USA). The area count for characteristic ion was extracted from the composite total ion peak in SIM mode (Fattorusso and Taglialatela-Scafati, 2007). Calibration curve of concentration range 1-8 µg/ml was constructed using standard swainsonine. The observed $[M+H]^+$ 174.36±0.21 intensities were plotted against the corresponding concentrations (inset of Fig. 4.8).

4.2.10. Analytical LC-MS validation of MSD purified broth fractions

The MSD purified broth fractions for swainsonine were further analyzed by analytical LC-MS (Triple quad 6410, Agilent Technologies Inc. USA) for validation. The instrumentation involved an analytical C18 column (4.6×250 mm) of particle size 5 A°, fitted in thermostatted oven and quaternary pump. The run programme and solvent systems were all being same (Section 4.2.9), except the flow rate (0.5 ml/min).

4.3. Results and Discussion

4.3.1. Solvent extraction, enzymatic quantification and MS analysis

A prior subtractive purification strategy was applied to precipitate endogenous proteins, sugars and nucleotides *etc.* in fermentation broth using organic solvents under chilled conditions, followed by various analytical procedures for swainsonine (Fig. 4.1). A maximum 2.5 fold purification was achieved using solvent A (acetone), with 7.16 µg/ml of swainsonine in extract supernatant. However, only 1.88 and 1.16 fold purification of swainsonine was observed with solvent B (5.19 µg/ml) and C (3.35 µg/ml), respectively (Table 4.1).

The purified fractions were further subjected to mass spectrometry analysis and compared with mass spectrum of swainsonine standard for characteristic MS1 counts (Fig. 4.2a). The average of swainsonine MS1 $[M+H]^+$ 174.36±0.21, ion counts were most abundant in solvent A purified broth (Fig. 4.2b). The mass spectrum for solvent B purified broth fraction was much noisy with number of unknown higher mass fragments (Fig. 4.2c), whereas solvent C purified fractions exhibited a comparatively clear mass spectrum but with insignificant average MS1 ion counts for swainsonine (Fig. 4.2d). There are certain extra peaks which may be hypothesized to be of glucose units $[M+Na]^+$; *m/z* = 203, or other such sodium or formate adducts of higher sugars in fermentation broth. The organic solvent extraction of swainsonine provided a simpler sample preparation procedure for subsequent LC based analysis by removing other contaminants as described by Sen *et al.* (2011).

4.3.2. Quantitative HPLC analysis and method optimization

The HPLC analysis of swainsonine was performed at an optimized mUV detection wavelength of 205 nm. Natural products with double bonds or unpaired

electrons can be detected at mUV wavelengths of~200 nm (Gao *et al.*, 2008). Hence, the mUV wavelength (205 nm) was considered for swainsonine detection. The partially purified broth fractions were HPLC analysed using different mobile phases and gradient runs with variable flow rates. The three different mobile phases with gradient proportions (program) were optimized for maximum peak resolution and minimal signal-to-noise ratio (S/N) for chromatographic analysis of swainsonine (Fig. 4.3). Method A resulted in a major peak between 2.12-2.65 min with two minor peaks thereafter between elution time 5.2-5.8 min. The elution of major peak before 3 min of initial run of water (Solvent-B) might have resulted in the poor chromatographic resolution of swainsonine due to its extreme hydrophilicity and polarity (Fig. 4.3a) (Stockigt *et al.*, 2002). Hence, swainsonine elution was further optimized using Method B and Method C, which resulted in a major chromatographic peak between elution time 5.59-6.13 min (Fig. 4.3b) and 5.85-6.23 min (Fig. 4.3c), respectively. The optimal gradient elution of products were obtained with Method C using acetonitrile (solvent-A) and 0.1 % TFA in water (Solvent-B) as mobile phase (Fig. 4.3c). The chromatographic separation of major peaks in partially purified broth fractions was further confirmed with standard swainsonine runs with elution time 5.95-6.52 min followed by MS analysis of the HPLC fractions (Fig. 4.3a-b). The chromatographic separation of swainsonine was further calibrated by reducing the flow rates to 0.25 ml/min from 5.0-10.0 min, to resolve the two separate peaks between t_e = 5.25-7.10 min. Hence, two separate peaks were resolved at t_e = 6.10-6.92 min (comparable to the elution time for swainsonine standard) and an unknown peak between 7.24-8.20 min (Fig. 4.4c). The corresponding peak fractions were further characterized using mass spectrometry analysis, with respective MS1 fraction $[M]^+$ 173.04, $[M+H]^+$ 174.30 and $[M+2H]^+$ 175.04 for swainsonine (insets of Fig. 4.4a-c). The HPLC

quantification of swainsonine was performed using the standard curve between swainsonine concentrations (µg/ml) and peak area (mAU.min) with regression equation:

$$Y = 8.34\,X + 16.27 \qquad Eq.1$$

Where Y = peak area for swainsonine, X = swainsonine concentration with regression coefficient R^2 = 0.98 (Fig. 4.5).

The average swainsonine concentration was determined to be 7.67±0.33 µg/ml. The MS-ESI+ characterization of the HPLC fractions provided the sensitive analysis method for swainsonine in *Metarhizium* fermentation broth. The characteristic MS1 $[M+H]^+$ *m/z* = 174 was observed in both standard and purified samples, which confirmed the presence of swainsonine.

4.3.3. Liquid chromatography-mass derived purification

The selective mass derived purification can be used directly with excellent recovery and purity without involving the tedious purification strategies (Kagan *et al.*, 2009). Here, the approach is based on unique fragmentation patterns requiring either a precursor MS1 ion scan or a neutral loss scan observed under MS/MS^2 to generate the species specific quantification methodology as reported by Chen *et al.,* (2012). Solvent system A extracted broth was selected for LC-MSD purification. Swainsonine MS1 fraction $[M+H]^+$ 174.36±0.21 was eluted at time (t_e) 4.91±0.04 min. The MS1 fractions were collected between t_e = 4.41-4.78 min in vial-1 and 4.80-5.15 min in vial-2. Native mass fractions of (*m/z* 173) were eluted between t_e = 5.66-6.14 min in vial-4 but at negligible signal counts (Fig. 4.6). The purified fractions were further analyzed and validated for swainsonine purification with analytical LC-MS system. Swainsonine was eluted as a single peak at t_e = 6.38±0.04 min (Fig. 4.7a) with

characteristic m/z = 174.10 under MS1 scan showing 3.74×10^5 counts (Fig. 4.7b), which further confirmed the precision of MSD purification method.

4.3.4. LC-MSD ESI-MS+ quantification

LC-MSD purification was simultaneously accompanied with mass derived quantification using the MS1 ion counts $[M+H]^+$ 174.36±0.21 in selected ion monitoring (SIM) mode. The standard curve with regression equation:

$$Y = 1281314\,X - 1290157 \qquad Eq.2$$

Where, Y = MS1 ion counts (intensity) for swainsonine, X = swainsonine concentration (µg/ml) with regression coefficient R^2 = 0.99 (inset of Fig. 4.8).

Swainsonine concentration was expressed as an average of three replicate runs with standard deviation values, 7.85±1.59 µg/ml (Fig. 4.8). The lack of any interfering peak under the SIM-chromatogram demonstrated the high selectivity of the method as described by John *et al.*, (2010). Thus, the mass derived technique was found to be more rapid with high accuracy, selectivity and sensitivity for both qualitative and quantitative analyses.

4.3.5. Comparative evaluation of conventional HPLC and mass derived quantitative methods

4.3.5.1. *Limit of detection*

The lower detection limit in conventional HPLC with mUV (205 nm) detection was as low as 1-2 µg/ml, whereas the same was 0.25-1 µg/ml in MSD-ESI+ mode with significant values of ion count $[M+H]^+$ 174.36±0.21.

4.3.5.2. *Linearity*

Both HPLC and MSD quantification showed the linear calibration curves in the closed concentration ranges of 1-10 µg/ml (HPLC) and 1-8 µg/ml, respectively.

4.3.5.3. *Selectivity and precision*

The selectivity in mUV-HPLC method was comparatively lesser with various non-specific peaks, whereas in MSD method only selected mass fractions $[M+H]^+$ 174.36±0.21 were observed in SIM-mode, proving its more specific nature. The methods' precision was indexed with the relative standard deviation (RSD) values for chromatogram data. The observed RSD values for peak area and MS1 ion counts were 4.31 % and 4.04 % in HPLC and MSD methods respectively. The RSD values for sample elution times were 1.25 % for HPLC and 0.79 % for LC-MSD methods, which implies the higher degree of precision for mass spectrometry based detection procedure.

4.3.5.4. *Inter-method accuracy*

The concentration determined from the HPLC and MSD based methods were marginally varied with RSD value of 1.63 %. Hence, the data exhibits a higher and admissible degree of accuracy between to swainsonine concentrations determined.

4.3.5.5. *Ruggedness*

Furthermore, the investigation of ruggedness under a confined range of different HPLC and MSD conditions proved the method to be stable and rugged.

4.4. Conclusions

In summary, the present study describes the comparative method development studies on the extraction, purification and qualitative analysis of swainsonine in *Metarhizium* fermentation broth. A cold precipitation based acetone-methanol solvent extraction procedure was satisfactorily developed with nearly 2.5 fold purification of the crude swainsonine from fermentation broth. The extraction efficiency was qualitatively assessed using the mass spectrometry analyses of the broth extracts, which further confirmed the superiority of acetone-methanol based extraction procedure. A straightforward and fairly sensitive UV-HPLC method was developed using mUV detection at wavelength of 205 nm. A linear gradient method was also developed for efficient chromatographic separation of swainsonine using acetonitrile and 0.1 % TFA in water, as optimal mobile phases. Swainsonine eluted as a separate peak at 6.10-6.92 min, which was confirmed for the characteristic MS1 species $[M+H]^+$ 174.30. Simultaneously, a LC-MSD preparatory cum quantitative method based on flow injection mass spectrometry was developed for swainsonine in broth extracts. The method showed high throughput preparatory efficiency with selective MS detection (selective ion monitoring) and fractionation of swainsonine from the mixture of compounds. Both the methods were validated and satisfactorily compared for different chromatographic parameters in terms of linearity, selectivity, precision and accuracy. Hence, the results concluded the high efficacy of the preparatory and analytical procedures of the efficient recovery of swainsonine from *Metarhizium anisopliae* ARSEF-1724 (UM8) fermentation broths for routine *in vitro* studies.

Table 4.1. Extraction of swainsonine from *Metarhizium anisopliae* ARSEF-1724 (UM8) with three different organic solvents.

Sample	Broth volume (ml)	Yield (µg/ml)	Total (µg)	Yield (%)	Fold purification
A. Acetone extraction					
Crude	85	2.86	243.10	-	-
Extract supernatant	6	7.16	42.96	17.67	2.5
Extract pellet	1	0.69	0.69	0.28	0.24
B. 2 % acetic acid in chloroform extraction					
Crude	90	2.75	247.50	-	-
Extract supernatant	6	5.19	31.14	12.58	1.88
Extract pellet	1	0.57	0.57	0.23	0.20
C. Ethanol extraction					
Crude	90	2.88	259.20	-	-
Extract supernatant	6	3.35	20.10	7.75	1.16
Extract pellet	1	0.04	0.04	0.01	0.01

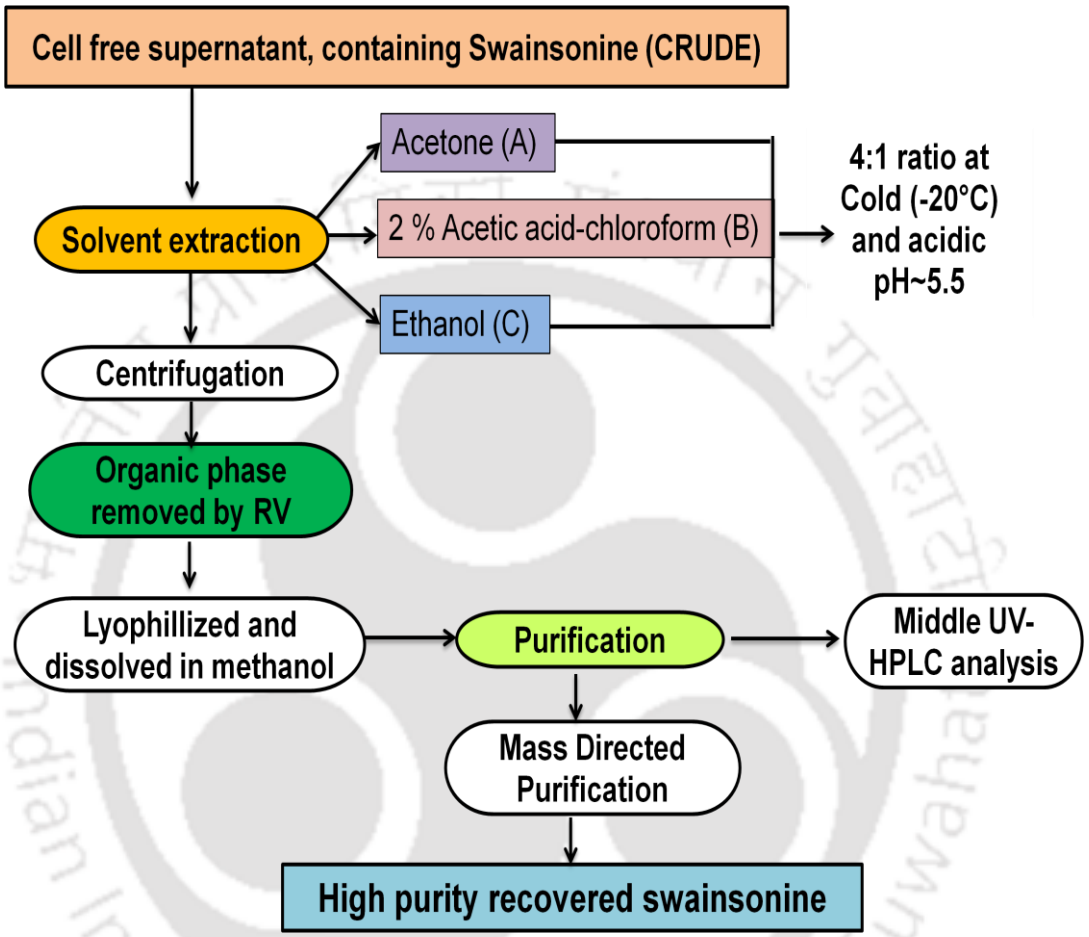


Fig. 4.1. Broad outline for the different purification and analytical procedures applied for swainsonine from *Metarhizium anisopliae* ARSEF-1724 (UM8) fermentation broth.

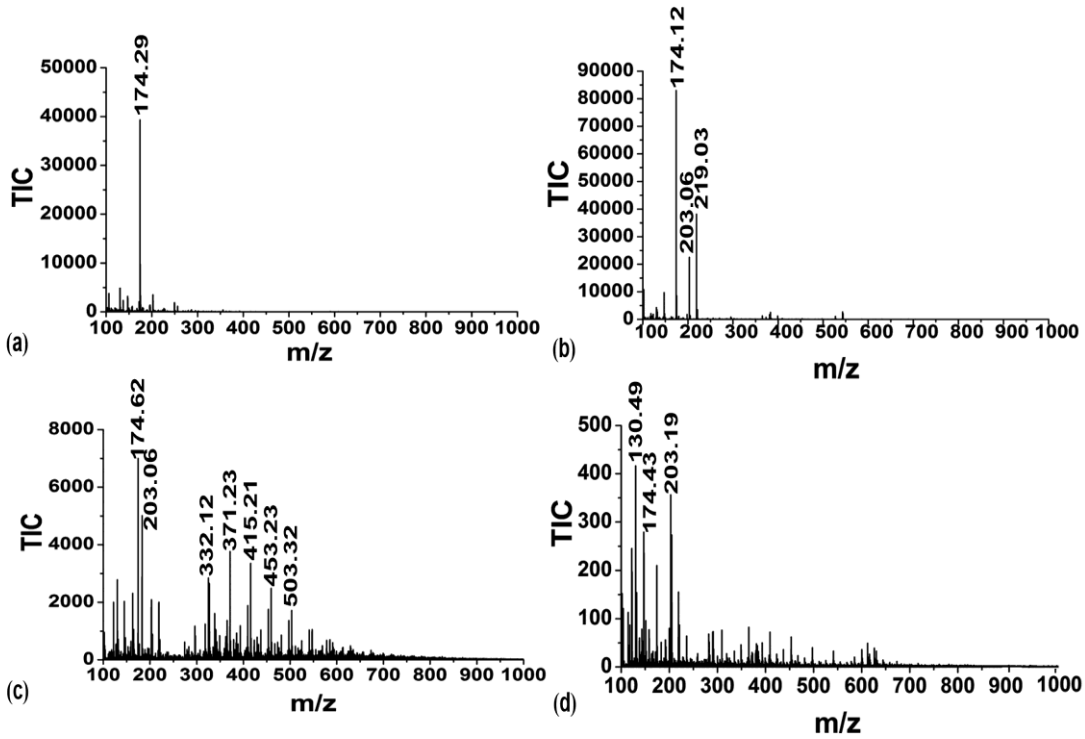


Fig. 4.2. Qualitative ESI-MS+ analysis of partially purified swainsonine from *Metarhizium anisopliae* ARSEF-1724 (UM8) fermentation broth, **(a)** swainsonine standard, **(b)** acetone extracted, **(c)** 2 % acetic acid-chloroform extracted and **(d)** ethanol extracted samples.

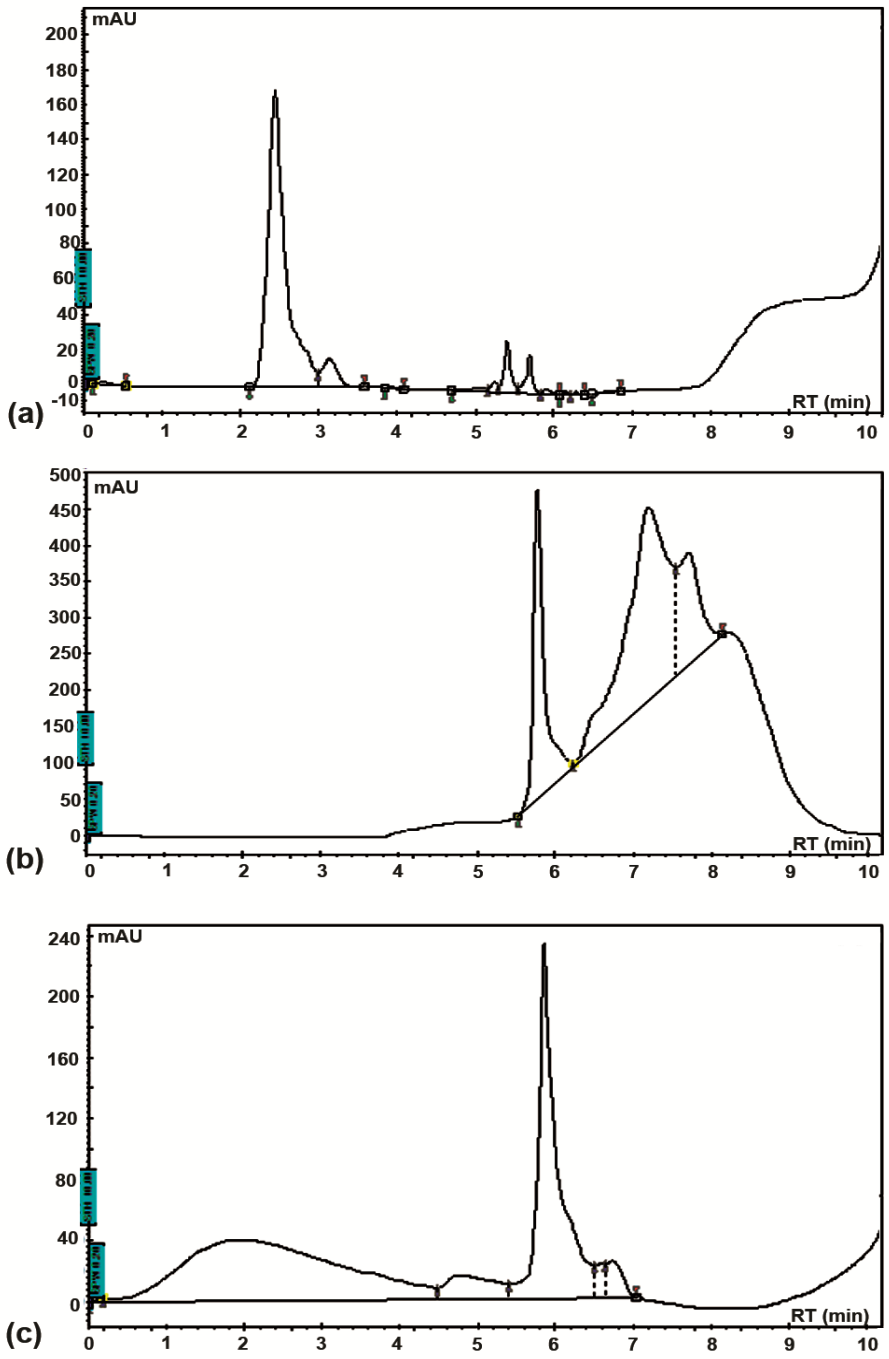


Fig. 4.3. HPLC chromatogram for partially purified broth samples showing major peaks with different mobile phases **(a)** 5 % methanol in ammonium acetate buffer (Solvent-A) and water (Solvent-B), **(b)** acetonitrile (Solvent-A) and water (Solvent-B), and **(c)** actetonitrile (solvent-A) and 0.1 % TFA in water (Solvent-B), at 205 nm for optimal chromatographic resolution.

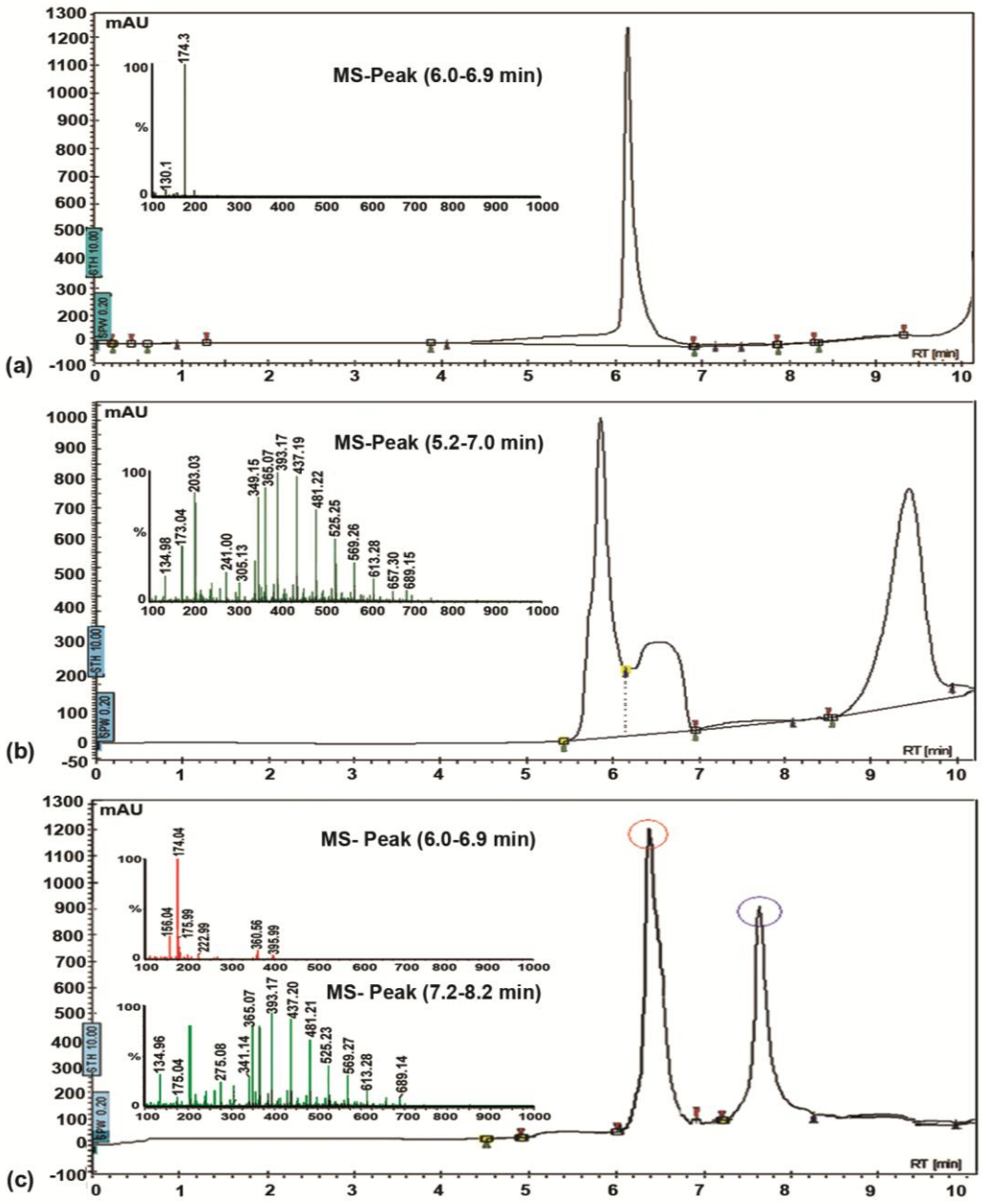


Fig. 4.4. HPLC chromatograms and corresponding mass spectrum (inset) for the major peaks collected **(a)** swainsonine standard at flow rates Fr = 1 ml/min from 0-5 min and 0.5 ml/min from 5-10 min **(b)** partially purified broth fraction at flow rates Fr = 1 ml/min from 0-5 min and 0.5 ml/min from 5-10 min, and **(c)** partially purified broth fraction at flow rates Fr = 1 ml/min from 0-5 min and 0.25 ml/min from 5-10 min, at 205 nm.

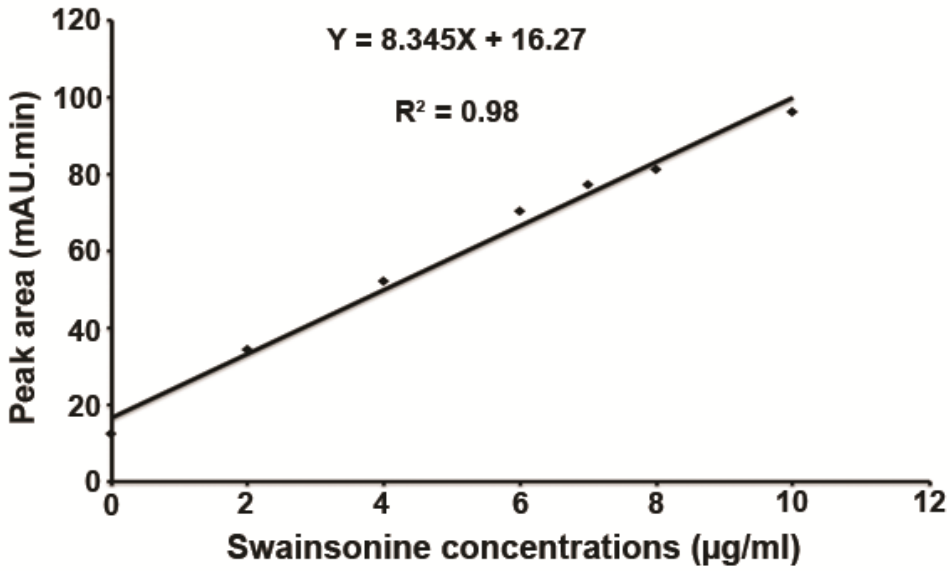


Fig. 4.5. HPLC standard curve for swainsonine with acetonitrile and 0.1% TFA in water as the mobile phase at middle UV wavelength range (205 nm).

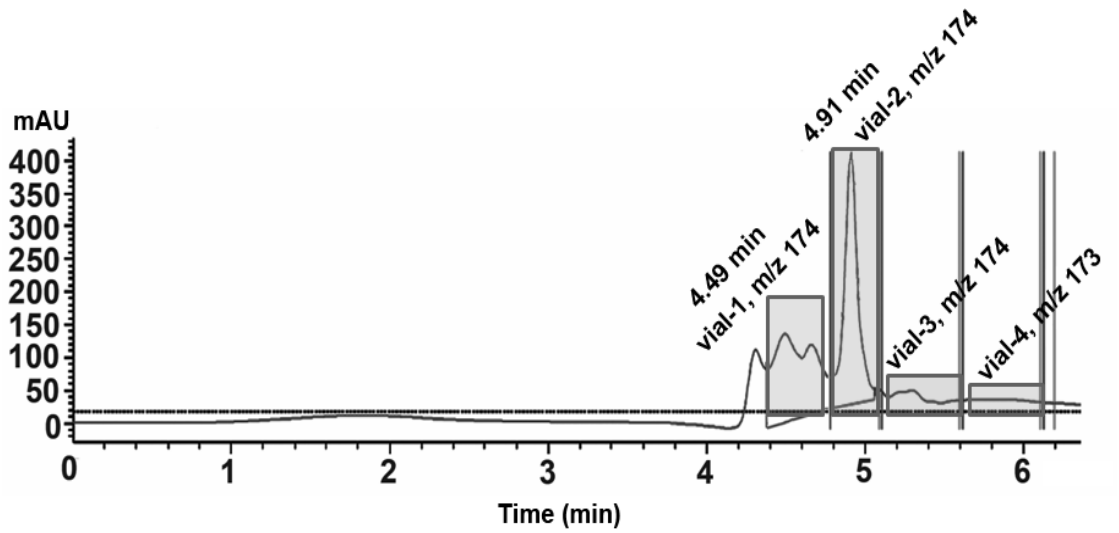


Fig. 4.6. DAD chromatogram of acetone extracted swainsonine fractions in LC-MSD purification.

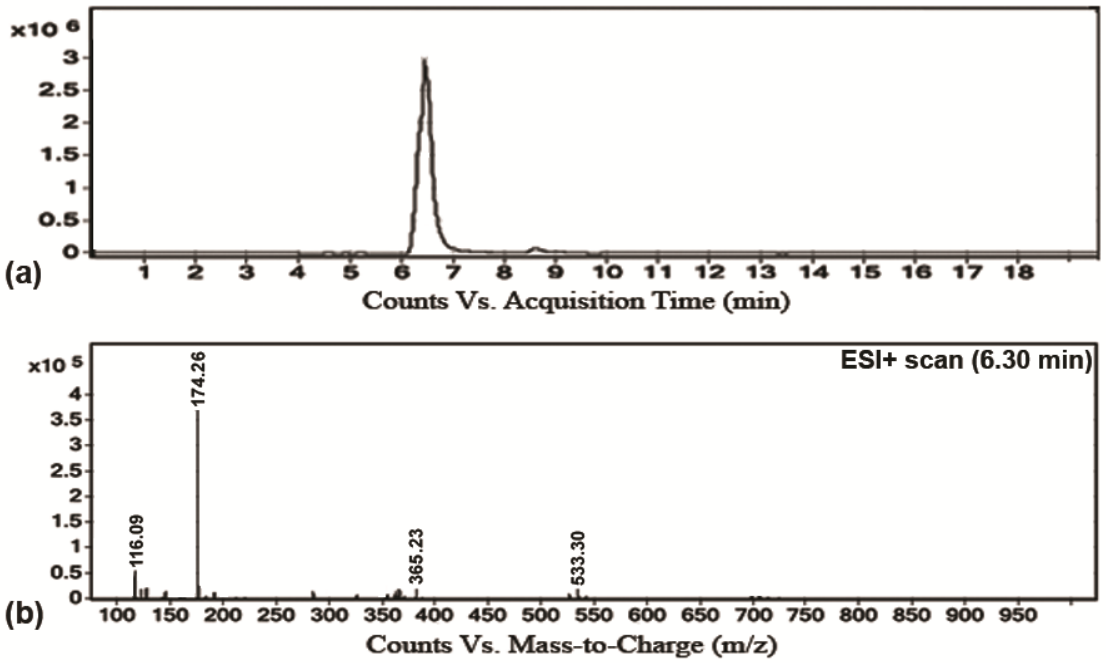


Fig. 4.7. LC-MS validation of MSD purified swainsonine from *Metarhizium anisopliae* ARSEF-1724 (UM8) **(a)** TIC chromatogram **(b)** ESI+ scan.

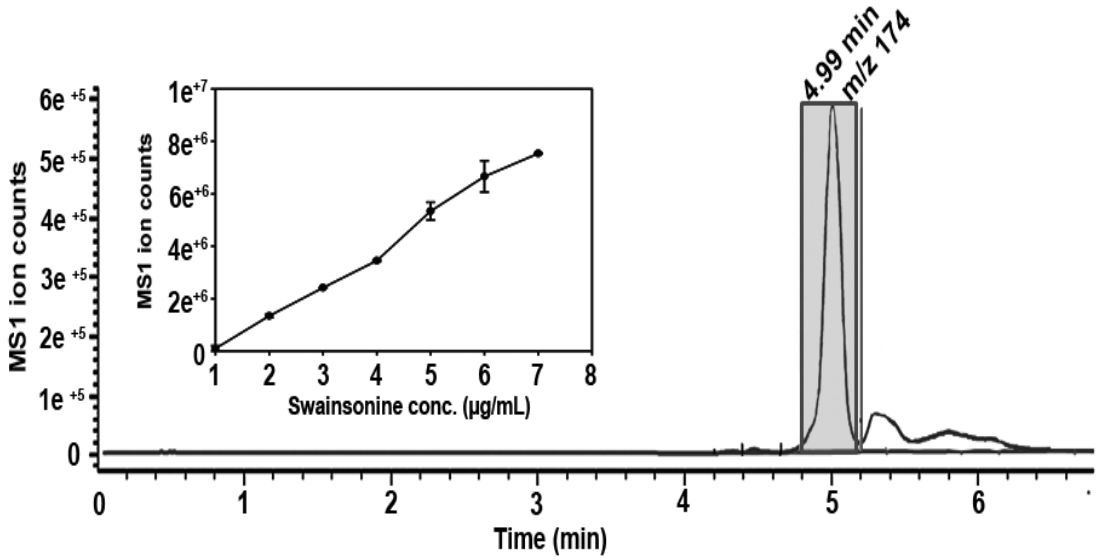


Fig. 4.8. Intensity ion counts vs. retention time (min) curve for mass directed quantification in SIM-mode. Calibration curve of swainsonine for precursor ion $[M+H]^+$ 174.36±0.21 as an inset figure.

CHAPTER 5

Analysis of *in situ* entomotoxic and anticancer activities of swainsonine

5.1. Introduction

The entomopathogenic fungus *Metarhizium anisopliae* is a well studied and applied species for microbial control of insect pests (Hoe *et al.*, 2009; Schrank and Vainstein, 2010). The genus *Metarhizium* in particular, have emerged as an excellent model organism to explore a broad array of questions in ecology and evolution, host preference and host switching, mechanisms of speciation and entomotoxicity. Interestingly, some of the strains have evolved as obligate parasites and are known to exploit living arthropods as a hub for growth and reproduction (Roy *et al.*, 2006; Vega *et al.*, 2009). While some pathogenic fungi have become highly specialized natural killers of arthropods, others are able to exploit both dead and living resources associated with plants and soils (St. Leger, 2008). The secondary metabolites with immunosuppressive or otherwise toxic functions may help fungi invading their animal hosts by overcoming cellular and humoral defence systems (Gillespie *et al.*, 2000). Here, the ability to form secondary metabolites may be maintained by the need to employ chemicals to withstand, or attack and overcome, the hosts' immune system. This strategy enables the fungus to consume an otherwise well protected resource, rather than producing metabolites to serve solely as chemical shields to fend off animal antagonists as in decomposer communities.

Metarhizium spp. produce a variety of secondary metabolites in several chemical classes, including cytochalasins C and D, myroridins, destruxins A, B and E, viridoxin, helvonic acid, 12-hydroxyovalicin, hydroxyfungerin, 7-desmethyl analogues of fusarin C and (8Z)-fusarin C, serinocyclins A and B and aurovertins. These metabolites are toxic to a broad range of animals and microbes, including insects, fungi, bacteria and viruses (Carlos *et al.*, 2010). Although, the secondary metabolites have no major role in growth, reproduction and development of an

organism but they critically influence the fungal survival, inter- and intra-species interactions and defence against predation or pathogens in the environment. The secondary metabolites from entomopathogenic fungus *Beauveria brongniartii* are recently been described to induce apoptotic effects in certain pest species, both *in situ* and *ex situ* (Fan *et al.*, 2013). Similarly, the entomotoxic effects of various fungal lectins of *Rhizoctonia* sp. (Hamshou *et al.*, 2013) and *Nomuraea* sp. (Tseng *et al.*, 2008) against *Lepidopterans*, have revealed the entirely new paradigm of modern bio-pesticides and their role in environment, health and agriculture. The evaluation of the insect suppressive effects of fungal secondary metabolites needs to be studied in a greater aspect of chemical ecology in fungi-arthropod interactions. The apparent induction of apoptosis in insect host hemocytes by the destruxin metabolites of *M. anisopliae* (Vilcinskas *et al.*, 1997; Gillespie *et al.*, 2000) and the cyclosporin A-mediated arrest of host detoxification mechanisms by *B. bassiana* (Podsiadlowski *et al.*, 1998) are two examples of how fungal secondary metabolites may act to incapacitate the immune systems of arthropod hosts (Gillespie *et al.*, 2000). However, many studies have described the therapeutic potentials of swainsonine, but its role in the entomopathogenic virulence of *Metarhizium* is still unexplored.

Swainsonine has attracted a great attention in the past two decades due to its potentially therapeutic biological activities. Swainsonine is a potent and specific inhibitor of lysosomal and cytosolic α-mannosidases, as well as Golgi α-mannosidase II. Its inhibition of the latter enzyme leads to the accumulation of hybrid type oligosaccharides and a decrease in glycoproteins containing complex side-chains. It has been used as an instrument drug to study glycoprotein, N-linked oligosaccharide since its initial extraction from the fruit of Australian *Swainsona canescens* and North American locoweeds (Dantas *et al.*, 2007; Li *et al.*, 2004; Ferrara *et al.*, 2006).

Swainsonine was shown to inhibit tumor growth and metastasis, enhance lethality of NK cell and LAK cells reduce the growth rate of human melanoma cells and stimulate proliferation and differentiation of bone marrow cells (Sun *et al.*, 2007; Wang *et al.*, 2003). The alkaloid has also been described for its apoptotic anticancer properties against cancerous A549 cells, antivirus and antitumor drug application (Li *et al.*, 2012; Santos *et al.*, 2011). Swainsonine has also been reported to reduce the growth rate of human melanoma, colon and gastric carcinoma cells and C6 glioma cells *in situ* and *ex situ* (Dennis *et al.*, 1989; Sun *et al.*, 2007; 2009).

In situ cytotoxicity tests are useful and necessary for screening purposes to define dose and time dependent cytotoxicity, considered primarily as the potential of a compound to induce cell death, in different cell types (Eisenbrand *et al.*, 2002). We, therefore, designed our studies to demonstrate the mode of entomotoxic action of swainsonine on a lepidopteran model, *Spodoptera frugiperda* (*Sf*-21) cell line. At the same time, the *in situ* anticancer activities of swainsonine were also assessed using human promyelocytic leukemic cell line (HL-60). These studies will further help in understanding the potential cytotoxic effects and applications of this established mannosidase inhibitor, swainsonine from *Metarhizium anisopliae* ARSEF-1724 (UM8).

5.2. Materials and methods

5.2.1. Microorganism and Cultivation conditions

Metarhizium anisopliae ARSEF-1724 (UM8) was procured from "The Plant Protection Unit, United States Department of Agriculture (USDA), New York, Ithaca. The culture was maintained in SDA slants at 4 °C as described in Section 2.2.1 of Chapter 2.

5.2.2. Chemicals and Reagents

All standards and cell culture related chemicals were purchased from Sigma-Aldrich, USA. The purified swainsonine (test samples) was prepared *M. anisopliae* fermentation broth (Section 4.3.1-4.3.3, Chapter 4). The swainsonine stock solutions were prepared by dissolving 1 mg/ml (5.78 mM) of standard and purified swainsonine (by appropriate dilutions) in Ca^{+2} and Mg^{+2} free phosphate buffer saline (PBS) and stored at -20 °C.

5.2.3. Cell culture and treatments

An early passage *Spodoptera frugiperda*, ovarian (*Sf*-21) and human promyelocytic leukemia (HL-60) cells were procured from National Centre for Cell Sciences (NCCS) Pune, India. These cells were cultured routinely in T-25 and T-75 flasks (BD biosciences). The *Sf*-21 cells were cultured in TNM-FH medium supplemented with 10 % heat inactivated fetal bovine serum (FBS) and maintained at 28 ºC in a non-humidified and non CO_2 incubator. On the other hand, HL-60 cells were cultured using RPMI 1640 medium supplemented with 20 % heat inactivated FBS and incubated at 37 ºC in a humidified incubator with 5 % CO_2 saturation.

5.2.4. MTT cell viability assay of *Sf*-21 and HL-60 cells

Cell viability was determined by a colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. The evaluation of the metabolic viability by MTT test is based on the ability of the mitochondria succinate tetrazolium reductase system to convert the yellow compound MTT to a blue formazan dye. The amount of dye produced is proportional to the number of live cells. Dose response curves were computer plotted after converting the mean data values to percentages of the control response. The 50 % inhibiting concentration (IC_{50}) was calculated from the dose response curves. The standard and MSD purified swainsonine were tested at concentrations ranging from 2-10 µM for 36 h (in case of *Sf*-21 cells) and 2-16 µM for 48 h (in case of HL-60 cells) with appropriate solvent controls. A 100 µl of 2×10^5 cells/ml were seeded into 96 well microtitre plates with 100 µl of swainsonine of varying concentrations in complete growth media for respective cells (*Sf*-21 and HL-60). A 20 µl of MTT solution (5 mg/ml in PBS) was added after the desired time points (12 h interval) into each well and the cells were incubated further for 4 h at stipulated incubation temperatures. The microtitre plate was centrifuged at 250*g* for 5 min at 4 °C. The media were removed gently and then 100 µl of DMSO finally added. The plate was then rotated on an orbital shaker for 10 min to dissolve the precipitate completely and absorbance was detected at 570 nm with reference wavelength of 660 nm using ELISA microplate reader (Tecan, Infinite 200, USA).

5.2.5. Tryphan blue cell death assay of *Sf*-21 and HL-60 cells

The cytotoxic effects of swainsonine were determined using the trypan blue (TB) dye exclusion assay. 100 µl of 2×10^5 cells/ml were seeded into 96 well microtitre plates with 100 µl of swainsonine at varying concentrations, 2-10 µM upto

36 h for *Sf*-21 cells and 2-16 µM upto 48 h for HL-60 cells. At the stipulated time point, the cell suspension was treated with tryphan blue dye (1:1) and counted using a neubauer hemocytometer under the inverted microscope (Nikon TS 100-F, Japan). The ratio of live to dead cells (cell viability) was determined, standard curves were prepared and 50 % cytotoxic concentrations (CC_{50}), *i.e.*, the concentrations of swainsonine that caused a 50 % decrease in cell viability, were derived. The cytotoxic effects of swainsonine were expressed as the mean percentage of TB stained dead cells in each treatment group from three independent experiments.

5.2.5. Statistical analysis

One-way ANOVA followed by Dunnett's test of pairwise multiple comparisons was used for MTT and Tryphan blue dye exclusion (TB) cell death data analysis. Cell viability plots were fitted in SPSS version 11 (Science Inc., Chicago, USA) with an exponential decay-linear combination equation to generate graphs. Each data point represented mean of multiple wells. The $p \leq 0.05$ was considered to be statistically significant in all cases.

5.2.6. Inverted phase contrast microscopy of *Sf*-21 and HL-60 cells

The cells (*Sf*-21 and HL-60) were immediately observed under inverted microscope (Nikon TS 100-F, Japan) at 40X magnification lens at stipulated time intervals.

5.2.7. Fluorescence microscopy and differential staining using Annexin V-FITC/ PI staining of *Sf*-21 and HL-60 cells

The PBS washed cells were suspended in binding buffer and incubated with Annexin V-FITC (5 µl) and propidiun iodide (PI) (10 µl), for 10 min in dark as per the

manufacturers' instructions (Sigma Aldrich, USA). The cells were then examined under fluorescence mode in inverted microscope (Nikon TS 100-F, Japan) at the 40X magnification.

5.2.8. Fluorescence activated cells sorting analysis

5.2.8.1. *Apoptosis analysis of Sf-21 cells*

Swainsonine induced apoptosis in *Sf*-21 cells was determined by flow cytometry using the Annexin V-FITC/PI apoptosis detection kit following the manufacturers' instructions. *Sf*-21 cells were treated with appropriate concentrations of swainsonine (IC_{50} value) in a 24 well plate for 36-48 h. The cells were then centrifuged (300*g* for 5 min at 4 °), washed in PBS and incubated with Annexin V-FITC (5 µl) and PI (10 µl) for cellular staining in 1X binding buffer (10X stock) and 250 µl of cell suspension (1 reaction) at room temperature for 10 min in dark. The stained cells were immediately FACS analyzed using a FACS Calibur instrument (BD Biosciences, USA) equipped with CellQuest 3.3 software. The early apoptotic cells stained with Annexin V-FITC, which give green fluorescence, are represented in the lower right quadrant of the FACS histogram, and the late apoptotic cells stained with both FITC and PI, which have red-green fluorescence, are represented in the upper right quadrant of the histogram.

5.2.8.2. *DNA cell cycle analysis of HL-60 cells*

Subconfluent HL-60 cells were treated with appropriate swainsonine concentrations (IC_{50}) in culture medium as described in Section 5.2.8.1 for 36-48 h. The cells were then harvested, washed with cold PBS, and processed for cell cycle analysis. The cells (2×10^5) were resuspended in 50 µl of cold PBS, to which chilled ethanol (450 µl) was added and then incubated for 1 h at 4 ºC. After centrifugation

(300*g* for 7 min at 4 °), the pellet was washed with cold PBS, suspended in 500 µl of PBS, and incubated with 5 µl RNase (20 µg/ml final concentration) for 30 min at room temperature. The cells were kept on ice for 10 min and incubated with PI (50 µg/ml final concentration) for 1 h in the dark. The cell cycle distribution of the cells of each sample was then determined using FACS Calibur instrument (BD Biosciences, USA). ModFit LT cell cycle analysis software was used to determine the percentage of cells in the different phases of the cell cycle.

5.2.9. Ultramorphodimensional analysis of *Sf*-21 cells

The ultramorphological examination of the swainsonine treated *Sf*-21 cells was performed using various microscopic techniques. An approximate of 5×10^5 cells were seeded in each of the 6-well culture plates in duplicate and treated with appropriate concentration of swainsonine (inhibitory concentrations or IC_{50}) with appropriate controls (untreated cells). The cells were harvested after appropriate time intervals, centrifuged (300*g* for 7 min at 4 °C) and washed twice with 0.22 µm filtered PBS to proceed further in each case of field emission-scanning electron microscopy (FE-SEM) and atomic force microscopy (AFM).

5.2.9.1. *Field emission-scanning electron microscopy*

The ultra-morphology of the swainsonine treated and control cells were examined using field emission scanning electron microscopy (FE-SEM) (Carl Zeiss, SIGMA VP, USA). The samples for FE-SEM analysis were prepared using the method described by Tang *et al.*, (2013). The PBS washed cell pellets were then fixed in 500 µl of 4 % paraformaldehyde and 2.5 % glutaraldehyde for 24 h at 4 °C. The pellets were again washed twice with filtered PBS, centrifuged (300*g* for 7 min at 4 °C) and treated with 1 % osmium tetroxide (OsO_4) in 0.1 M phosphate buffer. Then

again washed with filtered PBS and the pellets were finally ethanol dehydrated with gradients of 30 % (v/v), 50 % (v/v), 75 % (v/v), 90 % (v/v) and 100 % (v/v), for 10 min each. The pellets were then smeared evenly on a glass coverslip and dried in the dessicator upto a critical point. The samples were sputter-coated with gold film using a sputter coater (SC7620‘‘Mini’’, Polaron Sputter Coater, Quorum Technologies, Newhaven, England) before FE-SEM analysis.

5.2.9.2. *Atomic force microscopy*

The swainsonine treated *Sf*-21 cells were prepared for atomic force microscopy (AFM) as described in Section 5.2.9.1, except gold coating. The multimode AFM equipped with ambient air scanning probe microscope (Agilent Technologies 5500, USA) was used for the ultra-morphodimensional analysis of swainsonine treated and control *Sf*-21 cells. Images were scanned using the Picoscan-5 software in non contact mode. Topographic AFM images were collected in tapping mode using the rms amplitude of the cantilever as feedback signal operating in a saline liquid buffer. The silicon nitride probes were used with nominal spring constant around 2.5 N/m (NT-MDT, Russia) and cantilever length of 120 μm. The cantilever resonance frequency was about 30 kHz. The rms free amplitude of the cantilever was on the order of 15 nm and the relative set-point above 95% of the free amplitude. Images were recorded with a slow scan rate (below 1 Hz) and a resolution of 512×512 pixel per image was chosen.

5.3. Results and Discussion

In situ assays are important and useful tools in cytotoxicity assessment of various classes of environmental contaminants including fungal metabolites not only because they significantly reduce evaluation time, but also because they provide information about the mode of action of the toxicant.

5.3.1. Cytotoxicity analysis of swainsonine for *Sf*-21 cells

Sf-21 cell line is derived from a major crop pest, *Spodoptera frugiperda* a polyphagous insect which feeds on many different host plants. *In situ* cytotoxic evaluation of swainsonine against *Sf*-21 cells was performed with MTT cell viability and TB cell death assays. The MTT determined IC_{50} values for standard and MSD purified swainsonine were 3.90 µM and 5.27 µM, respectively. However, the TB assay showed slightly higher CC_{50} values of 6.91 µM and 8.67 µM for standard and MSD purified swainsonine, respectively (Fig. 5.1). All cytotoxicity evaluations were expressed for the data observed at 36^{th} h of post treatment incubation. The cell viability and cell death assays showed a significant decrease in both the parameters at as early as 12 h and the maximum at 36 h, suggesting the entomotoxic effects of swainsonine, as also reported for other *Metarhizium* metabolites by Skrobek and Butt, (2005) and Fornelli *et al.* (2004). The data also demonstrated the profound efficacy of swainsonine at micromolar concentrations with approximately 60 % of the treated cell population lost viability.

5.3.2. Cytotoxicity analysis of swainsonine for HL-60 cells

In situ cytotoxic evaluation of swainsonine of cancerous HL-60 cells was also performed with the MTT and TB assays. The cell viability and cell survival were differentially affected by different concentration of swainsonine at 48 h of post

treatment incubation. The MTT determined IC_{50} values for standard and MSD purified swainsonine fractions were 6.96 µM and 9.50 µM, respectively (Fig. 5.2). However, the swainsonine treatment was not shown significant cytotoxic effects on the HL-60 cell survival or live cell numbers, at the same concentration of swainsonine after 48 h. Hence, it can be concluded that swainsonine differentially affects the cell viability (cell proliferation) and cell death (cell survival) under *in situ* conditions for promyelocytic leukemic cells in contrast to the earlier reports (Li *et al.*, 2012, Li *et al.*, 2012).

5.3.3. Inverted phase contrast microscopy

5.3.3.1. *Microscopic examination of Sf-21 cells*

The microscopic analysis of swainsonine treated *Sf*-21 cells showed characteristic apoptotic features only after 12-36 h post treatment incubation. The phase contrast images showed characteristic membrane blebbing and released apoptotic bodies as early after 24 h of incubation (Fig. 5.3). This indicated preliminary insight towards the mitochondrial mediated apoptosis of *Lepidopteran* cell lines as also reported by Tseng *et al.* (2008).

5.3.3.2. *Microscopic examination of HL-60 cells*

The morphological analysis of swainsonine treated leukemic, HL-60 cells did not show any characteristic apoptotic or cell death features upto the first doubling time between 36-48 h. This indicated the lack of significant cell death due to swainsonine treatment, as also reported by Collins *et al.* (1977). The cell growth and counts were proportionally decreased with incubation time in treated as well as untreated cells, suggesting the ineffectiveness of the swainsonine for inducing the morphological markers of cytotoxicity through apoptotic or necrotic modes of cell death (Fig. 5.4).

5.3.4. Fluorescence microscopy and differential staining

The Annexin V-FITC/PI staining involves a differential staining method for the healthy and apoptotic cells in different phases of development. The Annexin V-FITC binding (green fluorescence) indicates the disintegration of cell membrane exposing the target molecules of phophatidylserine (PS) at early apoptotic phases and PI binding (red fluorescence), indicates the late apoptotic or necrotic phase of cell death with nuclear disintegration (Chiang *et al.*, 2011).

5.3.4.1. *Fluorescence microscopy of Sf-21 cells*

The fluorescence microscopy of swainsonine treated lepidopteran cells have also indicated a significant apoptotic features at 36th h of post treatment incubation. The qualitative analysis of fluorescent dye stained *Sf*-21 cells exhibited a comparatively higher number of cells in the late apoptotic or necrotic and few in the early apoptotic phase, suggesting the entomotoxic properties of swainsonine *in situ* (Fig. 5.5).

5.3.4.2. *Fluorescence microscopy of HL-60 cells*

The cytotoxic activity of swainsonine towards leukemic HL-60 cells was qualitatively assayed using fluorescent dyes, indicative of apoptotic biomarkers. The analysis showed that both the control (untreated) and swainsonine treated cells exhibited the similar fluorescent response at 48 h of post treatment incubation (Fig. 5.6). The results showed no signs of swainsonine induced apoptosis specifically in the treated cells, but the time dependent natural cell death or necrosis in both the control and treated cells. However, the results compelled us to investigate another closely linked but biologically distinct mode of cell cytotoxicity known as cell cycle deregulation, as described by Andrzej *et al.* (1989).

5.3.5. Fluorescence activated cell sorting analysis

5.3.5.1. *Analysis of apoptosis in Sf-21 cells*

The control and swainsonine treated *Sf*-21 cells were analysed for 36 h of post treatment incubation at every 12 h intervals. The FACS quadrant statistics showed that swainsonine treated cells in various apoptotic stages were found only after 24-36 h of post treatment incubation. The standard swainsonine treated cells showed a significant degree of cytotoxicity with approximately 30-35 % cells in the various apoptotic phases at 24-36 h. However, the cell damage was significantly increased in *Sf*-21 cells, treated with purified swainsonine with approximately 12 % and 42 % of the cell population undergone apoptotic cell death at 24 h and 36 h, respectively. (Fig. 5.7). The swainsonine treated cells might have encountered either apoptotic or necrotic or a combination of both, as also reported recently for human C6 glioma or A-549 cells (Sun *et al.*, 2009; Li *et al.*, 2012). The further evaluation of the swainsonine induced cytotoxicity can be better understood with various ultra-morphodimensional methods of analysis.

5.3.5.2. *Analysis of DNA cell cycle in HL-60 cells*

The cell cycle regulatory effects of swainsonine were evaluated to estimate its anticancer activities. The treatment of HL-60 cells with purified swainsonine samples resulted in a more significant S-phase arrest of 48.81 % and 60.72 % cells at 36 h 48 h of post treatment incubation, respectively. The treatment with equimolar concentrations of standard swainsonine showed the considerable effects only at 48 h of incubation with only 29.62 % cells arrested in the synthetic phase (Fig. 5.8). The response to irreparable DNA damage in mammalian cells often results in permanent cell cycle arrest or in many cases, cell death by apoptosis (Morgan 2007). Here, the

cell cycle analysis has revealed a higher percentage of cells are being arrested in the S-phase by the treatment with purified swainsonine, indicating a spectrum of similar compounds in the purified samples. Hence, it can be presumed that swainsonine might be influencing the DNA synthesis machinery, the specific cyclins or cyclin dependent kinases, *viz.*, cyclin A or E and cdk 2 as reported earlier in other *in situ* studies (Hung *et al.*, 1996; Hui *et al.*, 2005).

5.3.6. Ultramorphodimensional analysis of swainsonine induced apoptosis in *Sf*-21 cells

The morphological parameters of cells (treated and control) like stiffness, rigidness and cell membrane damage could be correlated well with *in situ* cytotoxicity of any inhibitory compound (Francis *et al.*, 2009). The treated *Sf*-21 cells might have undergone either apoptotic or necrotic or a combination of both the modes of cell death, which can be evaluated satisfactorily by the morphological, dimensional and mechanical examination using ultramicroscopic techniques like FE-SEM and AFM. The morphological analysis of swainsonine (at IC_{50} concentrations of standard and purified samples) treated cells showed characteristic apoptotic features only after 12-36 h post-treatment incubation. The FE-SEM imaging of swainsonine treated *Sf*-21 cells showed the disrupted cell membrane with characteristic cytoplasmic blebbings and apertures contrary to the control cells after 24-36 h of incubation (Fig. 5.9). The necrosis is characterized by the cytosolic swelling whereas apoptosis is marked by the cell shrinkage causing detectable changes in cell dimensions (Wang *et al.*, 2008). The cell membrane probation using AFM revealed the marked abrasions on cell surface and significant reduction in cell dimensions. The average height and average size (diameter) of swainsonine treated *Sf-21* cell population was reduced significantly by 4

µm in length and diameter (height and size were from 13 to 8 µm and 6 to 4 µm, respectively) as shown in Fig. 5.10. The ultramicroscopic examinations of swainsonine treated cell morphologies showed a severely damaged and punctured cell membranes compared to the control cells. The FE-SEM and AFM analysis together showed the enhanced roughness and decreased dimensions (height and volume) of the swainsonine treated *Sf*-21 cells compared to the control cells, which were significantly changed with incubation hours. These *in situ* entomotoxic studies of swainsonine from *Metarhizium anisopliae* ARSEF-1724 (UM8) may provide a new dimension towards the *in vivo* application studies in the field of biological pest control.

5.4. Conclusions

In summary, the present study describes the entomotoxic and therapeutic applications of swainsonine using various *in situ* methods. Here, the cytotoxic behaviour of the standard and MSD purified swainsonine from *Metarhizium anisopliae* ARSEF-1724 (UM8) were evaluated differentially towards lepidopteran, *Sf*-21 and human promyelocytic leukemic, HL-60 cells. In the context of entomotoxic potentials of α-mannosidase inhibitor, swainsonine was found to actively induce apoptosis in insect cells, confirming its contributory role in the virulence and chemical defence of *M. anisopliae* against arthropods. The *in situ* anticancer potential of swainsonine was differentially expressed in terms of influencing the cell viability and death. The compound was mildly effective in showing the anti-proliferative effects without causing cell death. However, cell cycle regulatory activities with significant S-phase arrest in swainsonine treated HL-60 cells. The alkaloid appears to effect or somehow interact with cyclins or cyclin dependent kinase, specific to a particular stage of cell cycle. Hence, the present study provides us an initial insight into the diverse biological activities of swainsonine. Further studies involving various signalling molecules regulating these cytotoxic effects would help in the better understanding of the mechanisms involved.

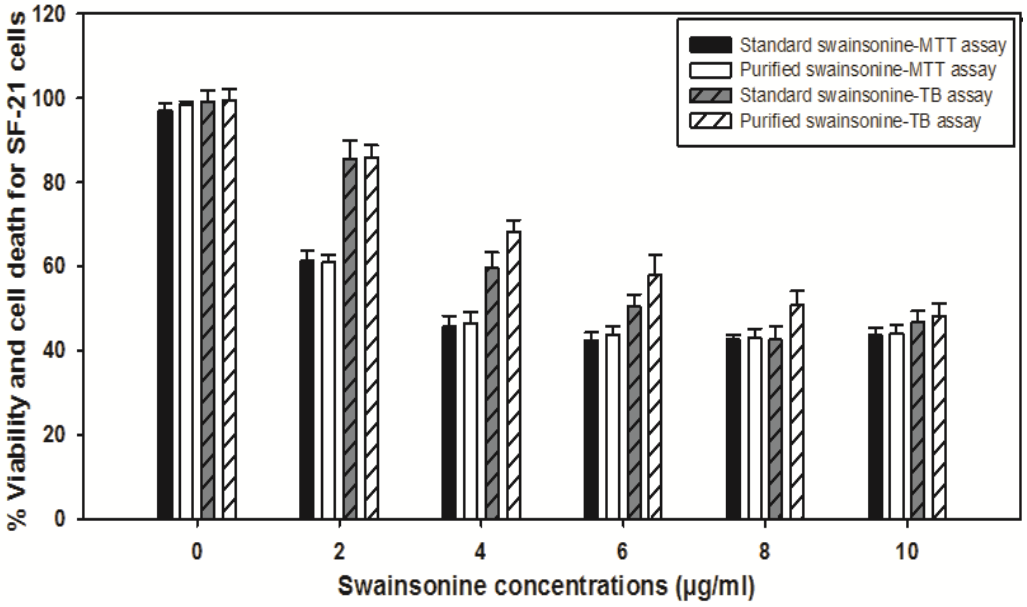


Fig. 5.1. MTT viability and TB dye exclusion cell death assays of *Sf*-21 cells measured after 36 h with different doses of standard and MSD purified swainsonine. One-way ANOVA with Dunnett's pair wise multiple comparisons with control indicated that purified swainsonine affected *Sf*-21 cell viability in a dose dependent fashion ($p < 0.001$). The regression values (R^2_{adj}) for standard and purified swainsonine are 0.99 and 0.97 in MTT assay and 0.99 and 0.98 for TB cell death assay, respectively. All data are average of three independent sets of experiments with standard deviation (±SD).

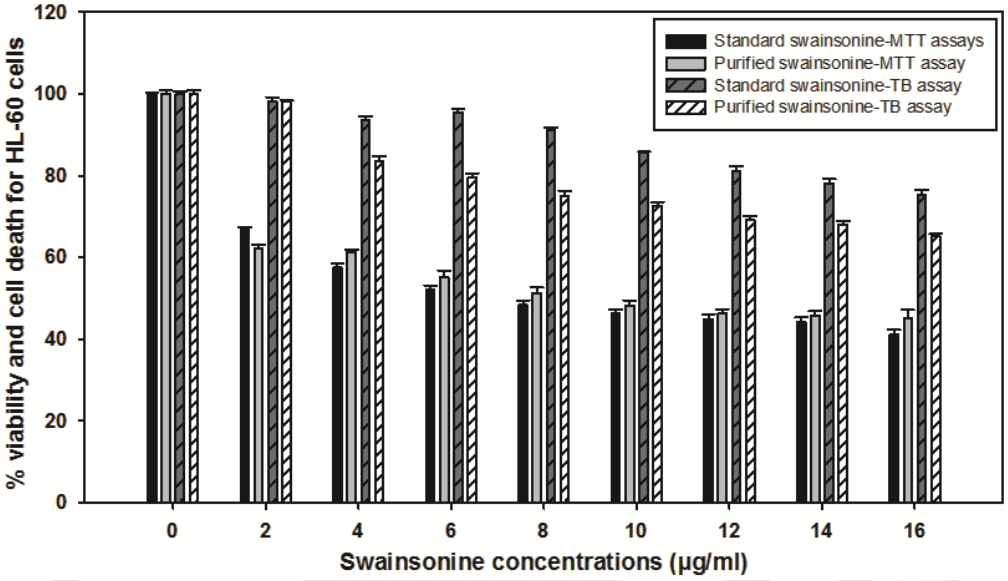


Fig. 5.2. MTT viability and TB dye exclusion cell death assays of HL-60 cells measured after 60 h with different doses of standard and purified swainsonine. One-way ANOVA with Dunnett's pair wise multiple comparisons with control indicated that purified swainsonine affected HL-60 cell viability in a dose dependent fashion ($p < 0.001$). The regression values (R^2_{adj}) for standard and purified swainsonine are 0.99 and 0.98 in MTT assay and 0.96 and 0.96 for TB cell death assay, respectively. All data are average of three independent sets of experiments with standard deviation (±SD).

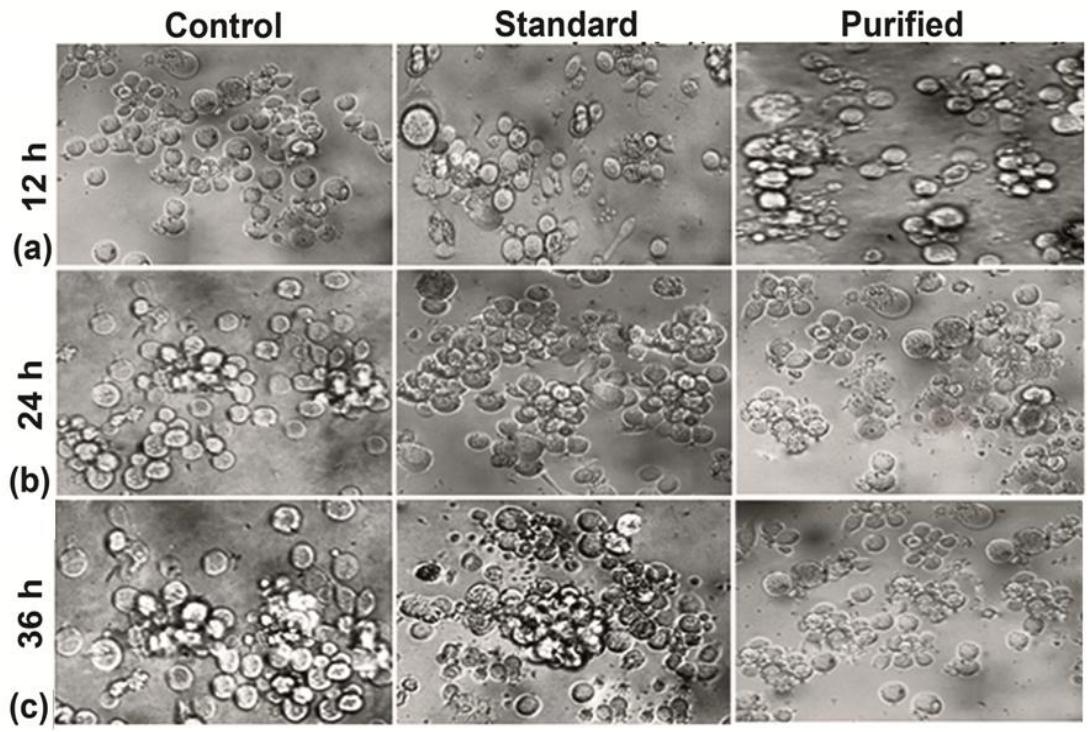


Fig. 5.3. The inverted phase contrast microscopic (40X) images for the control (untreated), standard (3.90 µM) and MSD purified (5.27 µM) swainsonine treated *Sf*-21 cells at **(a)** 12 h **(b)** 24 h, and **(c)** 36 h of post treatment incubation.

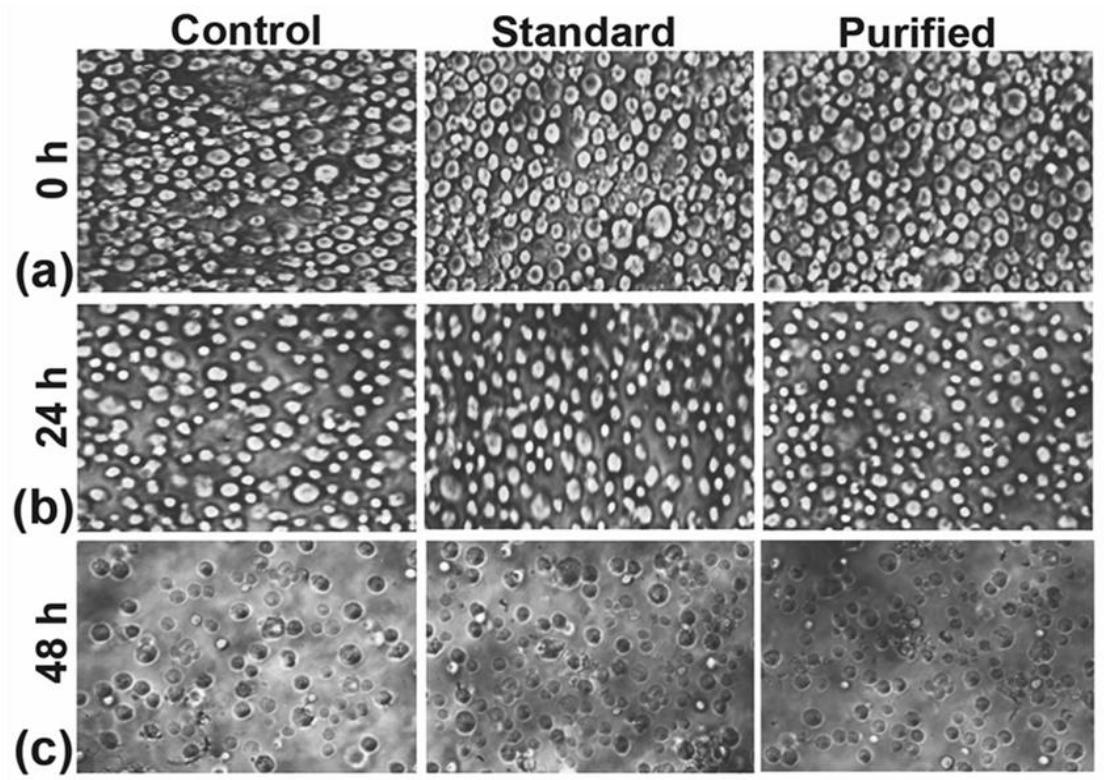


Fig. 5.4. The inverted phase contrast microscopic (40X) images for the control (untreated), standard (6.96 µM) and MSD purified (9.50 µM) swainsonine treated HL-60 cells at **(a)** 0 h **(b)** 24 h and **(c)** 48 h of post treatment incubation.

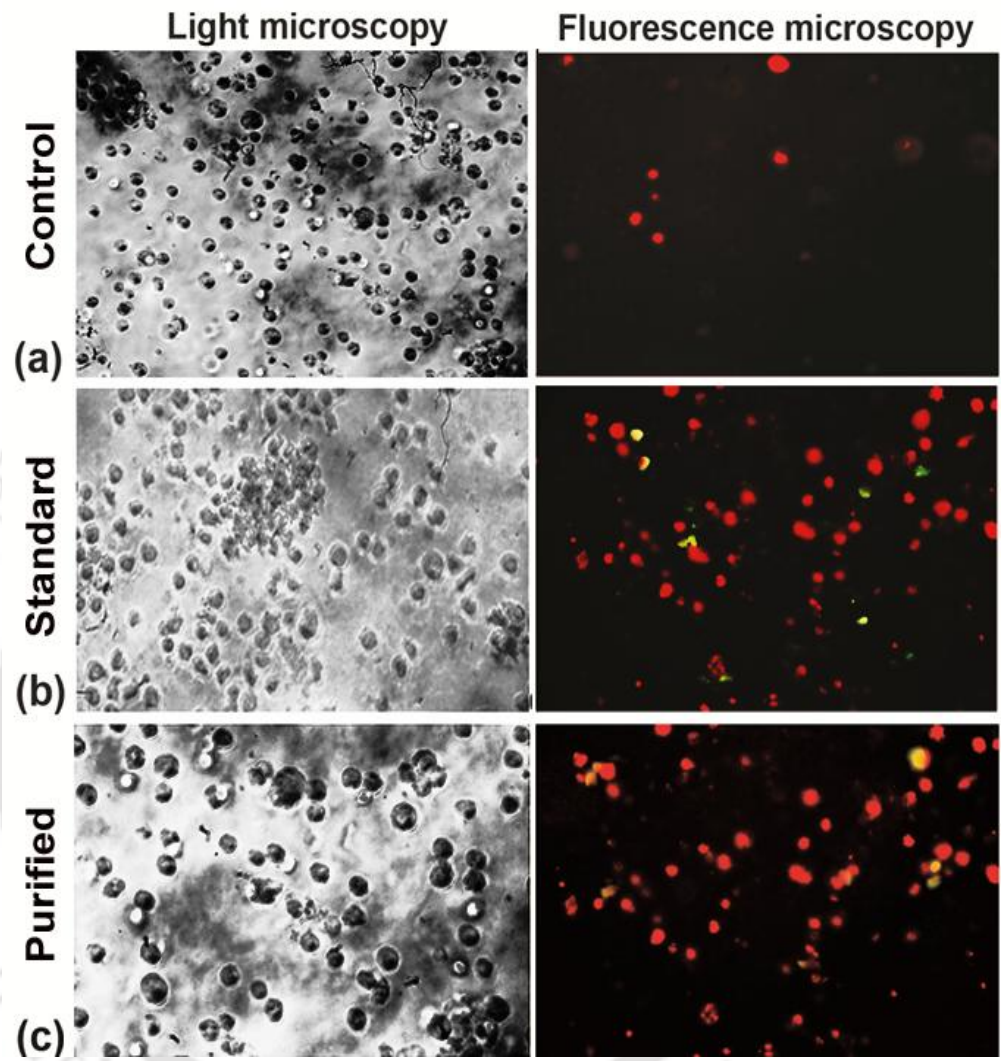


Fig. 5.5. Light and fluorescence microscopy at 40X magnification of **(a)** control (untreated) **(b)** standard (3.90 µM) and **(c)** purified (5.27 µM), swainsonine treated *Sf*-21 cells after 36 h of post treatment incubation.

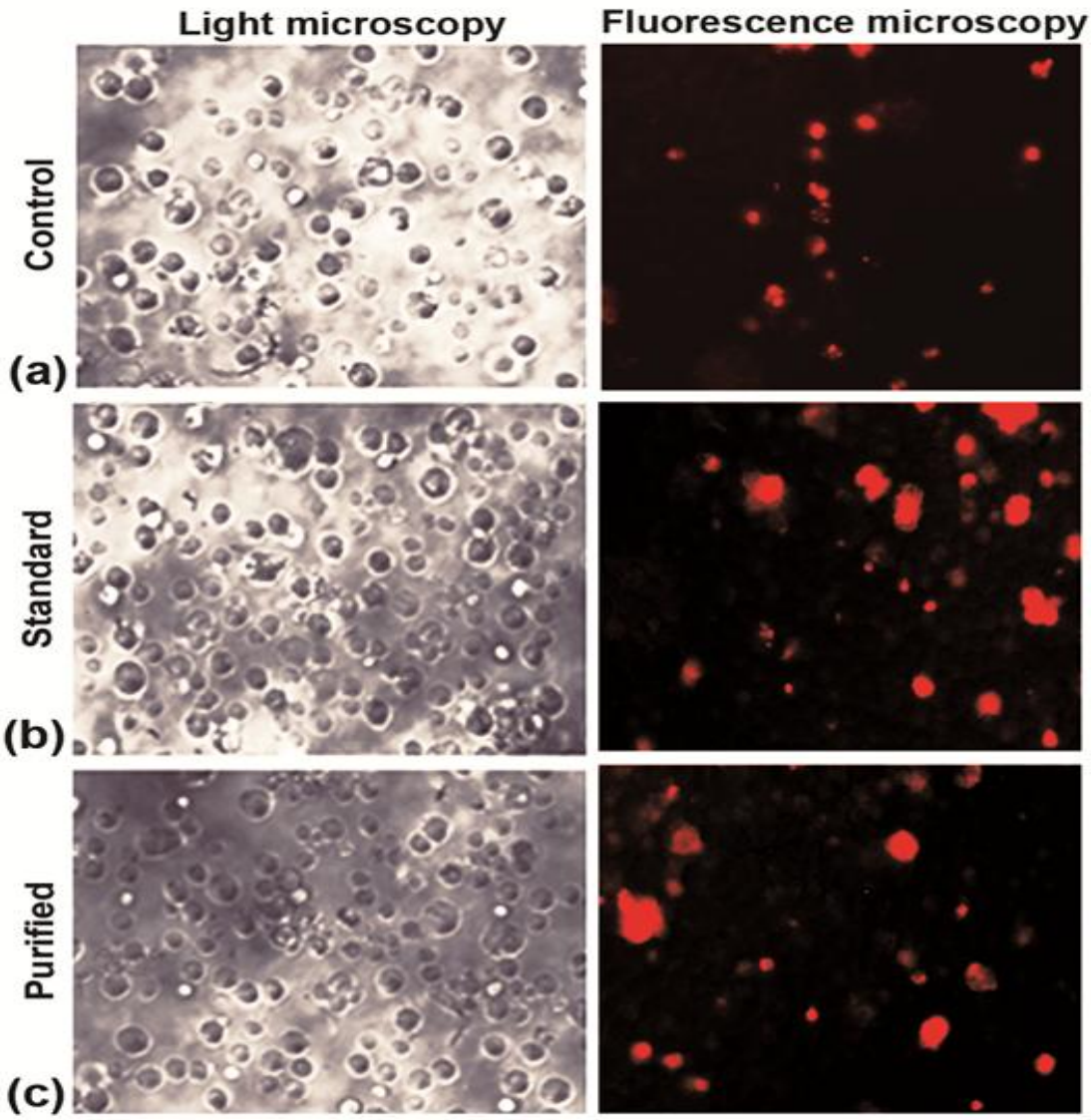


Fig. 5.6. Light and fluorescence microscopy at 40X magnification of **(a)** control (untreated) **(b)** standard (6.96 µM), and **(c)** MSD purified (9.50 µM), swainsonine treated HL-60 cells after 48 h of post treatment incubation.

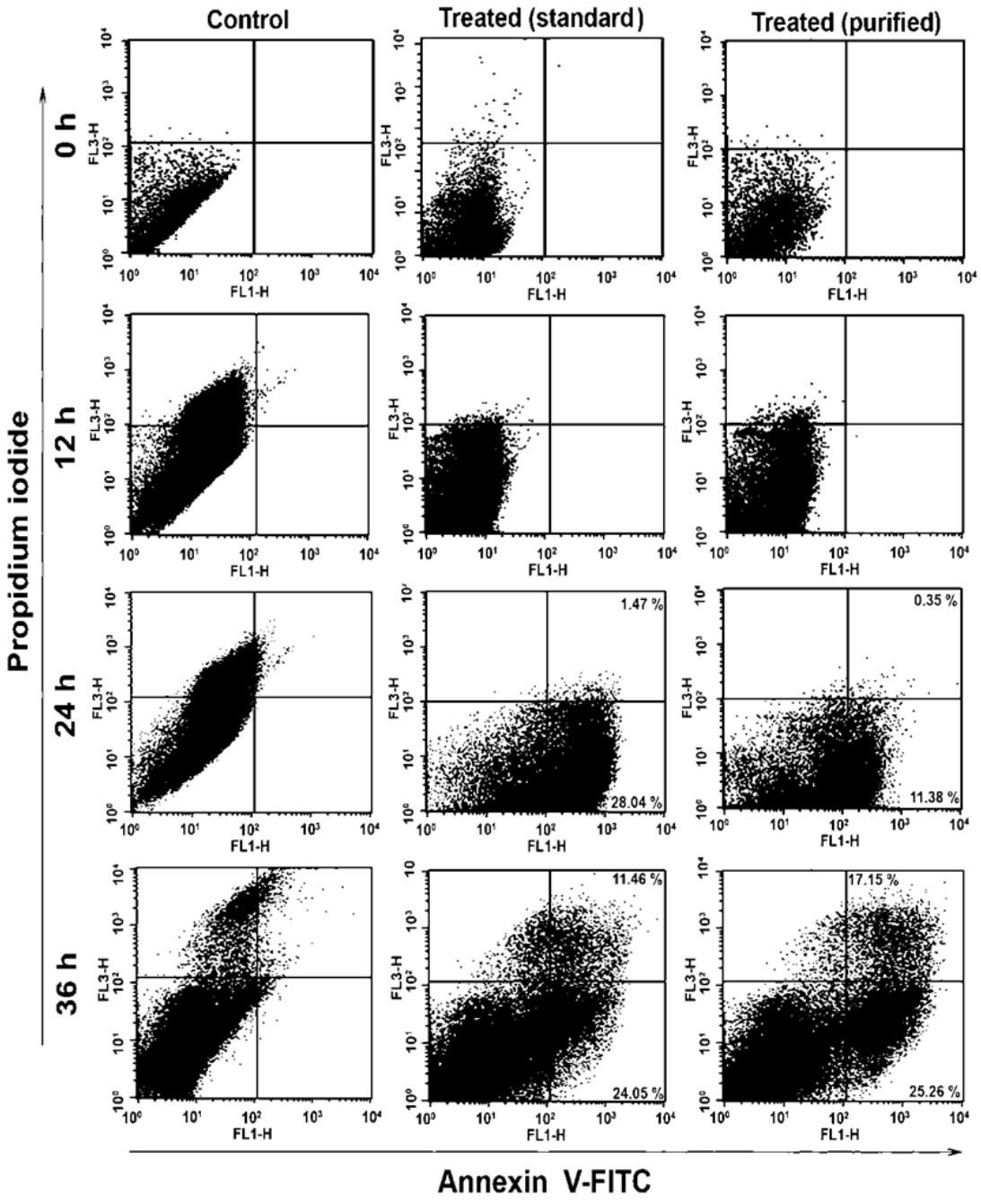


Fig. 5.7. FACS analysis of swainsonine (IC_{50} concentrations for standard and purified) treated induced apoptosis in *Sf*-21 cells for 36 h of post-treatment incubations. The data was representative for three different experiments at $p < 0.05$.

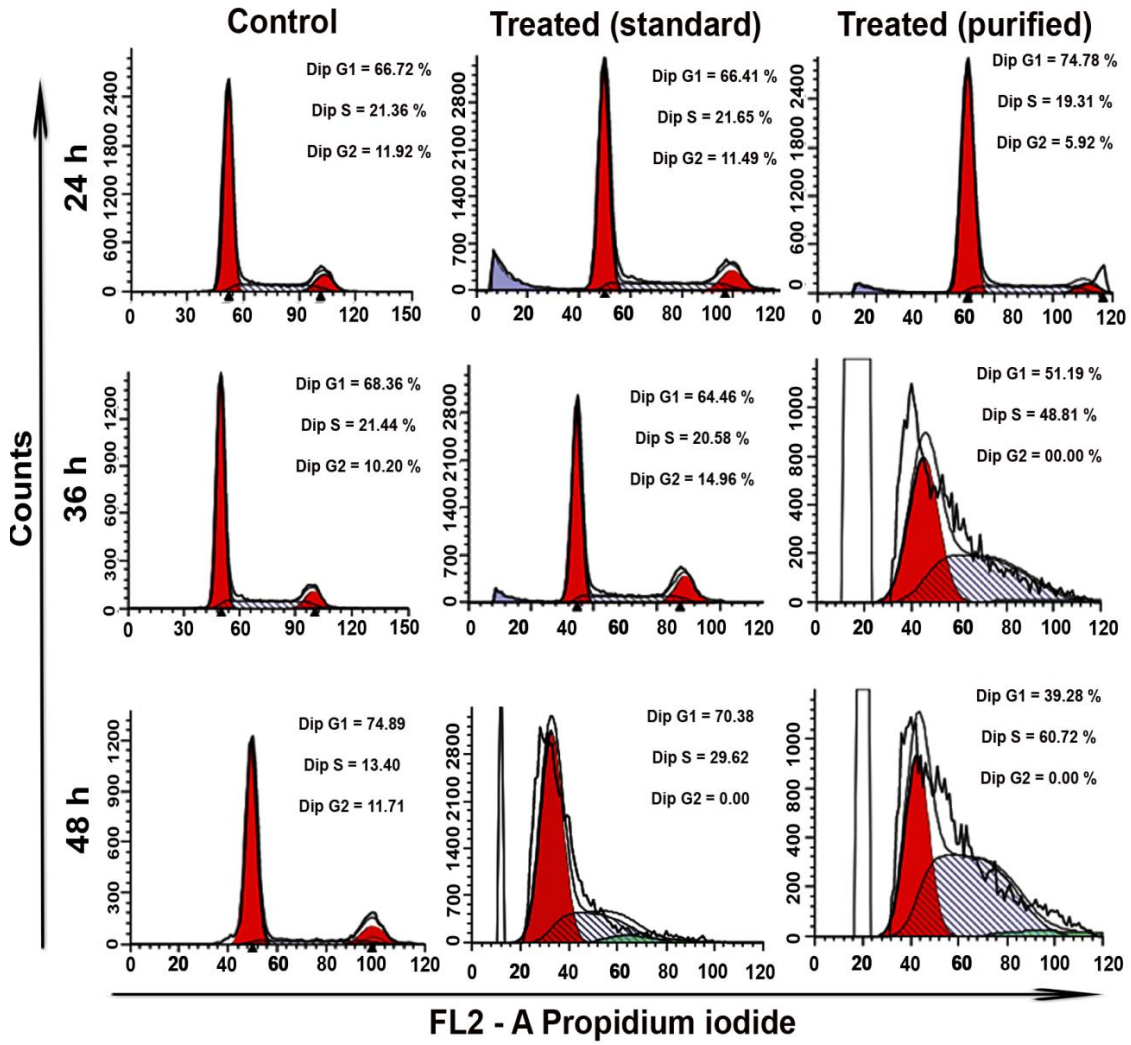


Fig. 5.8. Analysis of cells cycle progressions in control and swainsonine (at IC_{50} concentrations for standard and purified) treated HL-60 cells. The data was representative for three different experiments at $p < 0.05$.

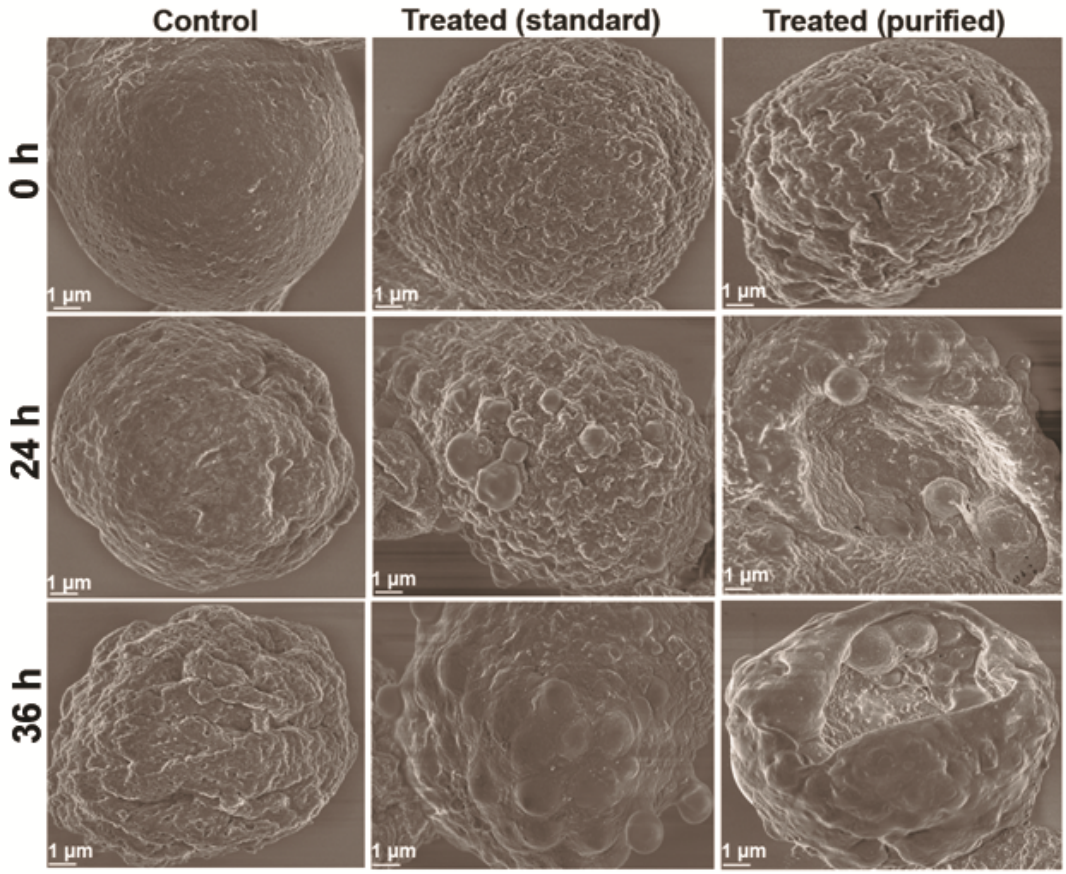


Fig. 5.9. FE-SEM micrograph (15KX) of control and swainsonine treated (at IC_{50} concentrations for standard and purified) *Sf*-21 cells at 0 h, 12 h and 36 h post treatment incubations.

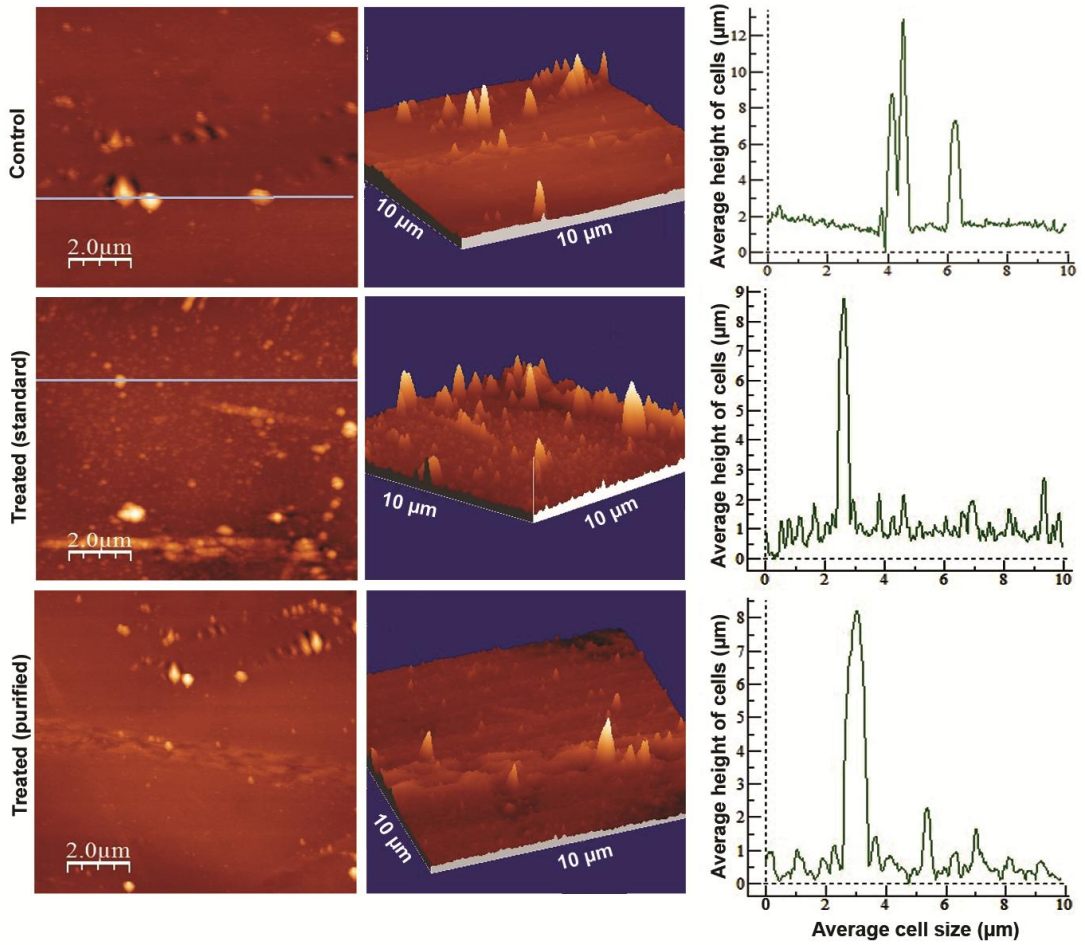


Fig. 5.10. AFM analysis of the control and swainsonine (at IC_{50} concentrations for standard and purified) treated *Sf*-21 cells after 36 h of post-treatment incubation. The full scan 2D image (first column), 3D image (second column) and average height-size curve (third column) for *Sf*-21 cells. The colour gradients-light to dark indicates the average height of cells under scan corresponding to their higher to lower topographies respectively.

REFERENCES

Abakarov, A., Sushkov, Y., Almonacid, S. and Simpson, R (2009). Thermal processing optimization through a modified adaptive random search. J. Food Eng. 93, 200-209.

Abdel-Fattah, Y. R. and Olama, Z. A. (2002) L-asparaginase production by *Pseudomonas aeruginosa* in solid-state culture: Evaluation and optimization of culture conditions using factorial designs. Process Biochem. 38, 115-122.

Abdul-Rahman, O.A., Munetomo, M. and Akama, K. (2013) An adaptive parameter binary-real coded genetic algorithm for constraint optimization problems: Performance analysis and estimation of optimal control parameters. Inf. Sci. 233, 54-86.

Achary, A., Hariharan, K. A., Bandhyopadhyaya, S., Ramachandran, R. and Jayaraman, K. (1997) Application of numerical modelling for the development of optimized complex medium for D-hydantoinase production from *Agrobacterium radiobacter* NRRL B-11291. Biotechnol. Bioeng. 55, 148-154.

Ahmad, Z., Mashitah, M. D., Siti Hatijah, M. and Rabiatul Adawish, M. N. (2010) Nonlinear process modeling of fructosyltransferase (FTase) using bootstrap re-sampling neural network model. Bioproc. Biosyst. Eng. 33, 599-606.

Ahmed (2005). Artificial neural networks for diagnosis and survival prediction in colon cancer. Mol. Cancer. 4, 29.

Alpert, A. J (1990) Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds. J. Chromatogr. A. 499, 177-196.

Amanullah, A., Justen, P., Davies, A., Paul, G.C., Nienow, A.W. and Thomas, C.R. (2000) Agitation induced mycelial fragmentation of *Aspergillus oryzae* and *Penicillium chrysogenum*, Biochem. Eng. J. 5, 109-114.

Amiri, B., Ibrahim, L. and Butt, T. M. (1999) Antifeedant properties of destruxins and their use with the entomogenous fungus *Metarhizium anisopliae* for improved control of crucifer pests. Biocontrol Sci. Technol. 9, 487-498.

Amiri-Besheli, B., Khambay, B., Cameron, S., Deadman, M. L. and Butt, T. M. (2000) Inter- and intra-specific variation in destruxin production by insect pathogenic *Metarhizium* spp., and its significance to pathogenesis. Mycol. Res. 104, 447-452.

Anderson, S. B., Gerritsma, S., Yusah, K. M., Mayntz, D., Hywel-Jones, N. L., Billen, J., Boomsma, J. J. and Hughes, D. P. (2009) The life of a dead ant: the expression of an adaptive extended phenotype. Am. Nat. 174, 424-433.

Andrzej, M., Kunicka, J.E., Melamed, M.R. and Darzynkiewicz, Z. (1989) Effect of swainsonine on stimulation and cell cycle progression of human lymphocytes. Cancer Res. 49, 2879-83.

Ashirua, D. A. I., Karub, K., Zlohb, M., Patelc, R. and Basit, A. W. (2011) Relative quantification of polyethylene glycol 400 excreted in the urine of male and female volunteers by direct injection electrospray-selected ion monitoring mass spectrometry. Int. J. Pharmaceutics. 414, 35-41.

Bandani, A. R., Khambay, B. P. S., Fauli, J., Newton, R., Deadman, M. and Butt, T. M. (2000) Production of efrapeptins by Tolypocladium species and evaluation of their insecticidal and antimicrobial properties, Mycol. Res. 104, 537-544.

Baptista, J. A., Goss, P., Nghiem, M., Krepinsky, J. J., Baker, M. and Dennis, J. W. (1994) Measuring swainsonine in serum of cancer patients: phase I clinical trial. Clin. Chem. 40, 426-430.

Benchapattarapong, N., Anderson, W.A., Bai, F. and Moo-Young, M. (2005) Rheology and Hydrodynamic Properties of *Tolypocladium inflatum* Fermentation Broth and Its Simulation. Bioprocess Biosyst. Eng. 27, 239-247.

Bhaskara, V. V., Middhab, A., Tiwaria, S. and Shivakumara, S. (2013) Liquid chromatography/tandem mass spectrometry method for quantitative estimation of polyethylene glycol 400 and its applications. J. Chromatogr. B. 926, 68-76.

Box, G. E. P., Draper, N. R. (1987) Empirical Model-Building and Response Surfaces. John Wiley & Sons, New York. ISBN-13: 978-0471810339.

Braun, K., Romero, J., Liddell, C. and Creamer, R. (2003) Production of swainsonine by fungal endophytes of locoweed. Mycol. Res. 107, 980-988.

Caldeira, A.T., Arteiro, J.M., Roseiro, J.C., Neves, J. and Vicente, H. (2011) An artificial intelligence approach to *Bacillus amyloliquefaciens* CCMI 1051 cultures: application to the production of anti-fungal compounds. Bioresour. Technol. 102, 1496-1502.

Cao, G. R., Li, S. J., Duan, D. X., Molyneux, R. J., James, L. F., Wang, K., Tong, C. (1992) The toxic principle of Chinese locoweeds (*Oxytropis* and *Astragalus*): toxicity in goats. In Poisonous Plants, LF James, RF Keeler, PR Cheeke, EM Bailey, MP Hegarty (eds). Iowa State University Press: Ames, IA; 117-121.

Carlos, A.C., Ana, L.A.C., Letícia, A.S., Thais, G., Donald, W.R., Norberto, P.L. and Gilberto, U.L.B. (2010) Fungal tyrosine betaine, a novel secondary metabolite from conidia of entomopathogenic *Metarhizium* spp. fungi. Fungal Biol. 114, 473-480.

Chen, J., Narayan, S. B., Edinger, A. L. and Bennetta, M. J. (2012) Flow injection tandem mass spectrometric measurement of ceramides of multiple chain lengths

in biological samples. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 883-884, 136-140.

Chet, I. (1987) Trichoderma application, mode of action and potential as biocontrol agent of soil borne plant pathogenic fungi. Innovative Approaches Plant Disease Control. Chet, I. (Ed.), JohnWiley and Sons, New York, NY, USA. pp. 137-160.

Chet, T., Schichler, H., Haran, S. and Appenheim, A. B. (1993) Cloned chitinase and their role in biological control of plant pathogenic fungi. Proceedings of the International Symposium on Chitin Enzymology. Senigalia, Italy. pp. 47-48

Chiang, J. H., Yang, J. S., Ma, C. Y., Yang, M. D., Huang, H. Y., Hsia, T. C., Kuo, H. M., Wu, P. P., Lee, T. H. and Chung, J. G. (2011) Danthron, an anthraquinone derivative, induces DNA damage and caspase cascades-mediated apoptosis in SNU-1 human gastric cancer cells through mitochondrial permeability transition pores and Bax-triggered pathways. Chem. Res. Toxicol. 24, 20-29.

Colegate, S. M., Dorling, P. R. and Huxtable, C. R (1979) A spectroscopy investigation of swainsonine: An a-mannosidase inhibitor isolated from *Swainsona canescens*. Aust. J. Chem. 32, 2257-2264.

Collins, S.J., Gallo, R.C. and Gallagher, R. E. (1977) Continuous growth and differentiation of human myeloid leukemic cells in suspension culture. Nature, 270, 347-349.

Cui, Y. Q., van der Lans, R. G. J. M. and Luyben, K. C. A. M. (1997) Effect of agitation intensities on fungal morphology of submerged fermentation, Biotechnol. Bioeng. 55, 715-726.

Dantas, A. F. M., Riet-Correa, F., Gardner, D. R., Medeiros, R. M., Barros, S. S., Anjos, B. L. and Lucena, R. B. (2007) Swainsonine-induced lysosomal storage disease in goats caused by the ingestion of *Turbina cordata* in northeastern Brazil. Toxicon. 49, 111-116.

Daniel, C. (1973) "One at a time plans". J. Am. Stat. Assoc. 68, 353-360.

de-Freitas-Junior, J. C., Bastos, L. G., Freire-Neto, C. A., Rocher, B. D., Abdelhay, E. S. and Morgado-Díaz, J. A. (2012) N-glycan biosynthesis inhibitors induce *in vitro* anticancer activity in colorectal cancer cells. J. Cell Biochem. 113, 2957-2566.

Dennis, J., Koch, K. and Beckner, D. (1989a) Inhibition of human HT29 colon carcinoma growth *in vitro* and *in vivo* by swainsonine and human interferon-α2. J. Natl. Cancer Inst. 81, 1028-1033.

Dennis, J., Laferte, S., Yagel, S. and Breitman, M. (1989b) Asparagine linked oligosaccharides associated with metastatic cancer. Cancer cells. 1, 87-92.

Dennis, J.W., Shar, R.N. and Ziser, L. (2002) Alkaloid halide salts of swainsonine and methods of use. US Patent. S.No. US 6, 395, 745, B1.

Desai, K. M., Survase, S. A., Saudagar, P. S., Lele, S. S. and Singhal, R. S. (2008) Comparison of artificial neural network (ANN) and response surface methodology (RSM) in fermentation media optimization: Case study of fermentative production of scleroglucan. Biochem. Eng. J. 41, 266-73.

Diener, U. L., Cole, R. J., Sanders, T. H., Payne, G. A., Lee, L. S. and Klich, M. A. (1987) Epidemiology of Aflatoxin formation by *Aspergillus flavus*. Annu. Rev. Phytopathol. 25, 249-270.

Donaldson, M. J., Brody, H., Adlard, M. W. and Bucke, C. (1990) High pressure liquid chromatography and pulsed amperometric detection of castanospermine and related alkaloids. Phytochem. Anal. 1, 18-21.

Dorling, P. R., Huxtable, C. R. and Colegate, S. M. (1980) Inhibition of lysosomal α-mannosidase by swainsonine, an indolizidine alkaloid isolated from *Swainsona canescens*. Biochem. Soc. 191, 649-651.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956) Colorimetric method for determination of sugars and related substances. Anal Chem. 28, 350-356.

Dumas, C., Matha, V., Quiot, J. M. and Vey, A. (1996) Effect of destruxins, cyclic depsipeptide mycotoxins, on calcium balance and phosphorylation of intracellular proteins in lepidopteran cell lines. Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 114, 213-219.

Dwek, R. A. (1996) Glycobiology toward understanding the function of sugars. Chem. Rev. 96, 683-720.

Eisenbrand, G., Pool-Zobel, B., Baker,V., Balls, M., Blaauboer, B. J., Boobis, A., Carere, A., Kevekordes, S., Lhuguenot, J. C., Pieters, R. and Kleiner, J. (2002) Methods of *in vitro* toxicology. Food Chem. Toxicol. 40, 193-236.

Elbein, A. D., Solf, R., Dorling, P. R. and Vosbeck, K. (1981) Swainsonine, an inhibitor of glycoprotein processing. Proc. Natl. Acad. Sci. USA. 78, 7393-7397.

El-Enshasy, H., Kleinea, J. and Rinas, U. (2006) Agitation effects on morphology and protein productive fractions of filamentous and pelleted growth forms of recombinant *Aspergillus niger*, Process Biochem. 41, 2103-2112.

Elibol, M. (2004) Optimization of medium composition for actinorhodin production by *Streptomyces coelicolor* A3 (2) with response surface methodology. Process Biochem. 39, 1057-1062.

Fan, J., Xie, Y., Xue, J., Zhang, Y. and Yang, Q. (2013) Cellular Apoptosis of Hemocytes from *Dendrolimus tabulaeformis* Tsai et Liu Larvae Induced with the Secondary Metabolites of *Beauveria brongniartii* (Sacc.) Petch. PLOS. 8(8):e71600. doi:10.1371/journal.pone.0071600.

Fattorusso, E. and Taglialatela-Scafati, O. (2007) Modern Alkaloids. Structure, Isolation, Synthesis and Biology. Reference Book-ISBN, 978-3-527-31521-5-Wiley-VCH, Weinheim.

Fellows, L. E., Bell, E. A., Lynn, D. G., Pilkiewicz, F., Miura, I. and Nakanishi, K. (1979) Isolation and structure of an unusual cyclic amino alditol from a legume. J. Chem. Soc., Chem. Commun. 977-978. DOI: 10.1039/C39790000977.

Fellows, L. E. and Fleet, G. W. J. (1989) In: Wagman, G.H., Cooper, R. (Eds.). Natural Products Isolation. Separation Methods for Antimicrobials, Antivirals and Enzyme Inhibitors, Elsevier, Amsterdam, pp. 539.

Ferrara, C., Briinker, P., Suter, T., Moser, S., Puntener, U. and Umana, P. (2006) Modulation of therapeutic antibody effecter functions by glycosylation engineering: influence of Golgi enzyme localization domain and co-expression of heterologous betal, 4-N-acetylgluco saminyltransferase III and Golgi alpha-mannosidase II. Biotechnol. Bioeng. 93, 851-861.

Fiolka, M. J. (2008) Immunosuppressive effect of cyclosporin A on insect humoral immune response. J. Invert. Pathol. 98, 287-292.

Fornelli, F., Minervini, F. and Logrieco, A. (2004) Cytotoxicity of fungal metabolites to lepidopteran (*Spodoptera frugiperda*) cell line (SF-9). J. Invertebr. Pathol. 85, 74-79.

Fox, J. D., and Robyt, J. F. (1991) Miniaturization of three carbohydrate analyses using micro sample plate reader. Anal. Biochem. 19, 593-596.

Francis, L. W., Gonzalez, D., Ryder, T., Baerm, K., Reesm, M., White, J. O., Conlan, R. S. and Wright, C. J. (2009) Progesterone induces nano-scale molecular modifications on endometrial epithelial cell surfaces; Biol. Cell. 101**,** 481-493.

Friedman, M. and Savage, L. J (1947) Planning Experiments Seeking Maxima, in Techniques of Statistical Analysis, McGraw-Hill, New York, pp. 365-372.

Galustian, C., Foulds, S., Dye, J. F. and Guillou, P. J. (1994) Swainsonine, a glycosylation inhibitor, enhances both lymphocyte efficacy and tumour susceptibility in LAK and NK cytotoxicity. Immunopharmacol. 27, 165-172.

Gao, X. F, Dan, M., Zhao, A. H, Xie, G. X. and Jia, W. (2008) Simultaneous determination of saponins in flower buds of *Panax notoginseng* using high performance liquid chromatography. Biomed. Chromatogr. 22, 244-249.

Gao, H., Liu, M., Liu, J., Dai, H., Zhou, X., Liu, X., Zhuo, Y., Zhang, W. and Zhang, L. (2009) Medium optimization for the production of avermectin B1a by *Streptomyces avermitilis* 14-12A using response surface methodology. Bioresour. Technol. 100, 4012-4016.

Garcia-Ochoa, F., Gomez-Castro, E. and Santos, V. E. (2000) Oxygen transfer and uptake rates during xanthan gum production. Enzyme Microb. Technol. 27, 680-690.

Gardner, D. R, Molyneux, R. J. and Ralphs, M. H. (2001) Analysis of swainsonine: extraction methods, detection and measurement in populations of locoweeds (*Oxytropis* spp.) J. Agric. Food Chem. 49, 4573-4580.

Gardner, D. R., Lee, S. T., Molyneux, R. J. and Edgar, J. A. (2003) Preparative isolation of swainsonine from locoweed: extraction and purification procedures. Phytochem. Anal. 14, 259-266.

Gardner D. R. and Cook, D. (2010) A Comparison of alternative sample preparation procedures for the analysis of swainsonine using LC-MS/MS. Phytochem. Anal. 22, 124-127.

Gavrilescu, M., Roman, R. V. and Efimov, V. (1993) The volumetric oxygen mass transfer coefficient in antibiotic biosynthesis liquids. Acta Biotechnol. 13, 59-70.

Gen, M. and Cheng, R. (2000) Genetic algorithms and engineering optimization. Wiley, New York.

Gerber-Lemaire, S. and Juillerat-Jeanneret, L. (2010) Studies toward new anti-cancer strategies based on α-mannosidase inhibition. Chimia-Zurich. 64, 634-639.

Gillespie, J. P., Bailey, A. M., Cobb, B. and Vilcinskas, A. (2000) Fungi as elicitors of insect immune responses. Arch. Insect Biochem. Physiol. 44, 49-68.

Goldberg, D. E. (1989) Genetic Algorithms in Search, Optimization and Machine Learning. Longman Publishing Co., New York. ISBN: 0201157675.

Goldberg, D. E. (1991) Real-coded genetic algorithms, virtual alphabets and blocking. Complex Syst. 5, 139-167.

Goss, P. E., Baptiste, J., Fernandes, B., Baker, M. and Dennis, J. W. (1994) A phase I study of swainsonine in patients with advanced malignancies. Cancer Res. 54, 1450-1457.

Grumbach, E. S., Wagrowski-Diehl, D. M., Mazzeo, J. R., Alden, B. and Iraneta, P. C. (2004) Hydrophilic interaction chromatography using silica columns for the retention of polar analytes and enhanced ESI-MS sensitivity. LC GC Magazine: North America-Solutions for Separation Scientists. 22, 1010-1023.

Guengerich, F., DiMari, S. J. and Broquist, H. P. (1973) Isolation and characterization of a 1-pyrindine fungal alkaloid. J. Ame. Chem. Soc., 95, 2055-2056.

Guo, Y., Xu, J., Zhang, Y., Xu, H., Yuan, Z. and Li, D. (2010). Medium optimization for ethanol production with Clostridium autoethanogenum with carbon monoxide as sole carbon source. Bioresour. Technol. 101, 8784- 8789.

Haaland, P. D. (1989) In Experimental Design in Biotechnology, Elsevier, New York.

Haider, M. A., Pakshirajan, K., Singh, A. and Chaudhry, S. (2008) Artificial neural network and genetic algorithm approach to optimize media constituents for enhancing lipase production by a soil microorganism. Appl. Biochem. Biotechnol. 144, 225-235.

Hakomori, S. I. (1996) Tumor malignancy defined by aberrant glycosylation and Sphingo (glyco) lipid metabolism. Cancer Res. 56, 5309-5318.

Hamaguchi, J., Nakagawa, H., Takahashi, M., Kudo, T., Kamiyama, N., Sun, B., Oshima, T., Sato, Y., Deguchi, K., Todo, S. and Nishimura, S. (2007) Swainsonine reduces 5-fluorouracil tolerance in the multistage resistance of colorectal cancer cell lines. Mol. Cancer. 6, 58.

Hamshou, M., Els, V. D., Silvia, C., Kaat, C., Gianni, V., Bart, G., Kris, G. and Guy, S. (2013) High Entomotoxicity and Mechanism of the Fungal GalNAc/Gal-specific *Rhizoctonia Solani* Lectin in Pest Insects. J. Insect Physiol. 59, 295-305.

Harris, E. M., Schneider, M. J., Ungemach, F. S., Hill, J. E. and Harris, T. M. (1988) Biosynthesis of the toxic indolizidine alkaloids slaframine and swainsonine in *Rhizoctonia leguminicola*: metabolism of I-hydroxyindolizidines. J. American Chem. Soc. 110, 940-949.

Haykin, S. (2008) Neural networks and learning machines, 3rd ed. Prentice-Hall, New Jersey.

Hendricks, J. A., Keliher, E. J., Marinelli, B., Reiner, T., Weissleder, R. and Mazitschek, R. (2011) *In vivo* PET-imaging of histone deacetylases by 18F-suberoylanilide hydroxamic acid (18F-SAHA)1. J. Med. Chem. 54, 5576-5582.

Hinaje, M., Ford, M., Banting, L., Arkle, S. and Khambay, B. (2002) An investigation of the ionophoric characteristics of destruxin A. Arch. Biochem. Biophys. 405, 73-77.

Hino, M., Nakayama, O., Tsurumi, Y., Adachi, K., Shibata, T., Terano, H., Kohsaka, M., Aoki, H. and Imanaka, H. (1985) Studies of an immunomodulator, swamsonine: I. Enhancement of immune response by swainsonine. In Vit. J. Antibiot. 38, 926-935.

Hoe, P. K., Bong, C. F. J., Jugah, K. and Rajan, A. (2009) Evaluation of *Metarhizium anisopliae* var. *anisopliae* (Deuteromycotina: Hyphomycete) isolates and their effects on subterranean termite *Coptotermes curvignathus* (Isoptera: Rhinotermitidae). Am. J. Agric. Biol. Sci. 4, 289-297.

Hohenschutz, L. D., Bell, E. A., Jewess, P. J., Leworthy, D. P., Pryce, R. J., Arnold, E. and Clardy, J. (1981) Castanospermine, a 1,6,7,8-tetrahydroxy-octahydro-indolizidine alkaloid, from seeds of *Castanospermum australe*. Phytochem. 20, 811-814.

Houston, K. M., Cushley, W. and Harnett, W (1997) Studies on the site and mechanism of attachment of phosphorylcholine to a filarial nematode secreted glycoprotein. J. Biol. Chem. 272, 1527-1533.

Hughmark. G. A. (1980) Power requirements and interfacial area in gas–liquid turbine agitated systems. Ind. Eng. Chem. Proc. Des. Dev. 19, 638-641.

Hu, G. and St. Leger, R. J. (2002) Field studies using a recombinant mycoinsecticide (*Metarhizium anisopliae*) reveal that it is rhizosphere competent. Appl. Environ. Microbiol. 68, 6383-6387.

Hui, K. P., Sit, W. H. and Wan, J. M. (2005) Induction of S phase cell arrest and caspase activation by polysaccharide peptide isolated from *Coriolus versicolor* enhanced the cell cycle dependent activity and apoptotic cell death of doxorubicin and etoposide, but not cytarabine in HL-60 cells. Oncol. Rep. 14, 145-55.

Hung, D. T., Jamison, T. F. and Schreiber S. L. (1996) Understanding and controlling the cell cycle with natural products. Chem. Biol. 3, 623-639.

Jackson, S. L. and Heath, I. B. (1993) Roles of Calcium Ions in Hyphal Tip Growth. Microbiol. Rev. 57, 367-382.

James, P. D. A., Edwards, C. and Dwason, M. (1991) The effects of temperature, pH and growth rate on secondary metabolism in Streptomyces thermoviolaceus grown in a chemostat. J. Gen. Microbiol. 137, 1715-1720.

Jegorov, A., Sedmera, P. and Matha, V. (1995) Biosynthesis of destruxins. Phytochemistry. 33, 1403-1405.

John, H., Eddleston, M., Clutton, R., Worek, F. and Thiermann, H. (2010) Simultaneous quantification of the organophosphorus pesticides dimethoate and omethoate in porcine plasma and urine by LC–ESI-MS/MS and flow-injection-ESI-MS/MS. J. chromatogr. B. 878, 1234-1245.

Justo, O. R., Calheiros, C. S., Perez, V. H. and Alegre, R. M. (2007) Screening of *Metarhizium* spp. strains for anticancer indolizidine alkaloid production and its rapid detection by MS analysis. Braz. J. Microbiol. 38, 780-784.

Kagan, M., Chlenov, M., Melnikov, S., McConnell, O., Bach, A. C., Carter, G., Failli, A., Caggiano, T.J., Shumsky, J.S. and Lubda, D. (2009) Normal-phase automated mass-directed HPLC purification of a pyrrolobenzodiazepine library with vasopressin agonist activity. J. Comb. Chem. 11, 1704-1719.

Kang, M. S. and Elbein, A. D. (1983) Mechanism of Inhibition of Jack Bean α-Mannosidase by swainsonine. Plant Physiol. 71, 551-554.

Kershaw, M. J., Moorhouse, E. R., Bateman, R. P., Reynolds, S. E. and Charnley, A. K. (1999) The role of destruxins in the pathogenicity of *Metarhizium anisopliae* for three species of insect. J. Invertebr. Pathol. 74, 213-223.

Kim, S. D. and Nho, H. J. (2004) Isolation and Characterization of α-glucosidase Inhibitor from the fungus *Ganoderma lucidum*. J. Microbiol. 42, 223-227.

Klein, J. L., Roberts, J. D, George, M. D., Kurtzberg, J., Breton, P., Chermann, J. C. and Olden, K. (1999) Swainsonine protects both murine and human haematopoietic systems from chemotherapeutic toxicity. Brit. J. Cancer. 80, 87-95.

Krasnoff, S. B., Keresztes, I., Gillilan, R. E., Szebenyi, D. M., Donzelli, B. G., Churchill, A. C. and Gibson, D. M. (2007) Serinocyclins A and B, cyclic heptapeptides from *Metarhizium anisopliae*. J. Nat. Prod. 70, 1919-1924.

Kucera, M. and Samsinakova, A (1968) Toxins of the entomophagous fungus *Beauveria bassiana*. J. Invert. Pathol, 12, 316-320.

Kudo, T., Nakagawa, H., Takahashi, M., Hamaguchi, J., Kamiyama, N., Yokoo, H., Nakanishi, K., Nakagawa, T., Kamiyama, T., Deguchi, K., Nishimura, S. I. and Todo, S. (2007) N-glycan alternations are associated with drug resistance in human hepatocellular carcinoma. Mol. Cancer. 6, 32.

Lefevre, T., Adamo, S. A., Biron, D. G., Misse, D., Hughes, D. and Thomas, F (2009) Invasion of the body snatchers: the diversity and evolution of manipulative strategies in host- parasite interactions. Adv. Parasitol. 68, 45-83.

Li, S. L., Lin, G., Chan, S. W. and Li, P. (2001) Determination of the major isosteroidal alkaloids in bulbs of Fritillaria by high-performance liquid chromatography coupled with evaporative light scattering detection. J. Chromatogr. A. 909, 207-214.

Li, B., Kawatkar, S. P., George. S., Strachan, H., Woods, R. J., Siriwardena, A., Moremen, K. W. and Boons, G. J. (2004) Inhibition of Golgi mannosidase II with mannostatin A analogues: synthesis, biological evaluation, and structure-activity relationship studies. Chembiochem. 5, 1220-1227.

Li, Z., Xu, X., Huang, Y., Ding, L., Wang, Z., Yu, G., Xu, D., Li, W., Tong, D. (2012) Swainsonine activates mitochondria mediated apoptotic pathway in human lung cancer A549 cells and retards the growth of lung cancer xenografts. Int. J. Bio. Sci. 8, 394-405.

Li, Z., Huang, Y., Dong, F., Li, W., Ding, L., Yu, G., Xu, D., Yang, Y., Xu, X. and Tong, D. (2012) Swainsonine promotes apoptosis in human oesophageal squamous cell carcinoma cells *in vitro* and *in vivo* through activation of mitochondrial pathway. J. Biosci. 37, 1005-1016.

Linek, V., Kordac, M., Fujasova, M. and Moucha, T. (2004) Gas-liquid mass transfer coefficient in stirred tanks interpreted throughmodels of idealised eddy structure of turbulence in the bubble vicinity. Chem. Eng. Process. 43, 1511-1517.

Mandli, A. R. and Modak, J. M. (2012) Evolutionary Algorithm for the Determination of Optimal Mode of Bioreactor Operation. Ind. Eng. Chem. Res. 51, 1796-1808.

Metz, B. and Kossen, N. W. F. (1977) The growth of molds in the form of pellets- a literature review. Biotechnol. Bioeng. 19, 781-799.

Metz, B., Kossen, N. W. F. and van Suijdam, J. C. (1979) The rheology of mold suspensions. Adv. Biochem. Eng. 11, 103-156.

Mohebbi, M., Barouei, J., Akbarzadeh, T. M. R., Rowhanimanesh, A. R., Habibinajafi, M. B. and Yavarmanesh, M. (2008) Modelling and optimization of viscosity in enzyme-modified cheese by fuzzy logic and genetic algorithm. Comput. Electron. Agric. 62, 260-265.

Mollier, P., Lagnel, J., Fournet, B., Aioun, A. and Riba, G. (1994) A glycoprotein highly toxic for *Galleria mellonella* larvae secreted by the entomopathogenic fungus *Beauveria sulfurescens*. J. Invertebr. Pathol. 64, 200-207.

Molyneux, R. J. and James, L. F. (1982) Loco intoxication: indolizidine alkaloids of spotted locoweed (*Astragalus lentiginosus*), Science. 216, 190-191.

Molyneux, R. J., James, L. F., Panter, K. E. and Ralphs M. H. (1989) The occurrence and detection of swainsonine in locoweeds. In James, L. F., Elbein, A., Molyneux, R. J. and Warren, C. D. (Ed.) Swainsonine and Related Glycosidase Inhibitors. pp. 100-117. Iowa State University Press, Ames.

Molyneux, R. J., James, L. F., Panter, K. E. and Ralphs, M. H. (1991) Analysis and distribution of swainsonine and related polyhydroxyindolizidine alkaloids by thin layer chromatography. Phytochem. Anal. 2, 125-129.

Molyneux, R.J., James, L.F., Ralphs, M.H., Pfister, J.A., Panter, K.P. and Nash, R.J. (1995) Polyhydroxy alkaloids glycosidase inhibitors from poisonous plants of global distribution: analysis and identification. In: Colegate, S.M., Dorling, P.R. (Eds.), Plant-Associated Toxins—Agricultural, Phitochemical and Ecological Aspects. CAB International, Wallingford, UK, pp. 107-112.

Molyneux, R. J., Gardner, D. R., James, L. F. and Colegate, S. M. (2002) Polyhydroxy alkaloids: chromatographic analysis. J. Chromatogr. A. 967, 57-74.

Molyneux, R. J., James, L. F., Panter, K. E. and Ralphs M. H. (2007) Analysis and distribution of swainsonine and related polyhydroxyindolizidine alkaloids by thin layer chromatography. Phytochem. Anal. 2, 125-129.

Moo-Young, M. and Blanch, H.W. (1981) Design of biochemical reactors: Mass transfer criteria for simple and complex systems. Advan Biochem Eng. 19, pp. 1-69.

Morgan, D.O. (2007) The DNA damage response. In: Lawrence, E. (Ed.), The Cell Cycle. Principles of Control. New Science Press Ltd., London, pp. 228-244.

Myokey, R., Samurai, A., Chang, C.F., Kodaira, Y., Takahashi, N. and Tamura, S (1969) Aspochracin, a new insecticidal metabolite of *Aspergillus ochraceus*. Part I. Isolation, struture and biological activities. Agric. Biol. Chem. J. 33, 1491-1494.

Nagata, Y. and Chu, K. H. (2003) Optimization of a fermentation medium using neural networks and genetic algorithms. Biotechnol. Lett. 25, 1837-1842.

Nanita, S. C., Pentz, A. M. and Bramble, F.Q. (2009) High-throughput pesticide residue quantitative analysis achieved by tandem mass spectrometry with automated flow injection. Anal. Chem. 81:3134-42.

Nash, R. J., Goldstein, W. S., Evans, S. V. and Fellows, L. E. (1986) Gas chromatographic method for separation of nine polyhydroxyalkaloids. J. Chromatogr. 366, 431-434.

Naveena, B. J., Altaf, M., Bhadriah, K., and Reddy, G. (2005) Selection of medium components by Plackett-Burman design for production of L (+) lactic acid by Lactobacillus amylophilus GV6 in SSF using wheat bran, Bioresour. Technol. 96, 485-490.

Nemr, A.E. (2000) Synthetic Methods for the Streoisomers of Swainsonine and its Analogues. Tetrahedron. 56, 8579-8629.

Nielsen, J., Johansen, C.L., Jacobsen, M., Krabben, P. and Villadsen, J. (1995) Pellet formation and fragmentation in submerged cultures of *Penicillium chrysogenum* and its relation to penicillin production. Biotechnol. Prog. 11, 93-98.

Ogut, A. and Hatch, R. T. (1988) Oxygen transfer into Newtonian and non-Newtonian fluids in mechanically agitated vessels. Can. J. Chem. Eng. 66, 79-85.

Olmos, E., Mehmood, N., Haj Husein, L., Goergen, J.L., Fick, M. and Delaunay, S. (2013) Effects of bioreactor hydrodynamics on the physiology of *Streptomyces*. Bioprocess Biosyst Eng. 36, 259-272.

Oredipe, O. A, Furbert-Harris, P. M, Laniyan, I., Green, W. R, White, S. L, Olden, K., Parish-Gause, D., Vaughn, T., Griffin. W. M. and Sridhar R. (2003) Enhanced proliferation of functionally competent bone marrow cells in different strains of mice treated with swainsonine. Int. Immunopharmacol. 3, 445-55.

Pal, S., St. Leger, R. J. and Wu, L. P. (2007) Fungal peptide Destruxin A plays a specific role in suppressing the innate immune response in Drosophila melanogaster. J. Biol. Chem. 282, 8969-8977.

Pastuszak, I., Molyneux, R. J., James, L. F. and Elbein, A. D. (1990) Lentiginosine, a trihydroxyindolizidine alkaloid that inhibits amyloglucosidase. Biochem. 29, 1886-1891.

Patrick, M. S., Adlard, M. W. and Keshavarz, T. (1993) Production of an indolizidine alkaloid, swainsonine by the filamentous fungus, *Metarhizium anisopliae.* Biotechnol. Lett. 15, 997-1000.

Patrick, M. S., Maxwell, W., Adlar, M. W. and Keshavarz, T. (1995) Swainsonine production in fed-batch fermentations of *Metarhizium anisopliae* in stirred-tank reactors. Biotechnol. Lett. 17, 433-438.

Patrick, M.S., Adlard, M.W. and Keshavarz, T (1996) Swainsonine production from batch cultures of *Metarhizium anisopliae* in stirred-tank reactors. Enzyme Microbial Technol. 18, 428-432.

Pazouki, M. and Panda, T. (2000) Understanding the morphology of fungi, Bioprocess Eng. 22, 127-143.

Pedras, M. S. C., Zaharia, L. I. and Ward, D. E. (2002) The destruxins: synthesis, biosynthesis, biotransformation, and biological activity. Phytochemistry. 59, 579-596.

Ping, L., Child, D., Meng-Li, Z., Gardner, D. R., Gui-Fen, L. and Guo-Dong, H. (2009) Culture and identification of endophytic fungi from *Oxytropis glabra* DC. Acta. Ecol. 29, 53-58.

Piyushkumar, M., Kiran, D. and Lele, S.S. (2007) Application of response surface methodology to cell immobilization for the production of palatinose. Bioresour. Technol. 98, 2892-2896.

Plackett, R. L. and Burman J. P (1946) The Design of Optimum Multifactorial Experiments, Biometrika. 33, 305-325.

Podsiadlowski, L., Matha, V. and Vilcinskas, A. (1998) Detection of a P-glycoprotein related pump in Chironomus larvae and its inhibition by verapamil and cyclosporin A. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 121, 443-450.

Prakasham, R. S., Rao, Ch. S., Rao, R. S., Lakshmi, G. S. and Sarma, P. N. (2007) L-asparaginase production by isolated *Staphylococcus* sp.-6A: design of experiment considering interaction effect for process parameter optimization. J. Appl. Microbiol. 102, 1382-1391.

Quesada-Moraga, E. and Vey, A. (2003) Intra-specific variation in virulence and *in vitro* production of macromolecular toxins active against locust among *Beauveria bassiana* strains and effects of *in vivo* and *in vitro* passage on these factors. Biocontrol Sci. Technol. 13, 323-340.

Roberts, D. W. and St. Leger R. J. (2004) *Metarhizium* spp., cosmopolitan insect-pathogenic fungi: mycological aspects, Adv. Appl. Microbiol. 54, 1-70.

Roberts, D. E., Gupta, S. and St. Leger R. J. (1992) Metabolite production by entomopathogenic fungi. Pesq. agropec. bras., Brasília. 27, 325-347.

Rosa, J. C., Neto, A. B., Hokka, C. O. and Badino, A. C. (2005) Influence of dissolved oxygen and shear conditions on clavulanic acid production by *Streptomyces clavuligerus*, Bioprocess Biosyst. Eng. 27, 99-104.

Roy, H. E., Steinkraus, D. C., Eilenberg, J., Hajek, A. E. and Pell, J. K. (2006) Bizarre interactions and endgames: Entomopathogenic fungi and their arthropod hosts. Annu. Rev. Entomol. 51, 331-357.

Samuels, R. I., Reynolds, S. E. and Charnley, A. K. (1988) Calcium channel activation of insect muscle by destruxins, insecticidal compounds produced by the entomopathogenic fungus, *Metarhizium anisopliae*. Comp. Biochem. Physiol. Part C: Comp. Pharm. 90: 403-412.

Santos, F. M., Latorre, A. O., Hueza, I. M., Sanches, D. S., Lippi, L. L., Gardner, D. R. and Spinosa, H. S. (2011) Increased antitumor efficacy by the combined administration of swainsonine and cisplatin *in vivo*. Phytomedicine. 18, 1096-1101.

Saul, R., Chambers, J. P., Molyneux, R. J. and Elbein, A. D. (1983) Castanospermine, a tetrahydroxylated alkaloid that inhibits beta-glucosidase and beta-glucocerebrosidase. Arch. Biochem. Biophys. 221, 593-597.

Schneider, M. J., Ungernach, F. S., Broquist, H. P. and Harris, T. M. (1983) (1S, 2R, 8R, 8aR)-1, 2, 8-Trihydroxyoctahydroindolizine (swainsonine), an α-mannosidase inhibitor from *Rhizoctonia legumicola*. Tetrahedron, 39, 29-32.

Schrank, A. and Vainstein, M. H. (2010) *Metarhizium anisopliae* enzymes and toxins. Toxicon. 56, 1267-1274.

Sen, D., Gosling, A., Stevens, G. W., Bhattacharya, P. K., Barber, A. R., Kentish, S.E., Bhattacharjee, C. and Gras, S. L. (2011) Galactosyl oligosaccharide purification by ethanol precipitation. Food Chem. 128, 773-777.

Shaheen, P. E., Stadler, W., Elson, P., Knox, J., Winquist, E. and Bukowski, R. M. (2005) Phase II study of the efficacy and safety of oral GD0039 in patients with locally advanced or metastatic renal cell carcinoma. Invest. New Drugs. 23, 577-581.

Shaikh, A. S., Tang, Y. J, Mukhopadhyay, A., Martín, H. G., Gin, J., Benke, P. I. and Keasling, J. D. (2010) Study of stationary phase metabolism via isotopomer analysis of amino acids from an isolated protein. Biotechnol Prog. 26, 52-56.

Shin, A. S., Hong, M. S. and Lee, J. (1996) Oxygen transfer correlation in high cell density culture of recombinant *E. coli*. Biotechnol. Tech. 10, 679-682.

Sim, K. L. and Perry, D. (1995) Swainsonine production by *Metarhizium anisopliae* determined by means of an enzymatic assay. Mycol. Res. 99, 1078-1082.

Singh, A., Majumder, A. and Goyal, A. (2008) Artificial intelligence based optimization of exocellular glucansucrase production from *Leuconostoc dextranicum* NRRL B-1146. Bioresour. Technol. 99, 8201-8206.

Singh, D. and Kaur, G. (2012**)** Optimization of different process variables for the production of an indolizidine alkaloid, swainsonine from *Metarhizium anisopliae*. J. Basic Microbiol. 52, 590-597.

Singh, D. and Kaur, G. (2013**)** Real encoded genetic algorithm and response surface methodology to optimize production of an indolizidine alkaloid, swainsonine from *Metarhizium anisopliae.* Folia Microbiol. 58, 393-401.

Sivapathasekaran, C., Mukherjee, S., Ray, A., Gupta, A. and Sen, R (2010) Artificial neural network modeling and genetic algorithm based medium optimization for the improved production of marine biosurfactant. Bioresour. Technol. 101, 2884-2887.

Skrobek, A. and Butt, T.M. (2005) Toxicity testing of destruxins and crude extracts from the insect-pathogenic fungus *Metarhizium anisopliae*. FEMS Microbiol. Lett. 251, 23-28.

St. Leger, R.J. (2008) Studies on adaptations of Metarhizium anisopliae to life in the soil. J. Invert. Pathol. 98, 271-276.

Stahl, E. and Muller, J. (1982) Parameters of preparative centrifugal thin-layer chromatography Chromatographia. 15, 493-497.

Stockigt, J., Sheludko, Y., Unger, M., Gerasimenko, I., Warzecha, H. and Stockigt, D. (2002) High-performance liquid chromatographic, capillary electrophoretic and

capillary electrophoretic-electrospray ionization mass spectrometric analysis of selected alkaloid groups. J. Chromatogr. A. 967, 85-113.

Strasser, H., Vey, A. and Butt, T. M. (2000) Are there any risks in using entomopathogenic fungi for pest control, with particular reference to the bioactive metabolites of *Metarhizium*, *Tolypocladium* and *Beauveria* species? Biocontrol Sci. Technol, 10, 717-735.

Sun, J. Y., Zhu, M. Z., Wang, S. W., Miao, S., Xie, Y. H. and Wang, J. B. (2007) Inhibition of the growth of human gastric carcinoma in vivo and in vitro by swainsonine. Phytomedicine. 14, 353-359.

Sun, J. Y., Yang, H., Miao, S., Li, J. P., Wang, S. W., Zhu, M. Z., Xie, Y. H., Wang, J. B., Liu, Z. and Yang, Q. (2009) Suppressive effects of swainsonine on C6 glioma cell *in vitro* and *in vivo*. Phytomedicine. 16, 1070-1074.

Taguchi, H. and Humphrey, A. E. (1966) Dynamic measurement of the volumetric oxygen transfer coefficient in fermentation systems. J. Ferm. Tech. 44, 881-889.

Tamerler, C., Adlard, M. W., Keshavarz, T. (1997) Production of swainsonine from *Metarhizium anisopliae* in stirred-tank and air-lift reactors. Biotechnol. Lett. 19, 1919-922.

Tamerler, C., Ullah, M., Adlard, M. W. and Keshavarz, T. (1998) Effect of pH on physiology of *Metarhizium anisopliae* for production of swainsonine. FEMS Microbiol. Lett. 168: 17-23.

Tamerler, C. and Keshavarz, T. (1999) Optimization of agitation for production of swainsonine from *Metarhizium anisopliae* in stirred tank and airlift reactors. Biotechnol. Lett. 21, 501-504.

Tang, M. K., Lian, Y. J., Chan, J. Y. H., Wong, S. N., Chen, E., Yao, Y., Gan, J., Xiao, L., Leung, H. C., Kung, H. F., Wang, H. and Lee, K. K. H. (2013) Promyelocytic Leukemia (PML) Protein Plays Important Roles in Regulating Cell Adhesion, Morphology, Proliferation and Migration. PLOS. doi:10.1371/journal.pone.0059477.

Terry, B. J., Liu, W. C., Cianci, C. W., Proszynski, E., Fernandes, P. and Meyers, E. (1992) Inhibition of herpes simplex virus type I DNA polymerase by the natural product oosporein, J. Antibiot. 2, 286-288.

Thomas, M. B. and Read, A. F. (2007) Can fungal biopesticides control malaria? Nat. Rev. Microbiol. 5, 377-383.

Tseng, C. P. and Montville, T. J. (1992) Enzymatic regulation of glucose catabolism by Lactobacillus plantarum in response to pH shifts in a chemostat. Appl. Microbiol. Biotechnol. 36, 777-787.

Tseng, Y. K., Wu, M. S. and Hou, R. F. (2008) Induction of apoptosis in SF-21 cell line by conditioned medium of the entomopathogenic fungus, *Nomuraea rileyi*, Through Sf-Caspase-1Signaling Pathway. Arch. Insect Biochem. Physiol. 68, 206-214.

Turner, N. W., Subrahmanyam, S. and Piletsky, S. A. (2009) Analytical methods for determination of mycotoxins: a review. Anal. Chim. Acta. 632, 168-180.

van't Riet, K. (1979) Review of measuring methods and results in non viscous gas–liquid mass transfer in stirred vessels. Ind. Eng. Chem. Process. Des. Dev. 18, 357-364.

Varki, A. (1993) Biological roles of oligosaccharides: all of the theories are correct. Glycobiology. 3, 97-130.

Vega, F. E., Goettel, M. S., Blackwell, M., Chandler, D., Jackson, M. A., Keller, S., Koike, M., Maniania, N. K., Monzon, A., Ownley, B. H., Pell, J. K., Rangel, D. E. N. and Roy, H. E. (2009) Fungal entomopathogens: new insights on their ecology. Fungal Ecol. 2, 149-159.

Vehovec, T. and Obreza, A. (2010) Review of operating principle and applications of the charged aerosol detector. J. Chromatogr. A. 1217, 1549-1556.

Vey, A., Hoagland, R. and Butt, T. M. (2001) Toxic metabolites of fungal biocontrol agents: In Fungi as Biocontrol Agents: Potential, Progress and Problems (Butt, T.M., Jackson, C.W. and Magan, N., Eds.), CAB International, Wallingford. pp. 311-345.

Vey, A., Matha, V. and Dumas, C. (2002) Effects of the peptide mycotoxin destruxin E on insect haemocytes and on dynamics and efficiency of the multicellular immune reaction. J. Invert. Pathol, 80, 177-187.

Vilaca, P. R., Badino, A. C. Jr, Facciotti, M. C. R. and Schmidell, W. (2000) Determination of power consumption and volumetric oxygen transfer coefficient in bioreactors. Bioprocess Eng. 22, 261-265.

Vilcinskas, A., Matha, V. and Gotz, P (1997) Effects of the entomopathogenic fungus *Metarhizium anisopliae* and its secondary metabolites on morphology and cytoskeleton of plasmatocytes isolated from the greater wax moth, *Galleria mellonella*. J. Insect Physiol. 43, 1149-1159.

Vilcinskas, A. and Gotz, P. (1999) Parasitic fungi and their interactions with the insect immune system. Adv. Parasitol. 43, 267-313.

Vishwanatha, K. S., Rao, A. G. A. and Singh, S. A. (2010) Acid protease production by solid state fermentation using *Aspergillus oryzae* MTCC 5341: optimization of process parameters. J. Ind. Microbiol. Biotechnol. 37, 129-138.

Wang, D. I. C., Cooney, C. L., Demain, A. L., Dunnill, P., Humphrey, A. E. and Lilly, M. D. (1979) Aeration and agitation. In: Fermentation and Enzyme Technology. John Wiley & sons; New York. Chapter 9, pp. 157-193.

Wang, S.W., Li, Y.R., Sun, J.Y., Xie, Y.H., Wang, S.H. and Zhao, Y.P. (2003) Inhibition effect of swainsonine to carcinoma. J. Med. Coll. PLA. 18: 191-192.

Wang, C. S., Skrobek, A. and Butt, T. M. (2004) Investigations on the destruxin production of the entomopathogenic fungus *Metarhizium anisopliae* in liquid and solid media. J. Invertebr. Pathol, 85, 168-174.

Wang, J., Hu, X. J., Tu, Y. and Ni, K. Y. (2006) Determination of spectinomycin hydrochloride and its related substances by HPLC-ELSD and HPLCMSn. J. Chromatogr. B. 834, 178-182.

Wang, C. and St. Leger R. J. (2007) A scorpion neurotoxin increases the potency of a fungal insecticide, Nat. Biotechnol. 25, 1455-1456.

Wang, X. P., Chen, T. S, Sun, L., Cai, J. Y., Wu, M. Q. and Mok, M. (2008) Live morphological analysis of taxol-induced cytoplasmic vacuoliazation in human lung adenocarcinoma cells. Micron. 39**,** 1216-1221.

Wang, Y., Hu, Y., Wang, J., Liu, Z., Yang, G. and Geng, G. (2011) Ultrasound-assisted solvent extraction of swainsonine from *Oxytropis ochrocephala* Bunge. J Medicinal Plants Res. 5, 890-894.

Watson, A. A., Fleet, G. W. J., Asano, N., Molyneux, R. J. and Nash, R. J. (2001) Polyhydroxylated alkaloids-natural occurrence and therapeutic applications. Phytochem., 56, 265-295.

Wessel, D. and Flügge, U. I. (1984) A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal. Biochem. 138, 141-143.

Weuster-Botz, D. (2000) Experimental design for fermentation media development: Statistical design of global random search? J. Biosci. Bioeng. v. 90, 473-483.

Whitley, D. (1988) Applying Genetic Algorithms to Neural Network Problems International Neural Network Society. pp. 230.

Wrodnigg, T. M., Steiner, A. J. and Ueberbacher, B. J. (2008) Natural and synthetic iminosugars as carbohydrate processing enzyme inhibitors for cancer therapy. Anti-Cancer Agents Med. Chem. 8, 77-85.

Xu, Y., Orozco, R., Wijeratne, E. M. K., Gunatilaka, A. A. L., Stock, S. P. and Molnar, I. (2008) Biosynthesis of the cyclooligomer depsipeptide beauvericin, a virulence factor of the entomopathogenic fungus *Beauveria bassiana*. Chem. Biol. 15, 898-907.

Xu, Y., Orozco, R., Kithsiri Wijeratne, E. M., Espinosa-Artiles, P., Leslie Gunatilaka, A. A., Patricia Stock, S. and Molnar, I. (2009) Biosynthesis of the cyclooligomer depsipeptide bassianolide, an insecticidal virulence factor of *Beauveria bassiana*. Fungal Genet. Biol. 46, 353-364.

Yang, G. D., Gao, R., Wang, Y., Li, J. C., Hu, Y. C., Kang, D. J., Li, Y. H., Li, H. L., Geng, G. X. and Wang, J. H. (2012) Determination of swainsonine in the endophytic *Undifilum* fungi by high-performance liquid chromatography with evaporative light-scattering detector. Toxicon. 60, 44-49.

Yoon, Y. and Kim, Y. H. (2012) The roles of crossover and mutation in real encoded genetic algorithms. In book: Bio-Inspired Computational Algorithms and Their Applications. Shangce Gao (ed.). In. Tech. ISBN: 978-953-51-0214-4.

Young, E., Lee, S.M. and Alper, H. (2010) Optimizing pentose utilization in yeast: the need for novel tools and approaches. Biotechnol. biofuels. 3, 24-36.

Zafar, M., Shashi, K., Kumar, S. and Dhiman, A. K. (2012) Optimization of polyhydroxybutyrate (PHB) production by *Azohydromonas lata* MTCC 2311 by using genetic algorithm based on artificial neural network and response surface methodology. Biocatal. Agr. Biotechnol. 1, 70-79.

Zhang, J., Lin, J. and Anderson, T. A. (2004) A flow injection analysis/mass spectrometry method for the quantification of polyethylene glycol 300 in drug formulations. Int. J. Pharm. 282, 183-187.

Zhang, Y., Jingliang, Xu., Yuan, Z., Xu, H. and Yu, Q. (2010) Artificial neural network-genetic algorithm based optimization for the immobilization of cellulose on the smart polymer Eudragit L-100. Bioresour. Technol. 101, 3153-3158.

Zhao, B. Y., Liu, Z. Y., Wang, J. J., Sun, L. S., Wang, Z. X. and Wang, Y. C. (2009) Isolation and NMR Study on Swainsonine from Locoweed, *Astragalus strictus*. Agric. Sci. (China). 8, 115-120.

Zmak, P. M., Podgornik, A., Podgornik, H. and Koloini, T. (2006) Impact of pellet size on growth and lignin peroxidase activity of *Phanerochaete chrysosporium*. World J. Microb. Biotechnol. 22, 1243-1249.

Zuzek, M., Friedrich, J., Cestnik, B., Karalic, A. and Cimerman, A. (1996) Optimization of fermentation medium by a modified method of genetic algorithms. Biotechnol. Tech. 10, 991-996.

List of Abbreviations

BCA	Biological Control Agents
IPM	Integrated Pest Management
ARSEF	Agriculture Research Service Collection of Entomopathogenic Fungi
USDA	United States Department of Agriculture
SDY/SDA	Sabouraud Dextrose Yeast Extract / Sabouraud Dextrose Agar
CZ	Czapek Dox
PDA	Potato Dextrose Agar
OFAT	One Factor At a Time
PB	Plackett-Burman
DOE	Design of Experiment
EVOP	Evolutionary operations
RSM	Response Surface Methodology
CCD	Central Composite Design
ANOVA	Analysis of Variance
ANN	Artificial Neural Networks
FFNN	Feed Forward Neural Network
GA	Genetic Algorithm
ANN-REGA	Artificial Neural networks-Real Encoded Genetic Algorithm
EA	Evolutionary Algorithm
EP	Evolutionary Programming
TLC	Thin Layer Chromatography
LC	Liquid Chromatography
HPLC	High Performance Liquid Chromatography
LC-MS	Liquid Chromatography-Mass Spectrometry
GC	Gas Chromaatography
GC-MS	Gas Chromatography-Mass Sperometry
HPIC	High Performance Ion Exchange
mUV	Middle Ultra Violet
DAD	Dioade Arrar Detector
ELSD	Evaporative Light Scattering Detector
TMS	Trimethylsilane
TFA	Trifluoroacetic acid
Fr	Flow rate
MS	Mass Spctrometry
MS-ESI+	Mass Spectrometry-Electrospray Ionization in positive ion mode
SIM	Selected Ion Monitoring
FIMS	Flow Injection Mass Spectrometry
LC-MSD	Liquid Chromatography-Mass Derived
MTT	3-(4, 5-dimethylthia-zol-2-yl)-2, 5-diphenyl tetrazolium bromide
TB	Tryphan Blue
LCB	Lactophenol Cotton Blue
PI	Propidium Iodide
Annexin V-FITC	Annexin V-fluorescein isothiocyanate
FACS	Fluorescence Activated Cell Sorting
FE-SEM	Field Emission-Scanning Electron Micrscopy
AFM	Atomic Force Microscopy
LC	Lethal concentration
CC	Cytotoxic Concentration
RMSE	Root Mean Squared Error

Mathematical Symbols and Greek Letters

Mathematical Symbols	
Fr	Flow rate for mobile phases in HPLC (ml/min)
lr	Learning rate for artificial neural networks
C_L	Dissolved oxygen concentration (mol/m^3)
C_L^*	dissolved oxygen concentration with mean gaseous oxygen concentration (mol/m^3)
N	Agitation rate (rpm)
Q	Aeration rate (vvm)
Pg	Gassed power requirement (W)
Pg/V_L	Gassed power required per unit volume (W/m^3)
K_La	Volumetric oxygen mass transfer coefficient (s^{-1})
F_g	Gas flow rate (m^3/s)
V_g	Gas superficial velocity (m/s)
*W*i	Impeller blade width (m)
Ni	Rotations per second (rps)
g	Acceleration due to gravity (m/s^2)
Di	Impellers diameter (m)
Dt	Tank diameter (m)
Wi	Width of impeller's blade (m)
X	Biomass concentration (g/l)
$QO_2.X$	OUR-Oxygen uptake rate (mg/l-h)

Greek Letters	
τ_{av}	Average shear stress (N/m^2)
γ_{av}	Average shear rate (s^{-1})
μ_a	apparent viscosity according to Ostwald de-Waele model (Pa.s)
μ_g	Gas viscosity (Pa.s)
η_{eff}	Eddy length (m)
ρ	Density (Kg/m^3)

Manuscripts published

Digar Singh and Gurvinder Kaur **(2014)** The antileukemic cell cycle regulatory activities of swainsonine purified from *Metarhizium anisopliae* fermentation broth. ***Natural Product Research*** **(Accepted)**.

Digar Singh and Gurvinder Kaur (**2014)** Swainsonine, a novel fungal metabolite: optimization of fermentative production and bioreactor operations using evolutionary programming. ***Bioprocess and Biosystems Engineering,*** **DOI 10.1007/s00449-014-1132-6.**

Digar Singh and Gurvinder Kaur **(2013)** Preparative cum quantitative mass directed analysis of swainsonine and its *in situ* activity against *Sf*-21 cell line. ***FEMS Microbiology Letters***, 347, 7-13.

Digar Singh and Gurvinder Kaur (**2013)** Real encoded genetic algorithm and response surface methodology to optimize production of an indolizidine alkaloid, swainsonine from *Metarhizium anisopliae*. ***Folia Microbiologica,*** 58, 393-401.

Digar Singh and Gurvinder Kaur (**2012)** Optimization of different process variables for the production of an indolizidine alkaloid, swainsonine from *Metarhizium anisopliae*. ***Journal of Basic Microbiology,*** 52, 590-7.

Digar Singh, Anita Sharma and Gurvinder Kaur (**2013)** Biochemical and molecular characterization of the bacterial endophytes from native sugarcane varieties of Himalayan region. ***3 Biotech,*** 3, 205-212.

Manuscripts under review

Digar Singh and Gurvinder Kaur (**2014)** Production, HPLC analysis and *in situ* apoptotic activities of swainsonine in lepidopteran, *Sf*-21 cell line. ***Biotechnology Progress*** (Revision submitted).

Conferences

1. **Digar Singh** and Gurvinder Kaur **(2013)**. Mass directed purification, quantification and *in vitro* cytotoxicity of an indolizidine alkaloid, Swainsonine from *Metarhizium anisopliae*. **5th Congress of European Microbiologists** (**FEMS-2013), Leipzig, Germany,** July 21-25, 2013.

2. **Digar Singh** and Gurvinder Kaur **(2012)** Swainsonine: production optimization and modeling, HPLC quantification and *in vitro* cell cycle regulatory activities in HL60 cell line. ICEHT-2012, **6th Annual convention of association of biotechnology and pharmacy (ABAP)**, SVU university, **Tirupati, Andra-Pradesh, India**. December 20-22, 2012.

3. **Digar Singh** and Gurvinder Kaur **(2011)** Media optimization and culture conditions for the enhanced production of Swainsonine from *Metarhizium anisopliae*. 51st Annual conference of **Association of Microbiologists of India (AMI),** BIT Mesra, **Ranchi, Jharkhand, India**. December 14-17, 2010.

Awards and Fellowships

1. Qualified **CSIR-(NET)-JRF** in (Dec-2009) a national level examination for research fellowship and lectureship with **AIR-249**, conducted by Ministry of Human Resource and Development, Govt. of India.

2. Qualified Graduate Aptitude Test Examination **(GATE) 2009** in **Life Sciences** with **95.5** percentile, conducted by Ministry of Human Resource and Development, Govt. of India.

3. **Best poster award** at ICEHT-2012, 6th annual Convention of Association of Biotechnology and Pharmacy. SVU University, Tirupati, Andhra Pradesh, India. December 20-22, 2012.

4. Awarded **"Travel fellowship under DBT-CTEP"** for attending 5th congress of European Microbiologists (FEMS-2013), Leipzig, Germany**,** July 21-25, 2013, by the **Department of Biotechnology**, Govt. of India.

VITAE

The author was born on January 5, 1986 in Pithoragarh district of Uttarakhand state (then UP), India. He passed the Higher and Senior Secondary Examinations conducted by the Central Board of Secondary Education, from Kendriya Vidyalaya Ranikhet, Almora, in the year 2001-2003. He completed B.Sc. with Chemistry, Botany and Zoology as major subjects from Govt. PG College Ranikhet, Kumaun University, Nainital in 2006. He did his M.Sc. in Microbiology from GB Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India in 2009.

Mr. Digar Singh joined the Ph.D. programme at the Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati, India, in the year July 2009. He received the Junior and Senior Research fellowships (JRF & SRF) from the Council of Scientific and Industrial Research (CSIR), New Delhi, India. He successfully completed the Ph.D. course work with 8.17/10 Cumulative Point Index (CPI). He gave the open Seminar (PhD Synopsis) on October 9, 2013 and presented his thesis work satisfactorily before the Doctoral Committee. He submitted the PhD thesis in December 2013.