**FERMENTATION, BIOSYNTHESIS, AND
IDENTIFICATION OF SECONDARY METABOLITES
FROM *PENICILLIUM* SPECIES**

Mark William Sumarah, B.Sc.

A thesis submitted to the Faculty of Graduate Studies in partial
fulfilment of the requirements for a degree of

Master of Science

Department of Chemistry

Carleton University

December 2002

National Library
of Canada

Acquisitions and
Bibliographic Services

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale
du Canada

Acquisitions et
services bibliographiques

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-79819-4

Canada

The undersigned hereby recommend to the Faculty of Graduate Studies the acceptance of this thesis submitted by Mark William Sumarah entitled:

Fermentation, Biosynthesis, and Identification of Secondary Metabolites from *Penicillium* Species

in partial fulfilment of the requirements for the degree of Master of Science.

Thesis Supervisor

Chairman of the Chemistry Department

I. ABSTRACT

The closely related fungi *Penicillium roqueforti* and *P. crustosum* occur on poorly ensiled feed. These species are known to produce a number of secondary metabolites including roquefortine and penitrem. In cooler dairy production areas, including Scandinavia, Western Europe, and North America cattle toxicosis have been associated with silage feed contaminated by these fungi. An attempt has been made to understand the fermentation conditions necessary to produce these metabolites. Studies of the different fermentation conditions have shown that the best yields were obtained on media of high osmotic tension, high sucrose content, and 20 - 30% O₂ saturation, while the form of nitrogen was not important. The maximum production rate of roquefortine and other metabolites occurred at day 6 and reached a maximum of 20mg/ L of filtrate at day 12. HPLC analysis revealed the presence of roquefortine, penitrem A and substantial amounts of unknowns in both the culture filtrate and mycelia. Specifically labelled ¹³C-sodium acetate was added to cultures to both resolve discrepancies in the biosynthesis of roquefortine and to produce enriched metabolites. Due to the sensitivity of the metabolites produced to UV light and acidic conditions, a new extraction technique and analytical method combining HPLC, LC/MS/MS, and LC/NMR was developed for the identification of major and minor metabolites.

ACKNOWLEDGEMENTS

I would first like to thank NSERC for their generous funding, which made this project possible.

I would like to thank my supervisor, Dr. J. David Miller (a fellow Maritimer) for many things; for giving me the opportunity to work in an excellent environment to do research, for generous funding throughout my degree, for his patience and help over the last few years and for teaching me a great deal about science.

I would especially like to thank Dr. Barbara Blackwell, Agriculture and Agri-Food Canada, first for her NMR expertise, and for analysing many samples. Additionally, I would like to thank her for her continued help with the project, for taking on the job of being a second supervisor and for always providing a good horse story.

Furthermore, I would also like to thank all the members of our laboratory group for their daily help, support and for providing an excellent environment to work in. Special thanks to Mark and Regina who have been with me since the start, have had a large impact on the success of the project, and who make working in our lab fun.

I would like to thank Dr. Laird Trimble, Merck Frosst Canada, for his expertise, assistance with the LC-NMR, for inviting me to Montreal to use his very expensive instruments, and letting me stay at his house (and eat all of his ice cream).

I would like to thank Dr. Paul Mayer, University of Ottawa for his assistance with the LC-MS part of the project, and for the use of his LC-MS.

I would also like to thank John Nikiforuk, Agriculture and Agri-Food Canada, for his assistance with the NMR and LC-MS analysis.

Finally, I would like to thank my family and Jennifer who understand very little of what I do but none the less continue to provide me with a great deal of support.

TABLE OF CONTENTS

I. Abstract	iii
Acknowledgements	iv
Table of Contents	v-vii
List of Figures	viii-x
List of Tables	xi
 II. Introduction	 1
II-A Fungal growth on silage and cattle toxicosis	1
II-B Fungi	4
II-C Fungal metabolism	6
II-C.1 Classes of secondary metabolites	9
II-D Fungal growth and secondary metabolism	10
II-D.1 Laboratory growth and regulation of secondary metabolism	11
II-E Expected fungal metabolites	12
II-E.1-1 Roquefortine	13
II-E.1-2 Toxicity	14
II-E.2-1 PR Toxin	16
II-E.2-2 Toxicity	17
II-E.3-1 Mycophenolic acid	18
II-E.3-2 Toxicity	19
II-E.4-1 Penitrem A	19
II-E.4-2 Toxicity	21
II-F Analysis of fungal metabolites	22
 III. Project Aim	 23
 IV. Experimental	 24
IV-A Fungal strains	24
IV-B Growth, extraction and analysis from shake flasks	24
IV-B.1 Extraction of shake flasks	25
IV-B.2 Analysis by thin layer chromatography (TLC)	26
IV-B.3 Modifications to the extraction procedure	28
IV-B.4 Roquefortine recovery and sensitivity to light tests	29
IV-C Growth and analysis from Roux bottles	29
IV-C.1 Growth	29

TABLE OF CONTENTS cont.

IV-C.2 New HPLC analysis for all mycotoxins	30
IV-D Maximizing roquefortine and other metabolite production	31
IV-D.1 Water tension – Glycerol	31
IV-D.2 Phosphorus content of media	31
IV-E Testing of all 4 strains in Roux bottles	32
IV-F Time study	32
IV-G Sugar analysis	32
IV-H Nitrogen analysis	33
IV-I Oxygen analysis	34
IV-J Final modifications to HPLC method	34
IV-K Isolation of major metabolites	35
IV-L Analysis of different media	35
IV-M Structure, ^{13}C Enrichment and Biosynthesis	36
IV-N LC/MS, LC/MS/MS, and LC/NMR	37
IV-O Analysis of <i>Penicillium</i> strains isolated from contaminated silage	39
V. Results	40
V-A Growth, extraction and analysis from shake flasks	40
V-A.1 Extraction method	40
V-A.2 Glycerol test- water activity	41
V-A.3 Phosphorus content	42
V-A.4 Growth and metabolite production on different media	42
V-B Growth of <i>P. crustosum</i> DAOM 215343 in Roux bottles	44
V-B.1 Sugar analysis	45
V-B.2 Nitrogen analysis	45
V-B.3 mycelia weight and roquefortine production	45
V-B.4 pH and dissolved oxygen analysis	46
V-C Analysis of different <i>Penicillium</i> strains for metabolite profiles	46

TABLE OF CONTENTS cont.

V-D Identification, biosynthesis and ^{13}C labelling of major metabolites	48
V-D.1 Roquefortine	48
V-D.2 Large unknown from filtrate – cyclopenin	62
V-D.3 Penitrem A	70
V-E Identification of minor metabolites	75
V-E.1 Roquefortine D	77
V-E.2 16-Hydroxyroquefortine	79
V-E.3 Penitrem B-F	79
V-E.4 Cyclopenol, Viridicatol, and Viridicatin	79
V-E.5 Unknown metabolites	80
VI. Discussion	82
VI-A Extraction and analysis	82
VI-B Growth conditions	83
VI-C Identification, biosynthesis and ^{13}C labelling of major metabolites	86
VI-D Identification of minor metabolites	90
Summary	95
Future Work	97
References	98

LIST OF FIGURES

Figure	Title	Page
1	<i>Penicillium roqueforti</i> and <i>P. crustosum</i> growing on corn and grass isolated from a farm in Quebec. (Silage showed no visible signs of mold but was producing a bad odour and cattle showed signs of distress.	1
2	Isolated strains of <i>Penicillium roqueforti</i> and <i>P. crustosum</i> (malt extract agar).	5
3	<i>Penicillium roqueforti</i> a viewed under a microscope.	6
4	Structure of roquefortine.	13
5	Structure of PR toxin.	16
6	Structure of mycophenolic acid.	18
7	Structure of penitrems A-F	20
8	Growth of <i>P. crustosum</i> in Roux bottles.	30
9	Diagram showing Varian LC-NMR system used for experiment.	39
10	Total analysis of <i>P. crustosum</i> (DAOM 215343) grown in Roux bottles	44
11	HPLC chromatogram for mycelia extract of <i>P. crustosum</i> DAOM 215343 indicating major metabolites.	47
12	HPLC chromatogram for filtrate extract of <i>P. crustosum</i> DAOM 215343 indicating major metabolites.	48
13	MS/MS of roquefortine indicating parent (390 M+1) and daughter ions.	49
14	¹ H NMR spectrum of roquefortine in CDCl ₃ .	50
15	¹³ C NMR spectrum of roquefortine in CDCl ₃ .	50
16	Gradient DQF-COSY spectrum of roquefortine.	51
17	Gradient HSQC spectrum of roquefortine.	52
18	Gradient HMBC spectrum of roquefortine.	53
19	¹⁵ N/ ¹ H correlation spectra for roquefortine (HSQC).	54
20	¹⁵ N/ ¹ H correlation spectra for roquefortine (HMBC).	54
21	NOESY spectrum of roquefortine.	55

LIST OF FIGURES (CONT.)

Figure	Title	Page
22	Structure of roquefortine with stereochemistry and proper numbering.	56
23	^{13}C NMR spectra of crude filtrate extract labelled with ^{13}C -1 acetate in CDCl_3 .	58
24	^{13}C NMR spectra of crude filtrate extract labelled with ^{13}C -2 acetate in CDCl_3 .	58
25	^{13}C NMR spectra of crude mycelia extract labelled with ^{13}C -1 acetate in CDCl_3 .	59
26	^{13}C NMR spectra of crude mycelia extract labelled with ^{13}C -2 acetate in CDCl_3 .	59
27	3-7 day ^{13}C -1 enriched roquefortine NMR spectrum (mycelia).	61
28	3-7 day ^{13}C -2 enriched roquefortine NMR spectrum (mycelia).	61
29	Incorporation pattern of ^{13}C -1 (★) and ^{13}C -2 (●) acetate into roquefortine.	62
30	MS/MS of cyclopenin indicating the parent ion (295 M+1) and daughter ions.	63
31	^1H NMR spectrum for cyclopenin.	64
32	^{13}C NMR spectrum for cyclopenin.	64
33	COSY spectrum of cyclopenin (LC/NMR stopped flow).	65
34	gHSQC for cyclopenin in CDCl_3 .	66
35	gHMBC for cyclopenin in CDCl_3 .	66
36	Structure of cyclopenin indicating stereochemistry and numbering pattern.	67
37	Day 3-7 ^{13}C -1 NMR spectrum from isolated cyclopenin (filtrate).	68
38	Day 3-7 ^{13}C -2 NMR spectrum from isolated cyclopenin (filtrate).	68
39	Incorporation pattern of ^{13}C -1 (★) and ^{13}C -2 (●) acetate into cyclopenin.	69
40	MS/MS spectrum of penitrem A indicating parent (634) and daughter ions.	70

LIST OF FIGURES (CONT.)

Figure	Title	Page
41	^1H spectrum of penitrem A in CDCl_3 .	71
42	^{13}C spectrum of penitrem A in CDCl_3 .	71
43	Structure of penitrem A indicating stereochemistry and number pattern.	72
44	Day 3-7 ^{13}C -1 NMR spectra for isolated penitrem A (mycelia).	74
45	Day 3-7 ^{13}C -2 NMR spectra for isolated penitrem A (mycelia).	74
46	Incorporation pattern of ^{13}C -1 (★) and ^{13}C -2 (●) acetate into penitrem A.	75
47	HPLC chromatogram of filtrate with cyclopenin and roquefortine removed.	76
48	HPLC chromatogram of mycelia with roquefortine removed.	76
49	MS/MS spectrum of roquefortine D indicating parent (392) and major daughter ions.	77
50	^1H NMR spectrum of roquefortine D from LC-NMR experiment (64 scans).	78
51	Structure of roquefortine D	78
52	Structure of a) cyclopenol, b) viridicatol, and c) viridicatin	80

LIST OF TABLES

Table	Title	Page
1	Initial HPLC conditions used for all experiments	31
2	Final HPLC conditions used for all experiments	35
3	Comparison of extraction solvents and pH conditions for roquefortine.	41
4	Test of Czapek-Dox medium containing variable amounts of glycerol.	41
5	Impact on roquefortine production, of reducing phosphorus content of media.	42
6	Roquefortine and cyclopenin production when grown on different media.	43
7	^1H , ^{13}C , and ^{15}N NMR Chemical Shift Assignments for Roquefortine	57
8	^1H and ^{13}C NMR Assignments for Cyclopenin	67
9	^1H and ^{13}C NMR Assignments for Penitrem A	73
10	Metabolites identified from each <i>Penicillium</i> strain tested	81

II. INTRODUCTION

II-A Fungal growth on silage and cattle toxicosis

In developed areas such as Eastern North America, Europe and Japan farm animals are either allowed to eat forage crops or are fed grass, maize, straw or hay from a stored source such as a silo. In both instances, fungal contamination is a major problem not only for spoilage of the feed (reduction in nutritional value), but because these fungi produce toxic metabolites that are potentially harmful to the animals (Scudamore KA and Livesey CT, 1998; Mills JT et al., 1995). Forage crops are potentially affected by completely different species of fungi than feed that has been stored in a silo. The fungal species associated with forage crops include *Fusarium*, *Alternaria*, *Cladosporium*, and *Claviceps*. Silage, on the other hand, is more likely to be contaminated with fungi from the genus *Aspergillus* or *Penicillium*, the latter of which will be the focus of this study.

Figure 1: *Penicillium roqueforti* and *P. crustosum* growing on contaminated animal feed isolated from a farm in Quebec. The silage showed no visible signs of mold, but was producing a bad smelling odor and also the cattle feeding on the grass and corn showed many signs of distress including reproduction problems, internal bleeding, and death.

In areas where silage is an important component of cattle feed, a number of phenomena have been observed. Cases have been reported involving animals that have suffered paralysis, abortions, internal bleeding and even death. Lesser symptoms including ill-thrift, mastitis and increased bacterial and parasite diseases have also been reported (Häggbloom P, 1990). All cases were associated with *Penicillium roqueforti* or *P. crustosum* and the metabolites they produce that were isolated from the feed, rather than low amounts of deoxynivalenol, zearalenone or fumonisin, which are commonly found in North American corn (Seglar B, 1997 and 1999). *P. roqueforti*, has been shown to grow in acidic environments and under very low oxygen tension levels (Boysen ME et al., 2000). These *Penicillium* species produce a variety of secondary metabolites, including roquefortine (roquefortine C), PR toxin, penitrem A and mycophenolic acid (Häggbloom P, 1990 and Roberts BA and Patterson DS, 1975). Studies from around the world have tested silage using the presence of one or more of these particular toxins as an indicator of fungal contamination and quality of the silage. Schneweis et al. (2000) used mycophenolic acid, a common immunosuppressant metabolite produced by *P. roqueforti*. PR toxin and roquefortine have also been used as indicators of fungal contamination (Nout MJR et al, 1993). Auerbach et al. (1998) showed that 89% of the visibly moldy silage in Germany contained *P. roqueforti* of which, 88% were found to be contaminated with roquefortine. Additionally, they found that 85% of the silage samples without visible mold were contaminated with *P. roqueforti*, of which 25% tested positive for the presence of roquefortine. None of the compounds listed above appear sufficiently toxic on their own to explain the observed symptoms, although the toxicology is far from complete.

When stored properly, silage should not show any signs of fungal contamination, and as a result will not cause any problems to the animals. Silage needs to be reasonably dry when it is stored as moisture levels above 15% will result in fungal growth (Scudamore KA and Livesey CT, 1998). Additionally, the use of organic acids produced by lactic acid bacteria along with low oxygen tension prevents the growth of spoilage molds (Scudamore KA and Livesey CT, 1998). Problems occur when these optimal conditions are not achieved or maintained throughout the entire storage phase. As feed is removed, the conditions inside the silo are constantly changing and even feed that was once properly stored can become contaminated.

In addition to the use of silage for animal feed, the use of recycled feed in the form of food waste has become popular in recent years. The interest in using recycled feed is twofold: first it provides a reduction in overall waste by utilizing materials that would normally end up in landfills, and secondly it provides a less expensive food source to feed farm animals. The use of food waste from households, commercial food outlets and also the food industry was examined. The results of the initial testing showed that household waste was contaminated with mold and mycotoxins and was unsuitable for animal feed. However the use of recycled feed such as bread from industrial sources was declared safe and continues to be used as an animal feed source. A study in the United States tested 1024 fungal isolates from recycled animal feed. All of the species identified belong to either the genus *Aspergillus* or *Penicillium*, with the latter dominating. The wide variety of species found, and especially *Penicillium* species is of interest because; as stated above, they are not the normal field fungal species associated with the feed and are reliable indicators of feed contamination, which has been linked with health problems in

humans and animals. Included in the different *Penicillium* species isolated were *P. crustosum* and *P. roqueforti*, the species of interest for the present study.

II-B Fungi

Fungi form one of the largest groups of organism that includes 70000-10000 described species, with an estimated 1.5 million in total (Hawksworth DL, 1991). This kingdom includes four subdivisions; the Ascomycetes, Basidiomycetes, Chitrids and the Zygomycetes which are mostly saprophytes that live in soil and water. The last group includes the molds, which can be parasites of animals, plants and even other fungi and are of particular interest to the present work (Levine KR and Miller JS, 1991).

In general, fungi are very difficult to classify. Identifications are normally made by comparing morphological features, which vary with the culture technique used to prepare them (Söderström B and Frisvad JC, 1984). This had led to a great deal of work in the development of new methods for the identification of fungi. Methods currently being used and tested include the use of gene sequences, metabolite profiles and even lipid profiles (Boysen ME et al., 2000; Dörge T et al., 2000). These advances have resulted in the separation of some species into new classes of fungi, which can not be differentiated by even the best mycologists. *P. roqueforti* was one of the species recently reclassified into three new sub-species, though the majority of these are still identified as the original *P. roqueforti* (Boysen ME et al., 1996 and 2000).

Penicillium roqueforti is a common saprophytic fungus that was originally identified by the American mycologist Charles Thom in 1906. It is widespread in nature and can be isolated from soil, decaying organic matter and plants. It is traditionally

identified in pure culture by its broad spreading colonies that grow to the edges of the plate (5-6 cm) in approximately 10 days at 25°C on Czapek medium. The colonies are characterized by heavy sporulation, a dark green centre surrounded by a thin white margin, a velvety appearance, and a greenish – blue underside (Figure 2). (Gravesen S et al., 1994).

P. roqueforti is the principal species used in the production of Roquefort cheese, and for this reason it has received a fair amount of attention. A number of studies have focused on this fungus because of its ability to produce a wide variety of fungal metabolites. The majority of the work was done in the late 1970's and early 1980's and was then more or less forgotten. The results of the extensive studies and a report by the Environmental Protection Agency (EPA) on *P. roqueforti* and its presence in cheese has led to the following conclusions: The majority of the secondary metabolites produced are unstable in cheese. The cheese producers are using strains that are classified as poor producers of toxins, and that the average person does not eat enough cheese to consume a harmful amount of toxins (Finoli C et al., 2001 and EPA, 1997).

Figure 2: *P. roqueforti* (left) and *P. crustosum* (right) isolates from contaminated silage.

Figure 3: *P. roqueforti* as viewed under a microscope (Norwegian fungus of the month).

Penicillium crustosum was first described in 1930 and was believed to be uncommon until the last two decades. It has now been recognized as a very common contaminate of food and animal feed (Pitt JI, 1979 and 2002). Research on strains of *P. crustosum* has also gained popularity because of its recognition as the major producer of penitrem A, a potent tremorgenic mycotoxin. *P. crustosum* is recognized by its rapid growth on both malt extract and CYA media. The fungus produces dull green colonies with a granular texture, and is also distinguished by the large number of conidia it produces (Raper KB and Thom C, 1949).

II-C Fungal metabolism

Secondary metabolism in fungi is an idea that has received much debate over the past four decades. This issue was best described by Demain (1989) when he stated that “It has always amazed me that the importance of secondary metabolites in ecological interactions between plant versus herbivore, insect versus insect and plant versus plant have been universally accepted, but their importance in microbial interactions has been

almost universally denied". The use of the term "secondary metabolism" indicates that fungal metabolism can be divided into both primary and secondary.

Primary metabolism is present in all organisms and is essential for growth and life. Primary metabolism has been described as the sum of an interrelated series of enzyme-catalyzed chemical reactions that include processes such as glycolysis, Krebs cycle, amino and nucleic acid production, and so on (Betina V, 1995). The intermediates and end products of these processes are called primary metabolites. These primary metabolites are then used by secondary metabolism in the production of secondary metabolites or compounds specific to the organism producing them.

Secondary metabolism is closely linked to primary metabolism and occurs in a wide variety of fungi and plants. Secondary metabolism differs from primary metabolism in fungi because it only occurs after a nutrient limitation, and only if the fungal cell possess the correct genes (Miller JD and Greenhalgh R, 1991). The fungus will only produce secondary metabolites when the primary growth of the cell is limited by the lack of an essential nutrient such as its carbon source (carbohydrates), nitrogen, or even phosphorus.

The reason for the production of these metabolites as stated above has been greatly debated. It was first believed that they were simply waste products of the cell. Others proposed the idea that they served no purpose and were only produced more or less for fun. It is also accepted that they are not essential for growth, but they may play other roles such as regulators in the cell. Currently the most widely accepted view is that they are used for defense and have been labeled with the term "interference competition" (Janzen D, 1977 and Wicklow DT, 1981). Fungi and plants use these often-potent

secondary metabolites as chemical warfare against other fungi, insects, animals and even bacteria.

Fungi live in established niches and function normally as long as nutrients are available. Problems occur when these niches overlap, which leads to competition for available nutrients. Competition can occur in a variety of ways, the first is through exploration competition, which involves an organism depleting the resource even though it does not reduce the probability that others will compete for the remaining resources. Interference competition involves some form of behavioral or chemical interaction between the organisms before the resource is used (Wicklow DT, 1981). These interactions among individuals influence the access of the competitor to the resource, thereby reducing the impact on the limited resource and increasing its own probability of survival. The general mechanism of interference in fungi is antibiosis, and involves the use of toxic metabolites or antibiotics. Additionally, fungi colonize resources such as fruit, making the resource unattractive to animals.

Fungal antagonism is a very common method of limiting competitor access to a resource. Early colonizers gain a foothold on the resources and then through the use of antibiotics they can slow and even prevent invasion by others. Fungal antagonism is limited to close-ranged encounters. Additionally, marine fungi have been shown to produce anti-fungal compounds with low water solubility. This idea is important because at first thought it is not logical because they would simply leach out into the environment as they are produced (Miller JD et al., 1985 and Strongman DB, et al., 1987).

II-C.1 Classes of secondary metabolites

Fungal secondary metabolites are classified into four main groups: polyketides, terpenes, shikimate acid derivatives and peptides. Polyketides are similar to fatty acids with the exceptions that they contain alternating CO – CH₂ groups versus just CH₂ groups for fatty acids and have greater synthetic capacity, as evident by the large variety produced. The biosynthesis of polyketides occurs when the acetate used in the production of fatty acids is combined with malonate. The resulting straight chain can undergo various reactions including oxidation, reduction, methylation and rearrangement to form a variety of different compounds. Polyketides can occur as toxins, immunosuppressants, antibiotics or antiparasites. Examples of important fungal polyketides include zearalenone and the aflatoxins (Blackwell BA et al., 1994 and Steyn PS, 1998).

Terpenes are the second major class of metabolites produced by fungi. They are closely associated with the formation of sterols in primary metabolism with both using mevalonic acid as the key intermediate in their formation. The most economically important fungal metabolites in this class are the trichothecenes (Steyn PS, 1998).

The shikimate pathway leads to the formation of the third major class of fungal metabolites. These metabolites are the result of shikimic acid being used for the formation of important primary metabolites such as tryptophan, tyrosine and phenylalanine. These compounds are then used to form secondary metabolites such as roquefortine, which is derived directly from tryptophan, histidine and dimethylallylpyrophosphate (Mantle PG et al., 1983 and Steyn PS, 1998).

The final class of fungal secondary metabolites are the peptides, these are the amino acid derived compounds. They can be formed from either a linear peptide, penicillin being a good example or cyclosporine which is a cyclic peptide.

II-D Fungal growth and secondary metabolism

Fungi undergo three main phases in their life cycle when grown under fermentation conditions. The first is growth phase, which has been defined for fungi as an increase in dry weight of the mycelium. During this stage, the fungal cells are provided optimal conditions for growth with careful attention to pH, temperature, oxygen content and nutrients (Miller JD and Blackwell BA, 1986). The conditions for optimal growth vary among fungal species but if provided the resulting growth stages are similar. The cells will rapidly increase in number during this stage, which corresponds to a rapid increase in mycelial dry weight and also with primary metabolism stage of the cells. If the optimal conditions could be provided indefinitely, the fungus could in theory continue in this stage forever.

The second stage occurs when one or more of the essential nutrients used for growth or primary metabolism are limited. Carbon, nitrogen and phosphorous are the most common limited resources (Wicklow DT, 1981). At this point the fungal cells slow down the growth phase, this is evident by a lack of increase in mycelia dry weight. The resulting restriction on growth leads to an accumulation of some primary metabolites. Secondary metabolism then begins by using these primary metabolites along with a series of enzymes to produce a wide variety of secondary metabolites.

The final stage involves the death of the cells, which leads to the release of nitrogen and other nutrients back into the medium for consumption by other cells. Growth conditions in a fermenter are unique because the cells are defined as “synchronous” meaning that all cells are more or less at the same stage at the same time. This is very different from still cultures or in nature where cells are at different stages at any given time.

II-D.1 Laboratory growth and regulation of secondary metabolism

Fungal strains that have been domesticated and grown under laboratory conditions often produce very different metabolite profiles than in nature. In order to achieve optimal metabolite production in the laboratory the conditions and factors that affect biosynthesis must be understood. Achieving this optimal level of production is very difficult, it has been said that if even one of the many conditions that affect growth and production of metabolites are wrong then the fermentation will probably fail. The major factors affecting a good fermentation include the physical characteristics such as the composition, pH and oxygen tension of the medium, the fungal strain being used, and its cellular metabolism.

The first major influence on the fermentation is the carbon source used in the medium. Carbon is very important because it provides the energy for all of the organism’s essential processes, and it is also needed for synthesis of proteins, lipids and nucleic acids. The source of carbon used in the medium can vary greatly and depends on the nature of the organism. Some fungi grow very rapidly and therefore may need a carbon source such as sucrose or maltose which is more difficult to process than glucose

or fructose. Other fungi may need an easier carbon source for quicker metabolism. Carbon is often the limiting factor in the fermentation, with no secondary metabolism until it has all been consumed by the organism. Both *P. crustosum* and *P. roqueforti* grow very well on Czapek-Dox medium, which contains sucrose as its carbon source.

Nitrogen is another important factor for metabolite production, especially for the biosynthesis of amino acids and metabolites derived from these primary metabolites. The nitrogen source in the media can be ammonia, nitrates or nitrites, and even amino acids. Nitrogen, like carbon, can also be the limiting factor in a good fermentation. The carbon to nitrogen (C/N) ratio of the medium used is also very important because it influence secondary metabolite production (Miller JD and Blackwell BA, 1986). The nitrogen sources in Czapek-Dox medium are sodium nitrate directly and small but significant nitrogen sources from the use of yeast extract in the medium. Experiments have shown that the growth and production of metabolites by *P. crustosum* and *roqueforti* are not greatly influenced by changing the nitrogen source used, and are unlikely to be the limiting factor.

Phosphorus is a key nutrient consumed by fungi for DNA, RNA and ATP. Phosphorus can be from a number of sources, but the most common form is PO_4^- .

Oxygen is an important factor for all fermentations because all filamentous fungi are aerobic. The oxygen tension in the growth medium affects the production of secondary metabolites. The fungi of interest for this study have the unusual property that they grow very well under reduced oxygen conditions.

Trace metals also play important roles in fungal growth, the present medium requires the addition of very small quantities of iron.

The pH of the medium is also very important for fungal growth and secondary metabolism. It has been shown for some species of fungi that a drop in pH will result in a complete stop of secondary metabolism (Blackwell BA et al., 1985). Additionally, many of the compounds produced by the species in the present study are very acid sensitive and require a neutral medium to retain their original form.

II-E Expected Fungal Metabolites

A search of the literature for both *P. roqueforti* and *P. crustosum* provided a list of possible mycotoxins that are produced by these species. This information was compared to case studies of toxicosis in cattle to determine which compounds should be receiving the focus of attention at the start of the project. The four main compounds were determined to be roquefortine, PR toxin, mycophenolic acid, and penitrem A (Frisvad JC and Filtenborg O, 1989; Müller HM, and Amend R, 1997; Frisvad JC and Samson RA, 1991).

II-E.1-1 Roquefortine:

Roquefortine (roquefortine C) is the major secondary metabolite produced by both *P. crustosum* and *P. roqueforti*. It has also been shown to be produced by other *Penicillium* species including *P. oxalicum*, *P. communi*, *P. corymbiferum*, *P. expansum* and *P. urticae*. Roquefortine is an alkaloid indole (tryptophan derived) mycotoxin that was independently discovered by both Scott and Kennedy (1976) from the mycelium of a strain of *P. roqueforti* and by Ohmomo (1975) from Roquefort cheese. Figure 4 shows the chemical structure of roquefortine with its correct stereochemistry.

Figure 4: Structure of roquefortine

The consensus in the literature is that the majority of roquefortine produced using surface cultures remains sequestered in the mycelia. It is derived biosynthetically from tryptophan, histidine and dimethylallylpyrophosphate and has been shown to be sensitive to light and to acidic conditions. Scott et al. (1979) found that 100 mg of roquefortine irradiated under nitrogen at a wavelength of 330nm for 6 hours yielded 78 mg of a photoisomer. They also showed that when roquefortine was refluxed with a methanol – 1N HCl solution for 3 hours, an acid-catalyzed de-isoprenylation reaction occurs resulting in a new product with a molecular formula $C_{17}H_{15}N_5O_2$. The issue of light sensitivity was also examined more recently by Noroozian et al. (1998) where they tested standards of roquefortine in different solvents and using different light sources. Their results indicated that roquefortine is least stable in ethyl acetate and that it has a half-life of approximately 50 min in diffuse sunlight.

Roquefortine has been isolated from cheese, silage for animal feed, contaminated beer, and was the source of an intoxication of dogs who consumed garbage in Alberta, and Ottawa Canada (Lowes N, 1995; Walter SL, 2002). It has been found in cheese at

concentrations up to 6.8 mg/kg (Scott PM and Kennedy BPC, 1976; Ware GW et al., 1980) found an average concentration of 0.42 µg/g from 12 samples of cheese tested. Roquefortine was also isolated as the major fungal metabolite from feed grain by both Auerbach et al. (1998) and Häggblom (1990).

II-E.1-2 Toxicity

A number of toxicity studies have been conducted on roquefortine. Schoch et al. (1983 and 1984) conducted mutagenic studies using the Ames test on strains of *P. roqueforti* used for cheese production. The results showed no signs of mutagenic activity toward either the fungus or roquefortine. Rats that received intraperitoneal injections of roquefortine showed a range of LD₅₀'s. Ueno and Ueno (1978), reported values of 15-20 mg/kg. Similar experiments performed on mice by Arnold et al. (1978) reported LD₅₀ values for male and female CR57 mice as 169 and 184 mg/kg respectively and 189 and 184 mg/kg for Swiss-Webster mice. Scott et al. (1976) referred to a French study on the neurotoxic activity of roquefortine in mice, similar symptoms were also observed in day-old cockerels dosed with roquefortine (Wagener RE et al, 1980). The results of these studies reporting neurotoxic properties for roquefortine have been contradicted by Arnold et al. (1978) who observed no neurotoxic activity and have argued that previous LD₅₀ values, which were 10 times lower than their data, may be incorrect.

The carcinogenic effect of *P. roqueforti* was studied using 800 rats that were fed both a strain of mold used for commercial cheese production and also the finished cheese product. The results of the experiment clearly indicated that there were no significant

differences between control and treated animals for either the final cheese products or for the starter strains used for their production (Frank HK et al., 1977).

Kopp-Holtwiesche and Rehm (1990) demonstrated that roquefortine possesses antibiotic properties towards gram-positive bacteria but not gram-negative. Bioassays were also performed using the eukaryotic organism *Tetrahymena thermophila*, the results indicated less sensitivity towards roquefortine than bacteria by a factor of two (Benitez L et al 1994).

An important study on the impact of roquefortine on 18 female sheep was conducted by Tuller et al. (1998). They dosed the animals by means of a gel capsule via a stomach tube with the equivalent to 0, 5, and 25 mg/kg of silage over a 16 – 18 day period. The results showed that no clinical signs of intoxication were recognized over the period of the experiment. Neither chemical (ALT, AST, GLDII, bilirubin or blood glucose) nor pathological factors (numbers of erythrocytes, leukocytes, thrombocytes, hemoglobin, or packed cell volume) were altered by the presence of roquefortine. The only observable changes were in the pH of the rumen fluid, which decreased by 0.5 units. Additionally, residual roquefortine could be detected unchanged in the rumen fluid, feces, liver, bile, kidneys, muscles, fat, lungs and heart.

II-E.2-1 PR Toxin:

PR toxin (*Penicillium roqueforti* toxin) was originally isolated from moldy grain and corn silage, but has received a great deal of research attention because of its presence in cheese. The major producer of PR toxin, a colourless crystalline compound, is *P. roqueforti*. It is produced biosynthetically from the pathway of acetyl-coenzyme →

mevalonate $\rightarrow$ dimethylallylpyrophosphate $\rightarrow$ farnesyl pyrophosphate and subsequent cyclizations (Steyn P, 1998). PR toxin has a number of related derivatives, which include PR oxidase, PR acid, and PR imines which are believed to play roles in the toxicity observed from *P. roqueforti* contamination (Chang SC et al., 1991a and 1991b).

Figure 5: Structure of PR toxin

II-E.2-2 Toxicity

PR toxin has been described as one of the most acutely toxic metabolites known to be formed by *P. roqueforti* (Scott PM, 1981). The LD₅₀ values for mice were determined to be between 72-140 mg/kg for oral and 2-5 mg/kg when delivered intraperitoneally (Arnold DL et al, 1978). Chen et al. (1982) also tested mice intraperitoneally and determined the LD₅₀ to be 5.8 mg/kg. They also examined rats that were both given PR toxin intraperitoneally (IP) and intravenously (IV), the LD₅₀ values were 11.6 and 8.2 mg/kg respectively. Wei et al. (1973) determined oral LD₅₀'s in rats to be 115 mg/kg and intraperitoneally 11 mg/kg. Additionally, Wei et al. (1973) reported on the pathology of the rats. Treated animals showed signs of internal trauma such as swelling of the stomach, edema of the lungs, brain, and kidneys, hemorrhaging of the kidneys as well as degenerative changes in the liver.

The mutagenicity of PR toxin was demonstrated using *Salmonella typhimurium* (Ueno Y and Ueno I 1978). The carcinogenic effects of PR toxin were examined by Polonelli et al. (1982) who showed that 2 out of 10 experimental mice developed tumors in half the time as 1 out of 10 control animals. The results indicate the possibility of PR toxin being a carcinogen but the results are very inconclusive.

PR toxin has also been shown to inhibit RNA and protein synthesis, the activity of DNA polymerases alpha, beta and gamma, mitochondrial respiration and oxidative phosphorylation in animal cells (Lee et al., 1984).

II-E.3-1 Mycophenolic acid:

Mycophenolic acid (MPA) was first isolated in 1913 from a strain of *Penicillium stoloniferum*, although its structure was not determined until 1948 by Birkinshaw (Figure 6). MPA has since been isolated from a number of other *Penicillium* species including *P. roqueforti* (Lapont P et al., 1979). It was originally believed that all strains of *P. roqueforti* produced MPA but Engel et al.(1982) showed that only 20 out of 80 strains tested were producers. The biosynthesis of mycophenolic acid is still not fully understood, but what is known is that the polyketide origin of the ring system is from methionine, and that the side chain is the result of the addition of a farnesyl unit (Turner WB and Aldridge DC, 1983). MPA is of particular interest because of its role as an immunosuppressant. It has model use as an anti-rejection drug for organ transplant patients in-conjunction with other immunosuppressant drugs such as cyclosporine (Gummert J et al., 1999). Its role as an immunosuppressant is perhaps important for the

present study because its presence in silage could lead to immunosuppression in cattle making them more susceptible to disease (Schneweis I et al., 2000).

Figure 6: Structure of mycophenolic acid

II-E.3-2 Toxicity

The toxicity of mycophenolic acid has also been extensively studied because of its importance as a drug. Its toxicity appears to be very low in comparison to other metabolites isolated from strains of these fungi. The LD₅₀ for rats was determined to be 2500 mg/kg when administered orally, and 500 mg/kg when administered intravenously (IV). Mice showed similar results 700 mg/kg orally, and 450 mg/kg when administered IV (Wilson et al., 1972). Chronic toxicity was also tested in rabbits for one year with daily oral doses ranging from 80 to 320 mg/kg with no apparent signs of toxicity (Adams E et al., 1975). Carter et al. (1969) showed that rats lost weight and were dead within nine weeks when given daily oral doses of 30 mg/kg, they also showed that rhesus monkeys who received 150 mg/kg daily developed abdominal colic, lost weight, and had bloody diarrhea after two weeks of feeding. The impact of MPA on humans was also tested, 35 patients received daily oral doses of 2.4 to 7.2 g for periods of 1 or 2 years. Common symptoms reported were cramps, nausea and diarrhea (Marinari R et al, 1977).

Additionally, mycophenolic acid has also been shown to have antibiotic activity against bacteria, dermatophytic fungi and it can also interfere with viral multiplication (Planterose DN, 1969). AMES test of MPA using *Salmonella* showed no mutagenic signs (Wehner FC et al., 1978).

II-E.4-1 Penitrem A:

Penitrem A and its related metabolites, penitrem's B-F (originally called tremorgens), are produced by a few *Penicillium* species but the major producer is *P. crustosum*. Penitrems were first reported in isolates of *P. cyclopium* (later changed to *P. crustosum*) collected from feed that was lethal to sheep, moldy horse feed and moldy peanuts (Wilson BJ et al., 1972 and Mantle PG et al., 1978). They are derived biosynthetically from the indole part of tryptophan, geranylgeranylpyrophosphate and two isopentenylpyrophosphate units (Steyn PS, 1998).

	R1	R2	
a)	Cl	OH	penitrem A
b)	H	H	penitrem B
c)	H	OH	penitrem E
d)	Cl	H	penitrem F

- a) R = Cl - penitrem C
- b) R = H - penitrem D

Figure 7: Structure of Penitrems A-F

II-E.4-2 Toxicity

The penitrems are of interest because they are powerful neurotoxins, and have been described as probably the most toxic compounds produced by any *Penicillium* species (Pitt JI, 2002). The penitrems have demonstrated these effects on a wide variety of test animals (EL-Banna AA and Leistner L, 1988; Arp LH and Richard JL, 1981). The LD₅₀ of penitrem A in mice was determined to be 1.05 mg/kg when doses intraperitoneally. Its effects on mouse liver composition and also on prenatal mice were tested by Hayes and Hood (1978) and Hayes et al. (1977). Sheep when given doses of 25 mg or more of penitrem A exhibit tremors. Penny et al., (1979) tested sheep and pigs with varying doses of penitrem A over a 14 week period. The sheep displayed similar symptoms to those observed in ryegrass staggers, pigs on the other hand were overall less susceptible, most importantly no histopathological changes were observed for either. Similar results were obtained from studies comparing sheep and pigs that were given doses of penitrem A (Day JB and Mantle PG, 1982; Peterson DW, et al., 1982).

Natural intoxication from penitrem A has been reported for dogs who experienced muscle tremors and seizures after eating moldy cream cheese. Similar symptoms were shown for a dog who consumed moldy walnuts, for a dog who consumed a moldy hamburger bun and for a dog following garbage consumption (Arp LH and Richard JL, 1979, Hayes AW et al., 1976, Hocking AD et al, 1988, and Walter SL, 2002). Penitrem A has also been implicated in the mortalities of dairy cattle fed moldy commercial feed, and it is also responsible for the intoxication of cattle fed moldy corn (Richard JL, 1990). A reported case of penitrem A intoxication has even been reported for a human who consumed contaminated beer (Cole RJ et al, 1983).

II-F Analysis of fungal metabolites.

There are a large variety of methods available for analyzing fungal metabolites. The majority of the methods used for analyzing those from *Penicillium* species were developed over two decades ago and involve TLC. Most methods are also only used for the analysis of one metabolite. Methods do exist for HPLC and mass spectral analysis but very few are effective at analyzing unknown compounds from fungal extracts. In addition, the higher resolution and better experimental techniques available on current NMR spectrometers permits easier and more accurate structure determination than was available for the previous work on these metabolites from the literature. The need exists for a refinement to the current analytical techniques used for studying secondary metabolites from *Penicillium* species.

III. PROJECT AIM

The first goal of this study was to determine the optimal growth conditions for the two species, *P. crustosum* and *P. roqueforti*, and to use this information in obtaining maximum metabolite yields. Many researchers have studied these species for a variety of reasons, but to our knowledge no one has reported systematically examining each growth factor to determine the optimal conditions. A better understanding of these conditions used by the fungus to produce toxic metabolites will hopefully provide some insight into maximizing their production. Additionally, the use of ^{13}C stable isotopes for labeling experiments was used to help provide information on the biosynthesis of the secondary metabolites found. This will also help in the understanding of the conditions needed for maximizing their production.

The next aim of the project was to develop an extraction method that was specific to *Penicillium* metabolites and takes into account the sensitivity of these metabolites to light and acidic conditions. Additionally, the use of new methods and technologies for analysis was to be examined. The goal was to develop a new HPLC method for detecting fungal metabolites that could be used for three analysis systems, HPLC-DAD, LC/MS and LC/NMR. This will provide a quicker more accurate method of screening fungal strains to determine their metabolite profiles. Additionally, it will provide a tool for identifying unknown metabolites without the use of isolation, which can result in changes to the metabolites.

IV. Experimental

IV-A Fungal strains

Four different fungal strains were tested throughout this project, all strains were stored at 5°C on 2% malt extract agar slants. The original test strain DAOM 229761 was identified as *Penicillium roqueforti* by Dr. J. David Miller. The strain was isolated from recycled animal feed (bread) implicated in a severe toxicosis of cattle near Moncton, New Brunswick and was supplied by the Canadian Food Inspection Agency. The second test strain DAOM 215343 was obtained from the culture collection at Agriculture Canada in Ottawa, Ontario. It was originally isolated from spruce lumber in Kirkland Lake, Ontario in 1990 and was identified as *Penicillium crustosum*. This strain was used for all labeling experiments due to its rapid growth rate and mycotoxin profiles. The remaining two test strains used were also obtained from the culture collection. DAOM 194930, *Penicillium crustosum* isolated in 1985 from barley feed (potential livestock problems), Winnipeg, Manitoba. DAOM 225571, *Penicillium roqueforti* isolated in 1998 from corn silage collected in St. Louis, Quebec.

IV-B Growth, extraction and analysis from shake flasks

A slant of *Penicillium roqueforti* DAOM 229761 was macerated in 50 ml of sterile distilled water, from which 2.5 ml or a 5% aliquot was used to inoculate each 250 ml Erlenmeyer flask. The flasks contained 50 ml of Czapek-Dox medium (30g/L sucrose, K₂HPO₄ 1g/L, yeast extract 5g/L, MgSO₄ 0.5g/L, NaNO₃ 3g/L, FeSO₄ 0.01g/L, KCl 0.5g/L and glycerol 10g/L) and were autoclaved for 12 min at 121°C. All flasks were incubated in the dark at 25°C on a New Brunswick Scientific triple tier rotary shaker

(220 rpm, 3.81 cm throw). All cultures were harvested after 7 days of growth and filtered through a Buchner funnel using Whatman #1 filter paper and vacuum pressure. The volume of filtrate from each flask was measured and the pH recorded before storing at 5°C for later processing. The remaining mycelium was washed with 2 times equal volumes of distilled water to remove unwanted sugars, dried using vacuum pressure and then wrapped in aluminum foil and stored in the freezer for later processing.

IV-B.1 Extraction of Shake Flasks

The filtrates were originally extracted two times with equal volumes of hexane (Fisher Scientific Canada) for 1 min in a separatory funnel for cleanup purposes. Both hexane fractions were separated from the filtrate and combined together. The filtrate was returned to the separatory flask and extracted again with 2 equal volumes of ethyl acetate (Fisher Scientific Canada) to extract out the mycotoxins from the aqueous filtrate, and once again the fractions combined together. Both the hexane and ethyl acetate extracts were then poured into round bottom flasks through a funnel containing filter paper and sodium sulphate powder (BDH Canada) to remove any water from the samples. The round bottom flasks were then dried to completion using a Caframo VV 2000 rotary evaporator set to 30°C and medium rotation. Each sample was then re-suspended in either hexane or ethyl acetate, dried under nitrogen in clean vials and dissolved again in an exact volume of solvent (3 ml).

The frozen mycelia were freeze-dried (24 to 48 hours) using a cryocool (Neslab CC-100) freeze dryer. The weight of each mycelial sample was recorded immediately after the mycelia were removed from the freeze-drier and were then placed into

Erlenmeyer flasks for extraction. As with the filtrate, the mycelia were first extracted with hexane. 50 ml of hexane was added to each of the flasks, which were then macerated with a polytron homogenizer, a stir bar was added and they were placed on stir plates for 1 hour. The procedure was repeated once more with hexane and both volumes of hexane were decanted off and combined together for analysis. The mycelia were then extracted with 2 equal volumes of ethyl acetate in the same manner as the hexane and they were combined for analysis, while the remaining mycelia were discarded. Both the hexane and ethyl acetate extracts were dried using the same procedure as for the filtrates and were re-dissolved using 3 ml of hexane or ethyl acetate and placed into clean 10 ml vials.

IV-B.2 Analysis by thin layer chromatography (TLC):

A comparison of TLC solvent systems was performed on both standards and sample extracts. A wide variety of solvent mixtures from the literature were tested which included; benzene/methanol 93:7 and 85:15, benzene/acetone 93:7, acetone/ethanol 20:3, 2:1, 20:15, 1:1 and 1:2, chloroform/ethanol 10:1, and toluene/ethyl acetate/formic acid 5:4:1. Comparisons of the different solvent systems were performed using identical standards and samples, where it was determined that the chloroform/ethanol 10:1 system provided the best separation and results.

Standards of roquefortine (minimum 90% were obtained from Sigma Canada) were used to quantify and evaluate mycotoxin production in the cultures. Different concentrations of the roquefortine standard in ethyl acetate were spotted on the bottom of TLC plates (250 μm silica, 60 Å, Whatman, New Jersey) along with the filtrate and

mycelium samples extracted above. The TLC plates were placed into development jars containing a 10:1 mixture of chloroform/ethanol, the solvent was allowed to travel to 1 cm from the top of the plate at which point the plate was removed from the jar and allowed to air dry. The plates were then sprayed with Ehrlich's reagent (1g of Ehrlich's reagent crystals in 100 ml of 95% ethanol) dried using a blow dryer, sprayed with a development reagent (1:1 v/v of sulfuric acid:ethanol) and once again dried. The mycotoxin spots appeared in less than 1 min and were compared to the standards for identification and quantification.

The growth of strain DAOM 229761 was tested at different cell concentrations to determine the impact of cell numbers on metabolite production. 250 ml Erlenmeyer flasks containing Czapek-Dox medium were inoculated with different inoculation concentrations (0.1%, 1%, 4%, and 5% v/v) and grown for 7 days as above. The samples were harvested, extracted and analyzed by TLC as described above. The results of the filtrate analysis for roquefortine indicated that more cells (lower oxygen content) corresponded to more metabolite production. The experiment was repeated using higher concentrations (8%, 10%, 12% and 16% v/v). The filtrate extracts were analyzed as above. The results indicated that with more cells there was an increase in metabolite production, but only to a level that was below those observed by other researchers. The need for higher levels of metabolite production led to the change from shake flasks to Roux bottles for future experiments.

Roquefortine analysis from shake flasks was also performed using high performance liquid chromatography (HPLC), to allow for more reliable concentration determinations. The growth, extraction and cleanup procedures were identical to the ones

used for TLC. The HPLC method used was a simplified method for determining roquefortine concentrations reported by Auerbach et al. (1998). A Varian Vista 5500 HPLC instrument attached to a Varian 2550 UV detector and a computer was used for the analysis. Analyses were performed using a Lichrosorb reverse phase C-18 column (250 X 4.6) supplied by Supelco, and an isocratic mobile phase consisting of 70:30 methanol/water (all solvents were HPLC grade and supplied by Fisher Scientific Canada) with 5.75 g of $(\text{NH}_4)_2\text{H}_2\text{PO}_4$ /L (BDH Canada). The solvents were degassed with helium, heated to 35°C and run at 1 ml/min. The UV detector was set at 328nm, resulting in a standard roquefortine peak at 6.5 min. The resulting peak was very broad so the method was changed to 85:15 methanol/water with 10g/L of buffer, resulting in much sharper and better-resolved sample peaks.

The growth of strain DAOM 229761 was also evaluated using rice cultures. Rice medium (50g of rice and 30ml of distilled water were autoclaved in 500ml Erlenmeyer flasks) was inoculated with spores and allowed to incubate at 25°C until the rice was completely covered in mold (10 days). The cultures were then harvested and extracted with chloroform to kill the spores and to extract metabolites. The chloroform extractions (4 x 50 ml) were combined together, dried by rotary evaporation and analyzed by TLC.

IV-B.3 Modifications to the extraction procedure

The efficiency of the extraction procedure using hexane and then ethyl acetate was examined. Comparisons were made using identical culture filtrates of strain DAOM 229761 for the normal method described above and extraction using just ethyl acetate. Additionally, both experiments were performed under acidic conditions (pH 4) by

addition of 1N HCl and under basic conditions (pH 12) by addition of 1N NaOH. The use of chloroform instead of ethyl acetate was also evaluated under the same conditions. The method that gave the highest roquefortine recovery was obtained with 2 equal volumes of chloroform and the filtrate pH adjusted to 12 with 1N NaOH. The use of more extractions proved to be of no value as greater than 95% of the extractable roquefortine was obtained in the first two extractions. Additionally, the change from ethyl acetate to methanol was made for dissolving all standards and for the re-suspension of the dried chloroform extracts for HPLC analysis.

IV-B.4 Roquefortine recovery and sensitivity to light tests

To check the efficiency of the new extraction method, normal medium was spiked with different concentrations (100mg/L, 10mg/L, 1mg/L, and 0.1 mg/L) of roquefortine standards and extracted under normal conditions. Additionally, roquefortine has been shown to be sensitive to light and exposure greatly reduces recovery (Noroozian E et al, 1998). Therefore the spiking experiment was performed in the light (normal laboratory conditions) and under reduced light (laboratory lights off, windows closed). The recoveries were significantly different (50 % in the light vs. 85% for reduced light). These results led to the second modification to the procedure, from this point on all experiments were performed under reduced light conditions. All cultures were grown in the dark (covered in aluminum foil), filtered and extracted under reduced light, and all samples and standards were stored in amber vials or vials covered in aluminum foil.

IV-C Growth and Analysis from Roux bottles

IV-C.1 Growth

Roux bottles (Figure 8) were used for the next step in the growth experiments. Using the procedure for shake flasks described above, these first stage cultures were inoculated and placed on the shaker for 72 hours. At this point they were macerated and used to inoculate 1 liter Roux bottles containing 200 ml of Czapek-Dox media at 5% v/v. The flasks were incubated at 25°C for 14 days in the dark. The Roux bottles were harvested, filtered and processed in the exact way as the modified method for shake flasks described above. The extraction procedure of 2 x 200 ml of chloroform at pH 12 was used.

Figure 8: Growth of *P. crustosum* in a Roux bottle.

IV-C.2 New HPLC analysis for all mycotoxins

A new method was adopted from Frisvad et al. (1987) to allow for the analysis of all mycotoxins contained in the fungal extracts (Table 1). The new HPLC analysis

procedure was performed on an 1100 series Hewlet Packard HPLC – diode array detector (DAD) with built in degasser, and four pumps. The solvent system used was a water/acetonitrile gradient containing 0.05% trifluoroacetic acid (all solvents and chemicals obtained from Fisher Scientific, Canada). A new Spherclone RP-C18 250 X 4.6 column (Phenomenex, California) was used for the analysis and the DAD was set to 254nm for the optimal absorption of all compounds. Additionally, the use of methanol for re-dissolving extracts was changed to acetonitrile, to allow for more consistency.

Table 1: Initial HPLC conditions for metabolite detection (Frisvad JC, 1987).

Time (min)	Water	Acetonitrile	Flow rate (ml/min)
0	70	30	1
2	70	30	1
27	10	90	1
30	70	30	1

IV-D Maximizing roquefortine and other metabolite production in Roux bottles

IV-D.1 Water tension - Glycerol

The impact of changing the glycerol content of the medium was examined using both strains DAOM 229761 and DAOM 215343 with the normal Roux bottle medium grown for 14 days with the 72 hours starter cultures for DAOM 229761 and 48 hour (due to its rapid growth) for DAOM 215343. Experiments were performed in triplicate using 0, 10, 20, 30, 40, and 50 mg/L of glycerol in Czapek-Dox medium. All cultures were harvested and processed as before and were analyzed for roquefortine content only (representative of all metabolites present) using the modified version of Noroozian et al., (1998). Yield was not increased with the addition of glycerol, indicating that a modification to the Czapek-Dox medium would not be required.

IV-D.2 Phosphorus content of media

An experiment was performed to determine the impact of altering the phosphorus content of the medium. Roux bottles containing normal Czapek-Dox medium were grown under normal conditions and compared to Roux bottles with 50% phosphorus and Roux bottles containing Czapek-Dox medium with 10% phosphorus. The samples were filtered, extracted and analyzed using the HPLC conditions for the detection of just roquefortine. The results of the experiment indicated that phosphorus content of the medium had little impact on roquefortine production and on the production of other metabolites.

IV-E Testing of all 4 strains in Roux bottles

The next experiment performed involved the use of all four strains discussed above to determine if any were better producers of roquefortine and other metabolites. All four strains were grown in triplicate for 14 days in the dark under the normal conditions and on Czapek - Dox medium without glycerol. The strains were extracted and analyzed using the modified Frisvad method to examine total metabolite profiles.

IV-F Time study

The production of roquefortine and other metabolites was then examined by analyzing cultures in triplicate every 3 days starting with day 3 and ending with day 21. For the time study, both strains DAOM 229761 and DAOM 215343 were tested. The metabolite production by both strains was tested using the modified Frisvad analytical method and the normal extraction procedure. The results of the time study indicated that

metabolite yield reached a maximum at day 12. All future cultures were incubated for 12 days only.

IV-G Sugar analysis

An analysis of the carbohydrate or sugar content of the medium was also performed on the same strains at the same time by removing 10 ml of each culture filtrate before extraction. Sugar analysis of samples was used to determine when the organisms consumed the sucrose, glucose and fructose. Carbohydrate analysis was performed using the method described by Miller and Blackwell (1986). A Waters high performance carbohydrate column was attached to the previously described Varian HPLC using a refractory index (RI) detector. Standards of the three sugars were obtained from Sigma Canada and were analyzed using 20 μ l injections, an isocratic mobile phase of 75:25 acetonitrile / water and a flow rate of 1.4 ml/min. Test samples were compared to standard curves for the three sugars to determine the concentrations of each sugar remaining at each day of the experiment. Day zero or blank medium (non-inoculated) was used to confirm the starting concentrations of each sugar.

IV-H Nitrogen analysis

The total remaining nitrogen in each sample from the time study was also examined using the method of Ferree and Shannon (2001), and Bachmann and Canfield (1996). Five milliliter aliquots of the filtrate were removed at each day and were tested to determine the total remaining nitrogen, with blank medium used as the starting concentration. The method uses the second derivative UV/visible absorption spectrum

for NO_3^- which occurs at $\sim 224\text{nm}$ and once digested is proportional to the total nitrogen remaining (Crompton WG et al., 1992; Suzuki N and Kuroda Rokuro 1987). Standard curves were produced from blank media containing different concentrations of nitrogen. Standards and samples were first diluted 100 times to help eliminate interferences and to bring the scale into the appropriate range. The volume of each standard and sample was initially 10 ml and these were placed into Teflon-lined screw cap vials for digestion by autoclave. One and a half milliliters of freshly prepared oxidizing agent (6g of low N potassium persulfate in 100 ml of 1.5 N NaOH) was added to each vial, and the vials were capped and vortexed at high speed for 1 min. The vials were then arranged on a test tube rack and placed into the autoclave for 60 min at 121°C . The vials were removed and allowed to cool in a Laminar flow hood, at which point they were acidified with concentrated sulfuric acid to below pH 2. Standards and samples were then transferred to the proper cuvettes and read on a Varian UV/visible spectrophotometer. The instrument was set to read the second derivative over the range 190 to 250nm. The standard curve obtained was then used to determine the total nitrogen concentrations of the cultures from the time study.

IV-I Oxygen analysis

The oxygen tension in Roux bottles was tested using a YSI model 57 dissolved oxygen meter. Samples from Roux bottles were measured in triplicate to determine the O_2 tension in mg/L over the length of the experiment. Initial readings were recorded and O_2 concentrations were recorded every day starting at day 6.

IV-J Final modifications to HPLC method

The HPLC method described above for the determination of all metabolites from both filtrate and mycelia was modified to eliminate acid degradation of roquefortine and other metabolites. The 0.05% trifluoroacetic acid was changed to 0.1% ammonium acetate and all other conditions remained the same. One additional change was the switch from a Phenomenex RP C18 column to a Phenomenex Synergi Max- RP (C12) 250 X 4.6 which gave better separation of metabolites. These final HPLC solvent conditions will be used for all experiments including prep HPLC, LC/MS and LC/NMR (Table 2).

Table 2: Final HPLC conditions used for all experiments.

Time (min)	Water	Acetonitrile	Flow rate (ml/min)
0	70	30	1
2	70	30	1
27	10	90	1
30	70	30	1

IV-K Isolation of major metabolites

Cultures of DAOM 215343 were grown in Roux bottles for 12 days under the above-described conditions, and were extracted and analyzed under the final HPLC conditions to confirm the presence of all metabolites. The filtrate and mycelia extracts were then directly injected onto a prep HPLC column packed with DEAE Sephadex A-25 supplied by VWR Canada. The column was attached to a specially designed Waters prep HPLC with controller, UV detector and computer. The HPLC conditions used were described above and the individual peaks for each of the major metabolites were collected into Erlenmeyer flasks wrapped in aluminum foil. They were then poured into

round bottom flasks and placed on the Caframo VV 2000 rotary evaporator and dried to completeness. The isolated compounds were then re-suspended in a minimal amount of hot acetonitrile and allowed to crystallize. The melting points of the compounds were tested along with HPLC analysis, MS/MS and NMR to confirm the identity and to check the purity of the compounds.

IV-L Analysis of different media

The standard Czapek-Dox medium used was modified in an attempt to slow down cell growth and to increase metabolite production. The normal Czapek-Dox medium was used without yeast extract for one test, and the other included no nitrogen and only 1g/L of yeast extract. The addition of more sucrose to the Czapek-Dox medium was tested using 60 and 150 g/L of sucrose. MYRO media (40g/L sucrose, 10g/L glycerol, 1g/L $(\text{NH}_4)_2\text{HPO}_4$, 3g/L KH_2PO_4 , 0.2g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5g/L NaCl) was also tested because of its ability to produce other fungal metabolites.

IV-M Structure, ^{13}C Enrichment and Biosynthesis

The first step of the NMR enrichment study was to properly characterize the major metabolites that have been identified from fungal cultures. ^1H , ^{13}C and ^{15}N experiments were performed on standards of roquefortine, cyclopenin and penitrem A. The standard sets of homonuclear (gradient DQF-COSY) and heteronuclear correlation (gradient HSQC and HMBC) were performed on a Varian 600 MHz spectrometer. The stereochemical assignments were made via coupling constants and a NOESY spectrum. The ^{15}N assignments were determined by using $^{15}\text{N}/^1\text{H}$ HSQC and HMBC spectra.

Maximum production of roquefortine determined from the time course study was between days 6 and 12. ^{13}C -1 and ^{13}C -2 sodium acetate (99.5% atom ^{13}C , obtained from CDN Isotopes Canada) was added under aseptic conditions to cultures of DAOM 215343 (5 for each label) grown in Roux bottles. The labeled acetate was added in equal amounts (1 ml per day) for a total addition of 1g of each label from day 7 to day 11 to coincide with the highest roquefortine production rate. The cultures were harvested and processed as above. A profile of the metabolites present in both the filtrate and mycelium were obtained using the final HPLC conditions (Table 2) to confirm the presence of each metabolite. The major metabolites from both the filtrate and mycelium were then isolated. The isolated metabolites were analyzed on Bruker 500 MHz instrument using identical acquisition parameters (number of scans, pulse length, and relax and delay) to permit direct comparison of peak intensities. The labeling experiments were then repeated using identical conditions but with addition of label from day 3 to day 7 for one experiment and from day 5 to day 9 for the other. These were performed in an attempt to induce greater incorporation of the label and to examine the impact of adding label during different stages of metabolite production.

IV-N LC-MS, LC-MS/MS, and LC-NMR

LC-MS was used to help in the determination of unknown compounds from both the filtrate and mycelia. A Hewlett Packard 1050 HPLC system (pump, inline degasser, UV detector, and computer) was attached to a triple quadrupole Micromass electrospray ionization (EI) mass spectrometer (MS) set for analysis in positive ion mode. The EI-MS was controlled using MassLynx software version 3.5, which was also used for analysis of

mass spectrum data. This allowed for the analysis of extracts under the identical HPLC conditions, including the column used for the quantification of the metabolites. The only difference here was that, after separation, the effluent passed into a 10:1 splitter. The majority of the flow was diverted into the UV detector as usual to provide a chromatogram of the sample mixture. The remaining portion was diverted through capillary tubing into the EI-MS. The sample entering the electrospray has been separated on the HPLC column and was compared to the complementary UV trace. Comparisons were made between both to determine the molecular weights of individual peaks from the samples for aid in identification of the minor metabolites. The same samples were then analyzed using LC-MS/MS to provide fragmentation patterns (daughters) of parent ions determined above to further aid the identification of minor unknown metabolites.

LC-NMR was also used for this project because the data obtained from the MS analysis was not always sufficient to identify the metabolites. LC-NMR was performed using the identical HPLC conditions used for HPLC and LC-MS analysis. A Varian 9010 HPLC system and 9050 UV detector with inline degasser and flow switch was used for the LC-NMR. It was attached to a Varian 600 MHz INOVA instrument and console using a Varian microflow triple resonance probe with a 120 μ l flow cell (60 μ l active volume). The whole system was controlled by Varian LC-NMR software to connect the two instruments and allow for proper communication (Figure 9). The principle is the same as for LC-MS with the exceptions that the flow is directed to the flow probe after passing through the UV detector. The major differences are with LC-NMR are a) it requires much more material (vs. small amounts for MS) and b) during NMR acquisition, the flow of the HPLC is stopped so that the metabolite of interest can be analyzed. The

instrument was operated in stop flow mode, where a minimum detection threshold is set on the HPLC. Once a peak crosses the threshold it signals the pump to stop at the appropriate time to allow the peak to enter the flow probe, where a proton spectrum can be acquired on that peak. Once the spectrum was acquired, the flow was restarted and the HPLC run was allowed to continue until the next peak of interest was ready for analysis by ^1H NMR. The information obtained from the LC-NMR experiments combined with the LC-MS data was used to identify minor metabolites without the need for isolation (Vogler B et al., 1998).

The LC-NMR block diagram indicating the LC system, the NMR system, interconnect lines, and the optional analyte collector.

Figure 9: Diagram showing Varian LC/NMR system used.

IV-O Analysis of *Penicillium* strains isolated from contaminated silage

The newly developed extraction and analysis methods were used to screen an additional nine strains that were isolated from corn and grass silage from a farm in Quebec. The silage was plated on MEA plates, from which three strains of *P. crustosum* and six strains of *P. roqueforti* were isolated. The nine fungal strains were grown as described above for 12 days, extracted and analyzed using the final conditions to determine their metabolite profiles.

V. RESULTS

V-A Growth, extraction, and analysis from shake flasks.

Initial work on growth, extraction and analysis from shake flasks (detailed results not reported here), yielded a number of key factors which guided further fermentations. Firstly under shake flask conditions, the yield of roquefortine and other metabolites were much lower than previously reported (Mantle PG et al., 1983; Scott PM et al., 1977). Secondly, low oxygen tension was shown to be important for metabolite yields and changing from shake flasks to Roux bottles would be required to help reduce oxygen tension to more favorable levels for metabolite production. The last important point was that the currently available extraction methods were not optimal for all of the metabolites produced by the species tested, this required further work on optimizing these extraction methods.

V-A.1 Extraction method

The effectiveness of different extraction solvents was examined over a range of pH, the results are displayed in Table 3. Quantities of roquefortine extracted from identical culture was used as the indicator of extraction success and the table shows that use of chloroform with the filtrate corrected to pH 12 was the most effective at extracting roquefortine from the cultures.

Table 3: Comparison of extraction solvents and pH conditions for roquefortine.

Extraction solvents : pH of filtrate	mg of roquefortine/L of filtrate (DAOM 229761)
ethyl acetate: pH 4	13.18
ethyl acetate: pH 8	12.58
ethyl acetate: pH 12	14.96
ethyl acetate and hexane: pH 8	13.26
ethyl acetate and hexane: pH 12	12.03
Chloroform: pH 4	9.17
Chloroform: pH 8	14.26
Chloroform: pH 12	21.28

V-A.2 Glycerol test – water activity

The addition of glycerol to Czapek-Dox medium was also employed as a method of reducing the water activity. The results for both strain DAOM 229761 and DAOM 215343 are displayed in Table 4 and indicated that the addition of glycerol to the medium does not significantly increase the production of metabolites and was therefore not added to future experiments.

Table 4: Test of Czapek-Dox medium containing different amounts of glycerol.

mg / L of glycerol added to Czapek-Dox medium	mg of roquefortine / L of filtrate (DAOM 229761)	mg of roquefortine / L of filtrate (DAOM 215343)
0	12.98	14.29
10	12.57	15.02
20	12.33	19.80
30	13.78	19.07
40	8.15	16.19
50	7.24	15.56

V-A.3 Phosphorus content

The impact on metabolite production, by the phosphorus content of the media was examined by comparing *P. crustosum* (DAOM215343) grown on normal Czapek-Dox medium with it grown on Czapek-Dox medium with 50% phosphorus and with 10% phosphorus. The results of the experiment are displayed in Table 5, and show that altering the phosphorus content of the media had no positive impact on roquefortine production.

Table 5: Impact of reducing the phosphorus content of media on roquefortine production.

g / L of K ₂ HPO ₄ added to Czapek-Dox medium	mg of roquefortine / L of filtrate (DAOM 215343)
1	13.58
0.5	9.91
0.1	12.83

V-A.4 Growth and metabolite production on different media.

The results of testing the growth of *P. crustosum* (DAOM 215343) on different media are displayed in Table 6. The mycelia dry weight was included as an indicator of how well the culture grew on the media. Additionally, the amounts of roquefortine and what was later identified as cyclopenin (please see section V-D.2) produced for each medium are expressed in mg / L of filtrate. The results indicate that increasing the sugar content of the Czapek-Dox media increased the amount of cells as indicated by the increase in dry mycelia weight, but did not lead to an increase in metabolite production. Altering the yeast extract and nitrogen content of Czapek-Dox medium reduced the cell growth significantly, but also did not increase metabolite production. The strain of *P.*

crustosum grew poorly on MYRO media and produced very small amounts of metabolites.

Table 6: Roquefortine and cyclophenin production when grown on different media.

Media tested (DAOM 215343)	Average mycelia weight g / 1L Roux bottle	mg of roquefortine / L of filtrate	mg of cyclophenin / L of filtrate
Czapek-Dox	2.69	17.51	22.52
Czapek-Dox with no yeast extract	0.23	0.79	0.00
Czapek-Dox with no nitrogen and 1g/L yeast extract	0.88	2.50	1.73
Czapek-Dox with 60 g/L sucrose	4.31	4.50	5.40
Czapek-Dox with 150 g/L sucrose	3.52	0.29	0.00
MYRO	0.30	0.36	0.18

V-B Growth of *P. crustosum* DAOM 215343 in Roux bottles.

The growth of *P. crustosum* DAOM 215343 was examined in Roux bottles to determine the optimal growth conditions for the major factors affecting metabolite production. The results are shown in Figure 10 and are discussed below.

Figure 10: Total analysis of *P. crustosum* (DAOM 215343) grown in Roux bottles.

Addition times of ^{13}C – 1&2 acetate: — — — — —

V-B.1 Sugar Analysis.

The results of the sugar analysis indicated that all of the sucrose from the initial medium was consumed by day 3 of the fermentation (Figure 10). Glucose and fructose appear due to enzymatic hydrolysis of the sucrose in the initial medium. The glucose and fructose concentrations increase to a maximum at day 3 and decrease to a non detectable level by day 6. The consumption of all of the available sugar by day 6 was coincidental with the maximum mycelia dry weight and a sharp increase in the rate of roquefortine production.

V-B.2 Nitrogen Analysis.

The results of the total nitrogen analysis indicated that the available nitrogen was reduced below the detection limit by day 3. The consumption of the nitrogen did not correlate with the rapid increase in roquefortine production, or with the maximum mycelium dry weight (Figure 10). Additionally, the amount of total nitrogen slightly increased at day 9 to levels above day 6. This slight increase was the result cell death and releasing nitrogen back into the medium from autolysis.

V-B.3 Mycelium weight and roquefortine production.

The increase in mycelial dry weight up to day 6 (Figure 10) of the fermentation corresponded to the rapid increase in cell numbers and the consumption of sugars. There was a steady reduction in mycelial dry weight from day 6 through to day 12, corresponding to cell death. Roquefortine production was detected at day 3, but does not achieve its maximum rate until between day 6 and day 9. The production of roquefortine

and other metabolites occurs for the most part between day 6 and 12 in Roux bottles under the described conditions. Roquefortine production reached a maximum of approximately 20 mg/l at day 12 and did not increase beyond that point, as was shown in cultures allowed to grow for up to 21 day (data not shown).

V-B.4 pH and dissolved oxygen analysis.

The initial pH of the medium was pH 7, this fell to pH 4.5 at day 3 and returned to pH 7 by day 6, where it remained. The oxygen tension of the medium just after inoculation was approximately 80% saturation. This dropped to 30% by day 6 where it remained to the end of the fermentation.

V-C Analysis of different *Penicillium* strains for metabolite profiles.

The initial four test strains used in this study (experimental section IV-A for descriptions) were grown in Roux bottles and harvested after 12 days so that they could be analyzed using HPLC-DAD to determine metabolite profiles. The resulting chromatograms for the mycelia and filtrate extracts were compared to that of a mixture of the four expected metabolites (roquefortine, penitrem A, PR toxin and mycophenolic acid) for identification purposes (Figures 11 and 12 show the chromatograms for DAOM 215343 which produced both the largest number and quantity of metabolites). Roquefortine was identified as the major constituent in both the filtrate and mycelia of all strains tested. Penitrem A was also found to be present in both the filtrate and mycelia of DAOM 215343 and DAOM 229761, with the majority occurring in the mycelia. None of the strains tested produced any detectable PR toxin or mycophenolic acid. The

chromatograms of DAOM 194930 and 225571 were similar with both strains only producing a few metabolites. DAOM 229761 and 215343 produced roquefortine, penitrem A and other minor metabolites, but DAOM 215343 also contained a large unknown peak in the filtrate at approximately 9.5 min and 11 or more minor unknown peaks, and as result became the focus of the study. The mycelia extract of DAOM 215343 also contains a peak at 9.5 min but is very small in proportion to the amount present in the filtrate, and at least 20 other minor unknown compounds that needed to be identified, were present in the mycelia.

The analysis of the 9 strains isolated from Quebec silage showed that roquefortine was the major metabolite found in the filtrate and mycelia extracts for both *P. roqueforti* and *P. crustosum*. Penitrem A was also present in the mycelia of most strains but in very low concentrations. No cyclopenin was detected in any of the *P. crustosum* strains but other amounts of minor known and unknown metabolites were detected.

Figure 11: HPLC chromatogram for mycelia extract of *P. crustosum* DAOM 215343 indicating major metabolites.

Figure 12: HPLC chromatogram for filtrate extract of *P. crustosum* DAOM 215343 indicating major metabolites.

V-D Identification, biosynthesis and ^{13}C labeling of major metabolites (DAOM 215343)

V-D.1 Roquefortine:

Roquefortine was one of the major metabolite produced by the strain of *P. crustosum* (DAOM 215343) that was used for the labeling studies. Roquefortine was initially identified by comparison of HPLC chromatograms to standards and was later confirmed by mass spectrum and NMR analysis. The mass spectrum analysis of roquefortine provided a molecular weight of 390 (M+1) with daughter ions of 322, 197, 192, 130, 108, and 69 (Figure13).

Figure 13: MS/MS of roquefortine indicating parent (390 M+1) and daughter ions.

The biosynthesis of roquefortine was examined through the addition of ^{13}C 1 & 2 labeled sodium acetate to cultures grown in Roux bottles. The first step was to properly characterize the molecule, this was accomplished by performing complete NMR assignments on a 5 mg standard of roquefortine (Sigma Canada) in CDCl_3 . Initial proton and carbon spectra were acquired (Figures 14 and 15). The proton spectrum shows a number of singlets and doublets with two singlets around 1 ppm that were assigned to the two methyl groups. In CDCl_3 , the exchangeable NH protons were observed at 8.95, 4.94, and 12.95ppm (not shown). The carbon spectra clearly indicated one resonance for each of the 22 carbons contained in roquefortine. To ensure assignments, the standard set of homonuclear (gradient DQF-COSY) and heteronuclear correlation (gradient HSQC and HMBC) were performed at 600 MHz (figures 16-18).

Figure 14: ^1H NMR spectrum of roquefortine in CDCl_3 .

Figure 15: ^{13}C NMR spectrum of roquefortine in CDCl_3 .

The gradient DQF-COSY for roquefortine is very simple and is displayed in Figure 16. The two isolated spin systems correspond to a) aromatic region derived from tryptophan (C 9-12), b) the allylic side chain C-24 and C-25, and c) C-15 and C-16. The

singlets at 5.62ppm and 6.27ppm were assigned to the protons on C-6 and C-17 respectively as the only two isolated protons of the molecule.

Figure 16: Gradient DQF-COSY spectrum of roquefortine.

The gradient HSQC spectrum (figure 17) for roquefortine was used to assign the protonated carbons. The spectrum clearly shows for example that the doublet 6.59ppm is attached to C-9 at 109.1ppm. The remaining protonated carbons were similarly assigned.

Figure 17: Gradient HSQC spectrum of roquefortine.

Once the protonated carbons are assigned the structural adjacencies must be determined from the gradient HMBC experiment, along with non-protonated carbons, the results are displayed in figure 18. The gradient HMBC confirms ^1H /long range carbons interactions, an example is the two methyl peaks with protons at 1.14 and 1.01ppm, which show correlation to C-14 (61.5ppm), C-23 (40.9ppm), and C-24 at (43ppm)in addition to the expected single bond correlations to 22.5 and 22.9ppm. H-15 shown correlation to all carbons that are both 2 and 3 bond away which was used to give the correct assignment for C-14 and allows for discrimination between C-13 and C-8. The assignment of C-12 vs. C-9 was confirmed by the correlation from H-12 to C-13, 8, and 14. Additionally, the coupling of H's assigned to C-11 and C-9 to the resonance at 129.1ppm confirms this carbon to be C-10.

Figure 18: Gradient HMBC spectrum of roquefortine.

Roquefortine is a unique metabolite in that it contains 5 discrete nitrogen atoms in different molecular environments. ^{15}N assignments were also made on the five resolved nitrogen atoms. This was done by observing direct and long range $^{15}\text{N}/^1\text{H}$ coupling using $^{15}\text{N}/^1\text{H}$ HSQC and HMBC spectra (figures 19 and 20), with the hope of being able to directly observe the incorporation of ^{15}N enriched precursors.

Figure 19: $^{15}\text{N}/^1\text{H}$ correlation spectra for roquefortine (HSQC).

Figure 20: $^{15}\text{N}/^1\text{H}$ correlation spectra for roquefortine (HMBC).

(^{15}N chemical shifts based on $\delta^{15}\text{N}/\delta^1\text{H}$ at transmitter offset)

The $^{15}\text{N}/^1\text{H}$ HSQC and HMBC spectra show that roquefortine contains 3 protonated and 2 quaternary nitrogens. The HSQC identified the protonated nitrogens as corresponding to H-7, H-2 and H-12 (not shown but corresponded to a nitrogen resonance at 167.3ppm). The HMBC confirmed the presence of the quaternary nitrogen through adjacent protons, the nitrogen at 259.2ppm showed correlation to H-20 and H-22 and was identified as N-21.

The NOESY spectrum was used to identify the $^1\text{H}/^1\text{H}$ through space interactions (Figure 21). The orientation of 2 protons on C-15 were determined through interactions with protons at C-12 and C-16 (α) and with those at C-24 C-6 and C-16 (β). Also, the assignment of C-26 vs. C-27 was determined.

Figure 21: NOESY spectrum of roquefortine.

The stereochemical assignments were made via coupling constants and a NOESY spectrum (Figure 21). The presence of NOE's between H-15 β and H-6, H-24, and H-12 and between H-15 α and H-16, H-12 and Me-26 and 27 resolved the stereochemistry of the 15 methylene function as well as determined the relative positions of the C-26 and C-27 methyl functions. The observation of an NOE between the H-24 proton and H-6 resolved the *Z* vs *E* protons of the terminal methylene function. The presence of an NOE between H-20 and the amine proton on position N-7 confirms the C-3, C-17 bond as *E*. In addition, the presence of an exchange peak in the NOESY spectrum between the amide proton at position N-2 and the amine proton at N-7 shows spatial proximity of these two substituents. These assignments (Table 7) resolved some minor discrepancies in the literature and confirmed the stereochemistry of roquefortine as determined by Scott et al. (1979) and Vleggaar and Wessels (1980).

Figure 22: Structure of roquefortine with stereochemistry and proper numbering.

Table 7: ^{13}C , ^1H and ^{15}N NMR Chemical Shift Assignments for Roquefortine (CDCl_3).

Position #	^{13}C (ppm from TMS)	^1H (ppm from TMS)	^{15}N (ppm)
1	166.7		
2		8.95 (br s)	136.8
3	121.5		
4	159.4		
5			146.7
6	78.4	5.62 (s)	
7		4.94 (br s)	83.0
8	149.8		
9	109.1	6.59 (d, 7.82)	
10	129.1	7.09 (dt, 7.65, 1.32)	
11	119.1	6.76 (dt, 7.51, 0.89)	
12	125.1	7.17 (dd, 7.65, 1.12)	
13	128.5		
14	61.5		
15	36.8	2.59 (α) (dd, 12.42, 5.89) 2.46 (β) (dd, 11.5, 12.42)	
16	58.8	4.05 (dd, 5.94, 11.5)	
17	111.3	6.27 (br s)	
18	125.3		
19		12.95 (br s)	167.3
20	136.8	7.70 (br s)	
21			259.2
22	135.2	7.27 (br s)	
23	40.9		
24	143.3	5.97 (dd, 10.88, 17.39)	
25	114.7	5.14 (<i>E</i>) (dd, 0.97, 10.83) 5.10 (<i>Z</i>) (dd, 0.90, 17.36)	
26	22.5	1.14 (s)	
27	22.9	1.01 (s)	

Specifically enriched sodium acetate (C-1 & C-2, 99.5% atom C) was added in 5 aliquots for different periods of time (Figure 10) to a final concentration of 1 mg per ml culture medium to cover the period of highest rate roquefortine production. 5 x 200 ml of media in Roux bottles were used per label. HPLC analysis showed that the addition of acetate had no detrimental effect on roquefortine production. The five Roux bottles were pooled giving extracts for NMR analysis containing approximately 15 mg of roquefortine

from the filtrate and 20 mg from the mycelia. NMR samples were made up to constant volume and run under identical conditions (# scans, pulse length etc.) to allow for direct comparison of peak intensities.

Figure 23: ^{13}C NMR spectra of crude filtrate extract labeled with ^{13}C -1 acetate in CDCl_3 .

Figure 24: ^{13}C NMR spectra of crude filtrate extract labeled with ^{13}C -2 acetate in CDCl_3 .

Figure 25: ^{13}C NMR of crude mycelia extract labeled with ^{13}C -1 acetate in CDCl_3 .

Figure 26: ^{13}C NMR of crude mycelia extract labeled with ^{13}C -2 acetate in CDCl_3 .

The roquefortine resonances from the crude extracts of both the filtrate and mycelia (Figures 23-26) were identified by comparison with the ^{13}C spectra obtained on the 5 mg roquefortine standard. The crude filtrate was dominated by resonances of the large unknown observed in the chromatogram and by roquefortine. The crude mycelial extract was dominated by the resonances from roquefortine and clearly shows enrichment in the methyl groups of roquefortine at 22.5 and 22.9ppm for the extract labeled with ^{13}C -2 acetate. More detailed results required purification of the individual enriched metabolites (Figures 27-28). Comparisons between the crude extracts and the later isolated compounds showed that no changes to the molecule occurred during the isolation. The isolation of the major metabolites allowed for the better detection of slight incorporation that may have been missed due to interference from other compounds in the crude extracts. The extracts were purified to remove interfering resonances and allow for easier identification of enrichments. Once the remaining resonances were identified, comparisons of intensities with spectra of unlabeled extracts showed that small incorporations (ca.1% or less) of the enriched acetate occur at C-1, C-23 and C-25 from the carboxyl of acetate, and at C-24, C-26 and 27 and C-16 from the methyl of acetate (Figure 29). This is consistent with the isoprenoid origin of the allyl side chain. Limited enrichment was observed for the portion of the molecule that arises from tryptophan and none from histidine as would be expected if these were formed early in the fermentation (before the acetate was added to the medium). Slightly different levels of incorporation were observed depending on the time of addition of the acetate, with day 3-7 and 5-9 additions being similar and the least incorporation occurring for the 7-11 day addition.

Figure 27: 3-7 day ^{13}C -1 enriched roquefortine NMR spectrum isolated from mycelia.

Figure 28: 3-7 day ^{13}C -2 enriched roquefortine NMR spectrum isolated from mycelia.

Figure 29: Incorporation pattern of ^{13}C -1 (★) and ^{13}C -2 (●) acetate into roquefortine.

V-D.2 Large unknown from filtrate - Cyclopenin:

The second major metabolite produced by the strain of *P. crustosum* studied above and found predominately in the filtrate was identified as Cyclopenin ($\text{C}_{17}\text{H}_{14}\text{O}_3\text{N}_2$). Cyclopenin was partially identified by its mass spectrum which gives a molecular weight ($M+1$) of 295 with major fragments (daughter ions) of 90, 146 and 177 and minor fragments of 118, 120, 132, 208 and 236 (Figure 30) (McCamish M and White JD, 1970).

Figure 30: LC/MS/MS of cyclophenin indicating the parent (295 M+1) and daughter ions.

Cyclophenin was later isolated and purified from culture filtrate to use as standards for mass spectrometry and NMR analysis, because standards of cyclophenin were not available for purchase. Initially NMR experiments were performed by LC/NMR on crude filtrate extracts, run in stopped flow mode to get some structural features of this unknown. The structure was confirmed on the isolated sample as above for roquefortine, the results of which are displayed in Table 8. Both the proton and carbon spectra (Figures 31 and 32) for the isolated cyclophenin are uncomplicated as expected from the structure (Figure 36). The proton spectrum does show one resonance for N-H group at 9.3ppm, a large N-Methyl resonance at 3.2ppm, and a single for the C-H at 4.2ppm. The ^{13}C spectrum shows the presence of an N-Methyl at 31.4ppm, two aromatic rings and some additional peaks due to minor contaminants from the isolation process. A COSY experiment performed in stop flow mode showed coupling relationships between the protons of the two independent aromatic rings.

Figure 31: ^1H NMR spectrum for isolated cyclophenin.

Figure 32: ^{13}C NMR spectrum for isolated cyclophenin.

Figure 33: COSY spectrum of cyclophenin (LC/NMR stopped flow).

The gradient HSQC spectrum for cyclophenin was acquired by LC/NMR (figure 34). The spectrum clearly indicates which resonances from the proton spectrum are attached to which carbon atoms. The HSQC spectrum for example shows that carbons 6-9 (from what is later determined to be the anthranilic acid derived portion of the molecule) have the aromatic protons at 7.00, 7.17, 7.20, and 7.60 respectively attached to them. The gradient HMBC spectrum (Figure 35) shows very few interactions which is consistent with the structure of the molecule. An example is the interaction of the proton on C-10 at 4.20ppm with the protons on C-14 and C-18.

Figure 34: gHSQC for cyclophenin, LC/NMR stopped flow mode CD₃CN/D₂O.

Figure 35: gHMBC for cyclophenin, LC/NMR stopped flow mode CD₃CN/D₂O.

Figure 36: Structure of cyclopenin indicating stereochemistry and numbering pattern.

Table 8: ^1H and ^{13}C NMR Assignments for Cyclopenin (CDCl_3)

Position #	^{13}C (ppm from TMS)	^1H (ppm from TMS)
2	167.6	
3	70.3	
5	166.0	
6	132.6	7.00 (dd)
7	125.3	7.17
8	120.8	7.20
9	133.1	7.60 (br t)
10	64.8	4.20 (s)
11	130.1	
12	126.8	
13	134.0	
14, 18	126.0	6.65 (d)
15, 17	128.1	7.24
16	129.1	7.33 (br t)
N-CH ₃	31.4	3.10 (s)

The enrichment pattern of cyclopenin was also observed as with roquefortine by comparing resonance intensities from labeled isolated metabolites with unlabelled ones (figures 37-38).

Figure 37: Day 3-7 ^{13}C -1 NMR spectrum for isolated cyclophenin from filtrate.

Figure 38: Day 3-7 ^{13}C - 2 NMR spectrum for isolated cyclophenin from filtrate.

V-D.3 Penitrem A

Penitrem A and its related metabolites B-F are the third most abundant compounds isolated from *P. crustosum* (DAOM 215343). The mass spectrum analysis of penitrem A was used to confirm its presence and provided a molecular weight of 634 (M+1) and only a few daughter ions (558 and 616 (major), 597, 546, 539, 370, and 296 (minor) (Figure 40).

Figure 40: MS/MS spectrum of penitrem A indicating parent (634) and daughter ions.

The biosynthesis of penitrem A was also studied by first characterizing the molecule using the complete set of NMR experiments, which were performed on a 5 mg standard sample, the results of which are displayed in Table 9. The protons spectra of penitrem A is characterized by 5 singlets corresponding to each of the 5 methyl functions on the molecule. The carbon spectrum is more complicated than the other molecules studied because of its size, but the spectrum still shows one resonance for each carbon found in the molecule. The results of the homonuclear and heteronuclear experiments are

not shown, but were used to confirm the structure of the molecule as described by de Jesus et al., 1983a and b).

Figure 41: ^1H spectrum of penitrem A in CDCl_3 .

Figure 42: ^{13}C spectrum of penitrem A in CDCl_3 .

Figure 43: Structure of penitrem A indicating stereochemistry and number pattern.

Table 9: ^1H and ^{13}C NMR Assignments for Penitrem A

Position #	^{13}C (ppm from TMS)	^1H (ppm from TMS)
1		7.83 (NH)
2	152.9	
3	119.9	
4	131.5	
5	126.0	
6	125.3	7.29 (s)
7	111.6	
8	120.3	
9	138.2	
10	34.3	3.69, 3.28 (d, $J_{AB}=17.3\text{Hz}$)
11	147.0	
12	45.9	2.96 (d, $J_{12,13} = 8.5\text{Hz}$)
13	24.2	2.39, 2.22
14	51.5	2.39
15	80.9	
16	75.7	
18	71.4	2.14 (d, $J_{18,19} = 8.1\text{Hz}$)
19	57.8	2.60
20	17.6	1.75, 1.85
21	30.3	1.5, 1.6
22	77.8	
23	64.7	
24	61.8	3.59 (d, $J_{24,25} = 2.8\text{Hz}$)
25	65.7	4.01
26	73.7	4.09
28	71.5	4.28 (t, $J = 9.0\text{Hz}$)
29	29.7	1.9, 2.35
30	27.8	1.48, 2.6
31	42.6	2.60
32	49.3	
33	107.9	5.04, 4.87
34	19.9	1.69 (s)
35	30.6	1.09 (s)
36	19.5	1.71 (s)
37	141.1	
38	112.4	5.15, 5.02
39	18.9	1.16 (s)
40	21.0	1.33 (s)

Figure 44: Day 3-7 ^{13}C -1 NMR spectra for penitrem A isolated from mycelia.

Figure 45: Day 3-7 ^{13}C -2 NMR spectra for penitrem A isolated from mycelia.

The results of the labeling experiment for penitrem A agree with those of roquefortine and cyclopenin that is the ring from tryptophan or the amino acid derived portion of the molecule received no label. Unlike roquefortine and cyclopenin, the majority of the molecule is derived from an acetate background and as a result label is found throughout the entire molecule (Figure 46).

Figure 46: Incorporation pattern of ^{13}C -1 (★) and ^{13}C -2 (●) acetate into penitrem A.

V-E Identification of minor metabolites

The minor metabolites found in both the filtrate and mycelium were also identified. This identification was performed by first analyzing filtrate and mycelial extracts containing both major and minor peaks by LC/MS and LC/NMR to obtain as much information as possible about the metabolites. This method was useful for the identification of some metabolites, but others required the isolation and removal of the major metabolites to enhance the signal of minor metabolites present in small quantities. The major peaks (roquefortine from both the filtrate and mycelium, cyclopenin from the

filtrate and penitrem A from the mycelium) were removed by isolated through prep-HPLC, leaving a mixture of minor unknown metabolites (HPLC chromatograms figures 47-48).

Figure 47: HPLC chromatogram of filtrate with cyclophenin and roquefortine removed.

Figure 48: HPLC chromatogram of mycelia with roquefortine and penitrem A removed.

V-E.1 Minor metabolite - Roquefortine D

The identification of roquefortine D is a good example of using LC/MS and LC/NMR to identify a metabolite without isolation, and in the presence of large amounts of other compounds. The analysis of the filtrate by HPLC-DAD show a small peak at 9.2 min (figure 12) that was too small, and not sufficiently resolved by HPLC from cyclopenin to isolate. Additionally, standards were not available for the compound making its identification very difficult. The same sample was then examined using LC/MS and the molecular weight ($M+1$) was determined to be 392. The fragmentation pattern was obtained by analyzing the same sample again using LC/MS/MS, the results showed daughter ions at 324, 198, 195, 130 and 110 (figure 49).

Figure 49: MS/MS of small unknown (roquefortine D) indicating parent (392) and major daughter ions.

LC/NMR was then used to determine the proton spectrum of the unknown peak at 9.2 min. The results of the ^1H NMR for the above peak clearly indicate the same resonances as present in roquefortine with a few minor differences. A new proton is present at 5ppm, which is consistent with the addition of a hydrogen atom on carbon 3. The proton on carbon 17 is no longer present in the spectrum of the small unknown, and also a new CH_2 has been formed at 3.3ppm (Figure 50).

Figure 50: ^1H NMR spectrum of roquefortine D from LC/NMR experiment (64 scans).

The molecular formula was determined to be $\text{C}_{22}\text{H}_{25}\text{N}_5\text{O}_2$, the only difference from roquefortine C was the addition of 2 hydrogen atoms upon breaking of the double bond between carbons 3 and 17 (Figure 51).

Figure 51: Structure of roquefortine D

V-E.2 16-Hydroxyroquefortine

16-hydroxyroquefortine was another minor metabolite identified using the above method. The HPLC-DAD analysis of the filtrate showed a small peak at 10.2 min on the opposite shoulder of the cyclopenin then roquefortine D was found. The LC/MS of the compound provided a molecular weight ($M+1$) of 406 which corresponded to a known compound. The MS/MS provided a spectrum with major daughter ions of 322, 193, and 129 and that looked very similar to roquefortine. The LC/NMR ^1H spectrum was useful for confirming the identity of the compound, because of both its similarity to roquefortine C and for comparison to NMR data in the literature.

V-E.3 Penitrem B-F

The five other known penitrems B-F (Figure 7) were also identified from the large number of peaks present in the HPLC-DAD chromatogram of the mycelia extract. Their identity of each was confirmed by LC/MS - MS/MS and LC/NMR. The resulting molecular weights ($M+1$: B = 584, C = 602, D = 568, E = 600, and F = 618) and fragmentation patterns along with the ^1H NMR data once analyzed against the data I obtained on penitrem A and compared to the literature provided enough evidence for their presence without the need to isolate them.

V-E.4 Cyclopenol, Viridicatol, and Viridicatin.

The 3 biosynthetically related compounds cyclopenol, viridicatol, and viridicatin (Figure 52) were also identified in the mycelia extracts by comparison of their mass

spectrum and NMR data to cyclopenin and to the literature. All three were present in small quantities only.

Figure 52: Structure of a) cyclopenol, b) viridicatol, and c) viridicatin

V-E.5 Unknown Metabolites.

In addition to the metabolites listed above a number of compounds were also detected for which no literature matches could be found. Mass spectrum and NMR data for one compound of interest indicated that it was related to roquefortine. The molecular weight was determined to be 462 and the MS/MS spectrum was very roquefortine-like in nature with the daughter ions matching roquefortine with the addition of a 73 mass unit. The ^1H LC/NMR spectrum also looked like the proton spectrum of roquefortine but due to the relatively small amounts of the compound, a carbon spectrum and 2D NMR data could not be acquired. The result was an unknown compound with a very similar structure and probably origin to roquefortine. The production of more materials in the future should lead to its proper characterization and identification.

From the 20 or more different peaks present in the initial chromatograms of the filtrate and mycelium of *P. crustosum* (DAOM 215343) 13 different known compounds were identified and are listed in Table 10 below. The remaining peaks or minor metabolites present could not be identified because of their low concentrations in the

extracts. Mass spectrum data was obtained on some compounds but it did not provide enough information to make a positive identification.

Table 10: Metabolites identified from each *Penicillium* strain tested.

Compound	Fungal Strains			
	<i>P. crustosum</i> (215343)	<i>P. roqueforti</i> (229761)	<i>P. crustosum</i> (194930)	<i>P. roqueforti</i> (225571)
Roquefortine – filtrate	17.51	13.79	5.75	5.86
Roquefortine – mycelia	8.79	4.25	1.02	0.67
Roquefortine D – filtrate	+	+	+	+
Roquefortine D – mycelia	++	+	+	+
16-hydroxyroq – mycelia	+	-	-	-
Penitrem A – filtrate	+	+	-	-
Penitrem A – mycelia	3.36	1.53	-	-
Penitrem B – mycelia	+	+	-	-
Penitrem C – mycelia	+	+	-	-
Penitrem D – mycelia	+	+	-	-
Penitrem E – mycelia	++	+	-	-
Penitrem F – mycelia	+	+	-	-
Cyclopenin – filtrate	22.52	-	-	-
Cyclopenin – mycelia	+	-	-	-
Cyclopenol – filtrate	+	-	-	-
Viridatol – mycelia	+	-	-	-
Viridicatin – mycelia	+	-	-	-

(++) = mg/L, (+) = µg/L, (-) = not detected

Grown in 1L Roux bottles (200 ml media) for 12 days on Czapek-Dox medium

mycelia extracts = mg/g

filtrate extracts = mg/L

VI. DISCUSSION

VI-A Extraction and analysis

The previously published extraction methods (Mantle PG et al. 1983; Scott PM et al. 1977) were inadequate for the purposes of this project. These methods used a variety of solvents but very few involve the use of a pH adjustment. The pH of the filtrate is very important because of the instability of the *Penicillium* metabolites under acidic conditions. Both roquefortine and penitrem A have been shown to rapidly degrade in acid. Roquefortine degrades by cleaving off the dimethylallylpyrophosphate derived portion of the molecule resulting in a new product with a molecular weight of 320 (Scott PM et al., 1979). Penitrem A has been shown to be extremely sensitive to acid degradation, even with only trace amounts of acid found in CDCl_3 . Degradation was recorded by changes to both its NMR and mass spectrum, and is characteristic of a colour change from clear to blue color in solution.

Also of importance for the extraction procedure, was the sensitivity to light of the metabolites. As previously stated, the results of the current study (a drop from 85 % recovery for a reduced light environment to 50 % recovery for extraction performed under normal light), and those of Scott et al. 1979 and Norozian et al. 1998 are conclusive in showing that roquefortine and probably other metabolites are sensitive to light. These results clearly indicate that the use of reduced light is required for proper extractions. The new method developed eliminates these concerns by performing all extractions in the dark and by adjusting filtrate to pH12 where there is a reduced risk of acid degradation of the metabolites.

The current methods of analyses were only useful for one or two metabolites at a time or were not optimized for *Penicillium* metabolites as with the case of the Frisvad (1987) method, which at the time of the work was the only published method for analysis of all metabolites. The Frisvad method also uses 0.05 % trifluoroacetic acid, which causes problems for the acid sensitive compounds produced by these species. The new HPLC conditions using an acetonitrile/water gradient with ammonium acetate provides a method of analyzing strains for their complete metabolite profiles.

V-B Growth Conditions

Roux bottles were used by other researchers, and were chosen to help improve roquefortine and other metabolite yields. The growth conditions in Roux bottles are very different than in shake flasks because of the stationary nature of the bottles. Roux bottles provided low but changing oxygen tension, with the hope that at some point during the fermentation the right conditions for metabolite production occur. The low oxygen saturation inside the Roux bottles is important because as previously discussed these species of fungi can grow well at low levels. This is evident from their growth in silage where oxygen saturation is often too low for most species of fungi to survive.

The addition of glycerol to the medium was also used as a means of lowering water activity and increasing metabolite yields. The use of glycerol in media is a common practice and its addition is often an effective way to increase metabolite production (Miller JD and Blackwell BA, 1986). As evident from the results of the

glycerol experiment, the addition of various amounts did not result in consistently higher yields, and its use was subsequently dropped.

The phosphorus content of the medium was reduced in an attempt to initiate and ultimately increase metabolite yields. As previously stated, fungi produce metabolites when they are stressed, and phosphorus has been shown to play an important role in secondary metabolite production for some fungal species (Iwai Y and Omura S, 1981). Changing the phosphorus content of the medium clearly indicated that it is not the limiting factor for metabolite production and as a result was left unchanged for future experiments.

Both species of fungi used in this study grew very well on the Czapek-Dox medium, which contained sucrose as its carbon source. The results of the sugar analysis indicated that carbon was most likely the limiting factor for secondary metabolite production by these organisms. This was evident because the consumption of the available sugars corresponded with the maximum mycelial dry weight and greatest increase in roquefortine production. A carbon limitation does not necessarily mean a complete exhaustion of all available carbon sources, but refers to the limitation of readily metabolizable carbon sources such as sucrose, glucose and fructose for this media. This limitation places a constraint on primary metabolism for this organism and as a result induces secondary metabolism. Carbon limitation is common for fungi that mainly produce secondary metabolites derived from amino acids, as is the case for the species used for this study (Miller JD, 2000).

The nitrogen content of the medium is thought to be important for secondary metabolite production because it is often the limiting resource for primary metabolism. The graph (figure 10) indicates that this is probably not the case for the strains examined in this study. The amount of total nitrogen in the culture is reduced to almost zero by day 3, which does not correspond to the maximum production of roquefortine. The available nitrogen was used up by day 3 in the production of amino acids which the organism stores for later use in metabolite production. This was evident from the labeling experiments (described later) which showed very little label in the amino acid derived portions of the metabolites, even when label was added early in the fermentation. Additionally, the amount of total nitrogen slightly increases at day 9, which at first seem to be an analytical error. This slight increase in total nitrogen present in the cultures is probably the result of cells dying and releasing stored nitrogen back into the medium (Wicklow D, 1981). This is common among fungi such as *Penicillium* which release nitrogen and other nutrients back into the environment for the benefit of the colony.

The pH of the fermentation can have an affect on the production of secondary metabolites. An example of this is the production of deoxynivalenol (DON), where a drop below pH4 results in an almost complete stop to its production (Blackwell BA and Miller JD, 1985). The initial change in pH from neutral to acidic does not appear to affect metabolite production and hence there is no evidence that it needs to be controlled through the addition of a base such as sodium hydroxide. There are two important points to consider when examining the lack of a pH response of the cultures. First is that these organism can tolerate acid conditions as is evident from there presence in silage treated

with lactic acid producing bacteria. The second point is that the organism returns that pH to neutral naturally before the maximum production of metabolites occurs. This is very important because as noted above both roquefortine and penitrem A are very acid sensitive.

Mycelial dry weight is important because it provides valuable information about the state of the organism. The graph (Figure 10) indicates the different life stages for the cells. The first is the rapid growth stage from day 0 – day 6 and corresponds to primary metabolism by the organism. During this stage very little secondary metabolite production occurs as evident by the low levels of roquefortine produced. The next stage occurs when the carbon source is exhausted and secondary metabolism commences, this corresponds to the drop in mycelial weights evident from day 6 – 12. During this stage the organism has greatly increased its secondary metabolite production, the slope of the roquefortine production graph demonstrates this point. The final stage involves the death of cells in the organism which corresponds to the reduced dry mycelial weight at days 9 and 12. The production of roquefortine and other metabolites drops off rapidly at day 12 which corresponds to the death of majority of the cells.

VI-C Identification, biosynthesis and ^{13}C labeling of major metabolites

Roquefortine

The roquefortine produced by DAOM 215343 was found in relatively equal amounts in both the filtrate and the mycelium. This is in contrast to the literature where most studies have reported the majority of roquefortine as being sequestered in the

mycelia (Mantle PG et al., 1983). The production of roquefortine by this strain, which was the most productive out of all four strains used for this study, reached a maximum of approximately 20 mg/l in the filtrate. Additionally, the mass spectrum data obtained for roquefortine was compared to the literature, and was helpful for not only identifying roquefortine but other minor structurally related compounds (Cole RJ and Cox RH, 1981).

The biosynthesis of roquefortine, examined through the addition of specifically enriched ^{13}C sodium acetate to cultures grown in Roux bottles, was carried out for two main reasons. The first was to examine some discrepancies in the literature regarding the exact biosynthesis of the molecule. The tryptophan and histidine origin are widely accepted, and have been confirmed by the addition of ^{14}C and ^2H labeled tryptophan, and histidine to cultures. The conflicting reports in the literature occur as to the incorporation of the hydrogen at C-6 and to the origin of the linking function C-1 and C16 (Barrow KD et al, 1979; Bhat B et al., 1990 and 1993). The second reason was to enrich metabolites with a view of increasing the possibility of finding toxic compounds that might be produced in low quantities.

Enrichment from the acetate was not observed for the portion of the molecule that arises from tryptophan and histidine even for cultures where label was added as early as day 3. This indicated that the amino acids used for the biosynthesis of these compounds must be produced very early in the fermentation and stored for later use. This is in contrast to other fungal metabolites that use precursors which are produced as they are needed for secondary metabolite production (Miller JD and Blackwell BA, 1986). Slightly different levels of incorporation were observed depending on the time of addition

of the acetate, with least label incorporated into the cultures where it was added from day 7-11. No differences in labeling pattern were seen between roquefortine from mycelia or filtrate. In both cases, label was primarily incorporated into the dimethylallyl-pyrophosphate portion arising from one molecule of mevalonic acid lactone, and secondarily into C-1 and C-6 of roquefortine (figure 29), which agrees with the results of Gorst-Allman (1982) who added double labeled ^{13}C -1 & C-2. Comparisons between the crude extracts and the later isolated compounds showed that no changes to the molecule occurred during the isolation. The isolation of the major metabolites allowed for the better detection of slight incorporation that may have been missed due to interference from other compounds in the crude extracts. While some incorporation was shown for roquefortine, greater enrichment might be achieved in submerged culture, specifically for the incorporation of ^{15}N enriched amino acids. This is due to the dynamics of surface culture biomass and problems associated with uptake of exogenously administered precursors.

Cyclopenin

The majority of the cyclopenin produced was released into the filtrate upon production, as almost none was found in the mycelium of the organism. Cyclopenin was a known metabolite produced by *P. crustosum* but was not expected in such high quantities. It has been more commonly isolated from cultures of *Penicillium cyclopium* and *P. viridicatum* (Frisvad JC and Samson RA, 1991).

Cyclopenin is derived biosynthetically from all the carbon atoms of anthranilic acid, L-phenylalanine and from the methyl group of methionine (Kirby GW and

Narayanaswami S, 1976). N-1 (Figure 36) is derived from anthranilic acid while N-4 is from L-phenylalanine (Nover L and Luckner M, 1969; Mohamed ZS et al., 1984). Cyclophenin like roquefortine received very little of the acetate label because of its amino acid origin. The enrichment pattern for the ^{13}C 1 & 2 sodium acetate is shown in figure 39. The only areas of cyclophenin to receive the label were the carbonyl carbons, which originate ostensibly from anthranilic acid and the N-CH₃. However, if anthranilic acid is formed by day 3 of the fermentation the reason these carbons receive label is not known. Once again improvements to the enrichment levels could be achieved by the use of specifically labeled amino acids.

The toxicity of cyclophenin is poorly understood, with very little vertebrate data available. Cyclophenin was administered to day old chicks at levels ranging from 25 to 500mg/kg of body weight. The results showed that only the 250 and 500mg/kg doses had any significant effects on the chicks and that all had recovered within 18 hours. The symptoms observed included drowsiness, prostration, ataxia and intoxication (Cutler HG et al, 1984). Cyclophenin was also tested by the same group against beans where it caused malformation of leaves and necrosis and stunting in corn but had no effect on tobacco plants. Kusano et al. (2000) showed that cyclophenin has very little biological activity when tested against the nematode *Pratylenchus penetrans*. Additionally, they showed that cyclophenin had no effect on the growth of rice seedlings. Cyclophenin was shown to have slight antibiotic activity against *Micrococcus pyogenes* var. *aureus* and *Escherichia coli* (Cole and Cox, 1981).

Penitrem A

The majority of the penitrems remained sequestered in the mycelium but small amounts were also detected in the filtrate which is in contrast to the report of Mantle et al. (1983) where they found 99% in the mycelium of the strain they tested. Penitrem A is a difficult compound to work with for a number of reasons, the first as previously noted is that it is very acid sensitive, and rapidly degrades with only trace amounts of acid in CDCl_3 . The results of the NMR assignments agreed with the literature as to the structure and configuration of penitrem A (Figure 40) (de Jesus EA et al., 1982, 1983a, b and c). The large amount of label found in penitrem A helped to answer the important question of the fate of the ^{13}C label. Initially, after only roquefortine was studied using NMR we believed that the incorporation of ^{13}C acetate was poor in all metabolites. The discovery of label throughout penitrem A and in related metabolites B-F in an approximate 6-1 ratio vs. roquefortine, explains why so little acetate was incorporated into the other metabolites. The level of enrichment is similar in cyclophenin, roquefortine and penitrem indicating that all metabolite are likely being synthesized at similar rates and at the same time. The labeling pattern is consistent with that observed by Mantle PG et al. (1983), de Jesus EA et al. (1982) and Laws I and Mantle PG (1989), where we also observed $^{13}\text{C}/^{13}\text{C}$ coupling resulting from adjacent labels though cyclization of rings.

VI-D Identification of minor metabolites

Roquefortine D

The identification of roquefortine D, a minor metabolite found in both the filtrate and mycelium of cultures of *P. crustosum* DAOM 215343 provides an excellent example

of the power of the new analytical technique describe in this study. The molecular weight was determined to be 392 (M+1) which agree with the literature (Ohmomo S et al., 1978). The identity was confirmed by MS/MS which showed the addition of 2 hydrogen atoms occurred on the histidine derived portion of the molecule, and the use of LC/NMR provided the structure of the metabolite as roquefortine D, without the need to isolate the compound. The configuration of the ^1H of C-3 was not determined.

A few factors need to be addressed regarding the use of LC/NMR, the retention times of the compounds are slightly longer on the LC/NMR system. This is due to a both the use of deuterated solvents for the mobile phase which have slightly different properties then the normal HPLC grade acetonitrile, and also the length of the tubing leading into the flow probe impacts on the retention times of the metabolites (Roberts JK and Smith RJ, 1994; Griffiths, 1995). Comparison of the new chromatograms from the LC/NMR system with the UV trace from the HPLC-DAD were almost identical, indicating that comparison could be made and conclusion drawn about compounds present in both.

16-Hydroxyroquefortine

16-hydroxyroquefortine was another minor metabolite identified using LC/NMR and LC/MS/MS. Very little is known about 16-hydroxyroquefortine, and it was only present in small quantities, but because of its similarity to roquefortine it may contribute to the overall toxicity.

Penitrem B-F

All 5 of penitrem B-F were also identified by comparison of their mass spectrum and NMR data to the literature. The importance of these tremorgenic compounds to the toxicity of cattle is unclear. No toxicity data has been reported on any of these but the biosynthetic origin and similarity to penitrem A indicate that they probably are of similar toxic nature to penitrem A and that their combined relative amounts make them important for future study.

Cyclophenol, Viridicatol, and Viridicatin.

These 3 biosynthetically related compounds were also identified but only in relatively low amounts. This was somewhat unexpected due to the fact that they are closely related to cyclophenin (Cole RJ and Cox RH, 1981), which was found in very high quantities. Cyclophenol is very similar to cyclophenin, and both act as biosynthetic precursors to viridicatol and viridicatin. The reason for this variance in amounts present is unclear, but raises a number of important questions about the conditions needed for their production and also their about their toxicity, of which little is known.

Unknown Metabolites.

In addition to the metabolites identified in Table 10, other compounds were also detected but could not be identified because of relative amounts and also a search of the literature provided no positive matches. The unknown roquefortine related metabolite with the molecular weight of 462 (M+1) was present in large enough quantities for identification for a known metabolite, but as indicated no literature matches could be

found. The result was an unknown minor metabolite that is very similar to roquefortine and that requires more material for identification in the future. In addition to this unknown metabolite, small quantities of other compounds which were probably penitrem derivatives were also detected.

Analysis of *Penicillium* strains to determine metabolite profiles.

The analysis of all strains used in this study showed that roquefortine was identified in both the filtrate and mycelium as a major metabolite. Penitrem A was also found to be present in two of the original four test strains, with the majority occurring in the mycelia as expected. PR toxin and mycophenolic acid, two expected metabolites from *Penicillium* species, were not detected in any of these four original strains. While this is common for *P. crustosum*, it is unusual for *P. roqueforti* and gives support to the need for more research on these two species of fungi (Frisvad JC and Filtenborg O, 1989).

To our knowledge only one other paper exists with data on Canadian strains of *Penicillium* species, and they were isolated from wood sources and only report presence and absence of compounds (Seifert KA and Frisvad JC, 2000). The results of the analysis of strains from Canadian sources indicated lower metabolite yield than strains from France and New Zealand (Scott PM et al. 1977, and Mantle PG et al. 1983). This result does not indicate a failure by us to achieve their yields, but indicates that Canadian strains are not as productive as others from around the world. The idea of strains from different areas producing different metabolites and different yields has been shown for a number of other fungal species. The metabolite production for *Fusarium graminearum*

was shown to be very different for strains from China vs. Mexican and other North American strains (Miller et al. 1991). Additionally, when fumonisin, a toxic mycotoxin was tested from corn samples collected from Ontario farms, the results indicated very low levels of fumonisin compared to corn from other countries around the world. The fungal species were then isolated from the corn and when grown in liquid culture the Canadian strains produced similar results to South African, European and Asian strains (Miller JD et al., 1995). The reason for the lower metabolite yields has to do with a number of factors including environmental and the relatively age of the problem of increased fungal contamination, caused by changes to the growth and storage of feed by farmers in these areas.

SUMMARY

1. Determined the optimal growth conditions for the strain of *P. crustosum* DAOM 215343 used in this study and gained a much better knowledge of what conditions are required for production of secondary metabolites. One major finding was that carbon is the limiting factor for secondary metabolite production and not nitrogen. Additionally, determined that Canadian strains of *Penicillium crustosum* and *roqueforti* are not as productive at producing mycotoxins as strains isolated from Europe, and New Zealand.
2. Developed an extraction method that accounts for the sensitivity of these compounds to acid conditions and to light. Also developed an analytical method combining three techniques not previously used for analysis of these fungal species (HPLC, LC/MS/MS and LC/NMR), this provided:
 - a) A method for the rapid screening of fungal strains to determine metabolite profiles.
 - b) A new method of identifying major and minor metabolites without the need for isolation, which can result in changes to the metabolites.
3. The use of trace ^{13}C acetate added to the cultures showed simultaneous synthesis of all major compounds and the origin of compounds was in agreement with published biosynthesis studies using NMR and radiolabels. Very little label was incorporated into amino acid derived portions of the molecules, even when label was added early in the fermentation. This point is important because it indicated that the organisms must produce these secondary metabolite precursors early and store them for later use. The label was effectively incorporated into all 3 metabolites simultaneously, and

provided evidence for the use of more complex labels and ^{15}N labels for future experiments.

4. The cause of the cattle toxicosis is unclear but is probably the result of synergistic effects of fungal metabolites, as the individual metabolites detected cannot account for the symptoms displayed by sick animals. It is also clear that more toxicology testing is required to better examine the individual metabolites, and to test groups of metabolites together.

FUTURE WORK

LC/NMR and LC/MS/MS need to be used to screen more isolates from contaminated silage to determine metabolite profiles. This will provide additional information as to the frequency of occurrence and identity of metabolites from *Penicillium* species isolated from silage. Additionally, the silage used to obtain the isolates needs to be analyzed for secondary metabolites to determine differences between raw material and isolated fungal strains. Once the strains have been screened the next step is to select the best strains as determined by the greatest production of secondary metabolites. These strains then need to be grown in the fermenter so that, larger quantities of the metabolites can be produced. Additionally, the growth in the fermenter will allow for the addition of ^{15}N labeled amino acids for more complete biosynthesis studies. The fermenter will also provide the materials necessary for proper toxicological testing, so that positive associations can be made between cattle symptoms and metabolites in their feed, which will ultimately help in the understanding of why these metabolites are causing the problems in cattle.

REFERENCES

1. Adams E (1975): "Long-term toxicity study of mycophenolic acid in rabbits". **Toxicology of Applied Pharmacology**. 34: 509-512.
2. Arnold DL, Scott PM, McGuire PF, Hawig J, and Nera EA (1978): "Acute toxicity studies on roquefortine and PR toxin, metabolites of *Penicillium roqueforti* in the mouse". **Cosmet. Toxicol.** 16: 369-371.
3. Arp LH, and Richard JL (1979): "Intoxication of dogs with the mycotoxin penitrem A" **JAVMA**. 175(6): 565-566.
4. Arp LH, and Richard JL (1981): "Experimental intoxication of guinea pigs with multiple doses of the mycotoxin, penitrem A". **Mycopathologia**. 73: 109-113.
5. Auerbach H, Oldenburg E, and Weissbach F (1998): "Incidence of *Penicillium roqueforti* and roquefortine C in silages". **Journal of Sci. Food Agric**. 76: 565-572.
6. Bachmann RW, and Canfield Jr. DE (1996): "Use of an alternative method for monitoring total nitrogen concentrations in Florida lakes". **Hydrobiologia**. 232: 1-8.
7. Barrow KD, Colley PW and Tribe DE (1979): "Biosynthesis of the neurotoxin alkaloid roquefortine". **J.C.S. Chem. Comm.** 225-226.
8. Benitez A, Martin-Gonzalez A, Gilardi P, Soto T, Rodriguez de Lecea J, and Gutierrez JC (1994): "The ciliated protozoa *Tetrahymena thermophila* as a biosensor to detect mycotoxins". **Letters in Applied Microbiology**. 19: 489-491.
9. Betina V (1995): "Differentiation and secondary metabolism in some prokaryotes and fungi". **Folia. Microbiol.** 40(1): 51-67.
10. Bhat B, Harrison DM, and Lamont HM (1990): "The biosynthesis of the tryptophan-derived mould metabolites roquefortine and aszonalenin". **J. Chem. Soc., Chem. Commun.** 1518-1519.
11. Bhat B, Harrison DM, and Lamont HM (1993): "The biosynthesis of the mould metabolites roquefortine and aszonalenin from L-(2, 4, 5, 6, 7-²H₅) tryptophan". **Tetrahedron**. 49(46): 10663-10668.
12. Blackwell BA, Miller JD, and Greenhalgh R (1985): "¹³C NMR study of the biosynthesis of toxins by *Fusarium graminearum*". **The Journal of Biological Chemistry**. 260(7): 4243-4247.

13. Blackwell BA, Miller JD, and Savard ME (1994): "Production of carbon14-labeled fumonisin in liquid culture". **Journal of AOAC International**. 77(2): 506-511.
14. Boysen ME, Jacobsson KG, and Schnürer J (2000): "Molecular identification of species from the *Penicillium roqueforti* group associated with spoiled animal feed". **Applied and Environmental Microbiology**. 66(4): 1523-1526.
15. Boysen ME, Skouboe P, Frisvad J, and Rossen L (1996): "Reclassification of the *Penicillium roqueforti* group into three species on the basis of molecular genetic and biochemical profiles". **Microbiology**. 142: 541-549.
16. Carter SB, Franklin TJ, Jones DF, Leonard BJ, Mills SD, Turner RW, and Turner WB (1969): "Mycophenolic Acid: An anti-cancer compound with unusual properties". **Nature** 223: 848-850.
17. Chang SC, Lu CY, Li SY and Wei YH (1991a): "Potentiation effect of corn extract on the production of eremofortin C, EC oxidase, and PR toxin by *Penicillium roqueforti*". **Proceedings of the National Science Council, ROC. Part B: Life Sciences**. 15(3): 153-159.
18. Chang SC, Wei YH, Wei DL, Chen YY, and Jong SC (1991b): "Factors affecting the production of eremofortin C and PR toxin in *Penicillium roqueforti*". **Applied and Environmental Microbiology**. 57(9): 2581-2585.
19. Chen FC, Chen CF and Wei RD (1982): "Acute toxicity of PR toxin, a mycotoxin from *Penicillium roqueforti*". **Toxicol**. 20: 433-441.
20. Cole RJ and Cox RH (1981): *Handbook of Toxic Fungal Metabolites*. Academic Press, New York.
21. Cole RJ, Dorner JW, Cox RH, and Raymond LW (1983): "Two classes of alkaloid mycotoxins produced by *Penicillium crustosum* thom isolated from contaminated beer". **J Agric Food Chem**. 31(3): 655-7.
22. Crumpton WG, Isenhardt TM, and Mitchell PD (1992): "Nitrate and organic N analyses with second-derivative spectroscopy". **Limnol. Oceanogr**. 37(4): 907-913.
23. Cutler HG, Crumley FG, Cox RH, Wells JM, and Cole RJ (1984): "The biological properties of cyclopenin and cyclopenol". **Plant and Cell Physiology**. 25(2): 257-263.
24. Day JB, and Mantle PG (1982): "A comparative study of sheep and pigs given the tremorgenic mycotoxins verruculogen and penitrem A". **Research in Veterinary Science**. 33: 183-187.

25. de Jesus AE, Gorst-Allman CP, Steyn PS, van Heerden FR, Vleggaar R, and Wessels PL (1983a): "Tremorgenic mycotoxins from *Penicillium crustosum*. Biosynthesis of penitrem A". **J. Chem. Soc. Perkin Trans. I.** 8: 1863-1868.
26. de Jesus AE, Hull WE, Steyn PS, van Heerden FR, Vleggaar R, and Wessels PL (1982): "High-field ^{13}C NMR evidence for the formation of 1,2 - ^{13}C acetate from 2- ^{13}C acetate during the biosynthesis of penitrem A by *Penicillium crustosum*". **J. Chem. Soc., Chem Commun.** 337: 837-838.
27. de Jesus AE, Steyn PS, van Heerden FR, Vleggaar R, and Wessels PL (1983b): "Tremorgenic mycotoxins from *Penicillium crustosum*. Isolation of penitrem A-F and the structure elucidation and absolute configuration of penitrem A". **J. Chem. Soc. Perkin Trans. I.** 8: 1847-1856.
28. de Jesus AE, Steyn PS, van Heerden FR, Vleggaar R, and Wessels PL (1983c): "Tremorgenic mycotoxins from *Penicillium crustosum*. Structure elucidation and absolute configuration of penitrem B-F". **J. Chem. Soc. Perkin Trans. I.** 8: 1857-1862.
29. Domain AL (1989): "Functions of secondary metabolites". In: Genetics and Molecular Biology of Industrial Microorganisms (eds. Hershberger CL, Queener SW and Hegeman H). American Society of Microbiology, Washington DC, USA: 1-11.
30. Dörge T, Carstensen JM, and Frisvad JC (2000): "Direct identification of pure *Penicillium* species using image analysis". **J Microbiol Methods.** Jul;41(2): 121-33.
31. El-Banna AA, and Leistner L (1988): "Production of penitrem A by *Penicillium crustosum* isolated from foodstuffs". **International Journal of Food Microbiology.** 7: 9-17.
32. Engel G, von Milczewski KE, Prokopek D, and Teuber M (1982): "Strain-specific synthesis of mycophenolic acid by *Penicillium roqueforti* in blue-veined cheese". **Applied and Environmental Microbiology.** 43(5): 1034-1040.
33. Environmental Protection Agency (EPA) (1997) Final Risk Assessment *Penicillium roqueforti*.
34. Ferree MA, and Shannon RD (2001): "Evaluation of a second derivative UV/visible spectroscopy technique for nitrate and total nitrogen analysis of wastewater samples". **Wat. Res.** 35(1): 327-332.
35. Finoli C, Vecchio A, Galli A, and Dragoni I (2001): "Roquefortine C occurrence in blue cheese". **J Food Prot.** 64(2): 246-51.

36. Frank HK, Orth R, Ivankovic S, Kuhlmann M, and Schmahl D (1977): "Investigations on carcinogenic effects of *Penicillium caseicolum* and *P. roqueforti* in rats". **Experientia**. 33: 515-516.
37. Frisvad JC, and Thrane U (1987): "Standardized HPLC of 182 mycotoxins and other fungal metabolites based on alkylphenone retention indices and UV-VIS spectra (DAD)". **J Chromatography**. 28:404(1): 195-214.
38. Frisvad JC, and Filtenborg O (1989): "Terverticillate *Penicillia*: chemotaxonomy and mycotoxin productions". **Mycologia**. 81(6): 837-861.
39. Frisvad JC, Samson RA (1991): "Filamentous fungi in foods and feeds: ecology, spoilage, and mycotoxin production." In Handbook of Applied Mycology Volume 3. Marcel Dekker Inc. New York.
40. Gorst-Allman CP, Steyn PS, and Vleggaar R (1982): "The biosynthesis of roquefortine. An investigation of acetate and mevalonate incorporation using high field NMR spectroscopy". **J. Chem. Soc., Chem. Commun**. 652-653.
41. Gravesen S, Frisvad JC, Samson RA and Munksgaard. 1994. Microfungi. Special-Trykkeriet Viborg a/s. Denmark.
42. Griffiths (1995): "Optimization of NMR and HPLC conditions for LC-NMR". **Analytical Chemistry**. 67(22): 142-145.
43. Gummert JF, Ikonen T, and Morris RE (1999): "Newer immunosuppressive drugs: a review". **J Am Soc Nephrol**. 10(6): 1366-1380.
44. Häggblom P (1990): "Isolation of roquefortine C from feed grain". **Applied and Environmental Microbiology**. 56(9): 2924-2926.
45. Hawksworth DL (1991): "The fungal dimension of biodiversity: Magnitude, significance, and conservation". **Mycological Research**. 95(6): 641-655.
46. Hayes AW, and Hood RD (1978): "Effects of prenatal administration of penicillic acid and penitrem A to mice". **Toxicon**. 16(1): 92-96.
47. Hayes AW, Phillips RD, and Wallace LC (1977): "Effect of penitrem A on mouse liver composition. **Toxicon**. 15(4): 293-300.
48. Hayes AW, Presley DB, and Neville JA (1976): "Acute toxicity of penitrem A in dogs". **Toxicology and Applied Pharmacology**. 35(2): 311-320.
49. Hocking AD, Holds K, and Tobin NF (1988): "Intoxication by tremorgenic mycotoxin (penitrem A) in a dog". **Australian Veterinary Journal**. 65(3): 82-85.

50. Iwai Y and Omura S (1981): "Culture conditions for screening of new antibiotics". **Japan Antibiotics Research Association**. 35: 123-160.
51. Janzen D (1977): "Why fruits rot, seeds mold, and meat spoils". **Amer. Natur.** 111: 691-713.
52. Kirby GW, and Narayanaswami S (1976): "Stereochemical studies on the biosynthesis of the $\alpha\beta$ -didehydro-amino-acid units of mycelianamide, cyclopenin, and cyclopenol". **J.C.S. Perkin 1**. 1564-1567.
53. Kopp-Holtwiesche B, and Rehm HJ (1990): "Antimicrobial action of roquefortine". **JEPTO**. 10(1-2): 41-44.
54. Kusano M, Koshino H, Uzawa J, Fujioka S, Kawano T, and Kimura Y (2000): "Nematicidal alkaloids and related produced by the fungus *Penicillium simplicissimum*". **Biosci. Biotechnol. Biochem.** 64(12): 2559-2568.
55. Lapont P, Debeaupuis JP, Gaillardin M, and Payen J (1979): "Production of mycophenolic acid by *Penicillium roqueforti* strains". **Applied and Environmental Microbiology**. 37(3): 365-368.
56. Laws I and Mantle PG (1989): "Experimental constraints in the study of the biosynthesis of indole alkaloids in fungi". **Journal of General Microbiology**. 135: 2679-2692.
57. Lee YHW, Fang SC, and WEI RD (1984): "The effects of *Penicillium roqueforti* toxin on the activity of rat hepatic DNA polymerases". **Toxicology**. 33: 43-57.
58. Levine JS and Miller KR (1991): *Biology. Discovering Life*. D.C. Heath and Company. Toronto, Canada.
59. Lowes N (1992): "Roquefortine identified as a major differential diagnosis in suspected strychnine poisoning in dogs". **Can Vet J**. 33: 193.
60. Mantle PG, Mortimer PH, and White EP (1978): "Mycotoxic tremorgens of *Claviceps paspali* and *Penicillium cyclopium*: a comparative study of effects on sheep and cattle in relation to natural staggers syndromes". **Research in Veterinary Science**. 24(1): 49-56.
61. Mantle PG, Perera KPWC, Maishman NJ and Mundy GR (1983): "Biosynthesis of penitrems and roquefortine by *Penicillium crustosum*". **Applied and Environmental Microbiology**. 45(5): 1486-1490.
62. Marinari R, Fleischmajer R, Schragger AH, and Rosenthal AL (1977): "Mycophenolic acid in the treatment of psoriasis: long-term administration". **Arch Dermatol**. 113(7): 930-2.

63. Miller JD (2000): "Screening for secondary metabolites". (eds) Hyde KD and Pointing SB: In *Marine Mycology: "A practical Approach. Fungal Diversity Research Series 1*, Fungal Diversity Press, Hong Kong 158-171.
64. Miller JD, and Blackwell BA (1986): "Biosynthesis of 3-acetyldeoxynivalenol and other metabolites by *Fusarium culmorum* HLX 1503 in a stirred jar fermentor". **Canadian Journal of Botany**. 64: 1-5.
65. Miller JD, and Greenhalgh R (1991): "Trichothecene chemotypes of three *Fusarium* species". **Mycologia**. 83(2): 121-130.
66. Miller JD, Jones EBG, Moharir YE, and Findlay JA (1985): "Colonization of wood blocks by marine fungi in Langstone harbour". **Botanica Marina**. 28: 251-257.
67. Mills JT, Seifert KA, Frisvad JC, and Abramson D (1995): "Nephrotoxicogenic *Penicillium* species occurring on farm-stored cereal grains in western Canada". **Mycopathologia**. 130: 23-28.
68. Mohamed ZS, Todorova-Draganova RW, and Luckner M (1984): "Nuclear inheritance of the biosynthesis of cyclopenin and cyclopenol in *Penicillium cyclopium*". **Zeitschrift für Allgemeine Mikrobiologie**. 24(9): 615-618.
69. Müller HM, and Amend R (1997): "Formation and disappearance of mycophenolic acid, patulin, penicillic acid and PR toxin in maize silage inoculated with *Penicillium roqueforti*". **Arch. Anim. Nutr.** 50: 213-225.
70. Noroozian E, Lagerwerf F, Lingeman H, Brinkman UATH, and Kerkhoff MAT (1998): "Determination of roquefortine C in blue cheese using on-line column-switching liquid chromatography". **Journal of Pharmaceutical and Biomedical Analysis**. 17: 1215-1223.
71. Nout MJR et al. (1993): "Fungal growth in silages of sugarbeet pulp and maize". **J Agric Sci Camb**. 121: 323-326.
72. Nover L, and Luckner M (1969): "On the biosynthesis of cyclopenin and cyclopenol, benzodiazepine alkaloids from *Penicillium cyclopium* westling". **European J. Biochem**. 10: 268-273.
73. Ohmomo S, Sato T, Utagawa T, and Matazo A (1975): "Isolation of festuclavine and three new indole alkaloid, roquefortine A, B and C from the cultures of *Penicillium roqueforti*". **Journal of Agric. Chem. Soc. Japan** 49: 615-623.
74. Ohmomo S, Oguma K, Ohashi T, and Matazo A (1978): "Isolation of a new indole alkaloid, roquefortine D, from the cultures of *Penicillium roqueforti*". **Agric. Biol. Chem**. 42(12): 2387-2389.

75. Penny RHC, O'Sullivan BM, Mantle PG, and Shaw BI (1979): "Clinical studies on tremorgenic mycotoxicoses in sheep". **The Veterinary Record**. 27: 392-394.
76. Peterson DW, Penny RHC, Day JB, and Mantle PG (1982): "A comparative study of sheep and pigs given the tremorgenic mycotoxins verruculogen and penitrem A". **Research in Veterinary Science**. 33: 183-187.
77. Pitt, J.I. (1979): "*Penicillium crustosum* and *P. simplicissimum*, the correct names for two common species producing tremorgenic mycotoxins". **Mycologia** 71: 1166-1177.
78. Pitt JI (2002): "Biology and ecology of toxigenic *Penicillium* species". **Advanced and Experimental Medical Biology**. 504: 29-41.
79. Planterose DN, 1969: "Antiviral and cytotoxic effects of mycophenolic acid". **J Gen Virol**. 4(4): 629-30.
80. Polonelli I, Lauriola L, and Morace G (1982): "Preliminary studies on the carcinogenic effects of *Penicillium roqueforti* toxin". **Mycopathologia**. 78: 125-127.
81. Raper KB and Thom C. 1949. A manual of the *Penicillia*., Williams Publishing, Baltimore.
82. Richard JL (1990): "Additional mycotoxins of potential importance to human and animal health". **Vet Hum Toxicol**. 32 Suppl:63-9; 69-70.
83. Roberts BA, and Patterson DS (1975): "Detection of twelve mycotoxins in mixed animal feedstuffs, using a novel membrane cleanup procedure". **Journal of the AOAC**. 58(6): 1178-1181.
84. Roberts JK, and Smith RJ (1994): "Use of liquid chromatography-nuclear magnetic resonance spectroscopy for the identification of impurities in drug substances". **Journal of Chromatography A**. 677:385-389.
85. Seifert KA and Frisvad JC (2000): "*Penicillium* on solid wood products" (eds) In Samson RA and Pitt JI "Integration of modern taxonomic methods for *Penicillium* and *Aspergillus* classification". Hardwood Academic Publishers, Canada. 285-298.
86. Schneweis I, Meyer K, Hörmansdorfer S, and Bauer J (2000): "Mycophenolic acid in silage". **Applied and Environmental Microbiology**. 66(8): 3639-3641.
87. Schoch U, Luthy J, and Schlatter C (1983): "Mycotoxins in mold-ripened cheese". **Mitt. Geb. Lebensmittelunters Hyg**. 74: 50-59.

88. Schoch U, Luthy J, and Schlatter C (1984): "Subchronic toxicity testing of mold-ripened cheese". **Z. Lebensm Unters Forsch.** 179: 99-103.
89. Scott PM (1981): "Toxins of *Penicillium* species used in cheese manufacture". **Journal of Food Protection.** 44(9): 702-710.
90. Scott PM, and Kennedy BPC (1976): "Analysis of blue cheese for roquefortine and other alkaloids from *Penicillium roqueforti*". **J. Agric. Food Chem.** 24(4): 865-868.
91. Scott PM, Kennedy BPC, Harwig J, and Blanchfield BJ (1977): "Study of conditions for production of roquefortine and other metabolites of *Penicillium roqueforti*". **Applied and Environmental Microbiology.** 33(2): 249-253.
92. Scott PM, Polonsky J, and Merrien MA (1979): "Configuration of the 3, 12 double bond of roquefortine". **Journal of Agric. Food Chem.** 27(1): 201-202.
93. Scudamore KA, and Livesey CT (1998): "Occurrence and significance of mycotoxins in forage crops and silage: a review". **Journal of Sci. Food Agric.** 77: 1-17.
94. Seglar B (1999) "Comparison of mycotoxins levels among problem and healthy dairy herds". Unpublished Data.
95. Seglar B (1997) "Case Studies that implicate silage mycotoxins as the cause of dairy herd problems". Unpublished Data.
96. Söderström B and Frisvad JC (1984): "Separation of closely related asymmetric *penicillia* by pyrolysis gas chromatography and mycotoxin production". **Mycologia** 76: 408-419.
97. Steyn PS (1998): "The biosynthesis of mycotoxins". **Revue. Méd. Vét.** 149(6): 469-478.
98. Strongman DB, Miller JD, Calhoun L, Findlay JA, and Whitney NJ (1987): "The biochemical basis for interference competition among some lignicolous marine fungi". **Botanica Marina.** 30: 21-26.
99. Suzuki N, and Kuroda Rokuro (1987): "Direct simultaneous determination of nitrate and nitrite by ultraviolet second-derivative spectrophotometry". **Analyst.** 112: 1077-1079.
100. Tuller G, Armbruster G, Wiedenmann S, Hanichen T, Schams D and Bauer J (1998): "Occurrence of roquefortine in silage – toxicological relevance to sheep". **J. Anim. Physiol. A. Anim. Nutr.** 80: 246-249.

101. Turner WB and Aldridge DC (1983): Fungal Metabolites II. Academic Press Inc. New York.
102. Ueno Y and Ueno I (1978): "Toxicology and biochemistry of mycotoxins". In: Toxicology, Biochemistry and Pathology of Mycotoxins. Halstead Press. Tokyo.
103. Vleggaar R and Wessels PL (1980): "Stereochemistry of the dehydrogenation of (2S) histidine in the biosynthesis of roquefortine and oxaline". **J. Chem. Soc Chem Comm.** :160-162.
104. Vogler B, Klaiber I, Roos G, Walter CU, Hiller W, Sandor P, and Kraus W (1998): "Combination of LC-MS and LC-NMR as a tool for the structure determination of natural products". **Journal of Natural Products.** 61(2): 175-178.
105. Walter SL (2002): "Acute penitrem A and roquefortine poisoning in a dog". **Canadian Veterinary Journal.** 43: 373-374.
106. Wagner RE, Davis ND, and Diener UL (1980): "Penitrem A and roquefortine production by *Penicillium commune*". **Appl. Environ. Microbiol.** 39: 882-887.
107. Ware GM, Thorpe CW, and Pohland AE (1980): "Determination of roquefortine in blue cheese and blue cheese dressing by HPLC with UV and electrochemical detectors". **Jour. Assoc. Off. Anal. Chem.** 63: 637-641.
108. Wehner FC, Thiel PG, van Rensburg SJ, and Demasius IC (1978): "Mutagenicity to *Salmonella typhimurium* of some *Aspergillus* and *Penicillium* mycotoxins". **Mutat Res.** 58(2-3): 193-203.
109. Wei RD, Still PE, Smalley EB, Schnoes HK, and Strong FM (1973): "Isolation and partial characterization of a mycotoxin from *Penicillium roqueforti*". **Appl. Microbio.** 25: 111-114.
110. Wicklow DT (1981): The fungal community. Its organization and role in the ecosystem. Marcel Dekker Inc. New York.
111. Wilson BJ, Hoekman T, and Dettbarn WD (1972): "Effects of a fungus tremorgenic toxin (penitrem A) on transmission in rat phrenic nerve-diaphragm preparations". **Brain Research.** 40: 540-544.