Protein Profiling of Wild-type *Neurospora crassa* Grown on Various Carbon Sources

by

Katie Allen

Submitted in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in the

Department of Biology

Program

YOUNGSTOWN STATE UNIVERSITY

December, 2010

Protein Profiling of Wild-type *Neurospora crassa* Grown on Various Carbon Sources

Katie Allen

I hereby release this thesis to the public. I understand that this thesis will be made available from the OhioLINK ETD Center and the Maag Library Circulation Desk for public access. I also authorize the University or other individuals to make copies of this thesis as needed for scholarly research.

Signature:

Katie Allen, Student Date

Approvals:

Dr. David Asch, Thesis Advisor Date

Dr. Gary Walker, Committee Member Date

Dr. Xiangjia Min, Committee Member Date

Peter J. Kasvinsky, Dean of School of Graduate Studies and Research Date

ABSTRACT

Neurospora crassa possesses characteristics that make an ideal model for eukaryotic organisms. *N. crassa* metabolizes preferred carbon sources such as dextrose, but has the ability to metabolize less preferred carbon sources such as quinic acid or glycerol. This study analyzes the protein profiles of wild-type *N. crassa* grown on 2% dextrose, 2% glycerol, and 0.3% quinic acid. To perform the study, *N. crassa* was grown on Vogels minimal media and shifted to various carbon sources. Protein was extracted from *N. crassa* tissue and ran on two-dimensional gel electrophoresis (2-DGE). The 2-D gels were imaged and analyzed utilizing PDQuest™. Protein spots of interest were excised from the 2-D gels and sequenced by capillary-liquid chromatography-nanospray tandem mass spectrometry. Protein identifications were determined by searching SwissProt and NCBI databases for homologous fungal sequences.

The study revealed that more protein was expressed on the preferred carbon source, dextrose, compared to the less preferred carbon sources, quinic acid and glycerol. Unique protein expression patterns were also generated for each of the carbon sources. The identified proteins found to be unique to dextrose included an ATP-dependent RNA helicase, an enolase, and a cytochrome c peroxidase. A probable pyridoxine biosynthesis protein was established to be unique to glycerol, while a peptidyl-prolyl cis-tans isomerase and a Cu-Zn superoxide dismutase were determined to be unique to quinic acid.

ACKNOWLEDGEMENTS

First and most importantly, I would like to thank my advisor, Dr. David Asch, for his time and guidance in the laboratory during my thesis project. I have been very fortunate to have had the opportunity to learn and pursue my Master's degree in his research laboratory. With his help in countless ways, it was possible for me to complete this research project. I would also like to thank my thesis committee members; Dr. Gary Walker, who has taken time to help and guide me through the analysis of my 2-D protein gels, and Dr. Min, who has provided me with valuable insight on my thesis project.

Special thanks to my parents, Gary and Judy Allen, for teaching me the value of education. I am truly grateful for their continued support, patience, and unwavering faith in me. With their encouragement during difficult times, it was possible for me to finish my thesis project as well as all my graduate classes. May both of you never again, at least for a few years, have to hear me complain about writing my thesis or studying for a graduate exam.

Last, but not certainly least, I would like to express my gratitude to all my friends in the Biology Department at Youngstown State University that made my graduate experience unforgettable. I would also like to express my thanks to a few graduate students/friends in the PGRG hall; Sumedha Sethi, who showed me all the 2-DGE techniques, and Melissa Fleeger, who helped make the pouring of "Big Bertha" gels a complete success.

TABLE OF CONTENTS

		Page Number
Introduction		1
I.	Kingdom Fungi	1
II.	Phylum Ascomycota	1
III.	*Neurospora crassa*	2
IV.	Quinic Acid	4
V.	History of the Quinic Acid (*qa*) Gene Cluster in *Neurospora crassa*	5
VI.	Organization of the Quinic Acid (*qa*) Gene Cluster in *Neurospora crassa*	10
VII.	Regulation of the Quinic Acid (*qa*) Gene Cluster in *Neurospora crassa*	13
VIII.	Carbon Source Metabolism in *Neurospora crassa*	17
IX.	Proteomics in Fungi	21
Materials and Methods		24
I.	Strains	24
II.	Tissue Growth	24
III.	Protein Isolation	31
IV.	Bradford Assay	31
V.	Two-Dimensional Gel Electrophoresis	32

VI. Imaging and Analysis 36

VII. Protein Spot Excision 36

VIII. In Gel Digestion 37

IX. Mass Spectrometry 38

Results 40

Discussion 104

References 111

LIST OF FIGURES

Page Number

1. The organization of the quinic acid (*qa*) gene cluster in *Neurospora crassa* 12

2. The current model for regulation of the quinic acid (*qa*) gene cluster in *Neurospora crassa* 15

3. Quinic acid (QA) metabolism in *Neurospora crassa* 20

4. Coomasie stained gels depicting *N. crassa* proteins from tissue grown in 2% dextrose resolved by two-dimensional gel electrophoresis (2-DGE) 43

5. Coomasie stained gels depicting *N. crassa* proteins from tissue grown in 2% glycerol resolved by two-dimensional gel electrophoresis (2-DGE) 45

6. Coomasie stained gels depicting *N. crassa* proteins from tissue grown in 0.3% quinic acid resolved by two-dimensional gel electrophoresis (2-DGE) 47

7. SYPRO stained gel depicting *N. crassa* proteins from tissue grown in 2% dextrose resolved by two-dimensional gel electrophoresis (2- DGE) 49

8. SYPRO stained gel depicting *N. crassa* proteins from tissue grown in 2% glycerol resolved by two-dimensional gel electrophoresis (2- DGE) 51

9. SYPRO stained gel depicting *N. crassa* proteins from tissue grown in 0.3% quinic acid resolved by two-dimensional gel electrophoresis (2- DGE) 53

10. Individual matchset of dextrose gels created using PDQuest™ 56

11. Individual matchset of glycerol gels created using PDQuest™ 58

12. Individual matchset of quinic acid gels created using PDQuest™ 60

13. Higher level matchset comparing master gels of dextrose and quinic acid 62

14. Higher level matchset comparing master gels of dextrose and glycerol 64

15. Higher level matchset comparing master gels of glycerol and quinic acid 66

16. Labeled proteins shown in the dextrose master gel were excised and sent for MS. A box denotes up-regulated proteins unique to dextrose, while a circle denotes proteins that are expressed at higher levels in a particular carbon source 69

17. Labeled proteins shown in the quinic acid master gel were excised and sent for MS. A box denotes up-regulated proteins unqiue to quinic acid, while a circle denotes proteins that are expressed at higher levels in a particular carbon source 71

18. Labeled proteins shown in the glycerol master gel were excised and sent for MS. A box denotes up-regulated proteins unique to glycerol, while a circle denotes proteins that are expressed at higher levels in a particular carbon source 73

19. Sequence of excised protein, S9 in Figure 16, is indicated in red. A portion of the protein sequence obtained from MS analysis indicated that the protein is an ATP-dependent RNA helicase (eIF4A) 93

20. Sequence of excised protein, S10 in Figure 16, is indicated in red. A portion of the protein sequence obtained from MS analysis indicated that the protein is an enolase 95

21. Sequence of excised protein, S12 in Figure 16, is indicated in red. A portion of the protein sequence obtained from MS analysis indicated that the protein is a mitochondrial cytochrome c peroxidase 97

22. A) Sequence of excised protein, S1 in Figure 18, is indicated in red. B) Sequence of excised protein, S2, is indicated in red. A portion of the protein sequence obtained from MS analysis indicated that the protein is a peptidyl prolyl cis-trans isomerase 99

23. A) Sequence of excised protein, S3 in Figure 18, is indicated in red. B) Sequence of excised protein, S4, is indicated in red. A portion of the protein sequence obtained from MS analysis indicated that the protein is a superoxide dismustase [Cu-Zn] 101

24. A) Sequence of excised protein, S20 in Figure 17, is indicated in red. B) Sequence of excised protein, S21, is indicated in red. C) Sequence of excised protein, S30 in Figure 18, is indicated in red. D) Sequence of excised protein, S31, is indicated in red. A portion of the protien sequence obtained from MS analysis indicated that the protein is a probable pyridoxine biosynthesis protein 103

LIST OF TABLES

		Page Number
1.	50x Vogels media	26
2.	Trace element solution	28
3.	Biotin solution	30
4.	Identification of proteins excised from *N. crassa* grown on dextrose, quinic acid or glycerol	76

INTRODUCTION

I. Kingdom Fungi

The Kingdom Fungi, one of five kingdoms of classification, has been regarded as a separate kingdom since 1783 by some researchers, but the concept was not accepted until 1959 when proposed by Whitaker (Hibbett et al., 2007). The fungal kingdom represents a large, diverse group of eukaryotic species that belong to the Domain Eukarya. This fungal group includes over 1.5 million members that range from simple yeast to mold and the members continue to increase every year as scientists work on the fungal taxonomy (Galagan et al., 2005). The fungal species are found widely dispersed throughout the world-wide environment and reside in places from soil, water bodies, mountain rocks, to sea water (Feofilova, 2001). The fungi are typically multi-cellular and filamentous; growing as branched threads or filaments commonly known as hyphae (Alexopoulos, 1962). Fungi obtain their nutrients and survive as saprobes, which infect living organisms, or parasites, which attack non-living organisms (Alexopoulos, 1962). Fungal species reproduce both sexually and asexually (Alexopoulos, 1962). In both asexual and sexual reproduction of fungi, the formation and dispersal of spores is seen.

II. Phylum Ascomycota

The Ascomycota, the largest phylum within the kingdom Fungi, comprises almost seventy-five percent all described fungi and contains more than 30,000 species (Trail, 2007). The members of the phylum, Ascomycota, are commonly referred to as “Sac Fungi” or “Higher Fungi.” The members range from important organisms such as baker’s yeast, cup fungi, powdery mildews, to the morels and the truffles (Alexopoulos,

1962). The characteristic that differentiates the Ascomycetes from all other fungi is the ascus, a sac-like structure that contains spores called ascospores (Alexopoulos, 1962).

III. *Neurospora crassa*

Neurospora, a genus of Ascomycete fungi, has been an ideal model organism used in a variety of biological research laboratories from genetics, molecular biology to biochemical genetics. Commonly referred to as "red bread mold", *Neurospora* was first described in 1842 as an orange growth contaminating bread in Paris bakeries. The orange growth, *Neurospora*, was first studied by scientists in an effort to control the infestation in the bakeries, but lead to the organism's life-long contribution in biological studies. At the turn of the century, a plant physiologist named F. A. F. C. Went started experimenting with an orange oncham fungus known now as *Neurospora*. The plant physiologist, Went, studied the effects of various substrates on enzymes such as tyrosinase and the effect of light on carotenoid production in *Neurospora*. In the mid-1920's, Bernard Dodge and C. L. Shear started to perform experimental crosses and studied the ascospores of orange mold from sugar cane. In the 1930's, the genetics of *Neurospora* were developed and it became known to geneticists by Dodge and Carl Lindegren (Perkins, 1992).

Neurospora, as it was recognized for its value and potential, was widely utilized in the genetics labs. In 1941, George Beadle and Edward Tatum employed *Neurospora* in their study of nutritional mutants and to propose their "one-gene one-enzyme" theory. By the late 1940's, students and well-known scientists such as Norman Giles, Francis Ryan and Sterling Emerson were interested in working with *Neurospora*. The utilization of *Neurospora* not only assisted in the establishment of the "one-gene one-enzyme" theory,

but it contributed many advances and essential contributions such as the mechanism of recombination and fungal chromosome behavior in meiosis (Perkins, 1992).

Neurospora continues today to be a valuable resource in the biological laboratories. Its continued use in the laboratories is attributed to the advantageous features that *Neurospora* posses. One advantageous feature of *Neurospora* is its simple nutritional requirements, which allows it to grow well on minimal media or adapt to other regimes such as carbon sources. It also can be easily maintained and grown in the laboratory in a variety ways. It can be grown on agar slants, solidified medium in petri dishes or flasks, and liquid cultures in Erlenmeyer flasks. Another important feature is that *Neurospora* stocks can be frozen, which allows for easy preservation and the avoidance of serial transfers. In addition, its rapid, linear growth is an advantage for many biological procedures. Other advantageous features of *Neurospora* include its small genome and haploid lifestyle which allows for easy genetic analyses (Davis, 2000).

There are a number of known *Neurospora* species, but *Neurospora crassa* is the most commonly studied. It is found naturally occurring in moist tropical and subtropical regions. It is also found in areas of fire devastated plant life. In 2003, the nuclear genome of *N. crassa* was completely sequenced. The sequenced genome was found to be approximately 38.6 Mb, which is distributed among seven (I-VII) linkage groups (Galagan et al., 2003). From this sequenced genome, it was predicted that *N. crassa* contained a total of 10,082 protein-coding genes (Galagan et al., 2003). A total of 1,336 predicted *Neurospora* proteins, 13%, were identified by similarity to known protein sequences from public databases. However, it was determined that the other 41% of the predicted proteins, a total of 4,140 proteins, in *N. crassa* contained no similarity to known

protein sequences from public databases (Galagan et al., 2003). Based on the predicted protein-coding sequences, it was determined that the remaining 46% of the protein-coding genes were found to be hypothetically conserved proteins (Galagan et al., 2003). Commonly referred to as filamentous fungi, *N. crassa* contains tubular, branched hyphae which grow by tip extension also known as apical growth. The continuous apical growth of the branched hyphae eventually forms a mycelium. A heterothallic organism, *N. crassa* can undergo both sexual and asexual reproduction. In the asexual cycle, the formation of conidia is observed whereas, the formation of ascospores is observed in the sexual cycle.

IV. Quinic Acid

Quinic acid, an aromatic carboxylic acid, is highly recognized as a natural product (Barco et al., 1997). It is found widely distributed throughout the plant kingdom and was first isolated in the 18th century (Barco et al., 1997). Although mainly isolated from plant sources, quinic acid was also found to be synthesized in a transgenic strain of *Escherichia coli* (Ran et al., 2001). The ability of quinic acid to be synthesized in a microbial source was dependent upon glucose, the starting material. Although not a preferred carbon source, quinic acid can serve as an alternate source of carbon in several microorganisms (Barco et al., 1997). These microorganisms are capable of using this aromatic acid as an alternative carbon source due to their ability of converting quinic acid to 3-dehydroquinate. Microorganisms also obtain energy from quinic acid as the sole carbon source because it is metabolized to multiple intermediates and eventually enters the Krebs cycle as succinate or acetyl-CoA (Kuswandi, 1992). Also involved in aromatic amino acid biosynthesis, quinic acid has emerged as a valuable chiral starting material in a

variety of molecules from antitumor agent esperamicin-A to immunosuppressant FK506. It is also known to be involved in the synthesis of neuraminidase inhibitor GS4101, which is used in the treatment of influenza (Draths et al., 1999).

V. History of the Quinic Acid (*qa*) Gene Cluster in *Neurospora crassa*

Present in the fungus *Neurospora crassa*, the quinic acid (*qa*) gene cluster has provided researchers with a model to study gene regulation in eukaryotic organisms. In *N. crassa*, the quinic acid (*qa*) gene cluster is responsible for controlling the utilization of quinic acid as the primary carbon source.

Researched extensively in laboratories since the late 1960's, the quinic acid gene cluster in *N. crassa* continues to be a useful model system. The quinic acid (*qa*) cluster was initially studied in the laboratory in response to *Neurospora* mutants that could not metabolize the sole carbon source, quinic acid. Shortly after, during the early 1970's, researchers discovered a cluster of four quinic acid (*qa*) genes in *N. crassa* engaged in the breakdown of quinic acid. Through biochemical analysis, three of the quinic acid genes (*qa-2, qa-3, and qa-4*) were found to encode inducible enzymes involved in the conversion of quinic acid (QA) to protocatechuic acid (PCA). However, the fourth quinic acid gene (*qa-1*) of *N. crassa* was proposed to act as a regulator and control production of the quinic acid (*qa*) enzymes (Patel et al., 1981).

Other early studies, provided by Rines, Giles and Chaleff, analyzed individual quinic acid (*qa*) genes and gene products of *N. crassa* through genetic and biochemical studies of quinic acid (*qa*) mutants. The initial mutant of quinic acid studied, *qa-2* was demonstrated by Rines to be deficient in C-DHQase (5-dehydroquinate hydrolyase) activity (Giles et al., 1985). These studies confirmed that the *qa-2* gene product encoded

a catabolic dehydroquinase, which converts 5-dehydroquinate (DHQ) to 5-dehydroshikimate (DHS). Later studies by Chaleff isolated *qa-3* mutants, which lacked activity for both SDHase (shikimic acid dehydrogenase) and QDHase (quinic acid dehydrogenase) (Chaleff, 1974). This study provided evidence that the *qa-3* gene product catalyzed both shikimate and quinate dehydrogenation. *Qa-4* mutants, originally isolated by Case, were demonstrated in studies to lack DHS-Dase (DHS dehydratase) activity (Chaleff, 1974). Through isolation and analysis of *qa-4* mutants, evidence provided that the *qa-4* gene product encoded 3-dehydroshikimate dehydratase which converts 5-dehydroshikimate to protocatechuic acid (Chaleff, 1974).

The *qa-1* gene, through biochemical and genetic studies, was proposed to be a single, regulatory gene that positively controlled expression of the quinic acid enzymes along with the inducer, quinic acid. Through other biochemical analyses, *qa-1* mutants were isolated and proposed to lack activity required for the enzymatic conversion of quinic acid to protocatechuic acid. Based on their complementation response with other quinic acid (*qa*) mutations, the *qa-1* mutants were divided into two distinct categories that were designated fast and slow. The mutants that displayed slow complementation were assigned *qa-1S*, while the others that displayed fast complementation were assigned *qa-1F*. From genetic mapping studies, evidence suggested that the *qa-1F* and *qa-1S* mutations occupied two distinct locations of the *qa-1* locus. Case and Giles later proposed that the regulatory gene of the quinic acid (*qa*) cluster, *qa-1*, had characteristics of a multimeric protein with two distinct domains (Case et al., 1975). This decision of *qa-1* to be a multimeric protein was based upon complementation of alleles between *qa-1*

mutations grown in the absence of the inducer, quinic acid (Case et al., 1975, Giles et al., 1985).

Through the development of recombinant deoxyribonucleic acid (DNA) technology, and the subsequent cloning of the quinic acid cluster, later studies analyzed the *qa-1* gene at the molecular level. These advances in cloning, in addition to transformation experiments revealed the existence of two regulatory genes in the quinic acid (*qa*) cluster, assigned *qa-1S* and *qa-1F*. The existence of two regulatory genes, *qa-1S* and *qa-1F*, was further supported by demonstration that each gene produced separate messenger ribonucleic acid (mRNA) (Patel et al., 1981). Through genetic studies, the *qa-1F* gene was found to encode an activator protein, whereas the *qa-1S* gene was found to encode a repressor protein. It was later proposed that the genes, *qa-1S* and *qa-1F*, had an interconnected regulation and both were required along with quinic acid to induce expression of the quinic acid genes (Giles et al., 1985). However, gene deletion experiments later conducted revealed that the *qa* genes were constitutively expressed even in the Δ*qa-1S* strain, which contained the deleted *qa-1S* gene (Case et al., 1992).

The *qa-1S* gene product, the repressor protein, is an 918 amino acid, 101 kD protein (Huiet et al., 1986). Through *qa-1S* gene deletion experiments, the protein was shown to function as a repressor molecule. As described earlier, when the *qa-1S* gene is disrupted, *qa* genes are constitutively transcribed at high levels (Case et al., 1992). Localization studies of *qa-1S* pleiotropic mutants, including both non-inducible and constitutive classes, provided potential evidence for functional domain locations within the repressor protein (Giles et al., 1991).

The *qa* mutants belonging to the semi-dominant, non-inducible class (*qa-1S*$^{-}$) were found to each contain a missense mutation, which causes them to encode functional proteins that act as superrepressors. These missense mutations were found to be located between codons 627 and 743, which propose that the repressor interacts with the inducer quinic acid at this particular region of the domain (Huiet et al., 1986). The constitutive (*qa-1S*^{c}) class of mutants each contains nonsense or frameshift mutations, which causes them to encode inactive repressors. The nonsense mutations were determined to be located at the carboxy terminus, which propose this region of the domain is where the repressor interacts with the target. To determine its target, researchers utilized repressor protein overexpressed in a baculovirus system to show binding of DNA to repressor protein. However, no evidence has shown binding of DNA, which implies the activator protein is the target of the repressor protein. From these studies, the repressor protein was proposed to have two functional domains: one domain that interacts with the target, the activator protein, and one domain that interacts with the inducer, quinic acid (Giles et al., 1985, Giles et al., 1991).

The *qa-1F* gene product, the activator protein, is an 816 amino acid, 89 kD protein (Geever et al., 1989). Through gene disruption studies, the protein was shown to function as an activator molecule. When the *qa-1F* gene is altered, the *qa* genes lack inducibility and are transcribed only at basal levels. Other studies as well have shown that the activator protein plays a vital role in transcription of the *qa* genes, as well as itself (Patel et al., 1985). DNA sequence analysis, sequence alignments of *qa-1F* with *qutA*, along with "domain swapping" studies of activator genes between *N. crassa* and *A. nidulans* have all provided potential evidence for functional domain locations within the

activator protein. From these studies, at least four functional domains were proposed: a DNA binding domain, a dimerization domain, a transcriptional activation domain, and a domain for *qa* repressor interaction. The protein dimerization domain of the activator was determined to be located between codons 296 and 562, whereas the DNA binding domain was proposed to be located in the region of the first 183 amino acids. The carboxy terminus of the activator protein, composed mainly of acidic residues, was proposed to provide activator interactions with the *qa* repressor as well as transcriptional factors (Giles et al., 1991).

Further analysis of the quinic acid (*qa*) gene cluster at the molecular level through cloning along with DNA-RNA hybridization studies later identified two additional structural genes in the *qa* cluster, *qa-x* and *qa-y*. The existence of five structural genes, rather than three, in the *qa* cluster was further supported by DNA sequencing data obtained from the *qa* genes (Geever et al., 1989). The two genes, *qa-x* and *qa-y*, were originally identified by researchers as quinic acid inducible transcripts with unknown functions. It was later proposed that one structural gene, *qa-x* or *qa-y,* encoded a permease in the *qa* gene cluster. The hypothesis that quinic acid uptake involved a permease was based on evidence from studies utilizing radioactive quinate (Giles et al., 1985, Patel et al., 1981).

Other later studies, provided by Geever and Case, proposed that *qa-y* gene product encoded a quinate permease which allows quinic acid into the cell. This theory was based on evidence from comparative and gene deletion studies (Geever et al., 1989, Case et al., 1992). Through comparative studies, the coding region of the *qa-y* gene was shown to display a high degree of similarity to other known quinate permeases such as

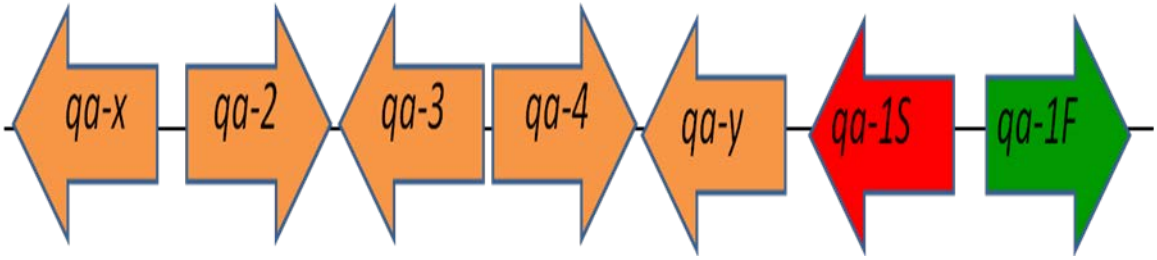
the *gutD* gene of *Aspergillus nidulans*. Later, through the utilization of gene deletion experiments, it was shown that strains containing the Δ*qa-y* deletion were not able to be grown on quinic acid in addition the *qa-2* and *qa-3* genes had low induction levels. The results from the gene deletion experiments were consistent with phenotypes produced by genes also involved in quinic acid transport (Case et al., 1992).

The last structural gene, *qa-x*, remains to have an unidentified function in the quinic acid (*qa*) gene cluster. It was hypothesized that the *qa-x* gene product encoded an enzyme responsible for catalyzing the conversion of chlorogenic acid to quinic acid (Giles et al., 1985). This theory, however, was later shown to be incorrect by demonstrating the capability of *qa-x* mutants to utilize chlorogenic acid as the sole carbon source (Case et al., 1992). The *qa-x* mutants also exhibited the ability to utilize quinic acid, but the production of a brown pigment was observed when grown in this carbon source (Case et al., 1992). The brown pigment produced by mutants in quinic acid remains unidentified, along with the correct gene function of *qa-x*.

VI. Organization of the Quinic Acid (*qa*) Gene Cluster in *Neurospora crassa*

The *qa* cluster consists of seven adjacent genes: five structural (*qa-2, qa-3, qa-4, qa-y, and qa-x*) and two regulatory (*qa-1F and qa-1S*). All seven *qa* genes of the quinic acid cluster are tightly linked on approximately 17.3 kb of DNA located on linkage group VII of *N. crassa*. As shown in Figure 1, the *qa* gene cluster is organized into three pairs of divergently transcribed genes (*qa-x/qa-2, qa-4/qa-3, and qa-1S/qa-1F*) and one un-paired gene (*qa-y*). The regulatory gene pair, *qa-1S/qa-1F*, is located at one end of the *qa* cluster, while the structural gene pairs (*qa-x/qa-2 and qa-4/qa-3*) occupy the remaining

Figure 1: The organization of the quinic acid (*qa*) gene cluster in *Neurospora crassa*.


qa-x
qa-2
qa-3
qa-4
qa-y
qa-1S
qa-1F

end. The single unpaired gene, *qa-y*, separates the regulatory gene pair from the structural gene pairs (Giles et al., 1991).

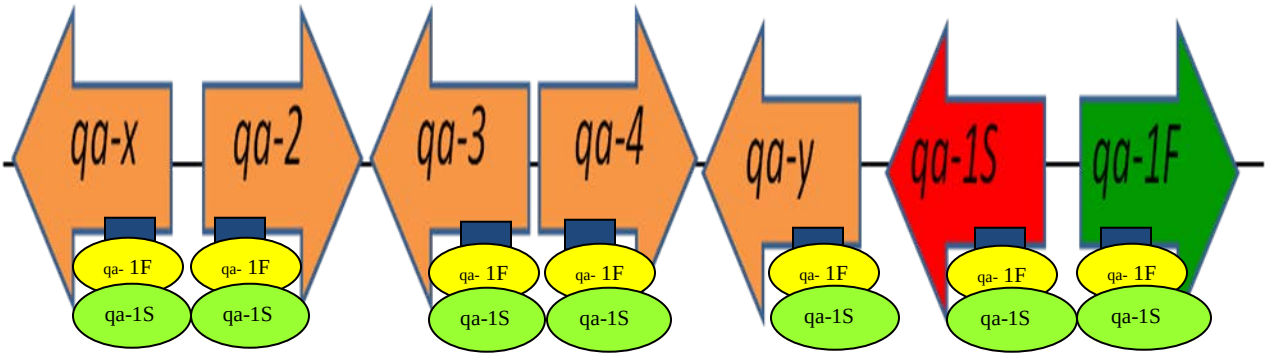
VII. Regulation of the Quinic Acid (*qa*) Gene Cluster in *Neurospora crassa*

The genetic regulation of the quinic acid (*qa*) gene cluster in *Neurospora crassa* is controlled at two levels, both of which occur at the level of transcription. The primary level of regulatory control involves the inducer, quinic acid, along with the *qa* regulatory proteins, *qa-1S* and *qa-1F*. The secondary level of *qa* gene regulation, catabolite repression, occurs in the presence of a preferred carbon source such as glucose (Giles et al., 1985, Giles et al., 1991).

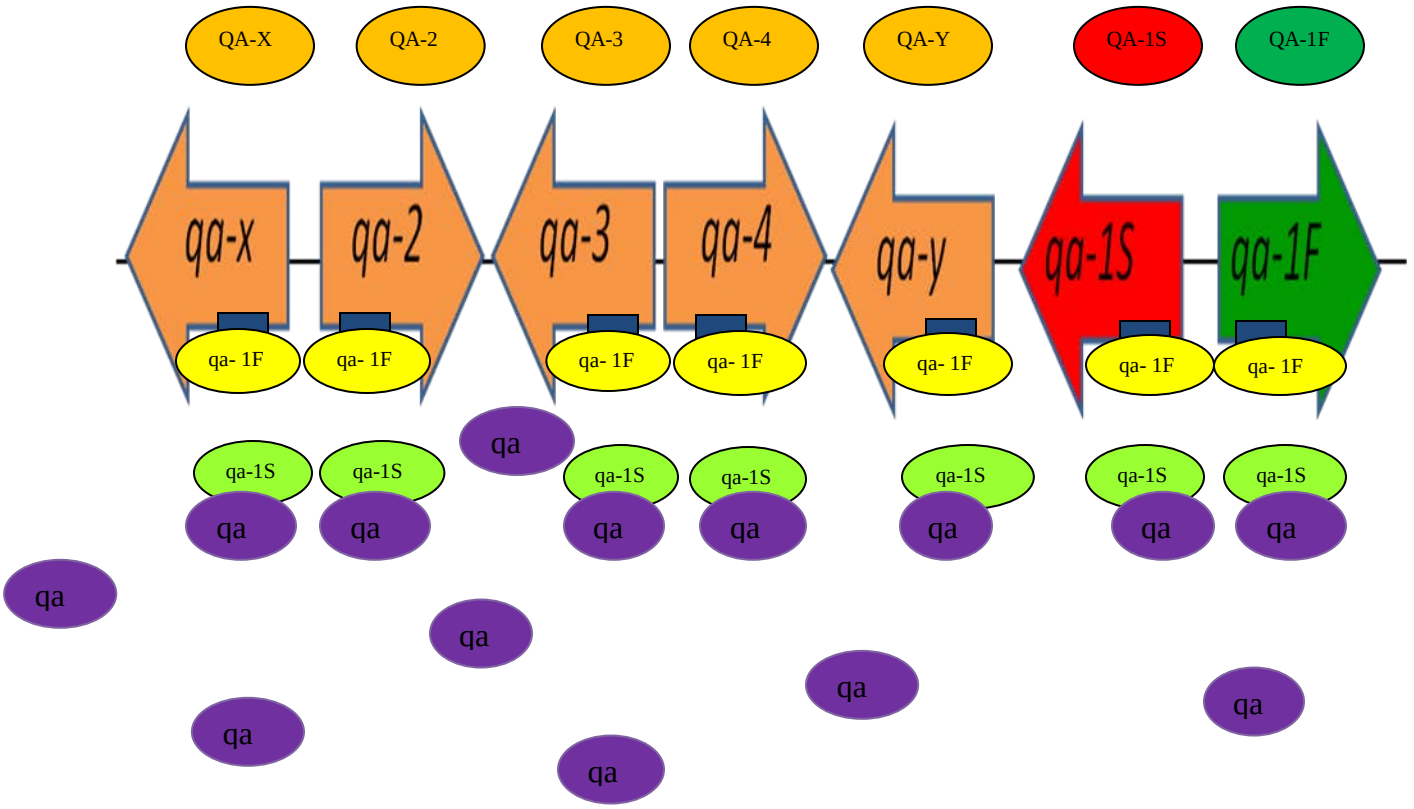
The primary level of control for regulation of the quinic acid gene cluster requires the actions of the repressor gene (*qa-1S*), the activator gene (*qa-1F),* their protein products, and the inducer quinic acid. The current model as shown in Figure 2, based on studies of *qa-1S* and *qa-1F* mutants, proposes that the *qa* repressor protein binds to the activation domain of the *qa* activator protein in the absence of quinic acid. The binding of the repressor to the activator regulates expression of the *qa-1F* activator encoding gene, which inhibits the production of the qa-1F activator protein as well as transcription of the other *qa* genes. The inhibition of the activator results in exceptionally low production levels of the quinic acid (*qa*) gene products. However, in the presence of quinic acid, the inducer, repression of the qa-1F activator protein is released by the binding of quinic acid to the qa-1S repressor protein. The qa-1F activator protein then binds to the sixteen base-pair DNA sequences of the *qa* genes. This binding results in up-regulation of all the *qa* genes, including *qa-1S* and *qa-1F*. However, it is also proposed that the activator protein, qa-1F, may be bound initially to the activation domain, but remains inactive due to the

Figure 2: The current model for regulation of the quinic acid (*qa*) gene cluster in *Neurospora crassa.*

A) Absence of Quinic Acid



B) Presence of Quinic Acid



bound repressor. In this case, activation of the *qa* system would occur with the introduction of the inducer, quinic acid (Giles et al., 1985, Giles et al., 1991).

Carbon catabolite repression offers a secondary level of control for gene regulation of the *qa* cluster in *N. crassa*. This second line of regulatory control decreases transcription of the *qa* genes in the presence of a preferred carbon source, such as glucose or sucrose. Under catabolite repression, in the presence of a preferred carbon source, the *qa* gene transcripts are expressed at an induction level of approximately 10% when quinic acid is the sole carbon source (Giles et al., 1991). However, the mechanism of catabolite repression on *qa* gene expression is not fully understood in *N. crassa*. Although the exact mechanism is unidentified, researchers have hypothesized possible causes of catabolite repression on the *qa* gene cluster. Based on Northern blot analyses, it was first proposed that *qa-x* played a possible role in effecting carbon catabolite repression (Giles et al., 1991). This theory was proposed because transcription of the *qa-x* gene appeared to be more strongly repressed by glucose than transcription of the other *qa* genes (Giles et al., 1991). However, *qa-x* gene deletion experiments later conducted revealed that catabolite repression was still occurring even in the absence of the *qa-x* gene (Arnett et al., 2009).

Through RNA blot and sequence analysis, researchers later proposed three additional possible causes of carbon catabolite repression on the *qa* cluster. First, in the presence of a preferred carbon source transcription of the *qa* genes is blocked because trans-acting repressors interact with cis-acting DNA sequences that are 5' to the gene cluster. Second, it was proposed that transcription of *qa-1F* is specifically repressed, which leads to transcription of other *qa* genes being repressed. The third possibility for

the cause of catabolite repression is direct inhibition of *qa-y* transcription, which reduces the amount of inducer present in the cell (Arnett et al., 2009).

Based on RNA blot quantitation, it was later revealed that four of the seven *qa* genes (*qa-x*, *qa-y*, *qa-3*, and *qa-1F*) were repressed in the presence of glucose. However, the largest effect was noticed on transcription of the *qa-y* gene. It was determined that *qa-y* was repressed ten-fold in the presence of glucose compared to when quinic acid was the sole carbon source. These results from the RNA blot indicate that the main mechanism of catabolite repression of the *qa* genes may be caused by the direct inhibition of *qa-y* transcription causing inducer exclusion. Through DNA sequence comparison, two related species of *N. crassa*, *N. africana* and *N. terricola*, were found to have highly conserved upstream *qa-y* regions. This region of *qa-y* conservation may indicate the existence of binding sites for catabolite repression mediators. From the study, the researchers proposed that inducer exclusion mainly mediates carbon catabolite repression of the *qa* genes in the presence of a preferred carbon source, such as glucose or sucrose. However, it is suggested that the major mechanism of catabolite repression, inducer exclusion, may be coupled with a minor mechanism such as the reduction of *qa-1F* activator expression (Arnett et al., 2009).

VIII. Carbon Source Metabolism in *Neurospora crassa*

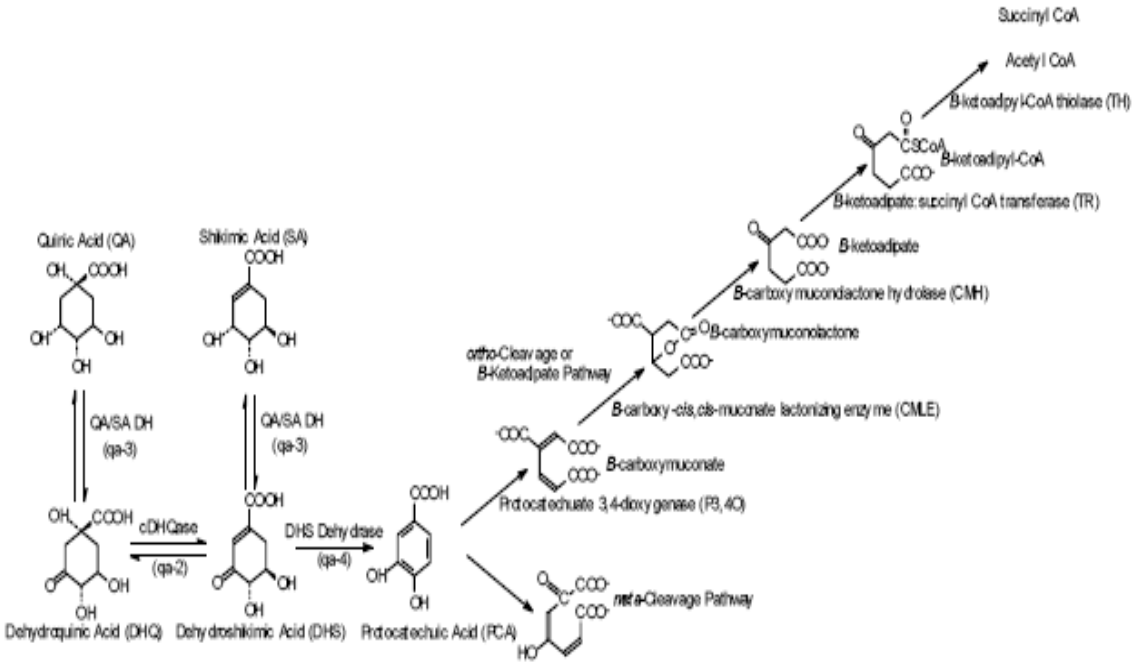
Metabolism occurs in all organisms from animals, yeast, bacteria to fungal species including *Neurospora crassa*. The process of metabolism occurs in a series of enzymatic steps or biochemical reactions that are systematized into particular metabolic pathways. These metabolic pathways establish which carbon substrates are able to be

utilized by the cell. For *N. crassa*, a variety of carbon sources, including preferred and non-preferred sources, can be metabolized in order to obtain energy, increase mycelial growth, and retain fungal structures. The most common and preferred carbon sources include glucose, sucrose, fructose, mannose, and maltose. However, some of the known poor or non-preferred carbon sources that *N. crassa* utilizes include substrates such as acetate, quinic acid, sorbose, and glycerol (Davis, 2000, Deacon, 2006).

A preferred carbon source such as glucose is metabolized as an energy source in *N. crassa* and other fungi by a central metabolic pathway of fungi known as the Embden-Meyerhof (EM) pathway or glycolysis. In this metabolic pathway, glucose is first phosphorylated into glucose-6-phosphate, which is catalyzed by a family of enzymes identified as hexokinases. A phosphoglucose isomerase then catalyzes the conversion of glucose-6-phosphate to fructose-6-phosphate. The fructose-6-phosphate is then converted to fructose-1, 6-biphosphate, which is spilt into glyceraldehyde-3-phosphate and dihydroxyacetone. Then, through a series of enzymatic steps the glyceraldehydes-3-phosphate and dihydroxyacetone are converted into pyruvic acid, an important intermediate of central metabolism. This intermediate, pyruvic acid, is then capable of being converted into acetyl-coenzyme A, the precursor for the Krebs cycle. Through these main metabolic pathways, glycolysis and the Krebs cycle, *N. crassa* is able to generate energy from simple sugars such as glucose (Deacon, 2006).

Quinic acid, a non-preferred carbon source, is utilized by *N. crassa* and other filamentous fungi as a carbon source via various pathways (Davis, 2000). As shown in Figure 3, the metabolism of quinic acid in *N. crassa* is catalyzed by three distinct

Figure 3: Quinic acid (QA) metabolism in *Neurospora crassa*.


Quinic Acid (QA)
Shikimic Acid (SA)
QA/SA DH
(qa-3)
QA/SA DH
(qa-3)
cDHQase
(qa-2)
DHS Dehydrase
(qa-4)
Dehydroquinic Acid (DHQ)
Dehydroshikimic Acid (DHS)
Protocatechuic Acid (PCA)
ortho-Cleavage or
B-Ketoadipate Pathway
meta-Cleavage Pathway
Protocatechuate 3,4-dioxygenase (P3,4O)
B-carboxymuconate
B-carboxy-cis,cis-muconate lactonizing enzyme (CMLE)
B-carboxymuconolactone
B-carboxymuconolactone hydrolase (CMH)
B-ketoadipate
B-ketoadipate: succinyl CoA transferase (TR)
B-ketoadipyl-CoA
B-ketoadipyl-CoA thiolase (TH)
Acetyl CoA
Succinyl CoA

inducible enzymes, which are the gene products of three structural genes (*qa-2*, *qa-3*, and *qa-4*) (Arnett, 2005). In the quinic acid catabolic pathway, quinic acid (QA) is first converted to 5-dehydroquinate (DHQ), which is catalyzed by quinic acid dehydrogenase (QDHase), the *qa-3* gene product. A catabolic dehydroquinase (C-DHQase), the *qa-2* gene product, then converts 5-dehydroquinate (DHQ) to 5-dehydroshikimate (DHS). The *qa-4* gene product, 3-dehydroshikimate dehydratase, at last converts 5-dehydroshikimate to protocatechuic acid (PCA). The protocatechuic acid can then be further broken down and metabolized until it enters the Krebs cycle as succinate or acetyl-CoA (Geever et al., 1987, Kuswandi, 1992).

Glycerol, also recognized as a non-preferred carbon source, is capable of being utilized as a sole carbon source by *N. crassa* and other filamentous fungi. The utilization of this carbon source, glycerol, requires the inducibility of two specific enzymes, a cytosolic glycerokinase and a mitochrondrial glycerol-3-phosphate dehydrogenase. In the glycerol metabolic pathway in *N. crassa*, the glycerol is first phosphorylated, which is catalyzed by the cytosolic glycerokinase. The resulting cytosolic glyceraldehyde-3-phosphate then undergoes mitochondrial oxidation, which is catalyzed by the glycerol-3-phosphate dehydrogenase. The resulting product, dihydroxyacetone phosphate, can then enter the glycolytic pathway through the actions of both a triose phosphate isomerase and an aldolase (Denor et al., 1978).

IX. Proteomics in Fungi

Proteomics, a newly emerging field in molecular genetics, is characterized as the analysis of cellular protein expression, function, and regulation in cells, tissues, and

organisms. This protein characterization could be used to describe the changes in the proteome at different time points throughout the cell cycle, and in response to many different environmental conditions to which the cell is exposed. This might include changes in temperature, light exposure, and nutrient limitations such as carbon substrates. In this field, researchers can study intracellular proteomics, subproteomics including membrane, mitochrondrial, and proteasome as well as secretome proteomics. Although proteomics has been widely studied in other organisms, such as bacteria and mammals, this field of research has just began to be used to study fungi. This field, fungal proteomics, is becoming recognized as an essential area of research because it can provide quantitative as well as a qualitative analysis of large numbers of fungal proteins. This essentially can provide a systematic viewpoint on fungal physiology, metabolic changes, virulence factors, and possibly a role in hypotheses generating tools (Kim et al., 2007, Bhadauria et al., 2007).

At the start of the twenty-first century, the beginning of proteomics importance in fungi as mentioned earlier, proteomic studies were only initiated in a few fungal species including the mycoparasistic fungi *Trichoderma harzianum, Trichoderma atroviride*, *Trichoderma reesei* and various *Aspergillus* species such as *A. fumigatus* and *A. nidulans*. However, sufficient progress was made in the field of fungal proteomics between 2002 and 2006; during this period more proteomic studies were performed in a number of different fungi including *Botrytis cinerea, Penicillium expansum, Phanerochaete chrysosporium, Pleurotus sapidus, Sclerotinia sclerotiorum,* and *Penicillium marneffei.* This progress was contributed to advancements in fungal sample preparation and extraction, MS tools and software as well as technology in bioinformatics. Along with

these advancements, some of the dominant tools in the field that have contributed to its development as well include two-dimensional electrophoresis (2DE), two-dimensional fluroscence difference gel electrophoresis (2D-DIGE), MALDI-TOF MS, and MALDI LIFT-TOF/TOF MS (Kim et al., 2007, Bhadauria et al., 2007).

Although significant progress has already been made in the field of fungal proteomics, researchers expect this field of study to expand even further in the near future. However, researchers will still struggle with a few challenges that exist in the field of proteomics such as reproducibility in protein separation and detection of low-abdunance proteins (Kim et al., 2007, Bhadauria et al., 2007).

MATERIALS AND METHODS

I. Strains

Wild-type *Neurospora crassa* 74A (FGSC NO. 2489) strain was obtained from laboratory stock collections and employed throughout the study.

II. Tissue Growth

Neurospora crassa 74A strain was inoculated into 50 mL Vogel's Minimal Media (Table 1) containing 1.5% agar and 2% sucrose in a 250 mL Erlenmeyer flask. The flask of *Neurospora crassa* was grown at 30°C in the incubator for 2 days, and then switched to fluorescent light at room temperature for approximately 14 days. The conidia were then harvested by adding 25 mL of 1 x Vogels to the flask of *N. crassa.* The conidial suspension was swirled around the flask and poured through sterile cheesecloth. The suspension was then divided into two equal portions which were added to 50 mL of 1x Vogels and 2% dextrose in a 250 mL Erlenmeyer flask. The cultures were grown overnight at 30°C in the shaker, shaking at approximately 150 rpm. The mycelia from the overnight culture were collected and filtered through Whatman® (1) filter paper. The mycelial pads were transferred to 50 mL of sterile water, washed, and re-filtered. The mycelial pads were then shifted to 50 mL of 1x Vogels with one of three carbon sources: 2% glycerol, 2% dextrose, or 0.3% quinic acid. The cultures were then grown for 3 hours at 30°C in the shaker, shaking at approximately 150 rpm. The mycelia obtained were filtered and the mycelial pads were frozen and stored at -70°C.

Table 1: 50x Vogels media.
Ingredients used to make 1 liter of 50x Vogels Media.

Table 1: 50x Vogels media

(Per liter)

Na_3 citrate- $5^1/_2$ H_2	150 g
KH_2PO_4 anhydrous	250 g
NH_4NO_3	100 g
$MgSO_4$- $7H_2O$	10 g
$CaCl_2$ anhydrous	5 g
Trace element solution	5 ml
Biotin solution	5 ml
H_2O	750 ml

Table 2: Trace element solution.
Ingredients used to make trace element solution.

Table 2: Trace element solution

(Per liter)

Citric acid $1H_2O$	5.0 g
$ZnSO_4$-$7H_2O$	5.0 g
$Fe(NH_4)$ $(SO_4)_2$ – $6H_2O$	1.0 g
$CuSO_4$ -$5H_2O$	0.25 g
$MnSO_4$ – $1H_2O$	0.25 g
H_3BO_3 anhydrous	0.05 g
Na_2MoO_4 – $2H_2O$	0.05 g
Chloroform	0.05 g
Water to make 100 ml	1.0 ml

Table 3: Biotin solution.
Ingredients used to make biotin solution.

Table 3: Biotin solution

(Per liter)

Biotin	5.0 mg
50% Ethanol	50 ml

III. Protein Isolation

The frozen mycelial pads were ground with liquid nitrogen, baked sand, and a mortar and pestle. The ground tissue was transferred to 1.5 mL Eppendorf tubes until approximately half-filled. To each Eppendorf tube, approximately 800 µL of lysis buffer (200mM Tris-HCL, pH 7.6, 10mM NaCl, 0.5mM deoxycholate, 40 µL protease inhibitor [Promega]) was added. The Eppendorf tubes were then vortexed for 1 minute and iced for 2 minutes, which was repeated three times. The tubes were then spun in the centrifuge for 10 minutes at 12,000 rpm at 4°C, after which the supernatant was collected and transferred to new 1.5 mL Eppendorf tubes. To the supernatant, 100 µL Trichloroacetic acid (Fisher Scientific) was added, in order to precipitate out the protein, and the solution was iced for 20 minutes. After icing, the tubes were gently inverted and spun in the centrifuge for 20 minutes at 12,000 rpm at 4°C. The Trichloroacetic acid was removed and the protein pellets were washed three times with acetone (Pharmco Products). The protein pellets obtained were dried for 20 minutes in the LABCONCO CentriVap DNA Concentrator speed-vac, resuspended and stored in MSB (2M thiourea, 7M urea, 4% w/v CHAPS, 1% w/v DTT) at -70°C.

IV. Bradford Assay

The Bradford Assays were performed to measure the concentration of protein obtained during the protein isolations. Eight standards and our protein samples were prepared in triplicate. The standards were prepared by pipetting the following into each test tube: 80 µL de-ionized H_2O, 20 µL 0.1M HCL, and 10 µL 2-DE buffer. Then, to each test tube varying concentrations of 10 mg/mL bovine serum albumin (BSA) was

added. Final concentrations of BSA are as followed in each tube: 0 µg/µl in test tube one, 0.09 µg/µl in test tube two, 0.14 µg/µl in test tube three, 0.18 µg/µl in test tube four, 0.23 µg/µl in test tube five, 0.27 µg/µl in test tube six, 0.32 µg/µl in test tube seven, and 0.36 µg/µl in test tube eight. The protein samples were prepared by pipetting the following into each test tube: 80 µL de-ionized H_2O, 20 µL 0.1M HCL, 10 µL 2-DE buffer, and 10 µL *N. crassa* protein solution. The standards and the samples were placed on the table at room temperature to sit for 5 minutes before adding the Bradford Dye (100 mg Brilliant Blue G-250, 50 mL 95% ethanol acid, 100 mL 85% w/v phosphoric acid). Four mL of Bradford Dye was added to each standard and sample tube. The final solution was mixed thoroughly on the vortex and allowed to sit for 5 minutes before taking the absorbance readings. The absorbance of the standards and the samples were measured using a Bio-Rad® Smart Spec™ Plus Spectrophometer.

V. Two-Dimensional Gel Electrophoresis

The *N. crassa* protein was obtained from the -70°C freezer. The passive rehydration was performed using Bio-Rad® ReadyStrip™ IPG strips. The IPG strips used in our passive rehydration had a pH gradient of 5-8 and two strip sizes, which were 7 cm and 17 cm. *N. crassa* protein samples and rehydration buffer (8-9.8M Urea, 0.5% CHAPS, 10mM DTT, 0-0.2% w/v Bio-Lytes, 0.001% Bromophenol Blue) were added to the rehydration/equilibration tray. For the 7 cm IPG strips, 125 µL of rehydration buffer and protein sample was used to provide a final protein concentration of 0.667 ng/µl. For the 17 cm IPG strips, 300 µL of rehydration buffer and protein sample was used to provide a final protein concentration of 0.667 ng/µl. The Bio-Rad® ReadyStrip™ IPG strips were placed, gel side down, on top of the rehydration buffer/protein sample in the

rehydration/equilibration tray. Any bubbles were removed if present under the IPG strips. The IPG strips were overlaid with mineral oil and the rehydration/equilibration tray was placed on the orbital shaker overnight.

The next day (approximately 20 hours), the rehydration/equilibration tray with the IPG strips was obtained from the orbital shaker and placed on the table in order to perform the first-dimension, isoelectric focusing (IEF). The Bio-Rad® Electrode Wicks were dipped in de-ionized H_2O, and placed over the wire electrodes in the Protean® IEF focusing tray. The IPG strips were obtained from the rehydration/equilibration tray and the mineral oil was blotted off the tips of the strips before transferring to the Protean® IEF focusing tray. Carefully, the IPG strips were placed over the wire electrodes of the IEF focusing tray with the gel side down and the positive (+) end of the strip matching the positive (+) electrode of the IEF focusing tray. The IPG strips were overlaid with mineral oil and the lid was placed on the IEF focusing tray. The IEF focusing tray was placed in the Bio-Rad® Protean IEF Cell. The Bio-Rad® Protean IEF Cell was turned on and programmed accordingly to the size of the IPG strips used. For the 7 cm IPG strips, the program was set at the preset method, linear ramping mode, 40,000 V-hr, and held at 500 V. For the 17 cm IPG strips, the program was set at the preset method, linear ramping mode, 60,000 V-hr, and held at 500 V.

The same day as isoelectric focusing (IEF) was performed; 12% poly-acrylamide gels were prepared in order to perform second dimension electrophoresis the following day. To prepare the 12% poly-acrylamide gels, the gel plates were cleaned with 70% ethanol and the poly-acrylamide solution was prepared. For 100 mL of solution: 12% acrylamide, 0.375 M Tris, 0.1% SDS, and later 0.1% ammonium persulfate, and 0.04%

TEMED was added to a beaker. After the poly-acrylamide solution was prepared, it was added in between the long and short gel plates and allowed to polymerize. For the 7 cm gels, the poly-acrylamide solution was added to each individual gel plate by using a sterile pipette. For the 17 cm gels, the Protean® plus Multi-Casting Chamber was used to add the poly-acrylamide solution. After the gels were polymerized, they were transferred to ½ X TGS buffer (For 1 x TGS buffer: 25mM tris, 192mM glycine, 0.1% SDS, H_2O, pH 8.6) and stored in the cooler until ready to perform second dimension electrophoresis.

The following day, after completion of the isoelectric focusing (IEF), the IEF focusing tray was obtained from the Bio-Rad® Protean IEF Cell and the IPG strips were removed from the IEF focusing tray. The IPG strips were blotted off, to drain the excess mineral oil, and placed in the rehydration/equilibration tray with the gel sides up. The IPG strips were then immersed in equilibration buffer I (6M urea, 2% SDS, 0.375M Tris-HCL, pH 8.8, 20% glycerol, 2% DTT) and placed on orbital shaker for 10 minutes. After 10 minutes, the IPG strips were transferred from equilibration buffer I to equilibration buffer II (6M urea, 2% SDS, 0.375M Tris-HCL, pH 8.8, 20% glycerol, 2.5% iodoacetamide) and were gently shaken on orbital shaker for 10 minutes. The IPG strips were then removed from equilibration buffer II and were transferred to 1 x TGS buffer for approximately 1-3 minutes. The 12% poly-acrylamide gels were obtained from the ½ X TGS buffer (25mM tris, 192mM glycine, 0.1% SDS, H_2O, pH 8.6) in the cooler and were filled with overlay agarose (0.5g agarose, 100 mL 1 X TGS buffer, 1 grain of bromophenol blue) to the top of the short plate. After the overlay agarose was added, the IPG strips were quickly placed directly on top of the 12% poly-acrylamide gels, with gel side up and positive (+) side on left, between the long and short plates. The gels were

then placed on the table or in the refrigerator for approximately 10 minutes until the agarose solidified. After the overlay agarose was solidified, the gels were placed into the electrophoresis cell in order to perform second dimension gel electrophoresis. The electrophoresis cell was then filled with 1 X TGS buffer, room temperature for 7 cm strips and chilled for 17 cm strips, and electrophoresis was started. For the 7 cm IPG strips, the gels were placed in the Bio-Rad® Mini-Protean® 3 Cell and were run on manual, constant milli-amps at 16 milli-amps per gel for approximately $2^{1/2}$ hours. For the 17 cm IPG strips, the gels were placed in the Bio-Rad® Protean® Plus Dodeca™ Cell and were run on manual, constant volts at 200 volts per run for approximately 8 hours. After electrophoresis, the gels were removed from the electrophoresis cell and stained. For the 7 cm IPG strips, the gels were stained with Comassie (0.25% Comassie Brilliant Blue R-250 [Sigma], 45% methanol, 10% acetic acid). For the 17 cm IPG strips, the gels were stained with SYPRO® purchased from Bio-Rad®.

To use Comassie stain, the gels were transferred to a staining container and were immersed in Comassie stain. The gels were kept in the staining container overnight, while being shaken on the orbital shaker. After being stained, the Comassie stain was removed from the staining container and the gels were then immersed in high de-stain (40% methanol, 10% acetic acid) for 1 hour while being shaken on the orbital shaker, in order to de-stain the gels. After 1 hour, the high de-stain was removed from the staining container and the gels were re-immersed in low de-stain (10% methanol, 6% acetic acid) for approximately two hours, while being shaken on the orbital shaker. The low de-stain was removed from the staining container and the gels were placed in de-ionized H_2O in

order for the gels to be scanned using the EPSON Scan Program and to be stored on the table top for future use.

To use SYPRO® stain, the gels were transferred to a staining container and immersed in fixing solution/high de-stain (40% methanol, 10% acetic acid) for approximately 1 hour to fix the gels. The fixing solution/high de-stain was then removed from the staining container and the gels were immersed in SYPRO® stain. The gels were kept in the SYPRO® stain overnight, while being gently shaken on the orbital shaker. The following day, the SYPRO® stain was removed from the staining container and the gels were re-immersed in de-ionized H_2O for 20 minutes.

VI. Imaging and Analysis

The SYPRO® stained gels were imaged using the Bio-Rad ChemiDoc XRS system. An ultraviolet transillumination lamp was then utilized to visualize the SYPRO® stained gels. The gel images were then analyzed using the Bio-Rad® PDQuest™ 2-D analysis software. In this software system, individual matchsets were first created for each experimental condition (glycerol, dextrose, and quinic acid). Protein spots were then automatically filtered, detected and matched among the individual matchsets. From the individual matchsets, higher level matchsets were then created to compare experimental conditions.

VII. Protein Spot Excision

In order to perform protein spot excision, the SYPRO® stained gels were placed directly on a moistened ultraviolet gel tray. The protein spots of interest were then excised from the illuminated SYPRO® stained gels utilizing a sterile Pasteur pipette. The

excised protein spots were then placed directly into a sterile eppendorf tube containing 5% v/v acetic acid. The eppendorf tubes containing the excised protein spots were then stored at -20ºC until submitted to the Ohio State University Mass Spectrometry and Proteomics Facility.

VIII. In Gel Digestion

At the Ohio State University Mass Spectrometry and Proteomics Facility, the protein samples were processed by the following procedure. First, the gels were digested with sequencing grade trypsin (Promega) or sequencing grade chymotrypsin (Roche) using the Multiscreen Solvinert Filter Plates (Millipore). Briefly, samples were then trimmed as close as possible to minimize background polyacrylamide material. The gel samples were then washed in nanopure water for 5 minutes. The wash step was then repeated twice before samples were washed with a 1:1 methanol/ammonium bicarbonate solution (methanol: 50 mM ammonium bicarbonate; v/v) for 10 minutes. The samples were then dehydrated with a 1:1 acetonitrile/ammonium bicarbonate solution (acetonitrile: 50 mM ammonium bicarbonate; v/v). Subsequently, the protein samples were rehydrated and incubated with a dithiothreitol solution (25 mM in 100 mM ammonium bicarbonate) for 30 minutes prior to the addition of iodoacetamide (55 mM iodoacetamide in 100 mM ammonium bicarbonate) solution. The protein samples were then incubated with iodoacetamide in the dark for 30 minutes. The samples were then washed again with two cycles of water and dehydrated using a 1:1 acetonitrile/ammonium bicarbonate solution (acetonitrile: 50 mM ammonium bicarbonate; v/v). The protease was then driven into the protein samples by rehydrating them in 12 ng/ml trypsin in 0.01% ProteaseMAX Surfactant for 5 minutes. After

rehydration, the samples were then overlaid with 40 ml of 0.01% ProteaseMAX Surfactant: 50 mM ABC and gently mixed on a shaker for 1 hour. The in gel digestion was finally stopped with the addition of 0.5% TFA.

IX. Mass Spectrometry

Capillary-liquid chromatography-nanospray tandem mass spectrometry (Nano-LC/MS/MS) was immediately performed on the digested samples to ensure high quality tryptic peptides with minimal non-specific cleavage. The Nano-LC/MS/MS was performed on a Thermo Finnigan LTQ mass spectrometer equipped with a nanospray source operated in a positive ion mode and the LC system was an UltiMate™ 3000 system (Dionex). To each protein sample, 5 µl of solvent A, 50mM acetic acid, and solvent B, acetonitrile, was first injected on to the µ-Precolumn Cartridge (Dionex) and then washed with 50 mM acetic acid. The injector port was then switched to inject and the peptides were eluted off of the trap onto the column. A 5 cm 75 µm ID ProteoPep II C18 column (New Objective, Inc.) packed directly in the nanospray tip was then used for chromatographic separations. The peptides were eluted directly off the column into the LTQ system using a gradient of 2-80%B over 45 minutes, with a flow rate of 300nl/min. The total run time was 65 minutes and the MS/MS was acquired according to standard conditions established in the laboratory. Briefly, a nanospray source operated with a spray voltage of 3 KV and a capillary temperature of 200^{o}C was used. The scan sequence of the mass spectrometer was based on the TopTen™ method; the analysis was programmed for a full scan recorded between 350 – 2000 Da, and a MS/MS scan to generate product ion spectra to determine amino acid sequence in consecutive instrument

scans of the ten most abundant peak in the spectrum. The CID fragmentation energy was set to 35%. Dynamic exclusion was enabled with a repeat count of 2 within 10 seconds, a mass list size of 200, an exclusion duration of 350 seconds, a low mass width of 0.5 and a high mass width of 1.5.

Data processing was performed following recommended guidelines. Sequence information from the MS/MS data was processed by converting the raw files into a merged file (.mgf) using an in-house program, RAW2MZXML_n_MGF_batch (merge.pl, a Perl script). The resulting mgf files were searched using Mascot Daemon (version 2.2.2; Matrix Science) against the full SwissProt database (version 57.5; 471472 sequences; 167326533 residues) or the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.hih.gov) database (version 20091013; 9873339 sequences; 3367482728 residues). The mass accuracy of the precursor ions were set to 2.0 Da given that the data was acquired on an ion trap mass analyzer and the fragment mass accuracy was set to 0.5 Da. Considered modifications (variable) were methionine oxidation and carbamidomethyl cysteine. Two missed cleavages for the enzyme were permitted. A decoy database was searched to determine the false discovery rate (FDR) and the peptides were filtered according to the FDR and proteins identified required bold red peptides. Protein identifications were checked manually and proteins with a Mascot score of 50 or higher with a minimum of two unique peptides from one protein having a -*b* or -*y* ion sequence tag of five residues or better were accepted.

RESULTS

To assess protein expression levels, identify proteins that are unique and others that are differentially regulated, our research studied protein profiles of *Neuospora crassa* grown on three different carbon sources: 2% dextrose, 2% glycerol, and 0.3% quinic acid. To initiate the study of protein profiles, the conidia of *N. crassa* were harvested and cultures were grown overnight. The mycelia from the overnight culture were collected and transferred to one of the three carbon sources: 2% dextrose, 2% glycerol, or 0.3% quinic acid. The resulting mycelial pads were homogenized as described earlier in order to isolate proteins from *N. crassa*. As stated in the methods, protein profiles were then generated by two-dimensional gel electrophoresis (2-DGE) from the whole proteins extracted from *N. crassa* grown on each of three carbon sources. All two-dimensional (2-D) gels utilized in the study were of 12% poly-acrylamide and were prepared in triplicate in order to ensure reproducibility. The orientation of the two-dimensional (2-D) gels were of an increasing pI from left to right and a decreasing moleucular mass from top to bottom.

The initial protein profiles generated in our expermient by performing a two-dimensional gel electrophoresis (2-DGE) were mini format, 7 cm, gels with a pI range of 5-8. All mini format 2-D gels utilized in the study were stained with coomasie in order to visualize protein spots. The resulting mini format 2-D gels are shown in Figures 4, 5, and 6. Figure 4 depicts representative images of mini 2-D gels in triplicate from *N. crassa* tissue grown in 2% dextrose. Figure 5 depicts representative images of mini 2-D gels in triplicate from *N. crassa* tissue grown in 2% glycerol. Figure 6 depicts representative images of mini 2-D gels in triplicate from *N. crassa* tissue grown in 0.3% quinic acid.

Once acceptable protein profiles were generated for the mini format 2-D gels, we began utilizing a large format 2-D gel for analytical purposes. The second protein profiles then generated were done by performing 2-DGE in large format, 17 cm, gels with a pI range of 5-8. All large format two-dimensional (2-D) gels utilized in the study were stained with SYPRO® in order to visualize protein spots under ultraviolet light. The resulting large format two-dimensional (2-D) gels are shown in Figures 7, 8, and 9. Figure 7 depicts a representative image of a large 2-D gel from *N. crassa* tissue grown in 2% dextrose. Figure 8 depicts a representative image of a large 2-D gel from *N. crassa* tissue grown in 2% glycerol. Figure 9 depicts a representative image of a large 2-D gel from *N. crassa* tissue grown in 0.3% quinic acid. Once acceptable results were generated for the large format 2-D gels, our research consisted of analyzing the 2-D gels.

In order to properly analyze the 2-D gels, as stated in the methods, the Bio-Rad® PDQuest™ 2-D analysis software was utilized in our study. Utilizing this software, individual matchsets were first created for each experimental condition in order to evaluate like trials. In our experiment, the different experimental conditions included the three sole carbon sources utilized to grow *N. crassa* tissue: 2% dextrose, 2% glycerol, and 0.3% quinic acid. All individual matchsets created for each experimental condition included 2-D gel images in triplicate and a master gel image (white). The individual matchsets created utilizing PDQuest™ for 2% dextrose, 0.3% quinic acid, and 2% glycerol are shown in Figures 10, 11, and 12. Figure 10 depicts a representative individual matchset generated from triplicate gel images of *N. crassa* tissue grown in 2% dextrose as the sole carbon source. Figure 11 illustrates a representative individual matchset generated from triplicate gel images of *N. crassa* grown in 2% glycerol as the

Figure 4: Coomasie stained gels (7 cm in length and pI range 5-8) depicting *N. crassa* proteins from tissue grown in 2% dextrose resolved by two-dimensional gel electrophoresis (2-DGE). Cells were homogenized as described in the Materials and Methods. Gels were done in triplicate.

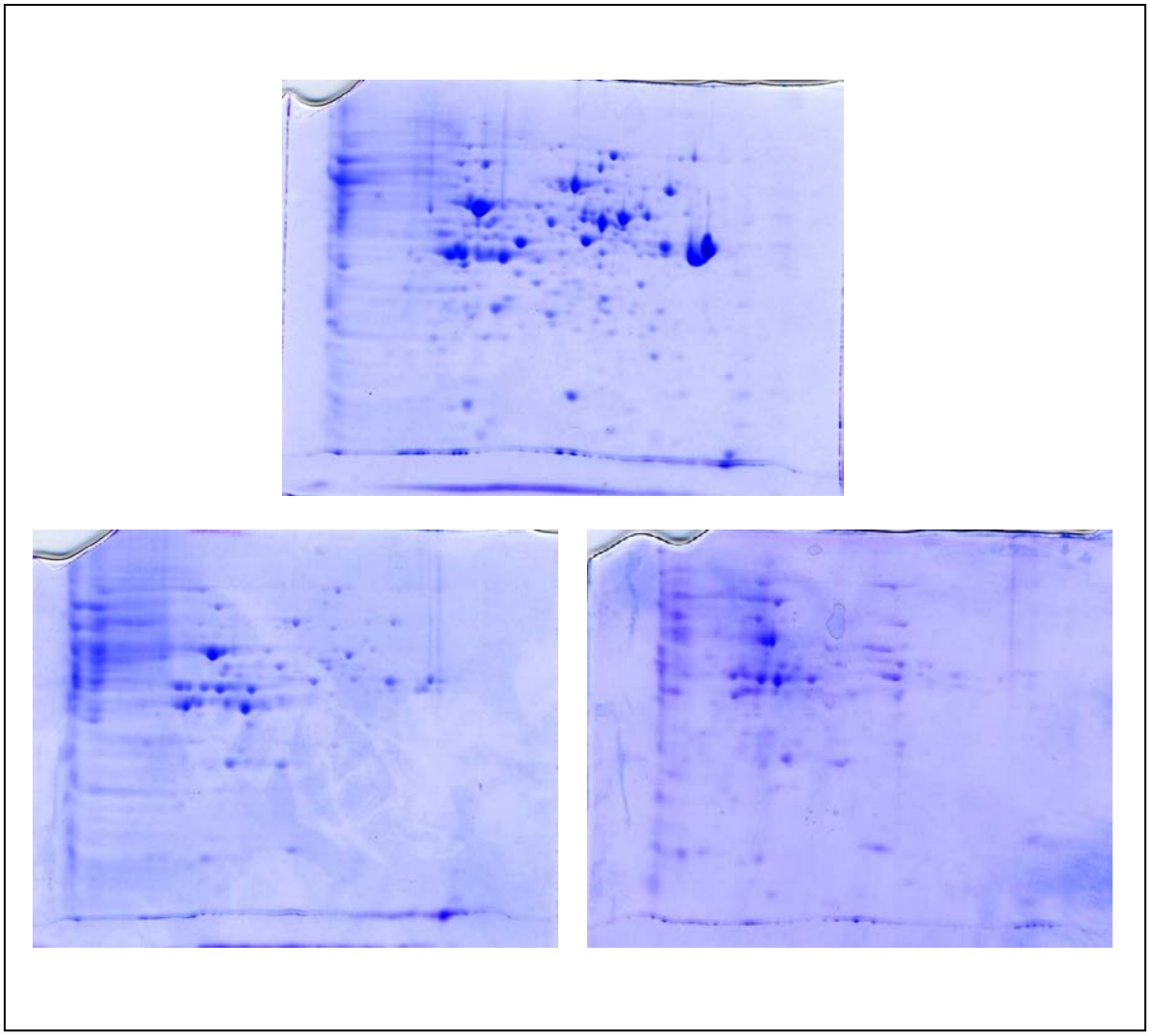


Figure 5: Coomasie stained gels (7 cm in length and pI range 5-8) depicting *N. crassa* proteins from tissue grown in 2% glycerol resolved by two-dimensional gel electrophoresis (2-DGE). Cells were homogenized as described in Materials and Methods. Gels were done in triplicate.

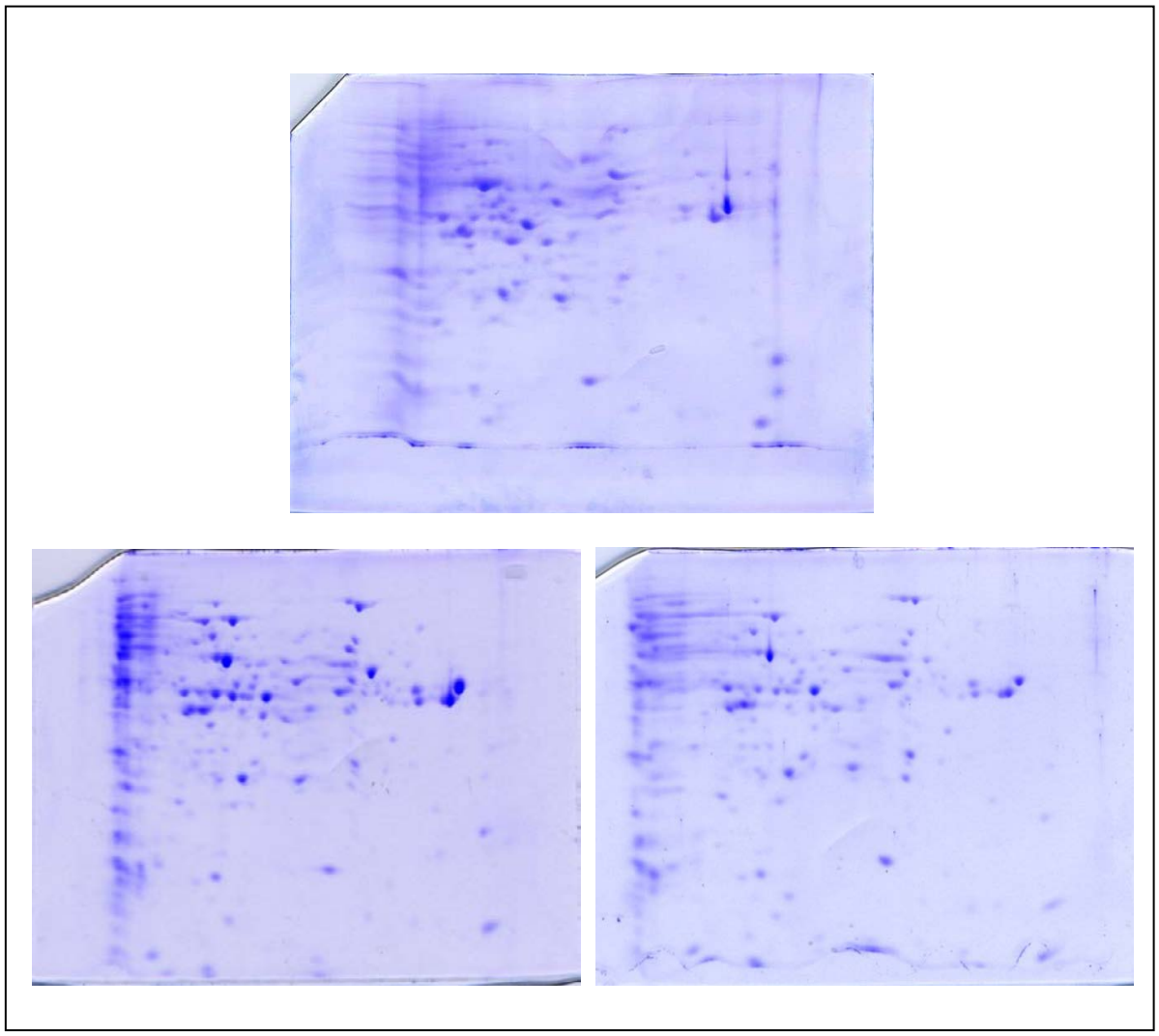


Figure 6: Coomasie stained gels (7 cm in length and pI range 5-8) depicting *N. crassa* proteins from tissue grown in 0.3% quinic acid resolved by two-dimensional gel electrophoresis (2-DGE). Cells were homogenized as described earlier in Materials and Methods. Gels were done in triplicate.

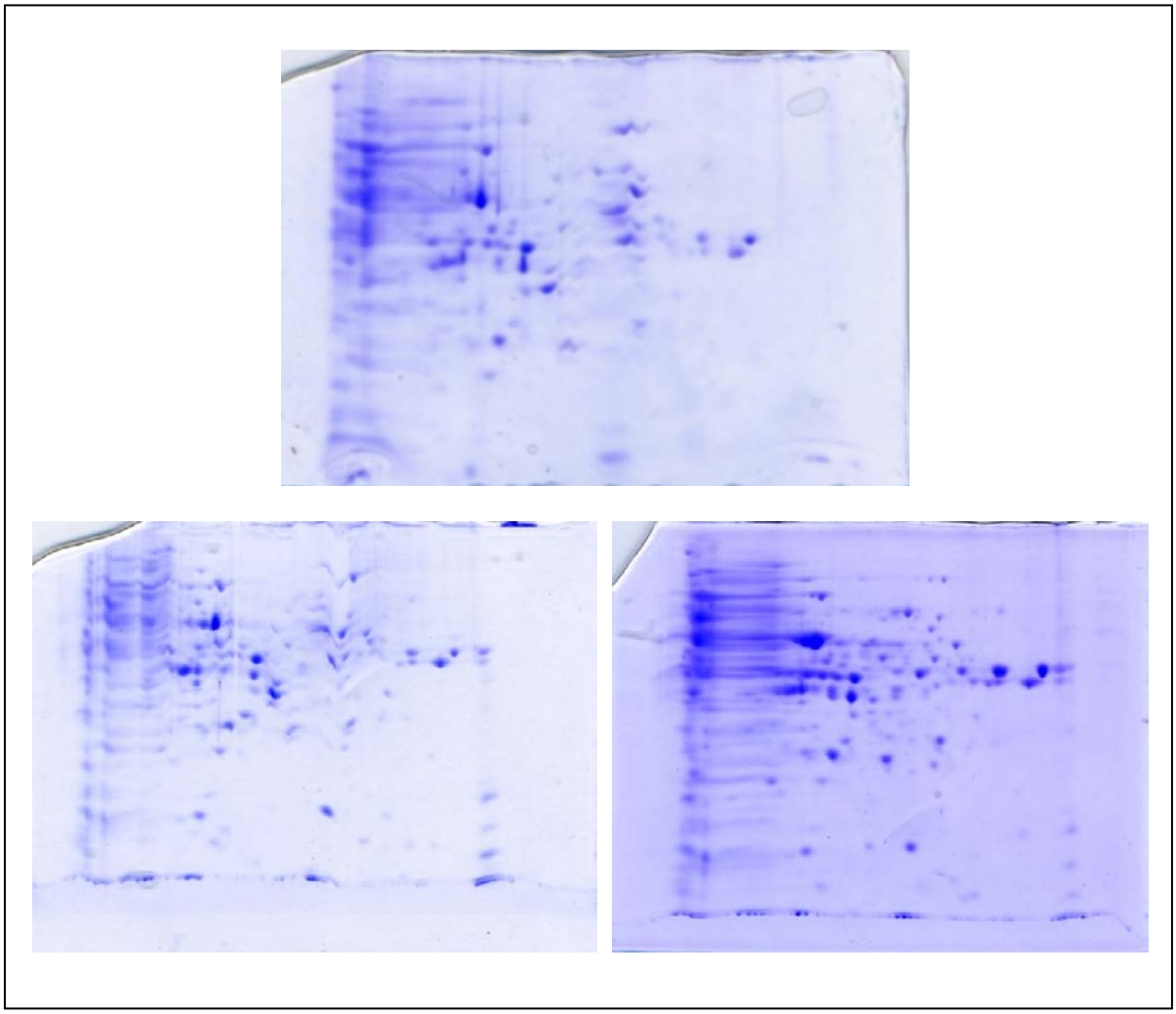


Figure 7: SYPRO stained gel (17 cm in length and pI range 5-8) depicting *N. crassa* proteins from tissue grown in 2% dextrose resolved by two-dimensional gel electrophoresis (2-DGE). Cells were homogenized as described earlier. Gels were done in triplicate.

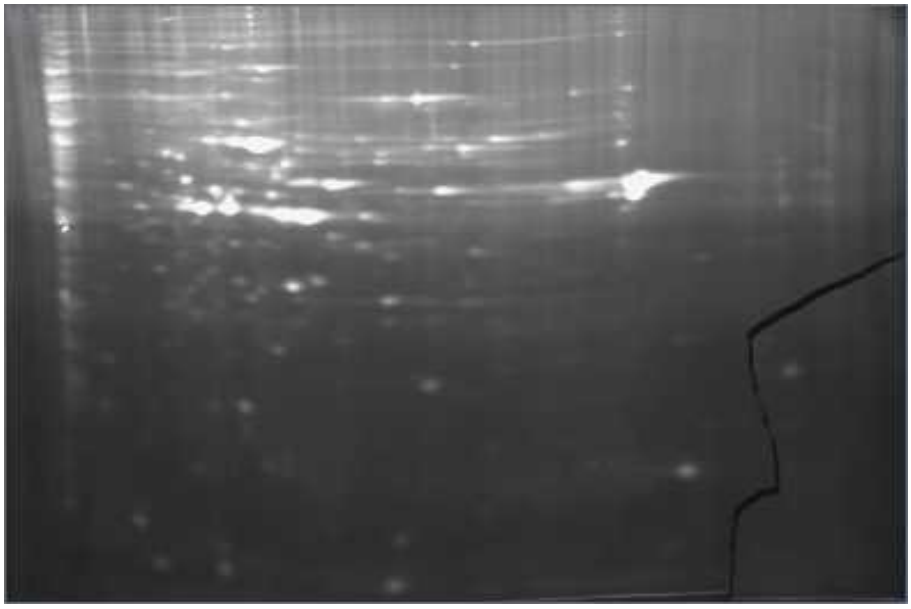


Figure 8: SYPRO stained gel (17 cm in length and pI range 5-8) depicting *N. crassa* proteins from tissue grown in 2% glycerol resolved by two-dimensional gel electrophoresis (2-DGE). Cells were homogenized as described earlier. Gels were done in triplicate.

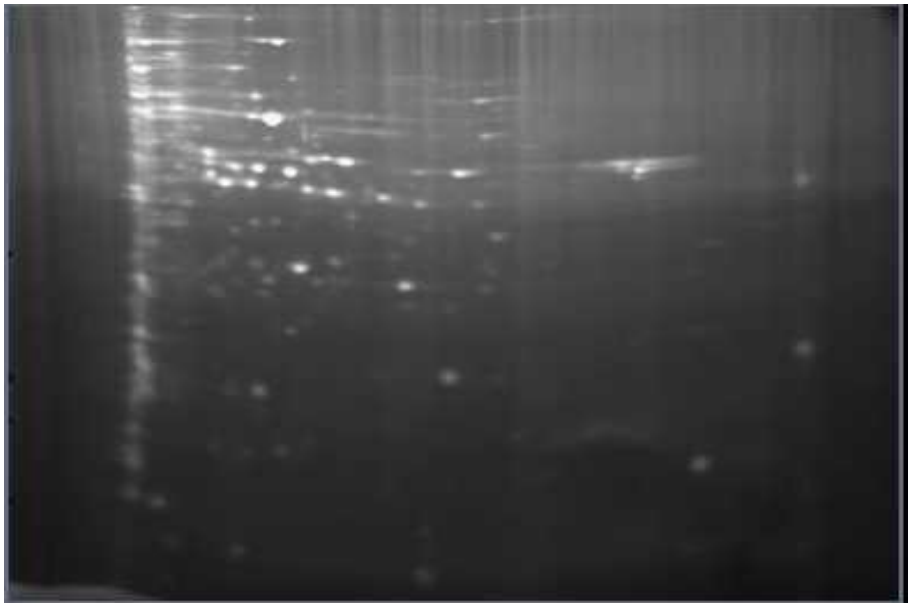
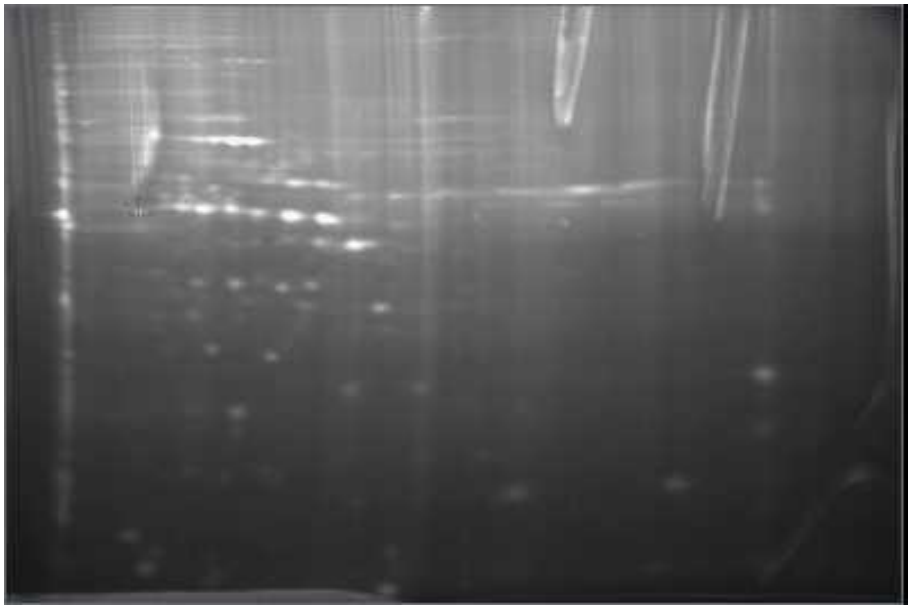


Figure 9: SYPRO stained gel (17 cm in length and pI range 5-8) depicting *N. crassa* proteins from tissue grown in 0.3% quinic acid resolved by two-dimensional gel electrophoresis (2-DGE). Cells were homogenized as described earlier. Gels were done in triplicate.



sole carbon source. Figure 12 depicts a representative individual matchset generated from triplicate gel images of *N. crassa* tissue grown in 0.3% quinic acid as the sole carbon source.

Once individual matchsets were generated, the master gel images (white) were then utilized to create higher level matchsets in order to compare and contrast the masters from each of the experimental conditions. All higher level matchsets generated in our study included the master gel images from two different experimental conditions and a new higher master gel image. The higher level matchsets created from utilizing PDQuest™ are shown in Figures 13, 14, and 15. Figure 13 depicts a representative higher level matchset generated from comparing the master gel images of dextrose and quinic acid. From the analysis of this higher matchset, there were 38 matched (shown as green letters) and 82 unmatched (shown as red circles) protein spots found between dextrose and quinic acid. Figure 14 illustrates a representative higher level matchset created from comparing the master gel images of dextrose and glycerol. From the analysis of this higher matchset, there were 62 matched (shown as green letters) and 58 unmatched (shown as red circles) protein spots found between dextrose and glycerol. Figure 15 depicts a representative higher level matchset generated from comparing the master gel images of glycerol and quinic acid. From the analysis of this higher matchset, there were 33 matched (shown as green letters) and 62 unmatched (shown as red circles) protein spots found between glycerol and quinic acid. From the anlaysis of all higher matchsets generated, there were approximately 31 matched protein spots found between glycerol, quinic acid, and dextrose. Once these results were obtained from the computational analysis of PDQuest™, our master gel images were then manually analyzed in order

Figure 10: Individual matchset of dextrose gels created using PDQuest™.

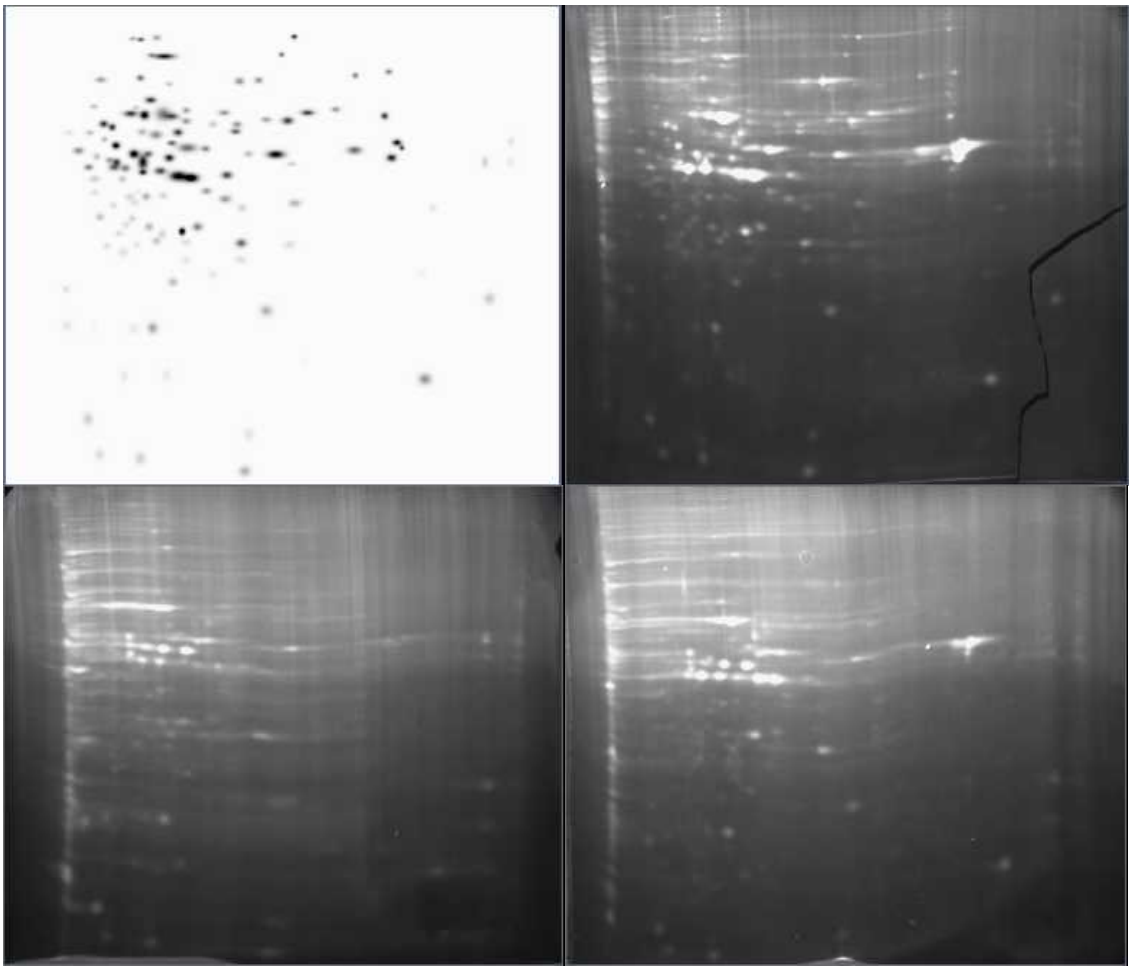


Figure 11: Individual matchset of glycerol gels created using PDQuest™.

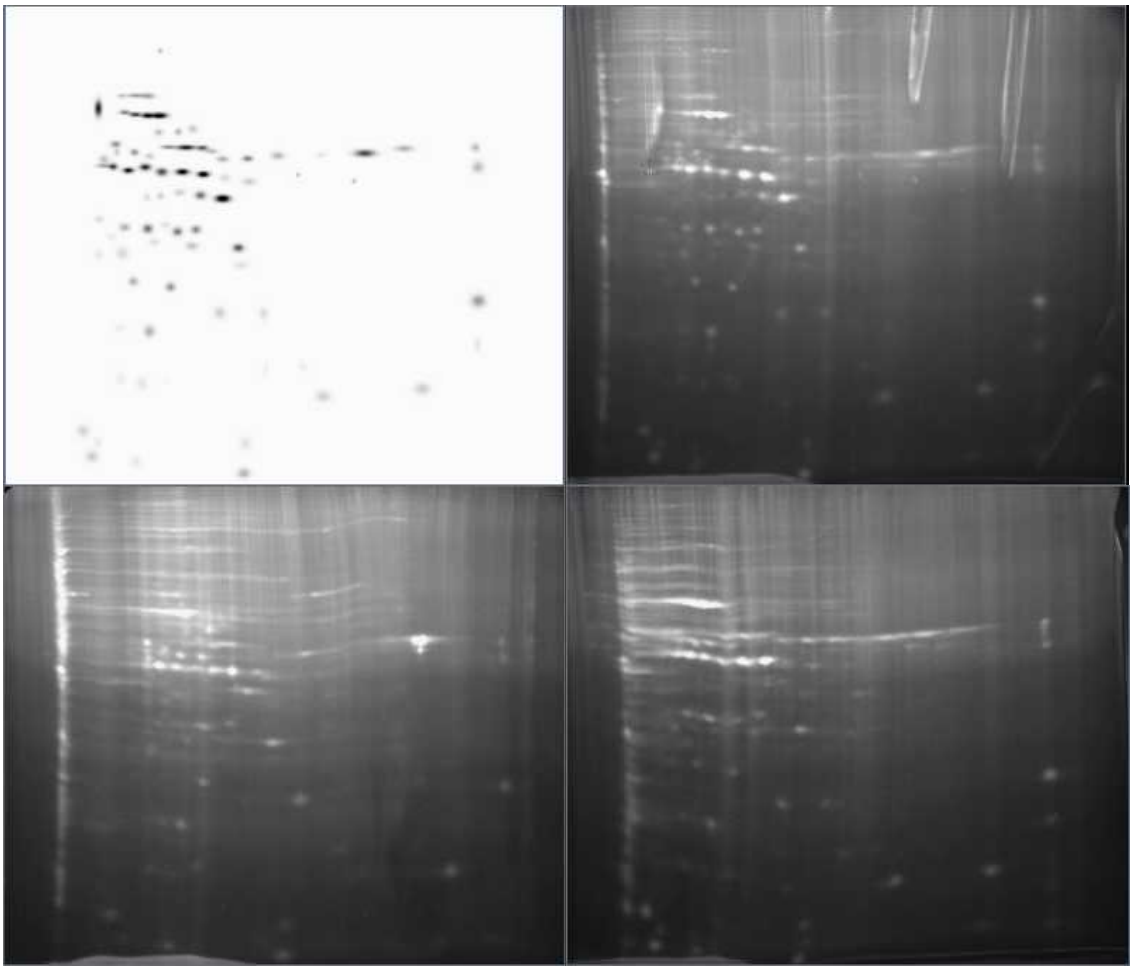


Figure 12: Individual matchset of quinic acid gels created using PDQuest™.

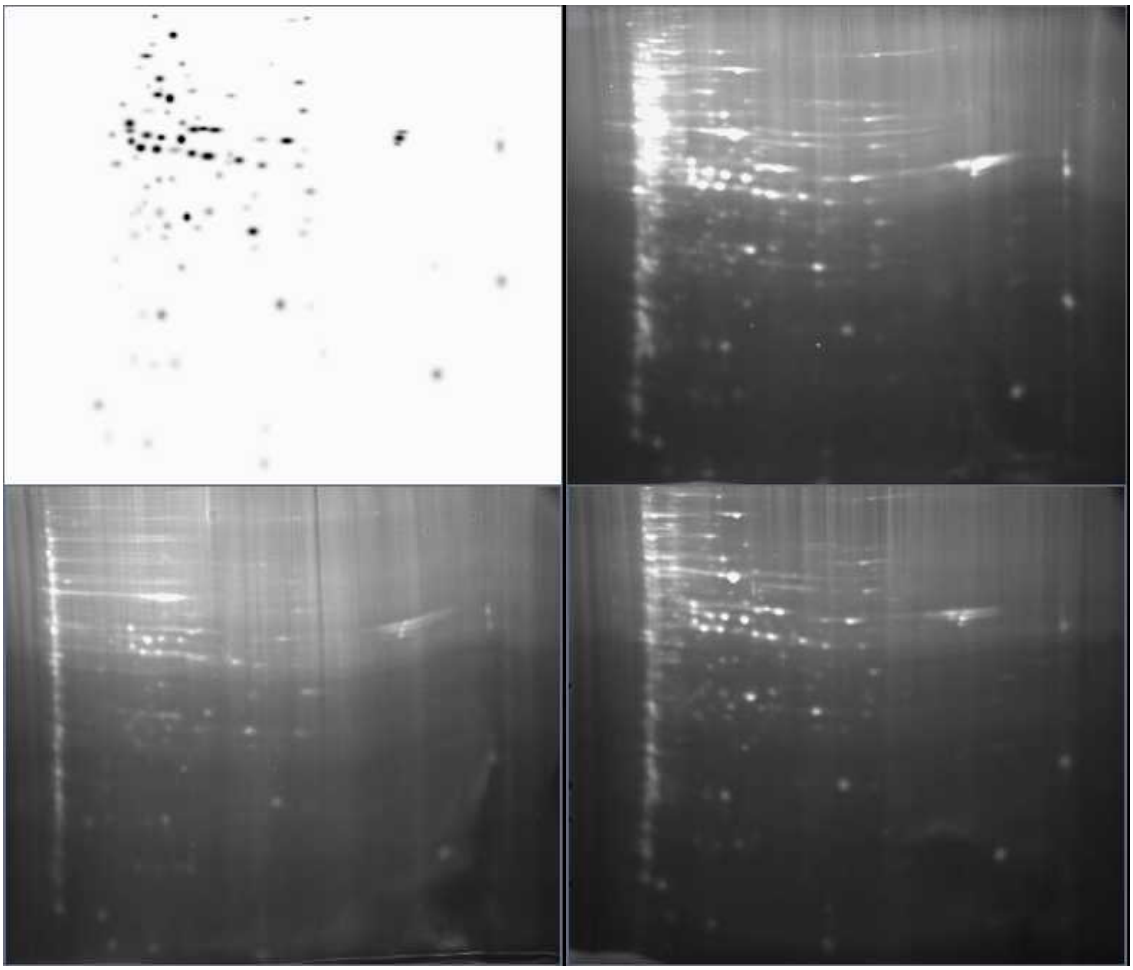
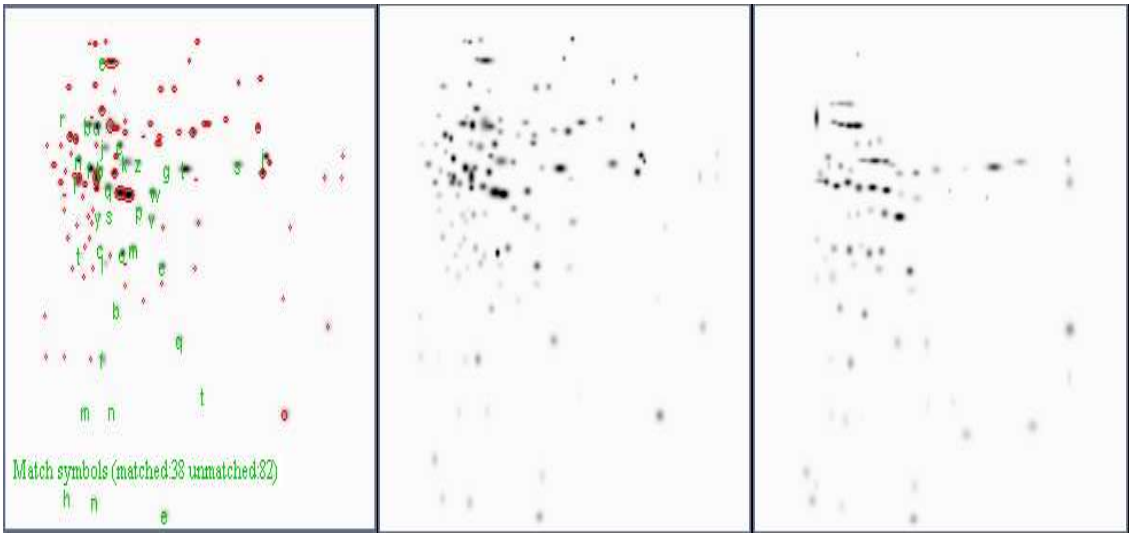


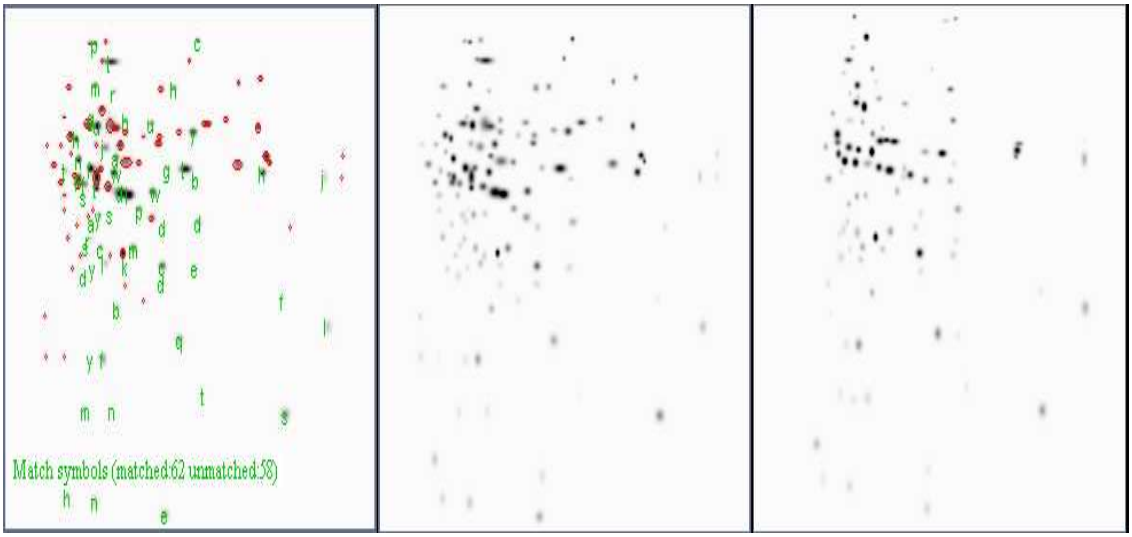
Figure 13: Higher level matchset comparing master gels of dextrose and quinic acid. Matched protein spots are shown as green letters and unmatched protein spots are shown as red circles.

.



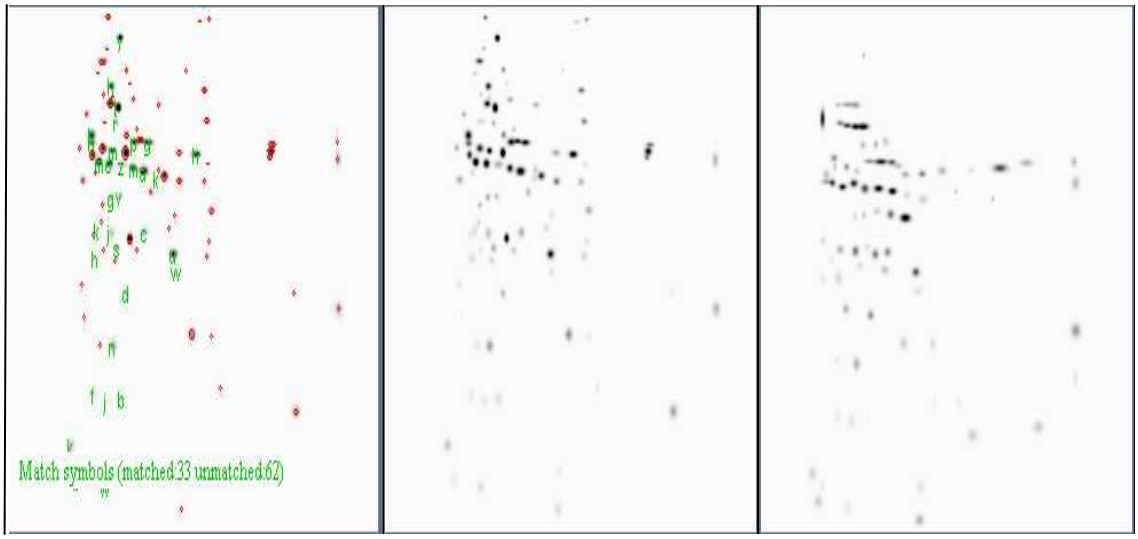
Higher Master	Dextrose	Quinic Acid

Figure 14: Higher level matchset comparing master gels of dextrose and glycerol. Matched protein spots are shown as green letters and unmatched protein spots are shown as red circles.



Higher Master	Dextrose	Glycerol

Figure 15: Higher level matchset comparing master gels of glycerol and quinic acid. Matched protein spots are shown as green letters and unmatched protein spots are shown as red circles.



Higher Master	Glycerol	Quinic Acid

to ensure that the protein spots were accuratally matched and unmatched among the different carbon sources. Based on these image analysis results, both computational and manual, it was established that each carbon source utilized in our study to grow *N. crassa* exhibited proteins that are unique as well as ones that are differentially expressed. Also, it was determined that more protein was being produced while grown in the preferred carbon source, dextrose, whereas less protein was being produced while grown in quinic acid. The image analysis results also revealed that unique patterns of protein expression are being generated for each different carbon source.

Once the 2-D gel images were completely analyzed by PDQuest™, protein spots of interest were excised from the large format 2-D gels in order to be sent to the Ohio State University Mass Spectrometry and Proteomics Facility for partial sequence determination and punitive identification. From our collective large 2-D gels, a total of 31 protein spots were selected and excised. The 17 cm 2-D gels displaying the excised spots are shown in Figures 16, 17, and 18. In Figure 16, eight protein spots were excised from the master 2-D gel image of *N. crassa* grown in 2% dextrose. Six of the spots, labeled as squares, were presumed to be unqiue to dextrose as the sole carbon source. The remaining two spots, labeled as circles, were presumed to be differentially expressed among dextrose and one of the other carbon sources. In Figure 17, eight protein spots are shown as excised from the master 2-D gel image of *N. crassa* grown in 0.3% quinic acid. Six of the spots, labeled as squares, were assumed to be unique to quinic acid as the sole carbon source. The remaining two spots, labeled as circles, were assumed to be differentially expressed among quinic acid and one of the other carbon sources. In Figure 18, four protein spots are shown as excised from the master 2-D gel image of *N. crassa*

Figure 16: The labeled proteins shown in the master gel were excised and sent for MS. Those labeled that are boxed (square) denote proteins that are essentially unique to dextrose as the sole carbon source. Those labeled that are circled denote proteins that are expressed at higher levels in a particular carbon source. Labeled proteins correspond to those listed in Table 4.

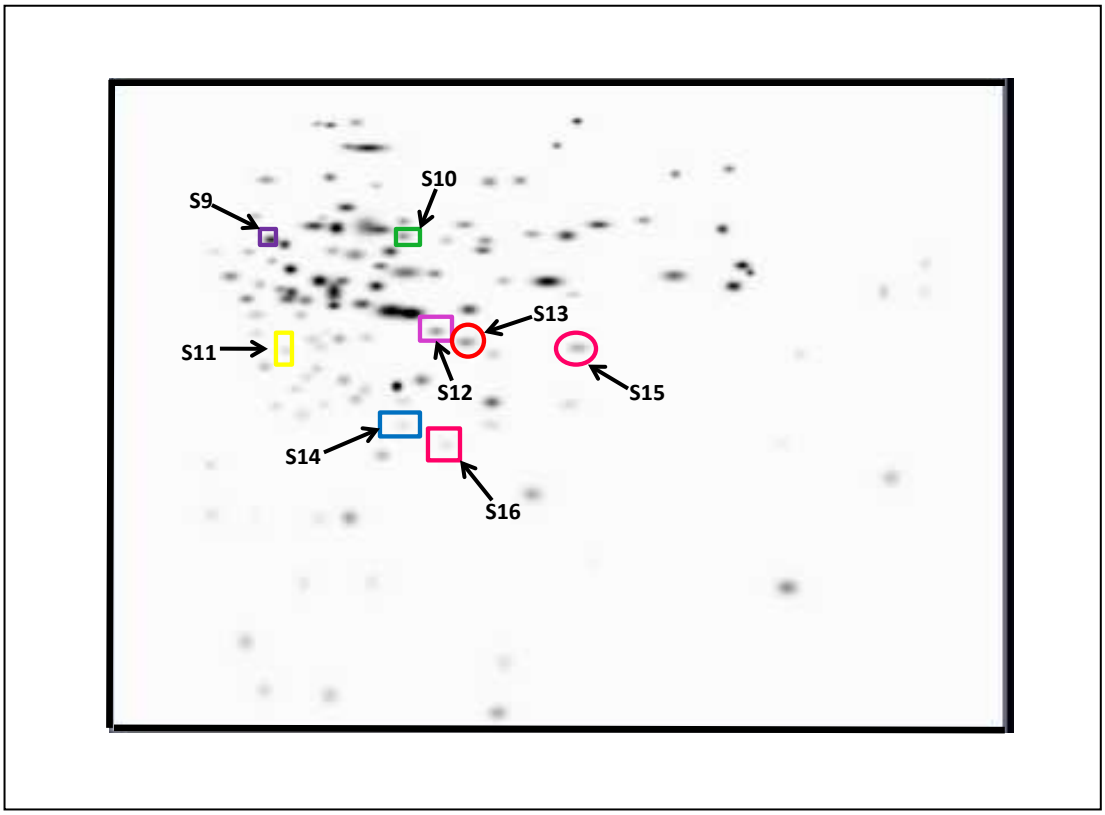

S9
S10
S11
S12
S13
S14
S15
S16

Figure 17: The labeled proteins shown in the master gel were excised and sent for MS. Those labeled that are boxed (square) denote proteins that are essentially unique to quinic acid as the sole carbon source. Those labeled that are circled denote proteins that are expressed at higher levels in a particular carbon source. Labeled proteins correspond to those listed in Table 4.

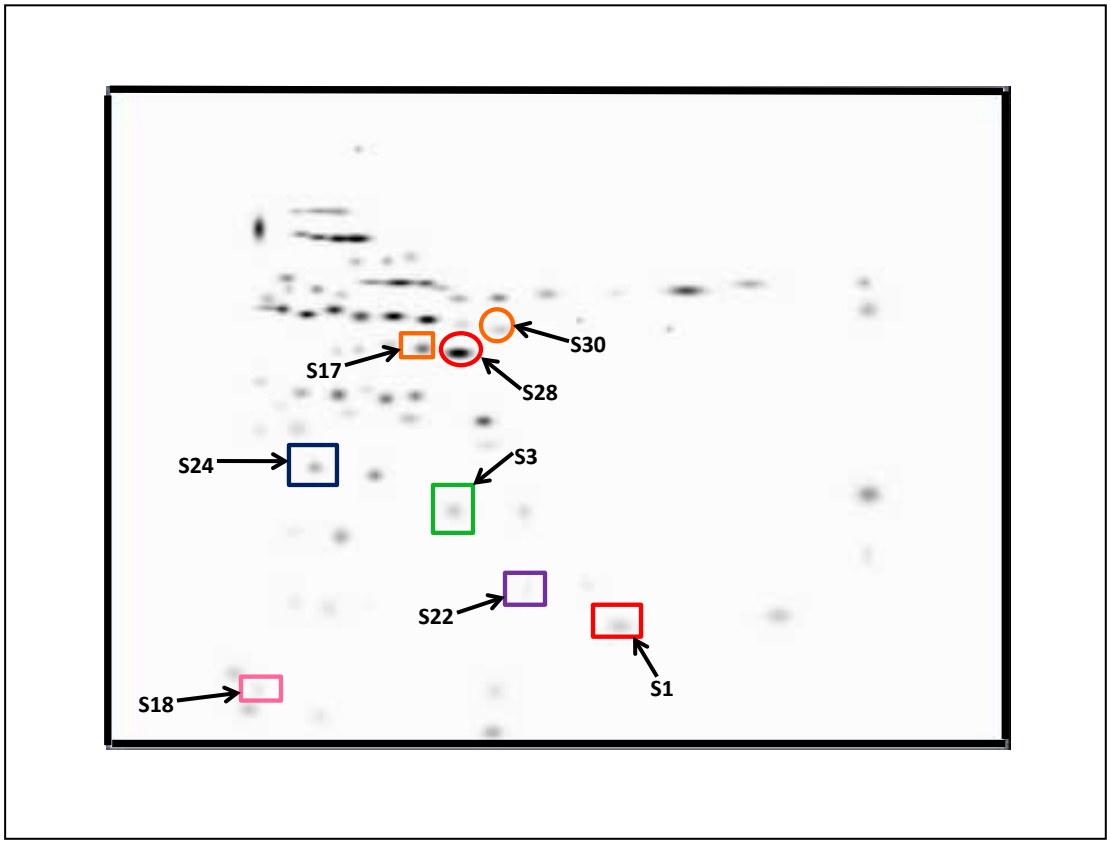
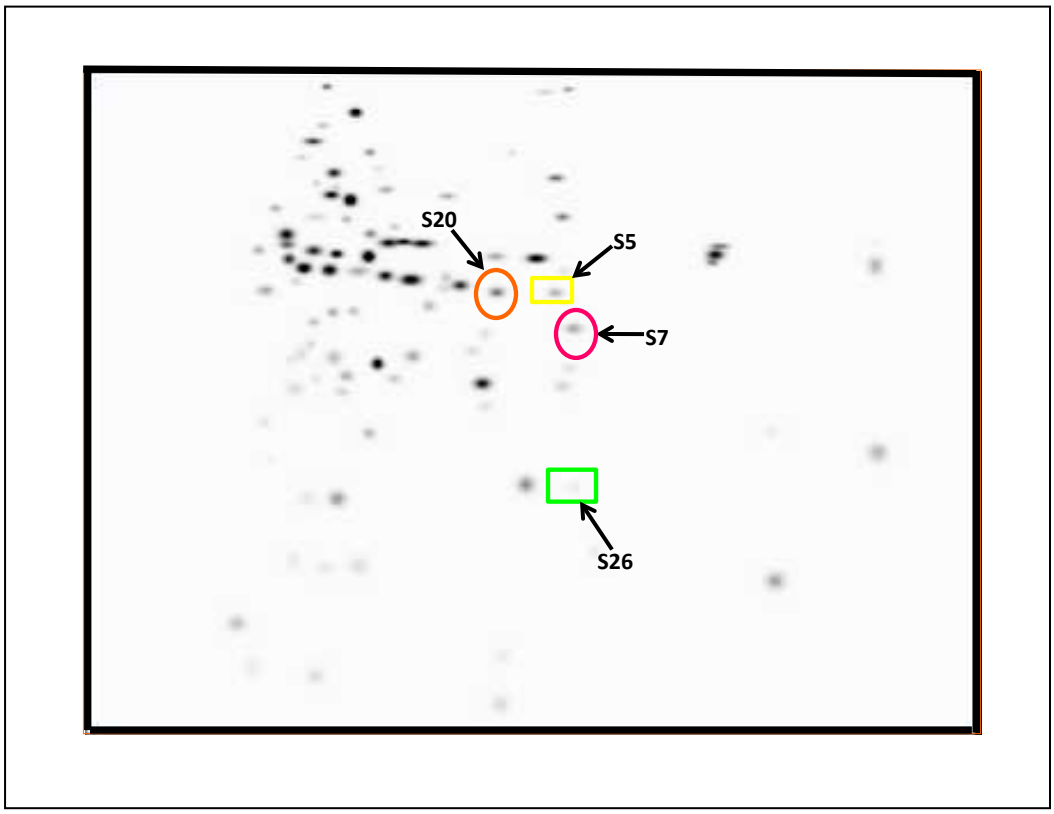

S30
S17
S28
S24
S3
S22
S1
S18

Figure 18: The labeled proteins shown in the master gel were excised and sent for MS. Those labeled that are boxed (square) denote proteins that are essentially unique to glycerol as the sole carbon source. Those labeled that are circled denote proteins that are expressed at higher levels in a particular carbon source. Labeled proteins correspond to those listed in Table 4.


S20
S5
S7
S26

grown in 2% glycerol. Two of the spots, labeled as squares, were presumed to be unique to glycerol as the sole carbon source. The remaining two spots, labeled as circles, were presumed to be differentially expressed among glycerol and one of the other carbon sources.

Once the excised spots were received at the Ohio State University, capillary-liquid chromatography-nanospray tandem mass spectrometry was performed in order to recover and sequence all the selected protein spots. The identifications and functions of all the spots were then determined by searching for homologies among fungal proteins within SwissProt and NCBI databases. The results of these analyses are shown in Table 4. In Table 4, all 31 excised protein spots, denoted S1-S31, are described in further detail with their protein name, NCBI accession, theoretical pI/Mr, experimental pI/Mr, sequence coverage, and their MS/MS peptide sequence. Each of the peptides listed in the table possessed an ion score above the lowest acceptable values (> 50) indicating identity or extensive homology.

Based on all spots analyzed, identifications were assigned to fourteen unique proteins as well as three proteins that were established to be up-regulated, differentially expressed among two different carbon sources. It was determined that six of the protein spots, S9, S10, S11, S12, S14, and S16, identified were essentially unique to dextrose as the sole carbon source. These proteins found to be unique to dextrose included an ATP-dependent RNA helicase (eIF4A), an enolase, a ribosomal protein, as well as a cytrochrome c peroxidase. Two of the other protein spots, S5 and S26, identified were concluded to be essentially unique to glycerol as the sole carbon source. These proteins that were found to be unique to glycerol included a 30s ribosomal protein and a probable

Table 4: Identification of proteins excised from *N. crassa* grown on dextrose, quinic acid or glycerol. Spot, spot number corresponds to labeled spots in Figs. 16, 17, and 18 (as indicated in parentheses). A (*) denote proteins that were duplicates; Carbon source, source protein spot was excised; Protein name, matched protein description; NCBI accession, accession number and submission version of matched protein from NCBI database; Theo. pI/Mr (kDa), theoretical isoelectric point and molecular mass based on amino acid sequence of the identified protein; Expt. pI/Mr (kDa), experimental isoelectric point and molecular mass estimated from the 2DGE gel; Species, the fungal species of the matched protein; Mascot score, score obtained from the Mascot search for each match; MS/MS Peptide Sequence, amino acid sequences of peptides identified by Nano-LC/MS/MS; SC, percent amino acid sequence coverage for the identified protein.

Spot (Fig.)	Carbon Source	Protein Name	NCBI Accesion	Species	Theo. pI/Mr (kDa)	Expt. pI/Mr (kDa)	Mascot Score	MS/MS Peptide Sequence	SC (%)
S1 (18)	Quinic Acid	Peptidyl-prolyl cis-trans isomerase	P20080	*Neurospora crassa*	6.74/13029	7.5/13000	656	M. TIPQLDGLQIEVQQEGQGTR. E R. RGDNVDVHYK. G R. RGDNVDVHYK. G R. RGDNVDVHYK. G K. KFDASYDR. G K. KFDASYDR. G K. KFDASYDRGEPLNFTVGQGQVIK. G K. FDASYDR. G K. FDASYDR. G K. FDASYDRGEPLNFTVGQGQVIK. G R. GEPLNFTVGQGQVIK. G R. GEPLNFTVGQGQVIK. G R. KLTIAPHLAYGNR. A R. KLTIAPHLAYGNR. A K. LTIAPHLAYGNR. A K. LTIAPHLAYGNR. A K. LTIAPHLAYGNR. A R. AVGGIIPANSTLIFETELVGIK. G R. AVGGIIPANSTLIFETELVGIK. G R. AVGGIIPANSTLIFETELVGIKGVQK. G R. AVGGIIPANSTLIFETELVGIKGVQK. G R. AVGGIIPANSTLIFETELVGIKGVQKGE. - R. AVGGIIPANSTLIGETELVGIKGVQKGE. -	78
S2 (*)	Quinic Acid	Peptidyl-prolyl cis-trans isomerase	P20080	Neurospora crassa	6.74/13029	7.5/13000	655	M. TIPQLDGLQIEVQQEGQGTR. E M. TIPQLDGLQIEVQQEGQGTR. E M. TIPQLDGLQIEVQQEGQGTR. E M. TIPQLDGLQIEVQQEGQGTR. E R. RGDNVDVHYK. G R. RGDNVDVHYK. G R. RGDNVDVHYK. G R. RGDNVDVHYK. G R. RGDNVDVHYK. G R. RGDNVDVHYK. G K. FDASYDR. G K. FDASYDR. G K. FDASYDRGEPLNFTVGQGQVIK. G K. FDASYDRGEPLNFTVGQGQVIK. G K. FDASYDRGEPLNFTVGQGQVIK. G R. GEPLNFTVGQGQVIK. G R. GEPLNFTVGQGQVIK. G R. KLTIAPHLAYGNR. A R. KLTIAPHLAYGNR. A R. KLTIAPHLAYGNR. A R. KLTIAPHLAYGNR. A R. KLTIAPHLAYGNR. A R. KLTIAPHLAYGNR. A K. LTIAPHLAYGNR. A K. LTIAPHLAYGNR. A K. LTIAPHLAYGNR. A R. AVGGIIPANSTLIFETELVGIK. G R. AVGGIIPANSTLIFETELVGIK. G R. AVGGIIPANSTLIFETELVGIK. G R. AVGGIIPANSTLIFETELVGIK. G	77

Spot (Fig.)	Carbon Source	Protein Name	NCBI Accesion	Species	Theo. pI/Mr (kDa)	Expt. pI/Mr (kDa)	Mascot Score	MS/MS Peptide Sequence	SC (%)
								R. AVGGIIPANSTLIFETELVGIK. G	
								R. AVGGIIPANSTLIFETELVGIK. G	
								R. AVGGIIPANSTLIFETELVGIK. G	
								R. AVGGIIPANSTLIFETELVGIK. G	
								R. AVGGIIPANSTLIFETELVGIK. G	
								R. AVGGIIPANSTLIFETELVGIK. G	
								R. AVGGIIPANSTLIFETELVGIK. G	
								R. AVGGIIPANSTLIFETELVGIK. G	
								R. AVGGIIPANSTLIFETELVGIKGVQKGE. -	
S3(18)	Quinic Acid	Superoxide dismutase [Cu-Zn]	P07509	*Neurospora crassa*	5.74/15989	6.75/21100	447	K. AVAVVRGDSNVK. G	67
								K. GTVIFEQESESAPTTITYDISGNDPNAK. R	
								K. GTVIFEQESESAPTTITYDISGNDPNAK. R	
								K. GTVIFEQESESAPTTITYDISGNDPNAK. R	
								K. GTVIFEQESESAPTTITYDISGNDPNAK. R	
								K. GTVIFEQESESAPTTITYDISGNDPNAK. R	
								R. HVGDLGNIETDAQGNAK. G	
								R. HVGDLGNIETDAQGNAK. G	
								R. HVGDLGNIETDAQGNAK. G	
								R. HVGDLGNIETDAQGNAK. G	
								K. GTVTDNLVK. L	
								K. GTVTDNLVK. L	
								K. GTVTDNLVK. L	
								K. GTVTDNLVK. L	
								K. GTVTDNLVK. L	
								K. GTVTDNLVK. L	
								R. TVWHAGTDDLGK. G	
								R. TVWHAGTDDLGK. G	
								R. TVWHAGTDDLGK. G	
								R. TVWHAGTDDLGKGGNEESLK. T	
								K. TGNAGPRPACGVIGISQ. -	
S4(*)	Quinic Acid	Superoxide dismutase [Cu-Zn]	P07509	*Neurospora crassa*	5.74/15989	6.75/21100	723	K. AVAVVRGDSNVK. G	74
								K. GTVIFEQESESAPTTITYDISGNDPNAK. R	
								K. GTVIFEQESESAPTTITYDISGNDPNAK. R	
								K. GTVIFEQESESAPTTITYDISGNDPNAK. R	
								K. GTVIFEQESESAPTTITYDISGNDPNAKR. G	
								R. HVGDLGNIETDAQGNAK. G	
								R. HVGDLGNIETDAQGNAK. G	
								R. HVGDLGNIETDAQGNAK. G	
								R. HVGDLGNIETDAQGNAK. G	
								R. HVGDLGNIETDAQGNAK. G	
								K. GTVTDNLVK. L	
								K. GTVTDNLVK. L	
								K. GTVTDNLVK. L	
								K. GTVTDNLVK. L	
								K. GTVTDNLVK. L	
								K. GTVTDNLVKLIGPESVIGR. T	
								K. GTVTDNLVKLIGPESVIGR. T	
								K. LIGPESVIGR. T	
								K. LIGPESVIGR. T	
								K. LIGPESVIGR. T	
								K. LIGPESVIGR. T	
								K. LIGPESVIGR. T	

Spot (Fig.)	Carbon Source	Protein Name	NCBI Accesion	Species	Theo. pI/Mr (kDa)	Expt. pI/Mr (kDa)	Mascot Score	MS/MS Peptide Sequence	SC (%)
								K. LIGPESVIGR. T	
								R. TVVVHAGTDDLGK. G	
								R. TVVVHAGTDDLGK. G	
								R. TVVVHAGTDDLGK. G	
								R. TVVVHAGTDDLGK. G	
								R. TVVVHAGTDDLGKGGNEESLK. T	
								R. TVVVHAGTDDLGKGGNEESLK. T	
								K. TGNAGPRPACGVIGISQ. -	
								K. TGNAGPRPACGVIGISQ. -	
								K. TGNAGPRPACGVIGISQ. -	
S5(17)	Glycerol	Probable pyridoxine biosynthesis protein	Q9C1K6	*Neurospora* crassa	5.84/32677	7.3/35050	584	M. TSTSTTTNGDSFTVK. A	35
								M. TSTSTTTNGDSFTVK. A	
								M. TSTSTTTNGDSFTVK. A	
								M. TSTSTTTNGDSFTVK. A	
								R. IAEEAGACAVMALER. I	
								R. IAEEAGACAVMALER. I	
								R. IAEEAGACAVMALER. I	
								K. EIQAAVTIPVMAK. A	
								K. EIQAAVTIPVMAK. A	
								R. NLGEALR. R	
								R. NLGEALR. R	
								R. NLGEALR. R	
								R. NLGEALR. R	
								R. IKEGAAMIR. T	
								R. IKEGAAMIR. T	
								K. EGAAMIRTKGEAGTGDVVEAVK. H	
								R. TKGEAGTGDVVEAVK. H	
								R. TKGEAGTGDVVEAVK. H	
								R. TKGEAGTGDVVEAVK. H	
								R. TVNAEIAQAK. A	
								R. TVNAEIAQAK. A	
								K. AALASGGELAVAK. M	
								K. AALASGGELAVAK. M	
								K. AALASGGELAVAK. M	
								K. AALASGGELAVAK. M	
								K. GISNAGMKPEER. M	
								K. GISNAGMKPEER. M	
								K. GISNAGMKPEER. M	
S6(*)	Glycerol	Probable pyridoxine biosynthesis protein	Q9C1K6	*Neuospora* crassa	5.84/32677	7.3/35050	845	M. TSTSTTTNGDSFTVK. A	50
								M. TSTSTTTNGDSFTVK. A	
								M. TSTSTTTNGDSFTVK. A	
								K. AGLAQMLK. G	
								K. AGLAQMLK. G	
								R. IAEEAGACAVMALER. I	
								R. IAEEAGACAVMALER. I	
								R. IAEEAGACAVMALER. I	
								K. EIQAAVTIPVMAK. A	
								K. EIQAAVTIPVMAK. A	
								R. ILEQLGVDYIDESEVLTPADDTYHVQK. D	
								R. NLGEALR. R	
								R. NLGEALR. R	
								R. NLGEALR. R	

Spot (Fig.)	Carbon Source	Protein Name	NCBI Accesion	Species	Theo. pI/Mr (kDa)	Expt. pI/Mr (kDa)	Mascot Score	MS/MS Peptide Sequence	SC (%)
								R. NLGEALR. R	
								R. NLGEALR. R	
								R. NLGEALR. R	
								R. NLGEALRR. I	
								R. IKEGAAMIR. T	
								R. IKEGAAMIR. T	
								R. TKGEAGTGDVVEAVK. H	
								R. TKGEAGTGDVVEAVK. H	
								K. GEAGTGDVVEAVK. H	
								K. GEAGTGDVVEAVK. H	
								R. TVNAEIAQAK. A	
								R. TVNAEIAQAK. A	
								K. AALASGGELAVAK. M	
								K. AALASGGELAVAK. M	
								K. AALASGGELAVAK. M	
								R. EIGADVELLK. K	
								R. EIGADVELLK. K	
								R. EIGADVELLKK. T	
								R. EIGADVELLKK. T	
								R. EIGADVELLKK. T	
								K. GISNAGMKPEER. M	
								K. GISNAGMKPEER. M	
								K. GISNAGMKPEER. M	
S7(17)	Glycerol	F- actin- capping protein subunit alpha	Q9C1K6	*Neurospora* crassa	6.10/29611	7.35/33100	437	K. ALTSSTPNLLNELGPAFQK. Y	33
								K. ALTSSTPNLLNELGPAFQK. Y	
								K. ALTSSTPNLLNELGPAFQK. Y	
								K. GLSAYVK. E	
								K. VAVVIVANK. Y	
								K. VAVVIVANK. Y	
								R. SLYIYDPSNNSIEGSIK. V	
								R. SLYIYDPSNNSIEGSIK. V	
								K. TVTATVSSGTGSGIAK. E	
								K. TVTATVSSGTGSGIAK. E	
								K. TVTATVSSGTGSGIAK. E	
								K. SFTSKSEGAFK. G	
								K. SFTSKSEGAFK. G	
								R. LGQDIGGGSSR. R	
								R. LGQDIGGGSSR. R	
S8(*)	Glycerol	F- actin- capping protein subunit alpha	Q9P5K9	*Neuospora* crassa	6.10/29611	7.35/33100	332	K. GLSAYVK. E	22
								K. GLSAYVK. E	
								K. VAVVIVANK. Y	
								K. VAVVIVANK. Y	
								R. SLYIYDPSNNSIEGSIK. V	
								R. SLYIYDPSNNSIEGSIK. V	
								R. SLYIYDPSNNSIEGSIK. V	
								K. TVTATVSSGTGSGIAK. E	
								K. TVTATVSSGTGSGIAK. E	
								K. TVTATVSSGTGSGIAK. E	
								K. SFTSLSEGAFK. G	
								K. SFTSLSEGAFK. G	
S9(16)	Dextrose	ATP- dependent	Q7RVBB	*Neuospora*	5.03/45044	5.8/39900	972	R. GIYAYGFERPSAIQQR. A	54

Spot (Fig.)	Carbon Source	Protein Name	NCBI Accesion	Species	Theo. pI/Mr (kDa)	Expt. pI/Mr (kDa)	Mascot Score	MS/MS Peptide Sequence	SC (%)
		RNA helicase eIF4A		crassa				R. GIYAYGFERPSAIQQR. A	
								R. GIYAYGFERPSAIQQR. A	
								K. GHDVIAQAQSGTGK. T	
								K. GHDVIAQAQSGTGK. T	
								K. GHDVIAQAQSGTGK. T	
								K. GHDVIAQAQSGTGK. T	
								K. TATFSISVLQK. I	
								K. TATFSISVLQK. I	
								K. TATFSISVLQK. I	
								K. TATFSISVLQK. I	
								R. ELAQQIQK. V	
								R. ELAQQIQK. V	
								K. ALQDGPQVVVGTPGR. V	
								K. ALQDGPQVVVGTPGR. V	
								K. ALQDGPQVVVGTPGR. V	
								K. ALQDGPQVVVGTPGR. V	
								K. TDSMKMFVLDEADEMLSR. G	
								K. TDSMKMFVLDEADEMLSR. G	
								K. MFVLDEADEMLSR. G	
								K. MFVLDEADEMLSR. G	
								K. MFVLDEADEMLSR. G	
								R. GFTEQIYDIFQLLPQSTQWLLSATMPQDVLEVTTK. F	
								K. KDELTLEGIK. Q	
								K. KDELTLEGIK. Q	
								K. KDELTLEGIK. Q	
								K. LDTLSDLYETVTITQAVIFCNTR. R	
								R. DFTVSAMHGDMDQAQR. D	
								R. DFTVSAMHGDMDQAQR. D	
								R. DLIMKEFR. S	
								R. VLIATDLLAR. G	
								R. GIDVQQVSLVINYDLPANR. E	
								R. GIDVQQVSLVINYDLPANR. E	
								R. GIDVQQVSLVINYDLPANR. E	
								R. GIDVQQVSLVINYDLPANR. E	
								R. GIDVQQVSLVINYDLPANR. E	
								R. GIDVQQVSLVINYDLPANR. E	
								R. GIDVQQVSLVINYDLPANR. E	
								R. KGVAINFVTADDVR. M	
								R. KGVAINFVTADDVR. M	
								R. KGVAINFVTADDVR. M	
								R. KGVAINFVTADDVR. M	
								R. KGVAINFVTADDVR. M	
								R. KGVAINFVTADDVR. M	
								K. GVAINFVTADDVR. M	
								K. GVAINFVTADDVR. M	
								K. GVAINFVTADDVR. M	
								K. GVAINFVTADDVR. M	
								K. GVAINFVTADDVR. M	
								K. GVAINFVTADDVR. M	
								K. GVAINFVTADDVR. M	
S10(16)	Dextrose	Enolase	Q7RV85	*Neuospora* crassa	5.21/47679	6.5/39950	739	K. AVQNVNEVIGPALIK. E	39
								K. AVQNVNEVIGPALIK. E	
								K. AVQNVNEVIGPALIK. E	

Spot (Fig.)	Carbon Source	Protein Name	NCBI Accesion	Species	Theo. pI/Mr (kDa)	Expt. pI/Mr (kDa)	Mascot Score	MS/MS Peptide Sequence	SC (%)
								K. FLIDLDGTPNK. T	
								K. LGANAILGVSLAVAK. A	
								K. LGANAILGVSLAVAK. A	
								K. LGANAILGVSLAVAK. A	
								K. GVPLYAHISDLAGTK. K	
								K. GVPLYAHISDLAGTK. K	
								R. LAFQEFMIVPSAAPTFSEALR. Q	
								R. QGAEVYQJLK. S	
								R. QGAEVYQJLK. S	
								K. YGQSAGNVGDEGGVAPDIQNPEEALDLITEAIEK. A	
								K. TQDIQIVADDLTVTNPLR. I	
								K. VNQIGTLTESIQAAK. D	
								K. VNQIGTLTESIQAAK. D	
								K. VNQIGTLTESIQAAK. D	
								K. VNQIGTLTESIQAAK. D	
								K. LNQJLR. I	
								K. LNQJLR. I	
								R. IEEELADNAIFAGEK. F	
								R. IEEELADNAIFAGEK. F	
								R. IEEELADNAIFAGEK. F	
S11(16)	Dextrose	60s ribosomal protein L7	Q7SBD5	*Neurospora crassa*	10.06/28727	5.9/32050	280	K. NDVLVPETLLK. K	26
								K. NDVLVPETLLK. K	
								K. QDGSFHIPAEAK. L	
								R. KILQLLR. L	
								R. KILQLLR. L	
								R. LLQINNGVFVR. V	
								R. VALTDNSIIEENLGK. Y	
								R. VALTDNSIIEENLGK. Y	
								R. VALTDNSIIEENLGK. Y	
								R. VALTDNSIIEENLGK. Y	
								K. LSNPTGGFR. T	
								K. LSNPTGGFR. T	
S12(16)	Dextrose	Cytochrome c peroxidase, mitochondrial	Q7SDV9	*Neurospora crassa*	8.54/39964	6.6/33200	556	K. FDDYQAVYNEIASR. L	31
								K. FDDYQAVYNEIASR. L	
								R. LEEKDDYDDGSYGPVLVR. L	
								R. LEEKDDYDDGSYGPVLVR. L	
								R. FAPESDHGANAGLK. A	
								R. FAPESDHGANAGLK. A	
								R. DFLEPVK. A	
								R. DFLEPVK. A	
								R. LPDASQAQDHLR. N	
								R. LPDASQAQDHLR. N	
								R. MGFNDQEIVALSGAHALGR. C	
								R. MGFNDQEIVALSGAHALGR. C	
								K. SLMMLPADMALIQDK. K	
								K. SLMMLPADMALIQDK. K	
								K. SLMMLPADMALIQDK. K	
								K. SLMMLPADMALIQDK. K	
								K. SLMMLPADMALIQDK. K	
								K. LFELGVPFAENSER. W	
								K. LFELGVPFAENSER. W	

Spot (Fig.)	Carbon Source	Protein Name	NCBI Accesion	Species	Theo. pI/Mr (kDa)	Expt. pI/Mr (kDa)	Mascot Score	MS/MS Peptide Sequence	SC (%)
S13(16)	Dextrose	Outer membrane porin F	P37726	*Pseudomonas* fluorescens	5.73/34461	6.75/32500	204	K. HIEDGFNPGAR. I K. HIEDGFNPGAR. I R. VELDVK. F R. VELDVK. F R. ITAVGYGESRPVADNATEAGR. A R. ITAVGYGESRPVADNATEAGR. A R. ITAVGYGESRPVADNATEAGR. A R. RVEASVEAQAQ. - R. RVEASVEAQAQ. -	15
S14(16)	Dextrose	50s ribosomal protein L6	Q4K548	*Pseudomonas* fluorescens	9.73/19170	6.45/26050	193	K. FAGQQLSVK. G R. ALVNNMVQGVSQGFER. K K. QLVGQVAAEIR. D K. QLVGQVAAEIR. D	20
S15(16)	Dextrose	F-actin-capping protein subunit alpha	Q9P5K9	*Neuospora* crassa	6.10/29611	7.25/33100	134	K. VAVVIVANK. Y K. TVTATVSSGTGSGIAK. E K. TVTATVSSGTGSGIAK. E	9
S16(16)	Dextrose	Peptidoglycan-associated lipoprotein	Q9C1K6	*Pseudomonas* putida	5.16/17879	6.65/24050	126	R. AITTFYFEYDSSDLKPEAMR. A R. AITTFYFEYDSSDLKPEAMR. A R. VVLEGNTDER. G R. VVLEGNTDER. G R. EYNMALGER. R R. EYNMALGER. R	23
S17(18)	Quinic Acid	Disulfide bond formation protein B	Q0BHC9	*Burkholderia* ambifaria	9.40/18992	6.5/33000	51	M. NDSTLALR. R M. NDSTLALRRER. R	6
S18(18)	Quinic Acid	Cold shock protein capA	P72188	*Pseudomonas* fragi	6.54/7011	5.75/9050	151	K. GFGFITPQGGGDDLFVHFK. A K. GFGFITPQGGGDDLFVHFK. A K. AIESDGFK. S K. AIESDGFK. S K. SLKEGQTVSFVAEK. G K. SLKEGQTVSFVAEK. G K. EGQTVSFVAEK. G K. EGQTVSFVAEK. G	64
S19(*)	Quinic Acid	Cold shock protein capA	P72188	*Pseudomonas* fragi	6.54/7011	5.75/9050	137	K. AIESDGFK. S K. EGQTVSFVAEK. G K. SLKEGQTSFVAEK. G	34
S20(17)	Glycerol	Probable pyridoxine biosynthesis protein	Q9C1K6	*Neurospora* crassa	5.84/32677	6.9/34950	974	M. TSTSTTTNGDSFTVK. A M. TSTSTTTNGDSFTVK. A K. AGLAQMLK. G K. GGVIMDVTTPAEAR. I K. GGVIMDVTTPAEAR. I R. IAEEAGACAVMALER. I R. IAEEAGACAVMALER. I R. IAEEAGACAVMALER. I R. IAEEAGACAVMALER. I R. IPSDIR. A R. IPSDIR. A K. EIQAAVTIPVMAK. A	56

Spot (Fig.)	Carbon Source	Protein Name	NCBI Accesion	Species	Theo. pI/Mr (kDa)	Expt. pI/Mr (kDa)	Mascot Score	MS/MS Peptide Sequence	SC (%)
								K. EIQAAVTIPVMAK. A	
								K. EIQAAVTIPVMAK. A	
								K. EIQAAVTIPVMAK. A	
								R. ILEQLGVDYIDESEVLTPADDTYHVQK. D	
								R. ILEQLGVDYIDESEVLTPADDTYHVQK. D	
								R. NLGEALR. R	
								R. NLGEALR. R	
								R. NLGEALR. R	
								R. NLGEALR. R	
								R. NLGEALR. R	
								R. IKEGAAMIR. T	
								K. EGAAMIRTKGEAGTGDVVEAVK. H	
								K. EGAAMIRTKGEAGTGDVVEAVK. H	
								R. TKGEAGTGDVVEAVK. H	
								R. TKGEAGTGDVVEAVK. H	
								R. TKGEAGTGDVVEAVK. H	
								R. TVNAEIAQAK. A	
								R. TVNAEIAQAK. A	
								R. TVNAEIAQAK. A	
								R. TVNAEIAQAK. A	
								R. TVNAEIAQAK. A	
								R. TVNAEIAQAKAALASGGELAVAK. M	
								K. AALASGGELAVAK. M	
								K. AALASGGELAVAK. M	
								K. AALASGGELAVAK. M	
								K. AALASGGELAVAK. M	
								K. AALASGGELAVAK. M	
								K. AALASGGELAVAK. M	
								K. AALASGGELAVAK. M	
								R. EIGADVELLK. K	
								K. GISNAGMKPEER. M	
								K. GISNAGMKPEER. M	
								K. GISNAGMKPEER. M	
S21(*)	Glycerol	Probable pyridoxine biosynthesis protein	Q9C1K6	*Neurospora* crassa	5.84/32677	6.9/34950	940	M. TSTSTTTNGDSFTVK. A	51
								M. TSTSTTTNGDSFTVK. A	
								M. TSTSTTTNGDSFTVK. A	
								K. AGLAQMLK. G	
								K. AGLAQMLK. G	
								K. GGVIMDVTTPAEAR. I	
								K. GGVIMDVTTPAEAR. I	
								R. IAEEAGACAVMALER. I	
								R. IAEEAGACAVMALER. I	
								R. IAEEAGACAVMALER. I	
								R. IAEEAGACAVMALER. I	
								K. EIQAAVTIPVMAK. A	
								K. EIQAAVTIPVMAK. A	
								K. EIQAAVTIPVMAK. A	
								K. EIQAAVTIPVMAK. A	
								K. EIQAAVTIPVMAK. A	
								R. ILEQLGVDYIDESEVLTPADDTYHVQK. D	
								R. ILEQLGVDYIDESEVLTPADDTYHVQK. D	
								R. NLGEALR. R	
								R. NLGEALR. R	

Spot (Fig.)	Carbon Source	Protein Name	NCBI Accesion	Species	Theo. pI/Mr (kDa)	Expt. pI/Mr (kDa)	Mascot Score	MS/MS Peptide Sequence	SC (%)
								R. NLGEALR. R	
								R. NLGEALR. R	
								R. NLGEALR. R	
								R. NLGEALR. R	
								R. IKEGAAMIR. T	
								R. IKEGAAMIR. T	
								R. TKGEAGTGDVVEAVK. H	
								R. TKGEAGTGDVVEAVK. H	
								R. TKGEAGTGDVVEAVK. H	
								R. TKGEAGTGDVVEAVK. H	
								R. TKGEAGTGDVVEAVK. H	
								R. TKGEAGTGDVVEAVK. H	
								K. GEAGTGDVVEAVK. H	
								R. TVNAEIAQAK. A	
								R. TVNAEIAQAK. A	
								R. TVNAEIAQAK. A	
								R. TVNAEIAQAK. A	
								R. TVNAEIAQAK. A	
								R. TVNAEIAQAKAALASGGELAVAK. M	
								K. AALASGGELAVAK. M	
								K. AALASGGELAVAK. M	
								K. AALASGGELAVAK. M	
								K. AALASGGELAVAK. M	
								K. AALASGGELAVAK. M	
								K. AALASGGELAVAK. M	
S22(18)	Quinic Acid	50s ribosomal protein L18	Q4K549	*Pseudomonas* fluorescens	10.27/12692	6.9/15500	112	K. VLASASTLDK. E	25
								K. VGQLVATR. A	
								K. VGQLVATR. A	
								K. AAGVSQVAFDR. S	
S23(*)	Quinic Acid	50s ribosomal protein L18	Q4K549	*Pseudomonas* fluorescens	10.27/12692	6.9/15500	133	K. VLASASTLDK. E	25
								K. VLASASTLDK. E	
								K. VGQLVATR. A	
								K. AAGVSQVAFDR. S	
								K. AAGVSQVAFDR. S	
S24(18)	Quinic Acid	DNA-binding protein HU-beta	P05384	*Pseudomonas* aeruginosa	9.99/9081	6.1/24400	206	K. SELIDAIAASADIPK. A	32
								K. SELIDAIAASADIPK. A	
								R. ALDAVIESVTGALK. A	
								R. ALDAVIESVTGALK. A	
S25(*)	Quinic Acid	DNA-binding protein HU-beta	P05384	*Pseudomonas* aeruginosa	9.99/9081	6.1/24400	180	K. SELIDAIAASADIPK. A	32
								K. SELIDAIAASADIPK. A	
								K. SELIDAIAASADIPK. A	
								R. ALDAVIESVTGALK. A	
								R. ALDAVIESVTGALK. A	
S26(17)	Glycerol	30s ribosomal protein S7	Q48D32	*Pseudomonas* syringae	10.15/17636	7.3/21500	133	K. ALDAIAPLVEVK. S	15
								K. ALDAIAPLVEVK. S	
								R. LAGELLDAAEGK. G	
								R. LAGELLDAAEGK. G	
S27(*)	Glycerol	30s ribosomal	Q48D32	*Pseudomonas*	10.15/17636	7.3/21500	139	K. ALDAIAPLVEVK. S	15

Spot (Fig.)	Carbon Source	Protein Name	NCBI Accesion	Species	Theo. pI/Mr (kDa)	Expt. pI/Mr (kDa)	Mascot Score	MS/MS Peptide Sequence	SC (%)
		protein S7		syringae				K. ALDAIAPLVEVK. S R. LAGELLDAAEGK. G	
S28(18)	Quinic Acid	Outer membrane porin F	P37726	*Pseudomonas* fluorescens	5.73/34461	6.75/32500	216	K. HIEDGFNPGAR. I K. HIEDGFNPGAR. I K. HIEDGFNPGAR. I K. HIEDGFNPGAR. I R. VELDVK. F R. ITAVGYGESRPVADNATEAGR. A R. ITAVGYGESRPVADNATEAGR. A R. RVEASVEAQAQ. - R. RVEASVEAQAQ. -	15
S29(*)	Quinic Acid	Outer membrane porin F	P37726	*Pseudomonas* fluorescens	5.73/34461	6.75/32500	241	K. HIEDGFNPGAR. I K. HIEDGFNPGAR. I K. HIEDGFNPGAR. I K. HIEDGFNPGAR. I R. VELDVK. F R. VELDVK. F R. VELDVKFDFDK. S R. ITAVGYGESRPVADNATEAGR. A R. ITAVGYGESRPVADNATEAGR. A R. RVEASVEAQAQ. - R. RVEASVEAQAQ. -	16
S30(18)	Quinic Acid	Probable pyridoxine biosynthesis protein	Q9C1K6	*Neuospora* crassa	5.84/32677	6.9/34950	834	K. GGVIMDVTTPAEAR. I K. GGVIMDVTTPAEAR. I R. IAEEAGACAVMALER. I R. IAEEAGACAVMALER. I R. IAEEAGACAVMALER. I K. EIQAAVTIPVMAK. A K. EIQAAVTIPVMAK. A K. EIQAAVTIPVMAK. A R. ILEQLGVDYIDESEVLTPADDTYHVQK. D R. ILEQLGVDYIDESEVLTPADDTYHVQK. D R. NLGEALR. R R. NLGEALR. R R. NLGEALR. R R. NLGEALR. R R. NLGEALRR. I R. IKEGAAMIR. T R. IKEGAAMIR. T R. IKEGAAMIR. T K. EGAAMIRTKGEAGTGDVVEAVK. H R. TKGEAGTGDVVEAVK. H R. TKGEAGTGDVVEAVK. H K. GEAGTGDVVEAVK. H R. TVNAEIAQAK. A R. TVNAEIAQAK. A K. AALASGGELAVAK. M R. EIGADVELLKK. T R. EIGADVELLKK. T K. GISNAGMKPEER. M K. GISNAGMKPEER. M	47

Spot (Fig.)	Carbon Source	Protein Name	NCBI Accesion	Species	Theo. pI/Mr (kDa)	Expt. pI/Mr (kDa)	Mascot Score	MS/MS Peptide Sequence	SC (%)
S31(*)	Quinic Acid	Probable pyridoxine biosynthesis protein	Q9C1K6	*Neurospora crassa*	5.84/32677	6.9/34950	965	M. TSTSTTTNGDSFTVK. A M. TSTSTTTNGDSFTVK. A K. AGLAQMLK. G K. AGLAQMLK. G K. GGVIMDVTTPAEAR. I K. GGVIMDVTTPAEAR. I R. IAEEAGACAVMALER. I R. IPSDIR. A R. IPSDIR. A K. EIQAAVTIPVMAK. A K. EIQAAVTIPVMAK. A K. EIQAAVTIPVMAK. A K. EIQAAVTIPVMAK. A R. ILEQLGVDYIDESEVLTPADDTYHVQK. D R. NLGEALR. R R. NLGEALR. R R. NLGEALR. R R. NLGEALR. R R. NLGEALR. R R. IKEGAAMIR. T R. TKGEAGTGDVVEAVK. H R. TKGEAGTGDVVEAVK. H K. GEAGTGDVVEAVK. H R. TVNAEIAQAK. A R. TVNAEIAQAK. A R. TVNAEIAQAK. A R. TVNAEIAQAK. A R. TVNAEIAQAK. A R. TVNAEIAQAKAALASGGELAVAK. M R. TVNAEIAQAKAALASGGELAVAK. M K. AALASGGELAVAK. M K. AALASGGELAVAK. M K. AALASGGELAVAK. M K. AALASGGELAVAK. M K. AALASGGELAVAK. M K. AALASGGELAVAK. M K. AALASGGELAVAK. M K. AALASGGELAVAK. M K. AALASGGELAVAK. M K. AALASGGELAVAK. M R. EIGADVELLKK. T K. TPEHALQR. A K. GISNAGMKPEER. M K. GISNAGMKPEER. M K. GISNAGMKPEER. M	59

pyridoxine biosynthesis protein. Six other protein spots, S1, S3, S17, S18, S22, and S24, identified were determined to be essentially unique to quinic acid as the sole carbon source. These proteins found to be unique to quinic acid included a peptidyl-prolyl cis-trans isomerase, a disulfide bond formation protein, and a superoxide dismutase. In contrast, one protein spot, S13 in dextrose and S28 in quinic acid, identified was established to be up-regulated, differentially expressed between dextrose and quinic acid. Another protein spot, S15 in dextrose and S7 in glycerol, identified was found to up-regulated, differentially expressed between dextrose and glycerol. The last protein spot, S30 in quinic acid and S20 in glycerol, identified was determined to be up-regulated, differentially expressed between glycerol and quinic acid. These protein spots found to be up-regulated, differentially expressed between the carbon sources included an outer memebrane porin F and a F-actin-capping protein.

Once all analyzed spots were identified and organized into categories of unique or differentially expressed, the fungal species of each protein spot's matched peptides were further observed in order to determine and analzye proteins from *N. crassa*. Based on these further observations, a total of seven unique and two differentially expressed protein spots were determined to have matched peptides from *N. crassa*. The Mascot scores for these *N. crassa* proteins ranged from 134 to 974 with sequence coverage, the percent of all amino acids from valid peptide matches to the total number of amino acids in the complete protein sequence, for each protein ranging from 9 to 78 percent. As mentioned above, a Mascot score (>50) indicates identity or extensive homology, however, the top scoring peptide matches represent higher sequence coverage.

Based on these Mascot scores, at this point we focused on further analyzing one differentially expessed *N. crassa* protein and five proteins which were unique to one of the three carbon sources utilized. These final six proteins choosen to be further analyzed included S1, S3, S9, S10, S12, S20, and S30. The matched peptide sequence obtained, indicated in red, for each of these six *N. crassa* proteins are shown in Figures 19, 20, 21, 22, 23, and 24. From these sequences, the identity and function of each *N. crassa* protein was determined. Additional characteristics of these proteins can be found in Table 4 as mentioned above.

One of five unique *N. crassa* proteins further observed, spot S9 as mentioned above was determined to be unique to dextrose as the sole carbon source. The matched peptide sequence, indicated in red, of protein spot S9 is shown in Figure 19. This portion of the protein sequence obtained from the MS analysis matches a *N. crassa* protein that is known to be an ATP-dependent RNA helicase (eIF4A). This protein functions in translation to unwind RNA secondary structures in the 5' UTR of mRNAs, which allows for efficient binding of the small ribosomal subunit and sbsequent scanning for the start codon. These results indicate that this ATP-dependent RNA helicase (eIF4A) is probably up-regulated during growth on a good carbon source like dextrose.

Another unique protein of *N. crassa* further observed, spot S10 as mentioned above was also determined to be unique to growth on dextrose. The matched peptide sequence, indicated in red, of protein spot S10 is shown in Figure 20. This portion of the protein sequence obtained from the MS analysis matches a *N. crassa* protein that is known to be an enolase. This protein functions as an essential glycolytic enzyme that

catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate. These findings indicate that this enolase is up-regulated in the presence of dextrose.

A third *N. crassa* protein unique to dextrose as the sole carbon source, was spot S12. The matched peptide sequence, indicated in red, of protein spot S12 is shown in Figure 21. This portion of the protein sequence obtained from the MS analysis matches a *N. crassa* protein that is known to be a mitochrondrial cytochrome c peroxidase. This protein functions to destroy radicals which are normally produced within the cells during aerobic respiration and are toxic to biological systems. This indicates that cytochrome c peroxidases in the mitochondria are important to the growth of *N. crassa* tissue on dextrose.

A *N. crassa* protein unique to quinic acid, protein spot S1 was also further analyzed. The matched peptide sequence, indicated in red, of protein spot S1 is shown in Figure 22(A). This portion of the protein sequence obtained from the MS analysis matches a *N. crassa* protein that is known to be a peptidyl-prolyl cis-trans isomerase. In Figure 22(B), the matched peptide sequence of protein spot S2, confirmed duplicate of S1, is shown in red. The protein sequence obtained from the MS analysis matches the same *N. crassa* peptidyl-prolyl cis-trans isomerase as its duplicate. This protein functions to catalyze the cis-trans isomerization of proline imidic peptide bonds in oligopeptides, which aids in the acceleration of protein folding. These results indicate that peptidyl-prolyl cis-trans isomerases are important to the growth of *N. crassa* on quinic acid as the sole carbon source.

The last unique *N. crassa* protein further observed, spot S3 as mentioned above was determined to be unique to quinic acid. The matched peptide sequence, indicated in

red, of protein spot S3 is shown in Figure 23(A). This portion of the protein sequence obtained from the MS analysis matches a *N. crassa* protein that is known to be a Cu-Zn superoxide dismutase. In Figure 23(B), the matched peptide sequence of protein spot S4, confirmed duplicate of S3, is shown in red. The protein sequence obtained from the MS analysis matches the same *N. crassa* Cu-Zn superoxide dismutase as its confirmed duplicate. This protein functions to destroy radicals that are normally produced within the cells during aerobic respiration and are toxic to biological systems. This indicates that superoxide dismutases [Cu-Zn] are important to the growth of *N. crassa* on the sole carbon source, quinic acid.

The one up-regulated, differentially expressed *N. crassa* protein further observed, spots S20 and S30, were determined to have expression differences between glycerol and quinic acid. Spot S20 was identified on the carbon source of glycerol whereas spot S30 was identified on quinic acid. The matched peptide sequence, indicated in red, of protein spot S20 is shown in Figure 24(A). This portion of the protein sequence obtained from the MS analysis matches a *N. crassa* protein known to be a probable biosynthesis pyridoxine. In Figure 24(B), the matched peptide sequence of protein spot S21, confirmed duplicate of S20, is shown in red. The protein sequence obtained from the MS analysis matches the same *N. crassa* probable biosynthesis pyridoxine protein as its duplicate. The matched peptide sequence, indicated in red, of protein spot S30 is shown in Figure 24(C). This portion of the protein sequence obtained from the MS analysis matches the *N. crassa* probable biosynthesis pyridoxine. In Figure 24(D), the matched peptide sequence of protein spot S31, confirmed duplicate of S30, is shown in red. The protein sequence obtained from the MS analysis matches the same *N. crassa* protein as its

duplicate and spot S20. This protein in all probability functions in the production of pyridoxine, commonly referred to as vitamin B6. These results indicate that the production of vitamin B6 is essential to the growth of *N. crassa* on both glycerol and quinic acid, but occurs at different expression levels.

Figure 19: Sequence of excised protein, S9 in Figure 16, is indicated in red. Spot S9 was determined to be unique to dextrose as the sole carbon source. A portion of the protein sequence obtained from the MS analysis indicated that the protein is an ATP-dependent RNA helicase (eIF4A), present in *N. crassa*.

- Spot “S9”: ATP-dependent RNA helicase (eIF4A)

```
  1 MATDKGLEDI PEGQIESNYD ETVDSFDEMN LKPELLRGIY AYGFERPSAI
 51 QQRAIMPVIK GHDVIAQAQS GTGKTATFSI SVLQKIDPSL KACQALILAP
101 TRELAQQIQK VVVAIGDFMN IECHACIGGT SVRDDMKALQ DGPQVVVGTP
151 GRVHDMIQRR FLKTDSMKMF VLDEADEMLS RGFTEQIYDI FQLLPQSTQV
201 VLLSATMPQD VLEVTTKFMR EPVRILVKKD ELTLEGIKQF YIAVEKEEWK
251 LDTLSDLYET VTITQAVIFC NTRRKVDWLT DKLTARDFTV SAMHGDMDQA
301 QRDLIMKEFR SGSSRVLIAT DLLARGIDVQ QVSLVINYDL PANRENYIHR
351 IGRGGRFGRK GVAINFVTAD DVRMMREIEQ FYSTQIEEMP MNVADLI
```

Figure 20: Sequence of excised protein, S10 in Figure 16, is indicated in red. Spot S10 was determined to be unique to dextrose as the sole carbon source. A portion of the protein sequence obtained from the MS analysis indicated that the protein is an enolase, present in *N. crassa*.

- Spot “S10”: Enolase

```
  1 MPISKIHARY VYDSRGNPTV EVDVVTELGL HRAIVPSGAS TGQHEACELR
 51 DGDKTKWGGK GVLKAVQNVN EVIGPALIKE NIDVKDQSKV DKFLIDLDGT
101 PNKTKLGANA ILGVSLAVAK AGAAEKGVPL YAHISDLAGT KKPYVLPVPF
151 MNVLNGGSHA GGRLAFQEFM IVPSAAPTFS EALRQGAEVY QILKSLAKKK
201 YGQSAGNVGD EGGVAPDIQN PEEALDLITE AIEKAGYTGQ VKIAMDVASS
251 EFYKEDVKKY DLDFKNPESD PSKWLTYEEL ANLYSELCKK YPIVSIEDPF
301 AEDDWEAWSY FYKTQDIQIV ADDLTVTNPL RIKKAIELKA ANALLLKVNQ
351 IGTLTESIQA AKDSYADGWG VMVSHRSGET EDVTIADIVV GIRSGQIKTG
401 APARSERLAK LNQILRIEEE LADNAIFAGE KFRKAVEL
```

Figure 21: Sequence of excised protein, S12 in Figure 16, is indicated in red. Spot S12 was determined to be unique to dextrose as the sole carbon source. A portion of the sequence obtained from the MS analysis indicated that the protein is a mitochondrial cytochrome c peroxidase, present in *N. crassa*.

- Spot “S12”: Cytochrome c peroxidase

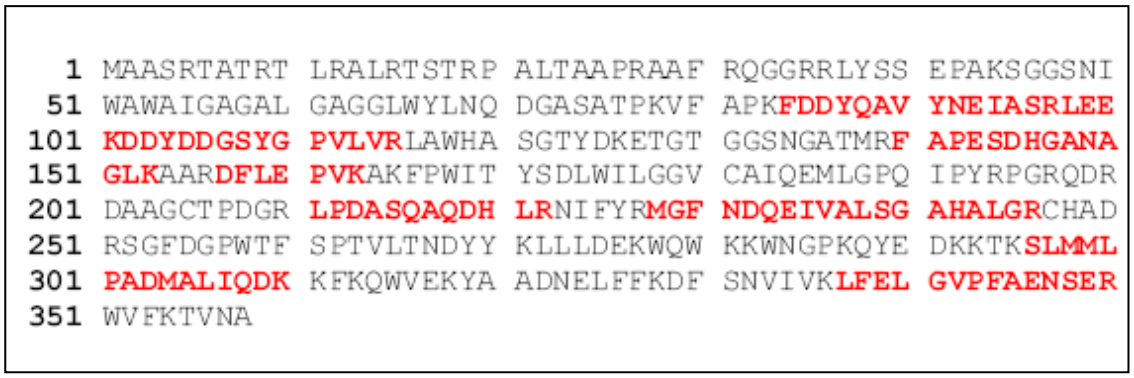


Figure 22: A) Sequence of excised protein, S1 in Figure 18, is indicated in red. B) Sequence of excised protein, S2 (duplicate of S1), is indicated in red. Spot S1 and S2 were determined to be unique to quinic acid as the sole carbon source. A portion of the protein sequence obtained from the MS analysis indicated that the protein is a peptidyl-prolyl cis-trans isomerase, present in *N. crassa*.

- Spot “S1”: Peptidyl-prolyl cis-trans isomerase

A)

```
  1 MTIPQLDGLQ IEVQQEGQGT RETRRGDNVD VHYKGVLTSG KKFDASYDRG
 51 EPLNFTVGQG QVIKGWDEGL LGMKIGEKRK LTIAPHLAYG NRAVGGIIPA
101 NSTLIFETEL VGIKGVQKGE
```

- Spot “S2”: Peptidyl-prolyl cis-trans isomerase

B)

```
  1 MTIPQLDGLQ IEVQQEGQGT RETRRGDNVD VHYKGVLTSG KKFDASYDRG
 51 EPLNFTVGQG QVIKGWDEGL LGMKIGEKRK LTIAPHLAYG NRAVGGIIPA
101 NSTLIFETEL VGIKGVQKGE
```

Figure 23: A) Sequence of excised protein, S3 in Figure 18, is indicated in red. B) Sequence of excised protein, S4 (duplicate of S3), is indicated in red. Spot S3 and S4 were determined to be unique to quinic acid as the sole carbon source. A portion of the protein sequence obtained from the MS analysis indicated that the protein is a superoxide dismutase [Cu-Zn], present in *N. crassa*.

- Spot “S3”: Cu-Zn superoxide dismutase

A)

```
  1 MVKAVAVVRG DSNVKGTVIF EQESESAPTT ITYDISGNDP NAKRGFHIHT
 51 FGDNTNGCTS AGPHFNPHGT THGDRTAEVR HVGDLGNIET DAQGNAKGTV
101 TDNLVKLIGP ESVIGRTVVV HAGTDDLGKG GNEESLKTGN AGPRPACGVI
151 GISQ
```

- Spot “S4”: Cu-Zn superoxide dismutase

B)

```
  1 MVKAVAVVRG DSNVKGTVIF EQESESAPTT ITYDISGNDP NAKRGFHIHT
 51 FGDNTNGCTS AGPHFNPHGT THGDRTAEVR HVGDLGNIET DAQGNAKGTV
101 TDNLVKLIGP ESVIGRTVVV HAGTDDLGKG GNEESLKTGN AGPRPACGVI
151 GISQ
```

Figure 24: A) Sequence of excised protein, S20 in Figure 17, is indicated in red. B) Sequence of excised protein, S21 (duplicate of S20), is indicated in red. C) Sequence of excised protein, S30 in Figure 18, is indicated in red. D) Sequence of excised protein, S31 (duplicate of S30), is indicated in red. Spot S20, S21, S30, and S31 were determined to be differentially expressed between quinic acid and glycerol. A portion of the protein sequence obtained from the MS analysis indicated that the protein is a probable pyridoxine biosynthesis protein, present in *N. crassa*.

- Spot “S20” and “S21”: Probable pyridoxine biosynthesis

A)

```
  1 MTSTSTTTNG DSFTVKAGLA QMLKGGVIMD VTTPAEARIA EEAGACAVMA
 51 LERIPSDIRA AGGVARMSNP SMIKEIQAAV TIPVMAKARI GHVTECRILE
101 QLGVDYIDES EVLTPADDTY HVQKDQFKAP FVCGCRNLGE ALRRIKEGAA
151 MIRTKGEAGT GDVVEAVKHI RTVNAEIAQA KAALASGGEL AVAKMAREIG
201 ADVELLKKTA ELGRLPVVNF AAGGVATPAD AALMMEFGCD GVFVGSGIFK
251 DAKTPEHALQ RARAVVKAVA NWNDYGALVE ACIEHGEAMK GISNAGMKPE
301 ERMAGRGW
```

B)

```
  1 MTSTSTTTNG DSFTVKAGLA QMLKGGVIMD VTTPAEARIA EEAGACAVMA
 51 LERIPSDIRA AGGVARMSNP SMIKEIQAAV TIPVMAKARI GHVTECRILE
101 QLGVDYIDES EVLTPADDTY HVQKDQFKAP FVCGCRNLGE ALRRIKEGAA
151 MIRTKGEAGT GDVVEAVKHI RTVNAEIAQA KAALASGGEL AVAKMAREIG
201 ADVELLKKTA ELGRLPVVNF AAGGVATPAD AALMMEFGCD GVFVGSGIFK
251 DAKTPEHALQ RARAVVKAVA NWNDYGALVE ACIEHGEAMK GISNAGMKPE
301 ERMAGRGW
```

- Spot “S30” and “S31”: Probable pyridoxine biosynthesis

C)

```
  1 MTSTSTTTNG DSFTVKAGLA QMLKGGVIMD VTTPAEARIA EEAGACAVMA
 51 LERIPSDIRA AGGVARMSNP SMIKEIQAAV TIPVMAKARI GHVTECRILE
101 QLGVDYIDES EVLTPADDTY HVQKDQFKAP FVCGCRNLGE ALRRIKEGAA
151 MIRTKGEAGT GDVVEAVKHI RTVNAEIAQA KAALASGGEL AVAKMAREIG
201 ADVELLKKTA ELGRLPVVNF AAGGVATPAD AALMMEFGCD GVFVGSGIFK
251 DAKTPEHALQ RARAVVKAVA NWNDYGALVE ACIEHGEAMK GISNAGMKPE
301 ERMAGRGW
```

D)

```
  1 MTSTSTTTNG DSFTVKAGLA QMLKGGVIMD VTTPAEARIA EEAGACAVMA
 51 LERIPSDIRA AGGVARMSNP SMIKEIQAAV TIPVMAKARI GHVTECRILE
101 QLGVDYIDES EVLTPADDTY HVQKDQFKAP FVCGCRNLGE ALRRIKEGAA
151 MIRTKGEAGT GDVVEAVKHI RTVNAEIAQA KAALASGGEL AVAKMAREIG
201 ADVELLKKTA ELGRLPVVNF AAGGVATPAD AALMMEFGCD GVFVGSGIFK
251 DAKTPEHALQ RARAVVKAVA NWNDYGALVE ACIEHGEAMK GISNAGMKPE
301 ERMAGRGW
```

DISCUSSION

A limited number of studies have focused on analyzing the effects on gene expression in *Neurospora crassa* when utilizing a variety of carbon sources (Aign et al., 2003, Logan et al., 2007). However, even fewer studies have focused on analyzing the expression levels of the proteome in *N. crassa* during growth in different carbon sources. The present study is one of the first to use two-dimensional gel electrophoresis (2-DGE) and MS sequencing in a proteomic approach to identify proteins expressed by *N. crassa* grown on different carbon sources including 2% dextrose, 2% glycerol and 0.3% quinic acid. Our goal of the project was to compare and contrast protein profiles of *N. crassa* grown on different carbon sources and identify individual proteins that are unique as well as play a significant role in the metabolism of filamentous fungus *N. crassa* grown in 2% dextrose, 2% glycerol, and 0.3% quinic acid.

The most relevant research studies to this present investigation that have been found include one study by Aign et al. and another by Logan et al. (Aign et al., 2003, Logan et al., 2007). In Aign's study, the researchers analyzed RNA levels extracted from *N. crassa* mycelia grown under different nutrient conditions utilizing microarray technology (Aign et al., 2003). The researchers in this study identified multiple subgroups of genes with similar transcript profiles including genes with stress response, amino acid biosynthesis, protein synthesis, and glycolysis (Aign et al., 2003). In Logan's study, the research team utilized microarray experiments to analyze *N. crassa* genes that were shifted from sucrose to quinic acid as the sole carbon source (Logan et al., 2007). These researchers determined that quinic acid caused increased transcription of genes involved in carbon metabolism, protein degradation, amino acid metabolism, and

ribosome synthesis (Logan et al., 2007). In both studies, the researchers demonstrated and identified RNA transcripts associated with carbon source utilization in *N. crassa*.

In our experiment, an initial study on the proteome of *N. crassa*, protein from mycelium grown in three different carbon sources was extracted. The carbon sources utilized in our experiment included a preferred carbon source, 2% dextrose, and two non-preferred carbon sources, 2% glycerol and 0.3% quinic acid. A number of different carbon sources have been shown to be metabolized by *N. crassa* (Davis, 2000). The protein profiles generated and the significant proteins involved in the metabolism of these sole carbon sources, however, remains to be determined.

In order to generate protein profiles of *N. crassa* grown on these three carbon sources, we employed two-dimensional gel electrophoresis (2-DGE). The protein profiles generated were analyzed after reproducible results were obtained for the 7cm as well as the 17cm two-dimensional (2-D) gels. On each 2-D gel, we observed between 65 and 100 protein spots. The difference between the numbers of protein spots observed was attributed to the different carbon sources utilized by *N. crassa*. As we expected, *N. crassa* expressed more protein when grown in 2% dextrose compared to 0.3% quinic acid or 2% glycerol. However, the lowest numbers of protein were expressed while *N. crassa* was grown in 0.3% quinic acid. These results were predicted because dextrose was the preferred carbon source, while quinic acid was the well-known less-preferred carbon source utilized by *Neurospora*. This observation is consistent with a previous study performed that has shown more genes are up-regulated in the presence of a minimal media containing only sucrose, while the up-regulation of metabolic genes occurs at a lower degree when grown in a less-preferred carbon source (Aign et al., 2003).

In addition to differences in the number of proteins expressed, it was observed that the protein profiles as well displayed unique patterns of protein expression for each of the sole carbon sources utilized by *N. crassa*. These unique patterns of protein expression that were observed on the 2-D gels are attributed to different proteins being up-regulated and down-regulated during the shift from dextrose to one of the other carbon sources: either quinic acid or glycerol. A number of different proteins would be expected to be up-regulated or down-regulated because the utilization of different carbon sources by *N. crassa* requires specific enzymes and the activation of particular metabolic pathways in order to induce growth and obtain energy.

In the present investigation, it was observed that each carbon source exhibited a unique pattern of protein expression, but to gain more knowledge on these protein profiles we wanted to determine which two expression patterns were the most similar. Among the three protein profiles produced and analyzed, as might be expected, it was determined that the protein profiles of dextrose and quinic acid exhibited the least similar patterns of protein expression. Whereas, the protein profiles of dextrose and glycerol displayed the most similar patterns of protein expression. In the analysis, it was expected that the resulting protein profiles of dextrose and quinic acid would exhibit the least similar patterns of protein expression. This result was predicted because dextrose was a well-known preferred carbon source of *N. crassa*, while quinic acid was a well-known less-preferred carbon source. Therefore, in order for *N. crassa* to utilize these substrates, different metabolic pathways are essential. It is known that dextrose is metabolized as an energy source by glycolysis also known as the Embden-Meyerhof pathway, while quinic acid is metabolized by various *qa* inducible enzymes which eventually enter the Krebs

cycle as succinate or acetyl-CoA (Kuswandi, 1992 and Deacon, 2006). However, it was not expected that the resulting protein profiles of glycerol and dextrose would exhibit the most similar protein expression patterns. This result was not predicted because we would have expected the protein profiles of glycerol and quinic acid to display the most similar patterns of protein expression due to both being less-preferred carbon sources of *N. crassa*. However, after further analyzing the metabolic pathways of these carbon sources, it was observed that dextrose as well as glycerol enters the Embden-Meyerhof (EM) pathway thus suggesting our results for the most similar patterns of protein expression are acceptable (Denor et al., 1978).

In our study, in addition to determining that different proteins are being up-regulated and down-regulated on the different carbon sources, we wanted to identify some of the individual proteins that were found to be essentially unique to each of the sole carbon sources as well as identify a few proteins that are differentially expressed. The protein spots of interest, a total of thirty-one, were excised and sent to undergo mass spectrometry in order to identify the proteins.

At the beginning of this analysis, we did not know which proteins to expect to be identified as well as established to be unique or up-regulated, differentially expressed among the sole carbon sources: dextrose, glycerol, or quinic acid. However, it would be reasonable to expect some of the identified proteins to be metabolic proteins related to the carbon sources utilized by *N. crassa* in our study. However, these metabolic proteins would only account for a low concentration of the total proteome. Some of the possible metabolic proteins that could be identified for dextrose as the sole carbon source include enzymes involved in aerobic respiration. Other metabolic proteins that could be identified

for quinic acid as the sole carbon source include enzymes involved in quinic acid metabolism (products of the *qa* genes) as well as other protein products involved in the Krebs cycle. Metabolic proteins that could be identified for glycerol as the sole carbon source were somewhat less clear but could be involved in the Krebs cycle.

In our study, however, only one of these metabolic proteins was identified as being significant for the utilization of dextrose, quinic acid, or glycerol by *N. crassa*. The metabolic proteins not being identified support the fact that these proteins only make-up a low concentration of the total proteome. The results from the mass spectrometry (MS) analysis assigned protein identifications to fourteen unique proteins as well as three proteins that were found to be up-regulated, differentially expressed.

The identified proteins that were found to be unique, in our study, to dextrose as the sole carbon source included an ATP-dependent RNA helicase (eIF4A), an enolase, a 60S ribosomal protein L7, a cytrochrome c peroxidase, an outer membrane porin F, and a 50s ribosomal protein L6. Out of these identified proteins, only the ATP-dependent RNA helicase (eIF4A), enolase, 60s ribosomal protein L7, and the cytochrome c peroxidase were clearly *N. crassa* proteins. As mentioned in the Results, we further analyzed the ATP-dependent RNA helicase (eIF4A), enolase, and the cytochrome c peroxidase. The ATP-dependent RNA helicase (eIF4A) could be up-regulated in dextrose because it's a good quality carbon source, therefore, promotes more proteins to be synthesized. The enolase being up-regulated in dextrose is logical because it's metabolized by the glycolytic pathway and the enolase functions to catalyze the ninth step of this pathway. In dextrose, the mitochondrial cytochrome c peroxidase may be up-regulated because a high

rate of aerobic respiration could lead to the production of peroxidases, therefore, the cells are utilizing this protein to detox the cell.

The proteins that were found to be essentially unique to quinic acid as the sole carbon source included a peptidyl-prolyl cis-trans isomerase, a Cu-Zn superoxide dismutase, a disulfide bond formation protein B, a cold shock protein capA, a 50s ribosomal protein L8, and a DNA-binding protein HU-beta. Out of these identified proteins, only the peptidyl-prolyl cis-trans isomerase and the Cu-Zn superoxide dismutase were clearly *N. crassa* proteins.

The identified proteins that were found to be essentially unique to glycerol as the sole carbon source inlcuded a probable pyridoxine biosynthesis protein and a 30s ribosomal protein S7. Out of these identified proteins, only the probable pyridoxine biosynthesis protein was clearly a *N. crassa* protein. The proteins that were found to be differentially expressed among the carbon sources included a F-actin-capping protein subunit alpha, an outer membrane porin F, and a probable pyridoxine biosynthesis protein. Out of these identified proteins, only the F-actin-capping protein subunit alpha and the probable biosynthesis protein were clearly *N. crassa* proteins. Form changes may account for the identification of two probable pyridoxine biosynthesis proteins in the glycerol carbon source.

To conclude the present investigation, we wanted to compare our results to the previous studies that were performed by Aign et al. and Logan et al. (Aign et al., 2003, Logan et al., 2007). As mentioned earlier, these two studies were determined to be the most relevant to our present investigation. After our investigation of these two studies, we found only one protein, the probable biosynthesis pyridoxine, that correlated to the

findings in these experiments. In Logan's study, some of the results revealed that protein degradation, ribosome biogenesis, and amino acid metabolism are turned off during the shift from glucose to quinic acid (Logan et al., 2007). It was also determined that protein modification genes which encode products that functions as kinases and phosphatases are turned on with quinic acid as the sole carbon source, however, putative mannosyl transferase enzymes are turned off during the shift to quinic acid (Logan et al., 2007). In Aign's study, they revealed that a number of genes were up-regulated in minimal media only containing sucrose (Aign et al., 2003). Some of these genes that were found to be up-regulated in the minimal media included the genes responsible for metabolism and biosynthesis of thiamine (Aign et al., 2003). Most of these differences could be due to the quantity of these gene products being low or the pI's of these proteins not being suitable for our experimental conditions.

REFERENCES

Alexopoulos, C. J. 1962. *Introductory Mycology.* New York: John Wiley and Sons.

Aign, V., Hoheisel, J. D. 2003. Analysis of nutrient-dependent transcript variations in *Neurospora crassa*. *Fungal Genetics and Biology*. 40: 225-233.

Arnett, D. R., Lorimer, H. E., Asch, D. K. 2009. Catabolite repression directly affects transcription of the *qa-y* ge of *Neurospora crassa. Fungal Genetics and Biology*. 46: 377-380.

Arnett, D. R. 2005. The quinic acid gene cluster in *Neurospora*: sequence comparison and gene expression. Ph.D. dissertation, Kent State University.

Barco, A., Benetti, S., De Risi, C., Marchetti, P., Pollini, G. P., Zanirato, V. 1997. D-(-)-quinic acid: a chiron store for natural product synthesis. *Tetrahedron: Asymmetry.* 8: 3515-3545.

Bhadauria, V., Zhao, W., Wang, L., Zhang, Y., Liu, J., Yang, J., Kong, L., Peng, Y. 2007. Advances in fungal proteomics. *Microbiological Research*. 162: 193-200.

Case, M. E., Giles, N. H. 1975. Genetic evidence on the organization and action of the *qa-1* gene product: a protein regulating the induction of three enzymes in quinate catabolism in *Neurospora crassa*. *Proceeding of National Academy of Science*. 72: 553-557.

Case, M. E., Geever, R. F., Asch, D. K. 1992. Use of gene replacement transformation to elucidate gene function in the *qa* gene cluster of *Neurospora crassa*. *Genetics.* 130: 729-736.

Chaleff, R. S. 1974. The inducible quinate-shikimate catabolic pathway in

Neurospora crassa: genetic organization. *Journal of General Microbiology*. 81: 337-355.

Davis, R. H. 2000. *Neurospora Contributions of a Model Organism*. New York: Oxford University Press.

Deacon, J. W. 2006. *Fungal Biology*. Hong Kong: Blackwell Publishing Ltd.

Denor, P. F., Courtright, J. B. 1978. Isolation and characterization of glycerol-3-phosphate dehydrogenase-defective mutants of *Neurospora crassa*. *Journal of Bacteriology*. 136: 960-968.

Draths, K. M., Knop, D. R., Frost, J. W. 1999. Shikimic acid and quinic acid: Replacing isolation from plant sources with recombinant microbial biocatalysis. *Journal of American Chemical Society.* 121: 1603-1604.

Feofilova, E. P. 2001. The kingdom fungi: Heterogeneity of physiological and biochemical properties and relationships with plants, animals, and prokaryotes. *Applied Biochemistry and Microbiology.* 37: 141-155

Galagan, J. E., et al. 2003. The genome sequence of the filamentous fungus *Neurospora crassa*. *Nature.* 422: 859-868.

Galagan, J. E., Henn, M. R., Cuomo, C. A., Ma, L., and Birren, B. 2005. Genomics of the fungal kingdom: Insights into eukaryotic biology. *Genome Research.* 15: 1620-1631.

Geever, R. F., Baum, J. A., Case, M. E., Giles, N. H. 1987. Regulation of the *qa* gene cluster of *Neurospora crassa*. *Journal of Microbiology.* 53: 343-348.

Geever, R. F., Huiet, L., Baum, J. A., Tyler, B. M., Patel, V. B., Rutledge, B. J., Case, M. E., Giles, N. H. 1989. DNA sequence, organization and

regulation of the *qa* gene cluster of *Neurospora crassa*. *Journal of Molecular Biology*. 207: 15-34.

Giles, N. H., Case, M. E., Baum, J., Geever, R., Huiet, L., Patel, V., Tyler, B. 1985. Gene organization and regulation in the qa (quinic acid) gene cluster of *Neurospora crassa*. *Microbiological Reviews*. 49: 338-358.

Giles, N. H., Geever, R. F., Asch, D. K., Avalos, J., Case, M. E. 1991. Organization and regulation of the *qa* (quinic acid) genes in *Neurospora crassa* and other fungi. *Journal of Heredity*. 82: 1-7.

Hibbett, D. S. et al. 2007. A higher-level phylogenetic classification of the fungi. *Mycological Research.* 509-547.

Huiet, R. L. and Giles, N. H. 1986. The qa repressor gene of Neurospora crassa: wild-type and mutant nucleotide sequences. *Proceedings of National Academy of Science.* 83: 3381-3385.

Kim, Y., Nandakumar, M. P., Marten, M. R. 2007. Proteomics of filamentous fungi. *Trends in Biotechnology*. 25: 395-400.

Kuswandi, Roberts, C. F. 1992. Genetic control of the protocatechuic acid pathway in *Aspergillus nidulans*. *Journal of General Microbiology*. 138: 817-823.

Logan, D. A., Koch, A. L., Dong, W., Griffith, J., Nilsen, R., Case, M. E., Schuttler, H., Arnold, J. 2007. Genome-wide expression analysis of genetic networks in *Neurospora crassa*. *Bioinformation*. 10: 390-395.

Patel, V. B., Schweizer, M., Dykstra, C. C., Kushner S. R., Giles, N. H. 1981. Genetic organization and transcriptional regulation in the *qa* gene cluster

of *Neurospora crassa*. *Proc. Natl. Acad. Sci. USA.* 78: 5783-5787.

Patel, V. B. and Giles, N. H. 1985. Autogenous regulation of the positive regulatory *qa-1F* gene in *Neurospora crassa*. *Molecular Cell Biology.* 5: 3593-3599.

Perkins, D. D. 1992. Neurospora: The organism behind the molecular revolution. *Genetics.* 130:687-701.

Ran, N., Knop, D. R., Draths, K. M., Frost, J. W. 2001. Benzene-free synthesis of hydroquinone. *Journal of American Chemical Society.* 123: 10927-10934.

Trail, F. 2007. Fungal cannons: explosive spore discharge in the ascomycota. *Federation of European Microbiology Societies.* 276: 12-18.