Proteomic Analysis of *Neurospora crassa* Using the Non-Preferred Carbon Source Acetic Acid

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ABSTRACT

The Ascomycete fungus, *Neurospora crassa*, has been used for over 90 years as an ideal model organism in a variety of fields ranging from genetics to molecular biology. *N. crassa* is able to use multiple different carbon sources under varying conditions. It metabolizes preferred carbon sources such as dextrose, but has the ability to metabolize non-preferred carbon sources such as acetic acid also. Little research has been done concerning protein expression using non-preferred carbon sources, but there has been growing interest in non-preferred or otherwise thought toxic nutrient sources, since NASA's discovery of bacteria able to use arsenic as a sole source of nutrition, which was thought to be toxic to all forms of life.

In this study, we analyze the protein profiles of wild-type *N. crassa* grown on two different carbon sources: 2% dextrose (preferred carbon source), and 10% sodium acetate (non-preferred carbon source). Wild-type *N. crassa* was grown on Vogel's minimal media and shifted to one of two carbon sources utilized in our study. Afterwards, protein was extracted from N. *crassa* tissue and two-dimensional gel electrophoresis (2-DGE) was performed. Following 2-DGE the gel images were examined using PDQuest™ 2-D analysis software.

From the 2-DGE analysis using PDQuest™, it was shown that acetic acid, a non-preferred carbon source, had a much higher number of protein spots expressed, in comparison to the preferred carbon source, dextrose. The total number of protein spots visualized for acetic acid was found to be 257 and the total number for dextrose was found to be 138. The two carbon sources were found to share only 121 proteins. This goes against current research that was previously performed, in which preferred carbon sources display greater numbers of proteins in relation to non-preferred carbon sources.

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Introduction

    I.   **Kingdom Fungi**

The fungal kingdom is one of the most diverse and large kingdoms in all of biology, second only to the Kingdom Animalia Phylum Arthropoda. Originally it was separated into its own kingdom 1783, but not officially accepted until 1959 when proposed by Whitaker (Hibbett, 2007). The kingdom has been rapidly expanding ever since. Thought only to contain between 6000 and 15000 species, the number has now ballooned to over an estimated 1.5 million species. (Galagan, 2005) There are four major groups of fungal organisms: ascomycetes, basidiomycetes, zygomycetes and chytrids. Fungal biomass is said to take up a great percentage of total mass in soil worldwide, where it accounts for up to 90% of the total soil biomass. (Feofilova, 2000)

Fungi are found all over the world, in a variety of locations and temperate zones, from the arctic to the sea. Most fungi share common traits, such as: being eukaryotic, having cells that typically grow apically as hyphae, but sometimes grow isotropically as yeasts; that they are heterotrophic, and usually haploid. The true fungi also possess cell walls composed primarily of chitin and glucans, and typically absorb soluble molecules through their cell walls and membranes. Fungi also are able to aid in the biochemical degradation and decomposition of living and non-living organisms and also are shown to maintain symbiotic relationships with prokaryotes, plants, and animals. (Galagan, 2005) Though these relationships can have positive outcomes for both organisms, either through biotransformation such as fermentation and the production of antibiotics it can also be harmful for one of the partners. More and more fungi are being discovered that are shown to be pathogenic, either to human or plant species, such as *Paracoccidioides brasiliensis*, a human pathogen that causes paracoccidioidomycosis in immune

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compromised humans or as is the case with *Magnaporthe grisea*, which is plant pathogen that causes rice blast disease in rice. (Silva, 2008 and Couch, 2002)

Scientific research into fungal cellular physiology and genetics is showing today that most fungi share common key characteristics with animal and plant cells, including multicellularity, cytoskeletal structures, development and differentiation, sexual reproduction, cell cycle, intercellular signaling, circadian rhythms, DNA methylation, and chromatin modification.(Galagan, 2005) These common traits that are continually being discovered between human and fungi, make the true understanding of these biological and genetic functions of vast important to how we understand biology. (Galagan, 2005)

II.   Phylum Ascomycota

The phylum Ascomycota is one of the largest and most diverse phylums in all of biology. It is said to account for 75% of all described fungal species. (Trail, 2007) It is commonly referred to as the sac fungi. Similar to other fungi, members of the phylum Ascomycota are heterotrophs and able to obtain nutritional resources from living and dead organisms. Famous Ascomycota fungi include: *Saccharomyces cerevisiae*, the yeast of commerce and foundation of the baking and brewing industries and *Penicillium chrysogenum*, producer of penicillin. Harmful and pathogenic fungi of this class include: *Aspergillus flavus*, producer of aflatoxin, the fungal contaminant of nuts and stored grain that is both a toxin and the most potent known natural carcinogen found in nature and *Cryphonectria parasitical*, which is primarily responsible for the demise of 4 billion chestnut trees in the eastern United States. (Alexopoulos, 1996). The sexual cycle of Ascomycota is primarily conserved throughout the different species present in the phylum. Most asci remain in a dikaryon state, with hyphae that are partitioned into cells that

contain two haploid nuclei. Once this occurs, karyogamy only occurs in connection with the formation of sexual spores. The newly formed diploid nucleus undergoes multiple rounds of mitosis, followed by meiosis, which in the end yields 4 or 8 haploid nuclei. These nuclei are then petitioned into individual ascospores. Many members of the phylum can also reproduce asexually through conidia.

III.   *Neurospora crassa*

The Ascomycete fungus, Neurospora, has been used for over 90 years as an ideal model organism in a variety of fields ranging from genetics to molecular biology. Usually referred to as " red/orange bread mold", Neurospora , was originally discovered and categorized in 1842 as an orange mold like growth that was contaminating bread in bakeries located throughout Paris, in the summer of that year. It was also used in Indonesia as an ingredient in the production of an orange cake called oncham. F. A. F. C. WENT, a Dutch plant physiologist stationed there at the time, was interested in studying the orange oncham fungus (i.e. Neurospora) and started experimenting with it. He used the oncham fungus and its culture in a series of studies on the effects of various substrates on enzymes such as trehalase, invertase and tyrosinase. In addition he also studied the effect of light on carotenoid production, which is responsible for the orange pigmentation present in Neurospora. (Perkins, 1992.) After Went's experimentation with Neurospora, Bernard Dodge and C.L. Shear continued his work and performed a variety of experiments using Neurospora. In their research they were able to isolate multiple organisms and additionally perform experimental crosses with Neurospora. Two species with eight-spored asci, *Neurospora crassa and Neurospora sitophila*, were identified and shown to be heterothallic, in that they only could be crossed with strains that had opposite mating types.(Perkins, 1992.) Dodge's enthusiasm eventually led him to do work with Carl Lindegren, a

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fellow plant pathologist, who after working with *N. Crassa*, determined it was a species well suited for work in genetics.(Perkins, 1992.) During the 1930's, Dodge and Lindegren developed the Genetics of Neurospora and showed the scientific community its value and how we can transfer the understanding of Neurospora's genetics to more complex organisms and how to utilize the genetic similarities it shares with plants and animals. (Horowitz, 1991.) In modern times, Neurospora plays an important role as a primary model organism, which contributes to the overall understanding of the genome defense systems, DNA methylation, mitochondrial protein import, circadian rhythms, post-transcriptional gene silencing and DNA repair. (Galagan, 2003.)

Neurospora is still used as a model organism in laboratories today. It has multiple advantageous characteristics that allow it to be widely and easily used. The primary advantage that it has that it is cheap and easily maintained, due to its simple nutritional requirements and also that it is able to adapt to a variety of carbon sources. In addition to Neurospora's simple needs, it is able to be grown on a defined medium, under controlled conditions. (Schmit, 1976). Also, due to that Neurospora species are obligate anaerobes, they are unable to grow in the gut, bladder, or in tissues, which makes it unlikely to have any pathogenic like effects on animals or human beings. (Davis, 2000)

Neurospora crassa is the main Neurospora species used today in molecular and genetic studies. This fungus usually grows in moist, high climate regions, with a mean temperature of around 35°C. It was also found to grow in regions of volcanic eruptions or slash-and-burn clearings of rain forest. (Davis, 2000) *N. crassa* has an asexual life cycle, where conidia are formed following mitosis of the haploid nuclei, and sexual life cycle, in which ascospores are formed from different mating types. (Schmit, 1976)
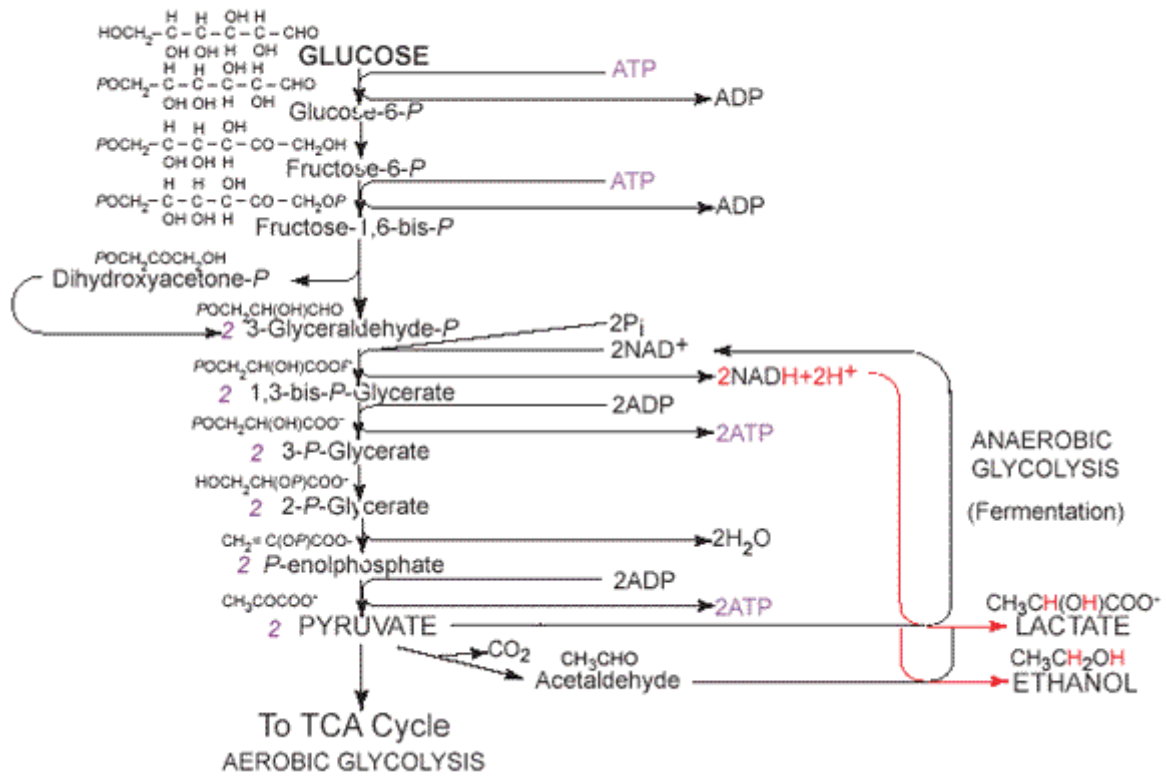
The genome of *N. crassa* was found to be 38.6 Mb, distributed among seven different linkage groups or chromosomes. (Galagan, 2003). The genome of *N. crassa* has been completely sequenced and shown to contain a total of 10,082 protein-coding genes (Galagan, 2003). Out of a total of 1,336 predicted *Neurospora* proteins, 13% were identified by similarity to known protein sequences from public access 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.(Galagan, 2003). Also present is evidence that for many of the *N. crassa* genes that do show homology to other known genes, that most of the homologs present are prokaryotic in origin and either deal with substrate degradation or secondary metabolism.(Hynes, 2003.)

IV.   Carbon Source Metabolism

Metabolism is a common place cellular process throughout biology. It is a biological process that uses a set of chemical interactions that happen in living organisms to extract energy from various sources to maintain life. Throughout the biological world, various organisms use specific metabolic pathways, which are able to designate which carbon substrates can be used by the cell. *N. crassa* utilizes a variety of carbon sources in metabolism. The preferred carbon sources are: glucose, sucrose, fructose, mannose, and maltose. (Schmit, 1976.) And the non-preferred carbon sources that *N. crassa* is able to use are: acetate, quinic acid, sorbose, and glycerol. (Schmit, 1976.)

Glucose is the preferred metabolite used as an energy source in *N. crassa* and most other organisms through glycolysis. (Fig.1) The first step in glycolysis is phosphorylation of glucose to form glucose 6-phosphate. Glucose 6-phosphate is then converted into fructose 6-phosphate by glucose phosphate isomerase. The reaction is then catalyzed by phosphofructokinase 1 by the

GLYCOLYSIS

GLUCOSE

ATP → ADP

Glucose-6-P

Fructose-6-P

ATP → ADP

Fructose-1,6-bis-P

Dihydroxyacetone-P

2 3-Glyceraldehyde-P   2P$_i$

2NAD$^+$

2 1,3-bis-P-Glycerate   2NADH+2H$^+$

2ADP

2 3-P-Glycerate   2ATP

2 2-P-Glycerate

2 P-enolphosphate   2H$_2$O

2ADP

2 PYRUVATE   2ATP

CO$_2$ CH$_3$CHO
Acetaldehyde

To TCA Cycle
AEROBIC GLYCOLYSIS

ANAEROBIC
GLYCOLYSIS

(Fermentation)

CH$_3$CH(OH)COO$^-$
LACTATE

CH$_3$CH$_2$OH
ETHANOL

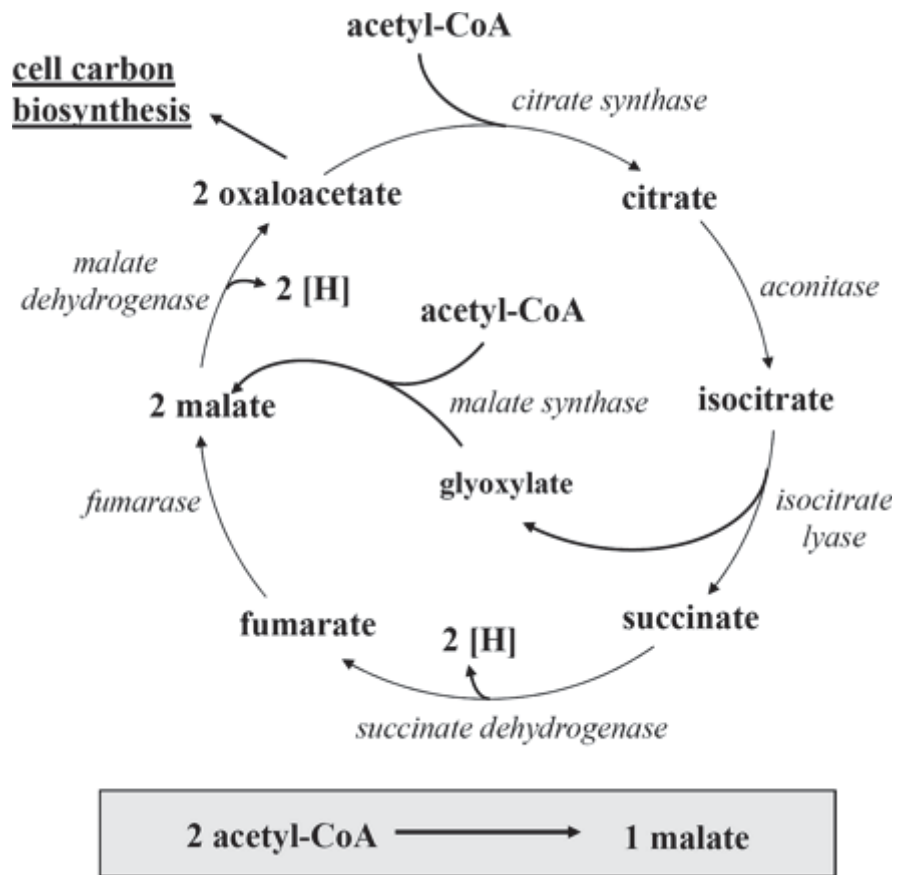**Figure 1. Glycolysis Cycle (Nicholson, 2002)**

hydrolysis of ATP. 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. Pyruvic acid, ale able to be converted into acetyl-coenzyme A, which is a key the precursor for the Krebs cycle.

The glyoxylate cycle is an alternate pathway to the Krebs cycle for oxidizing acetate to dicarboxylic acids, such as succinate, malate, and oxaloacetate. (Fig.2) The first steps of the glyoxylate cycle are *acetate to citrate to isocitrate*. The next step of the cycle is that isocitrate undergoes cleavage into succinate and glyoxylate by isocitrate lyase. At the same time malate synthase causes glyoxylate to condense with acetyl-CoA, which produces malate. Both Malate and oxaloacetate are then converted into phosphoenolpyruvate. Phosphoenolpyruvate is the substrate of phosphoenolpyruvate carboxykinase, the first enzyme in gluconeogenesis cycle. Most of the glyoxylate cycle enzymes are found in organelles called glyoxysomes. The primary function of the glyoxylate cycle is to use fats/lipids for the synthesis of carbohydrates. It has been shown through the research of Flavell and Woodward, that the enzymes of the glyoxylate cycle are present at high levels during growth on acetate. (Flavell, 1971.)Evidence that it is necessary in the germination of ascospores of Neurospora when stored fat is being used as the major source of carbon was presented also present by Flavell.(Flavell, 1968.)In the absence of available carbohydrates, the glyoxylate cycle is able to synthesize glucose from lipids via acetate generated in fatty acid β-oxidation.

V.   Acetate

Acetic acid, CH_3COOH, also known as ethanoic acid, is an organic acid that gives vinegar its sour taste and pungent smell. It was used in history as a way to preserve food by pickling, but its use in metal refining appear as early as ancient Greece. (Zoeller, 1992.) It is one of the simplest carboxylic acids, in that it is a proton donor and among the most common type of organic acids. In 1847, German chemist Hermann Kolbe synthesized acetic acid from inorganic materials for the first time. The experimental sequence was initially the chlorination of carbon disulfide to carbon tetrachloride. He then followed this by pyrolysis to tetrachloroethylene and aqueous chlorination to trichloroacetic acid, and concluded with electrolytic reduction to acetic acid. (Rocke, 1993.) Acetate is a derivative of acetic acid. It is a common anion in biology and is primarily used in the form of acetyl coenzyme A(A(CoA)).

acetyl-CoA

cell carbon biosynthesis

2 oxaloacetate   citrate

citrate synthase

malate dehydrogenase   2 [H]

acetyl-CoA

2 malate   *malate synthase*   isocitrate

aconitase

glyoxylate

fumarase   *isocitrate lyase*

fumarate   2 [H]   succinate

succinate dehydrogenase

2 acetyl-CoA ⟶ 1 malate

Figure 2. The glyoxylate cycle as proposed by Kornberg and Krebs 50 years ago. (Erb, 2007)

VI.   History of Acetate utilizing and non-utilizing genes in *Neurospora crassa*

Neurospora crassa is able to utilize acetate as a sole carbon source similar to most organisms. Under these growth conditions, acetate is used for gluconeogenesis through the A (CoA) and the glyoxylate cycle. In most eukaryotes, the glyoxylate cycle functions within the glyoxysome, which is a special organelle that has a varying biogenesis and enzyme content depending on organisms. (Cioni, 1981.)The glyoxysome of *N. crassa* contains the enzymes isocitrate lyase, malate synthases, and part of the cellular malate dehydrogenase (Thomas, 1988.) The known genes responsible for the utilization and non-utilization of acetate as a sole carbon source are the: *ace-1-9* and *acu-1-16*.

Studies on acetate utilization in Neurospora have been ongoing for the last 60 years. Originally identified in 1954, Strauss and Pierog were able to identify acetate utilizing mutants *ac-1, ac-2, ac-3, ac-4,* and *ac-5*, but the mutants were lost and cannot be confirmed. (Strauss, 1954.) It was not until 1979, through the work of Kuwana and Okumura, that these genes were truly identified and placed on the linkage map of *N. crassa*. Kuwana and Okumura discovered *ace-3* thru *ace-7* and also *ace-6*, which actually is allelic with a *suc* gene based on its location in the linkage group and the deficiency of pyruvate carboxylase activity.(Kuwana, 1979.) Acetate mutants *ace-1* through *ace-7* are auxotrophs that grow on 0.3% sodium acetate, as do *suc* mutants, which have been shown to grow better on acetate than succinate. Most of the *ace* mutants are shown to grow better when the provided carbon source present is maltose rather than sucrose. (Kuwana, 1979.) The mutant *ace-1*, is found on linkage group 2, and was first identified by Ralph W. Barratt in 1954, which discovered it requires acetate for expression and slows ascospore germination and maturation when expressed. (Barratt, 1954.) The mutants: *ace-2, ace-3, ace-4*, all encode for 3 different components in the pyruvate dehydrogenase complex, which is

needed to transform pyruvate into acetyl CoA. The *ace-2* gene located at linkage group 3encodes the dihydrolipoamide acetyltransferase component (E2) of the pyruvate dehydrogenase complex. Gene *ace-3* is located on linkage group 1 and shows homology to the α- and β-subunits of the pyruvate dehydrogenase E1 component under preferred carbon source conditions in *N. crassa.* The gene, *ace-4,* is located at linkage group 4 and also shows homology to the α- and β-subunits of the pyruvate dehydrogenase E1 component, similar to *ace-3*, but when *ace-4* is expressed, lipose acetyltransferase, a key to the formation of the pyruvate dehydrogenase complex, is not formed. (Okumura, 1979.) The gene, *ace-5,* is found on linkage group 5, but overall there is no information in respect to it, other than it requires acetate for expression. The gene, *ace-6,* is located on linkage group 1, and is usually not included in the list of acetate requiring mutants, in that it is deficient in pyruvate carboxylase activity and thus allelic with *suc*. The *ace-7* mutant is found on linkage group and is a structural gene for glucose-6-phosphate dehydrogenase (Kuwana, 1979.) After Kuwana and Okumura's research, there has been little further work in reference to the *ace* mutants of *N. crassa*. In 1983, Kuwana and Tanaka, found a new *ace* mutant, *ace-8*, which encodes a structural gene for pyruvate kinase. (Kuwana, 1987.) In 1990, the final gene *ace-9*, was discovered by Santosa and Kuwana, whom discovered that *ace-9* is located on linkage group 2 and has very weak activity in the pyruvate dehydrogenase complex (PDHC). (Santosa, 1990.) This places it with: *ace-2, ace-3,* and *ace-4*, in that it is the fourth gene shown to cause a deficiency in PDHC activity. (Santosa, 1990.)

A number of acetate non-utilizing mutants (*acu*) have also been isolated. These genes demonstrate single enzymes deficiencies and define genes that specifically are able to encode for enzymes that are involved in acetate metabolism. (Mizote, 1996.) To date, there have been 16 *acu* mutants discovered and located on the genome of *N. crassa*. The *acu* groups *acu-1* and *acu-*

4, are not yet associated with any a specific identifiable enzymatic defect, although they do show

a lower increases in specific activity on acetate for NADP-linked isocitrate dehydrogenase and

NAD-linked glutamate dehydrogenase.(Flavell, 1968.) The gene *acu-2* is found on linkage group

4 and *acu-7* is found on linkage group 3. Both *acu-2* and *acu-7* have a reduced level of

oxoglutarate dehydrogenase, which is involved in the maintenance of the supply of energy and

carbon fragments for various biosynthetic pathways when growing on acetate. (Flavell, 1968.)

The *acu-3* gene is found on linkage group 5 and is the structural gene for isocitrate lyase, one-of-

two enzymes involved in the glyoxylate bypass system. (Mizote, 1996.) The gene, *acu-5*, is

located on linkage group 2 and is a structural gene for acetyl coenzyme A synthetase. Expression

of Acetyl coenzyme A synthetase seems necessary for this enzyme to activate acetate to its

metabolizable form. The gene, *acu-6*, is located on linkage group 6 and has been identified to be

a structural gene for phosphoenolpyruvate carboxykinase, which is an enzyme in the lyase family

used in gluconeogenesis. This enzyme converts oxaloacetate into phosphoenolpyruvate and

carbon dioxide. (Beever, 1973.) The gene, *acu-8*, is located on linkage group 2 and has been

identified to be a structural gene for acetate permease. In addition, the mutant has also been

shown to be deficient in acetyl-CoA hydryolase and to accumulate acetyl-CoA when supplied

with acetate. (Connerton, 1992.) The gene *acu-9* is located on linkage group 7 and was found to

be a structural gene for malate synthase, which is needed in pyruvate metabolism and glyoxylate

and dicarboxylate metabolism. (Sandeman, 1991.) The genes: *acu-10, acu-11, acu-12,* and *acu-*

13 are all linkage group 2 and resistant to the to the metabolic toxin fluoroacetate, which occurs

in nature as an anti-herbivore metabolite. (Owen, 1992.) The last *acu* gene is *acu-14*. It is located

on linkage group 6 and has no complementation to any other *acu* mutants. Though there are no

deficits in overall enzyme levels present when expressed, the levels of acetyl-CoA synthase and isocitrate lyase are slightly less than normal. (Chaure, 1995.)

VII.   Proteomics in Fungi

The field of proteomics is fairly new in the overall study of molecular genetics. Proteomics is the systematic analysis of protein expression, function, and characterization of all proteins in an organism.(Pevsner, 2009.) There are a variety of different specialized fields of studying specific proteins, from secretome proteomics, which deals specifically with proteins secreted by the cell; to mitochondrial proteomics. Changes in the proteome occur at different rates and at different times in an organism, thus the study of the specific conditions under which these changes occur is very useful in our overall understanding of how and why these proteins are expressed. Proteomics in filamentous fungi is of huge interest, due to the relevance it has in the fields of antibiotics and antifungal drugs. Also due to that filamentous fungi are known human and plant pathogens, potential proteomic studies can led to cures and treatments to these pathogen, helping people the world over and solving issues of plant disease that occur in crops that are fiscally needed to support farmers and major food sources. (Kim, 2007.) The relevance of the use of proteomics in human fungal diseases is prevalent in the work that Lessing and his colleagues used to examine the protein profile of *Aspergillus fumigatus*, a invasive fungal human pathogen, in 2007, when they investigated the effect of H_2O_2 on, and the deletion of the transcription factor AF*yap1,* from *A. fumigatus* .(Doyle, 2011.) Their research determined that regulation of *yap1* was needed to protect *A. fumigatus* from reactive oxygen intermediates like H2O2, which is needed for virulence in a murine infection model. (Doyle, 2011.) Furthermore, the use of mass spectrometry (MS) in proteomics has multiple different applications that have the potential to

help in our overall understanding of proteins in general. Some of the multiple uses that MS has in reference to proteomics are: the analysis of protein complexes, the detection and quantification of post-translational modifications, protein identification in complex mixtures, protein quantification, and proteome profiling. (Oliveira, 2011.)

The field of fungal proteomics is hurtling toward an exponential explosion of information, which can be seen by the increased rate of publications and the relevant research conducted in conjunction with/through the use of the scientific field of bioinformatics. (Kim, 2007.)

VIII. The Non-Preferred Carbon Source Quinic Acid and the potential "blueprint" it provides for Acetic Acid gene regulation

Another non-preferred carbon source utilized by *N. crassa* is quinic acid. Quinic acid is similar to acetic acid, in that it is also able to be the sole carbon source for *N. crassa* when other preferred carbon sources are not present. When quinic acid is used as the primary catabolite, quinic acid is first converted to 5-dehydroquinate and then is catalyzed by quinic acid dehydrogenase. Then quinic acid dehydroquinase is converted into 5-dehydroquinate then to 5-dehydroshikimate. Finally, 3-dehydroshikimate dehydratase converts 5-dehydroshikimate into protocatechuic acid. From there protocatechuic acid is able to enter the Krebs cycle as either Succinyl-CoA or Acetyl-CoA. (Fig.3)
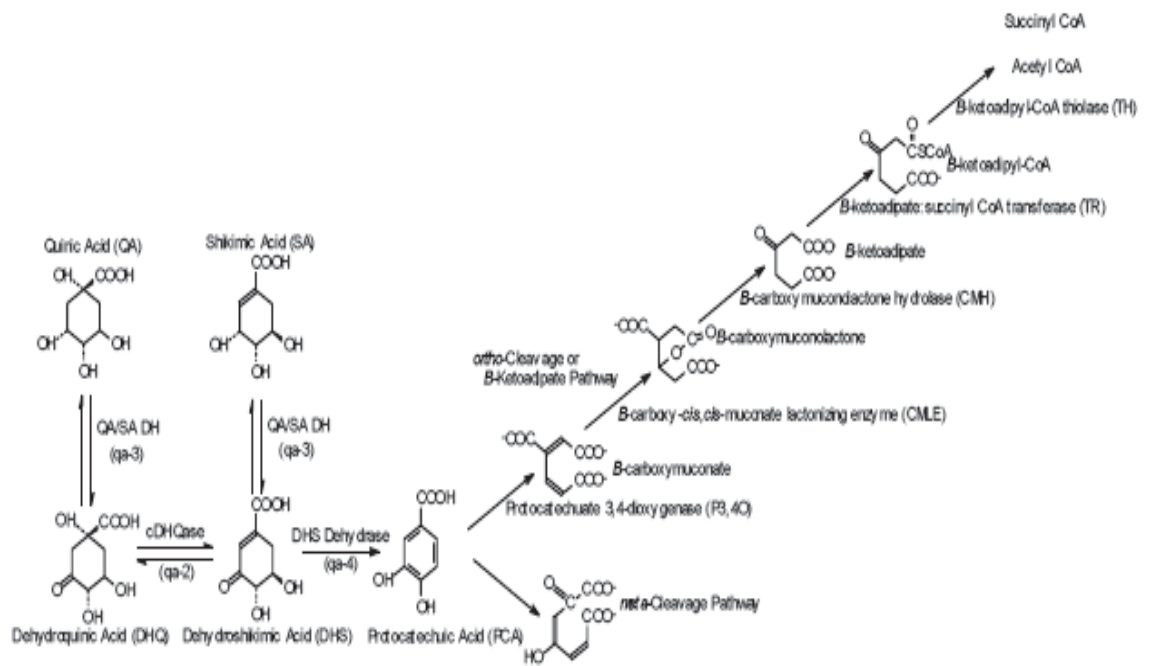
Quinic acid has been the focus of multiple research projects, mainly with interest into the *qa* gene cluster and its regulation and induction in the presence of quinic acid. The *qa* gene cluster is made up of 7 genes overall. Five structural genes, that are induced when quinic acid is present and two regulatory genes. The 5 structural genes are *qa-x, qa-2, qa-3, qa-4,* and *qa-y.*

(Geever,1989 & Case, 1992.) The *qa-2, qa-3*, and *qa-4* genes are primarily responsible for the conversion of quinic acid to protocatechuic acid, while qa-x has a unknown function and *qa-y* encodes as quinase permease, which allows quinic acid into the cell.(Chaleff, 1974.)

In addition, the quinic acid gene (*qa-1*) of *N. crassa* was proposed to act as a regulator and control production of the *qa* enzymes. (Patel, 1981.) The two regulatory genes present are *qa-1S* and *qa-1F*. Gene qa-1S acts as a quinate repressor protein. (Huiet, 1986.) while *qa-1F* acts as an activator protein in the presence of quinic acid. (Geever, 1989).From previous 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, 1985 & Giles, 1991.)

We hypothesize that those similar events that occur when quinic acid is used as a primary carbon source could also be possibly arising when acetic acid is used as a primary carbon source, due to that both are non preferred carbon sources and should have similar genes activated under these conditions. In addition, both the identified acetic acid genes and the identified quinic acid genes all code for respective parts their individual metabolic cycles, so it is possible that a gene for the regulation of acetic acid in response to inducer acetic acid could be identified. Furthermore, protein profiling comparisons could also confirm that similar events could be happening in reference to the possible up-regulation of particular possible starvation genes. Though different genes will be activated under each separate condition, quinic acid enables us to have a blueprint to help characterize and understand the potential outcome of the results obtained in this study.

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-carboxymucondactone hydrolase (CMH)

B-ketoadipate

B-ketoadipate: succinyl CoA transferase (TR)

B-ketoadipyl-CoA

B-ketoadipyl-CoA thiolase (TH)

Acetyl CoA

Succinyl CoA

Figure 3. The Quinic Acid Metabolic Pathway in *Neurospora crassa* (Knaggs, 1999)

Materials and Methods:

I. Strain

Wild-type *Neurospora crassa* 74A (FGSC NO.2489) strain was obtained from stock collections.

II. Mycelial Growth

Wild-type *N. crassa* 74A strain was originally inoculated into 50 mL of Vogel's Minimal Media, plus 1.5 % agar and 2% sucrose into 250 mL Erlenmeyer flasks. (Vogel, 1956) (Table 1)The flasks containing the *N. crassa* inoculate, were then grown at 30^oC in an incubator for 2 days and then moved under a fluorescent light source, at room temperature for duration of 14 days. Once 14 days passed, the conidia were harvested. Twenty-five mL of 1x Vogel's was added to each of the flasks. The conidial suspensions were then manually swirled around the flasks, and then these suspensions were poured through sterile cheesecloth to remove mycelia. The resulting suspensions were then divided into two equal portions to standardize overall conidial concentrations   Each conidial suspension was then added to 50mL of 1x Vogels and 2% dextrose in separate 250 mL Erlenmeyer flasks. The cultures were then grown for 24 hours at 30^oC in an orbital shaker at 150 rpm. After 24 hours passed, the mycelia were collected by vacuum filtration through Whatman®(1) filter paper. The resulting mycelial pads were transferred into a new flask that contained 50 mL of sterile distilled water, washed and re-vacuum filtered. Once refiltered, the mycelial pads were shifted to medium containing 50 mL of 1x Vogels, in combination with either 2% dextrose or 0.5% acetic acid. The new experimental cultures were grown for 3 hours in an orbital shaker at 30^oC at 150 rpm. After incubation for 3 hours, mycelia were collected again through vacuum filtration and the resulting mycelial pads were then frozen at -70^oC for storage.

III. Protein Isolation

To isolate protein, the mycelial pads were combined with liquid nitrogen and baked sand, then ground together with a mortar and pestle. Once the mycelial pad was ground sufficiently, the tissue was then divided into seven 1.5 mL Eppendorf tubes until the tubes were half full. Afterwards, 800 uL of lysis buffer (200mM Tris-HCL, pH 7.6, 10mM NaCl, 0.5mM deoxycholate, 40 µL protease inhibitor [Promega] was added to each Eppendorf tube. These Eppendorf tubes were then vortexed on high for 1 minute and then iced for two minutes. This process was repeated three more times. Once vortexing was complete, the tubes were placed into a microcentrifuge and spun for 10 minutes at 4°C at 12,000 rpm. Once centrifugation was completed, the resulting supernatant was collected and transferred to new 1.5 mL Eppendorf tubes and the original Eppendorf tubes were discarded. Following transfer of the supernatant, 100 uL of Trichloroacetic Acid (TCA) (Fisher Scientific) was added to the supernatant, to precipitate the protein out of solution and iced for 20 minutes. Following icing, the eppendorf tubes were inverted and spun in a centrifuge for 20 minutes at 4°C at 12,000 rpm. Following centrifugation, TCA was poured off and the collected protein pellets at the bottom of the Eppendorf tubes were washed a total of 3 times with acetone. Once washing was completed, the protein pellets were dried via a LABCONCO CentriVap DNA Concentrator speed vac for 20 minutes. This step was performed an additional time, if drying was incomplete and liquid was still obtained. Once drying was completed the protein pellets were resuspended in minimal suspension buffer (MSB) (2M thiourea, 7M urea, 4% w/v CHAPS, 1% w/v DTT) and placed in storage at -80°C.

IV. Bradford Assay

The Bradford protein assay was used to determine the total protein concentration of each sample from the protein isolations. A total of eight standards and one test tube containing a sample of the experimental protein, were prepared and tested in triplicate. The eight standard test tubes were composed of the following reagents: 80 µL de-ionized H_2O, 20 µL 0.1M HCL, and 10 µL 2-DE buffer. Following pippeting of the aforementioned reagents varying concentrations, 10 mg/mL of bovine serum albumin (BSA) was added to each standard test tube. The concentrations are as follows, 0.0 ug/ul 0.09 ug/ul 0.14 ug/ul , 0.18 ug/ul , 0.23 ug/ul , 0.27 ug/ul, 0.32 ug/ul and 0.36 ug/ul. The experimental protein samples were prepared the same as the standard test tube, except 10 uL of the experimental *N.crassa* protein sample was substituted for BSA. The collection of standards and the experimental protein sample were placed on the lab bench and allowed to settle for 5 minutes at room temperature. After the 5 minutes passed, 4mL of Bradford dye was added to each test tube and allowed to sit for an additional 5 minutes. These samples were then vortexed for 10 seconds to allow for mixing and were placed on the lab bench to sit again for 5 minutes prior to running the samples through a Bio-Rad® Smart Spec™ Plus Spectrophometer to measure the absorbance of each standard and sample.

V. Two-Dimensional Gel Electrophoresis

For two- dimensional gel electrophoresis to be performed, the *N.crassa* protein sample was taken from storage in the -80°C refrigerator, vortexed on and off for 10 seconds, and then allowed to settle for 2 minutes before passive rehydration was performed. For passive rehydration, Bio-Rad® ReadyStrip™ IPG strips were used that had a pH gradient of 5-8 and depending on the desired gel size, two different IPG strip lengths were used, either 7cm or 17 cm. The *N.crassa* protein sample was then combined with approximately, either 125 uL of

rehydration buffer for the 7cm IPG strips or 300 uL of rehydration buffer for the 17 cm IPG strips, in an equilibrium tray. The protein concentration for the 7 cm IPG strips was calculated to be 0.667 ng/μL, as recommended by the manufacturer. The protein concentration for 17 cm IPG strips was calculated to be 0.667 ng/μL, as recommended by the manufacturer. Once the *N.crassa* protein was combined with the rehydration buffer, the two were mixed with a pipette tip to thoroughly mix the two. Following mixing, Bio-Rad® ReadyStrip™ IPG strips were laid, gel side down, on top of the rehydration buffer-protein sample mix in individual lanes of the equilibration tray and 1 mL of mineral oil for the 7cm IPG strips and 3 mL of mineral oil was pipetted over the individual IPG strips. If any bubbles were present underneath the IPG strips, there were gently removed. The IPG strips were then placed on an orbital shaker at room temperature for 24 hours.

    After 24 hours the equilibration tray was removed from the orbital shaker and allowed to sit for 10 minutes. After the 10 minutes, the "loaded" IPG strips were then prepared to undergo isoelectric focusing (IEF), the first dimension of 2D gel electrophoresis. To perform IEF, Bio-Rad® Electrode Wicks were submerged in de-ionized water (H_2O) and with the use of sterilized tweezers, gently placed over the top of the wire electrodes at the positive and negative ends of the Protean® IEF tray. The "loaded" IPG strips were then taken from the equilibration tray and the tips of the strips were held up right and blotted off to allow for the excess mineral oil on the strips to run off. Once the majority of the mineral oil was removed, the "loaded" IPG strips were placed into the Protean® IEF tray, with the positive end of the IPG strip placed over the positive electrode and the negative end of the IPG strip placed over the negative electrode gel side face down. The IPG strips were then overlaid with 1 mL of mineral per lane for the 7 cm IPG strips and 3 mL per lane for the 17 cm IPG strips. Once the previous step was completed and no

bubbles were seen to be present under the IPG strips, the Protean® IEF tray was covered with the plastic cover and placed into the Bio-Rad® Protean IEF Cell to undergo IEF. The Bio-Rad® Protean IEF Cell was switched on and programmed according to IPG length. For the 7 cm IPG strips, the program was set to: preset method, liner ramping mode, 40,000 V-hr and held at a constant 500 V. For the 17 cm IPG strips, the program was set to: preset method, linear ramping mode, 60,000 V-hr, and held at a constant 500 V.

The following day, 12% poly-acrylamide gels were prepared in order to perform the second dimension of 2D gel electrophoresis. Twelve percent poly-acrylamide gels were prepared using 100 mL of solution: 12% acrylamide, 0.375 M Tris, 0.1% SDS, and later 0.1% ammonium persulfate, and 0.04% TEMED were all added to a 500 mL beaker. Once the poly-acrylamide solution was prepared, it was added into the Protean® plus Multi-Casting Chamber, which was filled with 12 glass spacer plates and 12 glass long plates, was used to add the poly-acrylamide solution. Prior to adding the 12% polyacrylamide solution, the entire Protean® plus Multi-Casting Chamber and gel plates were cleaned, using 70% ethanol to prevent contamination. After the gels polymerized, they were transferred to ½ X TGS buffer (1 x TGS buffer: 25mM tris, 192mM glycine, 0.1% SDS, H_2O, pH 8.6) and stored upright in the cooler until ready for use.

Once isoelectric focusing (IEF) was completed, the IEF focusing tray was removed from the Bio-Rad® Protean IEF Cell and the IPG strips were taken from the IEF tray and blotted off using sterilized tweezers and a sterile paper towel. This allowed for the mineral oil to drain off the IPG strip prior to getting treated with Equilibration buffers I and II. Once sufficiently free of mineral oil, the IPG strips were placed in the equilibration tray gel side up and immersed in equilibration buffer I (6M urea, 2% SDS, 0.375M Tris-HCL, pH 8.8, 20% glycerol, 2% DTT) for

a total of 10 minutes on an orbital shaker at room temperature. Once 10 minutes had passed, the

IPG strips were then 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 again placed on an

orbital shaker for 10 minutes at room temperature. Following the 10 minutes on the orbital

shaker, the IPG strips were removed from equilibration buffer II and transferred into another

equilibration tray that contained 1 x TGS buffer for approximately 2 minutes. While the IPG

strips were in the 1 x TGS buffer, he 12% poly-acrylamide gels were taken from storage, stood

upright, with the short plate facing forward and up; and overlay agarose (0.5g agarose, 100 mL 1

X TGS buffer, 1 grain of bromophenol blue) was added on top of the short plate. Before the

overlay agarose had time to solidify, the IPG strips were removed from the 1 x TGS buffer and

quickly placed directly on top of the 12% poly-acrylamide gels, between the long and short

plates, gel side up, and positive (+) side on left. The gels sat on the lab bench until the overlay

agarose fully solidified. Once the overlay agarose was fully solidified, the gels were placed into

the electrophoresis cell and filled to the top of the long plate with 1 X TGS buffer, at room

temperature for the 7 cm strips and chilled between 15°C and 18°C for 17 cm strips, and

electrophoresis was started. For the 7 cm IPG strips, the gels were ran in a Bio-Rad$^{\circledR}$ Mini-

Protean$^{\circledR}$ 3 Cell and were run on: manual program, at a constant rate of 16 milli-amps per gel for

approximately $2^{1/2}$-3 hours. For the 17 cm IPG strips, the gels were placed in the Bio-Rad$^{\circledR}$

Protean$^{\circledR}$ Plus Dodeca™ Cell and run on: manual program, at a constant voltage of 200 volts per

run for approximately 6-8 hours. Once electrophoresis was fully complete, the gels were

removed from the electrophoresis cell and stained accordingly. The 7 cm IPG strip gels were

stained with Comassie Blue stain (0.25% Comassie Brilliant Blue R-250 [Sigma], 45%

methanol, 10% acetic acid). And the 17 cm IPG strip gels were stained with SYPRO® Ruby Red stain from Bio-Rad®.

To Comassie stain, the gels were transferred to a plastic container and 300 mL of Comassie stain was added to fully immerse the gels. The staining containers with the gels in them, where then placed on an orbital shaker overnight at room temperature. After 24 hours passed, the Comassie stain was poured out of the plastic staining container and the gels were then immersed in 300 mL of high de-stain (40% methanol, 10% acetic acid) for 1 hour while being shaken on the orbital shaker at room temperature. After the 1 hour had passed, the high de-stain was poured from the plastic staining container and 300 mL low de-stain (10% methanol, 6% acetic acid) was added to fully immerse the gels for a duration of two hours, while being shaken on the orbital shaker at room temperature. After two hours, the low de-stain was poured from the plastic staining container and 300 mL of de-ionized H_2O was placed into the plastic staining for storage and to be transferred in order for the gels to be scanned using the EPSON Scan Program.

The SYPRO® Ruby Red stain was used on the 17 cm IPG strip gels. These gels were transferred to a large plastic staining container and immersed in 600 mL of fixing solution/high de-stain (40% methanol, 10% acetic acid) for 1 hour to fix the gels. After 1 hour, the fixing solution/high de-stain was then poured from the large plastic staining container and 600 mL of SYPRO® Ruby Red stain, was added till the gels were fully immersed and in no danger of sticking to the sides of the container. The gels were then left in the SYPRO® Ruby Red stain overnight on an orbital shaker at room temperature. After 24 hours, the SYPRO® Ruby Red stain was poured back into its original container and the gels were placed in de-ionized H_2O in a large plastic staining container for storage until needed.

VI.   **Gel Imaging and Analysis**

The large format 17cm gels that were stained using SYPRO® Ruby Red stain were then

imaged using the Bio-Rad ChemiDoc XRS imaging hardware in conjunction with Bio-Rad's 2-

Dimensrional Gel Analysis software PD-QUEST version 7.1. To image the gels, the gels were

taken from their individual storage containers and placed, one at a time, on the imaging platform.

Once aligned and the image was correctly focused, the gels were exposed to an ultraviolet

transillumination lamp, which activated the SYPRO® Ruby Red stain. This allowed the gel to be

imaged and the resulting gel images were analyzed using PD-QUEST analysis software. When

using the PD-QUEST software, the gel images and resulting protein profile of each individual

gel was able to be analyzed and compared amongst one another in each experimental group. The

two experimental groups in this case were the gels that represented the dextrose protein profile

and the acetic acid protein profile. Matchsets were then created for each experimental group and

landmarks unique to each protein profile were marked. Once landmarks were established, the

experimental matchsets were then matched again and higher level matchsets were created to

provide increased reliability. Once these higher level matchsets were completed for each

experimental group, the two groups were compared to one another and differential protein

expression was noted and individual landmarks unique to acetic acid were marked and assigned

an identification number. Once numbering was complete, protein spots of interest were ranked

according to level of priority to send out for identification.

VII.   **Protein Spot Excision**

The protein spots of interest were then excised according to priority. The total number of

protein spots excised per acetic acid SYPRO® Ruby Red stained gels was thirty spots that were

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found to be visually present on each gel. Each SYPRO® Ruby Red stained acetic acid gel, was individually placed directly on the Bio-Rad ChemiDoc XRS imaging platform. Ultraviolet light was then activated and transillumination of the gels occurred. The protein spots of interest were then visually identified and then cut out using the bulb-end of a sterile Pasteur pipette. The excised spots were then placed into the pre-numbered Eppendorf tubes, which contained 5% acetic acid solution to allow for transport and stored in the -80°C refrigerator.

Table I. Vogel's Minimal Media 50X (1 Liter)

Water	750 ml.
Na_3 citrate$\cdot$2H_2O	125 g.
NH_4NO_3, anhydrous	100 g.
$KH_2 PO_4$, anhydrous	250 g.
$MgSO_4\cdot7 H_2O$	10 g.
$CaCl_2\cdot2 H_2O$	5 g. in 20 ml. water; add dropwise.
trace elements solution*	5 ml.
biotin solution, 0.1 mg/ml	2.5 ml.

Table II. Trace Element Solution (100 ml) (Stored at Room Temperature)

Water	95 ml
citric acid·H_2O	5 g.
$ZnSO_4$·7 H_2O	5 g.
$Fe(NH_4)2(SO_4)2$·6 H_2O	1 g.
$CuSO_4$·5 H_2O	0.25 g.
$MnSO_4$·H_2O	0.05 g.
H_3BO_3	0.05 g.
Na_2MoO_4·2 H_2O	0.05 g.
Chloroform, as a preservative	1 ml

Table III. Biotin Solution (50 ml) (Stored Frozen)

Biotin	0.05 g.
Distilled Water	50 ml

Results

The preliminary goal of our research was to study the effect that acetic acid had on the proteome of *Neurospora crassa*, when acetic acid was used as the sole carbon source. To analyze our hypothesis, we performed two dimensional gel electrophoresis (2-DGE) using proteins from *N. crassa* grown on either dextrose or acetic acid as a sole carbon source. Twelve percent polyacrylamide gels were used to perform these experiments and *N. crassa* protein grown on each carbon source was run in triplicate to ensure that the results were able to be reproduced. The pH range used was 5-8 and the orientation for the gels when being observed is an increasing pI, from right to left, and the molecular mass decreases from top to bottom.

The initial protein profiles were the mini format 7 cm gels using IPG strips with a pH range of 5-8. These gels were run using 2-DGE and stained using coomasie blue stain in order to visualize the protein profiles of *N. crassa* grown on each carbon source. The resulting min format 7 cm gels are shown in Figures 4 and 5. Figure 4 shows the protein profile on 3 mini format 7 cm 2-D gels, in triplicate from the *N. crassa* tissue grown on 2% dextrose. Figure 5 shows the protein profile on 3 mini format 7 cm 2-D gels, in triplicate from the *N. crassa* tissue grown on 0.5% sodium acetate.

Once the protein profiles for dextrose and acetic acid were examined and protein expression was deemed acceptable and uniform across the min format 7 cm gels, the large format 17 cm gels were used to allow for greater visualization and analysis. The second set of protein profiles were generated using large format 17 cm gels using IPG strips with a pH range of 5-8. Each gel was run using two-dimensional gel electrophoresis and was stained for visualization using SYPRO® Ruby Red in conjunction with the Bio-Rad® XRS Chemidoc imaging system
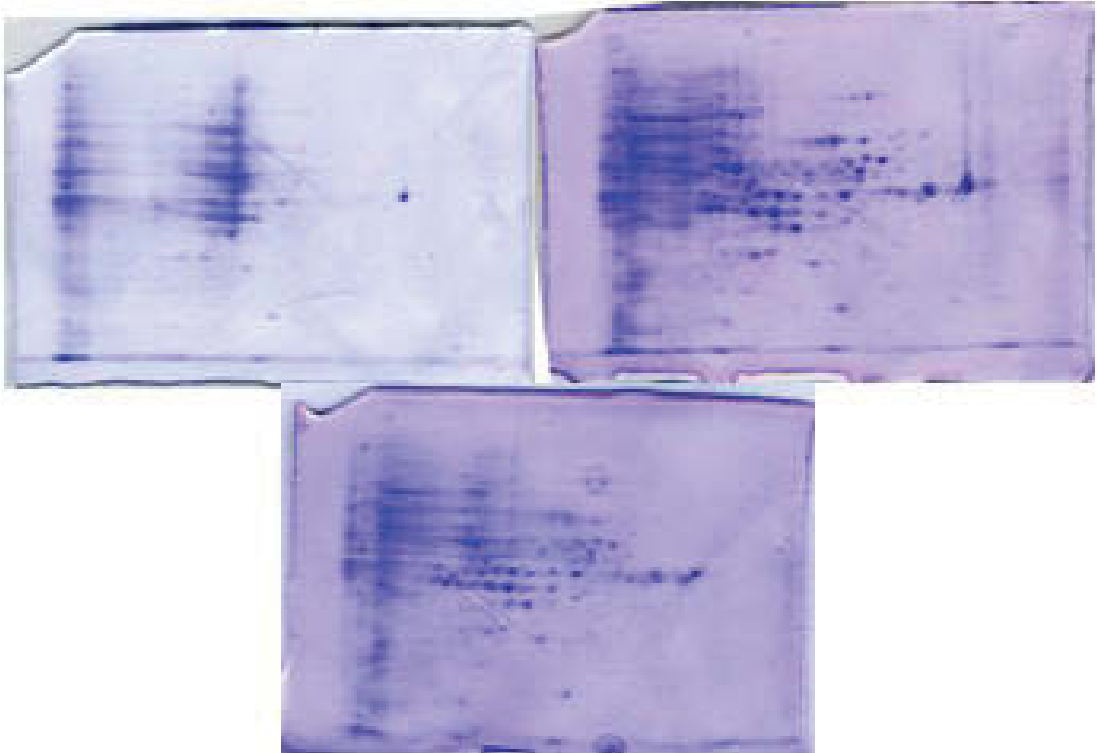
Figure 4. Protein profiles of *N. crassa* gown on Vogel's media plus 2% dextrose run on mini format 7cm 2-DGE stained with coomasie blue.
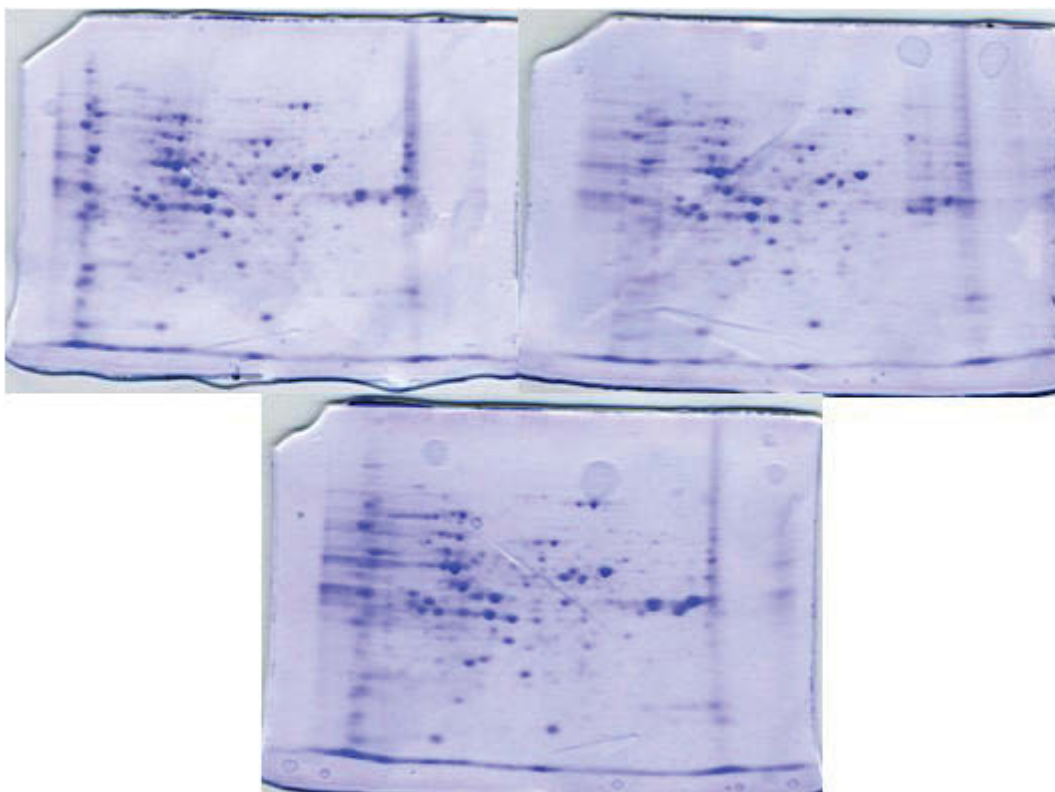
Figure 5. Protein profiles of N. crassa grown on Vogel's plus 0.5% Sodium acetate on mini format 7cm 2-DGE stained with coomasie blue.

using PD-QUEST version 7.1 and are shown in Figures 6 and 7. Figure 6 depicts the large

format 2-DGE protein profile from the *N. crassa* tissue that was grown on 2% dextrose. Figure 7

shows the resulting large format 17 cm 2-DGE protein profile from *N. crassa* tissue that was

grown on 0.5% sodium acetate. Each of the gels, from both carbon sources, was run in triplicate

to again allow for the reproducibility of results. Once these gels were analyzed and a consistent

protein profile was observed for each carbon source the large format 17 cm 2-D gels, were then

further analyzed using the PD-QUEST™ version 7.1 software.

To analyze each protein profile, we created individual matchsets using PD-QUEST™

comparing the two carbon sources to evaluate protein expression individually from each

experimental condition. The individual matchsets reflect the protein profiles of *N. crassa* grown

on each carbon source and electrophoresised on a 17 cm 2-D gel that was performed in triplicate

and a master (white) gel image. The master gel image shows a visual representation of the 3

experimental 2-D gel images combined to make a hypothetical "4th" master gel that has protein

spots that show the most reproducibility amongst the three experimental 2-D gels. The

individual matchsets created using PD-QUEST™ for 2% dextrose and 0.5% sodium acetate are

shown in Figures 8 and 9. Figure 8 shows the individual matchset created from triplicate gel

images from *N. crassa* using only 2% dextrose as a sole carbon source. Figure 9 shows the

individual matchset created from triplicate gel images from *N. crassa* using only 0.5% sodium

acetate as a sole carbon source.

Once each carbon source's individual matchset was analyzed and protein spots were

added to the master to show the best representation of the 3 individual carbon source 2- D gels,

higher level matchsets were created to allow for the comparison of master gel images between

the two different carbon sources: dextrose and acetic acid. This higher level master matchset

comparison then generated a new master gel image along with a side-by-side comparison between the master matchset gel image for dextrose and the master matchset gel image for acetic acid as displayed in Figure 10. From the higher level master matchset generated between dextrose and acetic acid, it was calculated that a total of only 121 protein spots were found to be similarly expressed on both dextrose and acetic acid. A total of 257 unique protein spots were found to be differentially expressed on acetic acid as a sole carbon source. Figure 11 shows a Venn diagram, which depicts the total number of unique protein spots in dextrose, the total number of protein spots in acetic acid, and the total number of proteins differentially expressed by both carbon sources.

Of the 257 unique acetic acid protein spots, 30 were chosen that had high reproducibility amongst all 3 triplicate acetic acid gels to be sent to Ohio State University for capillary-liquid chromatography-nanospray tandem mass spectrometry (Nano-LC/MC/MS). The 30 chosen unique spots were placed under an ultraviolet light source and the spots were manually removed using the sterile round-bulb end of a Pasteur pipette. These spots are currently being stored at -80° C in a 5% acetic acid solution, waiting to be sent out for identification.
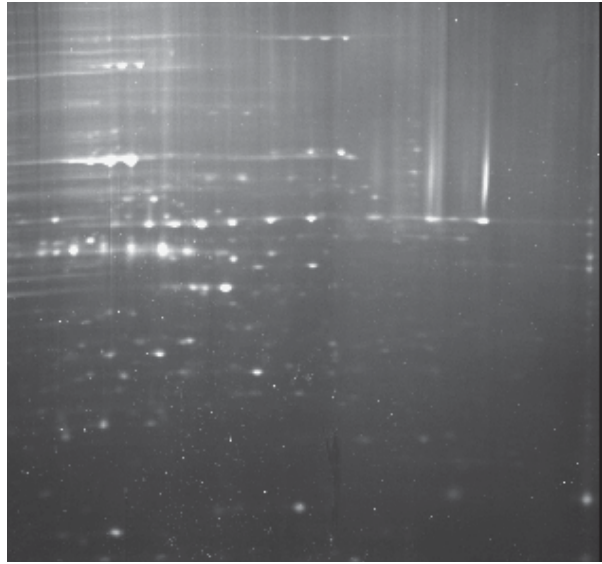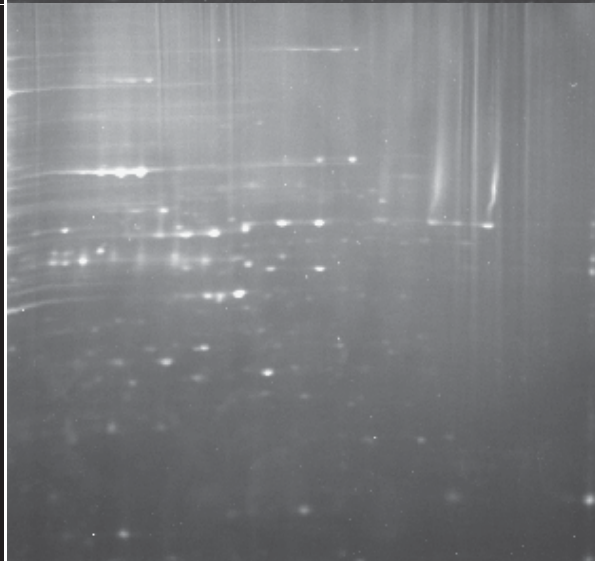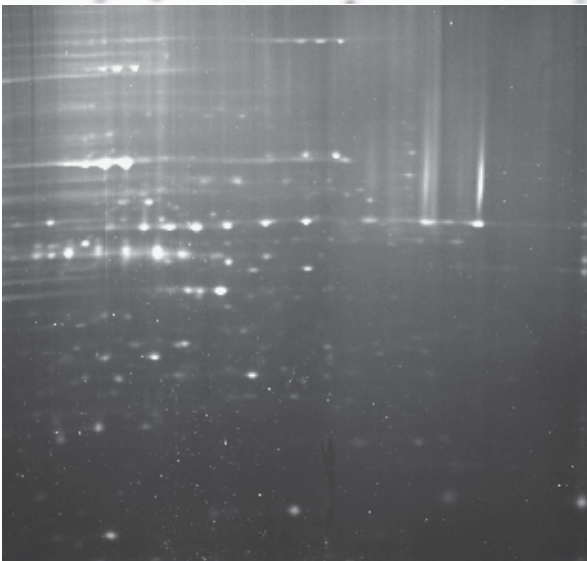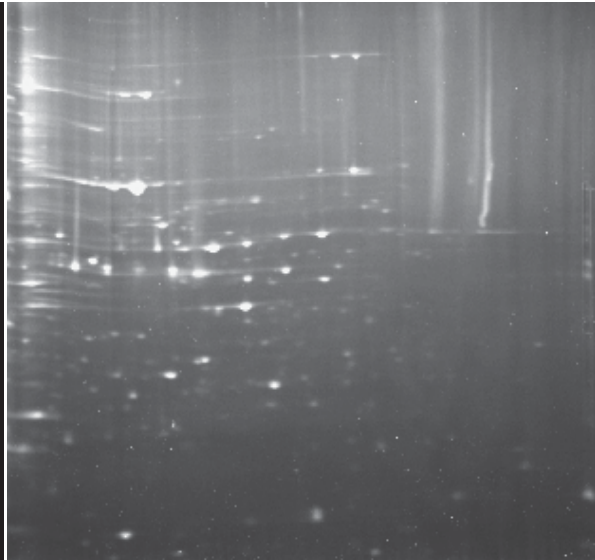
Figure 6. **Protein profile of *N. crassa* grown on Vogel's plus 2% dextrose on large format 17cm 2-DGE stained with SYPRO® Ruby Red stain.**
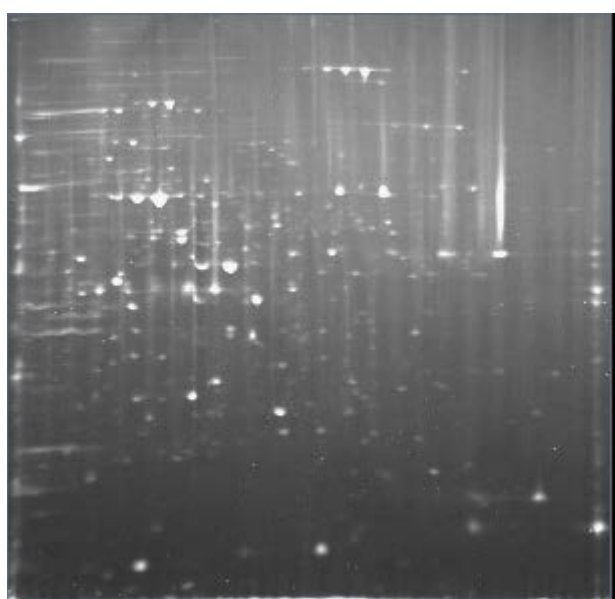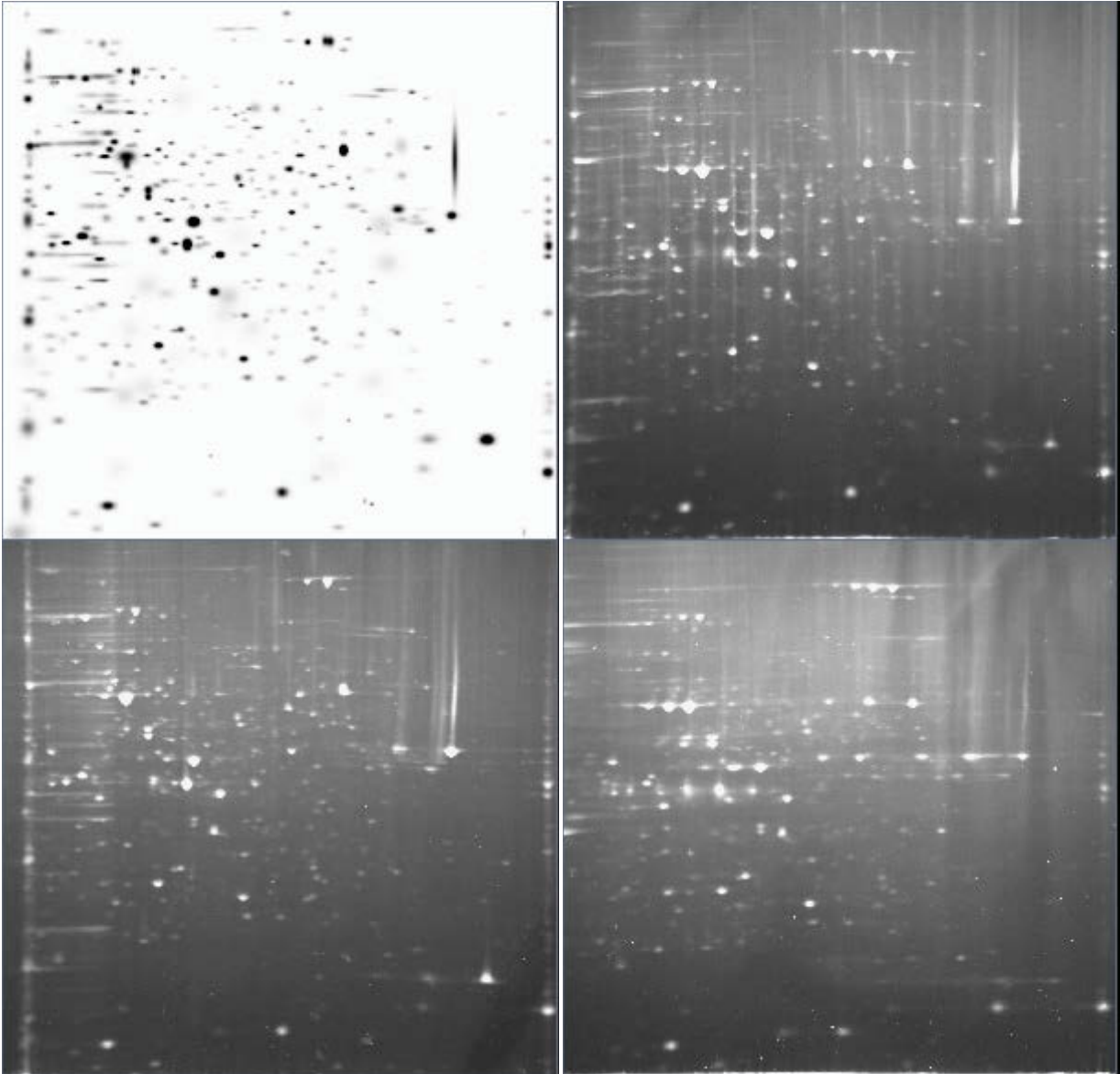
Figure 7. **Protein profile of *N. crassa* grown on Vogel's plus 0.5% sodium acetate on large format 17cm 2-DGE stained with SYPRO® Ruby Red stain.**

Figure 8. Protein profiles of N.crassa grown on Vogels plus 2% dextrose on large format 2-D gels (three dark gel pictures) with the constructed Dextrose gel master matchset (white w/ black spots).
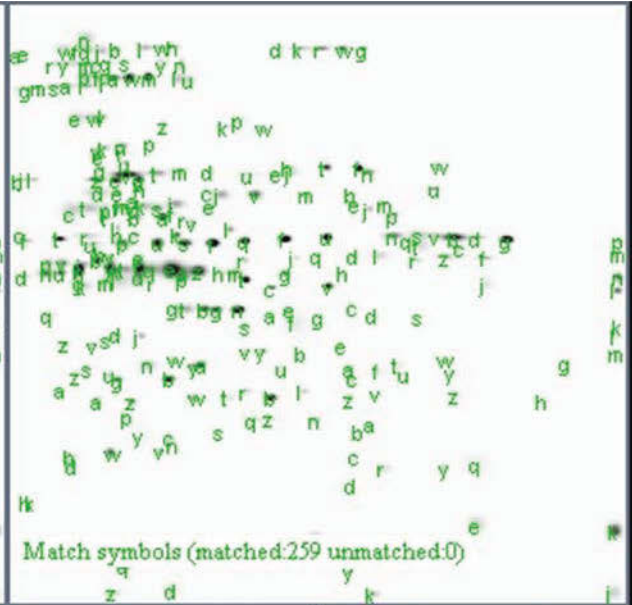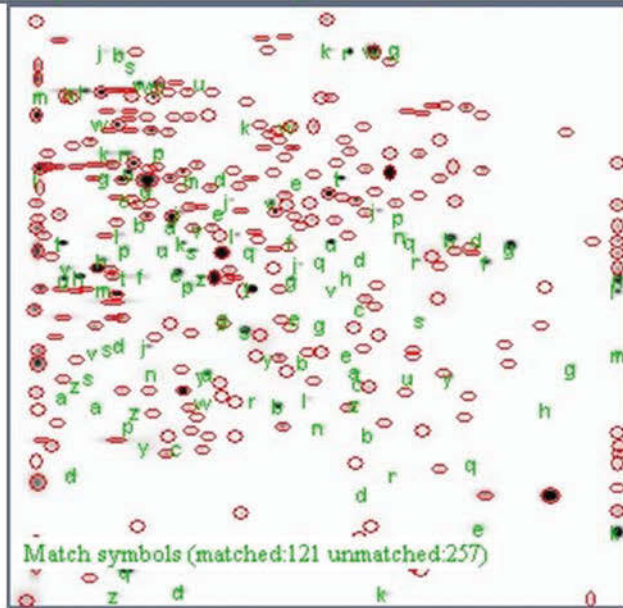
Figure 9. Protein profiles of N.crassa grown on Vogels plus 2% dextrose on large format 2-D gels (three dark gel pictures) with the constructed acetic acid gel master matchset (white w/ black spots).
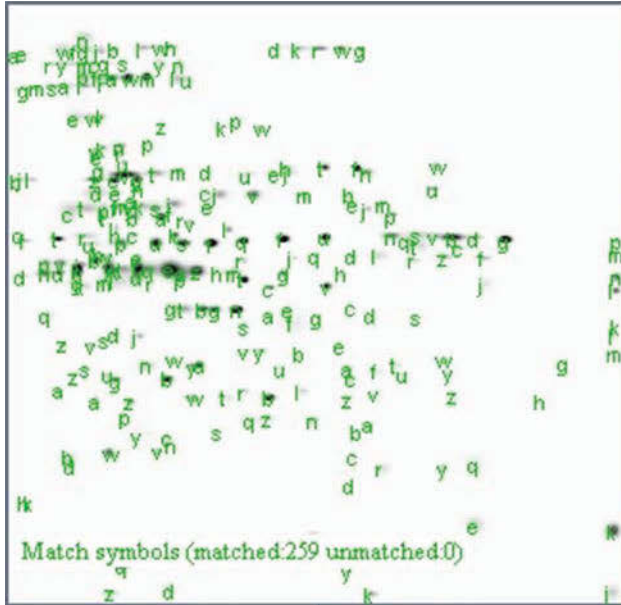
Master Matchset

Dextrose Matchset

**Acetic Acid Matchset**

Figure 10. Higher level matchset comparison between Dextrose master matchset (Upper left) and acetic acid master matchset (bottom middle). Red circles stand for unmatched protein spots in the acetic acid protein profile when compared to the dextrose protein profile. Green circles are matches between the acetic acid protein profile and the dextrose protein profile.
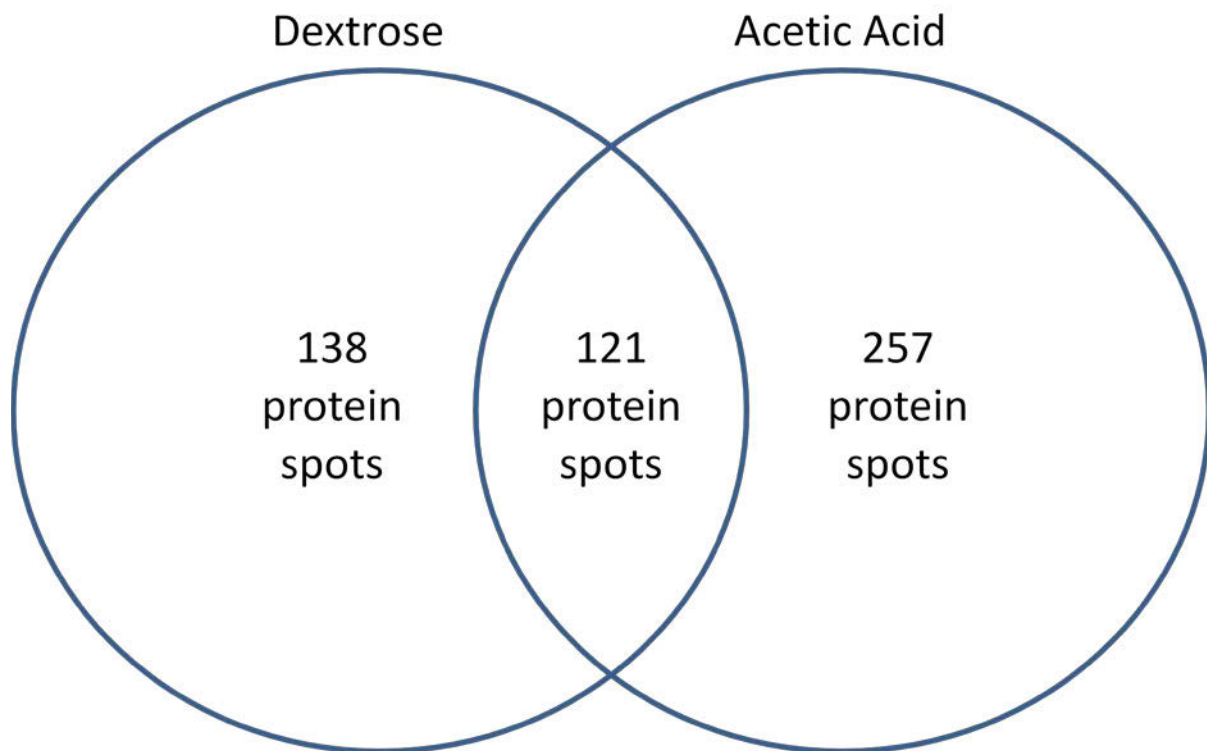
Figure 11. Venn diagram showing total number of proteins spots expressed on each separate carbon source and the total number of protein spots shared between both dextrose and acetic acid.

Discussion:

Since the induction of proteomic work with *Neurospora crassa*, there has been little research into carbon source utilization and it's effect on the Neurospora proteome. The current study is one of the first studies to utilize 2-dimensional gel electrophoresis (2-DGE) to analyze protein expression patterns between preferred and non-preferred carbon sources and how the resulting expression patterns relates to metabolic products. The main goal of this study was to analyze and identify individual, as well as unique proteins from the most preferred carbon source that *N. crassa* uses, dextrose, and compare and contrast it with one of the most non-preferred carbon sources, acetic acid.

In our study, *N. crassa* protein was extracted from mycelia grown on 2% dextrose and 0.5% sodium acetate. In earlier studies it was shown that *N. crassa* is able to use a variety of carbon sources, both preferred and non-preferred, to undergo normal growth, maintenance, and metabolism, though in the former a variety of different pathways are activated to undergo gluconeogenesis. (Schmit, 1976.) Currently there are minimal studies that involve protein profiling to attempt to identify gene expression in regard to metabolism.

Initially, we were unsure of what to aspect in regards to the protein profile of acetic acid. It was known from previous studies that the majority of identified genes in response to acetic acid utilization as a sole carbon source had to deal primarily with the pyruvate dehydrogenase complex, but we did not know which proteins would be unique, up-regulated, down-regulated, or differentially expressed when acetic acid was used as a sole carbon source in comparison to the dextrose protein profile. (Okumura, 1979; Kuwana, 1979; Santosa, 1990.)
To allow for protein expression, we used 2-dimensional gel electrophoresis (2-DGE) to examine the individual protein expression in *N. crassa* grown on the two carbon sources used: 2%

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dextrose and 0.5% sodium acetate. Once large (17cm) gels were run we analyzed the results using PD-Quest[TM], looking at the protein expression patterns in triplicate. Overall the generated protein profiles exhibited a vast difference in expression between carbon sources. In the dextrose protein profile, the total number of valid spots was determined to be 259 individual protein spots. While in the acetic acid protein profile, the total number of valid protein spots was determined to be 378. These results were not expected, due to that acetic acid is the non-preferred carbon source and protein activation and should exhibit little protein expression, but the observed protein expression is almost double the observed protein expression of the preferred carbon source, dextrose. Previous research concerning protein expression with non-preferred carbon sources yields conflicting results. In the previous study, using the non-preferred carbon sources quinic acid and glycerol, overall protein expression was greatly reduced when compared to the protein expression present in dextrose. (Allen, 2010.) Also in terms of protein expression patterns, there was little similarity in pattern when comparing dextrose and acetic acid. Again, this conflicts with a previous study that used the non-preferred carbon sources glycerol and quinic acid, which showed comparable similarity to the dextrose protein profile. (Allen, 2010.) Additionally again our results do not follow what is consistent with other previous studies, in that more genes are up-regulated in the prescence of minimal media containing only sucrose, while the up-regulation of metabolic genes occurs at a lower degree when grown in less-preferred carbon source, which is the exact opposite of what we have found. (Aign, 2003.) Now whether these proteins are starvation genes or not is still to be determined through mass spectrometry analysis.

Our research, also displays unique protein patterns between the two carbon sources, in addition to the different protein expression numbers. The unique protein patterns observed using

2-DGE is due to the specific up-regulated and down-regulated proteins being expressed when *N.crassa* is shifted from the preferred carbon source, dextrose, to the non-preferred carbon source, acetic acid. This is expected, due to that each different carbon source used undergoes specific metabolic pathways and enzymes to achieve gluconeogenesis. In the case of dextrose, the Krebs's cycle is used and in the case of acetic acid, the glyoxylate cycle is used to achieve this goal. This follows with previous research performed by Logan and associates, in their research dealing with sucrose and quinic acid, in that the pattern of gene expression in *N. crassa* is altered when shifted from sucrose to quinic acid, which allows for *N. crassa* to use a less preferred carbon source for metabolism and growth. (Logan, 2007.)

To conclude our study, through the use of PD-Quest[TM], we were able to identify 30 unique and differentially expressed proteins in the acetic acid protein profile. Each of the 30 proteins was found in triplicate and a similar level of protein expression was noted to further validate our findings. The identity of these specific proteins and how they relate to acetic acid metabolism, when used as a sole carbon source, is still to be determined through mass spectrometry analysis.

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