Differential Proteomics of *Penicillium marneffei*: A dimorphic fungal pathogen

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ABSTRACT

Penicillium marneffei can be characterized as an opportunistic pathogen. It is usually only pathogenic to individuals who are immunodeficient. The dimorphism of *P. marneffei* is what makes it an interesting organism to study because of its relationship to disease. In the lab, it grows as a mold at 25°C, but when isolated from an infected person or rat, only the single-celled yeast form is seen. This phenomenon can be simulated in the lab by growing cultures at room temperature (25°C) and body temperature (37°C). The explanation for this dimorphic switch can be arrived at by the study of changes in the protein makeup of the fungal cells. This proteomics approach to analyzing *P. marneffei* could illuminate the specific change necessary for dimorphism.

Growing the cultures at these two different temperatures and respectively isolating the protein is the starting point for this proteomics approach to studying the dimorphic growth of *P. marneffei*. Two dimensional gel electrophoretic analysis of the protein yields protein profiles of the cultures. Comparative analysis of the profiles identify proteins that are distinctly different between the two morphologies. These spots can be excised and the proteins can be sequenced using mass spectrometry.

The differential protein expression and subsequent identification provides a way in which to study the molecular mechanisms of *P. marneffei*. Using *P. marneffei* as a model for other infectious organisms that may share similar molecular mechanisms will aid in the understanding of dimorphism and its possible link to pathogenicity.

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List of Abbreviations

2-D 2-dimensional

2-DGE 2-dimensional gel electrophoresis

ACN acetonitrile

AIDS acquired immune deficiency syndrome

ambic ammonium bicarbonate

APS ammonium persulfate

BSA bovine serum albumin

CHAPS 3-[(3-Cholamidopropyl)dimethylammonio]propanesulfonic acid

DTT dithiothreitol

HIV human immunodeficiency virus

IAA iodoacetamide

IEF isoelectric focusing

IPG immobilized pH gradient

M molar

min minutes

MS mass spectrometry

MSB modified sample buffer

MW molecular weight

pI isoelectric point

RB rehydration buffer

RPM revolutions per minute

s second

SAB Sabouraud dextrose broth

SDS sodium dodecyl sulfate

SDS-PAGE sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

SOD superoxide dismutase

TCA trichloroacetic acid

TE Tris-EDTA

TEMED N,N,N',N,-tetramethylethylenediamine

TGS tris-glycine-SDS

TIC total ion count

TOF time-of-flight

Tris Trizma buffer

UV ultraviolet

V volts

Vhr. volt hour

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CHAPTER ONE:

Introduction

The Introduction:

Fungi are very diverse and interesting organisms. They are important in medicine and in public health. In recent years, there have been increasing incidences of infections due to pathogenic fungi (20). These pathogenic fungi have acquired the ability to enter and survive in a host. Often times, the pathogenicity of the fungus is thought to be related to dimorphism, that being the ability to grow in two different forms following a change in environment (20). *Penicillium marneffei* is one in this category.

Penicillium marneffei is endemic in Southeast Asia, especially Thailand, preying mostly on the immunocompromised individuals in this area. The largest group of immunocompromised individuals in this area is that whose members are infected with the acquired immunodeficiency syndrome (AIDS) and its predecessor, the human immune deficiency virus (HIV) (4). Penicilliosis marneffei, the infection caused by *P. marneffei*, was not recognized until greater numbers of HIV and AIDS patients showed signs of the disease. These include fever, weight loss, skin lesions and irregularities in respiration and digestion (7). *P. marneffei* was first discovered in infected bamboo rats in 1956, but it was not until about 1985 and an increase in the number of HIV and AIDS cases, that it was prevalent as a human pathogen (4, 7, 11). From 1988 to 2000, 1600 AIDS patients were infected with *P. marneffei*. With continued increase in HIV and AIDS cases, and 10% of these becoming infected with *P. marneffei*, by 1992, penicilliosis marneffei was classified as an AIDS-related indicator disease in Southeast Asia (4, 11, 14). In addition, *P. marneffei* is the third most common opportunistic pathogen in AIDS patients (4, 8, 11, 19).

Originally, it was thought that the bamboo rat served as a reservoir for *P. marneffei*. However, since there is little contact between humans and bamboo rats, and as they are rarely taken as food, this is unlikely (13). Yet, those individuals who are immunodeficient and more frequently exposed to animals, plants and soil are at an increased risk of developing penicilliosis marneffei (4). Therefore, the bamboo rat and the human are infected by a common environmental source (11). At the present time, the mode of transmission of *P. marneffei* has not been described. What is known is that *P. marneffei* infects its victims with airborne spores called conidia. These conidia enter the body through inhalation (4, 7, 20). Upon entering the comfortable body temperature of 37°C, this infectious agent morphs into the pathogenic yeast-like agent and causes disease (20).

The Invasion:

As previously mentioned, the conidia enter the body through respiratory passages. The first cells to respond to the invader are the pulmonary alveolar macrophages found in the lungs (15, 19). Although the macrophages engulf the conidia, the switch to the yeast phase is not prevented and the pathogenic form prevails inside these immune cells. In fact, the conidia convert into hyphae extracellularly and do not change into the yeast form until in the macrophages and thereafter, the hyphal network disappears (15, 19, 20) According to Roilides *et. al.*, macrophages change in at least three ways upon infection with *P. marneffei*. There is phagocytosis by the macrophages, where the conidia are allowed to morph into the yeast form, an oxidative burst producing measurable amounts of superoxide anion (O_2^-) and ultrastructural changes (19). The cell-mediated immune response in healthy individuals is the primary response and seems to be successful in

eradicating the disease. However, *P. marneffei* yeast cells are capable of dividing and killing macrophages in immunocompromised individuals and use this ability as a way to spread through the host (20).

The Switch:

Herein lays the interesting fact about *Penicillium marneffei*. Fungi usually grow either as filamentous mold cells or as yeast-like cells and are classified as such (2). Members of the Penicillia take the form of filamentous mold cells (4, 5). However, *P. marneffei* can switch from one growth state to the other. At 25°C it grows as the multinucleate filamentous cells with septate hyphae and apical extension and branching (Fig. 1.1a). At 37°C it grows as uninucleate single cells, resembling yeast and dividing by fission (Fig. 1.1b) (2, 10). This dimorphic nature makes *P. marneffei* very distinctive (12). Even more interesting is that it is the only species of the 150 among the Penicillia to portray this characteristic (2, 6, 7, 13). Upon inhalation of non-*P. marneffei* species, filamentous cells are seen instead of a transition to yeast-like cells (4). However, when *P. marneffei* is inhaled, it does convert into the yeast-like phase (4). This temperature sensitivity is of great interest because the switch to the pathogenic form requires incubation at 37°C, or body temperature. The virulence of *P. marneffei* depends on its ability to survive at 37°C in the yeast phase (11). Other Penicillia that only grow as a mold at 37°C rarely exhibit the capability to cause disease.

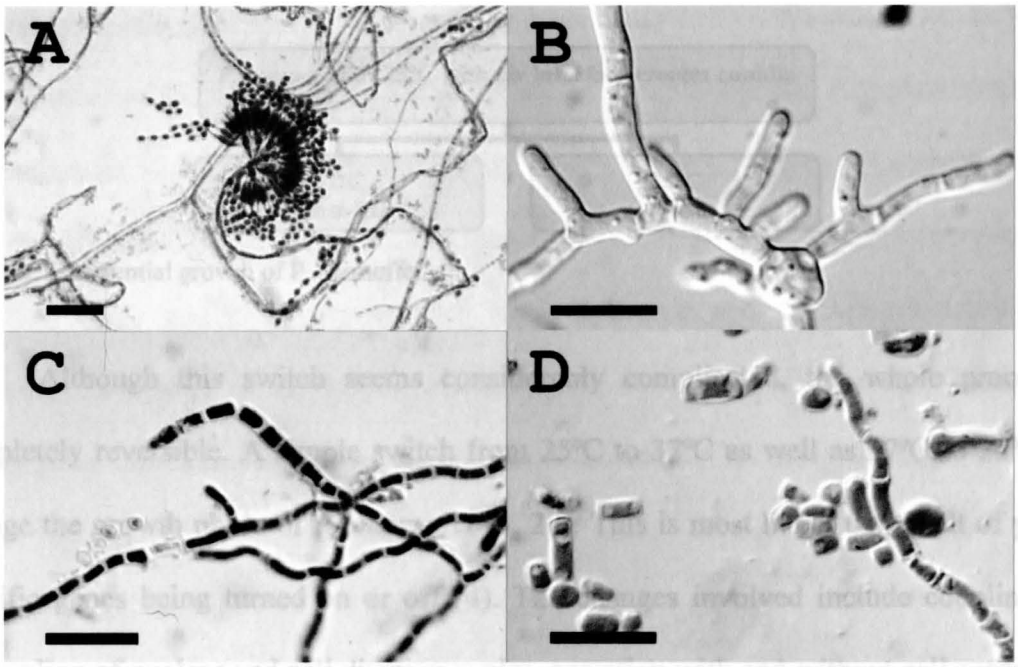


Fig. 1.1: Conidiophores in the mold phase produce conidia (a). Conidia incubated at 37°C transition into the yeast-like form at 24 hr, (b) form septa for later division by fission (c) and after a longer period of growth form into single-celled arthroconidia (d). (12)

To see the morphological changes unique to *P. marneffei*, the dormant spore is the initiating factor. At 25°C, *P. marneffei* grows by asexual reproduction (conidiation). This filamentous growth consists of multinucleate hyphae that, when exposed to air, differentiate to form asexual reproductive structures (conidiophores). These conidiophores produce the uninucleate spores (conidia) (2, 16). These spores can then be persuaded simply by a change of temperature to undergo filamentous vegetative growth at 25°C or unicellular yeast-like growth at 37°C (Fig. 1.2) (20). The intracellular pathogen is the growth found at 37°C. Specifically, at 37°C, the process of arthroconidiation occurs. In this type of cellular development septation and nuclear division are coupled (2, 16). In addition, cell size is decreased and the double septa that are found between cells allow cell separation and fission, producing uninucleate yeast cells (Fig. 1.1c-d) (2, 5, 13).

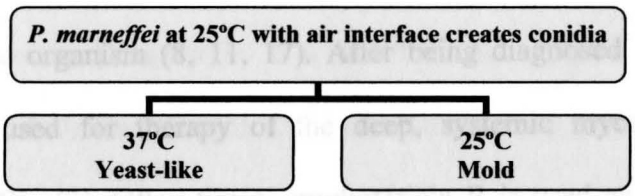


Fig. 1.2: Differential growth of P. marneffei

Although this switch seems considerably complicated, the whole process is completely reversible. A simple switch from 25°C to 37°C as well as 37°C to 25°C can change the growth phase of *P. marneffei* (4, 20). This is most likely the result of phase-specific genes being turned on or off (4). The changes involved include coupling and uncoupling of nuclear and cell division cycles, septation with and without cell separation, and directional and branching growth polarity (16, 20). The regulation of polarization alone is a drastic change for a cell. General fungal growth and development requires regulated changes in polarized growth (22). Therefore, dimorphic switching requires even more regulation of polarized growth in addition to regulation of morphology. Due to all of these required changes, the conidia must undergo a certain period of growth before gaining the ability to arthroconidiate (20).

The Treatment:

Fortunately for those immunocompromised individuals at risk of becoming infected with *P. marneffei*, there are very effective treatments to control the infection which is fatal if untreated. The first step to treatment of this disease is correct identification. By observation alone, it is difficult to identify penicilliosis marneffei because the symptoms present are similar to other types of infections presented by its immunocompromised, HIV infected host (5). Patients must be correctly diagnosed before an effective therapy can be chosen (8). Penicilliosis marneffei can be diagnosed using the

polymerase chain reaction (PCR) to amplify hypervariable DNA domains for molecular identification of the organism (8, 11, 17). After being diagnosed, a typical antifungal treatment can be used for therapy of the deep, systemic mycosis associated with penicilliosis marneffei (7). Often times, amphotericin B is used as the primary therapy and then other antifungal agents such as itraconazole and ketoconazole follow as maintenance therapy (4, 5, 7, 10). In one study, six out of eight patients responded favorably to this therapy with the symptoms disappearing in approximately two weeks (10).

The Purpose:

Considering the treatable nature of penicilliosis marneffei, the interest in it lies not in finding better treatments or diagnostic abilities, but in its relatedness to other organisms and it serving as a model for other pathogenic fungi. At the present time, there is a lack of understanding of fungal pathogenesis, the mechanism of disease (20). By studying *P. marneffei*, a pathogenic fungus, a better understanding may be in the near future (14). This is especially the case since fungal pathogenesis is often associated with dimorphism, another characteristic of *P. marneffei* (16).

Over the last decade, *P. marneffei* has not been the only fungal pathogen on the rise. Infections due to *Histoplasma capulatum, Candida albicans, Cryptococcus neoformans, Coccidioides immitus* and *Aspergillus fumigatus* have also increased in number within the immunocompromised population (16). For this reason, it is important to have a model for studying possible causes of pathogenicity. *P. marneffei* is a perfect candidate for this model because of its dimorphic nature and the precise mechanisms it uses for controlling the switch (2). Among the fungi, those that are dimorphic are

generally also pathogenic, having one form while outside the host and one form during invasion (2, 7). By using *P. marneffei* as a model for dimorphic fungal pathogenesis, it will be possible to study the specific molecular mechanisms that allow the dimorphic switching and therefore relay the virulence of the organism (4, 11). In turn, these processes may be linked to the acquisition of pathogenicity and virulence of other organisms (20). This seems a likely relation due to previous studies that have shown similarity between the internal morphology of *P. marneffei* and that of other pathogenic dimorphic fungi (7).

The Mechanisms:

There must be regulation of phase-specific genes involved in the dimorphic switching of *P. marneffei*. It has been postulated that some of these genes may be involved in energy metabolism (4, 11). Some studies have already been completed on specific genes to see how they influence this phenomenon. The genes studied were chosen from other organisms similar to *P. marneffei* because some genes are conserved between programmes and across species in one way or another (20). One of these organisms is *Aspergillus nidulans*, a monomorphic ascomycete fungus that incorporates conidiation mechanisms and involves a large multicellular structure, termed a conidiophore, through the differentiation of several specialized cell types (2). It was found that the *abaA* gene of *A. nidulans* can complement conidiation defects in the *P. marneffei* mutant. *abaA* as well as *brlA* have been found to play an important regulatory role in the conidiation of both organisms (2, 20). Although *abaA* show such an important role in conidiation, it does not seem to have an important role in yeast growth of *P. marneffei*. However, there may be another factor that works with *abaA* at 37°C to

regulate the nuclear division cycle (2). In addition, *brlA* is required in *A. nidulans* for the switch to the budding program.

There are many other genes and proteins that are of interest in the study of *P. marneffei*. The regulation of conidiation may involve *stuA* (6, 20). The dimorphic switching may incorporate cdc42p which is known to be involved in cytokinesis and polarized growth (22). Tup1 has been shown to control dimorphism in *Candida albicans* and asexual development in *Aspergillus nidulans*. The *P. marneffei tup1* homologue of *tupA* was found to repress both asexual development and yeast cell morphogenesis (16). This allows the mold form to become predominant. A large number of thiodiester-mediated non-ribosomal protein synthesis or reduced carbon-chain carboxylate intermediates could cause a modulation of the immune response allowing *P. marneffei* to infect its host by evading or modifying the immune response. Any or all of these mechanisms may be related to the pathogenicity of *P. marneffei*.

The Proteomic Approach:

Obviously, since the focus of the studies of *P. marneffei* thus far have been on gene expression at the RNA level, the next logical step is to look at the protein population that results from genomic expression. Observations of the protein population present in the different forms of *P. marneffei* can be made through the use of proteomics. Proteomics is the study of the protein constituents of a biological sample on a large scale (1). The proteome of an organism is the protein complement of its genome. Easily explained, it is the function aspect of genomics. Proteomics provides a description of the molecular basis of (patho) physiological processes (9). The study of proteomics utilizes two-dimensional polyacrylamide gel electrophoresis (2DGE) to separate a heterogeneous

protein mixture (9). First, the proteins are separated by their isoelectric points via electrophoresis on strips with immobilized pH gradients (IPG strips). This step is called isoelectric focusing (IEF). Next, the proteins are separated in the second dimension according to their molecular weights via polyacrylamide gel electrophoresis (SDS-PAGE). This two-dimensional approach to separating proteins creates a protein profile map of that sample. In addition, post-translationally and proteolytically modified proteins are distinctly separated from their 'parent' protein, which allows better identification and description of a gene product than the gene itself would (1). Subsequently, once protein maps have been obtained, individual spots (proteins) can be excised, digested and sequenced using mass spectrometry.

The isolated protein spot is digested with trypsin, which cleaves proteins specifically after lysines and arginines (25). The masses of these resulting peptides are measured by mass spectrometry and then correlated to amino acid sequences in databases (25). There are three steps to analysis with mass spectrometry. First, molecules are ionized in the ion source, then the mass analyzer separates them according to their mass-to-charge ratio and finally the separated ions are detected (25). After ionization, two spectra can be obtained by the use of a tandem mass spectrometer (26). The first spectrum is the result of the separated peptides originally cut by trypsin. The second spectrum is obtained from each previous peptide peak being further analyzed to acquire amino acid masses. These masses are fed into a database and the best fitting protein/s will be identified (Fig. 1. 3).

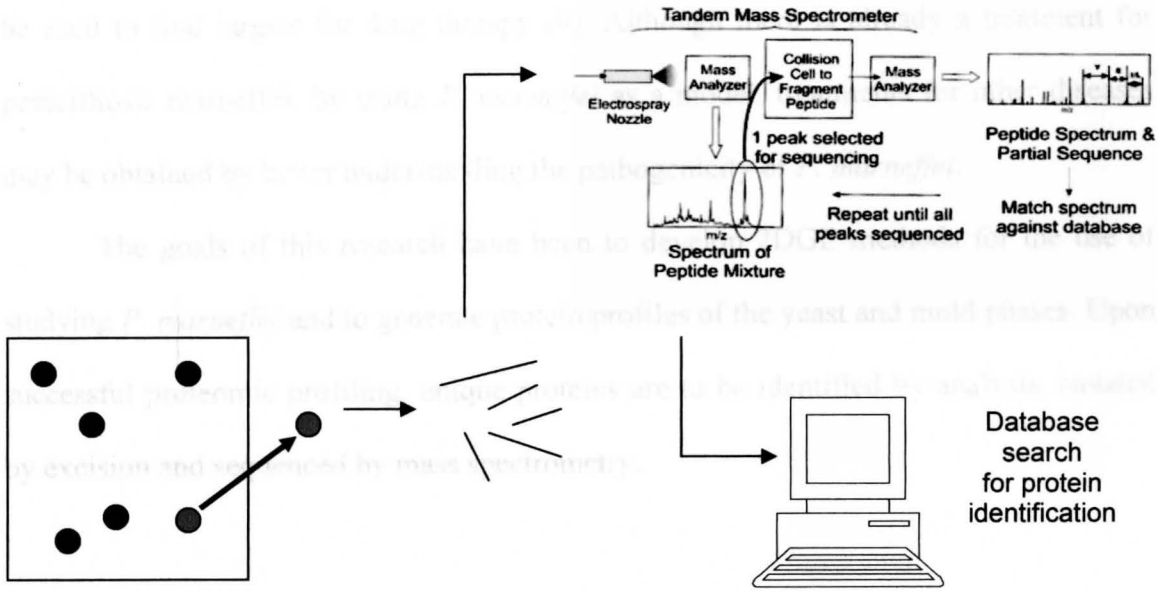


Fig. 1.3: Steps to protein identification. 2-DGE, spot cutting, trypsin digestion, mass spectrometry and database search. (TMS courtesy of http://chemlearn.chem.indiana.edu/bcce/BioanalApplic Electronic Spectroscopy.htm)

Proteomics becomes important in the study of *P. marneffei* because it is the metabolism, growth mechanisms and physiology that are of interest. Proteomics can lead researchers to keys in the important changes that occur during the phase transition. Metabolic signaling pathways can be better understood when linking the proteome to the genome by way of relating proteins identified with gene expression and post-translational modification (1, 3). Not only does exploring the proteome help in the understanding of the disease, but also in the possible therapies for the disease at hand (9). Genetics has given small insights on molecular mechanisms, but genes can produce many different products through different splicing events. Moreover, the protein can undergo post-translational modification that can change its function as well as result in many different forms of one protein. Any of these seemingly small changes or a conjunction of several changes could lead to the dimorphic switching seen in *P. marneffei*. Proteomics can also

be used to find targets for drug therapy (9). Although there is already a treatment for penicilliosis marneffei, by using *P. marneffei* as a model, treatments for other diseases may be obtained by better understanding the pathogenicity of *P. marneffei*.

The goals of this research have been to develop 2DGE methods for the use of studying *P. marneffei* and to generate protein profiles of the yeast and mold phases. Upon successful proteomic profiling, unique proteins are to be identified by analysis, isolated by excision and sequenced by mass spectrometry.

CHAPTER TWO:

Methods

Cell Growth and Collection

Penicillium marneffei, F4 strain, was grown on potato dextrose agar (PDA) 150 cm^2 cell culture flasks with vented caps at 25°C for 10 days. On the tenth day, the culture was eluted off of 2 plates using 10 mL of sterile water and a cell scraper. This culture solution was then filtered through glass wool between screened caps at 1000 rpm for 15-30s. This filtered out any hyphae and allowed only conidia to be present in the filtrate. The conidia in this solution was counted using a hemocytometer to allow for inoculation of 50 mL of Sabroud dextrose broth (SAB) in 500 mL Erlenmeyer flasks at 1 x 10^7 conidia/mL. The flasks were incubated in either a 25°C orbital air shaker or a 37°C orbital water bath for 24 hr. The cells were collected by centrifugation at 15,000 rpm for 15 min at 4°C, followed by two Tris-EDTA buffer (TE) (10 mM Tris, 1 mM EDTA, pH 8.0) washes centrifuged at 15,000 rpm for 15 min at 4°C.

Homogenization and Sample Preparation

Originally, a sonication protocol was used to lyse the cells and release their proteins. First, a 25 mg wet pellet of cells was obtained and 250 µL hot Tris-SDS buffer (100mM Tris-HCl, pH 7.0 + 1% SDS) was added. The cells were sonicated 3 x 30s, cooling in an ice slurry between sonication times. The sample was boiled for 5 min and then cooled in an ice bath for 5 min. The solution was diluted with 250 µL Modified Sample Buffer (MSB) (2M thiourea, 7M urea, 4% w/v 3-[(3-Cholamidopropyl)dimethylammonio]propanesulfonic acid, CHAPS, 1% w/v dithiothreitol DTT, 2% carrier ampholytes pH 3-10) and shook on a paddle shaker at room temperature for at least one hour. The solution was then spun at 16,000 x g, for 20

min at 4°C and the supernatant was transferred to a new tube which could then be frozen at -80°C (18).

After limited success with this protocol, a bead beat protocol for homogenization was adopted. This protocol involved obtaining a 400 mg wet pellet of cells. To this was added an equal amount of acid-washed glass beads and 800 µL lysis buffer (20mM Tris-HCl, pH 7.6, 10mM NaCl, 0.5 mM deoxycholate, 40µL/mL of protease inhibitor cocktail). The cells were then bead beat in a Mini-BeadBeater® (Biospec, USA) at 50 rpm for 4 min in 30s increments, cooling in an ice bath in between for 30s. The resulting slurry was centrifuged at 6000 x g for 10 min at 4°C. The supernatant was transferred to a pre-weighed 1.5 mL microcentrifuge tube and proteins were precipitated using 20% v/v trichloroacetic acid (TCA). These tubes were iced for 20 min and then centrifuged at 6000 x g for 20 min at 4°C. The supernatant was discarded and the pellet was washed 3x with 500 µL cold acetone centrifuging at 3000 rpm for 1 min at 4°C. Vacuum centrifugation was used to dry the pellet which was then resuspended in MSB according to pellet weight (5-15mg pellet in 500 µL MSB or a 16-30mg in 750 µL MSB) and could then be stored at -80°C (21).

To receive even better results, the previous bead beat protocol for homogenization was slightly modified. After the TCA precipitation, the solution was centrifuged at 2000 x g for 20 min at 4°C. This allowed for a looser pellet to be obtained for better solubilization of proteins. Vacuum centrifugation was no longer used to dry the pellet, but simple air drying in a biological safety hood sufficiently dried the protein pellets without further packing the pellet, again allowing for easier resuspension in MSB.

For additional analysis, at time six cell pellets were homogenized at the same time and pooled in the resuspension step. Instead of using MSB to resuspend a pellet of a precise weight, 250μL Tris-SDS was added. If sufficient resuspension was not achieved, more Tris-SDS was added.

This pooled fraction was run through a liquid IEF, Rotofor® System (BioRad) for pH fractionation along a gradient of pH 3-10. The resulting 20 fractions were pooled into 3 subsequent fractions, 1-7 representing the low approximate pH fraction, 8-13 representing the middle approximate pH fraction and 14-20, representing the high approximate pH fraction (30).

A second TCA precipitation was used to precipitate the proteins from these pooled fractions. The protein pellets were washed and dried as previously stated. This time, the pellets were resuspended in 250 μL MSB with shaking. The resulting homogenate could be stored at -80°C.

Protein Quantification

A modified Bradford Assay (24) was used to find the concentration of protein in each sample. Each tube contained 80 μL water, 10-20 μL 0.1 M HCl, 10 μL 2DE buffer (8.4 M urea, 2.4 M thiourea, 5% CHAPS, 25 mM spermine base, 50 mM DTT) (32), 4 mL of Bradford dye (100mg Coomassie Brilliant Blue G-250 dissolved in 50 mL 95% ethanol, 100 mL 85% (w/v) phosphoric acid was added, solution was diluted to 1 L with water) and protein (24). A standard curve of known Bovine Serum Albumin (BSA) protein amounts of 10-40 μg was used to quantify the sample proteins. 5-10 μL of sample protein was added to the sample tubes. The absorbencies were recorded at 595 nm. With

the creation of the standard curve using Microsoft Excel, the sample protein concentrations were calculated. When duplicate samples were run of 5 and 10 μL, these values were averaged (24) (Fig. 2.1).

Iso-electric Focusing

Passive rehydration was used to prepare immobilized pH gradient (IPG) strips to separate proteins by isoelectric point. 125 μL rehydration buffer (RB; 8M urea, 1% CHAPS, 15mM DTT, 0.2% BioLytes (BioRad), 0.001% bromophenol blue) was used for 7 cm strips and 300 μL was used for 17 cm strips. MSB was used for rehydration of pH 7-10 strips with pH 7-10 buffer added at 1/100 volume of strips. The buffer was added to a tray and the IPG strip was laid over it, covered with mineral oil and placed on an orbital shaker for 12-24 hours. The volume of protein needed to load 100-150 μg protein (for 7cm) or 200-255 μg protein (for 17 cm) was calculated using the previously obtained concentration. Electrode wicks (dampened by Millipore water), sample and IPG strips were loaded into the focusing tray according to instructions provided by manufacturer (27). The IPG strips were again covered with mineral oil. The IPG strips (both 7 and 17 cm) were focused at 20°C starting at 250V for 15 min. Both use a linear voltage ramp. For the 7 cm strips, this occurs over 2 hr to reach 4,000 V and are focused at 40,000 V-hr. For the 17 cm strips, this occurs over 3 hr to reach 10,000 V and are focused at 60,000 V-hr. The strips are then equilibrated in equilibration buffer I (6 M urea, 2% sodium dodecyl sulfate (SDS), 0.375 M Tris-HCl pH 8.8, 20% glycerol, 130 mM DTT) and equilibration buffer II (6M urea, 2% SDS, .375 M Tris-HCl pH 8.8, 20% glycerol, 135mM iodoacetamide) for 10 min each with orbital shaking (27, 28).

SDS-Polyacrylamide Gel Eletrophoresis

The equilibrated strips were then rinsed with 1x Tris-Glycine-SDS buffer (TGS) (3.02 g/L, 18.8 g/L, 1.0 g/L) and sealed onto either 10% or 12% polyacrylamide gels (Table 2.1). Melted overlay agarose (0.5% w/v low melt agarose in 1x TGS buffer with bromophenol blue) was added on top and allowed to solidify. The gels were made with Bio-Rad systems, either single cast or multicast up to 12 gels. The gels were loaded into either a Bio-Rad Mini-Protean (7 cm) or Bio-Rad Protean II XL (17 cm). A constant current of 14-16 mA was applied to the 7 cm gels and of 10-24 mA was applied to the 17 cm gels. These were run until the dye front reached the bottom (28).

At this point the gel was removed from the gel plates and stained. For colorimetric staining the gels were placed in Coomassie stain (.25%w/v Coomassie brilliant blue R-250, 45% methanol, 45% H_2O, 10% acetic acid). Enough was added to sufficiently cover the gel while shaking for at least 1 hr. The stain was then removed and the gel was destained in high destain (40% methanol, 10% acetic acid) for one hour and then low destain (10% methanol, 6% acetic acid) until spots were visible against a light blue background.

For fluorescent staining, the gels were placed in fixing solution (40% methanol, 10% acetic acid) for 1hr. This solution was removed and SYPRO® Ruby protein gel stain (Bio-Rad, Inc.) was added to stain the proteins 4-24 hrs while shaking. SYPRO® Ruby was removed and destain (10% methanol, 6% acetic acid) was added for 2hr. At this point the destain was removed and the gels could be stored in water until analysis (28, 29). In later experiments, the destain step of SYPRO® Ruby staining was omitted and gels were simply rinsed in H_2O after stain was removed.

Imaging and Analysis

Stained gels were imaged using a Bio-Rad ChemiDoc XRS system. For SYPRO® stained gels, a trans UV lamp of wavelength 365 nm was used to visualize proteins. Coomassie stained proteins were visualized using white light.

PDQuest software (Bio-Rad, Inc.) was then used to analyze the gels. Using this system filters, matches and detects spots in all gels. Triplicate runs of yeast and mold gels were initially compared. Later, triplicate runs of molds were compared, as were yeasts. With much time completing manual comparisons, this created a master yeast gel and a master mold gel, allowing them to then be compared and matched (31).

Spot Excision and Protein Sequencing

Protein spots of interest were excised from the gel by using a sterile Pasteur pipette to cut and retrieve the gel piece from a gel illuminated by UV light. The gel piece was then expelled into a sterile microcentrifuge tube.

In Gel Digestion:

To destain the gel pieces, they were covered with 25mM ammonium bicarbonate (ambic) and vortexed for 10 min. The solution was removed and this process was repeated twice. Gel pieces were dried by vacuum centrifugation for 20 min.

To reduce and alkylate, the gel pieces were covered with 100µL DTT solution (10mM DTT in 25 mM ambic) and incubated at 56°C for 45 min. The sample was cooled to room temperature and the solution was removed. The gel pieces were then coved with 100µL IAA (55mM iodoacetamide in 25mM ambic), incubated at RT for 45 min in the

dark, washed with 100μL ambic and vortexed for 10 min. Dehydration was carried out by adding 100μL ambic/ACN (25mM ambic in 50% acetonitrile/water) and vortexing for 5 min. The dehydration was repeated once. The sample was then dried by vacuum centrifugation.

For the digestion of protein the gel pieces were covered with 50μL trypsin (12.5ng/μL trypsin in 25mM ambic) and incubated on ice for 45 min. More trypsin was added if it was fully absorbed. Solution was then removed and the gel pieces were covered with 100μL ambic and incubated at 37°C overnight. 10μL TFA (2% TFA) was added and the supernatant was removed after 1-2 min. This supernatant was saved in a siliconized tube. For peptide extraction, 30μL ACN/formic acid (50% CAN/5% formic acid) was added to the gel pieces and sonicated for 20 min. The supernatant was removed and placed in tube with other supernatant. The ACN/formic acid step was repeated once. The combined supernatant was concentrated by vacuum centrifugation for a final volume of 20μL.

Mass Spectrometric Analysis

Samples were sent to The University of Cincinnati, Department of Chemistry for sequencing by mass spectrometry using standard MS protocols to identify proteins.

Database Search

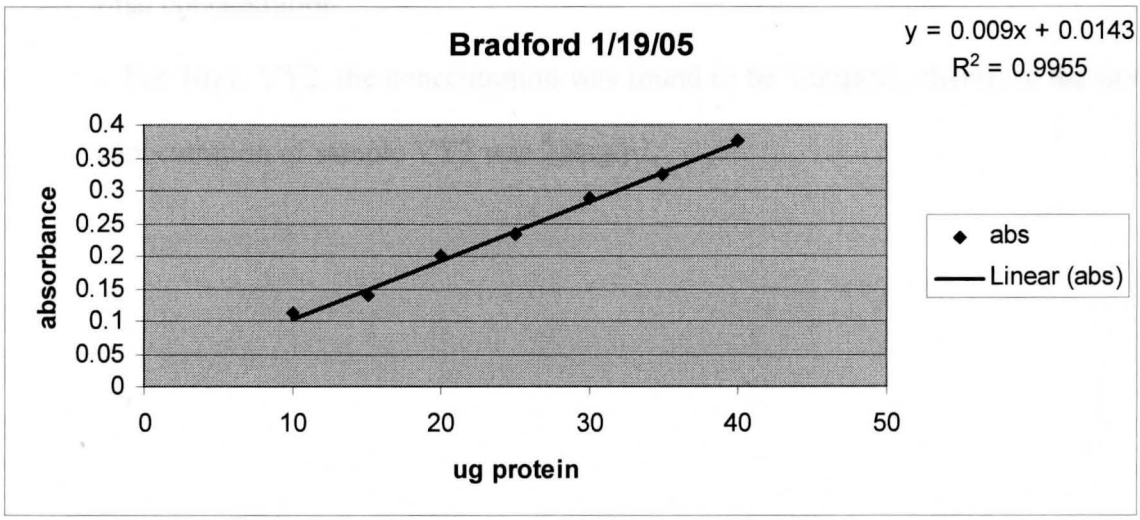
With the receipt of sequences from Cincinnati, a Mascot database search was run for protein identification (http://www.matrixscience.com).

Fig. 2.1 Example of Bradford assay. (a) Construction of standard curve. (b) Example calculations of protein concentrations

(a) BSA of 10.3 μg/μL was used to create the standard curve of 10-40 μg of protein

- 10μg BSA / (10.3μg/μL BSA) = .97 μL BSA solution

μg BSA	μL BSA solution	Absorbance
10	.97	.11067
15	1.5	.13919
20	1.9	.19968
25	2.4	.23296
30	2.9	.28872
35	3.4	.32473
40	3.9	.37667



(b) - The equation of the line is then used to translate the sample absorbencies to concentrations:

sample	abs
5ul V Y 2	0.14145
10ul V Y 2	0.28099
5ul V Y 3	0.1294
10ul V Y 3	0.28166
5ul V M 2	0.11483
10ul V M 2	0.23166
5ul V M 3	0.11852
10ul V M 3	0.21924

$y = 0.009x + 0.0143$

$x = (y - 0.0143) / 0.009$

- substitute the absorbance in for y

$x = (.14145 - .0143) / .009$

$x = 14.1\mu g$ protein

- divide by μL used gives concentration

$14.1\mu g / 5\mu L = 2.8\mu g/\mu L$

- These calculations were repeated for all samples. On replicate samples with differing amounts of protein, the two concentrations were averaged to give the final concentration.

- For 10μL VY2, the concentration was found to be 3.0μg/μL, therefore the final concentration of sample VY2 was 2.9μg/μL

Table 2.1 SDS-PAGE second dimension recipes

SOLUTION COMPONENTS	5mL	10mL	15mL	20mL	100mL
6%					
H_2O	2.85	5.8	8.65	11.6	58.0
40% acrylamide	0.75	1.5	2.25	3	15.0
1.5M Tris (pH 8.8)	1.3	2.5	3.8	5	25.0
10% SDS	0.05	0.1	0.15	0.2	1.0
10% ammonium persulfate	0.05	0.1	0.15	0.2	1.0
TEMED	0.004	0.008	0.012	0.016	0.08
8%					
H_2O	2.6	5.3	7.9	10.6	53.0
40% acrylamide	1.0	2.0	3.0	4.0	20.0
1.5M Tris (pH 8.8)	1.3	2.5	3.8	5	25.0
10% SDS	0.05	0.1	0.15	0.2	1.0
10% ammonium persulfate	0.05	0.1	0.15	0.2	1.0
TEMED	0.003	0.006	0.009	0.012	0.06
10%					
H_2O	2.35	4.8	7.15	9.6	48.0
40% acrylamide	1.25	2.5	3.75	5	25.0
1.5M Tris (pH 8.8)	1.3	2.5	3.8	5	25.0
10% SDS	0.05	0.1	0.15	0.2	1.0
10% ammonium persulfate	0.05	0.1	0.15	0.2	1.0
TEMED	0.002	0.004	0.006	0.008	0.04
12%					
H_2O	2.1	4.3	6.4	8.6	43
40% acrylamide	1.5	3	4.5	6	30
1.5M Tris (pH 8.8)	1.3	2.5	3.8	5	25
10% SDS	0.05	0.1	0.15	0.2	1
10% ammonium persulfate	0.05	0.1	0.15	0.2	1
TEMED	0.002	0.004	0.006	0.008	0.04
15%					
H_2O	1.725	3.55	5.275	7.1	35.5
40% acrylamide	1.875	3.75	5.625	7.5	37.5
1.5M Tris (pH 8.8)	1.3	2.5	3.8	5	25
10% SDS	0.05	0.1	0.15	0.2	1
10% ammonium persulfate	0.05	0.1	0.15	0.2	1
TEMED	0.002	0.004	0.006	0.008	0.04

CHAPTER THREE:

Results

All growth trials were completed as previously stated with changes to homogenization protocols noted below. All gels were run in triplicate, except where indicated, to ensure repeatability. These triplicates allowed matchsets to be created using PDQuest software. Also unless specified, all second dimension gels consisted of 10% acrylamide gels. In all figures, the gels are oriented in the same fashion - increasing pH from left to right and decreasing mass from top to bottom (See Fig. 3.1). Where differences in protein expression are indicated, a circle denotes its presence, whereas a square denotes the absence or decreased expression of a protein.

As stated in the methods, a sonication protocol was first used to homogenize the differentiated *P. marneffei* cells. Fig. 3.1 presents representative images of yeast and mold forms on mini, 7 cm gels, pH 3-10. Once good results were generated, large format, 17 cm, pH 3-10 gels were run. These experiments were performed in triplicate and one example pair is seen in Fig. 3.2a. This particular set of gels shows one very distinct differentially expressed protein as indicated in red. This spot was excised from the gel and sent to the University of Cincinnati for digestion and mass spectrometric analysis. The mass spectra obtained can be seen in Fig. 3.2b. The sequence obtained from this analysis was further analyzed by a Mascot database search. A small portion of the protein sequence that was obtained matches a protein found in *Aspergillus fumigatus* and *Emericella nidulans* (Fig. 3.2c). This protein is superoxide dismutase (SOD).

In another set of gels, the yeast phase of *P. marneffei* was homogenized using the sonication protocol, whereas the mold phase was homogenized using the bead beat protocol. IPG strips of pH 4-7 and 3-10 were used. The results of these experiments can be seen in Fig 3.3 and 3.4. These gels were the first to be accompanied by a series of pH

problems, indicated by protein spots being compacted in the middle and/or top portion of the gels. After a few trials of using sonication for the yeast phase and bead beat for the mold phase, a full switch of homogenizing both forms by bead beat was undertaken. Also at this time and in all trials thereafter, electrode wicks were used in the IEF focusing tray.

New gels of pH 3-10 were also accompanied by a continued pH problem, causing most of the proteins to reside in the top half of the gel (data not shown). However, aside from the pH problem, more consistent mold patterns were seen.

After resolving the pH problems, pH 4-7 gels were repeated (Fig. 3.5). It can be seen that there are some proteins present at all portions of the gel, so the pH problem had been resolved. However, there is no recognizable pattern on these gels. Possible protein degradation lead to obtaining fresh protein from fresh cells.

After obtaining new protein, pH 4-7 gels were run and can be seen in Fig. 3.6. The spot indicated in red was thought to be the SOD according to isoelectic point (pI) and mass, but was present in both mold and yeast forms. These spots were excised, digested then sent for sequencing relative to the yeast and mold forms, but the results were inconclusive. There were also other spots of interest found on these pH 4-7 gels. As indicated in blue in Fig. 3.6, there is a "trio" seen in the mold that is only a "duo" in the yeast. Indicated in green is a spot present in the yeast phase, but not in the mold phase. These spots were excised, digested, then sent for sequencing. However, no usable data recovered from these samples following analysis by mass spectrometry.

Subsequent to resolving the pH problem, gels of pH 3-10 were repeated and are seen in Fig. 3.7. No spot resembling the SOD can be seen. However, since it seems as though the whole proteome is slightly different after changing to a full bead beating

homogenization protocol, the protein spot indicated in red in the figure was thought to be the SOD. In other trials, this protein spot was present in the mold as well as the yeast (not shown). This protein spot was located close to the dye front and in some trials it may have actually fused with the dye front, making it undetectable. These spots were excised and digested then sent for sequencing. Again, no usable results were obtained from the mass spectrometric analysis.

In order to shift the proteins up in the gel and move them away from the dye front, 12%, pH 3-10 gels were run. This resulted in the rediscovery of the familiar protein constellation and the protein spot previously matched to the SOD sequence. This protein is indicated in red in Fig. 3.8. It has yet to be excised, digested or sequenced.

In order to better resolve and isolate the proteins further, 12%, pH 4-7 gels were run. Fig. 3.9 shows a representative pair of gels. The spot assumed to be SOD seemed to have faded into the crowded constellation of proteins. However, also present in these 12%, 4-7 gels were the "trio" spot in the mold indicated in blue. This spot was again excised and sent for digestion and sequencing. Results have yet to be obtained from this protein spot.

As stated in the methods, PDQuest software was used to analyze the gels. When trials were run in triplicate, matchsets were created to compare like trials and then to compare different trials. Four representative matchsets are given in Fig. 3.10a-d of 12%, 17cm, pH 4-7 gels. In Fig. 3.10a, a matchset was created using three trials of the mold form. In Fig. 3.10b, a matchset was created using three trials of the yeast form. The masters (in white) were then used to create higher level matchsets to further compare the mold to the yeast form. These matchsets are seen in Fig. 3.10c-d. From the analysis in

Fig. 3.10c, there were 101 unmatched spots found in the yeast when compared to the mold. From the analysis in Fig. 3.10d, there were 156 unmatched spots found in the mold when compared to the yeast. Analyses similar to these were completed for most triplicate trials.

In hopes of finding still more differentially expressed proteins, pH 5-8 gels were run (Fig. 3.11). A new spot of interest was found on the right portion of the gel and is indicated in orange in the figure. It was excised, digested then sent for sequencing. However, no usable results were obtained. Later it was realized that since the pH 4-7 and pH 5-8 gels overlap, the "trio" spot previously found in the mold pH 4-7 gels was also in the pH 5-8 gels. Therefore, that spot was excised and sent for digestion and sequencing as a replicate trial. Results have yet to be obtained from this protein spot.

New experimental trials continued to be conducted using pH 7-10 gels in which revealed at least one main differential spot (indicated in yellow; Fig. 3.12).

As indicated in the methods, another step was added to later trials. Liquid IEF (Rotofor®, Bio-Rad) was used to pre-fractionate whole protein samples into three pH fractions prior to gel IEF and SDS-PAGE. The three fractions were obtained by pooling the 20 pH fractions produced by the Rotofor®; 1-7 representing the low approximate pH fraction, 8-13 representing the middle approximate pH fraction and 14-20, representing the high approximate pH fraction. These three fractions were run on gels according to corresponding IEF pH ranges. In Fig. 3.13a-b, yeast and mold phase gels (7cm) show protein differences in the three pH ranges: 3-6, 5-8 and 7-10. Indicated in pink in Fig. 3.13a is the recognizable "trio" seen in earlier non-prefractionated trials of the mold phase.

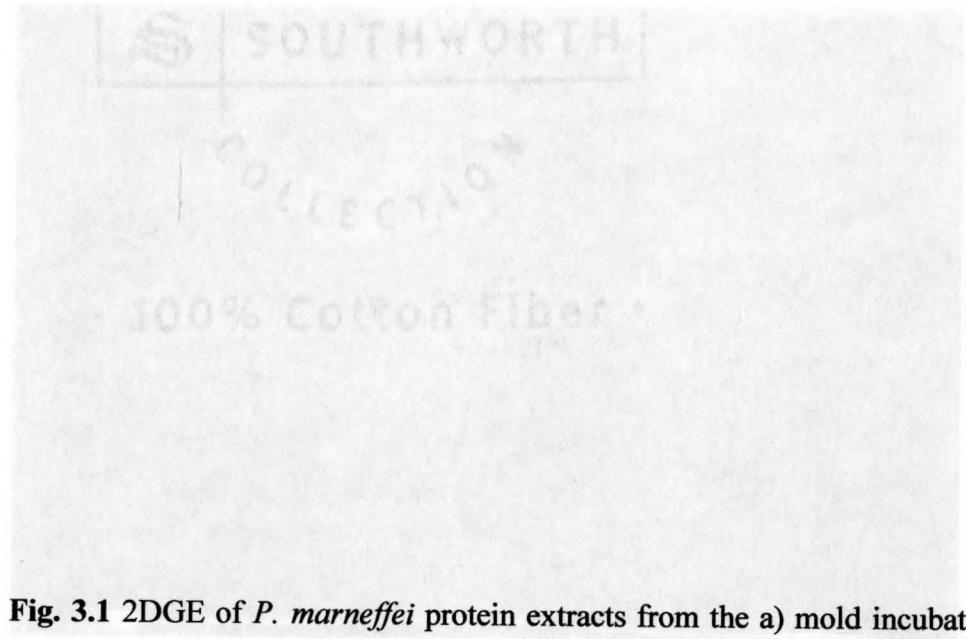
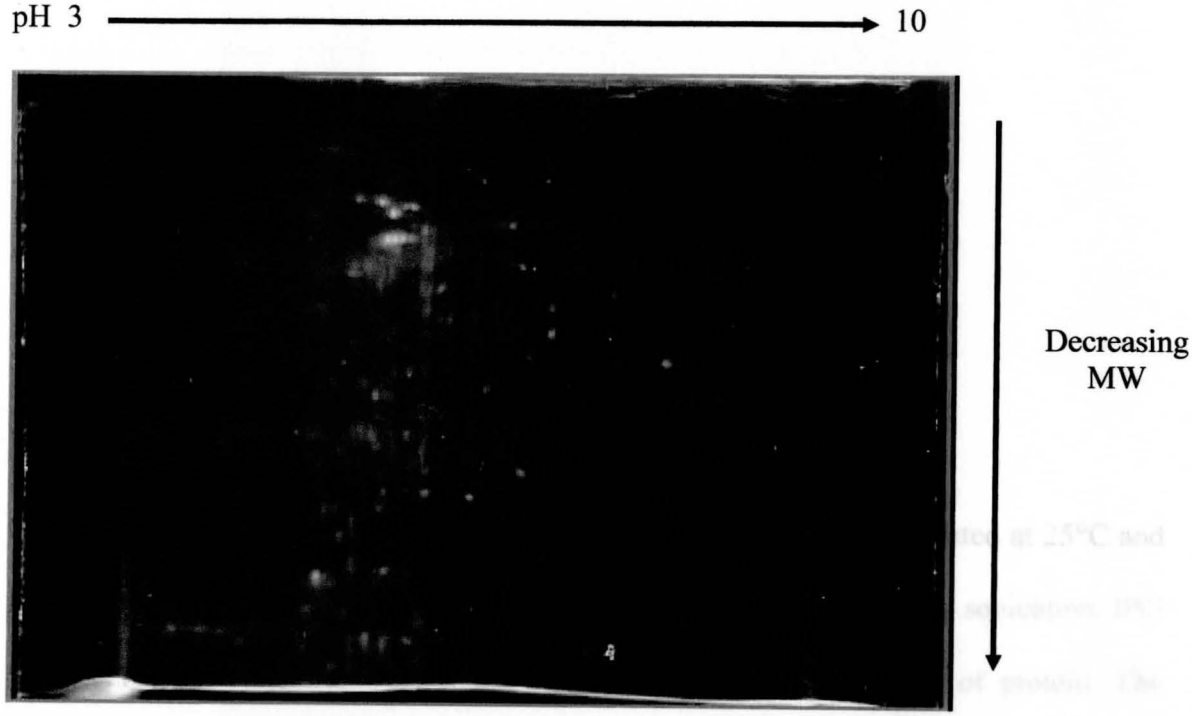
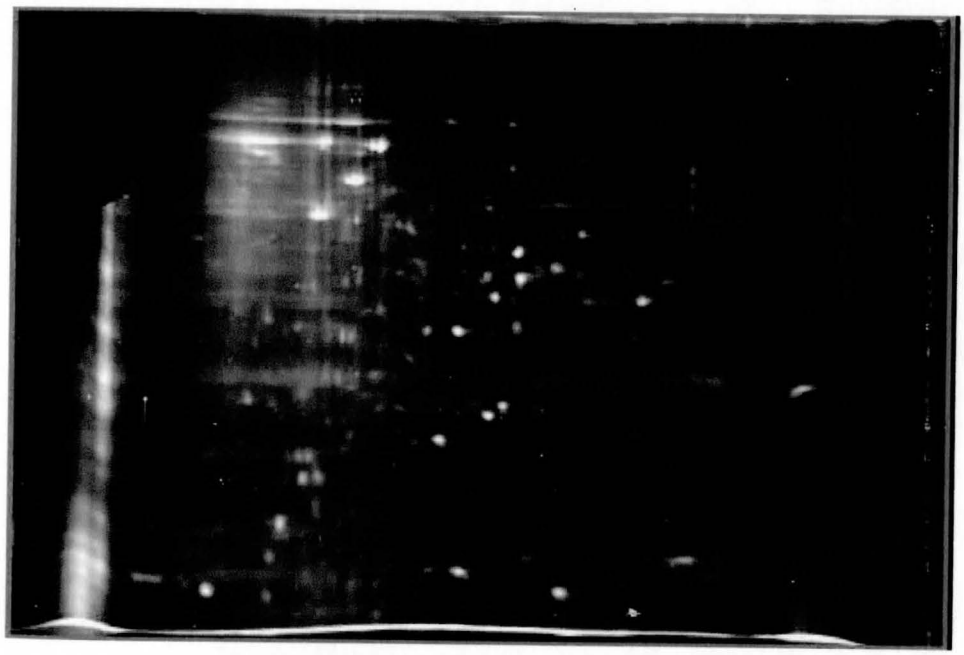


Fig. 3.1 2DGE of *P. marneffei* protein extracts from the a) mold incubated at 25°C and from b) yeasts generated by growth at 37°C. Cells were homogenized by sonication. IPG strips (7 cm in length and pH range 3-10) were loaded with 150µg of protein.



(a)



(b)

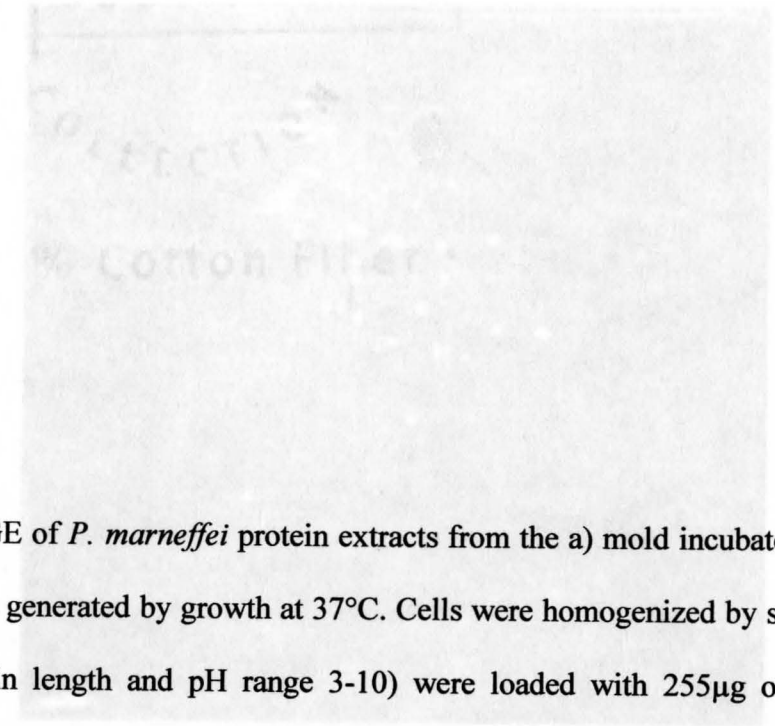
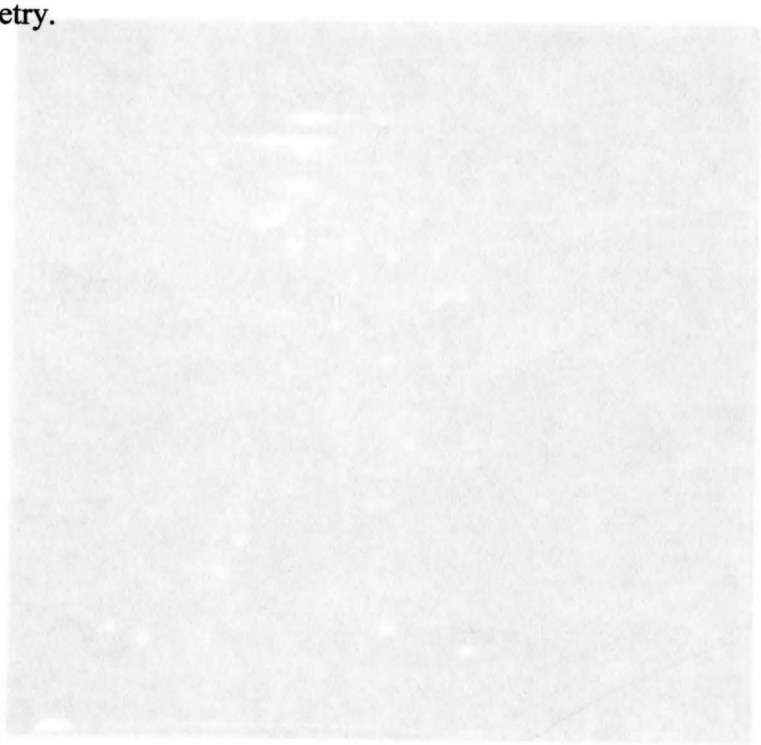
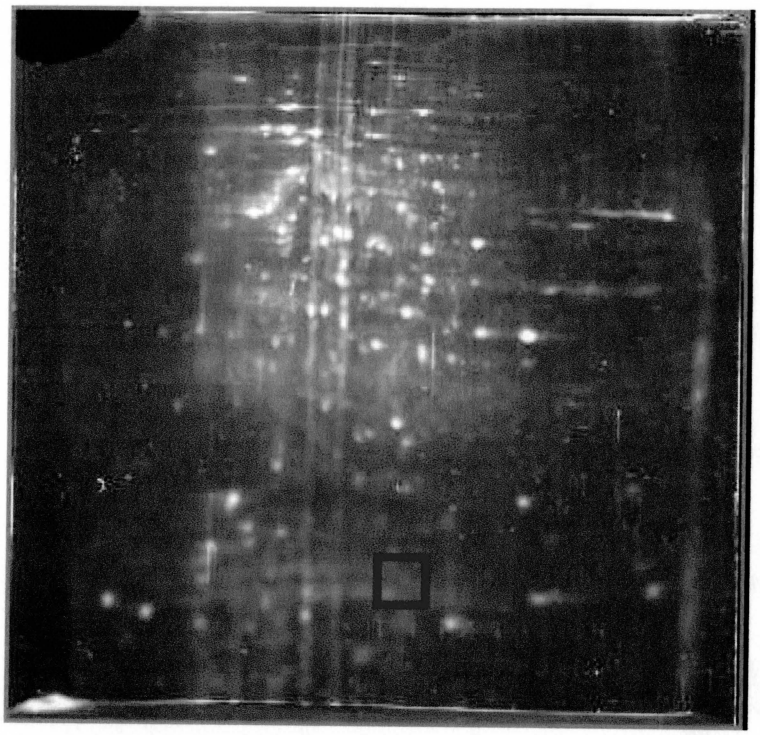
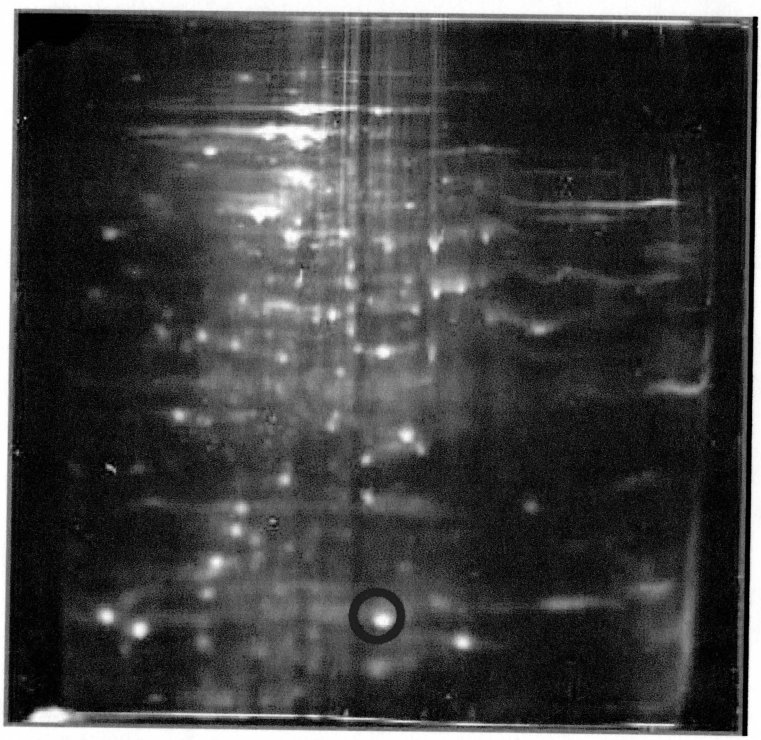


Fig. 3.2a 2DGE of *P. marneffei* protein extracts from the a) mold incubated at 25°C and from b) yeasts generated by growth at 37°C. Cells were homogenized by sonication. IPG strips (17cm in length and pH range 3-10) were loaded with 255μg of protein. The protein spot indicated in red was only found in the yeast form, excised and sequenced by mass spectrometry.





(a)



(b)

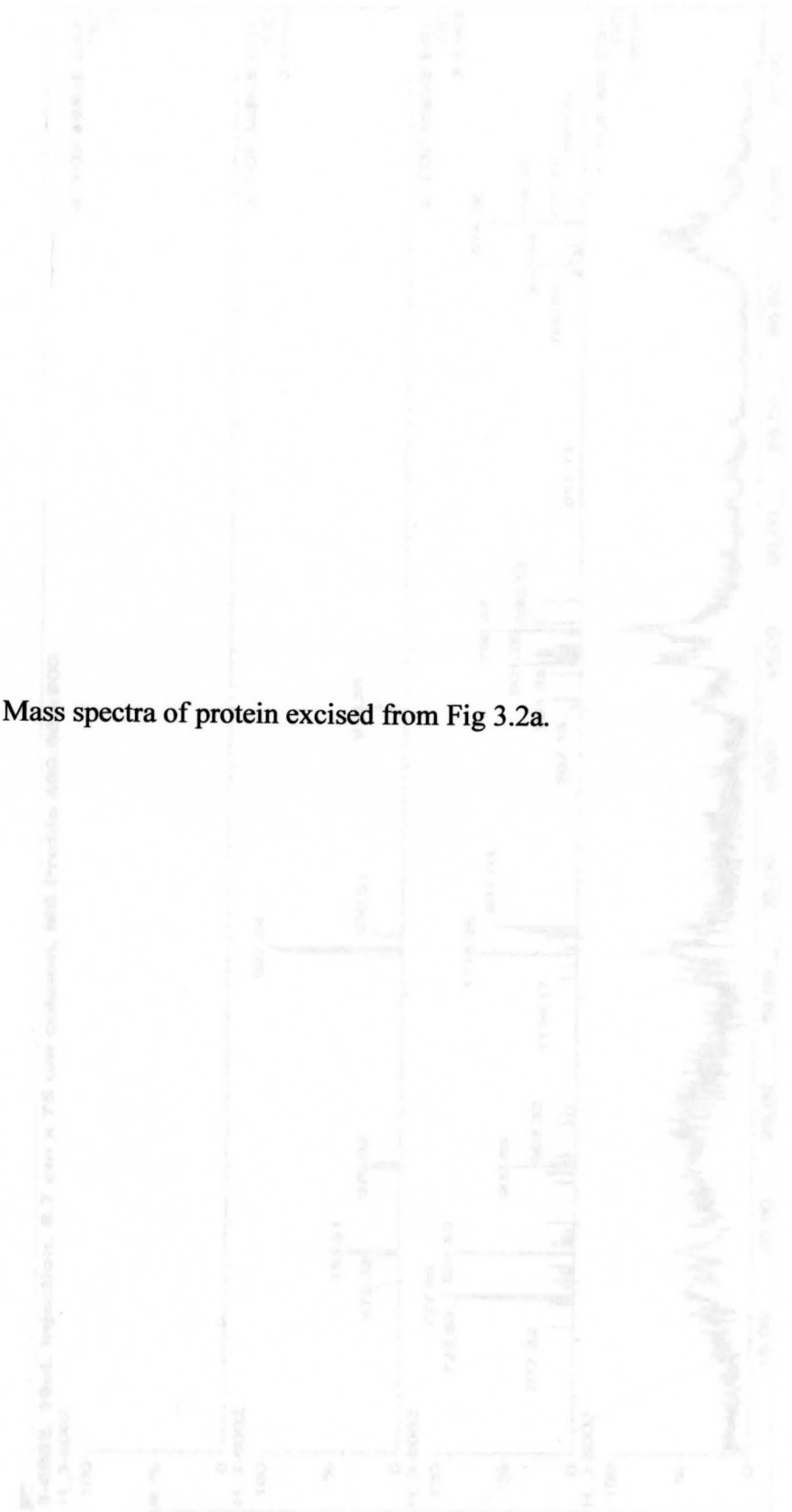


Fig 3.2b Mass spectra of protein excised from Fig 3.2a.

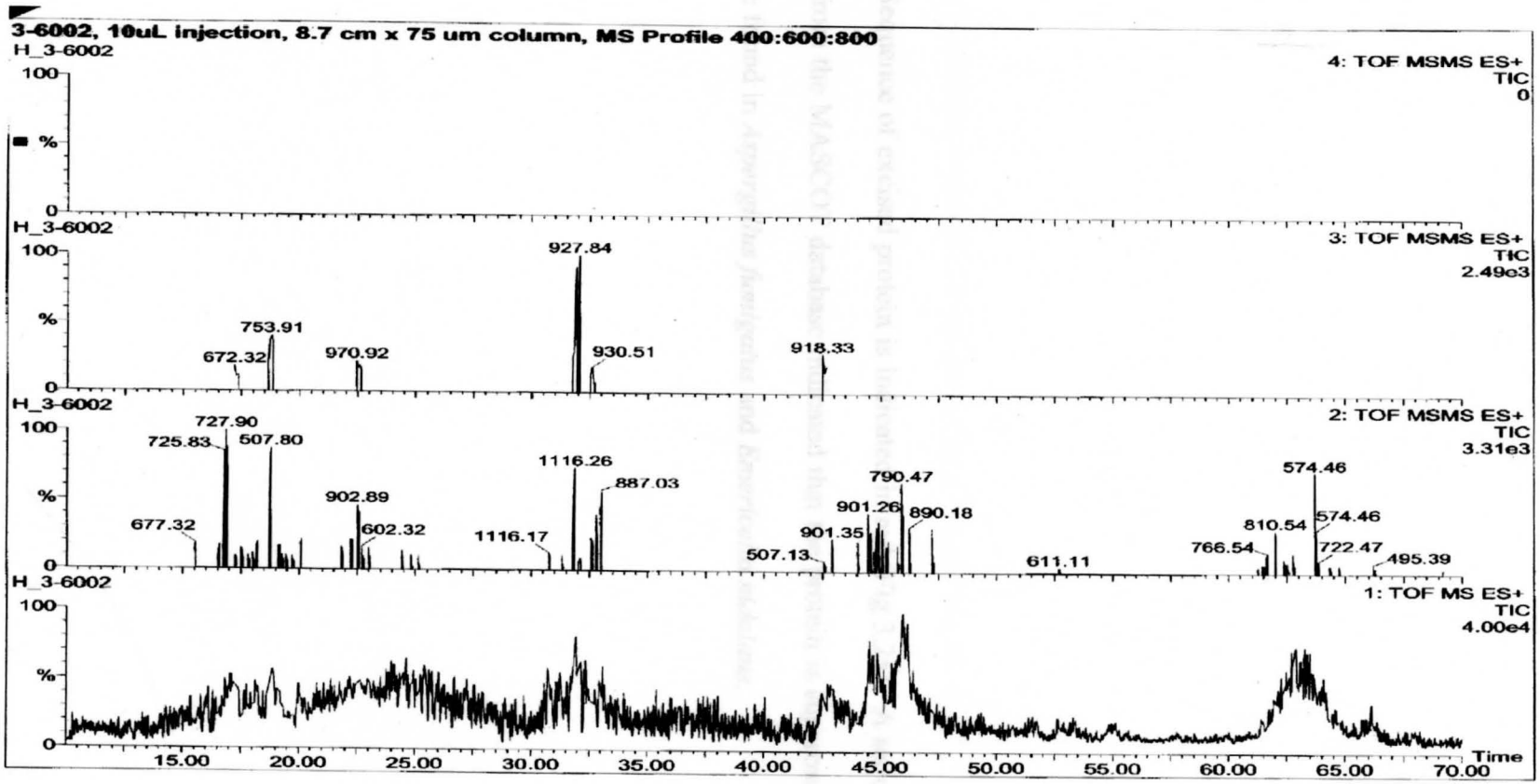

3-6002, 10uL injection, 8.7 cm x 75 um column, MS Profile 400:600:800
H_3-6002
4: TOF MSMS ES+
TIC
0
H_3-6002
3: TOF MSMS ES+
TIC
2.49e3
927.84
753.91
672.32
970.92
930.51
918.33
H_3-6002
2: TOF MSMS ES+
TIC
3.31e3
727.90
725.83
507.80
1116.26
887.03
790.47
574.46
902.89
901.26
890.18
677.32
602.32
1116.17
901.35
810.54
574.46
507.13
766.54
722.47
611.11
495.39
H_3-6002
1: TOF MS ES+
TIC
4.00e4
100
%
0
15.00
20.00
25.00
30.00
35.00
40.00
45.00
50.00
55.00
60.00
65.00
70.00
Time

Fig 3.2c Sequence of excised protein is indicated in red (Fig 3.2a). A sequence matched obtained from the MASCOT database indicated that the protein is superoxide dismutase, an enzyme found in *Aspergillus fumigatus* and *Emericella nidulans*.

1 MVKAVAVRGD SKVSGTVTFE QADENSNTTV SWNITGNDPN

41 AERGFHIHQF GDNTNGCTXA GPHFNPFGKT HGAPEDEVRH

81 VGDLGNFKTD AEGNSKGSKT DKLIKLIGAE SVLGRTLVVH

121 AGTDDLGRGD SEESKKTGNA GARPACGVIG IAA

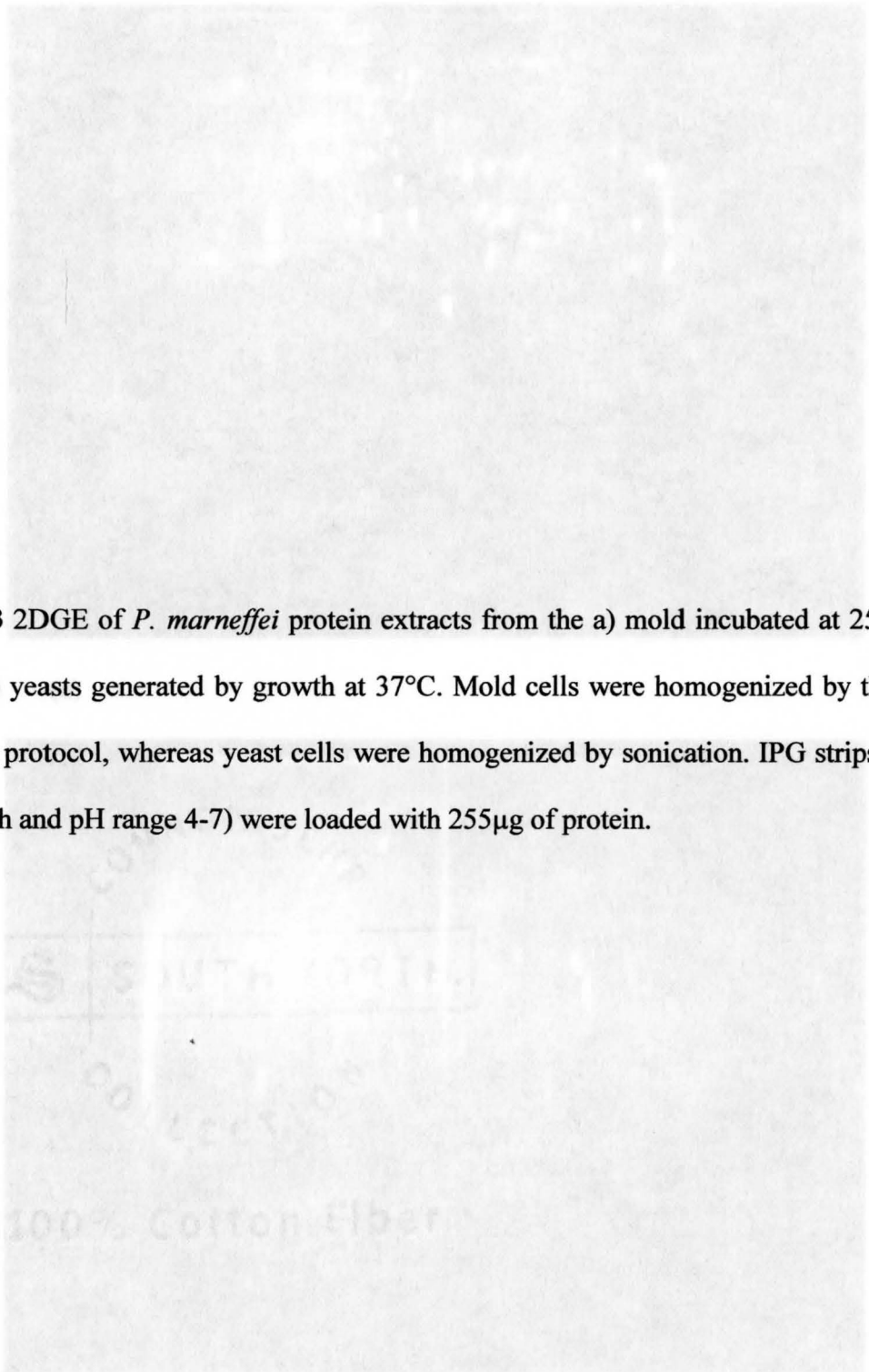
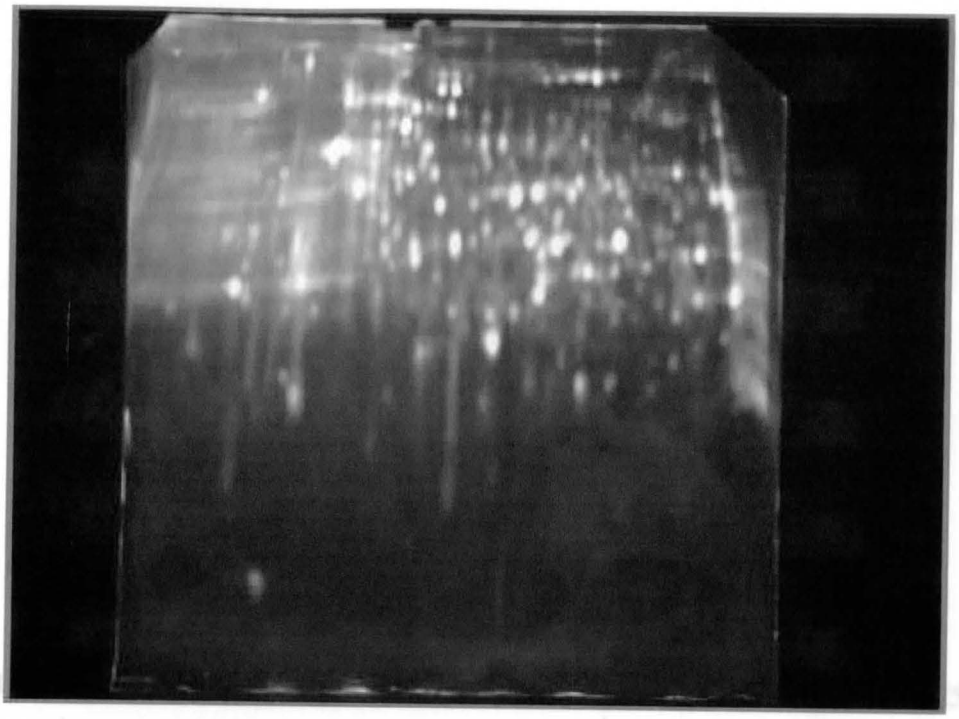
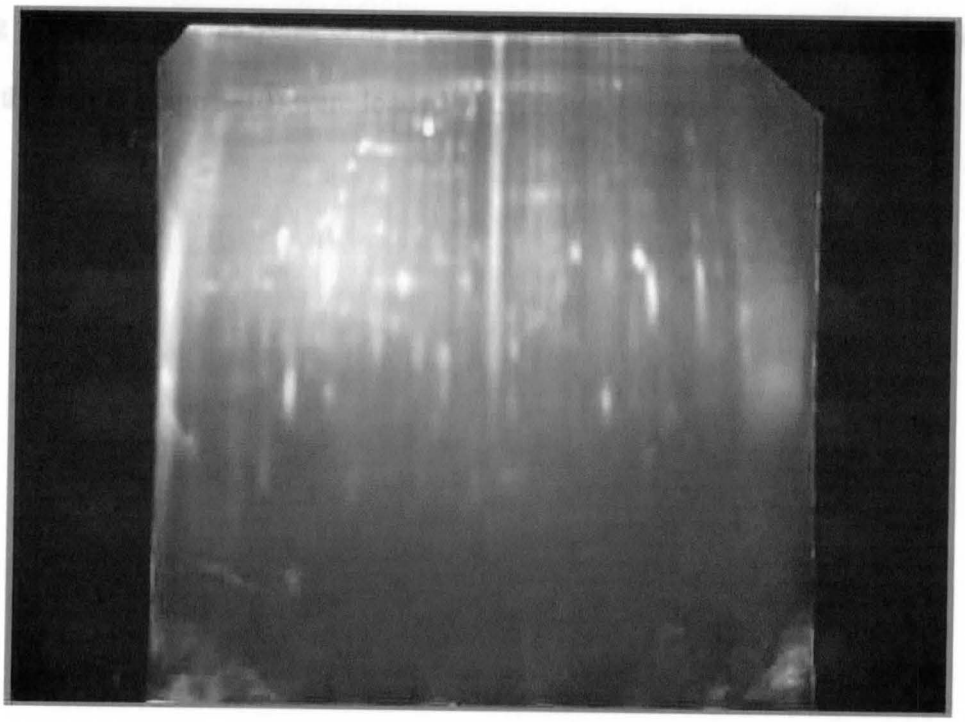


Fig. 3.3 2DGE of *P. marneffei* protein extracts from the a) mold incubated at 25°C and from b) yeasts generated by growth at 37°C. Mold cells were homogenized by the bead beating protocol, whereas yeast cells were homogenized by sonication. IPG strips (17cm in length and pH range 4-7) were loaded with 255µg of protein.



(a)



(b)

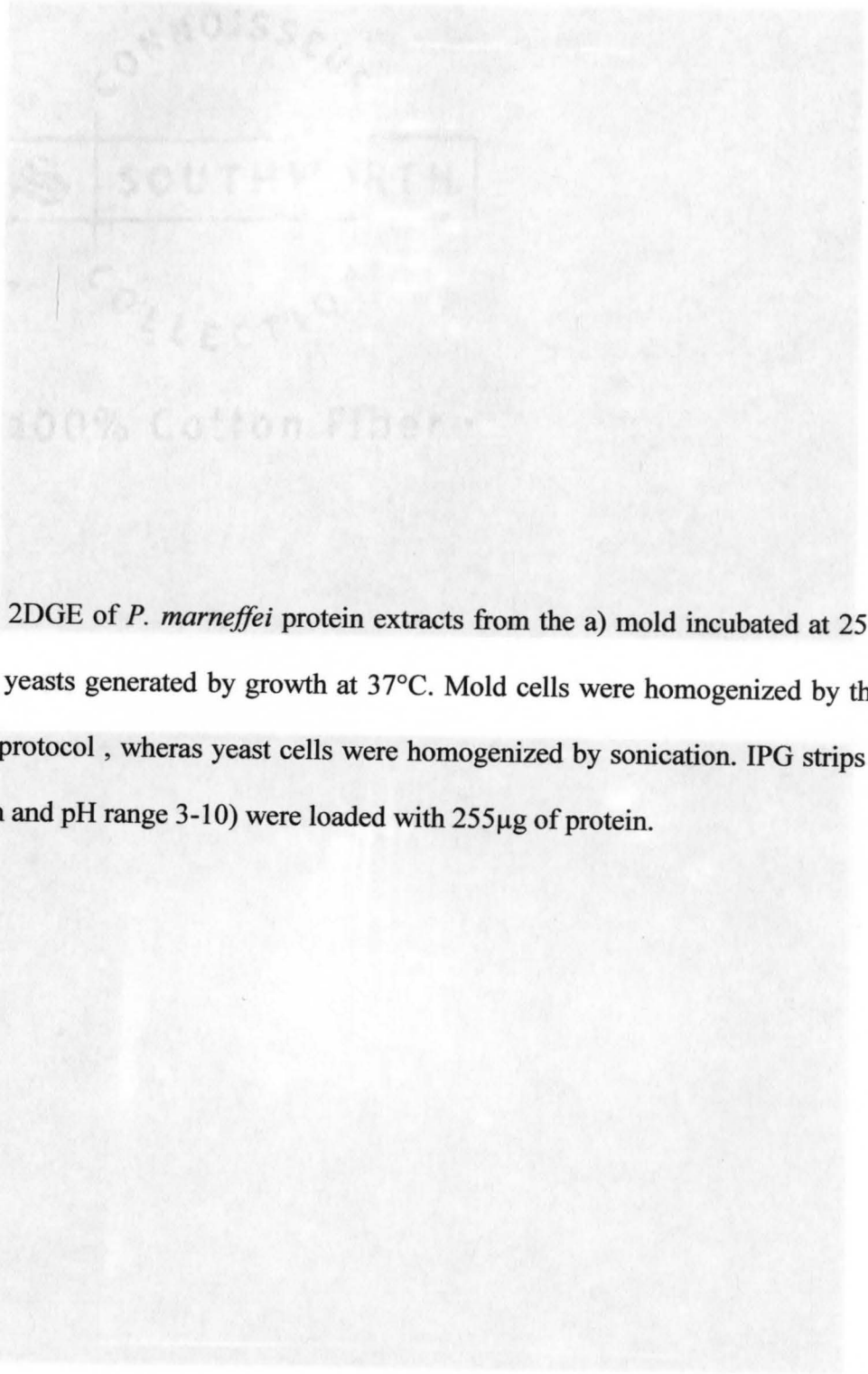
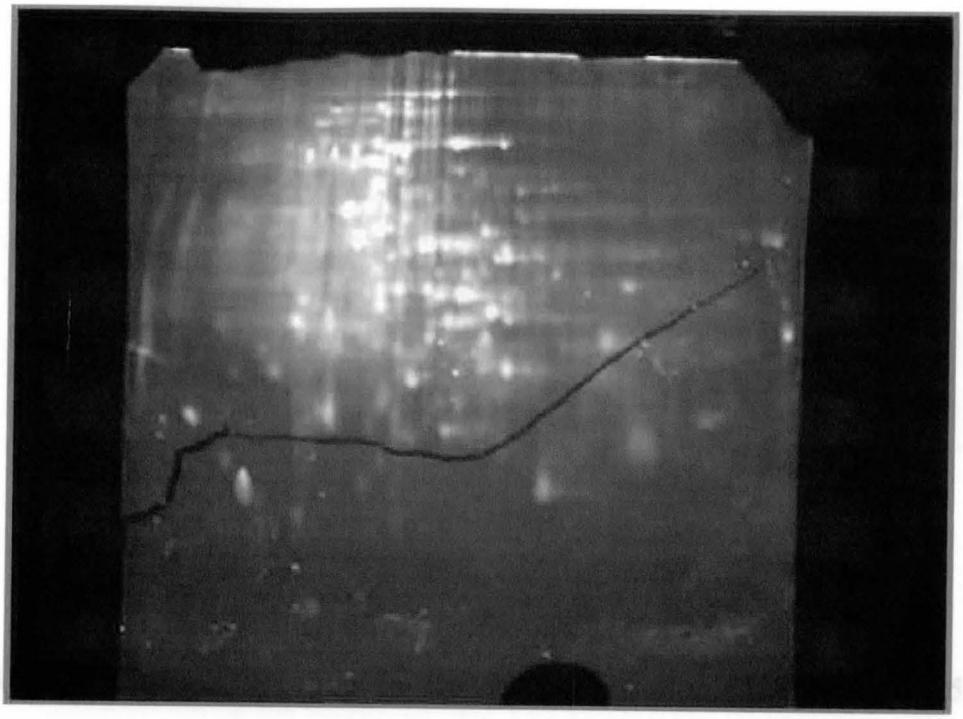
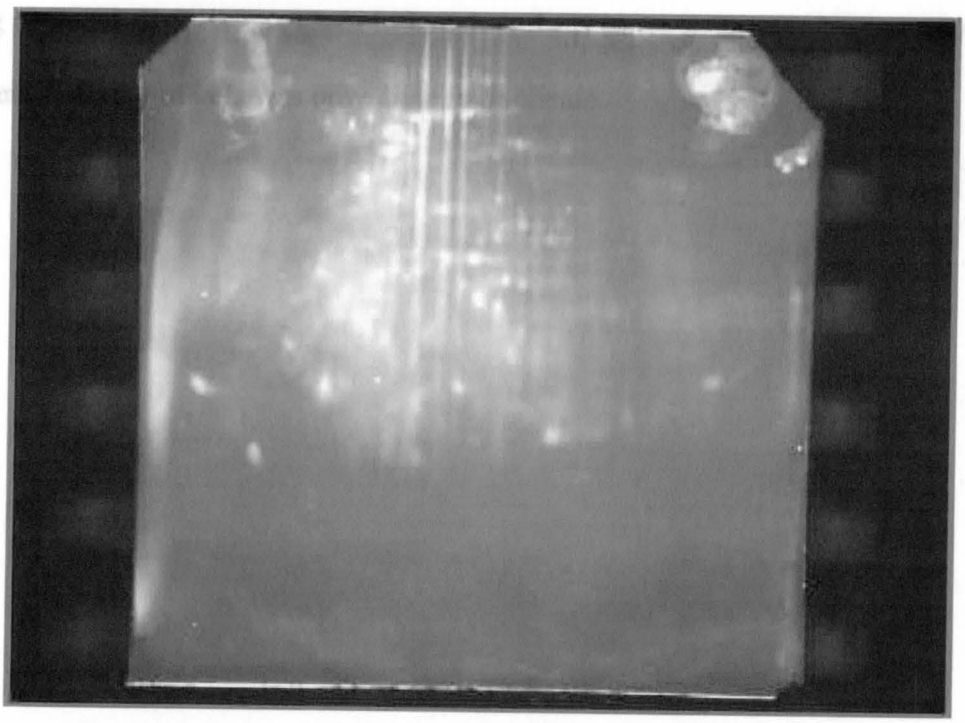


Fig. 3.4 2DGE of *P. marneffei* protein extracts from the a) mold incubated at 25°C and from b) yeasts generated by growth at 37°C. Mold cells were homogenized by the bead beating protocol , wheras yeast cells were homogenized by sonication. IPG strips (17cm in length and pH range 3-10) were loaded with 255µg of protein.



(a)



(b)

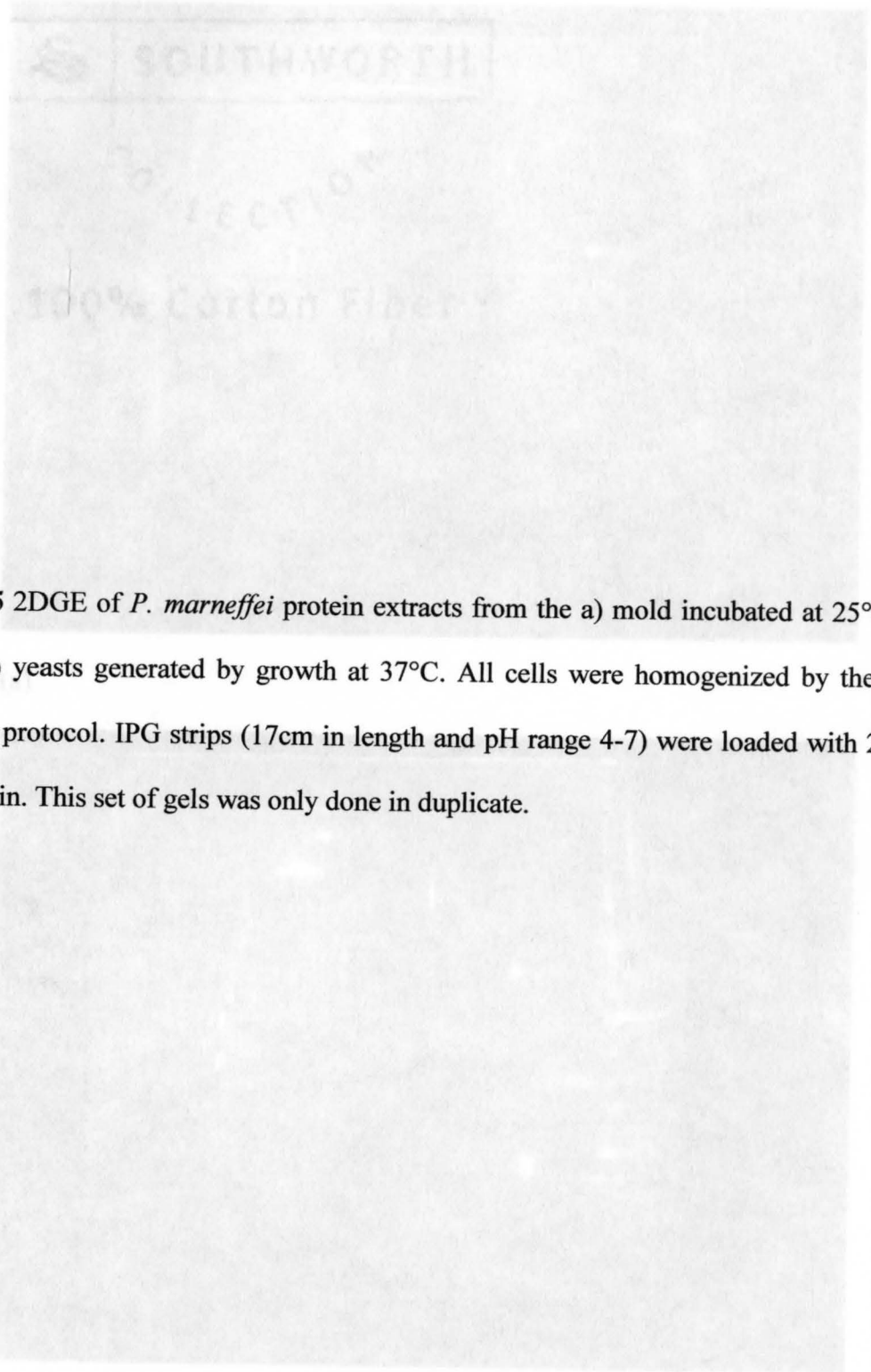
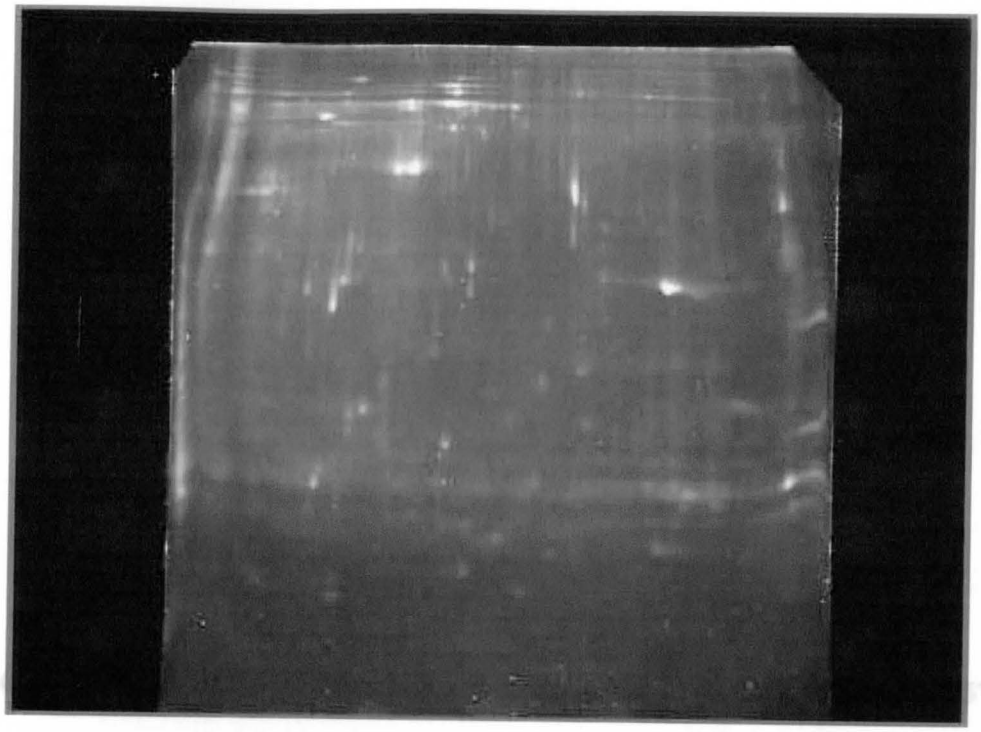
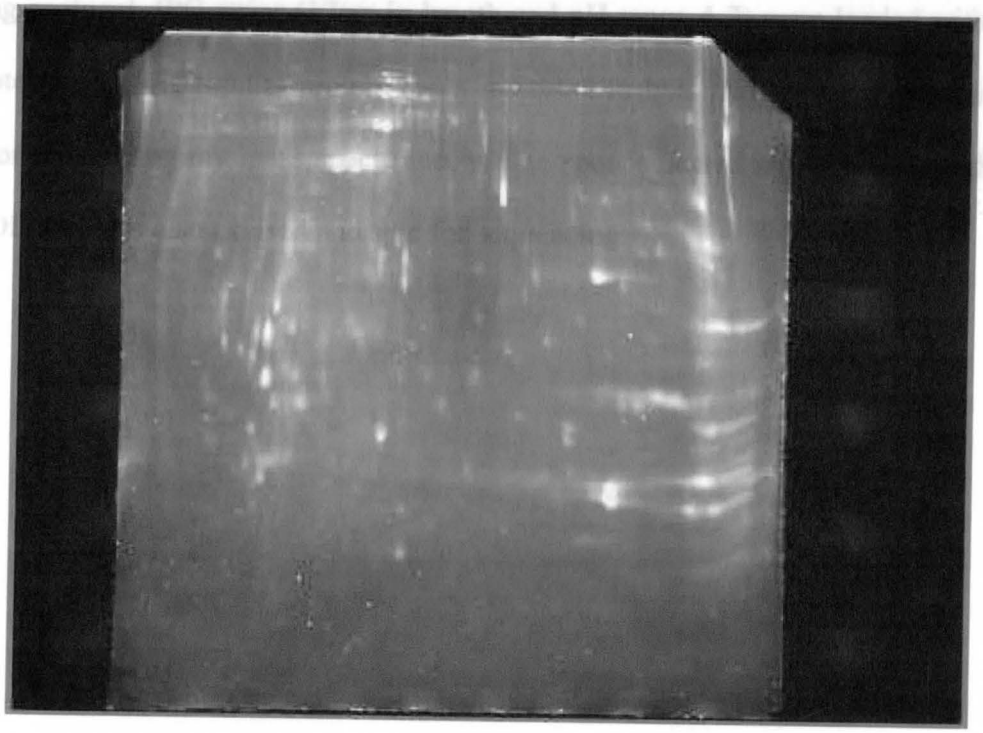


Fig. 3.5 2DGE of *P. marneffei* protein extracts from the a) mold incubated at 25°C and from b) yeasts generated by growth at 37°C. All cells were homogenized by the bead beating protocol. IPG strips (17cm in length and pH range 4-7) were loaded with 225µg of protein. This set of gels was only done in duplicate.



(a)



(b)

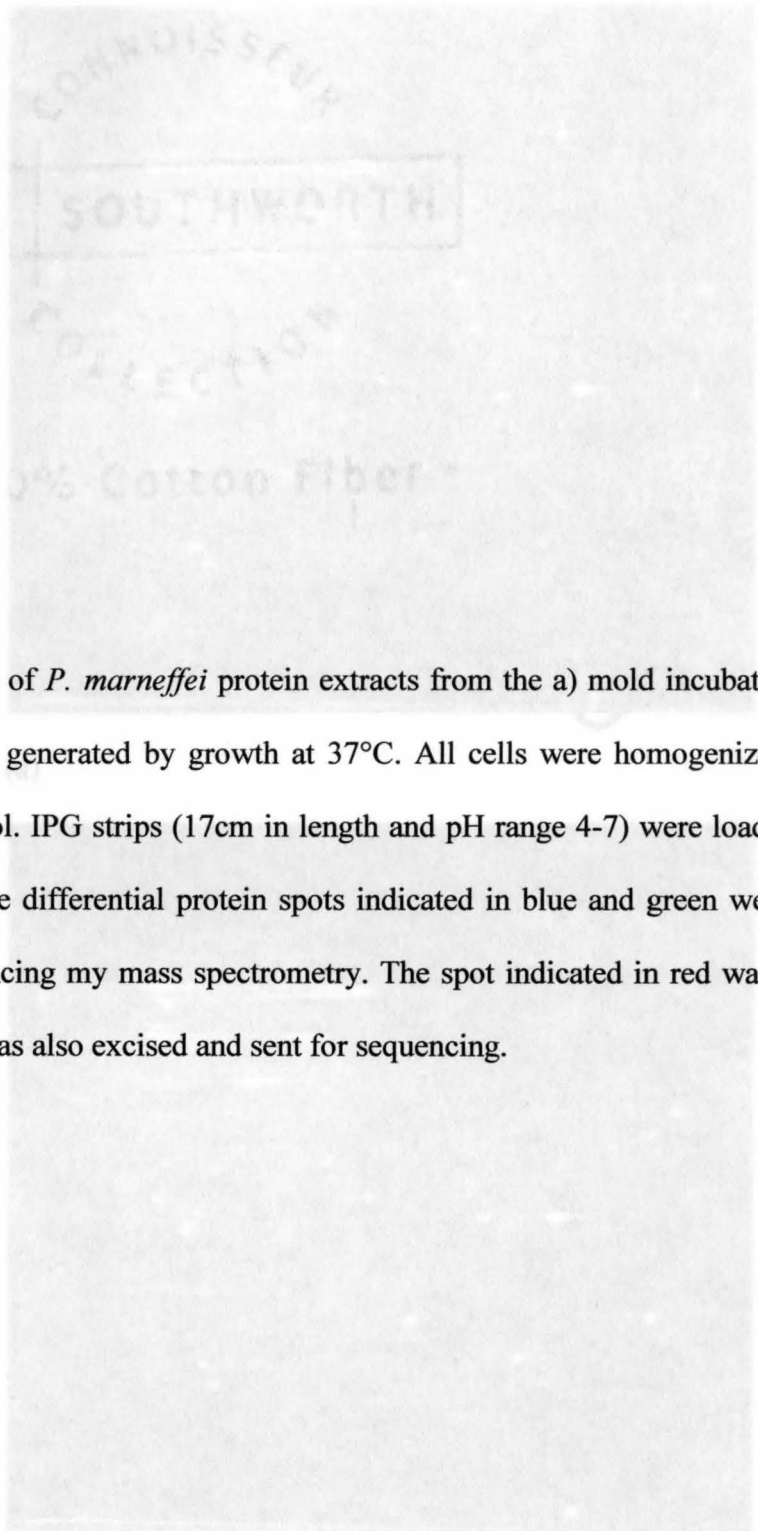
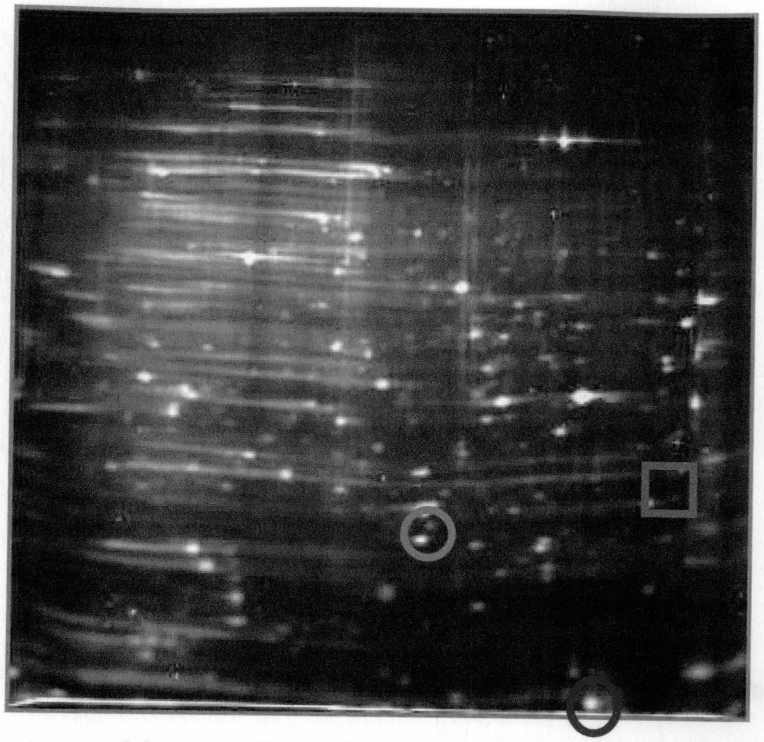
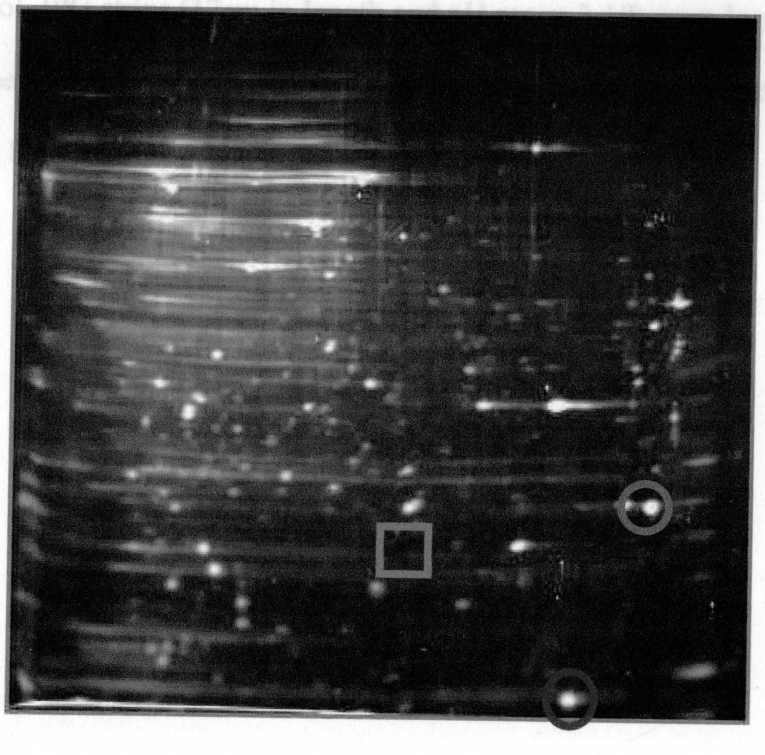


Fig. 3.6 2DGE of *P. marneffei* protein extracts from the a) mold incubated at 25°C and from b) yeasts generated by growth at 37°C. All cells were homogenized by the bead beating protocol. IPG strips (17cm in length and pH range 4-7) were loaded with 225µg of protein. The differential protein spots indicated in blue and green were excised and sent for sequencing my mass spectrometry. The spot indicated in red was thought to be the SOD and was also excised and sent for sequencing.



(a)



(b)

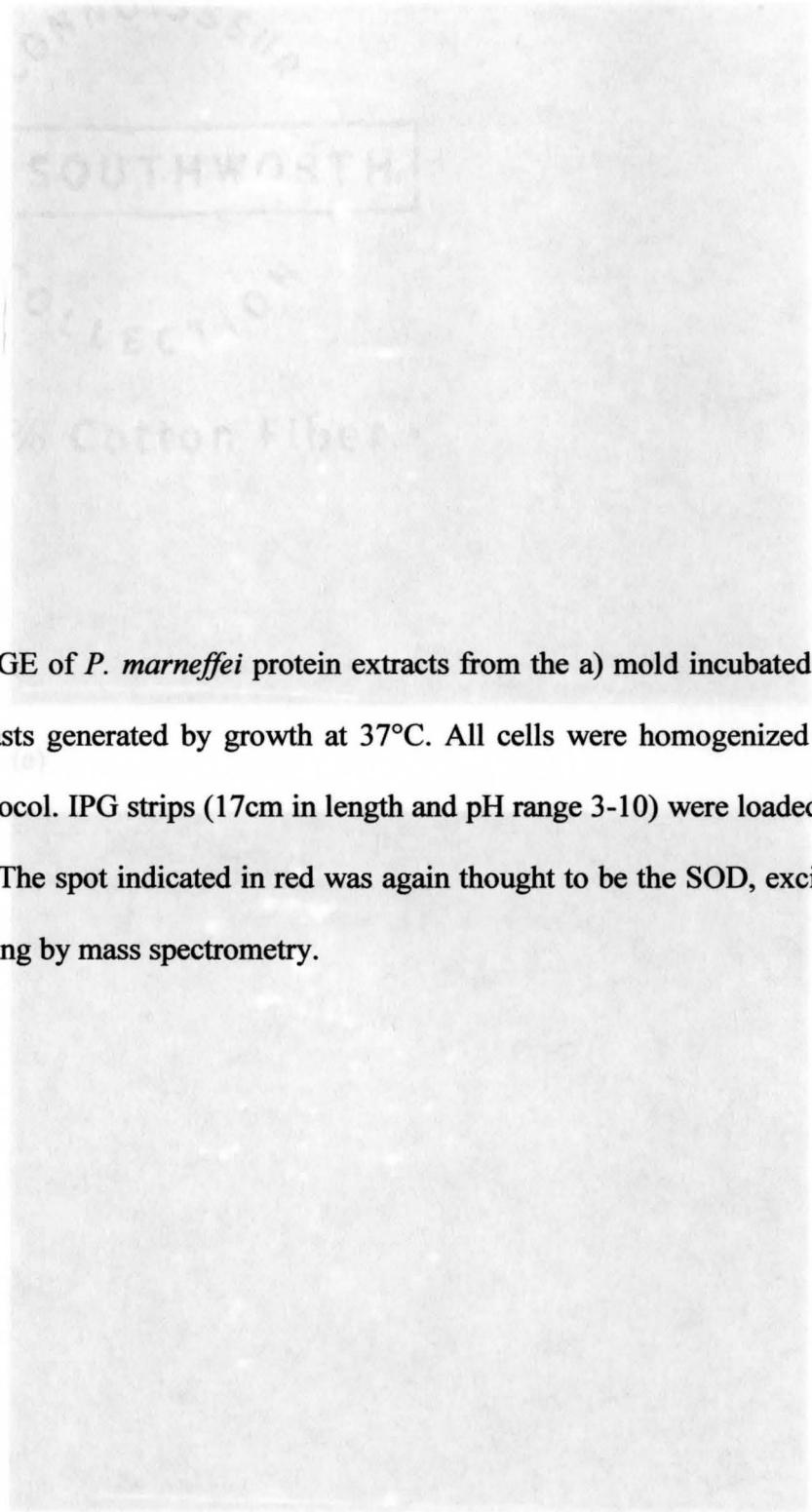
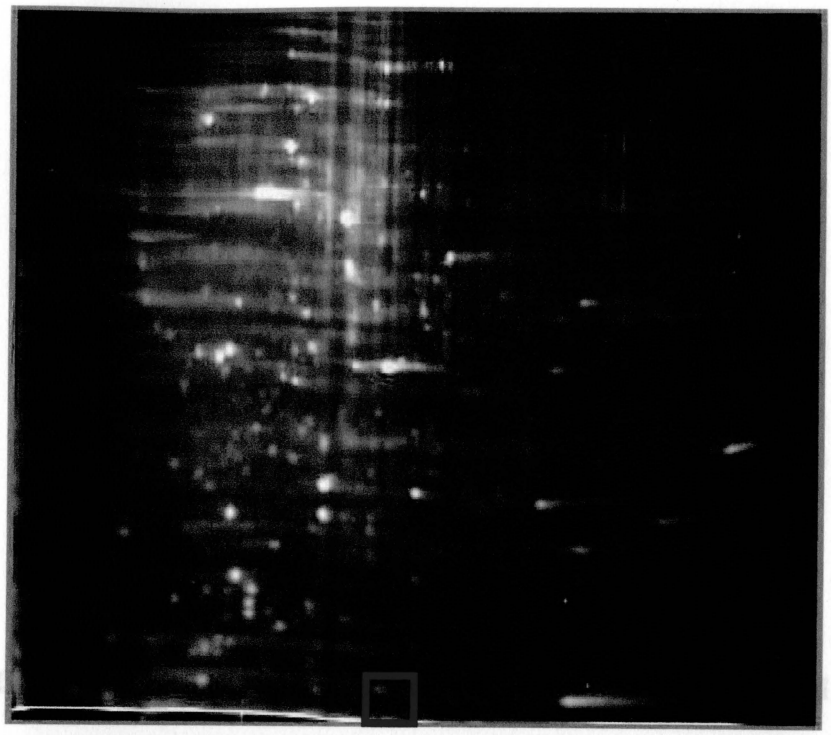
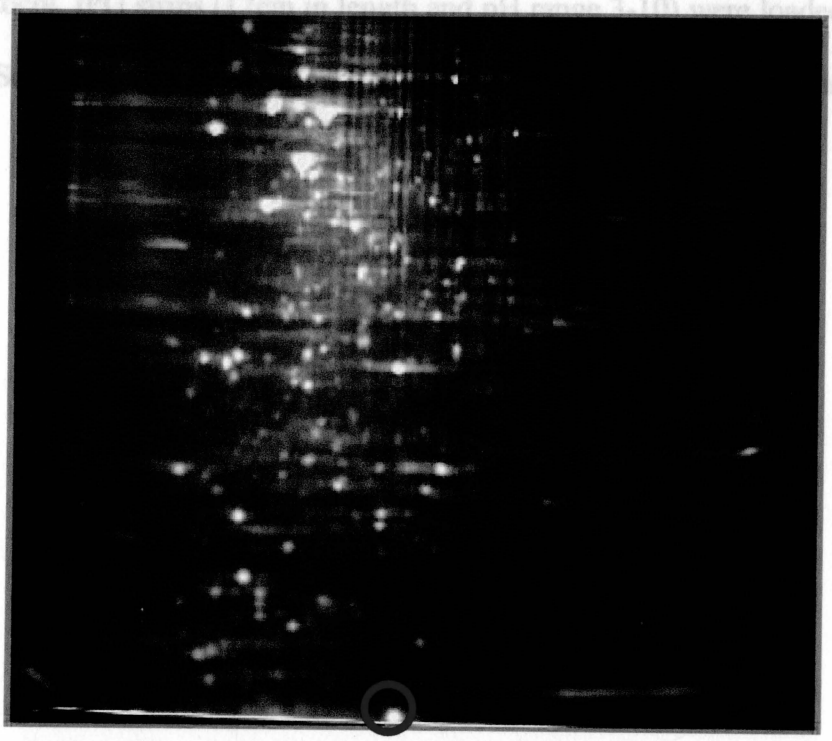


Fig. 3.7 2DGE of *P. marneffei* protein extracts from the a) mold incubated at 25°C and from b) yeasts generated by growth at 37°C. All cells were homogenized by the bead beating protocol. IPG strips (17cm in length and pH range 3-10) were loaded with 225µg of protein. The spot indicated in red was again thought to be the SOD, excised and sent for sequencing by mass spectrometry.



(a)



(b)

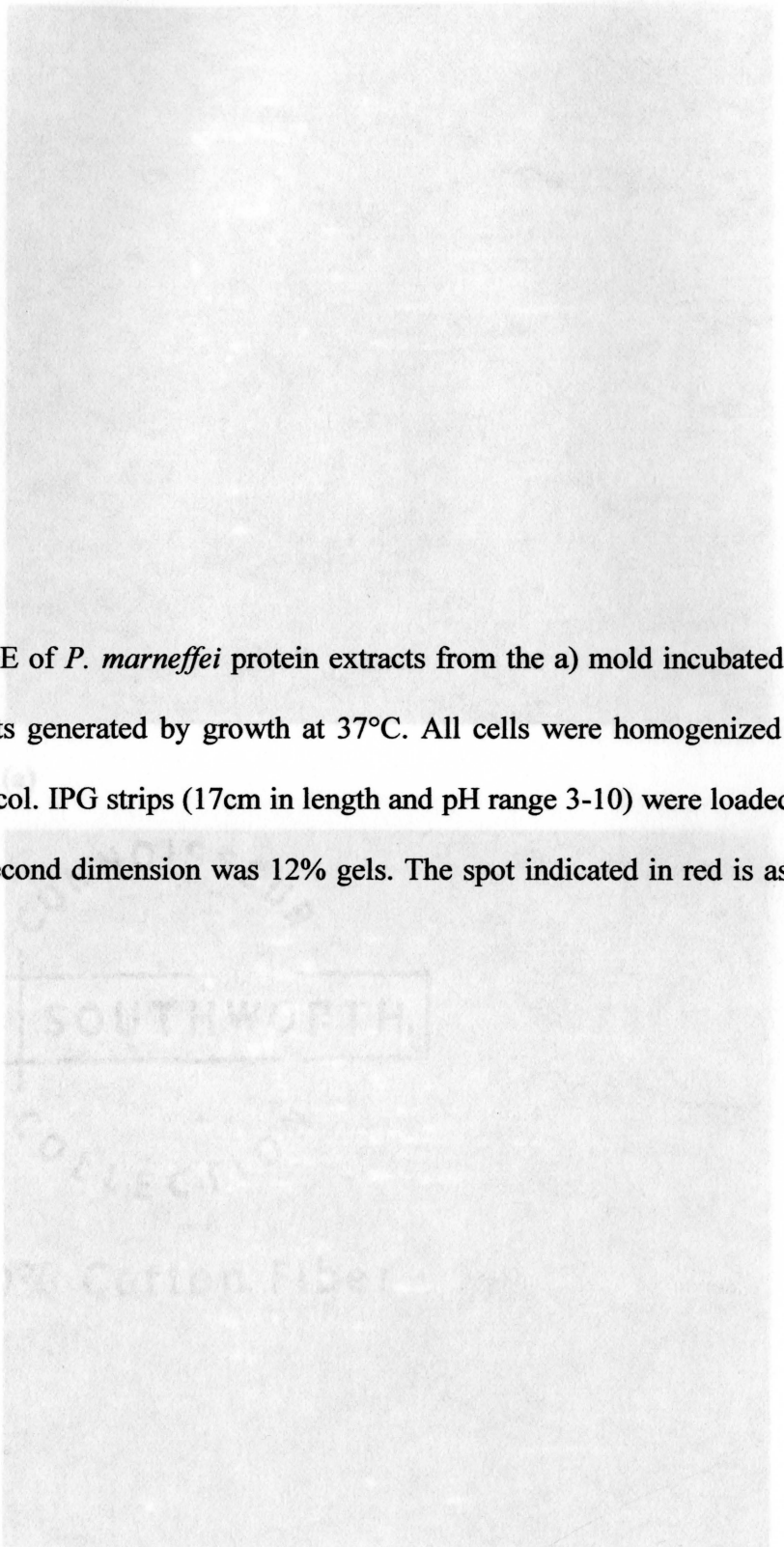
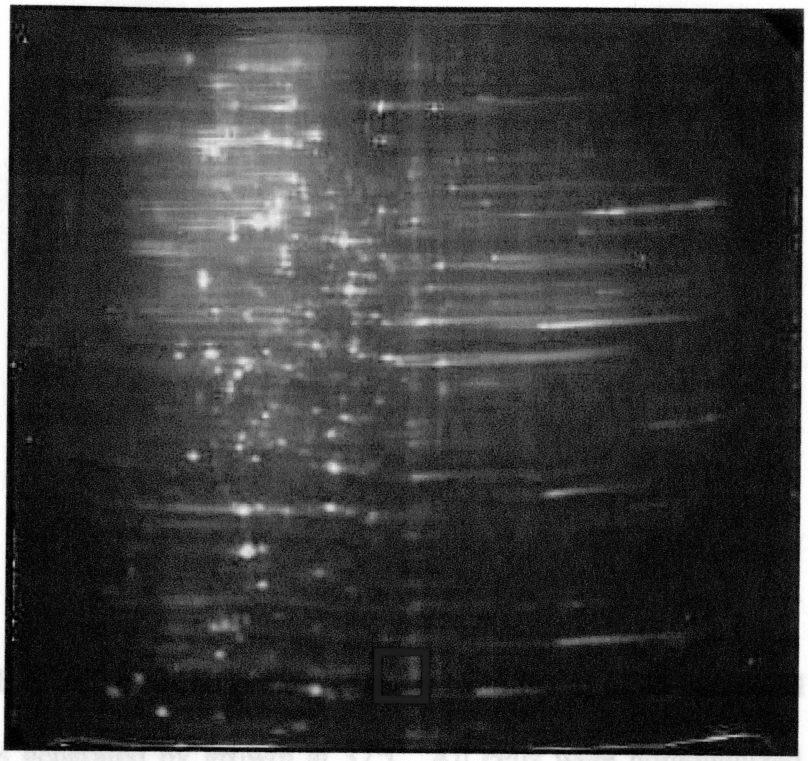
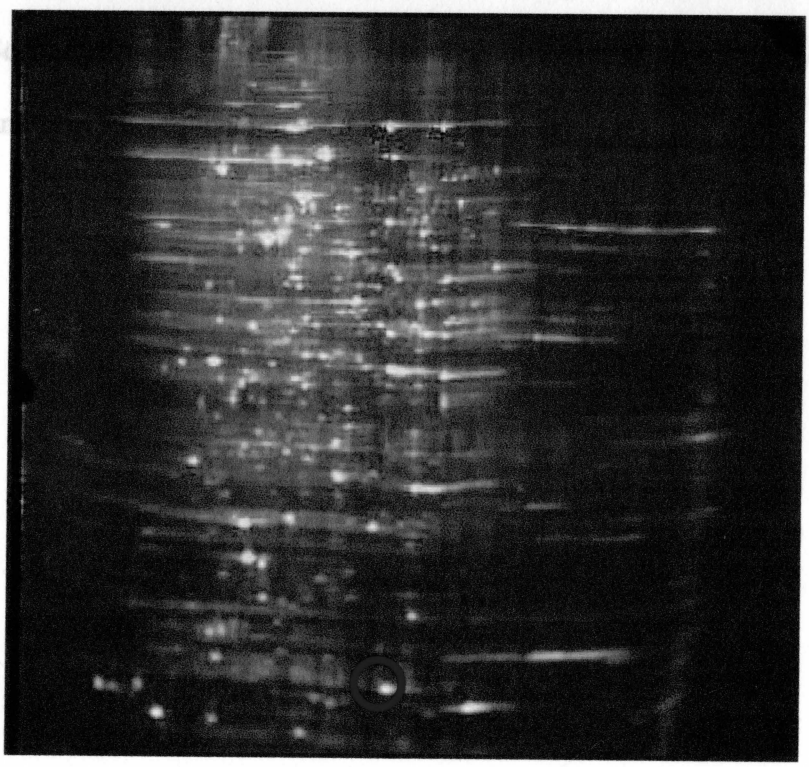


Fig. 3.8 2DGE of *P. marneffei* protein extracts from the a) mold incubated at 25°C and from b) yeasts generated by growth at 37°C. All cells were homogenized by the bead beating protocol. IPG strips (17cm in length and pH range 3-10) were loaded with 200μg of protein. Second dimension was 12% gels. The spot indicated in red is assumed to be the SOD.



(a)



(b)

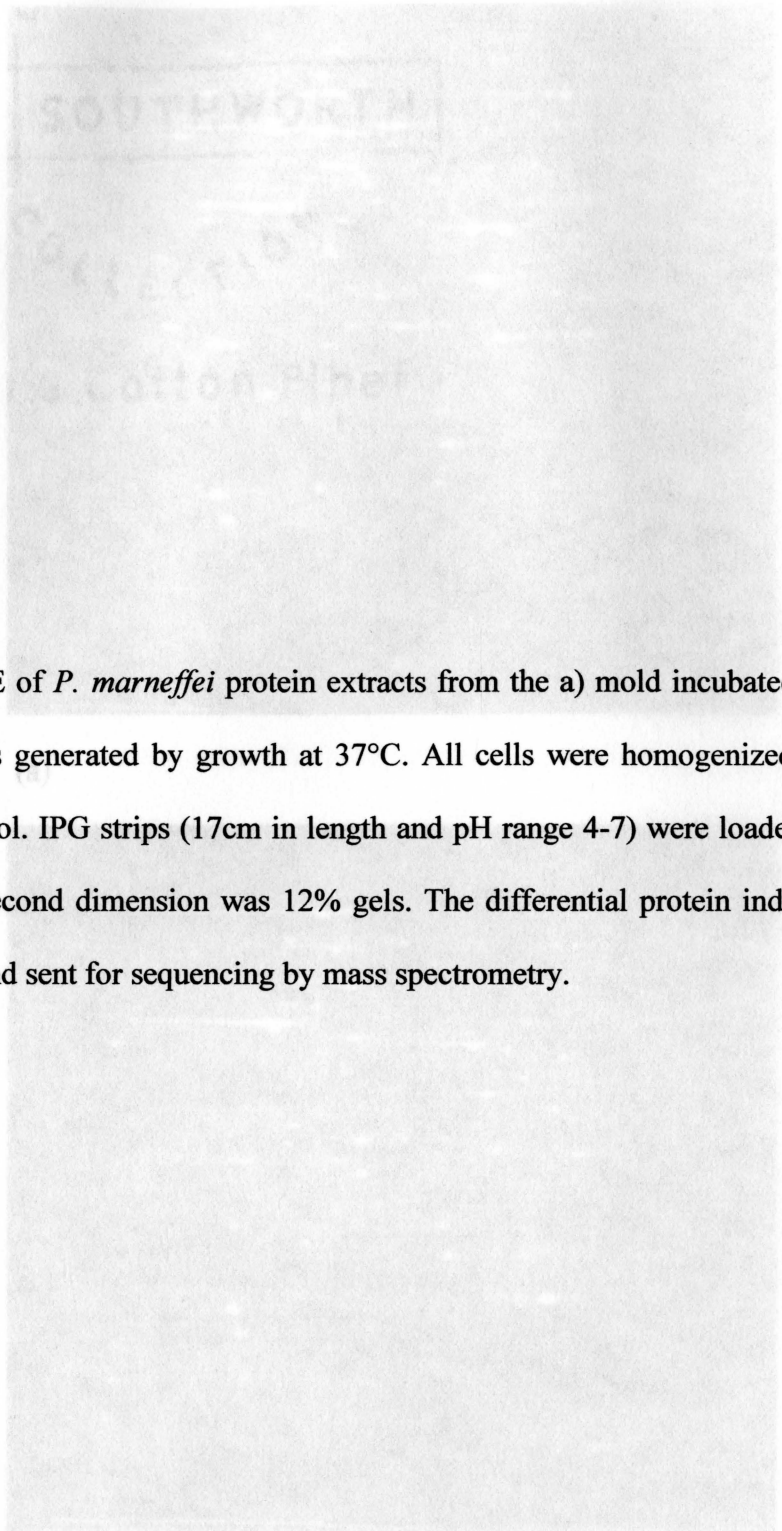
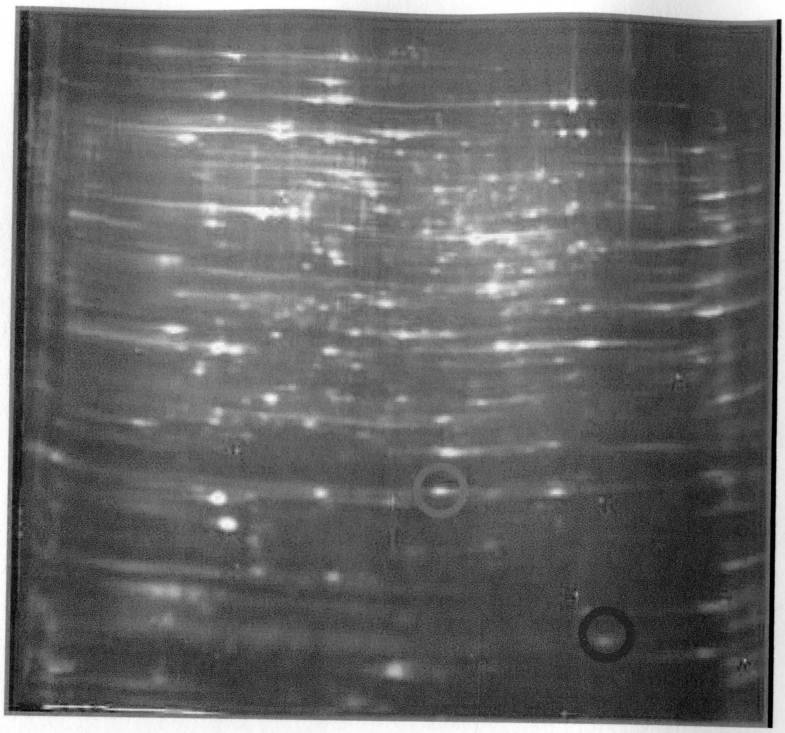
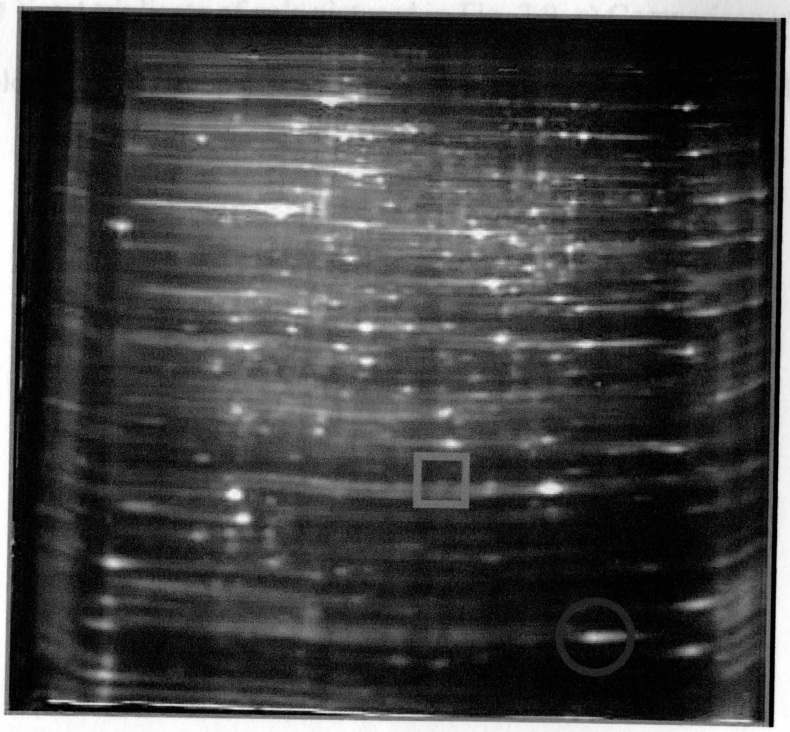


Fig. 3.9 2DGE of *P. marneffei* protein extracts from the a) mold incubated at 25°C and from b) yeasts generated by growth at 37°C. All cells were homogenized by the bead beating protocol. IPG strips (17cm in length and pH range 4-7) were loaded with 225µg of protein. Second dimension was 12% gels. The differential protein indicated in blue was excised and sent for sequencing by mass spectrometry.



(a)



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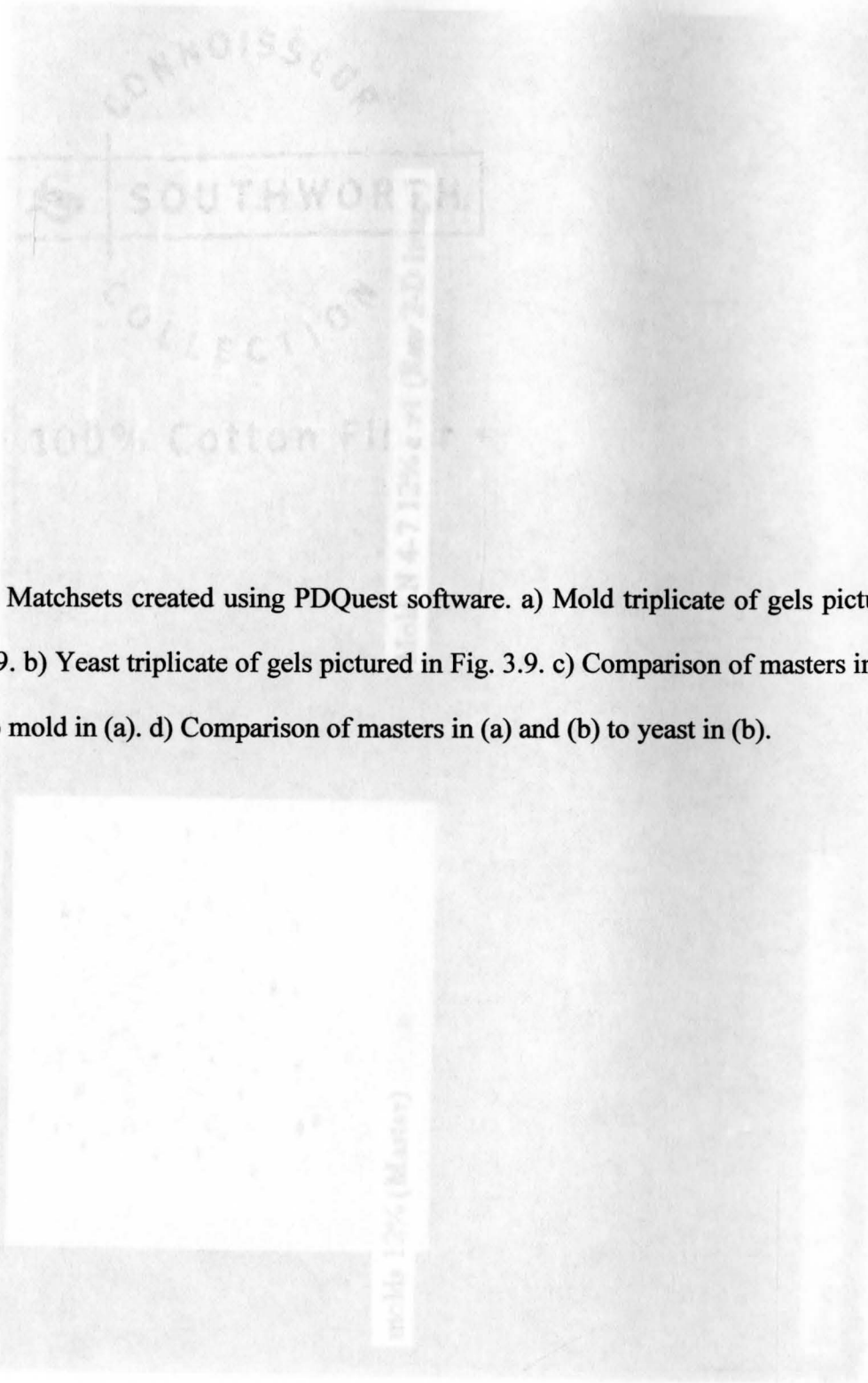


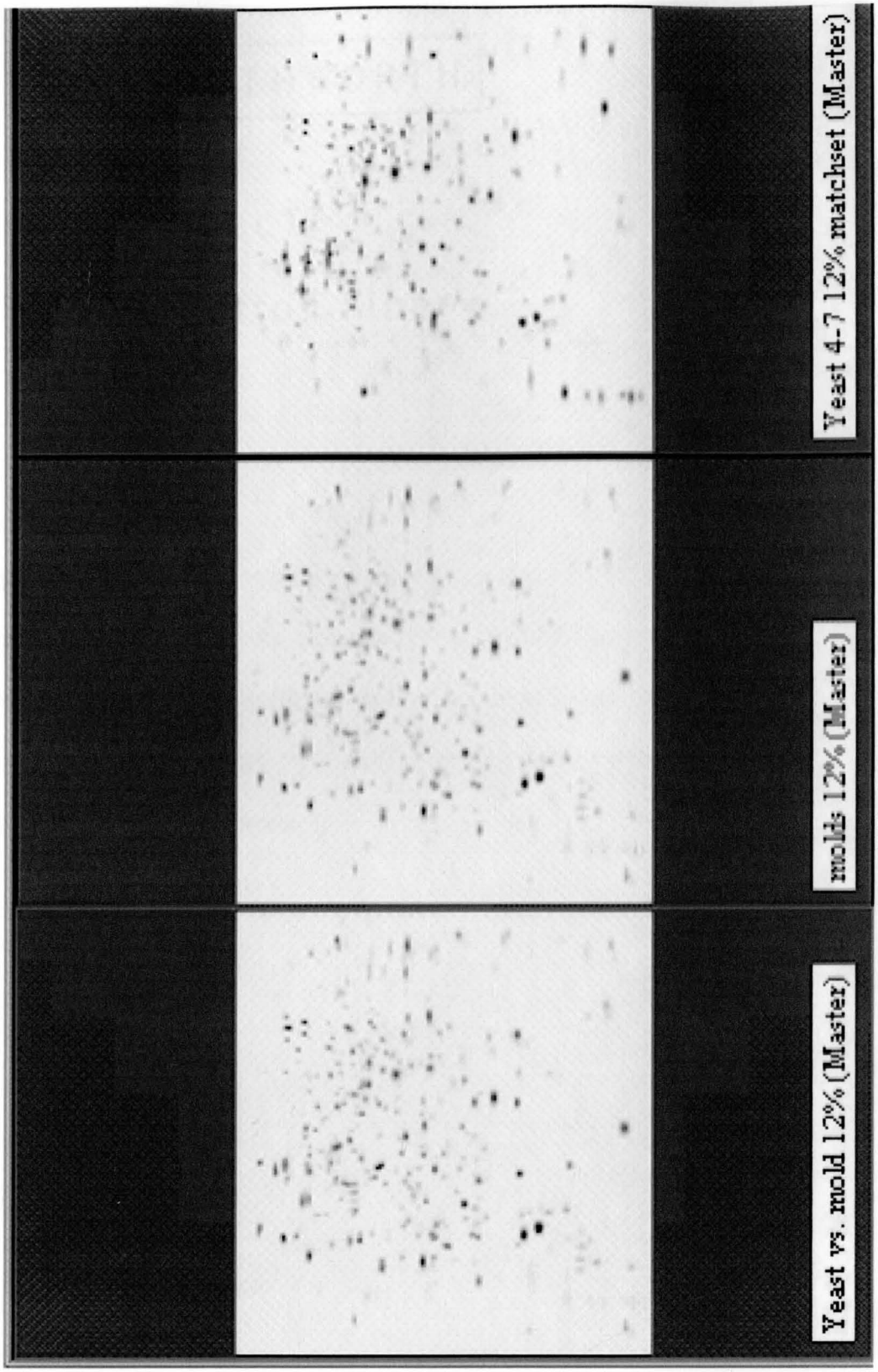
Fig. 3.10 Matchsets created using PDQuest software. a) Mold triplicate of gels pictured in Fig. 3.9. b) Yeast triplicate of gels pictured in Fig. 3.9. c) Comparison of masters in (a) and (b) to mold in (a). d) Comparison of masters in (a) and (b) to yeast in (b).

(a)

molds 12% (Master)
Mold N 4-7 12% c v1 (Raw 2-D Image)
Mold L 4-7 12% c v1 (Raw 2-D Image)
Mold M 4-7 12% c v1 (Raw 2-D Image)

(b)

Yeast 4-7 12% matchset (Master)
Yeast L 4-7 12% c v1 (Raw 2-D Image)
Yeast M 4-7 12% c v1 (Raw 2-D Image)
Yeast N 4-7 12% c v1 (Raw 2-D Image)


Yeast vs. mold 12% (Master)
mold: 12% (Master)
Yeast 4-7 12% matchset (Master)

(c)

(d)

HL Y v M matchset Yeast master
Yeast 4-7 12% matchset (Master)
molds 12% (Master)

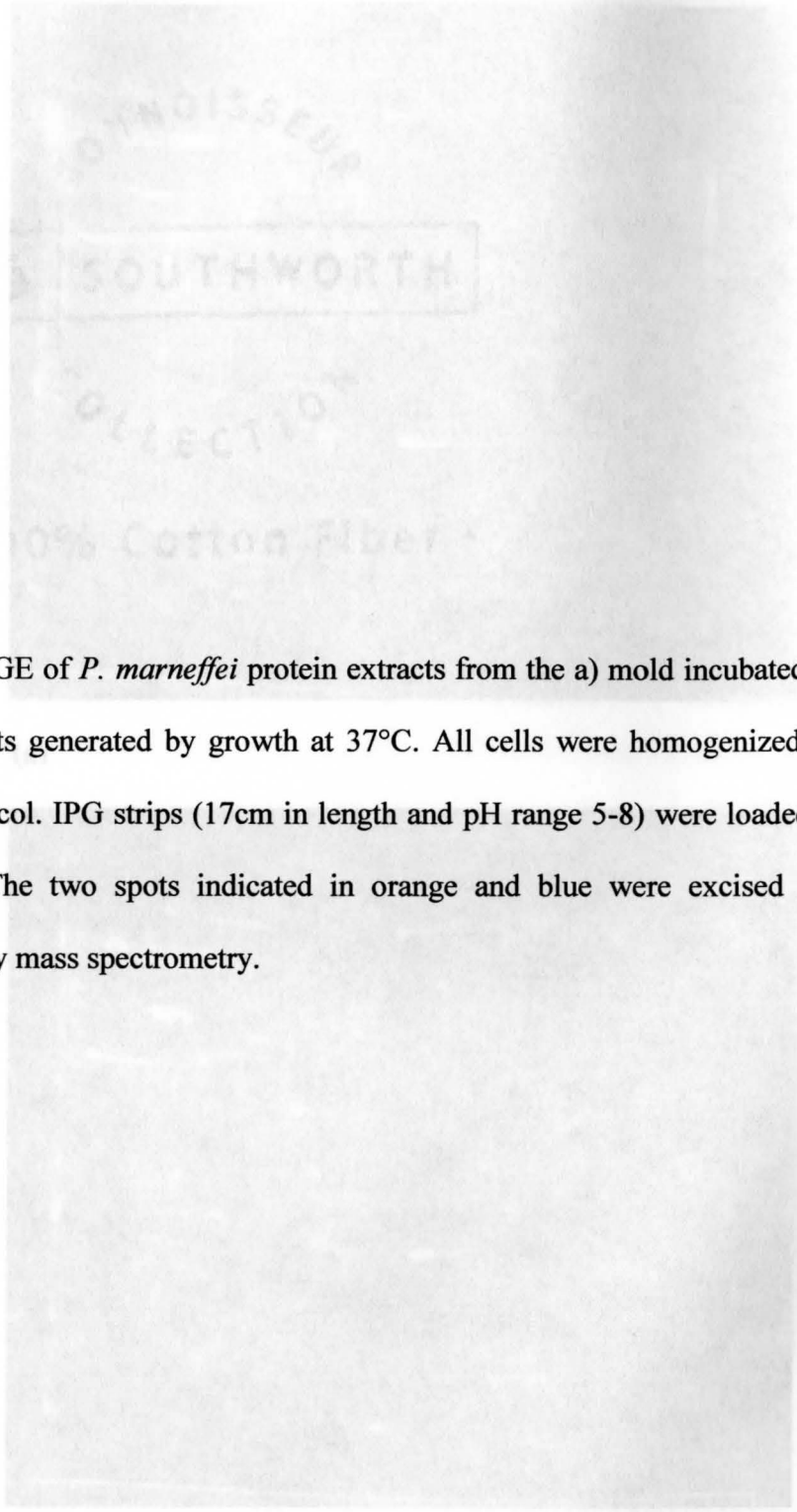
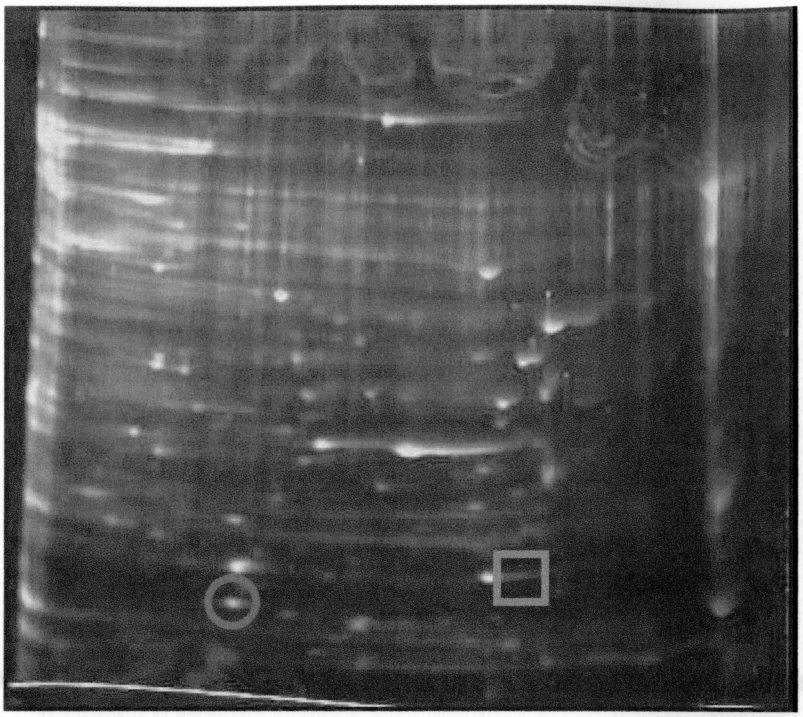
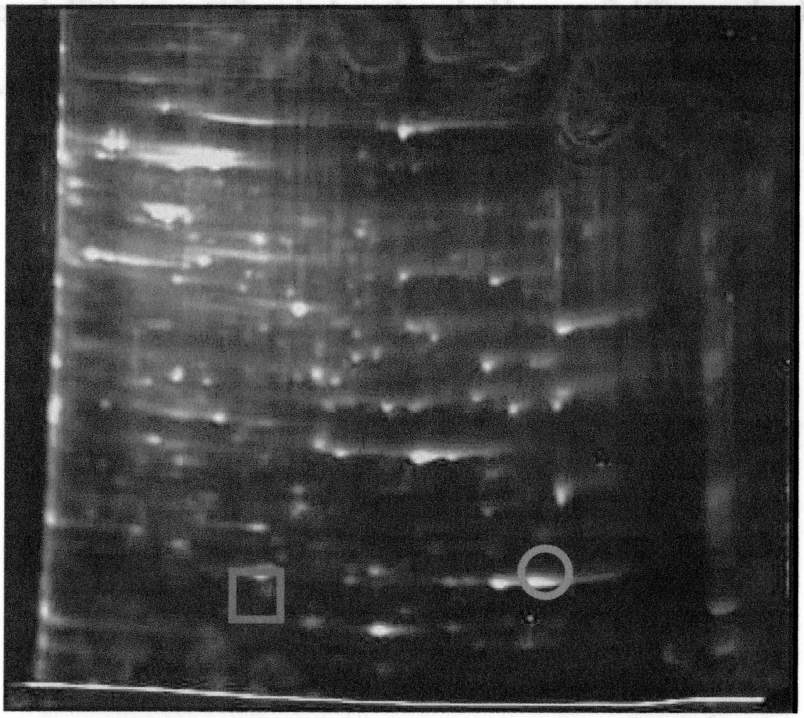


Fig. 3.11 2DGE of *P. marneffei* protein extracts from the a) mold incubated at 25°C and from b) yeasts generated by growth at 37°C. All cells were homogenized by the bead beating protocol. IPG strips (17cm in length and pH range 5-8) were loaded with 225µg of protein. The two spots indicated in orange and blue were excised and sent for sequencing by mass spectrometry.



(a)



(b)

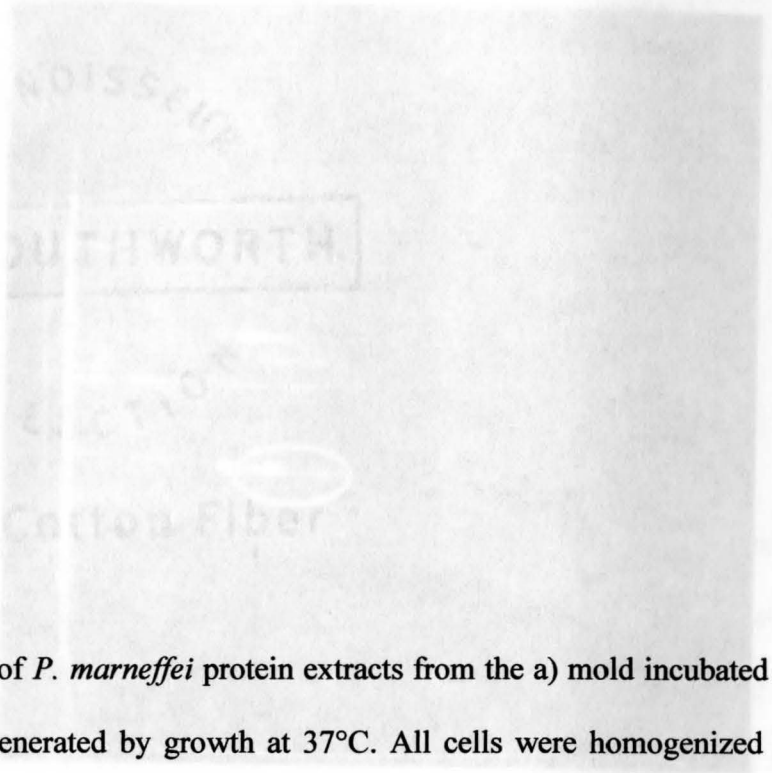
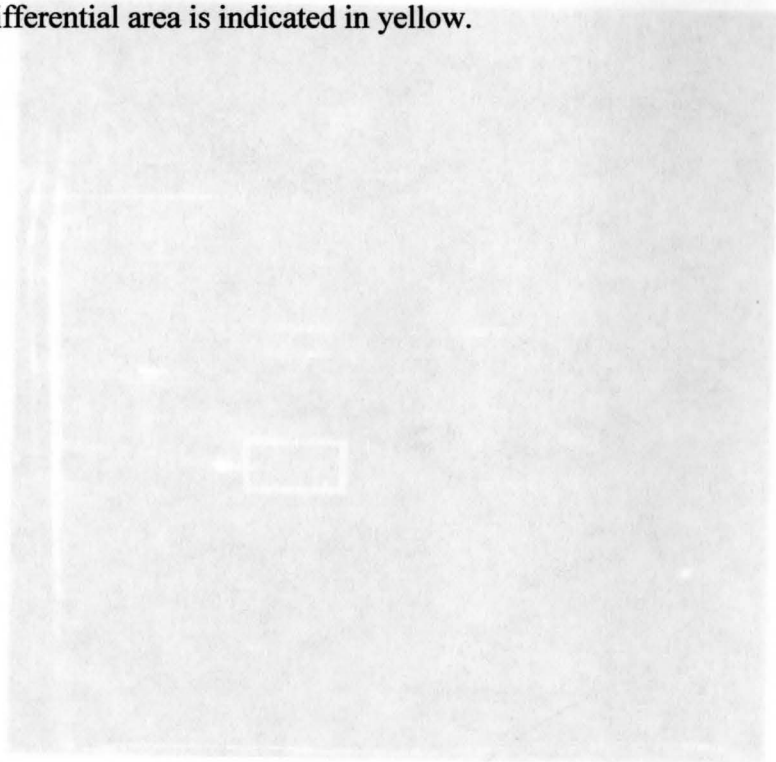
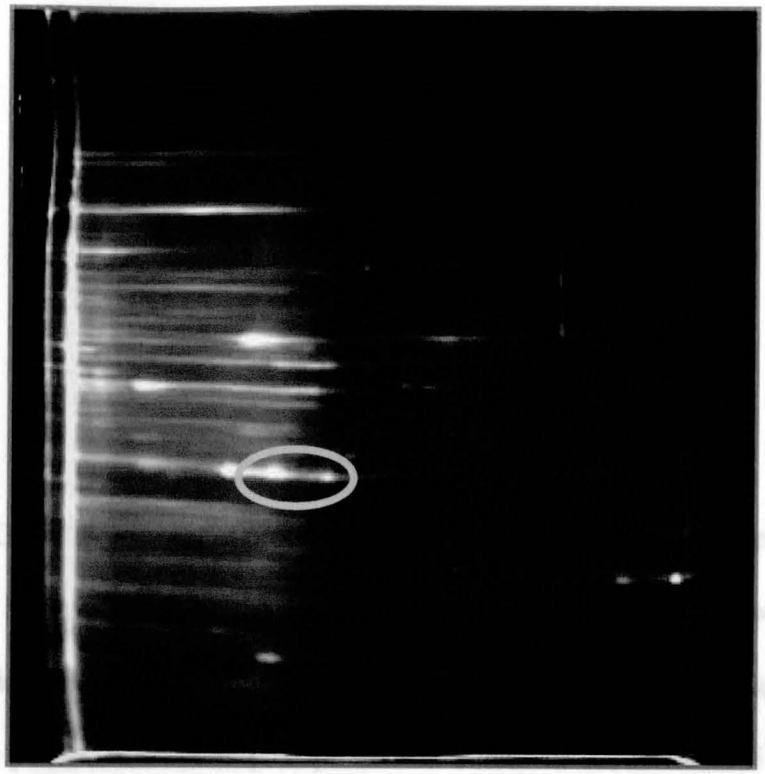
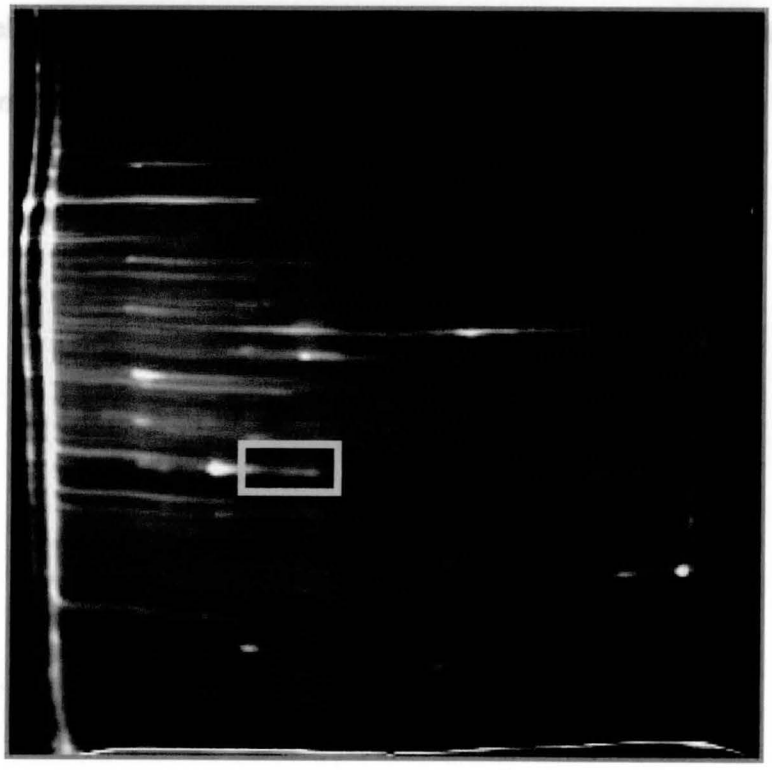


Fig. 3.12 2DGE of *P. marneffei* protein extracts from the a) mold incubated at 25°C and from b) yeasts generated by growth at 37°C. All cells were homogenized by the bead beating protocol. IPG strips (17cm in length and pH range 7-10) were loaded with 225µg of protein. A differential area is indicated in yellow.



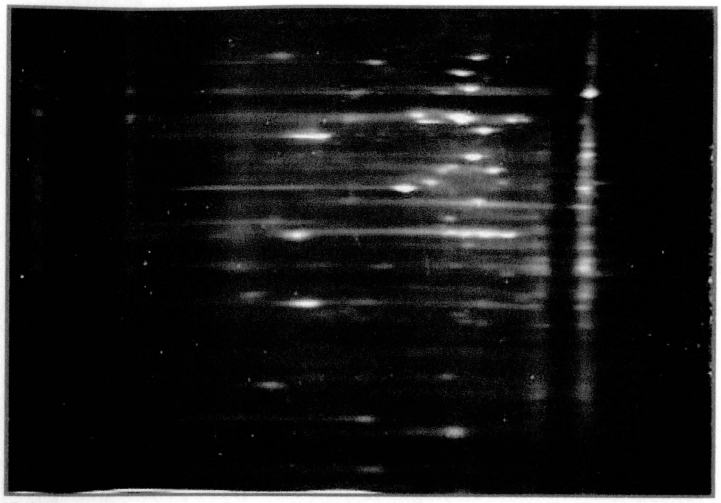


(a)

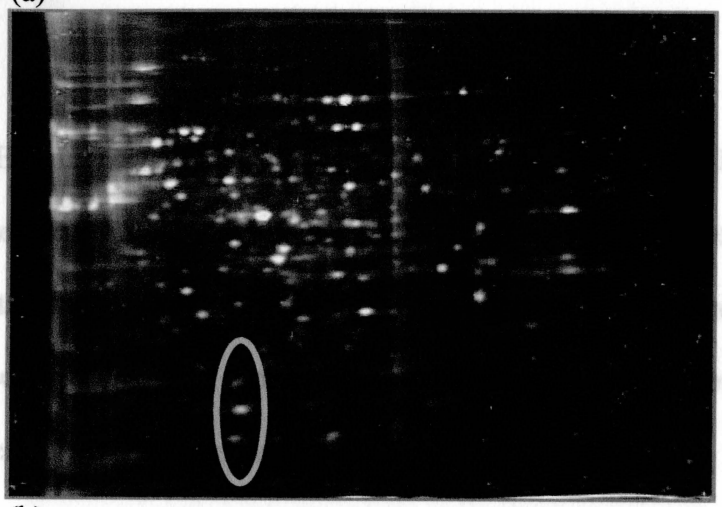


(b)

Fig. 3.13a 2DGE of *P. marneffei* protein extracts from the mold phase incubated at 25°C. All cells were homogenized by the bead beating protocol. Whole cell homogenate was run through liquid IEF prefractionation, the approximate low, middle and high pH fractions were pooled and TCA precipitated as stated in methods. IPG strips, 7cm in length, and pH range a) 3-6 b) 5-8 c) 7-10 were loaded with unknown amounts of protein. This set of gels was not completed in triplicate. The "trio" protein complex observed previously is indicated in pink.



(a)



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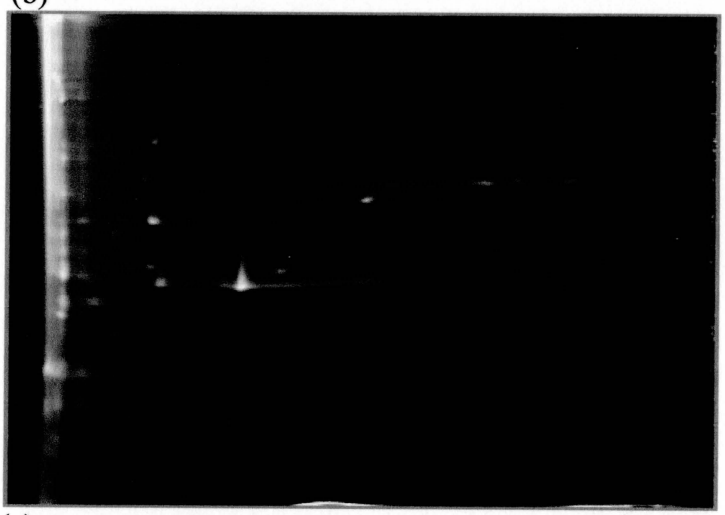


(c)

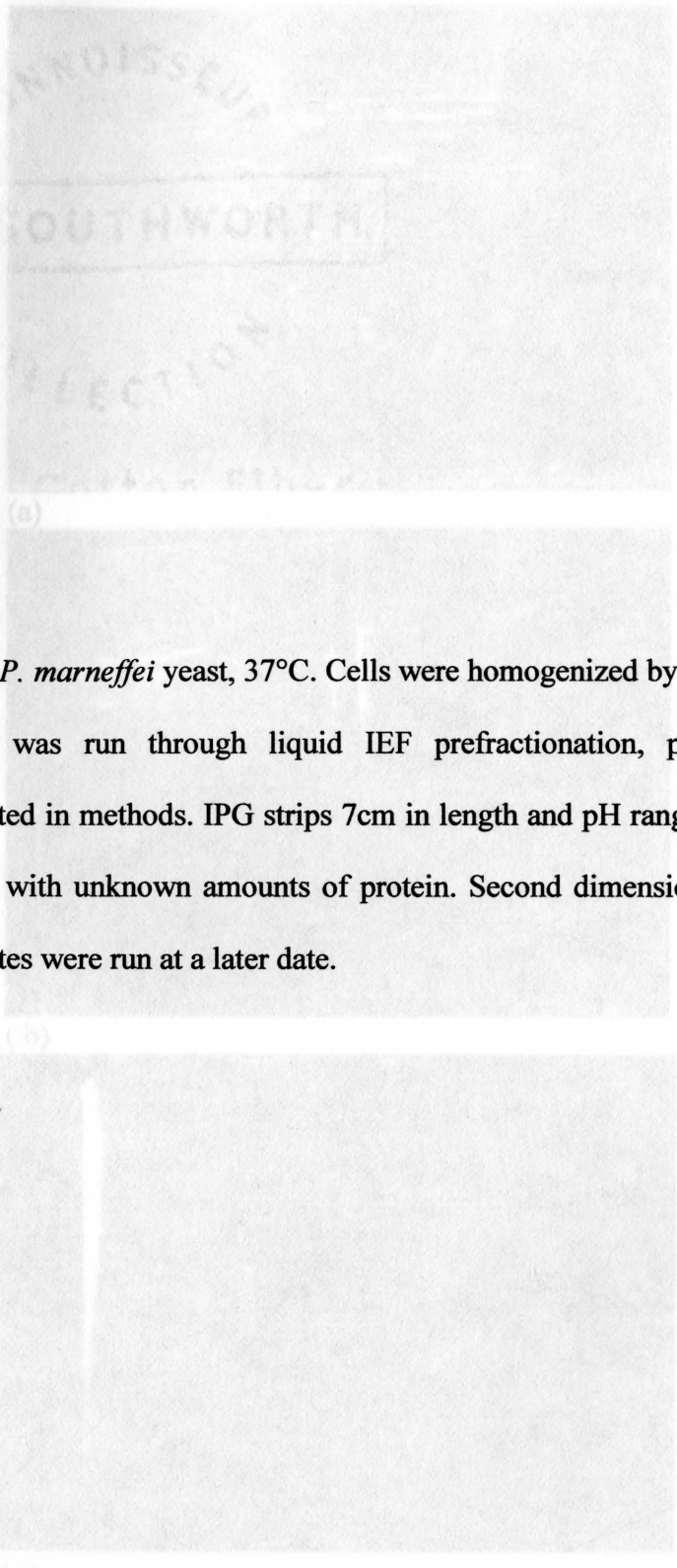
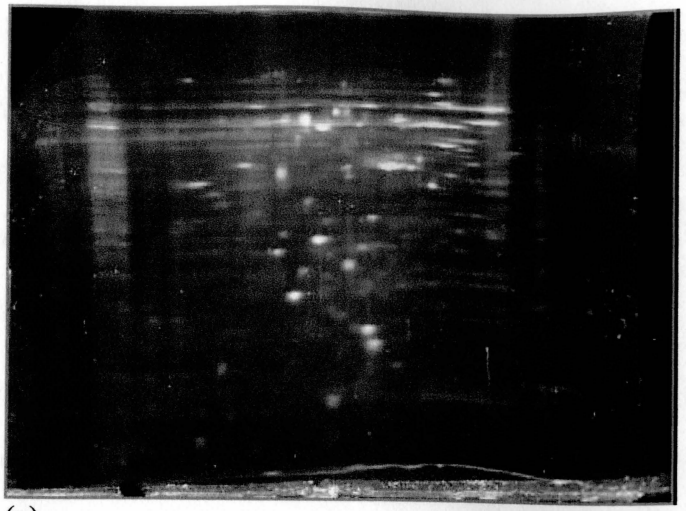
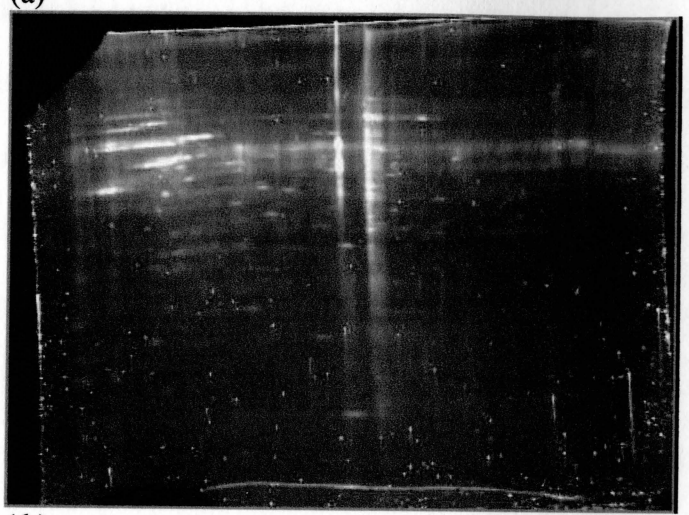


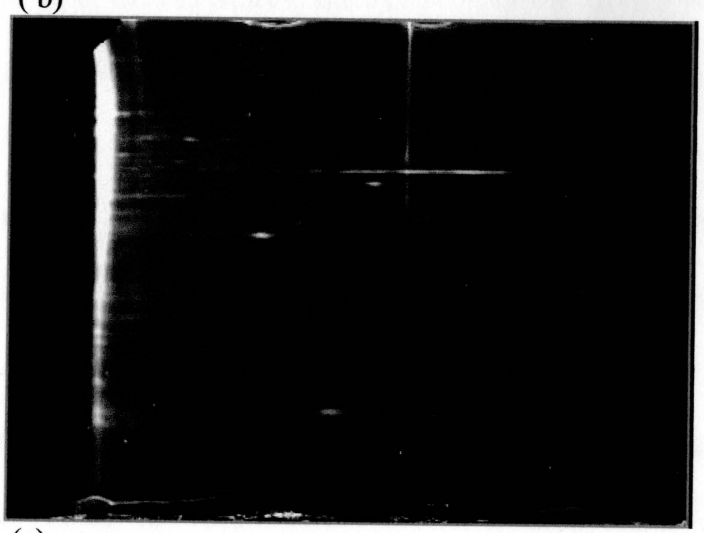
Fig. 3.13b 2DGE *P. marneffei* yeast, 37°C. Cells were homogenized by bead beat. Whole cell homogenate was run through liquid IEF prefractionation, pooled and TCA precipitated as stated in methods. IPG strips 7cm in length and pH range a) 3-6 b) 5-8 c) 7-10 were loaded with unknown amounts of protein. Second dimension was 12% gels. Only 10% duplicates were run at a later date.



(a)



(b)



(c)

CHAPTER FOUR:

Discussion

Throughout this research project all methods used were modified for efficiency as specified in the results. Common problems in the protocols used were also identified and addressed. In working out the protocols, reproducible proteome maps of *Penicillium marneffei* for both the mold incubated at 25°C and for the yeast generated by growth at 37°C were created. These proteome maps were the first of *P. marneffei* to be produced. Proteome maps were completed for several different pH ranges for better separation and resolution (Figs. 3.6-9, 3.11-12). Several protein spots in these proteome maps were identified as differentially expressed proteins.

The collection and growth protocol was the most tedious to refine. First, the right amount of culture needed to yield enough conidia to inoculate broth cultures needed to be determined. In growing any culture, the correct temperature, pH and media must first be established for optimum growth. This became evident when *P. marneffei* cultures seemed to change in gross appearance. The temperature of the environment and the pH, age and content of the media were assessed. It was found that the temperature of the environment and the pH of the water used in making the media had been varying beyond the tolerance of the organism. In addition, the expiration of the media was not conducive to optimum growth.

As discussed in the methods and results, due to poor reproducibility using the sonication method of cell homogenization, a switch to a bead beating protocol was undertaken for the yeast and mold forms of *P. marneffei*. This allowed better reproducibility and better protein yield. In addition, a later modification of air drying the protein pellets in a biological safety hood allowed for better protein solubilization.

The two-dimensional gel electrophoresis protocol was also accompanied with various changes. The most prevalent was that of the cleaning procedure for the gel plates. After finally working out a procedure for this (soap >1 hr, hot water rinse 1 hr, DI water rinse 1 hr) better spot definition and more reproducible proteomes were obtained. In addition, as stated in the results, electrode wicks were used after experiencing much horizontal streaking from the first dimension. These absorbed excess ions in the protein homogenate and thus decreased streaking. A gel multi-caster system was often used when running replicate trials to ensure decreased variability among trials. After obtaining reproducible proteome maps of pH 3-10, smaller pH ranges (pH 3-6, 4-7, 5-8, 7-10) were used to better resolve and spread out the protein spots.

A shelf life of frozen protein homogenate was determined after trying to use protein that was more than one month old. This protein produced unrecognizable protein patterns due to break down of peptide chains resulting in different positioning in the first and second dimension (Fig. 3.5). Following this realization, protein homogenates were discarded after one month.

Throughout this research project, the protein digestion and mass spectrometric analysis protocols were also varied so as to find the most efficient means to obtain significant results. On some occasions, the spot digestion was done at the University of Cincinnati (UC); other times the digestion took place at Youngstown State University (YSU). After mixed results with those samples digested at YSU, all further proteins were digested at the UC.

As protocols were being refined, data was being collected. Once the first differential protein spot (Fig. 3.2a) was identified, excised and sent to the UC for

digestion and mass spectrometric analysis, the resulting mass list was analyzed by a MASCOT database search (http://www.matrixscience.com). This searches for peptide fragment matches between the experimental protein and known proteins. The protein that matched with the excised protein was superoxide dismutase (SOD) found in *Aspergillus fumigatus* and *Emericella nidulans.* In previous studies it was shown that catalase/peroxidase activity, which is similar to the activity of SOD activity, peaked at 24 hrs in the yeast form of *P. marneffei* only (Table 4.1) (23).

Survival of *P. marneffei* requires that the hostile environment of the phagosome not be so hostile (33). Therefore, *P. marneffei* itself must utilize some mechanism to make the environment more hospitable. It is likely that SOD could be involved in that mechanism. SOD, catalase and peroxidase are antioxidants having the ability to prevent damage caused by oxygen radicals (33-34). In the past, antioxidants have been found to be associated with virulence (35). In the normal host, antioxidants are used to destroy harmful by-products of metabolism, such as H_2O_2. However, when oxygen radicals are used for defense, antioxidants can be used by the invader to preempt this attack. It has been hypothesized that fungal catalase ceases oxidative killing and is therefore determinant of fungal virulence (33-34). Since catalase, peroxidase and SOD work in conjunction in oxidative detoxification, it is possible that these three could also work in concert in the evasion of the oxygen radicals found in the phagosome, thus creating a hospitable environment in which *P. marneffei* could flourish. In addition, SOD in *A. fumigatus* has been found to be more efficient at body temperature (34). This phenomenon could explain the presence of SOD in the yeast phase of *P. marneffei* at 24 hours.

Unfortunately, these results were not repeatable since they were obtained prior to switching to the more reproducible bead beating homogenization protocol. After the switch, the proteome was slightly different due to more efficient breaking of cells and collection of more protein. The spot previously indicated as SOD was no longer seen in pH 3-10 gels, at least not in the same position. Assumptions were made as to the new position of the SOD by its brightness (indicated in red in Figs. 3.6-3.7). However, this spot began appearing in both the yeast form and the mold form in both 10% and 12% pH 4-7 gels (Figs. 3.6, 3.9). In 10% pH 3-10 gels seen in Fig. 3.7, it would appear that this spot is only in the yeast. However, it may be that in these gels the spots reside so close to the dye front that the spot in the mold has simply blended with the dye front and is undetectable.

The SOD was rediscovered in 12%, pH 3-10 gels (Fig. 3.8) as was the familiar protein constellation seen previously with the sonication protocol (Fig. 3.2a). Although excision, digestion and sequencing have yet to be performed, it is likely that sequencing will result in the identification of this spot as the SOD due to its pI positioning and brightness in comparison to early gels. Therefore, previous assumptions of what spot was the SOD in 10%, pH 3-10 gels and both 10% and 12%, pH 4-7 gels may have been incorrect.

The matchsets that are seen in Fig. 3.10 give analysis of gels run in triplicate. In Fig. 3.10c, 45% of the spots in the yeast gel were unmatched to the mold. In Fig. 3.10d, 55% of the spots in the mold gel were unmatched to the yeast. These spots are potentially differentially expressed and could possibly be involved in pathogenicity or the different growth phases of *P. marneffei.*

As seen in the results in pH 4-7, 5-8 and 7-10 gels in Figs. 3.9, 3.11-12, three different spots were identified as differential and are indicated in blue, orange and yellow. Further analyses on these spots are pending.

In all smaller pH range gels, accumulations at either end of the pH range are seen (for examples see Figs. 3.6, 3.9, 3.11-12). In the pH 7-10 gels, accumulation at the 7 end denotes all the proteins below pH 7. Similar, yet lesser findings can be seen on pH 4-7 and 5-8 gels. In the interest of resolving this problem, and cleaning up the smaller pH range gels, as indicated in the methods, an additional step was added to the separation. The Rotofor® (Bio-Rad, Inc.) liquid IEF system was used to pre-fractionate the whole protein fraction prior to gel IEF.

The liquid IEF system was incorporated for pre-fractionation of proteins into smaller pH ranges. This step was added to try and alleviate some of the streaking and accumulations seen in the smaller pH ranges when whole protein homogenate was used. When the twenty fractions were pooled into thirds, pH cutoffs should have been present. However, the cutoffs must have been more gradual than first imaged. As can be seen in 7 cm gels in Fig. 3.13, there is still much accumulations at the edges of these smaller pH range gels, although the streaking does seem to be decreased. There are many more trials to run before completely understanding the role of liquid phase IEF in *P. marneffei* research.

Despite these results, there was one interesting result found in the pre-fractionated protein runs. The "trio" seen previously in the pH 4-7 and pH 5-8 whole protein gels was also seen in this pre-fractionated pH 5-8 mold gel and is indicated in pink in Fig. 3.13a.

The "duo" normally seen in the yeast form is not seen in Fig. 3.13b due to a 2DGE problem.

Over the course of this research project, several questions were answered. Most protocols used were refined for efficiency while the use of liquid IEF has only begun to be understood. Clear and reproducible proteome maps of *P. marneffei* yeast and mold forms were created. Several differential spots were identified and one was sequenced and matched. In the course of answering these important questions, several other questions have arisen. First, will the newly rediscovered "SOD" in the 12%, pH 3-10 gels (Fig. 3.8) also be identified as SOD? Then, what are the identities of the newly found differential proteins? Investigations will need to be done as to what their role is in dimorphism and/or virulence of *P. marneffei*. The infection of the macrophage by *P. marneffei* and the proteins expressed in this mechanism are being studied. In addition, follow-up time course studies are being done on both the yeast and mold forms to verify and/or elucidate the meaning of the appearance of catalase/peroxidase activity at 24 hours. With continued study and protein identification, in the future, knockouts/mutants could be made in hopes of finding if the actions of these proteins are actually pertinent to the *in vivo* dimorphism and/or virulence of *P. marneffei*. Following this finding, the results can be related and the techniques can applied to the mechanisms of other dimorphic and pathogenic fungi causing infections in increased numbers of the immunosuppressed populations (4). Possible treatments and preventative medicine may then be soon to follow.

Table 4.1 Enzymatic assay of catalase/peroxidase activity from the cellular extracts of *P. marneffei* cultures of mold incubated at 25°C and of yeast generated by growth at 37°C (23).

Proteins Extracted from:	Catalase/Peroxidase Activity (Units per mg protein) at:			
	12 hours	24 hours	48 hours	72 hours
25°C	0.69 ± 0.12	0.65 ± 0.25	0.40 ± 0.24	0.38 ± 0.32
37°C	1.73 ± 0.38	15.06 ± 1.14	1.39 ± 0.79	0.81 ± 0.08

[a] The average activities (including standard deviation) are shown for two independent experiments. Only the difference in enzymatic activity of extracts prepared from 24 hours samples is statistically significant ($p = 0.003$).

References

1. Blackstock, W. P. & Weir, M. P. 1999. Proteomics: quantitative and physical mapping of cellular proteins. *Tibech.* 17:121-127.
2. Borneman, A. R., Hynes, M. J. & Andrianopoulos, A. 2000. The *abaA* homologue of *Penicillium marneffei* participates in two developmental programmes: conidiation and dimorphic growth. *Molecular Microbiology.* 38:1034-1047.
3. Cash, P. 2000. Proteomics in medical microbiology. *Electrophoresis.* 21:1187-1201.
4. Cooper, C. R., Jr. & Haycocks, N. 2000. *Penicillium marneffei*: An Insurgent Species Among the Penicillia. *J. Eukaryot. Microbiol.* 47: 24-28.
5. Cooper, C. R., Jr. & McGinnis, M. R. 1997. Pathology of *Penicillium marneffei*: An Emerging Acquired Immunodeficiency Syndrome-Related Pathogen. *Arch. Pathol. Lab. Med.* 121:798-804.
6. Borneman, A. R., Hynes, M. J. & Andrianopoulos, A. 2002. A basic helix-loop-helix protein with similarity to the fungal morphological regularotrs, Phd1p, Efg1p and StuA, controls conidiation but not dimorphic growth in *Penicillium marneffei. Molecular microbiology.* 44:621-631.
7. Drouhet, E. 1993. Penicilliosis due to *Penicillium marneffei*: A New Emerging Systemic Mycosis in AIDS Patients Traveling or Living in Southeast Asia. *J. Mycol.* 4:195-224.
8. Imwidthaya, P., Thipsuvan, K., Chaiprasert, A., Danchaivijitra, S., Sutthent, R. & Jearanaisilavong, J. 2001. *Penicillium marneffei*: types and drug susceptibility. *Mycopathologia.* 149:109-115.
9. Kellner, R. 2000. Proteomics. Concepts and Perspectives. *Fresenius Journal of Analytical Chemistry.* 366: 517-524.
10. Supparatpinyo, K., Chiewchanvit, S., Hirunsri, P., Uthammachai, C., Nelson, K. & Sirisanthana, T. 1992. *Penicillium marneffei* Infection in Patients Infected with Human Immunodeficiency Virus. *Clinical Infectious Diseases.* 14:871-874.
11. Yuen, K., Pascal, G., Wong, S. S. Y., Glaser, P., Woo, P. C. Y., Kunst, F., Cai, J. J., Cheung, E. Y. L., Medigue, C. & Danchin, A. 2003. Exploring the *Penicillium marneffei* genome. *Arch Microbiol.* 179:339-353.
12. Cooper, C. R., Jr. 1998. From Bamboo Rats to Humans: the Odyssey of *Penicillium marneffei. ASM news.* 64:390-397.
13. Pracharktam, R., Sriurairatna, S. & Jayanetra, P. 1992. Morphological Variation in Pathogenic Strains of *Penicillium marneffei. J. Med. Assoc. Thai.* 75:172-179.
14. Restrepo, A., Baumgardner, D. J., Bagaglp, E., Cooper, C. R., Jr., McGinnis, M. R., Lazera, M. S., Barbosa, F. H., Bosco, S. M. G., Camargo, Z. P. FR., Coelho, K. I. R., Fortes, S. T., Franco, M., Montenegro, M. R., Sano, A. & Wankes, B. 2000. Clues to the presence of pathogens fungi in certain environments. *Medical Mycology.* 38:67-77.
15. Chan, Y.& Chow, T. C. 1990. Ultrastructural Observations on *Penicillium marneffei* in Natural Human Infection. *Ultrastructural Pathology.* 14:439-452.

16. Todd, R. B., Greenhaigh, J. R., Hynes, M. J. & Andrianopoulos, A. 2003. TupA, the *Penicillium marneffei* Tup1p homologue, represses both yeast and spore development. *Molecular Microbiology*. 48:85-94.
17. Prariyachatigul, C., Chairprasert, A., Geenkajorn, K., Kappe, R., Chuchottaworn, C., Termsetjaroen, S. & Srimuang, S. 2003. Development and evaluation of a one-tube seminested PCR assay for the detection and identification of *Penicillium marneffei*. *Mycoses*. 46: 447-454.
18. Harder, A., Wildgruber, R., Nawrocki, A., Fey, S. J., Larsen, P. M. and Gorg, A. 1999. Comparison of yeast cell protein solubilization procedures for two-dimensional electrophoresis. *Electrophoresis*. 20: 826-829.
19. Roilides, E., Lyman, C. A., Sein, T., Petraitiene, R. & Walsh, T. J. 2003. Macrophage colony-stimulating factor enhances phagocytosis and oxidative burst of mononuclear phagocytes against *Penicillium marneffei* conidia. *FEMS Immunology and Medical Microbiology*. 36:19-26.
20. Andrianopoulos, A. 2002. Control of morphogenesis in the human fungal pathogen *Penicillium marneffei*. *Int. J. Med. Microbiol*. 292:331-347.
21. Nandakumar, M. P. & Marten, M. R. 2002. Comparison of lysis methods and preparation protocols for one – and two-dimensional electrophoresis of *Aspergillus oryzae* intracellular proteins. *Electrophoresis*. 23:2216-2222.
22. Boyce, K. J., Hynes, M. J. & Andrianopoulos, A. 2001. The *CDC42* Homolog of the Dimorphic Fungus *Penicillium marneffei* Is Required for Correct Cell Polarization during Growth but Not Development. *J. Bacteriology*. 183:3447-3457.
23. Husk, T., Kim, T., & Cooper, C. Unpublished data.
24. Bradford, M. M. 1976. A Rapid and Sensitive Method for the Quantitation of microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Analytical Biochemistry*. 72:248-254.
25. Nyman, T. A. 2001. The role of mass spectrometry in proteome studies. *Biomolecular Engineering*. 18:221-227.
26. Yates, J. R. 1998. Mass Spectrometry and the Age of the Proteome. *J. of Mass Spectrometry*. 33:1-19.
27. Bio-Rad ReadyStrip™ IPG Strip Instruction Manual.
28. 2-D Electrophoresis for Proteomics: A Methods and Product Manual. Bio-Rad.
29. Nishihara, J. C. and Champion, K. M. 2002.Quantitative evaluation of proteins in one- and two-dimensional polyacrylamide gels using a fluorescent stain. *Electrophoresis*. 23: 2203-2215.
30. Bio-Rad Rotofor® System Instruction Manual.
31. PDQuest 2-D Analysis Software User Guide. Bio-Rad.
32. Rabilloud, T. Ed. *Proteome Research: Two-Dimensional Gel Electrophoresis and Identification Methods.* Springer, New York, 2000.
33. Pongpom, P., Cooper, C. R., Jr., & Vanittanakom, N. 2004. Isolation and characterization of a catalase-peroxidase gene from the pathogenic fungus, *Penicillium marneffei*. *Medical Mycology*.
34. Hamilton, A. J. & Holdom, M. D. 1999. Antioxidant systems in the pathogenic fungi of man and their role in virulence. *Medical Mycology*. 37: 375-389.

35. Missall, T. A., Lodge, J. K., & McEwen, J. E. 2004. Mechanisms of Resistance to Oxidative and Nitrosative Stress: Implications for Fungal Survival in Mammalian Hosts. *Eukaryotic Cell*. 3:835-846.