Time-Course Based Proteomic Profiling of the Yeast form of *Penicillium marneffei*

by

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ABSTRACT

Penicillium marneffei is a thermal dimorphic fungus that is the causative organism for third most frequent opportunistic infectious disease of the AIDS patient group. It is the yeast form of the fungus that is pathogenic to humans. Proteins both structural and functional of this fungus are vital for its survival and causing pathogenesis in host. The proteins of this fungus are separated at a time-course of 12 hours and 24 hours by two-dimensional gel electrophoresis. In this technique proteins separate according to their net charge in the first dimension and molecular mass in the second dimension. Study of the proteins of an organism in part or in its entirety is called proteomics. Proteomes of 12 hour and 24 hour time course were compared for differential protein expression. Proteins spots unique to a time course were excised and sent for mass spectral analysis. The mass spectrum provides data about the structure of the protein from which the function can be derived. The ultimate goal of knowing the protein structure and function is to understand the molecular mechanisms of the fungal pathogenecity which aids in the development of new drugs caused by *Penicillium marneffei* and device new tests to accurately diagnose the disease.

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List of Abbreviations

ml milliliter

rpm revolutions per minute

EDTA ethylenediaminetetraacetic acid

NaCl sodium chloride

TCA trichloroacetic acid

2-DE two-dimensional electrophoresis

DTT dithiothreitol

CHAPS 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate

µl microliter

µg microgram(s)

nm nanometer

BSA Bovine serum albumin

IPG immobilized pH gradient

cm centimeter

IEF isoelectric focusing

SDS-PAGE Sodium dodecylsulfate-polyacrylamide gel electrophoresis

HCl hydrochloric acid

kDa kiloDalton(s)

ng nanogram(s)

PTM post-translational modification

LC liquid chromatography

MS mass spectrometry

UV ultraviolet

P.marneffei *Penicillium marneffei*

DNA deoxyribonucleic acid

^{0}C degree Celsius

mRNA messenger ribonucleic acid

M molar

V volt

V-hr volt-hour

hr hour

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Chapter-1

Introduction

Ecology of *Penicillium marneffei*

Penicillium marneffei belongs to the euascomycetes class of the fungal kingdom and is geographically restricted to Southeast Asia. The fungus probably exists as a saprophyte bearing numerous conidia in the soil, and can be found in the internal organs of bamboo rats belonging to the species *Rhizomys pruinosus* (1). Penicillium species play an active role in the biodegradation of organic substances in the soil (2).

Penicillium marneffei is found in the alveolar lavage fluid and blood samples of immunodepressed subjects particularly in HIV (Human deficiency virus) positive patients (3). Most of the Penicillium species are ubiquitous; interestingly *Penicillium marneffei* has never been isolated from nature. It is isolated from the organs of bamboo rats and is rarely found in the bamboo rat feces and burrows (2).

Eight strains of *Penicillium marneffei* have been recognized by antifungal susceptibility, chromosomal DNA restriction fragment-length polymorphism types and randomly amplified polymorphic DNA patterns (4). Pulsed-field gel electrophoresis with restriction enzymes is also a method to identify different strains of *Penicillium marneffei* (5). The strain of *Penicillium marneffei* used in our laboratory is *Penicillium marneffei* F4. The uniqueness of *Penicillium marneffei* is that it exhibits thermal dimorphism, unlike the more than 200 other species of Penicillium (6).

Growth and Development

In the laboratory *Penicillium marneffei* grows well on Sabourad dextrose agar medium. Known to be thermally dimorphic, the culture grows as mycelial (mould-like with hyphae) form when grown at 25 ^{0}C characterized by the production of a red-soluble

pigment that diffuses into the medium. At a temperature of 37 ^{0}C, the fungus assumes a yeast form, the colonies of which are beige-colored and do not produce any red pigment.

The mycelial form of the fungus is multinucleate and undergoes asexual development to produce uninucleate spores. While most yeast cells divide by budding, yeast cells of *Penicillium marneffei* divide by fission, which results in a transverse septum to form uninucleate yeast cells termed as arthroconidia. This feature differentiates *Penicillium marneffei* from other fungi. The yeast form of the fungus is pathogenic. Thermal dimorphism is due to changes in morphology and cell shape, a process termed phase transition (7).

A reciprocal shift of incubation temperatures (25 ^{0}C $\leftrightarrow$ 37 ^{0}C) reverses the direction of phase transition. This suggests that the expression of phase-specific genes control the morphogenic conversion (2). An interesting observation was the growth inhibition of *Penicillium marneffei* at a 0.015 to 0.25% concentration of galactose, when galactose is the sole carbon source. The yeast phase cells of *Penicillium marneffei* are normally elongated, but the cells grown beyond the perimeter of the zone of inhibition were rounded and reverted to normal shape after the addition of glucose as carbon source. This ballooning effect could be due to the synthesis of abnormal cell wall components (8).

The mycelial form of the fungus has additional food stored in the form of lipid droplets. These lipids are markedly reduced after the phase conversion which was indicated by staining with Sudan black B stain. This indicates that the dormant mycelial form reserves food material which serves as an energy source during phase transformation. Polar vacuoles are a characteristic feature in the cells of *Penicillium*

marneffei undergoing phase transformation. The yeast phase cells of *Penicillium marneffei* are elongate, the cell wall is single layered rather than double and the yeast phase cells are similar in several aspects to pathogenic dimorphic fungi like *B.dermatidis, H.capsulatum* (9).

Penicillium marneffei Genetics

The switch in mycelial-yeast forms requires regulated changes in cellular polarization and morphology. *cflA* gene in *Penicillium marneffei* encodes a highly conserved Cdc42p - like protein which is a Rho family GTPase in *Saccharomyces cerevisiae* and is needed for changes in polarized cell growth and pseudohyphal development (7).

cflA gene is predicted to encode a 192 - amino acid protein and spans an open reading frame of 819 base pairs, consisting of four exons and three introns. CflA in *Penicillium marneffei* is required for cell polarization ad determination of correct cell shape during yeast-like growth, but not required for dimorphic switching and plays no role during asexual development. The molecular mechanisms of polarity generation are not fully understood. The fungal dimorphism provides an excellent sculpt for the study of morphological changes during polarization and growth (7).

A homologue gene *abaA* of the fungus *Aspergillus nidulans* is responsible for the conidation and dimorphic growth. Deletion of *aba*A gene blocks asexual development at 25 ^{0}C resulting in defective conidiophores and at 37 ^{0}C the yeast form fails to switch from multinucleate to uninucleate form (10).

Pathology, Etiology and Treatment

The diseases caused by Penicillium species are collectively called penicilliosis. Penicilliosis caused by *Penicillium marneffei* is the third most frequent opportunistic infectious disease of the AIDS (Acquired immunodeficiency syndrome) patient group in Southeast Asia, trailing tuberculosis (37%) and cryptococcosis (25%) (2).

The route of transmission and the mechanism of action of *Penicillium marneffei* are not known well. It is believed that inhalation of conidia is a likely route to cause infections in line with other infectious moulds (5). So, it can be regarded as an air-borne infection (11) whose occurrence frequents the tropical rainy season than the dry season (12). The first cases of penicilliosis in HIV-positive individuals were reported in 1988 and *Penicillium marneffei* was first isolated in 1956 from infectious bamboo rats (2).

Penicilliosis caused by *Penicillium marneffei* is manifested by – diarrhea, anemia, skin lesions, weight loss, lymphadenopathy (abnormal enlargement of lymph glands), chronic productive cough, pulmonary infiltrates, pancytopenia, septicemia, hepatosplenomegaly, leucocytosis, intermittent fever with or without chills, pericarditis, multiple subcutaneous abscesses and papule like ulcers. The risk factor is AIDS. *Penicillium marneffei* is primarily a pulmonary pathogen that spreads through blood and is the cause for septicemia in more than half of the HIV patients (13).

The first step in causing the infection is the attachment of conidia to host tissues which may occur by adhesion to the extra cellular matrix proteins laminin and fibronectin via a sialic acid-dependent process. Laminin and fibronectin are glycoproteins present in the basement membrane of cells and become exposed after tissue damage. Immunofluorescence microscopy demonstrates the binding of laminin and fibronectin to

the surface of conidia. The presence of binding sites on the conidial surface was confirmed by plate adherence assay (11). The occurrence of *Penicillium marneffei* in liver suggests its capability to invade the reticuloendothelial system (14).

The presence of *Penicillium marneffei* can be diagnosed microscopically from biopsy specimens of – skin, bone marrow, blood, sputum, bronchoalveolar lavage, lung, liver, lymph nodes (13). The ability to invade the fore-mentioned organs is due to morphological transformation of conidia to parasitic yeast state. Its pathogenecity can be related to nutritional, thermal dimorphism (9).

Currently there is no vaccine available against *Penicillium marneffei*, but Mp1p (encoded by *Mp1* gene) antigen secreted from the cell wall given by intramuscular route offers best protection against Penicilliosis marneffei. This was proved by comparing the serum immunoglobulin levels produced by the administration of Mp1 DNA vaccines via oral, intramuscular and intraperitoneal routes. Intramuscular route produced the highest level of serum immunoglobulins (15).

Penicillium marneffei is highly susceptible to itraconazole, ketoconazole, miconazole and 5-fluorocytosine; moderately susceptible to amphotericin B and resistant to fluconazole (13). The risk of relapse underlines the importance of secondary prophylaxis in immunodepressed subjects (3). An effective treatment would be initial therapy with amphotericin B followed by maintenance regimen with itraconazole, fluconazole and ketoconazole. The success depends upon the rapid and accurate diagnosis of the organism (13). The availability of iron is crucial for the survival of *Penicillium marneffei* in human host. The human body's natural defense withholds the

availability of iron to the fungus through sequestration. Exogenous iron enhances the growth and iron chelators inhibit the extra cellular growth of *Penicillium marneffe*i (16).

Significance of the Analysis of the Fungal Proteome

Proteomics is the large scale characterization of the entire protein complement of a cell line, tissue or organism. A proteome can be defined as a total set of proteins in a cell line, tissue or organism. It is analogous to the word genome in genomics. In simple terms it is a "snapshot" of the protein environment at any given moment (17). A genome is a constant while a proteome is a variable. A proteome differs from cell to cell, and changes constantly through biochemical interactions, environment, and different stages of life cycle. For example, the human body has ~ 400,000 proteins for ~ 22,000 genes (almost 18 proteins for a single gene). So, the principle of 'one gene-one protein' is not justified. This implies that protein diversity cannot be fully characterized by gene-expression analysis alone, which is why proteomics is a good technique for characterizing cells and tissues.

Proteomics is a good separation technique as it resolves complex mixtures of proteins according to isoelectric point, molecular mass, solubility and relative abundance (18). Proteins are responsible for the phenotype of a cell, so studying genome alone would not yield total information about the mechanisms of disease, effects of environment on cell. The proteome yields information about protein function, protein modifications, protein localization and compartmentalization, protein-protein interactions, protein-signaling and protein-drug interactions. The proteome of the yeast form of *Penicillium marneffei* grown at different time courses is compared and the

information from this approach can identify novel proteins in signal transduction or identify disease-specific proteins. Protein identification provides immediate information that directs subsequent experimentation. The proteome information can be used to construct a 3-dimensional protein map of the cell indicating protein location in the cell. Three-dimensional protein maps provide information about protein regulation. Protein expression proteomics, structural proteomics, functional proteomics are different types of proteomics (17).

Studies show that there is poor correlation between mRNA and protein expression levels. This is because mRNA is subjected to posttranscriptional changes like alternative splicing, polyadenylation, mRNA editing and nearly 200 types of posttranslational modifications (17). The greatest advantage of proteomics is that proteins undergoing post-translational modifications can be readily located and studied. Post-translational modifications frequently appear as distinct rows of spots in the horizontal and/or vertical axis of the 2-DE gels. Appearance of partly homologous peptide sequence in more than one excised protein spots during mass spectrometry confirms this event (18). Phosphorylation changes in a cell can be studied by proteomics. The phosphoproteins of these cells (eg. normal and diseased) are radiolabeled in vivo with inorganic ^{32}P. The phosphoproteomes are then obtained from the cell lysates and examined by 2-dimensional electrophoresis and autoradiography (17).

Immobilized pH gradients, specialized pH gradients, and fluorescent dyes that have greater sensitivity for protein detection are some improvements made in proteomics over the years (17). The ambiguity of multiple proteins in a single protein spot can be resolved by the use of narrow range IEF gel strips also called zoom-in gels which are

available in the pH interval varying by one unit (18). A recent development is the development of a new technique called DIGE – difference gel electrophoresis. In this technique two protein samples are tagged with two different fluorescent dyes differing in excitation and emission wavelength and run in the same 2-DE gel. A third dye containing both protein samples can also be added that acts as an internal standard to normalize data by minimizing the experimental variation and increasing the confidence in matching spots. The differential fluorescent tagging is taken advantage to create two images which are then analyzed. This prevents the necessity to run multiple gels for different protein samples (17).

Proteomics is beneficial over LC-MS because molecular mass and isoelectric point information is lost in the latter (18). The combination of proteomics with yeast genetics is a powerful tool in studying in vivo cell signaling events. The functions of enzymes can be understood by this combined technique. For example an enzyme (which is a protein) is subjected to changes in structure or function by deletion of a portion of gene. The target protein – substrate of this enzyme can be recognized by studying the 2-DE protein maps of the normal and the mutant enzyme (17).

Chapter-2

Materials and Methods

Cell culture and Isolation

Conidia were initially grown in culture flasks with solidified potato dextrose agar medium (Difco laboratories, pH 5.6 ± 0.2) for a period of 8 days at room temperature (25^0C). This was done by swabbing the hyphal growth from an existing culture and streaking on fresh medium with a cotton swab moistened with sterile millipore water. Up to six such flasks were prepared under a biological safety cabinet sterilized by ultraviolet radiation and 70% ethanol. The screw caps were needed to remain air-tight. This step can be called sub-culturing.

The conidia were scraped in sterile millipore water. The conidial suspension was filtered by centrifugation at a speed of 900-1100 rpm and a temperature of 4^0C to remove unwanted waste and collect conidia. The number of conidia required for a milliliter of Sabourad broth medium is 10^7. The conidia were counted on a Neubars chamber (hemocytometer) and added to Sabourad broth medium (Difco laboratories, pH 5.6 ± 0.2). A sample calculation is explained in the appendix. The conidial suspension was centrifuged for 5 minutes at a speed of 3000 rpm to get a pellet which was added to the Sabourad broth medium. The Sabourad broth medium was incubated at a temperature of 37^0C in a rotary shaker water bath (Boekel Grant) for 12 hours and 24 hours.

The yeast cells of *Penicillium marneffei* were collected by ultracentrifugation (Sorvall) after a time period of 12 hours and 24 hours. The cells were washed with tris-EDTA buffer thrice. The centrifuge was run at a speed of 15000 rpm at a temperature of 4^0C for 15 minutes during every wash. The supernatant was discarded and the cell pellets were frozen at -83^0C till subsequent steps were performed.

Homogenization and Protein Quantization

Osmotic lysis, freeze-thaw cycling, detergent lysis, enzymatic lysis of the cell wall, sonication, grinding cells with or without nitrogen, high pressure, nitrogen cavitation, rotating blade homogenizer, homogenization with glass beads are the methods adopted for cell lysis (18). We used homogenization with glass beads technique to lyse the cells of *Penicillium marneffei* in our laboratory. Cell pellet with glass beads and lysis buffer (tris-HCl, NaCl, deoxycholate, protease inhibitor cocktail) was subjected to vortexing at a speed of 5000 rpm on a mini-bead beater (Biospec) for 4 minutes (30 seconds beat alternating with 30 second cooling on ice).

The solution from the above step was centrifuged (10 minutes, 6000g, 4^0C) and the supernatant was precipitated with 20% v/v ice-cold TCA (20 minutes on ice). The protein pellet was recovered by centrifugation and washed thrice with ice-cold acetone. This eliminates high amount of lipids that may interact with membrane proteins and consume detergents that are added in the next steps. Polysaccharides and nucleic acids are removed by acetone wash and TCA precipitation. The protein pellet was either air-dried or dried under vacuum in a centrifuge (Labconco). After cell disruption the protein pellet was solubilized in modified sample buffer (urea, thiourea, CHAPS, DTT). The solubilized protein sample was stored in frozen aliquots at -83^0C.

The protein samples were quantitated by modified Bradford assay method. Bovine serum albumin standard solution (8-12 µg/µl) was used to obtain standard absorbencies. BSA (10, 15, 20, 25, 30 µg) was taken in small test tubes to which 10µl of 2-DE buffer (urea, thiourea, CHAPS, spermine base, DTT), 10 µl of 0.1M Hydrochloric acid, 80 µl of distilled water, 4 ml of Bradford dye were added. A blank solution was

prepared with all the above contents except protein sample or standard BSA. Absorbencies were measured by an ultraviolet-visible spectrophotometer (Hewlett Packard Diode Array 8453) at a wavelength of 595 nm. A standard curve was created with which the protein concentrations were calculated (Fig. 4.1) (Table 4.1).

Two – dimensional Electrophoresis

An immobilized pH gradient (IPG) gel strip was rehydrated with 300 µl of rehydration buffer (urea, CHAPS, DTT, biolytes, bromophenol blue), overlaid with mineral oil overnight. 250 µg of protein solution of 12 hour and 24 hour *Penicillium marneffei* yeast cells was loaded into the wells of the IEF focusing tray and moist paper wicks (Bio-Rad) were placed on the terminals to absorb any excess ions that cause streaking. Previously rehydrated IPG gel strips were then placed into the wells of IEF focusing tray and overlaid with mineral oil to prevent frying. Isoelectric focusing was run in a PROTEAN IEF cell (Bio-Rad) run under the following conditions –

Start voltage – 0 V

End voltage – 10,000 V

Volt-Hours – 40-60,000 V-hr

Ramp – linear

Temperature - 20^0C

These conditions vary according to the size and pH range of the IPG gel strips. A higher focusing temperature can cause the risk of protein carbamylation and lower temperature can cause urea crystallization on the IPG gel strip. A temperature of 20^0C is ideal to run IEF. The protein load for IEF should not exceed 10 µg/µl (18). After the

first dimension was complete the excess mineral oil from the IPG gel strips was drained and they can be directly used to run SDS-PAGE or frozen at -83^{0}C till they are used.

The IPG gel strips were equilibrated before the second dimension was run. The IPG gel strips were soaked in equilibration buffer 1 (tris-HCl, SDS, DTT, urea, glycerol) and equilibration buffer 2 (tris-HCl, SDS, iodoacetamide, urea, glycerol) for a time period of 15 minutes each.

Equilibrated IPG gel strip was loaded on the polyacrylamide gel and covered with overlay agarose. This setup was placed in a tank (Bio-Rad) filled with 1X Tris-Glycine-SDS buffer and a current of 10 milliamperes per gel was applied. The second dimension was run at a temperature of 4^{0}C as the heat generated could degrade the proteins. The IPG gel strip itself acts as a stacking gel, so a separate stacking gel was unnecessary. The gels from the second dimension were removed from gel plates and protein spots on the gel were fixed by immersing in a solution of acetic acid, methanol and water for an hour. The fixing solution was removed; the gel was immersed in Sypro Ruby stain (Bio-Rad) and allowed to shake overnight on an orbital shaker. The gel was rinsed with copious amounts of water and water was re-added to store the gels.

Image Analysis

The 2-DE gels after destaining were imaged using a CCD (charge coupled device) camera that is available from Bio-Rad Laboratories. The CCD camera uses ChemiDoc XRS software for imaging and has a resolution of 1.3 mega pixels. It is supported by PDQuest software and has a radiation detector that converts signals from 2-DE gels into digital data. The 2-DE gel was carefully positioned on the imaging tray already

moistened with distilled water to allow easy movement of the gel without ripping it. Imaging-area dimensions and sharpness were set for a clear view of the gel image. The images were captured in presence of UV light and saved to the computer. The 2-DE gels are analyzed by using PDQuest 2-D software (version 7.1, compatible with Windows and Macintosh operating systems) purchased from Bio-Rad Laboratories. PDQuest is a software for imaging, analyzing and data basing 2-DE gels.

Spot Excision

Proteins spots of interest were excised using a sterile Pasteur pipette and put in labeled sterile microcentrifuge tubes. They were sent for mass spectral analysis.

Chapter-3

Results and Discussion

2-DE Gel Analysis

The research work compares the proteomes of the yeast form of *Penicillium marneffei* at various time-courses. All trials were performed in quadruplicates to exclude any errors and artifactual spots and to ensure reproducibility. The spots (proteins) on the gels (12 hour versus 24 hour) were compared one against other using PDQuest software. PDQuest uses sophisticated algorithms for spot detection and matching spots across gels. Initially coomassie stain was used but the use of Sypro Ruby stain provided gels with better spot resolution and clarity (Figure 3.1). The raw images contain horizontal and vertical streaks, speckles, overlapped spots which interfere with spot comparison. Spots can still be compared using raw images (Figure 3.2). Ellipses and boxes show the variation of the spot intensity with time (Figure 3.2). PDQuest software eliminates background noise and creates synthetic images for spot comparison. Filtered and Gaussian images are synthetic images. Raw, filtered and Gaussian images (with Gaussian spots) together are called a scanset (Figure 3.3.). Filtered image is a raw image whose background clutter has been removed A Gaussian spot is a 3-dimensional representation of an original spot. All analyses and comparisons in PDQuest are performed via Gaussian images.

To obtain high-quality results multiple gel images of a single time point were collected under a single header called a matchset. A master image and its member gels together are called a matchset. A master image is a synthetic image which is a representative of all the member gels and contains spot data from all the member gels. One of the member gels can be chosen as a master gel. Spot editing can be performed on a matchset. At this point there are two matchsets, one for 12 hour and the other for 24

hour (Figure 3.4, Figure 3.5). These matchsets are called lower level matchsets. Matchset information lists the total number of spots matched to all gels and the overall mean coefficient of variation of all the members of the matchset (Table 3.1, Table 3.2). The variation among the member gels of a matchset must be low for superior results. Match Rate 1 – Shows percentage of matched spots in the gel relative to the total number of spots on the gel. Match Rate 2 – Shows percentage of matched spots in the gel relative to the total number of spots on the master (19). Trial-10 of the 12 hr time-course and trial-13 of 24 hr time-course have a 100% match rate as they are the masters. In a lower level matchset comparison is done among members of the same time-course. PDQuest software assigns standard spot number (SSP) to every spot on the master image. SSPs are unique numbers; the lower left region of the image has low SSP numbers while the upper right region has high SSP numbers (Figure 3.6).

Comparison between the 12 hr and 24 hr trials is made with the higher level matchsets. Higher level matchsets were created from the master images (12 hr and 24 hr) of the lower level matchsets, which is why higher level matchsets have Gaussian images only and do not have raw and filtered images (Figure 3.7). Matched spots are displayed as alphabets in green, unmatched spots in red ellipses and green triangles represent landmarked spots (Figure 3.8). Landmarks are reference spots used to align and position the gels for matching. Landmarking is done on all the corners and middle of the gels. Spots those are well resolved and present in both member gels are chosen as landmarks.

Twelve hour trials have an average of 78 protein spots. All protein spots are in the pH range ~ 4 to ~ 8. The largest part of the spots are in the pH range ~ 5 to ~ 7, which indicates that *Penicillium marneffei* 12 hour proteins have isoelectric points in the

moderately acidic pH to neutral pH. The 12 hr trial has few proteins in the alkaline pH and there is only one protein spot in the pH ~9 range (Figure 3.9). 24 hr trials have an average spot count of 137, which is nearly an 80% raise when compared to the 12 hr trials. This clearly shows that the protein expression amplifies as the yeast ages from 12 hours to 24 hours. All the proteins in 24 hr trials have isoelectric points in the pH range ~ 4 to ~ 8 and vast part of the proteins fall in the pH ~ 5 to ~ 7 ranges. These observations are analogous to 12 hr trial. 24 hr yeast form appears to have somewhat more proteins in the alkaline pH than its counterpart 12 hr yeast. Out of the 141 protein spots in 24 hour trial, 68 spots matched with the 12 hr trial (Table 3.3). This indicates that *Penicillium marneffei* F4 yeast form after a 24 hour growth still conserves 87% of its proteins from that of 12 hour growth. This is still significant because the 24 hour trial has 73 unmatched spots, which means after a growth period from 12 to 24 hour nearly 50% of the proteins are new. Spots that vary distinctly i.e. up/down regulate, totally appear/disappear between time courses are indicated with ellipses and boxes in figure 3.9.

The appearance and disappearance of protein spots provides information about differential protein expression, the up/down regulation of spots provides quantitative information about the proteins. This type of proteomics is called protein expression proteomics. These unique proteins could have been synthesized newly or could be due to a post-translational modification. These unmatched spots are of significance to us because microscopical observation discloses that after 12 hr growth the *Penicillium marneffei* cells are not distinctly yeast forms, they are merely swollen conidia with tiny isotropic growth. There was not a great deal of success in taking the picture of the cells under microscope. After a 24 hr growth they were unambiguous yeast like cells. Since

the yeast form of this fungus is pathogenic, proteins unique to 24 hr trial may be the cause for dormant conidia assuming the pathogenic yeast form. Although there were many spots that differed between the 2-DE gels in the 12 hr and 24 time-courses, only five protein spots were excised as there was a 3 mm spot free radius around them making the excision easy for human hand (Figure 3.10). The mass spectral analysis results are pending. The mass spectrum provides peptide mass and aminoacid sequence of the excised spots. These data from unknown proteins when compared with known proteins in genomic databases should provide information about the structure and function. This information is valuable because it helps to understand the mechanisms by which *Penicillium marneffei* renders virulence and survives in the host. The structural information of the proteins also provides an ideal target for the development of new drugs or improving the efficacy of already existing ones. It also aids in improvising diagnostic tests. Nevertheless it could also help understand the mechanisms of other dimorphic and pathogenic fungi. Proteomics is an excellent research method because proteins which are the final products of gene expression are studied and understood.

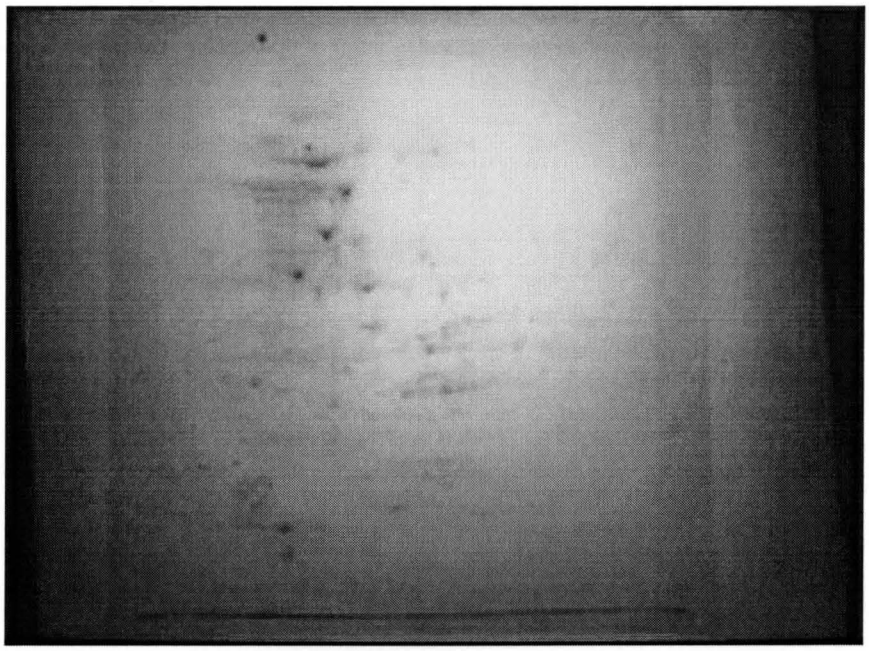
Future Work

Proteomics of *Penicillium marneffei* can also be performed at different time courses (36, 48, 72 hrs) and under different conditions like – treatment with drugs, inhibitors, poisons, chemical mediators, inducers, heat shock, stress etc. Using zoom-in IEF gel strips (narrow pH ranges) for IEF, cellular subfractionation and prefractionation of proteins in proteomics is also a good idea.

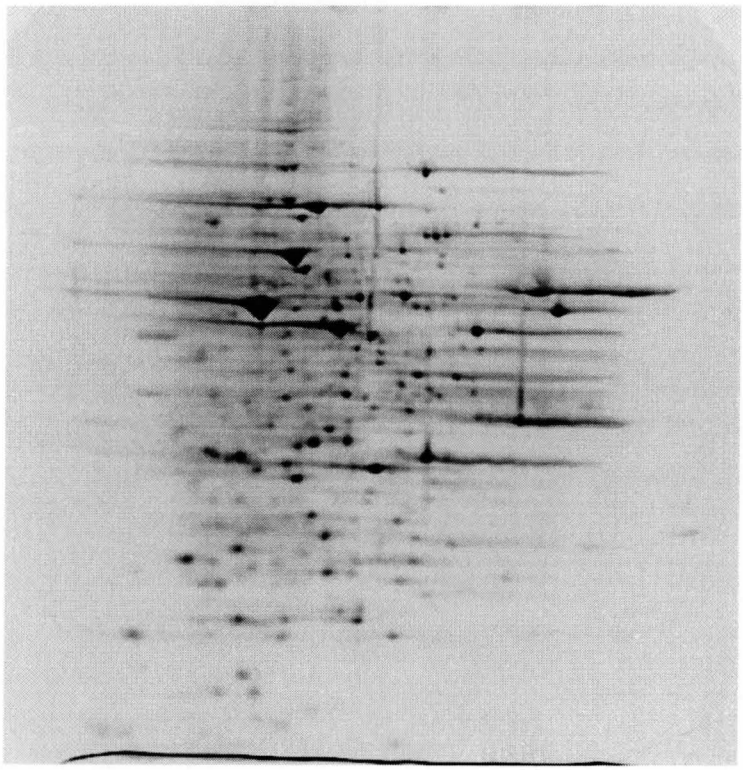
Draw Backs

As there is no equivalent of Polymerase chain reaction for proteins low-abundancy proteins are difficult to study as they might not show up in 2-DE gels. Proteomics is a time-consuming process as it is a multi-step process and less automated. The proteins need to be in their native state conformation to provide accurate results, but the denaturation and separation steps in proteomics may distort the proteins (17).

Figure: 3.1: Two-DE gel images of *Penicillium marneffei* F4 yeast proteins stained with Coomassie and Sypro Ruby stains.

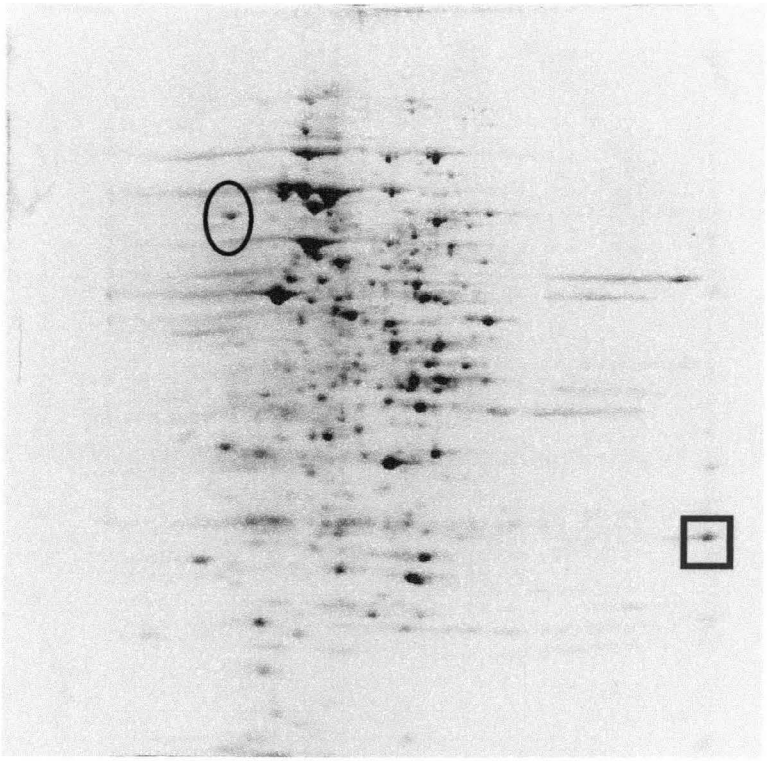


12 hr trial stained with coomassie

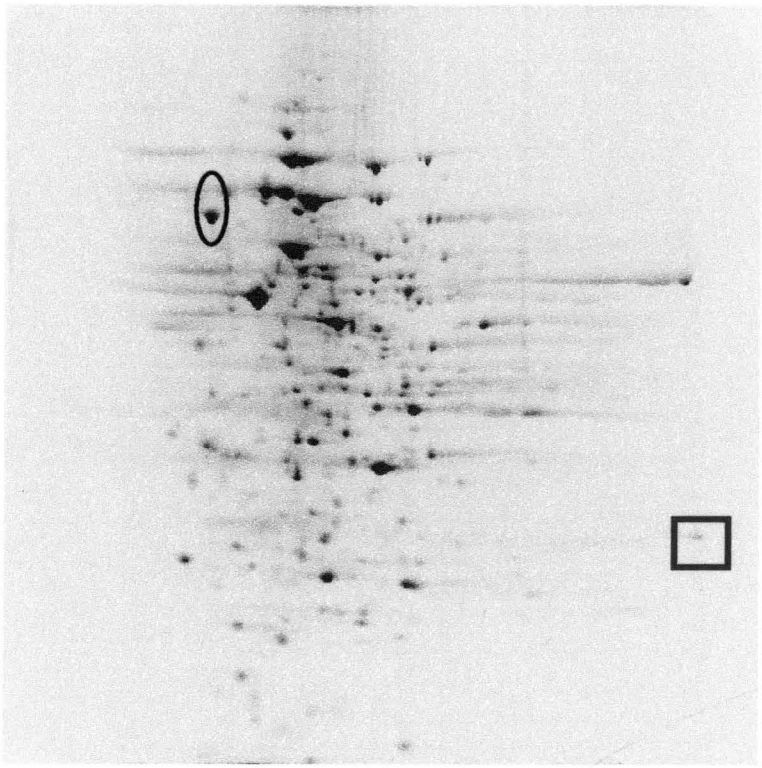


12 hr trial stained with Sypro Ruby

Figure: 3.2: Spot comparison between 12 hr and 24 hr *Penicillium marneffei* F4 yeast 2-DE raw gel images.



12 hr trial



24 hr trial

Figure 3.3: Scanset of a 12 hr trial showing raw, filtered and Gaussian images.

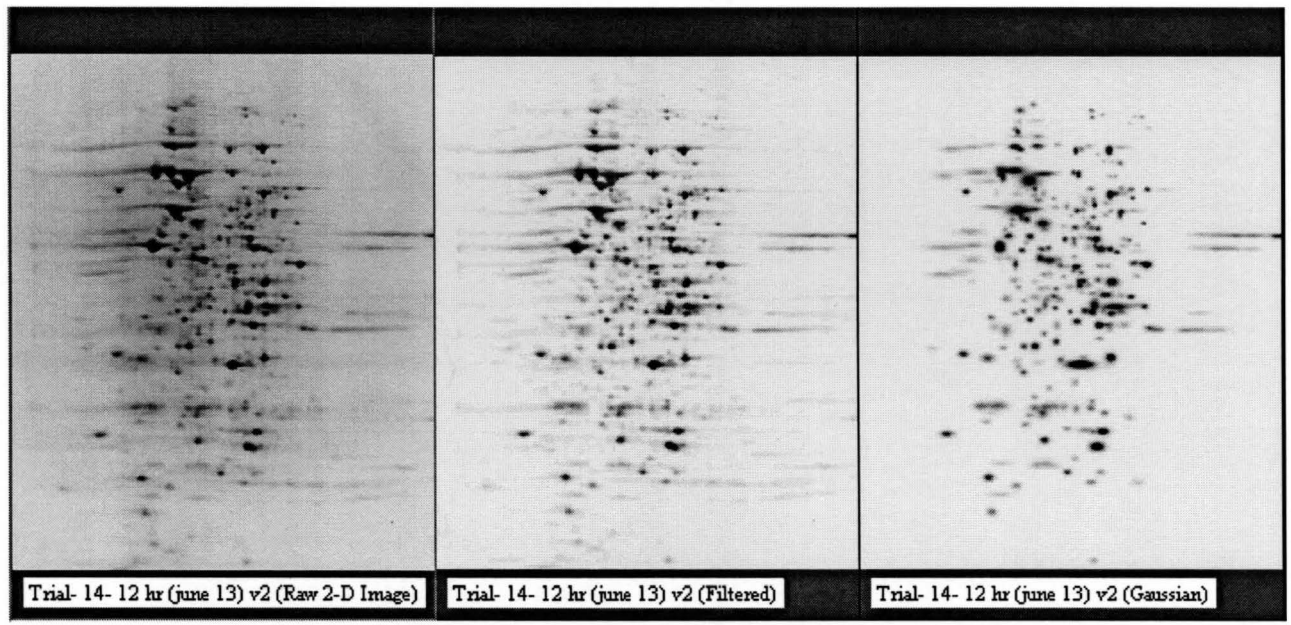

Trial- 14- 12 hr (june 13) v2 (Raw 2-D Image)
Trial- 14- 12 hr (june 13) v2 (Filtered)
Trial- 14- 12 hr (june 13) v2 (Gaussian)

Figure: 3.4: Twelve hour *Penicillium marneffei* F4 yeast matchset with master and member gels in subwindows.

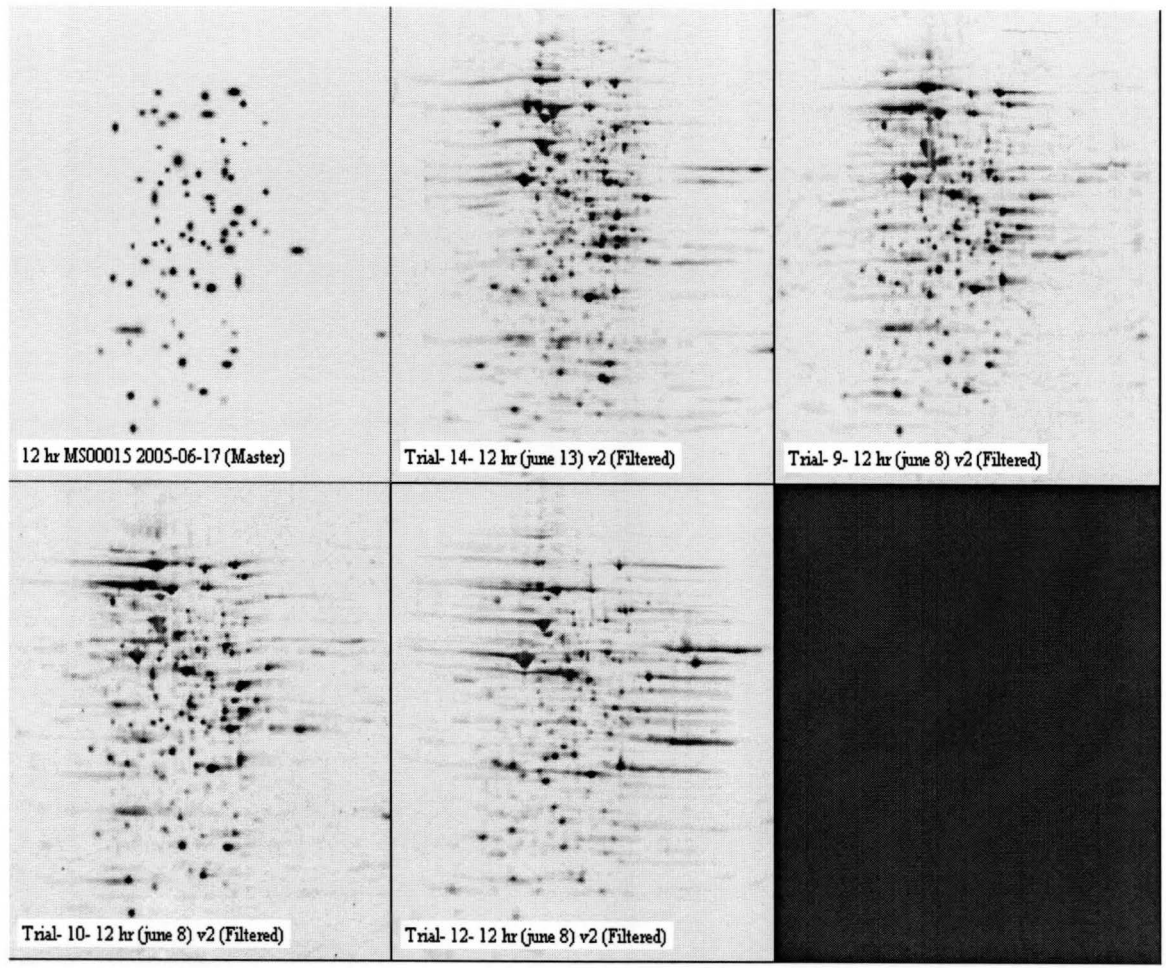

12 hr MS00015 2005-06-17 (Master)
Trial- 14- 12 hr (june 13) v2 (Filtered)
Trial- 9- 12 hr (june 8) v2 (Filtered)
Trial- 10- 12 hr (june 8) v2 (Filtered)
Trial- 12- 12 hr (june 8) v2 (Filtered)

Figure: 3.4: Twenty four hour *Penicillium marneffei* F4 yeast matchset with master and member gels in subwindows.

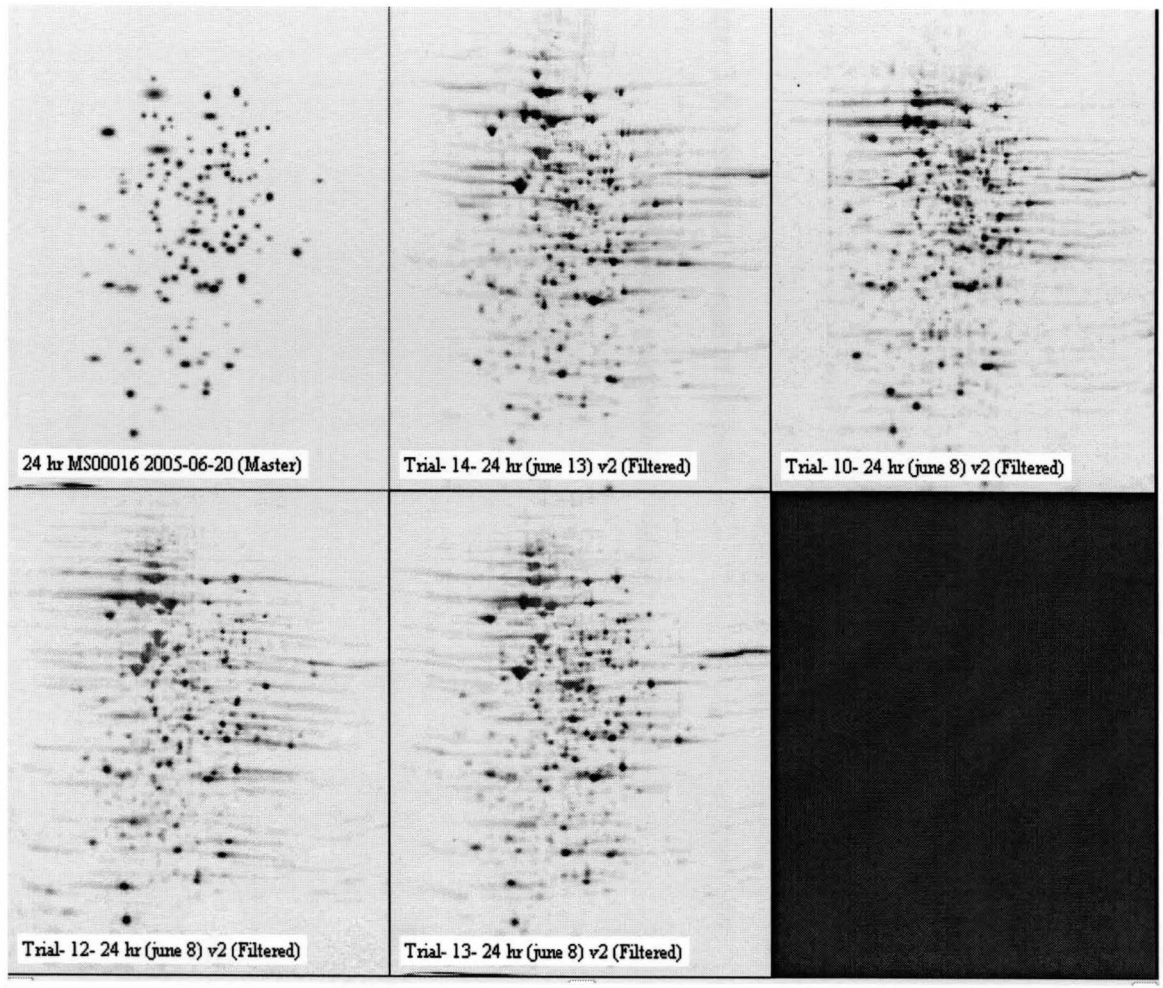

24 hr MS00016 2005-06-20 (Master)
Trial- 14- 24 hr (june 13) v2 (Filtered)
Trial- 10- 24 hr (june 8) v2 (Filtered)
Trial- 12- 24 hr (june 8) v2 (Filtered)
Trial- 13- 24 hr (june 8) v2 (Filtered)

Figure: 3.6: Matchset images of 12 hour and 24 hour *Penicillium marneffei* F4 yeast 2-DE gels with SSP numbers displayed.

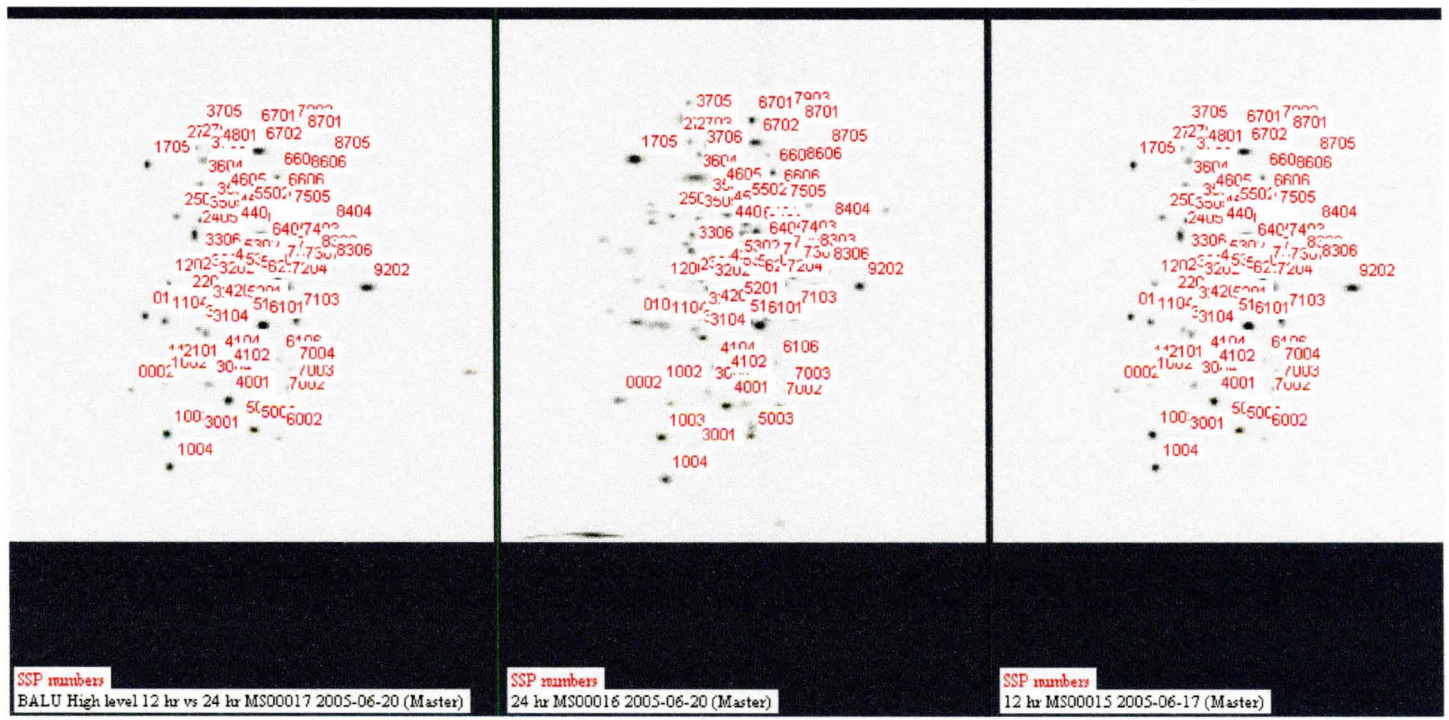

SSP numbers
BALU High level 12 hr vs 24 hr MS00017 2005-06-20 (Master)
SSP numbers
24 hr MS00016 2005-06-20 (Master)
SSP numbers
12 hr MS00015 2005-06-17 (Master)

Figure: 3.7: Higher level matchset images of 12 hour and 24 hour *Penicillium marneffei* F4 yeast 2-DE gels.

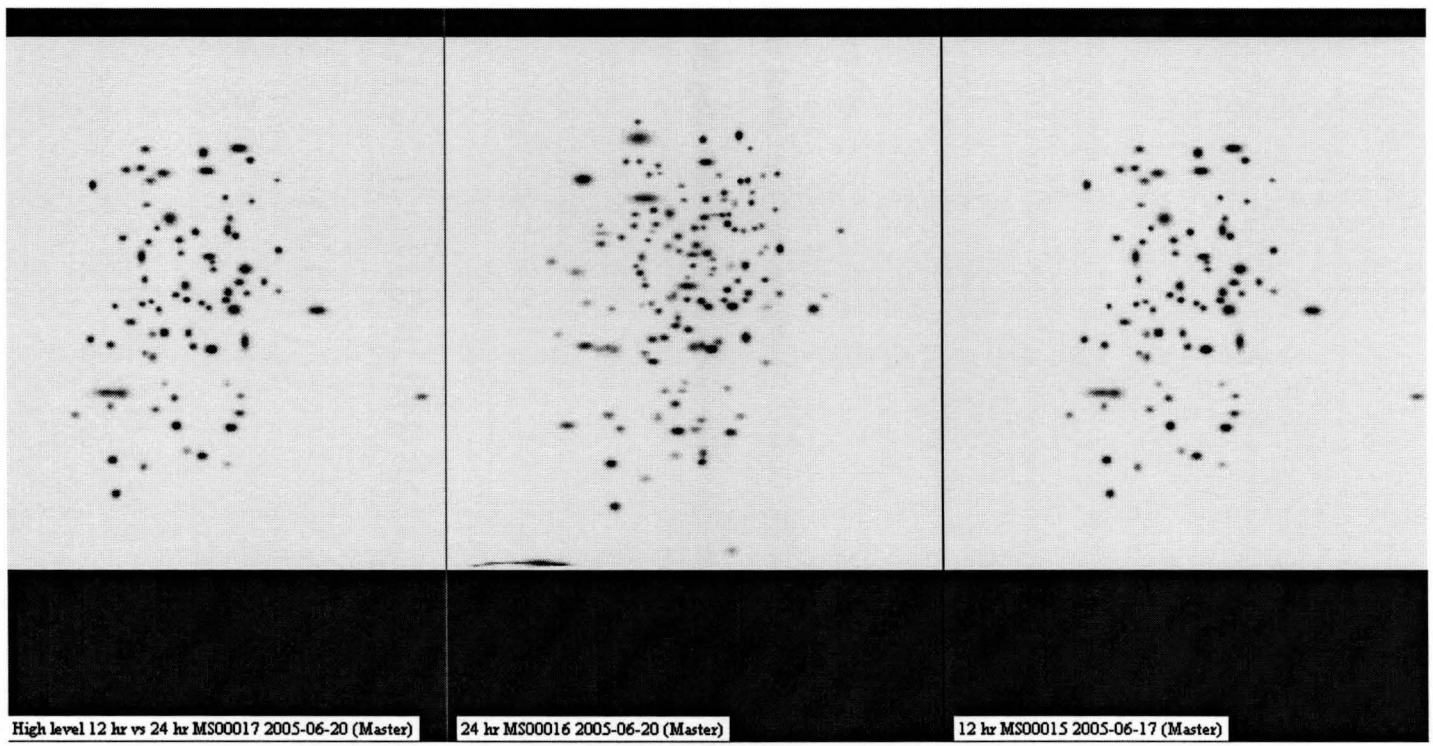

High level 12 hr vs 24 hr MS00017 2005-06-20 (Master)
24 hr MS00016 2005-06-20 (Master)
12 hr MS00015 2005-06-17 (Master)

Figure: 3.8: Higher level matchset images of 12 hour and 24 hour *Penicillium marneffei* F4 yeast 2-DE gels with matched, unmatched and landmark spots displayed.

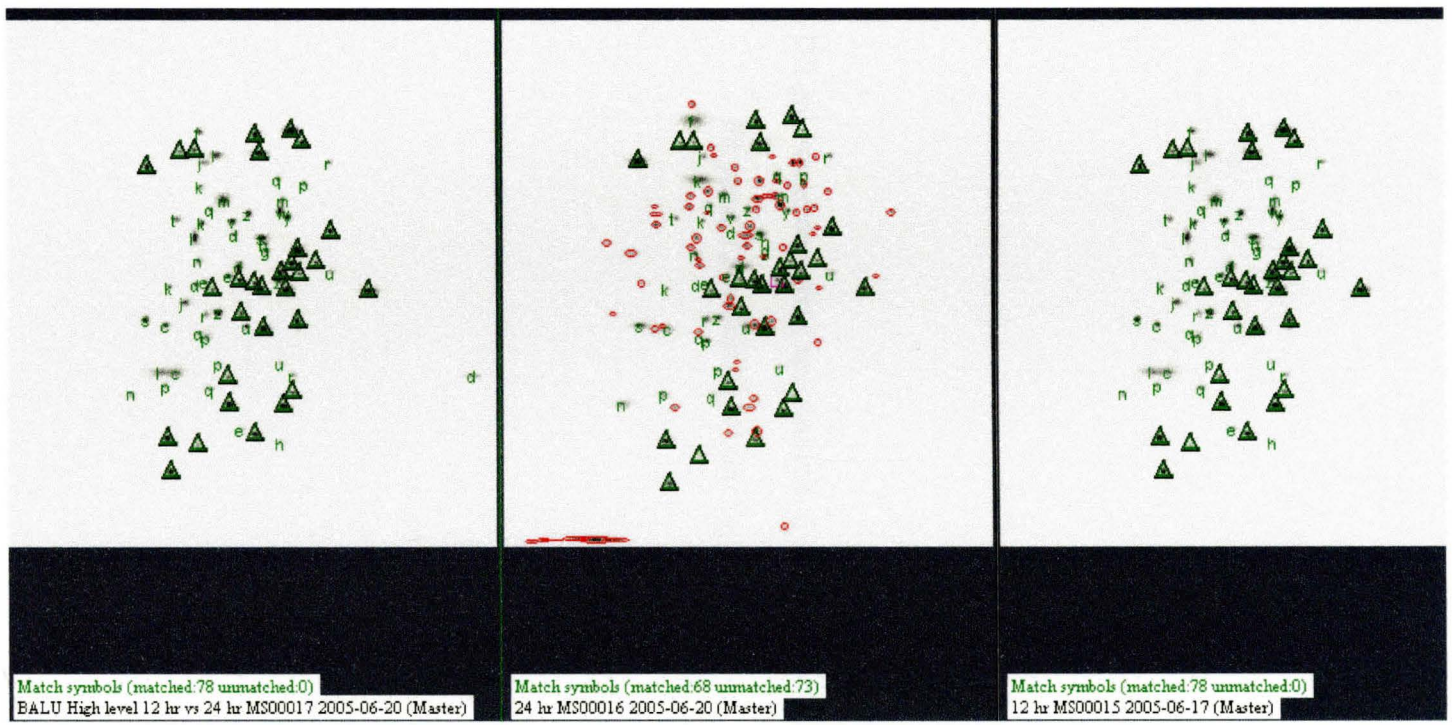

Match symbols (matched:78 unmatched:0)
BALU High level 12 hr vs 24 hr MS00017 2005-06-20 (Master)
Match symbols (matched:68 unmatched:73)
24 hr MS00016 2005-06-20 (Master)
Match symbols (matched:78 unmatched:0)
12 hr MS00015 2005-06-17 (Master)

Figure: 3.9: Two-DE gel image depicting spot variation between *Penicillium marneffei* F4 yeast 12 hr and 24 hr time courses.

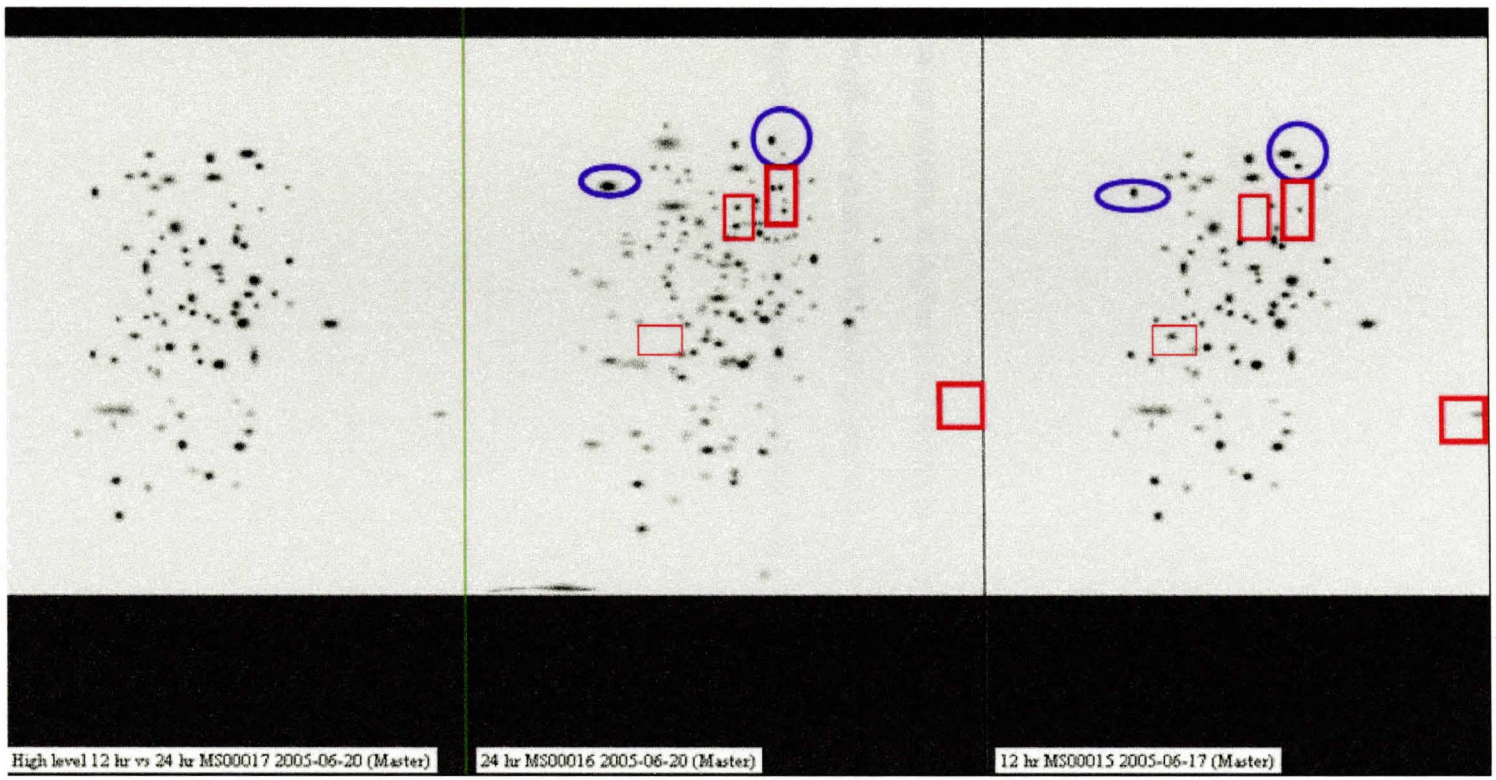

High level 12 hr vs 24 hr MS00017 2005-06-20 (Master)
24 hr MS00016 2005-06-20 (Master)
12 hr MS00015 2005-06-17 (Master)

Figure: 3.10: Close-up section of higher level matchset images of 12 hour and 24 hour *Penicillium marneffei* F4 yeast 2-DE gels displaying spots excised; left block is the master, central block is the 12 hr trial and the right block is 24 hr trial.


1705
1705
1705

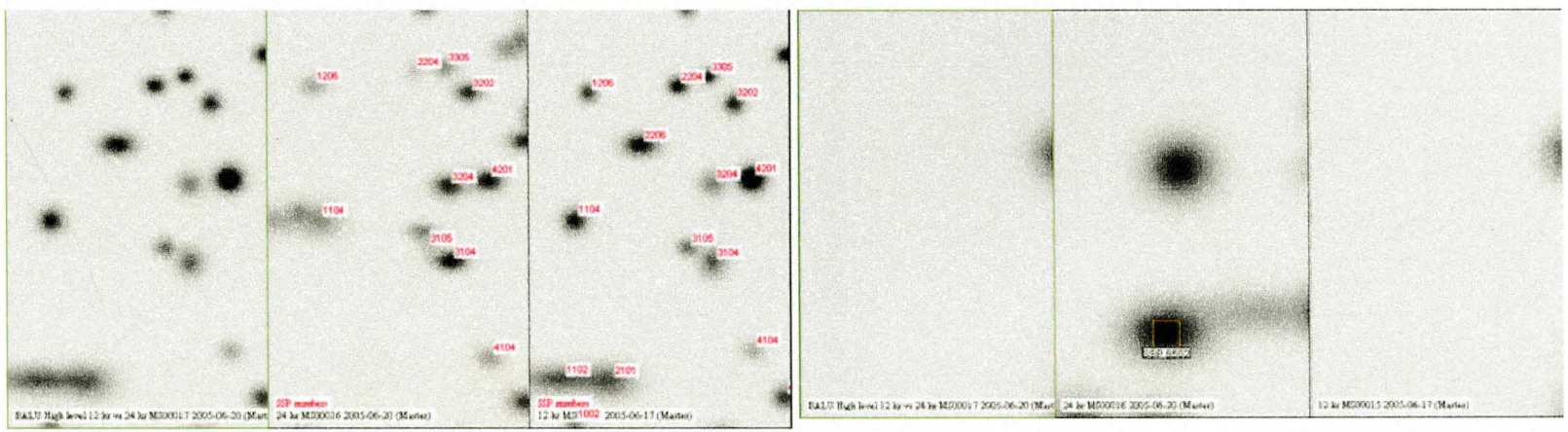

1206
2204
3305
3202
3204
4201
1104
3105
3104
4104
1102
2101
1206
2204
3305
3202
2206
3204
4201
1104
3105
3104
4104

Table: 3.1: Matching summary report of *Penicillium marneffei* F4 yeast 12 hr matchset.

Gel Name	Spots	Matched	Match Rate 1	Match Rate 2	Correlation coefficient	Match settings
Trial -14	81	21	25%	26%	0.602	Default match settings
Trial - 9	80	26	32%	33%	0.873	Default match settings
* Trial - 10	78	78	100%	100%	1.000	Default match settings
Trial - 12	71	23	32%	29%	0.403	Default match settings

Table: 3.2: Matching summary report of *Penicillium marneffei* F4 yeast 24 hr matchset.

Gel Name	Spots	Matched	Match Rate 1	Match Rate 2	Correlation coefficient	Match settings
Trial - 14	137	67	48 %	47%	0.816	Default match settings
Trial - 10	136	78	57%	55%	0.811	Default match settings
Trial - 12	133	72	54%	51%	0.763	Default match settings
* Trial - 13	141	141	100%	100%	1.000	Default match settings

Table: 3.3: Matching summary report of *Penicillium marneffei* F4 yeast higher level matchset.

Gel Name	Spots	Matched	Match Rate 1	Match Rate 2	Correlation Coefficient	Match Settings
24 hour	141	68	48%	87%	Not applicable	Default match settings
* 12 hour	78	78	100%	100%	Not applicable	Default match settings

* 12 hr - master

Chapter-4

Appendix

Sample calculation showing the number of conidia being added to the Sabourad broth medium

Number of conidia on both sides of hemocytometer = 207 + 235 = 442.

Average number of conidia on each side = 442/2 = 221.

Average number of conidia on each side x dilution factor x standard dilution factor =

10^7 conidia /ml x V_f

Average number of conidia on each side = 221,

Dilution factor = 100,

Standard dilution factor = 10000,

V_f = volume of Sabourad broth medium that can be inoculated with the conidia obtained.

From the above formula V_f = 420 ml.

The quantity of conidial suspension (V_i) required for inoculating 350 ml of Sabourad broth medium is calculated by the formula below –

Average number of conidia on each side x dilution factor x standard dilution factor x

V_i = 10^7conidia/ml x 350 ml.

From the above formula V_i = 16 ml.

16 ml of conidial suspension is required for 350 ml of Sabourad broth medium.

Protein quantization by modified Bradford assay

Concentration of standard BSA	Absorbance at 595 nm
10 µg	0.1904
15 µg	0.3256
20 µg	0.3797
25 µg	0.5036
30 µg	0.5687
5 µl of 12 hour protein sample	0.2457
10 µl of 12 hour protein sample	0.4348
5 µl of 24 hour protein sample	0.3210
10 µl of 24 hour protein sample	0.6228

Table: 4.1: Absorbencies of BSA standard and protein samples.

A graph is plotted with concentrations of standard BSA on x-axis and absorbencies on y-axis.

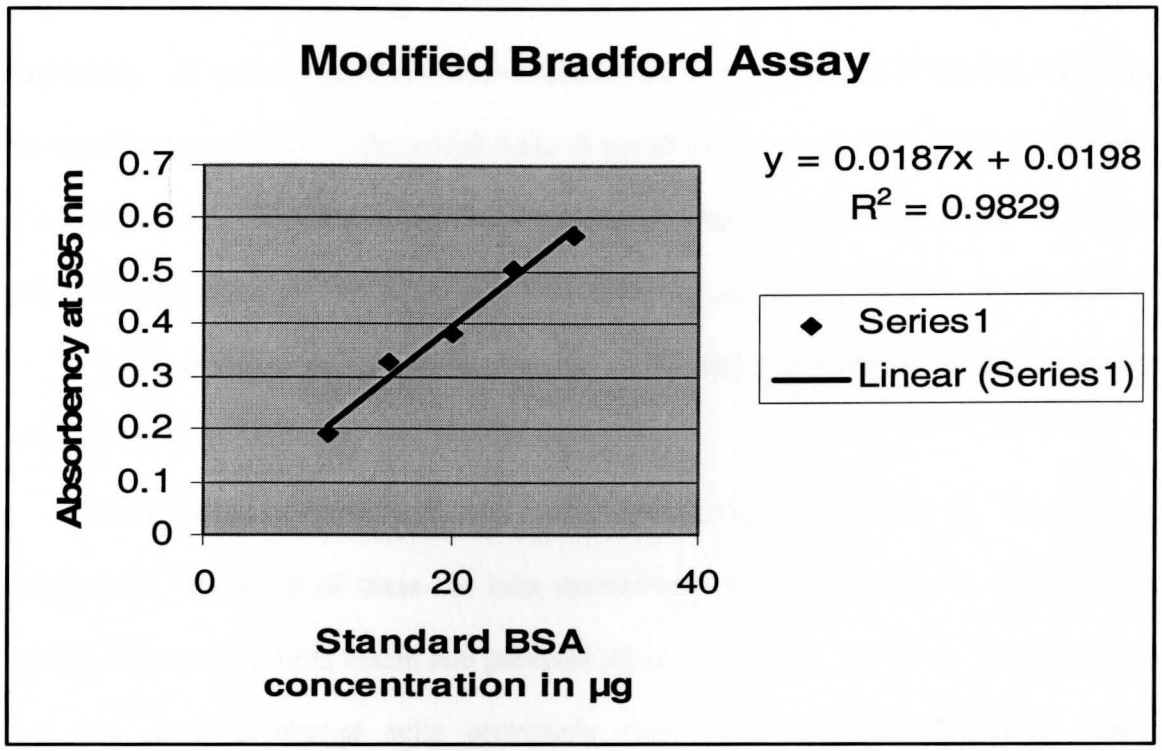


Figure: 4.1: Graph for the calculation of concentration of protein samples.

The protein concentrations of 12 hour and 24 hour *Penicillium marneffei* yeast protein samples can be calculated from the equation y = 0.0187x + 0.0198 obtained from the above graph.

Substituting the absorbencies (y) in the above equation the concentrations (x) of

12 hour protein sample is – 2.3 µg/µl,

24 hour protein sample is – 3.2 µg/µl.

Regression line (R)

A regression line is a line drawn through a scatterplot of 2 variables (here they are concentration and absorbency). The line is chosen so that it comes as close to the points as possible. R^2 values range from 0 to 1 and reveal how closely the estimated values for the trendline correspond to the actual data. A trendline is most reliable when its R^2 value is 1 or close to 1. R^2 value is also known as the coefficient of determination. The above graph has a R^2 value of 0.9829 which is very close to 1, revealing the accuracy of data.

The purpose of using various reagents, techniques in material and methods section is explained here-

EDTA used in washing of cells chelates any divalent metal ions like calcium and magnesium. Removal of these cat ions destabilizes the cell membrane. Also EDTA inhibits DNases to a little extent and prevents phosphorylation. The sonication technique that was used to disrupt cells previously was discontinued as the glass beads-homogenization method yielded higher (nearly twice) protein concentrations. This could be due to the fact that yeast cells have tough cell walls and require vigorous lysis conditions.

Lysis Buffer

Lysis buffer during cell disruption inhibits any proteases and phosphatases that could degrade proteins and result in artifactual spots and loss of high molecular mass proteins. Protease inhibitor cocktail commercially available from Sigma is a clear, faint pink solution in dimethyl sulfoxide. It has a broad specificity for the inhibition of serine, cysteine, aspartate and metalloproteases. Deoxycholate is a mild detergent that facilitates

the release of membrane proteins and enhances the interaction of proteins with TCA (trichloro acetic acid). The former protocol involved the use of TCA that was at room temperature, but the use of ice-cold TCA lead to a substantial rise in the amount of protein (~100 mg to ~200mg) precipitated. TCA precipitation curtails protein degradation and removes interfering compounds such as salts, polysaccharides and polyphenols that impede with electrophoretic separation. Polysaccharides intermingle with carrier ampholytes (amphoteric electrolytes) and cause streaking on 2-DE gels. They could in addition increase the viscosity of the solutions and obstruct the polyacrylamide gel pores causing erroneous separation of proteins on the second dimension. TCA precipitation as well eliminates high amount of lipids that may interact with membrane proteins and consume detergents that are added in the next steps.

Modified Sample Buffer

Urea and thiourea are chaotropes that destabilize proteins. Urea is capable in disrupting hydrogen bonds that results in denaturation and unfolding of proteins. Thiourea breaks hydrophobic interactions among polypeptides. CHAPS is a zwitterionic detergent. Detergents avert interactions between hydrophobic protein domains to shun loss of proteins due to aggregation and precipitation. DTT is a reducing agent.

Urea, detergents, carrier ampholytes and reducing agents like DTT hinder with the determination of protein concentration. Determination of the actual amount of protein in solubilized protein solution is critical to assess the second dimension polypeptide maps accurately both in quantity and quality. The protein samples are acidified by the addition of 0.1M Hydrochloric acid, which results in a stable linear relationship between protein concentration and absorbency (20).

Equilibration Buffer

The reducing agent – DTT cleaves intramolecular and intramolecular disulfide bonds to completely unfold the proteins. Iodoacetamide alkylates sulfhydryl groups and prevents their reoxidation. Also iodoacetamide alkylates free DTT if any, to check streaking in the second dimension. Urea and glycerol reduce the electroendosmotic effects to improve protein transfer (18). The equilibration step improves the transfer of proteins from the first dimension (IEF) to the second dimension (SDS-PAGE) permitting the separated proteins to fully interact with SDS. Close to 20% of the proteins are lost in the first equilibration step and the loss in the second step is marginal.

Techniques

Isoelectric focusing is the scheme of protein separation in the first dimension and gel electrophoresis is used to separate proteins in the second dimension. Isoelectric focusing is an electrophoretic method that separates proteins according to their isoelectric points. The isoelectric point is the specific pH at which the net charge of the protein is zero. This means the number of positive charges equal the number of negative charges. At isoelectric point a protein is stationary in an electric field (21).

Proteins are amphoteric molecules and can carry either a positive, negative or net zero charge depending upon the type of aminoacids they are composed. When a protein sample is subjected to electrophoresis through a solution/gel having a stable pH gradient, the protein moves to the position in the pH gradient where its net charge is zero (isoelectric point). A protein with a net positive charge moves to the cathode, progressively becoming less positively charged till it reaches a net zero charge. A protein

with a net negative charge moves towards the anode in the same manner. If a protein moves to a position different from its isoelectric point, the resulting electrophoretic forces due to a change in charge push it back to its isoelectric position. The proteins are focused about its isoelectric point that may be as small as 0.001 pH units (21). The immobilized pH gradient gel strips (Bio-Rad) used for the experiment were 17 cm long and have a pH range of 3-10. They are available in different sizes and pH ranges. The pores of the IPG gel are large enough to eliminate any sieving effect. Passive rehydration is a method where the IPG gel strips are rehydrated prior to isoelectric focusing. Active rehydration is performed in the IEF focusing tray under current immediately followed by isoelectric focusing without user intervention (22). Passive rehydration method was followed in my research work. Rehydration buffer has biolytes which are carrier ampholyte mixtures that improve protein solubility and ensure uniform conductivity during IEF without altering the pH gradient, bromophenol blue imparts color to the rehydration buffer.

The proteins are separated according to their molecular mass in the second dimension. Gel electrophoresis is the migration of charged molecules in solution in response to an electric field in a gel. The types of gels commonly used are polyacrylamide and agarose. The purpose of using polyacrylamide gels in the second dimension owes to their better resolving power than agarose gels. Polyacrylamide gels with their minute pore size can retard small molecules. 10% gels were used whose composition includes – acrylamide, tris buffer, SDS, ammonium persulfate, TEMED, water. Polyacrylamide is a polymer of acrylamide monomers cross-linked by N, N'-methylenebisacrylamide. Ammonium persulfate initiates polymerization, TEMED is a catalyst that enhances polymerization. Atmospheric oxygen is a free-radical that can

inhibit polymerization; this is the reason for casting polyacrylamide gels between glass plates.

SDS is an amphipathic molecule that is an anionic detergent. SDS binds to proteins and confers a negative charge to the polypeptide in proportion to its length. This masks the intrinsic charge of the protein and all the SDS treated proteins have identical charge-to-mass ratios. Proteins bind to SDS in the ratio of 1.4 gram of SDS : 1 gram of protein; i.e. one SDS molecule for 2 aminoacid residues. Electrophoresis of proteins in an SDS-PAGE gel separates them in order of their molecular masses. An SDS-PAGE gel has high molecular mass proteins on the top. The mass of the proteins decreases gradually towards the bottom of the gel. Porous gel acts as a sieve by retarding, in some cases completely obstructing the movement of large macromolecules while allowing smaller molecules to migrate freely. The relative mobilities of proteins vary linearly with the logarithmic molecular masses. So, in the second dimension migration is not determined by intrinsic electric charge of the protein, but by its molecular mass. The approximate range of molecular mass of proteins that can be separated by SDS-PAGE is 5 kDa to 500 kDa (21).

Staining

Different types of staining methods are – fluorescent and radioactive labeling, silver staining, reverse staining with metal cat ions like copper/zinc/potassium, coomassie blue staining in the decreasing order of their sensitivity. Sypro Ruby is a ruthenium-based dye that is sensitive enough to stain a protein spot as low as 1-2 ng in molecular weight (18). Sypro Ruby stained 2-DE gels appear transparent to the unequipped naked

eye. The protein spots on these gels appear only in presence of UV light, so UV transillumination is the mode of illumination in imaging these gels.

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