The application of proteomics to Pseudomonas putida F1

Han Hoang
The Application of Proteomics to
Pseudomonas putida F1

Han Hoang
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Approved: Paul A. Craig
Thesis Advisor

T. C. Morrill
Department Head

Department of Chemistry
Rochester Institute of Technology
Rochester, NY 14623-6503
The Application of Proteomics to
Pseudomonas putida F1

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Common Abbreviations

2DE - Two Dimensional Gel Electrophoresis
Aa - Amino Acid
BA - Benzoic Acid
DNA - Deoxyribonucleic acid
IEF - Isoelectric Focusing
IPG - Immobilized pH Gradient
MW – Molecular Weigh
P. putida - Pseudomonas putida
PEA - Phenylethylamine
pI – Isoelectric Point
RNA - Ribonucleic acid
SA - Succinic Acid
SDS-PAGE - Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
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Abstract

Through years of technology development, the drug industry has been able to synthesize many valuable medicines to provide better health care. However, any time a new medication (or industrial chemical for that matter) is made; it must be tested to ensure that it is not carcinogenic. For years, scientists have worked to design a screening method that is fast, efficient, and reliable. Currently, the most widely-used method used is the Ames test [1]. This test has both strengths and weaknesses which will be discussed.

In this project, Two Dimensional Electrophoresis (2DE) is used to monitor protein expression in *Pseudomonas putida* F1 grown on different carbon sources with the purpose of finding a set of carcinogenic indicator proteins, which will lead to a replacement for Ames test. 2DE provides a molecular approach to carcinogenesis, thus is more detailed and potentially more reliable. The result of the project as well as possible future directions for this research will be discussed.
Introduction

A- Central Dogma of Molecular Biology

The genetic information of an organism, called the genome, is found in the DNA of most organisms except for viruses. Nucleic acids (see Figure 1-1 below) are very large molecules with a backbone made of alternating sugar and phosphate units. Depending on the type of sugar, it is called DNA (with deoxyribose) or RNA (with ribose). Each of the sugar groups in the backbone is attached to a nucleotide base: Adenine (A), Thymine (T), Cytosine (C), and Guanine (G) in DNA and Uracil (U) replaces Thymine in RNA. [2]

Figure 1-1: Nucleic Acid Composition [3].

While there are only 4 different nucleotide bases in DNA, one nucleic acid chain may contain thousands to hundreds of thousands of bases. In the early 1950's, four
scientists, James Watson and Francis Crick at Cambridge University and Maurice Wilkins and Rosalind Franklin at King's College, discovered the double helical structure of DNA as demonstrated in Figure 1-2 B from data and X-ray pictures of the molecule. In DNA, the bases form complementary pairs, that hydrogen bond to one another as illustrated in Figure 1-2 A. A always pairs to T while G always pairs to C.

[2]

Figure 1-2: A) Double Stranded DNA Bonding [4]
B) Double Helices Diagram [5]

Unlike DNA, RNA is single-stranded. RNA contains the genetic information for some viruses and is also important in the production of protein in all living organisms [2]. While DNA mostly resides in the nucleus of eukaryotes, RNA can freely move around the cells. Therefore, it may act as a genetic messenger, passing on the information from DNA (in the nucleus) to other parts of the cell where it is used to help make proteins [2]. Proteins are polymers of amino acids [6]. Amino acids all have the general structure as shown in Figure 1-3:

![Generic Amino Acid](Figure 1-3: Generic Amino Acid (aa) [6])
The R in Figure 1-3 represents a functional group that varies depending on the specific amino acid. R can be simply a hydrogen atom, as in glycine, or a more complex organic group. While there are hundreds of thousands of different proteins that exist in nature, they are all made up of different combinations of the same 20 amino acids, with very few exceptions. Amino acids are linked together by peptide bonds as illustrated in Figure 1-4. In a peptide bond, the carboxylic acid end of one amino acid bonds to the amino group of the adjacent one. [6]

![Figure 1-4: Peptide Bond](image)

Polypeptides with defined biological functions are called proteins. Proteins serve many purposes in living organisms, from supporting cellular structure, catalyzing essential reactions such as cellular metabolism, to maintaining the genetic information in the DNA. The various functions of proteins require each of them to be extremely specific. [6]

There are four characteristics of the structure of a protein that are specific to its function. The first, called the primary (1°) structure of a protein is the amino acid sequence: the number and order of amino acid linked together. The secondary (2°) structure refers to localized interaction in the polypeptide backbone. Once coiled, the polypeptide will fold into its native structure, which is called the protein's tertiary (3°)
structure. Many proteins consist of multiple polypeptide chains. The interactions between the separate polypeptide chains in these multimeric proteins are called quaternary (4°) structure. [6]

Proteins are synthesized from the genetic information of nucleic acids as shown in Figure 1-5:

![Central Dogma](image)

Figure 1-5: Central Dogma of Molecular Biology [7].

In a coding region of a gene, each set of three DNA bases is called a codon, and it codes for one amino acid. Any change in DNA bases (mutation) may result in the incorrect synthesis of protein. This could greatly affect the organism if the protein is essential. Some changes of DNA may lead to a non-stop replication of the cells, or cancer. [8]

Even though cells have a DNA repair system, many conditions may result in change of DNA bases, from chemicals to ultraviolet light. Since drugs are made from
chemicals, they may have the potential to cause mutagenesis and carcinogenesis, it is necessary to examine any drug carefully before use, and the Ames test has been long used as a primary screening method for determining mutagenicity. [8]

B - Ames Test

The Ames test is named for its developer, Bruce Ames. It is used with the assumption that any substance that is mutagenic for the bacteria used in this test meaning that it causes changes in the genetic information, may also be carcinogenic. [1]

The bacterium used in the test is a mutant strain of *Salmonella typhimurium* that is unable to synthesize the amino acid histidine (a histidine auxotroph) from the ingredients in a minimal culture medium (a medium in which living organism has to synthesize all the essential amino acids on its own). The normal strain, on the other hand, is able to synthesize histidine when grown on the same medium. The mutation can be reversed, in a process called reverse mutation, as the mutated gene regains its function enabling the bacteria to grow on a medium lacking histidine. A typical Ames test is shown in Figure 1-6. Panel A shows the result of growing $10^9$ cells on the minimal medium. There are approximately 30 spontaneous revertants. In panel B, a disk containing a mutagen is placed in another plate containing $10^9$ cells. Several hundred colonies have formed as the result of a reverse mutation caused by the mutagen. [1]
Figure 1-6: Ames Test A) Negative result B) Positive result [8].

There are many types of mutant strains used to test for different classes of mutagens. A chemical that causes frameshift mutation (mutation in which one nucleotide base is deleted or inserted and all the codons shift by one nucleotide, resulting in a completely different protein) is most likely to reverse a frameshift mutant. Table 1-1 gives the popular strains used in the Ames test. [9]

Table 1-1 [9]: Different Bacterial Strains of Salmonella typhimurium Specie Used in the Ames Test

<table>
<thead>
<tr>
<th>Strain</th>
<th>Histidine mutation</th>
<th>Type of mutation</th>
<th>Main DNA target</th>
</tr>
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<tr>
<td>TA 97a</td>
<td>hisD6610</td>
<td>Frameshift</td>
<td>GC</td>
</tr>
<tr>
<td>TA 98</td>
<td>hisD3052</td>
<td>Frameshift</td>
<td>GC</td>
</tr>
<tr>
<td>TA 100</td>
<td>hisG46</td>
<td>Base pair substitution</td>
<td>GC</td>
</tr>
<tr>
<td>TA 102</td>
<td>hisG428</td>
<td>Base pair substitution</td>
<td>AT</td>
</tr>
</tbody>
</table>
Recently, the Ames test has also been modified by adding a rat liver homogenate (containing active liver enzymes) to better mimic human responses to mutagens. Liver enzymes, which are often responsible for detoxification, sometimes activate pro-mutagens (chemicals that are not mutagenic unless they are activated by metabolism), thus the addition of these enzymes enables the Ames test to detect mutagens, including those that are metabolically activated. [9] The Ames test is relatively simple and the ease of use and low cost of the test makes it invaluable for screening substances for carcinogenicity. Yet the results of the Ames test remain questionable because of the number of false results, even with the accommodation of a variety of testing strains, and the addition of rat liver homogenate, as well as other improvements that have been made.

A number of factors may contribute to false results in the Ames test [10]:

1. The Ames test utilizes bacterial strains, which are prokaryotic cells. They are different from mammalian cells in many aspects, uptake of chemicals, metabolism, chromosomal structure, and DNA repair processes. Even when all these aspects are accommodated, this test is still conducted in the lab and there are many other in vivo conditions that may not be accounted for. Thus, the test does not provide direct information regarding the mutagenicity and carcinogenicity of the substance in mammals in general or human beings specifically.

2. The specific nature of the endpoint detected should also be taken into consideration. Some substances take longer incubation times to have their mutagenic effects than others; the incubation time period may not be flexible enough to accommodate such variance.
3. Metabolic activation and bioavailability also greatly contribute to the false results from this test. Though rat liver homogenate and many other types of enzymes may be added to the growth medium yet, the substances may be metabolized in different locations of the body. A pre-mutagen may only convert to a mutagen in specific tissues or organs other than the liver.

4. The candidate compounds may also be highly toxic to bacteria in a way that is not seen in human beings. For example antibiotics would probably kill the tester strain, giving a negative Ames test, regardless of whether they have any mutagenic or carcinogenic effects on human beings.

5. Sometimes a drug that is screened may be derived from plant or animal tissues that may contain histidine or its derivatives that the bacteria can feed on. It is also possible that a chemical biodegradation pathway may generate histidine as an intermediate, eliminating the need for a back mutation of the histidine synthetic gene.

These are only a few factors that may contribute to false results of this test. Thus the Ames test, even though it is a useful tool, has limitations for screening mutagens and carcinogens. These limitations may result from the concept of monitoring mutations on the cellular level (is the bacteria alive or dead?), which is relatively superficial. It may be time to use new technology to develop a better method to monitor mutations on the molecular level specifically by exploring protein expression of an organism.
C - Two Dimensional Gel Electrophoresis and Proteomics

Proteomics is a young science which enables researchers the ability to analyze protein expression by organisms. The application of proteomics to the testing of mutagens and carcinogens may provide a more reliable means to monitor these concerns at the molecular level (protein expression) using two dimensional gel electrophoresis (2DE). This project is a step toward the development of a screening method for carcinogenicity based on 2DE.

a) Proteomics

Proteomics has emerged following the growth of genomics, the study of genomes. The proteome is defined as the protein complement encoded by a given genome at a specific time point under a specific set of growth conditions. Proteomics encompasses expression proteomics and functional proteomics. Expression proteomics involves the identification and quantification of all proteins encoded in the genome and assessment of their cellular localization and their post-translational modification. Functional proteomics involves the assay of protein interaction, the determination of the role of individual proteins in specific pathways and cellular structures, and the connection between protein structure and function. [11]

Two-dimensional gel electrophoresis is a core technology for expression proteomics. This technique is able to separate thousands of proteins in a reasonable time period. The first dimension of 2DE is isoelectric focusing (IEF), separation based on the charge behavior of a protein. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is used in the second dimension to separate proteins by their size.
(molecular weight, MW). 2DE is frequently used as a component of proteomics, in which proteins are isolated for further characterization by mass spectroscopy. It is used for two main purposes: (1) it can separate thousands of proteins in a single sample and (2) it can be used to evaluate differential expression of proteins by one organism or tissue under two different sets of conditions. [12]

In the Figure 1-7, 2DE is the central method for protein analysis. All preparation, from growing the cell to collecting the proteins to preparing the protein sample, is all focused on how to prepare the best sample for 2DE analysis. From 2DE, protein expression then can be analyzed using imaging software and if any proteins of interest are identified, the protein spot can be picked for protein characterization using mass spectrometry. If no proteins of interest are found, the process is repeated with growth different conditions to obtain another protein population.

![Diagram of Protein Analysis Process](image)

Figure 1-7: A General Protocol for Proteomics [13].
b) Two Dimensional Electrophoresis

With this powerful protein separation tool, protein expression by a given organism under different conditions can be monitored in great detail. Thus, if the protein expression profile in the presence of known carcinogens is monitored for an organism, it may be possible to find a set of proteins that are associated with carcinogenicity. This could then lead to an inexpensive, fast, and reliable screening method for carcinogens and mutagens.

1st dimension separation

The first dimension of a 2DE separation is based on Isoelectric Focusing (IEF), which involves electrophoresis in a pH gradient set up between a cathode and anode with the cathode at a higher pH than the anode. In an electrical field, non-charged molecules are unaffected (not moving) thus when a protein moves to the pH at which all its internal charges cancel each other (the isoelectric point, pI, where the net charge on the protein is zero), it stops migrating (Figure 1-8). Proteins are positively charged at pH values below their isoelectric pH (pI) and negatively charged when pH>pI. Therefore, proteins migrate to their pI in the pH gradient. Most proteins have a pI between 5 and 8.5. [12]
A pH gradient can be established by carrier ampholytes, a mixture of amphoteric (both acid and basic) species which form a pH gradient in the medium during electrophoresis. Ampholytes are derived synthetically and comprise a wide range of varying pI values. In agarose or polyacrylamide gels containing ampholytes, when an electric field is applied, the charged ampholyte molecules migrate until they reach the position according to their pI. As a result, a linear pH gradient will be built up between the electrodes during the pre-focus period (in which the pH gradient is made).

However, the resulting pH gradient is not stable since ampholytes are kept immobilized by their attraction to the two opposing electrodes; thus fluctuation in the current during a prolonged isoelectric focusing may lead to an inevitable shift of the pH gradient. This instability also contributes greatly to poor reproducibility. This problem is reasonably resolved by the use of an immobilized pH gradient (IPG). In IPG strips, the pH gradient is generated by a limited number of well-defined chemicals, called immobilines, which are co-polymerized with the acrylamide matrix. Immobilines are weak acids or bases that are polyacrylamide derivatives. Thus polymerization of immobilines creates a fixed pH gradient, thus eliminating any electrodic (cathode or
anode) drift. Reproducibility is therefore enhanced. IPGs also allow the generation of pH gradients of any desired range (broad, narrow or ultra-narrow) between pH 3 and 12. The pH interval may be chosen depending on the purpose of the experiment. For example, for screening purposes, a broad range interval (pH 3-10) should be used; for more detailed analysis, a narrow pH range interval is preferred, once the pI values for the protein(s) of interest are known. Different protein species migrate and focus (concentrate) at different isoelectric points. [14]

Figure 1-9: The Principle of Isoelectric Focusing [14].

Figure 1-9 is an illustration of a sample with two proteins P1 and P2 placed in the center of a pH gradient. P1 is positively charged and will migrate toward the cathode; P2 is negatively charged and will migrate toward the anode. As the proteins approach their
pI, they gradually become less and less charged. The proteins will concentrate at the position where their pH is equal to their pI. However, proteins cannot concentrate in an infinitely concentrated zone, therefore widening by diffusion is inevitable. The use of IPG strips and storage of completed IPG strips at -20° are means to eliminate such diffusion. [14]

2nd dimension separation

The second dimension in 2DE is SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE). Electrophoresis is simply the migration of charged species in an electrical field. In general, electrophoresis can be run in any support matrix such as paper, cellulose acetate, starch, agarose or polyacrylamide. At the end of the run, the matrix can be stained and used for scanning, autoradiography or storage. For 2DE, polyacrylamide is the matrix of choice. The porous polyacrylamide gel separates molecules by size. [12, 14]

Figure 1-10: Basic 2nd Dimension SDS-PAGE [8].
To prepare the samples for SDS-PAGE, proteins are denatured by coating with sodium dodecyl sulfate (SDS), an anionic detergent which denatures proteins by wrapping around the polypeptide backbone. SDS binds to proteins fairly specifically in a mass ratio of 1.4:1. In addition to denaturation using SDS, it is also necessary to reduce disulfide bridges in proteins before they adopt the random-coil configuration necessary for separation by size: this can be done with 2-mercaptoethanol or dithiothreitol. In denaturing SDS-PAGE separations, migration is strictly determined by molecular weight. [12, 14]

For this separation, a linear relationship exists between the logarithm of the molecular weight of an SDS-sample and its $R_m$ value, the ratio of the distance migrated by the molecule to that migrated by a marker dye-front, from which the MW of the unknown sample can be estimated. A simple way of determining relative molecular weight by electrophoresis ($Mr$) is to plot a standard curve of distance migrated vs. log$_{10}$ MW for known samples. From the curve the log $Mr$ of the sample can be calculated by measuring the distance an unknown protein migrated on the same gel. [12]

Staining

There are many available stains, each with different sensitivity. Several popular commercial stains are described below.

Zinc or copper [14, 15]: These are used in a negative staining technique in which the background of the entire gel is stained except parts where protein is concentrated because zinc and copper do not stain SDS, which has been used to coat the proteins, but do stain the gel matrix. By this method, proteins can be visualized against an appropriate
background. This technique is quick, easy and cheap and since the proteins are not stained there is no problem with downstream processing. However, in the case of thin gels, the gel matrix is too thin to enable this stain to give an effective contrast. This stain can detect protein in extremely low quantities, from 6-12ng.

*Coomassie blue* [14, 15]: There are several types of Coomassie blue stain and several related protocols. Similar to copper/zinc staining, they all are quick, easy, and cheap; however coomassie blue stain may lose its sensitivity if it is not fresh. Another disadvantage of these stains is that they are not specific and will also detect non protein components such as polysaccharides. Lower detection ranges from 36 to 47ng.

*Fluorescent dyes* [14]: There are many different types of fluorescent stain and most are easy and relatively quick to use. They are very economical. These dyes are both selective and quantitative. With fluorescent dyes, the intensity of the protein spot is directly correlated to the quantity of protein. However, this staining can not be viewed with the naked eye but only under UV light (which excites the fluorescent dye giving a positive image). Their lower limit of detection is 1-2ng for an individual protein spot.

*Silver* [14, 15]: This staining technique is considered time consuming, expensive, and requires high quality substrates, but can give excellent results. Recently, many new protocols have been described that are compatible with mass spectroscopy [15]. The great advantage of this type of staining is its sensitivity; however silver stain also lacks specificity and dynamic range, making quantification of protein in spots unreliable outside the normal detection ranges 0.5-1.2ng of protein.
Standardization

As with other separation methods, standardization plays an important role in 2DE. Generally, there are six molecular weight standards clearly labeled on every stained 2D gel along with one or more IEF internal standards. The standards may appear as thin lines across the gels. As shown in Figure 1-11 (below) the six molecular weight (MW) standards (in kilodalton) are myosin with MW of 220,000, phosphorylase of 94,000, catalase of 60,000, actin of 43,000, carbonic anhydrase of 29,000, and lysozyme of 14,000. There is also a single isoelectric focusing (IEF) standard, tropomyosin, to validate running of the first dimension. Tropomyosin appears as a doublet with lower spot of pI 5.2 and MW 33,000; the lower spot is always marked with an arrow. The same standards should be included for both Coomassie blue and silver-staining so proteins that stain differently by these methods can be located. [14, 15]

Figure 1-11: Molecular Weight and IEF Standards for 2DE [15].
Much research in proteomics has been done using 2-D gel technology; however problems in staining and visualizing as well as image analysis still concern researchers. Despite recent advances, some areas still rely heavily on the human eye to make statistical sense out of an image spot, which can lead to possible human error. A fully automated publicly available image analysis system has not yet been developed. Analysis is still the most time consuming process in proteomics. The gel image itself may also vary from gel to gel as a result of various discrepancies, such as over or under staining or variation in pore size. Such discrepancies can lead to serious problems in analysis. [16]

In addition to the reproducibility problems, 2DE has a limited ability to detect low abundance proteins and hydrophobic proteins as well as resolving at either molecular weight extreme. Another serious concern about this technique is its inability to directly couple with subsequent analysis using mass spectrometry. [11]

These shortcomings of 2DE have stimulated researchers to develop alternative technologies. Some of the most successful new developments are the use of multi dimensional liquid [11] phase-based chromatography linked with mass spectrometry. [17]

D – Comparison of Alternatives to Two Dimensional Gel Electrophoresis

Liquid Chromatography has a strong position in protein separation history. This technique separates mixtures of substances based on characteristics that influence their migration in specific stationary/mobile phases setting (size, charge, etc). As other techniques developed, such as 2DE, liquid chromatography fell from favor. Yet, recently
it has become popular to combine liquid chromatography with other powerful protein identification tools. For example, nanoflow capillary high-performance chromatography (cHPLC) has been used in conjunction with electrospray ionization tandem MS (HPLC-ESI-MS-MS). The use of multi-dimensional liquid chromatography techniques is attracting much attention in proteomics circles. The possibility of techniques that combines HPLC, Isoelectric focusing (IEF), chromatofocusing (CF), and capillary electrophoresis (CEF), may offer many options for the separation of much more complex mixtures of proteins and peptides. In multi-dimensional chromatography systems (MDCS), a variety of stationary phases (ion-exchange, in which proteins are separated by charge in a given pH; reversed-phase, which uses polarity for separation; or size exclusion, a size-based separation, can be combined in sequence or with capillary electrophoresis. With the development of isoelectric focusing, the later MDCS’s, with IEF as one dimension, are able to separate many more proteins. Another great advantage of this approach is the ability to directly link an MDCS with a mass spectrometer, thus proteins can be analyzed as they are separated. [11]

An MDCS provides a very powerful alternative method to study protein expression in an organism under different growth condition. Yet it has limitations when compared to 2DE, particularly when considering the application in this project. MDCS may separate and identify thousands of protein yet all the information that is analyzed (through mass spectrometry) may be important to certain study but may not contribute to a drug screening method [11]. Besides generating too much data, it also destroys the protein samples.

When compared to these other techniques, 2DE is also the least costly. The results can also be obtained more quickly. As a set of protein spots related to carcinogenicity is
determined, any substance can be studied by 2DE in a one-week period which will include time for the bacteria to grow in the medium that contains the substance of interest, 2DE separation, imaging, and analysis. Overall, 2DE is a less costly with a reasonable turn-around time, thus making it more appropriate for drug screening purposes.

E - *Pseudomonas putida* and aromatic compounds

One critical aspect of this project is selection of a suitable bacterial strain. In this project, *Pseudomonas putida* F1 is the strain of choice. *Pseudomonas putida* is a versatile bacterium very well known for its ability to metabolize aromatic compounds. This gram-negative soil bacterium is a rod-shape cell with flagella (Figure 1-12). *Pseudomonas putida* is also listed among microorganisms most commonly found in consumer products such as paints and solvents.

![Image of Pseudomonas putida](image)

Figure 1-12: *Pseudomonas putida* [19].

With its versatile metabolism as illustrated in Figure 1-13, *Pseudomonas putida* can resist the harmful effects of organic solvents and break many of these down to safe intermediary metabolites such as pyruvate and acetyl CoA. Polluted soil and groundwater
purification technology which depend on the remedial power of microbes, use this bacteria to render many toxic organic substances harmless. [20]

Figure 1-13: Proposed Metabolic Pathways of Pseudomonas putida [20].

The main factor that differentiates among Pseudomonas putida strains is the presence of extrachromosomal DNA, called plasmid(s) that is unique for each strain [21]. Each plasmid contains different genes that code for unique enzymes. For example, one strain, Pseudomonas putida KT2440, has been long used in fertilizer as a soil renovation microbe. Pseudomonas putida KT2440 metabolizes many organic compounds into simple carbon sources for vegetable growth. The strains KT2440 and F1, indeed, have identical genomes. However, they are different in that F1 has a TOL plasmid that enables
it to metabolize toluene and similar compounds, while KT2440 does not contain any plasmids. [22]

With strains that are already in use in agriculture as well as industrial waste management, *Pseudomonas putida* has been extensively studied. Its genome is sequenced as well as many of the plasmids found in the different strains. For *Pseudomonas putida Fl*, the TOL plasmid has been sequenced [21]. This microorganism has also been used in a study of the metabolism of toluene and similar organic compounds [23].

The complete genome (circular DNA) of *Pseudomonas putida* (strain KT2440) is comprised of 6181863 base pairs, which code for 5314 different open reading frames. Various metabolic pathways of *Pseudomonas putida* are also very well studied. [24]

2DE has been used to identify proteomic signatures for *P. putida* Fl grown on different carbon sources. One of the main attractions of this bacterial species is its ability to metabolize numerous aromatic compounds, such as toluene and phenol. Reardon et. al. performed 2DE analysis of protein production during growth of *P. putida* Fl on toluene, phenol, and a mixture of the two. They succeeded in identifying different proteomic signatures that are unique for each growth condition. [23]

Aromatic compounds contain a highly stable ring system that makes them difficult to metabolize. Many aromatic compounds are mutagenic due to their compact planar nature, which facilitate their movement within the cell and into the nucleus where they may interact with the DNA. Regardless of their potential health hazards, aromatic compounds are widely used commercially (in many drugs) and industrially (gasoline). [25]
*P. putida* species metabolize many aromatic compounds with a unique protein class called dioxygenases, which add oxygen atoms to aromatic rings [27]. A very well-known example is the Toluene Degradation pathway for *P. putida* F1 (Figure 1-14).

![Figure 1-14: Toluene Degradation Pathway (abbreviations are explained in text) [27].](image)

The toluene degradation pathway consists of seven enzymatic reactions. All the enzymes are synthesized from the TOL plasmid that is unique to *P. putida* F1. Toluene dioxygenase (TDO) catalyzes the formation of the cis-1,2-dihydrodiol from toluene (or similar substrates). Then the cis-dihydrodiol is dehydrogenated to 3-methylcatechol by an NAD+-dependent cis-toluene dihydrodiol dehydrogenase (TodD). 3-Methylcatechol undergoes meta-cleavage by 3-methylcatechol-2,3-dioxygenase (TodE) to form a yellow colored substance called 2-hydroxy-6-oxo-6-methylhexa-2,4-dienoate (6-methyl-HOHD) which is then hydrolyzed to acetic acid and 2-hydroxypenta-2,4-dienoate by 6-methyl-HOHD hydrolase (TodF). 2-Hydroxypenta-2,4-dienoate is transformed by a series of
enzymes - 2-hydroxypenta-2,4-dienoate hydratase (TodG), 4-hydroxy-2-oxovalerate aldolase (TodH) and acylating aldehyde dehydrogenase (TodI) - to produce pyruvate and acetyl-CoA. [27]

Many proteins are synthesized only when needed, therefore under different growth or environmental conditions, it is expected that protein expression will vary. For example, if the bacteria are grown on a carbon source such as succinic acid, none of the seven enzymes of the toluene degradation pathway would be synthesized in noticeable quantities. Yet if toluene or a similar substrate is used as the carbon source, the expression of these seven proteins will be very significant. Proteins which are specific for a particular growth condition are called a proteomic signature. This term is also defined as the subset of proteins whose alteration in expression is characteristic of a response to a defined condition or genetic change. Signatures are recognized after analyzing numerous profiles of samples from related and unrelated conditions. Signatures can include one or many proteins. [28]

Using 2DE to compare the protein expression of *P. putida* F1 on different carbon sources may lead to finding a proteomic signature that can serve as a bio-marker for drug screening as well as the drug development purposes. Because the *P. putida* F1 genome is known to be extremely well conserved through markedly adverse conditions, and its chromosomal genome is considered bio-safe (bio-safe rating is 1), proteomic signatures from this organism are most likely to be a response to the growth condition rather than genetic change. [23, 27]

In order to get the most complete protein expression as well as consistency in growth on the different carbon sources, a growth curve is necessary. A growth curve follows the
kinetics of cell growth. The simplest conditions for determining a growth curve are known as a batch culture: a single bacterial culture, introduced into and growing in a fixed volume with a fixed amount of nutrient. [30]

As shown in Figure 1-15, the growth curve has four main phases: lag, exponential growth (or log), stationary, and death phase. During the lag phase, bacteria become acclimated to the new environment and synthesize necessary proteins to replicate. Thus there is no significant growth during this phase. After this preparation, bacteria are then growing exponentially during the log phase. During this time, bacteria are replicating and nutrients are metabolized. Then growth enters the stationary phase, in which nutrients are exhausted and waste is accumulating. The growth rate has slowed to be equal to the death rate, thus there is no net growth during this period. When the nutrients are completely consumed and toxic waste products are predominant, the living bacteria population shrinks, and the growth curve enters the death phase. [30]

![Growth Curve Diagram](image)

Figure 1-15: Growth Curve Diagram [29].

When the growth curve has been determined for the compounds of interest, the mid-log (the middle point of log phase) times of the different compounds are used to
harvest cells for analysis. During mid-log phase, cells are growing, replicating, and dying all at the same time (but mostly growing and replicating); protein extracted from such samples would have the greatest variety of proteins. [30]

In this project, three organic compounds have been utilized as carbon sources for comparison purposes, one is a straight chain compound, succinic acid (SA; Figure 1-16), and two are aromatic compounds: benzoic acid (BA) and phenylethylamine (PEA; Figure 1-17). The two aromatic compounds are not known carcinogens but protein expression from these compounds may involve some dioxygenases or DNA repair/protection enzyme that could be proteomic signature of interest.

![Figure 1-16: Succinic Acid (by Chemsketch ACD)](attachment)

![Figure 1-17: Aromatic Carbon Sources (by Chemsketch ACD)](attachment)
Experimental

A - Bacterial Growth

This project includes bacterium; its growing is an important step. Methods for bacterial growth and sterile technique are described below. All chemicals used (except where indicated) are from J.T. Baker.

a) Plate-Streaking

Streaking a plate is a method used to obtain a single colony of growing bacteria. A single colony is necessary in bacteria growth because each colony theoretically grows from an individual cell, thus the genetic material is identical. The bacterial strain, *P. putida* F1, was obtained from American Type Culture Collection. After isolation of a single colony, a culture was grown on Luria medium. Stock cultures were prepared by dilution with an equal volume of sterile glycerol, snap frozen in liquid nitrogen and stored at -80°C; the stocks were then used to inoculate plates as described below at the start of each experiment. From the stock culture, bacteria can be transferred to an agar plate (of agar mixed with a selected carbon source and other minerals) by following these steps:

1. Sterilize the inoculating loop by running the entire wire through a flame until all parts glow bright red. Allow the wire to cool.

2. Submerge the wire loop in the liquid culture, then begin inoculating the first, or primary, quadrant of the agar plate. Streak lightly so that the agar surface is not scratched. Cover plate with lid.

3. Flame the loop, cool by touching an uninoculated portion of the surface.
4. Now rotate the plate. Open lid and streak again, following the diagram in Figure 2-1. Note that in this method, the cells from quadrant one are diluted more in each quadrant as streaking of the next quadrant only touches the end of the prior quadrant.

5. Flame loop; rotate plate, and repeat procedure for quadrants three and four.

![Diagram of streaking method](image)

Figure 2-1: Four Corner Plate Streaking Method Illustrated Streaking Plate [34].

This is called the "four corner" method, the most popular streaking technique [34]. However, any method of streaking designed to dilute cell density can be used.
b) Liquid Culture Inoculation [34]

A single colony from the agar plate is then used to inoculate a liquid culture of the desired volume. First, the inoculating loop is flame sterilized (as in streaking plate technique). Then, a single colony is picked from the plate, with the inoculating loop. The inoculating loop is submerged into the prepared media. After inoculation, the top of the media container (e.g., a flask) is passed through the flame to create an updraft to take air contaminants away from the container. If the inoculum is a liquid culture (obtained from a single colony), the exact volume of media needed can be transferred by sterile pipette. To ensure the sterility and purity of the cell growth, all glassware and media need to be autoclaved.

c) Growth Medium Preparation [23]:

The cultures for these experiments were all grown on Hutner's minimal media [23]. The media is prepared as follows. Note that all H₂O used in the preparation is deionized (D.I.).

Prepare one liter of solutions A and C according to the recipe below:

**Solution A:**

<table>
<thead>
<tr>
<th>per liter deionized H₂O:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂HPO₄</td>
</tr>
<tr>
<td>141.2 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
</tr>
<tr>
<td>136 g</td>
</tr>
</tbody>
</table>

(should give a pH = 7.25)

**Solution C:**

per liter deionized H₂O:
(NH₄)₂SO₄  200 g

Metals 44 Solution:

In 800 ml deionized water, dissolve the following:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>2.50 g</td>
</tr>
<tr>
<td>ZnSO₄ · 7H₂O</td>
<td>10.95 g</td>
</tr>
<tr>
<td>MnO₄ · 7H₂O</td>
<td>1.54 g</td>
</tr>
<tr>
<td>FeSO₄ · 7H₂O</td>
<td>5.00 g</td>
</tr>
<tr>
<td>CuSO₄ · 5H₂O</td>
<td>392 mg</td>
</tr>
<tr>
<td>Co(NO₃)₂ · 6H₂O</td>
<td>248 mg</td>
</tr>
<tr>
<td>Na₂B₄O₇ · 10H₂O</td>
<td>177 mg</td>
</tr>
</tbody>
</table>

Add a few drops of concentrated H₂SO₄ to retard precipitation. Dilute the solution with deionized water to a total volume of 1L. The solution should appear clear and lime green in color, and can be stored indefinitely in a brown glass bottle at room temperature in a closed cabinet.

Solution B: per liter deionized H₂O:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrilotriacetic acid</td>
<td>10 g</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>14.45 g (or 29.56 g MgSO₄ · 7H₂O)</td>
</tr>
<tr>
<td>CaCl₂ · 2H₂O</td>
<td>3.33 g</td>
</tr>
<tr>
<td>(NH₄)₆Mo₇O₂₄ · 4H₂O</td>
<td>9.25 mg</td>
</tr>
<tr>
<td>FeSO₄ · 7H₂O</td>
<td>99 mg</td>
</tr>
</tbody>
</table>
It is recommended that 1L of Solution B be prepared at a time with the following procedure:

1. Dissolve 10 g nitilotriacetic acid (NTA) in about 500 ml of water. (This is easier if the water is hot when adding the NTA. Heating the water to boiling is okay and seems to help.)

   **Safety Tip:** BE CAREFUL WITH NTA!! THIS COMPOUND IS KNOWN TO CAUSE HEREDITARY GENETIC DAMAGE!

2. Dissolve 29.56 g MgSO₄ · 7H₂O in about 100 ml water. Again, heat may help things to dissolve.

3. Dissolve 3.33 g CaCl₂ · 2H₂O in 50 ml water

4. Dissolve 9.25 mg ammonium molybdate and 99 mg FeSO₄ · 7H₂O together in 100 ml water.

5. Add the magnesium sulfate solution to the NTA solution slowly while stirring with a Teflon stir bar and be careful to avoid any clouding of the mixture. Once these are mixed, gradually add the calcium chloride solution. Then add the remaining solutions. The mixture should now be a yellow color. Finally, add 50 ml **Metals 44 solution**. The solution should now be a deeper yellow color.

6. Bring the volume up to a total volume of 1L.
At this point insert a pH probe into the liquid. Adjust the pH up to 6.7 with 10M NaOH. This must be done slowly, especially towards the end. A transitory precipitate will appear that eventually dissolves upon vigorous stirring. Let the precipitate dissolve before adding more NaOH. If the pH goes over 7, the solution will turn a green/gray color and a precipitate will form. It is possible to salvage this solution by adding a drop or two of concentrated sulfuric acid (H₂SO₄) and then again adding NaOH until the pH is 6.7. All medium component solutions should be vacuum-filtered through Whatman qualitative filter paper before storage in order to remove any particulate impurities that may be present.

To prepare 1 liter of Hutner's minimal medium, use the following volumes of solutions A, B, and C. Dilute to 1 L:

- Solution A ------------ 40 ml
- Solution B ---------- 20 ml
- Solution C --------- 5 ml

A heavy precipitate will form upon autoclaving. It will dissolve as the medium is stirred while cooling. It has been found that if the following components are autoclaved separately: (1) Water, (2) Solution A, and (3) Solutions B & C together; and then all combined after cooling, that no precipitate will form, saving a great deal of time.

The Hutner's minimal medium as described above does not include a carbon source. For our studies, we used carbon sources at 5 mM concentrations where possible. For example, in media containing succinic acid as carbon source, a 0.5 M solution of succinic acid was prepared from sodium succinate (solid) and filter sterilized. Different volumes of media can be prepared by mixing solutions A, B, C, carbon source, and water proportionally.
d) Growth Curve Measurement

Before examining the bacterial proteome, it was essential to prepare growth curves for *Pseudomonas putida* F1 on different carbon sources. Growth times, concentrations, and growing conditions differ greatly depending upon the specific carbon source used. The media used for growth is Huttner’s media. A minimal medium was selected to allow careful control of the carbon source for growth. Huttner’s medium is favored for the growth of *Pseudomonas* species because of the presence of many metal salts, which enable the enzymes in *Pseudomonas* species to catalyze a wide variety of degradative processes [34]. The following procedure was followed to obtain a growth curve of *P. putida* F1 with 5mM succinic acid as the carbon source. This procedure can be used as a guideline for other growth curves on other carbon sources. Please note that the growth rates may differ greatly but the analytical techniques should remain the same.

1. Prepare a 500 ml sample of the 5mM succinic acid growth media as described above.
2. Place a single colony of *P. putida* F1 in 5 ml of the prepared growth media and incubate for 24 hours at 30°C in a shaker to make a saturated cell culture.
3. Place 100 ml of the growth media into four side arm flasks. Label one of these flasks with a ‘B’ to use as a blank and set it aside.
4. Label the other three flasks samples 1 – 3 respectively.
5. Add 1 ml of saturated cell culture to each of flasks labeled 1-3.
6. Make sure to zero the Spectronics 20 instrument, setting the wavelength to 600 nm. While there is no sample in the Spectronics 20, set the needle to 0 by using
the dial on the left hand side of the instrument, then insert the blank. Using the
dial on the right hand side, with the blank still inserted, set the \%Transmittance
(\%T) to 95%. Setting \%T to 95% helps to include the fluctuation of the reading
(if it is greater than 100%).

7. Measure the \%T of the first sample by using the sidearm and repeat in triplicate.

8. Use the blank to zero the instrument again after the first three readings on
sample 1 have been completed.

9. Repeat steps 6 - 8 for each replicate sample.

10. Continue taking readings at least once every half-hour until growth ceases or the
\%T begins to increase.

After the data are obtained, convert \%T to absorbance and plot absorbance versus
time and determine the time needed for the bacteria to grow to the mid-log phase.

B - Reagent Preparation

a) Rehydration Buffer [31]

A series of stock solutions were also prepared for the 2DE experiments. The
rehydration buffer is important since it is used for both sonication and rehydration
processes to facilitate protein solubilization. The rehydration buffer can be prepared as
below:

1. Measure the following components (from Bio-Rad) and place into a 100ml
volumetric flask:

   i. 48.1g of Urea
ii. 4.0g of CHAPS (3-[(3-Cholamidopropyl) dimethylammonio] propanesulfonic acid

iii. 0.2ml of Ampholytes

iv. 0.77g of 50mM DTT (dithiothreitol)

2. After all of the components have been measured into the flask add approximately 50ml of distilled/deionized water to help dissolve them.

3. Shake vigorously until all of the solids have dissolved and the solution is clear.

4. Fill to line with D.I. water. (Total volume = 100ml).

5. Wrap the flask in foil and store in the refrigerator.

b) Equilibration Buffer [31]

The equilibration buffer helps to reduce interference by protein charges and hydrogen bonding; this facilitates protein movement in electrophoresis. To prepare the equilibration buffer, weigh the following components and place them in a 100ml volumetric flask:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Amount (final concentration)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>36 g Urea (6M)</td>
</tr>
<tr>
<td>20% SDS</td>
<td>10 ml (2%)</td>
</tr>
<tr>
<td>1.5M Tris/ HCl pH = 8.8</td>
<td>3.3 ml (0.05M)</td>
</tr>
<tr>
<td>50% Glycerol</td>
<td>40 ml (20%)</td>
</tr>
<tr>
<td>D.I water</td>
<td>Adjust to total volume of 100ml</td>
</tr>
</tbody>
</table>

c) Agarose

To make 50 ml of 0.5% low melting point agarose combine:

1. 0.83ml of 1.5M Tris buffer (from Bio-Rad Catalog # 161-0716)
2. 0.721 g of glycine (from Sigma Aldrich Catalog # G8898)
3. 0.25 g of low melting point agarose (from Bio-Rad Catalog # 161-3111)
4. 0.25 ml of 20% SDS (final concentration should be 0.1% SDS) (from Bio-Rad Catalog # 161-0418)
5. A trace amount of bromomphenol blue (from Bio-Rad Catalog # 161-0404)
6. Dilute to a total volume of 50 ml with D.I. water and mix to dissolve all components.

The prepared agarose may be stored in the freezer for an extended period of time. Immediately prior to use, the reagent must be thawed in a microwave.

**Safety Point:** Agarose is quick to boil. Loosen the cap before heating and heat for very brief periods of time until liquid (approximately 30 second increments). Use of goggles is also recommended.

---

C - Sample Preparation for 2DE

a) Growth of Bacteria

1. Mix a large amount (1 to 2 liters) of Hutner’s Growth Media (see page 34) and set aside.
2. Pick a single colony from a previously streaked cell plate and place it in 10 ml (for 1 liter of media prepared, 20 ml if 2 liter is used) growth media. Incubate the media in the orbital shaker at 37 °C for 24 hours.
3. Transfer the saturated culture to the 1 liter (or 2 liters) of fresh medium.

4. Stop at mid-log time (determined previously from growth curve) by reducing storing temperature to 4°C (in centrifuge or refrigerator).

b) Centrifugation of Bacteria Culture

1. After cell growth has been stopped, place all of the mid-log culture into 250ml centrifugation bottles, making sure that they all contain equal masses of solution (within 0.1 g) and that there is an EVEN number of centrifuge bottles.

2. Use the centrifuge (Sorvall Model RC 5CPlus) and rotor SA-800 (that will hold six 250 ml centrifuge bottles). This Centrifuge is temperature controlled and can accommodate forces of 12,000 x gravity.

3. Place bottles opposite each other in the instrument to ensure balance.

4. Close the instrument and adjust parameters. The rotor code should be set at 28. The temperature should be set to 4°C. RPM’s should be set to 8200, which brings the system to approximately 12,000 x gravity.

5. Spin for 30 minutes.

c) Sonication

1. After centrifugation is complete remove all containers.

2. Drain the supernatant back into cell growth container.
3. Collect all pellets after centrifugation with micro-spatula and transfer to a 50ml pre-weighed beaker.

4. Record the weight of the isolated pellets.

5. Add 2-3ml rehydration buffer to the pellets so that the mass in gram of protein is one third the volume of rehydration buffer in milliliters.

6. Place the beaker containing the cells in a bath of crushed ice.

7. Turn the sonicator (Ultrasonics Heat System Mod W-375, Convertor Mod C3) on and let it warm up for approximately 5 minutes.

8. Rinse sonicator tip with DI water, then with iso-propanol, and rinse again with water.


10. Carefully lower the sonicator tip into the beaker, making sure it does not touch the sides or the bottom of the beaker.

11. Sonicate the suspended pellet for 3 minutes, then let it stand on ice for 10 minutes.

12. Repeat step 11 two more times.

13. When sonication is complete, clean the tip by repeating step 8.

14. Microfuge – The final step of the sample preparation is to aliquot the sonicated protein sample into 2.0 ml disposable microfuge tubes in balanced portions. Place the samples in a microfuge instrument (Biofuge 13 Mod. 75004646/01 or equivalent). Microfuge for 5 hours under the following conditions: RPM = 13,000 and 4°C.
15. After the microfuge step is complete there should be a black pellet in the bottom of the microfuge tube. Transfer the supernatant to clean (new) microfuge tubes without disturbing the unwanted black pellet.

16. The isolated protein can now be stored in the freezer or used right away for the following procedures.

d) Protein Assay

The protein assay is an important step in the sample preparation because it dictates the volume of protein to be used during 2DE. The procedure for the protein assay that was used can be found on page 30 in Bio-Rad 2D Electrophoresis for Proteomics: A Methods and Product Manual [31]. From the protein concentration determined, the volume needed for one IPG strip is calculated for rehydration.

e) Ribonuclease Treatment

After determining the protein concentration, Ribonuclease treatment (RNase) can be performed to increase the purity of the protein mixture by digesting all the RNA that may still exist in the sample. RNase treatment can be done as follows:

1. After the protein assay has been completed, add 2mg of RNase per 1ml of protein sample contained in the clean microfuge tubes
2. Microfuge again for 20 minutes
3. Transfer to another clean microfuge tube
D- Rehydration and Isoelectric Focusing

a) Isoelectric Focusing Running Conditions

Table 2-1 contains the running conditions used in this work.

Table 2-1[23]: IEF Running Conditions

<table>
<thead>
<tr>
<th>IEF Step</th>
<th>16 hour run</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1 Voltage</td>
<td>300 V</td>
</tr>
<tr>
<td>Time, Ramp</td>
<td>6 min, Linear</td>
</tr>
<tr>
<td>Step 2 Voltage</td>
<td>300 V</td>
</tr>
<tr>
<td>Time, Ramp</td>
<td>4.5 hours, Linear</td>
</tr>
<tr>
<td>Step 3 Voltage</td>
<td>4000 V</td>
</tr>
<tr>
<td>Time, Ramp</td>
<td>5 hours, Linear</td>
</tr>
<tr>
<td>Step 4 Voltage</td>
<td>4000 V</td>
</tr>
<tr>
<td>Time, Ramp</td>
<td>6.5 hours, Linear</td>
</tr>
</tbody>
</table>
b) Rehydration

Remove the number of IPG strips needed (pH 3-10, 11cm) from the freezer.

1. Let the protein sample(s) thaw and mix to resuspend any undissolved urea.

2. Calculate the volume of protein sample needed for approximately 200 μg protein from the protein assay.

3. Transfer enough rehydration buffer into 1.5ml centrifuge tubes so that the total volume of buffer and protein will equal 185 μl.

4. Add protein sample and mix.

5. Take up the entire contents of the tube and apply to one well in a clean dry rehydration tray (see Figure 2-2) along the back corner of the well.

6. Gently peel off plastic backing of the IPG strip using forceps.

7. Lay the strip, gel side down, in the well such that the “+ pH 3-10” is legible and towards the left of the tray (when the sloped edge is on the right). Take care not to trap any air bubbles under the strip. This insures proper wetting of the strip and uniform distribution of proteins throughout the strip.

Figure 2-2: Rehydration Tray [36].
8. Overlay strips with approximately 2 – 3 ml mineral oil, cover the tray and let it sit at room temperature overnight (at least 12 hours and no more than 24 hours).

**NOTE:** Rehydration wells have numbers at the side so you can keep track of which strips and samples are in which wells. Be sure to record the number and description of the sample.

c) Isoelectric Focusing

1. Place a clean, dry focusing tray on the lab bench with the + to the bottom left side (see Figure 2-3).

![Figure 2-3: Isoelectric Focusing Tray](image)

2. Place paper wicks over the wire electrodes in the wells to be used (using forceps).

3. Pipet 8μl nanopure water onto each wick.

4. Using clean forceps, carefully remove 1 IPG strip from the rehydration tray and hold it vertically for about 7 – 8 sec. to allow
the mineral oil and rehydration buffer to drain, then place it gel side down in the focusing tray. (Making sure that polarities are correct.)

5. Repeat step 4 for all remaining strips.

6. Cover each strip with 2 – 3 ml fresh mineral oil (remove any air bubbles by moving the IPG strip)

7. Place the lid on the tray and carefully move the tray into the cell and close the cover.

8. Program the run as in table 2-1 and press start.

9. Upon completion, remove the strips from the focusing tray (allowing them to drain for 5 seconds) and place them in a clean rehydration tray.

10. At this point, the strips can be used for electrophoresis, or can safely be stored by freezing at -20°C.

E- Second Dimension Separation

1. For every two IPG strips, pipette two 10 ml portions of Equilibration buffer into 2 separate centrifuge tubes. Label tubes I and II for buffer I and buffer II.

2. Add 200mg of DTT to tube I. Shake to get the DTT into solution.

3. Add 4ml of this solution to each well containing a focused IPG strip, and place the tray on an orbital shaker for 10 minutes.

4. While the IPG strip(s) are in the orbital shaker:
   
   i. Place the bottle of agarose in the microwave for 30 second increments until it becomes liquid and slightly warm.
ii. Prepare the running buffer: Mix 100ml of Tris/Glycine/SDS Buffer (BioRad Catalog 161-0732) with 900ml of nano-pure water.

iii. Prepare the equilibration buffer II: Add 250mg of Iodoacetamide to the tube labeled buffer II.

5. Slowly pour off equilibration buffer I from IPG strips when they have finished shaking.

6. Add 4ml of buffer II to each tray, and place in an orbital shaker for another 10 minutes.

7. Take pre-cast gels out of packaging. Remove the green comb, and rinse the well (that the comb was covering) with nano-pure water. Remove the white sticker from the bottom of the gel.

8. Remove the strips from the shaker and pour off buffer II.

9. Pour the running buffer into a graduated cylinder.

10. Cut a 1cm wide strip of blotting paper and dry/blot out the well in the pre-cast gel.

11. Remove one of the IPG strips from the tray and dip it into the cylinder that contains the running buffer.

12. Place the IPG strip into the pre-cast gel, slightly above the well with the gel side out so the pH number on the IPG strip reads 3-10 from left hand side (the reading pH 3-10 is at the left and number 1 in 10 is upside down).

13. Fill the well in the gel to the top with liquid agarose.
14. Push the IPG strip into the well with the agarose and let it sit for approximately 2 minutes while the agarose hardens.

15. Repeat steps 11-14 for all remaining IPG strips.

16. Rinse the electrophoresis cell (see Figure 2-4) with distilled water.

![Electrophoresis Cell](image)

Figure 2-4: Electrophoresis Cell [36].

17. Inset pre-cast gels into the electrophoresis cell and pour the running buffer into each cell compartment up to the fill line.

18. Make sure the wires on the green top of the electrophoresis cell are completely submerged in the running buffer, which completes the electric circuit.

19. Place the top on the electrophoresis cell. Plug into an outlet. Set to 200 volts and set the run time for 52 minutes.

20. Check the gel at least every 15 minutes to see where the dye-line is on the gel. The dye line must not run off the bottom of the gel. Stop the run if the dye line approaches the bottom of the gel before it runs off.
F - Staining

* This procedure is taken from the BioRad Methods and Product Manual (page 38 - 39)

1. Remove pre-cast gel from electrophoresis cell. Break the plastic of pre-cast gel open by pressing the well against the lid of the electrophoresis cell.

2. Lay gel flat in a plastic container.

3. Pour D. I. water enough to cover the gels. Gently agitate the content to rinse the gels. Repeat it twice, each time for 10 minutes remove the running buffer down the drain.

4. Add sufficient Bio-Safe Coomassie Blue stain to cover the gels.

5. Allow the gels to soak in the stain for at least 90 minutes. (Color will continue to develop after 1 hour).

6. Rinse the gels with deionized water, with agitation, for approximately 3 minutes, decant the water and cover gel with fresh water and let sit for 10 minutes.

7. Step four should be repeated at least three times.

8. Gels can be placed in water to de-stain up to 24 hours.

9. Repeat the rinsing procedures until desired contrast between background and protein spots is achieved.
G - Drying and Storage

1. Remove the gel from aqueous conditions and place between two pieces of cellophane.
2. Place several pieces of large filter paper in the fume hood.
3. Place the cellophane wrapped gel on top of the filter paper, and cover it with additional pieces of large filter paper.
4. Cover it with several large books and leave it for at least 24 hours.
5. Transfer the dried gel to a plastic zip-top bag and seal it.
6. Label the bag with the date, operator initials, *Pseudomonas* strain, and carbon source.

H - Image Analysis

a) Image J and File Conversion

There are many electrophoresis gel analysis software packages available. The one used in this project is Non Linear Progenesis. This program allows analysis of multiple gels, detection of spots on gels, and comparison of the differences automatically and manually. In order to load gel images into the program, scanned gels in bitmat (.bmp) format need to be changed to .tiff using the Image J program (this can be downloaded from National Institute of Health Website [37]):

1. Open Image J
2. Select file, then open to open a gel image
3. After the image is opened, select Image, then change type to 8 bit;
4. Select the file again and Save as Tiff. Make sure to enter the name of the image with .tiff in the end

5. Save the converted files onto desktop

b) Analyzing Gel Images Using Progenesis [32]

Due to the manual drying process, gels were stretched and the automatic analysis was not able to warp and match gels. Automatic analysis was launched to detect spots on gels as follows:

1. Launch automatic analysis by double-clicking on Progenesis Workstation icon on the Desktop. If the automatic wizard does not appear, select Analysis from program menu, then Automatic analysis

2. Choose single stain analysis

3. Name the experiment using analyst initials, bacterial strain, and carbon source

4. Select the gel images from the directory

5. Follow the wizard and keep the default settings (Background Subtraction, etc), except to include the reference gel in the protocol, uncheck add spots to reference gels, and check synchronize spot number.

6. After the automatic analysis is performed, the experiment will be opened with the reference gel window open. Select Analysis, then warping to manually warp unmatched gels.
7. Select warped or overlaid images for display mode (located on the left frame under warping). The shift vector/pin can be inserted to match gels. Colors and contrast can be changed by clicking the color button on the left of the image window. Zoom can be changed by clicking the magnifying glass icon from the right bottom of the image window.

8. After manually warping, the image can be copied by clicking on View, then copied to the clipboard, and then pasted onto paint.
Data and Results:

A - Growth Curve for P. putida F1

a) Reproducibility Study

In this study, three different analysts performed growth-curve measurements on the same carbon source on different dates to evaluate the reproducibility. The media used was 4mM Succinic Acid in Hutner’s minimal media as described in Table 3-1.

Table 3-1: Preparation of the 4mM Succinic Acid Medium

<table>
<thead>
<tr>
<th>4 mM succinic acid in Hutner’s minimal media</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution</td>
<td>Volume (ml)</td>
</tr>
<tr>
<td>A</td>
<td>16</td>
</tr>
<tr>
<td>B</td>
<td>8</td>
</tr>
<tr>
<td>C</td>
<td>2</td>
</tr>
<tr>
<td>0.5 M Succinic acid</td>
<td>3.2</td>
</tr>
<tr>
<td>Water</td>
<td>370.8</td>
</tr>
<tr>
<td>Total</td>
<td>400</td>
</tr>
</tbody>
</table>

From 400 ml media total, triplicate samples of 100 ml were used (leaving a 100 ml surplus). Each sample measurement was taken three times for averaging and comparison purposes. The average of three measurements was calculated as well as the standard deviation. Then measurements of the growth curve from each technician were averaged and compared graphically.
As seen in Figure 3-1, a simple batch culture growth curve pattern was seen. The lag phase lasted approximately three hours, then between hours 4 to 6 is the log phase, the stationary phase lasts from hours 6 to 9, and the death phase was not observed. The three technicians' results are relatively consistent with a mid-log phase of 5 hours (±10 minutes). Following this demonstration of reproducibility, growth curve measurements of *P. putida* F1 on different carbon sources were determined.

![Growth curve reproducibility](image)

**Figure 3-1: Growth Curve Reproducibility of *P. putida* F1 on 4mM Succinic Acid**

Legend:  
HH: measurement by Han Hoang  
KF: measurement by Kelly Folkwes  
MP: measurement by Maryed Pratts

b) Succinic Acid-Benzoic Acid-Phenyl Ethyl Amine

To maintain consistency in the results, all carbon sources were used in fixed concentration of 5mM. Media containing different carbon sources were prepared with
total volumes of 400ml, with volumes of solutions A, B, and C as in described table 3-1.
The remaining ingredients for the respective growth curves are shown in Table 3-2:

Table 3-2: Amounts of Carbon Sources Used to Prepare Growth Media.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Quantity</th>
<th>Water added (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 M succinic acid</td>
<td>4.00 ml</td>
<td>370</td>
</tr>
<tr>
<td>Sodium Benzoate (MW=144)</td>
<td>0.288 g</td>
<td>374</td>
</tr>
<tr>
<td>Phenylethylamine (MW=121.18)</td>
<td>0.251 ml</td>
<td>373.749</td>
</tr>
</tbody>
</table>

Growth curves on these media were then performed using the same protocol for the growth curve of *P. putida* F1 on 4mM Succinic acid. The readings from each sample were averaged then compared graphically. Figure 3-2 shows the result for *P. putida* F1 on 5 mM SA.

Figure 3-2: Growth of *P. putida* F1 on 5mM Succinic Acid.
The triplicates are almost identical, again showing the reproducibility of the growth curves. The mid-log time for 5mM succinic acid was found to be 5 hours (±10 minutes), just as it was for 4mM succinic acid. Thus the growth curve seems not to depend heavily on concentration for succinic acid in this concentration range. The stationary phase and the death phase were not clearly distinguished because of the lack of data points, between 8 and 22 hours. Figure 3-3 shows the results of growing *P. putida* F1 on 5mM BA.

Figure 3-3: Growth of *P. putida* F1 on 5mM Benzoic Acid

The growth curve on benzoic does not follow the normal growth curve pattern. The log phase took off slowly but quite steadily yet the stationary phase did not seem to be defined. The death phase was as evident as the log phase. The beginnings and endings of each phase were well-defined and consistent. A mid-log phase time of 8.3 hours (±10 minutes) was determined.
The phenylethylamine study shown in Figure 3-4 had the longest growth curve. The consistency was not as good as the other two. There seems to be variability from 10.4 hours to 14.0 hours. Trial 1 grew faster than trial 2 yet log phase of these seems to stop at the same time, yet trial 3 is quiet different. There was no stationary phase in trial 2, yet trial 1 and three had approximately 2 hours of this phase. This inconsistency possibly caused a shift in mid-log time determination. Since two trials were in agreement, the mid-log phase was determined by trial 1 and 3 to be 9.3 hours (±10 minutes).

![Graph](image)

Figure 3-4: Growth of *P. putida* F1 on 5mM Phenylethylamine.

B - Sample Preparation and Protein Assay

a) Growth and Harvesting of *P. putida* F1

For protein extraction purposes, bacteria were grown in one liter batches, according to the method described in the Experiment section (pages 37-40). Media
containing different carbon sources were prepared in a total volume of one liter, containing 40 ml of solution A, 20 ml of solution B, and 5 ml of C with carbon source as shown in Table 3-3.

Table 3-3: Media Prepared for Protein Extraction Experiments

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Quantity</th>
<th>Water (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 M succinic acid</td>
<td>10 ml</td>
<td>925</td>
</tr>
<tr>
<td>Sodium Benzoate (MW=144)</td>
<td>0.72 g</td>
<td>935</td>
</tr>
<tr>
<td>Phenylethylamine (MW=121.18)</td>
<td>0.6275 ml</td>
<td>934.3725</td>
</tr>
</tbody>
</table>

Growth was stopped after 5 hours for the succinic acid cultures, 8.3 hours for the benzoic acid cultures, and 9.3 hours for the phenylethylamine culture. Methods for cell harvesting and protein recovery are described in the Experimental section.

c) Protein Assay

The BioRad protein assay, which is based on the Bradford method [31], was used to determine protein sample concentrations. This is necessary to ensure consistent protein loading on the IPG strips, which in turn improves run-to-run reproducibility. The calibration curve for a typical protein assay is shown in Figure 3-5.
Figure 3-5: Protein Standardization Curve

The curve shown in Figure 3-5 was used to measure protein concentrations in samples extracted from cells grown on BA and PEA. For analytical purposes, the desired protein load for an 11 cm IPG strip is 200 µg. Calculations for typical sample loads are shown in Table 3-4.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Total Concentration (µg/µl)</th>
<th>Volume for 1 strip (µl)</th>
<th>Total protein concentration (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA</td>
<td>18.5</td>
<td>11.0</td>
<td>203.0</td>
</tr>
<tr>
<td>PEA</td>
<td>21.5</td>
<td>9.5</td>
<td>203.5</td>
</tr>
</tbody>
</table>

Once the protein concentrations had been determined, samples were treated with RNase, applied to the IPG strips and separated by 2DE. Three gels were run from cells grown on each carbon source, and were used for further analysis.
C - Gels from *P. putida* F1 Cells Grown on Succinic Acid as the Carbon Source

Reproducibility is the major challenge when comparing different gels. The three SA gels were run under identical conditions from the same sample of SA protein extraction. Before obtaining these gels, much work had been done to improve the clarity of the gel background as well as the standard operating procedure, including changing IEF conditions, fixing SDS-PAGE running times, lengthening centrifugation time, and adding RNAse treatment. Reproducibility for these three gels was quite good. The software program, Progenesis [32], detected 394 spots on the reference gel (the gel with the most spots out of the three). Compared to complete list of 5614 proteins predicted from the open reading frames of *P. putida* F1, this number represents less than 10% of all possible proteins. However, only certain proteins are vital and present at high enough levels to appear in 2DE gel. Furthermore the stain used on the gels is quite reliable with a wide dynamic range of protein concentration correlation, but it is not very sensitive below 36 ng of protein [31].

In figure 3-6, the overlaid gels matched quite well. There are a few patterns which did not match, for example, the smallest proteins at the bottom right of the gels and also in the middle range (approximately from pH 5-7; indicated by arrow and box). There are a few mismatched spots in the gels yet it appears that the adjacent spots between the reference gel and SA gel 2 are the same proteins.
Figure 3-6: Overlaid SA Reference Gel (magenta) and SA Gel 2 (green). When magenta and green spots coincide, the color changes to black.

The comparison of the reference gel and SA gel 3 shown in Figure 3-7 is similar to the one in Figure 3-6. Yet there are two totally mismatched proteins on the high pH side that exist only in the reference gel (indicated using arrow). Overall, the reproducibility for gels from cells grown on SA was reliable. Some spots do not overlay yet should be the same protein spot (circled) while some streaking/ spots stand out as being mismatched.
D - Gels from *P. putida* F1 Cells Grown on Benzoic Acid as the Carbon Source

Gels from cells grown on BA were run under conditions identical to those described in the previous section. These gels were also subjected to the drying process which led to inconsistent shrinking, resulting in imperfect pattern matching again.

In Figure 3-8, considering how faint all the green spots are, it was evident that there was not as much protein loaded onto BA gel 2 as on the reference gel (based on size of overlaid protein spots). Nonetheless, the pattern generally matches up. The only one non-matched pattern in the middle of the gel (a green streak) is questionable. The patterns appear to be identical since the magenta color of most of the reference gel spots is quite darkened. The circles indicate protein spots that may be the same spots but do not overlay because of stretching.
The two gels in Figure 3-9 matched better than those in Figure 3-8. The intensities of protein spots are similar. The green streak in the middle still persists. Overall the reproducibility of the three gels from the BA sample is not as good as that of SA gels (overlaid gels do not have as many intense black spots). But the match is evident as the general protein spots pattern is found. It was noted that the lower molecular weight proteins sometimes ran off the gels even though all gels were run under the same conditions. Thus instrumental reproducibility of 2DE still needs to be improved.
Figure 3-9: Overlaid BA Reference Gel (magenta) and BA Gel 3 (green)

E - Gels from *P. putida* F1 Cells Grown on Phenylethylamine as the Carbon Source

The last set of three gels is from *P. putida* F1 cells grown on phenylethylamine as the carbon source. These gels do not have as many spots as those from SA samples. The backgrounds of these gels are reasonably clear.

In Figure 3-10, there is a resemblance of the middle streak (but in magenta this time) to the BA sample gels. Some spots on the PEA reference gel have quite clearly drifted (when compared to the PEA gel 2, the green protein spots in rectangle box at higher pH and low MW area). At the same time, the PEA reference gel appears to have a lower protein load based on the faintness of magenta spots (but black matched spots show
the superposition of proteins from reference and sample gels). There are noticeably mismatched spots between the two gels, such as the two dark pink spots (high MW around pH 5—indicated by the arrow), the corresponding green spots from PEA gel 2 may have smeared into streaking and are not clearly shown. The protein pattern at the bottom of the gel reappeared in PEA gel 3 (Figure 3-11). Thus those proteins appear to have eluted from the gel during SDS-PAGE in the reference gel.

Figure 3-10: Overlaid PEA Reference Gel (magenta) and PEA Gel 2 (green)
Figure 3-11: Overlaid PEA Reference Gel (magenta) and PEA Gel 3 (green)

The two gels in Figure 3-11 matched much better than those in Figure 3-10 yet it is obvious protein concentration of PEA gel 3 is much greater than that of the reference gel since the green (from PEA gel 3) overwhelms the magenta (from the PEA reference gel) in most of the spots. There is also a matching pattern of proteins at the bottom of the gels (low molecular weight) which was not seen in PEA gel 2. Overall the matching is quite good. Most spots can be seen in all three gels and the remaining spots appear on at least two gels.

Reproducibility between gels for samples grown on the same carbon source was acceptable; the standard operation procedure may have to be modified since some proteins migrated off the gels during electrophoresis.
Comparison of Gels from the Three Different Carbon Sources

Based on the evaluation of the gels grown on each carbon source, the reference gels were used to compare protein expression between cells grown on the different carbon sources. The goal was to determine a proteomic signature for BA and PEA when compared to SA.

In Figure 3-12, there are noticeable differences between the two gels. The SA reference gel has many more spots than the BA gel (some small spots from SA gel are boxed). Circled protein spots are those that clearly appear on the BA gel, but not on the SA gel. Additional mismatched spots result from slight shifting – they are present on both gels. The appearance of green streaking on the BA reference gel does limit proteomic signature detection with BA as the carbon source – clearer gels might reveal more of the BA proteomic signature.

Figure 3-12: Overlaid SA Reference Gel (magenta) and BA Reference Gel (green). Circle spots were found only in the BA reference gel and spots indicated with rectangles were unique to the SA gels.
In Figure 3-13, examination of the overlaid SA and PEA reference gels reveals a similar pattern of unique spots as was seen in the previous comparison (between SA and BA); these spots are again circled (PEA) and boxed (SA). Streaking is still an issue that prevents the identification of more unique protein spots. The identification of four protein spots as the proteomic signature for PEA is significant. The lack of the SA spots is also a part of the signature.

Figure 3-13: Overlaid SA Reference Gel (magenta) and PEA Reference Gel (green). Circle spots were found only in the BA reference gel and spots indicated with rectangles were unique to the SA gels.

Figure 3-14, on the other hand, compares the BA and PEA gels with each other. They are both aromatic compounds. Thus similar patterns of protein expression might be
expected. If the four proteins identified in the proteomic signatures for each of these compounds match, it could be the proteomic signature of aromatic compounds.

![Figure 3-14: Overlaid PEA Reference Gel (magenta) and BA Reference Gel (green). The circles indicate locations of the signature proteins for BA and PEA which were identified in Figures 3-12 and 3-13.](image)

As expected, the two gels align very closely. They both do not contain the SA proteomic signature. In addition, the four proteins identified in both proteomic signatures match up as well. As mentioned previously, the loss of low molecular weight proteins (that indicated in figure 3-11 in a rectangle) on the bottom of the gel prevents further comparison between the two proteomes.
Results and Discussion

A – Reproducibility

a) Growth Curve

The growth curve study for each carbon source is important to ensure the consistency of the protein sample content from each of the carbon sources. Bacteria use nutrients in growth media to synthesize building blocks (amino acid, carbohydrates, lipids and nucleotides) for macromolecular structures and replication, as well as vitamins and cofactors to support metabolic processes. Once enough proteins and nucleic acids have been synthesized, they divide. Thus the bacterial cell contains the most proteins and other building blocks just prior to division; in other words, this is the time when the protein expression is most complete. The growth curve study determines the time when most bacterial cells are at the stage before division. Thus the reliability of the growth curve is significant in protein expression results.

Reproducibility of the growth curve was tested for variation on the same carbon source starting from the same single colony culture (thus all bacteria are genetically identical) and measured by the same analyst. A second study was conducted to determine variation when different cultures from different colonies were measured by different analysts. The resulting standard deviations were within 10% between curves at each time point on the growth curves. Therefore, it can be concluded that the growth curve is reproducible and that the results obtained are reasonably precise and accurate. Thus, variability from growing cells in this method is 10% or less.
b) Sample Preparation

Sample preparation has the potential to contribute the most inconsistency to this analysis. After bacterial growth, the cells are fractured by sonication to release a cellular protein homogenate. In the beginning, Tris buffer (40 mM, pH 7.5) was used to suspend the cells and prepare the protein homogenate. The resulting gels had a great deal of horizontal streaking which was probably caused by contamination in the protein sample and poor solubility. The RNA and mineral salts can form a lattice which interferes with protein migration during electrophoresis [14]. The poor solubility was overcome by replacing the Tris buffer with rehydration buffer during lysis. Regarding the RNA problem, RNase was added to remove any remaining RNA in the sample. Experimentally, the use of rehydration buffer during sonication and RNase improved clarity of the final gels (see Appendix C). During sonication, the heat generated from vibration may have denatured some proteins while at the same time activating proteases (enzymes in cell that degrade other protein to amino acids) in the extract. These problems during sonication may have resulted in variation between protein samples derived from different incubations of the organism on the same carbon source. For the results reported in this project, only one sample of protein from each carbon source was used for 2DE, thus this variation was eliminated.

c) Two Dimensional Gel Electrophoresis

With all these variances minimized, the final gels were expected to be quite reproducible. The 2DE gels from the same samples matched reasonably well. Though the clarity of the gels varied between runs as did the amount of protein loaded (some gels
have inconsistent spot intensities). This may have resulted from the passive rehydration of the protein prior to IEF. One approach to overcome this problem would be to use active rehydration, where an electric field is applied to facilitate protein migration into the IPG strip. Another approach is to lengthen the solubilization period of protein in rehydration buffer so the mixture is homogenous. The most important factor is that the protein sample should be mixed thoroughly before it is applied to the IPG strips.

The IEF process was also modified to improve gel reproducibility as well as clarity. In the Bio-rad 2DE Manual [31], the recommended IEF program for 11 cm pH 3-10 IPG strips take 5 hours and 30 minutes to run, with reasonably high voltage used. The IEF instrument was re-programmed with longer focusing time – 16 hours – at lower voltage but with the same total volt-hour exposure. This slower and steadier approach results in better resolution of IEF, especially reducing horizontal streaking.

The second dimension, SDS-PAGE, also had some problems with reproducibility. Even though the SDS-PAGE run time was set for 52 minutes and we used pre-cast gels from Bio-Rad, the protein migration rate was inconsistent. In some gels, it was evident that some low molecular weight proteins eluted from the bottom of the gels despite run conditions being fixed. This instrumental inconsistency should be further investigated and improved.

Despite the occasional loss of low molecular weight proteins, and problems with streaking and background, the final gels coming from the same samples matched each other quite well. As mentioned in the results, most protein spots appear on all three gels, only a few appear on two out of three. Nonetheless, the problems with reproducibility, despite the changes made, clearly point to the need for further study on this topic.
B - Comparison of Different Carbon Sources

Both bacterial growth and protein expression changed when different carbon sources were used.

a) Growth Curve Comparison

Succinic acid, a simple four carbon molecule, gave the best growth and followed the typical growth curve pattern with lag, log, stationary, and death phases. The two aromatic compounds, BA and PEA, on the other hand, had little or no stationary phase with the death phase following immediately after log phase. *P. putida* F1 not only grows faster (as in time) but also better (as in cell density) in succinic acid. Phenylethylamine also seems to be a better growth carbon source for this organism compared to benzoic acid since the stationary phase was longer and maximum cell density was much higher as well.

The concentration of the carbon source was kept constant at 5 mM and this may also have affected the study. Succinic acid has only 4 carbon atoms compare to 7 in benzoic acid and 8 in phenylethylamine, yet there was less growth on the two carbon sources that had more carbons. Thus the number of carbon atoms in a molecule did not correlate with the energy or nutrition the bacteria could derive from it. When growth was stopped at mid-log phase for protein extraction, there were many more cells from succinic acid than from either phenylethylamine or benzoic acid. This could explain why there are many protein spots that exist in gels from the SA samples that were not found in proteins extracted from cells grown on PEA and BA. With higher cell density, proteins from different stages of bacteria life cycle are more likely to have a high enough concentration to be detected.
Regarding the non-typical growth pattern of PEA and BA, one possible reason for this occurrence may have been that the waste generated from metabolizing these compounds was toxic for the cell. This hypothesis can be examined by analyzing the media by GC/MS after the cell growth for any remaining carbon source and its metabolic intermediates or by varying the concentrations of the carbon sources to see if that affects growth.

d) Comparison of the Gels

From the results obtained, the proteins expressed in the SA gels were clearly different from those seen in the BA and PEA gels. Many of the protein spots match up in all three sets of gels; these probably represent the vital proteins (sometimes called ‘housekeeping” proteins) for *P. putida* F1. Many proteins in low concentration (estimation from the intensity and the size of protein spots) exist in SA gels but not BA and PEA gels. One reason for this was mentioned previously, relating to varied cell density from growth on different carbon sources. During the process of analysis of the gel images, there was also some difficulty. Due to manual drying of gels, which caused stretching, automatic warping and matching for comparison by Progenesis could not be utilized. As a consequence, the valuable statistical data that can be generated by this program were not available.

There may also be more signature proteins (other than those identified) within the streaking. The goal of the project was reasonably achieved with the detection of four proteins spots on BA and PEA gels that were not found on SA gels. These four proteins may represent a proteomic signature of aromatic compounds in *P. putida* F1.
From this result, further studies can be carried out to confirm or disprove the proposition that these four protein spots are in fact a proteomic signature for aromatic compounds. More aromatic compounds should be used as carbon sources for 2DE proteomic study of *P. putida* F1 to see if a similar pattern persists. Another suggested approach is to identify the four protein spots by mass spectrometer. Since the complete genome of *P. putida* F1 is known, identification of these proteins will be possible.

When BA gels and PEA gels were compared, similar patterns of protein expression were found. It was surprising how well they matched. The two reference gels of BA and PEA match up even better than some of the BA or PEA gels match with other gels from the same carbon source. This showed that there is still much room to improve the reproducibility in the separation, staining, drying, and imaging processes of 2DE.

Even though BA and PEA are both aromatic, and should express similar proteins (where the pathways merge into one), there should still be proteins unique for each pathway. The presence of streaking may have masked some of these proteins. Another explanation for this is also the sensitivity of the stain is not high enough to detect low abundance proteins. Very faint magenta/green spots are located in the pH 6-8 range. Further study could focus on this range (by using narrower range IPG strips) and use of a more sensitive stain, such as silver stain, to reveal more signature proteins for each carbon source.
C - Benzoate versus Phenylethylamine Pathway

A careful examination of the proposed metabolic pathways for the degradation of BA and PEA provides insight into the similarity of the BA and PEA gels (Figure 4-1).

Figure 4-1: Metabolic Pathways of PEA and BA (strain KT2440) [24].

In Figure 4-1, the two compounds enter two totally different metabolic pathways. BA goes to the catechol pathway while PEA is metabolized via the phenylacetate pathway. The pathways shown in Figure 4-1 are proposed for a different bacterial strain (P. putida KT2440 in Figure 4-1 vs. P. putida F1 in this project), however, the organisms are
closely related, with the only major difference being the presence of some additional extrachromosomal DNA in the form of the TOL plasmid in *P. putida* F1 [21]. Due to these similarities, it is reasonable to expect these two compounds should still be metabolized by the indicated pathways in *P. putida* KT2440. To metabolize these compounds, the TOL plasmid (which contains genes that encode enzymes for the degradation of Toluene) does not need to be expressed. The two pathways converge at the TCA cycle; succinic acid is a TCA cycle intermediate. Thus the proteomic signatures found are not likely to be TCA cycle enzymes since succinate also needs those, but may be other metabolic enzymes. Note that many steps are omitted in the two pathways shown in Figure 4-1 and that in each case the gene thought to encode the metabolic enzyme is listed, rather than the names of the enzymes themselves. Figures 4-2 and 4-3 show the proposed metabolic fate of BA and PEA in more detail.
Figure 4-2: Styrene Pathway *Pseudomonas putida* [39].
Figure 4-3: Benzoate Pathway of Pseudomonas putida [39].
A comparison of the BA and PEA pathways reveals many unique proteins that must be expressed to metabolize these different compounds. For example, for the BA pathway, enzymes from benzoate dioxygenase to 4-hydroxy-2oxo-valerate aldolase must be expressed at significant levels. The same is true for PEA metabolic enzymes. If the protein expression in the presence of BA and PEA reflected only elevated levels of these metabolic enzymes, then the proteomic signatures for each of these two compounds would be completely unique. Yet the results indicate at least four common signature proteins for BA and PEA.

These common signature proteins may be involved in protective mechanisms of the cells. Considering the toxic intermediates and metabolites generated from the two metabolic pathways (indicated by the pattern of growth curve), one would expect a defensive response from \textit{P. putida} F1. That defensive response may be proteins which are either DNA repair enzymes or enzymes that inactivate the generated toxins.

\textbf{D - DNA Repair Enzyme}

If the proteomic signature includes cell repair/protection enzymes, possible candidates include the DNA repair enzyme of \textit{P. putida}. DNA carries the genetic information and therefore living organisms must always possess protective mechanisms to guard and repair DNA from damage. Some passive protective mechanisms are the redundancy in DNA material (bases that exist in DNA which do not encode proteins or structural RNAs) or the degeneracy of the genetic code (most amino acids are coded by multiple codons in the genetic code). Bacteria also use active mechanisms to protect
themselves from their harsh environment. There are many conditions that can cause mutations and there are many types of mutation. Mutations may be caused by radicals, which are generated from metabolic intermediates such as hydrogen peroxide, and radiation. For survival, bacterial cells have proteins that detect changes in DNA and repair it. Most studies regarding DNA protective mechanism have been done on *E. coli* (*E. coli*) [34]. Only a few have been described for *P. putida* [34].

A search of the *P. putida* proteome, revealed a number of DNA repair enzymes that may contribute to the unique proteomic signature of *P. putida* F1 when grown on BA and PEA. These are listed in table 4-1 with their theoretical pI as well as their molecular weight (MW).

Two of detected possible signature proteins fall within the pI range and one of them may fall in the indicated Molecular Weight range. Yet there are some that have much higher molecular weight (MutS with MW of 951112.13) or much higher pI values (FPG with pI of 9.13). Since a protein molecular weight standard was not included in the SDS-PAGE, there is not enough supportive data to assign any of these DNA repair enzymes to the signature spots on the gels, but it is reasonable to speculate that the detected proteomics signature may have included DNA repair enzymes. The signature proteins should be analyzed and identified using mass spectrometry. This would have a high probability of confirming their true identity.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Organism Specification</th>
<th>Theoretical pl</th>
<th>MW</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPO4_PSEPK</td>
<td>Pseudomonas putida (strain KT2440)</td>
<td>8.85</td>
<td>39859.58</td>
<td>DNA polymerase IV (EC 2.7.7.7) (Pol IV).</td>
</tr>
<tr>
<td>FPG_PSEPK</td>
<td>Pseudomonas putida (strain KT2440)</td>
<td>9.13</td>
<td>30177.95</td>
<td>Formamidopyrimidine-DNA glycosylase (EC 3.2.2.23) (Fapy-DNA glycosylase).</td>
</tr>
<tr>
<td>LXA1_PSEPK</td>
<td>Pseudomonas putida (strain KT2440)</td>
<td>6.24</td>
<td>22147.65</td>
<td>LexA repressor 1 (EC 3.4.21.88).</td>
</tr>
<tr>
<td>LXA2_PSEPK</td>
<td>Pseudomonas putida (strain KT2440)</td>
<td>6.32</td>
<td>22461.82</td>
<td>LexA repressor 2 (EC 3.4.21.88).</td>
</tr>
<tr>
<td>MUTL_PSEPK</td>
<td>Pseudomonas putida (strain KT2440)</td>
<td>6.21</td>
<td>69745.25</td>
<td>DNA mismatch repair protein mutL.</td>
</tr>
<tr>
<td>MUTS_PSEPK</td>
<td>Pseudomonas putida (strain KT2440)</td>
<td>5.91</td>
<td>95112.13</td>
<td>DNA mismatch repair protein mutS.</td>
</tr>
<tr>
<td>RADC_PSEPK</td>
<td>Pseudomonas putida (strain KT2440)</td>
<td>7.14</td>
<td>25194.19</td>
<td>DNA repair protein radC homolog.</td>
</tr>
<tr>
<td>RECA_PSEPK</td>
<td>Pseudomonas putida (strain KT2440)</td>
<td>5.50</td>
<td>37544.89</td>
<td>RecA protein (Recombinase A).</td>
</tr>
</tbody>
</table>
Conclusion:

At the start of this project, no 2D gel electrophoresis had been performed by this research lab. The project started with a 2DE separation of a commercially supplied protein homogenate from *E. coli* and has progressed through a series of modifications to the procedures (from rehydration to IEF). Much progress has been made in improving the quality of the gels, to eliminate possible variation in the method, and to produce reproducible results.

To improve the quality of the background signal in the gels, the lysis buffer was changed from Tris buffer to rehydration buffer. RNase was used to eliminate the streaking due to the presence of high molecular weight RNA in the samples. The IEF focusing time was increased and voltage was lowered to improve separation quality, while maintaining the same total voltage x hours.

Reproducibility of different steps in this method has also been tested. The first step of producing a growth curve has proven to be reproducible and reliable. The second step of sample preparation has not yet been tested. This step can be evaluated by running 2DE with protein samples prepared under different conditions. The third step 2DE (separations of the proteins by 2DE) was sufficiently reproducible to allow of comparison of gels coming from a single protein preparation (either SA, BA, or PEA). Nevertheless, reproducibility of this method has only been tested when the experiment was carried out by one person. In order to strengthen the reproducibility of this method, more detailed testing has to be done with comparison of results from different analysts.
To summarize the results of this project, *P. putida* F1 cells were grown on three different sources: SA, BA, and PEA. Three gels from each of the carbon source were compared for reproducibility. Then reference gels (gels with the most spots) of the three samples were compared to each other, SA vs BA, SA vs PEA, and BA vs PEA. From the comparison of gels from SA with BA and PEA, four unique protein spots were found to be expressed in the BA and PEA samples, which were not present in the from SA protein samples. These four protein spots align well when BA and PEA gels were compared. From this result, it is reasonable to speculate that the detected spots are part of the proteomic signature for aromatic compounds in *P. putida* F1.

The original objective of this project was to detect a proteomic signature for aromatic compounds, specifically potentially mutagenic/carcinogenic compounds, using 2DE of the proteins expressed by *P. putida* F1. This objective was achieved. Though the two aromatic compounds are not known to be carcinogenic or mutagenic, if an aromatic proteomic signature can be established and the component proteins conclusively identified, the functionality of the proteins may help to create the connection to or facilitate the discovery of a proteomic signature indicating carcinogenic potential. In addition to this possibility, from growth curve results of the two aromatic compounds, the absence of stationary phase clearly indicates that the intermediates are toxic to the bacteria and that growth on these carbon sources was limited and short-lived.

The methods and results developed in this project provide a solid foundation for future developments in this area. The next project should seek to identify and characterize the signature proteins. Another project should focus on further study of the reproducibility issues previously mentioned. The effects of minor changes in growth time
(if it's not at the exact mid-log point) by using 2DE to analyze samples that were grown within different time periods could also be investigated. All of these suggested studies may improve the quality of this method and hopefully lead to a new carcinogenesis test that is more accurate and reliable than the Ames test.
References


23. Reardon, KF. Kim, KH. Two-dimensional electrophoresis analysis of protein production during growth of Pseudomonas putida F1 on toluene, phenol, and their mixture. Electrophoresis (02) 23, 2233-2241.


Website: [http://www.corrosion-club.com/images/bacteriagrowth.gif](http://www.corrosion-club.com/images/bacteriagrowth.gif)


32. Non-Linear, Inc. Progenesis. Give access to by Proteomic Lab of University of Rochester.

Website: [http://www.towson.edu/~cberkowe/medmicro/315lab3.html](http://www.towson.edu/~cberkowe/medmicro/315lab3.html)


Glossary

Adenine: a purine base, C₅H₅N₅, that is the constituent involved in base pairing with thymine in DNA and with uracil in RNA.

Agarose: a polysaccharide obtained from agar that is the most widely used medium for gel electrophoresis procedures.

Ames test: a test used to determine the mutagenic potential of a substance based on the mutation rate of bacteria that are exposed to the substance.

Ampholytes: molecules containing both acidic and basic groups.

Autoradiography: a technique that records image on a photographic film or plate produced by the radiation emitted from a radioactive labeled specimen.

Back mutation: A reversal process whereby a gene that has undergone mutation returns to its previous state.

Bacterium: Any of the unicellular microorganisms without a true nucleus of the class Schizomycetes.

Bioavailability: the degree to which or rate at which a drug or other substance is absorbed or becomes available at the site of physiological activity after administration.

Biodegradation: the process of decomposision by biological agents, especially bacteria.
Carbonic anhydrase: an enzyme that catalyzes dehydration (giving up water molecule) of carbonic acid.

Carcinogenesis: a process that cause cancer.

Centrifugation: an apparatus consisting essentially of a compartment spun about a central axis to separate contained materials of different specific gravities, or to separate colloidal particles suspended in a liquid.

Chromatofocusing: a unique new column chromatographic method for separating proteins according to their isoelectric points.

Codon: A sequence of three adjacent nucleotides constituting the genetic code that determines the insertion of a specific amino acid in a polypeptide chain during protein synthesis or the signal to stop protein synthesis.

Co-polymerize: to bond two or more types of monomers to form a polymer.

Cytosine: A pyrimidine base, $C_4H_5N_3O$, that is the constituent of DNA and RNA involved in base pairing with guanine.

Degradation: decomposition of a compound by stages, exhibiting well-defined intermediate products.

Denaturation: term used to describe the loss of native, higher-order structure of protein molecules in solution.
Deoxyribonucleic acid: molecule stored genetic information in most living organism (DNA).

Deoxyribose: a sugar, C₅H₁₀O₄, that is a constituent of DNA.

Dioxygenase: a class of enzyme that catalyzes the addition of oxygen molecule (O₂) to the carbon chair.

Disulfide bridges: a special bond between two cysteine residues whose sulfur atoms are less than 3 Angstroms apart.

Electrophoresis: A method of separating substances, especially proteins, and analyzing molecular structure based on the rate of movement of each component in a colloidal suspension while under the influence of an electric field.

Electrospray ionization tandem mass spectrometry: a mass spectrometry technique used for analysis of nonvolatile compounds such as proteins.

Expression proteomics: a type of proteomics that studies proteins expressed by a genome under different conditions.

Flagella: long, threadlike appendage, especially a whiplike extension of certain cells or unicellular organisms that functions as an organ of locomotion.

Frameshift mutation: a mutation in a DNA chain that occurs when the number of nucleotides inserted or deleted is not a multiple of three, so that every codon beyond the point of insertion or deletion is read incorrectly during translation.
Functional proteomics: a type of proteomics that studies protein structures and its function.

Gel electrophoresis: Electrophoresis performed in a gel composed of agarose, polyacrylamide, or starch.

Glycine: a sweet-tasting crystalline nonessential amino acid, C₂H₅NO₂ that is the principal amino acid occurring in sugar cane.

Glycosidase: An enzyme that hydrolyzes glycosidic bonds between monosaccharide components of a polysaccharide.

Gram-negative: of, relating to, or being a bacterium that does not retain the violet stain used in Gram's method.

Guanine: a purine base, C₅H₅ON₅, that is an essential constituent of both RNA and DNA.

Histidine: an essential amino acid, C₆H₉N₃O₂, important for the growth and repair of tissues.

Homogenate: something that has been made uniform in consistency.

Immobiline: buffering acrylamide derivatives.

Isoelectric point: the pH at which the electrolyte concentration of an amphoteric substance such as protein is electrically zero.

Liquid Chromatography: a technique for the separation of complex mixtures that rely on the differential affinities of substances for a liquid mobile medium and for a stationary adsorbing medium through which they pass, such as paper, gelatin.
Lysozyme: an enzyme occurring naturally in egg white, human tears, saliva, and other body fluids, capable of destroying the cell walls of certain bacteria and thereby acting as a mild antiseptic.

Metabolic activation: chemical alteration of a substance within the body, as by the action of enzymes, of relatively inert chemicals to biologically reactive metabolites.

Metabolism: The chemical processes occurring within a living cell or organism that are necessary for the maintenance of life. In metabolism some substances are broken down to yield energy for vital processes while other substances, necessary for life, are synthesized.

Metabolites: a substance produced by metabolism or a substance necessary for or taking part in a particular metabolic process.

Microarray: a technology that embedded genetic molecule on a chip to study gene expression.

Multi-dimensional chromatography system: a new combined method of chromatography that involve two or more traditional chromatography (affinity, charge, size, etc).

Mutagenesis: formation or development of a mutation.

Nanoflow capillary high-performance chromatography: an improved liquid chromatography with nanoflow pump.

Oligonucleotides: a short polymer of two to twenty nucleotides.
Pathway: A sequence of enzymatic or other reactions by which one biological material is converted to another.

Peptide bond: The chemical bond formed between the carboxyl groups and amino groups of neighboring amino acids, constituting the primary linkage of all protein structures.

Plasmid: A circular, double-stranded unit of DNA that replicates within a cell independently of the chromosomal DNA. Plasmids are most often found in bacteria and are used in recombinant DNA research to transfer genes between cells.

Polymerase: Any of various enzymes, such as DNA polymerase, RNA polymerase, or reverse transcriptase, that catalyze the formation of polynucleotides of DNA or RNA using an existing strand of DNA or RNA as a template.

Polypeptide: a peptide, such as a small protein, containing many molecules of amino acids, typically between 10 and 100.

Polysaccharides: Any of a class of carbohydrates, such as starch and cellulose, consisting of a number of monosaccharides joined by glycosidic bonds.

Post-translational modification: process of protein modification that happens after protein synthesis from RNA.

Prokaryote/prokaryotic cell: an organism of the kingdom Monera (or Prokaryotae), comprising the bacteria and cyanobacteria, characterized by the absence of a distinct, membrane-bound nucleus or membrane-bound organelles, and by DNA that is not organized into chromosomes.
Proteomics: a term in the study of genetics which refers to all the proteins expressed by a genome.

Purine: a double-ringed, crystalline organic base, C$_5$H$_4$N$_4$, not known to occur naturally, from which is derived the nitrogen bases adenine and guanine, as well as uric acid as a metabolic end product.

Pyrimidine: A single-ringed, crystalline organic base, C$_4$H$_4$N$_2$, that forms uracil, cytosine, or thymine and is the parent compound of many drugs, including the barbiturates.

*Pseudomonas aeruginosa*: a well-known deadly pathogen (especially with cystic fibrosis patients); in the same genus of *Pseudomonas putida*.

*Pseudomonas putida* F1: a well-known bacterium specie for its ability to degrade aromatic carbon.

Recombinase: an enzyme that catalyzes the exchange of short pieces of DNA between two long DNA strands, particularly the exchange of homologous regions between the paired maternal and paternal chromosomes.

Repressor: a protein that binds to an operator, blocking transcription of an operon and the enzymes for which the operon codes.

Retardation: something that retards; a delay or hindrance.

Ribonuclease: any of various enzymes that break down RNA. Also called *RNase*.

Ribonucleic acid: a molecule stored or transferred genetic information, RNA.
*Salmonella typhimurium*: bacterium specie used for Ames test.

Sodium dodecyl sulfate (SDS): a detergent that can dissolve hydrophobic molecules but also has a negative charge (caused by sulfate group) attached to it; SDS facilitate proteins solubilization by cover them with many negative charges.

Sonication: the process of dispersing, disrupting, or inactivating biological materials.

Spectroscopy: study of spectra, especially experimental observation of optical spectra.

Thymine: A pyrimidine base, C₅H₆N₂O₂, that is an essential constituent of DNA.

Transcriptome: a profile of possible RNA that can by transcribed from DNA.

Transversion: A point mutation in which a purine is replaced by a pyrimidine, or a pyrimidine is replaced by a purine.

Tropomyosin: Any of a group of muscle proteins that bind to molecules of actin and troponin to regulate the interaction of actin and myosin.

Uracil: A pyrimidine base, C₄H₄N₂O₂, that is an essential constituent of RNA.

*Vitro* condition: a term to describe a laboratory stimulated condition.

*Vivo* condition: a term to describe real life condition.