# DEVELOPMENT OF TWO-DIMENSIONAL GEL ELECTROPHORESIS PROCEDURES FOR ANALYSIS OF CUPRIAVIDUS NECATOR H16 SUBCELLULAR PROTEOMES

---

A Thesis

Presented to

the Faculty of the College of Science and Technology

Morehead State University

---

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

---

by

Smita Joshi

May 2011

Accepted by the faculty of the College of Science and Technology, Morehead State University, in partial fulfillment of the requirements for the Master of Science degree.

Douglas Dennis
Director of Thesis

Master's Committee: Douglas Dennis, Chair
David K. Peyton
Janelle M. Hare

6/17/2011
Date

DEVELOPMENT OF 2-D GEL ELECTROPHORESIS PROCEDURES FOR ANALYSIS OF CUPRIAVIDUS NECATOR H16 SUBCELLULAR PROTEOMES

Smita Joshi, M.S.
Morehead State University, 2011

Director of Thesis: Douglas Dennis

*Cupriavidus necator* H16 is a gram negative, chemolithoautotrophic bacteria known for the production of polyhydroxyalkanoates (PHAs). PHAs are polyester polymers and have promising characteristics that can be used as biodegradable plastic. In certain bacteria such as *C. necator* H16, the PHA is found in the inclusion bodies located in the cytoplasm of the bacteria. The inclusion bodies are covered with proteins called phasins. PhaP is the most abundant phasin and forms a network on the surface of inclusion body. In addition to being found on the surface of the inclusions, there is evidence that PhaP is also located at the perimeter of the cell as suggested by immunogold transmission electron microscopy studies. It is also noteworthy that a tat-like sequence (tat is the signal sequence that facilitates protein transfer into the cytoplasmic membrane or periplasm) exists upstream of the PhaP amino terminua in the gene sequence. Based on this prior research, we speculated that the location of the PhaP protein may be the cytoplasmic membrane of bacteria. To examine this hypothesis, we used 1-D gels and 2-D gel electrophoresis analysis of fractionated cellular components. To maximize the representation and resolution of proteins on the

two-dimensional gels, we developed different solubilization buffers by using various combinations of detergents and reducing agents and isoelectric focusing protocols some of which were already been tested in similar studies. Our data suggest that the solubilization buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 1 % ASB-14, 2 mM TBP, 0.5% carrier ampholytes and 0.02% bromophenol blue gives optimized protein representation in terms of higher as well as lower molecular weight proteins and also the resolution of those proteins. Our data also support the hypothesis that PhaP might be on the cytoplasmic membrane of bacteria however mass spectroscopic analysis is necessary to confirm the findings.

Accepted by: Douglas Dennis, Chair
David K. Peyton
Janelle M. Hare

# ACKNOWLEDGMENTS

I would like to express my sincere gratitude and appreciation to Dr. Douglas Dennis, the chairman of my thesis committee, for his continuous guidance, patience and support throughout the development of this thesis.

I am grateful to Professor Janelle Hare, Professor David Peyton, and Professor Geoffrey Gearner for their guidance and valuable suggestions which were very helpful in the preparation of this thesis. I also wish to thank Lynn Eisenhour for her help and guidance during the lab work.

This work was supported by a grant from the National Institute of Health (1R15GM085749-01) and by funding from the Kentucky Biomedical Research Infrastructure Network (KBRIN).

# TABLE OF CONTENTS

# List of Tables

# List of Figures

# CHAPTER 1. INTRODUCTION

## 1.1. Overview

*Cupriavidus necator* H16 is beta proteobacteria which can make polyhydroxyalkanoates (PHA) inclusion bodies. These rod shaped motile bacteria are from Burkholderiaceae family. They are mainly found in soil and water. In past, this bacterium was also known as *Hydrogenomonas eutropha, Alcaligenes eutrophus, Ralstonia eutropha, and Wautersia eutropha.*

Polyhydroxyalkanoates (PHAs) are a class of intracellular storage polymer synthesized in some bacterial species, but not all (Anderson *et al.*, 1990). PHAs have attracted interest because they can be formulated into biodegradable plastic (Anderson *et al.*, 1990, Reinecke *et al.*, 2009). The PHA polymer is synthesized in multiple intracellular granules in response to a nutrient deprivation, such as nitrogen-limitation, sulfur-limitation, and phosphate limitation. The more the carbon/nitrogen ratio, the more is the PHA production (Anderson *et al.*, 1990). The stored carbon is converted into a fatty acid polymer (PHA) until the necessary nutrients are available again to use that carbon source for energy. In the presence of these nutrients, the stored fatty acids are depolymerized (Anderson and Dawes, 1990). The PHAs are formed mainly in three forms – short chain length hydroxyalkanoic acids ($PHA_{SCL}$), which are consisted of 3-5 carbons in a monomer or medium-chain-length hydroxyalkanoic acids ($PHA_{MCL}$), which are consisted of 6-14 carbons in a monomer.

The third type SCL-MCL PHA copolymer is consisted of both SCL and MCL subunits (Nomura *et al.*, 2004).

The most studied bacteria in regards to PHA production are the Gram-negative *Cupriavidus necator* species (Anderson and Dawes, 1990, Verlinden, 2007). Though there is abundant literature on the various aspects of biosynthesis, molecular structure, intracellular and extracellular degradation of PHAs, very little is known about PHA inclusion structure. Both Transmission Electron Microscopy (TEM) and Atomic Force Microscopic (AFM) analyses have indicated that there are about 10-14 inclusions per cell and the average inclusion size is 300 -800 nm (Anderson *et al.*, 1990, Dennis *et al.*, 2003). Proteomic analyses have shown that the major surface protein on inclusions is PhaP (Wieczorek *et al.*, 1995, Pieper-Fürst *et al.*, 1995, York and Stubbe, 2002). PhaP is thought to be a structural protein because when the *phaP* gene is deleted the structure of the inclusion is altered (Steinbuchel *et al.*, 1995, Pieper-Fürst *et al.*, 1995). The other two proteins that have been located on the surface of the PHA inclusion are, the PHA synthase molecule, PhaC, and PhaR, a small protein that is thought to bind with PhaP (Potter *et al.*, 2005).

Though PhaP is by far the most dominant protein on the surface of the inclusion (Wieczorek *et al.*, 1995), little was known about its structure. At one time it was thought to cover the surface in a monolayer, but other studies have shown that there is insufficient PhaP in the cell to cover all of the inclusions (Tian *et al.*, 2005). Dennis *et al.* (2008) provided a possible explanation by using atomic force

microscopy that PhaP exists on the surface of the cell not as a uniform monolayer, but as a network.

How PhaP forms the network on the surface of the inclusion is not known. However, there are several clues that suggest that PhaP may embed itself into the cytoplasmic membrane of the bacteria and the nascent inclusion forms from the periplasmic spaces into the cytoplasm. For instance, Jendrossek *et al.* (2007) has determined that in *Caryophanon latum* very early inclusions are formed in close proximity to the cytoplasmic membrane, and may even be attached to the membrane. Also, it has been found that other intracellular inclusions, such as magnetosomes, bud from the cytoplasmic membrane into the cytoplasm (Komeili *et al.*, 2006). Another reason for suspecting the cytoplasmic location of PhaP is that, the Dennis lab has identified a putative twin arginine translocation (tat) sequence upstream of the amino terminus of PhaP. Twin-arginine translocation sequence is a membrane targeting sequence that transports folded proteins across the cytoplasmic membrane, or embeds them in the membrane (Berks *et al.*, 2005). In *C. necator*, this sequence is SRRLNLL, which matches well with the consensus tat sequence SRRXFLK (Wickner *et al.*, 2005). Because of this putative tat sequence it seems possible that PhaP may be embedded in the membrane and from there direct PHA synthesis as the inclusion buds into the cytoplasm.

Preliminary studies in the Dennis lab have indicated that the PhaP may be in the cytoplasmic membrane of *C. necator* H16. A Western blot of total membrane

fraction of *Cupriavidus necator* using anti PhaP antibody has indicated the presence of PhaP in that fraction. To provide more substantial support to the theory that PhaP is embedded in the cytoplasmic membrane, more detailed studies must take place. First, we must devise procedures that separate various subcellular fractions, such as the total cell protein, cytoplasm, cytoplasmic membrane, and outer membranes. The second technology that can be implemented to support this research is the development of protocol so that the proteins may be isolated from the subcellular fractions and analyzed via 2D gel electrophoresis, followed by Western blotting. Though 1 D gel will be able to provide evidence to support the presence of PhaP in certain fraction of the cell, two-dimensional gel electrophoresis analysis will yield a bigger picture of overall characteristics of the proteomes to be studied, such as the distribution of proteins in terms of molecular weight, acidity/alkalinity, and presence of isomers.

## 1.2. Two-dimensional gel electrophoresis analysis

Two-dimensional gel electrophoresis is considered a leading method to detect and identify the proteins present in a sample (O'Farrell 1975). In the first dimension, the proteins are separated on the basis of their isoelectric point (during isoelectric focusing stage) and in the second dimension, they are separated based on their molecular weight (during SDS-PAGE gel electrophoresis). Since both of these characteristics are independent of each other, this procedure facilitates good resolution of the proteins on the gel. The method, however, is somewhat challenging

since the samples vary according to their origin (prokaryotic or eukaryotic, gram positive/ gram negative) and present unique obstacles in carrying out the two-dimensional electrophoresis. Apart from their cell structure, the amalgamation of the proteins to be studied also creates a problem. These proteins differ in their location, hydrophilicity /hydrophobicity, isoelectric points, molecular weights and other characteristics. These characteristics consequently create challenges in making a unique universal solubilization buffer for all the cells. So an empirically developed unique 2-D electrophoresis protocol that will produce sufficient yield of good quality proteins is needed for *C. necator* H16 proteomic analysis. The suitable protocol also should be reproducible and compatible with the sample to be studied. (Gorg *et al.*, 2004). These methods should be easy to replicate and should not change the characteristics of the proteins to be studied.

## 1.3. **Sample preparation**

The first step for sample preparation is homogenization of the sample in which the cells are broken into fragments so that all the cellular structures are exposed to the chemicals used in the later stage of solubilization. Mechanical methods such as grinding, liquid homogenization methods such as using French press operation, homogenizers etc., freeze/thaw, and bead beater are used to lyse the cells. All of these methods have some advantages and disadvantages over the other methods. Depending on the nature and origin of the sample (microbial/plant tissue/mammalian tissue/animal tissue), the appropriate method should be chosen

which will offer better protein yield in both quantitative and qualitative manner (Gorg *et al.*, 2004).

The second step is to protect the released proteins. The protection of the proteins is necessary to eliminate the possibility of degradation of the proteins and artifacts on the gel (Rabilloud 2007, Berkelman 2008). When bacterial cells are fragmented, unwanted materials such as lipids, salts, and enzymes such as proteases and nucleases are also released that may interfere with the structure and other characteristics of the proteins and create artifacts on the gels. Polysaccharides and DNA may block the gel pores, small ionic molecules may create defects in IEF separation, and lipids may bind to proteins and affect their migration on the gel or solubility in the buffer (Berkelman 2008). So these interfering materials should be neutralized or removed from the sample. According to Gorg *et al.*, (2004), protease inhibitors are added to inactivate the proteases, trichloroacetic acid and acetone precipitation can be used to remove the salts and nucleic acids and DNase and RNase can be used to get rid of genetic material. A commercial clean-up kit can be used to make the sample free from salt and make it more concentrated as well. (Gorg *et al.*, 2004, Berkelman, 2008).

The third and final step in sample preparation is to make a solubilization buffer (also known as rehydration buffer) for the sample. It is the most important step because the contents of the solubilization buffer should interfere in the non-covalent interactions among the proteins in the sample and also be able to denature the proteins

(Rabilloud, 2007). As described by Gorg *et al.* (2004), the general solubilization buffers consist of some common chemicals that have been proven helpful in denaturing polypeptides. The intramolecular and intermolecular interactions among the polypeptides that may give rise to artifacts are reduced by denaturation.

A general recipe for solubilization buffer contains chaotropes, detergents, reducing agents and ampholytes. Chaotropes are the denaturing agents such as urea and thiourea. Urea is efficient in breaking hydrogen bonds whereas thiourea interferes in hydrophobic interactions and increases solubility of hydrophobic proteins (Hurkman *et al.*, 1986, Walter *et al.*, 2008). Detergents are amphipathic molecules. They are classified as non-ionic (no charge) or zwitterionic (both charges present but net charge is zero) detergents. CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate,Tergitol-type, **nonyl** phenoxypolyethoxylethanol-40(NP-40), Triton X-100 and sulfobetaines such as Amidosulfobetaine-14 (ASB 14) and SB 3-10 are used as detergents. These chemicals prevent loss of protein due to precipitation and aggregation caused due to the interactions among hydrophobic domains of the proteins. Reducing agents prevent the re-oxidation of disulfide bonds and thus help in protein unfolding (Walter *et al.*, 2008). Dithiothreitol (DTT) or dithioerythritol (DTE) or tributylphosphine (TBP) are used as reducing agents. DTT and DTE are not compatible with proteins with high cysteine content. Different combinations of these detergents and / or reducing agents provide a wide range of rehydration (solubilization) buffers to use for optimum quality of two-dimensional gel.

Optimization of all of these approaches:

1. 2-D gel protocol
2. Determination of appropriate solubilization buffer
3. Development of efficient cell fractionation procedure

must be done in order to accomplish the major aim of this project, localization of PhaP in the cell. This thesis describes our efforts in these areas. Taken together the application of these optimized procedures and the resulting data suggest that, indeed, PhaP is located in the cytoplasmic membrane.

# CHAPTER 2. MATERIALS AND METHODS

## 2.1. Materials

The following chemicals and instruments were used in this project.

Chemicals and buffers - Urea (Bio-Rad Catalog # 161-0731), thiourea (Sigma, Lot # 76H0105), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (Bio-Rad Catalog # 161-0460), Dithiothreitol (DTT) (Bio-Rad Catalog # 161-0611), iodoacetamide (Bio-Rad Catalog # 163-2109) Ampholyte (Bio-Lyte 3-10 Bio-Rad Catalog # 163-1112), ASB-14 (Sigma, A1346-1G), N-lauroylsarcosine (Sigma Aldrich product no. L5125-50G) Tributylphosphine (TBP) (Bio-Rad Catalog # 163-2101) were used. HALT protease inhibitor cocktail (Thermo Scientific catalog # 1860932),RNase 1 solution ( 5 Prime Catalog # 2500130), RQ RNase free DNase (Promega, M610 A- 25308622), Broad range protein marker (Promega, WI Catalog # V8491), prestained SDS-PAGE marker (Bio-Rad Catalog # 161-0305) were used for silver staining and the western blot analysis respectively. A silver staining kit (Bio-Rad Catalog #161-0449), Sypro Ruby stain and methylene blue were used to stain the gels.

Gels and instruments- Bio-Rad laboratory's 2D SDS-PAGE Standard ( Catalog # 161-0320), Ready Strip non-linear Immobile pH gradient (IPG) strips-11 cm (Catalog # 163-2016), Amicon centrifugal filter devices- Ultracel-3K membrane (Millipore, Catalog # UFC500324), Protean Isoelectric Focusing instrument (IEF

cell) (Bio-Rad Catalog # 165-4001), Ready Prep Protein Extraction Kit- Total protein ( Bio-Rad, Catalog # 163-2086 ), Ready Prep Protein Extraction Kit – Membrane II ( Bio-Rad Catalog # 163-2084) were used for two-dimensional electrophoresis analysis. Beckman Coulter XL-90 ultracentrifuge (Beckman coulter, Brea, CA Catalog # 233923) was used for ultracentrifugation. Genesys 20 (Thermo scientific Catalog # 4004-000) visible spectrophotometer, Tekmar Sonic Disruptor in medium probe was used for protein assays.

## 2.2. **Growth and preparation of bacterial cells**

A starter culture of *C. necator* in nutrient broth no. 2 (containing beef extract 10 gm., peptone 10 gm., and sodium chloride 5 gm. in 1liter water) (Difco Catalog # 234000) was inoculated from a fresh Nutrient Broth-2 (NB-2) plate. The culture was 50 ml in 250 ml flasks and was incubated at 30 $^{0}$C at 150 rpm for16-18 hours. An aliquot of this culture was used to inoculate 200 ml of NB-2 media in 1 liter flask. This was incubated as before and cells were harvested after 24 to 48 hours of growth by centrifugation at 3,000 rpm for 10 minutes in an Eppendorf centrifuge 5810 R machine ( Eppendorf Catalog # 5804 709.004). These growth conditions limit PHA production because carbohydrate in the form of sugar is limited and nitrogen is not limited. The recovered cells were washed with1 X Phosphate Buffer Saline (PBS) twice and the pellet was stored at 4 $^{0}$C for further use at most for two weeks.

## 2.3. Sample preparation

An RNase/ DNase stock solution was made according to the Michigan proteome consortium recipe as follows: 1 mg/ml DNase I, 0.25 mg/ml RNase, 500mM Tris/ HCl pH 7.0, 50 mM $MgCl_2$. Before proceeding to the homogenization step, a protease inhibitor cocktail containing combination of serine proteases, amido-peptidases, cysteine proteases, metallo-proteases, aspartic acid- proteases (10 µl /ml of sample) and nucleases (20 µl of stock solution/400 µl of cell lysate) were added in the sample to prevent protein degradation and contamination by nucleic acids, respectively. For the sample preparation, two methods of mechanical disruption of cells were used, bead-beating and sonication.

***Bead Beating***

When using bead beater homogenization method, the pooled pellet was suspended in about 7-8 ml of PBS and transferred to a 15 ml screw cap container. The container was then filled with about 7-8 ml (equal amount) of 0.1 mm of zirconia beads (Biospec Catalog # 11079101 z) and was placed in a beaker filled with ice to prevent the overheating of the sample during the homogenization process. The sample suspension was homogenized for 30 seconds with a 30 second gap in between two successive homogenization bursts. The total duration of homogenization was 5 minutes. It means that actual bead beater bursts were used for 2 and half minutes. After the homogenization, the sample was kept on ice for 2 minutes to let the beads settle. The homogenate was transferred into a 15 ml Eppendorf tube and centrifuged

at 8,000 rpm for 10 min to remove the unlysed cells and cell debris. The supernatant which contained both the total membrane and the cytoplasmic fraction was carefully collected and used for further analysis or stored at 4 $^{0}$C until used for no more than two weeks.

***Sonication***

The second method used to lyse the cells was sonication. The pooled pellet was suspended in 10 ml of 1X PBS and transferred into two 15 ml Eppendorf tubes. The tubes were placed in a beaker containing ice during the sonication to avoid heating of the sample. The sample was exposed to sonication under mid-sized probe for four seconds at 30% power and an eight seconds break was placed in between two successive sonication bursts. After the sonication, the unlysed cells and the cell debris were removed by sedimentation at 8,000 rpm in an Eppendorf 5810 R centrifuge with a fixed angle rotor for 10 min. This supernatant, which contained both the total membrane and the cytoplasmic fraction, was carefully collected and used for further analysis or stored at 4 $^{0}$C until used.

## 2.4. Isolation of subcellular fractions

### 2.4.1. Obtaining the whole cell fraction (total protein)

After the growth of cells, the suspension was centrifuged at 3,000 rpm for 10 minutes in Eppendorf Centrifuge 5810 R machine and the pellet was collected. The pellet was washed with 1X PBS buffer two times and the final pellet was used as total

cell/protein fraction. In some procedures, the Bio-Rad's total protein extraction kit (Bio-Rad Catalog # 163-2086), was used according to manufacturer's directions.

### 2.4.2. Isolation of cytoplasmic proteins

The collected total cell sample was homogenized by sonicator or bead beater method as described above. After removing unlysed cells and cell debris, by centrifugation at 8,000 rpm in an Eppendorf 5810 R centrifuge for 10 min, the supernatant was collected. The supernatant was then subjected to centrifugation at 27,000 rpm for an hour in Beckman Coulter XL-90 ultracentrifuge using an SW28.1 swinging bucket rotor and the supernatant was collected and stored as cytoplasmic fraction at 4 $^{0}$C until used for analysis.

### 2.4.3. Isolation of total membrane

After collecting the supernatant as the cytoplasmic fraction, the pellet from the 27,000 rpm centrifugation mentioned in the above section was suspended in 1X PBS and again subjected to centrifugation at 27,000 rpm for an hour in Beckman Coulter XL-90 ultracentrifuge (SW28.1 rotor) to remove any remaining cytoplasmic proteins from the pellet. The insoluble pellet was collected and stored as total membrane proteins at 4 $^{0}$C. The total membrane protein fraction was also isolated using Bio-Rad's membrane II kit (Catalog# 163-2084) by following manufacturer's instructions.

### 2.4.4. Isolation of outer membrane and isolation of cytoplasmic membrane

The isolation of outer membrane and cytoplasmic membrane was attempted using three procedures:

1. To solubilize the cytoplasmic membrane protein away from the total membranes N-lauroyl sarcosine was employed to wash the total membrane fraction, extracting the cytoplasmic membrane proteins from the mixture. This washing was followed by pelleting and washing of the outer membrane fraction that was not solubilized.

2. To extract outer membrane proteins, Sodium carbonate extraction method- Method described by Fujiki *et al.* (1982) and modified by Molloy *et al.* (2000) was used.

3. Separation of the cytoplasmic and outer membrane fractions by isopycnic sucrose density centrifugation.

These methods are described in detail below:

***N-lauroyl sarcosine treatment to obtain outer membrane***

25 µl of 5% sarkosyl in 10 mM HEPES, pH 7.4, was added to 225 µl of total membrane protein and the solution was incubated for 30 min on tilter at speed of 7 rpm. This solution was subjected to centrifugation in a SW28 swinging bucket rotor at 27,000 rpm at 15 $^{0}$C for an hour. The supernatant was designated as solubilized cytoplasmic membrane proteins and was stored at 4$^{0}$ C. The pellet was suspended in

4.5 ml of 1X PBS and 0.5 ml of 1% N-lauroylsarcosine was added to the solution. The sample was incubated for 30 min on tilter. The solution was then subjected to centrifugation in a SW28 swinging bucket rotor at 27,000 rpm at 4 $^0$C for one hour. The pellet was suspended in PBS and saved as an outer membrane fraction.

***Sodium carbonate extraction method***

A sodium carbonate extraction method described by Fujiki *et al.* (1982) and modified by Molloy *et al.* (2000) was used to extract outer membrane proteins.-The total protein sample was resuspended in 10 ml of 10 mM Tris (pH 7.4)-1 mM $MgCl_2$. 5 µl of RNase and 5 µl of DNase was added to sample and sonication was done as described earlier. The unlysed cells were removed by centrifugation at 8,000 rpm for 10 min (Eppendorf 5810 R fixed angle rotor), and the supernatant which contains a mixture of total membrane and cytoplasmic proteins was collected. This sample was then incubated in 10 ml of 0.2 M cold ($4^0$ C) sodium carbonate for an hour on ice stirring continuously. After incubation, the mixture was ultra-centrifuged at $4^0$ C, 27,000 rpm for an hour. The supernatant contained cytoplasmic proteins as well as loosely attached peripheral proteins and the insoluble outer membrane and cytoplasmic membrane proteins were in the pellet. The pellet was designated as total membrane fraction and washed again with 10 mM Tris (pH7.4) – 1 mM $MgCl_2$ and used for outer and cytoplasmic membrane separation through density gradient.

***Isopycnic sucrose density gradient method***

The method described by Filip *et al.* (1982) was used with few modifications. An isopycnic sucrose density gradient was made using 5 ml of 55% sucrose, 3 ml of 53% sucrose, 3 ml of 51% sucrose, 3 ml of 49% sucrose, 3 ml of 47% sucrose, 3 ml of 45% sucrose, and 8 ml of 35% sucrose in 100mM Tris pH 7.4. 10 ml of a bacterial total membrane sample collected by using the above mentioned procedures was loaded on the sucrose density gradient. The gradient was then subjected to centrifugation in the Beckman Coulter SW28 swinging bucket rotor at 27,000 rpm for 18 hours at 4 $^0$C. The bands appearing in the gradient were collected and washed with 1X PBS in ultracentrifuge machine SW28 swinging bucket rotor at 27,000 rpm at 4 $^0$C for an hour. The pellets were collected and suspended in 500 µl of 1X PBS for further analysis.

After ultra-centrifugation was complete, the upper band (cytoplasmic membrane) was observed at the interface of the 45% and the 35% layers and the lower band (outer membrane) was observed at the 51% and the 49% interphase. Both the membrane fractions were extracted and washed with 1X PBS in a ultracentrifuge machine SW28 swinging bucket rotor at27, 000 rpm at 4$^0$C for an hour. The pellets were collected and suspended in 500 µl of 1X PBS for further analysis. A BCA protein assay (Thermo Scientific product # 23236) was used to quantitate the proteins.

## 2.5. Protein quantification

The method described by Lowry *et al.* (1951) was used to quantitate the proteins in different cell fractions by using Bio-Rad's detergent compatible (DC) protein assay (catalog # 163-0116). Bovine serum albumin (Bio-Rad catalog # 163-0112) was used as a standard for these protein assays. For some procedures a BCA protein assay (Thermo Scientific product # 23236) was used to quantitate the proteins.

## 2.6. 1D SDS-PAGE

1D SDS-PAGE was performed according to protocol by using a Criterion cell (Bio-Rad catalog # 165-6001). After protein quantitation, various amounts of protein fractions (total cell, total membrane, cytoplasmic protein etc.) were suspended in $dH_2O$ and Lammelli dye was added. The samples were then denatured by boiling in 100 $^0$C water for 5 min and centrifuged at 13,000 x g for 3 min. A Prestained low range protein marker (Bio-Rad catalog # 161-0305) was used. The electrophoresis was carried out at 100 volts for 2 hours. Western blot analysis was done on the gel.

## 2.7. Generation of antibody to PhaP

Antibodies were generated using a commercial vendor, Pacific Immunology. The amino acid sequence of PhaP was sent to the vendor and they identified a 12-amino acid peptide sequence that was both immunogenic and likely to be exposed. The peptide was synthesized and used to immunize two rabbits. After 13 weeks, the

serum from the rabbits was collected and affinity purified. The antibody had an effective titer of 1:20,000 in ELISA. The amino acid sequence used to make the antibody was CAYYNDTRNSDADP.

## 2.8. **Western blot analysis**

Separated proteins from the gel (either one dimensional or two dimensional gel) were transferred to nitrocellulose membrane. Bio-Rad Transblot secondary transfer cell was used for transfer of proteins to the nitrocellulose membrane. Affinity purified peptide specific anti PhaP antibody was used as primary antibody with 1:1000 dilution. Western Breeze kit (Invitrogen catalog # WB7105) for Western blot was used for this analysis according to manufacturer's instructions.

## 2.9. **Two-dimensional gel electrophoresis analyses**

Various solubilization buffers were made using recipes from the literature (Twine et al, 2005, Gorg et al 2004). The buffer generally contained the combination of chaotropes (urea and thiourea), zwitterionic detergents (CHAPS and/or sulfobetaines), reducing agents (DTT and/or TBP), and carrier ampholytes.

The IPG strips were passively rehydrated with the appropriate concentration of protein (~50 μg) in about 250 μl of rehydration buffer for about 20-24 hours at room temperature. Isoelectric focusing was carried out in a protein IEF chamber (Bio-Rad, Hercules, CA) according to one of the two protocols. The Protocol I was according to Twine *et al.* (2005) as follows: 200 volts for one hour, 500 volts for one

hour, 6 hours ramp to 5000 volts, hold at 5000 volts for 12 hours. After this step, hold the focused IPG strips at 500 volts for 4 hours to prevent dissociation of proteins. This step can be interrupted any time since IEF is already completed by this stage and this step is used to provide leeway before processing the IPG strips. The second protocol was as follows: 150 volts for 1 hour, 6000 volts ramp for 5 hours, hold at 6000 volts for 12.5 hours, and 500 volts for 4 hours.

The IPG strips were equilibrated with the equilibration buffers. Buffer I contained 2% SDS, 50mM Tris/HCL pH 8.8, 6M urea, 20% glycerol and 1% DTT. Equilibration Buffer II contained 2% SDS, 50mM Tris/HCL pH 8.8, 6M urea, 20% glycerol and 4% iodoacetamide. The IPG strips were equilibrated for 15 min in each equilibration buffer, first in buffer I followed by equilibration in buffer II and were washed in 1 X gel running buffer ( 0.25M Tris base, 1.9M +glycine, 0.035M SDS to make 10 X running buffer) for one minute. The strips were then loaded on the precast 10-20% Tris-HCL gels. 1 µl of broad range protein marker (Bio-Rad, Hercules CA) was used for molecular weight determination. The electrophoresis was carried at 100 volts for 2 hours at room temperature. The gels were stained with silver stain (Bio-Rad) or with Sypro Ruby Stain (Bio-Rad) and the gel images were scanned and stored for further analysis.

# CHAPTER 3. RESULTS

## 3.1. Methods used for subcellular fractionation

Figure 1 represents different cell fractionation techniques used for subcellular proteomic study. Bacterial cells grown in culture were harvested and washed two times with 1 X phosphate buffer solution (PBS). The pellet was stored at 4 $^{0}$C and used as total protein sample. It was also used for further extraction of other subcellular fractions. After undergoing homogenization though sonication or a bead beater method, the unlysed cells were removed by centrifugation at 8,000 rpm for 10 min (Eppendorf 5810 R fixed angle rotor) and supernatant was recovered. The supernatant then was subjected to centrifugation at 27,000 rpm (Beckman SW28 rotor) for an hour and the supernatant was collected and stored as cytoplasmic proteins. To remove any unwashed cytoplasmic proteins from the total membrane fraction, the sample was subjected to centrifugation again as described earlier and the pellet was saved as total membrane sample. After this stage, N-lauroyl sarcosine treatment, isopycnic sucrose density gradient or sodium carbonate methods were used as described previously.

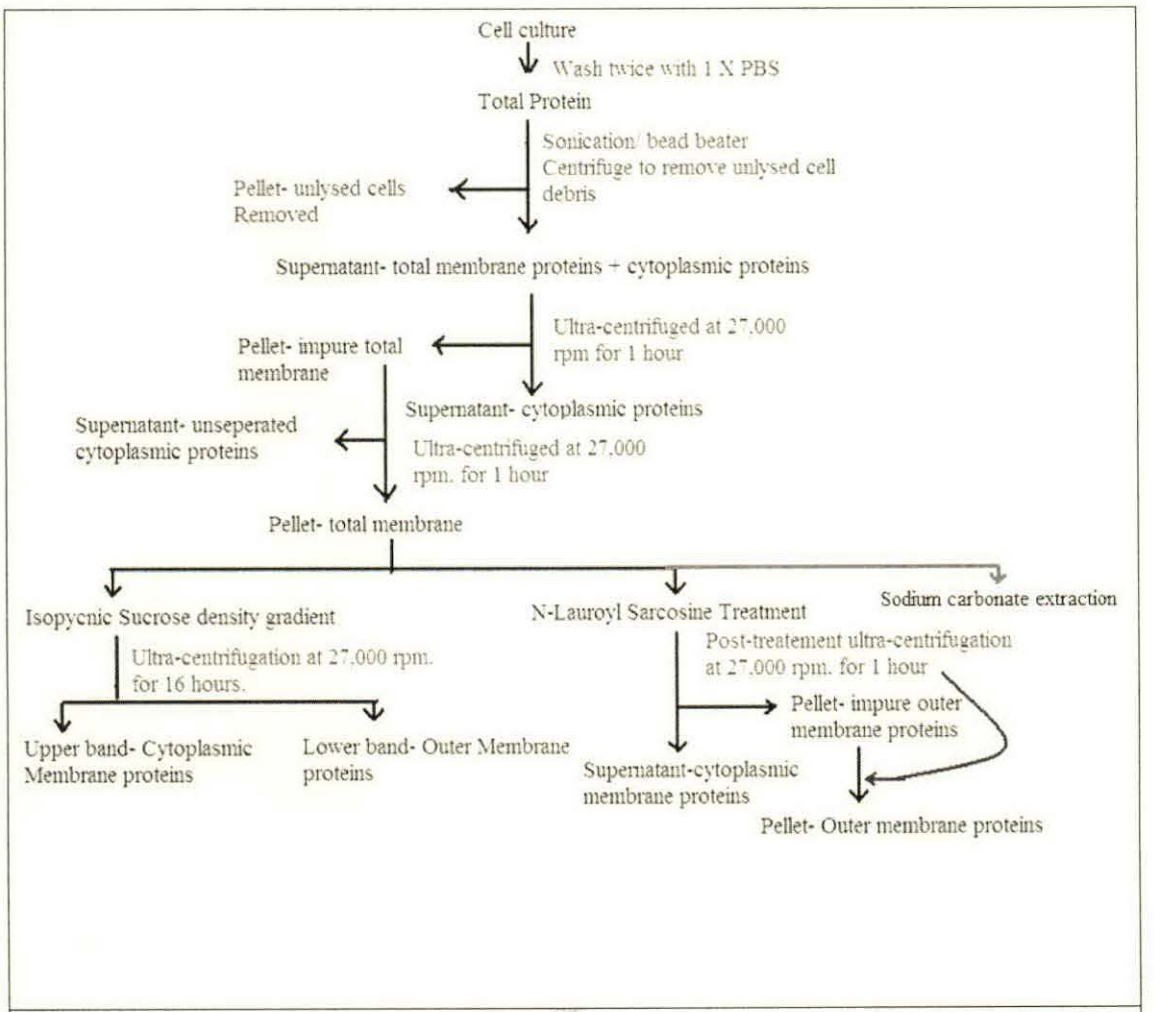
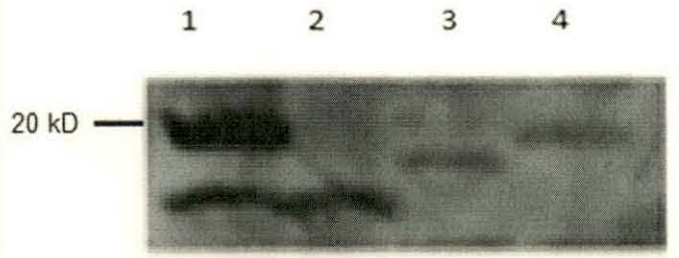



Figure 1. Schematic representation of methods used for subcellular fractionization

## 3.2. **Specificity of antibody against PhaP**

The specificity of our anti-PhaP antibody was tested using Western analyses. Total protein samples from the wild type *C. necator* H16, *phaP* mutant *C. necator* H16, wild type *E. coli* and recombinant *E. coli* with *PhaP* plasmid were used. PhaP is a 20 kD protein (Potter *et al.*, 2004). As expected, a band corresponding to this protein appeared in both positive controls (wild type *C. necator* H16 and *E. coli* with

*PhaP* plasmid). The negative controls – *PhaP* mutant C. necator H16 and wild type *E. coli* did not show the band. There were other bands that appeared on the gels such as two bands which appeared in both the wild type and *PhaP* mutant *C. necator* samples. One similar band appeared in wild type *E. coli* sample.




Lane 1- *C. necator* H16 wild type.

Lane 2 – *C. necator* H16 *PhaP* mutant

Lane 3- *E. coli* wild type

Lane 4 – Reconstituted *E. coli* with *PhaP* plasmid

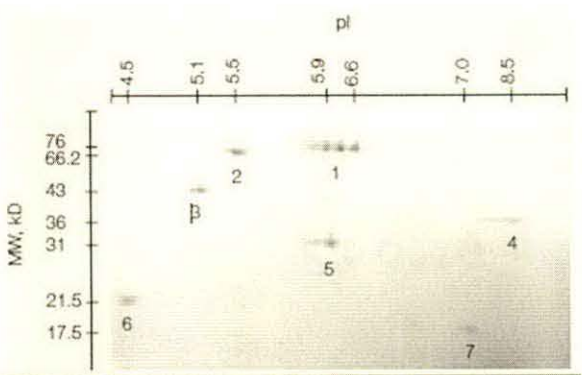
Figure 2. Western blot analysis was performed as described in methods and materials using affinity purified anti –PhaP antibody to study the specificity of the antibody. *C. necator* H16 wild type and the reconstituted *E. coli* were used as positive controls ( well 1 and well 4 respectively) and *C. necator* H16 *PhaP* mutant and the wild type *E. coli* were used as negative control for the presence of this protein.(well 2 and well 3 respectively).

This appearance of multiple bands might be because of the peptide used to make the antibody. The antibody was peptide specific. The peptide sequence was CAYYNDTRNSDADP. There is possibility that if other proteins in any of the samples tested share this peptide sequence with PhaP protein; they might show up on Western blot analysis. So that might be the reason why there were more bands at different molecular weights than expected. To find out the similarities among the samples, the Basic Local Alignment Search Tool (BLAST) database search was performed. However it did not yield any matches that show the similarity between the

peptide sequence used in anti-PhaP antibody and in *E. coli.* proteome. Hence, it is unclear why the lighter band appeared in the lane 3 representing wild type *E.coli.*

## 3.3. **Effects of reducing agents and isoelectric focusing protocols**

Different commonly used reducing agents (DTT and TBP) and two different isoelectric focusing protocols were tested to examine which one gives better results. A commercially available 2 D SDS-PAGE standard (Bio-Rad) was used to compare the images with each other. Figure 3 is a reference figure obtained from the manufacturer (Bio-Rad) shows the respective locations of the proteins used in the standard according to their isoelectric points and molecular weights.




**Constituent proteins of 2-D SDS-PAGE standards.**

| Protein | pI | MW (kD) |
|---|---|---|
| 1. Hen egg white conalbumin | 6.0, 6.3, 6.6 | 76 |
| 2. Bovine serum albumin (BSA) | 5.4, 5.5, 5.6 (empirically determined) | 66.2 |
| 3. Bovine muscle actin | 5.0, 5.1 (empirically determined) | 43 |
| 4. Rabbit muscle GAPDH | 8.3, 8.5 | 36 |
| 5. Bovine carbonic anhydrase | 5.9, 6.0 | 31 |
| 6. Soybean trypsin inhibitor | 4.5 | 21.5 |
| 7. Equine myoglobin | 7.0 | 17.5 |

Figure 3. Two-dimensional electrophoretic protein pattern of 2D-SDS PAGE standards. (www.bio-rad.com)

This standard was used to compare the quality of two-dimensional gels obtained from different solubilization buffers when the protein sample is constant.

The following four rehydration buffer combinations were used to study the effects of reducing agents on the abundance and quality of protein representation on the two-dimensional SDS- PAGE standard.

**Table 1. Different rehydration buffers used to study the effects of reducing agents such as DTT and TBP on the protein abundance and resolution**

| | A | B | C | D |
|---|---|---|---|---|
| 7 M urea | Present | Present | Present | Present |
| 2 M thiourea | Present | Present | Present | Present |
| CHAPS | 0.80% | 4% | Absent | 4% |
| ASB-14 | 1% | Absent | 0.50% | 0.50% |
| DTT | Absent | Absent | Absent | 1% |
| Ampholyte 3-10 | 0.50% | 0.50% | 0.50% | 0.50% |
| TBP | 2mM | 2mM | 2.5mM | Absent |

To find the suitable reducing agent for *C. necator* H16 proteomic analysis, the above mentioned four buffers were studied using a commercial two-dimensional SDS-PAGE standard. Isoelectric focusing protocols were also compared simultaneously (Figure 4A-E). The main purpose of using this standard was to evaluate the combinations of different reducing agents and IEF protocols as well as various types and concentrations of detergents against certain constant protein preparation. This will help to identify the problems with cell fractionation method and /or with the rehydration buffer recipes independently of each other. This two-

dimensional image of the standard was also used to evaluate the resolution of the protein spots expressed in a particular gel.

The gel images show that the solubilization buffer A gave very good results in terms of the resolution of spots (Figure 4A.). All the protein spots except number 5 (Bovine carbonic anhydrase) appeared on the gel. The isoforms in number 1 (Hen egg white conalbumin), number 3 (Bovine muscle actin) and number 4 (Rabbit muscle GAPDH) were also represented and the appearance was sharp and clear (Figure 3 and Figure 4A.). There was no horizontal or vertical streaking or other artifacts on the gel. On the other hand, solubilization buffer D containing 1 % DTT did not work out as well (Figure 4B.). The problems were particularly seen in the acidic area of the gel (which is on the left side of the reader, indicated by lower pH values on the isoelectric point range on the reference gel). Number 6 protein (Soybean trypsin inhibitor) barely appeared and also was in diffused form (Figure 3 and Figure 4B.). In general, acidic proteins did not appear as clear and distinct as they did in previous gels. For both of these gels, IEF protocol I was used.

In the second set of gels which were run using IEF protocol II, the gel representing rehydration buffer B ( Figure 4C) worked better as compared to the gel representing rehydration buffer D (Figure 4D). In the gel representing buffer B, alkaline proteins especially number 7 (equine myoglobin) appeared more distinctly than in gel representing using buffer D. 2 mM TBP was used as a reducing agent in solubilization buffer B while 1% DTT was used in buffer D. The rehydration buffer

combination was same for both the gels in Figure 4B. and Figure 4D however the IEF protocols were different. In that sense, IEF protocol I seems better than IEF protocol II. The last gel was run using the fourth protocol- rehydration buffer C (Figure 4E) and IEF protocol II. It contained 2mM TBP and it also worked very well. All the protein spots appeared distinctly (Figure 3 and Figure 4E). The rehydration buffers used contained different types as well as concentrations of the detergents. These detergents might have played role in better solubilization of the sample or its resolution on the gels affecting the quality of the results. Thus the presence of variable detergents in these solutions makes it difficult to evaluate the role of reducing agents. However, the rehydration buffers containing TBP as a reducing agent showed better results (Figure 4A, 4C, and 4E) than the buffer containing DTT as a reducing agent (Figure 4B and 4D) despite the detergents or IEF protocols used. The standard picture shows the IPG strip which was 7 cm (Figure 3). The IPG strips used for these experiments were 11 cm. So the pattern is little different than how it is represented on 7 cm IPG strip.

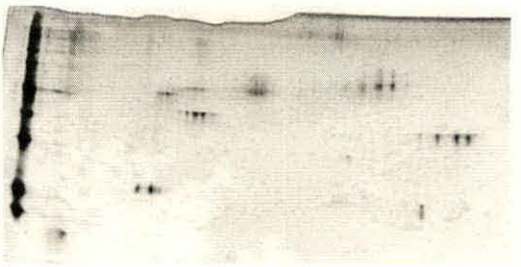


Figure 4A. 2D gel of *E. coli* protein standard using solubilization buffer A and IEF protocol I.

Figure 4B. 2D gel of *E. coli* protein standard using solubilization buffer D and IEF protocol I.

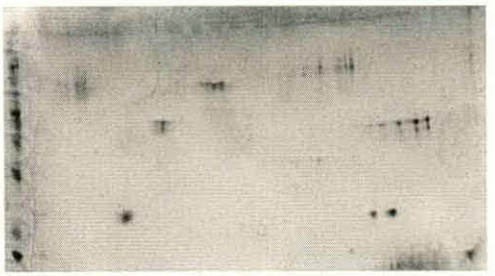


Figure 4C. 2D gel of *E. coli* protein standard using solubilization buffer B and IEF protocol II.

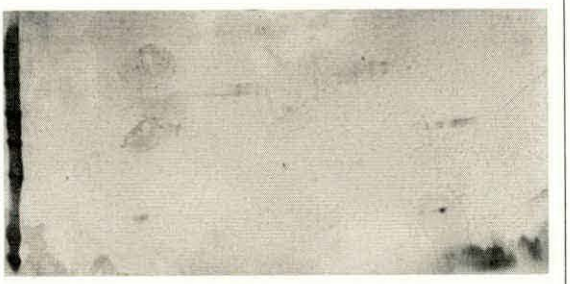


Figure 4D. 2D gel of *E. coli* protein standard using solubilization buffer D and IEF protocol II.

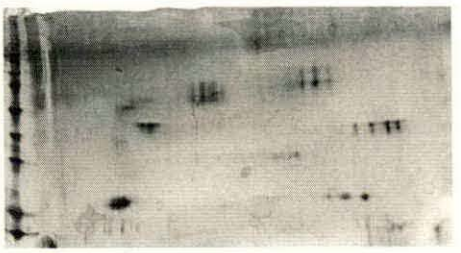


Figure 4E. 2D gel of *E. coli* protein standard using solubilization buffer C and IEF protocol II.

Figure 4A-E. Comparison of reducing agents and IEF protocols to produce optimum protein representation on two-dimensional gel.

## 3.4. IEF protocols

Two different isoelectric focusing protocols (Table 2) were compared. The protocols differed in the duration of the total run. The sequence of voltages and other times were kept consistent throughout the run.

There was not much difference found with the quality of gels and abundance of proteins represented in regard to the isoelectric focusing protocol used. First, the protocols used were not much different from each other in terms of total volt hours. The literature shows a wide range of protocols used from about 30,000 volt-hours (Molloy *et al.*, 2000) to around 90,000 volt-hours with not much variety in the results (Hasona *et al.*, 2005). It has also been found that though artifacts appear due to under focusing during the first dimensional run; over focusing is generally not a problem until total volt-hours run above the 100,000 volt- hours limit (Gorg *et al.*, 2004).

**Table 2. The evaluation of IEF protocols to analyze the effectiveness of first dimensional run**

| Steps | IEF protocol I | IEF protocol II |
|---|---|---|
| 1. | 200 volts for 1 hour | 150 volts for 1 hour |
| 2. | 500 volts for 1 hour | 6000 volts ramp for 5 hours |
| 3. | 5000 volts ramp for 6 hours | Hold at 6000 volts for 12.5 hours |
| 4. | 5000 volts for 10 hours | 500 volts for 4 hours |
| 5. | 500 volts for 4 hours | ---------- |

## 3.5. Comparison of homogenization methods

Methods of mechanical disruption of bacterial cells are very important to break open the cells. Sonication as well as the bead beater method was used to evaluate which homogenization method gives better yield of *C. necator* proteins. Earlier in our work we found that the bead beater method offered better results than the sonication method. In an experiment to assess the effectiveness of these methods, the cells were fractionated using either method as described in materials and methods section and different subcellular proteomes such as outer membrane and cytoplasmic membrane were isolated through isopycnic sucrose gradient based on their characteristic densities. These fractions were then used for western blot analysis using an affinity purified peptide specific anti-PhaP antibody. The results demonstrate that the cytoplasmic membrane fraction derived from the bead beater method contains the PhaP which does not appear in outer membrane fraction from the same sample. In sonicated sample, however, the protein appears in both the outer membrane and the cytoplasmic membrane fractions.

In another homogenization method comparison experiment, the total protein extract was either subjected to sonication or bead beating as described in the materials and methods section. The resulting samples were centrifuged at 8,000 rpm for 10 min (Eppendorf 5810 R fixed angle rotor) and unlysed debris was removed. A sucrose step gradient was made with 4 ml of 65% sucrose in 25 mM of Tris pH 8 on the bottom and 18 ml of 40 % sucrose in 25 mM of Tris pH 8 on top. The total

membrane sample treated with either of the two homogenization methods was placed on the prepared gradient. The gradients were subjected to centrifugation at 27,000 rpm for an hour at 4 $^{0}$C. After ultracentrifugation, the upper band was observed at the interface of sample and 40% sucrose. The lower band was at the interface of 65% sucrose and 40% sucrose. Both the bands as well as any material above them were collected. Four density gradients were made in 25 mM Tris pH 8.0 with 3 ml of each 70%, 58%, 52%, 48%, 40%, and 30% sucrose. Sonicated top, sonicated bottom, bead beater top and bead beater bottom samples collected from a previous experiment were loaded on the gradients. The gradients were subjected to centrifugation at 27,000 rpm for 16 hours. At the end of the ultracentrifugation, clear bands were observed at sonicated bottom, sonicated top and bead beater bottom sample gradients. It demonstrates that these fractions were well separated from each other based on their densities.

The bead beater top did not work out very well. That band was too diffuse. The samples were collected by piercing a hole at the bottom of the tube with a 25 gauge needle and every ml was collected and diluted by adding 2 ml of 25 mM Tris buffer. The mixture was vortexed and Optical Density was read at 600 nm (Figure 5). The measurement of optical density was used as the parameter for the presence of the cellular fraction at that density level. The more fractions (protein) present in the solution, the higher will be the optical density of the solution.

The graphs in Figure 5 showing these two different phases demonstrate that each fraction was contaminated with each other and illustrate the challenges of getting pure sample. This cause of impurity in membrane samples may affect some of the results.

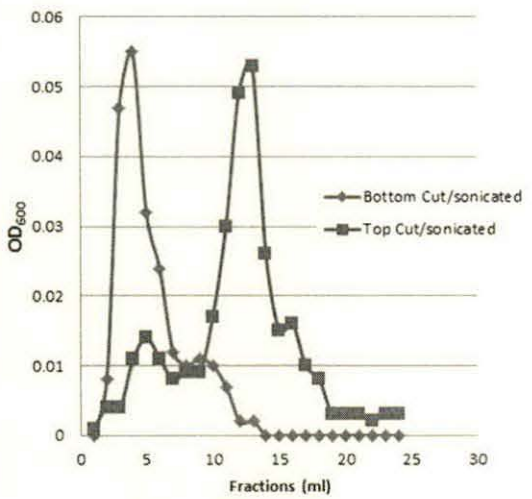



Figure 5A. The separation of outer membrane and cytoplasmic membrane bands in isopycnic sucrose gradient treated with sonication.

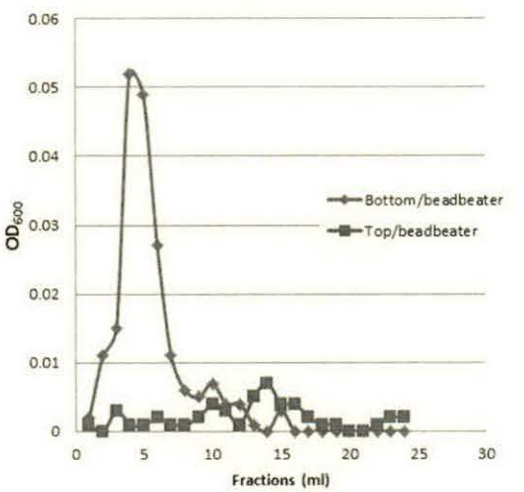



Figure 5B. The separation of outer membrane and cytoplasmic membrane bands in isopycnic sucrose gradient treated with Bead beater.

Figure 5A and B. Comparison of effectiveness of sonication and bead beater homogenization methods. Total membrane samples collected by using either sonication or bead beater method were loaded on 65%-40% sucrose density gradients, The respective bands were collected, purified and were loaded again on 70%, 58%, 52%, 48%, 40%, and 30% sucrose and subjected to centrifugation at 27,000 rpm for 16 hours. The fractions of gradients (every milliliter) were collected by piercing hole at the bottom and diluted 2X with 25 mM Tris pH 8. Optical readings were taken at 600 nm and the data was plotted.

## 3.6. Modification of the solubilization / rehydration buffer

To find the most suitable rehydration buffer which would optimize the solubilization and representation of proteins, various recipes of rehydration buffers were used. The chaotropic agents are the chemicals that denature the macromolecules such as DNA, RNA or proteins. These agents can disrupt the hydrogen bonds and hydrophobic bonds which lead to the unfolding and denaturation of proteins. Generally, a high concentration of urea (about 9 M urea) was used as a chaotropic agent (Potter *et al.*, 2004). Recently it has been found that use of 2 M thiourea efficiently helps to break hydrophobic interactions (Rabilloud *et al.*, 2008), so no change was made in the chaotropic contents of the trial rehydration buffers The chaotropic agents such as the urea and thiourea were uniformly included in the same concentration (7 M urea and 2 M thiourea) in all the combinations of solubilization buffers.

The next most important ingredient is the reducing agent. The reducing agents are necessary to break intra-molecular and inter-molecular disulfide bonds. Dithiothreitol (DTT) and tributyl phosphine (TBP) were used as reducing agents in different quantities to study how they affect the resolution and the abundance of proteins on the two-dimensional gel by using 2 D SDS-PAGE standard as described earlier (Figure 4A-4E)

The next important ingredient of the solubilization/ rehydration buffer is the detergent. These are generally amphipathic molecules with polar heads and non- polar

tail structure. They may be charged, uncharged or zwitterionic (both charged groups are present but no net charge on the particle). For two-dimensional electrophoresis analysis, uncharged or zwitterionic detergents are preferred since charged particles are more likely to interfere with the migration of proteins in the first dimensional run. In this experiment, two detergents 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) and sulfobetaines (ASB 14or SB 3-10) were studied for their compatibility for *C. necator* H16 proteomic analysis.

### 3.6.1. Effects of detergents

The following buffer recipes were used to evaluate the effects of detergents.

The first rehydration buffer tried was used by Molloy *et al.* (2000) to extract *E. coli* outer membrane proteins. It contained 7M urea, 2M thiourea, 1% ASB 14, 2 mM TBP, 0.5% ampholytes and trace amount of bromophenol blue.

The second rehydration buffer was previously used by Twine *et al.* (2005). It was modified by using 0.5% ASB 14 instead of using 1 % ASB-14 as was done in that study. The final recipe of this buffer contained 7M urea, 2M thiourea, 4% CHAPS, 0.5% ASB14, 1% DTT, 0.5% ampholyte and trace amount of bromophenol blue.

In the third buffer, DTT was replaced with TBP. It contained 7M urea, 2M thiourea, 4% CHAPS, 1% ASB14, 2mM TBP, 0.5% ampholyte 0.002% bromophenol blue.

In the next two buffers, the amount of CHAPS was only changed from 4% to 3% and 2% respectively to examine the difference in pattern. So the fourth rehydration buffer contained7M urea, 2M thiourea, 3% CHAPS, 1% ASB14, 2mM TBP, 0.5% ampholyte and trace amounts of bromophenol blue. The fifth rehydration buffer contained 7M urea, 2M thiourea, 2% CHAPS, 1% ASB14, 2mM TBP, 0.5% ampholyte and trace amount of bromophenol blue.

Table 3 is a summary of the contents and concentrations of all rehydration buffers used for detergent compatibility experiments.

**Table 3. Different solubilization buffers used to compare the effects of zwitterionic detergents- CHAPS and ASB14**

| | **Solubilization buffer** | | | | |
|---|---|---|---|---|---|
| | **I** | **II** | **III** | **IV** | **V** |
| Urea | 7M | 7M | 7M | 7M | 7M |
| Thiourea | 2M | 2M | 2M | 2M | 2M |
| CHAPS | -- | 4% | 4% | 3% | 2% |
| ASB 14 | 1% | 0.50% | 1% | 1% | 1% |
| DTT | -- | 1% | -- | -- | -- |
| TBP | 2mM | -- | 2mM | 2mM | 2mM |
| Ampholyte | 0.50% | 0.50% | 0.50% | 0.50% | 0.50% |
| Bromophenol Blue | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% |

SDS is a frequently recommended detergent for complete protein denaturation and solubilization. Consequently, 0.5% SDS was used in 100 mM Tris pH8.8 solution to evaluate the change in protein abundance on two-dimensional gel as compared to the CHAPS and sulfobetaines. (Figure 5A and 5B). The total cell pellet was sonicated

as described above in both the presence and absence of 0.5% SDS. 2-D electrophoresis was carried out. The results show that in the absence of SDS, the gel displayed a small number of proteins. On the other hand, the sample which was solubilized in SDS showed better representation and resolution of proteins especially low molecular weight proteins. SDS seems more effective in solubilization; however there was noticeable streaking on the gel which might have arisen due to incompatibility of SDS with the IEF procedure (Stanley *et al.*, 2003) because of its negative charge.

In rehydration buffer I, sulfobetain ASB 14 was used as a detergent (Figure 6). Significant vertical streaking was observed especially in the acidic region of the gel. Resolution was also not satisfactory. In basic regions, there was horizontal streaking and the proteins represented were not as abundant as expected. ASB 14 is known for its ability to solubilize more basic proteins (Stanley *et al.*, 2003). In this gel, however, very few basic proteins appeared.

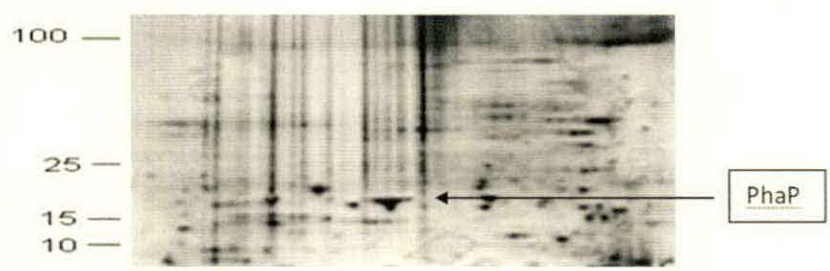



Figure 6. Total membrane fraction of *C. necator* H16 solubilized in 7M urea, 2M thiourea, 1% ASB14, 40 mM Tris base, 2 mM TBP, 0.5% ampholyte, 0.002% bromophenol blue. IEF protocol- 200 volts for 1 hour, 500 volts for 1 hour 5000 volts 6 hours ramp, hold at 5000 volts for 10 hours keep at 500 volts for 4 hours

Since using ASB 14 alone did not work satisfactorily in previous experiment, the combination of two detergents 4% CHAPS, 0.5% ASB14 was used (rehydration buffer II). The N-lauroyl sarcosine extraction method was used to extract cytoplasmic membrane and outer membrane proteins as described above and two-dimensional electrophoresis was done. The images show that the total protein gel failed to represent the abundance of proteins (Figure 7). Particularly high molecular weight proteins were absent. It suggests that there might be a problem with solubilization of the sample. The gel images of the cytoplasmic fraction (Figure 7B) and the total membrane fractions (Figure 7C.) were acceptable. The outer membrane fraction did not show many proteins (Figure 7D). Most of the proteins in this fraction were alkaline proteins. The horizontal and vertical streaking was present in total cell and the cytoplasmic cell fraction of sample. ASB 14 might have played a role in the abundance of proteins observed in the cytoplasmic fraction and the total membrane fraction of *C. necator* H16. Though same rehydration buffer was used for all of these samples, the results are markedly different. It shows that the proteins from different fractions of the same cell react differently to the composition of the rehydration buffer. This problem can be answered by using different rehydration buffers for each cell fraction to get maximum resolution of proteins. In this study however, one combination of detergent/detergents, reducing agent and IEF protocol was designed to get optimal results in all the fractions of the bacterial cells.

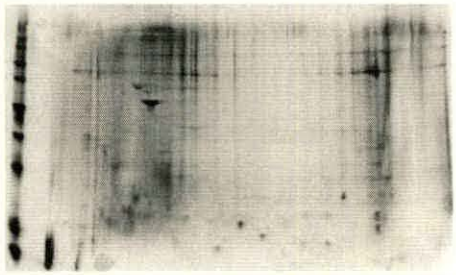


Figure 7A. H16 NB Whole cell Protein

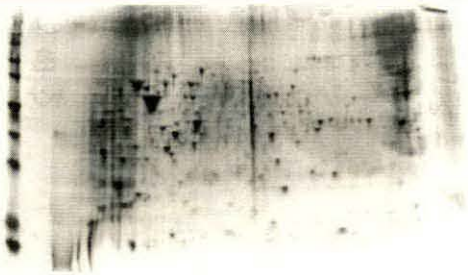


Figure 7B. H16 NB cytoplasmic proteins

Figure 7C. H16 NB total membrane

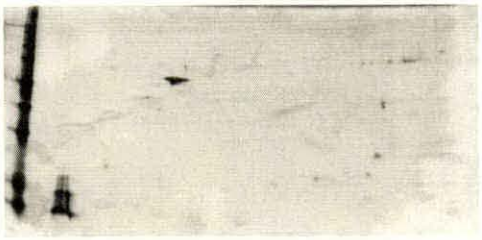


Figure 7D. H16 NB Outer membrane proteins

Figure 7A-D. *C. necator* H16 cells were grown on NB media and Bio-Rad's total protein extraction kit was used to obtain the total protein, total membrane protein and the cytoplasmic protein, were collected as discussed above. The membrane fraction was incubated in 1% N-Lauroyl Sarcosine for 30 min and subjected to centrifugation as described previously. The solubilization buffer contained 7M urea, 2M thiourea, 4% CHAPS, 0.5% ASB14, 1% DTT, 0.5% ampholyte and 0.002% of bromophenol blue. The IEF protocol used was150 v. for an hour, ramp to 6000 v. over a period of 5 hours hold at 6000 v for 12.5 hours, 500 v. for 4 hours. The equilibration and the second dimension run were carried out as explained above. The gels were silver stained according to manufacturer's directions.

Since this method failed to extract the total protein, another rehydration buffer recipe was designed. The total protein sample was extracted as described above and divided in three equal parts. Each part was then treated differently. The first pellet was treated with nucleases and protease inhibitors only. The second part was purified

and concentrated by using Amicon centrifugation method. The third part was treated purified and concentrated with Bio-Rad's CleanUp kit. (Figure 8A-8C).

All these samples were then solubilized with the buffer containing 4% CHAPS and 1% ASB14. The combination worked better than previously tested combinations. The quality of gels in terms of both, protein abundance and resolution was increased significantly. There was virtually no horizontal or vertical streaking in gels representing Amicon filtered samples (Figure 8B) and CleanUp kit treated sample (Figure 8C). Though untreated total protein sample (Figure 8A) showed some streaking, it was minor as compared to the gels previously run. Wide ranges of proteins were displayed and especially prominent presence of alkaline proteins was discerned for the first time.

In this experiment, sample 2 and sample 3 were purified and concentrated using Amicon centrifugal unit and Bio-Rad's CleanUp kit respectively. These techniques removed the salts and other impurities which might cause artifacts such as streaking on gels. Horizontal streaking generally takes place when the impurities or charged particles are present in the sample during IEF. The presence of these contaminants during the second dimensional run (SDS-PAGE) causes vertical streaking on the gels. Since all of the samples showed protein abundance and clarity of spots, the combination of rehydration buffer, especially detergents and the reducing agents, seems to be suitable for the sample preparation.

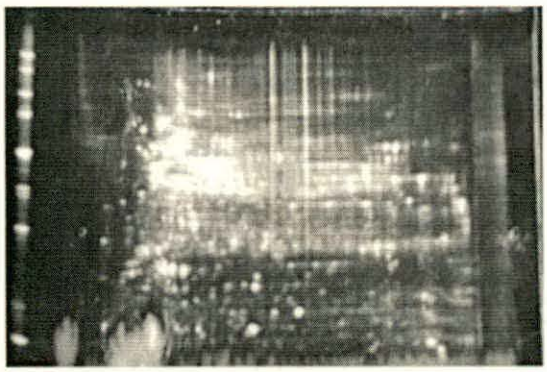


Figure 8A. Total protein sample

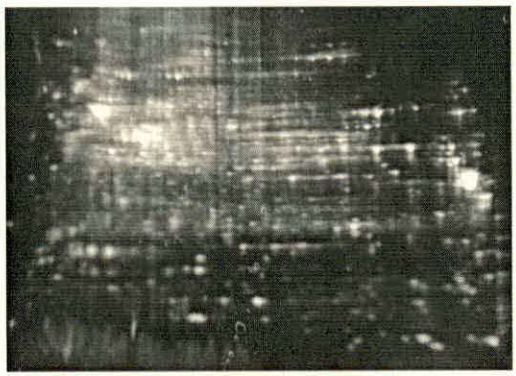


Figure 8B. Total protein sample concentrated using Amicon filters

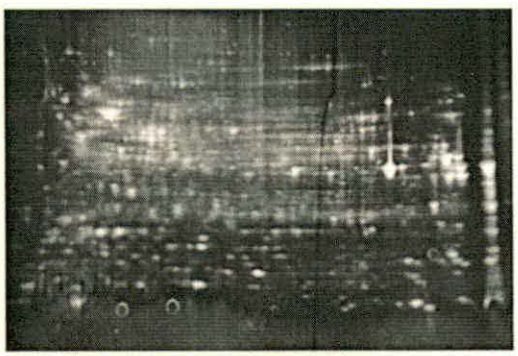


Figure 8C. Total protein sample treated with CleanUp kit (Bio-Rad)

Figure 8A-C. The total cell protein samples collected from the *C. necator* H16 cells as described above. After sonication, the sample was divided in three equal parts and processed as following-sample 1 was solubilized in buffer containing 7M urea, 2M thiourea, 4% CHAPS, 1% ASB14, 2mM TBP, 0.5% ampholyte and 0.002% bromophenol blue. (Figure A) Sample 2 was purified using Amicon centrifugal filter to remove the impurities. (Figure B). Sample 3 was treated with the BioRad's CleanUp kit to remove the salts and other contaminating particles to purify the product. After respective treatments, the same rehydration buffer used for sample 1 was used to solubilize the sample 2 and sample 3 (Figure C) The IEF protocol used was 150 v. for an hour, ramp to 6000 v. over a period of 5 hours hold at 6000 v for 12.5 hours, 500 v.for 4 hours. The gels were then stained with Sypro Ruby stain according to manufacturer's directions.

A different combination of detergents: 3% CHAPS and 1% ASB 14 was used to evaluate if it worked better than the combination of 4% and 1% respectively. The results show that there was noticeable vertical streaking in the total cell sample (Figure 9A). It might be due to the impurities in the sample such as lipids and salts or due to poor solubilization of proteins in the solubilization buffer or a combination of both. The protein abundance was observed only in total protein and not in cytoplasmic proteins (Figure 9B) or total membrane proteins (Figure 9C). The total membrane protein gel also shows the prominent front at the bottom. Low molecular weight proteins are virtually absent from the total membrane gel and are rare in the cytoplasmic protein fraction of the gel. These results show that the combination of detergents 3% CHAPS and 1 % ASB-14 is not as effective as previously tested combination of 4% CHAPS and 1% ASB-14.

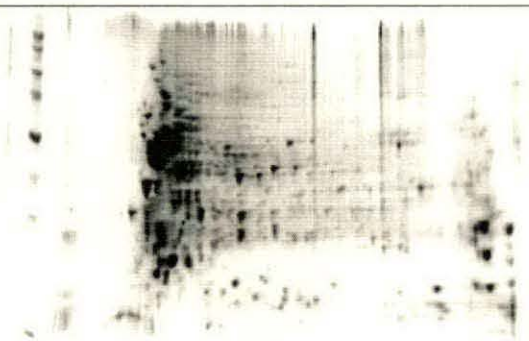


Figure 9A. *C. necator* H16 total cell proteins

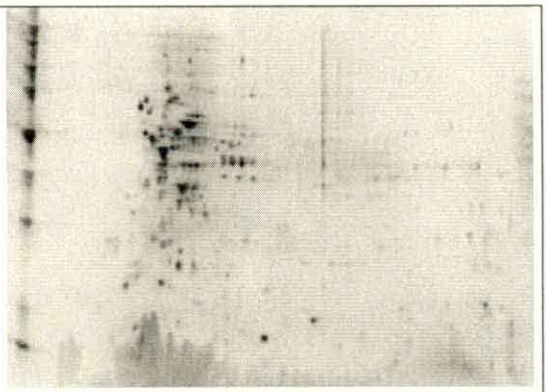


Figure 9B. *C. necator* H16 cytoplasmic proteins

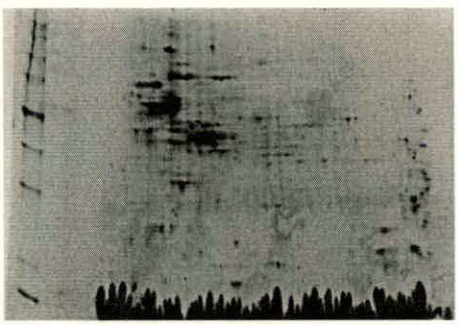


Figure 9C. *C. necator* H16 cytoplasmic proteins

Figure 9A-9C. The *C. necator* H16 cells were fractionated and subcellular proteins (total cell, cytoplasmic proteins and total membrane proteins) were isolated as described above. All the fractions were solubilized in a buffer containing 7M urea, 2M thiourea, 3% CHAPS, 1% ASB14, 2mM TBP, 0.5% ampholyte and trace amount of bromophenol blue. The IEF protocol used was 150 v. for an hour, ramp to 6,000 v. over a period of 5 hours hold at 6,000 v for 12.5 hours, 500 v. for 4 hours. The gels were then stained with Sypro Ruby stain according to manufacturer's directions.

In the next experiment, SDS was used as the detergent. Literature shows that SDS is very effective to solubilize proteins (Gorg, 2004) so it was used during the

homogenization process and later a different rehydration buffer (with different combination of detergents) was used to solubilize the sample.

In the gels shown in Figure 10, the sample treated with 0.5% SDS shows more protein abundance (Figure 10B) than the gel which was not treated with it (Figure 10A). SDS also caused significant vertical as well as horizontal streaking on the gel especially on the acidic/neutral portion of the gel. The abundance and or streaking cannot be totally attributed to presence of SDS since both of these observations may have been due to different solubilization buffers.

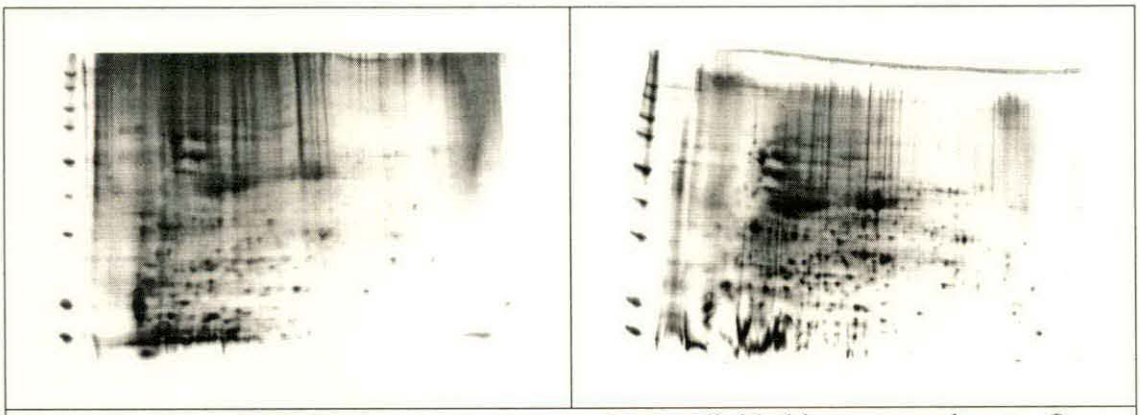


Figure 10A and B. The total cell protein sample was divided in two equal parts. One part was resuspended in 50 mMTris pH 8.8 and the other pellet was resuspended in 100 mM Tris pH 8.8 and 0.5% SDS. Protease inhibitor was added and sonication was done as described above. After sonication, the samples were centrifuged for 30 min at 10,000 rpm and total protein was retrieved. Two different solubilization buffers differing in the detergent content were used. The first rehydration buffer contained 7M urea, 2M thiourea, 4% CHAPS, 2.5mM TBP, 0.5% ampholyte and 0.002% bromophenol blue. The other buffer contained 7M urea, 2M thiourea, 2% CHAPS, 1% ASB14, 2.5mM TBP, 0.5% ampholyte and trace amount of bromophenol blue. RC-DC protein assay was done to quantitate the proteins as described above.1 D gel was used to determine the quantity of the protein to be loaded on two-dimensional gel. The equilibration and second dimension gel was run as described above. The IEF protocol used was 150 v. for an hour, ramp to 6000 v. over a period of 5 hours hold at 6000 v for 12.5 hours, 500 v. for 4 hours The gels were then stained with Silver stain according to manufacturer's directions.

Table 4 summarizes different solubilization buffers used and their results in the form of protein abundance, resolution and any artifacts such as streaking appeared on two-dimensional gels.

**Table 4. Summary of the different solubilization buffers used and the characteristics of the images observed**

| | **Solubilization buffer** | | | | |
|---|---|---|---|---|---|
| | **I** | **II** | **III** | **IV** | **V** |
| Urea | 7M | 7M | 7M | 7M | 7M |
| Thiourea | 2M | 2M | 2M | 2M | 2M |
| CHAPS | -- | 4% | 4% | 3% | 2% |
| ASB 14 | 1% | 0.50% | -- | 1% | 1% |
| DTT | -- | 1% | 50mM | -- | -- |
| TBP | 2mM | -- | -- | 2mM | 2mM |
| Ampholyte | 0.50% | 0.50% | 0.50% | 0.50% | 0.50% |
| Bromophenol Blue | 0.00% | 0.00% | -- | 0.00% | 0.00% |
| Results | Vertical streaking in acidic and neutral region of two-dimensional gel. | Protein abundance. No streaking | Protein abundance. Mild streaking | Vertical and horizontal streaking. Poor resolution | Prominent solvent front. Poor resolution. |

To reduce the streaking and to evaluate if another combination of CHAPS and ASB 14 will work better, the samples were treated with a commercial CleanUp kit. As the gels in Figure 11 show, this treatment did not improve the quality of SDS gel. However, the gel without SDS treatment had better resolution and abundance of proteins.

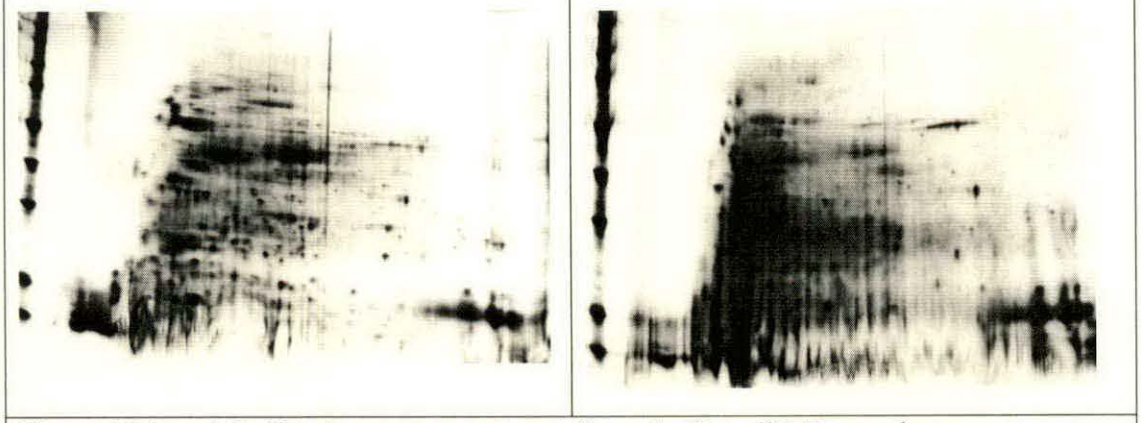


Figure 11A and B. Total proteins retrieved from the E. coli LB samples were suspended in 50 mM Tris pH 8.8 and in 100 mM Tris pH 8.8 and 0.5% SDS as described above. BioRad's CleanUp kit was used to make the samples more concentrated and free of contaminants. Solubilization buffer 7M urea, 2M thiourea, 2% CHAPS, 1% ASB14, 2.5mM TBP, 0.5% ampholyte and trace amount of bromophenol blue was used to solubilize the proteins and the IEF was run at 150 v. for an hour, ramp to 6000 v. over a period of 5 hours hold at 6000 v for 12.5 hours, 500 v. for 4 hours. The equilibration and second dimension run was carried out as explained above. The gels were then stained with Silver stain according to manufacturer's protocol.

BioRad's total membrane extraction kit (membrane II) was used with the total membrane sample. It gave good results with minimal streaking and better abundance of proteins (Figure 12A). Literature shows that PhaP has an isoelectric point of 6.0 and molecular weight around 20 kD (Potter *et al.*, 2004). The two-dimensional gel shows proteins which match this description. The spot appeared as a group of three proteins. Since the literature also supports the existence of homologs of PhaP (Potter *et al.*, 2004), there is a possibility that these proteins are homologs of each other. To ensure that the protein is PhaP, western blot analysis was done using anti PhaP antibody. The blot shows the presence of two distinct proteins (Figure 12B). The

distinct spot seems like PhaP (red arrow in Figure 12B) and the smaller spot (black arrow in Figure 12B) appears to be homolog of PhaP.

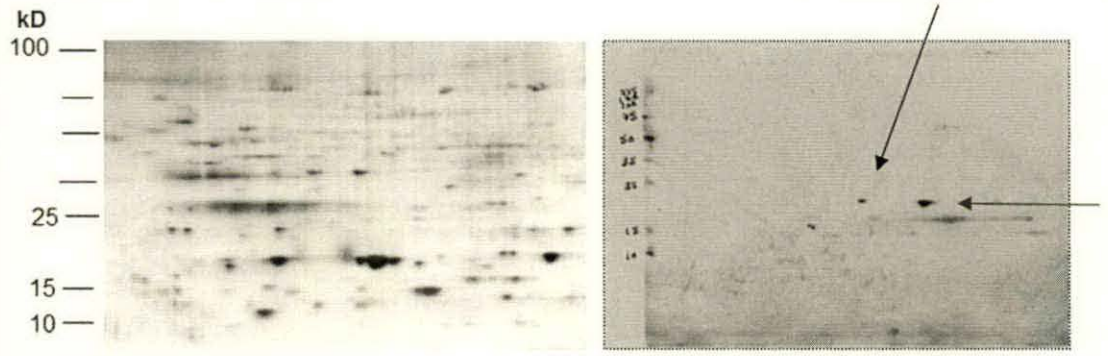



Figure 12A and B. Figure A is the image of total membrane protein extracted with the use of Bio-Rad's two-dimensional total membrane extraction kit (membrane II). The commercial rehydration buffer used for this experiment contained 7 M urea, 2M thiourea, 4% CHAPS, 50 mM DTT, 0.5% Ampholyte, and 0.002% bromophenol blue. IEF protocol used was150 v. for an hour, ramp to 6000 v. over a period of 5 hours hold at 6000 v for 12.5 hours, 500 v. for 4 hours. The gel was stained with silver staining according to manufacturer's directions. Figure B is the Western blot image of 2-D gel shown in Figure A. AntiPhaP antibody was used as primary antibody.

Once it was shown that the PhaP is in the total membrane fraction of the cell, we wanted to see if it is in the cytoplasmic membrane or outer membrane. So an experiment was done using different homogenization methods comparing sonication and bead beater methods as described previously. The total membrane samples were treated with N-Lauryl sarcosine and subjected to centrifugation. The cytoplasmic membrane proteins were solubilized and the other membrane proteins (outer membrane) were pelleted. The RC-DC protein assay was done on these samples to determine their concentration and decide the volume of the sample to be loaded on

the gels. The samples were mixed with Laemlli buffer, boiled in water bath for 5 min and centrifuged at maximum speed for 3 min and loaded on SDS- PAGE gel for one dimensional run. The western blot analysis was done with the anti-PhaP antibody. The gel in Figure 13 indicates that the PhaP is located in the inner membrane (cytoplasmic) fraction of the sample treated with bead beater method but failed to appear in the outer membrane. This supports the hypothesis that PhaP is in cytoplasmic membrane of bacteria. However in the sonicated sample, the results are not as clear. PhaP appeared in both the cytoplasmic membrane as well as in the outer membrane fractions. The band appeared in the cytoplasmic membrane is stronger than the faint band appeared in the outer membrane fraction. This band might be due to the incomplete solubilization of cytoplasmic membrane proteins in the sarkosyl solution and these impurities in outer membrane sample might have caused these results.

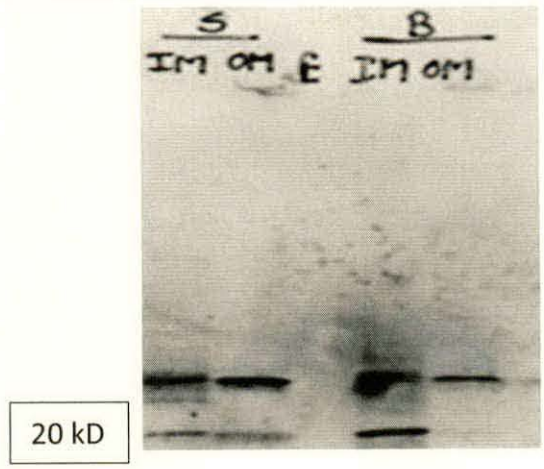



Figure 13. The western blot showing the inner (cytoplasmic) and outer membrane fractions obtained by the sonication and the bead beater methods respectively.

# CHAPTER 4. DISCUSSION

## 4.1. Analysis of results

Two-dimensional electrophoresis analysis was used as the main technique to study the proteomic features of different subcellular proteomes of *C. necator* H16 bacteria. Despite many limitations of the two-dimensional electrophoresis technique to represent the low abundance proteins, highly acidic or basic proteins, hydrophobic proteins, the 2DE remains the major technique for proteomic analysis. It is mainly because two-dimensional electrophoresis technique allows for visualization of a wide range of proteins at the same time and helps visualize the general proteomic nature of the organelle or an organism in terms of acidic/basic nature of proteins, high/low molecular weight etc. This method is also useful to study post-translational modifications. Among all protein samples, the membrane proteins present a more challenging class of proteins to be studied using the two-dimensional electrophoresis because of their hydrophobic nature (Molloy *et al.*, 1998, Rabilloud *et al.*, 2008). Various methods such as the differential solubilization method have been used to answer this problem for enrichment of the protein of interest and reduction in sample complexity (Molloy *et al.*, 1998, Lehner *et al.*, 2003).

The principal objective of this project was to find the combination of a rehydration buffers and an IEF protocol to maximize the protein representation and resolution on the two-dimensional gel for proteomic analyses. Our other goal was to

determine the location of phasin molecule PhaP in *C. necator*. To achieve these goals, different methods of cell disruption, various recipes of rehydration buffers and multiple IEF protocols were tested. The fractionation and isolation of *C. necator* H16 subcellular proteomes were performed by various methods described in literature (Filip *et al.*, 1973, Gorg *et al.*, 2004). No single perfect method was found for subcellular protein extraction and isolation due to the chemical nature of the cytoplasmic membrane, outer membrane and the peptidoglycan layer in between them. Some modifications were done to preexisting methods for good yield and better quality of the bacterial proteins in the sample. The total protein extraction method had been used frequently in literature, the cytoplasmic proteins and total membrane extraction was done using various methods, sodium carbonate method (Molloy *et al.*, 2004) was tried multiple times but both times did not give sufficient yield of proteins and was not pursued further. Surprisingly Hobb *et al.* (2009) also found that the sodium carbonate extraction method failed to offer purified outer membrane samples. Another method, the acetone precipitation method (Gorg *et al.*, 1997) was also used to remove the contaminants from the sample (mainly salts, nucleic acids, lipids etc.) but failed to offer acceptable protein concentration of the products so it was not used further. The cytoplasmic membrane and outer membrane extraction was achieved through sarkosyl treatment (Filip *et al.*, 1973) and isopycnic sucrose density gradient method (Hobb *et al.*, 2009). Since the methods followed were literature based, no separate controls were used to detect the contamination of one fraction with another.

The comparison study between sonication and bead beater method remained inconclusive. There are very few studies in literature that compare homogenization methods with each other. The superiority of sonication over French press extraction has already been demonstrated (Abram *et al.*, 2009). In our study however, the analysis for sonication and bead beater method was inconclusive at best. The bead beater method gave better results at first. But later experimentation demonstrated that it is very difficult to avoid the contamination of two membrane fractions with each other (Figure 5).

The detergents play a major role in the extraction of proteins and their solubilization in solution though out the isoelectric focusing stage. The ideal detergent should perform both of these functions (Gorg *et al.*, 2004, Twine *et al.*, 2005) without causing any artifacts on the gel. Our data suggest that the zwitterionic detergents such as CHAPS and sulfobetains such as ASB-14 work well together in the proportion of 4% CHAPS and 1% ASB than used individually (Figure 8A-8C). The use of another sulfobetain –SB 3-10 was not pursued because the literature shows that it is insoluble in buffers that contain more than 5 M urea and 2 M thiourea. This low concentration of urea is beneficial to obtain a good yield of low molecular weight proteins but fails to represent higher molecular weight proteins (Rabilloud *et al.*, 1997, Stanley *et al.*, 2003). Since the objective of this study was to construct a buffer that will offer optimal protein presentation and good resolution, using SB 3-10 would not have been very useful. On the other hand, amidosulfobetain-14 (ASB-14) has

good solubilizing power and has been found to facilitate the representation of membrane proteins. CHAPS is considered a moderately good detergent, but it is not very strong in any aspect of protein extraction or IEF. So it is advantageous to use it in combination with ASB-14 which may offer good solubilizing strength, but will not affect the quality of the gel because it will be in minor amount as compared to CHAPS.

The data comparing the compatibility of reducing agents suggest that TBP works better for *C. necator* H16 samples than DTT. However, the results are not conclusive. The solubilization buffers used for this analysis contained varied types and amounts of detergents. So there is a possibility that the presence of these different detergents might have caused the effects seen on the gel and protein presentation. However, the gels that represent the TBP as reducing agents gave consistently better results (Figure 4A, 4C, and 4E) than the gels that represent DTT ( Figure 4 B and 4D) despite the IEF protocol used. So it can be interpreted that TBP is a better reducing agent than DTT. Previous studies done by Molloy *et al.* also support this finding (Molloy *et al.*, 2000)

The study regarding the composition of solubilization buffers suggests that the buffer made up of 7M urea, 2M thiourea, 4% CHAPS, 1% ASB-14, 2.5mM TBP and 0.5% ampholytes is the most suitable for a proteomic study of *C. necator* H16 bacteria. This combination is not entirely new. Twine *et al.* (2005) have already tested a similar combination for the proteomic analysis of *Francisella tularensis* bacteria

and found that ASB-14 increases visualization of membrane proteins. It is difficult to predict if this formulation will be perfect for all membrane proteins in all organisms, but it certainly looks promising. As suggested by many scientists, an empirically derived unique solubilization buffer is necessary for two-dimensional electrophoresis because the nature of proteins changes often according to their origin (prokaryotic, eukaryotic cells), location in cell (membrane proteins more hydrophobic), isoelectric point and molecular weight (Gorg *et al.*, 2004). The varied characteristics of sample and proteins may not be compatible with the mostly accepted formulation of buffer. Even though these limitations exist, the above mentioned formula seems to provide optimum results for membrane proteins in *C. necator* H16

A few protocols for isoelectric focusing were also studied. The literature shows a wide range of protocols for IEF. The total volt hours used for two-dimensional electrophoresis range anywhere from 35,000 to 90,000 (Zhobi-Hasona, 2005) and in few instances up to 100,000 volt hours (Potter *et al.*, 2004). It was difficult to change one stage (either in voltage or in duration or in pattern, like linear or ramping) because of the time constraints. The change in only one stage in duration or voltage may make only minor changes in appearances of the gels Two IEF protocols were compared; however it is difficult to decide which worked better. Protocol I looks promising when two gels were run with same sample (Figure 3B and 3D). However, the difference is not very noticeable and since the total volt-hours

used were not very much different from each other, it seemed that there is a wide range of voltage ramping protocols that could be successfully employed.

The polyhydroxybutyrate granules (PHB) are the most studied polyhydroxyalkaonates (PHAs). There are at least four paralogs of PhaP on the surface of inclusion bodies (Potter *et al.*, 2004). The two-dimensional gel (Figure 10A) and the western blot of total membrane fraction (Figure 10B) strongly support the hypothesis that the PhaP is in membrane fraction. The appearance of three spots so close to each other on two-dimensional gel is consistent with the findings of Potter. There is a possibility that these spots represent the paralogs of PhaP.

After showing that the PhaP is in the total membrane fraction, further analysis was done to see if it is present in the outer membrane or in the cytoplasmic membrane. The separation of membrane proteins was very challenging technically. Sarkosyl treatment and isopycnic sucrose gradients were used. The principal problem with these methods was the outer membrane band and cytoplasmic membrane bands were not distinct enough to collect them individually without contamination. The second challenge was to get the sufficient concentration of proteins in these fractions. The low concentration samples did not work well and the proteins were very scarce due to progressive loss of proteins through all the steps from IEF to second dimensional run. While studying the efficiency of two different homogenization methods, it was determined that the PhaP is present on the cytoplasmic membrane but not on the outer membrane fractions derived by sarkosyl treatment (Figure 13). In a

sonicated sample however, the PhaP band appears more strongly in cytoplasmic membrane fraction than in the outer membrane fraction. During the sarkosyl treatment of total membrane fraction, the cytoplasmic membrane proteins are solubilized from the total membrane fraction and after ultracentrifugation, the pellet essentially contains total membrane proteins from which cytoplasmic membrane proteins are removed. In this case, there is a possibility that all the cytoplasmic membrane proteins were not completely solubilized and they appeared in outer membrane fraction. Though these data support the hypothesis that the PhaP is located on the cytoplasmic membrane of *C. necator* H16, it is not conclusive and further analysis is needed.

The tentative presence of PhaP on the cytoplasmic membrane supports the inclusion body formation by budding theory (Jendrossek, 2009) depicted in Figure 14. It shows that the PHAs enter the inclusion body from the periplasmic space and the bud is formed from the cytoplasmic membrane. This bud enters the cytoplasm as it grows in size and becomes ultimately enveloped with cytoplasmic membrane when it is fully formed. Since PhaP is on the cytoplasmic membrane, it appears on the surface of these inclusion bodies.

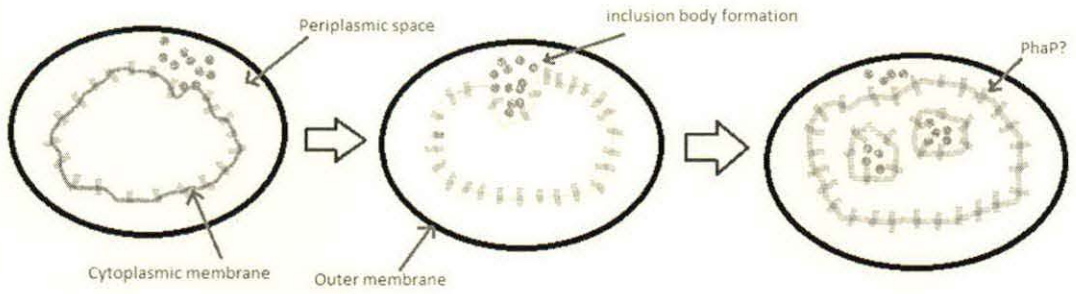



Figure 14. Schematic representation of the PhaP location theory

Though the structure and morphology of inclusion bodies has been studied in the past (Steinbuchel, A *et al* 1995, Dennis *et al*, 2003, Potter *et al,* 2004), the process of their formation is still under investigation. It will be beneficial to study if the above mentioned budding theory of inclusion formation is correct, and if it is, then the next question will be if it is typical in other bacteria that make PHA or if it is found only in certain bacteria. More knowledge of this process will provide us with more ways to manipulate the PHA production and ultimately produce more biodegradable plastic.

## 4.2. **Future Directions**

To support the data collected, the Matrix Assisted laser Desorption/Ionization – Time of Flight Mass Spectrometry (MALDI-TOF) analysis is necessary for protein identification and sequencing. This method will confirm the presence of PhaP on cytoplasmic membrane of *C. necator* H16.

Similar studies in other bacteria that make inclusion bodies and PHA are necessary to determine if the inclusion bodies are formed in similar fashion as they do in *C. necator* H16. If it is found that the formation of inclusion bodies from the cytoplasmic membrane is the typical, then may be this mechanism can be manipulated to get better yield of the PHAs and ultimately of the biodegradable plastic.

## REFERENCES

Anderson, A. J., and Dawes, E.A. (1990). Occurrence, metabolism, metabolic role, and industrial uses of bacterial Polyhydroxyalkanoates. *Microbiology Reviews*, 54 (4): 450-72.

Berkelman, T. (2008). Removal of interfering substances in samples prepared for 2DE. *2D PAGE Sample Preparation and Fractionation*. Totowa, NJ: Humana, 51-62.

Berks, B., Palmer, T., and Sargent, F. (2005). Protein targeting by the bacterial twin-arginine translocation (Tat) pathway. *Current Opinion in Microbiology,* 8 (2): 174-81.

Cordwell, S. J. (2006). Technologies for bacterial surface proteomics. *Current Opinion in Microbiology*, 9(3): 320-29.

Dennis, D., Liebig, C., Holley, T., Thomas, K.S., Khosla, A., Wilson, D., and Augustine, B. (2003). Preliminary analysis of polyhydroxyalkanoate inclusions using atomic force microscopy. *FEMS Microbiology Letters*, 226 (1): 113-119.

Dennis, D., Sein, V., Martinez E., and Augustine B. (2008). PhaP is involved in the formation of a network on the surface of polyhydroxyalkanoate inclusions in *Cupriavidus necato*r H16. *Journal of Bacteriology*, 190 (2): 555-563.

Frank, R., and Steinbüchel, A. (2009). *Ralstonia Eutropha* strain H16 as model organism for PHA metabolism and for biotechnological production of technically interesting biopolymers. *Journal of Molecular Microbiology and Biotechnology*, 16 (1-2): 91-108.

Filip, C., Fletcher, G., Wulff, J., and Earhart, C.F. (1973). Solubalization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium- lauryl sarcosinate. *Journal of Bacteriology*, 115 (3): 717-722.

Fujiki, Y., Hubbard, A.L., Fowler, S., and Lazarow, P.B. (1982). Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. *The Journal of Cell Biology*, 93 (1): 97–102.

Gorby, Y. A., Beveridge, T.J., and Blakemore, R.P. (1988). Characterization of the bacterial magnetosome membrane. *Journal of Bacteriology,* 170 (2): 834-841.

Gorg, A., Obermaier, C., Boguth, G., Csordas, A., Diaz, J.J., Madjar, J.J. (1997). Very alkaline immobilized pH gradients for two-dimensional electrophoresis of ribosomal and nuclear proteins. *Electropohresis*, 18 (3-4): 328-337.

Gorg, A., Weiss W., and Dunn, M.J. (2004) Current two-dimensional electrophoresis technology for proteomics. *Proteomics*, 4 (12): 3665-3685.

Hobb, R.I., B., Fields, J.A., Burns, C.M., and Thompson, S.A. (2009). Evaluation of procedures for outer membrane isolation from *Campylobacter Jejuni*. *Microbiology*, 155 (Pt 3): 979-88.

Hurkman, W. J., and Tanaka, C.K. (1986). Solubilization of plant membrane proteins for analysis by two-dimensional gel electrophoresis. *Plant Physiology*, 81(3): 802-806.

Imperi, F., Ciccosanti, F., Perdomo, A.B., Tiburzi, F., Mancone, C., Alonzi, T., Ascenzi, P., Piacentini, M., Visca, P., and Fimia, G. M. (2009). Analysis of the periplasmic proteome of Pseudomonas aeruginosa, a metabolically versatile opportunistic pathogen. *Proteomics*, 9 (7): 1901-1915.

Jendrossek, D., Selchow, O., and Hoppert, M. (2007). Poly(3-Hydroxybutyrate) granules at the early stages of formation are localized close to the cytoplasmic membrane in caryophanon latum. *Applied and Environmental Microbiology*, 73 (2): 586-593.

Lehner, I., Niehot, M., and Borlak, J. (2003). An optimized method for the isolation and identification of membrane proteins. *Electrophoresis*, 24 (11), 1795-1808.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *The Journal of Biological Chemistry,* 193 (1): 265-275.

Molloy, M. P., Herbert, B. R., Walsh, B. J., Tyler, M. I., Traini, M., Sanchez, J. C., Hochstrasser, D. F., Williams, K. L., and Gooley, A. A. (1998). Extraction of membrane proteins by differential solubilization for separation using two-dimensional gel electrophoresis. *Electrophoresis*, 19 (5): 837-844.

Molloy, M. P., Herbert, B.R., Slade, M.B., Rabilloud, T., Nouwens, A.S., Williams, K.L., and Gooley A.A. (2000). Proteomic analysis of the *Escherichia coli* outer membrane. *European Journal of Biochemistry*, 267 (10): 2871-2881.

Molloy, M. P., Phadke, N.D., Maddock, J.R., and Andrews, P.C. (2001). Two-dimensional electrophoresis and peptide mass fingerprinting of bacterial outer membrane proteins. *Electrophoresis*, 22 (9): 1686-96.

Neumann, L., Spinozzi, F., Sinibaldi, R., Rustichelli, F., Potter, M., and Steinbuchel, A. (2008). Binding of the major phasin, PhaP1, from *Ralstonia eutropha* H16 to poly(3-Hydroxybutyrate) granules. *Journal of Bacteriology*, 190 (8): 2911-2919.

Nomura, C. T., Taguchi, K., Taguchi, S., and Doi, Y. (2004). Coexpression of Genetically Engineered 3-Ketoacyl-ACP Synthase III (fabH) and Polyhydroxyalkanoate Synthase (phaC) Genes Leads to Short-Chain-Length-Medium-Chain-Length Polyhydroxyalkanoate Copolymer Production from Glucose in Escherichia Coli JM109. *Applied and Environmental Microbiology*, 70 (2): 999-1007.

O'Farrell, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. *The Journal of Biological Chemistry*, 250 (10): 4007-4021.

Pieper-Fürst, U., Madkour, M.H., Mayer, F., and Steinbüchel, A. (1995). Identification of the region of a 14-kilodalton protein of *Rhodococcus ruber* that

is responsible for the binding of this phasin to polyhydroxyalkanoic acid granules. *Journal of Bacteriology*, 177 (9): 2513-2523.

Posch, A. (2008). Sample Preparation Basics. *2D PAGE Sample Preparation and Fractionation*. Totowa, NJ: Humana, 3-63.

Potter, M., and Steinbuchel, A.. (2005). Poly (3-hydroxybutyrate) granule-associated proteins: impacts on poly (3-hydroxybutyrate) synthesis and degradation. *Biomacromolecules*, 6 (2): 552-560.

Potter, M., Muller, H., Reinecke, F., Wieczorek, R., Fricke, F., Bowien, B., Friedrich, B., and Steinbuchel, A. (2004). The complex structure of polyhydroxybutyrate (PHB) granules: four orthologous and paralogous phasins occur in *Ralstonia eutropha*. *Microbiology*, 150 (Pt 7): 2301-2311.

Potter, M., Muller, H., and Steinbuchel, A. (2005). Influence of homologous phasins (PhaP) on PHA accumulation and regulation of their expression by the transcriptional repressor PhaR in *Ralstonia eutropha* H16. *Microbiology*, 151 (Pt 3): 825-833.

Potter, M., Madkour, M.H., Mayer, F., and Steinbuchel, A. (2002). Regulation of phasin expression and polyhydroxyalkanoates (PHA) granule formation in *Ralstonia eutropha* H16. *Microbiology*, 148 (Pt 8): 2413-426.

Raberg, M., Reinecke, F., Reichelt, R., Malkus, U., Konig, S., Potter, M., Fricke, W.F., Pohlmann, A., Voigt, B., Hecker, M., Friedrich, B., Bowien, B., and Steinbuchel, A. (2008). *Ralstonia eutropha* H16 flagellation changes according to nutrient supply and state of Poly(3- Hydroxybutyrate) accumulation. *Applied and Environmental Microbiology*, 74 (14): 4477 - 4490.

Rabilloud, T., Chevallet, M., Luche, S., and Lelong, C. (2008). Fully denaturing two-dimensional electrophoresis of membrane proteins: a critical update. *Proteomics*, 8 (19): 3965-3973.

Rabilloud, T., Luche, S., Santoni, V., and Chevallet, M. (2007). Detergents and chaotropes for protein solubilization before two-dimensional electrophoresis. *Methods in Molecular Biology*, 355: 111-119.

Rhomberg, T.A., Karlberg, O., Mini, T., Zimny-Arndt, U., Wickenberg, U., Rottgen, M., Jungblut, P.R., Jeno, P., Andersson, S.G., Dehio, C. (2004). Proteomic analysis of the sarcosine-insoluble outer membrane fraction of the bacterial pathogen *Bartonella henselae*. *Proteomics*, 4 (10): 3021-3033.

Steinbuchel, A., Aerts, K., Babel, W., Follner, C., Liebergesell, M., Madkour, M.H., Mayer, F., Pieper-Furst, U., Pries, A., Valentin, H.E., and Wieczorek, R. (1995). Considerations on the structure and biochemistry of bacterial polyhydroxyalkanoic acid inclusions. *Canadian Journal of Microbiology*, 41 (1): 94-105.

Tian, J., He, A., Lawrence, A.G., Liu, P., Watson, N., Sinskey, A.J., and Stubbe, J.. (2005). Analysis of transient polyhydroxybutyrate production in *Wautersia eutropha* H16 by quantitative Western analysis and transmission electron microscopy. *Journal of Bacteriology*, 187 (11): 3825-3832.

Tian, P., Shang, L., Hong R., Mi, Y., Fan, D., and Jiang, M. (2009). Biosynthesis of polyhydroxyalkanoates: current research and development. *African Journal of Biotechnology*, 8 (5): 709-714.

Twine, S.M., Mykytczuk, N.C., Petit, M., Tremblay, T.L., Lanthier, P., Conlan, J.W., and Kelly, J.F. (2005). *Francisella tularensis* proteome: low levels of ASB-14 facilitate the visualization of membrane proteins in total protein extracts. *Journal of Proteome Research*, 4 (5): 1848-1854.

Marc, V., Atrian, S., Lorenzo, V.D., and Fernández, L.A. (2000). Engineering a mouse metallothionein on the cell surface of *Ralstonia eutropha* CH34 for immobilization of heavy metals in soil. *Nature Biotechnology*, 18: 661-665.

Verlinden, R.A., Hill, D.J., Kenward, M.A., Williams, C.D., and Radecka, I. (2007). Bacterial synthesis of biodegradable polyhydroxyalkanoates. *Journal of Applied Microbiology*, 102 (6): 1437-49.

Weiss, W., and Gorg A. (2008). Sample solubilization buffers for two-dimensional electrophoresis. Ed. Posch Anton. *2D PAGE Sample Preparation and Fractionation*. Totowa, NJ: Humana, 35-42.

Wickner, W., and Schekman, R. (2005). Protein translocation across biological membranes. *Science*, 310 (5753): 1452-1456.

Wieczorek, R., Pries, A., Steinbüchel, A., and Mayer, F. (1995). Analysis of a 24-kilodalton protein associated with the polyhydroxyalkanoic acid granules in *Alcaligenes eutrophus*. *Journal of Bacteriology*, 177 (9): 2425-2435.

York, G.M., Stubbe, J., Sinskey, A.J. (2002). The Ralstonia eutropha PhaR protein couples synthesis of the PhaP phasin to the presence of polyhydroxybutyrate in cells and promotes polyhydroxybutyrate production. *Journal of Bacteriology*, 184 (1): 59-66.

Zuobi-Hasona, K., Crowley, P.J., Hasona, A., Bleiweis, A.S, and Brady, L.J. (2005). Solubilization of cellular membrane proteins from *Streptococcus mutans* for two-dimensional gel electrophoresis. *Electrophoresis* 26 (6): 1200-205.