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## METABOLITE ANALYSIS OF CLOSTRIDIUM THERMOCELLUM USING CAPILLARY ELECTROPHORESIS BASED TECHNIQUES

Anup P. Thakur

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ABSTRACT OF DISSERTATION

Anup P. Thakur

Graduate School  
University of Kentucky  
2008

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ABSTRACT OF DISSERTATION

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A dissertation submitted in partial fulfillment of degree of Doctor of Philosophy in the  
college of Arts and Sciences at the University of Kentucky

By

Anup P. Thakur

Director: Dr. Bert C. Lynn

Professor of Chemistry

Lexington, KY

2008

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## ABSTRACT OF DISSERTATION

### METABOLITE ANALYSIS OF *CLOSTRIDIUM THERMOCELLUM* USING CAPILLARY ELECTROPHORESIS BASED TECHNIQUES

*Clostridium thermocellum* is a thermophilic bacterium that converts biomass to ethanol directly; however, high sensitivity of this bacterium toward ethanol limits its commercial utility. To elucidate the effect of ethanol on the growth of this bacterium a metabolite analysis of *C. thermocellum* was performed. The hypothesis of the project was that exogenous ethanol alters the metabolite profile of *C. thermocellum*. For metabolite analysis, capillary electrophoresis-electrospray ionization-mass spectrometry method (CE-ESI-MS) was developed due to highly polar and charged nature of metabolites. To increase the sensitivity of CE-ESI-MS, several parameters at the ESI interface were optimized. The application of 50% isopropanol as a sheath liquid increased sensitivity for metabolite analysis dramatically. Trimethylamine acetate (pH 10) was used as background electrolyte (BGE) due to its ability to separate the structural isomers of glucose phosphate.

For metabolite sample preparation, novel methods for quenching and CE compatible metabolite extraction protocols were developed. Newly developed protocols were applied to metabolite analysis of wild type (WT) and ethanol adapted (EA) strains of *C. thermocellum* grown in batch cultures. Significant differences were found in key intracellular metabolites such as  $\text{NAD}^+$  and pyruvic acid. Intracellular concentrations of  $\text{NAD}^+$  were low in EA cells compared to WT cells and pyruvic acid was only detected in EA cells. To further understand the effect of ethanol on metabolite fluxes, WT and EA cells were grown in increasing concentrations of ethanol and the metabolite profile for each ethanol treatment was obtained. Significant changes were found in intracellular metabolite concentrations. Metabolic data showed that the glycolysis process in WT cells was obstructed due to exogenous ethanol which was evident from accumulation of G6P. On the other hand, no such accumulation of G6P was observed in the EA strain; however pyruvate began to accumulate in EA strain. These changes in intracellular metabolite concentrations due to perturbation of exogenous ethanol supported the hypothesis. Also, this investigation revealed a correlation between ethanol and metabolite profile changes and was able to explain a possible mechanism of growth inhibition of *C. thermocellum* which will certainly help genetic engineers to develop superior strains of *C. thermocellum* for commercial cellulosic ethanol production.

Keywords: *C. thermocellum*, metabolomics, quenching, extraction, capillary electrophoresis-electrospray ionization-mass spectrometry.

Anup Thakur

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By  
Anup P. Thakur

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DISSERTATION

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Graduate School  
University of Kentucky  
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*Dedicated to*

My beloved Uncle  
*Arvind Thakur*

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## Chapter 1 : Introduction

Since, metabolites are at the final stage of the chain of events from genes to metabolism; any genetic, environmental or nutritional perturbation to the cell will be reflected by the changes in metabolite concentrations. A comprehensive metabolite analysis can help to understand the biochemical status of a cell and can unravel the mysteries of the cellular processes. A comprehensive metabolite analysis is termed metabolomics and it is an emerging 'omics' science which holds strong promise in finding the phenotypes and to discover the genotype-phenotype and genotype-environment relationships. Many analytical tools have been developed for comprehensive metabolite analysis; yet the analysis of highly polar and negatively charged metabolites is still very challenging. Several metabolites involved in the central metabolic pathway (ATP, glucose-6-phosphate, pyruvic acid, and  $\text{NAD}^+$ ) are negatively charged at neutral pH, because of presence of acidic moieties such as phosphate or carboxylate in their structures. Regular analysis platforms such as LC-MS and GC-MS can not be used to analyze these polar and charged metabolites, since these chromatographic techniques can't separate polar and charged molecules. Lack of a suitable analytical method to analyze the negatively charged metabolites hinders their quantification. Therefore, this thesis emphasizes development of methods to analyze polar and charged metabolites and application of these analytical methods to quantify metabolites from *Clostridium thermocellum*, a thermophilic anaerobe and model organism of the study.

*C. thermocellum* (CT) is a microorganism which produces ethanol from cellulose by the process of fermentation. Ethanol has many industrial applications and can be used in automobiles as a fuel. Ethanol produced from CT is cost effective, since cellulose is inexpensive. However, CT is highly sensitive to exogenous ethanol which hinders its commercial exploitation for ethanol production.<sup>1</sup> Recently new strains of CT which can grow in relatively higher concentration of ethanol were developed.<sup>2</sup> Although, ethanol adapted (EA) strain was able to grow in higher exogenous ethanol concentrations, its growth is slow relative to the wild type (ET) strain and its ethanol tolerance is lower than other ethanol producing microorganisms such as *yeast*. Therefore, to understand the basis of ethanol tolerance of the EA strain compared to WT strains, a metabolite analysis was carried out on WT and EA *C. thermocellum* strains. A metabolic comparison of

these two strains will help to unravel the mechanism of growth inhibition in WT cells and basis of ethanol tolerance in EA cell. Also, metabolite data can be used for genetic engineering to develop superior strains of *C. thermocellum* for improved and commercial production of ethanol from cellulose.

### **1.1 Study goal**

The goal of this project is to develop methods and sample preparation protocols for analysis of important metabolites involved in central metabolic pathway of *C. thermocellum* and further apply these newly developed methods and sample preparation protocols for intracellular metabolite analysis of WT and EA strains.

### **1.2 Study hypothesis**

The hypothesis of the project was that exposure of *C. thermocellum* to external stimulus such as ethanol alters intracellular metabolite concentrations.

### **1.3 Study rationale**

It is well known that any environmental and/or nutritional perturbation during cell growth can change the metabolism of a cell<sup>3</sup> and in turn external and internal metabolite concentrations. In other words, consumption and production of metabolites is directly related to the biochemical status of the cells. For example, Bothum and co-workers studied *C. thermocellum* cultures under varying external pressure and measured extracellular metabolites such as ethanol and acetate.<sup>4</sup> Ethanol and acetate are branch points originating from acetyl-CoA in a metabolic pathway of *C. thermocellum*. Therefore, the ratio of ethanol to acetate was expected to be constant under all the conditions. However, their study showed that growing *C. thermocellum* cultures under elevated hydrostatic pressure caused an increase in ethanol: acetate ratios > 100 fold compared to atmospheric pressure. This shift was the effect of increasing growth pressure of *C. thermocellum* cultures from atmospheric to 7 MPa. Such dramatic shifts in the ratios of ethanol to acetate due to variations in the pressure suggest that alterations in the growth environment of the cells dramatically shift the metabolic pathways and subsequently change the extracellular metabolite concentrations. Any changes in extracellular metabolite concentration can be linked to changes in the intracellular metabolite

concentrations of the cells. Therefore if a change in growth pressure (i.e. environment of the cells) causes change in extracellular concentrations (ethanol and acetate), it would also affect intracellular metabolite concentrations. Similarly, EA cells developed by growing WT cells in a different environment (i.e. increasing concentration of ethanol) would lead to changes in the intracellular metabolite concentrations of the cells.

#### **1.4 Specific goals**

- 1) The first objective of this project is to develop capillary electrophoresis (CE) based methods for analysis of acidic metabolites (total 12 metabolites) involved in the central metabolic pathway of *C. thermocellum*
- 2) The second objective is to evaluate and develop quenching and extraction protocols for cellulolytic and gram-positive bacteria from the class *clostridia*
- 3) Third and the final objective is to apply newly developed quenching and extraction protocols for metabolite analysis for WT and EA *C. thermocellum*

## **Chapter 2 : Background**

### **2.1 Fossil fuels**

Some hundreds of millions years ago, fossils of plants and animals were buried under the earth's crust. In the earth's crust, tremendous pressure and heat converted these remnants of plants and animals to oil, coal, and gas or what we know of today as fossil fuels. Human beings have been using fossil fuels as a source of the energy over a century and even in today's modern era, more than 80% of our energy comes from burning of fossil fuels.<sup>5</sup> The demand for fossil fuels is increasing and with the rate that fossil fuels are used today, it is feared that these natural reserves will be completely depleted in few decades.

In addition to depletion of these fuel sources, there are other problems caused by the widespread use of fossil fuels, such as the emission of greenhouse gases. Combustion of fossil fuels, which mainly consist of carbon and hydrogen, releases carbon-dioxide (CO<sub>2</sub>) and water (H<sub>2</sub>O) as the main byproducts. Therefore, the vast use of fossil fuels has resulted in emission of a tremendous amount of CO<sub>2</sub> to the atmosphere. Continuous emission of CO<sub>2</sub> has increased the atmospheric concentration of CO<sub>2</sub> from 280 to 380 ppm over last two centuries.<sup>6</sup> Since CO<sub>2</sub> is a greenhouse gas, rising concentrations of CO<sub>2</sub> in atmosphere has increased the green house effect, accounting for 20% of the total greenhouse gas effect.<sup>7</sup> Burning fossil fuels emits tremendous amounts of CO<sub>2</sub> into the atmosphere, of which 35% is emitted from burning of the gasoline alone.<sup>8</sup> Therefore, rapid depletion of the fossil fuel reserves, their non-renewable nature, and increasing greenhouse gas effects from their utilization has compelled researchers to pursue renewable and environment friendly sources of the energy.

### **2.2 Biofuels: Alternative sources of energy**

To replace fossil fuels, many other sources are available on the earth to generate energy such as solar power, wind power, hydrogen, ocean, and hydropower. However, there are no cost-effective ways of harnessing these energies. Another source of energy available is biomass. Biomass is basically living or dead biological material or a metabolic byproduct derived from a biological species. However, in a more general sense the plant matter is categorized as biomass. Biomass such as wood and grass has



been used as source of energy from ancient times to generate heat and to cook food. Biomass is the most abundant organic biological matter present on the earth today.<sup>9</sup> However when biomass is utilized directly to generate energy, it is inefficient (energy/unit volume) and difficult to transport. Therefore, biomass must be converted in to a more efficient and easily transportable form for effective utilization, so that it could be burned more efficiently and easily transported to desired destination. Therefore, by converting biomass to some volatile liquid, it could be burned efficiently and easily transported. Fuels derived in such a way from biological sources are termed biofuels.

A variety of microorganisms can be used to convert biomass to small organic molecules such as methane, ethanol or butanol and these organic molecules can be later combusted to generate energy. *Saccharomyces cerevisiae* (yeast) is the most commonly used microorganism to ferment sugar (derived from biomass) and to produce ethanol. The obtained ethanol could be used as fuels for vehicles, since ethanol can be combusted in gasoline engines to run automobiles. Ethanol can be found blended with gasoline at most of the gas stations in USA at a proportion of 10:90 (ethanol: gasoline).

### **2.2.1 Advantages of biofuels**

On combustion of biofuels, heat is produced and byproducts of the combustion reaction, CO<sub>2</sub> and H<sub>2</sub>O, are released into the atmosphere. Plants absorb the CO<sub>2</sub> from the atmosphere and convert it to plant matter via the process of photosynthesis. This plant matter can be then converted to biofuels such as ethanol, which when combusted releases CO<sub>2</sub> back into the atmosphere. In this way carbon is recycled. Since there is no net addition of CO<sub>2</sub> into the atmosphere, biofuels are considered carbon neutral. Combustion of fossil fuels also releases CO<sub>2</sub> into the atmosphere, however the release of CO<sub>2</sub> due to burning of fossil fuels can not get absorbed again to produce fossil fuel in a short period of time. By controlling the emission of CO<sub>2</sub> the greenhouse gas effects could be minimized. In addition to its carbon neutral nature, biofuels are non-toxic, degradable, and renewable source of the energy.<sup>10</sup>

### 2.2.2 Sources of biofuels

**A) Solid biofuels:** Prime examples of solid biofuels are wood, grass, cow dung, and charcoal which has been used since ancient times for household purposes. However, solid biofuels can not be directly used for transportation purposes in automobiles.

**B) Biogas:** Methane is the primary component of biogas. Biogas is obtained by anaerobic digestion of organic matter by anaerobes. Biogas is mainly used for household purposes such as heating and cooking.

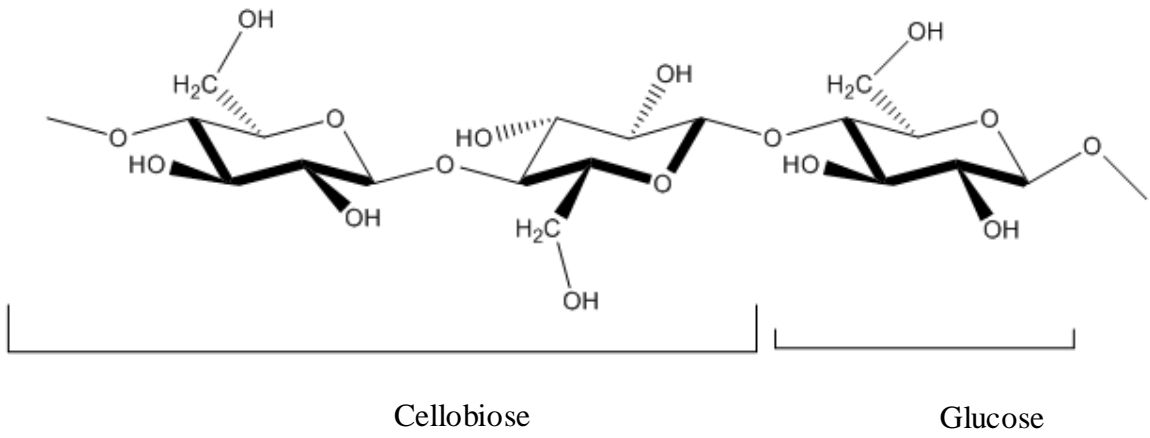
**C) Biodiesel:** Biodiesel is obtained by transesterification of vegetable oil and can be mixed with regular diesel in varying proportions. Biodiesel is most commonly used in Europe. The majority of engine manufactures recommend using 15% biodiesel blended with regular diesel.

**D) Bioalcohols:** Alcohol such as ethanol (more common) and butanol can be produced through fermentation of biomass by microorganisms.

**i) Biobutanol:** Supporters of biobutanol claim that butanol could be used in a similar fashion to gasoline in automobile engines. However as yet, no engine manufacturers support this claim. Butanol could be produced by the anaerobic fermentation using *Clostridium acetobutylicum*.<sup>11</sup>

**ii) Bioethanol:** Among all the biofuels used today, bioethanol is the most predominantly used biofuel for automobiles.<sup>12</sup> Ethanol is blended with gasoline at a maximum of 15%, since gasoline engines are not originally made to run on ethanol. A blend of 10:90 ethanol: gasoline is termed as E10, which is commonly found in USA. Recently some manufactures have started making engines which can run on gasoline or a blend of gasoline/ethanol or ethanol alone, such vehicle are known as flex-fuel vehicles.<sup>13</sup> Countries such as Brazil, USA, Sweden, Australia, and Iceland have large bioethanol production programs to make themselves less dependant on foreign oil. Brazil has the largest biofuel program of any other country and its bioethanol production peaked in 2005. In Brazil, sugar-cane (a source of simple sugar) is primarily utilized to produce ethanol via fermentation. Depending on atmospheric and environmental conditions different countries use different sources of crops (biomass) to produce sugar for ethanol production via yeast fermentation (corn in USA and wheat and sugar beet in Europe). However, the use of crops such as sugar-cane and corn raise their demand and in turn

increase their cost which may jeopardize food supplies. Therefore, even though bioethanol has many incentives to serve as a substitute for fossil fuels, it might risk the food supplies. Recently, other alternatives to the production of bioethanol have attracted the attention of many researchers to avoid putting food supplies at risk. Such alternatives are the utilization of biomass for production of ethanol. Since biomass is non-edible matter, its utilization for bioethanol production does not compete with food materials. Biomass is mostly composed of cellulose (about 50%) along with hemicellulose and pectin to a smaller extent.<sup>9</sup> Cellulose is a polysaccharide with molecular formula  $(C_6H_{10}O_5)_n$  and its structure is shown in Figure 2-1. Cellulose is a water insoluble polymer made up of cellobiose monomer in which 2 sugar molecules are connected by glycosidic  $\beta$ -1, 4 bonds. In contrast to cellulose, cellobiose is water soluble. Cellulolytic bacteria such as *Clostridium cellulolyticum* (*C. cellulolyticum*)<sup>14</sup> and *Clostridium thermocellum* (*C. thermocellum*)<sup>15</sup> have the ability to hydrolyze cellulose. The cellulolytic microorganisms contain a multi-enzymatic complex called cellulosome, which break cellulose into simple sugars.<sup>16</sup> Subsequently, these microorganisms can then carry out fermentation of the sugar molecules to produce ethanol, acetate, and lactate as the main products of the fermentation reaction. The biggest advantage of using cellulolytic bacteria for ethanol production is that due to one step conversion of cellulose to ethanol, final cost of ethanol produced is low compared to 2 step conversion of cellulose to ethanol (cellulose to glucose using cellulase and glucose to ethanol using yeast).<sup>17</sup> A commercialization bottleneck in ethanol production using cellulolytic bacteria mainly occurs because of the high sensitivity of these bacteria against ethanol and the low concentration of ethanol produced in fermentation broth.<sup>2</sup>



**Figure 2-1: Structure of cellulose**

### **2.3 *Clostridium thermocellum***

*Clostridium thermocellum* (*C. thermocellum*) is an thermophilic, anaerobic, cellulolytic bacterium which has the ability to grow on cellulosic biomass and produce ethanol.<sup>18</sup> However, at 5 g/L (0.5%) exogenous ethanol results in 50% growth inhibition of *C. thermocellum*.<sup>19, 20</sup> To increase the ethanol tolerance of *C. thermocellum*, sequential transfer of WT cultures into increasing concentrations of ethanol were performed. Such a process of sequential transfer to improve ethanol tolerance for *C. thermocellum* was documented by Gomez *et al* in 1980.<sup>1</sup> Using the technique of the sequential transfer, they were able to develop a new *C. thermocellum* strain which can grow in 25 g/L (2.5%) exogenous ethanol concentration. Recently, other authors documented that *C. thermocellum* was able to grow in ethanol concentration as high as 50 g/L (5%)<sup>2</sup> using the technique of sequential transfer such strains are refereed as an ethanol adapted (EA) strains. Although, ethanol adapted (EA) strains are able to grow in 5% (w/v) exogenous ethanol, it is not known how much ethanol is produced by EA strains. In theory, if the EA strains can make ethanol at all, it will make a maximum of 5% ethanol. Since exogenous ethanol concentration greater than 5% halts the growth of *C. thermocellum*. In comparison the final concentration of ethanol produced in commercial fermentation broth using *yeast* is typically between 10 to 20% (w/v).<sup>21, 22</sup> In addition to exogenous ethanol limits, EA strains grow relatively slow compared to WT strains for unknown reasons (see Figure 5-1). In order to improve the final ethanol yield from *C. thermocellum*, it is essential to improve its ethanol tolerance. The exact mechanism by which ethanol affects the growth of *C. thermocellum*, is not completely understood.

### **2.4 Effect of ethanol on cellular processes**

Ethanol has been reported to affect a variety of cellular processes and inhibit the normal functioning of a cell. Gomez *et al.* showed a correlation between growth inhibition of microorganisms and the partition coefficient of different alcohols with increasing carbon numbers, suggesting that the site of attack of alkanols is the cell membrane.<sup>1</sup> By partitioning in the cell membrane (composed of hydrophobic lipids) alkanols disrupt the cell membrane resulting in inhibition of cell growth. In addition to

disruption of the membrane, ethanol could affect other activities across the cell membrane. In yeast, ethanol affects transportation of phosphate (Pi), D-glucose, D-glucosamine, L-lysine, and L-arginine; however transport differences do not fully explain the growth retardation.<sup>23</sup> In *E. coli* lactose transport and sugar transport in erythrocytes was reported to be affected by ethanol.<sup>24</sup> Ethanol was also reported to affect lipid synthesis in *Escherichia coli*<sup>25</sup> and *Bacillus subtilis*,<sup>26</sup> respiration and oxidative phosphorylation in rat liver mitochondria,<sup>27</sup> and the activity of cyclic 2'-3'-nucleotide-3'-phosphohydrolase<sup>28</sup> and Na<sup>+</sup>/K<sup>+</sup>-ATPase in rat cerebral cortex.<sup>29, 30</sup> In such a variety of ways exogenous ethanol can affect cellular processes and disturbs normal cell functioning, it is essential to determine the exact mechanism by which it inhibits the growth of *C. thermocellum*. Comprehension of growth inhibition of *C. thermocellum* by ethanol would help to develop new strains of this bacterium which grow in higher concentration of ethanol. Strains with improved ethanol tolerance could make commercial production of ethanol possible in the near future. For strain improvement, it is essential to understand how EA strains grow in higher concentrations of ethanol compared to WT stain. To understand the difference between the two strains a metabolite analysis of both strains of *C. thermocellum* was initiated. A comprehensive metabolite analysis can help unravel the metabolite fluxes and assist in understanding the physiology of a cell.

## 2.5 Metabolomics

According to the 'central dogma' of molecular biology, DNA is transcribed into mRNAs, which are then translated into proteins.<sup>31, 32</sup> Many proteins function as enzymes and are organized into very elegant and complicated signal transduction pathways. Enzymes participate in many reactions and are involved in primary or intermediary metabolism. As a result, enzymes could degrade and/or produce small molecules<sup>33</sup> such as glucose-6-phosphate, and ATP which are termed metabolites. In addition, a variety of other small molecules are utilized and synthesized by DNAs<sup>34, 35</sup> and RNAs.<sup>36, 37</sup> Any disease, genetic or environmental perturbations may result in up or down regulation of the mRNA and proteins/enzymes in a cell leading to alterations in concentrations of metabolites which enzymes catalyze.<sup>38</sup> Therefore by comparing the concentrations of metabolites from diseased or treated sample to the control sample, pathological or

physiological state of the cell could be obtained. A correct physiological state can predict the phenotype. A global qualitative and quantitative study of metabolites contained in the cell, tissue, organ, or biological fluids is termed as metabolomics.<sup>39, 40</sup> Any alteration in the concentrations of these small molecules in diseased/treated sample relative to control are suggestive of genetic or environmental influences.<sup>41</sup> Metabolite analysis is very crucial in understanding the alterations in a system.

After the success of human genome project, comparison of genome sequences has proven to be a potential research tool to identify complex disease and traits by identifying genetic variants.<sup>42</sup> However, comparison of genomes of two populations can't always predict the physiological state of a cell, since genomic sequence does not reveal whether a gene is being expressed or silenced. Therefore for predicting the physiological state and identifying a phenotype, downstream products of DNAs such as mRNAs<sup>43</sup> and proteins<sup>44</sup> are identified routinely and were able to predict changes in a biological system due to genetic and/or environmental perturbations on many occasions. However due to limited sensitivity, detection of low abundance mRNA is difficult and complexity of RNAs hinders prediction of mRNA levels changes.<sup>31</sup> In proteomics analysis, the level of a particular protein (many of those acts as an enzyme) may not tell us its activity since many proteins get activated only after post-translation modifications (PTMs)<sup>45</sup> such as phosphorylation.<sup>46</sup> PTMs could turn on or off the activity of an enzyme, suggesting that different levels of the enzymes could have similar activities and vice versa. Therefore, it is more important to find out the activity of a protein rather than actual quantity of the protein in two samples, however PTMs has limited success because of lack of availability of appropriate techniques.<sup>47</sup> Nonetheless, the activity of the enzymes has a direct effect on the level of metabolites. As a result, metabolite levels tend to mirror changes in enzyme activity and are ultimately indicative of physiological conditions. Thus, identification and quantification of all metabolites in a biological system can divulge changes in the system and uncover the phenotypes. Moreover, it is well known that any minor change in enzyme levels or activity could have dramatic effects on the levels of the metabolites which enhance the sensitivity of metabolomics study.<sup>48</sup> For all these reasons, metabolite analysis will give a better picture of a phenotype of an organism which is crucial in understanding changes to biological systems.

Although global quantification is desired for metabolomics, in reality it is not possible to identify and quantify all metabolites in a cell. Therefore several different approaches are typically used in metabolite analysis. The first approach is metabolite finger printing, which is a global screening of all the metabolites to obtain a metabolite pattern or fingerprint. A fingerprint is obtained for a sample in response to disease, environmental or genetic perturbation and is compared with a control. Only metabolites which differ from control are identified. A second approach is metabolite profiling in which identification and quantification of predefined metabolites is performed, since quantification of all the metabolites is not always necessary. Metabolites involved in a particular metabolic pathway, or metabolites having similar chemical characteristics are identified and quantified. The third approach is targeted metabolite analysis, which is very similar to metabolite profiling except that the number of metabolites chosen for identification and quantification is very few in number, for e.g. single metabolite (precursor or product) of a biochemical reaction. The fourth and last approach is metabolite foot-printing in which metabolites present in growth media are determined. This approach is useful in determining the uptake of nutrients and/or excretion of metabolites.<sup>49</sup> Due to the potential of metabolomics, it will be utilized by many fields, especially in biotechnology, where metabolomics can help understand the metabolite fluxes and such information can be used to develop superior strains of microorganisms for industrial productions of desired products.

### **2.5.1 Metabolomics in biotechnology**

Microbes have long been used for the industrial production of chemicals, vitamins, amino acids, enzymes and antibiotics.<sup>50</sup> In the early 19<sup>th</sup> century, the chemist Chaim Weizmann, for the first time used microbial culture of *Clostridium acetobutylicum* for the industrial production of acetone.<sup>51</sup> However, the modern era of biotechnology was started in 1980's when United States Supreme Court ruled that genetically-modified microorganisms could be patented.<sup>52</sup> Since then microbes have been modified by random strain mutagenesis and selection procedures for product enhancement or to increase product specificity. There have been many success stories using random strain mutagenesis for biotechnologically derived products such as amino acids (glutamic acid and lysine)<sup>53</sup> and antibiotics (penicillin).<sup>54</sup> However, in random strain mutagenesis and



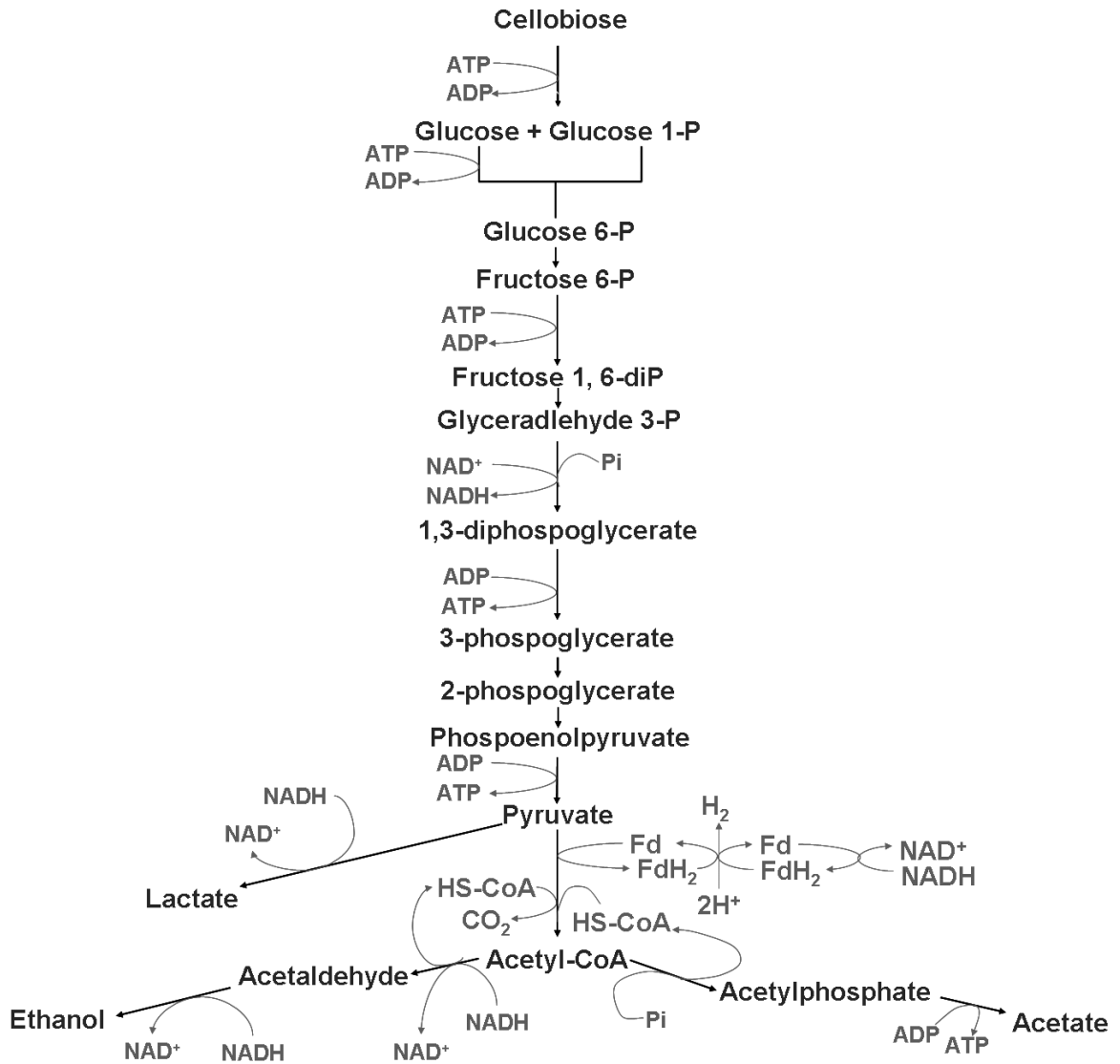
selection procedures, mutations are mostly uncharacterized; a very labor intensive process based on trial and error.<sup>50</sup> However, with advent of recombinant DNA technology it became possible to modify and altogether introduce a specific pathway(s) of interest such as production of insulin in *Escherichia coli* and *Saccharomyces cerevisiae* or to increase metabolic flux in a pathway for product enhancement.<sup>55</sup> Although most of the biotechnologically derived products are secondary products of metabolism and are not directly linked to the primary metabolism, their production rate and concentration is indirectly linked to primary metabolism. Therefore, for microbial engineering of superior strains, a firm understanding of primary cellular metabolism and its regulation is necessary. Such an understanding of basic metabolism could be derived from intracellular metabolite concentrations, which could be ultimately obtained from metabolite analysis.<sup>56</sup> In addition, using metabolic data bottlenecks in metabolic reaction networks can be identified.<sup>57</sup> Therefore, metabolomics is going to play a huge role in biotechnology where intracellular metabolite data will be used for strain improvement.

### **2.5.2 Advantages of metabolomics**

- a) The metabolome is relatively simple to study compared to the proteome and the genome.
- b) Individual molecules can be identified and quantified by GC/LC/CE-MS with the help of standards.
- c) No homology is needed like in protein or DNA sequencing.

### **2.6 Metabolite analysis of *C. thermocellum***

The goal of this research project is to quantify important metabolites involved in the central metabolic pathway of *C. thermocellum*. Figure 2-2 depicts the catabolism of cellulose by *Clostridium thermocellum*.



**Figure 2-2:** Catabolism of cellulose by *C. thermocellum*<sup>58</sup>

Nicotinamide adenine dinucleotide (NAD<sup>+</sup>), reduced nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate (NADP<sup>+</sup>), reduced nicotinamide adenine dinucleotide phosphate (NADPH), acetyl-CoA, adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), glucose-1-phosphate (G1P), glucose-6-phosphate (G6P), fructose-1, 6-diphosphate (FBP) and pyruvic acid (pyr) were chosen for analysis. Since this is an analysis of predefined metabolites, this approach is termed metabolite profiling. All selected metabolites for this study are involved in central metabolic pathway of *C. thermocellum* and have at least one phosphate group except pyruvic acid. Due to acidic properties of phosphate or carboxylate, all these metabolites are negatively charged at neutral pH. In addition to the acidic character of the metabolites, these metabolites are highly water soluble, since each metabolite contains at least 2 hetero atoms (N, O, or P) in their structures. Higher electronegativity of the hetero atoms compared to carbon renders these molecules highly polar and hence water soluble characteristics and analysis of highly polar and charged metabolites is challenging.

## **2.7 Sample preparation in microbial metabolite analysis**

Sample preparation in microbial metabolite analysis involves 2 main steps, quenching of cells followed by metabolite extraction.

### **2.7.1 Quenching**

Growth media may contain salts (buffers), yeast extract, and extracellular metabolites secreted by cells during growth, which may interfere with analysis. Therefore, it is often necessary to separate cells from the media in which they were grown using centrifugation. The whole process of centrifugation on an average takes a few minutes to complete. However, metabolism is very fast process and metabolite turn over rates are in seconds,<sup>59</sup> e.g. the turn over rate for ATP is 1.5 mM/second.<sup>60</sup> Therefore to get a snap shot of metabolite concentrations at a particular time point, it is necessary to instantaneously stop the metabolism or at least slow metabolic activity so that metabolite concentrations would not change over this time. This process of stopping or slowing metabolism is termed quenching. In the past, quenching has been achieved by immediately immersing cells in a cold methanol/buffer solution at or below -40°C.<sup>40, 60-65</sup>

The principle of quenching is that at low temperature the activity of enzymes will be minimal, resulting in instantaneous arrest of the metabolism. Cold methanol/buffer quench has been used on several occasions in the past<sup>55-60</sup> for metabolite analysis of *Escherichia coli*, *Saccharomyces cerevisiae* and *Aspergillus niger*. However, metabolite leakage is reported from microorganisms during the cold methanol/buffer quenching method.<sup>65</sup> Metabolite leakages are attributed to the cold shock phenomenon in which due to a sudden drop in the temperature of the cells, membrane can rupture and metabolites escape from the cell into the quenching solution. Wittmann *et al.* reported leakage of metabolites from *Corynebacterium glutamicum*<sup>65</sup> due to cold shock phenomenon of quenching. Similar observations were reported by Nielsen and co-workers during the quenching of *S. cerevisiae*<sup>66</sup> using cold methanol/buffer quenching solution. Such metabolite loss in quenching could underestimate intracellular metabolite concentrations. Losses due to leakage should be accounted to correct intracellular concentrations. Thus, quenching protocols should be carefully evaluated before applying to a new microorganism.

### **2.7.2 Extraction**

The washed cell pellet obtained from quenching is immediately transferred in an extraction solution for metabolites extraction. To be successful an extraction method should meet the following criteria.

- a) Metabolites should be stable and soluble in extraction solution
- b) The extraction method should extract all the possible metabolites
- a) The extraction solution should not interfere with analysis method

Although in theory it is required that an extraction method should extract all metabolites from the cells, metabolites are so diverse in nature that it is impossible for a single extraction method to extract all metabolites. Characteristics of metabolites vary from highly polar and water soluble such as sugar phosphates to highly non-polar and water insoluble such as lipids. Their molecular weight varies and is as low as pyruvic acid with MW of 88 to about 10 times higher, acetyl-coenzyme-A (MW 809). Some metabolites are acid unstable such as NADH, NADPH and some are base unstable such as NAD<sup>+</sup>, NADP<sup>+</sup>.<sup>67</sup> Therefore, a particular extraction method could only be suitable to extract a certain class of metabolites. Also, an extraction method might not be compatible with all

the methods of analysis. Extraction methods such as acid extraction cannot be used concurrently with capillary electrophoresis, since on neutralization of acid with base NaCl is formed which is detrimental to CE separation. Therefore, it is essential to carefully select an extraction method so that it wouldn't interfere in analysis and can extract the desired class of compounds. Several methods have been developed in the past for extraction of metabolites which are described as follows.

#### **A) Acid/base extraction**

In acid extraction, concentrated HCl<sup>68, 69</sup> or perchloric acid<sup>70, 71</sup> is directly added to cells, and cells are incubated at RT for 10 to 20 minutes followed by neutralization of acid with base such as NaOH or KOH. This extraction is only suitable for acid stable metabolites. Many metabolites are unstable at low pH (such as NADH, NADPH) and will be destroyed during the extraction. In base extraction, a strong base such as KOH is directly added to cells and incubated at RT for 10 to 20 min.<sup>67, 72</sup> The base is neutralized with acid such as HCl. Metabolites such as NAD<sup>+</sup> and NADP<sup>+</sup> are unstable in base extraction and are destroyed.

#### **B) Boiling ethanol extraction**

This protocol was originally developed by *Gonzales et al.* in which cells are added to a solution of ethanol/70 mM HEPES (75:25) and boiled for 3 minutes<sup>61</sup> and has been used on several occasions.<sup>73, 74</sup> The solution is subsequently cooled and concentrated for analysis.

#### **C) Chloroform/methanol extraction**

Cells are added to room temperature chloroform/methanol/water (50:25:25) solution, mixed well, and incubated for 10 minutes on ice. An aqueous layer is allowed to separate from the chloroform layer, the layer of interest is concentrated to increase the sensitivity, and metabolites are analyzed by the desired analysis method. The aqueous layer (methanol/water) contains all the polar and water soluble metabolites. The bottom/chloroform layer contains hydrophobic metabolites such as lipids.<sup>75</sup> Finally, extracted metabolites are analyzed using an appropriate method of choice.

### **2.8 Analytical platforms for metabolite analysis**

For small molecules FT-IR, NMR, GC-MS, LC-MS are common methods of analysis. Each method has its advantages and disadvantages. FT-IR is a relatively cheap

method of analysis of small molecules; however it suffers from low sensitivity and interferences. NMR is a nondestructive, noninvasive technique of analysis; however it suffers from interferences and low sensitivity as well. Recently, mass spectrometry (MS) based techniques are evolving as the analytical platforms for analysis of metabolites because of the high sensitivity of mass spectrometer. The number of applications of MS in metabolite analysis has expanded dramatically over last few years and today MS has evolved as one of the most important detectors in biotechnology. Especially with development of electrospray ionization (ESI) techniques, MS can be coupled to separation techniques such as liquid chromatography (LC), and online detection is possible. MS is a highly sensitive, versatile detector and provides structural information. Reversed phase liquid chromatography-mass spectrometry (RPLC-MS)<sup>76, 77</sup> and gas chromatography-mass spectrometry (GC-MS)<sup>78, 79</sup> are the most common techniques in analysis of small molecules. Hundreds of compounds can be resolved in the GC and LC and detected by MS, making these analytical techniques preferred methods for metabolite analyses. However RPLC and GC fail to separate acidic and highly polar metabolites. In RPLC, separation takes place on the basis of the hydrophobic interaction between analyte and stationary phase (C18); polar and charged metabolites are barely retained on the stationary phase therefore RPLC doesn't provide any separation of acidic and polar metabolites. In GC-MS separation is limited to only volatile compounds; therefore the poor volatility of polar and charged metabolites hinders their separation by GC. To increase the volatility of these metabolites derivatization of these metabolites prior to their injection on GC column is necessary. By using proper derivatizing agents volatility of polar and charged metabolites can be increased.<sup>80</sup> However, derivatization adds an additional step and the efficiency of the derivatization may vary for each analyte making the data less reliable. Derivatization processes are labor intensive and time consuming making GC-MS less favorable technique for analysis of polar and charged metabolites. Anion exchange liquid chromatography (AELC) is another suitable separation technique for charged metabolites, however to displace these charged analytes in AELC, high concentrations of salts must be used. Ensuing high salt concentration could result in suppression of ion intensity and increased LOD, when a mass spectrometer is used as a detector. Moreover, volatile mobile phase is generally not available to use in AELC-

MS.<sup>81</sup> Therefore, AELC must be ruled out for analysis of charged metabolites when MS is used as a detector.

For the separation of polar and charged metabolites, capillary electrophoresis (CE) has evolved as a suitable technique.<sup>82-85</sup> CE separates analytes on the basis of their charge and size, making it is the best technique available for separation of charged analytes. UV detector is a common form of detector used with CE; however with advent of electrospray ionization (ESI) it is possible to couple CE to MS online. High separation efficiency of CE combined with extreme detection sensitivity of MS suggests CE-ESI-MS is an ideal tool for the analysis of metabolites, especially charged metabolites.

## **2.9 Capillary Electrophoresis (CE)**

### **2.9.1 Setup of CE**

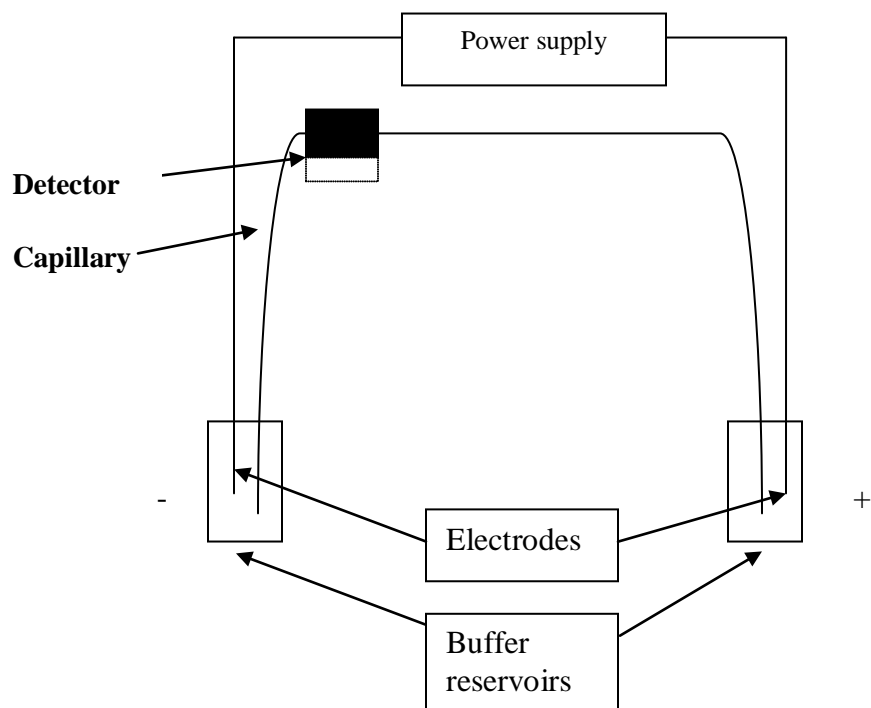
In capillary electrophoresis, two ends of a capillary [50-100 micron internal diameter (ID)] are placed in two separate buffer reservoirs. Each buffer reservoir has an electrode through which electrode potential is applied. Figure 2-3 shows the simplest setup of CE. Before the capillary is inserted in to the reservoirs, the outer layer of polyamide coating (about 25 cm from cathode end of capillary) is burned off (10-15 mm in length), which becomes the detection window for the UV detector at the cathodic end of the capillary.

### **2.9.2 Separation efficiency of CE**

CE is the simplest mode of capillary electrophoresis. The high resolution of CE is governed by the Van Deemter equation, which correlates plate height, H, to the velocity,  $v$ , of carrier liquid/gas.

$$H = A + (B/v) + Cv \quad \text{Equation 2-1}$$

In the equation above, term A represents eddy diffusion; B represents longitudinal diffusion; C represents mass transfer term of the analyte between mobile and stationary phase. Lower value of H results in a higher separation efficiency. This implies that more theoretical plates can be packed in given length along the axis of separation. Since, CE separation is carried out in a single phase of uniformly flowing liquid, consequently, there is no eddy diffusion so term A is absent.



**Figure 2-3:** Capillary electrophoresis instrument setup



Additionally, there is no mass transfer since there is no stationary phase so term C is absent. Hence, the only source of band broadening in CE is longitudinal diffusion of the analytes. Thus, in CE typically 50,000 to 500,000 theoretical plates can be obtained under ideal conditions.<sup>86</sup>

### 2.9.3 Principle of electrophoresis

In electrophoresis, ions migrate and separate under the influence of an applied field. Cations migrate towards the cathode and anions migrate towards the anode, and usually a UV detector is used for detection of analytes.

The force ( $F_E$ ), which is proportional to effective charge,  $q$ , and electric field strength,  $E$ , imparted by an electric field can be given as

$$F_E = qE \quad \text{Equation 2-2}$$

Frictional force ( $F_f$ ) which opposes the translational movement of the ion is proportional to electrophoretic velocity ( $v_{ep}$ ) of the ion and the frictional coefficient,  $f$ , which can be given as,

$$F_f = f v_{ep} \quad \text{Equation 2-3}$$

When the accelerating force is equal to frictional force the ion reaches the steady state velocity, therefore

$$F_E = F_f$$

$$qE = f v_{ep} \quad \text{from equations 2-2 and 2-3}$$

$$v_{ep} = (q/f) E$$

$$v_{ep} = \mu_{ep} E$$

where,  $\mu_{ep}$  is electrophoretic mobility of the ion, which is the proportionality constant between the velocity of the ion and the electric field strength. The electrophoretic mobility of the ion is directly proportional to the charge of the ion and inversely proportional to the frictional coefficient, which can be given as,

$$f = 6\pi\eta r \quad \text{Equation 2-4}$$

where,  $\eta$  is the viscosity of the surrounding medium and  $r$  is the radius of the ion. Since  $v_{ep} = (q/f)$ , as the hydrodynamic radius of the ion increases the electrophoretic velocity decreases. In addition to electrophoretic velocity of ions, electroosmotic flow exists in untreated fused silica capillary.

#### 2.9.4 Principle of electroosmosis in CE

A fused silica capillary is made from silicon oxide, which on hydrolysis forms silanol (Si-OH) groups. Therefore, the surface of the silica fused capillary is covered with silanol groups. The silanol groups are ionized inside the capillary and form a negative layer of Si-O<sup>-</sup> groups at pH > 3. When a buffer solution is passed through this capillary these negative ions are counterbalanced by the positive ions or protons from the buffer solution and form an electric double layer. As shown in Figure 2-4, the layer which is very close to the capillary wall is called as “stern layer” and the layer next to it is called as the “diffuse layer”. Protons from the diffuse layer travel toward the cathode, along with their movement they carry water molecules, resulting in electroosmotic flow. Since the electroosmotic flow and electrophoresis are complementary to each other, the equations will be same for both of the phenomenon.

$$v_{eo} = \mu_{eo} E \quad \text{Equation 2-5}$$

$\mu_{eo}$  = electroosmotic mobility and a constant of proportionality between electroosmotic velocity ( $v_{eo}$ ) and electric field strength ( $E$ )

Electroosmotic mobility ( $\mu_{eo}$ ) is proportional to the dielectric constant,  $\epsilon$ , of the medium, the zeta potential,  $\zeta$ , at the capillary-buffer interface and is inversely proportional to viscosity,  $\eta$ , of the medium.

$$\mu_{eo} = \epsilon \zeta / 4\pi \eta \quad \text{Equation 2-6}$$

#### 2.9.5 Apparent mobility of an ion

The apparent mobility,  $\mu_{app}$ , is the vector sum of electrophoretic mobility,  $\mu_{ep}$ , of the ion plus the electroosmotic mobility,  $\mu_{eo}$ , of the solution

$$\mu_{app} = \mu_{eo} + \mu_{ep} \quad \text{Equation 2-7}$$

The apparent velocity is directly proportional to apparent mobility,  $\mu_{app}$ , and electric field strength,  $E$ , across the capillary

$$v_{app} = \mu_{app} E \quad \text{Equation 2-8}$$

### 2.9.6 Principle of separation in CE

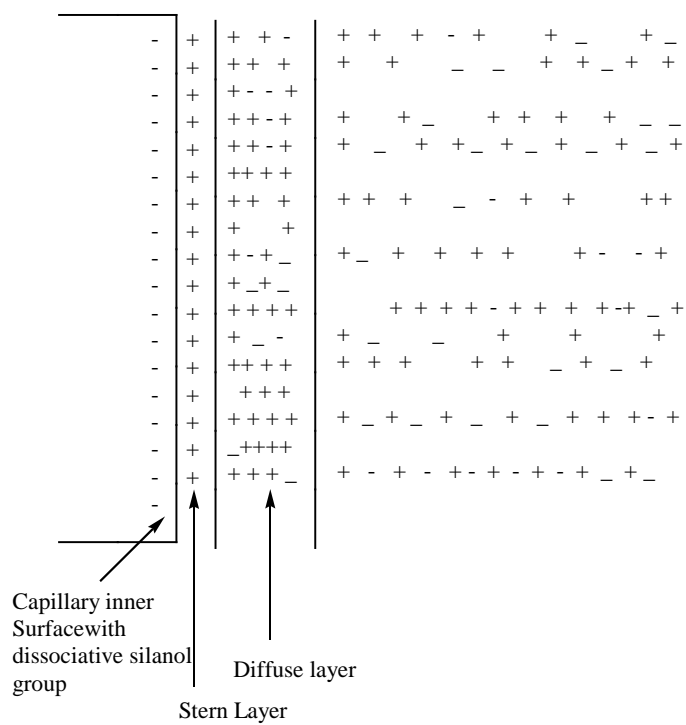
In CE, neutral molecules travel toward the cathode along with the electroosmotic flow and are not subject to separation. However, charged molecules (depending on the charge) can be separated. Cations will move in the same direction as the electroosmotic flow therefore,  $\mu_{ep}$  and  $\mu_{eo}$  will have the same sign and hence the  $\mu_{app} > \mu_{ep}$ . On the other hand anions will flow in the opposite direction of the electroosmotic flow, therefore,  $\mu_{ep}$  and  $\mu_{eo}$  will have opposite signs, and  $\mu_{app} < \mu_{ep}$ . Consequently, cations reach the detector first, followed by neutral species, and finally anions. Analytes with similar charge value and sign will be separated only on the basis of difference in size.

### 2.9.7 Advantages of CE

- a. High efficiency i.e. hundreds of components can be separated simultaneously
- b. Requires few microliters of sample and consumes a limited amount of reagents
- c. Can be used quantitatively

### 2.9.8 Sample injection

By applying high pressure at one end of the capillary, the sample can be injected hydrodynamically. Generally 100 mbar pressure is applied for 6 -12 seconds. The amount of sample injected is on order of nanoliters to picoliters. To determine the amount of sample injected on CE capillary, the length of the sample plug injected could be calculated by using Poiseuille's law<sup>87</sup> length of the plug could be converted to the sample volume.



**Figure 2-4:** Representation of electrical double layer inside the capillary

$$P.t. = \text{PLUG} \cdot 3200 \cdot \eta \cdot L/d^2 \quad \text{Equation 2-9}$$

where,

P = pressure difference in mbar

t = time in seconds

PLUG = Plug length in mm

$\eta$  = is viscosity of buffer in cP

d =  $\mu\text{m}$ , and L = length in cm

### 2.9.9 Joule Heating

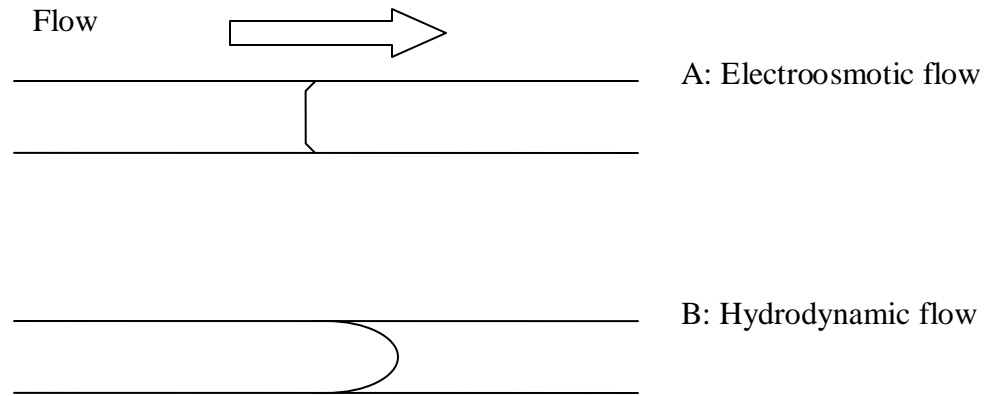
Joule heating is a consequence of the resistance of the solution to the flow current. The heat produced, H, is equal to the product of applied voltage between the electrodes (V); current (I); and time (t) for which current passed

$$H = VIt \quad \text{Equation 2-10}$$

The heat produced must be dissipated otherwise it reduces the efficiency of the CE. For this reason modern CE instruments have an integral cooling fan, which help to dissipate the heat produced. As the surface to area ratio is high in the capillary, heat is easily dissipated compared to conventional gel electrophoresis.

### 2.9.10 Flow in CE

Electroosmotic flow is distributed across the entire length of the system resulting in a negligible pressure gradient, except very close to the wall and very high resolution (Figure 2-5 A). On the other hand in liquid chromatography (LC) frictional forces are magnified at the interface of the wall of the capillary and mobile phase, resulting in a parabolic flow profile within the capillary (Figure 2-5B). Due to plug like flow in CE, efficiency of CE is much higher compared to LC.



**Figure 2-5** Electroosmotic flow (A) versus hydrodynamic flow (B)

## 2.10 Detectors in CE

Various detectors can be coupled to CE for detection of analytes separated and UV is the most common form of detector used. The UV detector in combination with CE has many advantages. It is very robust, relatively cheap, and easy to couple with CE. However, detection by UV is limited, since not all the analytes absorb the UV light. Analytes such as sugar phosphates do not absorb UV light, therefore the detector fails to detect compounds without a chromophore. Thus, for detection of such analytes, a mass spectrometer is an ideal detector. The greatest advantage of MS is its higher sensitivity compared to other detectors. MS is more sensitive than UV and can detect analytes such as sugar phosphates which can otherwise not be detected by UV detector.

In order to couple CE online to MS it is essential to transform the analyte ions in solution or liquid phase to the gaseous phase so that they could be injected in to the mass spectrometer. Transformation of the liquid/solution phase to the gaseous phase ion can be achieved by electrospray ionization (ESI) and it can be used to interface CE to the MS. However to set up CE-ESI-MS, first it is essential to understand CE separation, interface setup, ESI technique and operation of mass spectrometer independently.

## 2.11 Electrospray ionization (ESI)

In the 18<sup>th</sup> century Abbe Nollet illustrated the eletrospray phenomenon in human blood for the first time. He studied the electrostatic properties of liquids and observed that when a person is electrocuted with high voltage, the individual doesn't bleed but blood would spray from a cut given to the skin.<sup>88</sup> ESI has been widely used in several applications for a long time, such as in paint spraying; aerosol production for inhalant drug administration; spacecraft thrusters; and plasma desorption. The first detailed description of ESI was reported in 1917 by *Zeleni*.<sup>88</sup> ESI is the process by which liquid is pushed through a very small pressurized inner diameter (ID) metal capillary. In addition, an electrical potential is applied to the capillary, which causes the liquid to come out of the capillary in a thread like form, which further get disintegrated into much smaller particles and finally forms a fine mist of gaseous ions.

Although ESI is documented in literature a long time ago, it was further developed by John Bennet Fenn<sup>89-91</sup> for which he was rewarded with Nobel Prize in Chemistry in 2002.<sup>92</sup> ESI is referred to as a soft ionization technique since ions usually do not get fragmented in the process of ionization.

There are three major steps involved in the formation of gaseous ions in ESI (a) formation of a charged droplet at the tip of a capillary due to the electric potential applied to it (b) evaporation of solvent and/or disintegration of droplets causes the droplets to become much smaller and (c) formation of gas phase ions. A liquid containing analyte ions is pushed through a metal capillary and electric potential is applied to the capillary, due to electrostatic attraction the analyte ions along with solvent is pulled toward the opposite electrode and liquid expands to a shape of a cone (referred as Taylor cone) and the tip of the cone being the most stable point. As the liquid at the tip gets dragged to the counter electrode, it forms a shape of a filament and eventually breaks into individual charged droplets.<sup>93</sup> These charged droplets become smaller as solvent in droplet starts evaporating due to presence of a sheath gas. Sheath gas is a continuous stream of a gas passed coaxially to the ESI capillary and helps to nebulize the liquid forming a spray. Evaporation of the solvent molecules from the droplets causes these droplets to become much smaller and increases its charge density. Rising charge density increases columbic repulsion within the analyte ions in the droplet and when columbic repulsion surpasses surface tension, droplet explodes into much smaller drops and finally creates individual charged ions. Transformation of the droplets into the gaseous phase can be explained by either the ion evaporation model (IEM) or the charge residue model (CRM). According to IEM, ions are evaporated individually from the smaller charged droplets, since charge to surface ratio is very high.<sup>94</sup> However in CRM theory, when the charge to surface ratio of the droplet is very high, columbic repulsion surpasses the surface tension of the droplet to cause an explosion, forming individual gaseous ions.<sup>95</sup> However the validity of both theories cannot be explained with absolute certainty and both theories are still the topic of debate and open for discussion.<sup>96,97</sup> Once these ions are formed, they are guided through octapoles to the mass analyzer.



## 2.12 Interfacing CE to MS

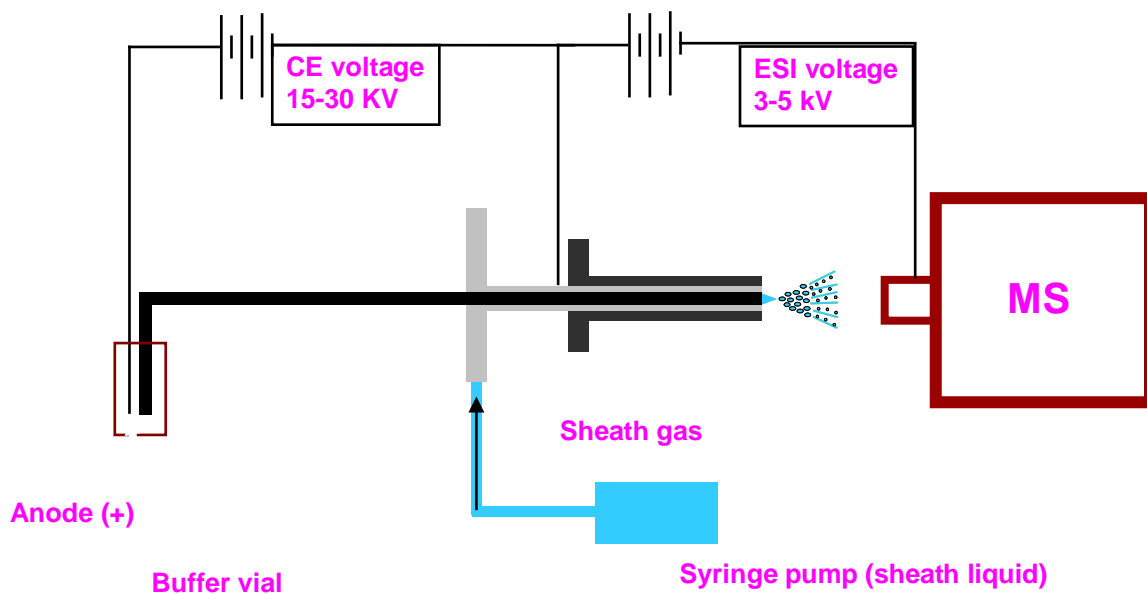
CE can be interfaced to MS by using an ESI interface. One end of the CE capillary is inserted in to the interface, where the ESI process will convert liquid coming from the CE capillary into its gaseous form and subsequently injects in to the mass spectrometer. However for a successful CE-ESI operation, the following factors must be considered

- 1) Electrical contact must be provided to the end of capillary which is inserted in ESI interface, which is difficult since outlet vial is not present toward this end
- 2) The low flow rate (50-100 nL/min) from the CE must be compensated with additional liquid to establish a stable ESI operation. For the stable ESI, few thousands of nL/min liquid flow is necessary. Therefore supplementary liquid must be added coaxially for the stable ESI operation.

The first issue could be resolved in a couple of ways. Voltage could be applied to the CE buffer via a supportive liquid (sheath liquid) which is termed sheath liquid interface. In a sheath liquid interface, the addition of a sheath liquid coaxially to the CE capillary provides good electrical contact and stability to the ESI which takes care of the second issue as well. The addition of a sheath liquid of few microliters/min is common in sheath liquid types of interfaces.<sup>98</sup> The stability of ESI in a sheath liquid interface is pronounced, making this combination more robust and reproducible and as a result, it is the most commonly found CE-MS interface.<sup>99-101</sup>

## 2.13 Polarity of CE-MS

For the analysis of anions, CE-MS is usually performed in the negative polarity mode in which the CE inlet vial is the cathode and outlet (MS end) is the anode.<sup>81</sup> In an untreated fused silica capillary, electroosmotic flow (EOF) always moves towards the cathode, which is the CE inlet vial in this polarity mode. For analysis of acidic analytes in CE-MS, it is essential to deprotonate them and form negative ions, which is subject to separation with high pH buffers. The high pH generates a strong EOF, which is traveling away from the MS. Since there is no vial at the MS end, EOF in a direction away from the MS would result in formation of a gap at the CE capillary exit. Any discontinuity in CE capillary buffer would cause a current drop and subsequent failure of CE-MS operation.



**Figure 2-6:** Sheath liquid interface and instrumentation of CE-ESI-MS

To overcome this problem, Soga *et al.* suggested the use of EOF reversal by using cationic coated capillaries which are available commercially named SMILE (+) capillary.<sup>81</sup> By reversing EOF towards the direction of the anode, they were able to prevent the current drop and went on to analyze 27 anionic intermediates from *Bacillus subtilis*. Nevertheless, the SMILE (+) capillary is not only expensive and has a short life span, it also fails to detect compounds such as acetyl-CoA, since such compounds are typically adsorbed on the capillary inner wall due to their hydrophobic and multivalent nature.<sup>102</sup> To overcome the problem of analyzing negative ions using an untreated fused silica capillary, one must perform CE-MS in positive polarity mode (inlet vial is the anode and MS or outlet end is the cathode). In this mode, the EOF could be consistently directed toward the MS end (cathode) using a buffer of high pH.<sup>103</sup> Followed by setting up a successful CE-ESI-MS setup it is essential to evaluate CE and ESI parameters for improved separation and higher sensitivity. Ionic strength and pH of the buffer affect the separation; however sheath liquid composition, sheath liquid flow rate, and sheath gas flow rate affect the sensitivity in CE-ESI-MS.

## **2.14 Parameters influencing CE-ESI-MS**

Although the sheath liquid interface is typically robust and reproducible, there are several parameters which are important to take in consideration in order for a CE-ESI-MS run to be successful and reproducible. Several parameters (for CE, ESI interface and MS) should be optimized to make CE-ESI-MS a sensitive technique.

### **2.14.1 Position of CE relative to MS**

It is absolutely essential that the capillary inlet (in CE buffer vial) should be at the same level as the capillary outlet (at MS entrance) to avoid any siphoning effect.<sup>104</sup> Any discrepancies in the level of these two ends would cause a siphoning movement of liquid in a particular direction, which could be detrimental for the success of the CE-ESI-MS run. A higher positioning of the inlet vial would cause the CE buffer to run toward the outlet vial, which would speed up the analysis time despite a decreased resolution/separation. A lower position of inlet vial would cause problems with the hydrodynamic injection of the sample.

#### **2.14.2 Position of ESI needle from the MS entrance**

Position of the ESI emitter from the MS entrance is important for sensitivity. Keeping the ESI emitter far away from the MS entrance would cause many ions to deviate from the path to the detector resulting in decreased sensitivity. However, the application of high voltage at the ESI emitter and keeping ESI emitter too close the MS would cause arcing.<sup>88</sup>

#### **2.14.3 Position of CE capillary inside the ESI emitter**

The position of the CE capillary inside the ESI emitter should be adjusted and maintained since at the junction of CE capillary and ESI emitter, sheath liquid and buffer coming from the CE capillary are constantly intermixing and are perpetually in flux.<sup>98</sup>

#### **2.14.4 CE separation conditions**

CE conditions such as ionic strength of buffer, pH, viscosity, length of the capillary, CE voltage, conditioning of the capillary and internal diameter (ID) of the capillary has an effect on separation and sensitivity. Improper conditioning of the CE capillary would cause variation in the EOF, which would result in fluctuation in ESI and a loss of reproducibility.

#### **2.14.5 Sheath gas conditions**

To assist the ESI, a neutral gas (such as Nitrogen) is passed through tubing coaxial to the CE capillary and sheath liquid and termed sheath gas. Sheath gas helps to nebulize the sheath liquid. It also sweeps any liquid in the ESI nozzle and keeps the interface as dry as possible.<sup>88</sup> A sheath gas flowing at an insufficient rate would cause the sheath liquid to flood the interface. Accumulation of sheath liquid inside interface may cause an electrical short circuit and could interrupt the ESI operation. A sheath gas coming out with a high flow rate would cause suction at the ESI tip, which would pull the buffer from CE capillary, negatively affecting CE separation.<sup>99</sup>

#### **2.14.6 Sheath liquid conditions**

Sheath liquid is usually a mixture of organic solvent and water, which is pumped hydrodynamically in to the ESI interface coaxially to the CE capillary. At the tip of ESI needle, sheath liquid and buffer unite and together get ionized by the ESI. Sheath liquid

provides electrical contact to the capillary end toward the MS and it also provides stability to the ESI. The purpose of adding organic solvents (methanol and acetonitrile) is to decrease the surface tension of the solution. Decreased surface tension gives rise to faster solvent evaporation, and higher sensitivity.<sup>99</sup> The rule of thumb is to add 50-75% of organic solvent. Addition of too much organic solvent would cause the sheath liquid to lose conductivity, rendering it unable to provide electrical contact to the capillary end at the MS. A sheath liquid also contains a small fraction of acid or base which helps in ionization of analyte ions. In the positive ion mode, a weak acid such as acetic acid is added to the sheath liquid which helps to protonate the basic analytes and thus enhances detection sensitivity.<sup>105</sup> Conversely, in negative ion mode, bases such as ammonia are added to deprotonate the analytes. Once the gaseous ions are formed, they are guided to mass spectrometer for analysis.

## **2.15 Mass spectrometer**

The mass spectrometer is an analytical instrument, which measures the mass to charge ratio of an analyte ion. The mass spectrometer contains an ion source, mass analyzer, and detector. The ion must be charged and in gaseous form to be detected by a mass spectrometer. Different techniques for the ionization of analyte ions are used such as electron impact (EI), electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and matrix assisted laser desorption ionization (MALDI). Once gaseous charged ions are formed, they are guided to the mass analyzer through ion gates. The mass analyzer can only detect mass to charge ratio of an ion. There are several types of mass analyzers such as sector, triple quadrupole, quadrupole ion trap, Fourier transform ion cyclotron resonance (FT-ICR), time of flight, linear ion trap and orbitrap. All of these work on the same principle of detection of mass to charge ratio, however each has its weakness and strengths. However, the ion trap mass spectrometer is versatile, robust, easy to use and relatively non-expensive mass analyzer compared to other mass analyzers.

### **2.15.1 Quadrupole ion trap (QIT) mass spectrometer**

The concept of QIT was originally developed by Wolfgang Paul and co-workers in 1950 at Bonn, Germany and used to determine the mass /charge ratio of ions.<sup>106</sup> In

1989, his pioneering work in QIT was awarded the Nobel Prize in Physics. QIT is about the size of a tennis ball and consists of a ring and two end cap electrodes with hyperbolic surfaces as shown in Figure 2-7. Initially, QIT was only able to isolate single value  $m/z$  because it was operated in “mass selective stability” mode. In “mass selective stability” mode, Radio Frequency (RF) and Direct Potential (DC) potential applied to the ring electrode is ramped which increases stability of a single value  $m/z$  ion resulting in its storage. Stored ions were detected by resonance absorption using an external power source<sup>107</sup> or they were ejected by applying DC potential to the end cap electrode and subsequently detected by electron multiplier tube.<sup>108</sup> Because of “mass selective stability” mode, QIT was very limited in its applications and remained confined mostly to the physics community for a long time. Hans Dehmelt, a physicist at the University of Washington, extensively studied properties of ions by isolating them using QIT.<sup>109</sup>

After this point, there was a continuous development in the operation of QIT; however the real breakthrough came when 2 major improvements were made to the operation of QIT by George Stafford and co-workers at Finnegan MAT in 1983. The improvement resulted in the sequential ejection of ions through the trap, called “mass selective instability” mode.<sup>110</sup> In this mode all ions created are trapped first and sequentially ejected out of the trap. The advantage of this method is that all the ions created can be stored. This allows mass analysis to be performed by sequentially ejecting one ion at a time, while in previous method only one  $m/z$  could be stored and analyzed at a time. The second improvement they made was an increase in resolution. Resolution was enhanced by introduction of helium gas within the trapping volume.<sup>111</sup> Ions collide with helium gas and slow down because of reduction in their kinetic energy and get trapped to form the ion packets. These ions then can be ejected out of the trap as packet compared to diffused ions, improving the resolution. These two major improvements made commercialization of the ion trap possible, successfully introducing the commercial ion trap mass spectrometer instrument in 1984. Followed by this, several areas of operation of QIT were developed such as external injection of ions,<sup>111</sup> extension of mass range,<sup>112</sup> tandem mass spectrometry operation,<sup>113</sup> and improvement in resolution<sup>114</sup> which resulted in increased application of mass spectrometry in variety of fields.



**Figure 2-7:** Ion trap mass spectrometer

### 2.15.2 Mathematical equations for QIT

As mentioned above, QIT consists of a ring electrode and two hyperbolic shaped end cap electrodes. A radio frequency (RF) or oscillating potential is applied to the ring electrode, which is termed the fundamental RF. The applied oscillating potential and parabolic geometry generates a saddle-like field and traps the ions. The strength of this electric field increases as the ion trajectory deviates from the center of the trap, forcing them to the center of the trap. The potential experienced by any ion inside the trap can be given by the equation below

$$\Phi(r, z) = \frac{(U - V \cos \omega t)}{2} \left[ \frac{r^2 - 2z^2}{r_0^2} \right] + \frac{(U - V \cos \omega t)}{2} \quad \text{Equation 2-11}^{115}$$

where,

$U$  = amplitude of dc potential applied to endcap electrodes

$V$  = amplitude of fundamental RF applied to ring electrode

$\omega$  = angular frequency of RF potential

$r_0$  = shortest distance between center of the trap and ring electrode

$z_0$  = shortest distance between center of the trap and endcap electrode

In a perfect quadrupole field,  $r_0 = \sqrt{2z_0}$ . However, sometimes in commercial quadrupole instruments geometry of the trap is stretched.

The force experienced by an ion due to the electric field is given by equation

$$\vec{F}(r, z) = \vec{E}(r, z) = -e\vec{\nabla}\Phi(r, z) = m\vec{a}(r, z) \quad \text{Equation 2-12}^{116}$$

According to Newton's law the force experienced by an ion is proportional to acceleration of an ion of charge  $e$  experienced due to this force. On substituting Equation 2-10 in the form of *Mathieu* equation in radial and axial direction, the following equations are generated,

$$a_z = \frac{-8eU}{mr_0^2 \omega^2}, \quad q_z = \frac{4eV}{mr_0^2 \omega^2} \quad \text{Equation 2-13}$$

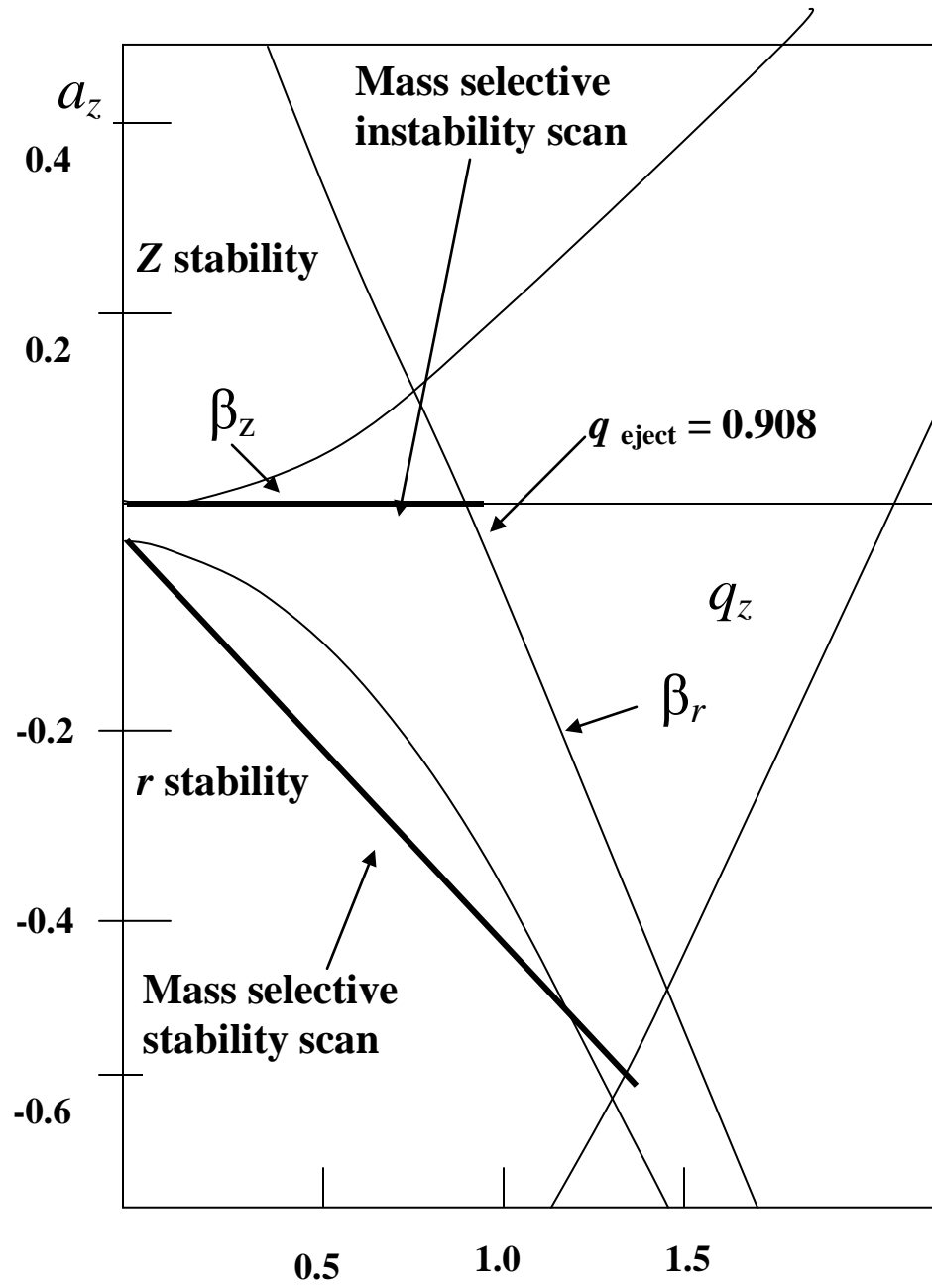


$$a_r = \frac{-a_z}{2}, \quad q_r = \frac{q_z}{2}, \quad \xi = \frac{\omega t}{2} \quad \text{Equation 2-14}^{117}$$

Ion trajectories of ions are determined by solutions to the Mathieu equations and regions of stability are decided by parameters  $a_z$  and  $q_z$ . Therefore the stability of ions depends on the mass of ion, its charge, size of the ion trap, RF frequency applied to ring electrode (fundamental RF or  $\omega$ ), amplitude of dc potential ( $U$ ), and amplitude of RF voltage ( $V$ ).

### 2.15.3 Operation of QIT

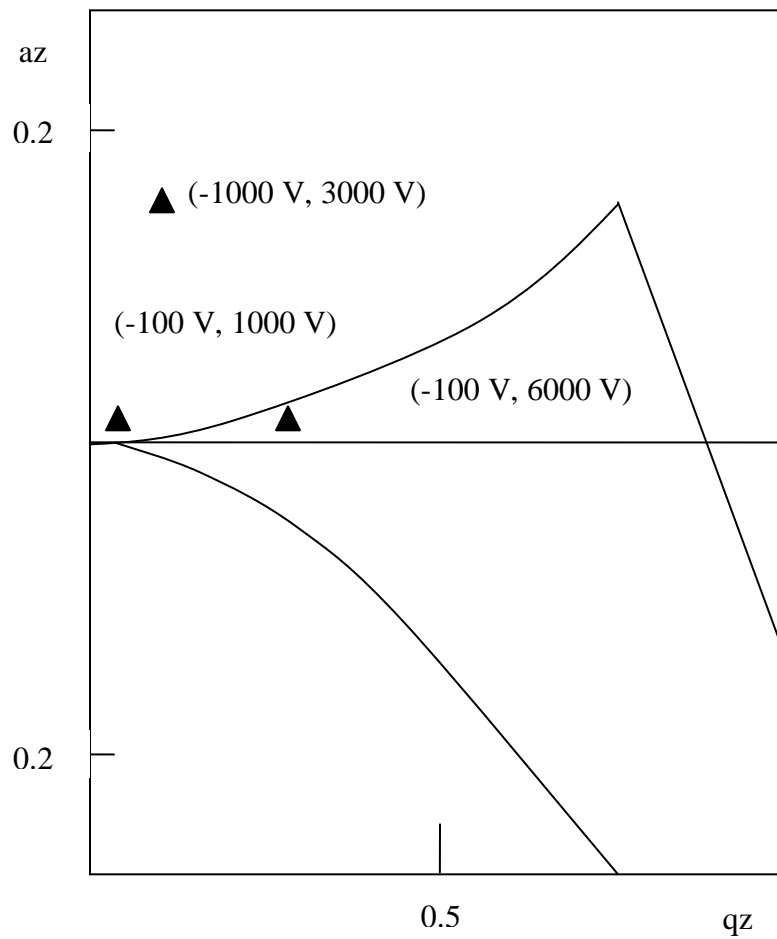
Once ions are generated by the API, they enter into ion optics after passing the skimmer. Ion optics consist of 2 octapoles and an interoctapole lens. Each octapole is made up of several cylindrical rods forming octapolar geometry. RF and DC voltages are applied to the octapole and act as an ion transmission device which transfers the ions to the mass analyzer. An RF voltage ( $\sim 2.45$  MHz, 400 V peak to peak) and a dc offset voltage (-10 V for positive ions and 10 V for negative ions) is applied to rods, which generates an electric field that regulates the velocity of the ions moving along the axis of the octapole. The two octapoles are separated by an interoctapole lens, which acts as a switch or gate for ions between the first octapole region and the analyzer region of the vacuum manifold. To transmit ions, typically -20 V or 20 V is applied to the interoctapole lenses for positive and negative ions, respectively. Once ions enter the mass analyzer, -300 V and 300 V is applied for negative and positive ions respectively to close the gate. Once this occurs mass analysis can be performed. During analysis, octapole voltages are turned off. Once ions enter the ion trap mass analyzer, they are stored and subsequently ejected out of the trap for detection. However, the stability of a particular ion in an ion trap depends on the size of ion, the charge of ion, fundamental RF ( $\omega$ ), amplitude of dc ( $U$ ), and RF ( $V$ ). Stability of an ion in ion trap can be explained by a stability diagram, emphasizing the region of axial and radial stability overlap.



**Figure 2-8:** Regions of stability in stability diagram

<b>dc (U)</b>	<b>RF (V)</b>	<b>a<sub>z</sub></b>	<b>q<sub>z</sub></b>
-100 V	1000 V	0.0108	0.0539
-1000 V	3000 V	0.108	0.162
-100 V	6000 V	0.0108	0.323

**Table 2-1: a<sub>z</sub> and q<sub>z</sub> values of a m/z = 1500 ions at varying RF and DC potentials**



**Figure 2-9:** Stability of ions at different RF and dc potential

An ion will be stable and trapped in those areas of diagram (Figure 2-8). If an ion of a particular  $m/z$  falls anywhere within the stability region, it will be securely trapped into the ion trap. Values of  $a_z$  and  $q_z$  can be changed by changing the amplitude of the RF and dc voltages, resulting in a change in the position of ions to the stability region. By using Equation 2-11, it is possible to calculate  $a_z$ ,  $q_z$ , and the DC potential at a given RF and to determine if the ion would likely to fall within the stability region. For example, Table 2-1 shows different RF and DC potentials applied to ion of mass 1500 and charge +1 and corresponding  $a_z$  and  $q_z$  Values. By marking the values of points ( $a_z$  and  $q_z$ ) on an  $a_z$  vs.  $q_z$  plot, it could be easily determined if the ion would fall within the stability region. After an ion is trapped successfully by selecting appropriate RF and DC voltages, it could be ejected out of the trap by ramping the amplitude of the RF potential. As the amplitude of the RF potential is ramped, the  $q_z$  value of ion increases. When it increases beyond 0.908, ions become unstable and are ejected out of the trap. At increasing amplitudes of RF, ion trajectories become unstable in the axial direction. However, ion trajectories remain stable in the radial direction during this process. Once, the ions are ejected out of the trap, they are detected by the electron multiplier tube. To maximize the effective mass range of analysis, the mass spectrometer is operated at  $a_z = 0$ , since at zero value of  $a_z$ ,  $q_z$  axis is longest within stability region. However, even with such high mass range, the ion of mass 1500 would have  $q_z$  value of 0.404 at amplitude of 7500V and would thus remain in the trap. Because of technical difficulty a voltage higher than 7500V can't be applied, making it impossible to eject the ion ( $m/z$  1500) out of the trap. Therefore, to eject such an ion resonance ejection is applied. Resonance ejection is achieved by applying RF voltage to the endcap electrode of increasing amplitude, during the ramping of the ring electrode RF voltage. At a certain point, the secular frequency of ion (the frequency at which an ion oscillates in the trap) is directly proportional to the frequency of the ring electrode, and matches the frequency of the RF voltage applied to ring electrode causing the ion to resonate. If the amplitude of the RF voltage on the endcap electrode is small, the ion will gain kinetic energy and move away from the center of the trap. However if the amplitude is large enough ions will be ejected from the trap. Such ejection of ion occurs because of the resonance effect and is called resonance ejection. This would appear as a hole in the stability diagram. Application of resonance ejection

increases the mass range significantly and in addition lowers the voltage requirement that normally would be required to eject such higher  $m/z$  ions.

#### **2.15.4 Damping Gas**

When the ion enters the mass analyzer cavity, they have higher kinetic energy. Collision with helium gas causes them to lose the kinetic energy thereby damping the amplitude of oscillation. The loss of kinetic energy causes ions to slow down, and eventually get trapped into the RF field of the ring electrode. Without a damping gas ions will not be trapped within RF field of the ring electrode. This whole process minimizes the loss of ions. In addition to this, helium gas helps to focus the ions to the center of the trap instead of allowing them to spread throughout the cavity which dramatically increases the mass spectral resolution.

Helium gas enters into the mass analyzer cavity through the nipple on exit endcap electrode. The flow of helium gas is maintained in such way that the pressure is approximately  $10^{-3}$  Torr (0.1 Pa) inside the mass analyzer cavity.

## Chapter 3 Methods development of capillary electrophoresis (CE) and capillary electrophoresis-electrospray ionization-mass spectrometry (CE-ESI-MS)

### 3.1 Introduction

Successful bioanalysis requires an analytical technique that provides a high degree of separation and accurate detection. Capillary electrophoresis (CE) and high performance liquid chromatography (HPLC) are the methods of choice for high performance separation. CE provides a much higher number of theoretical plates (high separation) compared to HPLC, mainly because of its uniform separation media. In CE, the separation media is liquid alone, while in HPLC the separation media is consists of layers of particles (beads) which are difficult to pack uniformly. Another reason that CE provides high separation is because of its plug like flow compared to laminar flow in HPLC. In a LC column, the mobile phase has maximum friction at the inner walls of the capillary while at the center of the column friction is minimum, which produces a parabolic flow and leads to zone broadening. In CE, there is no pressure gradient involved and the liquid moves due to the electroosmotic flow generating a plug like movement of the liquid and minimizes the band broadening. Plug like flow generates minimum distribution in velocity and produces narrower sample bands.

Once analytes are separated in CE, an array of detectors such as Ultra-violet (UV),<sup>118</sup> Laser Induced Fluorescence (LIF),<sup>119</sup> Diode Array Detectors (DAD),<sup>120</sup> and Mass Spectrometer (MS)<sup>99</sup> can be used. The most common detector for CE is UV, because it is relatively cheap, rugged, and easily coupled to CE. Although UV detectors have capacity to detect most of the metabolites selected in this study, they fail for analytes such as sugar phosphates, since sugar phosphates lack a chromophore. Therefore, for detection of sugar phosphates highly sensitive detectors such as mass spectrometers (MS) must be used. Thus the ultimate goal of this project is to develop a CE-ESI-MS method for twelve metabolites in the central metabolic pathway of *C. thermocellum*. Before coupling the CE to MS, it is essential to thoroughly understand CE operation and evaluate some basic parameters such as sample injection, capillary conditioning, and separation mechanism using more robust approaches of detection. Operation of CE in combination with UV is relatively easy compared to CE-ESI-MS. Therefore, basic CE parameters were evaluated using UV detection system.

For CE separation, buffer is very critical to achieve a good separation of analytes. Ionic strength of the buffer and buffer pH could have dramatic effect on CE separation. In CE-ESI-MS buffers should be volatile, since non-volatile buffer could crystallize during the ionization process and block CE capillary as well as MS orifice. In CE-ESI-MS, parameters such as the composition of sheath liquid and flow rate of sheath liquid and sheath gas could have dramatic effect on sensitivity. A careful attention to the parameters such as the position of the CE capillary inside the ESI needle, distance between the ESI needle and MS, and relative height of the CE vial is required for successful CE-ESI-MS operation. Therefore, a careful evaluation of above mentioned parameters is very important for a highly sensitive and reproducible CE-ESI-MS method.

## **3.2 Materials and methods**

### **3.2.1 Reagents**

Unless otherwise indicated, substrates and reagents were used without prior modification. Isopropanol, methanol, water, and hydrochloric acid were purchased from Fisher (Pittsburg, PA, USA). Ethanol was purchased from Aaper Alcohol and Chemical Company (Shelbyville, KY, USA). Trimethylamine was purchased from Acros (Geel, Belgium). Acetic acid was purchased from EMD Chemicals and Inc. (San Diego, CA, USA). Ammonium hydroxide, sodium hydroxide, NAD<sup>+</sup>, NADH, NADP<sup>+</sup>, NADPH, AMP, ADP, ATP, acetyl-CoA, glucose-6-phosphate, glucose-1-phosphate, fructose-1,6-diphosphate, pyruvic acid, and AMP (isotopically labeled) were purchased from Sigma (St. Louis, MO, USA).

### **3.2.2 CE instrument and optimized conditions**

Capillary electrophoretic separations were performed using PrinCE GPA 100 CE unit (Groton Biosystems, Boxborough, MA, USA) with UV detection at 260 nm. A fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of about 80 cm in length and 75 micron ID was used. The detection window was created in the fused silica capillary by burning a portion of polyamide coating of about 5 mm wide, and the distance from the cathode end to the detection window was about 26 cm.

Before using the capillary for the first time, it was flushed with 1 N NaOH for 30 min, 0.1 N NaOH for 30 min, water for 30 minutes, methanol for 30 minutes, and finally



with running buffer for 30 minutes. Prior to each CE run it was flushed with 0.1 N NaOH for 3 minutes, methanol for 3 minutes and finally with running buffer for 3 minutes. The CE was controlled using “prinCE” software.

Borate and phosphate buffer were prepared by taking required boric or phosphoric acid and adjusting the pH using a 0.1 M NaOH. Acetate buffer were prepared by taking required acetic acid and adjusting the pH using trimethylamine or ammonia hydroxide solution. Standards were prepared by making stock solution of 5 mg/ml in water for each metabolites and diluting with an appropriate volume of water. Samples were injected hydrodynamically, 50 mbar for 6 seconds. CE voltage was set to 21 kV for all separations.

### **3.2.3 CE-ESI-MS instrument and optimized conditions**

All CE-ESI-MS analyses were performed using LCQ classic (Thermo Electron Corporation, USA), and CE was interfaced to MS using CE/MS adapter kit (Thermo Electron Corporation, USA). Data acquisition on MS was performed using “Xcaliber” software. All CE conditions were same as above (section 3.2.1), unless noted otherwise.

Samples were injected by hydrodynamic injection, 100 mbar for 6 seconds. For separation in CE-MS, +21 kV was applied to the inlet electrode and outlet was set to ground. ESI-MS was conducted in negative ion mode and the applied voltage for ESI was 4 kV. Sheath liquid was delivered with a syringe pump at a flow rate of 3.5  $\mu$ L/min. Sheath gas was delivered at 1 (arbitrary unit). No hydrodynamic pressure was applied during the run for first 21 minutes, after that CE voltage was stopped in the middle of analysis and pressure of 100 mbar was applied for 5 minutes.

### 3.3 Results and discussions

#### 3.3.1 Method development of CE

##### 3.3.1A Sample injection

The total volume of a CE capillary is only a few microliters, thus CE is a microanalytical technique. To avoid excessive band broadening the amount injected should be less than 1-2% of the total capillary volume. Total volume of CE capillary can be calculated by using simple equation,

$$V = \pi r^2 L \quad \text{Equation 3-1}$$

where,  $V$  = Total volume,  $L$  = length in cm,  $r$  = internal radius of the capillary in cm

$$\begin{aligned} \text{Therefore, total volume (V)} &= 3.142 (0.0038 \text{ cm})^2 (80 \text{ cm}) \\ &= 3.142 (0.0000142) (80) \text{ cm}^3 \\ &= 0.00362 \text{ mL} \\ &= 3.62 \text{ }\mu\text{L} \end{aligned}$$

There are 2 ways samples can be introduced into the capillary, using hydrodynamic or electrokinetic injection. In hydrodynamic injection, the capillary end is inserted in sample vial and pressure is applied to push the sample in the capillary. In an electrokinetic injection the capillary end is inserted in the sample vial and a potential is applied for few seconds; the sample is introduced into the capillary by electroosmotic mobility of the buffer and electrophoretic velocity of the analytes. Both mechanisms act at the same time and depending on electrophoretic velocity, discrimination could occur among the injected analytes. Analytes with the higher electrophoretic velocities will be forwarded compared to analytes with the lower electrophoretic velocity. Therefore, hydrodynamic injection is usually the method of choice for sample introduction in CE.

In hydrodynamic injection, samples can be injected by generating a relative pressure difference at 2 ends of the capillary to drive the sample inside the capillary. The total amount injected by applying pressure of 50 mbar for 6 seconds is equal to 29.8 nL

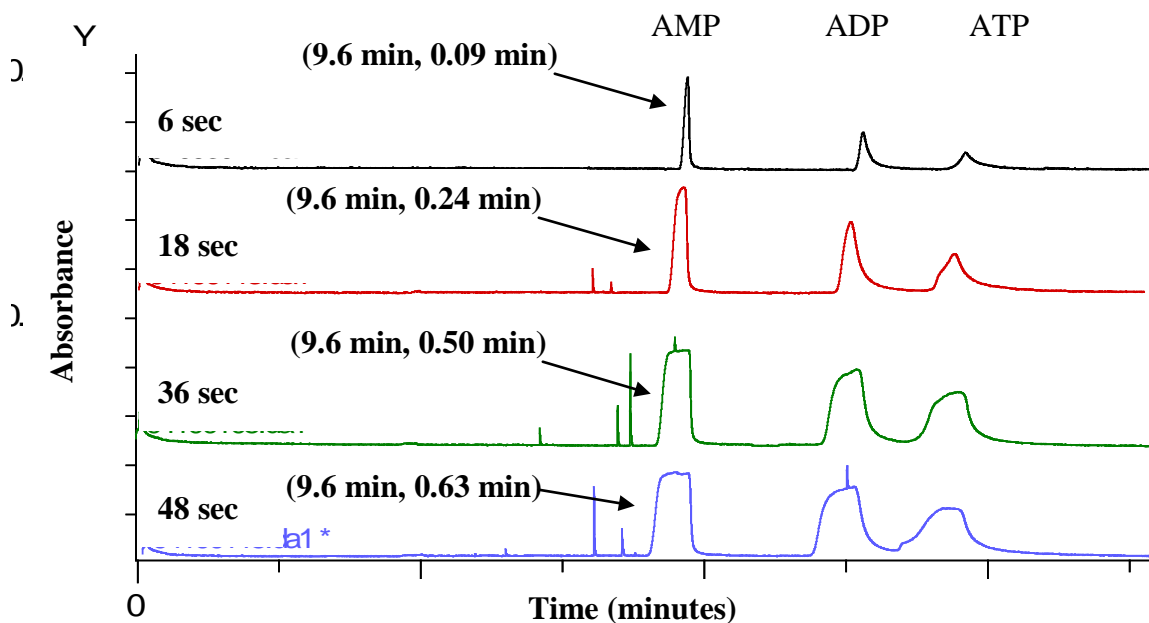
(using  $P.t. = PLUG.3200.\eta. L/d^2$  Equation 2-9), which is approximately 0.8% of the total capillary volume.

To assess the optimum volume of sample injected in CE capillary, a mixture of 3 analytes were injected onto the CE column at a pressure of 50 mbar for 6, 18, 36, and 48 seconds. These times were equivalent to 30, 90, 180, 240 nL for each injection, respectively. Electropherograms for all 4 injections are shown in Figure 3-1.

To calculate band broadening, efficiency is calculated for peak number 1 in each injection using peak widths at half height and retention times. Efficiency or number of theoretical plates obtained for injection volumes 30, 90, 180, and 240 nL were 61,666; 8726; 2044; and 1286, respectively. From the calculated theoretical plates and a visual inspection of Figure 3-1, it is clear that efficiency is inversely proportional to the amount of sample injected. Larger sample volumes resulted in band broadening; therefore samples should be restricted somewhere around 30 nL. However injection of low sample volume results in decreased sensitivity, therefore a compromise must be made between sample volume and sensitivity. An injection of 50 mbar for 6 seconds was determined to be optimum and selected for all CE analyses.

### **3.3.1B Buffer selection**

In CE, separation is based on charge to size ratio and selectivity depends on effective mobility of the analytes. The pH of the buffer determines the degree of ionization of the analytes and hence their effective mobility. Therefore, optimal resolution must be determined by varying buffer pH and ionic strength. An appropriate buffer for CE analysis is the buffer which generates minimum current and separates all the analytes. Since, CE current is directly proportional to the ionic strength of the buffer; increased ionic strength causes higher currents, Joule heating, and decreases resolution. To minimize the current, the ionic strength of the buffer should be kept below 100 mM. However low ionic strength (less than 5 mM) does not have enough buffering capacity, therefore an optimum ionic strength should be employed for good resolution and keep the current low. Seven metabolites  $NAD^+$ ,  $NADP^+$ , AMP, ADP, ATP, acetyl-CoA, and NADPH were selected as model metabolites for separation by CE and obtained electropherogram is shown in Figure 3-3. All the metabolites were separated in less than 7 minutes; however peaks such as  $NADP^+$  and AMP were not baseline resolved.



**Figure 3-2: Separation of standard metabolites using phosphate buffer**

Experimental conditions: Background electrolyte, 50 mM phosphate buffer (pH=8); fused silica capillary 75  $\mu\text{m}$  X 80 cm (100 cm total length); CE voltage, 21 kV; UV detection at 260 nm, hydrodynamic injection at 50 mbar for 6 seconds

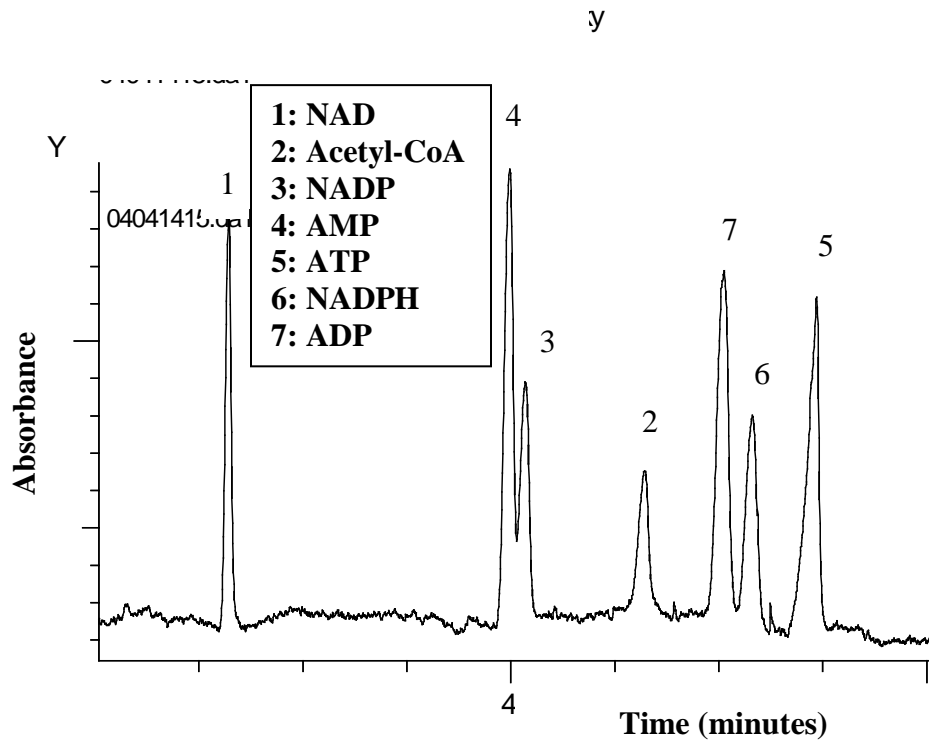
Since, UV detector can not distinguish any co-migrating peaks; baseline separation of analytes is required for accurate quantification. In addition, analysis of real samples numerous matrix peaks could degrade separation. Many parameters were evaluated to improve separation but all were in vain.

Alternate options are to use buffers with higher pH, since highly basic buffers enhance dissociation of the acidic metabolites and increase their charge state. More negative charge increases electrophoretic velocity of an analyte and results in increased migration times. Therefore, a borate buffer (pH 9.3), was evaluated. The resulting electropherogram is shown in Figure 3-4, which shows all analytes are baseline separated. Using the borate buffer resulted in change of migration orders. Migration order in the phosphate buffer was  $\text{NAD}^+$ , AMP,  $\text{NADP}^+$ , acetyl-CoA, ADP, NADPH, and ATP; however migration order in the borate buffer was  $\text{NAD}^+$ , acetyl-CoA,  $\text{NADP}^+$ , AMP, ATP, NADPH, and ADP. Such extreme differences in migration order can be explained by the concept of borate complexation. Since all of the 7 metabolites contain a pentose sugar borate interacts with vicinal diol moieties of these sugars and forms a complex.

Since boron possesses a negative charge, the newly formed complexes possess a higher negative charge. Formation of borate complexes not only adds more negative charge but it also increases their hydrodynamic radius and depending on the size of complex formed it could affect the electrophoretic velocity and apparent mobility of an analyte. As a result their migration order in borate buffer was extremely different compared to the phosphate buffer. Due to complete baseline separation of metabolites obtained in borate buffer, it was used for all CE analyses.

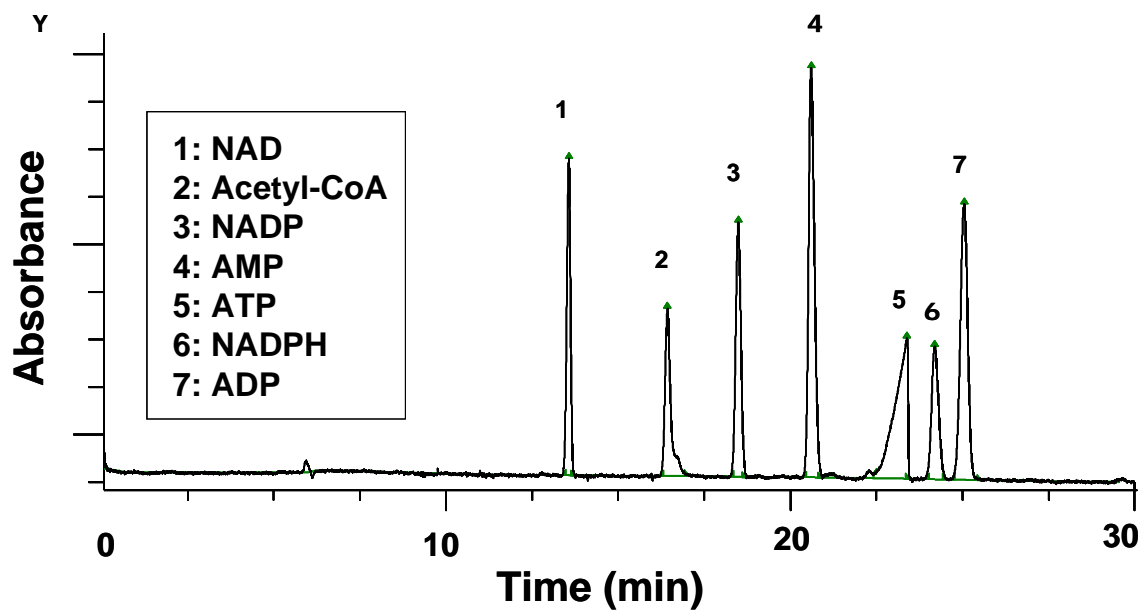
### **3.3.1C Reproducibility, LODs, and linear ranges**

Reproducibility of migration time, peak area, concentration limit of detection, mass limit of detections (LODs), and linear range of method for analysis of the 7 metabolites were determined. The obtained values are shown in Table 3-1. Highly reproducible data shows good choice of buffer and analytical conditions. The relative standard deviations (RSDs) for migration times with 3 consecutive injections ( $n = 3$ ) ranges between 1.9 to 3.3%. In CE, reproducibility of the migration time depends on preconditioning of CE capillary to a great extent.



**Figure 3-3: Separation of standard metabolites using phosphate buffer**

Experimental conditions: Background electrolyte, 50 mM phosphate buffer (pH=8); fused silica capillary 75  $\mu\text{m}$  X 80 cm (100 cm total length); CE voltage, 21 kV; UV detection at 260 nm, hydrodynamic injection at 50 mbar for 6 seconds



**Figure 3-4: Separation of standard metabolites using borate buffer**

Experimental conditions: Background electrolyte, 100 mM borate buffer (pH= 9.3); fused silica capillary, 75  $\mu\text{m}$  X 80 cm (100 cm total length); UV detection, 260 nm; hydrodynamic injection at 50 mbar for 6 seconds

Therefore, such high reproducibility of migration time suggests preconditioning method of CE capillary was efficient. Also, high reproducibility of peak area for all metabolites was observed. RSDs for 3 consecutive injections were below 10% except for ATP. The peak shape of ATP was relatively broad compared to other analytes possibly leading to the higher RSD. Broader and non-Gaussian peak shape affects integration and thus reproducibility. When the *Pka* of a molecule is close to the pH of the buffer, an analyte can switch between 2 different charge states which are migrating at different rates and appear a broad peak.

The concentration limits of detection for all metabolites analyzed were between 1.12 and 1.63  $\mu\text{M}$ , while mass limit of detection was between 35 and 53 fmoles with pressure injection of 50 mbar for 6 seconds at a signal-to-noise (*S/N*) ratio of 3. Since the amount of sample injected could be calculated using equations 3-1 and 3-2, the mass limit of detections for all metabolites could be calculated. Linear ranges up to 2 orders of magnitude were found and the correlation coefficient for all analytes were above  $R = 0.997$  (Figure 3-5).

### **3.3.2 Identification of metabolites in CE**

Metabolites separated by CE could be identified by comparing the migration times with the migration times of standards; however variation in migration time could hinder reliable detection of metabolites. Therefore, standards metabolites were spiked in samples for confirmation. Electropherograms obtained before and after spiking were compared for identification of metabolites. One such example is shown in Figure 3-6; top electropherogram is the sample and the bottom electropherogram is the sample spiked with  $\text{NAD}^+$ . Intensity of  $\text{NAD}^+$  is substantially increased after the spiking which confirms presence of  $\text{NAD}^+$  in the sample.



Analyte	% RSD peak area (n = 3)	% RSD migration time (n = 3)	LOD		Linearity range ( $\mu\text{M}$ )
			Concentraion ( $\mu\text{M}$ )	Mass (f moles)	
<b>NAD</b>	5.4	1.9	1.52	48.74	1.5 to 75.3
<b>AcCoA</b>	2.1	2.3	1.12	35.95	1.1 to 56.9
<b>NADP</b>	5.9	2.6	1.63	52.24	1.6 to 65.3
<b>AMP</b>	9.7	2.9	1.29	41.98	1.2 to 144
<b>ATP</b>	12.6	3.3	1.38	44.21	1.3 to 90.7
<b>NADPH</b>	7.1	2.3	1.28	41.23	1.2 to 59.9
<b>ADP</b>	8.7	3.2	1.43	45.88	1.4 to 117

RSD: Relative standard deviation

**Table 3-1: CE-UV reproducibility, limit of detections and linear ranges**

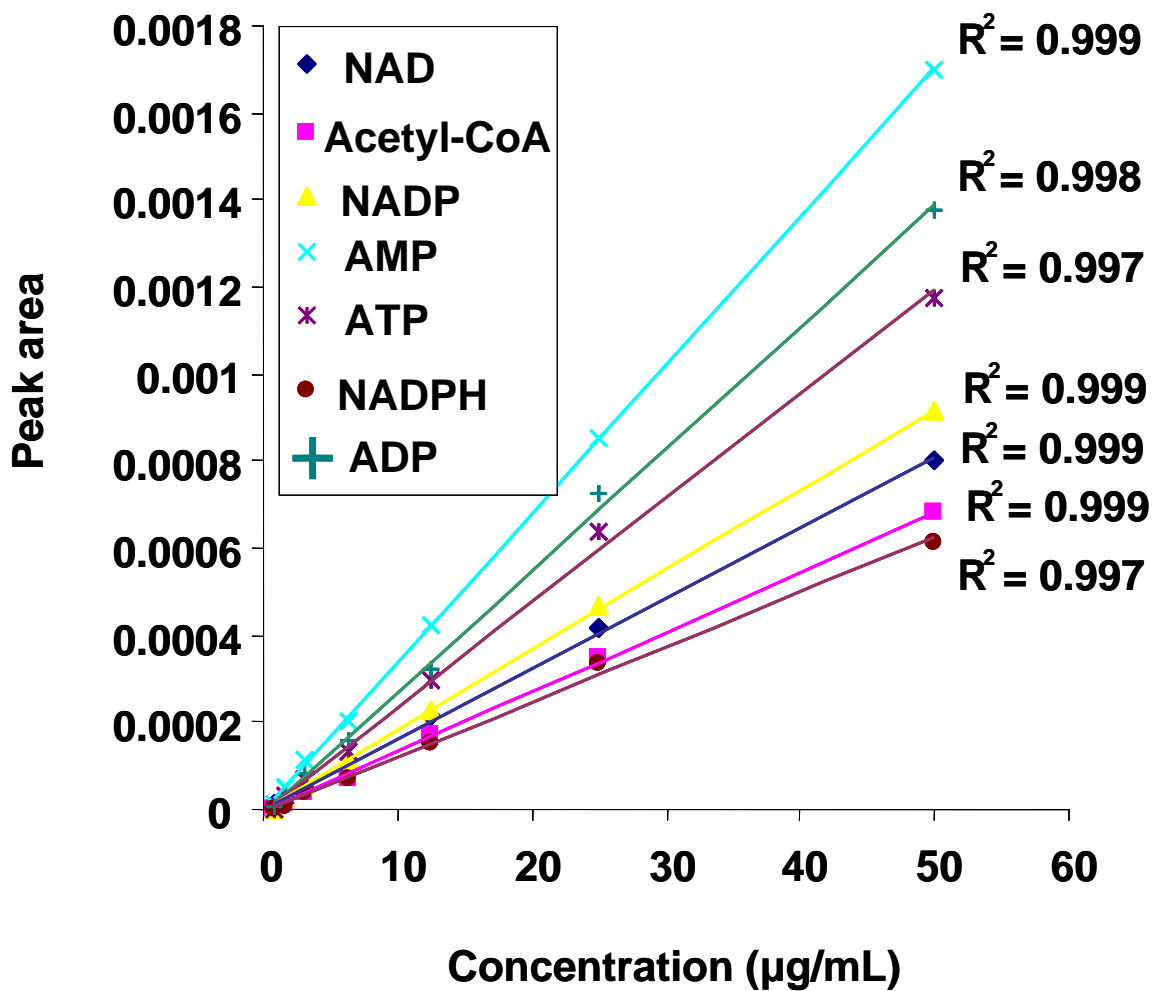
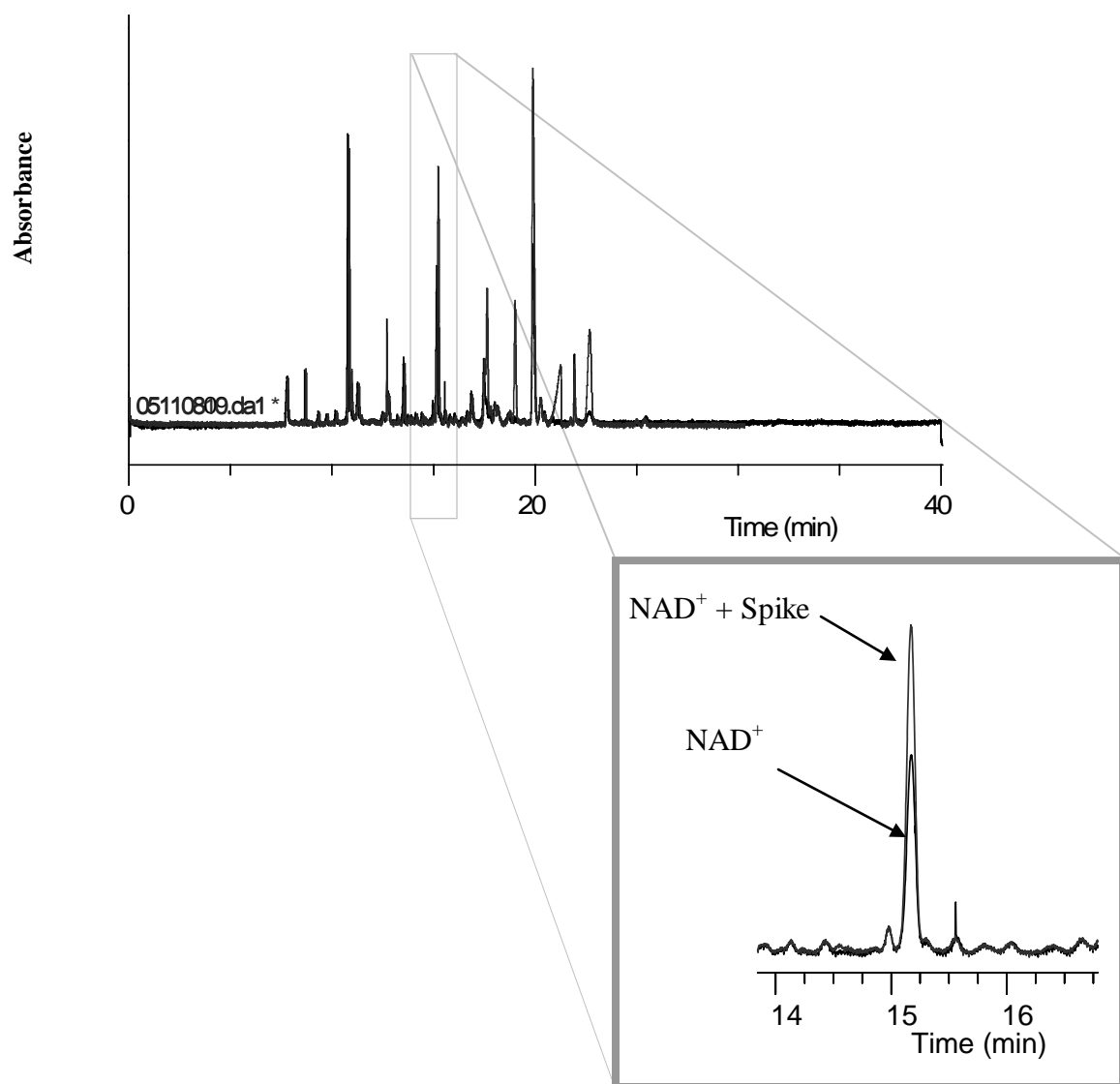


Figure 3-5: Calibration plots of standard metabolites

**Top:** Sample  
**Bottom:** Sample + NAD<sup>+</sup> spike



**Figure 3-6 Identification of peaks in CE by spiking**

### 3.3.3 Method development of CE-ESI-MS

#### 3.3.3A Coupling of CE to MS using ESI

In sheath-flow interface, sheath liquid provides stability to the ESI and electrical contact to the CE outlet and helps to complete the CE circuit. Due to ease of implementation and reliable nature of sheath-flow, this type of interface is used in this study. Before, evaluating CE and ESI parameters for separation and sensitivity, the following technical issues were evaluated during the coupling of the CE to MS.

##### *a) Distance between ESI interface and MS*

During coupling of the CE to MS, it was noticed that when ESI interface was too close to MS, arcing occurred and ESI operation was completely interrupted. This problem was circumvented by increasing the distance between ESI and MS. CE-ESI-MS is a three electrode system, in which CE outlet electrode and ESI emitter electrode (CE outlet/ESI emitter) is shared between CE and the ESI-MS circuit. Depending on the polarity and magnitude of the CE, CE outlet/ESI emitter, and ESI-MS, various redox reactions are occurring.<sup>121</sup> The electric field at the tip of the electrospray capillary can be calculated as

$$E = \frac{V}{r \cdot \ln\left(\frac{4 \cdot d}{r}\right)} \quad \text{Equation 3-2}^{88}$$

where,

$E$  = Electric field

$V$  = voltage difference between the spray capillary and counter-electrode

$r$  = radius of the spray capillary

$d$  = distance between the spray capillary and counter electrode

Since, the distance ( $d$ ) between the spray capillary and counter electrode is inversely proportional to the electric field, decreasing distance increased the applied electric field strength. Higher  $E$  values resulted in higher currents which caused arcing and electrical discharge at the ESI needle and interruption of the ESI operation.<sup>88</sup> To avoid the electrical discharge the distance between the ESI and the MS was increased and

at the same time care was taken not to keep ESI far away from the MS, since number of ions reaching the MS is inversely proportional to the distance between the ESI and MS.

***b) Position of CE capillary inside ESI emitter***

The position of the CE capillary also had an effect on ESI operation. The sheath liquid and buffer coming from CE converge at the tip of the CE capillary; therefore the position of CE capillary inside the ESI needle was crucial. On careful observation it was noticed that by keeping the tip of CE capillary beyond (~ 0.2-0.5 mm) the ESI needle rendered a stable ESI current and a successful CE-ESI-MS operation. CE and ESI current were used indicative of a stable ESI operation. Failure to position the CE capillary in this manner caused a drop in CE current and an interruption of CE-MS operation. This optimized position of CE capillary maintained a good electrical contact between CE and ESI, and also provide stable ESI operation.

***c) Tip of the CE capillary***

About a centimeter in length of polyamide coating at the tip of the CE capillary was removed before inserting into the ESI interface. Removal of polyamide coating helped in wetting the CE capillary and allowed good mixing of sheath liquid and buffer coming from the CE at the tip, which helped for a stabilized ESI operation.

**3.3.3B Buffer selection**

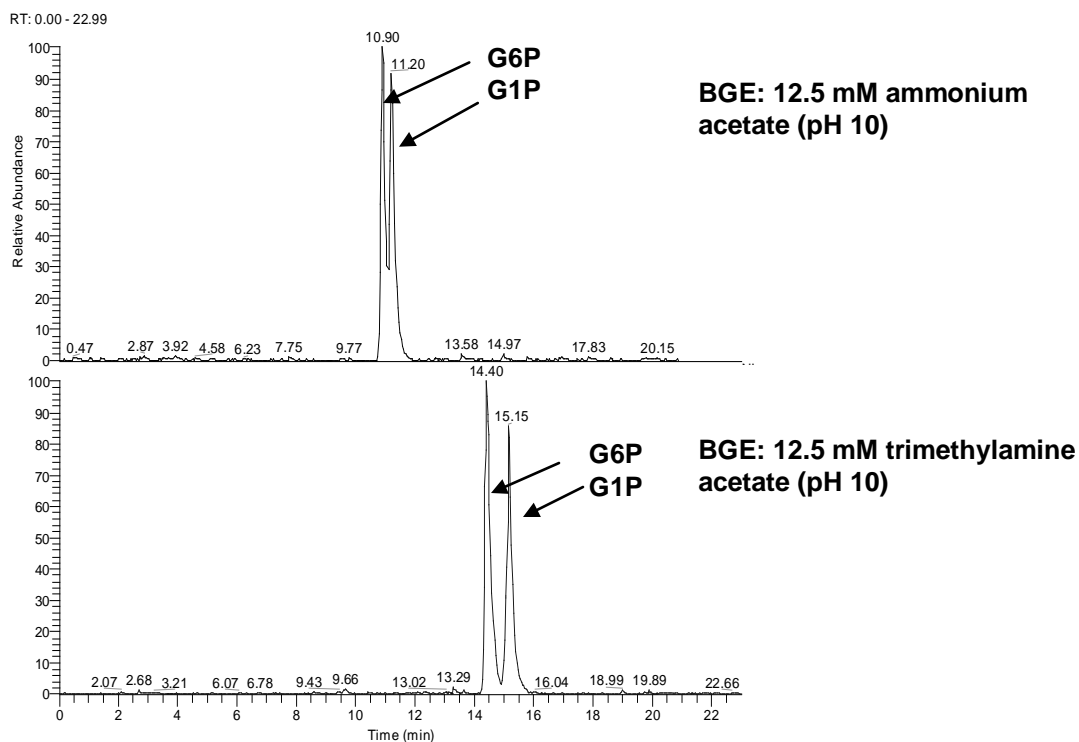
A non-volatile buffer can crystallize in the process of ESI and block the CE capillary as well as the MS orifice. Therefore, a volatile buffer was used for CE-ESI-MS. Ammonium acetate or ammonium formate is very common in CE-ESI-MS. As seen in CE method development, buffers play a vital role in separation of the metabolites, similarly selecting an appropriate buffer a good separation could be achieved in CE-ESI-MS.

Buffer plays a vital role in separation of analytes. Although, if a buffer could not resolve two peaks, the mass spectrometer has an ability to resolve analytes on the basis of mass to charge and resolve co-migrating peaks. However MS fails to distinguish between structural isomers such as glucose-1-phosphate (G1P) and glucose-6-phosphate (G6P). Therefore it is absolutely essential that structural isomers must be completely

separated on CE before they enter the mass spectrometer for their distinction. To evaluate the capacity of buffers to resolve structural isomers such as G1P and G6P, ammonium acetate (pH 10) and trimethyl ammonium acetate (pH 10) were evaluated. A mixture of these two structural isomers was separated in CE-ESI-MS using these 2 buffers and extracted ion electropherograms for  $m/z = 259$  are shown in Figure 3-7. Ammonium acetate (AA) buffer failed to completely separate structural isomers of glucose phosphates; however, a baseline separation of these two isomers was obtained on using trimethyl ammonium acetate (TMAA) buffer. In TMAA buffer, migration times were higher compared to AA buffer, since trimethyl amine molecules suppress the EOF. By slowing the EOF, apparent mobility of the analytes was increased which rendered enhanced resolution. TMAA buffer (pH 10) was selected for all CE-MS analyses.

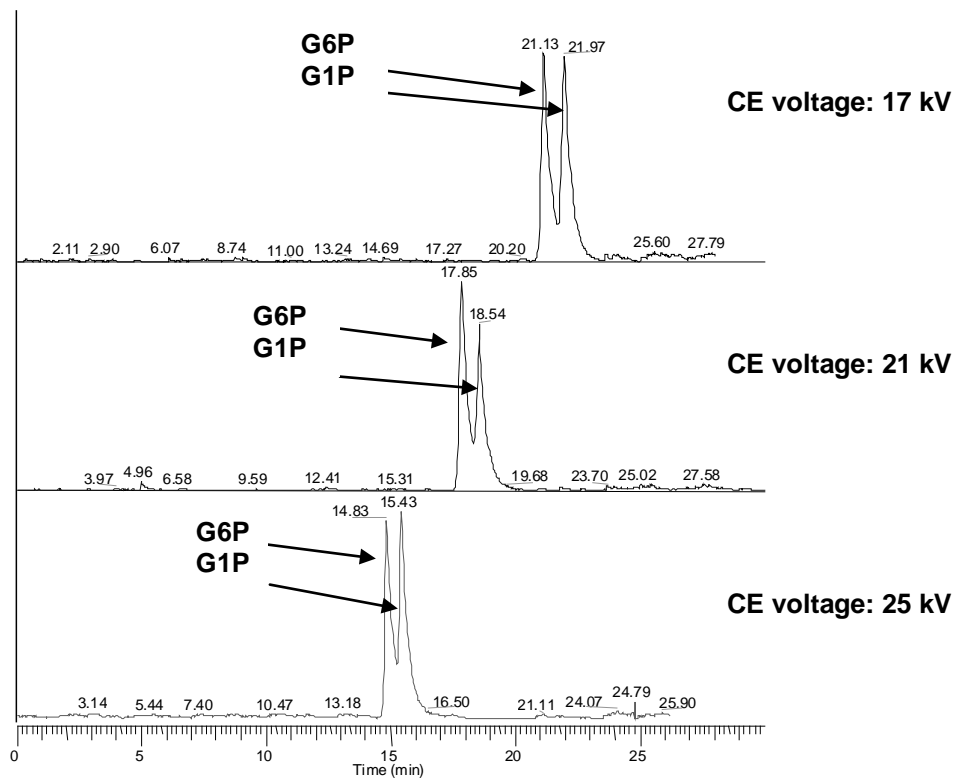
### **3.3.4 Effect of voltage on isomer separation**

The effect of variation of separation voltages on separation of G1P and G6P was also studied. Separations were carried out by keeping all parameters of CE-ESI-MS constant except the CE voltages. Voltages of 17, 21 and 25 kV were evaluated using these 2 structural isomers. The extracted ion electropherograms for  $m/z = 259$  are shown in Figure 3-8. No significant effect of voltage on these 2 structural isomers of sugar phosphate was observed. However, migration times were dramatically affected. For each increment of 4 kV, the migration time decreased about 3 minutes. In other words, with increasing voltages analysis time decreased. Although the separation of structural isomers was preserved at all the voltages applied, higher CE voltage produced elevated CE current (data not shown) which could be detrimental for CE separation. Therefore to keep current low, CE voltage was not increased beyond 21 KV for all analyses.



**Figure 3-7: Effect of buffer on separation of structural isomers**

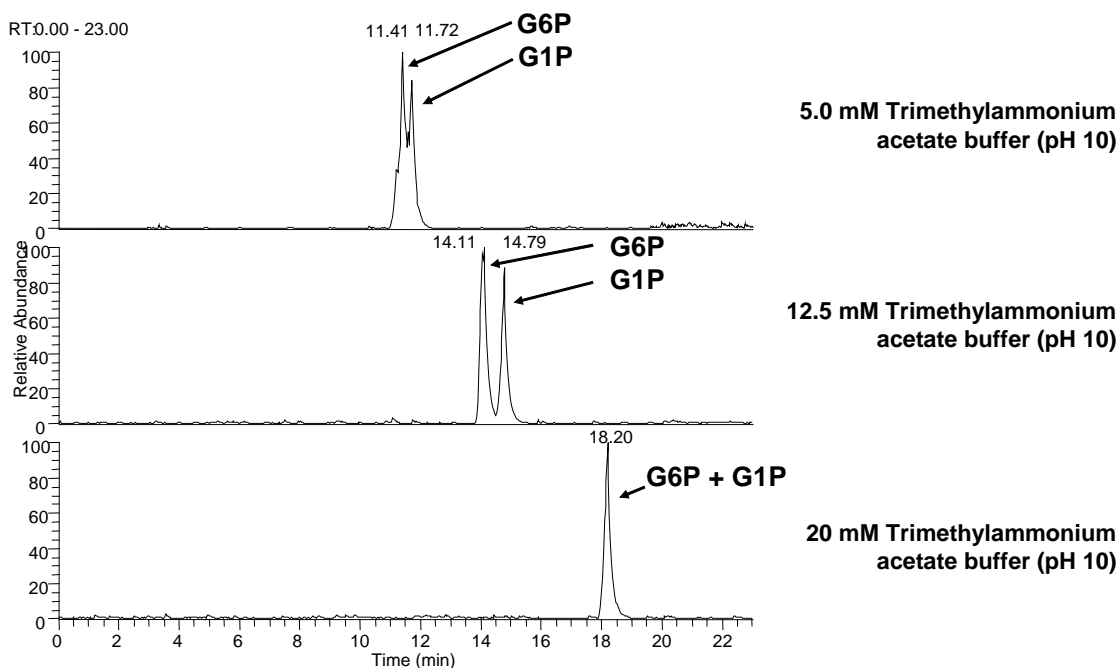
Experimental conditions: CE voltage, 21 kV; Sample injection, 100 mbar for 6 sec; ESI voltage, -4kV; untreated fused silica capillary 70  $\mu\text{m}$  X 80 cm; sheath liquid, 50% Isopropanol; Sheath liquid flow rate, 3.5  $\mu\text{L}/\text{min}$ ; sheath gas flow rate, 1 (arbitrary unit).



**Figure 3-8: Effect of CE voltage on separation of structural isomers**

Experimental conditions: BGE: 12.5 mM Trimethylamine acetate buffer (pH 10) Sample injection, 100 mbar for 6 sec; ESI voltage, -4kV; untreated fused silica capillary 70  $\mu$ m X 80 cm; sheath liquid, 50% Isopropanol; Sheath liquid flow rate, 3.5  $\mu$ L/min; sheath gas flow rate, 1 (arbitrary unit).





**Figure 3-9: Effect of ionic strength on separation of structural isomers**

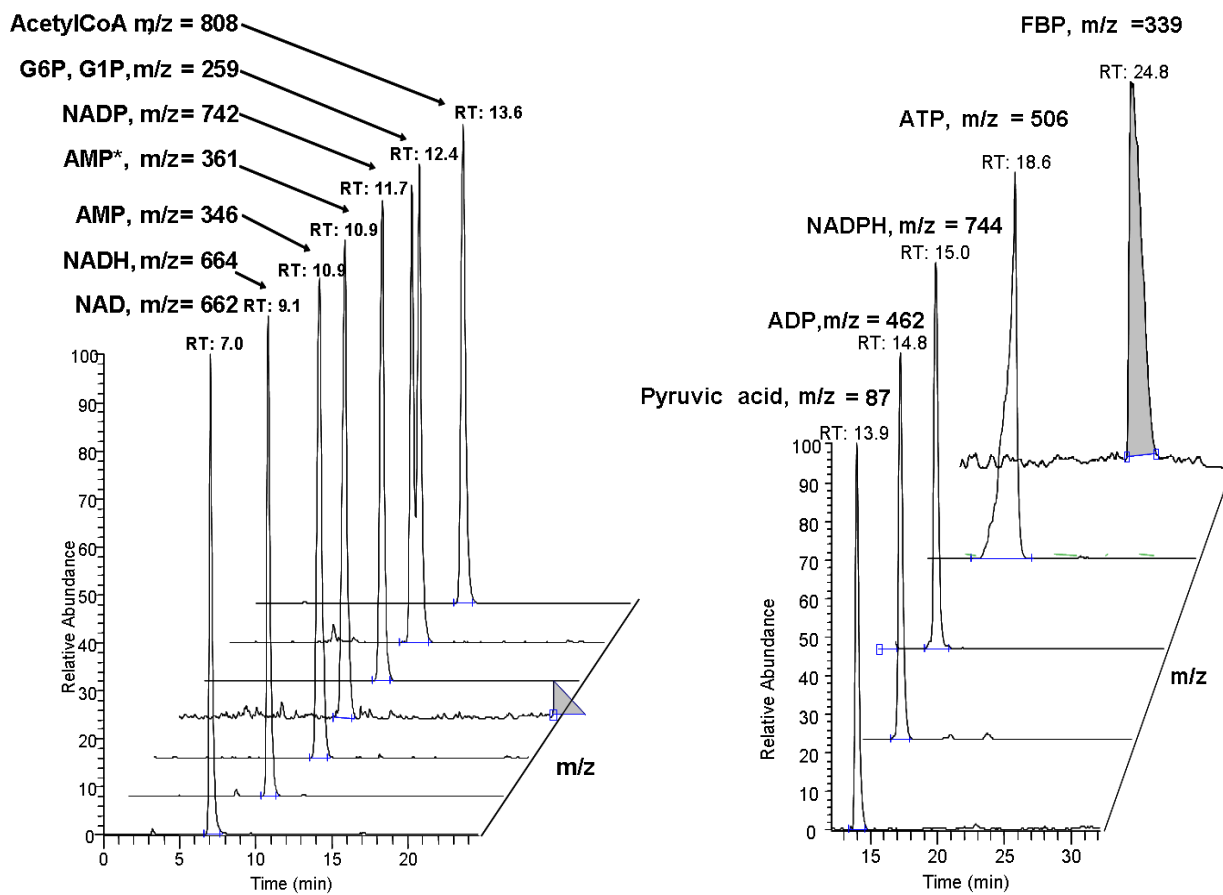
Experimental conditions: BGE: Varying; sample injection, 100 mbar for 6 sec; ESI voltage, -4kV; untreated fused silica capillary 70  $\mu\text{m}$  X 80 cm; sheath liquid, 50% Isopropanol; Sheath liquid flow rate, 3.5  $\mu\text{L}/\text{min}$ ; sheath gas flow rate, 1 (arbitrary unit).

### 3.3.5 Effect of ionic strength on isomer separation

Similar to the effect of CE voltages, effects of ionic strength of the buffer on the separation of the structural isomers were studied by keeping all other parameters constant except the ionic strengths of the running buffer. Separations were carried out using TMAA buffers having ionic strength of 5, 12.5, and 20 mM. Extracted ion electropherograms of  $m/z = 259$  for all three buffers is shown in Figure 3-9. At 5 mM ionic strength, separation of these 2 structural isomers was degraded and at 20 mM ionic strength separation was completely lost and these 2 peaks could not be resolved at all. However at 12.5 mM these 2 structural isomers were completely resolved. Decreasing the ionic strength of the buffer to 5 mM, EOF increased which resulted in decreased apparent mobilities and decreased separation efficiency, therefore resolution was lost. However, increasing ionic strength to 20 mM, resolution was completely lost due to increased Joule heating. Therefore, 12.5 mM TMAA buffer (pH 10) was used for all CE-MS analyses.

### 3.3.6 Separation of metabolites

A mixture of standard metabolites containing  $\text{NAD}^+$ ,  $\text{NADH}$ ,  $\text{NADP}^+$ ,  $\text{NADPH}$ ,  $\text{AMP}$ ,  $\text{ADP}$ ,  $\text{ATP}$ , acetyl-CoA, G1P, G6P, FBP, and pyruvate was readily separated by using TMAA buffer and applying conditions as described previously (Section 3.3.3B). Under these conditions, the migration time for fructose-bis-phosphate (FBP) was long compared to all of the metabolites and total analysis time was over 30 minutes (data not shown). Longer times on CE column cause longitudinal diffusion and zone broadening of FBP. Therefore to shorten the analysis time, CE voltage was turned off after 21 minutes, ESI operation and data acquisition on mass spectrometer was continued and a pressure of 100 mbar for 5 minutes was applied at the CE inlet. At the end of 21 minutes, all the metabolites were separated in CE and entered the mass spectrometer except the FBP and hydrodynamic injection applied after 21 minutes forced FBP to enter the mass spectrometer. Due to such hydrodynamic injection toward the end of the analysis, analysis time was reduced to less than 26 minutes and peak shape of FBP was much narrower.



**Figure 3-10: Extracted ion electropherogram of 12 standard metabolites**

Experimental conditions: BGE, 12.5 mM trimethylamine acetate (pH 10); rest of the conditions same as in Figure 3-7

Figure 3-10 shows separation of the 12 metabolites selected for this study. After separation of the metabolites, parameters were optimized to increase the sensitivity of CE-MS

### **3.3.7 Sensitivity enhancement of CE-ESI-MS**

#### **3.3.7A Effect of sheath liquid composition**

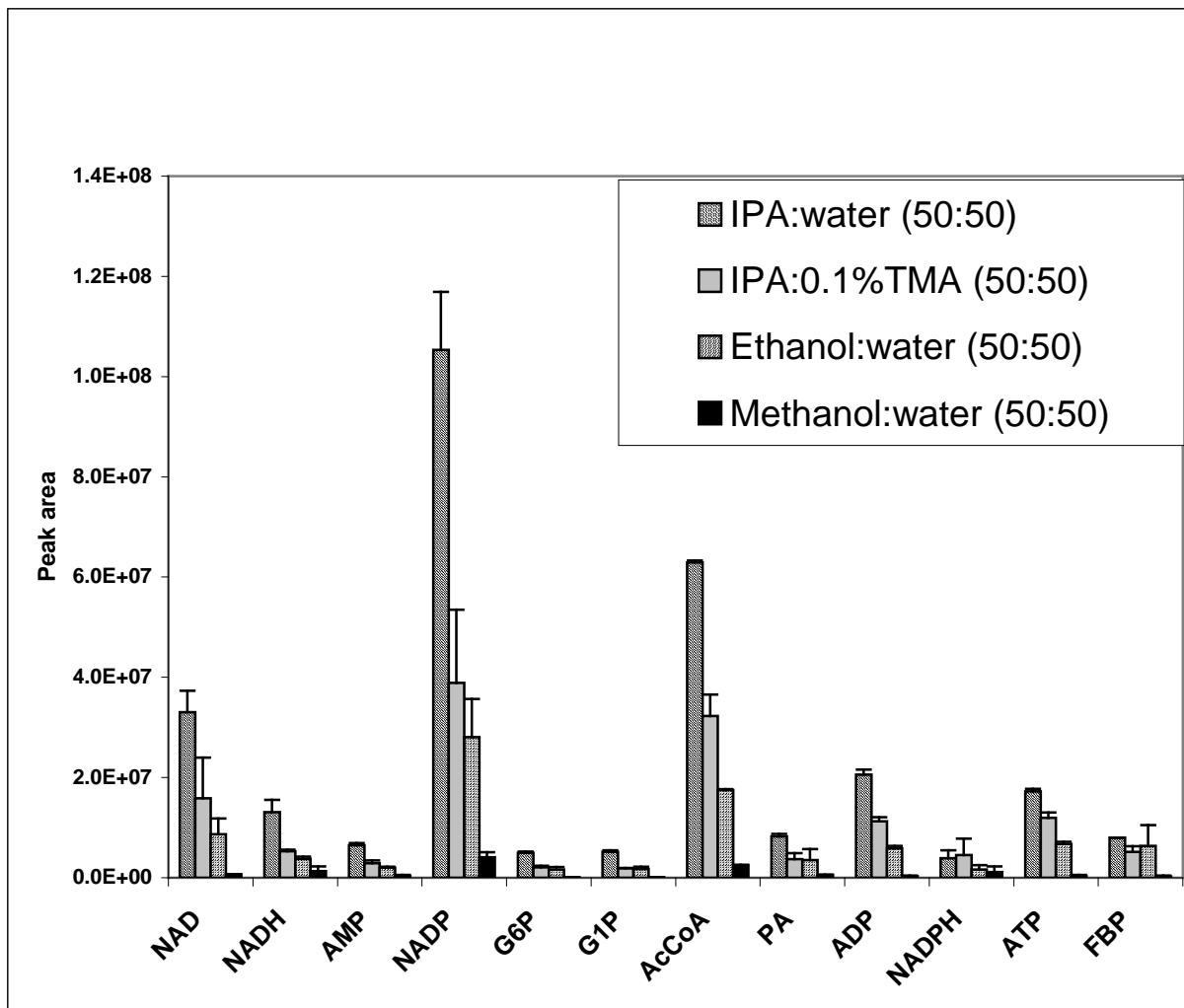
Sheath liquid is mainly comprised of water and organic solvents such as alcohols. Water is essential for dissolution of polar metabolites, and helps to conduct current and completes the CE circuit by providing a ground for the CE outlet. Moreover, the sheath liquid compensates for the low volume of liquid coming from the CE capillary and provided stability to the ESI. The organic solvent in sheath liquid reduces the surface tension of the liquid and assists in formation of ions.<sup>105</sup> Excessive concentration of organic liquids in sheath liquid could cause precipitation of metabolites at the tip of ESI needle because of highly polar nature of the metabolites selected in this study.

Methanol, ethanol and isopropanol were evaluated as sheath liquids by preparing a 50% solution of each alcohol and their effect on sensitivity was evaluated. It was observed that with increasing chain length of alcohol signal intensities of all the analytes increased (Figure 3-11). Addition of organic solvents certainly decreases the surface tension of the liquid and helps ion formation, however formation of ions is very complex process and other physicochemical parameters such as viscosity, volatility and pH may also affect it.<sup>105</sup> The purity of the alcohols and water used in sheath liquid also dramatically affected the sensitivity; therefore all the alcohols and water used were of highest purity. Other alcohols such as butanol were not available in high purity and therefore were not evaluated in this study. Organic solvents such as formaldehyde, acetonitrile, and acetone were also evaluated (data not shown), however signal intensities from these organic solvents were not comparable with intensities from alcohol. Signals for many analyte ions were lost when these organic solvents were used in sheath liquid, therefore only alcohol sheath liquid data is shown. A base such as trimethylamine was added to 50% IPA at 0.1%, and evaluated it as a sheath liquid to assess if the presence of base in sheath liquid helps to increase the intensity further by deprotonating the analyte ions in this negative ion mode detection. Surprisingly addition of trimethylamine in 50%

IPA sheath liquid decreased the intensity of all the analytes compared to 50% IPA sheath liquid. Decrease in intensity on addition of base to sheath liquid was attributed to the interaction of the base with the polyamide coating of CE capillary, which increased background and decreased the intensities of all analytes. Considering all above aspects, 50% IPA without any additional base was the choice of the sheath liquid over traditional 50% methanol sheath liquid.

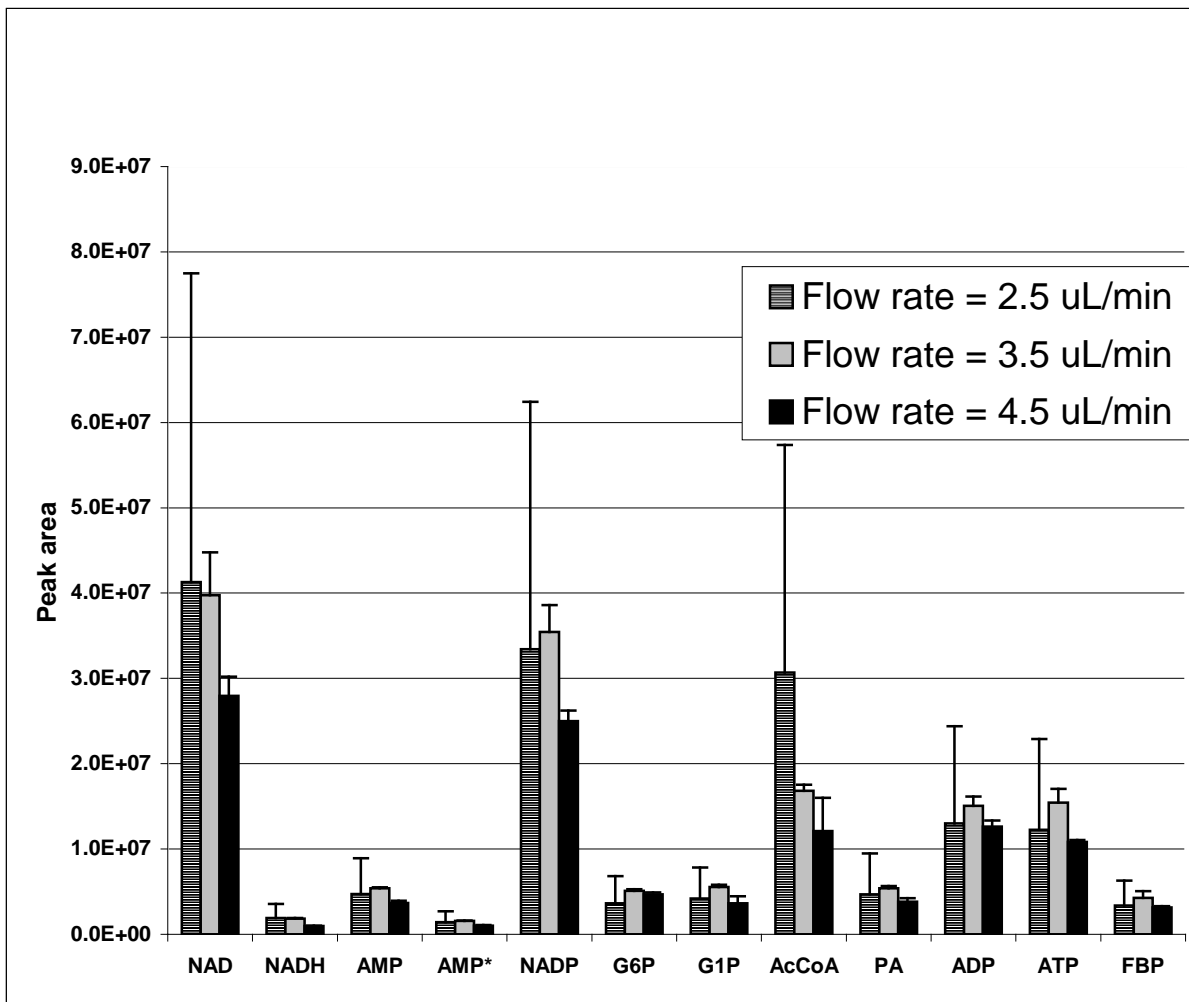
### **3.3.7B Effect of sheath liquid flow rate**

The low buffer flow (20-200 nL/min) from CE is usually compensated by providing a sheath flow rate at few microliters per minute.<sup>98</sup> A higher flow rate dilutes the analytes and decreases the sensitivity;<sup>122</sup> on the other hand low flow rates destabilize ESI.<sup>88, 123</sup> As can be seen from Figure 3-12, three different sheath liquid flow rates (2.5, 3.5, and 4.5  $\mu\text{L}/\text{minute}$ ) were evaluated. The intensity of all the ions was inversely proportional to the flow rate, since higher flow rates decrease ion concentration. Therefore, to achieve the highest intensity, the lowest possible sheath liquid flow rate was employed. Although, ion intensities obtained at 2.5  $\mu\text{L}/\text{minute}$  are higher than any other flow rates, at this flow rate ESI operation was not stable, which was observed from fluctuations in ESI current (data not shown) and high relative standard deviations of peak areas of all the metabolites at sheath liquid flow rate. Destabilization of the ESI at 2.5  $\mu\text{L}/\text{minute}$  flow could be because of the rate at which liquid entering into the ESI needle is probably much slower than the rate the liquid is being consumed for droplet formation in the ESI. Inefficient flow of liquid could lead to a liquid gap in the ESI needle which splits the CE circuit and interrupts CE-ESI-MS operation. At 3.5  $\mu\text{L}/\text{minute}$  sheath liquid flow rate ESI was much stable (smaller relative standard deviations of peak areas) and resulted in uninterrupted CE-MS. Therefore, to ensure reproducible results a compromise was made on the sensitivity and sheath liquid flow of 3.5  $\mu\text{L}/\text{minute}$  was used for all CE-ESI-MS analyses.



**Figure 3-11: Effect of sheath liquid composition on sensitivity**

Experimental conditions: BGE: 12.5 mM trimethylamine acetate (pH 10); sample injection, 100 mbar for 6 sec; ESI voltage, -4kV; untreated fused silica capillary 70  $\mu\text{m}$  X 80 cm; sheath liquid, varying; Sheath liquid flow rate, 3.5  $\mu\text{L}/\text{min}$ ; sheath gas flow rate, 1 (arbitrary unit)



**Figure 3-12: Effect of sheath liquid flow rate on sensitivity**

Experimental conditions: BGE: 12.5 mM trimethylamine acetate (pH 10); sample injection, 100 mbar for 6 sec; ESI voltage, -4kV; untreated fused silica capillary 70  $\mu$ m X 80 cm; sheath liquid, 50% Isopropanol; Sheath liquid flow rate, varying; sheath gas flow rate, 1 (arbitrary unit).

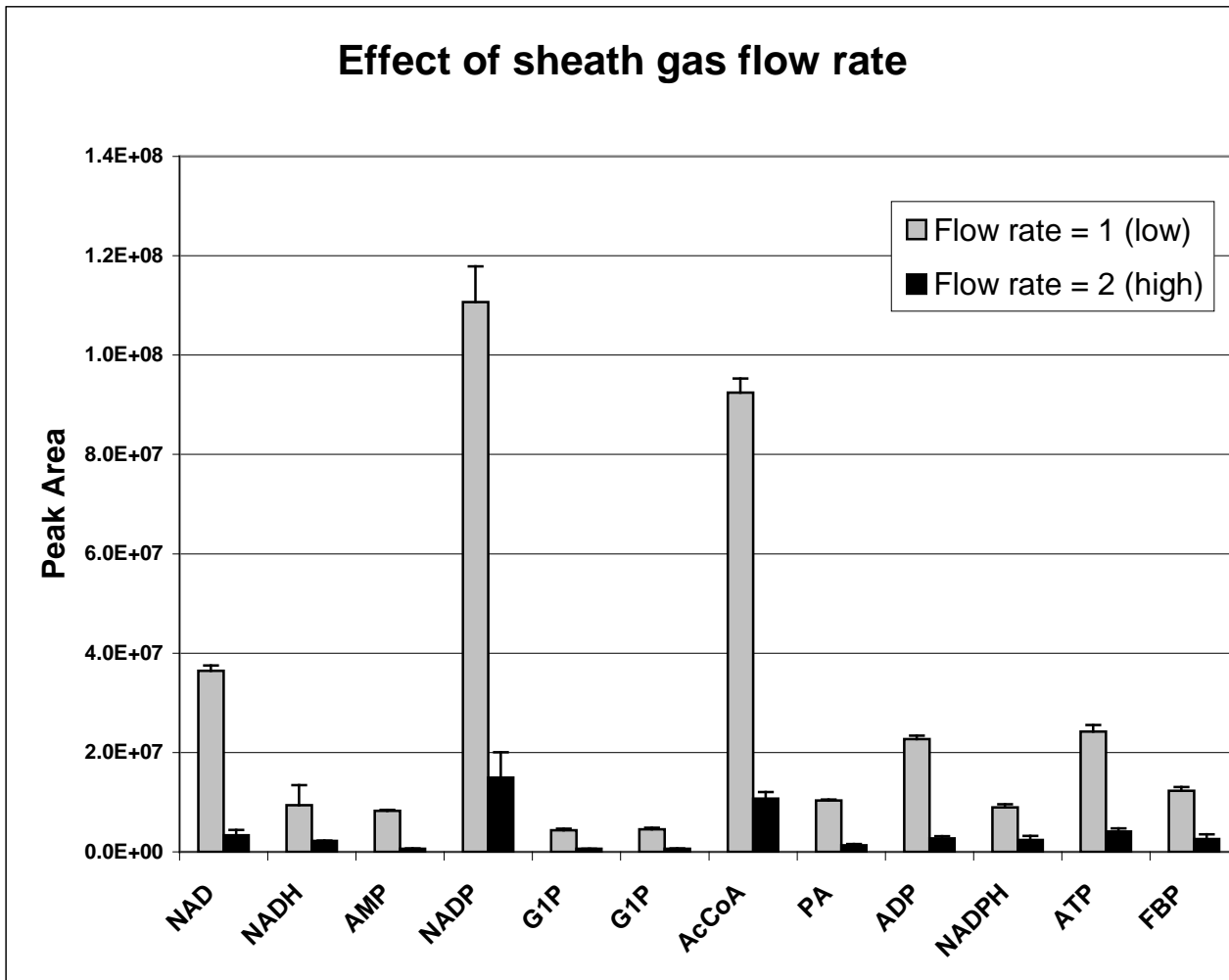
### **3.3.7C Effect of sheath gas flow rate**

Sheath gas flow rates of 1, 2, and 3 (arbitrary units) were evaluated, where 3 is the highest sheath gas flow rate. From Figure 3-13, it can be seen that increasing the sheath gas flow rate from 1 to 2, ion intensities for all the analytes were significantly decreased. Further increase of the sheath gas flow rate from 2 to 3, resulted in complete loss of signal. A sheath gas flow rate of 1 was used for all the analyses, since it provided higher sensitivity for all the analytes compared to sheath gas flow rate of 2 and 3. In a complete absence of sheath gas, the sheath liquid flooded the ESI nozzle and resulted in arcing which completely shut down the ESI operation.

### **3.3.8 Reproducibility, LODs, and linear ranges**

The reproducibility of migration time, peak area, concentration LODs and mass LODs, and linear ranges of method for analysis of the 12 metabolites were evaluated. The obtained data is shown in Table 3-2. The RSDs for migration times with 3 consecutive injections ( $n = 3$ ) were in the range of 0.4 to 4%. Also, high reproducibility for peak area was obtained and RSDs with 3 consecutive injections were 3 to 14%. Highly reproducible data shows good choice of a buffer and analytical conditions. The concentration LODs for all metabolites were between 0.1 and 2.2  $\mu\text{M}$  with pressure injection of 100 mbar for 6 seconds at a signal-to-noise ( $S/N$ ) ratio of 3. On converting amount of sample injected to moles of each analytes, mass limit of detection for all metabolites analyzed were between 6 and 130 fmoles with pressure injection of 100 mbar for 6 seconds at a signal-to-noise ration ( $S/N$ ) of 3. Response to the detector for all metabolites was linear and linear ranges as high as 3 orders of magnitude were obtained.





**Figure 3-13: Effect of sheath gas flow rate on sensitivity**

Experimental conditions: BGE: 12.5 mM trimethylamine acetate (pH 10); sample injection, 100 mbar for 6 sec; ESI voltage, -4kV; untreated fused silica capillary 70  $\mu$ m X 80 cm; sheath liquid, 50% Isopropanol; Sheath liquid flow rate, 3.5  $\mu$ L/min; sheath gas flow rate, varying.

<b>Analyte</b>	<b>%RSD for Peak area (n=3)</b>	<b>%RSD for migration time (n=3)</b>	<b>Conc LOD (µM)</b>	<b>Mass LOD (f moles)</b>	<b>Linear range (µM)</b>
<b>NAD</b>	13.6	2.3	0.59	35.3	0.5 to 75.4
<b>NADH</b>	8.4	2.5	0.13	8.0	0.1 to 70.5
<b>AMP</b>	8.0	2.5	0.55	32.9	0.5 to 288.2
<b>NADP</b>	8.3	2.8	0.25	14.9	0.2 to 130.7
<b>G6P</b>	6.4	2.7	1.28	77.0	1.2 to 328.9
<b>G1P</b>	6.4	2.9	1.28	77.0	1.2 to 328.9
<b>AcCoA</b>	6.7	2.9	0.11	6.5	0.1 to 113.9
<b>Pyruvic acid</b>	3.5	3.1	2.16	129.5	2.1 to 1136.4
<b>ADP</b>	9.5	3.1	0.44	26.7	0.4 to 234.2
<b>NADPH</b>	13.4	3.2	0.23	13.7	0.2 to 60
<b>ATP</b>	8.7	3.3	0.34	20.7	0.3 to 181.5
<b>FBP</b>	9.0	0.4	0.53	31.8	0.5 to 135.7

**Table 3-2: CE-ESI-MS reproducibility, limit of detection and linear ranges**

### **3.4 Conclusion**

CE and CE-ESI-MS methods for analysis of acidic metabolites were developed. For CE separation, borate buffer provided maximum resolution, and good reproducibility of method. RSDs for migration time and peak area were below 4 and 13% respectively. Concentration LODs were from 1 to 2 micromolar and mass limit of detection were from 35 to 53 femtomoles. Good linearity was achieved for all the metabolites.

In CE-ESI-MS, TMAA buffer proved to be an appropriate buffer for separation of all selected metabolites along with structural isomers of sugar phosphates. Also, ionic strength of the buffer has an effect on separation of structural isomers. RSDs for migration time and peak area for CE-MS were below 4 and 14% respectively. Concentration LODs were in submicromolar ranges and mass limit of detections were in few femtomoles. Over 3 orders of magnitude linear ranges were achieved for CE-MS separations. In conclusion, capillary electrophoresis based methods are very sensitive and reproducible methods for analysis of charged and polar metabolites.

## Chapter 4 : Development of quenching and extraction protocols for cellulolytic *Clostridia*: ethanol producing microorganisms

### 4.1 Introduction:

Microbial metabolite analysis involves four steps viz. growth of a microorganism, sampling, isolation of metabolites, and metabolite analysis. The first step is the cultivation of a microorganism which has been a routine procedure in the laboratories. The second step is quenching or arrest of the metabolism which instantaneously stops the metabolism, providing a snapshot of metabolites at a specific growth point. Quenching also permits separation of the growth media from the cells without changing the intracellular metabolite concentrations, since the metabolism is inactivated at this point. Traditionally quenching is achieved by inserting the cells in a cold (-40°C) methanol/buffer solution, which instantly drops the temperature of the cells and quenches the metabolism, since the enzymes are inactive at such a low temperature. Third step is the extraction of the metabolites which is achieved by using an extremely low or high pH solution or an organic solvent. After completion of extraction, solutions containing the metabolites are concentrated using vacuum evaporation. The last step is the analysis of metabolites using an appropriate technique. In the past enzymatic assays are widely used for metabolite analysis,<sup>69</sup> and more recently hyphenated techniques such as GC-MS,<sup>80</sup> LC-MS,<sup>124</sup> and CE-ESI-MS<sup>125</sup> are being utilized in metabolite analysis because of their extreme sensitivity and high throughput ability compared to enzymatic assay. Metabolite concentrations obtained using these techniques could be validated using traditional enzymatic assays.

Cellulolytic microorganisms such as *Clostridium cellulolyticum* and *Clostridium thermocellum* are highly important because of their ability to produce ethanol from inexpensive cellulosic biomass.<sup>14, 18</sup> Metabolite analysis of these microorganisms would benefit in development of superior strains for commercial ethanol production. Therefore, methods of metabolite quenching and CE compatible extraction protocols for cellulolytic microorganism were developed. Newly developed metabolite extraction protocol was validated using traditional acid extraction protocol (which is gold standard for extraction of metabolites such as ATP).

Enzymatic assays are widely used for metabolite analysis<sup>69, 126</sup> and have become the benchmark of the metabolite detection methods, therefore these techniques are ideal way to validate the intracellular concentration obtained by the modern analytical techniques. Enzymatic assays may require large volumes of samples for analysis depending on their sensitivity. However, the ATP enzymatic assay is very sensitive and able to detect few nanomoles of ATP in a sample, consequently requires very small volume (few  $\mu\text{L}$ ) of sample for analysis. Due to high sensitivity, ATP assay was preferred for validating ATP concentration obtained by CE methods. Details of the ATP enzymatic assay are described in the past.<sup>127, 128</sup>

## **4.2 Material and methods**

### **4.2.1 *Clostridium thermocellum* medium and culture conditions**

*C. thermocellum* cultures were grown in Dr. Herbert Strobel's lab at Department of Animal and Food Sciences, University of Kentucky. The medium and culture conditions as follows: *C. thermocellum* ATCC 27405 was obtained from the American Type Culture Collection and cultured in a medium that contained 10.8 mM  $\text{Na}_2\text{HPO}_4$ , 11.05 mM  $\text{KH}_2\text{PO}_4$ , 9.35 mM  $\text{NH}_4\text{Cl}$ , 3.79 mM  $(\text{NH}_4)_2\text{SO}_4$ , 0.44 mM  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.27 mM  $\text{CaCl}_2$ , 2.0 g/L yeast extract, 0.5 g of cysteine, 10 mL of a vitamin standard mixture (79), 5 mL of a modified micromineral mixture and 1 mg/L of the oxygen indicator resazurin. The stock micromineral mixture was modified to provide 10 mg of  $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$  and 1 mg of  $\text{Na}_2\text{SeO}_3$  per liter of medium. The basal medium was adjusted to pH 6.7 with 10% NaOH and autoclaved for 10 min to remove dissolved gases. Carbon dioxide was then immediately bubbled through the solution until it was cooled to room temperature and an anaerobic solution containing 4 g of  $\text{Na}_2\text{CO}_3$  was then added. The medium was anaerobically dispensed into culture tubes and bottles and sterilized by autoclaving for 20 min. This basal medium was supplemented with an anaerobic solution of cellobiose to provide a final concentration of 4 g/L. Cell cultures were grown at 55°C and harvested once the optical density had reached approximately 1.0 at 600 nm.

#### **4.2.2 Selection of ethanol-adapted strain**

*C. thermocellum* cultures were grown in Dr. Herbert Strobel's lab at Department of Animal and Food Sciences, University of Kentucky. The detailed protocols as follows: *C. thermocellum* was gradually adapted to increasing concentrations of exogenous ethanol using a serial transfer approach. Basal medium (10 ml in Balch tubes) was provided with 4 g/l cellobiose and sterile ethanol was added to achieve a concentration of 0.5% (w/v). The tubes were inoculated (10% inoculum) with an actively growing culture of strain 27405. Because preliminary experiments (data not shown) indicated that ethanol tolerance was more easily elicited at slightly lower temperatures than the optimal growth temperature of the wild-type (60°C), selection for ethanol adaptation and all subsequent growth of the organisms were conducted at 55°C. Optical densities (600 nm) were periodically recorded and once it had reached maximum, cultures were immediately transferred into fresh medium containing 0.5% ethanol. After growth of this secondary transfer, tubes containing 1.0% ethanol were inoculated with cells from the 0.5% ethanol incubation and treated similarly as described above. This cycle of transfers was continually repeated with increasing concentrations of ethanol. Cultures reliably grew in the presence of 5% ethanol.

#### **4.2.3 *Clostridium cellulolyticum* medium and culture conditions**

*C. cellulolyticum* cultures were grown in Dr. Herbert Strobel's lab at Department of Animal and Food Sciences, University of Kentucky. The detailed medium and culture conditions as follows: *Clostridium cellulolyticum* ATCC 35319 was obtained from the American Type Culture Collection. The organism was cultured in a medium that contained (L<sup>-1</sup>): K<sub>2</sub>HPO<sub>4</sub>, 2.21 g; KH<sub>2</sub>PO<sub>4</sub>, 1.50 g; (NH)<sub>2</sub>SO<sub>4</sub>, 1.3 g; MgCl<sub>2</sub> · 6H<sub>2</sub>O, 1.0 g; CaCl<sub>2</sub> · 2H<sub>2</sub>O, 0.15 g; FeSO<sub>4</sub> · 7H<sub>2</sub>O, 4.14 mg; yeast extract, 4.0 g; resazurin, 1mg; L-cysteine, 1.0 g. The basal medium was adjusted to pH 6.7 with 10% NaOH and autoclaved for 10 min to remove dissolved gases. Nitrogen was then immediately bubbled through the solution until it cooled to room temperature and the medium was anaerobically dispensed into culture bottles and sterilized by autoclaving for 20 min. This basal medium was supplemented with an anaerobic solution of cellobiose to provide a

final concentration of 4 g/L. Cell cultures were grown at 36° C and harvested once the optical density at 600 nm had reached approximately 1.0.

#### 4.2.4 ATP enzymatic assay

A luciferin-luciferase system was purchased from Sigma (product # FL-AAS); ATP dilution buffer was also purchased from Sigma (product # FL-AAB). Manufacturer given protocols were followed for enzymatic determination of ATP. ATP dilution buffer was prepared by dissolving Sigma # FL-AAB in 50 ml of water. Stock solution of ATP assay mix (luciferin + luciferase) was prepared by diluting ATP assay mix (luciferin + luciferase) with 5 ml of sterile water. The stock solution was diluted 10-fold with ATP dilution buffer. A luminometer LKB 1250 was used to measure the light intensity of bioluminescent reaction. Calibration curves were prepared by using standard ATP. Equal portions of sample and 10-fold diluted ATP assay mix was combined in a measuring tube (6 x 50 mm Kimble tube) and it was immediately put into luminometer to measure the light intensity.

**Calculation of intracellular ATP concentrations:** Once the concentration of sample was obtained from the intensity and calibration plot, intracellular concentration was calculated. For example, from calibration plot, obtained concentration of sample was 139.21 nM. Actual concentration is  $139.21 \times 50 = 6960$  nM, since the sample is diluted 50 times. Therefore, the amount of ATP in sample =  $6960 \text{ (nmoles/L)} \times 0.009 \text{ (L)}$

$$= 62.64 \text{ nmoles, since total volume of sample is 9 mL}$$

$$[\text{ATP}]_{\text{Intra}} = 62.64 \text{ nmoles}/10.8 \text{ }\mu\text{L, (since intracellular volume is 10.8 }\mu\text{moles)}$$

Therefore, intracellular concentration =  $5.8 \text{ nmoles}/\mu\text{L} = 5.8 \text{ mM}$

#### 4.2.5 CE and CE-ESI-MS instruments and optimized conditions

Same as per the experimental section of Chapter 3 (3.2.2 and 3.2.3)

#### 4.2.6 Separation of HEPES from $\text{NAD}^+$ for supernatant analysis

Two milliliter of water followed by 2 ml of methanol was passed through anion exchange cartridge by applying a vacuum. A solution of  $\text{NAD}^+$  was prepared by mixing standard  $\text{NAD}^+$  in 140 mM HEPES buffer (pH 7.5) to give a final concentration of 25  $\mu\text{g/ml}$ , and 0.8 mL of this solution was transferred through the anion exchange cartridge.

NAD<sup>+</sup> was eluted from the cartridge by passing 2 mL of 62.5 mM NH<sub>4</sub>OH solution, followed by 2 mL of 125 mM NH<sub>4</sub>OH. Eluted ammonium hydroxide was collected in 4 different fractions of about 1 mL each. Each fraction was concentrated by applying partial vacuum and analyzed using CE.

#### 4.2.7 Quenching

All quenching steps were performed at room temperature, unless otherwise noted. 20 ml cells were withdrawn from the growing cultures and immediately transferred to a 20 mL quenching solution. Depending on the experiment, quenching solution was 60% methanol/ 35 mM HEPES (pH 7.5) buffer, 35 mM HEPES (pH 7.5) buffer, or 35 mM Tris (pH 7.5) buffer at varying temperatures. Cells with quenching solution were rapidly centrifuged at the specific temperature of the quenching solution. After centrifugation supernatant was removed from the cell pellet and saved if required. To the cell pellet another 20 mL of quenching solution was added, cells were mixed well, and centrifuged again at previous centrifugation conditions. Supernatant was again removed from the cell pellet and saved if required. Cell pellet is ready for the next step which was an extraction step.

#### 4.2.8 Extraction

All extraction steps were performed at room temperature, unless otherwise noted. To the cell pellet obtained from the above quenching step an appropriate solution was added for extraction of the metabolites.

**Boiling ethanol extraction:** Cells were resuspended in 5 ml of a solution containing 90% ethanol/ 5 mM HEPES (pH 7.5) buffer and boiled for 10 minutes. Cooled on ice for 10 minutes and centrifuged at 14,000 rpm for 10 minutes at 5°C and supernatant was collected which contains the metabolites.

**Chloroform methanol extraction:** Cell pellet was resuspended in a solution of chloroform: water: methanol (2:1:1), mixed well, and incubated on ice for 10 minutes. Two layers were separated and top aqueous layer was saved which contained the polar metabolites.

**Ultra-cold chloroform methanol extraction:** Cells were resuspended in a solution of chloroform: water: methanol (2:1:1), mixed well, and frozen over night at -80°C. Then,



the next morning the solution was thawed, two layer were separated and top layer was saved which contains the polar metabolites.

**Sonication:** Cells were resuspended in 5 mL of 5 mM HEPES buffer (pH 7.5) and sonicated 4 times for 20 seconds at a frequency of 20 Kcycles/ second separated by 60 seconds. The solution was centrifuged at 14,000 rpm for 10 minutes at 5°C. The supernatant was collected which contained the metabolites.

**Hydrochloric acid extraction:** Cells were resuspended in 5 mL of 0.1 N HCl and incubated at 50°C for 10 minutes. The solution was cooled on ice for 10 minutes, neutralized using NaOH, and centrifuged at 14,000 rpm for 10 minutes at 5°C. A supernatant was saved which contained the metabolites.

#### 4.2.9 Sample processing

Solutions obtained from extractions are concentrated using partial vacuum and pass through a 10 KDa cut-off filters. The filter membrane was washed with 200 µL of water and 200 µL of methanol and filtrates were concentrated again. When 20 mL of 1 OD cells were used, samples were concentrated to 200 µL of final volume.

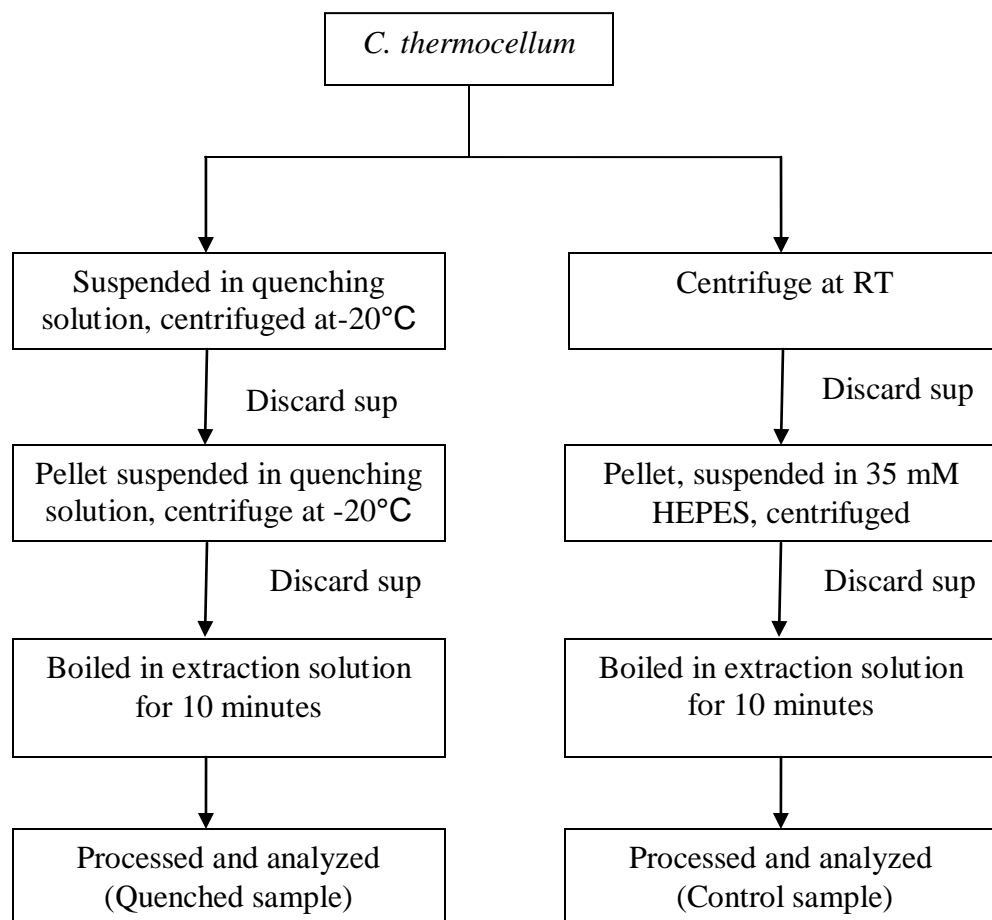
### 4.3 Results and Discussions

#### 4.3.1 Preliminary quenching study

For preliminary study of sampling, literature published metabolomics protocols were followed.<sup>129</sup> Cells were quenched using a solution composed of 60% methanol/ 35 mM HEPES buffer pre-cooled to -20°C and metabolites were extracted using boiling ethanol method<sup>61</sup> with slight modifications. However, cold shock of the quenching solution could compromise the cell integrity. Wittmann *et al.* reported such a cold shock phenomenon and metabolite leakage due to cold buffered methanol used in quenching solution.<sup>65</sup> Similar to this study, metabolite leakages during quenching were reported by Villas-Boas *et al.* and Winder *et al.* from yeast<sup>66</sup> and *Escherichia coli*,<sup>130</sup> respectively.

Therefore a positive control was established for comparison by processing the cells at room temperature and without using any quenching solution. Leakage of metabolites could be noticed by comparing the quenched samples against the control.

**Conditions:** All steps are performed at RT, unless otherwise noted  
**Quenching solution:** 60% methanol/ 35mM HEPES cooled to -20°C  
**Centrifugation:** 8,000 rpm for 8 minute  
**Extraction solution:** 90% ethanol/ 5 mM HEPES  
**Extraction conditions:** Boil cells for 10 minutes



**Figure 4-1** Sample preparation flow chart for metabolite analysis of *C. thermocellum*

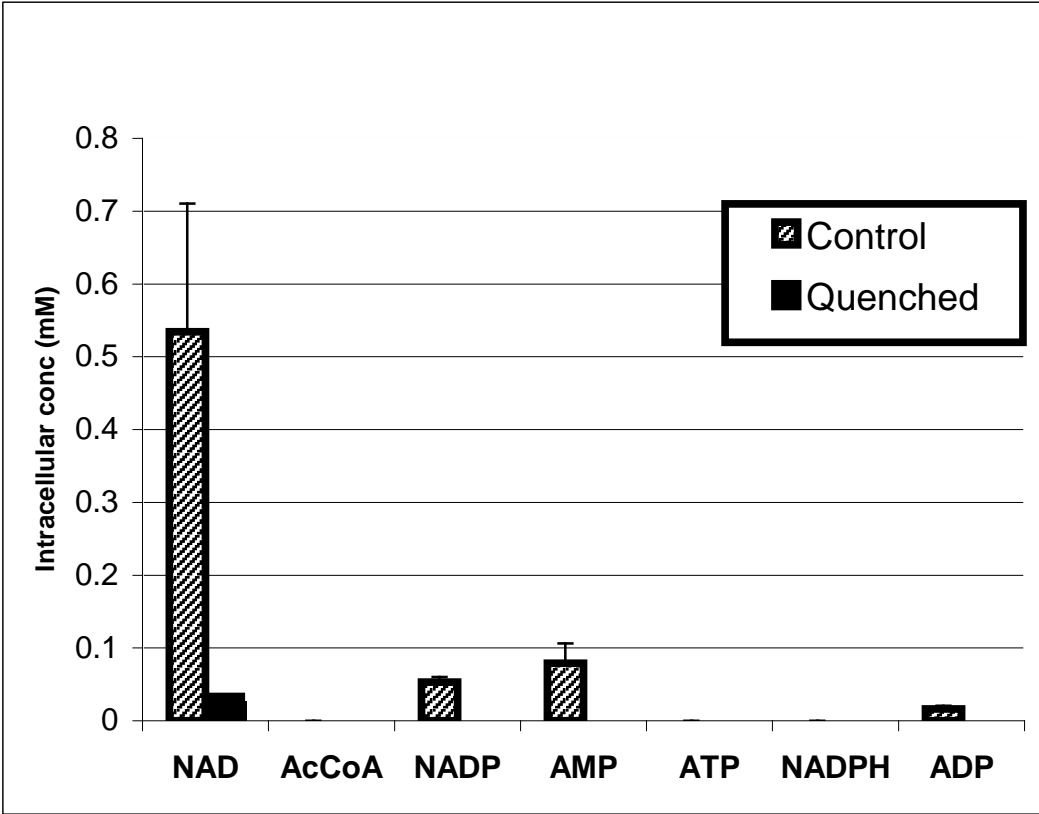


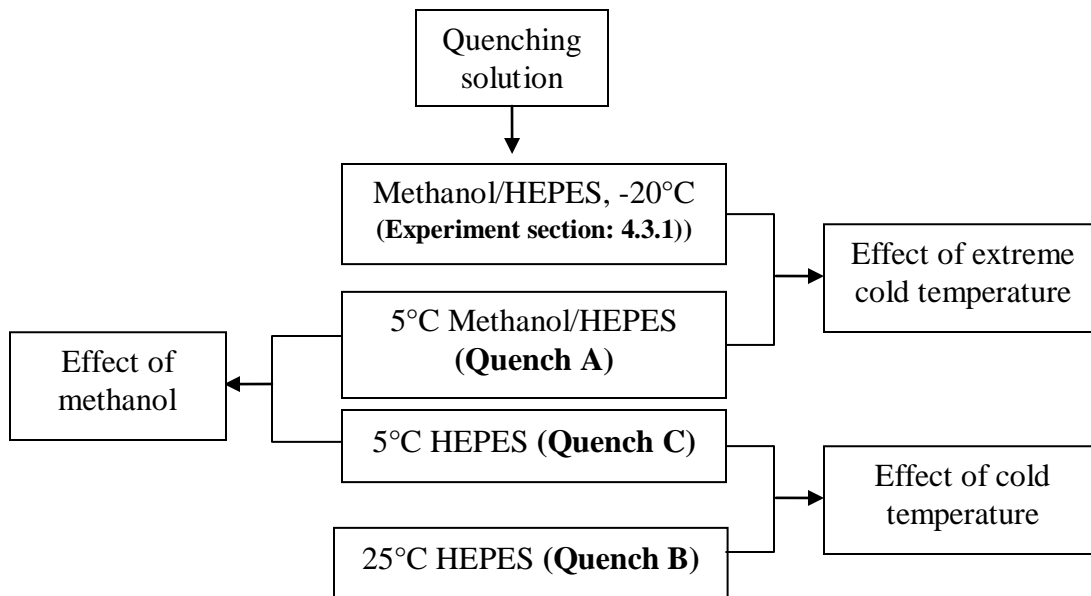
Figure 4-2: Intracellular metabolite concentrations of *C. thermocellum* control and quenched samples

A flow chart in Figure 4-1 summarizes these protocols. Seven metabolites were analyzed from both samples and obtained intracellular concentrations for the analyzed metabolites are shown in Figure 4-2.  $\text{NAD}^+$  was the only metabolite detected from quenched sample, while from control sample beside  $\text{NAD}^+$  metabolites such as  $\text{NADP}^+$ , AMP and ADP were detected.

This data suggests  $\text{NADP}^+$ , AMP, and ADP were completely lost in the process of quenching. Moreover, the concentration of  $\text{NAD}^+$  in quenched samples was ten times lower than control which suggested that  $\text{NAD}^+$  and other metabolites are probably leaking during the quenching process. A conclusion from this experiment could be drawn that quenching solution ( $-20^\circ\text{C}$  methanol/ HEPES) probably damages cell integrity leading to a substantial loss of metabolites. A sudden drop of  $75^\circ\text{C}$  in temperature could cause a cold shock to the cell and disrupt its integrity, very similar to shattering of a hot piece of a glass by immersing in the cold water. Due to partial hydrophobic character, organic solvents such as methanol could partition itself in the lipid bilayer membrane of the cell and disrupt the highly arranged lipid membrane. Disruption of cell the membrane could subsequently lead to metabolite loss. Therefore to find out whether cold temperature, methanol or combination of these two was causing the metabolite loss, further investigation was carried out.

#### **4.3.2 Effect of temperature or/and methanol on metabolite loss**

To decrease the temperature gap between the growth temperature of the cells and quenching solution, cells were quenched using a  $5^\circ\text{C}$  60% methanol/ 35 mM HEPES solution (Quench A) as opposed to  $-20^\circ\text{C}$  in the previous experiment. A positive control was established as per the previous experiment using a  $25^\circ\text{C}$  35 mM HEPES (Quench B) quench solution. To study metabolite loss exclusively due to the cold temperature of quenching solution, cells were quenched using  $5^\circ\text{C}$  35 mM HEPES solution (Quench C) and compared with Quench A. Figure 4-3 summarizes the purpose of different quenching solutions used in this experiment; Figure 4-4 explains the sample preparation protocols. All samples were analyzed using CE and intracellular concentrations for detected metabolites are shown in Figure 4-5. A total of 7 metabolites were analyzed, out of which  $\text{NAD}^+$ ,  $\text{NADP}^+$ , AMP, ADP were detected in samples from Quench B.



**Figure 4-3: Various quenching solutions to study the effect of temperature and methanol on metabolite loss in quenching**

**Conditions:** All steps are performed at RT unless otherwise noted

**Quench solutions:**

**Quench A:** 60% Methanol/ 35 mM HEPES (pH 7.5) at 5°C

**Quench B:** 35 mM HEPES (pH 7.5) at room temperature

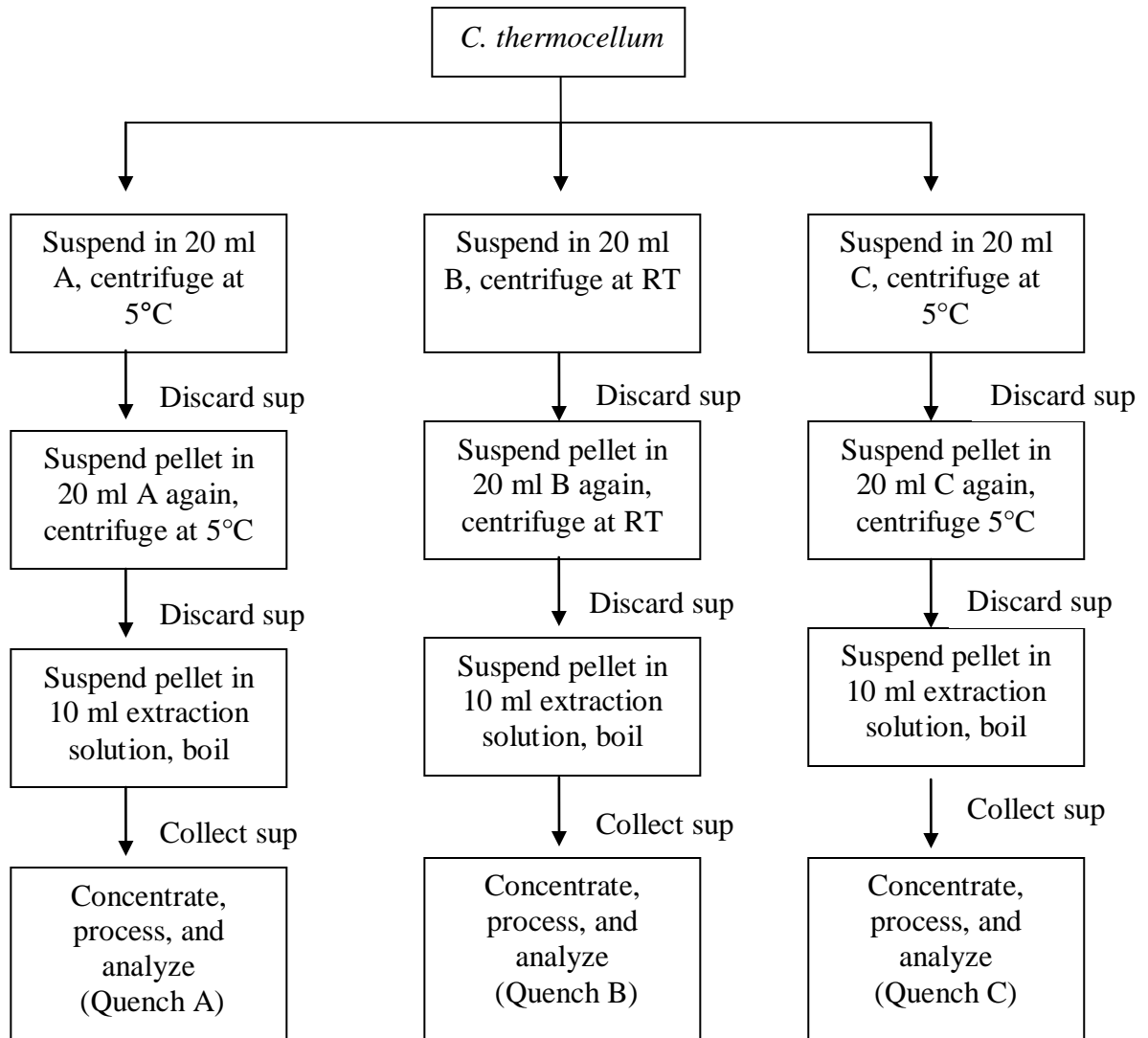
**Quench C:** 35 mM HEPES (pH 7.5) at 5°C

**Centrifugation condition:** 8,000 rpm for 8 minutes

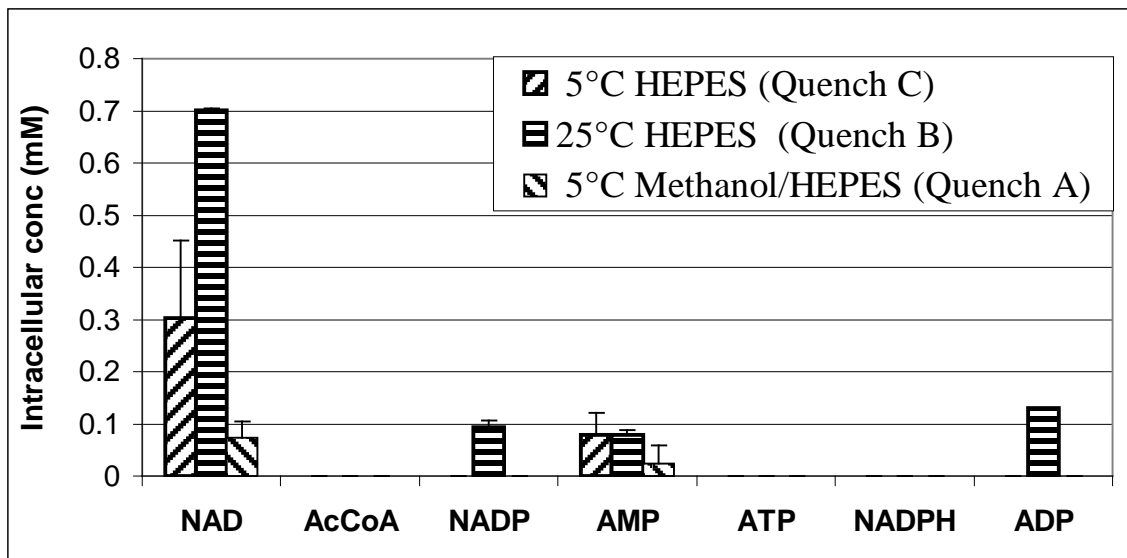
**Extraction solution:** 90% ethanol/ 5 mM HEPES (pH 7.5)

**Extraction condition:** Boil cell pellet in extraction solution for 10 min

**Sample processing:** Pass sample through 3,000 Da cutoff filter, collect filtrate



**Figure 4-4: Sample preparation protocols**



**Figure 4-5: Effect of various quenching solutions on intracellular metabolite concentrations obtained for *C. thermocellum***

In samples from Quench A and C,  $\text{NAD}^+$  and AMP were the only metabolites detected. The intracellular concentration of  $\text{NAD}^+$  in samples from Quench A was nine times less than samples from Quench B; in contrast in samples from Quench C, concentrations of  $\text{NAD}^+$  were two times less compared to samples from Quench B. These results suggest that quenching *C. thermocellum* using HEPES at  $5^\circ\text{C}$  instead of  $25^\circ\text{C}$  cause the loss of metabolites which is evident from low intracellular concentration of  $\text{NAD}^+$  and loss of metabolite  $\text{NADP}^+$  and ADP in sample from Quench C. The damaging effect of organic solvents such as methanol has been reported in the past on integrity of the cell membrane<sup>66</sup> and is not very surprising. However loss of metabolites due to HEPES at  $5^\circ\text{C}$  quenching solution was very surprising.

To examine whether metabolite loss was due to only cold temperature and not because of any intrinsic property of HEPES buffers at cold temperature leading to metabolite leakage, further investigation was carried out.

#### **4.3.3 Effect of Tris buffer on quenching**

To assure the loss of metabolite in the process of cold HEPES quenching was due to cold temperature alone and was not related to any specific property of HEPES buffer, HEPES buffer was substituted with Tris (trishydroxymethylaminomethane) buffer for quenching. Tris ( $\text{pK}_a = 8$ ) buffer was chosen because of its good buffering capacity in the natural pH range. Tris buffer ( $\text{pH } 7.5$ ) at  $25^\circ\text{C}$  (Quench A) and  $5^\circ\text{C}$  (Quench A) was used to quench the cells and Figure 4-6 summarizes the protocols applied. Both samples were analyzed using CE and obtained intracellular concentrations for the samples from two treatments are shown in Figure 4-7.  $\text{NAD}^+$ ,  $\text{NADP}^+$ , AMP, ADP were detected in samples from Quench A; however  $\text{NAD}^+$  and AMP were the only metabolites detected in samples from Quench B. The intracellular concentration of  $\text{NAD}^+$  was about three times less in samples from Quench B compared to samples from Quench A.

In conclusion, quenching the cells using cold Tris buffer at  $5^\circ\text{C}$  resulted in significant loss of metabolites, which is evident from lower intracellular concentrations of  $\text{NAD}^+$  and absence of metabolite  $\text{NADP}^+$  and ADP compared to samples from Quench A.



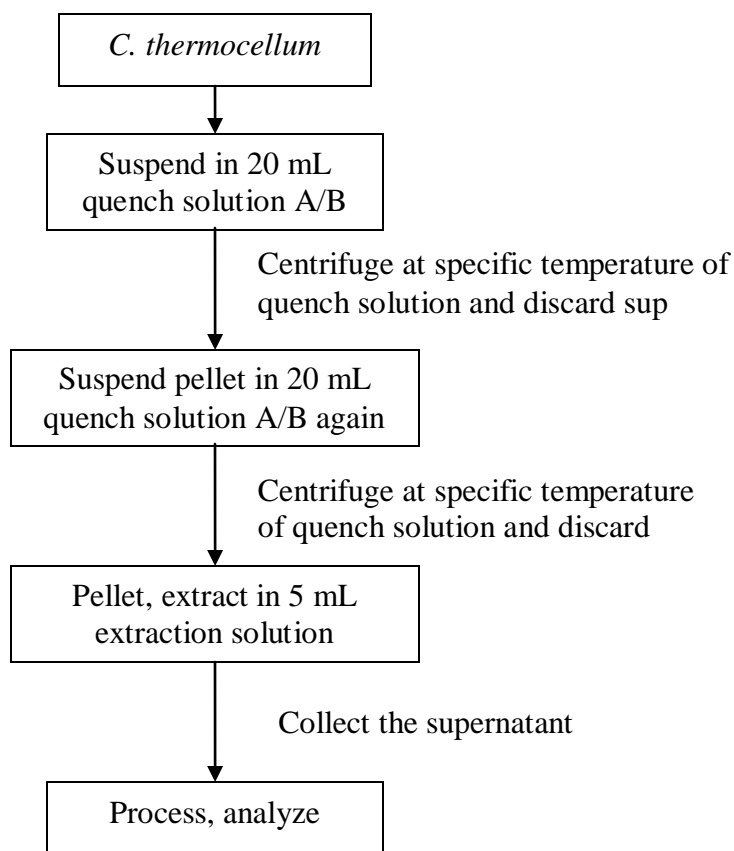
**Conditions:** All steps are performed at RT, unless otherwise noted

Quench solution A: 25°C 35 mM Tris buffer, pH 7.5

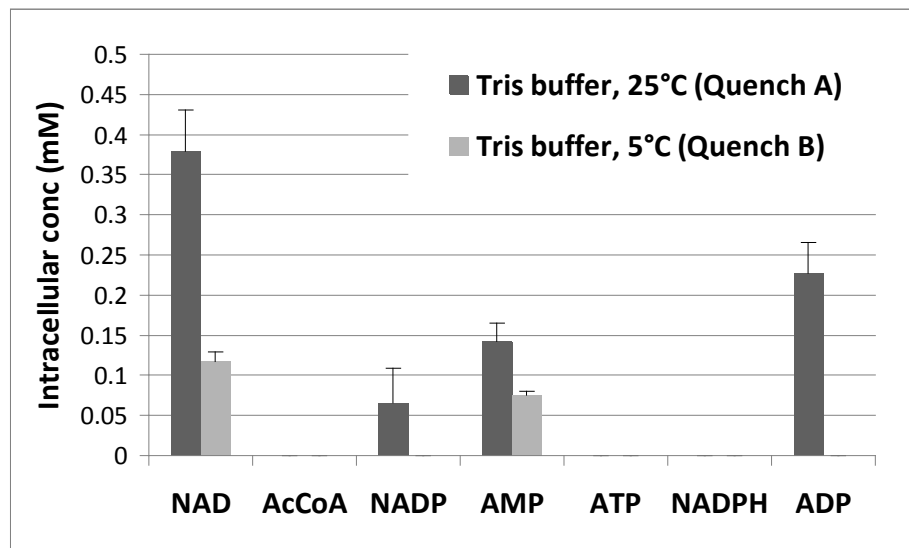
Quench solution B: 5°C 35 mM Tris buffer, pH 7.5

**Centrifugation:** 14, 000 rpm for 2 minutes

**Extraction conditions:** Boil cells in 90% ethanol/ 5 mM HEPES for 10 minutes



**Figure 4-6: Sample preparation flow chart**



**Figure 4-7: Effect of different quenching treatments on intracellular metabolite concentrations of *C. thermocellum***

Similar results were obtained in previous experiments (Section 4.3.2), in which samples from the 5°C HEPES quench treatment showed loss of NADP<sup>+</sup> and ADP, low intracellular concentrations of NAD<sup>+</sup> compared to the control samples from 25°C HEPES quench. These results combined with results from different tris buffer quenching suggest loss of metabolites is due to cold shock phenomenon alone and was unrelated to buffer properties.

#### **4.3.4 Supernatant analysis for evaluation of quenching protocols**

In all of the previous experiments it was seen that cold shock of quenching solution and/or methanol caused loss of metabolites. However, the results were indirect evidence, since metabolites escaped out of the cells were never detected directly. Therefore, NAD<sup>+</sup> was selected as a model metabolite and detected in quenching solutions (supernatants) to evaluate the leakage due to quenching. To avoid the interference of the salts from the media during analysis of supernatant, cells were separated from the media via centrifugation. Obtained cell pellet was suspended in required quenching solution which mainly contains HEPES buffer and methanol at varying composition and temperature. Supernatants were collected by centrifugation. For analysis of supernatants, HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] was removed from supernatant using anion exchange chromatography so that it would not interfere in CE analysis.

##### **A) Anion exchange chromatography for separation of HEPES from NAD<sup>+</sup>**

HEPES is a strong anion of sulfonic acid binds strongly to anion exchange resin and NAD<sup>+</sup> is a weak anion of phosphoric acid binds weakly to the anion exchange resin. Therefore in anion exchange chromatography, HEPES elutes after NAD<sup>+</sup>. To determine the recovery of NAD<sup>+</sup>, a known amount of NAD<sup>+</sup> was suspended in 140 mM HEPES solution and NAD<sup>+</sup> separated from HEPES using anion exchange chromatography; NAD<sup>+</sup> was recovered in 93.6 ± 10.4%.

**Conditions:** All steps are performed at RT unless otherwise noted

**Solution A:** 35 mM HEPES at 25°C

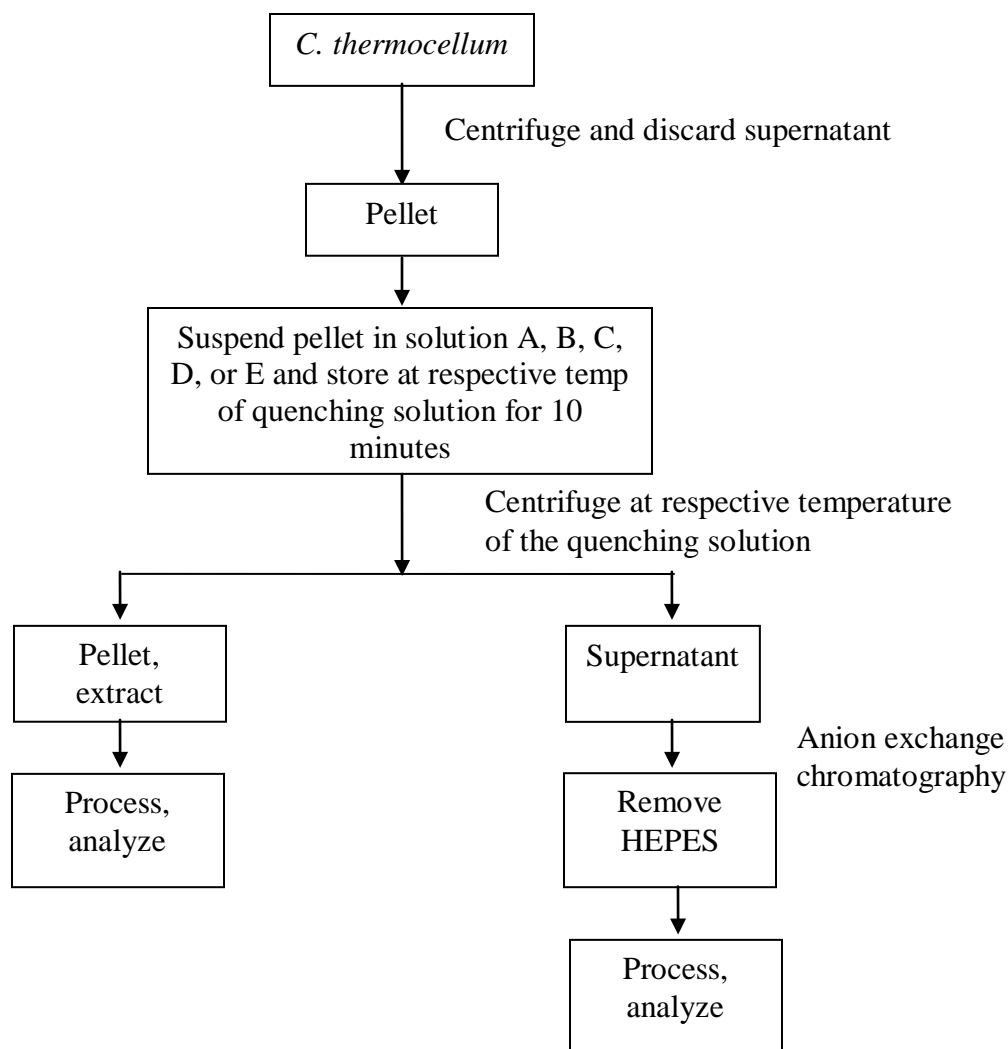
**Solution B:** 60% Methanol/ 35 mM HEPES at 25°C

**Solution C:** 35 mM HEPES at 5°C

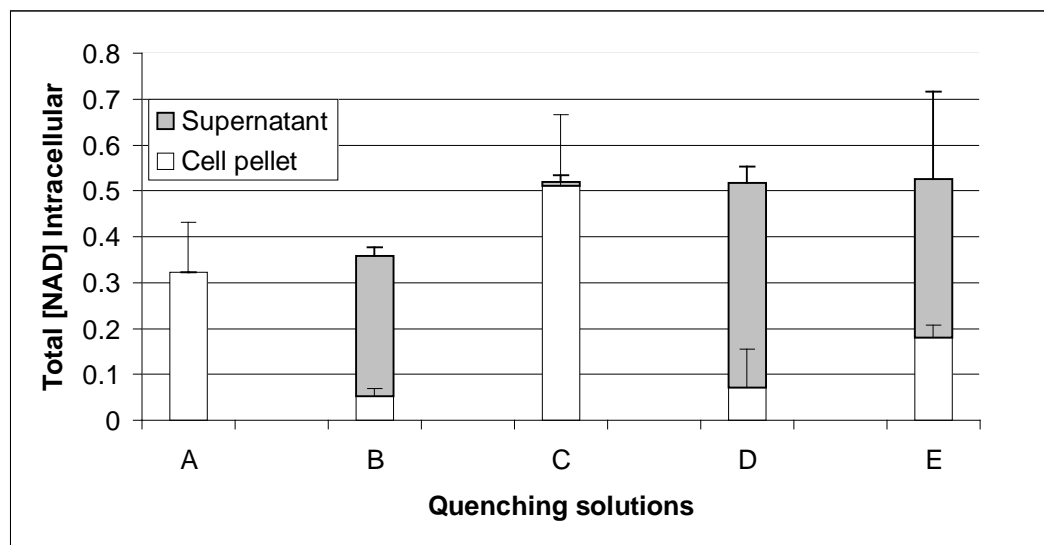
**Solution D:** 60% Methanol/ 35 mM HEPES at 5°C

**Solution E:** 60% Methanol/ 35 mM HEPES at -20°C

**Centrifugation:** 14,000 rpm for 2 minutes



**Figure 4-8: Sample preparation flow chart**



**Figure 4-9: intracellular concentration of NAD<sup>+</sup> in supernatant and cell extracts from different quenching treatments of *C. thermocellum***  
 Note: Height of the bar represents total intracellular NAD<sup>+</sup> concentration

Quenching solution	Cell pellet		Supernatant	
	[NAD <sup>+</sup> ] mM	% NAD <sup>+</sup> = $\left[ \frac{\text{Pellet}}{\text{Pellet} + \text{supernatant}} \right]$	[NAD <sup>+</sup> ] mM	% NAD <sup>+</sup> = $\left[ \frac{\text{Supernatant}}{\text{Pellet} + \text{supernatant}} \right]$
A	0.323 ± 0.108	100	ND	0
B	0.053 ± 0.015	15	0.304 ± 0.020	85
C	0.510 ± 0.150	98	0.010 ± 0.014	2
D	0.071 ± 0.082	14	0.445 ± 0.035	86
E	0.180 ± 0.028	36	0.345 ± 0.190	64

**Table 4-1: Intracellular concentration of NAD<sup>+</sup> in supernatant and cell pellet extracts from different quenching treatments of *C. thermocellum*, ND: Not detected**

## **B) Effect of quenching solutions on NAD<sup>+</sup> leakage**

Cells were centrifuged and media was discarded and resulting cell pellet was suspended in 5 different quenching solution including 35 mM HEPES at 25°C (A), 60% Methanol/ 35 mM HEPES at 25°C (B), 35 mM HEPES at 5°C (C), 60% Methanol/ 35 mM HEPES at 5°C (D), and 60% Methanol/ 35 mM HEPES at -20°C (E) for 10 minutes at the respective temperature of the quench solution. Cells were centrifuged and supernatants were saved and pellets from each treatment were extracted using boiling buffered ethanol method. NAD<sup>+</sup> was analyzed in supernatants as well as cell pellet. The procedure is summarized in Figure 4-8. Presence of NAD<sup>+</sup> in supernatant will provide impact of quenching solution on NAD<sup>+</sup> leakage and provide a direct evidence for leaking. Also from the amounts of NAD<sup>+</sup>, extent of leaking could be estimated. NAD<sup>+</sup> in the supernatants of various treatments were analyzed and amounts of NAD<sup>+</sup> in supernatants were divided by intracellular volume of the cells utilized, so that concentrations could be presented as intracellular concentration even though NAD<sup>+</sup> was present in supernatant. NAD<sup>+</sup> in cell pellet was also analyzed and obtained concentrations of NAD<sup>+</sup> in cell pellets as well as supernatants are shown in Figure 4-9.

Percentage of the NAD<sup>+</sup> inside the cell pellet and in the supernatant can be calculated by dividing the amounts of NAD<sup>+</sup> obtained by the total NAD<sup>+</sup> in the cell pellet and the supernatant as shown in Table 4-1. On analyzing supernatants, NAD<sup>+</sup> was not detected in supernatants from quenching A and C. The quenching solutions A and C are similar in terms of their composition and contain only HEPES solution (no methanol), however temperature of the treatment A and C was 25°C and 5°C, respectively. In contrast, a substantial amount of NAD<sup>+</sup> was detected in the supernatants of treatments B, D, and E. These 3 treatments were similar in composition and consisted 60% methanol/ 35 mM HEPES with temperatures of 25°C, 5°C, and -20°C, respectively. Percentages of NAD<sup>+</sup> in supernatants from treatments B, D, and E were 85, 84, and 64%, respectively, which suggests substantial amounts of NAD<sup>+</sup> escaped out of the cell in these quenching. Therefore, it can be concluded that regardless of temperature of the solution, methanol rendered NAD<sup>+</sup> leakage which was most probably due to damage to the cell membrane.

In summary, NAD<sup>+</sup> was detected in supernatants of all methanol containing quenching solutions, however NAD<sup>+</sup> was not detected in supernatants of 5°C and 25°C

HEPES quenching solutions, which suggests methanol is a key factor causing metabolites to leak from the cells. Therefore during quenching of *C. thermocellum*, methanol should not be used in quenching solution to avoid the leakage of metabolites.

#### **4.3.5 Filtration protocols**

From previous experiments, it is clear that methanol in quench solution as well as cold temperature cause NAD<sup>+</sup> and probably other metabolites to leak out of the cells. Therefore, neither methanol nor cold temperature could be used to quench the cells and hence sampling must be performed at the room temperature. Under such a scenario, the best option was to separate media as quickly as possible to keep the sampling time at a minimum and extract cells at room temperature which would provide very little time for metabolic changes to occur. The average sampling time for centrifugation was 12 to 15 minutes; therefore to speed up the sampling process, sampling by quick filtration was evaluated. Sampling time is the time between the cells taken out of the fermentation broth to the point immersed in the extraction solution.

For quick filtration, nylon and polyvinylidene fluoride (PVDF) were selected as choice of filter membranes with a pore diameter of 0.45 micron, since average diameter of *C. thermocellum* is 0.5 micron. However, sampling time could not be reduced using quick filtration, since the filtration does not reduce the sampling time substantially, these protocols were not applied for metabolite analysis. In fact, the filtration protocols were cumbersome (data not shown), since extensive washing for the filter membrane was required to remove any metabolites sticking to the filter membrane. Therefore, they were not utilized in any other analyses.

#### **4.3.6 Development of ambient temperature quenching method**

The investigation until this point suggests, cellulolytic *Clostridia* such as *C. thermocellum* can't be quenched using cold solutions, since below room temperature quenching solution resulted in metabolite loss. Therefore, it is essential to come up with a novel protocol for quenching of the cellular metabolism. Quenching of cellular metabolism is nothing but slowing down enzymatic activity by varying the physical conditions such as pH or temperature. Therefore, any action which affects enzymatic activity negatively is a good quenching process. To quench the metabolism advantage

can be taken that the *C. thermocellum* is a thermophile. Thermophilic bacteria grow well above the room temperature and it is well known that thermophilic enzymes are inactive at room temperature.<sup>131</sup> Therefore, bringing the temperature of the cells down to the room temperature from the growth temperature should make thermophilic enzymes significantly less active resulting in arrest of the metabolism. In other words, the metabolism of *C. thermocellum* can be quenched by bringing the temperature down from the growth temperature but not necessarily below room temperature. Figure 4-10 shows the activity of thermophilic enzymes at varying temperatures, which clearly shows about 80% activity of these thermophilic enzymes is lost at room temperature. Therefore, it can be hypothesized that metabolism of *C. thermocellum* is significantly slow at room temperature. To validate this assumption, a particular metabolite with relatively high turn over rate such as ATP (turnover rate 1.5 mM/sec) should be measured at two different points. First of all ATP should be measured right after the cells are taken out of the fermentation broth (sample  $t_0$ ), and secondly measuring the ATP after removing the media using centrifugation (sample  $t_1$ ). ATP concentration in sample  $t_1$  should not change even if the media is removed at the room temperature compared to  $t_0$ , since the activity of thermophilic enzymes is very low at room temperature. However, difficulties may arise when trying to detect ATP in *C. thermocellum*. First of all, in all the metabolite analysis of *C. thermocellum* performed so far, ATP was never detected, which is most likely due to lack of availability of an efficient extraction technique. To overcome this difficulty *C. thermocellum* was replaced with *Clostridium cellulolyticum* (*C. cellulolyticum*), another cellulolytic *Clostridia*. A method of ATP extraction (such as acid extraction) was developed for *C. cellulolyticum* by Petitdemange *et al.*<sup>67</sup> Using the acid extraction method, ATP could be reliably extracted from *C. cellulolyticum*. However, ATP can not be detected using CE since, acid extraction is incompatible with CE. Therefore enzymatic assay should be used to detect ATP, since salts do not interfere in enzymatic assays. *C. cellulolyticum* is very similar to *C. thermocellum* in many ways, such that both microorganisms are gram-positive, anaerobic, facultative, and originated from the class *Clostridia*. Therefore, it can be assumed that both microorganisms are similar in many biological characteristics and a new hypothesis was generated that

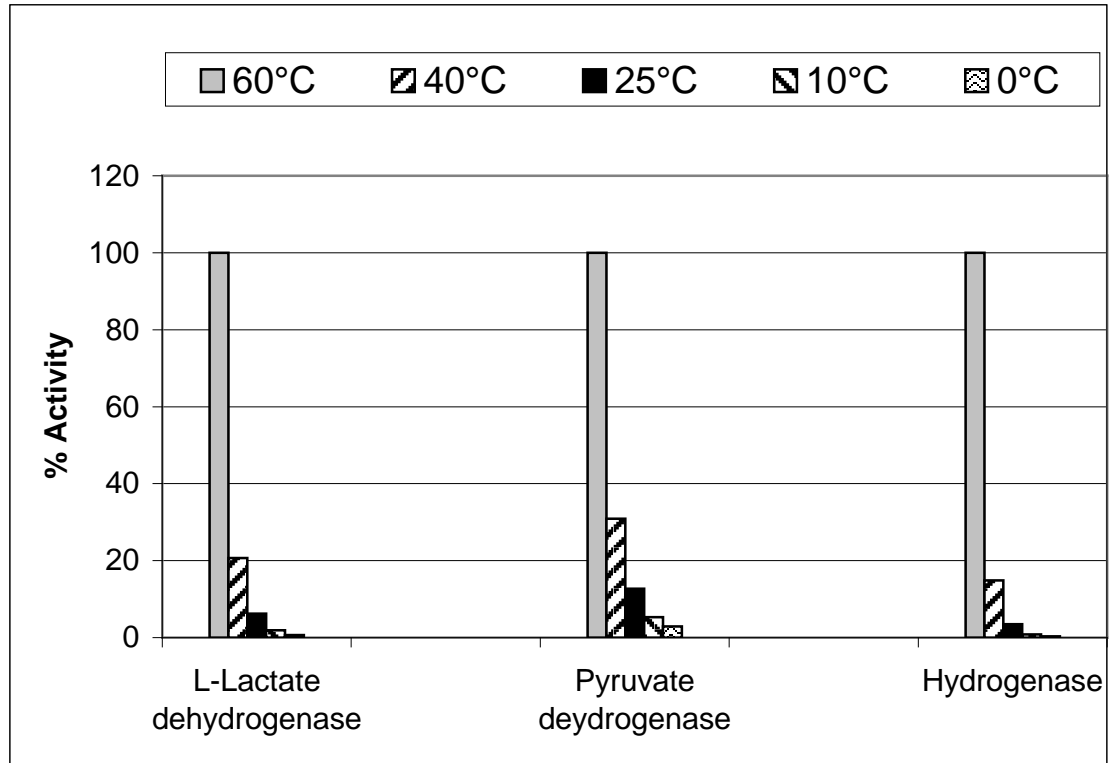


sampling of *C. cellulolyticum* at room temperature doesn't change intracellular ATP concentration.

To prove the hypothesis, *C. cellulolyticum* was processed 2 different ways (see Figure 4-12) and extracted using HCl. In the first sample, freshly growing cells were directly transferred to acid solution to extract the ATP and the extracted ATP was measured using enzymatic assay (this is referred sample  $t_0$ ). In second sample, cells were taken from the fermentation broth, centrifuged at room temperature to remove media, washed with an appropriate buffer, ATP is extracted using acid extraction and detected via enzymatic assay (this is referred sample  $t_1$ ). In sample  $t_0$ , intracellular ATP concentration represents concentration of ATP in actively growing cultures since ATP was extracted immediately after cells were taken out of the broth. Intracellular concentration of ATP in sample  $t_1$  will be very similar to samples  $t_0$ , since the metabolic activity in cells during the processing of sampling is significantly slow due to decreased activity of enzymes at ambient temperature. However, any variation in concentration of ATP in  $t_1$  compared to  $t_0$  will signify active metabolism and hypothesis will be rejected. In addition to sample  $t_0$  and  $t_1$  media was also analyzed for ATP.

#### **4.3.6.1 Enzymatic analysis of ATP**

Standard ATP solutions of 0, 4, 8, 16, 32, 64, 128, and 256 nM were prepared by diluting a 2000 nM ATP stock solution in to the ATP dilution buffer. Intensity for each concentration was measured using luminometer and a calibration plot of intensity versus concentration was generated as shown in Figure 4-11. ATP analysis was performed on the media and sample  $t_0$  and  $t_1$  and by using the calibration plot; concentrations of ATP were calculated as shown in Figure 4-13. Intracellular concentration of ATP obtained for samples,  $t_0$  and  $t_1$  were  $6.0 \pm 0.3$  and  $6.1 \pm 0.4$  mM, respectively; however no ATP was detected in media. There was no significant difference in the intracellular ATP concentrations of the sample  $t_0$  and  $t_1$ . These results suggest that, bringing *C. cellulolyticum* to the ambient temperature from its growth temperature of 37°C slows its metabolism substantially. Decreasing temperature by about 15°C substantially slows the activity of enzymes, which is the most likely reason that ATP concentration did not change while processing the sample.



**Figure 4-10 Effect of temperature on enzymatic activity of thermophilic enzymes<sup>132</sup>**

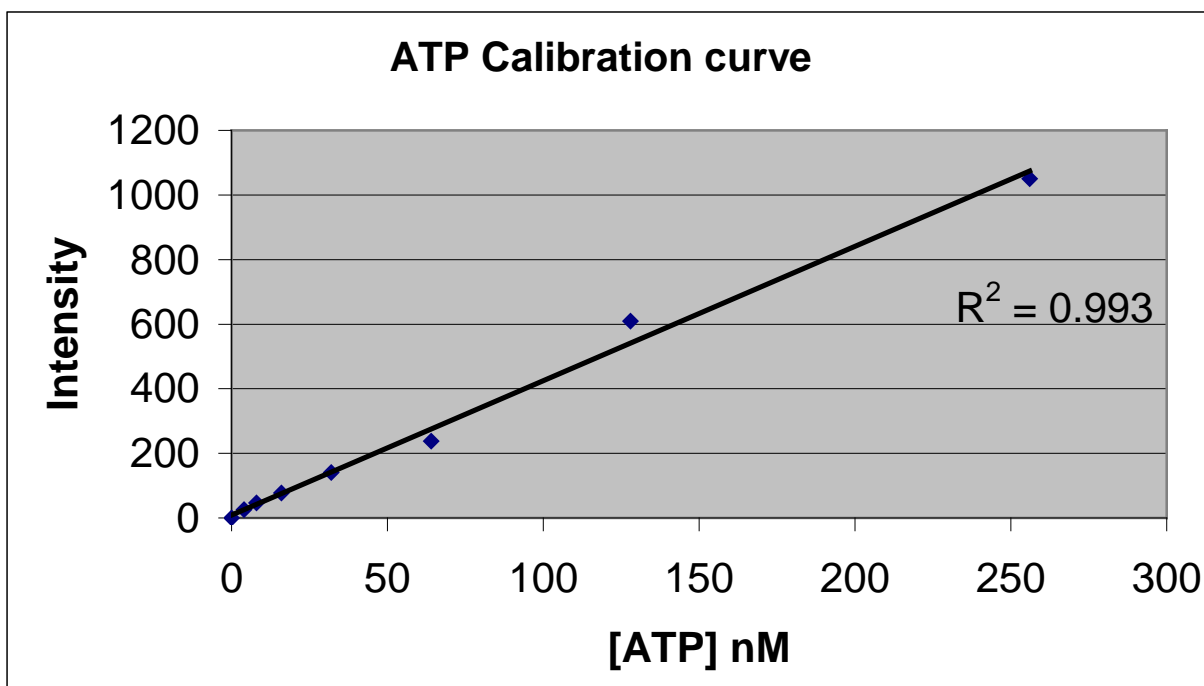


Figure 4-11: Calibration plot of ATP obtained using enzymatic assay

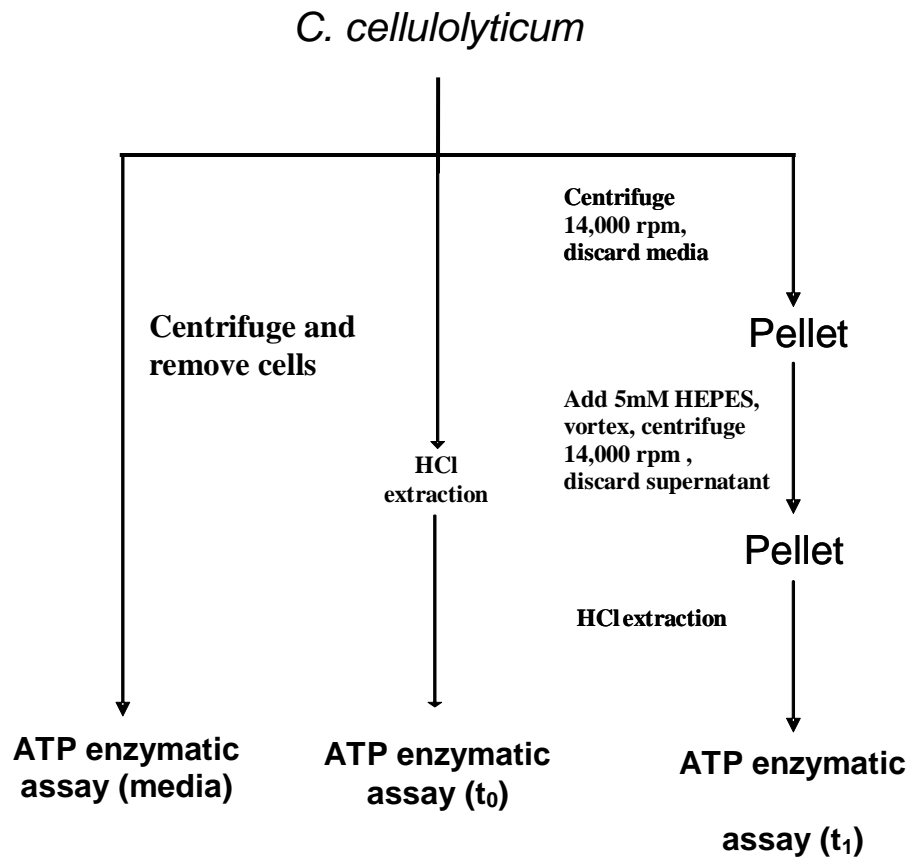
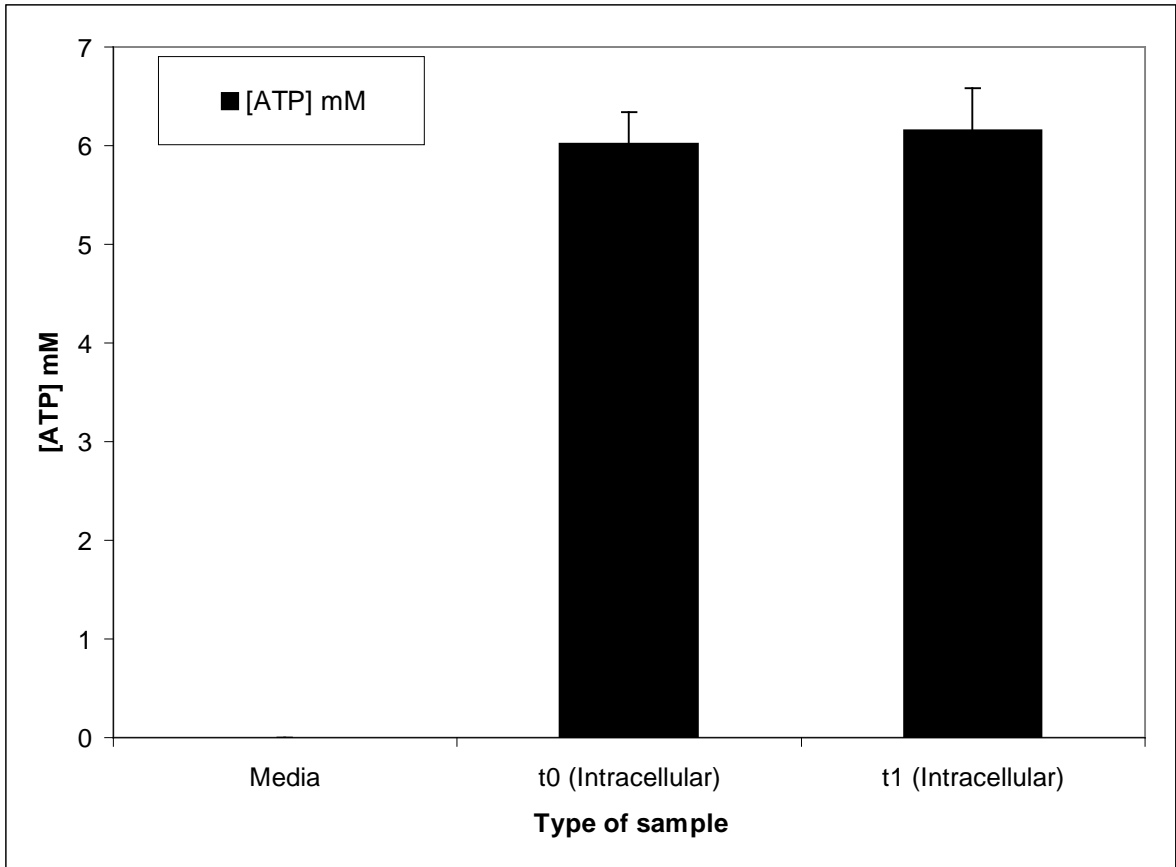


Figure 4-12: Sample preparation flow chart



**Figure 4-13: Intracellular ATP concentration of *C. cellulolyticum* obtained using enzymatic assay in different sample processing type (p = 0.07)**

Thus, performing the sampling at room temperature and keeping the sampling time short (few minutes), intracellular metabolite concentrations does not change. This developed quenching technique allows removal of media without affecting intracellular metabolite concentrations.

#### **4.3.7 Evaluation of extraction protocols**

After developing methods of quenching, next step in metabolite analysis is extraction of metabolites from the cells. An ideal extraction method should have high extraction efficiency and should be compatible with analysis method. Moreover, metabolites should be stable in the extraction solution during and after the extraction process. Extraction methods should be carefully evaluated for stability of metabolites and efficiency of extraction. Besides an extraction method to be compatible with CE, should leave minimum salts in the sample. Recently, new methods were developed using organic solvents to extract metabolites from bacterial cells as an alternative to acid/base extraction.<sup>70, 133</sup> By utilizing the organic solvents, formation of salts could be avoided and samples could be easily concentrated because of volatile nature of the organic solvents. However, methods of extraction using organic solvents were originally developed for gram-negative<sup>134</sup> bacteria and fungi.<sup>61</sup> Therefore, it is essential to evaluate extraction methods of organic solvents before their application for gram-positive bacteria to ensure efficient extraction of metabolites.

##### **4.3.7A Development of boiling buffered ethanol extraction method**

Recently a boiling ethanol extraction method was developed by Gonzalez and co-workers for metabolite extraction from yeast (*Saccharomyces cerevisiae*).<sup>61</sup> Since, these conditions are very harsh, it can be hypothesized that “*boiling ethanol will release the metabolites efficiently from cellulolytic organism*” According to Gonzalez and co-workers, presence of HEPES buffer in ethanol is essential for stability of metabolites during the boiling process and the concentration of HEPES determined to be 75 mM (final concentration). Since, they analyzed the extracted metabolites using enzymatic assays; presence of HEPES didn't affect the analysis of metabolites. However such buffered boiling ethanol protocol can't be utilized for CE analysis, since high concentration of HEPES would interfere in CE separation as well as in MS detection.

Particularly when samples are concentrated to increase the sensitivity of analytes, the final concentration of HEPES increases dramatically. To apply buffered boiling extraction protocols it is essential to reduce the HEPES concentration in extraction solution so that it would not affect the CE analysis; however at the same time decreased concentration of HEPES should not compromise the stability of metabolites in the process of boiling. Therefore, boiling ethanol/ HEPES protocol to be compatible with CE, amount of HEPES should be optimized. For optimization of HEPES concentration, 3 factors must be verified. The first factor is upper limit of the HEPES in the sample for CE analysis; second is the lower limit of the HEPES in boiling ethanol for stability of metabolite; and finally the stability of metabolites for a longer time period (15 to 20 minutes) in boiling ethanol/buffer solution.

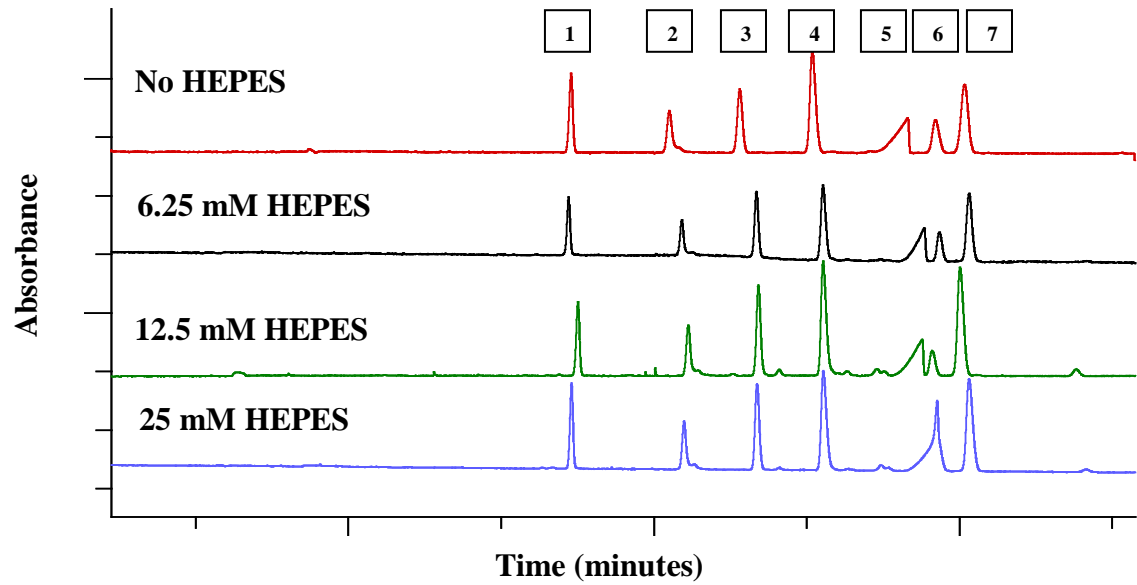
#### **Upper limit of HEPES in the sample for CE analysis**

To find out the upper limit of HEPES in the sample for CE separation, mixtures of 7 standard metabolites prepared in various concentrations of HEPES (0, 6.25, 12.5, and 25 mM) were analyzed by CE, and obtained electropherograms are shown in **Figure 4-14**. All analytes were baseline separated when samples containing 6.25 and 12.5 mM of HEPES were analyzed by CE. However, on analyzing the standard metabolites containing 25 mM HEPES by CE, the resolution between ATP and NADPH was completely lost and these two peaks appeared as one. Higher concentration of HEPES increased the conductivity of the solution, because of which resolution decreased. Therefore, the maximum concentration of HEPES allowed in a sample without affecting the resolution was 12.5 mM.

#### **Lower limit of HEPES in boiling ethanol for stability of metabolites**

In metabolite analysis, the extraction solution is usually concentrated by about 25 times to increase the sensitivity. Therefore, concentration of a 0.5 mM HEPES extraction solution by 25 times will increase the concentration of HEPES to 12.5 mM in the sample. As a result, concentration of HEPES in extraction solution should be close to 0.5 mM so that after concentrating the sample, final concentration of HEPES would not go beyond 12.5 mM and resolution on CE could be preserved. Thus, the stability of metabolites between 0 to 1 mM HEPES solution was investigated.

1. NAD<sup>+</sup>
2. Acetyl-CoA
3. NADP<sup>+</sup>
4. AMP
5. ATP
6. NADPH
7. ADP



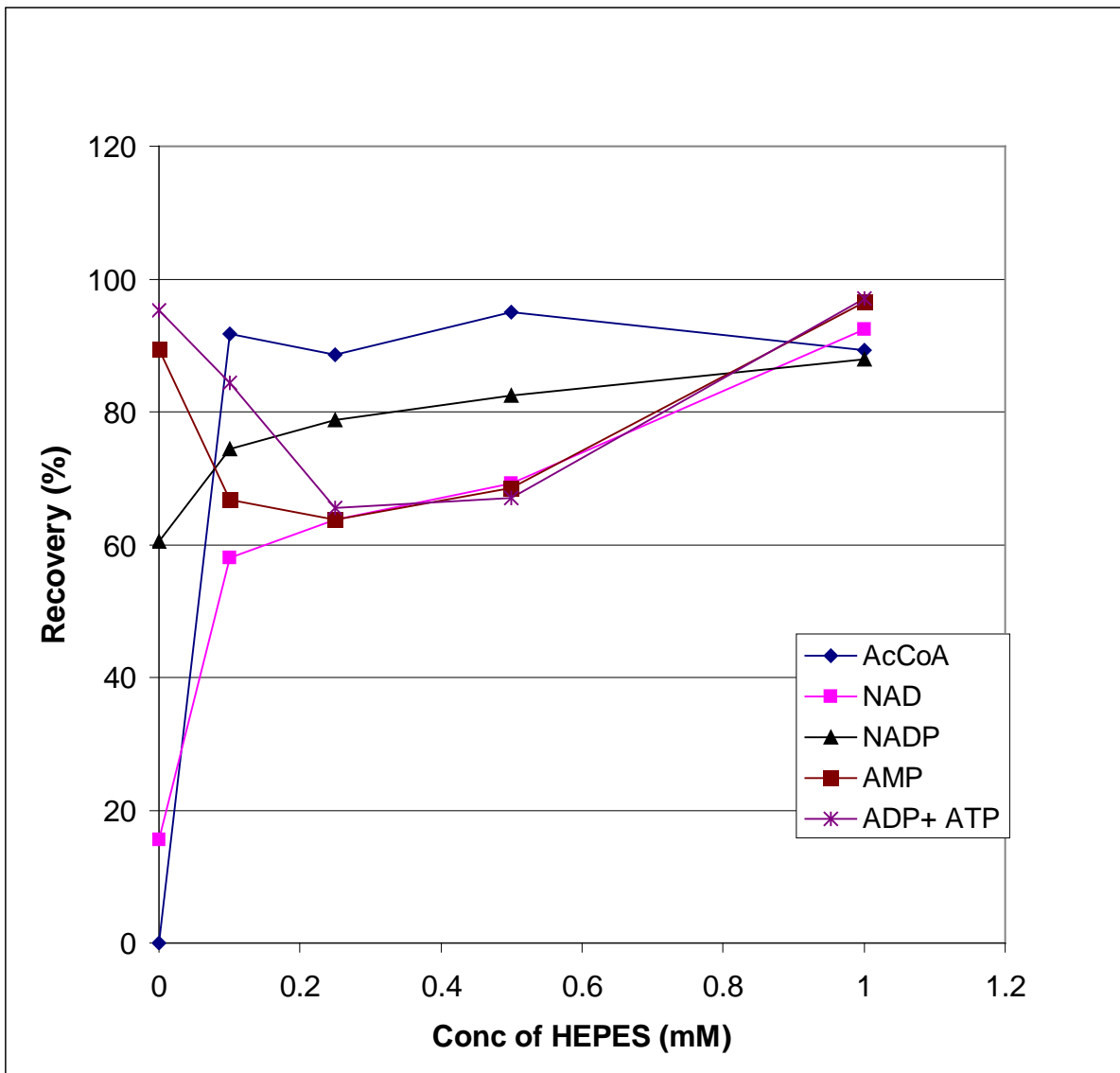
**Figure 4-14: Effect of increasing HEPES concentration in samples on separation**



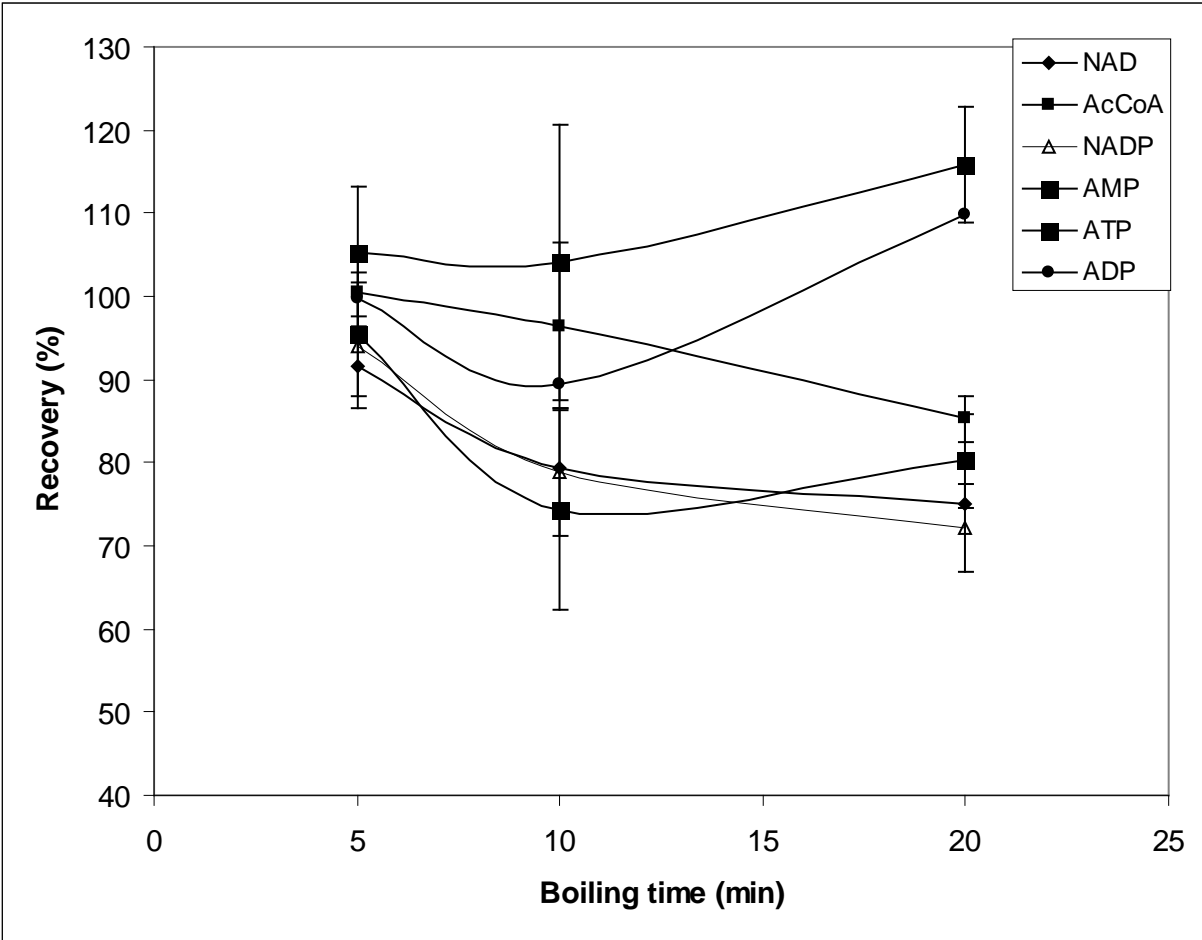
To examine the effect of HEPES concentration on stability of metabolites in boiling ethanol, a 90% ethanol/ HEPES solution, containing 0, 0.10, 0.25, 0.50, and 1.00 mM HEPES (final concentration) were prepared. To each solution, a mixture of  $\text{NAD}^+$ ,  $\text{NADP}^+$ , NADPH, Acetyl-CoA, AMP, ADP, and ATP was added in equal amounts and boiled for 3 minutes. After finishing the boiling, solutions were cooled on ice for 10 minutes, concentrated using partial vacuum by 25 times, and analyzed using CE. Recovery of each metabolite was plotted against the concentration of HEPES in boiling solution, and the obtained graph is shown in Figure 4-15. From this graph it can be seen that at 1 mM of HEPES, all the metabolites were recovered in more than 85%, however as concentration of HEPES decreased the metabolites become more unstable. At 0.1, 0.25, and 0.5 the recovery of all metabolites was still greater than 58%. However, in complete absence of HEPES recoveries of  $\text{NAD}^+$  and acetyl-CoA were less than 20%. However, adenosine phosphates (AMP, ADP, and ATP) were recovered in more than 60% regardless of HEPES concentrations in the boiling ethanol solution. Thus, 0.5 mM is the upper limit of HEPES since all the metabolites were recovered in more than 60% after boiling for 3 minutes.

#### **Effect of boiling time on stability of metabolites**

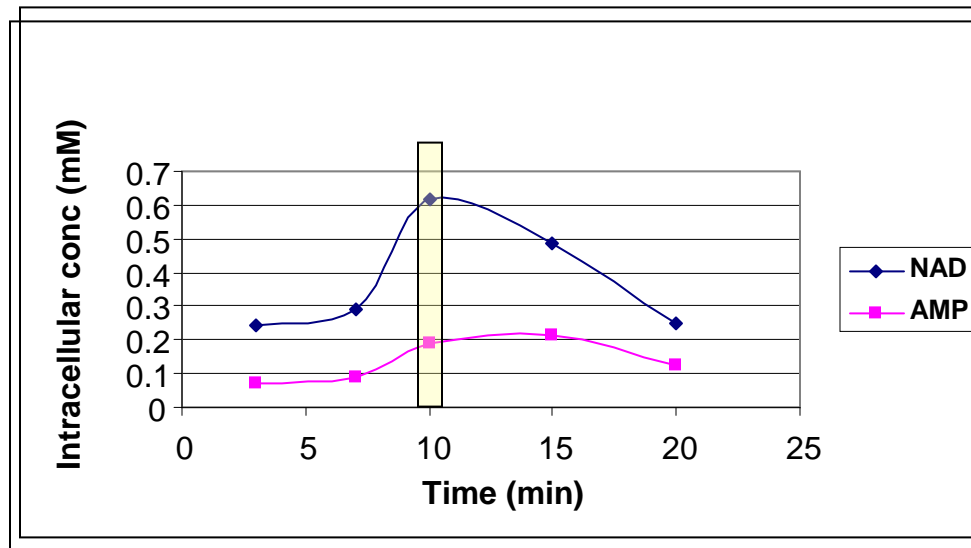
As shown before in absence of HEPES some metabolites could be unstable in boiling ethanol, therefore HEPES is certainly essential for metabolite stability in boiling ethanol. In the above experiments, it was shown that metabolites were stable in boiling 90% ethanol/ 0.5 mM HEPES (Final concentration) solution for 3 minutes. However, extraction of metabolite from cells could take longer than 3 minutes for efficient release of metabolites. Therefore, it was essential to test the stability of metabolites in boiling ethanol for longer period of time. To investigate effect of boiling time on stability of metabolites, a mixture of standard metabolites viz.  $\text{NAD}^+$ ,  $\text{NADP}^+$ , acetyl-CoA, AMP, ADP, and ATP was spiked in 90% ethanol/ 0.5 mM HEPES and boiled for 5, 10, and 20 minutes. After boiling, samples were concentrated and analyzed using CE and recovery of each metabolite was calculated. A plot of boiling time versus recovery of the metabolites is shown in Figure 4-16, which suggests metabolites are stable in boiling 0.5 mM 90% ethanol/ 0.5 HEPES solution for up to 20 minutes and recoveries for all metabolites were higher than 60%.



**Figure 4-15: Effect of HEPES concentration on stability of metabolites in boiling ethanol solution**



**Figure 4-16: Effect of boiling time on stability of metabolites**



**Figure 4-17: Effect of boiling time on release of metabolites from cells**

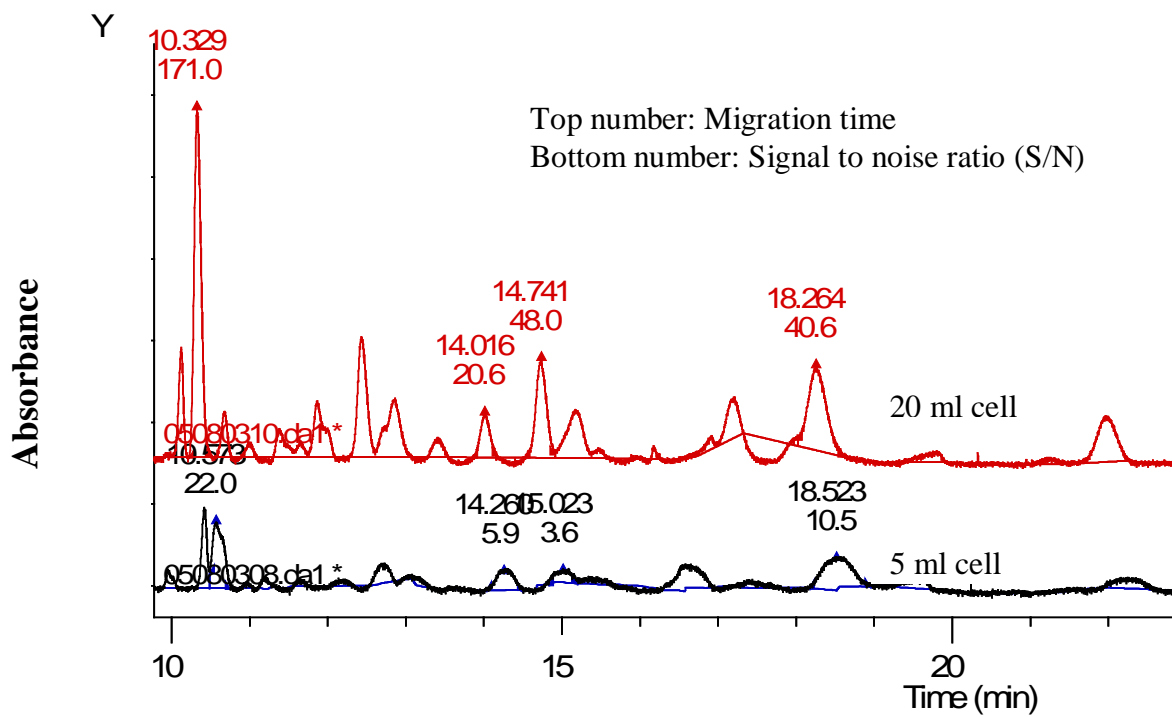


Figure 4-18: Effect of cell concentration on signal to noise ratio

#### **4.3.7B Effect of boiling time on release of metabolites**

To determine the extent of boiling for efficient release of metabolites, cells were separated from media and boiled in 90% ethanol/ 0.5 mM HEPES, pH 7.5 (final concentration) solution for 3, 7.5, 10, 15, and 20 minutes and samples were analyzed using CE.  $\text{NAD}^+$  and AMP were selected as model metabolites and their intracellular concentrations were obtained for all samples. Plots of intracellular concentrations of these metabolites versus boiling time were generated as shown in Figure 4-17. From the plot it can be seen that, with the increasing incubation time the concentration of  $\text{NAD}^+$  and AMP was increasing until 10 minutes. However, after 10 minutes the concentrations of  $\text{NAD}^+$  and AMP started decreasing most likely due to disintegration of these metabolites because of boiling for a longer period of time in the presence of the cellular matrix. Therefore, 10 minutes of incubation time was adequate to release the metabolites efficiently.

#### **4.3.7C Optimization of cell concentration**

In order for a metabolite to be detected, the amount of analyte should be above the limit of detection. Therefore, it is crucial to determine the cell concentration necessary so that metabolites could be detected. To determine the optimum cell concentration, 5 and 20 ml cells were quenched using ambient temperature centrifugation methods, washed, and obtained pellets were boiled in 90% ethanol/ 0.5 mM HEPES (final concentration) for 10 minutes. Supernatants containing metabolites were concentrated to a final volume of 200  $\mu\text{L}$  for both samples and analyzed using CE. Obtained electropherograms are shown in Figure 4-18; the top number on the peak shows migration time and the bottom number shows signal to noise ratio for that peak. When 5 ml cells were used only  $\text{NAD}^+$  was detected, however, when 20 ml cells were used, in addition to  $\text{NAD}^+$  metabolites such as  $\text{NADP}^+$ , AMP and ADP were detected (data not shown). Therefore, when 20 ml cells were harvested, final sample volume was set to 200  $\mu\text{L}$  or else when 2 ml cells were harvested final sample volume was set to 20  $\mu\text{L}$ .

#### 4.3.8 Evaluation of boiling ethanol extraction method

*C. cellulolyticum* were quenched using ambient temperature quenching method and metabolites were extracted using boiling ethanol and room temperature chloroform/methanol extraction methods. All the samples were analyzed using CE-ESI-MS for comparison and obtained intracellular concentrations are shown in Figure 4-19. All the metabolites detected in boiling ethanol extraction method were detected in room temperature chloroform/methanol methods as well, except  $\text{NAD}^+$  and  $\text{NADH}$ , which was surprising. Careful observation of mass spectra of extracted ion electropherograms for  $\text{NAD}^+$  and  $\text{NADH}$  showed that these peaks were suppressed due to HEPES in the sample. Left over HEPES during the washing step of the cell was the source of HEPES in the sample. HEPES ion has migration time close to the migration times of  $\text{NAD}^+$  and  $\text{NADH}$ . Therefore, prevalence of HEPES in sample left  $\text{NAD}^+$  and  $\text{NADH}$  undetected. However, on comparing the concentration of all the metabolites, it was observed that intracellular concentrations of AMP, G6P,  $\text{NADP}^+$ , ADP, ATP,  $\text{NADHP}$ , and FBP derived from room temperature chloroform/methanol extraction method were higher compared to boiling ethanol extraction method. Therefore, the hypothesis that harsh condition of boiling ethanol will extract metabolite efficiently was rejected. Boiling in ethanol, probably did not work that efficiently because ethanol is relatively polar and could not disrupt the lipid bilayer membrane effectively like chloroform, hence failed to extract metabolites efficiently.

Since, room temperature chloroform/methanol was more efficient, it was essential to minimize the HEPES concentration in washing step so that  $\text{NAD}^+$  and  $\text{NADH}$  can be detected and this extraction method could be used for metabolite analysis. During this optimization, in a serendipitous experiment, after transferring the cells in chloroform/methanol were stored at  $-80^\circ\text{C}$  overnight. Next morning, it was analyzed and surprisingly intracellular metabolite concentrations of all the metabolites were much higher compared to room temperature chloroform/methanol extraction, which prompted to evaluate the effect of cold temperature on metabolite extraction.

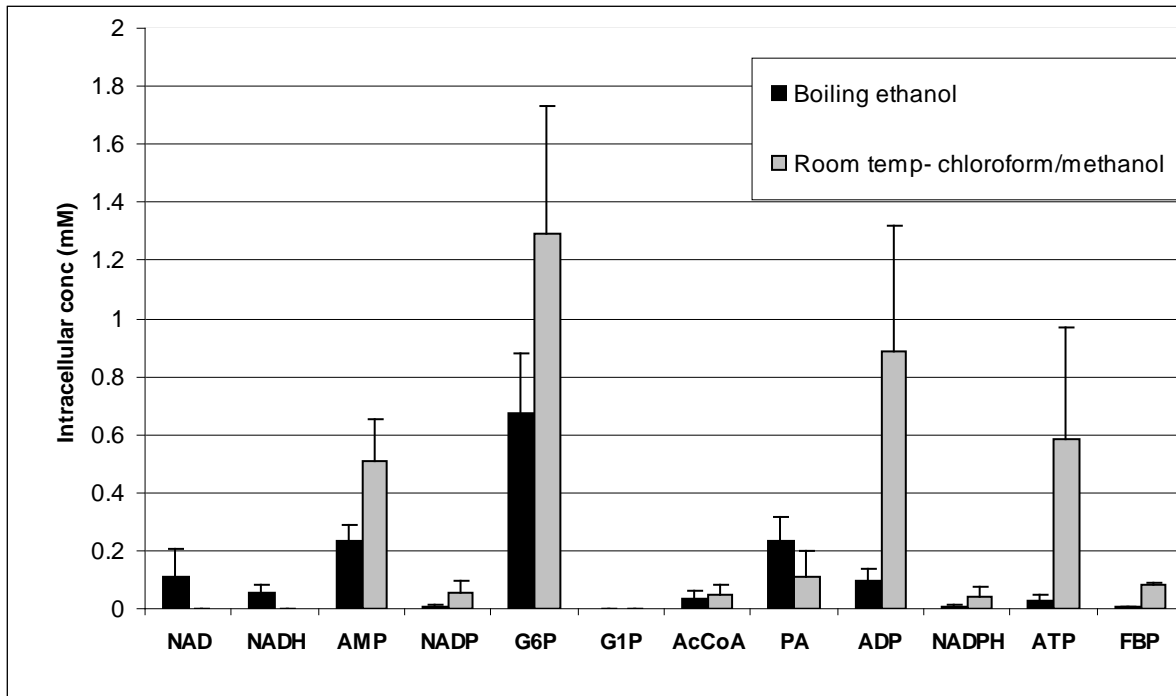
#### **4.3.9 Development of ultra-cold chloroform/methanol extraction method**

From previous observations it can be hypothesized that, extraction efficiency of cold chloroform/methanol will be higher than room temperature chloroform/methanol extraction. To evaluate the extraction efficiency of cold chloroform/methanol method, *C. cellulolyticum* cells were transferred in to a chloroform: methanol: water (2:1:1) solution and frozen at -80°C. This method is termed as ultra-cold chloroform/methanol solution (UCCM). Extraction efficiency of this method was compared with room temperature chloroform/methanol method. Concentrations of any particular metabolite derived from UCCM extraction method was always higher compared to room temperature chloroform/methanol extraction method (see Figure 4-20). In the UCCM method, chloroform disrupts the highly arranged lipid bilayer membrane which probably creates voids in the cell wall and during the freezing of solutions, water inside the cells expand which further help to break the lipid bilayer membrane and releases metabolites in the solution when cells are thawed. Since there is no freeze thaw cycle in the room temperature chloroform/methanol extraction method, it is less efficient than UCCM extraction method. The presence of chloroform is very important to disrupt the cell membrane. Freeze-thaw cycle further helps to rupture the cells and maximize the metabolite extraction.

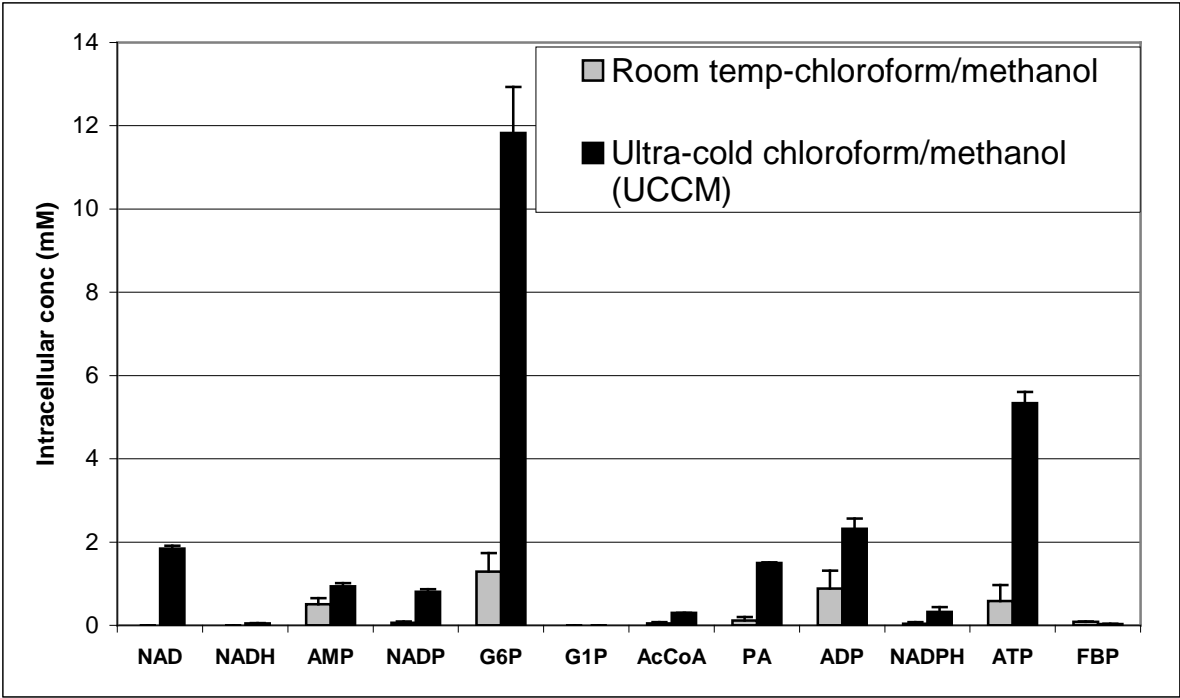
#### **4.3.10 Method validation**

To validate the newly developed UCCM extraction method and CE-ESI-MS analysis method, *C. cellulolyticum* cells were quenched using ambient temperature quenching method and acid extraction and UCCM extraction were carried out on the cells. Samples from the HCl extraction were analyzed using the enzymatic assay to detect ATP, however sample from the UCCM extraction was analyzed using the enzymatic assay and CE-ESI-MS to detect ATP (see Figure 4-21). Figure 4-22 shows the intracellular concentration of ATP in acid extraction method and UCCM extraction methods analyzed by enzymatic assay and CE-ESI-MS. Comparison of these 3 intracellular ATP concentrations showed no significant difference.

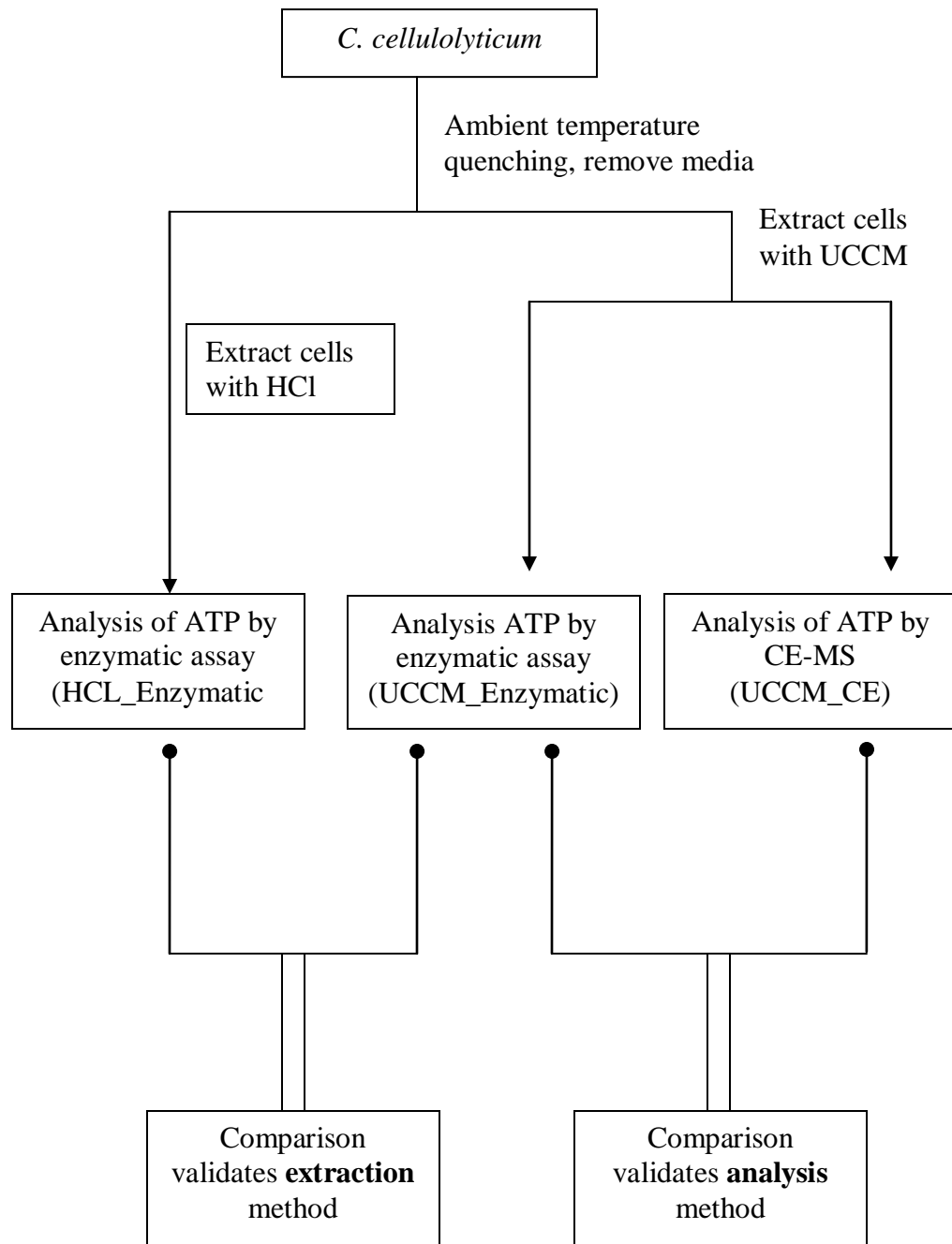




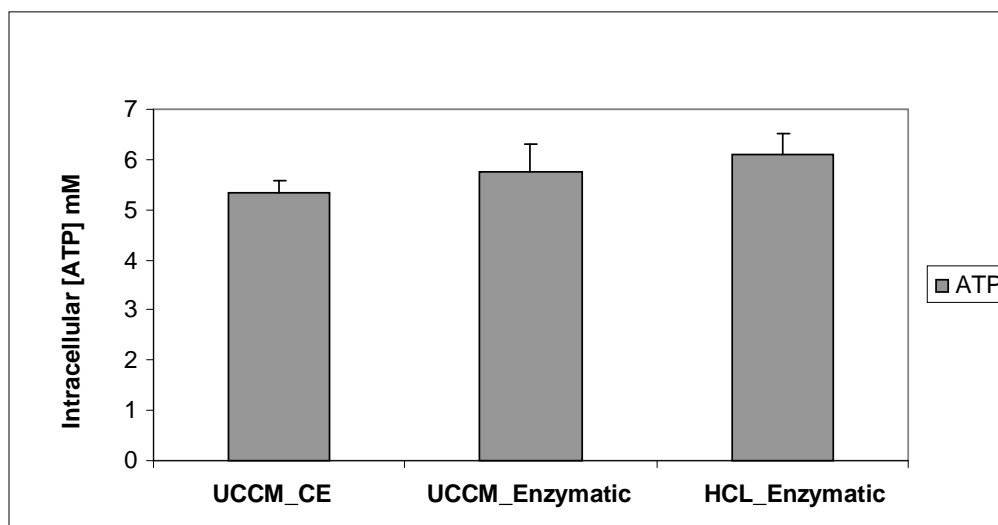
**Figure 4-19: Comparison of boiling ethanol extraction method with chloroform/methanol extraction method**



**Figure 4-20: Comparison of room temperature chloroform/methanol extraction method with ultra-cold chloroform/methanol (UCCM) extraction method**



**Figure 4-21 Sample preparation protocol for extraction and analysis method validations**



**Figure 4-22: Validation of extraction and analysis methods for ATP  
(ANOVA:  $p = 0.34$ )**

The comparison of ATP concentrations obtained using UCCM extraction method was equal ATP intracellular concentration obtained using traditional acid extraction method which suggests UCCM is an efficient and valid method of extraction of metabolites. The comparison of intracellular ATP concentrations obtained using enzymatic assay and CE-MS from UCCM extraction methods suggest CE-MS method is a valid technique of quantification of metabolites. Therefore, both CE-ESI-MS detection method and newly developed UCCM extraction method were validated using enzymatic assay and acid extraction protocols, respectively.

#### **4.4 Conclusions**

A thorough investigation of quenching and extraction protocols for cellulolytic clostridia was performed in this chapter. Application of literature published cold methanol quenching caused leakage of metabolites. Buffer precooled to 5°C also caused metabolite loss, which clearly demonstrates that cold temperature is not good method of quenching this type of microorganism. Methanol in quenching solution probably compromised membrane integrity and worsened the metabolite leakage. To overcome these obstacles in quenching, novel quenching protocols were established. In ambient temperature quenching, ATP concentration in the samples processed at room temperature was unchanged compared to ATP concentration in actively growing cells. Therefore, it was concluded that by bringing the cells to ambient temperature from its growth temperature metabolism can be quenched efficiently.

Followed by quenching protocols, metabolite extraction protocols were developed and evaluated. For boiling ethanol extraction method, presence of HEPES buffer was essential for stability of metabolites. Once the boiling ethanol protocol was developed, several parameters such as boiling time, efficiency of metabolites release, and cell concentration were optimized. Metabolites were extracted using the boiling ethanol extraction method and room temperature chloroform/methanol extraction method. Room temperature chloroform/methanol demonstrated higher efficiency for many metabolites than boiling ethanol extraction method. The effect of freezing the chloroform/methanol on metabolite extraction was also studied and in fact efficiency of ultra-cold chloroform/methanol extraction was higher than room temperature chloroform methanol.

Later study demonstrated that ultracold chloroform/methanol extraction protocol was as efficient as traditional acid extraction method, which is the benchmark of extraction methods. CE-ESI-MS method was also validated using enzymatic assay, which is the benchmark of metabolite analysis methods. In summary, room temperature quenching and ultra-cold chloroform/methanol extraction are efficient quenching and extraction methods for cellulolytic clostridia.

**Chapter 5 Application of developed quenching and extraction protocols for intracellular metabolite analysis of wild type and ethanol adapted *Clostridium thermocellum***

## **5.1 Introduction**

*Clostridium thermocellum* is one of the few microorganisms having the capacity to convert cellulose to ethanol. Since cellulose is an inexpensive starting material, final cost of ethanol produced is much lower compared to ethanol produced from simple sugar (corn) fermenting bacteria such as yeast. As ethanol is becoming more attractive as an alternative fuel for automobiles, cellulolytic bacteria such as *C. thermocellum* are receiving increased attention. However, the inefficient conversion of carbon to ethanol by *C. thermocellum* and low production yield of ethanol in fermentation broth has hindered its commercial exploitation. Unlike other ethanol tolerant microorganisms such as yeast which can tolerate greater than 10% of ethanol,<sup>21</sup> growth of *C. thermocellum* is strongly inhibited at relatively low ethanol concentrations (< 2%).<sup>1</sup> Ethanol concentrations as low as 0.5% (w/v) in a fermentation broth results in a 50% reduction in growth rate.<sup>20</sup> Ethanol is reported to affect variety of cellular processes in bacterial as well as mammalian cells which influence their normal functioning. Several mechanisms have been reported by which ethanol affects normal cellular functioning,<sup>25, 26</sup> however the exact mechanism by which ethanol causes growth inhibition in *C. thermocellum* is not completely understood. Recently, *C. thermocellum* cells with increased ethanol tolerance were developed, and are able to grow in as high as 5% ethanol (w/v).<sup>1</sup> To understand ethanol tolerance of EA cells, intracellular metabolite analyses were conducted on WT and EA *C. thermocellum* cells. A comparison of intracellular metabolite concentrations in these two strains should reveal the mechanism by which ethanol influences the cell growth of WT cells and the basis of the ethanol tolerance in EA cells. In addition, obtained metabolite data could be used by genetic engineers to develop superior strains using recombinant DNA technology to further increase ethanol tolerance for commercial production of ethanol from cellulose in consolidate bioprocessing.

Although metabolite profiling revealed differences in intracellular metabolite concentrations between the batch cultures of WT and EA strains, to fully understand the effect of ethanol and its tolerance by EA strain, ethanol perturbations were carried out on both strains and its effect on intracellular metabolite concentrations was studied. For ethanol perturbations, cells were grown in continuous mode in a bioreactor, since in the bioreactor ethanol concentration in the medium can be easily controlled and remained constant throughout the experiment. WT strain was perturbed with 0, 0.5, 1.0, and 5% exogenous ethanol (w/v), while EA strain was perturbed with 0, 1, 5 and 7% exogenous ethanol (w/v), and metabolite analysis was conducted.

## **5.2 Materials and Methods**

### **5.2.1 Organism and cultivation conditions**

Same as in Chapter 4 (section 4.2.1 and 4.2.2)

### **5.2.2 Continuous culture experiments**

Continuous cultures were grown by Dr. Satyakrishna Jujjuri in Dr. Barbara Knutson's lab at Department of Chemical engineering, University of Kentucky. Standard parameters were as follows: The exogenous ethanol continuous cultures of the wild-type (0 to 5% w/v ethanol) and ethanol-adapted (0 to 8% w/v ethanol) strains were performed at atmospheric pressure (0.1 MPa) and  $T = 55^{\circ}\text{C}$  in a 100-ml stainless steel Parr mini reactor (rated to 623 K, 20.7 MPa) continuously stirred using an impeller (300 rpm). Details of the continuous cultures are described in the past.<sup>4</sup>

The short procedure was as follows. After inoculation, the system was kept in batch mode for 24 h before initiating a continuous flow of medium. Deoxygenated medium containing cellobiose at 4 g/l was fed into the bioreactor *via* the syringe pump to maintain the desired dilution rate ( $D = 0.05 \text{ h}^{-1}$ ). Here dilution rate was selected based on the exogenous ethanol batch culture experiments of the WT and EA strains where maximum ethanol tolerance and similar growth rates were the selection criteria (data not shown). The continuous culture samples were collected from the reactor after 95% turnover and the culture steady state was verified by the cell density measurement, except for the highest exogenous ethanol concentrations of 5% for WT and 7% for EA where cultures were not at steady state and cells were completely washed out.



### 5.2.3 Sampling, quenching, and extraction

Once the cell density was measured, 2 ml of cells were withdrawn from the growing batch/continuous cultures and immediately centrifuged (14,000 rpm for 2 minute) at room temperature to quench the cells. Obtained cell pellet was washed with 1 ml of 5 mM HEPES buffer and the cell pellet was transferred into 4 ml of a chloroform: methanol: water (2:1:1) mixture. The mixture was vortexed well and immediately frozen at -80°C overnight; next morning it was thawed at room temperature. The top aqueous layer containing polar metabolites was separated from the chloroform layer and passed through 10,000 Da cut-off filter to remove any left over proteins and cell debris. The obtained filtrate was then concentrated to a volume of about 20  $\mu$ L and analyzed.

### 5.2.4 Spiking of metabolites

Before extraction, a mixture of known concentrations of standard metabolites containing  $\text{NAD}^+$ ,  $\text{NADP}^+$ , AMP, ADP, ATP, acetyl-CoA, G1P, G6P, FBP, pyruvic acid, and labeled AMP\*[Adenosine- $^{13}\text{C}_{10}$ ,  $^{15}\text{N}_5$  5'-monophosphate] was spiked in the extraction solution to determine any loss of metabolite during sample preparation protocols.

### 5.2.5 CE-ESI-MS conditions

Same conditions as per in Chapter 3 (Section 3.2.3)

### 5.2.6 Identification and quantification of metabolites

All the samples were analyzed using CE-ESI-MS and metabolites were identified by comparing their migration times and m/z values with the standards. Identity of all metabolites was confirmed by spiking a known amount of the standard into the cell extracts. Quantification was performed by interpolating the area of  $[\text{M-H}]^-$  peak for each metabolite against the calibration curve.

### 5.2.7 Calculation of intracellular volume

Concentration of each metabolite derived from calibration curve was converted to intracellular concentration by dividing it with the intracellular volume. Intracellular volume  $V_{Intra}$  is a function of the cell density (0.4 mg/mL), sample volume  $V_s$  (2 mL), protein content  $P$  (0.5 mg/mg of cell density), and specific cell volume  $V_{sp}$  (3 $\mu$ L/mg of protein),<sup>135</sup> which is governed by the formula below:

$$V_{Intra} = \text{cell density} \cdot V_s \cdot P \cdot V_{sp} \quad \text{Equation 5-1}$$

### 5.2.8 Statistical analysis

ABSTAT 1.96 was used for all statistical analysis. In ANOVA test, when at least one mean was significantly different than other two means, Tukey's test was performed as a *post hoc* test to compare all means to each other.

## 5.3 Results

### 5.3.1 Recovery of spiked metabolites

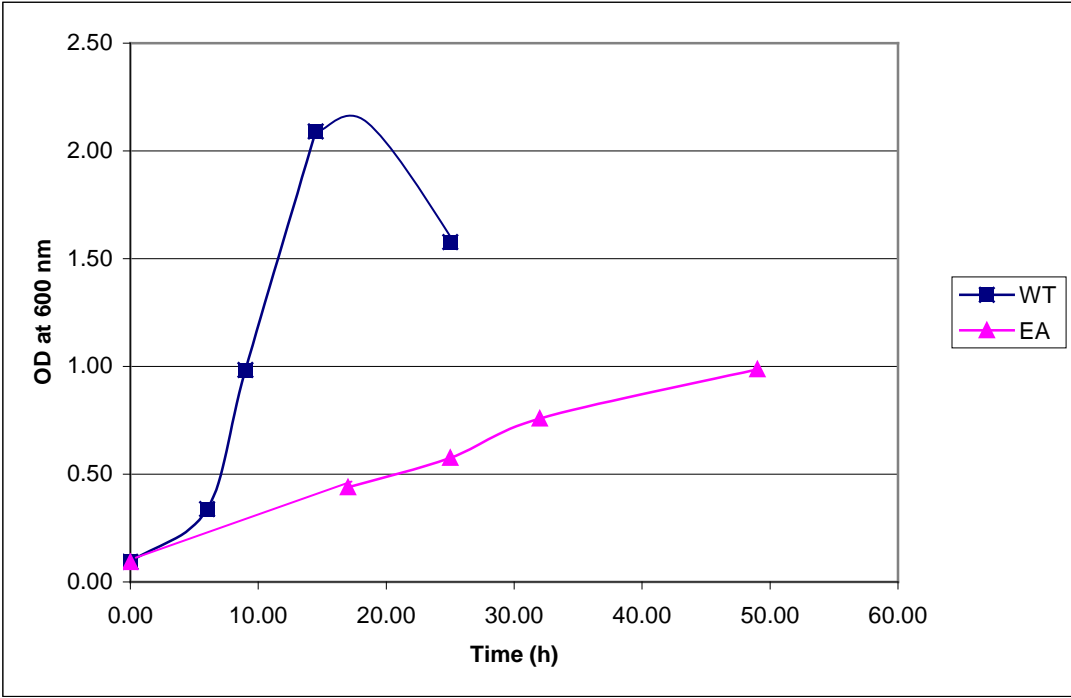
Recovery of all the spiked metabolites were higher than 100 percent because of presence of intracellular metabolites (data not shown) except NADH and NADPH. Recoveries of reduced nicotinamide adenine dinucleotides were less than 20% because of their unstable nature at room temperature. Therefore NADH and NADPH could not be quantified in this study. However, recovery of labeled AMP was  $103 \pm 2\%$  suggesting that sample processing was efficient.

### 5.3.2 Growth characteristics of WT and EA *C. thermocellum* in batch culture

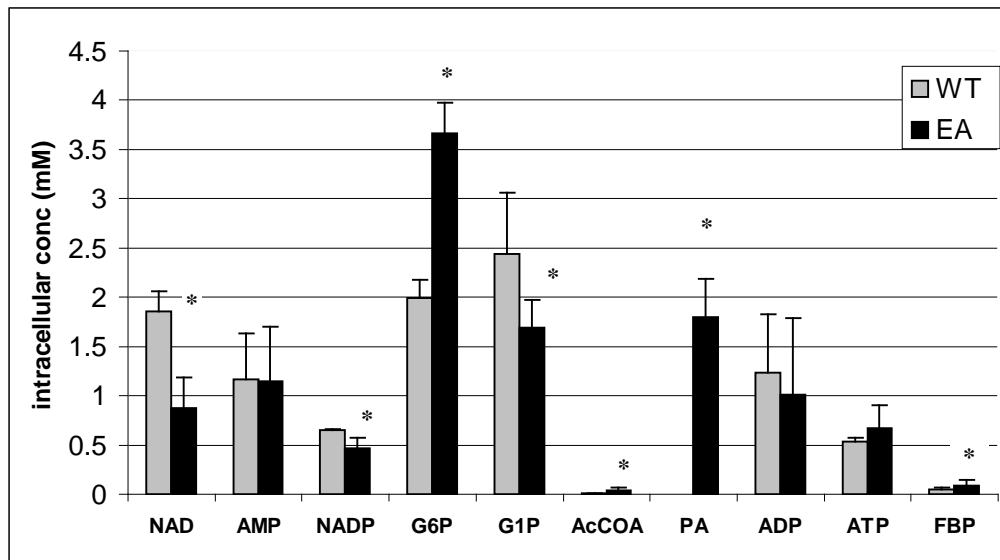
Growth characteristics of WT and EA *C. thermocellum* cultures were evaluated by measuring the optical density of the growth broth at 600 nm. Growth of WT cells was much faster (0.14 OD/hour) compared to EA cells (0.02 OD/hours). After 14 hours from the inoculation, cell density of WT cells reached a highest point and did not grow any further; however EA cells required 50 hours to reach the maximum cell density.

### 5.3.3 Intracellular metabolite analysis

Cell extracts of WT and EA *C. thermocellum* were analyzed by CE-ESI-MS. Pyruvic acid was only detected in samples from EA strain, however all other metabolites were detected in samples from both strains at varying concentrations. Obtained intracellular concentrations for the detected metabolites in samples from both strains were shown in Figure 5-2.



**Figure 5-1: Growth characteristics of WT and EA *C. thermocellum* batch cultures**

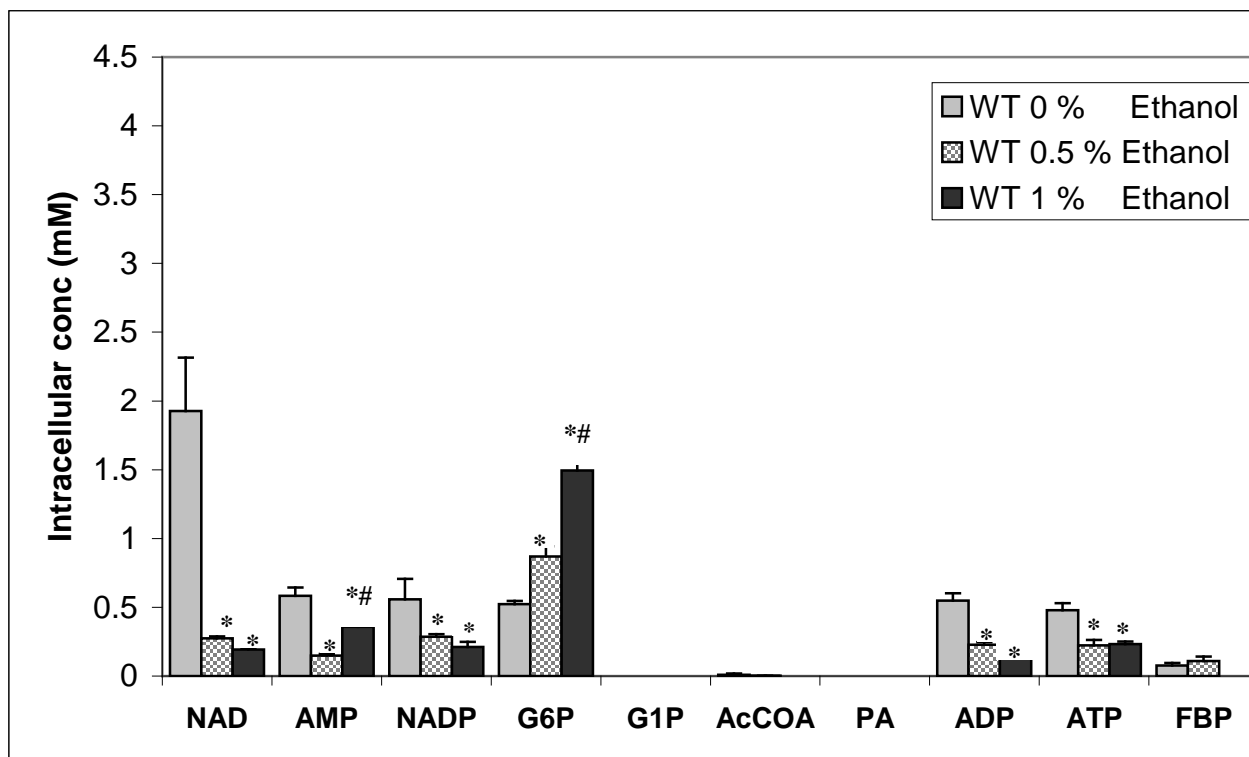


**Figure 5-2: Intracellular metabolite concentration of detected metabolites in cell extracts of WT and EA *C. thermocellum* in batch cultures (\*  $p < 0.05$ )**

On performing a paired Student's T-test for statistical analysis significant differences were noticed among intracellular concentrations of 6 metabolites. The intracellular concentrations of  $\text{NAD}^+$ ,  $\text{NADP}^+$ , and G1P were significantly lower in samples from EA strain compared to samples from WT strains; however intracellular concentration of G6P, acetyl-CoA, and FBP were significantly higher in samples from EA cells compared to samples from WT cells. As mentioned before, pyruvic acid was only detected in samples from EA cells and was below the LOD in samples from WT cells. No significant differences were noticed in intracellular concentrations of AMP, ADP, and ATP in samples from WT and EA strains.

#### **5.3.4 Intracellular metabolite analysis of WT *C. thermocellum* cultivated in a chemostat**

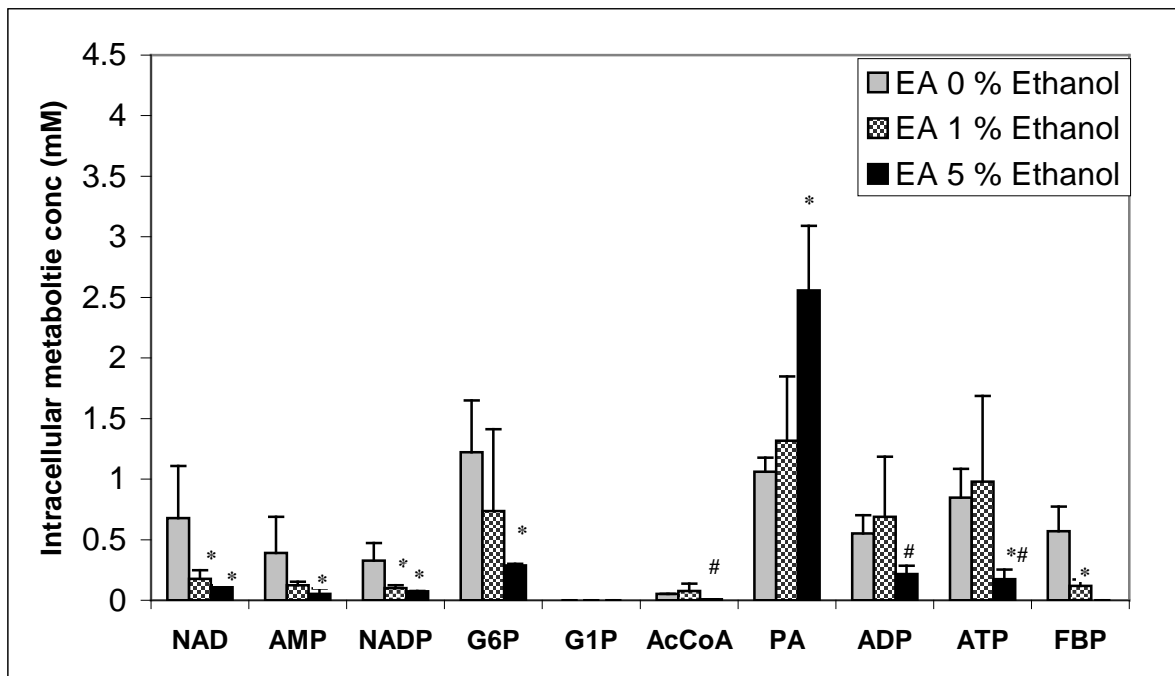
WT *C. thermocellum* cells were cultivated in chemostat at varying concentration of exogenous ethanol [0, 0.5, 1, and 5% ethanol (w/v)] and cell growth was monitored by measuring the optical density of the samples. WT cells were able to grow in all ethanol treatments, except at the 5% exogenous ethanol (w/v) treatment. The growth of WT strain is probably hindered at 5% ethanol concentration, which was not surprising due to increased toxicity at higher ethanol concentration. Cells were harvested once they reached steady state. CE-ESI-MS analysis was performed on the samples from 3 different ethanol treatments and obtained intracellular metabolite concentrations of the detected metabolites are shown in Figure 5-3. G1P and pyruvic acid were not detected in any of the samples. Acetyl-CoA and FBP were only detected in samples from 0% and 0.5% ethanol treatments and no significant differences were found among their intracellular concentrations.  $\text{NAD}^+$ ,  $\text{NADP}^+$ , AMP, ADP, ATP, and G6P were detected in all the samples from 3 ethanol treatments and significant differences were noticed in their intracellular concentrations. Intracellular concentrations of  $\text{NAD}^+$ , AMP,  $\text{NADP}^+$ , ADP, and ATP were significantly lower in samples from 0.5 and 1% ethanol treatment compared to samples from 0% ethanol treatment. No differences in intracellular concentrations of  $\text{NAD}^+$ ,  $\text{NADP}^+$ , ADP, and ATP were found among the samples from 0.5 and 1% ethanol treatments. The intracellular concentration of G6P was significantly different in all samples from 3 ethanol treatments (highest in 1% ethanol treatment and lowest in 0% ethanol treatment)



**Figure 5-3: Intracellular metabolite analysis of cell extracts of WT *C. thermocellum* cells cultivated in a chemostat at various ethanol concentrations in the growth media**

\*  $p < 0.05$  compared to 0% ethanol treatment

#  $p < 0.05$  compared to 0.5% ethanol treatment



**Figure 5-4: Intracellular metabolite analysis of cell extracts of EA *C. thermocellum* cells cultivated in a chemostat at various ethanol concentrations in the growth media**

\*  $p < 0.05$  compared to 0% ethanol treatment

#  $p < 0.05$  compared to 1% ethanol treatment

### 5.3.5 Intracellular metabolite analysis of EA *C. thermocellum* cultivated in a chemostat

EA *C. thermocellum* cells were cultivated in chemostat at various concentration of exogenous ethanol [0, 1, 5 and 8% ethanol (w/v)] and cell growth was monitored by measuring the cell density. Except at the 8% exogenous ethanol (w/v) concentration, the EA cells were able to grow in all other ethanol treatments. The growth of WT cells at 8% ethanol concentration was likely to be hindered due to increased toxicity from ethanol. For 0, 1, and 5% ethanol (w/v) treatments, cells were harvested after the steady state was reached. All the samples from these 3 ethanol treatments were analyzed using CE-ESI-MS and obtained intracellular metabolite concentrations of the detected metabolites are shown in Figure 5-4.

GIP was not detected in any of the samples from these 3 treatments. FBP was only detected in the samples from the 0 and 1% ethanol treatments and was significantly lower in the sample from the 1% ethanol treatment compared to the sample from the 0% ethanol treatment.  $\text{NAD}^+$ ,  $\text{NADP}^+$ , AMP, ADP, ATP, G6P, pyruvic acid, and acetyl-CoA were detected in all samples from 3 different ethanol treatments and significant differences were noticed in intracellular concentrations of these metabolites. Intracellular concentrations of the  $\text{NAD}^+$  and  $\text{NADP}^+$  were significantly lower in the samples from the 1% and 5% ethanol treatments compared to the samples from the 0% ethanol, however no significant differences in intracellular metabolite concentrations between the samples from the 1% and 5% ethanol treatments were observed for these 2 metabolites. Intracellular concentrations of AMP and G6P was significantly lower in the samples from the 5% ethanol treatment compared to the sample from the 0% ethanol treatment, however no significant difference were found between the samples from 0% and 1% ethanol treatments for these 2 metabolites. Intracellular concentrations of acetyl-CoA and ADP were significantly lower in the samples from 5% ethanol treatment compared to the samples from 1% ethanol treatment only, however no other differences were found for these 2 metabolites. Intracellular concentration of ATP was significantly lower in the samples from the 5% ethanol treatment compared to samples from the 0 and 1% ethanol treatments, however no significant difference were observed between the intracellular concentrations of ATP among the samples from the 0 and 1% ethanol treatments.



Intracellular concentrations of pyruvic acid were much higher in the 5% ethanol treatment compared to the 0% ethanol treatment only, however no difference was found of intracellular concentration of pyruvic acid among the samples from the 0 and 1% ethanol treatments.

#### **5.4 Discussion**

Analyses of bacterial metabolic responses to ethanol stress were carried out. Overall 3 objectives were accomplished from the metabolic data analyses; first, we investigated the reason for a slow growth of EA cells compared to WT cells in batch cultures; second, the mechanism of WT cell growth inhibition due to exogenous ethanol; finally, the basis of moderate (5%) ethanol tolerance of EA cells was elucidated. Intracellular metabolites from WT and EA batch culture samples were analyzed and the data showed no significant differences in the energy content (ADP + ATP) of these strains, which suggests that EA cells are not lacking in overall energy content. Therefore, lack of energy can be ruled out as a reason for the slow growth of EA cells compared to WT cells. Although there were significant differences in G1P and G6P in these two strains, these two molecules are readily inter-convertible inside the cell,<sup>136</sup> hence when the pool of G1P + G6P (WT:  $4.4 \pm 0.9$ ; EA:  $5.3 \pm 0.6$ ) were used, no significant differences were found. However, two key metabolites were found to be significantly different in these strains. Pyruvic acid was only detected in EA cell and intracellular concentration of  $\text{NAD}^+$  was significantly lower in EA cells compared to WT cells.

Formation of pyruvic acid is the last step in glycolysis. The net result of glycolysis is the conversion of a sugar molecule along with 2  $\text{NAD}^+$  and 2 ADP molecules to 2 pyruvate, 2 NADH, and 2 ATP molecules. The pyruvate subsequently is fermented to ethanol, acetate, and lactate in a series of steps. During the formation of lactate and ethanol, NADH is converted to  $\text{NAD}^+$ . Since fermentation of pyruvate regenerates  $\text{NAD}^+$ , this process is essential for glycolysis.

Pyruvic acid was only detected in EA, therefore it was hypothesized that, biochemical pathways downstream of pyruvate were kinetically slowed due to exogenous ethanol or some of the steps might be completely blocked in EA cells compared to WT cells. Decreased formation of lactate or/and ethanol due to obstruction in fermentation of pyruvate would lead to inefficient regeneration of  $\text{NAD}^+$ . This is likely the case in EA

cells, since intracellular levels of  $\text{NAD}^+$  were lower in EA cells compared to WT cells. Obstruction of pyruvate fermentation and its subsequent accumulation in EA cells was the probable reason for the slow growth of the EA cells compared to WT cells.

To extend the investigation and fully understand the effect of ethanol on *C. thermocellum*, WT and EA cells were grown in a stepwise increasing ethanol concentration and metabolite analysis was performed in response to changes in ethanol concentration. Considering the cellular metabolic processes resulting from activation, inhibition, and feed-back activities, a model was developed which was able to describe the modulation of the whole system induced by an external stress due to increasing concentrations of exogenous ethanol. This approach was able to interpret the experimental results in terms of metabolic response to exogenous ethanol in the *C. thermocellum*.

Figure 5-5 shows the hypothetical model for growth inhibition of WT cells due to ethanol. Step I in the model predicts the pathway for acetaldehyde to ethanol is slow or completely shut due to feedback inhibition of alcohol dehydrogenase (ADH) because of exogenous ethanol. ADH is an enzyme that carries reduction of acetaldehyde in presence of  $\text{NADH}$  to produce ethanol along with  $\text{NAD}^+$  as a co-product of the biochemical reaction. Since this pathway is slowed,  $\text{NADH}$  is not getting converted back to  $\text{NAD}^+$  at the required rate, reducing the overall availability of  $\text{NAD}^+$  in the cell. Step II of the model involves electrons generated in the process of conversion of pyruvate to acetyl-CoA. Two electrons and carbon dioxide gas are generated in the process of this conversion and these electrons can be deposited to  $\text{NAD}^+$  or protons to subsequently generate  $\text{NADH}$  or  $\text{H}_2$  gas, respectively. However, this model proposes that in WT cell, these electrons are shunted to  $\text{NAD}^+$  to generate  $\text{NADH}$ , since hydrogenase, an enzyme required for transfer of electrons to protons is probably present in low levels. Therefore, in step I presence of exogenous ethanol decreases the regeneration of  $\text{NAD}^+$  and  $\text{NAD}^+$  is being continuously utilized to deposit the electrons generated in step II. The result of this is a net decrease in  $\text{NAD}^+$  levels. These predictions are consistent with low levels of  $\text{NAD}^+$  seen in the WT samples from 0.5 and 1% ethanol treatments compared to samples from 0% ethanol treatment. The low availability of  $\text{NAD}^+$  may prevent some of the biochemical reactions from going in forward directions. For example, conversion of

glyceraldehyde 3-phosphate (G3P) to 1, 3-diphosphoglycerate in glycolysis requires inorganic phosphate (Pi) and  $\text{NAD}^+$ . Step III of the model predicts, low levels of  $\text{NAD}^+$  may slow down or completely stop the conversion of G3P to 1, 3 diphosphoglycerate, due to which products upstream of the pathway may start accumulating. One such product upstream of this pathway is G6P; increasing intracellular concentration of G6P in samples from 0.5 and 1% ethanol treatments compared to sample from 0% ethanol treatment of WT cells clearly suggest G6P is accumulating as the exogenous ethanol concentration increases. Accumulation of G6P signifies the obstruction in glycolysis which would eventually starve the cells for this carbon source and scarcity of carbon would lead to low energy content (ADP + ATP) of the cells. These predictions are consistent with the observations that higher G6P and lower ATP and ADP intracellular concentrations in samples from 0.5 and 1% ethanol treatments compared to samples from 0% ethanol treatments. Further increase of ethanol concentration beyond 1% would probably cause an additional increase in G6P and a further decrease in ADP and ATP content which would eventually lead to complete growth cessation. Therefore, this model along with intracellular metabolite data predicts, exogenous ethanol leads to blockage of ethanol production pathway, which decreases the overall  $\text{NAD}^+$  level of the cell. Insufficient  $\text{NAD}^+$  is an impediment in glycolysis and renders accumulation of G6P. Therefore, obstruction in glycolysis is the likely reason for growth inhibition of WT *C. thermocellum* in exogenous ethanol.

Similar to WT cells, a model for EA cells were also developed as shown in Figure 5-6. Analogous to model of WT cells, step I of the model for the EA cells also assumes acetaldehyde to ethanol pathway is inhibited in presence of exogenous ethanol due to feedback inhibition of ADH enzyme, resulting in diminished regeneration of  $\text{NAD}^+$ . The metabolic data clearly supports this prediction, since the intracellular concentration of  $\text{NAD}^+$  is significantly lower in samples from 1 and 5% ethanol treatments compared to samples from 0% ethanol treatment of EA cells. Step II of the model for EA cells suggests electrons generated in the process of conversion of pyruvic acid to acetyl-CoA are being deposited in to protons instead of  $\text{NAD}^+$  and produce hydrogen gas unlike WT cells where these electrons are deposited in  $\text{NAD}^+$  and NADH is produced. Shunting of electrons to protons is possible in EA cells, since hydrogenase, an enzyme required for

this process, is likely present in higher level in EA cells compared to WT cells. Indeed, in a separate WT and EA *C. thermocellum* membrane proteome analysis by Dr. Fu in our lab, hydrogenase was found to be about 2 times higher in EA cell compared to WT cells (manuscript in preparation by Dr. Fu), which strongly supports this hypothesis. Since, electron flow in step II is directed toward protons rather than  $\text{NAD}^+$  the demand for  $\text{NAD}^+$  decreases. Therefore,  $\text{NAD}^+$  is available to continue the glycolysis process unlike WT cells. This prediction is very consistent with the intracellular metabolite data of G6P; accumulation of G6P was not observed in EA cells on increasing the exogenous ethanol concentration. Uninterrupted glycolysis results in continued production of ATP molecules (2 ATP equivalent/ glucose equivalent) which is the main reason that that intracellular metabolite concentration of ATP didn't decrease from increasing the exogenous concentration from 0 to 1% in EA cells unlike WT cells. However increasing exogenous ethanol concentration further to 5%, the intracellular concentration of ATP decreased significantly in the sample from 5% ethanol treatment compared to the samples from 1 and 0% ethanol treatments of EA cells, which is mainly because of the maintenance energy required at 5% ethanol is higher due to unfavorable ethanol environment for the cell growth. Uninterrupted glycolysis in EA cells is the likely reason for its survival at higher ethanol (5%) concentration. However, at elevated exogenous ethanol concentrations (5%), obtained intracellular pyruvate is much higher, which suggests inefficient metabolism of pyruvate in presence of higher exogenous ethanol concentration. The obstruction in pyruvate metabolism is the likely reason EA cells could not grow at ethanol concentration greater than 5%.

Metabolite profile changes as a result of increase in exogenous ethanol explain that an obstruction of glycolysis due to exogenous ethanol is the mechanism of WT cell growth inhibition. However, higher level of hydrogenase enzyme and uninterrupted glycolysis is the basis of moderate (5%) ethanol tolerance of EA cells. However, as the exogenous ethanol concentration increases beyond 5%, intracellular pyruvate level in EA cells probably increases to such a level that it cells completely starve for this carbon source and results in growth cessation of EA cells. Intracellular metabolite data explains the relationship between the exogenous ethanol and shift in metabolic pathway, which is important for understanding the physiology of the biological systems.

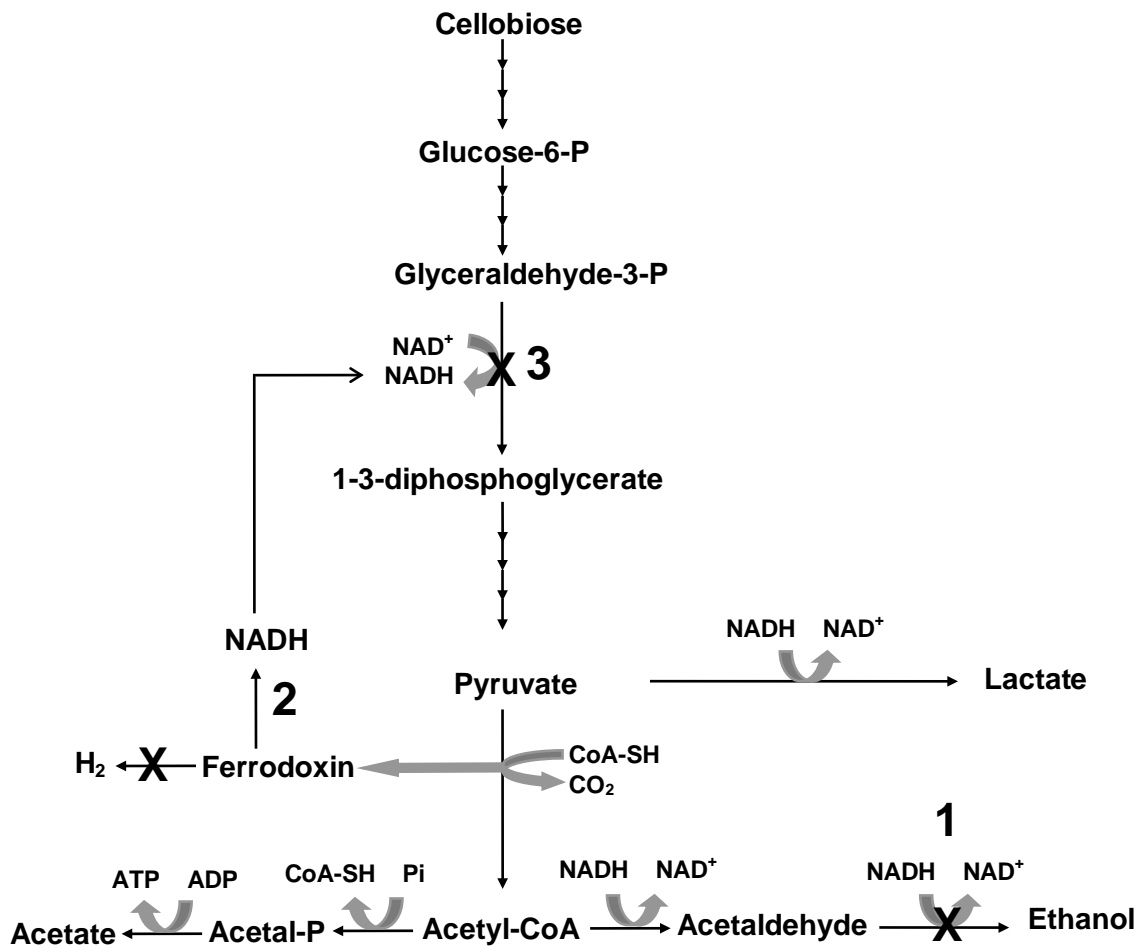
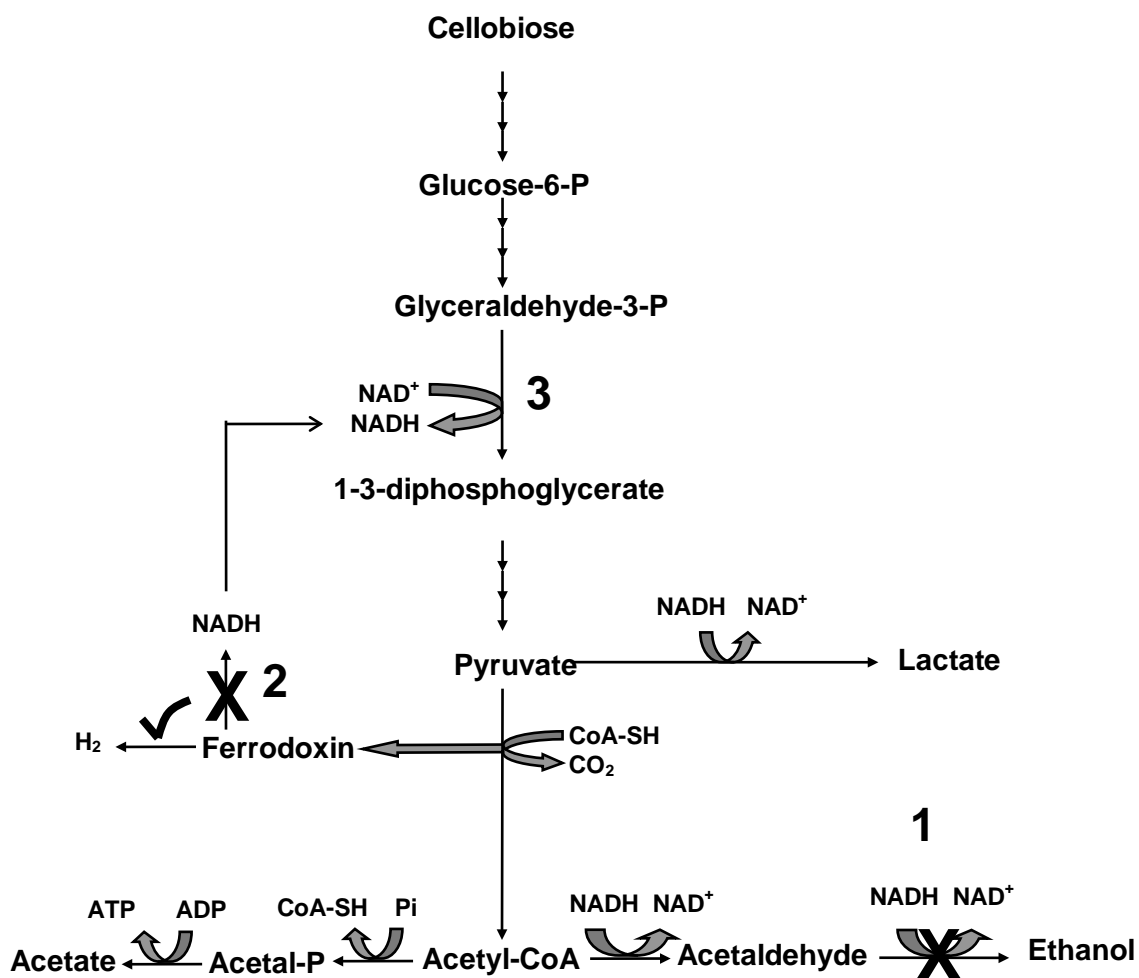


Figure 5-5 Hypothetical model of growth inhibition of WT *C. thermocellum* due to ethanol



**Figure 5-6: Hypothetical model of survival of EA *C. thermocellum* in moderate (~ 5%) exogenous ethanol**

## 5.5 Conclusion

Metabolite analysis of WT and EA *C. thermocellum* cells reveals interesting differences in metabolite profiles of these two strains. All 10 metabolites examined were found in both strains, except pyruvic acid was below LOD in WT strain. Some key metabolites such as  $\text{NAD}^+$  and pyruvic acid suggested vital differences in the metabolism of these two strains from batch culture samples. Since pyruvic acid was only detected in EA cells its accumulation in EA cells is probably the reason for its slower growth.

To study the effect of ethanol on metabolic fluxes, metabolite profile of WT and EA strains cultivated in chemostat in increasing concentration of exogenous ethanol was obtained, which revealed the basis of the growth inhibition of the WT cells and ethanol tolerance of the EA cell. Growth inhibition of the WT cells was the result of the depleted level of  $\text{NAD}^+$  because of exogenous ethanol. Low levels of  $\text{NAD}^+$  eventually lead to obstruction of glycolysis, which was evident from the accumulation of G6P in presence of exogenous ethanol. In EA cells depletion of  $\text{NAD}^+$  was also observed, although EA cells were able to circumvent the problem of low availability of  $\text{NAD}^+$  by minimizing  $\text{NAD}^+$  use and continued the glycolysis process. Unlike WT cells, accumulation of G6P was not observed in EA cells by increasing exogenous ethanol concentration, which suggests uninterrupted glycolysis was the basis of ethanol tolerance in EA cells. Metabolite analysis proved to be a valuable tool for understanding these complex biological systems.

## Chapter 6 : Conclusions

Metabolites are the end product of all intracellular processes; therefore any genetic alterations, protein flux changes, disease conditions, or environmental and nutritional changes can result in an alteration of intracellular metabolite concentrations. Analysis of these metabolites in a biological system provides an instantaneous snapshot of the physiological state of a cell and can predict the phenotypes. The science of analyzing these metabolites is termed metabolomics and could have tremendous impact on a variety of fields such as biotechnology, pharmaceutical, and disease research. For high throughput metabolite analysis analytical instruments consisting of a high throughput separation techniques combined with detectors such as mass spectrometer are the potential candidates. Although metabolomics certainly will benefit from high throughput analytical techniques, many challenges still exists to make metabolomics a complete success. The first challenge is the diverse nature of metabolites, due to the very different physicochemical properties, analysis of all metabolites using a single analytical technique would be merely impossible. Lipid molecules are highly non-polar, amino acids are moderately polar, sugars are highly polar, and nucleotide phosphates are acidic and polar. In addition, metabolite extraction protocols require optimization depending on the type of metabolites to be analyzed, therefore sample preparation protocols are not universal. Also, sample sources can vary [cell, fluid (urine or blood), and tissue], therefore sample preparation protocols require evaluation. The second challenge is the low intracellular concentration and large dynamic range of metabolites. Up to 3 orders of magnitude of range can be found within the same samples; therefore the dynamic range of an analytical technique should be very high and should be capable of detecting low level metabolites. The third challenge is analytical and biological variance; although analytical variance can be controlled to some extent biological variance is unavoidable. The fourth challenge is converting the obtained data to usable knowledge. Modern analytical instruments yield huge amounts of data in short periods of time and since metabolic pathways are highly complex, converting obtained metabolic data to useful knowledge can be challenging.



Due to all above mentioned impediments, using metabolomics for any application looks insurmountable; however strategies such as “divide and conquer” can be used to overcome these difficulties. Hypotheses can be generated for a particular study and instead of analyzing all the metabolites a set of predefined metabolites or metabolites involved in a particular pathway can be analyzed which are subject to change due to treatment(s). Moreover for analysis purposes, metabolites are usually divided in different categories depending on their physicochemical properties, which simplify the work to a great extent. Three main categories of metabolites are non-polar, polar, and charged and can be analyzed using one of the 3 separation methods such as GC, LC, or CE combined with mass spectrometer, respectively. The mass spectrometer is an ideal choice of detector in metabolite analysis due to its high sensitivity and addition dimension of separation ( $m/z$ ) it provides.

In this dissertation metabolites involved only in central metabolic pathway of *C. thermocellum* were selected for analysis. Capillary electrophoresis in combination with mass spectrometer was used because of charged and highly polar nature of these metabolites. A CE-ESI-MS method was developed which was able to detect few femtomoles of metabolites and produced dynamic range of more than 2 orders of magnitude. Due to high sensitivity of this method a few milligrams of cells were sufficient to detect most of the metabolites selected in this study. Intracellular metabolite data revealed that exogenous ethanol changes intracellular metabolite concentrations significantly and proved the hypothesis generate at the beginning of the investigation. From metabolic data it was observed that exogenous ethanol decreased overall  $NAD^+$  contain which obstructed glycolysis of WT *C. thermocellum* and as a consequence accumulation of G6P is observed. On the other hand in EA *C. thermocellum*, accumulation of G6P was not observed with increasing exogenous ethanol concentration which suggested the glycolysis process was not interrupted and was the likely reason for their survival in moderate (5%) exogenous ethanol. However at 5% exogenous ethanol, intracellular pyruvate was significantly higher compared to 0% exogenous ethanol in EA cells. Accumulation of pyruvate suggests cells are starving for this carbon source and further increase in exogenous ethanol will bring cell to complete halt due to lack of this carbon source.

To further validate the models developed in this study for interpretation of metabolic data and to extend this investigation, activity of enzymes such as ADH, hydrogenase in both 2 strains and the effect of ethanol concentration on the activity of these enzymes should be studied. Moreover, determination of metabolite end products such as lactate, acetate, and ethanol can provide insightful information, however determination of ethanol in presence of exogenous ethanol is very challenging. To distinguish ethanol produced by cells from exogenous ethanol, isotopic labeling is required; either exogenous ethanol or cellobiose can be labeled with  $^{13}\text{C}$ . The activities of these enzymes, metabolic end product concentrations, and intracellular metabolic data can help to further clarify the mechanism of growth inhibition of *C. thermocellum* due to exogenous ethanol. A clear understanding of effect of exogenous ethanol on cell growth will certainly help to develop superior strains of *C. thermocellum* using genetic engineering to improve its biotechnological application in consolidate bioprocessing (CBP) to produce ethanol. Ethanol produced through CBP can be much cheaper compared to other sources of ethanol and can replace the gasoline used for automobile transportation. Replacing gasoline with ethanol can reduce the green house gas emission and also provide solutions to the world energy crisis.

These developed CE-ESI-MS methods could also be utilized for intracellular metabolite analysis of many other microorganisms which are of huge importance in biotechnological industry for strains improvement. Genetically engineered microbes are highly essential for large scale production of vitamins, amino acids, enzymes, and biopharmaceuticals products. In addition to production of medicinal agents, the ability of microbes to produce solvents such as acetone, ethanol, and butanol can further foster the chemical industries. Therefore, intracellular metabolite data could tremendously benefit biotechnological industry to develop medicinal and chemical products which can have an immense impact on human health and lifestyle.

In addition to metabolic engineering, metabolite analysis can provide answers to problems related to health and disease research. Molecular pathways can be monitored via metabolite analysis and any molecular damages or perturbations can be studied during a disease condition or progression. Changes in molecular pathway will subsequently change the metabolite concentrations which become potential biomarkers for disease

diagnosis. Metabolomics approaches in biomarker identification are relatively non-invasive since metabolite analysis of urine and blood samples provide a tremendous amount of information about sickness and disease progression. In clinics, blood samples are routinely checked for limited number of metabolites, such as cholesterol and glucose which are biomarkers for heart disease and diabetes, respectively. By analyzing all possible metabolites in urine and blood samples compared to few metabolites checked today, a broader picture of a health state can be achieved. Early detection of disease could reduce the morbidity and mortality and prevent substantial medical expenditure and save many lives. Yang *et al.* were able to distinguish the population of patients with liver cancer compared to healthy volunteers using metabolite analysis. Urine samples from healthy and liver cancer patients were monitored for levels of 15 nucleosides using HPLC. They claimed their method was better than traditional method of tumor diagnosis alpha-fetoprotein, a tumor marker, in distinguishing the diseased samples from healthy samples (83% vs. 74%).<sup>137</sup> In a similar approach, Odunsi *et al.* analyzed the serum samples from 12 women previously diagnosed of benign ovarian cyst, 38 ovarian epithelium cancer (EOC) women, and 52 healthy women (19 premenopausal and 32 postmenopausal) using <sup>1</sup>H NMR. The metabolic patterns obtained using <sup>1</sup>H NMR could clearly distinguish EOC from healthy population, moreover the sera patterns of EOC patients could be clearly separated from the population of benign ovarian cyst.<sup>138</sup>

Metabolomics can be used for every disease one could imagine. A metabolite profile of an individual can be obtained (by analyzing blood and urine samples) and according to his/her metabolite profile a specific diet course, exercise routine, and required drug could be administered for curative purposes of common thing such as a weight loss to more complex problems such as a heart disease. Metabolomics could also provide an insight to what happens when a cell is infected with a virus and can help to develop antiviral therapies. Rabinowitz *et al.* identified the metabolite in human cells infected with *Cytomegalovirus* using liquid chromatography. Surprisingly they found metabolites involved in glycolysis, citric acid cycle, and pyrimidine nucleotide biosynthesis markedly increased.<sup>139</sup> This work represented metabolic environment of virally infected cells, and some of these metabolite could be eventually used as a target for antiviral therapy.

Metabolomics also has a potential in drug discovery, monitoring responses and toxicity of drugs against an illness, drug efficacy, and finding its mechanism in disease cure. Chanda *et al.* performed metabolite analysis of in-vitro hepatic and skin metabolism of Capsaicin, a pain killer, to find out the mechanism of action this drug.<sup>140</sup> Drug reaction mechanism can help develop superior drugs with better efficiency and lesser side effects. To avoid the drug toxicity effects personalized medicine is a new approach taken by FDA because many drugs cause adverse effect to some individuals even though these drugs were approved by FDA on the basis of clinical and toxicological data. This is mainly because every person is different in the manner he/she reacts to medicine; a particular drug might have varying effect and can benefit only some subjects and could cause adverse reactions to others. Liver injury is the most prevalent side effect of new drugs and sometime drugs can cause liver failure and required to pool it out of the market. One way to avoid the problem is to analyze the urine and blood sample from subjects to who drug is administered and define the metabolite profile. Metabolite profile will shed light on what measurable way people are different, and predict who shouldn't take the drug. Therefore, by eliminating the population at risk from that drug side effects could be avoided and other people can still benefit from it.

In drug discovery metabolomics can bring the overall cost down. Every year more than 5000 drugs are developed out of which only few make to the clinical trials. On average the cost of each drug is more than half a billion dollars. The main reason drugs fail is because of lack of efficacy of the drug and its impact on metabolic regulation of disease. Efficacy of the drug and metabolite regulations can be studied via metabolite analysis and in addition, any toxicity due to the drug can be detected at an early stage which substantially brings the cost down. Therefore, metabolomics can help bring the cost down of the drug and develop safer and quicker drugs.

In summary, metabolomics is going to play a big role in biotechnology, pharmaceutical, and healthcare industries. Moreover a holistic understanding of fundamental biology can be acquired by combining genomics, transcriptomics, proteomics, and metabolomics data. For comprehensive metabolite analysis new approaches of separation are essential, one separation method for all is somewhere the research should be focused. Although, it is impossible today to have single separation

method for all the metabolites, a method which can separate maximum number of metabolites should be developed. Mass spectrometry should be consistently challenged to increase the sensitivity and dynamic range. How low is good, though? The answer is lower than what we can detect today. From increasing use of mass spectrometry in studying metabolomics, it is clear that scientific community has a great belief that mass spectrometry has a bigger role to play in metabolomics. Careful attention should be given to analytical methods development, experimental design, data analysis, and data integration and interpretation for successful metabolomics investigations. Huge amounts of data will be generated in near future; therefore bioinformatics software are highly essential to convert the obtained data to readable information and will have a tremendous impact on the overall progress of metabolomics. Metabolomics certainly has implication in several biotechnological, pharmaceutical and healthcare related issues which will consequently benefit the environment and the lives of the humans to render this planet a beautiful place to habitat.

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## VITA

**Anup P. Thakur**

**Date of Birth: 2<sup>nd</sup> July, 1978**

**Place of Birth: Pune, India**

Anup Thakur is a native of India, and born to a family with a business background. He grew up in Muktainagar, a small town in Maharashtra province of India (300 miles away from Mumbai). He completed his primary and secondary education from a school in the same town, and went to North Maharashtra University, Jalgaon, India to pursue his bachelor's degree. He stood first in the class and graduated with distinction. Followed by bachelor's degree, he continued in the same University for his further education and received a Masters degree with major in Organic Chemistry and graduated with Distinction.

After graduation Anup joined National Chemical Laboratory (NCL), Pune, India as research assistant through very rigorous selection procedure. NCL is the premier research Institute in the Asia and one of its kinds. To pursue higher education, he moved to University of Kentucky. Under the supervision of Dr. Bert Lynn's, he started microbial metabolomics investigations. He gained extensive experience with capillary electrophoresis and mass spectrometry techniques. Using CE-MS he developed novel techniques for metabolite sample preparation, and applied for analysis of real microbial samples. Development of CE-MS as well as metabolite sample preparation were very challenging, since these both field are relatively new and complex. By applying CE-MS for metabolite analysis he carried out metabolite investigation of *C. thermocellum*.

Anup is recipient of travel grants to present his research in variety of conferences. He presented his research at annual American Society of Mass Spectrometry (ASMS) in 2006 and 2007, and American Society of Microbiology (ASM) in 2006.



## ***Publications***

- Williams T. I.; Combs J. C.; Thakur A. P.; Strobel H. J.; Lynn B. C. “A novel bicine running buffer system for doubled sodium dodecyl sulfate - polyacrylamide gel electrophoresis of membrane proteins” *Electrophoresis*, **2006**, 27, 2984 -2995
- Thakur A. P.; Nokes S.E.; Knutson B.; Strobel H.J. Lynn B.C. “Method development and sensitivity enhance of CE-ESI-MS for analysis of acidic metabolites ” (*To be submitted*)
- Thakur A. P.; Nokes S.E.; Knutson B.; Strobel H.J. Lynn B.C. “Development of novel methods of quenching and extraction for metabolite analysis of cellulolytic and gram positive bacteria” (*To be submitted*)
- Thakur A. P.; Nokes S.E.; Knutson B.; Strobel H.J. Lynn B.C. “Metabolite profile of wild type and ethanol adapted *Clostridium thermocellum* in batch culture” (*Manuscript in preparation*)
- Thakur A. P.; Nokes S.E.; Knutson B.; Strobel H.J. Lynn B.C. “Metabolite profile of wild type and ethanol adapted *Clostridium thermocellum* in continuous culture” (*Manuscript in preparation*)

## ***Presentations***

Jujjuri S.; Thakur A. P.; Strobel H.J.; Nokes S.E.; Knutson B.; Lynn B.C. “Metabolic profile of wild-type and ethanol- adapted *Clostridium thermocellum* in continuous culture” *American chemical Society (ACS)*, **2007**, Boston, MA

Thakur A. P.; Strobel H.J.; Nokes S.E.; Knutson B.; Lynn B.C. “Method development of capillary electrophoresis-electrospray ionization-mass spectrometry (CE-ESI-MS) for metabolic comparison of wild type and ethanol adapted strains of *Clostridium thermocellum*” *American Society for Mass Spectrometry Conference (ASMS)* **2007**, Indianapolis, IN

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